THEMATIC REVIEW

40 YEARS OF IGF1

Role of IGF-binding proteins in regulating IGF responses to changes in metabolism

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Abstract

The IGF-binding protein family contains six members that share significant structural homology. Their principal function is to regulate the actions of IGF1 and IGF2. These proteins are present in plasma and extracellular fluids and regulate access of both IGF1 and II to the type I IGF receptor. Additionally, they have functions that are independent of their ability to bind IGFs. Each protein is regulated independently of IGF1 and IGF2, and this provides an important mechanism by which other hormones and physiologic variables can regulate IGF actions indirectly. Several members of the family are sensitive to changes in intermediary metabolism. Specifically the presence of obesity/insulin resistance can significantly alter the expression of these proteins. Similarly changes in nutrition or catabolism can alter their synthesis and degradation. Multiple hormones such as glucocorticoids, androgens, estrogen and insulin regulate IGFBP synthesis and bioavailability. In addition to their ability to regulate IGF access to receptors these proteins can bind to distinct cell surface proteins or proteins in extracellular matrix and several cellular functions are influenced by these interactions. IGFBPs can be transported intracellularly and interact with nuclear proteins to alter cellular physiology. In pathophysiologic states, there is significant dysregulation between the changes in IGFBP synthesis and bioavailability and changes in IGF1 and IGF2. These discordant changes can lead to marked alterations in IGF action. Although binding protein physiology and pathophysiology are complex, experimental results have provided an important avenue for understanding how IGF actions are regulated in a variety of physiologic and pathophysiologic conditions.

Key Words

- ▶ bone
- ▶ cardiovascular
- ▶ diabetes (all)
- ▶ growth factor receptors
- ▶ growth factors

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Introduction

The IGF-binding proteins are a family of six proteins that contain specific amino acids in their amino and carboxyl terminus that mediate high-affinity IGF binding (Clemmons 2016). These regions of sequence are highly conserved in all six members of the family; therefore, the mechanism by which this high-affinity interaction with the IGFs is mediated is common among the six members

of the family. Importantly for understanding their role in regulating IGF1's metabolic effects is the observation that these proteins do not bind insulin; therefore, they have no effect on insulin's interaction with its receptor. The affinity of all of the forms of IGFBPs is higher than the IGF1 receptor and when IGF1 or II is bound to an IGFBP, it does not activate the receptor. Therefore, a major

function of these proteins is to regulate IGF bioavailability. Cleavage of IGFBPs by specific proteases results in a major reduction in their affinities, and this is accompanied by an increase in free IGF. Several investigators have prepared protease-resistant mutant forms of IGFBPs and infused them into animals or added them to cultured cells and shown that they can inhibit IGF actions (Conover *et al.* 1995, Nichols *et al.* 2007). The identification of proteases, such as PAPP-A that cleave IGFBPs has made it possible to modulate IGFBP cleavage and thereby assess the role of IGFBP proteolysis in regulating IGF1's metabolic actions (Mikkelsen *et al.* 2014).

In addition to their ability to regulate ligand–receptor interactions, the members of this family also have IGF1-independent effects. The preparation of mutant forms of each form of IGFBP that have reduced affinity but retain the ability to interact with other cellular proteins has allowed definitive testing of this hypothesis. However, the term IGF1-independent effects can be interpreted in two ways. The most conservative interpretation is effects that are mediated without IGF binding to the IGFBP. These can clearly be tested with nonbinding mutants. However, a broader interpretation includes effects that are mediated through protein–protein interactions that are not completely IGF independent in the sense that they may alter IGF signaling through the IGF receptor. Such an effect by definition would not be IGF independent. Therefore, using this definition to definitively prove that an IGF-binding protein effect is truly IGF independent requires the use of cells in which IGF synthesis has been inhibited or receptor activation has been attenuated following binding protein addition. Many of the published experiments have not utilized these controls; therefore, the conclusion that their effects are IGF independent often requires modification. Nevertheless, such experiments have led to an interesting line of research that is highly relevant to understanding the metabolic actions of IGFBPs. Some examples of these non-IGF-dependent effects include interaction with extracellular matrix proteins (Jones *et al.* 1993b), cell surface proteoglycans (Russo *et al.* 1997) or with specific cell surface receptors (Andress 1998) (Fig. 1). Additionally interactions with a variety of intracellular targets including PPAR gamma, RXR receptors, LIM protein family members, GRP 78, endoplasmic reticulum chaperone proteins, specific proteins in the nuclear membrane that mediate nuclear translocation and specific transcription factors have been identified (Martin & Baxter 2011). In some cases, the mechanism by which these protein–protein interactions modify IGF signaling through its receptor has been determined. These protein–IGFBP interactions are

Figure 1

IGFBPs have the capacity to function by one of multiple mechanisms some of which involve binding IGFs and some by interacting with other receptors or signaling systems.

often mediated through the linker region or C-terminal domain of IGFBPs. Unlike the carboxyl or amino terminus, the linker region has no sequence conservation among the six members of the family; therefore, protein–protein interactions that are mediated through this region can be specific for a single form of IGFBP. An example would be the heparin-binding domain sequence contained in IGFBP2, which interacts with proteoglycans (Russo *et al.* 1997). This sequence is only present in IGFBP2. Therefore, the metabolic consequences of this interaction are unique to this IGFBP. The C-terminal domain is the most highly conserved region; thus, the interactions mediated through this region may not be specific and may apply to multiple IGFBPs.

An additional concept that is important for understanding the ability of the IGFBPs to regulate metabolism is the distinction between locally secreted BPs (autocrine/paracrine effects) and IGFBP actions that are mediated via transport through the circulation (endocrine effects). The liver is the primary source of blood-borne IGFBPs and therefore identification of the factors that regulate hepatic synthesis has contributed to our understanding of their endocrine actions. In general, IGFBP3 and 5 account for much of the bound IGF in serum for two reasons; (1) they have very high affinity for IGF1 and II; (2) they bind to a third protein acid-labile subunit (ALS), which prolongs their half-lives and therefore extends IGF half-life much better than the other members of the family (Jones *et al.* 1993a). Therefore, in terms of understanding IGF transport to tissues, these two family members are extremely important. In contrast, since many of the metabolic actions of IGF1 and II are

mediated by the free peptide the importance of IGFBP1, 2, 4 and 6 is their ability to regulate free peptide availability. Analysis of binding capacity of serum shows that while IGFBP3 and 5 are saturated under normal physiologic conditions, IGFBP1 and 2 are unsaturated (Baxter 1994). Therefore, following acute changes in an IGFBP such as suppression of IGFBP1 after a high carbohydrate meal, there are major changes in free IGF1 (McCusker *et al.* 1989). Understanding how these acute changes in free IGF1 in blood translate into changes in free IGF1 in interstitial fluids and in receptor bioavailability is not well defined at present. This is an important area for future studies.

In contrast to endocrine actions, autocrine/paracrine effects are dependent primarily upon cell type-specific changes in the synthesis and secretion of specific forms of IGFBPs. For example, normal skin fibroblasts secrete primarily IGFBP3, 4 and 5, whereas vascular smooth muscle cells (VSMC) secrete primarily IGFBP2 and 4. Since these IGFBPs may have unique actions, this implies that this is one mechanism for mediating cell type specific changes. Several IGFBP actions that regulate metabolism are cell type specific; therefore, the metabolic factors that regulate IGFBP synthesis and/or degradation as well as posttranslational modifications that regulate their affinities can alter cellular metabolic responses.

There is minimal data regarding specific cell surface receptors that are linked to intracellular signaling mechanisms that bind to IGFBPs. Exposure of fibroblasts to the IGFBP3 was shown to trigger a phosphatase that altered IGF1 signaling, however, a specific receptor that mediated this interaction was not identified (Lewitt & Baxter 1991). Subsequent studies indicated that IGFBP3 could bind to the type V TGF- β receptor. This was later determined to be LRP-1 (Ricort & Binoux 2002). This resulted in LRP-1 phosphorylation and was associated with changes in cell behavior. However, the signaling elements that were activated were not determined. IGFBP3 has been shown to alter signaling through the TGF- β 1 and 2 receptors and deletion of the TGF- β 2 receptor inhibits the ability of IGFBP3 to activate SMAD proteins, a component of the TGF- β signaling pathway (Leal *et al.* 1999). This results in sphingosine kinase stimulation and transactivation of the epidermal growth factor receptor (Fanayan *et al.* 2002). IGFBP1 interacts directly with the α 5 β 1 integrin through its RGD-binding sequence (Fig. 1). This results in alterations in IGF action and direct stimulation of cell migration (Martin *et al.* 2014).

Recently, a tightly linked signaling mechanism coupled to a specific receptor for IGFBP2 has been

identified. IGFBP2 contains a unique heparin-binding domain in its linker region that is not contained in any of the other forms of IGFBPs. Specific amino acids in this region interact with a cell surface receptor termed receptor protein tyrosine phosphatase beta (RPTP β). Binding to RPTP β leads to inhibition of its polymerization and inhibition of its tyrosine phosphatase activity (Shen *et al.* 2012). A major target of RPTP β is PTEN an inhibitor of AKT activation. Following IGFBP2 engagement, there is enhanced PTEN tyrosine phosphorylation, which inhibits degradation of inositol 3 phosphate resulting in enhanced AKT activation. This signaling mechanism alters several IGFBP2 dependent metabolic functions.

Although the mechanism by which IGFBPs regulate the growth response to IGF1 and IGF II is beyond the scope of this review, it is important to point out that growth and metabolism are inextricably intertwined, therefore, understanding how the IGFBPs regulate metabolic responses is critical for interpreting their actions on somatic growth. The IGFs act as intermediaries between nutrient availability and growth regulation. Therefore, the actions of the IGFBPs in modulating these responses have both direct and indirect effects on cellular responses to changes in energy source availability. Many of the same nutritional variables that regulate IGF1 and II also coordinately regulate the IGFBPs. In contrast, in states of severe metabolic derangement, such as diabetes, regulation may become discordant, and this may result in pathophysiologic responses. The availability of transgenic and knockout animals has greatly contributed to our understanding of how these complex interactions influence organismal growth and metabolism.

Role of IGFBPs in regulating fat metabolism

Many of the publications regarding the effects of IGFBPs on fat metabolism have been derived from human or whole animal studies in which the serum concentration of the IGFBP is correlated with changes in fat mass or fat metabolism. Studies that have analyzed direct effects of IGFBPs on isolated adipocytes or fat tissue in animals are much less prevalent but some information is available regarding their direct actions.

IGFBP1

Direct addition of IGFBP1 to pre-adipocyte cultures inhibits proliferation and differentiation and IGFBP1 transgenic mice show reduced fat mass (Rajkumar *et al.* 1999, Siddals *et al.* 2002). Studies utilizing cells derived

from these animals have shown that IGFBP1 inhibits IGF1 stimulation of pre-adipocyte differentiation. When IGFBP1 transgenic mice were fed a high calorie diet, they gained significantly less fat mass compared to control mice (Rajkumar *et al.* 1999). IGF1 stimulates pre-adipocyte differentiation, thus, it was proposed that IGFBP1 functions by inhibiting IGF1's actions. The phosphorylated form of IGFBP1 was fivefold more effective than the non-phosphorylated form. The molecular explanation for this observation is that phosphorylation of IGFBP1 enhances its affinity for IGF1 sixfold (Jones *et al.* 1991). Since insulin inhibits IGFBP1 synthesis, the results support the conclusion that hyperinsulinemia that occurs in states of obesity and insulin resistance suppresses IGFBP1 leading to enhanced adipogenesis. Conversely, weight loss and restoration of more normal physiologic insulin secretion leads to an increase in IGFBP1, which inhibits adipogenesis (Wheatcroft & Kearney 2009). A specific receptor that is mediating these effects has not been identified, and they are presumed to be mediated only by changes in IGF1 receptor activation.

The initial studies of IGFBP1 in humans showed that it was markedly suppressed by insulin and that following ingestion of a mixed meal or carbohydrate, there was major suppression in serum IGFBP1 (Busby *et al.* 1988, Lewitt *et al.* 2010). Not surprisingly, follow-up studies showed that obese subjects particularly those who were hyperinsulinemic had a low IGFBP1 (Lewitt *et al.* 2010). Since IGFBP1 is produced almost solely in the liver, these changes are result of suppression of hepatic synthesis; therefore, any effect of IGFBP1 on fat mass accumulation would be mediated by changes in endocrine transport. Both obese and non-obese subjects are highly insulin responsive in terms of insulin-induced IGFBP1 suppression. Both acute and chronic caloric restriction results in highly significant increases in IGFBP1, and these changes correlate with fat mass loss (Reinehr *et al.* 2011, Henning *et al.* 2013). Similarly, refeeding an isocaloric diet after prolonged energy restriction results in a major decrease. The extent of IGFBP1 suppression in obesity can be profound since women who were greater than 107 kg over ideal body weight had undetectable IGFBP1 levels (Weaver *et al.* 1990). Overfeeding results in a substantial reduction in IGFBP1 that correlates with changes in increases in abdominal visceral fat (Ukkola *et al.* 2001). Longitudinal studies show that changes in IGFBP1 predict the development of metabolic syndrome. Specifically, children followed over time who developed metabolic syndrome had significantly lower IGFBP1 levels compared obese children who did not develop metabolic

syndrome (Reinehr *et al.* 2011). IGFBP1 correlates with several variables of metabolic syndrome including HDL cholesterol, triglycerides, proinsulin and measures of insulin sensitivity as well as PAI-1 (Mohamed-Ali *et al.* 1999). A study of adults who had been born for a small for gestational age showed that IGFBP1 was inversely related the body mass index, fasting glucose and insulin (van der Kaay *et al.* 2009). IGFBP1 predicted the fasting insulin and the insulin area under the glucose tolerance test curve in obese adolescents and phosphorylated IGFBP1 predicted the degree of liver fat content (Petäjä *et al.* 2016). There is also a longitudinal association between the degree of suppression of IGFBP1, and the development of markers of the metabolic syndrome in obese children before and after weight loss (Reinehr *et al.* 2011). Similarly, the change in IGFBP1 after glucose ingestion is an excellent predictor of hepatic insulin resistance (Kottronen *et al.* 2008). These data support the conclusion that there is a strong relationship among IGFBP1, fat mass and insulin sensitivity.

IGFBP2

Differentiating pre-adipocytes secrete predominantly IGFBP2 (Boney *et al.* 1994) and direct addition of IGFBP2 inhibits adipogenesis in 3T3-L1 cells (Wheatcroft *et al.* 2007). *In vitro* studies have shown that two regions of sequence within IGFBP2 termed heparin-binding domain-1 and heparin-binding domain-2 are the most important for inhibiting adipogenesis (Fig. 2). Specifically, addition of peptides containing those sequences was as effective as the native protein in inhibiting the response of pre-adipocytes to insulin-stimulated differentiation (Xi *et al.* 2013). That this effect was independent IGF binding to IGFBP2 was demonstrated by showing that IGFBP2 inhibited the effect of insulin, which does not bind IGFBPs and that these peptides that contain no IGF-binding activity were equipotent with native IGFBP2. Addition of IGFBP2 to human visceral pre-adipocyte cultures showed reduced fat cell differentiation and silencing IGFBP2 in visceral adipocytes was associated with enhanced

Figure 2

Schematic diagram of domains in IGFBP2 mutagenesis studies have shown that each of these domains has a distinct function.

adipogenesis but had it no effect and subcutaneous adipocytes (Russo *et al.* 2015). This is consistent with the transgenic overexpression data, which show a preferential effect on visceral fat. Wheatcraft *et al.* showed that transgenic mice overexpressing IGFBP2 were resistant to diet-induced fat accumulation and had enhanced insulin sensitivity compared to controls (Wheatcraft *et al.* 2007). The difference between visceral and subcutaneous adipose tissue was further amplified by Li and Picard who demonstrated that constitutive expression of IGFBP2 is preferentially decreased in obese mice in visceral fat (Li & Picard 2010). Overexpression of a form of IGFBP2 in which this HBD-1 sequence was altered showed that the mutant had no effect on fat mass, and these mice preferentially gained fat (Wiedmer *et al.* 2015). Studies in rodents show that leptin stimulates IGFBP2 expression in the liver and that these changes correlate with changes in body weight and glucose metabolism (Hedbacker *et al.* 2010).

Results from studies of IGFBP2-knockout mice also support the conclusion that IGFBP2 inhibits adipogenesis. Specifically, male IGFBP2 $-/-$ mice become obese (30% over ideal body weight) by age 3 months and their weight remains significantly greater than controls for one year (DeMambro *et al.* 2008). These changes occur on a normal diet suggesting that physiologic levels of IGFBP2 regulate fat mass. Administration of a peptide containing the HBD-1 domain resulted in significant attenuation of weight gain and inhibited gain of fat mass (Kawai *et al.* 2011). Taken together, the results strongly support the conclusion that IGFBP2 has direct effects on fat metabolism and that these effects are preferential for visceral fat. However, the receptor that is mediating these effects has not been definitively determined.

Human studies measuring IGFBP2 have focused primarily on obesity and metabolic syndrome. When obese, prepubertal children were compared to normal weight subjects IGFBP2 concentrations were significantly lower (Radetti *et al.* 1998). Since IGFBP2 is much less sensitive to suppression by insulin, it is possible that other hormonal variables are also suppressing IGFBP2. Cytokines that are increased in obesity, such as, IL-6 and TNF α inhibit IGFBP2 (Street *et al.* 2013). Another proposed mechanism that contributes to IGFBP2 suppression is hypermethylation of the gene, which occurs in response to high-fat feeding in mice (Kammel *et al.* 2016). In subjects who are overweight (BMI 28.9) IGFBP2 correlates independently with insulin sensitivity and IGF bioactivity suggesting that loss of suppression of IGF bioactivity in obesity may contribute to changes in insulin sensitivity (Nam *et al.* 1997, Arafat *et al.* 2009, Claudio *et al.* 2010).

As for IGFBP1 reversibility of the obese state is associated with upregulation of IGFBP2. Obese patients undergoing bariatric diversion showed a substantial increase in IGFBP2 in association with weight loss and improvement in metabolic syndrome parameters such as triglycerides and HDL cholesterol (Li *et al.* 2012). A longitudinal study showed that IGFBP2 predicted long-term changes in HDL cholesterol that occurred in association with the development of metabolic syndrome (Narayanan *et al.* 2014). Similarly IGFBP2 levels in adults correlate with the development of increasing VLDL triglycerides (Carter *et al.* 2014). Nam *et al.* reported that IGFBP2 concentrations were inversely related to free IGF1 in obese subjects suggesting that these effects are mediated through loss of suppression of IGF1-stimulated pre-adipocyte differentiation (Nam *et al.* 1997). Prepubertal and pubertal subjects who were born small for gestational age (SGA) had lower IGFBP2 levels compared to non-SGA children and later in life, IGFBP2 levels in post-SGA children continue to correlate with BMI, fat mass, triglycerides and insulin levels (de Kort *et al.* 2010). Therefore, like IGFBP1, IGFBP2 correlates with changes in fat mass, insulin sensitivity and metabolic syndrome, but the mechanisms that regulate these changes are more diverse.

IGFBP3

IGFBP3 interacts with the PPAR γ receptor and inhibits its dimerization with RXR thereby blocking ligand-induced transactivation of PPAR γ in pre-adipocytes (Chan *et al.* 2012). Additional studies showed that IGFBP3 activated Smad signaling in pre-adipocytes (de Silva *et al.* 2009). This signaling system is known to mediate the anti-lipogenic effect of TGF- β . IGFBP3 stimulates SMAD phosphorylation and knockdown of IGFBP3 *in vitro* impaired SMAD activation in response to TGF- β suggesting that TGF- β was working through IGFBP3 (de Silva *et al.* 2009). *In vivo* studies have been less definitive. Although transgenic mice overexpressing IGFBP3 had some reduction in body weight, analysis of the fat compartment was not reported (Yakar *et al.* 2009). One study demonstrated that overexpression of wild-type IGFBP3 had no effect on fat mass compared to control mice, but overexpression of a non-IGF-binding mutant of IGFBP3 was associated with increased fat mass (Nguyen *et al.* 2015b). This suggests that under physiologic conditions, IGFBP3 may be acting to attenuate the effect of IGF1 in stimulating pre-adipocyte differentiation. It is also possible that IGFBP3 was inhibiting fat accumulation by a mechanism independent of IGF binding. Interestingly, this group

reported that IGFBP3 and the non-IGF-binding mutant stimulated brown adipocyte differentiation (Nguyen *et al.* 2015a).

Population-based studies of IGFBP3 show that it correlates positively with total or LDL cholesterol, triglycerides and inversely with HDL cholesterol (Eggert *et al.* 2014). Similarly, the IGF1/IGFBP3 ratio correlates with the development of metabolic syndrome and insulin resistance (Eggert *et al.* 2014). Analysis of older men showed that IGF1/IGFBP3 had a U-shaped relationship with the odds of development metabolic syndrome (Savastano *et al.* 2011). Subjects in the lowest quartile and the highest quartile of IGFBP3 had the greatest probability. Notably IGFBP3 was positively associated with all five components metabolic syndrome. A strong positive correlation between IGF1, IGFBP3 and HDLC has been confirmed in elderly adults (Yeap *et al.* 2010). Analysis of 1291 subjects with cardiovascular risk factors showed that IGFBP3 correlated with triglycerides and total cholesterol. Follow-up over 10 years showed that increasing IGFBP3 was associated with increased cholesterol (Ceda *et al.* 1998).

IGFBP4

IGFBP4 is secreted by human pre-adipocytes prior to differentiation and its secretion does not appear to increase during differentiation *in vitro* (Boney *et al.* 1994). An important property of IGFBP4 is its ability to inhibit IGF1-stimulated angiogenesis (Contois *et al.* 2012). Angiogenesis is an important component of fat depot formation, and IGF1 stimulates endothelial cell sprouting *in vitro*. Addition of IGFBP4 blocked the effects of either insulin or IGF1 on adipose tissue sprouting (Gealekman *et al.* 2014). Furthermore, IGFBP4 levels were shown to decrease with aging in animals and with high-fat diet-induced adipose tissue expansion. Since IGFBP4 can block insulin-induced sprouting, this suggests that it is suppressing adipogenesis though its effects on angiogenesis and that its effect is not mediated through binding IGF1. A cell surface receptor that might mediate this effect has not been identified.

In summary, IGFBPs 1-4 modulate pre-adipocyte differentiation *in vitro*. Definitive studies in genetically manipulated animals are only present for IGFBP1 and 2 and suggest that these proteins, particularly IGF BP-2, function to directly inhibit pre-adipocyte expansion. This limitation of definitive understanding of molecular mechanisms by which the IGFBPs may alter pre-adipocyte differentiation has limited the amount of information that

can be drawn from human studies since these generally involve measuring changes that occur in serum IGFBPs after a particular metabolic manipulation. Human study data interpretation is also limited by the fact that very few studies have analyzed adipose tissue obtained from the subjects. Wherein this has been performed, the data have provided new and useful information.

The findings clearly support the conclusion that IGFBP1 and 2 function by regulating free IGF1 concentrations and thereby inhibiting the ability of IGF1 to stimulate pre-adipocyte differentiation. Additionally, both peptides have IGF1-independent effects that regulate fat mass accumulation and possibly insulin sensitivity. However the receptors and signaling mechanisms that mediate these effects have not been delineated. In contrast, IGFBP3 while capable of lowering free IGF1, is generally saturated therefore it appears to function predominantly by providing a consistent reservoir of IGF1 to stimulate pre-adipocyte differentiation and its net effects are the opposite of IGFBP1 and 2. This conclusion is consistent with the known mechanisms that regulate the synthesis and secretion of these forms of IGFBPs. The major challenge for the future is to dissect out those effects that are purely associative versus those wherein the specific form of IGFBP is the primary causal driver of the change in fat mass and/or insulin sensitivity.

Carbohydrate metabolism and diabetes

IGFBP1

IGFBP1 is regulated acutely by changes in carbohydrate metabolism. The primary source of IGFBP1 is hepatic synthesis. The IGFBP1 gene contains an insulin-sensitive response element that directly suppresses its transcription in response to increased hepatic insulin exposure (Suwanickul *et al.* 1993). As would be predicted following ingestion of a normal carbohydrate meal, there is suppression of IGFBP1, and following fasting, there is a 3- to 4-fold increase in IGFBP1 serum concentrations (Lewitt & Baxter 1991, Contois *et al.* 2012). Therefore, IGFBP 1 levels fluctuate throughout the day depending on food intake and fasting intervals. Glucocorticoids and hepatic nuclear factor-1 stimulate IGFBP1 synthesis (Bae *et al.* 2013). In states of significant insulin resistance such as type II diabetes, there is differential sensitivity to insulin actions, therefore, although glucose transport in skeletal muscle is markedly inhibited, the ability of insulin to suppress IGFBP1 synthesis in the liver is retained. Chronic caloric restriction also results in

sustained IGFBP1 increases and modest caloric restriction for period of two years resulted in the sustained 21% increase in IGFBP1 (Fontana *et al.* 2016). The suppression of IGFBP1 that occurs postprandially is not entirely due to insulin suppression of hepatic synthesis. Specifically ingestion of fructose, which induces minimal changes in insulin secretion results in a 35% reduction in IGFBP1 suggesting that stimulation of glycolysis also results in suppression of hepatic synthesis (Snyder & Clemmons 1990). This reduction in serum IGFBP1 in response to carbohydrate ingestion results in an increase in free IGF1 thereby enabling it to enhance insulin-stimulated glucose transport. The converse is also true since infusion of a high concentration of IGFBP1 into normal rats resulted in a modest rise in plasma glucose (Lewitt & Baxter 1991). In addition to regulating IGFBP1 concentrations, glucose and insulin regulate IGFBP1 phosphorylation (Nedić *et al.* 2011). Since phosphorylation increases the affinity of IGFBP1 for IGF1, this biochemical change regulates free IGF1 concentrations. IGFBP1 is phosphorylated by casein kinase-II, which is responsive to dietary manipulations such as leucine deprivation (Seferovic *et al.* 2009). Overexpression of IGFBP1 in transgenic mice was shown to attenuate the glucose-lowering effects of IGF1 and insulin. This was shown to be mediated in both skeletal muscle and adipose tissue (Crossey *et al.* 2000). The mice compensated by becoming hyperinsulinemic but elderly animals that could not mount an insulin response and had increased basal and postprandial glucose concentrations. This change was dependent upon IGFBP1 phosphorylation since transgenic overexpression of human IGFBP1, which could not be phosphorylated in mice did not result in changes in insulin or glucose (Sakai *et al.* 2001). Therefore, several studies support the conclusion that phosphorylated IGFBP1 regulates insulin responsiveness.

In type I diabetes, the absence of insulin results in marked increases in IGFBP1. Untreated type I diabetics had a 7-fold elevation, which decreased rapidly after insulin therapy (Bereket *et al.* 1995b). Not surprisingly, IGFBP1 levels fluctuate significantly in the subjects as a function of insulin dosage and insulin resistance. A study in Danish children showed that children at high risk of developing type I diabetes had higher IGFBP1 concentrations compared to controls and decreased residual beta cell function was associated with the degree of increase in IGFBP1 (Sorensen *et al.* 2015). A subset of type I patients with decreased methylation of the IGFBP1 gene had a greater increase in IGFBP1 (Gu *et al.* 2014).

IGFBP1 expression has been studied extensively in patients with type II diabetes. In older adults with modest glucose intolerance and relatively low IGFBP1 those who developed diabetes over a nine-year interval had a 57% increase in IGFBP1 at baseline as compared to a 25% increase in those who did not develop diabetes (Aneke-Nash *et al.* 2015). Because it is synthesized in the liver, IGFBP1 has been proposed as a specific marker of hepatic insulin sensitivity. Fasting IGFBP1 concentrations are inversely related to the liver fat content. Circulating IGFBP1 also correlates with hepatic insulin sensitivity (Kotronen *et al.* 2008). Obese subjects with impaired insulin sensitivity have significant reductions in IGFBP1 when compared to lean subjects. In contrast subjects with type II diabetes and similar degrees of insulin resistance had normal IGFBP1 concentrations (Gokulakrishnan *et al.* 2012). Based on those studies several investigators have examined whether IGFBP1 levels can predict the development of type II diabetes. Lewitt *et al.* reported that middle-age men with IGFBP1 in the lowest quartile as compared to those in the highest quartile had a 40-fold greater risk of developing type II diabetes after 10 years (Lewitt *et al.* 2008). A follow-up study in 664 nondiabetic subjects showed those in the lowest quartile at baseline had a 12.6% chance of developing type II diabetes in 7 years, whereas for those in the highest quartile it was 1.5% (Petersson *et al.* 2009). Therefore, the hazard ratio was 3.54. An additional study analyzed the relationship between fasting IGFBP1 and waist circumference in predicting the development of type II diabetes. Following an eight-year interval, subjects in the highest quartile for waist circumference and the lowest tertile for IGFBP1 all developed type II diabetes (Lewitt *et al.* 2010). These findings have been independently confirmed. Strickler *et al.* demonstrated that follow-up data from the nurses' health study showed fasting IGFBP1 levels predicted the risk with an odds ratio of 2.05/1, and this correlated with the ability of free IGF1 levels to predict diabetes development (Rajpathak *et al.* 2012). Recently, it was noted that obese pregnant women with low IGFBP1 levels at 24–28 weeks were more likely to develop gestational diabetes (Ramirez *et al.* 2014).

Part of the mechanism of suppression of IGFBP1 in obesity and insulin-resistant states may relate to gene methylation. IGFBP1 DNA methylation levels were higher in newly diagnosed type II diabetics compared to control subjects with normal glucose tolerance and methylation was shown to reduce IGFBP1 secretion (Gu *et al.* 2013). Whether IGFBP1 plays a causal role in type 2 diabetes development has not been determined. One study

showed that pioglitazone improves insulin sensitivity by inhibiting IGFBP1 synthesis thereby increasing free IGF1 and the change in IGFBP1 in subjects with established type II diabetes begun on pioglitazone correlates with a change in adiponectin (Arnetz *et al.* 2014). IGFBP1 was shown to increase beta cell regeneration by promoting alpha to beta cell transdifferentiation in mouse and human islets (Lu *et al.* 2016). The authors speculated that this contributed to the propensity of low IGFBP1 to predict the development of type II diabetes over time.

In summary, these findings suggest that suppression of IGFBP1 in response to obesity reduces insulin sensitivity leading to chronic impairment of glucose tolerance and possibly contributing to the development of type II diabetes.

IGFBP2

In contrast to IGFBP1, IGFBP2 is much less acutely responsive to changes in glucose and insulin concentrations. Specifically, following insulin infusion into rats, IGFBP2 levels decreased slowly compared to IGFBP1 and fasting results in a slow incremental increase in IGFBP2 that occurs over 36–48 h as compared to the acute change in IGFBP1 (Clemmons *et al.* 1991, Ooi *et al.* 1993). Although insulin can suppress IGFBP2 transcription, it is much less effective compared to IGFBP1. Thus, IGFBP2 levels appear to correlate well with fat mass and insulin sensitivity over prolonged periods but are not altered by acute fluctuations in insulin secretion that occur postprandially. Following nine days of fasting, the substantial decrease in insulin concentrations was associated with only a 1.7-fold increase in plasma IGFBP2 (Ooi *et al.* 1993). IGFBP2 levels are also very high in patients with anorexia nervosa (Argente *et al.* 1997). Extreme exercise has been shown to increase IGFBP2 (Berg *et al.* 2008). Nevertheless, IGFBP2 does play a role in postprandial glucose clearance since transgenic mice overexpressing a form of IGFBP2 in which the RGD sequence was mutated showed reduced glucose clearance as compared animals overexpressing wild-type IGFBP2, and this was associated with reduced levels of glucose transporters in the plasma membrane of muscle (Reyer *et al.* 2015). Prolonged fasting activates PPAR alpha, which binds to a PPAR-responsive element in the IGFBP2 promoter and upregulates IGFBP2 transcription (Kang *et al.* 2015). Additionally Kang *et al.* showed that metformin stimulates IGFBP2 expression through increases in PPAR alpha, and this response required activation of AMP kinase (Kang *et al.* 2016). As noted previously administration of leptin to insulin-resistant,

leptin-deficient mice resulted in stimulation of IGFBP2, and hyperinsulinemic clamp studies in these animals showed that IGFBP2 administration improved hepatic insulin sensitivity (Hedbacker *et al.* 2010).

During clamp studies in humans, high-dose insulin infusion decreased IGFBP1, but it increased IGFBP2 resulting in a decrease in IGF bioactivity. Basal IGFBP2 correlated with insulin sensitivity and was lower in those with impaired glucose tolerance (Arafat *et al.* 2009). A study of 294 subjects showed that a longitudinal increase in IGFBP2 was associated with an increase in HDL cholesterol in patients with established type II diabetes (Narayanan *et al.* 2014). Following initiation of very low calorie diets in women with type II diabetes and obesity, there was an increased expression of IGFBP2 in fat accompanied by an increase in plasma IGFBP2 concentrations (Touskova *et al.* 2012). IGFBP2 has also been compared to IGFBP1 for its ability to predict the development of type II diabetes. The risk of developing diabetes was increased 5-fold when IGFBP2 levels in the lowest quintile versus the top quintile in the nurse's health study as compared to a 2:1 risk ratio for IGFBP1 suggesting that it was a better predictor (Ramirez *et al.* 2014). When patients with untreated type I diabetes were compared to lean BMI-matched controls, there was no acute decrease in IGFBP2 levels during insulin therapy. In contrast, patients with reduced serum bicarbonate at presentation had a twofold elevation in IGFBP2, which returned to normal with insulin therapy (Bereket *et al.* 1995a).

In summary, high concentrations of IGFBP2 predict resistance to the development of diabetes. This has been shown in both obese, insulin-resistant, nondiabetic subjects followed prospectively over time as well as subjects with gestational diabetes. However, the molecular mechanism by which low levels of IGFBP2 predispose to diabetes has not been discerned. Furthermore, whether IGFBP2 has any direct effects on insulin-stimulated glucose transport is unknown. Although there is an associative relationship between changes in insulin sensitivity and changes in IGFBP2 whether IGFBP2 is directly affecting any of these properties or whether its ability to alter free IGF1 concentrations is contributing to changes in insulin sensitivity has not been determined.

IGFBP3

Similar to IGFBP2, there are minimal data regarding direct effects of IGFBP3 on glucose uptake or hepatic glucose output. IGFBP3 was shown to directly inhibit cytokine-induced insulin resistance in human aortic endothelial

cells (Mohanraj *et al.* 2013) but IGFBP3 knockout mice showed normal plasma insulin and glucose values (Ning *et al.* 2006, Yakar *et al.* 2009). Nevertheless, after high-fat feeding they developed fasting hyperinsulinemia and hyperglycemia but post prandial glucose tolerance was normal as was the response to a hyperinsulinemic clamp (Yamada *et al.* 2010). Clamp studies showed this change was not due to a change in insulin sensitivity but rather increased hepatic glucose output. In contrast, the IGFBP3, 4, 5 combined knockout animals had lower fasting glucose and enhanced insulin sensitivity (Ning *et al.* 2006). This change was replicated in ALS-knockout mice suggesting that the ternary complex of IGF, IGFBP3/5 and ALS is related to insulin sensitivity (Yakar *et al.* 2009). Transgenic mice overexpressing IGFBP3 developed glucose intolerance, and this effect was IGF1 independent (Silha *et al.* 2002). Mice showing the greatest increase in IGFBP3 developed fasting hyperglycemia and hyperinsulinemia. Aging male transgenic IGFBP3 mice show no change in fat mass but developed worsening glucose tolerance compared to controls (Nguyen *et al.* 2015b).

Several investigators have examined whether IGFBP3 levels like IGFBP1 and 2 would be useful in predicting the development of type II diabetes. Rajpathak *et al.* noted that IGFBP3 was positively associated with a trend to develop diabetes with an odds ratio of 2.0 and that the same group of subjects showed a relationship between free IGF1 values and diabetes development after 9 years (Ramirez *et al.* 2014). Other investigators have focused on the ratio between IGF1 and IGFBP3. Drogan *et al.* demonstrated that IGFBP3 was positively associated with development of type II diabetes but the ratio of IGF1/IGFBP3 was a stronger predictor of a trend (Drogan *et al.* 2016). The IGF1/IGFBP3 ratio also correlates with gestational diabetes development risk (Zhu *et al.* 2016). Aneke-nash *et al.* noted that older individuals with diabetes had a greater reduction in IGFBP3 over nine years as compared to nondiabetic controls (Aneke-Nash *et al.* 2017). Patients with severe type I diabetes and insulin deficiency have increased IGFBP3 proteolysis resulting in marked decreases in intact plasma IGFBP3 (Zachrisson *et al.* 2000). Two studies in type 1 diabetics have demonstrated a correlation between hemoglobin A-1C and IGFBP3 suggesting that it is related to glucose regulation (Kim & Lee 2015). Patients with severe type I diabetes who have insulin administered via intraperitoneal infusion show substantial increases in IGF1 and IGFBP3 that are greater than subjects receiving a subcutaneous infusion suggesting that portal delivery of insulin inhibits proteolysis (van Dijk *et al.* 2016). Palau *et al.* postulated that fat around the pancreas secretes

IGFBP3 to regulate beta cell proliferation. Specifically the adipose depot secreted IGFBP3 and blocking secretion or activation of the IGF1 receptor inhibited beta cell proliferation (Palau *et al.* 2012). Coverley *et al.* showed that casein kinase-II phosphorylates IGFBP3, and this induces resistance to proteolytic cleavage (Coverley *et al.* 2000). Whether this increased stability of phosphorylated IGFBP3 could preserve some beta cell function and residual insulin secretion in patients with type I diabetes has not been analyzed. Administration of IGFBP3 with IGF1 to patients with either type I or type II diabetes has been shown to improve insulin sensitivity and glucose regulation (Clemmons *et al.* 2000, 2005, Saukkonen *et al.* 2006). Whether this is mediated through direct effects of IGFBP3 on beta cell function or indirect effects on insulin sensitivity has not been determined.

In summary, IGFBP3 being the major carrier of IGF1 tends to reflect changes that occur in IGF1. In type I diabetes, the extent to which this alters IGF1 action has not been definitively determined. High concentrations of IGFBP3 apparently alter hepatic glucose output but the mechanism by which this occurs is undefined. IGFBP3 concentrations in obese insulin-resistant subjects have some value for predicting the subsequent development of type II diabetes but are less robust markers than IGFBP1 and 2.

IGFBP4

Unlike IGFBP1-3, IGFBP4 has been analyzed primarily at the tissue level in experimental animals with diabetes. Analysis in diabetic rats showed that IGFBP4 mRNA was significantly decreased in skeletal muscle and liver after the induction of diabetes (Chen & Arnqvist 1994). IGFBP4-knockout mice did not have altered glucose metabolism; however, the triple knockout of IGFBP3,4,5 showed significantly increased insulin secretion after glucose challenge but the extent to which this was due to IGFBP4 was not determined. IGFBP4 expression is increased in the presence of hyperglycemia in skeletal myoblasts in culture, and this inhibits myoblast differentiation in response to IGF1 (Grzelkowska-Kowalczyk *et al.* 2013). Han *et al.* studied IGFBP4 serum concentrations in diabetic rats and found minimal change (Han *et al.* 2006). Humans with type I and type II diabetes and showed no difference in serum IGFBP4 when compared to control subjects; however, patients with macroalbuminuria had higher IGFBP4 levels compared to those who did not have this complication (Jehle *et al.* 1998).

IGFBP5

IGFBP5-knockout animals did show mildly impaired glucose tolerance but no change in insulin resistance. Deletion of IGFBP5 expression also resulted in increased adiposity when the animals were fed a high-fat diet and in the absence of IGFBP5, these animals exhibited glucose intolerance (Gleason *et al.* 2010). Bach *et al.* implicated IGFBP5 in diabetic nephropathy progression. They noted that treatment with amino guanidine, which delays the development of diabetic nephropathy in experimental animals resulted in 75% reduction in IGFBP5 expression; however, they did not directly link this to alteration in kidney function (Bach *et al.* 2000). Giannini *et al.* noted that endothelial cells derived from micro vessels of diabetic patients preferentially expressed IGFBP2 and 5 and that adding IGFBP5 with IGF1-stimulated the growth of these cells (Giannini *et al.* 2001). They speculated that it might be involved in diabetic retinopathy. Arnqvist *et al.* using large vessel endothelium showed that the VEGF a major stimulant of retinopathy stimulated IGFBP5 expression by the cells (Dahlfors & Arnqvist 2000). In contrast, *in vitro* studies in beta cells suggest that IGFBP5 may stimulate their growth. Gleason *et al.* utilized beta cells transfected with a constituency active form of AKT. They analyzed genes that were preferentially synthesized by proliferating beta cells and noted that IGFBP5 was induced significantly (Gleason *et al.* 2010).

Human studies have shown that IGFBP5 levels are significantly reduced in both type I and type II diabetic patients and that the degree of reduction correlates with reduction in total IGF1 concentrations (Jehle *et al.* 1998). Interestingly, IGFBP3 exhibited the same relationship, but there was a negative correlation with IGFBP1 and 2. These findings suggest that the ternary complex components IGF1, IGFBP3 and IGFBP5 are reduced in the presence of diabetes and that these effects are dissociated from the changes in tissue IGFBP5 expression. Studies in humans have shown SNPs that alter IGFBP5 expression are associated with changes in adiponectin concentrations and that IGFBP5 concentrations correlate with adiponectin in women (Kallio *et al.* 2009). Similarly, analysis of candidate biomarkers for the development of gestational diabetes in pregnancy showed that IGFBP5 was one of four proteins with a high positive predictive value (Zhao *et al.* 2017).

IGFBP6

IGFBP6 concentrations are elevated in patients with type I diabetes and the degree of increase correlates with the

presence of complications. Patients in the upper quartile were 1.7 times more likely to develop complications than those in the lowest quartile (Sharma *et al.* 2016).

In summary, IGFBP concentrations are modulated significantly by changes in carbohydrate metabolism. These changes are mediated at the level of protein synthesis and secretion as well as posttranslational modifications and proteolysis. These changes can be secondary to changes in insulin secretion, insulin action or hyperglycemia. The IGFBPs definitely modulate IGF1's ability to alter insulin sensitivity and target cell responsiveness. Definitive determination of the role of the IGFBPs in directly modulating carbohydrate metabolism will require a much more in-depth understanding of how they function to alter cellular responsiveness.

Atherosclerosis

IGFBP1

Analysis at the cellular level showed that IGFBP1 can enhance or inhibit the effects of IGF1 on VSMC proliferation. The phosphorylation status of IGFBP1 is a major determinant of the response. Minimally phosphorylated IGFBP1 enhances the ability of IGF1 to stimulate proliferation, whereas heavily phosphorylated IGFBP1 binds to IGF1 and inhibits its interaction with the receptor, thus inhibiting proliferation (Jones & Clemmons 1995). Human studies suggest that IGFBP1 expressed in lesions can modify atherosclerosis. Gene expression profiles in 164 human carotid plaques demonstrated that IGFBP1 expression was significantly increased compared to unaffected artery, whereas IGF1 expression was unchanged (Wang *et al.* 2012). IGFBP1 expression was localized to macrophages and smooth muscle cells within these lesions. Expression correlated with IL-1 and IL-6, inflammatory markers whose expression is increased in atherosclerosis. The phosphorylation status was not measured but since expression was increased and phospho IGFBP1 has consistently been shown to inhibit IGF1 action. It is reasonable to assume most of the IGFBP1 in the carotid lesions was not phosphorylated. When plasma concentrations of the different phosphorylated forms of IGFBP1 were measured in 75 subjects, 36 of whom had ischemic heart disease, there was a correlation between low phosphate IGFBP1 and a predisposition to coronary artery disease risk (Borai *et al.* 2010). The ratio of non-phosphorylated to phosphorylated IGFBP1 was increased 2.5-fold in IHD and receiver-operator characteristic analysis showed a correlation of 0.75 for the

non-phosphorylated form. These findings are not consistent with the change in total serum IGFBP1 that is suppressed in obese, insulin-resistant subjects who are at increased risk for atherosclerosis (Gibson *et al.* 1996, Katz *et al.* 2016). Overexpression of IGFBP1 in obese mice reduced blood pressure and improved insulin sensitivity as well as insulin-stimulated nitric oxide generation. When these mice were crossed with an APOE^{-/-} mice there was reduced development of atherosclerosis (Rajwani *et al.* 2012). The phosphorylation status of IGFBP1 was not measured in this study, but the results suggest that overexpression resulted in a high concentration of heavily phosphorylate IGFBP1, which would be expected to inhibit the effects of IGF1 on lesion development. Total plasma IGFBP1 levels were positively associated with increased carotid intimal medial thickness in normal weight, healthy adults, but the association was negative in obese type 2 diabetic patients (Leinonen *et al.* 2002, Boquist *et al.* 2008). This suggests that defective insulin action may influence the results. Part of the explanation may be that plasma IGFBP1 is derived from endothelium rather than macrophages or smooth muscle cells. The protein-related transcriptional enhancer factor-1 (RTEF-1) is an important regulator of angiogenesis (Rubinow & Bornfeldt 2012). Under normal circumstances, insulin represses the transcription of RTEF-1. RTEF-1 binding to endothelial cells augments IGFBP1 expression. Mice in which RTEF-1 was knocked down in endothelial cells exhibited decreased serum IGFBP1 and had increased insulin resistance during high-fat feeding. RTEF-1 overexpression was associated with enhanced insulin sensitivity in response to high-fat feeding and upregulation of IGFBP1. Therefore, loss of RTEF-1 in insulin-resistant states could contribute to lowering IGFBP1. Unfortunately, the phosphorylation status of IGFBP1 was not documented in this study (Messmer-Blust *et al.* 2012).

In contrast to the long-term follow-up studies wherein low serum IGFBP1 concentrations correlate inversely with increased cardiovascular risk markers, measurement of IGFBP1 during acute coronary events shows that high levels predict increased cardiovascular morbidity and mortality. A recent study of 67 patients with unstable angina showed the absolute value of IGFBP1 predicted critical CAD with the specificity of 0.93. When combined with HDL values, the sensitivity was 0.82. The phosphorylation status was not determined (Zheng *et al.* 2017). Similarly, high IGFBP1 predicted increased risk of mortality in elderly women with the hazard ratio of 3/1 when compared to low IGFBP1 values (Maggio *et al.* 2013). IGFBP1 after oral glucose is a reflection of insulin sensitivity, and this

value correlates directly with increased cardiovascular risk and mortality in elderly nondiabetic adults (Kaplan *et al.* 2017). In summary, changes in total IGFBP1 (and possibly the non-phosphorylated form) within lesions predispose to lesion development. In contrast patients with multiple risk factors have low serum IGFBP1 but the degree to which this contributes to lesion development is unknown. Patients who develop vascular events have clear changes in IGFBP1 but a mechanism by which IGFBP1 leads to an increased incidence of events has not been identified.

IGFBP2

As for IGFBP1, IGFBP2 has direct effects on vascular smooth muscle. IGFBP2 binds RPTP β expressed on the surface of VSM and stimulates its polymerization resulting in enhanced PTEN tyrosine phosphorylation, thereby reducing its ability to inhibit AKT activation in response to IGF1 (Fig. 3) (Shen *et al.* 2012). These effects are IGF1 and IGFBP2 dependent and addition of IGFBP2 with IGF1 to VSMC stimulates proliferation. A study of 440 patients with peripheral vascular disease in the lower extremity showed that IGFBP2 was positively and significantly associated with peripheral vascular disease severity (Urbonaviciene *et al.* 2014). IGFBP2 concentrations were positively associated with a higher mortality rate including cardiovascular mortality even though the subjects had a better metabolic risk profile (van den Beld *et al.* 2012). A case-control study of 2023 subjects who had undergone coronary angiography showed that in comparison to multiple cardiovascular biomarkers, IGFBP2 was independently directly associated with myocardial infarction in elderly

Figure 3
Cooperative signaling by RPTP β and IGFBP2 receptor and IGF1. Signals generated by these two receptors converge to activate AKT.

adults (Halim *et al.* 2015). As for IGFBP1, there are conflicting epidemiological results for plasma IGFBP2 and atherosclerotic risk with several studies showing an inverse relationship (Ko *et al.* 2012, Carter *et al.* 2014, Narayanan *et al.* 2014). A recent study showed that IGFBP2 correlated inversely with pulse wave velocity and IGFBP2 was significantly lower in type II diabetics who had an increased risk for vascular disease (Olszanecka *et al.* 2017). An additional study showed that IGFBP2 correlated inversely with the presence of hypertension in perimenopausal women (Hjortebjerg *et al.* 2017). Overexpression of IGFBP2 in mice protected against obesity and increases in markers of atherosclerotic risk supporting the conclusion that low serum IGFBP2 would predispose to the development of risk factors (Wheatcroft & Kearney 2009).

In summary, it appears that locally secreted IGFBP2 has effects on vascular smooth muscle that are distinctly different from blood transported IGFBP2, which prior to lesion development appears to be negatively correlated with atherosclerotic risk factors. However, in later stages of lesion development, plasma IGFBP2 correlates directly with increased rates of myocardial infarction and cardiovascular mortality. These findings suggest that IGFBP2 has multi-functional effects on atherosclerotic lesion development and that more research is required to define the basic mechanisms mediating IGFBP2's effects on blood vessel cell types over time to discern the clinical implications.

IGFBP3

IGFBP3 has been extensively studied in epidemiologic analyses of atherosclerotic risk. Some *in vitro* studies support the hypothesis that IGFBP3 is functioning in vascular cells to inhibit IGF actions. In endothelium, IGFBP3 inhibits the ability of cytokines such as TNF to induce insulin resistance and early manifestations of atherosclerosis (Mohanraj *et al.* 2013). It also reduces ENOS and PKC zeta phosphorylation in vascular endothelium in diabetic animals (Zhang *et al.* 2015). However, in hyperlipidemic rabbits, there was increased IGFBP3 within developing lesions (Hirai *et al.* 2011). Studies in obese adolescents showed that IGFBP3 correlated directly with blood pressure, fasting insulin and C-reactive protein (Lam *et al.* 2010). Cross-sectional studies in adults show a positive correlation between IGFBP3 and total and LDL cholesterol and an inverse correlation with HDL (Kawachi *et al.* 2005, Lam *et al.* 2010, Eggert *et al.* 2014).

Several studies have examined the relationship between carotid atherosclerosis and serum IGFBP3 concentrations. Colao *et al.* showed that the IGFBP3 Z score and mean IMT were inversely correlated and that IGFBP3 Z scores and IGFBP3 was the second best predictor of IMT after age (Colao *et al.* 2005). This study was confirmed by Spilcke-Liss *et al.* who showed that after adjusting for confounding factors IGFBP3 was strongly associated with risk of increased IMT (Spilcke-Liss *et al.* 2011). In contrast, other studies analyzed hypertensive patients and found that IGFBP3 correlated positively with IMT and systolic blood pressure (Watanabe *et al.* 2003, Kawachi *et al.* 2005). Multiple logistic regression analysis revealed that IGFBP3 was positively associated with a 9 fold increase in risk for carotid plaque formation (Watanabe *et al.* 2003). Analysis by Kawachi *et al.* showed that IGFBP3 correlated with IMT independently of age, blood pressure and BMI (Kawachi *et al.* 2005). IGFBP3 is also associated with plaque instability and the relative risk ratio is 1.38 (Martin *et al.* 2008). In contrast, Kaplan *et al.* reported that IGFBP3 was inversely associated with a higher risk of nonfatal myocardial infarction (Kaplan *et al.* 2007). In studies analyzing patients with severe peripheral vascular disease, IGFBP3 concentrations were significantly lower compared to those with less severe disease (Brevetti *et al.* 2008).

Three studies have analyzed the relationship between IGFBP3 and outcomes. Low IGFBP3 levels predicted an unfavorable stroke outcome (Ebinger *et al.* 2015) and increased all-cause mortality (HR1.57) (Yeap *et al.* 2011). This was confirmed by Frederich *et al.* who showed that low IGFBP3 levels predicted increased cardiovascular mortality (HR 1.92) (Friedrich *et al.* 2009). In summary, high levels of serum IGFBP3 correlate with worsening metabolic profile as well as the early development of carotid atherosclerosis. In contrast, low IGFBP3 levels correlate with subsequent myocardial infarction and worse functional outcomes following vascular events. One mechanism by which low IGFBP3 might lead to worsening outcomes is the change in IGF1/IGFBP3 ratio. Although IGF1 can stimulate smooth muscle cell proliferation, it inhibits apoptosis and plaque hemorrhage thus an increasing ratio could mediate this beneficial effect. However, none of these studies have measured bioavailable IGF1; therefore, it is not possible to determine if this might provide an explanation.

IGFBP4

IGFBP4 has generally been classified as a negative regulator of VSMC proliferation, but this effect is dependent upon

context. VSMC release an IGFBP4 protease that degrades it thereby nullifying its ability to inhibit atherogenesis. Infusion of a protease-resistant mutant into pigs showed that the proliferative response that occurs in response to wounding was significantly inhibited (Nichols *et al.* 2007). Similarly, transgenic overexpression of PAPP-A, a protease that degrades IGFBP4, in mice accelerated atherosclerotic lesion development (Conover *et al.* 2010). Knockdown of the protease resulted in some resistance to the development of lesions (Harrington *et al.* 2007). There is additional supporting data in primates wherein estrogen treatment significantly stimulated the synthesis of IGFBP4 (Eyster *et al.* 2014). Since estrogen is a protective factor the authors concluded that this may be a mechanism by which estrogen inhibits lesion formation. Epidemiologic studies showed that in type I diabetics serum concentrations of the N-terminal and C-terminal fragments of IGFBP4 were significantly higher in patients with increased cardiovascular mortality suggesting that ongoing proteolysis of IGFBP4 was a risk factor for lesion development (Hjortebjerg *et al.* 2015). An additional study noted that IGFBP4 fragments were increased in patients with type II diabetes who had abnormal carotid artery remodeling as determined by MRI (Hjortebjerg *et al.* 2017).

IGFBP5

At the cellular level, IGFBP5 associates with extracellular matrix and enhances VSMC proliferation in response to IGF1 (Jones *et al.* 1993b). In contrast if the intact protein is present in the medium in quantities greater than IGF1, it inhibits VSMC proliferation. These effects are due in part to the fact that the affinity of IGFBP5 for IGF1 is lowered by extracellular matrix binding thus allowing IGF1 to be in better equilibration with its receptor. Almquist *et al.* demonstrated that large vessel endothelial cells from diabetic animals expressed IGFBP5 and that its synthesis could be stimulated by angiotensin-II, TGF-beta and VEGF (Dahlfors & Arnqvist 2000). Levels of IGFBP5 expression in VSM were found to be higher in SHR rats as compared to WKY and these rats have increased VSMC proliferation (Lee *et al.* 2013). Treatment of WKY rats with IGFBP5 increased IGFBP5 expression in VSM and further stimulated VSM proliferation. Incubation with angiotensin-II or IGF1 increased IGFBP5 transcription and siRNA transfection reduced VSMC proliferation (Lee *et al.* 2013). Pitavastatin inhibited IGFBP5 production in parallel with decreasing proliferation and migration (Ha *et al.* 2015).

Epidemiologic studies showed that high concentrations of IGFBP5 were present in 95 male patients with CAD as compared to age-matched controls who did not have CAD (Fischer *et al.* 2004). Following multi-variant analysis, IGFBP3, 5 and IGF1 were associated with the presence of CAD. Therefore, all of these studies support the conclusion that IGFBP5 is functioning to enhance IGF1's anabolic effects on VSMC.

Bone metabolism

The role of IGFBPs in regulating bone metabolism has been analyzed directly *in vitro* using osteoblast and osteoclast cultures, in genetically manipulated mouse models and indirectly in human studies. In general, *in vitro* studies have demonstrated that if an IGFBP is added in excess of IGF1 or II, their effects on osteoblast proliferation can be inhibited. Similar results have been generated for some forms of IGFBPs in inhibiting osteoblast differentiation and some forms have been shown to alter osteoclast differentiation. *In vivo* studies have been conducted using animals in which a form of IGFBP is overexpressed or knocked down and some studies have utilized direct injection of IGFBPs into bone or bone matrix.

IGFBP1

In vitro analysis showed that IGFBP1 was produced by human osteoblasts and that its production was stimulated by glucocorticoids and inhibited by insulin (Okazaki *et al.* 1994). Since glucocorticoids negatively regulate osteoblast division and mineralization, this was interpreted to mean that IGFBP1 was inhibiting the actions of IGF1. Similarly adding IGFBP1 to growth plate chondrocytes resulted in inhibition of cellular proliferation (Kiepe *et al.* 2002). During hypoxemia, which inhibits mineralization, IGFBP1 was induced significantly, and this was mediated by HIF α -1.

In heterochromatin-1-knockout mice that have significant dwarfism, there is a fourfold increase in IGFBP1 suggesting that it is inhibiting IGF1 stimulation of bone size (Garfinkel *et al.* 2015). Forced overexpression of IGFBP-1 in transgenic mice has given variable results; however, significant overexpression resulted in growth retardation and delayed mineralization (Kadaba *et al.* 1995). A recent paper demonstrated that FGF-21, which induces bone loss in experimental animals increased IGFBP1 production by the liver and administration of an anti-IGFBP1 antibody attenuated the bone loss that was induced by FGF-21 (Wang *et al.* 2015).

Human studies showed that serum levels of IGFBP1 were increased 4.1-fold in patients with idiopathic osteoporosis, and this occurred concomitantly with a 73% decrease in free IGF1 (Jehle *et al.* 2003). Analysis of IGFBP1 in IUGR twins showed increased levels correlated positively with markers of bone resorption and negatively with PINP a marker of bone formation (Bajoria *et al.* 2006). Analysis of the femoral neck bone mineral density (BMD) in elderly Swedish men showed a negative correlation with bone mineral density ($R=-0.37$) (Gillberg *et al.* 2002). Similarly IGFBP1 elevations were associated with increased fracture risk in 351 elderly women ($HR=1.46$) (Lundin *et al.* 2016). Bone histomorphometry analysis in 51 men with idiopathic osteoporosis showed that osteoid thickness correlated with the IGF1/IGFBP1 ratio. However, IGFBP1 was not shown to be an independent predictor of BMD in two other studies (Jassal *et al.* 2005, Pye *et al.* 2011). Taken together, the results support the conclusion that high serum IGFBP1 concentrations inhibit bone acquisition.

IGFBP2

Analysis of dental pulp cells showed that during differentiation IGFBP2 is upregulated and studies that analyzed temporal expression showed that abundant amounts were present in mid-differentiation (Alkharobi *et al.* 2016). Palermo *et al.* demonstrated that IGFBP2 plus IGF2 stimulated osteoblast differentiation as assessed by induction of alkaline phosphatase (Palermo *et al.* 2004). IGFBP2 undergoes proteolytic cleavage in extracellular fluids and a C-terminal fragment containing amino acids 167–289 stimulated proliferation of growth plate chondrocytes (Kiepe *et al.* 2015). Other studies show that if IGFBP2 associates with extracellular matrix and an equimolar concentration of IGF2 is added, IGFBP2 potentiates the effect of IGF2 on osteoblast differentiation (Conover & Khosla 2003). Additional studies suggest that IGFBP2 also stimulates osteoblast precursors. Expression of IGFBP2 in inducible brown adipose tissue enhanced osteoblast function and its depletion resulted in decreased osteoblast activity (Rahman *et al.* 2013). IGFBP2 interacts with the alpha 5 integrin in mesenchymal stem cells to stimulate osteogenic differentiation (Hamidouche *et al.* 2010). The anabolic role of IGFBP2 in osteoblast differentiation has recently been confirmed in both MC-3T3 cells and primary calvarial osteoblast cultures and its effects are mediated through induction of AMP kinase (Xi *et al.* 2016). Stimulation of differentiation is mediated

through the cell surface receptor RPTP β and concomitant engagement of RPTP β and the IGF1 receptor enhances the response (Shen *et al.* 2015).

Studies in animals have confirmed these findings. The infusion of equimolar concentrations of IGF2 and IGFBP2 to rats using the hindlimb suspension model showed that this complex stimulated bone formation (Conover *et al.* 2002). Similarly, ovariectomy, which is associated with loss of bone mineral, results in decreased serum IGFBP2 (DeMambro *et al.* 2015). Paradoxically, overexpression of IGFBP2 alone is associated with decreased BMD; however, under those circumstances, there is no concomitant increase in IGF1 and *in vitro* models wherein excess IGFBP2 is added without IGF1 show inhibition of osteoblast proliferation and ECM synthesis (Hoeflich *et al.* 1999). The mechanism underlying the requirement of concomitant exposure to IGF1 for IGFBP2 to be anabolic has been delineated. The ability of RPTP β to inactivate PTEN requires stimulation of the IGF1 receptor. This effect requires RPTP β binding to phosphorylated vimentin and IGF1 receptor activation stimulates vimentin phosphorylation (Fig. 3) (Shen *et al.* 2015). Therefore, failure to engage the IGF1 receptor results in refractoriness to IGFBP2 stimulation. The anabolic role of IGFBP2 has received further support from gene deletion studies. Knockdown of IGFBP2 yielded an important phenotype that was distinctly different from that noted with IGFBP2 overexpression (DeMambro *et al.* 2008). These mice had a 40% reduction in trabecular BMD at age 16 weeks and decreased femur length as well as decreased osteocalcin expression. Subsequent studies showed that IGFBP2 stimulated osteoblast proliferation in IGFBP2 $-/-$ mice, and it stimulated differentiation of preosteoblasts derived from these mice *in vitro* (Kawai *et al.* 2011).

Serum IGFBP2 concentrations peak during puberty concomitantly with IGF1 at the time of the maximal growth spurt. In contrast, major increases in IGFBP2 in the absence of a concomitant increase in IGF1 that occur in normal aging are associated with increases in bone turnover and bone loss (Amin *et al.* 2007). Very high IGFBP2 concentrations that occur in normal adults over 75 years correlate inversely with decreased BMD in the proximal femur (van den Beld *et al.* 2012). Other studies showed older patients with osteoporosis had an inverse correlation between IGFBP2 and BMD (Lebrasseur *et al.* 2012). Importantly, most of these studies did not analyze IGF1 and IGFBP2 as independent variables. Similarly, in patients with anorexia nervosa who have very low levels of serum IGF1, there is an inverse relationship between

IGFBP2 and BMD (Grinspoon *et al.* 2003). Since their actions are interdependent, the results clearly support the conclusion that IGFBP2 functions in bone are IGF1 dependent.

IGFBP3

Growth plate chondrocytes and primary osteoblasts secrete IGFBP3 (Malpe *et al.* 1997, Kiepe *et al.* 2005). Most *in vitro* studies support the hypothesis that IGFBP3 inhibits IGF1 stimulation of osteoblast proliferation. Specifically adding purified protein but not mutants that do not bind IGF1 inhibits cell division (Schmid *et al.* 1995). Additionally, culture conditions, such as hypoxia that stimulate IGFBP3, inhibit the differentiation of adipose-derived stem cells into osteoblasts (Kim *et al.* 2016). Conversely, other studies show that once osteogenic commitment has occurred agents such as TGF- β and vitamin D stimulate osteoblast differentiation via stimulation of IGFBP3 (Kveiborg *et al.* 2002). Recently matrix-associated IGFBP3 was found to localize IGF1 and the combination of matrix IGFBP3 plus IGF1 resulted in enhancement of IGF1-stimulated bone formation (Xian *et al.* 2012). This conclusion is consistent with *in vitro* studies, which showed that if IGFBP3 was added to osteoblasts then removed and IGF1 added subsequently, there was a greater response to IGF1 following pre-IGFBP3 incubation (Ernst & Rodan 1990).

In vivo studies show that if IGFBP3 is overexpressed it inhibits IGF1 action both in terms of osteoblast proliferation and bone formation (Silha *et al.* 2003). IGFBP3 overexpression was also found to inhibit BMP-2 stimulated differentiation *in vivo* (Zhong *et al.* 2011). In contrast, administration of recombinant IGF1 with IGFBP3 in equimolar concentrations improved osteopenia in rats following ovariectomy or bilateral sciatic neurectomy suggesting that the effect of overexpression of IGFBP3 alone was due to attenuation of IGF1 actions (Narusawa *et al.* 1995). An additional study showed this combination increased bone formation rate in lumbar vertebra of ovariectomized rats (Bagi *et al.* 1994). Direct injection of IGF1 plus IGFBP3 complex into bone resulted in localization of IGF1 in matrix and stimulated new bone formation as well as osteoblast differentiation (Xian *et al.* 2012). Mutagenesis of IGFBP3 resulting decreased matrix binding resulted in an inability of the injected IGF1 to enhance bone formation. Estrogen administration to ovariectomized monkeys induces IGFBP3 and administration of growth hormone increases IGFBP3

synthesis in bone in parallel with IGF1 (Ham *et al.* 2004, DiGirolamo *et al.* 2007). Similarly, PTH infusion enhances IGF1 induction of IGFBP3 and this response is attenuated in inflammatory conditions associated with decreased bone formation (Johansson *et al.* 1994).

Knockdown of IGFBP3 alone did not yield a specific bone phenotype however the combined knockdown of IGFBP3, 4 and 5 in mice reduced bone size but did not change BMD (Ning *et al.* 2006). This effect is likely mediated by reduction in the ternary complex of IGFBP3/5, ALS and IGF1. This conclusion is supported by studies which show that knockdown of ALS reduces the amount of IGF1 or in the ternary complex in serum and results in specific bone phenotype. ALS knockout mice have reduced bone size and BMD (Yakar *et al.* 2009). Administration of growth hormone does not increase growth or BMD, although there is a small increase in cross-sectional area in cortical bone (Kennedy *et al.* 2014). Ablation of ALS in mice over expressing growth hormone showed decreased skeletal size and trabecular bone (Liu *et al.* 2017).

Human studies have shown that postmenopausal women with increased risk of vertebral fractures have decreased IGFBP3 compared to women without fractures (Yamaguchi *et al.* 2006). Similarly postmenopausal women with low BMD had significantly lower levels compared to controls with normal BMD (Kim *et al.* 1999). Femoral neck osteoporosis was associated with lower levels of serum IGFBP3 however IGF1 and II were also reduced (Boonen *et al.* 1999). In older men higher levels of IGF1 and IGFBP3 were associated with higher BMD. In elderly man with idiopathic osteoporosis IGFBP3 was 46% lower than controls whereas, IGF1 levels were similar (Johansson *et al.* 1997). Pathophysiologic conditions such as chronic hepatitis C and inflammatory bowel disease are associated with decreased BMD and IGFBP3 (Raslan *et al.* 2010). Subjects that carry ALS mutations that reduce serum IGFBP3 have significantly lower BMD (Höglér *et al.* 2014).

In summary, systemic administration of IGFBP3, its enhanced production in bone or increased matrix association in the presence of adequate IGF1 is associated with a positive anabolic response. In contrast, conditions wherein there is high IGFBP3 and no concomitant change in IGF1 there is decreased bone formation as well as increased resorption. Therefore, the critical determinants of the response appear to be the stage of bone cell differentiation, the site of production, the ratio between IGF1/IGFBP3 and whether or not the IGFBP3 is associated with extracellular matrix.

IGFBP4

Analysis of osteoblasts *in vitro* has shown that these cells synthesize and secrete IGFBP4 (Amarnani *et al.* 1993). Initial studies concluded that IGFBP4 inhibited osteoblast proliferation and differentiation. Several factors that alter bone physiology including estrogen and vitamin D stimulate IGFBP4 synthesis (Scharla *et al.* 1993, Kassem *et al.* 1996). Factors that stimulate osteoblast differentiation such as, osteogenic protein-1 inhibit its synthesis (Yeh *et al.* 1997). Therefore it was concluded that IGFBP4 functions primarily as an inhibitor of differentiation and bone formation. Studies by Mohan and coworkers demonstrated that the addition of IGFBP4 *in vitro* inhibited IGF1 binding to its receptor and osteoblast proliferation (Mohan *et al.* 1995). Furthermore they showed that a protease secreted by osteoblasts cleaved IGFBP4 allowing IGF1 access to the receptor and alleviating IGFBP4 inhibition of IGF1-stimulated proliferation. They showed that IGFBP4 proteolysis was enhanced by the addition of IGF1 (Qin *et al.* 1999).

Transgenic overexpression of IGFBP4 specifically in osteoblasts resulted in marked inhibition of bone formation and global growth retardation (Zhang *et al.* 2003). Administration of IGFBP4 locally into bone in normal mice inhibited of IGF1 stimulated bone formation, however, when it was given systemically there was an increase in bone formation markers (Miyakoshi *et al.* 1999). A follow-up study revealed that systemic administration could increase bone formation parameters due to proteolysis of IGFBP4 since infusion of a protease-resistant form did not alter these markers (Miyakoshi *et al.* 2015). The role of proteolysis was further confirmed in transgenic mice overexpressing PAPP-A, a protease known to degrade IGFBP4. Bone formation was enhanced in these mice as was BMD (Clifton & Conover 2001). PAPP-A knockout mice had decreased bone size and BMD. In diabetic mice with severe osteopathy there is a significant decrease in serum IGF1 associated with an increase in intact IGFBP4 and reduced proteolysis which favors its sequestration (Ahmad *et al.* 2008). These findings suggest that IGFBP4 is sequestering IGF1 and eliminating its ability to stimulate osteoblastogenesis.

IGFBP4 deletion in mice results in a decrease in bone size however initial studies reported no change in BMD. A recent paper is revealed a complex mechanism by which IGFBP4 regulates skeletal growth in female mice during puberty (Maridas *et al.* 2017). This effect was sex specific. Both sexes showed reduce size at birth however between 8 and 16 weeks female mice showed a significant reduction

in the rate of acquisition of BMD compared to male mice. This effect was noted to be due to increased osteoclast number and osteoclastogenesis suggesting that IGFBP4 negatively regulates osteoclast number and activity in female mice. Notably the induction of IGFBP4 has been shown to be estrogen dependent therefore this may be an important function of IGFBP4 during normal female pubertal development.

Human studies have shown that IGFBP4 levels are increased in postmenopausal females but serum concentrations do not correlate with femoral neck BMD (Karasik *et al.* 2002). A second study demonstrated a correlation with PTH levels but again there was no significant correlation between increasing IGFBP4 during aging and decreasing BMD (Mohan & Baylink 1997). Additional studies demonstrated that growth hormone increased IGFBP4 and that at baseline IGFBP4 correlated with BMD but the change in IGFBP4 did not predict an increase in bone mass in response to growth hormone (Thorén *et al.* 1998). In summary, IGFBP4 directly inhibits IGF1 action on bone but high concentrations serve as a reservoir for IGF and exposure to proteases, such as PAPP-A, may result in high IGFBP4 concentrations being associated with an increase in osteoblast function.

IGFBP5

Several *in vitro* studies have proposed that IGFBP5 is an important trophic factor for bone. Analysis of aged osteoblast cultures showed that they produce less IGFBP5 as their regenerative capacity decreases (Kveiborg *et al.* 2000). Local application of IGFBP5 to mesenchymal stem cells in which IGFBP5 synthesis had been deleted restored osteoblast function and the ability to differentiate (Han *et al.* 2017). Treatment of osteoblasts with IGFBP5 *in vitro* enhanced alkaline phosphatase activity and cell proliferation (Andress & Birnbaum 1992). IGFBP5 expression was shown to be upregulated in growth plate chondrocytes during differentiation and overexpression rescued osteoblasts from apoptosis whereas exogenous addition facilitated cell death (Yin *et al.* 2004, Kiepe *et al.* 2006). IGFBP5 binds to hydroxyapatite and this localizes IGF1 in bone (Campbell & Andress 1997). A carboxyl terminal fragment of IGFBP5 that bound IGF1 with reduced affinity stimulated mitogenesis of osteoblasts and enhanced the response to IGF1 (Andress *et al.* 1993). Furthermore IGF1 itself stimulates IGFBP5 synthesis (Gabbittas & Canalis 1998). In contrast direct addition of IGFBP5 inhibited the ability of BMP -2 to induce osteoblast

differentiation and this effect was reduced if a mutant which bound IGF1 poorly was utilized (Mukherjee & Rotwein 2008). The addition of IGFBP5 *in vitro* was shown to inhibit vitamin D induced osteoblast differentiation (Schedlich *et al.* 2007). An additional study demonstrated that osteoblast-derived IGFBP5 negatively regulates bone accretion by stimulating osteoclast formation and activity (Kanatani *et al.* 2000). In part these paradoxical findings may be explained by the observation that IGFBP5 knockdown *in vitro* enhanced osteoblast apoptosis which was rescued by IGFBP5 induction but not by exogenous addition (Yin *et al.* 2004).

The importance of IGF1 stimulated IGFBP5 is emphasized by study in which heterozygous knockout IGF1 mice were found to have low IGFBP5 levels but following IGF1 administration this mRNA was specifically induced in osteoblasts. IGF1 also increased BMD and cortical thickness (Guerra-Menéndez *et al.* 2013). *In vivo* studies have shown that administration of equimolar amounts of IGF1 and IGFBP5 enhances cortical bone formation and BMD in mice (Bauss *et al.* 2001). Studies in IGF1 knockout mice showed that direct injection of IGFBP5 into bone stimulated calvarial bone mass accumulation (Miyakoshi *et al.* 2001). IGFBP5 binds avidly to extracellular matrix, therefore, it was postulated that the effects of direct injection are due to matrix association, thereby providing a slow release mechanism of IGF1. Administration of IGFBP5 to ovariectomized mice enhanced bone formation compared to OVX controls (Andress 2001). In contrast, osteoblast specific overexpression of IGFBP5 in transgenic mice resulted in a small degree of inhibition of bone mineral apposition that was transient (Devlin *et al.* 2002). In a separate experiment more intense overexpression that increased serum levels eight fold resulted in a 30% decrease in BMD (Salih *et al.* 2005). Knockdown of IGFBP5 did not result in a bone phenotype. However IGFBP5 knockdown was accompanied by an increase in IGFBP3 which could compensate for its loss in bone matrix as well as reduced ternary complex formation.

Human studies show that serum IGFBP5 declines with age and bone content decreases. Serum IGFBP5 correlates positively with femoral neck BMD. In osteoporotic patients there was a 52% reduction in IGFBP5 compared to controls (Jehle *et al.* 2003).

IGFBP6

IGFBP6 has been studied minimally in osteoblasts but it is believed to be a negative regulatory factor. Direct addition of IGFBP6 to osteoblast cultures inhibits osteoblast

differentiation and the protein decreases during osteoblast differentiation *in vitro* (Strohbach *et al.* 2008). Similarly cortisol which inhibits osteoblast differentiation stimulated IGFBP6 synthesis (Gabbitas & Canalis 1996). Analysis of nonunion fractures revealed that IGFBP6 synthesis was increased compared to control fractures and serum levels of IGFBP6 were increased 2.1 fold in adults with osteoporosis compared to age matched controls (Koh *et al.* 2011).

Skeletal muscle metabolism

IGFBP1

As for other body tissues both locally secreted IGFBPs and IGFBPs transported in the circulation regulate the development of muscle mass and its maintenance during pathophysiologic states. IGFBP1 is not synthesized in skeletal muscle but addition of IGFBP1 to myoblast cultures inhibits IGF1 stimulated protein synthesis (Frost & Lang 1999) and catabolic factors such as IL-1 stimulate IGFBP1 synthesis in liver in association with increased muscle breakdown (Cooney *et al.* 1999). Other catabolic stimuli such as TNF alpha IL-6, burn injury, alcohol and undernutrition stimulate IGFBP1 synthesis in liver resulting in accumulation of IGFBP1 in muscle (Fan *et al.* 1995, Gautsch *et al.* 1998, Lang *et al.* 2000, 2004). Infusion of IGFBP1 into rats inhibited basal and IGF1 stimulated muscle protein synthesis. Protein synthesis declined 20% when IGFBP1 increased 2–3 fold (Lang *et al.* 2003). This effect was muscle specific since protein synthesis rates did not decline in liver, kidney or heart. Transgenic overexpression was accompanied by reduced muscle mass and myostatin-knockout mice, which have increased muscle mass have suppressed IGFBP1 (Murphy 2000, Williams *et al.* 2011). Human studies show that serum concentrations correlate negatively with muscle mass. Older women who had higher IGFBP1 were catabolic and had low muscle mass compared to those who were obese and had suppressed IGFBP1 (Stilling *et al.* 2017).

IGFBP2

IGFBP2 is synthesized by both satellite cells (Ernst *et al.* 1996) and skeletal myoblasts (Rousse *et al.* 1998). High levels are present during proliferation and decline during differentiation (Rousse *et al.* 1998, Crown *et al.* 2000). Stewart and coworkers proposed that the actions of IGFBP2 were biphasic since addition of an IGFBP2 antibody early in the process facilitated differentiation but addition at a later phase inhibited differentiation (Sharples *et al.* 2013).

Overexpression of IGFBP2 in transgenic mice resulted in generalized growth inhibition. Administration of growth hormone to these mice resulted in less of an increase in muscle mass compared to controls (Rehfeldt *et al.* 2010). Muscle-specific overexpression was accompanied by a slower phenotype but no atrophy (Swiderski *et al.* 2016). This was associated with an increase in glycolytic metabolism. During pathophysiologic states such as the induction of diabetes or fasting in experimental animals expression of IGFBP2 in muscle decreases (Grzelkowska-Kowalczyk *et al.* 2013). In models of catabolic muscle wasting IGFBP2 expression is down regulated but upregulated during hypertrophy that occurs during the recovery phase after refeeding (Fuentes *et al.* 2015). Mechanical loading is associated with upregulation of IGFBP2 in association with IGF1 (Player *et al.* 2014). In elderly humans with reduced appendicular muscle mass and reduced BMD IGFBP2 is increased but these patients have low IGF1 therefore it is difficult to ascertain whether the effect is simply due to reducing free IGF1 further or a change in the IGF1/IGFBP2 complex (Amin *et al.* 2007, Lebrasseur *et al.* 2012). In summary, the studies support the conclusion that IGFBP2 can enhance IGF1 stimulated differentiation but the ratio between IGF1 and IGFBP2 ultimately determines the response.

IGFBP3

In vitro studies of IGFBP3 show that direct addition of IGFBP3 suppresses IGF1 stimulated myoblast proliferation but it also has effects that are IGF1 independent (Pampusch *et al.* 2003). Additionally WNT, a known inhibitor of cardiac muscle stem cell proliferation, exerts its inhibitory effect through induction of IGFBP3 (Oikonomopoulos *et al.* 2011). In contrast, Foulstone *et al.* reported that IGFBP3 synthesized by myoblasts in culture stimulated differentiation (Foulstone *et al.* 2003). *In vivo* IGFBP3 expression increases transiently in skeletal muscle during repair and after ischemic injury and these changes occur in muscle associated macrophages (Jennische & Hall 2000). IGF1 injection into muscle increases IGFBP3 synthesis in female rats but it decreased in males (Oliver *et al.* 2005). Administration of nandrolone, a potent anabolic, was associated with a 69% increase in IGFBP3 and a 50% increase in IGF1 (Lewis *et al.* 2002). Transgenic overexpression of IGFBP3 in mice did not increase muscle mass. When the complex of IGF1 and IGFBP3 was administered to rats made catabolic by hind limb suspension, it enhanced muscle mass accumulation and inhibited breakdown (Zdanowicz & Teichberg 2003).

Svanberg *et al.* showed that administration of this complex to nutritionally compromised rats increased muscle protein synthesis whereas administration of IGF1 alone did not (Svanberg *et al.* 2000). Booth *et al.* demonstrated that loading skeletal muscle enhanced IGFBP3 expression and aging was associated with a reduced expression response. Hind limb immobilization resulted in diminished IGFBP3 expression which increased during the recovery phase and these changes occurred primarily in type I fibers (Spangenburg *et al.* 2003).

Studies in humans show that normal diurnal variation in IGFBP3 correlates with changes in myogenic index and muscle fiber diameter (Burley *et al.* 2016). In resistance-trained athletes there is a greater increase in IGFBP3 if LVH was present compared to controls and this change in IGFBP3 was speculated to be related to the increased ventricular hypertrophy (Żebrowska *et al.* 2013). In Chinese elderly and IGFBP3 SNPs that resulted in lower serum IGFBP3 were associated with reduced skeletal muscle mass (Yang *et al.* 2015). Administration of the IGF1/IGFBP3 complex to elderly hip fracture patients improved muscle strength (Boonen *et al.* 2002). In summary, IGFBP3 appears to correlate with anabolic changes. Since excess IGFBP3 suppresses myoblast proliferation *in vitro* these changes are presumably due to concomitant increases in IGF1 which are necessary to promote skeletal muscle protein synthesis.

IGFBP4

IGFBP4 is expressed in skeletal muscle cells *in vitro* and it increases with differentiation (Ewton & Florini 1995). However forced overexpression inhibits differentiation (Damon *et al.* 1998). Direct addition to myoblast cultures inhibited IGF1 stimulated proliferation and differentiation (Ewton *et al.* 1998). *In vivo* studies in general have supported the conclusion that IGFBP4 inhibits IGF1 stimulated skeletal muscle proliferation and differentiation. IGFBP4 abundance was noted increase in muscle in aging mice (Oliver *et al.* 2005). Similarly nandrolone administration to rats which resulted in diaphragmatic hypertrophy decreased IGFBP4 expression by 40% (Lewis *et al.* 2002). Transgenic overexpression of PAPP-A which decreased intact IGFBP4 increased muscle mass in mice (Rehage *et al.* 2007). Protein restriction in rats during fetal development led to increased IGFBP4 expression in muscle (Bayol *et al.* 2004) and infusion of IL-6 which inhibits muscle growth during development increased IGFBP4 (Bodell *et al.* 2009). Denervation of muscle results in a rapid upregulation of IGFBP4 transcripts

(Bayol *et al.* 2000). In contrast, following skeletal muscle injury IGFBP4 expression was increased in connective tissue cells but not regenerating muscle cells during the period of rapid regeneration (Jennische & Hall 2000). Similarly the presence of diabetes or fasting which are catabolic resulted in decreased IGFBP4 expression which was restored with refeeding (Grzelkowska-Kowalczyk *et al.* 2013). Administration of clenbuterol which caused a 13% increase in soleus muscle mass in rats resulted in an 8 fold increase in IGFBP4 mRNA (Awede *et al.* 2002). Blocking Torc-1 activation in muscle which results in atrophy resulted in downregulation of expression of IGFBP4 (Fuentes *et al.* 2015). Skeletal muscle loading resulted in a 15% hypertrophy, a 60% increase in IGF1 and doubling of IGFBP4 mRNA (Awede *et al.* 1999). These results suggest that catabolic states increase IGFBP4 and decrease IGF1 whereas, during muscle hypertrophy there are increases in both proteins. These observations lead to the conclusion that IGFBP4 is anabolic in muscle if it is associated with a concomitant increase in IGF1 whereas, an increase in IGFBP4 alone results in a catabolic response.

Studies in humans have shown that the elderly have increases in serum IGFBP4 accompanied by declining IGF1 (Mohan & Baylink 1997, Karasik *et al.* 2002, Jehle *et al.* 2003). In contrast, testosterone injection increased IGF1 and IGFBP4 mRNA in skeletal muscle (Bamman *et al.* 2001). Exercise decreased in serum IGFBP4 concentrations but levels in muscle were not evaluated (Rosendal *et al.* 2002). Administration of prednisolone inhibited IGFBP4 proteolysis in muscle suggesting that free IGF1 release was impaired (Ramshanker *et al.* 2017). Based on the human studies it has been difficult to assign a definitive role for IGFBP4 in muscle cell hypertrophy. It has been proposed that increased IGFBP4 increases the supply of available IGF1 and if sufficient proteolytic activity is present this results in enhanced free IGF1 bioavailability and an anabolic response.

IGFBP5

IGFBP5 is expressed by skeletal myoblasts in culture and antisense RNA studies indicate that loss of IGFBP5 expression alters the timing of differentiation (Ewton *et al.* 1998). Expression in primary myoblasts increases during differentiation, however, there are conflicting data regarding its functional role in muscle. Over expression in cultured myoblasts inhibited myoblast differentiation and addition of exogenous IGF1 restored it. Antisense experiments suggested the main function was to properly time the response to IGF1 (James *et al.* 1996). Addition of

purified IGFBP5 inhibits differentiation but addition of IGF analogs that do not bind restores it (Pampusch *et al.* 2005, Mukherjee *et al.* 2008). Other investigators have noted that IGFBP5 is necessary for myoblast survival (Meadows *et al.* 2000). Ren and coworkers demonstrated that knockdown of IGFBP5 impaired mitogenesis and suppressed IGF2 expression (Ren *et al.* 2008). They determined that IGFBP5 had to bind IGF2 in order for differentiation to occur and in the absence of IGFBP5 there was minimal IGF2 expression. Exogenous addition of IGFBP5 to knockdown cells restored IGF2 production and differentiation. They proposed this feedback loop was required. This intricate requirement probably explains much of the conflicting data. Exposure of muscle cells to high glucose decreased IGFBP5 and inhibited differentiation (Grzelkowska-Kowalczyk *et al.* 2013). Inhibition of differentiation using TNF alpha could be partially rescued by omega-3 fatty acids which induced IGFBP5 expression (Saini *et al.* 2017).

In general *in vivo* studies have confirmed the *in vitro* observations. Dai *et al.* reported that IGFBP5 is expressed highly in myotomes during early development and suggested that it is important for early myogenic accumulation (Dai *et al.* 2009). Transgenic mice with a high level of IGFBP5 overexpression in early development had compromised muscle development (Salih *et al.* 2005). IGFBP5 knockout mice had no development abnormalities in muscle but compensatory changes in other IGFBPs were present (Ning *et al.* 2007). IGFBP3, 4, 5 knockout mice showed significant reduction in quadriceps muscle volume (Ning *et al.* 2006). During regeneration following injury there is increased expression of IGFBP5 in muscle (Jennische & Hall 2000). Cast immobilization significantly reduces IGFBP5 and reambulation is accompanied by an increase (Stevens-Lapsley *et al.* 2010). Sepsis downregulates IGFBP5 expression in muscle (Lang *et al.* 2006) as does malnutrition (Safian *et al.* 2012). In contrast mechanical loading decreases IGFBP5 expression but it increases IGF1 (Bodell *et al.* 2009, Payer *et al.* 2014). The results are consistent with the conclusion that local IGFBP5 generation in conjunction with IGF1 increase stimulates an anabolic response and that catabolic conditions reduce its expression. However the response to loading is not consistent with that model and suggests that following loading IGFBP5 inhibits the anabolic response to IGF1.

IGFBP6

IGFBP6 appears to regulate stem cell differentiation into muscle cells. Placental mesenchymal stem cells were forced to differentiate into muscle cells and the addition

of IGFBP6 enhanced their differentiation (Aboalola & Han 2017). Silencing IGFBP6 decreased differentiation suggesting an important role in muscle cell commitment. Both age and gender have been shown to regulate IGFBP6 expression by muscle and IGFBP6 was specifically decreased in females during aging in mice (Oliver *et al.* 2005). In models of regeneration following injury IGFBP6 was shown to be expressed in muscle cells during regeneration along with IGFBP5 (Jennische & Hall 2000).

Summary and conclusions

The IGFBPs function coordinately with the IGFs to regulate metabolic adaptations to changes in nutrition, stress and underlying disease states. Since the actions of the IGFs and insulin are interdependent, the IGFBPs indirectly influence insulin actions. Initially, viewed through the narrow focus of regulating IGF access to receptors the field of IGFBP research has been significantly expanded by new discoveries of IGFBP effects that are independent of their ability to bind IGFs. Many of these studies indicate that this area of investigation will provide explanations for cell type and tissue-specific actions of the IGFs and for a better understanding of how various tissue responses to these factors are coordinated. Additionally, since IGFBP synthesis and degradation can be regulated independently of the IGFs, some findings suggest that these experimental approaches will shed new light on the mechanism of how other important metabolic hormones that have major effects on metabolism, such as glucocorticoids or estrogen, influence IGF responsiveness. A major future need is for identification of the receptors and cellular proteins that bind to individual forms of IGFBPs and the signaling systems that are activated following these interactions. This information will be very useful in designing new therapeutic strategies for altering IGF actions in disease states. Overall, future IGFBP research is likely to continue to provide unique insights into understanding the role of this family of proteins in regulating the response to changes in the metabolic milieu.

Declaration of interest

Dr Clemmons is the guarantor of this review and as such has full access to all reported studies and takes full responsibility for the integrity of an accuracy of the review.

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