EML Erythroid and Neutrophil Differentiation Protocols

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[Abstract] Erythroid-Myeloid-Lymphoid cells (EML) are a multipotent haematopoietic cell line of mouse bone marrow origin capable of long-term maintenance *in vitro* in the presence of SCF (stem cell factor) (Tsai *et al*., 1994). The self-renewal capacity of the EML cell line is conferred by the presence of a dominant-negative retinoic acid receptor (RAR) originally delivered by retroviral transduction (Tsai *et al*., 1994), which arrests cells at an early progenitor stage blocked from normal progression into myeloid differentiation. The presence of the RAR trans-gene does not interfere with erythroid differentiation, and it is possible to capture a low percentage of early erythroid, but not myeloid, committed cells in maintenance cultures (Pina *et al*., 2012; Ye *et al*., 2005).

Cells can be driven into granulocytic/neutrophil differentiation through the use of high doses of retinoic acid (RA), which overcomes the differentiation block. It should be noted that these pharmacological doses of RA are not compatible with erythroid differentiation, and it is hence not viable to obtain robust erythroid and myeloid differentiation in the same assay. Indeed, colonies scored as mixed-lineage in CFC assays are a mixture of undifferentiated and erythroid cells (Tsai *et al*., 1994). Nevertheless, robust single-lineage erythroid and neutrophil differentiation can be obtained in liquid culture under defined cytokine conditions, as specified below.

Materials and Reagents

1. IMDM (powder or liquid)
2. Horse serum (HS) (may require batch testing if low cell viability)
3. Foetal bovine serum (FBS)
4. L-Glutamine
5. Penicillin/Streptomycin (P/S)
6. SCF-conditioned medium (SCF-CM) (see Notes)
7. Recombinant human erythropoietin (*e.g.* epoietin alpha, Amgen) (obtained through hospital pharmacy under appropriate local guidelines)
8. Recombinant mouse interleukin-3 (IL-3) (*e.g.* Pepro Tech, catalog number: 213-13)

9. Recombinant mouse granulocyte-monocyte colony-stimulating factor (GM-CSF) (*e.g.* Pepro Tech, catalog number: 315-03)
10. All-Trans Retinoic Acid (ATRA) (*e.g.* Sigma-Aldrich, catalog number: 302-79-4, reconstitute in ethanol)
11. Culture supplements and antibiotics (L-Glutamine and Penicillin/Streptomycin)
12. Monoclonal anti-mouse antibodies for flow cytometry
    a. C-kit/CD117 (clone 2B8) (*e.g.* PE-Cy7, eBioscience, catalog number: 25-1171) (suggested use at 1:100 dilution)
    b. CD34 (clone RAM34) (*e.g.* Alexa-Fluor 647, eBioscience, catalog number: 51-0341) (suggested use at 1:100 dilution)
    c. Mac-1/CD11b (clone M1/70) (*e.g.* PE, eBioscience, catalog number: 12-0112) (suggested use at 1:100 dilution)
    d. Gr1/Ly6C (clone RB6-8C5) (*e.g.* FITC, eBioscience, catalog number: 11-5931) (suggested use at 1:100 dilution)
    e. Sca-1/Ly6A/E (clone D7) (*e.g.* Pacific blue, BioLegend, catalog number: 122520) (suggested use at 1:50 dilution)

    Note: Stain on ice, in culture medium, for 20 min; wash with 10-20x volume of medium; pellet cells at 400 x g for 5 min. Re-suspend in 300-500 µl of medium for FACS analysis.
13. Trypan blue

Equipment

1. T175 tissue culture flasks with filtered cap (for production of conditioned medium)
2. T75 or T25 tissue culture flasks with filtered cap, or 6-well plates

   Note: For EML cultures, the size of the tissue culture vial is determined by the cell number seeded at the cell densities indicated in the protocol.
3. Cell culture incubator

Procedure

A. Maintenance culture conditions

1. EML cells maintenance culture conditions are IMDM with 5% HS, 2 mM L-glutamine and 1x penicillin/streptomycin (P/S) and 10-15% of SCF-conditioned medium (SCF-CM) obtained from BHK-MKL cells.

   Note: The original cultivars of EML cells were grown in medium supplemented with rat SCF at a final concentration of 200 ng/ml (Tsai et al., 1994).

2. Cells should be seeded at 2 x 10^4 cells/ml and split to the initial dilution when a cell density of 10^5-2 x 10^5/ml (absolute maximum) is reached. This typically means splitting the cultures every 2 or sometimes 3 days. Cultures die rapidly at higher cell densities.
   Note: The dead cell fraction in maintenance cultures, as judged by Trypan blue exclusion, should not exceed 5-8% (10% is acceptable if not indicative of a culture exhaustion trend.).
3. Cultures can be maintained for at least 20 passages post-thaw without any significant changes in biology.

B. Erythroid differentiation conditions

1. Use EML cells in logarithmic growth phase from a maintenance culture, and plate them at a seeding concentration of 2 x 10^4 cells/ml in fresh maintenance culture medium + recombinant human erythropoietin (EPO) at a final concentration of 10 U/ml.
2. EML erythroid differentiation cultures are very sensitive to cell crowding, so opt for increasing culture volumes rather than initial cell densities if high numbers of cells are required.
3. Culture for 2 days. After this initial period, wash cells 2-3x in IMDM with 5% HS + 2 mM L-glutamine and 1x P/S, and re-seed at 2 x 10^4 cells/ml in IMDM, 5% HS, 2 mM L-glutamine and 1x P/S, with 2.5% SCF-CM and 10 U/ml EPO.
4. Differentiate cells for a total of 7 days (so, 5 additional days), with fresh EPO (10 U/ml) added on day 5 to the existing culture.
5. It is expected to observe an average 20-fold expansion in cell numbers during the culture period, with cell viability decreasing gradually as cells differentiate, to a minimum of 40-50% at day 7.
6. The extent of cell differentiation can be determined by flow cytometry staining with C-Kit, Sca1 and CD34, with accumulation of differentiated $Sca1^{lo}CD34^{-}C\text{-}kit^{lo/-}$ cells (referred to as Kit^{-}, see Figure 1 for representative plot) (Pina *et al.*, 2012). It is expected that 20-30% of the culture become Kit^{-} by day 6 of differentiation.
7. When erythroid differentiation cultures are seeded with committed erythroid cells (*e.g.* sorted $Sca1^{lo}CD34^{-}$ cells cultured as per B1 above) (Pina *et al.*, 2012), 60-80% of the culture are Kit^{-} by day 4 of differentiation, reflecting faster differentiation kinetics from committed cells.
8. Not unexpectedly, cultures seeded with erythroid-committed cells are less proliferative, with a 10-fold expansion in cell numbers expected by day 4, after which the cultures plateau and become less viable.

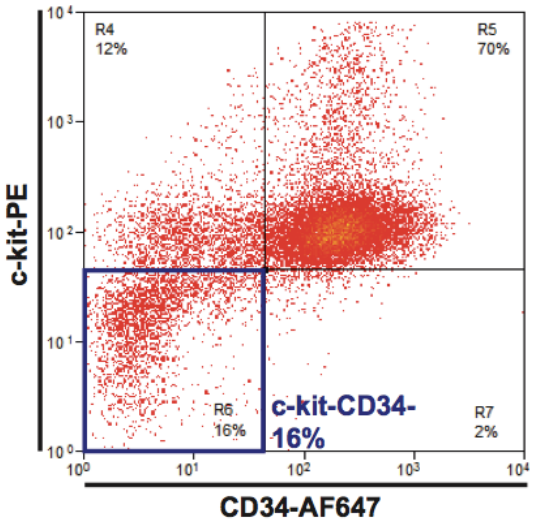

Figure 1. Erythroid differentiation of EML cells. A representative plot of flow cytometry analysis at day 4 is shown; c-kit$^-$CD34$^-$ cells are Sca1$^{lo/-}$. In this instance, cultures were seeded at day 0 with a population of cells with culture-reconstituting (or self-renewal) potential (Pina *et al.*, 2012).

C. Neutrophil differentiation conditions

1. Use EML cells in logarithmic growth phase from a maintenance culture, and plate them at a seeding concentration of 2 x 10^4 cells/ml in fresh maintenance culture medium + 10 ng/ml of recombinant mouse IL-3 and 10 µM ATRA.
2. Viability is less affected by cell crowding than in erythroid differentiation, and it is thus possible to seed cultures at higher densities (0.5-1 x 10^5) if high cell numbers are required.
3. After 2 days, wash cells 2-3x in IMDM with 5% HS + 2 mM L-glutamine and 1x P/S, and re-seed at 2 x 10^4 cells/ml in IMDM, 5% HS, 2 mM L-glutamine and 1x P/S, with 2.5% SCF-CM + 10 ng/ml of recombinant mouse GM-CSF and 10 µM ATRA.
4. Keep the culture growing for a total of 7 days (so, 5 additional days), with fresh GM-CSF (10 ng/ml) added on day 5 to the existing culture.
5. It is expected to observe an average 10-fold expansion in cell numbers during the culture period, with viabilities close to 80% or higher throughout the duration of the culture.
6. The extent of cell differentiation can be determined by flow cytometry after staining with Gr-1 and Mac-1 antibodies (Pina *et al.*, 2012), with accumulation of granulocytic Gr1$^+$Mac-1$^+$ cells to an expected proportion of 40-50% by days 4-5 of culture (see Figure 2 for representative plot). Proportions can drop thereafter, although absolute numbers of differentiated cells continue to increase.

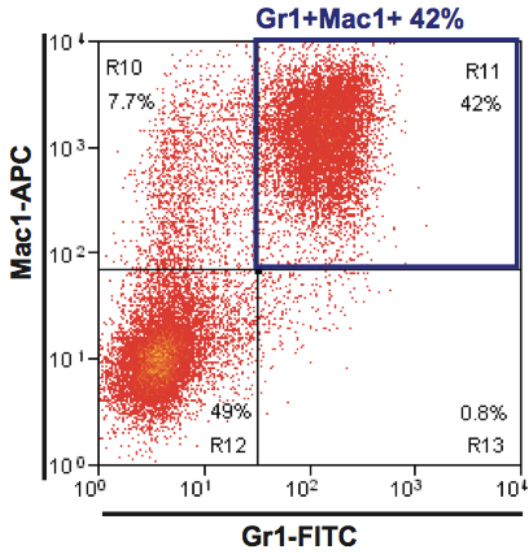

Figure 2. Neutrophil differentiation of EML cells. A representative plot of flow cytometry analysis at day 6 is shown.

Notes

1. SCF-conditioned medium (SCF-CM)
    a. One possible source are BHK cells expressing the murine kit ligand protein (MKL, or SCF).
    b. Conditioned medium can be produced in T175 flasks with a total production volume of 60 ml/flask.
    c. Culture the cells in IMDM with 10% FBS, 2 mM L-Glutamine and 1x Penicillin/Streptomycin until they reach 80% confluence.
    d. At this stage replace with fresh medium and culture for an additional 48 h, at which point the supernatant is collected, floating cells removed by pelletting at 400 *x g*, 5 min, and the medium filtered through a 0.2 µm mesh.
    e. Conditioned medium can be aliquoted and stored for several months at -20 °C without loss of activity.

Acknowledgments

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References

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