

A transitional stage in the commitment of mesoderm to hematopoiesis requiring the transcription factor *SCL/tal-1*

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SUMMARY

In this report, we describe the identification and characterization of an early embryoid body-derived colony, termed the transitional colony, which contains cell populations undergoing the commitment of mesoderm to the hematopoietic and endothelial lineages. Analysis of individual transitional colonies indicated that they express *Brachyury* as well as *flk-1*, *SCL/tal-1*, *GATA-1*, *βH1* and *βmajor* reflecting the combination of mesodermal, hematopoietic and endothelial populations. This pattern differs from that found in the previously described hemangioblast-derived blast cell colonies in that they typically lacked *Brachyury* expression, consistent with their post-mesodermal stage of development (Kennedy, M., Firpo, M., Choi, K., Wall, C., Robertson, S., Kabrun, N. and Keller, G. (1997) *Nature* 386, 488-493). Replating studies demonstrated that transitional colonies contain low

numbers of primitive erythroid precursors as well as a subset of precursors associated with early stage definitive hematopoiesis. Blast cell colonies contain higher numbers and a broader spectrum of definitive precursors than found in the transitional colonies. ES cells homozygous null for the *SCL/tal-1* gene, a transcription factor known to be essential for development of the primitive and definitive hematopoietic systems, were not able to form blast colonies but did form transitional colonies. Together these findings suggest that the transitional colony represents a stage of development earlier than the blast cell colony and one that uniquely defines the requirement for a functional *SCL/tal-1* gene for the progression to hematopoietic commitment.

Key words: Embryoid body, Mesoderm, Hematopoiesis, Endothelial, *SCL/tal-1*

INTRODUCTION

The hematopoietic and vascular systems of the mouse develop from extraembryonic splanchnopleuric mesoderm, cells of epiblast origin, which migrate through the primitive streak to the presumptive yolk sac between days 6.5 and 7.0 of gestation. Commitment to the hematopoietic and endothelial lineages within the yolk sac results in the development of discrete structures known as blood islands (Haar and Ackerman, 1971; Russel, 1979). These blood islands are organized into an outer layer of endothelial precursors, also known as angioblasts, surrounding an inner cluster of primitive erythroblasts, a population of large nucleated red blood cells that produce embryonic globins (Barker, 1968; Brotherton et al., 1979). The generation of a single hematopoietic lineage, primitive erythrocytes, within the yolk sac is the defining characteristic of the primitive hematopoietic system of the early embryo. The developing angioblasts of the blood islands rapidly form the first vascular network in the yolk sac through a process known as vasculogenesis. Yolk sac hematopoietic activity declines dramatically between days 10 and 12 of gestation, concomitant with the initiation of intraembryonic hematopoiesis, initially in the paraortic splanchnopleura

(P-Sp)/aorta-gonad-mesonephros (AGM) region and shortly thereafter in the developing fetal liver (Metcalf and Moore, 1971; Godin et al., 1993; Medvinsky et al., 1993; Muller et al., 1994). The transition from yolk sac to fetal liver defines the switch from the single-lineage primitive erythroid program to multilineage hematopoiesis which includes definitive erythropoiesis, myelopoiesis and lymphopoiesis.

While it has long been recognized that the yolk sac blood islands represent the first site of hematopoiesis and vascular development in the mouse embryo, the mechanisms regulating mesodermal commitment to these lineages as well as the relationship of these lineages to one another remain poorly understood. In recent years, these questions have become a major focus in developmental hematopoiesis and are being addressed using a number of different model systems. With respect to mesodermal development and commitment, a number of studies have implicated visceral endoderm as playing a key role at some stage in this process (Wilt, 1965; Miura and Wilt, 1969; Bielinska et al., 1996; Belaoussoff et al., 1998). Although its potential importance is recognized, the precise function of endoderm is not yet fully understood as some studies indicate that it is essential for the commitment of primitive ectoderm to mesoderm fates (Belaoussoff et al.,

1998) whereas others suggest that it plays a role in the formation and organization of yolk sac blood islands and vessels (Bielinska et al., 1996). In addition to the influence of cell populations such as endoderm, findings from studies using *Xenopus* animal cap induction assays, mouse knockouts and embryoid body differentiation have implicated specific molecules including activin, fibroblast growth factors and BMP4, in mesoderm induction, ventralization and blood formation (Smith and Howard, 1992; Hogan et al., 1994; Johansson and Wiles, 1995; Schulte-Merker and Smith, 1995; Winnier et al., 1995; Boucher and Pedersen, 1996; Huber et al., 1998; Xu et al., 1999).

Formation of blood islands in the yolk sac is noteworthy in that commitment of mesodermal precursors results in the simultaneous development of the hematopoietic and vascular lineages in immediate proximity to each other (Haar and Ackerman, 1971). This observation provided the basis for the hypothesis, first put forward in the early 1900s, that these lineages derive from a common bi-potential precursor, a cell termed the hemangioblast (Sabin, 1920; Murray, 1932; and reviewed in Risau and Flamme, 1995). While the identification and isolation of precursors with characteristics of the hemangioblast has been difficult, a number of findings do provide support for the existence of such a cell. The observation that the hematopoietic and endothelial lineages express a number of different genes in common, including *CD34* (Young et al., 1995), *flk-1* (Millauer et al., 1993; Yamaguchi et al., 1993; Eichmann et al., 1997; Kabrun et al., 1997), *flt-1* (Fong et al., 1996), *TIE2* (Takakura et al., 1998), *SCL/tal-1* (Kallianpur et al., 1994), *GATA-2* (Orkin, 1992) and *PECAM-1* (Watt et al., 1995) is one line of evidence in support of the hemangioblast. Additional indirect support has been provided by gene targeting studies in the mouse, which demonstrated that the disruption of *flk-1* (Shalaby et al., 1995, 1997), *SCL/tal-1* (Robb et al., 1995; Shivdasani et al., 1995) and *TGF β 1* (Dickson et al., 1995) all effect the development and growth of both the embryonic hematopoietic and endothelial lineages. Finally, studies in zebrafish have shown that the mutation *cloche* disrupts the development of hematopoietic lineages and endocardium in the embryo (Stainier et al., 1995).

More direct evidence supporting the existence of the bi-potential hemangioblast has come from studies using the in vitro differentiation of embryonic stem (ES) cells as a model of embryonic hematopoiesis. Using this system, we identified a precursor in ES cell-derived embryoid bodies (EBs) that, in response to vascular endothelial growth factor (VEGF), generates blast cell colonies with both hematopoietic and endothelial potential (Choi et al., 1998). The precursor that generates this bi-lineage colony, the blast colony-forming cell (BL-CFC) represents a transient population that develops prior to other hematopoietic populations within EBs and persists for approximately 24 hours. These characteristics of the BL-CFC strongly suggest that it represents the in vitro equivalent of the hemangioblast. In another study, Nishikawa et al. (1998a) demonstrated the presence of precursors with hematopoietic and endothelial potential in Flk-1⁺ populations isolated from differentiating ES cells. Given that the blast colony cells that we identified also express *flk-1*, these precursors are likely similar, if not identical, to the BL-CFC. Although cells with potential similar to the BL-CFC have not yet been identified in

the developing embryo, hematopoietic precursors expressing what are generally considered endothelial-specific markers have recently been isolated from the AGM region at day 11 of gestation (Nishikawa et al., 1998b). Whether or not these precursors also display endothelial potential remains to be determined.

Access to specific early developing cell populations, such as the BL-CFC in the ES/EB system, provides an experimental approach to identify and characterize key molecular events involved in the commitment of mesoderm to the hematopoietic and endothelial lineages. Several observations indicate that the *SCL/tal-1* gene (hereafter referred to as *SCL*), a member of the basic helix-loop-helix (bHLH) transcription factor family, plays an important role in these processes including the development of the putative hemangioblast. First, gene targeting studies show *SCL* to be essential for both primitive and definitive hematopoiesis as well as for the proper remodeling of the primary capillary plexus in the yolk sac (Robb et al., 1995; Shivdasani et al., 1995; Visvader et al., 1998). Second, overexpression of *SCL* in zebrafish embryos leads to expanded expression of both hematopoietic and endothelial markers (Gering et al., 1998). Third, overexpression of *SCL* in zebrafish *cloche* mutant embryos rescues defects in the hematopoietic and endothelial lineages (Liao et al., 1998). Given its role in early yolk sac hematopoiesis and possibly in hemangioblast development, we were interested in determining the function of *SCL* in the establishment of the BL-CFC and commitment to the hematopoietic and endothelial lineages within EBs. Here we report that *SCL*^{-/-} ES cells are unable to generate hemangioblast-derived blast colonies but do give rise to colonies that appear to represent a novel stage in the transition from mesoderm to hematopoietic and endothelial lineage commitment. These colonies, referred to as transitional colonies, are also generated by wild-type ES cells and are easily distinguished by morphological criteria from blast cell colonies and secondary embryoid bodies. The gene expression profile and developmental potential of the transitional colony suggests that it represents a stage of development earlier than the blast colony and one that spans progression from *SCL*-independent to *SCL*-dependent events.

MATERIALS AND METHODS

Growth and differentiation of ES cells

SCL^{-/-} and wild-type embryonic stem cells were maintained on gelatinized flasks in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal calf serum (FCS), penicillin, streptomycin, 1% supernatant containing leukemia inhibitory factor (LIF) and 1.5×10⁻⁴ M monothioglycerol (MTG; Sigma). 2 days prior to the onset of differentiation, cells were transferred to Iscove's modified Dulbecco's medium (IMDM) containing the above components. For the generation of EBs, ES cells were trypsinized into a single-cell suspension and plated at variable densities (300-4500 cells/ml) into differentiation media containing IMDM supplemented with 15% FCS, 2 mM L-glutamine (Gibco/BRL), 0.5 mM ascorbic acid (Sigma), and 4.5×10⁻⁴ M MTG in 60 mm Petri grade dishes. Cultures were maintained in a humidified chamber in a 5% CO₂/air mixture at 37°C.

Methyl cellulose colony assay

For the generation of blast cell and transitional colonies, EB cells were

plated at 0.5×10^5 – 1.5×10^5 cells/ml in 1% methylcellulose supplemented with 10% FCS (Hyclone), vascular endothelial growth factor (VEGF; 5 ng/ml) and 25% D4T endothelial cell conditioned medium (Kennedy et al., 1997). Colonies were scored following 4 days of culture. 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) plus the following cytokines: c-kit ligand (KL; 1% conditioned medium), VEGF (5 ng/ml), erythropoietin (2 U/ml), IL-11 (25 ng/ml), IL-3 (1% conditioned medium), GM-CSF (3 ng/ml), G-CSF (30 ng/ml), M-CSF (5 ng/ml), IL-6 (5 ng/ml), and LIF (1 ng/ml). Cultures were maintained at 37°C in 5% CO₂/air. Primitive erythroid colonies were scored at day 5–6 of culture, whereas definitive erythroid (BFU-E), macrophage, mast cell, granulocyte/macrophage and mixed colonies were counted at 7–10 days of culture. For the liquid expansion cultures, individual secondary EBs, transitional colonies or blast colonies were transferred to matrigel-coated (Collaborative Research) microtiter wells containing IMDM with 10% FCS (Hyclone), 10% Horse Serum (Biocell), VEGF (5 ng/ml), IGF-1 (10 ng/ml), Epo (2 U/ml), bFGF (10 ng/ml), IL-11 (50 ng/ml), KL (1% of the sup), IL-3 (1% conditioned medium), endothelial cell growth supplement (ECGS, 100 µg/ml; Collaborative Research), L-glutamine (1%), and 4.5×10^{-4} M MTG (Sigma). Following 4 days of growth, the entire contents of each well was harvested using trypsin and the cells cultured in 1 ml of methyl cellulose contain the above mixture of cytokines. LIF and c-kit ligand were derived from media conditioned by CHO cells transfected with LIF and KL expression vector, respectively (kindly provided by Genetics Institute). IL-3 was obtained from medium conditioned by X63 AG8-653 myeloma cells transfected with a vector expressing IL-3 (Karasuyama and Melchers, 1988). VEGF, GM-CSF, M-CSF, IL-6 and IL-11 were purchased from R&D systems.

Gene expression analysis

The gene expression analysis was carried out using the RT-PCR method described by Brady et al. (1990). Reverse transcription, poly(A) tailing and PCR procedures were performed as described, with the exception that the X(dT) oligonucleotide was shortened to 5'-GTTAACGCGAGAAATTC(T)₂₄-3'. The amplified products from the PCR reaction were separated on agarose gels and transferred to a Zeta-probe GT membrane (Biorad) or transferred to the membrane with a slot-blot apparatus (Schleicher & Schuell). The resulting blots were hybridized with ³²P randomly primed cDNA fragments (Ready-to-Go Labelling, Pharmacia) corresponding to the 3' region of the genes (for all except *βH1*). A *βH1*-specific probe was prepared by annealing two oligonucleotides, (5'-TGGAGTCAAAGAGGGCATCATAGACACATGGG-3', 5'-CAGTACACTGGCAATCCCATGTG-3') which share an 8-base homology at their 3' termini. This *βH1*-specific oligonucleotide was labeled with ³²P using a Klenow fill-in reaction.

In situ hybridization

Embryoid bodies and transitional colonies were harvested from methylcellulose with a micropipette, washed, and resuspended in a rat tail collagen solution that was subsequently gelled. The enveloped colonies were fixed in freshly prepared 4% paraformaldehyde and embedded in paraffin. Antisense and sense RNA probes were transcribed from a 946 bp fragment of *Brachyury*, a 724 bp fragment of *flk-1* (a gift of Dr Janet Rossant), a 1.7 kb fragment of *SCL* (a gift of Dr Stuart Orkin), and a 2 kb fragment of *GATA-1* (a gift of Dr Gerd Blobel) using a commercially available kit (Riboprobe; Promega) and [³³P]UTP (2000–4000 Ci/mmol; NEN Life Science Products). The transcribed riboprobes were reduced to an average size of 300 bp by limited alkaline hydrolysis then used for in situ hybridization. To evaluate co-localization of the different mRNAs, each of four consecutive 4 µm sections was placed on an individual slide and hybridized with one of the four riboprobes. In situ hybridization was

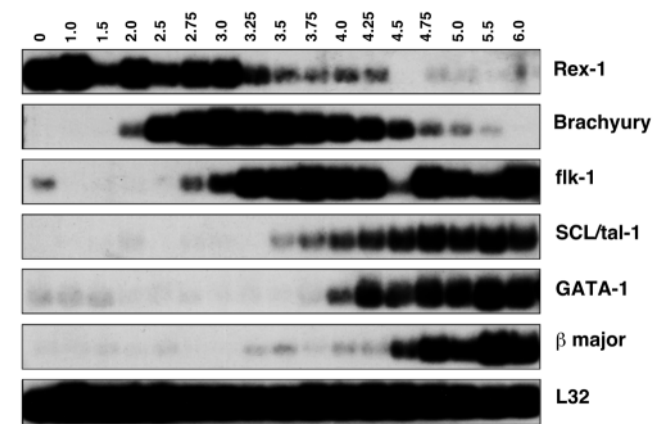
performed as previously described (Deterding and Shannon, 1995). Slides were dipped in NTB-2 emulsion, developed after a period of exposure appropriate for each probe and lightly counterstained with Hematoxylin.

RESULTS

Gene expression analysis during EB differentiation

We have previously reported the identification and characterization of an EB-derived precursor that gives rise to a blast cell colony with both hematopoietic and endothelial potential (Choi et al., 1998). This precursor, the blast colony-forming cell (BL-CFC), which has characteristics of the hemangioblast, is detected within EBs between days 2.75 and 4.0 of differentiation. Given that the endothelial and hematopoietic lineages are of mesodermal origin, we reasoned that mesoderm formation and the events leading to the development of the BL-CFC must be occurring in the differentiating EBs prior to this time. As an initial step in the characterization of molecular events associated with development of the BL-CFC and subsequent hematopoietic and endothelial commitment within the ES differentiation cultures, EBs were harvested at either 24, 12 or 6 hour intervals over a 6-day period and analyzed for gene expression patterns. The genes used for this analysis included *Rex-1*, encoding a zinc-finger transcription factor expressed in totipotent ES cells and downregulated upon their differentiation (Rogers et al., 1991); *Brachyury*, encoding a T-box transcription factor expressed initially in the primitive streak and ingressing mesoderm, and later in the notochord and posterior mesoderm (Herrmann et al., 1990; Wilkinson et al., 1990); *flk-1* (*VEGF-R2*), encoding vascular endothelial growth factor receptor tyrosine kinase 2 expressed in the earliest stages of endothelial and hematopoietic development (Millauer et al., 1993; Yamaguchi et al., 1993; Kabrun et al., 1997); *SCL*, encoding a helix-loop-helix transcription factor expressed in the developing endothelial and hematopoietic lineages (Kallianpur et al., 1994); *GATA-1*, encoding a transcription factor expressed in hematopoietic but not endothelial cells (Orkin, 1992); and *βmajor globin*, a gene expressed only in the erythroid lineage. As shown in Fig. 1, the expression patterns for these genes changed in a dramatic fashion throughout the 6-day EB time course and correlated well with our previous analysis and with the kinetics of hematopoietic commitment as defined by colony assays (Keller et al., 1993; Kennedy et al., 1997).

Rex-1 was found to be expressed in ES cells and then downregulated over the course of 5 days as these cells differentiated and formed EBs, a pattern consistent with previous findings (Rogers et al., 1991). *Brachyury* expression was first detected 48 hours following the onset of differentiation, indicating that mesoderm was induced early within the developing EBs. Expression of *Brachyury* was transient with peak levels found at approximately day 3 of differentiation followed by a gradual decline to undetectable levels by day 6. *flk-1* was first detected by day 2.75, approximately 18 hours following the onset of *Brachyury* expression, suggesting commitment to the first vascular and hematopoietic lineage precursors at or around this time. The onset of *flk-1* expression at day 2.75 is consistent with our



previous findings, which demonstrated the presence of cell surface FLK-1 expression and the emergence of the VEGF-responsive BL-CFC in the EBs approximately 6 hours later (Kabrun et al., 1997; Kennedy et al., 1997). *SCL* expression was found by day 3.5 of differentiation and *GATA-1* was present following a further 12 hours of culture (day 3.75-4.0), suggesting restricted commitment to the hematopoietic system in the EBs at this stage. The onset of expression of these two transcription factors was followed by the expression of *βmajor globin* at approximately day 4.5, defining the beginning of erythropoiesis. This pattern of gene expression observed within the EBs is consistent with the sequence of developmental events found in the mouse embryo whereby extraembryonic mesoderm gives rise to cells of both the hematopoietic and endothelial lineages in the developing yolk sac. The specific timing of the onset of expression of these genes highlights the precise temporal regulation of molecular programs within the differentiating EBs.

Developmental potential of *SCL*^{-/-} ES cells

The above expression analysis demonstrated that the onset of *flk-1* expression, which parallels the development of the bi-potential BL-CFC within the EBs (Kennedy et al., 1997; Choi et al., 1998) preceded *SCL* expression by approximately 18 hours. This sequence of events suggests that *SCL* might not be required for development of the BL-CFC, but rather may be involved in the commitment of these precursors to the hematopoietic lineages, a role consistent with its known requirement for embryonic hematopoietic development (Robb et al., 1995; Shivdasani et al., 1995). To determine the role of *SCL* in

Fig. 1. Gene expression analysis of developing EBs. EBs at the indicated times were pooled and 3' cDNA prepared by RT-PCR. Total cDNAs from the time course (approx. 1-2 µg each) were run side by side on a 1.5% agarose gel, blotted and probed separately with 3' probes from the indicated genes. The time of EB development is shown at the top of the figure. Undifferentiated ES cells are represented as time 0. Hybridization with a 3' probe from the L32 ribosomal protein gene was included to control for amounts of cDNA loaded for each time point.

hemangioblast development, *SCL*^{-/-} ES cells were assayed for their potential to generate the VEGF-responsive BL-CFC. As shown in Fig. 2A, *SCL*^{-/-} ES cells were incapable of generating typical blast cell colonies in response to VEGF, whereas the wild-type *SCL*^{+/+} cells did form such colonies. Although the *SCL*^{-/-} precursors did not generate blast colonies, they did give rise to colonies that clearly differed in morphology from secondary EBs. These colonies, which we will refer to as transitional colonies, have a more ruffled appearance than the secondary EBs, but are more compact and contain larger cells than the blast cell colonies (Fig. 2C). Transitional colonies were most readily detected in the *SCL*^{-/-} cultures. However, upon closer inspection, they were also observed in cultures from wild-type EBs. In addition to the *SCL*^{-/-} and wild-type ES cells analyzed here, other ES cell lines that we have tested including CCE and J1 are able to generate transitional colonies.

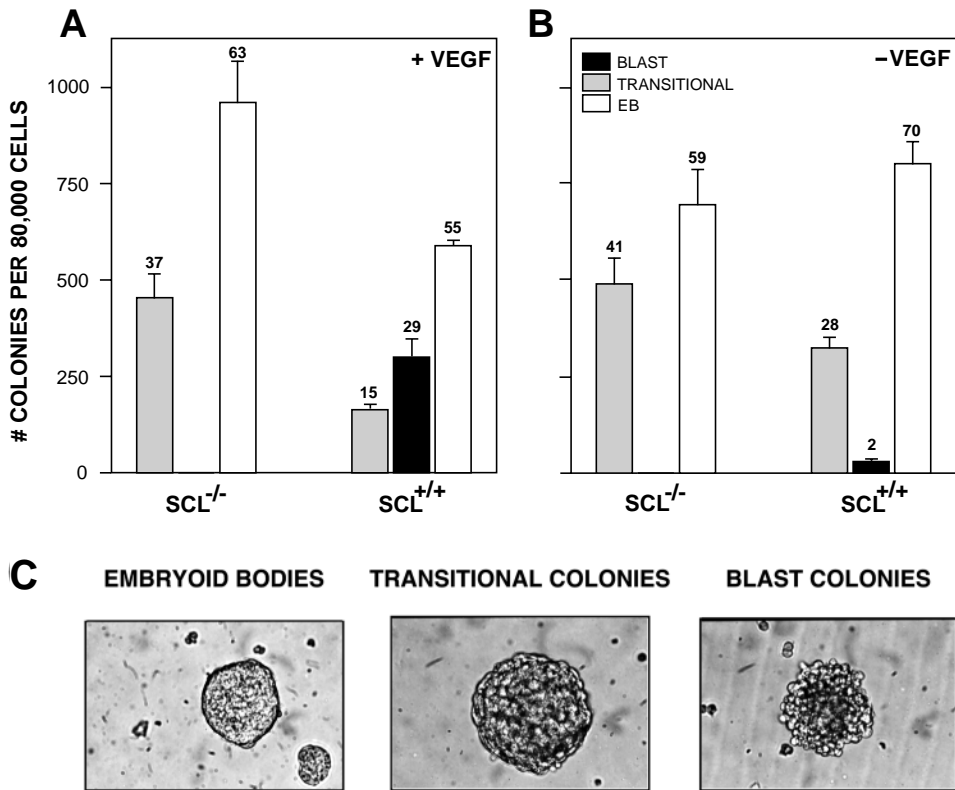


Fig. 2. Replating potential of *SCL*^{-/-} and *SCL*^{+/+} EBs. Day 3.25 EBs generated from the indicated ES cell lines were replated into methylcellulose in the presence or absence of VEGF plus appropriate growth factors and the number and type of colonies scored 4 days later. (A) *SCL*^{-/-} and *SCL*^{+/+} EBs replated in the presence of VEGF. (B) *SCL*^{-/-} and *SCL*^{+/+} EBs replated in the absence of VEGF. Bars represent standard error of the mean of 3 cultures. Numbers above the bars show the percentage of total within that group from a single representative experiment. (C) Morphology of a representative secondary EB, transitional colony and blast colony (original magnification ×200).

As a first step towards defining the relationship of the transitional colonies to the blast cell colonies, we analyzed their VEGF responsiveness. As shown in Fig. 2B, transitional colonies do not require VEGF for growth, as they were readily detected in wild-type cultures with no added cytokine. In fact, cultures without VEGF contained more transitional colonies than those with VEGF. The decrease in the number of transitional colonies with the addition of VEGF was observed consistently and the magnitude of this effect ranged from 1.3 to 4.0-fold [average of 2.2 ± 1.1 (s.e.m.)] in 7 independent experiments. As expected from our earlier findings (Kennedy et al., 1997), the reverse was true for the blast colonies, as their number increased dramatically with the addition of VEGF. The increase in blast colony numbers ranged from 7- to 30-fold in most experiments. The impact of VEGF on the number of secondary EBs was less dramatic than on transitional and blast colonies as we observed no change or a slight increase in their number in 3 of the 7 experiments. In the remaining 4 experiments, the number of secondary EBs did decrease; however, the size of this decrease was less than observed with transitional colonies (range 1.3-1.6 fold, average of 1.5 ± 0.1 -fold). In contrast to wild-type cultures, the number of *SCL*^{-/-} transitional colonies did not show a consistent change with the addition of VEGF. No change was observed in two experiments while a decrease of 1.2-fold was observed in the third.

The proportion of the different types of colonies generated from wild-type EBs did vary in the presence or absence of VEGF, as shown in Fig. 2. In cultures without VEGF, transitional colonies represented between 12% and 41% (average $29 \pm 3.3\%$ from 7 experiments) of the total number of colonies, blast colonies between 0 and 8% (average $3.3 \pm 1.2\%$) and secondary EBs between 50% and 80% (average $67.5 \pm 3.5\%$). With VEGF, the proportion of transitional colonies dropped to between 2 and 16% (average $13 \pm 2\%$), the blast colonies increased to between 17 and 51% (average $39 \pm 6\%$) and the secondary EBs decreased to between 31 and 69% (average $49 \pm 6\%$). As blast cell colonies did not develop in the *SCL*^{-/-} cultures, there was little change in the proportion of the different types of colonies that developed in the presence and absence of VEGF.

The observation that the number of transitional colonies decreased and the number of blast colonies increased with the addition of VEGF to wild-type cultures suggests that a subpopulation of BL-CFC and a subpopulation of precursors that generate the transitional colonies represent similar, if not identical, stages of development. These precursors appear to be *SCL* dependent as they are absent in the *SCL*^{-/-} cultures. Transitional colonies that do develop in the presence of VEGF likely derive from a more immature cell, possibly one that is *SCL* independent. Together these findings are consistent with the interpretation that transitional colonies develop from a population of precursors that exhibits a continuum of developmental potentials ranging from those more immature than the BL-CFC to those similar to BL-CFCs.

If this interpretation is correct, than precursors able to generate transitional colonies should be detected within the developing EBs before BL-CFCs. To test this, a kinetic analysis of transitional and blast cell colony development was carried out. As shown in Fig. 3, transitional colonies were detected at highest levels at the earliest time point tested (d2.0

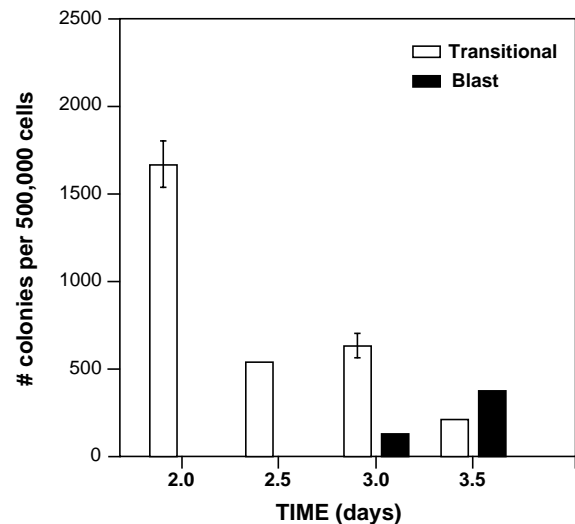


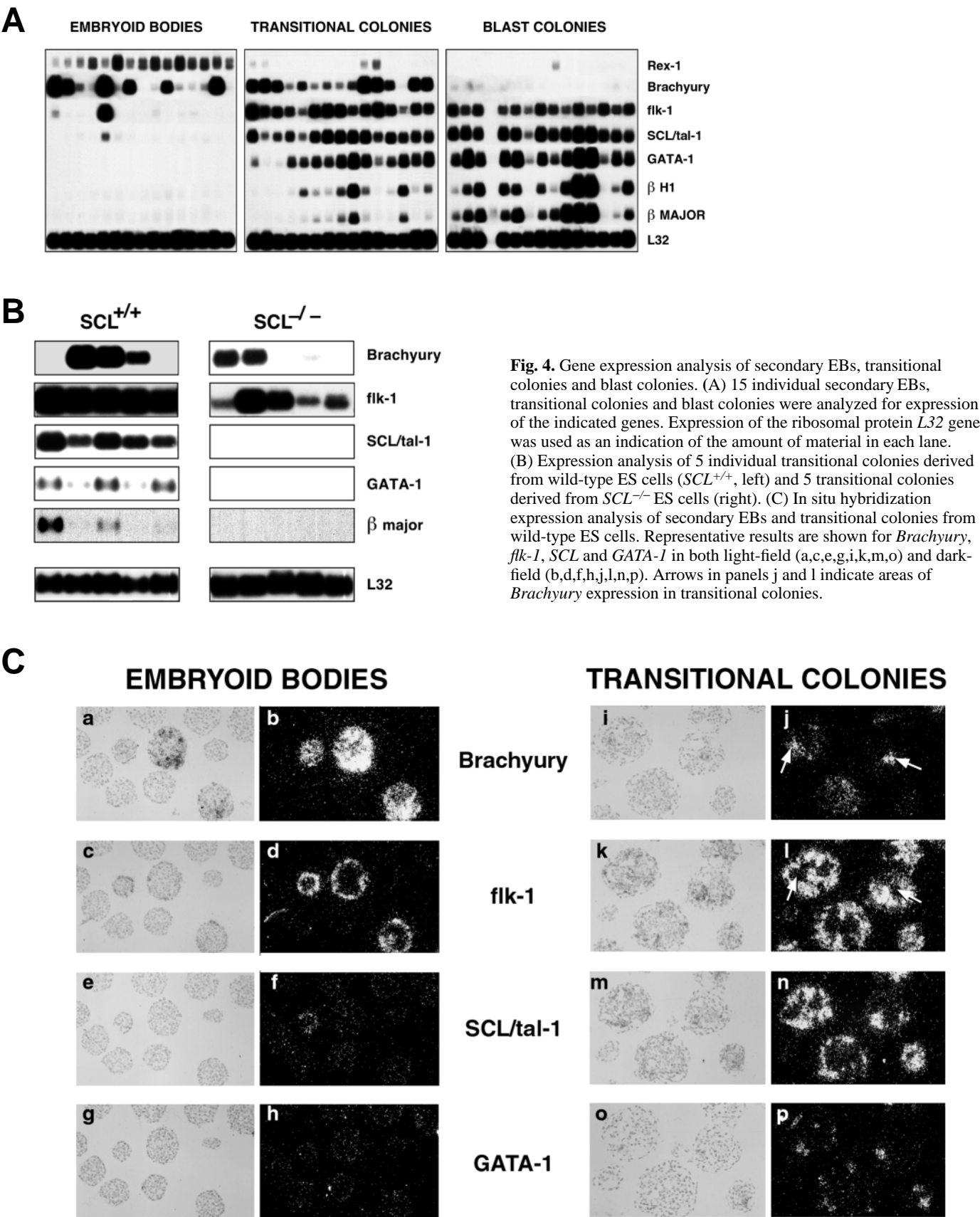
Fig. 3. Kinetics of transitional colony development. EBs differentiated for the indicated times were dissociated and replated in semi-solid medium with VEGF. Cultures were scored 4 days later for the number of blast and transitional colonies. Where visible, bars represent the standard error of the mean of 3 cultures.

primary EBs), and decreased steadily over the time course of the experiment. Blast colonies were not detected in the replated cultures of day 2 and 2.5 EBs, but were found in cultures of day 3.0 EB cells. These findings demonstrate that the precursor that generates the transitional colony develops within the primary EBs prior to the BL-CFC and support the interpretation that the transitional colony represents a developmental stage earlier than the blast cell colony.

Gene expression analysis of the transitional colonies

To further characterize the transitional colonies with respect to developmental status and their relationship to the secondary EBs and blast cell colonies, individual colonies of the three different types were subjected to gene expression analysis with the same panel of probes used for the primary differentiation cultures described in Fig. 1. All secondary EBs analyzed were positive for *Rex-1* and most also expressed variable levels of *Brachyury* (Fig. 4A). Few secondary EBs expressed *flk-1*, one expressed *SCL* and none expressed *GATA-1* nor the embryonic (*βH1*) or adult (*βmajor*) *globin* genes. This pattern of gene expression suggests that many of the secondary EBs have initiated mesoderm induction but most have not yet progressed to the early stages of hematopoietic and endothelial commitment. The expression pattern in these 4-day old secondary EBs differs from that observed in the primary EB cultures at day 4 of differentiation (Fig. 1). This difference is likely due to the fact that the primary cultures are relatively heterogeneous by day 4 of differentiation and consist of colonies with transitional morphology in addition to those of typical EB morphology.

In contrast to the secondary EBs, relatively few transitional colonies expressed *Rex-1*, almost all were *Brachyury* positive, and all expressed *flk-1* and *SCL*. Most transitional colonies expressed *GATA-1*, and more than half also expressed *βH1* and *βmajor* *globins*. Expression of *Brachyury* along with the later



lineage markers is consistent with the interpretation that an individual transitional colony encompasses developmental programs ranging from mesoderm to the early stages of hematopoietic and endothelial commitment and differentiation. These expression patterns also suggest that the extent of hematopoietic commitment in transitional colonies ranges from early (little *GATA-1* and no globin expression) to relatively late stages (*GATA-1* and globin expression). Blast colonies did not express significant levels of *Rex-1* or *Brachyury*. All expressed *flk-1*, *SCL* and *GATA-1*, and most expressed both globins. This pattern is consistent with our previous observations (Kennedy et al., 1997) and indicates that these colonies represent a postmesodermal stage of development and contain mixtures of hematopoietic and endothelial precursors. The expression profile of the three types of colonies clearly supports the interpretation that they represent a developmental progression with transitional colonies bridging the gap between the secondary EBs and the blast cell colonies.

Analysis of the *SCL*^{-/-} transitional colonies demonstrated that they all expressed *flk-1*, with a portion also expressing *Brachyury*. In contrast to the wild-type colonies, *SCL*^{-/-} transitional colonies did not express *SCL*, *GATA-1* or β major globin (Fig. 4B), a finding consistent with earlier studies (Elefanty et al., 1997). This pattern of *Brachyury* expression in which only a portion of the transitional colonies are positive is observed in some experiments and is consistent with the interpretation that these colonies contain cell populations that are undergoing rapid differentiation from mesoderm to cells of the hematopoietic and endothelial lineages. The expression patterns found in the *SCL*^{-/-} transitional colonies suggest that the earliest stages of hemangioblast commitment are occurring, but that subsequent developmental events are blocked.

The gene expression analyses indicate that the transitional colonies consist of mixtures of mesoderm and developing hematopoietic and endothelial precursors. To determine the spatial organization of the cells within the transitional colonies expressing these different genes, in situ hybridization was performed (Fig. 4C). Small clusters of *Brachyury*-expressing mesodermal cells were found in the center of the transitional colonies (Fig. 4Cj,l, arrows), surrounded by *flk-1*⁺ cells that likely represent the developing endothelial and possibly hematopoietic precursors. It is this outer layer of *flk-1*⁺ cells that apparently gives the transitional colonies their distinguishing morphology. Only a subpopulation of *flk-1*⁺ cells expressed *SCL* and even fewer expressed *GATA-1*. These expression patterns support the interpretation that the transitional colonies represent a spectrum of differentiating cells from an inner mesodermal core to the more peripheral populations that express both hematopoietic and endothelial markers. In the secondary EBs, *Brachyury* expression tended to be more evenly spread throughout the colony, while *flk-1* expression, when observed, was seen at the periphery.

The fact that transitional colonies contain mesoderm together with cells expressing genes indicative of the developing hematopoietic and endothelial lineages suggests that induction events involved in these early commitment steps are taking place within these colonies. In forming the first hematopoietic cells in the yolk sac blood islands there is increasing evidence for critical interactions between visceral endoderm and extraembryonic mesoderm (Belaousoff et al.,

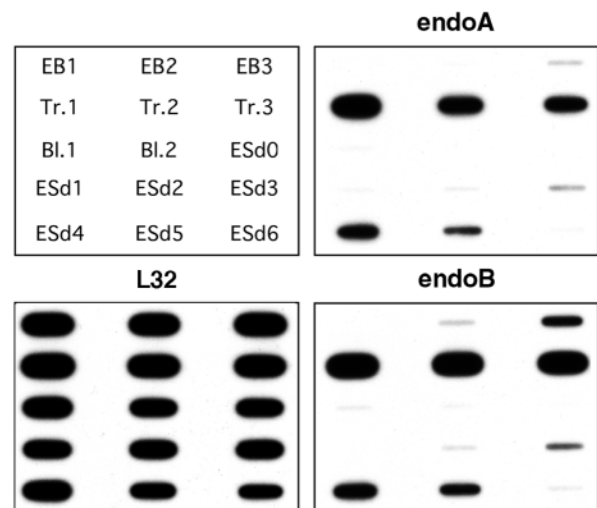


Fig. 5. Expression of extraembryonic endodermal cytokeratin type II (*endoA* and *endoB*). Hybridization of *endoA* and *endoB* 3'-end probes against total 3'-end cDNAs from a subset of secondary EBs, transitional colonies and blast cell colonies presented in Fig. 4A, as well as a subset from the primary EB time course in Fig. 1. The colony samples were selected to form a developmental series as determined by marker gene analysis and had the following expression profiles: EB1: *Rex-1*⁺, *Brachyury*⁻; EB2: *Rex-1*⁺, *Brachyury*^{lo}; EB3: *Rex-1*⁺, *Brachyury*⁺; Tr.1: *Brachyury*⁺, *Flk-1*⁺, *SCL*⁺; Tr.2: *Brachyury*⁺, *Flk-1*⁺, *SCL*⁺, *GATA-1*⁺; Tr.3: *Brachyury*⁺, *Flk-1*⁺, *SCL*⁺, *GATA-1*⁺, globin (β -H1 and β major)⁺; Bl.1 and Bl.2: *Flk-1*⁺, *SCL*⁺, *GATA-1*⁺, globin⁺. ESd0 represents undifferentiated ES cells and ESd1 through ESd6 are embryoid bodies differentiated for 1 through 6 days respectively. Samples were transferred to a nylon membrane using a slot blot apparatus. Expression of the ribosomal protein L32 gene was used as a control for the amount of material loaded in each lane.

1998). To determine if extraembryonic endoderm is present within the transitional colonies, we analyzed them for expression of the extraembryonic endodermal cytokeratin type II genes, *endoA* (Morita et al., 1988) and *endoB* (Singer et al., 1986). Both genes are expressed initially in the trophoblast and later in the epithelium formed by the yolk sac visceral endoderm (Duprey et al., 1985; Hashido et al., 1991). Our analysis also included three secondary EBs (EB1-EB3) representing slightly different stages of development as defined by gene expression, two blast cell colonies and the primary EB time course presented in Fig. 1. As shown in Fig. 5, the expression pattern of the two genes is almost identical, with the highest levels in the transitional colonies and very little, if any, expression in the blast colonies. In addition to the transitional colonies, a low level of *endoA* and *endoB* expression was detected in the most developmentally advanced secondary EBs (EB3). Both genes showed a transient expression pattern over the primary EB time course, with onset between days 2-3, peak levels at day 4 and extinction between days 5 and 6. This pattern is slightly delayed compared to that observed for *Brachyury* (Fig. 1). These analyses demonstrate that cells that express the endoderm markers *endoA* and *endoB* are present in the transitional colonies and further support the interpretation that these colonies span developmental stages in which mesoderm is induced to form the endothelial and hematopoietic lineages.

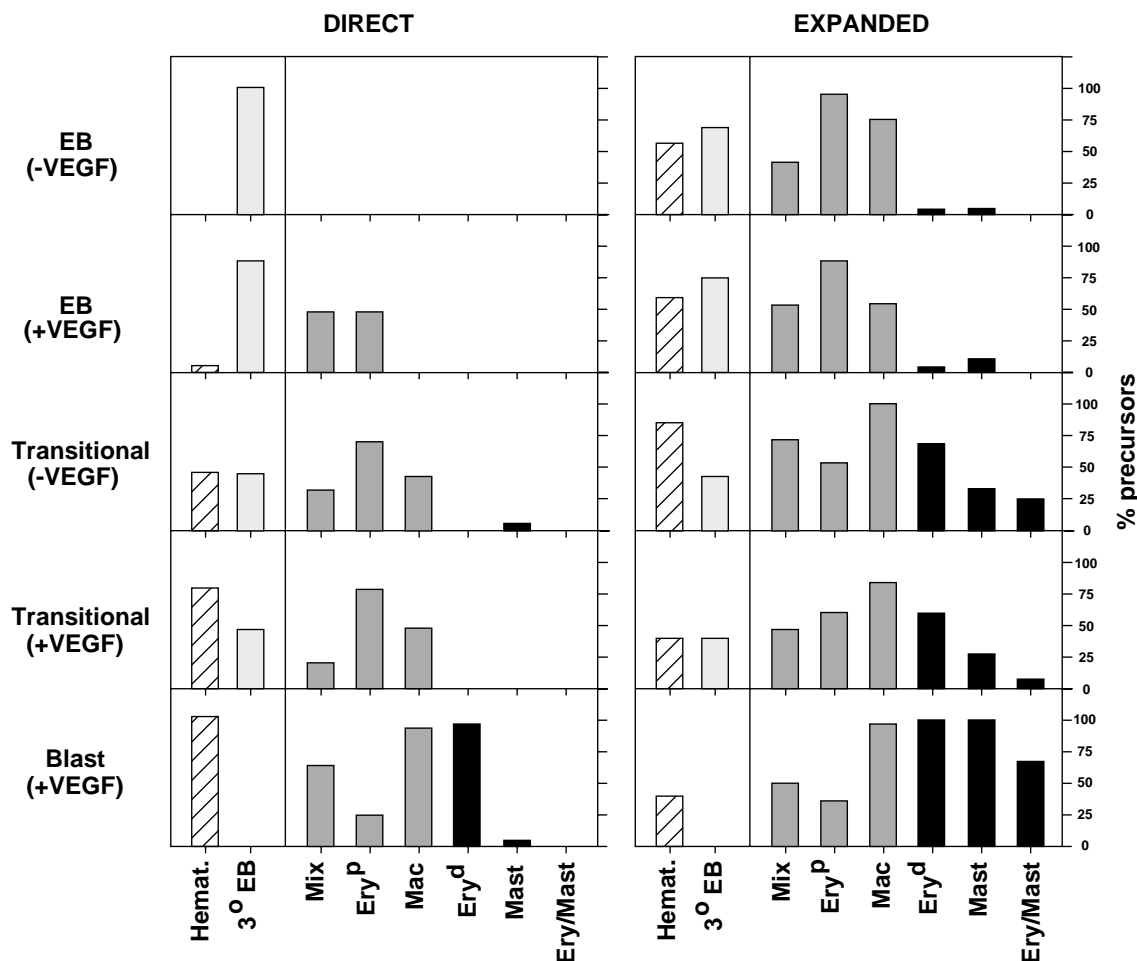


Fig. 6. Hematopoietic potential of secondary EBs, transitional colonies and blast cell colonies. The two bars to the left of the dividing line in each panel measure the proportions of the indicated cultures with hematopoietic precursors (hatched bars) and/or tertiary EBs (light gray bars). Cultures were scored positive for hematopoiesis if they contained one or more hematopoietic colonies. Percents may not equal 100 as some cultures gave rise to both tertiary EBs and hematopoietic colonies. The bars to the right of the dividing line in each panel represent the distribution of precursors in the cultures that were scored positive for hematopoiesis. Gray shaded bars represent the early stage precursors, while the black bars represent the more advanced definitive precursors. Twenty individual colonies were analyzed for each set of conditions. Mix, multilineage colonies consisting of cells of the definitive erythroid, macrophage, neutrophil and mast cell lineages; Ery^P, primitive erythroid colonies; Mac, macrophage colonies; Ery^d, definitive erythroid colonies; Mast, mast cell colonies; Ery/Mast, bi-lineage definitive erythroid and mast cell colonies. The left panel represents the potential following direct replating of the colonies (Direct) whereas the right panel is the potential following the 4-day expansion culture (Expanded).

Hematopoietic potential of transitional colonies

To further define the developmental stage represented by the EBs, the transitional colonies and the blast cell colonies, individual colonies of each type were analyzed for hematopoietic potential based on precursor content as assayed by colony growth in methylcellulose (Fig. 6). For this analysis, colonies were either dissociated and replated into the hematopoietic colony assay ('direct replating') or first cultured for 4 days in liquid and then replated in methylcellulose ('expanded replating'). This 4-day culture period was designed to expand and/or mature the hematopoietic potential of the colonies. As VEGF has been shown to impact the development of a subpopulation of transitional colonies, those generated in the presence and absence of this cytokine were assayed. Secondary EBs generated in both sets of conditions were also included in this analysis.

When replated directly into methylcellulose cultures, secondary EBs generated either in the presence or absence of

VEGF gave rise almost exclusively to tertiary EBs. When the secondary EBs were maintained in culture for an additional 4 days prior to replating, approximately 50% of them from cultures with or without VEGF generated hematopoietic precursors. Three different types of precursors were consistently detected upon replating the expanded secondary EBs: primitive erythroid (Ery^P), macrophage (Mac) and multipotential (Mix), which gave rise to multilineage colonies consisting of cells from the definitive erythroid, macrophage, neutrophil and mast cell lineages. This combination of precursors, which is similar to that observed at the onset of hematopoietic development in the primary EBs, is indicative of the early stages of hematopoietic commitment and therefore will be referred to collectively as early stage precursors.

Transitional colonies showed greater hematopoietic potential than the EBs in the direct replating assay, as a significant proportion were found to contain the same early stage

precursors as detected in the EB expansion cultures. VEGF appeared to influence the hematopoietic potential of the transitional colonies as a higher proportion (~75%) of those grown in the presence of the cytokine gave rise to hematopoietic colonies upon replating compared to those grown in its absence (~50%). Following the 4-day culture/expansion period, the transitional colonies generated a broader spectrum of precursors, which now included definitive erythroid (Ery^d), mast cell (Mast) and bipotential erythroid/mast (Ery/Mast) precursors in addition to the early stage precursors found in the direct replated cultures. The presence of definitive erythroid, mast cell and definitive erythroid/mast cell precursors indicates an expansion of the definitive hematopoietic program in these cultures. Again, VEGF appeared to influence the hematopoietic potential of the colonies; however, in this case, the effect was reversed compared to direct replating studies as a higher fraction (~75%) of those grown in the absence of VEGF contained hematopoietic precursors compared to those grown with VEGF (~50%). A portion of directly replated and expanded transitional colonies grown with or without VEGF generated colonies with an EB-like morphology. It is unclear, however, if these are true EBs or if they represent a colony comparable to secondary transitional colonies, as they develop in a broad mix of cytokines, conditions that differ significantly from the primary replated cultures.

Almost all of the blast colonies generated secondary hematopoietic colonies following direct replating. Analysis of these colonies indicated that the blast colonies contained the early stage precursors as well as definitive erythroid precursors. The definitive component of the blast cell colonies, including definitive erythroid, mast cell and definitive erythroid/mast cell, expanded considerably over the 4-day culture period. The drop in the frequency of blast cell colonies with hematopoietic potential following the expansion culture is likely due to the fact that the hematopoietic component of some of these colonies has matured beyond the precursor stage. Indeed, visual inspection of the cultures at this stage indicated that most had discernable mature hematopoietic cells prior to replating. In contrast to the secondary EBs and transitional colonies, replating of the blast colonies did not result in the generation of tertiary EBs. Taken together, the findings from this analysis confirm and support the interpretation that the transitional colonies represent a stage of development between the secondary EBs and the blast cell colonies. This relationship is most readily demonstrated by the fact that the 4-day culture period promotes the maturation of one type of colony to a developmental stage comparable to the next type of colony. For example, the hematopoietic precursor content of the expanded EBs was similar to that of the direct replated transitional colony and the expanded transitional colony was similar to that of the direct replated blast cell colony.

Given that the transitional colonies appear to represent a stage of development earlier than the blast colonies, and that they consist of a spectrum of cells ranging from mesoderm to endothelial and hematopoietic precursors, one would predict that they would also contain BL-CFC. To test for this, pools of transitional colonies were picked, the cells dissociated and replated in blast colony conditions. An aliquot of the population was also replated in conditions used in the above analysis to monitor the hematopoietic precursor content of the transitional colonies. Pooled blast cell colonies were picked and replated

Table 1. Generation of secondary colonies from transitional and blast colonies

Type of colony replated	Cell no. per colony	Secondary colonies per 1×10 ⁵ Cells				
		Blast	Ery ^p	Mac	Mix	Ery ^d
Transitional	750	166±15	1170±16	210±19	58±5	14±2
Blast	375	0.5±0.5	15±2	784±40	32±7	112±12

Numbers represent mean ± standard error. Transitional and blast colonies for replating were generated from day 3.25 primary EBs.

under the same two sets of conditions. As shown in Table 1, transitional colonies did generate blast colonies upon replating at a frequency of 166 per 1×10⁵ cells plated or approximately 1 BL-CFC per transitional colony. This frequency is not unexpected given the heterogeneity of the transitional colonies. The hematopoietic precursor content of the pooled colonies was as expected, consisting predominantly of the early stage precursors. To analyze the potential of the transitional derived-blast cell colonies, a representative sample was picked and replated in liquid cultures in conditions that support the growth of both the hematopoietic and endothelial lineages (Choi et al, 1998). Approximately 50% of these replated colonies generated both hematopoietic and adherent cells (endothelial) indicating that they were bi-lineage with a developmental potential similar to that of primary blast colonies (not shown). In contrast to the transitional colonies, pools of primary blast colonies did not generate significant numbers of secondary blast colonies, whereas they did give rise to the expected range of hematopoietic colonies. These analysis further demonstrate that the transitional colonies do indeed represent a stage of development earlier than the blast colonies. In addition they show that precursors of the blast colony, the BL-CFC, are generated during the formation of the transitional colonies.

The three colony types, secondary EBs, transitional and blast colonies, each assumed a unique morphology within 24 hours when plated intact into the liquid expansion cultures (Fig. 7A). Blast cell colonies generated both adherent and non-adherent cells, which we have previously shown to include endothelial cells and hematopoietic precursors, respectively (Choi et al., 1998). Transitional colonies also gave rise to two morphologically distinct cell types; an outer flat adhesive layer surrounding an inner compact core. EBs generated an adherent colony of cells with no obvious distinct subpopulations, similar to a colony of undifferentiated ES cells. Following 4 days of culture, both the adherent and non-adherent population of the blast cell colony expanded. The expansion of the non-adherent hematopoietic population is not obvious in the Fig. as these cells tended to float off the adherent layer when the culture dish was manipulated for photography. The adherent layer of the transitional colony also expanded over the 4-day culture period, whereas the inner core generated round non-adherent cells which, when assayed, were found to contain hematopoietic precursors (not shown). After 4 days of culture the EBs generated distinct subpopulations, which included an adhesive layer surrounding an inner core of cells that was somewhat more densely packed and morphologically more heterogeneous than that generated by the transitional colony. Transitional colonies generated from *SCL*^{-/-} ES cells also gave rise to the adherent and inner core populations (not shown). However, in this case, the core did not give rise to the round

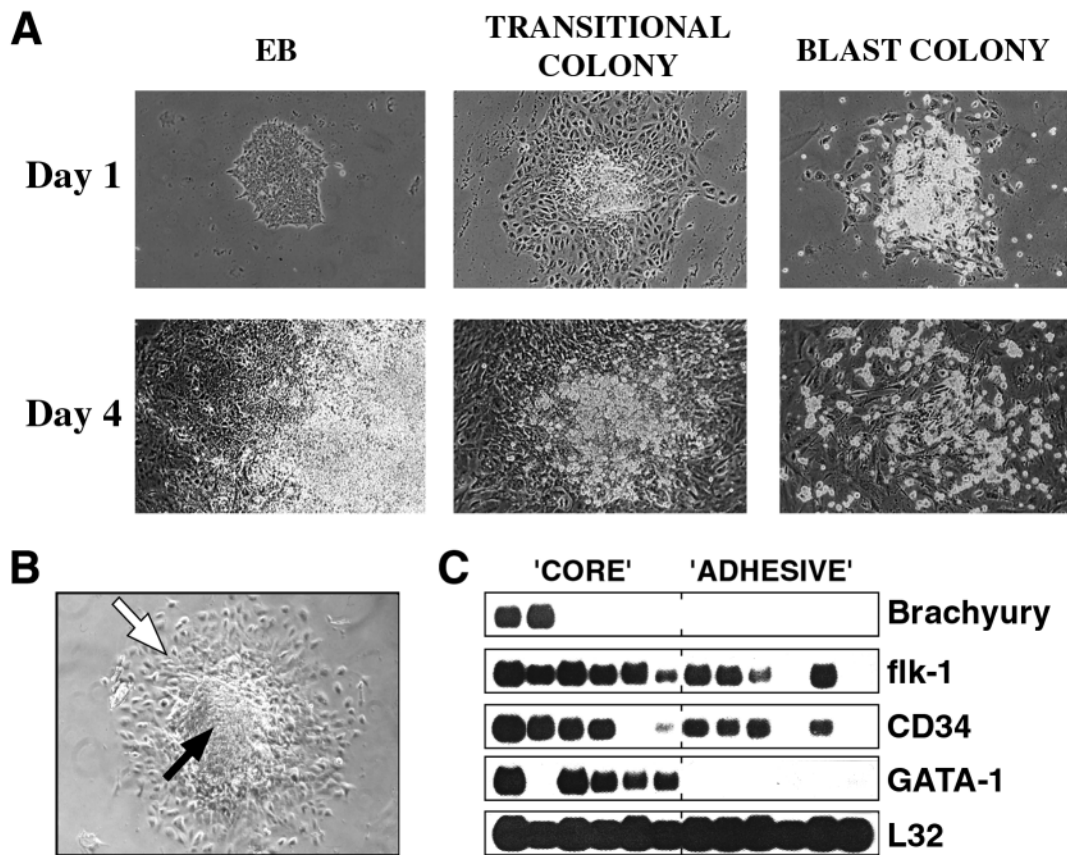


Fig. 7. Development of secondary EBs, transitional colonies and blast cell colonies in liquid culture. (A) Morphology of individual intact secondary EBs, transitional colonies and blast cell colonies following 1 and 4 days of culture in liquid medium. (original magnification at day 1, $\times 200$; at day 4, $\times 320$) (B) Segregation of a transitional colony into an inner core (black arrow) and outer adhesive population (white arrow) following 1 day of culture. (C) Expression analysis via 3' RT-PCR of the core and adhesive populations isolated from 6 transitional colonies following 1 day of culture.

nonadherent cells over the 4-day culture, a finding consistent with their inability to generate hematopoietic progeny.

To further characterize the adherent and core populations (depicted by white and black arrows respectively, Fig. 7B) from cultured transitional colonies, they were physically separated by micromanipulation and subjected to gene expression analysis. As shown in Fig. 7C, two of the six core samples analyzed expressed *Brachyury*, indicating that they retained some mesodermal potential following the 24-hour culture period. All six core samples expressed *flk-1*, and five out of six expressed *CD34* and *GATA-1*. None of the adherent cell populations expressed *Brachyury* or *GATA-1*, whereas four out of six analyzed express both *flk-1* and *CD34*. Analysis of the adherent cells from the *SCL*^{-/-} transitional colonies indicated that they also expressed *flk-1* (not shown). The findings from this analysis indicate that the core portion of the cultured wild-type transitional colonies represents a mixture of mesodermal cells as well as developing hematopoietic and endothelial populations. The expression profile of the adherent populations is consistent with them being of the endothelial lineage.

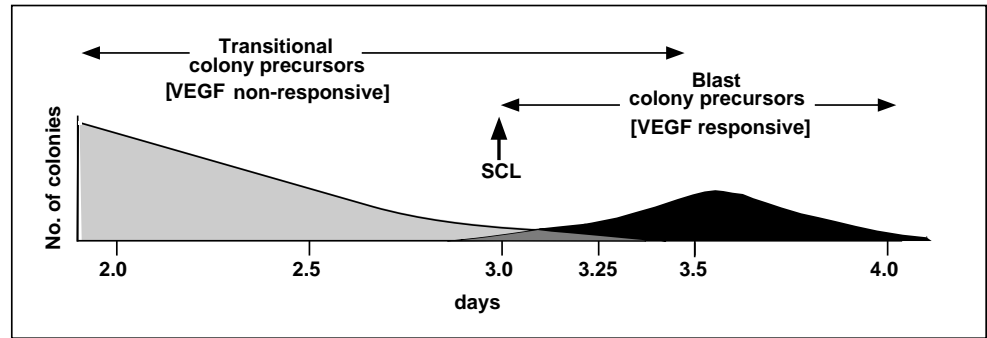
DISCUSSION

In this report, we describe the identification and characterization

of an embryoid body-derived colony that consists of developing cell populations representing the commitment of mesoderm to the hematopoietic and endothelial lineages. Gene expression analyses as well as functional studies strongly support the interpretation that the transitional colonies represent a developmental stage earlier than our previously described blast cell colonies but more advanced than an age-controlled secondary embryoid body. Analysis of the *SCL*^{-/-} ES cells indicates that the transitional colony uniquely defines the stage at which this gene plays an essential role in the development of the embryonic hematopoietic lineages.

The development of transitional colonies from *SCL*^{-/-} ES cells is consistent with the known role of this gene in embryonic hematopoiesis and vasculogenesis. *SCL*^{-/-} embryos do not generate any hematopoietic cells but do form early endothelial cells (Robb et al, 1995; Shivdasani et al, 1995). However, these *SCL*^{-/-} endothelial cells appear to have specific defects, as they are unable to undergo remodeling of the primary capillary plexus in the yolk sac (Visvader et al., 1998). Transitional colonies derived from *SCL*^{-/-} ES cells are unable to generate hematopoietic precursors but do form cells with endothelial characteristics. These endothelial cells could be identical to those that develop in the *SCL*^{-/-} embryos and may display similar defects in remodeling potential if assayed appropriately. At the present time, it is unclear if these early

Fig. 8. Model depicting transitional and blast cell colony development. Light and dark shaded area represent the stages of transitional and blast colony development respectively. The intermediate shaded area represents the stage of development when the transitional colony number is affected by VEGF. The proposed stage at which SCL is required is indicated.



developing endothelial cells present in the *SCL*^{-/-} transitional colonies represent a subpopulation distinct from those generated at later stages. The developmental potential and gene expression profile of the *SCL*^{-/-} and wild-type transitional colonies suggests that commitment to the hematopoietic and endothelial lineages through the hemangioblast takes place in several distinct stages. The initial stage, defined by the onset of *flk-1* expression and the establishment of the first population of endothelial cells, does not require *SCL*. The next stage involving the development of the earliest hematopoietic precursors and possibly other populations of endothelial cells is clearly dependent on a functional *SCL* gene. In the context of such a model, transitional colonies would be representative of the first stage of development whereas the blast cell colonies would more accurately reflect the second stage.

The in situ hybridization results demonstrate that transitional colonies exhibit a considerable degree of three-dimensional organization. Mesodermal cells expressing the transcription factor *Brachyury* are located towards the center of the colony, whereas cells expressing *flk-1* and/or *SCL* that likely represent the developing endothelial and hematopoietic precursors are positioned towards the periphery. The segregation of distinct populations within the transitional colonies, implicated by the in situ analysis, was confirmed by the liquid culture expansion experiments. Within 24 hours of culture, the transitional colony generated two distinct populations; (1) an adhesive layer of cells, which expressed a number of different endothelial markers, that formed around (2) a distinct compact cluster of cells, which expressed markers of mesoderm and developing hematopoietic and vascular cells. This structural organization may reflect important cell-cell interactions required within the developing colony for the induction of mesoderm to the hematopoietic and endothelial fates. In this regard, it is notable that cells expressing genes indicative of extraembryonic endoderm (*endoA* and *endoB*) were detectable within the transitional colonies, as endoderm-mesoderm interactions are thought to be important for specific mesodermal commitment events within the developing embryo. The combination of mesoderm, endoderm and developing blood cell and endothelial precursors within a single colony suggests that these structures could represent a rich source of molecules involved in the commitment of the hematopoietic and vascular lineages.

Most of the evidence presented in this study is consistent with the interpretation that the secondary EBs, the transitional colonies and the blast cell colonies represent a continuum of developmental stages in the generation of hematopoietic and

endothelial lineages during embryogenesis. While one must exercise caution when interpreting findings from in vitro model systems, it is possible to draw certain parallels between the stages of development represented by the three types of colonies and specific stages of development in the normal embryo. The secondary EBs contain cells ranging from pre-mesodermal (*Brachyury* negative) to the earliest stages of mesoderm commitment (*Brachyury* positive). With these expression profiles, the populations found within these colonies would be representative of the epiblast cells undergoing commitment to mesoderm early during gastrulation. Virtually all transitional colonies analyzed have undergone some level of hematopoietic and endothelial commitment as defined by gene expression patterns. The extent of hematopoietic differentiation ranges from early stages of development to those showing clear signs of erythropoiesis. While the transitional colonies do contain developing hematopoietic and endothelial populations, many cells are not yet fully committed to these programs as some retain mesodermal properties as defined by *Brachyury* expression. This mesodermal component appears to have potentials that extend beyond that of the hematopoietic and endothelial lineages as preliminary studies indicate that some transitional colonies are able to generate cardiomyocytes (unpublished observation). The potential of the transitional colonies suggests that they span the developmental spectrum from pre-yolk sac to the early stages of blood island development in the mouse embryo. Blast cell colonies, the most advanced of the three types of colonies, consist of developing endothelial and hematopoietic precursors and likely represent an entirely postmesodermal stage of development as they are unable to generate cardiomyocytes (Choi et al., 1998). Given this potential, they are similar to the early populations that are found within the yolk sac blood islands.

While many aspects of the three different colony types indicate that they represent distinct stages of development, it is clear that there is some overlap in potential as well, in particular between the transitional and blast cell colony. As the transitional colonies grow and develop, they give rise to BL-CFC as well as a subpopulation of precursors found in the blast cell colonies. Most evidence presented in this study would support the interpretation that the transitional colonies develop from precursors that represent a stage of development earlier than the BL-CFC as indicated in the model in Fig. 8. However, the observed effect of VEGF on certain transitional colonies from later stages of EB development suggests that they can derive from a broader spectrum of precursors ranging in

developmental status from a pre-BL-CFC to a stage similar, if not identical to, the BL-CFC. These precursors would develop within the primary EBs at approximately day 3.0 of differentiation, with the appearance of the BL-CFC. In the presence of VEGF, these precursors give rise to a blast cell colony, whereas in its absence, they retain the capacity to generate a transitional colony. While a subpopulation of BL-CFC may be identical to precursors that can also generate transitional colonies, the majority appear to represent a different stage of development as the increase in the number of blast colonies is often 5- to 15-fold greater than the decrease in the number of transitional colonies when VEGF is added to the cultures. The *SCL* gene appears to be essential for the maturation of the transitional precursor to the BL-CFC as no blast cell colonies are generated in replated cultures of *SCL*^{-/-} EBs and VEGF has no effect on the number of transitional colonies in these cultures.

In summary, the identification and characterization of the transitional colony further demonstrates the potential of the ES/EB system in defining unique developmental stages and in providing access to rare precursor populations representing them. These transitional colonies consist of different populations undergoing commitment to the hemangioblast and further to the developing hematopoietic and endothelial lineages. In addition, they represent the transition from SCL-independent to SCL-dependent differentiation. As such, access to these colonies will allow a molecular analysis of the commitment of mesoderm to the hematopoietic and endothelial systems and the role played by SCL in these processes.

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