

# Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse

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## SUMMARY

In this study, we have mapped the onset of hematopoietic development in the mouse embryo using colony-forming progenitor assays and PCR-based gene expression analysis. With this approach, we demonstrate that commitment of embryonic cells to hematopoietic fates begins in proximal regions of the egg cylinder at the mid-primitive streak stage (E7.0) with the simultaneous appearance of primitive erythroid and macrophage progenitors. Development of these progenitors was associated with the expression of *SCL/tal-1* and *GATA-1*, genes known to be involved in the development and maturation of the hematopoietic system. Kinetic analysis revealed the transient nature of the primitive erythroid lineage, as progenitors increased in number in the developing yolk sac until early somite-pair stages of development (E8.25) and then declined sharply to undetectable levels by 20 somite pairs (E9.0). Primitive erythroid progenitors were not detected in any other tissue at any stage of embryonic development. The early wave of primitive erythropoiesis was followed by the appearance of definitive erythroid progenitors (BFU-E) that were first detectable at 1-7 somite pairs (E8.25) exclusively within the yolk sac. The appearance of BFU-E was followed by the

development of later stage definitive erythroid (CFU-E), mast cell and bipotential granulocyte/macrophage progenitors in the yolk sac. *C-myb*, a gene essential for definitive hematopoiesis, was expressed at low levels in the yolk sac just prior to and during the early development of these definitive erythroid progenitors. All hematopoietic activity was localized to the yolk sac until circulation was established (E8.5) at which time progenitors from all lineages were detected in the bloodstream and subsequently in the fetal liver following its development. This pattern of development suggests that definitive hematopoietic progenitors arise in the yolk sac, migrate through the bloodstream and seed the fetal liver to rapidly initiate the first phase of intraembryonic hematopoiesis. Together, these findings demonstrate that commitment to hematopoietic fates begins in early gastrulation, that the yolk sac is the only site of primitive erythropoiesis and that the yolk sac serves as the first source of definitive hematopoietic progenitors during embryonic development.

Key words: Hematopoiesis, Yolk sac, Progenitor, Primitive streak, Definitive, Mouse

## INTRODUCTION

The hematopoietic system is established at specific sites in the embryo through a succession of developmental programs that result in the generation of distinct precursor colony-forming cells (CFC) that in turn produce all the differentiated blood cells that enter the circulation. The first hematopoietic cells in the mouse embryo arise in blood islands that form from extraembryonic mesoderm at the neural plate stage of development at embryonic day 7.5 (E7.5) (Haar and Ackerman, 1971). These primitive erythroblasts derived from the yolk sac represent the predominant mature blood cells in the early circulation and differ from erythrocytes derived later from the fetal liver and bone marrow. Primitive erythroblasts

are large, nucleated cells that synthesize embryonic globins (Barker, 1968; Steiner and Vogel, 1973). Definitive erythropoiesis is established in the liver beginning at 28-32 somite pairs (E9.5) (Houssaint, 1981), and results in the generation of small, enucleate erythrocytes that synthesize the adult forms of globin. In contrast to the yolk sac's restricted hematopoiesis, fetal liver hematopoiesis is multilineage and includes the differentiation of definitive erythroid, myeloid, megakaryocyte and lymphoid cells (Metcalf and Moore, 1971). Late in fetal life, hematopoietic activity initiates in the bone marrow which, shortly after birth, becomes the predominant site of blood cell production for the duration of postnatal life.

The early development of hematopoietic activity in the yolk sac led to the hypothesis that this tissue is the primary site of

hematopoietic development and that stem cells derived from it seed intraembryonic sites (Moore and Owen, 1967a). This concept gained initial support from studies in the chick demonstrating that yolk-sac-derived precursors were able to repopulate the hematopoietic system of recipient embryos (Moore and Owen, 1967b). Additional support was provided by studies in the mouse, which indicated that yolk sac, but not embryo proper, from precirculation embryos developed hematopoietic activity when maintained in culture for short periods of time (Moore and Metcalf, 1970) and from kinetic analysis, which demonstrated that progenitors develop in the yolk sac prior to the embryo proper (Wong et al., 1986a).

The yolk sac origin of hematopoiesis was challenged by embryo/yolk sac grafting experiments in the chick that provided convincing evidence that definitive hematopoiesis originated from an intraembryonic site and not from the yolk sac (Dieterlen-Lievre, 1975; Beaupain et al., 1979; Lassila et al., 1982). Studies on amphibian embryos have also defined two distinct sites of hematopoiesis, a ventral mesodermal site that gives rise to primitive erythrocytes and a dorsal lateral plate mesodermal site that gives rise to adult hematopoietic cells (Turpen et al., 1981). These findings led to the concept of two distinct sites of embryonic hematopoiesis, the yolk sac for the generation of primitive erythroblasts and an intraembryonic site for the establishment of definitive hematopoiesis. In recent years, putative intraembryonic sites of hematopoiesis have been identified in the mouse embryo. The region known at early somite-pair stages as the paraaortic splanchnopleura (P-Sp) and slightly later as the aorta-gonad-mesonephros (AGM) was found to contain multipotential cells as early as E8.5 (Godin et al., 1995), day 8 spleen colony-forming units (CFU-S8) by E9.5 (Medvinsky et al., 1996) and stem cells capable of repopulating adult recipients by E10.5 (Muller et al., 1994). These findings have been interpreted to further support the notion that embryonic hematopoiesis initiates in two sites, the yolk sac and the P-Sp/AGM (Dzierzak et al., 1998).

The identification of these intraembryonic hematopoietic sites has raised questions as to the ultimate potential of the yolk sac and its contribution to the hematopoietic system of the fetus and adult. It is well accepted that the yolk sac is the site of primitive erythropoiesis; however, other potentials of this tissue are not well resolved. While erythroid and macrophage progenitors have been found in unstaged mouse embryos as early as E7.5 (Wong et al., 1986a; Rich, 1992), it is not known if they are spatially restricted to the developing yolk sac or intraembryonic tissues. Furthermore, earlier developmental times and staged embryos during gastrulation have not been investigated, so that the origin of hematopoietic progenitors has not been elucidated.

In order to better define the onset of hematopoietic potential and the progenitor composition within the mammalian embryo, we mapped the spatial and temporal development of primitive and definitive hematopoietic precursors during gastrulation and early organogenesis in the mouse. We have identified two distinct waves of committed hematopoietic progenitors that arise prior to the onset of the circulation. The first is a transient primitive erythroid wave that emerges soon after the start of mesoderm formation in the proximal regions of the egg cylinder and remains confined to the yolk sac. It is followed by a second wave of definitive erythroid and myeloid progenitors that arise in the yolk sac and are found soon

thereafter in the bloodstream of the embryo proper. Our results indicate that the yolk sac serves as the exclusive source of committed hematopoietic progenitors prior to the onset of circulation and likely represents the predominant source of definitive hematopoietic progenitors that initially colonize the fetal liver.

## MATERIALS AND METHODS

### Mouse tissues

Timed pregnant outbred Swiss Webster mice were purchased from Taconic (Germantown, NY). At specified times, mice were killed by cervical dislocation and the uteri were removed from the peritoneum and washed with several changes of phosphate-buffered saline (PBS). Embryos were dissected free of decidual tissues and Reichert's membrane in IMDM/PBS with 0.2% delipidated BSA using #5 watchmakers forceps (Dumoxal; Electron Microscopy Science, Ft. Washington, PA). Vestiges of maternal blood cells were removed by multiple transfers of tissues into fresh medium during the dissection process. Presomite embryos (E6.5-E7.5) were staged and grouped according to established morphological criteria (Downs and Davies, 1993). Somite stage embryos (E8.25-E11.5) were grouped according to somite number.

Prestreak and early primitive streak embryos (E6.5) were maintained intact. Mid to late primitive streak (E7.0), and neural plate embryos (E7.5) were trisected into yolk sac, proximal embryo proper (pep) and distal embryo proper (dep, Fig. 1). After removal of the chorion, early somite-pair embryos (E8.5-E9.0) were separated into yolk sac, allantois, rostral embryo proper (headfolds, anterior somites, and heart = "head") and caudal embryo proper (posterior somites and tail region = "tail"). Later stage embryos were separated into extraembryonic membranes (yolk sac and amnion = "yolk sac"), rostral embryo proper (headfolds, branchial arches and heart = "head"), mid embryo proper (anterior somites to hindlimb region = "P-Sp/AGM"), caudal embryo proper (posterior somites and tail region = "tail") and blood. Most of the blood was derived from the larger vessels of the embryo proper. At E10.5 and E11.5, the liver was isolated separately from the remainder of the embryo proper. Dissected tissues obtained from each embryonic stage with corresponding developmental times are outlined in Table 1.

### Single-cell suspensions

Initial experiments were performed to optimize preparations of single-cell suspensions from mouse embryos. Treatment with either 0.25% collagenase (Sigma, St Louis, MO) plus 20% FCS for 2-3 hours or 2.5% trypsin EDTA (Sigma) for 3-5 minutes at 37°C with vigorous pipetting resulted in similar cell counts, viabilities and progenitor numbers. Cell counts and viability were determined after staining with eosin.

### Progenitor assays

Cells were plated in duplicate or triplicate at  $0.2-2 \times 10^5$  cells/ml in 1.0% methylcellulose supplemented with 10% plasma-derived serum (Antech, Tyler TX), 5% protein-free hybridoma medium (Gibco/BRL, Grand Island, NY), c-kit ligand (KL, 1% conditioned medium), VEGF (5 ng/ml), erythropoietin (2 U/ml), IL-11 (5 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 leukemia inhibitory factor (LIF; 1% conditioned medium). Cultures were maintained in a humidified incubator at 37°C in 5% CO<sub>2</sub> in air. Primitive erythroid, definitive erythroid (BFU-E), macrophage, mast cell and granulocyte/macrophage colonies were counted following 7-10 days of culture. CFU-E colonies were counted at 2-3 days of culture. LIF and KL were derived from media conditioned by CHO cells transfected with LIF and KL expression vectors (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 (Minneapolis, MN).

### Gene expression

Gene expression analysis was carried out using the poly(A)<sup>+</sup> RT-PCR method described by Brady et al. (1990). Individual pre-early streak embryos (E6.5), aliquots of the trypsinized tissues from later stage embryos or single micromanipulated primitive erythroblasts from E9.0-9.5 were transferred directly into first-strand PCR buffer. Reverse transcription, tailing and PCR procedures were performed as described, with the exception that the (dT)-x oligonucleotide was shortened to 5'-GTAACTCGAGAATTC(T)<sub>24</sub>-3'. The amplified products from the PCR reaction were separated on agarose gels and transferred to Z-probe GT membranes (Biorad). The resulting blots were hybridized with <sup>32</sup>P randomly primed cDNA fragments corresponding to gene 3' regions (for all except *βH1 globin*). To analyze *βH1 globin* gene expression, a probe was prepared by annealing two oligonucleotides, (5'-TGGAGTCAAAGAGGG-CATCATAGACACATGGG-3' and 5'-CAGTACACTGGCAATCC-CATGTG-3') which share an 8-base homology at their 3' termini. This *βH1 globin*-specific oligonucleotide was labeled with <sup>32</sup>P using a Klenow fill-in reaction.

## RESULTS

The specific stages of mouse embryogenesis analyzed in this study with their developmental times and cell counts are summarized in Table 1. The mouse conceptus (embryo proper and yolk sac) undergoes an approximate 8-fold increase in total cell number per day between E6.5 and E11.5 of development (Table 1). The rapid changes in embryonic growth and the wide intralitter and interlitter variation make accurate staging of the embryos critically important for the study of developmental events. Therefore, we carefully staged presomite embryos by morphologic criteria (Downs and Davies, 1993) and later embryos by somite counts. The accuracy of the staging and reproducibility of the tissue

dissections was confirmed by the low standard errors of the cell counts (Table 1).

### Commitment to hematopoietic fates begins during early gastrulation

Reasoning that commitment of embryonic cells to hematopoietic fates should precede the morphologic development of blood islands, we examined carefully staged embryos prior to E7.5 for the presence of hematopoietic progenitors. No progenitors were detected during the onset of mesoderm formation at prestreak and early primitive streak stages of development (E6.5, Fig. 1). Both primitive erythroid (EryP-CFC) and macrophage (Mac-CFC) progenitors were first detected shortly thereafter, at the mid-primitive streak stage (E7.0). Detailed analysis of distinct regions of the embryo revealed that these progenitors were spatially restricted to the proximal regions of the egg cylinder containing presumptive extraembryonic tissues (Fig. 1). EryP-CFC-derived colonies were compact, relatively small and homogeneous in size, and consisted of large nucleated cells expressing both embryonic (*βH1*) and adult (*βmajor*) globins (Fig. 2). The expression of *βmajor* globin in these colonies is most likely due to their coexpression by primitive erythroblasts and not to the presence of definitive erythroid cells (see below, Fig. 6). Primitive erythroid cells within the colonies showed signs of maturation by 3-4 days of culture, indicating that EryP-CFC represents a relatively advanced stage of differentiation, comparable in many respects to definitive CFU-E. Colonies generated from the yolk sacs of somite-pair stage embryos were only slightly smaller than those from presomite stage embryos, suggesting that EryP-CFC were a relatively homogeneous population with similar proliferative potential. Colonies generated by Mac-CFC comprised large vacuolated cells that express *c-fms*, a specific marker of this lineage (Fig. 2).

### Primitive erythropoiesis is transient

The primitive erythroid lineage expanded rapidly between mid-

**Table 1. Cell numbers at specific developmental stages from E6.5 to E11.5 of mouse embryogenesis**

Stage	Time (days)	Embryo No.	No. Exp	Cell counts (mean±s.e.m.×10 <sup>3</sup> )						
				TOTAL	YOLK SAC	PEP	DEP	HEAD	TAIL	ALLANTOIS
prestreak	6.5	23	3	1.3±0.2						
mid-streak	7	24	3	9.5±0.8	3.2±0.4	3.4±0.5	2.8±0.4			
neural plate	7.5	41	5	16.0±1.5	4.0±0.2	5.3±0.7	7.0±1.0			
				TOTAL	YOLK SAC	HEAD	TAIL	AGM	BLOOD	LIVER
1-8 sp	8.25	17	4	84±13	28±3	33±5	22±7	120	16*	
9-16 sp	8.5	10	2	112	62	32	14.5	860	360	
20-25 sp	9	7	1	387	61	116	74	120	16*	
26-29 sp	9.5	13	3	960±17	190±13	360±100	71±9	235±43	11±5	
30-35 sp	10	8	2	2550	227	855	245	860	360	
39-43 sp	10.5	6	3	6730±590	743±130	2000±300	576±12	1720±140	1600±30	89±5
58-60 sp	11.5	3	3	25500±2600	707±580	6600±900	2700±600	6920±1280	7520±2350	1050±110

The total number of mouse embryos (Emb No) and the number of independent experiments (No Exp) at each stage are also shown. sp, somite pairs; PEP, proximal embryo proper; DEP, distal embryo proper; AGM, aorta-gonad-mesonephros region. \*Blood fraction contaminated with embryonic cells during dissection.

		<u>EryP-CFC</u>	<u>Mac-CFC</u>
<b>Pre-streak (E6.5)</b>		0	0
		0	0
		0	0
<b>Mid-streak (E7.0)</b>		0.6	0.3
	yolk sac	3.0	0.5
		$6.3 \pm 0.3$	$3.7 \pm 0.3$
	pep	0.3	0.2
		0	0
	dep	$0.25 \pm 0.05$	$0.19 \pm 0.05$
		0	0
<b>Neural plate (E7.5)</b>		$17 \pm 1$	$1.2 \pm 0.4$
	yolk sac	$60 \pm 3$	$1.2 \pm 0.2$
		$48 \pm 2$	$9.3 \pm 0.9$
	pep	$0.5 \pm 0.5$	0
		$15 \pm 1$	$1.5 \pm 0.3$
	dep	$1.3 \pm 0.7$	0
		0	0
		0	0
		0	0
		0	0

**Fig. 1.** Distribution of hematopoietic progenitors (mean  $\pm$  s.e.m.) within mouse embryos at presomite stages. Results of three independent experiments each at pre/early streak (E6.5), mid-primitive streak (E7.0), and neural plate (E7.5) stages are shown. The dissection strategy of mid-streak and neural plate embryos are shown diagrammatically. Numbers without s.e.m. are derived from the mean of two cultures. pep, proximal embryo proper; dep, distal embryo proper; black, mesoderm; stippled, embryonic ectoderm.

streak (E7.0) and neural plate (E7.5) stages and remained localized to proximal regions of the yolk sac that contain developing blood islands (Fig. 1). Dramatic expansion of the primitive erythroid lineage was observed as the embryo proceeds from neural plate to early somite stages, with the number of EryP-CFC increasing more than 7-fold to reach 350-400 per yolk sac (Fig. 3). Subsequently, the number of EryP-CFC within the yolk sac declined sharply to undetectable levels by the 20-somite-pair stage (E9.0). Primitive erythroid progenitors were never detected at later stages of development in the yolk sac, nor were they found at any time within the embryo proper. Together, these findings demonstrate that primitive erythroid potential is extremely transient, persisting from E7.0 to 9.0, and is confined to the yolk sac, making it the most restricted, both temporally and spatially, of all the hematopoietic programs.

#### Definitive erythropoiesis originates in the yolk sac

Definitive erythroid progenitors were not detected at presomite stages of development (Fig. 1). However, low numbers of these cells were present in the yolk sac as early as the 2-6 somite pairs, prior to the establishment of circulation (Fig. 3, BFU-E). These progenitors consisted entirely of BFU-E, cells that represent the earliest identifiable stage of commitment to the definitive erythroid lineage. Yolk-sac-derived BFU-E generated large red colonies consisting of small cells, many of which are enucleated (Fig. 2A). The definitive nature of these cells was confirmed by gene expression analysis, which

demonstrated the presence of  $\beta$ major but no  $\beta$ H1 globin (Fig. 2B).

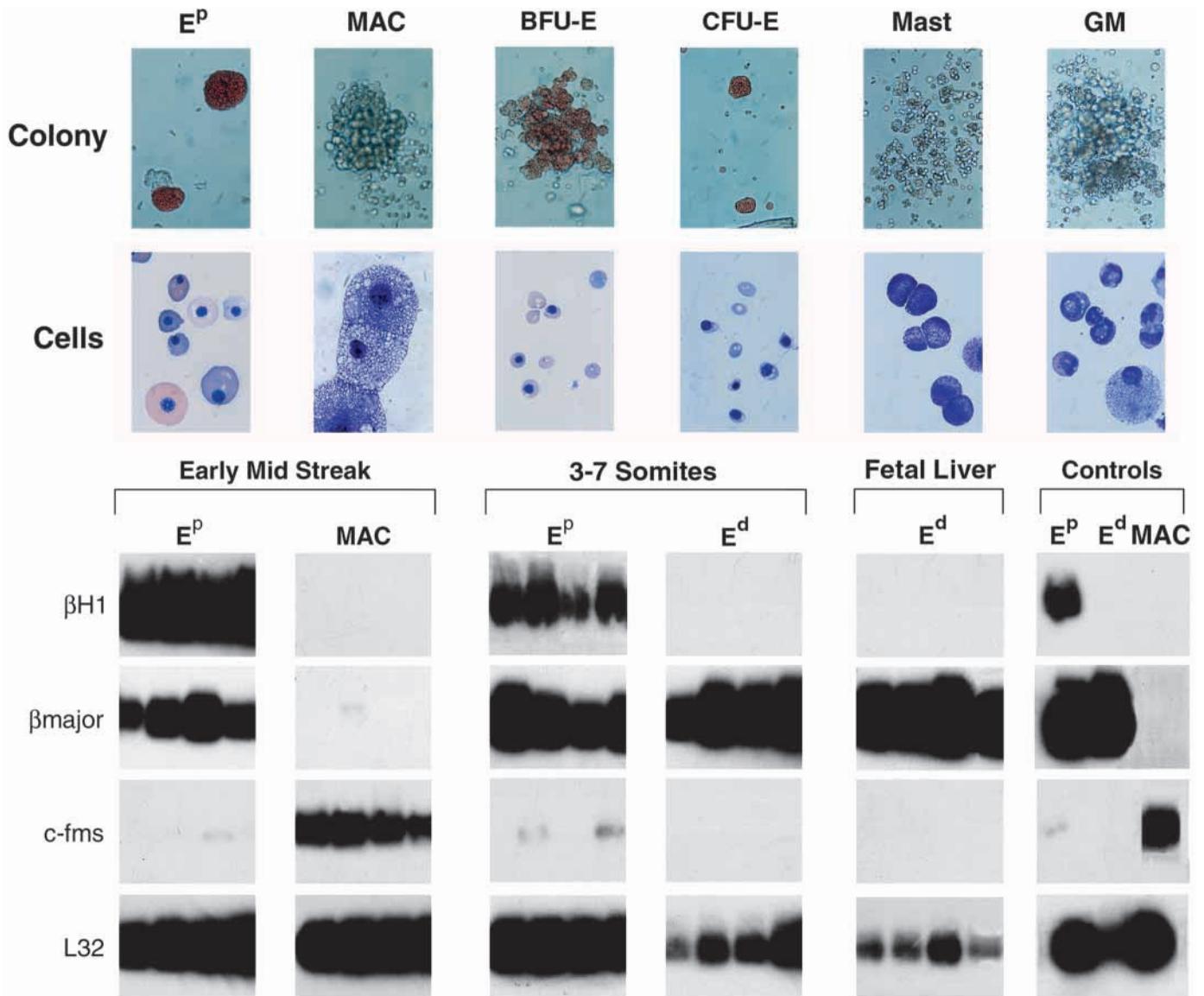
BFU-E were found exclusively in the yolk sac up to the 10-15 somite pairs by which time circulation has begun. By 20-25 somite pairs (E9.0), small numbers of these progenitors were present in the bloodstream and the embryo proper, although the majority were still localized to the yolk sac. Even at the 26-29 somite pairs (E9.5), there were still 10- to 40-fold more BFU-E in the yolk sac than in the embryo proper, a finding consistent with the notion that these definitive erythroid progenitors originate from this extraembryonic site (Fig. 3, BFU-E). Subsequently, BFU-E numbers decreased in the yolk sac and increased in the bloodstream and embryo proper. BFU-E were detected in the liver rudiment at the earliest stages examined (40-43 somite pairs), and their numbers expanded dramatically in this tissue (approximately 25-fold) over the next 24 hours (Fig. 3, BFU-E).

This wave of BFU-E progenitors in the yolk sac, bloodstream and liver is followed, both temporally and spatially, by the appearance of CFU-E. CFU-E represent a more advanced stage of development within the definitive erythroid lineage and generate small colonies consisting of enucleate erythrocytes (Fig. 2). CFU-E were first consistently detected at 26-29 somite pairs (E9.5) and were found predominantly in the yolk sac although lower numbers were present in the bloodstream and embryo proper (Fig. 3, CFU-E). By 40-43 somite pairs (E10.5), high numbers of CFU-E were found in both the bloodstream and the liver rudiment. During the next 24 hours, the number of CFU-E increased 18-fold in the liver, and decreased in the yolk sac and bloodstream (Fig. 3). Together, these findings are consistent with the interpretation that the definitive erythroid lineage develops initially within the yolk sac and differentiates from BFU-E to CFU-E stages in this extraembryonic environment.

A recent study has shown the allantois to be a hematopoietic tissue in the chick (Caprioli et al., 1998). We therefore analyzed isolated allantoises from 1-7 somite-pairs or 9- to 16-somite-pair embryos for progenitor activity to determine if it served a similar function in the mouse. No progenitors were detected (data not shown), demonstrating that the allantois has little, if any, hematopoietic potential in the mouse, consistent with the recent studies of Downs et al. (1998).

#### Myeloid progenitors originate in the yolk sac

Although macrophage progenitors could be detected as early as E7.0, their developmental pattern between E8.5 and E11.5 more closely paralleled definitive BFU-E than EryP-CFC. Mac-CFC increased in number in the yolk sac until 26-29 somite pairs (E9.5), and then steadily decreased thereafter (Fig. 3, Mac-CFC). As observed with the primitive and definitive erythroid progenitors, Mac-CFC were initially restricted to the yolk sac at precirculation stages (<8 somite pairs). Following the onset of circulation, low numbers could be found in the bloodstream and embryo proper, although even at 26-29 somite pairs (E9.5), the majority were still present in the yolk sac (Fig. 3). In contrast to definitive erythroid progenitors, macrophage progenitors were found in higher numbers in embryonic tissues compared to the bloodstream (Fig. 3) and were preferentially localized to the tail and P-Sp/AGM region prior to the development of the liver (see below). Like CFU-E, there was a massive (17.5-fold, average of three independent experiments)



**Fig. 2.** Morphology and gene expression analysis of hematopoietic colonies generated by different progenitor populations.  $E^d$  at 3-7 somite pairs are from BFU-E whereas those from fetal liver are from CFU-E. Each lane represents a single colony or a portion of a one colony. Control  $E^P$  represents primitive erythroid colonies grown from day 6 embryonic stem-cell-derived embryoid body (EB) precursors, control  $E^d$  are fetal liver-derived CFU-E and control MAC are macrophage colonies grown from day 6 EB-derived precursors. Ribosomal L32 expression served as a control. Cells were stained with May-Grunwald and Giemsa. Original colony and cell magnification are  $\times 100$  and  $\times 1000$ , respectively.

increase in Mac-CFC within the fetal liver between E10.5 and E11.5.

Progenitors of other definitive myeloid lineages were also detected in the yolk sac prior to liver colonization. Mast cell progenitors (Mast-CFC) could be detected in the yolk sac as early as the 10-15 somite pairs (Fig. 3, Mast-CFC, E8.5). These Mast-CFC, which gave rise to dispersed colonies consisting of cells with basophilic cytoplasmic granules (Fig. 2), were found predominantly in the yolk sac between E9 and E10. Unlike definitive erythroid and macrophage progenitors, the number of mast cell progenitors continued to increase in the yolk sac at E10.5 and E11.5. As observed with the macrophage progenitors, Mast-CFC were found in higher numbers in the embryo proper than in the blood; however, in this case, significant differences were only observed at the 58-60 somite-

pair stage of development (E11.5). Bipotential granulocyte/macrophage progenitors (GM-CFC) could also be detected within the yolk sac at the 10-15 somite-pair stage of development (Fig. 3, GM-CFC). These progenitors gave rise to small to medium size colonies that contain cells with both neutrophil and macrophage morphology (Fig. 2). Low numbers of GM-CFC persisted in both the yolk sac and embryo proper until 58-60 somite pairs at which time significant numbers were present in both blood and fetal liver.

#### Macrophage progenitors are differentially localized within the embryo proper

Our results support the interpretation that Mac-CFC, BFU-E, CFU-E, Mast-CFC and GM-CFC originate in the yolk sac and are found within the embryo proper only after the onset of the

circulation. Prior to fetal liver development, the number of BFU-E and CFU-E in the embryo proper does not exceed that found in the blood suggesting that they are not generated at any site other than the yolk sac and the fetal liver. In contrast, Mac-CFC and Mast-CFC were present in proportionally higher numbers within the embryo proper compared to other sites at E10.5 and E11.5, respectively. This observation suggests that they may originate from intraembryonic sites or they might preferentially migrate to these sites. To further define the distribution of intraembryonic hematopoietic progenitors, we divided the embryo proper into three sections: the "head" region containing head and heart, the "P-Sp/AGM" region between the heart and the tailbuds (and without the liver rudiment at E10.5 and E11.5), and the "tail" region containing caudal somites and tail. This physical separation is shown diagrammatically in Fig. 4 for the 20 somite-pair embryo. There were no differences in the absolute numbers of BFU-E and CFU-E in the head, P-Sp/AGM and tail regions between 20 and 60 somite pairs (E9.0-E11.5). In contrast, there were significant differences in distribution of Mac-CFC, with the highest numbers evident in the tail region at 20-29 somite pairs (E9.0-9.5) and in the P-Sp/AGM region at 30-43 somite pairs (E10-10.5). Interestingly, by E11.5, there were no differences in the distribution of macrophage progenitors between the head, P-Sp/AGM and tail regions. Mast-CFC showed no preferential localization during the early stages of development, but were present in higher numbers in the P-Sp/AGM region at 60 somite pairs. These findings strongly suggest that the P-Sp/AGM region is not a site of erythropoiesis, but may support limited macrophage and mast cell development or may be a site of selective homing and/or adhesion of these progenitors.

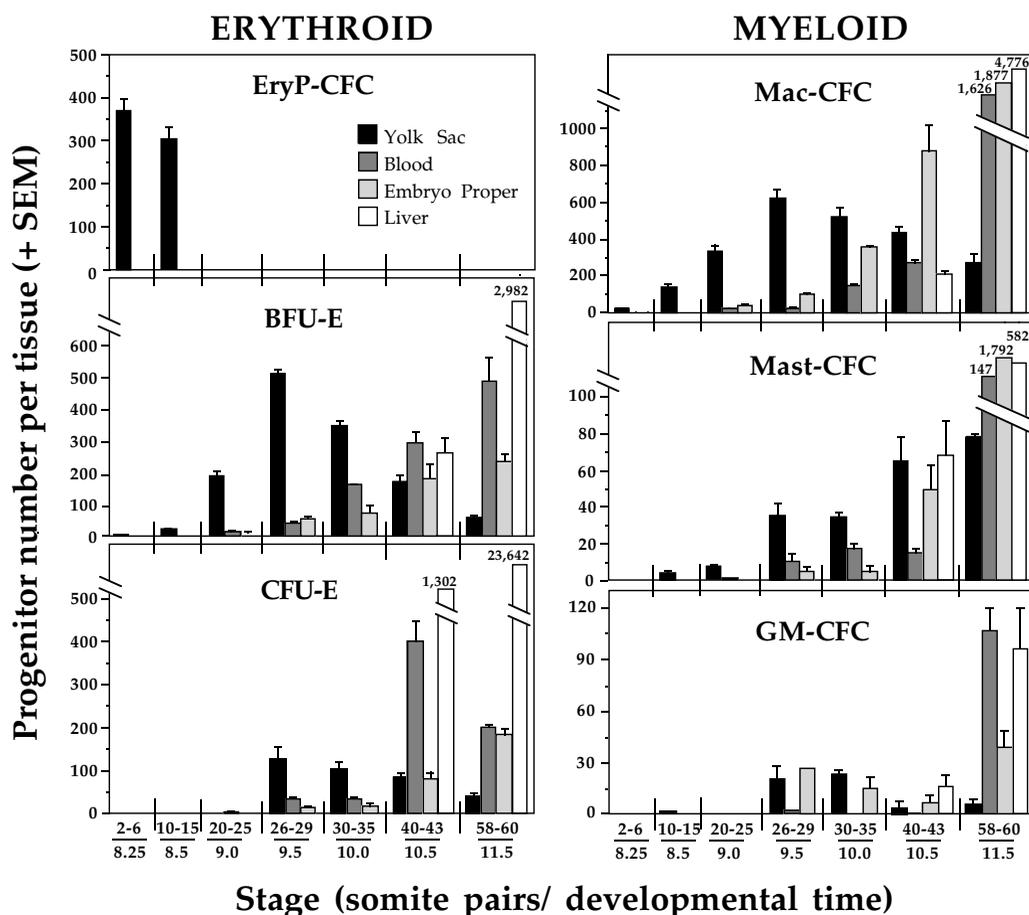
### Gene expression during hematopoietic ontogeny

To further characterize the onset of hematopoietic development, tissues from staged embryos were examined for the expression of genes known to play a role in embryonic hematopoiesis. These included *Brachyury*, coding for a transcription factor expressed in mesoderm cells along the primitive streak (Wilkinson et al., 1990); *flk-1*, a vascular endothelial growth factor (VEGF) receptor expressed in

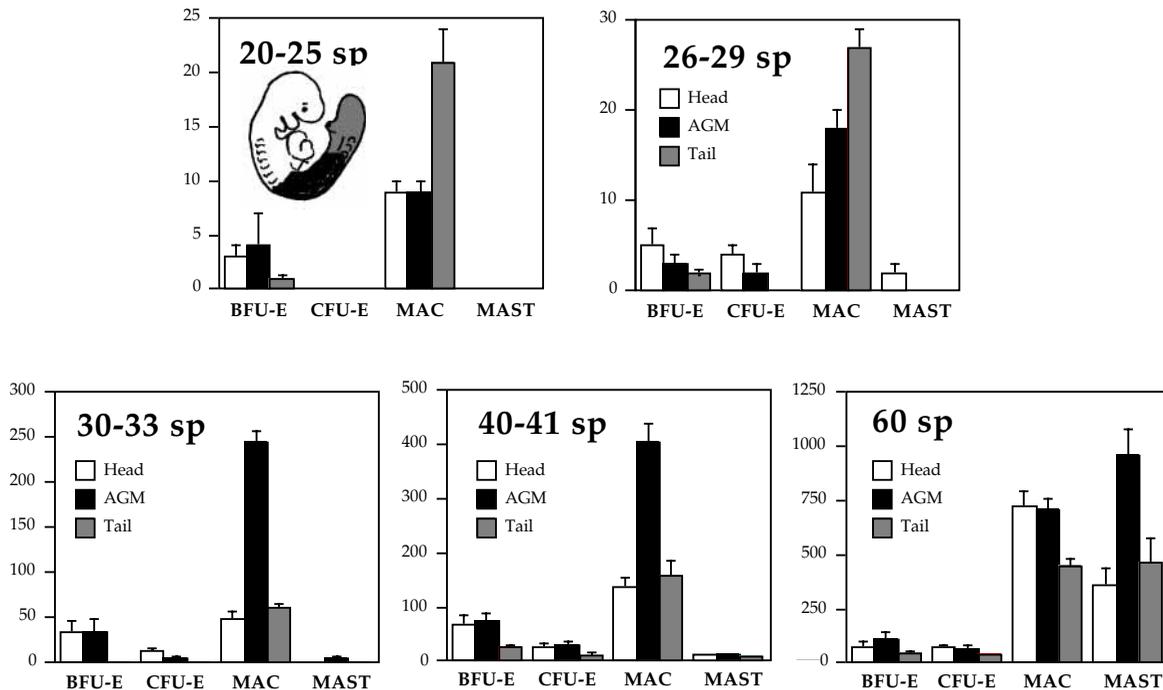
the earliest stages of both endothelial and hematopoietic development (Millauer et al., 1993; Yamaguchi, 1993); *SCL/tal-1*, a helix-loop-helix transcription factor expressed in both the endothelial and hematopoietic lineages and essential for hematopoietic development (reviewed by Begley and Green, 1999); *GATA-1*, a transcription factor required for both primitive and definitive erythropoiesis (Weiss et al., 1994);  *$\beta$ H1* and  *$\beta$ major globin*, embryonic and adult globins, respectively; *c-fms*, a macrophage-specific receptor tyrosine kinase; and *c-myb*, a transcription factor essential for definitive but not primitive hematopoiesis (Mucenski et al., 1991). Wherever possible, an aliquot of the same population of cells to be plated in the methylcellulose assay was used for the PCR analysis.

### Gene expression during the prehematopoietic to precirculation stages of development

The earliest site of hematopoietic development, as defined in the previous section by the presence of progenitor cells, was the proximal region of the egg cylinder in mid-streak embryos. No precursors were detected at the pre/early-streak stage of development, suggesting that commitment of embryonic cells



**Fig. 3.** Distribution of primitive erythroid (EryP-CFC), definitive erythroid (BFU-E and CFU-E), macrophage (Mac-CFC) and mast cell (Mast-CFC) progenitors (mean  $\pm$  s.e.m.) within various compartments of the mouse conceptus during somitogenesis (E8.25-E11.5). The liver was assayed separately from the remainder of the embryo proper at 40-43 somite pairs and 58-60 somite pairs. While results from a representative experiment are shown, multiple independent experiments outlined in Table 1 gave similar results.



**Fig. 4.** Distribution of erythroid (BFU-E and CFU-E), macrophage (MAC) and mast cell (MAST) progenitors (mean  $\pm$  s.e.m.) within the head, aorta-gonad-mesonephros (AGM), and tail regions of the embryo proper from E9.0 to E11.5. While results from a representative experiment are shown, multiple independent experiments outlined in Table 1 gave similar results. The liver is excluded from this analysis at 40-43 somite pairs and 58-60 somite pairs. The dissection of head, AGM and tail regions is shown diagrammatically at 20-25 somite pairs.

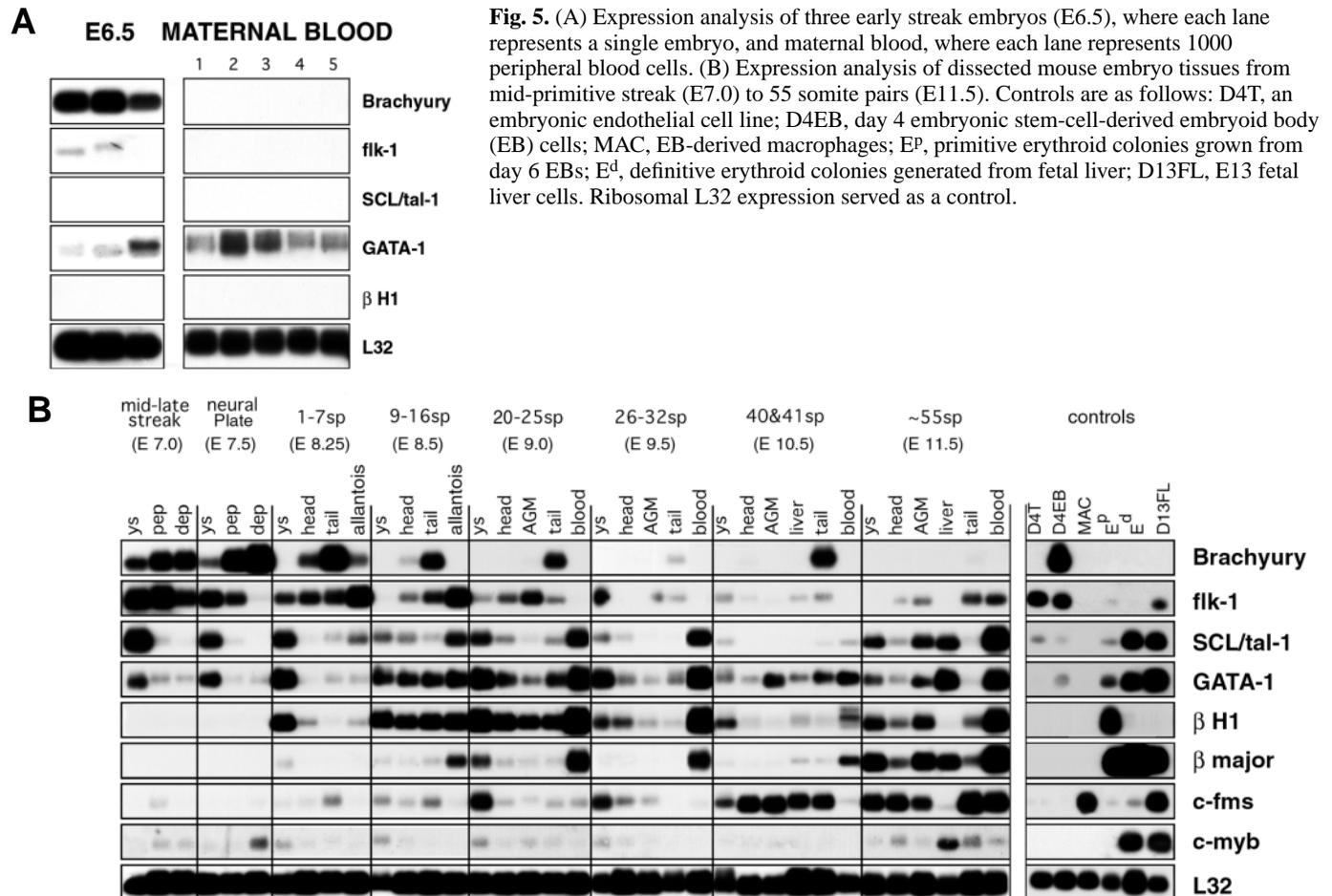
to a hematopoietic fate takes place within 12 hours of the onset of mesoderm formation. Expression analysis of three individual whole early streak embryos demonstrated readily detectable levels of *Brachyury* at this stage consistent with the onset of gastrulation (Fig. 5A). In addition, two of these embryos also expressed low levels of *flk-1*, suggesting the development of cells undergoing the earliest stages of specification to the endothelial and hematopoietic lineages. *SCL/tal-1* and  $\beta H1$  globin were not detected in any of the pre/early-streak stage embryos although low levels of *GATA-1* were found in two (Fig. 5A). The presence of *GATA-1* without *SCL/tal-1* and  $\beta H1$  globin is inconsistent with the onset of embryonic hematopoiesis and is likely indicative of contamination of these early stage embryos with maternal blood cells which also express *GATA-1* (Fig. 5A).

Mid- to late-streak embryos, which contain the first primitive erythroid and macrophage progenitors, expressed *SCL/tal-1* and *GATA-1* in addition to *Brachyury* and *flk-1*. Although all four genes were expressed at this stage, their localization within the developing embryo is quite different. Whereas *Brachyury* and *flk-1* were found in all three regions of the tri-sected embryos, *SCL/tal-1* and *GATA-1* were expressed predominantly in the presumptive yolk sac region of the egg cylinder that contains the hematopoietic progenitors.  $\beta H1$  globin,  $\beta$ major globin, *c-fms* and *c-myb* were not detected at significant levels in any region of primitive streak stage embryos. These patterns demonstrate that the onset of expression of *SCL/tal-1* together with *GATA-1* correlates with hematopoietic commitment to hematopoietic lineages. The lack of  $\beta H1$  globin indicates that the primitive erythroid precursors have not yet matured to the stage of globin expression.

The expression patterns of the three comparable regions of neural plate stage embryos (E7.5) were similar to the mid- to late-streak stage except that the distal egg cylinder (dep) no longer expressed *flk-1* and the yolk sac expressed lower levels of *Brachyury*. By 1-7 somite pairs,  $\beta H1$ -globin<sup>+</sup> cells were present in the yolk sac, indicating that the primitive erythroid lineage has matured to the stage of globin transcription. *Brachyury* was no longer expressed in the yolk sac, suggesting that mesodermal cells there have differentiated to hematopoietic and vascular lineages. The low levels of *c-myb* found in the yolk sac at early somite-pair stages are consistent with the establishment of definitive erythropoiesis. The allantois expressed *flk-1* and *SCL/tal-1*, indicative of the extensive vascular development ongoing in this tissue (Downs et al., 1998).

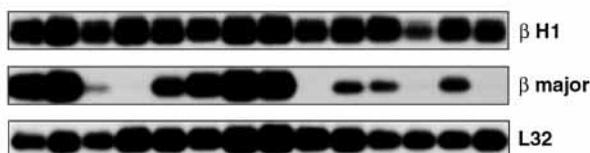
#### Expression from the onset of circulation to the fetal liver stage of development

As yolk-sac-derived blood cells begin to enter the circulation and embryo proper at 9-16 somite pairs (E8.5), *GATA-1*,  $\beta H1$  and, to a lesser extent, *SCL/tal-1* showed a broad pattern of expression in all tissues analyzed. The expression pattern of *Brachyury*, on the contrary, became more restricted to the caudal regions of the embryo proper, a finding consistent with its *in situ* hybridization pattern (Wilkinson et al., 1990). Shortly following the onset of circulation,  $\beta$ major globin expression could be detected, initially in the allantois (9-16 somite pairs, E8.5), and subsequently in the yolk sac and embryonic blood (20-25 somite pairs, E9.0). As definitive erythrocytes are not yet produced at this stage of development, it is likely that this  $\beta$ major globin expression originated from the primitive



erythroid population. To determine if this was the case, single primitive erythroblasts were isolated from the circulation of 22- to 30-somite-pair embryos and analyzed for the expression of  $\beta H1$  and  $\beta major$  globin. Out of a total of 93 cells expressing  $\beta H1$  globin, 71 (76%) were found to also express  $\beta major$  globin. An example of the expression pattern of 14 cells is shown in Fig. 6. The fact that  $\beta major$  globin was not expressed at significant levels at the onset of  $\beta H1$  globin expression in the yolk sac (1-7 somite pairs, Fig. 5B) suggests that  $\beta major$  expression may signify an additional maturation event in the primitive erythroid lineage.

Embryos undergoing the transition from extraembryonic to fetal liver hematopoiesis (40-41 somite pairs, E10.5), appeared to express less *flk-1*, *SCL/tal-1*, *GATA-1* and  $\beta H1$  globin than those at earlier stages of development. In contrast, the levels of *c-fms* were higher than in early embryos, a finding that correlates with the elevated numbers of macrophage progenitors at later developmental stages (Fig. 4). Gene



**Fig. 6.** Globin expression in single primitive erythroblasts isolated from E9.0-9.5 (22-30 somite pairs) embryos.

**Fig. 5.** (A) Expression analysis of three early streak embryos (E6.5), where each lane represents a single embryo, and maternal blood, where each lane represents 1000 peripheral blood cells. (B) Expression analysis of dissected mouse embryo tissues from mid-primitive streak (E7.0) to 55 somite pairs (E11.5). Controls are as follows: D4T, an embryonic endothelial cell line; D4EB, day 4 embryonic stem-cell-derived embryoid body (EB) cells; MAC, EB-derived macrophages; EP, primitive erythroid colonies grown from day 6 EBs; E<sup>d</sup>, definitive erythroid colonies generated from fetal liver; D13FL, E13 fetal liver cells. Ribosomal L32 expression served as a control.

expression patterns changed substantially in embryos at the 55-somite-pair stage (E11.5) as the fetal liver becomes an increasingly active hematopoietic organ. The developing liver expressed *SCL/tal-1*, *GATA-1*, *c-myb*,  $\beta major$  but no  $\beta H1$  globin, a pattern indicative of the onset and expansion of definitive hematopoiesis. It is worth noting that the region that contains the P-Sp/AGM did not show any substantial *SCL/tal-1* expression at any stage of development, with the exception of E11.5 following the onset of fetal liver hematopoiesis. Additionally, this area expressed very little *c-myb*. These findings are consistent with the progenitor analysis and indicate that this region is not a site of extensive hematopoietic differentiation.

## DISCUSSION

Our investigations of the postimplantation mouse embryo indicate that two waves of hematopoietic progenitors arise from extraembryonic membranes during gastrulation. The initial "primitive" wave consists predominantly of primitive erythroid progenitors which are very short-lived and are extinguished prior to liver rudiment colonization. The second "definitive" wave consists of definitive erythroid and myeloid (mast cell, GM and expanded macrophage) progenitors that originate in the yolk sac and are found soon thereafter in the bloodstream and within the embryo proper.

The precise staging of early embryos, combined with the use

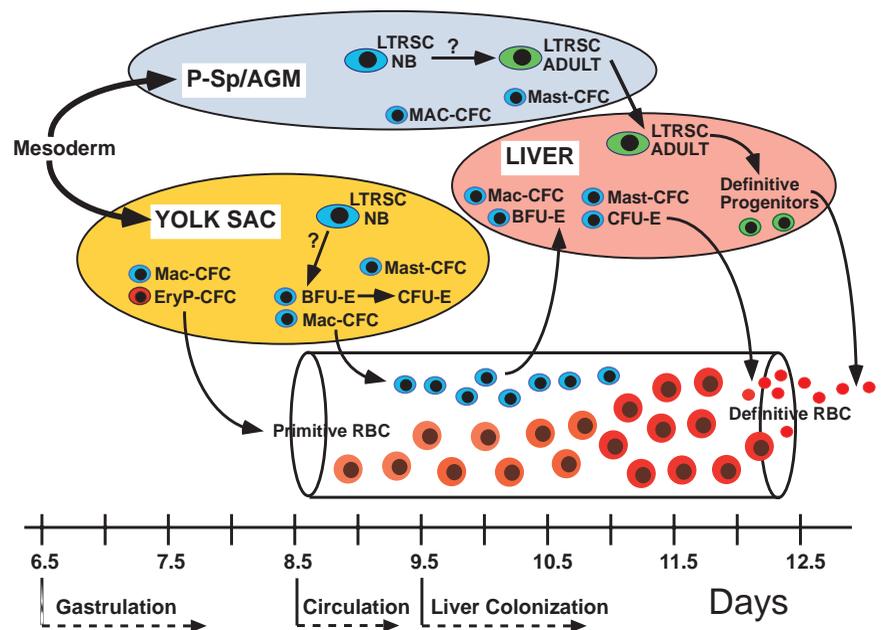
of culture conditions suitable for the growth and maturation of the primitive erythroid lineage has enabled us to generate the most precise kinetic analysis and most detailed characterization of the primitive hematopoietic program to date. While a number of previous studies have examined the mouse embryo for the presence of hematopoietic progenitors, none has specifically and systematically investigated the earliest stages of gastrulation and most have not analyzed the primitive erythroid lineage (Moore and Metcalf, 1970; Johnson and Barker, 1985; Wong et al., 1986a,b; Rich, 1992). The earliest analysis was carried out by Wong et al. (1986a) who demonstrated the presence of erythroid progenitors in whole E7.5 embryos. These progenitors, referred to as day 5 CFU-E, appear to be similar to the Eryp-CFC that we have analyzed, given that they generated colonies of cells that express embryonic globin. Although these previous findings clearly suggested an early onset of hematopoiesis, the precise developmental stage of the embryos and spatial localization of the progenitors were not ascertained in these studies.

Our findings have extended these studies and provided new evidence that the primitive erythroid lineage is unique and differs from other hematopoietic lineages in several ways. First, we have shown that primitive erythroid progenitors arise concomitantly and exclusively with macrophage progenitors. Eryp-CFC are first detected in mid-streak embryos shortly following the onset of mesoderm formation but prior to the morphological appearance of yolk sac blood islands. The development of primitive hematopoietic progenitors at the mid-streak stage is consistent with the reported timing of induction of embryonic cells to erythroid fates by visceral endoderm (Belaousoff et al., 1999). Second, the primitive erythroid program is extremely transient, being active for only 48 hours during the earliest stages of yolk sac development. As such, it is the only hematopoietic lineage whose development is localized to a specific tissue. Third, the homogeneous size of primitive erythroid colonies strongly suggests that Eryp-CFC develop as a synchronous wave. This is in contrast to the definitive erythroid lineage, which matures in a step-wise fashion through the BFU-E to the CFU-E stages. The developmental pattern of these progenitors in culture correlates well with the rapid development of yolk sac blood islands within the embryo and the subsequent generation of several million circulating primitive erythroblasts (Table 1).

The patterns of gene expression in gastrulating embryos (E6.5-E7.5) correlated well with the progenitor assays and with previous *in situ* hybridization studies (Silver and Palis, 1997; Yamaguchi et al., 1993). In particular, the upregulation of *SCL/tal-1* and *GATA-1* expression in the proximal region of the egg cylinder, precisely at the initiation of hematopoiesis is a striking example of a close relationship between molecular and cellular events. In addition, the localization

of *SCL/tal-1* and *GATA-1* expression to the region of progenitor cell development further confirms that the earliest stages of hematopoietic commitment occur in a temporally and spatially restricted region of the conceptus (Silver and Palis, 1997). The expression of these genes at the site of hematopoietic development is not unexpected given that *SCL/tal-1* is essential for both primitive and definitive hematopoiesis (reviewed by Begley and Green, 1999) and that *GATA-1* is required for the maturation of the primitive erythroid lineage (Weiss et al., 1994). The expression of *Brachyury* in these embryos is consistent with the known generation of mesoderm cells exiting the primitive streak (Wilkinson et al., 1990). The fact that *flk-1* is expressed in early streak embryos (E6.5) suggests that the initial stages of hematopoietic and endothelial lineage specification occurs during early gastrulation. The early expression of *flk-1* is consistent with its putative role in the migration of mesodermal cells and their subsequent development to the hematopoietic and endothelial lineages (Shalaby et al., 1995, 1997; Schuh et al., 1999). Mesoderm cells fated to hematopoietic and endothelial lineages might downregulate *Brachyury* and upregulate *flk-1* and *SCL/tal-1* as they migrate to extraembryonic sites.

In addition to primitive erythropoiesis, our data provide strong support for the concept that definitive hematopoiesis also initiates in the yolk sac. The most convincing evidence for this is the presence of both macrophage and definitive erythroid progenitors in the yolk sac at early somite stages (E8.25) prior to circulation. Even following the onset of circulation, the numbers of these progenitors was considerably higher in the yolk sac than in the embryo proper. Although Mast-CFC and GM-CFC do not develop until the beginning of circulation, the



**Fig. 7.** Model of early hematopoietic ontogeny in the mouse embryo. P-Sp/AGM, para-aortic splanchnopleura/aorta-gonad-mesonephros region; RBC, red blood cells; EryP-CFC, primitive erythroid progenitors; Mac-CFC, macrophage progenitors; BFU-E, burst-forming unit erythroid; CFU-E, colony-forming unit erythroid; Mast-CFC, mast cell progenitors; LTRSC: NB, longterm newborn-repopulating stem cells; LTRSC: ADULT, longterm adult-repopulating stem cells.

fact that they are found in the yolk sac prior to the embryo proper also suggests that they develop in this extraembryonic site. Our findings that definitive hematopoiesis develops in the yolk sac clearly demonstrates that this tissue is not solely a site for primitive erythropoiesis but rather appears to have broader functions in the generation of multiple hematopoietic lineages. This concept is supported by various studies in a number of different species. Evidence that definitive erythroid potential arises from the yolk sac has been found in avian embryos, including de-embryonated chick yolk sacs (Hagopian and Ingram, 1971), chick-quail chimeras (Beaupain et al., 1979), and chick-chick chimeras (Lassila et al., 1982). In amphibian embryos, ventral blood islands were shown to have both primitive and definitive potential early in development (Turpen et al., 1997). In the mouse, previous studies have documented the presence of erythroid and myeloid hematopoietic progenitors in the yolk sac prior to the embryo proper (Wong et al., 1986a). Further evidence that the mouse yolk sac is not restricted to primitive erythropoiesis is provided by the studies of Cumano et al. (1996), which demonstrated that yolk sacs isolated from precirculation embryos could generate both definitive erythroid and myeloid precursors when cultured for 48 hours. In addition to definitive erythroid progenitors, we have shown that macrophage, mast cell and bi-potential granulocyte-macrophage precursors also develop in the yolk sac. The yolk sac origin of these myeloid progenitors at E8.5-9.0 is consistent with the previous demonstration that mast cell progenitors are preferentially localized to the yolk sac at E9.5 (Sonoda et al., 1983).

The identification of multiple definitive lineages in the yolk sac strongly suggests the presence of multipotential precursors and possibly stem cells in this tissue. While our experimental conditions were not specifically designed to detect such cells, several studies indicate that they are present in the yolk sac. Using stromal cell co-cultures, Godin et al. (1995) identified precursors with erythroid, myeloid and lymphoid potential in the yolk sac as early as E8.5 (10-15 somite pairs). Recently, Yoder et al. (1997) demonstrated that *c-kit*<sup>+</sup>/*CD34*<sup>+</sup> cells isolated from E9.0 (15-20 somite pairs) yolk sacs provide long-term repopulation following transplantation into the liver of conditioned newborn pups. While these latter experiments were also carried out following the onset of circulation, the fact that 37-fold more *c-kit*<sup>+</sup>/*CD34*<sup>+</sup> cells were found in the yolk sac than in the embryo proper suggests that these cells are of extraembryonic origin. Finally, current studies have demonstrated the presence of high proliferative potential progenitors in the yolk sac, but not embryo proper, of precirculation embryos (J. P. and M. Yoder, unpublished data). Taken together, these findings indicate that the yolk sac possesses the potential to generate multiple definitive hematopoietic lineages.

The presence of both BFU-E and CFU-E suggests that definitive erythroid progenitors undergo some maturation in the yolk sac environment. However, the lack of circulating definitive erythrocytes prior to E12-13 (Brotherton et al., 1979; Steiner and Vogel, 1973), several days after colonization of the liver, suggests that the yolk sac environment is unable to support the late stages of definitive erythroid differentiation. This conclusion is supported by the experiments of Cudennec et al. (1981), where intraembryonic, but not yolk sac, tissues contain soluble factors that support definitive erythropoiesis in

20-25 somite-pair yolk sac explants. The generation of progenitors without complete maturation in the yolk sac strongly suggests that they are produced for export to another site. Two key observations in our study strongly support the hypothesis that these yolk-sac-derived progenitors are destined to colonize the developing liver and initiate the first phase of definitive hematopoiesis at this intraembryonic site. First, the sequence of events in which progenitors are first detected in the yolk sac and subsequently in the blood and fetal liver is consistent with migration from the yolk sac to the liver. Second, the concomitant presence of BFU-E and CFU-E in the early liver rudiment strongly suggests colonization rather than de novo generation from a more immature progenitor. In the latter scenario, one would expect BFU-E to precede CFU-E, as observed in the yolk sac.

Analysis of the distribution of progenitors in the different regions of the embryo demonstrated that the P-Sp/AGM region is not a site of active multilineage hematopoiesis. These findings are supported by the gene expression studies, which showed no *SCL/tal-1* expression at E9.5 and E10.5 and almost no *c-myb* expression at any stage in this region. The lack of *c-myb* expression is in contrast to previous studies in the chick embryo that demonstrated expression of this gene by *in situ* hybridization in para-aortic regions of the embryo proper (Vandembunder et al., 1989). As we have analyzed the entire region by RT-PCR, expression of *c-myb* by small clusters of cells may not be detected. The increased numbers of Mac-CFC and Mast-CFC transiently present in the AGM region are consistent with previous studies in the chick that identified progenitors in the wall of the aorta (Cormier and Dieterlen-Lievre, 1988). Their presence may reflect selective homing of yolk-sac-derived progenitors to this site or generation of these progenitors *in situ*. Given that progenitors from other lineages, in particular the definitive erythroid, are not localized to the AGM, it is more likely to be selective homing and/or adhesion rather than the development of only the macrophage and mast cell lineages. Our observations demonstrating a lack of substantial hematopoiesis in this region are not inconsistent with previous studies that have identified CFU-S, multipotential precursors and long-term repopulating stem cells in the P-Sp/AGM as the assay that we have used was not designed to detect these early stage cells. The lack of significant numbers of clonable progenitors does, however, indicate that these multipotential cells do not undergo significant differentiation in this environment.

Our data together with that from other studies support a model whereby the yolk sac serves two important functions that fulfill the rapidly changing hematopoietic requirements of the developing embryo (Fig. 7). As the initial demand is for erythrocytes, the first function of the yolk sac is the synthesis of a transient wave of primitive erythroid progenitors between E7.0 and E9.0 that go on to terminally differentiate within the developing vasculature. These primitive red cells generated in the yolk sac sustain the survival and growth of the embryo until the liver begins to release erythrocytes into the bloodstream at E12 (Copp, 1995). The relationship of EryP-CFC to the newborn-repopulating stem cell is unclear at this time. The second function of the yolk sac is the synthesis of definitive erythroid and myeloid progenitors between E8.5 and E10. We postulate that these definitive progenitors rapidly enter the circulation,

colonize the liver rudiment beginning at E9.5 and give rise to the first enucleate erythrocytes by E12. With this change in function, the yolk sac evolves from a hematopoietic tissue with erythroid-restricted potential to one that can generate multiple lineages. These yolk-sac-derived definitive hematopoietic progenitors may be derived from the newborn-repopulating stem cells that have been identified in this tissue (Yoder et al., 1997). As development progresses, stem cells of intraembryonic origin could seed the fetal liver and generate the next wave of blood-borne cells which would ultimately replace the yolk-sac-derived circulating cells.

The pattern of lineage development in the yolk sac described in this study, in which primitive erythroid precedes definitive erythroid and most myeloid progenitors, is similar to that found in embryoid bodies (EB) generated from embryonic stem (ES) cells in culture (Keller et al., 1993). The close developmental association of primitive and definitive hematopoiesis in the yolk sac raises interesting questions about the origin and relationship of these populations. Using the ES/EB system as a model of yolk sac development, we have addressed this question and have identified a VEGF-responsive precursor that develops earlier than any of the hematopoietic lineages and is able to generate both primitive and definitive hematopoietic as well as endothelial progeny (Kennedy et al., 1997; Choi et al., 1998). The potential of this EB-derived VEGF-responsive precursor is characteristic of the hemangioblast. Our current studies are aimed at identifying a comparable precursor population in the early embryo.

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