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To my family

What has been will be again, what has been done will be done again; there is nothing new under the sun. Is there anything of which one can say, "Look! This is something new"? It was here already, long ago; it was here before our time.

Ecclesiastes 1:9-10

ORIGINAL ARTICLES INCLUDED IN THIS THESIS

1. Adolfsson J, Mansson R, Buza-Vidas N, Hultquist A, Liuba K, Jensen CT, Bryder D, Yang L, Borge OJ, Thoren LA, Anderson K, Sitnicka E, Sasaki Y, Sigvardsson M, Jacobsen SE. **Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment**. Cell. 2005 Apr 22;121(2):295-306.

2. Liuba K, Pronk CJ, Stott S, Jacobsen SE. **Polyclonal T cell reconstitution of X-SCID recipients after in utero transplantation of lymphoid-primed multipotent progenitors** Blood. 2009 May 7;113(19):4790-8

3. Nygren JM, Liuba K, Breitbach M, Stott S, Thorén L, Roell W, Geisen C, Sasse P, Kirik D, Björklund A, Nerlov C; Fleischmann BK, Jovinge S, Jacobsen SE. **Myeloid and lymphoid contribution to non-haematopoietic lineages through irradiation-induced heterotypic cell fusion** Nat Cell Biol 2008 May;10(5):584-92

Abbreviations

Ag	Antigen
AGM	Aorta-gonads-mesonephros
BCR	B cell receptor
BM	Bone marrow
BMT	Bone marrow transplantation
CB	Cord blood
CD	Cluster of differentiation
CFU-S	Colony forming unit spleen
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
E	Erythroid
FACS	Fluorescence activated cell sorting
FL	Fetal liver
GM	Granulocyte/macrophage
GVHD	Graft versus host disease
HLA	Human Leucocyte Antigen
HSC	Hematopoietic stem cell
Ig	Immunoglobulin
IUHCT	In utero hematopoietic cell transplantation
LMPP	Lymphoid primed multipotent progenitor
LT-HSC	Long term hematopoietic stem cell
MHC	Major Histocompatibility Complex
Mk	Megakaryocyte
MPP	Multipotent progenitor
MSC	Mesenchymal stem cell
NK	Natural killer
NOD-SCID	Non-obese diabetic severe combined immune deficient
PB	Peripheral blood
P-Sp	Para-aortic splanchnopleura
SCID	Severe combined immune deficiency
ST-HSC	Short term hematopoietic stem cell
TCR	T cell receptor
YS	Yolk sac
X-SCID	X linked severe combined immune deficiency

Contents

BACKGROUND

Postnatal bone marrow transplantation (BMT) is the therapy of choice for a number of inborn hematologic, immunologic and metabolic disorders, although permanent neurological, musculoskeletal and other organ damage may be established at the age when BMT is performed. With recent advances in prenatal screening and molecular-based diagnostics, early knowledge of fetal disease is possible and the opportunity of prenatal therapeutic intervention has emerged. Fetal cell replacement therapy or in utero hematopoietic cell transplantation (IUHCT) is the proposed non-myeloablative alternative to allogeneic BMT, and represents the method through which variable amounts of natural or genetically modified cells from numerous sources (adult bone marrow, cord blood, fetal liver) can be transferred to the fetal recipient, in hope of achieving permanent prenatal correction of the disorder and preventing irreversible organ damage (1). The concept of IUHCT is based on a cumulus of potential advantages that prenatal delivery of healthy donor cells holds: pre-immune status of the fetal recipient and its tolerance for foreign antigen; availability of space for donor cells in the developing hematopoietic organs; competitive advantage of donor cells (at least for some disorders); delivery of larger cell numbers reported to the fetal size than could be delivered postnatally and thus increased levels of engraftment; potential prevention of disease symptoms and/or permanent organ damage; overriding treatment associated toxicity of the postnatal BMT (2). Although proof of principle has been achieved by successful correction of X linked severe combined immune deficiency (X-SCID)(3-5), the majority of target diseases that IUHCT has been applied to, have either failed to engraft or had inadequate engraftment for therapeutic relevance(2). In consequence, the clinical promise of IUHCT remains unfulfilled and understanding the complexity of the fetal microenvironment, the optimal cell source and cell population for transplantation, the barriers to engraftment and many other challenges remain (6).

The focus of this thesis was to address some of the issues raised by IUHCT current situation. Thus, we investigated the optimal cell source for transplantation in severe combined immune deficiencies by first identifying the earliest commitment/ differentiation step in adult murine hematopoiesis (Article I) and then evaluating the therapeutic potential of the newly identified progenitor for immune reconstitution in a model of fetal X-SCID transplantation (Article II). In Article III we examine in adult and fetal models the proposed plasticity of hematopoietic stem cells (HSC), a feature that confirmed holds promise for clinical BMT (or IUHCT) to non-hematopoietic disorders. In addition we explore the potential of human bone marrow (BM) and cord blood (CB) HSCs to engraft preimmune fetal sheep, a model of xenograft IUHCT proposed as optimal for physiological evaluation of human stem and progenitor cells (discussed as a separate section in the general presentation part).

OVERVIEW OF HEMATOPOIESIS

Definition of hematopoietic stem cells

Our current definition of hematopoietic stem cells (HSCs) originates in the early observations of Till and McCulloch (7), nearly half a century ago. In search of the most primitive cells residing in murine BM, they transplanted lethally irradiated mice (devoid of self hematopoietic cells) with donor BM and shortly after transplantation, observed numerous nodules (colonies) in the spleens of lethally irradiated recipients, numbers proportional with the BM cells injected. Morphologically these were sites of donor derived rapidly proliferating hematopoietic tissue, rich in blasts and mature cells of several myeloid lineages. Because the identity of the cells giving rise to the colonies was uncertain, they chose to call them colony forming units-spleen (CFU-S). Later they demonstrated that these colonies were indeed the progeny of single cells and that some CFU-S had self renewal capacities- generating new colonies when transferred to secondary irradiated hosts (8). These fundamental discoveries ground our definition of a hematopoietic stem cell (HSC) based on its cardinal properties, namely: **self-renewal** (ability to generate cells identical to the mother cell), **multipotentiality** (generation of specialized daughter cells) and **proliferation** (ability to generate large numbers of mature cells, sufficient for replacing the ablated host's entire blood compartment)(9).

Hematopoiesis

A life long process of supplying the organism with mature blood cells defines hematopoiesis. It is estimated that daily approximately 10^{12} blood cells are generated in order to balance the loss of aging or defective blood cells (10). In addition, an outer insult (infection, hemorrhage, hypoxia, foreign antigen) will cause the production of blood cells to increase immediately to meet the new demands. In order to support this high turnover and maintain flexibility, the hematopoietic system relies on tight regulatory mechanisms that act in correct sequence upon a relatively small number of HSCs (1 HSC in 10^4 BM cells) residing mainly in the adult BM (11). These rare cells are responsible throughout the lifetime of the organism both for proliferation and differentiation (through intermediate lineage commitment steps) into all the mature blood cells, but also for the maintenance of the own cell pool through self-renewal.

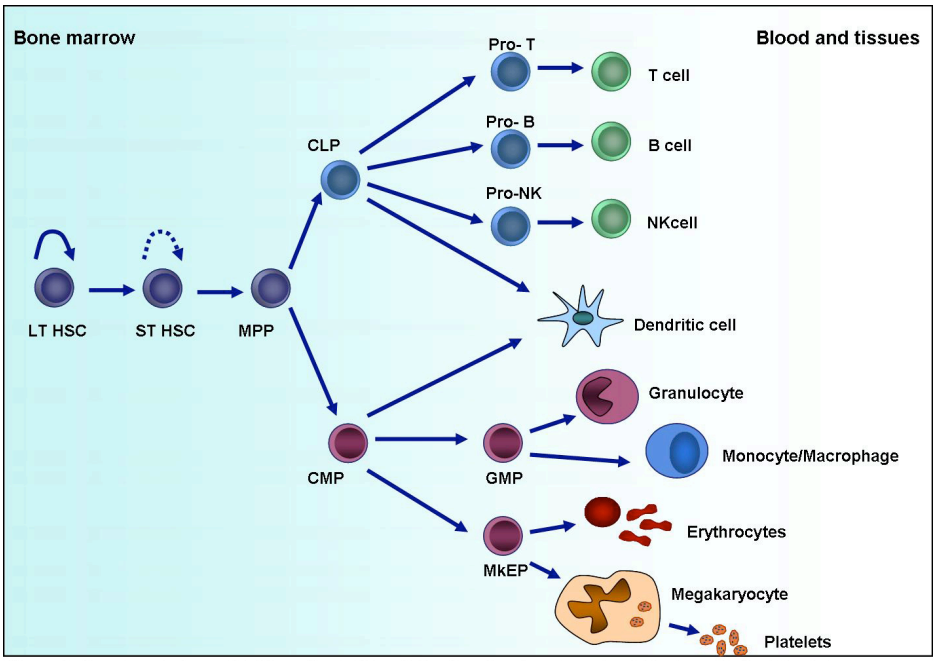

Figure 1. Hematopoiesis model. Hierarchical differentiation from long term hematopoietic stem cells (residing in bone marrow) into all the specialized blood cell types (distributed in the periphery), through steps of commited progenitors with limited self renewal capacity

In the adult, mature blood cells generated through hematopoiesis can be divided into three distinct compartments that are responsible for several life supporting functions (**Figure 1**).

First, red blood cells or **erythrocytes** – the most common blood cells, serve mainly for oxygen transportation from lungs to all the tissues via the blood stream. They lack nucleus and organelles, are small in size (6-8μm), biconcave in shape, mature from committed progenitors in about 7 days and have a life span of approximately 120 days before being destroyed in the spleen. Their frequency in a normal adult is estimated to approximately 2-3 x 10^{13}/L. Prenatal defects in erythrocyte lineage differentiation/production may result in hereditary forms of anemia like thalassemia, sickle cell disease, hereditary spherocytosis, Fanconi anemia.

Second, white blood cells or **leukocytes** are highly diversified nucleated cells that answer for protection of the body against infection and foreign antigen and can be found in blood, plasma as well as numerous tissues. Although less frequent (about 1% of total blood cell count, 4-11 x 10^{9}/L) their functions and numeric balance are extremely important for the well-being of the organism. Uncontrolled expansion of one line of white blood cells may cause leukaemia, while severe reduction/lack of a lymphocyte line may cause different forms of inherited immune deficiency. These cells are closer described in the following immune response section.

The third compartment, platelets or **thrombocytes** are small anuclear cytoplasmic bodies derived from megakaryocytes and are involved in bleeding prevention (hemostasis), a stepwise process leading to formation of blood clots (thrombi). Their lifespan is 7-10 days and are ultimately degraded in spleen and liver. Defects in platelet production alone may cause a rare disease called congenital amegakaryocytic thrombocytopenia (CAMT), while forms of Wiskott-Aldrichs syndrome (WAS) can also manifest with thrombocytopenia.

Immune response

The human body is shielded against insults from outward (pathogens) or inward (cancer cells) environment by a remarkable defence system, the immune response. In vertebrates, immunity is divided in two components: the non-specific (innate immunity; generates a rapid but unspecific response) and the specific (adaptive immunity; generates a custom tailored response to the pathogen), components that co-operate, through a tightly regulated interplay, to eliminate a foreign invader. Functionally, the immune system works in two steps. In the first step (recognition) it is able to specifically detect foreign molecules (proteins), chemical compounds (allergens), pathogens (bacteria, virus, fungi, parasites) and to differentiate these from body's own cells and proteins. In the second step (effector response) it enrols appropriate cells and molecules that effectively neutralize/eliminate pathogenic micro-organisms. Usually, a new encounter of the same pathogen will trigger a more rapid and aggressive immune reaction (memory response) that will disable the pathogen and prevent disease. Dysfunctions of the immune system result in the inability of the organism to generate appropriate immune response (immune deficiency syndromes), aberrant immune response against self antigen (autoimmune disease) or excessive immune response to foreign pathogen (allergic reaction).

Innate immunity, the first line of defence, is comprised of several elements: anatomical barriers as the skin and mucous membranes (non-pathogenic flora will out compete the pathogens on epithelial cell surface, cilia propels germs out from the body); physiologic barriers as temperature (fever will prevent growth of pathogens), low pH and chemical compounds (lysozyme- cleaves bacterial wall); inflammatory barriers as vasodilatation, increased permeability and efflux of vascular fluid rich in antibacterial components (i.e. C-reactive protein, histamine, kinins) and finally the phagocytic barrier of white blood cells (phagocytes, macrophages, dendritic cells, some lymphocytes) that engulf whole or fragmented extra-cellular particles (pathogenic microorganims) and annihilate the threat. Tissue specific macrophages derive from monocytes and are distributed throughout the body as Kupffer cells in the liver, alveolar macrophages in the lung, microglial cells in the brain, osteoclasts in the bone, histiocytes in conective tissue or mesangial cells in the kidney. Their main role is to recognize and eliminate pathogen through phagocytosis, process that mediates even antigen presentation and to secrete cytokines that orchestrate both the innate and adaptive immune responses (12). The dendritic cell, a specialized relative of the macrophage, presents antigens to lymphoid cells and stimulates adaptive immunity by cytokine secretion. Neutrophils, basophils and eosinophils (named after their affinity to basic or acid dye) are granulocytic cells indispensable in defense from

bacteria (neutrophils) and parasites (eosinophils). Basophils and mast cells are known to play a role in allergic response. The cells described above involved in innate immunity are all part of the myeloid branch of the hematopoietic development, with the exception of dendritic cells (subset derived from both lymphoid and myeloid pathways) and the natural killer (NK) cells (involved in defense against tumor and virus infected cells) developed in the lymphoid lineage.

Adaptive immunity however is ensured mainly by cells of the lymphoid lineage, B lymphocytes (generated in BM) and T lymphocytes (generated in thymus) which play separate roles in humoral and cell-mediated immune response, respectively.

B-cell development stages in BM start with a committed progenitor cell (pre-pro-B cell) continue through pro-B and pre-B cells and end in immature B cells (13). During their development in the BM, early B-lymphocytes undergo a stepwise genetically programmed process of random re-arrangement of the immunoglobulin chains and acquire surface expression of the B-cell receptor (BCR) capable of recognizing high numbers of unique antigen (Ag) epitopes. Through positive and negative selection based on BCR expression B-cells interacting with Ag may become inactivated (anergic) or undergo programmed cell death (apoptosis). Immature B cells that are not inactivated or eliminated may continue to develop into mature naive B cells, leave the BM, and enter peripheral lymphoid organs, where they become immune competent cells. Naïve B cells will bind specifically (clonal selection) an exogenous antigen, internalize and degrade it into peptides, form a peptide/ MHC-II complex and present it to the cell surface membrane. CD4 T helper cells carrying complementary shaped T cell receptor (TCR)/CD4 on the surface will bind to the presented complex and activate the B cell by CD40 interaction, into antibody secreting B cell (plasma cell) and memory cells. Activated B cells will undergo clonal expansion. Initially only IgM is produced, but with the help of CD4 T cells, B cells can further rearrange their Ig genes and switch to production of IgG, IgA or IgE. When the antigen is eliminated the clone undergoes regression, but remnants of the clones (memory cells) will be preserved for long time in the organism, resulting in faster and more efficient activation of the immune response (secondary immune response) at a new encounter with the same pathogen.

T cells on the other hand are generated in the thymus, apparently from migrating early BM progenitors that continuously seed the thymus and commit to T lineage in response to specific microenvironmental cues such as Notch signalling (14, 15). Similar to B cells, T lymphocytes are genetically programmed to produce a T cell receptor (TCR) with unique specificity. During T cell differentiation, naïve double positive CD4CD8 T cells undergo a process of positive and negative selection. Positive selection retains clones that react with MHC self antigen, negative selection eliminates clones that react strongly with MHC self antigen. As a result, successful T cell differentiation selects for MHC restricted TCR with low affinity for self antigens that are capable of recognizing aberrant over self-antigens. Cells that fall outside this range primarily die via programmed cell death (apoptosis). CD8 cytotoxic T cells express TCR capable of binding antigens presented in

combination with MHC-I molecules (recognized by the CD8), while CD4 helper T cells recognize antigens bound to MHC-II molecules (presented by B lymphocytes and antigen presenting cells of the innante immunity). Cytotoxic T cells are primarily involved in destruction of virus infected cells and tumor cells. Helper T cells regulate the activity of B lymphocytes and antigen presenting cells (macrophages). Another subset of T cells, regulatory T cells mediate suppression of the immune response.

It is estimated that the human body has the ability to recognize 10^7 or more different epitopes. In order to recognize this immense number of antigens, the organism generates 10^7 or more distinct clones of B and T-lymphocytes, each with a unique cell surface receptor, ready to bind any antigen the organism might encounter later in life. Clonal expansion as a result of pathogen recognition will not only provide immediate annihilation of the invader, but also confer long lived immune protection to that antigen through memory cells.

Ontogeny of hematopoiesis

During embryogenesis development of HSCs occurs in several sites (16). The first identifiable hematopoietic cells in mice appear at embryonic day 7 (E7) in the blood islets of the extra-embryonic yolk sac (YS) (17). This initial wave of blood cells is mainly comprised of nucleated erythroblasts and macrophages and originates from a common hematopoietic and endothelial precursor – the **hemangioblast** (derived from the mesodermal layer of the yolk sac) (18). The blood islands are thereby surrounded by endothelial cells that contribute to blood vessel formation (vasculogenesis) and connect to the embryo proper around embryonic day E8-E8.5. The hematopoietic precursors enter circulation and the YS disappears as hematopoietic organ by E10.5. Another site of hematopoietic emergence is the intra-embryonic region of the dorsal aorta, called paraaortic splanchnopleura (P-Sp) at E8.5-E10 or aorta-gonad-mesonephros (AGM) at E10-E11.5, as the embryonic organogenesis progresses (19). From the YS and/or AGM hematopoietic cells colonize the fetal liver (FL) (as it becomes the major hematopoietic organ until birth), spleen and thymus (19, 20). HSCs and progenitors expand exponentially in the FL (E12-E14) and towards the end of gestation migrate to the BM (main hematopoietic organ throughout life) (21).

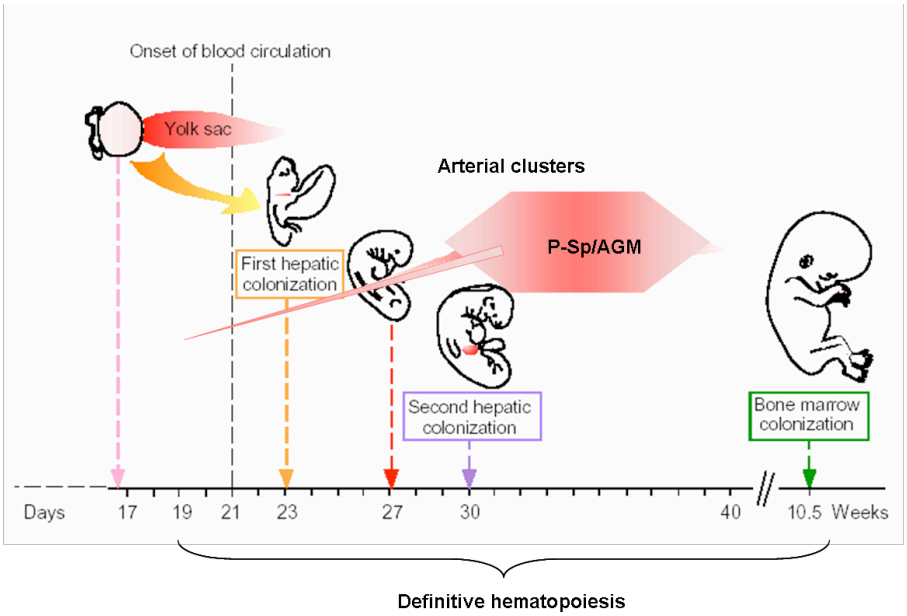

Figure 2. Schematic representation of spatial and temporal appearance of hematopoietic stem cells in the human embryo. (adapted from Tavian et al. 2005)

Whether adult HSC originate in the YS or AGM has been a subject for debate. In a recent study Samokhvalov et al.(22) demonstrate through non invasive *in vivo* lineage tracing an independent activation of the *Runx1* gene (23) (indispensable for definitive hematopoiesis) in the extra-embryonic blood islets at E7.5. These *Runx1* expressing YS cells migrate through umbilical veins into the dorsal aorta (E10.5) and can be later detected in the liver primordium, attesting long term lymphoid and myeloid contribution to the hematopoietic system. Based on this evidence, it was suggested that at least partially extra- and intra-embryonic blood cells share a common precursor in the YS and AGM region, and that the developmental fates of these precursors are determined by different extrinsic signals from extra- and intra-embryonic niches.

Human hematopoietic development **(Figure 2)** follows the conserved scheme of higher vertebrates, with an initial primitive hematopoiesis wave of myelo-eryhtroid blood cell formation in the YS shortly after gastrulation (E19), followed by an independent wave of rapidly expanding definitive $CD34^+$ HSCs generated in the AGM region at E27 and sustained to E40 (24). Interestingly, generation of myelo-lymphoid long term cultures is possible from pre-circulation day 19 P-Sp region, thus supporting intra-embryonic emergence of definitive hematopoiesis. The FL is then seeded by progenitors from the YS at the onset of circulation and from the P-Sp/AGM at E30 and serves as main hematopoietic organ until 20 weeks of gestation when the BM overtakes this function and harbours the HSCs throughout remaining prenatal and postnatal life (25).

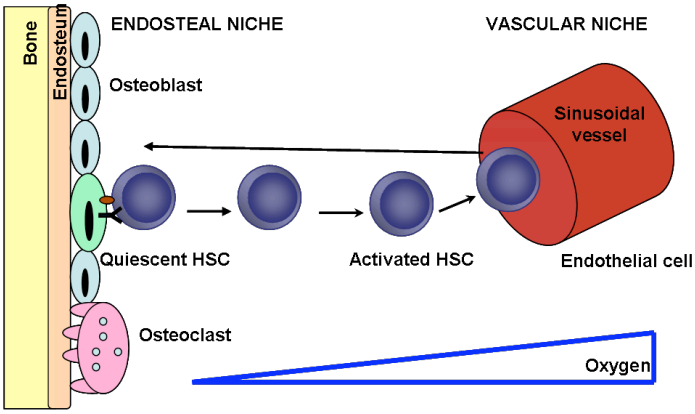

Figure 3. Hematopoietic niche. Quiescent HSC reside in the low oxygenated endosteal niche, under tight regulatory control from environment while activated HSC migrate towards the vascular niche and enter blood stream

Hematopoietic niche

In the late 70's Schofield (26) proposed that HSCs reside within fixed compartments in the hematopoietic organs and are regulated with regard to cell division, proliferation or maintenance by these supporting niches. Recent discoveries in worm and fruit fly invertebrate model systems provided *in vivo* evidence of the role the niche plays in stem cell maintenance, self renewal and asymmetric division of gonadal germ line stem cells (27, 28) but also indicated that the niche is an independent unit functioning even in absence of endogenous stem cells and is able to revert nearby progenitor cells (if not terminally differentiated) (29). These new insights stimulated studies in mammalian systems and specialized niches were soon identified for several stem cell types: skin/hair follicle, intestine, muscle, bone marrow-- to name a few (30, 31).

In mammalians, the bone and BM co-exist in close interaction, as the primitive blood cells need the shelter provided by the bone and the bone depends on the re-modelling contribution of osteoclasts (BM derived cells). Topographically the hematopoietic niche is situated in a poorly oxygenated area of the bone (protected from the oxidative stress of reactive oxygen species), adjacent to the endosteal bone re-modelling surface, rich in ionic calcium, populated by osteoblasts and osteoclasts **(Figure 3)**.Here HSCs attach to spindle-shaped osteoblasts through the N-cadherin/β-catenin complex (32). The increase in osteoblast numbers through either conditional inactivation of the bone morphogenic protein (BMP) receptor type IA (32) or through overexpression of the parathyroid hormone (PTH) and PTH- related protein receptor (33), results in linear increase in long term HSC numbers. Conversely, it was demonstrated that ablation of osteoblasts results in reduction of lymphoid, myeloid and erythroid progenitors as well as absolute HSC numbers (34). A number of signalling molecules are known to contribute to the niche functions. For example angiopoietin-1 is expressed in osteoblasts while Tie2 (tyrosine kinase recptor) is expressed in HSCs and endothelial cells. Angiopoietin promotes HSC quiescence and enables their adhesion to osteoblastic cells though Tie2

(35). Other signalling pathways implicated in maintenance and functions of the HSCs are SCF/c-kit, Jagged/Notch, Ca^{++} sensing receptor (present on the HSCs), various chemokines and integrins (36).

Because hematopoiesis continues even in absence of osteoblasts and can be maintained in hematopoietic tissues outside the BM it was proposed that another entity might support HSCs (34, 37). Evidence of the fact was recently provided by Kiel et al.(38) who showed that HSCs identified by the signalling lymphocyte activation molecule (SLAM) receptors family reside both at/near the endosteal surface of the bone and adjacent to the endothelium of the sinusoidal blood vessels within the BM and spleen. The function of the vascular niche however remains to be elucidated, as to whether it represents a transit station for circulating HSCs or it has specific regulatory functions (39). Recently, Scadden et al.(40) demonstrated through *in vivo* imaging of dye-labelled purified HSCs that cells of differing immunophenotype localize differently, with highest degree of stem cell enrichment in closest proximity to endosteum. This interesting data comes in support of the hypothesis proposed by Kopp et al. (41), in which the osteoblastic niche maintains the long term HSCs pool by providing a quiescent microenvironment, while the vascular niche acts as an intermediate step in HSCs homing (during ontogeny or different stress situations) and promotes proliferation and further differentiation **(Figure 3).**

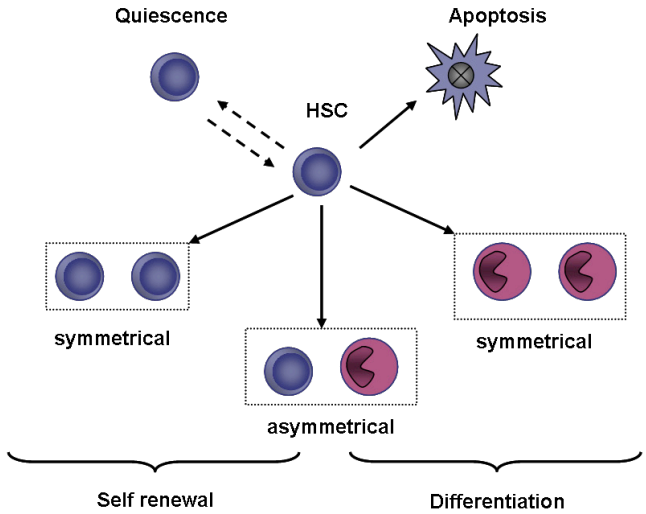

Figure 4. HSC potential fate decisions are determined by extrinsic and/or intrinsic regulatory factors.

Regulation of hematopoiesis

In response to physiological cues from the microenvironment, HSCs can undergo several fates during the lifetime of an individual: self renewal, differentiation and migration, quiescence and cell death (apoptosis) **(Figure 4)**. In fetal life there is a dramatic expansion of HSC numbers between days 13 and 16 of gestation (42). During adult life, in response to various types of stress (bleeding, infections) HSCs are rapidly activated to meet the increased demand of specialized blood cells production. In haematological malignancies, as a consequence of chemotherapy and bone marrow transplantation, HSCs expand and differentiate (43).

Self renewal is probably the most essential process of the HSCs as it is both required and sufficient to sustain hematopoiesis for life (44). As stem cells self-divide they can either generate an identical copy and a cell destined to an alternative fate (**asymmetrical** cell division), or two identical cells (**symmetrical** cell division) (**Figure 4**). Perpetuated symmetrical division will result in expansion of the stem cell pool (if the cells are identical to the mother cell) or depletion of HSCs (if the progeny has lost stem cell properties) (45, 46). Thus, by regulating the balance of symmetrical and asymmetrical divisions through intrinsic and/or extrinsic factors, the number of HSCs is kept at a constant level (steady state) or expanded (on demand-- in natural physiological needs or pathologically through aberrant signals in cancer)(47, 48).

Diferentiation is defined as the sequence of events through which primitive stem cells gradually loose their self renewal ability while undergoing a stepwise commitment process generating more mature and lineage restricted progenitors (49). Whether HSC fate decision is regulated through random events (stochastic model)(50) or deterministic

events is an ongoing debate (51). Further, fate decision can be mediated through extrinsic factors (outside the cell, such as cytokines, microenvironment of the BM niche) or intrinsic factors (pre-programmed in the cell genome) (52). These factors can in their turn can be indispensable (in which case their action translates as instructive) (53) or have a function that in their absence can be mediated by other players (in which case they are considered permissive)(54). The classical hematopoietic hierarchy describes a pluripotent long-term HSC, **LT-HSC** (a single cell can sustain life long hematopoiesis) followed by short term HSCs, **ST-HSC** (multipotent, but limited self renewal capacity) and downstream lymphoid or myeloid restricted progenitors (9) **(Figure 1)**. Support for this model comes from identification of two distinct subsets of hematopoietic progenitors, the common lymphoid progenitor (**CLP**)(55) and the common myeloid progenitor (**CMP**) (56), lacking myeloid and lymphoid lineage potentials, respectively. Similar populations have been identified in the human system, with a CMP described by Manz et al. (57) and a CLP identified in adult bone marrow (58) and cord blood (59). The CLP differentiates into cells of the lymphoid system- T, B and NK cells, while the CMP generates myeloid precursors of the megakaryocyte/erythroid, granulocyte and monocyte/macrophage lineages **(Figure 1).** These precursors have restricted lineage potential and no measurable self renewal capacity. Although this classical model of lineage commitment is widely recognized, there are alternative models proposed as well, for example the sequential model proposed by Singh (60) suggesting that commitment to a certain lineage could be a process of stepwise loss of lineage potentials in a multipotent HSC, process mediated primarily by a hierarchy of transcription factors. In Article I of the present thesis, we propose an alternative pathway to hematopoietic commitment based on the isolation of a novel lymphoid primed multipotent progenitor (**LMPP**) population, lacking significant myeloerythroid potential, but sustaining all other blood lineage developmental potentials (61, 62). New steps have been recently identified in the early myeloid commitment pathway as well, based on clear genetic and phenotypic delineation between the different progenitor subsets (63).

Maintaining **quiescence** is another highly important characteristic of the HSCs. Normal cell division cycle consists of distinct phases: cellular preparation of chromosomes for replication (G_1 phase), synthesis of DNA until all the chromosomes have been replicated (S phase), further growth and preparation for cell division (G_2 phase) and finally mitosis (M phase) which is the actual division into two daughter cells (64). Under phase transitions the cell cycle has several control stations (checkpoints) comprised of regulatory molecules cyclins that form complexes with cyclin dependant kinases (CDK) (65). This regulatory system is designed to monitor and prevent cell cycle progress if errors are detected. Thus, in defective cell cycle phases cyklin-CDK complexes will stop cell division until the DNA damage is repaired or the cell destroyed through programmed cell death (**apoptosis**). Errors escaping the control system may cause genetic mutations that lead to cancer (66). Following cell division, some cells exit the cell cycle into a resting G_0 phase. Cells in G_0 are either terminally differentiated (i.e. neuron), senescent or quiescent primitive cells that can re-enter G_1 and start cell division (67). HSCs are quiescent cells shown to cycle very slowly, only 8% leaving the G_0 phase on a daily basis. Nonetheless, within 60 days almost all HSCs had divided at least once (68). The quiescence state is

believed to serve two purposes: prevent exhaustion of HSCs by maintaining steady state hematopoiesis and protect HSCs from DNA alterations (and thus cancerous transformation) more likely to occur in an activated state (69).

Although the mechanisms are not completely elucidated, several regulatory pathways have been identified and the dominating current understanding is that HSCs fate decisions are a series of sequential molecular events governed by the integrated effects of intrinsic transcription factors and external signalling pathways initiated by regulatory cytokines (reviewed in (52)).

Plasticity

Based on their developmental potential, stem cells have been classified into totipotent (give rise to extra and intraembryonic cell types—i.e. the fertilized egg), pluripotent (give rise to all cell types of the organism—apply to embryonic cell lines), multipotent (give rise a subset of cell lineages resulting in a tissue or organ—such as HSCs), oligopotent (progenitors with restricted lineage potential in report to the multipotent stem cells) and unipotent (through maturation and division contribute to only one mature cell type) (70). Multipotent stem cells reside in a variety of tissues derived from all embryonal germ layers and are regarded as tissue specific, generating mature cell types corresponding to the tissue of origin. Most well characterized to date remains the HSC (44, 71), although mesenchymal stem cells (MSC) have also been prospectively isolated from adult BM (72). In addition stem cells in the nervous system, intestine, liver, pancreas, skeletal and cardiac muscle have been described to variable levels (73). With the exception of a few constantly renewing tissues (i.e. blood, skin, gut), adult organs exhibit limited turnover under steady state conditions. Nevertheless, in experimental injury/repair studies regenerative response is readily obtained for instance in liver and muscle tissue. Whether these regenerative responses are solely a result of endogenous tissue specific stem cell proliferation or whether cells of other tissues contribute to repair has been debated in recent years. Reports of BM contribution to non-hematopoietic tissues and of non-hematopoietic tissues generating blood cells raised the question of stem cell plasticity-- flexible lineage determination, conferring stem cells the ability to switch fate in relation to microenvironmental cues (74, 75)**(Figure 5)**. From the point of view of regenerative medicine this was very exciting news as it could potentially translate into new sources of stem cells for tissue repair and gene therapy. Given the fact that the mechanisms involved in stem cell plasticity have not entirely been identified, several explanations are possible.

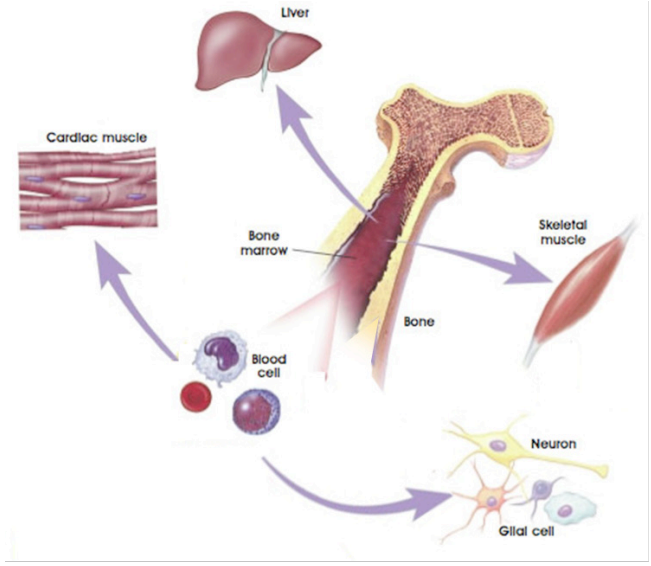

Figure 5. Proposed plasticity potential of HSCs. (adapted from Winslow et al., NIH 2001)

Trans-differentiation is one proposed mechanism by which stem cells could contribute to cell types of other lineages. According to this hypothesis the lineage switching would result either directly through activation of an alternative (otherwise dormant) differentiation program or through de-differentiation (shutting down lineage specification and returning to an earlier stem cell like stage) and re-differentiation (activation of a new lineage pathway). The reports supporting this mechanism are based on transplantations of more or less purified populations of bone marrow derived cells into lethally irradiated or injured mice, with tracing of donor derived cells in host tissues based on Y chromosome, viral vectors or transgenic markers (green fluorescence protein; GFP or β-galactosidase; lacZ). However recipient animals were lethally irradiated to obtain higher levels of donor blood reconstitution. In such experiments BM cells were claimed to contribute to liver parenchyma (76, 77), myocardium (78, 79) and skeletal muscle (80, 81), pancreas (82), intestinal epithelium (77) and central nervous system (83). Other reports suggested hematopoietic potential in non-hematopoietic neural and muscle tissues (84, 85). Although frequencies of blood derived cells in non hematopoietic tissues were highly variable (0.1-20 %) and tissue specific functions were not clearly demonstrated, the therapeutic promise drove premature clinical trials of BMT to non-hematological injuries (myocardic infarction) through injury site injections or HSC mobilization (86, 87). The clinical outcome however was below expectations. In addition, requirements for more stringent evidence of trans-differentiation (73, 88) and failure to reproduce original data in follow up studies (89-93) questioned the reliability of earlier reports.

Another explanation proposed for lineage conversion resides in heterogeneity of the transplanted population with possible multiple stem cells of different potentials or single rare pluripotent cells (73).

Although evidence of plasticity exists from some invertebrate and vertebrate model systems with extensive regenerative potential (the salamander forms new limbs after injury) (94), it is unclear whether this is a result of dedifferentiation of mature cells in close proximity to the wound or a result of activation of a pre-existing multipotent adult stem cell. Nonetheless, the amphibian is the only vertebrate exerting such capabilities, while in the remaining mammals embryonic developmental tissue specification appears irreversible. Considering the rare events of plasticity detected throughout tissues, strict criteria should be met in order to define genuine trans-differentiation: prospective isolation and transplantation of donor cells (preferably at clonal level) without culture dish manipulation; tracing of progeny in vivo and robust tissue engraftment; clear donor-specific and tissue-specific marking, with evidence of the donor cell integrated in the target tissue, acquiring tissue specific function; evidence of genetic silencing of original tissue programme and activation of target tissue gene profile; elimination of cell-to-cell fusion as possible mechanism. To date no reports have convincingly documented such trans-differentiation events, although claims of adult tissue stem cell plasticity continue to emerge (95, 96).

Finally, an explanation that has gained compelling evidence is that contribution of donor cells to other tissues is due to cell-to-cell fusion or **heterotypic cell fusion**. Cell fusion is the process by which two cells merge forming a hybrid cell with two nuclei. In practice, hybridoma cell lines (used to produce antibodies) are a result of cell fusion between myeloma cells and lymphocytes (97). In nature, cell fusion occurs during development as in zygote formation (98), skeletal muscle cell formation (99), osteoclast formation (100), as well as in disease—immune response (101) and tumorigenesis (73).

Recently, heterotypic cell fusion (fusion of two unrelated -heterotypic- cell types) has been reported during tissue regeneration *in vivo*. Blood cells have been shown to fuse with cardiomyocytes (93, 102), Purkinje neurons (102-104), hepatocytes (102, 105, 106) and skeletal muscle fibers (107) **(Figure 5)**. As a result, genetic and phenotypic conversion of the hematopoietic cells was documented with functional integration in the target tissue (105, 106). The identity of BM derived fusogenic cells is also unclear, although myeloid and macrophage lineage cells have been suggested as possible candidates (108, 109). Whether heterotypic cell fusion occurs as a random event or is a robust regenerative mechanism for adult tissue is still largely unknown. Clinical relevance of stem cell plasticity remains to be promoted subsequent to rigorous verification of regenerative potential in appropriate animal models. In Article III we addressed in part some of these questions (discussed separately).

CLINICAL APPLICATIONS OF HEMATOPOIETIC STEM CELLS

Bone marrow transplantation

Historically, basic knowledge of BMT curative potential emerged in mid last century with evidence of hematopoietic rescue of lethally irradiated mice transplanted with cells from same strain donors (110). Parallel with careful further laboratory investigations (7), BMT in leukaemic patients was attempted and the first successful isologous BMT between identical twins was reported 1959 (111), followed by HLA-matched allogeneic BMT report in 1970 (112). This pioneering work was performed by Dr E Donnall Thomas, who was awarded 1990 Nobel Prize in Medicine for his "discoveries concerning organ and cell transplantation in the treatment of human disease". His and others ground breaking work brought the field forward over the decades and presently BMT is a rather common and often life saving therapeutical approach for a wide variety of hematopoietic malignancies and non-malignant life threatening conditions (immune deficiencies, hemoglobinopathies or inborn errors of metabolism).

An inherent risk following allogeneic BMT (apart from cytopenia and risk of infections) to myeloablated individuals is development of **graft versus host disease (GVHD)**—that we know today is caused primarily by HLA-mismatching and reaction of donor derived allogeneic T lymphocytes against host cells (113). Due to the polymorphism of HLA only 30% of all patients will find suitable related donors (siblings that match somewhat satisfactory the host HLA system) and the large majority of 70% depend on allogeneic BMT. While one aspect of the reaction, namely graft versus leukaemia is a desired outcome attempts have been made to minimize the morbidity of the host. Transplantation of purified $CD34^+$ HSCs alleviates to some degree GVHD, however generation of sufficient mature cell types is relatively slow to and leaves the recipient in a large window of leukopenia with subsequent increased risk of infections (114). An alternative source is transplantation of enriched stem cells from peripheral blood (PB), shown to render faster myeloid recovery, probably due to a higher activation status after cytokine mobilization (115). Another possibility is collection of HSCs from unrelated FL and CB sources, indicated as more efficient in engraftment (116, 117) and less alloreactive due to their primitive nature (118). The disadvantage however lies in the low numbers of purified cells/ sample, usually insufficient for adult transplantations (119). This limitation could potentially be overcome by development of efficient *ex vivo* human HSC expansion assays (120). Other alternatives are co-transplantation of hematopoietic progenitors that at least in murine models rapidly generate mature blood cells and rescue immune competence in the host (121). The human identity of such progenitors however is not clearly defined. A final approach that has gained more recognition in later years is co-transplan-

tation with MSCs –immuno-modulatory players that efficiently suppress alloreactive T cells independent of HLA-matching (122-124).

Gene therapy

Permanent introduction of new genes into the genome of stem cells in general and HSCs in particular provides potential for therapeutic success in a vast number of monogenic hereditary diseases. In addition, gene therapy has curative potential in leukemias and solid tumor cancers if delivery of the transgene is achieved in the target cancer (stem) cells (125). The transfer vehicle for gene therapy usually is a viral vector and delivery of information to target cells can be done either *ex vivo* (best documented) or *in vivo* (direct delivery to target tissue). The advantage gene therapy holds over traditional BMT is that gene transfer can be done to patient's own cells, circumventing the search for a HLA matched donor. In addition, minimal myeloablation (if any) is sufficient for engraftment of genetically modified cells, thus avoiding the morbidity associated with cytopenia in classical BMT conditioning. Retroviral vectors are well characterized vehicles, used in the beggining for poof of principle gene marking, multiple drug resistance (MDR) targeting and most importantly gene therapy of a number of monogenic disorders (126, 127). However, the reported experimental success of retroviral gene transfer had little clinical benefit, as levels of transduction were disappointingly low for therapeutical purpose (128). In addition, for stable integration retroviral vectors depend on cycling cells, condition that is difficult to provide with simultaneous maintenance of stem cell properties in target human cells. An exception is represented by immune deficiency disorders (X-SCID and ADA) in which a selective advantage for transduced cells was observed and high levels of genetically corrected lymphocytes and long term immune reconstitution was achieved (129-131). Although in majority successful, three patients in the X-SCID trial developed T-cell leukemia at variable times after gene therapy, most likely due to vector integration in the LMO2 gene locus (132). The newly assessed risk of insertional mutagenesis prompted further evaluation of initial criteria before extensive and safe gene therapy can be applied routinely in clinical trials. Some of these criteria are high transduction efficiency albeit with limited copies per cell, precise integration, tropism to target tissue, reduced mutagenesis risk, selective engraftment of transduced cells. Efforts are underway to develop alternative viral vectors and address some of these issues (125).

In utero transplantation

Many congenital immunedeficiencies, haemoglobinopathies and some metabolic storage diseases can be treated by BMT. Others require life long treatment by blood transfusion or replacement therapy, while in some no definitive treatment has been identified. To avoid conditioning related morbidity and mortality of traditional BMT and irreversible disease related organ damage, a novel approach was proposed, namely the in utero hematopoietic cell transplantation (IUHCT). The rationale for IUHCT stands on several presumed advantages: the fetus undergoing normal development is immunologically tolerant and will accept cells of foreign antigen if presented at a certain gestational age; there is available space for donor cells, as the host own haematological compartment is expanding and new bone marrow of niches are created; the fetal size is relatively small such that, in comparison, larger doses of cells can be delivered than postnatally; disease related organ damage can be prevented if successful stable engraftment is achieved prenatally (1, 2). However, despite these unique opportunities offered by the fetal microenvironment, little therapeutic success was achieved in the clinic. To date, nearly 50 cases of IUHCT in human have been reported, with recipients diagnosed of immune deficiencies (bare lymphocyte syndrome, X-SCID and other forms of SCID, Ommen syndrome, chronic granulomatous disease (CGD), Chediak Higashi disease), red blood cells disorders (alpha and beta- thalassemia, sickle cell disease), metabolic storage diseases (globoid cell leukodystrophy, Hurler's syndrome, Niemann-Pick, metachromatic leukodystrophy) and osteogenesis imperfecta. Variable cell sources were used (unrelated fetal liver and bone marrow $CD34^+$, parental or sibling bone marrow, fetal blood, fetal or adult mesenchymal stem cells) and transplants were performed at different gestational ages (ranging week 13 to 37 of gestation), in some cases with repetitive transfers of donor cells(133).The results were highly disappointing, as chimerism was absent or too limited to have a therapeutic relevance. In one case fetal demise was caused by prenatal GVHD development.

Exceptionally though, several immune deficiency disorders were successfully treated by IUHCT. The first case of intrauterine transplantation was reported by Touraine et al. (134) in 1989 and it addressed a human fetus affected by bare lymphocyte syndrome (BLS). Three reports on X-SCID (3-5) and four cases of B cell deficient SCID (135) followed thereafter. All of these fetuses had survived to birth, were chimeric and had complete or split chimerism with donor derived lymphoid cells and a functioning immune system.

The poor outcome of the non-immune disorders argue for reconsideration of IUHCT initial advantages and identification of novel strategies to overcome the immune barrier, create space and competitive advantage for donor cells and augment their potential for proliferation and differentiation (6). Given the previous reports, fewer attempts to IUHCT have been made and many physicians were discouraged. However, proof of principle and effective correction has been achieved in immune deficiency disorders, apparently equally effective to postnatal transplantation, although no direct comparative studies of prenatal versus postnatal transplantation have been done (136). Clearly lessons

are to be learned from historical experience as well as experimental data and collaborative efforts should be made to identify optimal target diseases, cell sources and populations, gestational age and procedure protocol for IUHCT.

AIMS OF PRESENT STUDIES

- To identify and characterize at cellular and molecular level the earliest flt3 dependant commitment/differentiation step in adult murine haematopoietic hierarchy (Article I)
- To evaluate the ability of early lymphoid restricted multipotent progenitor to regenerate the immune system in X-SCID disease model at different ontogeny stages (Article II)
- To evaluate the ability of bone marrow cells to contribute to non-hematopoietic tissue regeneration after injury, in steady state and during normal development (Article III)
- To identify the cell lineage specification of bone marrow derived fusogenic cells (Article III)

METHODS FOR IDENTIFICATION AND CHARACTERIZATION OF HSC

HSCs are a population of highly infrequent and morphologically indistinguishable cells, interspersed in the BM among large numbers of differentiated blood cells at various stages of maturation. In order to identify, isolate, purify and evaluate these rare cells a number of methods have been developed throughout the years. One of the early strategies for enriching primitive hematopoietic cells is based on treatment with cytotoxic drugs such as 5- fluorouracil (5-FU) or hydroxyurea that kill actively dividing progenitors and mature cells but preserve quiescent HSCs in the BM (137, 138). Other approaches to isolate candidate HSCs utilized their functional properties, such as difference in metabolic state (139, 140), nucleic acid content (141, 142) and cell size or density of cell populations (143, 144).

The development of flow cytometry, **fluorescence-activated cell sorting (FACS)** and wide availability of fluorochrome-conjugated monoclonal antibodies against cell surface (or intracellular) markers account however for the breakthrough in isolation and identification of HSCs (71, 145, 146). In the **mouse adult bone marrow LT-HSCs** have been shown to lack surface antigens normally expressed by mature blood cell lineages (lineage negative, Lin^-) but express stem cell antigen-1 (Sca-1)(147) and low levels of Thy-1(71). Based on expression of the cytokine tyrosine kinase receptor (c-kit) the population was further enriched for stem cell activity (148). Later Osawa et al. (44) separated **$Lin^-Sca\text{-}1^+Kit^+$ (LSK)** population based on expression of cell surface adhesion molecule CD34 (149) and showed that the **$CD34^{low/-}$** single cells were capable of reconstituting the entire hematopoietic system of lethally myeloablated mice thus identifying LT-HSCs. Other markers as FLT3 (61, 62, 150), IL-7R (55) and CD150 (Slamf1)(38) were also used to subdivide hematopoietic stem cells and early progenitors.

The **human definitive HSCs** however are less defined with regard to their phenotype. Several cell surface antigens have been proposed as phenotypical markers of more or less homogenous human HSC populations (CD34, CD38, Thy1, Rho123, CD90, AC133) (151-153). Similar to the murine counter part, they lack cell surface expression of mature lineage markers and express low levels of Thy-1 (152), but unlike quiescent mouse HSCs, human HSCs express CD34 (154, 155). The cell surface antigen CD38 (156, 157) is another marker used, mainly in experimental settings, for prospective isolation of **$Lin^-CD34^+CD38^-$** candidate human HSCs which seem to retain most of the long term repopulating capacity (151). Positive selection and enrichment based on CD34 expression is routinely applied in clinical practice prior to patient BMT (158, 159).

Although an otherwise highly useful technology, one disadvantage of flow cytometry analysis/sorting is the requirement of a homogenous suspension of single cells, thereby

causing loss of information about the spatial relationship between different cells and disturbance of tissue architecture. Another consideration is that surface markers detected by flow cytometry may fluctuate depending on activation state of the cell (160, 161) or ontogeny stage (162-164). Therefore, a crucial step following the prospective isolation of a candidate stem cell population is to evaluate its cardinal properties of multipotentiality and self renewal, preferably at a single cell (clonal) level.

In vitro assays serve primarily for evaluation of multipotentiality, by observing the lineage potential, differentiation and proliferation capacity of single or limiting numbers of cells in presence of a variety of growth factors and culture media (120, 165). The outcome of these assays is measured in mature blood cells, and although they give qualitative information of the lineage potential and proliferation capacity in the test cells, they are limited by separate requirements for growth into different progeny. The simultaneous read-out of several potentials from the same cell remains problematic. Short time liquid or semisolid cultures with addition of exogenous growth factors can efficiently read out erythroid (E), granulocyte/macrophage (GM), megakaryocyte (Mk) and to some extent B lymphocyte potential. However, T lymphocyte potential has until recently been difficult to assess in absence of thymic stromal support (166). In addition, evaluation of primitive HSCs requires a long term culture system that allows read-out beyond the life time of co-cultured short-lived precursors.

An optimized alternative is co-culture on immortalized stromal cell layers, which provide through cell-to-cell contact and spontaneous or genetically engineered growth factor release a favourable microenvironment for the test cells. In practice, two types of long term in vitro culture for both human and mouse cells have gained recognition, namely the long-term culture-initiating cell (LTC-IC) (167) and the cobble stone area forming cell assay (CAFC) (168).

Another example of short term co-culture system that allows optimal readout of B and T cell potential is the OP9 and OP9 DL1 (Delta-1 Notch ligand) assay. The OP9 stromal cell line known earlier to support myeloid and B cell differentiation (169) was genetically engineered by Zuniga Pflucker et al. (170) to ectopically express the Notch ligand Delta like-1(171) and thereby enable T cell commitment and differentiation from co-cultured test cells. This type of cultures were employed in Article I and later modified into a switch-culture system that enables simultaneous readout of myeloid, B lymphoid and T lymphoid potentials from fetal liver candidate HSCs at clonal level (164).

In vivo transplantation models enable observation of transplanted cells and their progeny for an extended amount of time, thus giving information of their self renewal and long-term reconstitution capacities. Candidate murine HSCs and/or progenitors are most commonly evaluated through congenic transplantations, in which mouse strains separated by two isoforms (CD45.1 and CD45.2) of the CD45 pan-hematopoietic cell surface marker are used as donor, respectively host **(Figure 6).** Test cells can thus be transplanted in bulk or as single cells, through intravenous or intrafemoral injection, at variable numbers, into totally or partially myeloablated hosts, with or without radio-

protective competitive cell population. Qualitative contribution of donor cells towards different lineages can be repeatedly evaluated by **flowcytometry** of antibody stained PB and BM cells, usually up to 4 months. (Detection of donor cells at 16 weeks after transplantation is regarded as long term engraftment.) Self renewing capacity can be evaluated through serial transplantation—transfer of donor cells from primary recipient into secondary and tertiary myeloablated hosts, process that pushes HSCs in the test population to self renewing divisions in order to rescue hematopoiesis in the new host. Contribution of donor HSCs to non-hematopoietic tissues can also be assessed through immunohystochemical analysis.

Murine **in utero transplantation** is designed as a non-myeloablative approach to achieve hematopoietic chimerism and donor specific tolerance. The model has been employed in several studies of congenic and allogeneic transplantations (172). The technique implies laparotomy of anesthetized gestant females at embryonic day E14-16 and direct visualization of the fetal recipients through the uterine wall. Injections can be performed intraperitoneal (i.p.) (**Figure 6**), intrahepatic (i.h.), intravenous (i.v.) though the vitteline vein, intramuscular (i.m.) or intracranial, depending on study design and aims. Following injections, laparotomy wound is closed and gestant females are observed to term. Live born animals are analyzed thereafter for levels and types of donor cell engraftment, similarly to adult recipients (**Figure 6**). As the fetal transplantation procedure is often complicated by high mortality rates, the alternative **neonatal transplantation** approach has been undertaken in murine and rat animal models. Neonatal recipients are however more immunologically competent than the fetal recipients and conditioning is required for high levels of donor cell engraftment. Neonatal recipients can also be transplanted with hematopoietic cells i.v. (through the facial vein) or i.p., and analyzed at various time points thereafter.

In vivo evaluation of **human HSCs** has however obvious limitations, therefore xenogeneic (across species) transplantation models are needed. Through screening of several immune-deficient mouse strains Dick et al.(173) found that **non-obese diabetic (NOD) severe combined immune deficient (SCID)** mice yield highest levels of human engraftment. The NOD/SCID mice have apart from B and T cell deficiency a natural reduction of NK cells that enables higher tolerance to human xenograft donor cells and have quickly become the most used assay for investigation of human candidate HSCs. Marginal improvement of human engraftment was obtained through either genetic manipulation of the strain (beta 2 microglobulin NOD/SCID (174, 175) and NOD/SCID gamma chain deficient (176)), blocking antibody administration (177) or exogenous human cytokine treatment (178). In addition, the NOD trait apparently plays an important role, as strains with considerable higher immune deficiency features (i.e. rag 2 common gamma-chain deficient) are less permissive of human engraftment at adult age (179, 180).

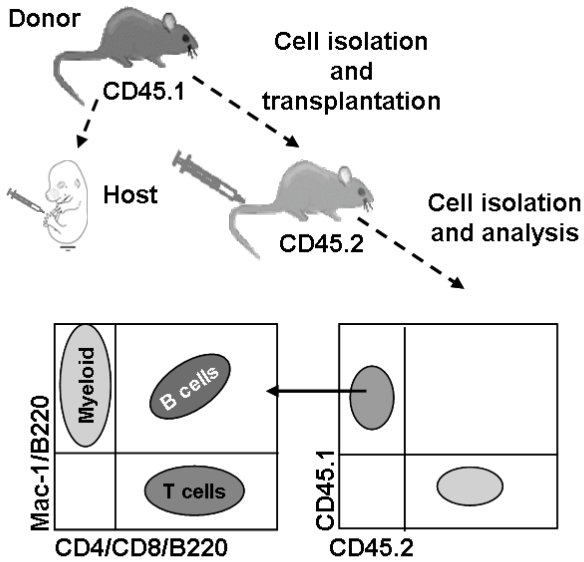

Figure 6. Schematic representation of congenic transplantation

Although a highly useful tool, the NOD/SCID model has certain limitations. To start, candidate human HSCs successfully engrafting and differentiating in the murine host (scid repopulating cells; SRC) are perhaps artificially selected exactly for their capacity to home, engraft and develop in a xenogeneic environment, thus maybe different to the human system (181). In contrast to murine counterpart, where single donor HSCs regenerate entire hematopoiesis, human HSCs have to be transplanted in high numbers (2x 10^4/ recipient) in order to detect donor derived contribution to host hematopoiesis (176). Further, the levels of human engraftment in mice transplanted with equal doses of test cells vary considerably and large numbers of animals are needed in order to reach reliable conclusions regarding homing and engraftment of investigated population. Another issue is the short life-time of the NOD/SCID mice that tend to develop thymic lymphomas and thus debilitate long term observation (182). Serial transplantation, although not reflecting steady state hematopoiesis, is a possibility to extend the evaluation of test cells. Finally, T cell development and terminal erythroid lineage differentiation are impaired in this mouse model, that is biased towards myeloid and B cell lineages (183).

Large animal models represent an alternative human xenogeneic transplantation assay. The advantages of using large animals are longer lifespan, possibility of repetitive sampling with collection of larger volumes of PB or BM aspirates and extended lineage differentiation. The drawbacks are however the high cost, resource demand and difficulties (practical and/or ethical) in setting up the models. Non-human primates are a practical model used by a few investigators for evaluation of both non manipulated and genetically engineered HSCs. (184) Xenograft human to preimmune dog fetus transplantation has also been reported (185), and another well known model is the **preimmune fetal sheep** transplantation assay, discussed in more detail in another section of this book.

SUMMARY AND DISCUSSION OF ARTICLES

Article I

Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment

Lineage commitment process in adult hematopoiesis from pluripotent long term repopulating HSCs to lineage restricted progenitors has been recently reviewed (153, 186). Several models for developmental pathways of stem cells have been proposed over the time, of which the classical view of strict separation of myelopoiesis and lymphopoiesis gained most recognition (9) (**Figure 1**). Presented below are the results of our experiments and interpretation of data in view of our and others recent findings.

In previous work from our laboratory, HSCs of LSK phenotype in adult mouse BM have been divided based on differential cell surface expression of CD34 (44) and fms like tyrosine kinase receptor Flt3/Flk2(61, 150) into long term repopulating HSC (LT-HSC; LSK $CD34^{-}FLT3^{-}$), short term repopulating HSC (ST-HSC; LSK $CD34^{+}FLT3^{+}$) and multipotent progenitors (MPPs; LSK $CD34^{+}FLT3^{+}$) (187). Through *in vivo* transplantation to lethally irradiated mice clear delineation of the radioprotective ability of these populations was demonstrated, as LT HSCs sustained hematopoiesis for the lifetime of the individual (self renewing), ST HSCs generated rapid myeloid and lymphoid reconstitution but were unable to sustain it for long term, while MPPs showed striking differences in lineage distribution with rapid and dominant lymphoid reconstitution, reduced myeloid reconstitution and lack of radioprotection of the host. Megakaryocyte erythroid (MkE) potential of the subpopulations was not evaluated at the time.

In Article I, we now identified the lymphoid primed multipotent progenitor (LMPP) as the highest 25% Flt3 expressing fraction of LSK $CD34^{+}$ cells, with prominent lympho-myeloid potential and little or no MkE potential. Several lines of evidence were employed to demonstrate the lineage restriction step from HSC to LMPP as suggested by loss of MkE potential. *In vitro* and *in vivo* data were supported by Q-PCR that showed downregulation of essential MkE associated genes (*Epor, Tpor and Gata1*) and upregulation of *IL7r* lymphoid gene, co-expressed with *GCSFr* myeloid gene as indicated by multiplex single cell RT-PCR. Whether the missing MkE lineage is derived directly from HSCs (expressing multiple MkE and myeloid genes) or whether a separate track exists for CMPs directly from HSCs (as suggested by the classical hierarchy) remains unclear.

Inversely to our data, Forsberg et al. (188) showed through *in vivo* transplantation of high numbers (500) of LSK $FLT3^{hi}$ cells contribution to MkE lineage. The authors brought

a relevant point with regard to interpretation of data in absence of lineage potentials, as possible limitation of the assays applied (given the fact that different conditions are required for different lineages) could be misleading. While Forsberg et al. show platelet and erythroid production from 500-1000 LSK FLT3^{hi} cells *in vivo*, we would argue that the data stands in agreement with our finding of 2-3% of LMPPs having residual MkE potential *in vitro* as well as *in vivo* (62). In further support of our data come recent studies from Yoshida et al. (189) and Lai and Kondo (190) who identify through different methods a similar restriction step in HSC commitment with generation of a granulocyte macrophage lymphoid progenitor (GMLP) that lacks MkE potential. Importantly, in a following study from our group, Luc et al.(191) show through separation of the LSK-FLT3^{hi} (LMPP) population based on cell surface expression of MPL (receptor for thrombopoietin), an important regulator of HSCs and Mk development, that the already low MkE potential of LMPPs is confined almost exclusively to the LSKFLT3^{hi} MPL^{hi} fraction of the LMPP, thus arguing further for the gradual downregulation of the MkE lineage programme and potential and concomitant gradual increase in lymphoid lineage priming. In addition, over 98% LSKFLT3^{hi} MPL^{hi} cells and all LSKFLT3^{hi} MPL^{-} cells lacked detectable MkE potential, supporting a GM/lymphoid commitment step and sustaining the presence of the functionally defined LMPP(191, 192).

The analysis of lineage commitment steps from HSCs to different lineage precursors is depending on prospective isolation based on phenotypical surface markers. However these methods are limited and sorted populations are highly heterogenous, as some surface antigens do not reflect lineage fate decision. It is desirable that HSCs and progenitor populations are classified based on transcriptional factor reporters that accurately reflect the lineage commitment a cell has made. Such analysis methods have recently emerged, and show distinct roles for GATA-1 and PU.1 transcription factors, that act instructively on directing HSCs towards the MkE and myeloid pathway respectively (193). In addition, Arinobu et al. (193) show not only GM potential in the PU.1+ population, but also robust lymphoid lineage potential and upregulation of *IL7r* to a subsequent CLP step, data in further support of a GMLP/LMPP commitment step.

Article II

Polyclonal T-cell reconstitution of X-SCID recipients after in utero transplantation of lymphoid-primed multipotent progenitors

X-linked severe combined immune deficiency (X-SCID) (194) represents half of the yearly diagnosed SCID (195, 196) and is manifested by profound lack of B and T cell function, although by number, B cells may be normal or elevated. Children with X-SCID are highly receptive to common and opportunistic pathogens and present with persistent, recurrent, severe infections. X-SCID is lethal unless treated by BMT. HLA-identical postnatal BMT has overall been very successful, although it is associated with significant morbidity and mortality (195). Further, only a minority of SCID patients have an available HLA-identical donor at birth. For these patients, it was considered a breakthrough when successful correction of the common gamma chain deficiency and T cell deficiency was achieved by retroviral mediated gene therapy (129). However, as some patients later developed T cell leukemia due to insertional mutagenesis (132, 197), gene therapy of X-SCID is currently approached with caution (198-200), and there is still need for development of alternative therapies.

IUHCT has been successfully provided for a limited number of X-SCID patients. Hematopoietic stem-progenitor cells enriched for CD34 antigen from haplo-identical BM cells or unrelated fetal liver cells collected early in gestation were transplanted in single or repetitive doses between 14 and 22 weeks of gestation. As a result, long-term donor-derived engraftment of T and NK cells has been achieved, with no observation of GVHD (3-5). Despite little or no evidence of stem cell engraftment, successful donor T and NK function was accomplished, reflecting a considerable competitive advantage of donor over host hematopoietic cells, in expansion and differentiation towards these lineages. While these cases support the main rationale behind IUHCT, namely that the fetal immune system is tolerant to foreign HLA early in gestation, IUHCT continues to be applied only in rare cases of X-SCID. This is partially due to limited prenatal screening and diagnostics and mainly due to arguments that postnatal transplantations have obtained very good results, although no direct comparative studies of IUHCT and post-natal BMT have been performed. Retrospective analysis of BMT to SCID patients clearly indicated that transplantation initiated in the neonatal period (first month of life) gives superior clinical benefits to BMT performed at later age, both with regard to T cell reconstitution and survival (201). Considering the enhanced benefit of neonatal transplantations and the obvious possibility of achieving effective results with IUHCT the question raises whether in the immune-barrier changes with age, whether the fetal/neonatal hematopoietic microenvironment is more permissive for homing and engraftment, or whether both are valid.

Early studies done in allogeneic mouse models have suggested that engraftment following fetal transplantations is not limited by an immune barrier (202-204) and suppor-

ted the idea of fetal immune tolerance. In contrast, a recent study provided compelling evidence for the existence of an immune barrier to allogeneic engraftment after IUHCT, demonstrating stable multilineage reconstitution in all congenic recipients, whereas allogenic IUHCT in over 70% of the cases resulted in failed long-term reconstitution (205). Although this may explain in part the poor outcome in reported non-immunological deficiencies transplanted in utero (6) and minimize the hematopoietic advantage of donor cells, successful IUHCT still provides the advantage of reduced susceptibility to postnatal infection by generating functioning immune system at birth (133, 206). To bear in mind, transplantation in adult mice with even small congenic differences is impaired by an immunological barrier (207-209). Thus, these apparent diverging findings may still reflect immunological and/or microenvironmental advantages of IUHCT as compared to postnatal transplantations, even in situations with minimal mismatch.

Our studies were designed to investigate following issues of relevance for IUHCT in X-SCID. First, whether or not engraftment with long-term HSCs (187) is a requirement to obtain stable lymphoid reconstitution in X-SCID recipients transplanted in utero (not addressed previously) and second, whether there is a potential non-immunological advantage for IUHCT versus postnatal (early and late) transplantation.
We transplanted fetal mice carrying the common gamma chain deficiency $\gamma c^{-/-}$(identical mutation to human X-SCID disease)(210) with congenic 200 LT-HSCs (187), 200 LMPPs (61, 62, 164) and 1000 LMPPs (calculated to reflect the natural ratio of the populations in vivo). When we analyzed these mice postnatally at various ages, we found sustained T and B cell reconstitution in PB, but also long term reconstitution of short-lived B and T cell precursors in BM and thymus. We demonstrated that the T cells were not a result of oligoclonal peripheral expansion, but had rearranged TCR in a comparable manner with wild type control mice. We also demonstrated that LMPPs are far superior to HSCs in providing rapid lymphoid reconstitution following IUHCT of X-SCID recipients, as LT-HSC-derived PB cells were remarkably low in the first weeks. However, based on secondary transplantation results (performed to address the known restricted self-renewal potential of LMPPs) and on the thymic profile regarding naïve T cells, it might prove essential to include LT-HSCs for securing stable engraftment.

When we compared the ability of equal doses of congenic HSCs as well as LMPPs to reconstitute lymphopoiesis in X-SCID recipients transplanted during fetal development (day 14-16 of gestation), neonatally (postnatal day 1-4) or in early adult life (4-6 weeks old) we found for the first time a potential non-immunological advantage for transplantations of non-conditioned X-SCID recipients in the prenatal (in utero) versus postnatal (early and late) stages, most evident for HSCs, but important also for LMPPs.

One limitation of this study is that we did not address the mechanism by which purified HSCs (or LMPPs) show competitive advantage in fetal over postnatal X-SCID recipients. We suggest however that the fetal micro-environment is more receptive to donor HSCs, and that more niches are available for HSCs and LMPPs to home. It is also possible that these cell populations have separate spaces designated in the bone marrow, and that displacement of host activated HSCs or commited LMPPs enables engraftment of

donor cells. Obviously, in this immunodeficiency model donor cells also hold a competitive advantage, advantage lacking in other inborn haematological or metabolic disorders.

Although we transplanted X-SCID recipients of different ages with equal cell doses, it is unlikely that normalising the cell dose to the body weight of the recipients would have corrected the differences, as both neonatal and adult recipients showed impaired reconstitution when compared to fetally transplanted mice. We are aware that conditioning the recipients through either irradiation or chemotherapy might have minimized the differences to large degree, but a major goal of our studies (and of BMT in general) was to reduce and preferably alleviate the need for cytotoxic treatment due to its serious side effects and resulting long term morbidity.

As thymic progenitors must be replaced daily from the BM to sustain active thymopoiesis (211), our results bear clinical relevance as fetally transplanted LMPPs have the ability to long-term replenish thymic progenitors, although probably less efficiently than HSCs. Our studies, including secondary transplantations, are most compatible with HSC reconstitution being essential for optimal and stable long-term replenishment of B and T lymphopoiesis in X-SCID recipients, but LMPP like cells appear required for rapid reconstitution and efficient contribution to mid- or long-term replenishment of polyclonal B and T lymphopoiesis.

It is in that regard of relevance that (CD34) enriched stem and progenitor cell populations also have been used successfully in clinical IUHCT of X-SCID (3, 4). Although human $CD34^+$ fraction is highly heterogenous, it is important to further subdivide it and hopefully identify a LMPP-like population applicable for clinical IUHCT (and postnatal transplantation) of X-SCID recipients, as one of the goals and claimed advantages of IUHCT in X-SCID is to achieve rapid immune-reconstitution postnatally (206).

In conclusion, our findings underscore the importance of two distinct populations of primitive multipotent stem and progenitors cells in achieving rapid and long-term reconstitution of the immune system in X-SCID recipients, thus clinically relevant in preparation of donor cell grafts. The identification of a lymphoid progenitor population of LMPP- equivalence in the human system might also be an attractive candidate for gene therapy of X-SCID, due to its higher cell cycling activity and susceptibility to retroviral transduction. We also provide evidence for IUHCT of HSCs giving superior T cell reconstitution in X-SCID recipients when compared to postnatal transplantations, although extrapolating these results to clinical application should be approached with caution.

Article III

Myeloid and lymphoid contribution to non-haematopoietic lineages through irradiation-induced heterotypic cell fusion

Early embryonic development separates generation of tissues and organs based on the three germ layers: ectoderm (skin and neural lineage), mesoderm (blood, bone, muscle cartilage and fat) and endoderm (respiratory and digestive tract). This separation is irreversible and allows further development of the embryo. Recent studies however have suggested that regeneration of non-haematopoietic and hematopoietic cell lineages can occur thorough transplantation across germ layer barriers (73). This newly found plasticity of adult cells was proposed as a viable alternative for therapeutical regeneration of injured/defective tissue (95, 96). Upon careful revision of these original reports and generation of new data it was showed that contribution to non-hematopoietic cells does occur, albeit through cell-to-cell fusion rather than transdifferentiation (93, 102, 105, 106, 212). In addition, blood derived fusion partners were suggested to originate in the myelo-monocytic cell lineage (109, 213).

In our study we aimed to address several relevant questions, first to investigate whether formation of heterotypic fusion hybrids is restricted to myelomonocytic cells, or whether it represents a random feature inducible even in lymphoid cells. Secondly, we investigated whether heterotypic cell fusion is a recurring event in steady state conditions or an injury induced process. Lastly, we examined whether lymphocytes contribute, through heterotypic fusion, towards the development of non-hematopoietic lineages during embryonic development at stages when homotypic cell fusion plays an important role in cell genesis of various organs (214).

By using donor mice in which hematopoietic lineage specific promoters regulate Cre recombinase expression we could identify cells committed to B cell (CD19)(215) and T cell (CD4) (216) lineages and their ancestry. Lineage depleted BM cells from these strains were transplanted into lethally irradiated Z/EG Cre-reporter mice and stable donor derived engraftment documented. Following such transplantations GFP expression is inducible by Cre mediated recombination and is indicative of cellular fusion between a hematopoietic lineage restricted donor cell with a recipient cell. We found rare lymphocyte derived fusion events in heart, skeletal muscle and liver. As the frequencies were extremely low (translating a possible low efficiency in cre-lox recombination), we further employed a model that enabled both high blood reconstitution levels and uniform labeling of donor cells. Transgenic "green" mice with β-actin (217) promoter driven GFP on wild type(wt) or $Rag1^{-/-}$ background (218) were used as donors for X-SCID (210) or wt mice, respectively. Thus high levels of donor derived lymphoid reconstitution (wt GFP^+ to X-SCID) generated readily detectable heterotypic cell fusion events in heart, skeletal muscle, liver and brain (Purkinje neurons), in absence of myeloid derived donor cells. Conversely, transplantation of $Rag1^{-/-}$ GFP^+ to wt irradiated mice generated exclusive myeloid reconstitution and fusion events with cardiomyocytes, muscle fibers, hepatocytes, albeit not with Purkinje neurons, in spite of high levels of GFP^+ microglia.

Further, to investigate steady state contribution to fusion events in adult, wt GFP$^+$ BM cells were transplanted to mice that have an inborn HSC deficiency (W^{41}/W^{41}) and allow high levels of PB and BM reconstitution without prior conditioning (219, 220). Despite stable high levels of donor derived myeloid and lymphoid cells in the PB, no evidence of cell fusion was detected in heart, skeletal muscle, liver or brain. However, when site specific injury was inflicted through myocardial infarction, muscle cardiotoxin or cryoinduced damage and brain toxin induced damage, contribution to cardiomyocytes, skeletal muscle fibers and Purkinje neurons, through blood--target tissue cell fusion at the injury site was efficiently detected. In contrast, when lethal total body irradiation was applied, fusion events were distributed randomly throughout the heart, muscle, liver and cerebellum.

As the mechanism by which these fusion events appeared after tissue injury (general or local) was not clear, we hypothesized that inflammation may play a significant role. Indeed, corticosteroid treatment of lethally irradiated mice reduced the frequency of fusion events nearly in a dose dependent manner, thus supporting at least partially inflammatory mediated mechanism for fusion hybrids.

Since homotypic fusion occurs normally during development, we transplanted in utero non-conditioned X-SCID deficient mice at embryonic day E14-E15 with BM derived and FL hematopoietic cells and assessed blood derived contribution to non-hematopoietic cells postnatally. Although fusion derived hepatocytes and Purkinje neurons with characteristic morphology and lineage marker expression were present, no heart or skeletal muscle fibers were detected. In contrast, no hepatocytes and few Purkinje neurons were found in neonatal X-SCID mice transplanted i.p. with wt GFP$^+$ cells, in spite of sustained lymphoid reconstitution levels and non-conditioned adult X-SCID mice transplanted with wt GFP$^+$ cells were also negative for heterotypic cell fusion. The ability of blood cells to contribute to hepatocytes and Purkinje neurons after IUHCT but not postnatally could be a result of a normally occurring physiological process, restricted to a narrow window during mid to late gestation. Another alternative could be that the extensive surgical procedure and manipulation of the fetus caused some degree of inflammation that in turn mediated cell fusion. This was addressed by genetic lineage tracing of B, T and pan-hematopoietic derived cells in offspring of CD19, CD4 and Vav –Cre expressing mice crossed with Z/EG reporter mice. No detectable heterotypic cell fusion was observed in tissues of the offspring, although analsyis was performed at two time points (neonatal and adult) to similar extent with the fetal transplanted mice. As expected, as a result of the cre/lox recombination, GFP was activated specifically in the different lineages.

Taken together, our data demonstrates through lineage fate-mapping that *in vivo* fusion of lymphoid and myeloid blood cells does not occur to an appreciable extent in steady-state adult tissues nor during normal development. Rather, fusion of blood cells with different non-haematopoietic cell types is induced by organ-specific injuries or whole-body irradiation, which has been used in previous studies to condition recipients of BMT. Our findings demonstrate that blood cells of the lymphoid and myeloid lineages contribute

to various non-haematopoietic tissues by forming rare fusion hybrids, but almost exclusively in response to injuries or inflammation (221).

Although we cannot exclude the possibility that rare heterotypic fusion events are formed during normal development (222, 223), our findings in lineage tracing models underscore the importance of using non-invasive methods to study the role of heterotypic fusion in normal physiology. In addition, the therapeutical relevance of such events appears limited to models of monogenetic deficiencies in with potential delivery of correcting gene through heterotypic cell fusion with genetically modified hematopoietic healthy donor cells. In such studies, the focus must be on either the mechanisms that enhance the fusion efficiency or identification of target cells with considerable proliferative potential and a competitive advantage in order to expand the rare and otherwise insignificant fusion event.

PREIMMUNE FETAL SHEEP XENOGRAFT TRANSPLANTATION OF HEMATOPOIETIC CELLS –the Lund experience

Introduction

The pre-immune fetal sheep model has been reported as a successful alternative xenograft assay and used for more than a decade in studies of human HSC (224, 225). The proposed advantages are lack of immune competence and hence ability to accept cells bearing foreign antigens, space for expansion of donor cells, long life span of recipient and possibility of extended follow up of transplanted cells through repeated bone marrow aspirations. Although promising, the model is not particularly widespread and the reported levels of achieved human engraftment vary largely. We aimed to establish the fetal sheep transplantation model for studies of human HSCs and progenitors and thereby identify optimal populations for fetal cell replacement therapies. The work was performed as a collaborative effort of members of the Hematopoietic Stem Cell Laboratory (Prof Sten Eirik Jacobsen, Dr. Lars Nilsson and Dr. Kristina Andersson) and of the Perinatal Research Group at the Department of Obstetrics and Gynecology (Prof Karel Maršál, Assoc. Prof Göran Lingman and the author) in Lund. The Perinatal Group had previously used the exteriorised fetal sheep model in studies regarding the effects of acute asphyxia on near term fetus, lung developmental studies (226-228) and experimentally induced preeclampsia (229). The group has significant experience of in utero surgery on fetal sheep as well as the availability at a farm outside Lund for breeding and housing of the animals, thus providing the necessary platform for these studies. As a result, three independent experiments were performed, with Swedish mixed-breed and Dorset pure-breed (1st and 2nd generation) gestant sheep. Adult BM-derived and CB (pooled, as well as individual source) CD34 enriched cells were used as donor cells. A separate group of sham transplanted animals was included for evaluation of transplantation procedure on term survival. Total number of experiments and fetal sheep transplanted/ experiment, dose size and distribution of donor cell source, sheep breed, gestational age, survival and transplantation outcome are summarized in **Table 1**.

Materials and methods

Sheep Adult (age 1-4 years) sheep of Swedish mixed breed or Dorset pure breed were time-mated. Gestation was assessed by transcutaneous ultrasound examination at 45-50 days after mating and nineteen ewes with 1 or 2 viable fetuses were enrolled in the study at gestational age (GA) 53-58 days.

Mice Non-obese diabetic/ severe combined immune deficient (NOD/LtSz-scid/scid) mice purchased from the Jackson Laboratories (Bar Harbor, ME, USA) were bred and maintained in pathogen-free conditions, and had unrestricted access to sterilized food and autoclaved acidified water. All animal experiments were approved by the local ethical committee.

Cell isolation and purity analysis BM aspirates from consenting adult donors and umbilical CB samples from term deliveries were collected in accordance with local ethical committee approval. Mononuclear cells (MNC) were isolated from pooled CB samples (experiment 1), individual BM samples (experiment 1 and 2) or un-pooled CB samples (experiment 3) and enriched for $CD34^+$ fraction as previously described (230). Briefly, the MNC layer was separated by density gradient centrifugation over Lymphoprep (Nycomed Oslo, Norway). Positive selection for CD34 expressing stem and progenitor cells from the MNC fraction was done using MACS (magnetically activated cell sorting) CD34 isolation kit (Miltenyi Biotech, Germany). Purity (90-95%, **Figure 7A**) was assessed on an aliquot of enriched cells at all times. Cells incubated with CD34-fluorescein isothiocyanate (FITC)- specific, CD38-phycoerytrin-specific and CD45-allophycocyanin(APC)-specific monoclonal antibodies or isotype matched control antibodies (all from Becton Dickinson, San Jose CA, USA) were analyzed by flow activated cell sorting (FACS) and viability was assessed by 7 amino-actinomycin D (Sigma Aldrich, Germany). CD34 enriched cells were transplanted either fresh (all in experiment 1 and experiment 2, and CB1, CB2 in experiment 3) or cryo-preserved for later use (CB3 in experiment 3).

Tail vein injections Sub-lethally irradiated (350cGy) six to eight weeks old NOD/SCID mice were injected each with 3-5 x 10^4 CD34 enriched CB cells through i.v. tail vein injection. Mice were given prophylactic Ciprofloxacin (100 μg/mL) in the drinking water until analysis and killed at 6 weeks after transplantation. Human reconstitution was evaluated in recipient BM.

In utero transplantation At GA 53-58 days of 149 days at term thirty three fetal sheep of generally anesthetized (Isofluran) gestant ewes were identified individually by transcutaneous or transuterine ultrasound (Acuson Aspen™, Mountain View CA, USA) and human $CD34^+$ cells ($2x10^5$/recipient) contained in a volume of 0,2ml IMDM with or without additional 5% FCS were injected under visual control (**Figure 7B** and **Table 1**) through a 22-gauge needle in the peritoneal cavity of each recipient. The needle was flushed with additional 0,2 ml IMDM and then swiftly removed. Fetal viability was confirmed directly after injection at all times.

Postnatal ovine sample collection Lambs were generally anesthetized with Ketamine (15mg/kg body weight) and local analgesia was provided with Xylocaine. BM aspirations were performed from an aseptic area corresponding to 1 dm^2 over posterior crista region, alternating sides for repetitive analysis. PB was collected from a front limb vein. Samples

were heparinized and transferred to pre-filled flasks of 10 ml IMDM + 1% FCS, transported without chilling (<30 minutes) and further processed in the laboratory.

FACS analysis NOD/SCID BM cells were collected at 6 weeks after transplantation and analyzed as previously described (230). Fetal sheep were analyzed at 48 hours after transplantation (PB, BM, fetal liver and peritoneal lavage) at 60 and 120 days after transplantation (PB, BM), subsequent to cytokine mobilization at 180 days after transplantation (PB, BM) as well as 270 days after transplantation (details described in **Table 1**). Isolation of MNC from sheep samples was done by density gradient centrifugation (Lymphoprep, Oslo, Norway). Cells were consequently washed twice in phosphate buffered saline (PBS) with 1% fetal calf serum (FCS) and incubated with myeloid lineage CD15/CD33 FITC, lymphoid lineage CD19 PE and pan-hematopoietic CD45 APC mouse anti-human monoclonal antibodies (BD, San Jose CA). Dead cells were excluded by 7-AAD (Sigma Aldrich, Gremany). Non-transplanted (negative controls) and mixtures of 0.1-0.5% human cells in mouse or sheep BM (positive controls) were included at all times. For each sample, a minimum of 500 000 viable BM cells were analyzed by a FACS Calibur (Becton Dickinson, San Jose CA). Animals were defined as positive for human engraftment if they showed at least 0.1% human reconstitution, including myeloid and lymphoid engraftment (minimum of 10 positive events each per 100 000 BM cells analyzed).

FISH analysis Interphase fluorescence in situ hybridization (FISH) analyses of human centromere were performed on cytospin slides prepared from PB, BM and FL from all FACS analyzed animals. Specific human and sheep probes were used and positive cytospin preparations of 0.1 to 0.5% human MNC mixed in non-transplanted sheep BM MNC served as control. A minimum of 1000 nuclei were analyzed/ sample.

Cytokine mobilization Fetal sheep at 180 days after transplantation (approximately three months after birth) were mobilized with human granulocyte colony stimulating factor (Neupogen G-CSF, Amgen) subcutaneously administered for 5 consecutive days at 5µg/kg (days 0-4)(231). PB and BM samples were collected from treated animals at day 3 (PB) and day 4 (PB, BM) respectively and analyzed by FACS.

Statistical analysis Results are presented as means (SEM) and statistical significance of differences between groups was determined using a two-tailed unpaired t-test. *P* values less than 0.05 were regarded as significant. (GraphPad Prism v.4; GraphPad software, San Diego, CA)

Experiment/ Cell source	Fetal transplants (cells/fetus)	Breed/GA Procedure	Live born/ (outcome, delivery)	Analyzed 48h (prenatal)	Analyzed 60 days (prenatal)	Analyzed 90-120 days	Mobilized G-CSF	Analyzed 180 days	Analyzed 270 days
Exp 1/ BM1 CD34⁺	2; 2 ($2x10^5$)	Mixed/58d Transuterine	1; 2 (natural; CS)			1; 2 negative; *		1; 1 negative (1 dead)	1; 1 negative
Exp 1/ BM2 CD34⁺	2; 2 ($2x10^5$)	Mixed/53d Transuterine	1; 0 (1 stillborn; maternal demise)			1 negative		1 negative	1 negative
Exp 1/ CB (pooled) CD34⁺	2; 2 ($2x10^5$)	Mixed/58d Transuterine	2; 2 (1 dead day 3; natural)			1; 2 negative		1; 2 negative	1; 2 negative
Exp 1/ sham	2; 2 none	Mixed/58d Transuterine	1; 2 (1 resorbed; natural)			1; 2 negative		1; 2 Negative	1; 2 negative
Exp 2/ BM CD34⁺ (no FCS)	1; 1; 2 ($2x10^5$)	Mixed/58d Transuterine	0; 1; 1 (analyzed 48h; 1 resorbed)	1 positive 0.16%		1;1 negative		1; 1 negative	
Exp 3/ CB 1 CD34⁺ (no FCS)	1; 2; 1 ($2x10^5$)	Dorset/56d Transcutaneous	0; 0; 1 (analyzed 48h; misscariage)	1 positive 0.22%		1 negative	Yes	1 negative	
Exp 3/ CB 2 CD34⁺ (no FCS)	1;2;2 ($2x10^5$)	Dorset/58d Transcutaneous	0; 0; 1 (2 analyzed 60d; 2 resorbed)		2 negative	1 negative	Yes	1 negative	
Exp 3/ CB 3 CD34⁺ (no FCS)	2; 1 ($2x10^5$)	Dorset/58d Transcutaneous	0; 0 (1 resorbed; 1 macrosom, died at birth)		1 negative				

Table 1. **Summary of preimmune fetal sheep transplantations with regard to donor cell source, outcome and analysis results.** Cells were delivered to the fetal recipients through ultrasound guided needle via the uterine wall (transuterine; laparotomized ewe) or via intact skin membrane (transcutaneous). CS= cesarean section. In experiments 2 and 3 cell suspensions were prepared free of fetal calf serum (FCS) and room tempered prior to injection. Survival to analysis: experiment 1: 12/16 (75%), in experiment 2: 3 /4 (75%), in experiment 3: 6/12 (50%)

Results and discussion

BM samples collected from consenting volunteers, pooled CB units and individual CB units (CB1-3) from term deliveries were highly enriched for $CD34^+$ hematopoietic stem/progenitor cells (154, 155) and FACS analyzed aliquots from each positively selected CD34 fraction showed high purity (98(±1,3) % of the total enriched cells) of viable (7AAD negative) hematopoietic (hu $CD45^+$) cells and 96(±2) % of the total analyzed cells were $CD34^+$ with 5(±1,2) % of total cells within $CD34^+CD38^-$ fraction (151, 232) **(Figure 7A)**. A summary of all three preimmune fetal sheep transplantation experiments is presented in Table 1. In experiment 1, FACS analysis of PB and BM at 90 days post-transplantation of 3 fetal sheep (marked with asterix in **Table 1**) recipient of adult bone marrow derived $CD34^+$ cells showed human $CD45^+$ and multilineage reconstitution at levels of 1%. At the same time, the negative control (only sheep MNC cells) included in the FACS analysis also indicated presence of human cells, at similar levels with the transplanted sheep, thus we suspected contamination of the reagents with human cells. To verify the finding, we repeated the analysis after two weeks and could not confirm the primary evidence of human engraftment (unpublished observation), thus analysis outcome in Table 1 is marked as negative.

Discussed in detail below are mainly the results of experiment 3 in which several parameters were optimized. Based on discussions with Prof Zanjani, Reno, NV in the latest experiment we used CB units isolated separately; excluded fetal calf serum (FCS) in injected cell suspension; transplanted recipients of pure breed (Dorset) and verified delivery of transplanted cells by analyzing recipient fetal sheep 48h post-transplantation- all to increase the probability of a successful outcome.

Briefly, in experiment 3, $CD34^+$ cells from two CB units (CB1, CB2) were directly transplanted into groups of 3 sub-lethally irradiated adult NOD/SCID mice (5×10^4 / recipient) and pre-immune fetal sheep (20×10^4 /recipient) respectively. Another CB unit (CB3) was thawed 48h after $CD34^+$ isolation and cells subsequently transplanted to 2 NOD/SCID mice (3×10^4/recipient) and 3 fetal sheep (20×10^4/recipient). The donor cell source was chosen based on evidence of higher SCID repopulating activity in human CB cells than adult BM (used in experiments 1 and 2) (233) and the specific cell dose was calculated based on previous reports to generate robust human engraftment (224, 234, 235).

In experiment 3, six out of twelve fetal sheep recipients (survival 50%) were available for analysis (5 were lost before term through spontaneous demise and resorbtion –as detected by ultrasound 60 days after transplantation; one was lost at birth due to fetal maternal disproportion). Although significant, the mortality rate is in line with previous reports on fetal transplantation at this gestational age (236). To confirm the correct delivery of HSCs to the fetal sheep one recipient of fresh $CD34^+$ cells was killed at 48h after transplantation and PB, BM, FL and peritoneal lavage cells were collected. As appropriate for the gestational age, FL was abundant in host hematopoietic cells (22×10^8 cells) and BM barely populated by hematopoiesis (45×10^3 cells). Presence of earlier transplanted human $CD45^+$ $CD34^+$ $CD38^+$ cells was detected in peritoneal lavage

(0, 22% viable human CD45+) but not in samples of FL, PB or BM (**Figure 7C**). The finding was confirmed by FISH on cytospins of analyzed cells and detection of human centromere, although at a lower frequency than indicated by FACS. In all analyses, negative (non-transplanted sheep) and positive (mixed in 0.1% and 0.5 % hu MNC into non-transplanted sheep MNC) were included.

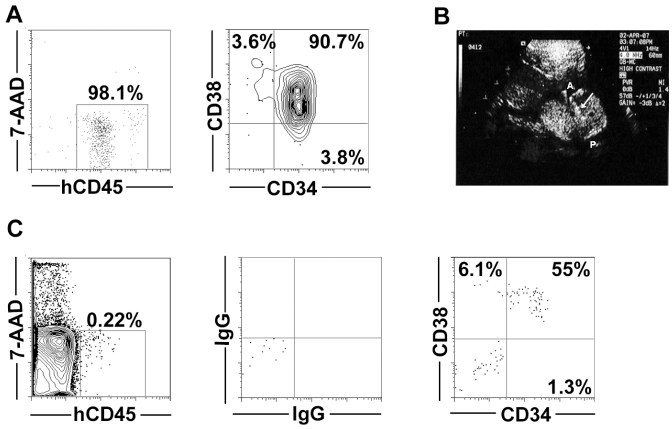

Figure 7. Efficient ultrasound guided xenograft cell transfer to unconditioned pre-immune fetal sheep
(A) Purity analysis was performed on an aliquot of cells from each individual cord blood (CB) sample after cell isolation and two-step enrichment for CD34 antigen. Shown here are representative FACS profiles from one purity analysis. Left panel shows frequency of viable (7AAD-) CD45+ cells of total nucleated cells; right panel shows frequency of CD34 single positive (SP), CD34CD38 double positive (DP) and CD38 single positive (SP) of total nucleated cells (B) Ultrasound scan of abdominal area (A-anterior, P-posterior) of a gestational day 56 pre-immune fetal sheep with donor cell suspension deposited intra-peritoneal (high echogenic area indicated by arrow). (C)One fetal sheep recipient was terminated 48 hours after ultrasound guided intra-peritoneal injection of 2x105 CD34+ highly enriched human CB cells and FACS analysis of cell suspension recovered from peritoneal lavage was performed to verify correct delivery of donor cells. Far left panel shows frequency of viable (7AAD-) donor-derived human CD45+ (hCD45+)cells of total nucleated cells, middle panel represents isotype control staining of gated donor derived CD45+ cells and far right panel shows frequencies of CD34 SP, CD34CD38 DP and CD38 SP of total donor derived human CD45+ cells.

Remaining fetal sheep recipients were divided for analysis as follows: three fetal recipients were killed at 60 days after transplantation and PB and BM analyzed; two fetal sheep were live born and sampled at 120 and 180 days after transplantation, the later after 5 day G-CSF mobilization.

Sub-lethally irradiated NOD/SCID mice transplanted with identical CB unit CD34+ cells as the pre-immune fetal sheep were killed 6 weeks after transplantation and total BM analyzed. The three groups showed appropriate levels of total human repopulation activity (13%), although significantly higher reconstitution was seen in mice transplanted with thawed cord blood CD34+ (34%) than fresh CD34+ (6,5%). In all recipients multi-lineage donor derived B- lymphoid CD19+ and myeloid CD15+CD33+ progeny was detected (**Figure 8A**). The higher engraftment capacity of CB3 sample (thawed CD34+ cells) most likely illustrates the previously described physiological variation in engraftment capacity between cord blood samples(233).

Three fetal sheep- recipients of CB2 (2 recipients) and CB3 (1 recipient) were killed and analyzed at 60 days after transplantation. None of these showed detectable human repopulation activity in PB or BM. Two fetal sheep recipients (0756 and 0757) were term delivered and analysis of PB and BM performed at 120 days after transplantation. FACS profiles of ovine recipients BM with insignificant human CD45 expression are shown alongside positive control sample (mixed in human MNC into non-transplanted sheep BM) (**Figure 8B**). Based on previous reports we expected human repopulation to be higher in BM than PB of transplanted sheep. As our negative readout could be due to very low levels of engraftment and inability to detect human cells, we aimed to mobilize possibly existing human WBC with G-CSF, reported to give 21-fold increase in cell count (231). Recipient sheep were analyzed at day 3 in PB and day 4 in PB and BM. Apparently positive human engraftment although at extremely low levels and with no lineage distribution was detected through this strategy. (**Figure 8C**) Human origin of these events (sorted based on $CD45^+$ expression and transferred to cytospin slides for FISH analysis) was not confirmed, as human centromere was only identified in the included positive control of 0.1% mixed in human cells (cut off level). (**Figure 8D**)

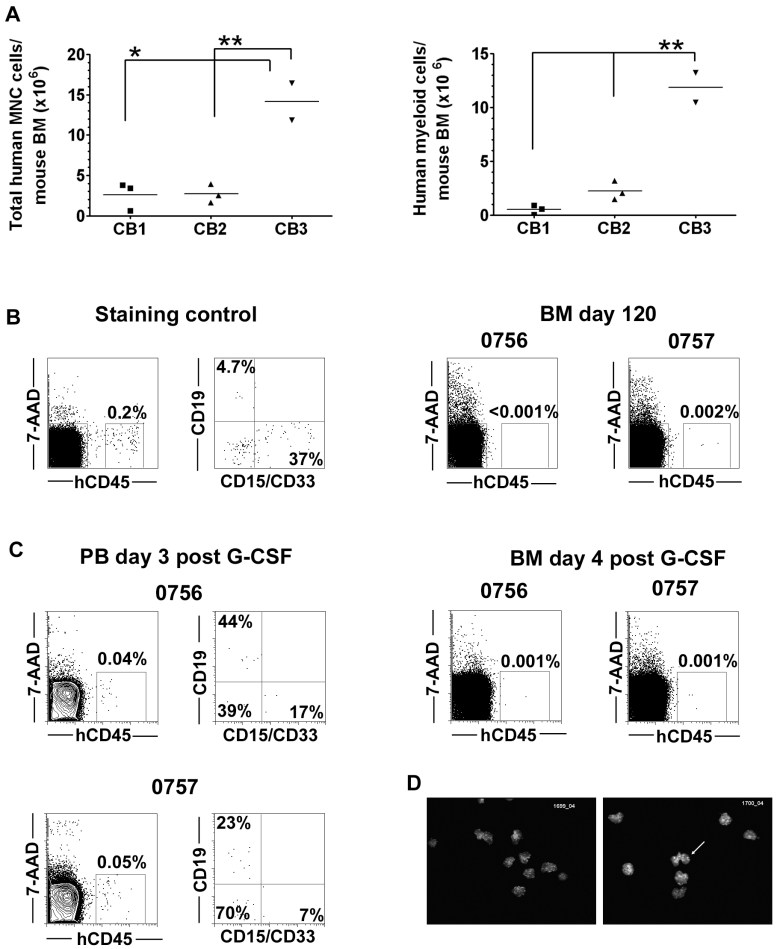

Figure 8. Highly enriched CD34$^+$ human cord blood cells provide robust multilineage reconstitution in adult NOD/SCID murine recipients but fail to engraft preimmune fetal sheep. **(A)** Bone marrow (BM) cells were collected 6 weeks after transplantation from (n=8) sub-lethally (350cGy) irradiated NOD/SCID adult recipients transplanted with 3-5x 10^4 fresh (CB1 and CB2) or thawed (CB3) CD34$^+$ cord blood cells. Total (hCD45$^+$) (left panel) and myeloid (CD15$^+$CD33$^+$)(right panel) human derived contribution to total mouse BM cells presented as absolute cell numbers (SEM) of total BM cells (2 femurs+ 2 tibiae) from three independent experiments. * P<0.05, ** P<0.005 **(B)** Representative FACS profiles of positive staining control sample prepared from non-transplanted age-matched sheep BM cells with 0.2% mixed in human MNC and BM samples of two individual fetal sheep recipients at 120 days after transplantation. Shown are frequencies of total viable human derived CD45$^+$ cells. Lymphoid (CD19$^+$) and myeloid (CD15$^+$CD33$^+$) lineage distribution is shown for positive staining control as indicator of functioning antibody binding. Transplanted sheep showed no detectable human donor derived lineage positive cells. **(C)** Two individual (0756 and 0757) fetal sheep transplanted with 2x10^5 CD34$^+$ cells and negative for human engraftment at repeated analysis time-points were mobilized with subcutaneous injections of human G-CSF (5µg/kg) for 5 days (day 0-day 4). Peripheral blood (PB) and BM were collected at day 3 and day 4 of treatment respectively. FACS profiles of PB (left panels) and BM (right panels) analysis are shown here. Numbers indicate frequencies of viable donor derived (CD45$^+$) positive cells in total nucleated cells and frequency of lymphoid (CD19$^+$) and myeloid (CD15$^+$CD33$^+$) lineage cells in total donor derived cells. **(D)** Fluorescence in situ hybridisation with specific human and sheep probes was used to analyze samples of BM from preimmune fetal sheep transplanted with human CD34$^+$ cells and positive samples prepared from non-transplanted age-matched sheep BM cells with mixed in human MNC were used and control. Left image shows only sheep cells in a fetal recipient with apparent FACS positive human CD45$^+$ cells; right panel shows positive control with human centromere indicated by arrow.

A number of questions are raised by the present findings and some will be discussed as follows. Eaves et al (237) transplanted identical source leukemic HSC populations from CML patients into NOD/SCID mice and pre-immune sheep and achieved various degrees of reconstitution, although leukemic progress was only observed in the murine recipients. Lewis et al (238) transferred fresh or *ex vivo* expanded CB $CD34^+$ cells to NOD/SCID and pre-immune fetal sheep recipients, although unclear if cells originated from the same CB unit were employed in these separate experiments. They report murine reconstitution levels at 6 weeks after transplantation of uncultured 50 000 $CD34^+$ cells around 4.7%, while fetal sheep transplanted with 100 000 uncultured $CD34^+$ had reportedly 4.8% human reconstitution at 60 days after transplantation and persistent to 180 days (reported 4.1%).

As we contemplate the striking difference we see in human engraftment between the NOD/SCID mice and the fetal sheep recipients, a number of arguments come to mind. At first, although the applied cell dose was calculated based on previous studies to produce detectable levels of engraftment (234, 235) it is also a fact that fetal sheep weight increases over 100-fold from approximately 40g at transplantation (mid-term gestation) to nearly 4000g at term. The increase in actual body mass, taken together with the exponential host hematopoietic expansion in fetal sheep compared to adult mice (constant in size, 20-30g) could argue that the human cells are undetectable due to dilution in the sheep blood/BM.

Another important and yet un-investigated factor might be the variation in sheep strain. Clearly, the trait of a xenograft recipient strain is important as shown in the mouse xenotransplantations. Lack of immune competence (although necessary) was insufficient to support high levels of human engraftment, and the non-obese diabetic background was required for successful human cell repopulation (239). Data from Young et al. (240) reporting xenograft human reconstitution below 0.8% when employing yet another sheep strain (Swiss White Alpine) could support our hypothesis. To keep in mind is also the age of the fetal recipient, as the immune system is probably less tolerant than expected. In this regard, the reported intracelomic route (241) might be a better option and allow for higher reconstitution levels as cells are delivered at an immunologically more immature gestational age, although human reconstitution levels via coelomic route (6-22%) did not exceed significantly those reported by Lewis et al. (238).

One of the proposed advantages of this model is that early transplanted donor cells would find available niche space for homing and differentiation in the developing hematopoietic environment of the fetus, however the lack /inability of conditioning the host and the natural competition from host hematopoietic cells could represent quite significant obstacles to donor engraftment (6). In this regard, evidence from Harrison et al. (242) indicating a relative excess of HSCs and progenitor cell during hematopoietic development in fetal life may be relevant, as human HSCs could simply be out-competed by host cells.

Given increasing evidence on MSCs' immuno-modulatory roles (123), co-transplantation with human test cells might improve BM niche homing, minimize host versus graft reaction and hopefully facilitate detectable levels of engraftment even in a suboptimal recipient strain.

In the end, a genetically more relevant alternative for human studies is the non-human primate xenograft model. Unfortunately, regardless numerous attempts made to enhance levels of reconstitution (through graft composition, fetal conditioning, different sources of donor cells) only donor microchimerism (<1%) has been observed, even with donor $CD34^+$ cells in order of 10^9/ recipient, probably due to immunological barriers as high as in the human fetus (243). Taken together, present data reflects the challenge of human *in vivo* assays in general and the fetal sheep xenograft transplantation model in particular, and highlights the variable outcomes depending on donor cell source, recipient strain and analysis methods applied.

GENERAL CONCLUSIONS

We identify in adult murine bone marrow an early commitment step from hematopoietic stem cells with multilineage potential towards a progenitor cell population with restricted granulocyte/monocyte and lymphoid potential and lacking megakaryocyte erythroid potential.

We demonstrate that lymphoid primed multipotent progenitors generate rapid and sustained lymphoid reconstitution in fetal X-SCID recipients. Self renewing hematopoietic stem cells appear however necessary for maintenance of lymphoid repopulation over the lifetime of an individual.
We also demonstrate that fetal microenvironment is more permissive to hematopoietic stem cells engraftment when compared to neonatal and adult transplantations.

We demonstrate that hematopoietic stem cell plasticity potential is largely retained to low levels of heterotypic cell fusion throughout ontogeny and suggest that the underlying mechanism is based on inflammation/injury in the target tissue.
We also demonstrate that not only myeloid, but also lymphoid cells are potent fusogenic partners with hepatocytes, Purkinje neurons and muscle cells.

FUTURE PERSPECTIVE

Better definition of human hematopoietic hierarchy with regard to long term repopulating cells and lineage commitment is a priority. In this respect, the recent contribution of Majeti et al. (244) may be relevant. The authors propose $Lin^-CD34^+CD38^-CD90^+CD45RA^-$ cells as LT-HSC based on their capacity to generate at very low numbers (10 cells) stable long term reconstitution of myeloid and B-lymphoid progeny in BM and T-cells in spleen, while maintaining secondary repopulation ability. Although $CD90^-CD45RA^-$ fraction also holds myelo-lymphoid potential in vitro and in vivo, the authors convincingly show that $CD90^+CD45RA^-$ fraction gives rise to the $CD90^-CD45RA^-$, while the later only expands within its phenotype. Thus the authors propose the $Lin^-CD34+CD38^-CD90^-CD45RA^-$ as a multipotent progenitor downstream of newly identified LT-HSC. The proposed common lymphoid progenitor (CLP) has been identified within the $Lin^-CD34^+CD38^+CD10^+$ phenotype in adult BM (58) and $Lin^-CD34^+CD38^+CD7^+$ phenotype in CB (59), although only B-, NK- and dendritic cell potential was demonstrated at single cell level in these populations, while T cell potential was investigated on a population basis. To further identify commitment steps from the LT-HSC to these lineage restricted progenitors and hopefully identify the human LMPP equivalent one depends however on readout of GM/lymphoid potential at a clonal level, and to date no *in vitro* or xenograft transplantation model offers those conditions.

We have tried to establish the fetal sheep xenograft transplantation model for evaluation of human hematopoietic cells, but were unable to reach detectable levels of human derived cells beyond 48 hours from transplantation. The poor outcome of the model reflects possible immunological barriers, potentially strain related, and motivates further investigation as it remains an attractive assay for both hematopoiesis and IUHCT studies.

Considering potential candidates for IUHCT, it is interesting to know that in Sweden only certain cases of inborn immune deficiencies (X-SCID, JAK3 deficiency, RAG1 and/or RAG2 deficiency, Omenn's syndrome, ADA (adenosine deaminase) deficiency and reticular dysgenesis) have been described. Although X-SCID is the most prevalent form, affecting over 50 per cent of all SCID patients worldwide, the estimated occurrence frequency in Sweden is of only 1-2 births/year (1-2:100 000 live births). Thus it becomes more imperative that a collaborative multicenter effort is undertaken to further develop and improve the therapeutical benefits of IUHCT for immune deficiencies. In the case of haemoglobinopathies and metabolic storage disease, enhanced chimerism and thus correction of disease might possibly be achieved through development of a non-toxic myeloablative method, through increased donor cell dose (tailored for stem and progenitor cell content, T reg cells, MSCs), manipulation of host niches for permissiveness to donor cells or manipulation of donor cells for competitive advantage over host cells, or improving host antigen presentation in early gestation in order to achieve donor tolerance.

Worth consideration is that although enzyme replacement therapies have been identified for certain lysosomal storage diseases and life long treatment is provided for patients, the procedure is both expensive and non-curative. In mucopolysaccharide (MPS) storage diseases for instance, enzyme replacement alone will not reverse disease related damage to the nervous system. BMT for MPS is curative, but it is most beneficial when undertaken early in life. Unfortunately, patients are often not diagnosed in infancy, thus treatment may be non-curative at a later stage. Prenatal screening and well standardized IUHCT of natural or genetically corrected cells could hold clinical promise in these cases. Likewise, methods of safely enhancing the rare heterotypic cell fusion events following IUHCT or BMT might prove beneficial in diseases like tyrosinemia or muscular dystrophy.

ETHICAL CONSIDERATIONS

Apart from pre-implantation diagnostics, accurate prenatal diagnosis is possible as early as 10-12 weeks of gestation, while the fetus is regarded viable from 22 weeks of gestation. Likely, fetal transplantation in the pre-immune stage would have to be performed shortly after diagnosis at 13-15 weeks of gestation. Thus, with disease detection possible long before legal limit for pregnancy termination, a few ethical considerations are evident. In considering fetal cell replacement therapy, the balance of potential benefit and harm, the autonomy of the patient and informed consent, the duty of the caregiver to the pregnant woman and the fetus are all issues that need to be discussed. It is important that fetal cell replacement therapy is recommended when it has a realistic chance of saving the life of the fetus or preventing serious and irreversible disease or disability. The information given should be balanced and maternal choice and assessment of risk should be respected. In early gestation, the legal duty of the obstetrician is primarily to the mother. In counselling, full information regarding the range of possible outcomes and the availability of support after birth should be presented to the couples. To bear in mind the risk for miscarriage following invasive in utero transplantation is estimated at 1-2%. In utero therapy can obviously never be undertaken without maternal consent, and nor should it be presented coercively as an option to avoid termination of pregnancy. Optimally, the potential of fetal therapy following diagnosis of target diseases should be first presented to couples after a decision for not terminating the pregnancy has already been taken. Finally, diagnostic and therapeutic procedures of unproven efficacy have to be undertaken with the voluntary informed consent of the pregnant woman and according to a clearly defined research protocol that has been approved by an appropriate research ethics committee.

SVENSK POPULÄRVETENSKAPLIG SAMMANFATTNING

Benmärgstransplantation (BMT) är en livräddande behandling för sällsynta medfödda immunbristsjukdomar, blodbildningssjukdomar och ärftliga ämnesomsättningssjukdomar. Förbehandlingen och återhämtningen efter BMT är ofta förknippad med ett antal komplikationer så som infektioner, reaktion av införda celler mot mottagaren eller i värsta fall döden. Dessutom kan ämnesomsättningsrubbningar genom pålagring av metaboliter orsaka neurologiska och organskador som inte är reversibla genom BMT efter födseln. Fostercellterapi eller in utero hematopoietisk cell transplantation (IUHCT) celler är ett alternativ till dessa sjukdomar, i dem fall tidig genetisk diagnos är utfört. Genom navelsträngs blodtransfusion kan man överföra blodbildande celler (från olika källor, i olika antal) till den drabbade fostret ett stor och på detta sätt förhoppningsvis uppnå korrektion av bristen före födseln. Bevis av principen har uppnåtts i immunbristsjukdomar, då foster transplanterade i livmodern återbildade immunförsvaret med blodkroppar av givarursprung. I andra sjukdomar dock har man inte lyckad uppnå tillräckligt stor antal givare celler för att bota mottagaren. Eftersom framgången med IUHCT har varit begränsad endast till immunbristsjukdomar, en del frågor kvarstår, så som vilka celler är mest optimala att överföra, hur fostrets inre miljö fungerar, hur mycket dennes utstötning mekanism påverkar utkomsten, osv. I denna avhandling försöker vi svara på frågan vilka celler är de mest optimala att transplantera genom att först hitta den tidigaste gren delningen från blodbildande stam celler till cell typer med mer begränsad utvecklingsförmåga och därefter använda denna tidiga cell typ i transplantation till mus foster med immunbristsjukdom. I arbete I fann vi en föregångarecell till vita blodkroppar som har begränsad självförnyelse och avsaknad av kapacitet till bildning av röda blodkroppar och blodplättar och som direkt nedstiger ifrån blodstamceller. I arbete II visade vi att denna föregångarecell ger snabbare upphov till immunförsvarets vita blodkroppar i immundefekta fostertransplanterade möss, dock kan långlivade blodstamceller vara nödvändiga för att uppehålla immunförsvaret livet ut. Dessutom fann vi att det är mer fördelaktigt att transplantera i fosterlivet, då högre återuppbyggande av immunförsvaretsceller uppnås på detta sätt än genom transplantation till nyfödda eller vuxna möss. I sista delen undersökte vi den såkallade blodbildande cellens plasticitet (förmågan att bidra till andra celltyper än blodceller), en egenskap som förhoppningsvis kan utnyttjas i terapeutiskt syfte också vid ämnesomsättning rubbningar. Vi visade att blodceller bidrar till muskelceller, leverceller och Purkinje celler i lillhjärnan genom en mekanism som kallas fusion (sammansmältning med celler i mål vävnaden). Cell fusion skedde med låg frekvens och endast i samband med orsakad skada (genom strålning, toxin eller dylikt) i vävnaden av vuxna möss. Blodceller transplanterade i fosterstadiet (men inte vid födseln) har också bidragit till leverceller och Purkinje celler, dock inte till muskelceller. Uppenbarligen kan blodceller bidra under en kort period av fosterutvecklingen till andra organ, dock till synes genom en inflammatorisk mekanism. Dessa fynd måste noggrant reproduceras och

betecknas med avseende till huruvida denna är en fysiologisk process som kanske går att manipulera i terapeutiska syften.

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Article I

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Identification of Flt3+ Lympho-Myeloid Stem Cells Lacking Erythro-Megakaryocytic Potential: A Revised Road Map for Adult Blood Lineage Commitment

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Summary

All blood cell lineages derive from a common hematopoietic stem cell (HSC). The current model implicates that the first lineage commitment step of adult pluripotent HSCs results in a strict separation into common lymphoid and common myeloid precursors. We present evidence for a population of cells which, although sustaining a high proliferative and combined lympho-myeloid differentiation potential, have lost the ability to adopt erythroid and megakaryocyte lineage fates. Cells in the Lin$^-$Sca-1$^+$c-kit$^+$ HSC compartment coexpressing high levels of the tyrosine kinase receptor Flt3 sustain granulocyte, monocyte, and B and T cell potentials but in contrast to Lin$^-$Sca-1$^+$c-kit$^+$Flt3$^-$ HSCs fail to produce significant erythroid and megakaryocytic progeny. This distinct lineage restriction site is accompanied by downregulation of genes for regulators of erythroid and megakaryocyte development. In agreement with representing a lymphoid primed progenitor, Lin$^-$Sca-1$^+$c-kit$^+$CD34$^+$Flt3$^+$ cells display upregulated IL-7 receptor gene expression. Based on these observations, we propose a revised road map for adult blood lineage development.

Introduction

All blood cell lineages derive from a common hematopoietic stem cell (HSC) responsible for life-long and balanced blood cell production, in man amounting to millions of cells per second in steady state (Ogawa, 1993). Although molecular pathways (cytokine receptors and transcription factors) regulating the development of the different blood cell lineages have been identified (Metcalf, 1993; Shivdasani and Orkin, 1996; Zhu and Emerson, 2002), the role of these and other pathways in governing hematopoietic lineage commitment remains elusive.

Developments in state of the art technologies enabling identification and prospective purification of HSCs and downstream progenitor cells at different stages of commitment (Akashi et al., 2000; Kondo et al., 1997; Matsuzaki et al., 2004; Osawa et al., 1996) have and will continue to play a key role in identifying the molecular mechanisms governing HSC self-renewal as well as lineage fate determination. The identification of common myeloid and lymphoid progenitors (CMPs and CLPs, respectively; Akashi et al., 2000; Kondo et al., 1997) lends support to the classical and currently prevailing model for hematopoietic commitment and blood lineage development, implicating that the first and decisive lineage commitment step of adult HSCs results in an immediate and complete separation of myelopoiesis and lymphopoiesis (Reya et al., 2001). Importantly, this model proposes that short-term HSCs (ST-HSCs) and multipotent progenitors (MPPs), although having reduced self-renewal capacity, sustain the full lympho-myeloid lineage potentials of long-term HSCs (LT-HSCs) (Reya et al., 2001).

Virtually all LT-HSC, ST-HSC, and MPP activities in adult mouse bone marrow (BM) have been shown to reside in the small Lin$^-$Sca-1$^+$c-kit^{hi} (LSK) HSC compartment (0.1% of all BM cells; Ikuta and Weissman, 1992; Li and Johnson, 1995; Spangrude et al., 1988; Weissman et al., 2001). Within the adult LSK compartment, LT-HSCs have been demonstrated to lack expression of CD34 as well as the cytokine tyrosine kinase receptor Flt3 (Adolfsson et al., 2001; Christensen and Weissman, 2001; Osawa et al., 1996), whereas ST-HSCs are LSKCD34$^+$Flt3$^-$ (Osawa et al., 1996; Yang et al., 2005). The LSKCD34$^+$Flt3$^-$ ST-HSC population gives rise to a third population of LSK cells, all coexpressing Flt3 and CD34 and representing as much as 50% of the LSK HSC compartment (Adolfsson et al., 2001). Upon transplantation LSKCD34$^+$Flt3$^+$ (hereafter called LSK Flt3$^+$) cells give primarily rise to rapid and robust lymphoid reconstitution (Adolfsson et al., 2001). A role for Flt3 in early lymphoid development has been further substantiated in studies of mice deficient in expression of Flt3 or its ligand, as these mice have reductions in early B and T cell progenitors as well as the CLPs (Mackarehtschian et al., 1995; McKenna et al., 2000; Sitnicka et al., 2002). Furthermore and in line with the documented synergistic interaction between Flt3 ligand (FL) and IL-7 on uncommitted progenitors (Veiby et al., 1996), mice double deficient in FL and interleukin-7 receptor α (IL-7Rα) expression lack mature B cells and evidence for B cell commitment in fetal as well as adult hematopoiesis (Sitnicka et al., 2003).

Although having a lymphoid-dominated short-term reconstitution potential, LSK Flt3$^+$ cells possess, at the single-cell level, a combined myeloid (granulocyte-monocyte; GM) and lymphoid (B and T cell) differentiation potential (Adolfsson et al., 2001) and should, therefore, according to the current model for HSC lineage commitment (Reya et al., 2001), also possess a megakaryocyte (Mk) and erythroid (E) differentiation potential. However, in contrast to LSKCD34$^+$Flt3$^-$ ST-HSCs and megakaryocyte/erythroid progenitors (MkEPs), LSK Flt3$^+$ cells lack in vivo day 8 clonogenic colony-forming unit spleen (CFU-S$_{d8}$) activity (Yang et al., 2005), typical for reconstituting cells capable of rapidly reconstituting myelo-erythropoiesis following lethal irradiation (Akashi

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et al., 2000; Na Nakorn et al., 2002; Osawa et al., 1996; Till and McCulloch, 1961).

Since the CD45 (common leukocyte antigen) congenic mouse model typically used to assess HSC reconstitution and lineage potentials (Bryder and Jacobsen, 2000) prevents direct evaluation of Mk and E lineage potentials (as platelets and erythrocytes do not express CD45), we here applied a number of complimentary in vitro and in vivo approaches to directly evaluate the Mk and E potentials of LSK Flt3$^+$ cells. Herein, we provide compelling evidence for a novel route for early blood lineage commitment and development, in that LSK Flt3$^+$ cells, although sustaining a high proliferative and combined lympho-myeloid differentiation potential, have lost the ability to significantly adapt Mk and E lineage fates and in agreement with this downregulate expression of a number of genes critically involved in development of these lineages.

Results

Single LSK Flt3$^+$ Lympho-Myeloid Stem/Progenitor Cells Lack Significant In Vitro Megakaryocyte and Erythroid Differentiation Potentials

We recently demonstrated that LSK Flt3$^+$ BM cells, although predominantly and efficiently reconstituting lymphopoiesis in vivo, have a combined B, T, and myeloid differentiation potential (Adolfsson et al., 2001). Specifically, using clonal assays, 95% of single LSK Flt3$^+$ cells possessed an M, 85% G, and 45% a combined B and T cell potential, demonstrating that a large fraction of LSK Flt3$^+$ cells have a combined macrophage, granulocyte, B, and T cell potential (Adolfsson et al., 2001). Here, to directly confirm their lympho-myeloid potential, highly purified (>97%) LSK Flt3$^+$ cells (25% highest Flt3 expressing LSK cells; Figure 1A; Adolfsson et al., 2001) were investigated at the single cell level for their combined B cell and GM potential. As much as 67% of all single LSK Flt3$^+$ cells demonstrated sufficient clonal growth to be evaluated in this assay, of which 70% revealed a combined B and GM potential (Figure 1B).

We also investigated the B cell potential of LSK Flt3$^+$ cells on the OP9 cell line that efficiently supports B cell development (Vieira and Cumano, 2004). When cultured on OP9 for 14–28 days, all single LSK Flt3$^+$-derived clones (40% cloning efficiency) produced B220$^+$CD19$^+$ B cells (Figure 1C).

The recent development of the OP9 cell line expressing the notch ligand delta like1 (OP9-DL1) has dramatically enhanced the efficiency by which cells with T cell potential can be detected from uncommitted progenitors in vitro (Schmitt and Zuniga-Pflucker, 2002; Vieira and Cumano, 2004). When cultured on OP9-DL1, as much as 91% of LSK Flt3$^+$ single cell-derived clones (50% cloning efficiency) contained CD3ϵ-, CD4-, and CD8α-expressing cells (Figure 1C). Noteworthy, in clones investigated, we concomitantly to T cell development frequently also observed production of Mac-1$^+$ myeloid cells (data not shown). In further support of their T cell potential, we also investigated by PCR the single LSKflt3$^+$-derived clones on OP9-DL1 for expression of the early T cell-restricted genes *CD3ϵ* and *LcK*. Whereas uncultured LSK Flt3$^+$ cells and OP9-DL1 cells lacked detectable expression of these genes, as much as 15 out of 16 (94%) investigated single LSK Flt3$^+$ clones derived on OP9-DL1 expressed both of these genes (Figure 1D), demonstrating efficient commitment of LSK Flt3$^+$ cells along the T-lineage pathway. We also analyzed the recombination status at the *TCRβ* locus and found that 11 out of 12 (92%) analyzed LSK Flt3$^+$-derived clones showed DJ rearrangements. These were mainly D_2-$J\beta_{2.1}$ but other recombination events could be detected as well (Figure 1E). Thus, a majority of LSK Flt3$^+$ cells possess a combined granulocyte, monocyte, B, and T cell potentials.

The current model for hematopoietic development suggests that the first lineage commitment step of HSCs leads to a strict separation of myelopoiesis (GM and megakaryocyte/erythroid; Mk and E lineages) and lymphopoiesis (Akashi et al., 2000; Kondo et al., 1997; Reya et al., 2001). According to this model, LSK Flt3$^+$ cells having a combined B, T, and GM potential should also possess a Mk and E developmental potential. Since the Mk and E lineages upon differentiation cease to express the pan-hematopoietic marker CD45, typically used to track all other blood cell lineages in vivo (Bryder and Jacobsen, 2000), we here first compared the in vitro Mk potential of highly purified LSK Flt3$^+$ and LSK Flt3$^-$ cells containing LSKCD34$^-$Flt3$^-$ LT-HSCs but predominantly LSKCD34$^+$Flt3$^-$ ST-HSCs (Yang et al., 2005). Strikingly, whereas as much as 57% of single LSK Flt3$^-$ cells produced Mk in response to a cytokine combination that included thrombopoietin (TPO), the primary regulator of Mk production (Gurney et al., 1994), only 2% of purified LSK Flt3$^+$ cells produced Mk under the same conditions (KL + FL + TPO + IL-3; KFT3) (Figure 2A). Furthermore, whereas almost 40% of LSK Flt3$^-$ cells produced Mk also in the absence of TPO (KL + IL-3 + EPO + IL-11; K3E11), no Mk development was observed from LSK Flt3$^+$ cells (Figure 2A). Testing of other cytokine combinations and culture conditions efficiently promoting Mk development of LSK Flt3$^-$ cells also failed to promote significant Mk development from LSK Flt3$^+$ cells (J.A. and S.E.J., unpublished data). The inability of LSK Flt3$^+$ cells to generate Mk in vitro was not due to different lineage differentiation kinetics of LSK Flt3$^+$ and LSK Flt3$^-$ cells, as single LSK Flt3$^-$ cells at high frequencies efficiently produced Mk by 8 and 10 days (Figure 2B) as well as 12–15 days (Figure 2A) following initiation of culture, whereas few or no Mk were observed at any time point from LSK Flt3$^+$ cells.

The Mk and E lineages are developmentally closely linked and share a common progenitor (Akashi et al., 2000). Thus, experiments were next designed to also investigate the erythroid differentiation potential of LSK Flt3$^+$ cells in vitro. To pursue this, we developed a new efficient and highly specific assay for directly investigating the erythroid potential of uncommitted progenitor/stem cells at the single cell level (see the Experimental Procedures). Single LSK Flt3$^-$ and LSK Flt3$^+$ cells were cultured under conditions and in a cytokine combination (KFT3E) efficiently supporting myelo-erythroid development, and after 14–15 days, clonally derived cells were investigated by FACS for generation of erythroid (TER119$^+$Gr-1/Mac-1$^-$) progeny. As much as 53% of single LSK Flt3$^-$ cells generated TER119$^+$Gr-1/

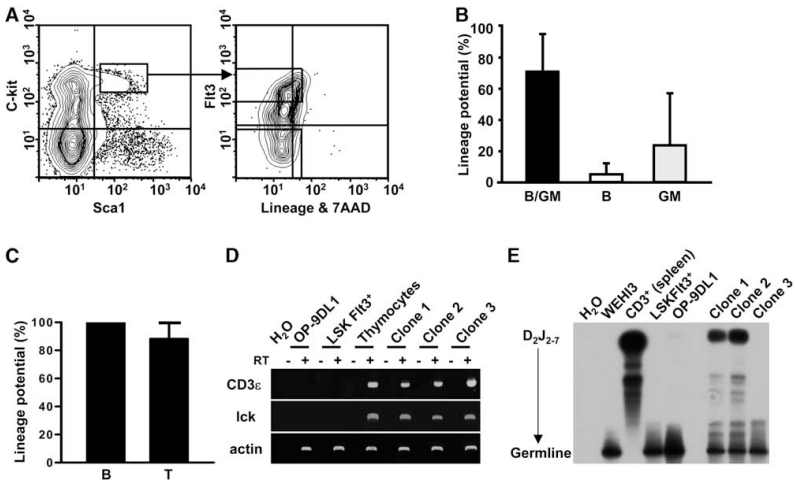

Figure 1. Combined Lympho-Myeloid Differentiation Potential of LSK Flt3+ Cells

(A) Sca-1+ BM cells expressing high levels of c-kit (left) were sorted into Lin−Flt3+ (with highest 25% Flt3 expression) and Lin−Flt3− populations as indicated (right).

(B) Frequencies of clonal single LSK Flt3+ cells demonstrating combined GM and B cell, B cell only, and GM potential only. Mean (SD) values of two experiments.

(C) B and T cell potential of single LSK Flt3+ cells evaluated after 14–28 days of culture on OP9 and OP9-DL1 stroma cells, respectively. Mean (SD) results from FACS analysis of a total of 34 clones on OP9 (from two experiments) and 104 clones grown on OP9-DL1 (from four experiments). Clones were picked at day 14–28 and analyzed by FACS. Clones, grown on OP9 stroma, which contained cells expressing both B220 and CD19 were considered to have B cell potential, and clones grown on OP9-DL1 stroma, if containing CD3ε-, CD4-, and CD8α-expressing cells were considered to have T cell potential. As much as 40% and 50% of LSK Flt3+ cells grown on OP9 and OP9-DL1, respectively, formed large enough clones to be analyzed.

(D) Ethidium bromide-stained agarose gel with PCR products of *ACTIN*, *LcK*, and *CD3ε* message from 1000 OP9-DL1 cells, 1000 uncultured LSK Flt3+ cells, thymocytes, or one twentieth of three representative (out of 16 analyzed) LSK Flt3+ single-cell clones derived on OP9-DL1.

(E) Autoradiogram of *TCRβ D-J2* rearrangements in WEHI-3 (myelomonocytic) cells, sorted CD3+ spleen cells, uncultured LSK Flt3+ cells, OP9-DL1 cells, and three representative (out of 12 analyzed) LSK Flt3+ clones derived on OP9-DL1.

Mac-1− erythroid progeny (Figures 2C and 2D). In contrast, whereas LSK Flt3+ cells efficiently generated myeloid (GM; Gr-1/Mac-1+TER119−) progeny, only 3% (one out of 34 cells investigated) produced erythroid progeny (Figures 2C and 2D). Thus, purified LSK Flt3+ cells lack (beyond the frequency of contaminating LSK Flt3− cells; 1%–3%; Experimental Procedures) signficant in vitro Mk and E differentiation potential.

LSK Flt3+ Lympho-Myeloid Stem/Progenitor Cells Lack Significant In Vivo Megakaryocyte and Erythroid Developmental Potential

The inability of most LSK Flt3+ cells to produce megakaryocytic and erythroid progeny in vitro could potentially be due to the utilized conditions not providing the optimal/unique signals required for Mk and E development from this stem/progenitor cell population. Thus, we next designed in vivo experiments to further investigate the Mk and E potentials of LSK Flt3+ cells. Since Mk progenitors, but not platelets or mature Mk, express the panhematopoietic marker CD45, we used the CD45 congenic mouse model (Bryder and Jacobsen, 2000) to compare the ability of transplanted LSKCD34+Flt3− ST-HSCs and LSK Flt3+ cells to generate Mk progenitors (CFU-Mk) following lethal myeloablation (Figure 3A). Importantly, complete myeloablation results in severe/lethal thrombocytopenia (Kempf et al., 1980; Uchida et al., 1998), thereby activating pathways promoting Mk development. One week following transplantation of 10,000 LSKCD34+Flt3− or LSK Flt3+ cells (CD45.1) in competition with 200,000 unfractionated BM cells (CD45.2), the spleens (and BMs; data not shown) of recipient mice (CD45.2) were highly and comparably reconstituted by (CD45.1+) LSK Flt3+ (mean reconstitution 77%) and LSKCD34+Flt3− (mean reconstitution 75%) cells. However, whereas more than 80 Mk progenitors were generated per transplanted LSKCD34+Flt3− cell at this time, Mk progenitors were almost undetectable among the cells derived from reconstituting LSK Flt3+ cells (Figure 3B), and mice transplanted with low numbers of LSKCD34+Flt3− cells, corresponding to the contamination within sorted LSK Flt3+ cells (1%–3% as determined by reanalysis), demonstrated that the very low numbers of Mk progenitors generated from purified LSK Flt3+ cells could be derived from contaminating LSKCD34+Flt3− cells (J.A. and S.E.J., unpublished data). In contrast, LSK Flt3+ cells produced GM progenitors following transplantation (>100 per transplanted cell), although less than LSK Flt3− cells (Figure 3C).

Experiments were next designed to also investigate

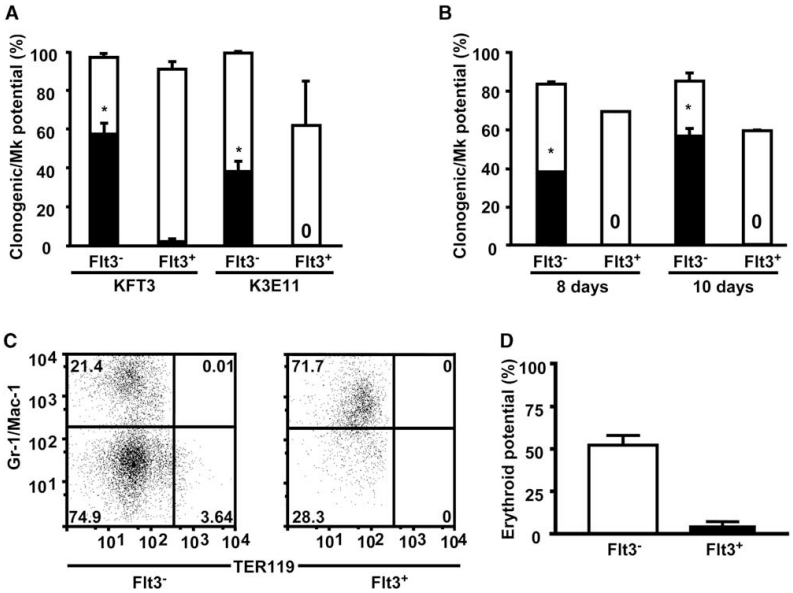

Figure 2. Lack of In Vitro Megakaryocyte and Erythroid Potential of LSK Flt3$^+$ Cells

(A) Single LSK Flt3$^+$ and LSK Flt3$^-$ cells were cultured for 12–15 days in the presence of the indicated cytokine combinations (Experimental Procedures). Bars show frequencies of LSK Flt3$^+$ and LSK Flt3$^-$ cells forming colonies (open bars) and Mks (filled bars). Results represent mean (SEM) values from 3–11 experiments.

(B) Clonal and Mk potential of single LSK Flt3$^+$ and LSK Flt3$^-$ cells cultured in KFT3 for 8 and 10 days. Mean (SD) values of two experiments. * $p < 0.05$, LSK Flt3$^+$ versus LSK Flt3$^-$ cells. 0 = no Mk formed.

(C) Representative profiles of TER119 and GR-1/Mac-1 expression on progeny of single LSK Flt3$^-$ (left) and LSK Flt3$^+$ (right) cells cultured for 14 days in the presence of KFT3E.

(D) Frequencies of single LSK Flt3$^-$ and LSK Flt3$^+$ cells producing erythroid (TER119$^+$Gr-1/Mac-1$^-$) progeny in vitro. Results represent mean (SD) values from 2 experiments, with a total of 30 and 34 clones, investigated for LSK Flt3$^-$ and LSK Flt3$^+$ cells, respectively.

the erythroid differentiation potential of LSK Flt3$^+$ cells in vivo, again taking advantage of lethally irradiated mice developing severe cytopenia and providing an environment permissive for E development. However, since mature erythrocytes lack expression of CD45, we utilized congenic mice expressing different hemoglobin isoforms as donors (Hbb^s) and recipients (Hbb^d) of LSK Flt3$^+$ and LSKCD34$^+$Flt3$^-$ cells (Figures 4A and 4B). Two

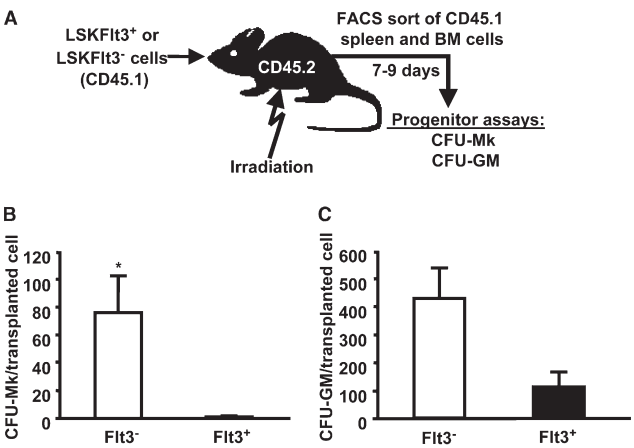

Figure 3. In Vivo Megakaryocyte and Granulocyte/Macrophage Potential of LSK Flt3$^+$ Cells

(A) Experimental design. Lethally irradiated (CD45.2) mice were transplanted with FACS-sorted LSK Flt3$^+$ or LSKCD34$^+$Flt3$^-$ (CD45.1) cells and congeneic (CD45.2) BM support cells. CD45.1$^+$ cells were sorted from spleen and BM 7–9 days posttransplantation and cultured in methylcellulose for an additional 8–12 days and scored for Mk (B) and GM (C) progenitors (CFU-Mk and CFU-GM, respectively). Mean (SEM) values of four experiments. * CFU-Mk $p < 0.05$, LSK Flt3$^+$ versus LSKCD34$^+$Flt3$^-$ cells. The difference in CFU-GM formation of LSK Flt3$^+$ and LSKCD34$^+$Flt3$^-$ cells did not reach statistical significance ($p = 0.11$).

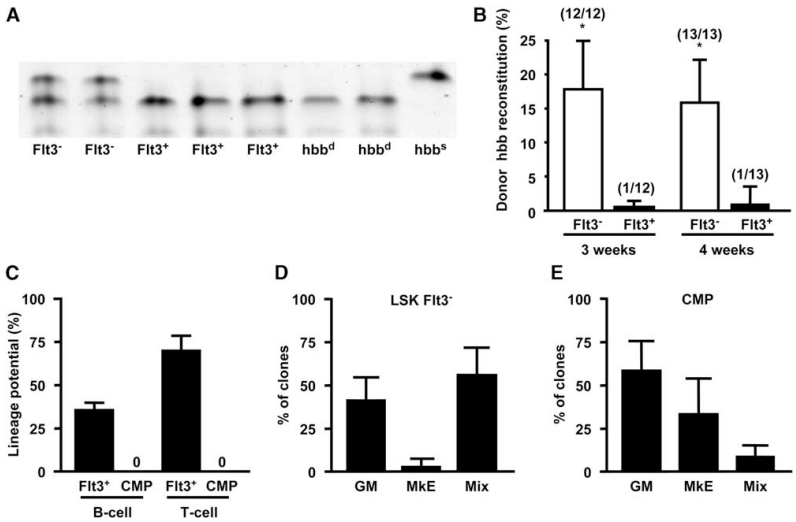

Figure 4. Myeloid Potentials of LSK Flt3+ Cells and CMPs

(A) Lethally irradiated (CD45.2, Hbb^d) mice were transplanted with 200 purified LSK Flt3+ or LSKCD34+Flt3− (CD45.1, Hbb^s) cells and 200,000 congeneic (CD45.2, Hbb^d) support BM cells. PB samples were analyzed at 3 and 4 weeks posttransplantation for recipient (Hbb^d) and donor (Hbb^s) hemoglobin reconstitution by electrophoresis. One representative example of electrophoresis gel with PB from two mice transplanted with 200 LSKCD34+Flt3− cells, three mice transplanted with 200 LSK Flt3+ cells, and three PB samples from nontransplanted mice of donor (Hbb^s) and recipient (Hbb^d) types.

(B) Mean (SD) values from two experiments with 12–13 mice in each group and time point. Numbers above bars show frequency of transplanted mice positive for donor-derived (Hbb^s) erythroid reconstitution. * $p < 0.05$, LSK Flt3+ versus LSKCD34+Flt3− cells.

(C) In vitro B cell (B220+CD19+) and T cell (CD4+CD8+) potential of single LSK CD34+Flt3+ cells and 10 Flt3− CMPs evaluated at 21–28 days of culture on OP9 and OP9-DL1 cell lines, respectively. Results are presented as mean (SD) percentages of single cells with B and T cell potential from three to five experiments. A total of 180 (OP9) and 300 (OP9-DL1) single LSK Flt3+ cells and 1440 (OP9) and 1440 (OP9-DL1) CMPs were investigated.

(D and E) Combined in vitro MkE and GM potential of single LSK Flt3− cells (D) and Flt3− CMPs (E). In each experiment, 360 to 600 single CMPs and LSK Flt3− cells were plated into conditions promoting both MkE and GM development. Results show mean percentages (SD) of analyzed clones containing GM or MkE lineages alone or in combination (Mix), from eight LSK Flt3− and five Flt3− CMPs experiments.

hundred LSK Flt3+ or LSKCD34+Flt3− (Hbb^s) cells were transplanted in competition with 200,000 BM cells (Hbb^d) into lethally irradiated recipients (Hbb^d). Three and four weeks posttransplantation, LSKCD34+Flt3− cells contributed substantially to E reconstitution in all (13/13) mice by as much as 18% and 16%, respectively, whereas only one of thirteen mice showed detectable E reconstitution from transplanted LSK Flt3+ cells (Figures 4A and 4B), although the contribution to other blood lineages (B, T, and myeloid cells) was as much as 14% ± 5% and comparable to that of LSKCD34+Flt3− cells (22% ± 14%). Thus, in contrast to LSKCD34+Flt3− cells, most LSK Flt3+ cells lack in vitro as well as in vivo Mk and E development potential.

Our finding that LSK Flt3+ cells sustain B, T, M, and G potential but lack significant Mk and E potential could be compatible with CMPs' (Akashi et al., 2000; Miyamoto et al., 2002) predominantly being derived from LSK Flt3− (or potentially LSK Flt3^{low}) cells that also have Mk and E potential. Thus, we next compared the combined GM and MkE potentials of single LSK Flt3− cells and CMPs. As recent studies had demonstrated that only the Flt3− fraction of CMPs is myeloid restricted (D'Amico and Wu, 2003), we purified Flt3− CMPs (Lin−IL-7Rα−Sca-1−c-kit+CD34+FcR−Flt3−; see Figure S1 in the Supplemental Data available with this article online). Importantly, no analyzed Flt3− CMPs generated B or T cells on OP9 (1440 cells total; n = 3) and OP9-DL1 (1440 cells total; n = 3), respectively (Figure 4C). Using culture conditions promoting both MkE and GM differentiation (Experimental Procedures and Figure 4), as much as 83% (n = 8) and 60% (n = 5) of single LSK Flt3− cells and Flt3− CMPs formed clones, respectively. Noteworthy, whereas 56% of analyzed LSK Flt3− clones had a mixed MkE and GM composition (Figure 4D), pure GM (59%) and MkE (33%) colonies dominated among Flt3− CMP clones analyzed, although 8% of Flt3− CMPs revealed a combined MkE and GM potential (Figure 4E). Another striking observation was that as much as 40% (n = 5) of all Flt3− CMP-derived clones represented small colonies (too small for cytospin analysis) composed exclusively of large megakaryocytes.

Downregulation of the Expression of Genes for Nonredundant Regulators of Megakaryocyte and Erythroid Development in LSK Flt3+ Cells

A number of cytokine receptors and transcription factors have been demonstrated to be critically involved in Mk and E development (Cantor and Orkin,

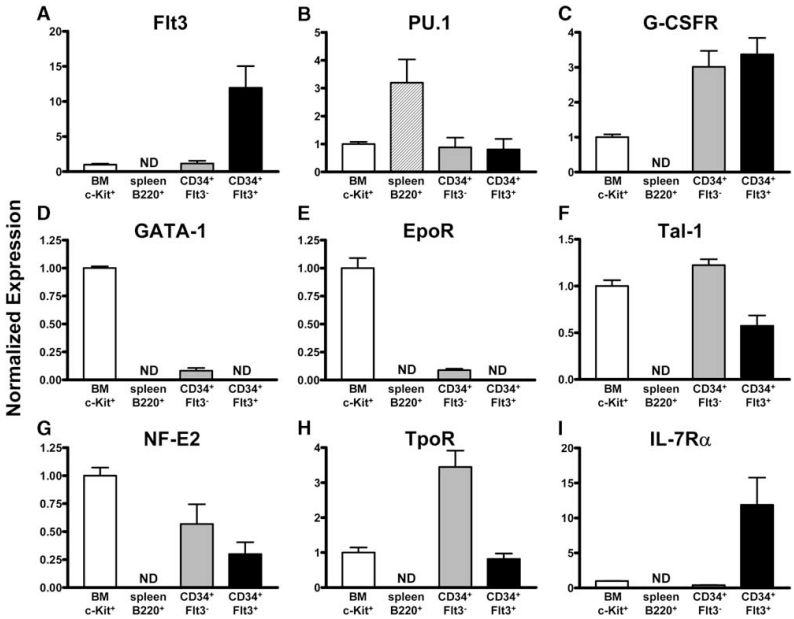

Figure 5. Downregulation of Essential Erythroid and Megakaryocyte Transcription Factors and Cytokine Receptor Genes and Upregulation of *IL-7Rα* mRNA Expression in LSK Flt3+ Cells

Quantitative PCR data of indicated genes in FACS-sorted LSKCD34+Flt3− and LSK Flt3+ cells. Lin−c-kit+ BM cells (containing committed progenitor cells of Mk, E, GM, and B lineages), and B220+ spleen cells (mature B cells) were used as controls for the PCR analysis, with the expression levels in Lin−c-kit+ cells set to 1. All data were normalized to the expression of *HPRT*. Results represent mean (SEM) values from at least two independent experiments, with PCR analysis in each experiment performed in triplicates. ND = no detectable expression after 45 cycles of PCR.

2002; Shivdasani and Orkin, 1996; Zhu and Emerson, 2002). In particular, Mk as well as E development are strictly dependent on the transcription factors *GATA-1* and *SCL/TAL-1* (Mikkola et al., 2003; Orkin et al., 1998; Shivdasani and Orkin, 1996). Importantly, the expression of these genes has not only been reported in E- and Mk-committed progenitors, but also in multipotent lympho-myeloid HSC populations with E and Mk potential, as has expression of the genes for the nonredundant hematopoietin receptors for Epo and Tpo (EpoR and TpoR, respectively) (Akashi et al., 2000; Miyamoto et al., 2000; Terskikh et al., 2003). Thus, any multipotent stem/progenitor cell population downstream of HSCs with E and Mk potential would be expected to express these genes. Using quantitative RT-PCR, we found in agreement with their sustained GM potential that LSK Flt3+ as LSKCD34+Flt3− ST-HSCs expressed high levels of mRNA for the transcription factor *PU.1* as well as the *G-CSFR* (Figures 5B and 5C). However, in support of their loss of E and Mk developmental potential, LSK Flt3+ cells, in contrast to LSKCD34+Flt3− cells, revealed no detectable gene expression of *GATA-1* and *EpoR* (Figures 5D and 5E) and had reduced expression of *SCL/TAL-1*, *NF-E2*, and *TpoR* (Figures 5F–5H). Most noteworthy, whereas LSKCD34+Flt3− ST-HSCs as expected expressed little or no mRNA for *IL-7Rα,* the expression was clearly upregulated in LSK Flt3+ cells (Figure 5I).

We also performed a multiplex single-cell RT-PCR analysis (Hu et al., 1997) to investigate whether LSK Flt3+ cells that had turned on *IL-7Rα* expression might still represent multipotent progenitors with sustained myeloid potential and gene expression or rather more lymphoid-restricted progenitors. Whereas a majority of single LSKCD34+Flt3− cells were found to express *G-CSFR* mRNA, none were *IL-7Rα*+ (Figure 6A). In contrast, 9% of single LSK Flt3+ cells were found to express detectable *IL-7Rα* mRNA (Figure 6B), and, noteworthy, the vast majority of these continued to coexpress the gene for the nonredundant myeloid *G-CSFR* (Figures 6B and 6C).

Thus, in contrast to upstream pluripotent LSKCD34+ Flt3− ST-HSCs and downstream committed E and Mk progenitors, LSK Flt3+ cells downregulate expression of transcription factors and cytokine receptors critically involved in development of the E and Mk lineages, sustain expression of GM-associated genes, and upregulate expression of the *IL-7Rα*, required for B and T cell development (Peschon et al., 1994).

Discussion

In the present studies, we provide compelling evidence for the existence in adult hematopoiesis of a prominent and potent lympho-myeloid stem/progenitor cell population within the LSK HSC compartment, which lacks

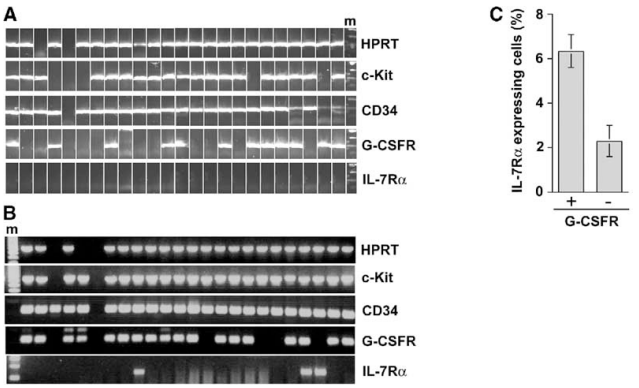

Figure 6. Multiplex Single-Cell RT-PCR Demonstrates Coexpression of *G-CSFR* and *IL-7Rα* in LSK Flt3+ Cells

Representative gel analysis data for expression of *HPRT*, *CD34*, *c-kit*, *G-CSFR*, and *IL-7Rα* in single LSKCD34+Flt3− cells (A) and LSK Flt3+ cells (B).

(C) Frequency (SD) of *G-CSFR* coexpression on *IL-7Rα*-expressing LSK Flt3+ cells. Only cells in which expression of *CD34* and *c-kit* as well as the housekeeping gene *HPRT* were detected were included for further analysis of *IL-7Rα* and *G-CSFR* expression. Mean (SD) values from two independent experiments, representing a total of three and two 96-well plates analyzed for LSKCD34+Flt3+ and LSKCD34+Flt3− cells, respectively. M = marker.

significant Mk and E developmental potential, contrary to what would be predicted from the classical and prevailing model for hematopoietic lineage commitment in adult hematopoiesis (Akashi et al., 2000; Kondo et al., 1997; Reya et al., 2001). That LSK(CD34+)Flt3+ cells (25% highest Flt3-expressing LSK cells), although sustaining a combined myeloid and lymphoid potential at the single cell level, have already undergone a first step of HSC lineage restriction through loss of Mk and E potential was substantiated by several lines of experimental evidence. First, in vitro and in vivo biological assays efficiently promoting Mk and E development from LSKCD34+Flt3− HSCs, including myeloablative conditioning activating endogenous pathways for Mk and E production, failed to promote significant development of these lineages from LSK Flt3+ cells beyond what could be expected from sorting impurities (1%–3%). Furthermore, in contrast to the LSKCD34+Flt3− ST-HSCs, LSK Flt3+ cells lacked detectable mRNA expression for *GATA-1* and *EpoR* and downregulated gene expression for *SCL/TAL1*, *NF-E2*, and *TpoR*. This combined with the fact that not only committed MkEPs (Akashi et al., 2000; Miyamoto et al., 2002) but also upstream HSCs (in agreement with previous studies; Miyamoto et al., 2002) express the genes for critical regulators of Mk and E development strongly support that LSK Flt3+ cells have undergone an irreversible lineage commitment step through downregulation of Mk and E development programs. That the first HSC lineage restriction step might involve simultaneous loss of Mk and E potentials is not so surprising in light of Mk and E lineages sharing a common progenitor, MkEPs (Akashi et al., 2000), as well as critical regulators of lineage development such as GATA-1 and SCL/TAL-1 (Cantor and Orkin, 2002; Mikkola et al., 2003; Shivdasani and Orkin, 1996). In contrast and in agreement with their sustained G and M potentials, LSK Flt3+ cells maintained similar expression levels of *PU.1* and *G-CSFR* as LSK Flt3− HSCs.

Contrary to the current model for hematopoietic development (Figure 7A), our findings do not support that the earliest lineage restriction or commitment event of adult HSCs results exclusively in a strict separation into common lymphoid and common myeloid differentiation pathways. The generation of LSK Flt3+ cells with G, M, B, and T cell, but little or no Mk and E potentials, would be more compatible with a model in which MkEPs and LSK Flt3+ cells are primarily generated through asymmetric cell divisions of LSKCD34+Flt3−/low HSCs (Figure 7B; Yang et al., 2005). A similar model has in fact previously been proposed based on a hierarchy of transcription factors, with PU.1-deficient HSCs giving rise to Mk and E progenitors, but not progenitors of the G, M, B, and T cell lineages (Singh, 1996). Recent studies of paired daughter cells of purified HSCs also support that MkEPs might develop from HSCs without a CMP intermediate (Takano et al., 2004). Furthermore, such a model is also compatible with the observation of myeloid (erythroid- and macrophage-like cells) emerging earlier in evolution (Hansen and Zapata, 1998) and ontogeny (Cumano and Godin, 2001) than lymphoid cells.

Alternative and more complex models of HSC lineage commitment are needed to reconcile the evidence for CMPs (Akashi et al., 2000; Miyamoto et al., 2002) and our findings of LSK Flt3+ lymphoid-primed multipotent progenitors (LMPPs). In one such model, LSKCD34+Flt3−/low ST-HSCs could upon asymmetrical cell divisions give rise to CMPs and LMPPs (Figure 7C). Importantly, our studies support the existence of myeloid-restricted CMPs with a combined MkE and GM potential (Akashi et al., 2000). However, when compared to LSK Flt3−, cells very few Flt3− CMPs were found to give rise to mixed MkE and GM colonies, and in agreement with recent studies (Nutt et al., 2005), the Lin−IL-7Rα−Sca-1−c-kit+CD34+FcR−Flt3− candidate CMP population gave rise to primarily pure GM or MkE colonies. Thus, although Flt3− CMPs represent one pathway for generation of Mk, E, and GM progenitors, it might not represent an obligate or even dominating intermediate for myeloid development from HSCs. Rather than a strict hierarchical model, our findings might be equally compatible with a commitment process occurring on a probabilistic or stochastic basis (Nakahata et al., 1982; Siminovitch et al., 1963) in which the probability of different commitment fates can be altered by intrinsic and extrinsic cues, differentially expressed within the LSK HSC hierarchy. If so, LSKFlt3+/high cells might potentially possess some residual MkE potential, although our findings of little or no MkE development in the utilized assays, would suggest that such a potential must be very restricted.

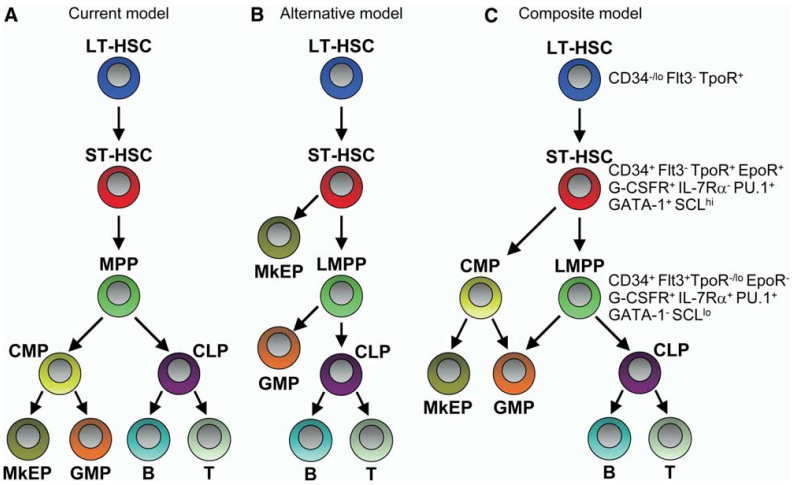

Figure 7. Current and Alternative Models for Hematopoietic Stem Cell and Blood Lineage Commitment

(A) Current model (Reya et al., 2001) for hematopoietic lineage commitment and development, postulating that the first lineage commitment step of HSCs results in a strict separation of myeloopoiesis and lymphopoiesis as supported through the identification of CMPs and CLPs, respectively (Akashi et al., 2000; Kondo et al., 1997).

(B) Alternative model, based on the present studies, in which a pluripotent HSC upon loss of Mk and E potential develops into a lymphoid primed multipotent progenitor (LMPP) that upon loss of GM potential generates the CLP (Adolfsson et al., 2001).

(C) Composite model, incorporating the experimental evidence for models (A) and (B). Also shown are expression of key genes in ST-HSCs and LMPPs based on Q-PCR data.

LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; MPP, multipotent progenitor; LMPP, lymphoid-primed multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage pronenitor; MkEP, megakaryocyte/erythroid progenitor; B, B cell; T, T cell.

Our findings are particularly intriguing in light of recent gene profiling studies of HSCs (with full myeloid and lymphoid lineage differentiation potentials), revealing wide expression of myeloid (MkE and GM) but not lymphoid gene programs (Delassus et al., 1999; Miyamoto et al., 2002). Combined with our finding, this observation strongly supports that HSCs might initially be primed to undergo myeloid (Mk, E, and subsequently GM) commitment (Figure 7B) and that lymphoid commitment through an LSK Flt3$^+$ LMPP stage depends on subsequent activation of lymphoid genes. In that regard, it is noteworthy that LSK Flt3$^+$ cells upregulate gene expression for *FLT3* and *IL-7R*α, the two cytokine receptors critically involved in lymphoid and B cell commitment (Peschon et al., 1994; Sitnicka et al., 2002). Furthermore, in agreement with their sustained G and M potential, multiplex single cell PCR analysis demonstrates that *IL-7R*α^+ LSK Flt3$^+$ cells also sustain expression of the *G-CSFR*.

In fetal (but not adult) hematopoiesis, a number of previous observations have implicated the potential existence of lympho-myeloid lineage-restricted progenitors, primarily with a combined B cell, monocyte (Cumano et al., 1992; Mebius et al., 2001), and in some cases also T cell (Lacaud et al., 1998; Lu et al., 2002) potential. However, as emphasized by others (Ema and Nakauchi, 2003; Katsura, 2002), definitive conclusions have been difficult to reach based on these studies, since they have been limited by the fact that the candidate progenitors in question have not been prospectively purified and characterized and therefore present at low frequencies and assayed along with other progenitors showing additional lineage potentials in the utilized assays. Thus, under such conditions it has not been possible to conclude whether or not progenitors showing restricted lineage development are in fact lineage restricted or whether it rather reflects the inability of the utilized assays to efficiently support the full lineage potentials of all progenitors investigated. For instance, the granulocyte potential of a multipotent progenitor might go unnoticed if investigated at the wrong time or under suboptimal conditions, as granulocytes are very short lived in vitro, whereas monocytes accumulate with time in such cultures. Thus, to prove the existence of other lineage-restricted progenitors, prospective purification must be combined with characterization of their lineage potentials in efficient in vitro and in vivo assays for all lineage potentials, as demonstrated for LSK Flt3$^+$ cells in the present studies. Prospective purification of such populations will also be required to obtain meaningful genetic information regarding the genetic determinants of lineage commitment.

In conclusion, we have in the adult mouse bone marrow LSK HSC compartment identified a prominent LSK Flt3$^+$ LMPP, which in contrast to true ST- and LT-HSCs lacks significant Mk and E potential but sustains other blood lineage developmental potentials. This repre-

sents the earliest known lineage commitment/restriction step of HSCs, leading us to propose that an alternative or complimentary road map to that of the classical hematopoietic hierarchy must guide adult blood cell lineage commitment and development. Its exact identification should greatly facilitate delineation of the molecular pathways regulating these and other HSC fate decisions.

Experimental Procedures

Mice

Wild-type C57BL/6(Hbb^s) mice were from M&B (Ry, Denmark) and C57BL/6-Hbb^d mice from Jackson Laboratories (Maine). All mice procedures were performed with consent from the local ethics committee at Lund University.

Hematopoietic Growth Factors

All cytokines were used at predetermined optimal concentrations, and all human cytokines utilized have been shown to be fully cross-reactive with mouse cells. For detailed information on cytokines see the Supplemental Experimental Procedures.

FACS Purification of Subpopulations of Lin$^-$Sca-1$^+$c-kit^{hi} Bone Marrow Cells and CMPs

All sorts were performed by immunomagnetic-based preenrichment followed by multicolor flow cytometric sorting as previously described (Adolfsson et al., 2001; Bryder and Jacobsen, 2000). Briefly, bone marrow cells were incubated in a cocktail of predetermined optimal concentrations of lineage (Lin) antibodies: purified RA3-6B2 (B220), RB6-8C5 (Gr-1), M1/70 (Mac-1), 53-6.7 (CD8), 53-7 (CD5), H129.19 (CD4), and Ter119, all from PharMingen (San Diego, California). Lin$^+$ cells were then depleted using sheep anti-rat IgG (Fc)-conjugated immunomagnetic beads (Dynal, Oslo, Norway). Lineage-negative/low (Lin$^{-/lo}$) cells were incubated with CyChrome-conjugated goat anti-rat antibody (Caltag Laboratories, Burlingame, California) and subsequently stained with FITC-conjugated rat anti-mouse E13-161.7 antibody (Sca-1), APC-conjugated rat anti-mouse 2B8 (c-kit), and PE- or biotin-conjugated rat anti-mouse Flt3 (A2F10.1) plus streptavidin-PE or isotype-matched control antibodies (all PharMingen). Cells were also stained with 7-amino actinomycin (7-AAD; Sigma-Aldrich Co., St. Louis, Missouri) to exclude dead cells. To exclude long-term stem cells (Osawa et al., 1996), Lin$^{-/lo}$ cells were in some experiments stained with FITC-conjugated rat anti-mouse RAM-34 antibody (CD34), APC-conjugated rat anti-mouse 2B8 (c-kit), PE-conjugated rat anti-mouse Flt3 (A2F10.1), and biotin-conjugated E13-161.7 (Sca-1) plus streptavidin-Tricolor (Caltag). For isolation of Flt3$^-$ CMPs (Akashi et al., 2000; D'Amico and Wu, 2003), Lin$^-$ BM cells were isolated and remaining Lin$^+$ cells visualized as described above. Cells were subsequently stained with 7-AAD, FITC-conjugated rat anti-mouse RAM34 (CD34), PE-conjugated rat anti-mouse 2.4G2 (CD16/32; FcγR), Tricolor-conjugated rat anti-mouse D7 (eBiosciences, San Diego, California; Sca-1) and Tricolor rat anti-mouse A7R34 (IL7Rα; eBiosciences), biotin-conjugated rat anti-mouse A2F10.1 (Flt3) plus streptavidin-PE-Texas-Red (Caltag), or isotype-matched control antibodies (all PharMingen unless otherwise indicated). To obtain high purity (reproducibly 97%–99% upon reanalysis) Lin$^{-/lo}$Sca-1$^+$c-kit^{hi}(LSK)Flt3$^{+/-}$ and Lin$^-$Sca-1$^-$c-kit$^+$IL7Rα^-FcγR^{lo}CD34$^+$Flt3$^-$ CMPs were sorted twice on a FACSVantage or FACSDiva Cell Sorter (Becton Dickinson).

Single-Cell Assay for Combined B Cell and Granulocyte/Macrophage Potential

LSK Flt3$^+$ cells were seeded in Terasaki plates at a concentration of one cell per well in 20 μl serum-free medium (X-vivo15; BioWhittaker, Walkersville, Maryland), supplemented with 1% detoxified Bovine Serum Albumin (StemCell Technologies Inc., Vancouver, Canada). Cells were cultured in kit ligand (KL) + Flt3 ligand (FL) + IL-7 for 6 days, at which time proliferating clones were divided, with one part of the cells continuing in KL + FL + IL-7 for an additional 9 days to establish their B cell potential through evaluation of B220$^+$CD19$^+$ cells produced in cultures (Veiby et al., 1996). The other part of the cells were switched to culture conditions promoting GM development (IMDM, BioWhittaker; supplemented with 20% FCS, BioWhittaker), and a cytokine cocktail composed of KL + FL + IL-3 + thrombopoietin (TPO) + granulocyte colony-stimulating factor (G-CSF) + granulocyte-macrophage colony-stimulating factor (GM-CSF) for an additional 9 days. GM potential was established by transferring individual colonies to slides in a cytospin centrifuge and examining cells morphologically after May-Grünwald/Giemsa-staining.

Clonogenic B and T Cell Assays on OP9 and OP9-Delta Like1 (OP9-DL1) Cell Lines

OP9 and OP9-DL1 cell lines (kindly provided by Drs. A. Cumano, Paris, and J.C. Zuniga Pflucker, Toronto) were maintained as described (Schmitt and Zuniga-Pflucker, 2002; Vieira and Cumano, 2004) in OptiMEM with L-Glutamine (Invitrogen Corporation, Carlsbad, California) supplemented with 10% FCS (Gibco Paisley, Scotland). Cell lines were trypsinized and prepared at a density of 2 × 10^4 cells/ml. Single LSK Flt3$^+$ cells and CMPs were deposited into wells containing OP9 or OP9-DL1 cell lines (supplemented with FL 50 ng/ml and IL-7 100 ng/ml in OP9 and FL 50 ng/ml in OP-DL1 cocultures and in some experiments with 25 ng/ml KL) by a single cell depositor unit coupled to a FACSDiva (Becton Dickinson), providing single cells in >98% of the wells and no wells with more than 1 cell. Clones were identified and picked at days 14, 21, and 28 and analyzed by FACS for presence of B cell (defined as B220$^+$CD19$^+$) and T cell (defined as CD3ϵ^+, CD4$^+$, and CD8α^+) committed progeny.

Reverse transcriptase and Polymerase chain reaction analysis of the clones generated on OP9-DL1 was preformed as described in the Supplemental Experimental Procedures.

Evaluation of In Vitro Megakaryocyte Potential of Single LSK Flt3$^{+/-}$ Cells

LSK Flt3$^+$ and LSK Flt3$^-$ cells were seeded in Terasaki plates at a concentration of one cell per well in 20 μl serum-free medium (as above) supplemented with KL + FL + TPO + IL-3 (KFT3) or KL + IL-3 + erythropoietin (EPO) + IL-11 (K3E11). Wells were scored for cell growth at different time points. Mk-containing colonies were identified by light microscopy and confirmed morphologically after transferring individual colonies to slides in a cytospin centrifuge and subsequent May-Grünwald/Giemsa-staining.

In Vitro Erythroid Potential Assay

Single LSK Flt3$^-$ and LSK Flt3$^+$ cells were seeded in Terasaki plates in 20 μl IMDM supplemented with 1% BIT 9500 (StemCell Technologies) and KL (50 ng/ml), FL (50 ng/ml), TPO (50 ng/ml), IL-3 (10 ng/ml), and EPO (5 U/ml). After 14–15 days of culture, clones were picked and stained with PE-conjugated rat anti-mouse TER119 and APC-conjugated rat anti-mouse Gr-1 (RB6-8C5) and Mac-1 (M1/70) (all from Becton Dickinson) as well as 7-AAD. Erythroid potential was defined by the presence of TER119$^+$Gr-1/Mac-1$^-$ cells. Culture-derived TER119$^+$Gr-1/Mac-1$^-$ cells were also sorted with a FACSDiva and subsequently stained with May-Grünwald/Giemsa to confirm erythroid identity by morphology (data not shown).

In Vitro Combined Myeloid Potentials of CMPs

LSK Flt3$^-$ cells and CMPs were deposited as single cells (using a single cell depositor; see above) into Terasaki plates in 20 μl medium (X-vivo15) supplemented with 10% FCS (Gibco) and 0.5% BSA, 0.1mM β-mercaptoethanol and cytokines, KL + FL + TPO (50 ng/ml each) + IL-3 (10 ng/ml) + EPO (5 U/ml), KL + FL + TPO + G-CSF (all 50 ng/ml) + IL-3 (10 ng/ml) + EPO (3 U/ml), or KL + FL + TPO + G-CSF (all 50 ng/ml) + IL-3 (10 ng/ml) + GM-CSF (20 ng/ml) + EPO (5 U/ml), all giving similar results. After initial optimization of growth conditions and kinetics, cell growth and multilineage (Mk, E, G, and M) differentiation (after May-Grünwald/Giemsa staining of clones containing over 100 cells) were established at day 13–14 for LSK Flt3$^-$ cells and at day 6–7 for CMPs.

In Vivo Megakaryocte and Granulocyte/Macrophage Potential Assay

Ten thousand LSK Flt3$^+$ or LSK Flt3$^-$ cells freshly isolated from C57BL/6 mice (CD45.1) were injected intravenously to lethally irradiated (975 cGy) C57BL/6 mice (CD45.2), along with 200,000 unfractionated congeneic (CD45.2) BM cells providing a competitor and survival population. Seven to nine days posttransplantation, spleen and BM cells were stained with CD45.1, CD45.2, and 7-AAD. LSK Flt3$^{+/-}$-derived (CD45.1$^+$CD45.2$^-$7-AAD$^-$) cells were sorted on a FACSDiva and evaluated for Mk progenitor potential in serum-free methylcellulose (M3226; StemCell Technologies Inc.) supplemented with KL + TPO and for colony forming unit-granylocyte/macrophage (CFU-GM) in FCS containing methylcellulose (M3134; StemCell Technologies Inc.) supplemented with KL + TPO + FL + IL-3 + G-CSF + GM-CSF. GM and Mk colony formation was evaluated following 8–12 days of in vitro incubation.

In Vivo Erythroid Potential Assay

Two hundred FACS-sorted LSK Flt3$^+$ or LSKCD34$^+$Flt3$^-$ cells from C57BL/6-Hbb^s (CD45.1) mice were transplanted into lethally irradiated C57BL/6-Hbb^d (CD45.2) mice, along with 200,000 unfractionated congeneic (C57BL/6-Hbb^d, CD45.2) BM cells. At 3 and 4 weeks after transplantation, peripheral blood (PB) was analyzed for Hbb^s and Hbb^d reconstitution (Harrison et al., 1988; Whitney, 1978). Briefly, PB was washed twice with 3 ml PBS (PAA Laboratories GmbH, Linz, Austria). Twenty microliters packed red blood cells were lysed and cystamine modified with 150 μl cystamine lyse solution for 30 min. The different Hbb isoforms were separated and visualized using a hemoglobin electrophoresis kit (P/N 4411780; Beckman Coulter Inc., Fullerton, California). Gel-pro analyser version 2.0 (Media Cybernetics, Silver Springs, Massachusetts) software was used for quantification of percentage hemoglobin reconstitution. In each experiment, PB from nontransplanted C57BL/6-Hbb^d (CD45.2) mice were used as a negative control and PB from nontransplanted C57BL/6-Hbb^s (CD45.1) mice as a positive control. The mean background of the electrophoresis assay was 4% (SD = 1%; n = 7).

Quantitative RT-PCR

LSKCD34$^+$Flt3$^{-/+}$ cells were FACSDiva sorted directly into 75 μl of buffer-RLT and frozen at –80°C. RNA extraction and DNase treatment was performed with the RNeasy Micro kit (Qiagen Inc., California) according to manufacturers' instructions for samples containing $\leq 10^5$ cells. Eluted RNA samples were reverse transcribed using SuperScript II and random hexamers (Invitrogen) according to protocol supplied by the manufacturer. Newly synthesized cDNA was diluted to approximately contain cDNA from 30 cells/μl and frozen at –20°C. Q-PCR reactions were performed by mixing 2× TaqMan universal PCR master mix, 20× Assays-on-Demand (primer/MGB-probe mix), RNase-free H20, and 5μl of cDNA for a final reaction volume of 25 μl. TaqMan Assays-on-Demand probes used are described in the Supplemental Experimental Procedures. All experiments were performed in triplicates, and differences in cDNA input were compensated by normalizing against HPRT expression levels.

Analysis of Single Cells by Reverse Transcriptase-Polymerase Chain Reaction

Multiplex single-cell RT-PCR analysis of LSK Flt3$^+$ and LSKCD34$^+$Flt3$^-$ cells was performed according to the methods described by Hu et al. (Hu et al., 1997). Single cells were deposited by a single-cell depositor coupled to a FACSDiva as described above (>98% of the wells contained single cells), into 96-well PCR plates containing 4 μl lysis buffer (0.4% Nonidet P-40, 65 μmol/l dNTPs, 25 μmol/l dithiothreitol, and 0.5 U/μl RNaseOUT (Invitrogene Corporation, California). Cell lysates were reverse transcribed using multiple (up to 6) pairs of gene-specific reverse primers and 50 U MMLV-RT per reaction in the buffer provided by the supplier (Invitrogene Corporation, California). The first-round PCR with 35 cycles was performed by addition of 40 μl PCR buffer and 1.25 U Taq polymerase (TaKaRa Bio Inc., Shiga, Japan). One microliter aliquots of first-round PCRs were further amplified using fully nested gene-specific primers. Aliquots of second-round PCR products were subjected to gel electrophoresis and visualized by ethidium bromide staining on ordinary agarose gels or E-gels (Invitrogene). The sequences for the external and internal oligonucleotide primers for *HPRT*, *CD34*, *c-kit*, and *G-CSFR* were kindly provided by Professor Tariq Enver, Weatherall Institute, Oxford, England. The external primer sequences used are described in the Supplemental Experimental Procedures. Empty wells were used as negative controls and never showed any signals for any of the investigated genes.

Statistics

The statistical significance of difference between groups was determined using the two-tailed paired Student's t test. For the progenitor data, the Student's t test was performed after log transformation of the data.

Supplemental Data

Supplemental Data include one figure and Supplemental Experimental Procedures and can be found with this article online http://www.cell.com/cgi/content/full/121/2/295/DC1/.

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Article II

Polyclonal T-cell reconstitution of X-SCID recipients after in utero transplantation of lymphoid-primed multipotent progenitors

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Although successful in utero hematopoietic cell transplantation (IUHCT) of X-linked severe combined immune deficiency (X-SCID) with enriched stem and progenitor cells was achieved more than a decade ago, it remains applied only in rare cases. Although this in part reflects that postnatal transplantations have overall given good results, there are no direct comparisons between IUHCT and postnatal transplantations of X-SCID. The proposed tolerance of the fetal immune system to foreign human leukocyte antigen early in gestation, a main rationale behind IUHCT, has recently been challenged by evidence for a considerable immune barrier against in utero transplanted allogeneic bone marrow cells. Consequently, there is need for further exploring the application of purified stem and progenitor cells to overcome this barrier also in IUHCT. Herein, we demonstrate in a congenic setting that recently identified lymphoid-primed multipotent progenitors are superior to hematopoietic stem cells in providing rapid lymphoid reconstitution after IUHCT of X-SCID recipients, and sustain in the long-term B cells, polyclonal T cells, as well as short-lived B-cell progenitors and thymic T-cell precursors. We further provide evidence for IUHCT of hematopoietic stem cells giving superior B- and T-cell reconstitution in fetal X-SCID recipients compared with neonatal and adolescent recipients. (Blood. 2009; 113:4790-4798)

Introduction

X-linked severe combined immune deficiency (X-SCID), the most frequent form of SCID (46%, ~1:20 000 births),[1,2] is caused by a mutation in the gene encoding the common gamma chain, shared by multiple cytokines with important functions in the immune system.[3] X-SCID is lethal unless treated by bone marrow transplantation (BMT). Human leukocyte antigen (HLA)–identical postnatal BMT has overall been very successful although associated with significant morbidity and mortality.[1] Further, only a minority of patients with SCID have an available HLA-identical donor at birth. For these patients, it was considered a breakthrough when successful correction of the common gamma-chain deficiency and T-cell deficiency was achieved by retroviral-mediated gene therapy.[4] However, as some patients later developed T-cell leukemia because of insertional mutagenesis of the retroviral vector in or near the *LMO-2* oncogene,[5,6] gene therapy of X-SCID is currently approached with caution,[7-9] and the need for development of alternative therapeutic approaches is still evident.

In utero hematopoietic cell transplantation (IUHCT) has been successfully applied to a limited number of patients with X-SCID. Using stem-progenitor CD34 antigen-enriched haploidentical bone marrow (BM) cells or unrelated fetal liver cells collected early in gestation, long-term donor-derived engraftment of T and NK cells has been achieved, with no observation of graft-versus-host disease.[10-12] Despite little or no evidence of stem cell engraftment, successful donor T and NK cell reconstitution was accomplished, reflecting a considerable competitive advantage over host hematopoietic cells, in expansion and differentiation toward these lineages. However, although these cases support the main rationale behind IUHCT, namely, that the fetal immune system is tolerant to foreign HLA early in gestation, IUHCT continues to be applied only in rare cases of X-SCID. The limited prenatal screening and diagnostic routines only partially account for this. Rather, it has been argued that postnatal transplantations have obtained very good results, although no comparative studies of IUHCT and postnatal transplantations have been performed. However, studies have clearly suggested that BMT of patients with SCID in the neonatal period (first month of life) gives superior results to transplantations later, with regard to both T-cell reconstitution and survival.[13] It does, however, remain unclear to what degree the improved results of early transplantations reflect changes in the immune barrier, with age and/or the fetal/neonatal hematopoietic microenvironment being more permissive for homing and engraftment.

Using mouse models, several previous studies supported that the engraftment after fetal transplantations is not limited by an immune barrier.[14-16] In contrast, a recent mouse study provided compelling evidence for the existence of an immune barrier to allogeneic engraftment after IUHCT, demonstrating stable multilineage reconstitution in all congenic recipients, whereas allogenic IUHCT in most cases resulted in failed long-term reconstitution.[17] If so, although there might still be strong clinical justifications for IUHCT,[18,19] such as reducing susceptibility to postnatal infections, the hematopoietic advantage of preimmune transplantations might be questionable. However, whereas most successful clinical IUHCT of X-SCID fetuses have been performed with stem and progenitor (CD34) enriched hematopoietic cells, the studies of Peranteau et al[17] involved IUHCT of whole BM transplants with major

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mismatch between the donor and recipient, and notably no mouse studies have compared the reconstitution of immune-deficient or wild-type (WT) recipients with purified stem/progenitor cells in the fetal, postnatal, and adult setting. Thus, although recent data clearly support the existence of an immune barrier after IUHCT, this barrier might be less significant than that in the postnatal setting. The fact that Peranteau et al[17] obtained consistent long-term multilineage chimerism in unconditioned fetal recipients of congenic BM cells, despite evidence of small congenic differences constituting a significant immunologic barrier in adult BMT,[20-22] could be compatible with immunologic and/or microenvironmental advantages of IUHCT compared with postnatal transplantations, even in situations with minimal mismatch.

The present studies were designed to investigate issues not previously addressed but of considerable relevance to IUHCT in X-SCID. First, we asked whether or not engraftment with long-term hematopoietic stem cells (HSCs)[23] is a requirement to obtain durable and polyclonal T- and B-cell reconstitution in fetal X-SCID ($\gamma c^{-/-}$) recipients. This was addressed by comparing the kinetics and longevity of not only peripheral B- and T-cell reconstitution but also reconstitution of short-lived B- and T-cell precursors in hematopoietic tissues, from purified (congenic) adult HSCs ($Lin^-SCA\text{-}1^+KIT^+CD34^-FLT3^-$)[23] and $Lin^-SCA\text{-}1^+KIT^+CD34^+FLT3^{hi}$ lymphoid-primed multipotent progenitors (LMPPs).[24-26] Importantly, unlike HSCs, LMPPs lack extensive self-renewal potential and represent the earliest identified lineage restricted MPPs, sustaining (at the single-cell level) combined granulocyte-monocyte (GM), B and T lineage potential, but little or no megakaryocyte-erythroid (MkE) potential.[25] Further, unlike HSCs, LMPPs express a combined GM and lymphoid transcriptional priming[25] and rapidly and robustly reconstitute B and T lymphopoiesis in lethally irradiated WT recipients, although in this setting lymphoid reconstitution does not remain stable in the long-term.[24] Second, we compared the ability of congenic HSCs as well as LMPPs to reconstitute lymphopoiesis in X-SCID recipients transplanted during fetal development, neonatally (postnatal days 1-4), or in early adult life (4-6 weeks old). Performing this comparative analysis in a congenic setting, and thereby reducing the immunologic barrier to a minimum, we could, for the first time, directly assess the potential nonimmunologic advantages for transplantations of nonconditioned X-SCID recipients in the prenatal (in utero) versus postnatal (early and late) stages.

Herein we demonstrate that LMPPs are far superior to HSCs in providing rapid lymphoid reconstitution after IUHCT of X-SCID recipients. LMPPs also sustain peripheral B cells and polyclonal T cells, as well as short-lived BM B-cell precursors and thymic T-cell precursors, although HSCs might prove essential for securing stable engraftment in the long term. Our findings underscore the importance of 2 distinct populations of primitive multipotent stem and progenitors cells in rapid and long-term reconstitution of the immune system in X-SCID recipients. Most notably, we provide evidence for IUHCT of HSCs giving superior T-cell reconstitution in X-SCID recipients compared with postnatal transplantations.

Methods

Mice

X-SCID (B6.129S4-*Il2rg*^{tm1Wjl}/J, CD45.2)[27] mice backcrossed to C57/Bl6 background for more than 10 generations were purchased from The Jackson Laboratory (Bar Harbor, ME). Congenic WT C57/Bl6 (CD45.1) mice were used as donors. Mice were housed in pathogen-free conditions and had unrestricted access to sterilized food and autoclaved acidified water. All experiments were approved by the Malmö/Lund Animal Ethics Committee of Sweden.

Isolation and purification of hematopoietic stem and progenitor cells

Hematopoietic stem and progenitor cell populations from 10- to 14-week-old C57Bl6 CD45.1 mice were isolated as previously described.[23,25] Briefly, BM cells were enriched for lineage (Lin) low/negative cells by immunomagnetic beads (sheep–anti-rat Dynabeads, Dynal Biotech, Oslo, Norway) through depletion of Lin^+ cells stained with purified B220/CD45R (RA3-6B2), CD4/L3T4 (H129.19), CD5 (53-7.3), CD8α (53-6.7), CD11b/Mac-1 (M1/70), Gr1 (RB6-8C5), and Ter119 (LY-76). Samples were subsequently incubated with polyclonal goat–anti-rat Tricolor (Caltag, San Francisco, CA), CD16/32 ($2.4G_2$; Fc block, to reduce unspecific staining), anti–mouse-CD34 fluorescein isothiocyanate (RAM34), -FLT3 phycoerythrin (PE) (AZF10.1), -SCA-1 biotin (E13-161.7), and -KIT allophycocyanin (2B8) or isotype-matched control antibodies. Biotinylated antibodies were visualized with streptavidin (Sav) PE TxR (Invitrogen, Carlsbad, CA). Dead cells were excluded by 7-amino actinomycin (7-AAD; Sigma-Aldrich, St Louis, MO). Cells were sorted on a FACSDiVa (BD Biosciences, San Jose, CA). BM cells were gated as $Lin^{-/lo}7\text{-}AAD^-$ and subsequently as $SCA\text{-}1^+KIT^+$. Within the $Lin^-SCA\text{-}1^+KIT^+$ (LSK) $CD34^+$ population, the 25% highest FLT3 expressing cells ($LSKCD34^+FLT3^{hi}$, purity reproducibly > 99%) and $CD34^-FLT3^-$ cells (purity reproducibly > 95%) were sorted as described[23,25] (see Figure 2A).

In utero transplantations

X-SCID mice were mated overnight and vaginal plugs checked the next morning (counted embryonic day E0.5). At E14 to E16, females premedicated with Temgesic (0.4 mg/kg) were anesthetized with isoflurane and a mid-abdominal incision was performed. Uterine horns were exposed and each fetus injected intraperitoneally with donor cells (5×10^5 unfractionated BM cells, 200 or 1000 $LSKCD34^+FLT3^{hi}$ and 200 $LSKCD34^-FLT3^-$) or phosphate-buffered saline (sham-injected group) in a total volume of 2 μL per fetus. Abdominal incisions were closed in 2 layers and females allowed to complete pregnancy to term. Peripheral blood (PB) samples were collected at indicated time points. BM, thymus, and spleen were collected at termination.

Neonatal transplantations

Newborn unconditioned X-SCID mice (postnatal day D1-4) were injected intraperitoneally with 200 $LSKCD34^+FLT3^{hi}$ or 200 $LSKCD34^-FLT3^-$ cells in a volume of 20 μL per mouse and returned to the mother until weaning. PB samples were collected at 3 to 4, 6, and 16 weeks after transplantation.

Adult transplantations

Young unconditioned X-SCID mice (4-6 weeks old) were injected with 200 LSK $CD34^+FLT3^{hi}$ or 200 LSK $CD34^-FLT3^-$ cells through intravenous tail vein injection. Hematopoietic reconstitution was evaluated in PB at 4, 6, and 16 weeks

Secondary transplantations

Sublethally (650 cGy) irradiated X-SCID mice (8-12 weeks old) received by tail vein injection 1 femur equivalent of BM cells harvested 16 weeks after IUHCT transplantation of either 1000 LSK $CD34^+FLT3^{hi}$ or 200 LSK $CD34^-FLT3^-$ cells. PB donor reconstitution in secondary recipients was evaluated by fluorescence-activated cell sorting (FACS) at 12 weeks after transplantation.

Analysis of donor reconstitution

PB was collected at indicated time points after transplantation and analyzed for donor-derived reconstitution as previously described.[23] BM, spleen, and

thymus were collected at 36 weeks after transplantation (in case of LMPPs and HCSs transplanted fetal recipients). We used rat–anti-mouse antibodies to differentiate host (CD45.2; clone104) from donor (CD45.1; A20) cell reconstitution, and defined B cells as $B220^+$ (clone RA3-6B2), T cells as $CD4^+$ (clone H129.19) and $CD8^+$ (clone 53-6.7), and myeloid cells as $Mac1/CD11b^+$ (clone M1/70). At 36 weeks after transplantation, a more extensive FACS analysis was performed in which antibodies against CD19 (1D3), IgM (R6-60.2), TCR β (H57-597), TCR-γ (GL-3), NK1.1 (PK136), CD49b (DX5), CD3 (17A2), CD44 (1M7), and CD25 (3C7) also were included. In the case of whole BM fetal recipients, BM was harvested from transplanted mice at 16 weeks after transplantation, and analysis of donor-derived LSK cells was performed. For all analyses, cell counts were measured on a KX21 Sysmex (Sysmex Europe, Hamburg, Germany). All antibodies were from BD Biosciences. FACS analysis was performed on a FACSCalibur or FACSDiva (BD Biosciences). Data obtained were subsequently analyzed using FlowJo Software (TreeStar, Ashland, OR).

TCR Vb rearrangement analysis

The Mouse Vβ TCR Screening Panel (BD Biosciences PharMingen, San Diego, CA), which contains prediluted fluorescein isothiocyanate-conjugated antibodies specific for mouse Vβ 2, 3, 4, 5.1 + 5.2, 6, 7, 8.1 + 8.2, 8.3, 9, 10^b, 11, 12, 13, 14, and 17^a T-cell receptors was used to identify polyclonal subsets of T cells in spleens after transplantation. Samples were prepared at a density of 10^6cells/antibody reaction and stained with donor marker CD45.1 (A20) PE and T lymphocyte markers CD4 (H129.19) PE Cy5, and CD8 (53-6.7) allophycocyanin (BD Biosciences) before specific staining with the Vβ antibodies. Analysis was performed on a FACSCalibur.

Statistical analysis

Results are presented as means (SEM), and statistical significance of differences between groups was determined using a 2-tailed unpaired *t* test. *P* values less than .05 were regarded as significant. Fisher exact test was used to analyze and compare the frequencies of survival and frequencies of positively reconstituted fetally transplanted mice (Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article) as well as frequencies of engrafted fetal, neonatal, and adult mice (GraphPad Prism version 4; GraphPad Software, San Diego, CA).

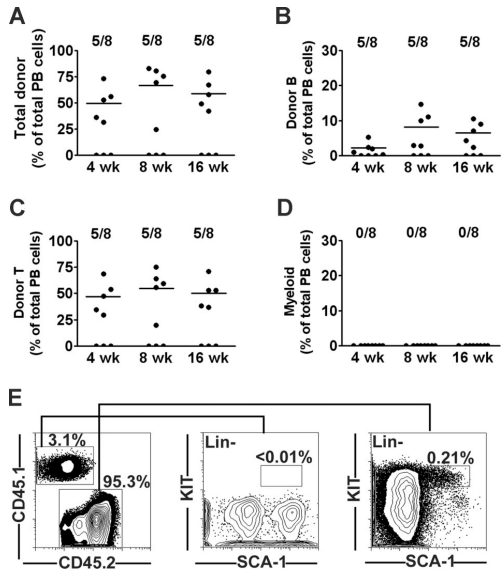

Figure 1. Long-term lymphoid but not myeloid or stem cell reconstitution in X-SCID mice BM transplanted during fetal development. Twenty-four fetal (E14.5) X-SCID (CD45.2) mice were transplanted with 5×10^5 whole BM cells from congenic (CD45.1) adult donors. Eight (33%) live born transplanted mice were analyzed for reconstitution at 4, 8, and 16 weeks after birth. Contribution of donor cells to (A) total nucleated cells, (B) B cells, (C) T cells, and (D) myeloid cells as percentage of total blood cells. Data points represent individual mice; horizontal bars represent mean values of positively engrafted mice (> 0.1% for each parameter). Results are from 2 independent experiments (E). FACS analysis of donor-derived LSK cells in BM of one representative animal at 16 weeks after birth. Left panel shows typical donor and host contribution to total BM cells. Middle and right panels show donor and recipient-derived LSK cells, respectively, gated as lineage negative, and investigated for expression of the HSC markers SCA-1 and KIT. Numbers represent percentages of total BM cells (0.01%; detection level).

Results

Undetectable myeloid and stem cell reconstitution after congenic transplantation of WT adult bone marrow cells to X-SCID fetuses

It has been suggested that the long-term engraftment and immune correction of X-SCID recipients transplanted in utero might require HSC reconstitution,[28,29] although direct evidence for this is limited. Thus, we first investigated the potential of unfractionated adult BM cells from CD45.1 C57Bl/6 mice to multilineage reconstitute lymphopoiesis and myelopoiesis in fetal (E14-E16) CD45.2 X-SCID mice (also on C57Bl/6 background). Of a total of 24 fetal recipients, 8 (33%) were alive at time of analysis; and of these, 5 (62%) were positive for donor engraftment at all analysis time points. At 4 weeks after transplantation of 5×10^5 CD45.1 congenic BM cells, donor-derived contribution to total peripheral blood (PB) white blood cells was as much as 49% (Figure 1A), of which B and T cells accounted for 2.1% and 46%, respectively. Eight weeks after transplantation, levels of donor chimerism increased to 66%, and B- and T-cell reconstitution reached 8.1% and 54%, respectively. These levels were sustained also in the long-term (16 weeks; Figure 1B,C). However, no evidence of significant (above background staining) myeloid reconstitution was found at any time point (Figure 1D). Because no previous IUHCT studies had directly investigated the potential reconstitution of the HSC compartment itself, we next analyzed the BM of transplanted mice for the presence of donor-derived HSCs as defined by a $Lin^-SCA\text{-}1^+KIT^+$ (LSK) phenotype.[25] Notably, and in seeming contrast to the high levels of chimerism in the periphery, the BM contained only 3.5% CD45.1 cells (Figure 1E), and no evidence of donor contribution to the LSK HSC compartment was observed (Figure 1E), whereas the host (CD45.2) frequency of LSK cells in BM was within the expected range. Thus, in agreement with earlier studies,[14,15] fetally transplanted WT congenic BM cells sustained B- and T-lymphoid reconstitution, without evidence for corresponding myeloid and/or HSC reconstitution.

Rapid and sustained lymphoid engraftment of X-SCID recipients transplanted with LMPPs in utero

As transplantation of unfractionated BM cells gave robust lymphoid reconstitution without evidence of HSC engraftment, we next addressed whether the non–self-renewing LMPPs could potentially promote and sustain immune reconstitution in nonablated X-SCID fetal recipients or whether HSCs are required for sustained reconstitution. Thus, highly purified CD45.1 HSCs ($LSKCD34^-FLT3^-$, 200 per recipient)[23] or LMPPs ($LSKCD34^+FLT3^{hi}$, 200 or 1000 per recipient)[25] (Figure 2A) were transplanted into fetal (E14-16) CD45.2 X-SCID recipients. Importantly, LMPPs were transplanted at the same cell number as

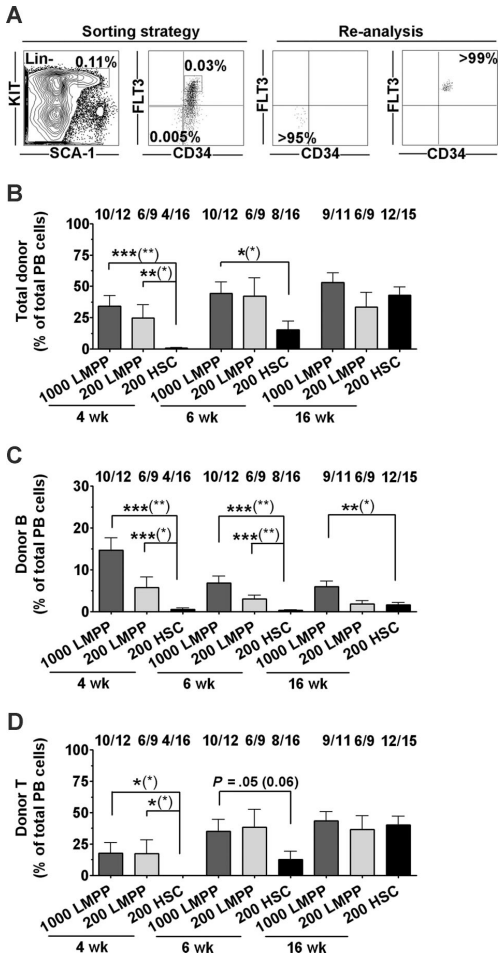

Figure 2. LSK CD34$^+$Flt3^{hi} LMPPs provide rapid and sustained B- and T-cell engraftment of fetal X-SCID recipients. (A) Sorting strategy and purity of sorted Lin$^-$SCA-1$^+$KIT$^+$CD34$^+$FLT3^{hi} LMPPs and Lin$^-$SCA-1$^+$KIT$^+$CD34$^-$FLT3$^-$ HSCs. Numbers in gates/quadrants indicate mean frequencies of total BM cells. (B-D) LMPPs (1000 or 200) and HSCs (200) were transplanted to fetal X-SCID recipients. PB analysis was performed at 4, 6, and 16 weeks after transplantation. (B) Donor-derived total reconstitution as percentage of total nucleated cells. Indicated above bars are frequencies of mice reconstituted (> 0.1% for each parameter) by transplanted test populations at the analysis time point, of all mice analyzed. (C) Donor-derived B lymphocytes as percentage of total PB cells. (D) Donor-derived T lymphocytes as percentage of total PB cells. All bar graphs represent mean (SEM) reconstitution levels of positively reconstituted mice. Statistical significance in reconstitution levels between LMPP and HSC transplanted recipients was evaluated, comparing only reconstituted mice, as well as comparing all recipients alive (positive and negative for reconstitution) at analysis time point (*P* values within parentheses). ***$P < .001$; **$P < .005$; *$P < .05$.

LSKCD34$^-$FLT3$^-$ HSCs to compare their reconstitution potencies on a cell-to-cell basis, but also at 5-fold higher numbers, as they are present in WT BM at a 5-fold higher frequency than LSKCD34$^-$FLT3$^-$ HSCs.[23,25]

LMPPs rapidly engrafted the majority of unconditioned fetal X-SCID recipients, and reconstituted mice showed at 4 weeks after IUHCT mean total reconstitution levels of 34% derived from 1000 LMPPs and 24% from 200 LMPPs, compared with only 0.6% from 200 HSCs (Figures 2B, S1A). At 6 weeks, mean total reconstitution levels were 44% and 42% for mice transplanted fetally with 1000 and 200 LMPPs, respectively, compared with the HSC recipient group with a mean reconstitution level of 15% (Figures 2B, S1B).

Notably, high levels of PB reconstitution were maintained at 16 weeks in SCID recipients transplanted with 1000 or 200 LMPPs (53% and 33%, respectively), whereas HSC-derived reconstitution increased to 43% (Figures 2B, S1C).

At 4 weeks, 200 LMPPs contributed substantially to reconstitution of the B- and T-cell lineages (mean of 5.7% and 17% of total blood cells, respectively; Figures 2C,D and S1A). These levels of LMPP-derived lymphoid reconstitution were in the case of B cells sustained and for T cells further improved (to 36% for 200 cells) at 16 weeks after transplantation. Although initially severely delayed in their reconstitution compared with LMPPs, HSCs contributed to 1.6% B cells and 40% T cells (of total PB cells) in X-SCID recipients at 16 weeks after IUHCT.

Previous studies of IUHCT in mouse models have been associated with considerable loss of transplanted recipients (typically 70%-80%), in part related to the surgical procedure itself and partially because of postnatal loss of mice resulting from cannibalism and neglect by the mothers.[15,16,30] In our studies, a total of 59 fetuses were transplanted with 1000 LMPPs; 34 fetuses received 200 LMPPs and 47 fetuses received 200 HSCs. Survival ratios at the time of weaning varied from 34% (200 HSC) to 20% (1000 LMPPs) and 26% (200 LMPPs; Table S1). These differences were not statistically different, and the loss of recipients appeared unrelated to transplanted cells, as in control experiments of sham- and BM-injected fetal recipients, comparable survival was observed (37% and 33%, respectively; Table S1).

Sustained generation of lymphoid progenitors and mature lymphocytes in fetal recipients of both HSCs and LMPPs

LMPPs have been demonstrated to have limited self-renewing capacity when transplanted in lethally irradiated WT hosts.[23-25] Herein we found LMPPs to not only engraft more rapidly and with higher efficiency than HSCs, but also to sustain high levels of B- and in particular T-cell reconstitution in unconditioned X-SCID fetal recipients up to 16 weeks after transplantation. To investigate the potential of LMPPs to not only sustain mature B and T progeny, which can also be maintained through peripheral expansion,[31-33] but also to replenish more short-lived T- and B-cell progenitors in the host hematopoietic tissues, we investigated the hematopoietic organs of reconstituted mice transplanted with 200 LMPPs or 200 HSCs, 36 weeks after transplantation. PB analysis at this time point showed comparable total reconstitution levels for mice transplanted fetally with as little as 200 LMPPs or the same number of HSCs, and also the total number of donor-derived (CD4$^+$ and CD8$^+$) T cells and (B220$^+$CD19$^+$IgM$^+$) B cells were comparable (Figure 3A,B). All circulating LMPP-derived CD4$^+$ and CD8$^+$ T lymphocytes expressed the beta chain of the T-cell receptor (TCR-beta; Figure 3B).

To compare the ability of LMPPs and HSCs to sustain active B lymphopoiesis in the BM, we also investigated, at 36 weeks after transplantations, the persistence of B-cell precursors in the BM. Notably, the absolute numbers of LMPPs and HSCs derived total B220$^+$ cells as well as B220$^+$CD43$^-$IgM$^-$ pre-B cells and CD43$^-$B220$^+$IgM$^+$ immature/mature B cells were comparable (Figure 3C), demonstrating the ability of as few as 200 LMPPs to sustain active B lymphopoiesis in the long term in X-SCID mice transplanted during fetal development.

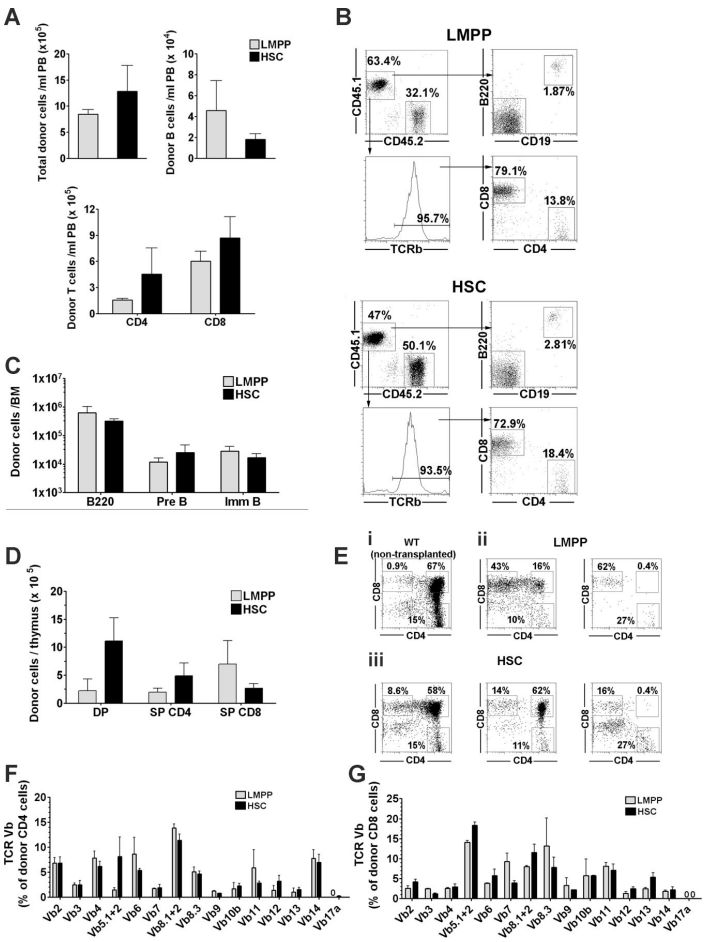

Figure 3. Long-term reconstitution of lymphoid progenitors and mature lymphocytes in unconditioned X-SCID mice after fetal transplantation of LMPPs and HSCs. (A-G) Fetal X-SCID mice transplanted with 200 LSK CD34$^+$FLT3^{hi} LMPPs (▭) or 200 LSK CD34$^-$FLT3$^-$ HSCs (■) were analyzed for donor-derived lymphoid progenitors and mature lymphocytes in PB, thymus, BM, and spleen 36 weeks after transplantation. (A) Mean (SEM) donor-derived contribution to total nucleated blood cells (top left panel), B cells (B220$^+$C19$^+$; top right panel), and T cells (CD4$^+$ and CD8$^+$; bottom panel) from 5 reconstituted mice. (B) FACS profiles of PB analysis from typical fetal X-SCID recipient transplanted with 200 LMPPs (top FACS plots) and 200 HSCs (bottom FACS plots). For each of the 2 populations, top panels show donor-derived CD45.1 (top left) B220$^+$CD19$^+$ B cells (top right). Bottom left panel shows frequency of TCR β^+ T cells among total CD45.1$^+$ cells, and bottom right panel shows distribution of TCR β^+ cells into CD4$^+$ and CD8$^+$ blood cells. Gated numbers represent frequencies of total donor cells. (C) Mean (SEM) donor contribution to total B220 cells (B220$^+$), pre B (B220$^+$CD43$^-$IgM$^-$), and immature/mature B cells (B220$^+$CD43$^-$IgM$^+$) in BM (per 2 femurs and 2 tibias) of 5 reconstituted mice. (D) Mean (SEM) donor contribution to DP, SP CD4, and SP CD8 cells in thymus from 5 mice, transplanted 36 weeks earlier. (E) Representative CD4 and CD8 FACS profiles of thymus (gated on CD45.1$^+$ donor cells) in mice transplanted with LMPPs (ii) and HSCs (iii) 36 weeks earlier, showing variable reconstitution of CD4$^+$CD8$^+$ DP thymocytes, and compared with same profiles in WT control (nontransplanted) mouse (i). Percentages for LMPP and HSC transplanted mice are relative to total donor cells. (F,G) TCR V-beta rearrangement of (F) CD4$^+$ and (G) CD8$^+$ donor (CD45.1$^+$) derived cells in spleen. Bar graphs represent mean (± SEM) percentage of cells (of 6 mice analyzed) expressing different V-β subtypes of total donor (F) CD4 and (G) CD8 cells. For all panels, all differences between LMPP and HSC transplanted mice were nonsignificant ($P > .05$).

Within the thymus, donor-derived reconstitution remained high in LMPP- and HSC-transplanted fetal recipients, respectively, at 36 weeks after transplantation. Importantly, in both cases, this reconstitution included naive double-positive (DP) CD4$^+$CD8$^+$ thymocytes and single-positive (SP) CD4$^+$ and CD8$^+$ thymocytes (Figure 3C,D), although the frequency of DP thymocytes was higher in HSC-transplanted recipients. Because thymic progenitors must be replaced daily from the BM to sustain active thymopoiesis,[34] this also established the ability of fetally transplanted LMPPs to long-term replenish thymic progenitors, although probably less efficiently than HSCs.

To establish that the sustained long-term peripheral T-cell reconstitution on LMPP transplantation was polyclonal and not the result of oligoclonal peripheral expansion, we investigated, at 36 weeks after transplantation, the expression of 15 different TCR V beta subtypes in CD4 as well as CD8 SP LMPP-derived T cells. All except one (TCR Vb17^a) of the subtypes analyzed could be found expressed in CD4$^+$ and CD8$^+$ T cells, with no difference observed between mice transplanted with LMPPs and HSCs during fetal development (Figure 3F,G), and with a repertoire of different TCR Vb subtypes comparable with that of splenic T cells in nontransplanted WT control mice, suggesting a polyclonal and balanced origin of T cells from low numbers of LMPPs transplanted into fetal X-SCID recipients.

Although the above data demonstrated the ability of low numbers of LMPPs to sustain B- and T-cell progenitors for considerable time, they also implied that reconstitution with self-renewing HSCs might in the long-term be required for stable B- and T-cell reconstitution in nonconditioned X-SCID recipients.

To compare the self-renewal ability of LMPP and HSC donor-derived BM cells in unconditioned X-SCID recipients, we performed secondary transplantation experiments. BM cells from a total of 11 fetal primary recipients of 1000 LMPPs or 200 HSCs with comparable B- and T-cell reconstitution levels at 16 weeks after transplantation (Figure 4) were transplanted into sublethally irradiated X-SCID adult mice. At 12 weeks after secondary transplantation, both total B- and T-cell donor-derived reconstitution levels were considerably higher in secondary HSC than LMPP recipients (Figure 4), despite fetal recipients being originally transplanted with 5 times more LMPPs than HSCs.

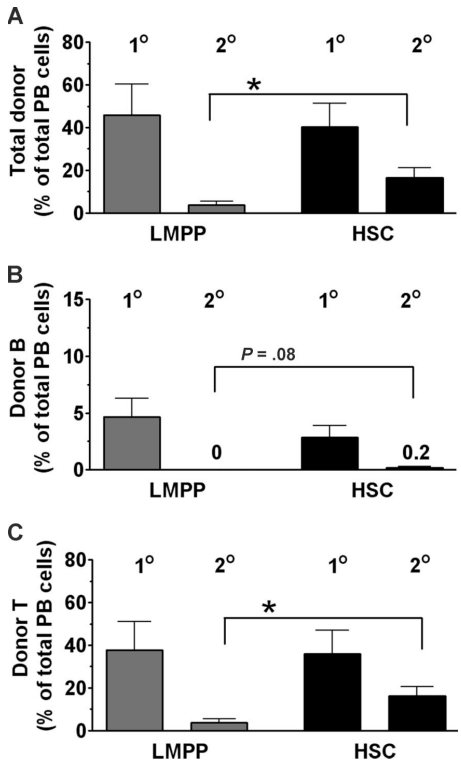

Figure 4. HSCs are superior to LMPPs in reconstituting unconditioned X-SCID recipients with self-renewing stem and progenitor cells. BM cells were collected 16 weeks after primary transplantation from (n = 11) unconditioned X-SCID fetal recipients transplanted with 1000 CD45.1$^+$ LMPPs (▭) or 200 HSCs (■). For each primary (first-degree) recipient, 1 femur equivalent was transplanted into 1 or 2 secondary (second-degree) sublethally (650 cGy) irradiated (8-10 weeks old) X-SCID (CD45.2) recipients (n = 18). Reconstitution analysis was performed at 12 weeks after second-degree transplantation. (A-C) Donor-derived contribution in first-degree and second-degree X-SCID recipients toward (A) total PB cells, (B) B lymphocytes, and (C) T lymphocytes, all presented as mean (SEM) percentages of total PB cells from 3 independent experiments. *$P < .05$.

Enhanced lymphoid reconstitution after fetal transplantation of X-SCID recipients with purified congenic HSCs

It has been proposed that the preimmune status and/or the microenvironment of the fetus might result in enhanced hematopoietic engraftment compared with postnatal transplantations.[14,15] However, previous comparative studies of stem cell transplantations of fetal, postnatal, and adult unconditioned recipients have all been performed in the allogeneic setting, and with whole BM transplants,[15,16] rather than with enriched stem/progenitor populations as in successful clinical IUHCT of patients with X-SCID.[10,11] Thus, we here also investigated the ability of purified congenic LMPPs and HSCs (in both cases, 200 cells per recipient) to reconstitute B and T lymphopoiesis at the early postnatal stage (neonatal mice D1-4) and in young adult (4-6 weeks old) unconditioned X-SCID recipients, compared with the results obtained through IUHCT.

Most X-SCID mice transplanted with 200 LMPPs at the neonatal stage showed significant donor-derived reconstitution at 4 (18 of 21) and 6 (16 of 20) weeks after transplantation (Figure 5A), but only 35% of neonatal X-SCID recipients of LMPPs sustained B- and T-cell reconstitution, at 16 weeks after transplantation (Figure 5B,C). Most notably, the reconstitution frequencies as well as reconstitution levels in neonatal X-SCID recipients of HSC transplants were much lower than in LMPP recipients, as only 2 of 8 neonatal recipients ever showed any reconstitution, and at 16 weeks only 1 of 6 mice showed evidence of very low levels B- and T-cell engraftment (Figure 5A-C). This was in contrast to fetal recipients, in which 16-week reconstitution frequencies and levels were comparable in recipients of LMPPs and HSCs (Figure 2B-D).

The results in young adult X-SCID recipients were similar to those of neonatal recipients, in that most LMPP recipients showed initial donor engraftment (Figure 5D-F), but only 27% and 45% were positive for B- and T-cell reconstitution, respectively, at 16 weeks after transplantation, and in adult recipients the long-term (16 weeks) results were comparable in HSC recipients (Figure 5D-F). Thus, for HSC transplantations, compared with neonatal and adult recipients, IUHCT was clearly superior, both with regard to B-cell (Figure 5G) and in particular for the critical T-cell reconstitution (Figure 5H) of X-SCID recipients. Although a similar tendency could be observed for recipients transplanted with LMPPs, the reconstitution levels achieved after IUHCT and in particular neonatal transplantations of LMPPs were much more comparable than observed after HSC transplantations (Figure 5G,H). Thus, IUHCT of purified HSCs results in superior B- and T-cell reconstitution in unconditioned X-SCID recipients, compared with neonatal and adult transplantations.

Discussion

Although neonatal hematopoietic transplantations of patients with SCID appear to give better outcome than later transplantations, neonatal and fetal transplantations have yet to be compared in patients with X-SCID in the clinical setting, largely because the clinical outcome with neonatal X-SCID transplantations has overall been very good,[13] accounting in part for the limited application of IUHCT in patients with X-SCID.[18,19] Using mice with the same γc-deficiency as patients with X-SCID, we provide here evidence for fetal transplantations of congenic HSCs giving considerably higher and more consistent B- and T-cell reconstitution than transplantations at the early postnatal stage or in young adult unconditioned X-SCID recipients, and for the critical T-cell lineage similar findings (although not statistically significant) were observed when transplanting LMPPs. Although our studies do not conclusively establish the mechanism for the competitive advantage of purified HSCs (or LMPPs) in fetal over postnatal X-SCID recipients, they do demonstrate that, even when using congenic donors and purified HSCs capable of crossing fully allogeneic barriers,[35] a considerable reconstitution advantage of HSCs is achieved through intrauterine transplantation. As we also demonstrate that HSC reconstitution might be critical for the long-term replenishment of T cells in X-SCID recipients, this strongly suggests that improved results with intrauterine transplantations of nonconditioned X-SCID recipients might at least in part be achieved because of a more favorable microenvironment in fetal hematopoietic tissues. Whether or not this specifically relates to enhanced access to suitable stem and progenitor niches remains to be investigated.

Although it is possible that normalizing the cell dose to the body weight of X-SCID recipients at different ages could have partially corrected the differences in reconstitution levels observed, we think this is unlikely to be a main reason for the observed differences in

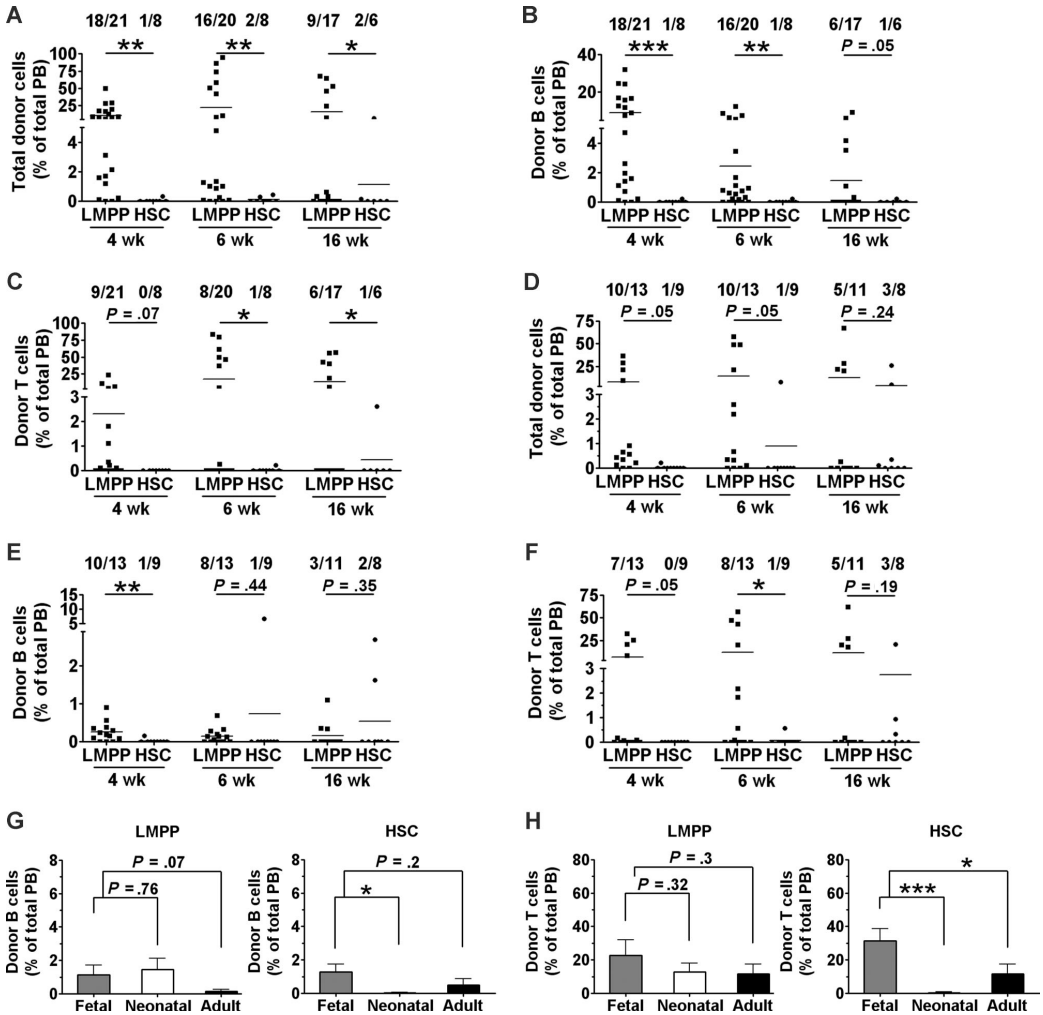

Figure 5. Fetal X-SCID recipients are more permissive to congenic HSC reconstitution of the T-cell lineage than postnatal and adult recipients. Neonatal (postnatal days 1-4) and young adult (4-6 weeks old) X-SCID mice were transplanted without any conditioning with 200 congenic LMPPs (LSK CD34$^+$ FLT3^{hi}) or 200 HSCs (LSK CD34$^-$ FLT3$^-$). PB reconstitution analysis of total, B cells, and T cells was performed at 3 to 4, 6, and 16 weeks after transplantation. Shown are results from 4 neonatal (A-C) and 3 adult (D-F) transplantation experiments. Numbers above bars indicate frequencies of transplanted mice with more than 0.1% total reconstitution at each time point. Statistical significance in reconstitution levels between LMPP and HSC transplanted recipients was evaluated, comparing all recipients alive (positive and negative for reconstitution) at analysis time point. ***$P < .001$; **$P < .005$; *$P < .05$. (G,H) Donor contribution of 200 congenic LMPPs (left panel) and 200 HSCs (right panel) to (G) B-cell and (H) T-cell reconstitution analyzed at 16 weeks after transplantation in fetal (▨), neonatal (□), and adult (■) recipients. Statistical differences comparing reconstitution levels in all transplanted and alive recipients are indicated above bars. ***$P < .001$; **$P < .005$; *$P < .05$.

reconstitution levels, in part because not only adult but also neonatal recipients transplanted with the same dose of HSCs showed impaired reconstitution compared with fetally transplanted mice, and importantly the high levels of reconstitution in fetally transplanted mice were sustained into adulthood.

Conditioning (with irradiation or chemotherapy) of the X-SCID recipients could also have minimized the differences seen in engraftment levels observed at different ages, but a major goal in the current studies, and in hematopoietic transplantations in general, is to reduce or preferably alleviate the need for cytotoxic conditioning because of its serious side effects and resulting long-term morbidity.

Although recent studies demonstrated the existence of a considerable immune barrier, also after intrauterine transplantations of allogeneic whole BM cells in WT recipients,[17] the data herein establish the superiority of intrauterine transplantations in immune reconstitution of unconditioned X-SCID recipients with purified HSCs. It is in that regard of relevance that (CD34) enriched stem and progenitor cell populations also have been used successfully in clinical IUHCT of X-SCID.[10,11] Whether or not such enrichment is required or beneficial beyond reducing potential graft-versus-host disease, however, remains to be established.

The observed striking differences in kinetics of B- and T-cell reconstitution by transplanted purified HSCs and LMPPs in

X-SCID recipients is of probable clinical relevance for IUHCT (and postnatal transplantation) of X-SCID recipients, and it also provides new insights into the biology and potential importance of LMPPs in replenishment of B- and T-cell progenitors in unconditioned recipients. From a clinical viewpoint, because one of the goals and claimed advantages of IUHCT in X-SCID is to achieve rapid immune reconstitution postnatally,[19] it would be important to ensure that multipotent progenitors equivalent to the LMPPs would be included in stem/progenitor cell-enriched grafts. In that regard, whereas transplantation of as few as 200 LMPPs gave high B- and T-cell reconstitution already 4 weeks after IUHCT, 200 HSCs gave limited B- and almost no T-cell reconstitution at 4 weeks, and also much less at 6 weeks after transplantation, compared with X-SCID recipients transplanted with LMPPs. This difference was even more striking after transplantations in neonatal and young adult X-SCID recipients. Importantly, this difference in kinetics of reconstitution was observed when transplanting the same number of LMPPs and HSCs. As the compartment of LSKCD34$^+$Flt3^{hi} LMPPs is approximately 5 times larger than LSKCD34$^-$Flt3$^-$ HSCs,[24-26] the relative importance of LMPPs over HSCs in rapid reconstitution of lymphopoiesis in X-SCID recipients is, if anything, underappreciated in these experiments.

Because the identity of human LMPPs has yet to be established, one risks depleting these multipotent progenitors in different enrichment methods, although they are probably part of the CD34$^+$ stem/progenitor compartment. Markers that might identify a human LMPP counterpart are probably largely distinct from that of mouse LMPPs as, for instance, Sca-1 does not exist in humans. Regardless, the present findings underscore the importance and relevance of seeking to identify and characterize the human LMPP or equivalent human multipotent progenitors.

In light of LMPPs possessing quite limited self-renewal ability when transplanted into lethally irradiated WT recipients,[25] it was of considerable interest that LMPPs in the long-term (16 and even 36 weeks after transplantation) after IUHCT sustained peripheral B and T cells at a high level, comparable with that of HSCs. Importantly, as mature B and T cells can be sustained in the long-term through peripheral expansion,[31-33] we investigated and demonstrated, as late as 36 weeks after transplantation, that IUHCT of as few as 200 LMPPs is sufficient to sustain replenishment of short-lived B-cell progenitors in the BM and short-lived T-cell progenitors in the thymus. The functional importance of this was further substantiated by the demonstration of polyclonal origin of LMPP-derived peripheral T cells at 36 weeks after transplantation. Thus, although our studies, including secondary transplantations, are most compatible with HSC reconstitution being essential for optimal and stable long-term replenishment of B and T lymphopoiesis in X-SCID recipients, LMPP-like cells appear required for rapid reconstitution and also efficient at contributing to mid- or long-term replenishment of polyclonal B and T lymphopoiesis. These conclusions were also supported by other recent studies[36] in which progenitors with full multilineage potential[37] were transplanted into adult $\gamma c^{-/-}$ mice also deficient for RAG_2 expression, giving a more severe immune deficiency than X-SCID. In this model, multipotent progenitors were demonstrated to be sufficient to give rapid and sustained reconstitution of peripheral B cells. However, these studies did not directly establish whether this also included maintenance of short-lived B-cell progenitors, and most importantly reconstitution of T-cell progenitors and polyclonally derived T cells was not directly demonstrated, although a functional antibody response that is T cell–dependent was demonstrated.[36] Because, in humans, isolated γc-deficiency results in reduced T cells but not B cells, whereas $\gamma c^{-/-}$ mice have reduced B and T cells, our demonstration of the ability of LMPPs to reconstitute in a rapid and sustainable manner polyclonal T cells is, from a clinical viewpoint, of particular significance.

The recent identification of LMPPs through different approaches[25,26,38-40] implicates a novel pathway in hematopoiesis in which MkE potential is lost before GM potential in the process of lineage restriction toward lymphopoiesis.[41] The present studies extend the potent ability of LMPPs to replenish lymphopoiesis to a clinically relevant setting. They also shed new light on the probable physiologic role of LMPPs as early lineage-restricted (compared with HSCs) but multipotent progenitors of B and T lymphopoiesis. Whereas the original studies of transplanted LMPPs in maximally myeloablated recipients demonstrated their lack of self-renewal ability,[23,24] the present studies demonstrate that as few as 200 LMPPs, despite this, have extensive ability to not only replenish peripheral B and T cells in lymphopenic mice but also their short-lived progenitors, at least for up to 36 weeks.

LMPPs might also prove to be attractive candidates for gene therapy of X-SCID, not only because of their potent ability to reconstitute X-SCID recipients but also in part because they are more actively cycling than HSCs, and might therefore be more accessible to transduction with viral vectors than HSCs. Because of their limited self-renewal ability, they might also prove less susceptible to leukemic transformation on transduction with γc-expressing viral vectors,[5] although this remains to be investigated.

The low survival rate in our fetal transplantation experiments is a considerable concern for these types of studies, although the survival rate was not lower than what has previously been reported after IUHCT transplantation in immune-deficient mouse models, typically ranging from 20% to 30%.[15,16,30] Recently, Peranteau et al[42] described a new transplantation procedure through the vitelline vein, apparently less traumatic, resulting in somewhat improved survival, but still only 35% to 37%. Our experience is in agreement with others in that the high loss of fetal transplant recipients is in part a consequence of the surgical procedure and transplantation of small mouse fetuses but is further aggravated by perinatal maternal cannibalism and neglect. Importantly, our control experiments with sham-transplanted fetuses demonstrated that the transplanted hematopoietic cells themselves have little or no influence on this mortality. More importantly, this high mortality appears to have limited relevance for human fetal transplantations. The expected loss in the human setting would be less than 3%, based on calculation of the cumulative risk of prenatal diagnosis ($< 1\%$) and fetal cord blood transfusion ($< 2\%$).[43] Even when retrospectively considering the report of a total of 4 procedure-related deaths of total 39 IUHCTs performed for various inborn hematologic/metabolic defects (1989-2001), the mortality does not exceed 10%.[18]

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Authorship

Contribution: K.L. designed and performed research, collected and analyzed data, and wrote the manuscript; C.J.H.P. performed research and collected and analyzed data; S.R.W.S. performed research and analyzed data; and S.-E.W.J. designed research, analyzed data, and wrote the manuscript.

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Article III

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Myeloid and lymphoid contribution to non-haematopoietic lineages through irradiation-induced heterotypic cell fusion

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Recent studies have suggested that regeneration of non-haematopoietic cell lineages can occur through heterotypic cell fusion[1–3] with haematopoietic cells of the myeloid lineage[2–6]. Here we show that lymphocytes also form heterotypic-fusion hybrids with cardiomyocytes, skeletal muscle, hepatocytes and Purkinje neurons. However, through lineage fate-mapping we demonstrate that such *in vivo* fusion of lymphoid and myeloid blood cells does not occur to an appreciable extent in steady-state adult tissues or during normal development. Rather, fusion of blood cells with different non-haematopoietic cell types is induced by organ-specific injuries or whole-body irradiation[1–10], which has been used in previous studies to condition recipients of bone marrow transplants. Our findings demonstrate that blood cells of the lymphoid and myeloid lineages contribute to various non-haematopoietic tissues by forming rare fusion hybrids, but almost exclusively in response to injuries or inflammation.

Heterotypic cell-fusion of bone marrow cells with cardiomyocytes, skeletal muscle, hepatocytes and Purkinje neurons has been proposed to occur normally in adult mice in the absence of organ-specific insults[2–6,11]. Such hybrids were suggested to originate from haematopoietic cells restricted to the myelomonocytic-committed lineage[3,6,7,10], although the potential contribution of lymphoid lineages has not been analysed. To investigate whether formation of heterotypic fusion hybrids can also originate from cells of the lymphoid lineages, we used mice in which lymphoid-lineage-specific promoters regulate Cre recombinase expression, to identify cells committed to B-cell (CD19) and T-cell (CD4) lineages and their ancestry. Bone marrow from these strains is transplanted into lethally irradiated Z/EG Cre-reporter mice. Following such transplantation, expression of green fluorescent protein (GFP) is inducible by Cre-mediated recombination, and can only occur following cellular fusion between a lymphoid-lineage-restricted Cre-expressing donor cell with a recipient cell. Nine weeks after transplantation, recipients had stable donor-blood reconstitution and the contribution to the cardiomyocyte, skeletal muscle, hepatocyte and Purkinje neuron lineages was investigated. Rare lymphoid-derived fusion events were detected in the heart (one cardiomyocyte), soleus muscle (two muscle fibres) and liver (three hepatocytes) of six CD19 and four CD4 Z/EG mice with bone marrow transplants. To our knowledge this is the first indication of a lymphoid-derived contribution to the formation of fusion hybrids with cells of non-haematopoietic origin. However, as only limited numbers of fusion events were observed in this model (and this could in part reflect the low efficiency of recombination), we further investigated the possibility for lymphoid-derived heterotypic cell fusion by using transplantation regimes that allow both high blood cell reconstitution and complete labelling with the donor-cell marker. Transplantation of Rag1$^{-/-}$GFP^{+} bone marrow cells reconstituting myelopoiesis, but not B and T lymphopoiesis[12,13] (Fig. 1a), into lethally irradiated congenic wild-type mice resulted in a reproducible contribution to cardiomyocytes, skeletal muscle fibres and hepatocytes 9 weeks after transplantation (Fig. 1b; Table 1). However, despite of high levels of bone-marrow-derived GFP^{+} microglia cells in the brain, no Rag1$^{-/-}$GFP^{+} Purkinje neurons were observed in any of the transplanted mice, suggesting that the reported haematopoietic contribution to Purkinje neurons[2,14] may have a non-myeloid and distinct origin from that of bone-marrow-derived microglia cells[15]. To investigate this further, *GFP* transgenic[13] bone marrow cells were

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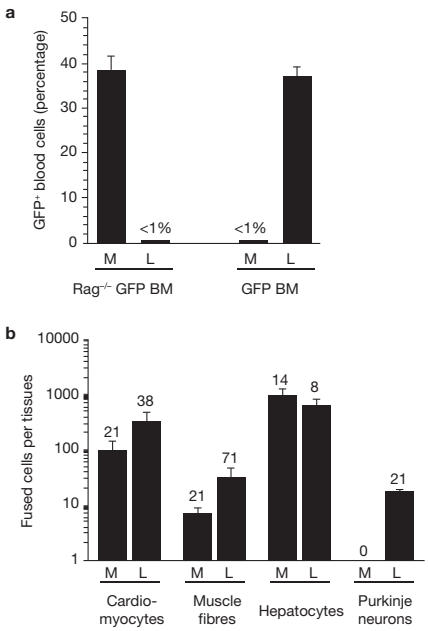

Figure 1 Both lymphoid and myeloid blood lineages contribute to non-haematopoietic cells through heterotypic cell fusion. (**a**) Myeloid (M) and lymphoid (L) reconstitution determined by FACS analysis of blood 4 weeks after transplantation in irradiated wild-type mice transplanted with Rag1$^{-/-}$ GFP$^+$ bone marrow (BM) and unconditioned (to allow lymphoid specific reconstitution[16]) X-SCID mice transplanted with GFP$^+$ bone marrow cells, respectively. (**b**) Contribution to cardiomyocytes, skeletal muscle fibres (tibialis anterior and soleus muscles), hepatocytes and Purkinje neurons 9 weeks after bone marrow transplantation. Numbers above bars represent actual numbers of cells found in 7 Rag1$^{-/-}$GFP transplanted mice (irradiated before transplantation) or 6 GFP$^+$ transplanted X-SCID mice (irradiated 4 weeks after transplantation, when lymphoid-restricted donor reconstitution was established). For a more detailed breakdown of data and estimation of frequencies, see Table 1. All mice in these experiments carried fusion hybrids in at least one of the investigated organs.

transplanted into non-irradiated X-linked severe combined immune deficiency (X-SCID) mice. This transplantation model results in sustained high levels of GFP$^+$ lymphoid but not myeloid reconstitution[16], which was confirmed by peripheral blood analysis (Fig. 1a) and assessment of myeloid cells in the bone marrow and liver of recipients (Supplementary Information, Fig. S1 and Supplementary Information, Methods). X-SCID recipients with donor-derived, lymphoid-restricted reconstitution were sub-lethally irradiated 4 weeks after transplantation; 5 weeks later, GFP$^+$ cardiomyocytes, skeletal muscle fibres, hepatocytes and Purkinje neurons were observed in all mice (Fig. 1b and Table 1). Taken together, these results suggest that lymphocytes possess a previously unrecognized ability (shared with myelocytes) to undergo heterotypic fusion with cardiomyocytes, skeletal muscle, hepatocytes and Purkinje neurons.

It has been proposed that the reported cellular fusion of haematopoietic cells with heterotypic cell types reflects a normal physiological phenomenon that can occur in adult mice[2–6,11], although studies have suggested that these events can be enhanced in muscle and liver by different stimuli[17–19]. Thus we wanted to investigate not only the potential influence but also the requirement of tissue conditioning in promoting the lympho-myeloid contribution to non-haematopoietic lineages. We therefore transplanted GFP transgenic bone marrow cells[13] into adult non-irradiated c-kit receptor-deficient (w^{41}/w^{41}) mice, to ensure that potential radiation-induced fusion would not complicate our analysis. Due to an intrinsic haematopoietic stem-cell (HSC) deficiency, these mice allow significant lympho-myeloid reconstitution from wild-type HSCs in the absence of irradiation-induced myeloablation[20]. Despite stable multi-lineage reconstitution of blood cells in these mice 4–6 weeks after transplantation (Fig. 2a), GFP$^+$ donor cells showed no contribution towards non-haematopoietic cell types 9 weeks after transplantation (Fig. 2b; Table 1). In contrast, if organ-specific injuries were inflicted when donor peripheral blood reconstitution was established in the non-irradiated w^{41}/w^{41} recipient mice (4–5 weeks after transplantation, Fig. 2a), non-haematopoietic donor-derived contribution was observed in the injured tissues 4–5 weeks following induction of injury (Fig. 2b–e; Table 1). Specifically, on induction of myocardial infarction[1], rare GFP$^+$ cardiomyocytes, determined as α-actinin-positive and CD45-negative, were observed exclusively in the infarction border zone (Fig. 2c), demonstrating that tissue injury can stimulate local contribution of blood-derived cells to cardiomyocytes. Furthermore, skeletal muscle damage caused by cardiotoxins or cryolesion[3,21] induced contribution towards α-actinin-positive and CD45-negative skeletal muscle fibres (Fig. 2d; Supplementary Information, Fig. S2). Finally, ibotenic acid, an excitatory amino acid and potent glutamate-receptor agonist, was injected into the brains of reconstituted, non-irradiated w^{41}/w^{41} recipients. This caused lesions through GABAergic-neuron toxicity[22]. Such injections of ibotenic acid did not stimulate contribution of haematopoietic cells to striatal neurons, despite severe injury to striatal structures and extensive infiltration of blood cells through temporary permeability of the blood-brain barrier. Rather, and unexpectedly, ibotenic injections caused a reproducible contribution of blood cells to GFP$^+$, calbindin-positive and CD45-negative Purkinje neurons throughout the cerebellum (Fig. 2e), associated with inflammatory infiltration of blood cells also in this region (Supplementary Information, Fig. S3). Lack of blood-cell-derived contribution at the site of injury suggests that heterotypic fusion with blood cells is specific to Purkinje neurons and inducible by inflammation. Thus, despite attraction of fusogenic blood cells to many tissues and cell types following injury-induced inflammation, hybrid formation seems to be restricted to a subset of cells implicated in syncytia formation[23] during development, and which therefore may also possess a particular propensity for heterotypic fusion.

The experiments in unconditioned w^{41}/w^{41} mice suggest that formation of heterotypic fusion hybrids derived from blood cells does not occur to an appreciable extent in steady-state, healthy adult subjects, although we cannot exclude the formation of rare, heterotypic-fusion hybrids at levels below those detectable in our assays (summarized in Table 1). This observation has important implications for interpretation of previous studies, which indicate that blood-cell-derived heterotypic cell fusion may be a normal phenomenon (in the absence of specific tissue conditioning); in these studies[2,3,5,6], reconstitution of traceable blood or bone marrow cells was facilitated by lethal whole-body irradiation[1–10], potentially promoting cell fusion in response to irradiation-induced tissue damage. To specifically address this important possibility, irradiated

Table 1 Lymphoid and myeloid contribution to non-haematopoietic cell lineages following irradiation and tissue-specific conditioning.

			% GFP+ blood reconstitution			GFP+ bone marrow derived non-haematopoietic cells											
						Cardiomyocytes			Muscle fibres[1]			Hepatocytes			Purkinje neurons		
				Distribution													
Group	GFP donor BM cells	Recipient	Total[2]	L	M	Number cells[3]	Frequency[4] (range)	Positive mice (%)	Number cells[3]	Frequency[4] (range)	Positive mice (%)	Number cells[3]	Frequency[4] (range)	Positive mice (%)	Number cells[3]	Frequency[4] (range)	Positive mice (%)
Myeloid reconstitution (irr. injury)	Rag1$^{-/-}$ β-actin	C57bl/6	44.9	0.5	86.0	21	1/127000 (1/52000-1/260000)	6/7 (86)	21	1/500 (1/300-1/1600)	6/7 (86)	14	1/38900 (1/18700-102600)	6/7 (86)	0	<1/704000	0/7 (0)
Lymphoid reconstitution (irr. injury)	β-actin	X-SCID	40.0	92.3	0.5	38	1/30800 (1/8900-1/195000)	5/6 (83)	71	1/100 (1/30-1/1400)	6/6 (100)	8	1/68100 (1/30500-1/91400	5/6 (83)	21	1/11200 (1/8200-1/20400)	6/6 (100)
No injury	β-actin	w^{41}/w^{41}	27.4	59.8	32.4	0	<1/3217500	0/9 (0)	1	1/100000	1/6 (7)	0	<1/3075000	0/8 (0)	0	<1/1530000	0/13 (0)
Myocardial infarction	β-actin	w^{41}/w^{41}	27.4	59.8	32.4	24	1/50000 (1/34500-1/100000)	6/7 (86)	N/A			N/A			N/A		
Muscle injury	β-actin	w^{41}/w^{41}	27.4	59.8	32.4	N/A			40	1/1300 (1/1000-1/2000)	8/8 (100)	N/A			N/A		
Brain injury	β-actin	w^{41}/w^{41}	27.4	59.8	32.4	N/A			N/A			N/A			44	1/4500 (1/3400-1/6700)	6/10 (60)
Irradiation injury	β-actin	w^{41}/w^{41}	86.2	64.6	31.4	104	1/50000 (1/34500-1/125000	7/8 (88)	42	1/2800 (1/2000-1/4300)	8/8 (100)	37	1/71400 (1/40000-1/333000)	7/8 (88)	61	1/15625 (1/11700-1/23300)	13/14 (93)
***In utero* transplant (no injury)**	β-actin[5]	SCID[6]	23.4	83,8	3.8	0	<1/858000	0/6 (0)	0	<1/60000	0/6 (0)	14	1/117600 (1/100000-1/142800)	4/6 (67)	63	1/11600 (1/4100-1/55600)	9/12 (75)
Neonatal transplant (no injury)	β-actin	SCID[6]	54.3	91.9	2.9	N/A			N/A			0	<1/1435000	0/7 (0)	2	1/250000 (1/76900-0)	3/13 (23)
Adult transplant (no injury)	β-actin	SCID[6]	48.4	90.3	2.9	0	<1/2574000	0/4 (0)	0	<1/40000	0/4 (0)	0	<1/820000	0/4 (0)	0	<1/408000	0/4 (0)

[1]Numbers from tibialis anterior and soleus were combined, except those from myeloid and lymphoid reconstitution experiments, where only tibialis anterior data are shown. [2]Mean total blood cell reconstitution of all mice in each transplantation group. [3]Total number of cells detected in each tissue from all mice. [4]Frequency of fusion hybrids of all cells in each tissue (compensated for GFP blood chimaerism, calculations and number of cells analysed in each tissue (explained in Methods, ranges are for positive mice, < frequencies in negative mice are based on number of mice and cells analysed per mouse). [5]Pooled data from both fetal liver and adult bone marrow donor cell transplantations. [6]Pooled data from both X-SCID- and NOD-SCID-recipient transplantations. Abbreviations: BM, bone marrow; irr., irradiation; L, lymphoid; M, myeloid; N/A, not analysed.

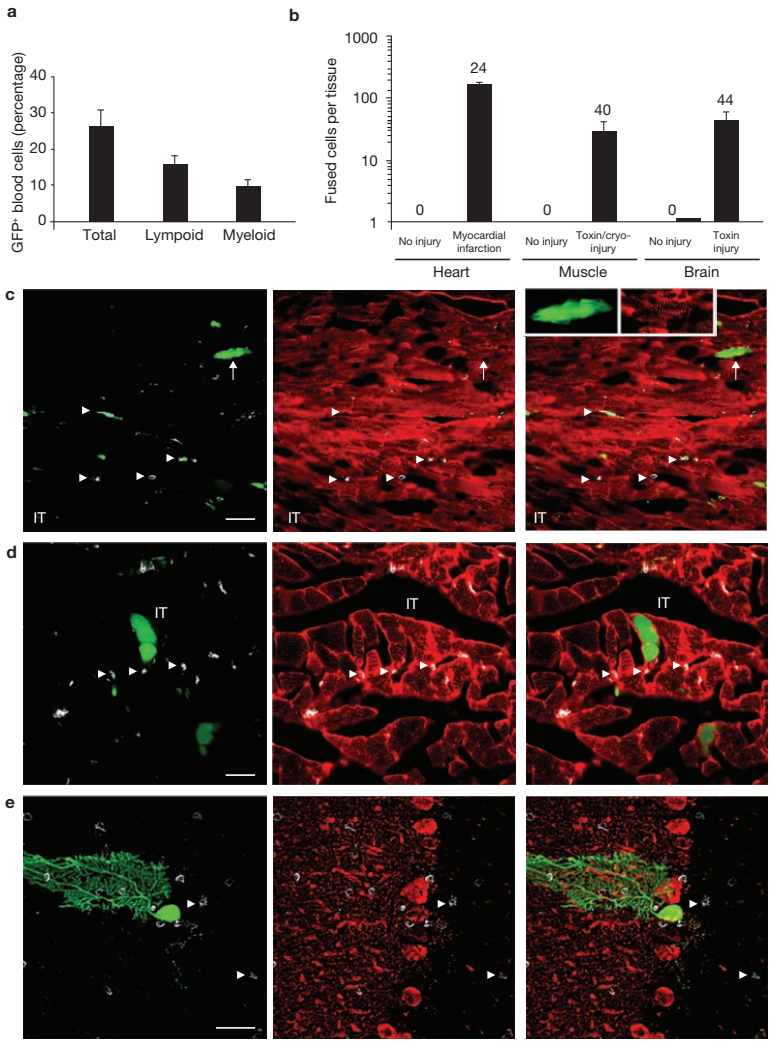

Figure 2 Contribution to heart, skeletal muscle and brain following tissue specific injuries. (**a**) Total, lymphoid and myeloid GFP$^+$ reconstitution in non-irradiated adult w^{41}/w^{41} mice 4–6 weeks after transplantation of GFP$^+$ bone marrow cells presented as a percentage of total blood cells. (**b**) Donor-cell contribution to non-haematopoietic cells in unconditioned and injured tissues evaluated 4–5 weeks after analysis of blood reconstitution and 4–5 weeks after induction of injury. Numbers above bars represent actual number of GFP$^+$ cells found in equally extensively analysed tissue injured mice (myocardial infarction; $n = 7$, muscle lesion; $n = 8$, brain lesion $n = 10$), and 15 unconditioned mice, from 3 experiments. For a detailed break down of data and frequency estimates, see Table 1. (**c**–**e**) GFP$^+$ (green, left panels) bone marrow derived cells expressing lineage markers (red, middle panels) but not pan-haematopoietic CD45 (white, arrowheads). Images merged in right panels. (**c**) GFP$^+$–α-actinin$^+$ (arrow and enlarged insets in right panel) cardiomyocyte in the border zone of the injured tissue (IT) following myocardial infarction. (**d**) GFP$^+$–α-actinin$^+$ soleus muscle fibres in the border zone of the injured tissue (IT) following cryo-injury. (**e**) GFP$^+$–calbindin$^+$ Purkinje neuron in the cerebellum following ibotenic acid injection (25 μg in 5 μl). Scale bars represent 100 μm (**c**, **d**) or 400 μm (**e**).

w^{41}/w^{41} recipients were reconstituted with GFP$^+$ bone marrow cells. In contrast to non-irradiated w^{41}/w^{41} recipients, GFP$^+$ cardiomyocytes, skeletal muscle fibres (soleus and tibialis anterior), hepatocytes and Purkinje neurons with characteristic morphology and lineage-marker expression were consistently observed throughout the tissues analysed in virtually all of the irradiated mice investigated 9 weeks after transplantation (Fig. 3a–e; Table 1; Supplementary Information, Fig. S4). As GFP$^+$ bone marrow cells were transplanted into recipients ubiquitously expressing the *lacZ* transgene (Rosa26)[24] we confirmed previous studies[1,2], in that the observed haematopoietic contribution occurred exclusively by

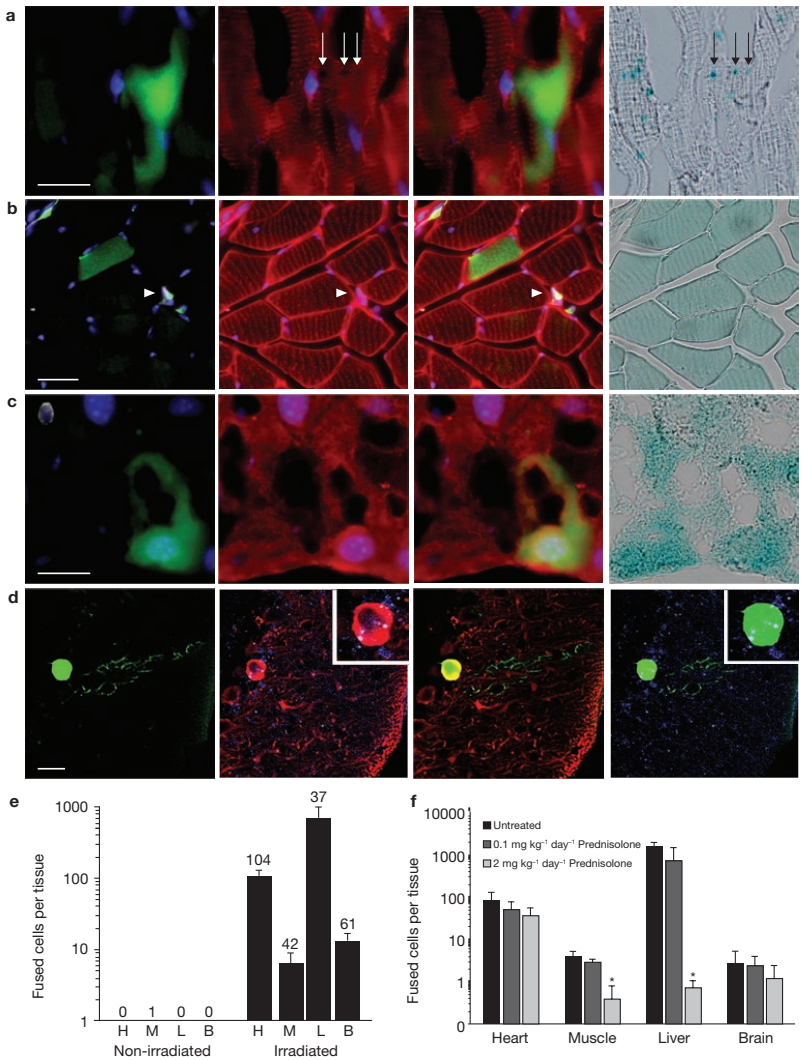

Figure 3 Evaluation of heterotypic blood cell fusion with cardiomyocytes, skeletal muscle fibres, hepatocytes and Purkinje neurons in adult mice 9 weeks after bone marrow transplantation. (**a**–**d**) Panel 1 (from left): donor-derived GFP$^+$ (green) and CD45$^-$ (white; arrowheads) cardiomyocyte (**a**), skeletal muscle fibre (**b**), hepatocyte (**c**) and Purkinje neuron (**d**) in irradiated w^{41}/w^{41} mice on Rosa26 background. Panels 2, 3: expression of tissue-specific markers (red) with CD45 staining (white; arrowheads in panels 2 and 3) or GFP signal (green, panel 3). Tissue-specific markers are α-actinin (**a**–**b**), albumin (**c**), and calbindin (**d**). Hoechst 33342 (blue) staining of nuclei in (**a**–**c**). Panel 4: cell fusion determined by detection of lacZ by either X-gal (blue-green, in **a**–**c**; white and black arrows in **a** indicate X-gal localization) or anti β-gal (blue dots) staining in GFP$^+$ cells. Staining was optimized for detection of antibody marker stains, GFP fluorescence and chromophoric X-gal detection of lacZ, allowing all analyses in one section. Note that balancing immunofluorescence and chromophoric detection results in weak but specific and characteristic[30] X-gal stains (Supplementary Information, Fig. S6). (**e**) Donor-derived GFP$^+$ non-haematopoietic cells derived through heterotypic cell fusion with or without lethal irradiation. Numbers above bars represents actual number of GFP$^+$ cells found, based on total number of cells screened in non-irradiated ($n = 15$) and in equally extensively analysed heart (H, $n = 8$), skeletal muscle (M, $n = 8$), liver (L, $n = 8$) and brain (B, $n = 14$) in irradiated mice in three independent experiments (>90% of mice carried fusion hybrids of one or more cell types investigated). For a detailed breakdown of data and frequency estimates, see Table 1. The single GFP$^+$ muscle fibre found in one of 15 analysed non-irradiated recipients represented a single event in all muscle tissues combined (soleus, tibialis anterior and panniculus carnosus), investigated in this study. (**f**) Effect on numbers of detected irradiation induced GFP$^+$ non-haematopoietic cells following treatment with antibiotics (125 mg l^{-1} ciprofloxacin and 185 mg l^{-1} amoxicillin) and varying doses (as indicated) of prednisolone acetate. Asterisks above bars represent statistically significant (Students *t*-test, muscle $P = 0.031$, liver $P = 0.008$) changes, compared with untreated mice ($P < 0.05$). Scale bars represent 100 μm (**a**–**c**) or 400 μm (**d**).

heterotypic cell fusion, as GFP$^+$ cells (Fig. 3a–d left panels) invariably co-expressed the *lacZ* transgene (Fig. 3a–d right panels). Furthermore, by transplantation of bone marrow cells from mice with muscle-specific (*α-actin* promoter) GFP expression[1], we also established reprogramming of the haematopoietic donor nuclei into a myogenic expression fate following the fusion event, by the presence of viable GFP$^+$ cardiomyocytes 9 weeks after transplantation (Supplementary Information, Fig. S4).

In mice with induced tissue-specific injuries, fusion hybrids were preferentially detected within regions of tissue damage or regeneration. In contrast, in irradiated mice, fused cells were distributed throughout the heart, skeletal muscle, liver and cerebellum. As the frequency of such events was very low for all investigated cell types, it proved essential to investigate a very large number of cells to be able to firmly conclude that irradiation is required for appreciable fusion to take place in these models. To demonstrate that this observation was reproducible in a large number of mice, as well as in multiple separate experiments, we also chose to investigate a reasonable number of cells per mouse in a large number of transplanted mice, rather than extensive cell numbers in a limited number of mice (summarized in Table 1). As for irradiated w^{41}/w^{41} recipients, a limited number of cells were investigated in each of the 21 mice in three separate experiments and, in 19 of these mice, fusion events were observed in one or more of the investigated cell types. In contrast, in 15 non-irradiated w^{41}/w^{41} mice only one GFP$^+$ muscle fibre in a single mouse was detected, and no GFP$^+$ cells in any of the other investigated tissues (Table 1). Thus, in irradiated w^{41}/w^{41} recipients a total of 244 fusion events (mean of 11.6 per mouse) were observed, in contrast to one (0.07 per mouse) in non-irradiated recipients. Although the reconstitution levels with GFP$^+$ blood cells were higher in irradiated than non-irradiated recipients, these data suggest that the blood-cell-derived fusion events observed in these cell lineages following establishment of bone marrow chimaeras is almost exclusively a consequence of irradiation. However, we cannot rule out the possibility that such fusion events can also occur at extremely low rates under steady-state conditions in the absence of irradiation or organ-specific injuries, particularly in the case of hepatocytes, as the GFP marker is variably expressed in liver cells of the β-actin–GFP mice (Supplementary Information, Fig. S6), a fact that could preclude detection of very rare events in our study.

A kinetic study demonstrated that blood-cell-derived hybrids in the heart were formed between 6 and 12 weeks (and not before) following transplantation and irradiation, and these fusion hybrids were sustained at stable levels until at least 24 weeks after transplantation (Supplementary Information, Fig. S4). The peak in the formation of fusion hybrids at approximately 12 weeks after transplantation, and of GFP$^+$ blood-cell infiltration of cardiac tissue at approximately 6 weeks after transplantation, substantiates the suggestion that the inducing effect of irradiation on heterotypic fusion hybrid formation is limited in time and that it is associated with whole-body lethal irradiation and blood-cell infiltration following bone marrow transplantation.

The mechanism underlying irradiation- and injury-induced blood-cell-derived heterotypic cell fusion was unclear but we hypothesized that local inflammation following tissue damage could have an important role. To address this issue, we investigated whether irradiation-induced heterotypic fusion could be reduced by inhibiting the inflammatory response to irradiation. Irradiated wild-type mice were transplanted with *GFP* transgenic bone marrow cells and treated with corticosteroids and antibiotics to suppress local inflammation and, potentially, secondary infections induced by post-irradiation tissue damage. Notably, corticosteroids decreased blood-cell-derived fusion hybrids (Fig. 3f). In agreement with this, we also repeatedly observed blood-cell-derived contribution to non-haematopoietic cells after bone marrow transplantation of unconditioned or sublethally irradiated, immunodeficient mice that had intestinal infections caused by the experimental conditions. However, when these infections were treated with antibiotics (Supplementary Information, Methods), the observed blood-cell contribution to cardiomyocytes, muscle fibres, hepatocytes and Purkinje neurons was completely blocked (Supplementary Information, Fig. S3). Taken together, our observations suggest that the formation of fusion hybrids following tissue injury or irradiation is, at least in part, mediated by the effects of inflammation. This hypothesis is supported by studies demonstrating that irradiation-induced inflammation promotes migration and infiltration of blood cells into injured tissues[25].

As our studies in adult mice demonstrated that lymphocytes contribute to non-haematopoietic cell lineages following tissue damage, we next investigated whether lymphocytes may contribute, through heterotypic fusion, to the development of non-haematopoietic lineages during embryogenesis, at stages when homotypic cell fusion plays a critical role in cell genesis of various organs[23]. As an initial approach we analysed unconditioned SCID mouse fetuses[16] transplanted *in utero* (14–15 days *post-coitum* (dpc)) with GFP$^+$ cells from either adult bone marrow or the fetal (14.5 dpc) liver, the primary haematopoietic organ at this stage of development. Six to eight weeks postnatally, GFP$^+$ lymphoid reconstitution[16] was verified and tissues screened for potential lymphoid-derived heterotypic fusion events. We found GFP$^+$ cells with characteristic morphology and phenotypic-marker expression of hepatocytes (Fig. 4a) and Purkinje neurons (Fig. 4b), but no GFP$^+$ cardiomyocytes or skeletal muscle cells (Fig. 4d; Table 1). GFP$^+$ hepatocytes and Purkinje neurons were found exclusively in mice with sustained GFP$^+$ lymphoid-restricted blood reconstitution of both adult bone marrow and fetal liver origin (Fig. 4c). In contrast, no GFP$^+$ hepatocytes and only rare GFP$^+$ Purkinje neurons were observed in SCID recipients transplanted at the neonatal stage (Fig. 4d), although such transplantation caused higher levels of lymphoid reconstitution (75 ± 4%) that should otherwise have increased the likelihood of detecting donor-derived rare fusion events. Furthermore, no GFP$^+$ non-haematopoietic cells were observed in non-irradiated adult SCID recipients intraperitoneally transplanted with GFP$^+$ adult bone marrow cells (Table 1). The ability of lymphoid cells to contribute to hepatocytes and Purkinje neurons exclusively following intrauterine transplantations could be compatible with blood-cell heterotypic fusion occurring during normal development. However, as the intrauterine transplantation represents a major surgical procedure likely to induce inflammation, it was essential to confirm this possibility by a non-invasive method. Thus, mice expressing Cre under control of T- and B-cell-specific, as well as pan haematopoietic (*Vav*) promoters, were used to more conclusively establish the potential contribution from haematopoietic committed cells towards non-haematopoietic cells during early embryogenesis and onwards. Such Cre-expressing mice were crossed with Z/EG reporter mice and their offspring (heterozygous for Cre and Z/EG) were investigated to the same extent as the intrauterine transplanted recipients at both neonatal (to limit evaluation to events occurring during fetal development) and adult stages, for evidence of heterotypic cell fusion of cells committed to either one of the two haematopoietic lineages with cells of the hepatocyte and Purkinje

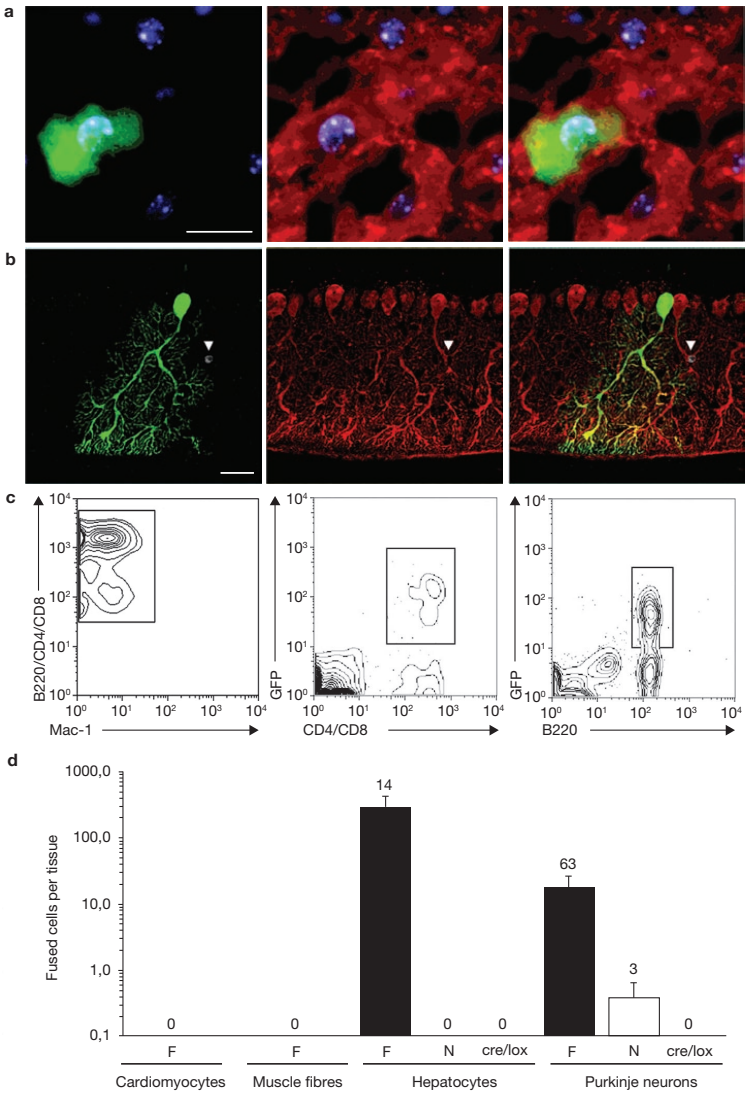

Figure 4 Lymphocytes contribute to hepatocytes and Purkinje neurons following *in utero* transplantation but not during normal embryonic development. GFP⁺ (green, left panels) cells expressing albumin and calbindin (red, middle panels) but not CD45 (white; arrowhead in **b**) in liver (**a**) and cerebellum (**b**), respectively. Hoechst 33342 (blue) staining of nuclei. (**c**) Lymphoid reconstitution 4–7 weeks postnatally in SCID mice transplanted with GFP⁺ bone marrow cells *in utero* (left) or in adult offspring from Z/EG crosses to either CD4cre (middle) or CD19cre (right) mice. Note the complete lineage specificity of GFP expression in the Z/EGxCD4cre and Z/EGxCD19cre crosses. (**d**) Haematopoietic-derived contribution to non-haematopoietic lineages. For a detailed break down of data and frequency estimates, see Table 1. Numbers above bars represent actual number of cells found in 12 mice in 5 *in utero* transplantations of adult bone marrow or fetal liver cells (F, data combined, 50% of mice contained GFP⁺ hepatocytes and 75% contained GFP⁺ Purkinje neurons), 7 mice in 2 neonatal (N; 23% of mice contained GFP⁺ Purkinje neurons) transplantations evaluated at 6–8 weeks of age and offspring from crosses of Z/EG reporter mice with either CD4 Cre ($n = 6$), CD19 Cre ($n = 6$) or Vav Cre ($n = 20$) mice (cre-lox) evaluated at 1–2 days or 8 weeks postnatally. Scale bars represent 100 μm (**a**) and 400 μm (**b**).

neuron lineages. Notably, no steady-state contribution (GFP⁺ cells) to hepatocytes or Purkinje neurons was detected in CD4 Cre ($n = 6$), CD19 Cre ($n = 6$) or Vav Cre ($n = 20$) offspring (Fig. 4d). This, together with our fetal transplantation experiments, further underscores the importance of using non-invasive methods to study the role of heterotypic fusion in normal physiology.

Although our study cannot exclude the possibility, reported previously[26], that rare heterotypic fusion hybrids are formed during normal development, our genetic lineage tracing models suggest that such events do not occur to an appreciable level under steady-state conditions. Further, although the existence of rare marked cells in postnatal tissues in such models could be compatible with rare blood-cell- derived fusion events, it is important to emphasize that conclusive evidence for their blood-cell origin is dependent on using a promoter to express Cre that is not also expressed in non-haematopoietic cell types (even in extremely small cell-subsets, as recently demonstrated[27]) at any time during development.

Herein, we have observed that not only myeloid cells but also lymphocytes contribute to various non-haematopoietic cell types through the formation of stable and reprogrammed fusion hybrids. The specific lymphoid lineage(s) involved in these processes remain to be established; however, as induction of such fusion events above detection levels depends on pre-conditioning, further investigation is relevant only for models in which the targeted tissues can be optimally conditioned. As fusion events appear to be exceptionally rare even under such conditions, the targeted cells should also possess considerable proliferative potential and a competitive advantage in order to expand the rare and otherwise insignificant fusion events. □

Note added in proof: a related manuscript by Johansson et al. *(*Nature Cell Biol. ***10,*** *575–583; doi:10.1038/ncb1720; 2008) is also published in this issue.*

METHODS

Mice. Experiments on genetically modified and haematopoietic deficient mice (for details see Supplementary Information, Methods) were approved by the ethics committees at Lund University and Bonn University.

Generation of bone marrow chimaeric adult mice. Whole bone marrow cells were transplanted by intravenous (tail-vein) injection into lethally irradiated or non-irradiated adult wild-type (900 rad), Rosa26 (900 rad), w^{41}/w^{41} (875 rad) or X-SCID (600 rad) recipients (all 8–12 weeks old). Haematopoietic reconstitution of the B cell, T cell and myeloid lineages was evaluated in peripheral blood 4–8 weeks post-transplantation (Supplementary Information, Fig. S5), as previously described[1]. Lymphoid-restricted reconstitution of non-irradiated X-SCID recipients was also verified by F4/80 antibody staining of monocytic descendants in the liver, as well as by granulocyte-macrophage progenitor-cell assays (CFU–GM) of recipient bone marrow cells at end point, as previously described[1] (Supplementary Information, Fig. S1).

Injury induction. Tissue-specific injuries to heart[1], skeletal muscle[3,21] and brain[22] were induced 4–5 weeks after bone marrow transplantation in mice with donor-blood reconstitution. Briefly, left ventricular myocardial infarction was induced by ligation of the left coronary artery in intubated adult mice anaesthetized with isoflourane. Skeletal muscle injuries were performed on isoflourane anaesthetized mice by injecting 25 μl of cardiotoxin (1 mg ml^{-1}; Sigma) to the right tibialis anterior muscle or by applying a copper probe (3-mm diameter, cooled in liquid nitrogen) twice for 10 s to the surgically exposed left soleus muscle. Brain toxin injury to GABAergic neurons was induced by injection of 0.5 μl of ibotenic acid (5 mg ml^{-1}; Sigma) unilaterally into the striatum (co-ordinates: AP +0.5 mm, L +1.7 mm, V –2.8 mm) into adult mice anaesthetized with halothane.

Cytokine mobilization. As in initial experiments we noted a higher frequency of heterotypic cell fusion in all tissues investigated following cytokine mobilization[1], such treatment was included in all experiments on adult mice (except in the kinetic studies). Bone marrow cells were mobilized to the blood by 5 daily cytokine injections (5 μg per mouse per day for each cytokine, starting 1 h after induction of injury or 4 weeks after bone marrow transplantation) with recombinant human fms-like tyrosine kinase-3 (flt3) ligand (FL) and recombinant murine granulocyte macrophage colony-stimulating factor (GM–CSF) (gifts from Immunex). Tissues were collected 4–8 weeks after cytokine treatment.

In utero **transplantation.** SCID mice with vaginal plugs (0.5 dpc) were anaesthetized with isoflourane at 14.5–15.5 dpc and a mid-abdominal incision was performed. Uterine horns were exposed and each fetus injected intraperitoneally with 5×10^6 GFP donor cells from adult bone marrow or fetal liver (14.5 dpc) with a Titanium 10 μl tapered, flexed-tip syringe (total volume of 2 μl). The abdominal incision was closed and females allowed to complete pregnancy. Haematopoietic reconstitution was evaluated as previously described[1] and tissues collected at 6–8 weeks of age. Recipient non-haematopoietic bone marrow reconstitution was excluded as no $GFP^+CD45^-Mac\text{-}1^-$ cells were observed in the bone marrow of transplanted mice before or after *in vitro* adherent cell cultures as described previously[28] (data not shown).

Phenotypical and morphological evaluation. Tissues (heart, skeletal muscle, liver and brain) were perfused *in situ*, post-fixed at 4°C with Stefanini solution, equilibrated in 20–30% sucrose and sectioned (8–40 μm). Sections were permeabilized and stained with X-gal (Sigma) or primary and secondary antibodies (Supplementary Information, Methods). Finally, sections were stained with Hoechst 33342 (Sigma). Specificity of all antibodies and the X-gal staining was verified by control antibodies and negative-and positive-control tissues. Expression of the GFP and *lacZ* transgenes in individual cells was detected in cryostat- or microtome-sectioned specimens using native GFP fluorescence microscopy and GFP-specific antibodies, and X-gal staining or β-gal-specific antibodies on cells in the same sections analysed by fluorescence and confocal microscopy (Supplementary Information, Fig. S6). To discriminate between auto-fluorescent skeletal muscle fibres and GFP^+ fibres, analysis of skeletal muscle samples was performed as described previously[29] to eliminate erroneous identification of auto-fluorescent cells as being GFP^+ (Supplementary Information, Methods). Images were taken using fluorescence and confocal microscopy. All data were confirmed independently by at least two investigators.

Statistics. Frequency of GFP^+ donor-derived non-haematopoietic cells in each tissue was determined by screening of representative sections. Equal amounts of tissue sections were investigated from irradiated and non-irradiated w^{41}/w^{41} mice, to an extent allowing detection of potential fusion events in both groups, despite the 2–3 fold lower level of haematopoietic reconstitution in non-irradiated mice (Supplementary Information, Fig. S5). In each mouse, approximately 214,500 cardiomyocytes, 3,500 tibialis anterior and 6,500 soleus muscle fibers, 205,000 hepatocytes and 102,000 Purkinje neurons were analysed (representing 1.7%, 100%, 0.4% and 50% of all cardiomyocytes, skeletal muscle fibers, hepatocytes and Purkinje neurons respectively, in each tissue). To calculate the frequency of cells derived through heterotypic cell fusion, the total numbers of GFP^+ cells found (numbers above bars in graphs) were divided by the total number of mice in each experiment and the total number of cells analysed in each tissue and then normalized to the level of blood reconstitution from GFP^+ donor cells, to represent the combined total number of heterotypic fusion events derived from both GFP^+ and GFP^- haematopoietic cells. Calculated frequencies were used to obtain estimates of the total number of fused cells in each tissue, based on an estimation of approximately 12.6×10^6 cardiomyocytes in the heart, 3,500 and 6,500 muscle fibres in tibialis anterior and soleus muscles, respectively, 50×10^6 hepatocytes in the liver and 204,000 Purkinje neurons in the brain. The GFP donor marker is extensively expressed in heart, muscles and brain, but only in approximately 50% of liver cells (Supplementary Information, Fig. S6); however this factor was not included in calculations of hybrid frequencies in recipient mice. All statistical analyses were performed using two-tailed Students *t*-test and *P* values less than 0.05 were regarded as significant. Data are presented as means ± s. e. m. throughout.

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS

J. M. N., K. L., L. T., C. N. and S. E. W. J. designed, performed and analysed bone marrow transplantation models; J. M. N., M. B., B. F. K. and S. E. W. J. designed, performed and analysed muscle injury models. J. M. N., S. S., D. K., A. B. and S. E. W. J. designed, performed and analysed brain injury models; J. M. N., M. B., W. R., B. K. J., S. J. and S. E. W. J. designed, performed and analysed heart injury models. J. M. N., K. L., S. J. and S. E. W. J. designed, performed and analysed irradiation injury models; J. M. N., M. B., S. S., C. G., P. S., B. K. F., S. J. and S. E. W. J. designed, performed and analysed fused cell characterization; J. M. N., K. L., S. S., D. K., A. B., S. J. and S. E. W. J. performed and analysed fetal and neonatal transplantation models; J. M. N. and S. E. W. J. wrote the manuscript and K. L., M. B., S. S. L. T., W. R., C. G., P. S., D. K., A. B., C. N., B. K. F. and S. J. reviewed the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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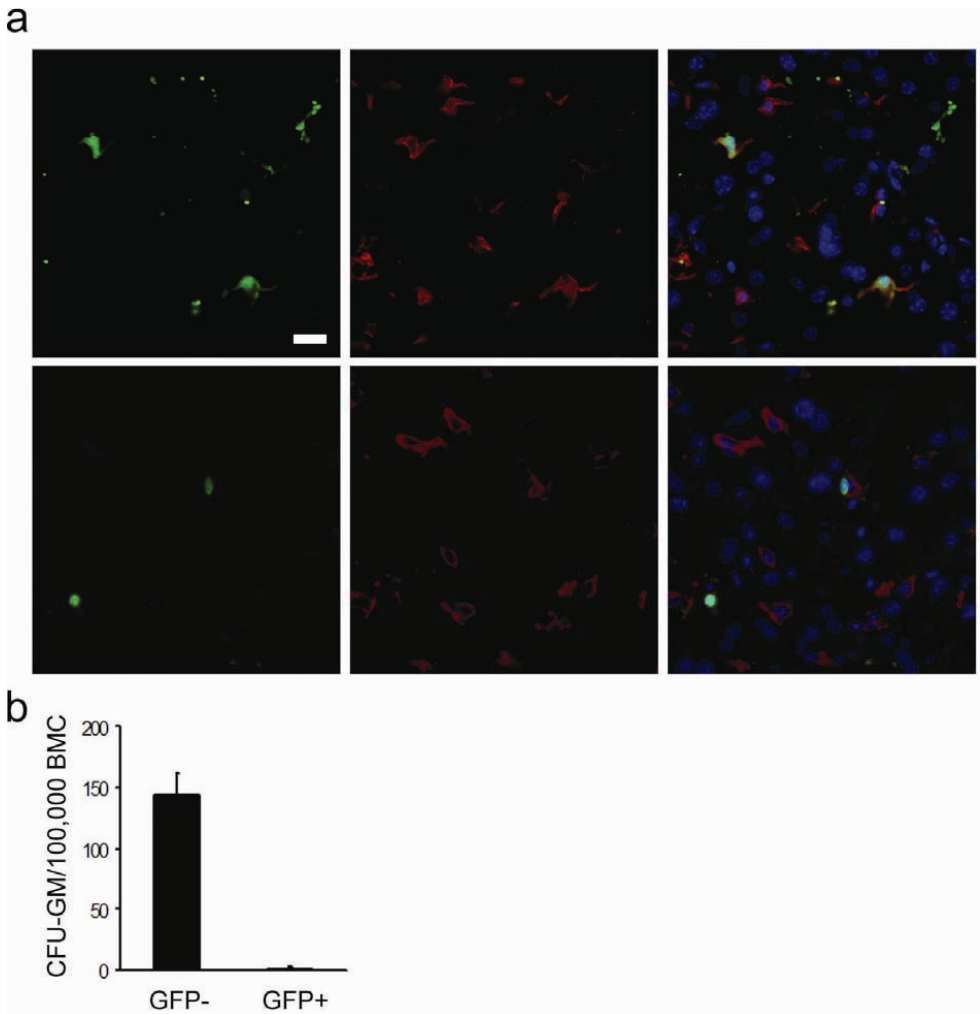

Figure S1 Verification of lymphoid restricted engraftment of GFP bone marrow cells in non-irradiated X-SCID recipients. (**a**) GFP$^+$ (green) donor derived cells investigated in the livers of Rag1$^{-/-}$GFP bone marrow recipients (myeloid restricted engraftment; upper panels) co-labelled with the macrophage/Kupffer cell marker F4/80 (red) whereas GFP$^+$ donor cells in livers of non-irradiated X-SCID recipients (lymphoid restricted engraftment; lower panels) did not. Hoechst staining of nuclei (blue). (**b**) Evaluation of donor derived (GFP$^+$) myeloid progenitor cells in the bone marrow of non-irradiated X-SCID recipients nine weeks after transplantation. Scale bar = 100 μm.

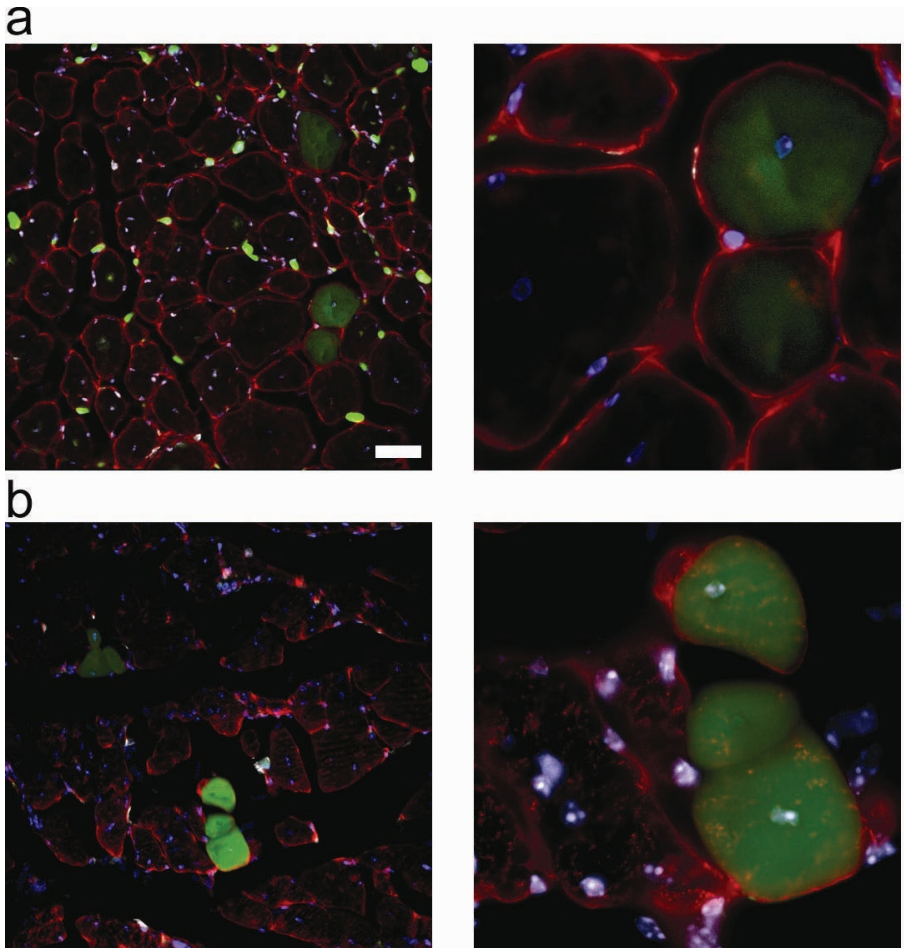

Figure S2 Cell fusion following tissue specific injuries to skeletal muscle. Non-irradiated w^{41}/w^{41} mice with significant (20±9.3%) multi-lineage reconstitution in blood following transplantation of GFP^+ BM cells were injured 4-5 weeks following transplantation in (**a**) tibialis anterior or (**b**) soleus muscles through cardiotoxin injection or cryo-injury, respectively. After 4-5 weeks GFP^+ (green) donor derived muscle fibres appeared in the border zone of the cryo-injured tissue or throughout the muscle of toxin injured mice and expressed α-actinin (red) but not CD45 (white). Lesioned areas in the skeletal muscle are characterized by the variety of fibre sizes, the wide spaces between them, damaged structural integrity, high numbers of infiltrating blood cells and central nuclei. Scale bar = 100 μm (**a** and **b**, left) and 25 μm (**a** and **b**, right).

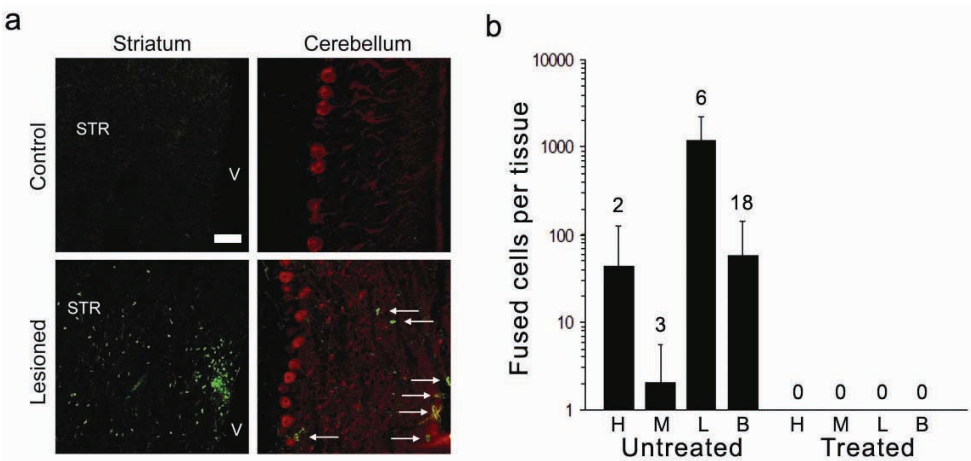

Figure S3 Inflammation as potential inducer of heterotypic cell fusion. (**a**) Infiltration of hematopoietic ($CD45^+$, green) cells to the striatum (STR) region adjacent to the ventricle (V), and cerebellum following ibotenic acid injection in the striatum but not in non-injected control mice. $CD45^+$ cells were found in the cerebellum (arrows) in close proximity to $calbindin^+$ Purkinje cells (red). (**b**) Estimated GFP^+ non-hematopoietic cells derived per tissue through heterotypic cell fusion in SCID mice suffering from intestinal infections. Numbers above bars represents actual number of GFP^+ cells found among total cells screened in the heart (H), skeletal muscle (M), liver (L) and brain (B), from equally extensively analyzed untreated (n=5) and treated (n=4) mice (as outlined in Supplementary Methods). Scale bar = 100 μm.

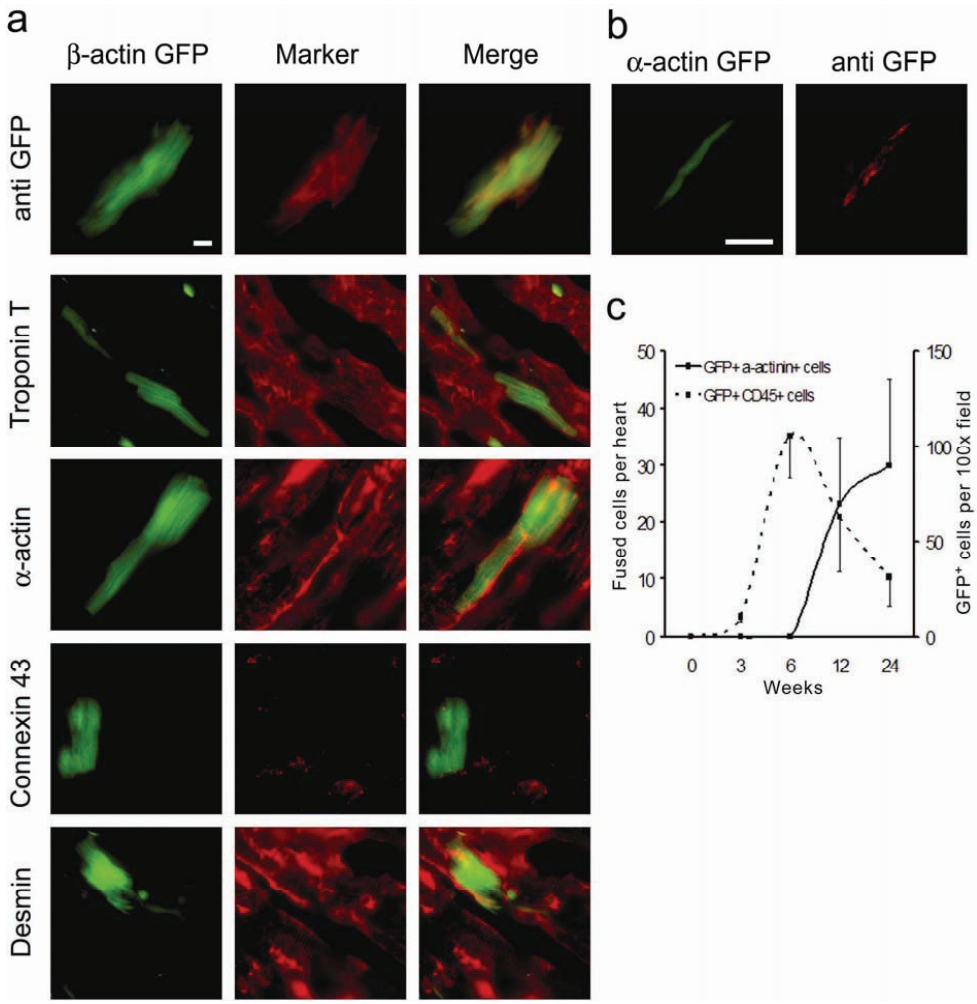

Figure S4 Characterization of cardiomyocytes derived through heterotypic fusion with donor bone marrow derived cells. (**a**) Donor derived GFP$^+$ (green) cardiomyocytes in irradiated recipient mice stained positive with anti-GFP and for the cardiac markers Troponin T, α-actin, Connexin 43 and Desmin. (**b**) Heterotypic fusion hybrids in wild type mice reconstituted with bone marrow cells expressing GFP under control of the α-actin promoter were positive for GFP determined by both native fluorescence and GFP specific antibodies. (**c**) Kinetics analysis of the formation of heterotypic fusion hybrids (GFP$^+$α-actinin$^+$) and infiltrating donor derived hematopoietic cells (GFP$^+$CD45$^+$) in hearts at 3, 6, 12 and 24 weeks after bone marrow transplantation and irradiation (3-4 mice analyzed at each time point, data are presented as means ±SD). Scale bars = 100 μm.

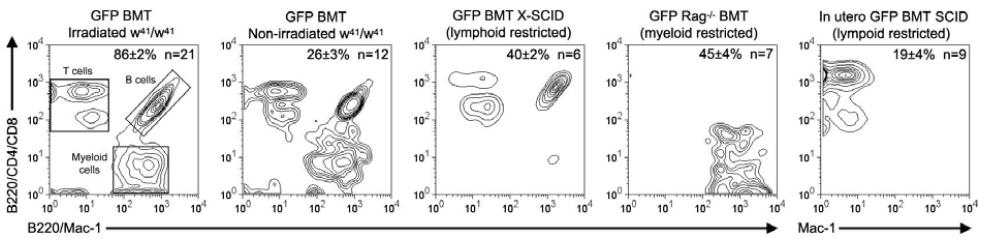

Figure S5 Lineage distribution of donor derived cells in peripheral blood of recipient mice 4-5 weeks following transplantation of GFP$^+$ bone marrow cells. From the left, plots show representative profiles of donor derived cells from transplantations from GFP$^+$ to irradiated w^{41}/w^{41} mice, GFP$^+$ to non-irradiated w^{41}/w^{41} mice, GFP$^+$ to X-SCID mice, Rag1$^{-/-}$GFP$^+$ to wt mice and GFP$^+$ to embryonic SCID mice. Numbers represent percent total donor derived reconstitution of indicated numbers (n) of mice.

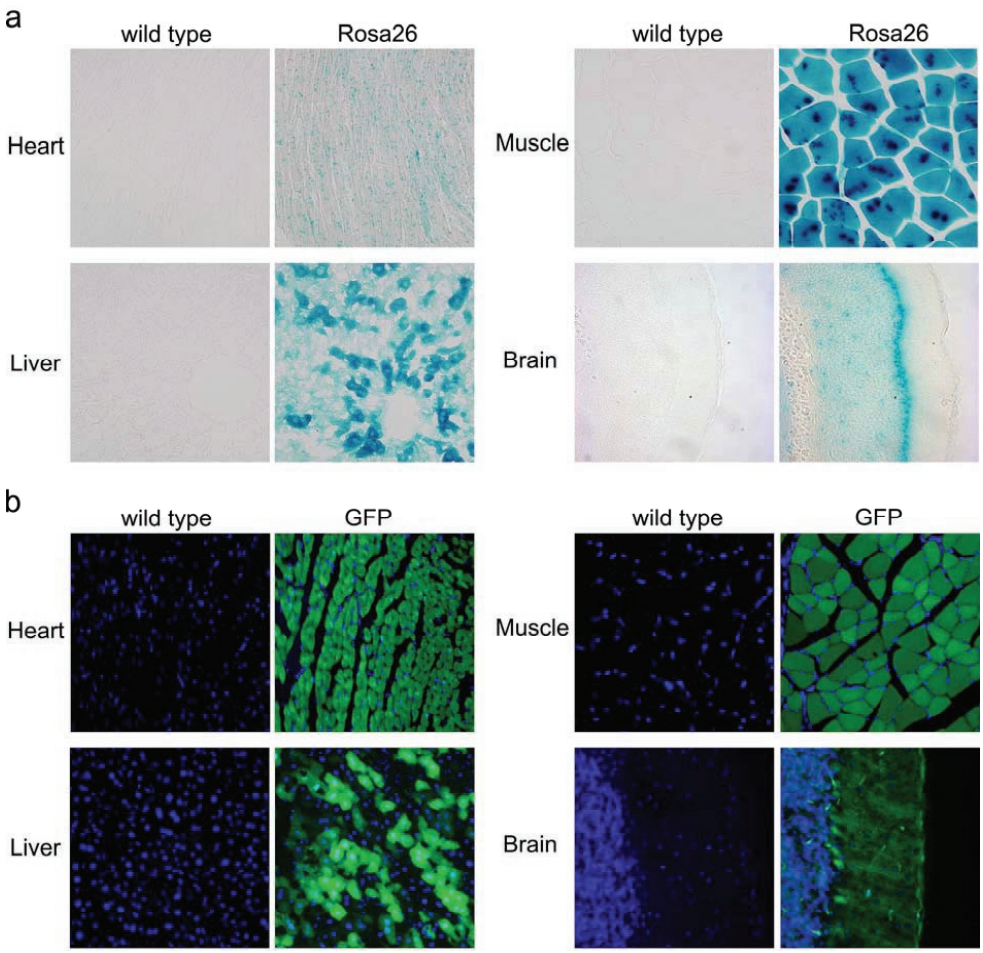

Figure S6 Extent of transgene expression and specificity of detection. (**a**) X-gal staining for lacZ on tissues from wild type and Rosa26 mice revealing virtual complete labelling of cardiomyocytes, smooth muscle fibres (soleus) and Purkinje neurons but only around 50% of hepatocytes. (**b**) GFP fluorescence in wild type and β-actin GFP mice revealing extensive GFP fluorescence in virtually all cardiomyocytes, smooth muscle fibres (soleus) and Purkinje neurons but only in around 50% of hepatocytes.

Supplementary Methods

Mice. LacZ (Rosa26; ref [1]) and α-actin[2] or β-actin[3] promoter driven GFP (on wild type or Rag1$^{-/-}$ background[4]) transgenic mice were all on C57Bl/6 background. Hematopoietic deficient mice were of w^{41}/w^{41} (ref [5]), X-SCID (ref [6]) and NOD-SCID (ref [7]) types. Hetero- or homozygous Cre expressing mice were genotyped by PCR, of DNA extracted from tail biopsies, as previously described (CD4 (ref [8]), CD19 (ref [9]) and vav (ref [10])) and mated with or transplanted to heterozygous Z/EG mice[11], genotyped by detection of lacZ with X-gal staining of tail biopsies, to induce Cre mediated recombination. Offspring were genotyped for presence of both Z/EG and Cre constructs. All mice were 8-14 weeks old at the time of transplantation.

Anti-inflammatory treatment. Irradiated recipient C57bl/6 mice were treated with ciprofloxacin (125mg/l), amoxicillin (185mg/l) and prednisolon-azetate (0.1 or 2.0 mg/kg/day) in the drinking water to prevent irradiation induced inflammation, starting from day of irradiation. To blunt inflammatory responses as a response to intestinal infections SCID mice were continuously treated with ciprofloxacin (125mg/l) and amoxicillin (185mg/l) from birth until termination.

Immunohistochemistry. Sections of heart, brain, liver and skeletal muscle tissues were permeabilized and stained with primary antibodies to pan-hematopoietic (CD45; Neomarkers, Fremont, California), cardiomyocte (α-actinin; Sigma, St. Louis, Missouri, α-actin; Sigma, cardiac troponin T; Neomarkers, connexin-43; BioTrend, Cologne, Germany, Desmin; Sigma), hepatocyte (albumin; Accurate Chemical, Westbury, New York) and Purkinje neuron (Calbindin-D-28k; Sigma) specific markers and GFP (Chemicon, Temecula, California or Abcam, Cambridge, UK). Primary antibodies were visualized with secondary antibodies conjugated with Cy2, Cy3 and Cy5 (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania), Alexa Fluor 555 (Molecular Probes, Eugene, Oregon) or Texas Red (Jackson ImmunoResearch Laboratories).

Skeletal Muscle analysis. Analysis of skeletal muscle was performed as previously described[12]. Briefly, tissue was fixated without glutaraldehyde to minimize auto-fluorescence and two fluorescence filter sets, a narrow band pass (BP 480/20 – BS 495 – BP 510/20) and a wide long pass (BP 470/40 – BS 510 – LP 515), was used. The GFP^{+} fibers displayed green emission under both filters whereas the auto-fluorescent fibers were yellow under the long pass filter.

Instruments and settings. The fluorescence and immunohistochemistry images were captured using either an Olympus BX51 (Olympus, Solna, Sweden) fluorescence microscope equipped with a mercury lamp, Olympus UplanFl 10x/0.3, UPlanFl 20x/0.5, UPlanFl 40x/0.75 and UPlanFl 100x/1.3

Oil objectives, DAPI (31000), GFP Band pass (41017), TexasRed (41004) and Cy5 (41008) filters (all from Chroma, Fuerstenfeldbruck, Germany) and a DP70 Olympus digital camera (DP Manager Software Version 1.1.1.71) or a Zeiss Axiovert 200M microscope (Carl Zeiss, Göttingen, Germany) equipped with a Zeiss ApoTome and a XBO75 fluorescence lamp, Zeiss PH1 Plan-Neofluar 10x/0.3, PH2 Plan-Neofluar 20x/0.5 and Plan-Neofluar 40x/1.3 Oil objectives, F36-500 HC-Set DAPI, F41-020 HQ-Set RS-GFP, F36-503 HC-Set TRITC, F46-006 ET-Set Cy5 and F51-024 Dualband FITC/TRITC (only for screening of EGFP fluorescence) filters (all from AHF Analysentechnik, Tübingen, Germany) and a Zeiss AxioCam MRm (Zeiss AxioVision software). Confocal images were collected sequentially on a Leica DM IRE3 confocal laser-scanning microscope (LeiCa, Wetzlar, Germany) using the Leica Confocal Software Version 2.77 (Leica). Raw TIFF or JPEG images were merged in Adobe Photoshop 8.0 (Adobe Systems, Edinburg, U.K.) without background subtraction. Linear adjustments of brightness or contrast was done using negative control (unstained or isotype control stained) tissue as reference and were applied to the whole image and did not obscure, eliminate, or misrepresent any information present in the original.

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