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-phosphate (M6P) receptor, as it binds a variety of M6P-containing glycoproteins, shuffling 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...
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: Kalscheuer 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 suppressor gene, i.e. loss of a single allele might lead to an oncogenic diathesis suppressor (De Souza et al., 1995; Hanksins 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; Hanksins 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 (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 lemurms 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
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 tissuespecific 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,
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

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.
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 tri-methylation. 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, 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 modifications constitute 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 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 (Caspar 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 imprint- ed 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.

References


