Modulation of hematopoietic and endothelial cell differentiation from mouse embryonic stem cells by different culture conditions

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Abstract

Embryonic stem (ES) cells can differentiate into many different somatic cells in culture. To better correlate hematopoietic and endothelial cell differentiation of ES cells in currently available protocols, we compared Flk-1, Scl, VE-cadherin, CD45, or Ter-119 expressing cells generated in embryoid bodies (EBs) and on OP9 cells. We report that the kinetics of Scl and Flk-1 expression were similar in EBs and OP9 cells, although Flk-1 expression was extended on OP9 cells. CD45+ and Ter-119+ cells developed more efficiently in EBs, while VE-cadherin+ cells developed largely on OP9 cells. Cell sorting and replating studies showed that Scl+ cells, not Flk-1+ or VE-cadherin+ cells, were enriched for primitive and definitive hematopoietic progenitors. Our studies indicate that optimal hematopoietic and endothelial cell differentiation occur in EBs and on OP9 cells, respectively. Regardless of the culture systems used, Scl is the most relevant marker for enriching primitive and definitive hematopoietic progenitors.

Introduction

There is great interest in generating different types of somatic cells from in vitro differentiated ES cells, as they can potentially be utilized for therapies for human diseases for which there are currently no effective treatments. Accordingly, many studies are aimed toward understanding mechanisms for maintaining the stem cell state and pathways leading to lineage specification. Successful generation and application of ES-derived somatic cells will require reproducible protocols to obtain desired cell types. There are currently two widely used protocols to generate blood and blood vessel cells in culture. In the first protocol, ES cells differentiate and form three-dimensional cell
masses called embryoid bodies (EBs). In the second protocol, ES cells differentiate on type IV collagen or stromal cells, such as OP9, in two-dimensional sheets.

In both the EB and OP9 culture systems, primitive erythroid cells develop prior to the definitive erythroid cell population. In addition, the putative common progenitor of hematopoietic and endothelial cells, the hemangioblast, has been reported to develop within EBs and on OP9 cells. Endothelial cells also develop in both culture systems. While these studies would argue that these two different methods could be utilized interchangeably, there has not yet been a systematic comparison of whether hematopoietic and endothelial cell development proceeds in parallel in these two culture systems. To better correlate hematopoietic and endothelial cell differentiation in EBs and the OP9 system, we compared Flk-1, Scl, CD45, Ter-119 and VE-cadherin expression and performed cell sorting and hematopoietic replating studies. We demonstrate that hematopoietic cells develop more efficiently in EBs, while endothelial cell maturation is better supported by OP9 cells. We further demonstrate that Scl, not VE-cadherin, is ideal for isolating hematopoietic progenitors.

**Study Design**

Scl+/hCD4 ES culture and hematopoietic colony assays were performed as described previously. EBs were formed in 20% FCS (pre-selected lot) in α-MEM with ascorbic acid (50 µg/ml), L-glutamine (2 mM) and MTG (4.5 x 10^{-4} M). OP9 differentiation was carried out in 20% FCS (pre-selected lot) in α-MEM. OP9 cells were treated with mitomycin C prior to ES seeding and differentiation, as the mitomycin C treatment
consistently gave a higher number of differentiated cells (Supplementary Figure 1). Cell staining and FACS analyses were carried out as described previously, \(^{10,11}\) except that differentiated cells were dissociated with collagenase (0.25%, Sigma) and anti-CD45 antibody used in this study was biotin labeled (Pharmingen).

**Results and Discussion**

To compare hematopoietic and endothelial cell differentiation in EBs and the OP9 system, we utilized Scl\(^{\text{hCD4}}\) ES cells.\(^{10}\) As a first step, we compared the kinetics of Flk-1, Scl and VE-cadherin expression. As previously reported\(^{10}\) and shown in Figure 1A, Flk-1 was readily detectable by day 2.75 in EBs. Flk-1\(^+\) cells continued to develop up to day 4, and declined thereafter. Cells expressing Flk-1 were also readily detectable on OP9 cells. A similar percentage of Flk-1\(^+\) cells was detected in EBs and OP9 cells up to day 4. However, Flk-1 expression was prolonged when ES cells were differentiated on OP9, such that Flk-1\(^+\) cells were still present at higher levels at later times (13.4% (19.3 ± 5.1) in day 6 EBs vs. 37.7 % (40.5 ± 3.4) in OP9, student t test value P< 0.01). Scl (i.e. hCD4) expressing cells developed slowly in EBs but expanded rapidly between days 4 and 5. About 60-70% of the total cells expressed hCD4 at days 5-7. On OP9, a higher percentage of cells expressed hCD4 at early time points (8% (7.7 ± 1.8) in day 3 EBs vs. 29% (28.6 ± 6.6) on OP9, P< 0.05). However, the percentage of hCD4\(^+\) cells detected at later times (days 5-7) was similar in both EBs and OP9. While a majority of hCD4\(^+\) cells present in days 5-7 EBs did not express Flk-1, a higher percentage of hCD4\(^+\) cells also expressed Flk-1 when ES cells were differentiated on OP9 cells. As hCD4-single positive cells were more abundant in EB cells, we examined CD45 and Ter-119 expression
patterns. As shown in Figure 1B, a higher percentage of CD45 (~7% (7.1 ± 1.1) in day 6 EBs vs. ~2.7% (3.5 ± 1.5) in OP9, P< 0.01) and Ter-119 (~4.5% (3.9 ± 0.5) in day 6 EBs vs. ~1.1% (1.9 ± 0.8) in OP9, P< 0.05) expressing cells was readily detectable in EBs. This suggests that hematopoietic differentiation was achieved more efficiently within EBs.

VE-cadherin expressing cells developed very poorly in EBs. On OP9, however, VE-cadherin expression was readily detectable. VE-cadherin was first detected at day 2.25 (data not shown) and about 10% (14.4 ± 3.6) of the cells at day 3 (Figure 1A) expressed VE-cadherin. VE-cadherin expression increased up to day 5 and then gradually decreased. By day 6, about 16% (16.7 ± 2) of the cells expressed VE-cadherin. More importantly, VE-cadherin+ cells at all stages (days 2.75-7) were always within the Flk-1+ hCD4+ cell population (Figures 1 and 2A). Our data suggest that endothelial progenitors will develop in EBs and OP9, but that endothelial cell maturation, based on VE-cadherin expression, occurs more efficiently in the OP9 system. Thus, the Flk-1 expressing cells present at later times on OP9 would represent mature endothelial cells. Consistent with this interpretation, Flk-1+ cells, sorted from OP9 culture 6 days after differentiation, showed poor endothelial cell replating (data not shown). As our previous studies demonstrated that Flk-1+ cells sorted from day 6 EBs exhibited a robust endothelial cell differentiation,10 we propose that endothelial progenitors developing on OP9 concomitantly differentiate into VE-cadherin+ endothelial cells, while endothelial progenitors developing in EBs cannot. OP9-derived factor(s) could play a role in this process. Alternatively, a simple two-dimensional culture condition could be sufficient.
To better define Scl and VE-cadherin expression in primitive erythroid and definitive hematopoietic progenitors,10, 12 ES cells differentiated on OP9 were collected on day 4, sorted for hCD4+VE-cadherin+, hCD4+VE-cadherin-, or hCD4+VE-cadherin- and replated for primitive erythroid colonies. As shown in Table 1, hCD4+VE-cadherin- cells, compared to hCD4+VE-cadherin+, generated a higher number of primitive erythroid colonies. To determine definitive hematopoietic potential, ES cells differentiated on OP9 for 6 days were initially sorted for VE-cadherin+ and VE-cadherin- cell populations and subjected to hematopoietic replating and globin gene analyses. As shown in Figure 2B and C, hematopoietic colonies developed from both cell populations. β-major globin gene was also expressed in erythroid cells developed from VE-cadherin- cell populations (Figure 2C). Subsequently, hCD4+Flk-1+VE-cadherin+, hCD4+Flk-1+VE-cadherin-, hCD4+Flk-1+VE-cadherin-, hCD4+Flk-1+VE-cadherin- and hCD4+Flk-1+VE-cadherin- cell populations, present in day 6 culture (Figure 1 and 2A), were sorted and replated. As shown in Figure 2D, definitive hematopoietic colonies developed from VE-cadherin- hCD4+Flk-1-, VE-cadherin-hCD4+Flk-1+ and VE-cadherin+hCD4+Flk-1+ cell populations. The common feature of these three cell populations was that they expressed hCD4. Thus, these studies suggest that Scl is the most relevant marker, not Flk-1 or VE-cadherin, for enriching primitive and definitive hematopoietic progenitors. Consistent with this interpretation, VE-cadherin knockout mice show relatively normal hematopoietic differentiation but defective endothelial cell development.13-15 Similarly, long-term repopulating potential of AGM-derived cells was found in both VE-cadherin+ and VE-cadherin- cell populations.16 Finally, Scl expression prior to the generation of VE-
cadherin expressing cells could rescue hematopoietic defects in $Scl^{-/-}$ ES cells.\textsuperscript{17} Collectively, our studies indicate that VE-cadherin expression alone is not sufficient for isolating hematopoietic progenitors and that Scl is the most reliable marker for tracking hematopoietic differentiation.

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SUPPLEMENTAL MATERIAL IS AVAILABLE ONLINE ONLY AT THE TIME OF FINAL PUBLICATION.

**References**


Figure Legends

**Figure 1.** Kinetic analyses of Flk-1, Scl, VE-cadherin, CD45, and Ter-119 expression. *In vitro* differentiated $Scl^{+/hCD4}$ ES cells during EB and OP9 differentiation (from day 2.75 to day 7) were subjected to FACS analyses for Flk-1, hCD4, VE-cadherin (A) and CD45, and Ter119 (B) expression. Numbers in a given box indicate the percentage of each population.

**Figure 2.** Hematopoietic cells develop from Scl expressing cells.

A. VE-cadherin expressing cells also express Flk-1 and Scl. $Scl^{+/hCD4}$ ES cells differentiated on OP9 for 6 days were subjected to FACS analyses for Flk-1, hCD4, and VE-cadherin expression. Based on the FACS data, the relationship between Flk-1, Scl, and VE-cadherin expressing cells is presented in Venn diagram.

B, D. $Scl^{+/hCD4}$ ES cells were differentiated on OP9 for 6 days and FACS-sorted for VE-cadherin$^+$ and VE-cadherin$^-$ cells (B) or hCD4$^+$Flk-1$^+$VE-cadherin$^+$, hCD4$^+$Flk-1$^+$VE-cadherin$^-$, hCD4$^+$Flk-1$^-$VE-cadherin$, hCD4$^+$Flk-1$^-$VE-cadherin, and hCD4 Flk-1$^+$VE-cadherin$^-$ cells (D), and subjected to hematopoietic replating (6 x 10$^4$ cells/ml, A; 5 x 10$^4$ cells/ml, B, respectively). The resulting hematopoietic colonies were counted 6 days later. Data are expressed as mean values ± SD. Three independent sortings show similar results. E, erythroid; M, macrophage; and E+M, erythroid/macrophage mixed colonies.
C. VE-cadherin is not a marker for definitive hematopoietic cells. ES cells were differentiated on OP9 for 6 days, sorted for VE-cadherin⁻ and VE-cadherin⁺ cells, and replated. Subsequently, erythroid/macrophage mixed colonies were subjected to globin gene analyses as described.¹⁰ As shown, erythroid cells developing from both VE-cadherin⁻ and VE-cadherin⁺ cell populations expressed β-major globin gene. N, H₂O, negative control; P, RNA from day 3 EBs, positive control. L32, ribosomal subunit protein is shown as a loading control.

**Table 1.** Primitive erythroid colonies develop from Scl⁺ cells.

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<thead>
<tr>
<th>Scl⁺/hCD4⁻</th>
<th>6 x 10⁴ cells/ml</th>
<th>Mean ± SD</th>
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<tbody>
<tr>
<td>hCD4⁻ VE-cadherin⁻</td>
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<td>hCD4⁺ VE-cadherin⁺</td>
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<td>hCD4⁺VE-cadherin⁻</td>
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ES cells were differentiated on OP9 for 4 days, sorted for hCD4⁻ VE-cadherin⁻, hCD4⁺VE-cadherin⁺, or hCD4⁺VE-cadherin⁻, and replated for primitive erythroid colonies (6 x 10⁴ cells/ml). The resulting primitive erythroid colonies were counted 4 days later. Data are expressed as mean values ± SD.
Zhang et al. Figure 1
Zhang et al. Figure 2
Table 1

<table>
<thead>
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<tbody>
<tr>
<td>Non-fractionated</td>
<td>425 ± 30</td>
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<tr>
<td>hCD4-VE-</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>hCD4+VE+</td>
<td>589 ± 61</td>
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<tr>
<td>hCD4+VE-</td>
<td>1636 ± 48</td>
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