Differentiating Embryonal Stem Cells Are a Rich Source of Haemopoietic Gene Products and Suggest Erythroid Preconditioning of Primitive Haemopoietic Stem Cells

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The difficulties associated with studying molecular mechanisms important in haemopoietic stem cell (HSC) function such as the problems of purifying homogeneous stem cell populations, have prompted us to adapt the murine ES cell system as an in vitro model of HSC generation and function. We now report that careful analysis of the time course of HSC generation in differentiating ES cells allows them to be used as a source of known and novel haemopoietic gene products. We have generated a subtracted library using cDNA from ES cells collected just prior to and just following the emergence of HSCs. Analysis of this library shows it to be a rich source of known haemopoietic and haemopoietic related gene products with 44% of identifiable cDNAs falling into these camps. We have demonstrated the value of this system as a source of novel genes of relevance to HSC function by characterizing a novel membrane protein encoding cDNA that is preferentially expressed in primitive haemopoietic cells. Intriguingly, further analysis of the known components of the subtracted library is suggestive of erythroid preconditioning of the ES cell-derived HSC. We have used dot-blot and in situ analysis to indicate that this erythroid preconditioning is probably restricted to primitive but not definitive HSC.

The hemopoietic stem cell (HSC) occupies a pivotal position within the hemopoietic hierarchy and it is at this cellular level that all hemopoietic function is ultimately regulated (1). It is also at this level that dysfunctions involved in the pathogenesis of leukemias and myeloproliferative disorders frequently arise (2). For these reasons, it is clear that a more complete understanding of the molecular mechanisms regulating the generation and function of haemopoietic stem cells is central to our appreciation of physiological and pathological stem cell function. Unfortunately, a number of practical limitations associated with studies on adult HSC have precluded in depth analyses of genes of relevance to stem cell function. Foremost among these difficulties is the problem of purifying homogeneous populations of stem cells for molecular analysis. To alleviate the problems associated with studies on adult HSC, we have recently turned our attention to the murine in vitro embryonal stem (ES) cell system and have been attempting to develop this as a tractable model of stem cell generation and function that may be more amenable to in depth molecular analyses.

Embryonal stem cells are totipotent cells derived from the murine blastocyst at 3.5 days post-coitum and are maintained in an undifferentiated state by in vitro culture in the presence of the differentiation inhibiting agent leukemia inhibitory factor (LIF) (3–5). Upon removal of the LIF, ES cells when cultured as aggregates, or embryoid bodies (EBs) will spontaneously commit in vitro to a range of embryological tissues including epidermis (6), neuronal and glial cells (7), muscle cells (8), and most notably haemopoietic cells (9). Haemopoiesis in differentiated EBs is typically represented by regions of hae moglobinization which are referred to as blood islands and which are composed principally of erythrocytes with small numbers of macrophages (9). This haemopoietic cell generation occurs in the absence of exogenous growth factors, however, addition of appropriate haemopoietic growth factors or use of supportive stromal cells during the differentiation process can lead to the generation of progenitor cells for all haemopoietic lineages in the developed embryoid body (10–16). This therefore suggests that at some point between being an ES cell with no direct haemopoietic potential and being a developed EB with the capacity to contain progenitors for all of the haemopoietic lineages, some of the ES cells within an EB must commit to the haemopoietic system, i.e. they must become haemopoietic stem cells. Indeed a number of studies has demonstrated the presence of cells at

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1 The abbreviations used are: HSC, haemopoietic stem cell; ES, embryonal stem; EB, embryoid body; PCR, polymerase chain reaction; LIF, leukemia inhibitory factor.
various stages of hemopoietic development, from hemangio-
blasts to primitive long-term and short-term repopulating stem 
cells as well as committed progenitors of the myeloid and 
lymphoid lineages, in the developing EBs (12, 14, 16–18).

We have used a range of in vitro and in vivo assays to 
demonstrate a reproducible temporal pattern of emergence of 
long-term repopulating and short-term repopulating HSC in 
EBs post-LIF removal in vitro. Thus, while at day 3 post-
initiation of differentiation no HSCs are detectable in the de-
veloping EBs, by day 4 long-term repopulating stem cells, and 
by day 5 short-term repopulating stem cells are detectable (19).
This stem cell generation requires no exogenous growth factors 
and precedes the emergence of mature hemopoietic cells or 
blood islands. The reproducible time frame of HSC emergence 
in this in vitro system and the ease of generation of large 
numbers of EB-derived cells has prompted us to examine the 
usefulness of the day 3/day 5 time frame as a source of known 
and novel hemopoietic gene products. We now report the char-
acterization of a subtracted library generated using cDNA from 
short-day 3 and day 5 embryoid bodies. Our results suggest that of 
the identifiable cDNAs within the subtracted library, ~46% are 
predominantly associated with hemopoietic or hemopoietic 
supportive cells confirming the usefulness of the ES cell system 
as a source of known hemopoietic gene products. Characteriza-
tion of a novel membrane encoding cDNA from the subtracted 
library has further emphasized the value of this system as a 
source of novel genes of relevance to hemopoiesis. In addition, 
analysis of the hemopoietic genes identified in the subtracted 
library is indicative of erythroid pre-conditioning of the primi-
tive hemopoietic stem cell.

EXPERIMENTAL PROCEDURES

ES Cell Culture and Embryoid Body Formation and Differentia-
tion—The EFC-1 ES cell line was routinely passaged and maintained in 
an undifferentiated state as described previously (19, 20). EBs 
were generated by hanging drop culture of 10 μl of ES cells at a concentration 
of 3 × 10^6/ml in the presence of LIF in a humidified, 5% CO₂ 
atmosphere. After 2 days, the EBs were harvested into a Petri dish, washed 
with PBS, and resuspended in fresh medium. The EBs were plated onto 
Matrigel-coated 100-mm diameter dishes and maintained for an additional 
3 days. At days 3 and 5 post-differentiation initiation, EBs were harvested and the 
presence of HSCs determined using the CFU-A assay as previously de-
scribed (19). The remaining EBs were processed for mRNA and cDNA 
generation as described below.

Isolation of mRNA and Generation of cDNA from the Day 3 and Day 
5 EBs—Large scale differentiation of EBs was carried out on a number 
of occasions until high level CFU-A generation was achieved between 
days 3 and 5 post-initiation of differentiation. EBs from this experiment 
were harvested and mRNA produced using a Stratagene Messenger 
RNA isolation kit. Aliquots (5 μg) of the mRNA samples were used to 
generate day 3 and day 5 full-length cDNA libraries in ZAP Express 
(Stratagene) according to manufacturers instructions.

Subtractive Hybridization—Subtractive hybridization was 
performed according to the method of Wang and Brown (21) with 
the modification of Balzer and Baumlein (22) which involves the use of 
alternative linkers on the driver and tracer cDNA populations thus 
minimizing driver cDNA carry-over which may complicate the analysis of 
the subtracted library. Briefly this method involved the preparation of 
mRNA from day 3 and day 5 EBs which was then converted to 
cDNA using the Invitrogen Copy Kit. cDNA from the 
full-length cDNA libraries of day 3 and day 5 EBs—
were ligated onto either the digested day 3 EB cDNA (ab linker pairing) 
or the day 5 EB-digested cDNA (cd linker pairing). These linkers serve 
both as PCR primer recognition site for amplification of the cDNA 
populations but also as restriction enzyme sites for cleaving prior to 
cloning into sequencing vectors, Thus, and as underlined above, the ab 
linker pairing is cleavable with EcoRI and the cd pairing with XbaI.
The linker ligated on the shorter 5' of the two primer pairs as 
PCR primer. Driver cDNAs were biotinylated using the Photobiotin 
reagent supplied by Vector Laboratories according to manufacturers 
instructions.

The subtractive hybridization involved a combination of short and 
long hybridizations and a simultaneous generation of both day 5 and 
day 3 sequence-enriched libraries (see Ref. 21 for a detailed discussion 
of the subtractive strategy). For each, biotinylated driver and nonbioti-
nylated tracer were mixed at a 20:1 molar ratio and co-precipitated. 
The mixture was then resuspended in 20 μl of 10 mM Tris, 1 mM EDTA, pH 
8, and boiled for 3 min. This was then mixed with an equal volume of 
2 × hybridization buffer (1.5 M NaCl, 50 mM Hepes, 10 mM EDTA, 0.2% 
SDS, pH 7.5) overlaid with mineral oil and then boiled for a further 3 
min. The denatured cDNA sample was then 
allowed to hybridize at 68 °C for 2 h (short hybridization) or 20 h (long 
hybridization) following which 9 volumes of 10 mM Tris, 1 mM EDTA, 
ph 7.5, prewarmed to 55 °C was added and the tubes incubated at 55 °C 
for 5 min. The aqueous phase was then transferred to a fresh tube to 
which 20 μl of streptavidin at 2 μg/ml (in 0.15 M NaCl, 10 mM Hepes, 1 
mM EDTA, pH 7.6) was added and the mixture incubated at room 
temperature for 20 min. Protein and protein-DNA complexes were 
removed by phenol/chloroform extraction followed by four further 
streptavidin incubation and extractions. Finally the subtracted 
material was subjected to two more phenol/chloroform extractions and one 
extractions with chloroform.

After 6 rounds of short and long subtractions (21), cDNA fragments 
were cloned into Bluescript (pSK+) and random clones selected for 
sequence. Sequencing was performed on an Applied Biosystems au-
tomated sequencer.

Northern and Southern Blotting—For Northern blotting, 1.4% form-
aldehyde/agarose gels were run with 2 or 20 μg of mRNA or total RNA, 
respectively, and blotted according to standard protocols onto Hy-
bond-N. Similarly for Southern blots, agarose gels with 5 μg of cDNA 
were run and blotted onto Hybond-N. All probes were labeled 
by random priming using the Amersham Pharmacia Biotech “Ready to 
Go” labeling kit.

Isolation of the Full-length cDNA for JB542—The full-length JB542 
cDNA was isolated from the day 5 EB cDNA library by PCR using two 
primers internal to the JB542 sequence and the flanking T3 and T7 
sites in pBR-CMV which can be rescued for the Zap Express λ vector. 
The primers used were: 1) 5′-TCCCGAGCTCTGTCCGCTCGCTCT-
GGTCTGGGCC-3′; 2) 5′-GGCCCGGAGACGAGATAATGGCTGCTGGA-
AAG-3′. Following sequencing of the full-length cDNA, it was re-derived from 
murine FDPcMx RNA by reverse transcriptase-PCR using PFU 
Tag polymerase and three independent clones were analyzed to verify 
sequence.

FDPcMx Culture and Differentiation—The murine FDPcMx cell 
line was maintained and induced to differentiate essentially as de-
scribed previously (23). Briefly, cells were maintained in Fischers 
medium with 10% donor horse serum and interleukin-3 and subcultured 
every 3–4 days. Neutrophil differentiation was induced by addition of 
interleukin-3, granulocyte macrophage-colony stimulating factor, and 
granulocyte-colony stimulating factor and the cells allowed to develop 
for 7 days. Monocytic/macrophage differentiation was induced by cul-
turing cells in interleukin-3, granulocyte-macrophage-colony stimulat-
ing factor, and macrophage-colony stimulating factor for 10 days. 
Following completion of the differentiation program cells were harvested 
and total RNA prepared using Trizol. The success of the differentiation 
was confirmed by morphological analysis of the cells following cyto-
spinning and staining with May Grunwald and Giemsa (data not shown).

Generation of cDNA from Lineage Marker-depleted Hemopoietic 
Cells—For generation of lineage-depleted cells, murine bone marrow 
cells were depleted of lineage marker-bearing cells using cell surface 
markers specific for B cells (B220), T cells (CD4 and CD5), macro-
phages/monocytes (Mac1), and granulocytes (Gr1). The residual sub-
population of cells that were negative for the above lineage markers 
were therefore enriched for primitive hemopoietic cells. This population
is referred to in the text as the lin- population. mRNA was generated from bone marrow and the lin- population using the Invitrogen microfast track mRNA kit and cDNA produced using the Invitrogen copy kit. Each of the cDNA preparations were sheared with AluI and linkers were ligated on to allow PCR amplification of the cDNA populations. It is important to note that different linkers were used for each cDNA preparation to minimize the chances of carry-over or cross-contamination between the different cDNAs during PCR. The cDNAs were amplified by PCR and used in Southern blotting experiments to assess gene expression. Success of the sorting and cDNA generation exercise has been confirmed previously (24).

Generation of RNA from Lineage Restricted Hemopoietic Cells—Separately, individual lineages were enriched using specific anti-surface marker antibodies. The antibodies used were TER-119 for erythrocytes (PharMingen), CD41 for megakaryocytes (PharMingen), Gr-1 for granulocytes (Cambridge Bioscience), CD11b for monocytes and macrophages (PharMingen), and CD3 for T-lymphocytes (Cambridge Biosciences). Cells were isolated using Dynabeads according to the manufacturers instructions, the cells washed, lysed, and RNA prepared using Trizol. JB542 expression was analyzed by PCR using the following primers which are expected to yield a product of 386 base pairs:

\[ 5'-CACCCTGATCCCGTGAGG-3' \]

and

\[ 5'-CCCTGATCACTTGGTG-3' \]

PCR was carried out for 30 cycles as described previously (24).

EB in Situ Hybridization—Procedures for in situ hybridization of EB sections were derived from hybridization protocols for mouse embryo sections (25, 26) and from whole mount in situ hybridization procedures on Ebs. Briefly, EBs allowed to differentiate in vitro for various periods of time were fixed in 4% paraformaldehyde, embedded into paraffin wax, and cut into 7-μm sections. Hybridization conditions were as described (25). Single-stranded riboprobes for a-globin were synthesized as run-off transcripts from linearized plasmid templates under standard conditions, essentially as described (27). Signal was visualized with alkaline phosphatase-conjugated anti-DIG antibodies and NBT/BCIP color substrate.

RESULTS

Generation of a Day 3/Day 5 Subtracted Library—To investigate the usefulness of differentiating ES cells as a source of known and novel hemopoietic gene products we have performed subtractive hybridization using cDNA populations obtained from day 3 and day 5 embryoid bodies. We have used intact embryoid bodies as our cDNA source in an attempt to ensure inclusion of both hemopoietic and stromal/supportive cell gene products in the subtracted library. Prior to the initiation of the subtractive hybridization, the successful generation of HSCs within the EBs was confirmed by measurement of “transiently engrafting” CFU-A stem cell activity in the day 5 EBs as described previously (19). Subtractive hybridization was carried out using the “Gene Expression Screen” method of Wang and Brown (21) as described under “Experimental Procedures.” The success of the subtractive hybridization is shown in Fig. 1a which demonstrates effective removal of common β-actin sequences after 4 rounds of subtraction and substantial up-regulation of hemopoietic specific a-globin gene sequences over the six rounds of subtraction. Slot-blot analysis (Fig. 1b) of a number of cDNAs from the subtracted library has demonstrated only low numbers (less than 10%) of non-day 3/day 5 differentially expressed cDNAs, thus further confirming the success and completeness of the subtraction. Also shown in Fig. 1b is differential expression of the primitive hemopoietic markers Scl (28, 29) and CD34 (30) confirming the usefulness of the day 3/day 5 time frame as a source of known genes of importance to hemopoietic stem cell generation and function.

Differentiating ES Cells Are a Rich Source of Known and Novel Hemopoietic Gene Products—We have sequenced 474 cDNAs from this subtracted library and data base searching has demonstrated 132 of these sequences to be identical to cDNAs already deposited in the non-EST data bases. Of these 132 cDNAs, which are representative of 80 discrete gene products, 61 (representative of 31 discrete gene products) are known to be primarily expressed in, and to function in, hemopoietic or hemopoietic supportive cells (Table I). Among the hemopoietic cell-associated sequences is a number characteristic of primitive hemopoietic cells and these include the transcription factors Ikaros (31) and GFI-1b (32), the surface markers CD34 (30), PECAM (33), and the integrins α4 and β3 (34) and the primitive cell associated GDP dissociation inhibitor (35). In addition, the transcription factor Tef identified in the library has a known role in HSC migration during development and may functionally relate to the CXCR4 chemokine receptor in this context (36, 37). The presence of these cDNAs in the subtracted library, along with the demonstration of differential expression of CD34 and Scl (Fig. 1b) further confirms the usefulness of the ES cell differentiation system as a source of known genes of importance to primitive hemopoietic cell function. The hemopoietic supportive cell-associated cDNAs include a number of sequences associated with bone marrow stromal cells such as the integrin ligands collagen, fibronectin, and laminin (34), endothelial cell-associated sequences such as VE-cadherin (38) and angiopoietin (39), and the adipocytic cDNAs Paf-1 and apolipoprotein-B. The remaining 71 (46 discrete gene products) identifiable sequences show no clear commonality in their tissue affiliations and appear to have little in common in either their known functions or expression patterns (Table II). Thus this preliminary sequencing data suggests that ~46% of identifiable cDNAs in the subtractive library are of relevance to the process of hemopoiesis with 38% being of hemopoietic cell origin and 8% being derived from presumed hemopoietic supportive cells. The fact that there is no other major tissue-

\[ \text{FIG. 1. Confirmation of the success of the subtractive hybridization.} \]

\[ a, \text{Southern blots of cDNA from alternate stages of the subtractive hybridization showing actin suppression and a-globin enrichment over the six rounds of subtraction.} \]

\[ b, \text{slot-blot analysis of day 3/5 differential expression of unknown and known cDNAs in the subtracted library.} \]

\[ \text{These blots were probed with clone specific probes labeled by random priming.} \]
specific gene expression represented in the library indicates that hemopoiesis is the predominant developmental process occurring in the EBs between days 3 and 5 of differentiation.

In addition to the known genes outlined in Tables I and II there are 78 ESTs for which no expression or functional data is yet available (Table III) and 264 sequences that currently have no counterparts in any publicly available data base. Of these 342 ESTs or unidentifiable sequences, it is likely, given the occurrence in the EBs between days 3 and 5 of differentiation.

TABLE I

List of known haemopoietic and haemopoietic related gene products in the subtracted library (for this and subsequent tables, numbers in brackets represent numbers of repeats of the individual sequences)

| Clone No. | Accession No. | Description |
|-----------|---------------|-------------|
| Haemopoietic |               |             |
| JB536     | NM_001773     | Murine CD34 |
| JB287     | L03547        | Murine Ikaros transcription factor |
| JB311     | AF017275      | Murine Gfi-1B transcription factor (2) |
| JB340     | L07916        | Murine GDP dissociation inhibitor (5) |
| JB38      | F19617        | Murine Elf-1 transcription factor |
| JB524     | Y07915        | Murine TEL transcription factor |
| JB218     | P06802        | Plasma cell membrane glycoprotein (PC-1) (4) |
| JB192     | P03979        | TCR γ-chain V region PT-gamma-½ precursor |
| JB140     | X07399        | Murine Procathepsin E |
| JB245     | L38933        | Murine erythrocyte membrane protein 4.2 (3) |
| JB110     | X78709        | Murine NRF1 transcription factor |
| JB71      | P04919        | Band 3 anion exchange protein (MEB3) |
| JB50      | X64594        | 50-kDa Erythrocyte plasma membrane glycoprotein |
| JB37      | NM_008816     | Murine PECAM |
| JB48      | M13268        | Murine β-aminolevulinic acid synthetase (2) |
| JB6       | S76831        | Murine tropomodulin (4) |
| JB442     | S23537        | Murine erythroid transcription factor NF-E2 (3) |
| JB40      | M26897        | Murine e-globin (4) |
| JB29      | M26894        | Murine β-H1-globin (8) |
| JB21      | S71555        | Murine β-globin (2) |
| JB532     | AF078905      | Murine α-4 integrin |
| JB195     | U34627        | Murine integrin β-3 subunit |
| JB534     | AF026509      | Murine CD34 |

Haemopoietic related

| Clone No. | Accession No. | Description |
|-----------|---------------|-------------|
| JB16      | M14243        | Murine pro-α1 type I collagen (3) |
| JB31      | M85212        | Murine tartrate resistant acid phosphatase |
| JB65      | Q09163        | Preadipocyte factor-1 |
| JB70      | M14081        | Apolipoprotein B |
| JB107     | X83590        | Murine VE-cadherin |
| JB226     | U43541        | Murine laminin β-2 (2) |
| JB342     | U82612        | Fibronectin |
| JB535     | AF125176      | Murine angiopoietin related |

450-base pair cDNA encoding a 134-amino acid peptide with a calculated molecular mass of 14,667 daltons. Interestingly, while this cDNA incorporates the full-length coding sequence for JB542, the size of the primary transcript for JB542 in tissues blots (see Fig. 3) is ~2.1 kilobases suggesting the presence of extensive 5’- and 3’-untranslated sequences. As mentioned, data base searching revealed similarities with members of an interferon inducible gene family typified by the 9-27/leu 13 (40) and 1-8D (41) proteins (Fig. 2) the roles for which remain to be elucidated. The highest levels of similarity is with a membrane protein of undetermined function from the marmal electric ray, Torpedo marmorata (42). EST data base searching with this full-length cDNA also reveals a close human homologue encoded by an embryo-derived EST (accession number AA463818) which displays ~81% identity with the murine protein (Fig. 2b). This human JB542 sequence is incorporated within a genomic sequence from chromosome 11q15 (accession number AF015416). The predicted murine protein is highly charged with acidic and basic residues accounting for 20% of the total sequence. The overall charge is +3. To investigate the likely orientation of the JB542 protein within the membrane we have performed Kyte-Doolittle analysis which reveals (data not shown) a potential transmembrane region, extending approximately from amino acids 40 to 60. There is a further potential transmembrane region predicted between amino acids 90 and 110 and the determination of the precise orientation of JB542 within the cell membrane awaits epitope tagging studies which are underway in our laboratory.

To attempt to implicate JB542 in hemopoietic cell function we have examined its expression in bone marrow, spleen, and a range of other tissues. Results from such tissue blot analysis demonstrates that expression of JB542 is predominantly seen in the brain and bone marrow with lower level expression in
testes and skeletal muscle (Fig. 3a). To further examine the expression of JB542 within the hematopoietic system and attempt to investigate any primitive cell-restricted expression patterns, we have adopted two cellular models. First, we have examined expression in the primitive murine hematopoietic cell line, FDCPmix, which displays many phenotypic similarities to murine transiently engrafting stem cells (23, 43). These cells can self-renew under the proliferative stimulus of interleukin 3 and can be induced to differentiate along a range of hematopoietic lineages following treatment with appropriate growth factors. For the purposes of the present study we have produced RNA from parental FDCPmix cells and from these cells following exposure to and differentiation down the macrophage or T cell lineages. These data therefore confirm the primitive cell expression further, we have separated hematopoietic cells into their component lineages using immunomagnetic techniques and have assessed JB542 expression in these sorted cell populations. As shown in Fig. 4, PCR again reveals preferential expression in lineage negative cells compared with lineage positive cells. The expression in lineage positive cells appears to be accountable for by maintained expression in erythrocytes, megakaryocytes, and granulocytes. In contrast expression is lost following commitment to and differentiation down the macrophage or T cell lineages. These data therefore confirm the primitive cell expression of JB542 but suggests that its down-regulation is lineage dependent.

Thus analysis of the expression patterns of JB542, a novel cDNA identified within the ES cell subtracted library has demonstrated its preferential brain and hematopoietic expression patterns and furthermore, has revealed that the hematopoietic expression is preferentially seen in primitive cell types. This identification of this novel hematopoietic cDNA confirms the

| Clone No. | Accession No. | Description |
|-----------|---------------|-------------|
| JB1       | U75215        | Mouse neutral amino acid transporter |
| JB2       | AJ006278      | Acetylglucosaminyltransferase-like protein (2) |
| JB3       | U24674        | Mouse Mxi1 |
| JB22      | D67076        | Mouse ADAMTS-1 |
| JB24      | X50561        | Prealbumin (2) |
| JB27      | U09816        | G$_{ML}$ activator protein |
| JB53      | Y12582        | Mouse calpain-like protease |
| JB69      | X95825        | Mono-ADP ribosyltransferase |
| JB76      | D49914        | Mouse seryl t-RNA synthase |
| JB112     | L25538        | Mouse folate-binding protein |
| JB113     | X52634        | tilm oncone |
| JB123     | L05781        | Cytosolic epoxide hydrolase |
| JB147     | X07201        | H19 gene (2) |
| JB153     | X84301        | MLE transposase (2) |
| JB157     | P54729        | BS4 protein |
| JB181     | X98330        | Cardiac ryanodin receptor |
| JB330     | U18869        | Mouse mitogen responsive phosphoprotein p96 |
| JB188     | J04970        | Carboxypeptidase M (2) |
| JB201     | U49968        | Lipoma preferred partner |
| JB209     | AB004109      | Phosphatidylinerase synthase |
| JB211     | U29726        | MAP kinase |
| JB215     | M80783        | Human B12 protein (TNFα induced) |
| JB227     | U80040        | Human nuclear Aconitase |
| JB240     | L09159        | Selenium-dependent/RHOA proto-oncogene (5) |
| JB256     | AB094231      | Selenium-dependent glutathione peroxidase (5) |
| JB274     | A5587         | Human TNF type-1 receptor associated protein (2) |
| JB279     | U53586        | Mouse Evi-5 |
| JB283     | D96675        | Human mRNA for A-U rich element-binding protein |
| JB289     | AF094211      | Mouse Ets homeodomain protein |
| JB326     | PM94279       | Mouse spindle pole body protein, spe97 homolog |
| JB331     | U48830        | Mouse subtilisin-like proprotein convertase-7 |
| JB349     | X84896        | Mouse ATP receptor (P2X gene) (12) |
| JB394     | U49393        | Mouse sarcoplasmic reticulum Calcium ATPase, SERCA 3b |
| JB399     | D10727        | Mouse NDPP-1 protein |
| JB422     | X57971        | Mouse gap junction gene, connexin 37 |
| JB436     | U24493        | Mouse tryptophan 2,3-dioxygenase |
| JB476     | Q13618        | Human Cullin homology 3 (Cul-3) |
| JB485     | U20238        | Mouse GTPase activating protein (GAP11) |
| JB488     | X99807        | Mouse selenoprotein P |
| JB495     | L25602        | Mouse bone morphogenetic protein 2 (BMP-2) |
| JB531     | M55154        | Mouse transglutaminase |
| JB532     | AF231120      | Murine iron-regulated transporter IREG1 |
| JB539     | M27073        | Murine protein phosphatase type 1 |
| JB540     | AP978776      | Human p38-binding protein |
| JB391     | NM008093      | Murine GATA-5 transcription factor |

**TABLE II**

List of known non-haemopoietic gene products in the subtracted library

| Clone No. | Accession No. | Description |
|-----------|---------------|-------------|
| JB1       | U75215        | Mouse neutral amino acid transporter |
| JB2       | AJ006278      | Acetylglucosaminyltransferase-like protein (2) |
| JB3       | U24674        | Mouse Mxi1 |
| JB22      | D67076        | Mouse ADAMTS-1 |
| JB24      | X50561        | Prealbumin (2) |
| JB27      | U09816        | G$_{ML}$ activator protein |
| JB53      | Y12582        | Mouse calpain-like protease |
| JB69      | X95825        | Mono-ADP ribosyltransferase |
| JB76      | D49914        | Mouse seryl t-RNA synthase |
| JB112     | L25538        | Mouse folate-binding protein |
| JB113     | X52634        | tilm oncone |
| JB123     | L05781        | Cytosolic epoxide hydrolase |
| JB147     | X07201        | H19 gene (2) |
| JB153     | X84301        | MLE transposase (2) |
| JB157     | P54729        | BS4 protein |
| JB181     | X98330        | Cardiac ryanodin receptor |
| JB330     | U18869        | Mouse mitogen responsive phosphoprotein p96 |
| JB188     | J04970        | Carboxypeptidase M (2) |
| JB201     | U49968        | Lipoma preferred partner |
| JB209     | AB004109      | Phosphatidylinerase synthase |
| JB211     | U29726        | MAP kinase |
| JB215     | M80783        | Human B12 protein (TNFα induced) |
| JB227     | U80040        | Human nuclear Aconitase |
| JB240     | L09159        | Selenium-dependent/RHOA proto-oncogene (5) |
| JB256     | AB094231      | Selenium-dependent glutathione peroxidase (5) |
| JB274     | A5587         | Human TNF type-1 receptor associated protein (2) |
| JB279     | U53586        | Mouse Evi-5 |
| JB283     | D96675        | Human mRNA for A-U rich element-binding protein |
| JB289     | AF094211      | Mouse Ets homeodomain protein |
| JB326     | PM94279       | Mouse spindle pole body protein, spe97 homolog |
| JB331     | U48830        | Mouse subtilisin-like proprotein convertase-7 |
| JB349     | X84896        | Mouse ATP receptor (P2X gene) (12) |
| JB394     | U49393        | Mouse sarcoplasmic reticulum Calcium ATPase, SERCA 3b |
| JB399     | D10727        | Mouse NDPP-1 protein |
| JB422     | X57971        | Mouse gap junction gene, connexin 37 |
| JB436     | U24493        | Mouse tryptophan 2,3-dioxygenase |
| JB476     | Q13618        | Human Cullin homology 3 (Cul-3) |
| JB485     | U20238        | Mouse GTPase activating protein (GAP11) |
| JB488     | X99807        | Mouse selenoprotein P |
| JB495     | L25602        | Mouse bone morphogenetic protein 2 (BMP-2) |
| JB531     | M55154        | Mouse transglutaminase |
| JB532     | AF231120      | Murine iron-regulated transporter IREG1 |
| JB539     | M27073        | Murine protein phosphatase type 1 |
| JB540     | AP978776      | Human p38-binding protein |
| JB391     | NM008093      | Murine GATA-5 transcription factor |
usefulness of the in vitro ES cell system as a valuable source of novel genes of relevance to primitive hematopoietic cell function.

Analysis of the ES Cell Subtracted Library Reveals Preferential Expression of Erythroid Lineage Gene Products—As mentioned above, there is a number of primitive cell-restricted cDNA sequences identified in the subtracted library confirming its value as a source of cDNAs of relevance to immature hematopoietic cell generation and function. In keeping with the previously reported absence of mature cells or lineage committed progenitors from the EBs at day 5 (19), there are no cDNA markers of mature myeloid cells and only two markers of the lymphoid lineages (T cell receptor γ and plasma cell membrane glycoprotein). Curiously, however, a large cohort of the identifiable cDNAs is representative of genes that are not characteristic of primitive hematopoietic cells but are more typically associated with maturing and mature erythrocytes. These erythroid genes include the transcription factors NRF1 (44) and NFE2 (45), the enzymes 5-ALAS (46) and procathepsin E (47), the surface markers (see Ref. 48 for a review of erythrocyte surface markers) erythrocyte membrane protein 4.2 (49), the tropomodulin structural gene (52) as well as multiple globin sequences. We have previously reported absence of erythroid progenitor cells and markers of mature myeloid cells and only two markers of the lymphoid lineages (T cell receptor γ and plasma cell membrane glycoprotein). Curiously, however, a large cohort of the identifiable cDNAs is representative of genes that are not characteristic of primitive hematopoietic cells but are more typically associated with maturing and mature erythrocytes. These erythroid genes include the transcription factors NRF1 (44) and NFE2 (45), the enzymes 5-ALAS (46) and procathepsin E (47), the surface markers (see Ref. 48 for a review of erythrocyte surface markers) erythrocyte membrane protein 4.2 (49), 50-kDa plasma membrane glycoprotein (50), and Band-3 anion exchange protein (51), the tropomodulin structural gene (52) as well as multiple globin sequences. We have previously demonstrated the absence of erythroid progenitor cells and markers of mature erythropoiesis in the day 5 EBs and have used benzidine staining to demonstrate that mature, benzidine-positive erythrocytes do not emerge in the EB differentiation system until day 8. The absence of evidence of maturing and mature erythrocyte cells in the EBs at day 5 has led us to tentatively conclude that while a multigenic program is evident in the differentiating ES cells, the relative wealth of erythroid genes is suggestive of erythroid lineage preconditioning of the ES cell-derived hematopoietic stem cell. It remains possible that this erythroid lineage gene expression is indicative of emergence of small numbers of committed erythroid cells in the EBs at day 5 that may be hard to detect using conventional bioassays. However, Northern blot (data not shown) and in situ (Fig. 5) analyses of α-globin expression in the developed EBs has revealed expression at time points even earlier than day 5. Indeed Fig. 5 shows that α-globin species are readily detectable in day 4 EBs and weakly in day 3 EBs. In our hands these time points are prior to the emergence of transiently engrafting stem cells or any identifiable committed progenitors. Indeed day 3, at which time low levels of globin expression is detectable, precedes the emergence of long-term repopulating stem cells in the ES cell system (19) and is more in keeping with the time of emergence of hemangioblasts (17).

In summary these data are consistent with the initiation of erythroid gene expression coincident with the emergence of the earliest detectable hematopoietic stem cells in the ES cell in vitro differentiation system. It is our contention that these data supports a model of erythroid preconditioning of the hematopoietic stem cell in the developing embryo.

Adul, Definitive HSCs Are Not Exclusively Preconditioned to Erythropoiesis—It is possible that this apparent erythroid preconditioning is a feature of primitive embryonic type HSCs and may therefore not be shared by adult definitive HSCs which have previously been reported to display a multigenic program of gene expression (53). To examine this issue we have arrayed man of the erythroid and primitive cell genes identified in the ES cell subtracted library (columns 1 and 2 and 3 and 4 of Fig. 6a, respectively) as well as genes that are more typically representative of differentiating and differentiated myeloid cells (columns 5 and 6 of Fig. 6a). We have used these arrays to examine gene expression in FDCPmix cells and lin− primary hematopoietic cells with a view to assessing the similarities in the multigenic gene expression pattern between these adult cells sources and the ES cell-derived HSC. RNA was prepared from FDCPmix cells, converted to cDNA, labeled, and used to probe the dot blots outlined on Fig. 6a. The results shown in Fig. 6b reveal that in the parental FDCPmix cell line, in contrast to the ES cell-derived hematopoietic cells, a multigenic program of gene expression is evident with as expected, a good representation of stem cell-associated gene expression along with expression of a number of erythroid and myeloid genes but with no clear predisposition to erythroid gene expression. A similar multigenic expression pattern without evidence of erythroid preconditioning is seen in primary murine lineage-negative cell populations (Fig. 6c) further indicating that, in contrast to the ES cell-derived HSCs, adult HSCs either from primary sources or as a self-renewing cell line, do not display significant erythroid preconditioning but instead exhibit a mul-

| Clone No. | Accession No. | Clone No. | Accession No. | Clone No. | Accession No. |
|----------|---------------|----------|---------------|----------|---------------|
| JB10     | AA200396      | JB11     | AA620185      | JB365    | AA671643      |
| JB13     | AA063843      | JB12     | AB002388      | JB408    | AA566848(2)   |
| JB14     | AA103447      | JB129    | AA409858(2)   | JB433    | AF037264      |
| JB19     | A273417       | JB136    | AA079440      | JB440    | AA08753       |
| JB20     | A557474*      | JB137    | AA496394      | JB452    | AA124089      |
| JB21     | AA179296      | JB138    | AA118441      | JB462    | AA26596       |
| JB30     | AA260787      | JB149    | AA402272*     | JB470    | AA959838      |
| JB32     | AA111447      | JB174    | Z53720*       | JB477    | AA56646       |
| JB33     | AA211856      | JB182    | AA516655      | JB501    | D98077        |
| JB34     | AA166323       | JB183   | AA638356      | JB516    | AA008578      |
| JB37     | AA553221      | JB193    | C81256        | JB517    | AV382276      |
| JB41     | AA075665*     | JB205    | AA412981(3)   | JB518    | AW495347      |
| JB42     | H53148        | JB207    | AA385214      | JB519    | A548540       |
| JB58     | AA591032      | JB210    | T32446*       | JB520    | AA387113      |
| JB59     | AA197359       | JB213    | AA451978*     | JB521    | AF276882      |
| JB63     | AA185198      | JB235    | AA555722      | JB125    | D80000        |
| JB64     | AA407284      | JB247    | AA171007      | JB128    | AB002368      |
| JB66     | WS9213        | JB254    | AA245632      | JB236    | D57448        |
| JB72     | AA129208       | JB290    | AA109999      | JB244    | D57448        |
| JB74     | H17599*        | JB299    | W13517        | JB309    | Q14999        |
| JB75     | AA253285*     | JB308    | AA681812      | JB337    | Q87743        |
| JB77     | AA125052       | JB312    | AA771001      | JB460    | AB002334      |
| JB84     | AA240945       | JB360    | AA537736      | JB522    | AB032974      |
| JB94     | AA065822       | JB363    | AA155047(2)   | JB526    | D86970        |
| JB527    | AV37824        |          |           |          |               |
tigenic expression program. These data is therefore in agreement with that of Hu et al. (53) who have used PCR to demonstrate a similar multigenic program in FDCPmix and sorted primary stem cells (53). The FDCPmix gene expression pattern changes on differentiation along the neutrophilic lineage with a loss of erythroid and stem cell gene expression and a concomitant increase in expression of some myeloid genes, most notably lysozyme (data not shown). These data suggests that the apparent erythroid specific preconditioning of the ES cell-derived stem cells may be an indication of their primitive rather than definitive nature and that definitive stem cells, either as a homogenous cell line or as an enriched population of lineage-negative cells display a more multigenic gene expression pattern. Further evidence pointing to the primitive nature of the EB-derived hemopoiesis is the predominance of fetal, or primitive, α- and β-globin species (Ref. 54 and Table I). These results therefore suggest that erythroid gene expression detected at the earliest time of emergence of primitive hemopoietic cells in the in vitro ES cell system is indicative of erythroid preconditioning of the primitive hemopoietic stem cell and may go some way to explaining the rapidity of mature primitive erythrocyte generation from primitive HSC and the apparent preference for erythroid differentiation in nongrowth factor-treated ES cells (9, 55).

**DISCUSSION**

The practical limitations inherent in studies using adult bone marrow stem cells have precluded directed analysis of the gene products involved in regulating stem cell production and function. For this reason we have turned our attention to the ES cell system as an in vitro model of developmental hemopoiesis. Other studies have also capitalized on the usefulness of this in vitro differentiation system to identify novel protein kinase cDNAs (56, 57) and to characterize a number of hemopoietic and developmental genes (58). We now report that precise definition of the time frame of emergence of primitive hemopoietic stem cells in the EBs (19) has allowed us to use this in vitro differentiation system to generate subtracted...
cDNA libraries that are abundant sources of both known and novel hemopoietic and related gene products. Our analysis of 474 sequences from the subtracted library suggests that approximately 46% of identifiable sequences are likely to be of relevance to the processes of hemopoiesis. The hemopoietically derived known genes include a number of markers of primitive cells such as CD34 (30) and additionally we have shown differential expression of SCL (28, 29), a transcription factor known to function at a most fundamental level in hemopoietic development (59, 60). Intriguingly, despite the absence of detectable mature hemopoietic cells in the EBs, a large number of the hemopoietic gene products differentially expressed in concert with the emergence of stem cells in the EBs are more typically associated with mature elements of the erythroid lineage. We have interpreted this as indicating that, within the multigenic program of the differentiating EBs, there is evidence of an erythroid preconditioning of the primitive cells generated in the EBs. It remains possible that small numbers of committed erythroid progenitors are present in the EBs at day 5, however, if this was the case these would be the only lineage committed cells present in the EBs and would again argue for some predisposition to erythroid development. We have used in situ hybridization and Northern blotting to demonstrate that globin gene sequences are detectable at day 4 and even as early as day 3 although only weakly. These time points precede the emergence of transiently engrafting stem cells in the in vitro differentiation system arguing for expression of globin in relatively uncommitted cells perhaps even hemangioblasts (17). This erythroid preconditioning appears to be a hallmark of primitive hemopoietic cells as both analysis of gene expression patterns in immature adult hemopoietic cells and also assessment of the precise nature of the globin species identified within the subtracted cDNA population attests to the primitive nature of the erythropoiesis being detected. It is intriguing that during development, primitive erythrocytes appear very rapidly following appearance of the first primitive HSC within the yolk sac (55) and in differentiating ES cells is the preferred hemopoietic lineage for terminal differentiation. This preconditioning seen in the EB-derived primitive HSC may help to account for this phenomenon, i.e. that the stem cells are generated with a specific predisposition for the erythroid lineage. Why should this pre-conditioning be lost in the adult HSCs? It is possible that the reason for the apparent pre-conditioning in the EBs and the earliest embryonic HSC is that they see no other growth factors at these early developmental stages and thus are free to spontaneously commit to their preordained lineage. In the later embryo and in the adult, the emergence of growth factor expression may subvert this preconditioning and allow a more multigenic program to be activated within the HSC. This phenomenon, i.e. that the stem cells are generated with a specific predisposition for the erythroid lineage, why should this pre-conditioning be lost in the adult HSCs? It is possible that the reason for the apparent pre-conditioning in the EBs and the earliest embryonic HSC is that they see no other growth factors at these early developmental stages and thus are free to spontaneously commit to their preordained lineage.

FIG. 3. JB542 is expressed in hemopoietic tissues with preferential expression in immature cells. a, expression of JB542 in murine tissues: RNA from the tissues shown was prepared and 20 μg of each run on a denaturing agarose gel. This gel was then blotted and the blot probed with JB542-specific probes. As indicated normalization was by comparison with the ethidium bromide stained 18 S ribosomal RNA band. b, expression of JB542 in primitive hemopoietic cells. FDCPmix cells were grown and allowed to differentiate along either the granulocytic or monocytic pathways. Total RNA was prepared and Northern blotted in an attempt to detect JB542 in parental or differentiated FDCPmix cells. c, also JB542 expression was examined in sorted primitive (lineage negative) murine hemopoietic cells. cDNA was prepared from lineage negative cells and from bone marrow and this was run out on an agarose gel and Southern blotted. Both the Northern and Southern blots in this figure were probed with random primed JB542 sequences.

FIG. 4. Examination of JB542 hemopoietic expression. Murine bone marrow cells were sorted into individual lineages and into lineage-negative (lin−) and lineage-positive (lin+) cell populations using immunomagnetic separation techniques. RNA was prepared from these cell populations and expression of JB542 assessed by PCR. PCR was allowed to run for 30 cycles following amplification. A digoxigenin-labeled probe (37) was hybridized and then transcribed and then used to detect JB542 expression by a colorimetric assay. Sections were sectioned and probed for α-globin expression using an in vitro transcribed digoxigenin-labeled probe.

FIG. 5. a-Globin is detected at the earliest time points of hemopoietic commitment in developing EBs. EBs at days 2, 3, 4, 5, 6, and 8 were sectioned and probed for α-globin expression using an in vitro transcribed digoxigenin-labeled probe.
likely that while limited numbers of HSC are detected by in vitro assay, hemopoietic gene expression is more widespread within the EB and a number of cells are expressing hemopoietic genes. It may be that only a few of the cells express the appropriate cohort of genes to allow them to be fully functional stem cells although further more rigorous analysis of EB HSC numbers is required before definitive conclusions can be reached.

We have also demonstrated the ES cell system to be a valuable source of novel hemopoietic genes and describe the cloning and characterization of JB542, a novel hemopoietic cell surface protein with expression patterns indicative of primitive hemopoietic cell expression. Embryologically this transcript is detectable by PCR between days 9.5 and 11.5 but the levels are to low to precise in situ localization (data not shown). A JB542 human homologue with 81% identity to the murine protein is also described and resides on human chromosome 11. We are currently raising antibodies to JB542 with a view to examining the specific clonogenic nature of the cells expressing this cell surface protein.

In conclusion, therefore, we have demonstrated the murine ES cell system to be a very valuable source of both known and novel hemopoietic and hemopoietic related genes. Furthermore analysis of known genes is suggestive of erythroid preconditioning of primitive hemopoietic stem cells.

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FIG. 6. Dot-blot analysis of erythroid, stem cell, and myeloid cell cDNA expression reveals a multigenic pattern of gene expression in FDCPmix and lineage negative primary cells. a, template showing the layout of the dot blots. Each dot has 100 ng of cDNA per spot. cDNAs are abbreviated as outlined in the text. Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are included as loading controls with actin being loaded at 50, 5, and 0.5 ng/spot and glyceraldehyde-3-phosphate dehydrogenase at 100, 10, and 1 ng/spot. Thus the differential intensity of the actin and glyceraldehyde-3-phosphate dehydrogenase spots correlates with these different loadings. B, dot-plot probed with radiolabeled total cDNA from FDCPmix cells. c, dot-plot probed with radiolabeled total cDNA from sorted lineage negative primary murine hemopoietic cells.
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