Removal of early differentiated cells from mouse pluripotent stem cell cultures by magnetic cell sorting

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Introduction

Mouse embryonic (mESCs) and induced pluripotent stem cells (iPSCs) can be maintained in a pluripotent state, e.g., by cultivation in serum-containing medium supplemented with leukemia inhibitory factor (LIF). Expression of the transcription factors Oct-4 and Nanog or the surface carbohydrate SSEA-1 are widely used to characterize pluripotency of mouse cells at the molecular level. However, expression dynamics of these markers are rather low, leaving transcript and/or protein expression detectable even several days after removal of LIF. Therefore, these markers have only limited potential for the discrimination between pluripotent and early differentiated cells. A cell surface marker that is absent on undifferentiated cells and rapidly up-regulated upon early differentiation would be a valuable tool for cell separation approaches aiming at the isolation of pluripotent stem cells.
In this study we have identified a dynamically regulated surface marker, which is expressed on a small subpopulation of mESCs (approx. 10%) under standard culture conditions and strongly upregulated to approx. 60% during the first days of differentiation. Identification of this surface marker was achieved by using a rat monoclonal antibody, referred to as Anti-Diff, that was previously generated in our laboratory. To enrich pluripotent stem cells from mESC cultures, we have established a magnetic cell sorting protocol based on MACS® Technology. Thereby, mESC culture quality, embryoid body (EB) formation, and generation of coat color chimera were improved.

Results

1 Dynamic expression regulation of the early differentiation marker in feeder-free mESC cultures

Dot plots and phase-contrast images of mESCs cultivated in the presence (+LIF) or absence of LIF (–LIF) for up to 3 days. Flow cytometric analyses showed an increasing number of differentiated cells upon LIF removal. In phase-contrast images most of the early differentiated cells can be identified by a fibroblast-like morphology. Standard cultivation (+LIF) generates low numbers of flat, fibroblast-like cells that are stained by Anti-Diff-PE (4–14%). Removal of LIF quickly increases the frequency of differentiated cells in the culture dish and Anti-Diff-PE-stained cells up to 60% at day 3.

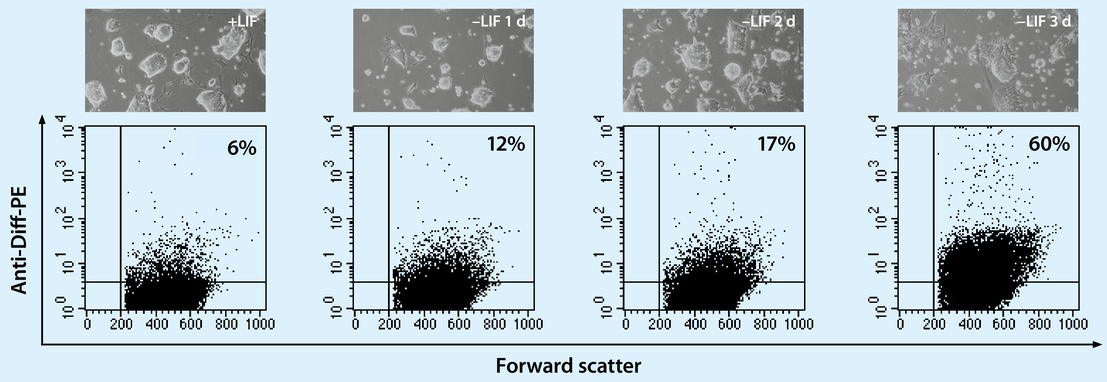


Figure 1

2 Efficient depletion of early differentiated cells from subpopulations of SSEA-1– and E-cadherin–positive cells in mESC cultures

Costaining of cells detected with our Anti-Diff-PE antibody and antibodies against two mESC surface markers (SSEA-1 and E-cadherin) was analyzed by flow cytometry (A–C, ori). As expected, during standard cultivation (+LIF) approx. 90% of the cells stained positive for SSEA-1. Among these, 4% costained with our Anti-Diff-PE antibody. Upon LIF removal the frequency of cells that were double-positive for SSEA-1 or E-cadherin and Anti-Diff-PE increased to 35%, indicating low dynamics of SSEA-1 and E-cadherin expression but strong up-regulation of the early differentiation marker.
In order to remove this subpopulation from mESC cultures and efficiently deplete early differentiated cells, we next established a magnetic cell sorting procedure. First, early differentiated cells were labeled with Anti-Diff-PE and Anti-PE MicroBeads (G). Then the cell suspension was loaded onto a MACS Column, which was placed in the magnetic field of a MACS Separator (H). The magnetically labeled early differentiated cells were retained within the column. The non-labeled pluripotent cells were recovered in the flow-through. This cell fraction (neg) was depleted of early differentiated cells.
As shown in figures 2D–F (neg) the purity of the enriched pluripotent cells reached 98%–99.6% after depletion of early differentiated cells.

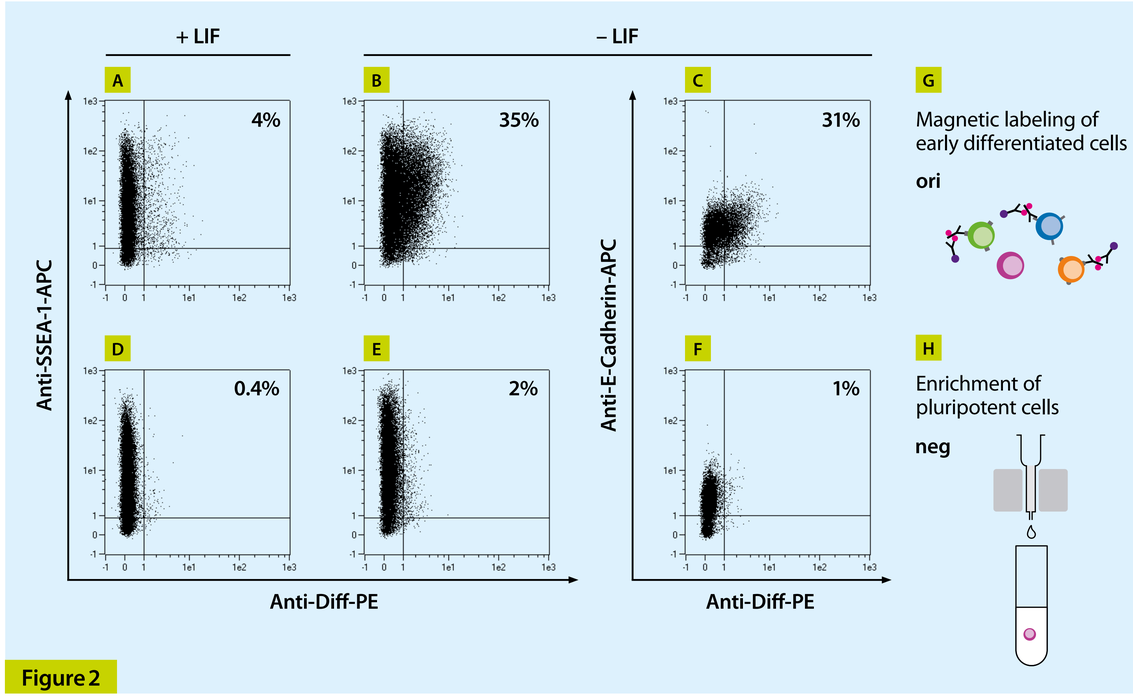


Figure 2

3 Efficient removal of cytokeratin 18–positive cells from mESC cultures by MACS Technology

To further characterize the population of cells that was depleted by magnetic cell separation, cells were plated before and after separation. Phase-contrast images of the cell population before separation (A, ori) indicate the occurrence of flat, fibroblast-like cells. As shown by immunofluorescence (B, ori), most of those early differentiated cells expressed cytokeratin-18. Upon depletion of early differentiated cells, fibroblast-like (C, neg) and cytokeratin-18–positive cells (D, neg) were efficiently removed from mESC cultures. In contrast, these early differentiated cells were enriched in the positive fraction after cell separation (E–F, pos). Additionally, molecular characterization of the enriched pluripotent stem cells by real-time PCR analysis revealed significant reduction of lineage markers such as N-cadherin (3-fold), prominin-1 (5-fold), and cytokeratin-18 (10-fold), whereas expression of Oct-4 and Nanog did not change significantly (data not shown).

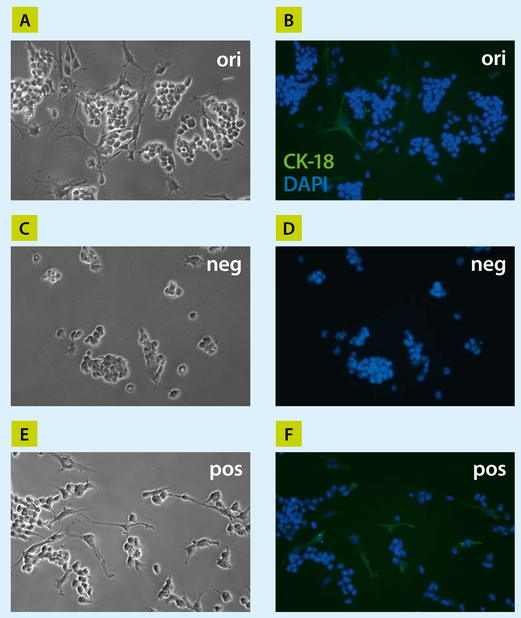


Figure 3

4 Improved embryoid body (EB) formation after depletion of early differentiated cells

Formation of EBs from non-separated (A, ori), enriched pluripotent (B, neg) and enriched differentiated cells (C, pos) was compared at day 4. Depletion of early differentiated cells before EB formation (B, neg) improves EB morphology as smooth and round EBs develop. In contrast, EBs formed from non-separated cells (A, ori) or enriched differentiated cells (C, pos) are irregularly shaped and sticky as they attach to the culture dish and single cells adhere on top.
In order to characterize the differentiation potential of the enriched pluripotent stem cells, flow cytometric analyses with antibodies against surface markers of germ layer–specific cell types were performed. As indicated in fig. 4D, formation of mesodermal (Flk-1), mesendodermal (CXCR4), and ectodermal (Prominin-1, PSA-NCAM) cells tends to be slightly improved after removal of early differentiated cells (neg) before embryoid body formation.

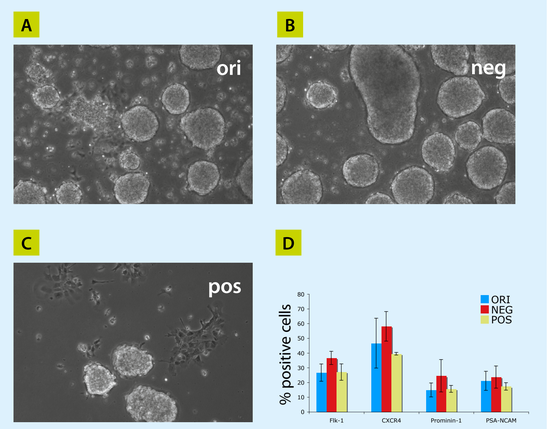


Figure 4

5 Improved generation of coat color chimera after depletion of early differentiated cells.

Transgenic mice were generated from blastocyst injections of mESC cultures. Experiments with either non-separated (ori) or separated mESCs (neg) were carried out. In 2 out of 5 experiments (ori) compared to 5 of 6 experiments (neg), coat color chimeric mice were born. This indicates a higher efficiency in generating coat color chimera from mESC cultures after depletion of early differentiated cells.

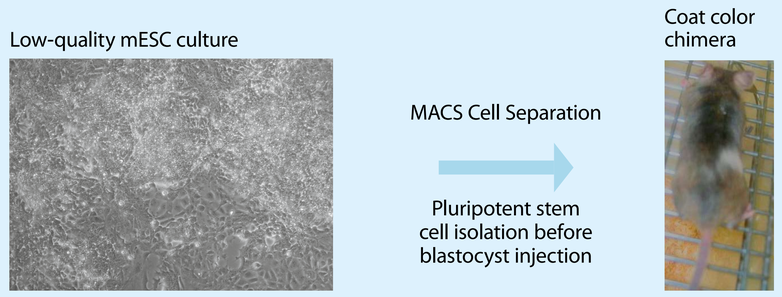


Figure 5

Conclusion

We have identified an antibody (Anti-Diff-PE) that labels early differentiated cells in feeder-free mouse pluripotent stem cell cultures and established a MACS Cell Separation strategy to remove these early differentiated cells.
We show that enrichment of mouse pluripotent cells improves:

- standard pluripotent stem cell cultures,
- formation of embryoid bodies,
- generation of coat color chimera.

The enrichment of pluripotent cells provides a major benefit in standardizing mouse pluripotent stem cell culture, conducting complex differentiation experiments, as well as for the generation of transgenic mice.