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Functional Analysis of Insulin-like Growth Factor Binding Protein -4 and -6 in Transgenic Mice

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CONTENTS

ABBREVIATIONS

aa	amino acid
ACTH	adrenocorticotropic hormone
ALS	acid-labile subunit
APS	ammonium persulfate
ATCC	American Type Culture Collection
α1AT	α1-antitrypsin (promoter)
bFGF	basic fibroblast growth factor
bp	base pair
BLG	β-lactoglobin (promoter)
BSA	bovine serum albumin
BW	body weight
CAT	chloramphenicol acetyltransferase
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CIAP	calf intestinal alkaline phosphatase
cm	centimeter
CMV	cytomegalovirus (promoter)
ConA	Concanavalin A
cpm	count per minute
CRE	cAMP responsive element
CRF	chronic renal failure
CSF	cerebrospinal fluid
DEPC	diethylpyrocarbonate
dl	deciliter
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
dpc	days post coitum
DTT	1,4-Dithio-DL-threitol
ECM	extracellular matrix
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid (disodium salt dihydrate)
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
ERE	estrogen responsive element
ES cells	embryonic stem cells
ESMS	electrospray ionisation mass spectrometry
FACS	fluorescent-activated cell sorting
FCS	fetal calf serum
FELASA	the Federation of European Laboratory Animal Science Associations
FFa	follicular fluid from androgen-dominant follicles
FFe	follicular fluid from estrogen-dominant growing follicles
FSH	follicle-stimulating hormone
g	gram
g	relative centrifugal force (RCF)
GFP	green fluorescent protein
GH	growth hormone

GHRA	GH receptor antagonist
GHRP-2	GH releasing peptide-2
GIT	gastrointestinal tract
GMA	2-hydroxyethyl methacrylate
h	hour
hCG	human chorionic gonadotrophin
HBD	heparin binding domain
HE	hematoxylin-eosin
HRP	horseradish peroxidase
ICM	inner cell mass
IFN-γ	interferon-γ
Ig	immunoglobin
IGF-I, -II	insulin-like growth factor-I, -II
IGF-IR	type I IGF receptor
IGF-IIR	type II IGF receptor
IGFBPs	IGF binding proteins
IGFBP-rPs	IGFBP-related proteins
IL-1, -2, -6	interleukin-1, -2, -6
IR	insulin receptor
kDa	kilodalton
kb	kilobase
LacZ	β-galactosidase
LPS	lipopolysaccharide
Luc	luciferase
M	molar
mA	milliampere
mg	milligram
MHC	major histocompatibility complex
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MMA	methyl methacrylate
MOPS	3-[N-morpholino]propanesulfonic acid
mRNA	messenger ribonucleic acid
MT	metallothionein-I (promoter)
nm	nanometer
ng	nanogram
NRL	nose-rump-length
OC	osteocalcin (promoter)
OP-1	osteogenic protein-1
PAGE	polyacrylamide gel electrophoresis
PAPP-A	pregnancy-associated plasma protein-A
PAS	Periodic-Acid-Schiff
proMBP	proform of eosinophil major basic protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF-BB	platelet-derived growth factor-BB
pg	picogram
PGK	phosphoglycerate kinase I (promoter)

PMSG	pregnant mare's serum gonadotrophin
PTH	parathyroid hormone
RA	retinoic acid
RARE	retinoic acid responsive element
rev.	review
RIA	radioimmunoassay
RNA	ribonucleic acid
rpm	revolution per minute
RT	room temperature; reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
s	second
SD	standard deviation
SDS	dodecyl sulphate sodium salt
SFCM	serum-free conditioned medium
SMP	smooth muscle α-actin promoter
SPF	specific pathogen-free
SPR	surface plasmon resonance
SRE	steroid responsive element
SSR	site-specific recombinase
T_3	triiodothyronine
T_4	thyroxine
TBS	Tris-buffered saline
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TG	transgenic
TGF-α, -β	transforming growth factor-α, -β
TIS	transcription initiation site
TNF-α	tumor necrosis factor-α
TPA	12-O-tetradecanoyl phorbol 13-acetate
UV	ultraviolet
ViSA	villous surface area
µCi	microcurie
µg	microgram
µl	microliter
µm	micrometer
WAP	whey acidic protein (promoter)
WT	wild-type
WT-1	Wilms tumor-1 protein

1 INTRODUCTION

The growth of a mammalian organism is effected predominantly by proliferative events outbalancing apoptosis and increasing total cell number, with some additional contributions by cell hypertrophy and deposition of extracellular matrix (ECM). Growth is brought about by the operation of cellular signaling pathways controlled by growth factors and hormones. In general, growth factors are produced by many tissues and exert mainly local (autocrine/paracrine) controls that predominate during embryogenesis, whereas hormones act systemically away from the sites of their production (Lupu *et al.* 2001).

Among the peptide growth factors, the insulin-like growth factors (IGF-I and IGF-II) are unique as they can act both locally as autocrine/paracrine growth factors, and systemically as a hormone (Cohick & Clemmons 1993; Mohan *et al.* 1996; Stewart & Rotwein 1996; Butler & LeRoith 2001). The IGFs have both mitogenic and metabolic actions that participate in the regulation of proliferation, differentiation, survival and specific functions of many cell types and tissues under different physiological and pathological conditions (Stewart & Rotwein 1996). The actions of IGFs, mediated via the type I IGF receptor (IGF-IR), are modulated by a family of six high-affinity IGF-binding proteins (IGFBP-1 to -6) by endocrine, autocrine and paracrine mechanisms (Clemmons 1997; Hwa *et al.* 1999; Mohan & Baylink 2002). Despite their structural similarity, each IGFBP has unique properties and exhibits specific functions. Some of the IGFBPs inhibit IGF actions, whereas others potentiate IGF effects. In addition, some IGFBPs show direct effects independent of IGF-binding (Rechler 1993; Jones & Clemmons 1995; Kelley *et al.* 1996; Clemmons 1997; Rajaram *et al.* 1997; Hwa *et al.* 1999; Duan 2002; Firth & Baxter 2002). The majority of the knowledge about these peptides is obtained from *in vitro* studies. Generation and analysis of transgenic mice overexpressing an individual IGFBP allows us to reveal the specific functions of the corresponding IGFBP *in vivo*. In the past decade, several transgenic mouse models for IGFBP-1, -2, -3 and -5 were established, which revealed some specific functions of the IGFBPs (rev. in Schneider *et al.* 2000; Silha & Murphy 2002). However, there is only one smooth muscle-specific transgenic mouse model of IGFBP-4 overexpression reported so far, and no IGFBP-6-overexpressing mice are available.

The objective of the study reported here was to investigate the consequences of IGFBP-4 and -6 overexpression on the growth and development of mice, thereby determining their specific roles *in vivo*. For this purpose, transgenic mice overexpressing murine IGFBP-4 and IGFBP-6 were generated. Transgenic IGFBP-4 was highly expressed in the lymphoid organs, whereas transgenic IGFBP-6 expression was high in the pancreas and large amounts of active IGFBP-6 were found in the lumen of the duodenum of transgenic mice. The effects of IGFBP-4 overexpression on the body and organ growth (particularly of spleen and thymus), the development of immune-related cells and the mitogenic response of splenocytes were evaluated. For IGFBP-6 transgenic mice, the consequences of IGFBP-6 excess on the body and organ growth (particularly of duodenum) and the glucose homeostasis were characterized.

2 REVIEW OF THE LITERATURE

2.1 Transgenic technologies in mice for studying gene function

In the past decade many declared aims of the genome projects have been achieved. The total genomic sequences of several relatively noncomplex organisms, such as *E. coli* (Blattner *et al.* 1997), yeast (Goffeau *et al.* 1996), *Caenorhabditis* (The C.elegans Sequencing Consortium 1998) and *Drosophila* (Adams *et al.* 2000), have already been determined. The human genome working draft sequence was completed in June 2000, with analyses published in February 2001 (Gustafsson *et al.* 1999a; Lander *et al.* 2001), and the high-quality sequence of the entire human genome will be finished by the end of 2003. Furthermore, a high-quality draft sequence of the mouse genome has also been generated at the end of 2002 (Waterston *et al.* 2002). However, these achievements are not the end of the road but rather the first step toward the functional understanding of the genome of human and other organisms. The determined linear nucleotide sequences remain only lists of A, C, G and T, unless they are given functional significance. The coding sequences of genes can be identified in a relatively reliable manner by computational methods, but the exact function of their protein products can rarely be determined without obtaining additional information by biochemical or biological methods. Thus, following sequencing, the next step must be to assign functions to the identified genes. The final goal of genome research today may look futuristic, but the knowledge of the function of every single gene and the interactions between them will finally allow us to understand the development and functioning of an organism as a whole.

Transgenic technology is one of the powerful strategies for determining gene function, because it enables rapid movement between genotype and phenotype through specific loss-of-function, overexpression or ectopic expression (Si-Hoe *et al.* 2001). The mouse represents the excellent model organism of choice for the analysis of gene function because of its anatomical, physiological, reproductive and genomic similarity to humans, the ability to manipulate the mouse genome in a random or targeted way, as well its high fecundity and relatively low maintenance costs (Denny

& Justice 2000; Malakoff 2000). Therefore, transgenic mice will play a pivotal role in studying mammalian gene function as we enter the post-genomic era.

2.1.1 Conventional transgenic technologies in mice

2.1.1.1 Pronuclear DNA microinjection

In most cases, a transgene resembles an expression cassette consisting of a gene driven by a promoter of choice. The classical transgenic procedure consists of randomly introducing such a transgene into the genome to generate an overexpression model (Brinster & Palmiter 1984). To this end, the DNA containing the transgene is microinjected into the male pronucleus of fertilized mouse zygotes. Subsequently, viable zygotes are implanted into the oviducts of pseudopregnant foster mothers. On the average, 10-30% of the resulting offspring bear the transgene in their genome. In general, a transgenic mouse line is established when the transgene is effectively transmitted to the following generations in a Mendelian way. Although the transgenic DNA is present in all cells, transgene expression is dependent on many factors such as the chosen promoter and enhancer elements, the number of integrated copies and the locus of integration.

This kind of transgenesis is employed most commonly for one of the following two purposes: (i) the forced expression of a recombinant protein to alter the physiology and/or morphology of the animal, or (ii) the analysis of transcriptional control mechanisms involved in regulatory pathways (Williams & Wagner 2000). To alter the phenotype of an intact animal, the transgene may encode a native protein, so that the experiment addresses the systemic effects at an altered protein level (overexpression) and the consequences of ectopic expression. Alternatively, the transgene is designed to encode a mutated protein that has been modified for a special purpose: to produce a constitutively active (gain-of-function mutant) or dominant-negative (loss-of-function mutant) form of a specific protein or to mimic a mutation observed in a human genetic disease.

Another common application is to identify transcriptional control elements that respond to developmental cues or physiological stimuli. In this application, the coding

region of a so-called “reporter gene”, such as the gene encoding β-galactosidase (LacZ), green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT) or luciferase (Luc), is linked to the segment of DNA thought to contain the regulatory elements of interest.

The main limitation of the classical transgenic approach is related to the uncontrolled integration of the transgene into the host genome. This random integration may influence the expression of genes situated close to the transgene, and the locus of integration may affect the expression of the transgene itself. Therefore, it is mandatory to generate several transgenic mouse lines with comparable transgene expression patterns that show identical phenotypes.

2.1.1.2 Targeted mutagenesis in mice

2.1.1.2.1 Knockout

Most targeting experiments performed to date were to generate null-mutant animals, commonly referred to as "knockout" mice (Thomas & Capecchi 1987). The principle of targeted gene disruption in the mouse is the following: a modified version of the gene of interest, a so-called targeting vector, is introduced into embryonic stem (ES) cells. A varying preparation of transfected ES cells will have replaced a wild-type allele of the endogenous gene with the targeting vector by homologous recombination. ES cell clones carrying the homologously recombined allele are then microinjected into blastocyst-stage embryos where they aggregate with the inner cell mass (ICM). After implantation into the uterus of foster mothers, these embryos will develop into chimeric mice. When the injected ES cells contribute to the formation of germ cells in chimeric mice, the ES cell genotype can be propagated to the next generations.

Since the first mouse with a targeted gene disruption was presented in 1989 (Thompson *et al.* 1989), thousands of targeted mutations have been described (see the Transgenic/Targeted Mutation Database: *http://tbase.jax.org*). Most targeted mice have been generated to characterize developmental and physiological functions of

genes *in vivo*. The approach is especially powerful in revealing the distinctive functions of genes encoding related proteins.

Sometimes the study of knockout mice reveals unexpected phenotypes and thereby highlights unsuspected functions of the disrupted gene (for examples see Huang *et al.* 1993; Wenger & Gassmann 1997). Although such discoveries are serendipitous, they should not be regarded as marginal. The targeted mutagenesis strategy has boosted the pace of knowledge in many fields by providing new insights into the functions of various genes.

2.1.1.2.2 Knockin

An interesting extension of the knockout approach is the so-called knock-in approach, in which the targeting vector contains, in addition to the selection cassette, a cDNA of interest inserted in-frame in an exon of the gene under study. The cDNA is thus expressed in place of the endogenous gene product. The main advantage of this approach is that it allows tight control of the expression of the cDNA of interest, because the cDNA is placed in the context of the complete set of *cis*-acting regulatory elements that normally control the expression of the endogenous gene. Furthermore, this approach avoids the position effect encountered in random gene-addition transgenesis. A common use of this approach is the targeted mutation of the LacZ reporter gene, which permits accurate definition of the expression pattern of endogenous genes (for examples see Schneider-Maunoury *et al.* 1993; Tajbakhsh *et al.* 1996). This approach has also proven to be a powerful tool for studying the functional relationships among members of a gene family and their potential abilities to functionally compensate for each other (for example see Hanks *et al.* 1998).

2.1.2 Conditional transgenic technologies in mice

Frequently, a genetic change has developmental consequences that either preclude or complicate studies on adult animals (e.g. embryonic lethality). Furthermore, conventional knockout strategies affect every cell in an animal, so that it is often impossible to distinguish primary and secondary changes in a complex phenotype. In order to tease out more precise information about the role of a gene in a specific cell

type at a critical stage of disease or development, more sophisticated approaches have been developed which extend and refine the possibilities of conventional transgenic technologies: conditional transgenic technologies, which allow flexible temporo-spatial control of gene expression or deletion by specific stimuli (rev. in Ryding *et al.* 2001).

2.1.2.1 Conditional overexpression

The ideal conditional overexpression system should allow the investigator to switch transgene expression on and off, rapidly, reversibly, at any point during development and postnatal life, and only in the desired cell type. Many systems have been developed harnessing the inherent responsiveness of specific promoters to various stimuli, such as tetracycline responsive system, ecdysone induction system and cytochrome P-450 induction system (rev. in Ryding *et al.* 2001).

2.1.2.2 Conditional knockout

Embryonic lethality in many conventional knockouts impedes attempts to study gene function in postnatal life. One way of avoiding this is to ablate specific genes at later stages of development or adulthood using recombinases. Two members of the α integrase family of site-specific recombinases (SSR), Cre and Flp, have proven invaluable for conditional transgenic use.

Cre recombinase of the P1 bacteriophage directs recombination between *loxP* sites. Its function is to maintain phage-encoding plasmids as monomers. In a similar manner the Flp integrase of *Saccharomyces cerevisiae* mediates recombination between FRT (FLP recombination target) sites within yeast plasmids. In each case, the only requirements for DNA rearrangement are the integrase and the recombination sites, no additional cellular factors are necessary. Both *lox*P and FRT sites are 34 bp DNA sequences comprising two 13 bp palindromes separated by an asymmetric 8 bp core. The recombinases catalyse DNA strand exchange between two aligned recombination sites, resulting in deletion, duplication, integration, inversion or translocation of

sequences, according to the orientation of the recombination sites and the number of molecules involved (Ryding *et al.* 2001).

The Cre-loxP system. Modification of a specific gene with *loxP* sites flanking the region of interest (so called 'floxed gene/segment') is achieved using standard gene targeting vectors in ES cells. Mice derived from these targeted ES cells can be bred to homozygosity for the floxed allele, and crossing with other mice transgenic for Cre recombinase under the control of a specific promoter allows time- or tissue-specific deletion of the floxed segment. In this way, the activity of the gene can be modified in a limited range of cells at a particular developmental stage. Crossing with a different Cre transgenic mouse line allows the generation of a different temporo-spatial pattern of recombination, and possible phenotype. Tissue specificity is determined by the choice of promoter driving Cre recombinase expression. Many tissue-specific Cre transgenic and floxed mice strains are becoming available (see *http://www.mshri.on.ca/nagy/cre.htm*). Several strategies have also been designed to control the timing of recombination, using inducible forms of Cre recombinase (Ryding *et al.* 2001). Cre-*loxP* has been used most extensively for small-scale DNA rearrangements affecting single loci to study the function of a single gene. However, it can also be used for generating megabase chromosome rearrangements, which provides a powerful means for functional analysis of complex genomic regions (Mills & Bradley 2001).

The Flp-FRT system. The recombination efficiency of Flp is inferior to that of Cre, and no ubiquitously expressing reporter lines for Flp are currently available. Thus, the use of Flp in transgenic mice is at a less advanced stage than that of Cre. However, an enhanced mutant form of Flp, Flpe, was developed, which exhibits fourfold greater activity than wild-type form, and maximally excises a target gene in a broadly expressing reporter line (Buchholz *et al.* 1998; Rodriguez *et al.* 2000). Moreover, use of Flp and Cre in combination allows sophisticated manipulation of loci, for example, removal of unwanted plasmid sequences from a conditional allele at the ES cell stage with Flp, and subsequently deletion by Cre *in vivo* (Moon & Capecchi 2000). A floxed hypomorphic allele could be knocked out completely using conditional Cre, or reverted back to wild-type using Flp (Meyers *et al.* 1998). Recently, a more efficient targeting method has been developed based on the combined use of the two

independent site-specific recombination systems described above (Lauth *et al.* 2002). The gene of interest is flanked by lox P on one side and FRT on the other side (called a froxed gene). ES cells carrying the froxed gene are transfected with a replacement plasmid harbouring the same sites and a plasmid expressing Cre and Flpe. This method, called *Froxing*, allows to unidirectionally deliver transgenes to a defined genomic locus, renders the introduced sequence more stable and allows for a higher integration rate without selection (14-15%), compared to systems that utilize only a single SSR.

2.1.3 Gene-trap mutagenesis in mice

Both transgenic and gene-targeting technologies require that the gene to be manipulated has been isolated and that the structure of the gene is known, at least partially. Another approach that allows the mutagenesis of uncharacterized genes is the gene-trap technology (Cecconi & Meyer 2000; Wiles *et al.* 2000). In this strategy, ES cells are electroporated with a so-called gene trap vector. These vectors are designed to integrate at random sites in the genome and to use the transcriptional activity of the endogenous target gene to drive a selection cassette and eventually a reporter gene. The integration of the gene trap vector may generate an insertional mutation. The gene trap vector also provides a tag to easily identify the targeted gene and the reporter gene may be used for an expression analysis, for example, for the identification of genes with specific expression patterns during embryogenesis. Mice generated from gene-trapped ES cells are subsequently used for the phenotypic analysis. The gene-trap technology is a powerful tool for combining large-scale random mutagenesis in the mouse with a rapid identification of the mutated gene and characterization of its biological functions.

2.2 Insulin-like growth factor (IGF) system

2.2.1 IGF peptides

In 1957, Salmon et al. found a growth hormone (GH)-dependent serum factor that stimulated sulfate incorporation into the ECM in rat cartilage. It was initially designated sulfation factor, and subsequently termed somatomedin C (Daughaday *et*

al. 1972), which ultimately was shown to be IGF-I (Rinderknecht & Humbel 1978a). In the 1960s, several laboratories made efforts to identify serum components with insulin-like effects on metabolism which were not neutralized by anti-insulin antibodies. These effects were designated nonsuppressible insulin-like activities (Froesch *et al.* 1963; Megyesi *et al.* 1974), which were ultimately shown to correspond to both IGF-I and IGF-II (Rinderknecht & Humbel 1976a). In the early 1970s, a polypeptide fraction with multiplication-stimulating activity for chicken embryo fibroblasts was described in calf serum (Pierson, Jr. & Temin 1972) and rat liver cell conditioned medium (Dulak & Temin 1973a; Dulak & Temin 1973b) This factor was then identified as IGF-II (Rinderknecht & Humbel 1978b). The initial purification of these disparate factors suggested that they had overlapping activities and the term *insulin-like growth factor* was proposed to signify their relationship to insulin and to emphasize their growth-promoting activities (Rinderknecht & Humbel 1976b). It is currently known that insulin-like peptides and their cellular receptors represent two superfamilies of regulatory proteins whose common ancestry extends back to early metazoan evolution. The involvement of these proteins in cellular anabolic processes is widespread throughout the animal world, where both ligand and receptor homologs are seen to regulate metabolite uptake, mitogenesis, synthetic and growth functions, and developmental processes (rev. in Kelley *et al.* 2002).

IGF-I and IGF-II are small single-chain peptides, approximately 7.5 kDa in size, which are structurally ~70% identical to one another and similar to pro-insulin. In addition to A and B domains, the IGFs also retain the C domain which is spliced out of insulin during pro-hormone processing, and contain an additional C-terminal D domain which is not found in insulin. C-terminal E peptides, which are generally cleaved posttranslationally, are also found in the IGF pro-peptides (rev. in Rechler & Nissley 1990; LeRoith & Roberts, Jr. 1993). Unlike insulin which is produced only by the pancreatic beta cells, IGFs are expressed in many cell types and tissues and act in endocrine, autocrine or paracrine manner to regulate cellular proliferation, survival and differentiation (rev. in Cohick & Clemmons 1993; Stewart & Rotwein 1996; Butler & LeRoith 2001).

IGF-I is a basic protein of 70 amino acids (aa). The human *IGF1* gene is located on chromosome 12. The major source of circulating IGF-I is the liver, but IGF-I is

widely expressed in most tissues, especially during postnatal development (Daughaday & Rotwein 1989). IGF-I was first identified as a mediator of GH actions. The primary regulator of *Igf1* transcription is GH. All species of IGF-I mRNAs increase after GH administration, although the magnitude of change varies with tissues and subclasses of mRNA (Roberts, Jr. *et al.* 1987). In addition to GH, hormonal, tissue-specific and developmental factors as well as nutritional status all modify IGF-I expression (Daughaday & Rotwein 1989). Null mutants for *Igf1* in mice show severe growth retardation with a highly variable, strain-dependent perinatal lethality (Baker *et al.* 1993; Liu *et al.* 1993; Powell-Braxton *et al.* 1993a; Powell-Braxton *et al.* 1993b) and also marked alteration in their nervous system (Beck *et al.* 1995) and reproduction (Baker *et al.* 1996).

IGF-II is a slightly acidic protein of 67 aa. The human *IGF2* gene is located on chromosome 11, contiguous with the insulin gene. In contrast to IGF-I, IGF-II is highly expressed embryonically (Stylianopoulou *et al.* 1988a; Lee *et al.* 1990). In rodents, IGF-II levels decrease postnatally (Moses *et al.* 1980; Daughaday *et al.* 1982), except in the choroids plexus and leptomeninges of the brain (Stylianopoulou *et al.* 1988b). In human, circulating IGF-II remains high in the adult (Rechler and Nissley 1990). Although IGF-II expression is also regulated hormonally, the trophic factors involved in the regulation of IGF-II expression are poorly characterized (Daughaday & Rotwein 1989). Inactivation of *Igf2* gene in mice produces growth-deficient but fertile and otherwise normal individuals (DeChiara *et al.* 1990). The growth deficiency of heterozygous mice carrying a disrupted paternal allele revealed that the *Igf2* gene is subject to maternal genomic imprinting. The imprinting of the *Igf2* gene has led to the argument for additional regulation of IGF-II at the level of gene dosage (DeChiara *et al.* 1991; Filson *et al.* 1993). Although IGF-II is primarily an important regulator of embryonic growth and differentiation in the rodent, transgenic mouse models revealed some functions of this peptide in growth, metabolism and tumorigenesis in postnatal life (rev. in Wolf *et al.* 1998).

2.2.2 IGF receptors

Type 1 IGF receptor (IGF-IR). Most of the IGF actions are mediated by IGF-IR. Like the insulin receptor (IR), the IGF-IR is a member of the tyrosine-kinase class of

growth factor receptors. The IGF-IR is expressed at the cell surface as a heterotetramer, comprised of two ligand-binding α and two transmembrane β subunits. The IGF-IR is similar in topography and sequence to the insulin receptor (IR) and shares more than 50% amino acid identity (Ullrich *et al.* 1986; Abbott *et al.* 1992; LeRoith *et al.* 1995). The IGF-IR binds IGF-I and IGF-II with high affinity, and insulin with considerably lower affinity. Ligand binding to extracellular cysteine-rich region within α-subunits of the IGF-IR induces activation of intracellular tyrosine kinase domain of each β-subunit. After autophosphorylation on intracellular tyrosine residues, the receptor is fully activated as a tyrosine kinase towards endogenous substrates (LeRoith 2000). Activation of the IGF-IR triggers intracellular events that regulate the cell cycle, apoptosis, cell mobility and gene expression (De Meyts *et al.* 1994). In addition to binding IGF-I, IGF-II and insulin, the IGF-IR has also been reported to interact with IGFBP-3 (Mohseni-Zadeh & Binoux 1997), but the significance of this binding is currently undefined. *Igf1r* knockout mice weigh 45% of normal at birth and die immediately afterwards due to respiratory failure (Baker *et al.* 1993; Liu *et al.* 1993). A targeted partial invalidation of the *Igf1r* gene exhibited a postnatal growth deficit of male hetero- and homozygous mice (Holzenberger *et al.* 2000). Patients with a deletion of the distal arm of chromosome 15 lack one copy of the *IGF1R* gene and exhibit both intrauterine and postnatal growth retardation (Siebler *et al.* 1995). Vast overexpression of human IGF-IR in mouse and rat fibroblasts has been found to cause IGF-dependent neoplastic transformation (Kaleko *et al.* 1990). This study highlights a potential role for the IGF-IR in tumorigenesis.

Type II IGF receptor (IGF-IIR). IGF-IIR is a single-chain membrane-spanning glycoprotein that also is known as the cation-independent mannose-6-phosphate (Man-6-P) receptor (Kornfeld 1992). The human *IGF2R* gene has been mapped to chromosome 6 (Laureys *et al.* 1988). In opposition to the *Igf2* gene, the expression of the *Igf2r* gene is paternally imprinted (Barlow *et al.* 1991). The mature human IGF-IIR contains 2451 amino acids that can be divided into three regions, a large 2264-residue extracellular domain, a 23-residue transmembrane region and a 164-residue C-terminal intracytoplasmic domain (Morgan *et al.* 1987; Oshima *et al.* 1988). The extracellular part of the IGF-IIR encodes a single binding site for IGF-II and two sites for Man-6-P-containing ligands, and the intracytoplasmic region regulates movement

among different cellular compartments. IGF-IIR binds IGF-II with high affinity but interacts minimally with IGF-I and insulin (Kornfeld 1992). The coordinated expression of IGF-II and IGF-IIR in most mammalian tissues and gene targeting experiments suggest a role of IGF-IIR in the control of extracellular IGF-II concentration by receptor-mediated endocytosis and subsequent degradation of the growth factor in lysosomes (Lau *et al.* 1994; Ludwig *et al.* 1996; Braulke 1999). Currently, there is no evidence that the IGF-IIR is involved in the IGF-signaling.

2.2.3 IGF-binding proteins

Unlike insulin, the IGFs in serum and other biological fluids are bound to specific IGF-binding proteins (IGFBPs) which were initially discovered as carrier proteins in serum (Zapf *et al.* 1975). The IGFBPs represent a family of six conserved proteins (IGFBP-1 to -6) that bind to IGFs with affinities similar to or greater than the IGF-IR. Six IGFBPs cloned in mammals share a 50% homologous protein sequence overall and up to 80% sequence homology among different mammalian species (Rechler 1993; Kelley *et al.* 1996). Human *IGFBP1* and *IGFBP3* genes are located on chromosome 7, *IGFBP2* and *IGFBP5* on chromosome 2, *IGFBP4* on chromosome 17, and *IGFBP6* on chromosome 12 (Allander *et al.* 1993; Allander *et al.* 1994; Ehrenborg *et al.* 1999; Hwa *et al.* 1999). The mammalian IGFBPs share a common domain organization, possessing a highly cysteine-rich amino (N)-terminal domain (12 cysteine residues), a cysteine-rich carboxy (C)-terminal domain (6 cysteine residues), and a central (L) domain with no cysteine residue except in IGFBP-4. The N- and C-domains are highly conserved among different IGFBPs in a given species, and the L-domain is highly variable (rev. in Duan 2002). Mutational studies suggest that both the N- and C-domains are involved in high-affinity IGF-binding (rev. in Clemmons 2001). IGF-binding may be modulated by IGFBP modifications, such as glycosylation, phosphorylation and proteolysis, and by cell surface or ECM association of the IGFBPs (rev. in Baxter 2000; Firth & Baxter 2002). Several low-affinity IGF binders, termed IGFBP-related proteins (IGFBP-rPs), have also been discovered that exhibit significant structural homology to the N-domain of the six high-affinity IGFBPs (rev. in Hwa *et al.* 1999). The functional significance of the IGFBP-rPs for the IGF system, if any, is currently unknown.

IGFBPs are present in serum and other biological fluids and are secreted by a broad spectrum of cell types. Numerous *in vitro* studies have demonstrated that IGFBPs have a diversity of functions depending on cell types, culture conditions, IGFBP dose and post-translational modification. They all can inhibit IGF actions by binding to IGFs and preventing the binding of IGFs to the IGF receptors (rev. in Clemmons 1997; Clemmons 1998; Baxter 2000), whereas some of them (e.g. IGFBPs-1, -3 and -5) also potentiate IGF actions presumably due to their cell surface or ECM association (rev. in Jones *et al.* 1993a; Mohan *et al.* 1995b; Clemmons 1998). In addition, some IGFBPs (e.g. IGFBP-3 and -5) have IGF-independent actions probably by signaling via IGFBP receptors (Oh *et al.* 1993; Leal *et al.* 1997; Andress 1998) or by nuclear localization and interaction with transcriptional modulators (Radulescu 1994; Liu *et al.* 2000; Amaar *et al.* 2002). The roles of IGFBPs *in vivo* are only partially defined. Transgenic and knockout technologies in mice provide important tools to alter level and tissue-specificity of expression of a particular IGFBP and – after extensive phenotypic analysis – draw conclusions regarding its functions. Although knockout mice have been produced for all IGFBPs, they showed only minor phenotypic alterations, probably due to functional compensation of the lacking IGFBP by the remaining ones (Pintar *et al.* 1995; Pintar *et al.* 1998; Wood *et al.* 2000). In contrast, transgenic mice overexpressing a particular IGFBP exhibited more clear phenotypes, which confirmed that IGFBPs are important regulators of IGF actions and also revealed some new functions (rev. in Schneider *et al.* 2000; Silha & Murphy 2002). A summary of the established IGFBP transgenic mouse models is shown in Table 2.1.

Table 2.1 Effects of overexpression of IGFBPs and ALS in transgenic mice

IGFBP	Transgene [a]	Transgene expression	Phenotypic manifestation	Ref
IGFBP-1	MT-hBP-1	Brain, heart, kidney, liver, lung, testes	Abnormal brain development.	1,2
	mPGK-rBP-1	Ubiquitous	Reduction in birth weight, postnatal growth retardation, reduction in litter size, impaired glucose tolerance.	3
	hα1AT-hBP-1	Liver	Reduced brain weight with structural alterations, reduced body weight gain, impaired glucose tolerance, reduced fecundity, proteinuria and glomerular lesions.	4
	entire hBP-1 [b]	Liver	Impaired glucose tolerance, abnor-	5

			malities in insulin action, growth retardation in early postnatal life.	
IGFBP-2	CMV-mBP-2	Ubiquitous	Reduced postnatal body weight gain.	6,7
IGFBP-3	mPGK-hBP-3	Ubiquitous	Reduction in birth weight, postnatal growth retardation, reduction in litter size, impaired glucose tolerance.	8
	CMV-hBP-3	Ubiquitous	Reduction in birth weight, postnatal growth retardation, catch-up growth after puberty, reduction in litter size, increased adiposity, impaired glucose tolerance and insulin sensitivity.	8
	MT-hBP-3	Kidney, intestine	Selective organomegaly, spleen, liver, heart.	9
	WAP-hBP-3	Mammary epithelial cells during late pregnancy and throughout lactation	Reduced size of alveoli at peak lactation, inhibition of the gland from undergoing programmed remodelling and apoptosis after weaning.	10
IGFBP-4	mSMP-rBP-4	Smooth muscle cells (SMC)	Smooth muscle hypoplasia, significant reduction in wet weight of SMC-rich tissues, including bladder, intestine, aorta, uterus and stomach, with no change in total body or carcass weight.	11
	mSMP-rBP4.7A [c]	SMC	Greater growth inhibition of SM than native IGFBP-4.	12
IGFBP-5	sBLG-hBP-5	Mammary epithelial cells during involution of the gland	Impaired mammary gland development, decreased mammary cell number and milk synthesis, premature cell death.	13
	rOC-rBP-5	Bone	Transient decrease in trabecular bone volume, impaired osteoblastic function, osteopenia.	14
ALS	CMV-hALS [d]	Ubiquitous	Modest postnatal growth retardation, reduction in litter size	15

[a] MT, metallothionein-I promoter; PGK, phosphoglycerate kinase I promoter; α1AT, alpha1-antitrypsin promoter; CMV, cytomegalovirus promoter; WAP, whey acidic protein promoter; SMP, smooth muscle α-actin promoter; BLG, β-lactoglobin promoter; OC, osteocalcin promoter; m, murine; r, rat; s, sheep; h, human. [b] A 35-kb fragment encompassing the entire human *IGFBP1* structural gene and its regulatory sequences that is responsive to normal hormonal stimuli. [c] BP4.7A, a protease-resistant IGFBP-4 mutant. [d] ALS, the acid-labile subunit that forms ternary complex with IGFBP-3 and IGF-I or IGF-II, responsible for transport of the majority of the IGF-I and IGF-II present in the circulation.1, Dai *et al.* 1994; 2, D'Ercole *et al.* 1994; 3, Rajkumar *et al.* 1995; 4, Gay *et al.* 1997; 5, Crossey *et al.* 2000; 6, Hoeflich *et al.* 1999; 7, Hoeflich *et al.* 2001; 8, Modric *et al.* 2001; 9, Murphy *et al.* 1995a; 10, Neuenschwander *et al.* 1996; 11, Wang *et al.* 1998; 12, Zhang *et al.* 2002; 13, Tonner *et al.* 2002; 14, Devlin *et al.* 2002; 15, Silha *et al.* 2001.

2.3 IGFBP-4

2.3.1 Genomic organization of the IGFBP-4 gene

The human *IGFBP4* gene is located on chromosome 17 (Allander *et al.* 1993) and spans about 15.3 kb long (Zazzi *et al.* 1998). According to the mouse genome sequence determined so far, the mouse *Igfbp4* gene spans 11.3 kb on chromosome 11 (*http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=16010*). The rat *Igfbp4* gene spans at least 12 kb of genomic sequence (Gao *et al.* 1993). The genes for human and rat IGFBP-4 are composed of four exons separated by three introns, which give them an arrangement similar to the genes of the other IGFBPs except for IGFBP-3 (Cubbage *et al.* 1990). The splice sites are highly conserved between human *IGFBP4* and rat *Igfbp4* genes, but the sizes of the introns vary slightly between the two species (Zazzi *et al.* 1998). Cell type-specific transcript sizes were documented in mouse cell lines which, when translated, suggest an additional non-IGF binding variant present in mouse cells (Glantschnig *et al.* 1998).

Alignment of the published rat (Gao *et al.* 1993), human (Dai *et al.* 1997) and mouse (Glantschnig *et al.* 1998) IGFBP-4 promoter sequences revealed an overall high evolutionary conservation, but some promoter regions show less conservation and vary between the three species. It is interesting that the human sequence differs from rodent sequences by a 12-bp insertion upstream to the transcription initiation codon (Dai *et al.* 1997). The IGFBP-4 promoter possesses a typical TATA box and a CAAT box. Several potential regulatory elements, such as cAMP responsive element (CRE), steroid responsive element (SRE), AP-1 binding site, Sp1 binding site, exist in the IGFBP-4 5' flanking regions of the three species (Gao *et al.* 1993; Dai *et al.* 1997; Glantschnig *et al.* 1998; Zazzi *et al.* 1998). These *cis*-regulatory binding sites provide the targets for a variety of local and systemic factors such as cAMP, parathyroid hormone (PTH) and various ligands of the steroid hormone receptor superfamily (such as glucocorticoids, retinoic acid, triiodothyronine, vitamin D), to regulate the expression of IGFBP-4 as discussed below.

Several *Alu* repeat sequences are clustered in the proximity (upstream) of the human *IGFBP4* gene, with an average of one *Alu* sequence per kilobase (Zazzi *et al.* 1998), which is a higher frequency than the normal distribution in the human genome (Houck *et al.* 1979). This indicates that the *IGFBP4* gene is a hot spot for *Alu* integration. High-density *Alu* regions are often sites of genomic instability (Calabretta *et al.* 1982) and show a higher frequency of sequence polymorphism (Batzer & Deininger 2002). Apart from the *Alu* repeat sequences, several polymorphic microsatellites were found within the boundaries of the human *IGFBP4* gene (Zazzi *et al.* 1998). One of these was used as a marker to locate the hereditary breast-ovarian cancer gene (Tonin *et al.* 1993). Another highly polymorphic microsatellite was found in the first intron of the human *IGFBP4* gene (Zazzi *et al.* 1998).

A typical cleavage site for poly(A) was found at the 3'-end of the human *IGFBP4* gene, however no conserved poly(A) addition signal was detected within the 30 bp upstream region. Nevertheless, within this region an AAAAAA and several AACAAA consensus sequences were found, which could form a degenerate poly(A) addition signal. The few described eukaryotic genes that do not contain a standard AAUAAA sequence are involved in alternative polyadenylation, but this does not seem to be the case for the human *IGFBP4* gene, since no variation in mRNA length has been reported and no alternate polyadenylation site was found within the *IGFBP4* gene (Zazzi *et al.* 1998).

2.3.2 The structure-function relationship of IGFBP-4

IGFBP-4 is the smallest one of the six IGFBPs. Human (h)IGFBP-4 contains 237 aa, and rat (r)IGFBP-4 consists of 233 aa. IGFBP-4 contains an N-linked glycosylation site and commonly exists in biological fluids as a doublet: a 24-kDa non-glycosylated form and a 28-kDa glycosylated form (Wetterau *et al.* 1999). IGFBP-4 is unique among the six IGFBPs by having two extra cysteine residues in the variable L-domain (Landale *et al.* 1995), which are linked to each other (Chelius *et al.* 2001). These unique properties of IGFBP-4 may be responsible for the distinctive biological behavior of this binding protein, i.e. solely inhibitory actions and lack of cell surface association.

2.3.2.1 IGF-binding

It is generally accepted that the IGF-binding site for the various high-affinity IGFBPs is located in the N-terminal region. Mutational analysis indicated that the IGF-binding activity of hIGFBP-4 is mainly determined by the N-terminal region Leu^{72}-Ser^{91}, and to a lesser extent by the C-terminal motif Cys^{205}-Val^{214} (Qin *et al.* 1998). Although the three-dimensional structure of most IGFBPs has not been determined, disulfide bridging in IGFBPs appears to be important for maintaining the secondary structure required for IGF-binding, since all six IGFBPs contain conserved cysteine residues in both the N-terminal and the C-terminal regions and reduced IGFBPs exhibit little or no IGF-binding activitiy (Landale *et al.* 1995; Qin *et al.* 1998; Neumann & Bach 1999). Direct evidence was provided by disruption of the disulfide linkages in the N-domain of rat IGFBP-3 which resulted in complete loss of IGF-binding ability (Hashimoto *et al.* 1997). There is evidence suggesting that the IGF-binding domain in hIGFBP-4 involves a hydrophobic motif (Leu^{72}-Met^{80}) located in the distal part of the conserved N-terminal region, and that the N-terminal Cys residues (Cys9 and Cys12) are more critical than the C-terminal Cys residues (Cys17 and Cys 20) for IGF-binding (Byun *et al.* 2001a). Eight disulfide linkages in rat and human IGFBP-4 have been determined, four in the N-terminal (two of them are present in all six IGFBPs), three in the C-terminal (present in IGFBP-2 and -6 as well) and one in the midregion (Chelius *et al.* 2001).

Although the IGF-binding is mainly determined by the N-terminal conserved cysteine residues, the six conserved C-terminal cysteine residues in IGFBP-4 are essential for high-affinity binding of IGFs (Qin *et al.* 1998; Standker *et al.* 2000; Byun *et al.* 2001a). There is also evidence that in IGFBP-3 (Spencer & Chan 1995) and IGFBP-2 (Forbes *et al.* 1998; Wang *et al.* 1988) the C-terminal region plays an important role in IGF-binding. The six C-terminal cysteine residues in IGFBP-4 are linked in the same manner as in IGFBP-2 and -6 (Chelius *et al.* 2001). Both IGFBP-2 and -6 share a binding preference for IGF-II and have the same C-terminal disulfide linkages, suggesting that a different disulfide linkage could conceivably influence IGF-II-binding preference (Forbes *et al.* 1998). However, IGFBP-4 binds IGF-I and IGF-II with similar affinities, thus the highly conserved three C-terminal disulfide linkages

either have no effect on the IGF-binding preference, or they are not the sole determinants.

2.3.2.2 Cell surface association and tissue distribution

Cell surface and ECM association is one of the possible mechanisms to alter the affinity of IGFBPs for the IGFs. Both IGFBP-1 and IGFBP-2 have an Arg-Gly-Asp (RGD) motif, which was shown to bind α5β1 integrin and consequently to mediate cell surface association of IGFBP-1 (Jones *et al.* 1993b). IGFBP-2 bound to the plasma membrane also in the absence of the RGD motif in IGFBP-2 (Hoeflich *et al.* 2002), indicating that additional mechanisms are involved in cell surface association of IGFBP-2. IGFBP-2 can bind to heparin, ECM and proteoglycans depending on the previous binding to IGF molecules (Arai *et al.* 1996). IGFBP-3 and -5 bind to distinct membrane receptors (Oh *et al.* 1993; Andress 1995; Leal *et al.* 1997; Andress 1998). However, there is no evidence for cell surface association of IGFBP-4 (Kelley *et al.* 1996), suggesting that IGFBP-4 exists primarily in a soluble extracellular form.

When IGFBP-3 was perfused through the isolated, beating rat heart, it crossed the microvascular endothelium and was distributed primarily in cardiac muscle. In contrast, perfused IGFBP-4 also crossed the microvascular endothelium of the rat heart, but was preferentially distributed in connective tissue (Bar *et al.* 1990; Boes *et al.* 1992). A small basic C-terminal region (heparin binding domain, HBD) of IGFBP-3 has been shown to be central to the ability of IGFBP-3 to bind to specific cells, such as endothelial cells (Booth *et al.* 1995; Knudtson *et al.* 2001). When this region was synthesized as 18-mer peptide (P3), P3 bound to endothelial cells (Booth *et al.* 1996). IGFBP-4 has no HBD and does not bind to endothelial cells (Booth *et al.* 1995). When the C-terminal 20 amino acids of IGFBP-4 region (P4) are replaced by the homologous P3, the generated chimeric IGFBP-4_3 bound specifically to endothelial cells, and it was distributed in the perfused rat heart similarly as the behavior of IGFBP-3 and different from that of IGFBP-4 (Knudtson *et al.* 2001), suggesting that the C-terminal region of IGFBP-4 is critical for its tissue distribution in the rat heart. These findings provide a novel potential mechanism of the tissue-specific actions of the IGFBPs.

2.3.2.3 Glycosylation

In 1991, Ceda and colleagues isolated two IGFBPs with apparent molecular weights (MW) of 28 and 24 kDa in the conditioned medium of B104 rat neuroblastoma cells (Ceda *et al.* 1991). Sequence analysis revealed that both proteins had identical N-terminal sequences and appeared to be two forms of IGFBP-4. Treatment of these IGFBPs with endoglycosidase-F reduced the MW of the 28-kDa IGFBP to 24 kDa. However, there was no change in the 24-kDa IGFBP. The data from this and other studies (Carr *et al.* 1994; Cheung *et al.* 1991) demonstrated that IGFBP-4 exists as both N-glycosylated and non-glycosylated protein. Further analysis revealed that a single N-linked glycosylation site is located at the midregion (Asn^{104}) of the rIGFBP-4 and that the glycosylation of IGFBP-4 does not affect its binding to IGFs (Chelius *et al.* 2001). Five different glycosylation isoforms of rIGFBP-4, isolated from rat serum, were recently identified (Chelius *et al.* 2002). All the identified oligosaccharides are biantennary and differ only in the number of sialic acid terminal residues and/or core modification with fucose. The physiological significance of the glycosylation in IGFBP-4 is unknown.

2.3.2.4 Proteolysis

Proteolysis is a major regulatory mechanism of IGFBP-4 functions. Each of the six IGFBPs can undergo proteolysis, which results in decreased affinity for IGFs. While some of the proteases can use multiple IGFBPs as a substrate, there are apparently proteases that are specific for individual IGFBPs (Maile & Holly 1999). An IGF-dependent IGFBP-4-specific protease was first reported in the media conditioned by both human and sheep dermal fibroblasts (Fowlkes & Freemark 1992), which was then identified as pregnancy-associated plasma protein-A (PAPP-A) (Lawrence *et al.* 1999b). This proteolytic activity has also been detected in the conditioned media from human osteoblasts (Qin *et al.* 1999b), vascular smooth muscle cells (Bayes-Genis *et al.* 2001), granulosa cells (Conover *et al.* 2001), trophoblast and decidualized endometrial stromal cells (Giudice *et al.* 2002), as well as in ovarian follicular fluid (Conover *et al.* 1999) and human pregnancy serum (Byun *et al.* 2001b).

PAPP-A was first isolated from human pregnancy serum (Lin *et al.* 1974) and belongs to the large metzincin family of metalloproteases (Lawrence *et al.* 1999a; Boldt *et al.* 2001). It cleaves IGFBP-4 at a single site, between Met^{135}/Lys^{136} in hIGFBP-4 (Byun *et al.* 2000; Laursen *et al.* 2002). IGFBP-4 cleavage by PAPP-A uniquely depends on the presence of IGF (Byun *et al.* 2000; Laursen *et al.* 2001). Recent data suggested that IGFs enhance the proteolysis by binding to IGFBP-4, but not by interaction with PAPP-A (Qin *et al.* 2000; Laursen *et al.* 2001). PAPP-A also cleaves hIGFBP-5 between Ser^{143}/Lys^{144}, which does not require the presence of IGF, but is slightly inhibited by IGF (Laursen *et al.* 2001). PAPP-A is secreted as a dimer of 400 kDa, but exists in human pregnancy serum as a 500 kDa covalent heterotetrameric 2:2 complex with the proform of eosinophil major basic protein (proMBP), which functions as an inhibitor of the proteolytic activity of PAPP-A (Overgaard *et al.* 2000). IGFBP-3, -5 and -6 can also inhibit IGFBP-4 proteolysis, likely through the homologous, highly basic heparin-binding domains in the C-termini of these IGFBPs (Fowlkes *et al.* 1997). Proteolysis of IGFBP-4 by PAPP-A enhances IGF bioavailability. Its physiological significance will be discussed below.

2.3.3 IGFBP-4 expression in vivo and its regulation

IGFBP-4 has been identified in all biological fluids, including serum, follicular fluid, seminal fluid, interstitial fluid and synovial fluid (Rajaram *et al.* 1997). It is the second most abundant IGFBP in adult rat serum after IGFBP-3. Northern blot analysis revealed that IGFBP-4 mRNA is widely expressed in adult rat tissues, including adrenal gland, testis, spleen, heart, liver, lung, kidney, stomach, hypothalamus and brain cortex, with liver being the site of the highest expression (Shimasaki *et al.* 1990). The expression of IGFBP-4 was also examined in rat small intestine (Shoubridge *et al.* 2001), smooth muscle (Smith *et al.* 2001), skeletal muscle (Jennische & Hall 2000), pancreas (Hill *et al.* 1999), uterus and placenta (Cerro & Pintar 1997), mouse spinal cord (Arnold *et al.* 2000), mouse and human thymus (Li *et al.* 1996), human prostate (Thomas *et al.* 2000), bone (Mohan *et al.* 1995a) and ovary (Zhou & Bondy 1993; el Roeiy *et al.* 1994) of several species. In the mouse embryo, IGFBP-4 transcripts were detected as early as day 11 in different regions, including telencephalon, mesencephalon, snout, tongue and differentiating sclerotomes. After 14 dpc, IGFBP-4 mRNA was undetectable in the brain, but clearly detectable in lung,

liver, kidney, intestine, vertebrae, ribs and incisivi (Schuller *et al.* 1993). IGFBP-4 protein was localized in telencephalon, mesencephalon, heart, liver, lung, tongue, blood vessels and kidney of 13.5-dpc-mouse embryo by immunohistochemical analysis (van Kleffens *et al.* 1999). These findings suggest that IGFBP-4 expression is developmentally regulated. Moreover, hormones, cytokines and other agents also regulate the expression of IGFBP-4 in a tissue-specific manner (see Table 2.2).

Table 2.2 Regulatory mechanisms for IGFBP-4 expression *in vivo*.

Agent	Tissue type/species	Effect	Reference
GH	Serum/zinc-deprived rat	s	Ninh *et al.* 1998
	Serum/postmenopausal woman	s	Kassem *et al.* 1998
	Serum/bGH transgenic mice	s	Blackburn *et al.* 1997
GHRA	Serum, liver/m	s	van Neck *et al.* 2000
GHRP-2	Plasma/b (high feed intake)	s	Lee *et al.* 2000
	Plasma/b (low feed intake)	n	Lee *et al.* 2000
T_3	Kidney, serum/hypothyroid rat	s	Voci *et al.* 2001
	Liver, serum/hypothyroid rat	s	Demori *et al.* 1997b
	Uterus/r	s	Bottazzi *et al.* 1996
T_4	Liver, mammary gland/r	s	Rosato *et al.* 2002
hCG	Mammary gland/r	i	Huynh 1998
	Ovary/r	s	Putowski *et al.* 1997
PMSG	Ovary/r	s	Putowski *et al.* 1997
FSH	Ovary/r	s	Putowski *et al.* 1997
Clenbuterol	Soleus muscle/r	s	Awede *et al.* 2002
Nandrolone	Diaphragm muscle/r	i	Lewis *et al.* 2002
Estrogen	Serum/constitutionally tall girls	s	Rooman *et al.* 2002
EB1089	Prostate/r	s	Nickerson & Huynh 1999
LPS	Serum/r	s	Soto *et al.* 1998

GHRA, GH receptor antagonist; GHRP-2, GH releasing peptide-2; T_3, triiodothyronine; T_4, thyroxine; hCG, human chorionic gonadotropin; PMSG, pregnant mare's serum gonadotropin; FSH, follicle-stimulating hormone; EB1089, vitamin D_3 analogue; LPS, lipopolysaccharide; b, bovine; h, human; m, mouse; r, rat; s, stimulation; i, inhibition; n, no effect.

2.3.4 IGFBP-4 expression in vitro and its regulation

Consistent with the widespread expression of IGFBP-4 *in vivo*, IGFBP-4 is expressed by various cell types *in vitro*, including fibroblasts, osteoblasts, myoblasts, epithelial cells, endothelial cells, chondrocytes and many kinds of tumor cells. The expression of IGFBP-4 *in vitro* is regulated by a large number of agents in a cell type-specific manner. The effects of these agents are summarized in Table 2.3.

Table 2.3 Regulatory mechanisms for IGFBP-4 expression *in vitro*.

Agent	Cell types/Species	Effect	Reference
ACTH	Adrenocortical cells/h	n	Fottner *et al.* 2001
ACTH and IGFs	Adrenocortical cells/h	s	Fottner *et al.* 2001
Activin	Granulosa cells/r	i	Choi *et al.* 1997
Androgen	Osteoblasts/h	i	Gori *et al.* 1999
	Granulosa cells/h	n	Greisen *et al.* 2002
Angiotensin II	Vascular smooth muscle cells/r	i	Anwar *et al.* 2000; Gustafsson *et al.* 1999b
bFGF	Multiple myeloma/h	n	Feliers *et al.* 1999
	Fetal lung fibroblasts/r	s	Price 1999
cAMP	Multiple myeloma/h	n	Feliers *et al.* 1999
	Vascular smooth muscle cells/h	s	Hayford *et al.* 1998
	Osteoblasts/r	s	Chen *et al.* 1998
	L6 myoblasts/r	s	McCusker & Clemmons 1998
	Articular chonodrocytes/h	i	Di Battista *et al.* 1997
Dexamethasone	Bone marrow stromal cells/r	s	Milne *et al.* 2001
	Bone marrow stromal cells/h	s	Cheng *et al.* 1998
	Vascular smooth muscle cells/h	s	Hayford *et al.* 1998
	TC3 pancreatic beta cells	s	Katz *et al.* 1997
	Fibroblasts/h,b	i	Conover *et al.* 1995
EGF	Fetal lung fibroblasts/r	s	Price 1999
Estrogen	MCF-7 breast cancer cells/h	s	Qin *et al.* 1999a
	SaOS-2 osteoblasts/h	i	Kudo *et al.* 1996 & 1997
	hFOB/ER9 osteoblasts/h	s	Kassem *et al.* 1996
FSH	Granulosa cells/r	i	Piferrer *et al.* 1997
GH	TC3 pancreatic beta cells	n	Katz *et al.* 1997
Glucagon	Ovarian thecal cells/b	n	Chamberlain & Spicer 2001
Glucose	Retinal endothelial cells/h	n	Giannini *et al.* 2001
Hypoxia	Vascular endothelial cells/b	n	Tucci *et al.* 1998
IGF-I	Retinal endothelial cells/h	i	Giannini *et al.* 2001
	Glomerular endothelial cells/h	i	Giannini *et al.* 1999
	Uterine myometrial cells	s	Huynh 2000
	Smooth muscle cells/h	i	Kuemmerle & Teng 2000
	Vascular smooth muscle cells/r	s	Gustafsson *et al.* 1999b
	Vascular smooth muscle cells/p	i	Duan & Clemmons 1998
	Vascular smooth muscle cells/h	i	Hayford *et al.* 1998
	L6 myoblasts/r	s	McCusker & Clemmons 1998
	Fibroblasts/h	i	Conover *et al.* 1995
	Fibroblasts/b	s	Conover *et al.* 1995
	SH-SY5Y neuroblastoma cells/h	s	Babajko *et al.* 1997
IGF-II	Marrow stromal cells/h	i	Thomas *et al.* 1999
	Adrenocortical cells/b	s	Fottner *et al.* 1999
	TC3 pancreatic beta cells	n	Katz *et al.* 1997
	Non-small cell lung cancer cells	i	Fottner *et al.* 1999
	SH-SY5Y neuroblastoma cells/h	s	Babajko *et al.* 1997
IL-1β	Ovarian granulosa cells	i	Chamoun *et al.* 1999
	Articular chonodrocytes/h	i	Di Battista *et al.* 1997

	Fetal lung fibroblast/r	s	Price *et al.* 2002
IL-6	Hepatocytes/r	s	Fernandez-Celemin & Thissen 2001
	Multiple myeloma/h	n	Feliers *et al.* 1999
IFN-γ	Multiple myeloma/h	s	Feliers *et al.* 1999
Insulin	Ovarian thecal cells/b	n	Chamberlain & Spicer 2001
	L6 myoblasts/r	s	McCusker & Clemmons 1998
	TC3 pancreatic beta cells	n	Katz *et al.* 1997
	SH-SY5Y neuroblastoma cells/h	s	Babajko *et al.* 1997
	Granulosa cells/h	s	Greisen *et al.* 2002
Insulin+glucagon	Ovarian thecal cells/b	i	Chamberlain & Spicer 2001
N-myc oncogene	SK-N-SH Neuroblastoma cells/h	s	Chambery *et al.* 1999
OP-1	Osteoblasts/r	i	Yeh *et al.* 1996
PDGF-BB	Fetal lung fibroblasts/r	s	Price 1999; Price 2001
	Multiple myeloma/h	n	Feliers *et al.* 1999
	Articular chonodrocytes/h	s	Di Battista *et al.* 1997
	SaOS-2 osteoblasts/h	s	Kudo *et al.* 1996 & 1997
RA	Sertoli cells/p	s	Bardi *et al.* 1999
	Neuroblastoma cells/h	i	Chambery *et al.* 1998
	Osteoblasts/m	s	Glantschnig *et al.* 1996
	HT-29 colon carcinoma cells	s	Corkins *et al.* 2002
T_3	Bone marrow stromal cells/r	s	Milne *et al.* 2001
	Sertoli cells/p	s	Bardi *et al.* 1999
	Primary hepatocytes/r	s	Demori *et al.* 1997a & b
	HepG2 hepatoma cells/h	n	Demori *et al.* 1997a
	Osteoblasts/m	s	Glantschnig *et al.* 1996
TGFα	Granulosa cells/r	s	Piferrer *et al.* 1997
TGFβ1	Glomerular endothelial cells/h	s	Giannini *et al.* 1999
	Multiple myeloma/h	n	Feliers *et al.* 1999
	Stromal-vascular cells/p	s	Richardson *et al.* 1998
Thrombin	Vascular smooth muscle cells/r	i	Anwar *et al.* 2000
TNF-α	Multiple myeloma/h	n	Feliers *et al.* 1999
	Fetal lung fibroblasts/r	s	Price *et al.* 2002
D_3	Bone marrow stromal cells/h	s	Kveiborg *et al.* 2001
WT-1	Embryonic stem cells/m	s	Wagner *et al.* 2001

ACTH, adrenocorticotropic hormone; bFGF, basic fibroblast growth factor; cAMP, cyclic adenosine monophosphate; EGF, epidermal growth factor; IL, interleukin; IFN, interferon; OP-1, osteogenic protein-1; PDGF-BB, platelet-derived growth factor-BB; RA, retinoic acid; TGF, transforming growth factor; TNF, tumour necrosis factor; D_3,1,25-dihydroxyvitamin D_3; WT-1, Wilms tumor-1 protein; b, bovine; h, human; m, mouse; p, porcine; r, rat; s, stimulation; i, inhibition; n, no effect.

2.3.5 Actions of IGFBP-4

Several lines of evidence suggest that IGFBP-4 functions as a purely inhibitory protein *in vitro and in vivo*. These inhibitory actions can be exerted via IGF-dependent and -independent pathways.

2.3.5.1 IGF-dependent actions

IGFBP-4 inhibits IGF-induced cell proliferation and differentiation in all cell types studied *in vitro* so far, including bone cells (Schiltz *et al.* 1993; Mohan *et al.* 1995b; Mohan & Baylink 2002), muscle cells (Damon *et al.* 1998a; Ewton *et al.* 1998; Gustafsson *et al.* 1999a), B104 rat neuroblastoma cells (Cheung *et al.* 1991), HT-29 human colon adenocarcinoma cells (Culouscou & Shoyab 1991), and M12 human prostate cancer cells (Damon *et al.* 1998b). These inhibitory actions of IGFBP-4 have been demonstrated to be IGF-dependent on the basis of the following facts: (i) IGFBP-4 had no effect or lower potency in blocking the biological activity of IGF analogues which have significantly (> 100-fold) reduced binding affinity to IGFBP-4 (Mohan *et al.* 1995b); (ii) IGFBP-4 inhibited the binding of IGF-I to purified IGF-IR *in vitro* (Mohan *et al.* 1995b).

Consistent with the *in vitro* data, IGFBP-4 is also a functional antagonist of IGF actions *in vivo*. For example, transgenic mice overexpressing IGFBP-4 selectively in smooth muscle cells exhibited smooth muscle hypoplasia (Wang *et al.* 1998; Zhang *et al.* 2002), which was in direct contrast to the smooth muscle hypertrophy induced by IGF-I overexpression (Wang *et al.* 1997). Moreover, a protease-resistant IGFBP-4 had more potency (Zhang *et al.* 2002) and the wet weight of selected smooth muscle tissues was reduced in the *Igf1/Igfbp4* double transgenic mice compared to the *Igf1* single transgenic mice (Wang *et al.* 1998), suggesting that these inhibitory effects of IGFBP-4 are IGF-I-dependent. In agreement with the above data, local administration of recombinant IGFBP-4 inhibited IGF-I-induced increases of bone formation in mice (Miyakoshi *et al.* 1999).

In contrast to local IGFBP-4 administration, systemic administration of IGFBP-4 increased bone formation (Miyakoshi *et al.* 1999; Miyakoshi *et al.* 2001). This was the only report regarding a growth-stimulatory effect of IGFBP-4, which was probably by increasing IGF bioavailability via an IGFBP-4 protease-dependent mechanism, since systemic administration of the native IGFBP-4, but not the protease-resistant IGFBP-4, increased the levels of serum free IGF-I, serum osteocalcin, serum and skeletal alkaline phosphatase and IGFBP-4 proteolytic activity in serum (Miyakoshi *et al.* 2001).

2.3.5.2 IGF-independent actions

In addition to IGF-dependent actions, IGF-independent actions of IGFBP-4 have been suggested, based on the following findings: (i) IGFBP-4 caused a marked inhibition of ceramide-induced apoptosis of Hs578T human breast cancer cells which lack a functional IGF-IR (Perks *et al.* 1999); (ii) IGFBP-4 inhibited human ovarian steroidogenesis in the presence of either the IGF-IR blocker αIR3 or excess IGFBP-3 to remove the effects of endogenous IGF action (Wright *et al.* 2002); (iii) Endogenous IGFBP-4 inhibited the mitogenic effects of IGF and insulin in HT-29 human colonic adenocarcinoma cells, which could not be compensated by the addition of an excess of IGF-I and insulin, but by the addition of an antibody against IGFBP-4 (Singh *et al.* 1994). However, unlike IGFBP-3 and -5, a specific receptor for IGFBP-4 has not been identified yet. Further studies are necessary to define the mechanism of IGF-independent actions of IGFBP-4.

2.3.6 Biological significance of IGFBP-4

2.3.6.1 Reproductive physiology

The expression pattern of IGFBP-4 and its regulation were extensively studied in human and animal reproductive organs at various reproductive stages. The findings in these studies indicate important roles of IGFBP-4 for reproduction.

Pregnancy. IGFs, as mitogenic peptides, are important for fetal and placental growth during pregnancy (Han & Carter 2000). In the human placenta, IGFs regulate

syncytiotrophoblast steroidogenesis (Nestler 1987; Nestler 1990), glucose and amino acid transport in the villi (Kniss *et al.* 1994) and the invasion of the extravillous trophoblast into the maternal decidua (Han *et al.* 1996; McKinnon *et al.* 2001). IGFBPs are expressed by the maternal decidua and may regulate IGF actions during pregnancy (Han *et al.* 1996). In this context IGFBP-4 is particularly interesting for several reasons: (i) IGFBP-4 is the second most abundant IGFBP in the placental bed (Han *et al.* 1996); (ii) PAPP-A is secreted by human trophoblast cells and decidualized endometrial stroma (Giudice *et al.* 2002) and is markedly increased in the maternal circulation as pregnancy progresses (Byun *et al.* 2000); (iii) in human pregnancy serum the majority of PAPP-A (> 99%) is found as a PAPP-A/proMBP complex (Oxvig *et al.* 1993). During pregnancy, rapid placental development and fetal growth obviously increases the need for growth-promoting factors such as IGFs. The mitogenic activity of IGFs at the local cellular level depends on the concentration of free IGFs that are able to interact with their receptors. The increased IGFBP-4 proteolytic activity resulting from uncomplexed PAPP-A may be required locally to increase the concentration of free IGFs for placental development and therefore for fetal growth during pregnancy, whereas the PAPP-A activity in maternal circulation is inhibited by complex formation with proMBP. The absence of PAPP-A expression in the placenta of pregnant patients with Cornelia de Lange syndrome, a condition involving incomplete fetal development and subsequent deformities (Westergaard *et al.* 1983), provides direct evidence for a role of PAPP-A in pregnancy. However, the roles of this complex system of enzyme (PAPP-A), substrate (IGFBP-4), inhibitor (proMBP) and cofactor (IGF-II) in the placenta and maternal circulation during human pregnancy deserve further investigation. Different from human, recent data showed that the IGFBP-4 proteolytic activity in murine serum is not increased during pregnancy, eventually due to the lower level of PAPP-A expression in the placenta (Qin *et al.* 2002; Soe *et al.* 2002). The significance of this difference between species is unknown, but this difference must be taken into consideration when the mouse is used as a model organism for the study of PAPP-A function.

Ovarian physiology. IGFs are produced by ovarian granulosa and theca cells, and mediate gonadotropin actions on ovarian cellular steroidogenesis and growth (Poretsky *et al.* 1999). Regulation of IGF actions within the ovarian follicle is particularly important in the processes of ovarian follicle development and follicle

atresia (Monget *et al.* 2002). As a potent inhibitor of IGF actions, IGFBP-4 appears to be particularly important in ovarian physiology (Iwashita *et al.* 1996; Poretsky *et al.* 1999). High levels of IGFBP-4 are present in small androgen-dominant follicles (Zhou & Bondy 1993; el Roeiy *et al.* 1994;) and in follicular fluid from androgen-dominant follicles (FF_a) that are growth-arrested or atretic (Cataldo & Giudice 1992; San Roman & Magoffin 1993). In contrast, IGFBP-4 is undetectable by ligand blot analysis in follicular fluid from estrogen-dominant growing follicles (FF_e). IGFBP-4 inhibits human ovarian steroidogenesis *in vivo* (Mason *et al.* 1998; Wright *et al.* 2002), and it has been suggested that IGFBP-4 inhibits follicle development by inhibiting IGF action in the ovary, and conversely, the loss of this inhibitory factor allows for increased bioavailable IGFs, which coincides with selection of the dominant follicle (Poretsky *et al.* 1999). The complexity of this process has become even more apparent with the finding of the IGFBP-4-specific protease PAPP-A activity in FF_e, but not in FF_a (Chandrasekher *et al.* 1995). PAPP-A is expressed in human and mouse ovaries, being restricted to healthy granulosa cells and granulosa-lutein cells (Hourvitz *et al.* 2000; Hourvitz *et al.* 2002). This restricted expression pattern and its co-expression with aromatase and LH receptor in granulosa cells from preovulatory follicles suggests that PAPP-A could be considered as a functional marker of follicular development (Mazerbourg *et al.* 2001). During terminal development of follicles to the preovulatory stage, degradation of IGFBP-4 by PAPP-A in the ovary may increase IGF bioavailability that further stimulates granulosa cell proliferation and steroidogenesis, and then actively participates in the selection of dominant follicles *in vivo*. In contrast, in atretic follicles, the IGFBP-4 degradation is inhibited by locally increased IGFBP-2 and -5 that contain a HBD in their C-terminal regions (Mazerbourg *et al.* 2000). The IGFBP-4 protease activity has been reported in human, ovine, bovine, porcine, equine (Mazerbourg *et al.* 2000; Mazerbourg *et al.* 2001) and mouse ovaries (Hourvitz *et al.* 2002), suggesting a well-conserved mechanism for this protease in ovarian function.

2.3.6.2 Bone formation

IGFs are the most abundant growth factors stored in the bone and regulate the proliferation and differentiation of bone cells (Bautista *et al.* 1990). As in other tissues, the local activity of IGFs in bone is modulated by IGFBPs. IGFBP-4 is one of

the major IGFBPs produced by bone cells (Mohan *et al.* 1995b) and has been proposed to function as an important regulator of bone formation. Evidence was provided by the following findings: (i) IGFBP-4 inhibited both basal and IGF-induced cell proliferation of MC3T3-E1 mouse osteoblasts and untransformed normal human bone cells (Mohan *et al.* 1995b) and the growth of embryonic chicken pelvic cartilage *in vitro* (Schiltz *et al.* 1993); (ii) a single local administration of IGFBP-4 inhibited IGF-I-induced increases in bone formation, whereas systemic administration of IGFBP-4 alone increased serum and skeletal levels of bone formation markers (osteocalcin and alkaline phosphatase) in mice (Miyakoshi *et al.* 1999; Miyakoshi *et al.* 2001). The latter stimulatory effect resulted from an increase of IGF bioavailability in the circulation via an IGFBP-4 protease-dependent mechanism (Miyakoshi *et al.* 2001); (iii) the serum level of IGFBP-4 was shown to increase with aging and to correlate positively with serum PTH levels. PTH upregulated IGFBP-4 expression in human osteoblasts *in vitro*, and serum IGFBP-4 levels were found to be increased during oral 1,25-dihydroxyvitamin D_3 therapy in psoriasis patients. These findings suggest that during calcium deficiency, the increase in serum PTH and 1,25-dihydroxyvitamin D_3 may not only stimulate bone resorption, but also inhibit bone formation by stimulating IGFBP-4 production in bone cells (Mohan *et al.* 1995a; Rajaram *et al.* 1997); (iv) sera from patients with chronic renal failure (CRF) usually contain high levels of IGFBP-4, which may contribute to decreased bone formation in renal osteodystrophy (Van Doorn *et al.* 2001).

2.3.6.3 Renal pathophysiology

IGFBP-4 is abundantly expressed in the kidney. A site-specific expression pattern of IGFBP-4 during nephrogenesis was described in human (Matsell *et al.* 1994), rat (Price *et al.* 1995a) and mouse (Lindenbergh-Kortleve *et al.* 1997), suggesting specific roles of IGFBP-4 in renal development and physiology. In addition, changes in IGFBP-4 abundance may be associated with pathological processes of the kidney. Upregulation of IGFBP-4 levels in serum correlated with the degree of renal dysfunction and growth retardation of children with CRF (Ulinski *et al.* 2000; Van Doorn *et al.* 2001), while downregulation of renal IGFBP-4 expression was reported in GH-induced rat hypersomatotrophy (Hise *et al.* 2001).

2.3.6.4 IGFBP-4 and cancer

IGFBP-4 is expressed in a range of cells of tumor origin, such as lung adenocarcinoma (Price *et al.* 1995b), non-small cell lung cancer (Noll *et al.* 1996), breast cancer (Qin *et al.* 1999a), colon carcinoma (Michell *et al.* 1997), follicular thyroid carcinoma (Bachrach *et al.* 1995), gastric cancer (Yi *et al.* 2001), glioma (Bradshaw *et al.* 1999), hepatoma (Scharf *et al.* 1998), myeloma (Feliers *et al.* 1999), neuroblastoma (Cheung *et al.* 1991; Babajko & Binoux 1996), osteosarcoma (Mohan *et al.* 1995b) and prostate cancer (Srinivasan *et al.* 1996; Damon *et al.* 1998b; Drivdahl *et al.* 2001).

In vitro and *in vivo* studies suggest that IGFBP-4 plays an important role in the growth regulation of a variety of tumors, possibly by inhibiting autocrine IGF actions or by as yet unknown IGF-independent mechanisms. Notably, in Coca-2 human colon carcinoma cells expression of IGFBP-4 mRNA was correlated with cell differentiation, indicating growth inhibitory effect in that cellular system (Hoeflich *et al.* 1996). Proliferation, anchorage-independent growth and tumor development in athymic nude mice were inhibited by overexpression of IGFBP-4 in M12 prostate cancer cells. Apoptosis was increased in the IGFBP-4-overexpressing cells, probably due to sequestrating IGF ligands (Damon *et al.* 1998b). Blocking of IGFBP-4 with antibodies enhanced both basal and IGF-stimulated growth of HT-29 human colonic carcinoma cells in both IGF-dependent and -independent manners (Singh *et al.* 1994). Recombinant IGFBP-4 caused marked inhibition of ceramide-induced apoptosis of Hs578T human breast cancer cells via an IGF-independent pathway (Perks *et al.* 1999).

2.4 IGFBP-6

2.4.1 Genomic organization of the IGFBP-6 gene

The human *IGFBP6* gene spans 4.7 kb on chromosome 12 (Ehrenborg *et al.* 1999). The rat *Igfbp6* gene is about 5.1 kb long, with a similar genomic organization as the human *IGFBP6* gene (Zhu *et al.* 1993). Comparison of human, rat and mouse IGFBP-6 sequences within the proximal promoter showed up to 90% similarity among the

three species. Four and two clustered transcription initiation sites (TIS) were found in the 5'-flanking regions of the human *IGFBP6* and rat *Igfbp6* genes, respectively. In each case, no TATA box was found, but Sp1 sites are clustered near the TIS of the IGFBP-6 promoter, like in other TATA-less promoters (Dusing & Wiginton 1994; Huber *et al.* 1998). Other putative responsive elements were also identified in the 5'-flanking region of the human *IGFBP6* gene, including retinoic acid response elements (RAREs), CAAT boxes, CACCC boxes, AP-1, AP-2, AP-3, C/EBPα, C/EBPβ, c-ets-2, EGR-2, HiNF-C, HSF, NF-1, NFκβ binding sites and a polypurine tract. Only a few of these sites are conserved in all three species (Dailly *et al.* 2001). The rat *Igfbp6* promoter contains an extended polyadenosine tract (Dailly *et al.* 2001) and a putative estrogen responsive element (ERE) (Zhu *et al.* 1993), which may play a role to shut off *Igfbp6* transcription by means of the estrogen receptor, since it was found that IGFBP-6 mRNA was expressed only by estrogen receptor-negative human breast cancer cells but not by estrogen receptor-positive cells (Sheikh *et al.* 1992). There is a line of evidence for retinoic acid (RA) being a strong stimulator of IGFBP-6 expression. Three putative RAREs with widely spaced half-sites were found in the human *IGFBP6* promoter region, but only the proximal one was functional, which is present in the human, rat and mouse IGFBP-6 proximal promoters, whereas the distal RAREs are not conserved. Each of the hexameric half-sites was shown to bind to retinoid receptors (Dailly *et al.* 2001). Moreover, several putative AP-2 binding sites were identified in the human *IGFBP6* promoter region. Interestingly, AP-2 production can be stimulated by RA treatment *in vitro*, and AP-2, as well as RA, has been shown to be developmentally regulated. AP-2 may play a role in vertebrate embryogenesis, however, there is no evidence for IGFBP-6 expression until late in fetal development (Ehrenborg *et al.* 1999).

In contrast to human *IGFBP4* gene without conserved poly(A) addition signal, a single polyadenylation consensus sequence was identified 1 kb downstream of the stop codon of the human *IGFBP6* gene (Ehrenborg *et al.* 1999).

2.4.2 The structure-function relationship of IGFBP-6

IGFBP-6 is an O-linked glycoprotein with 216 aa in human and 201 aa in rat, which differs from the other five IGFBPs in a number of aspects (rev. in Bach 1999): (i) the

highest binding affinity for IGF-II among the six IGFBPs; (ii) marked binding preference for IGF-II over IGF-I (20-100-fold higher than IGF-I); (iii) limited cell surface association; (iv) relatively low susceptibility to proteolysis; (v) lack of two and four of the 12 N-terminal cysteines in human and rat IGFBP-6. Is there a relationship between the structural features and its special actions? An increasing body of findings can be used, at least in part, to answer this question.

IGF-II binding preference. Surface plasmon resonance (SPR) studies revealed that the IGF-II-binding preference of IGFBP-6 is due to the lower dissociation rate of IGF-II, as compared to IGF-I (Marinaro *et al.* 1999b). Mutation of residues of hIGF-II that are important for binding to IGFBPs, such as Phe^{26}, Phe^{48}, Arg^{49} and Ser^{50}, decreased its binding to IGFBP-6 to a greater extent than to most other IGFBPs (Bach *et al.* 1993), suggesting that the precise structure of IGF-II is critical for its binding to IGFBP-6. On the other hand, the structural specificity of IGFBP-6 may also contribute to its IGF-II-binding preference. A chimera IGFBP-6_5, in which the C-domain of IGFBP-6 was replaced by the C-domain of IGFBP-5, retained the binding preference for IGF-II (Twigg *et al.* 1998). Since the non-conserved L-domains of the IGFBPs are not thought to be directly involved in IGF-binding, the structural determinants of the IGF-II-binding preference are therefore likely to reside in the N-terminus. The high-affinity IGF-binding site of IGFBP-5 has been localized to the N-terminal sequence containing Cys9 to Cys12, which is equivalent to Cys7 to Cys10 of hIGFBP-6 (Kalus *et al.* 1998). Since all IGFBPs share the same disulfide linkages in this region (Neumann & Bach 1999; Chelius *et al.* 2001), it is likely that this region confers high IGF-binding affinity to all IGFBPs, but does not contribute to the IGF-II-binding preference of IGFBP-6. Human and rat IGFBP-6 lack the adjacent cysteine pair that is a part of the $GC^5GC^6C^7$ motif present in the N-domains of IGFBP-1 to -5. Moreover, the disulfide linkage pattern of the first 6 N-terminal cysteines has been demonstrated to be inconsistent with other IGFBPs, such as IGFBP-1 and -4 (Neumann & Bach 1999; Chelius *et al.* 2001). These structural differences might be responsible for the distinctive IGF-binding characteristics of IGFBP-6. An alternate possibility is that the structures of the N-domains of IGFBP-6 and other IGFBPs are folded in a similar way that is stabilized by quite different disulfide linkages. In that case, assuming the remainder of the structure to be also similar in the six IGFBPs, the IGF-II-binding preference of IGFBP-6 could depend more on differences in the

primary sequence compared with other IGFBPs than on differences in three-dimensional structure, although both are likely to be influential (Neumann & Bach 1999).

Glycosylation. IGFBP-6 is O-glycosylated (Bach *et al.* 1992), but the effects of glycosylation on its properties are incompletely understood. The glycosylation sites of hIGFBP-6 were identified within the non-conserved midregion as Thr^{126}, Ser^{144}, Thr^{145}, Thr^{146} and Ser^{152} (Neumann *et al.* 1998). Electrospray ionisation mass spectrometry (ESMS) of glycosylated hIGFBP-6 expressed in Chinese hamster ovary cells revealed considerable heterogeneity of carbohydrate composition. Major glycoforms contained 8-16 monosaccharides, including N-acetylhexosamine, hexose and N-acetylneuraminic acid. One oligosaccharide chain contained 5-6 monosaccharides, while others contained 2-4 monosaccharides (Neumann *et al.* 1998). The rodent IGFBP-6 is glycosylated to a lesser extent (Bach 1999). O-glycosylation was reported to delay the clearance of IGFBP-6 from the circulation (Marinaro *et al.* 2000a), inhibit proteolysis of IGFBP-6 and the binding of IGFBP-6 to glycosaminoglycans and PC12 rat phaeochromocytoma cell membranes, but it does not directly influence the high IGF-II-binding and the IGF-II-binding preference of IGFBP-6 (Marinaro *et al.* 2000b).

Proteolysis. Proteolysis of IGFBP-6 has been described in a limited number of studies. Proteolytic activity against IGFBP-6 was found in media conditioned by NIH-3T3 fibroblasts (Claussen *et al.* 1995) and HaCaT keratinocytes (Marinaro *et al.* 1999a) after acidification, and Madin-Darby canine kidney (MDCK) cells at neutral pH (Shalamanova *et al.* 2001). The proteolytic activity in the NIH-3T3 cells was inhibited to variable extents by a range of protease inhibitors, suggesting that a cascade of proteases may be involved. It was also inhibited by IGFs. In contrast, the proteolytic activity in HaCaT keratinocytes was not regulated by IGFs, and it could only be inhibited by pepstatin A, suggesting that the proteolytic activity is a cathepsin D-like protease. However, another study suggested that IGFBP-6 was not cleaved by cathepsin D (Claussen *et al.* 1997). Moreover, 7S nerve growth factor at high concentrations also cleaves IGFBP-6 (Rajah *et al.* 1996). The relative paucity of described IGFBP-6 proteases may reflect protection of this glycoprotein from proteolysis by O-linked oligosaccharide chains. Although O-glycosylation can

partially protect IGFBP-6 from proteolysis by chymotrypsin and trypsin, both proteases can partly cleave glycosylated IGFBP-6 with different preferred cleavage sites (Marinaro *et al.* 2000b). Inferred major cleavage sites of chymotrypsin are Tyr^{74}, Leu^{97}, Tyr^{179}, Tyr^{186} and Phe^{195}/Tyr^{196} in hIGFBP-6, whereas trypsin preferentially cleaves hIGFBP-6 after Lys^{118} and Arg^{199}. The resulting fragments have lost the ability of IGF-II-binding.

Cell surface association. As noted in the IGFBP-4 section, the C-terminal heparin-binding domain (HBD) of IGFBP-3 is critical for the ability of IGFBP-3 to bind to endothelial cells (Booth *et al.* 1995). Human IGFBP-6 has a similar sequence (His^{191}-Arg^{209}) (Bach 1999). A synthesized 18-mer peptide based on this IGFBP-6 sequence (P6) mimics the properties of P3 (Booth *et al.* 1995; Booth *et al.* 1996; Booth *et al.* 2000). However, intact hIGFBP-6 does not bind to endothelial cell monolayers (Booth *et al.* 1995). Recently, a chimera IGFBP-6_3 was generated by replacement of the P6 region in IGFBP-6 with the homologous P3 region of IGFBP-3 (Boes *et al.* 2002). Unlike IGFBP-4_3 (Knudtson *et al.* 2001), IGFBP-6_3 did not bind to endothelial cells, suggesting that there may be other conformational or structural constraints of IGFBP-6 limiting its cell-association. For example, it is possible that O-linked carbohydrates mask the heparin-binding site (Marinaro *et al.* 2000b), since glycosylation of ECM proteins may affect binding to other matrix components (Varki 1993).

2.4.3 IGFBP-6 expression in vivo and its regulation

IGFBP-6 was originally isolated from cerebrospinal fluid (CSF) (Roghani *et al.* 1989), but it also exists in other physiological fluids, including serum, amniotic fluid, follicular fluid and human milk (Baxter & Saunders 1992; Ney *et al.* 1995; Van Doorn *et al.* 1999). The serum levels are about the same as those of IGFBP-2, higher than those of IGFBP-1 and less than 5 % of those of IGFBP-3.

IGFBP-6 expression is developmentally regulated. IGFBP-6 mRNA is widely expressed in the adult rat, with lung being the site of highest expression, but not in the developing fetal lung (Wallen *et al.* 1997). In the rat embryo, IGFBP-6 is expressed in the liver, nasal epithelium, cells surrounding cartilage and some muscle precursor

cells on embryonic day 14 as determined by *in situ* hybridization (Cerro *et al.* 1993). In the mouse fetus, IGFBP-6 mRNA is expressed at low levels in lung, liver, vertebrate and ribs in late gestation (Schuller *et al.* 1993). In contrast, IGFBP-6 mRNA is widely expressed in 14-18-week-old human fetuses at various sites, including skin, gut, heart, kidney, lung, brain, liver and skeletal muscle (Delhanty *et al.* 1993).

IGFBP-6 expression is also hormonally regulated in a tissue-specific manner. In rat white adipose tissue, levels of IGFBP-6 mRNA were decreased by hypophysectomy and partially restored by GH but not by IGF-I treatment (Peter *et al.* 1993). In fetal lung explant cultures, dexamethasone increased the abundance of IGFBP-6 mRNA (van de Wetering *et al.* 1997). In *Igf2r/Igf2*-deficient mice, a strong increase of IGFBP-6 immunoreactivity was observed in all cell types of the pancreatic islet, in the acinar cells and interlobular connective tissue of exocrine pancreas compared with controls (Braulke *et al.* 1999). Protein restriction had no effect on the levels of IGFBP-6 mRNA in the liver and kidney (Lemozy *et al.* 1994), but levels of IGFBP-6 in rat serum were increased by high-calorie total parenteral nutrition (Ney *et al.* 1995). IGFBP-6 levels in human serum are about 70% higher in men than women, decreased by 30% in pregnancy (Baxter & Saunders 1992) and increase gradually with aging (Van Doorn *et al.* 1999).

IGFBP-6 expression is regulated in some pathological conditions as well. Levels were reduced by 60% in patients with active acromegaly, but remained unchanged in hypothyroid patients (Baxter & Saunders 1992). Levels in blood from the umbilical cord and adults were similar. In children with CRF, serum IGFBP-6 levels were increased 5-20-fold. IGFBP-6 levels were inversely correlated with glomerular filtration rate, but were not correlated with height or changed by GH treatment (Powell *et al.* 1997). Serum IGFBP-6 levels are increased in some patients with non-islet cell tumor hypoglycemia, in which tumors overexpress a large metabolically active precursor form of IGF-II. Treatment with prednisolone or GH had no effect on the IGFBP-6 levels, but removal of the tumor normalized the IGFBP-6 levels (Baxter 1996). Furthermore, the levels of IGFBP-6 were significantly elevated in the CSF of patients with dementia of the Alzheimer type (Tham *et al.* 1993). In contrast, IGFBP-

6 levels were significantly decreased in the CSF of children with acute lymphoblastic leukemia, and remained low during 36 weeks of chemotherapy (How *et al.* 1999).

2.4.4 IGFBP-6 expression in vitro and its regulation

IGFBP-6 is expressed in a great number of cell types *in vitro*. These include transformed and non-transformed fibroblasts (Forbes *et al.* 1990; Martin *et al.* 1990), osteoblasts (Gabbitas & Canalis 1997), myoblasts (Ewton & Florini 1995), endothelial cells (Moser *et al.* 1992), epithelial cells (Cohick & Turner 1998), smooth muscle cells (Boes *et al.* 1996), skeletal muscle cells (Bayol *et al.* 2000), chondrocytes (De Los & Hill 2000), keratinocytes (Kato *et al.* 1995) and many tumor cells of different histogenetic origin. As described in Table 2.4, IGFBP-6 is regulated in a cell type-specific manner by a lot of agents *in vitro*. Furthermore, IGFBP-6 expression is also regulated during cell differentiation. In general, IGFBP-6 expression appears to be highest when cells are confluent and non-proliferating (rev. in Bach 1999).

Table 2.4 Regulatory mechanisms for IGFBP-6 expression *in vitro*

Agent	Cell type/Species	Effect	References
IGF-I	Vascular smooth muscle cells/h	s	Hayford *et al.* 1998
	PC12 phaeochromocytoma cells/r	s	Bach *et al.* 1997
	SH-SY5Y neuroblastoma cells/h	s	Babajko *et al.* 1997
	Osteoblasts/r	n	Gabbitas & Canalis 1997 McCarthy *et al.* 1994
	Fibroblasts/h	n	Conover *et al.* 1995
IGF-II	PC12 phaeochromocytoma cells/r	s	Bach *et al.* 1997 & 1998
	SH-SY5Y neuroblastoma cells/h	s	Babajko *et al.* 1997
	Osteoblasts/r	n	Gabbitas & Canalis 1997 McCarthy *et al.* 1994
D_3	HT-29 colon cancer cells/h	s	Oh *et al.* 2001
	SK-N-SH neuroblastoma cells/h	s	Chambery *et al.* 2000
	Prostate tumor cells/h	s	Drivdahl *et al.* 1995
T_3	SK-N-SH neuroblastoma cells/h	i	Chambery *et al.* 2000
	Bone marrow stromal cells/r	n	Milne *et al.* 2001
Dexamethasone	Vascular smooth muscle cells/h	s	Hayford *et al.* 1998
	PC12 phaeochromocytoma cells/r	i	Bach *et al.* 1997 & 1998
	Bone marrow stromal cells/r	n	Milne *et al.* 2001
	Fibroblasts/h	n	Conover *et al.* 1995
Glucocorticoids	Osteoblasts/r	s	Gabbitas & Canalis 1996a
RA	Bronchial epithelial cells/h	s	Sueoka *et al.* 2000a

	MCF-7 breast cancer cells/h	s	Martin *et al.* 1995
	Normal & transformed fibroblasts/h	s	Martin *et al.* 1994
	SK-N-SH neuroblastoma cells/h	s	Chambery *et al.* 2000
	Osteoblasts/r	s	Gabbitas & Canalis 1996b
TCDD	EL4 thymoma cells/m	s	Park *et al.* 2001
TPA	SK-N-SH neuroblastoma cells/h	s	Chambery *et al.* 2000
cAMP	Vascular smooth muscle cells/h	s	Hayford *et al.* 1998
	MCF-7 breast cancer cells/h	s	Martin *et al.* 1995
	PC12 phaeochromocytoma cells/r	s	Bach *et al.* 1998
	Normal & transformed fibroblasts/h	i	Martin *et al.* 1994
	Mammary epithelial cells/b	i	Cohick & Turner 1998
OP-1	Osteoblasts/r	i	Yeh *et al.* 1996
TGF-β1	Normal & transformed fibroblasts/h	i	Martin *et al.* 1994
	Osteoblasts/r	i	Gabbitas & Canalis 1997
bFGF	Osteoblasts/r	n	Gabbitas & Canalis 1997
PDGF BB	Osteoblasts/r	n	Gabbitas & Canalis 1997
Prostaglandin E2	Osteoblasts/r	n	McCarthy *et al.* 1994
hGH	Osteoblasts/r	n	McCarthy *et al.* 1994
Estradiol	MCF-7 breast cancer cells/h	s	Martin *et al.* 1995
Phorbol esters	HEC-1B endometrial carcinoma cells/h	s	Gong *et al.* 1992
N-myc oncogene	SK-N-SH neuroblastoma cells/h	i	Chambery *et al.* 1999

TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TPA, 12-O-tetradecanoyl phorbol 13-acetate; s, stimu-lation; i, inhibition; n, no effect.

2.4.5 Actions of IGFBP-6

A number of *in vitro* studies suggested that IGFBP-6 is a relatively specific inhibitor of IGF-II actions. Exogenously added IGFBP-6 from natural and recombinant sources inhibited IGF-II-induced proliferation, differentiation, survival and migration in a wide range of cell types including myoblsts (Bach *et al.* 1994; Bach *et al.* 1995; Ewton & Florini 1995), bronchial epithelial cells (Sueoka *et al.* 2000a), osteosarcoma cells (Yan *et al.* 2001), neuroblastoma cells (Babajko & Binoux 1996; Babajko *et al.* 1997; Grellier *et al.* 1998; Babajko *et al.* 2001; Grellier *et al.* 2002; Seurin *et al.* 2002), colon cancer cells (Leng *et al.* 2001; Oh *et al.* 2001; Kim *et al.* 2002a; Kim *et al.* 2002b) and rhabdomyosarcoma cells (Gallicchio *et al.* 2001). In most studies, IGFBP-6 did not inhibit IGF-I actions or did it with markedly decreased potency. In myoblasts, IGFBP-6 inhibited the actions of IGF-II and a series of IGF-II mutants in proportion to their binding affinities, suggesting that IGFBP-6 inhibits IGF-II actions by forming an IGF-IGFBP-6 complex that prevents IGF binding to the IGF receptor (Bach *et al.* 1994; Bach *et al.* 1995). Overexpression of IGFBP-6 inhibited

neuroblastoma (Babajko *et al.* 1997; Grellier *et al.* 1998; Babajko *et al.* 2001; Grellier *et al.* 2002; Seurin *et al.* 2002) and rhabdomyosarcoma (Gallicchio *et al.* 2001) cellular proliferation *in vitro* and xenograft growth *in vivo*, suggesting that IGFBP-6 may be a potential therapeutic agent for some cancers.

As for IGFBP-4, there is no evidence that IGFBP-6 can enhance IGF actions. This may be due to its relative resistance to proteolysis and lack of cell surface association. O-glycosylation contributes to both of these properties, so this post-translational modification may be important in maintaining the inhibitory properties of IGFBP-6. However, unexpected mitogenic and anti-apoptotic effects of IGFBP-6 were reported in the human osteoblastic osteosarcoma cell line Saos-2/B-10 which expresses little IGF-I and IGF-II (Schmid *et al.* 1999). The mechanism involved in these processes is not clear.

A number of studies suggested IGF-independent actions of IGFBP-6 based on the following findings: (i) IGFBP-6 inhibited des(1-3)IGF-I-induced proliferation of neuroblastoma cells (Babajko *et al.* 1997; Grellier *et al.* 1998) and (ii) IGFBP-6-induced apoptosis of non-small cell lung cancer cells was not affected by addition of IGF-I or -II (Sueoka *et al.* 2000b). However, neither a specific receptor nor mechanisms of IGF-independent actions of IGFBP-6 have been defined yet. Recent data suggested that IGFBP-6 inhibits osteoblast differentiation by an intracrine mechanism that may involve nuclear localization to modulate transcription of target genes (Strong *et al.* 2002). This may represent a pathway for IGF-independent actions of IGFBP-6 and indeed a novel paradigm for IGFBP actions in general.

2.4.6 Biological significance of IGFBP-6

From the information outlined above, it would seem that the main, if not exclusive, function of IGFBP-6 is to inhibit the actions of IGF-II. Since the actions of IGF-II have been studied to a far lesser extent than those of IGF-I and are therefore less completely understood, the biological significance of IGFBP-6 is still largely unknown.

Since IGF-II expression is maximal prenatally and largely extinguished in adult rodents, it has been suggested that IGF-II is mainly a fetal growth factor. However, IGF-II is the predominant IGF in the human circulation, suggesting that it has a continuing physiological role in the postnatal life of humans. Studies using transgenic mice overexpressing IGF-II also supported this contention (rev. in Wolf *et al.* 1998). Furthermore, IGF-II has some actions that are not shared by IGF-I. Evidence was provided by the following findings: (i) while all IGF-I actions were mediated by the IGF-IR, gene-targeting studies suggested that some prenatal actions of IGF-II were mediated through a receptor other than IGF receptors (Ludwig *et al.* 1996); (ii) IGF-II but not IGF-I stimulated proliferation of fibroblasts lacking IGF-IR through the IR *in vitro* (Morrione *et al.* 1997); (iii) IGF-II has been implicated as an autocrine growth factor in various tumors (Toretsky & Helman 1996), and systemic administration of IGF-I could not replace the action of the autocrine IGF-II in the oncogene-induced islet tumors of transgenic mice (Christofori *et al.* 1994).

Whether IGFBP-6 regulates these specific actions of IGF-II, needs to be clarified by further studies, as well as the possibility of IGFBP-6 regulating the actions of IGF-I. In addition, potential IGF-independent actions of IGFBP-6 should be taken into consideration.

3 ANIMALS, MATERIALS AND METHODS

3.1 Animals

Specific pathogen-free (SPF) C57BL/6 inbred mice were purchased from Elevage Janvier (Le Genest-Saint-Isle, France), and SPF NMRI outbred mice from Charles River Laboratories (Sulzfeld, Germany). They were maintained and bred under SPF condition in the facility on the 5th floor in the Gene Center of Munich. The health status of the facility was monitored based on the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) (Rehbinder *et al.* 1996). Non-SPF C57BL/6 inbred, NMRI outbred and B6D2F1 hybrid mice (Charles River Laboratories, Germany) were maintained under standard non-barrier conditions and had free access to standard rodent diets (#V1126 for breeding and #V1534 for maintaining; Ssniff, Soest, Germany) and tap water in the facilities on the 1st floor in the Gene Center. Mice used in expression studies and for phenotypic analysis were weaned at an age of three weeks, marked by ear piercing and housed in cages separated by sex. At the time of weaning, tail tips were clipped and frozen on dry ice for genotypic analysis. All experiments were carried out according to the German Animal Protection Law (Tierschutzgesetz; Genehmigungsaktenzeichen: 211-2531-31/96).

3.2 Cells and cell culture techniques

3.2.1 Cells

Murine NIH-3T3 fibroblasts (ATCC, Manassas, VA, USA) and human kidney epithelial cells (293 cells; ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Karlsruhe, Germany) with 10 % heat-inactivated (56 °C, 30 min) fetal calf serum (FCS; GIBCO) at 37 °C, 5% CO_2 and 100 % humidity. Cells were subcultivated (1:5) twice a week.

3.2.2 Freezing cell lines

After the culture medium was discarded, the cell layer was washed once with phosphate buffered saline (PBS) and incubated with trypsin-EDTA (0.05% trypsin and 0.02% EDTA, GIBCO) for 5 min. Trypsin action was stopped by addition of serum-containing medium and cells were spun down at 250 × *g* for 5 min. The pellet was resuspended in pre-cooled 1 ml DMEM + 20% FCS + 10% dimethylsulfoxide (DMSO; Sigma, Taufkirchen, Germany) and cells were frozen immediately in 1.5-ml or 1.8-ml cryo vials (Nunc, Kamstrup, Denmark) at -80 °C. After a few days they were transferred into liquid nitrogen.

PBS (pH 7.4):	NaCl	8.00 g
	KCl	0.20 g
	Na_2HPO_4	1.44 g
	KH_2PO_4	0.24 g
	bidistilled water	ad 1000 ml
	The buffer was autoclaved.	

3.2.3 Thawing cell lines

Cryo vials were removed from liquid nitrogen and incubated in a water-bath of 37 °C until the frozen medium had melted. The medium containing the cells was diluted in 5 ml medium and washed twice by centrifugation. The resulting cell pellet was resuspended in the appropriate culture medium.

3.3 Construction of expression vectors

3.3.1 Restriction enzyme digest

Restriction enzyme digests were performed in the recommended buffer and at the appropriate temperature for 90 min in a 20-100 µl volume. Usually, 10 units of enzyme (MBI, St. Leon-Rot, Germany) were used for each microgram of DNA.

3.3.2 Filling 5'- and 3'-protruding ends

Following the restrict enzyme digestion that generated the 5'- or 3'-protruding ends, the DNA were purified rapidly with the High Pure PCR Product Purification Kit (Roche, Mannhein, Germany) and eluted with 35 µl of Elution buffer (1 mM Tris-HCl, pH 8.5). The overhang ends were converted to blunt ends as follows: 10 µl of 5 × T4 DNA polymerase buffer, 5 µl of 1 mM of each dNTP and 2.5 µl of T4 DNA polymerase (5 units/µl, MBI) were added to 32.5 µl of the purified DNA, and incubated at 11 °C for 20 min. The reaction was stopped by heating at 70 °C for 10 min.

3.3.3 Dephosphorylation of 5'-ends

After the vector was blunt ended, it was purified as described in the section 3.3.2 and treated with Calf Intestinal Alkaline Phosphatase (CIAP; MBI) to remove the phosphate groups from the 5'-ends to prevent self-ligation of the vector. Add the following components directly to the purified DNA:

10 × CIAP reaction buffer	10 µl
CIAP (0.01 unit/pmol of ends)*	1-2 µl
bidistilled water	ad 100 µl

* A general formula for calculating the picomoles of ends of linear double-stranded DNA is: (µg DNA/kb size of DNA) × 3.04 = pmol of ends.

The mixture was incubated at 37 °C for 30 min. The reaction was stopped by heating at 85 °C for 15 min. The DNA was then purified as described in the section 3.3.2.

3.3.4 Extraction of DNA fragments from agarose gel

DNA fragments used for further manipulations were separated from other sequences by electrophoresis in a 1-2% TAE-buffered agarose gel. Ethidium bromide was added to permit visualization of nucleic acids under ultraviolet (UV) light.

6 × loading buffer at the appropriate volume was added to the samples, they were loaded into the slots and electrophoresis was performed using 1 × TAE buffer. Gel

was photographed under UV light at 366 nm (Eagle Eye II, Stratagene, Heidelberg, Germany), fragments were excised from the gel and weighed. DNA was extracted with the JETQUICK Gel Extraction Spin Kit (GENOMED, Bad Oeynhausen, Germany) and the concentration was estimated in a new agarose gel electrophoresis using 1 µg of the Lambda/*Hind*III+*Eco*RI marker (MBI) as a standard.

50 × TAE buffer:	Tris	242 g
	glacial acetic acid	57.1 ml
	0.5 M EDTA, pH 8	100 ml
	bidistilled water	ad 1000 ml

The buffer was sterilized by autoclaving.

6 × loading buffer:	glycerol	3 ml
	bidistilled water	7 ml
	bromphenol blue	one pinch

3.3.5 Ligation of DNA fragments

A molar vector : insert DNA ratio of 1:2 or 1:3 was used in every ligation. The amount of required insert was obtained using the following formula:

ng of insert = ng of vector x kb size of insert x molar ratio / kb size of vector

100 ng of vector DNA, appropriate quantity of insert DNA, 1 µl of 10 × ligation buffer and 1 µl (for overhang ends) or 2 µl (for blunt ends) of T4 DNA ligase (MBI) prediluted in the ligation buffer (1 unit/µl) were transferred in a sterile 1.5-ml centrifuge tube, and bidistilled water was added to a final volume of 10 µl. The ligation mix was incubated at 22 °C for 1-2 h or overnight.

3.3.6 Preparation of competent bacteria

A single colony of TOP10 *Escherichia coli* [genotype: *F¯ mcrA Δ(mrr-hsdRMS-mcrBC) ΦlacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG*; Invitrogen, CH Groningen, The Netherlands] from a plate grown

for 16-20 h at 37°C was picked and grown in 5 ml TYM medium (containing 50 µg/ml streptomycin; Sigma) at 37 °C overnight with vigorous agitation. 1 - 2.5 ml of this overnight culture was transferred to 250 ml fresh TYM medium (without streptomycin) and incubated at 37°C with vigorous agitation to an OD_{600} of 0.7 to 0.8 (about 2 - 4 h). After chilling for 10 min on ice in six 50-ml Falcon centrifuge tubes (40 ml culture/tube), cells were recovered by centrifugation at 800 × *g* and 4 °C for 10 min. The medium was completely removed. The pellet in each tube was carefully resuspended in 12 ml of ice-cold TfbI buffer and incubated for 10 min on ice. A new centrifugation was performed at 600 × *g* and 4 °C for 10 min. The supernatant was removed again. The pellet in each tube was then carefully resuspended in 1.6 ml of ice-cold TfbII buffer. Competent bacteria were aliquoted (200 µl) in 1.5-ml centrifuge tubes and stored at -80°C.

TYM medium:	bacto-tryptone	20 g
	bacto-yeast extract	5 g
	NaCl	5.8 g
	bidistilled water	ad 1000 ml

The mixture was shaken until the solutes had dissolved, the pH value was adjusted to 7.0 with 5 M NaOH and then the medium was autoclaved. 5 ml of 2 M $MgCl_2$ (presterilized by filtration) was added to an end concentration of 10 mM after the medium had cooled to RT.

TfbI buffer:	1 M potassium acetate (KOAc)	9 ml
	1 M $CaCl_2$	30 ml
	glycerol	45 ml
	bidistilled water	211 ml

The solution was autoclaved and then 5 ml of 3 M $MnCl_2$ (pre-sterilized by filtration) was added after the solution had cooled to RT.

TfbII buffer:	1 M MOPS	0.3 ml
	1 M KCl	3.0 ml
	1 M $CaCl_2$	3.2 ml
	glycerol	4.5 ml
	bidistilled water	19 ml

The buffer was autoclaved and stored at 4°C.

3.3.7 Transformation of bacteria

Competent bacteria were thawed on ice and 40 µl were transferred to a sterile centrifuge tube. 1 µl of plasmid (5 - 10 ng) or up to 5 µl of a ligation mixture were added. The ligation was mixed with a pipette, the tube was flicked once and incubated on ice for 30 min. The tube was transferred to a rack preheated to 37°C, incubated for exactly 30 s and then rapidly returned to ice and chilled for 5 min. 800 µl of SOC medium were added to each tube and cultures were incubated for 1 h at 37°C with vigorous shaking.

SOC medium:	bacto-tryptone peptone	20 g
	Bacto-yeast extract	5 g
	NaCl	0.5 g
	bidistilled water	ad 950 ml

The mixture was shaken until the solutes had dissolved. 10 ml of a 0.25 M KCl solution was added and the pH value was adjusted to 7.0, then the medium was autoclaved. The final medium additionally contains 100 mM $MgCl_2$ and 20 mM glucose.

The appropriate volumes (for plasmids: 5 and 50 µl; for ligations: 100 and 250 µl) of transformed competent cells were distributed onto agar-LB plates containing 50 µg/ml ampicillin (AppliChem, Damstadt, Germany). Using a sterile bent glass rod, the transformed cells were gently spread over the surface of the agar plate, which were subsequently inverted and incubated at 37°C. Colonies appeared within 12-16 h.

Luria-Bertani (LB) medium:	bacto-tryptone	10 g
	bacto-yeast extract	5 g
	NaCl	10 g
	bidistilled water	ad 1000 ml

The mixture was shaken until the solutes had dissolved, the pH value was adjusted to 7.0 with 5 M NaOH and then the medium was autoclaved.

Agar-LB plates:	LB medium	1000 ml
	agar, granulated	15 g

After autoclaving, swirl the solution to distribute the melted agar evenly. When the temperature reaches 50 °C, add the antibiotic and pour the plates directly from the flask in 90 mm Petri dishes. After the medium has hardened completely, invert the plates and store them at 4 °C.

3.3.8 Preparation of minipreps and midipreps

Colonies were picked with a tooth-pick, immersed in 5 ml LB medium with 50 µg/ml ampicillin and incubated for 12-18 h at 37 °C under constant agitation. 2 ml of the bacterial culture were transferred to a centrifuge tube and centrifuged at 15,000 × *g* for 30 s at 4°C. The remaining 3 ml bacterial cultures were stored at 4°C. The supernatant was discarded and the bacterial pellet was used to prepare minipreps using the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions. The concentration of the minipreps was determined with a spectrophotometer (Beckman, Palo Alto, CA, USA). 500 ng of minipreps were used for restriction enzyme analysis.

Following the identification of desirable clones in a miniprep, a midiprep was prepared. 1 ml of the miniprep culture was added to 100 ml LB medium with ampicillin (50 µg/ml) and incubated for 12-18 h at 37 °C under constant agitation. Midipreps were prepared with the Nucleobond AX100 Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

3.4 Expression vectors

3.4.1 The H-2K^b-mcIGFBP-4 and -6 constructs

The murine *Igfbp4* and *Igfbp6* cDNAs (mcIGFBP-4 and -6) were released from pGEM4Z-mIGFBP-4 and -6 (kindly donated by Dr. Drop, University of Rotterdam, The Netherlands), blunt ended with T4 DNA polymerase, and then subcloned into the blunted and dephosphorylated *Sal*I restriction site of the pUC-H2XXS mammalian expression vector (kindly provided by Prof. Pfeffer, Technical University of Munich, Germany). Location of the insert in sense direction was confirmed by restriction

enzyme digest. The injection fragments (5 kb for H-2K^b-mcIGFBP-4 and 4.9 kb for H-2K^b-mcIGFBP-6) were released from the vectors by a *Xho* I digest.

3.4.2 The CMV-mgIGFBP-6 construct

The murine *Igfbp6* genomic DNA (mgIGFBP-6) was released from pBSK-BP-6(6.0) (kindly donated by Prof. Braulke, University of Hamburg, Germany) by *Xho*I and *Sal*I digests, blunt ended and then subcloned between blunt ended *Eco*RI and *Xba*I sites of the mammalian expression vector pCMV-int, kindly provided by Dr. Kramer (Martinsried, Germany). The sense recombinant vector was identified by PCR (*Taq* DNA polymerase, Qiagen) using primers CMV#3 and mIGFBP-6#7 (see 3.7.1.2). The injection fragment (7.7 kb) was released from the vector by a *Spe*I / *Xho*I digest.

Figure 3.1 Schematic representation of the H-2K^b-mcIGFBP-4 and -6 vectors. H-2K^b promoter, mouse H-2K^b promoter (2.1 kb); mcIGFBP-4 or -6, mouse *Igfbp4* (1176 bp) or *Igfbp6* (1138 bp) complementary deoxyribonucleic acid (cDNA); beta-globin, a 1.7-kb human β-globin splice cassette including the last 20 bp of the exon 2, all of the intron 2 (850 bp*; shaded box*) and exon 3 (228 bp) and the polyadenylation signal sequence (593 bp). Transgene-specific PCR primers are indicated by *arrows*.

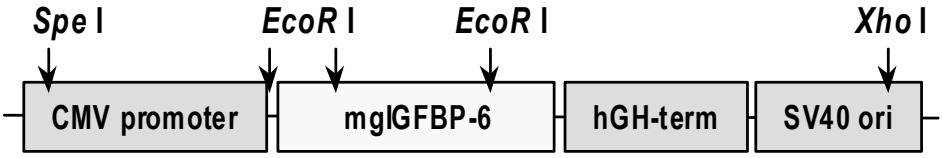

Figure 3.2 Schematic representation of the CMV-mgIGFBP-6 vector. CMV, cytomegalovirus immediate-early enhancer/promoter (763 bp); mgIGFBP-6, mouse *Igfbp6* genomic deoxyribonucleic acid (6 kb). hGH-term, transcription terminator and polyadenylation signal (624 bp) of the human growth hormone gene; SV40 ori, SV40 origin of replication (391 bp).

3.5 Transfection of cells

In order to determine whether the recombinant expression vectors are functionally active, they were stably or transiently transfected in the appropriate cells (H-2K^b-mcIGFBP-4 or -6 stably in NIH-3T3 cells; CMV-mgIGFBP-6 transiently in 293 cells). Cells were seeded into 6-well plates and allowed to grow to 60% confluence. 4 µg of recombinant expression vector and a mock vector lacking the *Igfbp4* and *Igfbp6* cDNA or the *Igfbp6* genomic DNA were transfected using the SuperFect Transfection Reagent (Qiagen). The transfection mixtures were pipetted as follows:

For stable transfection:

Vector	x µl (= 4 µg DNA)
SuperFect Transfection Reagent	9 µl
serum-free DMEM	ad 77 µl

For transient transfection:

Vector	x µl (= 4 µg DNA)
SuperFect Transfection Reagent	13.5 µl
serum-free DMEM	ad 83.5 µl

The components were mixed by pipetting and incubated at RT for 10 min. The cells were washed once with PBS. 800 µl DMEM with 10% FCS were added to each transfection mixture and pipetted into each well. The cells were incubated in this solution for 2.5 h at 37 °C and 5% CO_2. Thereafter, they were washed once with PBS and cultivated in 3 ml DMEM with 10% FCS for 48 h. For transient transfection, the cells were washed twice with PBS and 2 ml of serum-free DMEM were added. After two days of culture in the 2 ml serum-free DMEM, the serum-free conditioned media (SFCM) were collected, centrifuged at 600 × *g* and 4 °C for 10 min to remove cellular debris and stored in aliquots at -20 °C until use. Cells were lysed with 1 ml of TriPure® Isolation Reagent (Roche) to extract RNA. For stable transfection, selection was started by incubating the cells with selection medium [DMEM containing 10% FCS and 500 µg/ml geneticin (G-418 sulphate; GIBCO)]. The selection medium was renewed twice a week. After about two weeks, individual clones developed that had

stably integrated the vectors. They were picked, subcultured and frozen. The clones were subcultured in 6-well plates up to 90% confluence, the cells were washed twice with PBS and incubated with 2 ml of serum-free DMEM for 48 h. SFCM and RNA were prepared as described above.

3.6 Production of transgenic mice

3.6.1 Generation of transgenic mice by DNA-microinjection

Transgenic mice were generated by microinjection of the *Xho*I- or *Spe*I/*Xho*I-fragment, released from the expression vectors, into male pronuclei of F2 zygotes from C57BL/6 × DBA/2 F1 (B6D2F1) parents. The microinjected zygotes were implanted into the oviduct of pseudopregnant NMRI mice and carried to term. Founder transgenic mice were backcrossed to wild-type C57BL/6 mice for establishment and propagation of lines. Transgenic mice were identified by PCR using DNA recovered from tail clips. Transgene integration pattern was analyzed by Southern blot.

3.6.2 Generation of H-2K^b-mcIGFBP-4 SPF transgenic mice by embryo transfer

To analyse the potential effects of transgene expression on the immune system in the H-2K^b-mcIGFBP-4 transgenic mice, transgenic mice were produced under SPF conditions by embryo transfer. Male heterozygous H-2K^b-mcIGFBP-4 transgenic mice, generated by DNA-microinjection under standard non-barrier conditions, were mated with superovulated female C57BL/6 SPF mice, one-cell-stage embryos were obtained, transferred into the oviduct of pseudopregnant NMRI mice (SPF) and carried to term under SPF conditions. The transgenic offspring was backcrossed with C57BL/6 SPF mice and maintained under SPF conditions.

3.7 Identification of transgenic mice

3.7.1 Polymerase Chain Reaction (PCR)

3.7.1.1 Proteinase K digest of mouse tails

Tail tips were clipped at weaning and frozen at -80 °C. Fragments of 3-5 mm were cut and incubated at 56 °C overnight in 1.5-ml centrifuge tubes containing 100 µl of Kawasaki buffer and 6 µl proteinase K (Sigma, 20 mg/ml in bidistilled water).

Kawasaki buffer (pH 8.3): 20 mM Tris-HCl, pH 8.3
1.5 mM $MgCl_2$
25 mM KCl

The buffer was autoclaved. Tween-20 was added to an end concentration of 0.5% (v/v) after the solution had cooled to RT.The resulting buffer was kept at RT.

After the overnight digest, the samples were heated at 95 °C for 15 min to inactivate proteinase K, centrifuged at 15,000 × *g* and 4 °C for 5 min and the supernatant was transferred to a new tube. 2 µl of the supernatant were used as template for PCR.

3.7.1.2 PCR conditions

For the detection of construct integration, the following primers were used to amplify transgene-specific sequences:

H-2K^b-mcIGFBP-4:
mBP4#14 (sense): 5′-TAA GCC TGA GCC TTC TCG TG-3′
β-globin#1 (antisense): 5′-GGC AGC CTG CAC TGG TGG-3′

H-2K^b-mcIGFBP-6:
mBP6#6 (sense): 5′-CAG CTA GTT AGA AAG ATT GCT G-3′
β-globin#1 (antisense): 5′-GGC AGC CTG CAC TGG TGG-3′

CMV-mgIGFBP-6:
CMV#3 (sense): 5′-GTG TAC GGT GGG AGG TC-3′
mBP6#7 (antisense): 5′-CAG GCC ATC CCA GGT CAT-3′

To confirm the integrity of the DNA isolated from mouse tails, a sequence of the β-actin gene was amplified using the following primers:

β-actin#1 (sense): 5′-GGC ATC GTG ATG GAC TCC G-3′

β-actin#2 (antisense): 5′-GCT GGA AGG TGG ACA GGG A-3′

The reaction was prepared in 0.2-ml PCR reaction tubes (G. Kisker GbR, Steinfurt, Germany) on ice. The PCR was carried out with *Taq* DNA polymerase (5 units/µl; Qiagen) in a total volume of 20 µl:

DNA sample (template)	2.00 µl
10 x Qiagen PCR buffer	2.00 µl
5 x Q-solution	4.00 µl
25 mM $MgCl_2$	1.25 µl
1 mM dNTPs	1.00 µl
2 µM sense primer	1.00 µl
2 µM antisense primer	1.00 µl
Taq DNA polymerase	0.10 µl
bidistilled water	7.65 µl

The reaction was performed as following in a Biometra Uno II-Thermoblock (Göttingen, Germany): denaturation at 94 °C for 4 min, followed by 36 cycles of 94 °C for 1 min (denaturation), 60 °C for 1 min (annealing) and 72 °C for 2 min (extension); after a final 10-min extension at 72 °C, PCR products were cooled to 4 °C and mixed with 4 µl of 6 × loading buffer, separated in 1.2% TAE-buffered agarose gels with ethidium bromide and visualized under UV light.

3.7.2 Southern blot

3.7.2.1 Extraction of DNA from tail biopsies

0.5-1 cm of mouse tail tip fragments were used to extract the genomic DNA using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the Technical Manual. DNA concentration was estimated by measuring the OD of

100 µl of a 1:50 dilution of the samples at 260 nm and 280 nm in a spectrophotometer (Beckman). Ratios (260nm/280nm) between 1.6 and 2.0 were considered to indicate an appropriately pure sample.

3.7.2.2 Digestion and transfer of the DNA

10 µg of genomic DNA were digested with an appropriate restriction enzyme (100 units) in the recommended buffer in a volume of 50 µl overnight (at least 12 h). 10 µl of 6 × loading buffer were added and the cleaved DNA was separated in a 0.8% TBE-buffered agarose gel with a kb DNA ladder (MBI), using 1 × TBE as running buffer.

10 × TBE buffer:	Tris	121.1 g
	boric acid	51.35 g
	EDTA	3.72 g
	bidistilled water	ad 1000 ml

The gel was run until the bromophenol blue had reached at the end of the gel. Thereafter, the gel was photographed with a ruler and shaken for 30 min in 0.25 M HCl. After washing twice in distilled water, the gel was shaken for another 30 min in 0.4 M NaOH. The DNA was transferred to a Biodyne A Membrane (nylon membrane; Pall Gelman Laboratory, Ann Arbor, USA) by alkaline blotting: the gel was placed upside down over a Saran Wrap plastic film (Dow Chemical Company, Germany) and air bubbles were removed manually. It was overlaid with the nylon membrane, two sheets of gel blotting papers (GB002, 0.4mm thick; Schleicher & Schuell, Dassel, Germany) and a stack of tissue paper towels. Membrane and papers were cut to fit the gel size and the membrane was cut on the upper left corner to identify the orientation of the blot. At each step, air bubbles were removed with the help of a glass pipette. Finally, a glass dish of approximately 1 kg was placed at the top and capillary transfer took place for 18-24 h. Thereafter, the membrane was removed and rinsed in 5 × SSC for 1 min, the nucleic acids were crosslinked by exposition to UV light (120 J/cm^2) using a Crosslinker (Biometra, Göttingen, Germany).

3.7.2.3 Radioactive probe labeling

100 ng of cDNA were labeled with the HexaLabel™ DNA Labelling Kit (MBI) using [α-^{32}P]-dCTP (Amersham). The following components were added into a 1.5-ml centrifuge tube:

cDNA template	x µl (=100 ng)
hexanucleotide in 5 x reaction buffer	10 µl
deionized water	ad 41 µl

The tube was vortexed and spun down for 3-5 sec, incubated at 95 °C for 10 min, chilled on ice and spun down quickly. Thereafter, the following components were added in the same tube:

Mix C	3 µl
[α-^{32}P]-dCTP (10 µCi/µl)	5 µl
Klenow fragment, exo$^-$ (5 units/µl)	1 µl

The tube was shaken once, spun down for 3-5 sec and then incubated at 37 °C for 10 min. After the addition of 4 µl 0.25 mM dNTPs it was incubated again for another 5 min and the reaction was stopped by addition of 1 µl 0.5 M EDTA (pH 8.0). Unincorporated nucleotides were removed using a MicroSpin™ S-300 HR Column (Amersham). 5 µl of a 1:100 dilution in water were put into a scintillation vial and the number of counts per minute (cpm) was estimated in a multi-purpose scintillation counter (Beckman). The probe was stored on ice if used at the same day or kept at -20 °C for later application. The final cpm value was obtained with the following formula (the Cerenkov factor corrects the measurements without scintillation liquid):

$$\text{cpm/µl} = \text{cpm} \times 20\ \text{(dilution factor)} \times 1.55\ \text{(Cerenkov factor)}$$

3.7.2.4 Hybridization, washing and signal detection

The crosslinked membrane was placed into a glass tube and prehybridized with 12 ml Rapid-Hyb buffer (Amersham) at 65 °C for at least 30 min in a hybridization oven. During this time, sufficient probe to give a concentration of 2×10^6 cpm/ml in the 12 ml hybridization volume was incubated at 95 °C for 5 min and then chilled immediately on ice. The denatured probe was added directly to the prehybridization solution and hybridization occurred at 65 °C for 2 h or overnight. Subsequently, three wash steps were performed to remove unspecific radioactivity: 1 × 20 min at RT with 2 × SSC / 0.1 % SDS and 2 × 15 min at 65 °C with 1 × SSC / 0.1 % SDS.

20 × SSC (pH 7.0): 3 M NaCl
0.3 M sodium citrate
The buffer was autoclaved and kept at RT.

The hybridization solution with probe and the washing buffers were discarded into the radioactive waste. The membrane was sealed in a plastic bag and exposed either to a Storage Phosphor Screen (Molecular Dynamics, Krefeld, Germany) or to an X-ray film (Amersham). The membrane was not allowed to dry at any time. If background was still too high, the membrane was washed again more stringently (0.5 × SSC / 0.1% SDS or 0.25 × SSC / 0.1% SDS) and at higher temperatures (up to 70 °C).

3.8 Evaluation of gene expression at the RNA level

To avoid its degradation by nucleases, following attentions should be paid while working with the extraction, manipulation or analysis of RNA:
- gloves were worn all the time and changed frequently
- all equipment was autoclaved, glasses and magnet stirrers were sterilized at 180 °C for 8-10 h
- all solutions, except those containing Tris, were made with DEPC-H_2O (DEPC-treated water is used at a final concentration of 0.1 % diethylpyrocarbonate. 1 ml of DEPC was added into 1000 ml deionized water in a glass bottle. The bottle was shaken vigorously and incubated at 37 °C for about 2 h, then the solution was autoclaved and kept at RT.).

3.8.1 Extraction of RNA from tissues and cells

50-100 mg tissue samples stored at -80 °C were transferred directly into a 5-ml plastic tube (Falcon) containing 1 ml of the TriPure® Isolation Reagent (Roche) and homogenized with a tissue homogenizer (ART Labortechnik, Müllheim, Germany) at position D (23,500 rpm) for 1 min. The homogenizer was cleaned with 0.2 M NaOH and thereafter with DEPC-H_2O after homogenizing each sample. RNA was extracted according to the manufacturer's instructions and dissolved in 50-100 µl DEPC-H_2O according to the size of the pellets. RNA concentration was estimated with a spectrophotometer (Beckman).

Cells from a well of the 6-well plate were lysed in 1 ml of the TriPure® Isolation Reagent. RNA was extracted according to the manufacturer's instruction, resuspended in 60 µl of DEPC-H_2O, and the concentration was estimated as described above.

3.8.2 Reverse Transcription PCR (RT-PCR)

To get rid of DNA contamination in the RNA samples, 10 µg of total RNA were digested with RNase-free DNase I (Roche) at 37 °C for 30 min in a volume of 20 µl as following:

Total RNA	x µl (= 10 µg)
10 × DNase I reaction buffer	2 µl
RNase-free DNase I (10-50 units/µl)	1 µl
DEPC-H_2O	ad 20 µl

The solution was then incubated at 75 °C for 10 min to inactivate the enzyme, chilled on ice, spun down and stored at -20 °C for further use.

10 × DNase I reaction buffer: 250 mM Tris-HCl, pH 8.3
375 mM KCl
15 mM $MgCl_2$
The buffer was autoclaved, aliquoted and stored at -20 °C.

5 µl of the DNase I digests were used for reverse transcription with the M-MLV reverse transcriptase (GIBCO) at 37 °C for 1 h in a 20-µl volume as following:

DNase I digested RNA	5.0 µl
oligo dT (15mer, 500 ng/µl)	1.0 µl
dNTPs (10 mM)	2.0 µl
5 x first strand buffer	4.0 µl
DTT (0.1 mM)	2.0 µl
reverse transcriptase	0.1 µl
DEPC-H_2O	5.9 µl

The cDNA solution was then incubated at 95 °C for 10 min to inactivate the enzyme, chilled on ice, spun down and stored at -20 °C for further use. 2 µl of the cDNA solution were used as template in a PCR reaction as described in section 3.7.1.2.

3.9 Evaluation of gene expression at the protein level

3.9.1 Extraction of protein from tissues

Tissue samples stored at -80 °C were weighed, placed in 5 ml plastic Falcon tubes and homogenized in protein extraction buffer with a tissue homogenizer (ART Labortechnik) at position D for 1 min. For each 20 mg tissue, 500 µl extraction buffer were used. The homogenizer was cleaned with PBS after homogenization of each sample.

The homogenized samples were placed on ice, transferred to 1.5-ml centrifuge tubes, incubated at 95 °C for 5 min, chilled on ice and centrifuged at 15,000 × *g* and 4 °C for 5 min. The supernatants were aliquoted to new centrifuge tubes and stored at -20 °C until use. 10 µl of them was used for determination of protein concentration.

Protein extraction buffer:	1 M Tris (pH 7.5)	2 ml
	Triton X-100	2 ml
	5 × Laemmli buffer	20 ml
	bidistilled water	76 ml

5 × Laemmli buffer:	1 M Tris (pH6.8)	65.5 ml
	glycerol	100 ml
	0.5 M EDTA (pH8.0)	2.0 ml
	SDS	20 g
	bromophenol blue	0.1%
	bidistilled water	ad 200 ml

3.9.2 Determination of protein concentration

The protein concentration of the tissue extracts was estimated by the bicinchoninic acid (BCA; Sigma) protein assay. A set of protein standards of known concentration was prepared by serially diluting a bovine serum albumin (BSA; Sigma) stock solution (4 mg/ml in the BSA dilution buffer) with the BSA dilution buffer. 50 µl of the samples (1:5 diluted in PBS) and the standards were pipetted into 1.5 ml centrifuge tubes, 200 µl of a mixture of BCA and 4 % $CuSO_4$ (50:1) were added to each tube and mixed. 100 µl of the mixture was pipetted in duplicate into a 96-well plate. The plate was incubated at 37 °C for 30 min and the absorbance was measured at 562 nm with an ELISA reader (Spectra Max 250; Molecular Devices, Sunyvale, CA, USA). A standard curve was prepared by plotting the absorbance of standards versus protein concentration. The protein concentration of the samples was determined using the standard curve.

BSA dilution buffer:	protein extraction buffer	1 ml
	PBS	4 ml

The buffer was stored at 4 °C.

3.9.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

16 µl of the SFCM with 4 µl of 5 × Laemmli buffer or 50 µg of protein from the tissue extract were pipetted into centrifuge tubes, incubated at 95 °C for 5 min and chilled on ice.

The proteins were separated using the Mini Protean II System (BioRad, Munich, Germany). The separating gel (15% acrylamide) was prepared in an Erlenmeyer flask

under continuous agitation and poured into the gap between the glass plates of the system, leaving space enough for the stacking gel (about 2.5 cm). The separating gel was overlaid with bidestilled water to ensure an even surface.

Separating gel:	bidistilled water	2.35 ml
(for two gels)	1.5 M Tris-HCl (pH 8.8)	2.5 ml
	30% acrylamide	5.0 ml
	10% SDS	100 µl
	10% ammonium persulfate (APS)*	50 µl
	Temed	5 µl

* The 10% APS solution was stored at –20 °C in aliquots.

After complete polymerization (about 1 h), the water was discarded and the stacking gel (5% acrylamide) was prepared in the same way and loaded on the top of the separating gel. The comb was inserted taking care to not trap air bubbles under the teeth.

Stacking gel:	bidistilled water	4.2 ml
(for two gels)	0.5 M Tris-HCl (pH 6.8)	0.75 ml
	30% acrylamide	0.9 ml
	10% SDS	60 µl
	10% APS	60 µl
	Temed	5 µl

After complete polymerization, the comb was removed and the plates were mounted in the electrophoresis apparatus, which was filled with SDS-PAGE electrophoresis buffer.

10 x SDS-PAGE buffer:	Tris	30.3 g
	glycine	144 g
	SDS	10 g
	bidistilled water	ad 1000 ml

The buffer was stored at RT.

Samples were loaded and the electrophoresis was performed initially at 80 V for about 20 min and then at 120 V until the bromophenol blue left the separating gel at the bottom. A molecular weight standard (Low molecular weight range, M-3913; Sigma) was pipetted in the first slot for estimation of the protein size.

3.9.4 Electrophoretic blotting

The gel was removed from the electrophoresis chamber and the separated proteins were transferred to an Immobilon-P PVDF membrane (Millipore, Bedford, MA, USA) by semidry electrophoretic blotting in the MilliBlot-Graphite Electroblotter (MBBDGE001; Millipore). Six sheets of gel blotting paper (Schleicher & Schuell) cut to the same size as the gel (8.5 cm × 7 cm) were soaked in transfer buffer, stacked one on the top of the other on the bottom electrode and squeezed with a pipette to remove air bubbles. The PVDF membrane and then the gel were placed exactly over the paper stack and were covered with another six soaked gel blotting paper sheets. The upper electrode was placed and the system was connected to a power supply (POWER PAC 3000; BioRad). The transfer took place for 90 min at 60 mA for each gel (1 mA/cm^2).

10 × Transfer buffer:	Tris	58.2 g
	glycine	29.2 g
	SDS	3.7 g
	bidistilled water	ad 1000 ml

The buffer was stored at RT. Before use 20% of methanol were added.

After the transfer, the membrane was unambiguously labeled with a ballpoint pen and the molecular weight standard bands were marked. The membrane was stained with Ponceau red for 2 min under constant agitation, dried and stored at 4 °C.

Ponceau red:	Ponceau S (Sigma)	0.2 g
	glacial acetic acid	3 ml
	bidistilled water	ad 100 ml

3.9.5 [^{125}I]-IGF-II ligand blot

This methodology, first developed by Hossenlopp *et al.* (1986), has as major advantage over traditional Western immunoblot the possibility of detecting bioactive IGFBPs and a high sensitivity. The membrane containing the separated proteins was washed and hybridized in 50-ml Falcon plastic tubes in a hybridization oven as indicated bellow. All incubation steps were performed at 4 °C. The membrane was dried and IGFBPs visualized on Storage Phophor Image screens or X-ray film. The following steps were performed:

- washing with 10 ml Tris-buffered saline (TBS) solution with 3% Nonidet P40 for 30 min.

Tris-buffered saline (TBS):	NaCl	43.8 g
	Tris	6.06 g
	bidistilled water	ad 5000 ml

The pH value was adjusted to 7.4 with 1 N HCl and the buffer was stored at 4 °C.

- blocking in 5 ml TBS with 1% fish gelatin (Amersham) for at least 2 h.
- washing in 20 ml TBS with 0.1% Tween-20 for 30 min.
- incubation with 5 ml IGF-II tracer (Amersham) overnight.

IGF-II tracer in TBS:	fish gelatine	1%
	Tween 20	0.1%
	[^{125}I]-rhIGF-II	500,000 cpm/ml

- washing 2 × 30 min in 20 ml TBS with 0.1% Tween-20 and 3 × 30 min in 20 ml TBS.

3.9.6 Western immunoblot

The membrane containing the separated proteins was washed with TBS-T at RT for 10 min, and then incubated at RT for 60 min or at 4 °C overnight in a hybridization oven with blocking solution (TBS-T buffer with 3% fat-free milk powder).

TBS-T buffer:	Tris	2.42 g
	NaCl	8 g
	1 N HCl	3.8 ml
	bidistilled water	ad 1000 ml

The pH value was adjusted to 7.6, 0.5 ml Tween-20 was added (end concentration 0.05%) and the buffer was stored at 4 °C.

After blocking, incubation with the primary antibody diluted in blocking solution was performed at RT for 60 min. The membrane was then washed 3 times for 5 min at RT with TBS-T. Incubation with the secondary antibody diluted in blocking buffer took place at RT for 1 h. Finally, the membrane was washed 3 - 5 times with TBS-T at RT for 5 min. Detection was performed by incubating the membrane with 2 ml of the ECL Western blotting detection reagent (Amersham). The membrane was sealed under plastic and exposed to an ECL film (Amersham) or visualized with the Kodak Digital Image Station (440CF; NEN® Life Science Products, Inc., Zaventem, Belgium).

3.9.7 Immunohistochemistry

Mouse pancreata were separated from surrounding tissues, weighed and fixed in 4% PBS-buffered paraformaldehyde (pH 7.4) for 48 h. The fixed tissues were dehydrated in an ascending ethanol series and embedded in paraffin. Sections of 3 µm were cut and deparaffinized prior to immunohistochemical staining. IGFBP-6 immunodetection was performed using chicken anti-mIGFBP-6 antibodies and the HRP-coupled rabbit anti-chicken IgG according to the method reported by Putzer *et al.* (1998), or using rabbit anti-mIGFBP-6 polyclonal antiserum (GroPep Limited ABN, Thebarton, Australia) and HRP-coupled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) according to the method reported by van Kleffens *et al.* (1999).

3.10 Phenotypic analysis of transgenic mice

3.10.1 H-2K^b-mcIGFBP-4 transgenic mice

3.10.1.1 Analysis of body weight gain and organ weights

The body weight from selected litters was recorded once weekly from weaning to about three months of age. Eight- and twelve-week-old mice from selected groups were anesthetized with ether and were bled from the retro-orbital sinus. After measurement of the distance between nose and the base of the tail (nose-rump-length, NRL) to the nearest mm, they were killed by cervical dislocation and weighed. Organs and carcass were separated, blotted dry on paper towels and weighed to the nearest mg and 0.1 g, respectivly. Complete organs or parts of them were frozen on dry ice and then stored at -80 °C, or fixed in 4% PBS-buffered paraformaldehyde solution.

4% paraformaldehyde:

Paraformaldahyde (Sigma)	40 g
PBS	1000 ml
4 M NaOH	4 drops

The mixture was heated in a 50°C water bath until the solute was dissolved. The pH value was adjusted to 7.4 with 1 N HCl.

3.10.1.2 Morphometry of thymus and spleen

The thymus and spleen were released from the surrounding tissues and weighed to the nearest 0.1 mg. The volumes (V°) of the thymus and spleen were determined using the fluid displacement method according to Scherle (1970). Briefly, a 50-ml plastic cylinder filled with about 45 ml of 0.9% NaCl solution was placed on an analytical balance. Then the organ was hanged with a line and immersed completely in the NaCl solution without contact with the wall of the cylinder. The increased weight (g) of the NaCl solution after the organ immersion corresponds to the volume (cm^3) of the immersed organ, because the specific gravity of the 0.9 % NaCl solution is 1.0048 g/cm^3 ($\approx$ 1 g/cm^3). The specific gravity of the thymus and spleen was calculated

according to their weight and volume. Ten animals were used for the measurement (n = 10).

The organs were fixed in 4% PBS-buffered paraformaldehyde and embedded in paraffin. Cavalieri's principle (Gundersen & Jensen, 1987) was applied to estimate the volume of the paraffin-embedded thymus and spleen. First, the embedded organ was trimmed free of paraffin, and its length along the longitudinal axis was recorded. After positioning the first cut randomly within an interval of 1 mm, the organs were exhaustively sectioned perpendicular to their longitudinal axes into parallel slices of approximately 1 mm. These slices were placed with the right-hand cut-surface upward in tissue capsules and re-embedded in paraffin (Figure 3.3). Sections of 3 µm thickness representing systematic samples of the whole organs were prepared, stained with HE and subjected to morphometric evaluation.

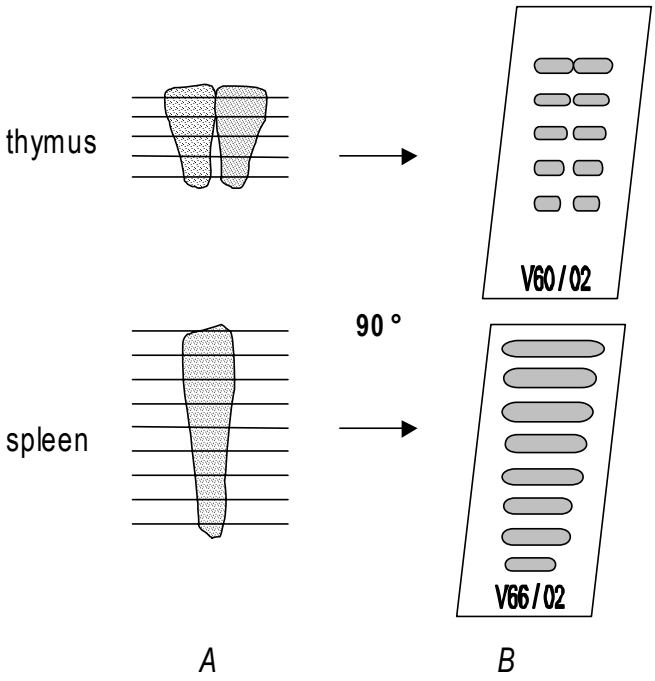

Figure 3.3: Schematic presentation of sectioning the organ into parallel slices (*A*) and the position of the histological sections on the glass slide for morpometrical studies (*B*).

Light microscopic planimetric evaluation was performed on a semiautomated image analysis system (Videoplan; Zeiss-Kontron, Eching, Germany) coupled to a microscope via a color video camera (Panasonic, Japan). A 2.5 × objective was used, providing a 90 × final linear magnification. An object micrometer (Zeiss) was used for calibration. The cross-sectional areas of organ structures of interest were determined on images displayed on a color monitor. These structures included the thymus, cortex and medulla of thymus, the spleen, red pulp (RP), white pulp (WP) and marginal zone (MZ) of the spleen. The following parameters were calculated:

The volumes of the thymus (th) and spleen (sp) before fixation:

$$V°_{(th\ or\ sp)} = W_{(th\ or\ sp)} / 1.10\ g/cm^3$$

Mean slice thickness:

$$T_{(th\ or\ sp)} = L_{(th\ or\ sp)} / N_{(th\ or\ sp)}$$

The volumes of the thymus and its compartments after embedding:

$$V'_{(a)} = T_{(th)} \times \sum A_{(a)}$$

The volumes of the spleen and its compartments after embedding:

$$V'_{(b)} = T_{(sp)} \times \sum A_{(b)}$$

The shrinkage factor of thymus or spleen:

$$Fs_{(th\ or\ sp)} = V°_{(th\ or\ sp)} / V'_{(th\ or\ sp)}$$

The real volumes of the compartments of thymus (corrected for embedding shrinkage):

$$V_{(a)} = V'_{(a)} \times Fs_{(th)}$$

The real volumes of the compartments of spleen (corrected for embedding shrinkage):

$$V_{(b)} = V'_{(b)} \times Fs_{(sp)}$$

where

$W_{(th)}$ and $W_{(sp)}$ = weight of thymus and spleen before fixation

1.10 g/cm^3 = the mean specific gravity of thymus and spleen determined as described above

a = thymus and its compartments (cortex and medulla)

b = spleen and its compartments (RP and WP + MZ)

$L_{(th\ or\ sp)}$ = length of the thymus or spleen (in mm)

$\sum A_{(a)}$ = sum of the cross-sectional areas of the thymus or its compartments

$\sum A_{(b)}$ = sum of the cross-sectional areas of the spleen or its compartments

$N_{(th\ or\ sp)}$ = number of slices per organ

3.10.1.3 Immunological analysis

To investigate the potential effect of transgene expression on the immune function, the following assays were carried out at the Institute of Medical Microbiology, Immunology and Hygiene, Technical University of Munich.

3.10.1.3.1 Sample preparation

Blood (400-600 µl) of 7-8-week-old mice was collected by bleeding from the retro-orbital sinus in a 1-ml lithium-heparin tube (Kabe Labortechnik GmbH, Nümbrecht-Elsenroth, Germany), centrifuged at 4500 × *g* for 5 min, and the plasma was recovered for ELISA analysis. The nucleated peripheral blood cells were obtained after removal of the erythrocytes by incubating the blood cells in 10 ml lysis buffer (140 mM NH_4Cl, 17 mM Tris-HCl, pH 7.2) for 5 min followed by two washing steps in FACS buffer (PBS containing 2 % FCS and 0.01 % NaN_3).

Eight-week-old mice from selected groups were anesthetized with ether and were killed by cervical dislocation. The thymus, spleen and subiliac lymph nodes were taken and cut into small pieces in FACS buffer, cells were pushed through a 100-µm nylon cell strainer (Becton Dickinson Labware, Le Pont de Claix, France) to obtain

single-cell suspensions. Subsequently, thymocytes and cells from the lymph node were washed twice in FACS buffer. Erythrocytes in the single-cell suspensions from spleen were removed as above and washed twice in FACS buffer. Suspensions of bone marrow cells were flushed from the tibiae with FACS buffer.

The cells of each sample were distributed into ten 1.4-ml linbro tubes (Integra Biosciences, Fernwald, Germany) pre-racked in a 96-linbro rack. All subsequent pipetting steps were performed with 12-channel pipettes, minimizing labor and pipetting errors.

3.10.1.3.2 Flow cytometry (FACS)

For immunofluorescence staining of the nucleated blood cells, thymocytes, splenocytes and cells from bone marrow and lymph node, Fc receptors were blocked by incubation with 10 µg/ml 4G8 rat anti-mouse Fc receptor for 5 min. After being washed with 300 µl FACS buffer, cells were incubated at 4 °C for 20 min with 30 µl of the respective antibody combination. The antibody-labeling panel consisted of a number of antibody combinations (Table 3.1).

Table 3.1 Panel of antibody combinations used in the flow cytometry

Blood:	1. CD4 FITC / CD19 PE / CD8 PerCP / CD3e Cy5
	2. lambda FITC / Kappa PE / CD5 PerCP / CD45 APC
	3. DX5 FITC / CD122 PE / CD8 PerCP / CD3e Cy5
	4. CD34 FITC / CD62L PE / CD4 PerCP / CD44 APC
	5. $H2K^k$ FITC / β7 intigrin PE / PI control / CD11b APC
Bone marrow:	1. CD43 FITC / IgM PE/B220 APC
	2. IgD FITC / B220 PE / CD24 bio Streptavidin Cy5
	3. AA4.1 FITC / B220 PE
	4. IgD FITC / IgM PE / CD45R APC
Thymus:	1. TCRβ FITC / CD8 PE / CD4 Cy5
	2. CD25 FITC / CD44 PE / CD4 PerCP / CD8 Cy5
	3. CD4 FITC / CD27 PE / CD8 PerCP / CD3 Cy5
	4. CD4 FITC / HSA PE / CD8 Cy5

Spleen:	1. IgD FITC / IgM PE / CD23 bio Strept. PerCP
	2. B220 FITC / CD24 bio Strept. PerCP
	3. AA4.1 FITC / IgM PE / CD24 bio Strept. PerCP
	4. B220 FITC / CD3e Cy5
	5. IgD FITC / IgM PE / CD23 bio Strept. APC
	6. IgD FITC / IgM PE / CD21 Cy5
	7. CD19 PE / CD23 bio PerCP / CD21 Cy 5
	8. CD1 PE / B220 APC
	9. CD1 PE / IgM bio Strept. Cy 5
Lymph node:	1. B220 PE / CD 3e Cy5
	2. IgD FITC / IgM PE / CD45R APC
	3. B220 FITC / IgM PE / CD23 bio Strept. PerCP / CD5 Cy5

After incubation, the cells were washed twice and measured on a FacsCalibur (Becton Dickinson, Mountain View, California, USA). The data were analyzed using the Attractors^{TM} and CellQuest softwares (Becton Dickinson) according to Flaswinkel *et al.* (2000).

3.10.1.3.3 Proliferation assay

Splenocytes were cultured in flat-bottom 96-well polystyrene microtiter plates (Nunc, Kamstrup, Denmark) at a density of 2×10^5 cells/ml in 200 µl RPMI 1640 medium (GIBCO) supplemented with 4 mM glutamine (GIBCO), 1 mM pyruvate (GIBCO), 50 µM 2-mercaptoethanol (Sigma), and 10 % heat-inactivated FCS (GIBCO), 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO). Splenocytes were stimulated either with 2 µg/ml Con A (Sigma), 10 µg/ml LPS (Sigma), 5 µg/ml anti-CD3 plus 10 U IL-2 (from Dr. Bauer, Technical University of Munich) or 5 µg/ml goat anti-mouse IgM antibody (Sigma). After 48 h of culture at 37 °C in an atmosphere of 5% CO_2 and 100% relative humidity, the cells were pulsed with 1 µCi of [^{3}H]-thymidine (Amersham) per well for 18 h of culture. The cells were harvested using a Micro 96 harvester (Skatron Instruments, Tranby, Norway). Incorporation of [^{3}H]-thymidine was quantified in a Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD, USA).

3.10.1.3.4 Basal immunoglobin ELISA

Basal immunoglobulin levels were determined by using sandwich ELISAs specific for IgM, IgG3 and IgA as described by Flaswinkel *et al.* (2000). Autoreactive antibody in plasma was also measured according to Flaswinkel *et al.* (2000).

3.10.2 CMV-mgIGFBP-6 transgenic mice

3.10.2.1 Analysis of body and organ weights

The body weight of mice was recorded once a week from 3-15 weeks of age. Four-month-old mice were anesthetized with ether, weighed and bled from the retro-orbital sinus. After measurement of the NRL, the mice were killed by cervical dislocation. The weights and gross dimensions of the gastrointestinal tract (GIT) were measured according to the methods described by Ogiolda *et al.* (1998). Briefly, the abdominal cavity was opened, and the caudal end of the duodenum was located and cut with scissors (the duodenum was identified by the absence of mesentery, whereas jejunum/ileum are supported by mesentery). Then stomach and duodenum as well as the other segments of the GIT were separated from adjacent organs and from the mesentery, respectively, and placed on an ice-cold glass plate. After the lengths of duodenum, jejunum/ileum, caecum and colon were recorded, small longitudinal incisions were made, and the luminal contents were flushed out with PBS. The stomach was removed from the duodenum at the pylorus, opened along the large curvature, flattened on a piece of paper, and the outline of the surface area was drawn onto the paper for planimetric evaluation. All other internal organs and the carcass were weighed to the nearest mg and 0.1 g, respectively. The empty GIT was fixed in 10% PBS-buffered formalin in a glass cylinder. After an overnight fixation, all GIT segments were blotted dry and weighed. Then they were immersed for a week in formalin solution and routinely processed for histological analysis. For expression studies, some mice from all groups were sacrificed as described above. The luminal contents of the various GIT segments were removed, transferred into individual centrifuge tubes, solubilized in Laemmli buffer and analyzed immediately as described above. The empty GIT segments were frozen on dry ice and thereafter kept

in -80 °C freezer. Samples from the other organs were frozen on dry ice and stored at -80 °C for RNA and protein isolation or fixed in 4% paraformaldehyde for histological analysis.

3.10.2.2 Morphometry of duodenum

3.10.2.2.1 Tissue preparation and sampling

The duodenum of each animal was longitudinally opened, randomly spun, flatted on a sheet of paper and fixed in 10% PBS-buffered formalin. Vertical sections were created according to Baddeley *et al.* (1986). Briefly, tissue samples (about 0.8 cm in length) were taken from four equidistant locations from proximal to distal duodenum. For each sample, a pair of mutually perpendicular stripes (about 5 mm in length) was taken, dehydrated and embedded in plastic in order to minimize shrinkage of tissue (Gerrits & Horobin 1996) according to the following procedure:

- Dehydrate and clear the samples using an automate (Shandon) at RT for 20 h: 1 × washing solution, 2 h; 2 × 50% ethanol, each 1 h; 2 × 70% ethanol, each 1 h; 2 × 96% ethanol, each 2 h; 3 × 100% ethanol, each 2 h; 2 × xylene, each 2 h.

  Washing solution:

Cacodylic acid sodium salt trihydrate ($C_2H_6AsNaO_2 \cdot 3H_2O$)	16.5 g
1 N HCl	6.23 ml
bidistilled water	1500 ml

  The pH value was adjusted to 7.2, then 105 g of D (+)-Sucrose ($C_{12}H_{22}O_{11}$) and 1.105 g $CaCl_2 \cdot 2H_2O$ were added and resolved, pH was adjusted to 7.2. The solution was stored at RT and prepared freshly every month.

- Incubate with GMA-MMA solution [2-Hydroxyethyl methacrylate (GMA): Methyl methacrylate (MMA), 1:1] for 18-24 h at 4 °C with gentle agitation.
- Incubate with solution A for 3-4 h (not longer than 4 h) at 4 °C with gentle agitation.

- The two tissue samples from the same location were placed on the bottom of a 12 ml embedding plastic cup, oriented perpendicular to surface, then the solution A containing 0.15% (v/v) N,N-dimethylaniline was poured into the cup. The cups were covered and placed in a pre-cooled water bath, which was subsequently placed at 4°C overnight. After the polymerization, the plastic-embedded tissue blocks were pulled out of the cups and stored in a -20°C freezer.

Solution A:	Benbzoyl peroxide (with 25 % water)	3.38 g
	MMA	200 ml
	GMA	600 ml
	Ethylene glycol monobutyl ether	160 ml
	Polyethylene glycol 400	20 ml

- 1.5 µm sections were cut normal to the horizontal plane, and PAS (Periodic-Acid-Schiff) staining was carried out as follows:
  A. Incubate with 1% periodic acid solution for 15 min.
  B. 3 × rinsing with bidistilled water.
  C. Incubate in fresh Schiff's reagent in dark room for 80 min.
  D. Wash with running water for 30 min.
  E. Dry on a 60°C stretching table.
  F. Incubate with Mayer's Haemalaun (hematoxylin) solution for 20 min.
  G. Wash with running water for 10 min.
  H. Rinse shortly in 1% HCl-ethanol solution.
  I. Wash with running water for 10 min.
  J. Dry on a 60°C stretching table.
  K. The sections were at last rinsed shortly in xylene and mounted with Eukitt mounting medium (O. Kindler GmbH & Co, Freiburg, Germany).

3.10.2.2.2 Morphometric analysis

Light microscopic morphometric evaluation was performed on a semiautomated image analysis system as described above. A 10 × objective was used, providing a 350 × final linear magnification. A cycloid test system consisting of 35 cycloid arcs and 70 test points (Fig. 7B in Baddeley *et al.* 1986) was photocopied on a transparent

sheet and superimposed over each test field displayed on the monitor screen (Figure 3.4). The vertical axis of the test system was aligned with the vertical direction on the section. Point counting was performed according to Baddeley *et al.* (1986). The number (P) of test points which hit the different layers of duodenum, and the number (I) of intersection points between cycloid arcs and villous surface were counted. Each vertical section was completely evaluated. With these data, the following parameters were calculated:

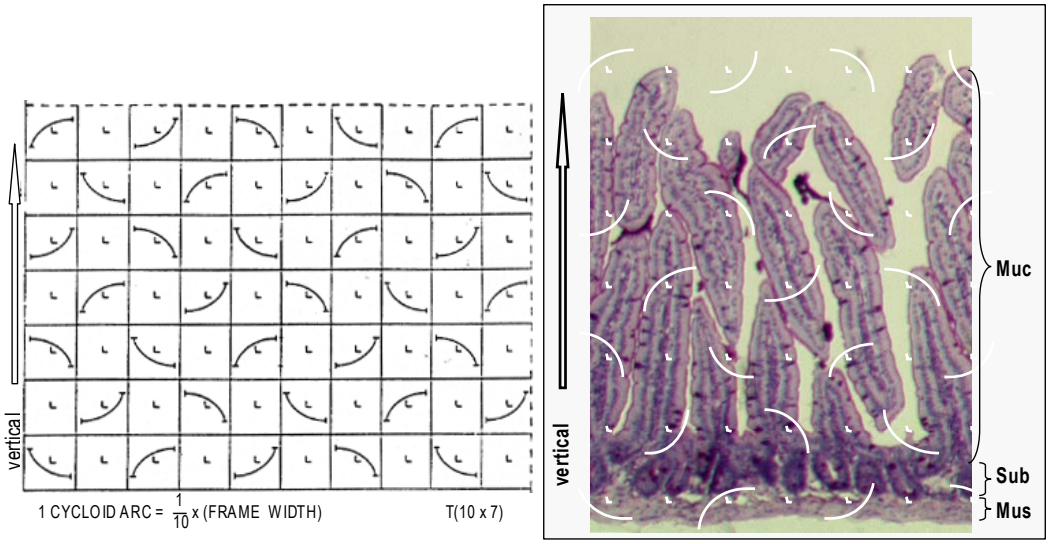

Figure 3.4 Schematic representation of the cycloid test system (*left panel*) superimposed over a test field of a vertical section of the duodenum (*right panel*). Muc, mucosa; Sub, submucosa; Mus, muscularis.

Fractional volumes of the 3 layers in duodenum (%):

$$Vv_{(x/duo)} = 100 \times \sum P_{(x)} / \sum P_{(duo)}$$

Total volumes of the 3 layers of duodenum (cm^3):

$$V_{(x)} = Vv_{(x/duo)} \times V_{(duo)}$$

Villous surface area density (cm^{-1}):

$$Sv = 2\,(p/l) \times M \times \sum I_{(muc)} / \sum P_{(muc)}$$

Total villous surface area (ViSA) of duodenum (cm^2):

$$ViSA = Sv \times V_{(muc)}$$

where

x = one of the three layers of the duodenum: mucosa (muc), submucosa (sub) or muscularis (mus).

$\sum P_{(x)}$ = total number of test points which hit the muc, sub or mus layer.

$\sum P_{(duo)}$ = total number of test points which hit the total duodenal wall.

p/l = the ratio of test points to test curve length = 70/(35 x 1.2 cm) = 1/0.6 (cm^{-1}).

M = the final linear magnification (x 350).

$\sum I_{(muc)}$ = total number of intersection points between cycloid arcs and villous surface.

$\sum P_{(muc)}$ = total number of test points which hit the mucosal wall.

$V_{(duo)} = W_{(duo)}$ (g) / 1.06 g/cm^3.

$W_{(duo)}$ = the weight of duodenum before fixation.

1.06 g/cm^3 = the mean specific gravity of the murine small intestine determined using the fluid displacement method (Ogiolda *et al.* 1998).

3.10.2.3 Scanning electron microscopy

Duodenum samples were taken from one- and two-month-old transgenic and control mice, opened longitudinally, washed three times in 0.9 % NaCl solution and fixed in 1 % glutaraldehyde solution. The specimens were then washed in PBS (pH 7.4), dehydrated in ascending concentrations of acetone, dried with a CPD-030 critical-point dryer (BAL-TEC, Schalksmuehle, Germany), and sputter coated (Balzers Union SCD-040, Balzers, Wiesbaden, Germany) with gold-palladium, mounted on a stub and then examined with a scanning electron microscope (DSM-950, Carl Zeiss, Oberkochen, Germany).

3.10.2.4 Serum glucose levels

Blood was extracted from the tail of 12 h-fasted animals and again after a period of 4 hours after the animals had free access to food. Glucose blood levels were measured with the Medisense Precision QID™ System (MediSense, Taufkirchen, Germany).

3.10.2.5 Serum glucagon levels

Serum glucagon levels were measured using serum from non-fasted mice with a commercial glucagon RIA kit (BioTrend GmbH, Cologne, Germany).

3.10.2.6 Glucose tolerance test

12 h-fasted animals received orally 250 µl of 1 M glucose solution/30 g body weight. Blood was collected from the tail vain immediately prior to glucose administration and after 10, 30, 60, 90 and 120 min. Blood glucose levels were measured with the Medisense Precision QID™ System.

3.10.2.7 Serum insulin levels

Blood was collected by bleeding from the retro-orbital sinus from 12 h-fasted animals and again after a period of 4 h after the animals had free access to food. Insulin serum levels were measured with a commercial insulin RIA kit (Insulin-CT, CIS Bio International, Gif Sur Yvette Cedex, France).

3.11 Statistics

The data were analyzed for significance of differences using the Student's t-test. A difference was considered to be statistically significant at $p < 0.05$.

4 RESULTS

4.1 Expression of mIGFBP-4 and -6 *in vitro*

4.1.1 Expression of mIGFBP-4 and -6 in murine NIH-3T3 fibroblasts

The constructs H-2K^b-mcIGFBP-4 and -6 were tested by stable transfection of murine NIH-3T3 cells. Several clones were established by G418 selection. Their genomic integration was analyzed by PCR (data not shown). The human β-globin splicing cassette allows the unambiguous identification of the transgene transcripts. RT-PCR revealed that the selected clones expressed transgene mRNA, which were 850-bp shorter than the corresponding sequences in the expression vectors due to the splicing of the 850-bp intron in the human β-globin splice cassette, whereas the untransfected parental cells (P) and mock clone (MC) did not (Figure 4.1 and 4.2). [^{125}I]-IGF-II ligand blotting analysis showed that a 24 kDa or 26 kDa protein was increased in the SFCM from pH-2K^b-mcIGFBP-4 or -6 transfected clones compared to P and MC

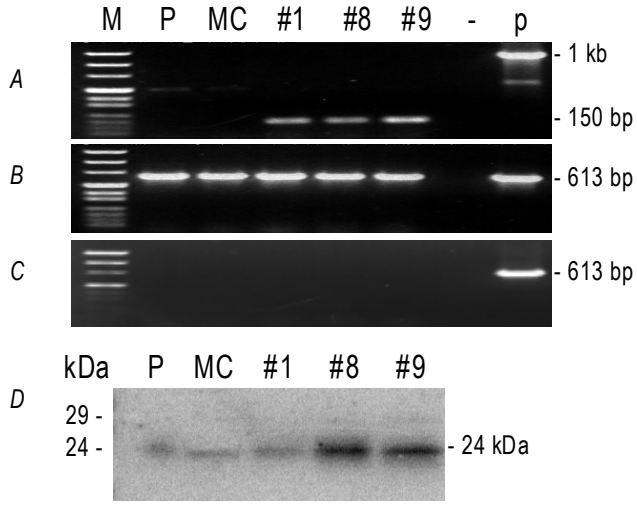

Figure 4.1 IGFBP-4 expression in NIH-3T3 fibroblasts. *A*. RT-PCR of H-2K^b-mcIGFBP-4. *B*. RT-PCR of β-actin to confirm the efficiency of the reverse transcription (RT). *C*. PCR of β-actin without RT to confirm the efficiency of DNase I-digest. *D*. [^{125}I]-IGF-II ligand blotting analysis of SFCM from untransfected cells (P), vector-only-transfected (MC) and H-2K^b-mcIGFBP-4-transfected clones. M, pUC Mix 8 DNA marker; -, water; p, plasmid pH-2K^b-mcIGFBP-4 (*A*) and pCR-TOP-beta-actin (*B, C*).

(Figure 4.1 and 4.2), which were identified as being IGFBP-4 or -6 by Western immunoblotting (Figure 4.7B and 4.15B).

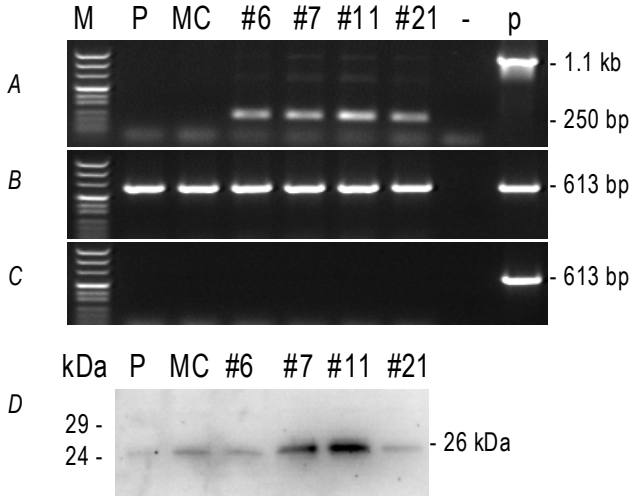

Figure 4.2 IGFBP-6 expression in NIH-3T3 fibroblasts. *A*. RT-PCR of H-2K^b-mcIGFBP-6. *B*. RT-PCR of β-actin to confirm the efficiency of the reverse transcription (RT). *C*. PCR of β-actin without RT to confirm the efficiency of DNase I-digest. *D*. [^{125}I]-IGF-II ligand blotting analysis of SFCM from untransfected cells (P), vector-only-transfected (MC) and H-2K^b-mcIGFBP-6-transfected clones. M, pUC Mix 8 DNA marker; -, water; p, plasmid pH-2K^b-mcIGFBP-6 (*A*) and pCR-TOP-beta-actin (*B, C*).

4.1.2 Expression of mIGFBP-6 in human 293 cells

To test the construct CMV-mgIGFBP-6, human 293 cells were transiently transfected with pCMV-mgIGFBP-6. [^{125}I]-IGF-II ligand blotting showed that a 26-kDa protein was detected in the SFCM from pCMV-mgIGFBP-6-transfected 293 cells (CMV-BP6) but not in the ones from the untransfected (P) and vector-only-transfected 293 cells (MK). This protein was identical to the IGFBP-6 expressed by the NIH-3T3 clone #11 described above (Figure 4.3).

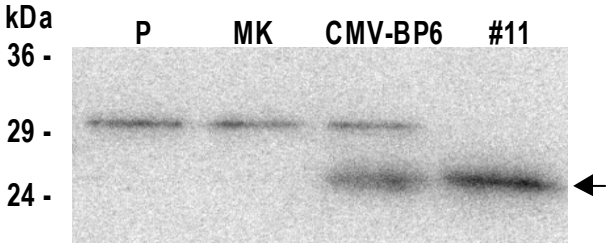

Figure 4.3 [^{125}I]-IGF-II ligand blotting analysis of SFCM from non-transfected (P), vector-only-transfected (MK) and pCMV-mgIGFBP-6-transfected 293 cells (CMV-BP6). The SFCM from pH-2K^{b}-mcIGFBP-6-transfected NIH-3T3 clone 11 (#11) was loaded as a positive control.

4.2 H-2K^{b}-mcIGFBP-4 transgenic mice

4.2.1 Identification of transgenic mice

One litter comprising 3 mice was obtained from microinjected zygotes. Two mice were identified as being transgenic founders by PCR (data not shown) and backcrossed with wild-type C57BL/6 mice. Both of them transmitted the transgene to offspring, producing two transgenic lines (L1 and L2). Genomic integration patterns of H-2K^{b}-mcIGFBP-4 in the both lines were analyzed by Southern blot analysis (Figure 4.4). In wild-type and transgenic animals, hybridization signals of the endogenous IGFBP-4 were detected at 8 kb and 2.7 kb after *Eco*RI digest. In addition to these bands, a 5 kb band, as large as the microinjection fragment, was detected in both transgenic lines. Furthermore, a band larger than 10 kb was detected in line 1 and a 10 kb band in line 2.

Two litters comprising 5 animals were obtained from embryo transfer of the line 2 F2-transgenic mice under SPF conditions. Three of the offspring (F3) were identified as being transgenic and mated with wild-type C57BL/6 SPF mice under SPF conditions to generate F4 animals for immunological analysis.

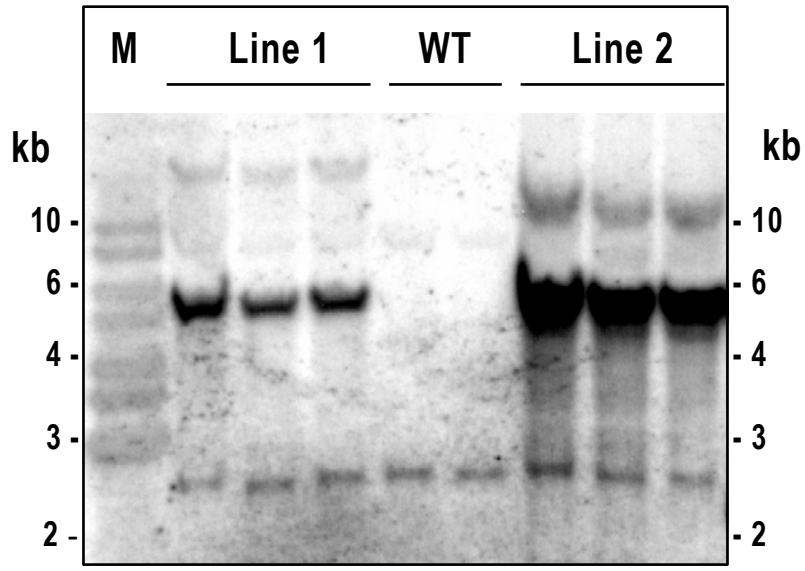

Figure. 4.4 Southern blot analysis of H-2K^b-mcIGFBP-4 transgenic (Line1 and Line 2) and control (WT) mice. M, kb DNA ladder.

4.2.2 Transgene expression

From both of the H-2K^b-mcIGFBP-4 transgenic lines, three transgenic (TG) and three control mice (WT) were selected for expression studies. Total RNA and protein were extracted from the following organs/tissues: adrenal gland, bladder, brain, heart, kidney, liver, lung, ovary/testis, pancreas, salivary gland, skeletal muscle, skin, spleen, stomach, small and large intestine and thymus. Except for pancreas, total RNA was successfully extracted from all organs/tissues. The RNA was degraded consistently in the pancreas (Figure 4.5). RT-PCR revealed ubiquitous expression of the transgene in all tested organs/tissues (Figure 4.6). However, elevated IGFBP-4 protein levels were detected only in spleen, thymus, lung and kidney by [^{125}I]-IGF-II ligand blotting and Western immunoblotting (Figure 4.7). IGFBP-4 overexpression was consistently higher in line 2 than in line 1.

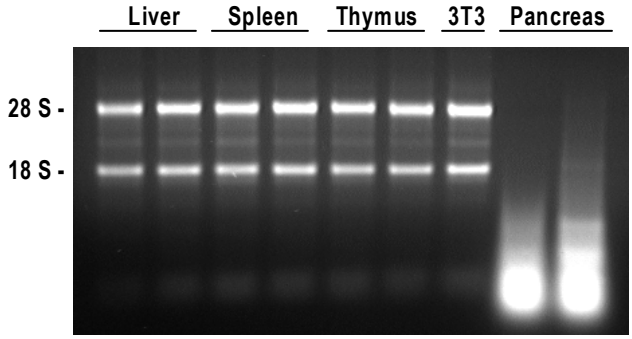

Figure 4.5 Agarose gel electrophoresis of total RNA extracted from liver, spleen, thymus and NIH-3T3 cells (3T3) using TriPure® Isolation Reagent.

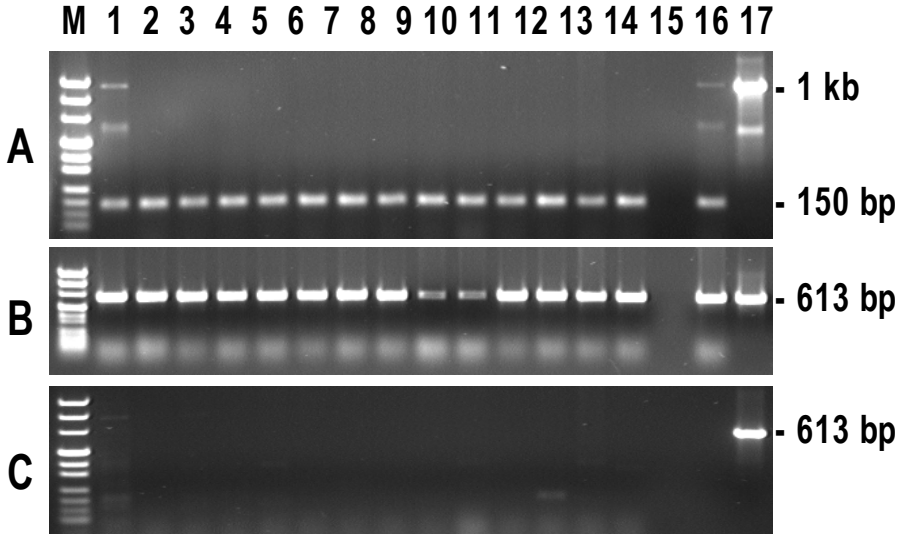

Figure 4.6 RT-PCR analysis of transgene mRNA expression in different tissues of H-2K^b-mcIGFBP-4 transgenic mice: adrenal gland (1), bladder (2), brain (3), heart (4), kidney (5), liver (6), lung (7), skeletal muscle (8), ovary (9), salivary gland (10), skin (11), spleen (12), testis (13) and thymus (14). 15, water. 16, pH-2K^b-mcIGFBP-4-transfected NIH-3T3 clone #9. 17, plasmids pH-2K^b-mcIGFBP-4 (*A*) or pCR-TOP-beta-actin (*B*,*C*). *A*. RT-PCR of H-2K^b-mcIGFBP-4. *B*. RT-PCR of β-actin to confirm the efficiency of the reverse transcription (RT). *C*. PCR of β-actin without RT to confirm the efficiency of DNase I-digest.

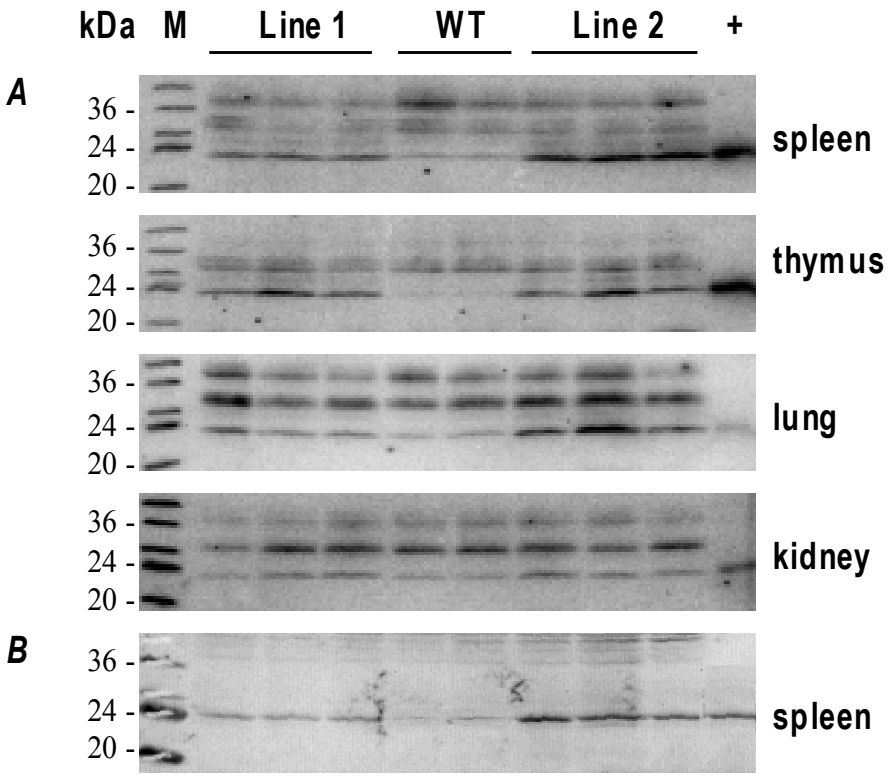

Figure 4.7 IGFBP-4 protein expression in several tissues of transgenic (Line 1 and Line 2) and control (WT) mice as determined by [^{125}I]-IGF-II ligand blotting (*A*) and Western immunoblotting (*B*). +, SFCM of H-2K^{b}-mcIGFBP-4-transfected NIH-3T3 clone #9. M, Sigma low-range protein marker.

4.2.3 Body weight gain and organ growth

Transgenic mice and non-transgenic littermates from both lines were weighed once a week from 3 to 10 weeks of age to detect a possible effect of H-2K^{b}-mcIGFBP-4 transgene expression on body weight gain. No significant difference was observed between TG and WT animals in both lines (Figure 4.8).

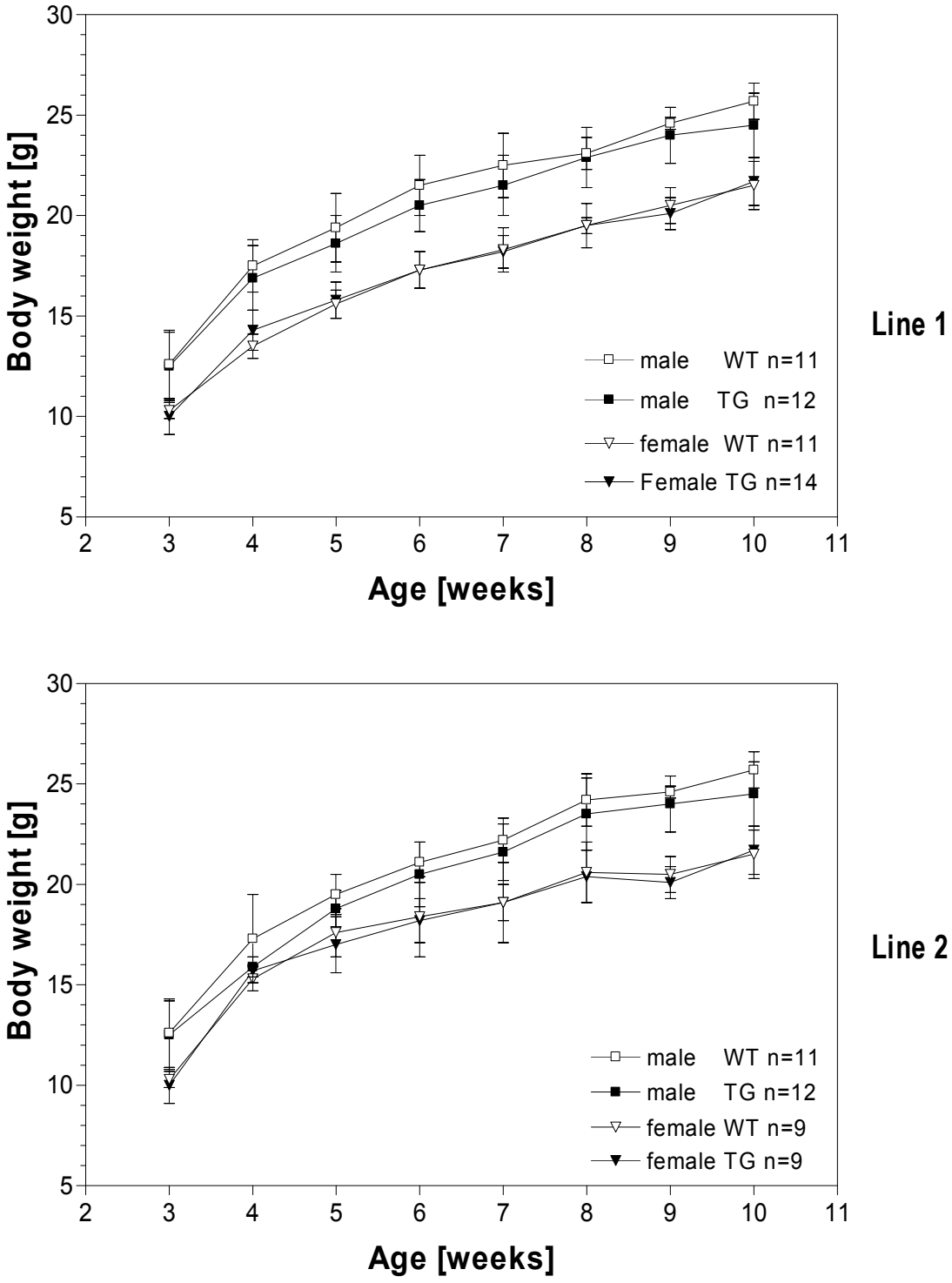

Figure 4.8 Body weight gain of H-2K^b-mcIGFBP-4 transgenic and control mice. The data are presented as mean ± SD.

NRL, body and organ weights were recorded in 8- and 12-week-old animals. There was no significant difference in NRL, body and organ weights between TG and WT mice. The only exception was the thymus of TG mice, which was significantly reduced in weight when compared to WT mice (Figure 4.9A). This reduction persisted when thymus weight was related to body weight (Figure 4.9B). Morphometric analysis revealed that the volumes of thymus and its cortex of TG mice were significantly reduced compared to WT littermates, whereas the volume of medulla of thymus was not different between TG and WT animals (Figure 4.10A). In contrast, there was no significant difference in weight (data not shown), total and compartment volumes of spleen between TG and WT mice (Figure 4.10B), although IGFBP-4 was also overexpressed in the spleen.

4.2.4 Flow cytometry

To investigate the potential effect of transgene expression in the thymus and spleen of H-2K^b-mcIGFBP-4 transgenic mice on the development of lymphocytes, the lymphocytes from the peripheral blood, thymus, spleen, lymph node and bone marrow of TG and WT mice (SPF) from line 2 were analyzed by flow cytometry (FACS), using antibodies against different markers expressed in the T- and B-cells and other populations of lymphocytes, as described in Material and Methods. No significant difference was found between the two groups (data not shown).

4.2.5 Basal plasma immunoglobin levels

To detect possible effect of transgene expression in the thymus and spleen of H-2K^b-mcIGFBP-4 transgenic mice (SPF) on the production of the humoral immuno-competence, the basal plasma immunoglobin (Ig) levels were measured using ELISAs specific for IgM, IgG3 and IgA. Plasma autoreactive antibody was also determined. Their levels of TG mice were not different from the ones of WT littermantes (data not shown).

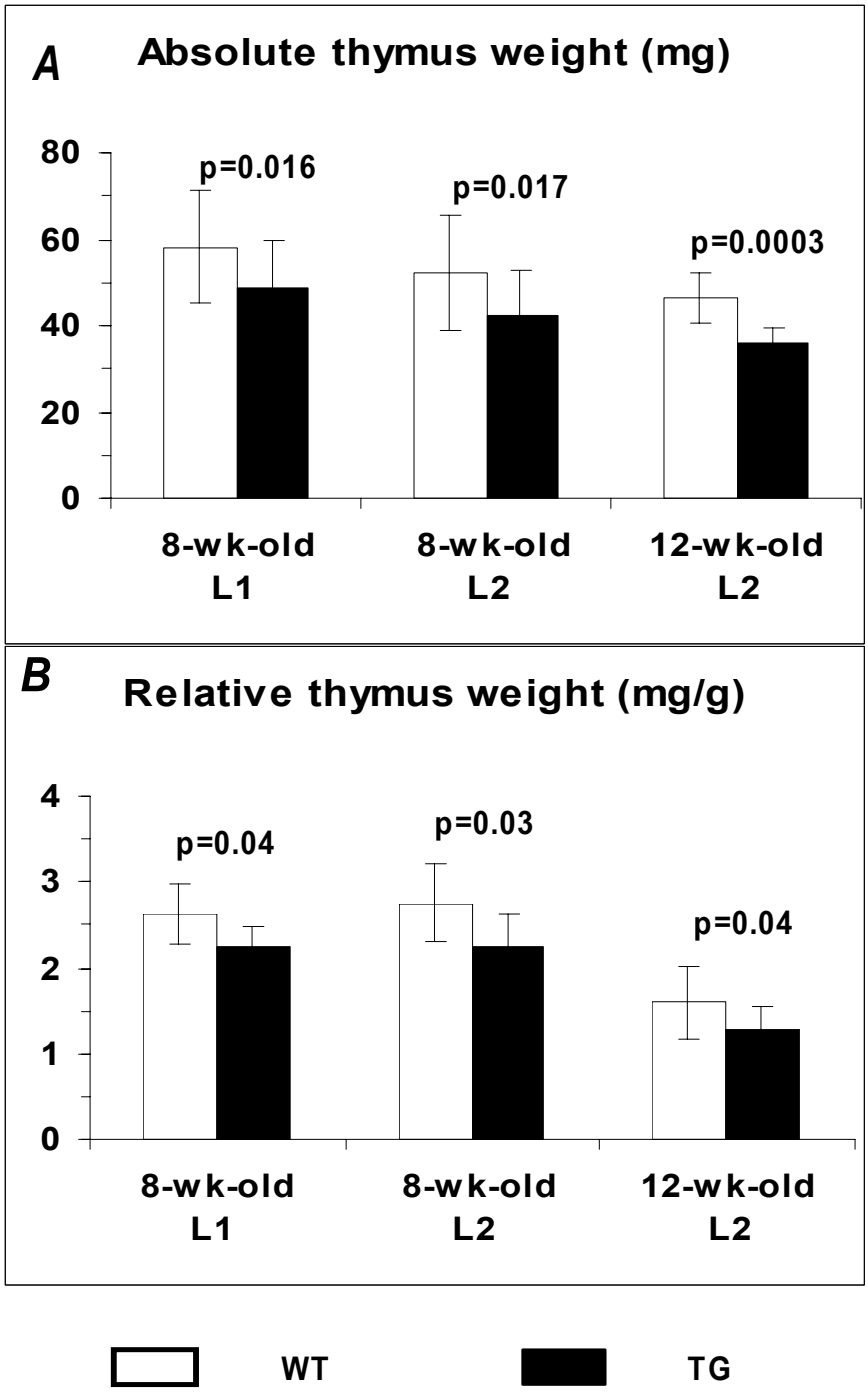

Figure 4.9 Reduced absolute (*A*) and relative (*B*) weight of thymus of H-2K^b-mcIGFBP-4 transgenic (TG) mice compared to control (WT) mice. 15-20 mice from each group were investigated. Data are presented as mean ± SD.

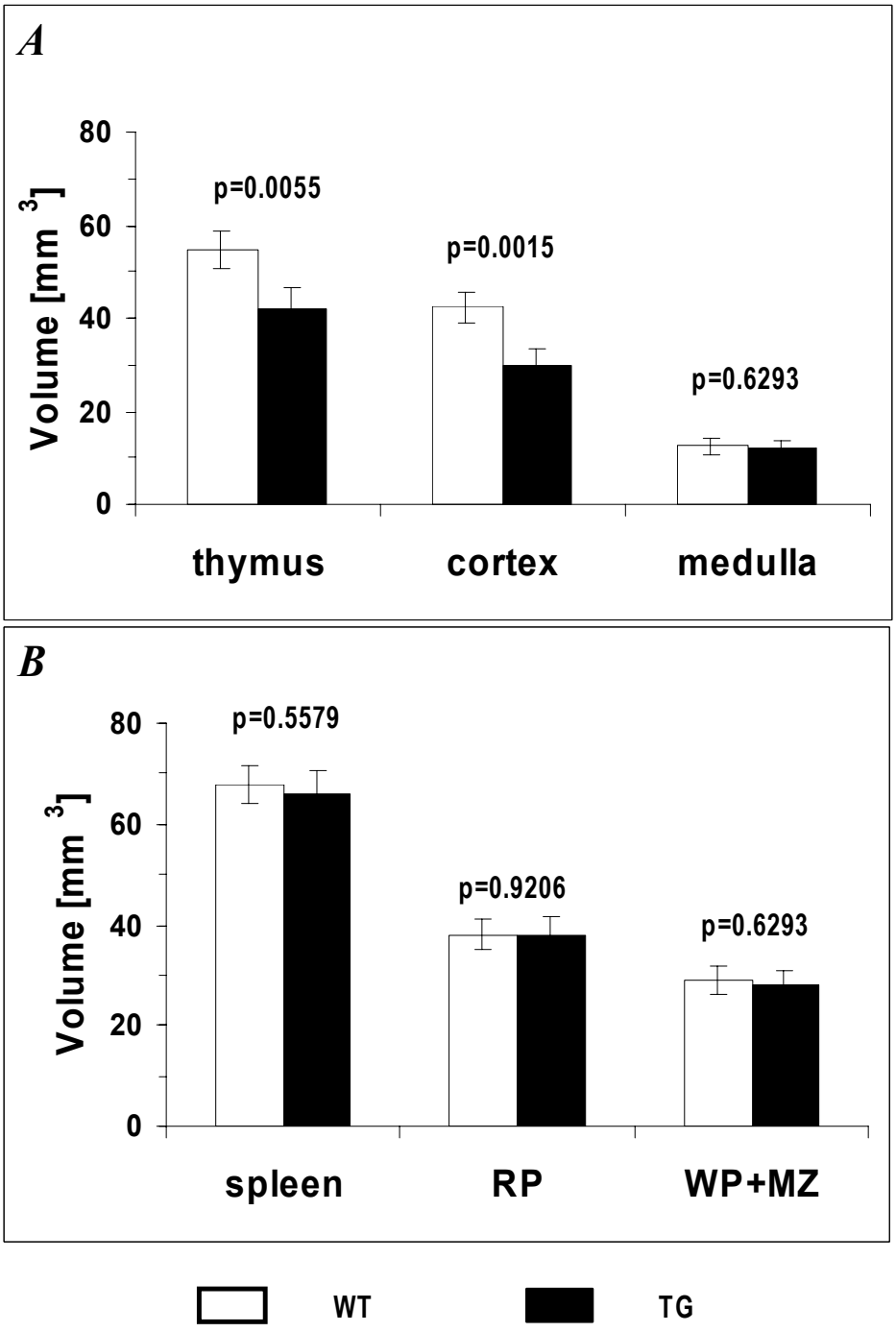

Figure 4.10 *A*. Thymus, cortex and medulla volumes of 8-week-old H-2K^b-mcIGFBP-4 transgenic (TG) mice (n = 4) and their wild-type (WT) littermates (n = 4). *B*. Volumes of spleen, red pulp (*RP*), white pulp and marginal zone (*WP+MZ*) of 8-week-old H-2K^b-mcIGFBP-4 TG mice (n = 4) and their WT littermates (n = 4). Data are presented as mean ± SD.

4.2.6 Mitogenic response of splenocytes

To ascertain the proliferative capacity of T- and B-cells in the IGFBP-4 transgenic mice, splenocytes were stimulated with ConA, anti-CD3 antibody, LPS and anti-IgM antibody/IL2 respectively. First results revealed that the proliferative activity of IGFBP-4 transgenic splenocytes was significantly reduced after ConA ($p < 0.05$) and LPS ($p < 0.01$) stimulation, but did not altered after anti-CD3 and anti-IgM/IL2 stimulation (Figure. 4.11). These findings await further confirmation.

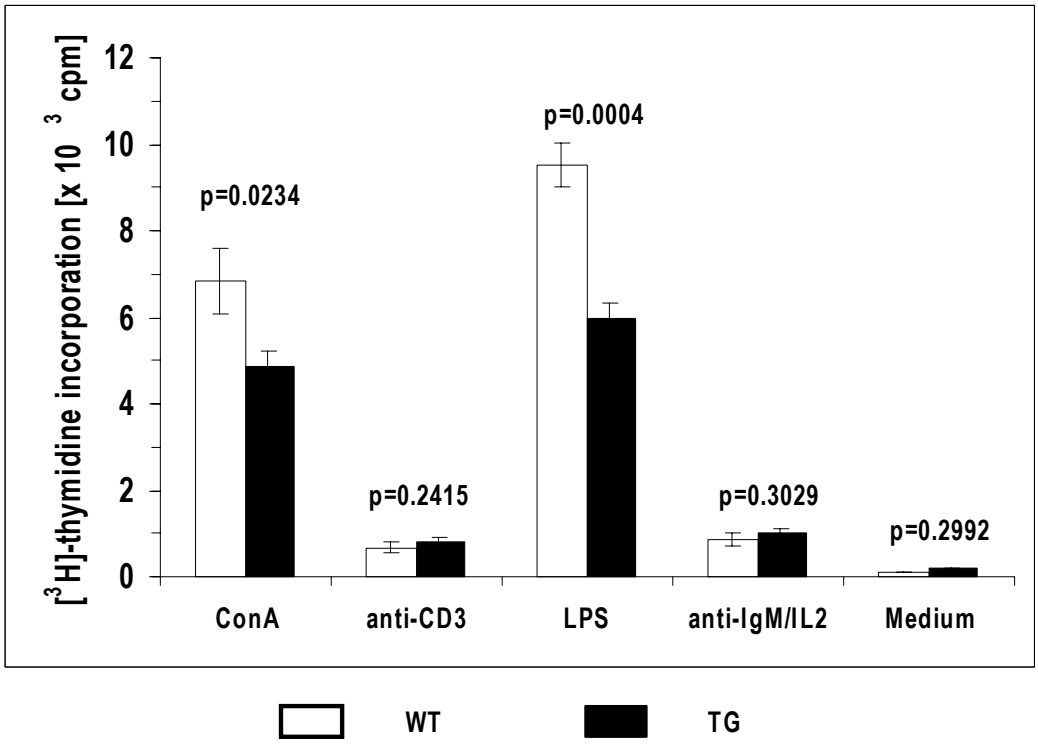

Figure 4.11 Proliferation assay of splenocytes from H-2K^b-mcIGFBP-4 transgenic (TG) and wild-type (WT) mice.

4.3 H-2K^b-mcIGFBP-6 transgenic mice

4.3.1 Identification of tramsgenic mice

Seven litters comprising 56 mice were obtained from microinjected zygotes. Ten mice were identified as being transgenic founders by PCR (some of them were shown in Figure 4.12), and transmitted the transgene to offspring, producing 10 transgenic lines.

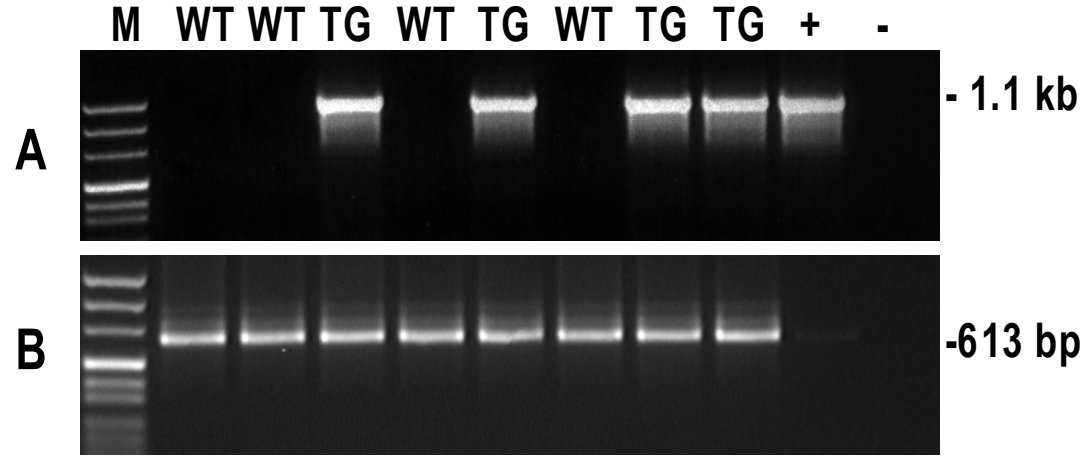

Figure 4.12. PCR analysis of H-2K^b-mcIGFBP-6 transgenic (TG) and wild-type (WT) mice. *A*, transgene-specific PCR using primers mBP6#6 and β-globin#1. *B*, β-actin PCR. M, pUC Mix 8 DNA marker. +, pH-2K^b-mcIGFBP-6 (*A*). -, water.

4.3.2 Transgene expression

Transgenic mRNA expression in H-2K^b-mcIGFBP-6 transgenic mice was screened by RT-PCR as described in the H-2K^b-mcIGFBP-4 transgenic model. Consistent with H-2K^b-mcIGFBP-4 transgenic mice, transgene was expressed ubiquitously at RNA level in all tissues of the H-2K^b-mcIGFBP-6 transgenic mice. The results of spleen, kidney, bladder liver and lung from lines 1 and 4 were shown in Figure 4.13. However, the IGFBP-6 protein levels were not increased in the TG mice as determined by [^{125}I]-IGF-II ligand blotting, when compared to the controls (data not shown).

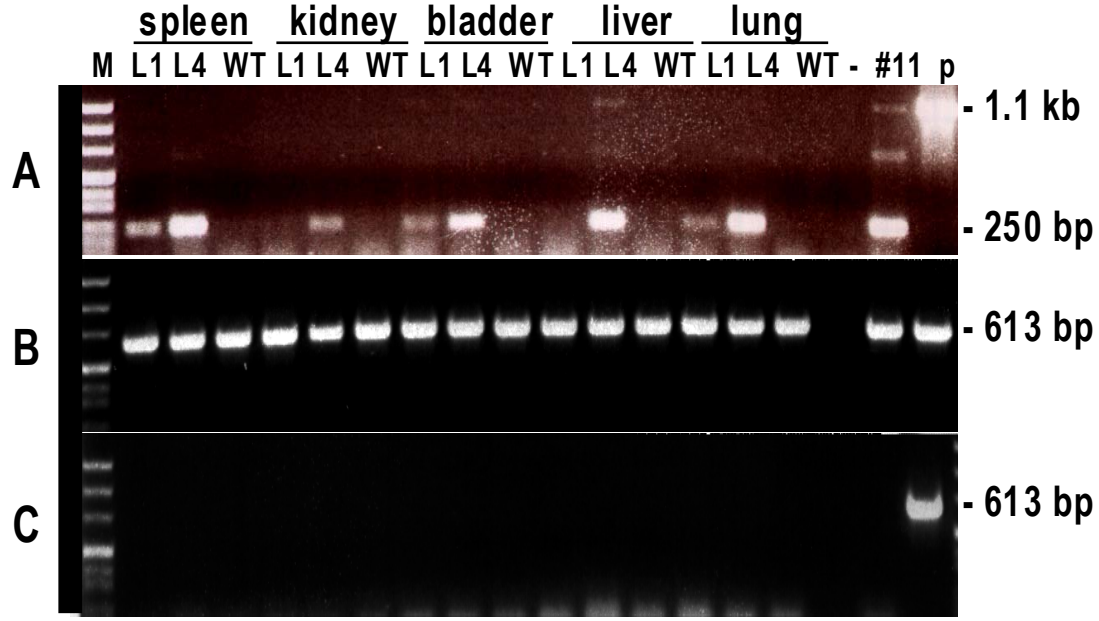

Figure 4.13 RT-PCR analysis of transgene expression in the H-2K^b-mcIGFBP-6 transgenic (L1, L4) and control (WT) mice. *A*. RT-PCR of H-2K^b-mcIGFBP-6. *B*. RT-PCR of β-actin to confirm the efficiency of the reverse transcription (RT). *C*. PCR of β-actin without RT to confirm the efficiency of DNase I-digest. -, water. #11, H-2K^b-mcIGFBP-6-transfected NIH-3T3 clone #11. p, plasmid pH-2K^b-mcIGFBP-6 (*A*) and pCR-TOP-beta-actin (*B, C*).

4.4 CMV-mgIGFBP-6 transgenic mice

4.4.1 Identification of transgenic mice

Ten litters comprising 45 mice were obtained from the microinjected zygotes. Five animals were identified as being transgenic founders by PCR (Figure 4.14) and mated with wild-type C57BL/6 mice. Four founders transmitted the transgene to offspring, producing four transgenic lines (lines 1, 2, 4 and 5).

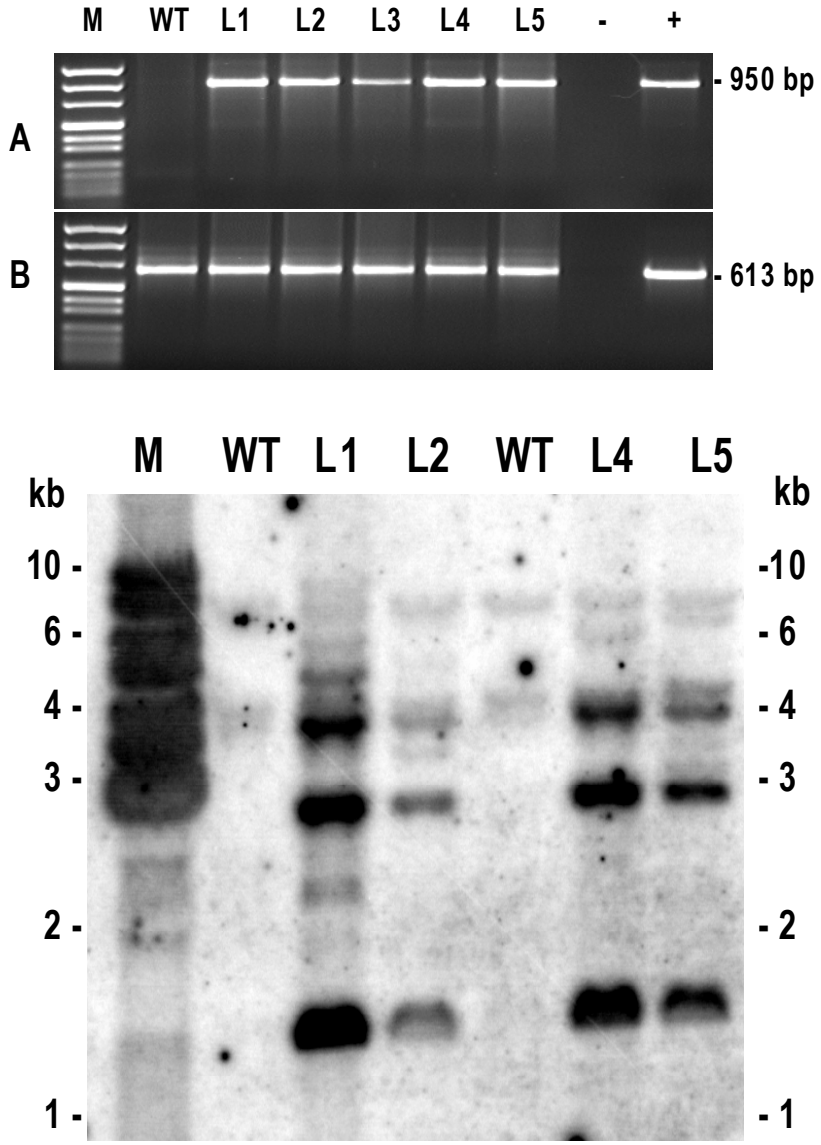

Figure 4.14 PCR identification of CMV-mgIGFBP-6 transgenic founders (*upper panel*) and Southern blot analysis of the TG and wild-type (WT) mice from the F1-generation (*lower panel*). *A*. transgene-specific PCR using primers CMV#3 and mBP6#7. *B*. β-actin PCR. M, pUC Mix 8 DNA marker (*upper panel*) and kb DNA ladder (*lower panel*). -, water. +, plasmids pCMV-mgIGFBP-6 (*A*) and pCR-TOP-beta-actin (*B*). The transgene in L3 was lost in the F1-generation.

Genomic integration patterns of CMV-mIGFBP-6 in the four transgenic lines were analyzed by Southern blot analysis (Figure 4.14). In all transgenic and wild-type mice, hybridization signals of the endogenous IGFBP-6 were detected at 8 kb and 3.8 kb after *EcoR* I digest. In addition to these bands, a 2.8 kb and a 1.5 kb fragment were detected in all four transgenic lines. Furthermore, two fragments of 5 kb and 2.3 kb were detected in line 1; a 3.5 kb band in line 2; a 6 kb band in line 4; and two bands of 7 kb and 4.5 kb in line 5

4.4.2 Transgene expression

Western ligand blot analysis revealed that the abundance of a 26-kDa protein, with can bind to [^{125}I]-IGF-II, was predominantly high in the pancreas, relatively low in the liver and lung, and undetectable in other organs of the CMV-mgIGFBP-6 transgenic mice from all lines. There was no difference between transgenic mice and controls in the levels of serum IGFBPs. Data are shown only for lines 2 and 5 which were further characterized (Figure 15A). The 26-kDa IGFBP detected in the pancreas extracts by ligand blot was IGFBP-6 confirmed by Western immunoblot using chicken anti-mIGFBP-6 antibodies (Figure 15B). To localize IGFBP-6 expression in the pancreas, immunohistochemical staining was performed. Two available anti-mouse IGFBP-6 antibodies were used in this study. Although there was strong background, IGFBP-6 immunoreactivity was stronger in exocrine pancreas than in the islets (data not shown). Interestingly, elevated levels of active IGFBP-6 were also detected in the luminal content of the duodenum, but neither in the luminal contents of other GIT segments, nor in the tissue extracts of any GIT segment of transgenic mice (Figure 16). This finding, together with the immunohistochemical staining pattern in the pancreas, suggests that the increased IGFBP-6 levels in the duodenum of transgenic mice originated from the exocrine pancreas.

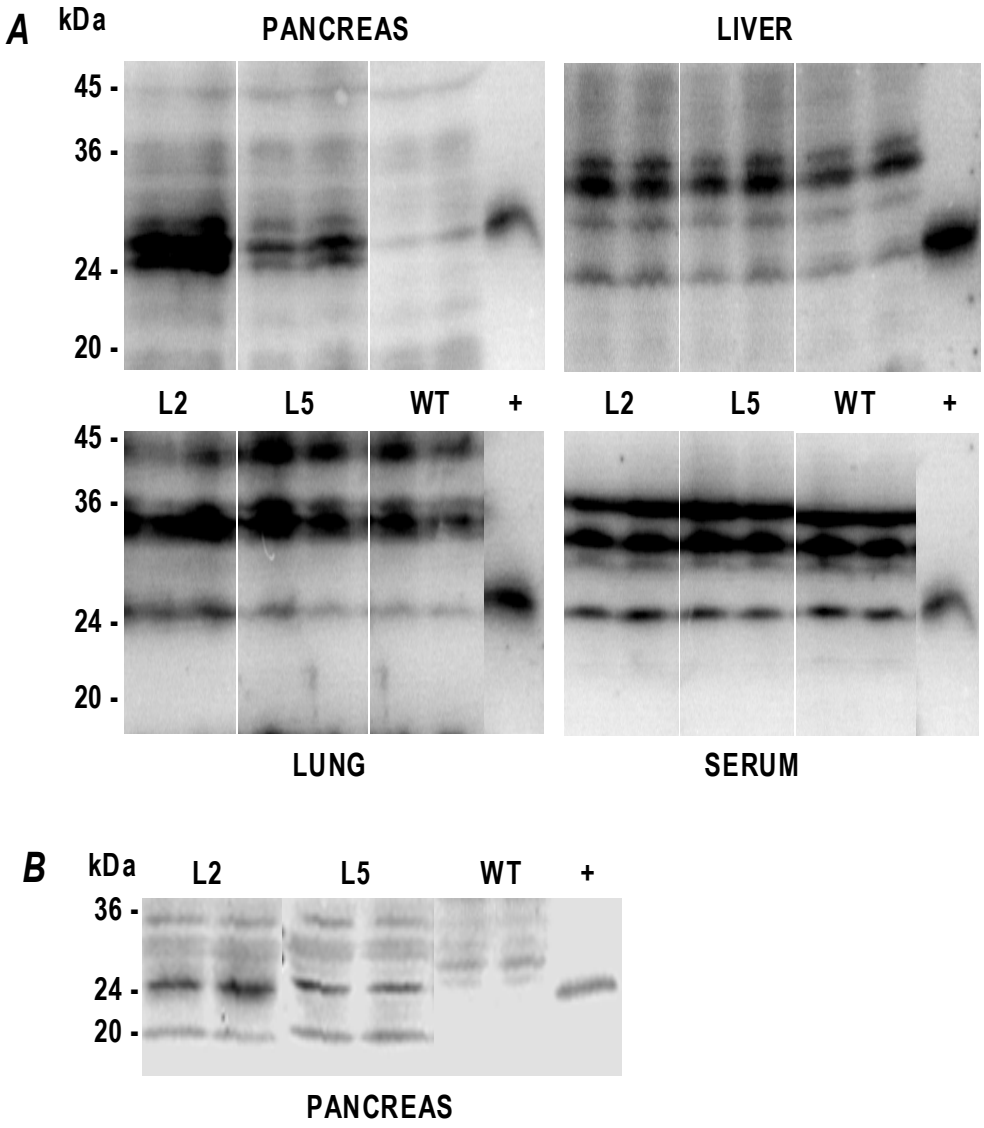

Figure 4.15 *Panel A*: [^{125}I]-IGF-II ligand blot analysis of IGFBP-6 expression in pancreas, liver, lung and serum from CMV-mgIGFBP-6 transgenic mouse lines L2 and L5 and control (WT) mice. *Panel B:* Western immunoblot analysis of IGFBP-6 expression in the pancreata of the TG and WT mice. The SFCM of the H-2K^{b}-mIGFBP-6-transfected NIH-3T3 cell clone #11 was loaded as IGFBP-6 protein positive control (+).

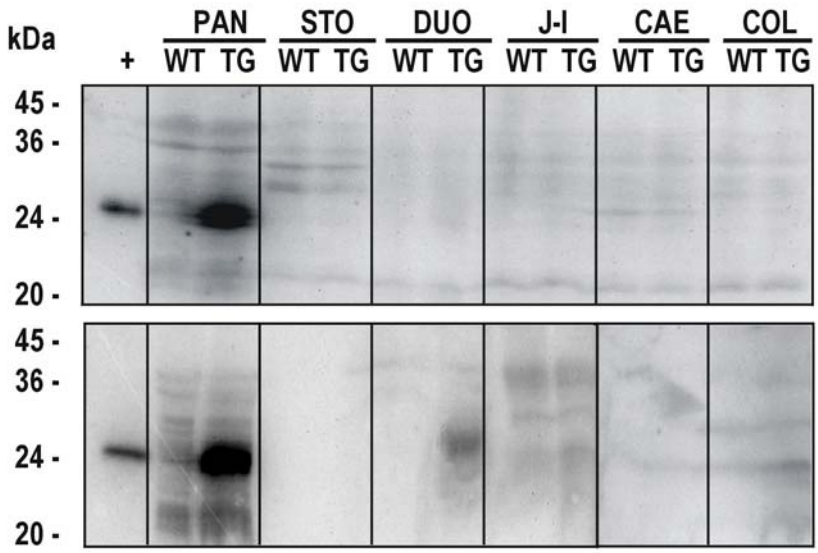

Figure 4.16. Detection of IGFBP-6 protein in the tissue extracts (*upper panel*) and luminal contents (*lower panel*) of different segments of the gastrointestinal tract from CMV-mgIGFBP-6 transgenic (TG) and control (WT) mice. Increased IGFBP-6 level was found only in the luminal content of duodenum of transgenic mice. The SFCN of the H-2K^b-mIGFBP-6-transfected NIH-3T3 clone #11 (+) and pancreas extracts (PAN) were also loaded as controls. STO, stomach; DUO; duodenum; J-I, jejunum/ileum; CAE, caecum; COL, colon.

4.4.3 Effects on glucose homeostasis

In order to investigate the potential effect of high IGFBP-6 levels in pancreas on glucose homeostasis, blood glucose levels and the corresponding serum insulin and glucagon levels were measured in fasted/refed transgenic and control animals, and a glucose tolerance test was also carried out. No significant differences between transgenic and control animals were found (Figure 4.17).

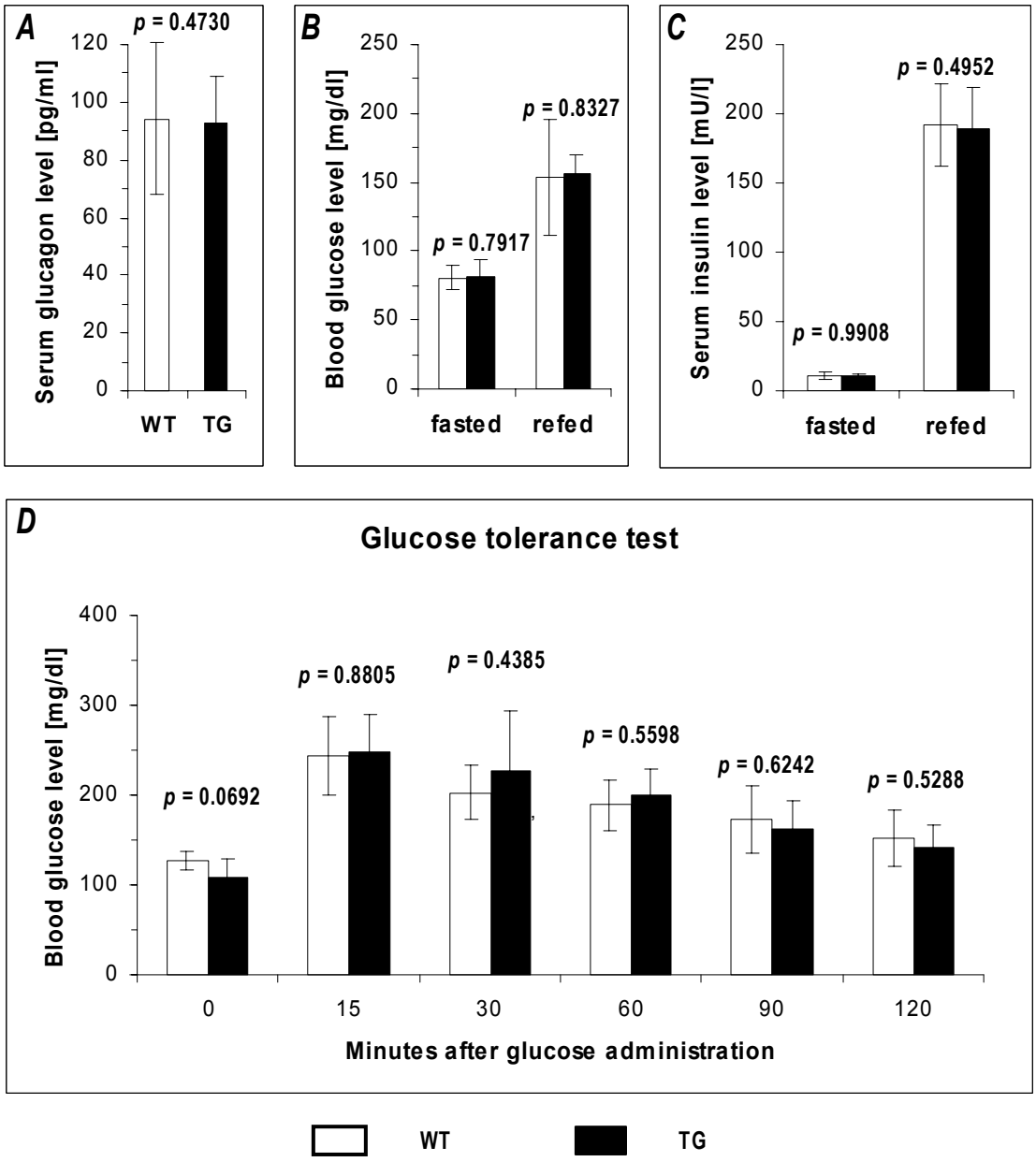

Figure 4.17. Effect of transgene expression on glucose homeostasis as determined by serum glucagon (*A*), glucose (*B*) and insulin levels (*C*) and by glucose tolerance tests (*D*) of CMV-mgIGFBP-6 transgenic (TG) and control (WT) mice. Data are presented as mean ± SD.

4.4.4 Effects on body and organ growth

Transgenic mice and nontransgenic littermates from lines 2 and 5 were weighed once a week from 3-15 weeks of age to detect possible effects of CMV-mIGFBP-6 transgene expression on body weight gain. No significant difference was observed between transgenic and control animals in both lines (Figure 4.18).

Body and organ weights were recorded in 4-month-old animals. There was no significant difference in NRL and organ weights between transgenic mice and their nontransgenic littermates. The only exception was the duodenum of transgenic mice, which was significantly reduced in length (L2: 17%; L5: 14%) and weight (L2: 32%; L5: 17%) when compared to controls (Figure 4.19). This reduction persisted when organ length and weight measurements were related to body weight (Figure 4.20). No significant difference between transgenic mice and controls was observed for the weights or lengths of other segments of the GIT. Morphometric analysis revealed that the fractional volume of mucosa in the duodenum of transgenic mice was significantly reduced, whereas those of submucosa and muscularis were significantly increased (Figure 4.21A). Accordingly, the absolute volume of the mucosa of duodenum was significantly decreased in transgenic mice by 31%, but those of submucosa and muscularis were not different between the two groups (Figure 4.21B). The mean total villous surface area (ViSA) of the duodenum of transgenic mice was significantly smaller (42%) than that of the nontransgenic littermates (Figure 4.22). Scanning electron microscopic observation suggested that the villous dimension of duodenum is smaller in transgenic mice than that in control mice (Figure 4.22).

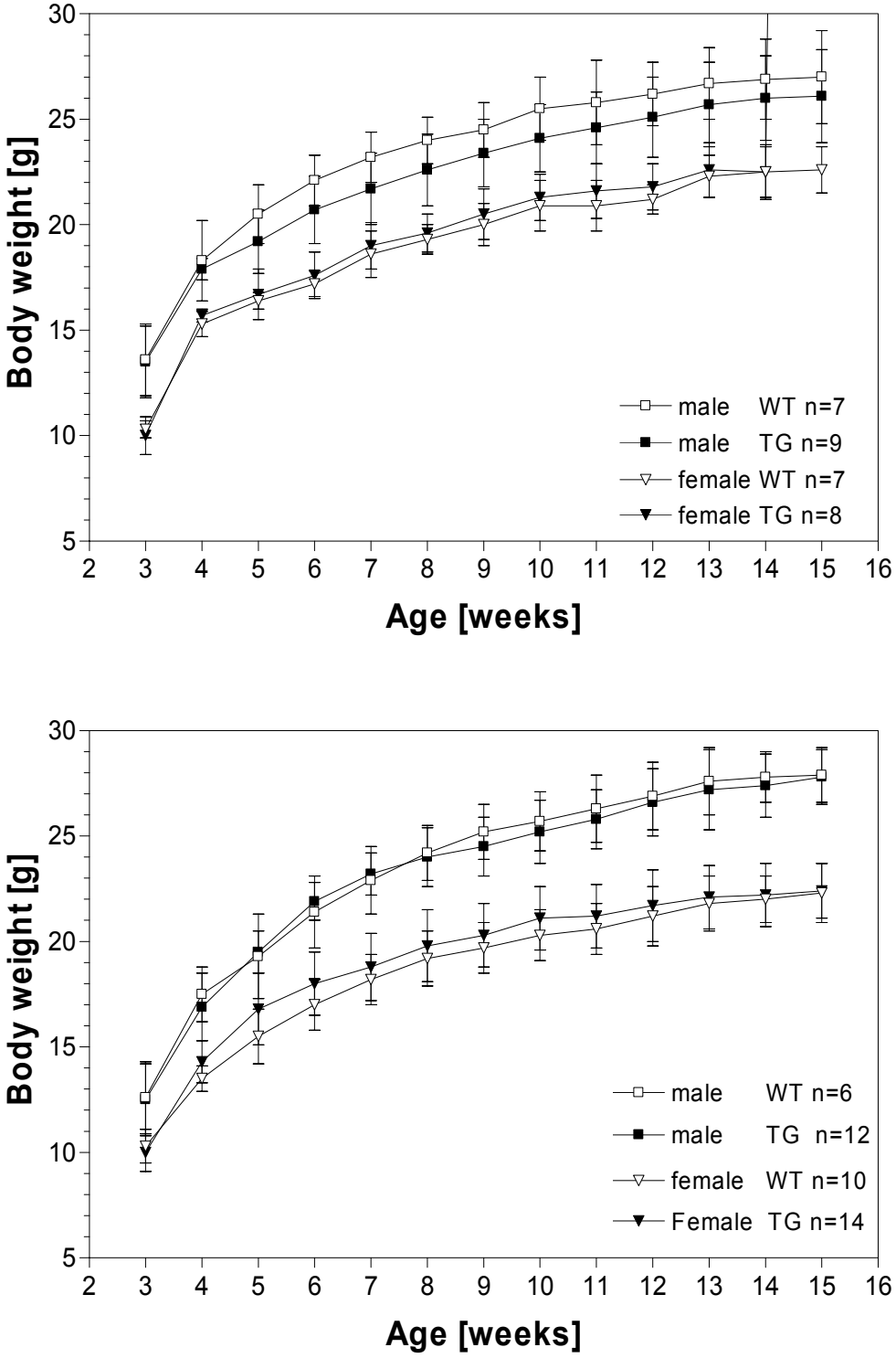

Figure 4.18 Body weight gain of CMV-mgIGFBP-6 transgenic (TG) and control (WT) mice. The data are presented as mean ± SD.

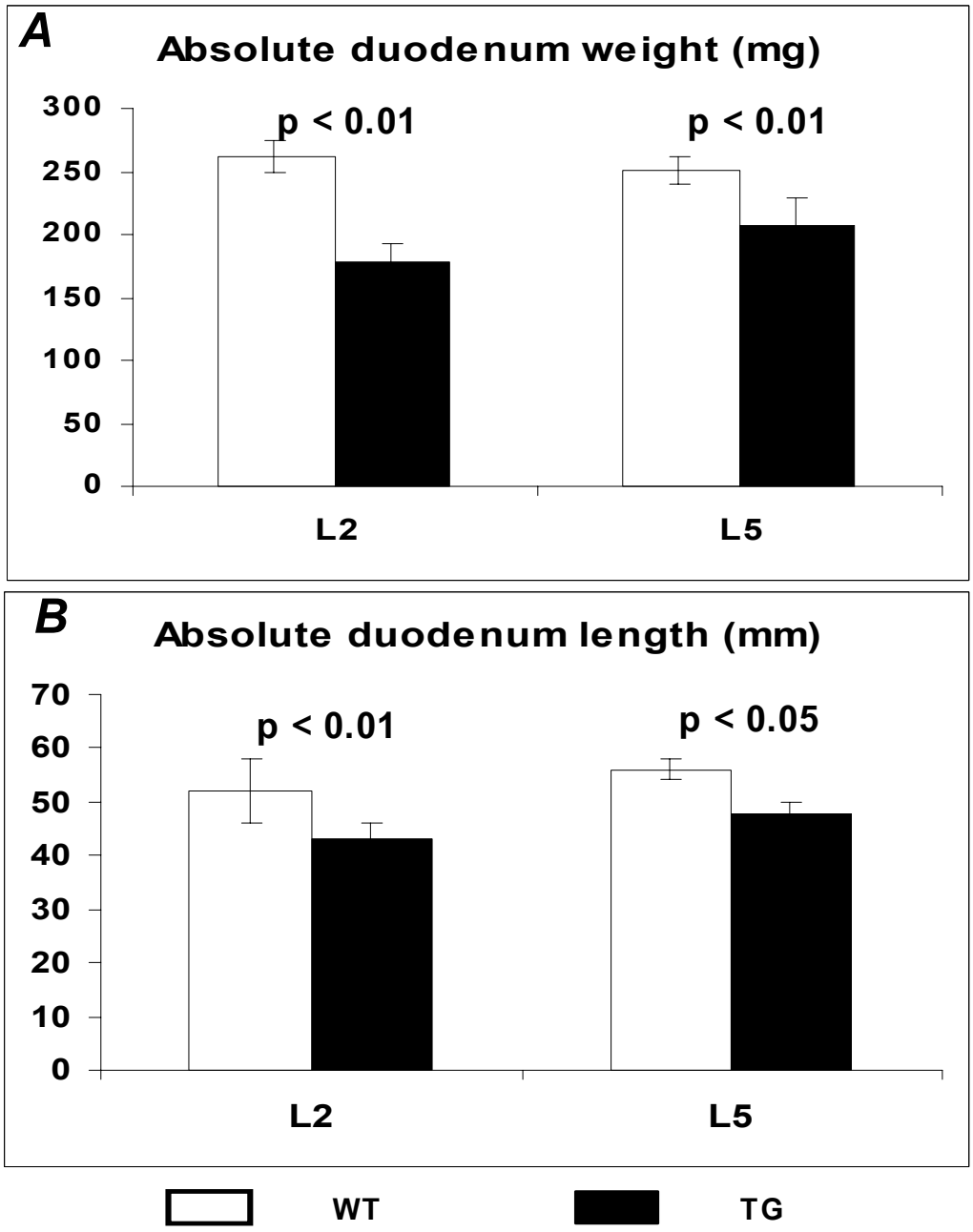

Figure 4.19 Absolute weight (*A*) and length (*B*) of duodenum of four-month-old transgenic (TG) and their wild-type (WT) littermates. 15-20 mice from each group were investigated. Data are presented as mean ± SD.

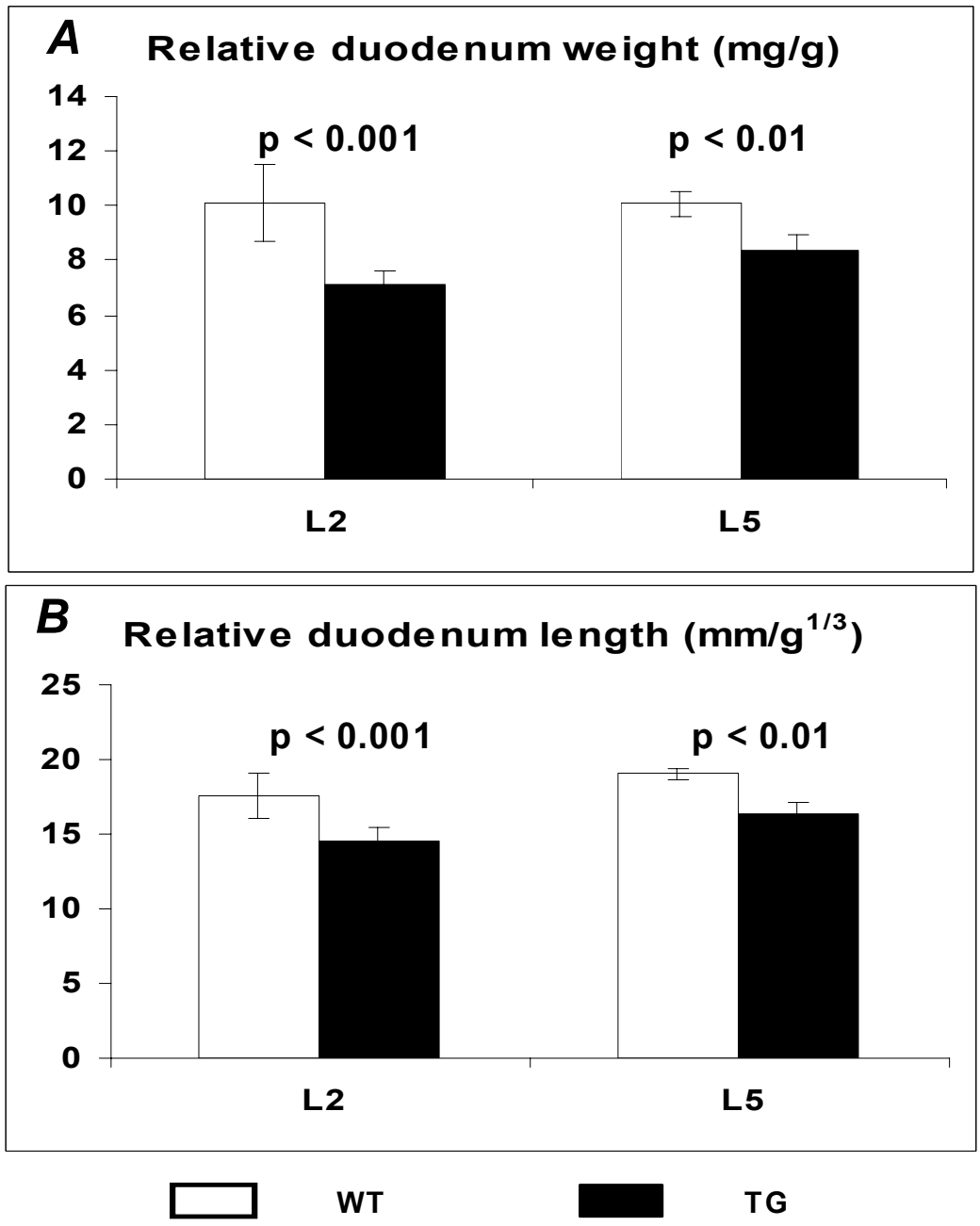

Figure 4.20 Relative weight (*A*) and length (*B*) of duodenum of four-month-old transgenic (TG) and their wild-type (WT) littermates. 15-20 mice from each group were investigated. To keep the same dimension, weight and length were separately related to body weight (BW), and $BW^{1/3}$, respectivly. Data are presented as mean ± SD.

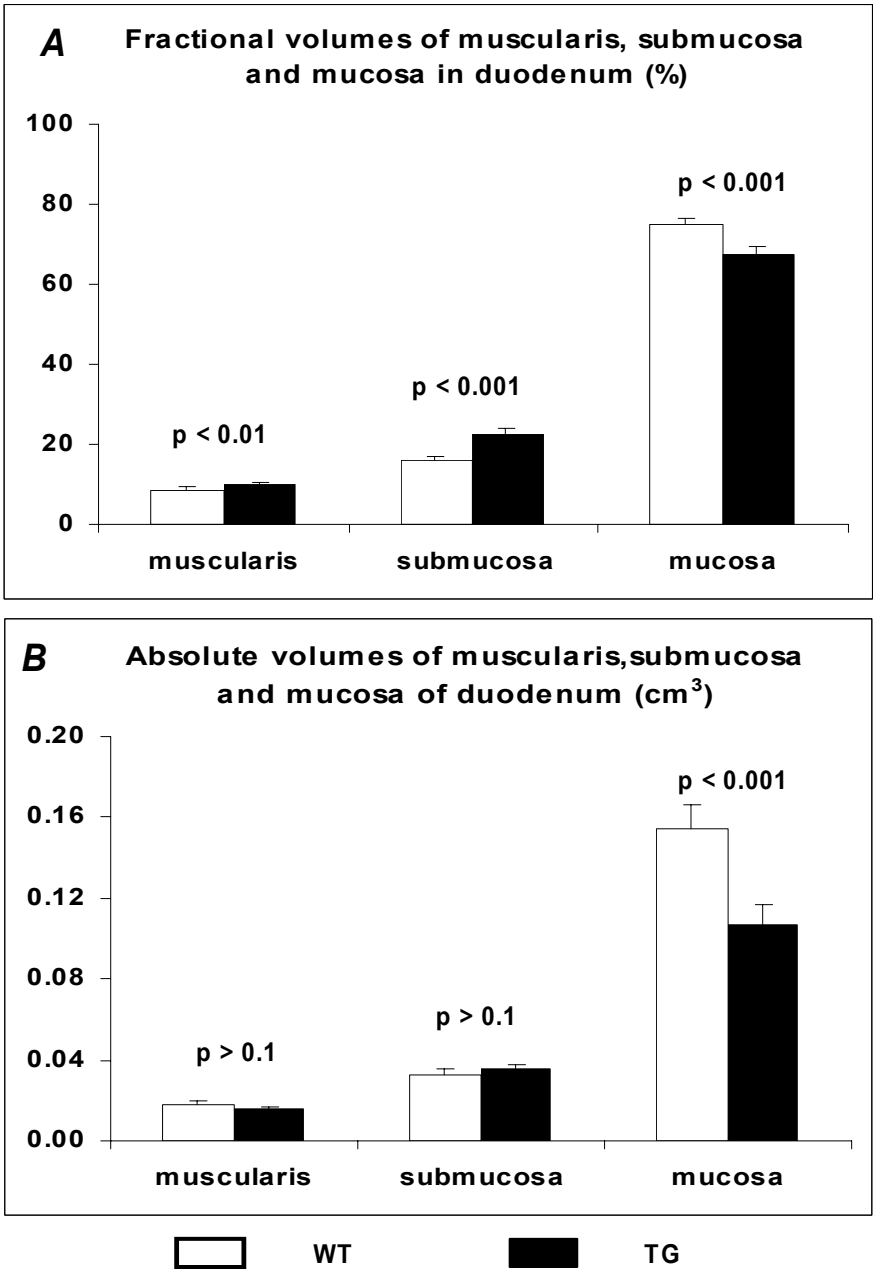

Figure 4.21 Fractional (*A*) and absolute (*B*) volumes of muscularis, submucosa and mucosa in/of the duodenum of four-month-old transgenic (TG) mice (n = 4) and their wild-type (WT) littermates (n = 4). Tissue samples were taken equidistantly from four locations covering duodenum, and the fractional volume of each layer was determined as described in Material and Methods. No significant difference was found among the four locations of each animal (data not shown). Data are presented as mean ± SD.

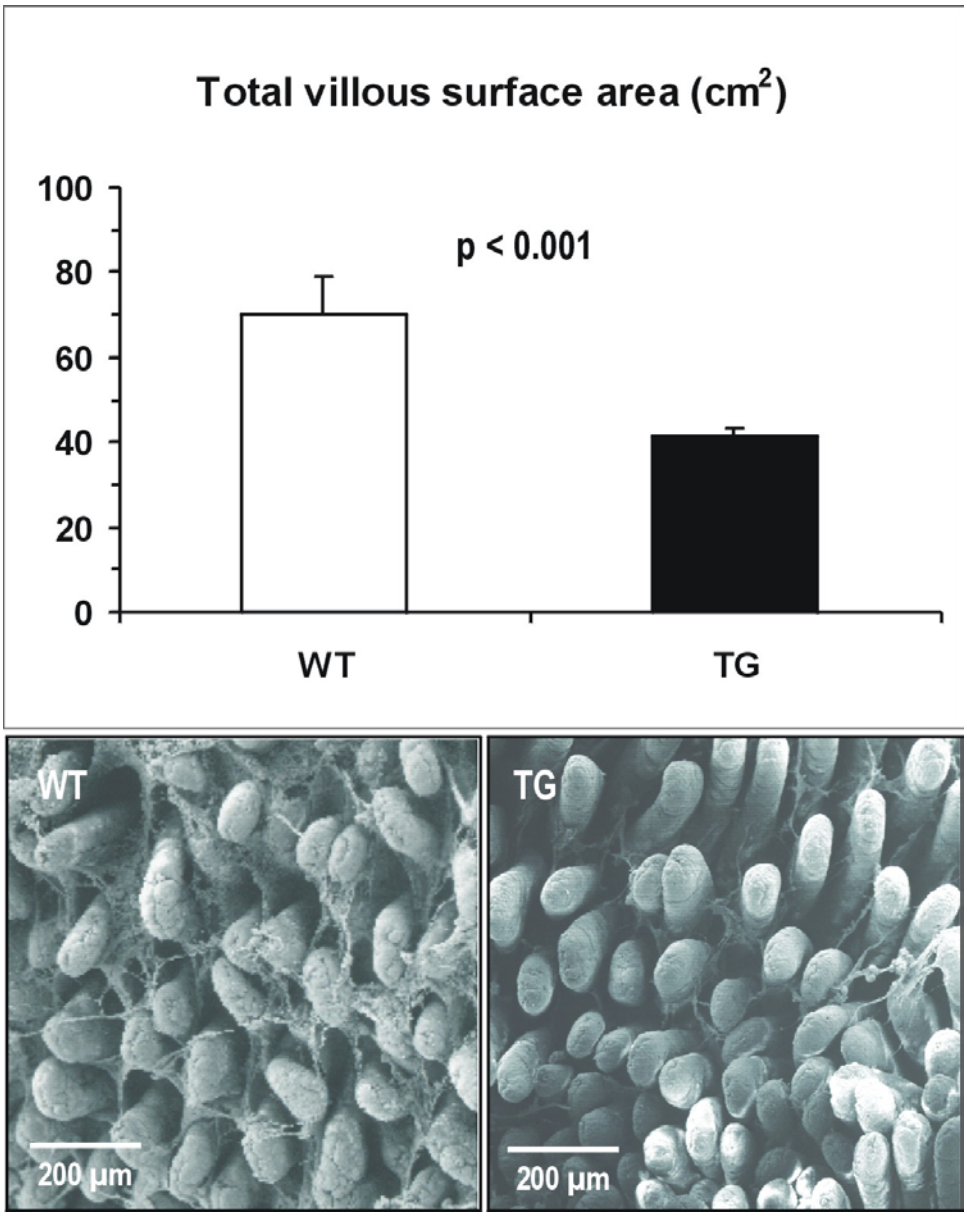

Figure 4.22. *Upper panel*: total villous surface area (ViSA) of the duodenum of 4-month-old female transgenic (TG) mice (n=4) and their wild-type (WT) littermates (n=4). ViSA was estimated from vertical sections as described in Materials and Methods. Data are presented as mean ± SD. *Lower panel*: scanning electron microscopy images of duodenal mucosa from a 2-month-old TG and a WT mouse. Note that the villous diameters appear to be reduced in the TG mouse.

5 DISCUSSION

5.1 Overexpression of IGFBP-4 in transgenic mice

5.1.1 Transgene integration

Initial identification of transgenic founders and transgenic mice in the subsequent generations was performed with PCR technique using the DNA from tail biopsies. This simple technique allows us to investigate a large number of animals in a very short time. However, transgene integration pattern can not be determined by PCR analysis. To this end, Southern blot analysis is necessary to determine the number of integration sites, the number of integrated copies, orientation of the copies within the integration site and the integrity of the construct.

Southern blot analysis showed that two bands of about 8 kb and 2.7 kb representing the endogenous *Igfbp4* were detected in both transgenic and wild-type mice, and a transgene-specific band of about 5 kb, as large as the microinjection fragment, represented in all transgenic mice from both lines. Additional bands, a > 10 kb only present in the mice from line 1 and a 10 kb band only present in the mice from line 2, represent the genomic regions that flanked the transgene construct in its integration site, indicating different integration sites in the two transgenic lines. From the Southern blot, we can see that there were more copies integrated in the genome of line 2 than in line 1. However, the exact number of the integrated copies was not determined, as we rather focused on the determination of the abundance of expression of the transgene and its biological activity.

5.1.2 Transgene expression

The transgene H-2K^{b}-mcIGFBP-4 was found to be transcribed and correctly spliced in all tissues tested, including adrenal gland, bladder, brain, heart, kidney, liver, lung, skeletal muscle, ovary, salivary gland, skin, spleen, testis and thymus. However, elevated protein levels were only detected in the spleen, thymus, lung and kidney by [^{125}I]-IGF-II ligand blot. The elevated protein in the spleen was confirmed to be IGFBP-4 by Western immunoblot using an antibody specific for IGFBP-4. The

biological activity of the transgenic IGFBP-4 was confirmed by its capability to bind recombinant human IGF-II in a Western ligand blot.

The expression pattern of the transgene was identical in the two different transgenic mouse lines, indicating that expression of the transgene H-2K^b-mcIGFBP-4 occurs independently of the integration sites. Except for spleen, thymus, lung and kidney, the IGFBP-4 protein levels were not elevated in the other tissues, suggesting that transgenic mRNA expression was high in the spleen, thymus, lung and kidney, and low in the others. This expression pattern was similar to the endogenous H-2K^b expression (Morello *et al.* 1986).

The H-2K gene is one of the class I genes of the major histocompatibility complex (MHC) of the mouse which encode the heavy chain of cell surface H-2 antigens. The MHC comprises a multigenic family including both classical H-2 (H-2K, H-2D and H-2L) and H-2 like (Qa-TL) genes (Weiss *et al.* 1984). Their expression is developmentally regulated: classical H-2 antigens are not expressed from the early embryonic stages of development, become active between embryonic day 11 and 13, and are expressed and present at the surface of all somatic cells (Morello *et al.* 1978). In the adult mouse, the endogenous H-2K^b mRNA expression is ubiquitous, although the levels are different among the tissues. Its expression is relatively high in lung, liver, spleen and lymph nodes, intermediate in thymus, kidney and heart, and very low in muscle, brain, pancreas and testicular germ cells (Morello *et al.* 1986). *Cis*-acting sequences required for establishing this developmental expression pattern are present in the H-2K^b gene itself, and distinct regulatory elements controlling the tissue specificity are restricted to the 2-kb upstream promoter sequence (Drezen *et al.* 1992).

The mouse H-2K^b promoter has been used by several investigators for generation of transgenic mice expressing hGH (Morello *et al.* 1986), c-myc (Morello *et al.* 1989), c-fos (Ruther *et al.* 1988), hIGF-II (Buul-Offers *et al.* 1995) and mutant forms of the TNFRp55 (Plitz *et al.* 1999). In these mice, a tissue specificity similar to the endogenous H-2K^b mRNA expression was observed. In the H-2K^b-mcIGFBP-4 transgenic mice reported here, transgene expression pattern resembled also the expression of the endogenous H-2K^b gene. Transgene expression was ubiquitous, being high in the spleen, thymus, lung and kidney, and low in the others. Unlike H-

2K^b-hGH and H-2K^b-hIGF-II transgenic mice, serum IGFBP-4 level was not increased in H-2K^b-mcIGFBP-4 transgenic mice, probably due to the low transgene expression in the liver. According to the property of the H-2K^b promoter as noted above, transgene expression under the control of this promoter should be high in the liver, as reported in the H-2K^b-hGH and H-2K^b-hIGF-II transgenic mice (Morello *et al.* 1986; Buul-Offers *et al.* 1995). This different expression in the liver is unknown. A possibility might be that the IGFBP-4 was degraded after its translation by protease in the liver. However, experimental evidence is needed to support this notion.

Comparison of transgene expression in both transgenic lines revealed that the expression in line 2 was higher than line 1. This result is positively correlated with the integration number of the transgene construct, suggesting a mechanism of gene expression regulated by gene dosage. Alternatively, this phenomenon could be due to a position effect.

5.1.3 Effect on body and organ growth

To investigate potential effects of transgene expression on body and organ growth, a large panel of allometric measurements was carried out as described in Materials and Methods. Except for the thymus, overexpression of IGFBP-4 during postnatal life did not affect body and organ growth. This is in contrast to findings in other IGFBP transgenic mouse models and probably related to the following facts: (i) Circulating IGFBP levels were not increased. (ii) Elevated tissue levels of IGFBP-4 were only observed in the spleen, thymus, lung and kidney of H-2K^b-mcIGFBP-4 transgenic mice. However, the postnatal weight of spleen, lung and kidney was not changed, indicating that IGFBP-4 has no major effect on growth of these organs. However, potential histological alteration of these organs of H-2K^b-mIGFBP-4 transgenic mice warrants further investigation. (iii) The exclusive effect of IGFBP-4 on growth of thymus might be the consequence of an interaction with a thymus-specific factor whose identity, however, remains to be unraveled.

The marked decrease in weight and volume of the thymus in the postnatal life is the outstanding feature of H-2K^b-mcIGFBP-4 transgenic mice. This is directly in contrast to the phenotype of H-2K^b-hIGF-II transgenic mice (Buul-Offers *et al.* 1995). Similar

to our H-2K^b-mcIGFBP-4 transgenic mice, IGF-II was highly expressed in spleen, thymus and liver in H-2K^b-hIGF-II transgenic mice, and only the postnatal growth of thymus was promoted by IGF-II overexpression. The absence of splenomegaly in H-2K^b-hIGF-II transgenic mice was proposed due to the upregulation of IGFBP-3 expression in the spleen by IGF-II overexpreesion (Smink *et al.* 1999). Whether the expression of some growth-promoting genes (such as the other components of the IGF system) was regulated by IGFBP-4 overexpression in the spleen, lung and kidney of H-2K^b-mcIGFBP-4 transgenic mice is unknown. Further expression studies need to be done.

In order to define the histological alteration in the thymus and spleen of H-2K^b-mcIGFBP-4 transgenic mice, histomorphometric analyses were performed. The transgenic mice showed a significant decrease in cortex volume compared to the controls, whereas the volume of the medulla was not altered. This finding is also directly in contrast to the increased cortex size of H-2K^b-hIGF-II transgenic mice (Van der Ven *et al.* 1997). No alteration in the architecture of the spleen of H-2K^b-mcIGFBP-4 transgenic mice was observed, which is in agreement with the unchanged weight and volume of this organ.

5.1.4 Effect on the immune system

The cross-talk between the endocrine and immune systems has been suggested by numerous studies, which have been summarized in many reviews (Blalock 1994; Madden & Felten 1995; Savino & Dardenne 1995; Besedovsky & del Rey 1996). Cytokines, the soluble factors secreted by the immune cells, exert biological actions on the endocrine system (Silva *et al.* 1998), conversely, a variety of hormones and peptide growth factors have receptors in the tissues of the immune system and modulate immune functions (Dorshkind & Horseman 2000). Increasing evidence indicates that GH and IGFs are not only involved in endocrine modulation of the development and function of the immune system but also act as cytokines to regulate local growth and differentiation (Buul-Offers & Kooijman 1998; Jeay *et al.* 2002). The components of the IGF system have been detected in lymphoid tissues. IGF-I mRNA and peptide are expressed by myeloid cells, particularly by macrophages in relatively large amounts (Arkins *et al.* 1993) and by peripheral lymphocytes in small

amounts (Arkins *et al.* 1993; Nyman & Pekonen 1993). Bone marrow stromal cells and thymic epithelial cells also produce IGF-I (Dorshkind 1990; Abboud *et al.* 1991; Timsit *et al.* 1992). IGF-II is the dominant peptide of the insulin family expressed by human and rat thymic epithelial cells (Geenen *et al.* 1993). IGF-IR are present on the majority of B cells, NK cells and monocytes as well as erythrocytes (Polychronakos *et al.* 1983; Catanese *et al.* 1986; Kozak *et al.* 1987; Tapson *et al.* 1988; Stuart *et al.* 1991; Kooijman *et al.* 1992). Different T-cell populations express different levels of IGF-IR (Kooijman *et al.* 1995b), suggesting that IGFs are associated with T-cell differentiation. Actually, there is increasing evidence indicating that both IGF-I and IGF-II affect the immune system. IGF-I augments the *in vitro* proliferation and differentiation of thymocytes and pro-B cells (Landreth *et al.* 1992; Gibson *et al.* 1993; Kooijman *et al.* 1995b). Systemic administration of IGF-I significantly increases the size and cellularity of both primary and secondary lymphoid organs in rodents (Binz *et al.* 1990; Beschorner *et al.* 1991; Murphy *et al.* 1992; Clark *et al.* 1993; Jardieu *et al.* 1994). IGF-I administration can also enhance immune response and alter lymphocyte survival and regeneration in thymus and spleen of the dexamethasone-treated rat (Hinton *et al.* 1995; Hinton *et al.* 1998). Administration of IGF-II also stimulates growth of thymus and spleen, but to a lesser extent than IGF-I (Buul-Offers *et al.* 1994; Conlon *et al.* 1995). Overexpression of IGF-I in mice stimulates T- and B-cell development and antigen specific IgG synthesis (Clark *et al.* 1993; Robbins *et al.* 1994). Overexpression of IGF-II in transgenic mice leads to a selective effect on thymic growth, increasing the thymic cellularity and stimulating the development of phenotypically normal T-cells but not mature B-cells (Buul-Offers *et al.* 1995; Kooijman *et al.* 1995a; Kooijman *et al.* 1997).

Some evidence indicates that IGFBPs modulate IGF actions in lymphoid tissues as they do in the circulation and many other tissues. Overexpression of IGFBP-1 in transgenic mice leads to inconsistent effects on spleen size (Dai *et al.* 1994; Murphy *et al.* 1995b; Rajkumar *et al.* 1995), overexpression of IGFBP-2 reduces spleen weight of male transgenic mice (Hoeflich *et al.* 1999), whereas overexpression of IGFBP-3 causes increased spleen size (Murphy *et al.* 1995b). Preliminary data suggest that IGFBP-2 knockout mice show no gross phenotype except for a reduced spleen size (Pintar *et al.* 1995). Some lymphocytes express IGFBPs. By RT-PCR, normal human peripheral lympocytes were shown to express mRNA for IGFBP-2 and

-3, and after mitogenic stimulation they additionally expressed IGFBP-4 and -5 (Grellier *et al.* 1995). IGFBP-4 to -6 are released by murine bone marrow stromal cells (Grellier *et al.* 1995). Sheep thymus cells also produce IGFBPs and IGFBP-3 protease in culture, and the secretion is increased by mitogen stimulation (Tonner *et al.* 1995). Human leukemic T and B lymphoblasts secrete IGFBP-2 and -4 (Neely *et al.* 1991), and their mRNA expression is affected by autocrine/paracrine IGF-II (Elmlinger *et al.* 1998). Thymic epithelial cells express different levels of IGFBP-2 to -6, with a predominance of IGFBP-4 (Kecha *et al.* 1999). IGFBP-4 has also been detected in murine thymic macrophages and in macrophage cell lines (Li *et al.* 1996). IGFBP-4 is thought to be a consistent inhibitor of IGF actions. Considering the predominant expression of the transgenic IGFBP-4 in lymphoid organs, we investigated the potential effect of IGFBP-4 on the development of immune-related cells and on mitogenic response of splenocytes.

To investigate the potential effects of transgene expression on the development of immune-related cells, the fractions of various cell types in the bone marrow, thymus, spleen, lymph node and blood were determined by flow cytometry using antibodies against different cell surface markers as described in Materials and Methods. Unlike in H-2K^b-hIGF-II transgenic mice, no difference was found in the parameters determined so far between H-2K^b-mcIGFBP-4 transgenic mice and control animals. This finding suggests that overexpression of IGFBP-4 in the thymus and spleen did not affect the development of the immune-related cells, although growth of the thymus was significantly inhibited by the transgene expression.

To ascertain the proliferative capacity of T- and B-cells in H-2K^b-mcIGFBP-4 transgenic mice, splenocytes were stimulated with mitogens Con A, anti-CD3, LPS and anti-IgM/IL2, respectively. The proliferative activity of the splenocytes was significantly reduced in transgenic mice after Con A and LPS stimulation, but not after anti-CD3 and anti-IgM/IL2 stimulus, when compared to the wild-type animals. Usually, Con A is thought to be a T-cell specific activator, and LPS is specific for B-cell proliferation. However, LPS can also stimulate the proliferation of macrophage (Paul 1993). Although the location of expression of the transgenic IGFBP-4 in thymus and spleen has not been characterized yet, the expression of endogenous MHC-I was shown to be high in the thymic epithelial cells and macrophages (Roitt 1991), as well

as in the marginal zone macrophages in the spleen (Kraal 1992). There is evidence indicated that transgenic IGF-II was highly expressed in the non-lymphocytic cells (monocyte/macrophage and epithelial cells) in the thymus and spleen of H-2K^b-hIGF-II transgenic mice (Van der Ven *et al.* 1997). Transgenic IGFBP-4 might also be expressed in the non-lymphocytic cells, such as macrophages, rather than in the T- and B-cells. This might be the reason why the development of T- and B-cells and the proliferative activity of splenocytes of transgenic animals after anti-CD3 and anti-IgM/IL2 stimulation were not affected. The reduced proliferative activity of the transgenic splenocytes after Con A and LPS stimulation might be due to the expression of IGFBP-4 in the macrophages, although it is unknown whether Con A can also stimulate the proliferation of other cells besides T-cells. Pre-purification of the lymphocytes from splenocytes may be necessary for the proliferation assay. Furthermore, detailed expression studies of IGFBP-4 in the thymus and spleen of H-2K^b-mcIGFBP-4 transgenic mice need to be done.

5.2 Overexpression of IGFBP-6 in transgenic mice

5.2.1 Transgene integration

As in the IGFBP-4 transgenic model, IGFBP-6 transgenic mice were initially identified by PCR analysis. Ten H-2K^b-mcIGFBP-6 transgenic founders were obtained, which all transmitted the transgene into the following generations, whereas one of the five CMV-mgIGFBP-6 transgenic founders lost the transgene construct in the F1 generation. This instability of the transgene integration is probably due to its integration site, whose flanking region in the genome may affect the stability of the transgene.

Southern blot analysis showed that two bands of about 8 kb and 3.8 kb representing the endogenous *Igfbp6* were detected in both transgenic and wild-type animals, because the genomic DNA sequence of mouse *Igfbp6* gene was used in the transgene construct. However, there were more copies of the two bands in transgenic mice than in wild-type mice. Two transgene-specific bands of 2.8 kb and 1.5 kb were present in all transgenic animals. The different density of these two bands was shown in the four

transgenic lines, suggesting different number of transgene copies were incorporated in the genome of the four lines. Additional bands of different length were represented in the offspring from the four transgenic founders, indicating that the four transgenic strains are independent with different integration sites.

5.2.2 Transgene expression

Consistent with the H-2K^b-mcIGFBP-4 transgenic mouse model, the transgene was expressed ubiquitously at the RNA level in the H-2K^b-mcIGFBP-6 transgenic mice. However, the IGFBP-6 protein level was not increased in serum and any tissue of the H-2K^b-mcIGFBP-6 transgenic mice from 10 lines, although the same expression vector (pUCH2XXS) was used in both transgenic models. The reason for this difference is unknown.

To investigate the specific function of IGFBP-6 *in vivo*, we established another IGFBP-6 transgenic mouse model (CMV-mgIGFBP-6), in which murine *Igfbp6* genomic DNA was cloned under the transcription control of the CMV promoter. Transgene expression was very high in pancreas, and relatively low in lung and liver. In the GIT, elevated levels of IGFBP-6 were detected only in the luminal contents of duodenum from transgenic mice, but neither in the lumen of other segments of the GIT nor in tissue extracts from flushed GIT segments. Based on the high transgene expression in the pancreas, which could be located to the exocrine part by immunohistochemistry, it can be assumed that the luminal IGFBP-6 originated from the pancreas and was excreted into the lumen of the duodenum. We cannot exclude that a proportion of the luminal IGFBP-6 was produced by the intestinal mucosa, although ligand blot analysis of the tissue extracts from preflushed intestinal samples did not support this possibility.

The fact that large amounts of active IGFBP-6 were detected by ligand blot analysis in the luminal contents of the duodenum indicates that the secreted IGFBP-6 was at least to some degree resistant to proteolysis, although large amounts of the serine proteases trypsin and chymotrypsin are produced by pancreatic acinar cells as zymogens and activated in the intestine (Mayer *et al.* 1999). In addition, they are also produced by the small intestine itself (Koshikawa *et al.* 1998). The relative resistance

of human IGFBP-6 to proteolysis has been shown to be related to O-glycosylation which inhibits proteolysis by trypsin and chymotrypsin, thus preserving IGF-binding capacity (Marinaro *et al.* 2000b). However, upon longer exposure, large amounts of trypsin and chymotrypsin were able to cleave also O-glycosylated IGFBP-6 (Marinaro *et al.* 2000b). In contrast to human IGFBP-6 which contains five O-glycosylation sites, mouse IGFBP-6 is O-glycosylated only at one site (Bach 1999). It is unclear whether the presence of active IGFBP-6 in the duodenum of transgenic mice is due to glycosylation-mediated protection from proteolysis. The distribution of immunoreactive IGFBP-6 fragments in different segments of the GIT remains to be determined.

5.2.3 Glucose homeostasis

Insulin is the central hormone in regulating blood glucose levels. However, other hormonal and non-hormonal factors are also important in blood glucose homeostasis. On a molar basis, the IGFs are present in the circulation in a 100-fold excess compared to insulin. Although the IGFs have only about 5% of the insulin-like activity of insulin, the vast majority of the insulin-like activity present in mammalian serum is due to the IGFs rather than insulin itself (Burgi *et al.* 1966). Several lines of evidence support the idea that the IGFs do indeed have some role in glucose homeostasis: (i) increased circulating levels of free IGFs induce hypoglycemia (Zapf 1994); (ii) glucose intolerance was observed in liver-specific *Igf1*-knockout mice (Yakar *et al.* 2001). As high-affinity IGF-binders, IGFBPs may modulate the insulin-like activity of IGFs in the circulation by alteration the concentration of free IGFs.

To investigate the potential effect of IGFBP-6 overexpression on glucose homeostasis, measurement of serum glucose, glucagon and insulin levels as well as a glucose tolerance test were performed. No significant difference was found between CMV-mgIGFBP-6 transgenic and control mice. This finding suggests that IGFBP-6 overexpression in the exocrine pancreas of transgenic mice has no effect on the production of insulin and glucagon, as well as on blood glucose homeostasis. In contrast, impaired glucose tolerance was observed in several transgenic mouse models overexpressing IGFBP-1 (Gay *et al.* 1997; Rajkumar *et al.* 1996) or IGFBP-3 (Silha *et al.* 2002). In one of the IGFBP-1 transgenic models an increased size and number

of pancreatic islets, with more beta cells and less alpha cells, was observed (Dheen *et al.* 1996). A common feature of these transgenic mice is a significant increase in the circulating levels of the corresponding IGFBP, which may increase the proportion of IGFBP-complexed IGF-I in serum and consequently attenuate the hypoglycemic effect of free IGF-I (Rajkumar *et al.* 1996; Silha *et al.* 2002). In our IGFBP-6 transgenic mice, no obvious alteration in the structure of pancreatic islets nor changes in the activity of any serum IGFBP were observed, which may explain why glucose homeostasis was not affected.

5.2.4 Effects on body and organ growth

To investigate potential effects of transgene expression on body and organ growth, a large panel of allometric measurements was performed as described in Materials and Methods. Except for parameters of growth of the duodenum (see below) no significant difference was observed between transgenic mice and controls in any of the parameters investigated. This is in contrast to findings in other IGFBP transgenic mouse models and probably related to the facts that (a) circulating IGFBP levels were not increased and (b) high tissue levels of IGFBP-6 were only observed in the pancreas of our CMV-mIGFBP-6 transgenic mice. However, pancreas weight was not altered, suggesting that IGFBP-6 has no major effect on growth and – based on the findings regarding glucose homeostasis – on endocrine functions of the pancreas. A potential reason for the latter finding could be the expression pattern of the transgene, which was almost exclusively in the exocrine pancreas. Nevertheless, detailed histological and ultrastructural studies of the pancreas of CMV-mIGFBP-6 transgenic mice still need to be done.

In contrast to pancreas, the weight and length of duodenum of the IGFBP-6 transgenic mice were significantly reduced, compared with their wild-type littermates. The reduction in weight of the duodenum was mainly attributable to a significantly smaller volume and surface area of the tunica mucosa in transgenic *vs.* control mice as determined by morphometric analysis. This finding suggests that luminal IGFBP-6 is a specific inhibitor of mucosal growth.

Gastrointestinal growth is regulated by many factors, such as nutritional, hormonal, pharmacological and luminal factors (Klein & McKenzie 1983). It has been well documented that IGF-I and IGF-II are very important for growth of the gut under physiological and pathological conditions (Lund 1998; MacDonald 1999). IGFs and their receptors are expressed in the intestinal tract of several mammalian species (Brown *et al.* 1986; Schober *et al.* 1990; Termanini *et al.* 1990; Winesett *et al.* 1995). Their expression is high in fetal and newborn tissues and decreases with age. The gut is one of the most responsive target tissue for the IGFs (Read *et al*. 1991). Oral or intraluminal administration of IGF peptides stimulates intestinal growth (rev. in MacDonald 1999), and IGF-I analogues such as des(1-3)IGF-I and long R^3-IGF-I with reduced or poor binding affinities for the IGFBPs have a higher potency (Garnaut *et al.* 2002; Steeb *et al.* 1994), suggesting that IGFBPs may be important regulators of IGF actions in the gut, as they are in other tissues and in the circulation.

IGFs and IGFBPs have been detected in human (Baxter *et al.* 1984; Corps *et al.* 1988; Elmlinger *et al.* 1999; Van Doorn *et al.* 1999), rat (Donovan *et al.* 1991; Philipps *et al.* 1991), porcine (Simmen *et al.* 1988; Donovan *et al.* 1994) and bovine milk (Collier *et al.* 1991). Recent data indicate that the components of the IGF system present in milk can, at least in part, arrive intact in the neonatal intestine (Elmlinger *et al.* 2002). Although their functional relevance has not been proven, oral intake of IGFs and IGFBPs may be important for the maturation of the neonatal gastrointestinal tract (GIT).

IGFBP-6 mRNA expression was demonstrated in the neonatal porcine small intestine, with decreasing levels after weaning (Tang *et al.* 2002). IGFBP-6 was detected in human milk at a concentration of 6-45 ng/ml (Van Doorn *et al.* 1999), which is relatively low compared to the concentration of IGFBP-2 (about 2 µg/ml) (Elmlinger *et al.* 1999). However, protease resistance of O-glycosylated IGFBP-6 might lead to relatively high levels of active IGFBP-6 in the gut. Thus, IGFBP-6 may take part in the regulation of gut growth and development. A recent study suggested that the mitogenic activity of IGF-II in human gastric epithelium was tightly inhibited by two locally produced IGF-II carriers, IGFBP-2 and -6 (Tremblay *et al.* 2001), providing direct evidence that IGFBP-6 can regulate GIT growth via an IGF-dependent mechanism. This observation and the findings of the present study are important in

the context of gastrointestinal growth in neonatal mammals, considering that IGFBPs in milk may at least in part arrive intact in the intestine. Whether IGFBP-6 regulates the bioavailability of IGF-I or IGF-II in the duodenum of our transgenic mouse model is not known.

In addition to IGF-dependent mechanisms, IGF-independent pathways of IGFBP-6 have to be taken into consideration to explain our present findings, considering the facts that (i) IGFBP-6 binds IGF-II with a great preference over IGF-I; (ii) the circulating levels of IGF-II are very low in the postnatal life of rodents, and (iii) a line of evidence suggests that IGFBP-6 may also act via an IGF-independent manner. These facts have been reviewed above in the section 2.4.

6 SUMMARY

Functional analysis of insulin-like growth factor binding protein -4 and -6 in transgenic mice

Insulin-like growth factors (IGF-I and IGF-II) are expressed in many cell types and tissues and act in endocrine, autocrine or paracrine manner to regulate cellular proliferation, survival and differentiation. IGF actions are initiated upon binding to the type I IGF receptor (IGF-IR) and are modulated through interactions with a family of six secreted IGF-binding proteins (IGFBP-1 to -6). Although the six conserved IGFBPs are structurally related, each of them has specific characteristics and may have specific functions. Most knowledge about the IGFBPs has been gained from the numerous *in vitro* studies, their specific roles *in vivo* are largely unknown.

Transgenic mice overexpressing a particular IGFBP allow us to investigate the specific functions of the corresponding IGFBP *in vivo*. To this end, IGFBP-4- and IGFBP-6-overexpressing models were established and analyzed in the present study.

First, an expression vector containing the murine H-2K^b promoter and a human beta-globin splicing cassette was used to construct the transgenes, to obtain ubiquitous expression of the mouse *Igfbp4* and *Igfbp6* cDNA. Two lines of H-2K^b-mcIGFBP-4 and ten lines of H-2K^b-mcIGFBP-6 transgenic mice were generated. The transgene was ubiquitously expressed at RNA level in both transgenic models, however, at protein level, transgene expression was only detected in the spleen, thymus, lung and kidney of both H-2K^b-mcIGFBP-4 transgenic lines, but in no organ of H-2K^b-mcIGFBP-6 transgenic mice. Phenotypic analyses of the H-2K^b-mcIGFBP-4 transgenic model revealed that overexpression of IGFBP-4 had no significant effect on the postnatal body and organ growth, except that the weight and volume of thymus in 8- and 12-week-old transgenic mice were significantly reduced ($p < 0.05$) compared to the controls. Histomorphometric analysis demonstrated that the volume of the thymic cortex was significantly decreased in transgenic mice ($p < 0.05$), whereas that of the thymic medulla was not changed. The fractions of various cell types in the bone marrow, thymus, spleen, lymph node and peripheral blood were determined by flow cytometry. No significant difference was found between

transgenic and control groups, suggesting that IGFBP-4 excess in the lymphoid organs did not affect the development of the lymphatic cells. The proliferative capacity of the splenocytes of transgenic animals was significantly reduced after Con A and LPS stimulation ($p < 0.05$), but not altered after the stimulation by anti-CD3 and anti-IgM/IL2. This is probably due to transgenic IGFBP-4 expression restricted in the non-lymphatic cells. However, detailed expression of the transgene warrants further investigation.

In order to realize IGFBP-6-overexpressing mice, a second construct was designed, namely CMV-mgIGFBP-6, in which the mouse *Igfbp6* genomic sequence was cloned under the control of the cytomegalovirus (CMV) promoter. Four independent lines of transgenic mice were generated. Transgene expression was high in the exocrine pancreas and relatively low in the lung and liver. The activities of serum IGFBPs were not different between transgenic mice and controls. In transgenic mice, high levels of active IGFBP-6 were detected in the luminal content of the duodenum, but neither in the luminal contents of other segments of the gastrointestinal tract (GIT), nor in tissue extracts of all GIT segments. Glucose homeostasis was not altered by IGFBP-6 expression. Postnatal body and organ growth was not affected in transgenic mice, except for the absolute and relative weight and length of duodenum which were significantly reduced in 4-month-old transgenic mice as compared to controls ($p < 0.05$). This reduction was mainly due to a significantly smaller volume and surface area of the tunica mucosa as determined by histomorphometric analsis. Our analysis of the first IGFBP-6 transgenic mouse model provides direct evidence for inhibition of intestinal growth by luminal IGFBP-6 excess. This finding is important in the context of neonatal intestinal growth of mammals, considering the fact that milk contains large amount of IGFBPs which may at least in part arrive intact in the intestine.

7 ZUSAMMENFASUNG

Funktionelle Analyse der Insulin-like Growth Factor-Bindungsproteine -4 und -6 in transgenen Mäusen

Die insulinänlichen Wachstumsfaktoren (IGF-I und IGF-II) werden in verschiedenen Zelltypen und Geweben exprimiert, und regulieren Zellproliferation, -überleben und -differenzierung durch endokrine, autokrine oder parakrine Mechanismen. Die Aktivitäten der IGFs werden durch den Typ 1 IGF-Rezeptor (IGF-IR) initiiert und durch die Interaktionen mit einer Familie von sechs IGF-Bindungsproteinen (IGFBP-1 bis -6) moduliert. Trotz ihrer strukturellen Ähnlichkeiten zeigt jedes IGFBP spezifische Eigenschaften und spielt eine spezifische Rolle. Weil die meisten Kenntnisse über die IGFBPs durch *in vitro* Studien gewonnen wurden, sind ihre spezifischen Funktionen *in vivo* weitgehend unklar.

Transgene Mäuse, in denen ein bestimmtes IGFBP überexprimiert ist, eignen sich zur funktionellen Analyse des entsprechenden IGFBP *in vivo* bedienen. Deshalb wurden IGFBP-4 und IGFBP-6 transgene Mausmodelle erstellt und analysiert.

Um eine ubiquitäre Expression der cDNA von *Igfbp4* oder *Igfbp6* zu erzielen, wurde ein Expressionsvektor, der den murinen H-2K^b Promotor und eine humane beta-globin Spleißkassette enthält, zuerst zum Aufbau transgener Konstrukte verwendet. Zwei Linien H-2K^b-mcIGFBP-4- und zehn Linien H-2K^b-mcIGFBP-6-transgener Mäuse wurden erstellt. Ubiquitäre transgene Expression wurde auf RNA-Ebene bei beiden transgenen Modellen erreicht. Erhöhte IGFBP-4 Proteinmengen konnten nur in Milz, Thymus, Lunge und Niere H-2K^b-mcIGFBP-4-transgener Tiere nachgewiesen werden, aber nicht bei den H-2K^b-mcIGFBP-6-transgenen Mäusen. Eine phänotypische Analyse des H-2K^b-mcIGFBP-4-transgenen Modells zeigte keinen Effekt der IGFBP-4-Überexpression auf das postnatale Körper- und Organwachstum, bis auf das Gewicht und Volumen des Thymus, das bei 8- und 12-Wochen alten transgenen Mäusen deutlich reduziert war ($p < 0,05$). Histomorphometrische Untersuchungen zeigten, dass das Volumen des Cortexes, aber nicht der Medulla, verkleinert ist. Die Fraktionen verschiedener Zelltypen aus dem Knochenmark, dem Thymus, der Milz, dem Lymphknoten und dem peripheren Blut wurden mittels der

Flowcytometrie untersucht. Es konnte kein Unterschied zwischen den IGFBP-4-transgenen und Wildtyp-Tieren gezeigt werden. Dieses Ergebnis gibt keinen Hinweis, dass die IGFBP-4-Überexpression eine Wirkung auf die Entwicklung der Lymphozyten hat. Nach der Con A- und LPS-Stimulation wurde die Proliferationskapazität der Milzzellen deutlich verringert, dagegen zeigte sich kein Unterschied zwischen den transgenen und Wildtyp-Mäusen bei der Proliferationskapazität nach der Stimulation mit anti-CD3 und anti-IgM/IL-2. Solche Phänotypen konnten infolge davon sein, dass das Transgen hauptsätzlich in den Nicht-Lymphozyten exprimiert wird. Jedoch muss das genaue Expressionsmuster des Transgens in den Lymphorgane weiter studiert werden.

Um die IGFBP-6-Überexpression *in vivo* zu erreichen, wurde ein zweites Transgenkonstrukt (CMV-mgIGFBP-6) kloniert, in dem eine genomische Sequenz des murinen *Igfbp6*-Gens unter der Transkriptionskontrolle des Cytomegalovirus (CMV)-Promotors steht. Vier unabhängige transgene Mauslinien wurden erstellt. Die Transgenexpression war sehr hoch im exokrinen Pankreas und relativ niedrig in Lunge und Leber der transgenen Tiere. Die Menge aller IGFBPs im Serum veränderte sich nicht. Interessanterweise wurde eine erhöhte Proteinmenge des aktiven IGFBP-6 im Lumeninhalt des transgenen Duodenums nachgewiesen, aber konnte nicht in den Lumeninhalten anderer Segmente des Magen-Darm-Traktes und auch nicht in den Gewebextrakten aller Segmente des Magen-Darm-Traktes nachgewiesen werden. Hinsichtlich der Glukosehomöostase und des postnatalen Körper- und Organwachstums konnte kein Unterschied zwischen den transgenen und wildtypen Tieren gezeigt werden. Eine Ausnahme waren das Gewicht und die Länge (absolute und auch relative Werte) des Duodenums 4-Monate-alten-transgener Mäuse, die im Vergleich zu Wildtyp-Tieren deutlich reduziert waren ($p < 0,05$). Morphometrische Untersuchungen zeigten, dass diese Reduzierung die Folge von einer Verkleinerung von Volumen und Oberfläche der Duodenummukosa war. Die Analyse des ersten IGFBP-6-transgenen Mausmodels hat einen direkten Hinweis gegeben, dass erhohte IGFBP-6-Aktivität im Lumen des Darmes das Darmwachstum verhindern kann. Diese Entdeckung ist wichtig im Kontext vom Darmwachtum bei der neonatalen Säugertieren, unter Berücksichtigung der Tatsache, dass die Milch eine grosse Menge von IGFBPs enthält, welche zumindest teilweise intakt den Darm erreichen können.

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