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NOTE

Magnetic separation of cells from developing embryoid bodies using magnetite cationic liposomes

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Embryoid bodies resemble post-implantation egg-cylinder stage embryos and are used to differentiate embryonic stem cells *in vitro*. In this study, we enriched mouse vasa homolog-positive germ cells from embryoid bodies after 8 d of differentiation using a magnetic separation method with magnetite cationic liposomes.

[**Key words:** Embryonic stem cells; Embryoid body; Germ cells; Magnetic separation; Magnetic force]

Mouse embryonic stem (ES) cells are pluripotent cells of an embryonic origin. Once ES cells are removed from anti-differentiation factors, such as leukemia inhibitory factor (LIF) or feeder cells such as mouse embryonic fibroblasts (MEFs), they spontaneously differentiate into endoderm, mesoderm and ectoderm germ layers (1). The most robust method for the differentiation of cell types is the formation of embryoid bodies (EBs) (2), in which ES cells spontaneously form three-dimensional multicellular aggregates of differentiated and undifferentiated cells similar to those in embryos at the egg-cylinder stage. Target cells can be isolated from EBs for characterization of specific cell types. Magnetic cell sorting is a simple method for preparative cell isolation, and widely used in research and clinical applications (3). Magnetic particles most frequently used for magnetic cell sorting are ferrites including magnetite Fe_3O_4. Magnetite cationic liposomes (MCLs) contain 10 nm magnetite nanoparticles and use the electrostatic interactions between MCLs and the cell membrane for magnetic labeling of cells (4,5). Various cell types can be labeled with MCLs because the cell surface is negatively charged. Based on MCL-labeled cell manipulation using a magnet, we reported the magnetic separation of target cells in co-culture systems using MCLs (6). In this method, mouse ES cells were co-cultured with STO feeder cells labeled with MCLs, which were magnetically removed from the target ES cells. Cell proliferation was inhibited using mitomycin C treatment and the magnetite within MCL-labeled cells was maintained throughout the 7 d culture period. However, the amount of magnetite within proliferating MCL-labeled cells diluted due to cell proliferation. Developing EBs contain a variety of differentiated and undifferentiated cell types with various growth rates. Therefore, we theorized that using MCL-labeled ES cells for EB formation would lead to varied magnetite levels within the cells during EB development and growth. Based on this hypothesis, we report whether a specific cell type can be isolated from developing EBs using magnetic separation of cells labeled with MCLs.

Mouse ES cell line 129sv (Chemicon, Pittsburgh, PA, USA) was cultured on mitotically inactivated MEF feeder cells treated with mitomycin C for 2 h. Cells were cultured on 0.1% gelatin (Nacalai Tesque, Kyoto, Japan)-coated tissue culture dishes (Greiner Bio-one, Frickenhausen, Germany) in growth medium composed of Knockout-DMEM™ (Invitrogen, Carlsbad, CA, USA) with 4 mM L-glutamine (Wako Pure Chemical Industries, Osaka, Japan), non-essential amino acids (NEAA; Invitrogen), 100 μM 2-mercaptoethanol (Millipore, Billerica, MA, USA), 100 U/ml penicillin G potassium (Wako), 50 μg/ml streptomycin sulfate (Wako), 15% Knock-out-serum-replacement (Invitrogen) and 1000 U/ml LIF (ESGRO; Millipore). Medium was replaced every day and ES cells were passaged every 2–3 d. MEFs were isolated from the fetuses of 14 d pregnant BALB/c mice and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS; Biowest, Miami, FL, USA) and 4 mM L-glutamine. Cells were cultured at 37°C in a 5% CO_2 incubator. MCLs were prepared with colloidal magnetite nanoparticles (average particle size 10 nm; Toda Kogyo, Hiroshima, Japan) and a lipid mixture of *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride, dilauroylphosphatidylcholine and dioleoylphosphatidyl-ethanolamine at a 1:2:2 molar ratio as described previously (5). Magnetite within cells and viable cell numbers were measured with the potassium thiocynate method and trypan blue exclusion, respectively. For magnetic labeling, MCLs were incubated for 2 h with an ES cell suspension (1×10^6 cells in 1 ml ES cell culture medium) at a net magnetite concentration of 100 pg/cell. For alkaline phosphatase (AP) staining, magnetically-labeled ES cells were cultured on feeder cells for 7 d, then 1×10^4 cells were replated and after a further 3 d culture, AP staining was performed as described previously (7). Briefly, cells were washed twice with phosphate buffered saline (PBS), fixed in 0.25% glutaraldehyde for 5 min at room temperature, then exposed to a solution containing naphthol AS-MX phosphate (Sigma) as a substrate and Fast Violet B Salt (Sigma) as a coupler for 20 min at 37°C

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TABLE 1. Primer sequences for RT-PCR analysis.

Target gene (product size)	Primer sequence
AFP (173 bp)	FW: 5′-TCG TAT TCC AAC AGG AGG-3′
	RV: 5′-AGG CTT TTG CTT CAC CAG-3′
Blimp-1 (310 bp)	FW: 5′-TGC TTA TCC CAG CAC CCC AC-3′
	RV: 5′-CTT CAG GTT GGA GAG CTG ACC G-3′
Bmp2 (249 bp)	FW: 5′-GGG ACC CGC TGT CTT CTA GTG TTG C-3′
	RV: 5′-TGA GTG CCT GCG GTA CAG ATC TAG CA-3′
Fragilis (460 bp)	FW: 5′-TTT GCT CCG CAC CAT GAA CCA-3'
	RV: 5′-GTG AAG CAC TTC AGG ACC GGA A-3′
GAPDH (150 bp)	FW: 5′-CTA CCC CCA ATG TGT CCG TC-3′
	RV: 5′-GCT GTT GAA GTC GCA GGA GAC-3′
GATA4 (207 bp)	FW: 5′-CTG GAG GCG AGA TGG GAC GGG ACA CTA C-3′
	RV: 5′-CCG CAG GCA TTA CAT ACA GGC TCA CC-3′
Mvh (192 bp)	FW: 5′-CGT TGA ATA CAG CGG GGA TTT-3′
	RV: 5′-GGA GGA AGA ACA GAA GAA CAG GAG ATA-3′
Nestin (425 bp)	FW: 5′-CGG GAG AGT CGC TTA GAG GTG C-3′
	RV: 5′-CAA GGG GGA AGA GAA GGA TGT TG-3′
Stella (454 bp)	FW: 5′-CAG CCG TAC CTG TGG AGA ACA AGA G-3′
	RV: 5′-AGC CCT GGG CCT CAC AGC TT-3′

in a humidified atmosphere containing 5% CO_2. AP-positive cells were counted using microscope images from five fields of view in three separate wells per sample. ES cells with or without magnetic labeling were used to form EBs with the hanging drop method (2). Briefly, ES cell droplets (7000 cells/ml in 15 μl ES medium without LIF) were placed on the lid of a bacterial grade 100 mm plastic dish (AsOne, Osaka, Japan). The lid was inverted and placed on the bottom half that was filled with PBS, and then incubated at 37°C in a 5% CO_2 incubator (designated day 0). On day 3, EBs were transferred into 96 well U-bottom plates (Sumitomo Bakelite, Tokyo, Japan) and cultured in DMEM with 15% FBS, NEAA, 100 U/ml penicillin G potassium and 50 μg/ml streptomycin sulfate. For magnetic capture, EBs were dispersed into single cells using trypsin, then resuspended in 1 ml ES cell medium and transferred to 1.5 ml sterile polypropylene tubes. Magnetic force was applied to the tubes using a cylindrical neodymium magnet with a 4000 G surface magnetic induction. The cell capture percentage was determined with the equation: Magnetic cell capture (%) = (the number of viable cells in

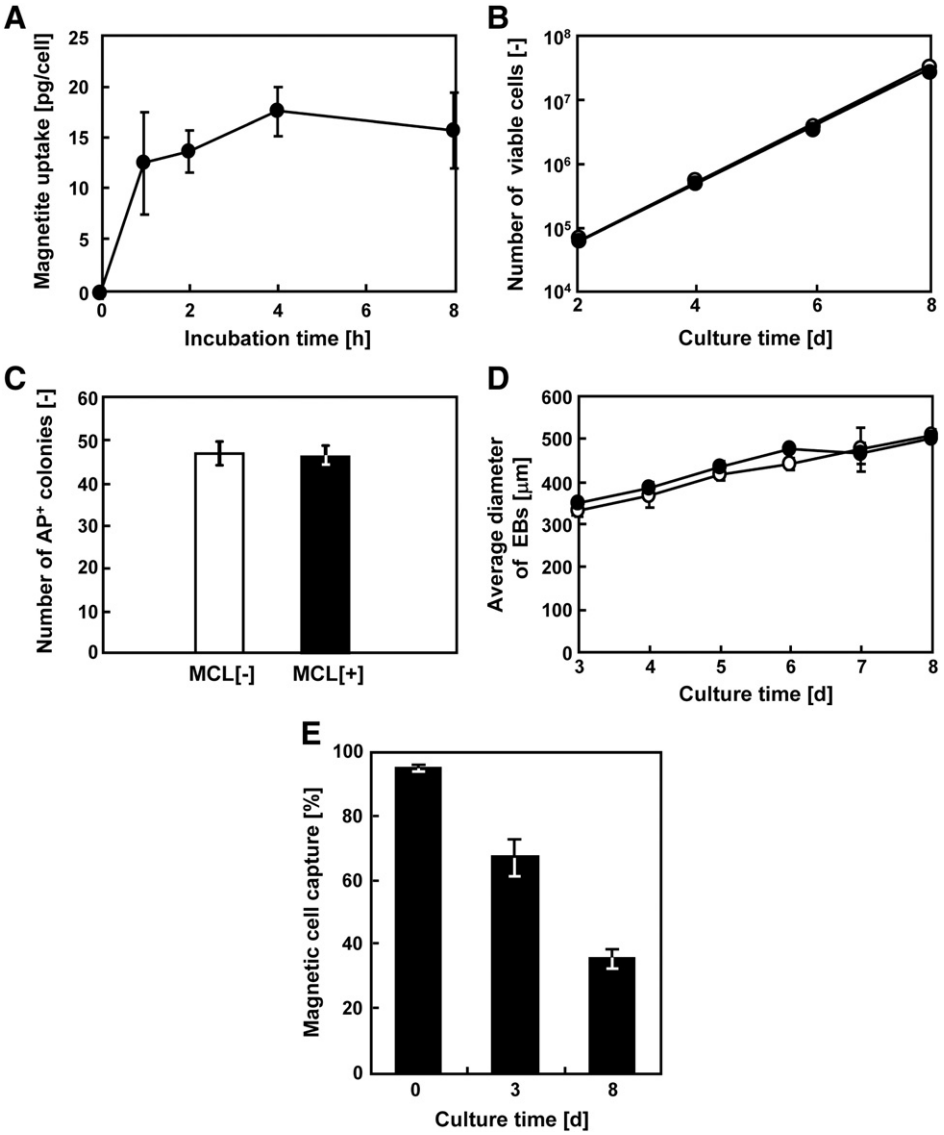

FIG. 1. Effect of magnetic labeling with MCLs on ES cells. (A) The uptake of magnetite particles by ES cells after MCL addition (100 pg/cell). (B) Time course of ES cell growth. Open circle, without magnetic labeling; closed circle, with magnetic labeling. (C) AP-positive ES cell colonies. Open column, without magnetic labeling; closed column, with magnetic labeling. (D) Time course of EB growth. Open circle, without magnetic labeling; closed circle, with magnetic labeling. (E) The percentage of magnetically captured cells from dissociated EBs. All data points represent the mean ± SD of triplicate experiments.