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ARTICLE

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 the maternally-inherited allele only (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 *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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Table 1. *M6P/IGF2R* SNPs

Species	Accession no.	Exon	SNP
Primates			
Ringtail lemur	AF339160	11	c.1229C→T
		12	c.1373G→T
		12	c.1394C→T
		13	c.1442A→G
		19	c.2572C→T
		39	c.5537C→T[a]
Humans	NM_000876	6	c.901C→G[a]
		9	c.1197A→G[a]
		12	c.1737A→G[a]
		16	c.2286A→G[a]
		34	c.5002A→G[a]
		40	c.6206A→G[a]
Dermoptera			
Colugo	AG339163	5	c.414A→C
		13	c.1512A→G
		22	c.2823A→G[a]
		22	c.2856C→T
		25	c.3363C→T
		26	c.3513C→T
		35	c.5001A→G
Scandentia			
Tree shrew	AF339161	5	c.360C→T
		10	c.1056C→T[a]
		34	c.4794C→T
		43	c.6252A→G
		48	c.7146A→G
		48	c.7256C→T
Artiodactyla			
Cow	AF342811	48	c.7475C→T
		48	c.7881A→G
		48	c.8672A→G[a]
Pig	AF342812	48	c.116A→C
		48	c.375C→T[a]
Sheep	AF353513	19	c.2401G→T[a]
		22	c.2761C→T

[a]SNPs used in this study.

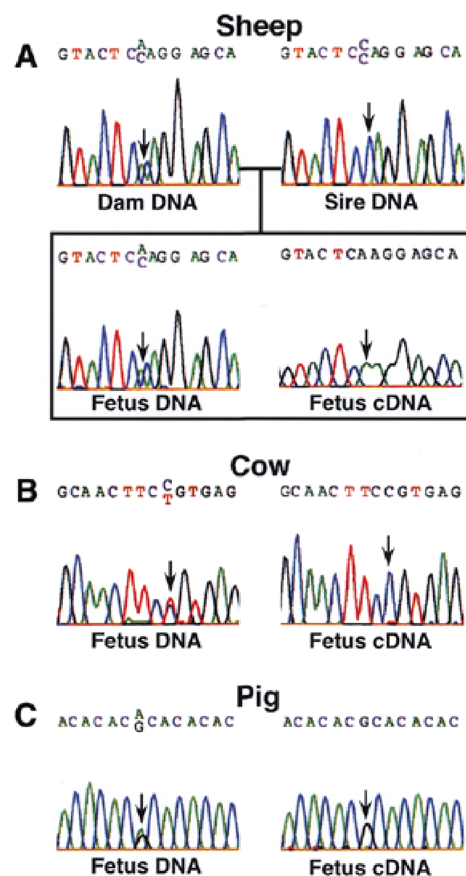

Figure 1. *M6P/IGF2R* imprinting in fetal sheep, cow and pig tissues. (**A**) The allelic expression of *M6P/IGF2R* in sheep was determined using fetal liver tissue heterozygous for a c.2401G→T transversion (arrow, fetus DNA) (sequenced in reverse direction). RNA analysis of this tissue demonstrates 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).

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 Prosimians

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

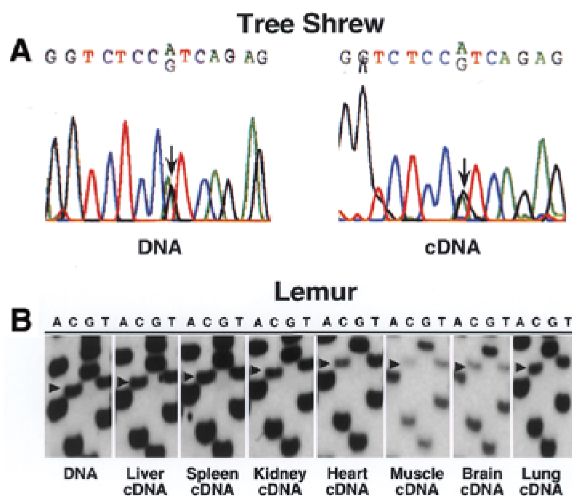

Figure 2. *M6P/IGF2R* expression in perinatal tree shrew and ringtail lemur tissues. (**A**) The allelic expression of *M6P/IGF2R* in the tree shrew was determined using fetal liver tissue heterozygous for a c.1056C→T transition (arrow, DNA) (sequenced in reverse direction). RT–PCR analysis of mRNA transcripts reveals that both the C and T alleles are equally expressed (arrow, cDNA) (sequenced in reverse direction). (**B**) The allelic expression of *M6P/IGF2R* in the ringtail lemur was determined using fetal tissues heterozygous for a c.5537C→T 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).

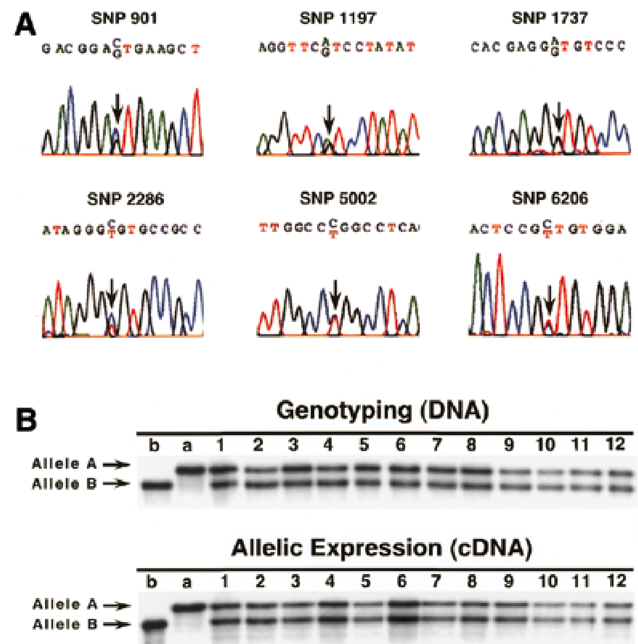

Figure 3. *M6P/IGF2R* expression in human fetal kidney tissue. (**A**) The allelic expression of *M6P/IGF2R* in first trimester human fetal kidney tissue was determined using samples heterozygous for c.901C→G transversion, c.1197A→G transition, c.1737A→G transition, c.2286A→G transition (sequenced in 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 tissue 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.

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 heterozygous for one or more of these polymorphisms. RT–PCR of these informative samples revealed biallelic expression, with no evidence of skewed allelic expression, in every informative human embryonic tissue studied, including those from the brain, kidney, heart, liver, lung and placenta. Representative results are shown for first trimester fetal kidney tissue using the six identified SNPs (Table 1 and Fig. 3A) and the tetranucleotide insertion/deletion polymorphism (Fig. 3B) (26).

The *M6P/IGF2R* imprinting status was also determined in pediatric Wilms' tumors. PCR amplification of genomic DNA demonstrated that 58% (7/12) of Wilms' tumors developed in patients heterozygous for one or more of the identified SNPs. RT–PCR of these informative tumor samples revealed biallelic *M6P/IGF2R* expression in all Wilms' tumors studied (Fig. 4).

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 kb tree shrew and 6.5 kb 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 tree shrew (Fig. 5C) and ringtail lemur (Fig. 5B) lacks an

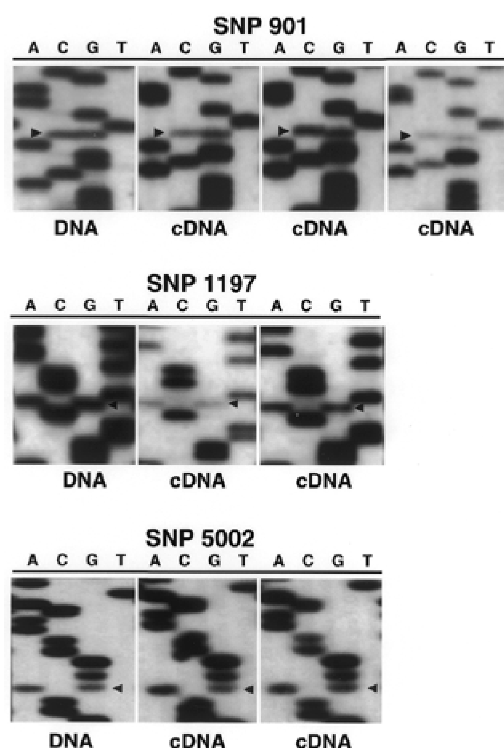

Figure 4. *M6P/IGF2R* expression in Wilms' tumors. The allelic expression of *M6P/IGF2R* in Wilms' tumor was determined using tumors from patients heterozygous for c.901C→G transversion ($n = 3$), c.1197A→G transition ($n = 2$) and c.5002A→G transition ($n = 2$) (arrowhead, DNA). RT–PCR analysis of mRNA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

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

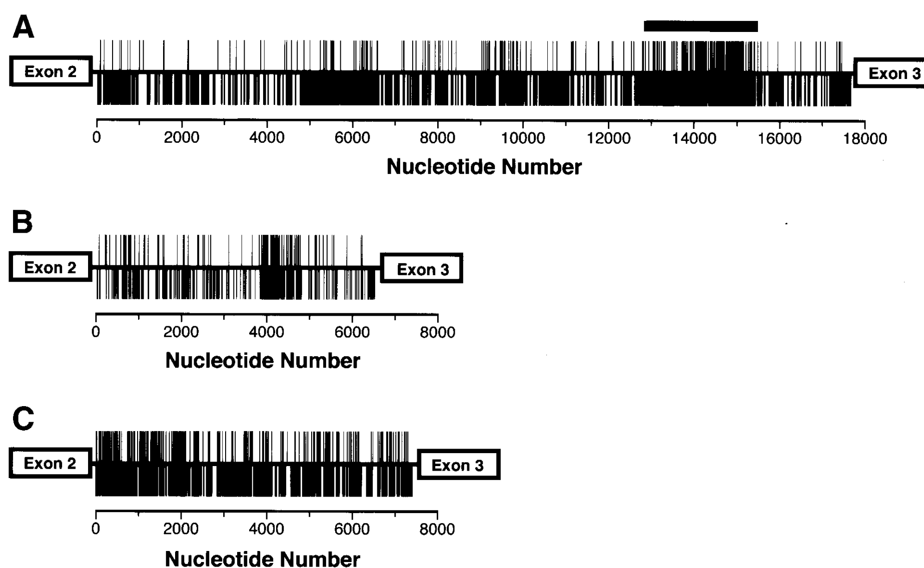

Figure 5. Distribution of CpG and GpC dinucleotides in human, ringtail lemur and tree shrew *M6P/IGF2R* intron 2. (**A**) The positioning of CpG and GpC dinucleotides in intron 2 of human *M6P/IGF2R* reveals a CpG island known to be differentially methylated (28; solid horizontal bar). (**B**) Ringtail 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.

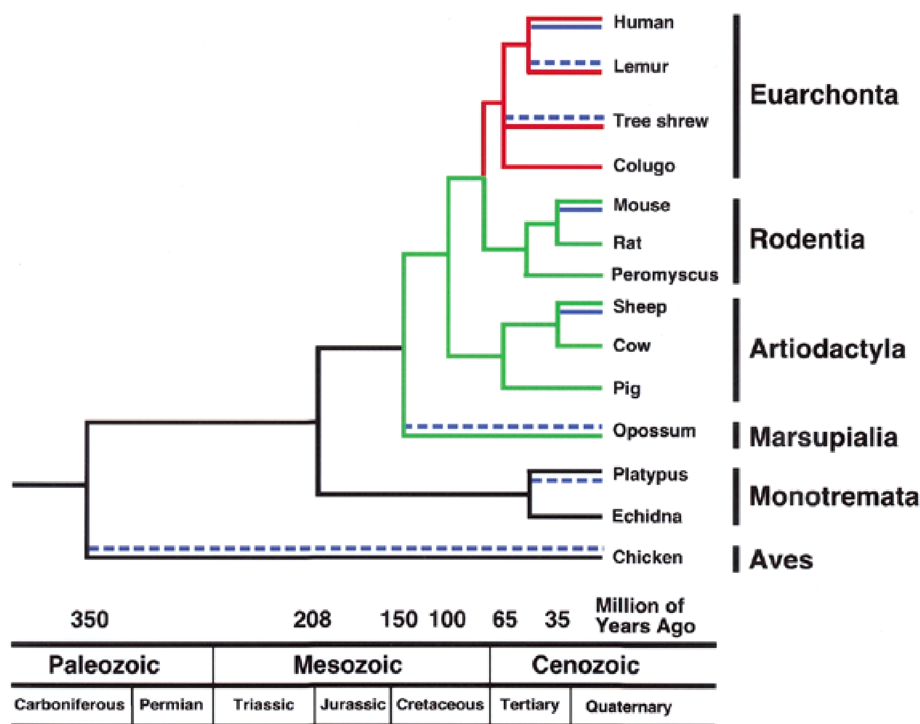

Figure 6. Phylogenetic distribution of *M6P/IGF2R* imprinting and intron 2 CpG isochores. Solid black line, *M6P/IGF2R* imprinting absent in extant species and presumably in ancestors; solid green line, *M6P/IGF2R* imprinting present in extant species and presumably in a common ancestor; solid red line, *M6P/IGF2R* imprinting absent in extant species but presumably was present in ancestors; solid blue line, *M6P/IGF2R* intron 2 CpG island present; dashed blue line, *M6P/IGF2R* intron 2 CpG island absent.

the ancestral therian mammalian set of imprinted genes included *M6P/IGF2R*.

We next determined the imprinting status of *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 *M6P/IGF2R* imprinting is not only absent in primates, but that this codominant parental expression extends to the close non-primate relatives. Thus, *M6P/IGF2R* imprinting was already absent in primate ancestors some 75 million years ago, a conservative estimate for the divergence of Euarchonta.

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 *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 *M6P/IGF2R* imprinting in primates and their close relatives.

It can be further inferred that if *M6P/IGF2R* imprinting is polymorphic in humans, ancestral imprinted alleles are still present in the human population, or else *M6P/IGF2R* imprinting is convergently re-emerging following its disappearance. To directly assess the imprint status of *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 *M6P/IGF2R* imprinting if 25–50% of human conceptuses are imprinted at this locus, as previously reported (20,21,29). Our analysis also predominantly included first trimester fetuses, in which *M6P/IGF2R* imprinting was reportedly most evident. We observed unambiguous biallelic *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 *M6P/IGF2R* predisposed children to this juvenile tumor (21). Interestingly, there are also no clinically recognized human syndromes associated with monoallelic expression of *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 *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 *M6P/IGF2R* (20–22) lies in the polymorphisms used for allelic analysis. All reported cases of imprinted *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 *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). The functional haploid state of *M6P/IGF2R* in mice, but not in humans, provides a plausible explanation of why mice are more susceptible to cancer than humans. It also brings into question the relevance of human carcinogen risk assessments that are based solely on mouse studies (40).

In vitro embryo culture and cloning of livestock often result in prenatal and perinatal lethality associated with large offspring syndrome (LOS). In sheep, LOS correlates with epigenetic changes in *M6P/IGF2R* and decreased gene expression (9). Our finding that *M6P/IGF2R* is in fact imprinted during sheep, pig and cow development indicates that livestock may be subject to reduced *M6P/IGF2R* expression through *in vitro* embryo manipulations that specifically alter epigenetic imprint programming thereby resulting in fetal overgrowth. Interestingly, human embryo development resulting from either *in vitro* fertilization or cloning may not be as adversely impacted by this problem since the *M6P/IGF2R* is not imprinted in humans. In support of this postulate, fetal overgrowth has not been reported to be a specific complication of human *in vitro* fertilization and embryo transfer procedures followed by gestational surrogacy (41).

The results of this study demonstrate a clear divergence of primates and their close relatives from artiodactyls, rodents and marsupials in *M6P/IGF2R* imprinting. They further show that DMR2 is poorly correlated with imprinting in therian mammals, thereby questioning the universal importance of this element in *M6P/IGF2R* imprint regulation. Thus, phylogenetic inference coupled with gene expression analysis provide evidence that male and female mammalian ancestors of humans resolved an epigenetic struggle at the *M6P/IGF2R* locus (42) that would otherwise have resulted in enhanced disease susceptibility and many more visits to the clinic.

MATERIALS AND METHODS

Tissue samples

Fetal sheep tissues from 10 individuals and respective parental blood samples were a kind gift of B.Freking (Clay Center, NE). Twenty-five fetal pigs from five different mothers and two fetal cow livers were obtained from Neese's Sausage (Burlington, NC) and Martin's Abattoir (Spivey's Corner, NC), respectively. Tree shrew tissue was obtained from an animal that died perinatally at Duke University (Durham, NC). A colugo heart specimen preserved at –80°C was provided by the Field Museum of Natural History (Chicago, IL). Kidney, liver and spleen from five adult ringtail lemurs, and a complete ringtail lemur conceptus that died perinatally, were obtained from the frozen tissue archives of the Duke University Primate Center. The Obstetrics and Gynecology Department of Duke University Medical Center provided 12 human placenta samples from term deliveries. Tissues from 75 unrelated human conceptuses were obtained from the NIH funded Birth Defects Research Laboratory at the University of Washington (Seattle, WA). Wilms' tumors from 12 different patients were obtained from the Duke Comprehensive Cancer Center frozen tissue bank facility.

Nucleic acid preparation

Genomic DNA and total RNA were extracted by homogenizing 100 mg of tissue in DNA-Stat 60 and RNA-Stat 60, respectively; the DNA and RNA were extracted according to the manufacturer's protocol (Tel-Test, Friendswood, TX). cDNA was prepared by reverse transcription of 1–5 µg of total RNA using an oligo-dT primer (SuperScript II, Life Technologies).

M6P/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 kb) 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 amplimers 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 amplimers at each polymorphic site. The ratios of maternal to paternal allelic band intensities in the DNA and cDNA were compared. Expression was considered to be biallelic if the DNA to cDNA allelic 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 amplimer size 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 for amplifying the non-human SNPs are available upon request. We analyzed a four-base insertion/deletion polymorphism (ACAA) in the 3′-UTR of human *M6P/IGF2R* (accession no. NM_000876, nucleotide position 8412–8415) (26) by PCR amplification using the forward primer p#2633 (5′-GTCAGTATTTTGCCGGCT-GGTGA-3′); and the reverse primer p#2634 (5′-ACACAAAT-CAATCTTTGGGCAGGTTG-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 kb human (accession no. AF348209) and 8.3 kb mouse (accession no. AF366566) *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. AY030099) were amplified from genomic DNA with PCR primers that hybridize in exons 2 and 3 (tree shrew forward primer int2F, 5′-CTCTGCGGAAACG-TGGGCATTAC-3′ and reverse primer int2R, 5′-CTGCAGC-TCAGGGTTGTGTTGAATTC-3′; ringtail lemur forward primer int2F, 5′-GGGCCATCAAGTGCTGTCTGTATG-3′ and reverse primer int2R, 5′-GTGTTGAATTCCAGAATAGATG-TGGTTGTACTTCTC-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, 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 30 s, 55°C for 30 s and 72°C for 30 s), and sequenced using radiolabeled terminator cycle sequencing (USB Corporation, Cleveland, OH).

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