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Paternal Expression of a Novel Imprinted Gene, *Peg12/Frat3,* in the Mouse 7C Region Homologous to the Prader–Willi Syndrome Region

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Paternally expressed imprinted genes (*Pegs*) were systematically screened by comparing gene expression profiles of parthenogenetic and normal fertilized embryos using an oligonucleotide array. A novel imprinted gene, *Peg12/Frat3,* was identified along with 10 previously known *Pegs*. *Peg12/Frat3* is expressed primarily in embryonic stages and might be a positive regulator of the Wnt signaling pathway. It locates next to the *Zfp127* imprinted gene in the mouse 7C region, which has syntenic homology to the human Prader–Willi syndrome region on chromosome 15q11-q13, indicating that this imprinted region extends to the telomeric side in the mouse.

Key Words: **genomic imprinting; Prader–Willi syndrome; *Peg12/Frat3;* paternally expressed gene.**

Among vertebrates, genomic imprinting is a mammalian-specific phenomenon in which functional differences between paternal and maternal genomes produce several parental-origin-specific phenotypes in development, growth, behavior, and some human genetic diseases and cancers (1–5). It is explained by the existence of two kinds of imprinted genes: paternally expressed genes (*Pegs*) and maternally expressed genes (*Megs*). To systematically isolate imprinted genes, a number of candidate genes were collected that showed differential expression in uniparental (parthenogenetic or androgenetic) and normal fertilized embryos using subtraction-hybridization and oligonucleotide arrays. So far, nine *Pegs* (*Peg1/Mest, Igf2, Peg3, Snrpn, Peg5/Nnat, Peg9/Dlk1, Ndn, Sgce, Impact*) have been identified by these methods (6–10). Using an improved version of GeneChip (Murine Genome U74 probe array), we obtained additional candidates and proved that *Peg12/Frat3* is a novel paternally expressed imprinted gene.

Peg12/Frat3 locates on the mouse 7C chromosomal region syntenic to human 15q11-q13, which is involved in Prader–Willi (PWS, MIM 176270) and Angelman (AS, MIM 105830) syndromes. PWS is a neurogenic disorder that is associated with significant developmental, behavioral, and mental problems, which result from a deficiency of the expression of paternally expressed imprinted genes in this region (5, 11). It is a well-conserved region in human and mouse and, so far, twelve *Pegs—Zfp127, Zfp127as, Magel2, Ndn, Sunrf, Snrpn, Pwcr1, MBII-85, MBII-52, MBII-13, Ipw,* Ube3aas—are known to be clustered in the mouse 7C region (http://www.mgu.har.mrc.ac.uk/research/imprinted/imprin.html).

Mice that fail to express all the *Pegs* in this region show phenotypes similar to PWS and serve as potential mouse models of PWS. These include mice with (i) maternal duplication/paternal deficiency of the 7C chromosomal region, (ii) a large deletion of the PWS/AS homologous region associated with a transgenic insertion, and (iii) a 42-kb deletion of the putative PWS imprinting center (IC) region (12–14). All of these model mice show characteristics similar to human PWS infants, such as feeding difficulties, decreased movement, and failure to thrive. Although they show 100% lethality within approximately one week (for example, 72% of IC mutation mice died within 48 h after birth and none survived past 9 days), why the model mice showed such a severe lethality remains unknown.

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Several gene-targeted mice for the *Pegs* in this region were analyzed. Mice deficient in *Snrpn* (14), *Snurf* (15), *Ipw* (cited in 16), and *Zfp127* (16) did not appear to differ from wild-type littermates. *Ndn* encodes a DNA-binding protein and is expressed in the nervous system; it is the only gene for which deficient mice showed neonatal lethality, although three independent groups have reported conflicting results on the lethal phenotypes: (i) Tsai *et al.* (17) observed no obvious phenotypic effect, (ii) Muscatelli *et al.* (18) reported neonatal and postnatal lethality before weaning (64% lethality in the N_2 generation), and (iii) Gerard *et al.* (19) reported the severest neonatal lethal phenotype within 30 h after birth (80% lethality in the N_2 generation). These differing degrees of lethality can be explained by the targeting strategy or differences in the genetic background to some extent. However, the complete lethality of the PWS mouse models does not seem to be explained simply by the lack of *Ndn* expression. Gerard *et al.* attributed the early neonatal lethality (within 48 h) associated with IC mutant mice mostly to the loss of function of *Ndn*, but the lethality at later stages (days 3–9) is not explained by *Ndn* deficiency. They also suggested that other imprinted genes cause additional lethality at later stages. Therefore, to fully understand the mechanisms of the lethality in these model mice, both more extensive screening of novel *Pegs* and functional analyses (gene targeting analysis) of *Pegs* in this region are necessary.

Here we report a novel paternally expressed *Peg12/Frat3* gene mapped to this region. Since *Peg12/Frat3* is expressed mainly in embryonic stages, especially in developing nerve systems, its potential contribution to the severe neonatal lethality in the PWS model mice is discussed.

MATERIALS AND METHODS

Mice and embryos. Parthenogenetic embryos were prepared as follows. Female (C57BL/6 × DBA/2)F_1 mice, 7–12 weeks of age, were superovulated with 5 IU of pregnant mare serum gonadotropin followed by 5 IU of human chorionic gonadotropin (hCG) 48 h later. Mature oocytes were collected from the ampullae of the oviducts 14–17 h after hCG injection and placed in CZB medium (20) containing 0.1 mg/ml bovine testicular hyaluronidase to remove cumulus cells from the oocytes. Cumulus-free oocytes were activated by treatment with 2.5 mM $SrCl_2$ in Ca^{2+}-free CZB containing 5 μg/ml cytochalasin B for 6 h. They were then transferred to fresh CZB medium and incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C for 72 h. Embryos that developed to the morula or blastocyst stages were transferred into the uteri of day 3 pseudopregnant ICR females. Recipient females were sacrificed on day 10 and live fetuses were retrieved from the uteri.

Two reciprocal F_1 hybrid mice, (C57BL/6 × JF1) F_1 and (JF1 × C57BL/6) F_1, were produced by an *in vitro* fertilization (IVF) method and natural mating, respectively. Samples were obtained from day 10 placenta, day 10 and day 13 embryos, neonatal brain, and adult tissues.

Comparative expression analysis using an oligonucleotide array. Twenty parthenogenetic and 15 fertilized control day 10 embryos were used to prepare cDNAs. mRNA was extracted from embryos and purified using an oligo(dT) cellulose method using a Fast Track mRNA isolation kit (Invitrogen Corp., Carlsbad, CA). The GeneChip Murine Genome U74 probe array set (A, B, and C) version 2 (Affymetrix, Inc., Santa Clara, CA) containing 6000 full-length murine cDNAs and 30,000 expressed sequence tags, was used in this analysis. cRNA preparation and GeneChip hybridization were carried out according to the manufacturer's protocols, and RNA transcript levels for the different genes were assessed and compared between parthenogenetic and normal control embryos using Affymetrix software.

Expression analysis using RT-PCR and polymorphism analysis. Genomic DNA and total RNA were extracted simultaneously from day 10 mouse embryos, neonatal brain, and adult tissues using ISOGEN (NipponGene, Tokyo, Japan), which is based on the acid guanidine thiocyanate-phenol–chloroform extraction method. Reverse transcription was carried out with 1 μg of the total RNA isolated from each sample. One thousandth of the resulting cDNA samples and 20 ng of genomic DNA samples were amplified by PCR using TaKaRa *ExTaq* DNA polymerase (TaKaRa Shuzo, Kyoto, Japan). Reaction mixtures contained 1× *ExTaq* buffer, 2.5 mM dNTP, 80 pmol of primers, and 2.5 units of *ExTaq* in a final reaction volume of 100 μl. The amplification conditions were 3 min at 85°C, followed by 24 (*β-actin* and *Gapdh*) or 30 (*Frat1* and *Peg12/Frat3*) cycles of 96°C for 15 s, 65°C for 30 s, 72°C for 1 min, with a final 3-min extension at 72°C. Primer sets were as follows: 5′-AAGTGTGACGTTGACATCCG-3′ and 5′-GATCCACATCTGCTGGAAGG-3′ for *β-actin*, 5′-CACTCTTCCACCTTCGATGC-3′ and 5′-CTCTTGCTCAGTGTCCTTGC-3′ for *Gapdh*, 5′-GAGGAGGAAACAGGAATGGAC-3′ (Peg12/Frat3-F) and 5′-ACACTCAATACCAGCCACCC-3′ (Peg12/Frat3-R) for *Peg12/Frat3*, and 5′-TAACAGCTGCAGTTCCCTGG-3′ and 5′-GGTCGCTCAGAGTGAACAGTG-3′ for *Frat1*. To verify imprinting, PCR products amplified with primers Peg12/Frat3-F and -R were digested with *Mbo*II restriction enzyme and separated by 1.5% agarose gel electrophoresis.

RESULTS

Screening Novel Imprinted Genes Using GeneChip

The expression profiles of approximately 36,000 genes in parthenogenetic embryos were compared with those of stage-matched normal control embryos using an oligonucleotide array. Candidate imprinted genes were selected that showed apparently lower expression in the parthenogenetic embryos than in the control embryos according to their rates of difference. The first seventeen candidates included nine previously known paternally expressed imprinted genes (*Peg1/Mest*, *Igf2*, *Peg3*, *Snrpn*, *Peg5/Nnat*, *Peg9/Dlk1*, *Ndn*, *Sgce*, and *Zac1*). The expression of the remaining candidates was further analyzed by RT-PCR to confirm the results of the GeneChip analysis. Figure 1A shows that *Frat3*, one candidate, was expressed only in control embryos and not in the parthenogenetic embryos. Another reported mouse *Frat* gene, *Frat1*, has 88% DNA sequence homology to *Frat3*. To confirm whether the expression difference detected with the GeneChip represented the *Frat3* gene itself, the expression of *Frat1* was also examined. Since no difference was observed in the expression of *Frat1*, the *Frat3* gene itself was a possible paternally expressed imprinted gene.

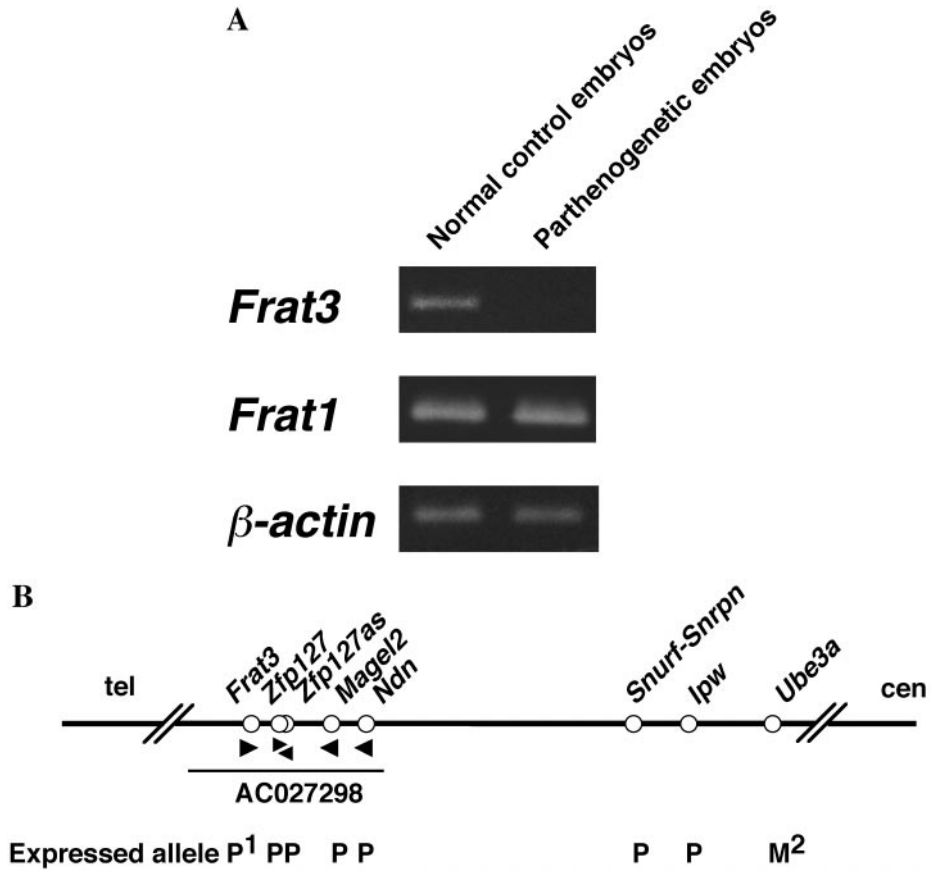

FIG. 1. (A) RT-PCR analysis of *Frat3* and *Frat1* in normal and parthenogenetic embryos. Poly(A)$^+$ RNA from pools of day 10 control and parthenogenetic embryos was used for RT-PCR. RT-PCR products for *Frat3, Frat1,* and *β-actin* were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. (B) Mapping *Frat3* in the mouse chromosome 7C region, a region of conserved synteny to the human Prader–Willi and Angelman syndrome region (15q11-q13). The location of the genomic sequence (AC027298) containing *Frat3* is shown. The orientations of the transcripts are indicated with arrowheads. The order of the mouse imprinted genes, *Zfp127, Zfp127as, Ndn, Snurf-Snrpn, Ipw, Ube3a,* is referred to in Ref. (13). The allele expressed is indicated for each gene. P, paternal; M, maternal. [Notes: (i) This paper verified imprinting of *Frat3.* (ii) *Ube3a* shows maternal expression in specific regions of the brain.]

Physical Mapping of Frat3 in the Mouse 7C Region

Previously, Jonkers *et al.* mapped *Frat3* to the mouse chromosome 7C region, which shows homology to human 15q11.2-q12 (21). For more precise physical mapping, database sequence searches using BLASTN were carried out, and revealed that the *Frat3* gene (GenBank Accession No. NM_013788) is included in the BAC complete genomic sequence (99.9% identical to the corresponding sequence at nucleotide positions 80, 913–983, and 552 of GenBank Accession No. AC027298) containing *Zfp127, Zfp127as, Magel2, Ndn,* which are imprinted genes in the mouse 7C imprinted region. Therefore, *Frat3* was mapped 40 kb telomeric to *Zfp127,* which is located at the telomeric edge of the imprinted gene cluster (Fig. 1B). Its location also suggests that *Frat3* is an imprinted gene in this domain. However, it should be noted that *Frat3* might exist only in the mouse genome, because there are no EST entries for human *FRAT3.*

Verification of Imprinting of Frat3

To verify the imprinting status of the *Frat3* gene, its expression was examined in subinterspecific F_1 mice between *M. musculus* (C57BL/6) and *M. molossinus* (JF1) at embryonic day 10. As shown in Fig. 2A, DNA polymorphism in the 3′-UTR of the *Frat3* gene was detected between these two strains. The *Mbo*II site in the JF1 allele was due to a 6-base deletion (TGGGTT) from the C57BL/6 allele. Therefore, these alleles could be distinguished by digestion with *Mbo*II; a 521-bp undigested PCR product was detected in the C57BL/6 samples (Figs. 2A and 2B, lane 1), while 338- and 177-bp digested DNA fragments appeared in JF1 samples (Figs. 2A and 2B, lane 2). This RFLP (restriction fragment length polymorphism) was used to test imprinting of *Frat3* in F_1 embryos derived from both matings between C57BL/6 females and JF1 males (B×J) and the reciprocal cross of JF1 females and C57BL/6 males (J×B). *Mbo*II digestion patterns of the

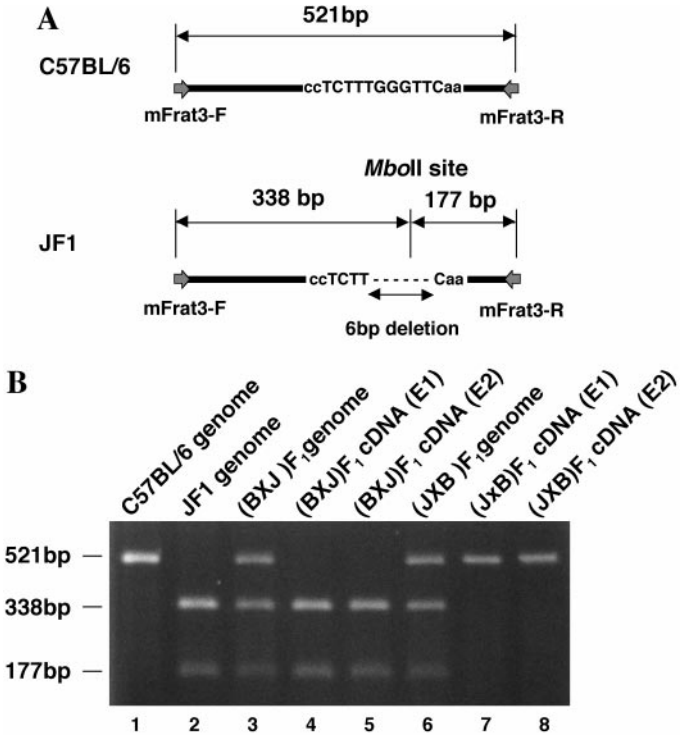

FIG. 2. (A) Schematic diagram of the *Mbo*II polymorphism in the 3′-UTR of *Frat3.* The PCR product amplified using primers Frat3-F and Frat3-R contained no *Mbo*II restriction site (TCTTC) for C57BL/6 and one site for JF1. The C57BL/6 and JF1 alleles could be distinguished by digestion with *Mbo*II. (B) Verification of *Frat3* imprinting in day 10 subinterspecific hybrid embryos by RT-PCR and RFLP analysis. PCR products with primers Frat3F and Frat3R from the C57BL/6 (lane 1), JF1 (lane 2), (C57BL/6×JF1) F_1 (lane 3), and (JF1×C57BL/6) F_1 (lane 6) genomes, and RT-PCR products from (C57BL/6×JF1) F_1 (E1, E2; lanes 4 and 5) and (JF1×C57BL/6) F_1 (E1, E2; lanes 7 and 8) day 10 embryos were digested with *Mbo*II, and separated by gel electrophoresis. Paternal expression of *Frat3* in mouse day 10 embryos was confirmed.

Frat3 RT-PCR products clearly showed that paternal alleles (JF1 allele in (B×J) F_1; B6 allele in (J×B) F_1) were exclusively expressed (Fig. 2B, lanes 4, 5, 7, and 8), and demonstrated that this gene was actually imprinted. Thus, this gene was named *Peg12/Frat3.*

Expression of Peg12/Frat3 in Various Tissues and Its Imprinting Status

Next, the pattern of *Peg12/Frat3* expression in adult tissues, neonatal brain, embryo, and placenta was examined. Due to its high homology to *Frat1* DNA sequences, RT-PCR was carried out using specific PCR primers for *Peg12/Frat3* and *Frat1* (Fig. 3A). *Peg12/Frat3* was highly expressed in the day 10 and day 13 embryos (lanes 12 and 10, respectively), and lesser expression was observed in the adult spleen, lung, neonate cerebrum, neonate cerebellum, and day 10 placenta (lanes 2, 4, 8, 9, and 11, respectively). No expression was detected in the adult liver, kidney, heart, cerebrum, or cerebellum (lanes 1, 3, 5, 6, and 7, respectively). These data suggest that *Peg12/Frat3* functions mainly in the embryonic period. Interestingly, the pattern of *Frat1* expression was very different from that of *Peg12/Frat3;* little signal was detected in the embryonic samples on days 10 and 13 (lanes 12 and 10, respectively). The highest expression was confirmed in the adult spleen (lane 2), followed by the neonate cerebrum (lane 8). Lesser expression was observed in the adult liver, lung, cerebrum, cerebellum, and neonate cerebellum (lanes 1, 4, 6, 7, and 9, respectively). There was no expression in the kidney, heart, or day 10 placenta (lanes 3, 5, and 11, respectively). Thus, unlike *Peg12/Frat3, Frat1* might function mainly in neonates and adults.

Allelic expression of *Peg12/Frat3* was further examined in the (C57BL/6×JF1) F_1 tissues where *Peg12/Frat3* expression was observed (adult spleen, lung, neonate cerebrum, neonate cerebellum, day 13 embryo, day 10 placenta, day 10 embryo) (Fig. 3B, lanes 4–10). With one exception, paternal expression was observed in all tissues examined (lanes 4–8 and 10). In the placental tissues, both the 177- and 338-bp bands from the paternal allele (JF1 pattern) were found together with the 521-bp band from the maternal allele (C57BL/6 pattern), indicating biparental expression of *Peg12/Frat3* in day 10 placenta, although paternal expression was slightly stronger than maternal expression (lane 9). The same result was obtained in the reciprocal (J×B) F_1 placental tissues (data not shown).

DISCUSSION

Eleven paternally expressed imprinted genes were detected using an oligonucleotide array (GeneChip, Affymetrix), including six *Pegs* previously isolated by subtraction-hybridization and oligonucleotide array methods, four known *Pegs* (*Sgce, Zac1, Ndn, Impact*), and the novel imprinted gene identified in this study, *Peg12/Frat3.* It is likely that the remaining candidates in this screening include some more imprinted genes. These results reconfirm that parthenogenetic and normal embryos are suitable materials for isolating *Pegs,* and that oligonucleotide arrays are a powerful tool for identifying novel imprinted genes. A similar approach with androgenetic embryos can be used to identify maternally expressed imprinted genes.

Peg12/Frat3 was reported as the second member of the mouse *Frat* family and shows 81% amino acid homology to the mouse Frat1 protein (21). The Frat proteins are thought to be a component of the Wnt signaling pathway. It has been proposed that Frat proteins activate the Wnt signal transduction pathway by stabilizing β-catenin via inhibition of GSK3 (glycogen synthase kinase-3) kinase activity (22, 23). Although the critical function of mouse *Peg12/Frat3* is still unknown, this gene likely acts as a positive regu-

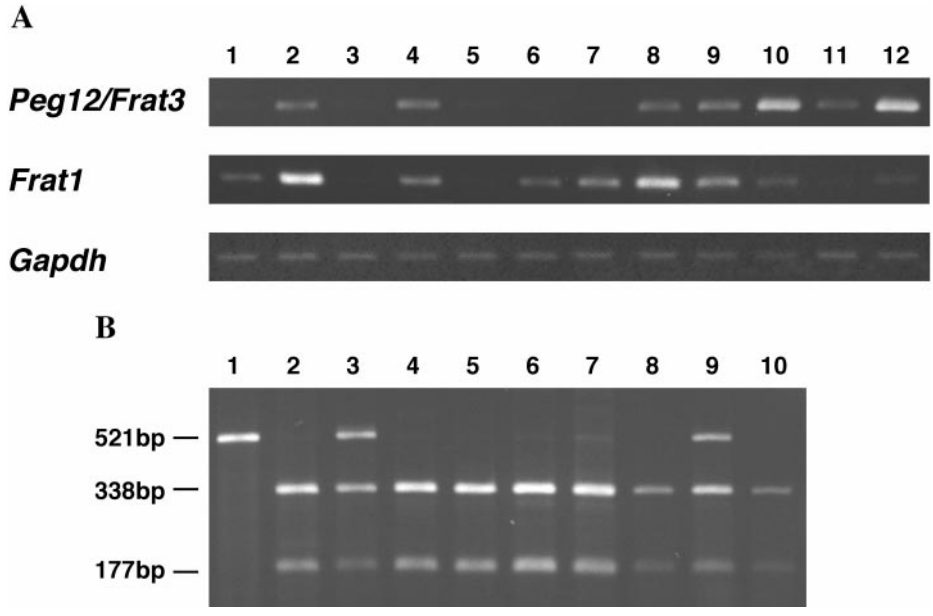

FIG. 3. (A) Expression analysis of the *Peg12/Frat3* gene in embryonic, neonatal, and adult mouse tissues. RT-PCR products of *Peg12/Frat3, Frat1,* and *Gapdh* were separated by gel electrophoresis and visualized by ethidium bromide staining. The expression was compared within the log-linear phase of the PCR for *Gapdh*. The samples are as follows: lane 1, adult liver; lane 2, spleen; lane 3, kidney; lane 4, lung; lane 5, heart; lane 6, cerebrum; lane 7, cerebellum; lane 8, cerebrum (neonate day 3); lane 9, cerebellum (neonate day 3); lane 10, day 13 whole embryo; lane 11, day 10 placenta; lane 12, day 10 whole embryo. (B) Allelic usage of *Peg12/Frat3* expression in various tissues. RT-PCR products (Peg12/Frat3 F-R) were digested with *Mbo*II. The samples are as follows: lane 1, C57/BL6 genome; lane 2, JF1 genome; lane 3, (C57/BL6×JF1) F_1 genome; lane 4, spleen cDNA; lane 5, lung cDNA; lane 6, cerebrum (neonate day 3); lane 7, cerebellum (neonate day 3); lane 8, day 13 whole embryo; lane 9, day 10 placenta; lane 10, day 10 whole embryo. Paternal expression was observed in all tissues examined, except day 10 placenta.

lator of the Wnt signaling pathway, in the same way as *Frat1*. The Wnt signaling pathway plays important roles in morphogenesis, including CNS patterning during development (24). Furthermore, mice deficient in *Dvl1*, one of the *Wnt* molecules, show abnormal social behavior and sensorimotor gating defects in spite of a lack of obvious morphological abnormalities in brain structure, suggesting that the Wnt pathway participates in complex behavioral phenomena (25).

It has been speculated that Frat3 compensates for Frat1 function, because *Frat1*-deficient mice show no abnormal phenotypes (21). However, the expression profiles of *Frat3* and *Frat1* differ in developmental stage and tissue; the former is most abundant in embryonic stages, while *Frat1* is expressed more strongly in adult tissues than in embryo, suggesting that each has its own specific function. It is possible that lack of *Peg12/Frat3* expression affects mostly phenotypes in embryonic stages. Expression of *Peg12/Frat3* was observed in neural tissues in E11.5 embryos (21), suggesting that *Peg12/Frat3* functions in the developing nervous system and that deficiency in this gene causes abnormalities in neonatal neural function. Given that the cause of the complete lethality associated with feeding difficulties and decreased movement of PWS model mice is attributed to abnormalities in the nervous system, the lack of *Peg12/Frat3* and *Ndn* expression might contribute to this phenotype. To gain more insight into the participation of *Peg12/Frat3* in the phenotype of these model mice and its critical function, it is important to analyze *Peg12/Frat3*-deficient mice and mice that are double deficient in *Frat1* and *Peg12/Frat3*. In humans, *FRAT2* was reported as a human *FRAT1* homologue located near *FRAT1* in chromosomal 10q24.1 (26, 27). However, there are no reports on mouse *Frat2*, implying that the organization of *Frat* family genes differs between human and mouse. Since humans may lack *FRAT3* in the PWS region, it is unclear whether *Peg12/Frat3* plays a role in the etiology of human PWS.

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