Comparison of two types of non-adherent plate for neuronal differentiation of mouse embryonic stem cells

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Abstract In vitro differentiation systems of mouse embryonic stem cells (ESCs) are widely used as tools for studies of cell differentiation, organogenesis, and regenerative medicine. We have studied the regulation of neuron-specific imprinting genes, *Ube3a* and its antisense transcripts (*Ube3a ATS*), using in vitro neuronal differentiation of F1 hybrid ESCs. Each different non-adherent plate used for embryoid body (EB) formation during differentiation is associated with different costs; notably, plates coated with 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer are more expensive than untreated polystyrene plates. Here, we assessed whether the polymer-coated plates gave better results than the untreated plates. The first stage of differentiation was performed in the MPC polymer-coated or untreated plates. The formed EBs were then passaged onto laminin-coated plates for further differentiation into neurons. Neither the neuron-specific imprinting status of *Ube3a* nor the expression levels of the neuron-specific markers *Ube3a ATS* and *Mtap2* differed between neurons prepared on untreated plates and those prepared on MPC polymer-coated plates. These results suggest

that the two non-adherent plates displayed almost the same characteristics for inducing neuronal differentiation of mouse ESCs and EB formation. Our study proved that untreated polystyrene plates are a cost-effective choice for EB formation in in vitro differentiation systems of mouse ESCs.

Keywords Embryonic stem cell · Neuronal differentiation · Imprinting · *Ube3a* · *Ube3a* antisense · *M. musculus molossinus*

Introduction

Mouse embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass (ICM) of a blastocyst (Nichols and Smith 2012). Like the ICM, which develops into an embryo, ESCs have the differentiation potential into three germ layers in vitro. Although ESCs retain pluripotency if maintained under appropriate culture conditions, they form embryoid bodies (EBs) when seeded on a culture whose conditions favor differentiation (Keller 1995).

In vitro neuronal differentiation of ESCs is useful for studying phenomena that occur in the early stages of neural development (e.g., neuron-specific imprinting). We have studied the allele-biased expression of *Ube3a* and its antisense transcripts (*Ube3a ATS*), both of which are neuron-specific imprinting genes, to understand complex features of mammalian gene

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regulation (Numata et al. 2011). The *Ube3a* gene, located in the imprinted domain *Snrpn-Ube3a* (Albrecht et al. 1997; Gabriel et al. 1998), is expressed maternally in the brain (especially in neurons) and biallelically in most other tissues (Albrecht et al. 1997; Rougeulle et al. 1997; Vu and Hoffman 1997; Yamasaki et al. 2003). *Ube3a* is accompanied by *Ube3a* *ATS*, which contributes to parts of large paternal non-coding RNAs that include exons upstream of *Snrpn* (U exons), *Snrpn*, snoRNAs (*Snord115* and *Snord116*), and *Ipw* (Chamberlain and Brannan 2001; Landers et al. 2004; Le Meur et al. 2005; Rougeulle et al. 1998). *Ube3a* *ATS* is expressed paternally in the brain (especially in neurons) and silences paternal *Ube3a* expression (Meng et al. 2012, 2013, 2015; Yamasaki et al. 2003). In a previous study, we developed an ESC differentiation system that recapitulates the neuron-specific maternal expression of *Ube3a* (*Ube3a* imprinting) (Kohama et al. 2012).

In this study, we assessed the cost-effectiveness of two types of non-adherent plate by using each with our ESC differentiation system: untreated polystyrene plates whose hydrophobic surfaces reduce adhesion of cells (Curtis et al. 1983); and 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer-coated plates that prevent cells from adhering to the plastic surfaces of the culture plates (Ishihara et al. 1990, 1999). The MPC polymer coating adds to the cost of the plates. Although both types of plate are useful for the formation of EBs from mouse ESCs (Koike et al. 2005; Konno et al. 2005), it is unclear whether the formed EBs mature into neurons that recapitulate *Ube3a* imprinting. Assessing the *Ube3a* imprinting of neurons prepared on these two plates may reveal beneficial information applicable to other in vitro ESC differentiation systems.

Materials and methods

Cell line and mouse strain

We used MB4, the same ESC strain as in our previous study (Kohama et al. 2012). MB4 was derived from a male embryo of an F1 hybrid between an *M. musculus molossinus* (MSM/Ms) mother and an *M. musculus domesticus* (C57BL/6) father. We used C57BL/6 to extract mouse tissues.

ESC culture

MB4 was cultured with Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 4500 mg/l D-glucose, 110 mg/l sodium pyruvate, 584 mg/l L-glutamine, 10 % fetal bovine serum (FBS: Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA), 1 × penicillin–streptomycin solution (Wako Pure Chemical Industries, Ltd.), 1 × minimal essential medium non-essential amino acid solution (MEM NEAA: Wako Pure Chemical Industries, Ltd.), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich Co. LLC., St. Louis, MO, USA), 1000 units/ml StemSure mouse leukemia inhibitory factor (Wako Pure Chemical Industries, Ltd.), and 1 μM PD0325901 (Focus Biomolecules, Plymouth Meeting, PA, USA, 10-2161) on mouse embryonic fibroblast (MEF) plates. MEF was derived from the 15-day postcoitus embryos of Slc:ICR (Japan SLC, Inc., Hamamatsu, Japan) and treated with mitomycin C (Sigma-Aldrich Co. LLC.) for 2 h to stop proliferation. MB4 was passaged every 2 days, using 0.25 % (w/v) trypsin-1 mM EDTA-4Na solution (Wako Pure Chemical Industries, Ltd.), which was diluted five-folds with phosphate-buffered saline [2.68 mM KCl, 1.47 mM KH₂PO₄, 8.09 mM Na₂HPO₄, and 136.89 mM NaCl].

Neuronal differentiation

Neuronal differentiation experiments were performed as described previously (Bouhon et al. 2006; Kohama et al. 2012) with a minor modification. Briefly, after trypsinization of MB4 cultured on MEF plates, cell mixtures were transferred to new cell culture dishes twice and incubated for 10 min after the first transfer and 15 min after the second transfer in a CO₂ incubator to deplete MEF. The cells were washed once with a chemically defined medium (Bouhon et al. 2005; Johansson and Wiles 1995; Kohama et al. 2012) (Kohjin Bio, Sakado, Japan) supplemented with 1 × penicillin–streptomycin solution, 1 × MEM NEAA solution, 64 μg/ml L-ascorbic acid 2-phosphate trisodium salt (Wako Pure Chemical Industries, Ltd.), and 14 ng/ml sodium selenite (Sigma-Aldrich Co. LLC.) (fCDM). The cells were seeded on non-adherent plates and cultured for 8 days at a concentration of 2000 cells/200 μl fCDM in each well for EB formation (neuronal differentiation step, D0–D8). Three quarters of the medium in each well was

changed on D4 during the neuronal differentiation step. We used two types of non-adherent plate—untreated polystyrene plates (PS: Watson Co., Ltd., Tokyo, Japan) and MPC polymer-coated polystyrene plates (MPC: NOF Co., Tokyo, Japan)—both of which were 96-well V-bottomed plates. The formed EBs were treated with Accumax (Innovative Cell Technologies, Inc., San Diego, CA, USA) for 6 min at room temperature, followed by trituration with a pipette. The tritured EBs were cultured for 9 days in fCDM supplemented with 2 % B-27 supplement (Gibco) and 1 % FBS on laminin-coated plates (neural maturation step, N0–N9). Half of the medium was changed every day after N2 during the neural maturation step.

RNA extraction and cDNA synthesis

RNAs were extracted from cultured cells and mouse tissues by using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) in accordance with the manufacturer's protocol. Briefly, after cell lysis or tissue homogenization with TRI Reagent, the samples were mixed with chloroform (5:1 v/v), shaken vigorously, and centrifuged at 12,000g for 15 min at 4 °C. The upper phases were transferred to new tubes and mixed with equal volumes of 100 % isopropanol. After incubation at room temperature for 10 min, the samples were centrifuged at 7500g for 10 min at 4 °C, washed with 70 % ethanol twice, and dissolved with appropriate amounts of UltraPure DNase/RNase-free distilled water (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA). The quality and quantity of the extracted RNAs were checked with a NanoDrop ND-1000 spectrophotometer (AGC Techno Glass Co., Ltd., Haibara-gun, Japan).

cDNAs were synthesized with SuperScript III reverse transcriptase (Invitrogen), using a T100 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in accordance with the manufacturer's protocol. Briefly, RNAs were mixed with 1 µl of a 10 mM deoxynucleotide triphosphate (dNTP) mixture and 1 µl of a 50 µM random hexamer primer or 0.2 µl of each gene specific primer (10 µM) (Table 1) in a total volume of 13 µl. After denaturation at 65 °C for 5 min, the samples were mixed with the reaction mixture, which consisted of 4 µl of 5 × First-Strand buffer, 1 µl of 0.1 M dithiothreitol, 0.25 µl of 200 units/µl SuperScript III reverse transcriptase, 1 µl of 40 units/µl RNaseOUT recombinant

ribonuclease inhibitor (Invitrogen), and 0.75 µl of sterile distilled water. The reaction program was 50 °C for 60 min and 70 °C for 15 min. Synthesized cDNAs were diluted five-folds for the experiment.

Allele-biased expression analysis

The allele-biased expression of *Ube3a* was measured by direct sequencing of the RT-PCR products. To amplify *Ube3a* pre-mRNA sequences that contain single nucleotide polymorphisms (SNPs), PCR was performed with a 20 µl volume scale, using a T100 thermal cycler. The reaction mixture consisted of 2 µl of cDNA, 2 µl of 10 × Ex Taq buffer, 1.6 µl of a 2.5 mM dNTP mixture, 1 µl of each 10 µM primer (Table 1), 0.1 µl of 5 units/µl TaKaRa Ex Taq (Takara Bio Inc., Kusatsu, Japan), and 12.3 µl of sterile distilled water. The reaction program comprised 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The PCR products were separated in a 1.0 % agarose gel, dissected from the gel, and purified with a FastGene Gel/PCR extraction kit (Nippon Genetics Co, Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. Purified PCR products were mixed with sequence primer (Table 1) and shipped to Eurofins Genomics K.K. (Tokyo, Japan) for sequence analysis. Peak signals were evaluated by using 4Peaks version 1.7.1.

Quantitative PCR

Quantitative PCR was performed in accordance with the manufacturer's protocol. Briefly, 1 µl of cDNA was mixed with 9 µl of premix, which consisted of 5 µl of SYBR Premix Ex Taq II (Takara Bio Inc.), 0.4 µl of each 10 µM primer (Table 1), and 3.2 µl of sterile distilled water. Relative expression was measured with a Thermal Cycler Dice real-time system (Takara Bio Inc.). The reaction program was 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. The assays were performed in duplicate. The mean values and standard deviations of relative expression, which were normalized to *Gapdh*, were calculated using two independent neuronal differentiation samples.

Results

Neuronal differentiation of ESCs

To evaluate the utility of untreated plates for neuronal differentiation of mouse ESCs, untreated and MPC

Table 1 Primers for strand-specific cDNA synthesis, allele-biased expression analysis, and quantitative PCR

Gene	Forward	Reverse
<i>Gapdh</i> ^{a,d}	tgtccgctcgtggaatctgac	cctgcttcaccaccttcttg
<i>Ube3a</i> <i>ATS</i> ^{a,d}	tagcagccagcttgccctttac	gactgtggccaagacttggtga
<i>Mtap</i> ^{2d}	ccaagacctctctcatcctc	gctctgcgaattggtctgac
<i>Ube3a</i> <i>G02</i> ^{b,c}	atcctgcagacttgaagaagc	atcatacatcattgggttacc
<i>NeuN</i> ^d	gtagagtctatgccgtgctga	cttcattgggtccgagaaggagac
<i>Ngn2</i> ^d	tctgcaggctgtgggaatttca	aaagaaggcgccggacaataggc
<i>Dcx</i> ^d	tggtcattgcttggctctgaaa	cttttgaggtgttggggaagcc
<i>Tubb3</i> ^d	tagaccgccagcgcaactat	gttcagggttccaagtccacc

^a For strand-specific cDNA synthesis (only reverse primers)

^b For PCR of allele-biased expression analysis

^c For sequence analysis of allele-biased expression (only forward primer)

^d For quantitative PCR

Fig. 1 Neuronal differentiation of ESCs. **a** Neuronal differentiation protocol. Undifferentiated ESCs were dispensed on non-adherent plates and cultured for 8 days to form EBs (D0–D8). The formed EBs were passaged onto laminin-coated plates with Accumax treatment and cultured for 9 days to form neurons (N0–N9, which corresponds to D8–D17). Two types of non-adherent plates were used in Step 2 in this study: untreated polystyrene plates and MPC polymer-coated plates. **b** Morphological features of N9 neurons. *Upper and lower images* were

observed under bright field with a $\times 4$ lens and under phase contrast with a $\times 10$ lens, respectively. The *rectangles* in the *upper images* are the visual fields of the lower images. *Black bars* in the *upper and lower images* indicate 250 and 100 μm , respectively. In this and all subsequent figures, N9 neurons prepared with untreated polystyrene plates and those prepared with MPC polymer-coated plates are denoted as N9 (PS) and N9 (MPC), respectively

polymer-coated plates were used in our neuronal differentiation protocol (Fig. 1a). Our protocol involves three steps: proliferation of ESCs ($\sim\text{D0}$), neuronal differentiation (D0–D8), and neural maturation (N0–N9, which corresponds to D8–D17) (Kohama et al. 2012). The ESCs cultured with serum and mitogen-free media on non-adherent plates formed EBs and differentiated into neural precursor cells (NPCs) during the neuronal differentiation step (Bouhon et al. 2005). The NPCs passaged onto laminin-coated plates further differentiated into

neurons with elongated neurites during the neural maturation step. The N9 neurons prepared on untreated plates and MPC polymer-coated plates during the neuronal differentiation step did not differ morphologically (Fig. 1b).

Ube3a imprinting

We checked *Ube3a* imprinting in N9 neurons by distinguishing two alleles with single nucleotide polymorphisms (SNPs) among mouse subspecies.

Our ESC strain MB4, derived from a male embryo of an F1 hybrid with a maternal genome inherited from MSM/MS (MSM) and a paternal genome inherited from C57BL/6 (B6) (Kohama et al. 2012), had the SNP termed G02 in an intronic region of *Ube3a*; the nucleotides at this SNP are cytosine and thymine in the MSM and B6 chromosomes, respectively (Fig. 2a). Sequence analysis of the RT-PCR products of D0 ESC samples showed the same levels of peaks for maternal cytosine and paternal thymine in G02, indicating biallelic expression of *Ube3a* before neuronal differentiation (Fig. 2a, b). In contrast, sequence analysis of the N9 neurons predominantly showed the maternal cytosine peak, indicating that these neurons acquired *Ube3a* imprinting through neuronal differentiation. Importantly, the N9 neurons prepared on untreated plates and MPC polymer-coated plates during the neuronal differentiation step did not differ in the level of *Ube3a* imprinting.

Expression of neuron-specific markers

Because the paternal silencing of *Ube3a* may be caused by the paternal expression of *Ube3a* ATS in neurons, we analyzed whether expression levels of

Ube3a ATS in N9 neurons correlated with *Ube3a* imprinting by using a specific primer complementary to *Ube3a* ATS to synthesize cDNA (Table 1). The relative expression level of *Ube3a* ATS in N9 samples increased to more than 300 fold of that in D0 (Fig. 3a). The levels of *Ube3a* ATS expression in the N9 samples were similar to those in the brain of an adult mouse. *Ube3a* ATS expression was very similar in the N9 samples prepared on untreated and MPC polymer-coated plates. The neuron-specific marker *Mtap2*, a cytoskeletal component of mature neurons, also showed an expression pattern similar to that of *Ube3a* ATS (Fig. 3b). The relative expression levels of two neuron-specific nuclear protein-coding genes *NeuN* (Kim et al. 2009; Mullen et al. 1992) and *Ngn2* (Lee 1997) in two N9 samples also increased more than 300 fold of those in D0 (Fig. 3c, d). Two neuron-specific microtubule-related genes *Dcx* (Francis et al. 1999; Walker et al. 2007) and *Tubb3* (Lee et al. 1990) increased more than 10^5 fold and 10 fold, respectively (Fig. 3e, f). The expression levels of these four neuron-specific markers in the brain sample were higher than those of the liver sample, although they were different from those of the N9 samples. These results suggest that neuronal differentiation is almost

Fig. 2 *Ube3a* imprinting. **a** Schematic representation of the mouse *Snrpn-Ube3a* domain. Paternal *Ube3a* expression in neurons is down-regulated by the neuron-specific expression of paternal *Ube3a* ATS. Paternally expressed non-coding RNAs, including alternative U exon transcripts, *Snrpn*, snoRNAs (*Snord115* and *Snord116*), *lpw*, and *Ube3a* ATS, contribute to parts of a large transcription unit. This paternal expression is regulated by the differentially methylated region (DMR) in *Snrpn* exon 1. Maternally and paternally inherited chromosomes are indicated as *Mat* and *Pat*, respectively. Genes, U exons, and snoRNAs are symbolized as rectangles, vertical bars, and triangles, respectively. Alleles that exhibit maternal and

paternal expression patterns are depicted in red and blue, respectively, whereas silenced alleles are depicted in black. The direction of transcription is shown by an arrow. Methylated and unmethylated DMRs are indicated as lollipops with black and white circles, respectively. Figures are not drawn to scale. The nucleotides at the SNP G02 of *Ube3a* pre-mRNA in maternal MSM and paternal B6 are cytosine and thymine, respectively. **b** Allele-biased expression of *Ube3a* transcripts. Direct sequencing of the RT-PCR products indicates allelic expression of *Ube3a* during neuronal differentiation. The SNP positions are indicated by arrows on peak signals and marked by circles on read sequences. (Color figure online)

Fig. 3 Expression of neuron-specific markers. Two independent neuronal differentiation experiments were performed to prepare the D0 and N9 samples. Liver and brain samples extracted from an adult mouse were used as negative and

positive controls, respectively. The y-axes in **a–f** represent relative expression of *Ube3a ATS*, *Mtap2*, *NeuN*, *Ngn2*, *Dcx* and *Tubb3*, respectively. *Gapdh* was used as a reference gene. Bars indicate the mean ± SD

identical for untreated and MPC polymer-coated plates.

Discussion

To assess the characteristics of morphologically indistinctive N9 neurons prepared on two different non-adherent 96-well plates, we compared the two samples in terms of their neuron-specific imprinting status and gene expression markers. *Ube3a* imprinting is an excellent index of the efficiency of neuronal differentiation because it could reflect the proportion of differentiated neurons in the cell population. Because *Ube3a* is expressed biallelically in most tissues except neurons (Albrecht et al. 1997; Rougeulle et al. 1997; Vu and Hoffman 1997; Yamasaki et al. 2003), the paternal signals observed in the N9 neurons produced by our in vitro neuronal differentiation system represent the proportion of non-neuronal

cells. Tissue specific markers such as *Ube3a ATS* and *Mtap2* are useful for assessing the presence of marker-positive cells; however, they have limitations in assessing the presence of marker-negative cells. Our in vitro neuronal differentiation system, which uses F1 hybrid ESCs, recapitulates the imprinted expression of *Ube3a*. Therefore, we utilized it to assess the plates used in neuronal differentiation.

In this study, *Ube3a* imprinting during neuronal differentiation did not differ between N9 neurons prepared on untreated plates and those prepared on MPC polymer-coated plates. The levels of neuron-specific marker genes such as *Ube3a ATS* and *Mtap2* expression were also indistinguishable between the two samples of N9 neurons. Our results indicate that neuronal differentiation of mouse ESCs and EB formation are nearly identical for the two non-adherent plates; this suggests that the hydrophobic surface of untreated polystyrene plates is sufficient for EB formation in our neuronal differentiation protocol.

Our differentiation protocol has characteristics similar to those of the “default model” of early embryonic cell differentiation (Hirose et al. 2012). Therefore, untreated polystyrene plates, which are more cost-effective than MPC polymer-coated plates, might become applicable to other in vitro ESC differentiation systems by using appropriate chemical compounds and biomolecules.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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