ENRICHMENT OF THERAPEUTIC HEMATOPOIETIC STEM CELL POPULATIONS FROM EMBRYONIC STEM CELLS

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ABSTRACT

Jennifer Bushman Gilner: Enrichment of Therapeutic Hematopoietic Stem Cell Populations from Embryonic Stem Cells.

(Under the direction of Suzanne Kirby, MD, PhD)

Embryonic Stem Cells (ESC) serve as a versatile and infinite source of primitive stem cells of any developmental lineage, made readily available for study or therapeutic application. The hematopoietic lineage in particular may benefit from such a level of accessibility since therapeutic applications from conventional sources of hematopoietic stem cells (HSC) are often limited by the inability to isolate or expand sufficient numbers of cells. However, the potential of alternative ESC-derived generation of HSC has not yet been fulfilled. Our fundamental goal in the work presented in this dissertation was to define and characterize a method for the isolation of transplantable HSC differentiated from ESC. Our initial study was designed to isolate early hematopoietic progenitors based on flow cytometric sorting of cells expressing a lineage-restricted fluorescent transgene. However, incomplete genetic regulation of the transgene at the necessary stage of differentiation precluded its use as a molecular marker of HSC. We then conducted studies in an adult source of HSC (i.e. bone marrow) to further characterize a stem cell enrichment method that separates a population of cells based on efflux of the DNA-binding fluorescent dye Hoechst

33342 (Side Population cells, SP). Our findings demonstrate that within the SP, the exposure of cells to commonly used cell surface marker antibodies can reduce the engraftment of HSC. Returning to our original goal of isolating ESC-derived HSC, we applied the SP sorting method to early stage embryoid bodies (EBs) differentiated from ESC. Our results show that the SP from early EBs contains long-term repopulating hematopoietic stem or progenitor cells, as evidenced by sustained (>16 weeks) lympho-myeloid engraftment of transplanted recipients. Furthermore, we have found that inclusion of a truncated erythropoietin receptor transgene in ESC used to derive the graft can improve the efficiency of transplantation in a ligand-dependent fashion. In summary, we have employed genetic and cell sorting techniques to successfully isolate and expand a population of HSC differentiated from ESC, and these studies may be useful in the design of future therapeutic applications from embryonic stem cells.

To Addison Elaine. You chose a most auspicious day, little one. Indeed, you have shared your first year so graciously. May everything this production has taken from you be redeemed to you a million times over through the doors this will open for me, for us.

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LIST OF ABBREVIATIONS

BM	Bone Marrow
EB	Embryoid Body
Epo	Erythropoietin
ESC	Embryonic Stem Cell
FACS	Fluorescence Activated Cell Sorting
GFP	Green Fluorescent Protein
HPRT	Hypoxanthine Phosphoribosyl Transferase
LIF	Leukemia Inhibitory Factor
LTR-HSC	Long-term Repopulating Hematopoietic Stem Cell
MHC	Major Histocompatibility Complex
PB	Peripheral Blood
SP	Side Population
tEpoR	Truncated Erythropoietin Receptor
UCB	Umbilical Cord Blood
YS	Yolk Sac

Chapter 1 LITERATURE REVIEW

A. HEMATOPOIETIC STEM CELLS

Defining the Phenotype of Hematopoietic Stem Cells— Hematopoietic stem cells (HSC) are functionally defined by their ability to both self-renew and differentiate into all lineages of the hematopoietic system (Fig. 1-1). Efficient isolation and manipulation of this rare cell population has important implications in both basic research and clinical use. Over the past several decades, many groups have sought to phenotypically define and purify long-term repopulating pluripotent hematopoietic stem cells. Protocols for the enrichment of HSC are based on: cell density [1, 2]; lectin binding [1]; dye exclusion [3, 4]; resistance to cytotoxic drugs, e.g. 5-fluorouracil [5-7]; adherence to plastic [8]; and, most commonly, expression of various combinations of cell surface antigens [9-16] (see also Table 1.1). Isolation of highly purified cells is critical for identifying the unique molecular mechanisms controlling HSC self-renewal and differentiation. Understanding the mechanisms for maintaining stem cell properties may improve our ability to manipulate and potentially expand HSC without losing their developmental potential. Furthermore, a precise definition of the HSC phenotype will facilitate the most efficient purification of therapeutic cells for transplantation.

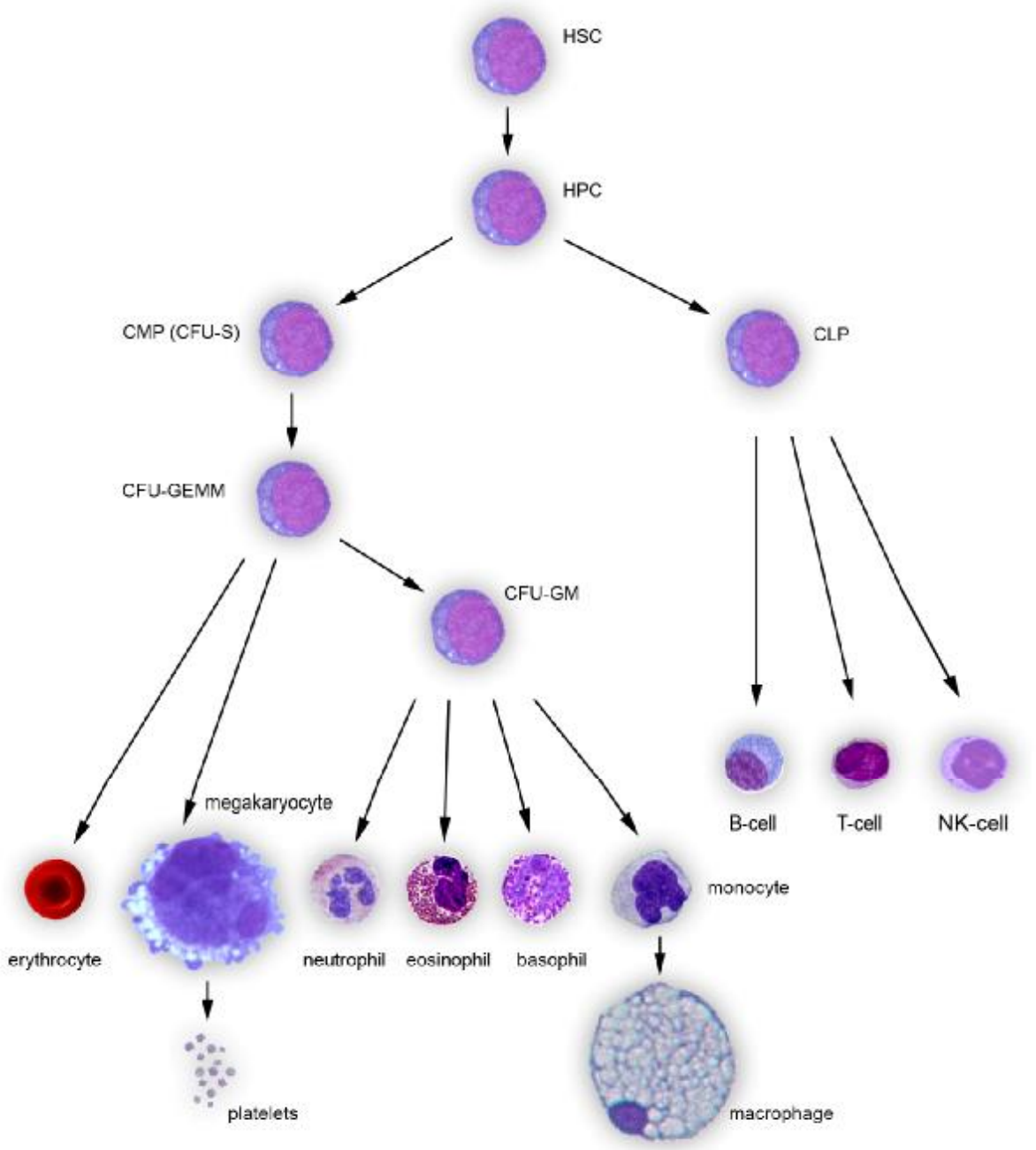

Figure 1-1 The Hematopoietic Stem Cell (HSC) gives rise to all lineages of the hematopoietic system.

Abbreviations: HPC, hematopoietic progenitor cell; CMP, common myeloid progenitor; CFU-S, spleen colony forming unit; CLP, common lymphoid progenitor; CFU-GEMM, colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte; CFU-GM, colony forming unit-granulocyte, macrophage, NK cell, natural killer cell

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Table 1.1 Methods used for isolation and enrichment of hematopoietic stem cells (HSC) from whole bone marrow (WBM)

HSC PHENOTYPE	RELATIVE % OF WBM	ENRICHMENT OF CFU-S (D12)	ENRICHMENT OF RADIOPROTECTION	ENRICHMENT OF LT REPOPULATION (WBM=1 IN 100,000)	REFERENCE
Low density, bind WGA, H-2K^{+}	nr	135-fold	180-fold	nr	[1]
Lin^{neg}	10-15% (5%[b])	nr	nr	nr	a) [9] b) [12]
Thy-1^{lo}Lin^{neg}	0.1-0.2%	200-fold	nr	nr	[9]
Thy-1^{lo}Lin^{neg}Sca-1^{+}	0.05%	1000-fold	1000-2000-fold (30 cells radioprotect)	2000-3000-fold[b]	a) [10] b) [17].
c-kit^{+}	5%	nr	nr	nr	[18]
Lin^{neg}Sca-1^{+}c-Kit^{+} (SKL)	0.08%	(80% are CFU-S)	100 cells needed[b]	100 cells rescue/reconst. (1000-2000-fold), 1 in 30 is CLTR[b]	a)[11] b) [19]
Rhodamine123^{neg} (WGA^{+}, 15-1.1^{neg})	0.25%	nr	30 cells radioprotect	1200-2000-fold	[4]
Lin^{neg}Sca-1^{+}c-Kit^{+}CD38^{+}	0.01%	CFU-S are defined by CD38$^{neg/low}$ fraction	nr	1 in 13 – 1 in 20	[12]
Lin^{neg}Sca-1^{+}c-Kit^{+}CD34^{neg}	0.004%	1.6% per 200 cells	nr	Single cell in 21% of recipients	[13]
Hoechst^{lo} (SP cells)	0.05-0.1%	200-fold	300-fold	1000-fold	[3]
Thy-1^{lo}Lin^{neg}Sca-1^{+}c-Kit^{+} (KTSL)	0.007%	1 in 61 cells	nr	80% of single cells are LT multipotent	[14]
CCE-25 (small, low density), c-kit^{neg}	nr	nr	nr	nr	[2]
Thy-1^{lo}Lin^{neg}Sca-1^{+}c-Kit^{+} Flk-2^{neg}	0.002%	nr	nr	nr	[15]
Hoechst^{lo}(SP), Endoglin^{+}	0.01% (20% of sp)	nr	nr	Enriches LTR 5-fold over SP alone	[16]

nr: not reported; CFU-S: colony forming unit – spleen; LTR: long-term repopulation; SP: side population; WGA: wheat germ agglutinin; CLTR: competitive long-term repopulation

Transplantation and Therapeutic Obstacles— Hematopoietic stem cell (HSC) transplantation has curative potential for many disease states, including hematopoietic or solid malignancies, congenital disorders, acquired bone marrow failure syndromes, and autoimmune diseases [20]. Currently, sources of cells for transplantation in humans are limited to the adult stem cell compartments in the bone marrow (BM), peripheral blood, and umbilical cord blood (UCB). There are three major obstacles to the success of transplantation therapy using these HSC sources. First, long-term repopulating hematopoietic stem cells are rare, so cell numbers are often limiting. Moreover, attempts to expand stem cell numbers *ex vivo* have been largely unsuccessful, resulting in loss of multilineage differentiation potential of the cells [21-23]. Lastly, the immunologic interaction between the donor and recipient cells following transplantation can lead to graft rejection or graft-versus-host disease, which may be a lethal complication of therapy [24]. These issues are exacerbated in the use of hematopoietic stem cells for gene therapy applications, where therapeutic (gene-corrected) cell numbers are critically low at the outset [25]. In addition, commonly employed methods of isolation or enrichment of HSC involving antibodies to cell surface markers may compromise the normal physiology of the cells, thereby reducing the effective cell number even further (discussed in Chapter 3).

Expansion of Hematopoietic Stem Cells— Initial approaches to expand HSC *in vitro* used various cytokine cocktails or co-culture with stromal cell lines (reviewed in [26]). These techniques have been deemed generally unsuccessful due to loss of repopulation potential [23] or homing ability of the cells [21]. An alternative approach has been through viral-mediated introduction of transgenes, such as genes that confer resistance to cytotoxic drugs like methotrexate [27], or genes that encode for transcription factors involved in cell

proliferation like *Gata-3* [28]. Undesirable side effects of the selective agents for drug-resistance transgenes limit their use in non-malignant disease. Regarding the transcription factor approach, *Hoxb4* emerged as a promising target for HSC expansion [29]. However, further studies have shown that prolonged expression of *Hoxb4* compromises differentiation of HSC to the lymphoid lineage [30-32]. Furthermore, the long-term effects of any transcription factor gene overexpression include the potential for malignant transformation. Our laboratory has had a long-standing interest in hematopoietic stem cell expansion through the use of transgenic expression of growth factor receptors. These receptors can serve as "non-toxic" transgenes, which optimally would give a benign and controllable expansion advantage to HSC without permanently altering the functions or developmental potential of the cells.

B. Use of a Truncated Erythropoietin Receptor as a Benign and Controllable Transgene for Stem Cell Expansion

Function and Structure of the Erythropoietin Receptor (EpoR)— As multipotent hematopoietic stem cells differentiate, erythroid progenitor cells up-regulate the lineage-specific erythropoietin receptor (EpoR), which confers responsiveness to the cytokine growth factor erythropoietin (epo) [33]. The peak of EpoR expression and epo responsiveness occurs in relatively mature precursors, which are termed colony forming units-erythroid (CFU-E) in *ex vivo* assays [33, 34]. Epo binding leads to homodimerization of the EpoR, which initiates intracellular signaling pathways that regulate survival, proliferation and differentiation of the committed erythroid progenitors [35-37].

The EpoR is a member of the hematopoietin receptor superfamily, which is itself a subgroup of the type 1 cytokine receptor family. Members of this family have a conserved

200 amino acid extracellular region consisting of an N-terminal domain with four conserved cysteine residues, and a C-terminal domain containing a Trp-Ser-X-Trp-Ser motif (WSXWS motif, where X represents any amino acid) (Fig. 1-2). Each of the family members also has a single transmembrane-spanning region. Ligand binding leads to rapid tyrosine phosphorylation of the cytoplasmic tail as well as associated signaling molecules, despite the fact that none of the receptors in this family possess intrinsic tyrosine kinase activity [38-41].

Signal Transduction from the EpoR— In the case of the EpoR, signaling pathways that have been implicated in the response to ligand binding include the Ras/mitogen activated protein kinase (MAPK) pathway, the phosphatidylinositol 3 kinase (PI3K) pathway (either directly or through the adapter proteins insulin receptor substrate (IRS-) 1 or 2), and the Janus kinase/signal transducer and activator of transcription (Jak/STAT) pathway (reviewed in [42]). However, a recent paper from Pelletier, *et al*. provides strong evidence based on the systematic analysis of over 40 mutant receptors that Jak2 is the sole direct signaling molecule downstream of EpoR required for biological activity [43]. Jak2 binding and activation by the EpoR requires two large domains in the membrane-proximal region, including the conserved Box1 and Box2 motifs as well as a glycine residue that is highly conserved amongst cytokine receptors. Another key feature of the EpoR cytoplasmic domain is the presence of eight tyrosine residues – most or all of which are phosphorylated following ligand binding [44]. The receptor can still transduce a proliferative signal when all eight tyrosine residues are mutated or removed, and importantly, removal of seven of the eight residues results in increased sensitivity of the receptor to epo [45].

Utility of a Truncated EpoR (tEpoR) Transgene— Previous studies in our laboratory explored the use of a truncated (epo-hypersensitive) mutant of the murine EpoR as a

transgene to impart a controlled proliferative advantage to hematopoietic stem cells both *in vitro* and *in vivo*. The mutant receptor used in these studies (as well as further work to be described in Chapter 4 of this dissertation) has a carboxy-terminal 91-amino acid truncation, and will be referred to as tEpoR [45, 46] (see also Fig. 1-2). Transgenic mice were generated by targeting the tEpoR to the hypoxanthine phosphribosyl transferase *Hprt* locus via homologous recombination in embryonic stem cells. The mice are healthy with no overt hematologic phenotype in the absence of exogenous epo administration. Treatment of these mice with epo results in the *in vivo* expansion of multipotent hematopoietic cells. Furthermore, the addition of epo to *in vitro* Dexter-type long term cultures of bone marrow cells from tEpoR transgenic mice results in the exponential expansion of trilineage hematopoiesis that can be maintained nearly twice as long as that from wild type bone marrow [46]. Competitive transplantation assays of tEpoR transgenic bone marrow proved that in the presence of exogenous epo, the tEpoR facilitates hematopoietic stem cell transplantation without stem cell depletion or malignant transformation [47]. Recently, a research group in Italy has further tested the utility of the same tEpoR transgene in the setting of viral transduction of both murine and human hematopoietic cells [48]. Consistent with the previous results from tEpoR transgenic mice, retrovirally transduced murine HSC acquired a repopulation advantage, and steady-state hematopoiesis in grafted recipients was unaffected as long as receptor levels were maintained at paraphysiological levels. Lentiviral tEpoR transgene transduction of human cord blood-derived CD34+ cells led to expanded clonogenic capacity *in vitro* as well as significant increases in the ability of tEpoR transgenic cells to repopulate immunodeficient mice in a xenotransplantation model [48]. Together these studies establish the tEpoR as a transgene capable of conferring a benign, controllable

proliferation advantage to hematopoietic stem cells in multiple settings, which may be useful for the isolation, expansion, and gene therapy of HSC.

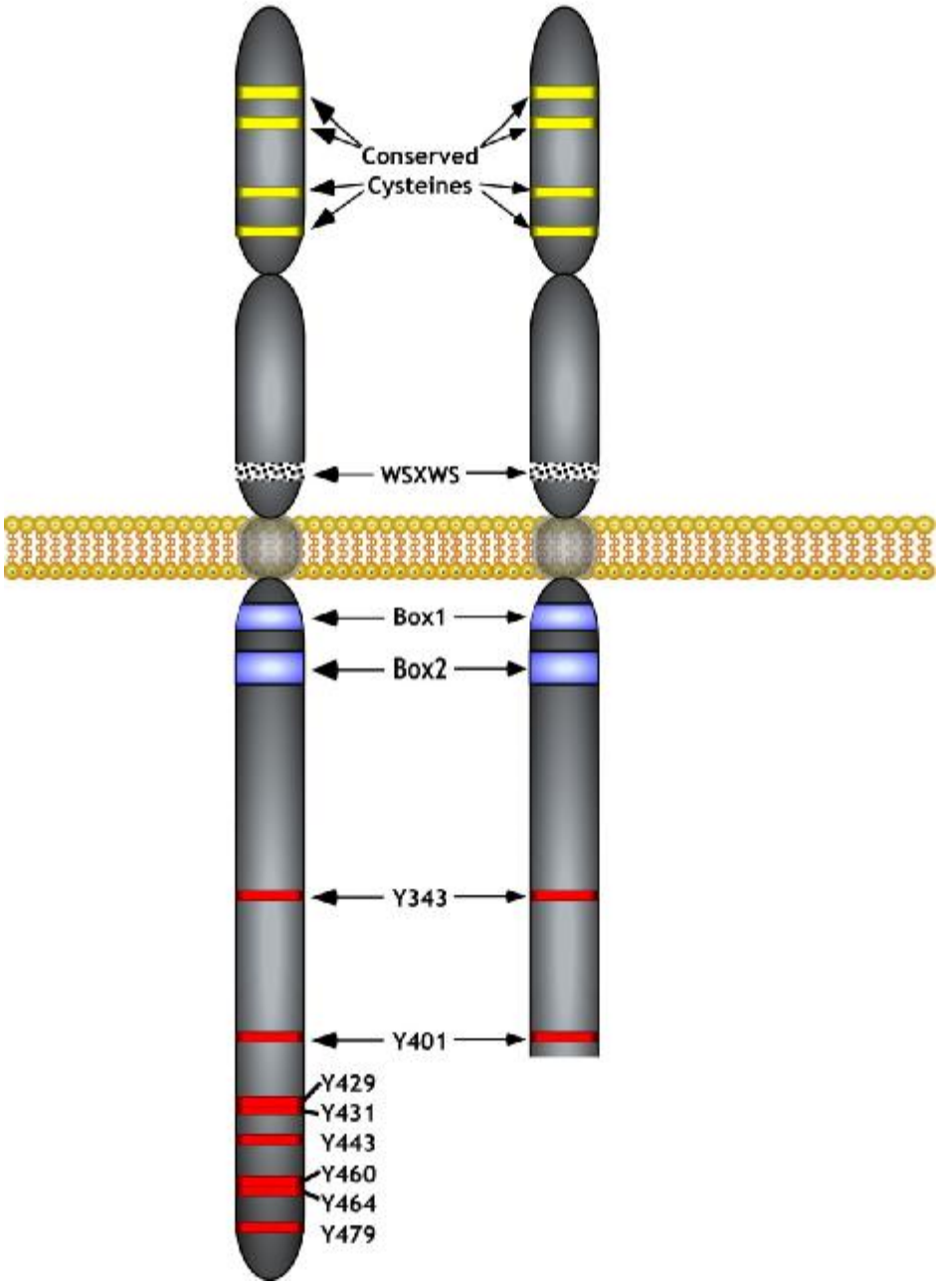

Figure 1-2 Schematic of the full-length and truncated Erythropoietin Receptors (EpoR and tEpoR)

C. Developmental Hematopoiesis in the Mouse Embryo

Gastrulation to Blood Formation— In the developing embryo, the process of gastrulation establishes the body plan of the organism through the formation of the three primary germ layers: endoderm, ectoderm and mesoderm. In the mouse this process begins approximately at embryonic day 6.5 (E6.5) [49]. The hematopoietic lineage arises out of the mesoderm layer through a common progenitor cell for endothelial and hematopoietic lineages, termed the hemangioblast [50]. The T-box transcription factor gene *Brachyury* (*T*) serves as a master regulator of genes required for the formation and differentiation of the mesoderm layer [51]. Molecular illumination of the mesoderm using a green fluorescent protein targeted to the *T* locus in combination with *in vitro* culture assays and gene expression profiling have defined the precise stage and localization of hemangioblastic commitment in the developing embryo [50]. A subset of mesoderm cells in the posterior primitive streak (a transient embryonic structure central to gastrulation [51]) demonstrate hemangioblast commitment through upregulation of a gene encoding the vascular endothelial growth factor (VEGF) receptor fetal liver kinase 1 (*Flk-1*) [50]. Genetic knock-out studies in embryonic stem cells and mice have established FLK1 as a molecular determinant of the hemangioblast [52] that likely functions by facilitating migration of committed progenitor cells to a hematopoietic-permissive environment [53]. Accordingly, $FLK1^+$ primitive streak progenitors then migrate to the extraembryonic yolk sac, where the earliest hematopoietic and endothelial cells arise in the blood islands around E7.5 [54, 55].

Ontogenic Sites of Hematopoiesis— This earliest wave of hematopoiesis in the yolk sac is predominated by primitive erythroid cells, characterized by large size, nuclear retention

and expression of the embryonic form of hemoglobin [56]. Definitive hematopoiesis, which generates definitive erythroid cells expressing adult globin genes, myeloid, megakaryocyte and lymphoid cells, arises in the fetal liver around E9.5 [57]. The classical view of hematopoietic ontogeny supposed that the yolk sac served as the origin of hematopoietic cells, which upon the establishment of the circulation around E8.5 would seed the fetal liver as a site for hematopoietic expansion [58], followed by migration to the bone marrow as the final site of hematopoiesis shortly after birth [57]. However, grafting studies in avian embryos provided evidence of a separate hematopoietic site within the embryo proper [59, 60], and in the mid 1990's several publications established the region of the developing mouse known as the para-aortic splanchnopleura (P-Sp, E8.5-9.5) / aorta-gonad-mesonephros (AGM, E10.5-11.5) as an autonomous site of hematopoietic development [61-64]. In the ensuing years there has been an ongoing debate over the true source of the definitive hematopoietic stem cell that seeds the fetal liver and eventually serves as the source of bone marrow hematopoiesis throughout the life of the animal (reviewed in [65, 66]). Initially, the requirement for repopulation of irradiated adult hosts deposed the theory of yolk sac origin for the definitive HSC [67, 68]. However, subsequent studies showed that exposure of yolk sac cells to a fetal liver environment or co-culture with AGM stroma could induce a presumed "maturation" in the cells that conferred repopulation ability [69, 70]. The most recent work in this field provides authoritative evidence, by way of an elegant inducible cell tracing method, that at least a portion of the definitive HSC of the adult mouse are indeed derived from *Runx1*-expressing yolk sac cells [71].

D. Embryonic Stem Cells as a Model of Hematopoietic Development

General Features of Embryonic Stem Cells— Embryonic stem cell lines (ESC) are derived from the inner cell mass of the preimplantation blastocyst-stage embryo [72, 73]. These cells represent an incredibly versatile biological tool in that they can be propagated indefinitely in culture while maintaining their totipotency. Leukemia inhibitory factor (LIF) is required to maintain murine ESC in an undifferentiated state in culture, whereas withdrawal of LIF leads to formation of embryoid bodies (EB) and differentiation [74]. EB formation recapitulates the early processes of *in vivo* development into the three primary germ layers: endoderm, ectoderm, and mesoderm. There have been reports of *in vitro* generation of multiple cell types from all three germ layers, and ESC can contribute to all cell types of an animal when re-introduced into a blastocyst-stage embryo [75]. The proliferation and differentiation capacities of ESC, combined with their versatility and relative ease of genetic manipulation make them an ideal source for cell-replacement therapies for many tissues and organs.

Generation of Hematopoietic Tissues from ESC— The first indication that ESC might model the development of the hematopoietic system came with the report that, in the absence of exogenous growth factors (other than those provided by serum), cystic EBs reliably develop blood islands similar to the blood islands of the E7.5 yolk sac [76]. Since that time, extensive exploration of the hematopoietic potential of ESC has ensued, making the hematopoietic lineage the most extensively characterized lineage from *in vitro* differentiation cultures of ESC [77, 78]. There are two basic differentiation schemes that have been developed for the generation of hematopoietic tissues from ESC; one involves the formation of EBs [79], and the other involves co-culture of the cells with a stromal line called OP9

[80]. The OP9 co-culture system allows for more control over the presence of specific growth factors or other influential molecules [77], and has been particularly useful in the generation of lymphoid lineages from ESC [80, 81]. However, it can be difficult to separate the stromal cells away from the desired differentiated populations, and the three-dimensional cell-cell interaction afforded by the EB system is necessary for accurate recapitulation of the *in vivo* processes of hematopoietic development [77]. Through careful study of the molecular and cell surface phenotypes as well as the developmental potential of the cultured cells over time, Keller *et al.* have established an efficient and reproducible EB-based protocol for the generation of primitive (erythroid, macrophage) and early definitive (erythroid, macrophage, megakaryocyte, neutrophil and mast) hematopoietic cell types in culture [79, 82, 83]. This differentiation protocol accurately mirrors the development of the hematopoietic system *in vivo*, as determined by gene expression [79, 84, 85], cell surface marker expression [86, 87], and kinetic analysis of lineage specification [88].

Advantages of Studying Hematopoietic Development in the ESC System— Because the ESC/EB differentiation system so closely recapitulates the developmental program of the early embryo, it has become a trusted model system for embryonic hematopoietic development. Yet, as an *in vitro* system, it allows access to rare numbers of early embryonic cells and stages of development that are otherwise inaccessible in the embryo [79]. As a result, several molecular markers of embryonic hematopoietic progenitors which differ from known adult HSC markers have been identified, such as FLK1 [86], VE-cadherin [87], and CD41 [89-91]. In addition, isolation of the elusive bipotential precursor of hematopoietic and vascular tissues, the hemangioblast, was first achieved in the *in vitro* ESC differentiation system [92, 93]. It was primarily through the study of culture conditions and molecular

markers for this *in vitro* hemangioblast that the identification of the hemangioblast in the developing embryo was possible [50]. Furthermore, this *in vitro* system has been highly complementary to the animal models for several molecules whose function could not be specifically characterized *in vivo* because their loss or mutation caused too severe a phenotype (e.g. *Gata-1* [94], *Gata-2* [95], *Vav* [96, 97]). The one area in which the ESC model system of hematopoiesis has not fulfilled its anticipated potential is in the derivation of transplantable HSC, which consequently is the underlying goal of the studies presented in this dissertation.

E. Embryonic Stem Cells as a Source of Differentiated Cell Types for Replacement Therapies

Therapeutic Promise of ESC— There is much excitement and anticipation regarding the therapeutic promise of ESC to treat pathologic conditions such as Parkinson's, Alzheimer's, diabetes, spinal cord injury, muscular dystrophy, and myocardial infarction. The media focus on these possibilities has been even greater since the isolation of human ESC lines in 1998 [98]. Recent reports demonstrate the isolation of transplantable ES-derived cells of all of the following types: endodermal precursors [99], hepatocytes [100], insulin-producing cells [101], dopamine neurons [102-104], neural precursors [105], microglia [106], and oligodendrocytes [107]. This research has met with varying levels of *in vivo* functional success, but overall has yielded evidence of many anticipated as well as some surprising characteristics of ES-derived tissues, such as the ability of certain cell types to engraft across major MHC barriers [99, 108]. A recurring problem in many of these reports is the incidence of teratoma formation in recipient animals due to the heterogeneous nature of transplanted cell populations [75]. Therefore, it is critical that any ESC-based therapeutic

proposals address the issue of isolating the desired cell type away from contaminating undifferentiated cells that may produce teratomas [77].

Hematopoietic Stem Cells from Embryonic Stem Cells— Despite the ever-growing body of knowledge regarding hematopoietic development from ESC since the discovery of yolk sac-like structures in EBs twenty-two years ago [76], there have been only about a dozen reports of successful generation of transplantable ES-derived hematopoietic cell types (See Table 1.2). Moreover, a recent review article suggested that numerous other studies along these lines have been conducted but not reported due to failure of engraftment of the cells [109]. Several of the earliest reports focused on the engraftment and/or functional maturation of lymphoid progenitor populations *in vivo*; thus the researchers either did not detect or did not assay for myeloid engraftment, and they did not conduct secondary transplants to test for self-renewal properties of engrafted populations [110-112]. The first group to report multilineage engraftment that was renewable in secondary transplants was Palacios *et al.* in 1995 [113]. In fact, this group saw the highest level of engraftment reported to date, with some recipients demonstrating complete replacement of their peripheral blood with donor-derived cells. They were also the first group to report an enrichment protocol for the transplanted cells – specifically, a lineage depletion step using magnetic bead-conjugated antibodies to seven different lineage markers, followed by a flow cytometry sorting step to isolate *P-glycoprotein*-expressing, lineage-negative cells. Despite the authors' reported success with this method, there have been no reports from other laboratories confirming the utility of this methodology in the generation of HSC. This lack of reproducibility may stem from the fact that certain culture conditions used in the differentiation of the cells in this report involve highly variable components that are not readily available to other researchers

(i.e. conditioned media designated "F" from a fetal liver stromal line and co-culture with the bone marrow stromal line RP010) [109, 113].

Two other reports described the isolation and moderately successful engraftment of a phenotypically defined cell population prior to transplantation. The first, from Potocnik *et al.*, defined a long-term lymphoid progenitor population by the expression of the fetal liver primitive hematopoietic marker AA4.1 [114], as well as the lack of expression of the early B cell marker B220 [115]. These authors reported a relatively low level (<15%) of donor chimerism, but showed evidence of functional lymphoid engraftment, as measured by immunoglobulin production of B cells and antigen-induced proliferation of T cells. The second phenotypic enrichment protocol involved the selection of cells expressing the stem cell factor receptor KIT in addition to the pan-hematopoietic marker CD45. The results of this study were notable for the fact that the authors saw relatively high levels of donor engraftment (>45%), even in the case of major histocompatibility complex (MHC)-mismatched mice. Furthermore, engraftment was enhanced in mice who were transplanted via direct intra-femoral injection despite lower numbers of cells injected [108]. These results suggest that the developmentally immature status of cells derived from ESC may be advantageous from the perspective of immunologic permissibility, but also problematic due to a possible lack of homing molecules necessary for engraftment in adult recipients.

A common condition to all of the preparations of ESC-derived HSC discussed so far is the prolonged maintenance of the cells in culture (7-30 days). Some of these studies make mention of the inability to transfer cells from earlier timepoints or different culture conditions due to the formation of teratomas in recipient mice [108, 111]. One study that challenges these claims reports multilineage engraftment from whole populations of

disaggregated day 4 EBs [116]. However, these results have been strongly criticized for the nonspecific characterization of donor-derived populations (reporting of the lineages of engrafted cells was based on forward and side scatter profiles rather than specific molecular markers) [109].

The remaining published reports of transplantable HSC are derived from genetically modified ESC. Several recent reports from Daley *et al.* illustrate a carefully planned approach to the derivation of definitive adult-repopulating HSC from ESC through the enforced activation so-called "homeotic selector" molecules [117] that may span the gap between primitive and definitive hematopoietic potential (i.e., HOXB4 [117], STAT5A [118], and CDX4 [119]). These studies were spawned by the results of their initial demonstration of multilineage engraftment of HSC derived from ESC that had been retrovirally infected with the chronic myeloid leukemia-causative oncogene *Bcr/Abl* [120]. The recipient mice in this primary study developed myeloproliferative disease due to oncogenic transformation of the graft. Moreover, their conclusions supported the existence of an "embryonic HSC" with both primitive erythroid potential and adult lymphomyeloid potential, thereby strengthening the hypothesis that repopulation activity is a function of the developmental environment of the cell.

Regardless of the extent of donor chimerism from ESC-derived hematopoietic cells, each demonstration of engraftment provides important insights into the derivation of HSC from ESC. Careful integration of the molecular and physiological lessons learned from each of these studies will bring the field gradually closer to the realization of the full HSC-producing potential of the ESC system.

Table 1.2 Summary of publications reporting the derivation of transplantable hematopoietic cells from embryonic stem cells

Source	Transgene	Culture Conditions				Graft Dose	Engr. (%) in 1° Txplts	Engr. (%) in 2° Txplts	Lymph / Myelo	Teratoma or other malignancy	Ref
		EBs?	Stroma	Cytokines	Days in Culture						
"ES fetus" D11-30EB	Oncogenes (retrovirus)	Y	None	-	11-30	10^7	0-72	ND	+/+	Y	[121]
Diffn Culture	None	N	RP010 (BM)	IL3, IL6, IL7	25	2-10x10^5	Y (%NR)	ND	+/-	N	[110]
D5-22EB	None	Y	None	-	5-22	0.8-36x10^5	≤ 4	ND	+/-	N	[111]
Diffn Culture	None	Y	ST2 (BM)	IL3, IL7, EPO	21	1-5x10^5	Y	ND	+/-	N	[112]
PgP^+Lin^{neg}	None	N	RP010 (BM)	IL3, IL6, "F"	25	4-6x10^4	≤ 100	Y (%NR)	+/+	NR	[113]
D4EB	None	Y	None	-	4	10^6 (+5x10^5 splenocytes)	Y (%NR)	ND	+/+	NR	[116]
$AA4.1^+$ $B220^{neg}$	None	Y	None	-	15	1-5x10^4	≤ 15	ND	+/(+)	NR	[115]
D5EB +culture	BCR/ABL	Y	OP9 ($MCSF^{neg}$)	IL3, IL6, SCF	30	4x10^6	20-55	Y (%NR)	+/+	MPD	[120]
D6EB +culture	HOXB4	Y	OP9 ($MCSF^{neg}$)	SCF, VEGF, TPO, FL	30	2x10^6	≤ 50	0-40	±/+	NR	[117]
KIT^+ $CD45^+$	None	N	None	SCF, IL3, IL6	7-10	1-10x10^5	45-90	ND	+/+	Y/N	[108]
Diffn Culture	STAT5A (constit)	N	OP9 ($MCSF^{neg}$)	-	7	1-2x10^6	5-20	Y (transient)	+/+	Y	[122]
D6EB +Culture	iSTAT5A	Y	OP9 ($MCSF^{neg}$)	SCF, VEGF, TPO, FL	30	1.75x10^6	≤ 1	ND	+/+	Y	[118]
D6EB +Culture	iCDX4 + HOXB4	Y	OP9 ($MCSF^{neg}$)	SCF, VEGF, TPO, FL	30	2x10^6	35-65	20-80	+/+	NR	[119]

Table 1.2 List of Abbreviations: EB, embryoid body; Engr., engraftment (expressed as percent donor chimerism in peripheral blood); Lymph, lymphoid; Myelo, myeloid; ND, not done; Diffn, differentiation; EPO, erythropoietin; PgP, P-glycoprotein; "F", conditioned media derived from the fetal liver stromal line FLS4.1 [123]; NR, not reported; $MCSF^{neg}$, indicates that the OP9 stromal cell line does not express the macrophage-colony stimulating factor protein; SCF, stem cell factor; VEGF, vascular endothelial growth factor; TPO, thrombopoietin; FL, FMS-like tyrosine kinase 3 ligand; constit, constitutively activated; i, inducible expression system

Chapter 2 SCL 3' ENHANCERS DO NOT RESTRICT TRANSGENE EXPRESSION WHEN TARGETED TO THE *Hprt* LOCUS OF EMBRYONIC STEM CELLS

A. ABSTRACT

The relative accessibility and ease of manipulation of embryonic stem cells (ESC) make them a potentially ideal source for the generation of long-term repopulating hematopoietic stem cells (HSC). However, an incomplete understanding of the molecular, cellular and environmental factors involved in HSC differentiation has limited the efficient generation of ESC-derived HSC with a repopulation potential comparable to conventional (adult-derived) sources of long-term repopulating HSC (LTR-HSC). A specific enhancer region downstream of the stem cell leukemia (*Scl*) gene has been used in murine models to direct the expression of several transgenes to embryonic sites of HSC formation, specifically to fetal and adult LTR-HSC. Thus, we hypothesized that fluorescent transgene expression controlled by this *Scl* enhancer would facilitate the isolation of HSC derived from ESC *in vitro* differentiation. Instead, we found that when targeted to the *Hprt* locus of murine ESC, neither the +19 nor the +18+19 *Scl* 3' enhancer sequence could properly restrict expression of the fluorescent transgene Venus. We observed widespread ectopic expression of Venus in non-*Scl*-expressing cells in both undifferentiated ESC and differentiated embryoid bodies (EB) generated from cell lines targeted with *Scl* enhancer-containing Venus constructs. Furthermore, integration of an *Scl* enhancer-containing construct in the reverse orientation with respect to *Hprt* transcription resulted in more robust Venus expression than if the Venus

construct (with or without an *Scl* enhancer) was integrated in the forward orientation. We noted some downregulation of Venus expression as the cells differentiated – particularly with the +18+19 enhancer element – which may indicate some later stage tissue-specific expression. Nonetheless, our studies demonstrate that additional regulatory elements are necessary to restrict Venus expression from a strongly permissive locus such as *Hprt* in ESC.

B. INTRODUCTION

Flow cytometry is a powerful tool for the identification, selection, and separation of discreet cell populations from complex mixtures of cell types. Traditional flow cytometry generally involves the use of fluorophore-conjugated antibodies directed against cell surface molecules to mark cell populations with known surface molecule profiles (reviewed in [124]). Staining techniques have also been developed for intracellular molecules, however these protocols require fixation and permeabilization of the cells and thus cannot be used to separate living cell populations [125]. For cell populations that do not have unique cell surface molecules, surface marker genes can often be genetically encoded for specific expression within the cells of interest. In recent years, the expanded repertoire of fluorescent molecule-encoding sequences has promoted combining flow cytometry with lineage-specific gene expression techniques. Using fluorescent transgenes as marker genes allows for the quick and easy identification and isolation of living cell populations by fluorescence activated cell sorting (FACS).

Embryonic stem cells are an excellent model system for exploring the full potential of lineage-restricted fluorescent transgenes. These totipotent cells are relatively easy to manipulate genetically, and *in vitro* differentiation protocols have been established for

numerous cell lineages (reviewed in [126]). Several groups have recently used ESC containing fluorescent transgenes to isolate endothelial [127, 128], cardiac [129, 130], neural [131] and endodermal [132] progeny after *in vitro* differentiation. In this study, we generated multiple embryonic stem cell lines carrying the fluorescent marker transgene Venus under the control of different enhancer elements from the stem cell leukemia (*Scl*) gene locus. Previous studies have demonstrated in murine models that these enhancer elements are sufficient to restrict transgene expression to cells of the hematopoietic and vascular lineages, including long-term repopulating hematopoietic stem cells (HSC) [133-135]. We targeted the Venus constructs to a specific locus (*Hprt*) in the ESC to control transgene copy number and integration site as well as to facilitate the direct comparison of Venus expression between transgenic cell lines [136].

Contrary to the results in transgenic mouse models, we found that the 3' *Scl* enhancer elements +19 or +18+19 were not sufficient to restrict Venus expression in undifferentiated ESC or in early stages of differentiation from ESC. These studies suggest that gene-targeted hematovascular-specific fluorescent transgene expression will require either additional *cis*-acting regulatory elements from the *Scl* locus or transcriptional regulatory elements from a different gene locus.

C. MATERIALS AND METHODS

Venus Expression Constructs and Gene Targeting— A plasmid containing the Venus fluorescent transgene and SV40 polyadenylation signal was kindly provided by Dr. A. Miyawaki (RIKEN Brain Science Institute, Saitama, Japan) [137]. The fluorescent gene was expressed from the SV40 minimal promoter, and for lineage-restricted expression vectors the

Scl gene 3' regions +19 (644 bp) or +18+19 (1 kb) were used as regulatory enhancer elements (generous gifts from Dr. B. Göttgens, University of Cambridge, United Kingdom). The Venus expression constructs were cloned into a modified version of the previously described *Hprt* targeting vector pSKB1 [138]. Targeting constructs were linearized with PmeI prior to electroporation. Cell culture, electroporation and selection of ESC were carried out by the University of North Carolina Animal Models Core Facility using the method described by Bronson *et al*. [138]. The ESC line used in these studies is a subclone of E14TG2a, a cell line derived from 129/Ola mice with an ~50 kb deletion in its *Hprt* gene [139] (Fig. 2-1).

In Vitro Differentiation— Undifferentiated embryonic stem cells were grown on gelatinized plates (0.1% gelatin, Sigma) in Glasgow Minimum Essential Medium (G-MEM, Gibco) supplemented with 500 U/mL ESGRO© (Millipore), 12% heat-inactivated ESC Qualified Fetal Bovine Serum (Gibco), 10 μM 2-mercaptoethanol (Sigma), 2 mM L-glutamine (Invitrogen), 100 μM nonessential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 100 U/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen). Hematopoietic differentiation of targeted ESC lines was carried out following the published method of Keller *et al* [83]. Briefly, EBs were generated by plating ESC in ultra-low binding 6-well plates (Corning) at a cell density of 50,000 cells/mL for day 3 EBs or 10,000 cells/mL for day 6 EBs. Differentiation medium consisted of Iscove-modified Dulbecco's medium (Gibco) supplemented with 15% Hematopoietic Differentiation Fetal Bovine Serum (Stem Cell Technologies), 2 mM L-glutamine (Invitrogen), 450 μM monothioglycerol (Sigma), 500 μg/mL holo-transferrin (Roche), and 50 μg/mL ascorbic acid (Sigma). Day 6 EB differentiation medium also included an additional 5% protein-free hybridoma medium (Invitrogen).

Flow Cytometric Analysis— Embryonic stem cells and embryoid bodies were disaggregated with 0.05% Trypsin / 0.02% EDTA (Gibco). Disaggregated cells were analyzed for Venus (YFP) fluorescence on a FACScan (BD Biosciences) or CyAn (Dako Cytomation) cytometer using Cytomation Summit software.

Microscopy of Targeted Cell Lines— Phase contrast and fluorescence microscopy of Venus-targeted undifferentiated ESC was carried out using an Olympus IMT-2 microscope. Digitized images were produced with an Optronics TAC470 camera and Scion Image software (NIH). Confocal images of day 3 EBs were captured on an Olympus FV500 microscope using a Zeiss 20X/0.5NA lens. Venus fluorescence was monitored using a 515 nm excitation laser and a 535-565 nm bandpass filter for emission. Stacks of single optical sections were processed into a single z projection using IMAGEJ software (NIH).

D. RESULTS

Targeting Scl-Venus Transgene Constructs to the Hprt Locus of Embryonic Stem Cells— We generated multiple constructs designed to restrict the fluorescent transgene Venus to hematovascular progenitor cells (hemangioblasts) upon differentiation of ESC *in vitro*. The 644 bp +19 *Scl* 3' enhancer element has been shown in transgenic mouse models to be sufficient to restrict transgene expression to hemangioblasts and their *Scl*-expressing progeny [135]. However, this minimal core enhancer has some dependence on integration site dependence for proper expression control, and it failed in directing β-galactosidase transgene expression to adult tissues (despite reliably directing a placental alkaline phosphatase transgene to adult tissues) [140]. In anticipation of similar expression fidelity issues in the ESC system, we also designed constructs incorporating a 1 kb fragment of the

+18+19 *Scl* 3' enhancer, which maintains proper *Scl*-controlled expression, but does not show integration site dependency (B. Göttgens, personal communication). Importantly, all other transcriptional control elements (promoter, polyadenylation sequence) were identical to those used in previous mouse transgenic studies showing properly restricted transgene expression. We also made a Venus only construct with no *Scl* enhancer elements to serve as a positive control with unrestricted Venus transgene expression. All Venus constructs are illustrated in Figure 2-1A.

We chose to target the Venus transgene constructs to the *Hprt* locus of mouse ESC using the single-copy chosen site integration method described by Bronson, *et al*. [138] (Fig. 2-1B). This targeting strategy eliminates transgene expression variability based on copy number or site of integration and allows for direct comparison of transgene expression between different transgene constructs. One important variable that remains when using this targeting method is the orientation of transgene construct integration with respect to the *Hprt* locus [141, 142]. Therefore, we designed two of our targeting constructs to direct Venus+19 integration both the forward and reverse (-Rev) orientation with respect to the direction of *Hprt* gene transcription (directionality of transgene integration is indicated by the large grey arrows in Figure 2-1A). Based on preliminary results demonstrating high-level unrestricted Venus expression from Venus+19-Rev targeted cells, as well as similar results from our collaborators in the laboratory of Dr. O. Smithies showing much stronger transgene expression in reverse-targeted ESC lines (D. Ciavatta and S. Hatada, personal communication), the remainder of our Venus constructs were only targeted in the forward orientation.

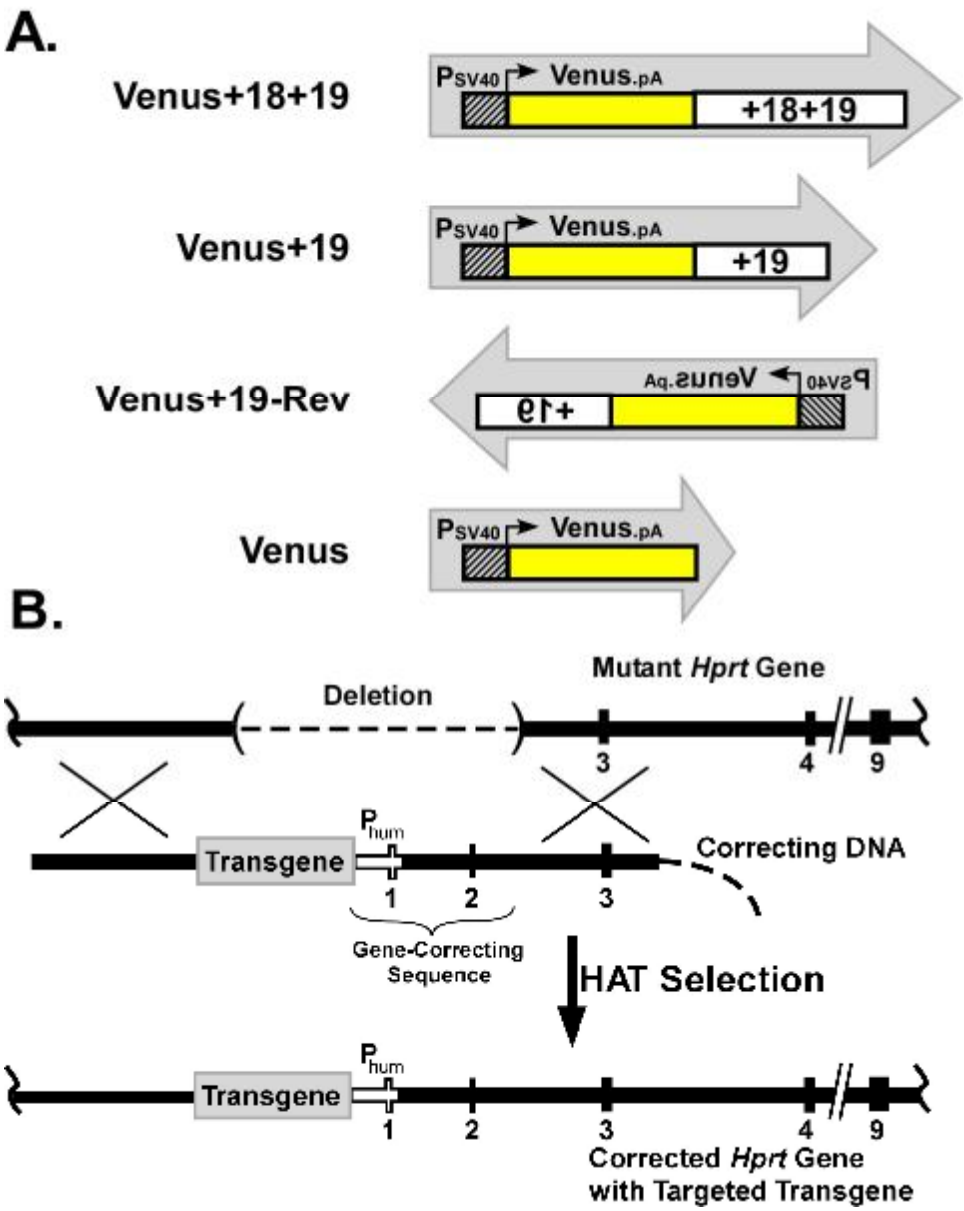

Figure 2-1 Transgene constructs and gene targeting to the *Hprt* locus.

A.) In all transgene constructs, the fluorescent protein gene Venus including the SV40 polyadenylation signal (solid yellow box) is driven by the SV40 minimal promoter (hatched box). Black arrows indicate the start site and direction of transcription. Open boxes labeled +18+19 (1 kb) or +19 (644 bp) represent the 3' *Scl* enhancer elements intended to restrict Venus expression to the normal *Scl* expression domain. Large grey arrows indicate the direction of transgene transcription with respect to *Hprt* gene transcription after targeting.

B.) (Top) The untargeted *Hprt* locus in the ESC line (E14Tg2a). The dashed line within parentheses represents the ~50 kb deletion that removes the *Hprt* promoter and first two exons. (Middle) The targeting construct includes a transgene from A.) and the missing promoter and exons to correct the *Hprt* gene, flanked by homologous sequences to direct recombination. Solid lines represent mouse sequences, and open lines indicate that in the correcting DNA the *HPRT* promoter (P_{hum}) and its first exon are derived from the human *HPRT* gene. (Bottom) Following HAT (hypoxanthine, aminopterine, thymidine) selection, only clones with a corrected *Hprt* locus, and thus an integrated transgene, will survive.

Scl-Venus Transgene Expression in Undifferentiated Embryonic Stem Cells— Much to our surprise, we saw widespread ectopic expression of the Venus transgene in undifferentiated ESC cultures from all targeted *Scl*-Venus cell lines. The undifferentiated cells showed normal ESC morphology and growth patterns in culture (see phase images, Fig. 2-2A), indicating that Venus expression did not have a detrimental effect on growth or maintenance of the ESC. However, considering that *Scl* is not normally expressed until the specification of hemangioblast progenitors from mesodermal precursors, we expected a complete lack of Venus expression at the undifferentiated ESC stage in the *Scl* 3' enhancer-containing clones [133]. On the contrary, nearly 100% of the cells from targeted ESC cultures display Venus fluorescence, as detected by flow cytometry, in comparison to untargeted ESC (Fig. 2-2, B and C). In forward-targeted cell lines, Venus transgene expression in *Scl* 3' enhancer containing lines was nearly indistinguishable from the positive control Venus-only cell line, suggesting that the +19 and +18+19 enhancer elements exert no control over Venus expression in undifferentiated ESC when targeted to the *Hprt* locus. Furthermore, Venus expression from the Venus+19-Rev transgene (69.35±30.09) was more than 3-fold higher than expression from the Venus+19 transgene (21.3±12.53), indicating orientation-dependent effects on transgene expression.

Scl-Venus Transgene Expression in Day 3 Embryoid Bodies— Targeted ESC lines were allowed to form embryoid bodies (EBs) *in vitro*, following the protocol described by the Keller *et al*. [82]. Using this differentiation scheme, multipotent hemangioblast progenitors are generated within the embryoid body after approximately 3 days of differentiation [92]. Our original goal in the creation of these ESC lines was to use *Scl*-restricted Venus expression to isolate hematopoietic stem cells from ESC. Thus, proper

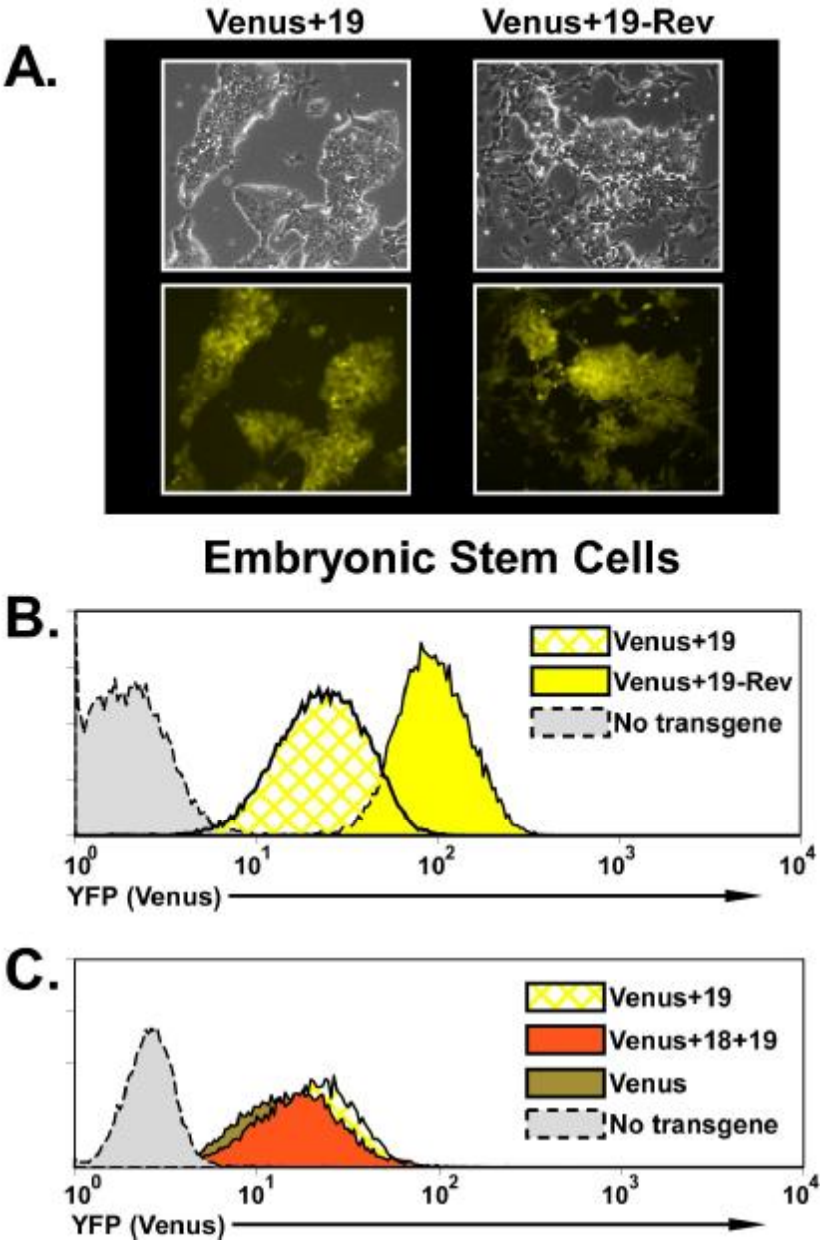

Figure 2-2 Venus expression in targeted, undifferentiated Embryonic Stem Cell (ESC) lines.

A.) Phase contrast images and fluorescence signal (pseudocolored yellow) from undifferentiated ESC cultures of cell lines targeted with the Venus+19 or Venus+19-Rev constructs, as indicated. Note that both cell lines show Venus expression in undifferentiated ESC, which do not normally express *Scl*, and that the expression appears brighter in the Venus+19-Rev cells.

B.) Flow cytometric verification of Venus expression in Venus+19 and Venus+19-Rev cells. Nearly 100% of live cells from undifferentiated cultures of both cell lines show some level of Venus fluorescence, and expression is much higher in the Venus+19-Rev cell line.

C.) Flow cytometric analysis of Venus fluorescence from targeted cell lines expressing Venus with no enhancer, the +19 enhancer, or the +18+19 enhancer, as indicated. There is almost complete overlap of the three cell populations, suggesting that the two enhancer elements exert no influence over Venus expression in undifferentiated ESC.

transgene expression at this stage was critical to our objectives. Unfortunately, day 3 embryoid bodies from all *Scl*-Venus targeted ESC lines continued to show widespread ectopic expression of Venus (Fig. 2-3). Confocal images of the 3-dimensional EBs illustrated fluorescent cells throughout the EBs, and as seen in the undifferentiated ESC, Venus expression was much brighter with the reverse-oriented transgene (Fig. 2-3A). Flow cytometric analyses of trypsin-disaggregated day 3 EB cells confirmed that there was continued ectopic expression of Venus at this stage of differentiation, particularly from the Venus+19 transgene. Interestingly, the Venus+18+19 transgene showed a small Venus-negative population, indicating at least partial control of Venus expression by the larger enhancer element as the cells differentiated.

Scl-Venus Transgene Expression in Day 6 Embryoid Bodies— Targeted ESC lines formed EBs as before and were allowed to differentiate for 6 days, at which point they contain multipotent hematopoietic progenitor cells of the myeloid and erythroid lineages [82]. EB cells were sampled every 24 hours during differentiation from day 3 to day 6 to track Venus transgene expression in the Venus+19 ESC line. Consistent with the findings of slight Venus downregulation in day 3 EBs, Venus expression continued to drop dramatically as the cells differentiated up to day 6 (Fig. 2-4A). The reduction in transgene expression was even more pronounced in Venus+18+19 cells, suggesting that the larger enhancer segment exerts more stringent control on Venus expression in later-stage EB cells. In contrast, the Venus+19-Rev transgene was still very bright in approximately 100% of cells at this stage of differentiation, indicating that the +19 enhancer element cannot overcome orientation-related influences on transgene expression from the *Hprt* locus.

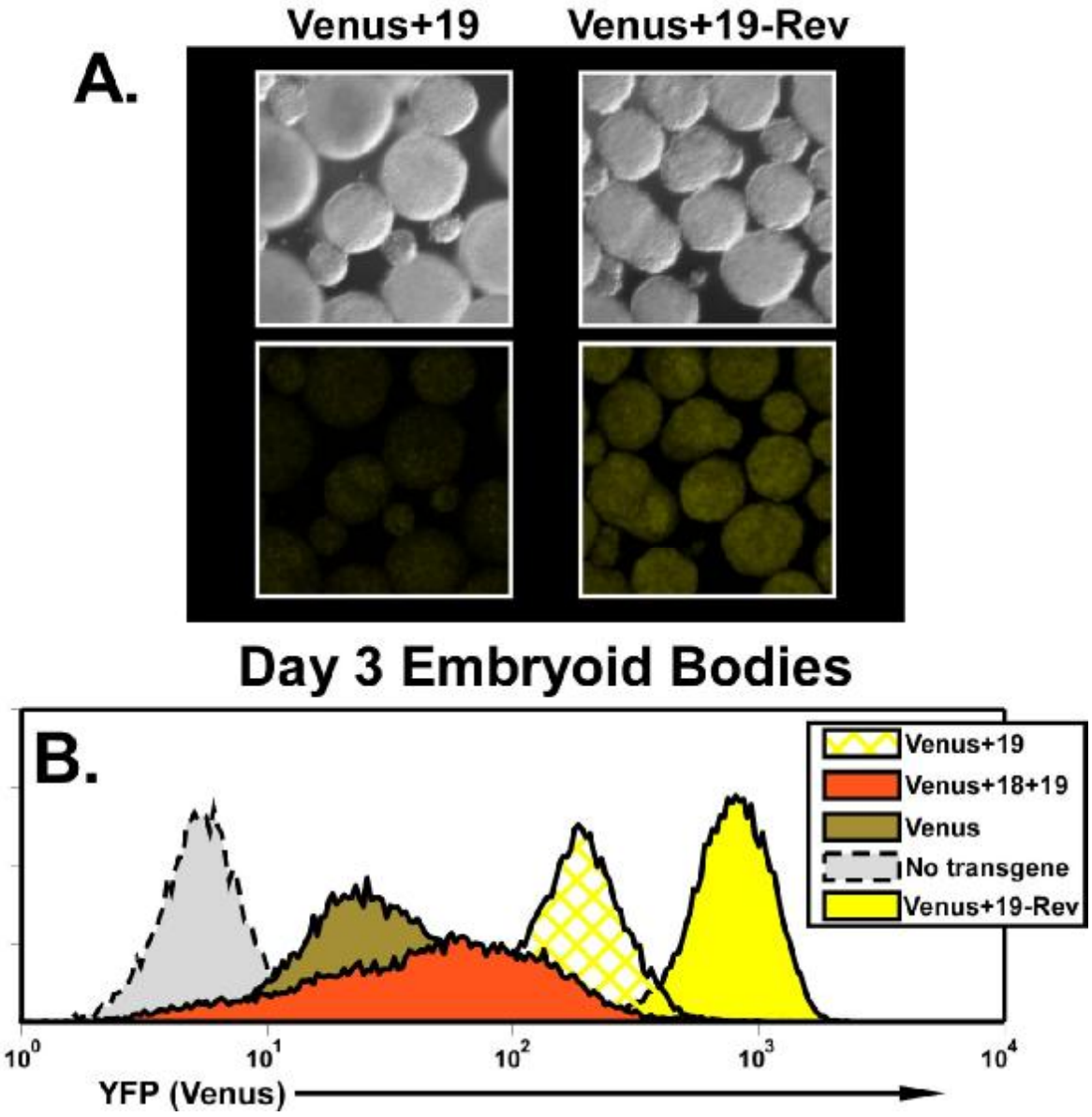

Figure 2-3 Venus expression in Embryoid Body (EB) cells after 3 days of differentiation.

A.) Phase contrast images and z projections of confocal images of the fluorescence signal (pseudocolored yellow) from day 3 embryoid bodies differentiated from cell lines targeted with the Venus+19 or Venus+19-Rev constructs, as indicated. Note that nearly all cells within the embryoid bodies show Venus expression and that again the expression appears brighter in the Venus+19-Rev cells.

B.) Flow cytometric analysis of Venus fluorescence from the day 3 embryoid bodies of cell lines expressing Venus with no enhancer, the +19 enhancer (For and Rev), or the +18+19 enhancer, as indicated. While the embryoid body cells from the +19 enhancer lines are still nearly 100% $Venus^{+}$ (and both brighter than the cells with Venus alone), the cells from the +18+19 enhancer line are beginning to show some downregulation of (or suppression of) Venus expression in a subpopulation of cells within the embryoid body.

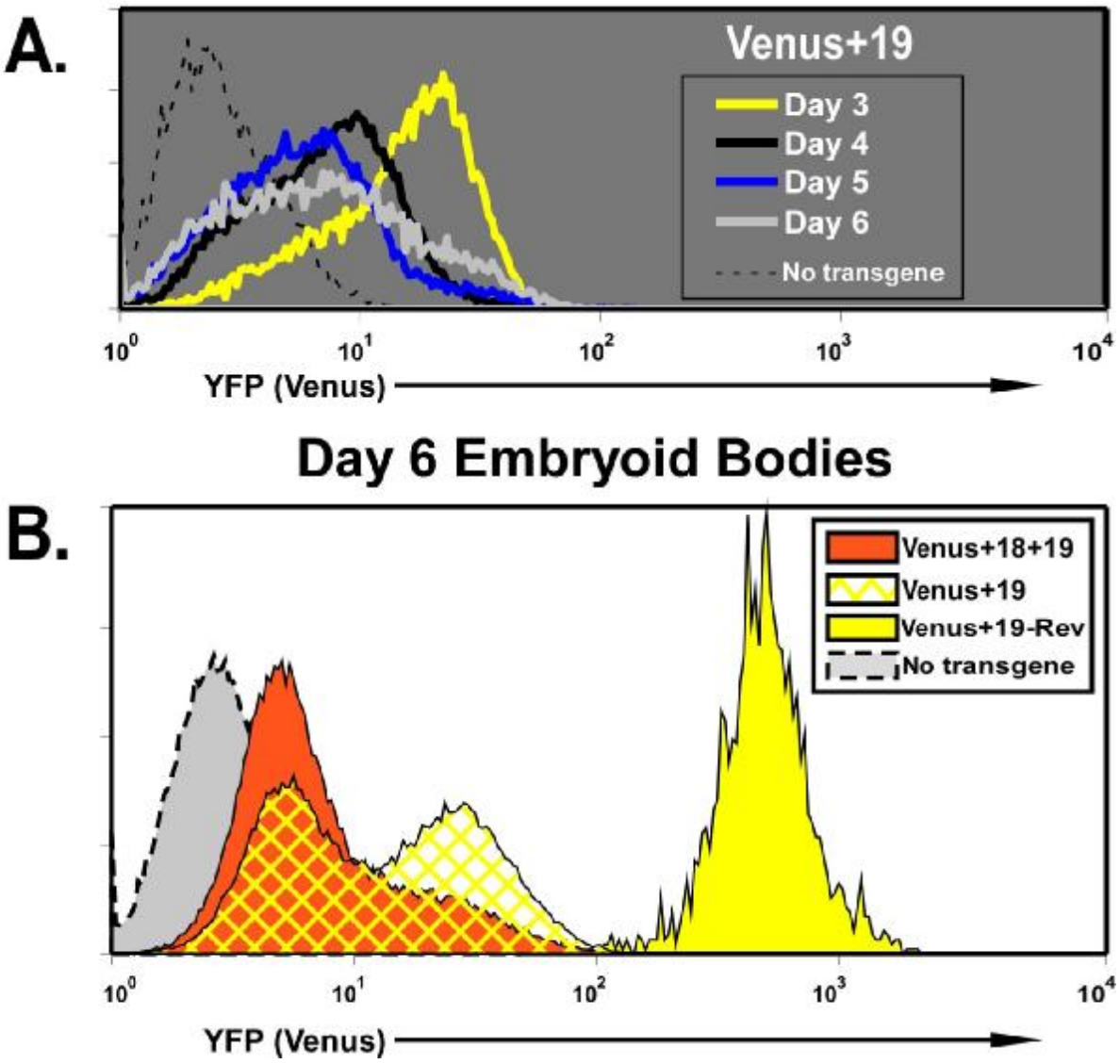

Figure 2-4 Venus expression in Embryoid Body (EB) cells after up to 6 days of differentiation.

A.) Progressive decline in Venus expression in Venus+19 embryoid body cells after 3, 4, 5, and 6 days of differentiation. As the cells differentiate, the Venus$^+$ population declines significantly, suggesting that in more differentiated cell types, the *Scl* enhancer element may exert some regulatory control over Venus expression.

B.) Flow cytometric analysis of Venus fluorescence from the day 6 embryoid bodies derived from cell lines expressing each of the three Venus-*Scl* enhancer constructs. Both the +19 and the +18+19 enhancer sequences appear to exert some control over Venus expression at this later stage, although the +18+19 enhancer is more stringent. When targeted in the Reverse orientation, the +19 enhancer sequence is unable to restrict Venus expression, even in day 6 EBs.

E. DISCUSSION

A major advantage of the embryonic stem cell system is that it allows access to and scalable production of rare or inaccessible cell populations. We planned to take advantage of this system for the identification and subsequent isolation of hematopoietic stem cells (HSC) from murine ESC. To that end, we hypothesized that lineage-restricted fluorescent transgene expression controlled by an enhancer element previously shown to direct transgene expression to long-term repopulating HSC [134] would facilitate FACS-based isolation of HSC from ESC. However, to our surprise, the *Scl* 3' enhancer element was not sufficient for lineage restriction of fluorescent transgene expression in ESC when targeted to the *Hprt* locus.

The transcriptional regulation of the *Scl* locus has been extensively characterized, resulting in the identification of five independent enhancers targeting expression to specific subdomains of normal *Scl* expression [133, 135, 143-145]. We were particularly interested in the 3' element that was shown to direct expression to hemagioblasts, hematopoietic stem and progenitor cells and endothelium [133-135]. This +18+19 enhancer element was originally characterized as a 5245bp fragment and the full-length enhancer has subsequently been used to direct HSC expression of multiple different transgenes in murine models [134, 146-149]. The present study represents the first description of the activity of this *Scl* 3' enhancer region in ESC, and notably, we have only characterized two core elements (644 bp +19 and 1 kb +18+19) and not the full-length 5245 bp +18+19 enhancer. Nonetheless, while these core elements have proven sufficient to restrict transgene expression to *Scl*-expressing cell populations in transgenic mice ([135, 140] and B. Göttgens, personal communication),

we found that they are unable to effect proper developmental restriction of transgene expression in the ESC system when targeted to the *Hprt* locus. We demonstrate here that *Hprt*-targeted Venus transgene constructs express strongly in all cells of undifferentiated ESC cultures even in the presence of +18+19 (1 kb) or +19 (644 bp) *Scl* 3' enhancer elements. This widespread ectopic transgene expression is gradually downregulated as the cells differentiate into embryoid bodies when the +18+19 enhancer is present, and to a lesser degree when the +19 enhancer is present, but only if it is targeted in forward orientation with respect to the *Hprt* locus.

While the ESC/EB system recapitulates developmental processes in many ways (reviewed in [150]), there clearly are transcriptional control mechanisms responsible for maintaining pluripotency in ESC that are no longer active in the developing embryo [151]. Thus, it stands to reason that transcriptional control elements which are sufficient to restrict transgene expression in whole mouse models may require additional *cis*-acting regulatory elements to effect similar lineage restriction in ESC. This phenomenon has been described in the case of a murine fetal liver kinase-1 promoter/enhancer (*Flk-1* p/e) element identified by Kappel *et al.* [152], which was shown to direct endothelial cell-specific expression in transgenic mice, but allowed ectopic transgene expression in undifferentiated ESC in two separate studies [128, 153]. In both cases, the ectopic activity was significantly reduced as the cells differentiated, which directly mirrors our findings with the *Scl* 3' enhancer elements. In the *Flk-1* p/e studies, the authors were interested in studying terminally differentiated cell types from the ESC system, thus the ectopic transgene expression had been abolished in the developmental stage in which they needed to make use of the transgene. In contrast, our study was intended to isolate a transient cell population known to exist in EBs between day 3

and 3.25 of differentiation [82]. At this stage of differentiation, EBs still contain many undifferentiated ESC, as evidenced by the ability to generate secondary EBs from cultures of dispersed day 3 primary EBs [82]. Therefore, widespread ectopic Venus expression in undifferentiated cells of the day 3 EBs precludes the use of the fluorescent transgene to isolate hemangioblasts or HSC at this stage, even if the *Scl* enhancer element properly restricts Venus expression in a subset of the cells.

A recent paper by Szutorisz *et al*. [154] describes a proteasome-based mechanism for transcriptional control in ESC that likely explains the phenomenon of differential enhancer activity in transgenic mice versus ESC that was noted in the studies described in this chapter. Their work illustrates a model of proteasome recruitment to and subsequent degradation of preinitiation complexes that are not stabilized by tissue-specific activators. This mechanism counteracts the permissive chromatin environment of ESC while maintaining pluripotency of the cells. In our ESC lines, it is likely that the *Scl* enhancer elements we employed lack the sequences that would be targeted by the proteasome complex in the endogenous *Scl* locus to prevent transcription in ESC.

In addition, it is possible that targeting the *Scl*-Venus transgenes to the *Hprt* locus may have exacerbated ectopic expression since it is a highly permissive locus that would not exert chromatin-based repression on the transgene. However, several groups have successfully produced lineage-specific expression of transgenes targeted to the same locus [155-157]. Also, since expression from this locus is not developmentally controlled, the ectopic transgene expression should not change upon differentiation of the ESC, which was not the case in our transgenic lines. On the other hand, the significant divergence in transgene expression in differentiated cells containing the Venus+19 transgene compared to

those containing the Venus+19-Rev transgene suggests that transgene orientation within the locus may influence ectopic expression. Previous studies have shown that within a specific locus, divergent expression of two transcriptional units is more permissive than a convergent or tandem arrangement [141, 142], so forward integration of the Venus transgene may help minimize ectopic expression from the *Hprt* locus.

In summary, lineage restriction of a fluorescent transgene for isolation of HSC from ESC may be possible with a few genetic modifications to the current approach. In order to maintain the benefits of *Hprt* targeting, including single-copy insertion to a known locus with efficient selection of targeted clones, it will be necessary to incorporate additional *cis*-acting regulatory elements capable of restricting transgene expression in undifferentiated ESC. A promising alternative approach has recently been described by members of the S. K. Bronson laboratory in which full bacterial artificial chromosomes (BACs) can be targeted as a single copy into the *Hprt* locus [158].

Chapter 3 ANTIBODIES TO STEM CELL MARKER ANTIGENS REDUCE ENGRAFTMENT OF HEMATOPOIETIC STEM CELLS

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A. ABSTRACT

Hematopoietic stem cells (HSC) have enormous potential for use in transplantation and gene therapy. However, the frequency of repopulating HSC is often very low; thus, highly effective techniques for cell enrichment and maintenance are required to obtain sufficient cell numbers for therapeutic use and for studies of HSC physiology. Common methods of HSC enrichment use antibodies recognizing HSC cell surface marker antigens. Because antibodies are known to alter the physiology of other cell types, we investigated the impact of such enrichment strategies on the physiology and lineage commitment of HSC. We sorted HSC using a method that does not require antibodies: exclusion of Hoechst 33342 to isolate side population (SP) cells. To elucidate the effect of antibody binding on this HSC population, we compared untreated SP cells to SP cells treated with the Sca-1^{+}c-$Kit^{+}Lin^{-}$ (SKL) antibody cocktail prior to SP sorting. Our findings revealed that HSC incubated with the antibody cocktail had decreased expression of the stem cell-associated genes *c-Kit*, *Cd34*, *Tal-1*, and *Slamf1* relative to untreated SP cells or to cells treated with polyclonal isotype control antibodies. Moreover, SKL antibodies induced cycling in SP cells and diminished

their ability to confer long-term hematopoietic engraftment in lethally irradiated mice. Taken together, these data suggest that antibody-based stem cell isolation procedures can have negative effects on HSC physiology.

B. INTRODUCTION

While many hematopoietic stem cell enrichment methods depend on the use of antibody cocktails to surface marker antigens, an alternative enrichment method exploits the ability of HSC to exclude the supravital fluorescent dye Hoechst 33342. This distinctive "side population" (SP) possesses both the phenotypic markers and the functional abilities of primitive stem/progenitor cells [3]. The dye exclusion-based approach is dependent on a functional property of HSC, whereas most of the antigens used in antibody-based enrichment schemes have been shown to be non-essential to HSC function [159-162]. In addition, expression of these antigens may vary with age [163], cell proliferation status [164], or may differ between species. Potential additional advantages to the dye exclusion-based enrichment are: inclusion of facilitating cells for engraftment of HSC, inclusion of progenitors for other cell types such as endothelial cells, cost savings, and the avoidance of antibody-induced physiologic changes in the HSC.

It is well documented that monoclonal antibodies may be used to activate or otherwise alter the homeostasis of certain cell types [165-173]. In hematopoietic cells, various monoclonal antibodies have been shown to induce cellular proliferation, differentiation, and lineage specification [165, 171]; induce cytokine secretion [166, 170] and apoptosis [167, 172]; and to activate cell signaling via tyrosine protein phosphorylation [168, 169, 173]. Furthermore, studies using monoclonal antibodies against

glycosylphosphatidylinositol (GPI) anchored proteins revealed that members of this class of proteins share the biological property of inducing cellular growth, likely through association with Src family protein tyrosine kinases [174, 175]. This data may be particularly pertinent to HSC isolation, as Sca-1 (Ly6a) and Thy1 are two GPI-anchored proteins that are frequently used to enrich long-term repopulating cells via antibody labeling and FACS sorting. Thus, we hypothesized that the antibody cocktails commonly used in HSC purification might alter the normal physiology of stem cells.

In this study, we investigated the effects of antibodies to stem cell surface marker antigens on HSC by comparison of SP-sorted HSC from untreated murine bone marrow cells versus bone marrow cells from the same animals that had been incubated with the SKL antibody cocktail prior to SP sorting. We observed significant changes in the expression of stem cell-related genes with SKL antibody treatment that appeared to correlate with a loss of functional repopulating stem cells, possibly by inducing HSC cycling, as has been previously reported [176]. Taken together, these results suggest that the antibodies used to label and sort HSC may alter their physiology, thereby effectively reducing the yield of actual long-term repopulating HSC.

C. MATERIALS AND METHODS

Animals— C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were maintained in the animal facility at the University of North Carolina, Chapel Hill, NC in accordance with IACUC standards. All animals were housed in autoclaved, micro-isolator cages and supplied with irradiated mouse chow and sterile water ad libitum. All mice used

were between 7 and 16 weeks of age at the initiation of the experiments, and mice were age-matched within each experiment.

For transplantation experiments, SP cells were isolated from the bone marrow of B6 mice expressing the enhanced GFP (eGFP) protein [177]. Bone marrow from B6.SJL-Ptprc^{a}-Pepc^{b}/BoyJ (CD45^{a}) mice (Jackson Laboratory, Bar Harbor, ME) – which are congenic with B6 mice, carrying the SJL allele for CD45^{a} – was used as a source of short-term engrafting cells.

Preparation of Bone Marrow Cells— Mice were euthanized and femurs and tibias were removed. The marrow cavities were flushed with either sterile HBSS+ (Hank's Balanced Salt Solution (HBSS) with 2% fetal calf serum (FCS), 10 mM HEPES, 100 U/mL penicillin and 100 μg/mL streptomycin) or sterile IMDM media supplemented with 5% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cell suspensions were filtered through a cell strainer to remove debris.

Hoechst 33342 Staining (+/- Antibody Staining) for SP Isolation— Bone marrow was harvested as above in cold HBSS+, and the resulting cell suspensions were centrifuged at 1500 rpm (~600*g*) for 12 minutes at 4°C. Cell pellets were resuspended in pre-warmed (37°C) RPMI medium containing 2% FCS, 10 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 μg/mL Hoechst 33342. Total nucleated cells (TNC) were counted and brought to a final concentration of 1 x 10^{6} TNC/mL. Cell suspensions were incubated at 37°C for 90 minutes and immediately centrifuged at 600*g* for 8 minutes at 4°C. Cell pellets were resuspended in 1 mL ice-cold flow suspension buffer (HBSS containing 2% FCS, 10 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 μg/mL propidium iodide). For samples treated with SKL antibodies, cells were suspended in 1mL ice-cold HBSS+ and

stained for 30 minutes on ice with the antibodies described below. Cells were then washed once with an excess of ice-cold HBSS+ and resuspended in 1 mL ice-cold flow suspension buffer.

Antibody Staining and FACS for SKL Isolation— Bone marrow was harvested as above and stained in a two-step procedure with FITC-conjugated c-kit (clone 2B8, 3.75 $\mu g/10^7$ cells), biotinylated Sca-1 (clone E13-161.7, 3 $\mu g/10^7$ cells), and PE-conjugated lineage marker antibodies (CD3:145-2C11, B220:RA3-6B2, TER119, Mac-1:M1/70, and Gr-1:RB6-8C5, 0.8 µg each/10^7 cells). The initial immunostaining was followed by incubation with a secondary antibody conjugated to streptavidin-PE-Cy5. (Antibodies were a gift from Dr. Lishan Su, UNC-Chapel Hill or purchased from Pharmingen, San Jose, CA). All incubations were done on ice and washes in ice-cold buffers. Aliquots of cells were stained individually for isotype controls.

Fluorescence Activated Cell Sorting (FACS)— Cell sorting was performed on a dual laser Modular Flow (MoFlo) cytometer (Cytomation, Inc.). For SP sorting, an Argon laser provided excitation of the Hoechst dye at 350 nm and emission was monitored simultaneously at 405 nm (blue fluorescence) and 670 nm (red fluorescence) for analysis. SP cells were sorted based on the gate shown in Figure 3-2. A small sample of cells was run back through the cytometer following each sort to check the purity of the SP sorting (Figure 3-2, right panel). For SKL sorting, excitation was provided by laser output at 488 nm and fluorescence emission was monitored as appropriate for each fluorochrome. For RT-PCR analysis in SP compared to SKL studies, samples of 100 cells were sorted into 0.2 mL microfuge tubes containing 5 µL cell lysis buffer (Cells-to-cDNA kit, Ambion, Austin, TX), moved immediately to dry ice, then stored at -70°C. For RT-PCR studies comparing

SP(+Ab) to SP(untreated), single cells or 20 cell pools were sorted (based on Hoechst dye exclusion, Figure 3-2) into 8-well mRNA Isolation GeneStrips containing 15 μL Working Lysis Buffer (RNAture, Irvine, CA), moved immediately to dry ice, then stored at -70°C. For transplantation experiments, defined numbers of SP cells (enough for final doses of 10, 20 or 50 cells per animal) were sorted into sterile tubes containing sterile IMDM media supplemented with 5% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 μg/mL Gentamicin.

Reverse transcription – polymerase chain reaction (RT-PCR)— For SP compared to SKL studies: Samples were lysed by heating to 80°C in a thermocycler block for 5 minutes and then cooled to 4°C. DNase I (0.07 U/μL) treatment was performed to degrade genomic DNA in the samples. Samples were incubated at 37°C for 30-45 minutes and then heated at 75°C for 5 minutes to inactivate the DNase I. Reverse transcription was performed by the protocol provided with the Cells-to-cDNA kit (Ambion, Austin, TX). The resulting cDNA was aliquoted into 5 individual 0.2 mL PCR tubes and stored at -20°C until PCR was performed. Amplification of specific genes was performed using enzymes and reagents supplied with the HotStarTaq Polymerase system (Qiagen). Table 3.1 lists all primers and PCR cycling conditions used. cDNA from murine bone marrow nucleated cells was used as a positive control for expression of all genes except the endothelial growth factor receptors Kdr (Flk-1) and Tek (Tie-2); for these genes cDNA from the endothelial cell line PY4.1 was used as the positive control [178].

For SP(+Ab) compared to SP(untreated) studies: Cell lysates were allowed to hybridize to GeneStrips Hybridization Tubes (RNAture, Irvine, CA) for 90 minutes at room temperature, then washed three times with wash buffer. Based on the solid-phase synthesis

protocol provided in the GeneStrips manual, cDNA was generated using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), dNTPs (Amersham Biosciences, Piscataway, NJ), and the immobilized poly(dT) on the Hybridization Tubes as primers. Since the synthesized cDNA was covalently linked to the GeneStrips tubes, several rounds (6-7) of PCR could be run on the same samples. Following cDNA synthesis, each sample was tested for the presence of template using HPRT as an internal control. Samples that tested negative for HPRT message were discarded, while samples that tested positive for HPRT were then assayed for other genes of interest. Figure 3-1 shows an example of results for the HPRT RT-PCR. Using these criteria, the number of samples analyzed was as follows: 20 SP NoAb – 40 samples (254 total PCR reactions); 20 SP +Ab – 40 samples (254 total reactions); Single SP NoAb – 55 samples (272 total reactions); Single SP +Ab – 49 samples (243 total reactions).

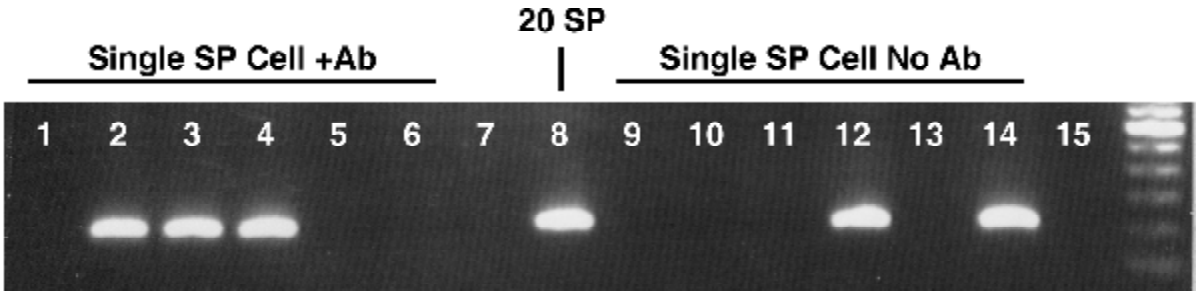

Figure 3-1 Representative *Hprt1* RT-PCR results for analysis of samples for Figure 3-3.

Following cDNA synthesis, all 20-cell-pool and Single-cell SP samples were tested for the presence of template using the *Hprt1* gene as an internal control. Negative samples (Lanes 1, 5, 6, 9-11, 13) were discarded, while positive samples (Lanes 2-4, 8, 12, 14) were further analyzed by PCR for multiple genes of interest. Lanes 7 and 15 are no-template controls

Samples were assayed for gene expression using a two-step nested PCR procedure. Round 1 of PCR was conducted in 50 μL using PCR Master Mix (Promega, Madison, WI), and gene-specific primers A and B listed in Table 3.2. Then 2 μL of the PCR product from

round 1 was used as a template for a second round of PCR using gene-specific primers C and D listed in Table 3.2. PCR conditions were as follows: 94°C 45"; (annealing temp., Table 3.2) 45"; 72°C 45"; 40 cycles. The PCR product from round 2 was then run on a gel containing ethidium bromide and scored as positive or negative for gene expression.

Real Time Quantitative RT-PCR— Pools of 200-500 SP cells were sorted (based on Hoechst dye exclusion) into 8-well mRNA Isolation GeneStrips containing 15 μL Working Lysis Buffer (RNAture, Irvine, CA). Cell lysates were allowed to hybridize to GeneStrips Hybridization Tubes for 90 minutes at room temperature, and then washed three times with wash buffer. Based on the solution-phase synthesis protocol provided in the GeneStrips manual, cDNA was generated using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), dNTPs (Amersham Biosciences, Piscataway, NJ), and Random Hexamer Primers (RNAture, Irvine, CA). The resultant cDNA was then subjected to quantitative real-time PCR analysis using the ABI Prism 7000 sequence detection system (Applied Biosystems (ABI), Foster City, CA). We used commercial TaqMan® Gene Expression Assays (ABI) as the gene-specific probe and primer sets for analysis of all genes studied. Real time PCR reactions were set up using TaqMan® Universal PCR Master Mix (ABI).

Data was analyzed using the $2^{-\Delta\Delta C}{}_T$ method as previously described [179]. Briefly, data is presented as the fold change in gene expression normalized to an internal control gene (Actb) and relative to a calibrator sample (untreated SP cells).

Transplantation— Wild-type B6 female mice ($CD45^b$) were lethally irradiated (9.5Gy total body irradiation from a ^{137}Cs source) and anesthetized with tri-bromo-ethanol (Avertin, Sigma-Aldrich, St. Louis, MO) for retro-orbital injection of the indicated dose of GFP+ SP cells plus 2 x 10^4 whole bone marrow cells from B6-$CD45^a$ mice. Since the

frequency of HSC in whole bone marrow is one in 10^5 cells, the B6-CD45^a whole bone marrow cells are considered to be short term repopulating (STR) cells. The transplanted animals were maintained in microisolator cages with autoclaved food and acidified (pH 2.0) sterile water supplemented with neomycin sulfate for the first 2 weeks after transplant.

Analysis for Donor Cell Repopulation— At various days following transplantation, mice were anesthetized using tri-bromo-ethanol and approximately 100 μL of peripheral blood was collected from the retro-orbital sinus into 1.5 mL eppendorf tubes containing 10 μL EDTA (25 mg/mL). Red blood cells were lysed using ACK lysis buffer (0.15 M NH_4CL, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4). The remaining cells were washed in PBS with 2% FCS and then stained with PE-anti-CD45^a (A20, Pharmingen, San Jose, CA). Dual-laser FACS analysis was performed with a FACScan machine (Becton Dickinson). Samples were gated based on high forward scatter (to avoid residual debris from ACK lysis step), and SP-derived cells were identified based on fluorescence intensity of eGFP, while B6-CD45^a-derived cells were identified based on fluorescence intensity of PE.

Cell Cycle Analysis— SP cells were sorted based on Hoechst dye efflux, then resuspended in 250 μL HBSS+ buffer with 0.1% sodium citrate and 50 μg/mL propidium iodide. Cells were incubated on ice for 10 minutes then analyzed using flow cytometry (488 nm excitation) and Modfit software (Verity Software House Inc., Topsham, ME) to determine the percentage of cells in each phase of the cell cycle.

Statistical Analysis— Two-tailed Student t-tests were performed for comparisons of quantitative RT-PCR results. Sigmastat software was used for statistical calculations.

Table 3.1 Primer sequences and PCR conditions for RT-PCR in SP compared to SKL studies

TRANSCRIPTION FACTORS	PRODUCT SIZE (bp)	CONDITIONS	(+) CONTROL
Tal1 [180]			
(f) 5'-CGC GGA TCC ACG GAG CGG CCG CCG AGC GCG	570	94°(45") 62°(45") 72°(60") 10% DMSO added	bone marrow
(r) 5'-CCG GAA TTC CGC GCC GCA CTA CTT TGG TGT G			
Gata1 **(hematopoietic)** [93]			
(f) 5'-ACC TGA AGG AGC TTG AAA TAG AGG C	290	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-CAT TGG CCC CTT GTG AGG CCA GAG A			
GROWTH FACTOR RECEPTORS - HEMATOPOIETIC	**PRODUCT SIZE (bp)**	**CONDITIONS**	**(+) CONTROL**
Kit **(Stem Cell Factor)** [181]			
(f) 5'-GGC TCA TAA ATG GCA TGC TC	~350	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-TAT CTC CTC GAG AAC CTT CC			
Mpl **(Thrombopoietin receptor)** [182]			
(f) 5'-GGC TCG AGA AGT GGC AAT TTC CTG CG	~400	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-GGC TCG AGG GGC TGC TGC CAA TAG C			
Il3ra [183]			
(f) 5'-GAA CAG ATT CCA CCA TGG CCT CCT TG	228	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-CTG TCT TCA CAG GCA TCA CCT CTG			
Epor **(Erythropoietin receptor)** [183]			
(f) 5'-AGG CCG CAC TGA GTG TGT TC	356	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-GGC TCC ACC ACA GAC AAC CA			
GROWTH FACTOR RECEPTORS – ENDOTHELIAL	**PRODUCT SIZE (bp)**	**CONDITIONS**	**(+) CONTROL**
Kdr **(*Flk-1*, VEGF receptor)** [93]			
(f) 5'-CAC CTG GCA CTC TCC ACC TTC	239	94°(45") 55°(45") 72°(45")	Py4.1 (endothelial)
(r) 5'-GAT TTC ATC CCA CTA CCG AAA G			
Tek **(*Tie-2*, Angiopoietin receptor)** [184]			
(f) 5'-CCT TCC TAC CTG CTA	441	94°(45") 57°(45") 72°(45")	Py4.1 (endothelial)
(r) 5'-CCA CTA CAC CTT TCT TTA CA			
MARKERS OF DIFFERENTIATION – HEMATOPOIETIC	**PRODUCT SIZE (bp)**	**CONDITIONS**	**(+) CONTROL**
Csf1r **(Monocyte specific/M-CSF receptor)** [93]			
(f) 5'-CTG AGT CAG AAG CCC TTC GAC AAA G	274	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-GAA ACA GCT TCC CGA AGA CTG AGT C			

***Hbb-b1* (β-major, Erythroid specific)** [93]			
(f) 5'-CTG ACA GAT GCT CTC TTG GG	578	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-CAC AAA CCC CAG AAA CAG ACA			
***Mpo* (Myeloid specific)** [181]			
(f) 5'-CGC TTC TCC TTC TTC ACT GG	~320	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-CTG CCA TTG TCT TGG AAT CG			
***Pecam1* (Platelet/endothelial cell adhesion, non-specific)**			
(f) 5'-GTC ATG GCC ATG GTC GAG TA	260	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-CTC CTC GGC ATC TTG CTG AA			
CHEMOKINE RECEPTORS	**PRODUCT SIZE (bp)**	**CONDITIONS**	**(+) CONTROL**
***Ccr1* (Mip-1α receptor)**			
(f) 5'-TGG AGT GGT GGG CAA TGT CCT	576	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-CGC ACG GCT TTG ACC TTC TTC			
***Ccr4* (Mip-1α receptor)**			
(f) 5'-GCC ATC TCG GAT TTG CTG TT	464	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-TGC GCA CTG CTC TGT TCT TCT			
***Ccr5* (Mip-1α receptor)**			
(f) 5'-GTG AAA CAA ATT GCG GCT CAG	597	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-GCG AAA CAG GGT GTG GAG AA			
***Cxcr4* (Stromal derived factor-1 receptor)**			
(f) 5'-TTG TCC ACG CCA CCA ACA G	521	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-CCC GAG GAA CCG ATA GAG GAT			
OTHER MARKERS OF HSC	**PRODUCT SIZE (bp)**	**CONDITIONS**	**(+) CONTROL**
***Cd34* (Stem cell activation marker)** [181]			
(f) 5'-TTG ACT TCT GCA ACC ACG GA	~300	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-TAG ATG GCA GGC TGG ACT TC			
***Fancc* (Fanconi Anemia group C protein – HSC maintenance)**			
(f) 5'-AAA GAC AGA GGA GAC GCC AC	463	94°(45") 58°(45") 72°(60")	bone marrow
(r) 5'-AAG AGC AAC ACA AAT GGT AAG G			
HOUSEKEEPING	**PRODUCT SIZE (bp)**	**CONDITIONS**	**(+) CONTROL**
***Hprt1* (Hypoxanthine phosphoribosyl transferase)** [184]			
(f) 5'-CAC AGG ACT AGA ACA ACC TGC	248	94°(45") 55°(45") 72°(45")	bone marrow
(r) 5'-GCT GGT GAA AAG GAC CTC T			

Table 3.2 Primer sequences and PCR conditions for RT-PCR in SP (+SKL Ab) compared to SP (Untreated) studies.

SP cell samples were assayed for gene expression using a two-step nested PCR procedure. Primers A and B (outer) were used for the first round of PCR; then the PCR product from round 1 was used as a template for a second round of PCR using primers C and D (inner).

GENE	PRIMER A	PRIMER B	PRIMER C	PRIMER D	ANNEAL TEMP
Tal1	AGCGCTGCTCTATAGCCTTA	CCTTCCTCCTCCTGGTCATT	ACTAGGCAGTGGGTTCTTTG	TGAGTAACTTGGCCAGGAAA	62
Gata1	CCAAGAAGCGAATGATTGTC	TCAAGGCTATTCTGTGTACC	CCCAATGCACTAACTGTCAA	AAAGGCATAAGATGGCTGAC	60
Runx1	GAAGAACCAGGTAGCGAGAT	TGGATCCCAGGTACTGGTAG	TTCAACGACCTCAGGTTTGT	ACTGGTAGGACTGGTCATAG	60
Tgif	CCAAGGAGTCAGTCCAGATT	AGAAAGGCCCATCCTTCAAT	ACTGGCTGTATGAACACAGA	GTCACAGTGGTATGGCAGAT	60
Kit	GATGCCAGTGCTTCCGTGAC	TCCCGAAGGCACCAGCTCCC	ACAGGAGCAGAGCAAAGGTG	CGACCACAAAGCCAATGAGC	62
Cd34	TTGACTTCTGCTACCACGGA	TAGATGGCAGGCTGGACTTC	ATCCCCATCAGTTCCTACCA	GTAGGCAGTATGCCAGTTGG	60
***Ly6a* (*Sca-1*)**	CTCAGGGACTGGAGTGTTAC	CTCCATTGGGAACTGCTACA	GGAGTGTTACCAGTGCTATG	TGCTACATTGCAGAGGTCTT	60
Il3ra	CTCAGAGCCACAGGAATACA	TAGCCACTGAAGTCACCAAG	GCCACAGGAATACAATGTCT	AAGTCACCAAGGCTGTCTTC	62
***Tek* (*Tie-2*)**	GGAGATGTGAAGCTCAGAAG	CTGCCTTCTTTCTCACACTG	TAGCCGTCCTTGTACTACTT	TCCTTGGAACCGATCACATA	60
Epor	CACCTATGACCACCCACATC	GCGAGATGAGGACCAGAATG	ACCACCCACATCCGATATGA	GACAGCGTCAAGATGAGAGG	60
Csf1r	CACAGATAAAATTGGAGCCTA	TGGAAGTTCATGGTGGCCG	CAAGCTGGTGCGGATTCGAG	TGCCAGCGTCTTGGAAGTCC	56
Mpo	CAATGACCCTCGAATCAAGA	AAGCATTGGTGAAGACATTG	CCAGGAACAACATCACCATT	GTACTGCGGTAGGTACTTCT	60
Pecam1	AGCAGTGAAGCTGTCTACTC	GGGTGGGAATGGCAATTATC	ACTACACCTGCAAAGTGGAA	ATGTCTCTGGTGGGCTTATC	62
Ccr1	TGGAGTGGTGGGCAATGTCC	CGCACGGCTTTGACCTTCTT	GCTCATGCAGCATAGGAGGC	GGGGAAATGAGGGCTACAGG	62
Cxcr4	AACCCTGCTTCCGGGATG	GCTGATGAAGGCCAGGATGA	CCTGCTTCCGGGATGAAA	ATGAAGGCCAGGATGAAC	60
Tert	AATACGGCAAGCTATCACTG	CTTGAGACGCTGGGTGAAAT	ATCACTGCAGGAACTGATGT	AGCTCTGGTACCCATGCTAT	60
Abcg2	GAACATCGGCCTTCAAAGAG	CATGGAACTGGCCGTATAAG	GCCTTCAAAGAGCCAGTCTA	CATCCACCGTCTTCTTCAGT	60
Hprt1	AGCTTTACTAGGCAGATGGC	GCAGTACAGCCCCAAAATGG	CGAAGTGTTGGATACAGGC	ACATCAACAGGACTCCTCG	60
Itga2b	AGCTGCATCTAAAGGCAGAG	CAGCTCCAACACATTGTCAG	CTGCAGCTGGACTTACAGAA	ATTGTCAGCACCGATTAGGA	60

D. RESULTS

Stem cell-related gene expression is altered in SP cells compared to SKL cells— In order to compare gene expression in two defined cell populations that are highly enriched for hematopoietic stem cell activity, RT-PCR analyses were performed with sorted samples of SP and SKL cells. Due to the rarity of these cell populations in bone marrow, we chose to examine the expression of select genes individually rather than in an array format. Considerable amplification of transcripts would be required to generate enough starting material for analysis by array, which can introduce significant bias due to variable amplification efficiency. Thus we studied the expression of 18 separate genes. Detection of a housekeeping gene, hypoxanthine phosphoribosyltransferase (*Hprt1*), was used as a positive control for each sample, so samples scoring negative for *Hprt1* were not included in the analysis. We analyzed a total of 231 PCR results from 48 SKL sorted samples and 224 PCR results from 46 SP sorted samples (each sample represents a pool of 20 cells). Each sample was scored as positive or negative for gene expression, and overall gene expression *frequency* was defined as the percentage of positive samples in all samples analyzed.

For this analysis, we focused on genes relevant to hematopoietic cell physiology, which can be organized into the following basic categories: transcription factors (*Tal1*, *Gata1*) [185], growth factor receptors (hematopoietic [38]: *c-Kit* [186], *Csf1r*, *Mpl*, *Epor*, *Il3ra*; vascular: *Kdr* (*Flk-1*) [187], *Tek* (*Tie-2*) [188]), markers of differentiation (*Pecam1* [189], *Csf1r* [190], *Mpo* [191], *Hbb-b1* [192]), chemokine receptors related to stem cell homing (*Cxcr4* [193]) or cycling (*Ccr5*, *Ccr4*, *Ccr1*) [194], and other markers of HSC activation (*Cd34* [195], *Fancc* [196]). Overall, the frequency of gene expression was lower in SP cells compared to SKL cells for all categories, with the exception of the chemokine

receptor category, which showed the opposite trend (Table 3.3). Within the chemokine receptor category, we observed similar expression frequency of *Cxcr4* in both cell populations, which is not surprising since this receptor has been shown to be required for homing of transplanted cells to the bone marrow [197]. In contrast, our data demonstrate a higher frequency of expression of the MIP-1α (*Ccl3*) receptors *Ccr1*, *Ccr4* and *Ccr5* in SP cells. From these results we made the general observation that the SP population and the SKL population clearly have different gene expression profiles with respect to genes important to hematopoietic stem cell physiology, but could not conclude a causal effect of antibody binding in inducing such gene expression differences.

Table 3.3 Differences in frequency[a] of gene expression in SKL compared to SP cells.

Genes more frequently expressed in SP Cells	Genes expressed with similar[b] frequency in SP and SKL cells	Genes more frequently expressed in SKL cells
Ccr5	*Cxcr4*	*Pecam1*
Ccr4	*Tek* (*Tie-2*)	*Csf1r*
Ccr1	*Kdr* (*Flk-1*)	*Mpo*
	Mpl	*Epor*
	Tal1	*Il3ra*
	Hbb-b1	*Kit*
		Gata1
		Cd34
		Fancc

[a]Each PCR sample (representing a pool of 20 cells) was scored as positive or negative for gene expression. Frequency is defined as the percentage of positive samples among all samples analyzed.

[b]Gene expression frequencies were scored as similar if the difference in percent positive samples was less than 15%.

The SKL antibody cocktail reduces the frequency of expression of stem cell-related genes in SP cells— The SP and SKL isolation protocols differ fundamentally in that the SKL protocol involves the use of antibodies that bind to cell surface molecules. We therefore hypothesized that the presence of these antibodies may actually give rise to some of the gene expression differences observed between the two HSC populations. However, the use of antibodies is not the only difference between SP and SKL isolation protocols, so any phenotypic differences seen by direct comparison of the two populations may not be solely due to the presence of the SKL antibody cocktail. *Thus, in all subsequent studies, we compare untreated cells sorted based on Hoechst dye exclusion (SP untreated) to the same population of cells that were incubated with the SKL antibody cocktail prior to sorting based on Hoechst dye exclusion (SP + SKL Ab).* Figure 3-2 illustrates the sorting gate used to isolate SP cells from whole bone marrow, and the right-hand panel shows a representative check of post-sort purity.

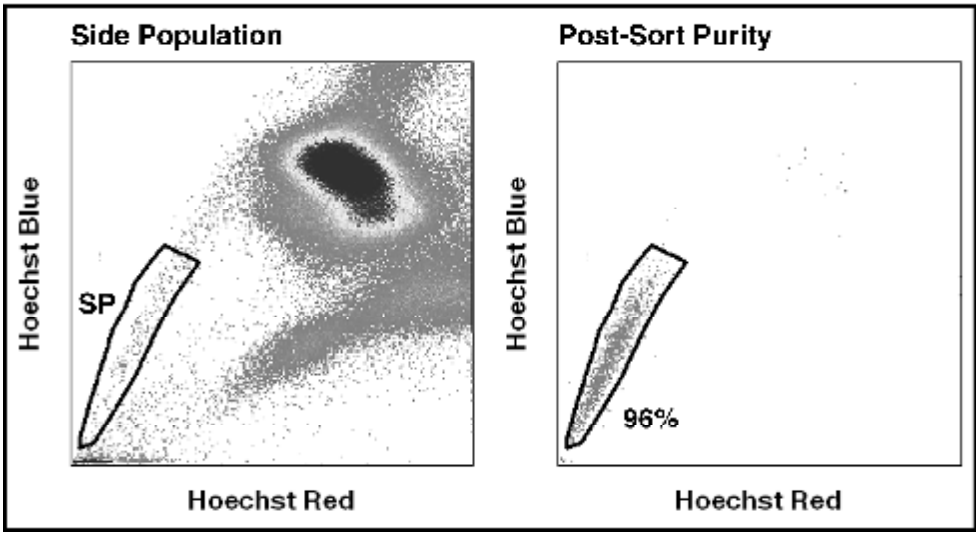

Figure 3-2 Sorting gate for Side Population (SP) cells based on Hoechst dye exclusion.

Whole bone marrow cells were incubated with 5 μg/mL Hoechst 33342. Flow cytometric emission monitoring at both 405 nm and 670 nm allows for selection of the Side Population of cells (gated, left panel), which exclude the Hoechst dye. The right panel demonstrates a post-sort assessment of purity.

To determine whether the presence of the SKL antibody cocktail alters gene expression within the SP population, we conducted RT-PCR analyses for hematopoietic cell-related genes on untreated SP cells and SKL antibody-treated SP cells. We studied the expression of nineteen different genes, which can be organized into the following general categories: transcription factors (*Tal1*, *Gata1*, *Runx1*, *Tgif*) [185, 198-200], growth factor receptors (*Kit*, *Il3ra*, *Tek*, *Epor*, *Csf1r*) [38, 186, 188], markers of differentiation (*Mpo*, *Pecam1*, *Csf1r*) [189-191], homing molecules (*Cxcr4*, *Itga2b*) [193, 201], molecules involved in cycling/self-renewal (*Ccr1*, *Tert*) [194, 202], and molecules implicated in stem cell phenotype and/or function (*Cd34*, *Ly6a* (*Sca-1*), *Abcg2*) [13, 203, 204]. Detection of a housekeeping gene, *Hprt1*, was used as a positive control for each sample, so samples scoring negative for *Hprt1* were not included in the analysis. Samples were scored as positive or negative for gene expression, then expressed as the percentage of positive samples among all samples analyzed (frequency). At least eight and as many as 22 RT-PCR samples were analyzed for each gene. We first analyzed the expression of these genes in cDNA samples collected from twenty-cell pools of sorted cells. Interestingly, we saw large differences in the frequency of expression of many of the genes studied between untreated SP cells and SP cells that had been treated with the SKL antibody cocktail (Figure 3-3 A). The antibody treated samples were generally less likely to express key HSC-related transcription factors *Tal1*, *Gata1*, and *Runx1* as compared to untreated samples. At the same time, antibody treated samples showed altered expression frequencies of *Epor*, *Csf1r*, *Mpo*, and *Pecam1*, which are markers of differentiated endothelial or hematopoietic cells. Moreover, the MIP-1α (*Ccl3*) receptor *Ccr1*, which may be important in maintaining quiescence in HSC, was less frequently expressed in the antibody-treated SP samples. In contrast,

expression of the integrin *Itga2b* and the SDF-1 (*Cxcl12*) receptor *Cxcr4*, which are both key molecules in HSC homing, was more frequent in the antibody treated samples than in untreated samples.

There were several genes in the study that were expressed with very high frequency in both treated and untreated twenty-cell pooled samples, therefore we analyzed gene expression at the single cell level to further elucidate any additional differences in expression frequency between untreated and antibody-treated SP cells (Figure 3-3 B). In these single-cell samples, we noted a considerable decrease in the frequency of expression of the HSC markers *Kit* and *Ly6a* (*Sca-1*) in the antibody-treated samples. Single-cell analysis also revealed an increase in *Abcg2* expression frequency in antibody-treated SP cells. Overall, these findings indicate that the presence of the SKL antibody cocktail results in distinct heterogeneity in expression of many genes important to hematopoietic stem cells and their progeny in the SP population. However, the net result of this gene expression heterogeneity is inconclusive as it is based on expression frequency alone. Thus, we used these results to direct both quantitative gene expression analyses and *in vivo* analysis of mice transplanted with antibody-treated versus untreated SP cells.

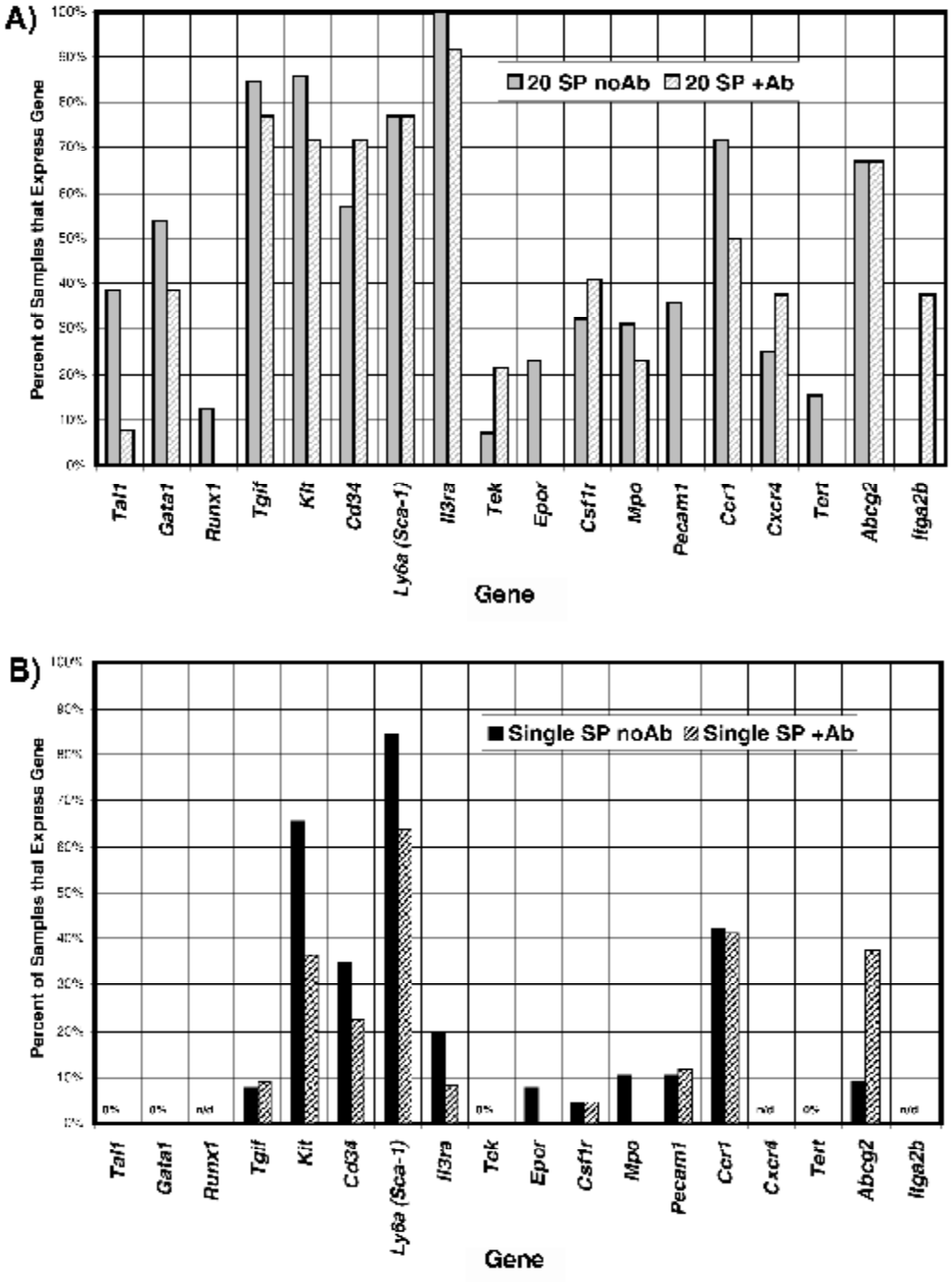

Figure 3-3 Frequency of gene expression in untreated and SKL antibody-treated SP cells.

RT-PCR analysis of cDNA samples from twenty cell pools (A) or single cells (B). Each sample was scored as positive or negative for expression of the indicated genes. Results are displayed as the percentage of positive results in all samples analyzed. The total number of samples analyzed for each gene and condition ranged from 8 to 22. The presence of the antibody cocktail results in distinct heterogeneity in expression of many HSC-relevant genes within the SP population. (n/d=analysis not done).

Quantitative studies confirm that reduced gene expression is specific to SKL antibody cocktail— To further confirm and quantitate the gene expression differences observed, we next conducted quantitative RT-PCR analyses on some of the key genes that were altered upon antibody treatment of the SP cells. As an additional control, we tested samples that were treated with the isotype controls of the SKL antibody cocktail to determine if any observed changes were specific to the SKL antibodies or simply a non-specific consequence of having antibodies in solution with the cells. It is notable that in these studies the approximate time from exposure to the antibodies until analysis of gene expression was a minimum of four hours, thereby allowing significant time for some alteration in gene expression if induced by antibody exposure. We found significant reductions in mRNA levels of *Kit*, *Cd34*, *Tal1*, and *Slamf1* from untreated SP samples to SKL antibody-treated SP samples ($p<0.05$ in all cases, Figure 3-4). Importantly, these significant reductions in message level were not seen in samples treated with isotype control antibodies, except in the case of *Cd34*. Yet, despite the small but significant ($p<0.05$) reduction in *Cd34* expression in the presence of isotype control antibodies, the SKL antibody-treated sample demonstrated a significant reduction in message level beyond the level in isotype-treated samples ($p<0.03$). Thus, the substantially decreased expression of genes critical to HSC physiology is specific to the SKL antibody cocktail and not a generalized antibody effect. Furthermore, we noted a large (greater than 2-fold) increase in p21 (*Cdkn1a*) transcript levels from untreated SP samples to SKL antibody-treated samples, which did not occur in isotype-treated samples (Figure 3-4). This result suggests a change in cell cycle status of the antibody-treated cells. Lastly, our results indicated no significant changes in transcript levels of several molecules

involved in hematopoietic cell homing and engraftment following treatment with the SKL antibody cocktail (Figure 3-4).

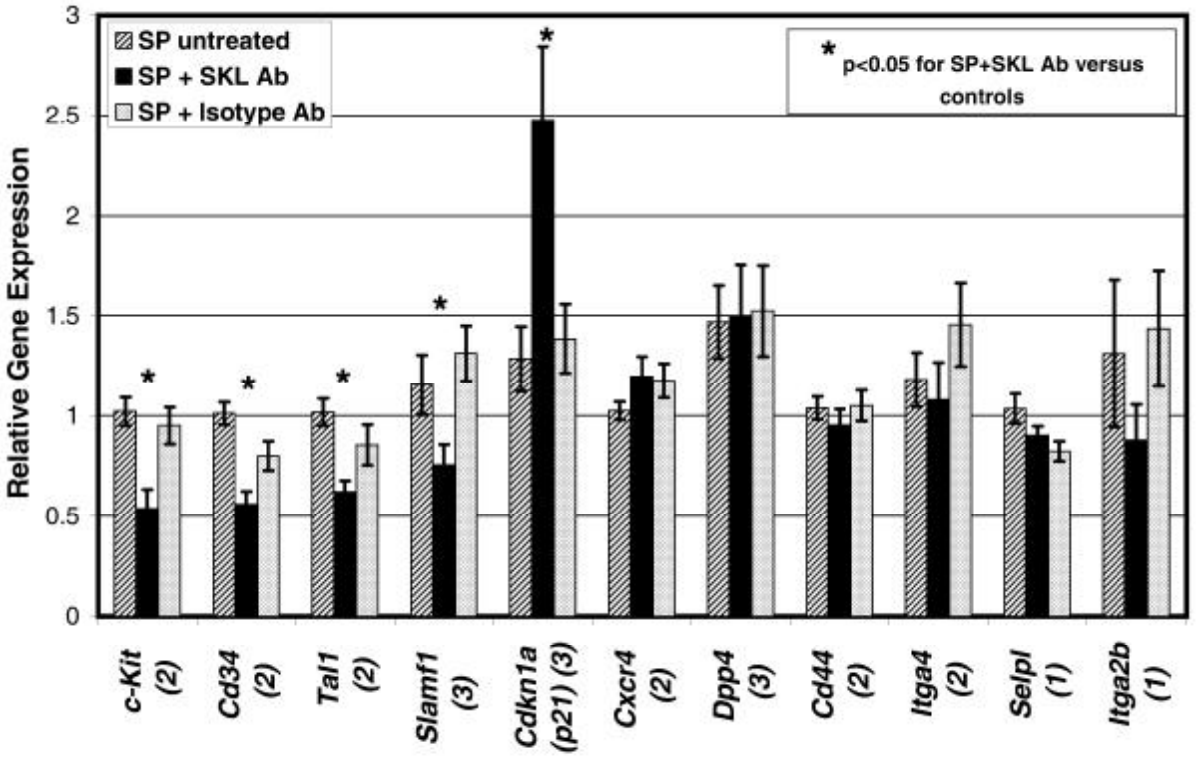

Figure 3-4 Quantitative gene expression in SP cells with or without antibody treatment.

QPCR analyses from pools of 200-500 cells are shown as the level of gene expression in the antibody-treated samples relative to the level of expression in the untreated samples. The numbers in parentheses indicate the number of individual cell sorts analyzed. The SKL antibody cocktail caused marked downregulation of several genes known to be important to hematopoietic stem cell physiology, which did not occur in isotype antibody-treated samples.

SKL antibody-treated SP cells show reduced engraftment of lethally irradiated recipients as compared to untreated SP cells— Given the nature of the genes whose expression was altered in the SKL antibody cocktail-treated SP cells, we hypothesized that the antibody-treated cells may have altered functional ability to repopulate the hematopoietic system in lethally irradiated recipients as compared to untreated SP cells. We sorted untreated or SKL antibody-treated SP cells from the BM of eGFP-transgenic mice, so that we could follow engraftment of the SP cells using GFP fluorescence. Transplant recipients

(CD45^b genotype) were lethally irradiated, and then given a mixture of BM cells containing 10, 20, or 50 SP cells (CD45^b genotype, GFP$^+$) and 2x10^4 whole bone marrow cells (CD45^a genotype) via retro-orbital injection (Figure 3-5 A). Following transplantation, peripheral blood was collected from recipient animals and analyzed by flow approximately once per month to monitor the percentage of eGFP$^+$ (SP-derived) cells as a measure of engraftment and hematopoietic repopulation by the transplanted cells (Figure 3-5 B).

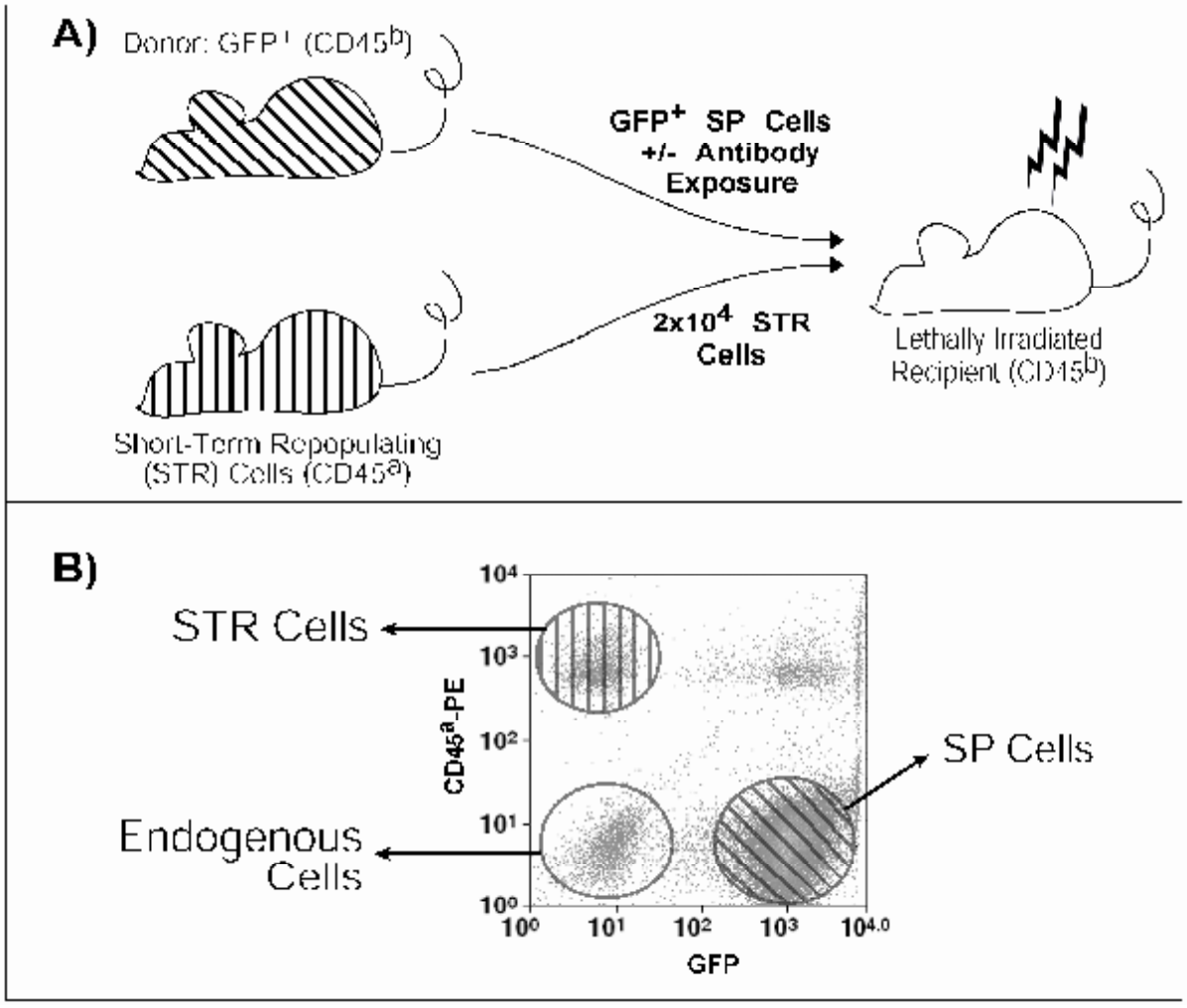

Figure 3-5 Transplantation and engraftment analysis for untreated versus SKL antibody-treated SP cells.

A) Lethally irradiated recipient animals (CD45^b genotype) received small numbers of untreated or antibody-treated GFP+ SP cells and 2x10e4 CD45^a unfractionated bone marrow cells.

B) Each month post-transplant, peripheral blood cells were harvested and analyzed by flow cytometry to determine their origin (SP donor, STR donor, or endogenous).

In mice receiving 20 SP cells, over the period of several months we noted that the percentage of SP ($eGFP^+$) engraftment was significantly lower in the animals that received SKL antibody-treated SP cells than in the animals that received untreated SP cells (Figure 3-6 A). In particular, many mice receiving antibody-treated cells died during the fourth month post-transplant, which suggests that a number of SP derived cells in those mice functioned as short-term progenitors and there were not enough long-term stem cells to keep the mice alive. Furthermore, the mice that survived past the fourth month showed very few SP-derived cells in their peripheral blood, suggesting either deficient engraftment or a possible loss of long-term repopulating potential after SP cell exposure to the SKL antibodies. Panel (B) of Figure 3-6 summarizes the engraftment of SP-derived cells in mice receiving all doses of SP cells. Once again, many of the mice died at around four months post-transplant when the short-term progenitor cells were exhausted, and the SP cell engraftment is significantly lower in surviving mice that received SKL antibody-treated SP cells. These findings indicate that a functional consequence of SKL antibody binding to SP cells is to significantly reduce their ability to stably engraft lethally irradiated mice, particularly when transplanting small numbers of cells.

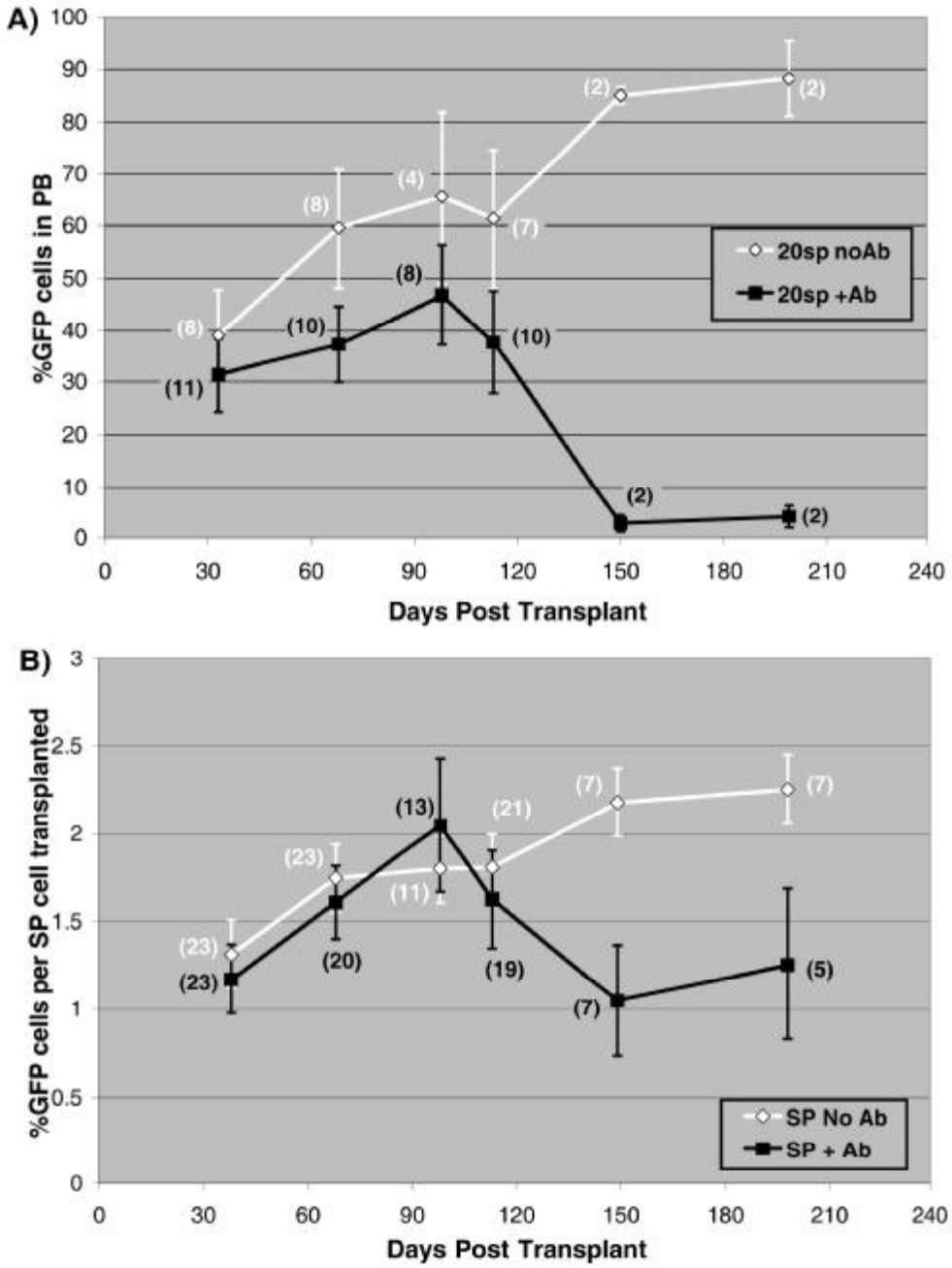

Figure 3-6 Engraftment of transplanted SP cells.

Flow cytometric analysis determined the percentage of recipient peripheral blood derived from the transplanted SP cells (GFP^+). (A) Values are shown as the mean ± SEM of the percentage of GFP^+ cells in all living mice at each timepoint (indicated by numbers in parentheses). The graph represents the average of two separate transplants in which the mice received a dose of 20 SP cells (+/- SKL Ab). Panel (B) summarizes all transplants performed, using multiple different doses of SP cells. Values are shown as the mean ± SEM of the percentage of GFP^+ cells as a function of the starting dose of SP cells. Beginning around four months post-transplant, the mice receiving SKL antibody-treated SP cells showed significantly reduced levels of SP-derived cells in their peripheral blood as compared to the mice receiving untreated SP cells.

The SKL antibody cocktail markedly increased the numbers of cycling SP cells— In order to explore a possible mechanism for the observed effects of the SKL antibody cocktail on the phenotype of SP cells, we conducted cell cycle analysis of the SP population with or without SKL antibody treatment. Untreated SP cells are usually a quiescent population, with over 90% of cells in G_0/G_1, approximately 8% of cells in S-phase, and 2% of cells in G_2/M (Figure 3-7 A). When these cells were exposed to the SKL antibody cocktail prior to Hoechst sorting, the percentage of cells in S-phase more than doubled to almost 20%, with a marked reduction of cells in G_0/G_1 (Figure 3-7 B). Importantly, when the cells were treated with isotype control antibodies prior to sorting, the cell cycle profile of isotype-treated cells was very similar to that of untreated cells (2% increase in S-G2M). These findings suggest that the SKL antibody cocktail induces cells within the SP population to enter the cell cycle, which may in part explain the changes in gene expression profile and stem cell functionality seen between untreated SP cells and SKL antibody-treated SP cells.

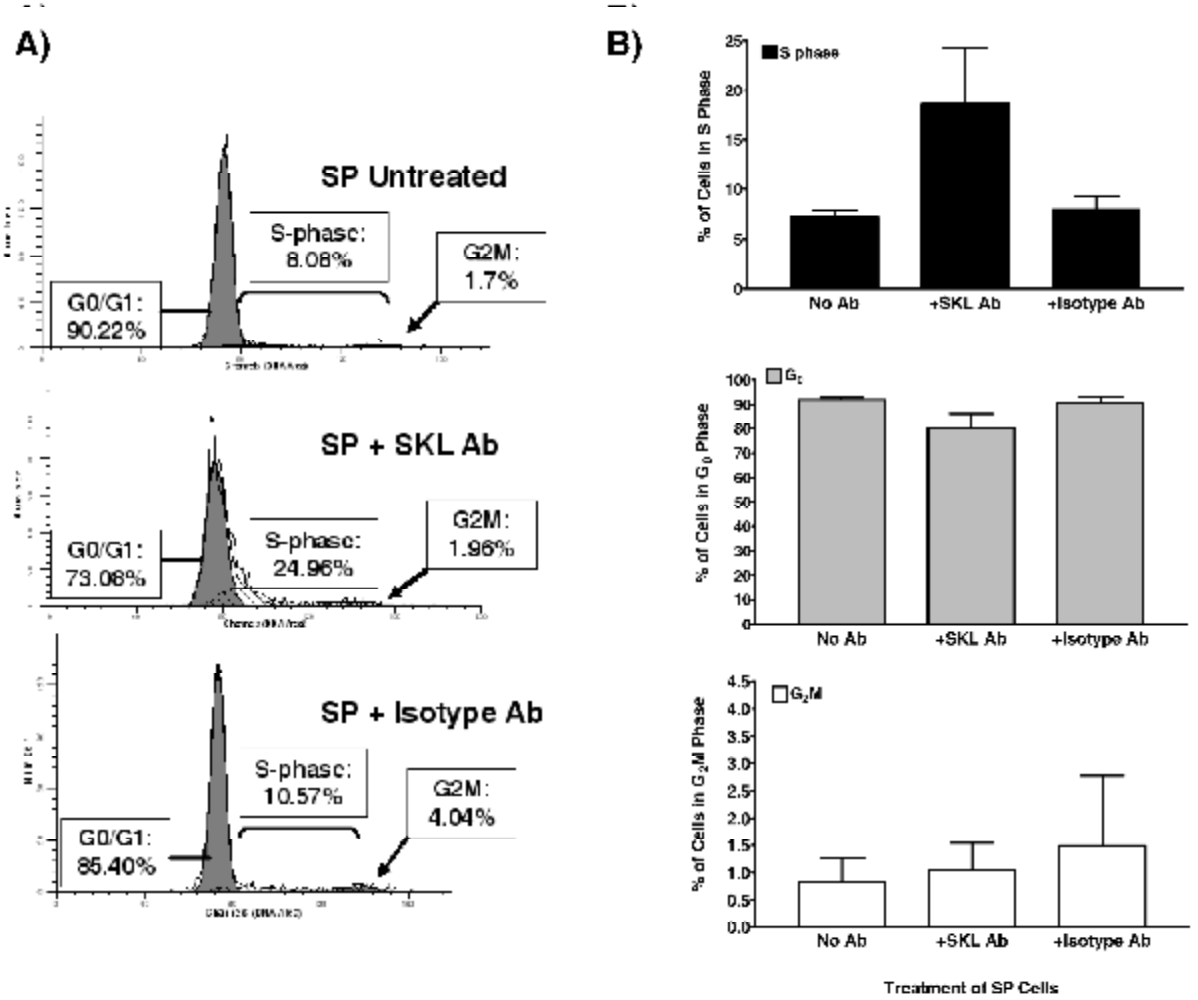

Figure 3-7 Cell cycle analysis of SP cells with or without antibody treatment.

A) Representative flow cytometric histograms showing the DNA content of SP-sorted cells with or without prior antibody treatment. Percentage numbers indicate the portion of the cell population in the designated phase of the cell cycle as determined by Modfit software.

B) Summary of cell cycle phase distribution of SP cells following the indicated antibody treatments. Note the large increase in S phase cells caused by the SKL antibody cocktail. Graphs represent the average of three separate experiments.

E. DISCUSSION

The two major determinants of successful hematopoietic stem cell transplantation are 1) repopulation of all components of the hematolymphoid system via multipotent differentiation of the stem cells; and 2) long-term maintenance of all cell types via self-renewal of the stem cells. Many different protocols have been established to isolate or enrich

the stem cells for transplants, and in all cases, the desired cell population comprises a small fraction of the original cell source. In addition, available stem cell numbers may be reduced depending on the source of the cells (i.e. umbilical cord blood) or by manipulation prior to transplantation (i.e. gene therapy). Therefore, it is important to understand the impact a chosen enrichment protocol will have on HSC function in order to maximize transplant efficiency.

We describe here the negative impact of certain antibodies to cell surface proteins on the long-term repopulating ability of HSC from the SP fraction of murine whole bone marrow. Our initial gene expression profile analysis comparing SKL-sorted cells to SP-sorted cells revealed many differences in the frequency of expression (percentage of samples positive for expression) of many genes related to HSC activation, homing, cycling, growth factor responsiveness, differentiation, and gene transcription. Overall, the SP population was less transcriptionally active with respect to HSC-related genes and showed less evidence of differentiated cells. The only genes studied which showed a significantly higher frequency of expression in SP cells were the chemokine receptors *Ccr1*, *Ccr4* and *Ccr5*, which bind to the common ligand MIP-1α (*Ccl3*). In several reports, MIP-1α has been shown to be a negative regulator of proliferation of hematopoietic stem cells [205-207]. The increased frequency of MIP-1α receptor expression in SP cells suggests that MIP-1α may play a substantial role in maintaining quiescence within the SP population. Furthermore, MIP-1α binding to CCR5 has been shown to inhibit chemotaxis through desensitization of CXCR4-mediated homing to SDF-1 (CXCL12) in bone marrow B cell progenitors [208]. Thus, the higher frequency of expression of proliferation- and migration-inhibitory chemokine

receptors coupled with lower frequency of expression of growth factor receptors and markers of differentiation suggest a more quiescent phenotype of SP cells compared to SKL cells.

The fundamental difference between SKL sorting and SP sorting of HSC is the use of antibodies to cell surface proteins. We hypothesized that antibodies within the SKL sorting cocktail are a cause of transcriptional changes in HSC which may lead to altered function. In order to directly test this hypothesis, we compared the effects of SKL antibody exposure versus no antibody exposure on a single HSC population (SP cells). We again found significant differences in the expression of a number of stem cell relevant genes that was not seen after exposure to irrelevant isotype control antibodies, suggesting some loss of the stem cell phenotype. Furthermore, through *in vivo* transplantation studies using small numbers of stem cells we show that exposure to the SKL antibody cocktail resulted in a significant loss of long-term repopulating potential of SP HSC.

Two general mechanisms that could explain the defect we saw in SKL antibody-treated SP cell long-term repopulating capability are: 1) a localization defect (including homing, migration, and engraftment in the niche); or 2) an alteration in intrinsic stem cell properties, i.e. multipotentiality and self-renewal. Our quantitative PCR results demonstrate that, at least at the transcriptional level, the SKL antibodies do not alter the expression of any known molecules important to HSC homing or migration. Therefore, a localization defect is unlikely to explain the reduced long-term repopulation shown by SKL-treated SP cells.

Our results support the idea of an intrinsic loss of stem cell properties caused by antibody exposure as demonstrated by significant changes in cell surface marker phenotype and cell cycle profile. Expression levels of *Kit*, *Slamf1*, and *Cd34* were significantly down-regulated in response to SKL antibody exposure, suggesting a possible loss of both long-term

and short-term repopulating HSC [18, 195, 209]. Cell cycle analysis showed that SKL antibody exposure resulted in a two- to three-fold increase in S-G2M phase cells that again was not seen with isotype control antibody exposure. Hematopoietic stem cells in S/G_2 demonstrate a shift in gene expression marked by down-regulation of transcription regulators and protein synthesis factors with concomitant up-regulation of cell cycle and chromatin remodeling genes [176]. Importantly, this shift is associated with a significant reduction in HSC engraftment [176, 210, 211]. In addition, several studies have shown detrimental effects of induced proliferation on HSC due to loss of developmental potential and/or self-renewal [21, 22].

Quantitative RT-PCR analysis revealed a significant up-regulation of p21 (*Cdkn1a*) expression upon SKL antibody exposure. While p21 in HSC is most often described by its role in maintaining quiescence by the inhibition of cell cycling [212], this protein has also been implicated in some cases as a promoter of cell cycling through its role as an adaptor or scaffold protein [213, 214]. In fact, growth factor treatments which signal through mitogen-activated protein kinase pathways have been shown to induce transcriptional activation of p21 via a p53-independent pathway [215, 216]. Furthermore, in a human myeloid cell line, increasing levels of p21 displaced p27 from cyclin-dependent kinase 2, leading to enhanced cell proliferation when stimulated by kit ligand [217]. These studies are in accordance with our findings showing increased p21 gene expression and increased cell cycling by exposing SP cells to the SKL antibody cocktail.

The potential impact of our findings on the possible detrimental effects of antibody-based enrichment strategies may be relevant to stem cell isolation in non-hematopoietic tissue types as well. Cell surface antigens useful for stem or progenitor cell isolation have

been identified in several other tissue types, including neural cells [218-220], stromal cells [221], endothelial cells [222, 223], skeletal muscle [224] and liver [225, 226]. Given that cells with the SP phenotype have also been identified in brain, skeletal muscle and liver [227], our results suggest that SP-based stem cell isolation may also have similar advantages over antibody-based enrichment in tissues other than bone marrow.

Chapter 4 SIDE POPULATION SORTING OF HEMATOPOIETIC STEM CELLS FROM tEpoR-TARGETED EMBRYONIC STEM CELLS

A. ABSTRACT

We have generated transgenic murine embryonic stem cell (ESC) lines expressing GFP in addition to a truncated erythropoietin receptor (GNtEpoR) with the goal of deriving hematopoietic stem cells (HSC) that may be fluorescently tracked *in vivo* and expanded with exogenous erythropoietin (Epo) administration. In this report, we describe the application of dye efflux-based sorting on day 3 embryoid bodies (EB) to identify a Side Population (SP), which we hypothesized would contain ESC-derived hematopoietic stem cells (HSC). Using a conditioned NOD.scid newborn mouse transplant model, we demonstrate long-term multilineage hematopoietic repopulation by ESC-derived cells. Furthermore, activation of the tEpoR transgene in these cells by exogenous administration of Epo improved the efficiency of transplantation, suggesting a role for this transgene in the expansion of ESC-derived hematopoietic progenitors. Lastly, we have conducted a focused stem cell microarray analysis on the SP-sorted day 3 EB cells grown in the presence or absence of Epo. The results of this analysis will be useful in characterizing the phenotype of ESC-derived HSC, and will clarify the relationship of these cells to HSC derived from classical embryonic- or adult-derived HSC.

B. INTRODUCTION

Despite more than two decades of study on hematopoietic differentiation from embryonic stem cells (ESC), the potential to generate therapeutic hematopoietic stem cell populations (HSC) from ESC has not yet been realized [77, 228]. Based on results from the small number of publications reporting engraftment of ESC-derived hematopoietic cells, it has proven exceptionally difficult to demonstrate engraftment under "normal" circumstances (i.e., without potentially variable long term culture conditions or enforced expression of oncogenes or transcription factors) [120, 228], (See also Table 1.2). There have been three published reports of phenotypic selection of cell populations for transplantation based on the expression of specific cell surface molecules [108, 113, 115]. While these groups report modest to high levels of donor cell engraftment (15-100%), molecular studies of hematopoietic development in the ESC system indicate that the cell surface phenotype of HSC from embryonic sources may differ significantly from that of HSC from adult sources [86, 87, 89]; thus it is difficult to predict which marker molecules will be useful in the enrichment of transplantable populations [77, 109]. Furthermore, we have previously reported that antibodies to common HSC surface marker antigens can limit engraftment of HSC from bone marrow, so it is feasible that the same may be true for embryonic HSC ([229], see also Chapter 3 of this dissertation).

An alternative to antibody-based selection of HSC is the isolation of cells with the capacity to efflux the fluorescent DNA-binding dye Hoechst 33342, known as the Side Population, or SP [3, 227]. SP sorting was first characterized as a way to isolate long term repopulating HSC from murine bone marrow [3], and has subsequently demonstrated utility

in the enrichment of stem or progenitor populations from several other adult tissues (reviewed in [227]). Moreover, an SP has been identified in undifferentiated ESC, in which dye efflux capacity is highly correlated with the percentage of chimerism achieved by the cells in blastocyst reconstitution experiments [230]. These findings suggest that the association between SP phenotype and stem cell function may endure in embryonic as well as adult-derived tissues.

Beyond the issue of HSC phenotype in the ESC model system is the recurrent theme that the adult bone marrow environment may not be suitable for proper homing or support of an HSC derived from an embryonic source [109, 120]. Likewise, a clear difference has been demonstrated between the engraftment abilities of embryo-derived yolk sac (YS) HSC versus adult-derived bone marrow (BM) HSC; namely that YS cells cannot engraft irradiated adult recipients unless they are exposed to specific environmental cues such as those provided by the neonatal liver [70] or aorta-gonad-mesonephros-derived stromal tissue [69]. Hematopoietic development within embryoid bodies (EB) kinetically and phenotypically mirrors that of hematopoiesis in the developing yolk sac [79, 88], thus it follows that requirements for engraftment may be similar between ESC-derived HSC and YS cells [231].

The current work describes the isolation of hematopoietic stem and progenitor populations from EB-based differentiation cultures of ESC. We demonstrate long-term multilineage engraftment of SP-sorted cells from day 3 EBs as well as unsorted cells from blasts-colony forming cell (BL-CFC) cultures, as defined by Keller *et al* [82, 83]. In both cases, engraftment was achieved through injection of the cells into *in utero* conditioned newborn mouse recipients. Furthermore, we provide evidence that ligand-dependent

activation of a truncated erythropoietin receptor (tEpoR) transgene can enhance the efficiency of engraftment of ESC-derived HSC.

C. MATERIALS AND METHODS

Transgene Constructs and Gene Targeting— Both transgenic constructs created for this study contained the humanized *Renilla reniformis* green fluorescent protein with nuclear localization signal (hrGN, Stratagene) to serve as a fluorescent cell marker. The GNtEpoR construct also included the coding sequence for an erythropoietin receptor with a carboxy-terminal, 91-amino acid truncation, which has been previously described [45, 46]. All transgenes were expressed from a 1.3-kb genomic fragment containing the human β-actin promoter [232]. The transgene constructs were cloned into a modified version of the previously described *Hprt* targeting vector pSKB1 [138]. Targeting constructs were linearized with PmeI prior to electroporation. Cell culture, electroporation and selection of ESC were carried out using the method described by Bronson *et al*. [138]. The ESC line used in these studies is a subclone of E14TG2a, a cell line derived from 129/Ola mice with an ~50kb deletion in its *Hprt* gene [139] (Fig. 4-1). Following gene targeting, Southern blot analysis was performed on BamHI-digested genomic DNA from ESC clones using a random primer-labeled RsaI probe from intron 3 of the mouse *Hprt* gene. Expression of the tEpoR transgene was also confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) using the following primer set: forward, CGCTGTTGCTGACGGTTCTGG; reverse, CTGAGAGGTTCTCACTGCATGG.

In Vitro Differentiation— Undifferentiated ESC culture conditions and EB formation were previously described in Chapter 2 of this dissertation. Hemangioblast colonies, primitive and definitive erythroid, and myeloid colonies were generated as described by

Keller *et al* [83]. Briefly, for hemangioblast colony formation, day 3 EB cells were disaggregated with 0.05% Trypsin / 0.02% EDTA (Gibco) and plated at 50,000 cells/mL in IMDM with 1% methylcellulose, 10% Hematopoietic Differentiation Fetal Bovine Serum (Stem Cell Technologies), 25% D4T cell conditioned media [92], and 5 ng/mL vascular endothelial growth factor (VEGF, R&D Systems). For formation of primitive and early definitive erythroid and myeloid colonies, day 6 EB cells were plated at 50,000 cells/mL in IMDM with 1% methylcellulose, 15% plasma-derived serum (Animal Technologies), 20 U/mL erythropoietin (Amgen), c-kit ligand (1% conditioned medium), thrombopoietin (2% conditioned medium), 5% protein-free hybridoma medium (Gibco), interleukin-3 (1% conditioned medium) and 5 ng/mL interleukin-11 (R&D Systems).

SP Isolation from day 3 EBs— Day 3 EB cells were disaggregated with 0.05% Trypsin / 0.02% EDTA (Gibco), then suspended at 10^6 cells/mL in prewarmed (37˚C) RPMI medium (Gibco) containing 2% fetal bovine serum, 10 mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin and 5 µg/mL Hoechst 33342. Cell suspensions were incubated at 37˚C for 90 minutes with agitation every 15 minutes. After the incubation period, cells were immediately centrifuged at 300*g* for 10 minutes at 4˚C. Cell pellets were resuspended in 2 mL ice-cold HBSS with 2% fetal bovine serum, 10 mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 µg/mL propidium iodide. For SP inhibitor studies, Hoechst dye incubation was carried out in the presence of 50 µM verapamil (Sigma) or 5 µM reserpine (Sigma). FACS sorting of side population cells was performed as described in Chapter 3 of this dissertation. A representative example of the SP sorting gate and post-sort purity are shown in Figure 4-3. For analysis of the cell surface phenotype of EB-SP cells, prior to SP sorting the cells were stained with a phycoerythrin (PE)-Cy5.5-conjugated anti-mouse

CD117 antibody (anti-c-KIT, clone 2B8, eBioscience) and an allophycocyanin (APC)-conjugated anti mouse Ly-6A/E antibody (anti-SCA-1, clone E13-161.7, BioLegend). Lineage-depleted bone marrow cells were prepared as a reference sample by using the Miltenyi Biotec Lineage Cell Depletion Kit according to package insert instructions.

Animals and transplantation of ESC-derived cells— Transplant experiments were conducted in NOD.scid mice (a generous gift from Dr. R. Tisch, UNC) using the neonatal transplant model described by Johnson and Yoder [233]. Briefly, timed matings were set up and on the morning of days E18 and E19, pregnant dams were given intraperitoneal injections of 15.5 mg/kg of busulfan (IVBusulfex, BioPharma). Within 24 hours of birth, recipient pups were injected via the facial vein with 2.5-20 x 10^3 EB-SP cells or 50-150 x 10^3 HB culture cells. Mice were housed in sterile microisolator cages with autoclaved food according to a protocol approved by the UNC Institutional Animal Care and Use Committee.

Flow Cytometric Analysis of Peripheral Blood of Recipient Mice— Approximately every four weeks for up to 18 weeks following transplantation, recipient mice were bled via the facial vein and peripheral blood was assayed for evidence of ESC-derived hematopoietic engraftment as measured by GFP fluorescence or expression of the MHC class I isotype H2Kb (using an R-phycoerythrin (PE)-conjugated anti-mouse H2Kb antibody, clone AF6-88.5, BD Pharmingen). After antibody staining but prior to flow cytometric analysis on a CyAn cytometer (Dako Cytomation), red blood cells were lysed using FACSLyse (Becton Dickinson). Residual red cell debris was eliminated from flow analysis by gating on cells expressing the pan-hematopoietic cell surface marker CD45 (using a peridinin chlorophyll-a protein (PerCP)-conjugated anti-mouse CD45 antibody, clone 30-F11, BD Pharmingen). To determine multilineage engraftment, peripheral blood cells were also stained with the

myeloid specific marker Gr-1 (PE-Cy7-conjugated anti mouse Ly-6G and Ly-6C, clone RB6-8C5, BD Pharmingen) as well as the lymphoid specific markers CD3 (T cells) and B220 (B cells) (APC-conjugated anti-mouse CD3ε, clone 145-2C11, eBioscience, and APC-conjugated anti-mouse CD45R/B220, clone RA3-6B2, BD Pharmingen). To reduce any non-specific antibody labeling, peripheral blood cells were incubated with anti-mouse CD16/CD32 (clone 2.4G2, BD Pharmingen) for five minutes prior to antibody staining.

Microarray Analysis of EB-SP cells, YS cells and Lin$^-$ BM cells— EB-SP cells and Lin$^-$ BM cells were prepared as previously described in this chapter. Yolk sac cells were dissected from day 9.5 embryos of C57BL/6J mice (The Jackson Laboratory) and disaggregated using Liberase Blendzyme-3 (Roche) as described by Johnson and Yoder [233]. Prepared cell samples were suspended in SuperAmp Lysis Buffer (Miltenyi Biotec) and frozen at -20˚C. Frozen samples were sent to Miltenyi Biotec for PIQOR™ Mouse Stem Cell Microarray Analysis following Amplification. The Miltenyi microarray and amplification services included RNA extraction, SuperAmp message amplification, microarray hybridization, and primary data analysis (determination of relative gene expression ratios).

D. RESULTS AND DISCUSSION

Generation of tEpoR-targeted ESC lines— We generated two different gene-targeted murine embryonic stem cell lines for the studies described in this report (Fig. 4-1). The first was designed to express a nuclear-localized red-shifted *Renilla reniformis* green fluorescent protein (GFP), abbreviated hrGN (Stratagene). The second cell line was engineered to express the same hrGN as well as a truncated erythropoietin receptor (tEpoR, described in

Chapter 1 of this dissertation) in tandem from the same locus, abbreviated GNtEpoR. To promote ubiquitous stable transgene expression, the engineered constructs were targeted by homologous recombination to the hypoxanthine phosphoribosyltransferase (*Hprt*) locus, and each gene was controlled by a β-actin promoter element [232] and an SV40 polyadenylation signal [234]. (Additional benefits of *Hprt* targeting have been discussed in-depth in Chapter 2 of this dissertation). The hrGN fluorescent protein was chosen as a molecular marker for these studies because previous work by our collaborators has demonstrated that it is non-toxic to ESC and does not alter the differentiation profile of the cells (S. Hatada, D. Ciavatta, and O. Smithies, personal communication). Southern blotting was performed on genomic DNA of targeted clones to confirm correct targeting of the transgenic constructs, as shown in Figure 4-1B, left panel. We also conducted reverse-transcriptase polymerase chain reaction (RT-PCR, Fig. 4-1B, right panel) to verify expression of the tEpoR in targeted clones. Both the Southern blot and the RT-PCR results include a clone in which the GNtEpoR transgene was integrated in the forward orientation with respect to *Hprt* expression (GNtEpoR-for). Since all subsequent experiments in this report were conducted with the reverse-oriented GNtEpoR only, these cells will be referred to simply as GNtEpoR (rather than GNtEpoR-rev) for the sake of brevity.

We assayed for hrGN expression by ultraviolet (UV) microscopy, and observed distinct foci of fluorescence, consistent with nuclear localization of the hrGN protein. To further determine the percentage of cells expressing hrGN, we performed flow cytometric analysis on disaggregated cells from undifferentiated ESC as well as day 3 EB. In both cases, at least 98% of the cells in culture demonstrated hrGN fluorescence (Fig. 4-1C, insets).

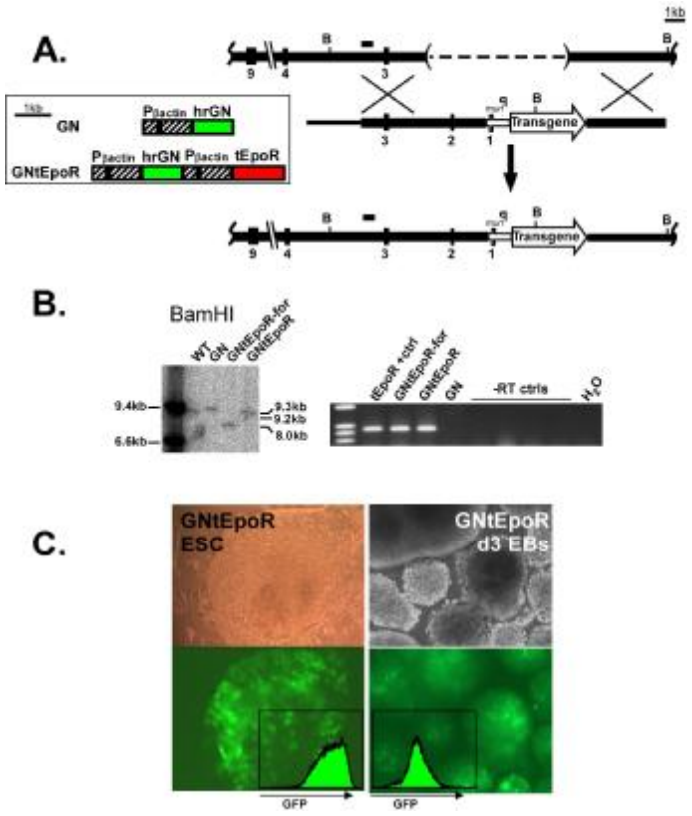

Figure 4-1 GFP and tEpoR transgene targeting to the *Hprt* locus of murine embryonic stem cells.

A.) The constructs depicted in the box at left were targeted separately into the mutant *Hprt* locus of E14Tg2a ESC, as illustrated by the schematic on the right. (*Left*) A 1.3-kb human β-actin promoter (hatched box) was used to drive expression of a nuclear-localizing red-shifted (*Renilla reniformis*) GFP (hrGN, green box) and a truncated erythropoietin receptor (tEpoR, red box); the small black rectangle marks the first exon of β-actin. (*Right*) The untargeted *Hprt* locus in the ESC line (E14Tg2a). The dashed line within parentheses represents the ~50 kb deletion that removes the *Hprt* promoter and first two exons. The targeting construct includes one of the transgene constructs and the missing promoter and exons to correct the *Hprt* gene, flanked by homologous sequences to direct recombination. Solid lines represent mouse sequences, and open lines indicate that in the correcting DNA the *HPRT* promoter (P_{hum}) and its first exon are derived from the human *HPRT* gene. Following HAT (hypoxanthine, aminopterine, thymidine) selection, only clones with a corrected *Hprt* locus, and thus an integrated transgene, will survive. The small black bar shows the location of the target sequence for the probe used in Southern blotting, and "B" markers indicate recognition sites for BamHI.

B.) (*Left*) The Southern blot resulting from BamHI digestion of targeted ESC DNA. Untargeted (WT) ESC gave a 9.2-kb fragment; cells targeted with the GN or the GNtEpoR construct gave a 9.3-fragment; and the 8.0-kb fragment was the result of integration of the GNtEpoR construct in the opposite orientation of that shown in panel A. (*Right*) Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed on mRNA from targeted ESC. Comparison to a known tEpoR-expressing cell line (tEpoR +ctrl) showed that cells targeted with the GNtEpoR construct express tEpoR, while those targeted with the GN construct do not. No reverse transcriptase was added to the (-RT ctrl) lanes, and H_2O indicates a no-template control.

C.) Phase contrast and fluorescent images of undifferentiated ESC (*left*) and day 3 embryoid bodies (EBs, *right*) from targeted ESC lines carrying the GNtEpoR transgenic construct. The insets at the bottom are flow cytometric histograms showing GFP fluorescence in the entire populations from disaggregated cultures of undifferentiated ESC or day 3 EBs.

A tEpoR transgene enhances the hematovascular potential of ESC in vitro— We next examined the *in vitro* hematopoietic differentiation capacity of our targeted ESC lines using the protocol described by Keller *et al* [83]. Within this culture system, the bipotential precursor of hematopoietic and endothelial lineages develops between day 3.0 and 3.25 of EB differentiation. Hemangioblast colonies (HB) derived from this precursor cell have an undifferentiated blast-cell morphology which can be readily distinguished from secondary embryoid bodies that develop in the cultures (Fig. 4-2A, schematic at left). The precise kinetics and predictability of hemangioblast colony formation makes this culture system a good assay for hematovascular potential of different ESC lines. Alternatively, a longer period of EB differentiation leads to the onset of primitive erythroid and early definitive hematopoiesis, allowing for assessment of multipotentiality of ESC lines (Fig. 4-2B, schematic at left). In the case of our GN and GNtEpoR ESC, there was no difference in baseline HB colony formation between the two cell lines or in comparison to untargeted ESC (Fig. 4-2A, grey bars). On the contrary, upon addition of erythropoietin (Epo) to the culture media, we saw a statistically significant 7.25-fold increase ($p<0.01$) in the ratio of HB to EB colonies from GNtEpoR ESC (8.63±1.11) as compared to untargeted cells (WT, 1.19±0.46) (Fig. 4-2A, red bars). The slight increase in HB formation in GN ESC (2.54±0.75) was not significant and $p<0.01$ for GNtEpoR +Epo versus GN +Epo as well. These results reinforce previous findings from our laboratory showing that in adult-type HSC from BM, ligand-dependent activation of the tEpoR can expand HSC populations [46] and confer a repopulation advantage in competitive transplantation assays [47]. Furthermore, this demonstrated tEpoR-dependent expansion of *in vitro* hematovascular potential suggests that

the ability of a tEpoR transgene to provide a benign and controllable proliferation advantage to adult-type HSC may also be translated to HSC derived from ESC.

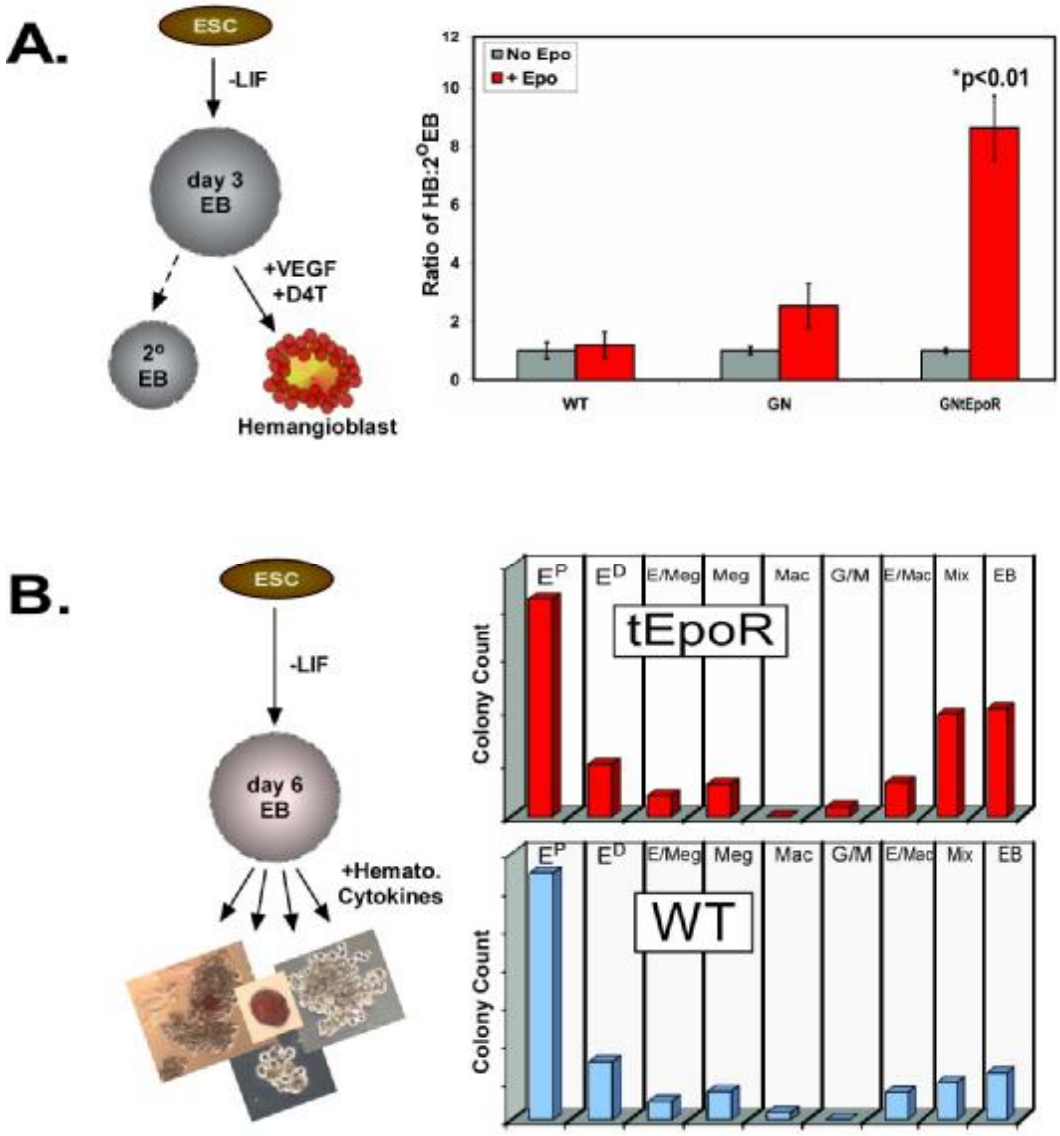

Figure 4-2 Effect of tEpoR on hematopoietic development during *in vitro* differentiation of targeted ESC.

A.) The hemangioblast assay was used to measure the hematovascular developmental potential of targeted ESC lines. The schematic (*left*) illustrates the basic culture conditions for generating hemangioblasts from ESC, as described by Keller, *et al* [83]. Removal of leukemia inhibitory factor (LIF) and non-adherent culture dishes allows the cells to differentiate in the form of embryoid bodies (EBs). Following three days of differentiation, the EBs are disaggregated and replated in methylcellulose medium with vascular endothelial growth factor (VEGF) and conditioned media from the embryonic endothelial cell line D4T. After approximately four days in culture, hemangioblast (HB) colonies form, which can be distinguished from secondary EBs morphologically. Cells were plated in triplicate and the results from two different experiments were combined to generate the graph shown at right. When erythropoietin (Epo) was added to the culture medium throughout the assay, GNtEpoR ESC showed a greater than 7-fold increase in hematovascular potential.

B.) Hematopoietic clonogenic potential of targeted ESC lines was tested with a similar *in vitro* assay, depicted at left. Differentiation was initiated as described in panel A, and after 6 days, the EBs were disaggregated and replated in methylcellulose media containing several hematopoietic cytokines including Epo (complete media is described within the chapter). After 5-7 days in culture, primitive and early definitive hematopoietic colonies were scored and the results are shown in the graph on the right. The presence of the tEpoR transgene does not alter the hematopoietic lineage potential of targeted ESC, as compared to untargeted ESC (WT). Colony types are defined as follows: E^P, primitive erythroid; E^D, definitive erythroid; E/Meg, erythroid/megakaryocyte; Meg, megakaryocyte; Mac, macrophage; G/M, granulocyte/macrophage; E/Mac, erythroid/macrophage; Mix, mixed-lineage colonies including granulocyte, erythroid, megakaryocyte and macrophage; EB, secondary embryoid body.

Side Population sorting from day 3 EBs— Based on the carefully described kinetics of lineage development within the Keller EB culture system [83], we reasoned that the multipotential self-renewing HSC should arise in this system very close to the time of hemangioblast precursor formation. We hypothesized that SP sorting of day 3 EBs differentiated via the Keller culture system would enrich for the hematopoietic or hemangioblastic progenitor cells known to reside within this structure. Since SP sorting from day 3 EBs has not previously been reported, we first had to characterize this cell population. The Hoechst red versus Hoechst blue profile of day 3 (d3) EBs (Fig 4-3A) was very similar to that of Hoechst-stained whole bone marrow, although the percentage of cells falling within the SP gate was generally much higher that what is seen in bone marrow (average from 32 SP sorts, 1.84%; range: 0.2%-5%; compared to reported BM SP of ~0.08% [3]). The SP phenotype is dependent upon the ATP-binding cassette (ABC) transporter protein ABCG2 [230], and enhanced by another member of this protein superfamily called ABCB1 [235, 236] (formerly known as P-glycoprotein or MDR1). We tested the effects of the ABCB1-specific inhibitor verapamil and the pan-ABC transporter inhibitor reserpine [237] on the presence of the SP population from d3 EBs. Verapamil had little to no effect on the appearance of the SP, but it was almost completely eradicated in the presence of reserpine (Fig. 4-3B). We also stained the cells with antibodies to KIT and LY6A to compare their cell

surface phenotype to that of lineage-depleted bone marrow (a population enriched for HSC). While there were similar percentages of KIT^+ and $LY6A^+$ single-positive cells within both cell types, the day 3 EB-SP cells had a much smaller population of double-positive KIT^+LY6A^+ cells (Fig. 4-3C). However, considering the fact that most of the events falling within the double-positive gate are only dimly positive for LY6A, it is possible that this result is partially an artifact of densely stained KIT^+ cells bleeding into the LY6A channel. Accordingly, the reported percentage of lineage-negative KIT^+LY6A^+ in bone marrow is less that 1% [11]. This issue could be resolved through the use of fluorochromes with less spectral overlap.

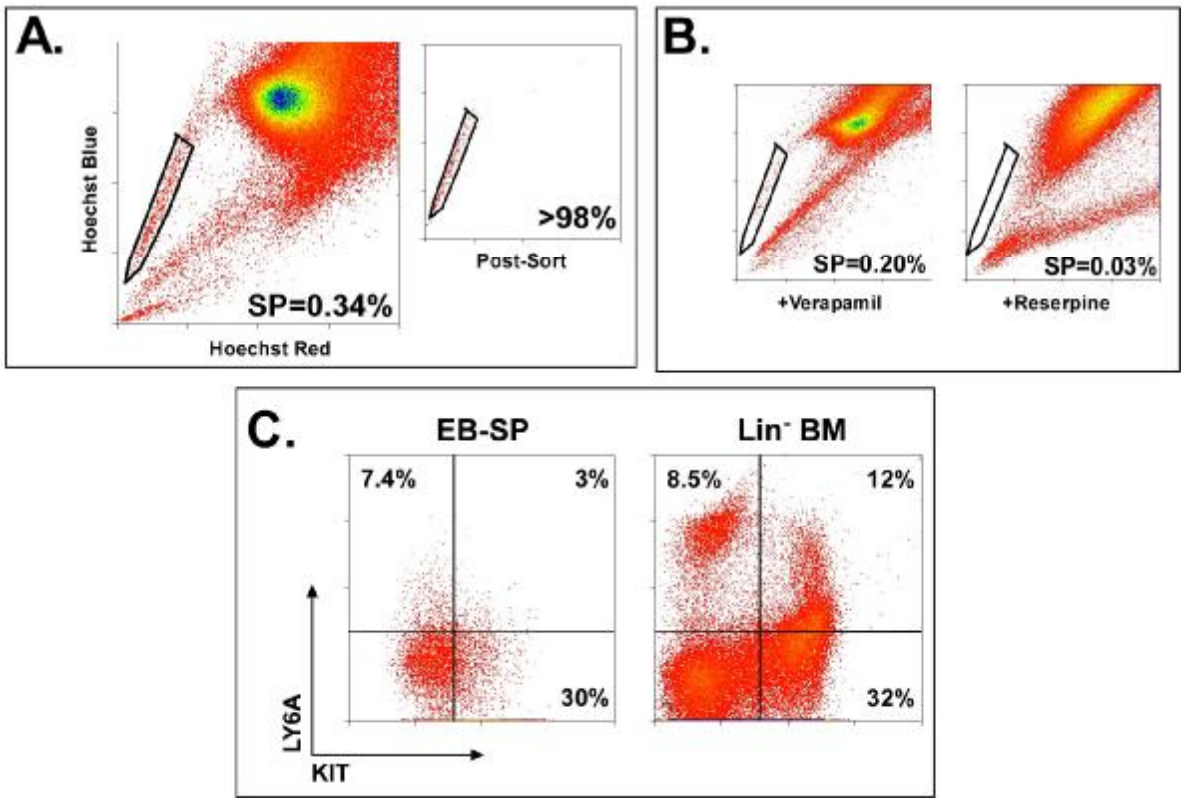

Figure 4-3 Characterization of Side Population (SP) sorted cells from GNtEpoR-targeted ESC.

A.) Day 3 EBs from GNtEpoR-targeted ESC were disaggregated with trypsin, then incubated with 5µg/mL Hoechst 33342. Flow cytometric emission monitoring at both 405 nm and 670 nm allows for selection of the Side Population of cells (SP cells, gated, left panel), which exclude the Hoechst dye. The right panel demonstrates a post-sort assessment of the purity of the sorted population.

B.) Disaggregated day 3 EBs were incubated with Hoechst 33342 in the presence of verapamil or reserpine, two multidrug transporter inhibitors with different molecular targets. Verapamil had only a minimal effect on the presence of the Side Population, but this population was eliminated in the presence of reserpine.

C.) (*Left*) Following Hoechst dye incubation, day 3 EB cells were stained with antibodies to the common HSC markers KIT and LY6A. The histogram is gated on the Side Population, and the percentage of cells stained by each marker is indicated in the respective quadrants. (*Right*) Lineage-depleted bone marrow (BM) was stained with the same antibodies to serve as a reference population.

The hrGN transgene is not sufficient for tracking engraftment of ESC-derived cells— To determine the ability of day 3 EB-SP cells to engraft and repopulate *in vivo*, we used the conditioned newborn transplant model described by Yoder *et al*. [70], and chose NOD.scid mice as our recipients in order to diminish the chance of graft rejection (Fig. 4-4A). We also transplanted some recipients with unfractionated cells from hemangioblast cultures to determine if these more mature blast colony cells could demonstrate stem cell properties *in vivo* (Fig. 4-4B). Initially, we assayed for donor cell engraftment by looking for GFP expression since both cell lines used to generate grafts were targeted with hrGN. However, after approximately 90% of the transplanted mice had reached the four week time point (with no evidence of engraftment as assayed by GFP expression), we concluded that the hrGN transgene may not be sufficient for tracking engraftment in this model.

Despite strong GFP fluorescence in immature cells from our targeted ESC (Fig. 4-4D), it is possible that some transcriptional silencing event took place as the cells differentiated to hematopoietic lineages. Tandem arrays of transgenes have been shown to generate regions of heterochromatin [238], which is one possible explanation for the loss of GFP signal. Alternatively, since the blood was fixed after collection and run through the flow cytometer at a later timepoint, it is possible that the fluorescence signal was lost prior to flow analysis. However, analysis of fresh (non-fixed) blood samples from mice known to be

engrafted (as detected by H2Kb expression, see below) also did not show GFP fluorescence, arguing for actual loss of hrGN expression rather than extinction of fluorescent signal.

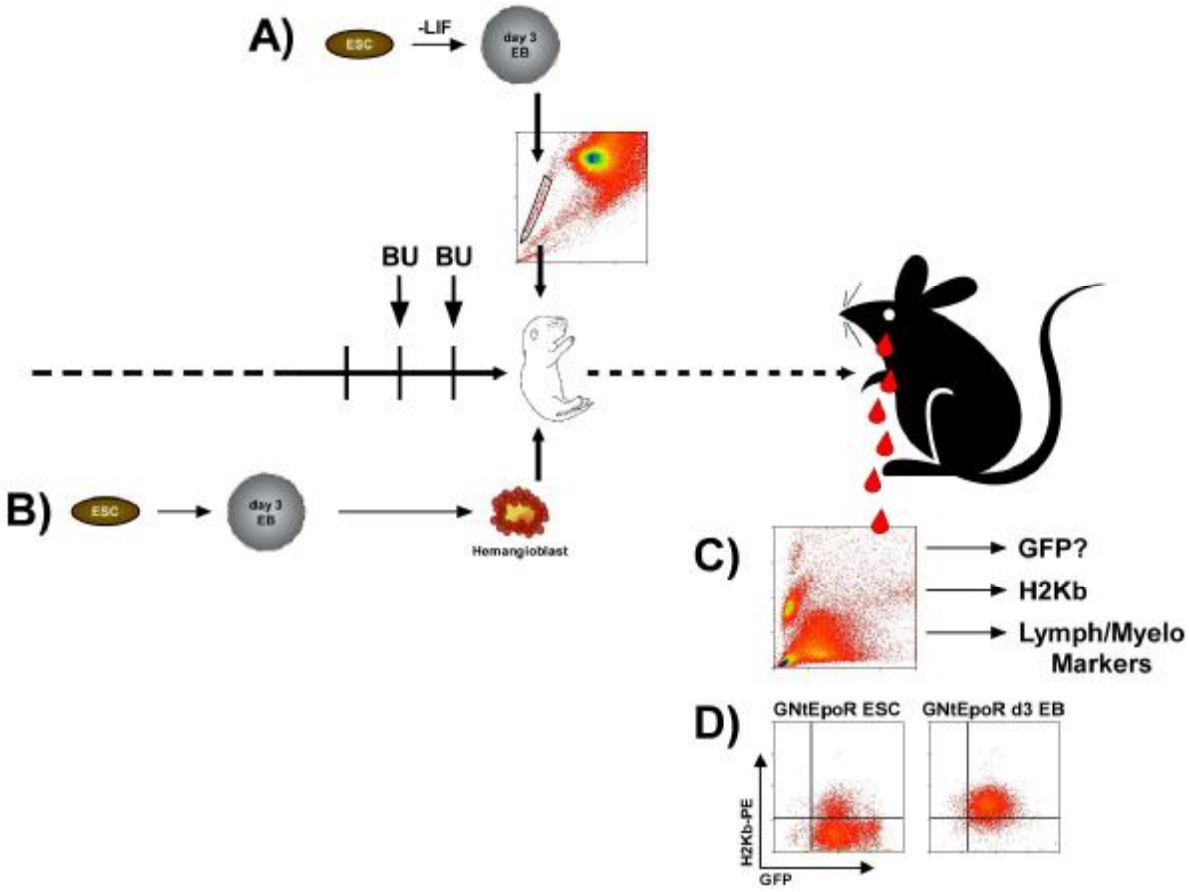

Figure 4-4 Transplantation scheme for ESC-derived cell populations injected into neonatal mice.

In utero conditioned NOD.scid neonates were used as recipients for ESC-derived hematopoietic stem and progenitor cells. The center of the figure depicts the developmental timeline of the recipient mouse. Timed pregnancies were set up and pregnant dams were injected intra-peritoneally with busulfan (BU, 15.5-22 mg/kg) two days and one day before delivery (day 18 and day 19 post-coitus). Within 24 hours of birth, recipient pups were injected via the facial vein with ESC-derived cells. After transplantation, recipient mice were bled every four to six weeks to assay for donor cell engraftment by flow cytometric analysis. (A) For EB-SP transplants, day 3 EBs were disaggregated and incubated with Hoechst 33342, then Side Population cells were sorted and injected into recipients. (B) For hemangioblast transplants, day 3 EBs were disaggregated and replated in methylcellulose media with VEGF and D4T, then after 4-6 days in culture cells were harvested for transplantation. (C) Peripheral blood of transplant recipients was analyzed by flow cytometry to assess engraftment. At the first (4 week) timepoint, blood was analyzed for GFP fluorescence. However, due to concerns over inadequate GFP expression from a single copy transgene, peripheral blood at all subsequent timepoints was stained for H2Kb, the class I major histocompatibility complex (MHC) antigen expressed only on donor cells; recipient cells express the H2Kd isotype of this gene. Blood from mice that showed donor cell engraftment after 12 weeks was also stained with antibodies to specific lymphoid and myeloid markers. (D) Representative histograms show GFP and H2Kb expression on GNtEpoR-targeted ESC (left) and day 3 EBs (right).

Transplantable Hematopoietic Progenitors from Side Population Sorted day 3 EBs and Unfractionated Hemangioblast Cultures— Following the conclusion that the hrGN transgene was insufficient for cell tracking, we converted to H2Kb detection as a marker for engraftment since the ESC lines (derived from 129 mice) express this major histocompatibility complex (MHC) isotype, while the recipient NOD.scid mice express the H2Kd isotype. At each time point assayed, a non-transplanted NOD.scid mouse was also bled to serve as a control for background nonspecific $H2Kb^+$ staining (Fig. 4-5C). Using the H2Kb detection method, we saw low to moderate levels of ESC-derived hematopoietic engraftment in all transplant groups. A small number of mice showed high levels (>30%) of engraftment after 3 months, but these mice did not cluster to any specific graft type. The complete engraftment results are presented in the charts in Figure 4-5A and B. Only mice that showed at least 1% donor chimerism (above background) on at least one time point were included on the charts. Recipient mice that never showed evidence of engraftment were not included on the charts, but a summary of all mice transplanted can be seen in Table 4.2.

In all surviving mice that showed engraftment after 12 weeks, we also tested for the presence of specific lymphoid (CD3 and B220) and myeloid (Gr-1) markers on $H2Kb^+$ donor-derived cells. Flow cytometric analysis of peripheral blood from a representative engrafted mouse is shown in Figure 4-5C. The presence of both lymphoid and myeloid donor-derived cells after four months in every transplant group we tested indicates that SP-sorted cells from day 3 EBs as well as cells from HB cultures contain long-term repopulating multilineage hematopoietic stem or progenitor cells. Rigorous definition of a stem cell requires evidence of self-renewal as assayed by secondary transplantation. Secondary transplants of bone marrow from engrafted primary recipients into irradiated secondary adult

hosts have been done, but at the time of dissertation preparation had not yet reached a time point for analysis.

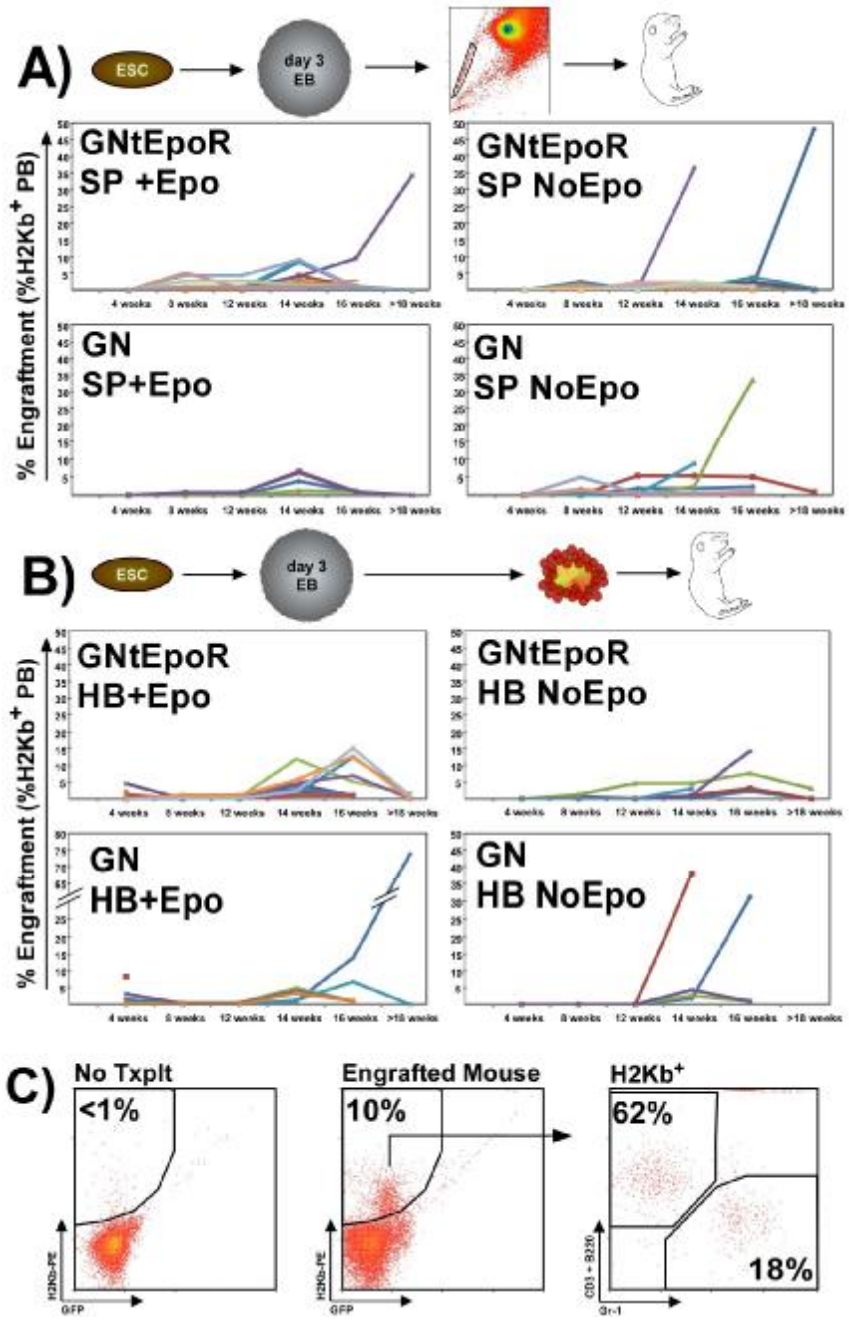

Figure 4-5 Engraftment of ESC-derived hematopoietic stem/progenitor cells.

Cells were prepared and transplanted as described in Figure 4-4. In some transplants (+Epo), erythropoietin was either added to the culture medium or injected into recipients following transplantation (or both). Only mice showing at least 1% peripheral blood donor cell chimerism on at least one timepoint are included on the graphs. **(A) EB-SP Cell Transplants**, **(B) Hemangioblast Cell Transplants**

C) Representative flow cytometric profile of peripheral blood from a mouse with multilineage ESC-derived engraftment. (*Left*) The H2Kb$^+$ gate was set based on antibody-stained blood from a NOD.scid mouse that did not receive a transplant. (*Middle, Right*) Only the H2Kb$^+$ gated cells were included on the far right histogram, which shows H2Kb$^+$ (ESC-derived) cells that stain positively for markers of both the lymphoid (CD3$^+$ for T cells and B220$^+$ for B cells) and myeloid (Gr-1$^+$) lineages.

Limitations of the transplant model— It is notable that the transplant procedure itself is rather hard on the mice as well as technically challenging, so many mice died before weaning and could not be included in the analysis. Furthermore, while the NOD.scid mouse is a good choice as a recipient in regards to avoidance of graft rejection, these mice have a very high incidence of thymic lymphomas [239], resulting in reduced lifespan (mean: 8.5 months). The occurrence of thymic lymphomas may have also been exacerbated in our transplant model by *in utero* busulfan conditioning. Indeed, a significant number of the mice (both engrafted and non-engrafted) harvested at the end of the transplant experiment (16-19 weeks post-transplant) were found to have thymic lymphomas. It is likely that many of the mice that died during the course of the experiment also harbored thymic lymphomas that may have been the cause of death, but the bodies were not autopsied, so we cannot be certain. In any case, interpretation of survival data as it relates to graft type is not useful in this transplant model because of the high background rate of death related to thymic lymphoma development.

Teratoma formation from transplanted ESC-derived HB cells— There were several mice that developed teratomas at the site of injection within 1-2 months following transplantation (see Table 4.1). These tumors were removed, preserved, sectioned, and examined histologically to confirm the presence of tissue types from all three germ layers. Notably, for transplants derived from the GNtEpoR ESC line, all of the mice that developed teratomas were from the unfractionated HB transplant groups. This result is not surprising

since the appearance of secondary embryoid bodies alongside HB colonies in these cultures indicates the persistent presence of undifferentiated ESC. It is somewhat surprising, however, that none of the GNtEpoR EB-SP cell recipients developed teratomas, since day 3 EBs are also known to harbor undifferentiated ESC. The lack of teratoma formation in the EP-SP group suggests that the SP sorting procedure excludes undifferentiated ESC at this stage of differentiation. However, in transplants derived from the GN ESC line, there were a small number of mice that developed teratomas from the EB-SP sorted cells. It is important to point out that the SP profile of the cells that gave rise to these teratomas was distinctly different from the majority of the SP profiles from day 3 EBs. Specifically, in the case of the teratoma-forming cells the SP percentage was extremely high (~4.5%) as compared to most SP sorts and the overall distribution of cells was shifted, resulting in an unclear demarcation between SP and non-SP cells. Thus, a critical detail about SP sorting is that dye efflux is a dynamic process which marks stem cell activity, but does not define a hematopoietic stem cell. So while SP sorting appears to be a highly successful method for elimination of undifferentiated cells, the ultimate goal of therapeutic application of these cells will require a more direct method of elimination of teratoma-forming cells.

Table 4.1 Teratoma formation in ESC-derived transplant recipients

Graft Genotype	Graft Phenotype	Teratoma, Teratocarcinoma, or Carcinosarcoma Formation (# out of total transplanted)
GNtEpoR	EB-SP	0/122
GN	EB-SP	6/112
GNtEpoR	HB	10/46
GN	HB	0/17

Effect of the tEpoR transgene on engraftment of ESC-derived hematopoietic progenitor cells— To test the ability of the tEpoR transgene to expand hematopoietic progenitors or facilitate engraftment of ESC-derived cells *in vivo*, we included Epo either in the culture media during cell differentiation, or administered it as subcutaneous injections into recipient mice following transplantation. Ligand-dependent activation of the transgene did not appear to affect the level of chimerism in recipient mice (Figure 4-5). On the other hand, the percentage of mice that showed some level of engraftment was higher in GNtEpoR-derived transplants with Epo as compared to those without Epo (Table 4.2). This trend was not evident in the GN-derived transplants, suggesting that the tEpoR may have a positive effect on transplantation efficiency from ESC-derived cells, consistent with the idea of tEpoR-dependent HSC expansion in the transplanted populations.

Table 4.2 Engraftment of ESC-derived hematopoietic stem/progenitor cells.

Graft Genotype	Graft Phenotype	Total Transplants	No. of Pups Analyzed (survived to weaning)		No. Engrafted (%)
GNtEpoR	EB-SP	122	53	18 +Epo	10 (56%) +Epo
				35 NoEpo	14 (40%) No Epo
GN	EB-SP	112	35	13 +Epo	4 (31%) +Epo
				22 NoEpo	8 (36%) No Epo
GNtEpoR	HB	46	38	16 +Epo	12 (75%) +Epo
				22 NoEpo	5 (23%) No Epo
GN	HB	17	15	10 +Epo	6 (60%) +Epo
				5 NoEpo	4 (80%) No Epo

Microarray Analysis to Compare GNtEpoR EB-SP to Standard Adult-Type and Embryonic-Type HSC— The ontogeny of the hematopoietic system illustrates a kind of spectrum of hematopoietic progenitor cell phenotypes. At the far left end of the spectrum is the YS HSC, which is the most primitive and the earliest to arise in the developing embryo. At the opposite end of this spectrum is the BM HSC, which is established last in development but maintains the animal throughout adult life. While the embryonic stem cell is obviously at the left end of this spectrum, it is unclear precisely where a hematopoietic stem cell differentiated from the ESC would land. Therefore, we conducted a molecular analysis (via microarray) of the hematopoietic stem/progenitor population we have identified through SP sorting of day 3 EBs. We chose a stem cell-focused microarray containing 912 genes related to the phenotype and function of stem cells derived from all three germ layers as well as undifferentiated ESC. The genes included in the array were assembled from literature screens, gene expression profiling data and bioinformatics-based homology screens. The gene expression profiles of our EB-SP HSC were compared back to cell populations at either end of the HSC phenotypic spectrum – namely, adult-type lineage-depleted BM cells and embryonic-type YS cells from day 9.5 embryos. A summary of gene expression changes is shown in Table 4.3. For all comparisons drawn, the vast majority of genes in the array were not significantly changed between the various HSC populations. However, there were a large number of stem cell genes that were significantly (>1.7-fold) up- or down-regulated in EB-SP cells with respect to expression in either adult-type BM HSC or embryonic-type YS HSC. Upon further study, these differentially regulated genes may prove useful in defining the molecular phenotype of transplantable HSC derived from ESC.

Table 4.3 Summary of gene expression results from the stem cell microarray

EB-SP Sample	No. of genes with significant change in expression (>1.7-fold)	Up-regulated / Down-regulated	No. of genes unchanged	No. of genes that could not be scored
Reference Sample: Lineage-Depleted Bone Marrow (Adult-type HSC)				
GNtEpoR No Epo	108	74 / 34	783	19
GNtEpoR +Epo	144	96 / 48	737	29
Reference Sample: day 9.5 Yolk Sac (Embryonic-type HSC)				
GNtEpoR No Epo	63	20 / 43	815	32
GNtEpoR +Epo	82	45 / 37	794	34

Genes whose expression was similarly changed in EB-SP differentiated both with and without Epo are of particular interest in this study, as they may include a set of genes specific to ESC-derived HSC (see Fig. 4-6A). Furthermore, genes whose expression was up- or down-regulated uniquely in the EB-SP +Epo sample may provide insight into the molecular mechanism of HSC expansion we observed in the transplants conducted with these cells. We found that differential gene expression across all genes assayed was much more pronounced in the comparison of EB-SP to Lin-depleted BM than it was in the comparison of EB-SP to YS (Fig. 4-6B, comparison of solid bars to hatched bars). As a rough estimation, this would suggest that EB-SP cells are more closely related to the YS HSC than they are to the BM HSC. This finding may have been predicted based on the close relationship between YS

hematopoiesis and hematopoietic development within the Keller *et al*. culture system [83, 92, 93].

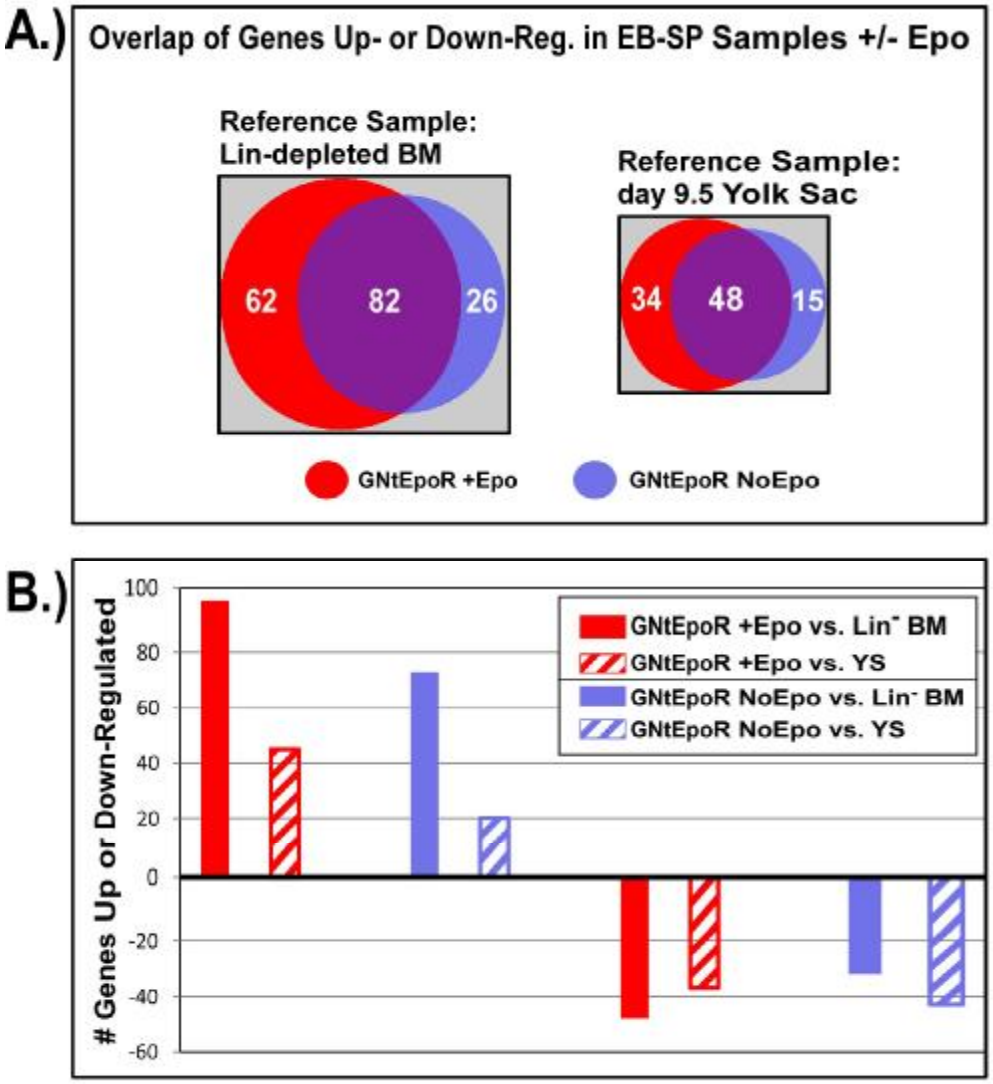

Figure 4-6 Summary of microarray results comparing GNtEpoR EB-SP to Lin-depleted BM and YS

A Stem Cell-Focused microarray analysis (912 genes) was conducted comparing GNtEpoR EB-SP cells (differentiated with or without Epo) to adult-type (Lin-depleted BM) and embryonic-type (YS) HSC populations. (A) The overlapping areas in the Venn diagrams indicate the number of genes whose expression changed significantly (>1.7-fold) in both GNtEpoR EB-SP samples (i.e., those differentiated with and without Epo).

(B) The chart summarizes the number of genes that were significantly up- or down-regulated (>1.7-fold change) for each comparison that was drawn. Solid bars represent comparison to Lin-depleted BM cells and hatched bars represent comparison to YS cells.

The microarray analysis we conducted included stem cell relevant genes from many different tissue types. However, our primary interest for the purposes of the current study was to focus on genes relevant to the hematopoietic system or to general stem cell properties, such as cell cycle quiescence. In addition, since the long-term goal of HSC isolation from ESC is to characterize cells capable of engraftment in adult settings, we concentrated on the differences between EB-SP and BM (setting aside EB-SP versus YS results for future studies). Accordingly, upon closer inspection of the microarray results comparing EB-SP to BM, we were intrigued by significant differential regulation of genes implicated in HSC homing (CXCR4 [240]), HSC quiescence (ITGB3 [241], CDKN1B [242]), cell cycle/aging (CDC42 [243], BUB3 [244]), and hematopoietic growth factor signaling (KIT [245], PAK1 [246]) in the EB-SP sorted cells (Summarized in Table 4.4). We are currently in the process of confirming these results by real-time RT-PCR so that we may further focus our studies on genes that may define the difference between ESC-derived HSC and adult-derived BM HSC.

Table 4.4 Genes of interest from stem cell microarray comparison of EB-SP to Lin-depleted BM

Genes of Interest (Fold Change wrt Lin-depleted BM)			
UPregulated		**DOWNregulated**	
KIT	+E: 39.12	TAL1	+E: -2.17
	NoE: 23.88		NoE: -1.54 (ns)
ITGB3	+E: 12.02	CDKN1B	+E: -2.33
	NoE: 20.31		NoE: -1.89
CXCR4	+E: 19.7	BUB3	+E: -2.86
	NoE: 20.69		NoE: -2.70
CDC42	+E: 18.65		
	NoE: 18.62		
PAK1	+E: 2.08		
	NoE: 1.99		

E. Future Directions

A microarray experiment is, by design, much more of a beginning than an ending. Preliminary analysis of our microarray results has revealed many exciting possibilities for exploration of the fundamental differences between embryonic- and adult-type HSC. The most pressing steps in further analysis at this point are: (1) confirmation of microarray results to validate the findings, and (2) analysis of the secondary transplants that we have conducted from the EB-SP cells. In the absence of engraftment in secondary transplants, EB-SP cells do not meet the strictest definition of HSC and can only be considered long-term repopulating hematopoietic progenitor cells. In the case of failed secondary transplants, the preliminary microarray results pertaining to cell cycle, self-renewal, and aging will be of particular interest for future studies.

If, on the other hand, we do see evidence of engraftment in secondary transplants, the microarray results, once confirmed, may be especially informative in translating our findings to an adult transplant model. We did transplant a small number (<30) of irradiated adult recipients with EB-SP cells at the same time that we conducted the experiments described in this chapter, and saw no evidence of engraftment over the four month analysis period (data not shown). This result mirrors the obstacles encountered in early attempts to transfer YS HSC to adult hosts [247]. Nonetheless, YS cells have been recently shown to contribute to adult hematopoiesis [71]. Likewise, in-depth molecular analysis of EB-SP cells compared to BM HSC may reveal the mechanistic basis of the lack of adult repopulation. Given the relative ease of genetic manipulation of ESC, it may be possible to alter any genes uncovered

in this analysis in order to facilitate the ultimate goal in these studies: adult engraftment by ESC-derived cells.

Chapter 5 PERSPECTIVES AND FUTURE DIRECTIONS

One of the great advances in modern medicine came with the advent of hematopoietic stem cell transplantation to alleviate or cure many of the diseases that affect the hematopoietic system. Over the past several decades, HSC transplantation has developed into a common and often successful treatment modality; however, there are still significant obstacles that must be overcome for this therapy to reach its full potential. First, HSC are a rare population in bone marrow, umbilical cord blood and peripheral blood, which are the only currently available sources of this cell type. Second, despite many years of research, attempts to expand HSC have been largely unsuccessful, commonly resulting in loss of self-renewal, multipotentiality or homing ability of the cells. Third, there are significant immunological hurdles in HSC transplantation, including allograft rejection, lack of available MHC-matched grafts, or post-transplant manifestations of graft-versus-host disease. Finally, the toxicity of currently available pre-transplant conditioning regimens is prohibitive for the application of HSC transplantation to non-life-threatening diseases.

Embryonic stem cells represent a potential alternative source of transplantable hematopoietic cells which may circumvent each of the aforementioned obstacles in HSC transplantation. There are many distinct advantages to the ESC system, including unlimited numbers of cells that maintain potency in culture, the ability to control differentiation to a particular tissue type, and relative ease of genetic manipulation, allowing for correction of genetic defects or introduction of therapeutic transgenes. Nevertheless, there are several key criteria which must be rigorously met before therapeutic transplantation of ESC-derived cells

can become a reality. Following induction of hematopoietic differentiation, there must be an efficient and robust protocol for isolation of the self-renewing, long-term, multilineage stem cell population which maintains the ability to engraft upon transplantation. Furthermore, there must be a reliable method for removal of any remaining undifferentiated cells which could give rise to teratomas.

The findings presented in this dissertation provide progress toward the ultimate goal of isolating transplantable hematopoietic stem cells differentiated from embryonic stem cells. In a mouse model system, we have demonstrated long-term multilineage hematopoietic engraftment from SP sorted day 3 embryoid body cells (EB-SP) as well as unfractionated hemangioblast culture cells (differentiated using the Keller method [83]). In the case of the EB-SP transplants, we achieved engraftment from the lowest number of ESC-derived cells that has been reported to date (range: 2.5-20x10^3 cells transplanted), suggesting that SP sorting significantly enriches for the stem cell population. Furthermore, prior to our study, the shortest differentiation culture time that has been reported to generate engraftable cells is four days [116], with culture time from all other similar studies ranging from 5-30 days (see Table 1.2). Minimal culture time is preferable for any proposed therapeutic application since the risk of contamination of the graft increases with the time in culture, thereby increasing the risks to the patient. Other groups attempted transplants from early stage EBs, but no engraftment results could be reported because all mice developed teratomas; thus another important advance in our study is the near complete elimination of teratoma formation by SP sorting from day 3 EBs. In addition to prolonged culture time, all but one of the published reports on ESC-derived cells in last 10 years have required enforced overexpression of transcription factor or signaling molecule genes to achieve hematopoietic engraftment. The

viral constructs used to introduce these genes, as well as the nature of the genes themselves are concerning for therapeutic application due to possible risk of malignant transformation. It is highly encouraging that, in our study, we were able to isolate a population of ESC-derived cells that could engraft under normal conditions (i.e. without enforced gene expression). One last point that makes our study unique is that the cells were transplanted into neonatal recipient mice. While adult engraftment will be necessary for any future therapeutic application, our demonstrated successful engraftment in neonates may reveal important characteristics of ESC-derived cells related to homing and engraftment abilities.

The next stage of the research presented here will focus on refining the selection methods for transplantable hematopoietic cells from ESC, developing further methods for reliable elimination of teratoma-forming cells, and improving expansion of the stem cell population. We intend to test combined positive/negative selection protocols that are designed to positively select transplantable hematopoietic stem cells (or their developmental precursors) and negatively select undifferentiated cells in the ESC differentiation culture. We have an ongoing collaboration with Dr. S. Hatada, who developed an elegant fluorescent protein based positive/negative selection system for gene-targeted cells which may be adaptable to our goals [248]. The positive selection may be achieved by a combination of SP sorting and the lineage-restricted fluorescent gene sorting described in Chapter 2 of this dissertation. Necessary adjustments to the previously described methods will be either the inclusion of additional regulatory elements from the Scl locus or the use of gene regulatory elements from a different gene whose expression is unique to early hematopoietic development, such as Tmtsp [249]. Similar lineage-restricted expression methods may be useful for negative selection of cells expressing genes unique to undifferentiated ESC.

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