Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality

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Murine embryos that inherit a nonfunctional insulin-like growth factor II/cation-independent mannose 6-phosphate receptor (Igf2r) gene from their fathers are viable and develop normally into adults. However, the majority of mice inheriting the same mutated allele from their mothers die around birth, as a consequence of major cardiac abnormalities. These mice do not express Igf2r in their tissues, are 25-30% larger than their normal siblings, have elevated levels of circulating IGF2 and IGF-binding proteins, and exhibit a slight kink in their tails. These results show that Igf2r is paternally imprinted and reveal that the receptor is crucial for regulating normal fetal growth, circulating levels of IGF2, and heart development.

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Development of the normal diploid mammalian embryo requires the expression of maternally and paternally inherited genomes. Diploid embryos that contain only paternally (androgones) or maternally (parthenotes or gynogenotes) derived genomes never develop to term, with the majority dying at or shortly after implantation (for review, see Solter 1988; Stewart 1993a). The underlying reasons for the abnormal development of these embryos are unknown, although evidence indicates that normal growth of the embryo and its tissues is affected by the parental origin of particular chromosomes. Androgenetic embryos show poor development of the embryo proper, but normal or even excessive growth of the trophectoderm [Barton et al. 1984; Kaufman et al. 1989]. In contrast, parthenogenesis is associated with poor growth of the trophectoderm but reasonable development of the embryo proper, at least to mid-gestation [Nagy et al. 1987; Fundele et al. 1989; Gardner et al. 1991].

Several genes in the mouse and human have been identified whose pattern of expression during embryogenesis is determined by parental origin [Barlow et al. 1991; Bartolomei et al. 1994]. A gene that is not expressed from the maternal allele is said to be maternally imprinted and one not expressed from the paternal allele is paternally imprinted. Seven imprinted genes have been identified to date. The genes encoding insulin-like growth factor II [Igf2, DeChiara et al. 1991], a U2 auxiliary factor-like splicing factor [U2afbs-rs] [Hayashizaki et al. 1994], a small nuclear ribonucleoprotein [snrnpn, Cattanach et al. 1992; Leff et al. 1992], and a zinc finger protein of the C3HC4 class [znf127] [M. Jong, A. Carrey, C.L. Stewart, and R.D. Nicholls, in prep.] have been shown to be maternally imprinted for most tissues analyzed, although Igf2 is expressed from both alleles within the leptomeninges and choroid plexus of the brain [Lee et al. 1990; DeChiara et al. 1991]. Two other genes, the IGF2/cation-independent mannose 6-phosphate receptor (Igf2r) and H19, an RNA of unknown function, are paternally imprinted, at least in late gestation embryos [Barlow et al. 1991; Bartolomei et al. 1994]. However, Igf2r expression can occur from the paternal allele early in development [Latham et al. 1994] and H19 expression can occur from the paternal chromosomes in human trophoblast or hydatidiform moles [Mutter et al. 1993]. The seventh gene, Xist, is preferentially expressed from the paternally inherited X chromosome in the extraembryonic endoderm and trophoblast of mouse embryos at the onset of X chromosome inactivation [Norris et al. 1994].

The role of imprinted genes in embryogenesis and their contribution to the phenotypes associated with androgenetic and parthenogenetic development is unknown, although at least three of these genes have been implicated in regulating cell proliferation. Mice lacking...
a functional paternal Igf2 allele are 30–40% smaller than their wild-type siblings [DeChiara et al. 1990, 1991], whereas the Igf2r allele has been postulated to regulate the availability of IGF2 [MacDonald et al. 1988; Czech 1989] and H19 transcripts have been shown to inhibit the proliferation of certain cell lines [Hao et al. 1993].

Previous studies have shown that embryos must inherit a functional T-associated maternal effect [Tme] locus through the maternal germ line for normal development to occur [Johnson 1974, 1975]. Embryos inheriting a defective Tme locus from their mothers, as in the chromosomal deletions Tbp or Tlub2, are edematous and die at mid-gestation [dryl5–16] [Johnson 1974; Kintanar-Alton 1982; Winking and Silver 1984]. Recent evidence suggests that one candidate gene for Tme is the Igf2r, which has been mapped to the Tbp and Tlub2 deletions and is paternally imprinted at least in mid-to-late gestation embryos [Barlow et al. 1991].

We sought to determine whether paternal expression of the Igf2r was necessary for early development and to what extent the lack of receptor expression contributed to the mutant Tme phenotype. To do this, we derived mice in which the Igf2r gene had been disrupted by targeted mutagenesis in embryonic stem (ES) cells with the subsequent introduction of the mutation into the germ line of mice.

Our results show that heterozygous offspring inheriting the mutated Igf2r allele from their fathers are viable, reach adulthood, and are fertile. In contrast, the majority of offspring inheriting the mutated allele from their mothers die at or near the time of birth. These mice are 25–30% larger than their normal siblings, have elevated levels of circulating IGF2 and several IGF-binding proteins, and exhibit a slight kink in their tails. Embryonic death appears to be a result of major cardiac abnormalities, including overgrowth of the ventricular myocardium and septal and valvular defects. These results show that Igf2r is paternally imprinted and reveal that the receptor plays a major role both in controlling IGF2 levels and regulating fetal growth and heart development.

Results

A deletion spanning exons 13–18 was introduced into the mouse Igf2r gene, with the deleted exons being replaced with a pgkNeopA expression cassette. A herpes simplex virus thymidine kinase [HSV-TK] cassette was inserted at the 3′ end as shown in Figure 1a [Stewart et al. 1992]. This construct was electroporated into W9.5 male ES cells established from a 129/Sv embryo. From a total of 90 clones analyzed, 5 were found to be homologous recombinants and therefore heterozygous for the mutated allele [Fig. 1b]. Karyotype analysis revealed all lines to have a normal diploid 40 XY chromosome composition. It was assumed that the possibility of mutating either the maternal or paternal allele would be equal, and thus all five ES clones were each separately injected into 20 recipient C57BL6/J blastocysts and transferred to pseudopregnant recipients [Stewart 1993b]. On the day equivalent to the fifteenth day of gestation [day of plug = day 1 of pregnancy], it was noted that all 10 recipients were visibly pregnant. However at term, only those recipients carrying blastocysts injected with two of the clones gave birth to viable chimeras that survived to adulthood. The remaining three clones produced offspring that were either stillborn or died shortly after birth, probably because the W9.5 cell line usually produces extreme ES-derived chimeras and in these three clones the maternal allele had presumably been disrupted (see below).

Four chimeric males, two from each of the viable clones, were paired with wild-type C57BL6/6 females. All transmitted the mutated allele to ~50% of their offspring, indicating that they were germ-line chimeras [Fig. 1b, Table 1]. Male and female m+/p− F1 heterozygotes [m+/p− or m−/p+, indicating that they were heterozygotes inheriting the mutated allele from their fathers or mothers, respectively] were then crossed with wild-type mice. The m+/p− F1 males transmitted the mutant allele to 45% of their offspring, with a mean litter size of seven. In contrast, the progeny of m+/p− F1 females were all [with 3 m−/p+ exceptions] wild type, with an overall mean litter size of four (Table 1). Thus, transmission of the mutated allele through the maternal germ line resulted in loss of the majority of the m−/p+ F2 offspring.

Phenotype of embryos inheriting the maternally transmitted mutated allele

Embryos from F1 m+/p− female × +/+/ male crosses were isolated between days 11 and 19 of gestation, and genotyping analysis showed that 46% of the embryos carried the mutated Igf2r allele. Between days 11 and 16 of gestation, all m−/p+ F2 heterozygous embryos [36/72 total analyzed] were viable and appeared normal [data not shown]. The majority of the embryos analyzed between days 17 and 19 of gestation, with the exception of eight [five m−/p+ heterozygotes and three m+/p+ that were dead at day 19 of gestation] were also viable and overtly normal (Table 2). Moreover the m−/p+ heterozygous embryos, from which the placenta and fetal membranes had been removed, were 25–30% heavier [wet weight] than their m+/p+ siblings and exhibited no signs of edema [Fig. 2a]. This increase in weight was noticeable by the seventeenth day of gestation and persisted throughout the remainder of development [Fig. 2a, b]. An increase in placental wet weight was also noted in the m−/p+ F2 heterozygotes. In contrast to wild-type placentas, which cease growing around day 17 of gestation [McLaren 1965], the placentas from the m−/p+ embryos continued growing until birth [Fig. 2c].

Seven pregnant m+/p− F1 heterozygous females, mated with wild-type males, were observed giving birth, and two others were delivered by cesarean section on the nineteenth day of gestation. All m−/p+ F2 heterozygous neonates [n = 26] were recognizable because they were larger than their wild-type siblings and had a slight kink at the tips of their tails [Fig. 2a]. Most of the m−/+ p+ heterozygotes failed to breathe and died. The re-
**Igf2r-deficient mice**

**Figure 1.** (a) Strategy for mutating the Igf2r gene by homologous recombination. A fragment of the gene, from exon 12 to 22, was used for construction of the targeting vector. Exons 13–18 were replaced with a pgkNeoNeo expression cassette and a HSV-TK expression cassette was linked to the 3' end. Digestion of the wild-type DNA with BglII results in detection of an 8.5-kb fragment when hybridized with an exon 11 probe. The same digest of the homologous recombinant results in detection of a 10.5-kb fragment. (b) (Lanes 1–5) DNA digests from the five homologous recombinant clones isolated, with lane 6 being a representative digest of wild-type 129/Sv DNA. (Lanes 7–10) Tail DNAs from four male chimeras, two from each of the two ES clones that resulted in the derivation of the viable chimeras. (Lanes 11–16) Tail DNAs from six F1 germ-line heterozygous offspring from one of the founder chimeras.

Remaining m−/p+ neonates had difficulty in breathing, were cyanotic, and unable to move effectively or suckle and died within 6–12 hr of birth. In contrast, their wild-type siblings all breathed spontaneously, started moving, and feeding.

Eight m−/p− homozygotes were also identified from crossing p+/m− heterozygotes. All eight developed to term but also died at birth and exhibited a slight kink in the tips of their tails. Thus, the homozygous p−/m− phenotype did not differ significantly from the p+/m− phenotype.

A histological examination of the m−/p+ heterozygous neonates revealed no overt abnormalities in most of the internal organs, which included the liver, lungs, gut,

| Female × Male | Generation | Adult | Percent | No. of litters | Litter size (mean) |
|---------------|------------|-------|---------|----------------|-------------------|
| m+/p+ B6 chimera | F₁ | wild type 28 | 33 | 54 | 8 | 7.6 |
| m+/p+ B6 m+/p− F₁ | F₂ | 35 | 29 | 45.3 | 9 | 7.1 |
| m+/p− F₁ m+/p+ B6 | F₂ | 85 | 3 | 3.4 | 21 | 4.2 |
| m+/p+ B6 m+/p− F₂ | F₃ | 17 | 18 | 51.4 | 5 | 7.1 |
| m+/p− F₂ m+/p+ B6 | F₃ | 29 | 0 | 0 | 8 | 3.6 |

Breeding of the Igf2r-deficient mice showing that all progeny (except three) that maternally inherit the mutated receptor gene do not survive to adulthood. B6(m+/p+) = wild-type C57BL6/J mice. The male founder chimeras were 100% germ-line chimeras. (m+/p−) An individual carrying a paternally inherited Igf2r gene.
The majority of the m−/p+ heterozygous embryos die at birth. (E) Embryonic day.

*No. viable/total no. of animals.

Expression of the Igf2r in the heterozygous offspring

Total RNA was prepared from wild-type and m−/p+F2 heterozygous progeny of heterozygous m+/p− F1 females crossed to wild-type males. Igf2r gene expression was analyzed by ribonuclease protection assay, using total RNA isolated from only the embryo proper. A probe to exon 11 was used to show that Igf2r mRNA was transcribed in embryos from days 11–16 and that 5- to 10-fold higher levels were detected in wild-type embryos.
Igf2r-deficient mice

Mice lacking the IGF2/M6P receptor have elevated levels of circulating IGF2- and IGF-binding proteins

One of the proposed functions of the IGF2R is to bind and remove IGF2 from the circulation (Czech 1989). Therefore, loss of expression of the receptor might be expected to result in elevated levels of IGF2. IGF2 was measured by radioimmunoassay, from serum collected from day 18 and 19 wild-type, heterozygous m −/p + and m −/p − embryos, and m −/p − homozygous null embryos. Concentrations of IGF2 in m +/p + embryos (26 analyzed) and in m +/p − heterozygotes (6 analyzed) ranged from 49 to 67 ng/ml, compared with 97−171 ng/ml in m −/p + heterozygotes (9 analyzed) and 1 m −/p − embryo, a 2- to 2.7-fold increase (Fig. 6). Similar results were also obtained from newborn mice (data not shown). Because IGF2 mRNA levels were similar in m −/p + and m +/p + embryos at various stages of development (Fig. 4c), the increase in circulating IGF2 protein was not secondary to a rise in the steady-state levels of IGF2 transcripts but could be attributed to a loss of normal clearance by the IGF2R or to an unknown secondary effect associated with IGF2R deficiency.

In blood and in other bodily fluids, IGF1 and IGF2 are complexed with the IGF-binding proteins (IGFBPs), a family of at least six structurally related secreted proteins. By binding the IGFs, IGFBPs may prolong growth factor half-life in the circulation, modify their local concentrations, and limit or promote their access to recep-

than in m −/p + heterozygotes [Fig. 4a]. Similar results were obtained using a probe to exon 36 [data not shown]. The Igf2r mRNA seen in the m −/p + F2 heterozygous embryos with the exon 11 probe could represent transcripts from the mutated maternal allele or from the normal paternal allele. To distinguish between these two possibilities, the same RNA was hybridized with a probe prepared from exon 16, which had been deleted in the mutated maternal allele. If the paternal allele was expressed, then a protected fragment would be detected in the m −/p + heterozygous embryos. The exon 16 probe detected IGF2R transcripts in RNA from wild-type embryos, but none were seen in the m −/p + heterozygotes. These results indicate that the paternal allele was silent from day 11 onward and that the low level of IGF2R transcripts detected in m −/p + heterozygous embryos, by the exon 11 probe, were derived from the mutated maternal allele [Fig. 4b]. Identical results were also obtained when total RNA isolated from the heart and lungs of the adult female m −/p + heterozygote was analyzed, demonstrating that this survivor did not reactivate the imprinted paternal allele in these tissues [data not shown]. Additional analysis of the same embryonic RNA showed relatively constant expression in age-matched m +/p + and m −/p + embryos of both IGF2R, and the cation-dependent mannose 6-phosphate receptor, which like the IGF2R, mediates the targeting of lysosomal enzymes [Fig. 4c,d].

To confirm that no IGF2R protein was produced in m −/p + heterozygotes, immunoblotting was performed on protein extracts prepared from skeletal muscle, liver, and heart of day 19 embryos, using a polyclonal antibody raised against the rat IGF2R. This antibody detected the ~250 kD IGF2R protein in wild-type tissues. However, no receptor protein was detected in any of the m −/p + heterozygous tissues [Fig. 5]. Similar results were also obtained using whole 13-day embryos [data not shown].

Mice lacking the IGF2/M6P receptor have elevated levels of circulating IGF2- and IGF-binding proteins

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Figure 3. (a) Morphology of a m+/p+ [left] and m−/p− [right] heart from 19-day fetuses. (b) Histological sections through a m+/p+ and m−/p+ hearts [not to scale]. In the m−/p+ heart [right] note the ventricular hyperplasia [1], sinusoids in and thinning of the intraventricular septum [3] and abnormal development of the tricuspid valve [2]. Mean wet weights of m+/p+ and m−/p+ hearts are shown [c], with the corresponding two- to threefold increase in total DNA content in m−/p+ hearts [d].
Figure 4. Expression of IGF2R, IGF2, and cation-dependent M6PR in m+/p+ and m−/p+ (abbreviated to +/+ and +/−, respectively) embryos from days 11–16 of gestation by RNase protection assay. (a) Analysis with an Igf2r exon 11 probe reveals 5- to 10-fold higher levels of the 165-bp protected fragment in the +/+ embryos, compared with the m−/p+ embryos. (b) Analysis with an exon 16 probe reveals that no Igf2r transcripts, as shown by the absence of 175-bp protected fragment, are expressed from the intact paternal Igf2r allele in the m−/p+ embryos. (c) Analysis with an Igf2 exon 5 probe showing similar Igf2 transcript levels in both the +/+ and m−/p+ embryos. (d) Analysis with a 238-bp cation-dependent M6Pr cDNA probe also showing similar transcript levels between the two groups.

tors (Cohick and Clemmons 1993). As shown by Western-ligand blot analysis (Fig. 7), serum levels of IGFBPs were elevated in m−/p+ heterozygotes and in homozygous null embryos compared with either of their m+/p+ or m+/p− heterozygous siblings. By densitometric analysis, the 42- and 45-kD IGFBP3 doublet was 4- to 6-fold more abundant in the m−/p+ or m−/p− embryos, and the 32- and 24-kD IGFBP bands were increased 2- to 2.5-fold. Therefore, circulating levels of IGF2 and the IGFBPs are increased in mice lacking a functional IGF2R.

Serum glucose levels in mice lacking a functional Igf2r are not changed.

In individuals harboring certain large mesenchymally
derived tumors and often in infants that have Beckwith-Wiedemann syndrome, clinically significant hypoglycemia is associated with excessive production and elevated serum levels of IGF2 (Engstrom et al. 1988; Daughaday 1990). Serum glucose levels were measured in 12 wild-type and 5 m−/p+ heterozygous embryos to determine whether glucose homeostasis was altered in mice lacking a functional Igf2r. Glucose values were similar in both groups [wild-type embryos, 4.9±1.9 mM; m−/p+ heterozygotes 3.1±1.6 mM, P<0.1 [not significant]]. Thus, hypoglycemia is apparently not one of the consequences of IGF2 excess in these fetal mice.

Discussion

Here we describe the effects of introducing a mutated and nonfunctional Igf2r gene into the germ line of mice by targeted deletion of exons 13–18. Mice inheriting this mutation through the paternal germ line are viable, phenotypically normal, and express the intact maternal allele. In contrast, the majority (97%) of heterozygotes that inherit the mutated allele through the maternal germ line die at or shortly before birth, and these mice produce no detectable IGF2R protein. Our results show directly that the Igf2r is paternally imprinted, thus confirming previous observations (Barlow et al. 1991) and also demonstrate that at least from day 11 of gestation onward the paternal allele is silent. Thus, disruption of the maternal allele results in mice that are “null” mutants for Igf2r expression. In addition, embryos homozygous for the mutation are indistinguishable from heterozygotes inheriting the mutated allele solely through the maternal germ line, indicating that any expression from the paternal allele in early gestation is not essential for development (Latham et al. 1994).

The majority of the m−/p+ heterozygotes die at birth, and death has been associated with cyanosis, an inability to breathe and failure of the lungs to inflate. Because these mice had markedly enlarged structurally abnormal hearts, with ventricular hyperplasia, as well as valvular and septal defects, a likely cause of death was heart failure. Consistent with this interpretation, the surviving adult male heterozygote had pathological changes in the lungs and other organs that resembled chronic congestive heart failure (Nugent et al. 1994).

Embryos lacking a functional Igf2r exhibited a 25–30% increase in body weight, together with an increase in placental growth rate. This was associated with markedly elevated levels of circulating IGF2 and a corresponding increase in circulating IGFBPs. The enhanced growth rate of the mutant embryos thus confirms a role for IGF2 as a major fetal growth factor and clearly complements the 30–40% decline in growth seen in the embryos lacking Igf2r (DeChiara et al. 1991). However it is not clear whether the increase in embryo weight was attributable to a direct effect of IGF2 on the fetal tissues or to an indirect effect through an enlarged placenta resulting in enhanced nutrient delivery to the growing embryo. The increase in IGF2 expression was not secondary to a rise in steady-state levels of IGF2 mRNA but appeared to result from the absence of Igf2r, thus supporting a role for this receptor in the clearance of IGF2 from the circulation (Czech 1989). Although not verified, the rise in circulating IGFBPs, particularly IGFBP3,
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could be secondary to elevated IGF2 levels. IGFBP3 expression has been shown to be stimulated by IGF1 (Martin and Baxter 1988) and excessive IGF2 action through the IGFR1 may lead to the same effect.

The causes of the disproportionate increase in the size of the heart and the observed cardiac abnormalities in IGFR2-deficient embryos are unknown. During normal development, the heart has the highest expression of the Igf2r of any fetal tissue, with the receptor comprising nearly 2% of the total detergent soluble protein in day 16 fetal rat hearts (Sklar et al. 1989). IGFR2 is also abundant in the heart (Lee et al. 1990), so it is possible that the lack of Igf2r expression potentiates a proliferative response to IGFBP3, with the final result being the marked hyperplasia observed. These abnormalities may also be caused by the loss of normal lysosomal enzyme targeting or lack of other functions of the IGFR2, such as processing of transforming growth factor-β1 (TGF-β1) to an active form (Rogers et al. 1990), as embryos with both IGFR2 deficiency and maternally inherited Tbp (hence, lacking Igf2r) exhibit only a partial improvement in viability (Fulton et al. 1993).

Our results contrast with previous reports describing the phenotype of embryos inheriting the Tbp deletion from their mothers (Johnson 1974; Kintanar-Alton 1982). Although these embryos lacked a functional Igf2r, the majority died at days 15–16 of gestation, with the occasional individual surviving to birth and sometimes even into adulthood. Most of these Tbp heterozygotes were overtly normal, apart from a hairpin tail and additional toes in some individuals, although all embryos were edematous at the time of death (Johnson 1974), and it has been suggested that this edema was secondary to a poor or defective circulatory system (Matzner et al. 1992). However, no cardiac or vascular abnormalities were reported in Tbp heterozygotes (Kintanar-Alton 1982). It is thus difficult to reconcile the differences between our results and those associated with maternal inheritance of Tbp as being solely attributable to lack of expression of the maternal Igf2r. However, variations in genetic background of affected mice and possibly haplo-insufficiency in the expression of other genes that map to the deletion (Schweier and Barlow 1992) might compound the lack of Igf2r expression in Tbp and could contribute to the distinguishable phenotypes at death. An additional possibility is that there is another, as yet unidentified gene, closely linked to the Igf2r that is similarly imprinted and is included in the Tbp deletion. The two genes may interact and produce the earlier onset of death in the affected Tbp embryos.

There are some phenotypic similarities between the few Tbp- and Igf2r-deficient individuals that survived birth and developed to adulthood, especially in the females. Survival of embryos that maternally inherit Tbp or Tlb2 can be enhanced by either introducing a paternally derived duplication of the Tbp region, TpCno (Tsai and Silver 1991) or by crossing Mus musculus domesticus females carrying Tbp with M. musculus musculus males (Forejt and Gregorova 1992). Both crosses result in a significant increase in the viability and development to adulthood of embryos maternally inheriting the Tbp deletion. The adult female progeny of both crosses developed urogenital abnormalities, including imperforate vagina, as did the surviving Igf2r-deficient females, indicating that these abnormalities arose from a lack of Igf2r expression. Although the cause of this defect is also unclear, relatively high levels of Igf2r mRNA transcripts are present in the urogenital region of developing mouse embryos (Matzner et al. 1992). Thus, some aspects of the phenotype associated with Tbp may be attributable to loss of Igf2r activity. It will be of interest to determine whether normal cardiac development occurs in these “rescued” offspring with Tbp, whether IGFBP3 levels are within the normal range, and whether the paternal Igf2r remains imprinted and not expressed.

In conclusion, our results have shown that the IGFR2 is critical for normal fetal development in the mouse. Our direct evidence confirms the suggestion that paternal imprinting of the receptor occurs and demonstrates that regulation of IGFBP3 levels by the IGFR2 is essential to prevent fetal overgrowth. Despite an attractive hypothesis for the opposite roles of IGFBP3 and the IGFR2 in balancing the needs of the embryo and mother during gestation (Haig and Graham 1991), it is unclear why Igf2r and the Igf2r should be imprinted while Igf1 and the Igf1r are not, as IGFBP3 is also important and the IGFBP3 receptor is essential for normal fetal growth (Liu et al. 1993).

Current evidence in humans indicates that the IGFBP3 is not as widely imprinted in tissues as it is in the mouse (Kalscheuer et al. 1993; Ogawa et al. 1993a). Furthermore, relaxation of maternal imprinting of IGFBP3 is thought to play a role in tissue overgrowth associated with Beckwith–Wiedemann syndrome (Feinberg 1993; Ogawa et al. 1993b; Ohlsson et al. 1993; Weksberg et al. 1993) and may also be a contributing factor in the development of certain perinatal neoplasms such as Wilms’s tumor (Ogawa et al. 1993c). Mice that have elevated levels of IGFBP3, as a consequence of lack of IGFBP3, may be
useful in determining whether an increase in this growth factor may result in a greater predisposition to growth enhancement and neoplastic changes.

Materials and methods

Preparation of the gene targeting vector

A 20-kb fragment of the mouse Igf2r gene containing exons 8–22 was isolated by screening a genomic library from the 129/Sv strain in λ FixII [Stratagene, La Jolla, CA] with a 32P-labeled probe derived from exon 11 and its adjacent introns [Szebenyi and Rotwein 1994]. The targeting vector was constructed using a pGKNeoβ expression cassette to replace exons 13–18, and an HSV-TK expression cassette was inserted at the 3’ end using standard molecular biological conditions [Sambrook et al. 1989].

Isolation of recombinant ES clones and derivation of mice carrying the mutated Igf2r

The ES line W9.5 was originally isolated from a male 129/Sv blastocyst by J. Mann [Washington University School of Medicine]. This was maintained, using standard conditions [Abbon-danzo et al. 1993], on primary mouse embryonic fibroblasts isolated from a transgenic mouse line that constitutively expresses the neomycin resistance gene [Stewart et al. 1987]. Approximately 3 × 10^7 ES cells in 0.8 ml of PBS were electroporated with 25 μg of linearized targeting vector, in a 0.4-cm Bio-Rad cuvette with a single pulse at 250 μF and 320 V. The cells were then plated onto 5 × 60-mm petri dishes containing irradiated fibroblast feeders. Selection with 350 μg/ml of G418 and 0.2 μM FIAU (Ocllassen) was initiated the next day. One week later the surviving colonies were picked and each colony placed into a well of a 48-well culture dish, preseeded with a feeder layer of blast feeders. Selection with 350 μg/ml of G418 and 0.2 μM FIAU (Ocllassen) was initiated the next day. One week later the surviving colonies were picked and each colony placed into a well of a 48-well culture dish, preseeded with a feeder layer of fibroblasts, as described previously [Kontgen and Stewart 1993]. Once confluency was reached, the clones were harvested, in pools of 3, DNA was prepared, digested with BglII, and analyzed by Southern gel electrophoresis, using a probe to exon 11 to distinguish between illegitimate and homologous recombinants.

Clones that were found to be homologous recombinants were expanded and a karyotype analysis performed to ensure that they were still euploid. Cells were injected into recipient C57BL/6J blastocysts, with subsequent transfer to pseudopregnant recipients as described [Stewart 1993b].

RNA isolation and analysis

Total cellular RNA was extracted from whole mouse embryos, dissected free from all surrounding fetal membranes and placental tissues, using RNAzol-B [Tel Test, Inc.] using the procedure of Chomczynski and Sacchi [1987]. Ribonuclease protection assays followed previously described protocols [Sambrook et al. 1989]. Single-stranded 32P-labeled antisense RNA probes were synthesized in vitro using linearized plasmid templates and T3 or T7 RNA polymerase. Plasmid templates contained exon 11 (165 bp), 16 (175 bp), or 36 (150 bp) of the mouse Igf2r gene [Szebenyi and Rotwein 1994], exon 5 of the mouse Igf2r gene [Rotwein and Hall 1990], or a 238-bp fragment of a mouse cation-dependent mannose 6-phosphate receptor cDNA [Szebenyi and Rotwein 1991]. Protected fragments were separated by electrophoresis on 6% polyacrylamide/7.5 M urea DNA sequencing gels, visualized by autoradiography, and quantified using a beta-scanner (Betagen, Thousand Oaks, CA).

Immunoblotting

Whole tissue protein extracts were prepared as described by Sklar et al. [1989] and quantitated using the BCA reagent [Pierce Chemical, Rockford, IL]. Proteins were separated by size using SDS-PAGE [7.5% resolving gel [Laemmli 1970]], transferred to PVDF membranes by electroblotting [Bio-Rad, Hercules, CA], and incubated with a 1:200 dilution of anti-Igf2r antisera no. 3637 [Kieß et al. 1987]. Following incubation with a goat anti-rabbit secondary antibody [1:1000 dilution], the Igf2r was detected by enhanced chemiluminescence [ECL detection kit, Amersham].

IGF2 radioimmunoassay

Serum was obtained from day 18–19 or newborn embryos by decapitation, collection of the blood on ice to prevent clotting, with subsequent dilution of the serum with an equal volume of Ca^2+/Mg^2+-free PBS. The serum was recovered by centrifugation. IGF2 concentrations were determined by RIA following acid ethanol cryoprecipitation [Daughaday 1987, Breier et al. 1991]. Recombinant human IGF2 was used as standard and as tracer. An equilibrium assay was established using monoclonal anti-rat IGF2 antibody at 2.5 ng/tube [Tanaka et al. 1989]. The antibody shows 100% cross-reactivity with human IGF2 and <10% reactivity with human IGF1 [Amano Enzymes USA Co., Troy, VA]. Maximum binding of added tracer was between 45% and 50% and recovery was >95%.

Ligand blots for IGFBPs

Serum (5 μl) from 18- and 19-day embryos was fractionated using SDS-PAGE [Laemmli 1970] under denaturing, nonreducing conditions. After blotting the proteins to nitrocellulose filters, subsequent steps followed the procedure of Hos-senlopp et al. [1986]. Blots were washed following overnight incubation with 4 × 10^5 cpm of [125I]-labeled IGF2 at 25°C and were exposed to X-ray film at −80°C for 16 hr.

Serum glucose measurements

Serum glucose levels were measured fluorometrically, using an enzyme-linked assay coupled to reduction of pyridine nucleotides [Passonneau and Lowry 1993].

Histological procedures

Tissues were fixed overnight in 4% paraformaldehyde, dehydrated, and embedded in paraffin wax. Sections were cut at 6 μm and stained with hematoxylin and eosin.

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