Cytotechnology (2018) 70:45–53
https://doi.org/10.1007/s10616-017-0126-z

METHOD IN CELL SCIENCE

Markers associated with neuron-specific *Ube3a* imprinting during neuronal differentiation of mouse embryonic stem cells

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Received: 15 January 2017 / Accepted: 19 July 2017 / Published online: 5 August 2017

Abstract Understanding gene expression in the brain requires allele-specific transcriptome analysis because of the presence of neuron-specific imprinted genes, which are expressed in a neuron-specific and parent-of-origin-specific manner. *Ube3a* is a neuron-specific imprinted gene with an expression pattern that changes from biallelic to maternal only (*Ube3a* imprinting) during differentiation. Because *Ube3a* imprinting occurs only in neurons, it has the potential to be a marker to assess the quality of neurons produced by in vitro neuronal differentiation of embryonic stem cells (ESCs). For the analysis of *Ube3a* imprinting, genetic polymorphisms between the two alleles are necessary to identify the parental origin of each. However, ESCs derived from commonly used inbred mouse strains have no genetic polymorphisms. To overcome this problem, we examined 10 markers of neurogenesis to determine whether they were associated with *Ube3a* imprinting. We measured the relative expression levels of these 10 gene markers and assessed the *Ube3a* imprinting status of 54 neuron samples differentiated under various in vitro conditions. Then we divided the samples into two groups depending on their *Ube3a* imprinting status and selected markers statistically associated with *Ube3a* imprinting. The identified markers included the antisense noncoding transcript of *Ube3a* and a mature neuron marker *Mtap2*, consistent with the markers we used empirically in our previous study to assess the quality of differentiated neurons. These findings provide new quality control criteria for differentiated neurons, and could also be applied to human ESCs and induced pluripotent stem cells.

Keywords *Ube3a* imprinting · Embryonic stem cell · In vitro neuronal differentiation · *Mus musculus molossinus* · *Mtap2* · Noncoding RNA · SNP ratio quantification

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Introduction

Neuronal precursors undergo numerous changes in gene expression as they differentiate into neurons (Kato et al. 2015). One of the temporal changes in gene expression during neural development is the allele-

biased expression of *Ube3a* (*Ube3a* imprinting), which we previously studied using mouse embryonic stem cells (ESCs) in vitro (Eitoku et al. 2016; Kohama et al. 2012). *Ube3a* is transcribed from both maternal and paternal alleles (biallelic expression) in all tissues except neurons, in which it is transcribed only from the maternal allele (maternal expression) (Albrecht et al. 1997; Rougeulle et al. 1997; Vu and Hoffman 1997; Yamasaki et al. 2003). The tendency of maternal *Ube3a* expression is mild in neuronal progenitor cells and is gradually reinforced during neural development (Kohama et al. 2012; Yamasaki et al. 2003). The mechanism underlying this time-dependent change is the silencing of paternal *Ube3a* expression by the neuron-specific antisense noncoding transcript of *Ube3a* (*Ube3a ATS*), which is expressed only from the paternal allele (Chamberlain and Brannan 2001; Kohama et al. 2012; Landers et al. 2004; Le Meur et al. 2005; Numata et al. 2011; Rougeulle et al. 1998; Yamasaki et al. 2003).

Because *Ube3a* imprinting is a product of neuron-specific gene regulation, we have utilized it as a marker to assess the quality of neurons produced by in vitro differentiation. In vitro neuronal differentiation systems have the advantage of permitting longitudinal analysis of the same cell population, enabling the study of temporal changes in gene expression. Our ESCs derived from F1 hybrids between two mouse subspecies enabled us to efficiently distinguish the alleles from each parent with single nucleotide polymorphisms (SNPs) (Kohama et al. 2012). However, this approach is difficult to apply to ESCs derived from inbred mice because of the lack of SNPs between the parents. Therefore, other ways to identify *Ube3a* imprinting status are needed to analyze temporal changes in in vitro neuronal differentiation systems derived from inbred mice.

In this study, we assessed 10 marker genes that are expressed during neurogenesis. Among these were *Ube3a ATS*, *Mtap2,* and *Ngn2*, which we had previously measured in a study of *Ube3a* imprinting (Kohama et al. 2012). From these 10 markers, we statistically selected those that were most strongly associated with *Ube3a* imprinting. These marker genes could be used to assess the quality of differentiated neurons and would be applicable not only to mouse ESCs derived from general inbred strains but also to those derived from other mammalian species, including humans, for longitudinal in vitro analyses for clinical or industrial applications.

Materials and methods

In vitro neuronal differentiation

For in vitro neuronal differentiation, we used a heterozygous F1 hybrid ESC line, MB4 (Kohama et al. 2012), and two homozygous F0 ESC lines, Mol/MSM-1 (Araki et al. 2009) and 6NK-7 (Ishikawa et al. 2016). MB4 was derived from a male embryo of an F1 hybrid between an *Mus musculus molossinus* (MSM/Ms [MSM]) mother and a *M. musculus domesticus* (C57BL/6 [B6]) father. Male MSM and B6 embryos were used to derive Mol/MSM-1 (MSM-ESC) and 6NK-7 (B6-ESC) lines, respectively.

We used 54 MB4 samples derived from neurons differentiated in combinations of the following conditions of ESC culture and in vitro neuronal differentiation. Each condition is described here briefly; detailed information can be obtained upon request. MB4 was cultured with two types of media: One was Dulbecco's modified Eagle's medium (DMEM: Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% fetal bovine serum (FBS: Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA); 1 × penicillin–streptomycin solution (PS: 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 (2-ME: Sigma-Aldrich Co. LLC., St. Louis, MO, USA); 1000 units/mL StemSure mouse leukemia inhibitory factor (LIF: Wako Pure Chemical Industries, Ltd.); and 1 μM PD0325901 (1i: Focus Biomolecules, Plymouth Meeting, PA, USA, 10-2161), which is a MEK inhibitor. The other was DMEM containing 15% knockout serum replacement (KSR: Gibco, Thermo Fisher Scientific Inc.), 1 × PS, 1 × MEM NEAA, 0.1 mM 2-ME, 1000 units/mL LIF, and 1 μM 1i. We used DMEM containing 4500 mg/L D-glucose, 110 mg/L sodium pyruvate, and 584 mg/L L-glutamine in both media. We used feeder and non-feeder plates to culture MB4. For feeder plates, mouse embryonic fibroblasts (MEFs) were derived from the 15-day postcoitus embryos of Slc:ICR mice (Japan SLC, Inc., Hamamatsu, Japan) and treated with mitomycin C (Sigma-Aldrich Co. LLC) for 2 h to stop proliferation. For non-feeder plates, polystyrene plates were coated with 0.1% gelatin. Every 2 days, MB4 was passaged by using 0.25% (w/v) trypsin-1 mM EDTA·4Na solution

(Wako Pure Chemical Industries, Ltd.), which was diluted five-fold with phosphate-buffered saline (PBS: 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.09 mM Na_2HPO_4, and 136.89 mM NaCl).

In vitro neuronal differentiation was performed as described previously (Bouhon et al. 2006; Eitoku et al. 2016; Kohama et al. 2012) with a minor modification. Briefly, after trypsinization of MB4, 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 × PS, 1 × MEM NEAA, 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). In the case of MB4 cultured on feeder plates, the MEFs were removed before this washing step. The cells were cultured for 8 days at a concentration of 1000, 2000, or 4000 cells per 200 μL fCDM in each well of non-adherent 96-well plates (neuronal differentiation step, D0–D8). On D4, 150 μL of the medium in each well was changed. On D8, the formed embryoid bodies were treated with Accumax (Innovative Cell Technologies, Inc., San Diego, CA, USA) for 6 min at room temperature, triturated with a pipette, and cultured for 9 days on laminin-coated plates (neural maturation step, N0–N9). Three types of fCDM-based media were used in the neural maturation step: (1) fCDM supplemented with 2% B-27 supplement (Gibco) and 1% FBS; (2) fCDM supplemented with 5 μM forskolin (Sigma-Aldrich Co. LLC.) and 3 μM CHIR99021 (Focus Biomolecules, 10-1279); and (3) fCDM supplemented with 5 μM forskolin, 3 μM CHIR99021, 5 μM DAPT (Sigma-Aldrich Co. LLC), and 2 μM all-trans-retinoic acid (Sigma-Aldrich Co. LLC). Half of the medium was changed every day after N2 during the neural maturation step.

RNA extraction and cDNA synthesis

RNA extraction was performed by using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) in accordance with the manufacturer's protocol. Briefly, cell lysates dissolved in TRI Reagent were mixed with chloroform (5:1 v/v), shaken vigorously, and centrifuged at 12,000*g* for 15 min at 4 °C. The upper phases were mixed with equal volumes of 100% isopropanol in new tubes and incubated at room temperature for 10 min. Then the samples were centrifuged at 7500*g* for 10 min at 4 °C, washed with 70% ethanol twice, and dissolved in UltraPure DNase/RNase-free distilled water (Invitrogen, Thermo Fisher Scientific Inc.). The quality and quantity of the RNAs were evaluated with a NanoDrop ND-1000 spectrophotometer (AGC Techno Glass Co., Ltd., Haibara-gun, Japan).

cDNA synthesis was performed by using SuperScript III reverse transcriptase (Invitrogen) in accordance with the manufacturer's protocol. Briefly, in the first step of the procedure, RNAs were denatured at 65 °C for 5 min using a T100 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The denaturation was conducted with 13 μL of a nucleic acid mixture containing 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 the second step, 7 μL of reaction mixture was added to the denatured RNA mixtures. The reaction mixture contained 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, and 1 μL of 40 units/μL RNaseOUT recombinant ribonuclease inhibitor (Invitrogen). The reaction program was 60 min at 50 °C and 15 min at 70 °C.

SNP ratio quantification by direct sequencing

Direct sequencing of the cDNA was performed to determine the SNP ratio of *Ube3a*. For amplification of *Ube3a* mRNA sequences that included SNP-863 and SNP-933 (Kohama et al. 2012; Numata et al. 2011), 2 μL of cDNA was used as the template in a 35-cycle PCR reaction with a T100 thermal cycler. The PCR conditions (20 μL final volume) were 1 × Ex Taq buffer, 0.2 mM dNTP mixture, 0.1 μL of 25 μU/μL TaKaRa Ex Taq (Takara Bio Inc., Kusatsu, Japan), and 0.5 μM of each primer (Table 1). The PCR program was 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C.

Purification of the PCR products was performed by using a FastGene Gel/PCR extraction kit (Nippon Genetics Co, Ltd., Tokyo, Japan) following the manufacturer's protocol. Sequence analysis of purified PCR products was performed by Eurofins Genomics K.K. (Tokyo, Japan) using a sequence primer (Table 1). The SNP ratio was evaluated by using 4Peaks version 1.7.1 (Nucleobytes Inc., Amsterdam, Netherlands).

Table 1 Primers and probes for SNP ratio quantification, strand-specific cDNA synthesis, and quantitative PCR

Gene	Forward	Reverse	Remarks
SNP-863_taqman[a]	tgatcctcatccctccaaga	gggaataatcctcactctctctaca	
SNP-863_FAM_probe[a]	aaaatgaaT(a)aga		
SNP-863_ROX_probe[a]	aaatgaaC(a)aga		
SNP-863_dir_seq[b,c]	ctgaggacattgaagctagccgaa	aagctcagaaccagtgcctcag	
SNP-933_dir_seq[b,c]	atcctgcagacttgaagaagc	atcatacatcattgggttacc	
Gapdh[d,e]	tgtccgtcgtggatctgac	cctgcttcaccaccttcttg	
Ube3a ATS[d,e]	tagcagccagcttgcctcttac	gactgtggcaaagacttggtga	
Mtap2[e]	ccaagaccttcctccatcctc	gctctgcgaattggttctgac	Microtubule-associated protein
Dcx[e]	tgttcattgcttgtggtcctgaaa	cttttgaggtgttggggaagcc	Microtubule-associated protein
Tubb3[e]	tagaccccagcggcaactat	gttccaggttccaagtccacc	β III tubulin
Huc[e]	cctgcaagttggttcgggata	aggccattgagggtgttgatg	RNA-binding protein
NeuN[e]	gtagagtctatgccgctgctga	cttcatggtccgagaaggagac	RNA-binding protein
Ngn2[e]	tctgcaggctgtgggaatttca	aaagaagggcgggacaataggc	Transcription factor
Tbr1[e]	tttcatcggcatcaaacggaga	tccgccaaaatcacatccacaa	Transcription factor
Nrxn1[e]	acacccttcgattggagctaga	tagccagcaaaaagggtctcag	Cell adhesion molecule
Ncam2[e]	aaaccacaccgctaacagaacc	agactggatgatgtcattggacac	Cell adhesion molecule

[a] For real-time TaqMan PCR assays of SNP ratio quantification (character noted in parentheses is the RNA that is digested specifically by RNase H if a DNA/RNA hybrid double strand is formed. If there is a mismatch at the RNA part or at the –1 position of RNA part, the chimeric probe is not digested.)

[b] For PCR of SNP ratio quantification

[c] For sequence analysis of SNP ratio quantification (only forward primer)

[d] For strand-specific cDNA synthesis (only reverse primers)

[e] For quantitative PCR

SNP ratio quantification by real-time TaqMan PCR assay

Real-time TaqMan PCR assays were performed in accordance with the manufacturer's protocol. Briefly, 1 μL of cDNA was used as the template in a 45-cycle PCR reaction with a Thermal Cycler Dice real-time system (Takara Bio Inc.). The PCR solution (10 μL final volume) included 2-fold-diluted CycleavePCR Reaction Mix (Takara Bio Inc.), each primer at a concentration of 0.2 μM, and two probes labeled individually with FAM and ROX, each at a concentration of 0.2 μM (Table 1). The PCR program was 45 cycles of 5 s at 95 °C, 10 s at 55 °C, and 20 s at 72 °C. The samples were measured in duplicate with two homozygous N9 samples of B6-ESC and MSM-ESC as positive controls, a cDNA-free sample as a negative control, linear pre-mixed samples of two homozygous N9 samples in 10 different B6/MSM ratios from 1.0 to 0.1 for standard curve generation, and an MB4 D0 sample as the reference for a SNP ratio of 1.0.

The SNP ratio was calculated by using the fluorescence normalization method shown in a previous study (Yu et al. 2006), with minor modification. Briefly, raw fluorescence data from each cycle were fitted to a four-parametric sigmoid function and normalized to background fluorescence:

$$f(Rx) = y_0 + \frac{a}{1 + e^{-\left(\frac{x - x_0}{b}\right)}}$$

where $f(R_x)$ is the raw fluorescence data of cycle x, y_0 is background fluorescence, a is the difference between the background fluorescence and the maximal fluorescence, b is the slope of the curve, and x_0 is the first derivative maximum of the sigmoid function. The background fluorescence was subtracted from each data point of raw fluorescence, and the subtracted fluorescence was divided by the background fluorescence for normalization.

The start and end of the exponential phase were determined as 80% of the second derivative maximum of the sigmoid function and the first derivative maximum x_0,

respectively. By using the normalized fluorescence data of FAM and ROX during the exponential phase, the linear regression equation $R_{ROX} = K_f \times R_{FAM} + R_0$ was generated. The slope of the regression line (K_f) was used for the SNP ratio calculation.

A standard curve was generated from the K_f of the sample series of the pre-mixed B6/MSM ratio from 1.0 to 0.1. The average K_f of each sample and the MB4 D0 sample was plotted against the standard curve. The SNP ratio was calculated by dividing the estimated B6/MSM ratio of each sample by that of the MB4 D0 sample. We used the SNP ratios determined by using this method, although they were almost the same (Pearson's correlation coefficient $[r] = 1.00$) as the simplified SNP ratios that were calculated by dividing the average K_f of each sample by that of the MB4 D0 sample. In the process of SNP ratio calculation, K_f of each sample was determined from the raw fluorescence data of each cycle by using R version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria), and the SNP ratio of each sample was evaluated by using Microsoft Excel for Mac 2011 (Microsoft Corporation, Redmond, WA, USA).

Quantitative PCR

Quantitative PCR was performed in accordance with the manufacturer's protocol. Briefly, 1 μL of cDNA was used as the template in a 40-cycle PCR reaction with a Thermal Cycler Dice real-time system. The PCR solution (10 μL final volume) included twofold-diluted SYBR Premix Ex Taq II (Takara Bio Inc.) and 0.4 μM of each primer (Table 1). The PCR program was 30 s at 95 °C, 40 cycles of 5 s at 95 °C, and 30 s at 60 °C. *Gapdh* was used as a reference gene for calculation of relative expression level. The samples were measured in duplicate. Relative quantities were evaluated by using the ΔΔCt method. Logarithmic relative expression levels of neuronal marker genes were used for the statistical analyses.

Statistical analysis

Fifty-four samples from various in vitro neuronal differentiation conditions were grouped into low- and high-imprinted groups at the 30th percentile of *Ube3a* imprinting status. The low-imprinted group contained 38 samples and the high-imprinted group contained 16 samples. The expression level of *Ube3a ATS* was compared between these two groups by using an unpaired Student's *t* test with a significance level of 0.05. In stepwise logistic regression analyses, the dichotomous *Ube3a* imprinting category was used as the dependent variable. Expression levels of neuronal marker genes were used as the independent variables. The significance level was set at 0.1 in a backward selection method. Predicted values calculated by using the final model were used for receiver operating characteristic (ROC) analysis. All data analyses were performed with STATA software version 13.1 for Mac (StataCorp LP, College Station, TX, USA).

Results

Ube3a imprinting

In a previous study, we assessed the *Ube3a* imprinting status with SNP-933 on exon 8 of *Ube3a* mRNA by direct sequencing of the cDNA samples. In the current study, we tried to quantify the SNP ratio by using a real-time TaqMan PCR assay; however, we were not able to design a TaqMan probe for SNP-933. Therefore, we assessed the SNP ratio with another SNP, SNP-863, which is located on exon 5 of *Ube3a* mRNA. Our F1 hybrid ESC line has thymine on the maternal allele and cytosine on the paternal allele at SNP-863 (Fig. 1a) . In our in vitro system, *Ube3a* is expressed biallelically in ESCs, and the peak levels of maternal thymine and paternal cytosine at the SNP-863 locus in ESCs are therefore the same (Fig. 1b). In neurons, the SNP ratio was determined as the ratio of peak paternal cytosine to peak maternal thymine, where a lower value represents a higher level of *Ube3a* imprinting. The Pearson's correlation coefficient (r) between the SNP ratios of the 54 neuron samples assessed by direct sequencing and those assessed by real-time TaqMan PCR assay was 0.91 (Fig. 1c). The correlation between the SNP ratios of the 54 neuron samples assessed by direct sequencing at SNP-863 and at SNP-933 was also $r = 0.91$; we therefore considered the SNP ratio assessed by real-time TaqMan PCR to be equivalent to the one assessed by direct sequencing. We used the SNP ratio assessed by real-time TaqMan PCR as the *Ube3a* imprinting status in the following analyses. The *Ube3a* imprinting status varied among the differentiated neuron samples and followed a roughly normal distribution (Fig. 1d). For the quality assessment, we divided these samples into two

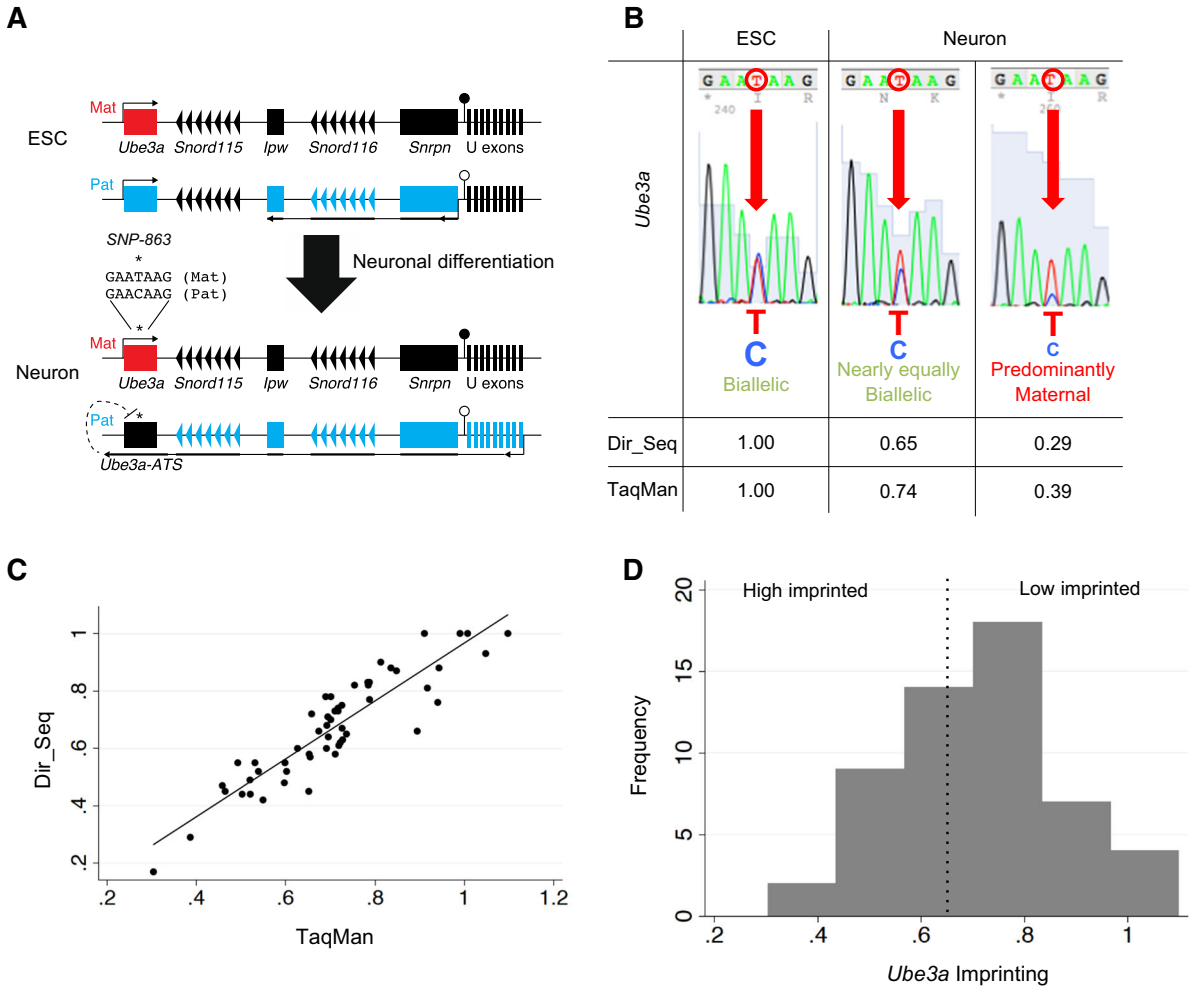

Fig. 1 *Ube3a* imprinting. **a** Schematic representation of *Ube3a* imprinting. Paternal *Ube3a* expression in neurons is silenced by the neuron-specific, paternal allele-specific, antisense noncoding transcript of *Ube3a* (*Ube3a ATS*). *Ube3a ATS* is transcribed as part of a large transcription unit containing alternative U exon transcripts, *Snrpn*, snoRNAs (*Snord115* and *Snord116*), and *Ipw*. Paternal expression of this large transcript is regulated by the differentially methylated region (DMR) in *Snrpn* exon 1. *Lollipops with black and white circles* indicate methylated and unmethylated DMRs, respectively. *Mat* and *Pat* in the figure indicate maternally and paternally inherited chromosomes, respectively. *Rectangles, vertical bars*, and *triangles* symbolize genes, U exons, and snoRNAs, respectively. *Red, blue, and black* indicate maternally expressed, paternally expressed, and silenced alleles, respectively. The direction of transcription is shown by an *arrow*. *Asterisks* indicate the position of SNP-863. The figures are not drawn to scale. **b** Allele-biased *Ube3a* expression. Direct sequencing of the cDNA reveals allelic expression of *Ube3a* during neuronal differentiation. *Arrows* on peak signals and *circles* on the sequences indicate the positions of SNP-863. Differentiated neurons show a range of imprinting, from nearly biallelic expression to overwhelming maternal expression. *Dir_Seq* and *TaqMan* indicate SNP ratios assessed by direct sequencing and real-time TaqMan PCR assay, respectively. **c** *Scatter plot* of SNP ratios assessed by direct sequencing and real-time TaqMan PCR assay. The regression equation is $y = 1.01 \times x - 0.04$. **d** Frequency distribution of *Ube3a* imprinting in the 54 samples. (Color figure online)

groups at the 30th percentile of *Ube3a* imprinting status, creating a high-imprinted group at values ≤ 0.652 and a low-imprinted group at values > 0.652. We used these two groups to determine neuronal marker genes that were associated with *Ube3a* imprinting.

Ube3a ATS and conventional neuronal marker expression

To assess the quality of the 54 neuron samples, we measured the expression levels of *Ube3a ATS* and nine

conventional neuronal marker genes that are expressed during neurogenesis. These markers encode the microtubule-associated proteins Mtap2 and Dcx (Francis et al. 1999; Walker et al. 2007), β III tubulin Tubb3 (Lee et al. 1990), RNA-binding proteins Huc and NeuN (Kim et al. 2009; Mullen et al. 1992), transcription factors Ngn2 (Lee 1997) and Tbr1, and cell adhesion molecules Nrxn1 and Ncam2. Among these genes, expression of *Mtap2*, *Nrxn1* and *Ncam2* was correlated most strongly ($|r| \geq 0.70$) with *Ube3a* imprinting (Fig. 2a). Expression of *Ube3a ATS*—the cause of *Ube3a* imprinting—was also correlated strongly with *Ube3a* imprinting ($r = -0.66$). The mean relative expression of *Ube3a ATS* was significantly higher in the high-imprinted group, indicating the validity of the *Ube3a* imprinting categorization (Fig. 2b). *Ube3a ATS* expression was correlated most strongly with that of *Mtap2* among the neuron marker genes (Fig. 2a). The expression levels of *Mtap2*, *Dcx*, *NeuN*, *Ngn2*, *Tbr1*, *Nrxn1*, and *Ncam2* were each highly correlated with those of at least 4 other genes in the group.

Markers associated with *Ube3a* imprinting category

To select the marker genes most associated with *Ube3a* imprinting category, we performed a multiple logistic regression analysis. In this analysis, we included *Ube3a* imprinting category as the dependent variable and all marker genes as independent variables and eliminated the independent variables by using a backward selection method. The significance level for removal from the model was 0.1. Four marker genes, *Ube3a ATS*, *Mtap2*, *Tubb3*, and *Tbr1*, remained in the final model (Table 2). The coefficients of *Ube3a ATS* and *Mtap2* were positive, whereas the coefficients of *Tubb3* and *Tbr1* were negative, indicating that higher expression levels of *Ube3a ATS* and *Mtap2* and lower expression levels of *Tubb3* and *Tbr1* were associated with higher *Ube3a* imprinting status. Using an ROC curve constructed from the values predicted in the final model (including *Ube3a ATS*, *Mtap2*, *Tubb3*, and *Tbr1*), we tested the ability to identify high- or low-imprinted neurons and found an area under the curve

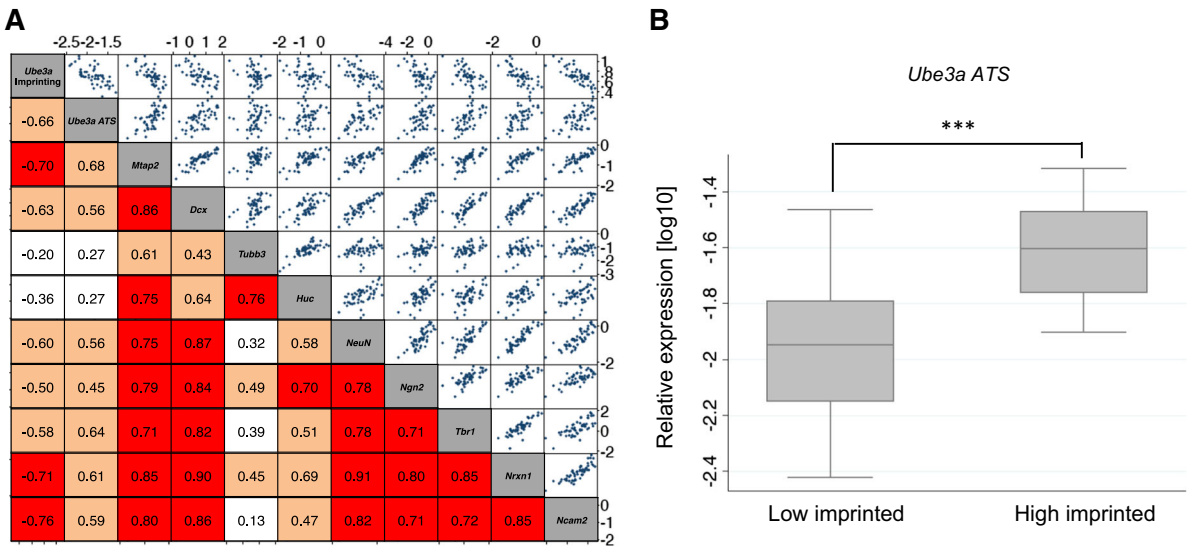

Fig. 2 Neuronal marker expression. **a** Scattering matrix of *Ube3a* imprinting and logarithmic relative expression of neuronal marker genes. Marker genes are indicated in the *gray boxes* on the diagonal line. *Scatter plots* are indicated in the right upper area. Pearson's correlation coefficients (r) are indicated in the left lower area, where a *red background* represents $|r| \geq 0.70$, *orange* represents $0.70 > |r| \geq 0.40$, and *white* represents $0.40 > |r|$. All of the coefficients were significant ($P < 0.05$) except for that between *Tubb3* and *Ncam2* ($r = 0.13$) and that between *Ube3a* imprinting and *Tubb3* ($r = -0.20$). **b** *Box plot* of the expression level of *Ube3a ATS*. The y axis represents relative expression of *Ube3a ATS*. From *top to bottom*, the *horizontal lines* of the *whiskers and boxes* represent the maximum, upper quartile, median, lower quartile, and minimum. $^{***}P < 0.001$. (Color figure online)

Table 2 Results of stepwise logistic regression analysis between *Ube3a* imprinting category and marker gene expression

Marker gene	Coefficient (95% CI)
Ube3a ATS	10.7 (1.9, 19.5)
Mtap2	12.8 (4.1, 21.6)
Tubb3	–4.3 (–7.8, –0.7)
Tbr1	–2.8 (–5.4, –0.1)

CI Confidence interval

(AUC) of 0.95 (Fig. 3). The AUC of the ROC curve constructed from only *Ube3a ATS* was 0.86. These two AUCs were significantly different ($P < 0.05$), indicating that adding *Mtap2, Tubb3*, and *Tbr1* to the model improved the accuracy of predicting the *Ube3a* imprinting status relative to using a model that included only *Ube3a ATS*.

Discussion

We evaluated 54 samples derived from various in vitro neuronal differentiation conditions to assess the association of selected neuronal marker genes with *Ube3a* imprinting status. We divided the sample set into two groups of *Ube3a* imprinting status at the 30th percentile. The expression level of *Ube3a ATS*—the cause of *Ube3a* imprinting—was significantly different between these two groups, indicating the validity of *Ube3a* imprinting categorization. Using this dichotomous category as a dependent variable, we performed stepwise logistic regression analyses. *Ube3a ATS*, *Mtap2*, *Tubb3*, and *Tbr1* remained in the final model as independent variables, indicating that these markers were associated with *Ube3a* imprinting. The pro-neural marker *Ngn2* and immature neuron marker *Dcx* were dropped from the final model, and the immature neuron marker *Tbr1* showed a negative association with *Ube3a* imprinting category. However, the mature neuron marker *Mtap2*, which encodes a microtubule-associated protein, was strongly positively associated with *Ube3a* imprinting category like *Ube3a ATS*, suggesting that *Ube3a* imprinting is associated with neuronal maturation, probably relating to neurite extension. In our previous study, we empirically used *Ube3a ATS*, *Mtap2,* and *Ngn2* as markers to evaluate the quality of differentiated neurons. Our current results statistically support the results of this empirical analysis. The ROC analysis indicated that *Ube3a ATS*, *Mtap2*, *Tubb3*, and *Tbr1* are useful for assessing the quality of differentiated neurons.

Although we used mouse ESCs for this study, in vitro neuronal differentiation is widely used for undifferentiated pluripotent cells—for example, induced pluripotent stem cells (iPSCs). High-quality iPSCs that support the development of entirely iPSC-derived animals exhibit higher expression levels of the imprinted noncoding transcript *Meg3* than low-quality iPSCs that do not support this development (Stadtfeld et al. 2010), indicating that imprinted, noncoding transcripts may reflect the quality of undifferentiated and differentiated cells in vitro. Because genome imprinting is a common form of gene regulation among mammals, including humans, our current strategy for assessing differentiated neurons could be applied to human ESCs and iPSCs in cases where analyzing allele-specific expression of imprinted genes is difficult because of the lack of parental SNP information.

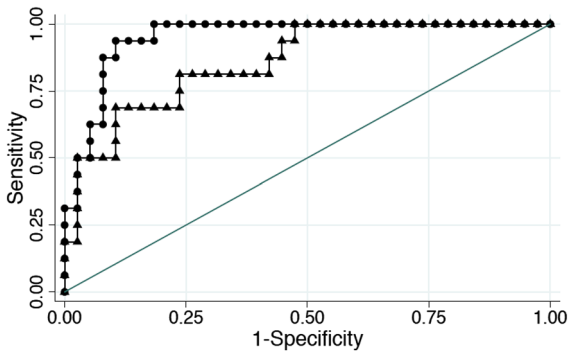

Fig. 3 Receiver operating characteristic (ROC) curves for assessment of *Ube3a* imprinting category. The line with the *black circles* is the ROC curve constructed from the values predicted in the final model of the logistic regression analysis (including *Ube3a ATS, Mtap2, Tubb3*, and *Tbr1*). The line with the *black triangles* is the ROC curve constructed from *Ube3a ATS* alone. The *x* and *y* axes are false positive rate (1—specificity) and true positive rate (sensitivity), respectively

Acknowledgements This work was financially supported by a Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (KAKENHI 24115703 and 26290063) and a JSPS Grant-in-Aid for Young Scientists (KAKENHI 16K19273). The authors thank Dr. K. Araki for embryonic stem cell lines and Ms. H. L. Le, M. Okabayashi, and Mr. H. Makino for technical assistance.

Compliance with ethical standards

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

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