Rescue of the T-associated maternal effect in mice carrying null mutations in \( \text{Igf-2} \) and \( \text{Igf2r} \), two reciprocally imprinted genes

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SUMMARY

In mice, only the paternal allele of the \( \text{Igf2} \) gene, encoding insulin-like growth factor II (IGF-II) is expressed due to parental imprinting. Interestingly, the \( \text{Igf2r} \) gene, which encodes one of the two known receptors (IGF2R) to which IGF-II binds with high affinity is also subject to imprinting, but in a reciprocal fashion. This observation raises the possibility that imprinting of these loci serves to regulate the ratios of the gene products, since IGF2R provides a mechanism for IGF-II turnover. To test this hypothesis, we crossed mice mutant for \( \text{Igf-2} \) with animals carrying the \( T^{hp} \) chromosomal deletion, which encompasses the \( \text{Igf2r} \) locus. Inheritance of the \( T^{hp} \) chromosome through the maternal germline results in a dominant lethal maternal effect (\( T^{\text{me}} \)). However, as we show here, \( T^{hp}/+ \) embryos that inherit the \( T^{hp} \) maternally are variably rescued to birth if they also lack IGF-II. Based on these data, the \( T^{\text{me}} \) phenotype can be viewed as a dominant effect resulting from an overabundance of IGF-II.

Key words: parental imprinting, insulin-like growth factor 2, T-maternal effect, mouse genetics
MATERIALS AND METHODS

Mice

The Igf-2 mutant animals were generated by targeted gene disruption in embryonic stem cells derived from 129/Sv/Ev inbred mice (DeChiara et al., 1990). Subsequently, the mutation has been maintained on this inbred background. For control crosses, we used wild-type 129/Sv/Ev animals from our own colony. C3H/HeSNJ mice carrying the hp allele were provided by Eva Eicher (Jackson Laboratories, Bar Harbor, ME).

DNA and RNA analysis

For genotyping, high molecular weight DNA was prepared from either tail or skin biopsies and analyzed by Southern blotting, as described (DeChiara et al., 1990). DNA samples were digested to completion with PvuII, transferred onto nylon membranes and hybridized with the probe pRB2 (a gift from Denise Barlow). This DNA fragment, derived from a cosmid clone containing the D17Rpl17 marker (Barlow et al., 1991), recognizes polymorphic differences between the Igf2r alleles carried by the 129/Sv and C3H mouse strains.

Total cell RNA was prepared by the guanidinium thiocyanate/cesium chloride procedure (Chirgwin et al., 1979). Reverse transcription PCR was employed to detect expression of the Igf-2 and Igf2r mRNAs. Since the mutation at the Igf-2 locus was generated by replacing the first coding exon (E2) with a neomycin-resistance cassette (DeChiara et al., 1990), expression of the maternal and paternal alleles can be monitored using primers specific for either the neo or the E2 sequence in combination with a 5′ primer. The RT-PCR analysis was performed as follows. Total RNA (200 ng) was reverse transcribed at 67°C (for β-actin and Igf-2 analyses) or at 56°C (for Igf2r samples) for 20 minutes with rTth Reverse Transcriptase (Perkin Elmer, Cetus). The samples were then primed with 20 pmoles of gene-specific 3′ oligonucleotide primer and 5 units of enzyme in a 20 μl reaction. After completion of first strand synthesis, 20 pmoles of the 5′ gene-specific primer were added and the mixture amplified for 34, 28, 20 and 20 cycles for Igf2r, neo, E2 and β-actin, respectively. All reactions were carried out according to the manufacturers’ recommendations. The PCR amplification products were analyzed on a 5% non-denaturing polyacrylamide gel and visualized by ethidium bromide staining.

The gene-specific oligonucleotide primers used were as follows: Igf2r 5′ primer: 5′-TGTACACTCTTCTCTTCTCTCTGTGCA-3′; 3′ primer: 5′-AGAGATGTTGTAGTAAAGACAGG-3′; Igf-2 5′ primer: 5′-TCTGTCTCTTCATCTTCTCCGCCCTC-3′; 3′ primer: 5′-CGGTCCGAACAGCCAAACTGAAAGTCTG-3′; neo 5′ primer: 5′-ATCCATCATGTTGAGGGCAGGCC-3′; 3′ primer (neo-specific): 5′-AGGCACCAAGGGTGTGATGGTGG-GAAT-3′; β-actin 5′ primer: 5′-AGGCACCAAGGGTGTGATGGTGG-GAAT-3′; 3′ primer: 5′-TTCACGTTGGCCTTAGGGTTCAGGG-3′.

RESULTS

Control genetic crosses

For our experiments, we used animals of two genetically defined strains of mice known to carry mutations at the Igf-2 and Igf2r loci, respectively. The first strain were inbred 129/Sv animals carrying a targeted mutation at the Igf-2 locus (DeChiara et al., 1990). We also used C3H mice carrying the hp form of chromosome 17 (Fig. 1). Transmission of the hp chromosome can be monitored phenotypically due to the absence of the semidominant T allele that affects tail development. Thus, hp/+ animals possess a ‘hairpin’ tail that is typically reduced in length and either blunt or slightly kinked at the tip (Johnson, 1974). Since penetrance of the Tme-associated lethality appears to be influenced by genetic background effects (Johnson, 1974; Tsai and Silver, 1991; Forejt and Gregorova, 1992), we first examined the possible contribution of background gene(s) that might complicate the interpretation of our experiment.

Reciprocal test crosses were set up between C3H Thp carriers and wild-type 129/Sv mice. Approximately 50% (23/43) of the viable pups recovered in crosses between 129/Sv females and C3H Thp/+ males, were readily classified as Thp heterozygotes. In the cross between C3H Thp/+ females and 129/Sv males, all 41 liveborn progeny exhibited...
a normal tail (Table 1). However, as the hairpin tail phenotype can be very subtle, genotypes of eleven randomly selected pups were confirmed using DNA probes that detect allelic polymorphisms in the Igf2r loci of C3H and 129/Sv strains. Southern analysis confirmed that these phenotypically wild-type animals had the predicted restriction pattern (data not shown). An additional five dead pups were recovered (approximately 11% of animals scored) that appeared to have hairpin tails. These animals were presumed to be \( T^{hp}/+ \) heterozygotes, although the advanced state of tissue decay precluded DNA analysis. Previous experiments similarly indicate that a small minority of embryos (5-10%) carrying a maternally derived \( Tme \) chromosome can reach an advanced gestational age (Johnson, 1974; McGrath and Solter, 1984). To confirm that the majority of the \( T^{hp} \) heterozygotes were indeed failing in utero, we recovered embryos at 15.5 days and 17.5 days post coitum. Four out of the eleven embryos examined were clearly developmentally abnormal and oedemic and displayed the hairpin tail phenotype. Genotyping of three of these abnormal embryos confirmed that they had all inherited the maternally derived \( T^{hp} \) chromosome. These results show that the \( Tme \)-associated lethality is observed in matings between 129/Sv and C3H inbred mouse strains at the expected frequency.

**Table 1. Genotypes of progeny obtained from crosses between C3H \( T^{hp/+} \) females and 129 Sv males**

<table>
<thead>
<tr>
<th>Experiment cross: ( C3H T^{hp/+} ) females×129/Sv Igf-2(^{-} ) males</th>
<th>Control cross: ( C3H T^{hp/+} ) females×wild-type 129/Sv males</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of litters scored</td>
<td>23</td>
</tr>
<tr>
<td>Number of pups available for scoring</td>
<td>114</td>
</tr>
<tr>
<td>Number of normal pups</td>
<td>76</td>
</tr>
<tr>
<td>Number of overt ( T^{hp/+} ) pups</td>
<td>38*</td>
</tr>
<tr>
<td>Killed for analysis</td>
<td>6</td>
</tr>
<tr>
<td>Stillborn</td>
<td>10</td>
</tr>
<tr>
<td>Dead after 24 hours</td>
<td>10</td>
</tr>
<tr>
<td>Dead after 48 hours</td>
<td>6</td>
</tr>
<tr>
<td>Surviving &gt;48 hours</td>
<td>6‡</td>
</tr>
</tbody>
</table>

*There was a significantly larger fraction of \( T^{hp/+} \) pups born in the cross between \( T^{hp/+} \) C3H females and Igf-2\(^{-} \) 129 Sv males \((P<0.005, \) Chi squared test of association).†Deaths recorded at 4, 5, 11, 15 and 35 days; one animal survived to adulthood.‡The observed offspring ratio departs significantly from the expected ratio of 1:1 with a \( P \) value of <0.001 (Chi squared test).

**Fig. 2.** Schematic representation of the proximal portion of chromosome 17. Genetic map showing the break points of the \( T^{hp} \) and \( Tme \) deletions that define the \( Tme \) region.

**Fig. 2.** Phenotypic appearance of live born pups. 1-day-old progeny obtained from a cross between C3H \( T^{hp/+} \) female and an Igf-2\(^{-/-} \) male. All of the progeny are growth deficient owing to the absence of the IGF-II growth factor. The animal that has a phenotypically normal tail carries the intact maternally derived copy of chromosome, while those with a vestigial tail have inherited the maternal \( T^{hp} \) chromosome.

**Variable rescue of the \( Tme \) embryonic lethality in an Igf-2 null background**

We next examined whether the embryonic lethality associated with inheritance of the \( T^{hp} \) chromosome through the female germline could be rescued in animals that additionally lack a functional Igf-2 gene product. Due to reciprocal imprinting, Igf-2 is expressed from the paternally inherited chromosome, while Igf2r transcripts are expressed from the maternally inherited chromosome. In the case of C3H \( T^{hp/+} \) females crossed to 129/Sv males homozygous for a null mutation at the Igf-2 locus, all of the resulting embryos lack a functional Igf-2 allele and are predicted to be phenotypically growth-deficient, but viable at birth. Half the progeny inherit the \( T^{hp} \) deletion and therefore lack the IGF2R/CI MPR. To determine whether this class of \( T^{hp/+} \) embryos survives to term, the progeny from these crosses were scored at birth for phenotypic alterations of the tail. As shown in Table 1, 33% of the pups scored at or shortly after birth had overt abnormalities of the tail. Interestingly, we observed a range of phenotypes including animals with the characteristic hairpin tail, as well as animals showing only a vestigial tail structure (Fig. 2).

Although the majority of the \( T^{hp/+} \) progeny were alive at birth, their longevity was severely compromised as compared with that of their normal littermates. Many of the \( T^{hp/+} \) animals died within the first week of postnatal life. The cause of death in these animals is unknown. A small percentage survived for periods ranging from between four days to five weeks (Table 1). Southern analysis was performed to confirm that the liveborn class of pups were indeed \( T^{hp} \) heterozygotes. As shown in Fig. 3, animals displaying the \( T^{hp} \) phenotype carried only the 129/Sv paternally derived Igf2r allele, while their phenotypically normal littermates possessed both C3H and 129/Sv alleles. A single
In mammals, the IGF2R is a bifunctional molecule that, in addition to binding IGF-II with high affinity, also serves as the cation-independent mannose 6-phosphate receptor (CI-MPR) involved in lysosomal enzyme targeting (reviewed by Nissley et al., 1991; Kornfeld, 1992). By contrast, the Xenopus and chicken CI-MPRs lack the IGF-II-binding site (Canfield and Kornfeld, 1989; Clairmont and Czech, 1989). Blocking of receptor function in cultured mammalian cells with antibodies directed against the IGF2R/CI MPR does not appear to impair the mitogenic effect of exogenously added IGF-II (Keiss et al., 1987). Thus, it seems likely that IGF-II exerts its growth-promoting functions via the IGF1R, and at present there is no compelling evidence that the IGF2R/CI-MPR participates in IGF-II signal transduction. Considerable data from cell culture experiments have suggested that the IGF2R/CI-MPR provides a turnover mechanism for the degradation of extracellular IGF-II via receptor-mediated endocytosis (Oka et al., 1985; Keiss et al., 1987; Nolan et al., 1990). One explanation for the Tme phenotype is that circulating IGF-II accumulates in embryos lacking the IGF2R/CI MPR, leading to inappropriate growth and/or dysfunction of specific tissue populations. Experiments presented here establish that the lethality associated with maternal transmission of the Tme locus is causally related to the Igf-2 gene product, as embryos that carry a maternally inherited Thp chromosome are rescued, if they additionally lack IGF-II.

The findings described above suggest that ligand binding and degradation by the IGF2R/CI MPR receptor affords a key regulatory mechanism by which the level of this growth factor is modulated in vivo. Moreover, the necessity to regulate precisely the growth-promoting function of IGF-II in the developing embryo provides a rationale to explain why these loci are subject to imprinting. Interestingly, overexpression of H19, a gene that is also imprinted (Bartolomei et al., 1991), cannot be tolerated in transgenic mice, leading to their failure at mid-gestation (Brunkow and Tilghman, 1991). In light of this, and also from the results presented here, it will be interesting to examine whether overexpression of either IGF-II or the IGF2R/CI-MPR affects embryonic growth and/or fetal survival.

In our experiments, we achieved only a variable rescue to birth of the class of Thp embryos (approximately 70% of the expected value). This reduced viability may, in part, be a consequence of an interaction between the dominant T mutation carried on the maternal Thp chromosome and the Igf-2 mutation. Extensive studies of alleles of the T
(brachyury) gene, determination of its expression pattern and chimera studies all indicate a key role for the protein product in regulating the formation of posterior mesoderm (Green, 1989; Wilkinson et al., 1990; Rashbass et al., 1991). Since IGF-II is expressed predominantly in embryonic mesodermal populations (Lee et al., 1990), the absence of this growth factor may exacerbate the effect of the semi-dominant T mutation. This could account for observation that approximately half of the T<sup>hp</sup>/+ progeny recorded in our experiments had only a vestigial tail structure (see Fig. 2). We presume that the most severely compromised animals die at or shortly after birth. Should this be the case, then we would expect an improved survival rate of Tme/+ progeny in matings between females carrying the smaller <sup>plab2</sup> deletion (which does not include the T locus) and Igf-2 null males. Interestingly, similar perinatal losses of males. Interestingly, similar perinatal losses of T<sup>hp</sup>/+ progeny have been recorded in genetic crosses using T<sup>hp</sup>/+ AKR/J males and wild-type C57BL/Gr and BALB/c/Gr females (Johnson, 1974), suggesting that additional genetic factors may affect the penetrance of the Tme locus.

Genetic rescue of the Tme lethality has previously been observed in two types of experiments. In the experiments of Tsai and Silver, viable <sup>plab2</sup>T<sup>Orl</sup> animals were obtained from matings in which the <sup>plab2</sup> chromosome was inherited through the female germ line (Tsai and Silver, 1991). The T<sup>Orl</sup> haplotype consists of a 3.5 cM deletion that includes T and an inverted duplication of the region that includes Tme (Silver et al., 1983; Tsai and Silver, 1991; Schweifer and Barlow, 1992). Since a gene-dosage effect could be excluded, it was proposed that the variable rescue occurred obtained as a consequence of a 'transvection' process by which the paternal Tme region present on the rearranged T<sup>Orl</sup> chromosome escapes imprinting (Tsai and Silver, 1991). In the experiment reported by Forejt and Gregorova, viable T<sup>hp</sup>/+ <sup>plab2</sup>T<sup>Orl</sup> offspring were recovered from crosses between T<sup>hp</sup>/+ females and males of three different inbred strains of Mus m. musculus (Forejt and Gregorova, 1992). In these T<sup>hp</sup>/+ progeny, the Igf2r gene retained the imprinting characteristics, suggesting that the Tme effect was not associated with imprinting of the Igf2r locus. To explain these results, it was necessary to propose the existence of a further imprinted Tme locus that escapes imprinting in this particular hybrid background, due to the inactivity of a paternally inherited 'imprinting gene' (imprintor-1). Our results, however, strongly contrast these findings. We observed variable rescue of a maternally transmitted Tme deletion that was not due to strain-specific variation in the imprinting status at this locus. In light of our findings, the simplest explanation for the rescue obtained by Forejt and Gregorova could be that the paternally derived Mus m. musculus IGF-II protein is present at lower levels or has a reduced mitogenic activity in a hybrid background such that it can be tolerated by the embryo. These alternative explanations are testable since they would predict that imprintor-1 would show genetic linkage to Igf-2.

Data presented in this report strongly suggest that the Tme-associated lethality is the consequence of the lack of expression of the Igf2r gene. In crosses between C3H T<sup>hp</sup>/+ females and 129/Sv males differing only at the Igf-2 locus, rescue of the maternal T<sup>hp</sup> embryos can be directly attributed to the absence of IGF-II. These results suggest that the Tme phenotype does not reflect the loss of function of the IGF2R/Cl MPR gene product per se, but more likely results from excessive levels of the IGF-II ligand. In conclusion, mouse strains that carry targeted mutations at specific genetic loci should prove useful in dissecting in vivo functional relationships between growth factors and receptors.

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