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Animal Model

Tissue-Specific Inactivation of Murine *M6P/IGF2R*

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The *mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R)* encodes a multifunctional protein involved in lysosomal enzyme trafficking, fetal organogenesis, tumor suppression, and T cell- mediated immunity. *M6P/IGF2R* is an imprinted gene in mice with expression only from the maternal allele. Complete knockout of this gene causes neonatal lethality, thus preventing analysis of its multifunctional role postnatally. To help elucidate the biological functions of M6P/IGF2R in adulthood, we generated both complete and tissue-specific *M6P/IGF2R* knockout mice using the *Cre/loxP* system. We confirm that complete *M6P/IGF2R* knockout results in fetal overgrowth and neonatal lethality. In contrast, tissue-specific inactivation of this gene in either the liver or skeletal and cardiac muscle gives rise to viable animals with no obvious phenotype. The successful creation of viable tissue-specific *M6P/IGF2R* knockout mouse models will now allow for detailed analysis of receptor function in a number of cellular processes including brain development, carcinogenesis, lysosomal trafficking, and T cell-mediated immunity. *(Am J Pathol 2003, 162:321–328)*

Both the 275-kd cation-independent mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R) and the 46- kd cation-dependent mannose 6-phosphate receptor (M6PR) function in the intracellular trafficking of lysosomal enzymes.[1,2] Homozygous *M6PR* null mice are both phenotypically normal and fertile, demonstrating that M6PR is not essential for either egg fertilization or organogenesis.[3,4] However, these animals exhibited defects in the targeting of multiple lysosomal enzymes, and increased levels of phosphorylated lysosomal enzymes are present in the body fluids. Thus, a physiological level of M6P/IGF2R does not compensate for M6PR loss, indicating that these two receptors target distinct subsets of lysosomal enzymes.

M6P/IGF2R encodes a multifunctional receptor that interacts with a diverse group of ligands not only intracellularly, but also at the cell surface.[1,5] Many of these ligands are glycoproteins that contain a mannose 6-phosphate (M6P) residue as a component of their N-linked oligosaccharide side chains. They include numerous lysosomal enzymes, the latent complex of transforming growth factor-β (TGF-β), and granzyme B.[1,2,6] In addition to binding M6P-modified glycoproteins, M6P/IGF2R also interacts specifically with several molecules through M6P-independent mechanisms. The best characterized of these is the mitogen insulin-like growth factor 2 (IGF2). It binds to a M6P-independent, high-affinity receptor binding site[7] that evolved with the appearance of Therian mammals.[8] Bound IGF2 is then internalized and transported to the lysosomes where it is degraded; M6P/IGF2R is subsequently recycled back to the membrane.[9] Thus, M6P/IGF2R controls the extracellular bioavailability of IGF2 and TGFβ, thereby regulating cell proliferation and apoptosis.

M6P/IGF2R function is critical for normal mammalian development. Mice that are deficient in M6P/IGF2R throughout fetal development die around the time of birth from a somatic overgrowth phenotype that is accompanied by severe skeletal, cardiac muscle, and lung abnormalities.[10–12] M6P/IGF2R-deficient mice can be rescued by a concomitant deficiency in IGF2 or the IGF1 receptor. In view of the diversity of ligands for M6P/IGF2R, it is surprising that this complex phenotype appears to result solely from the loss of the receptor's IGF2-binding function. This indicates that it is the failure to target IGF2 to the

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lysosomes, and the subsequent excess signaling through the IGF1 receptor that gives rise to the lethal phenotype. Furthermore, large offspring syndrome frequently observed in cloned animals is highly associated with epigenetic changes in gene regulation that decrease *M6P/IGF2R* expression.[13]

The variety and pleiotropic activities of the M6P/IGF2R ligand demonstrate that this receptor plays a key regulatory role in mammalian embryonic development, and indicate that altered receptor function could contribute to pathophysiology such as cancer. This postulate is supported by the finding that *M6P/IGF2R* loss of heterozygosity, coupled with intragenic loss-of-function mutations in the remaining allele, is a common early event in a number of human cancers.[14–18] Tumor cell growth is also inhibited when *M6P/IGF2R* expression is restored to normal and increased when gene expression is reduced.[19–21] The results of these mutational and functional studies clearly show that the *M6P/IGF2R* possesses the characteristics necessary to be classified as a tumor suppressor gene.[22]

M6P/IGF2R is also an imprinted gene in most viviparous mammals, and is expressed only from the maternal allele.[23] *M6P/IGF2R* imprinting evolved approximately 150 million years ago in a common ancestor to marsupials and Eutherian mammals,[8,24] but was subsequently lost about 75 million years ago in an ancestral progenitor to primates.[25] Thus, in contrast to mice, both copies of *M6P/IGF2R* are functional in humans.

It is important, therefore, to clarify the function of M6P/IGF2R in postnatal life since alteration of receptor activity may affect not only cancer formation and treatment,[26,27] but also wound healing,[28] autoimmune disease,[29,30] cognitive function,[31] and organ transplantation.[6] Although perinatal lethality in *M6P/IGF2R* knockout mice can be overcome by a deficiency in either IGF2 or IGF1 receptors, these animals are severely growth inhibited.[11] Therefore, *M6P/IGF2R* knockout mice are preferable for studying receptor function in postnatal animals.

In this report, we describe the generation of mice carrying a modified *M6P/IGF2R,* in which exon 10 is flanked by *loxP* sites (*floxed*). Exposure of the *floxed M6P/IGF2R* to *Cre*-recombinase expressed from either albumin (*Alb*) or muscle creatine kinase (*Ckmm*) promoters resulted in viable mice deficient in liver or skeletal and cardiac muscle M6P/IGF2R, respectively. The availability of a *M6P/IGF2R* conditional knockout mouse will finally enable the complex biological functions of this multifunctional receptor to be thoroughly investigated *in vivo*.

Materials and Methods

To circumvent the neonatal lethality observed in *M6P/IGF2R*-deficient mice generated using classical embryonic stem cell targeting methods,[10–12] we used the *Cre-loxP* system to create *M6P/IGF2R* null alleles in a tissue- and time-dependent manner.[32] Animals in this study were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture, the Department of Health and Human Services, and the National Institutes of Health. All mice were fed *ad libitum* on a diet of Purina Laboratory Chow and were maintained in an animal isolation facility on a standard 12-hour light/dark cycle. The study protocol was approved by the Administrative Panel on Laboratory Animal Care of Duke University.

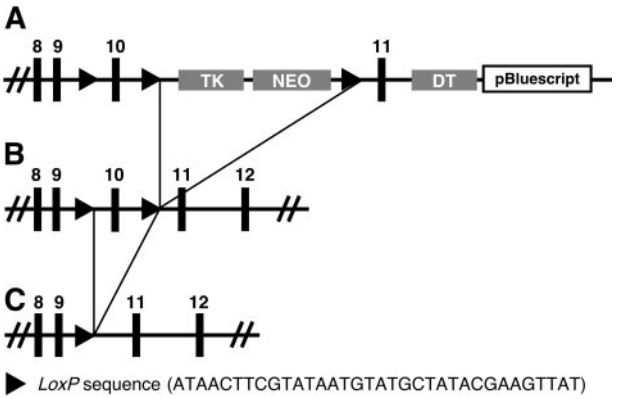

Figure 1. *LoxP* targeting of *M6P/IGF2R* exon 10. **A:** Generation of the *M6P/IGF2R LoxP* targeting construct involved the cloning of 7-kb of the mouse genomic DNA encompassing exons 8 through 11 and flanking intronic sequence into an appropriate *LoxP* vector. The *M6P/IGF2R LoxP* targeting construct was then transfected into Sv129 ES cells. Homologous recombinants were selected for using G418 (neomycin) and confirmed by PCR. **B:** *Cre*-recombinase plasmid (1 μg) controlled from the constitutively active CMV promoter was transfected into the ES cells which were then grown in the presence of gancyclovir to select for recombinants that had lost the TK/Neo cassette; PCR was used to confirm loss of the TK/Neo cassette. Recombinant ES cells were injected into the blastocoele cavity of the 3.5-day-old C57Bl/6J embryos before implantation into pseudo-pregnant mice. **C:** Expression of *Cre*-recombinase in mice with a *floxed* exon 10 results in its excision generating a M6P/IGF2R protein that encodes for a truncated receptor lacking the M6P and IGF2 extracellular binding domains. DT, TK, and Neo enable both positive and negative selection.

Construction of the Targeting Vector

A targeting vector was constructed to enable *Cre*-mediated deletion of *M6P/IGF2R* exon 10. The exon/intron splice junctions for exons 9–10 and exons 10–11 are categorized as type 2 (codon interrupted after the second nucleotide) and type 1 (codon interrupted after the first nucleotide), respectively.[33] Thus, deletion of exon 10 from the mouse genome will shift the coding sequence out of frame, resulting in a stop codon and truncation of the receptor 5′ of both mannose 6-phosphate binding sites, the IGF2 binding site, and the transmembrane region. Approximately 5-kb of mouse (Sv129) *M6P/IGF2R* genomic sequence (from intron 7 to intron 10) was cloned immediately upstream of a dual thymidine kinase, neomycin resistance cassette previously engineered to be flanked by two *loxP* sites (Figure 1). Approximately 2-kb of mouse genomic sequence corresponding to the remaining portion of *M6P/IGF2R* intron 10, exon 11, and intron 11 was then cloned downstream of the dual selection cassette. In two further modifications a third *loxP* site was introduced into intron 9 and a diptheria toxin selection cassette was inserted immediately downstream of the *M6P/IGF2R* sequence adjacent to intron 11.

Generation of Floxed M6P/IGF2R Mice

Mouse Sv129 embryonic stem (ES) cells were transfected with the linearized targeting vector and grown in selection medium containing G418 (neomycin). Only cells that have integrated the G418 resistance (*Neo*) cassette and lost the diptheria toxin (DT) cassette survive in this medium. The G418-resistant cells were then transiently transfected with a vector in which expression of *Cre* recombinase is directed from the constitutively active cytomegalovirus (CMV)-promoter to delete the phosphoglycerate kinase (PGK)-Neo and PGK-TK genes flanked by *loxP* sites (Figure 1A). Removing the thymidine kinase (TK) gene results in cells becoming resistant to gancyclovir. Gancyclovir-resistant cells were screened by polymerase chain reaction (PCR) (data not shown). Those that had undergone partial recombination, such that they had lost the TK-Neo selection cassette but maintained the *floxed* exon 10 of the *M6P/IGF2R,* were identified (11% of total). Following karyotype analysis, three clones were selected for micro-injection, and between 2 and 10 ES cells of individual clones were micro-injected into 3.5-day-old blastocysts isolated from C57Bl/6J donating females. Approximately 25 injected blastocysts were surgically implanted into the uterus of each of three pseudopregnant female mice. From the resultant litters, three chimeric females were obtained which were then backcrossed with C57Bl/6J males. Germ-line transmission of the *floxed* exon 10 sequence by one of the three chimeras was confirmed by PCR and sequence analysis (data not shown). Individuals heterozygous for the *floxed M6P/IGF2R* exon 10 were then crossed to generate homozygous animals. These were born at the expected Mendelian ratios and presented no obvious abnormalities.

Mouse Genotyping

A PCR-based assay was used to determine the presence of the *loxP* site in *M6P/IGF2R* intron 9. 100 ng of mouse genomic DNA was used as template in a PCR reaction (35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds) using the primers INT9F2 (5′-CCTTCCCTCCAGGCCGTTAC-3′) and INT9R1 (5′-GGTGAGGTCTCCATCTGAGTACC-3′).

Western Blot Analysis

Tissues were removed and homogenized in ice-cold dH_2O using a Caframo RZR1 stirrer homogenizer (Caframo, Ltd., Wiarton, Canada). The homogenates were then sonicated with a Misonix XL2020 sonicator (Misonix Inc., Farmingdale, NY) for 3 × 15 seconds at a setting of 10; the particulate matter was removed by centrifugation. Samples were then electrophoresed in a NuPAGE 4 to 12% gradient polyacrylamide gel (Invitrogen, Carlsbad, CA) and subsequently blotted onto nitrocellulose filters. M6P/IGF2R protein was then detected using a polyclonal rabbit antibody preparation against rat M6P/IGF2R[34] (kindly provided by C. Scott, University of Sydney), and visualized using a goat anti-rabbit antibody conjugated to horseradish peroxidase (Amersham, Piscataway, NJ).

Immunohistochemistry

Immunohistochemical staining for M6P/IGF2R was conducted as described previously.[35] Briefly, tissues were fixed overnight in Omnifix (Bovie Medical, Melville, NY), paraffin-embedded, and 5-μm sections were mounted on glass slides. Duplicate serial sections were mounted on each slide to allow for a negative control. Sections were de-paraffinized in Xylenes (2 × 5 minutes) and endogenous peroxidases were quenched by H_2O_2 (3% in methanol for 10 minutes). After gradually rehydrating the sections, the tissues were blocked (60 minutes at room temperature) in 10% goat serum (Vector Laboratories, Burlingame, CA) and 5% milk diluent (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Sections were then incubated overnight at 4°C in a humidified chamber with affinity-purified rabbit anti-bovine M6P/IGF2R antiserum (1:1800, kindly provided by P. Lobel, Rutgers University). Negative control sections were incubated with the same concentration of non-immune rabbit IgG. Tissue sections were immunoperoxidase-stained the next day using the Vectastain ABC Elite Kit per manufacturer's instructions (Vector Laboratories).

Statistical Analysis

Population means were compared by the unpaired *t*-test; a P value ≤ 0.05 was considered to be statistically significant.

Results

Cre-loxP Strategy for Creating a Functionally Null M6P/IGF2R Allele

M6P/IGF2R is imprinted in mice with expression predominantly from the maternally inherited allele.[23] Therefore, heterozygous mice inheriting a null *M6P/IGF2R* allele from their mothers are functionally equivalent to homozygous knockout individuals and display neonatal lethality.[10–12] In contrast, heterozygous individuals inheriting a null *M6P/IGF2R* allele from their fathers are functionally equivalent to wild-type mice. Previous *M6P/IGF2R* knockout mice retained selection cassettes, and such exogenous sequences can cause phenotypic effects in mice.[36] Therefore, it was important to determine whether the phenotype observed in the previous knockout studies is reproduced when global M6P/IGF2R deficiency during development is induced using the *Cre-loxP* strategy.

To accomplish this, a two-stage breeding protocol was devised. The first stage involved mating males of mixed genetic background, homozygous for the floxed *M6P/IGF2R* exon 10, with females from a transgenic line in which *Cre*-recombinase was expressed constitutively from the CMV promoter.[37] The maternally inherited *M6P/IGF2R* allele was normal in the resulting offspring while

the paternally inherited *M6P/IGF2R* allele was floxed. Since *Cre* recombinase is expressed in all cells, *Cre/loxP*-mediated excision of the paternally inherited exon 10 sequence occurred in every cell. Functionally, this has no effect since the paternal allele of *M6P/IGF2R* is normally silenced through genomic imprinting. Because *Cre* was expressed ubiquitously in these mice, half of their germ cells (ie, those containing the paternally inherited *floxed M6P/IGF2R* allele) have exon 10 deleted from the *M6P/IGF2R* and the other half (ie, those possessing the maternally inherited wild-type allele) retain an intact *M6P/IGF2R*.

Stage 2 involved breeding female progeny of the first breeding stage (in which 50% of the germ cells carry an inactivated copy of *M6P/IGF2R*) with wild-type male mice (C57Bl/6). This produced litters where 50% of the progeny inherited the deleted exon 10 *M6P/IGF2R* allele and 50% inherited a wild-type *M6P/IGF2R* allele. Consequently, half of the litter should be deficient in M6P/IGF2R throughout development, whereas the other half should be wild-type.

This two-stage breeding process produced small litters without mutant mice, indicating that mice inheriting a mutated *M6P/IGF2R* allele from their mothers were not viable. Timed pregnancies were therefore established, and embryos were harvested at day 18.5 of gestation. Genotyping and Western blot analysis showed that *M6P/IGF2R* protein was absent in individuals inheriting a deleted *M6P/IGF2R* exon 10 from their mothers (Figure 2C). Knockout embryos and their corresponding placentas were 36% and 31% larger than wild-type, respectively (Figure 2, A and B). Many of the knockout embryos also had an extra post-axial digit on fore and/or hind limbs, and the tails of mutant embryos were shorter and blunter than those of wild-type mice with a characteristic kink in the tip of the tail in some mutant embryos (Figure 2A).

Histological analysis of mutant animals indicated that the lungs were poorly developed, with a very low frequency of bronchiole structures observed (Figure 3,A and B). Immunohistochemical analysis of four knockout embryos confirmed the absence of M6P/IGF2R protein shown by Western blot. However, in two animals, intense heterogeneous immunostaining was observed in the lungs that co-localized with developing bronchiolar structures (Figure 3C). This staining may represent incomplete silencing of the wild-type paternally inherited *M6P/IGF2R* allele, as it is expressed at significant levels in early development.[38] Alternatively, it could result from re-expression of the paternal allele later in development. In summary, the phenotype of these mice is essentially identical to those of previous models of complete *M6P/IGF2R* deficiency.[10–12]

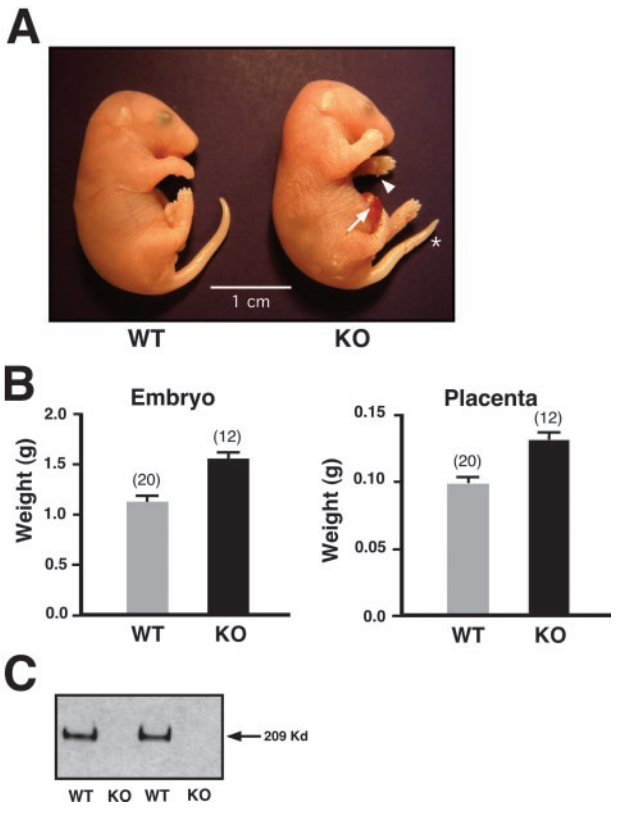

Figure 2. *Cre/loxP* strategy successfully creates a functionally null *M6P/IGF2R* allele. **A:** Wild-type (WT) and *M6P/IGF2R* knockout (KO) mice at 18.5 days of gestation. Embryos inheriting a deleted *M6P/IGF2R* exon 10 allele from their mothers (KO) exhibited an overgrowth phenotype. They also frequently displayed extra post-axial digits on the fore and/or hind limbs (**arrowhead**); shorter, blunter tails often with a characteristic kink in the tip (*); and extravasation of overgrown abdominal organs (**arrow**). **B:** Wet weight measurements demonstrate that knockout (KO) embryos ($n = 12$) and their corresponding placentas are significantly ($P < 0.0001$) larger than those in WT mice ($n = 20$); standard errors of the mean are shown. **C:** Western blot analysis performed on tail tips from the 18.5-day-old embryos confirmed the absence of M6P/IGF2R protein in KO mice.

Liver-Specific Knockout

To create a liver-specific *M6P/IGF2R* knockout, a transgenic line was used in which the promoter and upstream enhancer of the rat albumin gene directs liver-specific expression of *Cre* recombinase (*Alb-Cre*).[39] Female mice of mixed genetic background, heterozygous for the *floxed M6P/IGF2R* exon 10, were crossed with *Alb-Cre* males to create litters in which 50% of animals inherited a *floxed* allele maternally and 50% inherited a wild-type allele maternally. Since the paternal allele is normally silenced through imprinting, this strategy should produce liver-specific knockout animals and wild-type littermates at equal ratios. Western blot analysis was performed on protein extracts from liver, heart, skeletal muscle, kidney, and spleen of both genotypes. M6P/IGF2R protein was undetectable in the liver of the knockout animals at 3 ($n = 5$) and 12 ($n = 5$) months of age in comparison with wild-type littermates ($n = 5$) (Figure 4A). M6P/IGF2R levels in the other tissues of these knockout animals were at normal levels, confirming the tissue specificity of the *M6P/IGF2R* inactivation.

Knockout and wild-type mice were also examined for changes in body and organ weights and for any evidence of morphological changes at the cellular level. There were no significant differences in either average body weight or organ weight (liver, kidney, heart, and spleen) between knockout and wild-type animals (data not shown). Similarly, histological analysis of liver sections

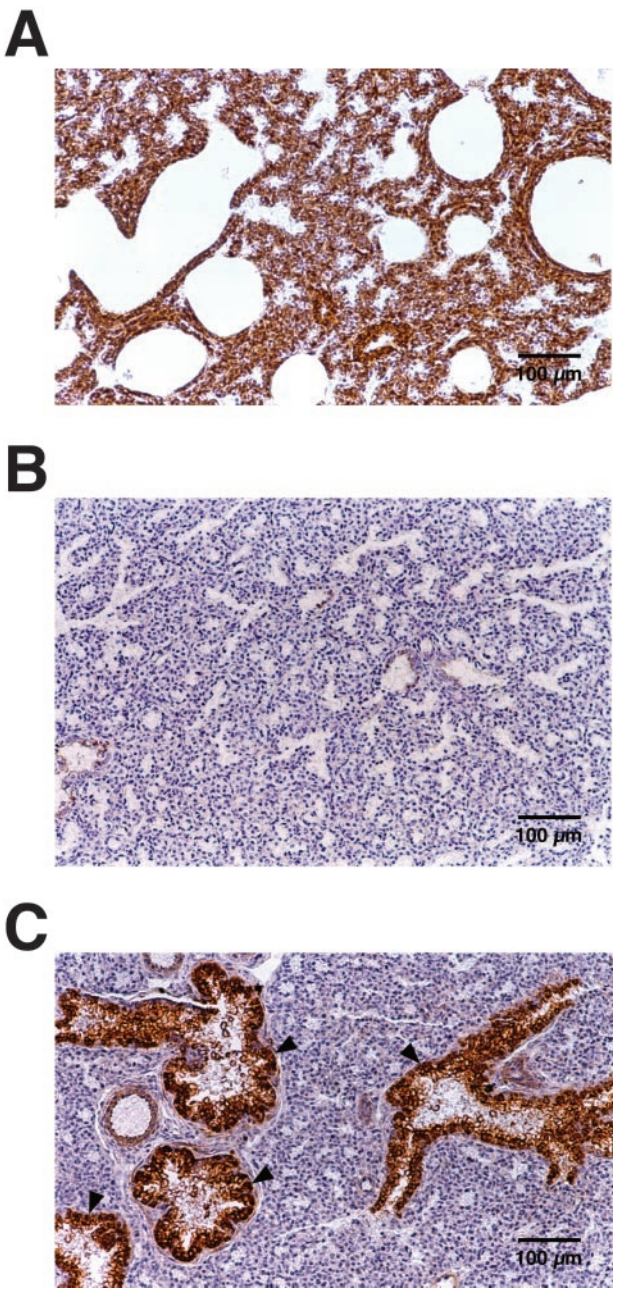

Figure 3. M6P/IGF2R immunohistochemical staining of lung at 18.5 days of gestation. **A:** Wild-type mouse lung, **B:** *M6P/IGF2R* knockout mouse lung, and **C:** *M6P/IGF2R* knockout mouse lung with reactivated receptor expression. **Arrowheads**; bronchial alveolar structures with expression of the normally silenced paternal *M6P/IGF2R* allele.

from knockout and wild-type animals did not appear significantly different (data not shown).

Despite our inability to detect significant M6P/IGF2R in the liver of knockout animals by Western analysis, it was possible that reactivation of the normally silent paternal *M6P/IGF2R* allele, as occasionally observed in the lung (Figure 3, A and B), could mask an abnormal liver phenotype. We addressed this possibility in a separate set of experiments. Breeding strategies were designed to produce mice that inherited *floxed M6P/IGF2R* alleles from both parents, together with the *Alb-Cre* transgene. These

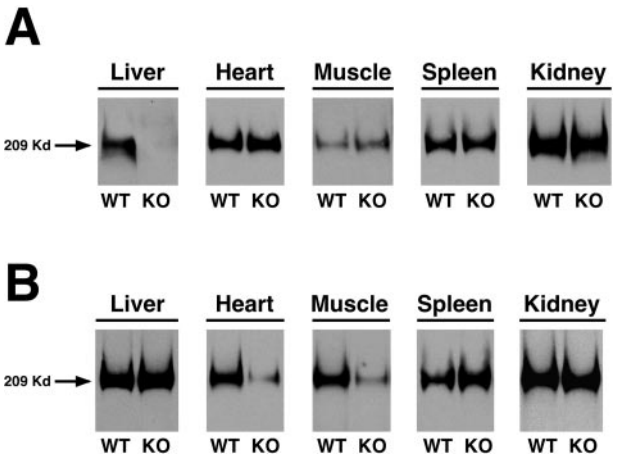

Figure 4. Western blot analysis of tissue-specific *M6P/IGF2R* knockout mice. **A:** Protein extracts were prepared from tissues of *M6P/IGF2R* liver-specific knockout (KO) ($n = 5$) and wild-type (WT) ($n = 5$) animals; representative samples are shown. 100 μg of total protein from each tissue was electrophoresed in a NuPAGE 4 to 12% gradient polyacrylamide gel, blotted onto nitrocellulose filters, and probed with a polyclonal M6P/IGF2R antibody**. B:** Protein extracts were prepared from tissues removed from *M6P/IGF2R* skeletal/cardiac KO ($n = 5$) and WT ($n = 5$) animals; representative samples are shown. 100 μg of total protein from each tissue was electrophoresed in a NuPAGE 4 to 12% gradient polyacrylamide gel, blotted onto nitrocellulose filters, and probed with a polyclonal M6P/IGF2R antibody. The blot labeled "Muscle" was exposed longer than the other tissues to reveal the faint band in the KO lane.

mice should not possess a potentially functional paternal *M6P/IGF2R* allele. Body and organ weights and liver sections of individuals inheriting two *floxed M6P/IGF2R* alleles were indistinguishable from the maternally inherited heterozygous mice at 3 and 12 months of age (data not shown). These findings indicate that the lack of abnormal liver phenotype in the heterozygous liver knockout animals was not due to reactivation of the paternal *M6P/IGF2R*.

Muscle-Specific Knockout

A transgenic line with *Cre*-recombinase under the control of the muscle creatine kinase promoter (*Ckmm-Cre*) was used to create mice in which *floxed M6P/IGF2R* exon 10 was deleted only in skeletal and cardiac muscle.[40] Female mice of mixed genetic background and heterozygous for the *floxed M6P/IGF2R* were crossed with *Ckmm-Cre* males. This created litters in which 50% of the animals inherited a *floxed* allele maternally and 50% inherited a wild-type allele maternally. This strategy was intended to produce functionally homozygous cardiac and skeletal muscle-specific *M6P/IGF2R* knockouts paired with wild-type littermates. M6P/IGF2R protein levels were reduced by approximately 90 to 95% in the cardiac and skeletal muscle of the *floxed* animals compared to their wild-type littermates (Figure 4B). Other tissues examined (liver, kidney, and spleen) showed normal levels of M6P/IGF2R in these knockout animals (Figure 4B), again demonstrating the tissue specificity of the knockout.

The presence of a small but significant level of M6P/IGF2R protein in the cardiac and skeletal muscle of knockout individuals (Figure 4B) may be due to either

expression of M6P/IGF2R protein in non-muscle cell types or reactivation of the silent paternal allele. To address the latter possibility, mice were bred such that both the maternal and paternal alleles were *floxed*. A low level of M6P/IGF2R protein, similar to that shown in Figure 4B, was still detected in the cardiac and skeletal muscle of homozygous knockout individuals (data not shown). This indicates that the residual receptor protein is derived either from cardiac cells in which *Cre*-recombinase was not expressed and/or in cells associated with the muscle tissue such as fibroblasts and vascular endothelial cells.

At 3 and 12 months of age, no gross phenotypic changes were observed in mice deficient in cardiac and skeletal muscle *M6P/IGF2R*, whether mice heterozygous or homozygous for the exon 10 deletion were examined. Additionally, body and organ weights of knockout individuals did not differ significantly from wild-type littermates while histological comparison of cardiac and skeletal muscle sections revealed no significant differences between knockout and wild-type mice (data not shown).

Discussion

The critical function of M6P/IGF2R in regulating IGF2 availability has been clearly shown by the lethal phenotype for mice deficient in receptor function during embryonic development, and their rescue from lethality by concomitant IGF2- or IGF1-receptor deficiency.[10–12] Unfortunately, because of this lethal phenotype, these mice are not useful for studying the role of this multifunctional receptor in postnatal life. The *Cre/loxP* recombinase system exploits the ability of *Cre* recombinase to direct excision of a DNA sequence that has been flanked by directly repeated copies of the *loxP* recombination site. Expression of *Cre* by an inducible or tissue-specific promoter therefore allows excision of the *loxP* flanked DNA.

We used the *Cre*/*loxP* technology in this study to create a transgenic line amenable to inactivating the *M6P/IGF2R* gene in a tissue-specific manner. To validate the ability of the *Cre*/*loxP* system to inactivate *M6P/IGF2R,* we mated wild-type male mice with female mice heterozygous for the *Cre*/*loxP*-mediated exon 10 deletion. Embryos that inherit a mutated *M6P/IGF2R* allele from their mother have a global deficiency of receptor throughout embryonic development, and should therefore reproduce the phenotype observed in previous reports of receptor deficiency.[10–12] As predicted, litters studied at embryonic day 18.5 contained wild-type embryos and embryos deficient in M6P/IGF2R. The latter embryos had the somatic overgrowth phenotype and skeletal abnormalities previously associated with maternal inheritance of a mutated *M6P/IGF2R* allele.[10–12] These results confirm the null status of the *floxed M6P/IGF2R*, and reaffirm its critical role in fetal development. Having confirmed that *Cre*-mediated excision of *M6P/IGF2R* exon 10 efficiently ablates receptor function, subsequent transgenic lines were then created in which *M6P/IGF2R* was knocked out in a tissue-specific manner.

We have previously reported genetic evidence that *M6P/IGF2R* behaves as a tumor-suppressor gene in a number of tissues including the liver.[14,41] To further elucidate the tumor suppressor role of M6P/IGF2R in the liver, we created liver-specific *M6P/IGF2R* knockouts by breeding *M6P/IGF2R floxed* mice with animals in which the liver-specific albumin promoter controls *Cre* recombinase expression.[39] Recombination induced by the expressed *Cre* is reported to be approximately 40% complete immediately after birth and increases steadily until about 6 weeks of age when recombination is complete.[42] In keeping with the known albumin promoter specificity and expression kinetics, M6P/IGF2R levels were 95 to 100% reduced in the liver of adult mice ($>$ 3 months) inheriting the *floxed* allele maternally. All other tissues examined, including kidney, spleen, heart, and skeletal muscle, had similar levels of receptor in the knockout animals compared to wild-type controls.

Human M6P/IGF2R is implicated in tumor suppression in the breast, gastrointestinal tract, liver, and lung.[14–18] The M6P/IGF2R is thought to contribute to tumor suppression by degrading IGF2, activating TGF-β, and mediating the action of cytotoxic T cells.[6,29] Therefore, the absence of these activities in M6P/IGF2R-deficient liver is predicted to increase susceptibility to hepatocellular carcinomas. We did not observe any gross histological abnormalities or any evidence of hyperplasia in the mutant livers at 3 and 12 months of age; however, the frequency of liver tumors in mice overexpressing IGF2 was not greater than that in control animals until they were over 18 months old.[43] Long-term carcinogenesis studies in mice with selective M6P/IGF2R deficiency in the liver, and other tissues, can now be conducted with the animals described in this report. They will allow, for the first time, a definitive assessment of the tumor suppressor activity of the M6P/IGF2R.

Viable mice deficient in M6P/IGF2R also represent an important model system for optimization of therapeutic approaches to inherited disorders of lysosomal metabolism. Numerous lysosomal storage disorders have been characterized in humans and, in most cases, are due to deficiency of a single acid hydrolase.[44] For example, Pompe disease (ie, glycogen storage disease II) is characterized by mutations in the gene coding for acid α-1,4-glucosidase (GAA) and abnormal accumulation of glycogen in skeletal muscle and heart (OMIM entry: no. 232300).[45] With a number of the recombinant enzymes now available for treatment purposes, a major challenge is to efficiently target the enzymes to the appropriate tissue *in vivo*. Because M6P residues are incorporated into most acid hydrolases during their biosynthesis, it may be feasible to exploit the endocytic activity of the M6P/IGF2R for tissue targeting.

To provide a model system for evaluating enzyme delivery *in vivo*, we generated mice deficient in skeletal muscle and heart *M6P/IGF2R* using the muscle creatine kinase promoter to drive expression of *Cre* recombinase. M6P/IGF2R levels in adult heart and skeletal muscle of mutant mice were reduced by up to 95% compared to control tissues, while receptor levels in other tissues of these animals remained normal. Despite the low levels of muscle M6P/IGF2R, there was no evidence of gross histological abnormalities in either heart or skeletal muscle. There were also no obvious signs of muscular dystrophy,

breathing or feeding difficulties, or muscular hyperplasia and/or hypertrophy. Therefore, these mice may represent a valuable model system for analysis of *in vivo* targeting of GAA and other lysosomal enzymes. Mice deficient in M6P/IGF2R in numerous other tissues may similarly be useful for evaluating enzyme therapy in other lysosomal disorders with different tissue involvement.

Cardiac *M6P/IGF2R* expression, which is high during embryogenesis, also persists at an elevated level in the adult rodent.[46] This indicates that this receptor plays a significant role in the heart postnatally. Recently, cultured cardiac cells were shown to bind, internalize, and activate prorenin in a M6P-dependent manner.[47] The relevance of these observations to the *in vivo* situation is currently unknown, but the renin-angiotensin system plays an important role in the regulation of blood pressure and salt and fluid homeostasis. Agents that interfere with angiotensin II formation, the ACE inhibitors in particular, are now widely used for the treatment of hypertension and heart failure.[48,49] If M6P/IGF2R is shown to contribute to angiotensin II formation *in vivo*, this knowledge may help in the development of alternative therapeutic strategies. Mice with cardiac deficiency of M6P/IGF2R, such as those described in this study should facilitate experiments that test the physiological relevance of M6P-dependent prorenin activation.

Interestingly, *M6P/IGF2R* in mice is imprinted in all tissues except for the brain where both alleles are expressed.[50] It is highly expressed in neurons of the forebrain, with the highest expression in the pyramidal cells, the polymorphic layers of the hippocampus, and the granule cell layer of the dentate gyrus; regions involved in emotional behavior, information processing, and memory formation.[51] These findings indicate that M6P/IGF2R may assist in the development of these brain functions. This postulate is reinforced by the identification of *M6P/IGF2R* as the first putative "IQ gene."[31] By comparing children with an IQ of 160 or higher to those with an average IQ, *M6P/IGF2R* was shown to be linked with general cognitive ability ("g"). The role of this receptor in the development of cognitive function can now be systematically assessed with *M6P/IGF2R* conditional knockout mice.

M6P/IGF2R functions in T cell activation and T cell-mediated apoptosis. Polyclonal antibodies to M6P/IGF2R block T cell differentiation at the $CD4^-$ and $CD8^-$ stage, indicating that this receptor is actively involved in the early steps of T cell differentiation.[52] A common feature of autoimmune diseases is the formation of autoantibodies targeted against a wide spectrum of cellular molecules. Interestingly, M6P/IGF2R has been identified as a novel target of autoantibodies in patients with autoimmune diseases.[30] Furthermore, M6P/IGF2R facilitates T cell activation by internalizing CD26/DPPIV (dipeptidyl peptidase IV), a cell surface T cell activation antigen.[29] Since a large number of $CD26^+$ T cells are found in inflamed tissues of patients with autoimmune disease, and M6P/IGF2R is a target for autoantibodies, studying the interaction between CD26 and the M6P/IGF2R may help better understand the pathogenesis of autoimmune diseases.

T cells and natural killer (NK) cells kill tumor cells by two major mechanisms involving the cross-linking of death receptors on the tumor cell surface and granule exocytosis. The major serine proteinase released from these granules is granzyme B. It enters a cell targeted for death through M6P/IGF2R-mediated endocytosis, and induces apoptosis on being released from the endosomes by the pore-forming ability of perforin.[6] Thus, M6P/IGF2R is not only essential for allogeneic cell rejection *in vivo*, but cancer cells may also avoid T cell-mediated immune surveillance by inactivating M6P/IGF2R and/or perforin function.[53] Thus, the efficacy of cancer immunotherapy may partly depend on the *M6P/IGF2R* mutation status in cancer. The *M6P/IGF2R* conditional knockout mouse provides a unique model in which to investigate the function of this receptor in both T cell activation and T cell-mediated apoptosis in cancer.

In summary, we have generated a transgenic mouse possessing a *M6P/IGF2R* modified by the presence of *loxP* sites. We have used the *Cre/loxP* system to generate mice deficient in *M6P/IGF2R* in a tissue-specific manner. The increasing availability of transgenic mice in which *Cre* recombinase expression is exquisitely controlled in a tissue- and/or age-dependent manner will allow for the elucidation of the normal physiological roles of this multifunctional receptor in postnatal life. Understanding the contribution of aberrant *M6P/IGF2R* expression to the pathophysiology of human diseases may ultimately lead to the development of novel therapeutic approaches.

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