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Magnesium chloride and polyamine can differentiate mouse embryonic stem cells into trophectoderm or endoderm

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ABSTRACT

Magnesium chloride and polyamines stabilize DNA and chromatin. Furthermore, they can induce nucleosome aggregation and chromatin condensation *in vitro*. To determine the effects of elevating the cation concentrations in the nucleus of a living cell, we microinjected various concentrations of mono-, di- and polyvalent cation solutions into the nuclei of mouse embryonic stem (ES) cells and traced their fates. Here, we show that an elevation of either $MgCl_2$, spermidine or spermine concentration in the nucleus exerts a significant effect on mouse ES cells, and can differentiate a certain population of the cells into trophectoderm, a lineage that mouse ES cells do not normally generate, or endoderm. It is hypothesized that the cell differentiation was most probably caused by the condensation of chromatin including the *Oct3/4* locus, which was induced by the elevated concentrations of these cations.

1. Introduction

ES cells, which are derived from the inner cell mass of blastocyst-stage embryos [1,2], have two unique characteristics. One is that they can be stably and indefinitely maintained as pure populations of undifferentiated cells. The other is that they have the capacity to give rise to any of the hundreds of different cell types comprising the body, thus providing exciting prospects for biomedical research and regenerative medicine [3–5]. In the process of ES cell differentiation, global changes occur in both the chromatin structure and gene expression. The major architectural chromatin proteins, such as core histones, linker histones and HP1, bind loosely to DNA and chromatin in ES cells, but they become immobilized upon differentiation [6]. Furthermore, they reportedly behave hyperdynamically during differentiation [6]. Genome-wide changes in histone modifications and DNA methylation also occur in the process of differentiation [7,8], causing changes in the gene expression profile. However, the means by which these global changes are induced remain elusive.

The extent of chromatin fiber folding and the conformations of nucleic acids can play crucial roles in gene expression [9–15]. They are strongly influenced by the concentration of metal cations in the solution and the temperature [16–31]. Metal cations are considered to participate in the global changes occurring in both chromatin structure and gene expression during ES cell differentiation, because it is well established that metal cations are absolutely required for chromatin condensation [16,18,21,26–29]. To our knowledge, however, all of the previous studies regarding these phenomena employed *in vitro* experiments.

In the current study, we examined the *in vivo* effects of actively introduced mono-, di- and polyvalent cations on the fate of mouse ES cells. The cation solutions were directly microinjected into the ES cell nuclei. We found that appropriate concentrations of Mg^{2+}, spermidine and spermine cations could induce the differentiation of mouse ES cells into either trophectoderm, a lineage that mouse ES cells do not normally generate [32], endoderm or cells with lineages that we could not identify, under the conventional culture conditions to maintain the pluripotency of ES cells.

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2. Materials and methods

2.1. Cell culture

Mouse ES cells (E14Tg2a) were cultured as described previously [33].

2.2. Measurement of the volume of a cell nucleus

Cells were stained with Hoechst 33342 (100 ng/μl). The z section images of a cell nucleus were captured at intervals of 0.2 μm, using an FV1000-ZDC confocal microscope (IX81, OLYMPUS). The 3D image of the nucleus was constructed using the FluoView1000-Analysis Software (OLYMPUS), and its volume was determined using the MetaMorph software (Molecular Devices).

2.3. Determination of the volume of solution for microinjection

To determine the appropriate volume of the solution for the microinjection, [γ-^{32}P]ATP [6,000Ci/mmol, Institute of Isotopes Co., Ltd. (HAS)] was used. Cells were spread onto dishes coated with BD Matrigel matrix (BD Biosciences) and cultured for 7 h. Each nucleus of 50 cells was microinjected manually with a volume of the [γ-^{32}P]ATP solution that allowed high cell viability (this volume was determined in preliminary experiments). The cells were then immediately washed with PBS (−) and collected onto filter paper, and the radioactivity was measured with a liquid scintillation counter (PerkinElmer). The above procedure was repeated in triplicate. Using the values and the calibration curve showing the relationship between the volume of the [γ-^{32}P]ATP solution and the radioactivity, the appropriate volume was determined to be 15 fl.

2.4. Microinjection into mouse ES cell nucleus

The aqueous solution used in the microinjection contained FITC-dextran (SIGMA; used for monitoring the injection) and either EDTA, sodium citrate, NaCl, KCl, $MgCl_2$, $CaCl_2$, spermine or spermidine, at the following concentrations: FITC-dextran, 1 mg/ml; EDTA, 10, 50, or 100 mM; sodium citrate, 10, 50, or 100 mM; NaCl, 10, 25, 50, 100, or 200 mM; KCl, 10, 25, 50, 100, or 200 mM; $MgCl_2$, 25, 50, 75, 100, 150, 200, or 300 mM; $CaCl_2$, 1, 5, 50, 100, or 150 mM; spermidine, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, or 2.0 mM; and spermine, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, or 3.0 mM. The control solution contained only the same concentration of FITC-dextran in sterile water. Using a Microinjector (IM-6, Narishige) and glass micropipettes prepared from glass capillaries, a 15 fl portion of each solution was manually injected into the nuclei of cells cultured on Matrigel-coated plates. At one hour after the microinjection, cells with FITC signals in their nuclei were further cultured for 5 days and then subjected to immunostaining. Mouse ES cells that were not microinjected were also cultured in parallel.

2.5. Immunostaining

To judge whether differentiation occurred, immunostaining was performed using mouse anti-Oct3/4 (1:1,000, sc-5279, Santa Cruz) and rabbit anti-Nanog (1:500, RCAB001P, ReproCELL) antibodies as the primary antibodies, and goat anti-mouse IgG-Alexa Fluor 546 (1:1,500, A11003, Life Technologies) and goat anti-rabbit IgG-Alexa Fluor 633 (1:1,500, A21070, Life Technologies) as the secondary antibodies, according to the procedure reported previously [31]. PBS/glycerol (1:1) was used to seal the cells.

Immunostaining to determine the cell types of the differentiated cells was performed, as follows. First, rat anti-Oct4/Pou5F1 (1:200, CE-052A, Cosmo Bio), goat anti-Brachyury (1:200, sc-

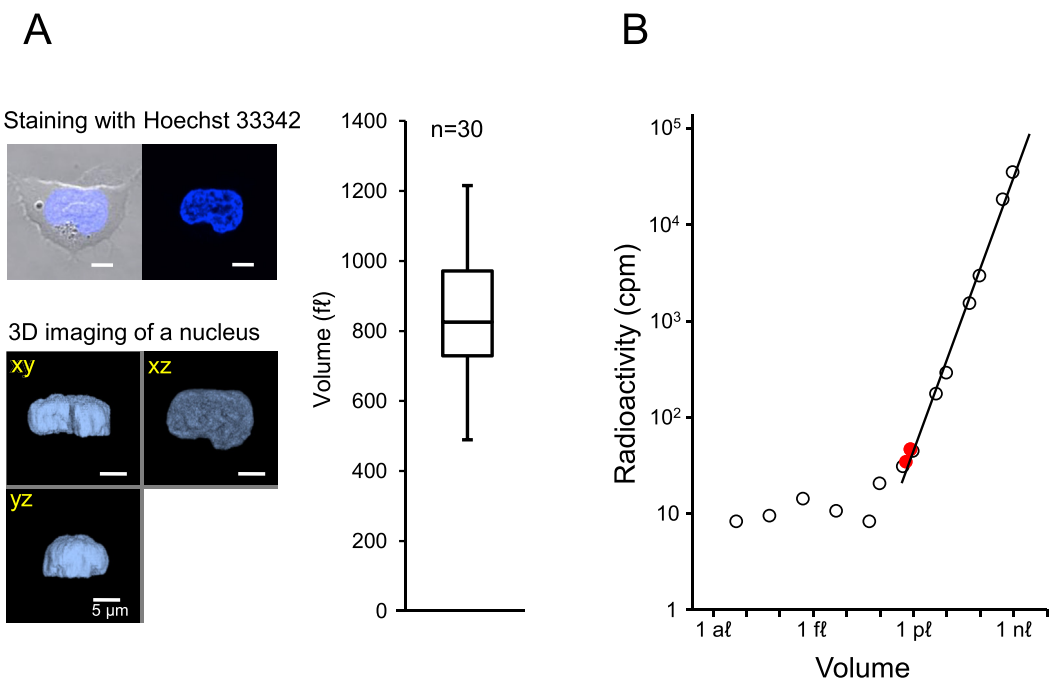

Fig. 1. Volume measurements. **(A) Volume of the ES cell nucleus**. Using 30 cells, the volume of the mouse ES cell nucleus was measured as described in the Materials and Methods. Upper left: an example of a mouse ES cell stained with Hoechst 33342. Lower left: an example of the 3D image of the nucleus. Right: the box plot for the nucleus volumes of 30 cells. **(B) Determination of the solution volume for the microinjection**. According to the procedure described in the Materials and Methods, 50 cells, each with a [γ-^{32}P]ATP-injected nucleus, were collected and the radioactivity was counted. The experiment was performed in triplicate. Each data point is shown as a red filled circle. White open circles indicate the radioactive solutions used to obtain the calibration curve. Using this curve, each datum was estimated to be 619 fl, 849 fl and 849 fl, and based on these values the average value of 15 fl/single nucleus was calculated.

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