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Mouse Mutants Lacking the Type 2 IGF Receptor (IGF2R) Are Rescued from Perinatal Lethality in *Igf2* and *Igf1r* Null Backgrounds

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The cation-dependent and cation-independent mannose 6-phosphate receptors (CD- and CI-MPRs) bind the phosphomannosyl recognition marker of lysosomal hydrolases, but in mammals the latter also interacts with insulin-like growth factor II (IGF-II). While IGF signaling is mediated by the type 1 IGF receptor (IGF1R), the type 2 receptor (IGF2R/CI-MPR) serves IGF-II turnover. Mouse mutants inheriting maternally a targeted disruption of the imprinted *Igf2r* gene, which is normally expressed only from the maternal allele, have increased serum and tissue levels of IGF-II and exhibit overgrowth (135% of normal birthweight) and generalized organomegaly, kinky tail, postaxial polydactyly, heart abnormalities, and edema. These mutants usually die perinatally, but a small minority can survive depending on genetic background and can occasionally reproduce, except for some females characterized by an imperforate vagina and hydrometrocolpos. Consistent with the hypothesis that lethality in the absence of IGF2R-mediated turnover is caused by excess of IGF-II overstimulating IGF1R, *Igf2r* mutants are completely rescued when they carry a second mutation eliminating either IGF-II or IGF1R. Normal embryonic development of the *Igf1r/Igf2r* double mutants, which differ from wild-type siblings only in the pattern of postnatal growth, appears to occur by signaling of IGF-II, being in excess, through a genetically identified unknown receptor, since triple mutants lacking IGF1R, IGF2R, and IGF-II are nonviable dwarfs (30% of normal size). In contrast with the *Igf2r/Igf2* double mutants, mice lacking IGF2R/CI-MPR and CD-MPR survive in an IGF-II null background at a very low frequency and only for a few postnatal weeks, indicating that the mannose 6-phosphate-mediated lysosomal enzyme trafficking is essential for viability.

INTRODUCTION

The mammalian family of insulin-like growth factors (IGFs) and their cognate receptors and binding proteins (IGFBPs) consists of two ligands with structural homology to proinsulin (IGF-I and IGF-II), two known receptors (type 1 and type 2; IGF1R and IGF2R), and at least six IGFBPs (reviewed by LeRoith, 1991; Schofield, 1992; Jones and Clemmons, 1995).

The IGFs are produced by many tissues and function in an autocrine/paracrine fashion (D'Ercole and Underwood, 1980; D'Ercole *et al.*, 1980, 1984), but they may also act as classical hormones, since they circulate in the plasma associated with IGFBPs (see, e.g., Guler *et al.*, 1988). The signaling of both of these ligands is mediated by IGF1R, a heterotetrameric ($\alpha_2\beta_2$) transmembrane glycoprotein with extracellular ligand-binding and intracellular tyrosine kinase domains (reviewed by Siddle, 1992; Moxham and Jacobs, 1992; LeRoith *et al.*, 1995). In contrast to IGF1R, the structurally unrelated IGF2R is a single-chain polypeptide (~300 kDa) with a repetitive extracellular domain (15 units) and a short intracellular tail devoid of tyrosine kinase activity (reviewed by Nissley *et al.*, 1991; Kornfeld, 1992; Ludwig *et al.*, 1995). In mammals, but not in other vertebrates, this receptor is bifunctional (Morgan *et al.*, 1987). In chickens and frogs it serves exclusively as the cation-independent

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mannose 6-phosphate receptor (CI-MPR; Canfield and Kornfeld, 1989; Clairmont and Czech, 1989), which is involved in the trafficking of lysosomal enzymes, while in mammals it also serves the turnover of IGF-II by receptor-mediated endocytosis (Oka *et al.*, 1985; Kiess *et al.*, 1987; Nolan *et al.*, 1990). Proteins possessing a mannose 6-phosphate recognition marker, but not IGF-II, are also bound by a second, cation-dependent receptor (CD-MPR; ~45 kDa), which functions in the same lysosomal pathway as IGF2R/CI-MPR (reviewed by von Figura, 1991; Ludwig *et al.*, 1995).

Previously, from the dwarfing phenotypes manifested after targeted mutagenesis of the mouse *Igf1, Igf2,* and *Igf1r* genes (DeChiara *et al.*, 1990; Liu *et al.*, 1993; Baker *et al.*, 1993; reviewed by Efstratiadis, 1994a), we demonstrated that the IGFs play growth-promoting roles *in vivo* through their interactions with IGF1R. Moreover, we showed that the *Igf1r* gene is essential, since its ablation results invariably in neonatal lethality. The existence of an additional, but still unknown signaling receptor (XR_e), which is distinct from IGF2R and apparently mediates only IGF-II action during mouse embryogenesis, has been inferred from the same genetic evidence (Baker *et al.*, 1993). As revealed by these gene knockout studies, and also by *in vitro* experiments with *Igf1r* null embryonic fibroblasts showing a 2.5-fold elongation of the normal cell cycle time (Sell *et al.*, 1994), the IGF signaling system influences the most important growth determinant: the rate of cellular proliferation that increases total cell number. Interestingly, in the case of the IGF gene system, growth regulation is also influenced by the epigenetic process of parental imprinting (reviewed by Efstratiadis, 1994b). Thus, the mouse *Igf2* and *Igf2r* genes are reciprocally imprinted; only the paternal *Igf2* allele is expressed in most tissues (DeChiara *et al.*, 1991), while the transcriptionally active *Igf2r* allele is the maternal one (Barlow *et al.*, 1991).

In mice, the *Igf2r* gene resides in the proximal region of chromosome 17 that has been eliminated in the partially overlapping deletion mutations T^{hp} (4 cM; Johnson 1974, 1975) and T^{lub2} (0.5 cM; Winking and Silver, 1984), both causing a dominant lethal maternal effect attributed to the absence of the imprinted genetic locus *Tme* (*T*-associated maternal effect). Heterozygous embryos inheriting maternally a chromosome with either of these deletions are characterized by edema and polydactyly and die at about Embryonic Day 15 (e15) and rarely later, whereas the heterozygous offspring are viable, if the deletion is transmitted paternally. Thus, it was suggested that *Igf2r* is the *Tme* locus (Barlow *et al.*, 1991). We reasoned that, if this is correct, the IGF2R turnover function would be absent and could result indirectly in *Tme* lethality, due to increased IGF-II levels harming the embryo by excessive signaling via IGF1R. To test this hypothesis, we generated double mutants inheriting paternally a disrupted *Igf2* allele and maternally a T^{hp} chromosome. Consistent with the prediction, these double mutants were brought to term, but the period of their postnatal survival was variable (Filson *et al.*, 1993). Since the T^{hp} deletion is large and involves many genes that could potentially affect viability, we decided to disrupt the *Igf2r* gene by targeting and to pursue a precise and more extensive genetic analysis of rescue in an *Igf2* null background. In particular, for stringent testing of the elements of our model it was important to show that rescue is complete; that the level of IGF-II ligand is indeed increased in *Igf2r* null mutants; and that IGF1R overstimulation is involved in lethality. Previous preliminary results were consistent with the latter point, as they indicated unexpectedly that a few double mutants nullizygous for the *Igf1r* gene and also carrying a maternally derived T^{hp} chromosome could variably survive, although each of the single mutations was lethal. While reexamining the rescue of mutants lacking both IGF1R and IGF2R using the defined *Igf2r* gene knockout, we also addressed the question whether survival is effected by the concomitant excess of IGF-II that sustains development opportunistically through overstimulation of the putative XR_e, as hypothesized. In parallel, considering that mutants nullizygous for the *M6pr* gene (encoding CD-MPR) exhibit a normal phenotype, with the exception of partial missorting of lysosomal enzymes (Ludwig *et al.*, 1993), we generated triple mutants lacking IGF2R/CI-MPR and CD-MPR in an *Igf2* null background, to examine the developmental significance of the mannose 6-phosphate trafficking pathway.

While this work was in progress, the phenotype of *Igf2r* null mutants, also obtained by gene targeting, was reported independently by two other groups (Wang *et al.*, 1994; Lau *et al.*, 1994).

MATERIALS AND METHODS

Mice

In addition to the *Igf2r* mutants that were generated specifically for this study (see below), we used mice carrying targeted mutations of the *Igf2, Igf1r,* and *M6pr* genes that have been described previously (DeChiara *et al.*, 1990; Liu *et al.*, 1993; Ludwig *et al.*, 1993).

Construction of Replacement Vectors

For targeting of the *Igf2r* gene, we used two different vectors. For their construction, we first screened a mouse phage λ genomic library (in the λFIX II vector) prepared from mouse strain 129/Sv DNA (Stratagene) using as a probe a 0.45-kb *Eco*RI fragment from *Igf2r* cDNA carrying 0.14 kb of 5′ noncoding region and also exons 1 and 2 and a portion of exon 3 (Ludwig *et al.*, 1994). Of the positive recombinant phages that were isolated, a clone containing a 22-kb insert that included exon 1 of the *Igf2r* gene (Fig. 1A) was selected and characterized by restriction mapping, Southern analysis, and partial DNA sequencing. A replacement vector (V1; Fig. 1A) was then constructed from subcloned genomic fragments in several steps, after replacement of a 0.44-kb *Xho*I–*Sal*I fragment of mouse sequence (consisting of 0.33 kb of 5′ flanking sequence and 38 of the 44 codons of exon 1) with a neomycin resistance gene (*neo*) cassette, placed in the same transcriptional orientation as that of the *Igf2r* promoter, for positive selection with the antibiotic G418. To enrich for targeted clones by negative selection with gancyclovir

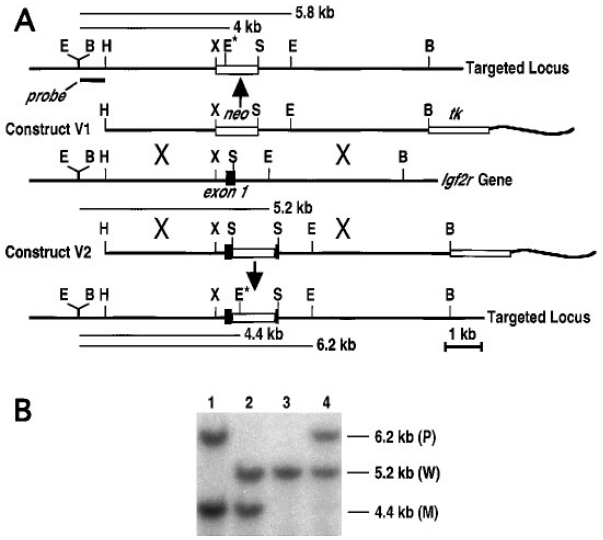

FIG. 1. Targeting of the mouse *Igf2r* locus. (A) The restriction map in the region of the first exon (black rectangle) of the *Igf2r* locus is shown in the middle and is flanked by the diagrams of two constructs (V1 and V2) that were used for gene disruption by homologous recombination (denoted by large X symbols). The wavy lines represents plasmid vector sequences. The structure of the locus after targeting (arrow) with each of the vectors is shown at the top and the bottom of the figure. The restriction sites are: *Eco*RI (E), *Bgl*II (B), *Xho*I (X), *Sal*I (S), and *Hin*dIII (H). The position of the probe that was used for Southern analyses of ES cell clones and mice is indicated, and the sizes (in kb) of the endogenous and targeted *Eco*RI genomic DNA fragments recognized by this probe are shown. An *Eco*RI site that is apparently methylated on the paternal allele becoming insensitive to digestion (see Materials and Methods) is denoted as E*. An example of animal genotyping by Southern analysis taking advantage of the E* site to assign the parental origin of *Igf2r* alleles is shown in (B). While a single 5.2-kb fragment corresponding to the wild-type (W) alleles is recognized by the probe in normal animals (lane 3), an additional 6.2-kb band (P; lane 4) of the allele mutated with the V2 construct is detected in paternal heterozygotes. In maternal heterozygotes, however, cleavage at the E* site is feasible generating a 4.4-kb fragment (M; lane 2). The Southern profile of a nullizygous *Igf2r* mutant is shown in lane 1.

(Mansour *et al.*, 1988), a thymidine kinase (*tk*) gene cassette was also included in the construct. The final product (cloned into pBluescript (SK^+); Stratagene) consisted of a 5′ *Hin*dIII–*Xho*I genomic fragment (3.1 kb); the 1.1-kb *Xho*I to *Sal*I *neo* cassette from plasmid pMC1neo polyA (Stratagene); a downstream *Igf2r Sal*I–*Bgl*II genomic fragment (4.8 kb); and the 1.85-kb *tk* cassette from plasmid pMC1tk (Thomas and Capecchi, 1990) placed in the same transcriptional orientation as that of the *neo* cassette. The second targeting vector (V2; Fig. 1A) was constructed similarly, except that a small *Not*I–*Sal*I fragment (codons 28 to 38 of exon 1) was replaced with the *neo* cassette.

Gene Targeting in ES Cells

*Not*I-linearized replacement vector DNA was introduced by electroporation as described (Liu *et al.*, 1993) into passage 12 129/Sv ES cells (CCE-33) grown on mitomycin C-treated G418-resistant STO cell feeder layers (Robertson, 1987). After drug selection, DNA of resistant clones was screened by Southern analysis using a 0.7-kb *Bgl*II–*Hin*dIII 5′ flanking probe (Fig. 1A). In nontargeted cells, this probe recognizes a 5.2-kb *Eco*RI fragment, while in successfully targeted cells an additional 4.0- or 4.4-kb *Eco*RI fragment (for vectors V1 and V2, respectively) is detected, corresponding to the disrupted allele (Fig. 1A). A *neo* probe was used to demonstrate that single-copy integration occurred in the targeted ES cells (not shown). Eight and six clones targeted with constructs V1 and V2, respectively, were obtained after screening 238 and 174 corresponding candidates.

Blastocyst Injections

To generate chimeras, cells of targeted clones were injected into host blastocysts from the C57BL/6J mouse strain that were transferred into pseudo-pregnant females, as described (Bradley, 1987). From the injected clones targeted with the V1 vector, we obtained only a single female germline transmitter. Obtaining female transmitters is a rare, but not unexpected occurrence. The injected ES cells are male, while the host blastocysts are either male or female. Usually there is conversion of the female blastocysts and the chimeras are male (27% of them are transmitters). However, if conversion does not occur (about 1/3 of the cases), XX/XY female chimeras are obtained, 11% of which are able to transmit the mutation (Bronson *et al.*, 1995). From the injected clones targeted with the V2 construct, we obtained 7 male germline chimeras. The derived progeny were either backcrossed to these transmitters or intercrossed. Transmission of the mutations introduced by targeting with the V1 and V2 constructs resulted in identical phenotypic manifestations. Therefore, our results obtained with either null mutation are described without discrimination.

Genotyping

For genotyping by Southern analysis with 5′ flanking and/or *neo* cassette probes, DNA was prepared as described (Hogan *et al.*, 1994) from embryos or the tail tip of 2-week-old mice. When the mutation was transmitted maternally, *Eco*RI digestion of genomic DNA produced the characteristic 4.0 (or 4.4)-kb fragment for the targeted allele, as in the case of ES cells. Unexpectedly, however, we observed that the size of this *Eco*RI fragment was invariably 5.8 (or 6.2) kb, when the targeted allele was inherited paternally (Fig. 1B). Since this increase in fragment size corresponded exactly to digestion at a downstream *Eco*RI site (Fig. 1A), we considered that the *Eco*RI site in the *neo* sequence may be insensitive to digestion. Although experimental verification by sequencing of genomic DNA was not sought, the interpretation that DNA methylation occurs differentially in the exogenous sequence of the inactive paternal allele is consistent with the occurrence of a CpG dinucleotide at the *neo* cassette *Eco*RI site (GAATT^{m}CG). Inhibition of *Eco*RI cleavage by such methylation has been described (reviewed by McClelland and Nelson, 1992). Thus, by serendipity, we were able to assign the paternal or maternal origin of the targeted allele in the genotyped progeny.

Histological Analyses

Dissected embryos or tissues were fixed overnight in 4% paraformaldehyde, 0.1 *M* phosphate buffer, pH 7.3, and then washed

for 24 hr at 4°C in 0.25 *M* sucrose, 0.2 *M* glycine, 0.1 *M* phosphate buffer, pH 7.3, before dehydration in 50, 70, and 100% ethanol. The tissue was cleared in toluene and embedded in paraffin. Paraffin blocks were sectioned at 6–8 μm and stained with hematoxylin and eosin. Immunohistochemical analysis was performed using a rabbit polyclonal antibody (KME686) raised against human recombinant IGF-II (Davenport *et al.*, 1988).

Measurements of Serum and Tissue IGF-II Levels

The IGF-II concentration in the serum of wild-type and *Igf2r* null e18.5 embryos was measured by a specific radioimmunoassay (RIA) as described (Davenport *et al.*, 1988). The final data represent averages of the values obtained from three dose replicates per sample, after determination of each value in duplicate. Pooled sera were diluted 1:25 with glycine–HCl buffer, pH 3.5, incubated at 4°C for 18 hr, and then filtered by centrifugation through a Centricon-10 microconcentrator apparatus (10-kDa cutoff; Amicon) that had been coated with bovine serum albumin (1% solution) and air dried. This centrifugation step was repeated after addition of 1 ml of RIA buffer into the sample reservoir of the microconcentrator, and the two filtrates were combined and further diluted to 1:100 of the initial serum. In RIA, using multiple volumes of filtrate, the displacement curves were parallel to those of human recombinant IGF-II. Ligand blot analyses (Camacho-Hubner *et al.*, 1991) showed that IGFBPs were undetectable in the filtrates, even when volumes twofold higher than those used for RIA were assayed.

To assess the relative level of IGF-II in mutant embryos, we have adapted published procedures (see, e.g., D'Ercole *et al.*, 1980; Arkins *et al.*, 1993) and developed a quantitative assay based on Western analysis, to derive mutant/wild-type ratios. For this purpose, embryos were dissected, snap frozen in liquid nitrogen, and stored at −70°C until use. Acid-soluble extracts were prepared by dounce homogenization in 1 *M* acetic acid on ice. Homogenates were incubated on ice for 2 hr and then centrifuged twice. The final supernatants were lyophilized, and the pellets were resuspended in 20 m*M* Tris, pH 8.0, 0.5% SDS by vortexing and boiling for 5 min. The insoluble material was pelleted and the supernatants were assayed for protein concentration (Bradford assay; Bio-Rad) in a 1:500 dilution. Samples were analyzed by SDS–PAGE through a 3.5% acrylamide stacking gel, a 10% spacer gel, and a 16% separating gel. A series of dilutions of human recombinant IGF-II standard (Gibco) were included to assess the linearity of the assay. The proteins were then electrotransferred overnight at 4°C to a PVDF membrane in 0.1 *M* CAPS, pH 11.0, 15% methanol, and the blots were stained with Ponceau S to verify equal loading and protein transfer. The membrane was blocked with 5% dry nonfat milk in Tris-buffered saline (TBS), 0.05% Tween 20 for 1 hr at room temperature. After washing, the membrane was incubated overnight at 4°C with anti-rhIGF-II monoclonal antibody (Amano) diluted 1:20 from a 10-μg/ml stock solution in TBS, 0.05% Tween 20, followed by an incubation first with biotinylated sheep anti-mouse IgG antibody (Amersham) diluted 1:1000 in TBS, 0.05% Tween 20 (1 hr at room temperature) and then with biotinylated horseradish peroxidase/streptavidin complex (Amersham) diluted 1:1000 in TBS, 0.05% Tween 20 for 30 min. After washing, the membrane was incubated for 15 sec with ECL detection reagents (Enhanced Chemilluminescence; Amersham) diluted 1:5 with water and then exposed to X-ray film for various times ranging from 30 sec to 10 min. The intensities of the bands corresponding to IGF-II (7 kDa), all within the linear range of the readings for the dilution series of the standard, were measured with a scanning densitometer. The derived arbitrary values were corrected for small differences in the total amount of protein between loaded aliquots and then averaged to calculate mutant to wild-type ratios.

Growth Curves

For staging of embryos, we considered the noon of the day of vaginal plug appearance as e0.5. For developmental analysis of growth, embryos and their placentas were dissected, patted dry with absorbent paper, and wet weights were determined with a microbalance. For determination of dry weights, preweighted embryos placed into tared tubes were dried by incubation at 60°C for 48 hr and 100°C for 24 hr (see Babiarz *et al.*, 1988).

Regression analyses (plots of weight versus age) by application of a Gompertz equation (see Laird *et al.*, 1965) were performed by a curve-fitting computer program (SigmaPlot) using all of the weight data from each class of embryos. In the Gompertz function $W = A \exp[-b \exp(-kt)]$, W and A are the weight at time t and the asymptotic weight, respectively, while b and k are constants. This mathematical treatment takes into consideration that the specific rate $(1/W) \times (dW/dt)$ of the exponential embryonic growth is not constant, but declines exponentially. Specific growth rates (γ) were calculated using the equation $\gamma = -k \ln(W/A)$. Postnatal growth curves are best fitted with a logistic equation $W = A/1 + \exp[-b(t - c)]$, where b and c are constants (Eisen, 1976). However, for the time period of 70 postnatal days that we have examined, growth is clearly triphasic. Thus, the weight data were fitted simultaneously using a computer program by summation of three logistic functions with different sets of A, b, and c values (Koops, 1986; Koops *et al.*, 1987).

RESULTS AND DISCUSSION

Breeding of Igf2r Mutant Mice

The paternal *Igf2r* allele is transcriptionally silent due to imprinting. Thus, paternal transmission of the *Igf2r* mutation that we generated by gene targeting did not affect the viability of heterozygous offspring (paternal heterozygotes; $Igf2r^{+/p-}$), which were indistinguishable from wild-type siblings (data not shown), in agreement with previous results (Wang *et al.*, 1994; Lau *et al.*, 1994). In contrast, progeny inheriting maternally a mutant allele (maternal heterozygotes; $Igf2r^{+/m-}$) usually died perinatally, although some of them were able to survive (see below).

To examine the *Igf2r* null phenotype in detail and also to test rigorously elements of our hypothesis concerning the mechanism of perinatal lethality (see Introduction), we first generated mice carrying combinations of mutations and then implemented a breeding program that included the following crosses: (a) $Igf1r^{+/-}Igf2r^{+/-}$ double heterozygotes were intercrossed; (b) female triple heterozygotes ($Igf2r^{+/-}M6pr^{+/-}Igf2^{+/-}$) were mated with male double mutants nullizygous for both *M6pr* and *Igf2;* and (c) female $Igf1r^{+/-}Igf2r^{+/-}$ double heterozygotes were mated with $Igf1r^{+/-}Igf2r^{+/-}$ $Igf2^{+/-}$ triple heterozygous males. The breeding data from these crosses are presented in Tables 1–3 and discussed in various sections below. We note for clarity that

TABLE 1
Results of Intercrosses between *Igf1r*+/−*Igf2r*+/− Mice

(A) Expected frequencies of gametes and offspring

Maternal gametes (0.25 each)	Paternal gametes (0.25 each)			
	1R 2R[a]	1R 2R	1R 2R	1R 2R
	+ +	− −	+ −	− +
1R 2R				
+ +	++/++	+−/+p−	++/+p−	+−/++
− −	+−/+m−	−−/−−	+−/−−	−−/+m−
+ −	++/+m−	+−/−−	++/−−	+−/+m−
− +	+−/++	−−/+p−	+−/+p−	−−/++

Offspring (0.0625 each)

(B) Data (492 genotyped mice from 65 litters)

Genotype 1R 2R	Number of mice	Observed frequency	Expected frequency[b]	Phenotype
++/++	44	0.089	0.0625	Class I: Normal
++/+p−	50	0.102	0.0625	W: 1.47 ± 0.019[c]
+−/++	96	0.195	0.125	
+−/+p−	83	0.169	0.125	
	273	0.555	0.375	
++/−−	6	0.012	0.0625	Class II: Lacking IGF2R
++/+m−	12	0.024	0.0625	W: 1.99 ± 0.021 (135% of normal)
+−/−−	49	0.100	0.125	Perinatal lethality (with some exceptions)
+−/+m−	42	0.085	0.125	
	109	0.221	0.375	
−−/++	15	0.030	0.0625	Class III: Lacking IGF1R
−−/+p−	18	0.037	0.0625	W: 0.7 ± 0.021 (47% of normal)
	33	0.067	0.125	Neonatal lethality
−−/−−	35	0.071	0.0625	Class IV: Lacking IGF1R + IGF2R
−−/+m−	42	0.085	0.0625	W: 1.4 ± 0.024 (normal)
	77	0.156	0.125	Survival

[a] 1R = *Igf1r*, 2R = *Igf2r*. The mutant paternal and maternal alleles of the imprinted *Igf2r* gene are indicated as p- and m-, respectively.
[b] Because of imprinting, the phenotypic Mendelian ratio of 9:3:3:1 for this cross becomes 3:3:1:1.
[c] Average birthweights (W; in g) ± SEM calculated from a subset of the progeny (70, 41, 23, and 26, for classes I–IV, respectively).

throughout the text we use the designation "*Igf2r* mutants" (manifesting a phenotype) to refer either to maternal heterozygotes ($Igf2r^{+/m-}$) or to nullizygous ($Igf2r^{-/-}$) mice, considering indiscriminately their wild-type and paternal heterozygous siblings as normal.

Phenotype of Mice Lacking IGF2R

Embryonic phenotypic features. In previous studies with 129/Sv × C57BL6/J hybrids, lethality of *Igf2r* maternal heterozygotes was either not observed during embryogenesis (Wang *et al.*, 1994) or detected only at e19.0 (Lau *et al.*, 1994). In contrast, in a similar genetic background or in a mixed background that also included an MF1 strain component we observed a significant incidence of embryonic lethality among *Igf2r* maternal heterozygotes examined at e17.5 and e18.5, but not in younger embryos (Table 4A).

The *Igf2r* mutants that we studied exhibited kinky tails (Fig. 2A) and postaxial polydactyly (Fig. 2B), as previously described (Wang *et al.*, 1994; Lau *et al.*, 1994), and were characterized by generalized organomegaly (Fig. 2C). However, with the exception of heart abnormalities (described separately below), histopathological analysis did not reveal any other impairment in organogenesis. In particular, the underdevelopment of the lungs described by Wang *et al.* (1994) was not evident in the specimens that we examined, in agreement with the observations by Lau *et al.* (1994). Measurements of cell sizes or counting of the number of cell nuclei present within a length of 100 μm in some tissues of e13.5 mutants did not reveal differences from wild-type (Tables 5A and 5B). Therefore, the organomegaly of the mutants can be attributed to an increase in cell number (hyperplasia) and not in cell size (hypertrophy). As a consequence, the mutants are 35–

TABLE 2
Results of Crosses between *Igf2r*+/−*M6pr*+/−*Igf2*+/− (Female) and *M6pr*−/−*Igf2*−/− (Male) Mice

(A) Expected frequencies of gametes and offspring

Maternal gametes (0.125 each)	Paternal gametes (1.0 each)
	2R M6 II[a]
	+ − −
2R M6 II	
+ + +	++/+−/+p−
+ + −	++/+−/−−
+ − +	++/−−/+p−
+ − −	++/−−/−−
− + +	+m−/+−/+p−
− + −	+m−/+−/−−
− − +	+m−/−−/+p−
− − −	+m−/−−/−−
	Offspring (0.125 each)

(B) Data (308 genotyped mice from 41 litters)

Genotype 2R M6 II	Number of mice		Observed frequency		Expected frequency		Phenotype (all dwarfs lacking IGF-II)
							Class I: Physiologically normal
++/+−/+p−	42	187	0.136	0.606	0.125	0.5	Lacking only IGF-II
++/+−/−−	57		0.185		0.125		
++/−−/+p−	46		0.149		0.125		Lacking CD-MPR+IGF-II
++/−−/−−	42		0.136		0.125		
+m−/+−/+p−	54	103	0.175	0.334	0.125	0.25	Class II: Surviving *Igf2r* null mutants in *Igf2* null background
+m−/+−/−−	49		0.159		0.125		
							Class III: Waddling gait
+m−/−−/+p−	11	18	0.036	0.059	0.125	0.25	Death within 4 postnatal weeks
+m−/−−/−−	7		0.023		0.125		Lacking CI-MPR+CD-MPR+IGF-II

[a] 2R = *Igf2r*, M6 = *M6pr*, II = *Igf2*. The mutant paternal allele of the imprinted *Igf2* gene is indicated as p−.

40% larger than normal at e18.5 and at birth (see Tables 1 and 3, and Figs. 2A and 3).

Measurement of the concentration of IGF-II in pooled sera from e18.5 mutant and wild-type embryos by a specific RIA revealed that the level of this factor was 4.4-fold higher in the mutants (Table 5F). This ratio is comparable to the 2- to 2.7-fold increase over the control value reported by Lau *et al.* (1994) for embryos of the same age, but the actual concentration was 6.8- to 12-fold higher in our mutants (1173 vs 97–171 ng/ml) and 4- to 5.4-fold higher in our controls (267 vs 49–67 ng/ml). This discrepancy could be attributed to different extraction methods or antibodies used in the assays. We note that the serum level of IGF-II that we measured in wild-type mice (267 ng/ml) agrees closely with independent RIA results reported for neonates of a different mouse strain (219 ng/ml; van Buul-Offers *et al.*, 1995). Analysis of protein extracts from whole embryos by Western blotting (see Materials and Methods) revealed that the amount of IGF-II was 2-fold higher in the mutants than in wild-type siblings at e12.5. This mutant to wild-type ratio declined to about 1.4 one day later (Table 5G). A more detailed investigation at the tissue level was precluded, due to inadequate sensitivity of immunohistochemical analysis. Examination of sections from e13.5 wild-type and *Igf2r* mutant embryos using a specific anti-IGF-II polyclonal antibody did not reveal striking differences in signal intensity or expression pattern (not shown).

Heart abnormalities. At e18.5, the average weight of the heart was 4-fold higher in the mutants than in wild-type embryos (Table 5C). Examination of coronal sections revealed that, in comparison with control siblings, the marked enlargement of the heart in the mutants was predominantly due to mural thickening of the left ventricle (38% increase) without cardiomyocyte hypertrophy, as well as cavity dilatation (89% increase, Table 5C; cf. Figs. 1D and 1E). Assuming that the heart is a hollow sphere, it can be calculated that the increase in dimensions measured in

TABLE 3
Results of Crosses between *Igf1r*$^{+/-}$*Igf2r*$^{+/-}$ (Female) and *Igf1r*$^{+/-}$*Igf2r*$^{+/-}$*Igf2*$^{+/-}$ (Male) Mice

(A) Expected frequencies of gametes[a] and offspring

	Paternal gametes							
	Nonrecombinant				Recombinant			
Maternal gametes	1R 2R II − + + (0.15)	1R 2R II − − + (0.15)	1R 2R II + + − (0.15)	1R 2R II + − − (0.15)	1R 2R II + + + (0.10)	1R 2R II + − + (0.10)	1R 2R II − + − (0.10)	1R 2R II − − − (0.10)
1R 2R								
+ +	+−/++/++	+−/+p−/++	++/++/+p−	++/+p−/+p−	++/++/++	++/+p−/++	+−/++/+p−	+−/+p−/+p−
+ −	+−/+m−/++	+−/−−/++	++/+m−/+p−	++/−−/+p−	++/+m−/++	++/−−/++	+−/+m−/+p−	+−/−−/+p−
− +	−−/++/++	−−/+p−/++	+−/++/+p−	+−/+p−/+p−	+−/++/++	+−/+p−/++	−−/++/+p−	−−/+p−/+p−
− −	−−/+m−/++	−−/−−/++	+−/+m−/+p−	+−/−−/+p−	+−/+m−/++	+−/−−/++	−−/+m−/+p−	−−/−−/+p−

(0.25 each)

(B) Data (115 genotyped embryos from 15 litters)

Genotype 1R 2R II	Number of embryos	Observed frequency	Expected frequency	Phenotype
++/++/++	3	0.026	0.025	Class I: Normal
++/+p−/++	7	0.061	0.025	W: 1.22 ± 0.041[b]
+−/++/++	9	0.078	0.0625	
+−/+p−/++	3	0.026	0.0625	
	22	0.191	0.175	
−−/++/++	4	0.035	0.0375	Class II: Lacking IGF1R
−−/+p−/++	4	0.035	0.0375	W: 0.56 ± 0.032 (45.9% of normal)
	8	0.070	0.075	
++/+m−/++	2	0.017	0.025	Class III: Lacking IGF2R
++/−−/++	3	0.026	0.025	W: 1.71 ± 0.075 (140% of normal)
+−/+m−/++	4	0.035	0.0625	
+−/−−/++	4	0.035	0.0625	
	13	0.113	0.175	
++/++/+p−	4	0.035	0.0375	Class IV: Lacking IGF-II
++/+p−/+p−	8	0.070	0.0375	W: 0.75 ± 0.025 (61.5% of normal)
+−/++/+p−	6	0.052	0.0625	
+−/+p−/+p−	5	0.043	0.0625	
	23	0.200	0.2	
−−/+m−/++	2	0.017	0.0375	Class V: Lacking IGF1R + IGF2R
−−/−−/++	4	0.035	0.0375	W: 1.28 ± 0.071 (104.9% of normal)
	6	0.052	0.075	
−−/++/+p−	3	0.026	0.025	Class VI: Lacking IGF1R + IGF-II
−−/+p−/+p−	5	0.043	0.025	W: 0.41 ± 0.014 (33.6% of normal)
	8	0.069	0.05	
++/+m−/+p−	7	0.061	0.0375	Class VII: Lacking IGF2R + IGF-II.
++/−−/+p−	6	0.052	0.0375	W: 0.90 ± 0.020 (73.8% of normal)
+−/+m−/+p−	7	0.061	0.0625	
+−/−−/+p−	6	0.052	0.0625	
	26	0.226	0.2	
−−/+m−/+p−	5	0.043	0.025	Class VIII: Lacking IGF1R + IGF2R + IGF-II
−−/−−/+p−	4	0.035	0.025	W: 0.42 ± 0.023 (34.4% of normal)
	9	0.078	0.05	

[a] Male partners were derived from a cross between *Igf1r*$^{+/-}$*Igf2r*$^{+/-}$ females and *Igf2*$^{-/-}$ males. The expected frequency of all recombinant paternal gametes is 40% (the *Igf1r* and *Igf2* genes are linked on mouse chromosome 7 at a genetic distance of 41 cM).

[b] Average weight (W; in g) ± SEM. Embryos were examined at e18.5.

the mutants will result in 4-fold larger wall volume in comparison with the controls.

Transverse midseptal sections showed persistence of early embryonic sinusoids that were virtually transmural in both ventricles and extended to the interventricular septum (cf. Figs. 1F and 1G). On the other hand, transseptal or valvular defects were not detected, in contrast with the results by Lau *et al.* (1994), who reported poor development of the tricuspid valve. In addition, these authors reported enlargement of the right, instead of the left, ventricle, but

TABLE 4

(A) Frequency of embryonic death in maternal *Igf2r* heterozygous mutants

Genetic background[a]	Embryonic age	Mutant embryos (total)	Dead mutant embryos
(F_1 × MF1) × (F1 × MF1)	e11.5–e16.5	41	0
F_2(i) × C57BL/6J	e11.5–e13.5	12	0
F_2(ii) × MF1	e14.5–e16.5	14	0
F_1 × C57BL/6J	e17.5	11	2
F_1 × C57BL/6J	e18.5	17	3
(F_1 × MF1) × (F1 × MF1)	e17.5	11	1
(F_1 × MF1) × (F1 × MF1)	e18.5	14	7
129/Sv × C57BL/6J	e14.5–e18.5	67	0[b]
129/Sv × C57BL/6J	e17.0	4	0[c]
129/Sv × C57BL/6J	e18.0	7	0[c]
129/Sv × C57BL/6J	e19.0	24	5[c]

(B) Frequency of survival of *Igf2r* mutants

			Genotype	
Genetic background	Wild-type mice	Surviving mutants	1R 2R	% Survivors
129/SV	109	0	++/−−	0[b]
129/Sv × C57BL/6J	331	5	++/+m−	1.5[b]
129/Sv × C57BL/6J	114	3	++/+m−	2.6[c]
(F_1 × MF1) × (F_1 × MF1)	273	32 (26)[d]		11.7 (9.5)
		0	++/−−	0
		2 (1)	++/+m−	0.7
		10 (7)	+−/−−	3.7
		20 (18)	+−/+m−	7.3
129/Sv × MF1	37	6	++/+m−	16.2

[a] F_1 = 129/Sv × C57BL/6J; F_2(i) = F_1 × C57BL/6J; F_2(ii) = F_1 × MF1.

[b,c] Data from Wang *et al.* (1994) and Lau *et al.* (1994), respectively, shown for comparison. In the latter study, 3/31 dead wild-type embryos were also recorded at e19.0

[d] Numbers in parentheses indicate long-term survivors.

it is unknown whether this was a consistent finding in several specimens (the published histological sections clearly show dilatation of both ventricular cavities). Cardiac enlargement and dilatation was also reported in T^{hp} maternal heterozygous mutants, which involved both the right atrium and ventricle and was accompanied by a ventricular septal defect, aortic stenosis, and pulmonary artery dilatation (Babiarz *et al.*, 1988). However, when the same mutants (with a different genetic background) were examined by another group, they exhibited only ventricular wall thickening, but bilaterally (Miyabara *et al.*, 1993). We feel that attempts to explain the aforementioned differences in pathological observations by resorting to *ad hoc* hypotheses are currently unwarranted.

A likely cause of the observed perinatal lethality is decompensated heart failure, according to the following considerations. In mice, the formation of the heart begins very early in embryogenesis (between e7.5 and e8.0; Kaufman and Navaratnam, 1981) and proceeds through a complex series of morphogenetic events (Virágh and Challice, 1981; DeRuiter *et al.*, 1992; reviewed by Icardo and Manasek, 1992; Brutsaert and Andries, 1992; Lyons, 1994). At e10, while the primitive heart is still a single tubular structure, the original endocardium and myocardium are separated by extracellular matrix (cardiac jelly) that is progressively reduced in thickness, as the endothelial endocardial cells invaginate into the inner (spongy) zone of the myocardium, while the outer myocardial layer remains compact. When the invading endocardial cells contact the myocardium, interlinked streaks of heart muscle invested with endothelium (trabeculae) begin extending from the ventricular wall into the interior of the chamber. During this trabeculation process, a network of intertrabecular spaces or sinusoids is formed, which communicate with the ventricular lumen and function as primitive vessels for diffusion, rather than circulation of blood serving the metabolic requirements of the myocytes. A more efficient system of irrigation, however, is provided by development of coronary capillaries in

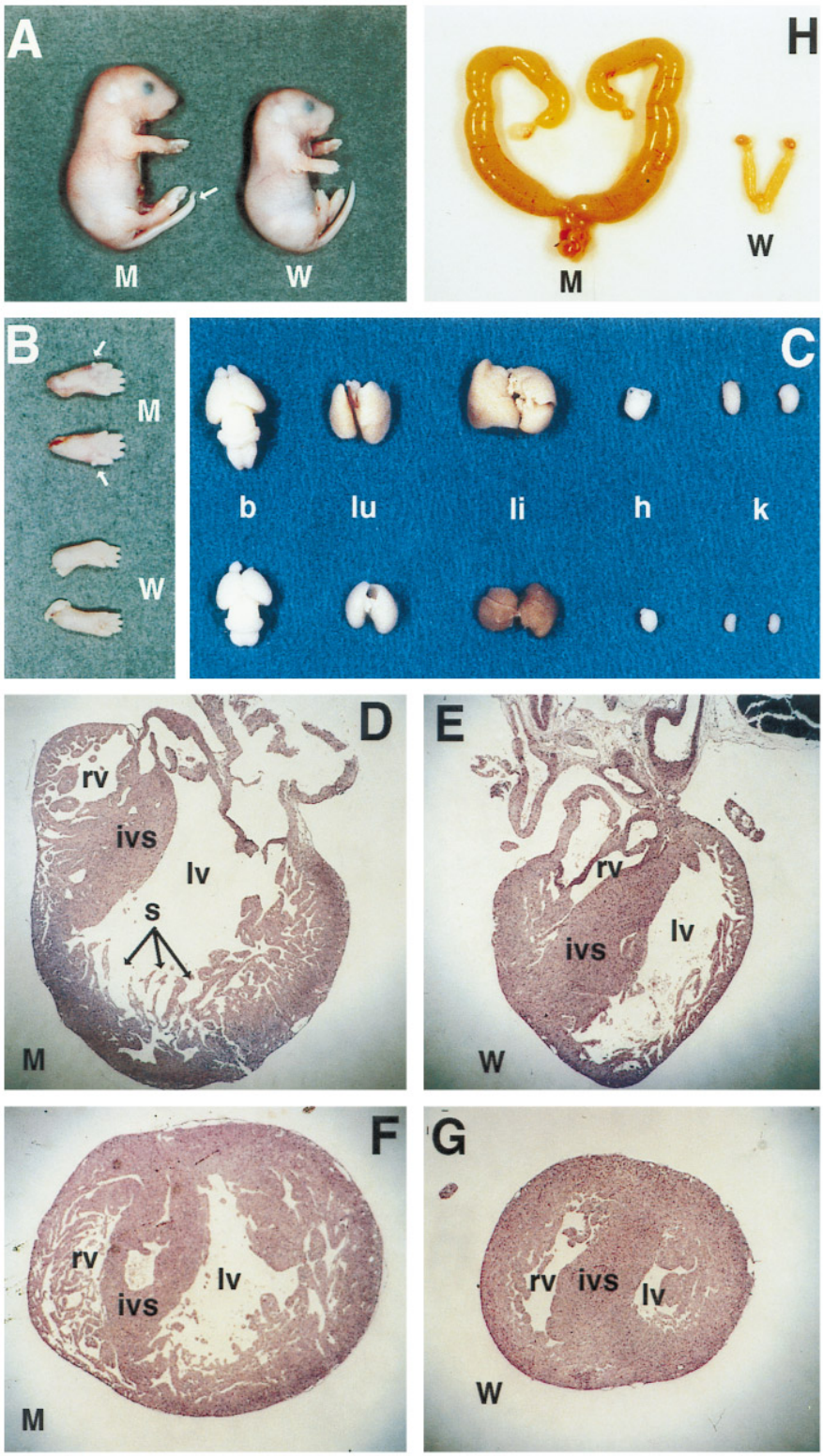

FIG. 2. (A) An e18.5 *Igf2r* mutant embryo (M) is compared with a wild-type (W) littermate. An arrow indicates the kinky tail of the mutant. (B) *Igf2r* mutants exhibit postaxial polydactyly (arrows). (C) The generalized organomegaly of an *Igf2r* mutant (top) is illustrated, in comparison with the sizes of normal organs (bottom). Fixed brain (b), lungs (lu), liver (li), heart (h), and kidneys (k) are shown. (D and E) Comparison of coronal sections of mutant and normal hearts, respectively. Corresponding transverse sections are shown in (F and G). In the mutant, prominent sinusoids (s) are present in the right and left ventricles (rv and lv) and in the interventricular septum (ivs). (H) Example of the enormous distention of the uterus filled with fluid because of the occurrence of an imperforate vagina in a subset of surviving *Igf2r* mutant females (M). A normal uterus (W) is shown for comparison.

TABLE 5
Measurements of Anatomical, Histological, and Physiological Parameters in Wild-Type and *Igf2r* Mutant Embryos

	Wild-type (W)	*Igf2r* Mutant (M)	M/W ratio	Δ[a]
(A) Cell sizes (μm; averages of 100 cells; e13.5)				
Cardiomyocytes (width)	7.7 ± 1.0	7.7 ± 0.8		−
Hepatocytes (diameter)	6.9 ± 1.3	6.8 ± 1.2		−
Mesenchymal cells (diameter)	7.6 ± 1.4	7.1 ± 1.0		−
(B) Number of nuclei (counted within 100 μm; averages of 5 optical fields; e13.5)				
Bronchial epithelium	30.2 ± 2.9	28.2 ± 2.4		−
Intestinal epithelium	30.2 ± 4.0	31.6 ± 3.0		−
(C) Weight (mg) and dimensions (μm) of the heart (e18.5)				
Weight	5.8 ± 0.7 (4)	24.2 ± 2.2 (7)	4.17	+
Transverse diameter	2025 ± 92 (4)	3333 ± 195 (6)	1.65	+
Left ventricle (wall thickness)	325 ± 27.0 (4)	454 ± 40.5 (6)	1.38	+
Left ventricular cavity (diameter)	519 ± 12.0 (4)	979 ± 38.4 (6)	1.89	+
Right ventricle (wall thickness)[b]	238 ± 36.0 (4)	275 ± 28.9 (6)	1.16	−
Right ventricular cavity (diameter)	344 ± 29.5 (4)	579 ± 46.3 (6)	1.68	+
Interventricular septum (width)	513 ± 46.2 (4)	742 ± 60.0 (6)	1.45	+
(D) Nucleated red blood cells[c] (% of total RBC)				
e15.0	8.33 ± 0.28 (2)	10.07 ± 0.51 (2)	1.21	−
e16.0	0.55 ± 0.29 (2)	2.51 ± 0.98 (2)	4.56	+
e17.0	0.16 ± 0.025 (2)	0.62 ± 0.054 (2)	3.88	+
(E) Water content (per unit dry weight)[d]				
e15.5	7.23 ± 0.052 (3)	7.16 ± 0.112 (6)	0.99	−
el6.5	6.31 ± 0.073 (9)	6.43 ± 0.146 (4)	1.02	−
e17.5	5.66 ± 0.088 (7)	6.07 ± 0.085 (11)	1.07	+
e18.5	5.24 ± 0.050 (11)	6.02 ± 0.284 (12)	1.15	+
(F) Serum IGF-II (ng/ml)[e]				
e18.5	267 ± 7	1,173 ± 58	4.39	
(G) IGF-II in embryonic extracts				
e12.5	(3)		2.1 ± 0.04[f]	
e13.5	(3)		1.4 ± 0.02	

[a] The presence (+) or absence (−) of a statistically significant difference (Δ) is indicated (Student's *t* test; $P < 0.05$). Mean values ± SEM were calculated from measurements of the number of specimens shown in parentheses.

[b] Although statistically not significant, the marginal increase in thickness of the right ventricular wall in the mutants can contribute to the overall increase in cardiac mass because of the enlargement of the chamber of a spherical organ.

[c] Nucleated RBC (90% at e12.0) progressively decline in number and disappear from the circulation by e17.5 (Kaufman, 1992).

[d] Water content per unit dry weight = (wet weight − dry weight)/dry weight. The average dry weights (g) in the four embryonic ages shown were: 0.0472, 0.0759, 0.146, 0.189 (wild-type) and 0.0695, 0.121, 0.168, 0.237 (mutant). In mice, as in all mammals, total body water content decreases with embryonic age (Altman and Dittmer, 1974).

[e] Pooled sera.

[f] Ratios were calculated from the results of Western analysis (see Materials and Methods), using as a normal value the average of densitometric readings obtained from three wild-type samples per embryonic age.

the epicardium that become functional at e13.5. Thus, in the embryonic heart, sinusoids and capillaries coexist for some time and may communicate to some degree. Later in development, the ventricular wall undergoes a process of compaction, during which most of the trabeculae are incorporated into the wall. Thus, the sinusoids regress and disappear, and the coronary system becomes the sole source of blood supply for the heart. Persistence of embryonic sinusoids analogous to that characterizing the *Igf2r* mutants has been described in some rare cases of heart abnormalities in humans (Chenard *et al.*, 1965; Feldt *et al.*, 1969; Jenni *et al.*, 1986; Allenby *et al.*, 1988; Amann and Sherman, 1992).

In rodents, the cardiomyocyte, which is the major cell type of cardiac tissue, proliferates during embryogenesis, but becomes permanently postmitotic a few days after birth (reviewed by Engelmann, 1993). Temporally, this pattern of

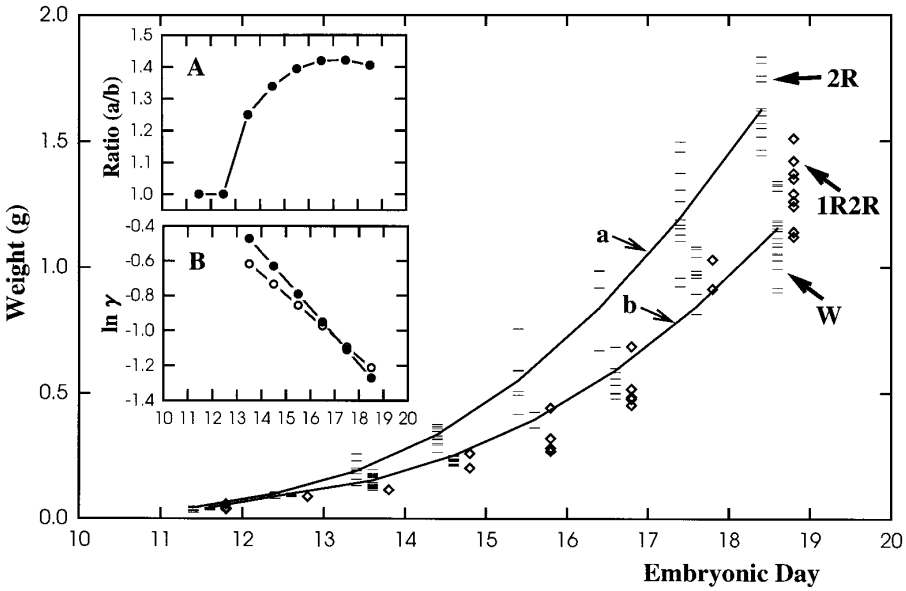

FIG. 3. Embryonic growth kinetics. Plot of weights of wild-type (W) and *Igf2r* mutant (2R) embryos versus gestational age. Each plot symbol (dash) represents a single embryo (in several cases dashes are superimposed). For comparison and clear display, the same symbol is used for both classes of embryos, and each group of data is presented in the form of two columns centered around a time point (as in histograms). Statistical analysis using Student's *t* test ($P < 0.05$) did not show significant differences in mean values between the two groups at e11.5 and e12.5. The curves represent the results of regression analyses using the Gompertz function (see Materials and Methods). The weights of double mutant embryos lacking both IGF receptors (1R2R), which were obtained from the same crosses, are also displayed (open diamonds), to indicate that their growth does not differ from wild-type (statistical differences were not detected). Insert (A) shows the *Igf2r* mutant to wild-type weight ratio at each time point, determined from the results of the regression analyses. In insert (B), the natural logarithm of the specific growth rate (γ) is plotted versus developmental age (for details, see text).

proliferation correlates with the developmental transcriptional down-regulation of *Igf2* (Soares *et al.*, 1986; Brown *et al.*, 1986), *Igf1r* (Werner *et al.*, 1989), and *Igf2r* (Sklar *et al.*, 1989, 1992; Senior *et al.*, 1990; Nissley *et al.*, 1993; Haskell and Tucker, 1994) in the heart. Although the level of cardiac expression of *Igf1r* is relatively low (Bondy *et al.*, 1990; Haskell and Tucker, 1994) and the exact cellular distribution of transcripts has not been studied in detail, *Igf2* is highly expressed in the myocardium and epicardium (Stylianopoulou *et al.*, 1988; Engelmann *et al.*, 1989a,b; Lee *et al.*, 1990), while the expression of *Igf2r* in these regions, and also in endothelium, is higher than that in any other tissue (Senior *et al.*, 1990; Matzner *et al.*, 1992; Nissley *et al.*, 1993). Since lysosomal activity in the heart is relatively low, the high level of IGF2R is likely to be important mainly for control of IGF-II turnover, as previously suggested (Senior *et al.*, 1990). Such fine tuning of IGF-II levels may be a requirement for unperturbed morphogenesis particularly in the heart, considering, for example, that the liver also expresses high levels of *Igf2* transcripts (Stylianopoulou *et al.*, 1988), but contains little *Igf2r* mRNA (Senior *et al.*, 1990; Matzner *et al.*, 1992). It seems, therefore, that the presumptive overstimulation of the available IGF1R by a significant excess of IGF-II in *Igf2r* mutants results in hyperproliferation, as evidenced by the observed dramatic myocardial hyperplasia, possibly bringing growth and morphogenesis out of pace. In this regard, the persistence of sinusoids may reflect a delay or block of morphogenetic events, potentially leading to inadequacy of the coronary circulation, even if it remains unaffected per se, to meet the metabolic demands of the heart that has grown excessively. As a consequence, poor ventricular contractility could lead to decompensated heart failure and perinatal death. The presence of edema is consistent with this putative condition.

T^{hp} mutants were consistently described as severely edematous (Johnson, 1974, 1975; Tsai and Silver, 1991; Winking and Silver, 1984; Filson *et al.*, 1993; Miyabara *et al.*, 1993), but only Babiarz *et al.* (1988) reported actual measurements of wet (ww) and dry (dw) weights for e15.0 mutant and wild-type embryos. From these data, we have calculated the average water content per unit dry weight per embryo [i.e., the ratio (ww − dw)/dw] and found that it was increased by about 18% in the mutants (edema). In our comparisons between *Igf2r* mutant embryos and normal siblings, however, we did not detect differences at e15.5 and e16.5, while the mutants were clearly edematous at e17.5 and e18.5, exhibiting an increase in water content by 7 and 15%, respectively, over the wild-type value (Table 5E). Therefore, in comparison with T^{hp} embryos, which usually die at midgestation, the development of edema may be de-

layed in *Igf2r* mutants, correlating with the perinatal time of their death. Previously, from analogous calculations using a relatively small set of dry weight data obtained exclusively from neonates, Wang *et al.* (1994) concluded that edema is absent from *Igf2r* mutants. In view of our analysis, this conclusion does not appear to be tenable.

An additional clue consistent with circulatory insufficiency during late developmental stages in the mutants, compared with control siblings, is the significant persistence of immature (nucleated) red blood cells in the embryonic circulation revealed from the analysis of blood smears (Table 5D), which suggests manifestation of anemia analogous to that previously detected in T^{hp} embryos (Babiarz *et al.*, 1988). Alternatively, the increase in nucleated red blood cells may be a direct effect of IGF-II excess, because this factor appears to stimulate erythropoiesis, at least under *in vitro* conditions (see Sanders *et al.*, 1993).

Postnatal survival of a minority of Igf2r null mutants. One of the phenotypic classes (class II) of progeny derived from intercrosses of $Igf1r^{+/-}Igf2r^{+/-}$ double heterozygotes (Table 1) were mutants lacking IGF2R and exhibiting overgrowth. Genotypically, these mutants were either $Igf2r^{+/m-}$ or $Igf2r^{-/-}$ and also either wild-type or heterozygous for the *Igf1r* gene. From such matings, the expectation was that the ratio between class II mutants and normal animals would be 1:1. However, while 273 normal animals were obtained, there were only 109 *Igf2r* mutants recovered (39.9% of the expected number; 109/273), which indicated a significant loss of dead neonates, due to maternal cannibalism. Interestingly, the deficit of mutants was not proportionate to the expected 1:1:2:2 ratios of the four class II genotypes ($Igf1r^{+/+}Igf2r^{-/-}$: $Igf1r^{+/+}Igf2r^{+/m-}$: $Igf1r^{+/-}Igf2r^{-/-}$: $Igf1r^{+/-}Igf2r\text{-}^{+/m-}$, respectively; see Table 1). Instead, the mutants recovered in the corresponding categories were 6, 12, 49, and 42. While the difference between 6 and 12 is not statistically significant (χ^2 test, $P > 0.1$), there is a significant paucity of *Igf2r* mutants possessing a wild-type *Igf1r* genotype. In fact, if only this latter genotype is considered, the ratio of the wild-type to heterozygous gene states is 1:5 [(6 + 12):(49 + 42)], rather than the expected 1:2 (see Table 1). On the other hand, when the *Igf2r* mutation is considered alone, there is no difference in frequency between maternal heterozygotes and nullizygous mutants [(12 + 42):(6 + 49) = 54:55]. Thus, it appears that the neonates lacking IGF2R die more rapidly and are not easily recovered, when the non-imprinted *Igf1r* gene is present in its normal double dosage than when it is represented by a single functional copy. This conclusion was strengthened by the observation that a similar deviation of the relative genotypic frequencies was detected among mutants that survived despite the lack of IGF2R (see below).

Of the recovered *Igf2r* mutants, 32 survived for various periods of time; 2 for 4 days and 1 each for 10, 11, 20, and 25 days, while the remaining 26 reached adulthood. Thus, assuming that a total of 273 mutants should have been observed, the overall frequency in a mixed genetic background that included 129/Sv, C57BL/6J, and MF1 components was 11.7% (32/273), while the frequency of long-term survival was 9.5% (26/273; Table 4B). However, this relatively high frequency was strongly dependent on *Igf1r* heterozygosity. Thus, only one each of the short- and the long-term survivors had an $Igf1r^{+/+}Igf2r^{+/m-}$ genotype (Table 4B). This is strong evidence that concomitant haploinsufficiency of IGF1R makes the *Igf2r* mutation more tolerable, consistent with our initial hypothesis (see Introduction) that excess of IGF-II in the absence of IGF2R leads to lethality by overstimulation of IGF1R.

Except for the influence of *Igf1r* heterozygosity, which is a special case, the survival of mutants lacking IGF2R, but possessing a wild-type *Igf1r* gene pair, is clearly dependent on the genetic background of the mouse strain carrying the mutation. Thus, previous studies indicated that no $Igf2r^{+/m-}$ survivors were obtained in an inbred 129/Sv background (Wang *et al.*, 1994), while the frequency of survival of 129/Sv × C57BL/6J hybrids was 1.5–2.6% (Wang *et al.*, 1994; Lau *et al.*, 1994; see Table 6). We found that a higher than 50% contribution of MF1 genetic background to outbred progeny increases the frequency of survival even more significantly. In six litters of progeny derived from matings between $Igf2r^{+/-}$ 129/Sv × MF1 females with wild-type MF1 males, we recovered 37 wild-type offspring and 6 $Igf2r^{+/m-}$ mutants, all of which survived to adulthood (frequency of survival: 16.2%; Table 4B).

Reproductive phenotype of surviving Igf2r mutants. Of 24 examined *Igf2r* mutants that survived and reached adulthood, 8 were males and 16 were females, 10 of which were characterized by an imperforate vagina, a phenotype previously described for a single female by Lau *et al.* (1994). At autopsy, while all other organs appeared normal, we observed a dramatic distention of the uterus (Fig. 2H), which filled the entire abdominal cavity, due to the accumulation of brown and slightly viscous fluid. On average (three measurements), the weight of the uterus was 3.72 ± 0.62 g, becoming 0.57 ± 0.07 g after complete drainage of liquid. Since the uterine weight of female siblings in diestrus was about 0.09 ± 0.016 g, we conclude that the uterus itself was enlarged about sixfold over the normal size. Examination of an additional mutant revealed an even more extreme case (uterine weight of 1.14 g with accumulation of over 10 ml of fluid).

Cases of imperforate vagina as an inherited mouse trait with variable degrees of penetrance leading to uterine distention have been described previously (Gowen and Heidenthal, 1942; Chase, 1944; Eisen *et al.*, 1989). More interesting, however, is the similarity of phenotypic features of our mutants to those manifested in the rare human hereditary Kaufman–McKusick syndrome, except that overgrowth at birth has not been reported (McKusick *et al.*, 1964; Dungy *et al.*, 1971; Kaufman *et al.*, 1972; McKusick, 1978; Robinow and Shaw, 1979; Goecke *et al.*, 1981; Knowles *et al.*, 1981; Jabs *et al.*, 1982; Farrell *et al.*, 1986; Vince and Martin, 1989; Schaap *et al.*, 1992; Pul *et al.*, 1994). Among several highly variable features, female patients are usually characterized by a combination of postaxial polydactyly and hy-

drometrocolpos (i.e., accumulation of fluid in the uterus and the vagina) that is secondary to vaginal atresia or to the presence of a transverse vaginal septum. However, even in affected families, hydrometrocolpos may not be accompanied by polydactyly and vice versa, while the characteristic postaxial hexadactyly may be bilateral or asymmetric for the hands, extending more rarely to three or all four limbs. In our *Igf2r* mouse mutants, hexadactyly, which is not always accompanied by imperforate vagina, is observed invariably, but appears more often in the forelimbs, occasionally unilaterally, and less often in the hindlimbs as well. Whether these correlations could be extended to include the heart, is unknown. Although congenital heart abnormalities have also been described in several cases of the Kaufman–McKusick syndrome on the basis of clinical signs, pathology reports are generally unavailable. Histopathological analysis of the heart in one of the *Igf2r* mutant survivors revealed a small degree of persisting sinusoids, which, however, were restricted to the right ventricle and were detected only in the most basal sections (closest to the valve ring; data not shown). Similar observations were made with one of the mutants lacking both IGF receptors (see below), suggesting that this condition is compatible with viability, although its potential pathophysiological impact cannot be assessed. Considering that the combination of the disparate features of hydrometrocolpos and polydactyly is unusual and unique to *Igf2r* mutant mice and Kaufman–McKusick patients, it is not unlikely that the genetic lesion in the clinical cases could be somehow related to dysfunction of the IGF system.

Several of the *Igf2r* mutants that survived to adulthood (males and also females with normal genitalia) were mated with wild-type mice to test for fertility. Six of the males and three of the females were proved to be fertile, while one male and two females were infertile.

Embryonic and postnatal growth of Igf2r mutants. Relative growth rates of wild-type and mutant embryos can be compared from plots of weight versus developmental age, which provide an overall index of the growth process. To handle the scattering of embryonic weight values in such growth curves, which is due to developmental asynchrony of embryos within and between litters, we subjected the data to regression analysis and then calculated respective specific growth rates (see Materials and Methods). As shown in Fig. 3, there is no difference in weights between *Igf2r* mutants and normal embryos at e11.5 and e12.5. After this age, however, the overgrowth of the *Igf2r* mutants becomes easily detectable, as the mutant to wild-type weight ratio increases quite abruptly from 1.0 at e12.5 to 1.25 at e13.5. This change is then followed by a slower progressive increase, and the weight ratio reaches a plateau value of approximately 1.4 at e16.5 (Fig. 3, inset A). These differences in wild-type and mutant growth patterns are a reflection of the corresponding specific growth rates, which decline exponentially with age as expected (see Materials and Methods), but differ in absolute values being higher in mutant embryos until e16.5 (Fig. 3, inset B). It is notable that the

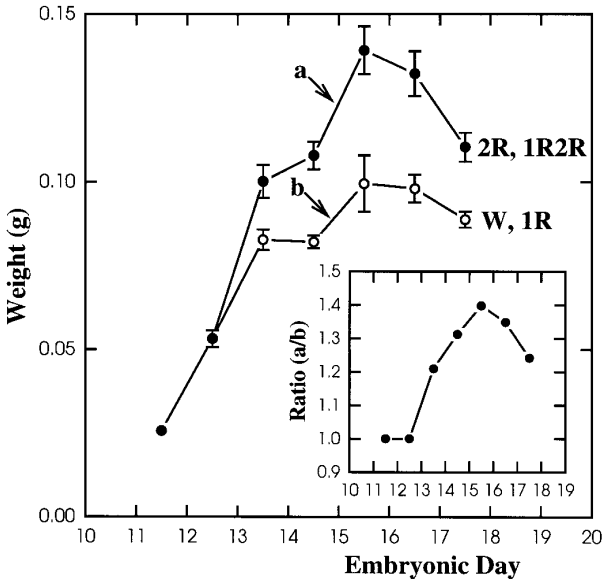

FIG. 4. Mean placental weights of the embryos in Fig. 3 are plotted versus gestational age. As expected, the placenta stops growing and actually loses weight toward the end of gestation (McLaren, 1965). The width of the vertical bars represents 2 SEM. Statistical analysis using Student's *t* test ($P < 0.05$) did not show significant differences in mean placental weights between wild-type controls (W) and mutants lacking IGF1R (1R), as previous results had indicated (Baker *et al.*, 1993). Similarly, the placental weights of mutants lacking only IGF2R (2R) or lacking both IGF receptors (1R2R) were statistically indistinguishable. Thus, only averages of the two respective groups (curves b and a) are shown, except for the e11.5 and e12.5 time points (averages of placental weights of all classes of embryos, which did not differ statistically). The inset shows the ratios (a/b) of mean values in the two curves at each time point. We note that, in comparison with a previous analysis (Baker *et al.*, 1993), the values for the e13.5 time point are unusually high, even if the known extensive variability in placental weights is taken into consideration.

opposite effects on growth caused by excess or complete absence of IGF-II in *Igf2r* and *Igf2* null embryos, respectively, are manifested with different growth kinetics. While the growth deficiency of *Igf2* null embryos is first detectable at e11.0 (Baker *et al.*, 1993), the overgrowth of *Igf2r* mutants becomes evident 2.5 days later.

Parallel determination of the weights of the placentas of *Igf2r* embryos indicated an increase in size in comparison with the wild-type controls, first detectable at e13.5 (Fig. 4). Since decrease in placental weight in *Igf2* null mutants appears at the same developmental age (Baker *et al.*, 1993), there is clearly a difference between the embryo and the placenta in the timing of the effects from the absence or excess of IGF-II. This can be attributed to different ligand/receptor signaling systems operating in the two components of the conceptus. While lack of either IGF ligand (IGF-I or IGF-II) or elimination of the function of the receptor serving

their signaling (IGF1R) are dwarfing mutations for the embryo, only the absence of IGF-II causes a decrease in placental size (Baker *et al.*, 1993). On the basis of this genetic evidence, we postulated that IGF-II is involved in placental growth via an interaction with an unknown receptor (XR_p). As other data have indicated (see below), this putative receptor appears to be distinct from the XR_e acting on the embryo proper, the presence of which was revealed from the growth deficiency of *Igf1r/Igf2* double mutants that is more severe than the phenotype manifested by either mutation acting alone (Liu *et al.*, 1993). Curiously, the placentas of T^{hp} embryos were found to be smaller than normal (84%) and exhibited paucity of spongiotrophoblasts accompanied with increased numbers of basophilic epithelial cells and trophoblastic giant cells (Babiarz *et al.*, 1988). In contrast, histopathological analysis of placentas from *Igf2r* mutants did not reveal deviations from normalcy (data not shown). At present, there is no straightforward explanation for this difference between the two classes of mutants.

In contrast to the narrow heterogeneity of embryonic growth data, analyses of postnatal growth curves showed that, after the first 2 weeks of age, there was extensive variability in the weights of wild-type animals, even between siblings (38 female mice from 16 litters were examined). Thus, in the absence of an invariant basis for comparison, and because we had the opportunity to examine only three growth curves of surviving *Igf2r* mutants, it is not possible to draw firm conclusions about the pattern of their relative postnatal growth. We simply note that, if only the average growth curves of wild-type mice are considered, two of the mutants exhibited similar growth patterns, while the growth of the third mutant animal was higher (see Fig. 5).

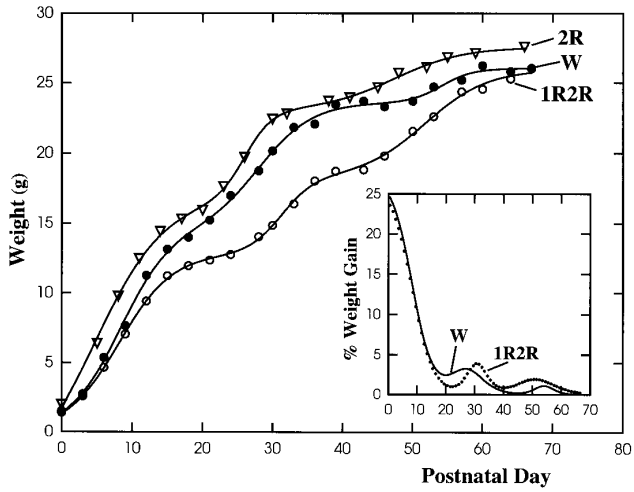

FIG. 5. Examples of postnatal growth curves (plots of weights versus time) of female wild-type (W) and double IGF receptor mutant (1R2R) siblings. In this particular case, despite retarded growth between p15 and p30, the double mutant became indistinguishable in weight from wild-type by 70 days of age (catch-up growth). The growth of the wild-type animal, which was average, is also compared with that of a surviving *Igf2r* mutant female (2R) from a different litter. The triphasic curves were fitted using a logistic equation (see Materials and Methods). The inset shows the percent daily weight gains of the wild-type and double receptor mutant mice calculated from the respective fitted curves as follows. The difference of weights at any two consecutive days was divided by the average of the two weights and the derived ratio was multiplied by 100. The catch-up growth of the double mutant in comparison with the control is evident from the difference in the middle portions of the two curves.

Survival of Igf2r Null Mice in Igf2 Null Background

Previously, consistent with our hypothesis that the lethality of mutants lacking IGF2R is the indirect consequence of excess IGF-II in the absence of turnover (see Introduction), we were able to rescue mutants carrying a maternally inherited T^{hp} deletion in an *Igf2* null background, but only partially (Filson *et al.*, 1993). However, it is now clear that, despite ample similarities, the *Igf2r* phenotype is less severe than that of the T^{hp} deletion mutants lacking additional genes. Thus, for a more critical test of our model, it was important to examine genetically whether the rescue of mutants carrying a defined *Igf2r* mutation is complete in an *Igf2* null background, especially in view of the experimental evidence that the level of IGF-II in *Igf2r* embryos is indeed higher than normal, as predicted from the proposed mechanism leading to lethality. At the same time, we sought to generate progeny lacking both the IGF2R/CI-MPR and CD-MPR functions, to examine the significance of the mannose 6-phosphate-dependent lysosomal enzyme trafficking for viability.

For genetic analysis, we first mated a female chimera transmitting partially the *Igf2r* mutation with male double mutants nullizygous for both *Igf2* and *M6pr* and, among the derived progeny, obtained a triple heterozygote female ($Igf2r^{+/m-}$; $Igf2^{+/p-}$; $M6pr^{+/-}$). This dwarf mouse (because of paternal transmission of the null *Igf2* gene) was viable, despite maternal transmission of the mutated *Igf2r* gene, and also fertile. Of 26 progeny (from five litters) obtained from matings of this triple heterozygote with a male nullizygous for *Igf2*, 13 were maternal heterozygotes for *Igf2r*, but survived to adulthood without exception. Because of their $Igf2^{+/p-}$ or $Igf2^{-/-}$ genotype (combined in this case either with a $M6pr^{+/+}$ or $M6pr^{+/-}$ genotype), all of the progeny exhibited a dwarfing phenotype, but lacked other abnormalities.

Next, to verify definitively the complete rescue of *Igf2r* maternal heterozygotes in an *Igf2* null background by examining a large number of animals, and also to generate mutants lacking both IGF2R/CI-MPR and CD-MPR, we mated female triple heterozygotes ($Igf2r^{+/m-}$; $Igf2^{+/p-}$; $M6pr^{+/-}$) with male double mutants nullizygous for both *Igf2* and *M6pr* (Table 2). The expectation from such crosses is that all progeny will be dwarfs (genotypically $Igf2^{+/p-}$ or $Igf2^{-/-}$), but of three classes obtained in a 2:1:1 ratio: (a) physiologi-

cally normal mice (wild-type for *Igf2r* and heterozygous or nullizygous for *M6pr*); (b) maternal *Igf2r* heterozygotes surviving in the absence of *Igf2*, and, therefore, indistinguishable in external appearance from the progeny of the previous class; and (c) mice lacking both of the mannose 6-phosphate receptors. The results (Table 2) showed that, in the absence of IGF-II, all *Igf2r* maternal heterozygotes (class II) were fully viable and were obtained at an approximately 1:2 ratio (103:187) with animals of class I that possessed a wild-type *Igf2r* gene.

Genetic Elimination of the Mannose 6-Phosphate Pathway

Among the progeny of the crosses described above, class III mutants lacking both CI- and CD-MPR in an *Igf2* null background were observed at a frequency significantly lower than expected, if they were fully viable (Table 2). Instead of obtaining a number of animals equal to that of their siblings belonging to class II (103) or half of the number in class I (187/2 = 93.5), only 18 double mutants were observed, i.e., 17.5% (18/103) or 19.3% (18/93.5) of the expected number. Since 5 of these mutants (5/18 or 28%) were found dead at birth, we assume that additional nonviable neonates were not recovered. Whether a fraction of the mutants lacking both mannose 6-phosphate receptors are also lost *in utero* is currently unknown.

The phenotype of class III animals, some of which escape perinatal lethality, and its potential dependence on genetic background are still under study. We note, however, that the liveborn class III mutants survive only for a period of time that does not exceed 3–4 postnatal weeks and exhibit a characteristic waddling gait, which is one of the striking features (due to joint stiffness) of the rare human hereditary diseases mucolipidosis II (ML II; I-cell disease) and III (ML III) (reviewed by Durand *et al.*, 1982; Watts and Gibbs, 1986; Nolan and Sly, 1989; McKusick, 1994). These disorders, which are clinically heterogeneous in skeletal and other abnormalities, differ in progression and severity of symptoms. While ML III is milder and permits survival to adulthood, the more severe and rapidly progressing ML II leads to death usually between 5 and 8 years of age. Although ML II and III appear to be manifestations of mutations at different loci, as suggested by cell-fusion complementation studies (see Shows *et al.*, 1982), they both exhibit invariably a deficiency of the enzyme *N*-acetylglucosamine-1-phosphotransferase that is necessary for the generation of phosphomannosyl residues. Thus, the newly synthesized lysosomal enzymes lacking a mannose 6-phosphate marker are not targeted to lysosomes, but secreted into the extracellular space. It is expected that the same will be observed in mice lacking the necessary receptors for binding of phosphomannosyl residues. It remains to be seen, however, whether our mutants simulate closely the overall phenotypic manifestations of ML II and/or ML III.

Survival of Double Mutants Lacking IGF1R and IGF2R

Previously, we examined a few double mutants lacking IGF1R and carrying a maternally derived T^{hp} deletion and observed that they were partially rescued from lethality, although each of the single mutations is lethal (Baker *et al.*, 1993). To explore further this unexpected phenomenon using the defined *Igf2r* null mutation, we intercrossed $Igf1r^{+/-}Igf2r^{+/-}$ double heterozygotes (Table 1). The ratio of normal animals to double mutants lacking both IGF receptors, all of which survived to adulthood, was 3.5:1 (273:77), rather than the expected 3:1, but this difference is not statistically significant (χ^2 test, $P > 0.2$).

During embryogenesis the growth of these double receptor mutants was practically indistinguishable from that of normal siblings (Fig. 3). To interpret their normal development and survival, we propose that the excess of IGF-II in the absence of IGF2R cannot cause lethality by overstimulation of IGF1R, which is also missing, but can sustain opportunistically embryonic development by signaling through a putative XR_e receptor. In this regard, to demonstrate that rescue is indeed attained by excess of IGF-II, we generated triple mutants lacking this ligand and both IGF receptors (see Table 3). The results showed that, in contrast with the double receptor mutants (class V; Table 3), normal embryonic growth cannot be sustained, if IGF-II is also absent (class VIII embryos; Table 3). In fact, these triple mutants (class VIII) exhibited only about 30% of normal weight and, thus, were phenotypicaly indistinguishable from double mutants lacking IGF1R and IGF-II (class VI; Table 3) or from embryos lacking both IGF ligands (IGF-I and IGF-II; Liu *et al.*, 1993).

Interestingly, while the double IGF receptor mutant embryos had normal weights, their placentas were larger than normal and did not differ statistically in size from those of mutants lacking only IGF2R (Fig. 4). In contrast, in all cases that IGF-II was absent (mutants lacking IGF-II; IGF-II and IGF1R; IGF-II and IGF2R; or IGF-II, IGF1R, and IGF2R), the placentas were small (about 67% of normal size at e18.5) and did not exhibit statistically significant differences in weight between the mutants. Considered altogether, the available data provide genetic evidence for the existence of two distinct unknown receptors, XR_e and XR_p, mediating the growth-promoting roles of IGF-II in the embryo and the placenta, respectively.

An additional class of mutants (VII; Table 3) obtained from the same crosses lacked both IGF2R and IGF-II. Unexpectedly, the weight of these double mutants at e18.5 was statistically significantly higher than that of embryos lacking IGF-II alone (about 74% vs 60% of normal; Table 3). From a limited number of observations (8 animals of each group), we ascertained that the *Igf2/Igf2r* double mutants continued to be about 20% heavier than the *Igf2* single mutants for at least 6 months of postnatal life. A possible, although not unique, interpretation of this difference is that IGF2R, in addition to its interaction with IGF-II, is involved

to some extent in IGF-I turnover. If this is correct, even a relatively small excess of IGF-I in *Igf2/Igf2r* double mutants could stimulate IGF1R beyond the degree allowable in single *Igf2* mutants retaining IGF2R function. We note in this regard that the current view from a few *in vitro* binding studies is that IGF-I either interacts with IGF2R at a level of 1% that of IGF-II or it is not recognized at all by this receptor (reviewed by Nissley *et al.*, 1991). Nevertheless, the situation may be different in whole embryos or tissue-specificities may exist, since IGF-I appears to interact quite strongly with IGF2R at least in the rat heart (Haskell and Tucker, 1994).

Postnatally, the growth of double mutants lacking both IGF receptors continued to be indistinguishable from wild-type for the first 2 weeks. However, between Postnatal Days 15 and 30 (p15–p30), very little increase in body size occurred in the mutants (Fig. 5), which exhibited their lowest relative weight (63.9 ± 2.8% of normal) at p30 (the growth curves of 19 double mutants and 32 wild-type mice from 14 litters were examined). In contrast, the growth of normal mice slowed down only temporarily after p15, as expected (see Boettiger and Osborn, 1938). Interestingly, after p30, catch-up growth was observed in the mutants, which by the age of 70 days either became 78.9 ± 1.8% of normal (15 mutants) or in some cases (4 mutants) attained a weight that was indistinguishable from that of wild-type siblings (see Fig. 5).

Surprisingly, both sexes of the double receptor mutants proved to be fertile, in contrast to the infertility of nullizygous *Igf1* mice (Baker *et al.*, 1996). We observed, however, that litter sizes were consistently small and that the mothers did not take care of the neonates, which could survive only by fostering. Since the observations on survival, growth, and fertility were made not only with $Igf1r^{-/-}Igf2r^{+/m-}$ mutants, but also with mice completely nullizygous for both IGF receptors ($Igf1r^{-/-}Igf2r^{-/-}$), any contribution of potential imprint relaxation of the paternal *Igf2r* allele to the phenotype is excluded, at least in this case.

Considering that the developmental extinction of *Igf2* gene transcription in all tissues of rodents (except in the choroid plexus and the leptomeninges) does not occur before at least 2 weeks of postnatal life (reviewed by Rechler and Nissley, 1990), the normal growth until p15 of mutants lacking both IGF receptors is not surprising, if XR_e continues to be expressed after birth. On the other hand, the catch-up growth observed with these mutants after p30 is puzzling, because IGF-II is not present any longer, while the mutants lack IGF1R. It is not obvious, therefore, how IGF-I action could be mediated, since IGF-I signals exclusively through IGF1R during embryogenesis, and the expectation was that the same would hold postnatally. Nevertheless, the data suggest that IGF-I should begin interacting after birth, perhaps opportunistically, with an unknown receptor (XR_e or other) to fulfill its functions on growth and reproduction. Any alternative model postulating that growth effectors other than those of the IGF system could play compensatory roles would be significantly more complex, because such compensation should have also occurred in the absence of IGF-I alone. However, in contrast to the catch-up growth and fertility of the mice lacking both IGF receptors, the infertile *Igf1* null mutants, which are born with 60% of normal body weight, become 30% of normal size at 8 weeks of age (Baker *et al.*, 1993, 1996). In addition, it cannot be ignored that growth hormone clearly requires the presence of IGF-I for mediation of at least some of its growth-promoting functions (for a review, see Scanes and Daughaday, 1995). Unraveling the identity of unknown receptors and exploring the mechanistic aspects of their relationships with the IGF ligands, which underlie the genetic observations that we have described, are new and challenging questions.

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