Non-imprinted \(Igf2r\) expression decreases growth and rescues the \(Tme\) mutation in mice

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SUMMARY

In the mouse the insulin-like growth factor receptor type 2 gene (\(Igf2r\)) is imprinted and maternally expressed. \(Igf2r\) encodes a trans-membrane receptor that transports mannose-6-phosphate tagged proteins and insulin-like growth factor 2 to lysosomes. During development the receptor reduces the amount of insulin-like growth factors and thereby decreases embryonic growth. The dosage of the gene is tightly regulated by genomic imprinting, leaving only the maternal copy of the gene active. Although the function of \(Igf2r\) in development is well established, the function of imprinting the gene remains elusive. Gene targeting experiments in mouse have demonstrated that the majority of genes are not sensitive to gene dosage, and mice heterozygous for mutations generally lack phenotypic alterations. To investigate whether reduction of \(Igf2r\) gene dosage by genomic imprinting has functional consequences for development we generated a non-imprinted allele (\(R2\Delta\)). We restored biallelic expression to \(Igf2r\) by deleting a critical element for repression of the paternal allele (region 2) in mouse embryonic stem cells. Maternal inheritance of the \(R2\Delta\) allele has no phenotype; however, paternal inheritance results in biallelic expression of \(Igf2r\), which causes a 20% reduction in weight late in embryonic development that persists into adulthood. Paternal inheritance of the \(R2\Delta\) allele rescues the lethality of a maternally inherited \(Igf2r\) null allele and a maternally inherited \(Tme\) (\(T\)-associated maternal effect) mutation. These data show that the biological function of imprinting \(Igf2r\) is to increase birth weight and they also establish \(Igf2r\) as the \(Tme\) gene.

Key words: Genomic imprinting, CpG island, DNA methylation, Mouse

INTRODUCTION

Imprinted genes are epigenetically silenced on one of the two parental chromosomes in diploid embryonic cells. More than 30 protein-coding genes, plus a growing number of non-coding RNAs, have now been shown to be imprinted in both mice and humans (Beechey and Cattanach, 2000; Jirtle, 2000). Approximately half of this number have been studied by gene inactivation in mice and the majority have been shown to influence the transfer of maternal resources to the embryo or neonate. These results, combined with the analysis of human imprinting disorders, have been used to support the proposal that the biological function of imprinting in mammals is to regulate the growth of the developing embryo in utero and of the neonate during the suckling period (Falls et al., 1999; Tilghman, 1999). An early, perceptive observation on the first two identified imprinted genes has been the basis for a hypothesis that imprinting evolved in mammals because of the conflicting interests of the maternal and paternal genome, in relation to the transfer of nutrients from the mother to her offspring (Haig and Graham, 1991; Moore and Haig, 1991). One prediction from this hypothesis is that paternally expressed imprinted genes would enhance fetal growth at maternal expense while maternally expressed genes would have the opposite action. The majority of imprinted genes do behave as predicted, but some fail to generate a growth phenotype when inactivated in mice, leaving open the possibility of other explanations for the evolution of imprinting in mammals (Hurst and McVean, 1998).

With the recent advances in understanding the imprinting mechanism (Reik and Walter, 1998) it has now become possible to design experiments to restore biallelic expression to imprinted genes by removing the defined imprint regulatory element. This type of experiment provides an additional tool to study the biological significance of imprinting. The absence or presence of a phenotype upon restoring expression of an imprinted gene to both parental gene copies would increase understanding of the function of imprinting in mammalian development.

The mouse \(Igf2r\) (insulin-like growth factor receptor type 2,
also called the cation-independent mannose-6-phosphate receptor) gene is specifically maternally expressed from the early postimplantation stage (Lerchner and Barlow, 1997). The mammalian Igf2r gene evolved from the ancestral vertebrate cation-independent mannose-6-phosphate receptor (Cl-MPR) gene, and subsequently acquired a binding site with affinity for Igf2 (Munier-Lehmann et al., 1996). Inactivating mutations of Igf2r in mice lead to increased systemic Igf2 levels that cause embryonic overgrowth and perinatal lethality (Lau et al., 1994; Ludwig et al., 1996; Wang et al., 1994). Consistent with this, overexpression of Igf2r in a tissue-specific manner was shown to cause a local reduction in organ size (Zaina et al., 1998). Thus, Igf2r can titrate extracellular levels of Igf2, and so regulate the growth-promoting function of Igf2.

We have previously shown that a CpG-island in intron 2 of Igf2r (named region 2) carries a maternal-specific methylation imprint that could act as an imprinting signal to maintain expression of the maternal allele (Stöger et al., 1993). Consistent with this, genomic DNA methylation is necessary for Igf2r expression from the maternal allele (Li et al., 1993). Region 2 is now known to contain the promoter of the Air (Antisense Igf2r RNA) transcript. Air is a large non-coding RNA transcribed only from the paternal allele, in the opposite direction to Igf2r, which overlaps the Igf2r promoter and the next upstream gene (Lyle et al., 2000; Wutz et al., 1997). Using yeast artificial chromosome (YAC) transgenes containing the entire Igf2r locus plus flanking DNA on a 300 kb fragment, we have previously identified region 2 as a key imprint regulatory element by showing that deletion leads to Igf2r expression independent of parental origin (Wutz et al., 1997). However, since this YAC carried an inactive Igf2r allele, this experiment did not test the biological consequences of biallelic Igf2r expression. In this study, we generate a non-imprinted Igf2r allele in mice by targeted deletion of region 2 to investigate the effect of loss of Igf2r imprinting on mouse development. We show that mice expressing Igf2r biallelically are viable and fertile, with a 20% reduced weight at birth that persists into adulthood. Complementation tests performed with mice carrying either the Igf2r null mutation or the Tme mutation demonstrated that paternal expression of the region 2 deleted allele is functionally equivalent to wild-type maternal expression.

**MATERIALS AND METHODS**

**ES cell culture and generation of chimaeric mice**

E14 ES cells (Doetschman et al., 1987) were grown on mouse embryonic fibroblasts in DMEM, 15% fetal calf serum and 250 units/ml LIF (Wang et al., 1994). 10×10⁶ cells were electroporated with 30 µg NotI linearized R2ΔNT targeting vector using a Gene Pulser (Biorad) set to 960 µF and 200 V, and selected in 0.2 µg/ml G418 (Sigma) for 8 days. Colonies were divided, one part was grown in a 96-well plate and the other was used for PCR analysis (Wang et al., 1994). Targeting of R2ΔNT was screened by nested PCR using the primer sets 5’-GCTGGGTTATCTCCCAATCACG-3’ and 5’-TATCCGGGACGTTTTACCC-3’; and 5’-CTGAGGGCTTACCCAGCATC-3’ and 5’-CTTACATGGGACCATGACG-3’, and correctly targeted clones (4 of 96) were expanded and confirmed by Southern analysis. Subsequently, the selection cassette was removed by transient transfection with 30 µg Cre expression plasmid (Gu et al., 1994) and selection in medium containing 2 µM gancyclovir.

**Plasmid construction and probes**

For the region 2 targeting vector a 1.5 kb Xbal-BamHI from Igf2r intron 2 and a 6 kb XhoI fragment containing parts of introns 2 and 3 were cloned into pBluescript (Stratagene). A Neo/Tk selection cassette flanked by loxP sites, constructed from plasmid pGHI (Gu et al., 1994) and PBS246 (Sauer, 1998), was inserted between the BamHI and the PacI sites. A 1.5 kb XhoI-Sall fragment containing the diphtheria toxin counter selection marker (Yagi et al., 1990) was inserted into the XhoI site giving the R2ΔNT targeting vector. Probe p1.5BX is a 1.5 kb Xbal-BamHI fragment and probe PM1K a 1 kb MinK-Prul fragment from Igf2r intron 2. Probe ISM5a is a 600 bp Smal fragment from region 1 (Stöger et al., 1993). Probe TL contains a 3’ part of the Igf2r cDNA (Wutz et al., 1997). Gapdh was detected with an 850 bp Xbal-AatI fragment of the cDNA.

**DNA, RNA analysis and PCR primers**

RNA was purified using the guanidinium thiocyanate/CsCl protocol (Sambrook et al., 1989) or Trizol reagent (Gibco), according to the manufacturer’s recommendations. Ribonuclease protection analysis was performed as described (Wutz et al., 1997). For the allele-specific RT-PCR analysis of Igf2r, 10 µg total RNA were reverse-transcribed using Superscript II (Gibco) and PCR was performed as described (Hu et al., 1998). The genotypes of mice were established from tail biopsies. The Igf2r null allele (Wang et al., 1994) was identified using an 800 bp Fse-BamHI fragment of the neomycin resistance gene. The Thp mutation was identified by the tail phenotype and confirmed by assessing the methylation patterns of region 1 and region 2. The R2Δ allele was identified by Southern analysis (see Fig. 1D) or by PCR using the primers 5’-CCCCATATGTTTTCAAGGACGTG-3’ and 5’-GGGACGTGGCGCTTTCAC-3’.

**Quantification of Igf2r mRNA expression**

RNA was extracted from E11.5 and E13.5 whole embryos (DNA from membranes was used to determine the genotype), or from 3-week-old heart (animal genotyped from liver DNA) using Trizol reagent (Gibco) according to the manufacturer’s instructions. Slot blots were prepared using 5 µg total RNA from each sample and hybridized with probe TL. The signal was detected using a FUJIX BAS 2000 Bio Imaging Analyzer and quantified using the Image reader (version 1.4) and Image Gauge (version 3.0) software (Fuji Film Co., Ltd). The slot blots were subsequently hybridized with a Gapdh probe to control for loading.

**RESULTS**

**Generation of mice lacking Igf2r region 2**

To investigate the effect of loss of Igf2r imprinting in vivo we removed region 2 by homologous recombination in ES cells. The targeting vector R2ΔNT was constructed to delete a 3.7 kb fragment containing the entire CpG-rich sequences of region 2, but potential sequences involved in splicing were left intact (Fig. 1A). In order to remove extraneous sequences from the targeted allele a neomycin/thymidine kinase selection cassette (NeoTk) flanked by loxP sites was used (Gu et al., 1994). E14 ES cells were transfected with the linearized R2ΔNT targeting vector, 96 neomycin-resistant colonies were screened by PCR, and four were identified to contain a targeted allele, which was confirmed by Southern analysis (Fig. 1B). Targeted ES cell clones were transiently transfected with a Cre recombinase expression plasmid to excise the selection cassette (Gu et al., 1994). ES cells selected with gancyclovir contained the region 2 deletion allele (R2Δ), which was identified by Southern analysis (Fig. 1C). Injection of two independently
Non-imprinted \textit{Igf2r} expression in mice

1883

derived ES clones into C57/Bl6 blastocysts yielded a total of 21 chimaeric mice with 60\% to 100\% contribution from the ES cells as judged by coat color. Chimaeras bred to C57/Bl6 and 129Sv females transmitted the \textit{R2}^D allele to their offspring (Fig. 1D). Mice inheriting the \textit{R2}^D allele from either parent or homozygous mutants were viable, healthy and fertile. Results obtained from the two independent ES clones were equivalent and the data are presented together in this manuscript.

Region 2 deletion restores endogenous paternal \textit{Igf2r} expression

To study the effect of region 2 deletion on the endogenous \textit{Igf2r} locus, we analyzed expression of \textit{Igf2r} and \textit{Air} from the R2\(\Delta\) allele in mice (Fig. 2). Slot blot and ribonuclease protection assay analysis of RNA from embryo and adult heart showed that \textit{Igf2r} was expressed from the R2\(\Delta\) allele (Fig. 2A,B).

To study the expression of the \textit{Air} RNA we used a 1.5 kb fragment from \textit{Igf2r} intron 2, which was not deleted on the R2\(\Delta\) allele. \textit{Air} expression was detected by slot blot analysis in RNA from mice with a maternally inherited \textit{Mus spretus} \textit{Igf2r} allele and a paternally inherited R2\(\Delta\) allele (lanes 1-3), or control wild-type mice inheriting a paternal (lane 4) or maternal (lane 5) \textit{Mus spretus} \textit{Igf2r} allele, and control \textit{Mus musculus} (lane 6) and \textit{Mus spretus} mice (lane 7). A 261 bp PCR product was obtained using a radioactively labeled primer and digested with \textit{HaeIII} to reveal the polymorphism (\textit{M. mus}, 138 bp; \textit{M. sp}, 98 bp).

(E) Methylation of region 1 was analysed by Southern analysis of \textit{StuI} and \textit{SmaI} digested DNA from mice inheriting the \textit{Igf2r} wild-type (+) or R2\(\Delta\) (\textit{D}) allele (maternally inherited allele is first) hybridized to probe I5Sma. A methylated \textit{SmaI} site is identified by the 1 kb band. (F) Region 2 methylation was assayed on \textit{PvuII} and \textit{MluI} digested DNA (as in E) using probe PM1K. A 3 kb fragment indicates a methylated \textit{MluI} site.
et al., 2000). In order to examine if deletion of region 2 affected splicing of \textit{Igf2r} intron 2, we performed a ribonuclease protection experiment using a probe spanning \textit{Igf2r} exons 2 and 3 (Fig. 2C). Using adult heart RNA from mice inheriting a R\(\Delta\) allele from their mother we detected the correct protected 3 (Fig. 2C). Using adult heart RNA from mice inheriting a R\(\Delta\) \(\Delta\) deletion of region 2 causes biallelic expression of \textit{Igf2r} if inherited from the father. These data demonstrate that was methylated if inherited from the mother and unmethylated \(\Delta\) II sites (Fig. 2F) and was found unchanged. Thus, region 2 indeed cause biallelic \textit{Igf2r} expression, we made use of a previously described restriction fragment length polymorphism between the \textit{Mus musculus} and \textit{Mus spretus} \textit{Igf2r} cDNA located in exon 48 (Hu et al., 1998). To distinguish between the two \textit{Igf2r} alleles we bred a \textit{Mus spretus} wild-type chromosome 17 into mice with a paternally inherited R\(\Delta\) allele. Allele-specific RT-PCR analysis of adult lung RNA showed that both the maternally inherited \textit{Mus spretus} and the paternally inherited R\(\Delta\) allele-derived \textit{Igf2r} messenger RNAs were produced in comparable amounts (Fig. 2D). No background expression of the paternal \textit{musculus} or \textit{spretus} \textit{Igf2r} allele was observed in control \textit{M. musculus} \textit{M. spretus} hybrid mice (Fig. 2D, lanes 4-5). Full expression of the maternal and paternal alleles in R\(\Delta\)+ mice was also supported by quantitation of the steady state \textit{Igf2r} mRNA levels following slot blot hybridization using E11.5 and 13.5 day embryos, and adult heart RNAs as described in Materials and Methods (data not shown). These results were as follows: E11.5 embryos: \(+/R\Delta\) (\(n=5\)) 1.7±0.4; control R\(\Delta\)+ (\(n=3\)) 1.0±0.1; \(+/+\) (\(n=3\)); E13.5 embryos: \(+/R\Delta\) (\(n=3\)) 1.8±0.3; \(+/+\) (\(n=3\)); heart of 3-week-old animals: \(+/R\Delta\) (\(n=2\)) 2.3±0.5; R\(\Delta\)/R\(\Delta\) (\(n=2\)) 2.4±0.1; \(+/+\) (\(n=6\)); (Gapdh was used to control for slot blot loading; wild-type expression level was set to 1, \(n\) is the number analyzed per genotype, values are means ± s.d.). Overall this analysis demonstrated that mice with a paternally inherited R\(\Delta\) allele expressed twice as much \textit{Igf2r} RNA as wild-type controls, which is attributable to equal expression of both the maternal and paternal \textit{Igf2r} alleles.

We next studied the methylation pattern of region 2 and of the \textit{Igf2r} promoter CpG island (region 1) that becomes specifically methylated on the paternal allele during late development, in various tissues of mice inheriting the R\(\Delta\) allele and in wild-type controls. The methylation of region 1 was assessed on the methylation sensitive \textit{SmaI} and \textit{NotI} sites and inversely correlated with expression of \textit{Igf2r} (Fig. 2E). Thus, region 1 was completely unmethylated in mice inheriting a R\(\Delta\) allele from their father, in both heterozygous and homozygous mutants. Methylation of region 2 on the wild-type allele in heterozygous R\(\Delta\) mutants was assessed on \textit{MluI} and \textit{HpaII} sites (Fig. 2F) and was found unchanged. Thus, region 2 was methylated if inherited from the mother and unmethylated if inherited from the father. These data demonstrate that deletion of region 2 causes biallelic expression of \textit{Igf2r} in animals inheriting the R\(\Delta\) allele from their father, which coincides with the absence of methylation on region 1. The wild-type \textit{Igf2r} allele in \textit{trans} is unchanged.

**Biallelic \textit{Igf2r} expression reduces growth in embryos, neonates and adults**

We used the R\(\Delta\) allele to study the effect of biallelic \textit{Igf2r} expression on growth by measuring weight. As expected, maternal inheritance of the R\(\Delta\) allele did not significantly affect the weight of animals compared to wild-type littermates (Fig. 3A). The average weight differed by less than 6%, which was statistically not significant. Paternal inheritance and hence biallelic \textit{Igf2r} expression caused a mean reduction of weight by 20% in animals heterozygous for the R\(\Delta\) allele, which was statistically significant (Fig. 3A). The weight difference became apparent between E15.5 and E17.5 (Fig. 3B) and continued after birth into adulthood (Fig. 3C). We also determined the weight of placentae of mutant and wild-type day 17.5 embryos and could not find a statistically significant difference (placental weight +/-R\(\Delta\): 0.082±0.009 g (\(n=11\)); +/-: 0.084±0.013 g (\(n=10\)); \(P\) (same weight) = 0.662), suggesting that the reduced weight arising from \textit{Igf2r} overexpression was not due to a limited amount of nutritional supply via the placenta, but due to a direct effect on embryonic growth.

**Complementation of the \textit{Igf2r} null allele and of \textit{Tme} with a non-imprinted \textit{Igf2r}**

We next performed two crosses to test if weight reduction in mice
inheriting a paternal R2Δ allele was caused by biallelic Igf2r expression as opposed to the absence of the Air transcript, and to test if expression from the paternal R2Δ allele was functionally equivalent to a wild-type maternal allele. Table 1 shows that a paternal R2Δ allele efficiently rescued a maternally inherited Igf2r null allele and these compound mutant mice were viable and fertile. The weight of compound heterozygous animals null allele and these compound mutant mice were viable that deletion of region 2 restores the normal expression level of Igf2r maternally inherited able to fully complement the loss of expression from the experiment shows that the paternally inherited R2 allele efficiently rescued a maternally inherited T hp and fertile. The weight of compound heterozygous animals null allele and these compound mutant mice were viable that deletion of region 2 restores the normal expression level of Igf2r maternally inherited able to fully complement the loss of expression from the experiment shows that the paternally inherited R2 allele efficiently rescued a maternally inherited T hp and fertile, and males bred to wild-type mice transmitted the T hp allele (Table 1). In contrast, we never observed live offspring of each genotype is listed and the combined data is given.

Table 1. Rescue of Tme by a non-imprinted Igf2r allele

<table>
<thead>
<tr>
<th></th>
<th>Thp/+</th>
<th>Thp/R2Δ</th>
<th>+/+</th>
<th>+/R2Δ</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Igf2r null allele</td>
<td>0</td>
<td>18</td>
<td>30</td>
<td>34</td>
<td>82</td>
</tr>
<tr>
<td>Combined rescue</td>
<td>Tme/+</td>
<td>Tme/R2Δ</td>
<td>+/+</td>
<td>+/R2Δ</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>47</td>
<td>55</td>
<td>50</td>
<td>152</td>
</tr>
</tbody>
</table>

Males heterozygous for the R2Δ allele (R2Δ/+) were crossed with females heterozygous for a Igf2r null mutation (+/−) or a Thp deletion (+/Thp).

The number of offspring of each genotype is listed and the combined data is given.

The data in this study suggest that these other antisense RNAs may also be linked to a functional imprint regulatory element.

Recently the Igf2r gene in the marsupial opossum has been shown to be imprinted and maternally expressed, yet to lack region 2 (Killian et al., 2000). The absence of any intron 2 CpG island corresponding to region 2 has cast doubt on its function in the endogenous mouse locus, despite previous transgenic experiments that demonstrated a role for region 2 (Wutz et al., 1997). The confirmation here, that region 2 is part of the imprint regulatory element at the endogenous locus, means that comparisons between opossum and mouse will be of great interest in identifying additional or different components of the imprinting mechanism. The fact that CpG island methylation is not linked to gene repression in marsupials, on the X chromosome at least (Loebel and Johnston, 1996), may indicate that neither methylation nor a CpG island is required to keep the maternal Igf2r allele active in marsupials. The alternative possibility that marsupials and placental mammals have distinct mechanisms imprinting Igf2r must also be investigated.

Igf2r is responsible for Tme

Maternal transmission of the Thp mutation, a 4 cM deletion on chromosome 17 encompassing Igf2r, leads to embryonic lethality between E13.5 and E18.5, due to absence of the Tme locus, in laboratory mouse strains. Paternal transmission of Thp is viable and fertile but displays a bent tail due to haploidy at the Brachyury locus (Johnson, 1975). We show that the non-imprinted R2Δ Igf2r allele can rescue the lethality associated with a maternally inherited Igf2r null mutation or a Thp deletion. Despite the reduced numbers of Thp/R2Δ offspring recovered (most likely due to additional deleterious effects of other genes within the large Thp deletion), this result supports the claim that Igf2r is the gene responsible for Tme. We cannot at this stage formally rule out that region 2 and Air regulate imprinting of other genes that may also contribute to the Tme phenotype.

DISCUSSION

We have generated an allele of Igf2r (R2Δ) that is not subject to genomic imprinting in order to test the biological function of imprinting at this locus. The decreased weight of mice with biallelic Igf2r expression (+/R2Δ), compared with wild-type mice that have imprinted maternal-specific expression (+m/+p), shows that Igf2r imprinting causes an increase in weight of embryos, neonates and adults.

A comparison of +/R2Δ mice with a reduced weight phenotype (that have biallelic Igf2r expression but no Air expression) with −/R2Δ mice with a wild-type body weight (that have paternal-specific Igf2r expression and no Air expression), indicates that the Air RNA plays no direct role in growth. These results support previous arguments (Lyle et al., 2000) that the non-coding Air transcript should not be considered as a 'gene', but part of the mechanism regulating the protein-coding Igf2r gene. Antisense RNAs have also been found overlapping three other imprinted protein-coding genes; UBE3A, KvLQT1 and GNAS1, in a similar fashion to the Air RNA (maternal expression of the coding gene and paternal expression of the cognate antisense non-coding transcript; Hayward and Bonthron, 2000; Lee et al., 1999; Rougeulle et al., 1998; Smilinich et al., 1999; Wroe et al., 2000). The data in this study suggest that these other antisense RNAs may also be linked to a functional imprint regulatory element.
genetic screen, employing crosses with Mus musculus wild mice, identified modifiers of the insulin-like growth factor pathway.

**Effect of Igf2r imprinting on growth**

It is generally assumed that the main function of Igf2r in embryonic growth is to regulate Igf2 levels. Igf2r also binds insulin and Igf1, though with lower affinity. It has been shown that the effect of insulin signalling on embryonic growth is small (Louvi et al., 1997), such that the insulin pathway might be neglected in the context of Igf2r function. Igf1 and Igf2 act synergistically in embryogenesis, and their effects seem to be pronounced at different developmental intervals. Igf2 but not Igf1 also regulates placental weight by signalling through InsR (Baker et al., 1993). We observed an effect of biallelic Igf2r expression on growth at E17.5. Previously, it has been shown that mutation of Igf2r leads to elevated levels of Igf2 from E12.5 onwards (Ludwig et al., 1996), and affects embryonic growth by E13.5. Although Igf2r affects the level of Igf2 by day 12.5 of gestation in wild-type embryos, biallelic Igf2r expression did not cause a weight reduction at this stage. We suggest that Igf2 is produced in excess between E12.5 and E17.5 such that Igf2r overexpression is not limiting Igf2 levels. Consistently, biallelic Igf2r expression did not affect placental weight, most likely because the placental growth phase ends around E16.5 in mouse (Baker et al., 1993). From this we conclude that imprinting, and consequently monoallelic expression, affects an increase in intrauterine growth only shortly before birth.

These data show that, in the mouse, region 2 is dispensable for expression of the maternal Igf2r allele, but mediates repression of the paternally inherited allele, thus enabling genomic imprinting of Igf2r. Methylation of region 2 on the maternally inherited allele is involved in inactivation of the repression mechanism, thus keeping the maternal Igf2r allele active (Li et al., 1993). The fact that region 2 can be removed without affecting expression of Igf2r implies that imprinting and expression are controlled by separate elements. This may suggest that imprinting was imposed in a modular fashion and future experiments are necessary to clarify this point.

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