

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

K. Yamasaki^{1,2}, K. Joh³, T. Ohta⁴, H. Masuzaki², T. Ishimaru², T. Mukai³, N. Niikawa¹, M. Ogawa⁵, J. Wagstaff⁶ and T. Kishino^{4,*}

¹Department of Human Genetics and ²Department of Obstetrics and Gynecology, School of Medicine, Nagasaki University, Nagasaki, Japan, ³Department of Biochemistry, Saga Medical School, Saga, Japan, ⁴Gene Research Center, Nagasaki University, Nagasaki, Japan, ⁵Laboratory for Cell Culture Development, Brain Science Institute, RIKEN, Saitama, Japan and ⁶Department of Pediatrics and Department of Biochemistry and Molecular Genetics, University of Virginia School of Medicine, VA, USA

Received December 2, 2002; Revised February 7, 2003; Accepted February 14, 2003

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 imprinted 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

*To whom correspondence should be addressed at: Nagasaki University Gene Research Center, 1-12-4 Sakamoto-machi, Nagasaki, Japan. Tel: +81 958497120; Fax: +81 958497121; Email: kishino@net.nagasaki-u.ac.jp

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 neurological 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* knock-out 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, MAP-2-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, paternal 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

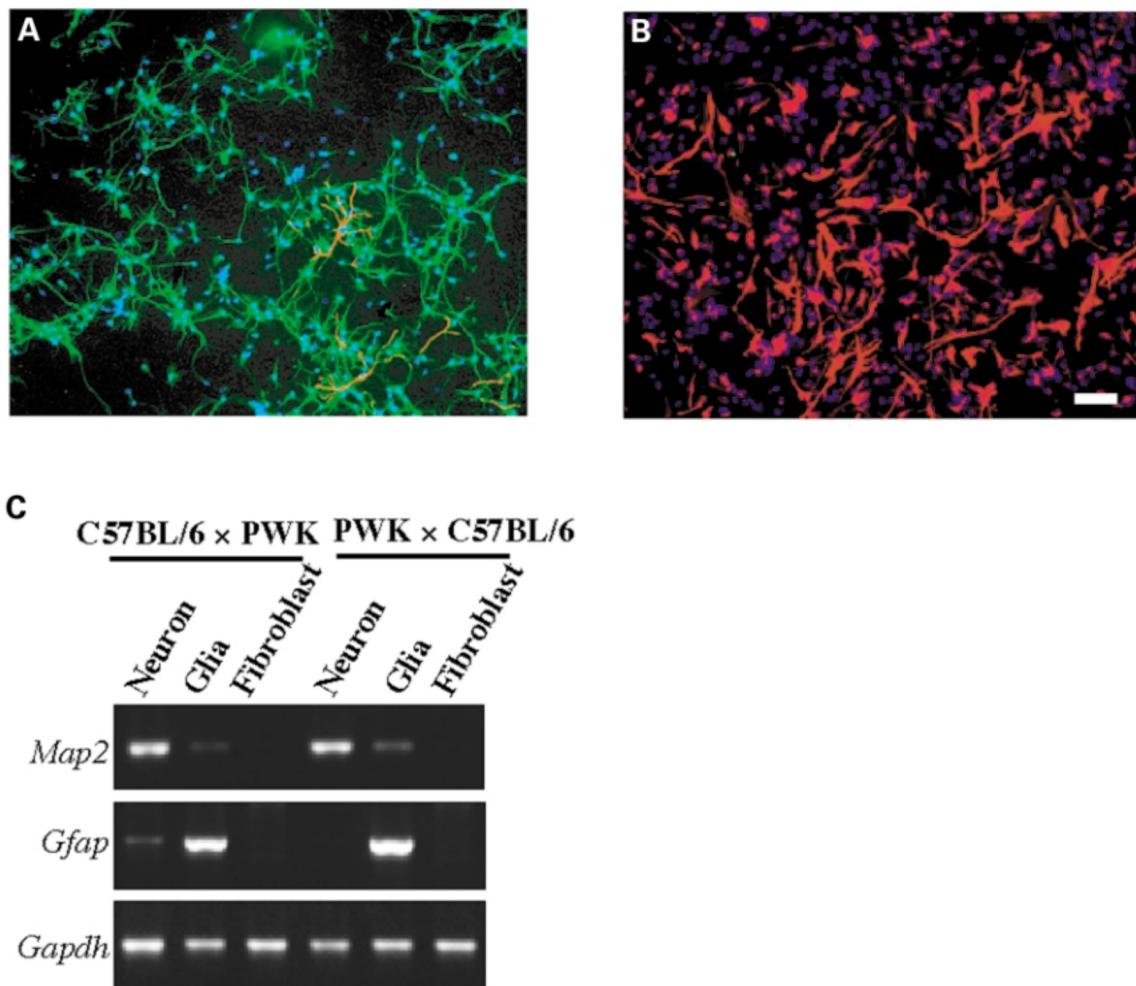


Figure 1. Evaluation of neurons or glial cells selectively grown from cultured cortical cells, by IF and RT-PCR. Brain cells derived from the E15–17 cerebrum were plated. The cells were double-stained for mouse monoclonal anti-MAP2 and rabbit anti-GFAP, and with DAPI. (A) Cells grown 5 days after the 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.

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).

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 16iR in m+/p+ and m-/p+ neurons, indicating that the longer product is paternally expressed antisense transcript of

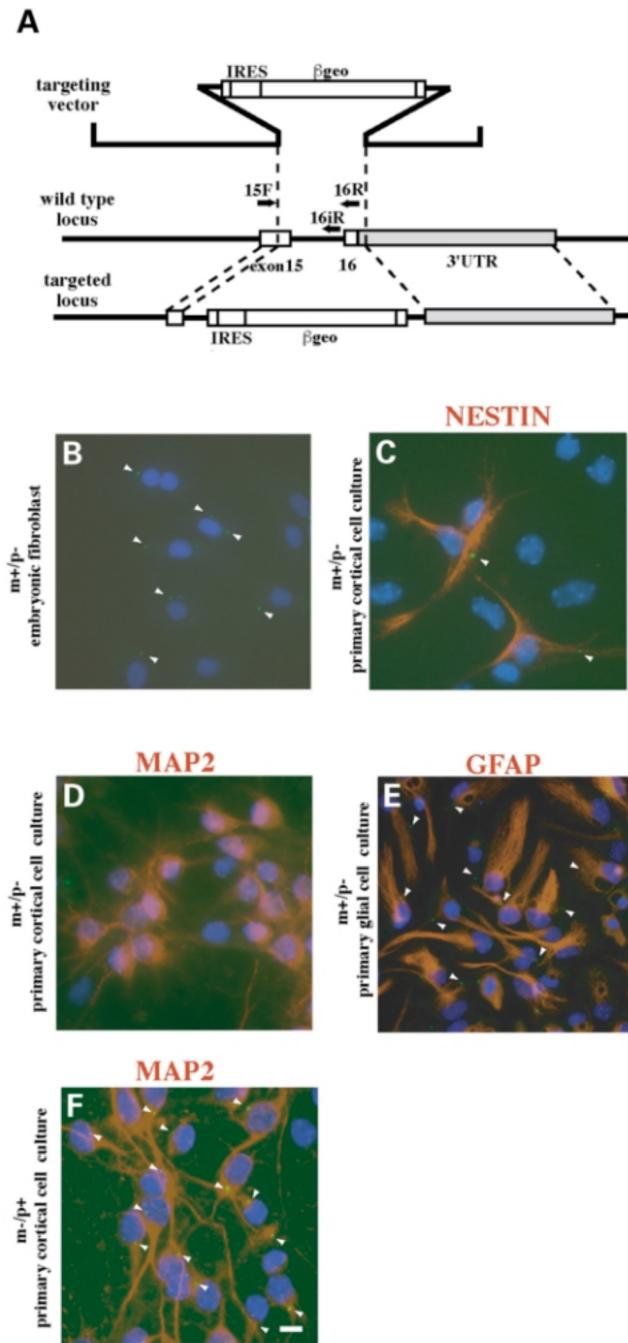


Figure 2. Imprinted *Ube3a* expression in *Ube3a*-deficient mice by IF. Primary cultured cells from *m*⁺/*p*[−] embryo (B, C, D, E) and from *m*[−]/*p*⁺ embryo at E15 (F) were double-stained for anti-β-galactosidase antibody (green) and cell marker antibodies (red), and also stained by DAPI (blue). (A) 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-βgeo 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.

Ube3a (Fig. 4A). We also evaluated the expression of *Ube3a* in cultured glial cells, and found that, in the *m*[−]/*p*⁺ sample, the sense transcript but not antisense transcript of *Ube3a* was detected (Fig. 4A). Such reciprocal imprinting of sense and antisense *Ube3a* transcripts in neurons but not in glial cells *in vitro* was supported by RT-PCR analysis using whole cortical tissues at different developmental stages. In *m*[−]/*p*⁺ whole cortices, both sense and antisense transcripts were detected, whereas in *m*⁺/*p*[−] only sense transcript was detected at E16 and adult (>P42) cerebrums, when progenitor cells/neurons and neurons/glial cells are main components, respectively (Fig. 4B).

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 unspliced 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

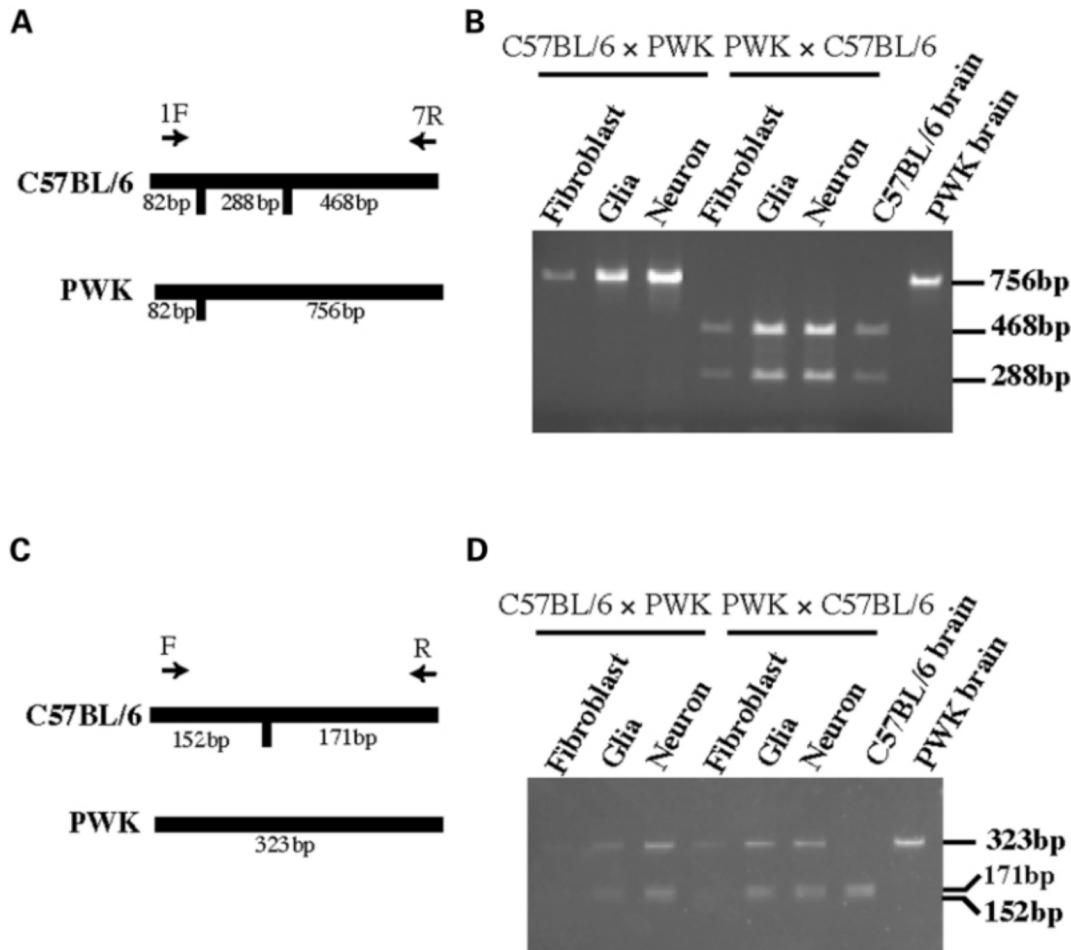


Figure 3. Expression analysis of *Snrpn* and *Gabrb3*. (A) Schematic representation of *Snrpn* exons including *AvaI* sites (vertical line). C57BL/6 and PWK alleles were diagrammed showing PCR products and fragment sizes. (B) Verification of *Snrpn* imprinting. RT-PCR products were digested by *AvaI*. 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*.

(Fig. 6A). 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

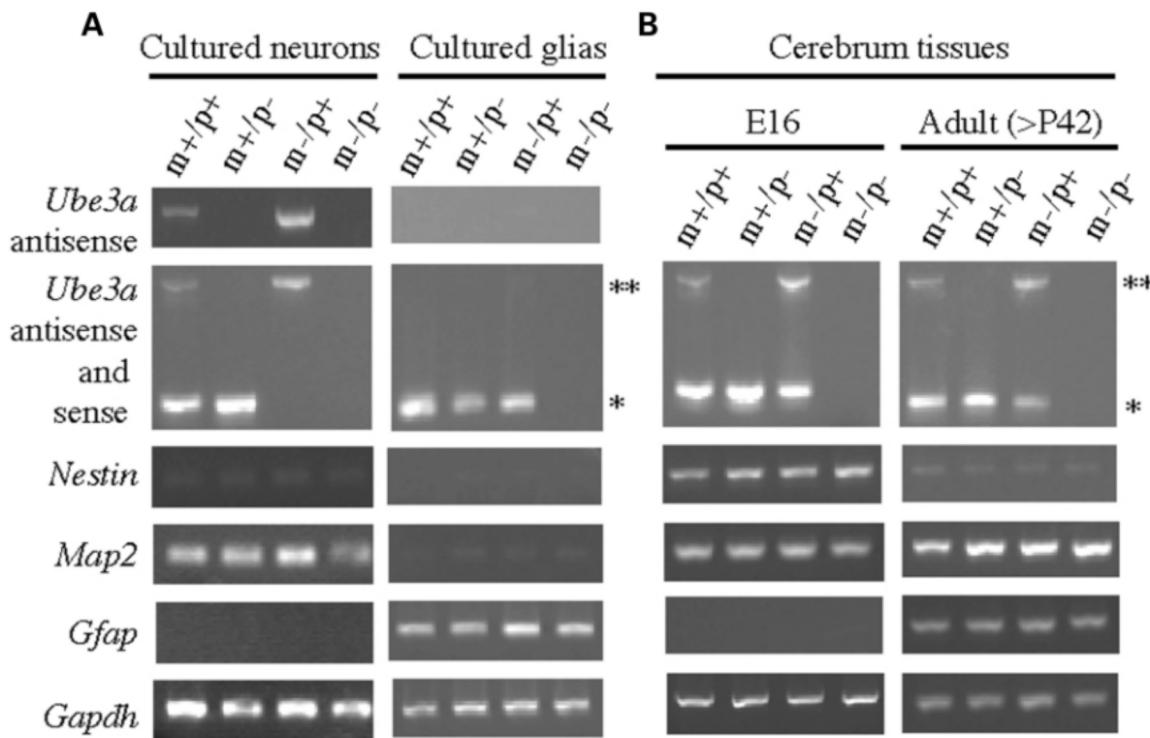


Figure 4. Imprinted *Ube3a* expression in *Ube3a* deficient mice by RT-PCR. (A) Cultured neurons (left) and glial cells (right) grown 5 days and 11 days after the initiation of culture in B-27 and G-5 medium, respectively; and (B) cerebral tissues from E16 embryos (left) and adult mice (right) were subjected 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.

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, hippo-

campus 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

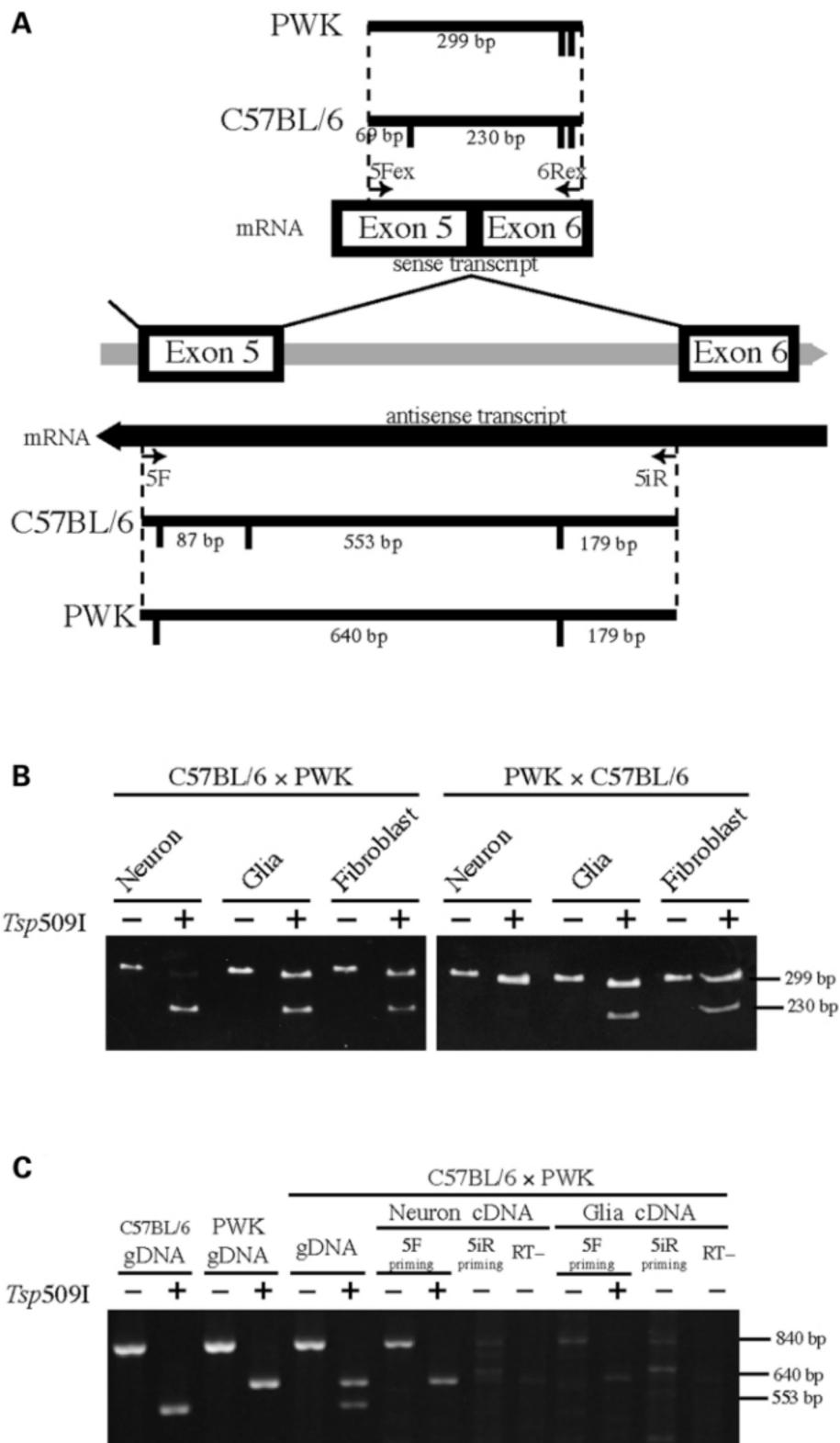


Figure 5. Imprinted *Ube3a* expression in F1 hybrids from reciprocal crosses between C57BL/6 and PWK. (A) Schematic representation of *Ube3a* exons 5 and 6 including restriction sites. C57BL/6 and PWK alleles are diagrammed showing PCR products, *Tsp509I* sites (vertical line) and fragment sizes. Exon 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.

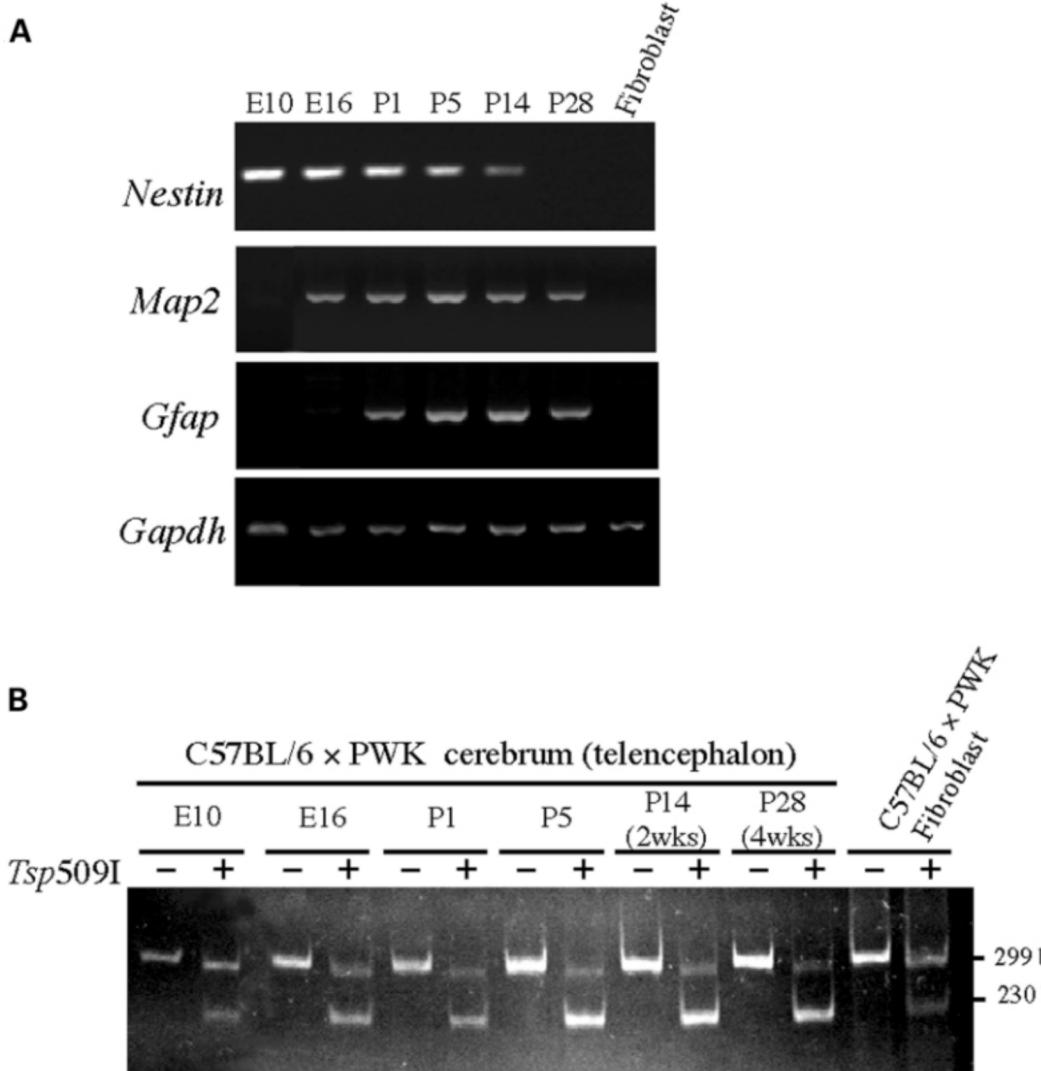


Figure 6. Evaluation of brain cells and imprinting status of *Ube3a* in telencephalon/cerebral cortices at developmental stages. The cDNA concentration was adjusted according to *Gapdh* amplification as an internal control. (A) Telencephalon/cerebral cortices from E10, E16, P1, P5, P14 and P28 embryos of F1 hybrids were used for RT-PCR. (B) Imprinting analysis of *Ube3a* in telencephalon/cerebral cortices. RT-PCR products were digested by *Tsp509I*. 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

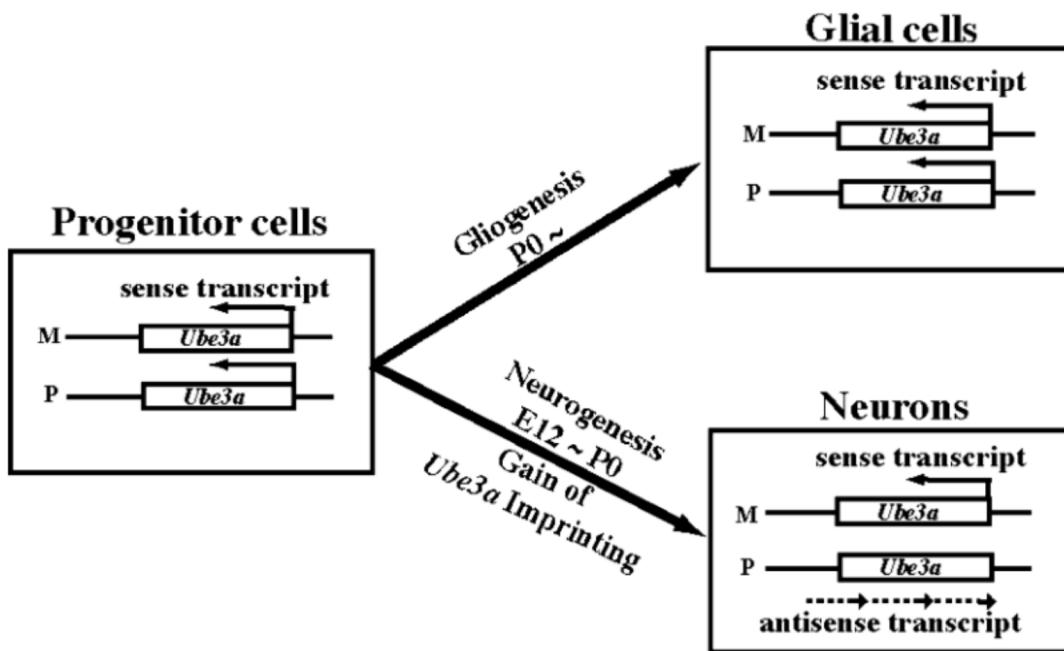


Figure 7. Summary of sense and antisense transcripts of *Ube3a* expressed in neural lineage. In neurons, the sense and antisense transcripts are expressed exclusively from the maternal (M) and paternal allele (P), respectively. In progenitor cells and glial cells, only sense transcript is expressed from both parental alleles.

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 mice 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 NeurobasalTM (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 1×10^6 cells/ml, and cultured in 5% CO₂ at 37°C.

Glial 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 1×10^6 cells/ml, and cultured overnight in 5% CO₂ at 37°C and medium was changed to NeurobasalTM 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 NESTIN (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'-GGAGTTCTGGGAAATTGTTCA-3', specific reverse primer 16iR for sense *Ube3a* in the knockout mice: 5'-AGGGAAAAACAGGACAATCATG-3', specific forward primer 5F for antisense *Ube3a*: 5'-CACATATGATG-AAGCTACGA-3', specific reverse primer 5iR for sense *Ube3a*: 5'-CAGAAAGAGAAGTGAGGTTG-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'-GAATGTAGAGGCAGAGAAAATC-3'; *Nestin* reverse, 5'-TCTTCAAATCTTAGTGGCTCC-3'; *Map2* forward, 5'-AGTCCCTCTCCCATCACCAGT-3'; *Map2* reverse, 5'-CTCTACTTACCCCCATCTCTT-3'; *Gfap* forward, 5'-AAGCTCCAAGATGAAACCAACCTGA-3'; *Gfap* reverse, 5'-GCAGATCTCGATGTCCAGGGC-3'; *Gapdh* forward, 5'-ACCACAGTCCATGCCATCAC-3'; and *Gapdh* reverse, 5'-TCCACCACCCCTGTTGCTGTA-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 using primers 15F and 16R in exons 15 and 16 in the knockout region, respectively (Fig. 2A). Primer 16R is 5'-GTTTACAGCATGCCAATCC-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 *SnRp* and *Gabrb3* amplification using the following primers: *SnRp* 1F, 5'-TTGGTTCTGAGGAGTGATTGC-3'; *SnRp* 7R, 5'-GCCTCCAAGTCTCGGACAGG-3'; *Gabrb3* forward, 5'-TGTCACTGGCGTGGAAAGGA-3'; *Gabrb3* reverse, 5'-CGATGGCTTGACATAGGGAAT-3'. The sense *Ube3a* strand was amplified using a primer pair: 5Fex, 5'-AATCCATCTCTTTGAAACTGAGGGT-3'; 6Rex, 5'-TTTGTAATTGAAATTATCACCATT-3'. The anti-sense strand of *Ube3a* was amplified using the same forward/reverse primers, 5F and 5iR, as those used for specific primings. PCR amplifications with primers 5Fex and 6Rex 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*, *Aval* and *HypCH4IV* for imprinting analysis of sense and antisense *Ube3a*, *SnRp* and *Gabrb3*, respectively, and electrophoresed in 2% agarose gel or 4% polyacrylamide gel.

ACKNOWLEDGEMENTS

T.K. was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare of Japan, and by a Grant-in-Aid for Scientific Research C (14570754) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1. Cattanach, B.M. and Beechey, C.V. (1990) Autosomal and X-chromosome imprinting. *Dev Suppl.*, 63–72.
2. Surani, M.A., Barton, S.C. and Norris, M.L. (1984) Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature*, 308, 548–550.
3. Tilghman, S.M. (1999) The sins of the fathers and mothers: genomic imprinting in mammalian development. *Cell*, 96, 185–193.
4. Huijbregts, J.M., Scheffner, M. and Howley, P.M. (1991) A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *EMBO J.*, 10, 4129–4135.
5. Huijbregts, J.M., Scheffner, M., Beaudenon, S. and Howley, P.M. (1995) A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc. Natl Acad. Sci. USA*, 92, 2563–2567.
6. Scheffner, M., Nuber, U. and Huijbregts, J.M. (1995) Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature*, 373, 81–83.
7. Kishino, T., Lalande, M. and Wagstaff, J. (1997) UBE3A/E6-AP mutations cause Angelman syndrome. *Nat. Genet.*, 15, 70–73.
8. Matsuura, T., Sutcliffe, J.S., Fang, P., Galjaard, R.J., Jiang, Y.H., Benton, C.S., Rommens, J.M. and Beaudet, A.L. (1997) De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome. *Nat. Genet.*, 15, 74–77.
9. Knoll, J.H., Nicholls, R.D. and Lalande, M. (1989) On the parental origin of the deletion in Angelman syndrome. *Hum. Genet.*, 83, 205–207.
10. Malcolm, S., Clayton-Smith, J., Nichols, M., Robb, S., Webb, T., Armour, J.A., Jeffreys, A.J. and Pembrey, M.E. (1991) Uniparental paternal disomy in Angelman's syndrome. *Lancet*, 337, 694–697.
11. Buiting, K., Saitoh, S., Gross, S., Dittrich, B., Schwartz, S., Nicholls, R.D. and Horsthemke, B. (1995) Inherited microdeletions in the Angelman and Prader-Willi syndromes define an imprinting centre on human chromosome 15. *Nat. Genet.*, 9, 395–400.
12. Nakao, M., Sutcliffe, J.S., Durtschi, B., Mutirangura, A., Ledbetter, D.H. and Beaudet, A.L. (1994) Imprinting analysis of three genes in the Prader-Willi/Angelman region: SNRPN E6-associated protein, and PAR2 (D15S225E). *Hum. Mol. Genet.*, 3, 309–315.
13. Rougeulle, C., Giatt, H. and Lalande, M. (1997) The Angelman syndrome candidate gene, UBE3A/E6-AP, is imprinted in brain. *Nat. Genet.*, 17, 14–15.
14. Vu, T.H. and Hoffman, A.R. (1997) Imprinting of the Angelman syndrome gene, UBE3A, is restricted to brain. *Nat. Genet.*, 17, 12–13.
15. Albrecht, U., Sutcliffe, J.S., Cattanach, B.M., Beechey, C.V., Armstrong, D., Eichele, G. and Beaudet, A.L. (1997) Imprinted expression of the murine Angelman syndrome gene, Ube3a, in hippocampal and Purkinje neurons. *Nat. Genet.*, 17, 75–78.
16. Jiang, Y.H., Armstrong, D., Albrecht, U., Atkins, C.M., Noebels, J.L., Eichele, G., Sweatt, J.D. and Beaudet, A.L. (1998) Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. *Neuron*, 21, 799–811.
17. Miura, K., Kishino, T., Li, E., Webber, H., Dikkes, P., Holmes, G.L. and Wagstaff, J. (2002) Neurobehavioral and electroencephalographic abnormalities in Ube3a maternal-deficient mice. *Neurobiol. Dis.*, 9, 149–159.
18. Bottenstein, J.E. and Sato, G.H. (1985) *Cell Culture in the Neuroscience*. Plenum Press, NY.
19. Barr, J.A., Jones, J., Glenister, P.H. and Cattanach, B.M. (1995) Ubiquitous expression and imprinting of Snrpn in the mouse. *Mamm. Genome*, 6, 405–407.
20. Nicholls, R.D., Gottlieb, W., Russell, L.B., Davda, M., Horsthemke, B. and Rinchik, E.M. (1993) Evaluation of potential models for imprinted and nonimprinted components of human chromosome 15q11-q13 syndromes by fine-structure homology mapping in the mouse. *Proc. Natl. Acad. Sci. USA*, 90, 2050–2054.
21. Chamberlain, S.J. and Brannan, C.I. (2001) The prader-willi syndrome imprinting center activates the paternally expressed murine Ube3a antisense transcript but represses paternal Ube3a. *Genomics*, 73, 316–322.
22. Bayer, S.A. and Altman, J. (1991) *Neocortical Development*. Raven Press, NY.
23. Lossie, A.C., Whitney, M.M., Amidon, D., Dong, H.J., Chen, P., Theriaque, D., Hutson, A., Nicholls, R.D., Zori, R.T., Williams, C.A. et al. (2001) Distinct phenotypes distinguish the molecular classes of Angelman syndrome. *J. Med. Genet.*, 38, 834–845.
24. Brewer, G.J. (1995) Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. *J. Neurosci. Res.*, 42, 674–683.
25. Jacobson, M. (1991) *Developmental Neurology*. Plenum Press, NY.
26. Qian, X., Shen, Q., Goderie, S.K., He, W., Capela, A., Davis, A.A. and Temple, S. (2000) Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. *Neuron*, 28, 69–80.
27. Evans, M.S., Collings, M.A. and Brewer, G.J. (1998) Electrophysiology of embryonic, adult and aged rat hippocampal neurons in serum-free culture. *J. Neurosci. Methods*, 79, 37–46.
28. Herzing, L.B., Cook, E.H.Jr. and Ledbetter, D.H. (2002) Allele-specific expression analysis by RNA-FISH demonstrates preferential maternal expression of UBE3A and imprint maintenance within 15q11–q13 duplications. *Hum. Mol. Genet.*, 11, 1707–1718.
29. Rougeulle, C., Cardoso, C., Fontes, M., Colleaux, L. and Lalande, M. (1998) An imprinted antisense RNA overlaps UBE3A and a second maternally expressed transcript. *Nat. Genet.*, 19, 15–16.
30. Runte, M., Huttenhofer, A., Gross, S., Kiefmann, M., Horsthemke, B. and Buiting, K. (2001) The IC-SNURF-SNRPN transcript serves as a host for multiple small nucleolar RNA species and as an antisense RNA for UBE3A. *Hum. Mol. Genet.*, 10, 2687–2700.
31. Edlund, T. and Jessell, T.M. (1999) Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. *Cell*, 96, 211–224.
32. Brewer, G.J., Torricelli, J.R., Evege, E.K. and Price, P.J. (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.*, 35, 567–576.

Copyright of Human Molecular Genetics is the property of Oxford University Press / UK and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.