THEMATIC REVIEW

40 YEARS OF IGF1

IGF-binding proteins

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Abstract

Insulin-like growth factor-binding proteins (IGFBPs) 1–6 bind IGFs but not insulin with high affinity. They were initially identified as serum carriers and passive inhibitors of IGF actions. However, subsequent studies showed that, although IGFBPs inhibit IGF actions in many circumstances, they may also potentiate these actions. IGFBPs are widely expressed in most tissues, and they are flexible endocrine and autocrine/paracrine regulators of IGF activity, which is essential for this important physiological system. More recently, individual IGFBPs have been shown to have IGF-independent actions. Mechanisms underlying these actions include (i) interaction with non-IGF proteins in compartments including the extracellular space and matrix, the cell surface and intracellular space, (ii) interaction with and modulation of other growth factor pathways including EGF, TGF-β and VEGF, and (iii) direct or indirect transcriptional effects following nuclear entry of IGFBPs. Through these IGF-dependent and IGF-independent actions, IGFBPs modulate essential cellular processes including proliferation, survival, migration, senescence, autophagy and angiogenesis. They have been implicated in a range of disorders including malignant, metabolic, neurological and immune diseases. A more complete understanding of their cellular roles may lead to the development of novel IGFBP-based therapeutic opportunities.

Key Words

- insulin-like growth factor
- binding protein
- regulation
- cellular actions
- protein structure

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The somatomedin hypothesis, which postulated that growth hormone activity was mediated by a serum factor, was published in 1957 (Salmon & Daughaday 1957). In the 1960s, several circulating somatomedin activities were identified and attributed to peptides sized 5~8 kDa that had both growth-promoting and insulin-like metabolic effects. A paradox of these early observations was that normoglycemia was maintained in vivo despite the circulating concentrations of somatomedins being sufficient to cause profound hypoglycemia. Following the purification of these small somatomedin peptides, it was observed that almost all of the circulating somatomedin activity was found in a number of chromatographic peaks with apparent molecular weights greater than 30~40 kDa and further studies indicated that this was due to binding by plasma-binding proteins (Megyesi *et al*. 1975, Zapf *et al*. 1975, Hintz & Liu 1977). The apparent hypoglycemia paradox could then be resolved if somatomedin activity was inhibited by association with these binding proteins. Of note, these early studies already demonstrated that insulin did not bind to these proteins.

In the following years, the somatomedin activities were identified as IGF1 and IGF2, and they were sequenced and cloned. IGF-binding proteins (IGFBPs) 1–3 were the

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first to be identified and purified, with IGFBPs 4–6 being subsequently described (Rechler 1993, Rajaram *et al.* 1997). By the early 1990s, all six members of this high-affinity IGFBP family had been cloned and a number of key structural and sequence similarities were identified. Additionally, the predominant 150 kDa serum complex was shown to consist of IGFs bound in a ternary complex with IGFBP-3 and an acid-labile subunit. Later, it was shown that IGFBP-5 but not the other IGFBPs could also form ternary complexes. In contrast, the 40 kDa serum binary complex was shown to contain IGFs bound to any of the six IGFBPs, and less than one percent of circulating IGFs was unbound. Although the main source of circulating IGFBPs was the liver, IGFBP expression was found to be widespread, suggesting a role in local regulation of IGF activity.

In the late 1990s, it was suggested that another six proteins with more limited homology to the IGFBPs were part of an IGFBP superfamily (Hwa *et al.* 1999). Four of these proteins were provisionally named IGFBPs 7–10 and subsequently all of them were named IGFBP-related proteins (IGFBP-rP) 1–6 (Hwa *et al.* 1999). However, none of these latter proteins was convincingly shown to modulate IGF activities and each had biological roles that were independent of the IGF system (Grotendorst *et al.* 2000, Yan *et al.* 2006), so this nomenclature and classification are rarely used now. This review will therefore focus exclusively on IGFBPs 1–6.

The IGF system in health and disease

IGF1 expression is regulated by growth hormone (GH) and, as articulated originally in the somatomedin hypothesis, it mediates many of the latter's effects. Although IGF2 is predominantly expressed prenatally in rodents, serum IGF2 levels are 3- to 4-fold higher than those of IGF1 in the adult human (Livingstone 2013). GH/IGF1 deficiency results in short stature in children, whereas GH/IGF1 excess causes the organ enlargement seen in acromegaly. Similarly, IGF2 deficiency was associated with prenatal and postnatal growth restriction (Begemann *et al.* 2015) and IGF1 overexpression, such as that seen in Beckwith-Wiedemann syndrome, was associated with overgrowth (Morison *et al.* 1996). Liver is the predominant source of circulating IGFs, but they are widely expressed in most tissues where they act locally. Autocrine and paracrine as well as endocrine actions are also implicated in many diseases including atherosclerosis, metabolic diseases and cancer (Clemmons 2007, Livingstone 2013).

The IGF1 receptor mediated most actions of IGF1 and IGF2 via its tyrosine kinase activity resulting in the activation of intracellular signaling pathways including MAP kinase and PI3 kinase/AKT (Adams *et al.* 2000). Some metabolic actions of IGFs were also mediated by the structurally related insulin receptor. Mitogenic actions of IGF2 but not IGF1 were also mediated by the insulin receptor A isoform (Belfiore *et al.* 2009), which may be especially relevant to development and cancer since it was preferentially expressed prenatally and often found in tumors. The IGF2/mannose 6-phosphate receptor was predominantly involved in clearance of IGF2 and also bound a range of structurally unrelated ligands (Brown *et al.* 2009).

IGFBPs

In keeping with its important physiological role, IGF activity was controlled by temporal and spatial regulation of IGF and IGF receptor levels (Clemmons 2007, Livingstone 2013). The IGFBP family provides an additional, predominantly extracellular mechanism to regulate IGF activity. The hallmark of IGFBPs is their binding of IGF1 and IGF2 but not insulin with high affinity. In most circumstances, they inhibit IGF actions by preventing binding to IGF receptors, but they may also potentiate their actions. Over the last two decades, IGF-independent actions of IGFBPs have also been described (Fig. 1).

IGFBPs prolong the circulating half-life of IGFs and regulate their movement into tissues. As mentioned earlier, more than 99% of circulating IGFs were found in complexes with IGFBPs (Rajaram *et al.* 1997, Firth & Baxter 2002). Unbound IGFs had a short circulating half-life of 10–12 min (Guler *et al.* 1989). The predominant ~150 kDa ternary complex contained ~75% of circulating IGFs and was too large to leave the circulation, thereby prolonging the latter's half-life to ~15 h (Guler *et al.* 1989). The importance of the ternary complex for IGF stability was exemplified by patients with mutations of the acid-labile subunit who had markedly decreased IGF1 and IGFBP-3 levels, resulting in variable and often mild growth impairment together with insulin resistance (Domene *et al.* 2011). The relatively mild growth deficit may have been due to unaltered local IGF synthesis and action in these patients. Apart from the ternary complex, most remaining serum IGFs were found in binary 40~50 kDa complexes with all six IGFBPs. Although these complexes could leave the circulation, they also prolonged IGF half-lives to 20–30 min (Guler *et al.* 1989). Additionally, studies from one laboratory showed that binding of IGFs to individual IGFBPs in binary

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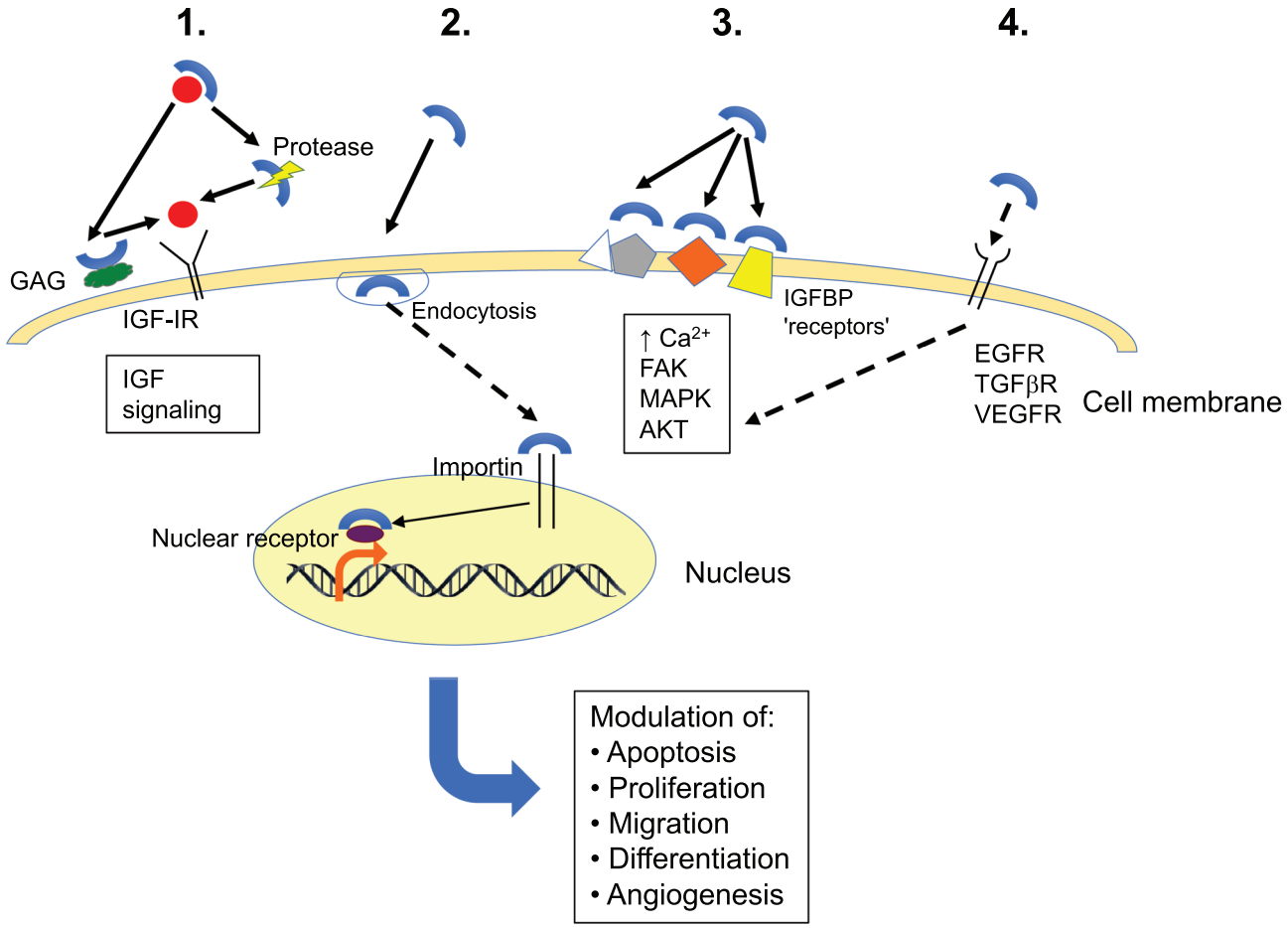

Figure 1
Cellular actions of IGFBPs. IGFBPs have IGF-dependent and IGF-independent actions. (1) All IGFBPs bind IGFs with high affinity and modulate IGF actions by regulating their availability to the IGF1 receptor (IGF-IR). Proteolytic cleavage and binding to cell-associated glycosaminoglycans (GAG) are mechanisms involved in IGF release from IGFBPs. (2) Some IGFBPs enter the nucleus and modulate transcription by binding to nuclear receptors. Mechanisms of cell entry of IGFBPs are incompletely understood but include endocytosis. (3) IGFBPs have IGF-independent actions that are mediated by interaction with cell surface 'receptors', only some of which have been characterized. Intracellular signaling pathways are activated by IGFBPs, resulting in a range of cellular outcomes. (4) Some IGFBPs interact and modulate other growth factor pathways. Adapted from a figure originally published in (Bach 2015).

complexes directed them toward specific extravascular tissue compartments (Boes *et al*. 1992, Sandra *et al*. 1998, Knudtson *et al*. 2001).

IGFBP genes

It is believed that the six mammalian *IGFBP*s arose from an ancestral chordate *IGFBP* gene that was duplicated locally, followed by expansion in the two episodes of early vertebrate tetraploidization, resulting in four gene pairs (Daza *et al*. 2011). The subsequent loss of one gene from two pairs resulted in six genes. Additional *IGFBP* genes present in teleost fish were due to a third tetraploidization. *IGFBP* genes have a conserved genomic structure, with each having four exons except *IGFBP3*, which also has a 3′ non-coding exon.

IGFBP structure

Human IGFBPs 1–6 each contain 216–289 amino acids divided into N-terminal, linker and C-terminal domains of approximately equal size (Bach *et al*. 2005). IGFBPs 1–5 have 18 conserved disulphide-linked cysteines, whereas IGFBP-6 has 16; IGFBP-4 has one additional disulphide-linked cysteine pair in its linker domain. There is a high degree of homology between IGFBPs in their N- and C-terminal domains, whereas the linker regions have little or no sequence homology.

The N-terminal domains of IGFBPs 1–5 contained six disulphide bonds and shared a conserved GCGCC motif that contributed to a rigid, ladder-like subdomain involving the first four disulphide bonds (Sitar *et al*. 2006). IGFBP-6 lacked this motif, and its disulphide linkages and

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structure in this subdomain differed from those of the other IGFBPs (Neumann & Bach 1999, Chandrashekaran *et al*. 2007). The remaining two N-terminal domain disulphide bonds were conserved in all IGFBPs and stabilized the structure of the high-affinity IGF-binding subdomain that consisted of a compact three-stranded β-sheet and short α-helix (Kalus *et al*. 1998, Zeslawski *et al*. 2001).

The C-terminal domains of IGFBPs contained three conserved disulphide bonds and adopted a thyroglobulin type 1 fold consisting of an α-helix followed by a three-stranded antiparallel β-sheet (Headey *et al*. 2004). A relatively large hydrophobic IGF-binding surface in the C-domain contributed to high-affinity IGF binding (Headey *et al*. 2004). The C-terminal domains of some IGFBPs also contain a highly basic region, which was involved in binding to glycosaminoglycans and a range of proteins, including the acid-labile subunit in serum and importins that mediated nuclear uptake (Bach *et al*. 2005). The C-terminal domains of IGFBP-1 and IGFBP-2 have integrin-binding Arg-Gly-Asp sequences (Bach *et al*. 2005). Many but not all IGF-independent actions of IGFBPs were mediated via their C-domains.

The non-conserved linker domains did not directly contribute to IGF binding, but they contained recognition sites for limited proteolysis, which decreased IGF-binding affinity and may therefore be an important physiological mechanism for releasing bound IGFs (Bunn & Fowlkes 2003). Linker domains are unstructured, making them accessible to proteases, and protease specificity for individual IGFBPs was conferred by unique recognition sequences within these domains. Since proteases are expressed in a tissue-specific manner, they may locally regulate release of free IGFs for receptor binding. PAPP-A proteases cleaved specific IGFBPs, and children with inactivating *PAPPA2* mutations were short in the presence of high circulating IGF1 and 2 levels as well as high levels of IGFBP-3 and -5, which are PAPP-A2 substrates (Argente *et al*. 2017). These findings imply that impaired growth was due to low free IGF levels secondary to defective IGFBP proteolysis. However, further studies are required to fully elucidate the physiological mechanisms resulting in the release of IGFs from IGFBP-containing complexes.

The linker domains also contain sites of post-translational modifications, including N- and O-glycosylation and phosphorylation. Although phosphorylation directly altered the binding affinities of IGFBP-1 and IGFBP-3, post-translational modifications also regulated IGFBP actions by modulating their stability, susceptibility to proteolysis, cell association and circulating half-lives (Firth & Baxter 1999, 2002, Marinaro *et al*. 2000*a*,*b*).

IGF-dependent actions of IGFBPs

Inhibition

All six IGFBPs inhibited IGF actions under most circumstances (Firth & Baxter 2002, Bach *et al*. 2005, Sitar *et al*. 2006). IGFBPs sterically hindered the interaction of IGFs with the IGF1 receptor, and they were potent competitive inhibitors because they bound IGFs with ~10-fold higher affinity than the IGF1 receptor. IGFBPs inhibited many IGF actions including proliferation, survival, migration, differentiation and nutrient uptake in a wide range of normal and malignant cell types *in vitro*. Overexpression studies also showed that IGFBPs inhibited IGF actions *in vivo*.

Potentiation

IGFBPs-1, -2, -3 and -5 may also enhance IGF actions (Firth & Baxter 2002). A common factor leading to potentiation was a decrease in IGF-binding affinity. Mechanisms that resulted in this included (i) cell association of the IGFBP by binding to glycosaminoglycans in the cell membrane and/or adjacent extracellular matrix; (ii) decreased phosphorylation of IGFBP-1 and (iii) proteolytic cleavage of IGFBPs. Cell association may have additionally resulted in the formation of a local reservoir of IGFs that could be released for optimal binding to the IGF1 receptor. However, further studies are required to fully elucidate the mechanisms underlying potentiation of IGF actions by IGFBPs.

IGF-independent actions of IGFBPs

Some IGFBPs also had actions that were independent of IGFs (Firth & Baxter 2002, Bach *et al*. 2005, Baxter 2014). In many instances, they inhibited cell proliferation, survival and migration but may have also enhanced these processes in a context-specific manner. IGF-independent actions were initially shown *in vitro* but they were subsequently demonstrated *in vivo*. The mechanisms underlying IGF-independent actions of IGFBPs are incompletely characterized. They include binding to a range of cell surface receptors, only some of which have been definitively identified, and activation

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of intracellular signaling pathways. For example, IGFBPs 2–6 increased intracellular calcium levels with some involvement of G-proteins, but specific receptors were not identified (Seurin *et al*. 2013). Following entry into the nucleus via importin-dependent mechanisms, IGFBPs also modulated gene transcription by interacting with nuclear receptors, although the pathways by which they entered or were retained in the cytoplasm are incompletely understood. IGFBPs also modulated other growth factor pathways. Further details of IGF-independent actions of individual IGFBPs are outlined below.

IGFBPs 1–6

IGFBPs modulate cell proliferation, survival, differentiation, migration and invasion. More recently, they were also shown to regulate senescence and autophagy as well as angiogenesis. Through these cellular effects, IGFBPs are implicated in a range of physiological and pathological processes, including those underlying metabolism, immune regulation, cancer and neurological disease. Although *IGFBP* mutations have not been identified in any diseases, altered expression was found in many as outlined below. A number of studies have investigated the association of *IGFBP* SNPs with a range of cancers but the results have been inconsistent (Li *et al*. 2010). An association between an *IGFBP3* SNP and hip osteoarthritis was also described (Evans *et al*. 2015), but further confirmatory work is required.

The following sections will provide an overview of each IGFBP (Table 1).

IGFBP-1

IGFBP-1 binds IGF1 and IGF2 with equal affinity, and it may inhibit or enhance IGF actions. When phosphorylated on serine residues in its linker domain, IGFBP-1 bound IGFs with increased affinity, thereby contributing to its inhibitory actions (Gupta 2015). In contrast, the non-phosphorylated form with lower binding affinity was associated with potentiation of IGF actions. IGFBP-1 has consensus phosphorylation sequences for protein kinase A (PKA), protein kinase C and casein kinases CK1 and CK2 (Gupta 2015). Further, CK2 and PKA phosphorylated IGFBP-1 *in vitro*, and it was suggested that CK2 may be responsible for increased IGFBP-1 phosphorylation during fetal growth restriction, as described below.

IGFBP-1 also has an Arg-Gly-Asp motif in its C-terminal domain that bound integrins and mediated at least some of its IGF-independent actions, including increased cell migration via binding to the α5β1 integrin (Jones *et al*. 1993). More recently, IGFBP-1 was shown to stimulate osteoclast differentiation and bone degradation in response to FGF21 via β1 integrin binding, and IGFBP-1 blockade was suggested as a potential therapeutic strategy to prevent bone loss (Wang *et al*. 2015).

Table 1 Some recent areas of IGFBP research.

IGFBP-1	Metabolism and diabetes, fetal growth
IGFBP-2	Tumourigenesis, obesity
IGFBP-3	Sphingosine kinase interactions, autophagy, nuclear actions
IGFBP-4	Bone biology, prenatal IGF targeting
IGFBP-5	Fibrosis, angiogenesis
IGFBP-6	Immune regulation, neuropathologies

IGFBPs have well-defined cellular actions that have been investigated over many years. While highlighting some recent areas of research, this table is not intended to be comprehensive.

Metabolism

IGFBP-1 was expressed in many organs, with high expression in liver, where it was involved in acute metabolic regulation of IGF activity (Hoeflich & Russo 2015). It was dynamically regulated by metabolic status, and insulin directly inhibited its gene transcription. Fasting therefore increased and feeding decreased plasma IGFBP-1 levels (Lewitt *et al*. 2014, Hoeflich & Russo 2015). IGFBP-1 levels were also increased in insulin-deficient subjects with type 1 diabetes, whereas they were inappropriately high relative to insulin levels in subjects with type 2 diabetes because of hepatic insulin resistance. Additionally, low fasting IGFBP-1 levels predicted the development of prediabetes and type 2 diabetes eight to 17 years later (Lewitt *et al*. 2014). IGFBP-1 promoted transdifferentiation of glucagon-producing α-cells to insulin-producing ß-cells within the pancreatic islet, thus increasing regeneration of the latter and providing a potential mechanism for the protective effect of IGFBP-1 against the development of diabetes (Lu *et al*. 2016). Another recent study showed that IGFBP-1 enhanced insulin sensitivity via its Arg-Gly-Asp sequence and focal adhesion kinase activation (Haywood *et al*. 2017). In contrast, IGFBP-1 blockade prevented FGF21-induced bone resorption but had no effect on insulin sensitization in mice (Wang *et al*. 2015).

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Non-alcoholic fatty liver disease is associated with insulin resistance and type 2 diabetes, and it was recently shown that phosphorylated IGFBP-1 levels were lower in subjects with higher liver fat content (Petaja *et al*. 2016).

Constitutive overexpression of IGFBP-1 resulted in fasting hyperglycemia, impaired glucose tolerance, growth restriction, glomerulosclerosis and abnormal brain development in mice (Silha & Murphy 2002, Wheatcroft & Kearney 2009). However, IGFBP-1 was not metabolically regulated in these models, and IGFBP-1 overexpression under its native promoter was protective against obesity-induced insulin resistance and glucose intolerance (Wheatcroft & Kearney 2009). Deletion of the IGFBP-1 gene had no effect on physiological growth or glucose regulation, but hepatic DNA synthesis was impaired following partial hepatectomy in these mice (Leu *et al*. 2003).

Fetal growth

IGFBP-1 plays an important role in placental function and fetal growth. Decidualised uterine endometrium and fetal liver were sites of high IGFBP-1 expression, and IGFBP-1 was the predominant IGFBP in the fetal circulation (Gupta 2015). It was postulated that IGFBP-1-regulated fetal growth by inhibiting IGF actions. For example, hypoxia stimulated IGFBP-1 expression, which contributed to impaired embryonic development (Kajimura *et al*. 2005). Additionally, both hypoxia and leucine deprivation increased IGFBP-1 phosphorylation, which may further contribute to IGF inhibition (Seferovic *et al*. 2009). Consistent with this, the extent of circulating IGFBP-1 phosphorylation was higher in growth-restricted fetuses (Gupta 2015).

IGFBP-2

IGFBP-2 binds IGF-2 with a slight preference over IGF1. In contrast to the other IGFBPs, it is neither glycosylated nor phosphorylated. IGFBP-2 inhibited IGF actions widely *in vitro* (Wheatcroft & Kearney 2009, Russo *et al*. 2015). Similar to IGFBP-1, IGFBP-2 has an Arg-Gly-Asp motif in its C-terminal domain, and binding to the α5β1 and αVβ3 integrins mediated cell association and some other IGF-independent actions (Russo *et al*. 2015).

Not all IGF-independent actions of IGFBP-2 were integrin dependent, since it supported the survival of hematopoietic stem cells via a pathway independent of both IGFs and its Arg-Gly-Asp sequence (Huynh *et al*. 2011). IGFBP-2 has a highly basic heparin-binding sequence in its linker domain, which contrasts with other IGFBPs that have a similar sequence in their C-domains. It bound to proteoglycans in the extracellular matrix and cell membrane, resulting in IGF-independent actions as well as modulation of IGF actions (Russo *et al*. 2015). This sequence also mediated its binding to receptor protein tyrosine phosphatase β (RPTP β), which together with IGF1 receptor activation by IGF1, enhanced osteoblast differentiation via biphasic regulation of AMPK and autophagy (Xi *et al*. 2016). IGFBP-2 binding to RPTP β was also required for optimal IGF1 signal transduction in vascular smooth muscle cells (Shen *et al*. 2015).

Cancer

Although most evidence suggested that other IGFBPs inhibit tumorigenesis, it appeared that IGFBP-2 predominantly promotes this process (Russo *et al*. 2015). Circulating IGFBP-2 levels correlated with established tumor markers and aggressiveness in many cancers including prostate, breast and ovary (Cohen *et al*. 1993, Hoeflich & Russo 2015, Hur *et al*. 2017, Russell *et al*. 2017). Hyperglycaemia increased chemoresistance of prostate cancer cells by enhanced histone acetylation resulting in increased *IGFBP2* expression (Biernacka *et al*. 2013). IGFBP-2 increased cancer cell proliferation, survival and migration/invasion via mechanisms involving integrins and other pathways including Wnt (Mehrian-Shai *et al*. 2007, Holmes *et al*. 2012, Baxter 2014, Russo *et al*. 2015). Of particular interest is the interaction of IGFBP-2 with phosphatase and tensin homolog (PTEN), a tumor suppressor that inhibits PI3 kinase/Akt signaling. PTEN downregulated IGFBP-2 expression in cancer cells, and high IGFBP-2 levels were associated with low PTEN levels in aggressive cancers (Zeng *et al*. 2015). In turn, IGFBP-2 suppressed PTEN activity via an integrin-mediated mechanism in normal and cancer cells, and interaction of IGFBP-2 with RPTP β on the cell surface also contributed to this inhibition.

As well as having a heparin-binding sequence, the linker domain of IGFBP-2 also contains a nuclear localization sequence, and IGFBP-2 transactivated *VEGF* gene expression and promoted angiogenesis in a neuroblastoma model (Azar *et al*. 2014). miR-126 decreased *IGFBP2* expression in breast cancer cells, thereby contributing to impaired metastatic endothelial cell recruitment and angiogenesis in an IGF-dependent manner (Png *et al*. 2012). Recently, IGFBP-2 was shown to

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increase EGF receptor (EGFR) levels, EGFR-STAT3 signaling and nuclear EGFR accumulation in glioma cells, resulting in enhanced migration and invasion (Chua *et al*. 2016). IGFBP-2 also promoted glioma stem cell expansion and survival (Hsieh *et al*. 2010). Hsp27 increased proliferation, migration and invasion of hepatocellular carcinoma cells via IGFBP-2 and induction of epithelial-to-mesenchymal transdifferentiation, suggesting an additional pro-tumourigenic mechanism (Hung *et al*. 2017).

Expression

IGFBP-2 was mainly expressed in liver, adipocytes and the reproductive and central nervous systems in adults, suggesting organ-specific functions (Wheatcroft & Kearney 2009). It played an important role in regulating IGF activity in the central nervous system (Chesik *et al*. 2007), and it may modulate behavior since it recently was shown to ameliorate a rat model of posttraumatic stress disorder via an IGF-independent mechanism (Burgdorf *et al*. 2017).

Metabolism

IGFBP-2 was shown to regulate metabolism and, more recently, adiposity (Wheatcroft & Kearney 2009, Sabin *et al*. 2011, Russo *et al*. 2015). Insulin decreased its expression in the liver, but the response was much slower than that of IGFBP-1. *Igfbp2* expression was increased by leptin (Hedbacker *et al*. 2010), and levels were inversely proportional to adiposity (Russo *et al*. 2015). IGFBP-2 levels were low in patients with type 2 diabetes but elevated in those with type 1 diabetes, which may be due to the differences in insulin sensitivity in these conditions. In the mouse, *Igfbp2* promoter hypermethylation and decreased expression early in life were associated with impaired glucose homeostasis and subsequent obesity and liver fat accumulation (Kammel *et al*. 2016).

Mice overexpressing *Igfb2* under the control of its native promoter had normal birth weight and early postnatal growth but were protected from diet-induced obesity and insulin resistance (Wheatcroft *et al*. 2007). In contrast, constitutive *Igfbp2* overexpression reduced postnatal body weight gain in transgenic mice (Hoeflich *et al*. 1999). Constitutive overexpression also impaired glucose tolerance and decreased GLUT4 glucose transporter translocation to the cell membrane via its Arg-Gly-Asp sequence, suggesting the involvement of integrins rather than the IGF1 receptor (Reyer *et al*. 2015).

Gene deletion

IGFBP-2 was widely expressed during fetal development. Knockdown of *igfbp2* in zebrafish embryos disrupted cardiovascular development and resulted in specific angiogenic defects (Wood *et al*. 2005). *Igfbp2*-knockout mice had decreased spleen and increased liver weights despite normal growth, suggesting specific roles in the growth of these organs (Wood *et al*. 2000). They also had gender-specific changes in bone turnover and architecture postnatally via both IGF-dependent and -independent mechanisms (DeMambro *et al*. 2008).

IGFBP-3

IGFBP-3 binds IGF1 and IGF2 with equal affinity, and it inhibited IGF actions in many cell types *in vitro* (Ranke 2015). However, it enhanced IGF actions in some studies (Martin *et al*. 2009), and cell association may have been be required. IGFBP-3 is N-glycosylated and may be phosphorylated (Coverley *et al*. 2000, Firth & Baxter 2002). Glycosylation inhibited cell association but had no effect on binding to IGFs or the acid-labile subunit. Phosphorylation also inhibited cell association, but, in contrast to IGFBP-1, decreased IGF-binding affinity (Schedlich *et al*. 2003).

As mentioned earlier, IGFBP-3 is the most abundant circulating IGFBP, and it was almost completely found within ternary complexes. Similar to IGF1, IGFBP-3 expression was GH dependent, so levels were decreased in GH-deficient patients and increased in acromegalic patients. Indeed, measurement of IGFBP-3 may have a secondary role in the diagnosis and monitoring of these conditions (Ranke 2015). IGFBP-3 levels were decreased in patients with non-alcoholic fatty liver disease, and *in vitro* studies suggested that this may contribute to the hepatic inflammation observed in this condition (Min *et al*. 2016).

Cancer

IGFBP-3 expression may be decreased or increased in cancer cells (Baxter 2014). Epigenetic regulation by hypermethylation of the *IGFBP3* promoter resulted in decreased expression, and this may be a marker of a more aggressive phenotype (Perks & Holly 2015). Histone acetylation also contributed to epigenetic regulation of *IGFBP3* in cancer (Perks & Holly 2015), and miR-21 contributed to glioblastoma tumorigenesis by downregulating *IGFBP-3* (Yang *et al*. 2014). *In vitro* studies showed that IGFBP-3 inhibited IGF-dependent

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tumourigenic processes including proliferation and survival (Baxter 2014). IGFBP-3 also promoted senescence, which may suppress tumorigenesis, most likely by inhibiting IGFs (Baxter 2014).

IGF-independent actions

IGFBP-3 had a number of IGF-independent actions, which also included inhibition of proliferation, survival and migration as well as modulation of angiogenesis. These actions were mediated via binding to a number of non-IGF proteins (Firth & Baxter 2002, Bach *et al*. 2005, Baxter 2014). These included cell membrane proteins such as caveolin and LRP1, extracellular matrix proteins such as fibronectin, endoplasmic reticulum proteins such as GRP78, nuclear receptors such as RXR-α and extracellular proteins such as plasminogen. A highly basic heparin-binding sequence within the C-terminal domain of IGFBP-3 mediated most non-IGF interactions, including binding to the acid-labile subunit.

Some IGF-independent actions of IGFBP-3 were mediated by interactions with other growth factor systems including TGF-β, EGF, BMP and Wnt (Firth & Baxter 2002, Martin *et al*. 2009, Zhong *et al*. 2011, Naspi *et al*. 2017). In many situations, IGFBP-3 was a tumour suppressor, but it may also have pro-tumourigenic actions; modulation of sphingolipids was proposed to underlie these apparently opposing effects (Baxter 2014). Binding of IGFBP-3 to GRP78, an endoplasmic reticulum protein, stimulated autophagy and promoted survival of breast cancer cells (Grkovic *et al*. 2013). IGFBP-3 lacks classical integrin-binding motifs but some of its effects may have been integrin-mediated through its interaction with integrin ligands such as fibronectin (Burrows *et al*. 2006, Yen *et al*. 2015).

Nuclear actions

Extracellular IGFBP-3 was endocytosed via a number of pathways, including those dependent on clathrin and/or caveolin. It entered the nucleus via interaction of a C-terminal bipartite nuclear localization sequence with importin-β (Schedlich *et al*. 2000, Baxter 2015). Within the nucleus, IGFBP-3 interacted with receptors including RXR-α, PPAR-γ, the vitamin D receptor and Nur77, leading to some of its effects on apoptosis, proliferation and differentiation. Nuclear IGFBP-3 also had a role in DNA damage repair (Liu *et al*. 2000, Lin *et al*. 2014, Baxter 2015), and it activated autophagy in bronchial epithelial cells by a mechanism involving translocation of the transcription factor Nur77 from the nucleus (Yin *et al*. 2017). Further studies are required to enhance our incomplete understanding of the cellular uptake and nuclear actions of IGFBP-3.

Angiogenesis

In addition to affecting tumourigenesis directly by its actions on cancer cells, IGFBP-3 also modulated angiogenesis. Decreased vessel formation via an IGF-independent mechanism contributed to IGFBP-3-induced inhibition of prostate cancer xenograft growth (Liu *et al*. 2007). IGFBP-3 also inhibited IGF1- and VEGF-induced endothelial cell proliferation and survival by an IGF-independent mechanism (Franklin *et al*. 2003). In contrast, another study showed that IGFBP-3 enhanced angiogenesis *in vitro* by an IGF-dependent mechanism involving activation of sphingosine kinase (Granata *et al*. 2007).

Stem cell biology

There is evidence of a role for IGFBP-3 in stem cell biology by IGF-dependent and -independent mechanisms. IGFBP-3 mediated the decreased adult cardiac progenitor cell proliferation induced by Wnt signaling in an IGF-dependent manner (Oikonomopoulos *et al*. 2011). It also inhibited IGF1-induced differentiation of human hematopoietic stem cells into pro-B-cells *in vitro* (Taguchi *et al*. 2006). In contrast, IGF-independent mechanisms were involved in the IGFBP-3-induced inhibition of mesenchymal chondroprogenitor cells proliferation (O'Rear *et al*. 2005) and increased differentiation of endothelial precursor cells into endothelial cells in oxygen-induced retinopathy (Chang *et al*. 2007, Lofqvist *et al*. 2007).

Gene deletion and overexpression

Igfbp3-knockout mice had normal growth and metabolism (Ning *et al*. 2006), likely related to functional redundancy with other IGFBPs. In contrast, *Igfbp3* overexpression impaired both prenatal and postnatal growth (Modric *et al*. 2001) together with decreased bone formation (Silha *et al*. 2003), insulin resistance and impaired glucose tolerance (Silha *et al*. 2002). In contrast, overexpression of a mutant IGFBP-3 that did not bind IGFs had no effect on physiological growth (Silha *et al*. 2005). *In vivo* models suggest that the inhibitory effects of IGFBP-3 on cancer were both IGF dependent and IGF independent.

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Igfbp3 deletion increased the number of metastases in a prostate cancer mouse model (Mehta *et al*. 2011), whereas overexpression of both wild-type and a mutant IGFBP-3 that does not bind IGFs attenuated prostate cancer growth (Silha *et al*. 2006). Further, IGFBP-3-mediated inhibition of lung tumourigenesis was predominantly IGF dependent although IGF-independent effects were also observed (Wang *et al*. 2017*b*).

IGFBP-4

IGFBP-4 binds IGF1 and IGF2 with equal affinity. It is N-glycosylated, which, similarly to the other IGFBPs, had no effect on IGF binding (Zhou *et al*. 2003). IGFBP-4 predominantly inhibited IGF actions *in vitro*. For example, it was recently shown that IGFBP-4 secreted by human mesenchymal stem cells inhibited the induction of regulatory T-lymphocytes by IGFs (Miyagawa *et al*. 2017). IGFBP-4 inhibited anchorage-independent growth of prostate cancer cells by an IGF-dependent pathway *in vitro*, and its overexpression delayed prostate cancer xenograft growth *in vivo* (Damon *et al*. 1998). Expression of IGFBP-4, which decreased clonogenicity of giant cell tumor-derived stromal cells, was epigenetically suppressed in these tumors; however, the IGF dependence of this effect was not studied (Fellenberg *et al*. 2013). Hypermethylation of the *IGFBP4* promoter leading to decreased expression was also found in 42% of lung adenocarcinomas (Sato *et al*. 2011).

Proteolysis

Proteolysis plays a large part in determining the IGF-dependent actions of IGFBP-4. It was proteolyzed by pregnancy-associated plasma protein-A (PAPP-A) (Lawrence *et al*. 1999), and IGFs enhanced this process, resulting in increased IGF bioavailability (Qin *et al*. 2000). Consistent with this, a protease-resistant mutant of IGFBP-4 inhibited smooth muscle growth more potently than wild-type IGFBP-4 *in vivo* (Zhang *et al*. 2002). Given the predominantly inhibitory role of IGFBP-4, it was surprising that deletion of the IGFBP-4 gene resulted in smaller mice (Ning *et al*. 2006), and it was postulated that coexpression of IGFBP-4 was required for optimal IGF-2-mediated fetal growth (Ning *et al*. 2008). Using these mice, it was further shown that IGFBP-4 was required for adipogenesis *in vivo* and for IGF signaling in adipocytes (Maridas *et al*. 2017*a*). Studies of double IGFBP-4/PAPP-A-knockout mice further suggested that IGFBP-4 proteolysis was necessary for most IGF-2-dependent fetal growth (Ning *et al*. 2008). It was recently shown that two coding variants of *STC2*, which encodes the PAPP-A inhibitor stanniocalcin-2, were associated with increased human adult height (Marouli *et al*. 2017). These variants were less effective in inhibiting PAPP-A *in vitro*, resulting in increased IGFBP-4 cleavage that presumably led to enhanced IGF bioactivity.

Bone

The role of IGFBP-4 in bone physiology has been studied in some detail. Osteoblast-specific *Igfbp4* overexpression decreased bone turnover and inhibited growth (Zhang *et al*. 2003), but IGFBP-4 infusion increased bone formation *in vivo* due to increased IGF1 bioavailability by a protease-dependent mechanism (Miyakoshi *et al*. 2001). These findings suggest that IGFBP-4 may have different endocrine and autocrine/paracrine roles in the regulation of bone growth. Consistent with this, it was recently proposed that IGFBP-4 regulated adult skeletal growth via systemic as well as tissue- and sex-specific regulation of osteoblast and osteoclast development (Maridas *et al*. 2017*b*). Further, IGFBP-4 promoted senescence of mesenchymal stem cells, implicating it in the impaired osteogenic differentiation observed with increasing age (Severino *et al*. 2013, Wu *et al*. 2017).

IGF-independent actions

Some IGF-independent actions of IGFBP-4 were mediated by its effects on the Wnt/β-catenin signaling pathway. IGFBP-4 acted as a cardiogenic growth factor by inhibiting Wnt signaling (Zhu *et al*. 2008), and it induced cardiomyocyte differentiation from stem cells by inhibiting β-catenin signaling (Xue *et al*. 2014). IGFBP-4 injection in rats also decreased ischemic injury after myocardial infarction by inhibiting β-catenin activation (Wo *et al*. 2016). A number of other studies also suggested IGF-independent actions of IGFBP-4, but the underlying mechanisms were not studied (Singh *et al*. 1994, Perks *et al*. 1999, Wright *et al*. 2002).

Angiogenesis

IGFBP-4 inhibited angiogenesis by both IGF-dependent and IGF-independent mechanisms. It inhibited angiogenesis induced by IGF1 and FGF-2 both *in vitro* and *ex vivo*, but by VEGF only *in vitro* (Moreno *et al*. 2006, Contois *et al*. 2012). The C-terminal domain of IGFBP-4 mediated its antiangiogenic effect in glioblastoma by

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inhibiting cathepsin B (Moreno *et al*. 2013). Expression of the αvβ3 integrin enhanced melanoma growth and angiogenesis *in vivo* and decreased IGFBP-4 levels, both by decreasing mRNA levels and increasing its p38 MAPK-dependent degradation by matrix metalloproteases (Contois *et al*. 2015); the IGF dependence of this finding was not assessed.

IGFBP-5

About half of circulating IGFBP-5 was found in ternary complexes with IGFs and the acid-labile subunit, with the remainder in binary complexes with IGFs or unbound (Baxter *et al*. 2002). It has a modest binding preference for IGF2 over IGF1 (Rajaram *et al*. 1997). O-glycosylation and phosphorylation of IGFBP-5 both inhibited binding to heparin but not IGFs or the acid-labile subunit (Graham *et al*. 2007).

As well as binding IGFs, IGFBP-5 also bound to a range of biomolecules, many of which are mediated by interaction with its C-terminal heparin-binding sequence. In the extracellular matrix, these included glycosaminoglycans and proteins including fibronectin, vitronectin and plasminogen activator inhibitor-1 (Firth & Baxter 2002, Beattie *et al*. 2006). As well as mediating its IGF-independent actions, these additional interactions may have determined whether IGFBP-5 inhibited or potentiated IGF actions. Binding of IGFBP-5 to glycosaminoglycans decreased its IGF-binding affinity, which may have contributed to potentiation of IGF actions. IGFBP-5 increased or decreased cell survival in a context-specific manner. Both IGF-dependent and IGF-independent mechanisms were involved in IGFBP-5-mediated apoptosis of mammary cells, the latter by enhancing plasmin generation via its interaction with tissue plasminogen activator (Sorrell *et al*. 2006).

IGFBP-5 interacted with a number of cell surface receptors. It inhibited TNF-α actions by binding to the latter's receptor via its linker domain (Hwang *et al*. 2011). IGFBP-5 also increased breast cancer cell adhesion via direct interaction of its heparin-binding region with the α2β1 integrin (Sureshbabu *et al*. 2012). IGFBP-5 activated the ERK MAP kinase pathway resulting in increased fibrosis, although a receptor was not described (Yasuoka *et al*. 2009).

Nuclear actions

IGFBP-5 entered to the nucleus via importin binding to a nuclear localization sequence in its C-terminal domain (Beattie *et al*. 2006). Within the nucleus, IGFBP-5 had transactivation activity via its N-terminal domain (Zhao *et al*. 2006) and it bound transcription factors such as FHL2 (Amaar *et al*. 2002) and the nucleolar protein nucleolin (Su *et al*. 2015). Nuclear IGFBP-5 bound to the vitamin D receptor and attenuated vitamin D-induced expression of bone differentiation markers (Schedlich *et al*. 2007). However, nuclear translocation and nucleolin binding were not required for IGFBP-5-induced fibrosis (Su *et al*. 2015).

Gene deletion and overexpression

Genetic models have helped determine the IGF-dependent and IGF-independent roles of IGFBP-5 in development. *Igfbp5*-knockout mice had a normal growth phenotype but delayed mammary gland involution after weaning (Ning *et al*. 2007). Global *Igfbp5* overexpression increased neonatal mortality, inhibited growth prenatally and especially prepubertally, impaired muscle development and decreased female fertility (Salih *et al*. 2004). Whereas these effects could have been due to IGF inhibition, these mice also had increased brain and liver weights, which may have been due to enhanced IGF actions. Overexpression of an IGFBP-5 mutant with minimal IGF binding also inhibited growth, suggesting the presence of IGF-independent effects *in vivo* (Tripathi *et al*. 2009). Decreased bone density that was more prominent in males was observed in mice overexpressing *Igfbp5*, and this effect was at least in part IGF independent (Salih *et al*. 2005).

IGFBP-5 was expressed widely in human tissues including bone, lung, testis, ovary, uterus and placenta. There is evidence for roles of IGFBP-5 in the physiology and pathology of bone and kidney, mammary gland involution and muscle cell differentiation (Schneider *et al*. 2002). Osteoblast-specific *Igfbp5* overexpression *in vivo* resulted in transient osteopenia (Devlin *et al*. 2002). Overexpression in mammary cells decreased survival and proliferation, and reduced milk synthesis *in vivo* (Tonner *et al*. 2002) and, as mentioned above, global IGFBP-5 deletion impaired mammary gland involution after weaning (Ning *et al*. 2007).

Cancer

IGFBP-5 inhibited or promoted tumor growth via IGF-dependent and IGF-independent mechanisms (Baxter 2014). The balance of sphingolipids (McCaig *et al*. 2002) as well as other context-specific differences may have been critical in determining which of these apparently contradictory effects was observed. One of the targets of

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miR-143/5 was *IGFBP5*, which inhibited IGF signaling during intestinal epithelial regeneration; this is particularly relevant since downregulation of this miRNA was implicated in colon cancer (Chivukula *et al*. 2014). Inhibition of angiogenesis was partially responsible for the inhibitory effect of IGFBP-5 on ovarian cancer xenograft growth (Rho *et al*. 2008). Supporting this finding, the C-terminal domain of IGFBP-5 and a peptide based on its heparin-binding site both inhibited VEGF signaling, angiogenesis and ovarian cancer xenograft growth (Hwang *et al*. 2016). Further, IGFBP-5 inhibited angiogenesis induced by activated coagulation factor Xa by increasing endothelial cell senescence *in vitro* (Sanada *et al*. 2016). Another study showed that a p53-dependent pathway mediated IGFBP-5-induced endothelial cell senescence (Kim *et al*. 2007). In contrast to these inhibitory effects, increased IGFBP-5 expression was associated with poor prognosis in some human tumors, including glioma, breast and ovarian cancers, suggesting a possible role as a tumor promoter in some circumstances (Baxter 2014).

IGFBP-6

Unlike the other IGFBPs, IGFBP-6 has a ~50-fold-binding preference for IGF2 over IGF1 (Bach *et al*. 2013). It therefore primarily inhibited the actions of IGF2, including cell proliferation, differentiation, migration and survival *in vitro*. It is O-glycosylated in its linker domain (Neumann *et al*. 1998), which inhibited cell association and prolonged its circulating half-life while having no effect on its IGF-binding affinity (Bach *et al*. 2013).

Cancer

Many cancers overexpress IGF2, and IGFBP-6 inhibited *in vivo* xenograft growth of two of these, neuroblastoma and rhabdomyosarcoma (Grellier *et al*. 1998, Gallicchio *et al*. 2001). Consistent with this, a number of studies showed that IGFBP-6 expression was lower in malignant cells than that in normal cells (Bach *et al*. 2013), including observations that lower levels were associated with poorer prognosis in nasopharyngeal (Chen *et al*. 2016) and gastric (Zeng *et al*. 2017) cancer. Increased methylation of the *IGFBP6* promoter may have contributed to decreased expression in the latter (Jee *et al*. 2009). Transcribed-ultraconserved regions (T-UCRs), a novel class of non-coding RNAs, have been implicated in cancer development, and one of these, Uc.416+A, promoted gastric cancer cell proliferation, possibly by inhibiting *IGFBP6* expression (Goto *et al*. 2016).

IGF-independent actions

Like the other IGFBPs, IGFBP-6 also had IGF-independent actions. Inhibition of proliferation and apoptosis by IGFBP-6 were both IGF dependent and IGF independent (Hale *et al*. 2000, Sueoka *et al*. 2000, Iosef *et al*. 2008). It entered the nucleus by binding to importins via a nuclear localization sequence in its C-terminal domain, and subsequently modulated cell proliferation, migration and survival (Iosef *et al*. 2008, 2010, Kuo *et al*. 2010). IGFBP-6 inhibited basal and VEGF-induced angiogenesis by an IGF-independent mechanism *in vitro* as well as inhibiting it in rhabdomyosarcoma xenografts and zebrafish embryos *in vivo* (Zhang *et al*. 2012). IGFBP-6 may limit the angiogenic response to hypoxia, which slowly increased its expression. IGFBP-6 enhanced cancer cell migration by an IGF-independent mechanism that included binding to cell surface prohibitin-2 and MAP kinase pathway activation (Bach *et al*. 2013, Fu *et al*. 2013). The above studies suggest that IGFBP-6 inhibited tumor growth via IGF-dependent and -independent effects on proliferation, survival and angiogenesis. In contrast, the IGF-independent promigratory effects of IGFBP-6 may have been pro-tumourigenic, so the role of IGFBP-6 in cancer requires further study.

Immune regulation

IGFBP-6 may also be involved in immune regulation. A recent study of infection in fish showed that proinflammatory cytokines increased the expression of *igfbp6* and *igfbp1*, which may have promoted immune system activation by limiting energy utilization for IGF-mediated growth (Alzaid *et al*. 2016). IGFBP-6 was required for pro-B-cell development *in vitro* (Taguchi *et al*. 2006). It may also have a role in the adaptive immune response, since dendritic cells exposed to hyperthermia increased expression of IGFBP-6, which promoted chemotaxis of monocytes and T-cells (Liso *et al*. 2017). IGFBP-6 also increased migration of T-cells from subjects with rheumatoid arthritis but not from controls, suggesting a possible role in autoimmune disease (Alunno *et al*. 2017).

Gene deletion and overexpression/CNS actions

As with most of the other IGFBPs, *Igfbp6*-knockout mice did not have an overt phenotype. In contrast, overexpression of *igfbp6* inhibited embryonic growth and development in zebrafish (Wang *et al*. 2009). Transgenic mice overexpressing *Igfbp6* in the brain also were smaller

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during the first postnatal month and had cerebellar and reproductive abnormalities as well as abnormal metabolic responses to a high-fat diet (Bienvenu *et al*. 2004, 2005). These findings suggest a number of roles for IGFBP-6 in the central nervous system. Further, *Igfbp6* expression was increased following a range of nervous system pathologies, including axonal transection (Hammarberg *et al*. 1998), hypoxic-ischemic injury (Beilharz *et al*. 1998) and demyelination (Wilczak *et al*. 2008). Increased *Igfbp6* expression after traumatic spinal cord injury may have contributed to neuronal apoptosis (Wang *et al*. 2017*a*).

Summary and future questions

As outlined in this review, IGFBPs are involved in a broad range of cellular processes through their IGF-dependent and IGF-independent actions. There is a degree of overlap in their modulation of IGF actions, and the normal or minimally impaired phenotypes following knockout of individual IGFBPs have been attributed to functional redundancy between them (Firth & Baxter 2002). In contrast, triple knockout of IGFBPs 3–5, which would reduce this redundancy, diminished postnatal growth and enhanced glucose metabolism; the absence of ternary complexes leading to an inability to stabilize IGF1 in the circulation may have contributed to these findings (Ning *et al*. 2006). Further, abnormal responses to metabolic and other insults in mice with individual IGFBP knockouts indicated potential roles in these processes.

In contrast to the presumed common mechanisms underlying IGFBP regulation of IGF actions, no such assumption can be made about the IGF-independent actions of individual IGFBPs. Clearly, further studies are needed to determine the mechanisms underlying these actions and to determine whether they are unique or common to multiple IGFBPs. Related to this, an important issue is the contribution of individual non-IGF ligands to IGFBP actions and how these are integrated. This will require quantitative analyses accounting for their relative binding affinities and abundance, as well as determining whether they compete for the same or similar binding sites. The actions of IGFBPs in the extracellular space and intracellular compartments including the nucleus, and the mechanisms underlying their movement between these compartments are another important issue. Finally, the balance between the IGF-dependent and IGF-independent actions of IGFBPs is a critical question, especially *in vivo*. Only by reaching a fuller understanding of IGFBP properties will it be possible to determine their role in physiology and in pathologies including malignant, metabolic, neurological and immune diseases. Ultimately, the potential of therapeutic approaches utilizing or modulating IGFBP actions can then be assessed.

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