Colloquium

Enhanced hematopoietic differentiation of embryonic stem cells conditionally expressing Stat5

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The signal transducer Stat5 plays a key role in the regulation of hematopoietic differentiation and hematopoietic stem cell function. To evaluate the effects of Stat5 signaling in the earliest hematopoietic progenitors, we have generated an embryonic stem cell line in which Stat5 signaling can be induced with doxycycline. Ectopic Stat5 activation at the point of origin of the hematopoietic lineage (from day 4 to day 6 of embryoid body differentiation) significantly enhances the number of hematopoietic progenitors with colony-forming potential. It does so without significantly altering total numbers or apoptosis of hematopoietic cells, suggesting a cell-intrinsic effect of Stat5 on either the developmental potential or clonogenicity of this population. From day-6 embryoid bodies, under the influence of Stat5 signaling, a population of semidifferent cells can be expanded on OP9 stromal cells that is comprised of primitive hematopoietic blast cells with ongoing, mainly myeloid, differentiation. When these cells are injected into lethally irradiated mice, they engraft transiently in a doxycycline-dependent manner. These results demonstrate that the hematopoietic commitment of embryonic stem cells may be augmented by a Stat5-mediated signal, and highlight the utility of manipulating individual components of signaling pathways for engineering tissue-specific differentiation of stem cells.

hematopoiesis

When differentiated as suspension aggregates (embryoid bodies, EBs), embryonic stem (ES) cells will readily give rise to differentiated hematopoietic cells (1) as well as colony-forming cells (CFCs) that can be assayed in secondary semisolid hematopoietic cultures (2). However, ES cell differentiation does not efficiently generate hematopoietic stem cells (HSCs) capable of repopulating the hematopoietic system of lethally irradiated adult recipients. In this regard, ES cells recapitulate the development of the earliest embryonic hematopoietic tissue, the yolk sac. Analysis of knockout mice has implicated several genes in the embryonic development of the definitive HSC (3–5); however, a detailed understanding of the extracellular signals that guide development from the pluripotent state to the lineage-restricted HSC state is lacking.

In an effort to evaluate signals that promote hematopoietic differentiation of pluripotent cells, as well as the generation of cells with hematopoietic repopulating potential, we sought to test the effects of Stat5 activation during in vitro differentiation of ES cells. Stats are cytoplasmic signal transducers that are recruited by ligand-activated receptors via Src homology 2-mediated interactions with receptor-bound Janus kinases. This interaction results in Stat protein phosphorylation, dimerization, and translocation to the nucleus where these proteins then function as a transcription factors, binding to and activating the transcription of target genes (6). Stat5 is encoded by two genes, Stat5a and Stat5b, with 95% sequence identity (7) and is activated by engagement of numerous hematopoietic and nonhematopoietic receptors (8–11). Stat5 signaling has been implicated in cellular proliferation (12, 13), resistance to apoptosis (14–16), and differentiation (17, 18).

Mice genetically null for both Stat5a and Stat5b display obvious defects in response to growth hormone and prolactin (15) and subletal defects in embryonic hematopoietic development (14). Although definitive HSCs develop in Stat5 knockout mice, they display a profound defect in competitive repopulation (19–21), suggesting that Stat5 may be interacting cooperatively and redundantly with other signal transducers in HSC regulation. In the classic HSC pathology, chronic myeloid leukemia, regulation of proliferation is disrupted by the oncogene Bcr/Abl, with concomitant activation of Stat5 (22–25). Moreover, dominant negative Stat5 mutants can block transformation by Bcr/Abl (26), indicating that inappropriate activation of Stat5 can have dramatic consequences for HSC regulation.

Because of the pivotal role of Stat5 signaling in hematopoiesis and HSC homeostasis, we selected this pathway for study during the earliest stages of hematopoietic specification in an in vitro system of ES cell differentiation. For this purpose, we have generated an ES cell line with a tetracycline-inducible, dominant-active allele of Stat5. We report that induction of Stat5 signaling during EB development dramatically enhances hematopoiesis. Furthermore, on OP9 stromal cell coculture, Stat5 promotes the expansion of a blast cell population from day-6 EBs. Cultures expanded in this way are rich in primitive, undifferentiated cells, with surface marker similarities to HSCs, and have the capacity to engraft lethally irradiated adult mice in a transient, Stat5-dependent manner.

Materials and Methods

Generation of Stat5CA Inducible ES Cells. The cDNA for the constitutively active mutant of Stat5 (H299R/S711F, a gift from T. Kitamura, University of Tokyo, Tokyo) was subcloned on an EcoR1–NotI fragment from murine stem cell virus (MSCV)–Stat5CA-iresGFP (16) into pLox. This was then co-electroporated along with pSalk-CRE (a gift from S. O’Gorman, The Salk Institute, San Diego) into the targeting cell line Ainv15. The targeting ES cell line and targeting plasmid, pLox, have been described (27). The resulting cell line was selected and expanded...
in 400 μg/ml G418 (Sigma). ES cells were maintained on irradiated mouse embryonic fibroblasts in DME/15% inactivated fetal serum (IFS)/0.1 mM nonessential amino acids (GIBCO/BRL)/2 mM glutamine/50 units/ml penicillin/50 μg/ml streptomycin (GIBCO/BRL)/0.1 mM 2-mercaptoethanol (Sigma)/1,000 units/ml leukemia inhibitory factor (PeproTech, Boston). To induce Stat5CA expression in ES cells, 2 μg/ml doxycycline (Sigma) was added to the culture medium.

**Bandshift Assay.** To generate whole cell extracts, cells were washed in ice-cold PBS and lysed in EMSA lysis buffer (150 mM NaCl/20 mM Tris-HCl, pH 7.4/1 mM EDTA/10 mM Na3VO4/1 mM MgCl2/1% Nonidet P-40/1 mM phenylmethyl-sulfonyl fluoride/10% glycerol) on ice for 10 min. Cells and lysate were scraped with a sterile cell scraper, collected, and spun for 10 min at max at 4°C on a benchtop centrifuge. Complementary oligonucleotides that contained a Stat5 consensus binding site from the β-casein promoter (5′-AGAGTTCTAGGAATTC-3′) were annealed and radiolabeled with [γ-32P]ATP by using T4 polynucleotide kinase (New England Biolabs). Approximately 20,000 cpm (0.2 ng) of probe was incubated with 20 μg of whole cell extract in 20 μl of 10 mM Hepes, pH 7.9/0.2 mM DTT/10% glycerol/0.05% Nonidet P-40/1 μg poly(dIdC) (Sigma) for 20 min at room temperature. Resulting DNA/protein complexes were resolved on a 5% nondenaturing polyacrylamide gel.

**EB Differentiation.** ES cells were trypsinized, collected in EBD [Iscove’s modified Dulbecco’s medium (IMDM)/15% IFS/200 μg/ml iron-saturated transferrin (Sigma)/4.5 mM monothioglycollate (Sigma)/50 μg/ml ascorbic acid (Sigma)/2 mM glutamine] and plated onto fresh T25 flasks (Corning) for 45 min to allow mouse embryonic fibroblasts to adhere. Nonadherent cells were collected and plated in hanging drops at 100 cells per 10-μl drop in an inverted bacterial Petri dish, and cultured for 2 days. They were then collected from the hanging drops and further cultured in 10 ml of EBD in slowly rotating 10-cm bacterial Petri dishes. At day 4, EBs were fed by exchanging half of their spent medium for fresh EBD. In some cultures, doxycycline was added at day 4 at 2 μg/ml to induce expression of Stat5CA.

**CFC Assay.** Day-6 EBs were dissociated by trypsinization, collected, and resuspended in IMDM/10% IFS at a concentration of 5 × 10^5 cells per ml. A total of 100 μl of this cell suspension was added to 1.5 ml of complete methylcellulose for murine colonies (StemCell Technologies, Vancouver, catalog no. 3434). Methylcellulose suspension cultures were not supplemented with doxycycline. EryP colonies were counted on day 6 of methylcellulose culture, all other colonies were counted at day 10.

**OP9 Coculture.** Day-6 EBs were trypsinized to a single cell suspension and plated on a semiconfluent monolayer of OP9 cells (a gift of T. Nakano, University of Osaka, Osaka) at a density of 200,000 cells per well of a six-well dish in IMDM, 10% IFS supplemented with 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin (GIBCO/BRL), 0.1 mM 2-mercaptoethanol (Sigma), cytokines [40 ng/ml vascular endothelial growth factor (VEGF), 40 ng/ml thrombopoietin (TPO), 100 ng/ml stem cell factor (SCF), and 100 ng/ml Flt-3 ligand], and doxycycline at 1 μg/ml. When cells became confluent, they were passaged by trypsinization onto fresh OP9.

**Fluorescent Staining and FACS Analysis.** Staining of day-6 EB cells. EBs were disaggregated by washing once with PBS followed by resuspension in 0.1% trypsin/PBS and pipetting for 30 s. Trypsin was blocked with IMDM/10% IFS, the cells were stained to remove clumps and collected by centrifugation. Annexin V–phycocerythrin staining was done at room temperature for 15 min according to the manufacturer’s specifications (CLONTECH). After staining, cells were transferred to 4°C, and 1 μl of FITC-conjugated CD41 antibody was added. After 20 min, samples were diluted with annexin V binding buffer containing propidium iodide and analyzed by FACS.

**Staining of OP9 cultures.** Cells were collected by trypsinization and resuspended in PBS containing 5% IFS. Samples of one million cells in 100 μl were blocked with 1 μl of Fe block (Pharmingen) and stained with 1 μl of phycocerythrin- or FITC-conjugated antibody for 20 min at 4°C. Samples were washed twice with PBS/5% IFS, and resuspended in PBS/5% IFS containing propidium iodide. All antibodies were purchased from Pharmingen. FACS analyses were performed on a Becton Dickinson FACSCalibur. Dead cells were excluded from phycoerythrin-stained cells by gating on FL2 vs. FL3.

**RT-PCR.** The following primers were used: actin(f) 5′-CTCTTTAATGTGCAACGACCGATTCTT-3′; actin(r) 5′-GCTGCAACGTGAAGTCCTG-3′; Stat3CA(a) 5′-GCAATCTTTGAGTGCTG-3′; Stat3CA(b) 5′-CTGGAATGTCCTG-3′; HIF-α 5′-TTCCTTTGAGTTCA-3′; HIF-β 5′-CTGGAATGTCCTG-3′;
β-maj(f) 5'-CTGACAGATGCTCTTTGGG-3'; β-maj(r) 5'-CACACCCCCAGAACGACA-3'. Cycle conditions were as follows: 2 min at 96°C; 30 cycles of 45 s at 95°C, 1 min at 60°C, and 45 s at 72°C; and then 5 min at 72°C.

**Retroviral Labeling with GFP.** GFP retroviral supernatants were produced by FUGENE transfection of 293 cells with pMSCViresGFP (28) and pCL-Eco, a packaging-defective helper plasmid (29). 293 cells were grown in DME/10% IFS, and medium was replaced on the day after transfection. Forty-eight hours after transfection, supernatants were collected, filtered, plated onto iStat5CA blast cells growing on OP9 at 3 ml per well of a six-well dish, supplemented with 4 μg/ml polybrene and cytokines (100 ng/ml SCF, 40 ng/ml VEGF, 40 ng/ml TPO, 100 ng/ml Flt-3 ligand), and spin-infected at 2,500 rpm for 90 min in a Beckman GS-6R centrifuge. After several days of growth, GFP-positive cells were separated by FACS and cultured on fresh OP9.

**Results**

**Stat5 Signaling During EB Development.** To generate an ES cell line with inducible Stat5 signaling, we made use of the Tet-On targeting cell line, Ainv15 (27). These cells constitutively express the reverse tetracycline transactivator from the ROSA26 locus, and have a tetracycline response element (TRE) integrated into the transcriptionally open chromatin 5’ to the HPRT gene on the X chromosome. Downstream of the TRE is a single LoxP site, into which we integrated, by Cre–Lox recombination, the cDNA for Stat5CA, a constitutively active mutant of Stat5a (a double mutant of H299R and S711F, also known as 1*6) (12b) (Fig. 1a). The resulting ES cell line, named iStat5CA, as well as its differentiated progeny, express this mutant cDNA when exposed to doxycycline. Expression results in binding of Stat5CA to DNA as measured by bandshifting activity against an oligonucleotide probe encoding a Stat5 DNA-binding consensus sequence, in the lystate of doxycycline-treated iStat5CA ES cells (Fig. 1b).

We used this cell line to generate EBs and applied doxycycline to the cultures for 48 h, from day 4 to day 6 of differentiation. This time window was chosen based on the kinetics of colony formation in EBs: the bipotent precursor to the hematopoietic lineage, the hemangioblast, peaks at day 3.75 and is no longer present by day 5 (30), whereas hematopoietic and endothelial lineages, the hemangioblast, peaks at day 3.75 and is no longer present by day 5 (30), whereas hematopoietic CFC, particularly mixed erythroid–myeloid colonies, first become detectable between days 5 and 6. Thus, Stat5 signaling was induced at the time of specification of the hematopoietic lineage. At day 6, the EBs were disaggregated into single cells and assayed for hematopoietic CFC content by plating in methylcellulose suspension medium with hematopoietic cytokines. As shown in Fig. 2a, exposure of EBs to doxycycline increased the numbers of all types of hematopoietic colonies assayed between 2- and 4-fold.

To investigate the mechanism of Stat5-mediated hematopoietic enhancement, we analyzed cells from doxycycline-treated or untreated day-6 EBs for apoptosis. Preliminary results (not shown) demonstrated that Stat5 activation modestly decreased the number of apoptotic (annexin V-positive) cells from day-6 EBs; however, a general reduction in apoptosis would increase CFC frequency only if the hematopoietic lineage were subject to higher levels of apoptosis than the other nonhematopoietic lineages that arise in a day-6 EB. To assay the levels of apoptosis in hematopoietic vs. nonhematopoietic cells, we stained EB cells with both annexin V and a pan-hematopoietic antibody. Studies of adult hematopoiesis commonly use the CD45 antigen as a pan-hematopoietic marker; however, this marker is not universally expressed by the earliest embryonic hematopoietic progenitors. The recent discovery that the adult megakaryocytic antigen CD41 is actually pan-hematopoietic (in very early embryos as well as in day-6 EBs (31, 32) prompted us to use this marker rather than CD45 for this purpose. To our surprise, this assay revealed that apoptosis in day-6 EBs is almost completely restricted to the nonhematopoietic population (Fig. 2b).

**Mouse Transplantation.** Two- to four-month-old 129Ola/Hsd (Harlan Breeders, Indianapolis) mice were exposed to 2 × 500 cGy of γ-irradiation, separated by 4 h, and injected with 1.75 × 10^6 cells in 500 μl of IMDM/10% IFS via lateral tail vein. Mice that received doxycycline were provided drinking water supplemented with 500 μg/ml doxycycline hydrochloride (Sigma) and 5% sucrose.

**Fig. 2.** Effects of Stat5 signaling during EB differentiation. EBs were grown for 6 days, either exposed or not exposed to doxycycline from day 4 to day 6. (a) CFC assay: day-6 EB cells were disaggregated and plated into methylcellulose suspension culture with hematopoietic cytokines. Filled bars denote colony number from doxycycline-treated EBs; open bars denote colony number from untreated EBs. Standard errors for three independent experiments are shown. (n = 3 for each bar; P < 0.05 for combined CFCs.) (b) Apoptosis assay: day-6 EB cells were disaggregated and stained with annexin V (y axis) to label apoptotic cells and anti-CD41 (x axis) to label hematopoietic cells. The percentage of cells falling into single- and double-positive quadrants is shown. (c) Hematopoietic compartment quantitation: day-6 EB cells were disaggregated and stained with antibodies to c-Kit (y axis) and CD41 (x axis). The percentage of cells falling within the double-positive rectangular gate is shown.
An alternative to a reduction in apoptosis would be overproliferation of hematopoietic cells in response to Stat5. Because hematopoietic CFCs from the day-6 EB are CD41 and c-Kit double positive (31, 32), we assayed the frequency of this population in the presence vs. absence of Stat5 induction (Fig. 2c). We observed only a very modest increase with doxycycline treatment, which was not sufficient to account for the increase in CFCs. We therefore conclude that Stat5 activation is either influencing development within the hematopoietic compartment, such that it contains a higher ratio of CFCs to more differentiated cells, or enhancing the clonogenicity of the CFCs that are present.

Stat5 Signaling During OP9 Stromal Cell Coculture of Day-6 EB Cells in Vitro. Cells from day-6 EBs that had been exposed to doxycycline for 48 h were also plated on OP9 stromal cells with a cytokine mixture tailored for HSC expansion, consisting of TPO, SCF, Flt-3 ligand, and VEGF. In the absence of doxycycline, there was minimal growth, whereas in the presence of doxycycline, there was a dramatic expansion of a semiadherent cell type growing attached to the OP9 feeder layer. These semiadherent cells could be passaged by trypsinization and expanded exponentially (Fig. 3a).

Whole cell protein extracts from cells growing on OP9 in the presence of doxycycline, but not in its absence, contained Stat5-specific DNA-binding activity (Fig. 3b). The intensity of the Stat5 bandshift was similar to that observed in the pro-B cell line BaF/3 growing in the presence of IL-3, but much less than the bandshift observed in BaF/3 cells infected with a retrovirus expressing Stat5CA (Fig. 3b). This demonstrates that the level of Stat5 activation achieved by exposure to doxycycline approximates the physiological level that hematopoietic cells experience when growing in the presence of cytokines.

The dominant cell type in the OP9 expansion cultures was a primitive hematopoietic blast; however, other cell types could also be detected, particularly differentiated myeloid cells (Fig. 3c). We analyzed these cells for surface antigen expression by flow cytometry (Fig. 4a and b). Consistent with the blast cell morphology, the majority of cells were negative for markers of hematopoietic differentiation. Of those cells that were positive for lineage markers, the majority expressed the myeloid markers, Gr-1 and Mac-1, but a small number expressed markers of lymphoid (B220) and erythroid (Ter-119) differentiation. We observed strong positivity for the hematopoietic stem cell markers c-Kit and Sca-1. The cells were negative for CD45 but positive for CD41, consistent with an early embryonic hematopoietic character, and the majority of both the c-Kit- and Sca-1-positive cells were double positive for CD41. The cells were also strongly positive for CD31, a marker displayed by hematopoietic stem cells, some differentiated hematopoietic cells, as well as endothelial cells. This expression is likely hematopoietic in origin as opposed to endothelial given the coexpression of CD41. This profile is suggestive of the expansion of an undifferentiated embryonic hematopoietic progenitor with many characteristics of the HSC, which undergoes concomitant differentiation mainly along the myeloid lineage in vivo.

We assayed globin gene expression in these cells and compared it to that seen in a similar cell population obtained by expression of HoxB4, which we have previously described (27). Whereas embryonic (β-H1) globin is almost undetectable in the HoxB4-expanded cells, it is clearly present in those expanded by Stat5. The embryonic globin signal is weak compared with adult (β-major) globin; however, its presence suggests that Stat5 signaling does not efficiently drive primitive to definitive hematopoietic switching in the same way that HoxB4 appears to.

Stat5-Dependent Engraftment in Vivo. To determine the capacity of these cells to undergo differentiation in vivo, they were marked with GFP by retroviral infection with the virus MSCViresGFP (28). Cells were then injected into 10 irradiated isogenic recipient adult mice, in two independent experiments, and the peripheral blood was sampled periodically for GFP positivity. We observed no engraftment in mice not treated with doxycycline, even at time points as early as 2 weeks. However, when mice were fed drinking water supplemented with doxycycline, we observed transient donor cell contribution to the peripheral blood, liver, spleen, and marrow, which was exhausted by 8 weeks after transplantation. Although we observed contribution to the spleen, we did not observe donor-derived CFU-S. Engraftment was best when OP9 cocultures were injected as soon as sufficient
cells were available, and was gradually lost with extensive passage in vitro. FACS analysis of the bone marrow of a typical recipient 1 month after transplantation is shown in Fig. 5. Although we observed low levels of GFP positive cells overall, they counterstained with markers representing differentiation into all three hematopoietic lineages: lymphoid (B220 and CD4), myeloid (Gr-1 and Mac-1), and erythroid (Ter-119), demonstrating that these cells have a broad differentiation potential in vivo. In one case, we observed an animal succumb to a GFP-positive myeloid leukemia 2 months after transplant, suggesting that at some frequency, continual activation of Stat5 signaling can result in the eventual outgrowth of a malignant population, as has been seen after retroviral transduction of Stat5CA in bone marrow transplant models (33). However, because donor cells were eventually lost by the majority of recipients, our results demonstrate that this cell population has limited self-renewal potential in vivo, even with maintenance of induction of Stat5 signaling.

Discussion
ES cells are competent to differentiate into cells of all embryonic and adult lineages, as evidenced by the derivation of chimeric animals from blastocysts injected with ES cells (34). This potency makes ES cells promising source material for regenerative medicine. However, putting this potential into practice in adults, as opposed to embryos, will require the derivation of adult-repopulating stem cells from ES cells in vitro. In the case of the hematopoietic system, this has proven to be more difficult than expected, given that ES cells will readily generate blood in vitro when differentiated as EBs (1). ES cells seem predisposed to an embryonic mode of blood differentiation akin to that of the early extraembryonic yolk sac, producing mainly primitive erythrocytes and myeloid progenitors (2, 35), but lacking adult-repopulating cells that are thought to arise in the embryo proper (36, 37).

The HSC for this primitive (embryonic) mode of hematopoiesis seems to have the latent potential to generate definitive (adult) lineages. When adult engraftment is enabled by transformation with Bcr/Abl, an oncogene with specific growth-promoting effects on the HSC, contribution to these lineages can be observed (38). Adult engraftment and multilineage hematopoiesis has also been observed when EB-derived cells are made to overexpress the transcription factor HoxB4 (27). This transcription factor has growth-promoting effects on the HSC similar to but less transforming than Bcr/Abl. HoxB4 also induces a switch in the expression pattern of several markers that distin-
is better able to suppress their differentiation drives expansion of the most primitive cells more efficiently, and to the HoxB4-expanded cells, the in vivo earliest phase of expansion. It may be the case that Bcr did observe lineage marker expression on some cells in the cells were negative for all lineage markers tested, although we et al. Kyba similarities to both the Bcr/Ab1-expanded cells and the HoxB4-expanded cells described here are closer in type to those expanded by Stat5 activation have similarities to both the Bcr/Ab1-expanded cells and the HoxB4-expanded cells that we have previously described. Morphologically, all three populations consist mainly of undifferentiated blast cells. By analysis of surface marker expression, the Stat5-expanded cells described here are closer in type to those expanded by HoxB4, in particular in their expression of CD31, CD41, and c-Kit, and in the presence of differentiating cells expressing myeloid lineage markers. The Bcr/Ab1-expanded cells were negative for all lineage markers tested, although we did observe lineage marker expression on some cells in the earliest phase of expansion. It may be the case that Bcr/Ab1 drives expansion of the most primitive cells more efficiently, and is better able to suppress their differentiation in vitro. In contrast to the HoxB4-expanded cells, the in vivo engraftment potential of the Stat5-expanded cells is quite distinct. Their engraftment is strictly doxycycline-dependent, and temporally limited, whereas HoxB4-expanded cells engraft without the need for maintenance of HoxB4 expression in vivo, and readily contribute to long-term hematopoiesis. It may be the case that Stat5 is driving self-renewal of lineage-committed progenitors or possibly short-term-repopulating HSCs in vitro, as opposed to long-term-repopulating HSCs. Given their maintenance of embryonic globin expression, it is also likely that the Stat5-expanded cells are not undergoing a primitive-definitive hematopoietic switch, and that their inability to reconstitute adult hematopoiesis is reflective of their similarity to the earliest hematopoietic progenitors of the yolk sac, which suffer a similar defect in adult repopulation.

Although genetic modification can enable engraftment, it should in principle be possible to guide the differentiation of unmodified ES cells into definitive HSCs. To achieve this, it will be necessary to recapitulate, in a temporally appropriate manner, all of the extracellular signals that an ES cell and its lineage-restricted progeny experience from the point of blastocyst injection to the point of differentiation to fetal liver-stage HSC. This is a daunting task, not only because of the large number of known extracellular signaling molecules and combinatorial possibilities, but also because the relevant molecules may not be known at present. Given that many extracellular signaling molecules share common intracellular mediators, the endeavor may be simplified by focusing on these signal transducers. Focusing on individual mediators has the additional advantage of bypassing or deconvoluting the complexity of cell surface receptor-mediated signaling, in which multiple pathways are commonly activated by binding of a single ligand. For example, signaling by the leukemia inhibitory factor receptor promotes ES cell self-renewal through activation of Stat3 (39); however, it also activates the mitogen-activated protein kinases, ERK1 and ERK2, resulting a counteractive signal that attenuates self-renewal (40). By interfering with or activating individual pathways, unique outcomes can be selected from the manifold of effects initiated by receptor activation.

We chose to study the effects of Stat5 activation on the process of hematopoietic differentiation of ES cells, in part because Stat5 is a major downstream mediator of Bcr/Ab1 signaling, which we have previously shown induces the expansion of an

Fig. 5. FACS analysis of bone marrow of a doxycycline-treated recipient mouse. Bone marrow cells were harvested 1 month after transplantation, stained with the indicated antibodies, and subjected to flow cytometry. GFP fluorescence is plotted on the x axis; antibody staining is plotted on the y axis. “iso” represents an isotype control nonspecific antibody. The upper left plot is from a control, uninjected mouse; all others are from the experimental mouse. In each plot, the percentage of double-positive cells is shown in the upper right quadrant. Double positives represent donor cells expressing a given antigen.
embryonic hematopoietic stem cell population from EBs (38), and in part because of the critical role that Stat5 plays in normal hematopoiesis by acting to transduce signals from a variety of cytokine receptors. Our results demonstrate that at the point of origin of the hematopoietic lineage, day 4 to day 6 of EB differentiation, the precursors of this lineage are competent to respond to Stat5 activation. We have determined that the enhanced hematopoiesis driven by Stat5 is not the result of the rescue of hematopoietic precursors that were destined for apoptosis, nor is it the result of over proliferation of the hematopoietic compartment. An attractive possibility is that Stat5 modulates the rate of progression from stem cell to committed progenitor (CFC) to differentiated progeny within the early embryonic hematopoietic compartment such that stem cells and progenitors accumulate to greater numbers in the presence of signaling.

The fact that Stat5 null embryos generate hematopoietic tissue means that Stat5 is not essential for hematopoietic development (15). However, the fetal anemia seen in these embryos, which has been attributed to a defect in erythropoietin signaling (14) might also be due in part to reduction of the stem cell pool in the absence of Stat5. It is noteworthy that Stat5 functions in a partially redundant manner in the regulation of the definitive HSC: Stat5 null HSCs are capable of repopulating irradiated partially redundant manner in the regulation of the definitive hematopoiesis by acting to transduce signals from a variety of and in part because of the critical role that Stat5 plays in normal embryonic hematopoietic stem cell population from EBs (38), the Alberta Heritage Foundation for Medical Research, the Canadian Institutes of Health Research, the State of Sao Paulo Research Foundation (FAPESP), the MIT Biotechnology Process Engineering Center, and the Burroughs Welcome Fund. G.O.D. is the Birnbaum Scholar of the Leukemia and Lymphoma Society of America.

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