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Consequences of postnatal insulin-like growth factor II overexpression in insulin-like growth factor I deficient mice

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Konsequenzen dauerhaft erhöhter Spiegel des Insulin-ähnlichen Wachstumsfaktors II in Insulin-ähnlichen Wachstumsfaktor I defizienten Mäusen

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MY FAMILY

Contents

Abbreviations

A	Ampere
ACTH	adrenocorticotropic hormone
ALS	acid-labile subunit
APS	ammonium persulfate
AU	arbitrary unit
BKVI	bovine keratin 10 gene
BMD	bone mineral density
bp	base pair
cDNA	complementary DNA
cm	centimeters
cpm	counts per minute
DEPC	diethylpyrocarbotate
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleoside triphosphates
DTT	dithiothriteol
e	embryonic stage
E	exon
EDTA	ethylene diamine tetraacetic acid
ES	embryonic stem
g	gram
g	relative centrifugal force (RCF)
GH	growth hormone
h	human
IGF-I; -II	insulin-like growth factor-I; -II
IGFBP	IGF binding protein
IGFBP-rP	IGFBP related protein
IGF-I, -IIR	IGF-I, -II receptor
kb	kilobase
kDa	kilodalton
l	liter
LID	liver-specific *igf1* gene-deletion
M	molar, marker
m	murine
M-6-P	mannose-6-phosphate
mA	milliampere
mg	milligram
ml	milliliter
MLV	murine leucemia virus
mm	millimeter
mRNA	messenger RNA
MSA	multiplication stimulating activity
MUP	major urinary protein

NSILA	nonsuppressible insulin-like activity
ng	nanogram
nn	neonatal
NRL	nose-rump-length
OD	optical density
P	promotor
PBS	phosphate-buffered saline
p.c.	post coitum
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEPCK	phosphoenolpyruvate carboxykinase
pn	postnatal
pQCT	peripheral quantitative computed tomography
RIA	radioimmunoassay
RNA	ribonucleic acid
rpm	revolution per minute
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TEMED	tetramethylethylendiamine
tg	transgenic
TRAMP	transgenic adenocarcinoma of mouse
U	Unit
UV	ultraviolet
V	volt
wt	wildtype
µg	micrograms
µl	microliters

1. Introduction and objectives

The size of an animal depends on the number and volume of the cells it contains, with some contribution by extracellular matrix and fluids. In mammals, growth (increase in size) begins at pre-implantational embryonic stages and lasts until a steady state is reached postnatally. Appropriate growth is controlled by hormones and growth factors regulating cellular signaling pathways.

In mice, the insulin-like growth factor system has been unequivocally identified as the major determinant of both embryonic and postnatal (here modulated by the growth hormone - GH) growth (Lupu et al., 2001). The IGF system consists of two ligands (IGF-I and IGF-II), two receptors (IGF-IR and IGF-IIR) and six high affinity IGF binding proteins (IGFBP-1 to -6). The single chain peptide growth factors IGF-I and -II are produced by several tissues and function in an autocrine/paracrine fashion and, since they circulate in the plasma bound to the IGFBPs, as classical hormones. Despite their structural homology, each growth factor has specific expression patterns and actions in regulating proliferation, differentiation, survival and specific functions of many cell types and tissues under different physiological and pathological conditions (Stewart and Rotwein, 1996).
The actions of both growth factors are mediated via the IGF-IR. In contrast, the structurally distinct IGF-IIR is not considered to play a role in IGF signal transduction, but is responsible for the turnover of IGF-II by receptor-mediated endocytosis, thereby reducing the levels of circulating IGF-II (Le Roith et al., 1995; Kornfeld et al., 1992).

To gain insight into the functions of the IGF system during embryogenesis and postnatal development several transgenic and knockout mouse models for the members of the IGF family have been established over the last decades.
Homozygous IGF-I deficient mice have ~60% of normal weight (N) at birth and become ~30% of N at an age of 2 months, indicating that IGF-I not only is necessary for normal embryonic growth, but also has a continuous function throughout postnatal development (liu et al., 1993). IGF-II nullizygotes are also born with ~60% but do not display a further size reduction, maintaining ~60% N at steady state, suggesting that this growth factor is only essential for normal embryonic growth (DeChiara et al., 1990).

Whereas adult humans display unaltered high levels of IGF-II, in mice and rats expression of IGF-II is shut down in almost every tissue after birth and very low levels of this growth factor

are detectable in postnatal life (Rotwein et al., 1991). Overexpression of IGF-II in transgenic mice resulted in disproportionate growth of specific organs but not in a significant increase in body size. In some cases an increased tumor formation could be detected, which is in line with the frequent observation of overexpression of IGF-II in a variety of human malignancies (Wolf et al., 1998).

The objective of the investigations reported here was to study the role of postnatally elevated IGF-II in the absence of IGF-I. Therefore we employed two available mouse models (IGF-I knockout and PEPCK-IGF-II transgenic mice) to generate mice harboring a homozygous deletion of the *Igf1* gene and overexpressing IGF-II at the same time. To test whether an overexpression of IGF-II is able to rescue phenotypic consequences of homozygous IGF-I deficiency in mice, body and organ growth, bone parameters (size, mineral density), and regulatory mechanisms of serum IGFBP and GH homeostasis were investigated.

2 Review of the literature

2.1 The insulin-like growth factor (IGF) system

2.1.1 Overview

The early history of insulin-like growth factors started in 1957 when William D. Salmon, Jr. and William H. Daughaday tried to set up an *in vitro* assay for growth hormone (GH). This assay should demonstrate a direct effect of GH on radioactively labeled sulfate (^{35}S-sulfate) uptake in cartilage of hypophysectomized rats. However, the addition of bovine GH turned out to be virtually inactive. Next, they compared the effects of incubation of these cartilage segments with diluted serum of normal or hypophysectomized rats. ^{35}S-sulfate uptake in the presence of normal serum was 200% greater than that in hypophysectomized rat serum, leading them to suggest that the effect of GH was attributable to an inducible "sulfation factor" (Salmon et al., 1957; Daughaday, 1992; Daughaday, 2000). Subsequently, the sulfation factor was renamed somatomedin C (Daughaday et al., 1972).
Independently, in the 1960s a serum activity was identified that lowered glucose levels even in the presence of anti-insulin antibodies (Froesch et al., 1963). These serum components were designated nonsuppressible insulin-like activity (NSILA). Additionally, a protein synthesized by the liver, which could enhance cell replication was described (Dulak and Temin, 1973; Nissley et al., 1976). It was termed multiplication stimulating activity (MSA). Initial purification of these three substances, revealed that they have overlapping activities. To indicate the relationship of these substances to insulin and to emphasize their growth promoting activities, the term insulin-like growth factor was proposed by Rinderknecht and Humbel (1976). With purification and sequencing of human IGF-I (Rinderknecht and Humbel, 1978a) and IGF-II (Rinderknecht and Humbel, 1978b), it became evident that somatomedin C was ultimately IGF-I, MSA corresponded to IGF-II and that NSILA included both growth factors. IGF-I and IGF-II are ~70% identical to one another, and their A and B domains are 50% identical to the A and B chains of human insulin, indicating the appropriateness of the nomenclature (Rinderknecht and Humbel, 1978a&b).

The IGF family (Figure 2-1) consists of two peptide ligands (IGF-I and -II), six high-affinity IGF binding proteins (IGFBP-1 to -6), two cell surface receptors, the IGF-I receptor (IGF-IR)

and the IGF-II/mannose-6-phosphate (M-6-P) receptor (IGF-IIR), and IGFBP proteases (Hwa et al., 1999).

IGF-I is a single chain basic protein of 70 amino acids and IGF-II is a slightly acidic single chain-peptide of 67 residues (Rinderknecht and Humbel, 1978a&b). Both peptides are ubiquitously expressed and their production is stimulated by a variety of hormones (Sara and Hall, 1990).

IGFs are integral components of multiple systems controlling both growth and metabolism. In addition to acting as classical hormones (they circulate in the plasma bound to the IGFBPs), they also exert paracrine as well as autocrine effects on cell cycle control and apoptosis (Jones and Clemmons, 1995).

Unlike insulin, circulating IGFs are bound to six IGFBPs, which elicit at least two functions: prolongation of the half-life of circulating IGFs and neutralization of their metabolic effects. IGFBPs themselves are regulated by protease activity and some may have IGF-independent actions. IGFBPs compete with IGF receptors for IGF binding since they have a considerably higher affinity for IGFs than the IGF-I receptor (Kelley et al., 1996; Rajaram et al. 1997; Hwa et al., 1999). In the circulation, ~75-80% of the IGFs are present in a complex of ~150 kDa, a smaller proportion of 20-25% is associated in binary complexes, and less than 1% is found in a free form. The ~50 kDa binary complex is made up of several IGFBP species (IGFBP-1 to -6) that are incompletely saturated with IGFs. The ~150 kDa complex is a ternary complex consisting of IGF-I or IGF-II plus IGFBP-3 or -5 and a non IGF binding component, the acid labile subunit (ALS) (Rajaram et al., 1997). In addition to the previously mentioned high-affinity binders, the existence of low affinity binders, such as IGFBP-related proteins and proteolysed IGFBPs is known. Contrary to IGFBPs, IGFBP-rPs and IGFBP fragments mediate biological effects mainly by IGF-independent mechanisms (Hwa et al., 1999).

The actions of IGFs are mediated primarily through the IGF-IR. The structurally distinct IGF-IIR, which is identical to the mannose-6-phosphate receptor (Morgan et al., 1987; Kiess et al., 1988; Kornfeld, 1992), binds IGF-II with a high affinity and interacts minimally with IGF-I. There is no evidence of interaction with insulin (Ballard et al., 1988). This receptor is not considered to play a role in IGF signal transduction but it is responsible for clearing and thereby reducing the levels of circulating IGF-II. Additionally, the IGF-II receptor plays a major role in transporting lysosomal enzymes among intracellular compartments.

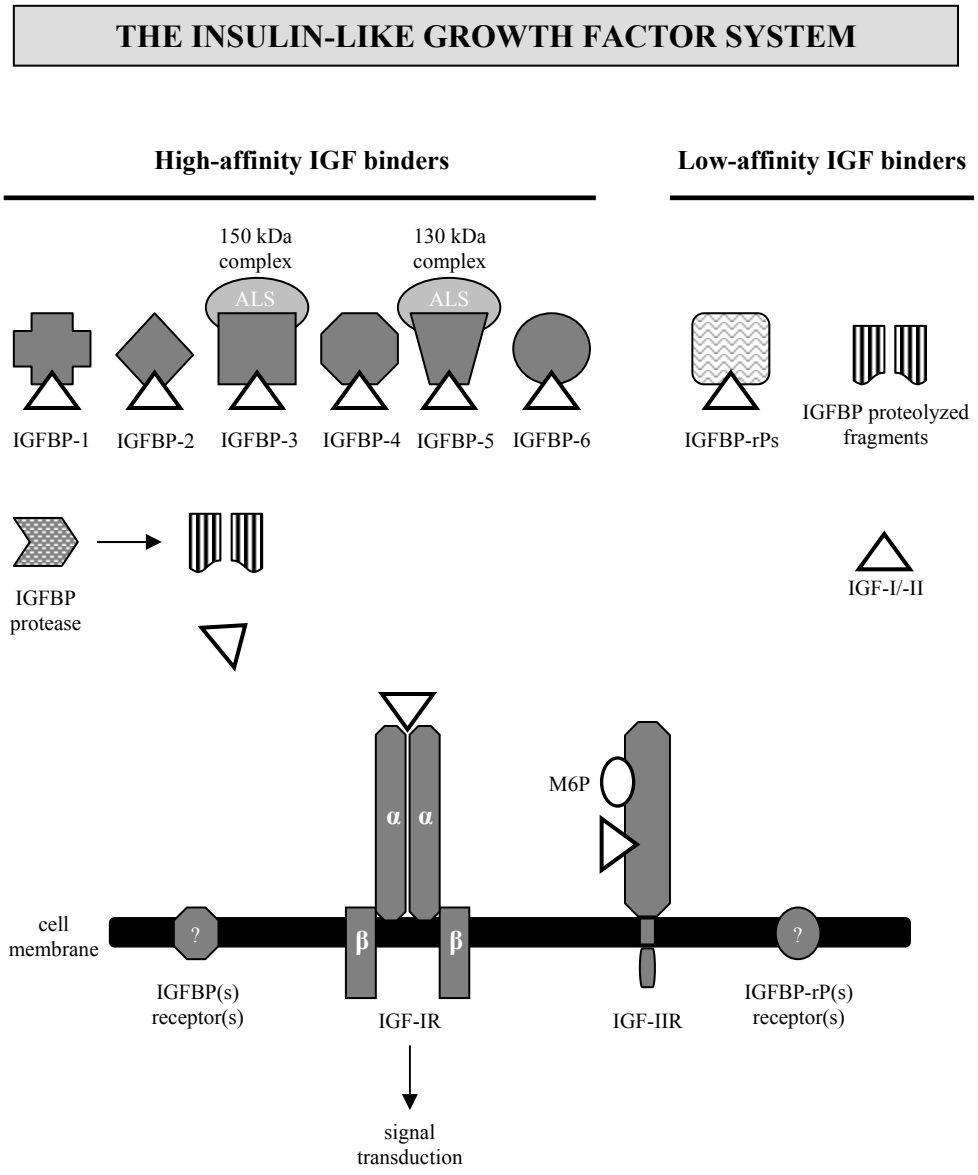


Figure 2-1: The IGF system (figure adapted from Hwa et al., 1999). The components of this system include the peptides IGF-I and -II, IGFBP-1 to -6, IGFBP-rPs, IGFBP proteases, IGF-I and -II receptors, and potential IGFBP(s) and IGFBP-rP(s) receptors. M6P, Mannose-6-phosphate. ALS, acid-labile subunit.

2.1.2 IGF-I

2.1.2.1 IGF-I gene

Since a potential site of regulation of IGF-I function is at the level of biosynthesis, the chromosomal localization of its gene is an important first step in understanding the role of IGF-I in the etiology of a disease or disorder. The human *IGF1* gene is a single copy gene, located on the long arm of chromosome 12 (Hoppener et al., 1985). The mouse *Igf1* gene is located on chromosome ten, whereas the rat *Igf1* gene is found on chromosome 7 (www.ensembl.org). In humans and rodents, the IGF-I gene spans >80 kb of chromosomal DNA and is composed of six exons and five introns (Rotwein, 1991). The expression of IGF-I is influenced by hormonal, nutritional, tissue specific, and developmental factors as described in chapter 2.1.3.3.

2.1.2.2 IGF-I protein

Through direct amino acid sequencing and by cDNA cloning and DNA analysis the structure of IGF-I is known for at least 10 species: 7 mammals (human, bovine, pig, sheep, guinea pig, rat, and mouse) and 3 nonmammalian vertebrates (chicken, xenopus, and salmon) (reviewed by Rotwein, 1991). The human peptide was the first to be purified and sequenced (Rinderknecht and Humbel, 1978a).

IGF-I is a ~7 kDa single chain polypeptide of 70 amino acids, containing a NH_2-terminal B domain of 29 residues, a C region of 12 amino acids, an A domain of 21 residues and a COOH-terminal D region of 8 amino acids. The IGF-I molecule is evolutionary well-conserved in vertebrates. There are only 5 amino acid substitutions among the IGF-I molecules across 7 different species (Rotwein, 1991).

2.1.2.3 Regulation of IGF-I expression

2.1.2.3.1 RNA

Despite the very simple and compact gene structure, regulation of *IGF1* gene expression is complicated. In mammals, several transcriptional or posttranscriptional mechanisms, such as the use of alternative promoters regulating transcription at multiple initiation sites, differential

RNA splicing, and variable RNA polyadenylation are responsible for the production of multiple IGF-I mRNA species. Thus, four IGF-I precursor proteins but only a single mature 70-amino acid IGF-I are produced (Rotwein, 1991; Hall et al., 1992; Rotwein et al., 1993).

IGF-I expression increases after birth and peaks during the prepubertal years. It is predominantly regulated by GH, enhancing its transcription in most tissues but also by other hormonal, nutritional, and developmental factors (Salmon and Daughaday, 1957; Roberts et al., 1986; Murphy et al., 1987; Mathews et al., 1986; Lowe et al., 1987 and 1988; Daughaday and Rotwein, 1989).

Several investigations revealed that a single injection of GH into GH-deficient hypophysectomized rats results in a rapid increase of IGF-I mRNA and thus enhanced levels of IGF-I after a few hours with a prompt return to baseline levels (Hynes et al., 1987; Hepler et al., 1990; Rotwein et al.1993). First, it was assumed that GH possibly increases IGF-I mRNA levels by selective effects on alternative RNA processing (Lowe et al., 1988) but this idea turned out to be wrong. Studies in GH-deficient adult rats showed that acute GH treatment does not regulate alternative splicing of IGF-I mRNAs, does not lead to preferential expression from one of the two IGF-I gene promoters and has no effect on selection of a polyadenylation site (Rotwein et al., 1993).

Additionally to GH, the expression of IGF-I is modulated by other hormones and trophic factors. Thyroid hormones were shown to have a modest potentiating effect on GH-stimulated IGF-I synthesis (Wolf et al., 1989; Tollet et al., 1990). Studies in isolated rat renal collecting ducts demonstrated an enhancement of *Igf1* gene expression by the epidermal growth factor (EGF) (Rogers et al., 1991). Parathyroid hormone treatment of osteoblast-enriched cultures from fetal rat bone causes a small but significant increase of IGF-I mRNA and thus IGF-I secretion, whereas treatment with cortisol inhibits this action (McCarthy et al., 1989 and 1990). Studies in rats showed that estradiol has a dose-dependent effect: acute treatment with estradiol profoundly enhances IGF-I mRNA abundance in the uterus, whereas a chronical therapy results in inhibition of hepatic IGF-I mRNA and IGF-I serum levels (Murphy and Friesen, 1988).

The nutritional status is an important nonhormonal regulator of IGF-I expression. Fasting of rats causes a decline of hepatic IGF-I mRNA and serum IGF-I levels, while refeeding neutralizes this effect (Elmler and Schalch, 1987; Straus and Takemoto, 1990). The decrease of liver IGF-I mRNA during fasting is mainly regulated at a posttranscriptional level (Zhang et al., 1998).

Furthermore, the expression of IGF-I is tightly regulated during development. IGF-I is essential for normal embryonic growth, as shown in studies of mouse models with a genetic ablation of *Igf1* as well as in a case study of a boy with a homozygous deletion of the *IGF1* gene (Powell-Braxton et al., 1993; Liu et al., 1993; Baker et al., 1993; Woods et al., 1996). On the contrary, the existence of nearly normal size infants with either congenital absence of the pituitary or deletions of the genes encoding GH or GH receptors demonstrated that GH is not crucial for normal intrauterine development (Le Roith et al., 2001). For example, newborns suffering of the Laron syndrome (a hereditary dwarfism resulting from defects in the GH receptor gene) are not significantly smaller (42-47 cm) than healthy babies (49-52 cm) but show a strong growth retardation during postnatal development (Laron, 2001). The same effect is shown in a mouse model for the Laron syndrome (Zhou et al., 1997).
In the rat IGF-I expression begins well before the developmental onset of GH action and GH receptors do not appear in the liver until ~2 weeks after birth (Mathews et al. 1989; Tiong and Herington, 1992). Dissimilarly, serum levels of IGF-I increase by nearly 20-fold during the first 14 days post natum, reaching 40% of the values found in adult animals (Daughaday et al., 1982; Donovan et al., 1991; Kikuchi et al., 1992).

2.1.2.3.2 IGF-I levels in the circulation

Liver-derived IGF-I, mostly regulated by GH, represents the main circulating source of this growth factor (Sjogren et al., 1999; Yakar et al., 1999). Circulating IGF-I feeds back centrally to inhibit synthesis and release of GH by the anterior pituitary, thus providing a regulatory negative feedback loop (Berelowitz et al., 1981; Sheppard and Bala, 1986; Becker et al., 1995). Studies on rat osteoblasts additionally suggest the presence of a peripheral negative feedback loop that allows IGF-I to limit locally the response of extrahepatic tissues to circulating GH (Leung et al., 1996).
In the circulation, most of the IGFs form binary complexes with one of the IGFBPs or ternary complexes with IGFBP-3 or -5 and ALS, acting as classical hormones (for further details see chapter 2.1.6).
Studies of IGF-I levels in healthy children showed that serum levels of IGF-I are only 50 % of adult values at birth and rise gradually during childhood to reach a peak at puberty, being at this point 2-3 fold higher as compared to average adult values. After puberty the levels slowly decline with age (Hall et al., 1980; Luna et al., 1983; Silbergeld et al., 1986; Yu et al., 1999b). The described rise and fall of IGF-I levels in circulation during and after teenage years is due

to an increased secretion of GH during puberty and a decreased release afterwards (Mauras et al., 1987; LeRoith et al., 1992).

2.1.2.4 Biological effects of IGF-I

2.1.2.4.1 Overview

IGFs are integral components of multiple systems controlling both growth and metabolism. In addition to endocrine effects exerted by circulating IGFs, locally produced IGFs exert paracrine as well as autocrine effects on cell cycle control and apoptosis, two functions also involved in tumorigenesis (Jones and Clemmons, 1995).

The most widely studied effect of the IGFs *in vitro* is the stimulation of DNA synthesis and cell replication, particularly using BALB/c-3T3 cells (mouse fibroblasts) for these experiments. IGF-I has been shown to function as a progression factor in the cell cycle by stimulating cells to traverse its successive phases. Moreover, it has been demonstrated that more than one growth factor is required for optimal growth in normal cells, since IGF-I could only elicit its effects after the cells had been primed with PDGF (Baserga and Rubin, 1993; Pardee, 1989). Apart from fibroblasts, IGF-I stimulates a mitogenic response in a wide variety of cells such as chondrocytes, osteoblasts, keratinocytes, thyroid follicular cells, smooth muscle cells, skeletal muscle cells, neuronal cells, mammary epithelial cells, mesangial cells, erythroid progenitor cells, thymic epithelium, oocytes, granulosa cells, spermatogonia, Sertoli cells and several cancer cell lines, showing enhanced proliferation after IGF-I treatment (for reviews see Lowe, 1991; Sara and Hall, 1990; Giudice, 1992; Mcauley, 1992; Cohick and Clemmons, 1993; Jones and Clemmons, 1995).

IGF-I also acts as a survival factor due to its capacity to inhibit apoptosis in certain cells (Sell et al., 1995; Parrizas et al., 1997; Ferrari et al., 1998). Additionally, the IGFs are known to promote differentiation of cells, such as myoblasts, osteoblasts, osteoclasts, chondrocystes, neural cells, and adipocytes (Florini and Magri, 1989; Mochizuki et al., 1992; Geduspan and Solursh, 1993; Pahlman et al., 1991; Sara and Hall, 1990).

Furthermore, the IGFs regulate the hormone secretion of many cell types. Hormone synthesis and secretion of ovarian granulosa and theca cells is stimulated by both insulin-like growth factors, whereas the thymulin secretion by thymic epithelium and the hormone secretion from Leydig cells and thyroid follicular cells are only stimulated by IGF-I (Giudice, 1992; Timsit et al., 1992; Lowe, 1991). IGF-I treatment on adrenal fasciculata cells increases the number of

adrenocorticotropic hormone (ACTH) receptors and potentiates steroid hormone secretion in response to ACTH (Penhoat et al., 1989).

In vivo actions of IGF-I have been studied by administration of this peptide to several animal species and humans, as well as in IGF-I knockout or overexpressing animal models. A short summary of the effects of administrated IGF-I observed in normal humans volunteers is shown in table 2-1. The observed effects are comparable to the effects seen in rats (reviewed in Jones and Clemmons, 1995).

Biological effects of IGF-I in various genetically engineered mouse models are described in chapter 2.2.

Table 2-1: Effects of IGF-I administration to humans (table from Jones and Clemmons, 1995).

Function	**IGF-I effect**
Glucose metabolism	↑ Glucose uptake ↓ Hepatic glucose production (human) ↑ Apparent insulin sensitivity Hypoglycemia (esp. with i.v. administration)
Fat metabolism	↓ Serum ketones ↓ Serum free fatty acids (human) ↓ Triglycerides
Protein metabolism	↑ Protein synthesis ↓ Nitrogen excretion ↑ Total body protein accretion ↑ Body/organ weight, esp. spleen, thymus, kidney Improved wound heeling
Renal function	↑ Glomerular filtration rate ↑ Renal plasma flow ↑ Speed of recovery from ischemic acute renal failure
Counterregulatory hormones	↓ GH ↓ Glucagon ↑ Catecholamines
Linear Growth	↑ In hypopituitary and GH-insensitive subjects
Miscellaneous side effects	Partoid enlargement and tendeness, headache, peripheral edema, tachycardia

2.1.2.4.2 Growth and development

IGF-I plays an important role in both embryonic and postnatal growth. Homozygous IGF-I knockout mice are born small (60 % of normal weight) and grow very poorly postnatally (Powell-Braxton et al., 1993; Baker et al., 1993; Liu et al., 1993). In contrast, lines of mice selected for high serum IGF-I levels show increased body weight (Blair et al., 1988). The idea of a GH-independent effect of IGF-I in embryonic growth is strongly supported by studies of GH and GH-receptor gene-deleted mice having relatively normal birth weights (Le Roith et al., 2001).

Generation of liver-specific IGF-I deficient mice (LID mice) demonstrated that the liver is the principal source of IGF-I in the circulation. In these mice, the concentration of IGF-I in the serum was reduced by 75%. Interestingly, the reduction of serum IGF-I concentration had no effect on postnatal body growth, indicating that autocrine/paracrine-produced IGF-I is more important than liver-derived IGF-I for body growth (Sjogren et al., 1999; Yakar et al., 1999).

In humans, circulating IGF-I levels also correlate with body size: constitutional tall children and adolescents have elevated levels (Gourmelen et al., 1984), whereas pygmy populations in Africa and from the Philippines display significantly decreased levels of this peptide (Baumann et al., 1989; Davila et al., 2002). Moreover, the description of a 15-year old patient carrying a homozygous partial deletion (exon 4 and 5) of the *IGF1* gene resulting in serum IGF-I levels below detection level demonstrated that IGF-I is also important for human fetal growth. This mutation was manifested with severe intrauterine and postnatal growth failure, sensorineural deafness and mild mental retardation (Woods et al., 1996). Recently, a new patient carrying a mutation in the *IGF1* gene was reported. Again pre- and postnatal growth failure and sensorineural deafness were the main clinical findings (Bonapace et al., 2003).

As a result of selective breeding, the domestic dog shows a very high degree of variation in body size. Evaluation of plasma IGF-I concentrations and comparison with body size in giant and miniature dog breeds revealed that these two parameters highly correlate (Eigenmann et al., 1988).

2.1.2.4.3 Bone metabolism

The mammalian skeleton (uniquely designed for its protective and structural roles) is a highly organized and physiologically active organ, consisting of an outer surface of cortical bone surrounding the inner trabecular elements. The trabecular skeleton is bathed in marrow,

whereas the cortical bone is nourished by perostial vessels and a series of canaliculi. The latter connect osteocytes to lining cells and osteoblasts. To preserve the integrity of the skeleton, both cortical and trabecular bone continuously undergo remodeling processes (Yakar and Rosen, 2003).

The IGF system is thought to play an important role in bone physiology, since several components of this system (IGF-I and -II, IGFBP-1 to -6, and IGFBP proteases) are stored abundantly in the skeletal matrix. Additionally, the IGF-IR is present in both osteoblasts and osteoclasts. During bone resorption the IGFs are released from storage, and in combination with circulating IGFs, influence the action of osteoblasts and osteoclasts during the process of remodeling (Yakar and Rosen, 2003). Liver-derived IGF-I, the main source of circulating IGF-I, is known to be critical for the modeling of bone, particularly cortical perostial bone growth, but is not required for the maintenance of trabecular bone in adult mice (Sjogren et al., 2002; Yakar et al., 2002).

Studies of genetically altered mouse models demonstrated that IGF-I has a great impact on longitudinal bone growth, perostial circumference, and bone mineral density (BMD). IGF-I deficient mice are significantly smaller compared to their wildtype littermates. Powell-Braxton et al. (1993) observed no histopathological difference in bone development, whereas the IGF-I deficient mice generated in the lab of Argiris Efstradiadis displayed smaller bones and delayed ossification (Liu et al., 1993; Baker et al., 1993). Further studies revealed a reduction in the size of hypertrophic chondrocytes in the proximal growth plate of the tibia and the distal femur. It is assumed that a decreased rate of production and maturation of hyperthropic zone chondrocytes leads to a significant reduction in the height of the spongiosa and thus to shorter bones in IGF-I deficient mice (Lupu et al., 2001). Bikle et al. (2001) showed a reduction of tibial bone formation rate and cortical thickness in homozygous IGF-I knockout mice. Interestingly, the trabecular bone volume (bone volume / total volume) is increased in the previously mentioned mice, showing a strong interaction with gender: male mice display a 23% and females an 88% higher value compared with wildtype controls as a result of increased connectivity, increased number and decreased spacing of the trabeculae. Furthermore, it was demonstrated that IGF-I in particular influences the increase of BMD during puberty. Deletion of the *Igf1* gene completely prevented perostial expansion during teenage years and the peak BMD was significantly reduced (Mohan et al., 2003). In accordance with this data, overexpression of IGF-I in osteoblasts increased peak BMD (Zhao et al., 2000).

2.1.2.4.4 Cancer

There is a lot of experimental evidence supporting a role for the insulin-like growth factor signaling system in the progression and maintenance of cancer. Moreover, the differential expression of its components in tumors relative to normal tissues makes this system an attractive target for therapeutic intervention (reviewed by Foulstone et al., 2005).
A correlation between circulating levels of IGF-I and IGFBP-3, and the relative risk of developing colon, breast, prostate, and lung cancer has been shown in various human epidemiological studies (Ma et al., 1999; Hankinson et al., 1998; Chan et al., 1998; Wolk et al., 1998; Yu et al., 1999a; Giovannucci et al., 2000). It is not only suggested that individuals with circulating levels of IGF-I at the high end of the normal range are more prone to develop certain cancers, but it is also suggested that IGF-I signaling stimulates growth of established cancers (Pollak, 2004). This hypothesis is supported by studies of mouse models (LID mice), in which circulating IGF-I levels are reduced, while tissue expression of IGF-I is normal. When compared with control mice, these mice show a lower risk for the development of colon and breast cancers and metastases (Yakar et al., 2004; Wu et al., 2002; Wu et al., 2003). Studies of another mouse model, the transgenic adenocarcinoma of mouse (TRAMP) corroborates the importance of IGF-I during the initiation and progression of primary prostate cancer (Kaplan et al., 1999).

2.1.3 IGF-II

2.1.3.1 IGF-II gene

The human *IGF2* gene spans 30 kb of chromosomal DNA on the distal short arm of chromosome 11. It consists of nine exons and has four promoters (Holthuizen et al., 1993; Sussenbach et al., 1993).
In rodents, the 12 kb spanning *Igf2* gene consists of six exons and has three promoters. The mouse *Igf2* gene is located on chromosome 7, whereas the rat *Igf2* gene is found on chromosome 1 (Rotwein and Hall, 1990; Soares et al., 1986). The *IGF2* genes of all three species form a linkage group regarding the position of the insulin gene, indicating that the *IGF2*/insulin locus was present prior to the mammalian radiation (Rotwein and Hall, 1990).

2.1.3.2 Parental imprinting

Genomic (or parental) imprinting is a phenomenon which leads to selective modification of specific genetic loci during the development and differentiation of the male and female germlines, and results in differential expression of these genes depending on their parental legacy (reviewed by Solter, 1988; Surani et al., 1990; Cattanach and Beechey, 1990).
Using a mouse model that carried a targeted disruption of *Igf2*, it could be shown that transmission of the mutation through the male germline resulted in growth-deficient heterozygous progeny. In contrast, the heterozygous offspring was phenotypically normal when the disrupted gene was transmitted maternally. Molecular analysis revealed that only the paternal allele is expressed in embryos, while the maternal is silent. Only the choroid plexus and the leptomeninges were excluded from the otherwise general influence of imprinting on IGF-II expression (DeChiara et al., 1991). Furthermore, genetic imprinting of the *IGF2* gene has been clearly demonstrated in rats and humans. In the latter, the existence of a promoter-specific imprinting status is indicated since in the adult liver IGF-II expression is biallelic and solely derived from P1 (also see chapter 2.1.4.4.1) (Holthuizen et al., 1999).
Relaxation or loss of *IGF2* gene imprinting, resulting in biallelic expression, occurs in the Beckwith-Widemann syndrome of somatic overgrowth, sporadic Wilms' tumor and several other cancers (O'Dell and Day, 1998).

2.1.3.3 IGF-II protein

The mature IGF-II peptide is a slightly acidic ~7 kDa 67 residue molecule, containing a NH_2-terminal B domain of 28 amino acids, a C domain of 12 residues, an A region of 21 amino acids, and a COOH-terminal D region of 6 amino acids (Rotwein, 1991). Like the IGF-I protein, it is highly conserved among different species and its sequence is known for at least 10 mammalian and 6 nonmammalian species. Interestingly, several human IGF-II proteins, such as a 69- or 70-amino acid IGF-II peptide, have been characterized but their biological significance is unknown. It is assumed that this observation is due to allelic variation in the *IGF2* gene, since the DNA sequence alteration responsible for the new amino acids does not occur near an intron-exon junction (Rotwein, 1999).

2.1.3.4 Regulation of IGF-II expression

2.1.3.4.1 RNA

Similarly to the *IGF1* gene, the gene encoding *IGF2* is a complex transcription unit, where multiple mRNAs are synthesized as a result of alternate promoter usage and alternative RNA polyadenylation (Rotwein, 1991). As previously mentioned, the human *IGF2* gene consists of nine exons (E1-9), of which E1-6 are non-coding leader exons, whereas E7, 8 and the first part of E9 code for the IGF-II precursor protein (Sussenbach et al., 1994). The rodent *Igf2* genes consist of six exons (E1-6): E1-3 are non-coding leader exons and E4-6 code for the IGF-II precursor protein (Rotwein and Hall, 1990; Holthuizen et al., 1993). The expression of the gene is developmentally regulated. The human *IGF2* gene is transcribed by four different promoters (P1 to P4). P1 is exclusively activated in adult liver tissues, whereas P3 is the most active promoter in fetal tissues. In fetal liver P2-P4 are active but they are shut off at birth. The major human promoters active in non-hepatic adult tissues are P3 and P4 (Holthuizen et al., 1993).

There are homologous promoters for human P2, P3 and P4 in mouse and rat, whereas a homologue for P1 is absent. In mice and rats, expression of IGF-II is shut down in almost every tissue shortly after birth and is believed to be a consequence of the lack of a homologue to human P1 (Holthuizen et al., 1993). During adult live IGF-II expression is maintained only in the exchange tissues of the brain (Stylianopoulou et al., 1988; Lee et al., 1990; DeChiara et al., 1991).

The expression of IGF-II is regulated at multiple levels: tissue-specific and developmental stage dependent transcription initiation, alternative splicing, usage of multiple polyadenylation sites, site-specific endonucleolytic cleavage of IGF-II mRNAs, and translational control (Sussenbach et al., 1989).

Hormonal regulation of IGF-II expression has been shown in several cases but their mechanisms of actions or the identification of specific responsive elements, which may be involved in regulation, are still elusive. Moreover, species differences seem to play an important role (reviewed in Holthuizen et al., 1999).

2.1.3.4.2 IGF-II levels in the circulation

In humans, circulating levels of IGF-II are low at birth and increase with age until puberty to remain at steady levels afterwards. The gender difference (only 2%) is minimal (Yu et al., 1999). In mice and rats, circulating IGF-II levels rapidly fall after birth and only very low levels are detectable throughout adult life, reflecting the expression pattern (Soares et al., 1985; Murphy et al., 1987; Rotwein et al., 1988; Wilson et al., 1987; Bautista et al., 1990; Ren-Qiu et al., 1993). Similar to IGF-I, most of the circulating IGF-II is bound in binary and ternary complexes (Rajaram et al., 1997).
IGF-II levels in the circulation are dependent on GH but this only becomes apparent at subnormal growth hormone levels as shown in studies of normal subjects and patients with growth disorders. GH deficiency leads to significantly decreased IGF-II levels, whereas oversecretion of GH does not increase the values of this peptide. It is assumed that normal GH levels already stimulate IGF-II production maximally (Zapf et al., 1981).

2.1.3.5 Biological effects of IGF-II

In vitro studies of mouse embryos from the 1-cell to the 64-cell stage demonstrated that IGF-II, but not IGF-I and insulin transcripts, are expressed before implantation (Rappolee and Werb, 1991). Additionally, supplementation of culture medium with recombinant IGF-II stimulates growth and metabolism of early mouse embryos (Harvey and Kaye, 1992).
IGF-II is known to be an important regulator of embryonic growth and differentiation, which has been elegantly demonstrated by DeChiara and colleagues, (1990), who showed that mice bearing a disrupted IGF-II allele display marked growth retardation. IGF-II deficient mice are only 60% of weight compared to their wildtype controls. During embryonic development the growth effects of IGF-II occur earlier than IGF-I effects. Subsequent analysis of mouse fetuses lacking expression of IGF-I, -II or IGF-IR revealed that IGF-II interacts with the IGF-IR from embryonic stage 11 to 12.5 onwards and with a second receptor, the insulin receptor, from embryonic day 13.5 onwards. At this stage, IGF-I comes into play as a stimulator of embryonic growth (Liu et al., 1993; Baker et al., 1993; Louvi et al., 1997).

Whilst IGF-II has a clearly established role in embryonic and fetal development, its role in postnatal life remains poorly understood. Similar to IGF-I, IGF-II has proliferative and antiapoptotic actions since its effects are exerted by the IGF-IR (Stewart and Rotwein, 1996).

Studies of prepubertal children with a constitutionally tall stature (CTS) demonstrated significantly increased serum IGF-II levels compared to their control groups, whereas the secretory state of GH was normal and serum levels of IGF-I, IGFBP-3 and ALS were not significantly altered. The increased IGF-II levels involve a significantly higher IGF/IGFBP molar ratio and, therefore, an increase in the potentially bioactive free form of the IGFs. These findings lead to the assumption that high circulating levels of IGF-II might be responsible for increased growth velocity, especially in early childhood, when the still immature GH-IGF-I axis has not yet fully assumed its predominat role in body growth (Garrone et al., 2002).

Furthermore, overexpression of IGF-II has been frequently observed in a variety of human malignancies such as breast, prostate, and colon cancer. Also, it is constantly found in pediatric cancers, like Wilms' tumor and rhabdomyosarcoma. IGF-II expression leads to an autocrine feedback loop stimulating the IGF-IR to stimulate cancer cell proliferation. In certain large tumors, particularly fibrosarcomas, rhabdosarcomas, and leimyosarcomas, the production of IGF-II may result in tumor-associated hypoglycemia (reviewed in Stewart and Rotwein, 1996; Le Roith and Butler, 1999; Moschos and Mantzoros, 2002).

To elucidate the effects of postnatal elevated IGF-II several mouse models overexpressing this peptide have been generated as detailed described in chapter 2.3. These models demonstrated that postnatal overproduction of IGF-II positively affects body and organ weights but has no effect on body growth. Only the mouse models with the highest transgene expression suffer from hypoglycemia, whereas moderate IGF-II expression does not lead to a decrease in serum glucose levels. Tumor formation has also only been observed in the mouse models with the highest IGF-II expression. Moreover, the different malignancies occurred after a very long latency and not all organs exposed to high concentrations of this peptide developed tumors (reviewed by Wolf et al., 1998). As shown in a study of transgenic mice overexpressing the simian virus-40 large T-antigen under the control of the insulin promoter strongly, it is very likely that IGF-II works as a second signal, maintaining the proliferation of neoplastic cells and is not a causative agent for tumor development (Christofori et al., 1994).

2.1.4 The IGF receptors

2.1.4.1 IGF-I receptor (IGF-IR)

The IGFs elicit their effects through activation of a specific high affinity cell-surface receptor, the insulin-like growth factor I receptor (IGF-IR). In structure and signaling, the IGF-IR is closely related to the insulin receptor, with greater than 50% overall amino acid homology. IGF-IR is a heterotetrameric glycoprotein composed of two α- and two β-subunits joined by disulfide linkages (Abbot et al., 1992). The α-subunit, containing 706 amino acids, is totally extracellular and mainly involved in ligand binding. The β-subunit, containing 627 amino acids, is composed of a short extracellular domain, a membrane spanning segment, and a large intracytoplasmatic region including a tyrosin kinase domain (Stewart and Rotwein, 1996). However, the IGF-IR is not a unique molecular entity. There is a large degree of heterogeneity that can be explained by a number of mechanisms, including potential primary structure variation, differential glycosylation, and hybrid formation with insulin receptors (Blakesley et al., 1999).

Ligand binding to the α-subunit initiates a conformational change that is transmitted to the intracellular domain leading to receptor autophosphorylation on several tyrosine residues by an intramolecular transmechanism. After autophosphorylation, the receptor is fully active as a tyrosine kinase towards endogenous substrates. Activation of IGF-IR triggers intracellular events that regulate the cell cycle, apoptosis, cellular motility and gene expression (De Meyts et al., 1994).

The IGF-IR binds IGF-I and IGF-II with a high affinity (~10^{-10} M) and insulin with a strongly (100-fold) lower affinity (Blakesley et al., 1996). This high affinity binding is dictated by the primary sequence of both the ligand and the receptor. Furthermore, the binding of IGFs to the IGF-I receptor is modulated by specific IGF- binding proteins (IGFBPs) (Blakesley et al., 1996).

The human IGF-IR protein is produced by mRNAs derived from the single 21-exon *IGF1R* gene, located on chromosome 15 (Abbot et al., 1992). The *IGF1R* gene is not subjected to parental imprinting (Liu et al., 1993). Expression levels of the IGF-IR are maximal at prenatal stages and decrease subsequently during postnatal development (Werner et al., 1989).

Genetic inactivation of the IGF-IR by targeted disruption of the *Igf1R* gene causes severe neonatal growth deficiencies as a consequence of hypoplasia in several tissues. Mice lacking

the *Igf1R* gene are only 45% of normal size and die shortly after birth because of respiratory failure (Liu et al., 1993).

2.1.4.2 IGF-II receptor (IGF-IIR)

The second receptor of the IGF system is the structurally distinct IGF-II receptor (IGF-IIR), which is identical to the mannose-6-phosphate receptor (Morgan et al., 1987; Kiess et al., 1988; Kornfeld, 1992). This single-chain membrane-spanning glycoprotein is devoid of tyrosin kinase activity. At present, there is no evidence for signal transduction mediated through the IGF-IIR (Stewart and Rotwein, 1996; Nissley, 1999).

The mature human IGF-IIR, containing 2,451 amino acids, can be divided into three regions: a large 2,264-residue extracellular domain, a 23-amino acid transmembrane region, and a 164-residue carboxyl-terminal intracytoplasmic domain (Morgan et al., 1987; Oshima et al., 1988). The extracellular domain is responsible for ligand binding, whereas the intracytoplasmatic region regulates movement among different cellular compartments (Stewart and Rotwein, 1996). The IGF-II receptor binds IGF-II with a high affinity but interacts minimally with IGF-I and not at all with insulin (Ballard et al., 1988). Binding and internalization of IGF-II at the cell surface result in degradation of this ligand. Thus, the IGF-IIR is able to regulate the IGF-II action by growth factor clearance (Kiess, 1999).

Harboring two functionally distinct carbohydrate recognition domains, the IGF-IIR is able to bind lysosomal enzymes and other proteins bearing a mannose-6-phosphate recognition sequence. Therefore IGF-IIRs play an important role in the clearance of lysomal enzymes from their extracellular environment (Marron-Terada et al., 1998; Marron-Terada et al., 2000).

The IGF-IIR is also found in the circulation, cleaved from the cell surface receptor. Particularly in the foetal plasma it binds a significant amount of IGF-II and may function as an IGF-II-specific binding protein (Gelato et al., 1989).

The IGF-IIR is highly conserved among different species, with ~80% identity being found among bovine, rat, mouse, and human receptors (Kornfeld, 1992; Stewart and Rotwein, 1996). In humans the *IGF2R* gene is located on chromosome 6 (Laureys et al., 1988). In mice, the *Igf2R* gene resides in the proximal region of chromosome 17 and is closely linked to the T-associated maternal effect (Tme) locus (Barlow et al., 1991; Szebenyi and Rotwein, 1994; Ludwig et al., 1996). Since genetic imprinting of this gene occurs in mouse and rats, but not in humans (Mills et al., 1998; Treacy et al., 1996), it provides one plausible explanation for

the enhanced sensitivity of mice to tumor formation (De Souza et al., 1997). Interestingly, opposite to IGF-II, the IGF-IIR is imprinted maternally (Barlow et al., 1991).
Genetic inactivation of the IGF-IIR by gene targeting results in fetal overgrowth, skeletal abnormalities and perinatal death due to overexposition of fetuses to IGF-II (Wang et al., 1994; Lau et al., 1994; Ludwig et al., 1996).

2.1.5 The IGF-binding proteins (IGFBPs) and the acid-labile subunit (ALS)

The insulin-like growth factor binding proteins (IGFBPs) comprise a family of six structurally and evolutionary related peptides (IGFBP-1 to IGFBP-6), which are expressed in a tissue specific manner and have different affinities for the IGFs. They are present in different concentrations in different body compartments and they can modulate the different IGF actions both positively and negatively. IGFBPs compete with IGF receptors for IGF binding since they have much higher affinities for IGFs ($k_d \sim 10^{-10}$ M) than the IGF-I receptor ($k_d \sim 10^{-8}$-10^{-9} M). Through this binding they form binary ~50 kDa complexes with the IGFs (Kelley et al., 1996; Rajaram et al. 1997; Hwa et al., 1999).
In circulation, ~75-80% of the IGFs are present in a complex of ~150 kDa, a smaller proportion of 20-25% is associated in ~50 kDa binary complexes, and less than 1% is found in form of ~7.5 kDa. The ~150 kDa complex is a ternary complex comprising of one molecule of IGF, IGFBP-3 or -5 and an 85kDA glycoprotein, the acid labile subunit (ALS) (Rajaram et al., 1997). However, the contribution of IGFBP-5 to the circulating ternary complex appears to be smaller compared to that of IGFBP-3 (Baxter et al., 2002).
ALS, mainly synthesized in the liver, is predominantly stimulated by the growth hormone (GH). Since the onset of ALS synthesis is one of the last events in the development of the circulating IGF system, high serum levels are almost exclusively found postnatally (Boisclair et al., 2001).
In adult animals, serum IGFs reach concentrations that are ~1000 fold that of insulin. ALS is a critical component that contributes to the development of this large reservoir by extending half-lives of IGFs from 10 minutes when in free form, and 30-90 minutes when in binary complexes, to more than 12 hours when bound in ternary complexes (Guler et al., 1989; Zapf et al., 1995).
A second important role of ALS, considering this large reservoir of bioactive IGFs in the circulation, is the prevention of non specific metabolic effects of the IGFs such as hypoglycemia. In contrast to free IGFs and IGFs bound to a ~50 kDa binary complex, the

~150 kDa complexes cannot traverse capillary endothelia and activate the insulin receptor (Zapf et al., 1995).

For their actions on target cells specific mechanisms must exist to release IGFs from ternary complexes. At least three mechanisms have been shown to alter the affinity of IGFBPs to IGFs, thus as: IGFBP proteolysis, phosphorylation, and adherence to the cell surface or to extracellular matrix (Clemmons, 1997).

IGFBPs have also been reported to be able to exert IGF-independent effects. To determine the *in vivo* significance of data, obtained using *in vitro* culture systems, several mouse models have been generated and investigated.

Further information about transgenic and knockout mice experiments for IGFBPs and ALS can be found in comprehensive reviews (Schneider et al., 2000; Boisclair et al., 2001; Silha and Murphy, 2002; Wolf et al., 2005).

2.1.6 The IGF-binding protein-related proteins

The discovery of several groups of cysteine-rich proteins sharing important structural and functional similarities with the IGFBPs has led to the proposal of an IGFBP superfamily, comprised of the IGFBPs and the IGFBP-related proteins (IGFBP-rPs) (Bork, 1993; Oh et al., 1996; Kim et al., 1997; Hwa et al., 1999). IGFBP-rPs bind IGFs but with substantially lower affinity than is the case with IGFBPs. It is most likely that IGFBP-rPs mediate biological effects mainly by IGF-independent mechanisms and may also act through IGF-dependent mechanisms (Hwa et al., 1999). Their activities are not very well understood but they may be important regulators of IGF functions and are object of intense research.

2.2 Genetically engineered mouse models for IGF-I and IGF-II

2.2.1 Overview

In order to study biological activities *in vivo* the mouse has been of particular interest among scientists from a wide range throughout the 20th century. This is due to its close genetic and physiological similarities to humans as well as to the ease with which its genome can be manipulated and analyzed. In the early days, biological studies involving alteration the mouse genome had to rely either on the appearance of spontaneous mutations or on the generation of chemically- and radiation-induced mutations and allophenic mice. Although

these studies played a major role in biomedical research, they are not without disadvantages. While spontaneous mutations are rare events, physical and chemical mutagenesis has an entirely random character and identification and characterization of the mutations are very time consuming. In the late 1980s, the merging of recombinant DNA methods with mammalian cell culture and embryo manipulation techniques provided a great breakthrough in biomedical research. Two new genetic technologies allowed producing custom-made mouse models. The first approach, transgenesis, consists in inserting foreign gene products in the mouse chromosomes to produce gain-of-function mutants. In the second approach, gene targeting, endogenous genes are disrupted, with results ranging from subtle mutations to complete loss of gene function (loss-of-function mutants). The former animals are called transgenic and the latter knockout mice (Jonas, 1984; Hafner and Müller, 2004).

To gain insight into the functions of IGF-I and -II during embryogenesis and postnatal development transgenic and knockout mice models have been established for both peptides (reviewed by Efstratiadis, 1998). IGF-I deficient and IGF-II transgenic mice are described in detail in chapters 2.2.2 and 2.2.3.

The investigation of IGF-I transgenic mice carrying human IGF-I coding sequences fused to the mouse metallothionein I promoter revealed that chronically elevated levels of IGF-I led to an increase in body weight as a result of selective organomegaly without an apparent increase in skeletal growth. Carcass weights are proportionally increased to body weight and also the spleen, pancreas, brain, and kidney exhibited significant overgrowth (Mathews et al., 1988; Quaife et al., 1989). Further studies showed that small bowel length and mass are greater in IGF-I transgenic mice compared to wildtype littermates (Ohneda et al., 1997). Overexpression of IGF-I was also targeted to specific tissues. For instance, constantly elevated levels of IGF-I in the heart are coupled with myocte proliferation in transgenic mice (Reiss, et al., 1996) and overexpression of IGF-I in the epidermis induces hyperplasia, dermal abnormalities, and spontaneous tumor formation (Bol et al., 1997). In the latter model, transgenic mice develop squamous papillomas (some of which converted to carcinomas) in a significant proportion compared to non-transgenic littermates (DiGiovanni et al., 2000).

IGF-II deficient mice are born with only 60% of normal weight but do not display further size reduction and have ~60% of normal weight at steady state. Otherwise they appear normal and are fertile. These results suggest that IGF-II is only essential for normal embryonic growth (DeChiara et al., 1990).

2.2.2 IGF-I deficient mice

In 1993, two independent laboratories generated IGF-I deficient mice by homologous recombination in embryonic stem cells. Powell-Braxton and collaborators created IGF-I deficient mice by inserting a neomycin gene at amino acid 15 of the major protein.

From birth on, heterozygous IGF-I deficient mice, displaying serum IGF-I levels 37% lower than normal, are 10- 20% smaller than their wildtype littermates and the size difference continues throughout growth. The size reduction is due to a decrease in organ, muscle, and bone mass in the absence of histological abnormalities. Both sexes are healthy and fertile and can be used for intercrossing to generate homozygous IGF-I deficient mice.

At birth, homozygous IGF-I deficient mice have only ~60% of the body weight of their wildtype siblings and the majority (>95%) dies perinatally, showing atelectatic lungs. The absolute size of homozygous knockout and wildtype mice is variably depending on the genetic background and litter size. Histopathological studies of embryonic mice and neonates revealed that the homozygous IGF-I knockouts display an underdevelopment of muscle tissue and that the lungs are less organized with ill-defined alveolae. No histopathological differences in bone development between these two groups can be found (Powell-Braxton et al., 1993).

The laboratory of Argiris Efstradiadis generated IGF-I deficient mice, using a slightly different strategy (insertion of the neomycin gene at amino acid 50). Although the overall phenotype is comparable, their mice do not seem to be so severely compromised. Heterozygous IGF-I deficient mice do not show any obvious phenotypic difference from wildtype siblings (Liu et al., 1993).

Homozygous IGF-I knockouts, showing a serum level of IGF-I beyond detection level, are also ~60% of normal birth weight. Although some of the dwarfs die shortly after birth, they are all able to breathe. Neonatal death occurs between 15 minutes and 6 hours after birth, for unknown reasons. Notably the survival rate depends on the genetic background (Liu et al., 1993). Studies of postnatal growth revealed that their relative size decreases progressively from 60% of normal birth weight to about 30% of the weight of their wildtype littermates at an age of 8 weeks, thereafter remaining at this level. Despite this highly decreased body weight, the homozygous IGF-I knockout mice appear proportionate in size and behave normally. Examination of the progress of long bone ossification in adult homozygous IGF-I deficient mice showed that it proceeds with a greatly reduced rate (Baker et al., 1993). Moreover, both sexes of these dwarfs do lack libido and are infertile. Female mutants possess

an infantile uterus exhibiting a strong hypoplasia especially in the myometrium. The primary cause of their infertility is thought to be their failing to ovulate even after administration of gonadotropins. Male mutants show reduced testes size and have only 18% of the normal level of spermatogenesis. Vas deferens of the epididymal duct, seminal vesicles and prostate are vestigial. Since capacitated sperms are able to fertilize wildtype eggs *in vitro*, the primary cause of infertility is thought to be the drastically reduced level of testosterone, resulting in a failure of androgenization and thus absence of mating behavior (Baker et al., 1996).

2.2.3 IGF-II transgenic mice

To elucidate the function of IGF-II in postnatal life, several mouse models overexpressing this growth factor have been generated over the last decades. Therefore, sequences coding human or mouse IGF-II have been placed under the transcriptional control of promoter elements from different genes, e.g. the mouse major urinary protein (MUP) gene, the bovine keratin 10 gene (BKVI), the mouse H-2K^b gene, or the rat phosphoenolpyruvate carboxykinase (PEPCK) gene (reviewed in Wolf et al., 1998). In MUP-IGF-II transgenic mice the tissue specific expression of the transgene starts 3 to 4 weeks after birth and can be detected in the liver, the preputial glands, and low in the adrenal glands. Serum IGF-II levels are ~30-fold higher compared to controls. Adult transgenic mice are smaller than their controls and have a significantly reduced body weight. The fat mass is reduced between 44 and 77%, being the major cause of the low body weight and fat mass. Also, their lean body mass is reduced. Transgenic animals develop hypoglycemia and hypoinsulinemia and show an incidence for a diverse spectrum of different tumors after a long latency (Rogler et al., 1994).

BKVI-IGF-II transgenic mice show transgene expression in skin, duodenum, ileum, appendix, colon, and uterus from embryonic stage 16.5 onwards. Serum levels are ~2- 4-fold higher compared to wildtype controls. Transgenic animals exhibit an overgrowth of skin and increased weights of appendix, colon, and uterus. They are leaner than their controls due to a lower lipid content of white and brown adipose tissue (Ward et al., 1994; Da Costa et al., 1994).

In H-2K^b-IGF-II transgenic mice, the transgene expression is barely detectable before birth but then can be found in thymus, spleen, and low in liver and kidney. Serum levels are ~6 - 8-fold increased. Transgenic mice display no alterations in body and organ growth except for a significant increase in the weight of the thymus (van Buul-Offers et al., 1995; Van der Ven et

al., 1997). In BKVI-IGF-II as well as in H-2K^b-IGF-II transgenic mice no incidence for tumor formation is found.

In the last few years, many additional transgenic mouse models overexpressing IGF-II under different promoters have been generated (Petrik et al., 1999; Devedjian et al., 2000; Moorehead et al., 2001; Moorehead et al., 2003; Zaina et al., 2002; Zaina et al., 2003). However, in none of these studies, a positive effect of IGF-II on postnatal growth was described.

In 1994, PEPCK-IGF-II transgenic mice were generated in our lab, as described in chapter 3.3.2. In heterozygous PEPCK-IGF-II transgenic mice, transgene-specific mRNA is found in liver, kidney, and several parts of the gut (duodenum, jejunum, ileum, and colon) from around birth onwards. Compared to wildtype controls, these mice show 2-3 fold higher serum IGF-II levels, even increasing after starvation. Serum IGF-II levels are significantly higher in male than in female mice, whereas no difference can be detected in different ages. Overall, serum IGF-I levels are lower in PEPCK-IGF-II transgenic mice than in wildtype siblings but sex related differences can be seen in both groups. Interestingly, transgenic males display significantly lower IGF-I levels than transgenic females, whereas wildtype males show significantly higher IGF-I levels than their female counterparts (Wolf et al., 1994). Serum IGF-II levels in these mice are lower than the physiological values in humans and do not have metabolic consequences such as hypoglycaemia (Wolf et al., 1994). Also no incidence for tumor formation is found. Investigations on body and organ growth in 4- and 12-week-old mice revealed that body growth is not significantly influenced by postnatally elevated IGF-II, but in transgenic animals the weight of kidneys and testis is increased at the age of 4 weeks, as well as the weight of the adrenal glands at an age of 12 weeks (Wolf et al., 1994; Weber et al., 1999). Studies of multiple bone parameters in PEPCK-IGF-II transgenic mice showed that moderate increased levels of IGF-II do not cause major changes in skeletal growth and turnover in mice (Wolf et al., 1995).

3 Animals, materials and methods

3.1 Animals

3.1.1 IGF-I knockout mice

The generation of IGF-I deficient mice by homologous recombination in embryonic stem (ES) cells has been previously described (Powell-Braxton et al., 1993). Briefly, gene targeting was obtained by insertion of a reverse orientated neomycin cassette in exon 3, leading to additional multiple stop codons in all IGF-I B chain reading frames. Targeted ES cells (129/Sv) were injected into C57BL/6J blastocysts, and the embryos were reimplanted into pseudopregnant females (CD1). Heterozygous offspring for the expected mutation was derived from parental chimeric mice and identified by Southern blot analysis using tail DNA. Mice homozygous for the IGF-I mutation were generated by intercrossing of heterozygotes. At birth, homozygous IGF-I knockout mice were 60% of weight of their littermates and until adulthood they only reached 30% of normal size. More than 95% of these pups died perinatally. Both sexes lacked libido and were infertile. Heterozygous IGF-I knockout mice were found to be slightly smaller (~10%) than their wildtype littermates but they were fertile and healthy.

The mice used for our experiment were kindly provided by Dr. Pieter A.F.M. Doevendans, University of Maastricht, Netherlands in cooperation with MD Cecilia Camacho-Hübner, Departments of Endocrinology and Chemical Endocrinology, St Bartholomew's Hospital, London, United Kingdom. We obtained two female and two male heterozygous IGF-I knockout mice on the original background. In our lab they were intercrossed with NMRI mice to obtain an outbred background of 50%.

3.1.2 PEPCK-IGF-II transgenic mice

PEPCK-IGF-II transgenic mice harbor fusion genes in which a human IGF-II complementary DNA is placed under the transcriptional control of the rat phosphoenolpyruvate carboxykinase (PEPCK) promoter. They were generated by pronuclear injection as previously described (Wolf et al., 1994). Briefly, a fragment including 550 bp of the PEPCK promoter, 120 bp of rat insulin II intron A sequences, the 768 bp human IGF-II cDNA, and 420 bp of the terminating sequences of the human GH gene was microinjected into pronuclei of zygotes from superovulated B6D2F1 mice (Charles River-WIGA) (Figure 3-1). Injected zygotes were

transferred to synchronized recipients (NMRI, Charles River-WIGA). Transgenic founder animals were mated to nontransgenic mice to set up transgenic lines. More than six generations were necessary to achieve over 98% NMRI background.

PEPCK-IGF-II transgenic mice were shown to overexpress IGF-II with serum IGF-II levels 2-3 times higher than those in controls. For the transgenic animals values in a range of ~130-160 ng/ml and ~30-60 ng/ml for the controls were detected. Transgene-specific messenger RNA was detected in liver, kidney and several parts of the gut (Wolf et al., 1994).

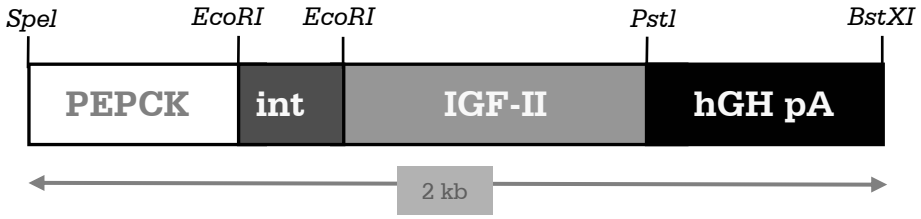


Figure 3-1: Schematic representation of PEPCK-IGF-II (Wolf et al., 1994). The construct includes the 550 bp PEPCK (phosphoenolpyrovate carboxykinase) promoter, 120 bp rat insulin II intron A sequences, the 768 bp human IGF-II cDNA and 420 bp of the terminating sequences of human GH gene.

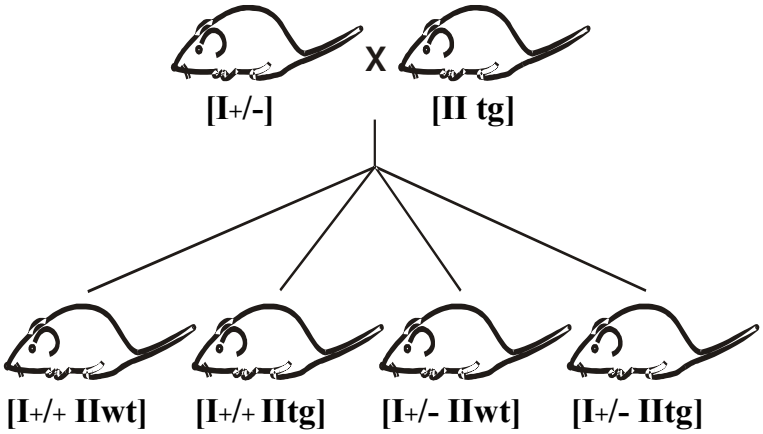
3.1.3 Crossbreeding

Since our aim was to study the role of postnatally elevated IGF-II in the absence of IGF-I we wanted to obtain mice lacking IGF-I and overexpressing IGF-II. Therefore the mating was done as described below.

Due to the infertility of IGF-I-deficient mice, two mating steps were necessary (Figure 3-2). First, heterozygous IGF-I knockout mice [I+/-] (50 % NMRI background) were mated with heterozygous PEPCK-IGF-II transgenic mice [IItg] (~100 % NMRI background), generating four different genetic groups: animals lacking one IGF-I allele and wildtype for the PEPCK-IGF-II transgene [I+/- IIwt], lacking one IGF-I allele and habouring the PEPCK-IGF-II transgene [I+/- IItg], wildtype for the IGF-I mutation and carrying the PEPCK-IGF-II transgene [I+/+ IItg], and completely wildtype [I+/+ IIwt]. In a second step, [I+/- IIwt] and [I+/- IItg] were mated to obtain two additional groups: homozygous IGF-I knockout and PEPCK-IGF-II wildtype mice [I-/- IIwt] and homozygous IGF-I knockout and PEPCK-IGF-II transgenic mice [I-/- IItg]. Thus, the resulting genetic background was nearly ~75% NMRI.

Mating Scheme

Step 1:



Step 2:

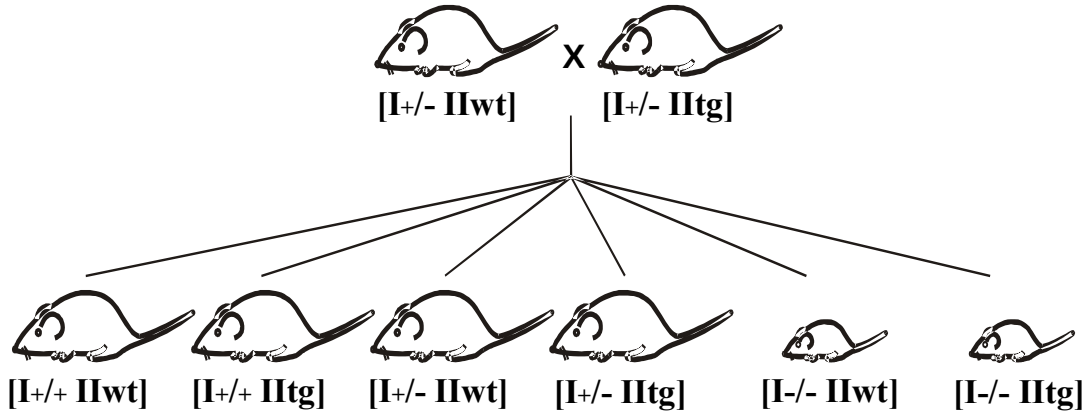


Figure 3-2: Mating scheme. The mating was done in two steps. In step 1, heterozygous IGF-I knockout mice [I+/-] were crossed with heterozygous PEPCK-IGF-II transgenic mice [IItg]. From the obtained offspring we mated the animals lacking one IGF-I allele and wildtype for PEPCK-IGF-II transgene [I+/- IIwt] with individuals lacking one IGF-I allele and carrying the PEPCK-IGF-II transgene [I+/- IItg].

3.1.4 Animal husbandry

All animals were maintained under specified pathogen-free conditions in a closed barrier system (sanitary trap system, sterile clothing, medical gloves, chemical material sluice, handing through autoclave, positive pressure ventilation) at 22°C, 65% humidity and a 12 h light / 12 h dark cycle.
Mice were housed separated by sex in open cages (Macrolon type II, type II long and type III) on static racks and had free access to a standard rodent diet (V1534 and V1126 for pregnant and lactating animals, Ssniff) and HCl-acidified tap water (pH 2.5-3).
Health monitoring screening programmes were based on the FELASA guidelines for breeding colonies and experimental units. Our programmes involved examinations at intervals of three months.
The animals were weaned at an age of four to five weeks, marked by ear piercing and tail tips were clipped and frozen on dry ice for genotype analysis.

3.2 Mouse genotyping

3.2.1 Proteinase K digests of mouse tail tips

Reagents:

EDTA	ethylene diamine tetraacetic acid, 0.5 M, pH 8.0
Proteinase K Solution	20 mg/ml dissolved in bidistilled H_2O
Isopropanol	100%
Ethanol	70%

Wizard® Genomic DNA Purification Kit (Promega) containing:
Nuclei Lysis Solution
RNase Solution
Protein Precipitation Solution
DNA Rehydration Solution

Assay procedure:

Tail tips (about 0.5 cm), stored at -80°C, were incubated overnight with gentle shaking in 1.5 ml centrifuge tubes containing 120 ml EDTA, 500 µl Nuclei Lysis Solution and 17.5 µl Proteinase K. On the next morning, 3 µl of RNase Solution were added, the samples were mixed by inverting 25 times and incubated for 25 minutes at 37°C. After cooling down to room temperature, 200 µl of Protein Precipitation Solution were added to the sample. The samples were vortexed at high speed for 20 seconds, chilled on ice for five minutes and then centrifuged at 20,800 x *g* for 4 minutes. The supernatant was transferred into a clean 1.5 ml centrifuge tube containing 600 µl isopropanol. The DNA became visible by gentle shaking and was pelleted by centrifugation at 20,800 x *g* for two minutes. After carefully decanting the supernatant, 600 µl of 70% ethanol were added to wash the DNA. The samples were inverted several times and then centrifuged at 20,800 x *g* for 2 minutes. Ethanol was carefully removed with a pipette tip and the pellet air-dried for 10-15 minutes. 50 µl of Rehydration Solution were added and the DNA was rehydrated by incubating at 65°C for one hour. DNA was stored at 2-8°C.

3.2.2 Determination of DNA concentration

For the determination of the DNA concentration, optical density (OD) of samples (each 100 µl at a dilution of 1:100) was measured against 100 µl bidistilled H_2O at a wavelength of 260 nm as well as 280 nm (Spectrophotometer, Beckmann). Ratios (260/280 nm) between 1.7 and 2.0 proved a sufficient purity of the samples. The DNA was diluted to a concentration of 100 ng/µl.

3.2.3 Principle of the Polymerase Chain Reaction (PCR)

PCR is an *in vitro* method that allows up to a billion fold amplification of a selected DNA sequence. The reaction uses two oligonucleotide primers that hybridize two opposite strands and flank the target DNA sequence that is to be amplified. In the presence of deoxyribonucleoside triphosphates (dNTPs), a heat-stable DNA polymerase catalyzes the elongation of the primers. A repetitive series of cycles involving template denaturation, primer annealing, and extension of the annealed primers by the polymerase results in exponential accumulation of a specific DNA fragment. Because the primer extension products

synthesized in a given cycle can serve as a template in the next cycle, the number of target DNA copies increases exponentially corresponding to each cycle.

3.2.4 PCR protocol for detecting the IGF-I knockout sequence

The following reagents were used for all PCRs described below:

Reagents:

Taq DNA polymerase Kit (Qiagen) containing:

PCR Buffer, 10x	TrisCl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$, pH 8.7 (20°C)
Q-Solution	5x concentrated
$MgCl_2$	25 mM
Taq Polymerase	5 U/µl, recombinant 94-kDa DNA polymerase, isolated from *Thermus aquaticus,* cloned in *E. coli*
dNTP Set	100 mM aquaeous solutions of dATP, dCTP, dGTP and dTTP each in a separate vial
Ethidium Bromide	0.1% solution in bidistilled H_2O
50x TAE running buffer	242 g Tris 57.1 ml Glacial Acetic Acid 100 ml EDTA, 0.5 M, pH 8.0 ad 1 l bidistilled H_2O
6x Loading Dye	30% Glycerol Bromophenol Blue

Sequences of the "IGF-I primers" detecting the wild type allele:

IGF-I s = 5′- GAC CAG TAG CAA AGG ACT TAC CAC - 3′

IGF-I as = 5′- CAC ACC TGG CTC CCC GAA AAT GAA - 3′

Sequences of the "Neo-Primers" detecting the disrupted allele (neomycin cassette):

Neo s = 5´- TGA CTG GGC ACA ACA GAC AAT CGG - 3´

Neo as = 5´- GTA GCC AAC GCT ATG TCC TGA TAG - 3´

Assay procedure:

For this PCR two pairs of primers were used, one detecting the wildtype allele (IGF-I sense and antisense oligonucleotide), the other detecting the disrupted allele (Neo sense and antisense oligonucleotide). For detecting the wildtype allele, primers were derived from sequences upstream of exon 3 and downstream of the neomycin insertion in this exon resulting in a 366 bp product. For detection of neomycin, primers 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 (Powel-Braxton et al., 1993).

The 20 µl reaction was prepared in 100 µl PCR reactions tubes on ice, containing:

DNA Template	1.00 µl (about 100 ng DNA)
IGF-I sense primer, 8 µM	1.00 µl
IGF-I antisense primer, 8 µM	1.00 µl
Neo sense primer, 2 µM	1.00 µl
Neo antisense primer, 2 µM	1.00 µl
dNTP Mix, 1 mM	3.00 µl
PCR Buffer, 10x	2.00 µl
Q-Solution	4.00 µl
$MgCl_2$, 25 mM	1.25 µl
Taq Polymerase, 5U/µl	0.10 µl
Bidistilled H_2O	4.65 µl

DNA of a hetero- and homozygous IGF-I knockout and of a wildtype mouse was used as a positive control and a non-template negative control (H_2O) to check for contamination.

The amplification took place in a Biometra Uno Cycler and was performed as follows:

1st step:	denaturation	94°C for 4 minutes
2nd step:	denaturation	94°C for 1 minute
3rd step:	annealing	66°C for 1 minute
4th step:	extension	72°C for 2 minutes
5th step:	extension	72°C for 10 minutes
6th step:	cooling at	4°C

Steps 2 to 4 were repeated 36 times before progression to the final extension step 5 and cooling down. Amplified products were electrophoretically separated on 2% TAE agarose gels. For this purpose 1 g agarose was mixed in 50 ml 1x TAE buffer and boiled. 4 µl ethidium bromide were added and the mixture was filled into a running chamber. After approximately half an hour the gel solidified and the chamber was filled up with 1x TAE running buffer. PCR products and a DNA standard (pUC Mix Marker, MBI) were mixed with 4 µl loading dye and were pipetted into the slots. The run was started at 90 V for 45 minutes. Amplified products were visualized under UV-light (Eagle Eye II, Strategene). Wild type animals showed a band at a size of 366 bp, homozygous IGF-I knockout mice at a size of 608 bp. Heterozygous IGF-I knockout mice showed both bands (Figure 3-3).

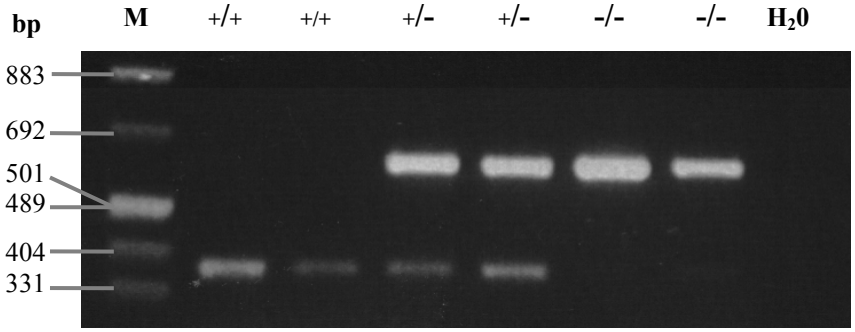


Figure 3-3: Detection of the IGF-I mutant allele. Homozygous IGF-I knockout animals (-/-) show a fragment at the size of 608 bp, wildtype animals (+/+) at the size of 366 bp. Heterozygous IGF-I knockout animals (+/-) show both fragments.

3.2.5 PCR protocol for detecting the PEPCK-IGF-II transgene

Sequence of the human IGF-II PCR sense primer:

hIGF-II #5 = 5' - ATG GGA ATC CCA ATG GGG AAG - 3'

Sequence of the human IGF-II PCR antisense primer:

hIGF-II #10 = 5' - CGG GGT CTT GGG TGG GTA GAG - 3'

Assay procedure:

For this PCR primers specific for the PEPCK-IGF-II construct were used. The 20 µl reaction was prepared in 100 µl PCR reaction tubes on ice, containing:

DNA template	1.00 µl (about 100 ng DNA)
hIGF-II #5 primer, 2 µM	1.00 µl
hIGF-II #10 primer, 2 µM	1.00 µl
dNTP Mix, 1 mM	2.00 µl
PCR Buffer, 10x	2.00 µl
Q-Solution	4.00 µl
$MgCl_2$, 25 mM	1.25 µl
Taq Polymerase, 5 U/µl	0.10 µl
Bidistilled H_2O	7.70 µl

DNA of a heterozygous PEPCK-IGF-II transgenic mouse was used as a positive control and a non-template control to check for contamination.

The amplification took place in a Biometra UnoII Cycler and was performed as follows:

1st step:	denaturation	94°C for 4 minutes
2nd step:	denaturation	94°C for 1 minute
3rd step:	annealing	63°C for 1 minute
4th step:	extension	72°C for 2 minutes
5th step:	extension	72°C for 10 minutes
6th step:	cooling at	4°C

Steps 2 to 4 were repeated 35 times before progression to the final extension step 5 and cooling down. Amplified products were electrophoretically separated on 2% TAE agarose gels as described before. Amplified products were visualized under UV-light. PEPCK-IGF-II transgenic mice showed a band at a size of 498 bp (Figure 3-4).

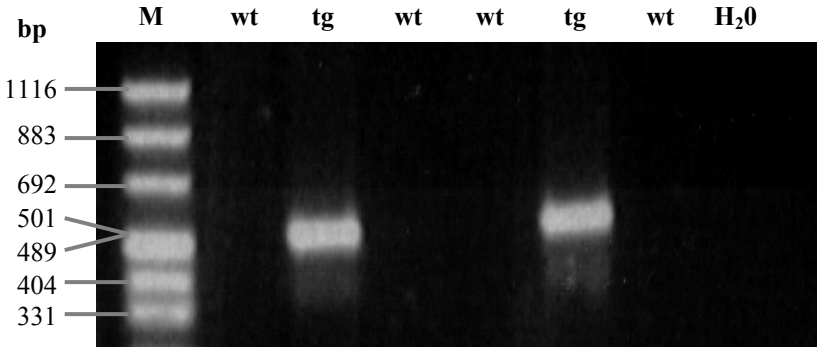


Figure 3-4: Detection of the PEPCK-IGF-II sequence. Transgenic animals show a fragment at a size of 498 bp.

3.3 Evaluation of gene expression at RNA levels

To determine the beginning of the transgenic IGF-II expression we investigated the RNA levels in liver and kidneys of embryos at day 17.5 and 19.5 p.c. as well as of pups at day four after birth.
We mated heterozygous PEPCK-IGF-II transgenic mice with NMRI. To obtain the embryonic stages day 17.7 and 19.5 pregnant mice were killed by cervical dislocation and the embryos were prepared from the uterus. Embryos and pups were killed by decapitation. Liver, kidneys as well as a piece of tail for genotyping were dissected and frozen on dry ice. The organ samples were stored at -80°C until further investigation. Genotyping was done as described in 3.2.5.

To avoid degradation by nucleases, following rules were observed with the extraction, manipulation or analysis of RNA:

- Disposable gloves were worn all the time and changed frequently
- All equipment was autoclaved, glasses and magnetic stirrers sterilized at 180°C for 8 h
- All solutions, except those containing Tris, were made with DEPC-H_2O (0.1 % diethylpyrocarbonate)

3.3.1 RNA extraction

Reagents:

TRIzol® Reagent, Invitrogen, Germany	mono-phasic solution of phenol and guanidine isothiocyanate
Chloroform	100%
Isopropanol	100%
Ethanol	75%, in DEPC-treated water
Bidistilled H_2O	RNase-free

Assay procedure:
Tissue samples (50-100 mg) stored at -80°C were added directly into 2 ml safe-lock tubes (Eppendorf) containing 1 ml of the TRIzol® Reagent and homogenized with a tissue homogenizer at 23500 rpm (ART Labortechnik). After each sample, the homogenizer was cleaned with bidistilled water and 0.2 M NaOH and shortly run in TRIzol® Reagent to avoid dilution of the samples. Homogenized samples were incubated for 5 minutes at room

temperature to permit the complete dissociation of nucleoprotein complexes. 0.2 ml chloroform were added. After vortexing the tubes vigorously for 15 seconds the samples were incubated at room temperature for 2 to 3 minutes and centrifuged at 12,000 x g for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separated into a lower red, phenol-chlorofrom phase, an interphase, and a colourless upper aquaeous phase. RNA remained exclusively in the aquaeous phase.

The aqueous phase was transferred into a fresh tube containing 0.5 ml isopropanol. Samples were mixed, incubated at room temperature for 10 minutes, and centrifuged at 12,000 x g for 10 minutes at 4°C, respectively. After removing the supernatant, 1 ml of 75% ethanol was added; the samples were mixed by vortexing and centrifuged at 7,500 x g for 5 minutes at 4°C. Afterwards, the ethanol was carefully removed with a pipette tip and the RNA pellet was air-dried for 5 to 10 minutes. Finally, the pellet was dissolved in RNase-free water.

RNA concentration was estimated in the same way as DNA concentration and calculated with the following formula:

RNA concentration (µg/ml): dilution factor x OD_{260} x 40 (unit factor)

3.3.2 Reverse Transcription PCR (RT-PCR)

Reagents :

10 x Reaction Buffer	Tris 10 mM, $MgCl_2$ 10 mM, pH 7.4, DEPC treated
DNase I	diluted 1:10 with bidistilled H_2O
Random Hexamer Primers	3 µg/µl in 3 mM Tris-HCl (pH 7.0), 0.2 mM EDTA
dNTPs	10 mM
5 x Reaction Buffer	M-MLV Reverse Transcriptase Buffer
DTT	10 mM (Dithiothriteol)
Reverse Transcriptase	200 U/µl (M-MLV Reverse Transcriptase)

Assay procedure:

A DNase I digest was performed with 5 µg of total RNA. The reaction mix was prepared on ice and contained the following:

RNA, 5 µg	16 µl
10 x Reaction Buffer	2 µl
DNase I	2 µl

The samples were incubated at 37°C for 30 minutes. Thereafter, the enzyme was inactivated by incubation at 75°C for 10 minutes.

10 µl of the DNase I digest (= 2.5 µg RNA) were used for reverse transcription. The reaction mix was prepared on ice and contained the following:

DNase digested RNA	10.0 µl
DTT	2.0 µl
Random Hexamer Primers	1.0 µl
dNTPs	2.0 µl
Reverse Transcriptase	0.1 µl
Bidistilled H_2O	0.9 µl

For reverse transcription samples were incubated at 37°C for 30 minutes. The enzyme was inactivated by incubation at 95°C for 10 minutes.

3.3.3 β-Actin PCR

Sequence of the β-Actin sense primer:

β-Actin #1 = 5’ - GGC ATC GTG ATG GAC TCC - 3’

Sequence of the β-Actin antisense primer:

β-Actin #2 = 5’ - CTC GGA AGG TGG ACA GGG - 3’

Assay procedure:

To confirm the correct reverse transcription from RNA into cDNA, a sequence of the house-keeping gene β-Actin was amplified. The 20 µl reaction was prepared in 100 µl PCR reactions tubes on ice, containing:

DNase digested RNA or	
cDNA	1.00 µl
β-Actin #1 primer, 2 µM	1.00 µl
β -Actin #2 primer, 2 µM	1.00 µl
dNTP Mix, 1 mM	2.00 µl

PCR Buffer, 10x	2.00 µl
Q-Solution	4.00 µl
$MgCl_2$, 25 mM	1.25 µl
Taq Polymerase, 5 U/µl	0.10 µl
Bidistilled H_2O	7.70 µl

Genomic DNA of a heterozygous PEPCK-IGF-II transgenic mouse was used as a positive control and a non-template control to check for contamination.

The amplification took place in a Biometra UnoII Cycler and was performed as follows:

1st step:	denaturation	94°C for 4 minutes
2nd step:	denaturation	94°C for 1 minute
3rd step:	annealing	60°C for 1 minute
4th step:	extension	72°C for 2 minutes
5th step:	extension	72°C for 10 minutes
6th step:	cooling at	4°C

Steps 2 to 4 were repeated 35 times before progression to the final extension step 5 and cooling down. Amplified products were electrophoretically separated on 2% TAE agarose gels as described before. Amplified products were visualized under UV-light. DNA contamination in the RNA preparations could be excluded since no bands were detected when RT-probes were loaded on a 2% agarose gel.

3.3.4 PEPCK-IGF-II PCR

The PEPCK-IGF-II PCR was performed as previously described (see chapter 3.2.5), using 3 µl of cDNA as a template.

3.4 Evaluation of gene expression at the protein level

3.4.1 Blood and serum collection

Blood samples from adult mice were obtained via bleeding from the retro-orbital sinus under ether anaesthesia. Embryos and pups were killed by decapitation and the blood was collected with a capillary tube from the cut.

Blood serum was collected from coagulated samples by centrifugation at 20,800 x *g* for 10 minutes at room temperature. The supernatant was transferred into a new plastic tube and stored at -20°C until further investigation.

3.4.2 Western ligand and immunoblot analysis

3.4.2.1 SDS-PAGE

Reagents:

Separating Gel (12%)	1.675 ml bidistilled H_2O
	1.25 ml Tris, pH 8.8, 1.5 M
	2 ml Acryl Amide, 30%
	50 µl SDS (Sodium Dodecyl Sulfate), 10%
	2.5 µl TEMED (N, N ,N'; N'-Tetrametylethylendiamine)
	25 µl APS (Ammonium Persulfate), 10%
Stacking Gel (5%)	1.75 ml bidistilled H_2O
	0.3125 ml Tris, pH 6.8, 0.5 M
	0.375 ml Acryl Amide, 30%
	1.25 µl TEMED (N, N ,N'; N'-Tetrametylethylendiamine)
	25µl APS (Ammonium Persulfate), 10%
Laemmli Buffer (2x)	12.5 ml Tris/HCl, 1 M, pH 6.8
	4 g SDS (Sodium Dodecyl Sulfate)
	20 ml Glycerine
	2 ml Bromophenol Blue, 1%
	ad 100 ml bidistilled H_2O
Running Buffer (10x)	30.3 g/l Tris, 48 mM
	144 g /l Glycine
	10 g /l SDS
	ad 1 l bidistilled H_2O
2-mercaptoethanol	100%

Assay procedure:

The serum proteins were separated using the Mini Protean II™ System. The separating gel (12% acrylamide) was prepared in an Erlenmeyer Flask under continuous agitation and poured into the gap between the glass plates of the system, leaving enough space for the stacking gel (about 2,5 cm). To ensure an even surface the stacking gel was overlaid with bidistilled water and kept for one hour at room temperature for polymerisation. After complete polymerisation, the water was discarded and the stacking gel (5% acrylamide, prepared in the same way as the separating gel) was loaded on top. The comb was inserted taking care not to trap air bubbles under the teeth. While keeping the gel for 30 minutes at room temperature for polymerisation the samples were prepared as follows: 1 µl serum, 9 µl bidistilled H_2O, 10 µl 2x Laemmli buffer and (only for Western immunoblot) 1 µl 2-mercaptoethanol were mixed, incubated at 95°C for 5 minutes, chilled on ice for one minute and kept at room temperature afterwards.

After complete polymerisation of the stacking gel, the comb was removed and the plates were mounted in the electrophoresis apparatus being filled up with running buffer. Samples and a standard marker (Protein Molecular Weight Marker, #SM0431, MBI) were loaded. Electrophoresis was performed initially at 100 V for 10 minutes and then at 160 V for approximately 60 minutes until the bromophenol blue left the separating gel at the bottom.

3.4.2.2 Electroblotting

Reagents:

Transfer Buffer (10x)	58 g Tris/l
	29 g/l Glycin
	3.7 g/l SDS
	ad 1 l bidistilled H_2O
Transfer Buffer	5 ml 10x Transfer Buffer
(for 1 gel)	32 ml bidistilled H_2O
	10 ml Methanol, 100%
Ponceau Red	2 g Ponceau S
	30 ml Acetic Acid
	ad 1 l bidistilled H_2O

Assay procedure:

Separated proteins were transferred to a nitrocellulose membrane by semidry electrophoretic blotting in the MilliBlot-Graphite Electroblotter (Millipore). The nitrocellulose membrane was incubated in 100% methanol for 10 minutes and in the complete transfer buffer for 30 minutes. Six sheets of Whatman paper cut to the same size as the gel were soaked in transfer buffer, stacked on top of each other on the bottom electrode and squeezed with a glas tube to remove the air bubbles. The nitrocellulose membrane and then the gel were placed exactly on the paper stack and were covered with another stack of six soaked Whatman paper sheets. The upper electrode was placed and the system was connected to a power supply. The transfer took place for 90 minutes at 60 mA for each gel ($1A/cm^2$).

After the transfer, the membrane was labeled with a pen and the molecular weight standard bands were marked. The membrane was stained with Ponceau red, dried and stored at 4°C.

3.4.2.3 Western Ligand Blot

Reagents:

Tris-buffered Saline (TBS)	1.21 g Tris 8.76 g NaCl ad 1 l bidistilled H_2O HCl ad pH 7.4
Tris-buffered Saline + 3% NP-40	485 ml Tris-buffered Saline, pH 7.4 15 ml Nonidet P-40,
Tris-buffered Saline + 0.1% Tween 20	500 ml Tris-buffered Saline, pH 7.4 500 µl Tween®-20,
Tris-buffered Saline + 1% fish-gelatin	5 ml Tris-buffered Saline, pH 7.4 50 µl Fish Gelatin
Tracer	5 ml Tris-buffered Saline, pH 7.4 50 µl Fish Gelatin 500,000 cpm/ml [^{125}I]-IGF-II Tracer

Assay procedure:

The major advantage of this method compared to traditional Western immunoblotting is the possibility of detecting bioactive IGFBPs. Ligand blot analysis of serum IGFBPs was performed using [^{125}I]-IGF-II as a tracer according to the method of Hossenlopp at al. (1986). The membrane containing the separated proteins was washed and hybridized in 50 ml conical tubes in a hybridization oven at 4°C according to the following scheme:

- Washing in Tris-buffered Saline + 3% NP-40 for 20 minutes
- Blocking in Tris-buffered Saline + 1% Fish Gelatin for 120 minutes
- Washing in Tris-buffered Saline + 0.1% Tween®-20 for 20 minutes
- Incubation with tracer overnight

The next day, blots were washed again according to the following scheme:

- 2x washing in Tris-buffered Saline + 0.1% Tween®-20 for 30 minutes
- 3x washing in Tris-buffered Saline for 30 minutes

The membrane was dried and IGFBPs were visualized on Storage Phosphor Image and X-ray films.

3.4.2.4 Western Immuno Blot

Reagents:

TBS-T	500 µl Tween®-20 ad 1 l TBS
Blocking Solution	2.5 g instant skimmed milk (spray dried) ad 1 l TBS-T
Primary Antibody	anti-mGH antibody from rabbit, diluted 1:20,000 in blocking solution (Dr. A.F. Parlow, National Hormone & Piatury Programme, USA)
Secondary Antibody	anti-rabbit antibody from goat, diluted 1:4000 in blocking solution (Cell Signaling Technology)

Assay procedure:

The membrane containing the separated proteins was dried at 37°C for 30 minutes and then incubated with blocking solution in a hybridization oven at room temperature for 60 minutes. After blocking, incubation with the primary antibody took place at room temperature for 60 minutes. The membrane was washed 3 times for 5 minutes at room temperature with TBS-T. Incubation with the secondary antibody occurred for 60 minutes at room temperature. The membrane was washed 3 times with TBS-T and finally once in TBS for 10 minutes at room temperature. Detection was performed by incubating the membrane with 2 ml of the Western blotting luminol detection reagent (Santa Cruz). The membrane was sealed under plastic and exposed to an ECL Hyper film.

3.4.3 Radioimmunoassay (RIA)

Specific radioimmunoassays of the blood serum for IGF-I, IGF-II and IGFBP-2 were kindly performed by Karin Weber (laboratory of Dr. Martin Elmlinger, University Childrens Hospital, Tuebingen, Germany) as previously described (Blum et al., 1988; Blum et al., 1992; Ranke et al., 2003). For all assays, dilution curves of mouse serum samples were linear, and paralleled those of human standards.

3.5 Analysis of body and organ growth

Body weight of selected litters (identified by palm-tattooing) was recorded daily, starting from day three or four after birth until day 45. From this date on, mice were weighed twice a week until day 60. At day 60 mice were killed by bleeding from the retro-orbital sinus under ether anaesthesia and the nose-rump-length was determined. The nose-rump-length was measured as the distance between nose and base of the tail. At necropsy, thymus, heart, lung, spleen, liver, kidneys, adrenal glands, pancreas, abdominal fat, testes and ovaries were dissected, blotted dry on tissue paper and weighed to the nearest mg. For bilateral organs, the paired weight was recorded. Carcasses were weighed to the nearest 0.1 g after removal of the organs, skin, head and tail.

3.6. Bone parameters

3.6.1 Bone preparation

Reagents:

Calcein Solution	10 ml NaCl
	140 mg $NaHCO_3$
	40 mg Calcein
Phosphate-buffered Saline (PBS)	8 g NaCl, 137 mM
	0.2 g KCl, 2.7 mM
	1.44 g Na_2HPO_4, 10 mM
	0.24 g KH_2PO_4, 2 mM
	ad 1 l bidistilled H_2O
	HCl ad pH 7.4
Paraformaldehyde, 4%	40 g Paraformaldeyde
	PBS ad 1 l
	250 µl NaOH, 5 M
	HCl, 37% ad pH 7.2-7.4

Assay procedure:

The injection of calcein was performed subcutaneously 72 and 24 hours before necropsy. Doses were calculated as follows: injection volume in µl = 5x body weight (g). The calcein application was done in accordance to the German law on Animal Protection and approved by the Bavarian Animal Research Authority (Az. 209.1/211-2531.2-29/03).

Both femurs and the lumbar vertebraes were isolated from the carcass and carefully defleshed. The right femur and the first lumbar vertebrae were fixed in 4% paraformaldehyde for 24 hours at 4°C, the left femur and the remaining lumbar vertebraes were conserved in 70% ethanol.

3.6.2 Bone histology

Bone histology and bone mineral density measurements, briefly described below, were kindly performed by the lab of PD Dr. Reinhold Erben, Institute of Animal Physiology, Veterinary Faculty, LMU Munich.
After washing overnight in 10% sucrose/PBS-solution at 4˚C, the fixed right femurs and first lumbar vertebraes were subsequently dehydrated and embedded undecalcified in methyl-methacrylate as previously described (Erben, 1997). Three-µm-thick sections were prepared using a HM 360 microtome (Microm, Walldorf, Germany), and stained with von Kossa/McNeal (Schenk et al., 1984).

3.6.3 Bone mineral density measurements

Bone mineral density (BMD) of the left femur was measured by peripheral quantitative computed tomography (pQCT) using a XCT Research M+ pQCT machine (Stratec Medizintechnik, Pforzheim, Germany). One slice (0.2-mm-thick) in the mid-diaphysis of the femur, and 3 slices in the distal femoral metaphysis located 1.5, 2.0, and 2.5 mm proximal to the articular surface of the knee joint were measured. BMD values of the distal femoral metaphysis were calculated as the mean over 3 slices. A voxel size of 0.070 mm and a threshold of 600 mg/cm³ were used for calculation of cortical BMD.

3.7 Statistical analysis

The average growth of individual groups was estimated by transforming body weight data to a weighing day of n x 3 by linear interpolation as described previously (Wolf et al., 1991).
Body weight and organ weight data as well as blood serum parameters were analyzed by the General Linear Models procedure (SAS Institute, Inc., Cary, NC), taking the effect of IGF-I status (+/+, +/-, -/-), IGF-II status (tg, wt) and sex into account. Means were compared by using LSD *post hoc* tests (SPSS, Inc. [Chicago, IL] program package).
Bone parameters were analyzed by 3-way ANOVA. The statistical model included IGF-I status, IGF-II status, sex, and the various interactions between these factors.

3.8 Reagents and Materials

Amersham Pharmacia GmbH **Freiburg, Germany**	Fish Gelatin (# RPN1636) Hyperfilm™ MP (# RPNK6K) ECL X-ray film (# RPN2103K) Electrophoresis Power supply EPS500/400
AppliChem **Darmstadt, Germany**	Ethylenediaminetetraaceticacid, (EDTA) (# A3353.1000)
ART Labortechnik **Mühlheim, Germany**	Tissue Homogenizer
Bachhofer **Reutlingen, Germany**	Hybridisation Oven
Beckman Coulter **Palo Alto, CA, USA**	Spectrophotometer DV 640
Becton Dickinson **Franklin Lakes, USA**	Falcon® BLUEMax™ conical tube 50 ml (# 352070) Falcon® BLUEMax™ conical tube 15 ml (# 352096)
Biometra **Göttingen, Germany**	Thermocycler UNO thermoblock Thermocycler UNO II thermoblock
BioRad **München, Germany**	Acrylamide/Bis Solution, 30% (# 161-0156) Ammonium Persulfate, (APS) (# 161-0700) Mini Protean II™ system Power Supply POWER PAC 300 TEMED (# 161-0800)
Brand **Melsungen, Germany**	Micro haematocrit Tubes (# 749311)
Carl Roth GmbH **Karlsruhe, Germany**	Acetic Acid Glacial (# 3738.1) DEPC (Diethylpyrocarbonate) (# K028.2) Diethyl Ether (# 8.222.70.5) Ethanol (# 9065.3) Glycerol (# 3783.1) Glycine (# 3908.2) Methanol (# 46271) Tris (# 4855.2)
Cell Signalling Technology	Anti-Rabbit Antibody (goat)
Eppendorf **Hamburg, Germany**	Centrifuge 5417R Mini Spin Plus

	Safe-lock Tubes, 1.5 ml (# 0030 121.848) Safe-lock Tubes, 2.0 ml (# 0030 120.084) Thermomixer 5436
G. Kisker GbR **Steinfurt, Germany**	PCR-Tube-Strips, 0.2 ml (# G002-A) PCR-Caps-Strips, 0.2 ml (# G004-A)
IKA-Works, Inc. **Wilmington, USA**	MS1 Minishaker (# 004124)
Invitrogen GmbH **Karlsruhe, Germany**	M-MLV Reverse Transcriptase (# 28025-013) M-MLV Reverse Transcriptase Buffer (# 18057-018) Random Hexamer Primers (# 48190-011) Ultra Pure™ Dithiothriteol, (DTT) (# 15508-013) Ultra Pure™ Agarose (# 15510-027)
Fermentas, MBI **St. Leon-Rot, Germany**	dNTP-Set (# R0181) Prestained Protein Ladder (# SM0671) Protein Molecular Weight Marker (# SM0431) pUC Mix Marker 8 (# SM0301) 6x Loading Dye (#R0611)
Fluka **Deisenhofen, Germany**	Isopropanol (# 33539) Nonidet® P40 (# 74385)
Heirler **Radolfzell, Germany**	Instant Skimmed Milk (spray dried)
Hormone and Pituitary Program, **(Dr. A.F. Parlow), USA**	Mouse Growth Hormone Antigen Rat Growth Hormone Antiserum (Rabbit)
Invitrogen **Germany**	TRIzol® (# 15596-018)
Merck **Darmstadt, Germany**	Bromophenol Blue Sodium Salt (# 1.11746.0005) Chloroform (# 1.02445.1000) di-Sodium Hydrogen Phosphate Anhydrous (# 1.06586.2500) Dodecyl Sulfate Sodium Salt (# 1.13760.1000) Ethidium Bromide 1% (# 1.11608.0030) Hydrochloric Acid, 37% (# 1.13386.2500) Potassium Chloride, (KCl) (# 1.04936.1000) Potassium Dihydrogen Phosphate (# 1.04873. 1000) Sodium Chloride (# 1.06404.1000)
Millipore **Bedford, MA, USA**	Immobilon-P PVDF membrane, 0.45 µm MiliBlot Graphite Electroblotter (# MBB DGE001)

Molecular Dynamics	Storage Phosphor Screen
MWG-Biotech **Ebersberg, Germany**	Gel chambers Power Supply PPS 200-1D
Pharmacia **Uppsala, Schweden**	Electrophoresis Power Supply EPS 500/400
Promega **Madison, USA**	Wizard® Genomic DNA Purification Kit (# A1125)
Qiagen **Hilden, Germany**	Taq DNA Polymerase Kit (# 201205)
Roche **Mannheim, Germany**	DNaseI (# 776751) Proteinase K (# 1092766) Random Hexamer Primers (# 1034731) Reverse Transcriptase AMV (# 1495062)
Santa Cruz Biotechnology, Inc. **Santa Cruz, California, USA**	Western Blotting Luminol Reagent (# sc-2048)
Schleicher & Schuell **Dassel, Germany**	Gel Blotting Paper (# GB002)
Schubert **Munich, Germany**	Protec Compact 45 (Film Processing machine)
Sigma **Taufkirchen, Germany**	2- Mercaptoethanol (# M-3148) Paraformaldehyde (# P-6148) Ponceau S solution (# P-7170) Tween® 20 (# P-7949)
Stratagene **Heidelberg, Germany**	Eagle Eye II
USV ELGA **Ransbach Baumbach, Germany**	Purelab™ Plus (# PL5121)

4 Results

4.1 Generation of double-mutant mice

Double mutant mice suitable for our experimental purposes were generated by a mating scheme in two steps (as described in chapter 3.1.3). In the first step heterozygous IGF-I knockout mice [I+/-] were mated with heterozygous PEPCK-IGF-II transgenic mice [IItg]. The obtained F1 generation included four different genetic groups on a ~75 % NMRI background: animals lacking one IGF-I allele and wildtype for the PEPCK-IGF-II transgene [I+/- IIwt], lacking one IGF-I allele and harbouring the PEPCK-IGF-II transgene [I+/- IItg], wildtype for the IGF-I mutation and carrying the PEPCK-IGF-II transgene [I+/+ IItg], and completely wildtype [I+/+ IIwt]. Subsequently, a mating with the following genotypes was performed: [I+/- IIwt] x [I+/- IItg]. The resulting offspring comprised animals with six different genotypes. In addition to the four genotypes already described ([I+/+ IIwt], [I+/+ IItg], [I+/- IIwt], and [I+/- IItg]), we obtained homozygous IGF-I knockout and PEPCK-IGF-II wildtype mice [I-/- IIwt] and homozygous IGF-I knockout and PEPCK-IGF-II transgenic mice [I-/- IItg].

All mice were genotyped by established PCR protocols detecting the IGF-I sequence or the PEPCK-IGF-II transgene as described in chapter 3.2.4 and 3.2.5. In total, we obtained 397 animals of 51 litters. Table 4-1 shows the genotype distribution of the obtained animals compared to the expected distribution according to the Mendelian inheritance principles.

Table 4-1: Expected versus observed distribution of the six different genotypes. In total 397 animals of 51 litters have been evaluated.

Distribution	**Total**	**Genotype**					
		[I+/+ IIwt]	**[I+/+ IItg]**	**[I+/- IIwt]**	**[I+/- IItg]**	**[I-/- IIwt]**	**[I-/- IItg]**
Expected	100%	12.5%	12.5%	25%	25%	12.5%	12.5%
Observed	100% (397)	13.1% (52)	18.9% (75)	31.5% (125)	22.4% (89)	5.3% (21)	6.8% (27)

The observed value for [I-/- IIwt] mice was 42.4 % and for [I-/- IItg] mice 54.4 % of the expected distribution, indicating a prenatal mortality rate of ~50%.

4.2 Expression analysis

4.2.1 Measurement of circulating levels of IGF-I and IGF-II

Radioimmunoassays were performed to measure the plasma levels of IGF-I and-II at day ~60 postnatal (Table 4-2). IGF-I was not detectable in [I-/- IIwt] and [I-/- IItg] mice. Reduced IGF-I levels were present in animals heterozygous for the IGF-I deleted allele ([I+/- IIwt], [I+/- IItg]), and the highest values were found in mice harbouring two intact IGF-I alleles ([I+/+ IIwt], [I+/+ IItg]). The presence of the IGF-II transgene resulted in reduced levels of IGF-I in the groups [I+/- IItg] and [I+/+ IItg]. A statistically significant increase in IGF-II expression could be seen in all animals harbouring the PEPCK IGF-II transgene ([I-/- IItg], [I+/- IItg], and [I+/+ IItg]) when compared to their IGF-II wildtype counterparts ([I-/- IIwt], [I+/- IIwt], and [I+/+ IIwt]). Thus, radioimmunoassays for serum levels of IGF-I and IGF-II demonstrated the expected expression of the two peptides reflecting the genotype.

Table 4-2: Serum levels (ng/ml) of IGF-I and IGF-II measured by RIA in 60-days-old mice. Data for males and females were combined and samples of 80 animals were investigated. Analysis of variance was used to evaluate statistically significant differences (Δ) between indicated groups (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$).

	genotype		**Δ**	**genotype**		**Δ**	**genotype**		**Δ**
	[I-/- IIwt]	**[I-/- IItg]**		**[I+/- IIwt]**	**[I+/- IItg]**		**[I+/+ IIwt]**	**[I+/+ IItg]**	
IGF-I	b	b	-	351.0 ± 18.3	271.4 ± 19.5	**	457.1 ± 17.7	375.9 ± 20.2	*
IGF-II	26.8 ± 8.5	209.2 ± 8.2	***	56.7 ± 10.7	146.2 ± 11.4	***	59.9 ± 10.3	123.6 ± 11.8	***
n	18	20		11	10		12	9	

b: below detection level. n: number of investigated animals.

4.2.2 RT-PCR

To determine the onset of transgenic IGF-II expression, total RNA from liver (Figure 4-1) and kidneys (Figure 4-2) (both major sites of endogenous PEPCK expression) of embryos at day 17.5 and 19.5 as well as of pups at day 4 after birth was extracted. RT-PCR was performed using genomic DNA of a PEPCK-IGF-II transgenic animal as a positive control. In both figures the first picture displays the PEPCK-IGF-II PCR using the transcribed cDNA as a template. The second picture shows a control PCR of the cDNA using the house-keeping gene β-Actin in order to confirm the correct reverse transcription and to compare the amounts of obtained cDNA. The third picture displays the control PCR for the DNase-I-digest of the RNA sample, confirming the absence of genomic DNA. As shown in figure 4-1, transgene expression of PEPCK-IGF-II in the liver could be detected from embryonic stage 19.5 onwards on. On day 4 after birth the obtained signal was visibly stronger.

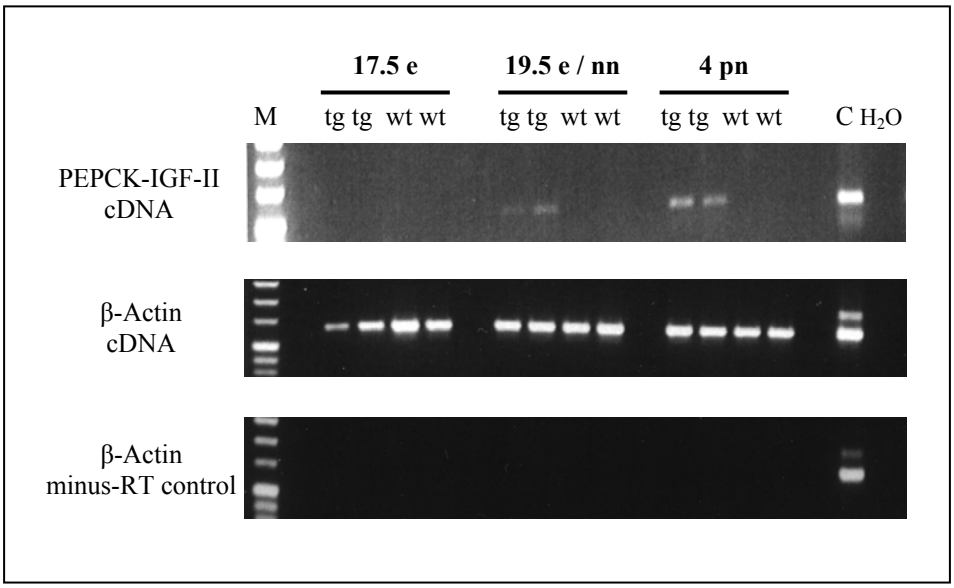


Figure 4-1: Expression analysis of the PEPCK-IGF-II transgene in the liver in different developmental stages by RT-PCR. M, marker. C, positive control (genomic DNA). H_20, negative control. nn, neonates. pn, postnatal.

Contrasting to the findings in the liver, PEPCK-IGF-II transgene expression could not be detected in the kidneys in any of the investigated stages (Figure 4-2).

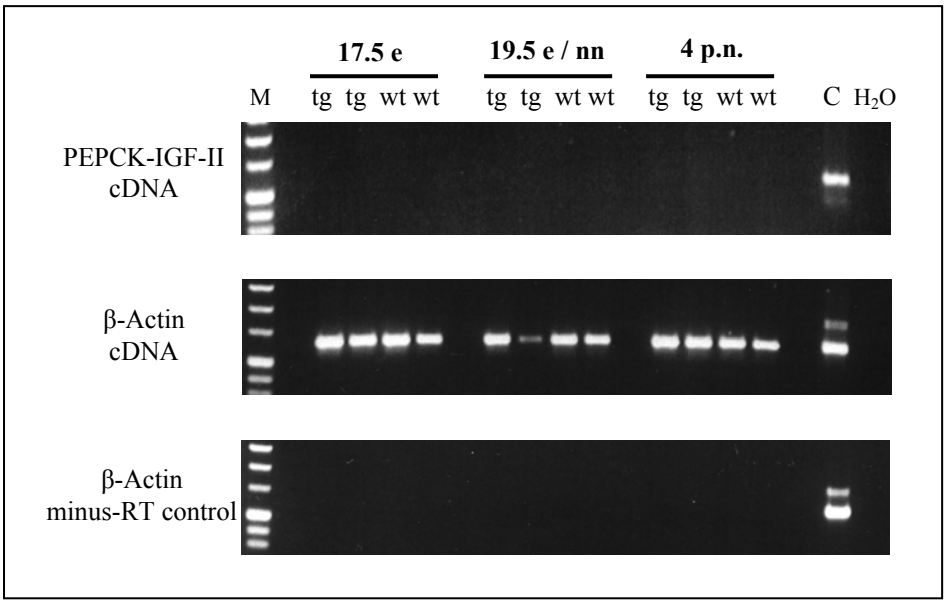


Figure 4-2: Expression analysis of the PEPCK-IGF-II transgene in the kidney in different developmental stages by RT-PCR. M, marker. C, positive control (genomic DNA). H_20, negative control. nn, neonates. pn, postnatal.

4.3 Body weight gain

To detect a possible effect on body weight gain of postnatally elevated levels of circulating IGF-II in the absence of IGF-I, selected litters were weighed daily from day 3 to 4 after birth until day 45. From this date on, body weight was recorded twice a week until day 60. The average growth of individual groups was estimated by combining data from males and females and transforming it to a weighing day of n x 3 by linear interpolation (as described in chapter 3.6).

In total, the data of 100 animals was analyzed distributed to the genotypes as follows: 10 [I+/+ IIwt], 21 [I+/+ IItg], 20 [I+/- IIwt], 18 [I+/- IItg], 14 [I-/- IIwt] and 17 [I-/- IItg].

Regardless of the presence of the PEPCK-IGF-II transgene, the homozygous IGF-I knockout animals displayed a body weight at day 3-4 after birth of ~50% as compared to their [I+/+] littermates. Figure 4-3 shows a six-day-old litter. Compared to their littermates, homozygous IGF-I deficient pups are not only significantly smaller but also display some developmental delay (e.g. a postponed emerging of the hair fibres through the epidermis and opening of the eyes).

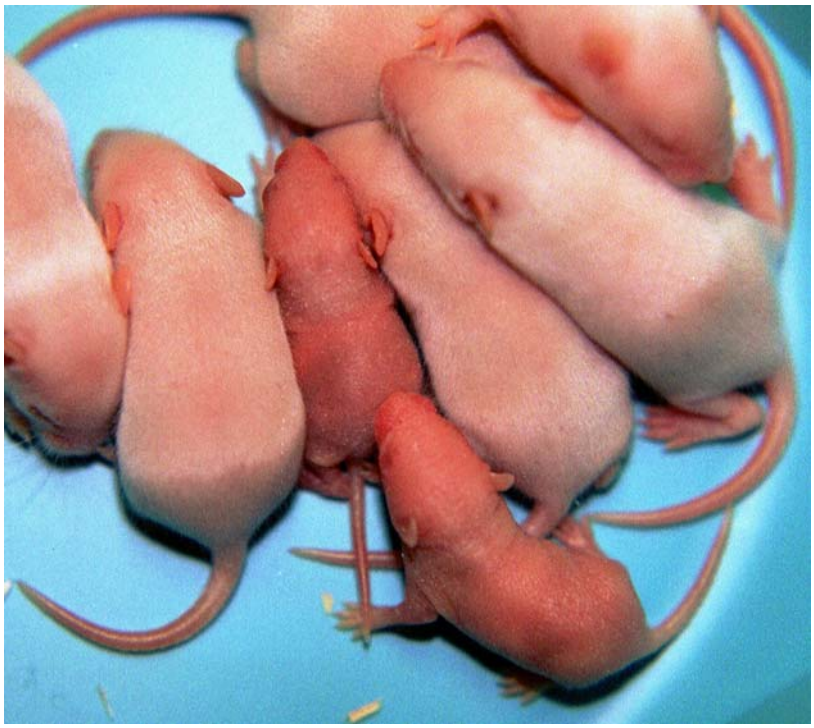


Figure 4-3: Six-day-old litter. Independent of the IGF-II transgene, homozygous IGF-I deficient pups are significantly smaller and are not synchronous in development compared to their control-littermates.

The postnatal growth curves in figure 4-4 indicated that in comparison with wildtype littermates [I-/- IIwt] and [I-/- IItg] mice continued to grow at a retarded rate after birth and reached ~38% of normal weight. The growth curves of [I-/- IIwt] and [I-/- IItg] mice are nearly identical, demonstrating that in the absence of IGF-I, elevated levels of circulating IGF-II have no effect on body weight gain. Also between [I+/- IIwt] and [I+/- IItg] animals no statistically significant difference in the increase in weight could be found. However, a slightly significant difference ($p<0.05$) in body weight gain could be seen between [I+/+ IIwt] and [I+/+ IItg] mice on day 12 to 24 and 39 to 45.

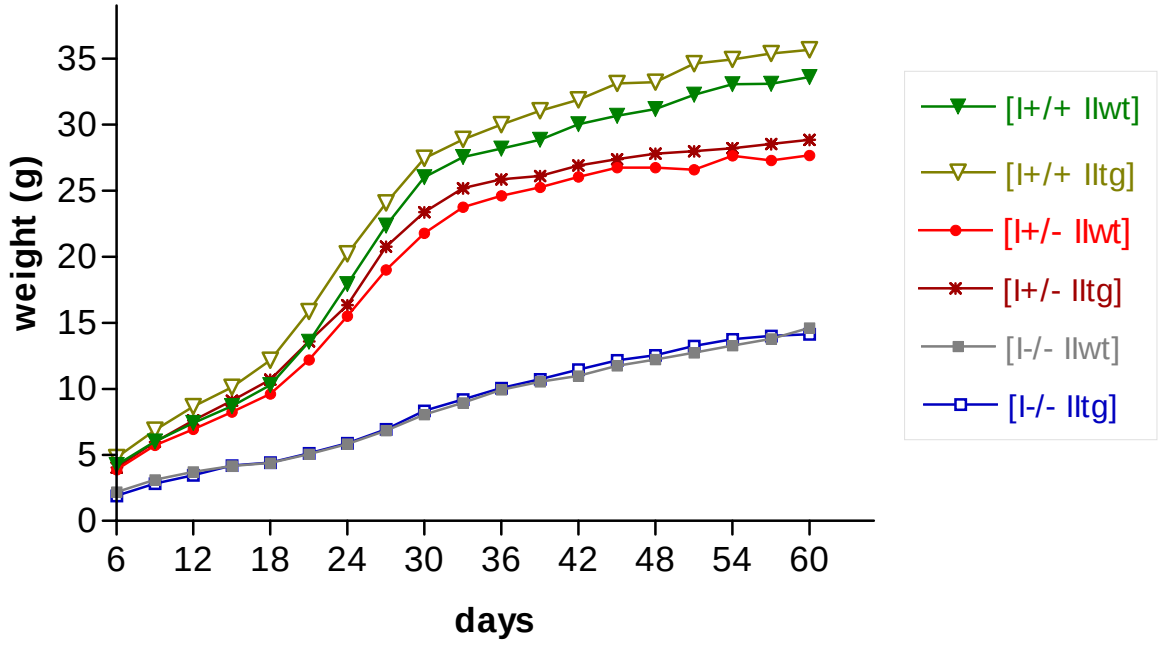


Figure 4-4: Growth curve from the early postnatal period to day 60. The data are presented as mean ± SD.

Figure 4-5 displays three female littermates at an age of 60 days. Whereas the wildtype littermate (left) is significantly taller compared to the two homozygous IGF-I deficient mice, no difference in body size can be seen between [I-/- IIwt] (middle) and [I-/- IItg] (right) mice, thus resembling the findings of the growth curve.

Figure 4-5: Three female littermates at an age of 60 days. Genotypes from left to right [$I_{+/+}$ IIwt], [I-/- IIwt], and [I-/- IItg].

4.4 Organ weight analysis

Organ weight, carcass weight and nose-rump length were determined at the age of 8 weeks. Since the focus of this work was to investigate the effect of postnatally elevated IGF-II in the absence of IGF-I, we excluded the animals heterozygous for the IGF-I mutation in further analysis. The following statistics were based on a total number of 46 animals distributed as follows: 10 [I+/+ IIwt], 13 [I+/+ IItg], 21 [I-/- IIwt] and 22 [I-/- IItg].

In table 4-3 and 4-4 the absolute and relative organ weights of [I-/- IIwt] animals compared to [I-/- IItg], and [I+/+ IIwt] versus [I+/+ IItg] are displayed. As shown in previous studies (Wolf et al., 1994), a significant difference in the absolute weight of kidneys ($p<0.01$) and adrenal glands ($p<0.05$) between [I+/+ IIwt] and [I+/+ IItg] mice was found. Furthermore, a significant difference ($p<0.05$) of the absolute weight of the lungs was observed between these groups. Except for these three organs (shown again in Figure 4-6), no effect of the IGF-II transgene could be detected in the absolute weight of individual organs.

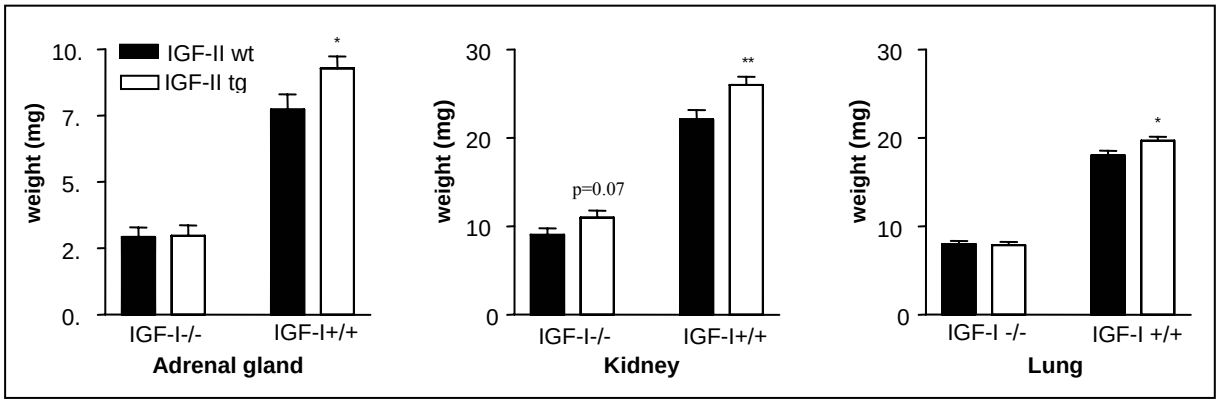


Figure 4-6: Absolute weights of the adrenal glands, kidneys and lungs in 60-day-old mice. Analysis of variance was used to evaluate statistically significant differences between indicated groups (*: $p<0.05$; **: $p<0.01$).

No significant differences could be detected when the relative organ weight of kidneys, lungs and adrenal glands of [I+/+ IIwt] and [I+/+ IItg] mice were compared.

The comparison of absolute kidney weight in [I-/- IIwt] and [I-/- IItg] mice revealed an increase which, however, did not reach statistical significance ($p=0.07$). When the relative weight of this organ was compared, a significant difference was found ($p<0.01$).

A	Weight (mg)			Weight (mg)		
Organs	[I-/- IIwt] mean ± SD	[I-/- IItg] mean ± SD	Δ	[I+/+ IIwt] mean ± SD	[I+/+ IItg] mean ± SD	Δ
Brain	311.57 ± 5.93	304.83 ± 8.19	-	458.20 ± 6.32	458.99 ± 5.73	-
Heart	105.45 ± 4.41	103. 01 ± 4.58	-	201.17 ± 6.46	188.40 ± 5.71	-
Lungs	79.84 ± 3.56	78.59 ± 3.70	-	180.20 ± 5.21	196.79 ± 4.60	*
Liver	987.50 ± 46.20	927.97 ± 47.96	-	1881.75 ± 67.63	1820.67 ± 59.73	-
Kidneys	90.28 ± 7.39	109.91 ± 7.68	-	220.77 ± 10.82	259.90 ± 9.56	**
Adrenal glands	2.94 ± 0.35	2.98 ± 0.39	-	7.74 ± 0.56	9.29 ± 0.45	*
Spleen	67.47 ± 3.98	65.99 ± 4.13	-	127.47 ± 5.82	124.15 ± 5.14	-
Pancreas	94.36 ± 5.03	92.65 ± 5.10	-	244.44 ± 7.12	236.45 ± 6.29	-
Carcass (g)	4.59 ± 0.16	4.75 ± 0.17	-	12.22 ± 0.23	12.31 ± 0.21	-
Body weight (g)	13.51 ± 0.46	13.82 ± 0.48	-	32.98 ± 0.68	33.30 ± 0.60	-
NRL (cm)	7.18 ± 0.08	7.14 ± 0.09	-	10.25 ± 0.12	10.21 ± 0.11	-

B	% Body weight			% Body weight		
Organs	[I-/- IIwt] mean ± SD	[I-/- IItg] mean ± SD	Δ	[I+/+ IIwt] mean ± SD	[I+/+ IItg] mean ± SD	Δ
Brain	2.46 ± 0.062	2.32 ± 0.085	-	1.41 ± 0.066	1.42 ± 0.060	-
Heart	0.78 ± 0.018	0.74 ± 0.018	-	0.61 ± 0.026	0.57 ± 0.023	-
Lungs	0.60 ± 0.012	0.57 ± 0.013	-	0.55 ± 0.018	0.59 ± 0.016	-
Liver	7.32 ± 0.234	6.74 ± 0.243	-	5.66 ± 0.342	5.44 ± 0.302	-
Kidneys	0.67 ± 0.024	0.79 ± 0.025	**	0.66 ± 0.035	0.76 ± 0.031	-
Adrenal glands	0.02 ± 0.002	0.02 ± 0.002	-	0.02 ± 0.003	0.03 ± 0.003	-
Spleen	0.50 ± 0.021	0.48 ± 0.022	-	0.40 ± 0.031	0.38 ± 0.027	-
Pancreas	0.70 ± 0.031	0.68 ± 0.031	-	0.75 ± 0.044	0.72 ± 0.038	-
Carcass	34.21 ± 0.704	34.73 ± 0.731	-	37.12 ± 1.031	37.06 ± 0.910	-
NRL (cm/g $^{1/3}$)	3.05 ± 0.028	3.01 ± 0.030	-	3.24 ± 0.041	3.22 ± 0.036	-

Table 4-3: Absolute (A) and relative (B) organ weights of [I-/- IIwt] compared to [I-/- IItg] mice and [I+/+ IIwt] compared to [I+/+ IItg] mice. Analysis of variance was used to determinate the absence (-) or presence (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$) of a statistically significant difference (Δ) between IGF-II wildtype (IIwt) and transgenic (IItg) mice.

Organ		I	II	Sex	I*II	I*II*Sex
		F	F	F	F	F
Brain	a	517.31 ***	0.20	1.35	0.32	0.04
	r	200.81 ***	0.88	41.08 ***	1.02	0.94
Heart	a	285.79 ***	2.02	34.99 ***	0.93	0.74
	r	61.75 ***	2.94	0.22	0.00	0.86
Lungs	a	639.25 ***	3.15	15.03 ***	4.26 *	1.36
	r	0.79	0.52	9.21 **	4.76 *	0.65
Liver	a	253.89 ***	1.16	57.21 ***	0.00	4.02 *
	r	27.12 ***	1.96	4.66 *	0.42	1.94
Kidneys	a	244.20 ***	10.72 **	97.83 ***	1.18	16.30 ***
	r	0.37	13.14 ***	45.32 ***	0.20	4.96 **
Adrenal glands	a	157.33 ***	3.21	75.87 ***	2.90	16.66 ***
	r	4.83 *	1.63	66.50 ***	2.23	5.12 **
Spleen	a	149.66 ***	0.25	1.50	0.04	3.84 *
	r	16.37 ***	0.71	1.28	0.00	3.77 *
Pancreas	a	610.05 ***	0.66	21.84 ***	0.28	2.08
	r	1.27	0.46	0.32	0.00	0.47
Carcass	a	1529.77 ***	0.42	49.69 ***	0.04	10.80 ***
	r	9.60 **	0.06	6.63 *	0.14	0.46
NRL	a	958.11 ***	0.19	7.78 **	0.00	0.22
	r	34.30 ***	0.57	9.58 **	0.06	1.32
Body weight	a	1197.33 ***	0.32	77.93 ***	0.00	7.89 ***

Table 4-4: Effects of IGF-I, IGF-II, sex and interactions between these factors on absolute and relative organ weights, nose-rump-length and body weight. Analysis of variance was used to evaluate effects of IGF-I (I; +/+ vs. -/-), IGF-II (II; wt vs. tg), sex and the various interactions of these factors on absolute (a) and relative (r) organ weights, nose-rump-length (NRL) and body weight. F values and their level of significance (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$) are shown.

4.5 Analysis of geometric and structural bone parameters

Geometric and structural bone parameters of 53 animals were investigated: 12 (7♀, 5♂) [I+/+ IIwt], 12 (5♀, 7♂) [I+/+ IItg], 12 (6♀, 6♂) [I-/- IIwt], and 17 (6♀, 11♂) [I-/- IItg].

At the femoral metaphysis, a predominantly trabecular bone site, the pQCT analyses showed slightly higher total volumetric BMD in male and female IGF-I knockout mice ([I-/- IIwt], [I-/- IItg]) relative to wildtype controls ([I+/+ IIwt], [I+/+ IItg]). In contrast, trabecular BMD was strongly reduced by 52% and 37% in male and female IGF-I knockout mice, respectively. Total cross-sectional area of the bone slices was reduced by approximately 70% in male and female IGF-I-ablated mice ([I-/- IIwt], [I-/- IItg]). Female mice had generally higher total but not trabecular BMD and lower cross-sectional area compared with male mice at the distal femoral metaphysis. At the femoral shaft, IGF-I-ablated mice ([I-/- IIwt], [I-/- IItg]) showed a pronounced reduction in bone size but unchanged total volumetric BMD compared with wildtype mice ([I+/+ IIwt], [I+/+ IItg]). Similar to the distal metaphysis, bone size of the shaft was reduced in female relative to male mice (Table 4-5).

At both the distal femoral metaphysis and the femoral shaft, 3-way factorial ANOVA revealed significant interactions between sex and IGF-I, i.e., the bone phenotype of male IGF-I knockout mice was more severe than that of female IGF-I knockouts relative to sex-matched wildtype controls (Table 4-5). However, most importantly, the presence or absence of the IGF-II transgene failed to show any significant effect on any of the geometric or BMD parameters in wildtype mice or in mice lacking IGF-I.

The pQCT findings were confirmed by bone histology. Femurs of IGF-I-ablated mice ([I-/- IIwt], [I-/- IItg]) were much smaller in size, and showed less cancellous bone together with reduced cortical bone thickness in comparison with wildtype controls ([I+/+ IIwt], [I+/+ IItg]) (Figure 4-7). Taken together, our findings confirm previous reports by other groups (Mohan et al., 2003; Wang et al., 2004) of an important role of IGF-I in bone growth and in cancellous bone homeostasis. In the presence or absence of IGF-I, an increase of the circulating levels of IGF-II was without effect on the skeleton in our mouse model and could not substitute for the skeletal functions of IGF-I in IGF-I-ablated mice.

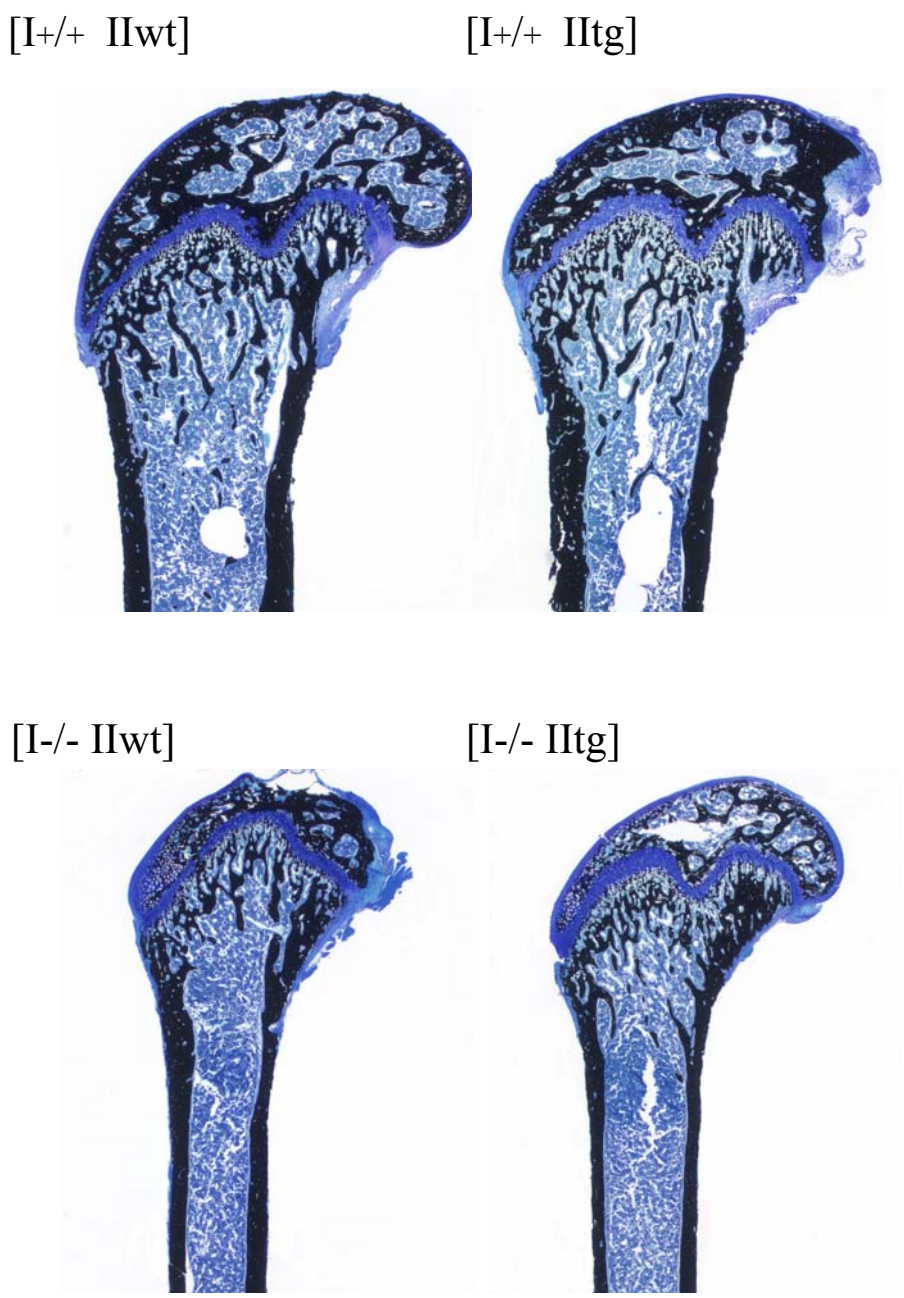


Figure 4-7: Histological sections of the femur stained with von Kossa/McNeal in 8-week-old mice. No histological differences could be detected when animals carrying the IGF-II transgene were compared to their control groups (IGF-I intact or deficient).

Variable		Genotype				3-way ANOVA					
		[I+/+ IIwt]	[I+/+ IItg]	[I-/- IIwt]	[I-/- IItg]	I	II	Sex	I * II	I * Sex	II * Sex
Femoral metaphysis											
Total BMD (mg/cm³)	Males	530±74	508±59	542±35	540±40	*	-	**	-	-	-
	Females	567±20	553±41	619±72	599±63						
Trabecular BMD (mg/cm³)	Males	297±46	276±45	144±45	156±21	***	-	-	-	*	-
	Females	260±36	260±41	163±27	193±40						
Cross-sectional area (mm²)	Males	5.32±0.46	5.09±0.32	1.53±0.24	1.71±0.32	***	-	***	-	**	-
	Females	4.45±0.26	4.42±0.49	1.38±0.24	1.47±0.20						
Femoral shaft											
Total BMD (mg/cm³)	Males	760±61	763±55	742±28	741±89	-	-	*	-	-	-
	Females	725±34	704±34	718±54	715±74						
Cross-sectional area (mm²)	Males	2.56±0.30	2.49±0.24	0.66±0.09	0.72±0.10	***	-	***	-	**	-
	Females	2.18±0.21	2.09±0.20	0.61±0.07	0.61±0.07						
Cortical area (mm²)	Males	1.42±0.25	1.40±0.15	0.39±0.05	0.43±0.08	***	-	***	-	**	-
	Females	1.17±0.12	1.09±0.13	0.36±0.05	0.35±0.03						
Cortical thickness (mm)	Males	0.30±0.04	0.30±0.02	0.16±0.01	0.18±0.03	***	-	***	-	*	-
	Females	0.27±0.02	0.25±0.02	0.16±0.02	0.15±0.01						

Table 4-5: Bone mineral density and geometric variables of the femur in 8-week-old mice. Analysis of variance was used to evaluate statistically significant differences between indicated groups (*: $p<0.05$; **: $p< 0.01$; ***: $p<0.001$). All values are means ± SD. BMD, bone mineral density. I, IGF-I. II, IGF-II.

4.6 Western ligand blot of IGFBPs

A Western ligand blot was used to evaluate the serum levels of IGFBPs in [I-/- IIwt], [I-/- IItg], [I+/+ IIwt], and [I+/+ IItg] mice (Figure 4-8).

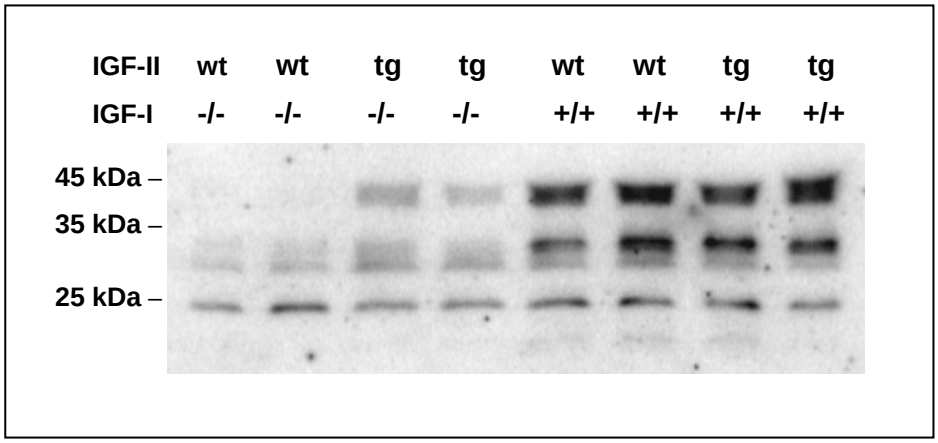


Figure 4-8: Western ligand blot analysis from serum samples using [125]IGF-II as a tracer.

While IGFBP-1 (~30 kDa) and IGFBP-4 (~24 kDa) levels were similar in all four groups, the levels of IGFBP-2 (~32 kDa) and IGFBP-3 (~39/43 kDa) varied significantly between the different groups. In [I-/- IIwt] the levels of IGFBP-2 (~32 kDa) and IGFBP-3 (~39/43 kDa) were strongly reduced but in the presence of IGF-II ([I-/- IItg]) it was partially restored. However, the values were still less than in the IGF-I wildtype animals ([I+/+ IIwt] and [I+/+ IItg]). A semiquantitave evaluation of the IGFBPs is shown in figure 4-9.

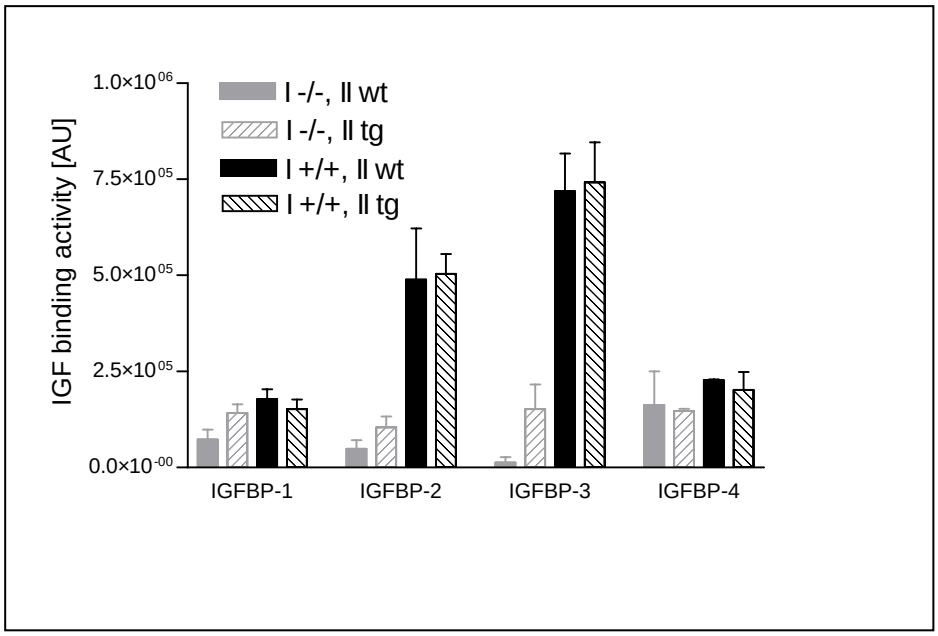


Figure 4-9: Semiquantitative evaluation of the individual IGFBPs presented in Figure 4-8.

4.7 Western immunoblot for growth hormone (GH)

Homozygous IGF-I deficient mice are known to have increased levels of GH. To demonstrate that the lack of an IGF-II effect on growth in our animals was not due to a loss of these elevated GH levels, a GH-Western immunoblot performed. We evaluated a large number of [I-/- IIwt] and [I-/- IItg] animals and observed the presence of extremely high GH levels in individual animals of both groups. Figure 4-10 shows a representative result using 1 µl serum of [I-/- IIwt], [I-/- IItg] and [I+/+ IIwt] mice. 4 ng of recombinant GH were used as a positive control.

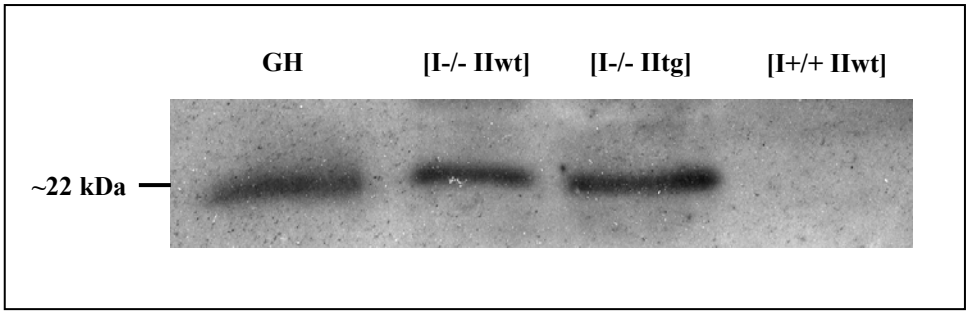


Figure 4-10: Growth hormone Western immunoblot. 4 ng recombinant GH were used as a positive control (first lane).

Thus, [I-/- IItg] animals still have elevated GH levels in spite of postnatally elevated IGF-II levels. This suggests that IGF-II cannot exert a similar negative feedback mechanism as IGF-I on GH release. The average GH levels in blood serum of wildtype mice were beyond the detection level for this assay.

5 Discussion

5.1 Generation of double-mutant mice

As described in chapter 4.1, we generated [I-/- IIwt] and [I-/- IItg] mice by a mating scheme in two steps. According to the Mendelian rules, the expected genotype distribution for [I-/- IIwt] and [I-/- IItg] animals would have been 12.5 % for each genotype. In this study the obtained genotype distribution was 5.3 % for [I-/- IIwt] and 6.8 % for [I-/- IItg] mice, indicating a mortality rate (before weaning) of 57.6 % and 46.6 %, respectively. A previous study of these homozygous IGF-I deficient mice on an intercross (129/Sv x C57BL/6) as well as on a C57BL/6J (N4) background in two different lines revealed a mortality rate of more than 95% (Powell Braxton et al., 1993). Liu et al. (1993) demonstrated that the survival frequency of homozygous IGF-deficient mice is dependent on their genetic background. On a 129/Sv x 129/Sv and a C57BL/6J x 129/Sv background they obtained a survival rate of 10 % and 16 %, respectively. On a MF1 x 129/Sv background the survival rate increased to 68 %, indicating that the intercrossing of an outbred strain (MF1) significantly decreases the mortality rate of homozygous IGF-I knockout mice (Liu et al., 1993).
This is consistent with our findings. First approaches in our lab to generate IGF-I deficient mice by mating of heterozygous animals ([I+/-] x [I+/-]) on the original genetic background revealed a mortality rate of homozygous IGF-I deficient mice of ~94 % (data not shown). On a 75 % NMRI outbred background the mortality rate of [I-/- IIwt] mice decreased to 57.6 %.
These findings were not really surprising since it is well known that outbred strains due to the higher level of heterozygosity have longer life spans, higher disease resistance earlier fertility and higher overall reproductive performance and most important a lower neonatal mortality as compared to inbred strains (Linder and Davisson, 2004).

The severe effects of the lack of IGF-I on the fertility of [I-/-] mice have been previously described (Baker et al., 1993) and include different alterations for both sexes. Female [I-/-] possess an infantile uterus and fail to ovulate. Their serum estradiol levels are reduced to 52.6 % of normal. Male mutants have reduced testis size and vas deferens of the epididymal duct; seminal vesicles as well as prostate are vestigal. Moreover, their levels of spermatogenesis are reduced and the serum concentration of testosterone is only 18 % of normal. Both sexes of [I-/-] mice lack libido and are infertile.

Although we did not evaluate the fertility of the different groups systematically, several [I-/-] animals from either genders, carrying or not the IGF-II transgene, were maintained for several weeks in cages containing fertile animals of the opposite sex. We neither observed mating behaviour nor obtained a pregnancy or litter where one of the parents were [I-/- IItg]. Therefore, although we cannot be sure that elevated levels of circulating IGF-II have no effect on the reproductive organs, we are able to conclude that it does not restore the fertility in IGF-I knockout mice.

5.2 Expression analysis

5.2.1 Circulating levels of IGF-I and IGF-II

Previous reports already demonstrated that the serum levels of IGF-I are intermediate in heterozygous and below detection level in homozygous IGF-I deficient mice harbouring a deletion of the gene (Powell-Braxton et al., 1993). Reduced levels of this growth factor were also described for a 15-year old patient carrying a homozygous partial deletion of the *IGF1* gene (Woods et al., 1996).

This is consistent with our findings. Plasma IGF-I levels measured by RIA in 60-day-old mice were below detection levels in [I-/- IIwt] and [I-/- II tg] mice. Intermediate levels were present in animals harbouring only one intact IGF-I allele ([I+/- IIwt], [I+/- IItg]) and the highest values were found in mice with two intact IGF-I alleles ([I+/+ IIwt], [I+/+ IItg]).

Animals harbouring the PEPCK-IGF-II transgene ([I-/- IItg], [I+/- IItg], and [I+/+ IItg]) displayed a significant increase in serum IGF-II level when compared to their wildtype counterparts ([I-/- IIwt], [I+/- IIwt], and [I+/+ IIwt]). Thus, radioimmunoassays for serum levels of IGF-I and IGF-II demonstrated the expected expression of the two peptides, reflecting the genotype.

Moreover, it was shown that the presence of the IGF-II transgene resulted in reduced levels of IGF-I in the groups [I+/- IItg] and [I+/+ IItg]. This has also been observed in a previous study on heterozygous PEPCK-IGF-II transgenic mice, which led to the assumption that the negative relationship between serum IGF-II and IGF-I levels might be due to displacement by IGF-II of IGF-I from IGFBPs (mainly the IGF/IGFBP-3/ALS-complex) leading to a rapid clearance of the growth factor because of the short half-life of free IGFs (Wolf et al., 1994).

5.2.2 Onset of PEPCK-IGF-II expression

Cytosolic phosphoenolpyruvate kinase (PEPCK) is a pace-setting enzyme for gluconeogenesis. The gene coding for PEPCK is expressed in many tissues and regulated developmentally as well as in a complex tissue-specific manner (Eisenberger et al., 1992; Short et al., 1992). Expression of the PEPCK gene starts in the kidney several days before birth and in the liver at birth (Eisenberger et al., 1992). Studies of mice harbouring a bovine GH gene controlled by a 460 bp of the rat PEPCK promoter demonstrated that the PEPCK/bGH gene was not transcribed in the liver of fetal mice until immediately before birth (embryonic stage 19). Transcription increased about 200-fold after birth (McGrane et al., 1990).

Previous studies of adult PEPCK-IGF-II transgenic mice revealed expression of this transgene in liver, kidney and several parts of the gut. In order to determine the onset of PEPCK-IGF-II expression at the major expression-sites, we performed an RT-PCR of total RNA from liver and kidneys of embryos at day 17.5 and 19.5 as well as of pups at day 4 postnatal. Transgene expression of PEPCK-IGF-II in the liver could be detected from embryonic stage 19.5 onwards. On day 4 postnatal the obtained signal was visibly stronger. These findings are consistent with the previous findings in PEPCK-bGH transgenic mice (McGrane et al., 1990).

Contrasting to the findings in the liver, PEPCK-IGF-II transgene expression could not be detected in the kidney in any of the investigated stages. A possible explanation for this difference is a postponed onset of expression in this organ. This may result from differences in the specific features of the PEPCK-IGF-II construct which included different heterologous intron and terminating sequences (Wolf et al., 1994).

However, the onset of the PEPCK-IGF-II transgene expression coincides with the time point when the expression of endogenous IGF-II is shut down in almost every tissue of the mouse. This specific expression pattern resulted to be very useful for the purpose of our investigation.

5.3 Body weight gain

To investigate the effects of postnatally elevated IGF-II on body weight gain, we recorded the body weight of selected litters daily starting from day 3 to 4 after birth. We did not start our weight records immediately after birth to avoid a further increase of the mortality rate of homozygous IGF-I deficient mice around this time. It is known that the handling of newborn

pups may cause maternal aggression and thus, increase the danger of the mother attacking the pups and killing or eating them (Poole, 1987).

Statistical analysis of the obtained data revealed that, regardless of the presence of the PEPCK-IGF-II transgene, mice harbouring a deletion of the *Igf1* gene exhibit a decreased postnatal growth rate. Homozygous ([I-/- IIwt], [I-/- II tg]) and heterozygous ([I+/- IIwt], [I+/- IItg]) IGF-I deficient mice displayed a significantly reduced weight as compared to IGF-I wildtype animals ([I+/+ IIwt], [I+/+ IItg]). When [I-/- IIwt] and [I-/- II tg] mice were compared to [I+/- IIwt] and [I+/- IItg] animals, also a significant reduction in body weight could be shown for the homozygous IGF-I knockout mice. However, visible consequences of the reduced body weight gain only could be observed in homozygous IGF-I deficient “dwarfs”. These findings are consistent with the initial description of the IGF-I knockout mice which were employed for our experimental design (Powell-Braxton et al., 1993).
Comparison of [I-/- IIwt] with [I-/- II tg] and [I+/- IIwt] with [I+/- IItg] mice demonstrated that elevated levels of IGF-II have no significant influence on body weight gain. Thus, an increase in the levels of circulating IGF-II is not able to rescue growth deficits in mice with a heterozygous or homozygous deletion of the IGF-I allele in the postnatal period.

In a previous study of PEPCK-IGF-II animals, a slight (even though not significant) increase in body weight could be observed in the IGF-II transgenic animals when compared to their wildtype controls (Wolf et al., 1994). This tendency could also be observed in our study. Moreover, the weight difference between [I+/+ IIwt] and [I+/+ IItg] animals reached statistical significance on day 12 to 24 and 39 to 45. A possible explanation for this observation is the different genetic background of the mouse strain employed in each study.

5.4 Organ weight analysis

IGF-I is well known to play an important role not only in fetal development but also in postnatal growth (reviewed in Efstradiatis, 1998). Postnatally elevated serum levels of IGF-II have already been shown to exert subtle time-specific effects on organ growth (Wolf et al., 1994). The initial description of the PEPCK-IGF-II transgenic mouse model employed in our study revealed a significantly greater kidney weight of the transgenic animals at the age of 4 weeks as compared to age-matched controls. At an age of 12 weeks, no significant difference in kidney weight could be observed but transgenic mice displayed significantly higher

weights of the adrenal glands than controls. Consistent with these findings, we observed that the absolute weight of kidneys and adrenal glands in our [I+/+ IItg] mice was significantly increased at an age of 8 weeks when compared to their controls ([I+/+ IIwt]). The effects of elevated IGF-II on the growth of kidneys and adrenal glands have been already comprehensively discussed in Wolf et al. (1994). Moreover, our studies indicate an additional effect of IGF-II on the lung, since we could detect a significant increase of the absolute weight in this organ. A possible explanation for this finding would be the different genetic background of the mice used in this study. Anyway, this is a very interesting finding which deserves further investigation. Specially, stereological analysis of this organ would help to clarify the specific sites of the size increase.
Interestingly, the relative organ weight of adrenal glands, kidneys and lungs was not elevated in the [I+/+ IItg] animals. The most probable explanation for this finding is that in the calculation of the relative organ weights, the significant increase of weight of these organs vanished due to the observed slight increase in body weight of [I+/+ IItg] mice.

Evaluation of the organ weight of the [I-/- IIwt] and [I-/- II tg] mice revealed no significant difference of the absolute organ weight between these two groups. Contrasting to this finding, the relative organ weight of the adrenal glands was significantly increased in the [I-/- II tg] animals. This is apparently intriguing but can be explained by the fact that the absolute weight of the kidneys was already increased in [I-/- IItg] mice but only reached borderline significance (p=0.07).

5.5 Bone parameters

The IGF system is thought to play an important role in bone physiology, especially in the regulation of BMD. In fact, the IGFs are profusely produced by osteoblasts and are the most abundant growth factors stored in bone (Yakar and Rosen 2003). To complement the analysis of growth presented here (based mainly on body and organ weights), we examined the geometric and structural properties of a long bone (femur), a system in which the IGF system has been extensively studied. pQCT analyses revealed an array of significant differences between [I-/-] and [I+/+] animals, regardless of the presence of the IGF-II transgene. Most of these alterations have been already described in previous studies (see below). The most obvious phenotype was a generalized reduction in bone size. In addition, at the femoral metaphysis, IGF-I knockout mice displayed a moderately higher total volumetric BMD but

strongly reduced trabecular BMD and cross-sectional area of bone slices. At the femoral shaft, total volumetric BMD was unchanged. Interestingly, 3-way factorial ANOVA identified significant interactions between sex and IGF-I (when compared to sex-matched wildtype control animals, bone parameters of male IGF-I knockout mice were more severely affected than those of females). This is a remarkable finding considering the strongly reduced levels of circulating sex hormones (Baker et al., 1996). Histological analyses of the femur confirmed these findings. In addition to a reduction in size, IGF-I knockout mice showed less cancellous bone and reduced cortical thickness when compared to control animals. More importantly, however, elevated circulating levels of IGF-II in [I-/- IItg] and [I$^{+/+}$ IItg] animals failed to exert any significant effect on any of the evaluated parameters when compared to the respective control groups.

Smaller bones and delayed ossification were among the first alterations observed in IGF-I knockout mice (Liu et al., 1993; Baker et al., 1993; Powell-Braxton et al., 1993). Further studies revealed, among other effects, a reduction in the size of hypertrophic chondrocytes in the femur (Lupu et al., 2001), in tibial bone formation rate (Bikle et al., 2001) and in femoral BMD (Mohan et al., 2003). In accordance with these data, overexpression of IGF-I in osteoblasts increased peak BMD (Zhao et al., 2000). Recently, Wang et al. (2004) compared the size and growth plate parameters in homozygous IGF-I deficient and homozygous growth hormone receptor knockout (GHR) mice. Animals lacking GHR displayed a stronger reduction in bone size and more severe effects in the growth plate as compared to the alterations observed in mice lacking IGF-I. Based on the observation that IGF-II is expressed in the germinal and proliferative zones of the growth plate (Shinar et al., 1993; Wang et al., 1995), the authors hypothesized that the effects of GH on chondrocyte generation and proliferation may be mediated by GH-induced IGF-II expression. In fact, the authors were able to measure increased levels of IGF-II in the growth plate of IGF-I knockout mice and reduced levels of this growth factor in GHR knockout animals (Wang et al., 2004). Although we did not evaluate growth plate parameters, our results are not in agreement with this theory. This difference can have at least two explanations. First, the potential growth-promoting effects of IGF-II in the growth plate are not strong enough to result in an increase in the structural parameters examined. Second, the growth-promoting effects of IGF-II in the growth plate are mediated exclusively by locally produced IGF-II, which may not be elevated in our experimental model.

5.6 Western ligand blot of IGFBPs

The six IGF-binding proteins (IGFBP-1 to -6) are expressed in a tissue-specific manner and act as modulators of IGF activity. Western ligand blot analyses represent a well-established method to detect bioactive IGFBPs in blood serum (Hossenlopp et al., 1986). Evaluation of the serum levels of IGFBPs in [I-/- IIwt], [I-/- IItg], [$I^{+/+}$ IIwt], and [$I^{+/+}$ IItg] animals revealed that the levels of IGFBP-1 and IGFBP-4 were similar in all four groups. This was not true for the levels of IGFBP-2 and IGFBP-3 which differed significantly between the groups. The levels of both binding proteins were strongly reduced in the [I-/- IIwt] mice but in the presence of IGF-II ([I-/- IItg]) their levels were partially restored. However, postnatally elevated levels of IGF-II in homozygous IGF-I deficient ([I-/- IItg]) mice were not able to completely compensate the lack of IGF-I, since their values of IGFBP-2 and -3 were still lower than in the IGF-I wildtype animals ([$I^{+/+}$ IIwt] and [$I^{+/+}$ IItg]).
These findings indicate that the IGFBPs are differentially regulated by IGF-I and IGF-II. While the IGFs may exert this effect by directly influencing the expression of these IGFBPs, a possible alternative explanation would be that they regulate the IGFBP levels by a post-translational mechanism, e.g. influencing their stability in blood circulation.

5.7 Growth hormone Western immunoblot

Both growth hormone and IGF-I are important for postnatal growth. GH partially mediates its growth promoting effect by upregulation of IGF-I expression in many tissues, especially the liver, but also exerts IGF-independent effects (Le Roith et al., 2001).
Growth hormone is synthesized and released from the anterior pituitary in a pulsatile manner. In wildtype mice the average value for GH in the blood serum is ~10 ng/ml for females and ~16 ng/ml for males (McLeod et al., 1991). IGF-I negatively regulates GH release through a feedback mechanism, inhibiting GH synthesis and release by the anterior pituitary. Lupu et al. (2001) already demonstrated that homozygous IGF-I deficient mice display increased levels of GH.
To exclude the possibility that the lack of an IGF-II effect on growth in our animals was due to a loss of these elevated GH levels, the blood serum of a large number of [I-/- IIwt], [I-/- IItg], and [$I^{+/+}$ IIwt] animals was evaluated by GH-Western immunoblot. For wildtype ([$I^{+/+}$ IIwt]) animals no signal could be detected. This was because the amount of GH in the wildtype serum was below detection limit of this assay. Moreover, it could be demonstrated

that, despite postnatally elevated IGF-II levels, [I-/- IItg] animals still have elevated GH levels.

Thus, the lack of growth effect of postnatally elevated levels of IGF-II in the absence of IGF-I is not caused by reduced levels of GH. Furthermore, these results suggest that circulating IGF-II cannot exert a similar negative feedback mechanism as IGF-I on GH release.

5.8 Final considerations

Our results support the notion that, in spite of a high degree of structural homology and a common signaling receptor, IGF-I and -II have distinct functions. This is particularly evident when we consider their role in animal growth (see Efstratiadis, 1998). During embryonic development when both growth factors are naturally present at high concentrations, one cannot compensate the growth impairment observed in the (experimentally induced) absence of the other one (knockout mice lacking IGF-I or -II are both born with a weight ~60% N). Previous studies involving administration of IGF-II or the generation of transgenic mice overexpressing this growth factor failed to show any effect on postnatal growth. We show that in mice, a species in which IGF-II expression is normally discontinued after birth, restoration of substantial circulating levels of this growth factor does neither stimulate body weight gain and skeletal development nor affects considerably the weight of individual organs even in the complete absence of IGF-I. The reasons for this striking observation can only be speculated on at the moment.

Since the actions of a growth factor obviously depend on its affinity to a specific receptor, an important point to be discussed concerns the affinity of the IGFs to the IGF-IR. Early studies suggested that the IGF-IR binds IGF-I with 10-30 times higher affinity than IGF-II (Heaton et al., 1984; Beguinot et al., 1985; Kiess et al., 1987; Ewton et al., 1987; Kadowaki et al., 1986). However, many subsequent reports indicate that the IGF-IR has an almost equal affinity for IGF-I and IGF-II (see, for instance, Steele-Perkins et al., 1988; Perdue et al., 1991). The reason for these differences may be related to the use of recombinant peptides of higher purity in latter studies but can also result from the presence of different receptor forms (discussed in Steele-Perkins et al., 1988). Species-specific differences may also play a role. Whatever the case may be, the IGFs can be considered to have a comparable affinity to the IGF-IR (Blakesley et al., 1996).

A much more probable explanation for our results is that, after activation of the IGF-IR, a different set of signaling pathways are initiated depending on which IGF activated the receptor. Little attention has been given to this issue by researchers so far but exactly this point may provide important clues for solving the “IGF-II enigma”.

6 Summary

Consequences of postnatal insulin-like growth factor II overexpression in insulin-like growth factor I deficient mice

Insulin-like growth factor I (IGF-I) and -II (IGF-II) are single chain peptides produced by many tissues, functioning in an endocrine, autocrine or paracrine fashion to regulate cellular proliferation, survival and differentiation. IGF actions are initiated upon binding to the insulin-like growth factor I receptor (IGF-IR) and are modulated through interactions with a family of six secreted IGF-binding proteins (IGFBP-1 to -6).
IGF-I is necessary for normal growth and differentiation during both, embryonic and postnatal development. IGF-II is a stimulator of fetal growth but its functions in the postnatal period are still unclear. Notably, expression of IGF-II is shut down shortly after birth in rodents (but not in humans).

Previous studies in phosphoenolpyruvate-carboxykinase (PEPCK)-IGF-II transgenic mice demonstrated that overexpression of IGF-II resulted in disproportionate growth of specific organs but a significant increase in body size was not observed. Homozygous IGF-I deficient mice were shown to be severely retarded in growth. The aim of this study was to test whether elevated levels of circulating IGF-II can rescue the dwarfism in IGF-I deficient mice and thereby function as a stimulator of postnatal growth in the absence of IGF-I.

For this purpose, we crossed heterozygous IGF-I deficient mice [$I^{+/-}$ IIwt] with heterozygous IGF-I deficient mice carrying PEPCK-IGF-II transgenes [$I^{+/-}$ IItg]. The resulting offspring comprised six different groups: homozygous IGF-I knockout and PEPCK-IGF-II wildtype mice [I-/- IIwt], homozygous IGF-I knockout and PEPCK-IGF-II transgenic mice [I-/- IItg], animals lacking one IGF-I allele and wildtype for the PEPCK-IGF-II transgene [$I^{+/-}$ IIwt], lacking one IGF-I allele and harbouring the PEPCK-IGF-II transgene [$I^{+/-}$ IItg], wildtype for the IGF-I mutation and carrying the PEPCK-IGF-II transgene [$I^{+/+}$ IItg], and completely wildtype [$I^{+/+}$ IIwt]. The genotype of all mice was determined by PCR.
Body weight of mice was recorded daily until the age of 8 weeks. The nose-rump length (NRL) and the weights of individual organs and of the carcass were recorded and the femurs and lumbar vertebras prepared for further investigations.

At an age of 8 weeks, mean serum concentrations of IGF-I were beyond detection level in [I-/- IIwt] and [I-/- IItg] mice, intermediate in [I+/- IIwt] and [I+/- IItg] animals and highest in [I+/+ IIwt] and [I+/+ IItg] mice. IGF-II levels were significantly increased in animals harbouring the PEPCK-IGF-II transgene ([I-/- IItg], [I+/- IItg], and [I+/+ IItg]) when compared to their wildtype counterparts ([I-/- IIwt], [I+/- IIwt], and [I+/+ IIwt]). This reflected the genotype, demonstrating the appropriateness of our experimental model.

Analysis of body weight data from day 3-4 after birth until day 60 revealed that in the absence of IGF-I, elevated levels of IGF-II have no effect on body weight gain. The same was found for the nose-rump length and the carcass. The weight of specific organs, however, was altered. Compared to the wildtype counterparts ([I-/- IIwt]), the relative kidney weight in [I-/- IItg] mice was significantly increased.

IGF-I is known to play an important role in bone growth and in cancellous bone homeostasis. Investigations of geometric and structural bone parameters showed that in the presence or absence of IGF-I, an increase in the circulating levels of IGF-II was without effect on the skeleton and could not substitute for the skeletal functions of IGF-I in IGF-I-ablated mice.

Homozygous IGF-I deficient mice are known to have elevated levels of growth hormone (GH). To demonstrate that the lack of effect on growth in our [I-/- IItg] animals was not due to a loss of these elevated GH-levels, a GH-Western immunoblot was performed, revealing that, despite elevated levels of IGF-II, increased levels of GH were still present in [I-/- IItg] animals.

Evaluation of the serum levels of IGFBPs by Western ligand blot analysis demonstrated that IGFBP-1 and IGFBP-4 levels were similar in all groups, whereas the levels of IGFBP-2 and IGFBP-3 were strongly reduced in [I-/- IIwt] animals. In the presence of IGF-II ([I-/- IItg]), they were partially restored but the amounts were still smaller than in the IGF-I wildtype animals ([I+/+ IIwt] and [I+/+ IItg]).

In summary, these results show that under our experimental conditions, IGF-II is not able to rescue the postnatal growth deficit of IGF-I knockout mice and apparently does not exert a negative feedback on the secretion of growth hormone. However, it could be demonstrated, that the IGFs have differentiated effects on the regulation of the expression/stability of individual IGFBPs.

7 Zusammenfassung

Konsequenzen dauerhaft erhöhter Spiegel des Insulin-ähnlichen Wachstumsfaktors II in Insulin-ähnlichen Wachstumsfaktor I defizienten Mäusen

Die in vielen Körpergeweben gebildeten Insulin-ähnlichen Wachstumsfaktoren I (IGF-I) und II (IGF-II) regulieren auf endokrinem, autokrinem oder parakrinem Weg Zellwachstum, -teilung und -tod. Ihre Wirkung vermitteln sie durch Bindung an die IGF-I-Rezeptoren. Im Blut zirkulieren sie meist gebunden an eines der sechs IGF-Bindungsproteine (IGFBP-1 bis -6), die ihre Halbwertszeit wie auch Verfügbarkeit für ihre Rezeptoren beeinflussen.
Sowohl in der embryonalen als auch postnatalen Entwicklung ist IGF-I von entscheidender Bedeutung für ein normales Wachstum. Zwar stimuliert auch IGF-II die vorgeburtliche Entwicklung, seine Funktionen nach der Geburt sind jedoch weitgehend unklar. Während bei Nagern die Expression von IGF-II kurz nach der Geburt eingestellt wird, können beim Menschen lebenslang hohe IGF-II-Spiegel im Blut nachgewiesen werden.

In früheren Untersuchungen PEPCK-IGF-II transgener Mäuse konnte gezeigt werden, dass eine Überexpression von IGF-II zu einem vermehrten Wachstum einzelner Organe führt, allerdings keine signifikanten Auswirkungen auf die Körpergröße hat. Bei homozygot IGF-I defizienten Mäusen hingegen war ein stark vermindertes Körperwachstum zu beobachten. Die vorliegende Studie sollte klären, ob erhöhte IGF-II-Spiegel im Blut die Zwergwüchsigkeit IGF-I defizienter Mäuse kompensieren und somit postnatales Wachstum in Abwesenheit von IGF-I stimulieren können.

Zu diesem Zweck wurden heterozygot IGF-I defiziente Mäuse [$I^{+/-}$ IIwt] mit heterozygot IGF-I defizienten / PEPCK-IGF-II transgenen Mäusen [$I^{+/-}$ IItg] verpaart. Aus dieser Kreuzung resultierten sechs verschiedene Gruppen: [I-/- IIwt], [I-/- IItg], [$I^{+/-}$ IIwt], [$I^{+/-}$ IItg], [$I^{+/+}$ IIwt] und [$I^{+/+}$ IItg] Mäuse. Der Genotyp sämtlicher Tiere wurde mittels PCR bestimmt und die Gewichtsentwicklung täglich bis zur achten Lebenswoche aufgezeichnet. Nach dem Tod der Tiere wurden Nasen-Rumpf-Länge, die Organgewichte und das Karkassengewicht ermittelt sowie die Oberschenkelknochen und Lendenwirbel für weitere Untersuchungen entfleischt.

Messungen der durchschnittlichen Serenspiegel für IGF-I und -II in acht Wochen alten Tieren zeigten, dass die ermittelten Werte den genetischen Status der Tiere widerspiegelten und somit die Eignung des eingesetzten Versuchsmodells bestätigten. Weder die statistische Analyse von Körper- und Karkassengewicht noch die der Nasen-Rumpf-Länge wies darauf hin, dass erhöhte IGF-II-Spiegel im Blut Gewicht und Wachstum in IGF-I defizienten Mäusen beeinflussen. Allerdings steigerte sich das relative Nierengewicht von [I-/- IItg] Mäusen im Vergleich zu ihren [I-/- IIwt] Kontrollen signifikant.

Frühere Studien genetisch veränderter Mäuse wiesen IGF-I als einen entscheidenden Faktor für Umfang, Dichte und Längenwachstum des Knochens aus. Die hier durchgeführten Untersuchungen verschiedener Knochenparameter verdeutlichten, dass unabhängig vom IGF-I-Status ein erhöhter Serumspiegel von IGF-II keine Wirkung auf das Skelett hat. IGF-II war also nicht in der Lage, die Funktionen von IGF-I in Bezug auf den Knochenstoffwechsel in IGF-I defizienten Mäusen zu übernehmen. Um auszuschließen, dass eine Reduzierung des bei [I-/- IIwt] Mäusen normalerweise stark erhöhten GH-Spiegels das mangelnde Wachstum in [I-/- IItg] Tieren bedingt, wurde ein GH-Western Immunoblot durchgeführt. Trotz eines erhöhten IGF-II-Spiegels blieb der GH-Spiegel in [I-/- IItg] Mäusen unverändert. Die Evaluierung der Serum-IGFBP-Spiegel mit Hilfe eines Western Ligandenblots ergab gleichbleibende IGFBP-1- und -4-Spiegel in allen Gruppen. Während in [I-/- IIwt] Tieren die IGFBP-2- und -3-Werte stark reduziert waren, steigerten sie sich in Anwesenheit von IGF-II ([I-/- IItg]), erreichten jedoch nicht das Niveau von Wildtyp IGF-I-Tieren ([$I^{+/+}$ IIwt] und [$I^{+/+}$ IItg]).

Unter den hier gegebenen experimentellen Bedingungen ist IGF-II nicht in der Lage, den postnatalen Zwergwuchs IGF-I defizienter Mäuse auszugleichen. Außerdem scheint IGF-II nicht die Ausschüttung des GH durch einen negativen Rückkopplungsmechanismus zu regulieren. Allerdings wurde eine unterschiedliche Wirkung der IGFs auf die Regulation der Expression / Stabilität der IGFBPs nachgewiesen.

8. Bibliography

Abbott, A. M., R. Bueno, M. T. Pedrini, J. M. Murray, and R. J. Smith, 1992: Insulin-like growth factor I receptor gene structure. *J Biol Chem*, **267,** 10759-63.

Baker, J., J. P. Liu, E. J. Robertson, and A. Efstratiadis, 1993: Role of insulin-like growth factors in embryonic and postnatal growth. *Cell*, **75,** 73-82.

Baker, J., M. P. Hardy, J. Zhou, C. Bondy, F. Lupu, A. R. Bellve, and A. Efstratiadis, 1996: Effects of an Igf1 gene null mutation on mouse reproduction. *Mol Endocrinol*, **10,** 903-18.

Ballard, F. J., M. Ross, F. M. Upton, and G. L. Francis, 1988: Specific binding of insulin-like growth factors 1 and 2 to the type 1 and type 2 receptors respectively. *Biochem J*, **249,** 721-6.

Barlow, D. P., R. Stoger, B. G. Herrmann, K. Saito, and N. Schweifer, 1991: The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature*, **349,** 84-7.

Baserga, R. and R. Rubin, 1993: Cell cycle and growth control. *Crit Rev Eukaryot Gene Expr*, **3,** 47-61.

Baumann, G., M. A. Shaw, and T. J. Merimee, 1989: Low levels of high-affinity growth hormone-binding protein in African pygmies. *N Engl J Med*, **320,** 1705-9.

Bautista, C. M., S. Mohan, and D. J. Baylink, 1990: Insulin-like growth factors I and II are present in the skeletal tissues of ten vertebrates. *Metabolism*, **39,** 96-100.

Baxter, R. C., S. Meka, and S. M. Firth, 2002: Molecular distribution of IGF binding protein-5 in human serum. *J Clin Endocrinol Metab*, **87,** 271-6.

Becker, K., S. Stegenga, and S. Conway, 1995: Role of insulin-like growth factor I in regulating growth hormone release and feedback in the male rat. *Neuroendocrinology*, **61,** 573-83.

Beguinot, F., C. R. Kahn, A. C. Moses, and R. J. Smith, 1985: Distinct biologically active receptors for insulin, insulin-like growth factor I, and insulin-like growth factor II in cultured skeletal muscle cells. *J Biol Chem*, **260,** 15892-8.

Berelowitz, M., M. Szabo, L. A. Frohman, S. Firestone, L. Chu, and R. L. Hintz, 1981: Somatomedin-C mediates growth hormone negative feedback by effects on both the hypothalamus and the pituitary. *Science*, **212,** 1279-81.

Bikle, D., S. Majumdar, A. Laib, L. Powell-Braxton, C. Rosen, W. Beamer, E. Nauman, C. Leary, and B. Halloran, 2001: The skeletal structure of insulin-like growth factor I-deficient mice. *J Bone Miner Res*, **16,** 2320-9.

Blair, H. T., S. N. McCutcheon, D. D. Mackenzie, J. E. Ormsby, R. A. Siddiqui, B. H. Breier, and P. D. Gluckman, 1988: Genetic selection for insulin-like growth factor-1 in growing mice is associated with altered growth. *Endocrinology*, **123,** 1690-2.

Blakesley, V. A. B., A.A., Koval, A.P.; Okubo, Y.; Le Roith, D., 1999: The IGF-I receptor function: Transducting the IGF-I signal into intracellular events. *Contemporary Endocrinology: The IGF System*, R. G. R. Rosenfeld, C.T., Ed., Humana Press, Inc.

Blakesley, V. A., A. Scrimgeour, D. Esposito, and D. Le Roith, 1996: Signaling via the insulin-like growth factor-I receptor: does it differ from insulin receptor signaling? *Cytokine Growth Factor Rev*, **7,** 153-9.

Blum, W., F. Gallaher, B. and M.B. Ranke, 1992: An IGFBP-blocked IGF-I RIA that measures what it pretends to measure: IGF-I. *74th Annual Meeting of the American Endocrine Society. 293.*

Blum, W. F., M. B. Ranke, and J. R. Bierich, 1988: A specific radioimmunoassay for insulin-like growth factor II: the interference of IGF binding proteins can be blocked by excess IGF-I. *Acta Endocrinol (Copenh)*, **118,** 374-80.

Boisclair, Y. R., R. P. Rhoads, I. Ueki, J. Wang, and G. T. Ooi, 2001: The acid-labile subunit (ALS) of the 150 kDa IGF-binding protein complex: an important but forgotten component of the circulating IGF system. *J Endocrinol*, **170,** 63-70.

Bol, D. K., K. Kiguchi, I. Gimenez-Conti, T. Rupp, and J. DiGiovanni, 1997: Overexpression of insulin-like growth factor-1 induces hyperplasia, dermal abnormalities, and spontaneous tumor formation in transgenic mice. *Oncogene*, **14,** 1725-34.

Bonapace, G., D. Concolino, S. Formicola, and P. Strisciuglio, 2003: A novel mutation in a patient with insulin-like growth factor 1 (IGF1) deficiency. *J Med Genet*, **40,** 913-7.

Bork, P., 1993: The modular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Lett*, **327,** 125-30.

Cattanach, B. M. and C. V. Beechey, 1990: Autosomal and X-chromosome imprinting. *Dev Suppl,* 63-72.

Chan, J. M., M. J. Stampfer, E. Giovannucci, P. H. Gann, J. Ma, P. Wilkinson, C. H. Hennekens, and M. Pollak, 1998: Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science*, **279,** 563-6.

Christofori, G., P. Naik, and D. Hanahan, 1994: A second signal supplied by insulin-like growth factor II in oncogene-induced tumorigenesis. *Nature*, **369,** 414-8.

Clemmons, D. R., 1997: Insulin-like growth factor binding proteins and their role in controlling IGF actions. *Cytokine Growth Factor Rev*, **8,** 45-62.

Cohick, W. S. and D. R. Clemmons, 1993: The insulin-like growth factors. *Annu Rev Physiol*, **55,** 131-53.

Da Costa, T. H., D. H. Williamson, A. Ward, P. Bates, R. Fisher, L. Richardson, D. J. Hill, I. C. Robinson, and C. F. Graham, 1994: High plasma insulin-like growth factor-II and low lipid content in transgenic mice: measurements of lipid metabolism. *J Endocrinol*, **143,** 433-9.

Daughaday, W. H., 2000: Growth hormone axis overview--somatomedin hypothesis. *Pediatr Nephrol*, **14,** 537-40.

Daughaday, W. H. and P. Rotwein, 1989: Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocr Rev*, **10,** 68-91.

Daughaday, W. H., I. E. Karl, and M. M. Karl, 1992: A failed assay opened a new door in growth hormone research. *Endocrinology*, **130,** 565-6.

Daughaday, W. H., K. A. Parker, S. Borowsky, B. Trivedi, and M. Kapadia, 1982: Measurement of somatomedin-related peptides in fetal, neonatal, and maternal rat serum by insulin-like growth factor (IGF) I radioimmunoassay, IGF-II radioreceptor assay (RRA), and multiplication-stimulating activity RRA after acid-ethanol extraction. *Endocrinology*, **110,** 575-81.

Daughaday, W. H., K. Hall, M. S. Raben, W. D. Salmon, Jr., J. L. van den Brande, and J. J. van Wyk, 1972: Somatomedin: proposed designation for sulphation factor. *Nature*, **235,** 107.

Davila, N., B. T. Shea, K. Omoto, M. Mercado, S. Misawa, and G. Baumann, 2002: Growth hormone binding protein, insulin-like growth factor-I and short stature in two pygmy populations from the Philippines. *J Pediatr Endocrinol Metab*, **15,** 269-76.

De Meyts, P., B. Wallach, C. T. Christoffersen, B. Urso, K. Gronskov, L. J. Latus, F. Yakushiji, M. M. Ilondo, and R. M. Shymko, 1994: The insulin-like growth factor-I receptor. Structure, ligand-binding mechanism and signal transduction. *Horm Res*, **42,** 152-69.

De Souza, A. T., T. Yamada, J. J. Mills, and R. L. Jirtle, 1997: Imprinted genes in liver carcinogenesis. *Faseb J*, **11,** 60-7.

DeChiara, T. M., A. Efstratiadis, and E. J. Robertson, 1990: A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature*, **345,** 78-80.

DeChiara, T. M., E. J. Robertson, and A. Efstratiadis, 1991: Parental imprinting of the mouse insulin-like growth factor II gene. *Cell*, **64,** 849-59.

Devedjian, J. C., M. George, A. Casellas, A. Pujol, J. Visa, M. Pelegrin, L. Gros, and F. Bosch, 2000: Transgenic mice overexpressing insulin-like growth factor-II in beta cells develop type 2 diabetes. *J Clin Invest*, **105,** 731-40.

DiGiovanni, J., D. K. Bol, E. Wilker, L. Beltran, S. Carbajal, S. Moats, A. Ramirez, J. Jorcano, and K. Kiguchi, 2000: Constitutive expression of insulin-like growth factor-1 in epidermal basal cells of transgenic mice leads to spontaneous tumor promotion. *Cancer Res*, **60,** 1561-70.

Donovan, S. M., L. C. Atilano, R. L. Hintz, D. M. Wilson, and R. G. Rosenfeld, 1991: Differential regulation of the insulin-like growth factors (IGF-I and -II) and IGF binding proteins during malnutrition in the neonatal rat. *Endocrinology*, **129,** 149-57.

Dulak, N. C. and H. M. Temin, 1973: A partially purified polypeptide fraction from rat liver cell conditioned medium with multiplication-stimulating activity for embryo fibroblasts. *J Cell Physiol*, **81,** 153-60.

Efstratiadis, A., 1998: Genetics of mouse growth. *Int J Dev Biol*, **42,** 955-76.

Eigenmann, J. E., A. Amador, and D. F. Patterson, 1988: Insulin-like growth factor I levels in proportionate dogs, chondrodystrophic dogs and in giant dogs. *Acta Endocrinol (Copenh)*, **118,** 105-8.

Eisenberger, C. L., H. Nechushtan, H. Cohen, M. Shani, and L. Reshef, 1992: Differential regulation of the rat phosphoenolpyruvate carboxykinase gene expression in several tissues of transgenic mice. *Mol Cell Biol*, **12,** 1396-403.

Emler, C. A. and D. S. Schalch, 1987: Nutritionally-induced changes in hepatic insulin-like growth factor I (IGF-I) gene expression in rats. *Endocrinology*, **120,** 832-4.

Erben, R. G., 1997: Embedding of bone samples in methylmethacrylate: an improved method suitable for bone histomorphometry, histochemistry, and immunohistochemistry. *J Histochem Cytochem*, **45,** 307-13.

Ewton, D. Z., S. L. Falen, and J. R. Florini, 1987: The type II insulin-like growth factor (IGF) receptor has low affinity for IGF-I analogs: pleiotypic actions of IGFs on myoblasts are apparently mediated by the type I receptor. *Endocrinology*, **120,** 115-23.

Ferrari, G., M. C. Minozzi, A. M. Zanellato, and B. Silvestrini, 1998: GM1, like IGF-I and GDNF, prevents neuronal apoptosis. *Ann N Y Acad Sci*, **845,** 408.

Florini, J. R. and K. A. Magri, 1989: Effects of growth factors on myogenic differentiation. *Am J Physiol*, **256,** C701-11.

Foulstone, E., S. Prince, O. Zaccheo, J. L. Burns, J. Harper, C. Jacobs, D. Church, and A. B. Hassan, 2005: Insulin-like growth factor ligands, receptors, and binding proteins in cancer. *J Pathol*, **205,** 145-53.

Froesch, E. R., H. Buergi, E. B. Ramseier, P. Bally, and A. Labhart, 1963: Antibody-Suppressible and Nonsuppressible Insulin-Like Activities in Human Serum and Their Physiologic Significance. An Insulin Assay with Adipose Tissue of Increased Precision and Specificity. *J Clin Invest*, **42,** 1816-34.

Garrone, S., G. Radetti, M. Sidoti, M. Bozzola, F. Minuto, and A. Barreca, 2002: Increased insulin-like growth factor (IGF)-II and IGF/IGF-binding protein ratio in prepubertal constitutionally tall children. *J Clin Endocrinol Metab*, **87,** 5455-60.

Geduspan, J. S. and M. Solursh, 1993: Effects of the mesonephros and insulin-like growth factor I on chondrogenesis of limb explants. *Dev Biol*, **156,** 500-8.

Gelato, M. C., C. Rutherford, R. I. Stark, and S. S. Daniel, 1989: The insulin-like growth factor II/mannose-6-phosphate receptor is present in fetal and maternal sheep serum. *Endocrinology*, **124,** 2935-43.

Giovannucci, E., M. N. Pollak, E. A. Platz, W. C. Willett, M. J. Stampfer, N. Majeed, G. A. Colditz, F. E. Speizer, and S. E. Hankinson, 2000: A prospective study of plasma insulin-like growth factor-1 and binding protein-3 and risk of colorectal neoplasia in women. *Cancer Epidemiol Biomarkers Prev*, **9,** 345-9.

Giudice, L. C., 1992: Insulin-like growth factors and ovarian follicular development. *Endocr Rev*, **13,** 641-69.

Gourmelen, M., Y. Le Bouc, F. Girard, and M. Binoux, 1984: Serum levels of insulin-like growth factor (IGF) and IGF binding protein in constitutionally tall children and adolescents. *J Clin Endocrinol Metab*, **59,** 1197-203.

Guler, H. P., J. Zapf, C. Schmid, and E. R. Froesch, 1989: Insulin-like growth factors I and II in healthy man. Estimations of half-lives and production rates. *Acta Endocrinol (Copenh)*, **121,** 753-8.

Hafner, M. and W. Müller, 2004: Generation of mouse mutants by sequence information driven and random mutagenesis. *The Laboratory Mouse*, H. Hedrich, Ed., Elsevier Academic Press, pp 85-93.

Hall, K., G. Enberg, M. Ritzen, H. Svan, L. Fryklund, and K. Takano, 1980: Somatomedin A levels in serum from healthy children and from children with growth hormone deficiency or delayed puberty. *Acta Endocrinol (Copenh)*, **94,** 155-65.

Hall, L. J., Y. Kajimoto, D. Bichell, S. W. Kim, P. L. James, D. Counts, L. J. Nixon, G. Tobin, and P. Rotwein, 1992: Functional analysis of the rat insulin-like growth factor I gene and identification of an IGF-I gene promoter. *DNA Cell Biol*, **11,** 301-13.

Hankinson, S. E., W. C. Willett, G. A. Colditz, D. J. Hunter, D. S. Michaud, B. Deroo, B. Rosner, F. E. Speizer, and M. Pollak, 1998: Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *Lancet*, **351,** 1393-6.

Harvey, M. B. and P. L. Kaye, 1992: IGF-2 stimulates growth and metabolism of early mouse embryos. *Mech Dev*, **38,** 169-73.

Heaton, J. H., N. L. Krett, J. M. Alvarez, T. D. Gelehrter, J. A. Romanus, and M. M. Rechler, 1984: Insulin regulation of insulin-like growth factor action in rat hepatoma cells. *J Biol Chem*, **259,** 2396-402.

Hepler, J. E., J. J. Van Wyk, and P. K. Lund, 1990: Different half-lives of insulin-like growth factor I mRNAs that differ in length of 3' untranslated sequence. *Endocrinology*, **127,** 1550-2.

Holthuizen, P. E., C. B. Cleutjens, G. J. Veenstra, F. M. van der Lee, A. M. Koonen-Reemst, and J. S. Sussenbach, 1993: Differential expression of the human, mouse and rat IGF-II genes. *Regul Pept*, **48,** 77-89.

Holthuizen E.P., P. H. Steenbergh and J. S. Sussenbach, 1999: Regulation of IGF gene expression. *Contemporary Endocrinology: The IGF System*, R. G. Rosenfeld and. C.T. Roberts Jr., Eds., Humana Press, Inc., pp 36-61.

Hoppener, J. W., P. de Pagter-Holthuizen, A. H. Geurts van Kessel, M. Jansen, S. D. Kittur, S. E. Antonarakis, C. J. Lips, and J. S. Sussenbach, 1985: The human gene encoding insulin-like growth factor I is located on chromosome 12. *Hum Genet*, **69,** 157-60.

Hossenlopp, P., D. Seurin, B. Segovia-Quinson, S. Hardouin, and M. Binoux, 1986: Analysis of serum insulin-like growth factor binding proteins using western blotting: use of the method for titration of the binding proteins and competitive binding studies. *Anal Biochem*, **154,** 138-43.

Hwa, V., Y. Oh, and R. G. Rosenfeld, 1999: The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocr Rev*, **20,** 761-87.

Hynes, M. A., J. J. Van Wyk, P. J. Brooks, A. J. D'Ercole, M. Jansen, and P. K. Lund, 1987: Growth hormone dependence of somatomedin-C/insulin-like growth factor-I and insulin-like growth factor-II messenger ribonucleic acids. *Mol Endocrinol*, **1,** 233-42.

Jonas, A. M., 1984: The mouse in biomedical research. *Physiologist*, **27,** 330-46.

Jones, J. I. and D. R. Clemmons, 1995: Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev*, **16,** 3-34.

Kadowaki, T., S. Koyasu, E. Nishida, H. Sakai, F. Takaku, I. Yahara, and M. Kasuga, 1986: Insulin-like growth factors, insulin, and epidermal growth factor cause rapid cytoskeletal reorganization in KB cells. Clarification of the roles of type I insulin-like growth factor receptors and insulin receptors. *J Biol Chem*, **261,** 16141-7.

Kaplan, P. J., S. Mohan, P. Cohen, B. A. Foster, and N. M. Greenberg, 1999: The insulin-like growth factor axis and prostate cancer: lessons from the transgenic adenocarcinoma of mouse prostate (TRAMP) model. *Cancer Res*, **59,** 2203-9.

Kelley, K. M., Y. Oh, S. E. Gargosky, Z. Gucev, T. Matsumoto, V. Hwa, L. Ng, D. M. Simpson, and R. G. Rosenfeld, 1996: Insulin-like growth factor-binding proteins (IGFBPs) and their regulatory dynamics. *Int J Biochem Cell Biol*, **28,** 619-37.

Kelley, K. M., K. E. Schmidt, L. Berg, K. Sak, M. M. Galima, C. Gillespie, L. Balogh, A. Hawayek, J. A. Reyes, and M. Jamison, 2002: Comparative endocrinology of the insulin-like growth factor-binding protein. *J Endocrinol*, **175,** 3-18.

Kiess, W., 1999: Molecular Biology of the IGF-II/Mannose-6-Phosphate Receptor. *Contemporary Endocrinology: The IGF-System*, R. G. R. Rosenfeld, C.T, Ed., Humana Press, pp 89-110.

Kiess, W., G. D. Blickenstaff, M. M. Sklar, C. L. Thomas, S. P. Nissley, and G. G. Sahagian, 1988: Biochemical evidence that the type II insulin-like growth factor receptor is identical to the cation-independent mannose 6-phosphate receptor. *J Biol Chem*, **263,** 9339-44.

Kiess, W., J. F. Haskell, L. Lee, L. A. Greenstein, B. E. Miller, A. L. Aarons, M. M. Rechler, and S. P. Nissley, 1987: An antibody that blocks insulin-like growth factor (IGF) binding to the type II IGF receptor is neither an agonist nor an inhibitor of IGF-stimulated biologic responses in L6 myoblasts. *J Biol Chem*, **262,** 12745-51.

Kikuchi, K., D. P. Bichell, and P. Rotwein, 1992: Chromatin changes accompany the developmental activation of insulin-like growth factor I gene transcription. *J Biol Chem*, **267,** 21505-11.

Kim, H. S., S. R. Nagalla, Y. Oh, E. Wilson, C. T. Roberts, Jr., and R. G. Rosenfeld, 1997: Identification of a family of low-affinity insulin-like growth factor binding proteins (IGFBPs): characterization of connective tissue growth factor as a member of the IGFBP superfamily. *Proc Natl Acad Sci U S A*, **94,** 12981-6.

Kornfeld, S., 1992: Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annu Rev Biochem*, **61,** 307-30.

Laron, Z., 2001: Insulin-like growth factor 1 (IGF-1): a growth hormone. *Mol Pathol*, **54,** 311-6.

Lau, M. M., C. E. Stewart, Z. Liu, H. Bhatt, P. Rotwein, and C. L. Stewart, 1994: Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes Dev*, **8,** 2953-63.

Laureys, G., D. E. Barton, A. Ullrich, and U. Francke, 1988: Chromosomal mapping of the gene for the type II insulin-like growth factor receptor/cation-independent mannose 6-phosphate receptor in man and mouse. *Genomics*, **3,** 224-9.

Le Roith, D. and A. A. Butler, 1999: Insulin-like growth factors in pediatric health and disease. *J Clin Endocrinol Metab*, **84,** 4355-61.

Le Roith, D., L. Scavo, and A. Butler, 2001: What is the role of circulating IGF-I? *Trends Endocrinol Metab*, **12,** 48-52.

Le Roith, D., D. Clemmons, P. Nissley, and M. M. Rechler, 1992: NIH conference. Insulin-like growth factors in health and disease. *Ann Intern Med*, **116,** 854-62.
Le Roith, D., C. Bondy, S. Yakar, J. L. Liu, and A. Butler, 2001: The somatomedin hypothesis: 2001. *Endocr Rev*, **22,** 53-74.

Lee, J. E., J. Pintar, and A. Efstratiadis, 1990: Pattern of the insulin-like growth factor II gene expression during early mouse embryogenesis. *Development*, **110,** 151-9.

Leung, K., I. A. Rajkovic, E. Peters, I. Markus, J. J. Van Wyk, and K. K. Ho, 1996: Insulin-like growth factor I and insulin down-regulate growth hormone (GH) receptors in rat osteoblasts: evidence for a peripheral feedback loop regulating GH action. *Endocrinology*, **137,** 2694-702.

Linder, C.C. and M.T. Davisson, 2004: Strains, stocks and mutant mice. *The Laboratory Mouse*, H. Hedrich, Ed., Elsevier Academic Press, pp 25-44.

Liu, J. P., J. Baker, A. S. Perkins, E. J. Robertson, and A. Efstratiadis, 1993: Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell*, **75,** 59-72.

Louvi, A., D. Accili, and A. Efstratiadis, 1997: Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. *Dev Biol*, **189,** 33-48.

Lowe, W. L., Jr., 1991: Biologic actions of the insulin-like growth factors. *Insulin-like growth factors: molecular and cellular aspects.*, D. Le Roith, Ed., CRC Press, pp 49-85.

Lowe, W. L., Jr., C. T. Roberts, Jr., S. R. Lasky, and D. LeRoith, 1987: Differential expression of alternative 5' untranslated regions in mRNAs encoding rat insulin-like growth factor I. *Proc Natl Acad Sci U S A*, **84,** 8946-50.

Lowe, W. L., Jr., S. R. Lasky, D. LeRoith, and C. T. Roberts, Jr., 1988: Distribution and regulation of rat insulin-like growth factor I messenger ribonucleic acids encoding alternative carboxyterminal E-peptides: evidence for differential processing and regulation in liver. *Mol Endocrinol*, **2,** 528-35.

Ludwig, T., J. Eggenschwiler, P. Fisher, A. J. D'Ercole, M. L. Davenport, and A. Efstratiadis, 1996: Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. *Dev Biol*, **177,** 517-35.

Luna, A. M., D. M. Wilson, C. J. Wibbelsman, R. C. Brown, R. J. Nagashima, R. L. Hintz, and R. G. Rosenfeld, 1983: Somatomedins in adolescence: a cross-sectional study of the effect of puberty on plasma insulin-like growth factor I and II levels. *J Clin Endocrinol Metab*, **57,** 268-71.

Lupu, F., J. D. Terwilliger, K. Lee, G. V. Segre, and A. Efstratiadis, 2001: Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. *Dev Biol*, **229,** 141-62.

Ma, J., M. N. Pollak, E. Giovannucci, J. M. Chan, Y. Tao, C. H. Hennekens, and M. J. Stampfer, 1999: Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3. *J Natl Cancer Inst*, **91,** 620-5.

Macaulay, V. M., 1992: Insulin-like growth factors and cancer. *Br J Cancer*, **65,** 311-20.

MacLeod, J. N., N. A. Pampori, and B. H. Shapiro, 1991: Sex differences in the ultradian pattern of plasma growth hormone concentrations in mice. *J Endocrinol*, **131,** 395-9.

Marron-Terada, P. G., M. A. Brzycki-Wessell, and N. M. Dahms, 1998: The two mannose 6-phosphate binding sites of the insulin-like growth factor-II/mannose 6-phosphate receptor display different ligand binding properties. *J Biol Chem*, **273,** 22358-66.

Marron-Terada, P. G., M. K. Hancock, D. J. Haskins, and N. M. Dahms, 2000: Recognition of Dictyostelium discoideum lysosomal enzymes is conferred by the amino-terminal carbohydrate binding site of the insulin-like growth factor II/mannose 6-phosphate receptor. *Biochemistry*, **39,** 2243-53.

Mathews, L. S., G. Norstedt, and R. D. Palmiter, 1986: Regulation of insulin-like growth factor I gene expression by growth hormone. *Proc Natl Acad Sci U S A*, **83,** 9343-7.

Mathews, L. S., B. Enberg, and G. Norstedt, 1989: Regulation of rat growth hormone receptor gene expression. *J Biol Chem*, **264,** 9905-10.

Mathews, L. S., R. E. Hammer, R. R. Behringer, A. J. D'Ercole, G. I. Bell, R. L. Brinster, and R. D. Palmiter, 1988: Growth enhancement of transgenic mice expressing human insulin-like growth factor I. *Endocrinology*, **123,** 2827-33.

Mauras, N., R. M. Blizzard, K. Link, M. L. Johnson, A. D. Rogol, and J. D. Veldhuis, 1987: Augmentation of growth hormone secretion during puberty: evidence for a pulse amplitude-modulated phenomenon. *J Clin Endocrinol Metab*, **64,** 596-601.

McCarthy, T. L., M. Centrella, and E. Canalis, 1989: Parathyroid hormone enhances the transcript and polypeptide levels of insulin-like growth factor I in osteoblast-enriched cultures from fetal rat bone. *Endocrinology*, **124,** 1247-53.

McCarthy, T. L., M. Centrella, and E. Canalis, 1990: Cortisol inhibits the synthesis of insulin-like growth factor-I in skeletal cells. *Endocrinology*, **126,** 1569-75.

McGrane, M. M., J. S. Yun, A. F. Moorman, W. H. Lamers, G. K. Hendrick, B. M. Arafah, E. A. Park, T. E. Wagner, and R. W. Hanson, 1990: Metabolic effects of developmental, tissue-, and cell-specific expression of a chimeric phosphoenolpyruvate carboxykinase (GTP)/bovine growth hormone gene in transgenic mice. *J Biol Chem*, **265,** 22371-9.

Mills, J. J., J. G. Falls, A. T. De Souza, and R. L. Jirtle, 1998: Imprinted M6p/Igf2 receptor is mutated in rat liver tumors. *Oncogene*, **16,** 2797-802.

Mochizuki, H., Y. Hakeda, N. Wakatsuki, N. Usui, S. Akashi, T. Sato, K. Tanaka, and M. Kumegawa, 1992: Insulin-like growth factor-I supports formation and activation of osteoclasts. *Endocrinology*, **131,** 1075-80.

Mohan, S., C. Richman, R. Guo, Y. Amaar, L. R. Donahue, J. Wergedal, and D. J. Baylink, 2003: Insulin-like growth factor regulates peak bone mineral density in mice by both growth hormone-dependent and -independent mechanisms. *Endocrinology*, **144,** 929-36.

Moorehead, R. A., J. E. Fata, M. B. Johnson, and R. Khokha, 2001: Inhibition of mammary epithelial apoptosis and sustained phosphorylation of Akt/PKB in MMTV-IGF-II transgenic mice. *Cell Death Differ*, **8,** 16-29.

Moorehead, R. A., O. H. Sanchez, R. M. Baldwin, and R. Khokha, 2003: Transgenic overexpression of IGF-II induces spontaneous lung tumors: a model for human lung adenocarcinoma. *Oncogene*, **22,** 853-7.

Morgan, D. O., J. C. Edman, D. N. Standring, V. A. Fried, M. C. Smith, R. A. Roth, and W. J. Rutter, 1987: Insulin-like growth factor II receptor as a multifunctional binding protein. *Nature*, **329,** 301-7.

Moschos, S. J. and C. S. Mantzoros, 2002: The role of the IGF system in cancer: from basic to clinical studies and clinical applications. *Oncology*, **63,** 317-32.

Murphy, L. J. and H. G. Friesen, 1988: Differential effects of estrogen and growth hormone on uterine and hepatic insulin-like growth factor I gene expression in the ovariectomized hypophysectomized rat. *Endocrinology*, **122,** 325-32.

Murphy, L. J., G. I. Bell, M. L. Duckworth, and H. G. Friesen, 1987: Identification, characterization, and regulation of a rat complementary deoxyribonucleic acid which encodes insulin-like growth factor-I. *Endocrinology*, **121,** 684-91.

Nissley, P., 1999: Type 2 IGF receptor mediated events. *Contemporary Endocrinology: The IGF System*, R. G. R. Rosenfeld, C.T., Ed., Humana Press, pp 165-198.

Nissley, S. P., J. Passamani, and P. Short, 1976: Stimulation of DNA synthesis, cell multiplication, and ornithine decarboxylase in 3T3 cells by multiplication stimulating activity (MSA). *J Cell Physiol*, **89,** 393-402.

O'Dell, S. D. and I. N. Day, 1998: Insulin-like growth factor II (IGF-II). *Int J Biochem Cell Biol*, **30,** 767-71.

Oh, Y., S. R. Nagalla, Y. Yamanaka, H. S. Kim, E. Wilson, and R. G. Rosenfeld, 1996: Synthesis and characterization of insulin-like growth factor-binding protein (IGFBP)-7. Recombinant human mac25 protein specifically binds IGF-I and -II. *J Biol Chem*, **271,** 30322-5.

Ohneda, K., M. H. Ulshen, C. R. Fuller, A. J. D'Ercole, and P. K. Lund, 1997: Enhanced growth of small bowel in transgenic mice expressing human insulin-like growth factor I. *Gastroenterology*, **112,** 444-54.

Oshima, A., C. M. Nolan, J. W. Kyle, J. H. Grubb, and W. S. Sly, 1988: The human cation-independent mannose 6-phosphate receptor. Cloning and sequence of the full-length cDNA and expression of functional receptor in COS cells. *J Biol Chem*, **263,** 2553-62.

Pahlman, S., G. Meyerson, E. Lindgren, M. Schalling, and I. Johansson, 1991: Insulin-like growth factor I shifts from promoting cell division to potentiating maturation during neuronal differentiation. *Proc Natl Acad Sci U S A*, **88,** 9994-8.

Pardee, A. B., 1989: G1 events and regulation of cell proliferation. *Science*, **246,** 603-8.

Parrizas, M., A. R. Saltiel, and D. LeRoith, 1997: Insulin-like growth factor 1 inhibits apoptosis using the phosphatidylinositol 3'-kinase and mitogen-activated protein kinase pathways. *J Biol Chem*, **272,** 154-61.

Penhoat, A., D. Naville, C. Jaillard, P. G. Chatelain, and J. M. Saez, 1989: Hormonal regulation of insulin-like growth factor I secretion by bovine adrenal cells. *J Biol Chem*, **264,** 6858-62.

Perdue, J. F., T. R. LeBon, J. Kato, B. Hampton, and Y. Fujita-Yamaguchi, 1991: Binding specificities and transducing function of the different molecular weight forms of insulin-like growth factor-II (IGF-II) on IGF-I receptors. *Endocrinology*, **129,** 3101-8.

Petrik, J., J. M. Pell, E. Arany, T. J. McDonald, W. L. Dean, W. Reik, and D. J. Hill, 1999: Overexpression of insulin-like growth factor-II in transgenic mice is associated with pancreatic islet cell hyperplasia. *Endocrinology*, **140,** 2353-63.

Pollak, M. N., 2004: Insulin-like growth factors and neoplasia. *Novartis Found Symp*, **262,** 84-98; discussion 98-107, 265-8.

Poole, T. B., 1987: Raising and defining laboratory animals. *The care and management of laboratory animals*, T. B. Poole, Ed., Longman Scientific and Technical, pp 2-7.

Powell-Braxton, L., P. Hollingshead, C. Warburton, M. Dowd, S. Pitts-Meek, D. Dalton, N. Gillett, and T. A. Stewart, 1993: IGF-I is required for normal embryonic growth in mice. *Genes Dev*, **7,** 2609-17.

Quaife, C. J., L. S. Mathews, C. A. Pinkert, R. E. Hammer, R. L. Brinster, and R. D. Palmiter, 1989: Histopathology associated with elevated levels of growth hormone and insulin-like growth factor I in transgenic mice. *Endocrinology*, **124,** 40-8.

Rajaram, S., D. J. Baylink, and S. Mohan, 1997: Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. *Endocr Rev*, **18,** 801-31.

Ranke, M. B., K. J. Osterziel, R. Schweizer, B. Schuett, K. Weber, P. Robbel, A. Vornwald, G. Blumenstock, and M. W. Elmlinger, 2003: Reference levels of insulin-like growth factor I in the serum of healthy adults: comparison of four immunoassays. *Clin Chem Lab Med*, **41,** 1329-34.

Rappolee D.A. and Z. Werb, 1991: Endogenous insulin-like growth factor II mediates growth in preimplantation mouse embryos. *Modern concepts of insulin-like growth factors*, E. M. Spencer, Ed., Elsevier Science Publishing, Inc.

Reiss, K., W. Cheng, A. Ferber, J. Kajstura, P. Li, B. Li, G. Olivetti, C. J. Homcy, R. Baserga, and P. Anversa, 1996: Overexpression of insulin-like growth factor-1 in the heart is coupled with myocyte proliferation in transgenic mice. *Proc Natl Acad Sci U S A*, **93,** 8630-5.

Ren-Qiu, Q., T. Ruelicke, S. Hassam, G. K. Haselbacher, and E. J. Schoenle, 1993: Systemic effects of insulin-like growth factor-II produced and released from Wilms tumour tissue. *Eur J Pediatr*, **152,** 102-6.

Rinderknecht, E. and R. E. Humbel, 1976: Amino-terminal sequences of two polypeptides from human serum with nonsuppressible insulin-like and cell-growth-promoting activities: evidence for structural homology with insulin B chain. *Proc Natl Acad Sci U S A*, **73,** 4379-81.

Rinderknecht, E. and R. E. Humbel, 1978a: The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *J Biol Chem*, **253,** 2769-76.

Rinderknecht, E. and R. E. Humbel, 1978b: Primary structure of human insulin-like growth factor II. *FEBS Lett*, **89,** 283-6.

Roberts, C. T., Jr., A. L. Brown, D. E. Graham, S. Seelig, S. Berry, K. H. Gabbay, and M. M. Rechler, 1986: Growth hormone regulates the abundance of insulin-like growth factor I RNA in adult rat liver. *J Biol Chem*, **261,** 10025-8.

Rogers, S. A., S. B. Miller, and M. R. Hammerman, 1991: Insulin-like growth factor I gene expression in isolated rat renal collecting duct is stimulated by epidermal growth factor. *J Clin Invest*, **87,** 347-51.

Rogler, C. E., D. Yang, L. Rossetti, J. Donohoe, E. Alt, C. J. Chang, R. Rosenfeld, K. Neely, and R. Hintz, 1994: Altered body composition and increased frequency of diverse malignancies in insulin-like growth factor-II transgenic mice. *J Biol Chem*, **269,** 13779-84.

Rotwein, P., 1991: Structure, evolution, expression and regulation of insulin-like growth factors I and II. *Growth Factors*, **5,** 3-18.

Rotwein, P., 1999: Molecular biology of IGF-I and IGF-II. *Contemporary Endocrinology: The IGF-System*, R. R. a. C. Roberts, Ed., Humana Press, Inc., pp 19-35.

Rotwein, P. and L. J. Hall, 1990: Evolution of insulin-like growth factor II: characterization of the mouse IGF-II gene and identification of two pseudo-exons. *DNA Cell Biol*, **9,** 725-35.

Rotwein, P., D. P. Bichell, and K. Kikuchi, 1993: Multifactorial regulation of IGF-I gene expression. *Mol Reprod Dev*, **35,** 358-63; discussion 363-4.

Rotwein, P., S. K. Burgess, J. D. Milbrandt, and J. E. Krause, 1988: Differential expression of insulin-like growth factor genes in rat central nervous system. *Proc Natl Acad Sci U S A*, **85,** 265-9.

Salmon, W. D., Jr. and W. H. Daughaday, 1957: A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. *J Lab Clin Med*, **49,** 825-36.
Sara, V. R. and K. Hall, 1990: Insulin-like growth factors and their binding proteins. *Physiol Rev*, **70,** 591-614.

Schenk, R., A.Olah and; W. Hermann, 1984: Preparation of calcified tissue for light microscopy. *Methods of calcified tissue preparation*, G. Dickson, Ed., Elsevier.

Schneider, M. R., H. Lahm, M. Wu, A. Hoeflich, and E. Wolf, 2000: Transgenic mouse models for studying the functions of insulin-like growth factor-binding proteins. *Faseb J*, **14,** 629-40.

Sell, C., R. Baserga, and R. Rubin, 1995: Insulin-like growth factor I (IGF-I) and the IGF-I receptor prevent etoposide-induced apoptosis. *Cancer Res*, **55,** 303-6.

Sheppard, M. S. and R. M. Bala, 1986: Insulin-like growth factor inhibition of growth hormone secretion. *Can J Physiol Pharmacol*, **64,** 525-30.

Shinar, D. M., N. Endo, D. Halperin, G. A. Rodan, and M. Weinreb, 1993: Differential expression of insulin-like growth factor-I (IGF-I) and IGF-II messenger ribonucleic acid in growing rat bone. *Endocrinology*, **132,** 1158-67.

Short, M. K., D. E. Clouthier, I. M. Schaefer, R. E. Hammer, M. A. Magnuson, and E. G. Beale, 1992: Tissue-specific, developmental, hormonal, and dietary regulation of rat phosphoenolpyruvate carboxykinase-human growth hormone fusion genes in transgenic mice. *Mol Cell Biol*, **12,** 1007-20.

Silbergeld, A., A. Litwin, S. Bruchis, I. Varsano, and Z. Laron, 1986: Insulin-like growth factor I (IGF-I) in healthy children, adolescents and adults as determined by a radioimmunoassay specific for the synthetic 53-70 peptide region. *Clin Endocrinol (Oxf)*, **25,** 67-74.

Silha, J. V. and L. J. Murphy, 2002: Insights from insulin-like growth factor binding protein transgenic mice. *Endocrinology*, **143,** 3711-4.

Sjogren, K., M. Sheng, S. Moverare, J. L. Liu, K. Wallenius, J. Tornell, O. Isaksson, J. O. Jansson, S. Mohan, and C. Ohlsson, 2002: Effects of liver-derived insulin-like growth factor I on bone metabolism in mice. *J Bone Miner Res*, **17,** 1977-87.

Sjogren, K., J. L. Liu, K. Blad, S. Skrtic, O. Vidal, V. Wallenius, D. LeRoith, J. Tornell, O. G. Isaksson, J. O. Jansson, and C. Ohlsson, 1999: Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. *Proc Natl Acad Sci U S A*, **96,** 7088-92.

Soares, M. B., D. N. Ishii, and A. Efstratiadis, 1985: Developmental and tissue-specific expression of a family of transcripts related to rat insulin-like growth factor II mRNA. *Nucleic Acids Res*, **13,** 1119-34.

Soares, M. B., A. Turken, D. Ishii, L. Mills, V. Episkopou, S. Cotter, S. Zeitlin, and A. Efstratiadis, 1986: Rat insulin-like growth factor II gene. A single gene with two promoters expressing a multitranscript family. *J Mol Biol*, **192,** 737-52.

Solter, D., 1988: Differential imprinting and expression of maternal and paternal genomes. *Annu Rev Genet*, **22,** 127-46.

Steele-Perkins, G., J. Turner, J. C. Edman, J. Hari, S. B. Pierce, C. Stover, W. J. Rutter, and R. A. Roth, 1988: Expression and characterization of a functional human insulin-like growth factor I receptor. *J Biol Chem*, **263,** 11486-92.

Stewart, C. E. and P. Rotwein, 1996: Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. *Physiol Rev*, **76,** 1005-26.

Straus, D. S. and C. D. Takemoto, 1990: Effect of fasting on insulin-like growth factor-I (IGF-I) and growth hormone receptor mRNA levels and IGF-I gene transcription in rat liver. *Mol Endocrinol*, **4,** 91-100.

Stylianopoulou, F., A. Efstratiadis, J. Herbert, and J. Pintar, 1988: Pattern of the insulin-like growth factor II gene expression during rat embryogenesis. *Development*, **103,** 497-506.

Surani, M. A., R. Kothary, N. D. Allen, P. B. Singh, R. Fundele, A. C. Ferguson-Smith, and S. C. Barton, 1990: Genome imprinting and development in the mouse. *Dev Suppl***,** 89-98.

Sussenbach, J. S., 1989: The gene structure of the insulin-like growth factor family. *Prog Growth Factor Res*, **1,** 33-48.

Sussenbach, J. S., R. J. Rodenburg, W. Scheper, and P. Holthuizen, 1993: Transcriptional and post-transcriptional regulation of the human IGF-II gene expression. *Adv Exp Med Biol*, **343,** 63-71.

Sussenbach J. S. , R. R. J. T., Scheper W. and Holthuizen P., 1994: Transcriptional and post-transcriptional regulation of the human IGF-II gene expression. *Current directions in insulin-like growth factor research*, D. L. R. a. M. K. Raizada, Ed., Plenum Press, pp 63-71.

Szebenyi, G. and P. Rotwein, 1994: The mouse insulin-like growth factor II/cation-independent mannose 6-phosphate (IGF-II/MPR) receptor gene: molecular cloning and genomic organization. *Genomics*, **19,** 120-9.

Timsit, J., W. Savino, B. Safieh, P. Chanson, M. C. Gagnerault, J. F. Bach, and M. Dardenne, 1992: Growth hormone and insulin-like growth factor-I stimulate hormonal function and proliferation of thymic epithelial cells. *J Clin Endocrinol Metab*, **75,** 183-8.

Tiong, T. S., J. L. Stevenson, and A. C. Herington, 1992: Regulation of prolactin receptor gene expression by thyroid hormone status in the rat. *J Mol Endocrinol*, **8,** 63-72.

Tollet, P., B. Enberg, and A. Mode, 1990: Growth hormone (GH) regulation of cytochrome P-450IIC12, insulin-like growth factor-I (IGF-I), and GH receptor messenger RNA expression in primary rat hepatocytes: a hormonal interplay with insulin, IGF-I, and thyroid hormone. *Mol Endocrinol*, **4,** 1934-42.

Treacy, E., C. Polychronakos, M. Vekemans, P. Eydoux, S. Blaichman, H. Scarpelli, M. Ross, Y. Xu, and V. M. Der Kaloustian, 1996: Translocation between chromosomes 6 and 15 (45,XX,t(6;15)(q25;q11.2)) with further evidence for lack of imprinting of the insulin-like growth factor II/mannose-6-phosphate receptor in humans. *J Med Genet*, **33,** 42-6.

van Buul-Offers, S. C., K. de Haan, M. G. Reijnen-Gresnigt, D. Meinsma, M. Jansen, S. L. Oei, E. J. Bonte, J. S. Sussenbach, and J. L. Van den Brande, 1995: Overexpression of human insulin-like growth factor-II in transgenic mice causes increased growth of the thymus. *J Endocrinol*, **144,** 491-502.

Van der Ven, L. T., P. J. Roholl, M. G. Reijnen-Gresnigt, R. J. Bloemen, and S. C. van Buul-Offers, 1997: Expression of insulin-like growth factor II (IGF-II) and histological changes in the thymus and spleen of transgenic mice overexpressing IGF-II. *Histochem Cell Biol*, **107,** 193-203.

Wang, Z. Q., M. R. Fung, D. P. Barlow, and E. F. Wagner, 1994: Regulation of embryonic growth and lysosomal targeting by the imprinted Igf2/Mpr gene. *Nature*, **372,** 464-7.

Wang, E., J. Wang, E. Chin, J. Zhou, and C. A. Bondy, 1995: Cellular patterns of insulin-like growth factor system gene expression in murine chondrogenesis and osteogenesis. *Endocrinology*, **136,** 2741-51.

Wang, J., J. Zhou, C. M. Cheng, J. J. Kopchick, and C. A. Bondy, 2004: Evidence supporting dual, IGF-I-independent and IGF-I-dependent, roles for GH in promoting longitudinal bone growth. *J Endocrinol*, **180,** 247-55.

Ward, A., P. Bates, R. Fisher, L. Richardson, and C. F. Graham, 1994: Disproportionate growth in mice with Igf-2 transgenes. *Proc Natl Acad Sci U S A*, **91,** 10365-9.

Weber, M. M., C. Fottner, P. Schmidt, K. M. Brodowski, K. Gittner, H. Lahm, D. Engelhardt, and E. Wolf, 1999: Postnatal overexpression of insulin-like growth factor II in transgenic mice is associated with adrenocortical hyperplasia and enhanced steroidogenesis. *Endocrinology*, **140,** 1537-43.

Werner, H., M. Woloschak, M. Adamo, Z. Shen-Orr, C. T. Roberts, Jr., and D. LeRoith, 1989: Developmental regulation of the rat insulin-like growth factor I receptor gene. *Proc Natl Acad Sci U S A*, **86,** 7451-5.

Wilson, D. M., J. A. Thomas, T. E. Hamm, Jr., J. Wyche, R. L. Hintz, and R. G. Rosenfeld, 1987: Transplantation of insulin-like growth factor-II-secreting tumors into nude rodents. *Endocrinology*, **120,** 1896-901.

Wolf, M., S. H. Ingbar, and A. C. Moses, 1989: Thyroid hormone and growth hormone interact to regulate insulin-like growth factor-I messenger ribonucleic acid and circulating levels in the rat. *Endocrinology*, **125,** 2905-14.

Wolf, E., A. Hoeflich, and H. Lahm, 1998: What is the function of IGF-II in postnatal life? Answers from transgenic mouse models. *Growth Horm IGF Res*, **8,** 185-93.

Wolf, E., R. Kramer, W. F. Blum, J. Foll, and G. Brem, 1994: Consequences of postnatally elevated insulin-like growth factor-II in transgenic mice: endocrine changes and effects on body and organ growth. *Endocrinology*, **135,** 1877-86.

Wolf, E., K. Rapp, W. F. Blum, H. Kolb, and G. Brem, 1995: Skeletal growth of transgenic mice with elevated levels of circulating insulin-like growth factor-II. *Growth Regul*, **5,** 177-83.

Wolf, E., R. Wanke, W. Hermanns, G. Brem, F. Pirchner, and I. von Butler-Wemken, 1991: Growth characteristics of metallothionein-human growth hormone transgenic mice as compared to mice selected for high eight-week body weight and unselected controls. I. Body weight gain and external body dimensions. *Growth Dev Aging*, **55,** 225-35.

Wolf, E., M. R. Schneider, R. Zhou, T. M. Fisch, N. Herbach, M. Dahlhoff, R. Wanke, and A. Hoeflich, 2005: Functional consequences of IGFBP excess-lessons from transgenic mice. *Pediatr Nephrol*, **20,** 269-78.

Wolk, A., C. S. Mantzoros, S. O. Andersson, R. Bergstrom, L. B. Signorello, P. Lagiou, H. O. Adami, and D. Trichopoulos, 1998: Insulin-like growth factor 1 and prostate cancer risk: a population-based, case-control study. *J Natl Cancer Inst*, **90,** 911-5.

Woods, K. A., C. Camacho-Hubner, M. O. Savage, and A. J. Clark, 1996: Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. *N Engl J Med*, **335,** 1363-7.

Wu, Y., S. Yakar, L. Zhao, L. Hennighausen, and D. LeRoith, 2002: Circulating insulin-like growth factor-I levels regulate colon cancer growth and metastasis. *Cancer Res*, **62,** 1030-5.

Wu, Y., K. Cui, K. Miyoshi, L. Hennighausen, J. E. Green, J. Setser, D. LeRoith, and S. Yakar, 2003: Reduced circulating insulin-like growth factor I levels delay the onset of chemically and genetically induced mammary tumors. *Cancer Res*, **63,** 4384-8.

Yakar, S. and C. J. Rosen, 2003: From mouse to man: redefining the role of insulin-like growth factor-I in the acquisition of bone mass. *Exp Biol Med (Maywood)*, **228,** 245-52.

Yakar, S., P. Pennisi, H. Zhao, Y. Zhang, and D. LeRoith, 2004: Circulating IGF-1 and its role in cancer: lessons from the IGF-1 gene deletion (LID) mouse. *Novartis Found Symp*, **262,** 3-9; discussion 9-18, 265-8.

Yakar, S., J. L. Liu, B. Stannard, A. Butler, D. Accili, B. Sauer, and D. LeRoith, 1999: Normal growth and development in the absence of hepatic insulin-like growth factor I. *Proc Natl Acad Sci U S A*, **96,** 7324-9.

Yakar, S., C. J. Rosen, W. G. Beamer, C. L. Ackert-Bicknell, Y. Wu, J. L. Liu, G. T. Ooi, J. Setser, J. Frystyk, Y. R. Boisclair, and D. LeRoith, 2002: Circulating levels of IGF-1 directly regulate bone growth and density. *J Clin Invest*, **110,** 771-81.

Yu, H., M. R. Spitz, J. Mistry, J. Gu, W. K. Hong, and X. Wu, 1999b: Plasma levels of insulin-like growth factor-I and lung cancer risk: a case-control analysis. *J Natl Cancer Inst*, **91,** 151-6.

Yu, H., J. Mistry, M. J. Nicar, M. J. Khosravi, A. Diamandis, J. van Doorn, and A. Juul, 1999a: Insulin-like growth factors (IGF-I, free IGF-I and IGF-II) and insulin-like growth factor binding proteins (IGFBP-2, IGFBP-3, IGFBP-6, and ALS) in blood circulation. *J Clin Lab Anal*, **13,** 166-72.

Zaina, S., L. Pettersson, A. B. Thomsen, C. M. Chai, Z. Qi, J. Thyberg, and J. Nilsson, 2003: Shortened life span, bradycardia, and hypotension in mice with targeted expression of an Igf2 transgene in smooth muscle cells. *Endocrinology*, **144,** 2695-703.

Zaina, S., L. Pettersson, B. Ahren, L. Branen, A. B. Hassan, M. Lindholm, R. Mattsson, J. Thyberg, and J. Nilsson, 2002: Insulin-like growth factor II plays a central role in atherosclerosis in a mouse model. *J Biol Chem*, **277,** 4505-11.

Zapf, J., H. Walter, and E. R. Froesch, 1981: Radioimmunological determination of insulinlike growth factors I and II in normal subjects and in patients with growth disorders and extrapancreatic tumor hypoglycemia. *J Clin Invest*, **68,** 1321-30.

Zapf, J., C. Hauri, E. Futo, M. Hussain, J. Rutishauser, C. A. Maack, and E. R. Froesch, 1995: Intravenously injected insulin-like growth factor (IGF) I/IGF binding protein-3 complex exerts insulin-like effects in hypophysectomized, but not in normal rats. *J Clin Invest*, **95,** 179-86.

Zhang, J., D. Chrysis, and L. E. Underwood, 1998: Reduction of hepatic insulin-like growth factor I (IGF-I) messenger ribonucleic acid (mRNA) during fasting is associated with diminished splicing of IGF-I pre-mRNA and decreased stability of cytoplasmic IGF-I mRNA. *Endocrinology*, **139,** 4523-30.

Zhao, G., M. C. Monier-Faugere, M. C. Langub, Z. Geng, T. Nakayama, J. W. Pike, S. D. Chernausek, C. J. Rosen, L. R. Donahue, H. H. Malluche, J. A. Fagin, and T. L. Clemens, 2000: Targeted overexpression of insulin-like growth factor I to osteoblasts of transgenic mice: increased trabecular bone volume without increased osteoblast proliferation. *Endocrinology*, **141,** 2674-82.

Zhou, Y., B. C. Xu, H. G. Maheshwari, L. He, M. Reed, M. Lozykowski, S. Okada, L. Cataldo, K. Coschigamo, T. E. Wagner, G. Baumann, and J. J. Kopchick, 1997: A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). *Proc Natl Acad Sci U S A*, **94,** 13215-20.

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