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DEVELOPMENT AND DISEASE

Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation

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SUMMARY

The hematopoietic and endothelial lineages derive from mesoderm and are thought to develop through the maturation of a common progenitor, the hemangioblast. To investigate the developmental processes that regulate mesoderm induction and specification to the hemangioblast, we generated an embryonic stem cell line with the green fluorescent protein (GFP) targeted to the mesodermal gene, brachyury. After the in vitro differentiation of these embryonic stem cells to embryoid bodies, developing mesodermal progenitors could be separated from those with neuroectoderm potential based on GFP expression. Co-expression of GFP with the receptor tyrosine kinase *Flk1* revealed the emergence of three distinct cell populations, GFP$^-$Flk1$^-$, GFP$^+$Flk1$^-$ and GFP$^+$Flk1$^+$ cells, which represent a developmental progression ranging from pre-mesoderm to pre-hemangioblast mesoderm to the hemangioblast.

Key words: ES cell, Brachyury, Mesoderm, Hemangioblast

INTRODUCTION

The first hematopoietic and endothelial precursors arise from extra-embryonic mesoderm and differentiate to form the blood islands in the yolk sac of the early embryo (Moore and Metcalf, 1970). The close spatial and temporal development of these lineages within the blood islands provided the basis for the hypothesis that they arise from a common progenitor, a cell known as the hemangioblast (Sabin, 1920; Wagner, 1980). Indirect evidence in support of the concept of the hemangioblast has come from studies demonstrating that the hematopoietic and endothelial lineages express a large number of different genes in common and that some of these genes are essential for both blood cell and vascular development (Anagnostou et al., 1994; Asahara et al., 1997; Fina et al., 1990; Kabrun et al., 1997; Kallianpur et al., 1994; Millauer et al., 1993; Porcher et al., 1996; Robb et al., 1995; Shalaby et al., 1995; Yamaguchi et al., 1993; Young et al., 1995).

Direct demonstration for the existence of a progenitor with hemangioblast properties has been provided by experiments using a model system based on the in vitro differentiation potential of embryonic stem (ES) cells (Choi et al., 1998; Nishikawa et al., 1998). Following the initiation of differentiation in culture, ES cells will form colonies known as embryoid bodies (EBs), that generate hematopoietic and endothelial progeny in a temporal pattern recapitulating the development of these populations in the yolk sac (Keller et al., 1993; Palis et al., 1999; Vittet et al., 1996). Analysis of early EBs, prior to the hematopoietic and endothelial commitment stages, revealed the presence of a progenitor with hemangioblast potential. In response to VEGF, these progenitors generate blast colonies that display both hematopoietic and endothelial potential (Choi et al., 1998). Kinetic studies demonstrated that this progenitor or blast colony-forming cell (BL-CFC) represents a transient population that is present within the EBs for approximately 36 hours, between day 2.5 and 4 of differentiation, preceding the onset of primitive erythropoiesis. The characteristics of the BL-CFC, namely its early development and its potential to generate primitive and/or definitive hematopoietic as well as endothelial progeny, suggests that it represents the in vitro equivalent of the yolk sac hemangioblast. More recent studies have shown that most BL-CFC express Flk1 (VEGF receptor 2; Kdr – Mouse Genome Informatics)) and that a subpopulation of Flk1$^+$ cells also expresses the transcription factor Scl (Chung et al., 2002; Faloon et al., 2000). The early development of the BL-CFC, prior to hematopoietic commitment, suggests that it could be a direct descendent of a mesodermal progenitor, or possibly a subpopulation of mesoderm. In the ES/EB model system, mesoderm, as defined by expression of the T-box gene brachyury, is induced within 48 hours of the onset of differentiation and persists until day 4 (Robertson et al., 2000).

This mesodermal window overlaps with the onset of Flk1 expression, initiated as early as day 2.5 of differentiation and with the BL-CFC stage of development, typically found between day 2.5 and 4.0 of differentiation.

In the mouse embryo, mesoderm is generated from the epiblast or embryonic ectoderm through the process of gastrulation that is initiated at approximately day 6.5 of gestation (reviewed by Tam and Behringer, 1997). At the onset of gastrulation, the epiblast cells in the region that defines the posterior part of the embryo undergo an epithelial to mesenchymal transition and form a transient structure known as the primitive streak from which the mesoderm emerges. The newly formed mesoderm migrates away from the primitive streak, moves laterally and anteriorly and is patterned into various populations with distinct developmental fates. Brachyury is expressed in all nascent mesoderm and downregulated as these cells undergo patterning and specification into the derivative tissues including skeletal muscle, cardiac muscle and connective tissues in addition to blood and endothelium (Herrmann, 1991; Kispert and Herrmann, 1994).

The first mesodermal cells to develop within the embryo contribute predominantly to the extra-embryonic tissues, giving rise to the hematopoietic and vascular cells of the yolk sac (Kinder et al., 1999). Hematopoietic progenitors are first found in the developing yolk sac as early as day 7.0 of gestation, ~12 hours after the beginning of gastrulation (Palis et al., 1999). Flk1 is expressed in the yolk sac at this stage and is essential for the establishment of the blood cell and vascular lineages (Schuh et al., 1999; Shalaby et al., 1995). Although the yolk sac hemangioblast has not yet been identified, the rapid commitment to the hematopoietic and endothelial lineages following the induction of mesoderm suggests that this putative in vivo progenitor should also be closely related to mesoderm.

Our understanding of hematopoietic and endothelial development has been greatly enhanced by the identification and characterization of cell populations representing the earliest stages of commitment towards these lineages (Nishikawa et al., 1998; Chung et al., 2002; Faloon et al., 2000; Lacaud et al., 2002; Robertson et al., 2000). By contrast, however, developmental stages earlier than the hemangioblast remain difficult to study as there are few known cell surface markers that enable one to isolate these populations or subsets of progenitors within these populations. To access pre-hemangioblast cell populations and define their relationship with respect to the BL-CFC, we targeted the GFP cDNA to the brachyury locus. In this report, we show that GFP is an effective marker for the mesodermal populations that develop within the EBs. Analysis of GFP and Flk1 expression led to the identification of three subpopulations $GFP^{-}Flk1^{-}$, $GFP^{+}Flk1^{-}$ and $GFP^{+}Flk1^{+}$ that represent a developmental progression from pre-mesodermal cells to the hemangioblast.

MATERIALS AND METHODS

Construction of the targeting vector

The 'arms' of the targeting vector homologous to brachyury sequences were isolated from a 129/Ola strain BAC library (Genome Systems). The final targeting construct consisted of: (1) a 'short arm' of homology encompassing nucleotides –1243 to +3 of the brachyury gene (the adenine in the ATG start codon counting 1); (2) a GFP mini gene as described in the 'Results' section; (3) a loxP flanked neomycin resistance gene (Gu et al., 1993); (4) a 'long arm' of homology encompassing nucleotides +364 to +7515 of the brachyury gene; and (5) the HSV thymidine kinase gene for negative selection. A detailed description of all cloning steps giving rise to the final targeting vector can be obtained directly from H.J.F. (joerg.fehling@medizin.uni-ulm.de).

Generation of brachyury/EGFP knock-in ES cells

Mouse embryonic stem cells (E14.1, 129/Ola) were electroporated with the *Not*I-linearized targeting vector. Clones that had undergone a homologous recombination event were identified by PCR with one primer (5′-CAGGTAGAACCCACAACTCCGAC-3′) annealing to genomic sequences in the 5′ region of the brachyury gene, upstream of the 'short arm of homology', the other primer (5′-CCGGACACGCTGAACTTGTGGC-3′) to the 5′ region of EGFP. Correctly targeted clones were confirmed by Southern blot analysis. Out of 384 singly selected and 80 doubly selected colonies, four and three correctly targeted clones were identified, respectively. Two positive clones (#164 and #201) were transiently transfected with a modified Cre recombinase expression vector (H.J.F., unpublished) to excise the *neo* gene. Neo-deficient clones were identified due to loss of G418 resistance. The intactness of the targeted locus before and after Cre-mediated excision of Neo was confirmed by Southern blot analysis. The absence of the *neo* cassette in Cre-treated G418-sensitive clones was verified by Southern blotting using the Neo cassette as probe (not shown).

ES cell growth and differentiation

ES cells were maintained on irradiated embryonic feeder cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% fetal calf serum (FCS), penicillin, streptomycin, LIF (1% conditioned medium) and 1.5×10^{-4} M monothioglycerol (MTG; Sigma). Two days prior to the onset of differentiation, cells were transferred on gelatinized plates in the same media. For the generation of EBs, ES cells were trypsinized and plated at various densities in differentiation cultures. Differentiation of EBs was carried out in 60 mm petri grade dishes in IMDM supplemented with 15% FCS, 2 mM L-glutamine (Gibco/BRL), transferrin (200 μg/ml), 0.5 mM ascorbic acid (Sigma), and 4.5×10^{-4} M MTG. For reaggregation, sorted cells were cultured in the media used to differentiate EBs. Cells were cultured for 20 hours at a density of 4×10^{5}/ml in ultra low attachment 24-well plates (Costar). Cultures were maintained in a humidified chamber in a 5% CO_2/air mixture at 37°C.

Colony assays

For the generation of blast cell colonies (BL-CFC assay), EB cells were plated in 1% methylcellulose supplemented with 10% FCS (Summit), vascular endothelial growth factor (VEGF; 5 ng/ml), Kit ligand (KL; 1% conditioned medium), IL6 (5 ng/ml) and 25% D4T endothelial cell conditioned medium (Kennedy et al., 1997). For the growth of hematopoietic precursors, cells were plated in 1% methylcellulose containing 10% plasma-derived serum (PDS; Antech), 5% protein-free hybridoma medium (PFHM-II; Gibco-BRL) and the following cytokines: KL (1% conditioned medium), TPO (5 ng/ml), erythropoietin (2 U/ml), IL11 (25 ng/ml), IL3 (1% conditioned medium), GM-CSF (3 ng/ml), G-CSF (30 ng/ml), M-CSF (5 ng/ml) and IL6 (5 ng/ml). Cultures were maintained at 37°C, 5% CO_2. LIF and Kit ligand were derived from media conditioned by CHO cells transfected with LIF and KL expression vectors, respectively (kindly provided by Genetics Institute). IL3 was obtained from medium conditioned by X63 AG8-653 myeloma cells transfected with a vector expressing IL3 (Karasuyama and Melchers, 1988). VEGF, GM-CSF, M-CSF, G-CSF, TPO, IL6 and IL11 were purchased from R&D Systems.

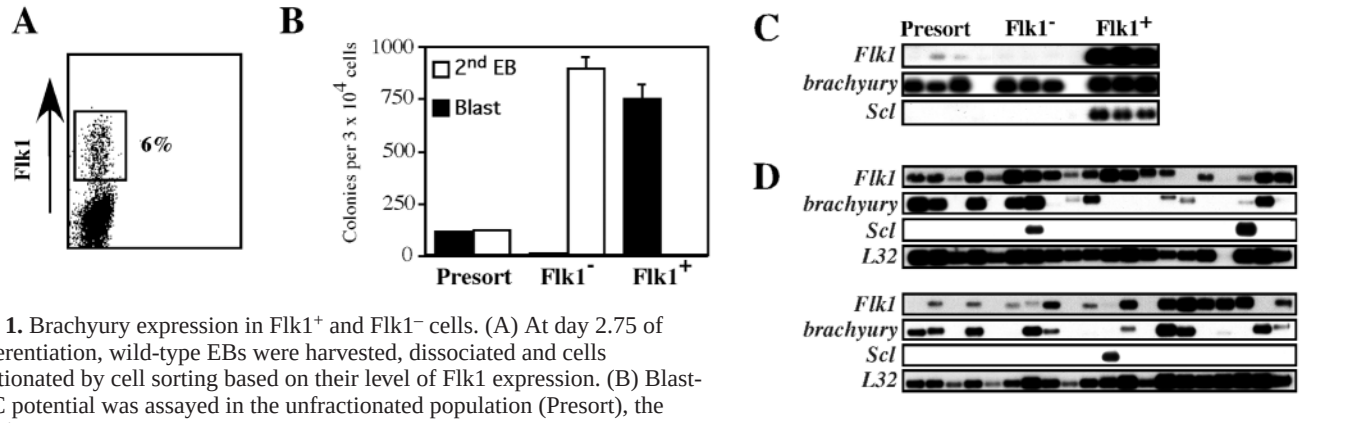

Fig. 1. Brachyury expression in Flk1$^+$ and Flk1$^-$ cells. (A) At day 2.75 of differentiation, wild-type EBs were harvested, dissociated and cells fractionated by cell sorting based on their level of Flk1 expression. (B) Blast-CFC potential was assayed in the unfractionated population (Presort), the Flk1$^+$ and Flk1$^-$ fractions. Blast cell colonies (Blast) and secondary EBs (2nd EBs) were scored following 4 days of culture. Data are presented as the mean number of colonies from three dishes. Bars, where visible, represent s.d. of the mean. (C) Expression analysis of subpopulations positive and negative for Flk1. 3′ cDNA from pools of sorted cells (1000 cells per lane) was prepared by RT-PCR and total cDNAs were separated on a 1.5% agarose gel, blotted and probed separately with 3′ probes from the indicated genes. (D) Expression analysis of single cells. One-hundred individual cells from the Flk1$^+$ sorted fraction were picked using a mouth pipette and deposited directly into the lysis buffer. 3′ cDNA was prepared and analyzed as described above. A representative expression pattern for 40 such individual cells is shown here. Hybridization with a 3′ probe from the L32 ribosomal protein gene was included to control for amounts of cDNA in each lane.

Neuronal differentiation

Both GFP positive and negative populations were isolated from day 2.5 EBs by cell sorting. Pre-sort and sorted cells were reaggregated at 10^5 cells/ml in ultra low attachment 24-well plates (Costar) in IMDM supplemented with 15% serum replacement media (Gibco BRL). Twenty-four hours later, the reaggregated EB-like structures were moved to 60 mm petri dishes in the same medium and cultured for an additional 3.5 days. At this stage, the EBs were harvested and transferred to gelatin-coated dishes for the evaluation of neurite formation or to gelatin-coated cover slip for specific staining. Four days later, the proportion of EBs that generated neurites was scored. For immunohistochemical staining, EBs were fixed in 2% paraformaldehyde for 20 minutes, washed twice in PBS, permeabilized in 0.2% Triton X-100/PBS, washed in 10% FCS, 0.2% Tween 20/PBS, and then blocked with 10% FCS/PBS for 10 minutes. EBs were incubated for 1 hour with an antibody against the neuronal class III β-tubulin (TuJ1; Babco). Bound antibodies were visualized using a secondary Cy3-conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch Laboratories).

Gene expression analysis

The preparation and analysis of 3′ UTR cDNA was performed as previously described (Robertson et al., 2000). For gene-specific PCR, total RNA was extracted from each sample with an RNeasy mini kit and treated with Rnase-free DNase (Qiagen). Two micrograms of total RNA was reverse-transcribed into cDNA with random hexamer using an Omniscript RT kit (Qiagen). The PCR reactions were performed with 2.5 U of Taq polymerase (Promega), PCR buffer, 2.5 mM $MgCl_2$, 0.2 μM of each primers and 0.2 mM dNTP. Cycling conditions were as follows: 94°C for 5 minutes followed by 35 cycles of amplification (94°C denaturation for 1 minute, 60°C annealing for 1 minute, 72°C elongation for 1 minute) with a final incubation at 72°C for 7 minutes. PCR was carried out using the following gene specific oligonucleotides: β-actin, 5′ATG AAG ATC CTG ACC GAG CG3′ (sense) and 5′TAC TTG CGC TCA GGA GGA GC3′ (antisense); brachyury, 5′CAT GTA CTC TTT CTT GCT GG3′ (sense) 5′GGT CTC GGG AAA GCA GTG GC3′ (antisense); Runx1, 5′CCA GCA AGC TGA GGA GCG GG3′ (sense) 5′CGG ATT TGT AAA GAC GGT GA3′ (antisense); Flk1, 5′CAC CTG GCA CTC TCC ACC TTC3′ (sense) 5′GAT TTC ATC CCA CTA CCG AAA G3′ (antisense); Nodal, 5′CCG TCC CCT CTG GCG TAC ATG3′ (sense) 5′GAC CTG AGA AGG AAT GAC GG3′ (antisense); Pax6, 5′GCT TCA TCC GAG TCT TCT CCG TTA G3′ (sense) 5′CCA TCT TTG CTT GGG AAA TCC G3′ (antisense); Rex1, 5′CGT GTA ACA TAC ACC ATC CG3′ (sense) 5′GAA ATC CTC TTC CAG AAT GG3′ (antisense); Fgf5, 5′AAA GTC AAT GGC TCC CAC GAA3′ (sense) 5′CTT CAG TCT GTA CTT CAC TGG3′ (antisense); Bmp2, 5′GAA TCA GAA CAC AAG TCA GT3′ (sense) 5′GTT TGT GTT TGG CTT GAC GC3′ (antisense); Bmp4, 5′TGT GAG GAG TTT CCA TCA CG3′ (sense) 5′CAG CGA AGG ACT GCA GGG CT3′ (antisense); Wnt3a, 5′GGA ATG GTC TCT CGG GAG TTT G3′ (sense) 5′AGG TTC GCA GAA GTT GGG TGA G3′ (antisense); *Wnt8a*, 5′CTG CCT GGT CAG TGA ACA ACT TC3′ (sense) 5′GAG TCT GGA GAT TTT TTC CCC G3′ (antisense).

Flow cytometry and cell sorting

EBs were harvested, trypsinized and the single cell suspension analyzed on a Facscalibur flow cytometer (Becton Dickinson) or sorted on a Moflo cell sorter (Cytomation Systems). Staining with mAb Flk1 bio, Kit-PE or CD31-bio (PharMingen) was performed as previously described (Kouskoff et al., 2000).

RESULTS

The mesodermal marker brachyury is expressed in Flk1 positive cells

As a first step in defining the relationship between mesoderm and the BL-CFC, we analyzed the expression pattern of the brachyury gene in Flk1$^+$ cells isolated from developing EBs. The majority of BL-CFC was found in the Flk1$^+$ fraction of day 2.75 EBs, confirming the earlier findings of Faloon et al. (Faloon et al., 2000) (Fig. 1A,B). Expression analysis revealed that brachyury was present in both the Flk1$^+$ and Flk1$^-$ populations, indicating that the BL-CFC may represent a subset of mesoderm (Fig. 1C). The helix-loop-helix transcription factor *Scl* (*Tal1* – Mouse Genome Informatics) (Begley et al., 1989) was expressed only in the Flk1$^+$ fraction, consistent with the presence of BL-CFC and cells undergoing commitment to the hematopoietic lineages.

To define more accurately the co-expression pattern of these genes, 100 individual Flk1$^+$ cells were analyzed. As shown in

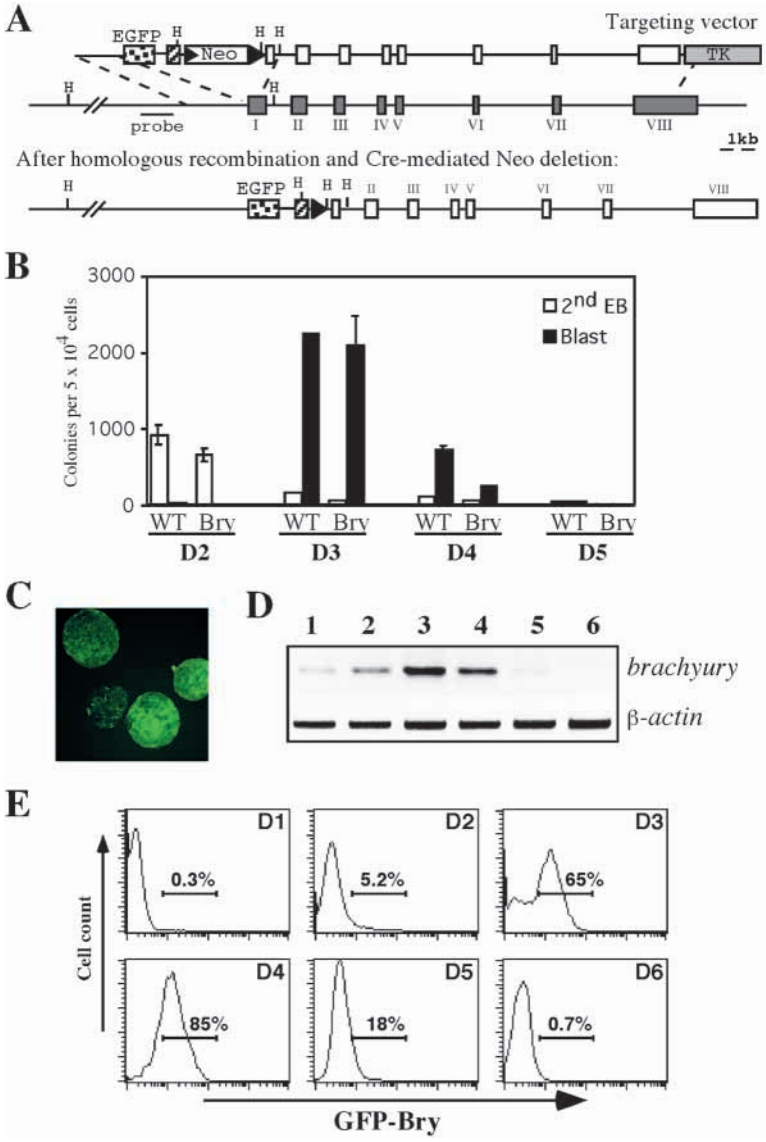

Fig. 2. Generation of GFP-Bry ES cells. (A) Schematic structure of the mouse brachyury gene locus and the targeting vector. LoxP sites are indicated by black triangles on either side of the Neo gene. The hatched box indicates an exon derived from plasmid pBK-CMV, which contains the SV40 polyadenylation signal. TK: Herpes simplex thymidine kinase gene. H: *Hin*cII. (B) Blast-CFC potential: EBs from both ES cell lines were harvested on the indicated days of differentiation and cells assayed for BL-CFC. Colonies were scored after 4 days of culture and were referred to as 2nd EB (secondary embryoid bodies) and Blast (blast colonies). Data are presented as the mean number of colonies from three dishes. Bars, where visible, represent s.e. of the mean. (C) Fluorescence microscopy of Bry-GFP EBs at day 3 of differentiation. Wild-type EBs did not show detectable levels of fluorescence (not shown). Photos were taken with a Hamasura camera at 100× magnification. (D) EBs from the GFP-Bry ES cell line were harvested daily between day 1 and 6 of differentiation. Cells were used for expression analysis of brachyury by RT-PCR using gene specific primers and (E) flow cytometric analysis for detection of the GFP protein.

Fig. 1D, which represents 40 of these single cells, different patterns were observed, the most common being co-expression of *Flk1* (*Kdr* – Mouse Genome Informatics) and brachyury. Cells expressing either *Flk1* or brachyury represented a smaller subset of the population. *Scl*-expressing cells were the least abundant. Of the 100 cells analyzed, 91 showed expression of the ribosomal gene *L32*, indicating the presence of amplified material in the sample. Out of these 91 cells, 43 expressed both *Flk1* and brachyury, and of these, three also expressed *Scl*. Although a number of cells expressed either *Flk1* or brachyury, we interpret these patterns with caution, as the lack of a signal on single cells analysis can reflect differences in cell cycle status. The high frequency of cells co-expressing *Flk1* and brachyury adds support to the notion that the BL-CFC is closely related to mesoderm. To directly address the question of whether or not the BL-CFC expresses brachyury, we targeted the GFP cDNA to the brachyury locus to enable us to isolate cells that express this gene.

Development and characterization of an ES cell line carrying GFP targeted to the brachyury locus

Two different vectors were designed for targeting GFP to the brachyury locus. The vector that gave the highest level of expression contained a mini gene locus, which consisted of the GFP cDNA, followed by a splice donor site, an artificial intron and an exon encoding the SV40 polyadenylation signal sequence to prevent transcription of regions downstream of the brachyury gene (Fig. 2A). A translational stop codon was positioned downstream of the artificial intron, as it has been reported that primary transcripts with stop codons preceding intronic sequences can be recognized as aberrant messages, thereby rendering them subject to rapid degradation (Maquat, 2002). The vector was designed to replace approximately two-thirds of the first exon of the brachyury gene with the GFP expression cassette, resulting in the disruption of the targeted brachyury allele. The targeting construct was electroporated into E14.1 ES cells and several positive clones were identified. The Neo selection marker was subsequently removed from the targeted ES cells by Cre/loxP-mediated recombination (Gu et al., 1993) to minimize the impact of foreign DNA sequences and ensure that expression of the inserted GFP cassette was under the control of native brachyury regulatory elements. Two GFP targeted, Neo-deleted ES clones, referred as GFP-Bry cells, were differentiated in vitro and analyzed for GFP expression. EBs generated from these two clones expressed readily detectable levels of GFP when observed under a fluorescence microscope (Fig. 2C) and were used in the subsequent analyses. The second vector, which did not result in significant GFP expression after targeting, consisted of an insertion of a simple GFP cDNA instead of the minigene into the same segment of the brachyury locus (not shown). The comparison of these vectors highlight the importance of vector design as relatively small differences in targeting constructs resulted in significant differences in levels of GFP expression.

For the targeted GFP-Bry ES cells to be a reliable model for studying mesoderm induction and subsequent specification to the hemangioblast and to the hematopoietic and endothelial lineages, the inactivation of one allele should not be detrimental to these developmental processes. Although the

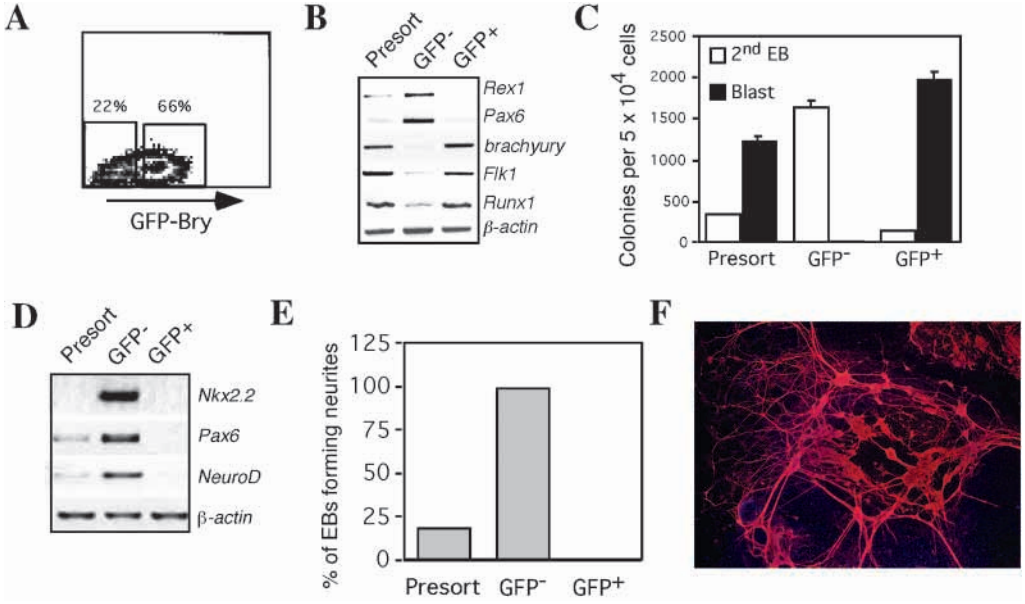

Fig. 3. Analysis of GFP$^+$ and GFP$^-$ EB-derived cells. (A) At day 3.5 of differentiation, GFP-Bry EBs were harvested, dissociated and cells fractionated by cell sorting based on their GFP expression level. (B) RNA extraction was performed on the GFP$^-$, GFP$^+$ as well as the starting population (presort). Gene expression patterns were analyzed by RT-PCR using specific primers for each of the indicated genes. (C) Blast-CFC potential of the presort, GFP$^-$ and GFP$^+$ populations. Colonies were scored after 4 days of culture. Data are presented as the mean number of colonies from three dishes. Bars, where visible, represent s.e. of the mean. The purity of sorted cells was ~95%. (D) Expression analysis of day 6 reaggregated EBs generated from day 2.5 EB presort, GFP$^+$ and GFP$^-$ cells. (E) Neurite potential of reaggregated EBs generated from GFP$^+$ and GFP$^-$ populations. Data are presented as the % of EBs that form neurites. (F) Immunostaining demonstrating the expression of neuronal class III β-tubulin (TuJ1) on neurites that develop from EBs derived from GFP$^-$ cells.

viability and near normal development of the T heterozygous mouse clearly indicates that there are no significant hemizygous effects on mesoderm development (Herrmann, 1991), it is possible that this targeting could impact the in vitro differentiation potential. To address this question, we assayed the BL-CFC potential of the GFP-Bry cells and compared it with that of wild-type cells. EBs generated from the GFP-Bry cells were indistinguishable from those that developed from wild-type ES cells with respect to morphology and size (not shown). As indicated in Fig. 2B, no significant differences were observed in the kinetics and numbers of blast colonies generated by the GFP-Bry ES cells when compared with wild-type cells. Analysis of the blast colonies indicated that their hematopoietic and endothelial potential was similar to those of wild-type colonies (not shown). Taken together, these findings demonstrate that heterozygosity at the brachyury locus does not alter the specification and development of the hematopoietic program during EB differentiation.

Correlation between expression of the targeted GFP and transcription of the endogenous brachyury gene

As a marker of mesoderm formation, GFP expression must reflect the expression pattern of the endogenous gene. To determine if this is the case, EBs derived from GFP-Bry ES cells were harvested at daily intervals over a 6-day differentiation period and analyzed for brachyury transcription by RT-PCR and for GFP expression by flow cytometry (Fig. 2D,E). As shown in Fig. 2D, brachyury expression was detected between day 2 and 4 of differentiation, with the highest levels present at day 3, consistent with the previously described pattern for this gene (Robertson et al., 2000). FACS analysis revealed the presence of low numbers of GFP$^+$ cells as early as day 2 of differentiation. The number of GFP$^+$ cells increased dramatically over the next 48 hours, representing 65% and 85% of the total day 3 and 4 EB populations, respectively. Following this peak, the number of GFP$^+$ cells dropped sharply to undetectable levels in day 6 EBs. The high levels of GFP detected by FACS analysis demonstrate that a large proportion of the day 3-4 EB cells express brachyury indicative of extensive mesoderm development in our differentiation conditions. The findings from this comparative analysis strongly suggest that GFP expression faithfully recapitulates brachyury expression in differentiating EBs and as such, provides a unique marker for the identification and isolation of cells expressing this gene.

Segregation of mesoderm and neuroectoderm lineages by GFP expression

In early development, brachyury expression is restricted to the primitive streak and nascent mesoderm in the region that will define the posterior part of the embryo. The epiblast cells in the anterior region of the embryo acquire a neuroectoderm fate and do not express brachyury (Herrmann, 1991). To determine if brachyury expression could also distinguish these primary germ cell populations within the ES differentiation cultures, EBs were fractionated into GFP$^+$ and GFP$^-$ populations by cell sorting and assayed for mesoderm and neuroectoderm potential (Fig. 3). GFP$^+$ and GFP$^-$ populations were isolated from day 3.5 EBs and subjected to gene expression and BL-CFC analysis (Fig. 3A). As shown in Fig. 3B, cells expressing brachyury segregated to the GFP$^+$ fraction indicating that it is possible to isolate mesodermal cells based on GFP expression. Genes associated with the earliest stages of hematopoietic and endothelial commitment, including *Flk1* and *Runx1* (Okuda et al., 1996; Wang et al., 1996), co-segregated with brachyury to the GFP$^+$ fraction. By contrast, markers of primitive ectoderm (*Rex1*; *Zfp42* – Mouse Genome Informatics) (Rogers et al., 1991) and neuroectoderm (*Pax6*) (Walther and Gruss, 1991) were detected only in the GFP$^-$ population. BL-CFC analysis

indicated that most progenitors were found in the GFP$^+$ fraction. Segregation of the BL-CFC potential to GFP$^+$ fraction demonstrates that it contains mesodermal derivatives and that the BL-CFC itself may retain some level of brachyury expression (Fig. 3C).

To evaluate the neuroectoderm potential of the GFP populations, day 2.5 EBs were fractionated, the cells from each population allowed to reaggregate to form EB-like structures and further cultured as described in the Materials and Methods. Day 2.5 EBs were used for this analysis as they contain significantly more neuroectoderm potential than later stage EBs in culture conditions selected to promote hematopoietic development. The reaggregated cells were cultured for an additional 3.5 days in suspension and then replated to assess their potential to generate neurites. At the end of the 6-day culture, EBs generated from the GFP$^-$ population expressed genes indicative of neural development, including *Nkx2.2* (Briscoe et al., 1999), *Pax6* (Walther and Gruss, 1991) and *Neurod1* (previously *NeuroD*) (Bang and Goulding, 1996) (Fig. 3D). By contrast, none of these genes was expressed in the EBs derived from GFP$^+$ cells. Almost all of the reaggregated EBs generated from the GFP$^-$ population developed neurite outgrowths that expressed the neuronal class III β-tubulin (TuJ1) (Fig. 3E,F). Consistent with the gene expression profile, none of the EBs from the GFP$^+$ fraction formed neurites. The findings from these analyses clearly demonstrate that it is possible to separate mesodermal cells from those that display neuroectoderm potential based on GFP expression under the control of the brachyury regulatory elements. Taken together with the findings from the kinetic analysis, these data demonstrate that the strategy of targeting a selectable marker to the brachyury locus was successful and that in this context, GFP provides a unique marker with which to track and isolate cells with mesoderm potential.

Temporal expression of brachyury relative to markers indicative of ES cell differentiation and BL-CFC development

As shown in Fig. 2, GFP is expressed in a dynamic temporal pattern that reflects expression of the endogenous brachyury gene. To further define the stages of ES differentiation to mesoderm and subsequent specification to the hemangioblast lineages, we compared the kinetic of GFP expression with that of CD31, Kit and Flk1. Although Kit and CD31 are best known for their expression on hematopoietic (Ogawa et al., 1991) and endothelial (Vecchi et al., 1994) populations from fetal and adult tissues, they are also expressed on undifferentiated ES cells (Robson et al., 2001; Vittet et al., 1996). As both are downregulated after the onset of ES cell differentiation, their expression patterns can be used to track the early commitment steps in the formation of EBs. Undifferentiated GFP-Bry ES cells did not express significant levels of GFP or Flk1, but did have high levels of CD31 (Fig. 4A) and intermediate level of Kit (Fig. 4B). Day 2 EBs contained a small population of GFP$^+$ cells, a fraction of which also expressed Flk1. The overall levels of CD31 on day 2 EBs were somewhat reduced compared with that found on the undifferentiated ES population. The expression patterns of all markers changed dramatically over the next 24 hours. At day 3 of differentiation, more than 40% of the EB population expressed GFP with no Flk1, while 18% of the cells expressed both GFP and Flk1.

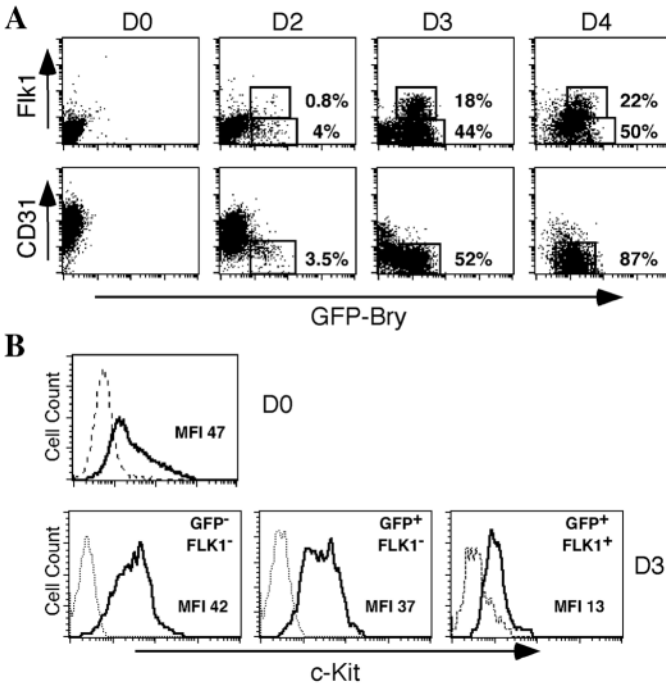

Fig. 4. GFP-Bry expression relative to Flk1, CD31 and Kit. (A) At the indicated time points, GFP-Bry EBs were harvested and dissociated. The cells were stained with Flk1, c-Kit or CD31 mAb and analyzed by flow cytometry. (B) The level of Kit marker was assessed for each subpopulation delineated by Flk1 and GFP-Bry expression at day 3 of differentiation. The mean fluorescence intensity (MFI) is given for each graph. At day 0 of differentiation, the ES cells expressed Kit. The broken line in this graph represents the unstained control.

CD31 levels were significantly reduced on the entire EB population and none was expressed on the GFP$^+$ cells. Three colors analysis revealed that the double positive GFP$^+$Flk1$^+$ cells had low level of Kit expression (Fig. 4B). Both the GFP$^+$Flk1$^-$ and the GFP$^-$Flk1$^-$ populations had intermediate levels of Kit. By day 4 of differentiation, the EB cells appear to have down regulated the levels of GFP and Flk1 expression, although a significant portion of the populations still expressed both markers. Relatively few cells expressed CD31 (Fig. 4A) or Kit (not shown) at this stage of development. These analyses clearly demonstrate that using the GFP-Bry ES line together with cell-surface markers, it is possible to track the differentiation of ES cells to brachyury-positive mesoderm and subsequently to cell populations that express Flk1 together with brachyury.

GFP and Flk1 delineate specific subpopulations of mesoderm

Expression of GFP and Flk1 on day 3 EB cells delineates three distinct populations designated as GFP$^-$Flk1$^-$, GFP$^+$Flk1$^-$ and GFP$^+$Flk1$^+$ (Fig. 5A). In some experiments, we observed a small GFP$^-$Flk1$^+$ population at early stages of differentiation. When analyzed, this population appeared to represent dead and/or dying cells that had non-specifically bound the secondary streptavidin reagent. Analysis of BL-CFC potential of the three populations revealed the expected pattern of segregation. The majority of these progenitors were present in the GFP$^+$Flk1$^+$ population, whereas the secondary EBs that

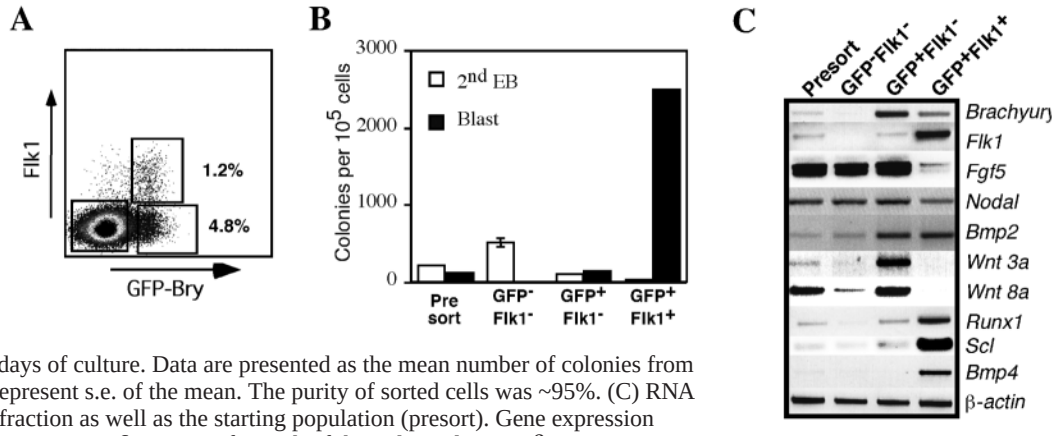

Fig. 5. Blast potential and gene expression patterns of Flk1/GFP-Bry subpopulations. (A) At day 2.5 of differentiation, GFP-Bry EBs were harvested, dissociated and cells fractionated by cell sorting based on their respective GFP and Flk1 expression levels. (B) Blast-CFC potential of the GFP$^-$Flk1$^-$, GFP$^+$Flk1$^-$ and GFP$^+$Flk1$^+$ populations. Colonies were scored following 4 days of culture. Data are presented as the mean number of colonies from three dishes. Bars, where visible, represent s.e. of the mean. The purity of sorted cells was ~95%. (C) RNA extraction was performed on each fraction as well as the starting population (presort). Gene expression patterns were analyzed by RT-PCR using specific primers for each of the indicated genes. β-actin expression was used to equilibrate the relative amount of each cDNA.

develop from residual ES cells segregated to the GFP$^-$Flk1$^-$ fraction (Fig. 5B). The GFP$^+$Flk1$^-$ cells did not generate significant numbers of blast colonies or secondary EBs. Gene expression analysis revealed striking differences between the populations, consistent with the observed difference in biological potential. As expected, *Flk1* was restricted to the GFP$^+$Flk1$^+$ fraction, whereas brachyury was present in both GFP$^+$ fractions, although at lower levels in the population that co-expressed Flk1. *Nodal* and *Fgf5*, genes found in the epiblast and early gastrulating embryo (Haub and Goldfarb, 1991; Hebert et al., 1991; Varlet et al., 1997), were expressed in the GFP$^-$Flk1$^-$ and GFP$^+$Flk1$^-$ fractions, but downregulated in GFP$^+$Flk1$^+$ cells. Two members of the Wnt family, *Wnt3a* and *Wnt8a* that are expressed in the primitive streak and early mesoderm (Bouillet et al., 1996; Takada et al., 1994; Yamaguchi et al., 1999) were restricted to the GFP$^+$Flk1$^-$ subpopulation. *Bmp2* and *Bmp4*, genes encoding factors that play crucial roles during mesoderm formation and specification (Hogan, 1996), displayed interesting patterns of expression. *Bmp2* was detected in both GFP-positive fractions, whereas *Bmp4* expression was restricted to the GFP$^+$Flk1$^+$ cells. Finally, genes associated with the hematopoietic lineages, *Runx1* (Okuda et al., 1996; Wang et al., 1996) and *Scl* (Begley et al., 1989) were expressed predominantly in the GFP$^+$Flk1$^+$ fraction, consistent with the fact that this fraction contains the BL-CFC.

Taken together, these findings demonstrate that expression of Flk1 and GFP delineates three subpopulations within day 3.0 EBs that display distinct developmental potential as defined by gene expression profiles and progenitor cell content. The potential of these populations are consistent with the hypothesis that they represent a developmental progression from pre-mesoderm cells, defined as GFP$^-$Flk1$^-$ cells to pre-hemangioblast mesoderm represented by the GFP$^+$Flk1$^-$ fraction to the hemangioblast, defined as GFP$^+$Flk1$^+$.

Tracking the induction of mesoderm and its specification to the BL-CFC

If the three fractions defined by GFP and Flk1 represent distinct steps within a developmental program, then it should be possible to demonstrate that those representing the early stages are able to give rise to the more mature populations. To address this issue, each fraction was isolated from day 3 EBs and allowed to reaggregate at high cell density in culture for 20 hours (Fig. 6A). Although cell numbers did not change significantly during this time, the developmental potential of the populations did. In the reaggregated presort control, the GFP$^+$Flk1$^+$ fraction increased in size from 16% to 40% of the total population, the GFP$^+$Flk1$^-$ fraction remained relatively constant in size whereas the GFP$^-$Flk1$^-$ fraction decreased in size during this time. After the 20-hour culture period, the GFP$^-$Flk1$^-$ sorted population gave rise to a significant number of GFP$^+$Flk1$^-$ cells (27% of the total culture) and also to a small emerging population (3.5%) that expressed Flk1. In this same period, the GFP$^+$Flk1$^-$ fraction generated a large GFP$^+$Flk1$^+$ population that represented 66% of the total cells recovered from the reaggregation culture. A subpopulation of cells did not acquire Flk1 and retained relatively high levels of GFP. Expression of both Flk1 and GFP was downregulated after culture of the GFP$^+$Flk1$^+$ fraction. Analysis of the BL-CFC and hematopoietic progenitor potential of the fractions prior to (pre-culture) and following (post-culture) the culture revealed changes that were consistent with the changes observed in Flk1 and GFP expression. Prior to culture, most of the blast colony-forming potential was found in the GFP$^+$Flk1$^+$ fraction (Fig. 6B). After culture, the BL-CFC potential of the two GFP$^+$ fractions changed dramatically. The GFP$^+$Flk1$^-$ population acquired the potential to generate blast colonies, a finding consistent with the fact that these cells upregulated Flk1 expression level during this time period. The GFP$^+$Flk1$^+$ population, conversely, lost BL-CFC activity but developed significant hematopoietic potential during this culture step (Fig. 6C). These changes in progenitor cell potential together with the downregulation of both Flk1 and GFP within this population are an indication of maturation beyond the hemangioblast stage to the early hematopoietic stage of development. The majority of hematopoietic progenitors that developed in the reaggregation culture of the GFP$^+$Flk1$^+$ fraction were of the primitive erythroid lineage, the earliest population to develop in EBs. The GFP$^-$Flk1$^-$ fraction contained no BL-CFC prior to or following culture, consistent with the lack of significant numbers of Flk1-expressing cells in either population.

The findings from this experiment support the concept that populations defined by GFP and Flk1 expression in day 3 EBs

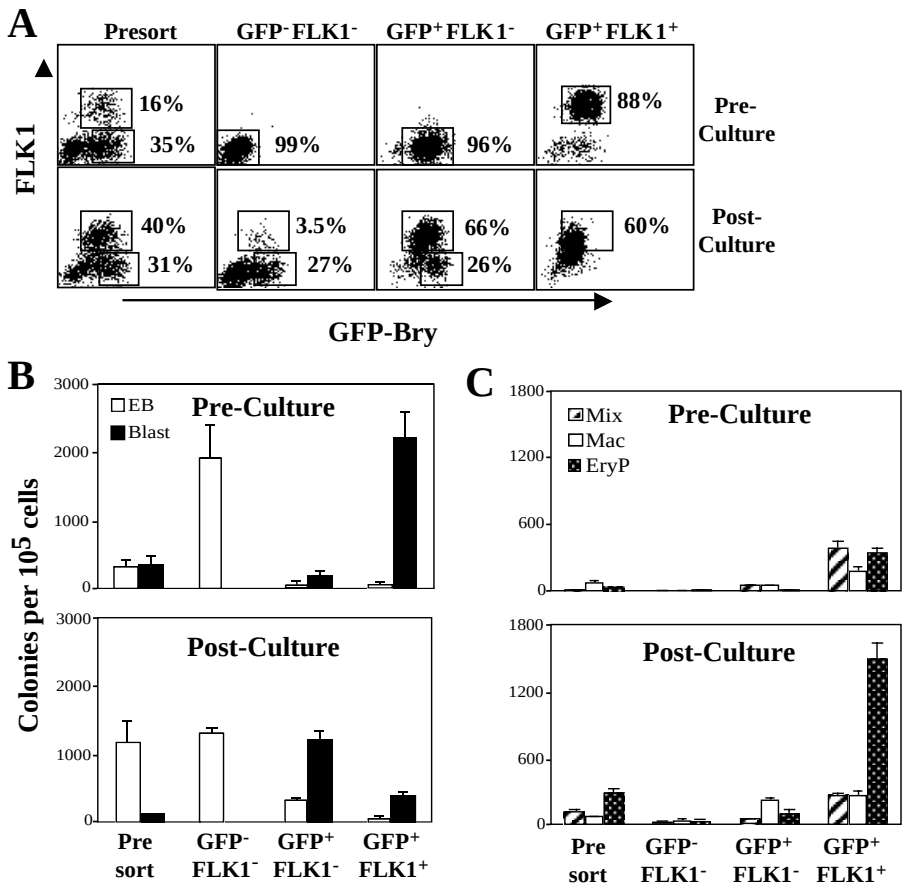

Fig. 6. Relationship between the GFP$^-$Flk1$^-$, GFP$^+$Flk1$^-$ and GFP$^+$Flk1$^+$ subpopulations. (A) At day 3 of differentiation, GFP-Bry EBs were harvested, dissociated and cells fractionated by cell sorting based on their respective GFP and Flk1 expression levels (pre-culture). Each subpopulation was allowed to reaggregate in culture for 20 hours. At this stage, EB-like structures were harvested, dissociated and stained for Flk1 expression (post-culture). (B) Blast-CFC potential of the GFP$^-$Flk1$^-$, GFP$^+$Flk1$^-$ and GFP$^+$Flk1$^+$ populations immediately after cell sorting (pre-culture) or after the 20-hour culture step (post-culture). Colonies were scored after 4 days of culture. (C) Hematopoietic potential of the same fractions as above. Cells were replated in methylcellulose cultures containing cytokines appropriate for hematopoiesis differentiation. Colonies were scored after 6 to 8 days of culture and designated as follows: Mac, macrophages; Mix, multi-lineage; EryP, primitive erythroid. Data are presented as the mean number of colonies from three dishes. Bars, where visible, represent s.e. of the mean.

represent distinct stages of ES cell differentiation to mesoderm and to the earliest stages of hematopoietic and endothelial development, as defined by the BL-CFC.

DISCUSSION

By targeting GFP to the brachyury locus, we have established a model system with which to study the development of mesoderm and its specification to the hematopoietic and vascular lineages after ES cell differentiation in culture. Using this approach, we have demonstrated that it is possible to separate cells with mesoderm potential from other cell types, including those of the neuroectoderm lineage. Our data highlight the power of such a strategy for isolating the primary germ cell lineages as they develop in this model system. By combining GFP and Flk1 expression, we were able to isolate and characterize three distinct populations that define a pre-mesoderm stage of development as GFP$^-$Flk1$^-$, a pre-hemangioblast mesoderm stage as GFP$^+$Flk1$^-$ and the hemangioblast stage as GFP$^+$Flk1$^+$.

One of the original goals of this targeting approach was to further characterize the developmental relationship between the BL-CFC and mesoderm by determining whether or not this hemangioblast-like progenitor expressed brachyury. The outcome of this study clearly demonstrates that the entire Flk1$^+$ population at the BL-CFC stage of development expresses GFP. Although all BL-CFC are GFP$^+$, the RT-PCR analyses in Fig. 5 indicate that the levels of brachyury are reduced compared with the GFP$^+$Flk1$^-$ population suggesting that these progenitors are downregulating brachyury as they differentiate to the hematopoietic and endothelial lineages. Previous findings demonstrating that progeny of the BL-CFC no longer express brachyury (Kennedy et al., 1997; Robertson et al., 2000) are consistent with this interpretation. Collectively, these observations would position the BL-CFC at a stage of development that represents mesodermal cells committed to the hematopoietic and vascular lineages.

In the early mouse embryo, the first mesodermal cells generated within the primitive streak migrate to the extra-embryonic region where they differentiate and form the hematopoietic and endothelial lineage of the blood islands (Moore and Metcalf, 1970; Kinder et al., 1999). Although most cells within the primitive streak are brachyury positive, expression is rapidly downregulated as they exit the streak and begin migrating (Wilkinson et al., 1990). Flk1 is expressed in these migrating cells and subsequently in the blood islands and the developing vasculature of the yolk sac (Shalaby et al., 1995). Given that brachyury is widely expressed in the primitive streak, it is assumed that the Flk1$^+$ cells and ultimately the hematopoietic and endothelial lineages derive from brachyury expressing mesoderm. The findings in this report formally demonstrate that brachyury$^+$ mesodermal progenitors do indeed give rise to the BL-CFC and to cells of the hematopoietic lineage within the ES/EB model system.

In addition to providing further characterization of the BL-CFC, the GFP-Bry ES cell line has enabled us to segregate the Flk1$^-$ fraction of day 3.0-3.5 EBs into GFP$^-$ and GFP$^+$ populations, representing cells with pre-mesoderm and pre-hemangioblast mesoderm potential, respectively. The strongest evidence in support of this interpretation is provided by the experiment using reaggregation cultures in which each isolated

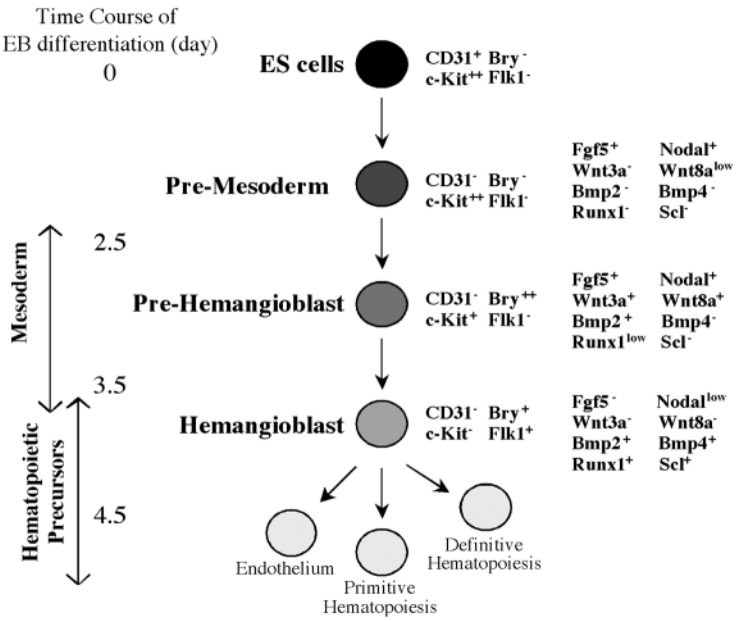

Fig. 7. Model of ES cell differentiation to the BL-CFC.

population was found to differentiate rapidly to the subsequent stage of development. One of the most striking developmental changes was observed with the GFP$^+$Flk1$^-$ population that contained little, if any, BL-CFC potential prior to culture. After culture, a substantial number of the cells upregulated Flk1 and with this change in expression the population acquired the capacity to generate blast cell colonies. This is an important observation as it enables one, for the first time, to access the immediate progenitors of the BL-CFC.

If the GFP$^+$Flk1$^+$ population is indicative of cells emerging from the primitive streak, then the GFP$^+$Flk1$^-$ population should represent cells within the primitive streak, whereas the GFP$^-$Flk1$^-$ population would be equivalent to the pre-gastrulation, pre-streak stage of development. The gene expression profiles of the three populations are consistent with this interpretation. Two of the most restricted expression patterns were observed with the Wnt genes that are upregulated with the onset of brachyury expression and downregulated with the acquisition of Flk1. In the early mouse embryo, expression of *Wnt3a* and *Wnt8a* are overlapping with that of brachyury, both are expressed in the primitive streak and then downregulated with migration and patterning associated with the formation of extra-embryonic mesoderm of the yolk sac (Bouillet et al., 1996; Takada et al., 1994; Yamaguchi et al., 1999). Studies in the chick embryo (Marvin et al., 2001) and in *Xenopus* (Schneider and Mercola, 2001) have implicated *Wnt3a* and *Wnt8* as important molecules in the specification of mesoderm to a hematopoietic fate. When expressed in cells of the cardiac crescent, *Wnt3a* displayed the potential to respecify these cells to a hematopoietic fate. Conversely, when the function of both was blocked in cells fated to the hematopoietic lineage, these cells acquired cardiac potential. Expression of both *Wnt3a* and *Wnt8a* in the GFP$^+$Flk1$^-$ population that contains cells undergoing commitment to the BL-CFC suggests that these factors could have a similar role in the specification of hematopoietic mesoderm in the mouse. The expression pattern of *Fgf5* and *Nodal* is also consistent with the assigned developmental potential of the three populations. In the mouse embryo, *Fgf5* is initially expressed in cells of the epiblast and following gastrulation is found in cells that form the primitive streak and subsequently in the paraxial subpopulation of mesoderm (Haub and Goldfarb, 1991; Hebert et al., 1991). *Nodal* is expressed in the epiblast and primitive endoderm, the primitive streak and ultimately in a subset of cells found in the node (Varlet et al., 1997). Expression of both genes in the GFP$^-$Flk1$^-$ and GFP$^+$Flk1$^-$ populations but not in the GFP$^+$Flk1$^+$ cells would be consistent with a progression from epiblast-like cells to cells representing the primitive streak and finally specification to the hemangioblast.

The isolation of cell populations based on brachyury expression described here together with our previous studies and findings from others provide the basis for a model of mesoderm induction and specification as outlined in Fig. 7. Based on surface marker analysis, the undifferentiated ES cells can be defined as CD31$^+$ Kit$^+$ brachury$^-$ Flk1$^-$. Over a 2.5-3.0-day period of differentiation, the ES cells differentiate and give rise to three different populations, the most immature of which is considered as pre-mesoderm and represented as GFP$^-$Flk1$^-$. The majority of these cells has downregulated CD31 (Fig. 4), but retain some expression of Kit. This population also expressed *Fgf5*, *Nodal* and low levels of *Wnt8a*. The next stage of development, the pre-hemangioblast mesoderm has upregulated brachyury (GFP$^+$) and continues to express Kit. *Fgf5*, *Nodal*, *Wnt3a*, *Wnt8a* and *Bmp2* are expressed at readily detectable levels at this stage. The most mature population, the hemangioblast, continues to express some brachyury, has upregulated Flk1 and downregulated Kit. These cells no longer express *Fgf5* or the Wnt genes, but do express *Bmp2* and *Bmp4* as well as *Runx1* and *Scl*.

Access to populations that represent the pre-hemangioblast stage of development as outlined in Fig. 7 provides a unique approach for studying the induction of mesoderm and its specification to the hematopoietic and vascular lineages. Although previous studies have implicated a role for factors such as BMP4, FGF and activin in the induction of mesoderm in EBs, they were unable to define the precise stage at which these molecules exerted their effect (Faloon et al., 2000; Johansson and Wiles, 1995). With the GFP-Bry ES cells, we will be able to assess the role of these and other factors on distinct developmental steps, the first being the induction of mesoderm as defined by the development of the GFP$^+$Flk1$^-$ population and the second being the specification of mesoderm to the hemangioblast lineages as characterized by the progression to GFP$^+$Flk1$^+$ cells. In addition to defining the role of known factors on mesoderm induction and specification, the large numbers of mesodermal cells accessible with the GFP-Bry model will provide a novel approach for defining new factors and genes that regulate these developmental steps.

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