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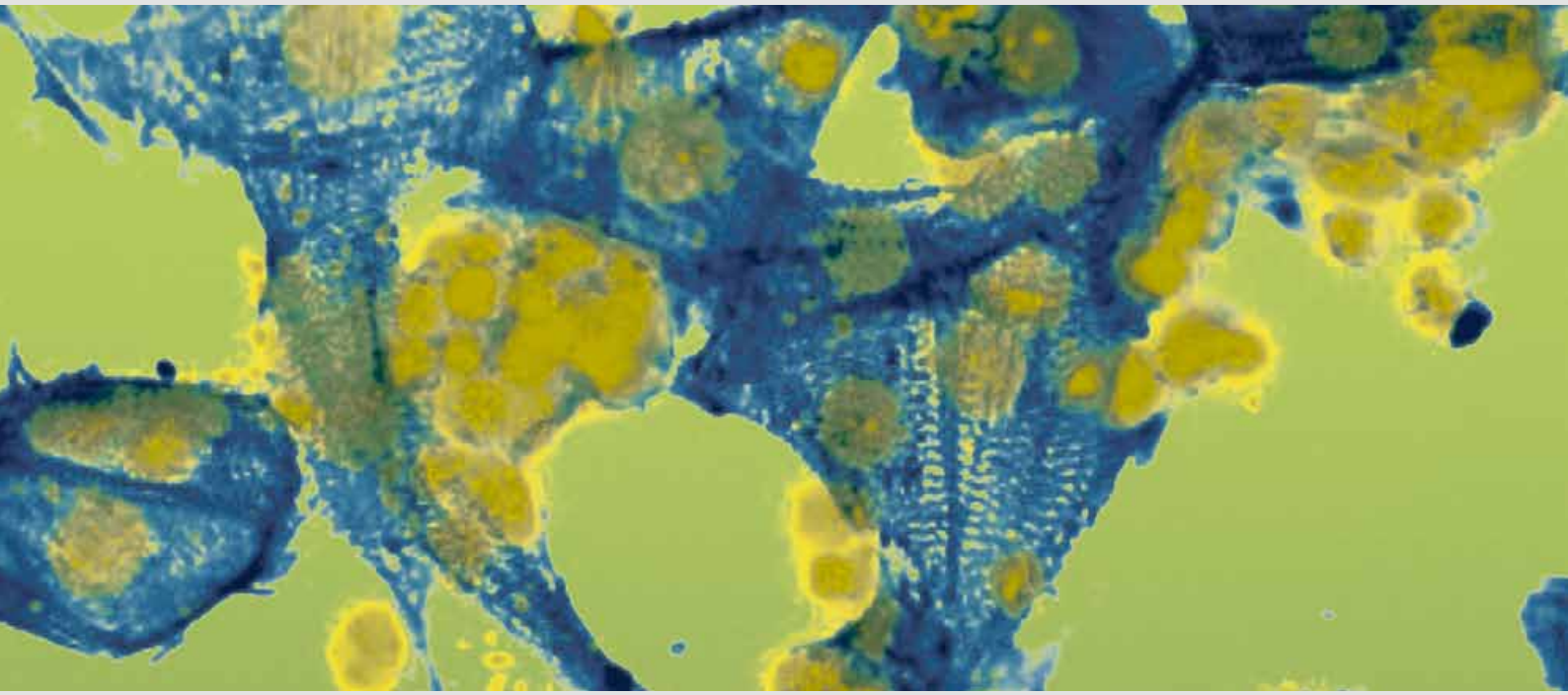

Use of magnetically enriched pluripotent stem cells increases chimerism rate after blastocyst injection and enables the use of inbred ES cell lines for tetraploid complementation


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Introduction

Transgenic mouse models are a powerful tool for disease modeling and studying gene function *in vivo*, either during development or in the adult organism. The generation of transgenic mice via blastocyst injection of pluripotent stem cells or tetraploid complementation requires high-quality pluripotent stem cells. However, regardless of whether feeder cells are present or not, standard culture conditions cannot fully prevent spontaneous differentiation of pluripotent embryonic stem cells (ESCs).

In order to deplete mouse embryonic fibroblasts (mEFs) and generate a homogeneous population of pluripotent stem cells, we used MACS® Technology.

The quality of enriched ESCs was evaluated by detection of alkaline phosphatase (AP) activity and nuclear Oct-4 expression, and compared to unseparated ESCs. For the assessment of the cells' pluripotency, we employed enriched pluripotent ESCs and unseparated ESCs for micro-injection into diploid and tetraploid blastocysts and compared their capacities to produce offspring.

Materials and methods

Cell culture

For this study, we used ESCs derived from BALB/c[1] and C57BL/6 (B6-RMCE) mice. Cells were cultured in DMEM containing LIF (1,000 U/mL) and 20% FCS (B6-RMCE) or 15% FCS (BALB/c). Cells were cultured on a layer of mEFs where indicated.

Combined feeder cell removal and purification of pluripotent mESCs

Magnetic enrichment of pluripotent ESCs and depletion of mEFs was achieved by using a special protocol combining Feeder Removal MicroBeads, mouse and the Pluripotent Stem Cell Isolation Kit, mouse (special protocol available at www.miltenyibiotec.com/protocols).

AP activity and Oct-4 immunofluorescence staining

AP activity was detected by using the Alkaline Phosphatase Detection Kit (Millipore) according to the manufacturer's protocol. For Oct-3/4 staining we used a primary monoclonal mouse anti-Oct3/4 (C-10) antibody (Santa Cruz Biotechnology) and a secondary Goat Anti-Mouse IgG-Rhodamine antibody (Dianova) according to the manufacturers' instructions. Cells were analyzed by light microscopy or fluorescence microscopy.

Generation of transgenic mice

Mouse blastocysts were isolated, cultured, and microinjected with enriched or unseparated pluripotent ESCs by using standard methods[2]. Tetraploid mouse blastocysts were prepared by electrofusion[3]. Diploid or tetraploid blastocysts were transferred into mouse uteri according to standard protocols[2]. Chimerism of mice derived from diploid blastocysts was evaluated by coat color.

Results

Morphological and phenotypic characterization of enriched and unseparated ESCs

We purified pluripotent ESCs by using the special protocol for combined feeder cell removal and enrichment of pluripotent stem cells and plated them onto gelatin-coated dishes. The next day, we evaluated their morphology and compared it with the morphology of unseparated cells. Figure 1A demonstrates that the enriched ESCs gave rise

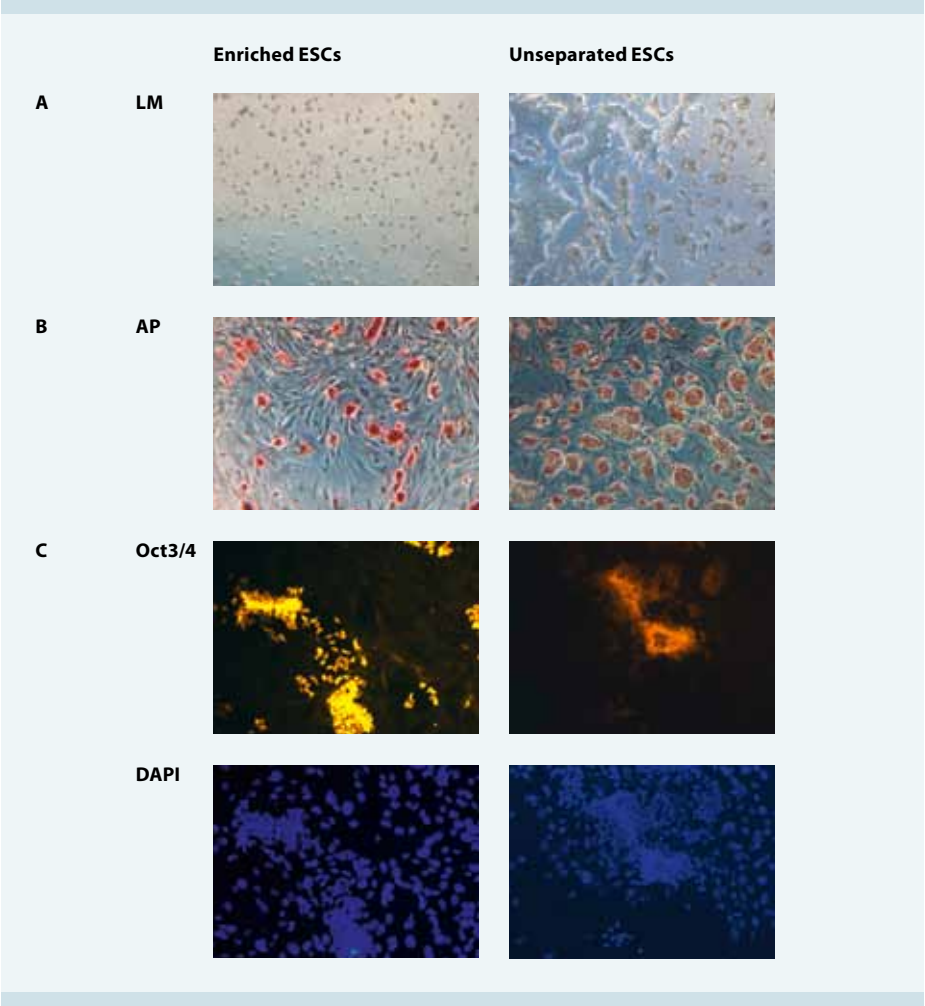

Figure 1 Morphological and phenotypic characterization of enriched and unseparated ESCs. Enriched or unseparated BALB/c ESCs were plated onto gelatin-coated dishes in a 12-well plate (2×10^5 cells per well) in the absence (A) or presence (B) of mEFs. One day after plating, the cells were analyzed by light microscopy (A). Two days after plating, the cells were analyzed for AP activity as described in materials and methods (B). (C) Enriched or unseparated B6-RMCE ESCs were seeded onto a glass slide (4×10^4 cells per slide). After two days the cells were stained with a mouse Oct3/4 (C-10) antibody and a Goat Anti-Mouse IgG-Rhodamine antibody, and viewed by fluorescence microscopy. Cell nuclei were stained with DAPI.

to small, single, circular-shaped colonies. In contrast, the unseparated cells showed a flat, differentiated phenotype and only few single colonies were left. To evaluate the quality of the enriched cells, we utilized an assay detecting AP activity. Non-differentiated stem cells are characterized by high AP activity, indicated by strong red staining in the assay. Figure 1B demonstrates that enriched ESCs derived from BALB/c mice showed distinct dark-red staining two days after separation, reflecting a high percentage of non-differentiated cells. Unseparated cells displayed a flattened morphology with only weak AP staining, indicating that the number of non-differentiated cells was low.

We also checked the cells' Oct-3/4 expression. Oct-3/4 is a transcription factor, which is expressed in non-differentiated ESCs, and whose expression ceases when differentiation progresses. Figure 1C shows bright fluorescence in 70–80% of the enriched B6-RMCE ESCs, whereas only 20–30% of the unseparated cells showed Oct-3/4 staining. Taken together, these results demonstrate that the enriched ESC populations showed a high level of homogeneity and that a large proportion of the population retained a non-differentiated state.

Capacity of enriched ESCs to generate chimeric mice via injection into diploid blastocysts

We also evaluated pluripotency of the enriched BALB/c or B6-RMCE ESCs, i.e., their capacity to give rise to chimeric mice. To this end, we injected 20 ESCs into diploid blastocysts for transfer into the uteri of pseudopregnant mice. For comparison, we used unseparated cells. Table 1 depicts that the use of enriched or unseparated BALB/c cells generated similar numbers of pups per blastocyst. However, the number of chimeric pups and the percentage of male pups showing >50% chimerism were higher with enriched ESCs. Enrichment of B6-RMCE ESCs led to an increase in the number of pups per blastocyst and in the percentage of male pups showing >50% chimerism (table 1).

ESC type	Number of injected blastocysts	Number of transfers	Number of pregnancies	Number of pups	Pups/ blastocyst	Number of chimeric pups	Number of male pups (>50% chimeric)	% male pups (>50% chimeric)
BALB/c, unseparated	18	1	1	3	0.16	3	2	66.7
BALB/c, enriched	33	2	2	5	0.15	5	5	100
B6-RMCE, unseparated	32	2	1	5	0.16	3	1	20
B6-RMCE, enriched	32	2	2	12	0.38	3	3	25

Table 1 Capacity of enriched ESCs to generate chimeric mice via diploid blastocysts. Twenty BALB/c or B6-RMCE ESCs were injected into diploid blastocysts. Sixteen to eighteen blastocysts were transferred into a pseudopregnant mouse. Chimeric pups were identified by coat color.

ESC type	Number of injected blastocysts	Number of transfers	Number of pregnancies	Number of ESC-derived mice	% ESC-derived mice per blastocyst
BALB/c, unseparated	120	6	1	1	0.8%
BALB/c, enriched	120	6	3	8	6.7%
B6-RMCE, unseparated	120	6	3	0	0%
B6-RMCE, enriched	120	6	2	2*	1.7%

Table 2 Capacity of enriched ESCs to generate viable pups via tetraploid complementation. Twenty BALB/c or B6-RMCE ESCs were injected into tetraploid blastocysts. Twenty blastocysts were transferred into a pseudopregnant mouse. Pups were delivered by C-section. *C-section was performed in one of two mice.

Capacity of enriched ESCs to generate viable pups via tetraploid complementation

Blastocysts generated by tetraploid complementation give rise to pups that are entirely derived from the ESCs used for complementation. We tested enriched BALB/c or B6-RMCE ESCs for their capacity to form viable offspring and compared them to the corresponding unseparated ESCs. Table 2 shows that enriched BALB/c or B6-RMCE ESCs yielded higher numbers of viable pups than unseparated cells.

Conclusion

- Both BALB/c and B6-RMCE ESCs could be enriched efficiently by using the special protocol combining feeder cell removal and enrichment of pluripotent stem cells.
- Enriched pluripotent ESCs retained their undifferentiated phenotype as indicated by high AP activity and Oct-3/4 expression levels two days after separation.
- Enriched ESCs used for injection into diploid blastocysts (20 cells/blastocyst) led to a higher percentage of male pups with >50% chimerism than unseparated cells.
- Enriched ESCs gave rise to viable pups from both BALB/c and B6-RMCE ESCs by tetraploid complementation. This is particularly remarkable since ESCs derived from these mouse strains are generally unable to produce viable offspring via tetraploid complementation[4].

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