# Multipotential hematopoietic progenitor cells from embryos developed *in vitro* engraft unconditioned $W^{41}/W^{41}$ neonatal mice

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Background and Objectives. The first hematopoietic stem cells (HSC) in the mouse able to give rise to the adult hematopoietic system emerge at embryonic day (E) 10.5 in the intraembryonic aorta-gonads-mesonephros (AGM) region, as demonstrated by transplantation into irradiated adult recipients. It has been shown by transplantation into conditioned neonatal or hematopoietic mutant adult recipients that less potent multipotential hematopoietic progenitors exist in the mouse embryo at E9, one day earlier than the appearance of HSC. Studies of the lineage relationships of multipotential hematopoietic progenitors and HSC in the mouse embryo have been complicated by inaccessibility due to *in utero* development. Attempts are being made to create an *in vitro* whole mouse embryo culture system to access the developing mouse embryo for such studies of hematopoietic cell emergence during early and mid-gestational stages. The aim of this study was to compare the development of multipotential hematopoietic progenitors in early *in utero* and *in vitro*-developed mouse embryos.

Design and Methods. To test hematopoietic progentior/stem cell activity in the mouse embryonic tissues obtained from genetically marked *in utero* and *in vitro*-developed embryos, transplantations were performed using unconditioned neonatal $W^{41}/W^{41}$ (c-kit hematopoietic mutant) recipients. Long-term donor-cell reconstitution in transplanted mice was measured by (i) semiquantitative polymerase chain reaction and (ii) flow cytometry on peripheral blood and hematopoietic organs.

Results. Our experimental data show that multipotent hematopoietic progenitors from *in utero*-developed embryos engraft unconditioned $W^{41}/W^{41}$ neonates. Furthermore, *in vitro*-developed whole embryos also contain early multipotent hematopoietic progenitor cells that are able to yield high-level, long-term engraftment of $W^{41}/W^{41}$ neonates.

Interpretations and Conclusions. The *in vitro* culture of whole mouse embryos during mid-gestational stages allows for the normal growth of multipotential hematopoietic progenitors that can be assayed by transplantation into $W^{41}/W^{41}$ neonatal recipients. Thus, *in vitro*-developed whole embryos can be used with confidence in future studies to examine the lineage relationships of multipotential hematopoietic progenitors and HSC.

Key words: hematopoietic stem cells, $W^{41}/W^{41}$, neonates, development, AGM, embryo culture.

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Over the last ten years, hematopoietic stem cell (HSC) development has been studied using several *in vivo* transplantation models. The first generation of HSC with engraftment potential equivalent to adult bone marrow HSC has been shown in the aorta-gonad-mesonephros (AGM) region at embryonic day (E)10.5 using irradiated adult recipients.[1,2] Prior to E10.5, no adult repopulating HSC were detected. However, multipotential hematopoietic progenitors have been detected in earlier stage yolk sac (E9 YS) and para-aortic splanchnopleura (E9 Sp) when tested in busulfan-conditioned neonatal recipients by intravenous injection or injection into the hematopoietically active neonatal liver.[3-5] These cells are thought to represent an early type of HSC that, through further inductive interactions, will acquire adult bone marrow homing and/or high proliferative properties. Similarly, multilineage or single lineage repopulating cells were observed when pre-E10.5 YS or Sp cells were injected into Rag-/-:γc-/- and W locus (receptor tyrosine kinase c-kit) lymphopoietic/hematopoietic mutant (adult or embryonic stage) recipients.[6-12] It is now of great interest to further study and manipulate multipotential hematopoietic progenitors in mouse embryos developed *ex utero*. *In vitro* culture conditions for whole mouse embryos have been established and allow for overt normal development over a 24-48 hour period.[13] However, to date no studies have been performed to determine whether hematopoietic development is normal in embryos cultured for more than 12 hours.[14] The $W^{41}/W^{41}$ mouse strain offers advantages as a transplant recipient for further studies on embryonic onset of

multipotential hematopoietic progenitor cell activity. Previously, it has been shown that neonates with severe W mutations (in the absence of irradiation) can be effectively engrafted with donor fetal liver HSC.[15] However, while the most severe mutations of the W locus affect both hematopoiesis and fertility,[16,17] the mild $W^{41}$ mutation (a single amino acid mutation in the c-kit kinase domain) affects only hematopoiesis.[18] Thus, it is possible to cross $W^{41}/W^{41}$ homozygous mutant parents to obtain complete litters of homozygous neonates for transplantation assays.

The objective of this study was to examine and compare multipotent hematopoietic progenitor activity in pre-E10.5 embryos developed *in utero* and *in vitro*, in order to determine whether *in vitro* whole embryo cultures allow the normal growth of these rare progenitors. We used unconditioned $W^{41}/W^{41}$ newborn mice as transplant recipients. The resulting hematopoietic reconstitution of neonatal $W^{41}/W^{41}$ recipients with cells from embryonic tissues was tested at 2 to 12 months post-transplantation by polymerase chain reaction (PCR) analysis for donor cell genetic markers and by flow cytometric analysis.

## Design and Methods

### Mice and embryo generation

$W^{41}/W^{41}$ neonates were obtained from $W^{41}/W^{41}$ mating pairs. C57BL/6 or (129Sv×C57BL/6)F1 adult mice (2-4 months old) from our facility were used as recipients for secondary transplantation experiments. Timed matings of C57BL/6 females with actin-EGFP male transgenic mice[19] were performed for the generation of embryos. The day of vaginal plug discovery was considered embryonic day 0 (E0). Embryos were genotyped by EGFP fluorescence. This transgenic line was maintained on a C57BL/6 background. All mice were housed in the Erasmus Medical Center Animal unit according to the institution's guidelines, with food and water provided ad libitum. Pregnant dams were sacrificed by cervical dislocation at E8 until E10 and embryos were isolated from the uterus as described previously.[20] Embryos were staged by counting the number of somite pairs.

### Embryo culture

Whole embryos were cultured *in vitro* as previously described.[13,21] Briefly, E8, E8.5 or E9 embryos were dissected so as to remove the mural trophoblast and Reichert's membrane while maintaining the integrity of the extra-embryonic membranes (amnion and yolk sac with the ectoplacental cone). Pools of 2-5 E8 or E8.5 embryos or 1-4 E9 embryos were cultured in a glass roller flask. E8/E8.5 embryos were cultured in Hank's buffered salt solution (HBSS) containing 50% rat serum, and E9 embryos in 100% rat serum with glucose levels adjusted to 10mM. Rat serum came from adult male and female rats and was prepared immediately by centrifugation after blood collection. Only those lots that gave maximal embryo growth were used. Oxygenation of the cultures varied between 20% $O_2$ (E8/E8.5) and 40% $O_2$ (E9). Cultures were maintained in a 38°C incubator for 24 hours. Embryos were harvested from the cultures and assessed for growth and viability under a dissection microscope. Only those embryos demonstrating overtly normal physiology and blood circulation were further processed. Dissection of tissues and preparation of cell suspensions for injection was performed as described previously.[20]

### Transplantation

Cell suspensions (20 µL in phosphate-buffered saline) were injected into livers of $W^{41}/W^{41}$ neonates (within 24 hours after birth) with a pulled glass pipette (Narishige Scientific Instrument Ltd., GD-1; 1×90 mm). At 2, 6 and 12 months post-transplantation, donor cell contributions were analyzed by semi-quantitative polymerase chain reaction (PCR) and flow cytometry for the EGFP transgene. In some cases, bone marrow cells obtained from positive engrafted primary recipients were transplanted into secondary recipients (129Sv×C57BL/6)F1 or C57BL/6 irradiated (9 Gy).[20]

Briefly, genomic DNA was isolated from peripheral blood of transplanted mice: 100 ng of DNA was used for PCR with EGFP primers 5'AAACGGCCACAAGTTCAGCG3' and 5'GGCGGATCTTGAAGTTCACC3' and myogenin primers 5'TTACGTCCATCGTGGACAGC3' and 5'TGGGCTGGGTGTTAGTCTTA3'. DNA was subjected to an initial 5-minute denaturation at 92°C followed by 30 cycles of denaturation (1 min at 92°C), annealing (2 min at 55°C) and elongation (2 min at 72°C). Reactions were run on an ethidium bromide stained agarose gel. EGFP and myogenin fragment sizes are 440 bp and 250 bp respectively. The percentage donor-cell contribution was determined by a dilution series of transgenic and normal genomic DNA from which a standard curve was established. Ethidium-stained fragments were analyzed by ImageQuant using the myogenin fragment as the DNA normalization control.

### Flow cytometric analysis

Flow cytometric analysis was performed to detect hematopoietic cells in the embryos developed *in utero* and *in vitro* and also to detect the presence and lineage of EGFP-positive cells in recipient mice, as described previously.[20,21] Phycoerythrin-conjugated

**Table 1.** Presence of multipotential hematopoietic progenitors in embryos developed *in utero* or in an *in vitro* embryo culture.

| | *Somite pairs* | *ee injected* | *Number repopulated/Number transplanted (% donor cell engraftment)* | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | *Sp/AGM* | | | *Yolk Sac* | | |
| | | | *2 months* | *6 months* | *12 months* | *2 months* | *6 months* | *12 months* |
| *In utero* | | | | | | | | |
| E9.0 | 17-21 | 0.5-1 | 1/7 (1) | *1/7 (8) | 1/7 (1) | 0/6 | 0/6 | 0/6 |
| E9.5 | 22-26 | 2-3 | 1/2 (50) | 1/2 (50) | 1/2 (50[a]) | 0/2 | 0/2 | 0/2 |
| E10.0 | 30-34 | 1-2 | 0/11 | 0/10 | 1/10 (1) | 0/7 | 0/6 | 1/7 (1) |
| *In vitro* whole embryo culture | | | | | | | | |
| E8 to 9 | 15-22 | 1-2 | 0/9 | 0/9 | 0/9 | 0/8 | 0/8 | 0/8 |
| E8.5 to 9.5 | 25-29 | 2 | 0/2 | 0/2 | 1/*2 (1) | 0/2 | 0/2 | 0/2 |
| E9 to 10 | 26-34 | 1 | 1/13 (10) | ND | 1/12 (27[b]) | 3/11 (80,10,1) | ND | 2*/9 (85[c],10) |

| *Secondary transplantation** | *Number repopulated/Number transplanted (% donor cell engraftment)* | | | |
|---|---|---|---|---|
| # of injected cells | 5×10[6] | 3×10[6] | 1.3×10[6] | 0.6×10[6] |
| E9.5 AGM[a] | 3/3 (92-100) | – | 2/2 (45-100) | 2/2 (30-72) |
| E9 to E10 AGM[b] | – | 2/3 (3, 7) | – | – |
| E9 to E10 YS[c] | – | 3/3 (15, 21, 16) | – | – |

*Embryos were obtained directly from pregnant females (in utero) or cultured (in vitro) for 24 hours. More than 80% of in vitro-cultured, whole embryos were viable and met the criteria for obvious normal development. Only these embryos were used in the transplantation experiments. Tissues were dissected and single cell suspensions injected into the liver of unconditioned newborn W[41]/W[41] recipients. Peripheral blood DNA was obtained at 2, 6 and 12 months post-transplantation and tested by semi-quantitative PCR for the presence of the EGFP transgene. Secondary transplantations were performed with varying doses of bone marrow cells isolated from the recipients indicated by the asterisks [a,b,c]. Cells from [a]3 pooled AGM (22-26 sp), [b]1 AGM (33 sp) and [c]1 YS (32 sp) were injected into primary neonatal recipients. Some tertiary repopulation was also observed (not shown). Sp: somite pairs; ee: embryo equivalents.*



B220 (CD45R, RA3-6B2), CD4 (GK1.5), CD8 (53-6.7), Mac1 (CD11b, M1/70), TER119 and Gr1 (8C5) specific antibodies (Pharmingen) were used to detect mature lineage hematopoietic cells. Single cell suspensions were stained with antibodies for 30 minutes on ice, washed twice in phosphate-buffered saline with 10% fetal calf serum, and filtered through a nylon mesh prior to screening. 7AAD was used to exclude dead cells during analysis on a FACScan (Becton-Dickinson). Ten thousand events were analyzed.

## Results

### Multipotential hematopoietic progenitors engraft W[41]/W[41] newborn recipients

Sp/AGM and YS tissues were dissected from E9, E9.5 and E10 EGFP transgenic embryos, prepared as single cell suspensions and injected into unconditioned W[41]/W[41] neonates. At 2, 6 and 12 months post-transplantation, donor cell engraftment was measured by PCR (EGFP transgene) or flow cytometric analysis on peripheral blood samples. As shown in Table 1, engraftment (12 months post-transplantation) was observed in the peripheral blood of 4 out of a total of 34 W[41]/W[41] recipients injected. Low-level engraftment from E9 Sp, E10 AGM or E10 YS cells (approximately 1%) was found in three recipients. Interestingly, high-level engraftment was observed as early as 2 months and up to 12 months post-transplantation in one recipient receiving E9.5 Sp cells (3 embryo equivalents; ee).

By flow cytometric analysis we found high-level multilineage donor contribution in the circulation of this recipient at 12 months post-transplantation (61% of B220$^+$, 43% of CD4$^+$/8$^+$, 53% of Mac1$^+$, 34% of TER119$^+$, 27% of Gr1$^+$ were donor-derived). This recipient was then sacrificed and its hematopoietic tissues were examined. Figure 1A shows high-level engraftment ranging from 13% to 78% in bone marrow, thymus, lymph node and spleen in the granulocyte (Gr-1$^+$), macrophage (Mac-1$^+$), T lymphoid (CD4$^+$/8$^+$; may include dendritic cells) and B lymphoid (B220$^+$) lineages. To test for the self-renewal of donor-derived HSC, bone marrow cells (5, 1.3 and 0.6×10$^6$ cells) were transplanted into irradiated secondary adult recipients. FACS of peripheral blood more than 4 months post-transplantation showed high-level engraftment in all recipients (Table 1). This

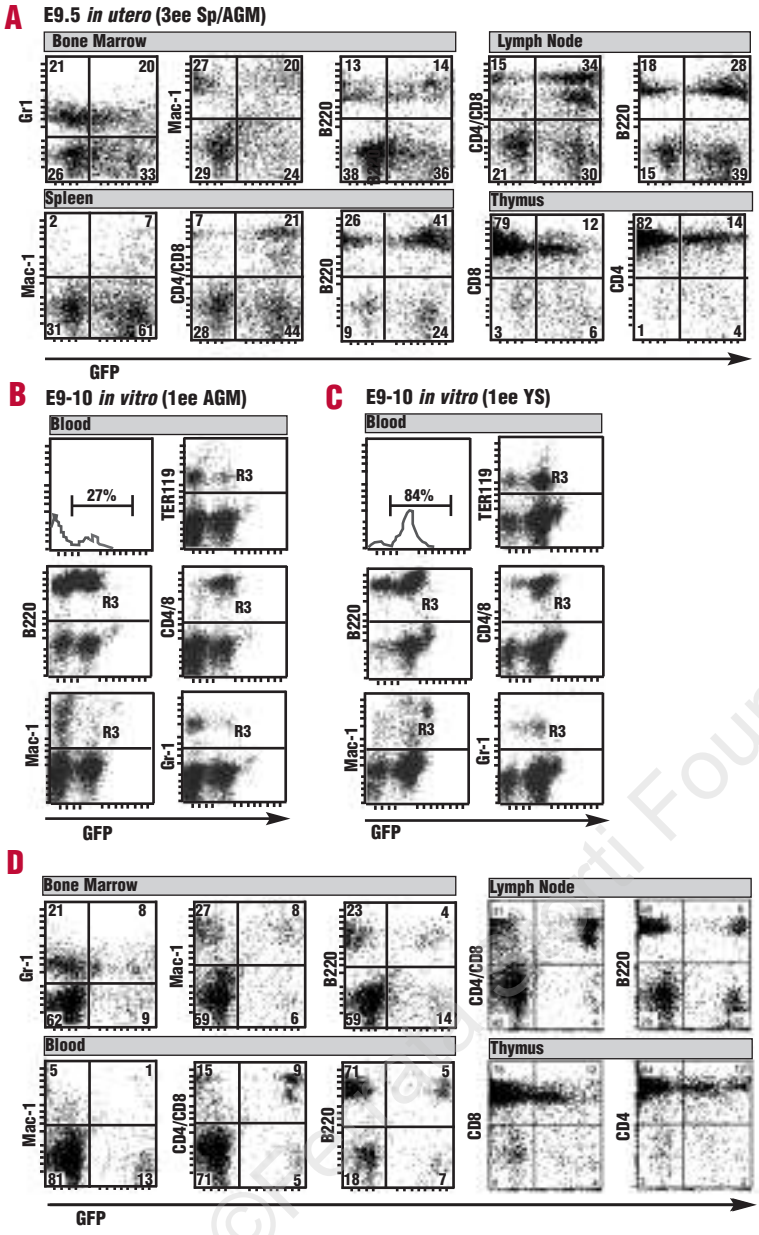


Figure 1. Flow cytometric analysis showing high-level, multilineage repopulation of unconditioned newborn W41/W41 recipients more than 12 months post-transplantation. Bone marrow, thymus, lymph node, spleen and/or blood cells were incubated with fluorescent antibodies specific for granulocytes (Gr-1), macrophages (Mac-1), T lymphocytes (CD4 and CD8), B lymphocytes (B220) and erythroid lineage cells (TER119) and fluorescence profiles measured on a FACScan (Becton-Dickinson). A. Tissue engraftment of a recipient injected with Sp/AGM cells from pooled *in utero* developed embryos (E9.5, 22-26 sp, 3 ee injected). B. Peripheral blood engraftment of a recipient injected with AGM cells from a whole embryo, *in vitro*-cultured from E9 to E10 (33 sp, 1 ee injected). C. Peripheral blood engraftment and D. Tissue engraftment of a recipient injected with YS cells from a whole embryo, *in vitro*-cultured from E9 to E10 (32 sp, 1 ee injected). Percentages of donor ($EGFP^+$) and recipient ($EGFP^-$) lineage specific cells within the total viable population are indicated in the upper quandrants. sp: somite pairs; ee: embryo equivalents.



engraftment was multilineage as determined by DNA PCR and flow cytometric analysis on cells obtained from hematopoietic tissues (*not shown*). Thus, as measured by transplantation into W41/W41 neonates, the E9.5 Sp region of *in utero*-developed embryos contains a small number of potent long-term multipotential hematopoietic cells with self-renewal abilities.

### *In vitro-cultured whole embryos contain multipotential hematopoietic progenitors*

Given the inaccessibility of mammalian embryos for *in utero* manipulation, it is of great interest to culture whole mouse embryos *in vitro* to follow their development. After a culture period of 24-48 hours, embryos are typically scored for viability and normal development by several anatomical landmarks - neural tube closure, somite pairs, circulating blood, etc. To determine whether hematopoietic development is maintained in *in vitro*-cultured mouse embryos, we performed flow cytometric analysis. E9 embryos were obtained and used for flow cytometric analysis or cultured overnight to the E10 stage and then analyzed. Sp/AGM and YS tissues were dissected and cells stained with antibodies specific for hematopoietic markers: CD45 for all hematopoietic cells, Mac-1 for macrophages, Ter119 for erythrocytes (the most common circulating hematopoietic cells at this stage) and Sca-1 for mainly hematopoietic stem cells. As shown in Table 2, there are relatively abundant levels of hematopoietic cells in both the AGM and the yolk sac of cultured embryos. While there are slight

**Table 2.** Percentages of cells positive for hematopoietic cell surface marker expression before and after whole embryo culture (E9 to E10).

| | Sp/AGM | | YS | |
|---|---|---|---|---|
| | % before culture (E9) | % after culture (E10) | % before culture (E9) | % after culture (E10) |
| CD45 | 3.2 (1.6-5.0) | 1.9 (1.2-2.5) | 10.4 (9.4-11.7) | 20.7 (20.0-21.3) |
| Mac-1 | 1.6 (1.2-2.3) | 0.5 | 6.6 (6.3-6.9) | 10 |
| Ter119 | 2.4 | 1.6 | 9.1 | 12 |
| Sca-1 | 1.5 (1.4-1.5) | 0.6 (0.3-0.8) | 2.1 (1.0-3.0) | 2.6 (0.6-4.5) |

*Percentages are the average of 1-4 flow cytometric analyses (range).*

decreases in the percentages of some hematopoietic cells in the Sp/AGM region of the embryo proper, yolk sac percentages of some hematopoietic cells are slightly increased during the culture period. Thus, despite the substantial growth of the embryo body during the 24-hour culture period, the hematopoietic cell content of the AGM and YS is generally maintained.

To test more specifically whether hematopoietic development in *in vitro*-cultured mouse embryos is comparable to that of *in utero*-developed embryos, we tested for the presence of multipotential hematopoietic progenitors by transplantation into unconditioned $W^{41}/W^{41}$ recipients. E8, E8.5 and E9 EGFP transgenic whole embryos were cultured for 24 hours. Only viable, anatomically normal embryos that developed to the E9, E9.5 and E10 stages (respectively) were used for transplantation. Sp/AGM, and YS tissues were dissected and cell suspensions were injected into unconditioned $W^{41}/W^{41}$ neonates. Three out of a total of 42 injected recipients were found to contain at least 10% donor-derived cells in the peripheral blood at 12 months post-transplantation (Table 1). One recipient was engrafted with E10 AGM cells and two recipients were engrafted with E10 YS cells that were obtained from E9 embryos cultured for 24 hours (26 to 34 somite pairs; sp). Flow cytometric analysis revealed multilineage hematopoietic engraftment in the peripheral blood (Figures 1B and 1C) and the hematopoietic tissues of these recipients (Figure 1D). Furthermore, transplantation of primary bone marrow into irradiated adult secondary recipients yielded long-term multilineage repopulation (Table 1). Hence, we conclude that AGM and YS multipotential hematopoietic progenitors develop under *in vitro* whole embryo culture conditions and that these cells are self-renewing.

## Discussion

These results represent two major advances in the study of developmentally early, multipotential, self-renewing hematopoietic cells. First and most importantly, the 24-hour *in vitro* whole embryo culture system allows for the development of these immature hematopoietic stem cells. When the 12-month engraftment results are considered, the numbers of multipotential hematopoietic progenitors in the *in vitro*-cultured embryos appear to be equivalent (or slightly increased) to those in the *in utero*-developed embryos. Cells from the *in utero*-developed embryos engrafted 3% (1/34) of the injected $W^{41}/W^{41}$ neonatal recipients to high levels (greater than or equal to 10% donor-derived hematopoietic cells) while cells from *in vitro*-cultured embryos were able to engraft 7% (3/42) of $W^{41}/W^{41}$ neonatal recipients to these high levels. We show that both *in utero* and *in vitro* developed progenitors are capable of long-term repopulation, producing all hematopoietic lineages. Furthermore, these cells lead to repopulation of secondary (and even tertiary, *not shown*) recipients, confirming that they possess self-renewal capacity, an important property of stem cells. Thus, our results demonstrate for the first time that the 24-hour *in vitro* whole embryo culture system effectively allows for the development and/or maintenance of these complex hematopoietic progenitors and that this system can be used for further progenitor manipulation and fate mapping studies.

While we would have used the *in vitro* culture system for the study of the development of adult repopulating HSC within the AGM and YS (and the subsequent colonization of the fetal liver by these cells) our studies are limited here to neonatal repopulating multipotential hematopoietic progenitor cells. Disappointingly, the *in vitro* whole embryo culture system does not promote the normal development of embryos past the 34 to 35 somite pair stage (mid E10), the time during which the first adult repopulating HSC are generated. It is currently thought that during mid-gestation the placenta takes over important functions leading to further embryo growth and perhaps even to the *de novo* generation of adult type HSC.[22,23,20] Thus, until the *in vitro* whole embryo culture system is further improved, fate mapping is restricted to only the early multipotential hematopoietic progenitors.

The second advance of these studies is the demonstration that unconditioned $W^{41}/W^{41}$ neonates can be used (although not as efficiently as busulfan-conditioned neonates) as transplantation recipients for the detection of multilineage repopulating cells from early stage embryos. Whether from *in utero* or *in vitro*-developed embryos, the high levels of repopulation we found in $W^{41}/W^{41}$ recipients contrast with those result-

ing from transplantation of early AGM cells into Rag-/-:γc-/- adult recipients.[7] In Rag-/-:γc-/- adult recipients, pre-E10.5 AGM cells (2.5-3 ee of explant cultured Sp [0-10 sp] or AGM [30-35 sp]) yielded only low levels (1-5%) of granulocytic repopulation. In the more comprehensively studied busulfan-conditioned neonate transplantation model,[4] repopulation with E9 Sp and YS was much higher (ranging from 8-27%), was multilineage, and was more comparable to the levels we observed in W41/W41 neonatal recipients. The frequency of repopulated W41/W41 neonatal recipients we observed was much lower than that reported for busulfan-conditioned neonatal recipients.[4] Although the reason for this is not yet known, it may be due to less endogenous hematopoietic cell competition in conditioned neonates than in the W41/W41 neonates. Busulfan conditioning is thought to reduce the number of endogenous hematopoietic progenitors to negligible levels. Future comprehensive studies are necessary to determine the actual hematopoietic progenitor content of W41/W41 embryos. The results of such studies may reveal that mild conditioning of W41/W41 neonates will yield a higher frequency of repopulation.

In addition, progenitor cell enrichment[21,24] by flow cytometric sorting may more efficiently reveal multipotential hematopoietic cells when injected into W41/W41 neonatal recipients. Notwithstanding the efficiency of the W41/W41 neonatal transplantation model, the transplantation results reported here, together with the demonstration that *in vitro*-cultured embryos contain multipotential hematopoietic progenitors, represent advances in the study of the development of the adult hematopoietic system. The next major challenge is to generate a functional fate map by marking the presumptive hematopoietic precursor cells in whole embryo cultures with low density lipoproteins labeled with a fluorescent dye (LDL-DiI).

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