An Evolutionary Scenario for Genomic Imprinting of Impact Lying between Nonimprinted Neighbors

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Abstract

Mouse Impact is the sole imprinted gene mapped to chromosome 18 to date. Despite its remarkable evolutionary conservation, human IMPACT was shown to escape genomic imprinting. Here we identified Hrh4 and Osbpl1 as the distal and proximal nearest neighbors of Impact, respectively, and found that both genes are expressed biallelically. Thus, in contrast with most imprinted genes, Impact fails to show apparent physical clustering with other imprinted genes. Since Impact not only lies in an intergenic region but also consists of 11 exons, it does not seem to be an imprinted gene generated by a retrotransposition. Hazardous effects of overexpressed Impact, a genomic segment containing paralogues of Hrh4 and Osbpl1 but not of Impact, and enhanced promoter activity in the mouse led us to propose an alternative model. This model assumes that segmental duplication followed by enhancement of the promoter activity in the lineage to mouse is responsible for the species-specific imprinting of Impact.

Key words: Impact; genomic imprinting; species-specific imprinting; segmental duplication; dosage compensation

1. Introduction

Genomic imprinting is an epigenetic modification leading to exclusive or highly skewed expression of a specific parental allele. The imprint must be erased and established during gametogenesis, and the memory, i.e. the parental origin, is maintained throughout the life of the offspring. Although over 50 mammalian imprinted genes have been discovered since 1991, why and how they are imprinted remains largely unexplained.

We previously developed a systematic screening for imprinted genes using mRNA display PCR to identify a paternally expressed gene Impact, which is preferentially expressed in brain to encode a protein of unknown function but of remarkable evolutionary conservation. It is the first and the sole imprinted gene mapped to mouse chromosome 18, which had been suggested to bear at least one imprinted gene by a genetic study using mice with a Robertsonian translocation chromosome. Subsequently, we and others isolated the cDNA for its human orthologue IMPACT and mapped it to human chromosome 18q11.2, a region syntenic to the mouse Impact locus. Intriguingly, we revealed that human IMPACT is expressed from both alleles,4 it is of particular interest to identify its neighbors and examine their allelic expression status.

In the present study, we analyzed allelic expression and
methylation of the genes identified as the nearest neighbors of Impact as well as its human counterpart. Based on these observations, we propose a model for the evolution of imprinting of Impact.

2. Materials and Methods

2.1. Screening of mouse and human BAC libraries

BAC clones for mouse Impact (200P19 and 365M4) and human IMPACT (457A4 and 558E15) were identified by PCR screening as described previously. The BAC clones 244P4 and 531H5 were obtained by additional rounds of screening using sequence-tagged sites (STTs) developed from the sequence of 365M4. The primers for a 230-bp STS, leading to the identification of 244P4, were 5′-CAG ATG ACT AAC CCT GTT CA-3′ and 5′-CCT AGG GTA TAA GCA ACT AC-3′. The primers for a 319-bp STS, leading to the identification of 531H5 were 5′-GCT TGT TAA CAT GTC AAC TTT C-3′ and 5′-GCA GTT GAG ACA TTG CAT TAG T-3′.

2.2. Annotations of the genes

Annotations for mouse, rat, and human genes were obtained from NCBI Map Viewer Web sites (http://www.ncbi.nlm.nih.gov/mapview/). Fugu Genome Browser on Ensembl Web sites (http://www.ensembl.org/Fugu) was used to identify pufferfish Impact and Hrh4 through tblastn search with the amino acid sequences of human IMPACT and HRH4 as queries.

2.3. Northern blot hybridization

For Northern hybridization, we used filters containing poly(A)+ RNAs isolated from multiple tissues (Clontech). Probes were purified by agarose gel electrophoresis and labeled with [α-32P]dCTP (Amersham) using RadPrime DNA Labeling System (Invitrogen). The filters hybridized with the probe were appropriately washed and exposed to Imaging-Plates (Fuji Film) for analysis by Fuji BioImaging Analyzer BAS2000 system. The 793-bp probe for mouse Osbpl1 was amplified from mouse brain cDNA with 5′-TGC AGA AGG GCT CAA CAA TG-3′ (forward) and 5′-AGT CCT CTT CCG ACT TGG AC-3′ (reverse). The 762-bp Osbpl1b-specific probe was amplified from mouse brain cDNA with 5′-GGT CGC TGA CAT CGA CTG TA-3′ (forward) and 5′-GCT TGA GTC AGG TGT TTG CAT TAG-3′ (reverse).

2.4. DNA methylation analysis by Hpa II-McrBC PCR

The Hpa II-McrBC PCR assay was performed as described. Human peripheral blood samples used in this study were collected after informed consent of 14 Japanese individuals in accordance with ethical guidelines. Native genomic DNAs were digested with Hpa II or McrBC (New England Biolabs) for overnight, treated with phenol/chloroform, and precipitated by ethanol. Subsequently, the digests were used as templates for PCR. The PCR primers for the Osbpl1a CpG island are 5′-GCT ACA GCC AGG ATC CCT TA-3′ (forward) and 5′-CCT GGG CTG GGT CCT GAA GA-3′ (reverse). Those for the Osbpl1b CpG island are 5′-TGC TGC CGC CCC TCT TTC AC-3′ (forward) and 5′-ACC CAC GCG GGC CGC CTG TCT CA-3′ (reverse), which were also used for direct sequencing. Those for the HRH4 promoter were 5′-GCC TAG GAA TGT AAA GAC GAG-3′ (forward) and 5′-CCA GCC AGA CAA TTC TGA CA-3′ (reverse). Those for the OSBPL1A CpG island were 5′-CAG GCT GCG CAA AGG TGA CT-3′ (forward) and 5′-GCC GCC TCT GAA GAG CGG AT-3′ (reverse). Those for the OSBPL1B CpG island were 5′-GGG AGT GCC AGC CAG AGT T-3′ (forward) and 5′-GGC ACG CAG CTG AAG ATC TG-3′ (reverse).

2.5. Bisulfite treatment-based methylation analysis

Native genomic DNAs were denatured with 0.3 M sodium hydroxide at 37°C for 15 min. The bisulfite (Sigma) solution, which was adjusted to pH 5 with sodium hydroxide, and freshly prepared hydroquinone (Sigma) were added to the denatured DNAs to the final concentrations of 3.2 M and 0.5 mM, respectively. The reaction mixes were overlaid with 100 µl of mineral oil, and incubated at 55°C overnight. The treated DNAs were purified by Wizard DNA Clean-Up System (Promega) followed by desulfonation in 0.3 M sodium hydroxide for 15 min. Finally, the DNAs were precipitated by ethanol and used as templates for PCR. Primers used for the upstream region of Hrh4 were 5′-ATT TAG TGA TGG TTG GGG TTA GTT AAA T-3′ (forward) and 5′-AAA TTC TAC CAA CCA CAT ACT AAA CCT A-3′ (reverse). Those for the promoter region of Hrh4 were 5′-CCT ACA TCA CCT CTA AAC TTC TCT AAA A-3′ (forward) and 5′-TTA AAA AT TTT TTT TGA TTA TAA GGA GAA ATT-3′ (reverse). Those for the Osbpl1b CpG island were 5′-TCA CCC CCA CCT CCC CCA CCC AAA TCT-3′ (forward) and 5′-GGG TTA GGA GTA AGG AGG GTT TYG GGA A-3′ (reverse). The latter was also used for direct sequencing of the PCR products. The PCR was performed with Platinum Taq DNA Polymerase (Invitrogen) which was complexed with antibody in order to inhibit polymerase activity until heat denaturation.

2.6. RT-PCR analysis

Total RNAs were prepared by homogenizing frozen tissues in TRIZOL Reagent (Invitrogen) followed by subsequent steps according to the supplier’s recommendations. We used QIAGen RNA Blood Mini Kits (Qiagen) for the extraction from whole blood. Following DNaseI treatment, reverse transcriptase reactions were performed using oligo-dT primer. The PCR for Hrh4 was performed
using primers 5′-AAT ATT GTC CTC ATT AGC TAC GAT CG-3′ (forward) and 5′-TGG TTG CTT TGT CAC ACA AAG TAT CT-3′ (reverse), and the products were directly sequenced with 5′-TGG CTT TGC ACA ATT GTC CTT TCA AC-3′. Those for Osbp1a were the same as the one used for Northern blot hybridization. The products were sequenced with the forward primer. Those for Osbp1b were 5′-GTA GTC GAG CAT GTG TTG AA-3′ (forward) and 5′-CAG TTC ACA TCA GGA GGA GGA TT-3′ (reverse), and were sequenced with the forward primer. The products were digested with Alu I overnight. The corresponding genomic region was amplified with 5′-CTG GGC AAC TAA CAG TGT TA-3′ (forward) and 5′-AAG CTG AGC ATG TTC AAT CA-3′ (reverse), and sequenced with 5′-GTG TGT GCC AAA GGC TT-3′. Those for Hrh4 were 5′-TAC CTG TCA TCA CCT GCT-3′ (forward) and 5′-GGG CAG ACC TGA TCA TTC AGG-3′ (reverse), and sequenced with 5′-ACT TCA AAC CAT ACT GGG GTC TT-3′ and 5′-CTT GGT TCT TGA GGA AAA CA-3′. Those for OSBPL1 were 5′-GAA GAG GAC TGG AAG ACG AG-3′ (forward) and 5′-TGA TAC TTA CAT GAG TGC AAC-3′ (reverse), and sequenced with 5′-CAG TTT TCT GCA GTC AGT ATC-3′.

2.7. Quantitative RT-PCR of mouse Impact and human IMPACT

The RT-PCR was performed using oligo-dT primer and IMPACT primers 5′-GAT GAC TGT GCC AAA ATA TTT TGT ATT AG-3′ (forward), 5′-TCT CTT ATT TTT TCC ACC CAC-3′ (reverse), or commercially available Gapd primers (Perkin Elmer) with the following thermal cycling parameters: 94°C for 60 sec followed by 20/25/30/50 cycles at 94°C for 20 sec, 58°C for 40 sec, 72°C for 10 sec, and a final extension at 72°C for 180 sec. We used 50 ng of total RNA isolated from B6 brain or 0.5-ng of human brain poly(A)+ RNA (Clontech) for the assay. Control PCR was performed using a 1-pg plasmid that has a 366-bp (B6) or 381-bp (human) cDNA fragment as the template.

3. Results and Discussion

3.1. Identification of the genes lying next to Impact/IMPACT

To obtain genomic clones containing the Impact/IMPACT locus, we screened mouse and human bacterial artificial chromosome (BAC) libraries by PCR. By subcloning and partial sequencing, we constructed BAC contigs covering the loci for mouse and human (Fig. 1). Combining these and public data, we identified the histamine H2 receptor gene (Hrh4/HRH4) and oxysterol binding protein-like 1 gene (Osbp1/OSBPL1) as the distal and proximal nearest neighbors of Impact/IMPACT, respectively.

3.2. Allelic expression and methylation analyses of mouse Hrh4 distal to Impact

To assess whether mouse Hrh4 is imprinted, we performed an RT-PCR assay using F1 hybrid mice generated by reciprocal crosses between Mus musculus molossinus JF1/Msf (JF) and M. musculus domesticus C57BL/6J (B6). A G/A single nucleotide polymorphism (SNP) can be used to distinguish the two parental alleles. Because this gene is preferentially expressed in immune tissues, we extracted total RNAs from the spleens of four different mice, JF, B6, and their reciprocal F1 hybrid mice, namely (JF×B6)F1 and (B6×JF)F1. All of the PCR primers for RNA analyses in this study were designed so that each amplicon spans an exon-intron boundary for the prevention of amplification from contaminated genomic DNAs. Direct sequencing of the RT-PCR products showed that mouse Hrh4 is expressed biallelically (Fig. 2A). We detected the expression of Hrh4 mRNA in the brain of some, but not all, of the hybrid mice. Since Impact is highly expressed in brain, we used these mice to examine the allelic expression status of Hrh4 in the brain and found that it is expressed equally from both alleles (data not shown). Thus, although Impact and Hrh4 are physically close (~5 kb apart), their allelic expression status is distinct.

Next we examined the allelic methylation status of Hrh4. In contrast to Impact, the Hrh4 gene lacks CpG islands. We cloned and sequenced PCR products from bisulfite-treated DNA (Fig. 2B). While we found that ~1.4-kb upstream region is hypermethylated in both spleen and brain, the proximal promoter region was shown to escape methylation in spleen but not in brain. We failed to find any clone of methylated allele from spleen, and found no clone of unmethylated allele from brain. We thus concluded that both alleles of the promoter region of Hrh4 escape methylation in spleen but are methylated in brain.

3.3. Allelic expression and methylation analyses of mouse Osbp1 proximal to Impact

The human OSBP genes were surveyed extensively, and OSBP1 was shown to produce two very different transcripts, the shorter one named OSBP1A and the longer one called OSBP1B. Although mouse Osbp genes were recently identified, Osbp1b, whose promoter should be close to Impact, has not been reported. To know whether Osbp1b identified proximal to Impact is indeed transcribed, we performed Northern blot hybridization using a probe derived from the open reading frame (ORF) of mouse Osbpl1a. It detected a 3-kb mRNA in liver, heart, brain and kidney, whereas testis contains an additional shorter transcript (Fig. 3A). Longer exposure of the same blot revealed another band of 4-kb long, which may be the transcript of Osbpl1b (Fig. 3A).

On the other hand, we noticed that a model refer-
Figure 1. Overview of the mouse Impact (A) and human IMPACT (B) loci. Physical distance is indicated by a short bar (50 kb) in the middle. Each gene is shown as a solid box. Arrows above and below the boxes denote the directions of transcription for maternal and paternal expression, respectively. Summary of the DNA methylation analysis is also shown by hashed circles (hypermethylation) and white circles (hypomethylation). As for the promoter region of Hrh4, the methylation status in spleen is shown because this gene is preferentially expressed in this tissue. Note that the region is hypermethylated in brain where the gene is inactive. The locations of the BAC clones are depicted in shaded bars. Their names are written on the left and their approximate sizes in parentheses. The Osbpl2-Hrh3 regions are indicated below the BAC contigs for both mouse and human.

Figure 2. Allelic expression and DNA methylation of mouse Hrh4. (A) Direct sequencing was performed using RT-PCR products amplified from mRNAs of parents (upper) and F1 hybrids (lower). The positions of the polymorphism between JF and B6 are indicated by small arrow (position 1020 of the sequence GenBank accession no. AF358859). (B) Bisulfite genomic sequencing was performed for the upstream region (−1.5 kb to −1.3 kb) and the promoter region (−0.5 kb to −0.3 kb) of the gene using genomic DNAs extracted from brain and spleen. Each row of circles corresponds to each clone of bisulfite PCR products. Open and closed circles stand for unmethylated and methylated C residues in CpGs, respectively. No sign was observed for allele-specific methylation.
Figure 3. Northern blot hybridization and allelic expression analyses of mouse Osbpl1. (A) The radio-labeled probe from a region common to Osbpl1a and Osbpl1b was hybridized to poly(A)+ RNAs isolated from various tissues. The middle panel, which detected a longer transcript, is the result of a 16-hr exposure of the same blot as the upper one, which was exposed for 3 hr. The probe derived from a region specific to Osbpl1b, shown in the lower panel, detected only the longer transcript. (B) Allelic expression analysis of Osbpl1a was performed by direct sequencing of the RT-PCR products. Arrows indicate the T/C SNP found at position 771 of the sequence deposited with GenBank accession no. BC057194. (C) Alu I sites in the 326-bp fragment amplified from the Osbpl1b mRNA are shown above the gel. JF allele has an additional Alu I site at position 352 of the sequence with GenBank accession no. AY536214, that gives a 76-bp fragment instead of a 99-bp one in B6. PCR products amplified from brain cDNAs of JF, B6, (JF × B6)F1, and (B6 × JF)F1 (from left to right, numbered 1 to 4, respectively) were digested with Alu I and subjected to polyacrylamide gel electrophoresis. Note that small fragments were not resolved on the gel used. (D) Allelic expression was also examined by direct sequencing. The results of (JF × B6)F1 and (B6 × JF)F1 are shown under genomic DNA sequencing chromatograms of JF, B6, (JF × B6)F1, and (B6 × JF)F1. These suggest that mouse Osbpl1b is preferentially expressed from the paternal allele. (E) The preference of the paternal allele was estimated by direct sequencing. JF and B6 mRNAs were mixed at gradual ratios illustrated in this figure. Subsequently, RT-PCR and direct sequencing were performed. This result demonstrates that sequencing chromatograms are reliably used for quantification of mRNA. As a result of sequencing more than 30 samples, we estimated that the quantity of the expressed maternal allele is two-thirds of that of the paternal one.
ence sequence (GenBank accession no. XM_140455) was mapped to the promoter region of mouse Impact by NCBI RefSeq Project. We designed a forward primer in the model sequence and a reverse primer in Osbpl1a, and performed RT-PCR using mouse brain RNA as template. Consequently, we identified a long transcript that can encode a hypothetical protein consisting of 950 amino acids. The deduced primary sequence (GenBank accession no. AY536214) is 92% identical and 98% similar to human OSBPL1B. Northern blot hybridization using a probe derived from a region unique to this transcript did detect the 4-kb, but not the 3-kb, mRNA (Fig. 3A). These results indicate that the 4-kb band is indeed the mouse Osbpl1b mRNA, which is expressed in various tissues including spleen and lung where the shorter Osbpl1a mRNA is barely detected (Fig. 3A).

While mouse Osbpl1a and Osbpl1b transcripts are expressed in various tissues (Fig. 3A), we used brain RNAs to analyze allelic expression, because both forms of Osbpl1 as well as Impact are abundantly expressed in brain. First, we designed primers around the 3′ untranslated region (UTR) that is involved in both the Osbpl1a and Osbpl1b mRNAs. However, since the quantity of the Osbpl1a transcript is ~20 times larger than that of Osbpl1b in brain (Fig. 3A), the latter is negligible in this assay. The 793-bp RT-PCR product obtained with these primers contains a T/C SNP that allows us to discriminate the two parental alleles. Direct sequencing of the RT-PCR products from the F1 hybrid mice revealed the presence of both T and C alleles in the transcripts (Fig. 3B). These results indicate that Osbpl1a is expressed biallelically (Fig. 3B).

Next we designed Osbpl1b-specific primers, one in exon 3 and the other in exon 7, that give a 326-bp RT-PCR product. This fragment has a T/C SNP that leads to a restriction fragment length polymorphisms (RFLP) when digested with Alu I (Fig. 3C). The results of the RT-PCR RFLP assay showed that Osbpl1b was also expressed from both chromosomes. Intriguingly, we noticed that the expression of the paternal allele was slightly stronger than that of the maternal one. This inclination was more clearly shown by direct sequencing of the RT-PCR products of F1 hybrid mice generated by reciprocal cross between B6 and JF (Fig. 3D). Similar results were obtained with F1 mice between JF and ICR (data not shown). To evaluate the skewed expression quantitatively, we mixed the JF and B6 brain RNAs at various ratios and subjected the mixed RNAs to RT-PCR followed by direct sequencing. Comparing these results with those from reciprocal F1 hybrid mice, we estimated that the amount of the expressed maternal allele is approximately two thirds of the paternal one (Fig. 3E).

We also examined methylation status of CpG islands at the promoters of Osbpl1a and Osbpl1b by Hpa II-McrBC PCR assay. In this assay, genomic DNA digested with Hpa II or McrBC was used as template for PCR. While the cleavage of CCGG site by Hpa II is blocked by methylation, McrBC selectively cuts methylated sequence (RmCN40–80RmC).11 Hence, PCR products amplified from Hpa II- and McrBC-digested DNA are derived from hypermethylated and hypomethylated alleles, respectively. As shown in Fig. 4A, both B6 and JF alleles...
of the CpG island at the *Osbpl1b* promoter were amplified from the McrBC-treated DNA but not from the *HpaII*-digested DNA, strongly suggesting that both alleles of this island escape methylation. This notion was further reinforced by the results of bisulfite sequencing, in which all of the CpGs were converted to TpGs (Fig. 4B), indicating that the CpGs were modified to UpGs upon bisulfite treatment as they had escaped methylation. Hence, we cannot attribute the skewed expression of *Osbpl1b* to differential DNA methylation. Since the promoter of *Osbpl1b*, which shows skewed expression, is much closer to *Impact* than that of *Osbpl1a*, which supports even biallelic expression, it is conceivable that an allele-specific difference in the chromatin structure of *Impact* somehow affects the function of the former. We also obtained results indicating that neither allele of *Osbpl1a* promoter is methylated (data not shown). We thus conclude that both alleles of the two CpG islands of *Osbpl1* gene escape methylation.

### 3.4. Imprinting analysis of the nearest neighbors of *human IMPACT*

While we previously reported biallelic expression of human *IMPACT*, it would be of importance to assess the imprinting status of adjacent genes. To examine the imprinting status of human *HRH4*, we searched polymorphisms in the transcribed region. Fortunately, we found a G/A SNP and an informative Japanese family. This SNP alters an amino acid residue from histidine to arginine. We performed RT-PCR using RNA isolated from peripheral blood cells as the template. The products were directly sequenced (Fig. 5A). The result shows that the cDNA contained both maternal and paternal alleles, although expression of the latter may be slightly higher than the former. Because there is no CpG island in the *HRH4* locus, we targeted the promoter region for DNA methylation analysis. The result of *HpaII*-McrBC PCR assay suggests that both chromosomes are methylated (data not shown). We thus assume it as a non-imprinted gene, although we cannot completely exclude a possibility of loosely imprinted expression of *HRH4* in other tissues and other individuals.

As for human *OSBPL1*, we found two Japanese families that are informative on a C/G SNP in the 3′ UTR which is transcribed not only as *OSBPL1A* but also as *OSBPL1B*. We extracted total RNAs from their blood cells and performed allelic expression analysis. The results showed that they are expressed from both alleles, although we could not distinguish the long and short transcripts (Fig. 5B). The locations of the two CpG islands of *OSBPL1* are conserved between mouse and human. We performed DNA methylation analysis for the two islands by *HpaII*-McrBC PCR assay. It demonstrated that both islands escape methylation biallelically: they are usual unmethylated CpG islands (data not shown). We thus concluded that both *OSBPL1A* and *OSBPL1B* are not imprinted.
3.5. Evolution of imprinting of Impact

As described above, it is unlikely that the locus around Impact/IMPACT forms a clear imprinted cluster. Hence we may assume Impact as a putative solitary imprinted gene. Some genes have been reported as solitary imprinted genes, e.g. U2af1-rs1, Nuat, Nap15, and Peg13. These are located within singular introns of other nonimprinted genes, Murr1, Blec, Herc3, and KIAA1882, respectively. Since these imprinted genes tend to have fewer and smaller introns, retrotransposition has been considered a common mechanism for the formation of such solitary ones. It is intriguing to note that all of these genes presumably generated by retrotransposition are paternally expressed like Impact. However, these genes and Impact display remarkable differences in their genomic organization. First, Impact has at least 10 introns and is much longer than other solitary imprinted genes. Second, Impact has no homologous genes in the genome except several processed pseudogenes in human. Third, Impact does not reside within other genes but lies between two distinct genes, Hrh4 and Osbpl1. These observations indicate that Impact is a unique solitary imprinted gene that was not generated by a retrotranspositional event.

When considering non-retrotranspositional origin of Impact, a genomic segment attracted our attention. The segment resides on human chromosome 20q13.3 and contains both HRH3, displaying the highest homology with HRH4, and OSBPL2, the closest parologue of OSBPL1, in a head-to-head orientation similar to the IMPACT locus on chromosome 18q11.2 (Fig. 1). Moreover, OSBPL1 and OSBPL2 are flanked by LAMA3 and its parologue LAMA5, respectively, in a tail-to-tail manner. It thus seems that these were generated by duplication. However, the segment on chromosome 20 lacks any parologue of IMPACT. Since retrotranspositional insertion of IMPACT into the segment on chromosome 18 is highly unlikely and a syntenic fragment containing Hrh4 and Impact can be found even in the puffer fish genome (data not shown), it seems that the duplicated copy of IMPACT is deleted from the segment on chromosome 20.

In this context, it is intriguing to note a recently proposed hypothesis that underscores a role for duplications in the evolution of imprinting. According to this hypothesis, duplication leads to monoallelic expression and subsequently imprinted expression of the resulting paralogues for a simple basis of dosage compensation. This model is based on their observation that the paralogues of imprinted genes are often in close vicinity to other imprinted genes. This is true for the Osbpl1-Hrh4 locus, because Osbpl5, a parologue of Osbpl1, and Htr2a, another amine receptor similar to Hrh4, are maternally expressed genes on mouse chromosomes 7 and 14, respectively.

We assume that an ancient segment containing Hrh-Impact-Osbpl-Lama was duplicated. However, since the increased dosage of Impact was hazardous, one of the copies was deleted from the genome, leaving a segment that eventually evolved to Hrh3-Osbpl2-Lama5. Indeed, we previously demonstrated that overexpression of Xenopus homologue of Impact in oocyte inhibits normal embryonic development. We also showed that the over-
expression of \textit{YIH1}, the yeast homologue of \textit{Impact}, confers growth defect under amino acid starvation stress.\textsuperscript{19} This may be the reason why only a single copy of \textit{Impact} was retained in the vertebrate genomes, which, on the other hand, contain multiple paralogues of \textit{Hrh4} and \textit{Osbp1}.

If the deletion of a duplicated copy of \textit{Impact} restored the original level of gene dosage, it does not have to be imprinted. Indeed, we found that \textit{Impact} is not imprinted in most vertebrates including frog, pig, monkey and human (in preparation). Then, why is \textit{Impact} imprinted in mouse? One possibility is that the promoter activity was enhanced in the lineage leading to mouse. To control its dosage, mouse might exploit monoalectic expression, which eventually led to imprinted expression.

To examine whether the promoter activity is indeed enhanced in mouse, we compared the expression level of \textit{Impact} and \textit{IMPACT} by an RT-PCR assay using a single common primer pair for amplification of both genes. Although the primer sequences are derived from human \textit{IMPACT} and not identical to the sequence of mouse \textit{Impact}, the primer pair can amplify the latter as efficiently as the former (Fig. 6). The results of the assay clearly indicated that the expression of mouse \textit{Impact} is much stronger than that of human \textit{IMPACT} (Fig. 6). Similarly, our preliminary analysis indicated that the expression of \textit{Impact} is significantly higher in rat and rabbit, both bearing imprinted \textit{Impact}, than in monkey and pig, both bearing non-imprinted \textit{Impact} (manuscript in preparation).

Based on these observations, we assume that the species-specific imprinting of \textit{Impact} evolved as a consequence of adaptations to increased gene dosage, which was first induced by segmental duplication and next by enhancement of the promoter activity in the lineage to rodents and rabbit. While the mechanism leading to the enhanced promoter activity remains elusive, it is intriguing to note the promoter sequence is considerably diverged between mouse and human: the human promoter, but not the mouse one, constitutes a conventional nonmethylated CpG island.\textsuperscript{4} Furthermore, the first intron of the mouse gene, but not its human counterpart, has a differentially methylated CpG island containing characteristic tandem repeats, which are often found near imprinted genes.\textsuperscript{4} It is thus possible that this island played a role in the evolution of imprinting via enhancement of the expression of \textit{Impact}.

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