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Cross-species clues of an epigenetic imprinting regulatory code for the *IGF2R* gene

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Abstract. The epigenetic marks on the *IGF2R* gene that encodes a receptor responsible for IGF-II degradation consist of differentially methylated DNA in association with multiple modifications on the associated histones. We review these epigenetic marks across various species during the evolution of *IGF2R* imprinting. Both *IGF2* and *IGF2R* genes are imprinted in the mammal lineage that diverged from Monotremata approximately 150 million years ago. While *IGF2* is consistently imprinted in all mammals following its divergence, *IGF2R* imprinting disappears in the Euarchonta lineage, including human species, approximately 75 million years ago. Differential DNA methylation marks on the two parental alleles correlate with imprinting in all imprinted genes including *IGF2R*. While the DNA methylation marks in the *IGF2R* promoter region 1 (DMR1) correlate with *IGF2R* allelic expression, the DNA methylation marks in the intron region 2 (DMR2) fail to correlate with *IGF2R* imprinting status in a number of species. Human *IGF2R* and mouse neuronal *Igf2r* are not imprinted despite the presence of DMR2. We have noted that human *IGF2R* is not imprinted in more than 100 informative samples including various tumor tissues. Furthermore, opossum (Marsupialia) *IGF2R* is consistently imprinted despite the absence of DMR2. These lines of evidence indicate that DNA methylation marks in DMR2 are neither necessary nor sufficient for consistent imprinting of *IGF2R* across species. Histone modification marks, however, correlate more consistently with the tissue-specific and species-specific imprinting status of *IGF2R* in human and mouse. Acetylated histone H3 and H4 and methylated lysine 4 of H3 (H3-K4Me) associate with transcriptionally active alleles while tri-methylated lysine 9 of H3 (H3-K9Me3) marks the silenced alleles. In the mouse, an antisense non-coding transcript called *Air* is transcribed from DMR2 on the paternal allele, and this imprinted transcript plays a central role in *Igf2r* imprinting. Mouse *Igf2r* imprinting depends on an *Air* RNA while the existence of *AIR* in other species is unknown. Overall, DNA methylation, histone acetylation, and histone methylation play a vital role in coordinating *IGF2R* allelic expression across all species. Rare monoallelic or skewed allelic expression of human *IGF2R* and their biological importance warrants further rigorous study.

The *mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R)*, hereafter referred to simply as *IGF2R*, is a large gene composed of 48 exons over 138 kilobases on human chromosome 6q26. The protein encoded by *IGF2R* is not involved in hormone signaling, but instead one of its primary functions is to decrease the concentration of the potent mitogen, IGF2 (insulin-like growth factor 2). After the receptor binds IGF2, the hormone-receptor complex is internalized and the growth factor is degraded. This receptor is a multifunctional protein, which is also known as the cation independent mannose-6-phosphase (M6P) receptor, as it binds a variety of M6P-containing glycoproteins, shuttling them to the lysosome (Lau et al., 1994; Wang et al., 1994; Ludwig et al., 1996; Melnick et al., 1998; Motyka et al., 2000).

Igf2r was the first gene shown to be imprinted in the mouse (Barlow et al., 1991). It is maternally expressed in all adult

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mouse tissues except for brain, where it is transcribed from both parental alleles (Wang et al., 1994; Hu et al., 1999). Interestingly, *IGF2R* was initially found to be not imprinted in humans (Kalscheuer et al., 1993); however, preferential expressions of the maternal *IGF2R* allele in placenta and in several tissues in early fetal life were subsequently reported, but monoallelic expression was seen in only a small minority of individuals who were sampled (Xu et al., 1993; Oudejans et al., 2001). The *IGF2R* locus is also one of many loci linked to susceptibility to insulin dependent diabetes mellitus (designated *IDDM8*). An SNP in this gene showed transmission distortion, but only in maternally transmitted alleles, suggesting a link to type 1 diabetes that is dependent on imprinted gene expression (McCann et al., 2004).

While *IGF2R* is imprinted in eutherians (mice: Barlow et al., 1991; rats: Mills et al., 1998; and pigs, cows, and sheep: Killian et al., 2001c) and marsupials (opossum: Killian et al., 2000), it is expressed biallelically in more ancestral monotremes (platypus and echidna) and aves (chickens) (Killian et al., 2000; Nolan et al., 2001). Moreover, there is evidence that imprinting at the *IGF2R* locus was lost approximately 75 million years ago in a common ancestor that gave rise to the near primate and primates in the Euarchonta group of eutherian mammals (Killian et al., 2001c). Thus, despite carrying some of the epigenetic marks that are considered to be components of imprinting, the experimental data are most parsimonious with human *IGF2R* being biallelically expressed in the majority of humans in both normal and pathologic tissues (Killian et al., 2001c).

There is also evidence that the *IGF2R* functions as a tumor suppresser gene, i.e. loss of a single allele might lead to an oncogenic diathesis suppressor (De Souza et al., 1995; Hankins et al., 1996). Inactivation of *IGF2R* may represent an early mechanism involved in liver, breast, lung, prostate, and gastrointestinal malignancies. A series of pioneer studies by Jirtle's group (De Souza et al., 1995; Hankins et al., 1996; Yamada et al., 1997; Kong et al., 2000; Jamieson et al., 2003), and by others (Piao et al., 1997; Oates et al., 1998) have clearly shown that loss of heterozygosity (LOH) or mutation of *IGF2R* occurs frequently in both premalignant dysplastic lesions and tumors. Gain of monoallelic expression of *IGF2R* has also been observed in a subset of Wilms' tumors (Xu et al., 1997); however, we did not observe this phenomenon in a study of more than 100 normal and neoplastic tissues, including Wilms' tumor (Killian et al., 2001c; Li et al, unpublished).

The evolutionarily divergent imprint status of *IGF2R* prompted us to further investigate the epigenetic marks involved in regulating imprinting at this locus. In this review, we summarize the DNA methylation and histone modification marks in the human and mouse *IGF2R* genes, and analyze the limited epigenetic data available for mammalian species ancestral to eutherians. Further information on mouse *Igf2r* imprinting is presented in this special issue (Regha et al., 2006). Recent reviews on imprinting evolution in general, and in the context of DNA methylation dynamics have been published (Geiman and Robertson, 2002; Murphy and Jirtle, 2003; Wilkins and Haig, 2003; Wilkins, 2005).

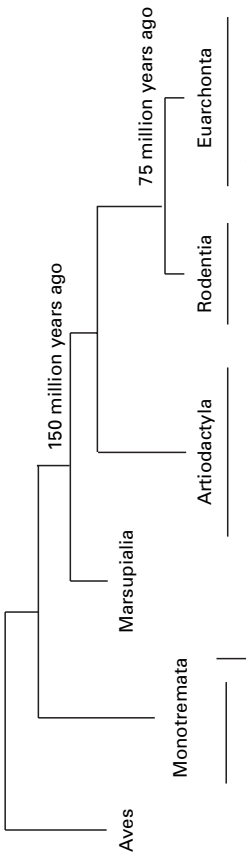
Evolution of *IGF2R* imprinting differs from that of *IGF2* imprinting

IGF2 and the gene encoding the receptor responsible for its degradation, *IGF2R*, are imprinted in most mammals. The evolutionary onset of the imprinting of both *IGF2* and *IGF2R* dates from the Jurassic era, approximately 150 million years ago (Killian et al., 2001c) (see Fig. 1). While neither *IGF2* nor *IGF2R* are imprinted in egg-laying animals (e.g., chickens of the Aves family) and the egg-laying mammals (e.g., platypuses and echidnas of the Monotremata clade) (O'Neill et al., 2000; Nolan et al., 2001; Yokomine et al., 2001), both genes are monoallelically expressed in marsupials (e.g., Virginia and gray short-tailed opossums: Killian et al., 2000, 2001a; O'Neill et al., 2000). *IGF2* is consistently imprinted in all mammals after the divergence from Monotremata (Killian et al., 2001b; Weidman et al., 2004). In contradistinction, *IGF2R* imprinting is maintained in artiodactyls (e.g., sheep, cow, pig: Young et al., 2001, 2003) and in rodents (mouse and rat), but biallelic expression of the gene appeared in the Euarchonta lineage which includes lemurs and humans following its divergence (Killian et al., 2001a, c) (Fig. 1). The potential mechanisms underlying the disappearance of *IGF2R* imprinting from the primate lineage over 75 million years ago are not well understood. Changes in some of the epigenetic marks at the *IGF2R* locus, including the differentially methylated regions of DNA (DMRs), histone modifications, and the presence/absence of *IGF2R* antisense message may underlie this evolutionary loss of *IGF2R* imprinting.

DNA methylation: allelic DNA methylation marks correlate with *IGF2R* imprinting in a number of species, but not in humans or opossums

Imprinting of mouse *Igf2r* depends upon the presence of an intron CpG island and an antisense transcript, *Air* (Wutz et al., 1997). The CpG island in intron 2 (region 2) of *Igf2r* is methylated on the maternal allele, which constitutes the differentially methylated region 2 (DMR2); DMR1 lies in the promoter region of the gene. DMR2 harbors a 113-bp methylation-imprinting box consisting of a de novo methylation signal (DNS) and an allele-discrimination signal (ADS) that may be involved in the establishment of its methylation (Birger et al., 1999); however, the mouse methylation-imprinting box has not been demonstrated in other species.

The presence of DMR2 correlates with *IGF2R* imprinting in rodents and in artiodactyls (sheep, cow, and pig). The absence of DMR2 also corresponds with the absence of *IGF2R* imprinting in ringtail lemur and tree shrew (Euarchonta), as well as the platypus (Fig. 1) (Killian et al., 2001b, c). In contrast, humans present an exception to this simple correlation: despite the presence of DMR2, human *IGF2R* is not imprinted in the great majority of the population (Kalscheuer et al., 1993; Smrzka et al., 1995; Riesewijk et al., 1996, 1998), The opossum (Marsupialia) presents yet another exception to this simple correlation: despite the absence of DMR2, opossum *IGF2R* is consistently imprinted (Killian et al., 2001c). This



Evolution in *IGF2R* imprinting and DNA methylation

		Allelic expression		Binding function of IGF2R protein		Region 2	
		IGF2	*IGF2R*	M6P-	IGFII-	Allelic DMR2	Presence of *Air*
Euarchonta	Human	Pat	Bi-allelic	+	+	Present	Absent
	Lemur	(Mono)	Bi-allelic	+	+	Absent	nd
	Tree shrew	(Mono)	Bi-allelic	+	+	Absent	nd
Rodentia	Mouse	Pat	Mat	+	+	Present	Present
	Rat	Pat	Mat	+	+	Present	nd
Artiodactyla	Sheep	Pat	Mat	+	+	Present	nd
	Cow	Pat	Mat	+	+	nd	nd
	Pig	Pat	Mat	+	+	nd	nd
Marsupialia	Opossum	(Mono)	(Mono)	+	+	Absent	nd
Monotremata	Platypus	Bi-allelic	Bi-allelic	+	No-site	Absent	nd
	Echidna	Bi-allelic	Bi-allelic	+	No-site	Absent	nd
Aves	Chicken	Bi-allelic	Bi-allelic	+	No-site	Absent	nd

Fig. 1. Divergent evolution in *IGF2* and *IGF2R* imprinting and phylogenetic distribution of DMR2/*Air*. The evolutionary onset of *IGF2* and *IGF2R* imprinting dates to approximately 150 million years ago, thus imprinting at these two loci evolved with the advent of live birth in Therian mammals (Killian et al., 2001b). Approximately 75 million years ago imprinting of *IGF2R* (i.e. maternal, monoallelic expression) disappeared in the Euarchonta lineage that includes the primates. Thus, while *IGF2R* is biallelically expressed in humans, *IGF2* is imprinted and expressed only from the paternal allele in all Therian mammals investigated. *IGF2R* encodes a receptor that contains a binding site (+) for M6P-containing glycoproteins in all species, whereas the binding site (+) for IGF2 is only present in Therian mammals. The presence of the allelic DMR in region 2 has not been verified in some species (nd = not determined). *Air* is present in mouse, absent in human, and has not been determined in other species. The horizontal line dividing the mammalian clades denotes the evolutionary onset of *IGF2* and *IGF2R* imprinting.

dissociation between *IGF2R* imprinting and the existence of a DMR2 is also evident in the Rodentia family, where *Igf2r* is biallelically expressed in CNS even in the presence of DMR2 (Wang et al., 1994; Hu et al., 1999). These lines of evidence indicate that DMR2 is neither necessary nor sufficient for imprinting of *IGF2R*. Thus, we speculated that histone modifications, rather than the DNA methylation, would be more consistently correlated with the imprint status of *IGF2R* (Vu et al., 2004).

Histone code: histone modifications mark the tissue-specific and species-specific imprinting status of *IGF2R* in human and mouse

Nucleosomes consist of DNA wrapped around an octamer of histones (four heterodimers of canonical histones H2A, H2B, H3, and H4). The extension of the histones' amino-terminal tails away from the central core allow for various modifications. These post-translational modifications include reversible acetylation, phosphorylation, ADP-ribosylation,

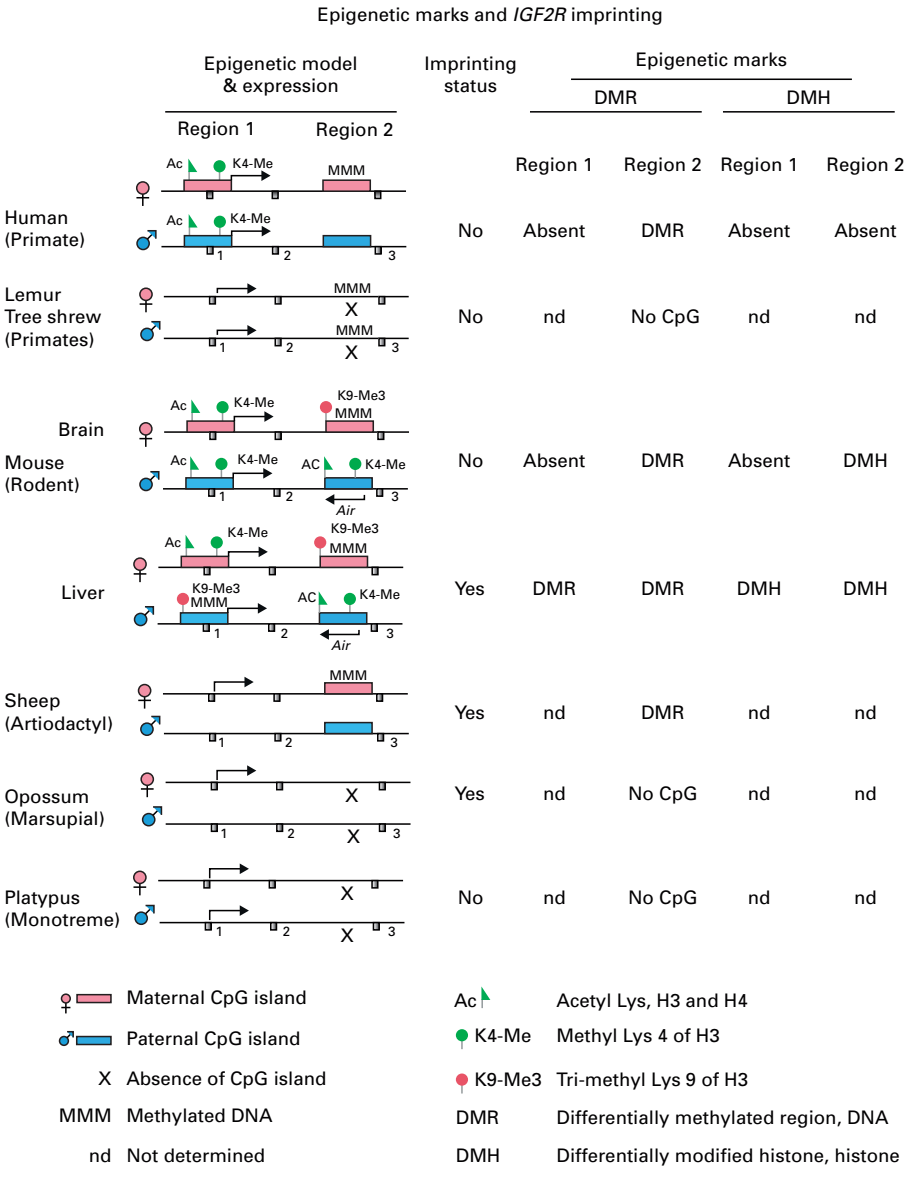


Fig. 2. DNA methylation and histone modification of *IGF2R*. Acetyl lysine (H3-Ac, H4-Ac) and methyl lysine 4 (H3-K4Me) mark the active allele in region 1 and region 2 of human and mouse *IGF2R*. DNA methylation and methyl lysine 9 (H3-K9Me) mark the silenced allele. Trimethyl lysine 9 (H3-K9Me3) marks the differentially methylated region 2 in mouse *Igf2r*. Differentially modified histone (DMH) of the two parental alleles correlates with *IGF2R* and *Air* imprinting in human and in mouse (brain and peripheral tissues). Histone modification has not been studied (nd = not determined) in species other than human and mouse. DMR in region 1 is present in mouse liver (DMR), but is absent in mouse brain and humans (absent), and has not been determined in other species. DMR in region 2 is present in human, mouse, and sheep (DMR); and absent (No CpG) in other species. Yamasaki and colleagues (2005) have recently established neurons and glial cells from primary cortical cultures. Glial cells demonstrated *Igf2r* imprinting with the epigenetic marks identical to those of peripheral tissues (shown here as the epigenetic model of mouse liver). Cultured neurons demonstrated a relaxation of *Igf2r* imprinting and its epigenetic model was compatible with the model of the mouse brain in this figure without the presence of *Air*. Lack of transcriptional activator(s) might correlate with the absence of the mouse *Air* in the neurons.

ubiquitination, and methylation. These modifications are specific for a particular histone and for specific amino acid residues. In particular, methylation of lysine and arginine represents a relatively stable modification. The array and combination of these histone modifications have been postulated to constitute a distinct histone code, which may govern the interaction between various proteins including the transcriptional initiation complex with target DNA sequences (Jenuwein and Allis, 2001; Spotswood and Turner, 2002; Turner, 2002; Grewal and Moazed, 2003). In a recent review, we have summarized the study of the histone code through the prism of the regulation of genomic imprinting (Hoffman and Vu, 2005).

We and others have used quantitative real-time PCR and chromatin immuno-precipitation (ChIP) assays with specific antibodies against various acetyl- and methyl-histones to study the tissue-specific and species-specific imprinting status of *IGF2R* in human and mouse (Fournier et al., 2002; Yang et al., 2003; Vu et al., 2004). These results are summarized in Fig. 2. Acetylated histone H3 (Lys9 and Lys14) and acetylated histone H4 (Lys5, 8, 12, and 16) are enriched in the human *IGF2R* promoter regions of both parental alleles of human *IGF2R*, correlating with the biallelic expression of *IGF2R* in human. In murine peripheral tissues such as liver, acetylated histones are enriched predominantly in the *Igf2r* promoter region of the maternally expressed allele. In contrast, the acetylated histones are present in both parental alleles in mouse brain (CNS) or in primary cultured neurons (Yamasaki et al., 2005), correlating with the murine tissue-specific biallelic expression of *Igf2r*. Allele-specific histone-acetylation was also observed in the mouse DMR2 that harbors the promoter for the antisense transcript *Air*, which is paternally expressed in CNS and in peripheral tissues. There is no differential histone acetylation between the parental alleles in

the human DMR2, and no human *IGF2R* antisense transcripts have been detected.

Methylation of Lys4 (H3-K4Me) and Lys9 (H3-K9Me) marks the active and silenced alleles, respectively. The patterns of histone methylation of the two parental alleles are similar to those of histone acetylation (Fig. 2). The lysine residues of histone H3 may be modified by mono-, di-, or trimethylation. Tri-methyl Lys9 (H3-K9Me3), but not di-methyl Lys9 (H3-K9Me2), specifically marks the suppressed maternal allele in the mouse DMR2 (region 2) harboring the antisense *Air* (Vu et al., 2004). In contrast to the allele-specific histone methylation in the mouse DMR2, there is no differential histone methylation between the two parental alleles in the human DMR. In summary, the available data indicate that histone modifications accurately mark the expressed and repressed alleles of the *IGF2R* in human and mouse.

IGF2R antisense: mouse *Igf2r* imprinting depends on an *Air* RNA while the existence of *AIR* in other species is unknown

In the mouse, an antisense non-coding transcript called *Air* is transcribed from DMR2 on the paternal allele, and this imprinted transcript plays a central role in *Igf2r* imprinting (Sleutels et al., 2002). *Air* is required for silencing *Igf2r* and two other maternally expressed, protein-coding genes on the paternal chromosome, *Slc22a2* and *Slc22a3.* Truncating 96% of the *Air* transcript by inserting a polyadenylation site disrupts *Igf2r/Slc22a2/Slc22a3* imprinting, but retains the methylation of DMR2 and the imprinting status of the truncated *Air* (Sleutels et al., 2002). Deleting or replacing the *Igf2r* promoter does not disrupt *Air/Slc22a2/Slc22a3* imprinting, suggesting an intrinsic *cis*-silencing property of the *Air* RNA (Sleutels et al., 2003). The mechanism whereby *Air* regulates imprinting, either through transcription of the *Air* gene or by direct involvement of *Air* RNA, has not yet been determined.

While the potential role of *IGF2R* antisense transcripts in other species has not been examined, a putative and functional *AIR*, if it exists, should be maternally imprinted and should originate in an intronic CpG island (DMR2). As shown in Fig. 2, the CpG island in region 2 is not present in all species. In addition to mouse and rat, human and sheep have the intronic DMR, but only the sheep maintains imprinting of *IGF2R* (Young et al., 2003). Human *AIR* RNA has not been detected in normal tissues or in placenta (Vu and Hoffman, 2000; Oudejans et al., 2001). Furthermore, the presence of an imprinted antisense *Air* transcript by itself does not absolutely determine the imprinting status of the *Igf2r*. In the mouse CNS, *Igf2r* is expressed biallelically despite the expression of the imprinted *Air* transcript (Hu et al., 1999). However, in primary cell cultures of mouse neurons, the levels of the *Air* transcript are very low or undetectable while *Igf2r* is not imprinted (Yamasaki et al., 2005).

Coordinating the epigenetic marks: the roles of histone modification over DNA methylation

DNA methylation and histone modification represent the major epigenetic marks of *IGF2R*. The presence of *IGF2R* DMR regions is limited to a few species, and histone modification data are limited to histone acetylation, H3-K4Me and H3-K9Me. Nevertheless, the study of human/mouse *IGF2R* suggests that enrichment of parental allele-specific histone acetylation and histone methylation in the promoter and DMR regions, rather than the presence of DMR2 or antisense transcripts, correctly identifies the species-specific and tissue-specific imprinting status of *IGF2R* in human and in mouse (Fig. 2).

How do these epigenetic marks coordinate to govern *IGF2R* imprinting? Histone modifications (H3-K9Me3) may interact with DNA methylation to mark a specific promoter on one parental allele for silencing of transcription. Transcription activation of the other parental allele requires histone acetylation and H3-K4Me in the promoter, in conjunction with the absence of H3-K9Me3 and the absence of DNA methylation. Therefore, the presence of repressive epigenetic marks such as H3-K9Me3 and DNA methylation is sufficient to silence the promoter, while their absence only frees its promoter from the suppression. The un-suppressed allele would require further active modifications, such as histone acetylation and H3-K4Me, and transcriptional activators for transcription to occur. The lack of active histone modifications in the human *IGF2R* therefore correlates with the absence of the *IGF2R* antisense transcript, *AIR*. Lack of transcriptional activator(s) might correlate with the absence of the mouse *Air* in neurons from primary cortical cultures (Yamasaki et al., 2005). The epigenetic model for the neurons would be similar to the model of the mouse brain in Fig. 2 without the presence of *Air*.

In conjunction with DNA methylation, histone modification constitutes a heritable epigenetic signature that is faithfully replicated in daughter cells after cell division (for a recent review, see Hoffman and Vu, 2005). How do these epigenetic marks establish and maintain as an 'imprint mark' during development? The mouse DMR2 methylation is acquired in the postnatal growing oocyte along with other paternally expressed genes *Snrpn*, *Peg1,* and *Peg2*. As shown in the establishment of methylation in the *Snrpn* DMR, after complete erasure of previous methylation, a de novo methylation mark is acquired asynchronously with epigenetic memory of parental origin (Lucifero et al., 2004), suggesting a role of epigenetic memory in the absence of DNA methylation. Histone modifications may serve as an epigenetic memory in the absence of DNA methylation. The histone modification patterns directly correlate with differential expression patterns of *IGF2R* in both human and mouse. DNA methylation does not control imprinted expression of all of the genes found in a cluster of imprinted genes located at the imprinting center 2 (IC2) on mouse chromosome 7 (Caspary et al., 1998; Tanaka et al., 1999). In embryonic stem (ES) cells and in extra embryonic tissues (placenta), the imprinting of distal genes (~700 kb region flanking the IC2) does not de-

pend upon DNA methylation, but is consistently associated with H3-K9Me and H3-K27Me in the silenced allele, and H3-KAc and H3-K4Me in the expressed allele (Umlauf et al., 2004).

Conclusion and future development

In summary, we have examined only a part of the gamut of epigenetic marks governing *IGF2R* imprinting in human and mouse. Overall, DNA methylation, histone acetylation, and histone methylation play a vital role in coordinating *IGF2R* allelic expression. The association of other modifications, including histone phosphorylation (H3-Serine10) in the active allele, H4-K20Me and H3-K27Me in the silent allele (Schotta et al., 2004; Martens et al., 2005), requires further study. In addition, the role of histone ADP-ribosylation (Yu et al., 2004) and the potential for chromatin looping (Murrell et al., 2004; Horike et al., 2005) may need to be addressed.

Finally, we have found that human *IGF2R* is not imprinted in more than 100 informative samples including various tumor tissues. The finding of rare monoallelic or skewed allelic expression may correlate with familial clustering of abnormal DNA methylation ratios (Sandovici et al., 2003). The possibility of slightly skewed ratios in allelic expression or DNA modifications and their biological importance warrants further rigorous study.

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