IGF-I is required for normal embryonic growth in mice

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IGF-I is a pleiotropic hormone reported to affect linear growth, glucose metabolism, organ homeostasis, and the immune and neurologic systems. In contrast to IGF-II, IGF-I is expressed at low levels embryonically and has been thought to be more important for postnatal growth and development. To investigate the role of IGF-I in normal development we generated mice with an inactive IGF-I gene by homologous recombination in ES cells. Heterozygous mice are healthy and fertile, but they are 10-20% smaller than wild-type littermates and have lower than normal levels of IGF-I. The size reduction is attributable to a decrease in organs and muscle and bone mass. However, all tissues appear histologically normal. At birth homozygous mutant mice (IGF-I$^{-/-}$) are <60% body weight of wild type. Greater than 95% of IGF-I$^{-/-}$ pups die perinatally. Histopathology is characterized by underdevelopment of muscle tissue. Lungs of late embryonic and neonates also appeared less organized with ill-defined alveolae. IGF-I appears to be essential for correct embryonic development in mice.

[*Key Words*: IGF-I; homologous recombination; embryonic development; mouse]

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Insulin-like growth factors (IGF-I and IGF-II) are involved in several aspects of growth and development. Their identification originates with the observation of an activity that could induce sulfate incorporation into cartilage (Salmon and DuVall 1970a,b). This activity is modulated by the level of growth hormone (GH), leading to the idea that the growth-promoting effects of GH are mediated by the stimulation of the synthesis and release of a somatomedin (the somatomedin hypothesis) (Daughaday 1972). Independently, an activity was identified that lowered glucose levels even in the presence of anti-insulin antibodies (nonsuppressible insulin like activity, NSILA) (Oelz et al. 1970; Megyesi et al. 1974; Rechler et al. 1974). Additionally, a protein synthesized by the liver, mitogenic for fibroblasts, and termed multiplication stimulating activity (MSA) was identified (Nissley et al. 1976). With their purification and cloning, all of these activities were found to be attributable to IGF-I (Jansen et al. 1983; Ullrich et al. 1984) and IGF-II (Bell et al. 1984; Ullrich et al. 1984; Whitfield et al. 1984). More recently, the IGFs have also been implicated in organ and soft tissue regeneration [kidney (Moran et al. 1991; Mulroney et al. 1991), pancreas (Smith et al. 1991), muscle (Edwall et al. 1989; Levinovitz et al. 1992)], lipid formation and breakdown (Lewis et al. 1988; Smith et al. 1988), skeletal muscle innervation (Caroni and Grandes 1990), erythropoiesis (Kurtz et al. 1988), and in some aspects of immune function (Binz et al. 1990; Baxter et al. 1991; Beschorner et al. 1991; Landreth et al. 1992; Timsit et al. 1992).

The actions of the IGFs are mediated primarily through the type-I IGF receptor. At high concentrations IGF-I will also bind to the structurally related insulin receptor. The mannose-6-phosphate receptor was identified as a receptor for IGF-II (Pohlmann et al. 1987; MacDonald et al. 1988; Oshima et al. 1988), although the importance of this receptor for the action of the IGFs is still unclear. In addition, there are at least six IGF-binding proteins, which are structurally related to each other but are quite distinct from the IGF-I receptor. The expression of each of these binding proteins is hormonally and developmentally regulated and has a distinct organ- and tissue-specific pattern of expression. The function of these binding proteins has not been fully resolved. They have been reported to both inhibit and enhance the actions of IGF-I, and they can clearly alter the biodistribution of the IGFs (for review, see Drop et al. 1992; Rechler and Brown 1992).

Transgenic mice overexpressing IGF-I (Mathews et al. 1988) and mice lacking a functional IGF-II (DeChiara et al. 1990, 1991) gene have been used to begin to resolve some of the questions concerning the biology of the IGFs. The IGF-I transgenic mice have demonstrated that high levels of IGF-I can enhance body growth, although this was not as dramatic as that seen in GH transgenic mice (Palmiter et al. 1982). Mice lacking a functional

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IGF-II gene are viable, demonstrating that the IGF-II protein is not essential for development and survival, although they are significantly smaller than normal (De Chiara et al. 1990, 1991).

In this report we demonstrate that mice lacking a functional IGF-I gene progress through prenatal development and are born. These IGF-I-deficient mice are significantly smaller than their normal littermates, they have a severe muscle dystrophy, and the majority (>95%) of these mice die at birth.

Results

Generation of IGF-I-deficient mice

An 18-kb genomic clone encompassing the 5′ end of the murine IGF-I locus (containing exons 1, 2, and 3) was isolated from a DBM EMBL3 library (Clontech). Sequences were identified corresponding to a 157-bp exon of the rat gene (Shimatsu and Rotwein 1987), encoding the leader sequence and the majority of the B chain of the protein. An artificial *Xho*I site was engineered into this exon, interrupting the coding sequence that interrupts amino acid 15 of the mature protein. An insertion vector was constructed in which a phosphoglycerate kinase (PGK)–neomycin resistance gene expression cassette (Tybulewicz et al. 1991) was inserted at this point, in the opposite orientation with respect to the transcription of IGF-I (Fig. 1A). The targeting vector contained 3.5 kb of homology 5′ of the selectable marker and 6.5 kb of 3′ homology. This construct introduces multiple stop codons in all reading frames in the middle of the B chain of IGF-I. A cytomegalovirus (CMV)-driven thymidine kinase cassette was placed next to the 5′ end of the IGF-I homologous sequences to permit negative selection against nonhomologous integration events (Mansour et

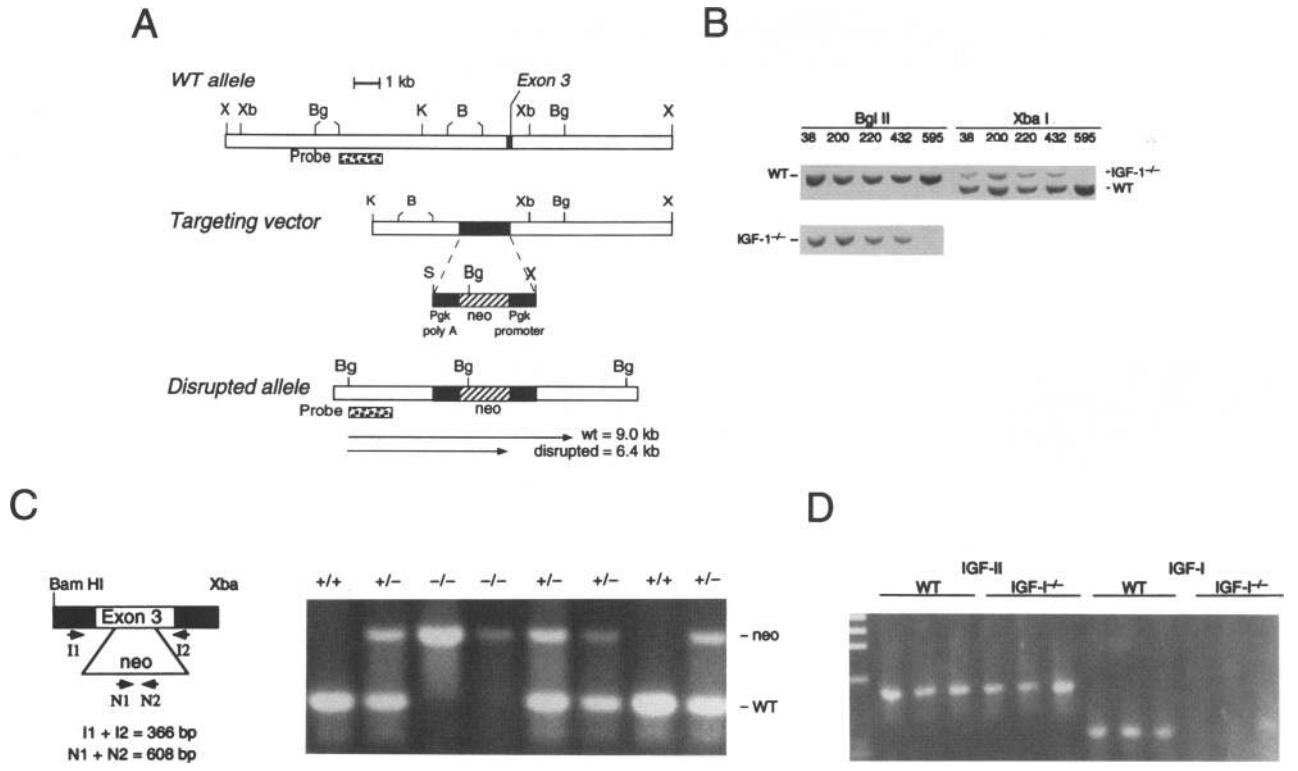

Figure 1. Targeting vector, screening strategy, and analysis of targeted cell lines and mutant animals. (*A*) Diagram of wild-type allele, targeting vector, and disrupted allele. The open box represents IGF-I genomic sequence, the solid box represents exon 3, and the hatched box represents the neomycin cassette. The dotted box represents the sequences used as a probe for Southern analysis. (B) *Bam*HI; (Bg) *Bgl*II; (K) *Kpn*I; (S) *Sal*I; (Xb) *Xba*I; (X) *Xho*I. (*B*) Southern blot of five G418-resistant cell lines, digested with *Bgl*II or *Xba*I and probed with a fragment external to the targeting construct. Fragment sizes are shown. The introduction of a new *Bgl*II site in the neomycin cassette results in a smaller hybridizing band in the correctly targeted clones (38, 200, 220, and 432) while digestion with *Xba*I results in a larger mutant band. Clone 595 is an example of a clone where the vector insertion was not at the IGF-I locus. (*C*) Diagram of primers for PCR analysis (not to scale) and the results of PCR genotyping of a representative litter (containing wild type +/+, heterozygotes +/−, and homozygous mutant −/− pups) generated by intercrossing heterozygote parents. A product generated using I1 and I2 (wild-type primers) indicates the presence of at least one wild-type allele, and a product using N1 and N2 (neomycin primers) indicates at least one disrupted allele. (*D*) Reverse transcriptase PCR of total mRNA from three neonate wild type and three IGF-I$^{-/-}$ pups, demonstrating that IGF-II message is present but that IGF-I mRNA is undetectable in the IGF-I$^{-/-}$ animals. The band visible in the last track is an artifact. It migrates with the wrong molecular weight and does not hybridize with probes specific for IGF-I sequences.

al. 1988). AB.1 embryonal stem (ES) cells were transfected with this construct, and clones were isolated based on resistance to G418. DNA extracted from these clones was screened for homologous recombination by Southern blot analysis (Fig. 1B). The presence of the *Bgl*II restriction site in the neomycin resistance gene results in a 6.5-kb *Bgl*II band in addition to the 9-kb wild-type band when DNA from clones with a disrupted IGF-I allele are hybridized with sequences flanking the targeting vector. Of 82 G418-resistant colonies, 1 contained homologously recombined sequences. This frequency increased to 1:11 when additional selection with FIAU was used to select against thymidine kinase expression (data not shown). Recombinant clones were injected into C57BL/6J blastocysts, and the embryos were reimplanted into pseudopregnant recipient females. Chimeric mice derived from two independent clones transmitted the mutation to offspring. Heterozygous animals were identified by Southern blot analysis using tail DNA and interbred so as to produce animals in which both IGF-I alleles were inactivated. Polymerase chain reaction (PCR) analysis was used to confirm the genotype (Fig. 1C). The absence of a product derived from the wild-type allele identified homozygous mutant mice (IGF-I$^{-/-}$). Reverse transcription and PCR amplification of IGF-I and IGF-II message from wild-type and IGF-I$^{-/-}$ mice shows no detectable mRNA from the IGF-I allele in the mutant mice (Fig. 1D).

Phenotype of IGF-I heterozygous mice

Heterozygotes were found to be smaller than their wild-type littermates. The size difference was detectable at birth and continued throughout the growth of the mice (Fig. 2A). Upon necropsy there was a corresponding reduction in the wet weights of most of the organs. Although there was individual tissue variation, most of the organs were 10–20% smaller in the heterozygotes (data not shown). No abnormalities or obvious differences were detected in a complete set of tissues on histological examination. Serum IGF-I levels were determined and found to be 37% lower in the heterozygotes (Fig. 2B). There were no significant differences in serum components (albumin, alkaline phosphatase, cholesterol, creatine kinase, creatinine, electrolytes, globulins, glucose, insulin, total protein, triglycerides, urea nitrogen, and other liver and kidney function tests) or blood cell profiles (data not shown). Tetracycline labeling of the growth plate showed no significant differences between heterozygotes and wild types in 129/Sv×C57Bl/6 F_1s (data not shown). Male and female heterozygotes were fertile and healthy and were intercrossed to generate homozygous IGF-I$^{-/-}$ mice.

Phenotype of homozygous IGF-I-deficient mice

Initially all IGF-I$^{-/-}$ mice were found dead at birth. These homozygotes were <60% of the body weight of

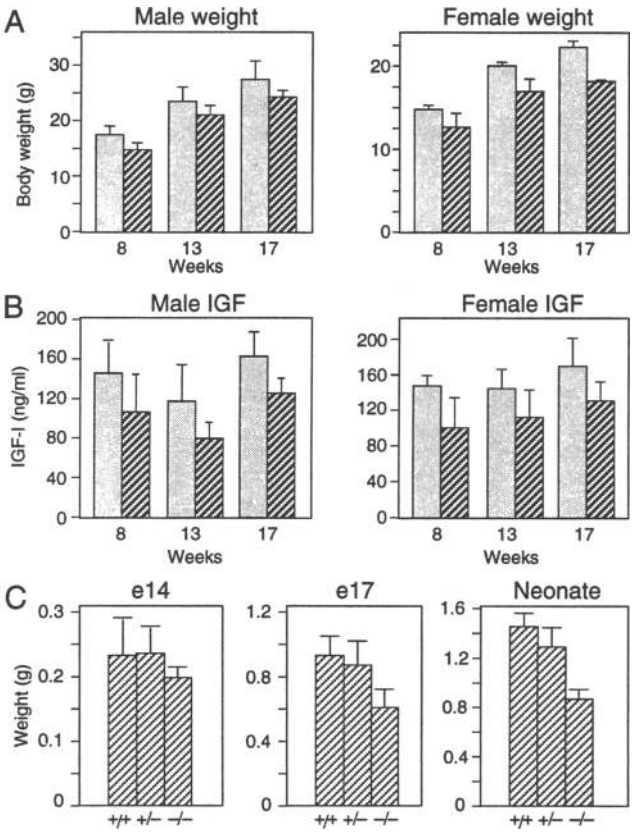

Figure 2. Weights and serum IGF-I levels of wild-type, heterozygote, and IGF-I$^{-/-}$ animals. (*A*) Weights of heterozygous and wild-type animals at 8, 13, and 17 weeks of age: males, 8 wild-type and 7 heterozygous siblings; females, 6 wild-type and 5 heterozygous. Males, $P<0.02$ for $+/=$ vs. $+/-$ at 8 and 17 weeks; Not significant (N.S) at 13 weeks. Females, $P<0.05$ (8 weeks); $P<0.01$ (13 weeks); $P<0.001$ (17 weeks). The wild-type ($+/+$) animals are represented by the shaded bars, and the heterozygous ($-/+$) animals are represented by the hatched bars. (*B*) Serum IGF-I levels of the same animals as in *A*. There is some interassay variation. All animals were bled orbitally after weighing, and serum was stored at −20°C and assayed for total IGF-I within a week. Males, N.S. (8 weeks); $P<0.05$ (13 weeks); $P<0.01$ (17 weeks). Females, $P<0.01$ (8 weeks); N.S. (13 weeks and 17 weeks). The wild-type ($+/+$) animals are represented by the shaded bars, and the heterozygous ($-/+$) animals are represented by the hatched bars. (*C*) Weights of neonates and embryonic mice. Neonates, $P<0.001$ for $+/+$ vs. $+/-$ and for $+/-$ vs. $-/-$. e14, N.S. for all comparisons. e17; $P<0.005$ for $+/+$ vs. $-/-$ and for $+/-$ vs. $-/-$.

their wild-type siblings, and their lungs were not inflated. Upon dissection and analysis of viscera it was found that the decrease in weight of the viscera was very small and probably could not account for the total decrease in body weight of the IGF-I$^{-/-}$ animals. When expressed as percent body weight, the livers and brains of the IGF-I$^{-/-}$ were found to be ~150% of the weight of the wild-type animals (IGF-I$^{-/-}$ brain, 9.68% of body weight; liver, 6.58%; wild-type brain, 6.22% of body weight; liver, 4.47%). Pregnant mice were sacrificed at

embryonic day 18.5 (e18.5), and the embryos were examined. The homozygous mutant (IGF-I$^{-/-}$) mice were found to be alive at e18.5 in that their hearts were beating and they appeared grossly normal but smaller than the heterozygous or wild-type littermates. Embryos were also examined from e13.5 to e17.5. At e17.5 the heterozygotes were 94% (not statistically significant) and the IGF-I$^{-/-}$ pups were 64% ($P<0.0025$), the size of their wild-type littermates. At all earlier stages there were no differences in body weights between the wild types and the heterozygotes; the IGF-I$^{-/-}$ mice were generally smaller at all stages examined, but this was not statistically significant earlier than e15.5. The absolute size of the IGF-I$^{-/-}$ pups and wild types was variable depending on the genetic background and litter size.

Histopathology of IGF-I-deficient mice

The primary alteration noted in the neonates based on histopathologic examination was a generalized muscular dystrophy that was seen most easily in the diaphragm, heart, and tongue (Fig. 3). The muscles appeared less organized, and there appeared to be a decrease in the amount of myofibrils. The degree of cellularity did not appear decreased; however, quantitative methods were not employed. The lungs in the IGF-I$^{-/-}$ were not inflated (ataletic). The alveolar septae appeared to be more cellular than those of the wild-type neonates; however, this observation was complicated by the presence of atelectasis.

Similar changes were observed in the day 17.5 embryos; however, the differences between the IGF-I$^{-/-}$ and wild type were less marked (Fig. 4). The lungs of the IGF-I$^{-/-}$ were more cellular, with less evidence of alveolar separation and definition than the wild-type controls. At e13.5 and e15.5, muscle changes were detected in some but not all of the IGF-I$^{-/-}$ animals. There was no difference in the appearance of the lungs at these

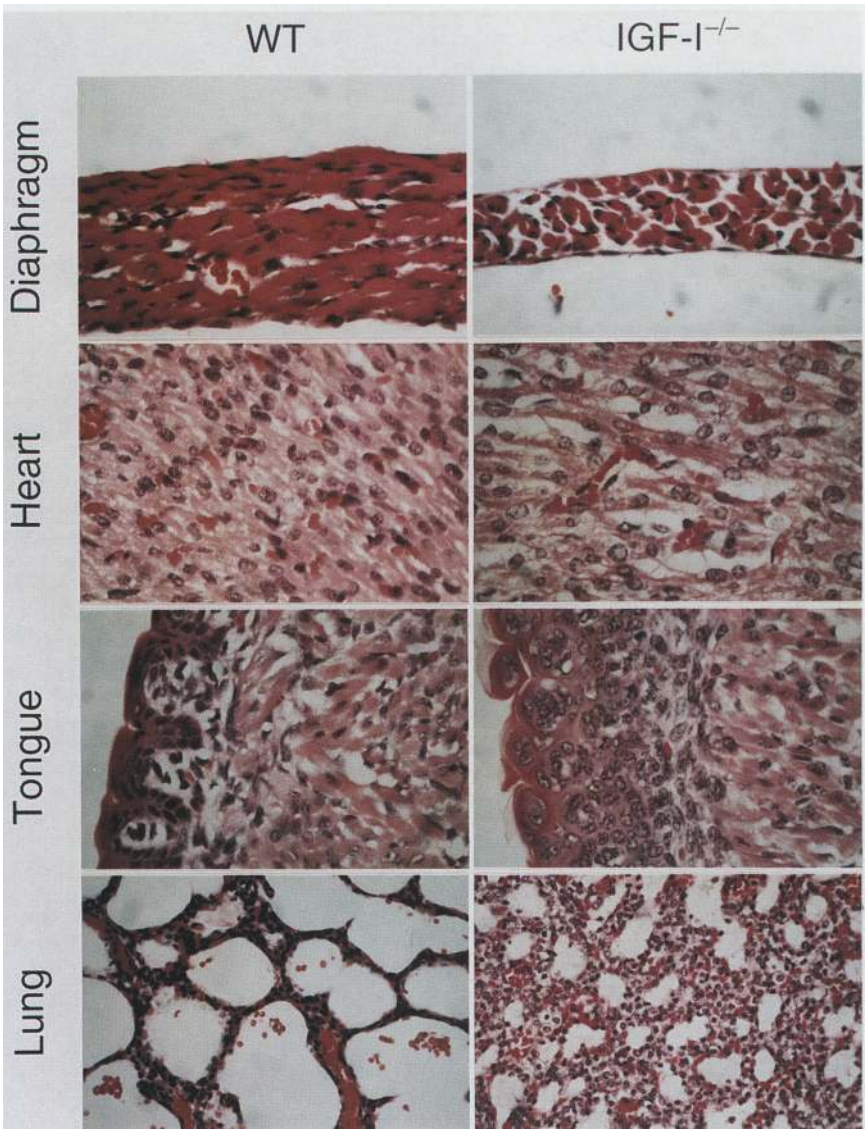

Figure 3. Histological examination of tissues from neonate animals. Comparisons of diaphragm (magnification, 34.8×), heart (magnification, 34.8×), tongue (magnification, 34.8×), and lung (magnification, 17.4×) histology of wild-type (*left* column) and IGF-I$^{-/-}$ (*right* column) animals are shown. The muscles in the diaphragm, heart, and tongue have decreased amounts of myofibrillary material, and there is increased vacuolization of the myofibers. In contrast, the epithelial layer of the tongue appears more cellular but is less organized in the IGF-deficient animals. The lungs of the IGF-I$^{-/-}$ neonates are not inflated, and there is an increased cellularity and decreased organization of the alveolar septae.

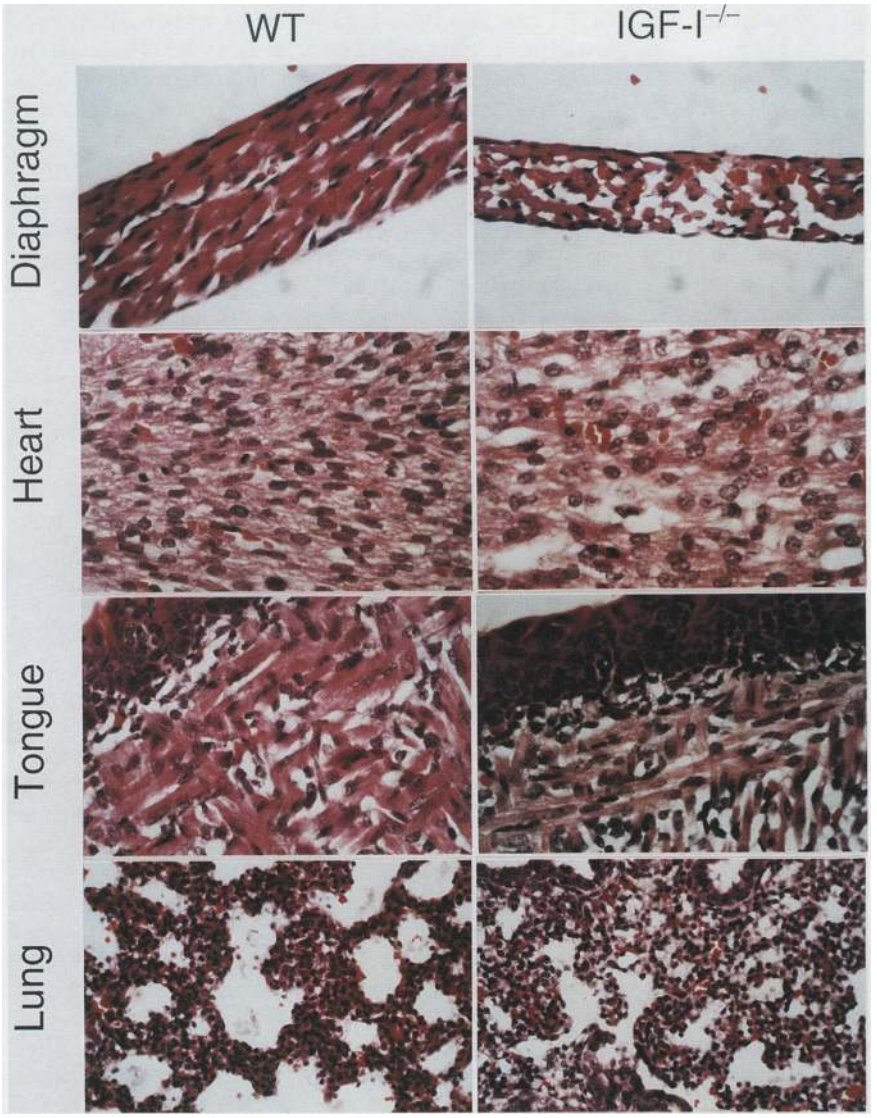

Figure 4. Histological examination of tissues from e17.5 animals. Comparisons of diaphragm (magnification, 34.8×), heart (magnification, 34.8×), tongue (magnification, 34.8×), and lung (magnification, 17.4×) histology of wild-type (*left* column) and IGF$^{-/-}$ (*right* column) animals are shown. Although the changes are not as dramatic in the 17 mice as compared with the neonates, the muscles (diaphragm, heart, and tongue) of the IGF-I$^{-/-}$ fetuses are less organized and have more vacuolization of the myofibers. The lungs of the e17 IGF-I$^{-/-}$ fetuses show less alveolar separation and appear more cellular.

stages. In addition, in some animals from all ages the epidermis appeared hypoplastic with a decrease in the thickness of the cell layers, particularly the strata spinosum. Remaining organ systems showed no detectable abnormalities at any of the stages.

Discussion

We have disrupted the mouse IGF-I gene by inserting a copy of the neomycin gene with its own promotor and polyadenylation sites into the start of the IGF-I-coding sequence. Expression from the targeted IGF-I gene is not detectable by reverse transcriptase PCR in homozygous IGF-I$^{-/-}$ mice (Fig. 1D). Two lines of mice carrying the same mutation at the IGF-I locus have been generated from independently isolated cell clones. In both lines the phenotype is indistinguishable; that is, reduced muscle mass in the heterozygotes and decreased size, and myodystrophy and substantial perinatal mortality in the homozygotes.

Serum levels of IGF-II in the rat are highest in the fetus and decline rapidly after birth (for review, see Daughaday and Rotwein 1989). In contrast, serum concentrations of IGF-I are low in the fetal and early postnatal periods compared with adult levels, and they increase markedly during puberty (Daughaday and Rotwein 1989). These data led to the hypothesis that IGF-II functions as the predominant fetal growth factor, whereas IGF-I is more important in postnatal life (Daughaday and Rotwein 1989). The observation that rat IGF-I mRNA increases approximately ninefold between e11 and e13 while remaining eightfold lower than adult mRNA levels (Rotwein et al. 1987) indicates some role for IGF-I in development. As the IGF-II gene has been inactivated with no effect on viability (DeChiara et al. 1990, 1991) and as IGF-I is essential for normal prenatal growth and substantially affects viability (this report), it is clear that the

relative importance of the IGFs in pre- and postnatal development may have to be revised.

Based on the results reported here, IGF-I has a profound effect on the size of embryonic and postnatal mice. Heterozygosity is associated with a 37% decrease in circulating IGF-I levels and a reduction in mean body weight at birth (heterozygotes 88% of wild type) at 2 months of age (heterozygous males, 85% of wild type; heterozygous females, 87% of wild type) and at 4–6 months (heterozygous males, 83% of wild type; heterozygous females, 81% of wild type). In all cases, the difference between the wild-type and heterozygous body weight is statistically significant on both intercross (129/Sv×C57Bl/6) and on C57BL/6J (N4) genetic backgrounds. At birth, homozygous mutant mice are 58% of the weight of the wild types in both the intercross and C57BL/6J (N4) backgrounds. We have found that the majority of homozygous mutants die at birth. However, ~5% of the homozygous mutant mice survive beyond birth for up to 4 months. This "leakiness" has been seen on both the intercross and the C57Bl/6 (N4) background and in the two independent lines. Thus, it could be considered that the deleterious effects of the lack of IGF-I lead to a syndrome that is at the borderline of survival; however, a small percentage of mice, for reasons as yet unknown, do survive. Although we do not have enough surviving animals to describe them fully here, the few animals that did survive were <45% of the size of their wild-type siblings at 8 weeks of age.

The IGF-$I^{-/-}$ mice exhibit a generalized decrease in muscle mass and a delay in muscle maturation. IGF-I has been shown to stimulate myogenic differentiation (Florini et al. 1991a; for review, see Florini et al. 1991b). It increases the levels of myogenin in vitro leading to terminal myogenic differentiation. Recently described mutations in the mouse myogenin gene have a muscle phenotype similar to but more severe than that described in this paper (Nabeshima et al. 1993; Hasty et al. 1993). These mice are also nonviable. It is not clear whether the myodystrophy is the primary cause of the perinatal mortality in the IGF-$I^{-/-}$ mice. We are investigating whether the timing or level of myogenin expression is altered in the IGF-$I^{-/-}$ background.

The poorly developed lungs in the IGF-I-deficient embryos and neonates clearly demonstrate the important role of IGF-I in normal lung maturation. It has been shown previously that IGF-I is synthesized by fetal lung explants (Snyder and D'Ercole 1987; Davenport et al. 1988). Furthermore, use of an anti-IGF-I receptor antibody has demonstrated that exogenous IGF-I is important for the proliferation and synthesis of extracellular matrix by lung-derived fibroblasts (Goldstein et al. 1989). This apparent role of IGF-I as a lung cell mitogen is also consistent with the many studies linking IGF-I to proliferation of lung tumor cells (for review, see Guillemin et al. 1991; Moody and Cuttitta 1993). Future experiments in IGF-I-deficient mice will attempt to define whether the lung abnormality is primarily a consequence of a failure of proliferation or of a deficiency in extracellular matrix deposition.

IGF-I has also been reported to be important in bone growth (for review, see Schmid et al. 1991). There was no histopathological difference in bone development between wild-type and IGF-$1^{-/-}$ neonates. We did not see any statistically significant difference in the growth plates of (C57Bl/6J×129/Sv) F_2 wild types and heterozygotes. However, the effects of the mixed genetic background may be masking subtle changes. 129/Sv mice are ~15% larger than C57Bl/6J mice. Skin changes were not present in all of the embryonic and neonatal IGF-$1^{-/-}$ animals, which may also reflect differences in genetic background. We are currently developing inbred strains to minimize these variables. The histopathological effects appear later than would be expected from the stage at which embryonic IGF-I mRNA levels increase in the rat (Rotwein et al. 1987). Experiments are in progress to determine whether transcriptional and structural components of various tissues, including muscle and lung, are affected by the abscence of IGF-I.

Based on correlations between expression and development and on the effects of IGF-I on organ recovery following damage, it has been suggested that IGF-I is involved in the development of other organ systems. However, there are no histologically obvious abnormalities in, for example, the kidney or the central nervous system of the IGF-I-deficient mice. If IGF-I is important in the development of these organs, it is possible that, in the absence of this factor, others can adequately compensate.

Following submission of this manuscript, a description of mice with an insertion in the IGF receptor and of mice with an insertion in the IGF-I gene was published (Baker et al. 1993; Liu et al. 1993). Although the overall phenotype is comparable, the mice described by Liu et al. do not appear to be compromised as severely as the mice described in this report. We clearly see a weight difference in the heterozygous mice that is detectable at birth and is still evident at 3 months of age; this weight difference correlates with a decrease in the circulating IGF-I levels in the heterozygotes. Liu et al. report that heterozygous animals are phenotypically normal. We also see severe and obvious changes in muscle and lung development in the homozygous mutant embryos and a perinatal death that was accompanied by an inability to breath; Liu et al. report that the IGF-I-deficient animals are able to breathe at birth but die a short time later. Finally, Liu et al. report that 16% of their homozygous animals (C57Bl/6×129) survive; we find that <5% of our animals survive and that these few animals do not survive beyond 7 months of age. It is unlikely that these differences are attributable to background genetics, as in both cases intercrosses between (C57Bl/6J×129/Sv) F_2 mice were used. One possible source of the difference is the position within the IGF-I gene at which the neomycin cassette was inserted. In our case, we interrupted the gene at amino acid 15 of the mature protein; Liu et al. inserted the neomycin gene at amino acid 50.

In summary, we believe that IGF-I plays a fundamentally very important role in embryonic development and is important for viability in the mouse.

Materials and methods

Construction of targeting vector

A murine DBM genomic library, packaged in EMBL3 phage (Clontech), was screened using a random prime radiolabeled DNA probe specific to murine IGF-I exon 3, generated by PCR. Two overlapping λ clones were isolated. The larger of these (λigf1.3a) containing an 18-kb fragment encompassing the 5′ end of the mIGF-I gene, was subcloned into the *SalI* site of puc118. The insert was characterized by restriction mapping and sequencing of intron/exon junctions. Subclones of fragments containing exon 3 were engineered using PCR to insert a unique *XhoI* cloning site, interrupting the protein-coding sequence. A PGKneopA (Tybulewicz et al. 1991) was inserted in the antisense direction into this *XhoI* site. These constructs were extended at both ends with adjacent genomic sequences to give a total of 9.5 kb homology. The vector also contained one copy of a CMV-driven TKpA cassette for negative selection (Mansour et al. 1988), inserted upstream of the homologous sequences. Plasmid DNA was prepared by cesium chloride banding, linearized with *KpnI*, and used for electroporation.

Cell culture and transfection

To inactivate one endogenous IGF-I allele, early passage (p13–15) AB2.1 ES cells (McMahon and Bradley 1990) were electroporated with *KpnI*-digested IGF–Ineo DNA in a Bio-Rad Gene Pulser. Each electroporation was done on 10^7 cells with 20–30 μg of DNA at 0.23 kV and 250 mF and plated onto two 90-mm dishes with mitomycin C-inactivated SNL76/7 feeder cells (McMahon and Bradley 1990). Selection with G418 (400 μg/ml) was applied 24 hr after electroporation. In some experiments FIAU (0.2 mM) was also added 24 hr after electroporation. Resistant colonies were picked after 10 days and replica cultured for freezing and screening by Southern analysis.

Blastocyst injection and mouse breeding

Approximately 15 targeted ES cells were injected into the blastocoel of 3.5 day postcoitus C57BL/6J blastocysts for several of the targeted clones. The injected blastocysts were transferred to CD1 pseudopregnant females and allowed to develop to term. The extent of chimerism was determined according to the degree of agouti coat color contribution to the mice at 1 week of age. The male chimeras were bred to C57BL/6J female mice to assay germ-line transmission of the manipulated ES cells. Germ-line transmission was determined by the presence of agouti coat color in the progeny. The presence of the mutation was initially determined by Southern blot analysis of mouse tail genomic DNA and subsequently by PCR.

To assay the effect of the mutation in alternative genetic backgrounds heterozygous males were bred with C57BL/6J females for several generations before the intercrossing of heterozygotes to generate mice homozygous for the IGF-$I^{-/-}$ mutation.

DNA analysis

ES cell clones were lysed in 100 mM NaCl, 50 mM Tris (pH 7.5), 10 mM EDTA (pH 8.0), 0.5% SDS, and 1 μg/ml of proteinase K at 50°C for >3 hr. The DNA was precipitated with 2.5 volumes of EtOH at room temperture and spooled from the tube using a sterile disposable inoculation loop, rinsed with 70% EtOH, air-dried, and resuspended in TE. To determine the genotype of the mice, genomic DNA was extracted from tail clips or placentas. For the tail extraction procedure, pieces of tail were lysed overnight in lysis buffer [50 mM Tris (pH 8.0), 7.5 mM EDTA (pH 8.0), 100 mM NaCl, 0.5% SDS, 1 mg/ml of proteinase K added fresh]. The next day, the lysate was adjusted to 100 mM potassium acetate and 1.5 volume of chloroform was added, mixed by inversion, and placed at 4°C for >15 min. The chilled extract was microcentrifuged for 10 min, the aqueous phase was removed to a clean tube, 2.5 volumes of EtOH was added, and the DNA was spooled out and resuspended in TE. Genotyping was carried out using Southern analysis or PCR.

RNA preparation

For neonate RNA preparations, eviscerated carcasses were ground in 7.5 M guanidine HCl, 100 mM KOAc (pH 5.0), and the nucleic acids precipitated with 0.6 volume of EtOH for >1 hr at −20°C. The precipitate was resuspended in fresh guanidine HCl solution and extracted with an equal volume of phenol/$CHCl_3$ twice and reprecipitated.

Southern analysis

Genomic DNA (10–20 μg) was digested with *BglII* or *XbaI* (Boehringer Mannheim) and fractionated by agarose gel electrophoresis. DNA was transferred to Zeta-probe (Bio-Rad) membranes in 0.4 M NaOH, rinsed in 2× SSC (0.3 M NaCl, 0.3 M sodium citrate), and baked for 1 hr at 80°C. Filters were hybridized with radiolabeled probes in 1.5× SSPE, 1% SDS, 0.5% fat-free milk, and 200 μg/ml of single-stranded DNA for 18 hr at 65°C and washed at high stringency (0.2× SSC) before autoradiography.

PCR

DNA samples were amplified for 35 cycles in 100-μl reactions in PCR buffer containing 1 mM of each dNTP and 40 U/ml of *Taq* DNA polymerase and oligonucleotide primers. For detection of the wild-type allele, primers were derived from sequences upstream of exon 3 (I1 = 5′-GACCAGTAGCAAAGGACTTACCAC and 3′ of the neomycin insertion in the exon (I2 = 5′-AAGTAAAAGCCCCTCGGTCCACAC) resulting in a 366-bp product. For detection of neomycin, primers (N1 = sense 5′-TGACTGGGCACAACAGACAATCGG and antisense N2 = 5′-GTAGCCAACGCTATGTCCTGATAG) producing a 608-bp fragment were used. To give bands of an equal intensity in the heterozygotes, a ratio of 4:1 IGF-I/neo primer was used in each reaction. For analysis of mRNA, first-strand cDNA was primed and 25 cycles of PCR were carried out using primers specific for murine IGF-I (sense 5′-GTCTTCACACCTCTTCTACC and antisense 5′-CCTTCTGAGTCTTGGGCATGTCAG = 320-bp product) and IGF-II (sense 5′-GGGAAGTCGATGTTGGTGCTTCTC and antisense 5′-ACTGATGGTTGCTGGACATCTCCGAAG = 525-bp product).

Histological analysis

Complete gross necropsies were done on eight (three female, five male) IGF-I heterozygotes and seven (three female, four male) wild-type adult mice. A complete set of tissues, including adrenal, bone, brain, cervix, epididymis, eye, heart, kidney, large intestine, liver, lung, lymph node, sciatic nerve, ovary, pancreas, prostate, salivary gland, skeletal muscle, small intestine, spleen, stomach, testis, thymus, thyroid, tongue, trachea, urinary bladder, and uterus, was collected in 10% neutral buffered formalin, processed routinely to paraffin, sectioned, and examined by light microscopy. Sections of pituitary were also examined from a subset of the animals.

Neonates and 17.5, 15.5, and 13.5 day embryos were also examined by light microscopy. The neonates were fixed intact in

10% neutral buffered formalin. The majority of the animals were processed on an extended processing schedule to ensure adequate infiltration. Animals were then sectioned sagitally at 50-μm intervals to allow for morphologic examination of all organ systems. All sections were examined by light microscopy.

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