THE ACTIONS OF IGFBPS ON GROWTH AND GLUCOSE METABOLISM IN TRANSGENIC MOUSE MODELS

by

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ABSTRACT OF THE THESIS

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Metabolism in Transgenic Mouse Models

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The roles of insulin/IGF signaling in growth control and glucose metabolism have been extensively studied. To date, the growth inhibitory effects of IGFBPs and impaired glucose homeostasis caused by IGFBP-3 have been mostly studied *in vitro*. Our study, here, provides the first *in vivo* evidence by targeted gene deletion to show that IGFBP-2, -3, and -5 have inhibitory effects on growth, whereas IGFBP-4 and -6 have no effect. However, only male IGFBP-3 KO mice have increased body weight, indicating a gender difference in the system. IGFBP-4 mice, in either C57BL/6J or 129S6 background, show no difference from wild type animals. Disrupting the ternary complex of IGF/IGFBP by knocking out IGFBP-3 and -5 at the same time has a tremendous effect in growth of female mice. As a result, double KO mice gain even more weight than single IGFBP-3 or -5 KO mice. Disruption of the most abundant IGFBP-3 in circulation results in enhanced glucose tolerance companied

by increased insulin secretion and insulin sensitivity. This makes IGFBP-3 a potential target in treating obesity and type 2 diabetes. Lastly, the effect of IGFBPs in traumatic brain injury was studied and it shows that, following brain injury, IGFBP-2 mRNA expression is upregulated at the injury site with the consequence that IGFBP-2 KO mice have significantly increased neurogenesis around the subventricular zone (SVZ). The role of IGFBP-2 in proliferation of stem cells in the CNS is further confirmed by quantitative PCR which shows a 28-fold upregulation in cultured neurospheres from the postnatal brain compared to Stratagene's Universal Mouse Reference cDNA.

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DEDICATION

This work is in memory of my father, Bohong Qin,

who's no longer with us, but part of him resides in me and

his figure and spirit will live with me and strengthen me forever.

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Introduction

Insulin

The characterization of insulin's primary amino acid sequence in the 1950s (Ryle et al., 1955) is a breakthrough in peptide research. Several years later, its tertiary structure was determined by X-ray crystallography and a radioimmunoassay for insulin was developed. As the first hormone identified, insulin, is a peptide hormone composed of 51 amino acids and two peptide chains, A-chain (21aa) and B-chain (30aa). Synthesized in the pancreas within the β-cells of the islets of Langerhans, insulin circulates in the blood stream in an active form of monomer, and is stored in the body as an inactive hexamer in the presence of zinc ions (Fig1.1, Brannigan and Wilkinson, 2002). At micromolar concentrations, it can also dimerize.

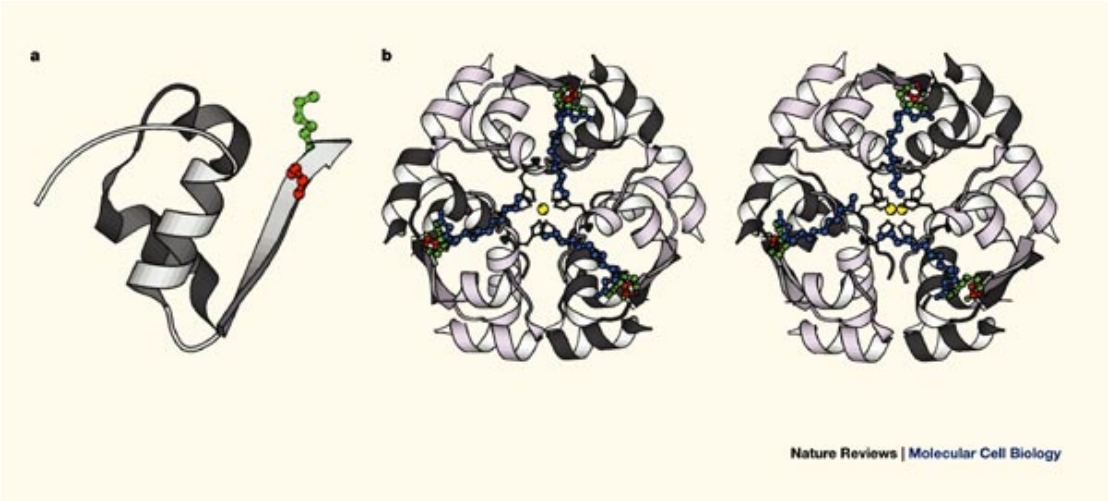


Fig.1.1. Tertiary structure of insulin monomer (a) and hexamer (b) in the presence of zion ions (yellow). Adapted by permission from Macmillan Publishers Ltd: [*Nat Rev Mol Cell Biol*] (Protein engineering 20 years on), copyright (2002).

Insulin has well-known metabolic effects. In addition to its primary function as a regulator for blood glucose levels, insulin also stimulates protein synthesis,

glycogenesis, and lipogensis, and inhibits proteolysis, glycogenosis, and lipolysis (Saltiel and Kahn, 2001; Shabanpoor et al., 2009). Insulin deficiency or resistance leads to dysregulation of these processes and causes elevated glucose and lipid levels (Arafat et al., 2010; Kumar et al., 2010).

The insulin receptor, IR, belongs to a receptor tyrosine kinase (RTK) family. It is a transmembrane glycoprotein complex consisting of two extracellular α-subunits and two transmembrane β-subunits with phosphorylation sites and tyrosine kinase activity. α- and β-subunits function as allosteric enzymes in which the α-subunit inhibits the tyrosine kinase activity of the β-subunit. Binding of insulin to the α-subunits suppresses this inhibition and leads to the activation and autophosphorylation of the β-subunits. As a result, a series of signaling cascades are triggered. The activated insulin receptor tyrosine kinase phosphorylates IRS-1 and recruits PI3K (p85/p110 dimer) to the plasma membrane. Activation of PI3K recruits PDK-1 which in turn phosphorylates and activates Akt/PKB and atypical PKC (aPKC). Other pathways include Ras/MAPK cascade and CAP/Cbl pathway and together they regulate glucose, protein and lipid metabolism (Fig1.2, Saltiel and Kahn, 2001; Khan and Pessin, 2002). IR not only binds to insulin, but also binds to IGF-I and IGF-II, with much lower affinity (Jones and Clemmons, 1995).

Insulin/IR signaling pathway plays a variety of roles in brain function, including learning and memory (Dou et al., 2005). Unlike its peripheral counterpart, neuronal insulin/IR signaling doesn’t regulate glucose metabolism, but more likely modulates the learning and memory process, including regulation of neurotransmitter release

(Figlewicz and Szot 1991), intracellular Ca^{2+} release and neuropeptide secretion (Jonas et al., 1997), and activation of downstream molecules such as Shc/Erk1/2 (Zhao et al., 1999; van der Heide et al., 2006). However, this modulatory effect may be compensated by other signaling pathways, such as IGF-IR system, as cytosolic IGF-IR was increased in streptozotocin induced diabetes mellitus (DM) rats (Dou et al., 2005).

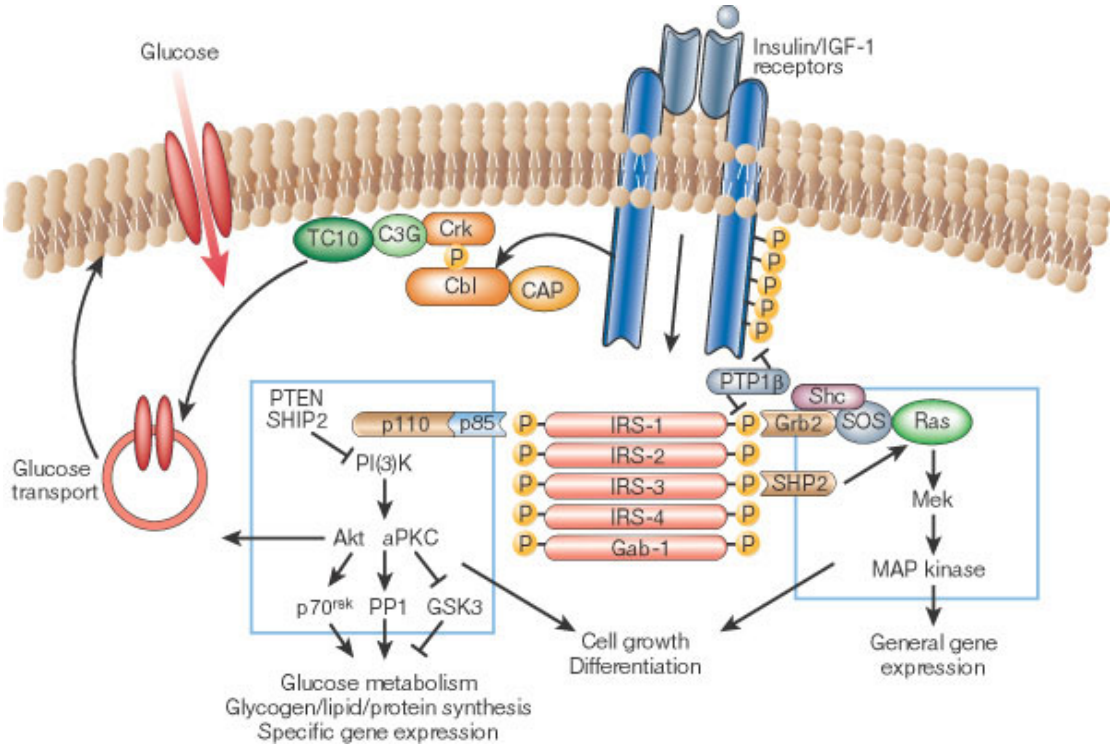


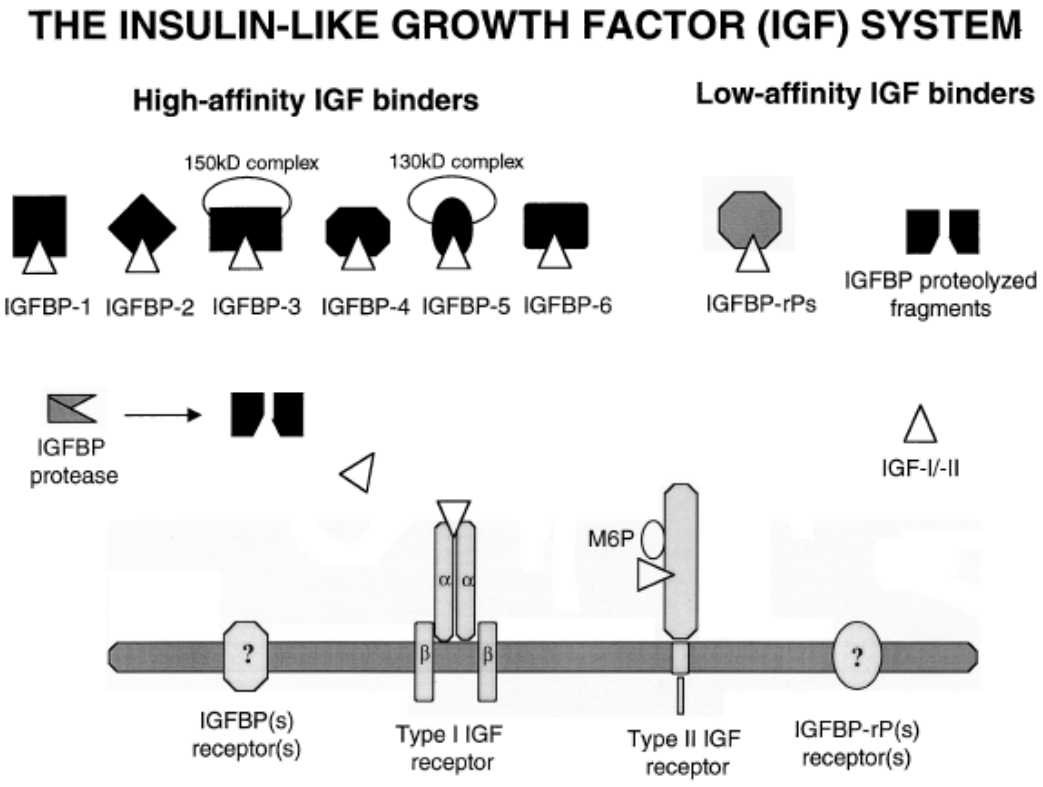
Fig.1.2. The classic insulin signaling pathway. Upon insulin receptor autophosphorylation, a number of cellular proteins such as Shc and members of IRS family are phosphorylated and interact with signaling molecules through the SH2 domain, which stimulates a series of singling pathways, resulting in the translocation of glucose via GLUT-4. Adapted by permission from Macmillan Publishers Ltd: [*Nature*] (Insulin signalling and the regulation of glucose and lipid metabolism), copyright (2001).

IGF system

The insulin-like growth factor (IGF) system has well characterized functions in cell proliferation, differentiation, and survival. In general, the IGF system is

composed of: two ligands (IGF-I and IGF-II), two IGF receptors (IGF-IR and IGF-IIR or type I and II IGF receptor), at least 6 IGF-binding proteins (IGFBPs), IGFBP related proteins (IGFBP-rPs), and IGFBP proteases (Fig1.3, Hwa et al., 1999).

Initially named as NSILA-1 and NSILA-2 because of their nonsuppressible insulin-like activity, it was 1976 when the sequencing of IGF-I and IGF-II revealed that they share ~48% homology with proinsulin. IGF-I was also known as "sulfation factor" and "somatomedin-C".



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Fig.1.3. The IGF system. Components of the IGF system include IGF-I and IGF-II, type I and type II IGF receptors, IGFBP-1 to -6, IGFBP-rPs, and IGFBP proteases.

IGF-I and IGF-II are single chain peptides, about 7 kDa in size, consists of A, B, C, D domains. The A- and B- domains share 50% sequence homology with the A-

and B- chains of insulin. Originally thought to be synthesized in the liver, circulate in blood, and act at distant target sites, now it's well recognized that IGFs are expressed in many non-hepatic tissues. Therefore, both IGF-I and IGF-II act as endocrine as well as autocrine/ paracrine factors (LeRoith et al., 2001; Frystyk, 2010). After conditional deletion of IGF-I, Govoni et al. (2007) have shown that local disruption of IGF-I causes postnatal lethality and a dramatic reduction in body weight and bone accretion. Disruption of the IGF system has a wide-spectrum effect. A large amount of *in vitro* and *in vivo* evidence has pointed out the pathologic role of IGF system in various diseases, including diabetes (Dunger et al., 2004; López-Bermejo et al., 2006; Rajpathak et al., 2009), various cancer types (Pavelić et al., 2007; Camidge et al., 2009) , and cardiovascular disease (Colao et al., 2004; Colao, 2008).

IGF-I

Growth hormone (GH) stimulates circulating (liver produced) as well as peripheral (locally produced) IGF-I (Frystyk, 2010). Other factors regulating IGF-I synthesis include sex steroids and nutritional status. In humans, IGF-I levels fluctuate with age, being relatively low at birth, increasing by 2-3 fold during puberty and slowly declining at adulthood. During postnatal growth, IGF-I is the primary IGF and deletion of *Igf-1* causes 40% reduction of birth weight compared to wild type mice (Liu et al, 1993; Baker et al., 1993). Overexpression of IGF-I in transgenic mice resulted in 30% increase in body weight over control mice (Mathews et al., 1988). Recent genetic studies using bitransgenic mice also demonstrate that endocrine IGF-1 action contributes about 30% of adult body size

and sustains postnatal growth (Stratikopoulos et al., 2008).

Unlike insulin, which circulates free in the blood stream, 99% of IGFs circulate in a complex with IGF binding proteins (IGFBPs). In human, 80% of IGF-I is carried by IGFBP-3/acid labile subunit (ALS) complex. Binding of IGFBPs prolongs IGF half-life and therefore regulates the availability and bioactivity of IGF. IGF-I is released from the complex when IGFBPs are proteolyzed or bind to extracellular matrix. By targeted deletion of *Pappa* gene (which increases IGF-I bioavailability by degrading IGFBPs), Conover et al. (2010) have shown that PAPP-A KO mice have significantly prolonged lifespan with delayed fetal neoplasias and decreased onset of age-related degenerative changes. Site-directed mutagenesis in the C-terminal ECM binding region of rat IGFBP-5 has identified two residues, Gly203 and Gln209, as important determinants of IGF-I binding affinity (Bramani et al., 1999).

Most IGF actions are mediated by the IGF-I receptor, IGF-IR, although IGFs also bind to insulin and IGF-II receptors. IGF-IR has a high degree of homology with the insulin receptor, IR, and is the only IGF receptor with IGF-mediated signaling functions. Binding to both IGFs, IGF-IR shows greater preference to IGF-I. Similar to insulin signaling, IGF-I binding to the extracellular α-subunits leads to the activation of the tyrosine kinase activity of the intracellular β-subunits, and subsequently stimulates a series of downstream transduction pathways, including Ras/MAPK, PI3K/PKB, and PI3K/mTOR/S6K (Oldham and Hafen, 2003). Activation of these signaling pathways results in various cellular responses, such as cell proliferation, differentiation, and survival.

IGF-II

The synthesis of IGF-II is GH-independent and it is paternally expressed in the placenta and fetus. It plays a vital role in placental development and fetal growth as mice lacking *Igf2* showed 60% of normal body weight (Liu et al., 1993). Although overexpression of *Igf2* in mice didn't increase body weight or size significantly, it did cause 25% overgrowth in kidney (Moerth et al., 2007). Excessive IGF-II also causes visceromegaly, placentomegaly, omphalocele, and cardiac and adrenal defects, which are features of the Beckwith–Wiedemann syndrome (BWS), a complex human genetic disorder associated with abnormal chromosome 11 where *Igf2* gene is located (Eggenschwiler et al., 1997).

Being identical to the cation-independent mannose-6-phosphate (M6P) receptor, IGF-IIR is structurally and functionally distinct from IGF-I and insulin receptors. It binds IGF-II with high affinity, IGF-I with much lower affinity, and doesn't bind insulin at all. Unlike IGF-IR, IGF-IIR doesn't have intrinsic signaling transduction capability and its main role is to bind and mediate IGF-II turnover by endocytosis. IGF-IIR also binds M6P which is critical in mediating cell proliferation (Scott and Firth, 2004) and sorting lysosomal enzymes (Gary-Bobo et al., 2007).

IGFBPs

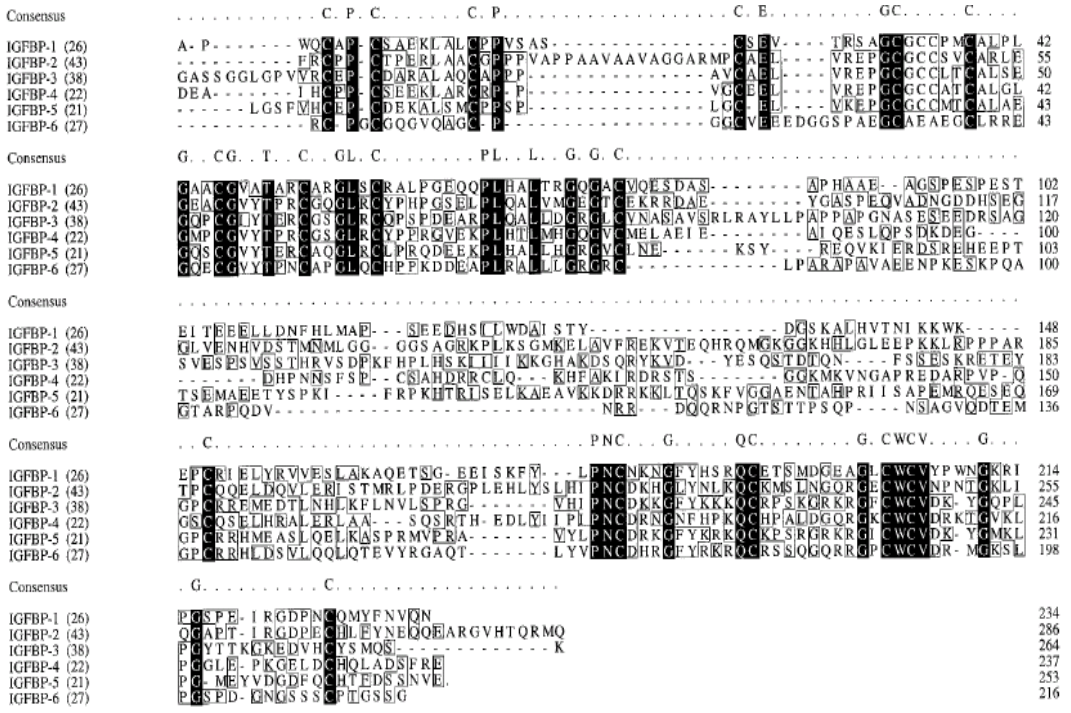
IGF-I and IGF-II exist in circulation in nanomolar concentrations (Heemskerk et al., 1999), compared to insulin detected at picomolar levels (Mizutani et al., 2008), which is sufficient to cause hypoglycaemia if without the binding of IGFBPs. In fact, more than 99% of IGFs are bound to IGFBPs and about 75~80% are found in a 150

kDa ternary complex with the acid labile α-subunit (ALS) and IGFBP-3, the predominant IGFBP (Sujatha et al., 1997). As a result, IGFs are sequestered from binding to their own receptors and IGF actions are inhibited. IGFBP-5, but not other IGFBPs, is also found in the ternary complexes (Salih et al., 2004). In addition, all six IGFBPs can be found in the free form or in 50kDa binary complexes with IGFs. They exit the circulation very quickly, compared with much confined ternary complexes.

IGFBPs are a family of secreted proteins, share similar molecular structure and bind to IGFs with high affinity equal or greater than that with IGF receptors.

The amino acid sequences of mouse IGFBPs share a high degree of homology (73-97%) with corresponding human IGFBPs, and even higher with rat IGFBPs (93-99.6%) (Schuller et al., 1994). Composed of 216-289 amino acids, the molecular mass of mature proteins is between 22.8-31.4 kDa. All IGFPBPs have a highly conserved N-terminal domain, a conserved C-terminal domain, and a variable central domain. The N-terminal domain contains 12 conserved cysteine residues forming 6 disulfide bonds which serve as the major IGF-binding sites. Exceptions are IGFBP-4 and -6 which have 14 and 10 cysteine residues at the N-domain respectively. The C-terminal domain is also conserved, with 6 cysteine residues and 3 disulfide bonds. In addition to IGF-binding, the C-terminal also contains nuclear localization signal and ALS-binding sites for IGFBP-3 and -5, and integrin-binding site for IGFBP-1 and -2 (Fig1.4, Hwa et al., 1999; Firth and Baxter, 2002). Recent studies have identified residues within N-terminal and motifs within C-terminal domains

necessary for interaction with retinoid X receptor-alpha (RXR-α) and retinoic acid receptor-alpha (RAR-α) which is essential to maintain normal growth and cell proliferation (Schedlich et al., 2007). Using NMR spectroscopy and mutagenesis, Headey et al. (2004) identified IGF-II binding site in the C-terminal domain of IGFBP-6. The structure of IGFBP-2 C-terminus was also solved by NMR spectroscopy. Analysis of the backbone structure of IGFBP-2 C-terminal domain has identified a pH-dependent heparin binding site which may bind to glycosaminoglycans in acid ECM, resulting in cancer cell proliferation (Kuang et al., 2006).



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Fig.1.4. Sequence alignment of human IGFBP-1 to -6. Shaded solid black indicates consensus amino acid residue, boxed amino acid residues indicate matched residues.

The central domain separating the N- and C- domains shows little conservation among all IGFBPs. It contains various post-translational sites, including glycosylation, phosphorylation, and proteolysis.

With the development of antibodies and cDNA probes for IGFBPs, various studies (western blotting, ligand blotting, RIA, and *in situ* hybridization) suggest that the expression of IGFBPs follows a very specific spatial and temporal pattern during development. All cells and tissues express at least one form of IGFBPs, and each cell or tissue synthesizes different combinations of IGFBPs. Fibroblasts synthesize IGFBP-3, -4, and -5, and epithelial cells usually synthesize IGFBP-2 and -4. IGFBP-1 expression is restricted to the liver, whereas other IGFBPs are expressed in many peripheral tissues, such as lung, heart, kidney, and brain, and circulate in extracellular fluids, including serum, amniotic fluid, placenta, cerebrospinal fluid (CSF), and lymph (Rajaram et al., 1997, Table 1). The tissue expression of IGFBPs also changes during fetal to adult transition. In rats, IGFBP-2 is abundant in fetal liver but is extremely low in adult liver, IGFBP-4 is low in fetal liver but becomes very high in adult (Rechler, 1993). It's not completely understood how the expression pattern changes over time and why it occurs in different tissue regions. But nevertheless, this adds in the autocrine/paracrine functions of IGFBPs and makes the regulation more dynamic.

IGFBP-rPs

In the past few years, a number of cysteine-rich proteins were identified to share some structural similarities with IGFBPs: they have highly conserved and

cysteine-rich N-terminus, but vary in the midregion and C-terminus. In vitro assays suggest these proteins can bind to IGFs, albeit at much lower affinity than that with IGFBPs. Thus these proteins are subgrouped into a family of proteins called IGFBP-related proteins, IGFBP-rPs (Hwa et al., 1999). Just like the diverse functions IGFBPs have in normal and diseased conditions, IGFBP-rPs have been reported to play roles in cell proliferation, differentiation, and tumorigenesis (Ferry et al., 1999).

Table 1. General characteristics of human IGFBPs

IGF binding protein	No. of amino acids	Molecular mass (kDa)	Tissue expression	Distribution in biological fluids
IGFBP-1	234	25.3	liver	amniotic fluid
IGFBP-2	289	31.4	liver, kidney, brain, lung,	CSF, resum
IGFBP-3	264	28.7	kidney, liver, lung, heart	serum, follicular fluid
IGFBP-4	237	25.9	liver, brain	serum, follicular fluid
IGFBP-5	252	28.5	liver, kidney, lung, heart, low in brain	bone matrix
IGFBP-6	216	22.8	liver, kidney, heart, low in brain	CSF, serum

Modulation of IGF actions by IGFBPs

Binding of IGFBPs modulates IGF actions in both positive and negative directions, depending on the cell type, culture environment, method of administration and experimental endpoints. Indeed, IGFBPs have been reported to potentiate and inhibit IGF actions both *in vitro* and *in vivo*. In general, IGFBPs perform three functions:

(1) Endocrine functions of IGFBPs

Since IGFBPs have higher affinity for IGFs than IGF receptors, most IGFs (~75%) in circulation are bound in a 150 kDa ternary complex with IGFBP-3 and ALS. A small amount of IGFs (~25%) are associated with other IGFBPs in a 50 kDa binary complex. Less than 1% of IGFs are free circulating. The ternary complex not only serves as an IGF reservoir, but also increases the half life of IGFs significantly, from 20 min to 15 h, because of its inability to cross the vascular endothelial barrier (Rajaram et al., 1997).

In addition to function as carrier proteins, IGFBPs in serum regulate the endocrine functions of IGFs by regulating IGF bioavailability. Unlike the ternary complex, the binary complex has small molecular mass, 50 kDa, therefore it can cross the vascular endothelium rapidly, becoming available to local tissues (Mohan and Baylink, 2002).

Although the insulin-like effect of IGFs is only 5% of insulin (Guler et al., 1987; Frystyk et al., 1997), the concentration of IGFs is 1000-fold higher than that of insulin (Mohan and Baylink, 2002). The net effect of IGFs' insulin-like action is therefore 50 times higher than insulin alone due to the abundance. However, IGFBP binding keeps IGFs from binding to their own receptors. As a result, the insulin-like, hypoglycemic effects of IGFs are prevented and glucose homeostasis is maintained.

(2) Autocrine/paracrine regulation of IGFs

Characterization of the types and amounts of IGFBPs in different biological fluids reveals that the extracellular fluids of certain tissues are enriched with specific

IGFBPs. For example, IGFBP-1 is the major IGFBP present in amniotic fluid. In cerebrospinal fluid, IGFBP-2 and -6 are found at high concentrations. IGFBP-3 is the most abundant IGFBP in serum and follicular fluid (Rajaram et al., 1997). In addition, IGFBPs are expressed in different cells/tissues (Table 1). Because of the local production and regulation of IGFBPs in different tissues, IGFBPs act as autocrine/paracrine regulators of IGF actions in a positive or negative way.

The IGF-dependent inhibitory modulation of IGF actions by IGFBPs is well documented. *In vitro* studies using IGF analogs with reduced affinity for IGFBP-4 and coincubating IGFBP-4 and IGFs suggest that IGFBP-4 inhibits IGF actions by preventing the binding of IGFs to the receptors (Mohan et al., 1995). The same inhibitory effect is also tested *in vivo* which shows blocked bone formation in mice.

The stimulatory modulation of IGF actions by IGFBPs (i.e. IGFBP-1,-3, and -5) is also reported. Nonphosphorylated IGFBP-1 has been shown to enhance IGF-I-induced DNA synthesis, whereas phosphorylated IGFBP-1 inhibits IGF-I effects. The increased IGF effect by IGFBP-3 is caused by cell association of IGFBP-3 which increases IGF concentration near the receptor and allows them to bind. IGFBP-5, on the other hand, stimulates IGF action by binding to the extracellular matrix (Mohan and Baylink, 2002).

(3) IGF-independent functions

In addition to modulate IGF actions in an endocrine and/or autocrine/paracrine fashion, it’s becoming increasingly clear that IGFBPs may exert intrinsic bioactivity in the absence of IGFs. IGF-independent actions of IGFBPs include effects on cell

migration, cell growth and apoptosis. Except IGFBP-4 and -6 which are generally considered to act directly through IGF binding, all other IGFBPs have been reported to have IGF-independent effects.

Studies have identified that IGFBP-1 has stimulatory effect on CHO cell migration by binding to α5β1-integrin receptor (a fibronectin receptor). This effect is IGF-independent as the addition of IGF-I to the culture medium had no effect on the migration of the cells (Jones et al., 1993). IGFBP-5 has been shown to stimulate bone formation through IGF-independent mechanisms. Studies on the mechanism by which IGFBP-5 increased the binding of ^{125}I-IGF tracer to bone cells suggest that IGFBP-5 could facilitate IGF binding by a mechanism in which IGFBP-5 has cell surface binding sites independent of IGF receptors (Mohan et al., 1995). Treatment of osteoblasts from IGF-I knockout mice with recombinant human IGFBP-5 promoted cell growth and alkaline phosphate activity. *In vivo* injection of IGFBP-5 to IGF-I knockout mice increased alkaline phosphate activity and osteocalcin, markers of bone formation. In contrast, local administration of IGFBP-4 had no significant effect on bone formation in IGF-I knockout mice (Miyakoshi et al., 2001).

Recently, using quantification (microarrays and real-time qPCR) and qualification (western blot and immunoassay) methods together with apoptosis assays, IGFBP-2 has been found to induce the expression of genes responsible for cell proliferation, migration and apoptosis in an IGF-I-independent breast cancer cell line, Hs578T, indicating IGF-independent apoptotic effect of IGFBP-2 (Frommer et

al., 2006). The *in vivo* IGF-independent effects of IGFBP-5 were first reported by Tripathi et al. (2009). Both wild type and mutant IGFBP-5 mice inhibit growth and overexpression of wild type IGFBP-5 could rescue the lethal phenotype of IGF-II R null mice.

Indeed the majority of studies on IGF-independent effects of IGFBPs come from IGFBP-3. Cohen and colleagues demonstrated that IGFBP-3 transfection with mouse fibroblasts reduced cell growth rate remarkably and this inhibitory effect couldn't be restored by insulin (Cohen et al., 1993). Studies of a 16 kDa proteolytic fragment of IGFBP-3, which has no affinity for IGFs, have revealed that this fragment inhibits the mitogenic effects of IGF-I on chick embryo fibroblasts (Zadeh & Binoux, 1997).

In addition, IGFBP-3 has also been shown to induce apoptosis independent of IGF. Using a differential cloning approach, *IGFBP-3* gene was identified to be a novel target gene for p53 (Buckbinder et al., 1995). Rajah et al. (1997) provided the first demonstration that in the p53 negative prostate cancer cell line, PC3, treatment of recombinant IGFBP-3 induced apoptosis in a dose-dependent manner. In addition, in mouse fibroblasts lacking functional IGF-I receptor, treatment of exogenous IGFBP-3 and transfection with IGFBP-3 induced apoptosis. Using a human IGFBP-3 mutant, 6m-hIGFBP-3, which has no binding affinity for IGFs, Hong et al. (2002) showed it induced apoptosis in PC-3 cells to the same extent as wild type hIGFBP-3. Recent studies have found out that IGFBP-3 leads to caspase-dependent apoptosis through a death receptor-mediated pathway in MCF-7 human breast cancer cells. Additional caspase activity studies revealed that the growth inhibitory effect of

IGFBP-3 is the main result of its apoptotic activity (Kim et al., 2004). The same group just identified a novel cell death receptor, IGFBP-3R, a single-span membrane protein that binds to IGFBP-3 exclusively. In vitro studies demonstrate IGFBP-3R mediates IGFBP-3 induced caspase-dependent apoptosis in various cancer cell lines. Knockdown of IGFBP-3R attenuates IGFBP-3 induced apoptosis and overexpression of IGFBP-3R enhanced IGFBP-3 effects (Ingermann et al., 2010).

Regulation of IGF-binding affinity

One of the most important features of IGFBPs is their high affinity for IGF-I and IGF-II. IGFBP-3 is the most abundant binding protein in circulation and it has the highest affinity. High-affinity IGFBPs prevent receptor association. The importance of changes in binding affinity is that it provides a reservoir of IGF. When the affinity is lowered under some circumstance, IGF is released from the binding complex enabling growth stimulation and anabolic functions. There are at least three post-translational modifications that have been shown to affect the IGF binding affinity of IGFBPs.

(1) Proteolysis

Proteolytic cleavage has been shown to occur with IGFBP-2, -3, -4, and -5 (Table 2). It produces IGFBP fragments with highly reduced (with IGFBP-3 and -5) or no affinity (with IGFBP-2 and -4) for IGFs, resulting in the potentiation of IGF actions (Jones and Clemmons, 1995). IGFBP proteases are calcium-dependent serine proteases, and have been detected in a number of body fluids and culture media.

Some of these are very specific to a given IGFBP. One of the first identified proteases was a pregnancy-associated IGFBP-3 specific protease. Incubation of intact IGFBP-3 with the protease, followed by ligand blotting, confirmed that the protein size shifted from 46 kDa to 30 kDa (Clemmons, 1997). Pregnancy-associated plasma protein-A (PAPP-A) was identified as IGF-dependent, IGFBP-4 specific protease, and it is produced by a number of cell types, including fibroblasts, granulose cells and osteoblasts (Boldt and Conover, 2007).

Table 2. Post-translational modifications and binding preference of IGFBP

Structural characteristics	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
Proteolysis	-	+	+	+	+	NA
Phosphorylation	+	-	+	-	+	NA
Glycosylation	-	-	N	N	O	O
Heparin binding	-	+	+	-	+	NA
ECM adhereance	-	-	+	-	+	-
IGF preference	I=II	II	I=II	I=II	II	II

+, presence of feature; -, absence of feature; N, N-linked glycosylation; O, O-linked glycosylation; NA, not available.

(2) Phosphorylation

Phosphorylation sites have been identified in IGFBP-1, -3, and -5. Phosphorylated human IGFBP-1 increased binding affinity for IGF-1 by six-fold. IGFBP-1 is phosphorylated at serines 101, 119, and 169 (Jones et al., 1991) and the number of phosphates per IGFBP-1 molecule affects its affinity for IGF-I (Jones et al., 1993). Hoeck and Mukku (1994) identified two phosphorylation sites, serines

101 and 111, on IGFBP-3. Conversely, phosphorylation doesn't influence IGF binding affinity of IGFBP-3. Mutants at these two sites, S101A and S111A, not being phosphorylated or glycosylated, were functionally indistinguishable from wild type IGFBP-3.

(3) Binding to cell surfaces and extracellular matrix

IGFBP-1,-3, and -5 have been reported to adhere to cell surfaces or extracellular matrix (ECM). IGFBP-1 binds to α5β1-integrin receptor which is associated with stimulation of cell migration in CHO cells (Jones et al., 2003). IGFBP-3 and -5 adhere to different molecules on cell surfaces and in ECM. Increased cell surface (IGFBP-3) and ECM (IGFBP-5) adherence has been shown to be associated with decreased IGF-I binding affinity and increased mitogenic response. IGFBP-3 binding to cell surfaces results in a ten-fold reduction in affinity for IGF-I, whereas IGFBP-5 binding to ECM results in an eight-fold reduction in affinity which is required for IGF potentiation (Clemmons, 1997). Recently, a group has identified a novel cell death receptor, IGFBP-3R, which binds to IGFBP3 and mediates caspase-dependent apoptosis in cancer cells (Ingermann et al., 2010).

IGF system in glucose metabolism

Type 2 diabetes has greatly caught public attention because of its strong association with obesity and life-threatening complications, such as various cardiovascular diseases, neuropathy, nephropathy, skin problems, hearing problems, and blindness. In spite of the controversial pathogenesis, it's generally accepted that

type 2 diabetes is primarily caused by insulin resistance, namely impaired insulin sensitivity. Nevertheless, there is fairly large amount of literature suggesting impaired insulin secretion is the main genetic factor predisposing to type 2 diabetes (Gerich, 1998).

There is no doubt that insulin plays a vital role in glucose metabolism. Due to structural and functional similarities with insulin, IGF system is likely to have insulin-like effects on glucose metabolism (Table 3, Rajpathak et al., 2009). In fact, studies have shown that IGF-I can promote glucose uptake in certain peripheral and hepatic tissues (Laager et al., 1993; Sherwin et al., 1994), augment glucose oxidation and nonoxidative glucose disposal, similar to insulin (Elahi et al., 1993; Russel-Jones et al., 1995). Although the effect of IGF-I is only 4-7% of that of insulin, it still makes IGF-I an important player in glucose homeostasis given that the concentration of IGF-I is 100-fold greater than insulin. In addition, IGF-I also suppresses endogenous glucose production (Elahi et al., 1993; Russell-Jones et al., 1995), though much less effectively than insulin. A study of free fatty acid (FFA) and glucose metabolism shows that suppression of endogenous glucose production by IGF-I is not impaired in type 2 diabetic subjects, indicating normal hepatic sensitivity to IGF-I (Pratipanawatr et al., 2002). This leads to the potential therapy of type 2 diabetes using IGF-I. Numerous studies have shown that exogenous IGF-I administration can reduce serum glucose levels. This effect is not only observed in healthy individuals (Schmid et al., 2005), but also in type 2 diabetic patients (Pratipanawatr et al., 2002). During these studies, IGF-I induced serum glucose

reduction was accompanied by improved insulin sensitivity.

As part of the IGF system, IGFBPs are predicted to have some roles in glucose metabolism. Among 6 IGFBPs, IGFBP-1 has been extensively studied. Serum levels of IGFBP-1 are strongly correlated with insulin sensitivity (Maddus et al., 2006). Endogenous IGFBP-1 levels correlate inversely with free IGF-I levels (Nyomba et al., 1997).

Though the relationship of insulin with IGFBP-2 is not as strong as that with IGFBP-1, IGFBP-2 is the principle binding protein secreted by differentiating preadipocytes (Boney et al., 1994), suggesting its potential role in the development of obesity. Recent studies have shown that transgenic mice overexpressing human IGFBP-2 have improved insulin sensitivity and are protected from obesity compared to wild type litter mates. Even under high-fat/high-energy diets, IGFBP-2 is still protective against obesity and insulin resistance (Wheatcroft et al., 2007). Studies on human subjects further demonstrates IGFBP-2 as independent predictor of insulin sensitivity and IGF-I bioactivity (Arafat et al., 2009). Microarray profiles of liver mRNA have identified IGFBP-2 as a leptin-regulated gene. Following IGFBP-2 treatment delivered by an adenovirus, diabetic symptoms were reversed in insulin-deficient mice and insulin resistant, diet-induced mice (Hedbacker et al., 2010).

Though IGFBP-3 is the most abundant IGFBP in circulation, much less is known about its role in glucose homeostatis. IGFBP-3 binds to a nuclear receptor, RXR-α, which interacts with PPAR-γ, a nuclear protein involved in the regulation of glucose

metabolism (Yamanaka et al., 1999; Schedlich et al., 2000). Transgenic mouse studies have indicated that overexpression of human IGFBP-3 cDNA is associated with significant body weight reduction compared to wild type mice (Modric et al., 2001). These transgenic mice have also shown fasting hyperglycemia and impaired glucose tolerance and insulin resistance (Silha et al., 2002).

Table 3. Association between IGF system and type 2 diabetes. License obtained from John Wiley and Sons, number 2403910598017.

	Cross-sectional associations		Predicted prospective association with type 2 diabetes	Remark
	Obesity/pre-diabetes[b]	Type 2 diabetes		
Total IGF-I	Normal levels	Normal levels	Low levels associated with increased risk of diabetes	IGF-I has insulin-like effects on glucose and FFA uptake as well as other effects that may compensate for increasing insulin resistance. Total IGF-I levels are normal despite GH hyposecretion, due to production by adipocytes, and insulin stimulation of hepatic IGF-I synthesis.
Free IGF-I	Elevated levels	Elevated levels	Low levels associated with increased diabetes risk (stronger association than that for total IGF-I)	Free IGF-I may be more bio-available than bound IGF-I, and may, as above, compensate for insulin resistance. If correct, those with insulin resistance who have low free IGF-I levels will be at an increased diabetes risk.
IGFBP-1	Reduced levels	Reduced levels	High levels associated with increased risk of diabetes	IGFBP-1 reduces the bio-availability of IGF-I. Insulin, however, down-regulates IGFBP-1, increasing free IGF-I levels in the face of insulin resistance; a potential compensatory mechanism. Cross-sectionally, though, low IGFBP-1 falsely appears to be associated with diabetes.
IGFBP-2	Reduced levels	Reduced levels	High levels associated with increased risk of diabetes	Similar to IGFBP-1.
IGFBP-3	Elevated levels	Elevated levels	High levels associated with increased risk of diabetes	IGFBP-3 reduces the bio-availability of IGF-I, increasing risk of diabetes. Insulin does not, however, regulate IGFBP-3; hence, reverse causality is not an issue with IGFBP-3 (unlike with IGFBP-1 and -2, above).

[a]Cross-sectional data reflect not only the effects of the IGF-axis parameter (e.g. IGF-I) on diabetes, but also the effects of the diabetes on the parameter (reverse causality). For example, the high free IGF-I levels observed in people with type 2 diabetes may not be because high IGF-I causes diabetes but because increases in free IGF-I are, in theory, a compensatory (protective) mechanism in the face of increasing insulin resistance.
[b]Prediabetes includes impaired glucose tolerance and impaired fasting glucose.

(Rajpathak et al., 2009)

Traumatic Brain Injury (TBI)

Traumatic brain injury, referred to as TBI, is a form of acquired brain injury caused by external damage to the brain. According to the CDC, each year there are approximately 1.5 million people in the US suffering from a traumatic brain injury.

Symptoms of TBI may vary depending on the severity of the injury. Mild TBI causes cognitive problems such as headache, unconsciousness, memory loss, and thinking problem. Others include blurred vision, ringing ears, vomiting, fatigue, and behavioral changes. The effects of severe TBI are profound. Patients with severe TBI have the same symptoms as mentioned above which only last longer and may cause some long-term social and behavioral deficits.

Following injury neurons and glials die within days due to induced apoptosis. IGF-I and IGFBPs, however, are upregulated around the injured area, suggesting the role of IGF-I in brain recovery. IGF-I has been shown to be associated with microglia and astrocytes surrounding the infarction area (Beilharz et al., 1998). IGF-1 is also induced after hypoxia-ischemia (Guan et al., 2003). Induction of IGFBP-2, -3, -4, and -5 have been reported following brain injury and are hypothesized to localize IGFs to the injury site, thereby providing neuroprotective functions. In infant rats, the expression of IGFBP-2 and -3 peaks around the same time as IGF-I, 3-5 days after hypoxia-ischemia. The expression of IGFBP-5 occurs later, around 7 days following injury, similar to IGF-II (Guan et al., 2003). IGFBP-2 preferably binds to IGF-II and is the major IGFBP in the CNS (Lee et al., 1992). However, it remains unelucidated how IGFBP-2 affects cell proliferation and apoptosis at the injury site.

Methods

Animals

Congenic C57BL/6J IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, and IGFBP-6 KO mice were generated by heterozygous mating after backcrossing WT and KO mice on 129S6/C57BL/6J mixed background to C57BL/6J background for at least 10 generations. All mice were maintained in the accredited UMDNJ vivarium facility with 25°C room temperature and 12-hour light-dark cycle.

Generation of IGFBP-2 knockout mice

An EcoRI fragment including exon 4 was cloned between neo and HSV-TK gene of the KO vector. Another EcoRI fragment was inserted into the 3' end of the neo sequence of the KO vector. This targeting construct was then introduced into CCE embryonic stem cells with G418 and gancyclovir resistant ES clones as a result. Genomic southern screening of DNA extracted from the ES clones identified target lines with majority of exon 3 replaced by the neomycin cassette. ES cells from the target lines were microinjected into C57BL/6J blastocysts and transferred to pseudopregnant females to produce germline transmitting chimeras (Wood et al., 2000). Male chimeras were bred with C57BL/6J females to generate IGFBP-2 deficient mice. IGFBP2 heterozygous mice on a mixed 129/C57 background were crossbred with pure C57BL/6J mice ten generations to obtain F10 heterozygous. Interbreeding of the F10 heterozygous mice gave rise to IGFBP-2 WT and KO inbred mice on a pure C57BL/6J background.

Generation of IGFBP-3 knockout mice

A 3.6kb *Sac*II / *Kpn*I fragment from exon 1 to exon 3 was cloned between neo and HSV-TK gene of the KO vector. Another 8.5kb *Xho*I fragment was inserted into the 3' end of the neo sequence of the KO vector. This targeting construct was then introduced into CCE embryonic stem cells with G418 and gancyclovir resistant ES clones as a result. Genomic southern screening of DNA extracted from the ES clones identified target lines with partial exon 1 replaced by the neomycin cassette. ES cells from the target lines were microinjected into C57BL/6J blastocysts and transferred to pseudopregnant females to produce germline transmitting chimeras. Male chimeras were bred with C57BL/6J females to generate IGFBP-3 deficient mice (Ning et al., 2006). IGFBP-3 heterozygous mice on a mixed 129/C57 background were crossbred with pure C57BL/6J mice ten generations to obtain F10 heterozygous mice. Interbreeding of the F10 heterozygous mice gave rise to IGFBP-3 WT and KO inbred mice on a pure C57BL/6J background.

Generation of IGFBP-4 knockout mice

A 5kb *Hind*III/*Bst*XI fragment including intron 1 was cloned between neo and HSV-TK gene of the KO vector. Another 2.5kb *Bst*XI/*Nru*I fragment was closed into the 3' end of the neo sequence of the KO vector. This targeting construct was then introduced into CCE embryonic stem cells resulting in G418 and gancyclovir resistant ES clones. Genomic southern screening of DNA extracted from the ES clones identified a target line with exon 1 replaced by the neomycin cassette. ES cells from the target line were microinjected into C57BL/6J blastocysts and

transferred to pseudopregnant females to produce germline transmitting chimeras. Male chimeras were bred with C57BL/6J females to generate IGFBP-4 deficient mice (Ning et al., 2006). IGFBP-4 heterozygous mice on a mixed 129/C57 background were crossbred with pure C57BL/6J mice twelve generations to obtain F12 heterozygous mice. Interbreeding of the F12 heterozygous mice gave rise to IGFBP-4 WT and KO inbred mice on a pure C57BL/6J background.

Generation of IGFBP-5 knockout mice

A 9kb *Eco*RI /*Sal*I fragment from intron 1 to exon 4 was cloned between neo and HSV-TK gene of the KO vector. Another 1.3kb *Pvu*II/*Eco*RV fragment was inserted into the 3' end of the neo sequence of the KO vector. This targeting construct was then introduced into CCE embryonic stem cells with G418 and gancyclovir resistant ES clones as a result. Genomic southern screening of DNA extracted from the ES clones identified target lines with partial exon 1 replaced by the neomycin cassette. ES cells from the target lines were microinjected into C57BL/6J blastocysts and transferred to pseudopregnant females to produce germline transmitting chimeras. Male chimeras were bred with C57BL/6J females to generate IGFBP-5 deficient mice (Ning et al., 2006). IGFBP-5 heterozygous mice on a mixed 129/C57 background were crossbred with pure C57BL/6J mice ten generations to obtain F10 heterozygous. Interbreeding of the F10 heterozygous mice gave rise to IGFBP-5 WT and KO inbred mice on a pure C57BL/6J background.

Generation of IGFBP-6 knockout mice

A *Hind*III fragment from intron 1 to exon 4 was cloned between neo and

HSV-TK gene of the KO vector. Another *Eco*RI/*Sma*I fragment was inserted into the 3' end of the neo sequence of the KO vector. This targeting construct was then introduced into CCE embryonic stem cells with G418 and gancyclovir resistant ES clones as a result. Genomic southern screening of DNA extracted from the ES clones identified target lines with partial exon 1 replaced by the neomycin cassette. ES cells from the target lines were microinjected into C57BL/6J blastocysts and transferred to pseudopregnant females to produce germline transmitting chimeras. Male chimeras were bred with C57BL/6J females to generate IGFBP-6 deficient mice (Grewal's thesis). IGFBP-6 heterozygous mice on a mixed 129/C57 background were crossbred with pure C57BL/6J mice ten generations to obtain F10 heterozygous. Interbreeding of the F10 heterozygous mice gave rise to IGFBP-6 WT and KO inbred mice on a pure C57BL/6J background.

Generation of IGFBP-35 double knockout mice

After IGFBP-3 and IGFBP-5 single KO mice were generated on a pure C57BL/6J background, they were crossbred to produce IGFBP-35 double heterozygous mice, IGFBP-35 +/- +/-. These heterozygous were interbred to produce IGFBP-35 double KO mice, IGFBP-35 -/- -/-.

Southern blot

Mouse tail was cut and digested overnight at 55°C with 10μg/μl Proteinase K. After phenol/chloroform extraction, tail DNA was digested with SacI (for IGFBP-3 genotyping), BamHI (for IGFBP-4 genotyping), and EcoRI (for IGFBP-5 genotyping), HindIII (for IGFBP-6 genotyping) respectively, and run on 0.7%

agarose gels and transferred overnight to Hybond membranes (Amersham Pharmacia, Arlington Heights, IL). Membranes were prehybridized in Rapid Hyb for 1 hour at 65°C and then incubated overnight at 65°C in the same hyb solution with ^{32}P-labedled probes. The next day membranes were washed with 1X SSC/0.1% SDS three times at 65°C for 1.5 hour and exposed to Kodak film. IGFBP3 heterozygous showed a 6.9kb wild type band and a 6kb mutant band. IGFBP4 heterozygous showed a 9kb wild type band and a 5.5kb mutant band. IGFBP5 heterozygous showed a 4kb wild type band and a 2kb mutant band.

PCR genotyping

Mouse tail was cut and digested overnight at 55°C with 10μg/μl Proteinase K. After boiling for 5 minutes, 2μl DNA was used as a template in PCR. PCR was performed using 5μl 5X Green GoTaq reaction buffer (Promega), 1.5μl each of forward and reverse primers (final concentration of 0.1μM), 0.2μl Plantinum Taq polemerase (Roche), and 10μl sterile water to attain a final reaction volume of 25μl. Thermal cycling conditions were as follows: hot start at 94°C for 3 min; 35 cycles of amplification, each cycle consisted of, 30s denaturation (94°C), 45s primer annealing (62°C), 45s primer extension (72°C) and a final extension of 5min at 72 °C. BP4 WT sequence size is 222bp, neo sequence size is 492bp.

Primers used: BP4 forward **GGTTGCGAGGAGTTGGTG**

BP4 reverse **AGAGGTCTGCAGG CTTTCCT**

neo forward **AGGATCTCCTGTCATCTCACCTTGCTCCTG**

neo reverse **AAGAACTCGTCGAGAAGGCGATAGAAGGCG**

Growth curve measurement

C57BL/6J congenic WT and KO mice were obtained from heterozygous mating to examine the effect of IGFBPs on growth. Litters were weighed from Day 0 up to Week 12. All animals were maintained on normal diet and group-housed in a clean facility with less than 5 animals per cage.

Glucose tolerance test

Age-matched, two to three month old male WT and IGFBP-3 KO mice were fasted for 16 hours (5:30pm-9:30am) followed by intraperitoneal injection of glucose (2g/kg body weight). Whole blood was collected from the tail vein at indicated time points (0, 15, 30, 60, 90, 120min) after glucose injection. Blood glucose levels were measured using an Accu-check blood glucose monitor (Roche, Indianapolis, IN).

Plasma insulin level measurements

To measure insulin levels during the glucose tolerance test, mice were fasted for 16 hours (5:30pm–9:30am) followed by intraperitoneal injection of glucose (2g/kg body weight). Blood was collected in EDTA-containing tubes and then centrifuged to obtain plasma. Insulin levels were measured at 0, 2, 5, 10, 15, and 30 min using an insulin ELISA kit (Linco, St. Charles, MO), following the protocol provided by the manufacturer.

Insulin tolerance test

Age-matched, two to three month old male WT and IGFBP-3 KO mice were fasted for 6 hours (9:30am-3:30pm) followed by intraperitoneal injection of bovine insulin (0.75U/kg body weight, Sigma, St.Louis, MO). Whole blood was collected

from the tail vein at indicated time points (0, 15, 30, 60min) after insulin injection. Blood glucose levels were measured using an Accu-check blood glucose monitor (Roche, Indianapolis, IN).

In-situ hybridization

Fresh frozen cryostat brain sections were mounted onto TESPA coated microscope slides and stored at –80°C until use. Sections were fixed in 4% paraformaldehyde in PBS, dehydrated in ethanol series, acetylated in 0.25% acetic anhydride/50mM triethanolamine, washed in 0.2X saline sodium citrate (SSC), dehydrated in ethanol series, and prehybridized at room temperature. Then, sections were hybridized overnight at 50°C with 2X 104cpm/µl 35S-labeled antisense RNA probes, washed in 50% formamide/10mM dithiothreitol/1x SSC, RNase treated in 100µg/ml Rnase A, washed in 0.5X SSC, dehydrated, and subjected to autoradiography. 35S-labeled antisense RNA probes were transcribed using SP6, T7, or T3 RNA polymerases in the presence of [35S]UTP from linearized plasmids pRBP1–501 [rat BP1: nucleotide (nt) 486–892] (56), pG3-2-11 (rat BP2: nt 502-1087) (57), pRBP3-AR (rat BP3: nt 163–861) (58), pRBP4-SH (rat BP4: nt 435–878) (59), pGEM3Z/mBP5, 2–3 (mouse BP5: nt 512–988) (60), pRBP6-PP (rat: BP6 nt 229–475) (61).

Immunohistochemistry

For BrdU nonfluorescent immunostaining, sections were first rinsed three times with 1X PBS for 5 min to remove OCT. In order to denature DNA, sections were incubated in Trypsin (0.1g $CaCl_2$ and 0.1g Trypsin/100ml 0.1M Tris) at 37°C for 20

min, rinsed in 1X PBS for 5 min, incubated in 2 N HCl for 30 min and rinsed with 1X PBS. To block endogenous peroxidases sections were treated with 3% H_2O_2/Methanol for 10 min. Sections were kept in 0.05% TBS/5% normal horse serum (NHS) for 1 hr and then incubated in anti-BrdU antibodies (1:100, BD Biosciences) in 0.05% TBS overnight at 4°C. After 3 rinses in 1X PBS, sections were incubated with antimouse secondary antibodies (1:300, Vector Laboratories, Burlingame, CA) in 0.05% TBS for 1 h. Sections were rinsed again in 1X PBS 3 times, and an avidinbiotin peroxidase complex (ABC, Vector Laboratories, Burlingame, CA) was applied for 1 hr according to the manufacturers instructions. As substrate for the peroxidase reaction, diaminobenzidine (Sigma, St.Louis, MO) was applied for 3 min 30 sec. Slides were finally rinsed in water and stained with methyl green.

Primary neurosphere assay

P5 mouse pups were decapitated and their brains removed. Under aseptic conditions, a cut was made 2 mm from the anterior pole of the brain. A second cut was made approx. 3 mm posterior to the first cut. The region enclosed between the cortex and the ventricle containing the SVZ was removed and placed in fresh PGM [1 mM MgCl2 and 0.6% dextrose in PBS (pH 7.3)]. The tissue was mechanically minced using forceps and then enzymatically dissociated using 0.05% trypsin/EDTA at 37°C for 7 min. The trypsin was inactivated by the addition of an equal volume of newborn calf serum and the tissue was resuspended in ProN media [DMEM (Dulbecco's modified Eagle's medium)/F12 (1:1) media containing 10 ng/ml d-biotin, 25 μg/ml insulin, 20 nM progesterone, 20 mM Hepes, 100 μM putrescine, 5 ng/ml

selenium, 50 μg/ml apo-transferrin and 50 μg/ml gentamycin] and triturated in ProN. The triturated suspension was passed through a 40 μM Nitex screen and the cells were collected by centrifugation at 200 g for 2 min and washed with ProN. The cells were counted with 0.1% Trypan Blue dye under a haemocytometer and plated at 5×104 cells/ml in ProN medium supplemented with 2 ng/ml EGF or 1 ng/ml FGF (fibroblast growth factor)-2 or as stated. The cells were cultured at 37°C in 5% CO2 incubators and fed every 2 days by removing approx. half of the medium and replenishing with fresh medium (Alagappan et al., 2009).

Quantitative PCR

Cell culture samples were snap frozen and thawed in 0.5 ml of Trizol reagent. After adding 100 μl of chloroform, the samples were centrifuged at 9300g rpm for 15 min at 4°C. The aqueous phase was then transferred to a new tube. After adding 250 μl of 70% EtOH, the aqueous phase was applied to an RNeasy Mini-spin column (Qiagen, Valencia, CA) to remove RNA contamination, according to the instructions of the manufacturer. The concentration of total RNA was determined by measuring optical density on the Nanodrop Spectrophotometer ND1000 (Thermo Scientific, Wilmington, DE). RNA samples were stored at –80°C until needed. cDNA was reverse transcribed from RNA using Superscript III RT kit (Invitrogen, Carlsbad, CA), following the protocol provided by the manufacturer (info provided by Ziegler).

Real-time PCR was performed using an ABI 7900HT (Applied Biosystems, Foster City, CA). RT products were diluted to 40ng/μl, and 2.5μl was used for each real-time PCR. Each RT product was run in triplicate. Real-time PCR was performed using a

real-time PCR kit provided with a SYBR Green fluorophore (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions and using 600 nm of each primer. After a 2-min incubation at 50°C and a 10-min incubation at 95°C, amplification was performed using the following cycle: 95°C for 15 sec, and 60°C for 1 min, 40 times. The relative abundance of target cDNA within a sample set was calculated from a serially diluted (standard curve) cDNA. Before analysis, real-time PCR data were normalized using GAPDH transcript abundance in the samples. After amplification, a dissociation curve was obtained using the following protocol: 15 sec of holding at 95°C, followed by 15 sec dropped to 60°C, and finally 15 sec going back to 95°C. The amplification profile was analyzed by SDS 2.2 (Applied Biosystems, Foster City, CA). Primer sequences are shown in Table 4.

Table 4. Primer sequence

Gene of interest	Forward primer	Reverse primer
IGFBP-2	**GGTACCTGTGAAAGGAGACGCG**	**ACTGCTACCACCTCCCAACATG**
IGFBP-3	**ATAAGAAGAAGCAGTGCCGCC**	**TACGTCGTCTTTCCCCTTGGT**
IGFBP-4	**CCTCTTCATCATCCCCATTCCA**	**TGTCTTCCGATCCACACACCAG**
IGFBP-5	**AAGACAGCACAACTTCAGTTCA**	**TCACAGGGAAGGATGTTTGAGT**

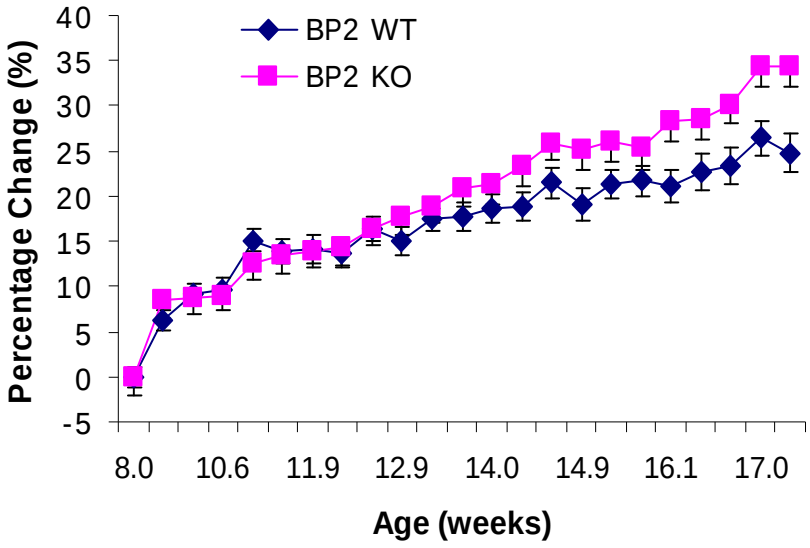
Statistics

Two-tailed t-test was applied in the analysis of growth curve measurement, glucose tolerance test, and insulin tolerance test. P value of less than 0.05 indicates test significance.

Results

To study the effects of IGFBPs on growth in a pure genetic background (C57BL/6J), growth curves were constructed for each single knockout strain (IGFBP-2 to IGFBP-6) and double knockouts (IGFBP-35) in both genders.

IGFBP-2 KO mice are significantly heavier than wild type littermates starting at 15wk (Fig 2.1). Male IGFBP-3 KO mice become significantly heavier than WT mice from 6.5wk to 13wk (Fig 2.2A, WT n=11, KO n=9, $p<0.05$). Body weights of IGFBP-3 females, on the other hand, don't differ significantly between KO and WT mice (Fig 2.2B, WT n=13, KO n=11).



By courtsy of Huang & Peng

Fig.2.1. Growth curve of BP2 C57BL/6J mice. WT n=6, KO n=4.

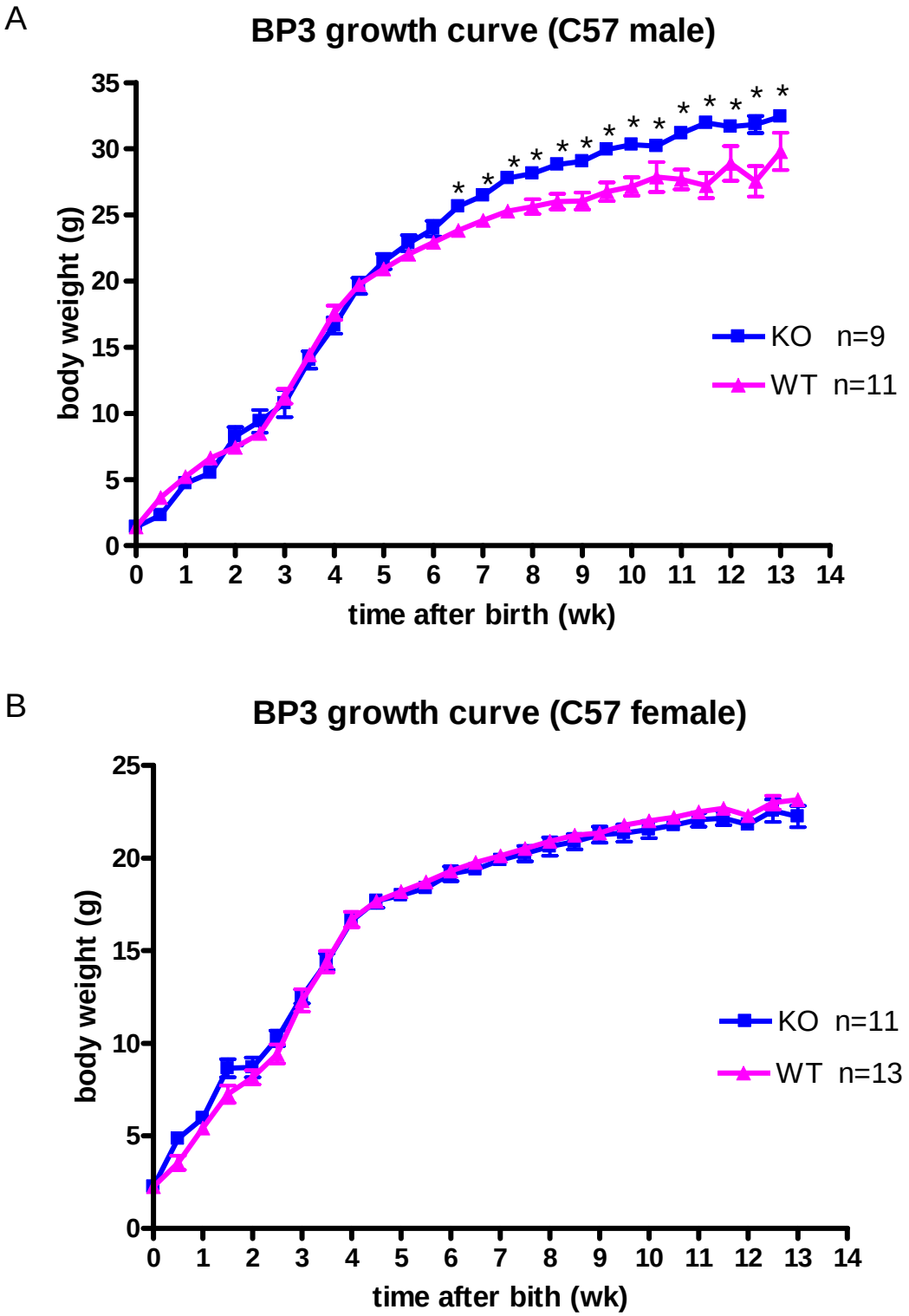


Fig.2.2. Growth curve of BP3 C57BL/6J mice. A, in males, from 6.5wk on, KO mice have significantly higher weight than WT littermates, $p<0.05$. B, in females, on the contrast, there is no significant difference in body weight between KO and WT mice.

Because our lab previously reported significant body weight reduction in IGFBP-4 single KO mice on a mixed C57/129S6 background which probably masked the phenotype caused by gene deficiency, this time the growth curve was measured in crossed 129S6 F4 generations mating as well as on a pure C57BL/6J background. Interestingly, growth curves of both genders of 129S6 mice don't show any difference (Fig 2.3A, 2.3B, male: HET n=11, KO n=2; female: HET n=15, WT n=7, KO n=4). Similarly, in C57BL/6J mice, both IGFBP-4 KO and WT don't differ in body weight (Fig 2.4A, 2.4B, male: WT n=11, KO n=6; female: WT n=22, KO n=24).

In both male and female IGFBP-5 mice, KO animals are significantly heavier than WT littermates after 8wk and 6wk respectively (Fig 2.5A, 2.5B, male: WT n=11, KO n=5; female: WT n=5, KO n=8, $p<0.05$). IGFBP-6 single KO mice have almost the same body weight as WT in both genders (Fig 2.6A, 2.6B, male: WT n=17, KO n=13; female: WT n=37, KO=17).

Comparison of male IGFBP-35 double knockout to WT and single IGFBP-3, -5 knockout mice shows significantly increased body weight in double KOs after 6wk (Fig 2.7A, $p<0.05$) and no significant difference in body weight among single and double knockout animals (Fig 2.7A, 2.7B). In females, IGFBP-35 double knockout mice are significantly heavier than WTs and single KOs after 5wk (Fig 2.8A, 2.8B, $p<0.05$).

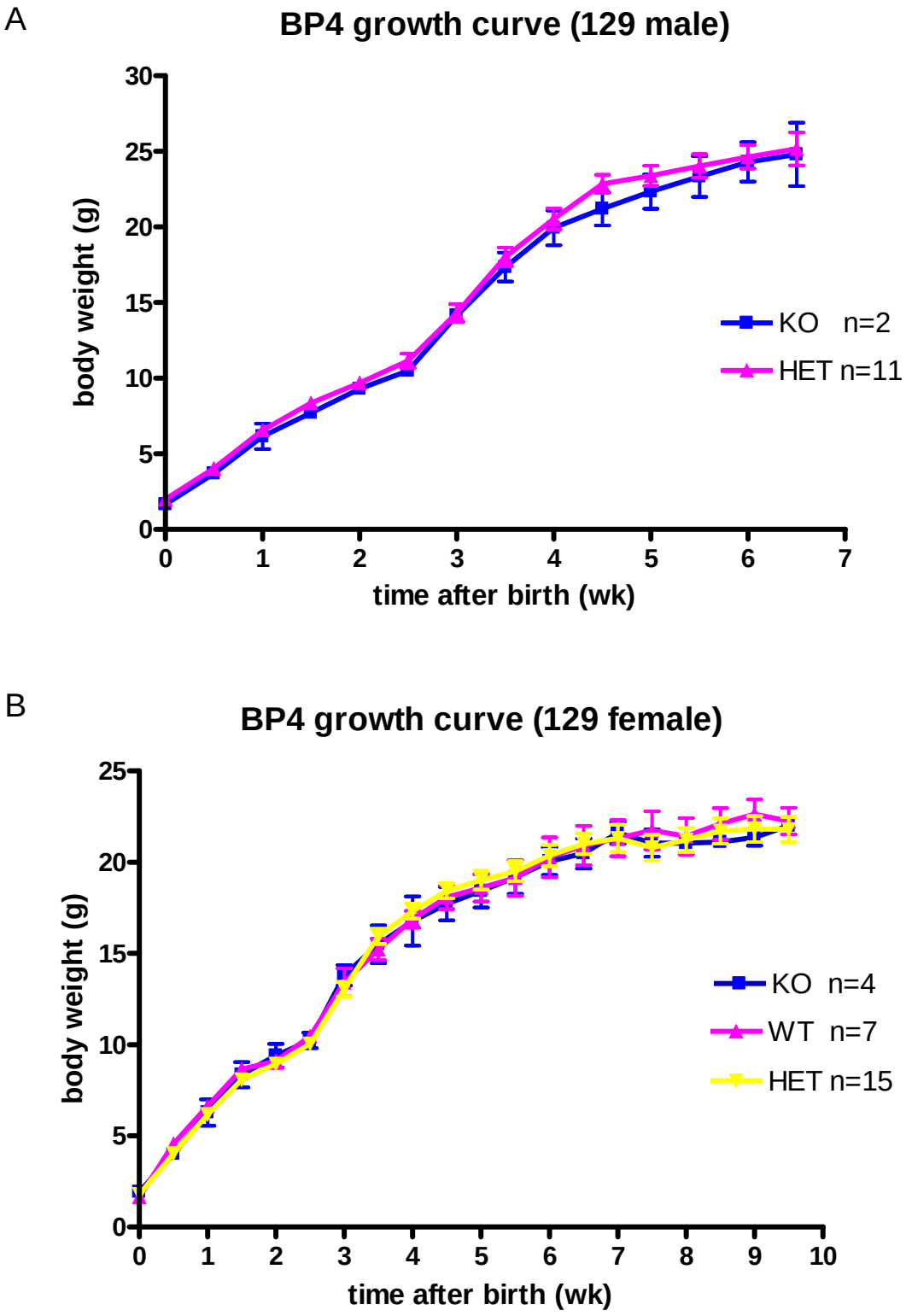
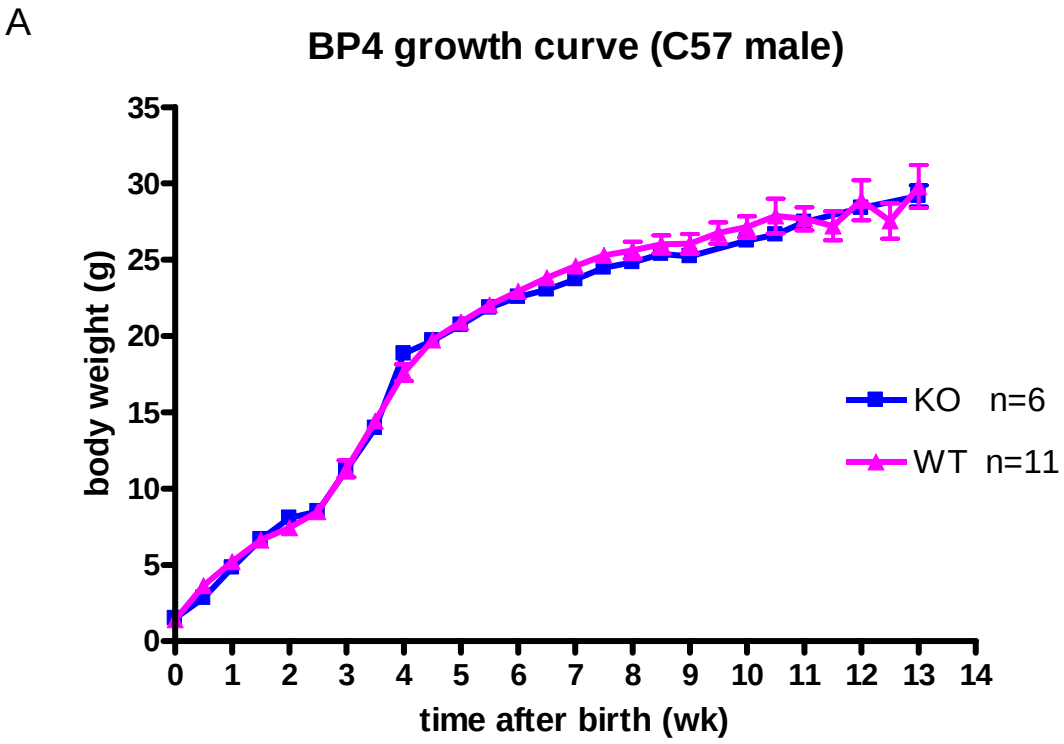


Fig.2.3. Growth curve of BP4 129S6 F4 mice. A, in males, there is no significant difference in body weight between knock out and heterozygous mice. KO n=2, WT n=11. B, in females, no significant difference in body weight is observed from brith to 9.5wk among KO, WT, and heterozygous mice.



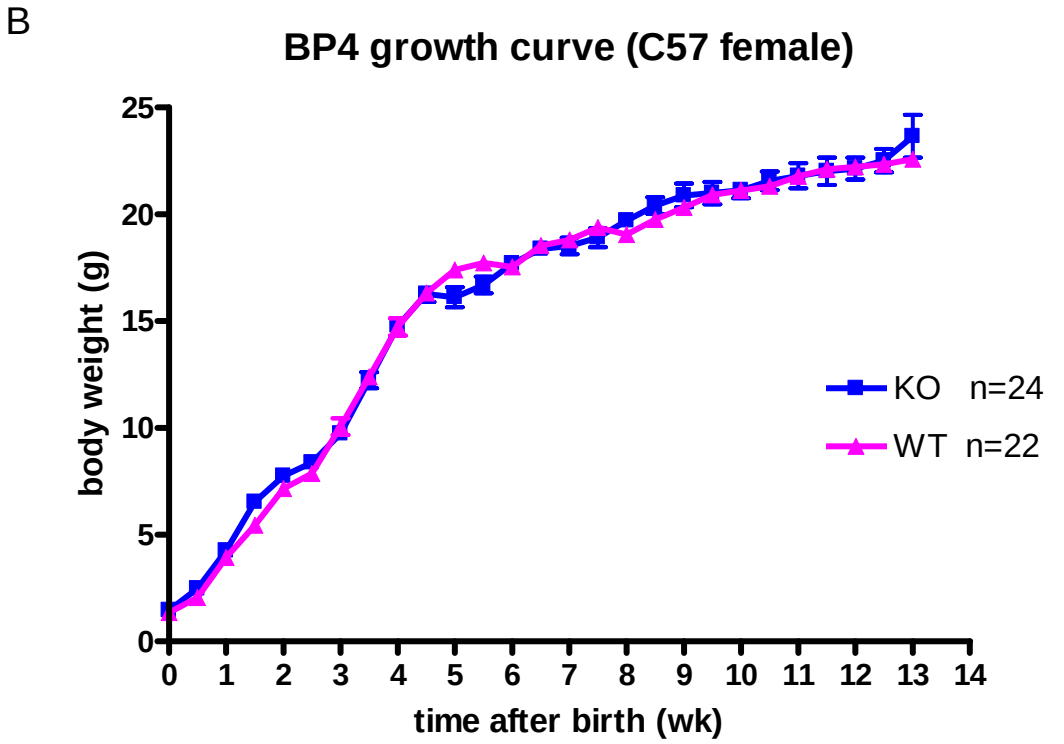


Fig.2.4. Growth curve of BP4 C57BL/6J mice. There is no statistical difference between KO and WT body weight in males (A) and in females (B).

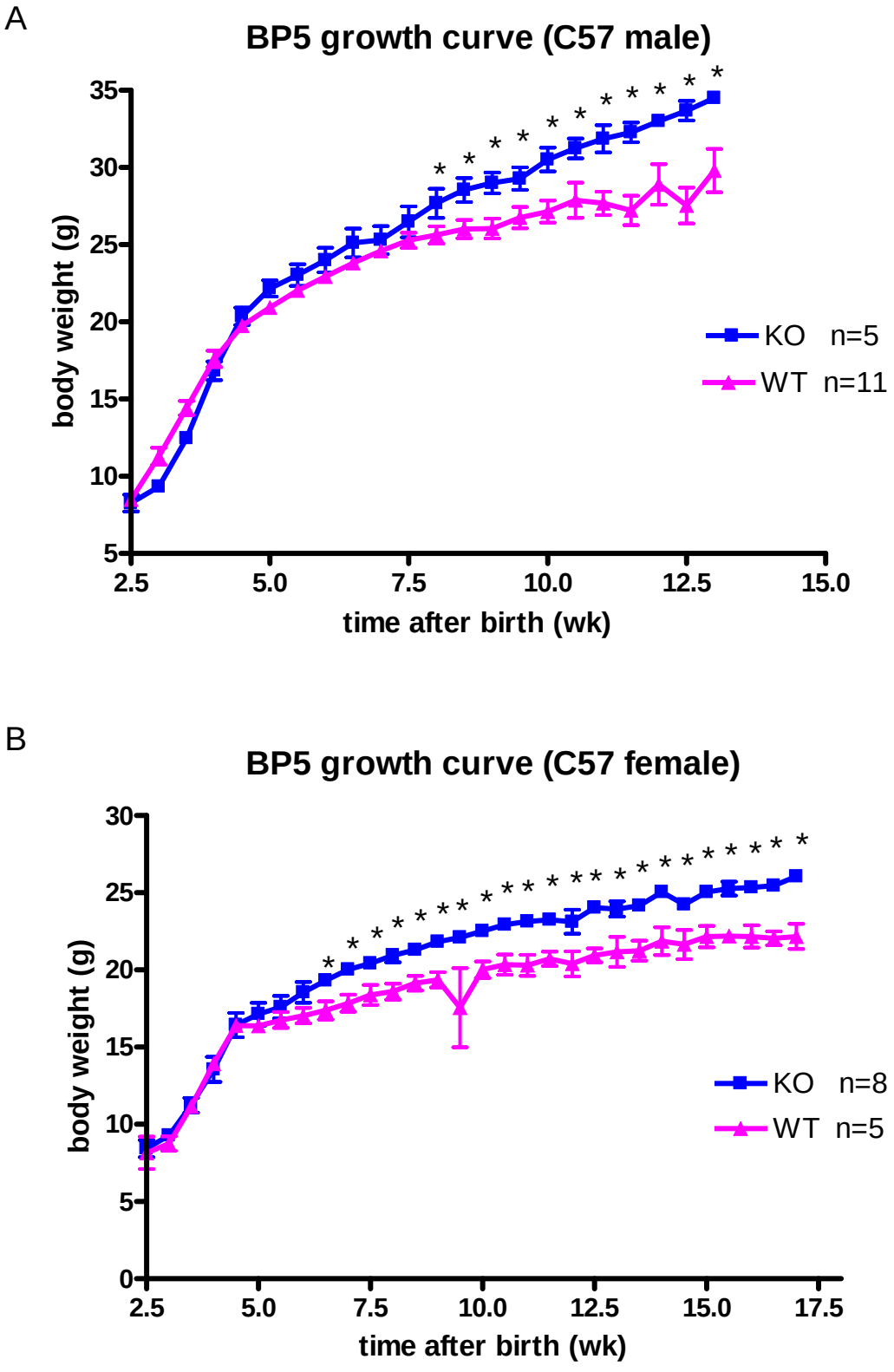


Fig.2.5. Growth curve of BP5 C57BL/6J mice. A, in males, significant difference in body weight between KO and WT mice is detected after 8wk ($p<0.05$). B, in females, KO mice are significantly heavier ($p<0.05$) than WT mice after 6wk.

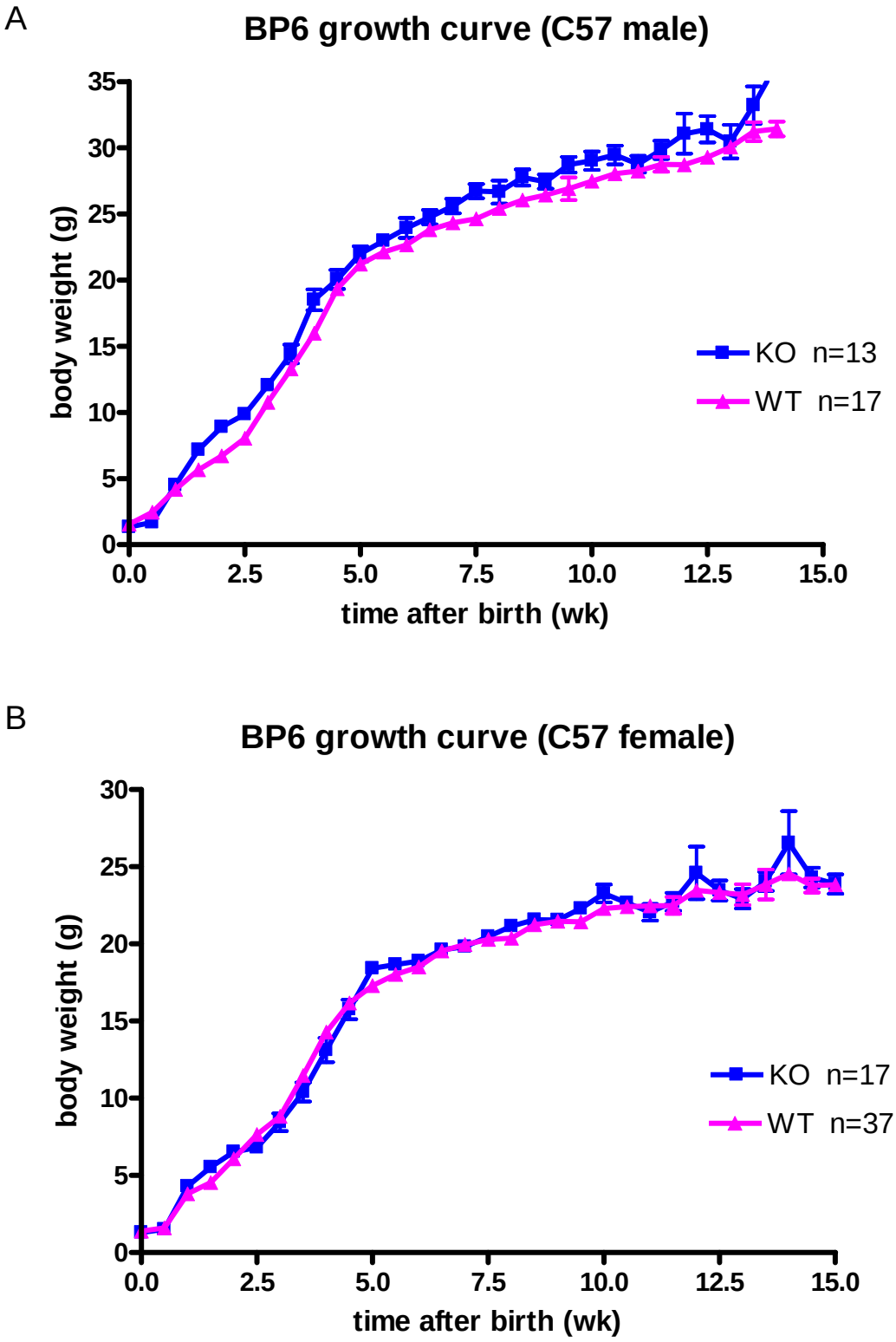


Fig.2.6. Growth curve of BP6 C57BL/6J mice. In both males (A) and females (B), there is no significant difference in body weight between WT and KO mice.

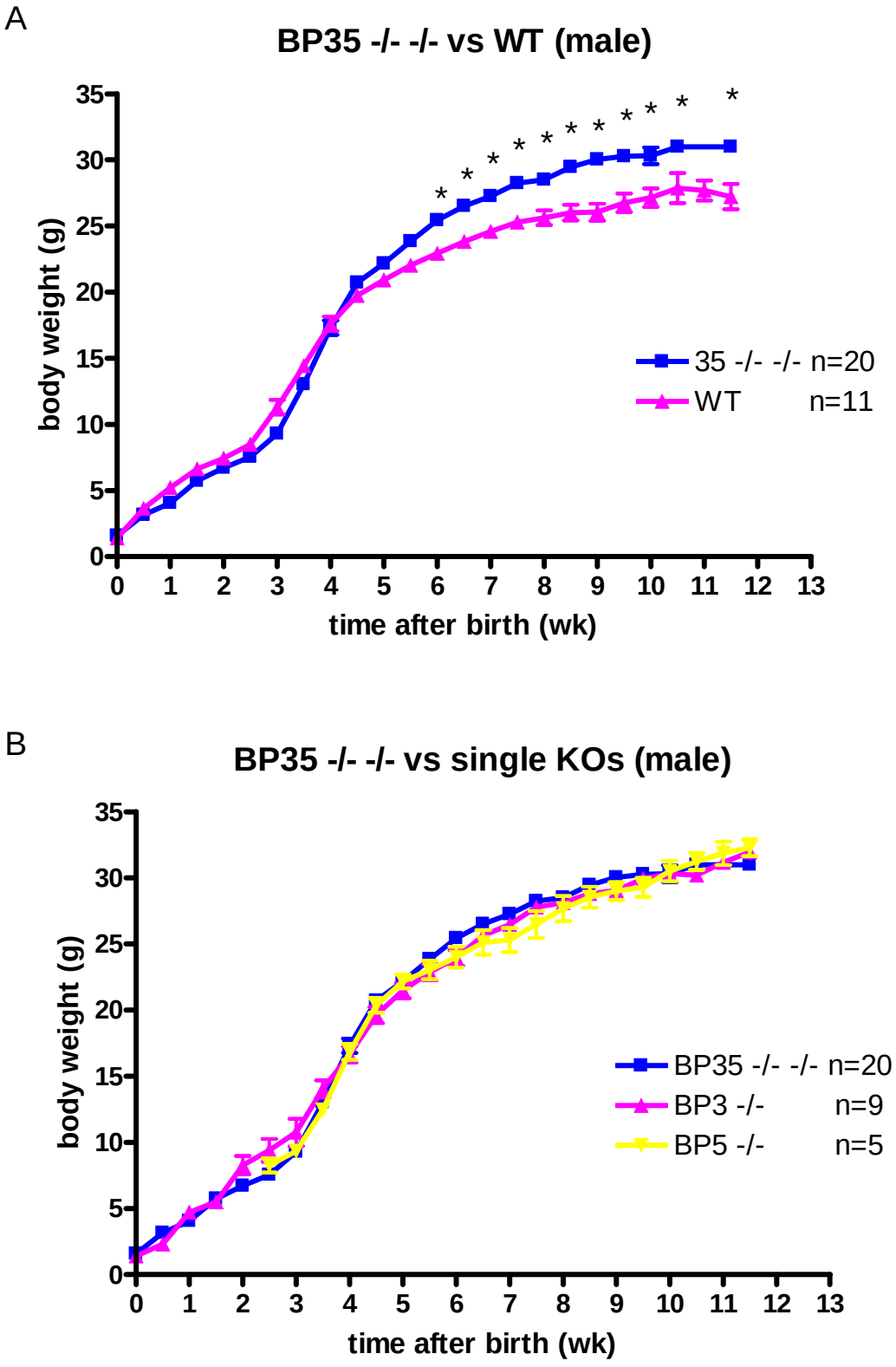


Fig.2.7. Growth curve of male BP35 C57BL/6J mice. A, after 6wk, IGFBP35 double knockout mice weigh statistically more than WT mice ($p<0.05$). However, compared to single KOs (B), there is no significant difference in body weight among single IGFBP-3, IGFBP-5 knockouts and IGFBP-35 double knockouts.

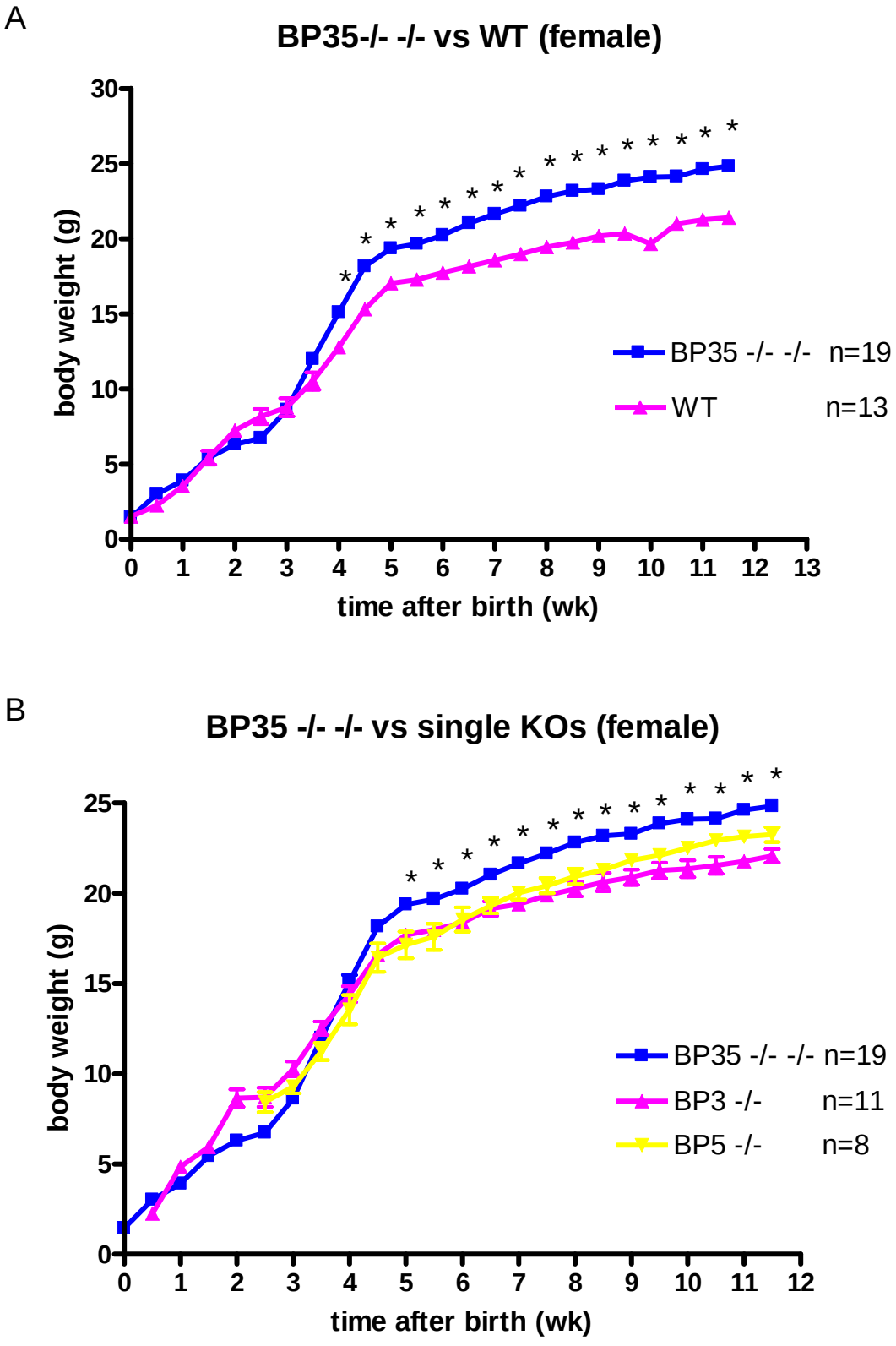


Fig.2.8. Growth curve of female C57BL/6J mice. A, there is significant difference in body weight between double KO and WT after 4wk, $p<0.05$. B, IGFBP-35 double knockouts are significantly heavier than single IGFBP-3, IGFBP-5 knockouts, $p<0.05$. Single IGFBP-3 and IGFBP-5 KOs have no body weight difference.

Glucose tolerance test in BP3 mice shows that IGFBP-3 KO mice have enhanced glucose tolerance compared to WT littermates. After overnight fasting and 2g/kg body weight i.p. injection of glucose, IGFBP-3 KO mice have a significantly reduced blood glucose level at 15, 30, and 60min (Fig 2.9, n=4 for each group, $p<0.05$ at 15, 60min, $p<0.01$ at 30min).

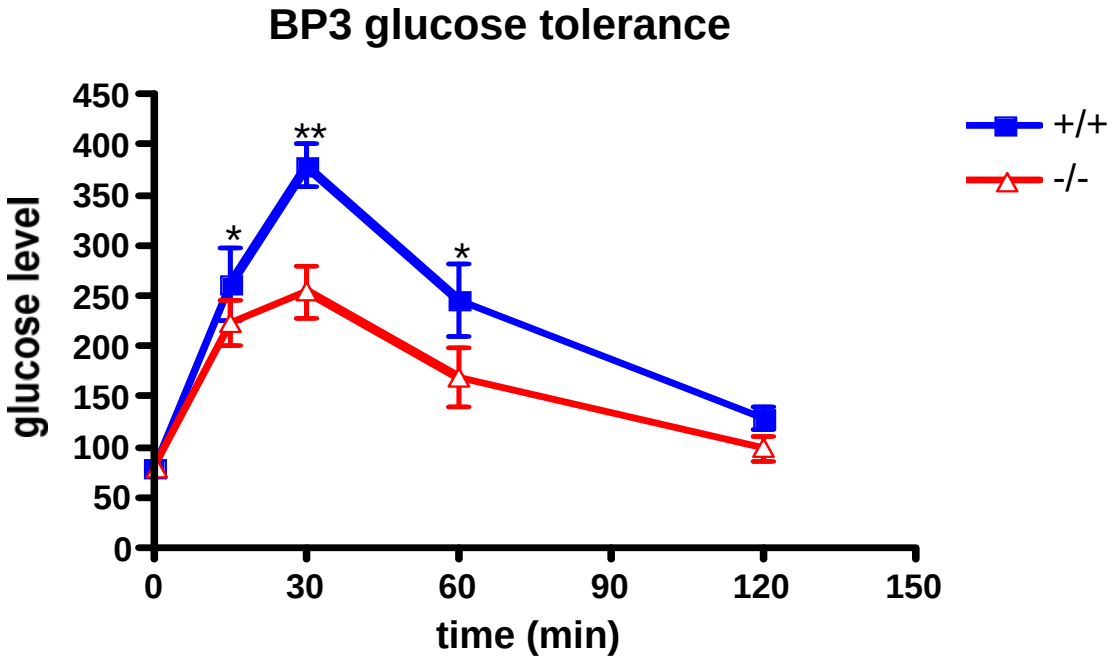


Fig.2.9. Glucose tolerance test in IGFBP-3 mice. At 15, 30, 60min, KO mice have significantly reduced glucose level compared to WT mice. *, $p<0.05$, **, $p<0.01$.

To test blood insulin level, mice were fasted overnight followed by a 2 g/kg i.p. glucose injection. Plasma was taken from the tail vein at indicated time points and assayed for insulin concentration by ELISA. Fig 2.10 shows at 2 and 15 min, IGFBP-3 KO mice have increased insulin secretion than WT ($p<0.05$).

To test insulin sensitivity, the insulin tolerance test was performed and it shows after 6 hr fasting, IGFBP-3 KO mice have significantly lowered glucose level at 30 and 60 min upon i.p. injection of 0.75U/kg insulin (Fig 2.11, $p<0.05$ at 30, 60 min).

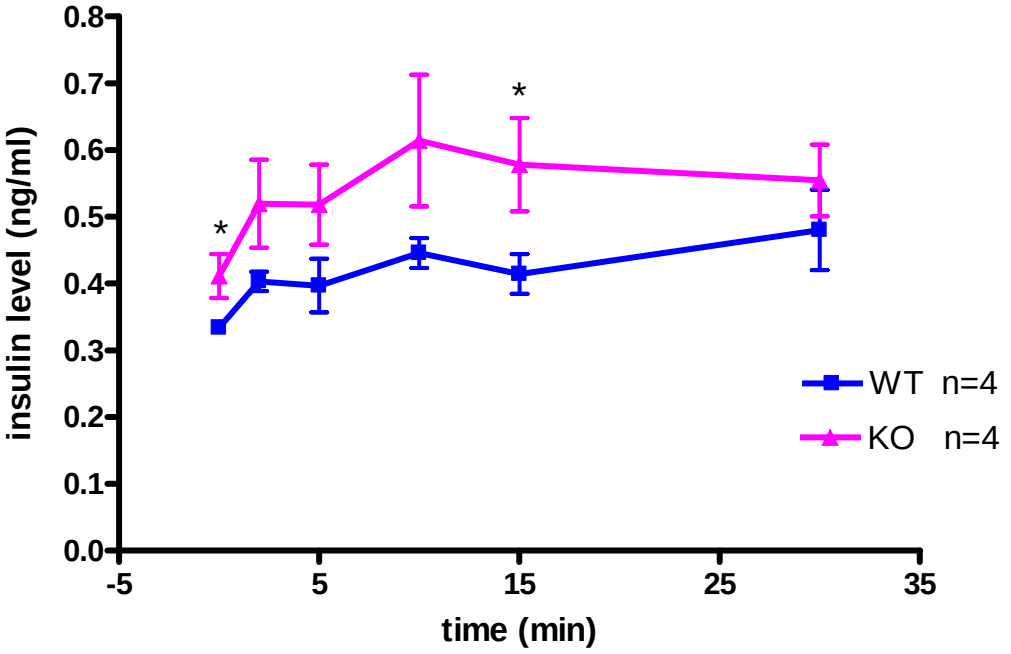


Fig.2.10. Blood insulin level after glucose injection. N=4 for WT and KO mice.

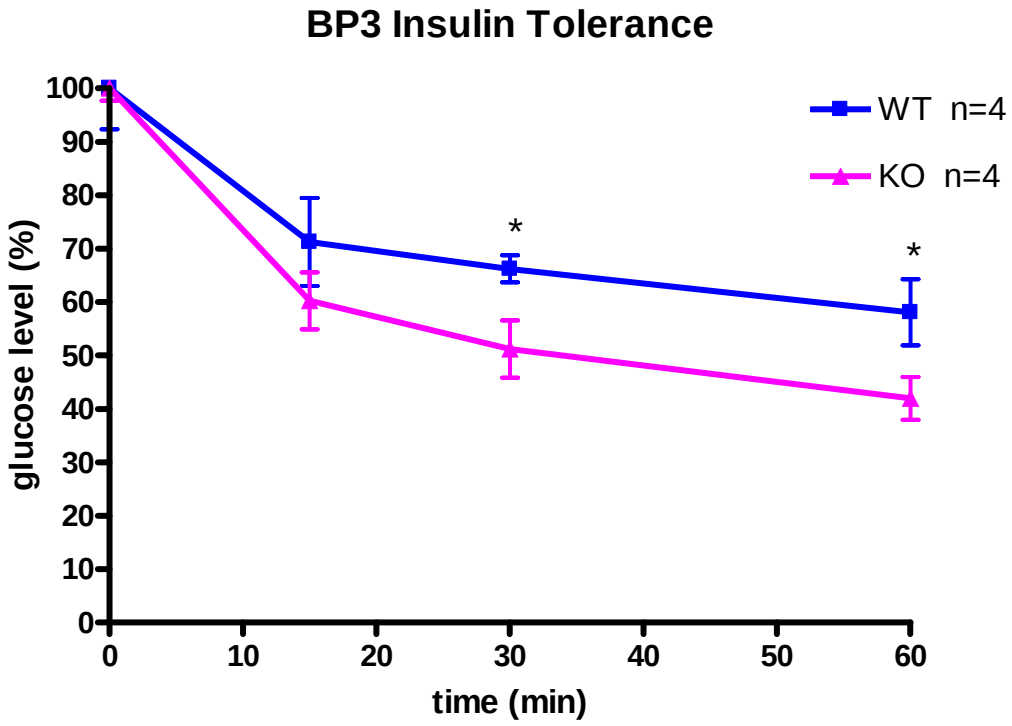


Fig.2.11. Insulin tolerance test in IGFBP-3 mice. After 6 hr fasting followed by a 0.75 U/kg i.p. insulin injection, blood glucose level was monitored at different time points in WT and IGFBP-3 KO mice (n=4 each). KO mice have significantly reduced glucose level ($p<0.05$) at 30 and 60 min.

Mice were injured as previously described. After 3 days and 7 days recovery, brains were sliced and stained with methyl green. Fig 2.12 and 2.13 show the injured brain and arrows indicate injury site.

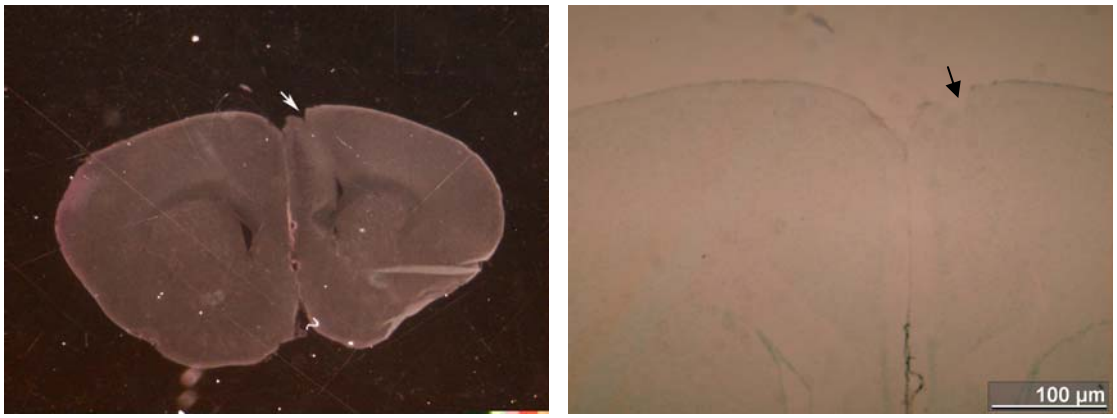


Fig.2.12. Injured mouse brain 3 days after moderate TBI. The left panel is a whole view and the right is a close-up view of the injury site. Arrows indicate the injury site.

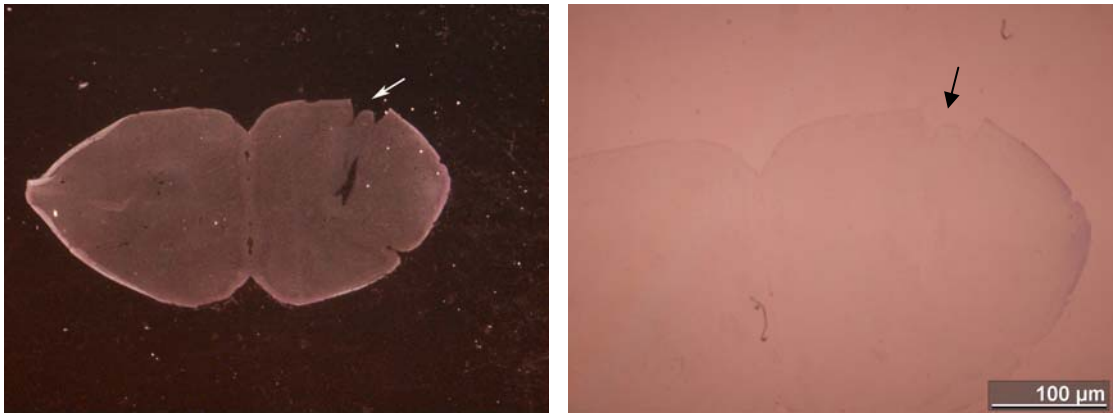


Fig.2.13. Injured mouse brain 7 days after moderate TBI. The arrows in both pictures indicate the injury site.

In situ hybridization was performed in WT mice following brain injury. After 3 days and 7 days recovery, IGFBP-2 has a significantly increased expression around the injury site (Fig 2.14, panels B, C, D compared to A). The expression of other IGFBPs, however, doesn't have a detectable increase (data not shown).

Immunohistochemistry staining with BrdU indicates 3 days after TBI brain injury, neurogenesis is increased along the SVZ in IGFBP-2 KO mice (Fig 2.15).

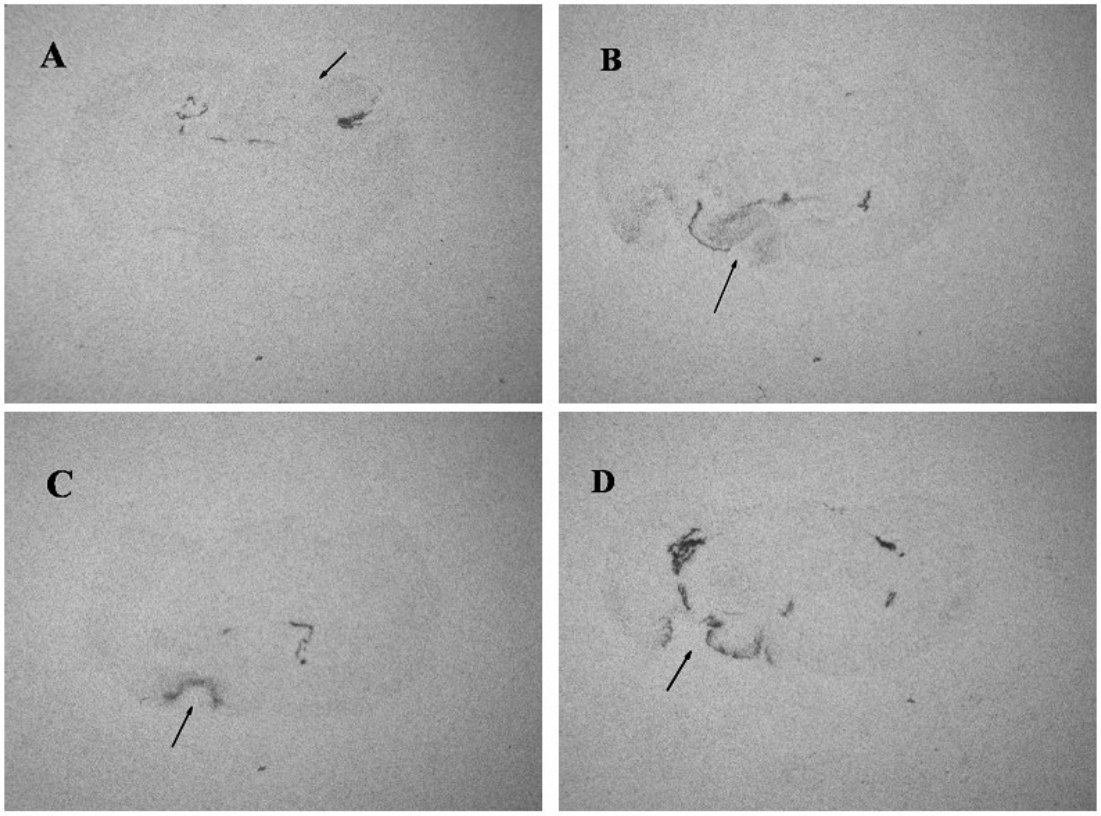


Fig.2.14. BP-2 expression 1, 3, 7 days after TBI. A, 1 day after TBI. B, 3 days after TBI. C, D, 7 days after TBI, arrow indicates the injury site. Similar pattern was observed in three animals.

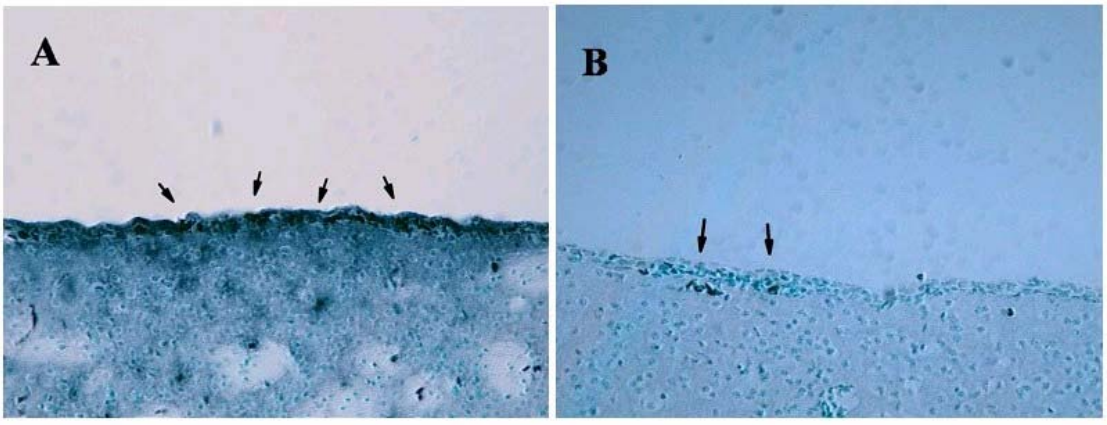


Fig.2.15. BrdU labeling of the SVZ in brain injured mice. IGFBP-2 KO mice had significantly increase BrdU+ cells (indicated by the arrows) along the SVZ. A, IGFBP-2 KO mouse B, WT mouse. Due to limited number of animals, quantification is not available at this time. However, the same results were seen three times.

Real-time quantitative PCR data indicates that in cultured neurospheres, compared to Stratagene's Universal Mouse Reference cDNA, IGFBP-2 expression is increased by 28-fold, IGFBP-3 has 12-fold increase, IGFBP-5 is doubled, whereas IGFBP-4 is decreased by about 8-fold (Fig 2.16).

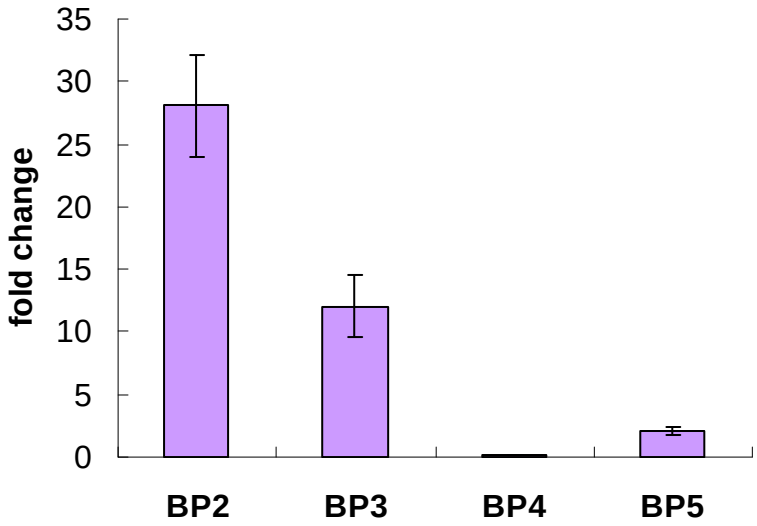


Fig.2.16. IGFBPs expression in neurospheres. qPCR was done in triplicates and repeated twice.

Discussion

An organism's phenotype is the result of genotype-environment interaction. In this sense, genetic background plays an important role in phenotype determination. Transgenic mice phenotypes generally depend on the background strains used in their creation. Indeed, study of strain differences has become a popular area since recent neural and behavioral studies with mice have yielded different results in different labs (Wahlsten et al., 2006). By comparing the behavior of mice kept under different housing conditions, lab environment and different experimenters, Lewejohann et al. (2006) showed that these variables affect the outcome of behavioral phenotypes. The effect of strain differences has also been studied in glucose homeostasis (Goren et al., 2004).

Growth, the main characteristic of childhood and adolescence, has a similar pattern in the majority of the individuals. It is regulated by the interaction of environmental signals with endogenous neuroendocrine responses to the genetic programs that determine the body plan. Genetic background and GH-IGF axis are the factors that directly influence growth. Pituitary GH acts on growth mainly through the regulation of the IGF system. Therefore, IGFs are essential for growth and metabolism and their bioavailability is tightly controlled by six IGF-binding proteins (IGFBPs).

All IGFBPs have growth-inhibitory functions by competitively binding to IGFs and preventing their binding to the receptors. To date, genetic loss-of-function studies using transgenic mouse models have yielded limited phenotypes due to the

redundancy of IGFBPs and compensation from remaining IGFBPs after disruption of any single IGFBP. IGFBP-1 knockout mice have normal development, although they have abnormal liver regeneration after hepatectomy (Leu et al., 2003). Our lab previously reported that IGFBP-2 knockout mice have no specific phenotype despite selective alternations in organ sizes (Wood et al., 2000). Ning et al. (2006) showed IGFBP-3 and -5 single knockout mice have no difference in body weight from wild type mice, although IGFBP-4 knockout mice show modest growth impairment (10-15% growth reduction compared to wild type animals). IGFBP-4 has a unique dual function in regulating IGF activities *in vivo*. Depending on the cellular context, it can affect growth in both positive and negative directions (Ning et al., 2008).

Interestingly, after back-crossing to C57BL/6J for at least 10 generations, three out of five single IGFBP knockouts display growth inhibition. Male IGFBP-2 (-/-), IGFBP-3 (-/-), and IGFBP5 (-/-) mice are all significantly bigger than wild types (Fig 2.1, 2.2A, 2.5A, 2.5B). This effect is gender specific, as female IGFBP-3 (-/-) mice show normal growth compared to wild type littermates (Fig 2.2B). IGFBP-6 has no effect on growth (Fig 2.6A, 2.6B). IGFBP-4(-/-) mice, in both C57BL/6J and 129S6 background, are no different from wild type mice (Fig 2.3A, 2.3B, 2.4A, 2.4B).

IGFBP-35 double knockout mice are significantly heavier than wild type mice indicating that interruption of the most dominant IGFBPs has a substantial effect on growth (Fig2.7A, 2.8A). However, compared to IGFBP-3 and IGFBP-5 single knockouts, male double KO mice show no body weight difference and female

double KO mice are even bigger than single knockouts (Fig 2.7B, 2.8B).

Taken together, we provide the first *in vivo* evidence of the effects of single IGFBP gene on growth. IGFBP-2, -3, and -5 all inhibit growth, whereas IGFBP-4 and -6 have no effect on growth. Our mouse model of IGFBP-3 here confirms with another transgenic model of overexpressing IGFBP-3 with a significant reduction in body weight (Modric et al., 2001). Targeted deletion of the most abundant IGFBPs, IGFBP-3 and -5, results in a tremendous effect on growth. This effect becomes much more severe than IGFBP-3 and -5 single KO in female mice.

Elevated levels of IGFBP-3 has been reported to be associated with Type 2 diabetes and obesity, here we provide the first *in vivo* evidence that mice lacking of IGFBP-3 have reduced glucose level (Fig 2.9) which is associated with increased insulin secretion (Fig 2.10). Indeed, our *in vitro* study by stimulating isolated pancreatic islets with glucose also show higher insulin level in IGFBP-3 KO mice (data not shown). In addition, the enhanced glucose tolerance could also be explained by increased insulin sensitivity (Fig 2.11).

Traumatic brain injury stimulates upregulation of IGFBPs around the wounded area, here we find out that IGFBP-2 mRNA expression is significantly elevated at the injury site (Fig 2.14), other IGFBPs, however, don't have visible change (data not shown). Real-time qPCR also verifies that IGFBP-2 is most abundant in the CNS, with 28-fold increase in neurospheres in contrast to a universal control (Fig 2.16). BrdU staining further demonstrates that after the brain injury, IGFBP-2 knockout mice have significantly induced neurogenesis around the SVZ (Fig 2.15). These

indicate that brain injury causes IGFBP-2 upregulation which inhibits neurogenesis.

Future Perspectives

Our gene knockout mice (IGFBP-2 to -6 and IGFBP-35) demonstrate that at least IGFBP-2, -3, and -5 inhibit growth. However, growth curve of IGFBP-2 is a combined result of both genders which can't distinguish whether the increased body weight is due to the absence of the gene or gender effect. Growth curve of IGFBP-4 in 129 male mice is only measured from 2 KO mice till 6.5wk which apparently needs more animals to be measured for an extended period of time. Nevertheless, we provide the first *in vivo* evidence of the physiological role of single IGFBP in growth and development. However, it still would be worthy of testing the growth inhibitory effects from the other way around, in transgenic mice overexpressing single IGFBPs.

Enhanced glucose tolerance and elevated insulin secretion and sensitivity in IGFBP-3 KO mice provide *in vivo* evidence of IGFBP-3 action in glucose homeostasis. In future, we need to extend the *in vitro* study, further testing insulin secretion in isolated islets of Langerhans. It's also worthy of testing if disruption of IGFBP-3 could rescue diabetic symptoms in db/db mice.

Traumatic brain injury is still an on-going project in the lab. Our preliminary data show increased IGFBP-2 around the injury site and increased neurogenesis in IGFBP-2 KO mice. In future, this increasing trend needs to be quantified. It also raised a question whether IGFBP-2 would be associated with apoptosis. Meanwhile, studying of the changes of neurons and glials around injury site using various neuronal and glial markers, such as NeuN and GFAP would provide further information after the brain injury.

References

Alagappan, D., Lazzarino, D.A., Felling, R.J., Balan, M., Kotenko, S.V. and Levison, S.W. 2009 Brain injury expands the numbers of neural stem cells and progenitors in the SVZ by enhancing their responsiveness to EGF. *ASN Neuro* **1**: 95-111.

Arafat, A.M., Möhlig, M., Weickert, M.O., Schöfl, C., Spranger, J. and Pfeiffer, A.F. 2010 Improved insulin sensitivity, preserved beta cell function and improved whole-body glucose metabolism after low-dose growth hormone replacement therapy in adults with severe growth hormone deficiency: a pilot study. *Diabetologia* **in press**.

Arafat, A.M., Weickert, M.O., Frystyk, J., Spranger, J., Schöfl, C., Möhlig, M. and Pfeiffer, A.F. 2009 The role of insulin-like growth factor (IGF) binding protein-2 in the insulin-mediated decrease in IGF-I bioactivity. *J Clin Endocrinol Metab* **94**: 5093-5101.

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

Beilharz, E.J., Russo, V.C., Butler, G., Baker, N.L., Connor, B., Sirimanne, E.S., Dragunow, M., Werther, G.A., Gluckman, P.D., Williams, C.E. and Scheepens, A., 1998 Co-ordinated and cellular specific induction of the components of the IGF/IGFBP axis in the rat brain following hypoxic–ischemic injury. *Mol Brain Res* **59**: 119–134.

Boldt, H.B. and Conover, C.A. 2007 Pregnancy-associated plasma protein-A (PAPP-A): a local regulator of IGF bioavailability through cleavage of IGFBPs. *Growth Horm IGF Res* **17**: 10-18.

Boney, C.M., Moats-Staats, B.M., Stiles, A.D. and D'Ercole, A.J. 1994 Expression of insulin-like growth factor-I (IGF-I) and IGF-binding proteins during adipogenesis. *Endocrinology* **135**: 1863-1868.

Bramani, S., Song, H., Beattie, J., Tonner, E., Flint, D.J. and Allan, G.J. 1999 Amino acids within the extracellular matrix (ECM) binding region (201-218) of rat insulin-like growth factor binding protein (IGFBP)-5 are important determinants in binding IGF-I. *J Mol Endocrinol* **23**: 117-123.

Brannigan, J.A. and Wilkinson, A.J. 2002 Protein engineering 20 year on. *Nat Rev Mol Cell Biol* **3**: 964-970.

Buckbinder, L., Talbott, R., Velasco-Miguel, S., Takenaka, I., Faha, B., Seizinger, B.R. and Kley, N. 1995 Induction of the growth inhibitor IGF-binding protein 3 by

p53. *Nature* **377**: 646-649.

Camidge, D.R., Dziadziuszko, R. and Hirsch, F.R. 2009 The rationale and development of therapeutic insulin-like growth factor axis inhibition for lung and other cancers. *Clin Lung Cancer* **10**: 262-272.

Clemmons, D.R. 1997 Insulin-like growth factor binding proteins and their role in controlling IGF actions. *Cytokine Growth Factor Rev* **8**: 445-62.

Cohen, P., Lamson, G., Okajima, T. and Rosenfeld, R.G. 1993 Transfection of the human IGFBP-3 gene into Balb/c fibroblasts: a model for the cellular functions of IGFBPs. *Growth Regul* **3**: 23-26.

Colao, A. 2008 The GH-IGF-I axis and the cardiovascular system: clinical implications. *Clin Endocrinol (Oxf)* **69**: 347-358.

Colao, A., Vitale, G., Pivonello, R., Ciccarelli, A., Di Somma, C. and Lombardi, G. 2004 The heart: an end-organ of GH action. *Eur J Endocrinol* **151 (Suppl 1)**: S93-S101.

Conover, C.A., Bale, L.K., Mader, J.R., Mason, M.A., Keenan, K.P. and Marler, R.J. 2010 Longevity and Age-Related Pathology of Mice Deficient in Pregnancy-Associated Plasma Protein-A. *J Gerontol A Biol Sci Med Sci* **in press**.

Dou, J.T., Chen, M., Dufour, F., Alkon, D.L. and Zhao, W.Q. 2005 Insulin receptor signaling in long-term memory consolidation following spatial learning. *Learn Mem* **12**: 646-655.

Dunger, D., Yuen, K. and Ong, K. 2004 Insulin-like growth factor I and impaired glucose tolerance. *Horm Res* **62 (Suppl 1)**:101-107.

Eggenschwiler, J., Ludwig, T., Fisher, P., Leighton, P.A., Tilghman, S.M. and Efstratiadis, A. 1997 Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes. *Genes Dev* **11**: 3128-3142.

Elahi, D., McAloon-Dyke, M., Fukagawa, N.K., Sclater, A.L., Wong, G.A., Shannon, R.P., Minaker, K.L., Miles, J.M., Rubenstein, A.H., Vandepol, C.J., Guler, H.P., Good, W.R., Seaman, J.J. and Wolfe, R.R. 1993 Effects of recombinant human IGF-I on glucose and leucine kinetics in men. *Am J Physiol Endocrinol Metab* **265**: E831-E838.

Ferry, R.J., Jr., Cerri, R.W. and Cohen, P. 1999 Insulin-like growth factor binding proteins: new proteins, new functions. *Horm Res* **51**: 53-67.

Figlewica, D.P. and Szot, P. 1991 Insulin stimulates membrane phospholipids metabolism by enhancing endogenous α 1/adrenergic activity in the rat hippocampus. *Brain Res* **550**: 101-107.

Firth, S.M. and Baxter, R.C. 2002 Cellular actions of the insulin-like growth factor binding proteins. *Endocr Rev* **23**: 824-854.

Frommer, K.W., Reichenmiller, K., Schutt, B.S., Hoeflich, A., Ranke, M.B., Dodt, G. and Elmlinger, M.W. 2006 IGF-independent effects of IGFBP-2 on the human breast cancer cell line Hs578T. *J Mol Endocrinol* **37**: 13-23.

Frystyk, J. 2010 Exercise and the growth hormone-insulin-like growth factor axis. *Med Sci Sports Exerc* **42**: 58-66.

Frystyk, J., Grofte, T., Skjaerbaek, C. and Orskov, H. 1997 The effect of oral glucose on serum free insulin-like growth factor-I and –II in health adults. *J Clin Endocrinol Metab* **82**: 3124-3127.

Gary-Bobo, M., Nirdé, P., Jeanjean, A., Morère, A. and Garcia, M. 2007 Mannose 6-phosphate receptor targeting and its applications in human diseases. *Curr Med Chem* **14**: 2945-2953.

Gerich, J.E. 1998 The genetic basis of type 2 diabetes mellitus: impaired insulin secretion versus impaired insulin sensitivity. *Endocr Rev* **19**: 491-503.

Goren, H.J., Kulkarni, R.N. and Kahn, C.R. 2004 Glucose homeostasis and tissue transcript content of insulin signaling intermediates in four inbred strains of mice: C57BL/6, C57BLKS/6, DBA/2, and 129X1. *Endocrinology* **145**: 3307–3323.

Govoni, K.E., Wergedal, J.E., Florin, L., Angel, P., Baylink, D.J. and Mohan, S. 2007 Conditional deletion of insulin-like growth factor-I in collagen type 1alpha2-expressing cells results in postnatal lethality and a dramatic reduction in bone accretion. *Endocrinology* **148**: 5706-5715.

Guan, J., Bennet, L., Gluckman, P.D. and Gunn, A.J. 2003 Insulin-like growth factor-1 and post-ischemic brain injury. *Prog Neurobiol* **70**: 443-462.

Guler, H.P., Zapf, J. and Froesch, E.R. 1987 Short-term metabolic effects of recombinant human insulin-like growth factor I in healthy adults. *N Engl J Med* **317**: 137-140.

Headey, S.J., Keizer, D.W. , Yao, S., Brasier, G., Kantharidis, P., Bach, L.A. and Norton, R.S. 2004 C-terminal domain of insulin-like growth factor (IGF) binding protein-6: structure and interaction with IGF-II. *Mol Endocrinol* **18**: 2740–2750.

Hedbacker, K., Birsoy, K., Wysocki, R.W., Asilmaz, E., Ahima, R.S., Farooqi, I.S. and Friedman, J.M. 2010 Antidiabetic effects of IGFBP2, a leptin-regulated gene. *Cell Metab* **11**: 11-22.

Heemskerk, V.H., Daemen, M.A. and Buurman, W.A. 1999 Insulin-like growth factor-1 (IGF-1) and growth hormone (GH) in immunity and inflammation. *Cytokine Growth Factor Rev* **10**: 5-14.

Hoeck, W.G. and Mukku, V.R. 1994 Identification of the major sites of phosphorylation in IGF binding protein-3. *J Cell Biochem* **56**: 262-273.

Hong, J., Zhang, G., Dong, F. and Rechler, M.M. 2002 Insulin-like growth factor (IGF)-binding protein-3 mutants that do not bind IGF-I or IGF-II stimulate apoptosis in human prostate cancer cells. *J Biol Chem* **277**: 10489-10497.

Hwa, V., Oh, Y. and Rosenfeld, R.G. 1999 The Insulin-Like Growth Factor-Binding Protein (IGFBP) Superfamily. *Endocr Rev* **20**:761-787.

Ingermann, A.R., Yang, Y.F., Paisley, T.E., Han, J., Mikami, A., Garza, A.E., Mohanraj, L., Fan, L., Idowu, M., Ware, J.L., Kim, H.S., Lee, D.Y. and Oh, Y. 2010 Identification of a novel cell death receptor mediating IGFBP-3-induced antitumor effects in breast and prostate cancer. *J Biol Chem* **in press**.

Jonas, E.A., Knox, R.J., Smith, T.C., Wayne, N.L., Connor, J.A. and Kaczmarek, L.K. 1997 Regulation by insulin of a unique neuronal Ca^{2+} pool and of neuropeptide secretion. *Nature* **385**: 343-346.

Jones, J.I., Busby, W.H. Jr, Wright, G., Smith, C.E., Kimack, N.M. and Clemmons, D.R. 1993 Identification of the sites of phosphorylation in insulin-like growth factor binding protein-1: Regulation of its affinity by phosphorylation of serine 101. *J Biol Chem* **268**: 1125-1131.

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

Jones, J.I., D'Ercole, A.J., Camacho-Hubner, C. and Clemmons, D.R. 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**: 7481-7485.

Jones, J.I., Gockerman, A., Busby, W.H., Jr., Wright, G. and Clemmons, D.R. 1993 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**: 10553-10557.

Khan, A.H. and Pessin, J.E. 2002 Insulin regulation of glucose uptake: a complex interplay of intracellular signalling pathways. *Diabetologia* **45**: 1475-1483.

Kim, H.S., Ingermann, A.R., Tsubaki, J., Twigg, S.M., Walker, G.E. and Oh, Y. 2004 Insulin-like growth factor-binding protein 3 induces caspase-dependent apoptosis through a death receptor-mediated pathway in MCF-7 human breast cancer cells. *Cancer Res* **64**: 2229-2237.

Kuang, Z., Yao, S., Keizer, D.W., Wang, C.C., Bach, L.A., Forbes, B.E., Wallace, J.C. and Norton, R.S. 2006 Structure, dynamics and heparin binding of the C-terminal domain of insulin-like growth factor-binding protein-2 (IGFBP-2). *J Mol Biol* **364**: 690-704.

Kumar, A., Lawrence, J.C. Jr, Jung, D.Y., Ko, H.J., Keller, S.R., Kim, J.K., Magnuson, M.A. and Harris, T.E. 2010 Fat cell-specific ablation of rictor in mice impairs insulin-regulated fat cell and whole body glucose and lipid metabolism. *Diabetes* **in press**.

Laager, R., Ninnis, R. and Keller, U. 1993 Comparison of the effects of recombinant human insulin-like growth factor-I and insulin on glucose and leucine kinetics in humans. *J Clin Invest* **92**: 1903-1909.

Lee, W.H., Javedan, S. and Bondy, C.A. 1992 Coordinate expression of insulin-like growth factor system components by neurons and neuroglia during retinal and cerebellar development. *J Neurosci* **12**: 4737-4744.

LeRoith, D., Bondy, C., Yakar, S., Liu, J.L. and Butler, A. 2001 The somatomedin hypothesis: 2001. *Endocr Rev* **22**: 53-74.

Leu, J.I., Crissey, M.A., Craig, L.E. and Taub, R. 2003 Impaired hepatocyte DNA synthetic response posthepatectomy in insulin-like growth factor binding protein 1-deficient mice with defects in C/EBP beta and mitogen-activated protein kinase/extracellular signal-regulated kinase regulation. *Mol Cell Biol* **23**: 1251-1259.

Lewejohann, L., Reinhard, C., Schrewe, A., Brandewiede, J., Haemisch, A., Görtz, N., Schachner, M. and Sachser, N. 2006 Environmental bias? Effects of housing conditions, laboratory environment and experimenter on behavioral tests. *Genes Brain Behav* **5**:64- 72.

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

López-Bermejo, A., Khosravi, J., Fernández-Real, J.M., Hwa, V., Pratt, K.L., Casamitjana, R., Garcia-Gil, M.M., Rosenfeld, R.G. and Ricart, W. 2006 Insulin resistance is associated with increased serum concentration of IGF-binding protein-related protein 1 (IGFBP-rP1/MAC25). *Diabetes* **55**: 2333-2339.

Maddux, B.A., Chan, A., De Filippis, E.A., Mandarino, L.J. and Goldfine, I.D. 2006 IGF-binding protein-1 levels are related to insulin-mediated glucose disposal and are a potential serum marker of insulin resistance. *Diabetes Care* **29**: 1535-1537.

Mathews, L.S., Hammer, R.E., Behringer, R.R., D'Ercole, A.J., Bell, G.I., Brinster, R.L. and Palmiter, R.D. 1988 Growth enhancement of transgenic mice expressing human insulin-like growth factor-I. *Endrocrinology* **123**: 2827-2833.

Mizutani, F., Ohta, E., Mie, Y., Niwa, O. and Yasukawa, T. 2008 Enzyme immunoassay of insulin at picomolar levels based on the coulometric determination of hydrogen peroxide. *Sensors and Actuators B: Chemistry* **135**: 304-308.

Miyakoshi, N., Richman, C., Kasukawa, Y., Linkhart, T.A., Baylink, D.J. and Mohan, S. 2001 Evidence that IGF-binding protein-5 functions as a growth factor. *J Clin Invest* **107**: 73-81.

Modric, T., Silha, J.V., Shi, Z., Gui, Y., Suwanichkul, A., Durham, S.K., Powell, D.R. and Murphy, L.J. 2001 Phenotypic manifestations of insulin-like growth factor-binding protein-3 overexpression in transgenic mice. *Endocrinology* **142**: 1958-1967.

Moerth, C., Schneider, M.R., Renner-Mueller, I., Blutke, A., Elmlinger, M.W., Erben, R.G., Camacho-Hübner, C., Hoeflich, A. and Wolf, E. 2007 Postnatally elevated levels of insulin-like growth factor (IGF)-II fail to rescue the dwarfism of IGF-I-deficient mice except kidney weight. *Endocrinology* **148**: 441-451.

Mohan, S. and Baylink, D.J. 2002 IGF-binding proteins are multifunctional and act via IGF-dependent and –independent mechanism. *J Endocrinol* **175**: 19-31.

Mohan, S., Nakao, Y., Honda, Y., Landale, E., Leser, U., Dony, C., Lang, K. and Baylink, D.J. 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**: 20424-20432.

Ning, Y., Schuller, A.G., Bradshaw, S., Rotwein, P., Ludwig, T., Frystyk, J. and Pintar, J.E. 2006 Diminished growth and enhanced glucose metabolism in triple knockout mice containing mutations of insulin-like growth factor binding protein-3, -4, and -5. *Mol Endocrinol* **20**: 2173-2186.

Ning, Y., Schuller, A.G., Conover, C.A. and Pintar, J.E. 2008 Insulin-like growth factor (IGF) binding protein-4 is both a positive and negative regulator of IGF activity in vivo. *Mol Endocrinol* **22**: 1213-1225.

Nyomba, B.L., Berard, L. and Murphy, L.J. 1997 Free insulin-like growth factor I (IGF-I) in healthy subjects: relationship with IGF-binding proteins and insulin sensitivity. *J Clin Endocrinol Metab* **82**: 2177-2181.

Oldham, S. and Hafen, E. 2003 Role of insulin-like growth factors in embryonic and postnatal growth. *Trends Cell Biol* **13**: 79-85.

Pavelić, J., Matijević, T. and Knezević, J. 2007 Biological & physiological aspects of action of insulin-like growth factor peptide family. *Indian J Med Res* **125**: 511-522.

Pratipanawatr, T., Pratipanawatr, W., Rosen, C., Berria, R., Bajaj, M., Cusi, K., Mandarino, L., Kashyap, S., Belfort, R. and DeFronzo, R.A. 2002 Effect of IGF-I on FFA and glucose metabolism in control and type 2 diabetic subjects. *Am J Physiol Endocrinol Metab* **282**: E1360-E1368.

Rajah, R., Valentinis, B. and Cohen, P. 1997 Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-beta1 on programmed cell death through a p53- and IGF-independent mechanism. *J Biol Chem* **272**: 12181-12188.

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

Rajpathak, S.N., Gunter, M.J., Wylie-Rosett, J., Ho, G.Y., Kaplan, R.C., Muzumdar, R., Rohan, T.E. and Strickler, H.D. 2009 The role of insulin-like growth factor-I and its binding proteins in glucose homeostatis and type 2 diabetes. *Diabetes Metab Res Rev* **25**: 3-12.

Rechler, M.M. 1993 Insulin-like growth factor binding proteins. *Vitam and Horm* **47**: 1-114.

Russell-Jones, D.L., Bates, A.T., Umpleby, A.M., Hennessy, T.R., Bowes, S.B., Hopkins, K.D., Jackson, N., Kelly, J., Shojaee-Moradie, F. and Jones, R.H. 1995 A comparison of the effects of IGF-I and insulin on glucose metabolism, fat metabolism and the cardiovascular system in normal human volunteers. *Eur J Clin Invest* **25**: 403-411.

Ryle, A.P., Sanger, F., Smith, L.F. and Kitai, R. 1955 The disulphide bonds of insulin. *Biochem J* **60**: 541556.

Salih, D.A., Tripathi, G., Holding, C., Szestak, T.A., Gonzalez, M.I., Carter, E.J., Cobb, L.J., Eisemann, J.E. and Pell, J.M. 2004 Insulin-like growth factor-binding protein 5 (Igfbp5) compromises survival, growth, muscle development, and fertility in mice. *Proc Natl Acad Sci U S A* **101**: 4314-4319.

Saltiel, A.R. and Kahn, C.R. 2001 Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414**: 799-806.

Schedlich, L.J., Graham, L.D., O'Han, M.K., Muthukaruppan, A., Yan, X., Firth, S.M. and Baxter, R.C. 2007 Molecular basis of the interaction between IGFBP-3 and retinoid X receptor: role in modulation of RAR-signaling. *Arch Biochem Biophys* **465**: 359-369.

Schedlich, L.J., Le Page, S.L., Firth, S.M., Briggs, L.J., Jans, D.A. and Baxter, R.C. 2000 Nuclear import of insulin-like growth factor-binding protein-3 and-5 is mediated by the importin beta subunit. *J Biol Chem* **275**: 23462-23470.

Schmid, C., Bianda, T., Zwimpfer, C., Zapf, J. and Wiesli, P. 2005 Changes in insulin sensitivity induced by short-term growth hormone(GH) and insulin-like growth factor I (IGF-I) treatment in GH-deficient adults are not associated with changes in adiponectin levels. *Growth Horm IGF Res* **15**: 300-303.

Schuller, A.G., Groffen, C., van Neck, J.W., Zwarthoff, E.C. and Drop, S.L. 1994 cDNA cloning and mRNA expression of the six mouse insulin-like growth factor binding proteins *Mol Cell Endocrinol* **104**: 57-66.

Scott, C.D. and Firth, S.M. 2004 The role of M6P/IGF-II receptor in cancer: tumor suppression or garbage disposal? *Horm Metab Res* **36**: 261-271.

Shabanpoor, F**.,** Separovic, F. and Wade, J.D. 2009 The human insulin superfamily of polypeptide hormones. *Vitam Horm* **80**: 1-31.

Sherwin, R.S., Borg, W.P. and Boulware, S.D. 1994 Metabolic effects of insulin-like growth factor I in normal humans. *Horm Res* **41 (Suppl 2)**: 97-102.

Silha, J.V., Gui, Y. and Murphy, L.J. 2002 Impaired glucose homeostasis in insulin-like growth factor-binding protein-3-transgenic mice. *Am J Physiol Endocrinol Metab* **283**: E937-E945.

Stratikopoulos, E., Szabolcs, M., Dragatsis, I., Klinakis, A. and Efstratiadis, A. 2008 The hormonal action of IGF1 in postnatal mouse growth. *Proc Natl Acad Sci U S A* **105**: 19378-19383.

Tripathi, G., Salih, D.A., Drozd, A.C., Cosgrove, R.A., Cobb, L.J. and Pell, J.M. 2009 IGF-independent effects of insulin-like growth factor binding protein-5 (Igfbp5) in vivo. *FASEB J* **23**: 2616-2626.

van der Heide, L.P., Ramakers, G.M. and Smidt, M.P. 2006 Insulin signaling in the central nervous system: learning to survive. *Prog Neurobiol* **79**: 205-221.

Wahlsten, D., Bachmanov, A., Finn, D.A. and Crabbe, J.C. 2006 Stability of inbred mouse strain differences in behavior and brain size between laboratories and across decades. *Proc Natl Acad Sci U S A* **103**: 16364-16369.

Wheatcroft, S.B., Kearney, M.T., Shah, A.M., Ezzat, V.A., Miell, J.R., Modo, M., Williams, S.C., Cawthorn, W.P., Medina-Gomez, G., Vidal-Puig, A., Sethi, J.K. and Crossey, P.A. 2007 IGF-binding protein-2 protects against the development of obesity and insulin resistance. *Diabetes* **56**: 285-294.

Wood, T.L., Rogler, L.E., Czick, M.E., Schuller, A.G. and Pintar, J.E. 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**: 1472-1482.

Yamanaka, Y., Fowlkes, J.L., Wilson, E.M., Rosenfield, R.G. and Oh, Y. 1999 Characterization of insulin-like growth factor binding protein-3 (IGFBP-3) binding to human breast cancer cells: kinetics of IGFBP-3 binding and identification of receptor binding domain on the IGFBP-3 molecule. *Endocrinology* **140**: 1319-1328.

Zadeh, S.M. and Binoux, M. 1997 The 16-kDa proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 inhibits the mitogenic action of fibroblast growth factor on mouse fibroblasts with a targeted disruption of the type 1 IGF receptor gene. *Endocrinology* **138**: 3069-3072.

Zhao, W.Q., Chen, H., Xu, H., Moore, E., Meiri, N., Quon, M.J. and Alkon D.L. 1999 Brain insulin receptors and spatial memory. Correlated changes in gene expression, tyrosine phosphorylation, and signaling molecules in the hippocampus of water maze trained rats. *J Biol Chem* **274**: 34893-34902.