Functional analysis of IGFBP-2 overexpression in mouse liver myofibroblasts: Therapeutic implication for liver fibrogenesis

Dissertation

zur Erlangung des Doktorgrades

der Matematisch-Naturwissenschaftlichen Fakultäten

der Georg-August-Universität Göttingen

vorgelegt von

RAJESWARA RAO PANNEM

aus Jandrapet, Indien

Göttingen 2007

D7
Referent: Prof. Dr. Rüdiger Hardeland
Korreferent: Prof. Dr. Detlef Doenecke
Tag der mündlichen Prüfung:

Contents

Abbreviations

Ab	antibody
Akt	PKB, homologue of v-Akt
ALS	acid labile subunit
APS	ammonium persulphate
BCA	bicinchoninic acid
bp	base pair
BrdU	5-bromo-2´-deoxyuridine
BSA	bovine serum albumin
cDNA	complementary DNA
CM	conditioned media
cpm	counts per minute
CsCl	cesium chloride
ddH_2O	double distilled water
DTT	dithiothreitol
ECM	extracellular matrix
ERK	extracellular signal regulated kinase
FAK	focal adhesion kinase
g	gravity
GAG	glycosaminoglycans
GBSS	Gey's balanced salt solution
GH	growth hormone
GITC	guanidine isothiocyanate
HBD	heparin-binding domain
HC	hepatocyte
HSC	hepatic stellate cell
Ig	immunoglobulin
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IGFBP-3R	insulin-like growth factor binding protein 3 receptor
IGFBP-rP	IGFBP-related proteins
IGF-II/M6-PR	insulin-like growth factor II/mannose 6-phosphate receptor
IGF-IR	insulin-like growth factor I receptor
IR	insulin receptor
IRR	insulin receptor related receptor
IRS	insulin receptor substrate
kb	kilobase
KC	Kupffer cell
kDa	kilodalton
KLH	keyhole limpet hemocyanin
LB	Luria Bertani
LMF	liver myofibroblast
M6P	mannose 6-phosphate
MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
NLS	nuclear localization signal
OD	optical density

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI3-K	phosphatidylinositol 3-kinase
PMSF	phenylmethanesulfonyl fluoride
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	room temperature
RXRα	retinoid X receptor α
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	sinusoidal endothelial cell
Shc	Src-Homology-Collagen protein
SMA	smooth muscle actin
TβR-V	type V TGF-β receptor
TAE	Tris/Acetate/EDTA buffer
TEMED	tetramethyl ethylene diamine
TGF-β	transforming growth factor β

List of Figures

Summary

Liver fibrosis is characterized by abnormal accumulation of extracellular matrix (ECM) proteins which are secreted from the cells of the fibroblast lineage during chronic liver injury. Different liver cell populations are involved in this process: activated hepatic stellate cells (HSCs) as well as portal and perivascular liver myofibroblasts (LMFs) which represent morphologically and functionally different fibroblast populations. Several lines of evidence demonstrate that in contrast to LMFs, HSCs undergo spontaneous apoptosis both *in vitro* and *in vivo*, in parallel with their activation. Therefore, LMFs appear to be an essential cell type with fibrogenic potential in the liver. The IGF system including the insulin-like growth factors I and II (IGF-I, -II), their receptors (IGF-I receptor, IGF-IR; IGF-II/mannose 6-phosphate receptor, IGF-II/M6-PR) and six high affinity IGF binding proteins (IGFBPs) participate in the regulation of growth and differentiation of cells of the fibroblast lineage, possibly contributing to the fibrogenic process. Significantly increased levels of IGFBP-2 in sera and hepatic tissue from patience suffering from liver cirrhosis of different etiology (Holt et al., 1996; Holt et al., 1997; Kratzsch et al., 1995; Ross et al., 1996; Wolf et al., 2000). In general, IGFBP-2 has been shown either to inhibit or to enhance the effects of IGF-I in certain cell types studied. However, the precise role of IGFBP-2 overexpression in liver fibrogenesis is unknown. The aim of the present work was to examine the relationship between IGFBP-2 overexpression and cellular functions of LMFs. For this purpose, LMFs were isolated from the livers of wild type (wt) and CMV-IGFBP-2 transgenic (IGFBP-2 (+/-)) mice.

LMFs were obtained by outgrowth of primary hepatocytes isolated from wt and IGFBP-2 (+/-) mice. Expression of IGF-I, IGF-IR, IGF-II/M6PR, IGFBP-2 and -3 messenger RNA (mRNA) was investigated by Northern blot hybridization and quatitative real-time (RT)-PCR. IGF-IRβ protein expression was confirmed by Western immunoblotting. IGFBP secretion was evaluated by [^{125}I]-IGF-I ligand blot. Determination of DNA synthesis in mLMF was assessed by means of BrdU incorporation assay and [^{3}H]-thymidine incorporation assay. mRNA expression of fibulin-2 and Fibronectin 1 was evaluated by quantitative RT-PCR.

IGFBP-2 (+/-) mLMFs showed an approximately four to five-fold increased expression of IGFBP-2 mRNA as compared with wt mLMFs during different time points of culture (days 2 to 5) that was confirmed at protein level by [^{125}I]-IGF-I ligand. In wt mLMFs, the expression of IGFBP-3 mRNA was low at day 2 of culture but high at day 5 of culture whereas IGFBP-3 mRNA expression was reversibly decreased from high levels at day 2 of culture to low levels at day 5 of culture in IGFBP-2 (+/-) mLMFs. The expression of IGF-I, IGF-IR and IGF-II/M6-PR mRNA was increased in IGFBP-2 (+/-) mLMFs compared with wt mLMFs. In wt mLMFs, addition of IGF-I dose-dependently reduced IGFBP-2 mRNA and protein levels whereas in IGFBP-2 (+/-) mLMFs IGF-I showed a stimulatory effect on IGFBP-2 mRNA and protein levels. The IGF-I-dependent stimulation of IGFBP-3 mRNA and protein levels in IGFBP-2 (+/-) mLMFs were less pronounced than in wt LMFs. In contrast, the IGF-I-dependent decrease of IGF-IR mRNA and protein levels were not significantly different in wt and IGFBP-2 (+/-) mLMFs. Functionally, IGF-I dose-dependently stimulated DNA synthesis in wt mLMFs whereas in IGFBP-2 (+/-) mLMFs IGF-I-induced DNA synthesis was abrogated compared to untreated controls. Similarly, in wt LMFs, IGF-I stimulated mRNA expression of fibulin-2 and fibronectin 1, two of the ECM proteins deposited during liver fibrosis whereas IGF-I-induced mRNA expression of fibulin-2 and fibronectin 1 was inhibited compared to untreated controls in IGFBP-2 (+/-) mLMFs.

Together, the data of present study demonstrate that overexpression of IGFBP-2 in LMFs is associated with alterations of DNA synthesis and of biosynthesis of ECM components in these cells. Our data point to a regulatory role of IGFBP-2 overexpression during liver fibrogenesis and indicate IGFBP-2 as a potential target in antifibrotic therapy.

1. Introduction

1. 1. Insulin-like Growth Factors

Insulin-like growth factors (IGFs, IGF-I and IGF-II) are fundamental cell modulators with an evolutionary conserved role synchronizing tissue growth, development and function. Although IGF-I and IGF-II are structurally related polypeptides that share a 62% homology at amino acid level with each other and share a 40% homology with proinsulin (Furstenberger and Senn, 2002), both IGF-I and IGF-II act in a distinct way as a cell regulators. Unlike insulin and other peptide hormones that are resided and released from the specific gland when needed, the IGFs are expressed widely throughout most tissues in the body. They are stored out of the cells in complex with soluble binding proteins (Holly and Perks, 2006) and circulate in approximately 1,000-fold higher concentrations than most other known peptide hormones (D'Ercole and Wilkins, 1984). These physiological properties indicate a multifunctional role of the IGFs in the organism compared with the more specific metabolic function of insulin.

1.1.1. IGF-I

IGF-I is a 70-amino acid peptide with a molecular mass of about 7649 Da (Adamo et al., 1993; Rinderknecht and Humbel, 1978) organized into four peptide domains: A, B, C, and D. Domains A and B are similar in structure to the A and B chains of insulin (49% sequence homology). A shortened “connecting” peptide with 12 residues (positions 30 to 41) compared to 30 to 35 in proinsulins shows no homology to proinsulin C peptide. An octapeptide sequence at the COOH-terminal end is also a feature not found in proinsulin. The IGF-I prohormone also contains a C-terminal E peptide that is cleaved in the Golgi apparatus before secretion (Daughaday and Rotwein, 1989). The number of differences in amino acid positions between IGF-I and insulin suggests that duplication of the gene of the common ancestor of proinsulin and IGF occurred before the time of appearance of the vertebrates (Rinderknecht and Humbel, 1978).

Although the liver is by far the major site of IGF-I production, it is also known to be synthesized by almost any tissue in the body (Rosen, 1999). Clearly, both tissue and circulating growth factor concentrations are critical in defining the relationship between IGF-I and cell activity. However, IGF-I gained characteristic features of both circulating hormone and a tissue growth factor, synthesis of IGF-I from the liver is complex. Serum IGF-I levels are affected by many factors (Pollak et al., 2004). GH is the principal regulator of IGF-I production in the liver and secretion into the bloodstream. IGF-I in the bloodstream then exerts feedback regulation on the hypothalamus and pituitary gland, reducing GH secretion from the anterior pituitary gland (Tannenbaum et al., 1983).

IGF-I is considered to be more essential for postnatal growth and development because the secretion of IGF-I is low during embryo development. In addition, this hormone is essential for organogenesis, as has been shown in mice with targeted disruption of the *IGF-I* gene (Baker et al., 1993; Powell-Braxton et al., 1993). However, the organ specific disruption of liver IGF-I production in mice decreases serum IGF-I levels by almost 75%, but surprisingly it showed only minor effect on postnatal growth (Sjogren et al., 1999; Yakar et al., 1999). Thus, the local production of IGF-I also plays a major role in the growth of tissues.

Transgenic studies have shown that overexpression of IGF-I results in muscle cell differentiation and myofiber hypertrophy (Coleman et al., 1995). Further, it is deciphered that the *IGF-I* gene is spliced to yield different isoforms that have different modes of action. In human skeletal muscle alternative splicing of *IGF-I* gene produces IGF-IEa, IGF-IEb and IGF-IEc (termed as IGF-IEb in rodents) splice variants (Hameed et al., 2003). The muscle IGF-IEa promotes an increase in cellular mass and induces the myoblast to fuse and form myotubes. In contrast, in rodents IGF-IEb increases cellular proliferation and inhibits terminal differentiation and produces more myoblasts for secondary myotube formation.

1. 1. 2. IGF-II

Insulin-like growth factor -II (IGF-II) is a small mitogenic peptide and is one of the most ubiquitous growth factors in the mammalian embryo, where it plays an

important role in regulating fetal growth. This was demonstrated when transgenic mice with a disrupted *IGF-II* gene showed fetal growth retardation. The *IGF-II* gene shows a complex structural organisation in all species analyzed. It consists of at least nine exons in man and sheep, and six exons in rat and mouse. Its expression is regulated in a developmental and tissue-specific manner, involving differential promoter usage and alternative splicing, as well as differential RNA processing site. Ability of translation of the different promoter transcripts is variable and growth-dependent, and the translated product is also subject to posttranslational modification. The *IGF-II* mRNA population originates from the use of four promoters in man and sheep and three promoters in rodents. During fetal life three promoters are active both in human and rodents, with promoter P3 in humans and promoter P3 in rodents (which corresponds to P4 in human) being predominantly used. Transcription from these promoters is repressed during adult life and a fourth promoter becomes activated in human liver. No homologue to human promoter P1 has been identified in rodents, but is present in the ovine and baboon *IGF-II* genes. Furthermore, imprinted antisense transcripts are expressed in the mouse *IGF-II* gene (Otte et al., 1998).

IGF-II is subject to epigenetic modifications (Otte et al., 1998). In most of the tissues of normal subjects, IGF-II is produced only from the paternal allele whereas the maternal allele is transcriptionally silent (DeChiara et al., 1991; Reik et al., 2000) indicating the genomic imprinting of the *IGF-II* gene. Genomic imprinting is a mode of gene regulation in which two parental alleles are differentially expressed. Most of genes are expressed equally from both parental alleles, whereas imprinted genes are expressed exclusively or preferentially from either the paternal or maternal allele. The allelic expression of imprinted genes is dependent on whether the allele is inherited from the egg or sperm, because differential epigenetic marking occurs during gametogenesis (Barlow, 1995; Surani, 1998; Tilghman, 1999). It is regulated by a number of variables that includes an inverted repeat, matrix attachment regions, and several differentially methylated regions (Lawton et al., 2007).

Serum concentrations of IGF-II are 5- and 3.5-folds higher than IGF-I levels in human fetus and adult, respectively (Bennett et al., 1983). Circulating IGF-II levels are relatively stable after puberty and not regulated by GH. IGF-II has proliferative

and antiapoptotic actions similar to IGF-I since its effects are mediated *via* the IGF-IR (O'Dell and Day, 1998). Moreover, IGF-II assists in the supply of nutrients to the developing fetus (Constancia et al., 2002) and plays a critical role in communication between cells of fetal trophoblasts and maternal decidual cells at the maternal-fetal interface (Fazleabas et al., 2004). However, IGF-II plays a fundamental role in embryonic and fetal growth, its role in postnatal period of life is less important as it is substituted by IGF-I. A possible explanation for the relatively lower importance of IGF-II in postnatal life might be the 2- to 15-fold lower affinity of IGF-II for the IGF-IR and the equal or greater affinity of IGFBPs for IGF-II than for IGF-I. The combination of higher affinity to binding proteins and lower affinity to the receptor results in relatively more IGF-I than IGF-II interacting with the IGF-IR.

1. 2. Receptors

1. 2. 1. IGF-IR, Hybrid IGF-IR/IR, IR and IRR

Gene knockout studies revealed that the biological effects of both IGF-I and IGF-II are mediated through the IGF-IR, a type 2 tyrosine kinase receptor that shares an approximately 60% structural homology with insulin receptor (IR) at the amino acid level (Ullrich et al., 1986). The IGF-IR and IR are heterodimers, each composed of two extracellular α-subunits and two transmembrane β-subunits with short cytoplasmic tail localized primarily intracellularly (Steele-Perkins et al., 1988). Both subunits are linked together by disulfide bonds and form a tetramer (β-α-α-β) which is N-linked glycosylated and transported to plasma membrane (Carlberg et al., 1996; Dricu et al., 1997; Jansson et al., 1997). The intracellular region of the β-subunit contains a cytoplasmic tyrosine kinase domain. Upon ligand binding, tyrosine residues in the β-subunits of IGF-IR undergo auto-phosphorylation (Brodt et al., 2000). This results in the binding of signaling proteins to this cytoplasmic domain. Adaptor proteins such as the insulin receptor substrate (IRS) and Shc proteins bind and transmit signals downstream of the receptor via the Ras/Raf/MAPK pathway and the phosphotidylinositol 3-kinase (PI3-K)/Akt pathway which then mediate cell proliferation, metabolism and cell survival (Adamo et al., 1992; Dupont and LeRoith, 2001; LeRoith et al., 1995).

Conversely, mutations at amino acid level in IGF-IR are associated with growth retardation in humans and mice (Abuzzahab et al., 2003; Kawashima et al., 2005; Raile et al., 2006; Walenkamp et al., 2006). Recently, NIH-3T3 cells overexpressing a mutant form of the *IGF-IR* gene, in which arginine at 481 is substituted by glutamine leads to reduced levels of IGF-IR β-subunit phosphorylation, as well as ERK1/2 and Akt phosphorylation that is accompanied by decreased cell proliferation.

The complexity of IGF signaling is further increased by the formation of hybrid receptors by the dimerization of IGF-IR and IR hemireceptors. Such IGF-IR/IR hybrid receptors have a high affinity for IGF-I, but a very low affinity for insulin. Thus, the presence of a significant number of hybrid receptors may selectively diminish the responsiveness of the cell to insulin, but not to IGF-I. The signaling potential of hybrid receptors is increased by the presence of various IR isoforms. It was recently demonstrated that IGF-IR/IR-A hybrid receptors bind IGF-I, IGF-II, and insulin, whereas IGF-IR/IR-B hybrids bind IGF-I with high affinity, IGF-II with low affinity, and do not bind insulin (Pandini et al., 2002).

In addition, the IR is involved in regulation of some of the mitogenic actions of IGF-II (Louvi et al., 1997; Morrione et al., 1997). The IR is expressed as two variably spliced isoforms (IR-A and IR-B), which differ by the presence or absence of a 12-amino acid sequence 717-729 encoded by *IR* exon 11. IGF-II potentiates the expression of A-isoform at high levels in fetal and neoplastic tissues, and binds to IR-A with similar affinity than insulin (Frasca et al., 1999), and plays a primary role in embryonic growth.

IRR is the only known orphan receptor of the IR family. IRR transcripts are predominantly found in kidney, neural tissues, stomach, and pancreatic β-cells. IRR is ability to bind all the different insulin-like peptides whereas its biological significance remains unclear (Nakae et al., 2001).

1. 2. 2. IGF-II/M6-PR

The IGF-II/M6-PR is a multifunctional transmembrane glycoprotein that consists of a 300 kDa single polypeptide chain, with a large extracellular domain capable to bind

M6-PR-containing ligands, IGF-II, and IGF-I with lower affinity (Brown et al., 2002; Kiess et al., 1988; MacDonald et al., 1988; Morgan et al., 1987), and a small cytoplasmic domain (Braulke et al., 1988; Kornfeld, 1992). There is no enzymatic activity found in the cytoplasmic tail of the IGF-II/M6-PR as in tyrosine kinase-linked receptors and the IGF-II/M6-PR is not considered to have any major role in IGF signal transduction (Braulke, 1999). However, the IGF-II/M6-PR is involved in transportation of M6-PR-bearing glycoproteins including lysosomal enzymes from trans-Golgi network or cell surface to lysosomes (Kornfeld, 1992; Le Roith et al., 2001; Wang et al., 1994) as well as in clearing and thereby reducing the levels of IGF-II (Baker et al., 1993).

The expression of IGF-II/M6-PR is developmentally regulated, with the receptor being highly expressed in fetal and neonal tissues and the expression declining postnatally (Nissley et al., 1993). The knockout of this receptor results in fetal overgrowth and neonatal death (Wylie et al., 2003) in mice and those effects can be rescued by a concomitant lack of IGF-II or the IGF-IR. This indicates that the failure of targeting IGF-II for lysosomal degradation *via* the IGF-II/M6-PR which is accompanied by subsequent excessive signaling through the IGF-IR that finally results in the lethal phenotype. By contrast, tissue-specific inactivation of this gene in the liver or skeletal and cardiac muscle results in viable animals with no obvious phenotype. It explains that the failure to clear extracellularly produced IGF-II by lysosomal degradation *via* the IGF-II/M6-PR in those tissues is compensated by expression of the same receptor in other tissues where it clears IGF-II.

The *IGF-II/M6-PR* gene is known to be imprinted (Killian et al., 2000) in viviparous mammals whose maternal expression hampers proliferation, and reduce long term growth of primary embryonic fibroblasts. Interestingly, in mice the IGF-II/M6-PR is imprinted in all tissues except the brain where IGF-II/M6-PR is biallelically expressed (Vu and Hoffman, 2000). It is highly expressed in neurons of the forebrain, in regions involved in emotional behavior, information processing and memory formation (Couce et al., 1992). It indicates that the IGF-II/M6-PR plays a crucial role in the development of these brain functions. This finding is pointed to the IGF-II/M6-PR as one of the putative “IQ genes” in line with the observation that in children the IGF-II/M6-PR expression positively correlated with general cognitive ability.

The *IGF-II/M6-PR* gene is believed to act as tumor suppressor gene. Loss of IGF-II/M6-PR function is associated with tumor progression, and vice versa overexpression of IGF-II/M6-PR resulted in tumor regression of choriocarcinoma *in vivo* and *in vitro* (O'Gorman et al., 2002; Zaina and Squire, 1998). As the IGF-II/M6-PR interacts with an extensive array of ligands including lysosomal proteases and growth factors, its deficiency is therefore likely to have a complex phenotype with impact on multiple aspects of the malignant phenotype, including cell death, angiogenesis and metastasis.

1. 3. IGFBPs

The IGFBP gene family comprises six well characterized members that encode six multifunctional high affinity proteins termed IGFBP-1 to IGFBP-6 (Firth and Baxter, 2002). IGFBPs coordinate and modulate the biological activity of IGF in several ways: 1) transport IGF in plasma and control its diffusion and efflux from the vascular space; 2) increase the half-life and regulate clearance of the IGFs; 3) provide specific binding sites for the IGFs in the extracellular and pericellular space; and 4) modulate, inhibit, or facilitate interaction of IGFs with their receptors (Russo et al., 2005a). Furthermore, IGFBPs bind to IGFs with sufficiently higher (Kd, 10-11 to 10 -10 mol/L) affinity to ensure that virtually all circulating IGF is bound to IGFBPs. The six specific IGFBPs are structurally related to a larger group of proteins that share less homology but clearly form more distant relatives of a superfamily of proteins that evolved from a common ancestral gene. These distant relatives include CTGF (Connective tissue growth factor), CYR61 (Cysteine rich protein) and NOV (Nephroblastoma overexpressed gene) collectively called CCN proteins. Like IGFBPs, the CCN proteins are all cysteine-rich modular proteins with many pleiotropic actions on cell functions similar to the intrinsic actions of IGFBPs. It seems likely that the IGFBPs evolved as part of this family of cell regulatory proteins and then acquired an ability to bind IGFs and modulate IGF activity which complemented their original functions (Holly and Perks, 2006).

1. 3. 1. Structure of IGFBPs

IGFBPs share a highly conserved structure that consists of three domains of approximately equal size. The conserved amino-terminal domain contains six disulfide bonds in all IGFBPs except IGFBP-6 that contains five. The IGF-binding residues in the amino terminal domain of IGFBP-3 and -5 are identified by mutagenesis studies. In addition, amino-terminal fragments of IGFBP-3 revealed IGF-I-independent abrogation of proliferation. The conserved carboxyl-terminal domain contains three disulfide bonds in all IGFBPs, formed by the pairing of adjacent cysteines within the domain. Proteolysis and mutagenesis studies of this domain in different IGFBPs demonstrated IGF-binding sites within this domain. This indicates the role of amino and carboxyl-terminal domains in formation of an IGF-binding pocket for IGF binding. The carboxyl domain of IGFBP-1 and -2 also contains Arg-Gly-Asp (RGD) integrin-binding sequences. In addition to IGFBP-2 an important 18-residue basic motif with heparin-binding activity has also been identified in IGFBP-3 and -5 that are involved in interaction with the serum glycoprotein ALS (acid-labile subunit) and other ligands such as plasminogen activator inhibitor-1 and transferrin, cell and matrix binding, and nuclear transport. The central domain reveals essentially no structural conservation among all IGFBPs. There are no disulfide bonds detected in this region of all IGFBPs except an intradomain bond in IGFBP-4. In addition, for posttranslational modification sites such as N-linked glycosylation, phosphorylation and proteolytic cleavage have also been identified in this region (Firth and Baxter, 2002).

1. 3. 2. Localization of IGFBPs

IGFBPs are present in serum and in a variety of biological fluids including amniotic, follicular, cerebrospinal, and seminal fluid, as well as milk (Mohan and Baylink, 2002). IGFBPs have also been identified in the extracellular environment and inside the cell, and play distinct physiological roles in growth and development. There is less information on the exact relationship between IGFBPs in the circulation and those in the cellular environment. It is believed that IGFBPs may be differentially targeted to different tissues depending on both their primary structure and their posttranslational modifications. In some cases, endogenous IGFBPs from circulating ternary complexes may be found at low concentration in the tissues, as first implied by comparison of IGFBPs in serum and lymph (Binoux and Hossenlopp, 1988). Using

exogenous IGFBPs, Boes *et al.* (Boes et al., 1992) demonstrated in an isolated perfused heart model that IGFBP-4, after crossing the capillary endothelium, preferentially localizes to connective tissue rather than cardiac muscle, the exact distribution depending on the glycosylation state. In contrast, IGFBP-1, -2, and -3 are preferentially localized to cardiac muscle. IGFBP-3 injected intravenously appears initially in the liver (40% of injected dose) and kidney (4%), within 5 min after administration (Arany et al., 1996).

All six IGFBPs are also found in the circulation in the free form or in bound binary and/or ternary complexes with IGFs. In the circulation the majority of IGF is associated with IGFBP-3 and -5 in a ternary complex that also contain ALS, a leucine-rich-glycoprotein (Baxter et al., 1989; Baxter et al., 2002; Twigg et al., 1998; Twigg et al., 2000). The ternary complexes appear to be essentially confined to the vascular compartment whereas free or binary-complexed IGFBPs are believed to exit the circulation rapidly (Guler et al., 1989; Lewitt et al., 1994; Young et al., 1992). In addition to their localization in the circulation, distinct IGFBPs e.g. IGFBP-2, -3, -4 and -5 are identified in the extra- or pericellular compartment and inside the cell of various tissues with different biological functions.

1. 3. 3. Regulation of IGFBPs production

Local abundance of IGFBPs is regulated in a developmentally specific and tissue specific manner (Ferry et al., 1999b), both via transcriptional and post-translational mechanisms such as proteolysis. In terms of IGFBP expression, it is known that most tissues produce more than one IGFBP, although a given tissue may express one or two IGFBPs more abundantly than others. Studies on regulation of IGFBP expression in various cell types have provided evidence that IGFBP production is under the control of both systemic hormones and local regulators. For example, expression of both IGFBP-4 and IGFBP-5 in osteoblasts is known to be regulated by a variety of systemic hormones including growth hormone (GH), parathyroid hormone, glucocorticoid, 1,25 dihydroxyvitamin D3 and local growth factors, including IGFs, platelet derived growth factor (PDGF), bone morphogenetic proteins, transforming growth factor- β (TGF -β) and interleukins (Mohan and Baylink, 2002). In addition, systemic administration of growth hormone increases circulating levels of IGF-I, IGFBP-3, and ALS which are essential components in formation of the major

ternary complex involved in transportation of IGFs from the circulation to the target tissues. In postnatal mammals, plasma IGFBP-1 levels are regulated in accordance with metabolic status through effects of metabolic hormones on hepatocyte IGFBP-1 gene transcription. For instance the metabolic hormone insulin plays a primary role by strongly inhibiting IGFBP-1 production (Frystyk, 2004; Lee et al., 1993; Unterman et al., 1991). Moreover, IGF-I acquires the capability in regulation of IGFBP-2 and -3 in different cell types (Bale and Conover, 1992; Fleming et al., 2005). Studies on the molecular mechanisms by which hormones and growth factors regulate expression of IGFBPs provide evidence for a complex regulation involving both transcriptional and post-transcriptional mechanisms.

1. 3. 4. IGF availability by post-translational mechanisms of IGFBPs

The large quantities of IGFs bound with greater affinity to the IGFBPs than to the cell receptor require mechanisms to release IGFs from the IGFBPs for actions in tissues. IGFBPs can undergo a variety of posttranslational modifications that can have profound effects on IGFBP structure/function, and, hence, availability of IGFs and their action. There are at least four major posttranslational mechanisms that have been shown to influence the IGF binding affinity: i) glycosylation ii) phosphorylation iii) proteolysis and iv) differential localization of IGFBPs to the cell surface or extracellular matrix.

1. 3. 5. Glycosylation of IGFBPs

The post-translational modification of proteins by glycosylation confers heterogeneity in the structure and conformation of proteins resulting in different physical and biochemical properties (Lis and Sharon, 1993; Opdenakker et al., 1993). Carbohydrate units on glycoprotein can regulate the folding, conformation and intracellular traffic and localization of the protein; they may act as recognition determinants or contribute to binding sites in protein–protein, protein–cell and cell–cell interactions. In addition, they can confer protection against proteolysis and may act as clearance markers and hence determine the lifetime of the glycoprotein in the circulation. Some IGFBPs (IGFBP-1, -3, -4, -5, and -6) can be glycosylated. Among these, IGFBP-3 and -4 are N-glycosylated, and IGFBP-1, -5 and -6 are O-glycosylated (Firth and Baxter, 1999). Glycosylation of IGFBP-3 has no significant effect on the binding of IGF-I (Sommer et al., 1993) or ALS (Firth and Baxter, 1999).

However, decreased or non-glycosylation of IGFBP-3 increased its cell surface association and susceptibility to proteolytic degradation whereas glycosylation of IGFBP-6 decreased its cell surface association (Marinaro et al., 2000) and resistance to proteolytic degradation. This implies that glycosylation sites are essential for proteolytic cleavage and cell surface association, and are hindered by addition of carbohydrate moiety by glycosylation to prevent them from proteases (Bach, 1999).

1. 3. 6. Phosphorylation of IGFBPs

Protein phosphorylation and dephosphorylation are two major mechanisms for the regulation of protein function, providing a fast and reversible response to changing conditions by either activating or inactivating certain proteins. The role of phosphorylation in protein regulation has been studied for many intracellular enzymes, and for many proteins involved in intracellular signaling pathways (Cohen, 1985; Cohen, 1992; Roach, 1991). Less is known about the role of phosphorylation in regulating the activity of secreted proteins, especially those with no demonstrated catalytic activity. Three of the six IGFBPs, IGFBP-1, -3, and -5, are phosphorylated predominantly at serine residues in the central region (Coverley and Baxter, 1997). Phosphorylation and dephosphorylation status of human IGFBP-1 determines higher or lower binding affinity for IGF (Jones et al., 1991), thus leading to inhibition or potentiation of IGF effects (Scharf et al., 2004), respectively. Phosphorylation of IGFBP-1 is catalyzed by casein kinase and occurs only on serine residues of IGFBP-1 located in acidic regions of the molecule. Conversely, phosphorylation status does not appear to influence the IGF binding affinity of IGFBP-3. Thus, the issue of how alterations in the phosphorylation of some IGFBPs can influence certain functional features remains to be established (Mohan and Baylink, 2002).

1. 3. 7. Proteolysis of IGFBPs

IGFBP proteases are capable of cleaving intact IGFBPs and IGFBP-IGF complexes to form IGFBP fragments that have either significantly reduced or no affinity for IGFs, and to release IGFs from IGFBP-IGF complexes. IGFBP proteases have been demonstrated in a number of body fluids and cell culture conditioned media (Conover, 1995; Maile and Holly, 1999; Rajaram et al., 1997). Some of these IGFBP proteases have been shown to be relatively specific to a given IGFBP, in that they degrade one IGFBP at a greater potency than they do other IGFBPs. The proteases e.g.

pregnancy-associated plasma protein-A (PAPP-A) produced by fibroblasts, granulosa cells and osteoblasts, and is present in both normal and pregnancy serum, and complement C1s produced by human osteoblasts are relatively specific to IGFBP-4 and -5, respectively.

In addition, a number of other proteases e.g. plasmin, cathepsin D and prostate specific antigen (PSA) degrade IGFBPs and other proteins (Conover, 1995; Fowlkes et al., 1995; Maile and Holly, 1999; Rajah et al., 1995; Rajaram et al., 1997). Moreover, a circulating protease which is mainly responsible for limited cleavage of IGFBP-3 produces a fragment with lower affinity of IGF. Even a small decrease in affinity could result in a shift in the complex equilibrium with the IGF re-equilibrating to other IGFBPs that are present and which are generally not cleaved by the same protease. These other IGFBPs only form binary complexes, and therefore, have greater ability to transport the IGFs to target tissues (Holly and Perks, 2006). The role of these proteases in regulation of the effective concentration of a given IGFBP in local body fluid depends on a number of factors like concentration of the protease, relative potency with which the protease degrades a given IGFBP, presence or absence of other potential substrates, presence or absence of activators and inhibitors of the protease. In some conditions, IGFs might act as important modulators of IGFBP protease activity. For example, IGFs bind to IGFBP-4, and induce conformational change that lead to exposure of cleavage site for PAPP-A. In contrast, the proform of eosinophil major basic protein (proMBP) binds to PAPP-A, thereby blocking IGFBP-4 proteolysis. Thus, IGFBP proteolysis provides a mechanism for regulation of IGFBP degradation (Mohan and Baylink, 2002) and of IGF bioavailability.

1. 3. 8. Differential localization of IGFBPs to the cell surface or extracellular matrix (ECM)

It has been postulated that a number of IGFBPs can associate with ECM or cell surface via glycoproteins, collagens, integrins (Jones et al., 1993a; Jones et al., 1993b). IGFBP-1, -2, -3 and -5 have been reported to bind to cell surface or ECM (Mohan and Baylink, 2002; Russo et al., 1997; Russo et al., 2005b). Binding affinity of IGFs to IGFBPs is less when they are bound to cell surface or ECM. For example, IGF-I affinity of IGFBP-3 bound to the human fibroblast cell line was reduced by 40-

fold compared with IGFBP-3 in solution (Conover and Powell, 1991). IGFBP-5 binding to the ECM resulted in an eightfold reduction in IGF binding affinity compared with IGFBP-5 in solution (Jones et al., 1993a). However, the IGFBPs retain their ability to bind IGF-I in these locations. On the other hand, this leads to fixation of IGFs in the ECM of certain tissues. For instance, the stored IGFs in ECM of bone may be released during the osteoclastic bone resorption phase of bone remodeling to stimulate nearby osteoblasts during the bone formation phase of remodeling (Jones et al., 1993b). Thus, even the reduced IGF binding affinity of the IGFBPs bound in the ECM or cell surface bound IGFBPs has been implicated in IGFBP modulation of IGFs availability and their actions.

1. 3. 9. Physiological functions of IGFBPs

The physiologic roles of the IGFBPs can be divided into IGF-dependent and IGF-independent activities.

1. 3. 9. 1. IGF-dependent actions of IGFBPs

The term “IGF-dependent” functions of IGFBPs have been used to define functions of IGFBPs, both positive and negative, that are directly linked with IGF bioactivities. Because the affinity of IGFBP towards IGF is an important determinant in mediating the inhibitory or stimulatory effect of an IGFBP, a number of conditions that regulate IGF binding affinity of a given IGFBP, including phosphorylation, proteolysis and binding to cell surface or extracellular matrix proteins could influence the potency of an inhibitory or stimulatory IGFBP. *In vivo* and *in vitro* studies demonstrated that all IGFBPs have growth-inhibitory effects by competitively binding IGFs and preventing their binding to the IGF-IR. The most powerful evidence supporting this sequestration mechanism has come from studies using the IGF-I analog des-(1-3)-IGF-I (des-IGF-I) which binds to IGF-IR with similar affinity than IGF-I, but has a significantly reduced affinity to IGFBPs. In the human promyeloid cell line HL-60, addition of IGFBP-3 to serum-free media inhibited cell proliferation induced by IGF-I and IGF-II but not by des-IGF-I (Li et al., 1997). In 1997, Mohseni-Zadeh and Binoux (Mohseni-Zadeh and Binoux, 1997) implicated that IGFBP-3 may directly inhibit IGF binding to the IGF-IR, an alternative to inhibition through extracellular sequestration. Moreover, generation of mutated forms of IGFBPs with reduced affinities for IGFs was used to study the effects of the mutants on IGF bioactivities. This was most clearly

demonstrated in the case of IGFBP-4, an IGFBP known to inhibit the mitogenic effect of IGFs on bone cell growth. Mutations in human IGFBP-4 in line with greatly reduced affinity for IGF-II resulted in an inability of the mutant IGFBP-4 to inhibit IGF-II-induced human osteoblast proliferation (Qin et al., 1998). In addition, Hoeflich et al. (2001) reported that giant GH transgenic mice with increased IGF serum levels reduced growth kinetics when mated with IGFBP-2 transgenic mice, implicating that IGFBP-2 is also inhibitory to IGF-I action *in vivo*.

Other *in vitro* studies have also demonstrated that a number of IGFBPs e.g. IGFBP-1, -3, and -5 can also increase IGF actions by enhancing IGF-I binding to IGF-IR. In this regard, phosphorylation or nonphosporylation of IGFBP-1 altered the cellular effects of IGF-I in cultured smooth muscle cells or fibroblasts. The potentiating effect of IGFBP-1 was not seen with insulin, which does not bind to IGFBP-1, suggesting that the effect of IGFBP-1 is mediated via an IGF-dependent mechanism. Notably, cell surface or ECM association of IGFBP-3 and -5 have been indicated to increase the cellular effects of IGF-I. The less affinity of IGFBP-3 and -5 for IGF-I caused by cell association would facilitate a slow exchange of IGF-I between the receptor and IGFBP-3 and -5, and avoid downregulation of IGF-I receptor by excess IGF-I (Mohan and Baylink, 2002).

1. 3. 9. 2. IGF-independent actions of IGFBPs

Besides the effects of IGFBPs that involve altering the storage, transport and delivery of IGFs and inhibiting and enhancing their cellular actions, each of the IGFBPs also has ‘IGF-independent’ actions that are not dependent upon the binding and modulation of IGF activity. However, in many instances, these actions are not entirely IGF- independent because the intrinsic action of the IGFBP can be modulated when an IGF is bound to the IGFBP by altering the affinity of IGFBPs to cell surface. As a result, there will be complex interactions *in vivo* with IGFBPs modulating the mitogenic effects of the IGFs and the intrinsic actions of the IGFBP which in turn being modulated by the IGFs. To date, the molecular mechanisms of the IGF-independent actions of IGFBPs are incompletely understood. Conceptually, IGFBPs are believed to exert their direct actions on target cells in three ways: 1) by activation of cell surface receptors or membrane-bound proteins that initiate intracellular signaling responses; 2) by direct transportation to the cytoplasmic

compartment where they modulate biological effects of intracellular proteins; 3) by direct translocation to the nucleus where *via* interaction with transcription factors (nuclear receptors) they induce their effects directly on gene expression.

1.3.9.2.1 IGF-independent actions of IGFBPs via interaction with integrins and cell binding sites

IGF-independent actions of some IGFBPs have been defined in several *in vivo* and *in vitro* systems with the feature of cell surface association. The recognized Arg-Gly-Asp (RGD) sequence of IGFBP-1 directly interacts with α5β1 integrin receptor that leads to cell migration of smooth muscle cells and trophoblasts in the placenta (Jones et al., 1993b). Mutation of RGD sequence in IGFBP-1 prevented binding to the α5β1 integrin, and failed to show an increased cell migration. Like IGFBP-1, interaction of RGD motif of IGFBP-2 with α5β1 integrin receptor results in dephosphorylation of focal adhesion kinase (FAK) followed by inhibition of proliferation, and increasing cell de-adhesion in human breast cancer cells (Schutt et al., 2004). Besides, the heparin binding domain (HBD) which is believed to interact with cell surface is observed in IGFBP-2, -3, and -5. The binding of IGFBP-2 to cell membrane proteoglycans (cell-binding site) in the IGF-rich olfactory bulb rat brain (Russo et al., 1997) is mediated via the heparin binding domain *in vivo*. This indicates the capability of IGFBP-2 to interact with α5β1 integrin receptor and HBD motif in growth regulatory effects of IGFBP-2 through IGF-independent manner.

Although the other IGFBPs do not possess conventional integrin recognition sequences, it has been demonstrated that a number of IGFBPs can alter key integrin actions and signaling pathways. Moreover, activation of integrins can dramatically alter IGFBP actions. Exposure of human breast cancer cells to IGFBP-3 significantly increased cell attachment to plastic, collagen, laminin indicating activation of integrins and an increased dephosphorylation of FAK which is completely independent of IGF. In contrast, the effects of IGFBP-3 on cell attachment and apoptosis could be blocked by the presence of a sub-apoptotic dose of disintegrin (fibronectin). Exposure of cells to IGFBP-5 also affected integrin actions and apoptosis, with opposing effects to those of IGFBP-3, but again activation of integrin receptors by fibronectin completely reversed the actions of IGFBP-5. These data suggest that IGF-independent action of IGFBP-3 and -5 may be mediated through

unidentified interactions with integrin receptors. Recently, association of non-classical receptor sequence of IGFBP-3 and -5 to β1 integrin was explained, and they are known to bind with a variety of ligands of integrin receptors including fibronectin, plasminogen and fibrin. This explains the interaction of IGFBP-3 and -5 directly with integrin receptors or indirectly by binding with integrin ligands (Holly and Perks, 2006).

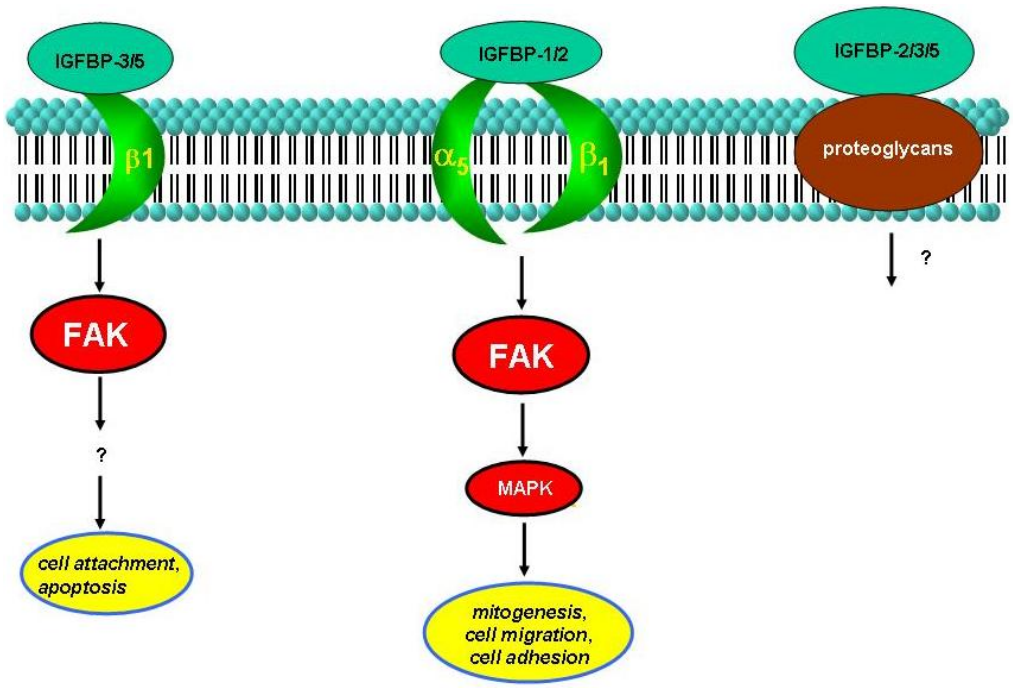

Figure 1. IGF-independent IGFBP actions via interaction with integrins and cell binding sites. IGFBP-3 and -5 are capable to bind integrin β1 receptor on cell surface, and initiate FAK signaling in cell attachment and apoptosis. IGFBP-I and -2 bind to α5β1 integrin and transduce signals through FAK followed by MAPK, and exert a role in mitogenesis, cell migration, and cell adhesion. Moreover, binding of IGFBP-2, -3, and -5 to proteoglycans on cell surface with undemonstrated signaling and their functional role has been proposed.

It is also believed that intracellular signals generated by binding of IGFBPs to integrin receptors interact with intracellular signals of IGF-IR which determine the ultimate signals delivered to the nucleus (Nam et al., 2002). In this regard, it has been demonstrated that ligand occupancy of α5β3-integrin receptor is required for full activation of the β subunit of IGF-IR and its signal transduction element, IRS-1, by IGF-I stimulation. It is possible that cells may contain a protein mediating the interaction between α5β3-integrin and IRS-1 or between α5β3-integrin and the tyrosine kinase subunit of the IGF-IR (Maile et al., 2001; Zheng and Clemmons,

1998). Thus, the binding of IGFBPs to cell surface play an important role in mediating their cellular effects and IGFs.

1.3.9.2.2 IGF-independent activities of IGFBPs via interaction with putative receptors

Other than the distinct integrin interactions of IGFBP-1, -2, -3 and -5, some mechanisms of the intrinsic actions of the IGFBPs are less clear. Putative IGFBP receptors have been demonstrated for IGFBP-3 and -5 (Andress, 1998; Oh et al., 1993) that could mediate their intrinsic actions. An association of IGFBP-3 with the transforming growth factor-β type V receptor (Leal et al., 1997) has also been reported. The binding of IGFBP-5 to its receptor, for example, may stimulate an IGFBP-5 signaling pathway that may interact with the IGF-I signaling pathway at one or more points to amplify the IGF-I signaling (Mohan et al., 1995). At present, all of these associations are intrinsic interactions of IGFBPs with their putative 'IGFBP receptors' that provide hope to gain IGF-independent actions, but links between these putative receptors and intracellular signaling events and consequent altered cell functions have yet to be established.

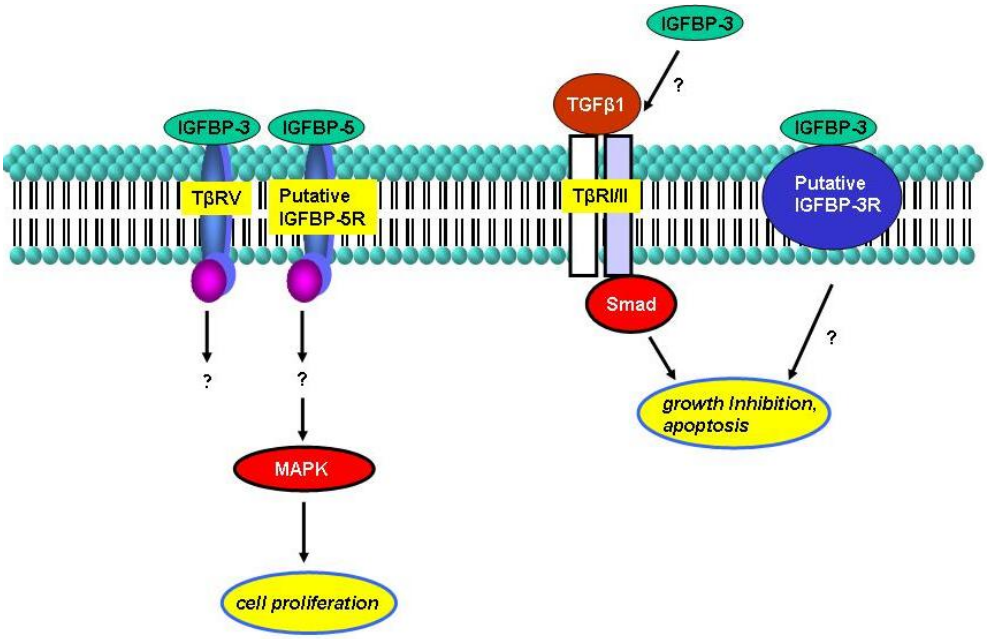

Figure 2. IGF-independent IGFBP actions via interaction with putative receptors in a variety of cell types. Putative TβRV and IGFBP-5R (420-kDa) have been proposed as serine/threonine kinase receptor for IGFBP-3 and IGFBP-5, respectively. IGFBP-5 bound putative IGFBP-5R is involved in cell proliferation through the activation of MAPK. IGFBP-3 stimulates TGF-β signaling via the Smad pathway, and this activity requires TβRII and is enhanced in the presence of TGF-β. Binding of IGFBP-3 to putative IGFBP-3R with undetermined signaling that might be involved in growth inhibition and apoptosis has been proposed.

1.3.9.2.3 Nuclear localization of IGFBPs and their IGF-independent actions

Some of the IGFBPs are known to localize inside the cell environment and exert their intrinsic actions in various biological processes. Recently, nuclear localization of IGFBP-3 and -5 has been reported (Jaques et al., 1997; Schedlich et al., 2000). Nuclear transport of IGFBP-3 and IGFBP-5 was not unexpected because both IGFBP-3 and IGFBP-5 possess basic C-terminal nuclear localization signals (NLS) and the DNA-binding domains of several transcription factors. This nuclear import of IGFBP occurs through a NLS-dependent pathway, mediated by the importin β nuclear transport factor (Butt et al., 1999). Addition of fluorescent IGF-I and IGFBP-3 in combination or alone in media from proliferating opossum kidney cells resulted in the colocalization of both in the nucleus suggesting that IGFBP-3 may carry IGF-I to the cell nucleus, and provides another regulation mechanism for IGF action. Furthermore, nuclear localization of IGFBP-3 (Radulescu, 1994) modulated the mRNA levels of bcl-2 in IGFBP-3-transfected MCF-7 cells, raising the possibility that the regulation of gene expression due to localization of IGFBP-3 in the nucleus may effect its apoptotic function (Butt et al., 2000). Recently, it was demonstrated that nuclear IGFBP-3 induces apoptosis and is targeted to ubiquitin/proteasome-dependent proteolysis (Santer et al., 2006). It has been shown that IGFBP-3 and the nuclear retinoid X receptor α (RXRα) bind each other within the nucleus, and IGFBP-3-induced apoptosis was abolished in RXRα-knockout cells. IGFBP-3 and RXR ligands were additive in inducing apoptosis in prostate cancer cells. IGFBP-3 enhanced RXR response element and inhibited signaling *via* retinoic acid receptor response element. RXRα-IGFBP-3 interaction has led to modulation of the transcriptional activity of RXRα and was essential for mediating the effects of IGFBP-3 on apoptosis (Liu et al., 2000). Nevertheless, in spite of the absence of a classical nuclear localization signal which is thought to be the essential for nuclear transportation of IGFBP-3 and -5, nuclear import of IGFBP-2 has been demonstrated in oxidant exposed lung adenocarcinoma cells (Besnard et al., 2001). Furthermore, intact IGFBP-2 and IGFBP-2 fragments were identified in the peri/nuclear fractions isolated pancreas, stomach, and brain of IGFBP-2 transgenic mice. This suggests that novel functions of IGFBP-2 and/or IGFBP-2 fragments can be presumed in the nuclear environment under normal and elevated IGFBP-2 expression levels (Hoeflich et al., 2004).

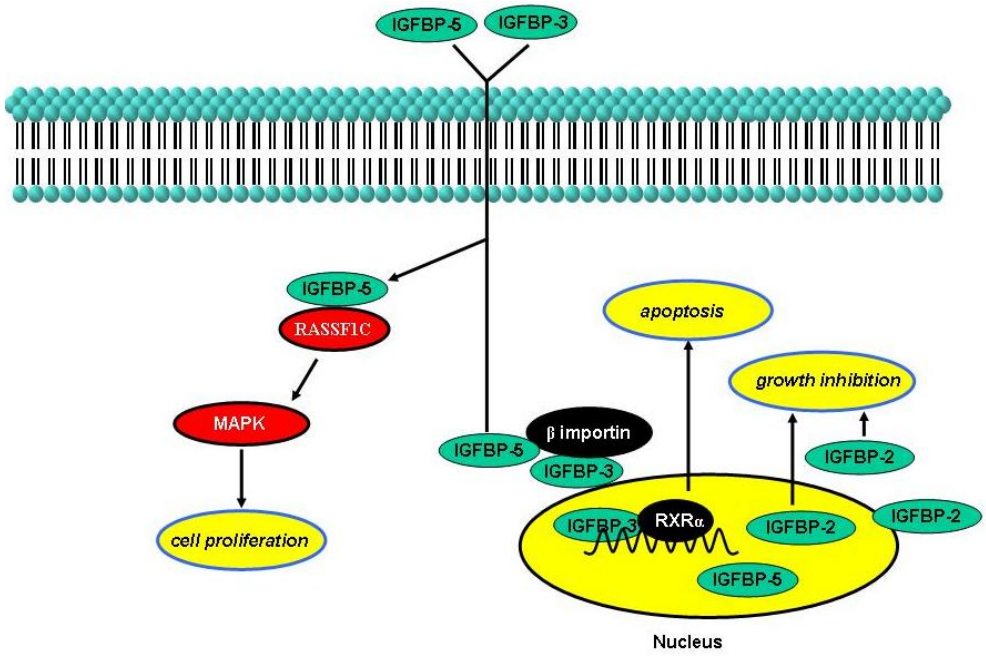

Figure 3. IGF-independent IGFBP actions by differential localization of IGFBPs within the cells. IGFBP-3 and IGFBP-5 present in extracellular space are diffused into cytoplasm from where they are translocated to the nucleus by importin β. IGFBP-3 forms a complex with nuclear partner, RXRα in nucleus. Thus, formed complex is involved in up-regulation of genes essential for apoptosis. Moreover, cytoplasmic IGFBP-5 has also been interacted with Ras-Association Domain Family 1 Protein (RASSF1C) that further activates MAPK, and displays a significant role in cell proliferation. IGFBP-2 is localized in cytoplasm, on surface of nucleus, and in nucleus. IGFBP-2 present in cytoplasm and nucleus are participated in growth inhibition.

1. 4. Role of liver in biosynthesis of IGF axis components

The liver plays a crucial role in the IGF homeostasis (Baruch, 2000; Scharf and Braulke, 2003; Scharf et al., 2001) because it is the main source of various IGF axis components such as circulating IGF-I, IGFBP-1 and ALS. In rat liver, the biosynthesis of IGF system components is attributed to different cell population. Hepatocytes (HCs) have been demonstrated to secrete IGFBP-1, -2, -4 and ALS (Scharf et al., 2001). IGFBP-3 was exclusively expressed in nonparenchymal cells namely Kupffer cells (KCs), hepatic stellate cells (HSCs), sinusoidal endothelial cells (SECs) and liver myofibroblasts (LMFs) (Novosyadlyy et al., 2004; Scharf et al., 2001). In contrast to other IGFBPs, IGFBP-2 is the only IGFBP that was detected in all the different liver cells studied so far (HCs, KCs, SECs, LMFs and HSCs). Interestingly, despite the wide distribution of the IGF-IR throughout the body, the IGF-IR expression is almost undetectable in hepatocytes, the cells with the highest levels of IGF-I expression (Caro et al., 1988; Hartmann et al., 1990; Novosyadlyy et

al., 2006a). In contrast, presence of the IGF-IR has been demonstrated in non-parenchymal liver cells such as HSCs (Brenzel and Gressner, 1996; Caro et al., 1988; Scharf et al., 1998a), SECs (Scharf et al., 1998a; Zimmermann et al., 2000; Zindy et al., 1992), Kupffer cells (Zimmermann et al., 2000; Zindy et al., 1992) and LMFs (Novosyadlyy et al., 2004; Zindy et al., 1992). Moreover, production of the IGFs by these cells has also been observed (Novosyadlyy et al., 2004; Pinzani et al., 1990; Scharf et al., 1998a; Zindy et al., 1992).

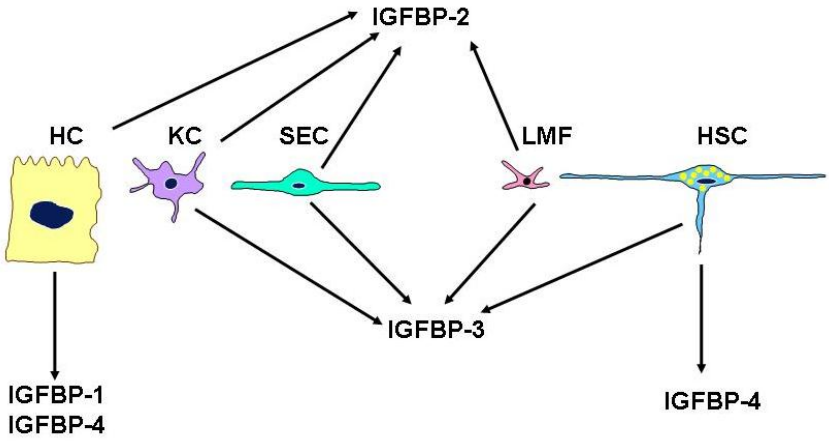

Figure 4. Cellular distribution of IGFBPs in liver. Hepatocytes (HCs) and hepatic stellate cells (HSCs) are involved in synthesis of IGFBP-4 whereas IGFBP-1 is exclusively expressed only by HCs. Kupffer cells (KCs), sinusoidal endothelial cells (SECs), myofibroblasts (MFs) and HSCs are participated in biosynthesis of IGFBP-3. In contrast to other IGFBPs, IGFBP-2 is unique since it is the only IGFBP that is secreted by all types of cells within the liver except HSCs.

1. 5. Liver fibrogenesis

Liver fibrosis is defined as the abnormal accumulation of ECM in the liver. Its endpoint is cirrhosis, which is responsible for a significant morbidity and mortality of the affected patients. Cirrhosis is an advanced stage of fibrosis, characterized by the formation of regenerative nodules of liver parenchyma separated by fibrotic septa. This occurs after longstanding chronic liver injuries caused by a number of variables including viral infections (hepatitis B and C), alcohol abuse, drugs, helminthic invasions, metabolic diseases due to overload of iron and copper, autoimmune destruction of hepatocytes and bile duct epithelium, or congenital abnormalities. Hepatic fibrosis results from the aberrant synthesis (fibrogenesis) and degradation (fibrolysis) of ECM components (Friedman, 1993; Ramadori et al., 1998; Zindy et al., 1992). This process in the liver is characterized by a three- to six-fold overall increase and deposition of the ECM components with their subsequent molecular

reorganization resulting in an altered composition of fibrotic matrix. Advances in the isolation and characterization of liver cells, in conjunction with progress in molecular biology, have led to important new insights into the cellular basis of hepatic fibrosis.

It is known that HSCs (vitamin A-rich cells), also known as Ito cells, are considered as one of the key effectors of the fibroproliferative response in the liver (Friedman, 1993; Friedman, 1999; Ramadori et al., 1998; Saile and Ramadori, 2007). Both *in vivo* and *in vitro* these cells undergo a phenotypic transition from a quiescent, vitamin A-rich phenotype (quiescent HSCs) to myofibroblast-like phenotype (activated HSCs), cells with high proliferative and fibrogenic abilities (Friedman, 2000; Saile and Ramadori, 2007). However, several independent groups have clearly demonstrated that in addition to transdifferentiation, HSCs undergo spontaneous apoptosis both *in vitro* and *in vivo* (Fischer et al., 2002; Friedman, 2000; Iredale et al., 1998; Saile et al., 1997; Taimr et al., 2003). Therefore, it is difficult to assume that dying cells are responsible for fibroproliferative process in the liver. Moreover, transdifferentiation of one clearly identified HSC to myofibroblast has never been shown *in vitro.* Thus, it appears likely that myofibroblast-like cells involved in hepatic fibrogenesis may also arise from another cell type within the liver. Recent data have demonstrated that activated HSCs and liver myofibroblasts (LMFs), despite their common features, represent morphologically and functionally different fibroblast populations. Moreover, it has been shown that the rate of synthesis of the ECM proteins fibronectin and type I collagen by LMFs is higher than in HSCs during a fibrillar matrix synthesis, suggesting similar but not identical roles of these cells during fibrogenesis (Fischer et al., 2002; Knittel et al., 1999b). Furthermore, HSCs and LMFs are present in normal and diseased livers in distinct anatomical compartments and respond differentially to tissue injury. Acute liver injury results in most exclusive increase in the number of HSCs, while in chronically injured livers both HSCs and LMFs are involved in fibrogenesis (Knittel et al., 1999b).

At present, the precursor pool of LMFs is not identified, in spite of very important clinical relevance. The precursors of these cells could be resident cells of the fibroblast lineage in the liver such as portal fibroblasts, periductal fibroblasts, vascular myofibroblasts, and “second layer “cells or capsular fibroblasts. Portal fibroblast, residing under the normal conditions in the portal mesenchyme, can be

responsible for periportal fibrosis. Periductal fibroblasts, which constitute a distinct subpopulation of mesenchymal cells in the portal tract, have been suggested to proliferate and transdifferentiate in response to bile duct ligation, causing periductal, periductular and periportal “biliary“ type of fibrosis. In schistosomiasis, vascular smooth muscle cells or vascular myofibroblasts situated in the wall of portal vein branches and portal arteries were thought to perpetuate to matrix-producing cells, thereby leading to periportal fibrosis as well. So called “second layer” cells are myofibroblasts located around the centrolobular vein. They were suggested to cause typical “alcoholic” type of pericentral fibrosis. Finally, capsular fibroblasts detected in Glisson’s capsule can also be a potential source of ECM in the liver (Cassiman et al., 2002; Knittel et al., 1999a; Ramadori and Saile, 2002).

1. 6. IGF axis in Liver fibrogenesis

It has been shown that during perpetuation of liver cirrhosis, the homeostasis of IGF axis is severely disturbed. The possible role of IGF axis in the pathogenesis of liver cirrhosis is not completely known. Patients with end-stage liver cirrhosis had reduced IGF-I, IGF-II and IGFBP-3 serum levels (Cassiman et al., 2002; Moller et al., 1995; Scharf et al., 1996), which were associated with adverse clinical outcome and complications of advanced cirrhosis such as malnutrition, insulin resistance, impaired immunity and osteoporosis. In addition, overexpression of IGFBP-1, -2, and -3 was reported in liver tissue during end-stage liver disease and liver cirrhosis (Holt et al., 1997; Holt et al., 1996; Ross et al., 1996; Scharf et al., 1996).

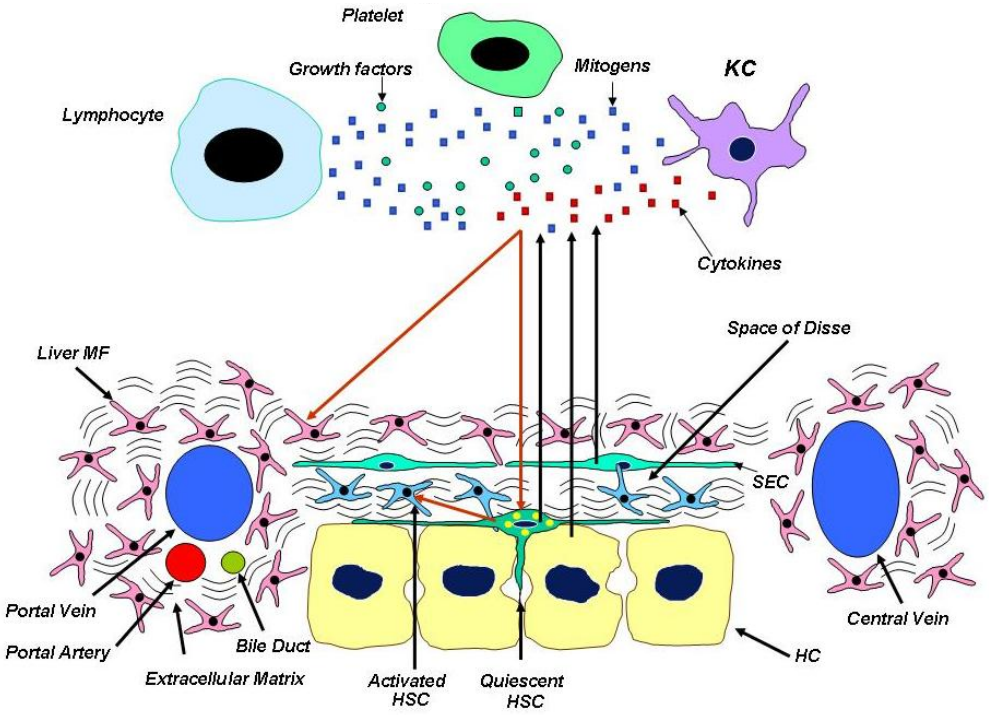

Figure 5. Liver fibrogenesis. During chronic liver injury, mitogens from hepatocytes (HCs) due to membrane damage, and cytokines and growth factors by activated Kupffer cells (KCs)/macrophages, sinusoidal endothelial cells (SECs), hepatic stallate cells (HSCs), platelets and lymphocytes were released at the site of necrosis. Among these growth factors, IGF axis components act on quiescent, vitamin A-rich HSCs located in the space of disse, and transdifferentiate to myofibroblast-like phenotype (Activated HSCs) with high proliferative potential and ability to synthesis excessive amounts of extracellular matrix (ECM) components during perpetuation of liver fibrogenesis. However, activated HSCs simultaneously undergo apoptosis. MFs are a second resident cell population of fibroblastic lineage located in periportal and pericentral areas within the liver. These are morphologically and functionally distinct from HSCs and are activated by released IGF axis components, and undergo high proliferation and synthesize ECM components abnormally during liver fibrogenesis. In contrast to HSCs, MFs are resistant to apoptosis.

Intriguingly, recent *in vivo* studies have demonstrated that exogenous IGF-I improved liver function, and reduced oxidative liver damage and fibrosis in rats with experimental liver cirrhosis (Castilla-Cortazar et al., 1997; Holt et al., 1996). Alternatively, recent studies have shown that proliferation of HSCs and accumulation of type I collagen, the principal ECM protein, by these cells *in vitro* is stimulated in response to IGF-I (Castilla-Cortazar et al., 1997; Gentilini et al., 1998; Gentilini et al., 2000; Pinzani and Marra, 2001; Scharf et al., 1998a; Svegliati-Baroni et al., 1999). Therefore, it is believed that due to chemotactic, mitogenic and fibrogenic activity of IGF I released locally during hepatic injury trigger HSCs and possibly LMFs, thereby leading to their activation, proliferation as well as to collagen production and, finally, to perpetuation of fibrogenic response within the liver.

1. 7. Aim of the study

Recent studies clearly identified two morphologically and functionally different fibroblast lineage cell populations within the liver: HSCs and LMFs. Clearly, more detailed insights into the mechanism regulating proliferation and ECM synthesis of myofibroblasts in the liver will allow the identification of new molecular targets and the development of new therapeutic modalities for more specific, effective, less harmful modes of treatment capable to cease a progression of liver cirrhosis. Some of these targets could be components of the IGF axis. In liver cirrhosis hepatic expression and circulating levels of IGFBP-2 are significantly increased and positively correlate with the severity of disease (Holt et al., 1996; Kratzsch et al., 1995; Pinzani and Marra, 2001; Ross et al., 1996; Scharf et al., 1996). Since IGFBP-2 has generally been demonstrated either to inhibit or to potentiate the IGF effects depending on the cell type studied, the role of IGFBP-2 in liver fibrogenesis is unclear. Therefore, in the current work mLMFs were isolated from livers of wild type (wt) and IGFBP-2 transgenic (IGFBP-2(+/-)) mice and were used as *in vitro* model to study the role of IGFBP-2 in cellular functions of mLMFs. In detail, the following issues were addressed:

1) to assess whether IGFBP-2 is overexpressed in LMFs isolated from CMV-IGFBP-2 transgenic (IGFBP-2 (+/-)) mice liver compared with LMFs from wild-type (wt) mice liver;
2) to study the expression and regulation of other IGF axis components in wt and IGFBP-2 (+/-) mLMFs;
3) to evaluate the role of IGFBP-2 overexpression on DNA synthesis and biosynthesis of ECM in wt and IGFBP-2 (+/-) mLMFs;

2. Materials

2.1. Animals

Generation of transgenic mice overexpressing mouse IGFBP-2 under the control of the cytomegalovirus (CMV) promoter was reported before (Hoeflich et al., 1999). The wild type (wt) and transgenic mice overexpressing mouse IGFBP-2 (IGFBP-2 (+/-)) were obtained from Institute of Animal Breeding and Biotechnology, München, Germany. All these male adult mice kept at 19-23°C under standard conditions with 12-hour light/dark cycles and access to fresh water and food *ad libitum*. All animals received humane care in accordance with the institution's guidelines, the German Convention for Protection of Animals and the National Institutes' of Health guidelines.

2. 2. Bacterial Strain and Vectors

E. coli DH5α strain (Stratagene, Heidelberg, Germany) was used for plasmid transformation. The phagemid pBluescript SK+ (Stratagene, Heidelberg, Germany) was used for molecular subcloning of rat IGF-I, IGFBP-2 and IGFBP-3 specific cDNA fragments. The pGEM®-4Z and pGEM®-3Z vectors were used to subclone rat IGF-IR and IGF-II/M6-PR specific cDNA fragments (Promega Mannheim, Germany).

2.3. cDNA inserts and Oligonucleotides

A 700 bp *EcoRI-HindIII* fragment of rat IGF-I cDNA, 265 bp *EcoRI-SmaI* fragment of rat IGF-IR cDNA, 500 bp *EcoRI-BamHI* fragment of rat IGF-II/M6-PR cDNA, 397 bp *EcoRI-HindIII* insert of clone pRBP2-501 coding for rat IGFBP-2, 699 bp *ApaI-BamHI* insert of clone pRBP3-AR encoding rat IGFBP-3 and an oligonucleotide *5´ AAC GAT CAG AGT AGT GGT ATT TCA CC 3´* complementary to 28 S rRNA (MWG Biotech, Ebersberg, Germany) was used to quantify Northern blots.

2. 4. Antibodies

2. 4. 1. Primary Antibodies

Anti-IGF-IRβ Ab

For the detection of the IGF-IRβ by Western immunoblotting, an affinity purified rabbit polyclonal antiserum raised against a peptide mapping at the carboxy terminus

of the IGF-IRβ of human origin was used (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Species reactivity: mouse, rat, human.

Anti-Smooth muscle α-actin (SMAα) Ab
For the detection of the SMAα by immunocytochemistry, monoclonal anti-SMAα Ab derived from the hybridoma produced by the fusion of mouse myeloma cells and splenocytes from an immunized mouse was used (Sigma, Munich, Germany).

Anti-collagen 3 Ab
For the detection of the collagen 3 by immunocytochemistry, rabbit anti-bovine collagen type 3 polyclonal antiserum was used (Paesel and Lorei, Hanau, Germany).

2. 4. 2. Secondary Antibodies

Peroxidase-Conjugated Swine Anti-Rabbit immunoglobulins, Peroxidase-Conjugated Rabbit Anti-Mouse immunoglobulins, Peroxidase-Conjugated Rabbit anti-Mouse immunoglobulins, Peroxidase-Conjugated Swine anti-Rabbit immunoglobulins were used against a primary antibody of rabbit and mouse origin respectively which were bound to a protein of interest by Western immunoblotting and Immunocytochemistry, (DAKO, Copenhagen, Denmark).

2. 5. Proteins, Enzymes and Protein Standards

Bovine serum albumin (BSA) (PAA, Linz, Austria); Collagenase H (Roche, Mannheim, Germany); DNase I (Roche, Mannheim, Germany); Glucose oxidase (Sigma, Munich, Germany); recombinant human IGF-I (GroPep, Adelaide, Australia); recombinant rat PDGF-BB (Sigma, Munich, Germany); Pronase E (Merck, Darmstadt, Germany); Rainbow™ colored protein molecular weight markers, Protein A sepharose 4 fast flow (Amersham Biosciences, Freiburg, Germany); Restriction enzymes with buffers (Boehringer Mannheim, Mannheim, Germany); recombinant human TGF-β1 (PeproTech, Rocky Hill, NJ, USA).

2. 6. Protease and Phosphatase Inhibitors

Antipain (Sigma, Munich, Germany), Benzamidine (Sigma, Munich, Germany), Chymostatin (Sigma, Munich, Germany), Leupeptin (Sigma, Munich, Germany), Phenylmethanesulfonyl fluoride (PMSF) (Sigma, Munich, Germany), Pepstatin A, (Sigma, Munich, Germany), Sodium fluoride (Merck, Darmstadt, Germany), Sodium orthovanadate (Sigma, Munich, Germany), Tetrasodium pyrophosphate (Sigma, Munich, Germany), β-glycerophosphate (Sigma, Munich, Germany).

2. 7. Detergents

CHAPS (Roche, Mannheim, Germany), Nonidet P-40 (NP-40) (USB, Cleveland, OH, USA), Sodium dodecyl sulfate (SDS) (Roth, Karlsruhe, Germany), Triton X-100 (Serva, Heidelberg, Germany), Tween 20 (Serva, Heidelberg, Germany).

2. 8. Amplication, Detection, Purification and Synthesis and Systems (Kits)

5-Bromo-2´-Deoxy-Uridine Labelling and Detection kit III (Roche, Mannheim, Germany), Agarose Gel DNA Extraction Kit (Roche, Mannheim, Germany), BCA Protein Assay (Pierce, Bonn, Germany), EndoFree™ Plasmid Maxi Kit (Qiagen, Hilden, Germany), NEBlot® Kit (New England Biolabs, Schwalbach, Germany), SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Bonn, Germany), cDNA preparation Kit, Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen™ GmbH, Karlsruhe, Germany).

2. 9. Stock solutions

All stock solutions were prepared either in double distilled water (ddH_2O) or (for experiments with RNA) in RNase-free water; the pH values of stock solutions were adjusted at 25°C.

Ammonium persulphate (APS) 10% (The solution was aliquoted and stored at −20°C), Citric acid 0.25 M (The solution was stored at room temperature (RT)), EDTA 0.5 M (pH was adjusted with 5N NaOH to 8.0, sterile filtered and stored at RT), 10X Phosphate-buffered saline (PBS) (pH was adjusted with HCl to 7.3 and stored at RT), SDS 20%, Sodium acetate 2 M (pH was adjusted with acetic acid to 5.4 and

stored at RT), Sodium citrate 0.25 M (pH was adjusted with 0.25 M citric acid to 7.0 and stored at RT), Tris-HCl 2 M (pH was adjusted with NaOH to 7.4. The solution was sterile filtered and stored at 4°C).

2. 10. Chemicals

All chemicals were of analytical grade and obtained from commercial sources as indicated: [α-^{32}P]-labeled deoxy-cytidine-triphosphate (specific activity 3,000 Ci/mmol), sodium [^{125}I] iodide (carrier-free, specific activity 16.85 mCi/μg), [^{3}H]-thymidine (Amersham Biosciences, Freiburg, Germany); Glycine, nonfat dried milk powder (AppliChem, Darmstadt, Germany); fetal calf serum, trypan blue, trypsin (Biochrom, Berlin, Germany); Ampicillin (Boehringer, Mannheim, Germany); Re-Blot Plus (strong antibody stripping solution) (Chemicon, Temecula, CA, USA); Bactoagar, bacto-trypton, yeast extract (Difco Laboratories, Detroit, MI, USA); Ampuwa® water (Fresenius, Bad Homburg, Germany); 123 kb DNA ladder, agarose, guanidine isothiocyanate, low melting point agarose (Invitrogen, Karlsruhe, Germany); 6x loading dye (MBI Fermentas, St. Leon-Rot, Germany); Acetic acid, acetone, bromophenol blue, chloramine T, ethanol, 37% formaldehyde, formamide, glycerol, methanol, β-mercaptoethanol, penicillin G, sodium bisulphite, sodium fluoride, streptomycin (Merck, Darmstadt, Germany); Pentobarbital sodium (Narcoren®) (Merial, Hallbergmoos, Germany); Nycodenz® (Nyegaard, Oslo, Norway); Dulbecco´s modified Eagle medium (DMEM), L-Glutamine (PAA, Linz, Austria); Cesium chloride (Paesel and Lorei, Frankfurt, Germany) Trifluoroacetic acid (Pierce, Bonn, Germany); Fish sperm DNA (Roche, Mannheim, Germany); Acetonitril, hydrogen peroxide, Rotiphorese Gel 30 (30% acrylamide stock solution with 0.8% bisacrylamide in proportion 37.5:1), Sodium chloride, Tris base (Roth, Karlsruhe, Germany); Ammonium persulfate, tetramethyl ethylene diamine (TEMED), Tris-HCl, Triton X-100, Tween 20 (Serva, Heidelberg, Germany); Citric acid, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), fish gelatine, EDTA, EGTA, ethidium bromide, glucose, HEPES, MOPS, N-lauroylsarcosyl, sodium acetate, sodium citrate (Sigma-Aldrich Chemie, Munich, Germany); QuikHyb® Hybridization Solution (Stratagene, Heidelberg, Germany); Scintillation liquid (Zinsser Analytic, Frankfurt, Germany); Potassium chloride, Magnesium sulphate, sodium hydrogen carbonate, Sodium dihydrogen phosphate monohydrate, calcium chloride 2-hydrate crystals, Potassium

dihydrogen phosphate, Sodium hydrogen carbonate, Glucose (Merck, Darmstadt, Germany).

2. 11. Other Materials and Tools

Petri dishes (100 mm) for bacterial cultures (Greiner, Frickhausen, Germany); 96-well microtiter plates (Nunc, Naperville, IL, USA); Animal cell culture dishes (100 and 150 mm) (Becton Dickinson Labware, Lincoln Park, NJ, USA); Hybond N nylon membrane, disposable NICK columns prepacked with Sephadex® G-50 DNA grade (Amersham Biosciences, Freiburg, Germany); Hybridization glass tubes (Biometra, Göttingen, Germany); Latex powder-free gloves (Kimberly-Clark, Zaventem, Belgium); Nitrile gloves Nitra Tex (Ansell, Kulim, Malaysia); Nitrocellulose Transfer Membrane (Sartorius, Göttingen, Germany); Polyallomer thin-walled centrifuge tubes (5 ml) (Beckman, Munich, Germany); Safe-Lock tubes (0.2, 0.5, 1.5 and 2 ml) (Eppendorf, Hamburg, Germany); Scintillation vials (5 ml) (Zinsser Analytic, Frankfurt, Germany); Serological pipettes (2, 5, 10, 25 ml), transfer pipettes, plastic tubes (15 and 50 ml) (Sarstedt, Nümbrecht, Germany); Sterile filter Nalgene, 0.2 µm (Sartorius, Göttingen, Germany); Sterile filter pipette tips (Biozym, Oldendorf, Germany); Whatman 3MM paper (Schleicher and Schuell, Dassel, Germany); X-ray films Hyperfilm™ (Amersham Biosciences, Freiburg, Germany); CL-Xposure TM film (Pierce, Bonn, Germany).

2. 12. Instruments

Automatic pipettes, type Reference® (Eppendorf, Hamburg, Germany); Beckman model J2-21 centrifuge (Beckman, Munich, Germany); Centricon T-2070 ultracentrifuge (Kontron Instruments, Neufahrn, Germany); Eppendorf bench-top centrifuge, type MiniSpin 5415C (Eppendorf, Hamburg, Germany); Hettich Mikro Rapid/K centrifuge, Hettich Rotina 3850 centrifuge, Hettich Rotina 48RS centrifuge, Hettich Rotixa/RP centrifuge (Hettich, Tuttlingen, Germany); Minifuge GL centrifuge (Heraeus-Christ, Osterode, Germany); Sigma 3K30 centrifuge (Sigma Laboratory Centrifuges, Osterode, Germany); Electrophoresis power supply Power Pac 300 (Bio-Rad, Munich, Germany); Electrophoresis power supply ST305 (Invitrogen, Karsruhe, Germany); Gamma-counter (Mini-instruments, Burnham-on-Crouch, UK); Gas controlled incubators (Heraeus-Electronic, Hannover, Germany); Geiger hand

gamma-counter (Berthold, Oak Ridge, TN, USA); Hybridization oven (Biometra, Göttingen, Germany); Incubator with shaking for bacterial culture, model 3-25 (New Brunswick Scientific Co., Edison, New Jersey, USA); Liquid scintillation counter Wallac 1409 (EG&G, Turku, Finland); Microplate reader MRX (Dynatech Laboratories, Chantilly, VA, USA); Microscope Axiovert 25 (Zeiss, Oberkochen, Germany); Mini-vertical gel electrophoresis unit Hoefer Mighty Small II (Amersham Biosciences, Freiburg, Germany); pH-Meter (Glas-Gerätebau, Bovenden, Germany); Rocking platform (Biometra, Göttingen, Germany); Scanning densitometer (Molecular Analyst, Bio-Rad Hercules, CA, USA); Sonicator Sonoplus HD 70 (Bandelin, Berlin, Germany); Standard dual cooled gel electrophoresis unit Hoefer SE 600 (Amersham Biosciences, Freiburg, Germany); Sterile bench (The Baker Company, Sanford, ME, USA); Thermomixer 5436 (Eppendorf, Hamburg, Germany); Thermostat (Heraeus, Hanau, Germany); Transfer electrophoresis unit Hoefer TE 50X (Amersham Biosciences, Freiburg, Germany); Transfer electrophoresis unit Mini Trans-Blot® (Bio-Rad, Munich, Germany); Ultra-pure water system Milli-Q (Millipore, Molsheim, France); UV spectrophotometer, RNA/DNA Calculator GeneQuant II (Pharmacia Biotech, Freiburg, Germany); Vortex, with platform, (Schütt Labortechnic, Göttingen, Germany); X-ray film cassettes 10x18 (Siemens, Munich, Germany); X-ray film-developing machine SRX-101A (Konica Europe, Hohenbrunn, Germany).

3. Methods

3. 1. Cell Biology Methods

3. 1. 1. Isolation of Mouse Hepatocytes

Liver Perfusion

Mice were anesthetized by intraperitoneal injection of Pentobarbital-sodium and fixed on the preparation desk. The abdomen was disinfected with 70% ethanol and opened by longitudinal incision along *linea alba* up to *processus xyphoideus* giving access to the liver sinus and portal vein. After laparotomy, the *vena portae* was canulated, *vena cava inferior* was ligated above the diaphragm to prevent flow of the perfusion media into the circulation. Then the *vena cava inferior* was cut beneath the liver and canulated as well. The liver was perfused first in a non-recirculative mode through the portal vein with 150-200 ml of CO_2-enriched preperfusion medium at a flow rate of 30 ml/min until the liver was free from blood. Further, in order to break down sufficiently an extracellular matrix, the liver was perfused in a recirculative mode with collagenase perfusion medium until it started to soften (about 7-11 min).

Krebs-Ringer stock solution

	For 1 l	Final concentration
NaCl	7 g	120 mM
KCl	0.36 g	4.8 mM
$MgSO_4x7H_2O$	0.296 g	1.2 mM
KH_2PO_4	0.163 g	1.2 mM
$NaHCO_3$	2.016 g	24.4 mM
ddH_2O	to 1 l	

The solution was equilibrated with carbon dioxide and pH was adjusted to 7.35.

Preperfusion medium

	For 1 l	Final concentration
EGTA	95.1 mg	0.25 mM
Krebs-Ringer stock solution	to 1 l	

Collagenase perfusion medium

	For 100 ml	Final concentration
HEPES	360 mg	15 mM
$CaCl_2x2H_2O$	58.8 mg	4 mM
Collagenase	50 mg	
Krebs-Ringer stock solution	to 100 ml	

The medium was prepared directly prior to isolation, equilibrated with carbon dioxide for 30 min and finally sterile filtered.

Preparation of primary hepatocyte culture

After perfusion, the liver was excised and transferred to a sterile glass beaker filled with culture medium M199 and supplements. Glisson's capsule was carefully removed and discarded. To obtain a cell suspension, the tissue was disrupted mechanically using sterile forceps. Connective tissue and the remaining liver capsule as well as big cell aggregates were removed by filtration of the primary cell suspension through a nylon mesh (pore size 79 µm). Nonparenchymal cells and cell debris were removed by numerous selective sedimentations (20 x *g*, 2 min, and 4°C) in wash medium. The cell pellet was resuspended in culture medium, and hepatocytes were then separated by Percoll® density gradient centrifugation (1500 x *g,* 5 min, RT). Finally, purified hepatocytes were resuspended in serum-enriched M199 with supplements, plated onto 35- and 60-mm Falcon plastic dishes at a density 1 x 10^6 and 2 x 10^6 cells per dish, respectively, and incubated at 37^0C in 5% CO_2 atmosphere and 95% humidity.

Wash medium

	For 1 l	Final concentration
HEPES/NaOH pH 7.4	4.77 g	20 mM
NaCl	7.00 g	120 mM
KCl	0.36 g	4.8 mM
$MgSO_4x7H_2O$	0.30 g	1.2 mM
KH_2PO_4	0.16 g	1.2 mM
BSA	4.00 g	0.4%
ddH_2O	to 1 l	

Percoll® solution

Percoll®	43.1 ml
Gradient buffer	6.5 ml

Gradient buffer for Percoll® solution

	For 100 ml	Final concentration
NaCl	8.18 g	1.4 M
KCl	0.37 g	50 mM
$MgCl_2$	0.163 g	8 mM
Na_2HPO_4	0.28 g	16 mM
KH_2PO_4	0.054 g	4 mM
ddH_2O	to 100 ml	

pH was adjusted to 7.35; the solution was then sterile filtered.

M199 with supplements (stock medium)

Solution A

	For 500 ml
M199 with Earle's salts without $NaHCO_3$, in powder	1 bottle (for 1 l)
BSA	2.0 g
HEPES	3.6 g
ddH_2O	to 500 ml

pH was adjusted to 7.35, and the solution was equilibrated with carbon dioxide under the control of pH.

Solution B

	For 500 ml
$NaHCO_3$	1.5 g
ddH_2O	to 500 ml

The solution was equilibrated with carbon dioxide and pH was adjusted to 7.35. *Solution A* and *Solution B* were then mixed and equilibrated with carbon dioxide under the control of pH. Finally, the medium was sterile filtered.

Serum-enriched M199 with supplements

	For 100 ml
FCS	4 ml
Dexamethasone stock	100 µl
Insulin stock	10 µl
Penicillin/streptomycin stock	1 ml
M199 with supplements (stock medium)	to 100 ml

Penicillin/Streptomycin stock

	For 100 ml
Penicillin G (sodium salt)	0.64 g
Streptomycine sulfate	1.17 g
NaCl	0.9 g
ddH_2O	to 100 ml

The penicillin/streptomycin stock was sterile filtered, aliquoted and stored at –20°C.

Dexamethasone stock solution (100 µM)

	For 100 ml
Dexamethasone	3.92 g
0.9% NaCl	to 100 ml

Dexamethasone was first dissolved in 0.3 ml of ethanol and then adjusted with 0.9% NaCl to a final volume of 100 ml. The stock solution was then sterile filtered, aliquoted and stored at –20°C.

Insulin stock solution (10 µM)

	For 100 ml
Insulin	6 mg
BSA	100 mg
0.9% NaCl	to 100 ml

Insulin was dissolved at pH 2.5, neutrilized and then BSA was added. The stock solution was then sterile filtered, aliquoted and stored at –20°C.

3. 1. 2. Isolation of Liver Myofibroblasts

Out growth of Liver Myofibroblasts from primary culture of hepatocytes

LMFs were isolated by outgrowth from primary hepatocytes culture. 24 hours after plating, hepatocytes were washed in sterile PBS and new culture medium (serum-enriched DMEM with supplements) was then added. In the presence of DMEM and

serum hepatocytes were dying, whereas LMFs contaminating hepatocytes culture were proliferating. To eliminate dead hepatocytes, every two days the cultures were rinsed in PBS and medium was replaced. Further, cultured LMFs were grown until confluence and were then released from the culture dishes by trypsinization and replated at split ratio 1:3 or 1:4. Culture medium was replaced 2 days after passaging and then twice a week. Cells were cultured at 37^0C in 5% CO_2 atmosphere and 95% humidity. Cells were subcultured for several passages. Cells from passages 7 to 36 were used for further studies.

3. 1. 3. Treatment of Cultured Liver Myofibroblasts

Before the addition of stimuli, cultures of mice LMFs were washed in PBS and were then growth-arrested under serum reduced conditions by addition of DMEM supplemented with 0.3% FCS (serum-reduced medium, SRM) for 1 hour at 37^0C in 5% CO_2 atmosphere and 95% humidity, and thereafter cells were incubated in SRM supplemented with certain hormones and growth factors. After treatment, cell-free supernatants were collected and frozen at -20^0C, and cells were rinsed in cold PBS and immediately were used for RNA isolation and protein extraction. Alternatively, in order to study the phosphorylation of the signaling intermediates, cell starvation and treatment of cells were performed under serum-free conditions using serum-free DMEM (serum-free medium, SFM).

Solutions and Media for Isolation, Culture and Treatment of Liver myofibroblasts

Serum-enriched DMEM with supplements	Penicillin/streptomycin stock -1ml, FCS -15ml, L-Glutamine -1ml, DMEM (with low glucose) -100ml
Serum-reduced DMEM with supplements	Penicillin/streptomycin stock -1 ml, FCS -0.3 ml, L-Glutamine1 ml, DMEM (with low glucose) -100ml
Serum-free DMEM with supplements	Penicillin/streptomycin stock -1 ml, L-Glutamine -1ml, DMEM (with low glucose) -100 ml

3. 2. Molecular Biology Methods

3. 2. 1. Transformation of E. coli

Competent E. coli DH5α cells capable to take up linear or circular (plasmid) double stranded DNA were used for transformation. The competent bacteria (100 µl) were thawed on ice. Then 100 ng of plasmid DNA were added directly to the competent cells and the mixture was incubated on ice during 30 min. Cells were subjected then to heat shock by incubating at 42°C for 2 min and subsequently cooled down on ice for 2 min. Afterwards, 300 µl of SOC medium were added to the cells followed by 40 min incubation at 37°C under continuous shaking at 225 rpm. After cooling down on ice, transformed cells (50 µl) were spread over a LB-ampicillin agar dish and incubated for 18 hours at 37°C.

Media and solutions used for E. coli transformation

SOC medium

0.5% yeast extract, 2% bacto-trypton, 10 mM NaCl, 20 mM glucose, 10 mM $MgSO_4$, 10 mM $MgCl_2$, 2.5 mM KCl.

Luria Bertani (LB) medium

Bacto-trypton -10 g, Yeast extract -5 g, NaCl -5 g, ddH_2O, 1L. pH was adjusted with NaOH to 7.3, the medium was autoclaved and stored at 4°C. Prior to use, ampicillin was added to the medium at the final concentration of 50-100 µg/ml.

LB-ampicillin agar dishes

Bactoagar was added to the LB medium to a final concentration of 1.5% followed by autoclaving. Subsequently, the medium was cooled down to 55°C and ampicillin was added to the final concentration of 50 µg/ml. This medium was poured onto 10 cm sterile Petri dishes and left undisturbed for about 30 min to solidify. LB-Agar dishes were stored in the dark at 4°C.

Ampicillin stock solution

10% ampicillin solution for 10 ml was prepared and adjusted pH to 7.0 with HCl, and 500 µl aliquots was stored at −20°C.

3. 2. 2. Purification of Plasmid DNA

Purification of plasmid DNA from transformed bacteria was performed using EndoFree^{TM} Plasmid Maxi Kit (Qiagen). A single colony of transformed E. coli was scraped from LB-ampicillin agar dish and inoculated into 2 ml of ampicillin-containing LB medium, followed by incubation for 12 hours at 37°C with vigorous shaking at 300 rpm. This starter culture was diluted 1:1,000 in LB medium, and bacteria were grown at 37°C for 12-16 hours with vigorous shaking at 300 rpm followed by centrifugation at 6,000 x g for 15 min at 4°C. The supernatant was discarded and bacterial pellet was resuspended in 10 ml of P1 buffer containing RNase A. To lyse the cells, 10 ml of P2 buffer were added, mixed gentlely and incubated at 5 min at RT. Thereafter, 10 ml of chilled P3 buffer were added to the lysate that was transferred to the barrel of QIAfilter Cartridge and kept there for 10 min at RT. During this incubation, floating layer of the precipitate was formed on top of the lysate. Subsequently, the lysate was cleared by passing the liquid through the filter of the QIAfilter Maxi Cartridge using the plunger. After addition of 2.5 ml of ER buffer, the filtered lysate was incubated on ice for 30 min, and then was applied onto a Qiagen-tip 500 column equilibrated with 10 ml of QBT buffer that was followed by washing twice with 30 ml of QC buffer. For all subsequent steps endotoxin-free reagents and plastic ware were used. Plasmid DNA eluted with 15 ml of QN buffer was mixed with 10.5 ml of room-temperature isopropanol and centrifuged immediately at 15,000 x g for 30 min at 4°C. The supernatant was discarded and the DNA containing pellet was washed from precipitated salt with 5 ml of endotoxin-free, 70% ethanol, followed by centrifugation at 15,000 x g for 10 min. The supernatant was carefully decanted. The DNA pellet was air-dried for 5-10 min and then resuspended in a required volume (usually 100-500 µl depending on the amount of DNA) of endotoxin-free TE buffer. The concentration of DNA was measured by taking OD at 260 nm (DNA) and 280 nm (protein) was measured.

3. 2. 3. Restriction Digestion of Plasmid DNA

In order to isolate required cDNA from the whole plasmids, digestion of plasmid DNA with restriction enzymes was performed. Typically, preparative reaction contained 20 µg of plasmid DNA in a final volume of 60 µl. The amount of each component

required for restriction reaction was calculated in advance. The following chart was used as a guide:

RNase/DNase-free water (Ampuwa®)	to 60 µl
Reaction buffer, 10x	6 µl
Plasmid DNA	20 µg
Restriction enzyme I	100 U
Restriction enzyme II	100 U

In a sterile 1.5 ml tube placed on ice the above mentioned components were added one by one and the reaction mixture was then incubated at appropriate temperature (usually at 37°C) for 4 hours. Afterwards, the mixture was subjected to agarose gel electrophoresis. The following restriction enzymes and buffers were used for isolation of certain cDNA inserts in the current work:

cDNA of interest	Enzyme I	Enzyme II	Buffer
IGF-I	EcoRI	HindIII	B
IGF-IR	EcoRI	BamHI	B
IGF-II/M6-PR	EcoRI	BamHI	B
IGFBP-2	EcoRI	HindIII	B
IGFBP-3	ApaI	BamHI	A

3. 2. 4. Agarose Gel Electrophoresis of DNA

For preparation of 0.75% and 1.25% gels, 187.5 mg and 375 x g of low melting point agarose were mixed with 25 ml of 1x TAE buffer and dissolved by microwaving followed by addition of 8 µl of ethidium bromide (10 mg/ml). Then the agarose solution was poured onto the gel casting plate. During gel polymerization, the samples were prepared for loading. For analytical gels, 10 µl of respective DNA sample were mixed with 2 µl of 6x loading Dye Solution. For preparative gels, 60 µl of DNA sample (product of restriction digestion reaction) were mixed with 12 µl of 6x loading Dye Solution. In parallel, molecular weight DNA marker was also processed by mixing of 3 µl of the 123 kb DNA ladder, 7 µl of nuclease free water and 2 µl of 6x

Loading Dye Solution. After polymerization, the gel was placed into an electrophoresis chamber filled with 1x TAE buffer, the samples and molecular weight marker were loaded (12 µl per well), and the electrophoresis was carried out at a constant voltage of 60 V. Finally, DNA bands on the ethidium bromide-stained gel were visualized by ultraviolet shadowing and cDNA of interest was cut out with a sharp scalpel from the gel, transferred to a clean, preweighed reaction tube and saved at -20^{0}C for further manipulations.

20x Tris/Acetate/EDTA (TAE) buffer

	For 1 l	Final concentration
Tris base	122 g	1 M
Sodium acetate	32 g	0.4 M
EDTA	14 g	40 mM
ddH_2O	to 1 l	

After EDTA was completely dissolved, pH was adjusted with acetic acid to 7.4; the buffer was autoclaved and stored at RT.

1x TAE buffer

	For 1 l
20x TAE buffer	50 ml
ddH_2O	to 1 l

3. 2. 5. Agarose Gel DNA Extraction

To isolate cDNA fragments from excised TAE agarose gel portions for subsequent random primed labelling, the agarose gel DNA extraction kit (Roche) was used. The procedure was started by addition of agarose solubilization buffer to the reaction tube containing electrophoretically separated DNA fragment. Usually, 3 µl of agarose solubilization buffer was applied per 1 mg of gel. Subsequently, 10 µl of the resuspended silica suspension were added to the tube. The sample was incubated at 60 ^{0}C for 10 min and vortexed every 2 min. After centrifugation for 30 sec at maximal speed in a table top centrifuge, the supernatant was discarded, and the matrix containing DNA was resuspended by addition of 500 µl of nucleic acid binding

buffer. After centrifugation (30 sec, maximal speed) the supernatant was discarded, and the pellet was washed with 500 µl of washing buffer followed by centrifugation and discarding the supernatant, and the pellet was air-dried for 15 min at RT. Afterwards, 25 µl of Tris-HCl were applied on the pellet and incubated at 60 ^{0}C for 10 min, and vortexed every 2 min. After centrifugation (30 sec, maximal speed) the supernatant containing the eluted DNA was transferred to a new reaction tube. Typically, two elution cycles were performed (2 x 25 µl). Finally, the DNA concentration was measured, and the extracted DNA insert was further stored at -20^{0}C.

3. 2. 6. Radioactive cDNA Labelling

Radioactive random primed DNA labelling was performed to label specific cDNA fragments restricted from the respective plasmids coding for rat IGFBP-2, IGFBP-3, IGF-I, IGF-IR and IGF-II/M6-PR.

DNA Labelling by Random Priming Reaction

Random primed DNA labelling was performed using NEBlot® Kit (New England Biolabs) according to manufacturer's instructions. 50-100 ng of template DNA were dissolved in nuclease free water to the final volume of 33 µl. The DNA was denatured by heating at 95°C for 5 min and subsequently cooled down on ice for 5 min. The following reagents were added to the DNA in the indicated order: 5 µl of 10x labelling buffer (includes Random Octadeoxyribonucleotides), 2 µl of dATP, 2 µl of dTTP, 2 µl of dGTP, 5 µl of [α-^{32}P]-dCTP (50 µCi), 1 µl of DNA polymerase I − Klenow Fragment (5 units). The mixture was incubated at 37°C for 1 hour; the reaction was then stopped by addition of 5 µl of 0.2 M EDTA, pH 8.0.

Purification of Labelled DNA

Synthesized labelled cDNA probe was separated from non-incorporated nucleotides by gel filtration on Sephadex® G-50 using Pharmacia NICK Column (Pharmacia Biotech). Initially, column was equilibrated with 3 ml of 1x TE buffer, pH 8.0. After the entire volume of buffer had entered the gel, random priming reaction mixture was applied onto the column, followed by addition of 400 µl of 1x TE buffer. The flow-through was collected in the tube placed under the column and kept for further measurement of radioactivity. A new reaction tube was placed under the column and

the purified labelled DNA was eluted with 400 µl of 1x TE. Finally, the β-radioactivity in the obtained sample was measured, and labelled DNA was either immediately used for hybridization or stored at −20°C for further experiments.

3. 2. 7. Isolation of total RNA

Isolation of total cellular RNA was performed from monolayers of cultured mice liver cells was according to the method of Chirgwin (Hoeflich et al., 1999). Culture dishes with both frozen and alive liver cells were placed on ice and 3 ml of GITC-buffer with freshly added β-mercaptoethanol (10 µl per ml). Plates were scraped with a disposable scraper and lysate was transferred to a prechilled RNase-free 15 ml tube, and homogenized by passing through 24G injection needle connected to the syringe. This cell lysate was carefully layered onto 2ml of CsCl buffer already added in 5-ml polyallomer ultracentrifuge tube. The samples were centrifuged overnight (14-20 h) at 35,000 rpm in a Kontron TST55 rotor at 20°C. Thereafter, the supernatants were carefully removed and transparent gelatin-like RNA pellets were reconstituted in 200 µl of RNase-free water. This mixture was transferred to sterile 1.5 ml reaction tubes containing 2M sodium acetate, pH 5.4 (20 µl per sample). Further, 400 µl of absolute cold ethanol was added and kept overnight at –20°C. RNA precipitates were sedimented by centrifugation (12,000 rpm, 30 min, and 4°C). Supernatants were discarded and pellets were washed with 150 µl of ice-cold 70% ethanol to remove all traces of sodium acetate. After subsequent centrifugation (12,000 rpm, 30 min, 4°C) the supernatants were discarded and the pellets were air-dried at RT. Finally, the pellets were reconstituted in 100 µl of RNase-free water. The concentration of the RNA was measured by taking OD at 260 and OD at 280 nm by the spectrophotometer (GeneQuant II, Pharmacia Biotech). The ratio between OD at 260 nm and OD at 280 nm characterized the RNA purity.

GITC buffer

	For 200 ml	Final concentration
Guanidine isothiocyonate	94.53 g	4 M
0.25 M sodium citrate	20 ml	25 mM
N-lauroylsarcosyl	1 g	0.5%
RNase-free H_2O	to 200 ml	

The solution was sterile filtered and stored in the dark at 4°C. β-mercaptoethanol was added briefly before RNA isolation at the quantity of 10 µl per 1 ml of GITC buffer.

CsCl buffer

	For 200 ml	Final concentration
Cesium chloride	192 g	5.7 M
0.25 M sodium citrate	20 ml	25 mM
0.5 M EDTA	40 ml	100 Mm
RNase-free H_2O	to 200 ml	

pH was adjusted with 0.25 M citric acid to 7.5; the solution was sterile filtered and stored at RT.

3. 2. 8. Northern Blot Analysis

Northern blot analysis is a technique for quantitative evaluation of gene expression. Under RNase-free conditions, total RNA is separated in a denaturing formaldehyde/ agarose gel, blotted by capillary transfer to a nylon membrane and fixed by UV cross-linking. Specific transcripts are identified by autoradiography after hybridization with a specific radiolabelled cDNA probe.

Preparation of RNA Samples

10-15 µg of total RNA were mixed with 7.5 µl of sample buffer and denatured by heating at 65°C for 10 min and cooled down on ice and centrifuged at 10,000 rpm, 1 min, RT). Finally, each sample was mixed with 3 µl of loading buffer, centrifuged (10,000 rpm, 1 min, RT) and loaded onto the gel.

Formaldehyde/Agarose Gel Electrophoresis of RNA

For preparation of 1% denaturing formaldehyde/agarose gel, 1 g of agarose was mixed with 72.2 ml of RNase-free water (Ampuwa®), dissolved by microwaving and then slightly cooled down to approximately 70°C. Subsequently, 10 ml of 10x running buffer 16.7 ml of 37% formaldehyde, and 8 µl of ethidium bromide (10 mg/ml) were added as well. The solution was mixed, poured onto the casting plate and left for polymerization under the fume hood. Thereafter, prepared RNA samples were loaded onto the gel, and the electrophoresis was performed at a constant voltage of 80 V. After electrophoresis, equal loading and integrity of RNA were evaluated by ultraviolet shadowing in the UV transilluminator built-in Eagle Eye™ system (Stratagene), the gel was then photographed and immediately subjected to RNA transfer.

RNA Transfer to Nylon Membrane

Separated RNA samples were transferred to nylon membranes using capillary transfer system. A plastic tray was filled with 500 ml of 20x SSC buffer and covered with a glass plate. Oblong Whatman 3MM filter paper was soaked in 20x SSC buffer and raped over the glass plate with edges submerging in the buffer. The gel was carefully placed on the filter and covered with 2x SSC buffer. Bubbles were carefully removed. Four strips of paraffin were placed in contact with the gel edges to prevent direct absorption of buffer by paper towels as well as to prevent evaporation of buffer. Wetted piece of nylon membrane was placed on the exposed gel surface followed by placing of two additional sheets of Whatman 3MM paper wetted in 2x SSC buffer. Finally, paper towels were stacked on top of Whatman papers to a height of approximately 8 cm, covered with another glass plate and pressed with a 1 kg blotting weight. The transfer was carried out overnight. After transfer, RNA was cross-linked to the membrane by ultraviolet waves in the Stratalinker™ 180 system (Stratagene).

Hybridization of RNA with Radiolabelled cDNA Probe

After cross-linking, the nylon membrane was rinsed with RNase-free water and placed into the hybridization tube. To prevent unspecific binding, the membrane was first prehybridized in QuikHyb® hybridization solution at 68°C for 2 hours. Radiolabelled probe (1,500,000-3,000,000 cpm/ml) was mixed with a double volume

of fish sperm DNA and denatured at 95°C for 5 min. After cooling down on ice, the DNA probe was applied into QuikHyb® solution inside the hybridization tube. The tube was placed back in the hybridization oven, and hybridization was carried out at 68°C for 2 hours. After hybridization, the membrane was washed once in 2x SSC/0.1% SDS for 10 min at RT, then twice in 0.1x SSC/0.1% SDS for 15 min at 43^0C (IGF-IR, IGF-II/M6-PR cDNA), 55 ^{0}C (IGF-I, cDNA) or 60^0C (IGFBP-2, IGFBP-3, cDNA) and, finally, twice in 2x SSC/0.1% SDS for 10 min at RT. To visualize 28S rRNA, the membrane was prehybridized in QuikHyb® hybridization solution at 42°C for 2 hour followed by overnight hybridization at 42°C with the labelled oligonucleotide specific for 28S rRNA and washed three times in 2x SSC/0.1% SDS for 10 min at 37^0C. After washing, membranes were wrapped in a saran wrap, placed in X-ray film cassette and autoradiographed during various exposure times.

3. 2. 9. cDNA Amplification Method

The first strand cDNA synthesis

Total cellular RNA isolated from mice liver myofibroblasts was reverse transcribed into cDNA using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen™ GmbH, Karlsruhe, Germany). A quantity (1 µg) of total RNA and 1 µl (40 U/µl) of RNase inhibitor (Roche, Mannheim, Germany) were then mixed with RNase inactivated sterile double distillated water to the total volume of 10 µl and heated at 65°C for 10 min followed by the transfer on ice and brief centrifugation. The samples were then mixed with 8 µl of first strand buffer (5x), 8 µl of 40 mmol deoxy nucleotide triphosphates mix (40 mmol each dATP, dGTP, dCTP and dTTP at neutral pH), 4 µl of 0.1 M DTT, 2 µl (200 U/µl) of Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT) and 8 µl of pd (T) 5-PO primers (Amersham Bioscience) to achieve a final reaction volume of 40 µl. The RT reaction was carried out at 37°C for 1 hour followed by inactivation of reverse transcriptase at 94°C for 5 min and brought up to final volume of 156.25 µl.

Quantitative real-time PCR

The Quantification of mRNA expression of extracellular matrix proteins was evaluated by quantitative real-time PCR using ABI PRISM® 7000 Sequence Detection System (AB Applied Biosystems, Darmstadt, Germany) and Syber® green as dsDNA specific fluorescent dye. Amplification mixes contained 2.5 µl (16 ng) first

strand cDNA solution, 13.5 µl 2x Platinum® SYBR® Green qPCR superMix-UDG contained ROX reference dye (Invitrogen™), 1.5 µl (7.5 pmol) of each forward and reverse primers, and brought up to final reaction volume of 25 µl with RNase inactivated sterile double distillated water.

The primer sequences used were as follows:

mouse IGFBP-2,	sense: 5'-GCG GGT ACC TGT GAA AAG AG-3'
	antisense: 5'-CTG CTA CCA CCT CCC AAT AT-3'
mouse IGFBP-3,	sense: 5'-CAG GCA GCC TAA GCA CCT AC-3',
	antisense: 5'-GCA TGG AGT GGA TGG AAC TT-3'
mouse IGF-IR,	sense: 5'-CCG CGC CAG TTT TGA TG-3'
	antisense: 5'-AGG CAA GGC CCT CTC GTT-3'
mouse IGF-II/M6-PR,	sense: 5'-TGA ACT CCC ATC CCA TAT TCA CT-3'
	antisense: 5'-GGT CCC TTA TTG AGC AAG CCT-3'
mouse IGF-I,	sense: 5'-ATG AGT GTT GCT TCC GGA GCT-3'
	antisense: 5'-AGC GGG CTG CTT TTG TAG G-3'
mouse fibulin-2,	sense: 5'-TGC ACA TAT CTT CCG CAT CG-3'
	antisense: 5'-TGA GTC TGC GTG TGA CGA AGT-3'
mouse fibronectin 1,	sense: 5'-TGG CTG AAG TCG CAA GGA AA-3'
	antisense: 5'-TCC CCG TTT GAA TTG CCA C-3'
mouse β-actin,	sense: 5'-TGT TAC CAA CTG GGA CGA CA-3'
	antisense: 5'-GGG GTG TTG AAG GTC TCA AA-3'

The amplification was performed at 95-60°C for 45 cycles. The relative expression of mRNA was expressed in percentage of control after normalization against mouse endogenous β-actin.

3. 3. Immunochemical and Biochemical methods

3. 3. 1. Protein Extraction from Cultured Cells

Preparation of Cell Lysates

The whole procedure was performed at 4°C to prevent proteolytical degradation of proteins. After respective treatment, cells were washed with ice-cold PBS, placed on ice and then solubilized in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% CHAPS, pH 7.4) supplemented with protease and phosphatase inhibitors

(500 and 1500 µl of buffer per 100 mm and 150 mm dish, respectively) followed by scraping and transferring lysate to prechilled 2.0 ml reaction tubes on ice, and kept for 30 min and vortexed every 5 min. For better homogenization cell lysates were passed through 24G injection needle connected to the syringe followed by centrifugation at 10,000 x g for 10 min at 4°C, and the supernatants containing cellular proteins were then transferred to new 2.0 ml reaction tubes. Protein concentrations were determined by BCA protein assay (Pierce) using bovine albumin as a protein standard.

Measurement of Protein Concentration

Measurement of protein concentration was performed by using BCA TM Protein Assay Kit (Pierce) according to manufacturer instructions. To measure protein concentration, 25 µl of each standard or unknown sample were applied into its own microplate well in triplicate. Subsequently, 200 µl of working reagent (mixed 50 parts of BCA reagent A and 1 part of BCA Reagent B) were added to each well, and the plate was gently and thoroughly shaken for 30 sec followed by incubation at 37^0C for 30 min. The plate was then cooled down to RT, and the absorbance was measured at 570 nm on a plate reader.

3. 3. 2. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A tris/glycine SDS polyacrylamide gel system was performed according to the method of Laemmli (Laemmli, 1970). For [^{125}I]-IGF-I ligand blot and immunoblotting analysis, gels of standard sizes were cast, and proteins were electrophoretically separated in the standard dual cooled gel electrophoresis unit Hoefer SE 600 (Amersham Biosciences). The units were assembled according to manufacturer's instructions. In general, a discontinuous gel system was used, which was composed of 4% (polyacrylamide concentration) stacking gel and resolving gel with one of the following percent (%).

Resolving and stacking gels were prepared as described below.

Resolving Gel

	6%	10%	12.5%
Rotiphorese Gel 30	6.3 ml	10.5 ml	13.1 ml
Buffer for resolving gel	7.5 ml	7.5 ml	7.5 ml
10% APS	250 µl	250 µl	250 µl
TEMED	25 µl	25 µl	25 µl
ddH_2O	to 30 ml	to 30 ml	to 30 ml

Stacking Gel

	4%
Rotiphorese Gel 30	1.3 ml
Buffer for stacking gel	2.5 ml
10% APS	100 µl
TEMED	10 µl
ddH_2O	to 10 ml

Prepared resolving gel solution was poured inside the gel sandwich and overlaid carefully with water to create a barrier for oxygen, which inhibits polymerization. After polymerization (about 30 min at RT) the water was aspirated, and the monomer solution of stacking gel was applied over the polymerized resolving gel. The wells were formed by appropriate comb inserted into the sandwich. The gel was polymerized for approximately 20 min. Subsequently, the comb was removed, the wells were rinsed with water, and traces of water were carefully aspirated. The samples were loaded into the wells (100 µl per well in standard gel and 30 µl per well in mini-gel) and were carefully covered with cathode buffer. Thereafter, precooled cathode and anode buffers were poured into the upper and lower buffer chamber, respectively. The gel electrophoresis unit was then fully enclosed and connected to a suitable power supply. Proteins were size-fractionated at a constant current (8 mA per gel in the standard dual cooled gel electrophoresis unit Hoefer SE 600, and 25 mA per gel in the mini-vertical gel electrophoresis unit Hoefer Mighty Small II).

Rainbow™ colored protein marker (Amersham Biosciences) was used as a molecular weight standard.

3. 3. 3. Electrophoretic Transfer of Proteins

After size-fractionation of proteins by SDS-PAGE, electrophoretic transfer was performed essentially as described by Towbin (Chirgwin et al., 1979). Gel was delicately transferred on to the transblot sandwich according to the manufacturer's instructions. The assembled transfer cassette was inserted into the transblot cell filled with precooled transfer buffer. Proteins were then electroblotted at a constant current of 350 mA for 2 hours at 4^0C.

3. 3. 4. Iodination of IGF-I

IGF-I was iodinated by the chloramines-T method to a specific activity of about 60-80 μCi/μg. In brief, 1 mCi $Na^{125}I$ (usually 10 μl) was added to the reaction tube containing 10-15 μg of IGF-I. Iodination reaction was started by addition of 10 μl of 0.4 mg/ml chloramine-T working solution and stopped after 10-15 seconds by addition of 20 μl of 12.6 mg/ml sodium bisulphite. The reaction mixture was then diluted with 100 μl chromatography buffer (buffer A) and loaded into HPLC column equilibrated with buffers A and B in order to separate [^{125}I]-labelled IGF-I from unreacted product. The column was then run at a flow rate of 1.5 ml/min and 1.5 ml fractions were collected. Finally, the gamma-radioactivity of each sample was measured, and fractions with highest radioactivity rates were selected and stored at -20^0C. The quality of [^{125}I]-labelled IGF-I was checked by ligand blot analysis with human and rat serum samples.

<u>0.4M sodium phosphate buffer</u>

	For 100 ml	Final concentration
di-Sodium hydrogen phosphate dihydrate	7.12 g	0.4M
Sodium dihydrogen phosphate	4.66 g	0.4M
ddH_2O	to 100 ml	

pH was adjusted to 7.4. The solution was stored at RT.

<u>Chloramine-T working solution</u>

	For 1 ml	Final concentration
Chloramine-T	4 mg	4 mg/ml
0.4M sodium phosphate buffer	1 ml	

The solution was freshly prepared before iodination.

Sodium bisulphite working solution

	For 1 ml	Final concentration
Sodium bisulphate	12.6 mg	12.6 mg/ml
0.4M sodium phosphate buffer	1 ml	

The solution was freshly prepared before iodination.

Buffer A

	For 1 l	Final concentration
Trifluoroacetic acid	1 ml	0.1%
HPLC H_2O	to 1 l	

The solution was stored at RT.

Buffer B

	For 1 l	Final concentration
Acetonitril	700 ml	70%
Trifluoroacetic acid	1 ml	0.1%
HPLC H_2O	to 1 l	

The solution was stored at RT.

3. 3. 5. [^{125}I]-IGF-I Ligand Blot Analysis

To determine which IGFBPs are secreted by cultured mice liver myofibroblasts, samples of conditioned media (CM) were analyzed by [^{125}I]-IGF-I ligand blot analysis according to (Towbin et al., 1979), with slight modifications. For SDS-PAGE, 750 µl culture supernatant was precipitated with 1200 µl of absolute ethanol and kept at -80^{0}C followed by centrifugation (14,000 rpm, 15 min, 4^{0}C) in a bench-top centrifuge. The supernatant was then discarded, the pellet was air-dried, resuspended in 100 µl of non-reducing sample electrophoresis buffer, heated at 95^{0}C for 5 min and loaded onto 10% gel. Electrophoretic separation of proteins and their transfer to

nitrocellulose membranes were performed as described above. Molecular weight standard was processed in parallel. After transfer, the nitrocellulose membranes were blocked in Tris-buffered saline supplemented with 1% fish gelatine at RT for 2 hours with slight agitation, and were then incubated with 8,000,000 cpm of [^{125}I]-IGF-I diluted in 30 ml of washing buffer at 4^{0}C overnight. Finally, the membranes were washed in washing buffer (three times, 10 min on each occasion), air-dried, wrapped and exposed to X-ray films at -80^{0}C for various exposure times.

3. 3. 6. Western Blot Analysis

25 µg of proteins extracted from whole cell lysates of cultured mice liver myofibroblasts were denatured in reducing sample buffer by boiling at 95°C and further subjected to 6% SDS-PAGE. Electrophoretic separation of proteins and their transfer to nitrocellulose membranes were performed as described above. After transfer, the membrane was incubated overnight on rocking platform with blocking buffer at 4°C. Subsequently, the blot was incubated with primary antibody diluted in antibody dilution buffer for 3 hours at RT, followed by washing in TBST buffer (six times, five min on each occasion). Thereafter, the membrane was incubated with HRP-conjugated secondary antibody diluted in antibody dilution buffer for 1 hour at RT. The membrane was then washed as before and incubated for 5 min at RT with working solution of SuperSignal® West Pico Chemiluminescent Substrate (Pierce). The membrane was placed between two sheets of transparent plastic films and exposed to X-ray films during different time intervals.

Primary antibodies were used in the following dilutions

Anti-IGF-IRβ (1:100)

Secondary HRP-conjugated antibodies were used in the following dilutions

Peroxidase-conjugated swine anti-rabbit Ig (1:5,000)

3. 3. 7. Immunocytochemistry

Mice LMFs cultured on Lab-Tek chamber slides were washed in PBS and then fixed in 4% paraformaldehyde (20 minutes) at RT. Then the cells were washed with PBS. The cells were permeabilized with methanol (5 min), again washed in PBS, air-dried and frozen at -20^{0}C. To block endogenous peroxidases, after washing in PBS cells

were incubated in Dako Real™ peroxidase-blocking solution for 10 min at 37^{0}C. Cells were rinsed again with PBS, and nonspecific binding was blocked by incubation with mixture of bovine calf serum (1% BSA in PBS) and swine serum in 1:10 ratio for 30 minutes. The slides were washed with PBS, placed in a humidified chamber, and then cells were incubated with primary antibodies for overnight at 4^{0}C. Dilutions of primary antibodies are indicated below. Thereafter, cells were washed three times for 5 min in PBS followed by incubation with secondary peroxidase-conjugated antibodies for 60 min at RT also performed in a humidified chamber. Secondary antibodies were preabsorbed with rat serum to avoid nonspecific binding. For this purpose, 10 µl of rat serum were added to 50 µl of secondary antibody and incubated for 60 min at 37°C, followed by centrifugation for 5 min at maximum speed in a bench-top centrifuge to pellet precipitate. Afterwards, 50 µl of supernatant were transferred to a new reaction tube and mixed with 350 µl of PBS and 600 µl of γ-globulin-free serum. After incubation with secondary antibodies for 60 min, cells were washed as described above, and immune complexes were visualized by diaminobenzidine, which upon oxidation forms a brown end-product at the site of the target antigen. For this purpose cells were incubated with the mixture of diaminobenzidine (0.5 mg/ml) and H_2O_2 (0.01%) in 1:50 for 10 minutes at RT. Finally, cells were washed 2 times for 5 min in distilled water and counterstained in hematoxilin solution for 2 min at RT. After additional washing steps (2 x 5 min in slightly warm a tap water), the slides were covered with Kaiser's glycerol gelatine and mounted with cover-slips. Images were acquired using a light microscope (Axioskop, Carl Zeiss), digital photocamera Canon EOS D60 (Canon) and software Remote Capture 2.5 (Canon).

Diaminobenzidine working solution

	For 100 ml	Final concentration
Diaminobenzidine	50 mg	0.5 mg/ml
33% hydrogen peroxide	33 µl	0.01%
PBS	to 100 ml	

The solution was prepared briefly before use. First, diaminobenzidine was dissolved in PBS by stirring for 60 min at RT in the dark. The solution was filtered, and, finally, hydrogen peroxide was added.
Primary antibodies were used in the following dilutions:

Mouse monoclonal anti-SMAα	1:250
Rabbit anti-bovine collagen-3 polyclonal antiserum	1:100

Secondary HRP-conjugated antibodies were used in the following dilutions:

Peroxidase-conjugated rabbit anti-mouse Ig	1:200
Peroxidase-conjugated swine anti-rabbit Ig	1:200

3. 3. 8. Determination of DNA Synthesis

BrdU Labelling and Detection

DNA synthesis in cultures of mLMFs was evaluated by BrdU Labelling and Detection Kit III (Roche). mLMFs were plated on 96-well microtiter plates and cultured for 2 days. After starvation in serum-reduced DMEM (SRM) for 2 hours, cells were incubated with certain growth factors (IGF-I (0.1, 1.0, 10, 100 nM/L), PDGF-BB (10 ng/ml) and TGF-β (10 ng/L)) diluted in SRM for 36 hours. Control wells were incubated with SRM alone. Cells were labelled with 10 µmol/L of BrdU for 36 hours. Later, cells were washed in culture medium supplemented with 10% serum, fixed with precooled ethanol/HCl solution at -20^{0}C for 30 min, washed again, and then incubated with nucleases (100 µl per well) at 37^{0}C for 30 min to digest partially cellular DNA. After washing, wells were incubated with monoclonal HRP-conjugated anti-BrdU Fab-fragments (100 µl per well) at 37^{0}C for 30 min, rinsed three times in washing buffer, the conjugate bound to an incorporated BrdU was visualized by soluble chromogenic substrate (ABTS substrate supplemented with enhancer; 100 µl per well), and the absorbance was measured using an ELISA reader at 405 nm with a reference wavelength at 490 nm.

Thymidine incorporation Method

DNA synthesis in mLMFs was assessed by incorporation of [^{3}H] thymidine. In brief, for [^{3}H] thymidine incorporation culture plates containing mLMFs (50000 cells/ml) were washed twice with PBS and growth arrested in 1ml of SRM for 1 hour followed by incubation with 1 µCi [^{3}H] thymidine diluted in 1ml of SRM per plate in the presence or absence of IGF-I (10 nmol/L) for 6 and 12 hours. Cells were washed 3

times with PBS and trypsinised at 37^0 C followed by collection of cells and centrifugation. Cells were then mixed with 1 ml of 10% Trichloro acetic acid (TCA). This cell suspension was passed through the filter, and that was followed by passing of 0.5 ml of 5% TCA five times and 0.5 ml of ethanol five times through the filter. Filter was kept for drying, placed in 20 ml of scintillation liquid and measured β-radioactivity.

3. 3. 9. Statistical Analysis

All experiments were replicated two- to three times. Autoradiographs of Western ligand, Western immunoblot and Northern blots were scanned (BIO-RAD, Hercules, CA). After background subtraction, densitometry of individual bands was analyzed by ImageJ software (Version 1.34 s, NIH, Bethesda) according to the instructions of the manual. The relative densities of the bands were expressed as a percentage of control. The proliferation assays were performed in triplicates using at least three individual mLMF isolations. Means ± standard deviation (SD) were indicated relative to the control. Statistically significant differences of experiments with multiple comparisons were determined by ANOVA.

4. Results

4. 1. Characterization of LMFs

In addition to hepatic stellate cells (HSCs), liver myofibroblasts (LMFs) are described as a second resident cell population of fibroblastic lineage involved in liver fibrogenesis. Mouse LMFs (mLMFs) were isolated from livers of wild type (wt) and IGFBP-2 transgenic (IGFBP-2 (+/-)) mice by outgrowth from primary culture of mouse hepatocytes. Outgrowth of mLMFs was achieved when serum-enriched medium was added to hepatocytes, which are normally cultivated under serum-free conditions. Within 5-10 days, mLMFs became predominant in these cultures and formed a confluent cell layer after prolonged cultivation followed by passing of cells for subsequent studies.

mLMFs in culture demonstrated a fibroblast-like morphology characterized by a spindle-shaped appearance or a myofibroblast-like morphology characterized by a cross-striated morphology. Further cytological characterization was done by immunostaining of mLMFs for cytoskeletal and extracellular matrix proteins such as smooth muscle α-actin (SMAα) and collagen-3. Both wt and IGFBP-2 (+/-) mLMFs displayed immunoreactivity to collagen-3 and SMAα. The immunoreactivity of collagen-3 and SMAα was appeared to be more in wt mLMFs than in IGFBP-2 (+/-) mLMFs **(Fig. 6)**.

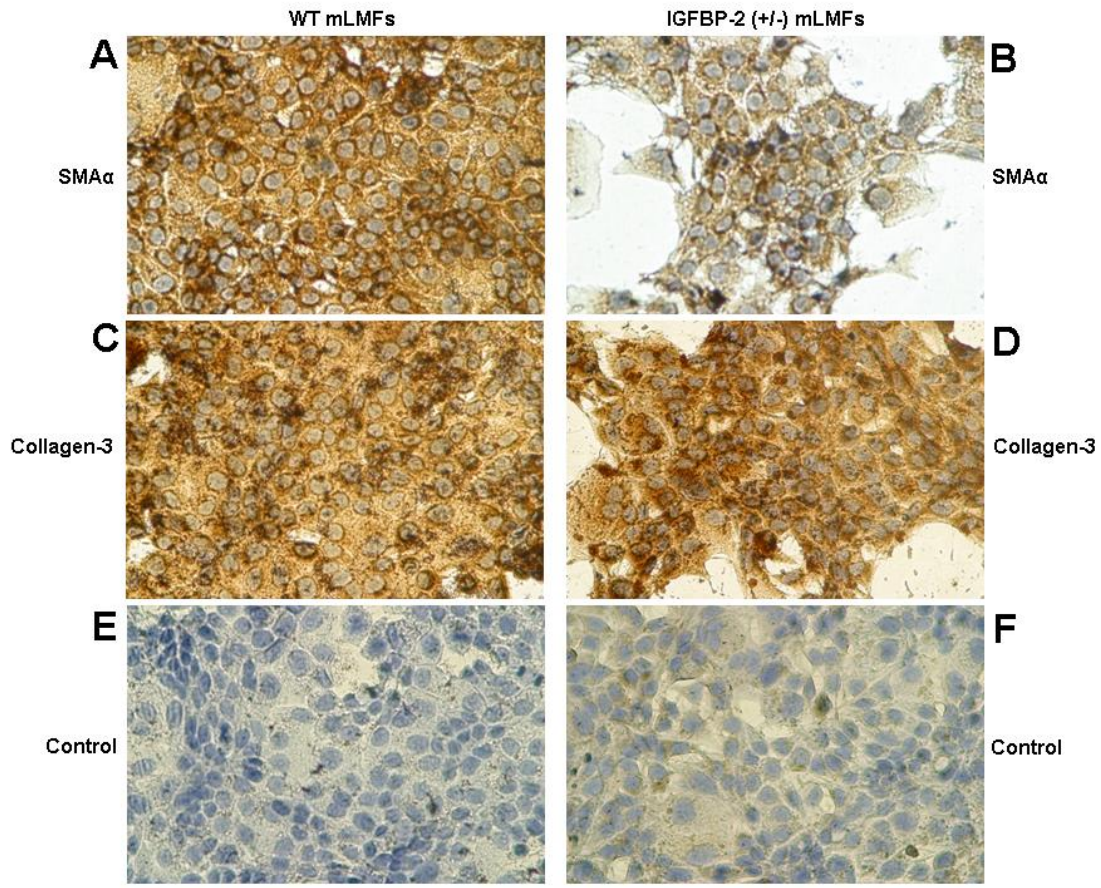

Figure 6. Immunocytochemical detection of smooth muscle α-actin (SMAα) and collagen-3 in mLMFs. Antibodies against SMAα in wt **(A)** and IGFBP-2 (+/-) mLMFs **(B)**, and collagen-3 in wt **(C)** and IGFBP-2 (+/-) **(D)** mLMFs were used, detected by peroxidase-labelled second antibodies. Controls were obtained by omitting primary antibodies in wt **(E)** and IGFBP-2 (+/-) **(F)** mLMFs. Original magnification x 20 **(A, B, C, D, E, F)**; Immunoreactive material was detected by the peroxidase staining protocol.

4. 2. Expression of IGF axis components in wt and IGFBP-2 (+/-) mLMFs

The mRNA expression of IGF axis components such as IGF, IGFBPs and IGF receptors was evaluated by Northern blot and quantitative real-time PCR analysis of total RNA isolated from wt and IGFBP-2 (+/-) mLMFs at different time points of culture (days 2 to 5). Northern blot analysis of total RNA from both wt and IGFBP-2 (+/-) mLMFs revealed messenger RNA (mRNA) species of IGFBP-2 at 1.6 kb and of IGFBP-3 at 2.5 kb **(Fig. 7A)**. The expression of IGFBP-2 mRNA transcripts were increased in wt (2.08 ± 1.1-fold increase in day 5 cultures relative to day 2 cultures) (soild bars) and IGFBP-2 (+/-) (1.13 ± 0.06-fold increase) (open bars) mLMFs with time of culture. In IGFBP-2 (+/-) mLMFs (open bars), an approximately four to five-fold increased IGFBP-2 mRNA expression was observed compared with wt mLMFs (solid bars) that was significantly different **(Fig. 7B)**. In contrast, expression of

IGFBP-3 mRNA was reversibly decreased in IGFBP-2 (+/-) mLMFs (0.41 ± 0.19-fold decrease in day 5 cultures relative to day 2 cultures) (open bars) whereas IGFBP-3 mRNA transcripts were increased in wt mLMFs (6.47 ± 2.99-fold increase in day 5 cultures compared to day 2 cultures) (solid bars) from days 2 to 5 **(Fig. 7C)**.

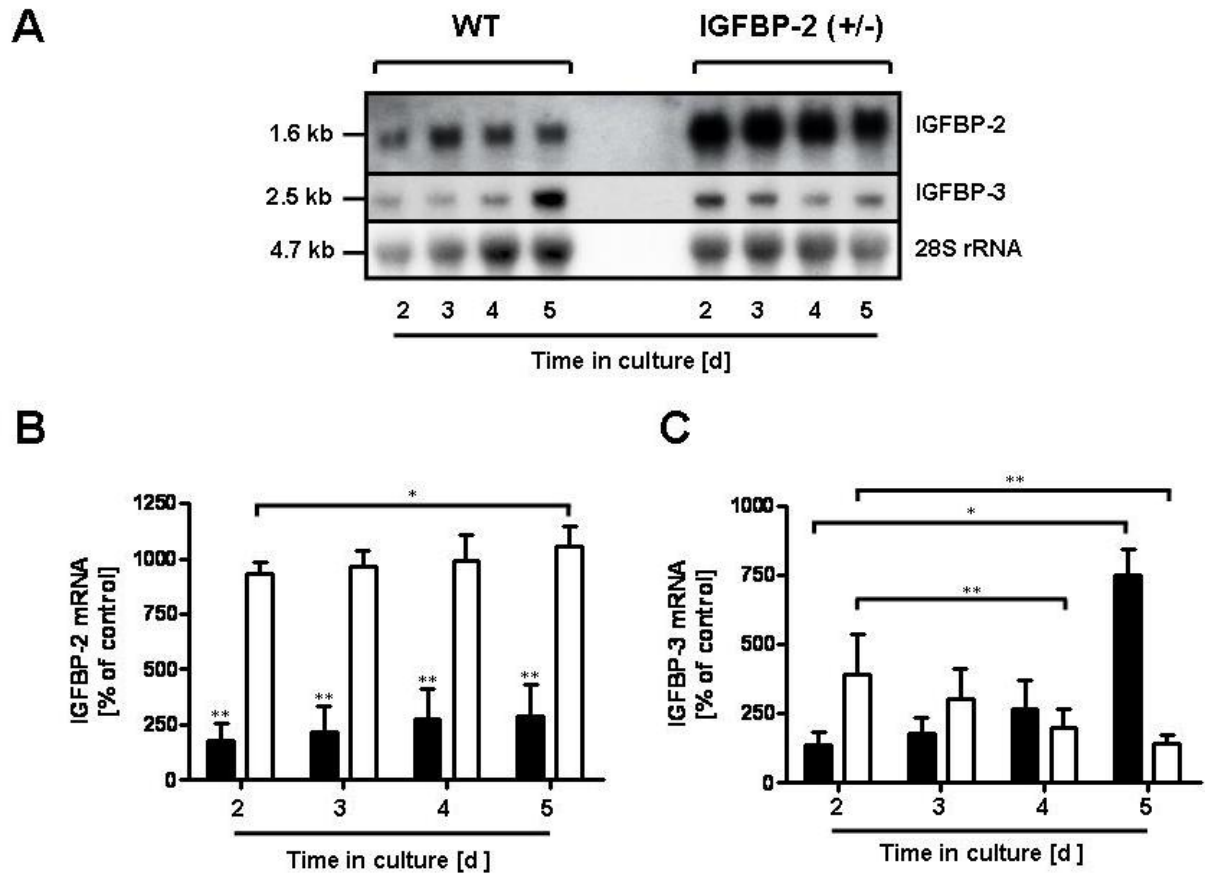

Figure 7. Expression of insulin-like growth factor binding protein (IGFBP) -2 and -3 mRNA in mLMFs analysed by Northern blotting. 10-15 µg of total RNA isolated from wt and IGFBP-2 (+/-) mLMFs at days 2 to 5 of culture were separated by 1% agarose gel electrophoresis, transferred to nylon membranes, and hybridized with ^{32}P-labeled complementary DNA (cDNA) specific for rat IGFBP-2 and -3 mRNA. Equal loading of RNA was demonstrated by rehybridization of 28 S rRNAs with a radiolabeled oligonucleotide cDNA after stripping of the membranes. **A:** Representative Northern blots of IGFBP-2 and -3 mRNA in wt and IGFBP-2 (+/-) mLMFs. The sizes of the hybridization bands (in kb) were indicated on the left. **B, C:** Densitometric analysis of IGFBP-2 mRNA in wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs, and IGFBP-3 mRNA in wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs, respectively. Hybridization signals of IGFBP-2 and -3 mRNA were densitometrically analysed and indicated as percent increase or decrease (means ± SD) compared with the respective wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs at day 2 of culture (n = 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences in IGFBP-2 and -3 mRNA expression compared with the respective wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs at day 2 of culture were indicated over the horizontal lines: *, $P < 0.05$; **, $P < 0.01$ (Student's t test). Statistically significant differences in IGFBP-2 mRNA expression between wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs relative to the respective cultures of wt mLMFs (solid bars) (days 2 to 5) were given over the bars: **, $P < 0.01$ (Student's t test).

Quantitative real-time PCR analysis of total RNA from wt and IGFBP-2 (+/-) mLMFs revealed expression of IGFBP-2 and -3 mRNA in wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs during different time points of culture (days 2 to 5). IGFBP-2 mRNA transcripts were pronounced in wt (1.62 ± 3.74-fold increase in day 5 cultures compared to day 2 cultures) (soild bars) and IGFBP-2 (+/-) (2.18 ± 2.10-fold) (open bars) mLMFs. Moreover, an approximately four to five-fold increased expression of IGFBP-2 mRNA was detected in IGFBP-2 (+/-) mLMFs (open bars) compared with wt mLMFs (solid bars) that was significantly different **(Fig. 8A)**. In contrary, IGFBP-3 mRNA levels were declined in IGFBP-2 (+/-) mLMFs (0.76 ± 0.65-fold decrease in day 5 cultures relative to day 2 cultures) (open bars) whereas expression of IGFBP-3 mRNA was increased in wt mLMFs (5.4 ± 4.51-fold increase in day 5 cultures compared to day 2 cultures) (solid bars) from days 2 to 5 **(Fig. 8B)**. All these effects of IGFBP mRNA expression in quantitative real-time PCR were similar at Northern blot analysis.

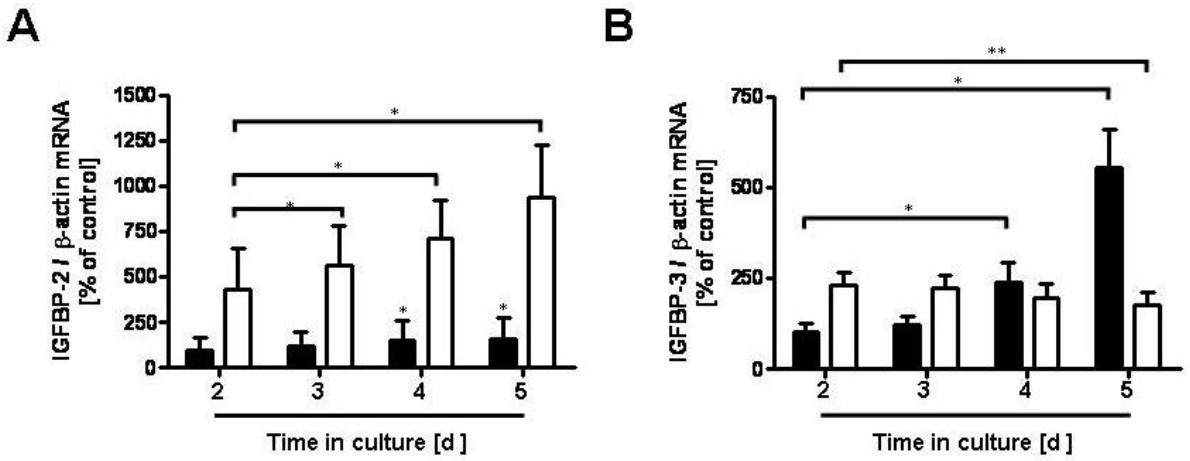

Figure 8. Expression of IGFBP-2 and -3 mRNA in mLMFs analysed by quantitative real-time (RT)-PCR. 1 µg of total RNA isolated from wt and IGFBP-2 (+/-) mLMFs (days 2 to 5) was reverse transcribed to cDNA using Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT). 16 ng of cDNA were used to determine IGFBP-2 and -3 mRNA expressions by quantitative RT-PCR amplification. **A, B:** Representative quantitative RT-PCRs of IGFBP-2 mRNA in wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs, and IGFBP-3 mRNA in wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs, respectively. The relative expression of IGFBP-2 and -3 mRNA was expressed as percent increase or decrease (means ± SD) compared with the respective wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs at day 2 of culture (n = 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs) after normalization against mouse endogenous β-actin. Statistically significant differences in IGFBP-2 and -3 mRNA expressions relative to the respective wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs at day 2 of culture were depicted over the horizontal lines: *, $P < 0.05$; **, $P < 0.01$ (Student's t test). Statistically significant differences in IGFBP-2 mRNA expression between wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs compared with the respective cultures of wt mLMFs (solid bars) (days 2 to 5) were given over the bars: *, $P < 0.05$ (Student's t test).

Northern blot analysis of total RNA from LMFs revealed mRNA species for IGF-IR at 11 kb, and IGF-II/M6-PR at 9 kb which were detected in wt and IGFBP-2 (+/-) mLMFs **(Fig. 9A)**. The IGF-IR mRNA in wt (2.18 ± 1.76-fold increase relative to day 2 cultures) (solid bars) and IGFBP-2 (+/-) (2.49 ± 0.56-fold) (open bars) mLMFs, and IGF-II/M6-PR mRNA transcripts in wt (7.72 ± 1.61-fold) (solid bars) and IGFBP-2 (+/-) (3.68 ± 0.3-fold) (open bars) mLMFs increased with time of culture (days 2 to 5). In IGFBP-2 (+/-) mLMFs (open bars), the expression of IGF-IR mRNA was more pronounced than wt mLMFs (solid bars) whereas IGF-II/M6-PR mRNA in IGFBP-2 (+/-) mLMFs (open bars) was increased compared with wt mLMFs (solid bars) that was significantly different showing maximal levels at days 4 and 5 of culture **(Fig. 9B, C)**.

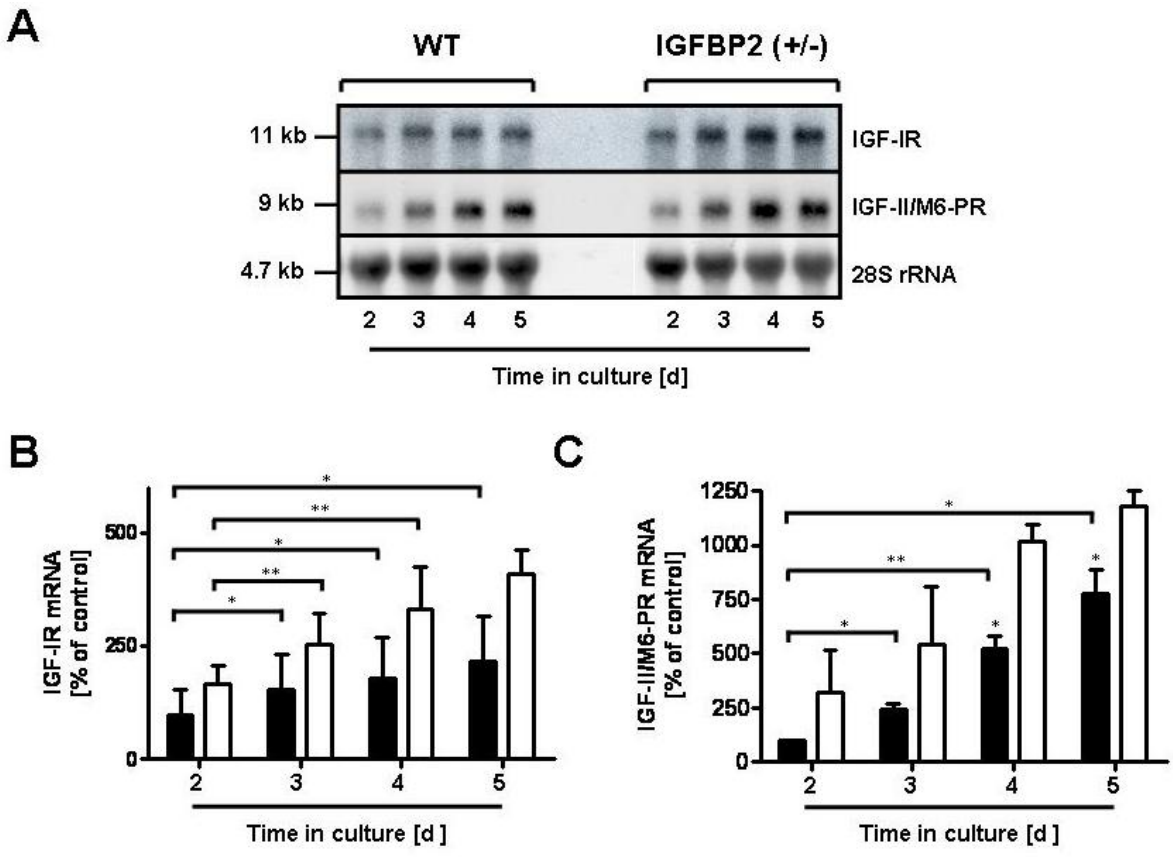

Figure 9. Expression of IGF-IR and IGF-II/M6-PR mRNA in mLMFs analysed by Northern blotting. Total RNA (10-15 µg) gained from wt and IGFBP-2 (+/-) mLMFs (days 2 to 5) were subjected to agarose gel electrophoresis, blotted, and hybridized with a radiolabeled cDNA specific for rat IGF-IR and IGF-II/M6-PR mRNA. **A:** Representative Northern blots of IGF-IR and IGF-II/M6-PR mRNA in wt and IGFBP-2 (+/-) mLMFs. **B, C:** Densitometric analysis of IGF-IR mRNA in wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs, and IGF-II/M6-PR mRNA in wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs, respectively. After autoradiography, hybridization signals corresponding to the 11 kb IGF-IR and 9 kb IGF-II/M6-PR were densitometrically analyzed and given as percent increase or decrease of IGF-IR and IGF-II/M6-PR mRNA (means ± SD) compared with wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs at day 2 of culture (n = 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences in IGF-IR and IGF-II/M6-PR mRNA expressions relative to the respective wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs at day 2 of culture were indicated over the horizontal lines: *, $P < 0.05$; **, $P < 0.01$ (Student's t test). Statistically significant differences in IGF-IR and IGF-II/M6-PR mRNA expressions between wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs compared with respective cultures of wt mLMFs (solid bars) (days 2 to 5) were given over the bars: *, $P < 0.05$ (Student's t test).

In addition, quantitative real-time PCR evaluation of total RNA from both wt and IGFBP-2 (+/-) mLMFs cultured at days 2 to 5 showed pronounced levels of IGF-IR in wt (2.02 ± 1.37-fold increase compared to day 2 cultures) (solid bars) and IGFBP-2 (+/-) (2.54 ± 2.52-fold) (open bars) mLMFs, and of IGF-II/M6-PR mRNA in wt (1.89 ± 0.75-fold) (solid bars) and IGFBP-2 (+/-) (2.07 ± 1.05-fold) (open bars) mLMFs **(Fig. 10A, B)**. In IGFBP-2 (+/-) mLMFs (open bars), increased expression of IGF-IR mRNA was significantly different compared to wt mLMF (solid bars) from days 2 to 5

of culture **(Fig. 10A)**. The expression of IGF-II/M6-PR mRNA in IGFBP-2 (+/-) mLMFs (open bars) was enhanced than wt mLMFs (solid bars) displaying a maximum at days 4 and 5 of culture **(Fig. 10B)**.

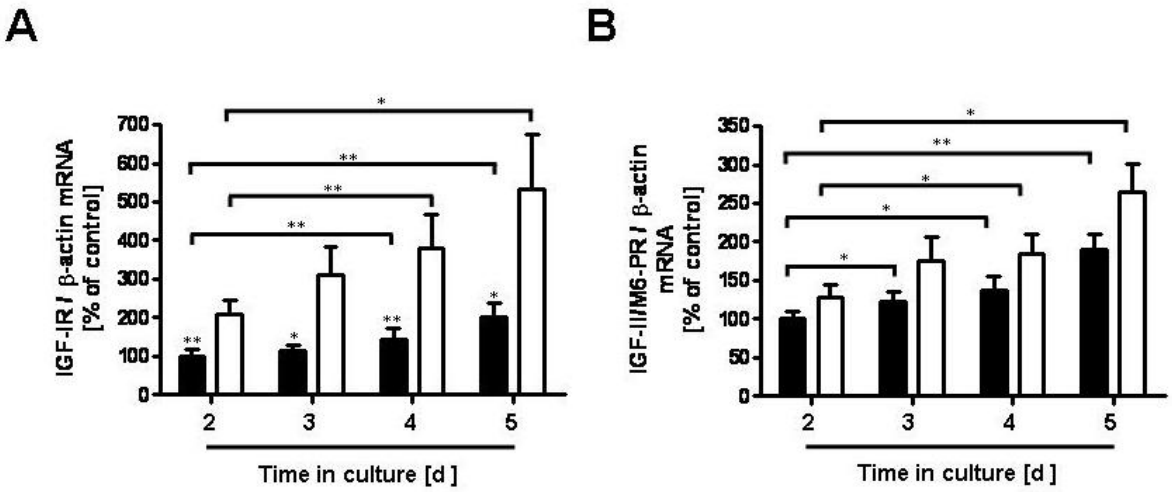

Figure 10. Expression of IGF-IR and IGF-II/M6-PR mRNA in mLMFs analysed by quantitative RT-PCR. Reverse transcription of 1 µg of total RNA isolated from wt and IGFBP-2 (+/-) mLMFs (days 2 to 5) to cDNA was performed with M-MLV RT. Biosynthesis of IGF-IR and IGF-II/M6-PR mRNA was evaluated by quantitative RT-PCR amplification with 16 ng of cDNA. **A, B:** Representative quantitative RT-PCRs of IGF-IR mRNA in wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs, and IGF-II/M6-PR mRNA in wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs, respectively. The relative expression of IGF-IR and IGF-II/M6-PR mRNA was given as percent increase or decrease (means ± SD) compared with the respective wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs at day 2 culture (n = 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs) after normalization against mouse endogenous β-actin. Statistically significant differences in IGF-IR and IGF-II/M6-PR mRNA expressions relative to the respective wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs at day 2 culture were given over the horizontal lines: *, $P < 0.05$; **, $P < 0.01$ (Student's t test). Statistically significant differences in IGF-IR and IGF-II/M6-PR mRNA expressions between wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs compared with respective cultures of wt mLMFs (solid bars) (days 2 to 5) were indicated over the bars: *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

Total RNA obtained from wt and IGFBP-2 (+/-) mLMFs at different time points of culture was analysed for IGF-I mRNA expression by real-time PCR. Wt mLMFs (solid bars) expressed steady-state level of IGF-I mRNA whereas IGFBP-2 (+/-) mLMFs (2.39 ± 2.74-fold increase compared to day 2 cultures) (open bars) displayed increased level of IGF-I mRNA that was significantly different relative to wt mLMFs showing a maximal levels at days 4 and 5 of culture **(Fig. 11)**. Northern blot analysis in both wt and IGFBP-2 (+/-) mLMFs also revealed faint bands of IGF-I mRNA transcripts at 7.5 kb, 2 kb and 1.0 to 1.5 kb. In line with data from quantitative real-

time PCR, Northern blot analysis showed similar expression patterns of IGF-I mRNA in wt and IGFBP-2 (+/-) mLMFs (data not shown).

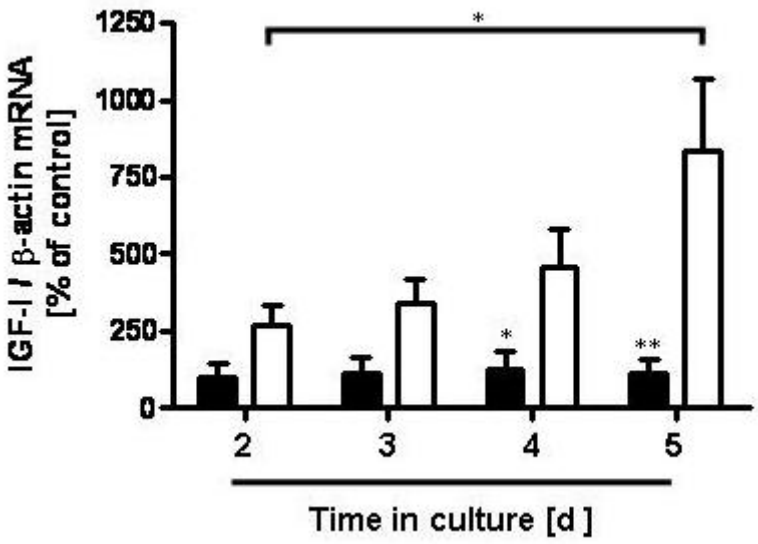

Figure 11. Expression of IGF-I mRNA in mLMFs by quantitative RT-PCR. Wt and IGFBP-2 (+/-) mLMFs at different time points of culture (days 2 to 5) were harvested for RNA isolation. cDNA was prepared with 1 µg of total RNA using M-MLV RT. For evaluation of IGF-I mRNA expression, 16 ng of cDNA was utilized by quantitative RT-PCR amplification. The relative expression of IGF-I mRNA after normalization against mouse endogenous β-actin was expressed as percent increase or decrease (means ± SD) compared with respective wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs at day 2 culture (n = 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences in IGF-I mRNA expression relative to the respective wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs at day 2 culture were depicted over the horizontal lines: *, $P < 0.05$ (Student's t test). Statistically significant differences in IGF-I mRNA expression between wt (solid bars) and IGFBP-2 (+/-) (open bars) mLMFs compared with respective cultures of wt mLMFs (solid bars) (days 2 to 5) was given over the bars: *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

4. 3. Regulation of IGFBP mRNA

The effect of mitogenic growth factors such as IGF-I and PDGF-BB as well as profibrogenic cytokines such as TGF-β on IGFBP mRNA expression was studied by Northern blotting. In wt mLMFs, addition of IGF-I time- (data not shown) and dose-dependently reduced IGFBP-2 mRNA (0.51 ± 0.01-fold decrease relative to untreated controls) **(Fig. 12)**. In contrast, in IGFBP-2 (+/-) mLMFs IGF-I stimulated IGFBP-2 mRNA expression (1.6 ± 0.6-fold increase relative to untreated controls). The IGF-I-dependent stimulation of IGFBP-3 mRNA in IGFBP-2 (+/-) mLMFs (1.18 ± 0.04-fold increase relative to untreated controls) was less pronounced than in wt mLMFs (4.1 ± 0.17-fold) **(Fig. 12 and 13)**. In all cases, IGF-I displayed maximal effects at a concentration of 100 nmol/L. PDGF-BB had no effect on IGFBP-2 and -3

mRNA expressions both in wt and IGFBP (+/-) mLMFs. TGF-β stimulated IGFBP-2 and -3 mRNA expressions which was not significantly different in wt and IGFBP-2 (+/-) mLMFs **(Fig. 13)**.

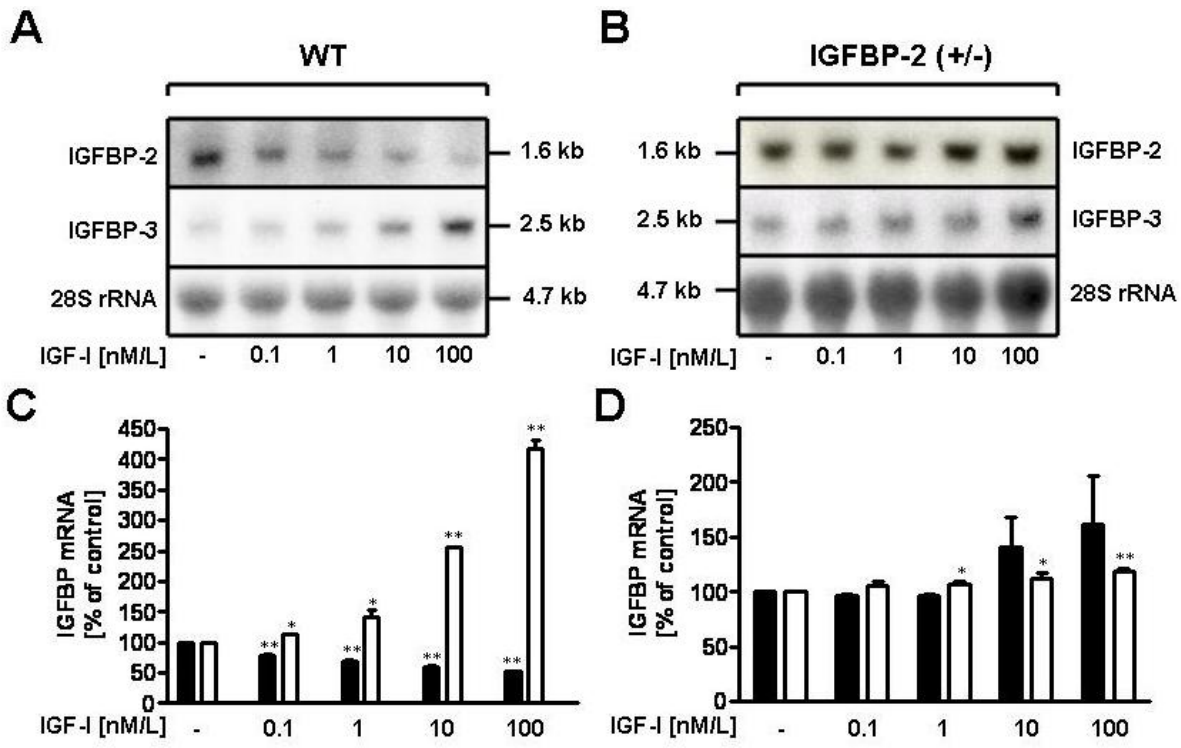

Figure 12. Effect of IGF-I on IGFBP-2 and -3 mRNA expressions in mLMFs analysed by Northern blotting. Wt and IGFBP-2 (+/-) mLMFs were cultured in serum-reduced medium (SRM) in the presence of IGF-I at concentrations ranging from 0.1 to 100 nmol/L for 36 hours (h). Control cells were cultivated in SRM alone. 10-15 µg of isolated RNA from these cells were size-fractioned by gel electrophoresis, blotted, and probed for IGFBP-2 and -3 mRNA. **A, B:** Representative Northern blots of IGFBP-2 and -3 mRNA in wt and IGFBP-2 (+/-) mLMFs. **C, D:** Densitometric analysis of IGFBP-2 and -3 mRNA in wt and IGFBP-2 (+/-) mLMFs. Hybridization signals were densitometrically analysed and indicated as percent increase or decrease (means ± SD) of IGFBP-2 (solid bars) and -3 (open bars) mRNA compared with respective untreated controls (n = 2 to 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences in IGFBP-2 (solid bars) and -3 (open bars) mRNA expressions compared with respective untreated controls were given: *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

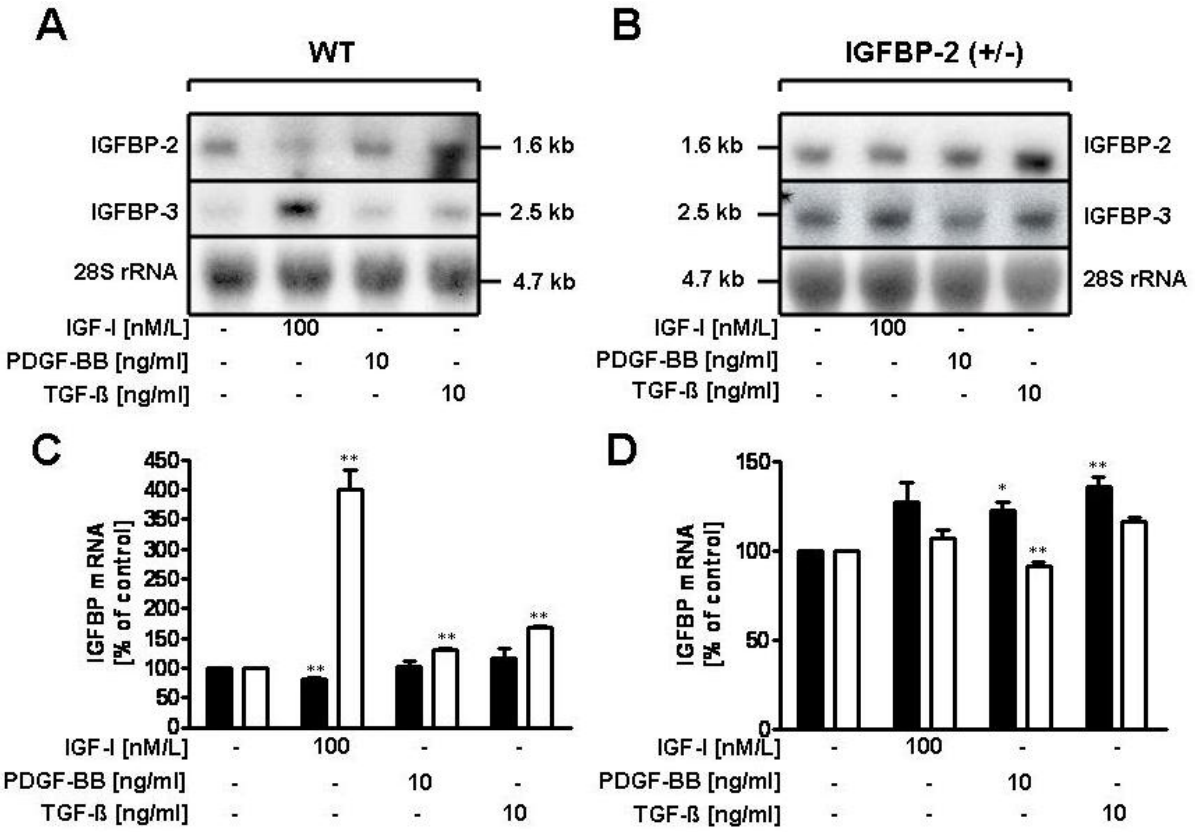

Figure 13. Effect of IGF-I, PDGF-BB and TGF-β on IGFBP-2 and -3 mRNA expressions in mLMFs analysed by Northern blotting. Cultivation of wt and IGFBP-2 (+/-) mLMFs was performed with SRM in the presence or absence of IGF-I (100 nmol/L), PDGF-BB (10 ng/ml) and TGF-β (10 ng/ml) for 36 h. Total RNA (10-15 µg) isolated from these cells were used to evaluate the expression of IGFBP-2 and -3 mRNA by Northern blot analysis. **A, B:** Representative Northern blots of IGFBP-2 and -3 mRNA in wt and IGFBP-2 (+/-) mLMFs. **C, D:** Densitometric evaluation of IGFBP-2 and -3 mRNA in wt and IGFBP-2 (+/-) mLMFs. After autoradiography, the relative densities of hybridization signals were densitometrically examined and given as percent increase or decrease (means ± SD) of IGFBP-2 (solid bars) and -3 (open bars) mRNA compared to respective untreated controls (n = 2 to 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences in IGFBP-2 (solid bars) and -3 (open bars) mRNA expressions relative to respective untreated controls were depicted: *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

4. 4. Regulation of IGFBP secretion

Presence of IGFBP in CM of mLMFs was determined by ligand blotting using [^{125}I]-IGF-I as a tracer **(Fig. 14)**. These experiments demonstrated protein bands at 31 kDa and at 41-45 kDa representing IGFBP-2 and -3, respectively, in CM of wt and IGFBP-2 (+/-) mLMFs. In line with the results of IGFBP mRNA expression, abundance of IGFBP-2 protein was approximately four to five-fold increased in IGFBP-2 (+/-) mLMFs compared with wt mLMFs. Maintenance of wt mLMFs with IGF-I dose-dependently decreased IGFBP-2 (0.84-fold decrease relative to untreated controls) whereas in IGFBP-2 (+/-) mLMFs IGF-I showed a stimulatory effect on

IGFBP-2 secretion (1.4 ± 0.2-fold) **(Fig. 14 and 15)**. The IGF-I dose-dependent stimulation of IGFBP-3 in IGFBP-2 (+/-) mLMFs (1.16 ± 0.04-fold increase compared with untreated control) was less pronounced than in wt mLMFs (1.6 ± 0.2-fold) **(Fig. 14 and 15)**. In these experiments IGF-I showed maximal effects at concentrations of 100 nmol/L. PDGF-BB (10 ng/ml) had no effect on IGFBP-2 and -3 mRNA expression both in wt and IGFBP-2 (+/-) mLMFs whereas TGF-β (10 ng/ml) stimulated IGFBP-2 and -3 expression which was not significantly different in wt and IGFBP-2 (+/-) mLMFs **(Fig. 15)**. The effect of IGF-I, PDGF-BB and TGF-β on IGFBP secretion was similar to their effect at mRNA level.

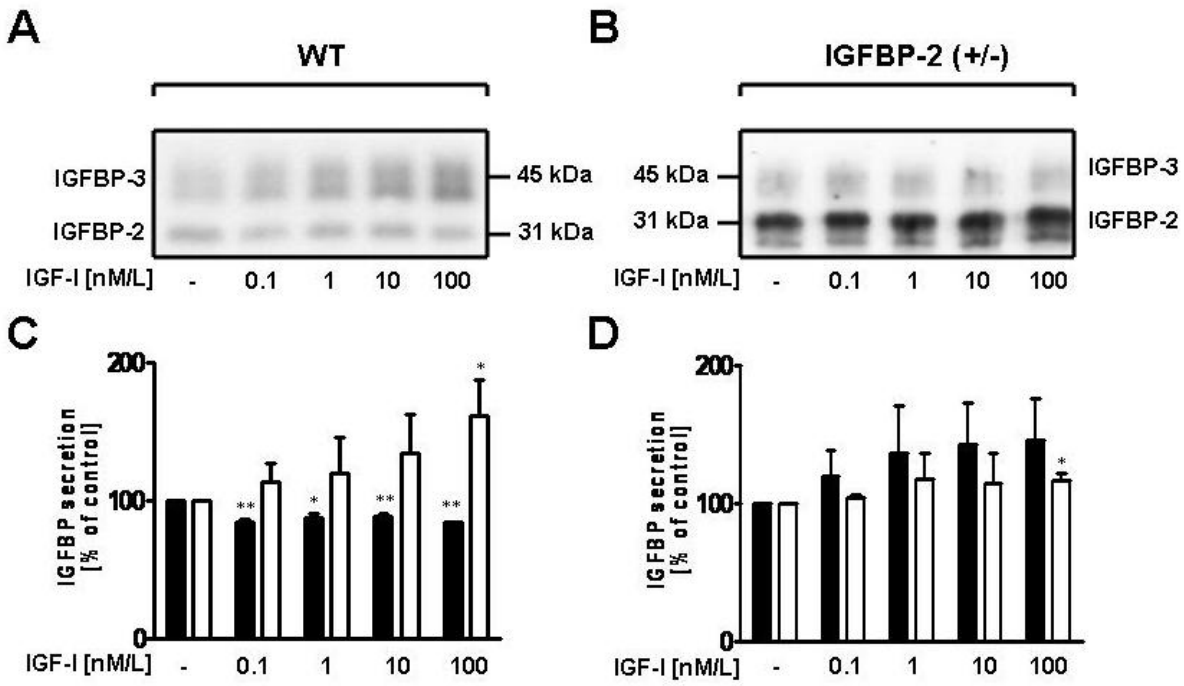

Figure 14. Effect of IGF-I on IGFBP-2 and -3 secretions in mLMFs analysed by Western ligand blotting. Wt and IGFBP-2 (+/-) mLMFs were kept under serum-reduced conditions in the presence of IGF-I at specified concentrations for 36 h. Equal amounts of proteins extracted from cultured medium (CM) were size-fractioned by SDS-PAGE under non-reducing conditions, transferred to nitrocellulose membranes. For ligand blotting, membranes were incubated with [^{125}I]-IGF-I and exposed to X-ray films. The positions of molecular mass standards were indicated on the left. **A, B:** Representative Western ligand blots of IGFBP-2 and -3 in wt and IGFBP-2 (+/-) mLMFs. **C, D:** Densitometric analysis of IGFBP-2 and -3 in wt and IGFBP-2 (+/-) mLMFs. Ligand binding signals were quantified by densitometrical analysis. The results were indicated as percent increase or decrease (means ± SD) of IGFBP-2 (solid bars) and -3 (open bars) relative to respective untreated controls (n = 2 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences in IGFBP-2 (solid bars) and -3 (open bars) secretion compared with respective untreated controls were given: *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

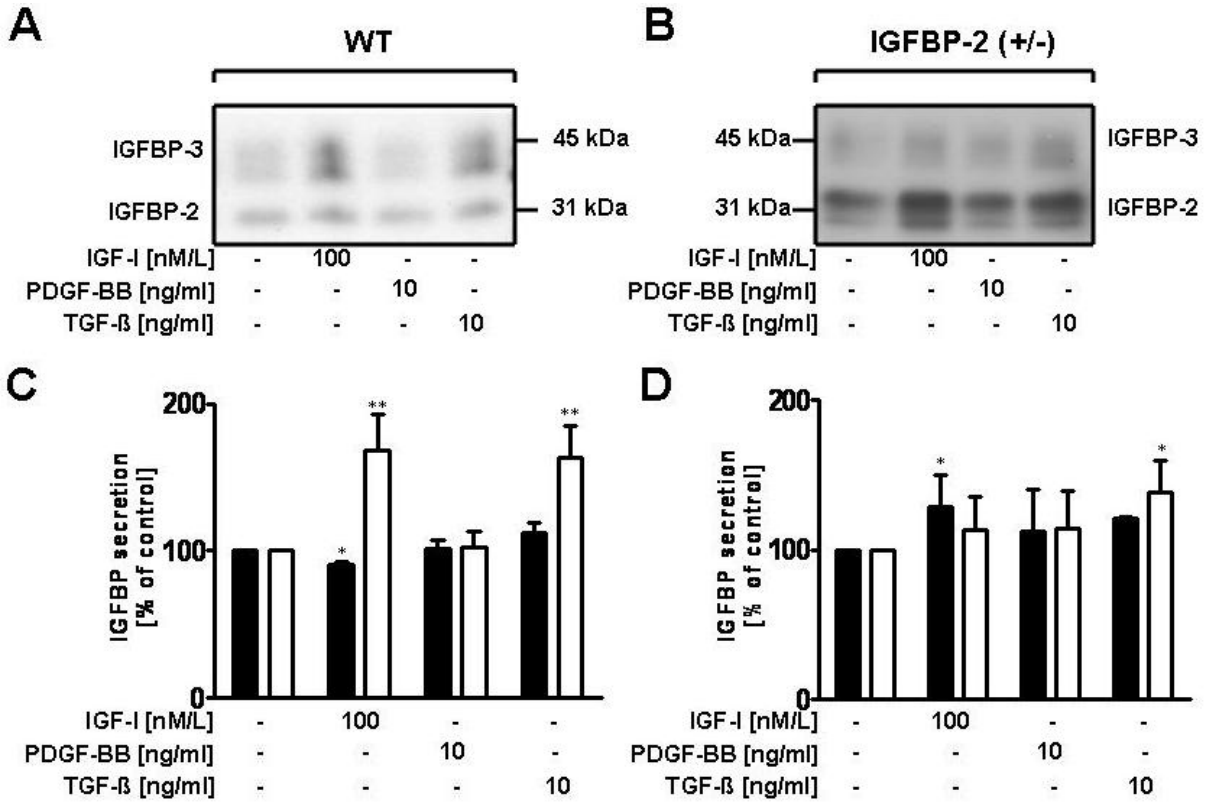

Figure 15. Effect of IGF-I, PDGF-BB and TGF-β on IGFBP-2 and -3 secretions in mLMFs analysed by Western ligand blotting. Maintenance of wt and IGFBP-2 (+/-) mLMFs was achieved under serum-reduced conditions in the presence or absence of IGF-I (100 nmol/L), PDGF-BB (10 ng/ml) and TGF-β (10 ng/ml) for 36 h. 40 µg of wt and IGFBP-2 (+/-) mLMFs CM was processed under non-reducing conditions for ligand blotting with [^{125}I]-IGF-I. **A, B:** Representative Western ligand blots of IGFBP-2 and -3 in wt and IGFBP-2 (+/-) mLMFs. **C, D:** Densitometric analysis of IGFBP-2 and -3 in wt and IGFBP-2 (+/-) mLMFs. After autoradiography, the relative densities of ligand binding signals were densitometrically evaluated and given as percent increase or decrease (means ± SD) of IGFBP-2 (solid bars) and -3 (open bars) relative to respective untreated controls (n = 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences in IGFBP-2 (solid bars) and -3 (open bars) secretions compared with respective untreated controls were indicated: *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

4. 5. Regulation of IGF-IR mRNA

To determine whether the IGF-IR of wt and IGFBP-2 (+/-) mLMFs is regulated differently by growth factors, mLMFs were cultivated in the presence or absence of IGF-I, PDGF-BB and TGF-β. Addition of IGF-I dose-dependently decreased IGF-IR mRNA that was not significantly different in wt (0.67 ± 0.07-fold decrease compared with untreated controls) and IGFBP-2 (+/-) mLMFs (0.57 ± 0.03-fold) **(Fig. 16 and 17)** with maximal effects at 100 nmol/L. PDGF-BB (10 ng/ml) had no effect on IGF-IR mRNA expression both in wt and IGFBP-2 (+/-) mLMFs whereas TGF-β (10 ng/ml) stimulated IGF-IR mRNA expression which was not significantly different in wt and IGFBP-2 (+/-) mLMFs **(Fig. 17)**.

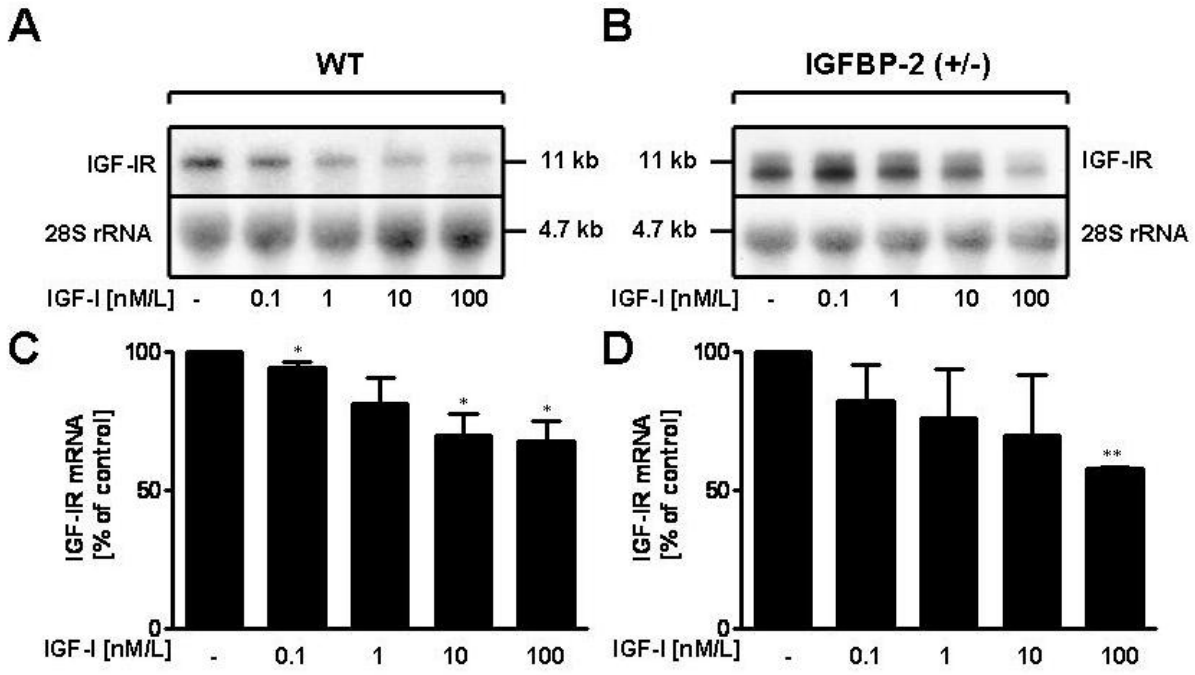

Figure 16. Effect of IGF-I on IGF-IR mRNA expression in mLMFs analysed by Northern blotting. 15 µg of total RNA obtained from wt and IGFBP-2 (+/-) mLMFs cultivated under serum-reduced conditions in the presence of increasing doses of IGF-I (0.1, 1.0, 10, 100 nmol/L) for 36 h were subjected to Northern blot analysis for IGF-IR mRNA with ^{32}P-labeled cDNA specific for rat IGF-IR mRNA. **A, B:** Representative Northern blots of IGF-IR mRNA in wt and IGFBP-2 (+/-) mLMFs. **C, D:** Densitometric examination of IGF-IR mRNA expression in wt and IGFBP-2 (+/-) mLMFs. The relative densities of bands were expressed as percent increase or decrease of IGF-IR mRNA (means ± SD) compared with untreated controls (n = 2 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences in IGF-IR mRNA expression relative to untreated controls were indicated: *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

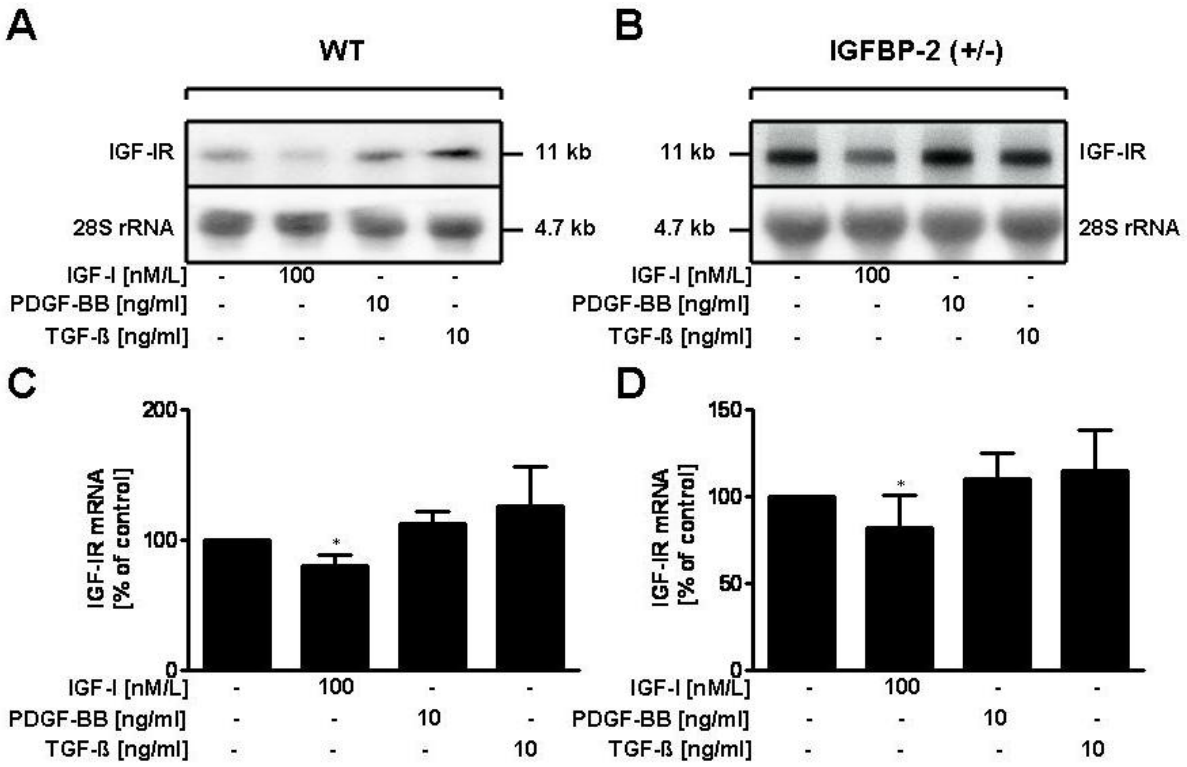

Figure 17. Effect of IGF-I, PDGF-BB and TGF-β on IGF-IR mRNA expression in mLMFs evaluated by Northern blotting. Wt and IGFBP-2 (+/-) mLMFs were grown in serum-reduced conditions in the presence or absence of IGF-I (100 nmol/L), PDGF-BB (10 ng/ml) and TGF-β (10 ng/ml) for 36 h. 10-15 µg of total RNA from these cells were resolved by 1% agarose gel electrophoresis, blotted and probed for IGF-IR mRNA. **A, B:** Representative Northern blots of IGF-IR mRNA in wt and IGFBP-2 (+/-) mLMFs. **C, D:** Densitometric examination of IGF-IR mRNA expression in wt and IGFBP-2 (+/-) mLMFs. After autoradiography, hybridization signals corresponding to the 11 kb IGF-IR mRNA were quantified by densitometrical analysis. The results were expressed as percent increase or decrease of IGF-IR mRNA expression (means ± SD) relative to untreated controls (n = 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences in IGF-IR mRNA expression compared with untreated controls were depicted: *, $P < 0.05$ (Student's t test).

4. 6. Regulation of IGF-IRβ protein

Immunoblot analysis of total protein isolated from IGF-I, PDGF-BB and TGF-β treated wt and IGFBP-2 (+/-) mLMFs revealed an IGF-IRβ specific protein band at 97 kDa **(Fig. 18 and 19)**. Addition of IGF-I dose-dependently decreased IGF-IRβ protein that was not significantly different in both wt (0.23 ± 0.01-fold decrease compared with untreated controls) and IGFBP-2 (+/-) mLMFs (0.16 ± 0.01-fold) with maximal effects at 100 nmol/L. PDGF-BB (10 ng/ml) had no effect on IGF-IRβ protein expression in both wt and IGFBP-2 (+/-) mLMFs. TGF-β (10 ng/ml) slightly stimulated IGF-IRβ protein in wt (1.2 ± 0.2-fold increase relative to untreated controls) and in IGFBP-2 (+/-) mLMFs (1.1 ± 0.1-fold) **(Fig. 19)**. The regulatory effect

of IGF-I, PDGF-BB and TGF-β on IGF-IR was similar at the level of mRNA as well as protein expression.

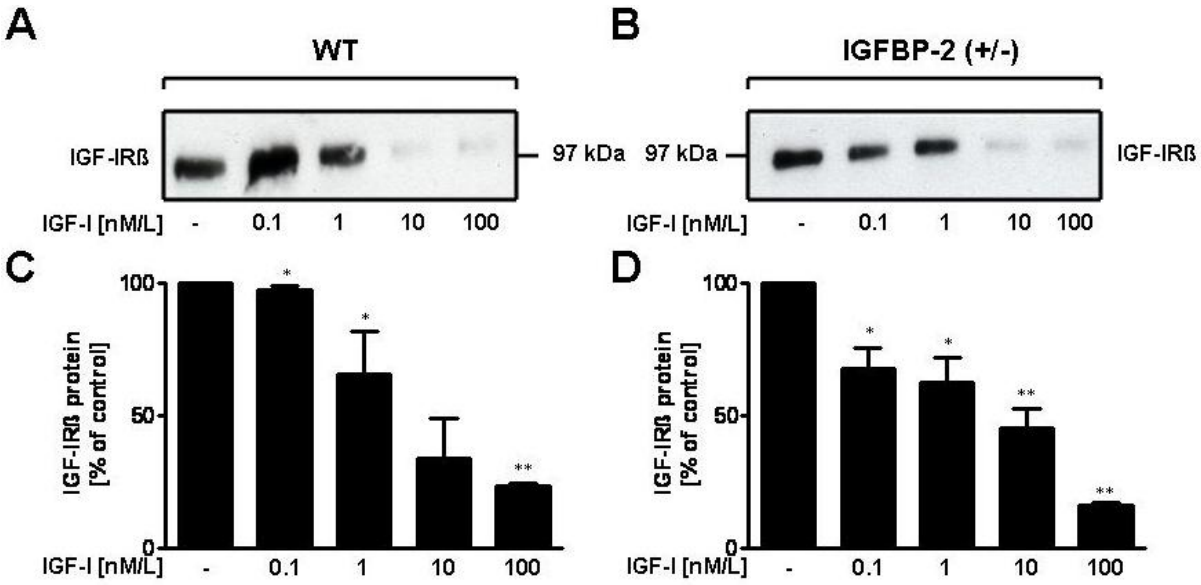

Figure 18. Effect of IGF-I on IGF-IRβ protein expression in mLMFs analysed by Western immunoblotting. Cultivation of wt and IGFBP-2 (+/-) mLMFs was performed under serum-reduced conditions in the presence of IGF-I at specified concentrations for 36 h. 25 µg of proteins from whole cell lysates were size-fractioned by SDS-PAGE under reducing conditions, transferred on to the membrane, and incubated with antibodies raised against IGF-IRβ protein. **A, B:** Representative Western blots of IGF-IRβ protein in wt and IGFBP-2 (+/-) mLMFs. **C, D:** Densitometric analysis of IGF-IRβ protein in wt and IGFBP-2 (+/-) mLMFs. Antigen-antibody interaction signals corresponding to the 97 kDa IGF-IRβ protein were densitometrically quantified and expressed as percent increase or decrease of IGF-IRβ protein (means ± SD) compared to untreated controls (n = 2 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences in IGF-IRβ protein expression relative to untreated controls were given: *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

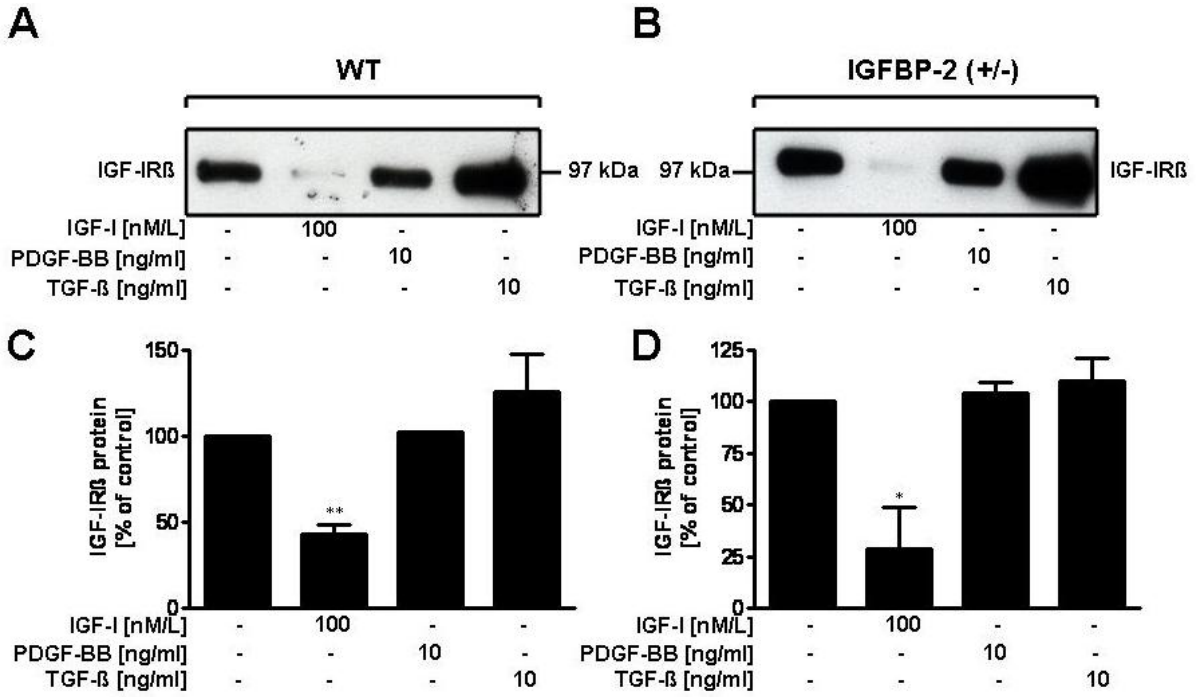

Figure 19. Effect on IGF-I, PDGF-BB and TGF-β on IGF-IRβ protein in mLMFs analysed by Western immunoblotting. Proteins (25 µg) from wt and IGFBP-2 (+/-) mLMFs incubated in SRM in the presence or absence of IGF-I (100 nmol/L), PDGF-BB (10 ng/ml) and TGF-β (10 ng/ml) for 36 h were separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes. For immunoblotting, membranes were incubated with antibodies raised against IGF-IR β protein. **A, B:** Representative Western blots of IGF-IRβ protein in wt and IGFBP-2 (+/-) mLMFs. **C, D:** Densitometric analysis of IGF-IRβ protein in wt and IGFBP-2 (+/-) mLMFs. The relative densities of bands were expressed as percent increase or decrease of IGF-IRβ protein (means ± SD) compared with the untreated controls (n = 2 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences in IGF-IRβ protein expression compared with untreated controls were indicated: *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

4. 7. Regulation of IGF-II/M6-PR mRNA

The competence of wt and IGFBP-2 (+/-) mLMFs grown in presence or absence of IGF-I in modulating the expression of IGF-II/M6-PR mRNA was evaluated by Northern blot analysis. In wt and IGFBP-2 (+/-), the addition of increasing doses of IGF-I had no effect on IGF-II/M6-PR mRNA expression. Steady state level of IGF-II/M6-PR mRNA was similar in both wt and IGFBP-2 (+/-) mLMFs **(Fig. 20 and 21)**. Simultaneously, modulatory effects of mitogenic growth factor such as PDGF-BB and profibrogenic growth factor such as TGF-β on IGF-II/M6-PR mRNA expression was analysed in wt and IGFBP-2 (+/-) mLMFs. PDGF-BB and TGF-β had neither stimulatory nor inhibitory effect on IGF-IIR/M6-PR mRNA expression in both wt and IGFBP-2 (+/-) mMLFs **(Fig. 21)**.

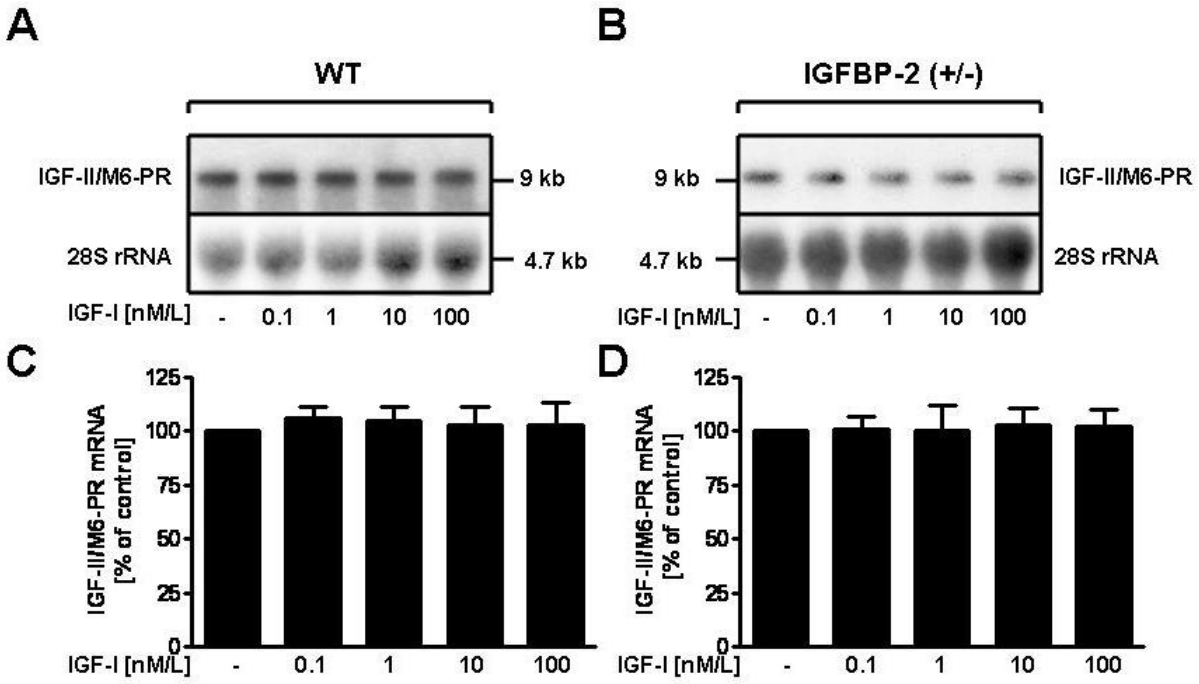

Figure 20. Effect of IGF-I on IGF-II/M6-PR mRNA expression in mLMFs analysed by Northern blotting. After starvation in SRM for 1 h, wt and IGFBP-2 (+/-) mLMFs were maintained in the presence of IGF-I (0.1, 1.0, 10, 100 nmol/L) for 36 h. Total RNA (10-15 µg) isolated from these cells was size-fractioned by gel electrophoresis, blotted, and probed for IGF-II/M6-PR mRNA. **A, B:** Representative Northern blots of IGF-II/M6-PR mRNA in wt and IGFBP-2 (+/-) mLMFs. **C, D:** Densitometric evaluation of IGF-II/M6-PR mRNA in wt and IGFBP-2 (+/-) mLMFs. Results were expressed as percent increase or decrease of IGF-II/M6-PR mRNA expression (means ± SD) compared with untreated controls (n = 2 to 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs).

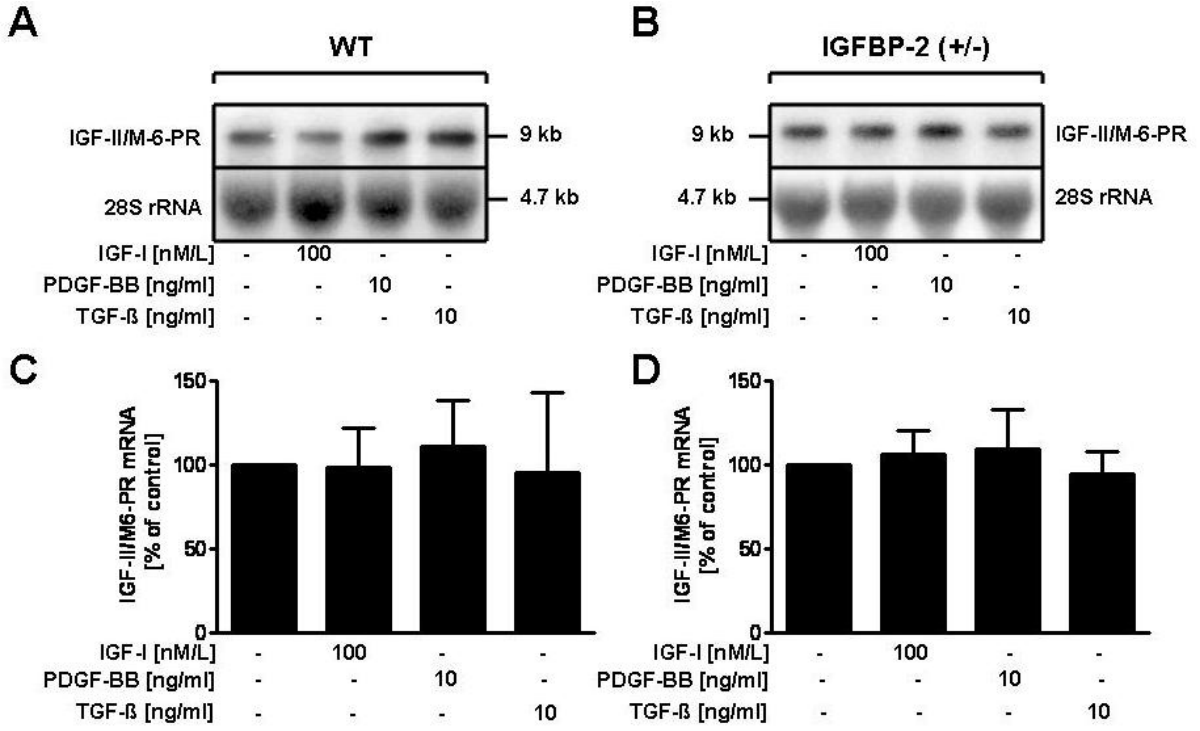

Figure 21. Effect of IGF-I, PDGF-BB and TGF-β on IGF-II/M6-PR mRNA expression in mLMFs analysed by Northern blotting. Wt and IGFBP-2 (+/-) mLMFs were cultivated with SRM in the presence or absence of IGF-I (100 nmol/L), PDGF-BB (10 ng/ml) and TGF-β (10 ng/ml) for 36 h. Expression of IGF-II/M6-PR mRNA was determined by Northern blot analysis in which 10-15 µg of total RNA were resolved by gel electrophoresis, blotted, and hybridized for IGF-II/M6-PR mRNA. **A, B:** Representative Northern blots of IGF-II/M6-PR mRNA in wt and IGFBP-2 (+/-) mLMFs. **C, D:** Densitometric studies of IGF-II/M6-PR mRNA in wt and IGFBP-2 (+/-) mLMFs. The relative densities of bands were expressed as percent increase or decrease of IGF-II/M6-PR mRNA expression (means ± SD) compared to untreated controls (n = 2 to 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs).

4. 8. Regulation of DNA synthesis in mLMFs

To evaluate the functional consequences of overexpression of IGFBP-2 in mLMFs, the effect of IGF-I, PGDF-BB and TGF-β on DNA synthesis in wt and IGFBP-2 (+/-) mLMFs was studied by BrdU incorporation assay. IGF-I dose-dependently stimulated DNA synthesis in wt mLMFs (1.6 ± 0.2-fold increase relative to untreated controls) whereas in IGFBP-2 (+/-) mLMFs IGF-I-dependent DNA synthesis was reduced (0.56 ± 0.1-fold) **(Fig. 22 and 23)**. In wt mLMFs, PDGF-BB stimulated DNA synthesis was more pronounced than in IGFBP-2 (+/-) mLMFs. TGF-β at 10 ng/ml led to an abrogation of DNA synthesis in wt mLMFs (0.74 ± 0.06-fold decrease relative to untreated controls) that was more prominent than in IGFBP-2 (+/-) mLMFs (0.93 ± 0.08-fold) without reaching statistical significance **(Fig. 23)**.

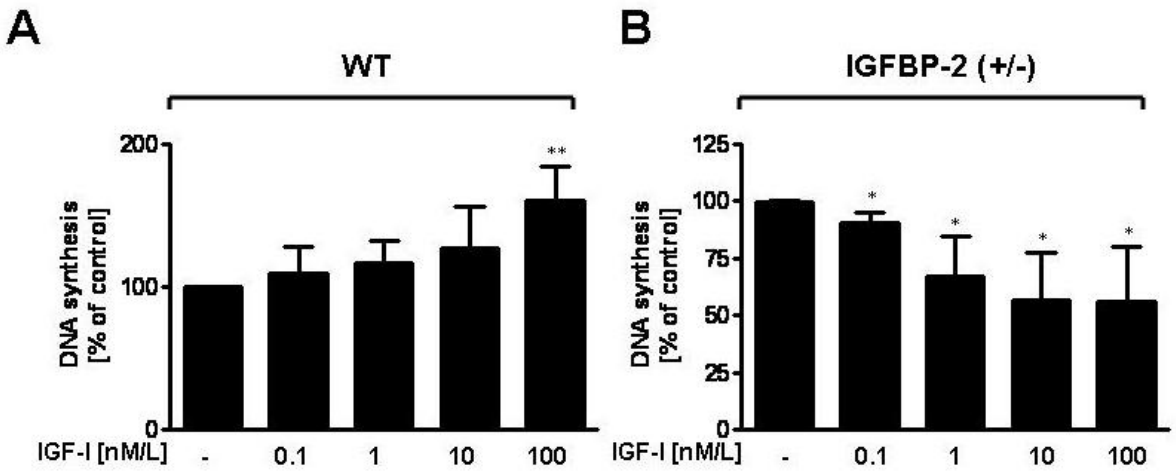

Figure 22. Effect of IGF-I on DNA synthesis in mLMFs analysed by BrdU incorporation assay. After starvation in SRM for 2 h, wt and IGFBP-2 (+/-) mLMFs were incubated with simultaneous addition of IGF-I at specified concentrations (0.1, 1.0, 10, 100 nmol/L) and 5-bromo-2'-deoxyuridine (BrdU) for 24 h. Cultivation of control cells was performed only with serum-reduced medium and BrdU. **A, B:** Representative BrdU incorporation assays in wt and IGFBP-2 (+/-) mLMFs. Synthesis of DNA was expressed as percent increase or decrease of incorporation (means ± SD) relative to untreated controls (n = 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences relative to untreated controls were indicated: *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

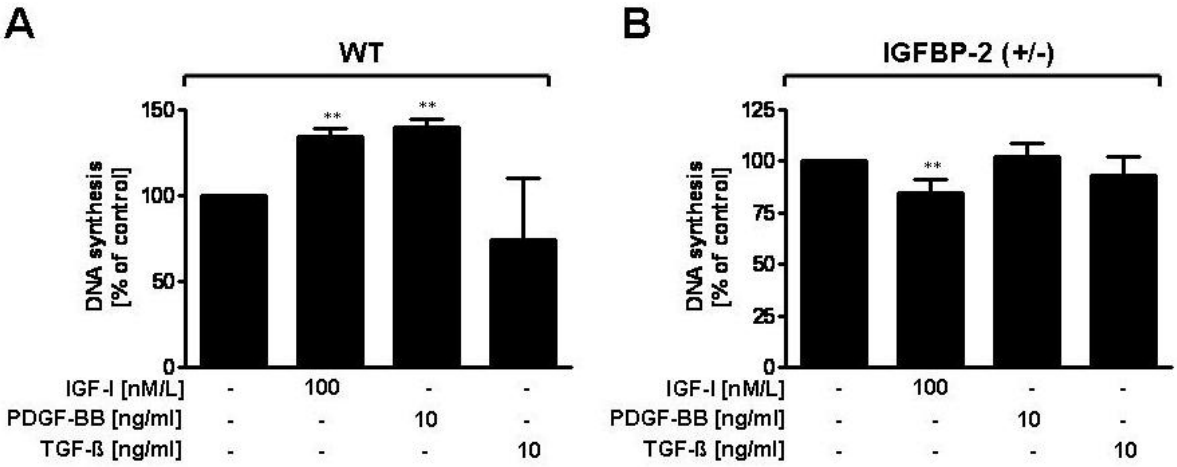

Figure 23. Effect of IGF-I, PDGF-BB and TGF-β on DNA synthesis in mLMFs analysed by BrdU incorporation assay. After starvation in SRM for 2 h, wt and IGFBP-2 (+/-) mLMFs were maintained first in the presence or absence of IGF-I (100 nmol/L), PDGF-BB (10 ng/ml) and TGF-β (10 ng/ml), and then simultaneously pulse-labeled with BrdU for 24 h. **A, B:** Representative BrdU incorporation assays in wt and IGFBP-2 (+/-) mLMFs. Results were expressed as percent increase or decrease of incorporation (means ± SD) relative to untreated controls (n = 2 to 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences relative to untreated controls were indicated: **, $P < 0.01$ (Student's t test).

Furthermore, the functional role of IGFBP-2 overexpression on DNA synthesis in wt and IGFBP-2 (+/-) mLMFs was also verified by means of [^{3}H]-thymidine incorporation

at 6 and 12 hours after addition of IGF-I. Incubation of wt mLMFs with IGF-I (10 nmol/L) stimulated DNA synthesis at 6 (1.42 ± 0.16-fold increase relative to untreated controls) and 12 hours (2.0 ± 0.48-fold) whereas IGF-I-dependent DNA synthesis was inhibited in IGFBP-2 (+/-) mLMFs at 6 (0.87 ± 0.1-fold decrease compared with untreated controls) and 12 hours (0.71 ± 0.02-folds) of cultures **(Fig. 24)**.

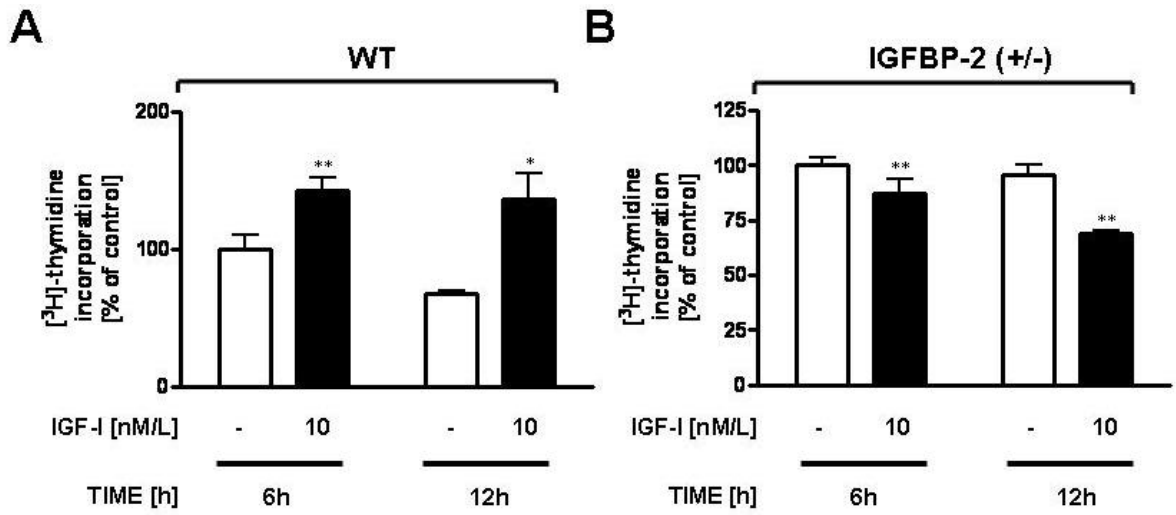

Figure 24. Effect of IGF-I on DNA synthesis in mLMFs evaluated by [^{3}H]-thymidine incorporation assay. Wt and IGFBP-2 (+/-) mLMFs were pulse-labeled with [^{3}H]-thymidine under serum-reduced conditions for 6 h and 12 h. The effect of IGF-I on DNA synthesis of wt and IGFBP-2 (+/-) mLMFs was evaluated by simultaneous addition of IGF-I at concentration of 10 nmol/L. **A, B:** Representative [^{3}H]-thymidine incorporation assays in wt and IGFBP-2 (+/-) mLMFs. Results were expressed as percent increase or decrease of incorporation (means ± SD) relative to untreated controls (n = 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences relative to untreated controls were indicated: *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

4. 9. Regulation of fibulin-2 and fibronectin-1 mRNA

To study whether IGFBP-2 overexpression affects synthesis of extracellular matrix (ECM) proteins, abundance of fibulin-2 and fibronectin-1 (FN-1) mRNA in wt and IGFBP-2 (+/-) mLMFs was determined by real-time (RT)-PCR. To evaluate whether fibulin-2 and FN-1 mRNA expression in wt and IGFBP-2 (+/-) mLMFs is differentially regulated by growth factors, mLMFs were cultivated in the presence or absence of IGF-I, PDGF-BB and TGF-β. IGF-I dose-dependently stimulated fibulin-2 (1.76 ± 0.12-fold increase relative to untreated controls) mRNA in wt mLMFs whereas in IGFBP-2 (+/-) mLMFs IGF-I abrogated fibulin-2 (0.4 ± 0.1-fold decrease relative to untreated controls) mRNA expression **(Fig. 25 and 26)**. PDGF-BB (10 ng/ml) had no

effect on fibulin-2 mRNA in both wt and IGFBP-2 (+/-) mLMFs. The profibrogenic cytokine TGF-β at 10 ng/ml stimulated fibulin-2 mRNA in wt mLMFs (2.0 ± 0.4-fold increase relative to untreated controls), but decreased its expression in IGFBP-2 (+/-) mLMFs (0.63 ± 0.09-fold decrease relative to untreated controls) **(Fig.26)**. In addition, IGF-I (100 nmol/L) (1.36 ± 0.39-fold increase compared with untreated controls), PDGF-BB (10 ng/ml) (1.49 ± 0.67-fold increase) and TGF-β (10 ng/ml) (1.38 ± 0.34-fold increase) stimulated synthesis of fibronectin-1 mRNA in wt mLMFs **(Fig. 27)**. In contrast, IGF-I (0.78 ± 0.40-fold decrease relative to untreated controls) and PDGF-BB (0.69 ± 0.31-fold) were decreased FN1 mRNA in IGFBP-2 (+/-) mLMFs whereas TGF-β had no effect.

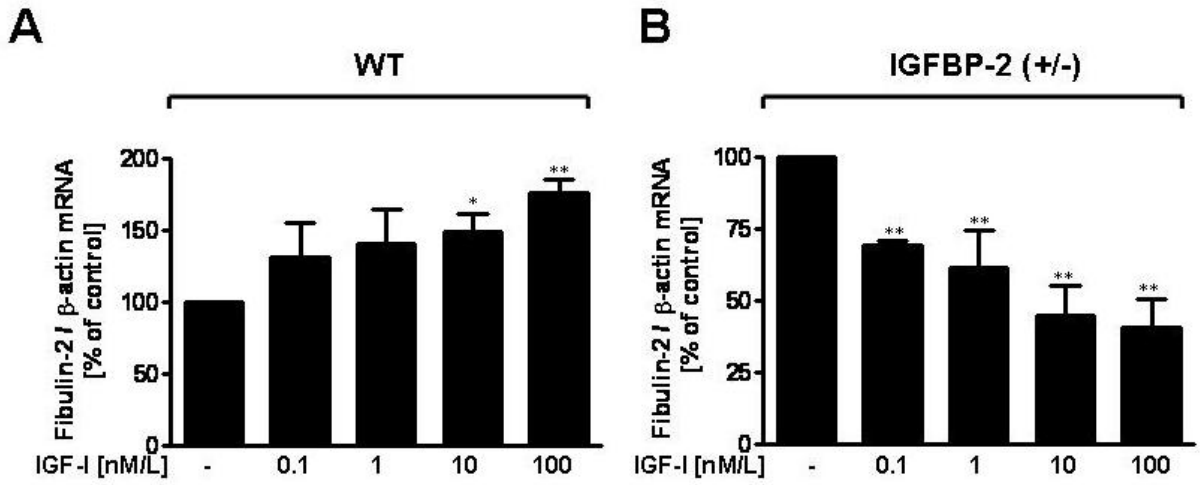

Figure 25. Effect of IGF-I on fibulin-2 mRNA expression in mLMFs analysed by quantitative RT-PCR. Wt and IGFBP-2 (+/-) mLMFs were cultivated with SRM in the presence of IGF-I at concentrations ranging from 0.1 to 100 nmol/L for 36 h. Expression of fibulin-2 mRNA was determined by quantitative RT-PCR amplification with 16 ng of cDNA prepared by reverse transcription of total RNA (1 µg) using M-MLV RT. **A, B:** Representative quantitative RT-PCRs of fibulin-2 mRNA in wt and IGFBP-2 (+/-) mLMFs. The relative expression of fibulin-2 mRNA was expressed as percent increase or decrease (means ± SD) compared to untreated controls (n = 2 to 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs) after normalization against mouse endogenous β-actin. Statistically significant differences relative to untreated controls were indicated: *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

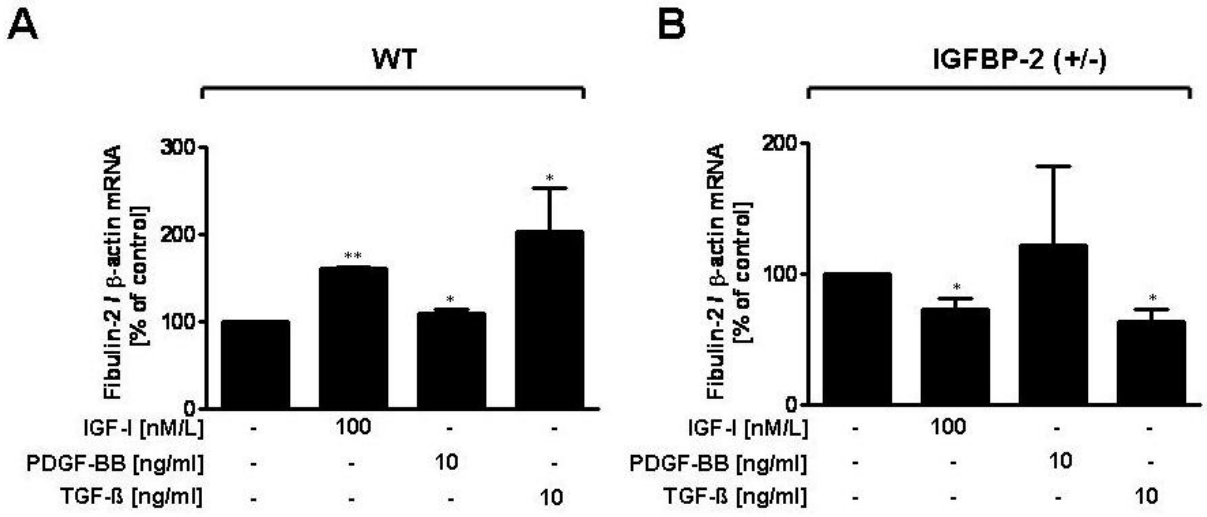

Figure 26. Effect of IGF-I, PDGF-BB and TGF-β on fibulin-2 mRNA expression in mLMFs analysed by quantitative RT-PCR. Wt and IGFBP-2 (+/-) mLMFs were incubated in SRM in the presence or absence of IGF-I (100 nmol/L), PDGF-BB (10 ng/ml) and TGF-β (10 ng/ml) for 36 h. Total RNA (1 µg) isolated from these cells were reverse transcribed to cDNA. 16 ng of cDNA were then used to study fibulin-2 mRNA by quantitative RT-PCR amplification. **A, B:** Representative quantitative RT-PCRs of fibulin-2 mRNA in wt and IGFBP-2 (+/-) mLMFs. The results were given as percent increase or decrease (means ± SD) of fibulin-2 mRNA expression compared to untreated controls (n = 2 independent isolations of wt and IGFBP-2 (+/-) mLMFs) after normalization against mouse endogenous β-actin. Statistically significant differences relative to untreated controls were indicated: *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

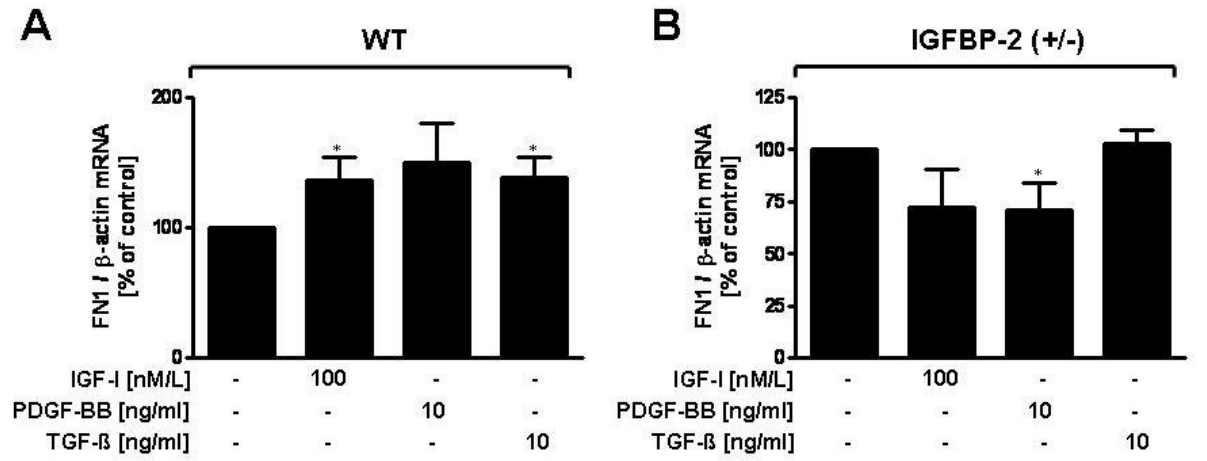

Figure 27. Effect of IGF-I, PDGF-BB and TGF-β on fibronectin-1 mRNA expression in mLMFs analysed by quantitative RT-PCR. Wt and IGFBP-2 (+/-) mLMFs were grown in serum-reduced conditions in the presence or absence of IGF-I (100 nmol/L), PDGF-BB (10 ng/ml) and TGF-β (10 ng/ml) for 36 h. Assessment of fibronectin-1 mRNA by quantitative RT-PCR amplification was performed as mentioned in Fig. 2. **A, B:** Representatives quantitative RT-PCRs of fibronectin-1 mRNA in wt and IGFBP-2 (+/-) mLMFs. After normalization against mouse endogenous β-actin the relative expression of fibronectin-1 mRNA was expressed as percent increase or decrease (means ± SD) compared to the respective untreated controls (n = 3 independent isolations of wt and IGFBP-2 (+/-) mLMFs). Statistically significant differences relative to untreated controls were indicated: *, $P < 0.05$ (Student's t test).

5. Discussion

Liver fibrosis results from excessive accumulation of ECM proteins which are secreted from cells of the fibroblast lineage during chronic liver injury (Hossenlopp et al., 1986; Ramadori and Saile, 2004). Morphologically and functionally different liver cell populations are involved in this process: activated HSCs and portal and perivascular LMFs (Bataller and Brenner, 2005; Cassiman et al., 2002; Knittel et al., 1999a; Knittel et al., 1999b). Among them, LMFs apparently play a crucial role in liver fibrogenesis since, in contrast to HSCs, LMFs do not undergo spontaneous apoptosis. The IGF system including IGF-I and -II, IGF-IR, IGF-II/M6-PR and six high affinity IGFBPs is involved in the regulation of growth and differentiation of cells of the fibroblast lineage, possibly contributing to the fibrogenic process (Novosyadlyy et al., 2004; Pinzani and Rombouts, 2004; Scharf et al., 1998a). Interestingly, in liver cirrhosis, hepatic expression and circulating levels of IGFBP-2 are significantly increased and positively correlated with the severity of disease (Kratzsch et al., 1995; Novosyadlyy et al., 2006b; Ross et al., 1996; Scharf et al., 1996). In general, IGFBP-2 has been demonstrated either to inhibit or to potentiate the IGF effects depending on the cell type studied, but the role of IGFBP-2 in liver fibrogenesis is unclear. Therefore, in the current work mLMFs isolated from livers of wild type (wt) and IGFBP-2 transgenic (IGFBP-2 (+/-)) mice were used as *in vitro* model to study the functional role of IGFBP-2 in cellular functions of mLMFs.

IGFBP-2 (+/-) mLMFs showed an approximately four to five-fold increased IGFBP-2 mRNA expression compared to wt mLMFs which was significantly different at different time points of culture (days 2 to 5). In contrast, in IGFBP-2 (+/-) mLMFs expression of IGFBP-3 mRNA was high at day 2 of culture but low at day 5 of culture whereas IGFBP-3 mRNA was low at day 2 of culture but high at day 5 of culture in wt mLMFs. The expression levels of IGF-I, IGF-IR and IGF-II/M6-PR mRNA were increased in IGFBP-2 (+/-) mLMFs compared to wt mLMFs at days 2 to 5. Moreover, IGF-I dose-dependently stimulated DNA synthesis as well as expression of ECM proteins such as fibulin-2 and fibronectin-1 in wt mLMFs whereas those effects were abrogated in IGFBP-2 (+/-) mLMFs. These data implicate a role of IGFBP-2 in cellular proliferation and in biosynthesis of ECM proteins in mLMFs *in vitro* and most likely during liver fibrosis.

So far, knowledge on the functional role of increased IGFBP-2 during liver cirrhosis (Holt et al., 1996; Ross et al., 1996; Wolf et al., 2000) is not available. In the present study, LMFs isolated from livers of wt and IGFBP-2 (+/-) mice expressed IGFBP-2 and -3 during different time points of culture (days 2 to 5). An approximately four to five-fold increased expression of IGFBP-2 was observed in IGFBP-2 (+/-) mLMFs compared to wt mLMFs **(Fig. 7, 8)**. In rats, the liver is the main source of circulating IGFs (Kratzsch et al., 1995; Mathews et al., 1986) and of IGFBP-1 to -4 that has been reported to be expressed in a cell-specific manner (Novosyadlyy et al., 2005; Scharf and Braulke, 2003; Scharf et al., 2001; Schwander et al., 1983). In contrast to other IGFBPs, IGFBP-2 is unique since it is the only IGFBP that was secreted by different cells in primary culture, e.g. hepatocytes (HCs), Kupffer cells (KCs), sinusoidal endothelial cells (SECs) and LMFs (Novosyadlyy et al., 2005; Novosyadlyy et al., 2006b; Scharf and Braulke, 2003; Scharf et al., 2001), but not by HSCs (Novosyadlyy et al., 2006b). Moreover, secretion of IGFBP-3 was demonstrated in rat LMFs. Of interest, the data presented herein indicate the secretion of similar species of IGFBPs in both rat and mouse LMFs. Expression of IGFBP-3 mRNA in wt mLMFs was low at day 2 of culture but higher at day 5. In IGFBPP-2 (+/-) mLMFs, an inverse time course of IGFBP-3 mRNA was observed with high levels at day 2 and low levels at day 5 of culture **(Fig. 7, 8)**. This indicates that an increasing expression of IGFBP-2 in IGFBP-2 (+/-) mLMFs could decrease IGFBP-3 mRNA expression with the time of culture in these cells. Thus, it is possible that increased expression of IGFBP-2 may acquire an antagonistic effect on IGFBP-3 expression which is decreased in LMFs. Moreover, an increased expression of hepatic IGFBP-2 mRNA in growth reduced mice selected for low body weight was associated with increased IGFBP-2 but decreased IGFBP-3 serum levels *in vivo* (Rogers et al., 1996). On the other hand, targeted disruption of IGFBP-2 increases expression of IGFBP-3 in sera of adult IGFBP-2 null male mice (Hoeflich et al., 1998). This indicates that overproduction or deletion of one IGFBP is associated with a compensatory decrease or increase of other IGFBPs. Consistent with these *in vivo* studies, the current study indicates that overexpression of IGFBP-2 in IGFBP-2 (+/-) mLMFs is associated with decreasing IGFBP-3 mRNA expression. Nevertheless, the mechanism by which IGFBP-2 overexpression decreases IGFBP-3 in mLMFs is unclear.

Studies in different cell lines have shown that the biosynthesis of IGFBPs is under the control of hormones, growth factors and cytokines such as IGFs, PGDFs and TGF-β (Baxter, 2000; Clemmons, 1998; Collett-Solberg and Cohen, 2000; Ferry et al., 1999a; Hwa et al., 1999; Kelley et al., 1996; Poretsky et al., 1999; Rajaram et al., 1997; Rechler, 1993; Wood et al., 2000). Regulation of IGFBPs is of special importance since local concentrations of IGFs and their bioavailability in the tissue environment are modulated by the presence of high affinity IGFBPs. In the present study, exogenously added IGF-I decreased IGFBP-2 mRNA and protein in wt mLMFs whereas in IGFBP-2 (+/-) mLMFs IGF-I increased IGFBP-2 levels. Conversely, IGF-I-dependent induction of IGFBP-3 mRNA and protein was more prominent in wt mLMFs than in IGFBP-2 (+/-) mLMFs **(Fig. 12, 13, 14, 15)**. Notably, in primary cultures of rat liver cells IGF-I decreased IGFBP-2 in HCs (Clemmons, 2001), but increased IGFBP-2 and -3 in LMFs (Scharf et al., 1996), and only IGFBP-3 in HSCs. In contrast, treatment of primary bovine mammary fibroblasts with IGF-I did not alter IGFBP-2 but slightly increased IGFBP-3 mRNA (Novosyadlyy et al., 2004). All these data suggest a differential regulation of IGFBPs biosynthesis by IGF-I in a cell-, tissue- and species-specific manner. Nevertheless, it is likely that overexpression of IGFBP-2 in IGFBP-2 (+/-) mLMFs could inverse IGF-I dose-dependent downregulation of IGFBP-2 that was observed in wt mLMFs, and decrease IGF-I-stimulated IGFBP-3 levels compared to wt mLMFs. These effects might be to due to IGF-independent effect of extracellular IGFBP-2 via autocrine and paracrine mechanisms through cell surface attachment.

Following liver injury, in addition to IGF-I, other mitogenic growth factors such as PDGF-BB, and profibrogenic growth factors such as TGF-β are known to be released from resident or infiltrating inflammatory cells. These growth factors have been reported to be involved in regulating proliferation and ECM deposition of LMFs and HSCs as well as in regulation of IGFBP production (Casini et al., 1993; Fleming et al., 2005; Gentilini et al., 1998; Gressner and Bachem, 1995; Novosyadlyy et al., 2004; Pinzani, 1995). In the present study, PDGF-BB did not alter expression of IGFBP-2 and -3 whereas TGF-β stimulated biosynthesis of IGFBP-2 and -3 **(Fig. 13, 15)**. These effects were not significantly different in wt and IGFBP-2 (+/-) mLMFs. In line with the present data, PDGF-BB had no effect on regulation of IGFBPs in human HSCs whereas TGF-β stimulated both IGFBP-2 and -3 in rat LMFs but only IGFBP-3

in human HSCs (Gentilini et al., 1998; Novosyadlyy et al., 2006b). These studies indicate the consistency in PDGF-BB and TGF-β induced effects on IGFBP-2 and -3 in wt and IGFBP-2 (+/-) mLMFs.

Although overwhelming knowledge on biosynthesis and function of IGF-I and IGF-IR is available, the precise role of IGF-I and IGF-IR in the context of IGFBP-2 overexpression in the pathogenesis of liver cirrhosis is unclear. The liver has been demonstrated to be the principle source of circulating IGF-I in body (Novosyadlyy et al., 2004). In several studies, IGF-I have been shown to be synthesized from parenchymal and non-parenchymal cells present in rat liver including LMFs, and to exert mitogenic effect through IGF-IR present on HSCs, SECs, KCs, (Brenzel and Gressner, 1996; Scharf et al., 1998b; Sjogren et al., 1999; Zimmermann et al., 2000; Zindy et al., 1992) and LMFs (Baserga, 1995). Moreover, Novosyadlyy *et al.* (2004) demonstrated steady state expression of IGF-I and IGF-IR mRNA in rat LMFs during different time points of culture. In the present study, IGFBP-2 (+/-) mLMFs revealed increased levels of IGF-I and IGF-IR compared with wt mLMFs *in vitro* **(Fig. 9, 10, 11)**. Similarly, in C6 glioma cells overproduction of IGFBP-2 was associated with increased expression of IGF-I mRNA and peptide *in vitro* (Novosyadlyy et al., 2004) suggesting an agonistic role of IGFBP-2 overproduction on increased expression of IGF-I and IGF-IR mRNA in IGFBP-2 (+/-) mLMFs.

Furthermore, a decrease in total serum IGF-I levels has been identified in patients with liver cirrhosis as well as in animal models of experimental liver cirrhosis (Bradshaw et al., 1999; Buzzelli et al., 1993; Castilla-Cortazar et al., 2000; Caufriez et al., 1991; Conchillo et al., 2007; Mirpuri et al., 2002; Scharf et al., 1996). Hepatocytes are known to be the main source of IGF-I in the liver (Gayan-Ramirez et al., 1998). During chronic liver injury, hepatocytes undergo necrosis in line with a decrease in the production of IGF-I. At the same time, the expression of IGF-I is increased in nonparenchymal liver cells such as HSCs, KCs, SECs and LMFs which, however, can not compensate the loss of IGF-I biosynthesis in HCs (Scharf et al., 2001) leading to low serum concentrations in cirrhotic patients. Therefore, from the present data it can be speculated that overexpression of IGFBP-2 has a positive correlation with increased IGF-I and IGF-IR mRNA in IGFBP-2 (+/-) mLMFs *in vitro*. Further studies on IGF-I and IGF-IR expression levels in parenchymal and remaining

nonparenchymal liver cells of wt and IGFBP-2 (+/-) mice are necessary to extend the knowledge on the correlation between IGF-I and IGF-IR expression, and IGFBP-2 overexpression in physiological and pathophysilogical conditions of the liver.

Novosyadlyy *et al.* delineated a downregulation of the IGF-IR by its primary ligand IGF-I in rat LMFs (Novosyadlyy et al., 2005). Similarly, a downregulation of IGF-IR mRNA and its protein by exogenous IGF-I was observed in both wt and IGFBP-2 (+/-) mLMFs which was not significantly different in both cell lines **(Fig. 16, 17, 18, 19)**. The data of present study demonstrated that IGFBP-2 overexpression did not influence the IGF-I-induced downregulation of IGF-IR mRNA expression.

In LMFs cultured in the presence of PDGF-BB, no alterations of IGF-IR mRNA expression in both wt and IGFBP-2 (+/-) mLMFs were detectable. IGFBP-2 overexpression had no influence on PDGF-BB mediated IGF-IR mRNA expression and its protein biosynthesis compared with wt mLMFs **(Fig. 17, 19)**. In rat LMFs, PDGF-BB was capable to slightly increase the IGF-IR and to abolish the IGF-I-induced downregulation of the IGF-IR (Novosyadlyy et al., 2004). Knock out studies of *IGF-IR gene* in mouse embryo fibroblasts demonstrated a central role of the IGF-IR for PDGF-BB-dependent cell proliferation since IGF-IR-deficient fibroblasts failed to proliferate in response to PDGF (Novosyadlyy et al., 2006b). The present data suggest that despite increased IGFBP-2 during chronic liver injury, PDGF-BB could maintain IGF-I-induced biological actions through the IGF-IR by preventing IGF-I-dependent downregulation of the IGF-IR during fibrogenesis.

There is little information available on modulation of IGF-IR by TGF-β. Tsukazaki *et al.* demonstrated upregulation of IGF-IR by TGF-β in cultured rat articular chondrocytes (DeAngelis et al., 1995). Consistent with these data, in the present study, TGF-β stimulated IGF-IR mRNA and its protein levels in both wt and IGFBP-2 (+/-) mLMFs that was not significantly different **(Fig. 17, 19)**. This indicates that regulation of the IGF-IR by TGF-β is mediated by IGF-IGFBP-independent mechanisms. Furthermore, TGF-β has the capability of inducing IGF-I mRNA in various cell culture systems, for example, in intestinal fibroblast (Tsukazaki et al., 1994). In summary, these data suggest that in addition to the TGF-β specific effects

via different TGF- receptors TGF-β might play a crucial role during liver fibrogenesis via induction of both IGF-I and IGF-IR.

Besides differential expression of IGF-IR, all the different parenchymal and nonparenchymal liver cells have been reported to synthesis the IGF-II/M6-PR (Novosyadlyy et al., 2004; Schmitz et al., 1995; Simmons et al., 2002; Waguri et al., 2001; Zindy et al., 1992). De Bleser *et al*. and Scharf *et al.* reported an increased expression of IGF-II/M6-PR on activated rat HSCs *in vivo* and *in vitro*, respectivey (Scharf and Braulke, 2003; Scharf et al., 1998a). The data presented herein showed the presence of the IGF-II/M6-PR mRNA in both wt and IGFBP-2 (+/-) mLMFs. Steady state mRNA levels of the IGF-II/M6-PR were higher in IGFBP-2 (+/-) mLMFs than in wt mLMFs **(Fig. 9, 10)**. Converging data pointed to an important role of increased expression of the IGF-II/M6-PR in targeting of mannose-6-phosphate-bearing glycoproteins including IGF-II, and consequently in growth inhibition of various tumors *in vivo* and *in vitro* (de Bleser et al., 1995; O'Gorman et al., 2002; Zaina and Squire, 1998). Moreover, IGF-II/M6-PR is capable to bind latent TGF-β facilitating its cleavage into the active form that then acts as a growth inhibitor to most cell types including rat HSCs (Dennis and Rifkin, 1991; Hebert, 2006). Armendariz-Borunda *et al.* described release of TGF-β in the necrotic centrilobular areas of the liver after CCl_4 treatment (Issa et al., 2001). Consistent with this, overexpression of IGF-II/M6-PR was observed in patients with cirrhotic liver tissue (Armendariz-Borunda et al., 1993) and animal models of liver cirrhosis (Fan et al., 2001). Therefore, during chronic liver injury, increased levels of IGFBP-2 may enhance IGF-II/M6-PR in line with an activation of latent TGF-β to its active form that might in turn promote fibrogenesis.

PDGF-BB has been demonstrated to involve in upregulation of IGF-II/M6-PR mRNA in HSCs. Moreover, it is known that TGF-β is involved induction of IGF-II/M6-PR by inducing PDGF β receptor (Braulke et al., 1988; Pinzani et al., 1995) to which PDGF-BB binds and induces IGF-II/M6-PR thereby activation of latent TGF-β to its active form that eventually promotes liver fibrosis. In contrary, in addition to IGF-I, PDGF-BB and TGF-β did not alter the expression of IGF-II/M6-PR mRNA in wt and IGFBP-2 (+/-) mLMFs **(Fig. 20, 21)**.

Prior studies revealed that high local concentration of IGF-I triggers DNA synthesis and proliferation in both rat HSCs (Pinzani et al., 1995; Saile et al., 2004; Scharf et al., 1998a) and LMFs (Novosyadlyy et al., 2006b; Saile et al., 2004) isolated from rat liver. In accordance with these data, the current study showed IGF-I dose-dependent stimulation of DNA synthesis in wt mLMFs that was abrogated in IGFBP-2 (+/-) mLMFs **(Fig. 22, 23, 24)** indicating the inhibitory role of IGFBP-2 in IGF-I-induced DNA synthesis of IGFBP-2 (+/-) mLMFs. In line with the data of present study, overexpression of IGFBP-2 inhibited the proliferation of human embryonic kidney fibroblasts which was reversed by exogenous addition of IGF-I (Novosyadlyy et al., 2004; Wolf et al., 2000). Further, purified IGFBP-2 has been reported to abrogate IGF-I-induced [^{3}H]-thymidine incorporation into chick embryo fibroblasts (Hoflich et al., 1998) demonstrating IGFBP-2 inhibitory role on IGF-I-induced proliferation *in vitro.* GH transgenic mice which had 2- to -3 fold increased serum IGF-I reduced growth rates when crossed with IGFBP-2 transgenic mice *in vivo.* These studies indicate the consistence in the present data of inhibition in IGF-I-induced DNA synthesis in IGFBP-2 (+/-) mLMFs. Moreover, these studies suggest that the possibility for sequestration of IGF-I to increased IGFBP-2, which might be in soluble, ECM- and/or membrane-bound form. Thereby that leads to formation of the inactive IGF-I/IGFBP-2 complex which does not allow IGF-I to bind IGF-IR (Jones and Clemmons, 1995), which in turn might exert IGFBP-2 inhibitory effect on IGF-induced DNA synthesis in IGFBP-2 (+/-) mLMFs. However, the potency of inhibitory action of IGFBP-2 on IGF-I actions is dependent on ratio of IGF-I and IGFBP-2 within the local cellular environment. According to the data presented herein, the inhibitory effect of IGFBP-2 on IGF-I-induced DNA synthesis was maximum at 100 nM/L concentration of IGF-I. The incubation of rat LMFs with equimolar amounts of exogenous IGF-I and IGFBP-2 resulted in inhibition of cellular proliferation in rat LMFs whereas high amount of IGF-I and low IGFBP-2 potentiated DNA synthesis in rat LMFs (Novosyadlyy et al., 2004). In addition, reduction in weight of the liver observed in IGFBP-2 transgenic mice (Hoeflich et al., 1999) was reversed in IGF-I transgenic mice *in vivo* (Mathews et al., 1988) implicating that the ratio of IGF-I and IGFBP-2 is critical for IGFBP-2 inhibitory action on IGF-I-induced DNA synthesis in IGFBP-2 (+/-) mLMFs. However, IGF-independent effects are also attributed to IGFBP-2 in inhibition of cell proliferation in certain types of cells. For example, membrane-bound IGFBP-2 reduced cell proliferation in tumor cells (Mathews et al., 1988). Although

studies on IGFBP-2 independent action without binding to IGF-I are less in fibroblasts, one can expect that overexpression of IGFBP-2 in IGFBP-2 (+/-) mLMFs can exert inhibitory effect on IGF-I-induced DNA synthesis by binding to cell surface in these cells as reported for IGFBP-3 in IGF-I-receptor-null fibroblast cells (Valentinis et al., 1995)

Several other studies reported nuclear import of IGFBP-2 and its interaction with the cyclin-dependent kinase inhibitor p21CIP/WAF which is a positive regulator of IGF-I-induced proliferation in certain cell types (Besnard et al., 2001; Schutt et al., 2004). The presence of IGFBP-2 in peri/nuclear fractions isolated from different tissues of IGFBP-2 transgenic mice littermates were reported *in vivo* (Terrien et al., 2005). Therefore, it points that together extra- and intra-cellular IGFBP-2 may involve in mitoinhibitory effect on IGF-I-induced DNA synthesis in IGFBP-2 (+/-) mLMFs. Nevertheless, intracellular localization of IGFBP-2 in IGFBP-2 (+/-) mLMFs and combined effect of extra- and intra-cellular IGFBP-2 are remained to answer. Taken together, these data indicate that overexpression of IGFBP-2 has inhibitory role in proliferation of LMFs during the development of liver fibrosis.

In addition, abundance of fibulin-2 and fibronectin-1 as characteristic ECM proteins have been acquired a significant attention in liver fibrogenesis. Converging evidence pointed the expression of collagen type IV and fibronectin in HSCs and LMFs but only fibulin-2 in cultured liver myofibroblasts (Ramadori and saile, 2004). The mitogenic growth factor such as IGF-I stimulated the proliferation and type I collagen, the principal ECM protein, in HSCs *in vitro* (Gentilini et al., 1998; Ramadori and Saile, 2004; Scharf et al., 1998b; Svegliati-Baroni et al., 1999). Therefore, it is believed that due to its chemotactic, mitogenic and fibrogenic activity IGF-I which is released locally during hepatic injury triggers LMFs, thereby leading to their activation, proliferation as well as ECM production, and finally to perpetuation of fibrogenic response within the liver. Intriguingly, in the data of present study IGF-I-induced fibulin-2 and fibronectin-1 mRNA in wt mLMFs whereas those effects were abrogated in IGFBP (+/-) mLMFs **(Fig. 25, 26)**. So, it points that overexpression of IGFBP-2 in IGFBP-2 (+/-) mLMFs has inhibitory role on IGF-I-induced secretion of ECM components in these cells. Russo et al (Pinzani and Marra, 2001) demonstrated the ability of IGFBP-2 to bind different ECM components including collagen and

fibronectin. Moreover, in accordance with the present study of characterization of ECM components such as collagen-3 in wt and IGFBP-2 (+/-) mLMFs, the basal level expression of collagen-3 was appeared to be more in wt mLMFs than in IGFBP-2 (+/-) mLMFs. So, presumably increased levels of IGFBP-2 could interact with cell surface, probably by decreasing chances of binding to ECM components, which in turn inhibits IGF-I-induced fibulin-2 and fibronectin 1 mRNA in IGFBP-2 (+/-) mLMFs. Thus, overproduction of IGFBP-2 has inhibitory role in IGF-I-induced biosynthesis of fibulin-2 and fibronectin 1 mRNA as IGFBP-2 inhibitory role in IGF-I-induced DNA synthesis in IGFBP-2 (+/-) mLMFs. Nevertheless, further experiments are necessory to evaluate IGF-I-induced fibulin-2 and fibronectin 1 at protein levels in wt and IGFBP-2 (+/-) mLMFs.

It is known that normal liver tissue is lacking a significant expression of PDGF-BB. Conversely, in liver cirrhosis, the release of PDGF-BB significantly increases and correlates with the progression of disease (Pinzani et al., 1996; Russo et al., 2005b). In addition, PDGF-BB was shown to be involved in DNA synthesis of human HSCs (Zhang et al., 2003) and rat LMFs (Adachi et al., 2005). Consistent with these findings, the data of the current study showed that PDGF-BB stimulated DNA synthesis was more pronounced in wt mLMFs than in IGFBP-2 (+/-) mLMFs **(Fig. 23)**. Therefore, that overexpression of IGFBP-2 decreases PDGF-BB stimulated DNA synthesis in IGFBP-2 (+/-) mLMFs. Moreover, PDGF-BB stimulated secretion of endogenous IGF-I in rat HSCs (Novosyadlyy et al., 2006b). Therefore, it is likely that a decrease in PDGF-BB stimulated DNA synthesis in IGFBP-2 (+/-) mLMFs might be mediated through IGF-I-dependent IGFBP-2 action. Regarding modulatory effects of PDGF-BB on ECM proteins in LMFs, information is scarce. In the present study, PDGF-BB had no effect on fibulin-2 mRNA in wt and IGFBP-2 (+/-) mLMFs whereas PDGF-BB stimulated fibronectin 1 mRNA in wt mLMFs but PDGF-BB induced fibronectin 1 mRNA expression was abrogated in IGFBP-2 (+/-) mLMFs **(Fig. 26, 27)**. Thus, the data of the current study indicate that overexpression of IGFBP-2 has inhibitory effect of PDGF-BB induced fibronectin 1 mRNA in IGFBP-2 (+/-) mLMFs.

TGF-β has been demonstrated to play a role in several fibrotic disorders including liver cirrhosis (Pinzani et al., 1990). TGF-β has been demonstrated to inhibit proliferation in various cell types, for example, in rat HSCs (Border and Noble, 1994)

and liver portal fibroblasts (Saile et al., 1999) *in vitro*. In the present data, the inhibitory effect of TGF-β on DNA synthesis in wt mLMFs was more prominent than in IGFBP-2 (+/-) mLMFs **(Fig. 23)** indicating overexpression of IGFBP-2 diminish the TGF-β induced inhibitory effect on DNA synthesis of IGFBP-2 (+/-) mLMFs. Similarly, TGF-β has been demonstrated to inhibit DNA synthesis through increasing endogenous IGFBP-2 production in mink lung epithelial cells (Wells et al., 2004). Consistent with this report, TGF-β induced IGFBP-2 in mLMFs in the present study **(Fig. 14, 16)**. This indicates that the inhibitory effect of TGF-β on DNA synthesis in mLMFs could be mediated by increasing endogenous IGFBP-2 levels in these cells. In addition, studies of hepatic fibrogenesis in experimental models of liver fibrosis as well as in human liver diseases clearly indicated the predominant role of TGF-β in stimulating fibrogenesis (Castilla et al., 1991; Dong et al., 2002). TGF-β has been shown to markedly enhance the expression of ECM proteins in cultured fibroblasts. Ramadori *et al.* (1998) reported TGF-β upregulation of matrix gene expression including fibronectin 1 in activated HSCs. In line with these studies, in the present data, TGF-β stimulated the expression of fibulin-2 and fibronectin 1 mRNA in wt mLMFs, whereas TGF-β induced fibulin-2 mRNA was inhibited but there was no effect on TGF-β induced fibronectin 1 mRNA in IGFBP-2 (+/-) mLMFs **(Fig. 26, 27)**. This indicates that overexpression of IGFBP-2 has an inhibitory effect on TGF-β induced fibulin-2 mRNA, but not on fibronectin 1 mRNA in IGFBP-2 (+/-) mLMFs. Further experiments are necessary to elucidate the mechanism by which IGFBP-2 regulates the TGF-β induced ECM production in mLMFs.

In summary, our data demonstrate that overexpression of IGFBP-2 in LMFs was involved in abrogation of proliferation and of biosynthesis of ECM components in LMFs *in vitro*. Thus, it can be hypothesized that together with functional properties of multiple growth factors released during chronic liver injury from nearby parenchymal and nonparenchymal cells, and cells recruited at the site of injury, overexpression of IGFBP-2 might play a pivotal role in inhibition of proliferation and of biosynthesis of ECM components in LMFs during developement of liver fibrosis *in vivo*.

6. References:

Abuzzahab MJ, Schneider A, Goddard A, Grigorescu F, Lautier C, Keller E, Kiess W, Klammt J, Kratzsch J, Osgood D, Pfaffle R, Raile K, Seidel B, Smith RJ, Chernausek SD. 2003. IGF-I receptor mutations resulting in intrauterine and postnatal growth retardation. N Engl J Med 349(23):2211-2222.

Adachi T, Togashi H, Suzuki A, Kasai S, Ito J, Sugahara K, Kawata S. 2005. NAD(P)H oxidase plays a crucial role in PDGF-induced proliferation of hepatic stellate cells. Hepatology 41(6):1272-1281.

Adamo M, Roberts CT, Jr., LeRoith D. 1992. How distinct are the insulin and insulin-like growth factor I signalling systems? Biofactors 3(3):151-157.

Adamo ML, Neuenschwander S, LeRoith D, Roberts CT, Jr. 1993. Structure, expression, and regulation of the IGF-I gene. Adv Exp Med Biol 343:1-11.

Andress DL. 1998. Insulin-like growth factor-binding protein-5 (IGFBP-5) stimulates phosphorylation of the IGFBP-5 receptor. Am J Physiol 274(4 Pt 1):E744-750.

Arany E, Zabel P, Hill DJ. 1996. Rapid clearance of human insulin-like growth factor binding protein-3 from the rat circulation and cellular localization in liver, kidney and stomach. Growth Regul 6(1):32-41.

Armendariz-Borunda J, Katai H, Jones CM, Seyer JM, Kang AH, Raghow R. 1993. Transforming growth factor beta gene expression is transiently enhanced at a critical stage during liver regeneration after CCl4 treatment. Lab Invest 69(3):283-294.

Bach LA. 1999. Insulin-like growth factor binding protein-6: the "forgotten" binding protein? Horm Metab Res 31(2-3):226-234.

Baker J, Liu JP, Robertson EJ, Efstratiadis A. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75(1):73-82.

Bale LK, Conover CA. 1992. Regulation of insulin-like growth factor binding protein-3 messenger ribonucleic acid expression by insulin-like growth factor I. Endocrinology 131(2):608-614.

Barlow DP. 1995. Gametic imprinting in mammals. Science 270(5242):1610-1613.

Baruch Y. 2000. The liver: a large endocrine gland. J Hepatol 32(3):505-507.

Baserga R. 1995. The insulin-like growth factor I receptor: a key to tumor growth? Cancer Res 55(2):249-252.

Bataller R, Brenner DA. 2005. Liver fibrosis. J Clin Invest 115(2):209-218.

Baxter RC. 2000. Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. Am J Physiol Endocrinol Metab 278(6):E967-976.

Baxter RC, Martin JL, Beniac VA. 1989. High molecular weight insulin-like growth factor binding protein complex. Purification and properties of the acid-labile subunit from human serum. J Biol Chem 264(20):11843-11848.

Baxter RC, Meka S, Firth SM. 2002. Molecular distribution of IGF binding protein-5 in human serum. J Clin Endocrinol Metab 87(1):271-276.

Bennett A, Wilson DM, Liu F, Nagashima R, Rosenfeld RG, Hintz RL. 1983. Levels of insulin-like growth factors I and II in human cord blood. J Clin Endocrinol Metab 57(3):609-612.

Besnard V, Corroyer S, Trugnan G, Chadelat K, Nabeyrat E, Cazals V, Clement A. 2001. Distinct patterns of insulin-like growth factor binding protein (IGFBP)-2 and IGFBP-3 expression in oxidant exposed lung epithelial cells. Biochim Biophys Acta 1538(1):47-58.

Binoux M, Hossenlopp P. 1988. Insulin-like growth factor (IGF) and IGF-binding proteins: comparison of human serum and lymph. J Clin Endocrinol Metab 67(3):509-514.

Boes M, Booth BA, Sandra A, Dake BL, Bergold A, Bar RS. 1992. Insulin-like growth factor binding protein (IGFBP)4 accounts for the connective tissue distribution of endothelial cell IGFBPs perfused through the isolated heart. Endocrinology 131(1):327-330.

Border WA, Noble NA. 1994. Transforming growth factor beta in tissue fibrosis. N Engl J Med 331(19):1286-1292.

Bradshaw SL, D'Ercole AJ, Han VK. 1999. Overexpression of insulin-like growth factor-binding protein-2 in C6 glioma cells results in conditional alteration of cellular growth. Endocrinology 140(2):575-584.

Braulke T. 1999. Type-2 IGF receptor: a multi-ligand binding protein. Horm Metab Res 31(2-3):242-246.

Braulke T, Causin C, Waheed A, Junghans U, Hasilik A, Maly P, Humbel RE, von Figura K. 1988. Mannose 6-phosphate/insulin-like growth factor II receptor: distinct binding sites for mannose 6-phosphate and insulin-like growth factor II. Biochem Biophys Res Commun 150(3):1287-1293.

Brenzel A, Gressner AM. 1996. Characterization of insulin-like growth factor (IGF)-I-receptor binding sites during in vitro transformation of rat hepatic stellate cells to myofibroblasts. Eur J Clin Chem Clin Biochem 34(5):401-409.

Brodt P, Samani A, Navab R. 2000. Inhibition of the type I insulin-like growth factor receptor expression and signaling: novel strategies for antimetastatic therapy. Biochem Pharmacol 60(8):1101-1107.

Brown J, Esnouf RM, Jones MA, Linnell J, Harlos K, Hassan AB, Jones EY. 2002. Structure of a functional IGF2R fragment determined from the anomalous scattering of sulfur. Embo J 21(5):1054-1062.

Butt AJ, Firth SM, Baxter RC. 1999. The IGF axis and programmed cell death. Immunol Cell Biol 77(3):256-262.

Butt AJ, Firth SM, King MA, Baxter RC. 2000. Insulin-like growth factor-binding protein-3 modulates expression of Bax and Bcl-2 and potentiates p53-independent radiation-induced apoptosis in human breast cancer cells. J Biol Chem 275(50):39174-39181.

Buzzelli G, Dattolo P, Pinzani M, Brocchi A, Romano S, Gentilini P. 1993. Circulating growth hormone and insulin-like growth factor-I in nonalcoholic liver cirrhosis with or without superimposed hepatocarcinoma: evidence of an altered circadian rhythm. Am J Gastroenterol 88(10):1744-1748.

Carlberg M, Dricu A, Blegen H, Wang M, Hjertman M, Zickert P, Hoog A, Larsson O. 1996. Mevalonic acid is limiting for N-linked glycosylation and translocation of the insulin-like growth factor-1 receptor to the cell surface. Evidence for a new link between 3-hydroxy-3-methylglutaryl-coenzyme a reductase and cell growth. J Biol Chem 271(29):17453-17462.

Caro JF, Poulos J, Ittoop O, Pories WJ, Flickinger EG, Sinha MK. 1988. Insulin-like growth factor I binding in hepatocytes from human liver, human hepatoma, and normal, regenerating, and fetal rat liver. J Clin Invest 81(4):976-981.

Casini A, Pinzani M, Milani S, Grappone C, Galli G, Jezequel AM, Schuppan D, Rotella CM, Surrenti C. 1993. Regulation of extracellular matrix synthesis by transforming growth factor beta 1 in human fat-storing cells. Gastroenterology 105(1):245-253.

Cassiman D, Libbrecht L, Desmet V, Denef C, Roskams T. 2002. Hepatic stellate cell/myofibroblast subpopulations in fibrotic human and rat livers. J Hepatol 36(2):200-209.

Castilla-Cortazar I, Garcia M, Muguerza B, Quiroga J, Perez R, Santidrian S, Prieto J. 1997. Hepatoprotective effects of insulin-like growth factor I in rats with carbon tetrachloride-induced cirrhosis. Gastroenterology 113(5):1682-1691.

Castilla-Cortazar I, Garcia M, Quiroga J, Diez N, Diez-Caballero F, Calvo A, Diaz M, Prieto J. 2000. Insulin-like growth factor-I reverts testicular atrophy in rats with advanced cirrhosis. Hepatology 31(3):592-600.

Castilla A, Prieto J, Fausto N. 1991. Transforming growth factors beta 1 and alpha in chronic liver disease. Effects of interferon alfa therapy. N Engl J Med 324(14):933-940.

Caufriez A, Reding P, Urbain D, Golstein J, Copinschi G. 1991. Insulin-like growth factor I: a good indicator of functional hepatocellular capacity in alcoholic liver cirrhosis. J Endocrinol Invest 14(4):317-321.

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18(24):5294-5299.

Clemmons DR. 1998. Role of insulin-like growth factor binding proteins in controlling IGF actions. Mol Cell Endocrinol 140(1-2):19-24.

Clemmons DR. 2001. Use of mutagenesis to probe IGF-binding protein structure/function relationships. Endocr Rev 22(6):800-817.

Cohen P. 1985. The role of protein phosphorylation in the hormonal control of enzyme activity. Eur J Biochem 151(3):439-448.

Cohen P. 1992. Signal integration at the level of protein kinases, protein phosphatases and their substrates. Trends Biochem Sci 17(10):408-413.

Coleman ME, DeMayo F, Yin KC, Lee HM, Geske R, Montgomery C, Schwartz RJ. 1995. Myogenic vector expression of insulin-like growth factor I stimulates muscle cell differentiation and myofiber hypertrophy in transgenic mice. J Biol Chem 270(20):12109-12116.

Collett-Solberg PF, Cohen P. 2000. Genetics, chemistry, and function of the IGF/IGFBP system. Endocrine 12(2):121-136.

Conchillo M, Prieto J, Quiroga J. 2007. [Insulin-like growth factor I (IGF-I) and liver cirrhosis]. Rev Esp Enferm Dig 99(3):156-164.

Conover CA. 1995. Insulin-like growth factor binding protein proteolysis in bone cell models. Prog Growth Factor Res 6(2-4):301-309.

Conover CA. 1996. Regulation and physiological role of insulin-like growth factor binding proteins. Endocr J 43 Suppl:S43-48.

Conover CA, Powell DR. 1991. Insulin-like growth factor (IGF)-binding protein-3 blocks IGF-I-induced receptor down-regulation and cell desensitization in cultured bovine fibroblasts. Endocrinology 129(2):710-716.

Constancia M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, Stewart F, Kelsey G, Fowden A, Sibley C, Reik W. 2002. Placental-specific IGF-II is a major modulator of placental and fetal growth. Nature 417(6892):945-948.

Couce ME, Weatherington AJ, McGinty JF. 1992. Expression of insulin-like growth factor-II (IGF-II) and IGF-II/mannose-6-phosphate receptor in the rat hippocampus: an in situ hybridization and immunocytochemical study. Endocrinology 131(4):1636-1642.

Coverley JA, Baxter RC. 1997. Phosphorylation of insulin-like growth factor binding proteins. Mol Cell Endocrinol 128(1-2):1-5.

D'Ercole AJ, Wilkins JR. 1984. Affinity labeled somatomedin-C-binding proteins in rat sera. Endocrinology 114(4):1141-1144.

Daughaday WH, Rotwein P. 1989. Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. Endocr Rev 10(1):68-91.

de Bleser PJ, Jannes P, van Buul-Offers SC, Hoogerbrugge CM, van Schravendijk CF, Niki T, Rogiers V, van den Brande JL, Wisse E, Geerts A. 1995. Insulinlike growth factor-II/mannose 6-phosphate receptor is expressed on CCl4-exposed rat fat-storing cells and facilitates activation of latent transforming growth factor-beta in cocultures with sinusoidal endothelial cells. Hepatology 21(5):1429-1437.

De Mellow JS, Baxter RC. 1988. Growth hormone-dependent insulin-like growth factor (IGF) binding protein both inhibits and potentiates IGF-I-stimulated DNA synthesis in human skin fibroblasts. Biochem Biophys Res Commun 156(1):199-204.

DeAngelis T, Ferber A, Baserga R. 1995. Insulin-like growth factor I receptor is required for the mitogenic and transforming activities of the platelet-derived growth factor receptor. J Cell Physiol 164(1):214-221.

DeChiara TM, Robertson EJ, Efstratiadis A. 1991. Parental imprinting of the mouse insulin-like growth factor II gene. Cell 64(4):849-859.

Dennis PA, Rifkin DB. 1991. Cellular activation of latent transforming growth factor beta requires binding to the cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor. Proc Natl Acad Sci U S A 88(2):580-584.

Dong F, Wu HB, Hong J, Rechler MM. 2002. Insulin-like growth factor binding protein-2 mediates the inhibition of DNA synthesis by transforming growth factor-beta in mink lung epithelial cells. J Cell Physiol 190(1):63-73.

Dricu A, Wang M, Hjertman M, Malec M, Blegen H, Wejde J, Carlberg M, Larsson O. 1997. Mevalonate-regulated mechanisms in cell growth control: role of dolichyl phosphate in expression of the insulin-like growth factor-1 receptor (IGF-1R) in comparison to Ras prenylation and expression of c-myc. Glycobiology 7(5):625-633.

Dupont J, LeRoith D. 2001. Insulin and insulin-like growth factor I receptors: similarities and differences in signal transduction. Horm Res 55 Suppl 2:22-26.

Fan ZR, Yang DH, Cui J, Qin HR, Huang CC. 2001. Expression of insulin like growth factor II and its receptor in hepatocellular carcinogenesis. World J Gastroenterol 7(2):285-288.

Fazleabas AT, Kim JJ, Strakova Z. 2004. Implantation: embryonic signals and the modulation of the uterine environment--a review. Placenta 25 Suppl A:S26-31.

Ferry RJ, Jr., Cerri RW, Cohen P. 1999a. Insulin-like growth factor binding proteins: new proteins, new functions. Horm Res 51(2):53-67.

Ferry RJ, Jr., Katz LE, Grimberg A, Cohen P, Weinzimer SA. 1999b. Cellular actions of insulin-like growth factor binding proteins. Horm Metab Res 31(2-3):192-202.

Firth SM, Baxter RC. 1999. Characterisation of recombinant glycosylation variants of insulin-like growth factor binding protein-3. J Endocrinol 160(3):379-387.

Firth SM, Baxter RC. 2002. Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 23(6):824-854.

Fischer R, Cariers A, Reinehr R, Haussinger D. 2002. Caspase 9-dependent killing of hepatic stellate cells by activated Kupffer cells. Gastroenterology 123(3):845-861.

Fleming JM, Leibowitz BJ, Kerr DE, Cohick WS. 2005. IGF-I differentially regulates IGF-binding protein expression in primary mammary fibroblasts and epithelial cells. J Endocrinol 186(1):165-178.

Fowlkes JL, Thrailkill KM, Serra DM, Suzuki K, Nagase H. 1995. Matrix metalloproteinases as insulin-like growth factor binding protein-degrading proteinases. Prog Growth Factor Res 6(2-4):255-263.

Frasca F, Pandini G, Scalia P, Sciacca L, Mineo R, Costantino A, Goldfine ID, Belfiore A, Vigneri R. 1999. Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. Mol Cell Biol 19(5):3278-3288.

Friedman SL. 1993. Seminars in medicine of the Beth Israel Hospital, Boston. The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies. N Engl J Med 328(25):1828-1835.

Friedman SL. 1999. Cytokines and fibrogenesis. Semin Liver Dis 19(2):129-140.

Friedman SL. 2000. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. J Biol Chem 275(4):2247-2250.

Frystyk J. 2004. Free insulin-like growth factors -- measurements and relationships to growth hormone secretion and glucose homeostasis. Growth Horm IGF Res 14(5):337-375.

Furstenberger G, Senn HJ. 2002. Insulin-like growth factors and cancer. Lancet Oncol 3(5):298-302.

Gayan-Ramirez G, van de Casteele M, Rollier H, Fevery J, Vanderhoydonc F, Verhoeven G, Decramer M. 1998. Biliary cirrhosis induces type IIx/b fiber atrophy in rat diaphragm and skeletal muscle, and decreases IGF-I mRNA in the liver but not in muscle. J Hepatol 29(2):241-249.

Gentilini A, Feliers D, Pinzani M, Woodruff K, Abboud S. 1998. Characterization and regulation of insulin-like growth factor binding proteins in human hepatic stellate cells. J Cell Physiol 174(2):240-250.

Gentilini A, Marra F, Gentilini P, Pinzani M. 2000. Phosphatidylinositol-3 kinase and extracellular signal-regulated kinase mediate the chemotactic and mitogenic effects of insulin-like growth factor-I in human hepatic stellate cells. J Hepatol 32(2):227-234.

Gressner AM, Bachem MG. 1995. Molecular mechanisms of liver fibrogenesis--a homage to the role of activated fat-storing cells. Digestion 56(5):335-346.

Guler HP, Zapf J, Schmid C, Froesch ER. 1989. Insulin-like growth factors I and II in healthy man. Estimations of half-lives and production rates. Acta Endocrinol (Copenh) 121(6):753-758.

Hameed M, Orrell RW, Cobbold M, Goldspink G, Harridge SD. 2003. Expression of IGF-I splice variants in young and old human skeletal muscle after high resistance exercise. J Physiol 547(Pt 1):247-254.

Hartmann H, Schmitz F, Christ B, Jungermann K, Creutzfeldt W. 1990. Metabolic actions of insulin-like growth factor-I in cultured hepatocytes from adult rats. Hepatology 12(5):1139-1143.

Hebert E. 2006. Mannose-6-phosphate/insulin-like growth factor II receptor expression and tumor development. Biosci Rep 26(1):7-17.

Hoeflich A, Reisinger R, Schuett BS, Elmlinger MW, Russo VC, Vargas GA, Jehle PM, Lahm H, Renner-Muller I, Wolf E. 2004. Peri/nuclear localization of intact insulin-like growth factor binding protein-2 and a distinct carboxyl-terminal IGFBP-2 fragment in vivo. Biochem Biophys Res Commun 324(2):705-710.

Hoeflich A, Schmidt P, Foll J, Rottmann O, Weber MM, Kolb HJ, Pirchner F, Wolf E. 1998. Altered growth of mice divergently selected for body weight is associated with complex changes in the growth hormone/insulin-like growth factor system. Growth Horm IGF Res 8(2):113-123.

Hoeflich A, Wu M, Mohan S, Foll J, Wanke R, Froehlich T, Arnold GJ, Lahm H, Kolb HJ, Wolf E. 1999. Overexpression of insulin-like growth factor-binding protein-2 in transgenic mice reduces postnatal body weight gain. Endocrinology 140(12):5488-5496.

Hoflich A, Lahm H, Blum W, Kolb H, Wolf E. 1998. Insulin-like growth factor-binding protein-2 inhibits proliferation of human embryonic kidney fibroblasts and of IGF-responsive colon carcinoma cell lines. FEBS Lett 434(3):329-334.

Holly J, Perks C. 2006. The role of insulin-like growth factor binding proteins. Neuroendocrinology 83(3-4):154-160.

Holt RI, Crossey PA, Jones JS, Baker AJ, Portmann B, Miell JP. 1997. Hepatic growth hormone receptor, insulin-like growth factor I, and insulin-like growth factor-binding protein messenger RNA expression in pediatric liver disease. Hepatology 26(6):1600-1606.

Holt RI, Jones JS, Stone NM, Baker AJ, Miell JP. 1996. Sequential changes in insulin-like growth factor I (IGF-I) and IGF-binding proteins in children with end-stage liver disease before and after successful orthotopic liver transplantation. J Clin Endocrinol Metab 81(1):160-168.

Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S, Binoux M. 1986. Analysis of serum insulin-like growth factor binding proteins using western blotting: use of the method for titration of the binding proteins and competitive binding studies. Anal Biochem 154(1):138-143.

Hwa V, Oh Y, Rosenfeld RG. 1999. The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev 20(6):761-787.

Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawley S, Hovell C, Arthur MJ. 1998. Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. J Clin Invest 102(3):538-549.

Issa R, Williams E, Trim N, Kendall T, Arthur MJ, Reichen J, Benyon RC, Iredale JP. 2001. Apoptosis of hepatic stellate cells: involvement in resolution of biliary fibrosis and regulation by soluble growth factors. Gut 48(4):548-557.

Jansson M, Hallen D, Koho H, Andersson G, Berghard L, Heidrich J, Nyberg E, Uhlen M, Kordel J, Nilsson B. 1997. Characterization of ligand binding of a soluble human insulin-like growth factor I receptor variant suggests a ligand-induced conformational change. J Biol Chem 272(13):8189-8197.

Jaques G, Noll K, Wegmann B, Witten S, Kogan E, Radulescu RT, Havemann K. 1997. Nuclear localization of insulin-like growth factor binding protein 3 in a lung cancer cell line. Endocrinology 138(4):1767-1770.

Jones JI, Clemmons DR. 1995. Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16(1):3-34.

Jones JI, D'Ercole AJ, Camacho-Hubner C, Clemmons DR. 1991. Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: effects on affinity for IGF-I. Proc Natl Acad Sci U S A 88(17):7481-7485.

Jones JI, Gockerman A, Busby WH, Jr., Camacho-Hubner C, Clemmons DR. 1993a. Extracellular matrix contains insulin-like growth factor binding protein-5: potentiation of the effects of IGF-I. J Cell Biol 121(3):679-687.

Jones JI, Gockerman A, Busby WH, Jr., Wright G, Clemmons DR. 1993b. Insulin-like growth factor binding protein 1 stimulates cell migration and binds to the alpha 5 beta 1 integrin by means of its Arg-Gly-Asp sequence. Proc Natl Acad Sci U S A 90(22):10553-10557.

Kawashima Y, Kanzaki S, Yang F, Kinoshita T, Hanaki K, Nagaishi J, Ohtsuka Y, Hisatome I, Ninomoya H, Nanba E, Fukushima T, Takahashi S. 2005. Mutation at cleavage site of insulin-like growth factor receptor in a short-stature child born with intrauterine growth retardation. J Clin Endocrinol Metab 90(8):4679-4687.

Kelley KM, Oh Y, Gargosky SE, Gucev Z, Matsumoto T, Hwa V, Ng L, Simpson DM, Rosenfeld RG. 1996. Insulin-like growth factor-binding proteins (IGFBPs) and their regulatory dynamics. Int J Biochem Cell Biol 28(6):619-637.

Kiess W, Blickenstaff GD, Sklar MM, Thomas CL, Nissley SP, Sahagian GG. 1988. Biochemical evidence that the type II insulin-like growth factor receptor is identical to

the cation-independent mannose 6-phosphate receptor. J Biol Chem 263(19):9339-9344.

Killian JK, Byrd JC, Jirtle JV, Munday BL, Stoskopf MK, MacDonald RG, Jirtle RL. 2000. M6P/IGF2R imprinting evolution in mammals. Mol Cell 5(4):707-716.

Knittel T, Kobold D, Piscaglia F, Saile B, Neubauer K, Mehde M, Timpl R, Ramadori G. 1999a. Localization of liver myofibroblasts and hepatic stellate cells in normal and diseased rat livers: distinct roles of (myo-)fibroblast subpopulations in hepatic tissue repair. Histochem Cell Biol 112(5):387-401.

Knittel T, Kobold D, Saile B, Grundmann A, Neubauer K, Piscaglia F, Ramadori G. 1999b. Rat liver myofibroblasts and hepatic stellate cells: different cell populations of the fibroblast lineage with fibrogenic potential. Gastroenterology 117(5):1205-1221.

Kornfeld S. 1992. Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. Annu Rev Biochem 61:307-330.

Kratzsch J, Blum WF, Schenker E, Keller E. 1995. Regulation of growth hormone (GH), insulin-like growth factor (IGF)I, IGF binding proteins -1, -2, -3 and GH binding protein during progression of liver cirrhosis. Exp Clin Endocrinol Diabetes 103(5):285-291.

Lawton BR, Obergfell C, O'Neill RJ, O'Neill MJ. 2007. Physical mapping of the IGF2 locus in the South American opossum Monodelphis domestica. Cytogenet Genome Res 116(1-2):130-131.

Le Roith D, Bondy C, Yakar S, Liu JL, Butler A. 2001. The somatomedin hypothesis: 2001. Endocr Rev 22(1):53-74.

Leal SM, Liu Q, Huang SS, Huang JS. 1997. The type V transforming growth factor beta receptor is the putative insulin-like growth factor-binding protein 3 receptor. J Biol Chem 272(33):20572-20576.

Lee PD, Conover CA, Powell DR. 1993. Regulation and function of insulin-like growth factor-binding protein-1. Proc Soc Exp Biol Med 204(1):4-29.

LeRoith D, Werner H, Beitner-Johnson D, Roberts CT, Jr. 1995. Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocr Rev 16(2):143-163.

Lewitt MS, Saunders H, Phuyal JL, Baxter RC. 1994. Complex formation by human insulin-like growth factor-binding protein-3 and human acid-labile subunit in growth hormone-deficient rats. Endocrinology 134(6):2404-2409.

Li YM, Schacher DH, Liu Q, Arkins S, Rebeiz N, McCusker RH, Jr., Dantzer R, Kelley KW. 1997. Regulation of myeloid growth and differentiation by the insulin-like growth factor I receptor. Endocrinology 138(1):362-368.

Lis H, Sharon N. 1993. Protein glycosylation. Structural and functional aspects. Eur J Biochem 218(1):1-27.

Louvi A, Accili D, Efstratiadis A. 1997. Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. Dev Biol 189(1):33-48.

MacDonald RG, Pfeffer SR, Coussens L, Tepper MA, Brocklebank CM, Mole JE, Anderson JK, Chen E, Czech MP, Ullrich A. 1988. A single receptor binds both insulin-like growth factor II and mannose-6-phosphate. Science 239(4844):1134-1137.

Maile LA, Badley-Clarke J, Clemmons DR. 2001. Structural analysis of the role of the beta 3 subunit of the alpha V beta 3 integrin in IGF-I signaling. J Cell Sci 114(Pt 7):1417-1425.

Maile LA, Holly JM. 1999. Insulin-like growth factor binding protein (IGFBP) proteolysis: occurrence, identification, role and regulation. Growth Horm IGF Res 9(2):85-95.

Marinaro JA, Neumann GM, Russo VC, Leeding KS, Bach LA. 2000. O-glycosylation of insulin-like growth factor (IGF) binding protein-6 maintains high IGF-II binding affinity by decreasing binding to glycosaminoglycans and susceptibility to proteolysis. Eur J Biochem 267(17):5378-5386.

Mathews LS, Hammer RE, Behringer RR, D'Ercole AJ, Bell GI, Brinster RL, Palmiter RD. 1988. Growth enhancement of transgenic mice expressing human insulin-like growth factor I. Endocrinology 123(6):2827-2833.

Mathews LS, Norstedt G, Palmiter RD. 1986. Regulation of insulin-like growth factor I gene expression by growth hormone. Proc Natl Acad Sci U S A 83(24):9343-9347.

Mirpuri E, Garcia-Trevijano ER, Castilla-Cortazar I, Berasain C, Quiroga J, Rodriguez-Ortigosa C, Mato JM, Prieto J, Avila MA. 2002. Altered liver gene expression in CCl4-cirrhotic rats is partially normalized by insulin-like growth factor-I. Int J Biochem Cell Biol 34(3):242-252.

Mohan S, Baylink DJ. 2002. IGF-binding proteins are multifunctional and act via IGF-dependent and -independent mechanisms. J Endocrinol 175(1):19-31.

Mohan S, Nakao Y, Honda Y, Landale E, Leser U, Dony C, Lang K, Baylink DJ. 1995. Studies on the mechanisms by which insulin-like growth factor (IGF) binding protein-4 (IGFBP-4) and IGFBP-5 modulate IGF actions in bone cells. J Biol Chem 270(35):20424-20431.

Mohseni-Zadeh S, Binoux M. 1997. Insulin-like growth factor (IGF) binding protein-3 interacts with the type 1 IGF receptor, reducing the affinity of the receptor for its ligand: an alternative mechanism in the regulation of IGF action. Endocrinology 138(12):5645-5648.

Moller S, Juul A, Becker U, Flyvbjerg A, Skakkebaek NE, Henriksen JH. 1995. Concentrations, release, and disposal of insulin-like growth factor (IGF)-binding proteins (IGFBP), IGF-I, and growth hormone in different vascular beds in patients with cirrhosis. J Clin Endocrinol Metab 80(4):1148-1157.

Morgan DO, Edman JC, Standring DN, Fried VA, Smith MC, Roth RA, Rutter WJ. 1987. Insulin-like growth factor II receptor as a multifunctional binding protein. Nature 329(6137):301-307.

Morrione A, Valentinis B, Xu SQ, Yumet G, Louvi A, Efstratiadis A, Baserga R. 1997. Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. Proc Natl Acad Sci U S A 94(8):3777-3782.

Nakae J, Kido Y, Accili D. 2001. Distinct and overlapping functions of insulin and IGF-I receptors. Endocr Rev 22(6):818-835.

Nam T, Moralez A, Clemmons D. 2002. Vitronectin binding to IGF binding protein-5 (IGFBP-5) alters IGFBP-5 modulation of IGF-I actions. Endocrinology 143(1):30-36.

Nissley P, Kiess W, Sklar M. 1993. Developmental expression of the IGF-II/mannose 6-phosphate receptor. Mol Reprod Dev 35(4):408-413.

Novosyadlyy R, Dargel R, Scharf JG. 2005. Expression of insulin-like growth factor-I and insulin-like growth factor binding proteins during thioacetamide-induced liver cirrhosis in rats. Growth Horm IGF Res 15(5):313-323.

Novosyadlyy R, Dudas J, Pannem R, Ramadori G, Scharf JG. 2006a. Crosstalk between PDGF and IGF-I receptors in rat liver myofibroblasts: implication for liver fibrogenesis. Lab Invest.

Novosyadlyy R, Dudas J, Pannem R, Ramadori G, Scharf JG. 2006b. Crosstalk between PDGF and IGF-I receptors in rat liver myofibroblasts: implication for liver fibrogenesis. Lab Invest 86(7):710-723.

Novosyadlyy R, Tron K, Dudas J, Ramadori G, Scharf JG. 2004. Expression and regulation of the insulin-like growth factor axis components in rat liver myofibroblasts. J Cell Physiol 199(3):388-398.

O'Dell SD, Day IN. 1998. Insulin-like growth factor II (IGF-II). Int J Biochem Cell Biol 30(7):767-771.

O'Gorman DB, Weiss J, Hettiaratchi A, Firth SM, Scott CD. 2002. Insulin-like growth factor-II/mannose 6-phosphate receptor overexpression reduces growth of choriocarcinoma cells in vitro and in vivo. Endocrinology 143(11):4287-4294.

Oh Y, Muller HL, Pham H, Rosenfeld RG. 1993. Demonstration of receptors for insulin-like growth factor binding protein-3 on Hs578T human breast cancer cells. J Biol Chem 268(35):26045-26048.

Opdenakker G, Rudd PM, Ponting CP, Dwek RA. 1993. Concepts and principles of glycobiology. Faseb J 7(14):1330-1337.

Otte K, Choudhury D, Charalambous M, Engstrom W, Rozell B. 1998. A conserved structural element in horse and mouse IGF2 genes binds a methylation sensitive factor. Nucleic Acids Res 26(7):1605-1612.

Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R, Belfiore A. 2002. Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. J Biol Chem 277(42):39684-39695.

Pinzani M. 1995. Novel insights into the biology and physiology of the Ito cell. Pharmacol Ther 66(2):387-412.

Pinzani M, Abboud HE, Aron DC. 1990. Secretion of insulin-like growth factor-I and binding proteins by rat liver fat-storing cells: regulatory role of platelet-derived growth factor. Endocrinology 127(5):2343-2349.

Pinzani M, Gentilini A, Caligiuri A, De Franco R, Pellegrini G, Milani S, Marra F, Gentilini P. 1995. Transforming growth factor-beta 1 regulates platelet-derived growth factor receptor beta subunit in human liver fat-storing cells. Hepatology 21(1):232-239.

Pinzani M, Marra F. 2001. Cytokine receptors and signaling in hepatic stellate cells. Semin Liver Dis 21(3):397-416.

Pinzani M, Milani S, Herbst H, DeFranco R, Grappone C, Gentilini A, Caligiuri A, Pellegrini G, Ngo DV, Romanelli RG, Gentilini P. 1996. Expression of platelet-derived growth factor and its receptors in normal human liver and during active hepatic fibrogenesis. Am J Pathol 148(3):785-800.

Pinzani M, Rombouts K. 2004. Liver fibrosis: from the bench to clinical targets. Dig Liver Dis 36(4):231-242.

Pollak MN, Schernhammer ES, Hankinson SE. 2004. Insulin-like growth factors and neoplasia. Nat Rev Cancer 4(7):505-518.

Poretsky L, Cataldo NA, Rosenwaks Z, Giudice LC. 1999. The insulin-related ovarian regulatory system in health and disease. Endocr Rev 20(4):535-582.

Powell-Braxton L, Hollingshead P, Warburton C, Dowd M, Pitts-Meek S, Dalton D, Gillett N, Stewart TA. 1993. IGF-I is required for normal embryonic growth in mice. Genes Dev 7(12B):2609-2617.

Qin X, Strong DD, Baylink DJ, Mohan S. 1998. Structure-function analysis of the human insulin-like growth factor binding protein-4. J Biol Chem 273(36):23509-23516.

Radulescu RT. 1994. Nuclear localization signal in insulin-like growth factor-binding protein type 3. Trends Biochem Sci 19(7):278.

Raile K, Klammt J, Schneider A, Keller A, Laue S, Smith R, Pfaffle R, Kratzsch J, Keller E, Kiess W. 2006. Clinical and functional characteristics of the human Arg59Ter insulin-like growth factor i receptor (IGF1R) mutation: implications for a gene dosage effect of the human IGF1R. J Clin Endocrinol Metab 91(6):2264-2271.

Rajah R, Katz L, Nunn S, Solberg P, Beers T, Cohen P. 1995. Insulin-like growth factor binding protein (IGFBP) proteases: functional regulators of cell growth. Prog Growth Factor Res 6(2-4):273-284.

Rajaram S, Baylink DJ, Mohan S. 1997. Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. Endocr Rev 18(6):801-831.

Ramadori G, Knittel T, Saile B. 1998. Fibrosis and altered matrix synthesis. Digestion 59(4):372-375.

Ramadori G, Saile B. 2002. Mesenchymal cells in the liver--one cell type or two? Liver 22(4):283-294.

Ramadori G, Saile B. 2004. Portal tract fibrogenesis in the liver. Lab Invest 84(2):153-159.

Rechler MM. 1993. Insulin-like growth factor binding proteins. Vitam Horm 47:1-114.

Reik W, Constancia M, Dean W, Davies K, Bowden L, Murrell A, Feil R, Walter J, Kelsey G. 2000. Igf2 imprinting in development and disease. Int J Dev Biol 44(1):145-150.

Rinderknecht E, Humbel RE. 1978. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. J Biol Chem 253(8):2769-2776.

Roach PJ. 1991. Multisite and hierarchal protein phosphorylation. J Biol Chem 266(22):14139-14142.

Rogers J, Wiltrout L, Nanu L, Fant ME. 1996. Developmentally regulated expression of IGF binding protein-3 (IGFBP-3) in human placental fibroblasts: effect of exogenous IGFBP-3 on IGF-1 action. Regul Pept 61(3):189-195.

Rosen CJ. 1999. Serum insulin-like growth factors and insulin-like growth factor-binding proteins: clinical implications. Clin Chem 45(8 Pt 2):1384-1390.

Ross RJ, Chew SL, D'Souza Li L, Yateman M, Rodriguez-Arnao J, Gimson A, Holly J, Camacho-Hubner C. 1996. Expression of IGF-I and IGF-binding protein genes in cirrhotic liver. J Endocrinol 149(2):209-216.

Russo VC, Bach LA, Fosang AJ, Baker NL, Werther GA. 1997. Insulin-like growth factor binding protein-2 binds to cell surface proteoglycans in the rat brain olfactory bulb. Endocrinology 138(11):4858-4867.

Russo VC, Gluckman PD, Feldman EL, Werther GA. 2005a. The insulin-like growth factor system and its pleiotropic functions in brain. Endocr Rev 26(7):916-943.

Russo VC, Schutt BS, Andaloro E, Ymer SI, Hoeflich A, Ranke MB, Bach LA, Werther GA. 2005b. Insulin-like growth factor binding protein-2 binding to extracellular matrix plays a critical role in neuroblastoma cell proliferation, migration, and invasion. Endocrinology 146(10):4445-4455.

Saile B, DiRocco P, Dudas J, El-Armouche H, Sebb H, Eisenbach C, Neubauer K, Ramadori G. 2004. IGF-I induces DNA synthesis and apoptosis in rat liver hepatic stellate cells (HSC) but DNA synthesis and proliferation in rat liver myofibroblasts (rMF). Lab Invest 84(8):1037-1049.

Saile B, Knittel T, Matthes N, Schott P, Ramadori G. 1997. CD95/CD95L-mediated apoptosis of the hepatic stellate cell. A mechanism terminating uncontrolled hepatic stellate cell proliferation during hepatic tissue repair. Am J Pathol 151(5):1265-1272.

Saile B, Matthes N, Knittel T, Ramadori G. 1999. Transforming growth factor beta and tumor necrosis factor alpha inhibit both apoptosis and proliferation of activated rat hepatic stellate cells. Hepatology 30(1):196-202.

Saile B, Ramadori G. 2007. Inflammation, damage repair and liver fibrosis--role of cytokines and different cell types. Z Gastroenterol 45(1):77-86.

Santer FR, Bacher N, Moser B, Morandell D, Ressler S, Firth SM, Spoden GA, Sergi C, Baxter RC, Jansen-Durr P, Zwerschke W. 2006. Nuclear insulin-like growth factor binding protein-3 induces apoptosis and is targeted to ubiquitin/proteasome-dependent proteolysis. Cancer Res 66(6):3024-3033.

Scharf JG, Braulke T. 2003. The role of the IGF axis in hepatocarcinogenesis. Horm Metab Res 35(11-12):685-693.

Scharf JG, Dombrowski F, Novosyadlyy R, Eisenbach C, Demori I, Kubler B, Braulke T. 2004. Insulin-like growth factor (IGF)-binding protein-1 is highly induced during acute carbon tetrachloride liver injury and potentiates the IGF-I-stimulated activation of rat hepatic stellate cells. Endocrinology 145(7):3463-3472.

Scharf JG, Dombrowski F, Ramadori G. 2001. The IGF axis and hepatocarcinogenesis. Mol Pathol 54(3):138-144.

Scharf JG, Knittel T, Dombrowski F, Muller L, Saile B, Braulke T, Hartmann H, Ramadori G. 1998a. Characterization of the IGF axis components in isolated rat hepatic stellate cells. Hepatology 27(5):1275-1284.

Scharf JG, Schmidt-Sandte W, Pahernik SA, Ramadori G, Braulke T, Hartmann H. 1998b. Characterization of the insulin-like growth factor axis in a human hepatoma cell line (PLC). Carcinogenesis 19(12):2121-2128.

Scharf JG, Schmitz F, Frystyk J, Skjaerbaek C, Moesus H, Blum WF, Ramadori G, Hartmann H. 1996. Insulin-like growth factor-I serum concentrations and patterns of insulin-like growth factor binding proteins in patients with chronic liver disease. J Hepatol 25(5):689-699.

Schedlich LJ, Le Page SL, Firth SM, Briggs LJ, Jans DA, Baxter RC. 2000. Nuclear import of insulin-like growth factor-binding protein-3 and -5 is mediated by the importin beta subunit. J Biol Chem 275(31):23462-23470.

Schmitz F, Bresciani R, Hartmann H, Braulke T. 1995. Effect of insulin-like growth factor II on uptake of arylsulfatase A by cultured rat hepatocytes and Kupffer cells. J Hepatol 22(3):356-363.

Schutt BS, Langkamp M, Rauschnabel U, Ranke MB, Elmlinger MW. 2004. Integrin-mediated action of insulin-like growth factor binding protein-2 in tumor cells. J Mol Endocrinol 32(3):859-868.

Schwander JC, Hauri C, Zapf J, Froesch ER. 1983. Synthesis and secretion of insulin-like growth factor and its binding protein by the perfused rat liver: dependence on growth hormone status. Endocrinology 113(1):297-305.

Simmons JG, Pucilowska JB, Keku TO, Lund PK. 2002. IGF-I and TGF-beta1 have distinct effects on phenotype and proliferation of intestinal fibroblasts. Am J Physiol Gastrointest Liver Physiol 283(3):G809-818.

Sjogren K, Liu JL, Blad K, Skrtic S, Vidal O, Wallenius V, LeRoith D, Tornell J, Isaksson OG, Jansson JO, Ohlsson C. 1999. Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. Proc Natl Acad Sci U S A 96(12):7088-7092.

Sommer A, Spratt SK, Tatsuno GP, Tressel T, Lee R, Maack CA. 1993. Properties of glycosylated and non-glycosylated human recombinant IGF binding protein-3 (IGFBP-3). Growth Regul 3(1):46-49.

Steele-Perkins G, Turner J, Edman JC, Hari J, Pierce SB, Stover C, Rutter WJ, Roth RA. 1988. Expression and characterization of a functional human insulin-like growth factor I receptor. J Biol Chem 263(23):11486-11492.

Surani MA. 1998. Imprinting and the initiation of gene silencing in the germ line. Cell 93(3):309-312.

Svegliati-Baroni G, Ridolfi F, Di Sario A, Casini A, Marucci L, Gaggiotti G, Orlandoni P, Macarri G, Perego L, Benedetti A, Folli F. 1999. Insulin and insulin-like growth factor-1 stimulate proliferation and type I collagen accumulation by human hepatic stellate cells: differential effects on signal transduction pathways. Hepatology 29(6):1743-1751.

Taimr P, Higuchi H, Kocova E, Rippe RA, Friedman S, Gores GJ. 2003. Activated stellate cells express the TRAIL receptor-2/death receptor-5 and undergo TRAIL-mediated apoptosis. Hepatology 37(1):87-95.

Tannenbaum GS, Guyda HJ, Posner BI. 1983. Insulin-like growth factors: a role in growth hormone negative feedback and body weight regulation via brain. Science 220(4592):77-79.

Terrien X, Bonvin E, Corroyer S, Tabary O, Clement A, Henrion Caude A. 2005. Intracellular colocalization and interaction of IGF-binding protein-2 with the cyclin-dependent kinase inhibitor p21CIP1/WAF1 during growth inhibition. Biochem J 392(Pt 3):457-465.

Tilghman SM. 1999. The sins of the fathers and mothers: genomic imprinting in mammalian development. Cell 96(2):185-193.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76(9):4350-4354.

Tsukazaki T, Usa T, Matsumoto T, Enomoto H, Ohtsuru A, Namba H, Iwasaki K, Yamashita S. 1994. Effect of transforming growth factor-beta on the insulin-like growth factor-I autocrine/paracrine axis in cultured rat articular chondrocytes. Exp Cell Res 215(1):9-16.

Twigg SM, Kiefer MC, Zapf J, Baxter RC. 1998. Insulin-like growth factor-binding protein 5 complexes with the acid-labile subunit. Role of the carboxyl-terminal domain. J Biol Chem 273(44):28791-28798.

Twigg SM, Kiefer MC, Zapf J, Baxter RC. 2000. A central domain binding site in insulin-like growth factor binding protein-5 for the acid-labile subunit. Endocrinology 141(1):454-457.

Ullrich A, Gray A, Tam AW, Yang-Feng T, Tsubokawa M, Collins C, Henzel W, Le Bon T, Kathuria S, Chen E, et al. 1986. Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. Embo J 5(10):2503-2512.

Unterman TG, Oehler DT, Murphy LJ, Lacson RG. 1991. Multihormonal regulation of insulin-like growth factor-binding protein-1 in rat H4IIE hepatoma cells: the dominant role of insulin. Endocrinology 128(6):2693-2701.

Valentinis B, Bhala A, DeAngelis T, Baserga R, Cohen P. 1995. The human insulin-like growth factor (IGF) binding protein-3 inhibits the growth of fibroblasts with a targeted disruption of the IGF-I receptor gene. Mol Endocrinol 9(3):361-367.

Vu TH, Hoffman AR. 2000. Comparative genomics sheds light on mechanisms of genomic imprinting. Genome Res 10(11):1660-1663.

Waguri S, Kohmura M, Kanamori S, Watanabe T, Ohsawa Y, Koike M, Tomiyama Y, Wakasugi M, Kominami E, Uchiyama Y. 2001. Different distribution patterns of the two mannose 6-phosphate receptors in rat liver. J Histochem Cytochem 49(11):1397-1405.

Walenkamp MJ, van der Kamp HJ, Pereira AM, Kant SG, van Duyvenvoorde HA, Kruithof MF, Breuning MH, Romijn JA, Karperien M, Wit JM. 2006. A variable degree of intrauterine and postnatal growth retardation in a family with a missense mutation in the insulin-like growth factor I receptor. J Clin Endocrinol Metab 91(8):3062-3070.

Wang ZQ, Fung MR, Barlow DP, Wagner EF. 1994. Regulation of embryonic growth and lysosomal targeting by the imprinted Igf2/Mpr gene. Nature 372(6505):464-467.

Wells RG, Kruglov E, Dranoff JA. 2004. Autocrine release of TGF-beta by portal fibroblasts regulates cell growth. FEBS Lett 559(1-3):107-110.

Wolf E, Lahm H, Wu M, Wanke R, Hoeflich A. 2000. Effects of IGFBP-2 overexpression in vitro and in vivo. Pediatr Nephrol 14(7):572-578.

Wood TL, Rogler LE, Czick ME, Schuller AG, Pintar JE. 2000. Selective alterations in organ sizes in mice with a targeted disruption of the insulin-like growth factor binding protein-2 gene. Mol Endocrinol 14(9):1472-1482.

Wylie AA, Pulford DJ, McVie-Wylie AJ, Waterland RA, Evans HK, Chen YT, Nolan CM, Orton TC, Jirtle RL. 2003. Tissue-specific inactivation of murine M6P/IGF2R. Am J Pathol 162(1):321-328.

Yakar S, Liu JL, Stannard B, Butler A, Accili D, Sauer B, LeRoith D. 1999. Normal growth and development in the absence of hepatic insulin-like growth factor I. Proc Natl Acad Sci U S A 96(13):7324-7329.

Young SC, Miles MV, Clemmons DR. 1992. Determination of the pharmacokinetic profiles of insulin-like growth factor binding proteins-1 and -2 in rats. Endocrinology 131(4):1867-1873.

Zaina S, Squire S. 1998. The soluble type 2 insulin-like growth factor (IGF-II) receptor reduces organ size by IGF-II-mediated and IGF-II-independent mechanisms. J Biol Chem 273(44):28610-28616.

Zhang BB, Cai WM, Weng HL, Hu ZR, Lu J, Zheng M, Liu RH. 2003. Diagnostic value of platelet derived growth factor-BB, transforming growth factor-beta1, matrix metalloproteinase-1, and tissue inhibitor of matrix metalloproteinase-1 in serum and peripheral blood mononuclear cells for hepatic fibrosis. World J Gastroenterol 9(11):2490-2496.

Zheng B, Clemmons DR. 1998. Blocking ligand occupancy of the alphaVbeta3 integrin inhibits insulin-like growth factor I signaling in vascular smooth muscle cells. Proc Natl Acad Sci U S A 95(19):11217-11222.

Zimmermann EM, Li L, Hoyt EC, Pucilowska JB, Lichtman S, Lund PK. 2000. Cell-specific localization of insulin-like growth factor binding protein mRNAs in rat liver. Am J Physiol Gastrointest Liver Physiol 278(3):G447-457.

Zindy F, Lamas E, Schmidt S, Kirn A, Brechot C. 1992. Expression of insulin-like growth factor II (IGF-II) and IGF-II, IGF-I and insulin receptors mRNAs in isolated non-parenchymal rat liver cells. J Hepatol 14(1):30-34.

Publications

Novosyadlyy R, Dudas J, **Pannem R**, Ramadori G, Scharf JG.
Crosstalk between PDGF and IGF-I receptors in rat liver myofibroblasts: implication for liver fibrogenesis. Lab Invest, 86:710-723 (IF: 3.702)

Viereck V, Siggelkow H, **Pannem R**, Braulke T, Scharf JG, Kubler B.
Alteration of the insulin-like growth factor axis during in vitro differentiation of the human osteosarcoma cell lines HOS 58. J Cell Biochem. 2007 September 1; 102(1): 28-40

Conferences

Oral Presentation

The Third International Congress of GRS and IGF society, 11-15 November, 2006, Kobe, Japan
Functional analysis of IGFBP-2 overexpression in mouse liver myofibroblasts: implications for liver fibrogenesis.

Rajeswararao Pannem, Andreas Hoeflich, Eckhard Wolf, Jens-Gerd Scharf

Acknowledgements

This study was performed in the Division of Gastroenterology and Endocrinology at the Department of Internal Medicine of Georg-August-University of Göttingen during years 2004-2007.

I am thankful to Deutsche Forschungsgemeinschaft (German Research Foundation) and University Hospital of Göttingen, which provided financial support of my work in the framework of the Postgraduate Program “Clinical, Cellular and Molecular Biology of Internal Organs”.

I express my particular gratitude to my scientific supervisor, Professor Jens-Gerd Scharf, for his outstanding supervision, support and contribution to my success.

I am indebted to Professors Andreas Hoeflich and Eckhard Wolf (Institute of Animal Breeding and Biotechnology, Munich, Germany) for providing mice for experimental purpose during my study.

I would like to thank sincerely Professor Giuliano Ramadori, director of the Division of Gastroenterology and Endocrinology, for inviting me to Göttingen and for providing excellent research facilities with a stimulating atmosphere. I am also greatful to Professor Gerhard Burckhardt, director of the Graduiertenkolleg 335, for giving me a chance to join the Postgraduate Program and for all the help and support during my PhD studies.

I am thankful to Professors Rüdiger Hardeland and Detlef Doenecke, for being official reviewers of the thesis. Their constructive criticism and suggestions are highly appreciated.

I am thankful to PD Dr. Michael Hoppert, Professors Ralf Ficner, Dieter Heineke and Ivo Feussner who involved in Ph. D. committee for evaluation of my thesis and abilities.

I would like to thank my colleague Drs. Jozsef Dudas from the Division of Gastroenterology and Endocrinology for his valuable suggestions during the study. I

am also grateful to the technical personnel of the Division of Gastroenterology, in particular to Sieglinde Zachmann, Elke Neumann, Renate Klages, Sandra Georgi, Anke Herbst, Sonia Heyroth and christen Hoffmann, for their significant help during these years. I would also like to thank Yeruva sunil, Peddireddi Sudhakar, Pemmasani Kayani, Chalaganti Rahgu, Goutham Ganjam, Boppana Sridhar, Boppana Sailaja, Ayodhya Kiran, Ayodhya Madumathi, D.V. Krishna Pantakani, Karthikeyan Radhakrishan, Ajay Kunwar, Saggu Durga Prasad, Vijay, and Puja who shared the ups and downs during my stay in Germany.

Finally, I owe my deepest gratitude to all my family members for their love, help, support, patience, encouragement and all the comfort and joy in our family.

Curriculum vitae

	Personal Data
Name	Rajeswara Rao
Family name	Pannem
Date of Birth	25.07.1979
Place of Birth	Jandrapet
Country	India
	Education
1985-1990	Elementary School Education, Jandrapet, India
1990-1995	Secondary School Education, B. V & B. N. High School Jandrapet, India
1995-1997	Intermediate Education, N. M Junior College, Chirala, India
1998-2001	Bachelor of Science (Human Genetics-Biochemistry-Biotechnology), Andhra University, Visakhapatnam, India
2001-2003	Master of Science in Biotechnology, Nagarjuna University, Guntur, India
2003-2004	Teaching assistant, Nagarjuna University, Guntur. India
2004-2007	Joined in Graduiertenkollegs 335 “Clinical, cellular and Molecular Biology of Internal Organs” in University Hospital, Göttingen for my doctoral study in Goettingen