COVER LEGEND

Ringtail lemur by San Francisco Bay artist, Pat Sherwood. Killian et al. demonstrate in this issue genomic imprinting divergence in the primate lineage roughly 75 million years ago. The divergence in M6P/IGF2R imprinting between primates and sister mammals, such as rodents and artiodactyls, provides an explanation for animal differences in carcinogen susceptibility. Moreover, it predicts that the success of in vitro embryo procedures such as cloning will be species-dependent.
Divergent evolution in M6P/IGF2R imprinting from the Jurassic to the Quaternary

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M6P/IGF2R imprinting first appeared approximately 150 million years ago following the divergence of prototherian from therian mammals. Although M6P/IGF2R is clearly imprinted in opossums and rodents, its imprint status in humans remains ambiguous. It is also still unknown if M6P/IGF2R imprinting was an ancestral mammalian epigenotype or if it evolved convergently. We report herein that M6P/IGF2R is imprinted in Artiodactyla, as it is in Rodentia and Marsupialia, but that it is not imprinted in Scandentia, Dermoptera and Primates, including ringtail lemurs and humans. These results are most parsimonious with a single ancestral origin of M6P/IGF2R imprinting followed by a lineage-specific disappearance of M6P/IGF2R imprinting in Euarchonta. The absence of M6P/IGF2R imprinting in extant primates, due to its disappearance from the primate lineage over 75 million years ago, demonstrates that imprinting at this locus does not predispose to human disease. Moreover, the divergent evolution of M6P/IGF2R imprinting predicts that the success of in vitro embryo procedures such as cloning may be species dependent.

INTRODUCTION

M6P/IGF2R encodes for a multifunctional protein involved in lysosomal enzyme trafficking, fetal organogenesis, tumor suppression and T-cell mediated immunity (1–7). The essential role of M6P/IGF2R in gestational fetal growth control is evidenced by gene knock-out models in mice (2–4,8), as well as fetal overgrowth in sheep attributable to gene underexpression (9). Parentally imprinted M6P/IGF2R alleles invaded the ancestral mammalian gene pool over 100 million years ago, resulting in the expression of only the maternally-inherited allele (10). M6P/IGF2R imprinting evolved concurrently with receptor acquisition of an IGF2 binding site which is present in marsupials and eutherians, but not in amphibians, avians and monotremes (10–15). Thus, genetic and epigenetic modifications of M6P/IGF2R directed at controlling offspring growth occurred in therian mammals following their divergence from prototherians.

Although M6P/IGF2R is known to be imprinted in opossums and rodents (10,16,17), its imprint status in other mammals is uncertain. Humans were initially reported to differ from rodents by expressing both parental M6P/IGF2R alleles (18,19); however, subsequent studies provided evidence that M6P/IGF2R imprinting is a polymorphic trait in humans (20–22). The involvement of this receptor in fetal organogenesis, cellular growth suppression and T-cell mediated programmed cell death predicts that those individuals who inherit an imprinted M6P/IGF2R would be predisposed to fetal overgrowth, teratogenesis and carcinogenesis because of gene haploinsufficiency. It is therefore important to resolve the issue of whether the M6P/IGF2R is imprinted in the human population.

In this investigation the phylogenetic breadth of M6P/IGF2R imprinting in eutherian mammals was explored outside the superordinal clade that includes rodents and primates. We report herein that the M6P/IGF2R is imprinted in the artiodactyls (i.e. sheep, cows and pigs), as it is in opossums (10) and rodents (16,23,24). In contrast, M6P/IGF2R imprinting is absent in all members of the Euarchonta clade, a taxonomic grouping that includes Scandentia (i.e. tree shrew), Dermoptera (i.e. colugo/‘flying lemur’) and Primates (i.e. ringtail lemur and humans). An ancestral origin followed by a more recent lineage-restricted loss of M6P/IGF2R imprinting is most parsimonious with this phylogenetic distribution. Since M6P/IGF2R imprinting disappeared in the primate lineage over 75 million years ago, and is totally absent in extant primates, imprinting at this locus does not predispose to human pathology. The defined evolutionary divergence of M6P/IGF2R imprinting during development also predicts that the success of in vitro embryo manipulations such as cloning may in part be species dependent.

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RESULTS

M6P/IGF2R imprinting analysis in Artiodactyla

The sheep M6P/IGF2R ortholog was cloned and sequenced. Comparison of RT–PCR transcript sequences from 10 individual fetuses uncovered single nucleotide polymorphisms (SNPs) in exons 19 and 22 of the coding sequence (Table 1). RT–PCR amplimers containing these SNPs demonstrated monoallelic M6P/IGF2R expression in 5/5 heterozygous informative fetuses (Fig. 1A). Genotyping of parents demonstrated that the expressed allele is of maternal origin. Amplification of exon 48 and the 3’-untranslated region (3’-UTR) from cow and pig genomic DNA revealed the presence of three cow and two pig SNPs in exon 48 (Table 1). RT–PCR amplimers containing SNPs from fetal cows (Fig. 1B) and pigs (Fig. 1C) consistently revealed monoallelic M6P/IGF2R expression.

M6P/IGF2R imprinting analysis in Scandentia, Dermoptera and Primates

RT–PCR amplification of ~3 kbp of tree shrew and ringtail lemur M6P/IGF2R cDNA revealed several SNPs in each

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*SNPs used in this study.

Figure 1. M6P/IGF2R imprinting in fetal sheep, cow and pig tissues. (A) The allelic expression of M6P/IGF2R in sheep was determined using fetal liver tissue heterozygous for a c.2401G→T transversion (arrow, fetus DNA) (sequenced in reverse direction). RNA analysis of this tissue demonstrates exclusive expression of the T allele (arrow, fetus cDNA) (sequenced in reverse direction). The maternal genotype is G/T (arrow, dam DNA) (sequenced in reverse direction) and the paternal genotype is G/G (arrow, sire DNA) (sequenced in reverse direction), demonstrating that the expressed T allele in the fetus is inherited from the mother. (B) The allelic expression of M6P/IGF2R in cows was determined using fetal liver tissue heterozygous for a c.8672A→G transition (arrow, fetus DNA) (sequenced in reverse direction). RT–PCR analysis of mRNA transcripts reveals that only the G allele is expressed (arrow, fetus cDNA; sequenced in reverse direction). (C) The allelic expression of M6P/IGF2R in pigs was determined using fetal liver tissue heterozygous for a c.375C→T transition (arrow, DNA) (sequenced in reverse direction). RT–PCR analysis of mRNA transcripts reveals that only the C allele is expressed (arrow, fetus cDNA) (sequenced in reverse direction).
species (Table 1). Tree shrew and ringtail lemur fetal tissue were unavailable because of the scarcity of these protected species; however, tissues were obtained from animals that died perinatally. RT–PCR amplification of RNA isolated from tree shrew liver (Fig. 2A), and brain, heart, kidney, liver, lung, muscle and spleen tissues from ringtail lemurs (Fig. 2B) demonstrated equal transcription from both parental alleles. Kidney, liver and spleen were also obtained from five informative adult ringtail lemurs, and all tissues demonstrated biallelic M6P/IGF2R transcription (data not shown). Heart tissue from a single adult colugo also manifested biallelic M6P/IGF2R expression (data not shown).

**M6P/IGF2R imprinting analysis in humans**

There is difficulty in interpreting the results from the often used dinucleotide repeat and insertion/deletion polymorphisms in the 3’-UTR of human M6P/IGF2R (25,26). The results of expression analyses based on these polymorphisms are potentially misleading. Technical difficulties associated with the use of these polymorphisms (27), and the potential use of differential polyadenylation signals compromise a clear interpretation of the results obtained. Therefore, we utilized six SNPs within the coding sequence of the human M6P/IGF2R (27) in addition to the 3’-UTR tetranucleotide insertion/deletion polymorphism (26) to investigate M6P/IGF2R imprinting in humans. M6P/IGF2R imprinting was studied in a number of tissues from 75 conceptuses (i.e., 40 first trimester and 35 second trimester conceptuses); 12 term placentas were also investigated. PCR amplification of genomic DNA demonstrated that 58% (7/12) of the placental samples and 61% (46/75) of the conceptuses were biallelic (homozygous 57 bases; lane a, homozygous 53 bases). RT–PCR analysis of mRNA transcripts (lanes 1–12, cDNA) reveals that both alleles (A and B) are equally expressed.

CpG analysis of M6P/IGF2R intron 2 in Euarchonta

M6P/IGF2R intron 2 was PCR amplified directly from tree shrew and ringtail lemur genomic DNA. The 7.3 kbp tree shrew and 6.5 kbp lemur amplimers were sequenced, and then analyzed for the presence of a CpG island motif by the CpGPlot bioinformatics software. The M6P/IGF2R in both the human and tree shrew intron 2 contains a CpG island motif (Fig. 4).

**Figure 2. M6P/IGF2R expression in perinatal tree shrew and ringtail lemur tissues.** (A) The allelic expression of M6P/IGF2R in the tree shrew was determined using fetal liver tissue heterozygous for a c.1056C→T transition (arrow, DNA) (sequenced in reverse direction). RT–PCR analysis of mRNA transcripts reveals that both the C and T alleles are equally expressed (arrowhead, cDNA). (B) The allelic expression of M6P/IGF2R in the ringtail lemur was determined using fetal tissues heterozygous for a c.901C→G transition (arrowhead, DNA). RT–PCR analysis of mRNA transcripts (cDNA) from liver, spleen, kidney, heart, muscle, brain and lung reveals that both the C and T alleles are equally expressed (arrowhead, cDNA).

**Figure 3. M6P/IGF2R expression in human fetal kidney.** (A) The allelic expression of M6P/IGF2R in first trimester human kidney tissues was determined using samples heterozygous for c.901C→G transversion, c.1197A→G transition, c.1737A→G transition, c.2286A→G transition (sequenced in reverse direction), c.5002A→G transition (sequenced in reverse direction) and c.6206A→G transition (sequenced in reverse direction). RT–PCR analysis of mRNA transcripts reveals that both alleles are equally expressed at the six SNP locations used (arrows). (B) The allelic expression of M6P/IGF2R in human fetal kidney tissues was determined using samples heterozygous for a 4-nucleotide insertion/deletion (ACAA) in the M6P/IGF2R 3’-UTR (lane a, homozygous 57 bases; lane b, homozygous 53 bases). RT–PCR analysis of mRNA transcripts (lanes 1–12, cDNA) of informative samples (lanes 1–12, DNA,) reveals that both alleles (A and B) are equally expressed.

**Figure 4.** CpG analysis of M6P/IGF2R intron 2 in Euarchonta. The CpG island motif was identified in the M6P/IGF2R intron 2 of tree shrew and ringtail lemur.
intron 2 CpG island, whereas the homologous region in the human M6P/IGF2R (Fig. 5A) contains such a motif (28,29). Using bisulfite sequencing analysis, we screened for differential methylation within intron 2 of the ringtail lemur (Fig. 5B) and tree shrew (Fig. 5C) M6P/IGF2R. All CpG dinucleotides analyzed for both species were fully methylated (data not shown). Thus, there was no evidence of the hemi-methylation that is characteristic of differentially methylated regions (DMRs) associated with imprinted genes. The phylogenetic distributions of M6P/IGF2R imprinting, and of a differentially methylated CpG island in intron 2 are shown in Figure 6.

**DISCUSSION**

It was initially reported that M6P/IGF2R was exceptional among imprinted genes in that allelic silencing was not conserved between rodents and humans (18,19); however, subsequent reports provided evidence that M6P/IGF2R imprinting is polymorphic within the human population (20–22). The recent discovery that both M6P/IGF2R and IGF2 are imprinted in marsupials indicates that imprinted alleles are often stable over an extended period of time once they invade the genome (10,30). Therefore, the possibility of human M6P/IGF2R imprinting in a subset of people is an enigma that needs to be resolved because the clinical implications of polymorphic imprinting of this cell growth suppressor are significant (31). In addition, the absence of imprinting in humans and other mammals would shrink the known phylogenetic breadth of M6P/IGF2R imprinting to marsupials and rodents, thereby increasing the probability of convergent evolution of imprinting in these species.

To address these important issues, we first determined the phylogenetic distribution of M6P/IGF2R imprinting in eutherian mammals. Artiodactyla was found to be an additional mammalian order in which members are imprinted at the M6P/IGF2R locus during development. The most parsimonious interpretation of the presence of M6P/IGF2R imprinting in marsupials, rodents and artiodactyls, three distantly-related mammalian orders, is that

**Figure 4.** M6P/IGF2R expression in Wilms’ tumors. The allelic expression of M6P/IGF2R in Wilms’ tumor was determined using tumors from patients heterozygous for c.901C→G transversion (n = 3), c.1197A→G transition (n = 2) and c.5002A→G transition (n = 2) (arrowhead, DNA). RT–PCR analysis of mRNA transcripts from all seven tumors reveals that both alleles are equally expressed at the three SNP locations used (arrowhead, cDNA).

**Figure 5.** Distribution of CpG and GpC dinucleotides in human, ringtail lemur and tree shrew M6P/IGF2R intron 2. (A) The positioning of CpG and GpC dinucleotides in intron 2 of human M6P/IGF2R reveals a CpG island known to be differentially methylated (28; solid horizontal bar). (B) Ringtail lemur intron 2 lacks a CpG island, and all CpG dinucleotides analyzed within this region were fully methylated. (C) Tree shrew intron 2 lacks a CpG island, and all CpG dinucleotides analyzed within this region were fully methylated.
the ancestral therian mammalian set of imprinted genes included the \textit{M6P/IGF2R}.

We next determined the imprinting status of \textit{M6P/IGF2R} in the closest extant relatives of primates, Scandentians (i.e. tree shrews) and Dermopterans (i.e. colugos), and the most distant primate relatives of hominoids, the Prosimians (i.e. ringtail lemurs). We observed equal expression from both parental alleles in the tree shrew, colugo and ringtail lemur. These findings indicate that \textit{M6P/IGF2R} imprinting is not only absent in primates, but that this codominant parental expression extends to the close non-primate relatives. Thus, \textit{M6P/IGF2R} imprinting was already absent in primate ancestors some 75 million years ago, a conservative estimate for the divergence of Euarchonta. \textit{M6P/IGF2R} is thus exceptional among analyzed imprinted loci in that imprinting of this gene is not highly conserved between the mammalian orders. This is in contrast to \textit{IGF2}, where imprinting appears to be a universal trait among wild-type therian mammals (30,32,33). Therefore, these mammalian evolutionary investigations support the postulate that the combined effects of natural selection do not tolerate or favor \textit{M6P/IGF2R} imprinting in primates and their close relatives.

It can be further inferred that if \textit{M6P/IGF2R} imprinting is polymorphic in humans, ancestral imprinted alleles are still present in the human population or else \textit{M6P/IGF2R} imprinting is convergently re-emerging following its disappearance. To directly assess the imprint status of \textit{M6P/IGF2R} in humans during development, we analyzed multiple tissues from 46 unrelated human conceptuses. This large number of samples could easily detect the presence of \textit{M6P/IGF2R} imprinting if 25–50\% of human conceptuses are imprinted at this locus, as previously reported (20,21,29). Our analysis also included predominantly first trimester fetuses, in which \textit{M6P/IGF2R} imprinting was reportedly most evident. We observed unambiguous biallelic \textit{M6P/IGF2R} expression in every informative fetus. Epigenetic allele silencing was likewise not found in Wilms’ tumors, in contrast to the results of previous investigators that concluded that polymorphic imprinting of \textit{M6P/IGF2R} predisposed children to this juvenile tumor (21). Interestingly, there are also no clinically recognized human syndromes associated with monoallelic expression of \textit{M6P/IGF2R}; this is incompatible with reports of nearly half of all conceptuses being imprinted at this locus during development (20,21). The totality of our results are therefore consistent with \textit{M6P/IGF2R} imprinting not being a polymorphic epigenotype in the human population.

A plausible explanation for the discrepancy between our results and those reporting polymorphic imprinting of \textit{M6P/IGF2R} (20–22) lies in the polymorphisms used for allelic analysis. All reported cases of imprinted \textit{M6P/IGF2R} expression involved the use of distal 3'UTR polymorphisms, and none of these results has been confirmed using SNPs within the coding sequence. These 3'-UTR polymorphisms can be particularly problematic for allelic analysis because of technical difficulties with PCR amplification (27). The potential use of allele-specific differential polyadenylation signals could also result in apparent monoallelic expression even when the coding region of the gene is expressed from both parental alleles. Tissue culture is also known to alter \textit{M6P/IGF2R} expression (9,28).
The SNPs and in vivo tissues used in this study circumvent these potential problems.

The murine M6P/IGF2R contains a 1.5 kb CpG island in intron 2 (DMR2) that is methylated in mouse oocytes but not in sperm, and was predicted to play a role in imprint inheritance (34–36). This postulate was supported by the demonstration that deletion of the DMR2 results in biallelic M6P/IGF2R expression in the mouse (8). Nevertheless, M6P/IGF2R is biallelically expressed in mouse brain even though DMR2 remains differentially methylated (37). The marsupial opossum M6P/IGF2R is also imprinted, as it is in rodents and artiodactyls, but intron 2 entirely lacks a CpG island or any differentially methylated CpG dinucleotide sequences. In contrast, we now provide evidence that the human M6P/IGF2R is not imprinted even though a differentially methylated CpG island is present in intron 2 (28,29). Other members of Euarchonta such as the tree shrew and ringtail lemur, however, lack both M6P/IGF2R imprinting and the presence of DMR2. Thus, the presence of a DMR in intron 2 of the M6P/IGF2R is neither indicative of nor specific for imprinting at this locus.

The absence of a DMR2 in lemur and tree shrew is most parsimonious with human and mouse DMR2 not being orthologous, and human DMR2 therefore not being a vestige of ancestral M6P/IGF2R imprinting. This idea is supported by the lack of sequence and organizational homology between human and mouse DMR2. Moreover, human DMR2 cannot represent a remnant of an ancestral imprinting motif since the imprinted marsupial M6P/IGF2R does not contain a DMR2 (10). These findings are consistent with DMR2 having evolved convergently in select eutherian mammals after the ancestral establishment of M6P/IGF2R imprinting, and also subsequent to the disappearance of M6P/IGF2R imprinting in Euarchonta. The existence of a DMR in intron 2 of the unimprinted human M6P/IGF2R indicates that such regions are not always associated with imprinted genes, and that they may be widespread in mammalian genomes and/or these elements are mobile. Further investigations of DMR distribution in the human genome are needed to resolve these issues.

The marked species difference in M6P/IGF2R imprinting between humans and the other viviparous mammals such as mice has important biological consequences. In accord with the Knudson two-hit model of tumor suppressor gene inactivation (38), only a single mutation is required to negate M6P/IGF2R tumor suppressor function in mice since one allele is already inactivated by the epigenetic phenomenon of imprinting. In contrast, two mutational events are required to inactivate this gene in humans (31,39). A partial transcript (SuperScript II, Life Technologies). cDNA was prepared by reverse transcription of 1–5 µg of total RNA using an oligo-dT primer (SuperScript II, Life Technologies).

N6P/IGF2R ortholog identification

Tree shrew (Accession no. AF339161), colugo (Accession no. AG339163), ringtail lemur (Accession no. AF339160) and human (Accession no. NM_000876) M6P/IGF2R cDNA sequences have been characterized previously. A partial M6P/IGF2R transcript (~7 kbp) from sheep (Accession no. AF353513) was amplified and sequenced with the use of cross-species RT–PCR primers as described previously (10,43). Pig (Accession no. AF342812) and cow (Accession no. AF342811) 3′-UTR M6P/IGF2R sequence,
not previously characterized, were obtained by a combination of EST database analysis and sequencing of 3'-RACE products (Roche Molecular Biochemicals, Indianapolis, IN).

Genomic imprint analysis

Imprinting was determined by comparing genomic PCR and RT–PCR amplifiers of regions containing known SNPs (44). SNPs were identified directly in exon-spliced RT–PCR products of tree shrew, colugo, ringtail lemur and human M6P/IGF2R as sequence dimorphisms in ABI Prism sequence histograms (ABI 377 sequencer; PE Biosystems, Foster City, CA) since M6P/IGF2R expression is biallelic in these species. In contrast, sheep, pig, and cow M6P/IGF2R SNPs were identified by alignment of amplified and sequenced transcripts from multiple individual fetal liver cDNA preparations since this gene is monoallelically expressed in these species. All SNPs were confirmed by repeat PCR amplifications, and the sequencing of these products in both the forward and reverse directions. PCR primers were designed to amplify the genomic DNA surrounding each SNP used in this study. Monoallelic versus biallelic expression was determined by comparing the RT–PCR and genomic PCR amplifiers at each polymorphic site. The ratios of maternal to paternal allele band intensities in the DNA and cDNA were compared. Expression was considered to be biallelic if the DNA to cDNA allele band intensity ratios were equal (i.e. allelic ratio of 0.7–1.3). The absence of genomic DNA in RT–PCR products was confirmed by gel extraction and by direct sequencing across spliced exon–exon junctions. SNP locations for human and non-human M6P/IGF2R orthologs are provided in Table 1. The PCR primers used to amplify the regions containing human SNPs have been described previously (27,45); the PCR primers used to amplify the regions containing known SNPs (44) by PCR amplification using the forward primer p#2633 (5'-GTCAGATTTTTCGCCGCTGGTA-3') and the reverse primer p#2634 (5'-ACACAAATCATCATTGGGCCAGGTTG-3').

CpG analysis of M6P/IGF2R intron 2

M6P/IGF2R intron 2 sequences for chicken (Accession no. AF305581), platypus (Accession no. AF151171), and opossum (Accession no. AF225877) have been characterized previously (10,32). The complete 18 kbp human (Accession no. AF348209) and 8.3 kbp mouse (Accession no. AF365596) M6P/IGF2R intron 2 were established from bacterial artificial chromosome sequences. The complete M6P/IGF2R intron 2 sequence for tree shrew (Accession no. AF339164) and ringtail lemur (Accession no. AY039099) were amplified from genomic DNA with PCR primers that hybridize in exons 2 and 3 (tree shrew forward primer int2F, 5'-CCTCTGCGAACC- TGGGCATTAC-3' and reverse primer int2R, 5'-GTGTTGAATTCCAGAATAGATG- TGGTTGACTTCTC-3'). Amplimers were gel extracted and sequenced on an ABI Prism DNA sequencer. CpG island analysis was performed with CpGPlot (http://bioweb.pasteur.fr/ seqanal/interfaces/cpgplot.html).

Methylation analysis using bisulfite sequencing

Genomic DNAs were treated with sodium bisulfite to convert all unmethylated cytosines to uracils, leaving methylated cytosines intact. Sodium bisulfite treatment was performed using the CpGenome DNA modification kit according to the manufacturer’s protocol (Intergen, New York, NY). 200 ng of bisulfite-treated DNA was empirically determined to avoid stochastic PCR amplification bias due to a limiting number of starting DNA molecules, and was therefore used as template for PCR amplification. Primer sequences used for amplification of CpG-containing regions are available upon request from the authors. Bisulfite-treated DNA was amplified using one round of PCR (40 cycles of 95°C for 30s, 55°C for 30s and 72°C for 30s), and sequenced using radiolabeled terminator cycle sequencing (USB Corporation, Cleveland, OH).

ACKNOWLEDGMENTS

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REFERENCES


