Neurons but not glial cells show reciprocal imprinting of sense and antisense transcripts of $Ube3a$

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The human $UBE3A$ gene shows brain-specific partial imprinting, and lack of a maternally inherited allele causes Angelman syndrome (AS), which is characterized by neurobehavioral anomalies. In several AS model mice, imprinted $Ube3a$ expression is detected predominantly in the hippocampus, cerebellar Purkinje cells and the olfactory bulb. Therefore, imprinting of mouse $Ube3a$ is thought to be region-specific with different levels of silencing of the paternal $Ube3a$ allele in different brain regions. To determine cell types of imprinted $Ube3a$ expression, we analyzed its imprinting status in embryonic brain cells by using primary cortical cell cultures. RT–PCR and immunofluorescence were performed to determine the allelic expression of the gene. The $Ube3a$ gene encodes two RNA transcripts in the brain, sense and antisense. The sense transcript was expressed maternally in neurons but biallelically in glial cells in the embryonic brain, whereas the antisense transcript was expressed only in neurons and only from the paternal allele. Our data present evidence of brain cell type-specific imprinting, i.e. neuron-specific imprinting of $Ube3a$ in primary brain cell cultures. Reciprocal imprinting of sense and antisense transcripts present only in neurons suggests that the neuron-specific imprinting mechanism is related to the lineage determination of neural stem cells.

INTRODUCTION

Genomic imprinting is a mode of gene regulation causing genetic nonequivalence of the mammalian maternal and paternal genomes. Most genes are expressed equally from both parental alleles, whereas imprinted genes are expressed exclusively or preferentially from either the paternal or the maternal allele. Which allele is expressed is dependent upon parental inheritance because of a differential epigenetic marking that occurs during gametogenesis. To date, there is a growing number of genes for which imprint expression is either tissue-specific, specific to developmental stage, species-specific, promoter-specific or partial. Imprinted genes are recognized to play important roles in a considerable number of human congenital syndromes and tumorigenesis (1–3).

The human $UBE3A$ gene is such an imprinted gene that is implicated in a human congenital syndrome, Angelman syndrome (AS) (MIM 105830), characterized by severe neurologic abnormalities and distinctive behavior. $UBE3A$ was originally identified as a cellular protein that mediates the interaction of the human papillomavirus (HPV) E6 oncoprotein with p53 (4). $UBE3A$ is a member of a class of functionally related E3 ubiquitin–protein ligases defined by a carboxy-terminal ‘hect’ (homologous to the E6-AP carboxyl terminus) domain (5). $UBE3A$ is now thought to play roles both in defining the substrate specificity of ubiquitin transfer and in directly catalyzing ubiquitin transfer to substrates (6). We and others (7, 8) found $UBE3A$ mutations in patients with AS, a disorder that can also be caused by maternal deletion of 15q11–q13 (9), paternal uniparental disomy (UPD) of chromosome 15 (10) or an ‘imprinting defect’ (11) that changes parent-specific patterns of epigenetic modification and gene expression in 15q11–q13. AS shows an imprinted mode of inheritance, consistent with a

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gene active exclusively or preferentially on the maternal chromosome 15. The fact that the absence of a functional maternal copy of UBE3A causes AS, whose phenotype is restricted to neurobehavioral anomalies, indirectly suggests brain-specific imprinting of UBE3A. Although expression of UBE3A was initially shown not to be imprinted in cultured human fibroblasts and lymphoblasts (12), direct evidence of brain-specific imprinting of UBE3A was demonstrated in human fetal brains and AS deletion brains (13,14). RT–PCR analysis revealed that, although UBE3A is not imprinted in most human tissues, it is imprinted in the brain, with preferential but not exclusive expression of the maternal allele. It remains unclear whether imprinting is brain cell type-specific or region-specific.

In several AS model mice, imprinted Ube3a expression in the brain has been analyzed histologically (15–17). Albrecht et al. (15) used in situ hybridization in mice with paternal UPD of the region of mouse chromosome 7 containing Ube3a to demonstrate very low levels of Ube3a transcript in the hippocampus, cerebellar Purkinje cells and the olfactory bulb compared with wild-type mice. Ube3a maternal-deficient knockout mice reported by us (17) and others (16) showed a number of subtle neurologic abnormalities consistent with those of AS humans. No histopathological abnormalities were seen in Ube3a maternal-deficient mice; however, they revealed dramatically reduced Ube3a expression in the hippocampus and dentate gyrus compared with those of Ube3a paternal-deficient mice (16,17). These data suggest that imprinting of Ube3a is restricted to some brain regions, where its inappropriate expression may cause neurologic abnormalities. To determine the mechanism leading to this brain-specific and region-specific imprinting, identification of the brain cell type(s) with imprinted expression of Ube3a is necessary, because previous data for Ube3a imprinting in the mouse brain was based on in situ hybridization, which did not permit sufficiently high resolution to show imprinting status at the single-cell level.

To characterize imprinting status of Ube3a in brain cells, we performed primary brain cell culture from E15 (embryonic day 15) products of Ube3a knockout mice (17) and E15–17 products from reciprocal crosses between the C57BL/6 and PWK strains (divergent strains of Mus musculus). In vitro primary cultures have been useful tools for study of neurons and glial cells. Methods have been developed for primary culture using completely defined serum-free media that optimize survival and growth of either neurons or glial cells (18). In the present paper, neurons and glial cells were separately cultured in the primary culture system and quantitated by immunofluorescent staining (IF) and RT–PCR. In addition to the expression analysis of Ube3a-deficient mice by IF, RNA from the cultured neurons or glial cells from Ube3a-deficient mice and reciprocal crosses between C57BL/6 and PWK was used for imprinting analysis.

RESULTS

Evaluation of neurons and glial cells in primary cultures

Cerebral cortices and skin tissues were prepared from E15–17 embryos and used for primary cultures. Neuronal cells grown selectively in the B-27 medium were confirmed by IF using an antibody against MAP2 (microtubule-associated protein 2) as a neuronal cell marker. Likewise, the glial cells grown in the G-5 medium were confirmed immunohistochemically for a glial cell marker, GFAP (glial fibrillary acidic protein). Five days after initiation of the culture with B-27, MAP2-positive neurons were growing almost exclusively with a negligible number of GFAP-positive cells (Fig. 1A), while in the G-5 medium cortical glial cells proliferated and differentiated and cortical neurons survived initially but began to degenerate thereafter. At 11 days, the cultures with G-5 consist almost entirely of GFAP-positive astrocytes (Fig. 1B). Similar results were obtained in the Map2 and Gfap expression analysis by RT–PCR, i.e. the B-27 cultures were composed primarily of neurons with a few glial cells and the G-5 cultures were of glial cells (Fig. 1C).

Maternal Ube3a expression in Ube3a-deficient mice by IF

We have previously described maternal expression of Ube3a in Ube3a-deficient mice with a lacZ-IRES transcriptional reporter (17). LacZ staining of the adult brain demonstrated maternal-specific expression of Ube3a in some brain regions including hippocampus and dentate gyrus (17). In the neonatal brain, Ube3a was highly expressed in whole cerebrum, but we could not identify the cell type with maternal Ube3a expression by LacZ immunofluorescence and cell type-specific markers (data not shown). To see the expression of Ube3a in neurons and glial cells, we used primary cultures of embryonic brain (E15) from Ube3a-deficient mice. Cells in primary cultures from paternal-deficient mice with only maternally inherited Ube3a allele (m+/p−) and maternal-deficient mice (m−/p+) were stained by anti-β-galactosidase antibody and cell marker antibodies: NESTIN for progenitor cells, MAP2 for neurons and GFAP for glial cells. β-Galactosidase from the lacZ-IRES transcriptional reporter was stained as green dots in the cytoplasm. In m+/p− products, β-galactosidase was detected in all of the embryonic fibroblasts, progenitor cells and glial cells, but was not detected in neurons (Fig. 2B–E). Although β-galactosidase is not strongly stained by IF, maternal transmission of the targeted allele leads to β-galactosidase expression in all cultured cell types except neurons. In m−/p+ products, all of the cultured cells including neurons expressed β-galactosidase (Fig. 2F), indicating β-galactosidase was expressed in all of the cell types examined from the maternal Ube3a promoter.

Imprinting is maintained in primary brain cell cultures

To evaluate the imprinting stability in primary cultures, we investigated imprinting status of the Snrpn and Gabrb3 genes located near Ube3a on mouse chromosome 7, by RT–PCR. As the Snrpn gene is known to be expressed most strongly in the brain and exclusively from the paternal allele (19) and Gabrb3 is not imprinted in the brain (20), they were used as positive markers (data not shown). To see the expression of Ube3a in neurons and glial cells, we used primary cultures of embryonic brain (E15) from Ube3a-deficient mice. Cells in primary cultures from paternal-deficient mice with only maternally inherited Ube3a allele (m+/p−) and maternal-deficient mice (m−/p+) were stained by anti-β-galactosidase antibody and cell marker antibodies: NESTIN for progenitor cells, MAP2 for neurons and GFAP for glial cells. β-Galactosidase from the lacZ-IRES transcriptional reporter was stained as green dots in the cytoplasm. In m+/p− products, β-galactosidase was detected in all of the embryonic fibroblasts, progenitor cells and glial cells, but was not detected in neurons (Fig. 2B–E). Although β-galactosidase is not strongly stained by IF, maternal transmission of the targeted allele leads to β-galactosidase expression in all cultured cell types except neurons. In m−/p+ products, all of the cultured cells including neurons expressed β-galactosidase (Fig. 2F), indicating β-galactosidase was expressed in all of the cell types examined from the maternal Ube3a promoter.

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and negative controls for an imprinted gene, respectively. We
used F1 hybrids from reciprocal crosses between the C57BL/6
and PWK strains. Allelic expression of Snrpn was analyzed
using an AvaI digestion polymorphism. The C57BL/6 strain
has two AvaI sites and the PWK strain has one such a site
between exons 1 and 7 (Fig. 3A). RT–PCR products by primers
1F and 7R, followed by AvaI digestion, revealed that
Snrpn was expressed only from paternal allele in both neurons
and glial cells in primary cultures (Fig. 3B). The Gabrb3
gene was previously demonstrated not to be imprinted in the
brain (20). A HpyCH4IV polymorphic site was used for allelic expression
of Gabrb3 (Fig. 3C). RT–PCR revealed that the Gabrb3 gene
was expressed in both neurons and glial cells and a small
amount of Gabrb3 expression was detected even in embryonic
fibroblasts. In all of these experiments, the Gabrb3 gene
was expressed equally from the maternal and paternal alleles
(Fig. 3D).

**Figure 1.** Evaluation of neurons or glial cells selectively grown from cultured cortical cells, by IF and RT–PCR. Brain cells derived from the E15–17 cerebrum were plated. The cells were double-stained for mouse monoclonal anti-MAP2 and rabbit anti-GFAP, and with DAPI. (A) Cells grown 5 days after the initiation of culture in B-27 medium. Most cells were stained for MAP2 (green) and a few glial cells for GFAP (red). (B) Cells grown for 11 days in G-5 medium. Most cells in the figure were labeled with GFAP (red), but a very few MAP2-labeled neurons were observed in other views. Bar, 100 μm. (C) RT–PCR of cultured cells (neurons) in B-27, those (glial cells) in G-5 medium, and embryonic fibroblasts. Each cDNA concentration was adjusted for Gapdh amplification as an internal control. A small amount of GFAP cDNA was detected in RT–PCR products from neuron cultures.

**Reciprocal imprinting of sense and antisense Ube3a transcripts in Ube3a-deficient mice by RT–PCR**

Expression of Ube3a in cultured neurons was assayed by
RT–PCR using primers 15F and 16R in the Ube3a-deficient mice.
In cultured neurons, RT–PCR using oligo-dT-primed cDNA
generated two products; one is the expected product of 267 bp
(Fig. 4, one asterisk) detected in m+/p+ and m+/p− samples and
the other detected in m+/p+ and m−/p+ sample is the
longer product 1265 bp in size (Fig. 4, two asterisks), the same
size as genomic PCR product containing intron 15. To know
whether the longer product in m+/p+ and m−/p+ neurons
reflects premature mRNA or antisense transcript of Ube3a
from the paternal allele, strand-specific RT–PCR was performed.
The longer product was detected in cDNA primed by 15F but
not by 16R in m+/p+ and m−/p+ neurons, indicating that the
longer product is paternally expressed antisense transcript of
Figure 2. Imprinted Ube3a expression in Ube3a-deficient mice by IF. Primary cultured cells from m+/p− embryo (B, C, D, E) and from m−/p+ embryo at E15 (F) were double-stained for anti-β-galactosidase antibody (green) and cell marker antibodies (red), and also stained by DAPI (blue). (A) Schematic illustration of Ube3a targeting construct (top), genomic locus (middle), and targeted allele (bottom) (17). A part of exons 15 and 16 was substituted by the IRES-geo cassette. (B) Fibroblasts from E15 m+/p− embryo express β-galactosidase in all cells. (C, D) Cortical cells from E15 m+/p− embryo, grown 1 day (C) and 5 days (D) after the initiation of culture in B-27 medium were stained by anti-NESTIN (red) and anti-MAP2 (red) antibodies, respectively. Only NESTIN-positive progenitor cells express β-galactosidase. (E) Cortical cells (glial cells) from E15 m+−/p− embryo, grown 11 days in G-5 medium, stained by anti-GFAP(red) antibody express β-galactosidase. (F) Cortical cells (neurons) from E15 m−/p+ embryo, grown 5 days in B-27 medium stained by anti-MAP2 (red) antibody express β-galactosidase. Arrowheads point to β-galactosidase-positive dots (green). Bar, 10 μm.

Reciprocal imprinting of sense and antisense Ube3a transcripts is maintained in wild-type mice

We have demonstrated by IF that the maternal Ube3a promoter was active in all cultured neurons from m−/p+ cerebrum and that the paternal Ube3a promoter was inactive only in neurons from m+−/p− cerebrum. The exclusive maternal expression of Ube3a in neurons was also confirmed by RT–PCR, a more sensitive assay than IF. To exclude the possibility that the deleted region in Ube3a-deficient mice with lacZ substitution may affect the imprinting status, we used F1 hybrids from reciprocal crosses between C57BL/6 and PWK. Allele specific expression of the sense and antisense transcripts of Ube3a was analyzed using a polymorphic site in exon 5, where C57BL/6 has two Tsp509I sites and PWK has one Tsp509I site (Fig. 5A) (21). RT–PCR was performed using primers 5Fex and 6Rex in exons 5 and 6, respectively. RT–PCR products digested by Tsp509I revealed that in cultured cortical neurons, the sense transcript of Ube3a was exclusively expressed from the maternal allele, whereas in cultured glial cells and fibroblasts, it was biallelically expressed (Fig. 5B). We also examined the imprinting status of the antisense transcript of Ube3a, which Chamberlain et al. have already verified in unspecific form of Ube3a in the mouse brain by RT–PCR across intron 5 using strand-specific cDNA (21). The strand specific cDNA primed only by 5F was amplified in RNA from total cerebral cortices and cultured neurons but not glial cells (Fig. 5C), indicating that the antisense transcript of Ube3a was not only exclusively expressed from paternal allele, as expected from the previous report by Chamberlain et al. (21), but was transcribed specifically in neurons.

Imprinting status of Ube3a in the telencephalon/cerebral cortices at developmental stages

To examine whether neuron-specific imprinting of Ube3a in vitro reflects the partial imprinting in the brain in vivo, telencephalon/cerebral cortices were prepared from F1 hybrid mice at E10, E16, P1, P5, P14 and P28, and used for RT–PCR assay. Before imprinting analysis, brain cDNA from these developmental stages was evaluated by RT–PCR using primers for Nestin as a marker for progenitor cells, Map2 for neurons, and Gfap for astrocytes, after normalization of cDNA concentration for Gapdh expression. As previously reported (22), Gfap became faintly positive around E16, and was clearly detected from birth onwards, whereas Map2 was not positive at E10, but clearly positive at E16. Nestin was positive for several weeks after birth.
The imprinting status of Ube3a in the whole telencephalon/cerebral cortices was analyzed. By quantitative analysis of electrophoretic bands for each allele, we found that Ube3a expression of maternal and paternal alleles was equal in E10 telencephalon where neurogenesis had not yet commenced, whereas partial imprinting became obvious after birth (Fig. 6B). The antisense transcript of Ube3a was not detected by strand-specific RT–PCR in E10 telencephalon (data not shown).

DISCUSSION

The role of human UBE3A in AS has been well established (23). Although no pathological abnormalities are recognized in the brain of AS patients, most AS symptoms are related to brain dysfunction, suggesting that UBE3A expression in the central nervous system (CNS) is affected in AS. Defining cell-types in the brain where Ube3a is imprinted is important for our understanding of the mechanism of imprinting in CNS and as well as of its neuronal maintenance and function. We have developed a cell culture model with which brain cell-type-specific imprinting of mouse Ube3a can be characterized. To minimize the survival of glial cells in the neuron culture, we used serum-free B27 medium, which allows a yield of >99% embryonic neurons 4 days after initiation of culture (24). In our primary neuron culture, a very small number of glial cells were detected with IF and RT–PCR, and a negligible amount of glial cell cDNA not analyzable for gene expression was detected by semi-quantitative RT–PCR (Fig. 1). Conversely, a negligible number of neurons were also detected in the glial cell culture. Another limitation of the primary neuron culture system is maturation or aging in cultured cells. In the mouse cerebral cortex, neurogenesis commences around stage E12, peaks around E15, and finishes around birth (22,25); cortical astrocytes are first detected around E16 and oligodendrocytes around birth, but the vast majority of both glial cell types are produced during the first postnatal month (26). In our primary cultures, cerebral cortices were removed from E15–17 embryos and cultured for 5 and 11 days in B-27 medium for neurons and G-5 medium for glial cells, respectively. Therefore, cells

Figure 3. Expression analysis of Snrpn and Gabrb3. (A) Schematic representation of Snrpn exons including Aval sites (vertical line). C57BL/6 and PWK alleles were diagrammed showing PCR products and fragment sizes. (B) Verification of Snrpn imprinting. RT–PCR products were digested by Aval. The same cDNA was used as in Figure 1C. (C) Schematic representation of Gabrb3 exons including an HpyCH4IV restriction site (vertical line). C57BL/6 and PWK alleles were diagrammed showing PCR products and fragment sizes. (D) Verification of biallelic expression of Gabrb3. RT–PCR products were digested by HpyCH4IV.
harvested were adjusted at ages equivalent for P0-2 for neurons and P6-8 for glial cells. Although the embryonic neurons cultured are reported to retain a clear morphological and electrophysiological phenotype (27), limitations of culture periods and difficulties in pure neuronal cell cultures from adult brain still prevents the analysis of matured or aged neurons in the primary culture system. As far as cells in this culture system are concerned, they represent neurons and glial cells in the neonatal period. Imprinting status of the gene in whole cortices from embryos and adult mice can be interpreted consistent with our experiments using primary brain cell culture system (Figs 4 and 6). However, considering the limitations of the in vitro culture system mentioned above, expression and imprinting status of brain cells, depending on their functions in development, might be analyzed directly in the future.

Using this culture system, we have presented evidence for neuron-specific imprinting of Ube3a by IF (Fig. 2) and RT–PCR (Figs 4 and 5). We also demonstrated that the imprinting status of a known imprinted gene, Snrpn, as well as a non-imprinted gene, Gabrb3, were stable in our primary culture system (Fig. 3). Our data indicated that Ube3a is imprinted only in neurons and not imprinted in glial cells in brain culture, in contrast with previous data by in situ hybridization (15). Albrecht et al. reported patterns of mouse Ube3a expression in the wild-type embryonic brain and in the adult brains with partial paternal UPD encompassing Ube3a. Expression of Ube3a in the neuro-epithelium was detected since stage E8.5, and the gene was highly expressed at E15.5 in the olfactory bulb, nasal epithelium, cerebral cortex, hippocampus and some other brain regions (15). On the other hand, the expression was lower in the adult brain with partial paternal UPD than in the wild-type embryonic brain. Lower expression in the brain with paternal UPD may have reflected lower expression from the paternal allele than the maternal allele. According to the expression level of Ube3a in the paternal UPD brain, Albrecht et al. divided the brain into three regions: (i) an undetectable region, as in the hippocampus; (ii) a moderately or slightly reduced region as in the cerebral cortex; and (iii) a region indistinguishable from the normal region as in the anterior commissure, optic chiasma and other regions (15). They concluded that imprinting of Ube3a was region-specific, which implies different relative activities of the Ube3a alleles in different brain regions. Although neuron-specific imprinting demonstrated in our study is restricted in cultured cortical neurons and never excludes the possibility of region-specific imprinting, plausible explanations for the discrepancy between studies by us and by Albrecht et al. include: (i) neuron-specific imprinting of Ube3a might lead to region-specific imprinting in brain areas with higher density of neurons, but to less specific imprinting in lower density areas; (ii) imprinting status in the adult brain in vivo might be originally different from that in the embryonic brain in vitro, depending on their functions in the brain; (iii) RT–PCR is much more sensitive than in situ hybridization to detect gene expression, i.e. relatively low expression in the brain of wild-type mice with paternal UPD might be difficult to be analyzed for imprinting status by in situ hybridization. Lower Ube3a expression in glial cells than in neurons may explain partial imprinting shown by RT–PCR.

**Figure 4.** Imprinted Ube3a expression in Ube3a deficient mice by RT–PCR. (A) Cultured neurons (left) and glial cells (right) grown 5 days and 11 days after the initiation of culture in B-27 and G-5 medium, respectively; and (B) cerebral tissues from E16 embryos (left) and adult mice (right) were subjected to RT–PCR assay. Sense (*) and antisense (**) transcripts of Ube3a in exons 15 and 16 were detected by RT–PCR in oligo-dT primed cDNA, whereas only antisense transcript was amplified by strand specific RT–PCR using 15F primed cDNA (left top). Each cDNA concentration was adjusted for Gapdh amplification as an internal control.
Figure 5. Imprinted Ube3a expression in F1 hybrids from reciprocal crosses between C57BL/6 and PWK. (A) Schematic representation of Ube3a exons 5 and 6 including restriction sites. C57BL/6 and PWK alleles are diagrammed showing PCR products, Tsp509I sites (vertical line) and fragment sizes. Exon 5 in C57BL/6 has one additional Tsp509I site compared to that in PWK. Horizontal small arrows show primers used for PCR. (B) Verification of Ube3a imprinting in cultured neurons, glial cells and fibroblasts. Each cDNA concentration was normalized by Gapdh expression in Figure 1C. (C) Verification of Ube3a antisense transcripts. Strand-specific RT-PCR was performed using oligo-dT priming, specific 5F priming for antisense RNA and 5iR priming for unspliced sense RNA. Plus and minus signs mean with and without Tsp509I digestion, respectively.
assay in the whole cerebral cortex (Fig. 6B). Although cultured embryonic neurons are reported to retain a clear morphological and electrophysiological characters, studies by in situ hybridization of the embryonic or neonatal brain with paternal UPD will clarify whether Ube3a imprinting in the brain is neuron-specific or region-specific in vivo.

Recently, Herzing et al. (28) reported that UBE3A was imprinted in fibroblasts and neural precursor cells by RNA-FISH, whereas previously reported RT–PCR results in human fibroblasts demonstrated equal biallelic UBE3A expression (12). Total RNA from cycling cell population may only reflect the accumulation of RNA from cells with unequal biallelic UBE3A expression detected by RNA-FISH, resulting in almost equal biallelic expression by RT–PCR assay. Interestingly they suggested that exclusive imprinted UBE3A expression may be related to neuronal maturation, because preferential maternal expression is observed in undifferentiated neural cells, becoming exclusively maternal as the neurons differentiate. If degree of imprinted expression of UBE3A in the cell depends on each cell cycle stage, RNA-FISH data may support our conclusion of exclusive maternal expression only in neurons, which stay in G0 phase after differentiation from the progenitor cells.

We also showed neuron-specific expression of antisense Ube3a, which was previously reported to be paternally expressed in the brain and under the control of the imprinting center at the Prader-Willi syndrome critical region (PWS-IC) (21). In the human brain, Rougeulle et al. (29) detected a 20-kb paternally-expressed, intronless UBE3A antisense RNA fragment, which was recently reported to overlap the 3'UTR of a hypothetical transcript extending from SNURF-SNRPN to Ube3a (30). In addition to neuron-specific expression of antisense Ube3a, our RT–PCR study has not detected any neuron-specific isoforms of Ube3a and the bisulfite sequencing study has not found any differences in DNA methylation at the Ube3a promoter regions.
between parental alleles (data not shown). Although the role of the antisense transcript is unknown, our finding that the antisense Ube3a is expressed paternally in neurons, only where the sense Ube3a is maternally expressed, suggests that the antisense transcript expressed in neurons is closely related to neuron-specific imprinting of Ube3a (Fig. 7).

Our finding of neuron-specific reciprocal imprinting of sense and antisense transcripts of Ube3a raises some questions. Ube3a is not imprinted in telencephalon at E10 (Fig. 6), where the brain tissue consists of progenitor cells, which will differentiate to neurons by birth and glial cells after birth (Fig. 7). Then, when do progenitor cells acquire imprinting of Ube3a and what are the epigenetic factors that control Ube3a imprinting in neurons? Specification of cell lineages in the developing brain is thought to be regulated in part by epigenetic modifications of cell-type-specific genes, besides cell-external cues including various cytokines (31). Further investigations using the primary brain cell culture system will help us to elucidate the underlying epigenetic mechanisms of neuron-specific imprinting of Ube3a.

MATERIALS AND METHODS

Ube3a knockout mice

Ube3a-deficient mice with a lacZ-IRES transcriptional reporter were generated in a C57BL/6 background (Fig. 2A) (17). Paternal-deficient mice with only maternally inherited Ube3a allele (m+/p−) were produced by matings of male heterozygotes with female wild-type mice (C57BL/6), and maternal-deficient mice (m−/p+) were vice versa. Homozygous Ube3a-deficient mice (m−/p−) and wild-type mice (m+/p+) were produced by matings of female heterozygotes with male heterozygotes. Mice and embryos genotyped by PCR and Southern blotting were used for further analysis (17). Cerebral cortices were prepared from E16 embryos and P5 mice.

F1 hybrid mice of reciprocal crosses between C57BL/6 and PWK

C57BL/6 female mice were crossed with PWK male mice (C57BL/6 × PWK), and vice versa (PWK × C57BL/6). Telencephalon/cerebral cortices and embryonic fibroblasts were prepared from E10–18 embryos, P1, P5, P14 and P28 products of reciprocal crosses between C57BL/6 and PWK.

Tissues used

Embryos were removed from the uterus of timed pregnant mouse and placed in Petri dishes containing ice-cold HEPES. Cerebral cortices were freed from meninges. Cerebral cortices and/or embryonic fibroblasts were prepared from embryos and neonatal/adult mice. Tissues were used for RNA extraction or primary cultures. All procedures were approved by the Ethics Review Committee for Animal Experimentation of the Animal Center for Medical Research, Nagasaki University.

Primary cultures

Fetal cerebral cortices without meninges were dissociated by mechanical trituration and trypsinized with 0.25% trypsin with EDTA at 37°C for 10 min. Fetal calf serum (FCS; Bio Whittaker) was then added to dissociate cells, followed by filtration through sterile nylon sieve (pore size, 100 μm). Filtered cells were collected by centrifugation at 1200 rpm for
10 min. The cell pellet was resuspended in optimal media for growth of neurons or glial cells.

**Neuronal cultures.** The cell pellet from cerebral cortices was resuspended in Neurobasal™ (Gibco BRL) supplemented with 1 mM L-glutamine and B-27 supplement (Gibco BRL) to ensure selective growth of cortical neurons (24,32). Cells from the embryonic cerebral cortex were plated on polyethyleneimine-coated 3.5 cm plastic dishes at a density of 1 × 10⁶ cells/ml, and cultured in 5% CO₂ at 37°C.

**Gli cell cultures.** The cell pellet from cerebral cortices was resuspended in Dulbecco’s modified Eagle’s medium MEM (DMEM; Sigma) supplemented with 10% FCS. Cells were plated on polyethyleneimine-coated 3.5 cm plastic dishes at a density of 1 × 10⁶ cells/ml, and cultured overnight in 5% CO₂ at 37°C and medium was changed to Neurobasal™ with 1 mM L-glutamine and G-5 supplement (Gibco BRL). After 5–7 days in the primary culture, glial components grown were dislodged enzymatically with 0.25% trypsin and subcultured on new polyethyleneimine-coated plastic dishes. Cultures were maintained in 5% CO₂ at 37°C for a total of 11 days.

**Embryonic fibroblast culture.** Fibroblasts were derived from E15–17 embryos and cultured in DMEM supplemented with 10% FCS. Cells were plated on plastic dishes and maintained in 5% CO₂ at 37°C.

**IF**

The cells cultured on plastic dishes were fixed with 4% paraformaldehyde in PBS and subjected to immunofluorescent staining. The following primary antibodies were used: mouse monoclonal anti-MAP2 (microtubule-associated protein 2) antibody, rabbit polyclonal anti-MAP2 antibody (Chemicon), rabbit polyclonal anti-GFAP (anti- glial fibrillary acidic protein) antibody (Dako), rabbit anti-serum to NETIN (a gift from M. Ogawa), and mouse monoclonal anti-β-galactosidase antibody (Promega). Secondary antibodies were Alexa 488-conjugated goat anti-mouse IgG antibody and Alexa 568-conjugated goat anti-rabbit IgG antibody (Molecular Probe). The cells were counterstained with DAPI to identify nuclei. Signals were viewed under a Zeiss Axioskop fluorescence microscope and images were acquired with a PXL cooled CCD camera (Photometrics).

**cDNA synthesis**

Total RNA was isolated from cultured cells and tissues with RNeasy (Qiagen) according to the manufacturer’s protocol. The cDNA was generated from total RNA by SUPERSCRIPT II RNase H-reverse transcriptase (Gibco BRL) primed with oligo (dT)₁₂–₁₈ or specific forward or reverse primers. The first-strand cDNA was synthesized at 42°C for 50 min. Then, mRNA-cDNA chains were denatured and the reverse transcriptase activity was arrested by heating at 70°C for 5 min. As a control, an identical reaction was carried out without reverse transcriptase. Primers for specific primings were as follows: specific forward primer 15F for antisense Ube3a in the knockout mice: 5’-GGAGTCTGGAAATTTGTC-3’, specific reverse primer 16iR for sense Ube3a in the knockout mice: 5’-AGGGAAAAACAGCAATGCTG-3’, specific forward primer 5F for antisense Ube3a: 5’-CACAATGTAGAAGCTACGA-3’, specific reverse primer 5iR for sense Ube3a: 5’-CAGAAAGAAGATGAGGTGTTG-3’ (21).

**Polymerase chain reaction (PCR)**

The cDNA obtained was used to perform PCR for Nestin, Map2, Gfap and Gapdh using the following primers: Nestin forward, 5’-GAATTGAGAGCAGAAGAAACT-3’; Nestin reverse, 5’-TCTTCAATCTTATGTTGCTTC-3’; Map2 forward, 5’-AGTCTCCCTCTCCCATCACAGT-3’; Map2 reverse, 5’-CTCA- TACCTTACCCCCATCCTCT-3’; Gfap forward, 5’-AAGC- TCCAGAGTAAGAAACCAAGCTGA-3’; Gfap reverse, 5’-GGATCTGTAGTGTCAGGC-3’; Gapdh forward, 5’-ACCACAGTCCATCCATCAC-3’; and Gapdh reverse, 5’-TCCACACACCCTTGCTGTA-3’. For a semi-quantitative PCR, optimal template cDNA concentrations were determined according to Gapdh amplification. PCR products were amplified through 28 cycles of 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C.

**Imprinting analysis by RT–PCR**

In the knockout mice, Ube3a expression was analyzed by using primers 15F and 16R in exons 15 and 16 in the knockout region, respectively (Fig. 2A). Primer 16R is 5’-GTTTACAGCATGCCAATC-3’. Antisense transcript of Ube3a in the knockout region was amplified using the same forward/reverse primers, 15F and 16iR, as those used for specific primings. PCR amplifications with primers 15F and 16R/16iR were performed through 30 cycles of 30 sec at 94°C, 30 sec at 55°C and 60 sec at 72°C. In the F1 hybrid mice from reciprocal crosses between C57BL/6 and PWK, allele specific expression of sense and antisense transcripts of Ube3a was analyzed using a polymorphic site in its exon 5, where the C57BL/6 strain has two Tsp509I sites and the PWK strain has one Tsp509I site (Fig. 2A) (21). The cDNA from cultured cells and tissues was subjected to PCR for Snrpn and Gabrb3 amplification using the following primers: Snrpn 1F, 5’-TGTGCTTGAGAGTGAAGTTCG-3’; Snrpn 3R, 5’- CGCTTCACACTGCCTGGCG-3’; Gabrb3 forward, 5’-TGTCATGTCGCGGGAAGGA-3’; Gabrb3 reverse, 5’- CGATGGGCTGTGACATGGAAT-3’. The sense Ube3a strand was amplified using a primer pair: 5’Fex, 5’-AACTCCTCTCGTTGAAACAGG-3’; 6’Rex, 5’-TTGTGAAATTCGATTTTACTACCAT-3’. The sense Ube3a strand was amplified using the same forward/reverse primers, 5F and 5iR, as those used for specific primings. PCR amplifications with primers 5Fex and 6’Rex were performed through 30 cycles of 30 sec at 94°C, 30 sec at 58°C and 30 sec at 72°C, by other primer pairs through 30 cycles of 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C. Each PCR product was then digested with Tsp509I, Avai and HypCtAIV for imprinting analysis of sense and antisense Ube3a, Snrpn and Gabrb3, respectively, and electrophoresed in 2% agarose gel or 4% polyacrylamide gel.
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