Expression and Imprinting Analysis of AK044800, a Transcript from the *Dlk1-Dio3* Imprinted Gene Cluster during Mouse Embryogenesis

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Recent advances of induced pluripotent stem cells (iPSCs) has demonstrated that full development potential is closely related with the expression state of noncoding RNAs (ncRNAs) of the *Dlk1-Dio3* imprinted gene cluster. However, few of them, especially the long noncoding RNAs (lncRNAs), have been characterized in detail. AK044800 is a transcript from the *Dlk1-Dio3* imprinted region with little known information. This study reports original data on the expression pattern of AK044800 during embryogenesis. Expression analysis showed that AK044800 was specifically expressed in the brain at mid-gestation, E9.5 and E11.5. And at E15.5, its expression was mainly concentrated in the forebrain. In the late-gestation stage (E18.5), AK044800 expression was weaker in the brain and began to emerge in some other tissues during this period. Notably, the expression of AK044800 was biallelic in the brain, unlike other noncoding transcripts from this imprinted region. In addition, its expression was dependent on inbred mouse strains. This may be the first lncRNA that has been identified with a different expression between inbred mouse strains. This study may provide useful clues for further investigations of expression regulation and functions of lncRNAs of the *Dlk1-Dio3* imprinted region.

INTRODUCTION

The *Dlk1-Dio3* region is an imprinted gene cluster located on the mouse distal chromosome 12 and on the human chromosome 14. The domain is approximately 1 Mb long, and contains three imprinted protein-coding genes, *Dlk1*, *Rtl1* and *Dio3*, expressed from the paternal chromosome. In contrast, genes expressed from the maternal chromosome are all imprinted noncoding RNAs (ncRNAs), including several long noncoding RNA (lncRNA) genes, *Gtl2*, *anti-Rtl1*, *Rian*, *Mirg* and numerous small nucleolar RNAs (snoRNAs) and microRNAs (da Rocha et al., 2008). Studies on these imprinted genes revealed a range of developmental processes capable of affecting growth and differentiation of some tissues, as well as modulating postnatal neurological and metabolic functions (Charalambous et al.,

2007; Wilkinson et al., 2007). In mouse, the uniparental disomy embryo causes growth retardation, hypotrophic immature skeletal muscle and even prenatal death (Tevendale et al., 2006).

Recent investigations indicated that maternally expressed noncoding transcripts originating from the imprinted *Dlk1-Dio3* gene cluster were closely related to full development potential of pluripotent stem cells (iPSCs) (Liu et al., 2010; Stadtfeld et al., 2010). Most iPSC lines showed low expression of maternal transcripts (for example, *Gtl2*, *Rian* and some microRNAs) and failed to generate all-iPSC mice. In contrast, iPSC clones with normal expression of the maternal *Dlk1-Dio3* cluster contributed to yielding viable all-iPSC mice. These findings suggest the potential functional roles of the noncoding maternal transcripts from the *Dlk1-Dio3* cluster on the establishment of full pluripotency in iPSCs. Multiple novel transcripts have been identified from this region (Hagan et al., 2009). However, our knowledge of these lncRNAs is still limited.

AK044800 is an unknown transcript whose locus is near the bottom of the *Dlk1-Dio3* imprinted region. In order to gain more information about this transcript during embryo development, we carried out expression pattern analysis. Results showed AK044800 was specifically expressed in embryo brain, indicating a potential role during mouse brain development. Unexpectedly, imprinting analysis revealed that AK044800 expression was biallelic and relied on mouse strains. This study allowed us to characterize the expression pattern of AK044800 during embryogenesis and gain a better understanding of the lncRNA expression differences between inbred mouse strains.

MATERIALS AND METHODS

Animals

The mouse strains C57BL/6J (B6), ICR and DBA/2J (DBA) were purchased from Slac Laboratory Animal Company (China). For timed mating, the day of the vaginal plug was E0.5. Conceptuses from crosses between B6, ICR and DBA heterozygotes were obtained at E15.5 for imprinting analysis. B6 and ICR mice were mated, and embryos were acquired at different stages for expression analysis. For additional experiments, certain tissues such as the developing brain, tongue, heart,

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Fig. 1. Schematic representation of AK044800 in the *Dlk1-Dio3* domain on mouse chromosome 12. (A) AK044800 is transcribed from the bottom of the *Dlk1-Dio3* imprinted gene cluster near *Dio3*. (B) Sequence analysis of AK044800 by ORF Finder in both sense and anti-sense showed it lack long (>100 amino acids) ORFs. Maternally expressed genes are in red and paternally expressed genes are in blue.

lung, liver and kidney, were dissected. All animal experiments carried out in this study were approved by the Rules for Animal Experiments 2011 published by the Chinese Government (China).

Nucleic acid purification, RT-PCR and quantitative real-time PCR (qRT-PCR)

Genomic DNA from the embryo brain was isolated according to the standard phenol-chloroform method. Total RNA was purified using Trizol (Invitrogen) according to the manufacturer's instructions and also DNase-treated (TaKaRa) for at least 60 min to remove contaminating gDNA. Approximately 2 µg of RNA was reverse-transcribed with M-MLV reverse transcriptase (Promega), using random primers according to the reverse transcript protocol. Quantitative real-time PCR was performed with the SYBR Green PCR master mix kit following the manufacturer's instructions (Applied Biosystems) using 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 45 s. The primer sequences are given in the Supplementary Table. The β -actin was performed using 27 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, and this was used as a loading control.

Digoxigenin (DIG)-labeled RNA probe preparation

The mouse cDNA PCR fragment for AK044800 was cloned into the pBluescript II KS (+) phagemids T-vector by using the DNA Ligation Kit (Taraka) according to the manufacturer's protocol. Primer reactions and sequences are given in the Supplementary Table. Approximately 25 µg of plasmid DNA was linearized with the appropriate restriction enzymes and purified by phenol-chloroform extraction. Approximately 1 µg was transcribed by T7 RNA polymerase (Roche) in the presence of DIG-labeled UTP (Roche) to generate sense and antisense DIG-labeled probes. The *in vitro* transcription was carried out at 37°C for 2 h,

incubated with DNase for 15 min to remove the plasmid DNA and purified by precipitation with 4 M LiCl.

In situ hybridization

The embryos for the whole-mount in situ hybridization were fixed in 4% paraformaldehyde and incubated at 4°C overnight. The next day, they were washed in PBS with 0.1% Tween 20 and dehydrated through a graded series of ethanol. To prepare the sections for hybridization, the embryos were also fixed in 4% paraformaldehyde and then embedded in paraffin wax using standard protocols. They were sectioned at 10 µm and adhered on to slides. The general in situ hybridization procedure for tissue sections was carried out as described previously (Olivier and Walter, 1998).

RESULTS

Genomic structure and characters of AK044800

AK044800 was originally reported as a cDNA isolated from the 9.5 day old parthenogenetic mouse embryo, with its size being 2694 bp without introns (Fig. 1A). It is located on the distal region of the imprinted *Dlk1-Dio3* cluster upstream to *Dio3*. In order to identify whether AK044800 has coding potential, we analyzed its sequence to find the open reading frame (ORF) using ORF Finder (<http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi>). Analysis results showed the lack of long (>100 amino acids) ORFs in both sense and antisense sequences of AK044800 (Fig. 1B). Since there remained the possibility of encoding short peptides, further comparison analysis was performed by BLASTp search of the NCBI, which revealed that each presumptive peptide of 12 potential ORFs were not homologous with any known proteins, suggesting it might act as a noncoding RNA.

Fig. 2. Expression analysis of AK044800 during embryo development by *in situ* hybridization. *In situ* hybridization with AK044800 RNA probe was performed at E9.5 (A), E11.5 (B) and E15.5 (C). Significant signals were found in the brain at all of these stages, and signals were mainly concentrated on the forebrain at E15.5. fb, forebrain; hb, hindbrain; mb, midbrain; to, tongue. Scale bars represent 1.0 mm.

Fig. 3. Expression of AK044800 in organs at different embryonic stages. Relative expression levels of AK044800 in different tissues were detected during embryogenesis (A) and postnatal development (B) by semi-quantitative RT-PCR. RT-PCR products of AK044800 (33 cycles) were compared to that of β -actin derived from the same tissues (27 cycles).

The expression of AK044800 during mouse embryogenesis

To ascertain spatiotemporal patterns of AK044800 during mouse embryo development, we assayed its expression by *in situ* hybridization. At E9.5 and E11.5, strong signals were observed only in the brain including the forebrain, midbrain and hindbrain (Figs. 2A and 2B). At E15.5 during embryo development, prominent expression was still found in the brain, but signals were mainly concentrated in the forebrain (Fig. 2C). Weak signals were also detected in other organs such as the tongue.

Subsequently, RT-PCR analysis was performed in diverse tissues at E12.5, E15.5 and E18.5. The results showed high expression of AK044800 in the brain and almost no expression in other tissues at mid-gestation (E12.5 and E15.5), which confirmed the previous data (Fig. 3A). However, at late gestation

(E18.5), the expression of AK044800 in the brain was decreased and emerged in some other organs including the tongue, heart, lung, liver and kidney (Fig. 3A). At postnatal day 1 (P1), the expression was weaker in the brain and scarcely detected at postnatal day 10 (P10) (Fig. 3B). Since the dramatic expression of AK044800 in the developing brain, we examined the embryonic brain from E9.5 to E19.5 by qRT-PCR. The results showed a wave expression pattern with high expression during the mid-stage brain development after implantation (Fig. 4).

AK044800 is the strain-dependent biallelic expression in mouse embryo brain

As AK044800 is located in an imprinted cluster, the following study was carried out to determine whether its expression is allele-specific. A polymorphism-based approach and a sequencing-based method were used to analyze the imprinting status

Fig. 4. Expression of AK044800 during the development of embryo brain. Total RNA was obtained from the brain at each embryo stages (E9.5 to E19.5). qRT-PCR was performed and the expression of AK044800 was normalized by β -actin. Data were obtained using at least three embryos for each stage from two different litters and were represented as mean $\pm$ s.d.

of AK044800 in the brain of E15.5. B6 females were mated with DBA males to generate BDF1 mice and reciprocally crossed to generate DBF1 mice. A T/C single-nucleotide polymorphism (SNP) of the AK044800 locus was identified by separately sequencing the B6 and DBA transcripts from the original parents,

which was used to distinguish transcription between maternal and paternal alleles in BDF1 and DBF1. Unexpectedly, the sequencing results showed AK044800 expression was all derived from the B6 original allele (Fig. 5A). Another SNP (A/C) chosen to perform the above experiment achieved the same result (Fig. 5B).

To further confirm the results, the same strategy was carried out on DBA and ICR mouse. In DBA but not in ICR mouse, there is a deletion mutation of base "A" in the AK044800 locus. Sequencing results from DIF1 or IDF1 showed that the AK044800 transcript was only derived from the ICR original allele if it was the direct or reciprocal cross, which is comparable to the previous data (Fig. 5C). These results strongly indicated that AK044800 is not expressed in the embryo brain of DBA mouse, but is expressed in B6 and ICR mice. This presumption was validated by RT-PCR analysis of the AK044800 expression in the brain of the three strains (Fig. 5D). For imprinting analysis, B6 females were mated with ICR males to generate BIF1. The transcripts can be distinguished according to the base difference of 'TG' in B6 and 'A' in ICR. The sequencing results showed that AK044800 expression is not imprinted, but were biallelic in the brain of E15.5.

DISCUSSION

In recent years since the discovery of the *Dlk1-Dio3* imprinted

Fig. 5. Allele-specific expression analysis of AK044800 in the brain at E15.5. The region containing the polymorphism was amplified by RT-PCR. The allelic expression of AK044800 was determined by sequencing PCR products. The black vertical arrows point to SNP sites. (A) Sequencing chromatograms of genome DNA obtained from E15.5 brain of B6, DBA and their F1 generation (BDF1), and cDNA from the same tissues of F1 generation (BDF1 and DBF1) between B6 and DBA. (B) Another SNP site between B6 and DBA was detected by sequencing with the same strategy. (C) Imprinting analysis by using a deletion mutation between DBA and ICR. (D) Expression analysis of AK044800 in E15.5 brain by RT-PCR. (E) Imprinting analysis by using a sequence mutation between B6 and ICR.

cluster, the organization of the imprinted domain has been characterized in more detail (da Rocha et al., 2008; Hagan et al., 2009; Lin et al., 2007; Tierling et al., 2006). Multiple unidentified transcripts within this region have been found, including AK044800. In this study, we revealed the specific spatiotemporal expression patterns of AK044800 in the mouse brain during embryogenesis. The *in situ* hybridization results revealed that AK044800 was universally expressed in the embryo brain at an early stage (E9.5 and E11.5), while at E15.5, it was mainly restricted in the forebrain. In the same imprinted cluster, lncRNA Gtl2 (McLaughlin et al., 2006; Schuster-Gossler et al., 1998), Rian (Gu et al., 2011; 2012) and Mirg (Han et al., 2012) were also highly expressed in the developing forebrain, suggesting roles of lncRNAs from the *Dlk1-Dio3* imprinted cluster in regional neuronal development and differentiation.

Besides the lncRNAs from the *Dlk1-Dio3* region, many transcripts from other lncRNA loci also exhibit developmentally regulated expression patterns in the brain. Using a computational analysis of *in situ* hybridization data, the Allen Brain Atlas identified 849 lncRNAs out of 1,328 examined, which showed specific expression patterns in adult mouse brain (Mercer et al., 2008). In addition, a large number of lncRNAs have been observed to undergo a dramatic change during neural differentiation in the developing brain and to be expressed at specific stages of cellular differentiation (Mercer et al., 2010). Such regulated expression patterns can perhaps be attributed to lncRNA loci tending to cluster near brain-expressed protein-coding genes and to the transcription factor-encoding genes associated with development (Guttman et al., 2009; Ponjavic et al., 2009). RNA structure may be widely involved in transcriptional and translational regulatory mechanisms in central nervous system development (Qureshi et al., 2010), and may ultimately play a role in brain function (Qureshi and Mehler, 2012).

Since AK044800 shows the features of noncoding RNA, we initially speculated it was an imprinted transcript, as with the other noncoding transcripts within the *Dlk1-Dio3* cluster. However, the imprinted analysis revealed its biallelic expression in embryo brain. This may be reasonable because AK044800 is in the vicinity of *Dio3*, the bottom gene of this imprinted cluster, which has been demonstrated by previous studies to be an incomplete imprinting transcript (Tsai et al., 2002; Yevtodiyeenko et al., 2002). Furthermore, the antisense transcript of *Dio3* known as *Dio3-as*, is also biallelically expressed without a preference for alleles (Hagan et al. 2009). It seems that the control of imprinted expression becomes quite weak at the bottom region of the *Dlk1-Dio3* imprinted cluster. However, the exact molecular mechanism is still unknown.

It is noteworthy that AK044800 expression in the brain depends on the particular mouse strains. In this study, we only detected AK044800 expression in the B6 and ICR mouse and not in the DBA. As far as we know, this may be the first identification of the differences in lncRNA expression across inbred mouse strains. B6, ICR and DBA are three of the most commonly inbred mouse strains containing an abundance of different genotypes and phenotypes. For example, many neuroscientific studies have proved that B6 has greater sensitivity to drug reward (such as alcohol and narcotics) than DBA due to the expression differences of a subset of genes in the brain (Giardino et al., 2012; Gieryk et al., 2010; Jamensky and Gianoulakis, 1997; 1999; Peirce et al., 1998). Recent research has also revealed approximately 5-7% microRNAs expression differences in the hippocampus (Parsons et al., 2008), and a further study indicated the differences of drug addiction between B6 and DBA were also associated with microRNAs (Tapocik et

al., 2012). However, little is known about the lncRNA expression differences across mouse inbred strains.

In conclusion, the results of this study help clarify the nature of AK044800 expressed during embryogenesis. Firstly, *in situ* hybridization results showed it was specifically expressed in the embryo brain. Secondly, the analysis of imprinted status demonstrated its expression was biallelic. And finally, its expression depended on inbred mouse brains. These findings may provide further insights into the roles of lncRNAs in embryo brain development and the genetic heterogeneity between inbred mouse strains.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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