CURRENT PERSPECTIVES ON INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN (IGFBP) RESEARCH

EDITED BY: Andreas Hoeflich, John Pintar and Briony Forbes

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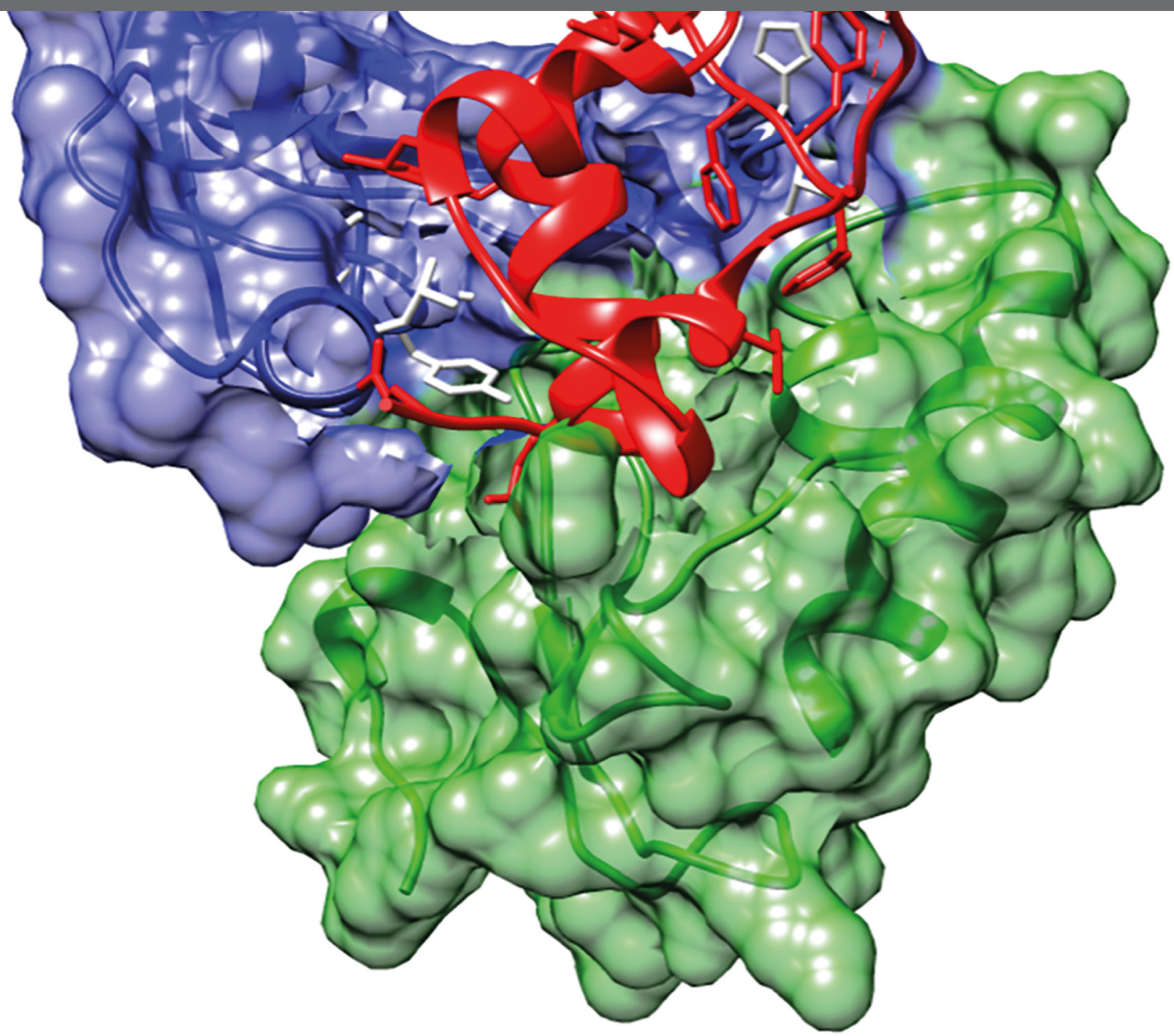

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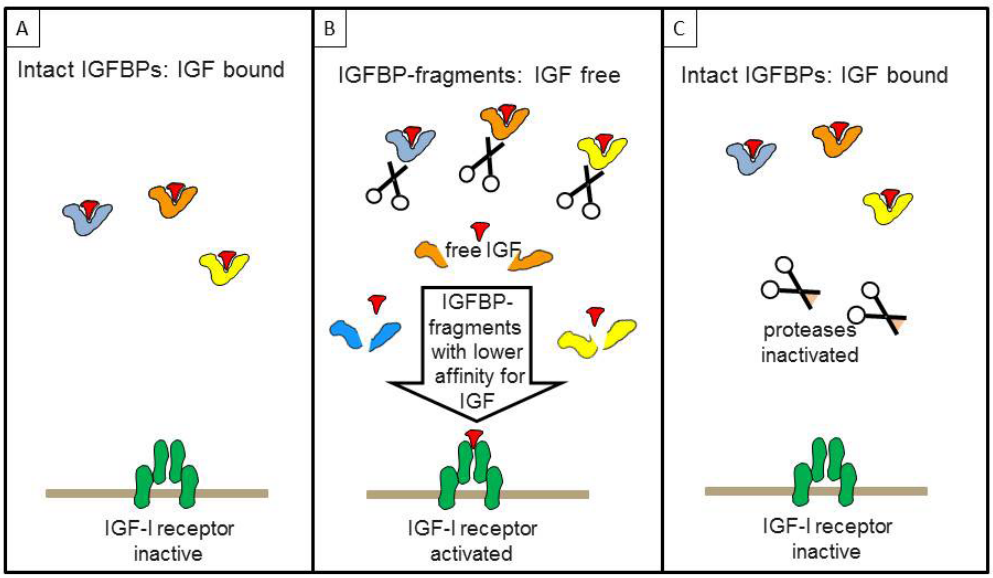
CURRENT PERSPECTIVES ON INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN (IGFBP) RESEARCH

Topic Editors:
Andreas Hoeflich, Leibniz-Institute for Farm Animal Biology (FBN) Dummerstorf, Germany
John Pintar, Rutgers Robert Wood Johnson Medical School, United States
Briony Forbes, Flinders University, Australia



Growth regulation by the control of the structural integrity of IGFBPs. In the presence of intact IGFBPs, IGFs are predominantly bound in dimeric or ternary complexes (A). In the presence of IGFBP-proteases (e.g. PAPP-A) distinct IGFBPs can be proteolyzed giving rise to IGFBP-fragments with lower binding activity (B). Free IGFs are considered as the biocative form of the IGFs having free access to the IGF-I receptors. In the presence of IGFBP-proteases structural integrity of IGFBPs can be retained by inhibitors (e.g. stanniocalcins; C). Image by Andreas Hoeflich. Cover image: Briony Forbes.

The family of IGFBPs has been developed by the duplication of genes and genomes and contributes to genetic and functional diversity. Due to the different protein domains present in the molecule, IGFBPs can be seen as mediators of tissue-specific IGF-functions. However, IGFBPs also have IGF-independent functions both inside and outside the cell. These diverse genetic, molecular and functional aspects of IGFBPs are discussed within this Research Topic. Accumulating data provide evidence for the regulation of IGFBP-functions by proteases, which may acutely regulate bioactivity of the IGFs. However, during proteolytic degradation IGFBP-fragments with novel functions can also be formed and are located both intra- and extracellularly. Distinct IGFBP-fragments can even be found in the perinuclear compartment or within the nucleus, where they can impact on gene expression. Several contributions presented in the current Research Topic particularly stress the relevance of structural aspects in IGFBP research. The current lack of comprehensive structural information is dramatically limiting the biomarker potential of particular IGFBPs. Finally, the Research Topic also provides novel functions of the IGFBP family from model organisms, farm

animals and humans. Thereby, the biomarker potential not only relates to normal and malignant growth but also to metabolism and animal welfare.

One important aim of the Research Topic is to encourage next generation IGFBP research reflecting subject-individual, conditional, and hormonal parameters but also additional structural aspects of IGFBPs.

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Editorial: Current Perspectives on Insulin-Like Growth Factor Binding Protein (IGFBP) Research

Andreas Hoeflich[1]*, *John Pintar*[2]* and *Briony Forbes*[3]*

[1] Institute of Genome Biology, Leibniz-Institute for Farm Animal Biology (FBN), Dummerstorf, Germany, [2] Department of Neuroscience and Cell Biology, Rutgers Robert Wood Johnson Medical School, Piscataway, NJ, United States, [3] College of Medicine and Public Health, Flinders University, Bedford Park, SA, Australia

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Editorial on the Research Topic

Current Perspectives on Insulin-Like Growth Factor Binding Protein (IGFBP) Research

The insulin-like growth factor binding proteins (IGFBPs), as high affinity IGF binding partners, are the principal regulators of IGF-1 and IGF-2 action. Accordingly, effects of IGFBPs can be observed on the levels of growth and differentiation, development, metabolism, and lifespan. The diversity of IGFBP-actions arises due to time-, sex-, and tissue-specific expression of the six distinct IGFBPs (IGFBP-1 to−6), which have redundant functions as seen from the analysis of double-, triple-, or quadruple IGFBP-knockout mice. The complexity of IGFBP functions is related not only to their roles as IGF carriers within the circulation but also to actions within the extracellular space and in distinct subcellular compartments, such as the cell nucleus. IGFBP functions have been attributed to structural motifs in the three conserved IGFBP subdomains, with specific residues being posttranslationally modified by glycosylation or phosphorylation to regulate IGFBP action. In addition, multiple binding partners inside and outside the cell have been identified that regulate IGFBP functions, including their IGF-independent activities. Furthermore, an in-depth understanding is emerging of the role of IGFBP proteolysis in the regulation of both IGF-dependent and IGF-independent actions through generation of potentially bioactive IGFBP-fragments. Accordingly, proteolytic degradation of IGFBPs as a physiologically relevant mechanism in disease has been revealed both in a malignant context but also in other acute or chronic pathophysiological conditions. Finally, the IGFBPs e.g., as sensors of GH/IGF-status have tremendous biomarker potential. Measurement of IGFBP-3/IGFBP-2 ratios provides ultimate sensitivity for the GH-status of a given cellular system. Similarly, detection of intact and fragmented IGFBPs may provide an indication of disease status. Accordingly, for the future we may expect an evolution of IGFBP-related diagnostic approaches, which extend to the characterization of both structural and functional properties of IGFBPs and their fragments in preclinical and clinical research.

In the present Research Topic we present both original work and reviews on the potential roles of IGFBPs in organisms ranging from fish, farmed animals (pigs and cows) to human. One of the most intriguing questions discussed by Allard and Duan is why there are so many IGFBPs. Because the different IGFBPs have evolved from one common ancestor, it is thought that the IGFBPs were used to increase functional biodiversity with respect to IGF-dependent and independent functions. This increase of diversity seems to be true particular in teleost fish where up to 4 copies may have arisen from IGFBP-2 due to genome duplications (Garcia de la Serrana and Macqueen). Evidence continues to accumulate for active and biologically significant proteolytic fragments of a number of IGFBPs. Some of this evidence is summarized in

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Edited and reviewed by:
Pierre De Meyts,
de Duve Institute, Belgium

***Correspondence:**
Andreas Hoeflich
hoeflich@fbn-dummerstorf.de
John Pintar
pintar@rwjms.rutgers.edu
Briony Forbes
briony.forbes@flinders.edu.au

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reviews on the role of IGFBP-proteases in bone (Beattie et al.) and for ovarian folliculogenesis (Mazerbourg and Monget). As mentioned above, IGFBPs may serve as biomarkers for a number of clinical conditions. However, there is need for additional structural information on the IGFBPs before this goal can be achieved, as in the example of cardiovascular diseases (Hoeflich et al.). The biomarker potential of IGFBPs was also critically discussed for autoimmune diseases because IGFBPs by IGF-dependent or -independent mechanisms also control proliferation of immune cells (Ding and Wu). Interestingly, IGFBP-5 was identified as an effector of cell senescence under the control of inflammatory signals and fibrinolysis and pro-coagulation (Sanada et al.). During pulmonary fibrosis, IGFBP-5 can increase its own expression which may increase the fibrotic effect in primary human lung fibroblasts by increasing expression of extracellular matrix proteins (Nguyen et al.). The assessment of either isolated IGFBPs or more complex IGFBP-signatures revealed novel biomarker potential for IGFBPs in different experimental systems ranging from malignant cells to farm animals. Accordingly, nuclear presence of IGFBP-3 in triple-negative breast cancer cells indicates a worse prognosis (Julovi et al.). Interestingly a link between obesity and malignant growth in prostate cancer cells was suggested as hyperglycemia was demonstrated to increase the expression of IGFBP-2 (Holly et al.). By contrast in farm animals, serum concentrations of IGFBP-2 and−3 were identified as novel biomarkers for intermediate stress conditions (Wirthgen et al.) or the maintenance of pregnancy (Mense et al.). Finally also gender aspects have to be considered since two independent contributions discussed the potential roles of IGFBPs for male (Safian et al.) and female (Spitschak and Hoeflich) reproductive development.

The different contributions *par excellence* provide substantial evidence that functional analysis of IGFBPs and IGFBP-related biomarker research contains huge potential but is only in its infancy. Future studies on the roles of IGFBPs need to take not only sex, age, and gender into consideration but also the metabolic conditions, subcellular localization, and IGFBP structural information. Taken together this would also lead to subsequent improvement in analytical method standardization.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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IGF-Binding Proteins: Why Do They Exist and Why Are There So Many?

*John B. Allard and Cunming Duan**

Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI, United States

Insulin-like growth factors (IGFs) are key growth-promoting peptides that act as both endocrine hormones and autocrine/paracrine growth factors. In the bloodstream and in local tissues, most IGF molecules are bound by one of the members of the IGF-binding protein (IGFBP) family, of which six distinct types exist. These proteins bind to IGF with an equal or greater affinity than the IGF1 receptor and are thus in a key position to regulate IGF signaling globally and locally. Binding to an IGFBP increases the half-life of IGF in the circulation and blocks its potential binding to the insulin receptor. In addition to these classical roles, IGFBPs have been shown to modulate IGF signaling locally under various conditions. Although members of the IGFBP family share significant sequence homology, they each have unique structural features and play distinct roles. These IGFBP genes also have different modes of regulation and distinct expression patterns. Some IGFBPs have been found to bind to their own receptors or to translocate into the interior compartments of cells where they may execute IGF-independent actions. In spite of this functional and regulatory diversity, it has been puzzling that loss-of-function studies have yielded relatively little information about the physiological functions of IGFBPs. In this review, we suggest that evolution has tended to retain an array of IGFBPs in order to facilitate fine-tuning of IGF signaling. We explore the emerging explanation that many IGFBP functions have evolved to allow the targeted adjustment of IGF signaling under stressful or irregular conditions, which would likely not be revealed in a standard laboratory setting.

Keywords: insulin-like growth factor, insulin-like growth factor-binding protein, insulin-like growth factor 1 receptor, insulin-like growth factor signaling, evolution

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Edited by:
Andreas Hoeflich, Leibniz-Institut für Nutztierbiologie (FBN), Germany

Reviewed by:
Taisen Iguchi, National Institute for Basic Biology, Japan
Tom Ole Nilsen, University of Bergen, Norway
Isabel Navarro, Universitat de Barcelona, Spain

***Correspondence:**
Cunming Duan
cduan@umich.edu

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INTRODUCTION

The insulin and insulin-like growth factor (IGF) signaling pathway is highly conserved among the metazoans. Many invertebrates have large numbers of insulin-like peptides (ILPs); for instance, the *Caenorhabditis elegans* genome contains around 40 (http://wormbase.org), and the *Drosophila melanogaster* genome contains 8 (http://flybase.org). In vertebrates, the ancestral insulin-like gene has diverged into insulin, IGFs-1 and -2, and several ILPs including relaxin and relaxin-like peptide (1). Insulin primarily acts in an endocrine fashion to regulate metabolism, whereas IGFs have a variety of roles as endocrine, paracrine and autocrine factors that promote cell growth, proliferation, differentiation, survival, etc. Both IGF-1 and IGF-2 bind to the IGF-1 receptor (IGF1R), which is expressed in almost all cells, with hepatocytes being an important exception in mammals. The liver secretes IGF-1 into the circulation in response to growth hormone (GH) stimulation (2, 3). Local tissues also secrete IGF-1 in response to GH, and this paracrine/autocrine IGF-1 acts together with the endocrine IGF-1 (mostly liver derived) to mediate the global growth promoting effects of GH (4). In addition to their role in regulating fetal, neonatal, and postnatal growth, IGFs

are also involved in diverse processes including wound healing (5), development of CNS and other tissues (6), regulation of protein, carbohydrate, and lipid metabolism (7), neuroprotection (8), aging (9), etc.

The diverse functions of this central hormonal pathway require that robust regulatory mechanisms be in place to avoid inappropriate regulation and/or dysfunction in different tissues and at different times. We now understand that IGF signaling is regulated by a family of specific IGF-binding proteins (IGFBPs) of which there are six distinct types in vertebrates. These IGFBPs share significant sequence homology and they are capable of binding IGFs with equal or greater affinity than the IGF1R. In fact, in both the circulation and in local tissues, most IGFs can be found bound to an IGFBP (10–13). In this review, we discuss the complex interplay of both overlapping and unique functions by which IGFBPs influence IGF signaling.

THE IGFBP FAMILY

The IGFBP family is evolutionarily ancient and highly conserved in vertebrates (11, 14–16). The six types of IGFBPs have been designated IGFBP-1 through IGFBP-6. Mammals generally possess one gene that belongs to each of the six types, and humans follow this pattern (**Table 1**). Some vertebrate species occasionally lack one or more of the types, and others have more than one IGFBP gene that can be classified within one type (**Table 1**). It is believed that the IGFBP family evolved *via* successive rounds of whole genome duplications. Notably, many teleost fish possess two copies of each of the six types of IGFBPs (**Table 1**), which is attributable to the third round of whole genome duplication that they are believed to have undergone following their divergence from the other vertebrates (16–21). Salmonid fish experienced an additional round of genome wide duplication and can have four copies of each IGFBP (22–24). We discuss the evolution of the IGFBP family in more detail in a later section.

All IGFBPs generally have approximately 200–300 amino acids and share a conserved structure consisting of a highly cysteine-rich N-terminal domain that is highly conserved among the IGFBP family and across species, a linker domain whose sequence is variable, and a cysteine-rich C-terminal domain that is also evolutionarily conserved (**Figure 1A**). The N- and C-terminal domains are globular and are structurally stabilized by multiple disulfide bonds between the conserved cysteine residues. Both of these domains participate in forming the IGF-binding site. The central linker domain is unstructured and serves to tether the N- and C-terminal domains together but also provides a location for functional motifs (10, 25).

Many of the functions of different IGFBPs are made possible by their unique collection of functional motifs (**Table 2**). These functional motifs include binding sites for heparin, components of the extracellular matrix and cell surface proteoglycans; proteolytic cleavage sites; sites of post-translational modifications including glycosylation, etc. In addition, IGFBP-2, -3, -5, and -6 all contain functional nuclear localization sequences by which they are imported into the cell nucleus in certain cell types as we discuss below.

It should be noted that a number of proteins belonging to the CCN (cyr61, ctgt, and Nov) protein family have been reported to contain sequences similar to the IGFBP N-domain and were once named IGFBP 7–12. It was later recognized that these were not IGFBPs and were renamed as IGFBP-related proteins and classified as part of a broader superfamily with the IGFBPs (26). The latter nomenclature has been questioned because these CCN family proteins not only lack high-affinity IGF-binding abilities but are also structurally no more related to IGFBPs than to von Willebrand factor, thrombospondin, or growth factor cysteine knots (27). These CCN family proteins will not be discussed in this review.

TABLE 1 | The IGFBP gene repertoire in selected vertebrate species.

	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	Total
Human	1	1	1	1	1	1	6
Mouse	1	1	1	1	1	1	6
Dog	1	1	1	1	1	1	6
Opossum	1	1	1	1	2	0	6
Chicken	1	1	1	0	1	0	4
Zebrafish	2	2	1	0	2	2	9
Medaka	2	2	1	1	2	1	9
Tetraodon	2	2	2	1	2	1	10
Fugu	2	2	2	1	2	1	10
Stickleback	2	2	2	1	2	2	11
Salmon	4	4	4	2	4	4	22

IGFBP BIOLOGICAL ACTIONS

In this section, we discuss a selection of the vast literature on the many reported biological actions/activities of IGFBPs that have been reported in gain-of-function studies *in vivo* or *in vitro*. Each species has its own standard nomenclature for gene and protein names. In this article, we deal with a large number of species. To increase the readability, we will use the same symbol for each IGFBP name. Whenever required, the species name is added to avoid confusion.

Endocrine Actions of IGFBPs

In extracellular environments, most IGFs are bound with IGFBPs, either in a binary complex or a ternary complex (**Figure 1B**). The vast majority of IGFs in the serum are bound to an IGFBP. IGFBP-3 is the most prevalent in adult serum with a concentration of around 100 nM/L, while all of the other IGFBPs are present at concentrations of less than 20 nM/L (25, 28). About 75–80% of serum IGFs were found in a ternary complex of about 150 kDa consisting of an IGF, IGFBP-3 (or less often IGFBP-5) and a glycoprotein called acid labile subunit (ALS). The remaining 20–25% of IGFs were complexed with one of the other IGFBPs (25, 28). Unbound IGFs have a half-life of less than 10 min (29). Binding to an IGFBP increases IGF half-life in the circulation to around 25 min, but the binary complexes are able to rapidly leave the circulation (29). Most of the circulating IGFs are present in the IGF-IGFBP3/5-ALS ternary complex (30, 31). The addition of ALS increases the molecular size of the complex and this has the effect

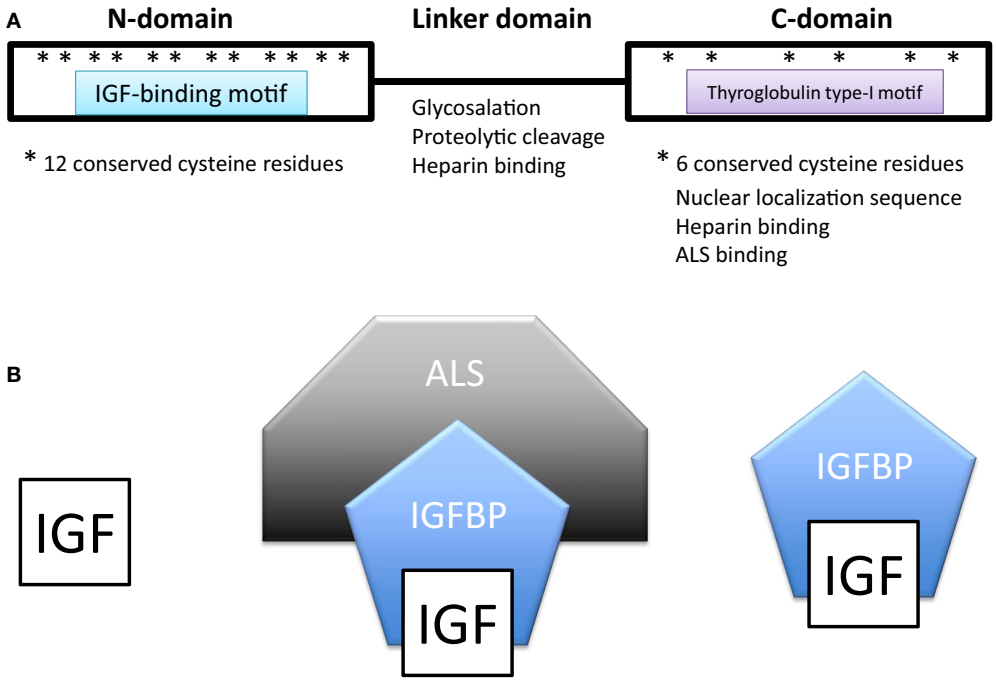


FIGURE 1 | **(A)** Domain structure of insulin-like growth factor-binding proteins (IGFBPs). IGFBPs contain conserved N- and C-terminal domains and a variable linker domain between them. The N-domain contains an insulin-like growth factor (IGF)-binding motif and the C-domain contains a thyroglobulin type-I repeat. The N-domain usually contains 12 conserved cysteine residues and the C-domain contains 6. **(B)** In extracellular environments, most IGFs are bound with IGFBPs, either in a binary complex containing one IGF and one IGFBP or a ternary complex consisting of an IGF, IGFBP-3 (or less often IGFBP-5), and a glycoprotein called acid labile subunit (ALS).

of preventing the bound IGF from leaving the capillaries thereby confining it within the circulation (32). The ternary complex thereby greatly prolongs the half-life of bound IGFs to about 16 h or more, forming a long-lasting reservoir of IGFs in the circulation (28). Deletion of the ALS gene in mice results in a 60% reduction in circulating IGFs and a 15–20% reduction in postnatal growth (33).

Insulin-like growth factors are sufficiently structurally similar to insulin that they can cross react with the insulin receptor (IR) (34). Another important function of circulating IGFBPs is to prevent the potential interaction of IGFs with the IR, which is crucial since IGF concentrations are high enough in the serum to cause hypoglycemic effects even given their lower affinity for the IR (25, 35).

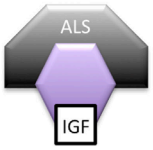
Insulin-like growth factor-binding protein-3 is produced in the liver and in other tissues and secreted into the serum, and its hepatic expression level is regulated by GH (36). This ensures that as the amount of secreted IGF-1 increases in response to GH stimulation, there will be an increased quantity of IGFBP-3 to absorb it in the circulation. IGFBP-1 is also synthesized in the liver and its expression and secretion are highly regulated by catabolic factors and hormones. For example, hepatic IGFBP-1 expression level is highly induced by starvation, hypoxia, and stress (37, 38). Its expression is reduced by insulin and increased by glucocorticoids (39, 40). These regulatory mechanisms serve to promote IGFBP-1 expression in response to starvation and catabolic conditions, including amino acid shortages and hypoxia (41, 42). The functional role of IGFBP-1 in these conditions is to reduce the rate of development and growth by binding to IGFs and inhibiting IGF activity (37, 38).

Local Actions of IGFBPs

While the bulk of circulating IGFBP-3 and IGFBP-1 are produced in the liver, IGFBP-3 and other IGFBPs are also expressed in many peripheral tissues (43, 44). The importance of local IGF-1 is supported by the finding that deletion of IGF-1 specifically in the liver resulted in an 80% reduction in circulating endocrine IGF-1 but no change in postnatal growth (45). Biochemical and cell culture studies suggest that IGFBPs generally bind IGFs with equal or higher affinity than the IGF1R and can inhibit IGF signaling by sequestration of the ligands (12, 13, 25, 46) (see **Figure 2A**). An example of this behavior is found in vascular smooth muscle cells (VSMCs) where IGFBP-4 acts to block IGF-1 from interacting with the IGF1R and thereby inhibits IGF-1-stimulated DNA synthesis (47). When IGFBP-4 was overexpressed in various tissues in mice, it resulted in hypoplasia of the affected tissue, suggesting that this may be a common action in different cell types (48).

Some IGFBPs have been shown to potentiate IGF signaling. Several proteases are known to cleave IGFBPs, and the resulting proteolytic fragments have greatly reduced binding affinity for IGFs. This leads to the liberation of IGFs from the IGF/IGFBP complex and increases the amount of IGFs available for IGF1R binding, thereby converting the inhibition of IGF signaling into an enhancement (**Figure 2B**). The proteases pregnancy-associated plasma protein A (PAPP-A) and PAPP-A2 are specific IGFBP

TABLE 2 | Structural and biochemical characteristics of the six distinct types of IGFBPs.

	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
						
Size (kDa)	25	31	43-45	24	29	28-30
Glycosylation			✔	✔	✔	✔
Binds to ALS			✔		✔	
Binds to Cell surface or ECM		✔	✔		✔	
Heparin binding domain		✔	✔		✔	✔
RGD sequence	✔	✔				
Nuclear localization sequence		✔	✔		✔	✔
Transactivation activity		✔	✔		✔	

proteases (49). IGFBP-4 is cleaved by PAPP-A when bound to IGF. This results in the release of the IGF ligand from the complex and a consequent increase in IGF ligand available for binding to the IGF-1R (50). PAPP-A knockout mice were about 40% smaller than wild-type littermates, which is consistent with the idea that PAPP-A cleaves inhibitory IGFBPs and thereby promotes IGF action (51). IGFBP-4 knockout mice were paradoxically slightly smaller than wild-type littermates, but mice null for both IGFBP-4 and PAPP-A were not smaller than IGFBP-4 knockout mice, indicating that the growth-promoting effects of PAPP-A likely result from the cleavage of IGFBP-4 (52). A number of other proteases have also been found to cleave various IGFBPs (53–55).

The potentiating action of some IGFBPs can occur when the IGFBP binds to the target cell's surface proteoglycans and/or extracellular matrix components, resulting in a concentration of local IGF that can then be released to the IGF1R (**Figure 2C**). It has been reported that IGFBP-5 undergoes a reduction of affinity for IGFs when it binds to certain extracellular matrix components, allowing it to deliver and then release IGF ligands at target sites (56, 57). Differentiating myoblasts provide one example of the interplay between a locally produced IGFBP and autocrine/paracrine IGF signaling. During myogenesis, IGF-2 is produced locally at high levels and this is required for myoblast differentiation (58). Prior to the onset of IGF-2 secretion, there is an increase in the expression and secretion of IGFBP-5. The secreted IGFBP-5 potentiates IGF-2 signaling and increases myoblast cell differentiation by binding to IGF-2 and promoting its interaction with the IGF-1R (59).

Insulin-like growth factor-binding proteins have also been reported to act locally under certain pathological conditions. A good example is the role of IGFBP-5 in the progression of atherosclerosis. IGFBP-5 is normally produced and secreted by VSMCs but its expression is upregulated in the VSMCs found within atherosclerotic plaques (60). Immunostaining for IGFBP-5 protein was dense within atherosclerotic plaques and especially around calcified areas (61). Locally secreted IGFs have been suggested to play an important role in atherogenesis by promoting the VSMC proliferation and their migration into the area of the arterial wall known as the intima (62, 63). These actions are promoted by local IGFBP-5 (57). Interestingly, a protease resistant IGFBP-5 mutant actually inhibited VSMC proliferation and migration, suggesting a mechanism by which IGFBP-5 is normally cleaved in order to present the IGF ligands to the IGF-1R on the surface of VSMCs (56). IGFBP-5 also binds to certain extracellular matrix proteins that are enriched in atherosclerotic lesions. It was reported that these interactions enhanced the mitogenic effects of IGFs on VSMCs (53, 55, 64). These studies support a model in which local IGFBP-5 is concentrated within atherosclerotic lesions (by both increased local expression and secretion and by binding to locally enriched ECM components), where it then acts to concentrate and deliver IGFs to the IGF1R on local VSMCs.

Studies suggest that IGFBP-4 may be involved in inhibiting atherosclerosis. A protease-resistant IGFBP-4 mutant was able to inhibit atherosclerotic lesion development in hypercholesterolemic pigs (65). When the PAPP-A protease that cleaves IGFBP-4 was knocked down in a mouse model of atherosclerosis (ApoE KO), there was decreased formation of atherosclerotic lesions (66, 67). When PAPP-A expression was transgenically increased locally within VSMCs in artery walls in ApoE KO mice, there

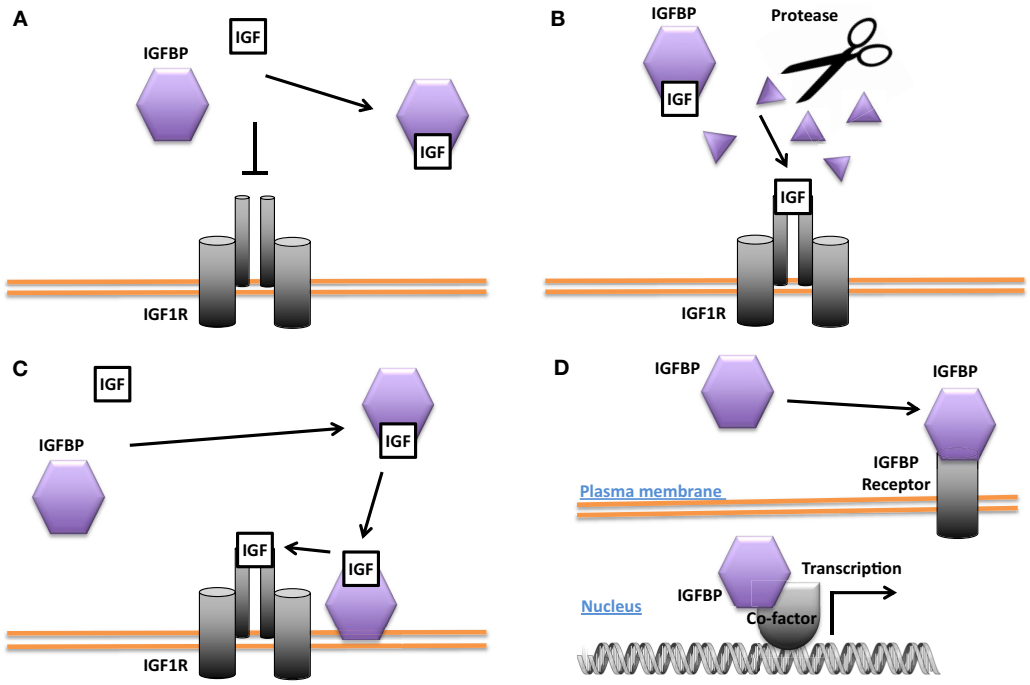


FIGURE 2 | Different modes of Insulin-like growth factor-binding protein (IGFBP) actions. **(A)** Inhibition of insulin-like growth factor (IGF) signaling by sequestering IGFs away from the IGF-1 receptor (IGF1R). **(B)** Promotion of IGF signaling by proteolytic cleavage of the IGFBP and liberation of IGFs from the IGF/IGFBP complex for binding to the IGF1R. **(C)** Enhancement of IGF signaling by concentrating IGF locally and increasing IGF availability for binding to the IGF1R. **(D)** IGF-independent actions. Some IGFBPs have been shown to be capable of translocating into the nucleus in certain cells and may affect gene transcription directly or indirectly. Some IGFBPs have been found to bind to cell surface proteins that may act as IGFBP receptors.

was a substantial increase in atherosclerotic plaque formation that was associated with an increase in local IGF-1 availability (68). Targeting PAPP-A in ApoE KO mice with a monoclonal antibody that inhibited its proteolytic activity resulted in a 70% reduction in aortic plaque burden (69). These results suggest that the proteolysis of IGFBP-4 by PAPP-A releases IGF-1 that acts locally to promote atherosclerotic plaque formation.

IGF-Independent Actions

Several IGFBPs have been reported to have cellular actions that are independent of their IGF binding (**Figure 2D**). Some IGF-independent actions are mediated by binding to cell surface proteins. For example, the integrin-binding RGD motif found in IGFBPs 1 and 2 allows them to promote cell migration and influence cell adhesion, respectively (70, 71). IGFBP-5 and -3 possess functional nuclear localization sequences and can enter the nucleus (72, 73). The nuclear localization of IGFBP-3 and -5 was found to be mediated by importin beta (72). Locally produced IGFBP-5 was found to stimulate porcine VSMC migration by an IGF-independent mechanism (74). IGFBP-5 was shown to possess transactivation activity (73). This transactivation activity was mapped to the N-domain and was also demonstrated in the N-domains of IGFBPs-2 and 3 (75). Nuclear localization and transactivation activity are also present in zebrafish IGFBP-3 and -5 (21, 76). In *cephalochordate* amphioxus, which diverged from the vertebrates approximately 520 million years ago, there is a single IGFBP-like gene. The amphioxus IGFBPs contains a functional nuclear localization signal and a transactivation domain (77). The lamprey IGFBP3, a jawless agnathan vertebrate, has been reported to possess both IGF-dependent action and the transactivation activity. The conservation of IGFBP transactivation activity across eons of evolution suggests that it likely has an important function. Along this line, several studies have found roles for nuclear IGFBPs in altering transcription in cancer cells (78, 79), but the physiological role(s) of the endogenous IGFBPs in the nucleus remain unclear.

Other IGF-independent actions have been reported that do not apparently involve nuclear localization. Paracrine IGFBP-4 was shown to promote differentiation of cardiomyocytes by inhibiting Wnt signaling in an IGF-independent manner (80). The physiological relevance of this effect was supported by the fact that knockdown of IGFBP-4 in Xenopus embryos resulted in cardiac defects attributable to impaired cardiomyogenesis (80). On the other hand, IGFBP-4 knockout mice have no cardiac phenotype (81). The lack of phenotype may be due to genetic redundancy and/or compensation by other IGFBPs. Another example is the antagonization of bone morphogenic protein signaling by IGFBP-3 in zebrafish (76). It has been reported that human IGFBP-6 has antiangiogenic activity when tested using *in vitro* assays. This action is independent from IGF binding because an IGFBP-6 mutant with 10,000-fold lower binding affinity for IGFs was as potent as the wild-type human IGFBP-6 in inhibiting angiogenesis (82). Interestingly, IGFBP-6 was found to be able to bind vascular endothelial growth factor (VEGF)

and coincubation with IGFBP-6 abolished VEGF-stimulated angiogenesis. This antiangiogenic action of IGFBP-6 was demonstrated *in vivo* in a tumor model by transplanting human Rh30 rhabdomyosarcoma cells stably transfected with IGFBP-6 into BALB/c nude mice (82). Expression of zebrafish IGFBP-6b had similar effects, indicating that this antiangiogenic activity is evolutionarily conserved (82).

Some IGFBPs may have cell surface receptor-mediated IGF-independent actions (**Figure 2D**). Exogenous IGFBP-3 was reported to inhibit cultured cell growth by an IGF-independent mechanism (83, 84). This effect was shown to be related to the binding of IGFBP-3 to the type 5 transforming growth factor β (TFGβ) receptor (85). This receptor was then shown to be identical to the low-density lipoprotein receptor-related protein-1 (LRP-1) (86). LRP-1 is known to be responsible for the uptake and clearance of various molecules from the circulation (87). The downstream mechanisms by which the interaction of IGFBP-3 with LRP-1 may lead to growth inhibition remain unclear. IGFBP-2 has been shown to bind to a receptor called receptor protein tyrosine phosphatase β (RPTPβ), which triggers a signal transduction cascade that leads to reduced PTEN phosphatase activity and a consequent enhancement of IGF-1-induced Akt pathway activation (88). This interaction between IGFBP-2 and RPTPβ was shown to be responsible for the ability of IGFBP-2 to trigger osteoblast differentiation (89). This role of IGFBP-2 was independent of IGF-binding and a 13-residue peptide corresponding of IGFBP-2's heparin-binding domain 1 was shown to mediate its binding to RPTPβ (88, 89).

Loss-of-Function Studies

Given the numerous biological actions of IGFBPs found in gain-of-function studies, it was surprising that little or no phenotypic change was observed when individual IGFBP genes were deleted in mice (81, 90–93). IGFBP-1 knockout mice were indistinguishable from their wild-type littermates and no embryonic lethality was observed (91). IGFBP-2 knockout mice were phenotypically normal with the exception of minor gender specific changes in bone structure and minor changes in the weights of spleen and liver in adult males (90, 92). IGFBP-3 knockout mice were also normal (81). Deletion of the IGFBP-4 gene in mice resulted in a mild 10–15% reduction in prenatal growth, which is somewhat paradoxical given that overexpression of IGFBP-4 also reduces growth (81). IGFBP-5 knockout mice were also phenotypically normal (81). Genetic deletion of IGFBP genes using CRISPR-Cas9 or TALEN in zebrafish have also resulted in little or no alteration in phenotype. Zebrafish IGFBP-3 knockout fish are morphologically normal and their growth rate and developmental speed are comparable to their siblings. Likewise, IGFBP-5a and -5b knockout zebrafish are morphologically indistinguishable from their wild-type siblings when kept under optimized lab conditions (unpublished data).

When IGFBP-3, -4, and -5 were knockout together in mice, there was a 25% reduction in body growth, decreased fat accumulation and quadriceps muscle mass, expanded pancreatic islets, and enhanced glucose homeostasis (81). These triple mutant mice were viable (81). Considering that knockout of IGF-1 itself results in a 60% reduction in prenatal growth followed by perinatal lethality for over 95% of mutant pups (94), the phenotype of the triple IGFBP-3/4/5 knockout mice can be viewed as relatively moderate.

The lack of substantial phenotypes in these IGFBP mutant mice and the finding that these animals can survive without three out of the six IGFBPs suggests a high degree of functional redundancy and/or genetic compensatory mechanisms. Indeed, elevated levels of IGFBPs-1, -3, and -4 were found in the IGFBP-2 knockout mice, supporting the notion that the lack of IGFBP-2 may be compensated for by upregulation of other IGFBPs (90).

Genetic redundancy among paralogous genes is a widespread phenomenon and can result in the masking of phenotypes in loss-of-function studies (95, 96). One study of the *Drosophila* genome suggested that when gene duplications occur, only 4% of the resulting paralogs survive (97). One explanation for the stable retention of redundant paralogous genes is that genes with redundant functions may also acquire functions that are unique to themselves. This can result in the coselection of the redundant functions with the unique functions in a model referred to as the "piggyback" mechanism (98, 99). In this model, whenever it is the case that most mutations tend to inactivate both the redundant and non-redundant functions simultaneously, redundant functions can then be retained in both gene duplicates. Unique functions could be obtained by gain-of-function mutations, but it is more common for complementary inactivating mutations to cause ancestral functions to be partitioned between the duplicates in the process of subfunctionalization (100). Redundant functions can be maintained in both duplicates when at least one unique function is maintained in each duplicate (100).

WHY ARE THERE SO MANY IGFBPs?

Why has evolution favored the retention of so many IGFBP genes? One potential explanation is that, given the crucial importance of the IGF pathway in determining central life history traits such as body size and longevity, it may be that even relatively minor fine-tuning of IGF signaling levels would be strongly selected for. A possible example comes from the zebrafish IGFBP genes. In zebrafish, there are two IGFBP-1 genes, being paralogs of mammalian IGFBP-1 (17). Zebrafish IGFBP-1a and -1b have similar expression patterns and regulatory responses, but IGFBP-1a has a higher affinity for IGFs than IGFBP-1b, which may allow more graded inhibition of IGF signaling during catabolic conditions than was possible with only a single IGFBP-1 gene (17). The zebrafish genome also contains two IGFBP-2 genes. In this case, the IGFBP-2a and -2b proteins have similar biological activities (18, 101). However, these two paralogous genes exhibit distinct spatiotemporal expression patterns. During embryogenesis, IGFBP-2a mRNA is found in the lens and the brain boundary vasculature; it subsequently becomes highly expressed in the liver. IGFBP-2b is detected initially in all tissues at low levels, but later becomes abundant in the liver (18). In the adult stage, liver has the highest levels of IGFBP-2a mRNA, followed by the brain. IGFBP-2b mRNA, on the other hand, is only detected in the liver (18, 101). The two zebrafish IGFBP-5 genes have diverged both

in gene expression patterns and protein functions. Zebrafish IGFBP-5a and -5b are expressed in spatially restricted, mostly non-overlapping domains during early development (21). The IGF-binding site is conserved in both zebrafish IGFBP-5a and -5b, and they are both secreted and capable of IGF binding (21). While zebrafish IGFBP-5b has transactivation activity, no such activity is found with IGFBP-5a (21). Given their divergence in both expression patterns and cellular actions, zebrafish IGFBP-5a and 5b may regulate IGF-signaling within their respective domains in subtly differing ways. This may provide enhanced fine-tuning of IGF signaling as compared with a single IGFBP-5 gene.

A second possible explanation is that genetic compensation is responsible for masking what would otherwise be more significant phenotypes. It has been recognized recently that permanent genetic deletions (knockouts), often result in a less severe phenotype than transient reductions in expression (knockdowns) (102). The mechanisms responsible for this phenomenon remain unclear but a number of hypotheses have been proposed, including the idea that related or unrelated genes could be upregulated in the permanent mutants (102). When zebrafish IGFBP-3 was deleted, for example, no phenotypes were detected. However, when zebrafish IGFBP-3 was knocked down using antisense morpholinos, it resulted in defects in the development of the pharyngeal skeleton and inner ear (103).

Another possible explanation is that, in addition to their somewhat overlapping functions of transporting and protecting IGFs in the circulation, the individual IGFBPs are also involved context-dependent regulation of IGF signaling in specific cell types and under specific stressful or aberrant conditions. Flexible and versatile modes of regulation such as these would be highly advantageous for organisms in the wild and would be strongly selected for, despite being unlikely to produce observable phenotypes under optimized laboratory conditions. One example is the role of IGFBP-1 in responding to catabolic conditions by throttling back growth and developmental rate in order to conserve scarce resources (38, 42, 104). Another example is the specific role of IGFBP-1 in liver regeneration. IGFBP-1 knockout mice exhibited normal growth but were found to have impaired liver regeneration (91). Their liver cells were highly sensitive to induction of apoptosis by treatment with Fas agonist. This effect could be ameliorated by pretreatment with IGFBP-1 (105), suggesting that IGFBP-1 has a crucial but conditional role in protecting the liver when facing injury and healing. The role of IGFBP-5 in mammary gland remodeling is a further example. The IGFBP-5 knockout mice had normal body growth and normal mammary gland development under standard laboratory conditions. However, these mutant mice exhibited delayed mammary gland involution and enhanced alveolar bud formation after ovariectomy and estradiol/progesterone treatment (106). Another example is provided by zebrafish IGFBP5a, which is specifically expressed in a specific type of epithelial cell (ionocytes) on the larval yolk sac skin that are responsible for transporting Ca^{2+} ions. When wild type larvae are raised in embryo solution containing a very low calcium concentration, these ionocytes rapidly proliferate *via* a mechanism that requires the activation of IGF signaling in these cells (107). This allows increased calcium import and is necessary for survival under these conditions. This proliferation is blunted in the IGFBP5a knockout fish larvae, causing lethality. However, under optomized and calcium-rich conditions, these mutant fish are indistinguisable from their wild type siblings. This

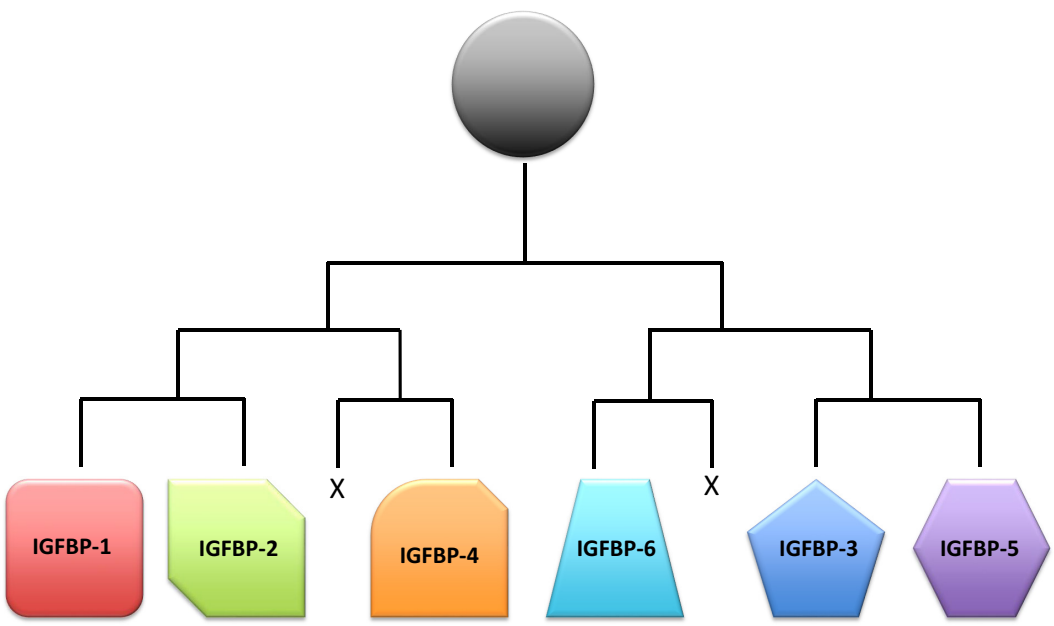


FIGURE 3 | Schematic representation of a proposed scenario of the insulin-like growth factor-binding protein (IGFBP) family evolution. A single ancestral IGFBP gene was duplicated in an early chordate. This duplication was followed by two successive rounds of chromosomal duplications or tetraploidization events in early vertebrates. Of the eight IGFBPs that resulted from this process, two were subsequently lost, leaving six types of IGFBPs that are seen in modern vertebrates.

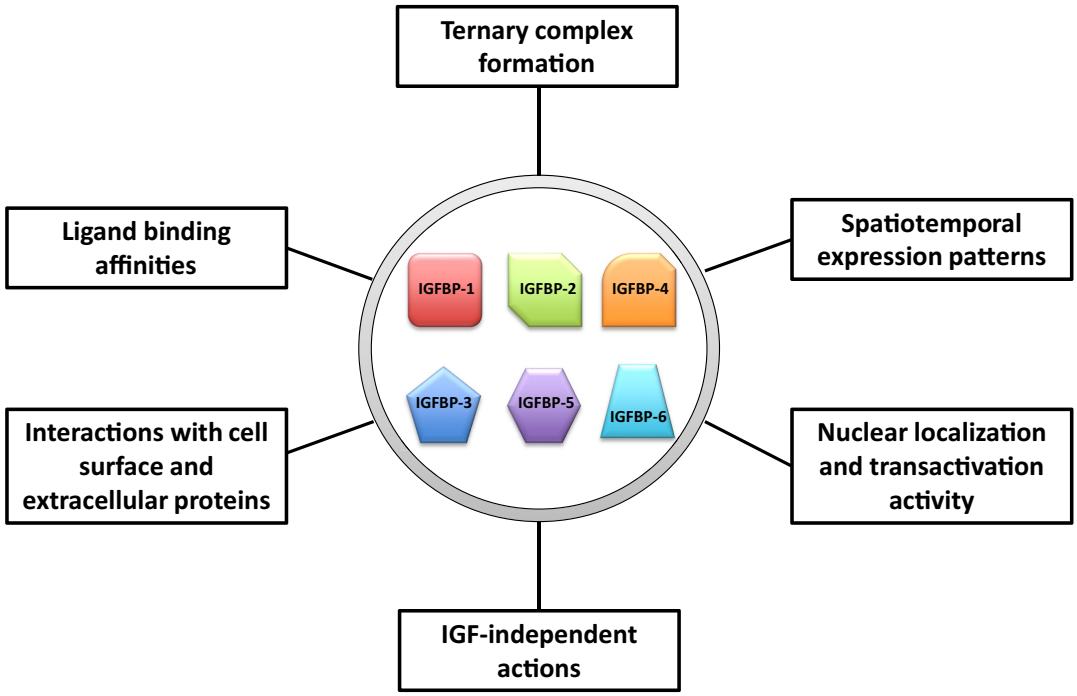


FIGURE 4 | Major attributes of insulin-like growth factor-binding proteins (IGFBPs) that may help to give rise to the increased flexibility and versatility in their abilities to regulate insulin-like growth factor (IGF) actions.

suggests that IGFBP-5a is critical for calcium ionocytes to activate a conditional proliferation program in order to maintain calcium homeostasis.

As always in biology, the question of why there are so many IGFBPs can only be fully understood in the context of evolutionary history. Based on analyses of phylogenetic relationships, the surrounding chromosomal regions in which modern IGFBPs sit, and IGFBP sequences from a large number of species, the evolution of the IGFBP family in early vertebrate ancestors has been reconstructed (16). An ancestral IGFBP sequence in an ancient early chordate was duplicated resulting in two adjacent IGFBP sequences in a chromosomal region that also bore the homeobox (HOX) genes. These two original genes were then duplicated along with the entire genome in the two successive rounds of tetraploidization that occurred in early vertebrates (108), resulting in eight IGFBP genes. It is thought that two of them were lost, resulting in the six types of IGFBPs seen in mammals and most other vertebrate classes (**Figure 3**). In many teleost fish, another round of tetraploidization occurred, resulting in a further doubling of IGFBP genes (16, 109). Some of these additional duplicates were subsequently retained in modern fish. Indeed, there is substantial variation in numbers of IGFBP genes between fish species (**Table 1**). The salmonid fish, whose common ancestor underwent a fourth round of whole genome duplication, exhibit the largest known repertoire of 22 IGFBP genes (22, 23). The preservation and evolutionary conservation of most of the IGFBP gene duplicates implies that these genes might have aquired unique evolutionarily adaptive roles, either by developing new functions opportunistically (neofunctionalization) or by retention of a subset of the parent gene's original functions in each duplicate (subfunctionalization). This is in agreement with the idea that fine tuning of IGF signaling is strongly adaptive to the extent that perhaps even small changes in the regulation of IGF signaling would be sufficient to account for the conservation of additional IGFBP genes to provide these regulatory advantages.

The acquisition of IGF-independent actions of IGFBPs presents an intriguing question. One possible explanation is that they were present in the ancestral IGFBP gene. A comparative study suggested that the single amphioxus IGFBP has a functional nuclear localization sequence and transactivation activity but lacks the ability to bind modern IGFs (77). Both IGF-dependent and IGF-independent actions appear to have been present in the earliest vertebrates as indicated by the fact that an IGFBP from sea lamprey exhibited both IGF-dependent and -independent actions (110). Therefore, the IGF-binding function of IGFBPs may have been acquired later in evolution.

CONCLUDING REMARKS AND PROSPECTS

We propose that IGFBPs provide a set of tools with which evolution has acted to increase the flexibility and versatility in the regulation of the IGF signaling system. An ancestral IGFBP gene has diversified into a number of IGFBP genes, which have both overlapping and unique expression patterns and functions. These IGFBPs can be viewed as different tools that all apply leverage but also provide individual context specific advantages. A number of attributes of IGFBPs may help to give rise to the increased

flexibility and versatility in their abilities to regulate IGF actions. These include: (1) distinct spatiotemporal expression patterns of these IGFBP genes, (2) differences in their ligand-binding affinity and selectivity, (3) different roles in the circulation including formation of the ternary complex with ALS, (4) different abilities to interact with cell surface proteins, extracellular proteins, and other growth factors, (5) different subcellular localization, and (6) various IGF-independent activities (**Figure 4**). The existence of multiple IGFBPs can contribute to the fine-tuning of IGF signaling both globally and locally, and under various physiological and pathological conditions. The involvement of IGFBPs in mammary gland growth, liver regeneration, and atherogenesis, and the adaptive proliferation of calcium ionocytes in zebrafish are all examples of this sort of process. It is plausible that more IGFBPs will be found to participate in other roles of this type. A great deal of work has identified many roles for IGFBPs in cancer cells despite the fact that IGFBPs are not commonly mutated in human cancers (12). Given the involvement of IGFBPs in tissue remodeling and conditional proliferation of certain cell types, it is not surprising that their physiological actions would be coopted by cancer cells in order to facilitate the needs of tumor growth.

Much has been learned in recent decades about the cell type-specific actions of IGFBPs but many questions remain unanswered. One major question is, why do several of the IGFBPs have the ability to enter the cell nucleus? Although certain IGFBPs have a functional nuclear localization motif and a transactivation domain that are both evolutionarily conserved, the physiological functions of the nuclear IGFBPs remain unknown. Another area of inquiry for future research will be to identify additional stressful conditions that IGFBPs have evolved to respond to. It will also be of great interest to identify pathological processes that depend on the misregulation of IGFBP(s) to increase or decrease IGF signaling, or on inappropriate activation of their IGF-independent actions. We also have much more to learn regarding the evolutionary history of the IGFBPs in early vertebrates and the nature of its IGF-independent functions. This may shed light on the complex biology of modern IGFBPs.

CRISPR/Cas9-based genetic editing technology will allow the generation of mutant animals whose endogenous IGFBP genes are directly mutated to disrupt individual functionalities such as IGF-binding, nuclear translocation, or interaction with cell surface proteins, to allow assessing the roles of those capabilities individually or collectively under physiological conditions *in vivo*. The CRISPR-Cas9-based approaches will allow the physiological roles of redundant paralogs to be determined much more readily by enabling the generation of multiple knockouts at reasonable cost. Increasing our understanding of IGFBPs will yield insights into the array of biological processes to which IGF signaling is linked, including many that are crucial for human health and diseases.

AUTHOR CONTRIBUTIONS

CD conceived the article. JA and CD wrote the manuscript.

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REVIEW
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Insulin-Like Growth Factor-Binding Proteins of Teleost Fishes

Daniel Garcia de la Serrana[1] and Daniel J. Macqueen[2]*

[1] School of Biology, Scottish Oceans Institute, University of St Andrews, St Andrews, United Kingdom,
[2] School of Biological Sciences, University of Aberdeen, Aberdeen, United Kingdom

The insulin-like growth factor (Igf) binding protein (Igfbp) family has a broad range of physiological functions and a fascinating evolutionary history. This review focuses on the Igfbps of teleost fishes, where genome duplication events have diversified gene repertoire, function, and physiological regulation—with six core Igfbps expanded into a family of over twenty genes in some lineages. In addition to briefly summarizing the current state of knowledge on teleost Igfbp evolution, function, and expression-level regulation, we highlight gaps in our understanding and promising areas for future work.

Keywords: insulin-like growth factor binding protein, teleost fish, genome duplication, physiology, comparative biology, gene family evolution

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****Correspondence:***
Daniel Garcia de la Serrana
dgdlsc@st-andrews.ac.uk

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INTRODUCTION

The insulin-like growth factor (Igf) binding protein (Igfbps) are highly studied, especially in mammals, and a vast literature has emerged on their roles as mediators of Igf signaling events, and diverse functions that extend beyond Igf regulation. This review focuses on the Igfbp family of teleost fishes, which remains poorly understood compared to the mammalian system. Our goal is to summarize the status of knowledge on teleost Igfbps in an evolutionary context, considering work on gene function and physiological regulation, in addition to phylogenetics and genomics. Our coverage of literature is non-encompassing, and we point the reader to additional reviews. The scope of the review is exclusive to the "true" Igfbps, which each bind Igfs with high affinity, rather than the broader proposed superfamily containing Igfbp-related proteins [reviewed in Ref. (1)], that are distantly related in both sequence and function (2). We also assume that the reader has prior knowledge of the core genetic components of the Igf system, where comprehensive reviews with a non-mammalian focus already exist (3–6).

ORIGINS OF THE CORE Igfbp SUBTYPES

Gene duplication and subsequent divergence is central to the evolutionary "narrative" of the Igfbp family. While it is long-established that many vertebrates possess six ancestral subtypes (Igfbp-1, -2, -3, -4, -5, and -6), with the primary cDNAs first reported over 25 years ago [e.g., Ref. (7, 8)], their evolutionary origins were elucidated more recently. An important study reported in 2011 (9), built on past work revealing linkage between Igfbp genes and Hox clusters [e.g., Ref. (10); Hox clusters being well-established markers of genome duplication events], to present a realistic scenario for the origin and expansion of core Igfbp subtypes. The hypothesis is that an ancestral Igfbp gene was duplicated in tandem during an early stage of vertebrate evolution to produce a pair of Igfbp genes (9, 10). Subsequently, two genome duplication events in the ancestor to extant vertebrates (11) led one gene to give rise to Igfbp-1, -2, and -4, and the other to Igfbp-3, -5, and -6. A single Igfbp is present in amphioxus, a chordate that did not undergo the same duplications, and this

molecule failed to bind Igf-I or Igf-II, indicating that Igf-binding is either a vertebrate-specific function (12), or was secondarily lost. The same study confirmed that Igf-independent functions had evolved before vertebrates (12).

It is also important to remember that the diversification of the core vertebrate Igfbp system occurred alongside expansions in other key gene families within the Igf system, including both hormones (13) and receptors (14). It now seems certain that the early vertebrate genome duplication events were crucial for the evolution of distinct insulin and Igf systems [e.g., Ref. (15)]. For the remainder of this review, we focus on the Igfbp system of teleosts, where additional genetic expansions—some dramatic—have been recently characterized.

EXPANSIONS IN THE TELEOST Igfbp GENE REPERTOIRE

A further round of genome duplication occurred in the ancestor to extant teleost fishes (i.e., around half of known vertebrate species) 300–350 million years ago (11). This led to retention of duplicated copies (paralogs) for all the core Igfbp subtypes barring Igfbp-4, where one paralog was lost early (9, 16). In different lineages that have not experienced further genome duplication events, the number of Igfbps retained is variable, but always higher than mammals and most non-teleosts. For example, zebrafish (*Danio rerio*), the most studied teleost in terms of Igfbp function, retains nine unique genes. This includes paralog pairs for Igfbp-1 (17), -2 (18), -5 (19), and -6 (20), along with a single Igfbp-3 copy and no Igfbp-4 gene, owing to lineage-specific losses (9, 16). The phylogenetic relationships of teleost Igfbp paralogs have been established using robust methods (9, 16). An "-a"/"-b" nomenclature common to different teleosts is preferred (e.g., "Igfbp-1a" and "-1b") (16), as it acknowledges a common ancestral origin from the same duplication event, while accommodating zebrafish nomenclature [e.g., Ref. (17–20)].

Several teleost lineages experienced additional rounds of genome duplication. This includes a well-studied event ~95 million years ago in the salmonid ancestor (21, 22) that caused dramatic genetic expansions within the Igf system (summarized in **Figure 1**). For example, we reported in 2013 that salmonids retain at least 19 unique Igfbp genes, with salmonid-specific paralogs of *igfbp-1a*, *-1b*, *-2b*, *-3a*, *-3b*, *-5b*, *-6a*, and *-6b* (16). We proposed a nomenclature with either "1" or "2" after the "a" and "b" teleost symbols (e.g., "*igfbp-1a1*" and "*-1a2*"). Several of these Igfbp pairs are highly divergent compared to the genome-wide average for paralogs retained from the salmonid genome duplication event (**Figure 1**; e.g., sharing <80% amino acid identity, compared to an average of ~93% across thousands of paralog pairs) (23). This points to functional divergence at the protein level that remains entirely unexplored.

More recently, an improved understanding of the complexities of genome evolution following the salmonid genome duplication, which was a spontaneous genome doubling event

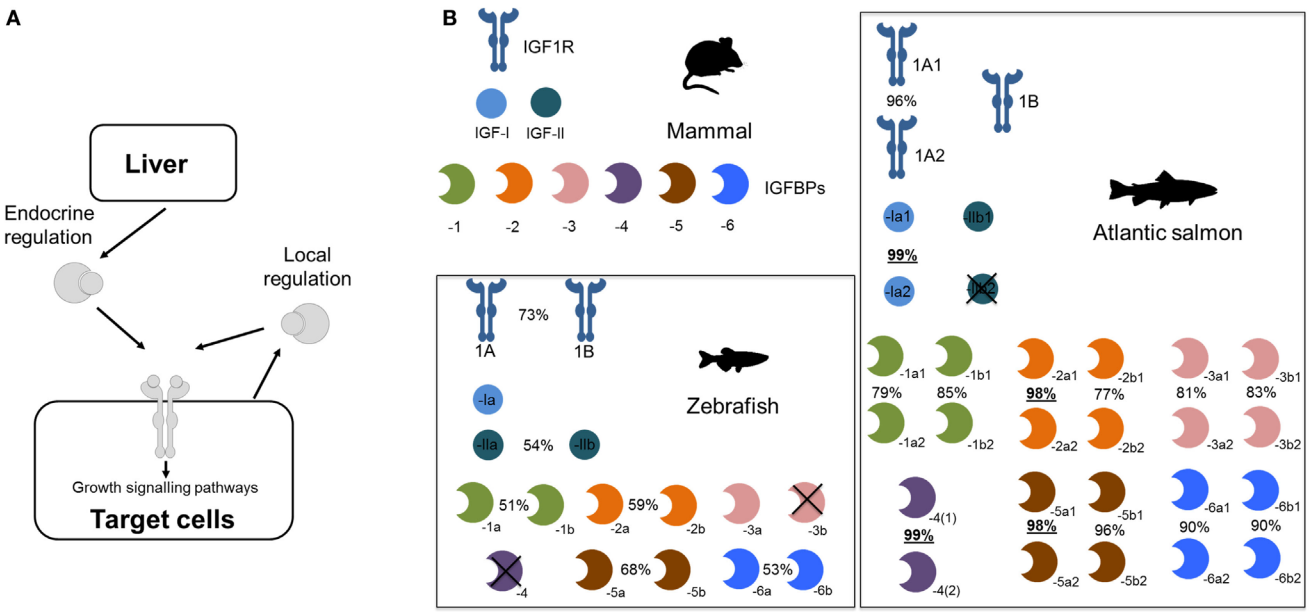

FIGURE 1 | Expansion of the core insulin-like growth factor (Igf) system—including Igfbps—during teleost evolution. **(A)** Simplified depiction of Igf system. **(B)** Core Igfbp system components (i.e., proteins encoded by distinct genes) in different vertebrate groups, contrasting a typical mammalian system with that of two teleost lineages. For teleosts, % identity is shown for paralogous amino acid sequences. For Atlantic salmon, the underlined % identities highlight paralog pairs residing in regions of the genome that experienced a delay in cytological rediploidization after genome duplication (24), a process required for paralogs to diverge in sequence on distinct chromosomes—hence, these genes have had less evolutionary time to diverge, leading to extremely high identity. Phylogenetic relationships of the Igfbp families from these different lineages, along with another group of teleosts that experienced a separate lineage-specific genome duplication event, are depicted in **Figure 2**.

(or "autotetraploidization") (21, 24), led to the discovery of salmonid Igfbp paralog pairs for *igfbp-4*, *igfbp-2a*, and *igfbp-5a* (24), which share extremely similar sequences and were previously unrecognized or ignored as alleles (see **Figure 1**; legend contains additional information). Thus, some salmonid species, including the commercially important Atlantic salmon (*Salmo salar*), possess 22 unique *igfbp* genes, with 11 paralog pairs (**Figure 1**), some of which may have evolved adaptively (25). Remarkably, every possible Igfbp paralog generated from the salmonid-specific genome duplication was evidently maintained, despite the genome-wide paralog retention rate being around 50% in the same species (21). We and others have also identified expansions to other core gene components of the Igf system due to the salmonid genome duplication, including Igf-I [e.g., Ref. (24, 26)], Igf-II (25), and Igf-1R (27). These paralogs remain of substantial interest, but we are at an early stage of understanding their roles in salmonid biology.

Additional lineage-specific genome duplication events have occurred in several teleost groups, including the ancestor to goldfish (*Carassius auratus*) and common carp (*Cyprinus carpio*). This event is younger than the salmonid-specific genome duplication event, occurring around 8–12 million years ago (28, 29). It also involved a distinct mechanism ("allotetraploidization"), where two species hybridized before genome duplication (28). This event created a large set of paralogs (28), some of which are known to have experienced functional divergence [e.g., Ref. (29, 30)]. However, no accompanying expansions to Igfbp repertoire are yet reported. To explore this knowledge gap, we performed a bioinformatic and phylogenetic analysis, revealing that common carp retains at least 17 unique *igfbp* genes, including paralog pairs for *igfbp-1a*, *-1b*, *-2b*, *-3a*, *-5a*, *-5b*, *-6a*, and *-6b* (see **Figure 2**; methods provided therein). To avoid confusion with the salmonid-specific paralogs, we suggest "α"/"β" is added to the existing teleost nomenclature when these duplicates are studied in the future (e.g., *igfbp-1a*α/*-1a*β) (**Figure 2**). The results confirm that salmonids are not unique among teleosts in retaining a highly expanded Igfbp repertoire. In fact, as many vertebrate groups have experienced lineage-specific genome duplication events, both fishes and non-mammalian groups, including anuran frogs [e.g., Ref. (31)], it seems likely that many other species possess expanded Igfbp repertoires, contributing additional complexity to their growth regulation.

PHYSIOLOGICAL ROLES OF TELEOST Igfbps

Many studies have investigated the physiological roles of Igfbp genes in the teleost lineage. Barring a few model species (e.g., zebrafish), it has been historically challenging to perform functional analyses in most teleosts, although this is changing in light of emerging genome-editing methods (see Perspectives and Future Work). Hence, while in mammals, Igf-dependent and Igf-independent functions have been widely demonstrated, the majority of studies in teleosts have failed to reach similar levels of functional insight. In fact, most work has focused on expression-level regulation of *igfbp* genes or proteins under a diverse set of experimental stimuli. It is also important to note that most teleost Igfbp research has focused on aquaculture species of high commercial value, including the salmonid, perciform (perch-like fish), pleuronectiform (flatfish), cypriniform (carp spp. and relatives), and siluriform (catfish) groups. This has led to a bias toward physiological processes relevant to commercial production, especially growth, muscle development, stress, and disease resistance. In this section, we briefly summarize the literature on teleost Igfbp function and regulation, considering the core vertebrate subtypes separately. We make attempts to distinguish Igfbp paralogs according to the evolutionary histories and nomenclature described above, although this is often not possible as many studies failed to distinguish paralogs, especially for the most recently discovered genes.

Igfbp-1: A Negative Regulator of Teleost Growth

In mammals, Igfbp-1 is mainly produced in the liver and secreted to circulation, where it acts to limit Igf signaling in catabolic contexts, such as fasting, stress, and hypoxia (39). It is widely considered a negative regulator of somatic growth, reproduction, and development (4, 40); and interacts with cell surface integrins to stimulate cellular motility (41). It has Igf-dependent and Igf-independent functions, along with important roles in the regulation of metabolism [reviewed in Ref. (42)].

In salmonids, Igfbp-1a and Igfbp-1b are two of the three major circulatory Igfbps (43), first identified by molecular weight (20–15 and 28–32 kDa, respectively) (4, 44). It is likely that similar molecular weight Igfbps detected in others teleosts plasma are Igfbp-1 orthologs (45–49). Igfbp-1 encoding genes, as in mammals, are mainly expressed in teleost liver (16, 50–55). In zebrafish embryos, *igfbp-1a* mRNA is expressed during early development; while *igfbp-1b* is expressed later, after which time both paralogs become restricted to liver (17, 50). At the functional level, both Igfbp-1a and -1b of zebrafish can bind to Igf-I and Igf-II, but Igfbp-1b had a lower affinity for each hormone, and a lesser ability to downregulate Igf-I signaling (17). In other species, it has been reported that *igfbp-1a* genes are expressed in non-hepatic tissues, but typically at lower levels than in liver (16, 51, 52, 54–58). This supports the hypothesis that Igfbp-1a evolved more localized functions than Igfbp-1b (6).

Several teleost studies have reported protein or transcript level upregulation of Igfbp-1 genes during catabolic a process, which probably serves to downregulate growth by sequestering Igfs from Igf-1Rs, allowing allocation of resources to metabolic processes essential for survival. Consistent with these findings, overexpression of *igfbp-1a* (and *igfbp-1a* in zebrafish) in cypriniform embryos (17, 54, 59) caused growth and developmental retardation. Nutrient deprivation has been shown to increase circulatory Igfbp-1 proteins and *igfbp-1* gene expression in liver (for both teleost paralogs, when distinguished) and skeletal muscle *igfbp-1a* expression, which is reversed by a return to anabolic conditions (50, 51, 55, 60–65). It has also been shown that dietary amino acid deficiency can upregulate *igfbp-1* gene expression through a not well-described mechanism (64). Teleost *igfbp-1* genes are also negatively regulated by growth hormone (Gh)

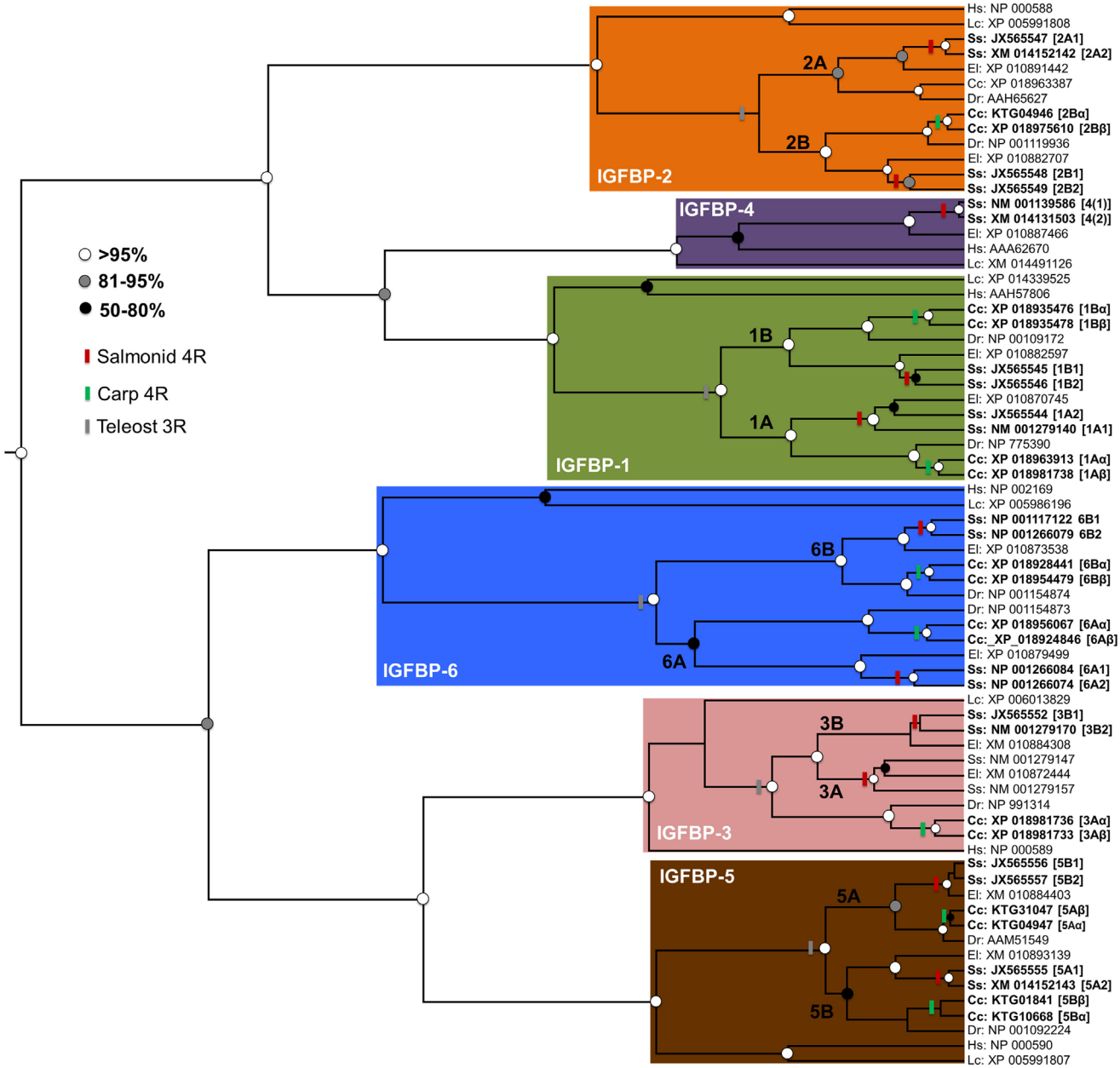

FIGURE 2 | Independent evolutionary expansions to the Igfbp family of teleosts. A phylogenetic analysis was performed, including 71 unique Igfbp amino acid sequences from a standardized set of taxa: Human *Homo sapiens* ("Hs"), coelacanth *Latimeria chalumnae* ("Lc"), zebrafish *Danio rerio* ("Dr'"), common carp *Cyprinus carpio* ("Cc"), Atlantic salmon *Salmo salar* ("Ss"), and northern pike *Esox lucius* ("El," from a sister lineage to salmonids that did not undergo the salmonid-specific genome duplication). Accession numbers are given for all sequences, which were gathered from the NCBI RefSeq database, facilitated by BLAST analyses (32). The sequences were aligned using Mafft V.7 (33) with default settings. Alignment quality filtering was done using the Guidance2 algorithm (34) to remove the least confidently aligned regions. This led to a high-confidence alignment of 212 amino acids positions (SI file 1). The alignment was used in Bayesian phylogenetic analysis, using methods published elsewhere (35). Briefly, this was done in BEAST v. 1.8 (36) using the best-fitting amino acid substitution model (JTT+G+I), determined by maximum likelihood *via* the IQ-tree server (37), along with a relaxed molecular clock model (38), allowing probabilistic estimation of the trees root [consistent with previous studies (9, 16)]. The tree is annotated to show genome duplication events in teleost evolutionary history, including in the teleost ancestor ("3R") and additional events in the salmonid and carp lineages. The nomenclature for salmonid and carp paralogs is given as described in the text. Branch support values (posterior probability) are highlighted by circles placed on nodes, with colors matching a legend. Minor inconsistencies in branching patterns in some Igfbp clades (e.g., relating to the 3R or salmonid 4R) compared to other studies (16), can be explained by the short alignment length. Nonetheless, the tree demonstrates independent expansions within the salmonid and carp Igfbp repertoire, additional to paralogs retained in many other teleosts.

and Igf-I, consistent with a negative role in growth regulation (44, 46, 48, 53, 57, 62).

Igfbp-1 expression in teleosts is also modulated by hormones others than the Igfs. For instance, *igfbp-1a* evidently plays a role in zebrafish sexual maturation, in a way that seems to differ from its classical anti-proliferative role. Specifically, *igfbp-1a* expression increases in response to T_3 and the follicle stimulating (Fsh) hormones (well-known for stimulating spermatogonia proliferation) (58). The female sex hormone 17β-estradiol also increased Igfbp-1 secretion in striped bass liver explants (46).

Stress is also known to induce *igfbp-1* gene expression in teleosts. Cortisol, the main stress hormone in vertebrates (66, 67), and other synthetic glucocorticoids such as dexamethasone increases both Igfbp-1a and Igfbp-1b circulatory levels, as well as gene expression in liver and cultured myotubes (43, 57, 62, 68, 69). Environmental stressors, such as hypoxia, confinement, temperature, heavy metals, and salinity, were also found to increase *igfbp-1* expression (17, 49–53, 56, 61, 70, 71). It is also possible that *igfbp-1* upregulation in response to food deprivation (see above) is related to a rise in cortisol, as some studies have found increasing levels of circulatory glucocorticoids in response to fasting (70, 72, 73). There is also emerging evidence from salmonids that *igfbp-1a1* upregulation during infection serves a role in linking growth to innate immunity, potentially promoting downregulation of growth in favor of a more effective immune response (74). This expression response represents an example of salmonid-specific divergence in the regulation of Igfbp paralogs, as *igfbp-1a2* was unaltered by infection in the same study (74).

Igfbp-2: A Major Circulatory Igfbp in Teleosts

In mammals, Igfbp-2 is highly expressed during embryonic stages, and more lowly expressed in adult tissues, with highest levels in liver, adipocytes, the central nervous and reproductive systems, heart, and kidney (75). Mice embryos overexpressing *Igfbp-2* show a reduced growth rate, likely through reduced Igf availability (76). However, Igfbp-2 deletion in mice embryos does not have any significant effect on growth or development (77), which may indicate compensatory effects with other Igfbps. While the functional roles of Igfbp-2 remain relatively poorly established, recent studies have linked it to several pathological states. For example, *Igfbp-2* may act as a tumor promoter (78) by suppressing epidermis growth factor receptor nuclear signaling (79). There is also increasing evidence that Igfbp-2 plays a role in mammalian bone formation (80).

Igfbp-2b is the third main circulatory Igfbp in salmonids and probably other teleost species (41 kDa form) and the main Igf carrier (6, 81, 82). For a long time, Igfbp-2b was wrongly considered to be Igfbp-3 (83) due to its similar physiological regulation to Igfbp-3 in mammals [e.g., Ref. (83)]. Teleost *igfbp-2* genes are expressed in a range of tissues [e.g., Ref. (16, 84–86)] with zebrafish *igfbp-2a* and *igfbp-2b* having different spatiotemporal patterns during early development, and each being expressed in liver in adults (18). In adult salmon, *igfbp-2a* was expressed across multiple tissues, with especially high abundance in liver, whereas *igfbp-2b1* and *igfbp-2b1* were liver-restricted (16). Overexpression of *igfbp-2a* and *igfbp-2b* causes a reduction in growth and developmental rate in early-stage zebrafish (18, 87), suggesting an equivalent role to that observed in mammals. Similar to Igfbp-1, past work has suggested a role for Igfbp-2 in teleost sexual maturation, with *igfbp-2* mRNA being expressed in pre-ovulatory ovaries of rainbow trout, and upregulated in response to female sex hormones (83).

Mixed results exist on the regulation of teleost *igfbp-2* genes by nutritional status. For example, some past studies showed that *igfbp-2* genes are downregulated or unchanged in liver and skeletal muscle of fasted fish (55, 88–90), which does not support an obvious role in growth inhibition. By contrast, a significant increase of *igfbp-2* expression was observed in fasted zebrafish (91), although this same effect was not clearly observed in a later study of zebrafish that distinguished *igfbp-2a* and *-2b* (65). In Atlantic salmon, a significant decrease in *igfbp-2a* (formerly "*igfbp-2.1*") expression was reported in skeletal muscle during refeeding after a period of restricted food intake, suggesting an inhibitory role on growth (90, 92). Similarly, all three tested *igfbp-2* paralogs (*igfbp-2a*, *-2b1*, and *-2b1*) were downregulated in Atlantic salmon liver upon post-fasting refeeding, again suggesting an inhibitory role on growth (16). However, such data have not been replicated *in vitro* where neither amino acid deprivation nor addition of Igf-I and amino acids to Atlantic salmon cultured myotubes modified the expression of the same paralogs (57).

Moreover, the regulation of *igfbp-2* expression by Gh does not clearly support a growth inhibitory role common to teleosts. While a study in zebrafish embryos reported that Gh inhibits *igfbp-2* expression (91), work in Atlantic salmon demonstrated an increase in circulating Igfbp-2b in response to Gh (48, 81, 93). By contrast, treatment with dexamethasone, known to enhance catabolism, led to an increase in *igfbp-2a* expression in salmon skeletal muscle myotubes (57). Despite not distinguishing teleost paralogs, recent work revealed upregulation of skeletal muscle *igfbp-2* expression in fine flounder (*Paralichthys adspersus*), concomitant to a rise in blood cortisol (94). Differences in Igfbp-2 expression across studies suggest a complex role for this Igfbp family member in teleost growth, dependent on both physiological and species context.

Igfbp-3: Divergent Physiological Regulation across Teleost Species

Igfbp-3 is the main carrier of circulating Igf in mammals, forming a tertiary structure with the acid-labile subunit (ALS) that increases Igf half-life and regulates Igf bioavailability (95). Igfbp-3 has anti-proliferative effects in many mammalian cell types, preventing the interaction of Igf-I and Igf-II with Igf-1R, and it also has Igf-independent roles (96). In this respect, Igfbp-3 directly interacts with two-cell surface receptors independently of Igf-I, Lrp1, and Tmem29, which mediates its anti-proliferative effects (97, 98). However, mammalian Igfbp-3 can also enhance cellular proliferation in some conditions, through both Igf-dependent and Igf-independent mechanisms (99, 100).

In contrast to mammals, teleost Igfbp-3 proteins are not considered major circulatory Igfbps (6). In fact, there exists no

known association between Igfbp-3 proteins—or indeed any teleost Igfbp subtype—and ALS (6), highlighting fundamental differences in the way Igfs are regulated in circulation. In zebrafish, the single *igfbp-3a* (16) gene has important roles in early development, showing dorsalizing effects in embryos through an Igf-independent interaction with bone morphogenic protein 2b (101), one of few studies demonstrating an Igf-independent role for a teleost Igfbp. The four distinct *igfbp-3* paralogs of salmonids (*igfbp-3a1*, *-3a2*, *-3b1*, and *-3b2*) were lowly expressed in 11 tested adult Atlantic salmon tissues (and each absent in liver), although *igfbp-3a1* was among the most abundant of all Igfbp family member genes in heart (16) and the only detected *igfbp-3* gene in primary myotube culture (57). Conversely, *igfbp-3b* of adult fine flounder was reported as more highly expressed in liver (the main route for Igfbp to circulation) than several other tested tissues, while *igfbp-3a* was not considered in the same study (88).

Studies in zebrafish, flounder, and yellowtail reported a significant increase in the expression of *igfbp-3* genes in liver and/or muscle in response to fasting (55, 88, 102), which may act to restrict Igf signaling. However, on the other hand, studies in salmonids have reported no changes in muscle *igfbp-3* gene expression in response to food deprivation (57, 89) with an increase in *igfbp-3a1* expression in liver during post-fasting refeeding, more consistent with a growth-promoting function (16). Similarly, in coho salmon (*Oncorhynchus kisutch*), *igfbp-3a1* muscle expression was increased by Gh transgenesis (103), again supporting a growth-promoting role. However, stress caused a downregulation of *igfbp-3b* gene expression in skeletal muscle of fine flounder (94), which is inconsistent with a role in growth inhibition.

Overall, there is a relatively limited body of research on teleost Igfbp-3 genes, leaving their roles unclear in many species, with the available evidence suggesting functional divergence among different lineages.

Igfbp-4: Growth-Promoting Role in Some Teleosts?

In mammals, Igfbp-4 is expressed in many cell types and tissues, where it is often considered to inhibit Igf action (104, 105). However, it is also considered to have growth-promoting roles during early embryogenesis, where it enhances Igf-II activity (106). Some studies have reported Igf-independent actions for Igfbp-4, including in relation to the inhibition of apoptosis (104, 105) and cardiogenesis (107).

In teleosts, *igfbp-4* was expressed in most tissues for each species investigated, including Atlantic salmon (16), tiger pufferfish (*Takifugu rubripes*) (108) and fine flounder (88). Moreover, in Atlantic salmon, *igfbp-4* was described as showing high abundance in gill (108). Atlantic salmon was recently shown to retain two highly similar Igfbp-4 paralogs (see **Figures 1** and **2**), which show conserved regulation across tissues (24). In tiger pufferfish, fasting caused upregulation of *igfbp-4* expression in several tissues, consistent with an inhibitory role on growth (108). In addition, the expression of recombinant pufferfish Igfbp-4 in zebrafish embryos resulted in significant growth retardation (108). However, these findings contrast studies of several species (including salmonids and fine flounder), where *igfbp-4* expression in response to nutritional status manipulation suggested a growth-promoting role. Such work revealed no change in *igfbp-4* expression during fasting (57, 65, 88–90, 92), but upregulation in response to subsequent refeeding (57, 65, 88–90, 92, 109–111). A study of Arctic charr (*Salvelinus alpinus*) showed that dwarf populations with highly restricted growth had lower constitutive *igfbp-4* expression in muscle than populations reaching larger body size (112). A growth-promoting role for *igfbp-4* in salmonids was also supported by a strong positive correlation between *igfbp-4* and several pro-myogenic gene markers during *in vitro* myogenesis in Atlantic salmon (110). Studies of Igfbp-4 expression in response to stress also suggest a growth-promoting role. For instance, addition of dexamethasone to Atlantic salmon myotubes (57), and stress confinement in fine flounder (94) induced a significant reduction in *igfbp-4* expression. Conversely, an increase in *igfbp-4* expression was reported in skeletal muscle during maturation-induced atrophy in rainbow trout (113).

Overall, the available evidence suggests that the physiological role of Igfbp-4, when conserved, differs across species and physiological contexts, though for some lineages, particularly salmonids, a growth-promoting function is implicated.

Igfbp-5: Conserved Roles in Muscle Growth

Igfbp-5 is the most conserved Igfbp family member. In mammals, it forms a ternary complex with ALS, similar to Igfbp-3, although much more circulating Igf is carried by Igfbp-3-ALS (114). Igfbp-5 represents an essential regulator of many processes in mammalian bone, kidney, mammary gland, and skeletal muscle (114) and can assert both stimulatory and inhibitory effects (depending on cell type) through Igf-dependent or Igf-independent routes. For instance, it has growth factor-like actions, stimulating bone growth in Igf-I knockout mice (115), and smooth muscle cell migration (116). There is also evidence that Igfbp-5 can translocate into the nucleus (117) and have nuclear functions (118). It is thought that Igfbp-5 cellular internalization is achieved by interaction with membrane proteins such as heparin sulfate proteoglycans, and that the Igfbp-5 N-terminal region has an Igf-independent transcriptional activity (118). Furthermore, Igfbp-5 can interact with transcription co-activators such as the four and half Lim domains 2 (119).

In zebrafish and grass carp embryos, *igfbp-5a* and *igfbp-5b* have distinctive patterns of expression during early development, suggesting evolutionary divergence in regulation (19, 120), which has also been demonstrated at the functional level (19). In adult zebrafish, *igfbp-5a* was expressed at high levels in brain and gill, and lower levels in several other tissues, but was absent in liver or skeletal muscle; while *igfbp-5b* was ubiquitously expressed. Similarly, in other studied teleost species *igfbp-5* genes were reported to show a broad tissue distribution, with differences noted among species and paralogs (51, 52, 55, 63, 120), including three paralogs in Atlantic salmon (16, 24).

The importance of *igfbp-5* genes for muscle differentiation and growth in teleosts is well established. *Igfbp-5* expression has been studied across *in vitro* myogenesis, with both teleost paralogs

(when distinguished) detected from early stages (i.e., myogenic progenitor cells) through to fully differentiated myotubes (57, 110, 111). In Atlantic salmon, both *igfbp-5a* (formerly: *igfbp5.1*) and *igfbp-5b* (formerly: *igfbp5.2*) showed highest expression in early-stage myoblasts, which decreased during myogenic differentiation (110). Using the same *in vitro* models, it was observed that pro-growth stimuli such as amino acids and Igfs increase *igfbp-5* gene expression (111, 113), including both *igfbp-5a* and *igfbp-5b* paralogs distinguished in salmonids (57, 110). However, *igfbp-5* paralogs appear to have different patterns of expression in response to catabolic signals. For instance, while amino acid deprivation had no effect on the regulation of any *igfbp-5* paralog in Atlantic salmon myotubes (57, 113), dexamethasone reduced *igfbp-5a* expression, while simultaneously increasing *igfbp-5b1* (57). A past study of rainbow trout skeletal muscle recorded no change in *igfbp-5* gene expression in response to fasting or re-feeding (89), though it was unclear which paralog was measured. Similarly, *igfbp-5a* and *igfbp-5b* muscle expression did not change in response to short- or long-term fasting in Atlantic salmon (90, 92). However, in grass carp, *igfbp-5a* and *igfbp-5b* expression decreased in skeletal muscle during fasting, while both paralogs were upregulated in liver, and upon injection of Gh in both tissues (120). In Gh transgenic coho salmon, *igfbp-5b1* was significantly upregulated (103).

There is also emerging evidence that *igfbp-5* genes play a role in ionic homeostasis and Igf regulation in zebrafish (121), stickleback [e.g., Ref. (122)] and Atlantic salmon gills (123) with Igfbp-5a acting to regulate calcium influx in zebrafish gills (121) and being differentially expressed and under divergent selective pressures in marine vs. freshwater sticklebacks (122, 124).

To sum up, the available evidence suggests that Igfbp-5 genes play conserved functions in multiple aspects of teleost biology, with roles most clearly demonstrated in myogenesis, muscle growth, and gill function. There is also considerable evidence that both teleost and salmonid-specific Igfbp-5 paralogs have evolved divergent roles.

Igfbp-6: A Growth Inhibitor with Emerging Roles

Igfbp-6 represents a special case among the Igfbp family. In mammals, it has a 50-fold binding preference for Igf-II over Igf-I (125, 126) (a unique feature among Igfbps), but also shows differences in key protein motifs, with three disulfide bonds in the N-terminal region instead of the 4 found in Igfbp-1 to 5 (127). Igfbp-6 is a relatively specific inhibitor of Igf-II actions and, therefore, regulates processes where Igf-II is involved such as proliferation, survival, migration, and differentiation (125, 126). Igfbp-6 also has known Igf-independent actions (125, 128), including the inhibition of fibroblast proliferation (129), cancer cell migration (130), and apoptosis (131). The gene has a broad tissue expression distribution in mammals, including lung, liver, and the gastrointestinal tract.

While differences in the affinity of Igfbp-6 proteins for Igf-II and Igf-I are yet to be confirmed in teleosts, the main underlying structural features are conserved (16). In zebrafish adults, *igfbp-6a* was highly expressed in muscle, and almost undetectable in other tissues, while *igfbp-6b* was only abundant in brain, heart, and muscle (20). In adult fine flounder, *igfbp-6b* was most highly expressed in heart, gills, and the gastrointestinal tract (88). In adult Atlantic salmon, neither *igfbp-6a1* nor *-6a2* were notably expressed across a panel of 11 tissues, while *igfbp-6b1* and *6b2* were each broadly expressed, with the latter being especially highly expressed in gill, brain, and spleen (16). Both *igfbp-6b1* and *6b2* were also reported as being highly expressed in Atlantic salmon gills, where they were dynamically regulated during smoltification (123).

The overexpression of either zebrafish *igfbp-6* paralog caused a significant reduction of embryonic growth (20), suggesting a role in growth inhibition. Studies of *igfbp-6* gene regulation in skeletal muscle support a similar role in other species, though some conflicting data exist. For example, a study in Atlantic salmon reported no change in *igfbp-6b* expression in response to food intake manipulation (92), while another reported downregulation of *igfbp-6b* in tilapia skeletal muscle in response to feeding after a period of fasting (53). Similar results were observed in fine flounder skeletal muscle, where *igfbp-6b* expression decreased in response to feeding immediately post-fasting, although expression then increased during long-term refeeding (88). However, *igfbp-6b* was repressed in fine flounder skeletal muscle in response to stress (94), which is less consistent with a negative role in growth. Conversely, in Atlantic salmon myotubes treated with dexamethasone, *igfbp-6a1* was downregulated, while *igfbp-6b2* was upregulated, highlighting complex functions that cannot be easily interpreted without functional data (57).

Recent work also implies a novel role for *igfbp*-6 genes in linking growth and immune regulation in teleosts. Alzaid et al. observed a significant increase of *igfbp-6a2* in primary immune tissues of rainbow trout following a bacterial infection, and provided evidence that immune-responsive *igfbp-6a2* upregulation was stimulated by immune signaling pathways driven by pro-inflammatory cytokines (27). Past work in salmonids has also shown that pro-inflammatory cytokines can promote the expression of *igfbp-6* genes in skeletal muscle cell cultures (132) and *in vivo* (103), which may be linked to the balancing of energetic allocation toward effective immune function.

In summary, Igfbp-6 genes of teleosts are rather understudied, and it is difficult to draw overarching conclusions about their roles and functions at this time.

PERSPECTIVES AND FUTURE WORK

Our current understanding of the Igfbp repertoire of different teleosts has benefited greatly from recent expansions to genomic resources. We can now be confident in the existence of many teleost paralogs, which are expressed and presumably functional. However, our understanding of the functions and regulatory control of these genes remains highly fragmented across teleosts as a group and remains highly underdeveloped compared to mammals. It is becoming increasingly clear—perhaps with the exception of Igfbp-1—that teleost and mammalian Igfbp orthologs have evolved distinct expression-level regulation. This points to distinct functional roles in the regulation of growth in

teleosts compared to mammals, which may be related to differences in growth dynamics, for example, indeterminate growth in teleosts. Moreover, there is also evidence that Igfbp orthologs from different species have evolved distinct regulation and hence, potentially functions, during teleost evolution. This can be speculatively linked to the additional functional flexibility or redundancy linked to Igfbp duplication events, which has allowed divergent regulation of paralogs to evolve under different physiological contexts.

It is also clear that differences in the expression of homologous Igfbp genes across teleost species are often difficult to interpret. In many cases, this may be linked to the historic ignorance of paralogous genes, either by considering one paralog in pair, or detecting signals from both paralogs in gene expression analyses. Hence, a fuller understanding of Igfbp genes will be possible in the presence of high-quality reference genomes, where all genes are properly annotated and can then be distinguished experimentally. The evidence for divergent regulation of Igfbp paralog expression is overwhelming, even for genes with very similar coding sequences (24), suggesting gene expression studies should make every effort to distinguish Igfbp paralogs.

An additional priority for future research should be to characterize the individual protein-level functions of all teleost Igfbp paralogs in multiple species extending beyond model organisms. While it has classically been challenging to perform functional analyses in non-model teleosts, the research landscape is rapidly changing. For example, genome editing using engineered CRIPSR/Cas9 systems has been demonstrated *in vivo* for various large commercial species, including salmonids (133) and catfishes (134), as well as in teleost cell culture (135). Hence, while even 5 years ago, the full repertoire of Igfbp genes was not even recognized in many teleosts, we can look forward to a future where every paralog within a species has its function cataloged by such approaches, even in lineages with hugely expanded Igfbp gene families. This will open the door for associating protein-level functional divergence in Igfbp paralogs with evolutionary changes in gene expression regulation, generating a fuller picture of the biological roles of this fascinating gene family in teleosts.

AUTHOR CONTRIBUTIONS

DGS and DJM wrote the manuscript and prepared the figures. DM built the phylogenetic tree showed in the manuscript.

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REVIEW
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Insulin- like Growth Factor-Binding Protein Action in Bone Tissue: A Key Role for Pregnancy- Associated Plasma Protein-A

James Beattie[1], Hasanain Al-Khafaji[1], Pernille R. Noer[2], Hanaa Esa Alkharobi[3], Aishah Alhodhodi[1], Josephine Meade[1], Reem El-Gendy[1,4] and Claus Oxvig[2]*

[1] Division of Oral Biology, Leeds School of Dentistry, Level 7 Wellcome Trust Brenner Building, University of Leeds, St James University Hospital, Leeds, United Kingdom, [2] Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark, [3] Department of Oral Biology, Dental College, King AbdulAziz University, Jeddah, Saudi Arabia, [4] Department of Oral Pathology, Faculty of Dentistry, Suez Canal University, Ismailia, Egypt

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Edited by:
Andreas Hoeflich, Leibniz-Institut für Nutztierbiologie (FBN), Germany

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Leon Bach, Monash University, Australia
Cunming Duan, University of Michigan, United States

***Correspondence:**
James Beattie
j.beattie@leeds.ac.uk

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The insulin-like growth factor (IGF) axis is required for the differentiation, development, and maintenance of bone tissue. Accordingly, dysregulation of this axis is associated with various skeletal pathologies including growth abnormalities and compromised bone structure. It is becoming increasingly apparent that the action of the IGF axis must be viewed holistically taking into account not just the actions of the growth factors and receptors, but also the influence of soluble high affinity IGF binding proteins (IGFBPs). There is a recognition that IGFBPs exert IGF-dependent and IGF-independent effects in bone and other tissues and that an understanding of the mechanisms of action of IGFBPs and their regulation in the pericellular environment impact critically on tissue physiology. In this respect, a group of IGFBP proteinases (which may be considered as ancillary members of the IGF axis) play a crucial role in regulating IGFBP function. In this model, cleavage of IGFBPs by specific proteinases into fragments with lower affinity for growth factor(s) regulates the partition of IGFs between IGFBPs and cell surface IGF receptors. In this review, we examine the importance of IGFBP function in bone tissue with special emphasis on the role of pregnancy associated plasma protein-A (PAPP-A). We examine the function of PAPP-A primarily as an IGFBP-4 proteinase and present evidence that PAPP-A induced cleavage of IGFBP-4 is potentially a key regulatory step in bone metabolism. We also highlight some recent findings with regard to IGFBP-2 and IGFBP-5 (also PAPP-A substrates) function in bone tissue and briefly discuss the actions of the other three IGFBPs (-1, -3, and -6) in this tissue. Although our main focus will be in bone we will allude to IGFBP activity in other cells and tissues where appropriate.

Keywords: insulin-like growth factor-binding protein-4, bone, pregnancy-associated plasma protein-A, proteolysis, insulin-like growth factor-binding protein-5

Abbreviations: IGF, insulin-like growth factor; IGFBP, IGF-binding proteins; IGF1R, IGF1 receptor; hDPC, human dental pulp cells; PAPP-A, pregnancy-associated plasma protein-A; STC, stanniocalcin; BMD, bone mineral density; hOB, human osteoblasts.

INTRODUCTION

The insulin-like growth factor (IGF) axis comprises two polypeptide growth factors (IGF1 and IGF2), two cell surface receptors [IGF1 receptor (IGF1R) and IGF2R], and six soluble high-affinity IGF-binding proteins (IGFBP-1–6). Ancillary proteins associated with the IGF axis include various IGFBP proteinases that cleave IGFBPs into fragments with greatly reduced IGF-binding affinity, thus regulating the partition of IGFs between IGFBPs and cell surface receptors (1). IGFs are present at high concentrations in bone matrix (2), and disruption of the IGF1 gene compromises skeletal growth in mice (3) and humans (4). In addition to an anabolic role in mature bone, IGFs also stimulate differentiation of osteoblasts in developing bone tissue and regulate the balance between bone accretion and resorption which occur throughout life (5–7). IGFBP-4 is abundantly expressed in bone tissue (8), and the role of this IGFBP in regulating bone metabolism has been extensively investigated (9–11). In recent years, the activity of a specific IGFBP-4 proteinase, pregnancy-associated plasma protein-A (PAPP-A), has also been investigated in bone and other tissues (12–14). IGFBP-2 and IGFBP-5 are also significantly active in bone tissue demonstrating both IGF-dependent and IGF-independent effects (15–18). Additionally, signaling pathways associated with IGFBP-2 and IGFBP-5 action in osteoblasts and bone tissues have been recently reported (19–22). In this review, we touch on each of these topics and also briefly on the actions of the other IGFBPs (IGFBP-1, -3, and -6) in bone cells and tissues.

INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN-4

Insulin-like growth factor-binding protein-4 was first identified as an inhibitory IGFBP in medium conditioned by the TE89 human osteosarcoma cell line (23) and then cloned from cDNA libraries of various tissues in human and rat (24, 25). It is a 237-residue protein sharing the 3-domain structure previously described for other IGFBPs. Early studies showed that IGFBP-4 inhibited IGF2-stimulated thymidine uptake in primary cultures of human osteoblasts (hOB) (26) and in the MC3T3-E1 mouse osteoblast cell line (27) and inhibited IGF1-stimulated aminoisobutyrate uptake in bovine fibroblasts and in the rat neuronal B104 cell line (28, 29). This inhibitory activity *in vitro* led to the hypothesis that IGFBP-4 generally displayed anti-anabolic and anti-proliferative effects. In confirmation of this, overexpression of IGFBP-4 in a malignant prostate epithelial cell line decreased the proliferative response to IGF1 and delayed tumor development when transfected cells were transplanted into nude mice (30). *In vivo* data also supported an IGF-inhibitory role for IGFBP-4. Tissue-specific overexpression of IGFBP-4 in smooth muscle cells using an α-actin promoter caused smooth muscle hypoplasia (31) and a similar strategy using a protease resistant form of IGFBP-4 (pr IGFBP-4) (see below) resulted in transgenic mice with decreased internal smooth muscle mass in stomach, bladder, and aorta (32). Importantly, with respect to this review, IGFBP-4 overexpression in osteoblasts decreased bone formation and compromised global skeletal growth (11). Some epidemiological data also supported an inhibitory role for IGFBP-4 with increased levels in a cohort of female patients with age-related osteoporotic fractures of the hip and spine (33). Although this evidence suggested an inhibitory role for IGFBP-4, other reports indicated an anabolic role for IGFBP-4. Therefore, systemic administration of IGFBP-4 to mice increased bone tissue markers (osteocalcin and alkaline phosphatase) in serum and skeletal tissues (10). Additionally, IGFBP-4 knockout (KO) mice exhibited prenatal growth retardation, suggesting that IGFBP-4 may be required for full growth promoting effects of IGF2 in the fetus (34). IGFBP-4 KO mice also showed gender dependent changes in skeletal phenotype with female mice having reduced bone mineral density (BMD) along with other features associated with osteopenia (9). Clearly, further research is required to definitively establish the role of IGFBP-4 in bone tissue physiology. In this respect, the observation of IGFBP-4 proteolysis by fibroblast and bone cell cultures has attracted much interest as a means of regulating the activity of IGFs in bone and other tissues, and we provide a short summary of this area in the following section.

IGFBP-4 PROTEOLYSIS

Addition of IGF1 to cultures of human fibroblasts reduced the levels of a 24 kDa IGFBP in conditioned medium and development of specific antibodies confirmed this species as IGFBP4 (35, 36). IGF1-dependent downregulation of IGFBP-4 occurred independently of IGF1R activation and was not associated with changes in IGFBP4 mRNA levels, suggesting a direct post-translational regulation of IGFBP-4. Shortly thereafter, IGF-induced decreases in IGFBP-4 protein levels were shown to be due to the presence of a proteolytic activity in fibroblast-conditioned medium which in cell-free assays was activated by IGF1 or IGF2 (37). IGFBP-4 was cleaved into two discrete fragments by this protease, suggesting a specific cleavage point within the protein (38). The cleavage site was identified at the peptide bond M135-K136 within the central domain of IGFBP-4 producing 14 and 18 kDa protein fragments (29). These data were used to engineer protease-resistant IGFBP-4 mutants that have proven useful in the further study of the biological significance of IGFBP-4 proteolysis (29, 39). This became apparent when intact, but not cleaved IGFBP-4, was shown to inhibit [^{3}H] aminoisobutyric acid uptake into bovine fibroblasts with the inference that cleaved IGFBP-4 fragments did not bind IGF1. Further study indicated that IGF2 was a more potent activator of IGFBP-4 cleavage than IGF1 and IGF2 pre-treatment of human dermal fibroblast cultures increased sensitivity of cell cultures to IGF1. The concept of IGF2-mediated IGFBP4 cleavage as a route for increasing sensitivity to IGF1 (40) may be significant as IGF1 and IGF2 are usually present together in the pericellular environment, suggesting a complex interaction between the growth factors to regulate anabolic responses.

Primary cultures of hOB expressed an IGFBP-4 protease activity identical to that described for fibroblasts (41), and pre-treatment of osteoblast cultures with IGF2 also increased sensitivity to IGF1-stimulated [^{3}H] thymidine incorporation (42). Subsequently, IGFBP-4 protease activity has been reported in human endometrial stromal cells (43) and in porcine aorta-derived smooth muscle cells (44), suggesting that proteolysis of

IGFBP-4 may have widespread biological significance. At around this time, a landmark study identified PAPP-A as the enzyme responsible for IGF-dependent cleavage of IGFBP-4 in fibroblast-conditioned medium (34). PAPP-A was also shown to cleave IGFBP-5 in an IGF-independent manner (45). Identification of PAPP-A as the IGF-dependent IGFBP-4 proteinase caused a paradigm shift in this area of IGF research. Whereas previously IGFBP-4 had been viewed mainly as an IGF-inhibitory IGFBP in tissue culture studies, co-expression of PAPP-A in cell culture could negate this inhibitory effect. Furthermore, the "activation" of PAPP-A by IGFs suggested possible positive feedback loop whereby growth factor action could be enhanced. Further aspects of function, structure, and regulation of PAPP-A activity are discussed below.

PREGNANCY-ASSOCIATED PLASMA PROTEIN-A

Functional Aspects

Pregnancy-Associated Plasma Protein-A was partially purified from human fibroblast-conditioned medium by Lawrence et al (46), and its identity was confirmed by mass spectroscopy. By using polyclonal anti-PAPP-A antibodies, IGFBP-4 protease activity in fibroblast-conditioned medium could be completely inhibited, suggesting that PAPP-A may be the only IGFBP-4 protease expressed by these cells. PAPP-A isolated from fibroblast cultures was found to be identical to the enzyme described in pregnant serum (46–48), showing both IGF dependency and the same site of proteolytic cleavage in the central domain of IGFBP-4 (see above). Identification of PAPP-A allowed some elegant transgenic studies highlighting the importance of this enzyme. Transgenic mice with a collagen I promoter–PAPP-A construct overexpressed PAPP-A specifically in osteoid tissue causing increased calvarial BMD (14). In double transgenic mice overexpressing PAPP-A and a pr IGFBP-4, bone phenotype was similar to single pr IGFBP-4 transgenics, showing decreased calvarial thickness and BMD compared to WT mice. This provided strong evidence that *in vivo* anabolic effects of PAPP-A were due to IGFBP-4 proteolysis, most likely resulting in an increase in the local bioavailability of IGF (49). In confirmation of this, PAPP-A KO mice showed reduced femur BMD and blunted responses to the anabolic actions of parathyroid hormone (12). In a clinical context, PAPP-A has been proposed as a target for anti-proliferative therapies in various cancers. Studies in an ovarian cancer tissue model (50) and using xenografts of adenocarcinoma A549 cells (45) showed that antibody-mediated inhibition of PAPP-A activity decreased tumor growth presumably because pericellular IGF remains bound to IGFBPs leading to a reduction in free IGF in the local tumor environment. This may be important as current anti-IGF-based strategies have proved disappointing in clinical trials. Anti-IGF1R strategies are hampered by hyperinsulinemia secondary to elevated GH levels as a result of impaired IGF-1 feedback at the level of the pituitary (51). This may lead to increased mitogenic signaling by elevated insulin levels through the insulin receptor (IR). IGF1R blockade may also result in IGF1 signaling through the IR or through hybrid IGF1R/IR isoforms which are known to exist in many tissues (52) and which may not be blocked by anti-IGF1R-directed monoclonal antibodies. See the study by Yee (53) for an excellent review of the abovementioned arguments. In contrast, the use of anti-PAPP-A-directed antibodies would not be associated with these complications acting only to inhibit IGF1 release from pericellularly proteolysed IGFBP:IGF complexes.

Structural Aspects

Although PAPP-A was isolated over four decades ago from pregnancy serum (54), it was only after the cloning and expression of this large (1,547 residues) protein that detailed work on protein structure began (55). PAPP-A belongs to the metzincin superfamily of metalloendopeptidases containing a Zn-binding motif and a highly structurally conserved Met-turn (56). PAPP-A associates with the cell surface through two of five short consensus repeat modules within the C-terminus of the protein, and membrane-bound PAPP-A remains catalytically active. This may ensure release of IGF in the vicinity of cell surface IGF1R (57). Under reducing conditions, PAPP-A migrates as a 200 kDa protein although in pregnancy serum (and some other biological fluids) it is primarily present as a disulfide-bound dimer associated covalently with another disulfide bound dimer of the proform of eosinophil major basic protein (proMBP) in a 2:2 heterotetrameric complex (58, 59). The structure of the heterotetrameric PAPP-A:proMBP complex identifies a disulfide bridged dimer of PAPP-A covalently bound to a disulfide-bridged dimer of proMBP *via* two interchain disulfide bridges (60). In this configuration, PAPP-A is inactive with the proMBP dimer binding at or close to the active site of PAPP-A, suggesting that steric inhibition of enzyme activity may result. Both PAPP-A and proMBP are extensively glycosylated, and under non-denaturing gel electrophoresis conditions, the complex runs as a large (>500 kDa) molecular weight species. A mutagenic analysis of the substrate IGFBP-4 suggested that the C-terminal domain of IGFBP-4 conferred the IGF dependence for PAPP-A cleavage of IGFBP-4 (61). In addition, this same study showed that the region between the Zn-binding domain and the Met turn motif of PAPP-A was important for proteolytic activity toward the IGFBP-4:IGF1 complex. Availability of recombinant PAPP-A allowed confirmation that the rate of IGFBP-4 proteolysis is enhanced by binding of IGFs to IGFBP-4 (62), and detailed kinetic analysis confirmed IGF2 as a more potent activator of proteolysis than IGF1. The effect of IGFs on IGFBP4 proteolysis was associated with changes in both affinity (K_m) and turnover rate (K_{cat}). This study also confirmed IGFBP-5 as a PAPP-A substrate although proteolysis of IGFBP5 was not IGF dependent (63). Further mutational analysis suggested that the Lin12-Notch repeat modules present in the C-terminal of PAPP-A are responsible for the differential requirement of IGFBP-4 and IGFBP-5 for IGF during PAPP-A-mediated proteolysis (64, 65).

Regulation of PAPP-A Activity

Relatively few agents have been shown to influence PAPP-A activity. IGFBP-4 proteolysis was inhibited following treatment of fibroblast cultures with phorbol esters. The attenuation of this

effect by prior treatment with actinomycin D or cycloheximide suggested PCK-regulated expression of an inhibitor of IGFBP-4 proteolysis (66). Such an inhibitory activity was also reported in SV40-transformed hOB cells, suggesting that the process of cellular transformation may be associated with inhibition of IGFBP-4 proteolysis (67). The finding that phorbol esters and/or SV40-mediated transformation increased the expression of proMBP – a covalent inhibitor of PAPP-A (see above) – suggested at least one route by which these agents may act to inhibit IGFBP-4 cleavage in fibroblast cultures (68).

An early study reported stimulation of IGFBP-4 proteolytic activity in the rat neuronal B104 cell line by glucocorticoids (69) and following identification of PAPP-A as an IGFBP-4 proteinase, the synthetic glucocorticoid dexamethasone was shown to increase enzyme activity in primary cultures of rat vertebral osteoblasts (13). PAPP-A mRNA levels were not altered by dexamethasone treatment, suggesting a post-transcriptional mechanism by which enzyme activity was increased. In contrast to the above, TGFβ increased PAPP-A mRNA levels approximately 12-fold in hOB cultures, and this was associated with increased PAPP-A activity in conditioned medium (70). The demonstration of increased IGF2-mediated IGFBP-4 cleavage following TGFβ treatment of hOB cultures (71) may be of particular significance given the fact that IGF2 and TGFβ are two of the most abundant growth factors present in bone matrix, and a co-ordinated action of TGFβ and IGF2 in bone matrix to increase local availability of IGF may occur. Osteoblasts secrete IGF peptides endogenously (IGF2 > IGF1) and, despite the fact that the IGFBP-4 levels in osteoblast-conditioned media are typically an order of magnitude higher that the IGF2 levels, endogenous IGF2 can stimulate the proteolysis of concurrently expressed IGFBP-4 protein in osteoblast cultures (72, 73).

Recently, two novel protein inhibitors of PAPP-A activity have been described. These are members of the stanniocalcin family (STC1 and STC2) and were first identified as regulators of Ca homeostasis in teleost fish (74, 75). However, in the context of the mammalian IGF axis, their status as PAPP-A inhibitors indicates that these proteins are negative growth regulators. Overexpression of STC1 or STC2 resulted in growth retardation in transgenic mice (76, 77), whereas KO of STC2 causes increased growth (78). Molecular mechanisms of STC1 and STC2 inhibition of PAPP-A differ with STC2 forming a disulfide-bonded covalent complex with PAPP-A and STC1 forming a high-affinity non-covalent complex with the enzyme. Nonetheless, both STC1 and STC2 potently inhibit PAPP-A which may cause an increased concentration of IGF bound in complex with IGFBP-4 (and IGFBP-5, see below) and hence less bioavailable IGF in the pericellular environment. In agreement with this, STC2 inhibited PAPP-A-stimulated IGF1R phosphorylation in transfected cells exposed to IGF1:IGFBP-4 complexes (74). A recent study using whole exome sequencing of a large human cohort reported two separate single amino acid mutations of STC2 leading to compromised inhibition of PAPP-A. The fact that these alleles strongly associated with increased height in the sampled population is of particular interest (79). A diagrammatic representation of the IGFBP-PAPP-A-STC axis is presented in **Figure 1**.

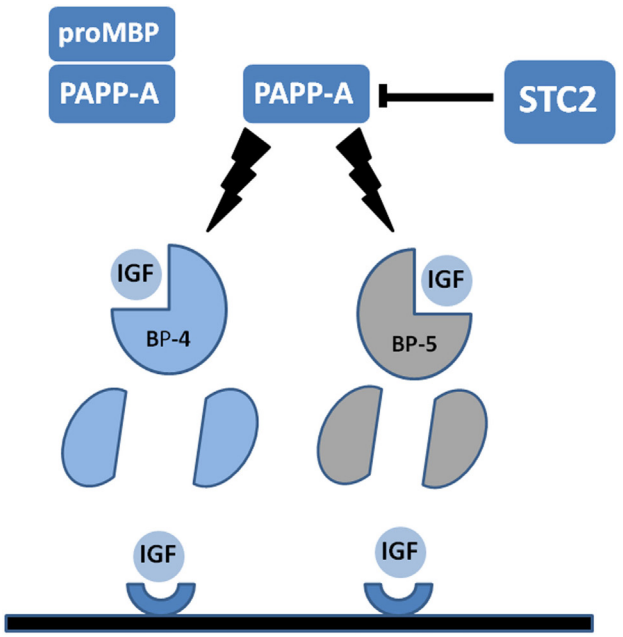

FIGURE 1 | Diagrammatic representation of pregnancy-associated plasma protein-A (PAPP-A) activity in the insulin-like growth factor (IGF) axis. PAPP-A is present in serum and some other biological fluids covalently complexed with the pro-form of eosinophil major basic protein (proMBP). In this form PAPP-A is inactive. Uncomplexed PAPP-A acts to cleave IGF-binding protein-4 (IGFBP-4) and IGFBP-5 into fragments with reduced IGF-binding affinity. IGFBP-4 but not IGFBP-5 requires binding of IGF for PAPP-A cleavage. Proteolysis of IGFBP substrates releases IGFs to allow interaction with cognate cell surface receptors. Recently, discovered stanniocalcins (STC1 and STC2) act to inhibit PAPP-A activity. STC2 is shown, and this inhibitor forms a covalent bond with PAPP-A to inhibit the proteolytic activity. See the text for further details.

PAPP-A IN OTHER SPECIES

Pregnancy-associated plasma protein-A has also been cloned from a mouse cDNA library (80). Although murine (m) PAPP-A shares 91% homology with the human enzyme and cleaves IGFBP-4 in an IGF-dependent manner, mPAPP-A activity is not elevated in pregnant serum or in placenta. In addition, a variant mPAPP-A containing a 29-residue insert (PAPP-Ai) was also isolated. Interestingly, this PAPP-A isoform was a less efficient IGFBP-4 protease than the shorter variant of the enzyme. The significance of these differences between murine and human PAPP-A remains to be resolved, although PAPP-A null mice are 40% smaller than littermates, suggesting a role for PAPP-A during embryogenesis (81). This may be due to diminished IGFBP-4 cleavage and, therefore, reduced IGF availability in the developing fetus. In agreement with this, IGFBP-4 cleavage is absent in fibroblast cultures derived from these null mice. PAPP-A is present in multiple other species, including zebrafish, and interestingly, the absence of PAPP-A in this species causes a developmental delay, which is independent of proteolytic activity (82).

PREGNANCY-ASSOCIATED PLASMA PROTEIN-A2

Overgaard et al. described the cloning of a metalloprotease from placental cDNA libraries with homology to PAPP-A. This protein, which cleaves IGFBP-5 (and IGFBP-3), was named PAPP-A2 (83) and is present in human pregnancy serum where it releases IGF1 from the IGF1:IGFBP-5 complexes (84). PAPP-A2 appears as a monomer of 200 kDa in non-reducing gel electrophoresis and in contrast to PAPP-A does not bind to proMBP or associate with cell surfaces. Cleavage of IGFBP-5 by PAPP-A2 is not IGF-dependent, but as IGFBP-5 has been reported to have both IGF-dependent and IGF-independent effects in hOB cultures (see below), PAPP-A2 activity may also have major relevance in bone cell physiology. In agreement with this, homozygous PAPP-A2 KO mice show decreased post-natal growth along with reduced body length (85). Similarly, conditional PAPP-A2 KO in osteoblasts decreased body mass and bone length, although other tissue sources of PAPP-A2 may be involved in appropriate post-natal growth (86). *PAPP-A2* may represent a quantitative trait locus regulating body shape in mice (87, 88). Recently, two separate families (of Palestinian and Spanish ancestry) were found to have two different inactivating PAPP-A2 mutations that result in growth retardation in homozygous children (89, 90). Further analysis of affected individuals indicated significant increases in IGF1 in ternary ALS complexes with reduced free serum IGF1. In addition, affected individuals showed moderate microcephaly, mild BMD effects, and thin long bones. This phenotype was presumably associated with the inability of mutant PAPP-A2 to proteolyse IGFBP-3 and IGFBP-5 substrates.

OTHER IGFBs IN BONE METABOLISM

Insulin-Like Growth Factor-Binding Protein-5

Insulin-like growth factor-binding protein-5 is also present at high concentrations in bone matrix and has been associated with both inhibitory and stimulatory activities in bone cells and tissues. IGFBP-5 was reported to have IGF-dependent and IGF-independent effects in bone tissue, although the literature is conflicted in this area. IGFBP-5 was shown to enhance IGF-stimulated mitogenesis in hOB cultures (91, 92) and to stimulate the differentiation of two osteoblast cell lines in an IGF-independent fashion (93, 94). In ovariectomized rats, daily subcutaneous injection of IGFBP-5 increased osteoblast proliferation (95) and enhanced the association of IGF1 with bone cells possibly *via* specific cell-surface binding sites for IGFBP-5 (26, 96) or through a specific IGFBP-5 receptor on osteoblast membranes (97, 98). Disappointingly, however, a specific IGFBP-5 receptor has not been isolated or characterized further. Signaling studies suggest that the actions of IGFBP-5 in osteoblasts involve Ras association family isoform C activation of Erk-1/2 (19). The association of IGFBP-5 with four and a half LIM domain protein within the nucleus of U2 osteosarcoma cells has also been reported although the functional significance of this observation remains unknown (99). Although all the abovementioned findings are consistent with a stimulatory role for IGFBP-5 action in bone tissue (IGF-dependent or independent), some authors have reported contrary findings. For example, IGFBP-5 was reported to inhibit IGF1-stimulated proliferation in the U2 human osteosarcoma cell line (100), and transgenic mice overexpressing IGFBP-5 from the osteocalcin promoter showed decreased trabecular bone formation and reduced rates of mineral deposition during the first few weeks of post-natal life (15). Stromal cells isolated from transgenic animals also showed decreased levels of osteogenic markers. Constitutive overexpression of IGFBP-5 in the mouse osteoblast precursor cell line MC3T3-E1 also decreased osteogenic marker expression and delayed formation of mineralized nodules under osteogenic culture conditions (16). Finally, addition of exogenous wtIGFBP-5 or overexpression of IGFBP-5 from an adenovirus promoter inhibited osteoblast differentiation and growth of mouse metatarsal bones in short-term culture (20).

Although IGFBP-5 is cleaved in an IGF-independent manner by PAPP-A and PAPP-A2 (see above), it is also a substrate for other proteolytic enzymes. Matrix metalloproteinase-1 and -2 (MMP-1 and MMP-2) were shown to degrade IGFBP-5 in a time-dependent fashion in medium conditioned by the mouse MC-3T3-E1 cell line (101), and the complement component C1s was identified as an IGFBP5-specific protease in human dermal fibroblast-conditioned media (102). Following on from this, Mohan et al. described a disintegrin and metalloprotease-9 as an IGFBP-5 protease expressed in the U2 human osteosarcoma cell line (103). Although the importance of IGFBP-5 cleavage may (as for IGFBP4) lie with the regulation of free pericellular IGF concentrations, this is somewhat complicated by the observations of IGF-independent actions of IGFBP-5 described earlier. Clearly, these may also be impacted by IGFBP-5 cleavage. Further work is required to establish the role of IGFBP-5 in osteoblast differentiation and in bone tissue metabolism in general.

Finally, there are reports of broad-spectrum proteolytic enzyme families, which degrade IGFBP-5 (and other IGFBPs). These include plasmin (104), thrombin (105), the serine proteases cathepsin G, and elastase (106), although questions of specificity and biological relevance related to these proteases remain largely unanswered.

Insulin-Like Growth Factor-Binding Protein-2

The literature describes both IGF-dependent and IGF-independent effects of IGFBP-2 in osteoblast cultures and bone tissues. An early study using a unilateral disuse osteoporosis model in the rat showed that osmotic minipump delivery of IGF2/IGFBP-2 complexes prevented the decrease in BMD in affected femurs associated with this model (107). A subsequent report from the same group showed that IGF2/IGFBP-2 complexes bound to heparin-Sepharose and that it was suggested that such complexes may associate with ECM components in bone tissue potentially increasing the local concentration of IGFs (108). In agreement with the abovementioned findings, IGFBP-2 potentiated IGF2-induced increases in ALP activity in cultures of rat tibial osteoblasts (109), and we have demonstrated the same effect of IGFBP-2 on IGF1-stimulated ALP activity in

differentiating human dental pulp cells (110). Studies in IGFBP-2 KO mice indicated gender-specific differences in osteogenic phenotype with increased cortical thickness and periosteal circumference in female mice but reduced cortical bone area and trabecular volume in male KOs (111). Although difficult to rationalize, it clearly suggests interplay between the IGF axis and other hormone systems. This same group also reported impaired osteoclastogenesis in bone marrow cells derived from *igfbp2−/− mice* and a transfection study in these cells and indicated that both the IGF and heparin-binding domain (HBD) of IGFBP-2 were required for osteoclast generation (112). This description of IGF-independent effects of IGFBP-2 *in vitro* was confirmed in concurrent studies demonstrating restoration of osteogenic phenotype in *igfbp2−/−* bone marrow cells by addition of a HBD peptide derived from IGFBP-2. In addition, *in vivo* administration of HBD peptide restored osteoblast number in *igfbp2−/−* mice (17). Recently, studies in the mouse MC-3T3 pre-osteoblast cell line showed that IGFBP-2 can bind and inhibit the activity of receptor phosphotyrosine phosphatase β causing increased levels of phosphorylated PTEN, activation of Akt, and stimulation of osteogenesis (17, 18). Further reports from this laboratory highlight the importance of the scaffold/adaptor protein IRS-1, PKCζ, and early activation of AMP-dependent protein kinase in the osteoblast differentiation of primary rat calvarial cells and the differentiating MC-3T3 cell line (21, 22). It should be noted that IGFBP-2 is also a PAPP-A substrate, although this IGFBP is cleaved less efficiently than IGFBP-4 and IGFBP-5 (113).

IGFBP-1, -3, AND -6

Although IGFBP-1, -3, and -6 have all been reported to be expressed in osteoblasts and to be present in bone tissue (114), there are fewer data describing the functions of these three IGFBPs. IGFBP-1 was expressed at low levels in primary hOB cultures under regulation of glucocorticoid and insulin although the physiological relevance of this effect in bone tissue has not been established (115). A recent prospective study (10-year follow-up) in a cohort of elderly women reported a positive correlation between serum IGFBP-1 and osteoporotic fracture, suggesting an IGF-independent osteopenic effect of IGFBP-1 (116). Further data are required on IGFBP-1 and its effects (if any) on bone physiology.

A very early study reported inhibition of IGF1-stimulated DNA synthesis in two osteoblast cell lines by intact IGFBP-3 (117). This inhibitory effect on both IGF1- and IGF2-stimulated DNA synthesis was confirmed in cultures of rat calvarial cells (118). Although these data suggest an inhibitory role for IGFBP-3 in bone metabolism, other *in vivo* data (119) and cross-sectional studies in a cohort of female patients with postmenopausal osteoporosis suggest an anabolic role for IGFBP-3 in maintaining bone density (120).

Insulin-like growth factor-binding protein--6 mRNA was expressed in primary osteoblast cultures derived from fetal rat calvaria (121), and the expression of both mRNA and protein was upregulated in a dose-dependent fashion by cortisol or retinoic acid treatment (122, 123) cultures. Conversely, IGFBP-6 expression was negatively regulated by TGFβ1 in the same cell culture system (124). IGFBP-6 shows a higher affinity for IGF2 than IGF1. Accordingly, it was shown to be a more potent inhibitor of IGF2-stimulated DNA and glycogen synthesis in hOB cells than IGF1 (125). This inhibitory effect of IGFBP-6 was confirmed in the SaoS2 human osteosarcoma cell line using a stable antisense transfection strategy to demonstrate that the anti-differentiative activity of all-trans retinoic acid (Vitamin D) was at least partly mediated *via* IGFBP-6 (126). More recently, IGFBP-6 has been shown to interact with the thyroid hormone receptor alpha1 and to inhibit the tri-iodothyronine-induced increase in osteoblast marker expression in the human U2-OS osteosarcoma cell line (127). In contrast to these reports, the inhibitory effect of IGFBP-6 attenuated by intracellular interaction with the LIM mineralizing protein in both human and mouse osteoblastic cells (128), and one study reported a stimulatory effect of IGFBP-6 on DNA synthesis and mitogenesis in the human osteosarcoma Saos-2/B-10 cell line (129). As for IGFBP-1 and IGFBP-3, the role of IGFBP-6 in osteogenesis and bone tissue physiology has been underreported, and further studies are required to elucidate the role of these 3 IGFBPs in osteogenesis and bone physiology.

CONCLUSION

Six decades have passed since the initial description of the anabolic role of IGF1 in skeletal tissue (130). In the intervening years, much progress has been made in defining the actions of IGF1 and IGF2 and other members of the IGF axis in bone tissue at all stages of development. This review has focused specifically on the function of IGFBPs in osteogenic tissues – both IGF-dependent and IGF-independent. However, the IGF axis acts in a co-ordinated fashion and is integrated with other hormonal systems and growth factor axes to regulate skeletal tissue development and maintenance. It is anticipated that in this and other aspects of IGF axis physiology, many important observations will be made in the near future.

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All the authors contributed to the writing and editing of the manuscript.

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Corrigendum: Insulin- like Growth Factor-Binding Protein Action in Bone Tissue: A Key Role for Pregnancy- Associated Plasma Protein-A

James Beattie[1], Hasanain Al-Khafaji[1], Pernille R. Noer[2], Hanaa Esa Alkharobi[3], Aishah Alhodhodi[1], Josephine Meade[1], Reem El-Gendy[1,4] and Claus Oxvig[2]*

[1] Division of Oral Biology, Leeds School of Dentistry, Level 7 Wellcome Trust Brenner Building, University of Leeds, St James University Hospital, Leeds, United Kingdom, [2] Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark, [3] Department of Oral Biology, Dental College, King AbdulAziz University, Jeddah, Saudi Arabia, [4] Department of Oral Pathology, Faculty of Dentistry, Suez Canal University, Ismailia, Egypt

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****Correspondence:***
James Beattie
j.beattie@leeds.ac.uk

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REVIEW
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Insulin-Like Growth Factor Binding Proteins in Autoimmune Diseases

Huihua Ding[1] *and Tianfu Wu*[2]*

[1] Department of Rheumatology, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China,
[2] Department of Biomedical Engineering, University of Houston, Houston, TX, United States

Insulin-like growth factor binding proteins (IGFBPs) are a family of proteins binding to Insulin-like growth factors (IGFs), generally including IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5, and IGFBP6. The biological functions of IGFBPs can be classified as IGFs-dependent actions and IGFs-independent effects. In this review, we will discuss the structure and function of various IGFBPs, particularly IGFBPs as potential emerging biomarkers and therapeutic targets in various autoimmune diseases, and the possible mechanisms by which IGFBPs act on the pathogenesis of autoimmune diseases.

Keywords: IGFBPs, autoimmune diseases, biomarkers, therapeutic targets, metabolism

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Alain Couvineau,
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Wendie Cohick,
Rutgers University, The State University of New Jersey, United States

***Correspondence:**
Tianfu Wu
twu13@central.uh.edu

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INTRODUCTION

Insulin-like growth factor binding proteins (IGFBPs) are a group of secreted proteins which serve as transport proteins for insulin-like growth factors (IGFs) with high affinity, regulating the bioavailability and function of IGFs. The IGFBP family consists of six IGFBPs, namely IGFBP1 through IGFBP6, however other proteins with low binding affinity to IGFs were incorrectly named as IGFBP7, IGFBP8, IGFBP9 etc., a consequence of belonging to the IGFBP-related protein (IGFBP-rPs) family (1, 2). Due to the conserved protein structure and high binding affinity to IGFs, only IGFBP1 through IGFBP6 are considered true IGFBPs. The eponymous function of IGFBPs is achieved through binding to IGFs thus regulating their biological activity; however, in pathological conditions, especially under cancer status, the role of IGFBPs in IGF-independent pathways has prompted increasing attention (3). In autoimmune diseases, IGFBPs are also known to play a role in the development of diseases both through IGF-dependent and IGF-independent pathologies (4, 5). In this review, we focus on the six true IGFBPs and their roles in autoimmune diseases, including their potential roles as biomarkers and therapeutic targets.

THE IGFBP FAMILY: STRUCTURE AND FUNCTION

The IGFBP family comprises six structurally similar proteins with high affinity to IGFs. The six proteins, with a mass of ∼24 to 50 kDa (216–289 amino acids), share a highly conserved structure with three domains of similar sizes: the conserved N-terminal cysteine rich region and the C-terminal cysteine rich region connected by a less structural and less conserved linker region (6, 7) (**Figure 1**). High-affinity IGFs binding capacity requires both the N- and C-terminals, with relative IGFs-binding affinities differ among IGFBPs (6). The linker domain is highly variable and unique to each IGFBPs; It's involved in the proteolysis of IGFBPs by several proteases, with cleavage sites located in the domain (7–9). The proteolysis of IGFBPs results in IGFs release, which provides a mechanism for the post-translational modification of IGFBPs. Other post-transcriptional modifications, such as glycosylation and phosphorylation, have also been

implicated in this region which can modulate the function of IGFBPs (10–12). The linker domain also indirectly plays a role in high affinity IGF binding through inter-domain movement control during ligand binding (13).

The biological function of IGFBPs can be classified as IGFs-dependent actions and IGFs-independent effects. Both IGF-I and IGF-II are protein hormones structurally and functionally similar to insulin, which play extensive roles in growth and development. When binding to insulin-like growth factor 1 receptor (IGF1R), IGFs activate the intracellular IGF signaling pathway and promote cell proliferation and differentiation as well as inhibit cell apoptosis (14, 15). In blood, most IGFs (∼75%) circulate as a 150 kDa ternary complex containing IGFs bound to IGFBP3 and a glycoprotein called acid labile subunit (ALS) (16–18). The rest of IGFs (∼25%) are bound to the six IGFBPs to form a 50 kDa binary complex. It is through this binding that IGFBPs regulate the bioavailability of IGF-I and IGF-II in a range of ways. Firstly, they transport and store IGFs in circulation such that IGFs are more stable with an extended half-life of several hours in ternary complex and several minutes in a binary complex (16). Secondly, IGFBPs modulate the action of IGFs in both positive and negative manners. Since IGFBPs have higher affinity to IGFs than IGF1R, the binding of IGFBPs to IGFs sequestrate IGFs from their receptors inhibiting the effects of IGFs (19–21). This inhibitory effect has been widely proved in the case of IGFBP4 in both *in vitro* and *in vivo* studies (22–24). On the other hand, some IGFBPs (IGFBP1, IGFBP3, IGFBP5) may stimulate IGF actions in certain circumstances.

The IGF independent action of IGFBPs include effects on cell adhesion and migration, cell growth and apoptosis (19, 25). IGFBP1 was reported to increase cell migration of Chinese hamster ovary (CHO) cells, which was mediated through the binding of RGD motif in C-terminal of IGFBP1 to $\alpha_5\beta_1$ integrin (26). In human carotid plaques, the expression of IGFBP1 is significantly increased and IGFBP1 has been proven to stimulate smooth muscle cells proliferation through ERK1/2 activation (27). Both the intact and proteolyzed form of IGFBP3 have been demonstrated to have IGF-independent growth-stimulatory and inhibitory effects in several cell lines including a range of cancer cell lines (28–34). IGFBP3 also protects against retinal endothelial cell apoptosis through inhibition of TNF-α production (35). In the respiratory system, IGFBP3 treatment reduces airway inflammation and hyper-responsiveness via activation of IGFBP3 receptor pathway (36). IGFBP5 fragments were first shown to stimulate osteoblast mitogenesis in the absence of IGF-I and recombinant human IGFBP5 stimulated osteoblast proliferation without the aid of IGF-I (37–39). Using genetic engineering, Pell JM et al proved that IGFBP5 played an important role in cell proliferation and apoptosis both *in vitro* and *in vivo* via an IGF-independent mechanism (40, 41). Due to the different structures of various IGFBPs, the expression, binding kinetics and dynamics may vary in a dose-dependent manner at temporal and spatial levels and may all contribute to the specific functions of IGFBPs at different physiological situations.

IGFBPS AS BIOMARKERS IN AUTOIMMUNE DISEASES

The past few decades have witnessed a growing interest in the exploration of biomarkers in human diseases. This growth is fueled by the biological functions of biomarkers as they can be used as diagnostic tool to improve the accuracy of diagnosis, possible early detection of diseases, monitoring markers allowing for elucidation of disease activity and complications. Furthermore, they can also be used as prognostic markers allowing for prediction of possible patient outcomes.

IGFBP family members have been indicated to be involved in the development and progression of tumors and may be useful prognostic biomarkers in various malignancies (3). Recent studies also validated IGFBPs' role in the diagnosis and prognosis prediction in some solid tumor including nasopharyngeal carcinoma, ovarian cancer, pancreatic cancer, etc. (42–46). Despite the huge development of IGFBPs as biomarkers in cancer, there have been a number of studies focusing on the utility of IGFBPs as biomarkers in autoimmune diseases (**Table 1**). Despite the advances of researches on IGFBPs as biomarkers in cancer, there have been several studies focusing on the utility of IGFBPs as biomarkers in autoimmune diseases (**Table 1**), most of which were investigating the diagnostic role of IGFBPs in the disease. While the potential role of IGFBPs as diagnostic biomarkers has been summarized below, the mechanism of action for these molecules has not been widely investigated. Here we summarized studies investigating the potential roles of IGFBPs in the diagnosis and monitor of autoimmune diseases.

Type 1 Diabetes Mellitus (T1DM)

Type 1 diabetes mellitus (T1DM) is an organ-specific autoimmune disease in which the insulin-producing pancreatic β cells are destroyed by the immune system. Due to the close relationship to insulin, the system of IGF and its binding proteins has been first explored as biomarkers in diabetes, especially T1DM. Serum IGFBP1 levels were consistently reported to be increased in T1DM population including prepubertal and pubertal individuals (47–52). In children with new onset diabetes, serum IGFBP1 served as a differential diagnostic marker for T1DM and T2DM (50). In young T1DM patients, IGFBP1 levels were increased independent of pubertal status (52). Besides being a diagnostic marker, serum IGFBP1 level was a good indicator of complications related to T1DM. In T1DM patients complicated with microalbuminuria, serum IGFBP1 level has been reported to be significantly increased (53). It's also a biomarker for diabetic retinopathy in T1DM but not T2DM patients (54). Serum IGFBP2 levels were also reported as an increased biomarker in T1DM (53, 55). In a recent study, Zhi et al. used a proteomic approach to identify IGFBP2 as a potential diagnostic biomarker for T1DM (56). However, in diabetic retinopathy, IGFBP2 levels have been reported to be either increased or decreased in T1DM patients (51, 54). IGFBP3, the most abundant type of IGFBPs, has been widely studied as biomarker in T1DM. In T1DM patients, serum

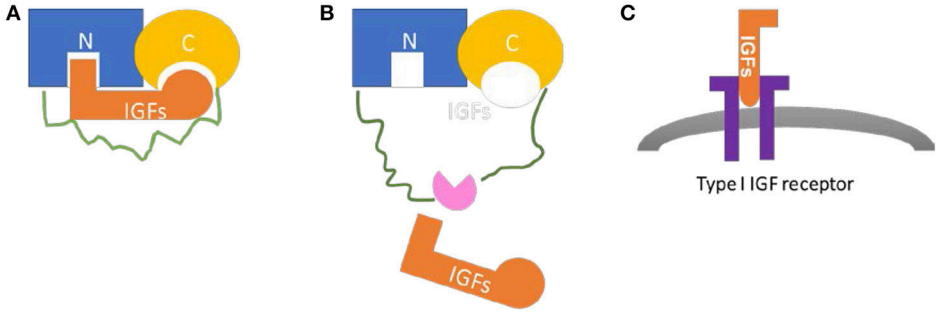

FIGURE 1 | Common structure and biological function of IGFBPs. **(A)** The N-terminal region (blue) and the C-terminal region (yellow) are connected by a linker region (green). Both N-domain and C-domain contains a binding site for IGFs (orange). **(B)** The proteolysis of IGFBPs by various proteases (pink) occurs in the linker region or other post-translational modifications, may result in IGFs release. **(C)** Once released from IGFBPs, IGFs bind to IGF receptors (purple) to exert their physiological effects.

TABLE 1 | Summary of IGFBPs as biomarkers in autoimmune diseases.

Autoimmune disease	GFBP1	IGFBP2	IGFBP3	IGFBP4	IGFBP5	IGFBP6	References
Type I diabetes	↑	↑/↓	↑/–	↑		↓	(47–63)
Multiple sclerosis	↑/–	↑/–	↑/↓/–				(64–70)
Rheumatoid arthritis		↑	Synovial fluid ↑, Serum ↑/↓				(71–83)
Juvenile idiopathic arthritis			Synovial fluid ↓, Serum↓				(84–86)
Systemic lupus erythematosus		↑		↑			(87–90)
Systemic sclerosis			↑		↑		(91–93)
Inflammatory bowel disease		↑					(94)

↑, means increased in serum; –, means no change; ↓, means decreased in serum.

IGFBP3 levels have reported to be significantly decreased in different populations, which was partially explained by increased proteolysis (47, 48, 53, 55, 56). However, in T1DM women during pregnancy, serum IGFBP3 levels have been implicated to either increase or decrease, which might be explained by the different testing time period (57, 58). IGFBP3 level also worked as a severity marker for T1DM. It correlated with HbA1c, total cholesterol, and LDL-cholesterol levels (59) and inversely correlated with blood pressure (60). Besides, patients complicated with autoimmune thyroiditis and coeliac disease had significantly lower serum concentration of IGFBP3 (61). More interestingly, IGFBP3 levels were significantly increased in the tears of diabetic patients, indicating the potential contribution to pathogenesis of ocular complications in diabetes (62). IGFBP4 was only reported in one study to be significantly increased in prepubertal T1DM children (47). More recently, higher IGFBP4 fragment levels was reported to be associated with cardiovascular mortality rates in T1DM patients, which made it a potential prognostic biomarker (63). Decreased IGFBP6 was reported to be related with diabetic retinopathy in both T1DM and T2DM (54).

Multiple Sclerosis (MS)

Multiple sclerosis (MS) is a demyelinating autoimmune disease in which the immune system damages the myelin sheath of the nerve cells in the central nervous system. The first study investigating IGFBPs concentrations in MS patients revealed no differences in concentrations of IGFBP1, IGFBP2, and IGFBP3 in circulation and cerebrospinal fluid between MS patients and controls (64). Later on, with the enlargement of study population size, Al-Temaimi et al. reported an increased IGFBP1 level in female MS patients (65). Consistent with this, IGFBP1 as well as IGFBP6 were proven to be overexpressed in oligodendrocytes at the edges of demyelinated plaques, indicating a pathogenic role of them in the development of MS (66). Other studies focused on the serum concentration of IGFBP3 in MS, but the results lacked consistency (67–70). This inconsistency of IGFBP3 levels in MS patients may be due to the differences of sample size and patients' demographic characteristics. However, IGFBP3 level correlated with disease severity score, relapsing-remitting disease pattern and treatment strategy, indicating the involvement of IGFBP3 in the pathogenesis of MS (67, 69, 70). Serum IGFBP2 was reported to be increased in MS patients compared to healthy control (69).

Rheumatoid Arthritis (RA)

Rheumatoid arthritis (RA) is systemic inflammatory disease that primarily affects the joints. The profile of IGFBPs were first characterized in the synovial fluid of RA patients, in which IGFBP2, IGFBP3, and IGFBP4 levels were significantly elevated compared to normal individuals or osteoarthritis patients (71–74). IGFBP3 levels in the synovial fluid of RA patients correlated with systemic C-reactive protein (CRP) levels, indicating the

involvement of IGFBP3 in inflammation (73). Unlike the synovial fluid, IGFBPs profiling in circulation of RA patients remains controversial. Early studies demonstrated a decreased serum level of IGFBP3 in RA patients (73, 75, 76), which correlated with habitual exercise level (75). However, other groups didn't observe the effect of dynamic exercise on serum IGFBP3 (77, 78). Toussirot et al. compared serum IGFBP3 levels in corticosteroid-treated RA patients, non-RA patients under corticosteroids treatment, and healthy population (79). No significant differences were observed. More recently, a number of studies have demonstrated an increased IGFBP3 levels in RA patients, which correlated with serum CRP level (80–82). The inconsistency of circulating IGFBP3 levels in RA patients could be due to the heterogeneity of RA population as well as differences in disease status and treatment since the treatment strategy for RA has been changed dramatically over time. Other than IGFBP3, serum IGFBP2 and IGFBP1 have been reported to increase in RA population (81, 83). In juvenile idiopathic arthritis, the expression of IGFBP3 was decreased both in the circulation and synovial fluid (84–86).

Systemic Autoimmune Diseases

Systemic autoimmune diseases such as systemic lupus erythematosus (SLE), systemic sclerosis, inflammatory bowel disease and idiopathic pulmonary fibrosis have also been reported to be associated with IGFBPs (87–91, 94, 95). In SLE, IGFBP2, IGFBP4 and IGFBP6 were discovered by array-based proteomic screening as diagnostic biomarkers for lupus (89). Later validation studies using a larger study population confirmed the role of IGFBP2 as a diagnostic biomarker for SLE as well as lupus nephritis (88, 90).

Moreover, IGFBP2 is a potential disease monitoring biomarker for renal function and renal histopathologic changes in lupus nephritis (88). Another SLE marker, IGFBP4, was mainly increased in lupus nephritis patients, which makes it

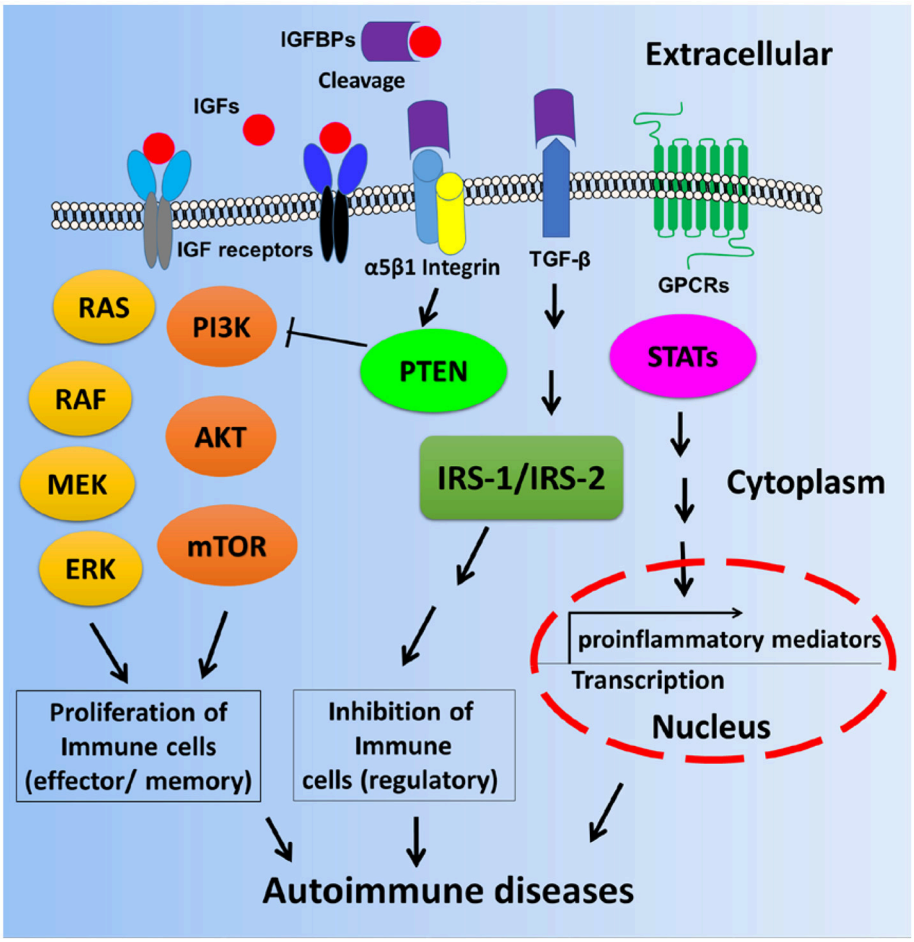

FIGURE 2 | Scheme of possible mechanisms by which autoimmune diseases are mediated by IGF-IGFBP signaling pathways. Immune cell proliferation may be partly dictated by IGF-IGFBP pathway. The inhibition of regulatory immune cells or the control of gene transcription of proinflammatory cytokines may be mediated by IGF-independent pathways such as IGFBP3, TGF-β, and GPCR etc. STAT: Signal transducer and activator of transcription including STAT1, STAT2, STAT3, and STAT4.

a good indicator for renal pathology chronicity changes (87). In systemic sclerosis, serum IGF-1 and IGFBP-3 levels were significantly elevated compared to SLE patients or healthy control (91). Yasuoka et al. demonstrated the overexpression of IGFBP5 in animal models of systemic sclerosis could induce fibrosis in the lung and the skin (92, 93). In inflammatory bowel disease patients, serum proteome profiling revealed elevated IGFBP2 levels in untreated patients and suppressed by steroids treatment, indicating the pro-inflammatory effect of IGFBP2 (94). Serum IGFBP1 and IGFBP2 are elevated in idiopathic pulmonary fibrosis patients and IGFBP2 level was significantly reduced by anti-fibrotic therapy (95).

All these studies suggest that IGFBPs are indicative of disease activity, although larger cohort of patients are still needed to validate these findings. Nevertheless, it is expected that these IGFBPs molecules may be involved in the pathogenesis of these autoimmune diseases and may serve as disease biomarkers to monitor flares or track drug responses.

IGFBPS IN AUTOIMMUNE DISEASES: POSSIBLE MECHANISMS

Although IGFBPs have been proved as potential biomarkers in a variety of autoimmune diseases, the underlying mechanism remains unveiled. Given that the biological function of IGFBPs can be divided into IGFs-dependent and independent effects, the underlying mechanism of IGFBPs in autoimmune diseases can also be divided into IGFs-dependent and IGFs-independent mechanisms. Immune regulation of IGF-I has been reviewed in detail elsewhere (96). A recent genome-wide association study (GWAS) in SLE patients suggested that serum IGF-1 levels were increased with SLE disease severity, and SLE may be affected by a modulation of the IGF-1 signaling pathway and +3179G/A IGF-1R polymorphism (97).

The following studies suggested that IGFBPs have direct effects on immunity and inflammation. Peripheral blood mononuclear cells (PBMC) mainly consist of lymphocytes and monocytes, representing cells of both the innate and adaptive immune systems. Early study demonstrated an inhibitory effect of IGFBP1 on the proliferation of activated PBMCs (98). However, an increase of IGFBP2 expression was indicated in activated human PBMCs and exogenous IGFBP2 treatment of enhanced the proliferation of human PBMCs, suggesting the involvement of IGFBP2 in lymphocyte proliferation (98). Intra-tracheal administration of IGFBP5 in mice induced mononuclear cell infiltration in the lung and *in vitro* IGFBP5 can stimulate migration of PBMCs (93). This monocyte/macrophage system contributes to autoimmune diseases and inflammation by phagocytosis and antigen presentation. Furthermore, several studies have demonstrated the impact of different IGFBPs on monocyte/macrophage. An increase of IGFBP3 expression was first reported to be related with increased monocyte apoptosis stimulated by lipopolysaccharide, which was proved to be IGF independent (99). Recently, IGFBP3 was demonstrated to inhibit monocyte-endothelial cell adhesion through down-regulate ICAM-1 expression under hyperglycemic condition, which can inhibit retinal inflammation (100). In the RA synovium, IGFBP3 was produced by macrophage (82). In a mouse model of lung fibrosis, IGFBP5 induced migration of activated CD4+T cells and monocytes, indicating a chemoattractant activity of IGFBP5 for immune cells (101). In the same model, monocytes treated with IGFBP5 acquired a mesenchymal phenotype *in vitro* and *in vivo* (101). *in vitro* culture of human hematopoietic stem cells revealed an inhibitory effect of IGFBP3 and a stimulatory effect of IGFBP6 on the development of pro-B-cell, which might be IGF-1 dependent (102). IGFBP3 was also proved to support the development and maintenance of naïve CD8+ T cells, indicating the beneficial impact of IGFBP3 in maintaining a health immune system (103). IGFBP2 supplement can maintain vigorous hematopoietic cell expansion and CD34+ phenotype (104).

These interesting findings suggest that IGFBPs may be involved in the pathogenesis of various autoimmune diseases, either via IGF-1 dependent signaling pathways or IGF-1 independent signaling pathways as depicted in **Figure 2**. Based on current knowledge, it is more apparent that IGF-IGFBP signaling axes may dictate cell proliferation and affect immune cell function or tissue damage. However, it is uncertain whether IGFBPs expression could impact proinflammatory pathways such cytokine production in the context of autoimmune diseases. Therefore, more delicate mechanistic studies are warranted in order to uncover the molecular and cellular basis and function of IGFBPs in autoimmune diseases.

LOOK INTO THE FUTURE

In the past decade, there has been a marked change in the way biomarker is discovered since the advent of high throughput techniques such as genomics, proteomics and metabolomics etc. The discovery of biomarkers used to be mechanism-driven, in which researchers choose candidate biomarkers based on their involvement in the pathogenesis of the disease to validate their role as biomarkers for disease diagnosis, disease monitoring, and prognostic evaluation. The investigation of IGFBPs in T1DM was a good example of this mechanism-driven strategy since IGFBP family is involved in insulin regulation, leading to the hypothesis that they might play a pathogenesis role in the development of T1DM. Most of the studies investigating the use of IGFBPs as biomarkers in autoimmune diseases in this review were based on the mechanism-driven strategy. Only two studies used proteomic technique to discover potential biomarkers for T1DM (56) and SLE (89). The untargeted global proteomic biomarker discovery is also called data-driven strategy, in which researchers use high throughput platforms to find potential biomarker from thousands of molecules regardless of their pathogenic role in the development of the disease (105).

Currently studies on IGFBPs role as biomarkers in autoimmune diseases have the following pitfalls. Firstly, most of the studies used conventional strategies instead of "omics" based high-throughput techniques. Future studies should adopt the more efficient and less biased strategy to discover biomarkers in autoimmune diseases. Secondly, almost

all the studies adopted retrospective case-control study design, in which selection bias cannot be avoided. In the future, prospective studies are highly recommended to validate the existing IGFBPs role as biomarkers in autoimmune diseases. In addition, some IGFBPs such as IGFBP2 were proved to be increased in several different autoimmune diseases including T1DM, MS, RA, SLE, and IBD. It is not clear whether IGFBP2 is a general marker for autoimmunity or it is specific to certain kind of autoimmune diseases. A direct comparison of IGFBP2 levels in patients with these diseases should be helpful in clarifying this issue. Thirdly, the pre-analytical conditions including sample collection and processing for various studies were different, which partially explain the inconsistency of some studies on the validation of the same biomarker in different study population. To overcome this, a standardized protocol for sample handling or biobanking should benefit future studies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Current IGFBP-Related Biomarker Research in Cardiovascular Disease—We Need More Structural and Functional Information in Clinical Studies

Andreas Hoeflich[1]*, *Robert David*[2,3] *and Rikke Hjortebjerg*[4,5]*

[1] Department of Genome Biology, Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany, [2] Department of Cardiac Surgery, Reference and Translation Center for Cardiac Stem Cell Therapy, Rostock University Medical Center, Rostock, Germany, [3] Department Life, Light and Matter, Interdisciplinary Faculty, Rostock University, Rostock, Germany, [4] Medical Research Laboratory, Department of Clinical Medicine, Faculty of Health, Aarhus University, Aarhus, Denmark, [5] The Danish Diabetes Academy, Odense, Denmark

Cardiovascular diseases are the leading cause of death around the world and the insulin-like growth factor (IGF)-system has multiple functions for the pathological conditions of atherosclerosis. IGF binding proteins (IGFBPs) are widely investigated as biomarkers for pathological disorders, including those of the heart. At the tissue level, IGFBP-1 to -6 decrease bioactivity of IGF-I and -II due to their high affinity IGF-binding sites. By contrast, in the circulation, the IGFBPs increase biological half-life of the IGFs and may therefore be regarded as positive regulators of IGF-effects. The IGFBPs may also exert IGF-independent functions inside or outside the cell. Importantly, the circulating IGFBP-concentrations are regulated by trophic, metabolic, and reproductive hormones. In a multitude of studies of healthy subjects and patients with coronary heart diseases, various significant associations between circulating IGFBP-levels and defined parameters have been reported. However, the complex hormonal and conditional control of IGFBPs may explain the lack of clear associations between IGFBPs and parameters of cardiac failure in broader studies including larger populations. Furthermore, the IGFBPs are subject to posttranslational modifications and proteolytic degradation by proteases, upon which the IGFs are released. In this review, we emphasize that, with the exception of IGFBP-4 and in sharp contrast to the preclinical studies, virtually all clinical studies do not have structural or functional information on their biomarker. The use of analytical systems with no discriminatory potential toward intact vs. fragmented IGFBPs represents a major issue in IGFBP-related biomarker research and an important focus point for the future. Overall, measurements of selected IGFBPs or more complex IGFBP-signatures of the family of IGFBPs have potential to identify pathophysiological alterations in the heart or patients with high cardiovascular risk, particularly if defined cohorts are to be assessed. However, a more thorough understanding of the dynamic IGF-IGFBP system as well as its proteases and protease inhibitors in both normal physiology and in cardiovascular diseases is necessary.

Keywords: IGFBP, PAPP-A, IGFBP-fragment, mortality, cardiovascular diseases, IGF

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Pierre De Meyts,
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Cheryl Conover,
Mayo Clinic, United States
Claire Perks,
University of Bristol, United Kingdom

***Correspondence:**
Rikke Hjortebjerg
rikke.hjortebjerg@clin.au.dk
Andreas Hoeflich
hoeflich@fbn-dummerstorf.de

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INTRODUCTION

In Europe (1) and in the US (2), cardiovascular diseases are heading the statistics on causes of death. It is well-known that the insulin-like growth factor (IGF)-system actively contributes to the pathological conditions of atherosclerosis, including activation of smooth muscle cells and macrophages, angiogenesis, and restenosis (3). First of all, IGF-I and IGF-II are potent stimulators of smooth muscle cell proliferation (4, 5). In human arterial smooth muscle cells, IGF-I has been identified as a potent effector of chemotaxis (4). IGF-I has also been shown to increase the release of proinflammatory cytokines and low-density lipoprotein (LDL) uptake, which facilitate atherosclerosis and plaque instability (3). In addition, it is thought that the IGF-system supports accumulation of extracellular matrix in the vessel walls (3), which may occur by the control of matrix degrading enzymes (6). Under conditions of reduced IGF-I concentrations, levels of matrix proteins (actin and procollagen 3A1) are decreased, whereas matrix metalloproteinase levels (MMP-3 and−13) are elevated in smooth muscle cells (6). Supplementation by IGF-I normalizes both matrix proteins and matrix degrading enzymes (6). Interestingly, IGF-I concentrations in *in vitro* cultivated smooth muscle cells are affected when cell culture medium conditioned by macrophages is used.

IGF bioavailability is strictly regulated by six high-affinity IGF binding proteins (IGFBPs) that are ubiquitously produced in most tissues. The IGFBPs bind the IGFs on a 1:1 molar basis and prevent receptor activation, but they also serve to prolong IGF half-life. IGFBP-3 is the most abundant IGFBP in adult serum with a concentration of approximately 3,000 ng/mL, whereas the remaining IGFBPs circulate at concentrations of 20–500 ng/mL (7). Due to their affinities and high concentrations, <1% of IGF is circulating in the free form (7, 8). The acid labile subunit (ALS) is found almost exclusively in the circulation and binds to preformed complexes composed of IGF and IGFBP-3 or IGFBP-5. Due to the size of the ternary complex, approximately 80% of all IGF-I is sequestered in the intravascular compartment. By contrast, the IGFs are predominantly bound to the IGFBPs in binary complexes within tissues (9). Consequently, the IGFBPs create a reservoir of readily available IGF (primarily IGFBP-3 and -5) and control tissue-specific efflux and distribution (primarily IGFBP-1,-2, and -4) (1, 2). Thus, the IGFBPs serve as important determinants of IGF actions and like the IGFs, the IGFBPs have been suggested to play a role in the pathogenesis of atherosclerosis. Of note, in the fetal and adult human heart, IGFBP-3 appears to be expressed at high levels (10), and in the developing rat heart, mRNA for IGFBP-3,−4, and−5 has been demonstrated (11). Importantly, based on the GeneCards^{R} database entries, all IGFBPs can be detected in the normal human heart. Accordingly, several IGFBPs have been suggested as attractive cardiovascular markers, although local vs. systemic effects of IGFBPs in the heart have not been resolved and causal relationships between IGFBP perturbations and the development of atherosclerosis remain to be firmly established (12). While the biomarker potential of growth hormone (GH) or IGF-I in heart failure has been discussed just recently (13), the present review for the first time addresses the biomarker potential of all IGF binding proteins (IGFBPs) in cardiovascular diseases. In order to understand the potential effects of local IGFBP-expression in the heart, the discussion of clinical studies is extended by concepts and hypotheses derived from selected functional studies of IGFBPs in the heart and entire circulatory system. Cardiovascular disease is a group of diseases that includes both the heart and blood vessels, and of which most are caused by atherosclerosis, where the inside of an artery narrows due to the accumulation of an atherosclerotic plaque. Ischemic heart disease [IHD, alternatively coronary artery disease (CAD) or coronary heart disease (CHD)] is the umbrella term for stable and unstable angina, acute myocardial infarction (AMI, commonly known as heart attack), and sudden cardiac death. AMI and unstable angina defines the acute coronary syndrome (ACS). Stroke is the subtype caused by a disruption in the flow of blood to part of the brain either due to blood vessel occlusion (ischemic stroke) or rupture (hemorrhagic stroke). Congestive heart failure is the end stage of several circulatory diseases and characterized by abnormal myocardial function and insufficient ability to maintain blood flow.

On a final note, it should be acknowledged that research in the area is still in its early phases, and larger and more comprehensive investigations are needed to fully assess the biomarker potential of the IGFBPs as well as potential redundancy function within the IGFBP family. Moreover, it is pivotal to appreciate the relation of cause and effect. Most theories derive from findings in cross-sectional studies and such observations should be considered as hypothesis generating as they cannot give evidence of causality. Finally, in all paragraphs to come, it is urged to remember that numerous studies have been performed using assays with undocumented specificity toward the IGFBPs, and our evaluation and interpretation of studies should be seen in the light of that.

Abbreviations: ACS, acute coronary syndrome; ALS, acid labile subunit; AMI, acute myocardial infarction; CAD, coronary artery disease; CASC, cardiac atrial appendage stem cell; CHD, coronary heart disease; CT, carboxyl-terminal; CV, cardiovascular; CVD, cardiovascular disease; ECG, electrocardiogram; GH, growth hormone; HDL, high-density lipoprotein; IGF, insulin-like growth factor; IGFBP, IGF binding protein; IHD, ischemic heart disease; IMT, intima-media thickness; LDL, high-density lipoprotein; MACE, major adverse cardiac events; MI, myocardial infarction; MMP, matrix metalloproteinase; NSTEMI, no elevation of the ST segment; NT, amino-terminal; PAPP-A, pregnancy-associated plasma protein-A; STEMI, elevation of the ST segment; T2D, type 2 diabetes; TNF, tumor necrosis factor.

IGFBP-1

Much attention has focused on IGFBP-1 as a partaker in metabolic diseases, as it is negatively regulated by insulin, glucose, and GH (14). In prediabetic patients, IGFBP-1 is reduced, but as the disease progresses, so does pancreatic secretory capacity, resulting in chronic insulin deficiency as well as increased IGFBP-1 levels. Thus, the biomarker potential of circulating IGFBP-1 is largely biased by conditional and age-related insulin or GH-insensitivities. In the heart, IGFBP-1

expression is differentially regulated by insulin (15). In smooth muscle cells, IGFBP-1 expression (**Table 1**), which is increased by interleukins and TNFalpha, exerts IGF-dependent as well as—independent effects on cell proliferation (16). Because higher concentrations of IGFBP-1 were found in aortic plaques, IGFBP-1 was discussed in the context of plaque stability (16). However, mice overexpressing IGFBP-1 present with reduced blood pressure and increased vascular nitric oxide production, and the overexpression prevents vascular endothelial dysfunction in the mice on high calorie diet [107]. In patients with AMI, significant reductions in serum IGFBP-1 (∼40 ng/ml) when compared to healthy subjects (∼70 ng/ml) have been demonstrated (20). Likewise, in patients with type 2 diabetes (T2D), decreased circulating concentrations of IGFBP-1 were correlated with cardiovascular risk factors such as low high-density lipoprotein (HDL) cholesterol or high blood pressure (17). These correlations have been confirmed by several consecutive studies (18, 19). However, in patients with heart failure, IGFBP-1 levels have been shown to be increased, although not associated with outcome (21), and in 112 patients with unstable angina, IGFBP-1 levels correlated with ACS disease severity and are higher in patients with multivessel disease than those with single-vessel (22). High circulating IGFBP-1 was also significantly associated with morbidity and cardiovascular mortality in a study including more than 500 diabetic patients with AMI (23). Thus, it was concluded that metabolic control and hepatic insulin resistance are related to fatal events in diabetic CVD patients. Notably, in the lowest or highest IGFBP-1 tertile, IGFBP-1 concentrations ranged between 2 and 24 or 43 and 677 μg/l, respectively (23). In diabetic patients, IGFBP-1 concentrations were associated with those of copeptin, which independently predicted myocardial events, and this could in part explain the prognostic value of IGFBP-1 for heart failure or AMI (56). However, the same association was not found in a more recent study by the same authors (24). On the tissue level, sonography of carotid arteries in type 2 diabetic patients revealed a highly significant negative correlation between IGFBP-1 serum concentrations and thickness of the combined intimal and media compartments (25). Irrespective of diabetes, high IGFBP-1 serum concentrations were correlated with and thus predictive for an increased risk of cardiovascular and coronary heart disease mortality in elderly men (26). Likewise, in survivors of a previous (first) AMI, higher circulating IGFBP-1 concentrations predicted heart failure as demonstrated by a prospective study (27) which included male and female subjects between 45 and 70 years of age. Interestingly, higher IGFBP-1 concentrations were informative for mortality in subjects with no history of heart failure after a follow-up of 8 years. Serum IGFBP-1 concentrations were also significantly increased in patients with critical CAD when compared to patients with less severe CAD (22). In combination with HDL cholesterol, IGFBP-1 serum concentrations were more sensitive and specific for the prediction of CAD (22). Lower IGFBP-1 and IGF-I serum concentrations were associated with an increased risk of IHD later in life or with higher cardiovascular disease mortality in men and women at an age between 51 and 98 years (28). Nevertheless, the authors also concluded that assessment of IGFBP-1 and IGF-I could be used for the identification of adult subjects at an increased risk of fatal IHD as well as for the selection of an appropriate intervention strategy. The reasons underlying the contradictory biomarker information of IGFBP-1 are not directly evident, because the follow-up periods were 9–13 years in one study (28) and 8 years in the later study (27). A possible explanation may be deduced from the fact that the study by Janszky et al. was restricted to the risk of heart failure but not to all-cause mortality or mortality related to cardiovascular disease. In 335 elderly male subjects (70–89 years of age), IGFBP-1 was not associated with increased prevalence of cardiovascular mortality risk (29). Concentrations of IGFBP-1 in the circulation furthermore were not correlated with the prevalence of coronary complications in aged subjects (30). Collectively, these findings suggest that IGFBP-1 may predict future cardiovascular mortality and morbidity, but perhaps more importantly, it may serve as a marker of hyperinsulinemia, which precedes subsequent development of insulin resistance and CVD.

IGFBP-2

IGFBP-2 has been established as a marker of the metabolic syndrome and therefore, it has been suggested that low concentrations of IGFBP-2 could be a useful biomarker for the assessment of cardiovascular risk factors (31). It is the second most abundant binding protein in circulation and is also metabolically regulated, albeit not as rapidly as IGFBP-1. Indeed, IGFBP-2 levels are reduced in obese subjects and in T2D, and low levels associate with elevated fasting glucose, serum triglycerides, and LDL cholesterol (31). As a particular advantage of IGFBP-2 as compared to IGFBP-1, circulating IGFBP-2 concentrations are less prone to post-prandial alterations. This suggests IGFBP-2 to represent a more robust biomarker than IGFBP-1 (31). In a cross-sectional study that included 310 study members at an age between 63 and 82 years, circulating IGFBP-2 concentrations were negatively correlated with arterial intima-media thickness (IMT), whereas IGF-II levels were positively associated with IMT (35). Conversely, IGFBP-2 concentrations in plasma were about 2-fold increased in 273 cases of fatal IHD and a strong association between IGFBP-2 and death/AMI was described (32). In 99 patients with T2D and 99 controls, IGFBP-2 was inversely associated with pulse wave velocity, which is a measure of arterial stiffness and thus, the degree of atherosclerosis (33). However, these cross-sectional studies reflect associations and give no evidence of causality. In a study by Hedbacker et al. (57), the authors demonstrated a direct beneficial effect of IGFBP-2 on cardiovascular risk factors in excessively obese (ob/ob) and diabetic mice. IGFBP-2 overexpression resulted in a 3-fold increase in hepatic insulin sensitivity and a reduction in plasma glucose, liver triglycerides, and hepatic steatosis. As a result, the diabetic phenotype was remedied. The same reduction in plasma glucose was observed upon overexpression of IGFBP-2 in wild-type mice, and there was a trend for reduction in insulin levels in both models. Considering this association between low IGFBP-2 and multiple CVD risk factors, IGFBP-2 may serve as a robust biomarker for the identification of individuals with high cardiovascular risk. However, IGFBP-2 is also a biomarker of mortality in elderly subjects (34), possibly explained by an

TABLE 1 | Biomarker potential of IGFBPs in ischemic heart disease.

IGFBP-	Patient information	Biomarker association	Method	References
1	Aortic plaques	SMC proliferation (+) plaque stability (+)	mRNA	(16)
1	Diabetic	CV risk factors: insulin (–), blood pressure (–)	RIA	(17, 18)
1	Aged (m: 70–89 y)	CV risk factors (–)	IFA	(19)
1	AMI	IGFBP-1 (–)	IRMA	(20)
1	Heart failure	Heart failure (+)	RIA	(21)
1	CAD	Severity of CAD (+)	ELISA	(22)
1	Diabetic and AMI	Morbidity and CV mortality (+)	RIA	(23)
1	ACS	Copeptin as a marker of AMI (no effect)	RIA	(24)
1	Diabetic	Carotid IMT thickness (+)	IFA	(25)
1	Aged (m: 65–84 y)	CV and CHD mortality (+)	IFA	(26)
1	AMI survivor (45–70 y)	Heart failure (+)	RIA	(27)
1	Healthy (45–70 y)	Heart failure (+)	RIA	(27)
1	CAD	Severity of CAD (+)	ELISA	(22)
1	Aged (51–98 y)	CVD mortality (–)	IRMA	(28)
1	Male (70–89 y)	Cardiovascular mortality risk (no effect)	IFA	(29)
1	Aged	Coronary complications (no effect)	ELISA	(30)
2	Diabetic	Cardiovascular risk factors (–)	RIA	(31)
2	IHD	Death/MI (+)	n.p.	(32)
2	Diabetic and controls	Carotid-femoral pulse wave velocity (–)	IDS-iSys	(33)
2	Aged (≥80 y)	Mortality (+)	RIA	(34)
2	Aged (63–82 y)	Arterial IMT (–)	ELISA	(35)
3	AMI	IGFBP-3 (+)	IRMA	(20)
3	Healthy	IHD later in life (+)	RIA	(36)
3	CHD	IGFBP-3 (+)	EIA	(37)
3	Male	Total cholesterol (+), LDL (+)	IRMA	(38)
3	Hypertension	Carotid atherosclerosis (+)	RIA	(39)
3	Aged (63–82 y)	Plaque instability (+)	RIA	(35)
3	Moderate IHD	Ischemic heart failure (–)	RIA	(40)
3	Adult (40–60 y)	CHD (–)	ELISA	(41)
3	Aged (≥65 y)	Incident coronary events (–)	ELISA	(30)
3	MI	IGFBP-3 (–)	ELISA	(42)
3	CHD	IGFBP-3 (–)	ELISA	(43)
3	Adult/aged (≥45 y)	carotid IMT (–)	CIA	(44)
3	Female (51–68 y)	MI (no effect)	SIA	(45)
3	Subjects (45–79 y)	CAD (no effect)	RIA	(46)
3	Subjects (40–79 y)	Mortality (no effect)	RIA	(47)
3	STEMI/NSTEMI	ACS (no effect)	RIA	(48)
4	Diabetic and controls	Carotid artery remodeling (NT-IGFBP-4) and accelerated atherosclerosis	TR-IFMA	(33)
4	ACS	PAPP-A (+)	n.a./WIB	(49–51)
4	MI-suspected	MACE (NT-/CT-IGFBP-4 +)	IA	(52)
4	CVD	long-term outcome (NT-/CT-IGFBP-4: no effect)	IA	(53)
4	Diabetic	CV mortality (NT-/CT-IGFBP-4 +)	TR-IFMA	(54)
4	STEMI	CV mortality (NT-/CT-IGFBP-4 +)	TR-IFMA	(55)
5	CHD	IGFBP-5 (+)	RIA	(37)
5	CHD and healthy controls	Apo A1 (+) HDL-C (+)	RIA	(37)

ACS, acute coronary syndrome, AMI, acute myocardial infarction; CAD: coronary artery disease; CHD, coronary heart disease; CIA, chemiluminescence immunoassay; CT-/NT-, carboxyl-/amino-terminal); CV, cardiovascular; CVD, cardiovascular disease; EIA, enzyme immunoassay; H/LDL, high-/low-density lipoprotein; IA, immunoassay; IDS-iSYS, Immunodiagnostic Systems (automated immunoassay analyzer); IFA, immunofluorometric assay; IHD, ischemic heart disease; IMT, intima-media thickness; IRMA, immunoradiometric assay; MACE, major adverse cardiac event; MI, myocardial infarction; n.a., not applicable; n.p., not provided by the authors; N/STEMI, no/elevation of the ST segment; PAPP-A, pregnancy associated plasma protein A; RIA, radioimmunoassay; SIA, 2-step sandwich-type immunoassay; TR-IFMAs, time-resolved immunofluorometric assay; WIB, Western immunoblot.

association between high serum IGFBP-2 and low physical function. Thus, it might be important to also assess the functional relevance of increased circulating IGFBP-2 levels for CVD in the future.

IGFBP-3

Due to its abundance in the circulation and role as the primary IGF carrier, substantial attention has been paid to IGFBP-3. Surprisingly, little is known about its regulation with regards to cardiovascular disease. In the human heart, IGFBP-3 is expressed throughout lifetime both on mRNA and protein levels and IGFBP-3 mRNA expression in the heart is higher when compared to the liver at fetal as well as adult stages (10). Interestingly, higher IGFBP-3 protein expression was identified by Western immunoblotting in the ischemic as compared to the hypertrophic or dilated heart (10). Intact IGFBP-3 is secreted by cardiac atrial appendage stem cells (CASCs) and thought to be related to the regenerative potential due to cardiac angiogenesis and possibly also to some extent cardiomyogenic differentiation in the ischemic heart as demonstrated also by Western immunoblotting (58). After heart transplantation, expression of IGBP-3 and IGF-I mRNA was quantified in end-stage dilated ($n = 11$) or ischemic hearts ($n = 12$) (59). Compared to healthy control hearts ($n = 10$), expression of IGFBP-3 mRNA was significantly increased in both pathological conditions, whereas IGF-I mRNA was elevated only in the dilated heart (59). Expression of IGFBP-3 was further discussed in the context of hypoxia and a pathophysiological function of the IGF-system was proposed for the heart. In fact, in cultivated H9c2 myocardial cells, hypoxia increased expression of IGFBP-3, which was responsible for the induction of apoptosis due to inhibition of IGF-signaling via protein kinase B (60). However, on the local level, IGFBP-3 exerted antiproliferative effects on the Wnt signaling pathway in cardiac progenitor cells (61). Thereby, the authors demonstrated that the antiproliferative effect of IGFBP-3 depends on an intact IGF-binding site.

Increased Concentrations of IGFBP-3

In contrast to IGFBP-1, IGFBP-3, and IGF-I levels significantly increased after AMI (20). The authors were able to specify a threshold of 137 ng/ml for the serum concentrations of IGF-I: lower concentrations were correlated with a worse prognosis in AMI patients, whereas higher concentrations of IGF-I after AMI were associated with improved functional parameters detected by echo cardiography (e.g., left ventricular mass or ejection fraction) (20). It was suggested that high IGF-I immediately after AMI fueled myocardial remodeling, and that IGFBP-3, which generally reflects total IGF-I levels, increased in parallel. In a prospective study, high IGFBP-3 and low IGF-I serum concentrations in healthy subjects were correlated with an increased risk of developing IHD later in life (36). In partial agreement, higher levels of IGFBP-3 but also higher serum concentrations of IGF-I were described in patients with CHD (37). Under conditions of AMI, an elevation of the ST segment (STEMI) on the electrocardiogram (ECG) is considered to characterize a more severe form of the ACS when compared to patients with no elevation of the ST segment (NSTEMI). In a longitudinal study of 747 White and 544 Black young males over a period of 10 years, an increase of IGFBP-3 serum concentrations over time was associated with significant increases of total cholesterol and LDL cholesterol (38). This study did not confirm previous cross-sectional studies describing associations of IGFBP-3 and IGF-I with hypertension, but rather revealed a link between IGFBP-3 and lipid concentrations in young males. In hypertensive patients, a role of IGFBP-3 in the formation of carotid atherosclerosis was suggested (39). In elderly patients (63–82 years of age), higher circulating concentrations of IGFBP-3 or IGF-I were positively or negatively associated with plaque instability, respectively (35). The authors concluded that IGF-I and IGFBP-3 might represent functional biomarkers for prediction of the risk for plaque rupture or therapeutic targets for stabilization of atherosclerotic plaques. In fact, systemic concentrations of IGFBP-3 and IGF-I were increased after application of the angiotensin-converting enzyme-inhibitor Fosinopril, and the beneficial effects of treatment in IHD were discussed in the context of the IGF-system (62).

Conditions of Decreased Circulating IGFBP-3

In addition to IGF-I and IGFBP-3, secretion of GH was also impaired in patients with ischemic heart failure (40). The authors observed patient-individual GH-sensitivity related to the degree of left ventricular dysfunction (40). In male patients between 40 and 60 years of age (41) and in adults of both genders older than 65 years (30), lower concentrations of IGFBP-3 or IGF-I in serum were associated with CHD. In patients with AMI, serum levels of IGFBP-3, IGF-I, and IGF-II were decreased immediately after AMI, but returned to their normal range 1 week after coronary intervention (42). Similarly, in 90 patients with a diagnosed CHD, reduced levels of circulating IGFBP-3 and IGF-I were described (43). The results are in principal agreement with a previous report on reduced IGF-I concentrations in patients with AMI (63). In both studies, serum concentrations of GH were increased under conditions of acute cardiac ischemia. Low concentrations of IGFBP-3 and high circulating levels of IGF-I were further associated with an increased thickness of the intima-media (44).

Lack of Biomarker Value for IGFBP-3 Concentrations

A prospective study in women at 51–68 years of age revealed no direct relationship between IGFBP-3 or IGF-I and a risk of AMI later in life (45). Systemic effects of IGFBP-3 or IGF-I on the development of CAD were excluded by a prospective study observing more than 1,000 cases and more than 2000 controls over a mean period of 6 years (46). From this study and from an additional meta-analysis assessing 31 single nucleotide polymorphisms present in the *IGF1* and *IGFBP3* genes, the authors concluded that neither circulating IGFBP-3 or IGF-I nor the corresponding genes were causative for the development of CAD in human populations. At a later time point, a single nucleotide polymorphism upstream of the *IGF1* genomic region was identified in patients at an increased risk of developing CAD

(64). With respect to IHD, an association between mortality and circulating concentrations of IGFBP-3, IGF-I, or IGF-II was also excluded by a large prospective Japanese study including more than 39,000 subjects between 40 and 79 years of age (47). In a Turkish study including 20 STEMI patients, 10 NSTEMI patients, and 20 healthy controls, IGFBP-3 was not affected by ACS (48). Instead, IGF-I was severely reduced in ACS patients with elevations of the ST segment on the ECG (48). Therefore, IGF-I serum concentrations were suggested to represent potential biomarkers of myocardial necrosis in the STEMI group of ACS patients.

Age-Related Biomarker Potential of IGFBP-3

Interestingly, age-related discrepancies exist in the IGF- and IGFBP-concentrations after AMI as analyzed by RIA (65). Accordingly, younger subjects (<50 years of age) are characterized by higher absolute concentrations and lower daily variations of circulating IGF-I when compared to more aged subjects (>50 years of age), which may be related to higher regenerative capacities in younger subjects after AMI (65). Age-related differences of IGF-I and IGFBP-3 in the context of differential prognosis after AMI also might indicate an involvement of GH during functional recovery of the infarcted heart. The inclusion of structural information (intact vs. fragmented IGFBPs) could be used in order to assess age-related control of IGF-bioactivity.

IGFBP-4 AND PAPP-A

IGFBP-4, also an abundant IGFBP, has been suggested in numerous studies to be directly involved in the inhibition of atherosclerosis (33, 52, 54, 55, 66, 67). As demonstrated on the level of mRNA and protein, intact IGFBP-4 is produced by a variety of cell types and represents the major IGFBP secreted by myoblasts (68–70). It is considered to attenuate IGF activity in most physiological contexts and inhibits proliferation and differentiation during the transition from myoblasts to myotubes (68). Studies suggest that intact IGFBP-4 modulates cardiac development and cardiomyocyte differentiation (70) and is actively involved in the development of atherosclerosis, with high expression levels in aorta lesional areas (66). Moreover, mice with an overproduction of IGFBP-4 present with smooth muscle hypoplasia (71). IGFBP-4 has a preferential affinity for IGF-II over IGF-I and transports them to peripheral tissues, where proteases cleave IGFBP-4 into low binding-affinity fragments (72–74). The enzyme pregnancy-associated plasma protein-A (PAPP-A) has been identified as the principal, if not only, protease responsible for this IGF-dependent cleavage of IGFBP-4 (75, 76), and PAPP-A levels strongly correlate with the levels of IGFBP-4 proteolytic fragments (54). PAPP-A tethers to cell surfaces, and thus, release of the IGFs occurs in close proximity to the IGF-IR and increases IGF bioavailability primarily at local sites (77). Interestingly, balloon injury of porcine coronary arteries has been shown to massively increase the expression of PAPP-A and the subsequent degradation of IGFBP-4 in the neointimal and medial layers, peaking at 4 weeks after treatment, thus causing possible hyperplasia and coronary restenosis (71). In a study of patients with T2D and healthy controls, IGFBP-4 fragment levels were associated with the normalized wall-index, which is a measure of carotid artery remodeling and accelerated atherosclerosis (33). In murine models, deletion of the PAPP-A gene resulted in an 80% reduction in atherosclerotic area, whereas transgenic overexpression of PAPP-A accelerated plaque progression (66, 78). Inhibition of the PAPP-A substrate binding site with a neutralizing monoclonal PAPP-A antibody caused a 70% reduction in plaque area (79). Importantly, when PAPP-A is deprived of its proteolytic activity, intact IGFBP-4 levels increase and diminish the actions of IGF-I. Collectively, the findings suggest that high PAPP-A and low IGFBP-4 levels may exacerbate the pathophysiological processes underlying plaque development. Under conditions of myocardial damage, PAPP-A concentrations are also increased in the circulation (49). In 2001, Bayes-Genis et al. were the first to demonstrated that PAPP-A was ubiquitously expressed in human eroded atherosclerotic plaques, and serum levels were elevated in patients with ACS (50). This initiated a large interest to study PAPP-A as a candidate marker of plaque burden and CVD. However, it was later revealed that administration of heparin to AMI patients, which is part of the standard initial treatment, results in a rapid increase in PAPP-A concentrations, possibly through a displacement of cell surface attached PAPP-A (51). Instead, Postnikov et al. proposed that quantification of the PAPP-A generated, cleaved amino-terminal (NT) and carboxyl-terminal (CT) fragments of IGFBP-4 could be reflective of PAPP-A enzymatic activity and hereby serve as prognostic biomarkers (12, 52). In patients with symptoms of IHD, both fragments were increased in the case of short-term cardiac events (coronary bypass, AMI, or cardiac death) (52). However, in a follow-up study by the same authors, neither IGFBP-4 fragments nor PAPP-A sufficed to predict the long-term outcome in patients with stable cardiovascular disease (53). The varying results may relate to differences in follow-up period and number of patients and hence, events. Recently, the fragments were shown to prognosticate cardiovascular mortality in 330 type 1 diabetes patients without cardiovascular disease at baseline during 12 years of follow-up (54) and in 656 patients with STEMI followed for 5 years (55). Importantly, it was also verified that the IGFBP-4 fragments, unlike PAPP-A, were unaffected by heparin treatment of coronary patients (80). Interestingly, accumulating evidence argues for multiple IGF-independent functions of IGFBP-4. In IGF-insensitive colorectal cancer cells, recombinant human IGFBP-4 was able to efficiently block colony formation (81), and in P19CL6 or in embryonic stem cells, knockdown of IGFBP-4 was shown to alter cardiomyogenesis and cardiac regeneration (70). The mechanism was mediated through inhibition of canonical Wnt signaling and not through IGF. In mice, an IGFBP-4 variant blocked translocation of beta-catenin to the cell nucleus in the ischemic heart (82). Injection of IGFBP-4/H95P directly after AMI prevented beta-catenin related DNA-damage in cardiomyocytes and reduced infarct size 4 weeks after AMI in the mouse (82). Because IGFBP-4/H95P is lacking the intact IGF-binding domain, the

protective effect of mutated IGFBP-4 in the ischemic heart is IGF-independent.

IGFBP-5

IGFBP-5 is produced and secreted by numerous cell types, including vascular smooth muscle cells, and its expression is increased within atherosclerotic plaques (83). Furthermore, atherosclerotic arteries exhibit staining of IGFBP-5 staining along intimal plaques (84). In a cross-sectional study of 95 male CHD patients and 92 healthy controls, elevations of IGFBP-5 and acid labile subunit (ALS) were measured in serum (37). In addition, IGF-I, IGF-II, IGFBP-3, and ALS were increased under conditions of CHD in the patients (37). In serum, concentrations of IGFBP-5 were significantly correlated with the levels of IGF-I and -II or IGFBP-3 (37). The increased concentrations of IGFBPs including IGFBP-5 and ALS could be causative for the larger amounts of IGF-I and -II in CHD patients (37).

Under ischemic conditions, local expression of IGFBP-5 mRNA, which succeeds the initiation of IGF-II mRNA expression, is thought to terminate cardioprotection exhibited by IGF-II (85–87). In fact, a dual role has been suggested for IGFBP-5, with positive effects on IGF-I-stimulated differentiation of myoblasts *in vitro*, yet inhibitory effects on IGF-II activity (68). Thus, the association of IGFBP-5 with atherosclerosis may indicate a direct stimulatory effect of IGFBP-5 on smooth muscle cell proliferation and plaque formation.

IGFBP-6

IGFBP-6 exerts both IGF-dependent and -independent effects in various cell types (88). In endomycardial biopsies from 12 patients, IGFBP-6 mRNA expression was increased after explantation of a left ventricular assist device when compared to biopsies sampled during implantation (89). Because other proteins in the IGF-system, including IGF-I, IGF-II, and IGFBP-4, were also increased after reverse remodeling of the heart, it was concluded that local effects of the IGF-system are active during heart recovery (89)—yet, a direct link to IHD remains unestablished. However, increased expression of IGFBP-6 after prolonged hypoxia has been observed in vascular endothelial cells (90). Notably, the *IGFBP6* gene promotor contains several hypoxia response elements, which could explain the increased expression of IGFBP-6 (90). IGFBP-6 levels were also increased after hypoxia in vascular endothelial cells (90). However, since expression of IGFBP-1 was increased as an earlier response to hypoxemic conditions (91, 92) than IGFBP-6, this may be interpreted as an example of molecular job sharing between distinct members of the IGFBP-family.

SUMMARY AND CONCLUSIONS

So far, clinical studies have demonstrated positive or negative associations and a number of studies have also concluded that the IGFBPs show no prognostic value in the assessment of cardiovascular risk. Certainly, one reason for the controversies present in the literature could be the fact that the IGF system is involved in so many biological processes, and hence, the complex nature and multifunctional properties of the IGFBPs. Firstly, alterations in IGFBP levels may represent a phenomenon caused by the disease *per se*. However, most patients receive medical therapy, which affects several components of the IGF-system and constitutes a significant confounder. The effect of heparin on the IGFBP-4 protease PAPP-A is an example of that. Acute patients often also present with an altered peripheral tissue perfusion, allowing leakage of tissue-localized IGFBPs. Furthermore, IGFBP levels may also fluctuate due to compensatory mechanisms or be affected by age or metabolism. It is well-established that IGFBPs are controlled by both GH and insulin, and under conditions of acute or prolonged illness, altered secretory patterns of GH and insulin can increase or decrease levels of IGFBPs (65, 93). Secondly, assessment of IGFBP biomarker potential highly depends on accurate measurements of the IGFBP. Thus, it is important to know of the factors that can potentially skew these measurements and it is crucial that immunoassays are based on antibodies that recognize all clinically relevant forms of the analyte. Unfortunately, most studies use immunoassays without information on target epitopes, and it is well-known that many assays exhibit cross-reactivity toward structurally similar IGFBPs or are unable to discriminate between various IGFBP forms (80). Since the IGFBPs can be proteolytically degraded by proteases, resulting in circulating IGFBP fragments, it is crucial that the assays can distinguished between intact and fragmented IGFBP. Several proteases have been identified, and although these proteases, like most other members of the IGF system, appear under the strict control of paracrine and endocrine influences, our knowledge on factors that control protease activity and tissue distribution is far from sufficient (12). Additionally, the IGFBPs are subject to post-translational modifications such as phosphorylation and glycosylation and may also be partially truncated or in complex with other proteins. Indeed, it is known that the glycosylation patterns of many proteins are altered in various disease states (94, 95), and it is likely that both proteolysis and post-translational modifications of the IGFBPs are influenced by an acute setting and differs from that of stable patients (96). Thus, the use of analytical systems with no discriminatory potential toward intact or modified IGFBPs represents a major issue in IGFBP-related biomarker research, and we prospectively need to include structural and functional information on the IGFBPs. This issue, which is not restricted to CVC patients, further raises concerns regarding the reliability and consequently, the utility of IGFBPs, and at present, identification of patients with high CVD risk cannot solely rely on IGFBP measurements.

It should be noted that research in the area is still in its early phases, and we advocate that larger and more comprehensive investigations are implemented before evaluating the biomarker potential of the IGFBPs. Investigations so far have mostly been limited to cross-sectional studies and animal studies, and cautious interpretation is necessary, as data should be confirmed by longitudinal and mechanistic studies. Accordingly, broader population studies including more complex settings of pathophysiological conditions in their subjects may come to the

conclusion that no associations are present between circulating IGFBPs and cardiovascular parameters. We speculate that the inclusion of patients with a higher degree of overlap with respect to their pathophysiology could improve the assessment of the biomarker potential of the IGFBPs.

Our understanding of the IGFBPs has advanced throughout recent years, and the clear link between the IGFBPs and a number of pathological conditions has preserved the interest in identifying novel biomarkers that may help improve future diagnosis and prognosis. However, studies regarding CVD have been varying and discrepant, and it remains unknown to what extent IGFBP measurements possess clinical value, in particular in the absence of structural and functional information on the IGFBPs. Further understanding of the IGF system in both normal physiology and specifically in CVD is necessary to avoid misinterpretations.

AUTHOR CONTRIBUTIONS

AH wrote the manuscript and prepared the table; RD revised the manuscript; RH wrote the manuscript.

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Conflict of Interest Statement: AH is related to Ligandis UG.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Increased Concentrations of Insulin-Like Growth Factor Binding Protein (IGFBP)-2, IGFBP-3, and IGFBP-4 Are Associated With Fetal Mortality in Pregnant Cows

*Kirsten Mense[1], Julia Heidekorn-Dettmer[2], Elisa Wirthgen[3], Yette Brockelmann[2], Ralf Bortfeldt[1], Sarah Peter[1], Markus Jung[1], Christine Höflich[4], Andreas Hoeflich[3] and Marion Schmicke[2]**

[1] Institute for the Reproduction of Farm Animals Schoenow, Bernau, Germany, [2] Clinic for Cattle, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany, [3] Institute of Genome Biology, Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany, [4] Ligandis UG, Gülzow-Prüzen, Germany

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***Correspondence:**
Marion Schmicke
marion.schmicke@tiho-hannover.de

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Insulin-like growth factors (IGFs) play a critical role in fetal growth, and components of the IGF system have been associated with fetal growth restriction in women. In human pregnancy, the proteolytic cleavage of insulin-like growth factor binding proteins (IGFBPs), particularly IGFBP-4, releases free IGF for respective action at the tissue level. The aim of the present study was to determine IGFBP-2, IGFBP-3, and IGFBP-4 concentrations by Western ligand blotting during pregnancy until day 100 in cows and to compare these concentrations with those of non-pregnant cows and cows undergoing embryonic/fetal mortality. Therefore, two study trials (I and II) and an *in vitro* study were conducted. In study I, 43 cows were not pregnant, 34 cows were pregnant, and 4 cows were undergoing fm. In study II, 500 cows were examined, and 7 cases of pregnancy loss between days 24–27 and 34–37 after artificial insemination (AI, late embryonic mortality; em) and 8 cases of pregnancy loss between days 34–37 and 54–57 after AI (late embryonic mortality and early fetal mortality; em/fm) were defined from the analyses of 30 pregnant and 20 non-pregnant cows randomly selected for insulin-like growth factor 1 and IGFBP analyses. *In vitro* serum from pregnant ($n = 3$) and non-pregnant ($n = 3$) cows spiked after incubation with recombinant human (rh) IGFBP-4 for 24 h, and IGFBP-4 levels were analyzed before and after incubation to detect proteolytic degradation. The IGFBP-2, -3, and -4 concentrations did not decline during early pregnancy in cows, while IGFBP-4 concentrations were comparable between pregnant and non-pregnant cows, irrespective of low proteolytic activity, which was also demonstrated in cows. Interestingly, cows with em or fm showed distinct IGFBP patterns. The IGFBP-2 and -3 concentrations were higher ($P < 0.05$) in cows with fm compared to pregnant. The IGFBP-4 levels were significantly higher in cows developing fm. Thus, distinct differences in the circulating IGFBP concentrations could be associated with late embryonic and early fetal losses in cattle.

Keywords: IGF-1, binding protein, bovine, pregnancy, gestation, embryonic mortality

INTRODUCTION

The somatotropic axis is a key regulatory pathway for dairy cows, and a growth hormone insensitivity plays a role in energy partitioning to facilitate milk production after calving (1). During lactation, a recoupling of the somatotropic axis occurs as milk yield decreases. The concentration of insulin-like growth factor-binding proteins (IGFBP)-2 and IGFBP-4 was shown to be elevated throughout the early lactation period (2). Dairy cows should achieve a new pregnancy within 2–3 months of calving in order to maintain high milk production. Therefore, the gestational period begins during lactation. Insulin-like growth factors (IGFs) play a crucial role not only for metabolic adaption but also in fetal growth. In this regard, the local IGF system, comprising insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) and their respective IGFBPs are expressed in the endometrium prior to implantation and in the placenta (3–6). These are important as well as maternal IGF-1 and IGFBPs in the blood circulation. Here, we focus on maternal IGFBP concentrations because the bioavailability of IGF-1 in the blood is regulated by the binding of IGF-1 to six high-affinity binding proteins (IGFBP1-6) (7, 8). In the blood, the most abundant binding protein is IGFBP-3. This binding protein forms a complex with IGF-1 and the acid-labile subunit that restricts IGF-1 to the blood stream, representing an IGF-1 blood reservoir. The second most abundant binding protein in blood is IGFBP-2. The protein transports IGF-1 through the endothelium to cells expressing the respective IGF-receptors (IGFR) (8). Both IGFBP-3 and IGFBP-2 are mainly produced in the liver. IGFBP-4 is also detectable in serum and is described as an inhibitor of IGF activity (8, 9). In human pregnancy, the proteolytic cleavage of IGFBPs, particularly IGFBP-4, releases free IGF for respective action at the tissue level (10, 11). The responsible enzyme for IGFBP-4 proteolysis in women is pregnancy-associated plasma protein-A (PAPP-A), which is expressed in high amounts in the human placenta and released into the blood and, therefore, acts on maternal blood IGF-1 bioavailability (12). We previously showed that in heifers, the maternal IGFBP-4 concentration decreases towards day 18 of pregnancy and speculated that the proteolytic cleavage of binding proteins is the underlying reason for this decrease (13). However, whether proteolytic activity is present during pregnancy in cattle in maternal blood circulation, similar to humans, has not yet been investigated. Interestingly, high IGFBP-4 concentrations have previously been linked to fetal growth restriction in human gestation (14, 15), and less proteolytic cleavage might be due to higher IGFBP-4 and lower IGF-1 levels. In cows, also substantial differences in metabolic adaption throughout lactation may be responsible for differences in peripheral IGFBP-2 or IGFBP-4 concentrations and, therefore, play a role in pregnancy maintenance or serve as biomarker. Moreover, it was previously indicated in dairy cows that a higher peripheral IGFBP-3 concentration was associated with a lower fertility rate (16).

Therefore, the aim of the present study was to examine whether the maternal IGFBP concentration in blood decreases during early pregnancy in cows, to compare the IGFBP-4 concentration between pregnant and non-pregnant cows to examine cows undergoing embryonic/fetal mortality (fm) and to determine whether proteolytic activity for IGFBPs can be detected in pregnant cows.

MATERIALS AND METHODS

Animals

To address the research questions, two subsequent experimental studies and *in vitro* testing were conducted. In the first experiment (Study trial I), pregnant and non-pregnant pluriparous cows from one farm were investigated until day 100 of pregnancy. In four animals, embryonic/fm occurred, and a specific IGFBP pattern was found in the blood of these animals. Therefore, a second sampling period (Study trial II) was utilized to compare the cattle suffering from em or em/fm among pregnant or non-pregnant cows. Further, we conducted *in vitro* analyses (*in vitro* trial) to determine whether the proteolytic degradation of IGFBP-4 is also detectable in cows.

Study Trial I

Pluriparous Holstein Friesian cows from the Brunckhorst/Romundt GbR (Vahlde, Germany) were used. In total, 260 cows were maintained in a free-stall housing system with straw-bedded maternity pens. The study animals were selected out of 105 lactating and pluriparous animals (mean 305-milk yield of 9,000 kg). All the cows used for the present study had physiological calving without any dystocia or obstetrical assistance needed. The cows were fed a total mixed ration (consistent of corn and grass silage, grinded corn and wheat, beer marc, feed fat, minerals, lime, salt, and yeast) and provided free access to water and additional concentrate based on the daily milk yield *via* transponders. The study was performed in accordance with the German legislation on animal welfare (Lower Saxony Federal State Office for Consumer Protection 279 and Food Safety, AZ 33.9-42502-05-14A487).

Study design

Only pluriparous cows> 1 lactation were enrolled in the present study, and initially, the general health condition was examined. Subsequently, behavior, posture, body temperature, and body conditioning score (BCS) (17) were recorded. Additionally, the milk yield during the previous lactation was documented. A gynecological examination was performed to assess uterine health, and the cycle stage was determined *via* transrectal ultrasonography (HS 101V, HONDA Electronics, Tokyo, Japan). If the cows showed estrous symptoms (mounting, standing heat, mucus discharge), then day 0 was defined, and a blood sample was drawn from the coccygeal vessel directly into tubes with EDTA and in one tube without any anticoagulants to acquire serum (Sarstedt, Nümbrecht, Germany). The cows were subsequently artificial insemination (AI, day 0). On day 18 ± 1 after AI, the cows were gynecologically examined again, and a second blood sample was obtained. On day 42 ± 1 after AI, a pregnancy diagnosis was performed by transrectal ultrasonography and analysis of pregnancy-associated glycoproteins in blood samples. If the cows were diagnosed as pregnant, two subsequent blood samples were obtained, as described above

(days 60 ± 3 and 100 ± 3). When pregnancy was clearly diagnosed on day 42 ± 1, but the cow was non-pregnant on either day 60 ± 3 or day 100 ± 3, then the animal was defined as undergoing fm. Therefore, three groups of animals were compared in this study trial (p, pregnant; np, non-pregnant; and fm, fetal mortality).

Study Trial II

In total, 500 pluriparous Holstein Friesian cows from "Gut Schönerlinde/Wansdorf, Wandlitz" were examined four times after artificial insemination. The study was performed in accordance with the German legislation on animal welfare (state office of Brandenburg for work protection, consumer protection, and physical health; AZ 2347-29-2014). Estrous was detected by determination of estrous symptoms (mounting, standing heat, and mucus discharge) and additional rectal examination of the uterus and, if necessary, the ovaries following AI; this examination was performed by a herd insemination technician. After AI (days 10–14), the animals were clinically investigated, the BCS was documented, and blood samples were collected from the coccygeal vein. Subsequently, blood samples were collected on days 24–27 and 34–37 after AI, in all the animals and additionally on days 54–57 in pregnant cows. A pregnancy diagnosis was performed by using ultrasonography (My Lab®, Esoate, Italy) and analysis of PAG in the blood on days 24–27, 34–37, and 54–57. If on days 24–27, a pregnancy clearly was diagnosed but the cow was not pregnant on days 34–37, the cow was defined as late embryonic mortality (em). Blood samples were taken on day blood samples were collected on days 10–14, 24–27, and 34–37 but not on days 54–57. If on days 34–37, a pregnancy was clearly diagnosed but the cow was not pregnant on days 54–57, the animal was defined as late embryonic mortality or early fetal mortality (em/fm).

Laboratory Analyses

Pregnancy-Associated Glycoproteins

Semi-quantitative PAG analysis was performed using a commercially available PAG-ELISA (IDEXX Bovine Pregnancy Test, IDEXX, Westbrook, ME, USA). The samples were analyzed according to the manufacturer's instructions, and the optical density (OD) was interpreted with regard to Ref. (18). The PAG-ELISA is based on a system with an indirect sandwich ELISA, and the PAG OD was photometrically measured (SLT Spectra, SLT Lab Instruments GmbH, Salzburg, Austria).

Insulin-Like Growth Factor I

The total serum IGF-1 concentration was determined using a commercial RIA according to the manufacturer's instructions (IGF-I IRMA A15729, Beckman Coulter, CA, USA), as previously described in cattle (19). This method is based on the "sandwich" principle, and the detection antibodies were mouse monoclonal antibodies against 2 epitopes of IGF-I. The intraassay CV was 5.1%, and the interassay CV was 9.3%.

Insulin-Like Growth Factor-Binding Proteins

Quantitative Western ligand blotting of serum IGFBP-2, IGFBP-3, and IGFBP-4 levels was performed to detect the IGFBP concentration of functional binding proteins as described before (20). The lower and upper detection limits differed between the individual runs of BP analyses and were 195–12,500 ng/ml for IGFBP-2, 391–250,000 ng/ml for IGFBP-3, and 195–12,500 ng/ml for IGFBP-4 for Study trial I and 130–8,333 ng/ml for IGFBP-2, 260–16,000 ng/ml for IGFBP-3, and 260–16,666 ng/ml for IGFBP-4 for Study trial II. **Figure 1** shows an example of a Western Ligand Blot of a pregnant and non-pregnant cow. All Western ligand blot results were normalized and presented as percent of the respective BP concentration of non-pregnant cows on day 0 (Study trial I) or days 10–14 (Study trial II).

In Vitro Protease Testing

Serum from pregnant ($n = 3$) and non-pregnant ($n = 3$) cows was diluted 1:10 with sterile phosphate-buffered saline, spiked with recombinant human (rh) IGFBP-4 (100 ng/µl) + rhIGF-1 (0.5 ng/µl) and incubated for 24 h at 39°C under soft agitation. Endogenous serum IGFBPs and rhIGFBP-4 were analyzed in duplicate as area under the curve using Western ligand blotting, as described in Section "Insulin-Like Growth Factor Binding Proteins". Proteolytic degradation was detected before (A) and after incubation (B) using the value before incubation as 100%. As a positive control for the high proteolytic degradation of IGFBPs, human serum from a pregnant woman was available, which was treated similar to bovine serum. The human serum was available from one of the researchers, therefore, no ethical approval was necessary according to the ethics committee of the Hanover medical school.

Statistical Analyses

Statistical analyses were performed using the statistic software R (21). Each variable was initially tested within the groups (p, np, em, and em/fm) for deviation from the normal distribution using the Shapiro–Wilk and Anderson–Darling tests. In some cases,

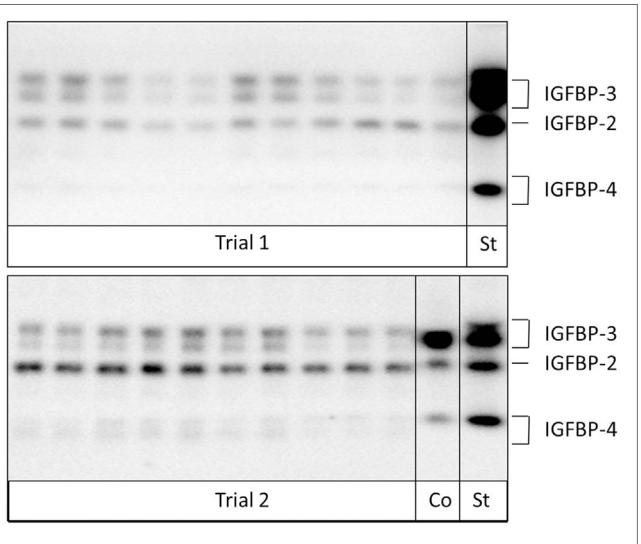

FIGURE 1 | Analysis of insulin-like growth factor-binding proteins (IGFBPs) in samples from pregnant and non-pregnant cows by Western ligand blotting. Due to protein glycosylation, IGFBP-3 and -4 can be detected as band doublets (Co: internal control; St: human recombinant IGFBP-standard).

the data differed from the normal distribution; however, due to the small sample sizes, standardized QQ-plots were additionally consulted to evaluate normality criteria. The QQ-plots displayed reasonable agreement of the sample quantiles with that from a standardized normal distribution; hence, parametrical tests were deemed appropriate. Additionally, the response variables were evaluated for the homogeneity of variances across these groups using the Brown–Forsythe test. Minor inhomogeneities of variances were tolerated due to the balanced study design and by applying a lower significance level. A two-factor analysis of variance (ANOVA) was conducted using the following model: $y \sim x1 + x2 + x1^{*}x2$, where y denotes the response variable of the measured traits and $x1$, $x2$ the explanatory variables group and time. Moreover, the interaction $x1^{*}x2$ was considered in the model. If the ANOVA results for explanatory variables indicated a significant influence on the response variable, then further investigation by using a *post hoc* test (Tukey's HSD) was conducted. A P-value of <0.05 was considered significant.

RESULTS

Study Trial I

Animals

Out of 105 examined cows, 43 non-pregnant and 34 pregnant cows were enrolled in the blood sampling and analyses. In 3 cases, the cows were diagnosed as pregnant on day 60 and non-pregnant on day 100, and in another cow, pregnancy was lost between days 42 and 60 after AI. These four cases were defined as fm.

Laboratory Analyses

As expected, pregnant animals had high PAG levels until day 42 of pregnancy. The PAG values of pregnant and non-pregnant pluriparous cows were comparably low between days 0 and 18. There was no significant difference between pregnant cows and cows with fm; however, PAG levels were numerically lower in cows with fm than those in pregnant animals (**Figure 2A**).

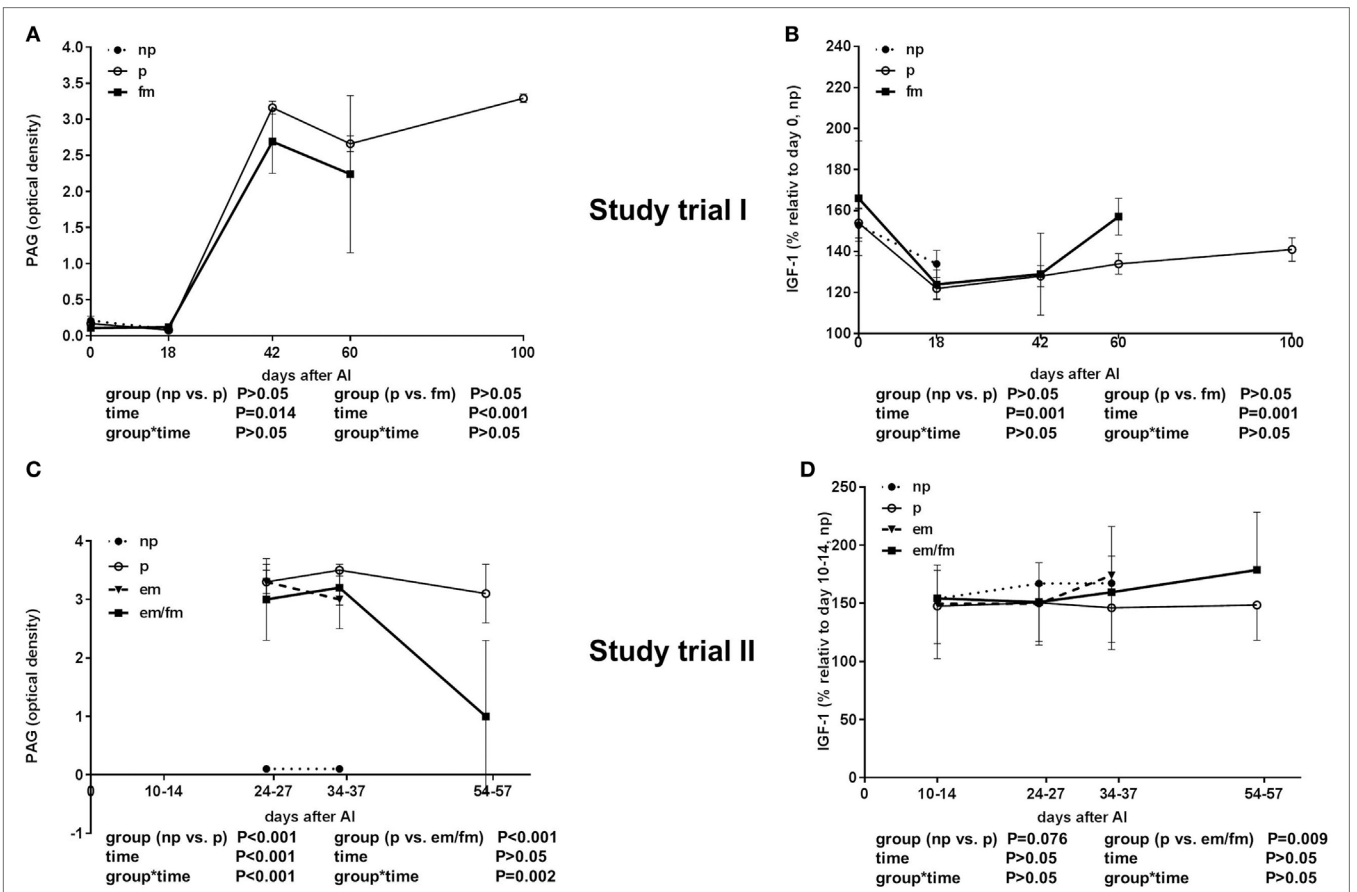

FIGURE 2 | Semi-quantitative pregnancy-associated glycoproteins (PAG) values given in optical density (OD) units and insulin-like growth factor 1 (IGF-1) concentrations. **(A,B)** Study trial I in not pregnant [np, $n = 43$, day of AI (0) and 18 days after AI] and pregnant (p, $n = 34$, day of AI, and 18, 42, 60 and 100 days after AI) pluriparous Holstein–Friesian cows as well as cows with fetal mortality (fm, $n = 4$, day of AI and until day 60 after AI). **(C,D)** Study trial II in not pregnant (np, $n = 20$, 10–14 (IGF-1), 24–27, 34–37 days after AI) and pregnant (p, $n = 30$, 24–27, 34–37 and 54–57 days after AI) Holstein–Friesian cows as well as cows with embryo mortality (em, $n = 7$, 24–27, 34–37 days after AI) or late embryo/fetal mortality (em/fm, $n = 8$, 24–27, 34–37, and 54–57 days after AI). Values are given as mean ± SEM.

The IGF-1 concentration was decreased in all the groups from days 0 to 18 and thereafter steadily increased from days 18 until 100 of gestation ($P < 0.001$). No differences between pregnant and non-pregnant cows, and cows with fm were detectable in the total IGF-1 concentrations; however, between days 42 and 60, an abrupt increase in the total IGF-1 in the cows suffering from fm was measured (**Figure 2B**).

The IGFBP-2 concentration was lower in pregnant cows and highest in cows with fm. This distinct difference was visible on the day of insemination (**Figure 3A**). Additionally, the IGFBP-3 concentration was higher in cows with fm than that in pregnant cows (**Figure 3B**). IGFBP-4 was higher in cows with fm ($P = 0.0076$) if compared to pregnant and non-pregnant cows. Within the group of cows with fm, IGFBP-4 decreased from days 0 to 18 and thereafter increased until day 60 of pregnancy. In non-pregnant cows, IGFBP-4 decreased until day 42 after AI but increased later (**Figure 3C**). The IGFBP-3/IGFBP-2 ratio sharply decreased between days 0 and 18 and increased thereafter steadily until day 100 of pregnancy (**Figure 3D**).

Study Trial II

Animals

Out of 500 examined cows, 203 were pregnant on days 24–27 after AI, and 284 were non-pregnant; in 15 cows with a positive pregnancy diagnosis on days 24–27 after AI, no pregnancy was detectable at either days 34–37 or 54–57 after AI. Therefore, two groups of pregnancy loss were defined: pregnancy loss between days 24–27 and 34–37 after AI = late embryonic mortality (em, $n = 7$) and pregnancy loss between days 34–37 and 54–57 after AI = late embryonic mortality and early fetal morality (em/fm, $n = 8$). A total of 13 animals were excluded from the study due to various illnesses. To analyze a balanced subset, $n = 30$ pregnant and $n = 20$ non-pregnant cows were chosen by random for IGF-1 and IGFBP analyses.

Laboratory Analyses

As expected, the PAG levels, displayed in OD, were high in pregnant animals and low in non-pregnant animals in Study trial II.

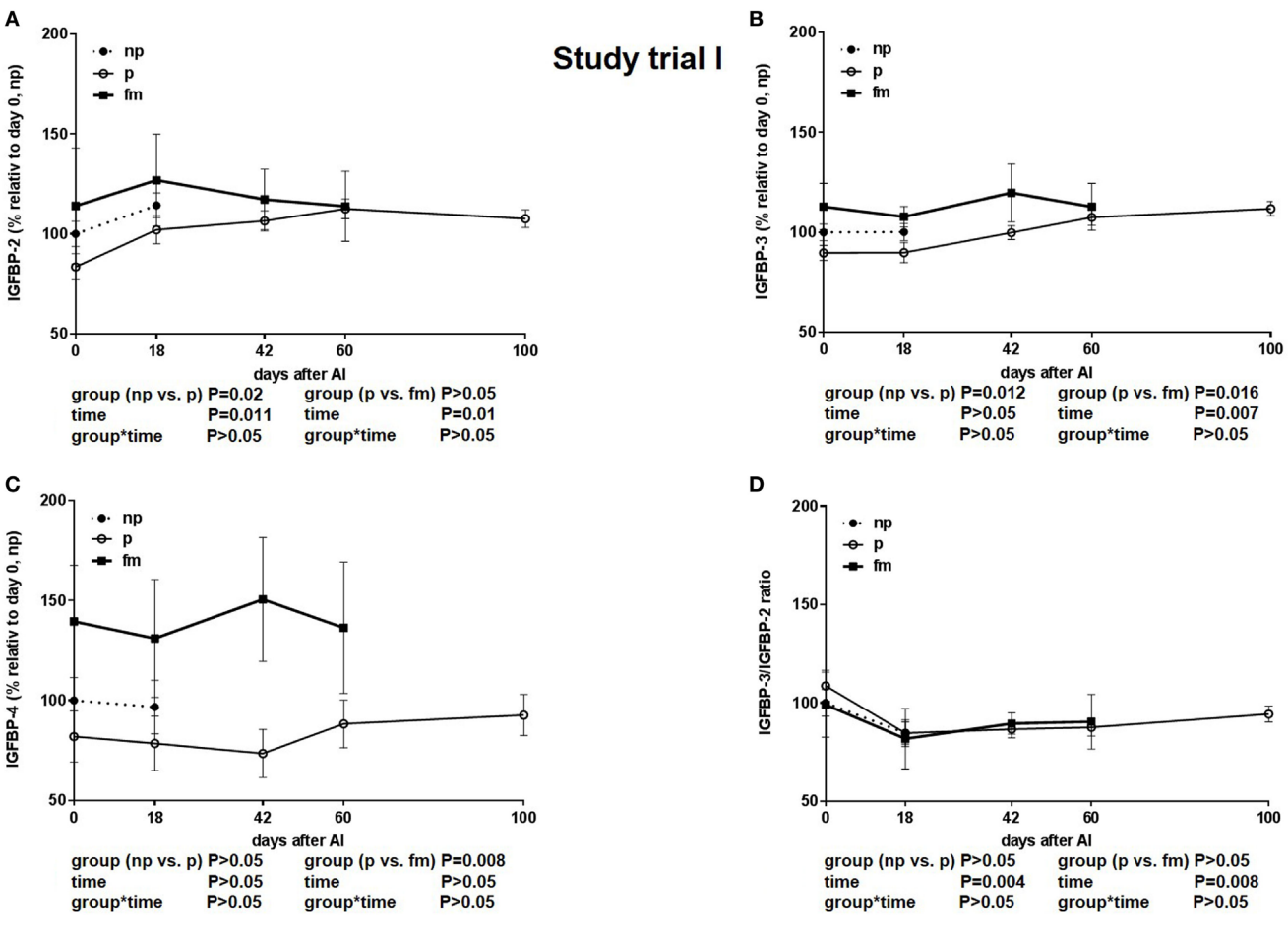

FIGURE 3 | Study trial I: insulin-like growth factor-binding protein (IGFBP) **(A)**: -2, **(B)**: -3 and **(C)**: -4 concentration and **(D)**: IGFBP-3/IGFBP-2 ratio in not-pregnant [np, $n = 43$, day of AI (0) and 18 days after AI] and pregnant (p, $n = 34$, day of AI, and 18, 42, 60 and 100 days after AI) pluriparous Holstein–Friesian cows as well as cows with fetal mortality (fm, $n = 4$, day of AI and until day 60 after AI). Values are given in% relative to day 0 of non-pregnant cows. Values are given as mean ± SEM.

The PAG OD decreased between days 34–37 and 54–57, clearly indicating pregnancy loss (**Figure 2C**).

The total IGF-I concentrations were tended to be lower in pregnant cows if compared to non-pregnant cows or cows with em ($P = 0.0076$; **Figure 2D**). The IGFBP-2 concentration was significantly higher in cows with em and em/fm compared to pregnant and not pregnant cows (**Figure 4A**). IGFBP-3 differed between pregnant and cows with em/fm. The latter had higher IGFBP-3 concentrations than did pregnant and not pregnant cows (**Figure 4B**). IGFBP-4 was clearly higher in cows with em/fm compared to the other groups (**Figure 4C**). The IGFBP-3/IGFBP-2 ratio was clearly lower in cows with em/fm than in pregnant cows (**Figure 4D**).

In Vitro Trial

The proteolytic degradation of rhIGFBP-4 was detected in pregnant cows (25–75% reduction of IGFBP-4) in all trimesters. The rhIGFBP-4 was 100% degraded in human serum. No proteolysis of rhIGFBP-4 was detected in non-pregnant cows. Moreover, no proteolysis of endogenous serum IGFBP-2 and IGFBP-3 in pregnant cows was obvious compared to that in the human control, where nearly all binding proteins were absent during pregnancy (**Figure 5**).

DISCUSSION

Both study trials were independently conducted in 2015 and 2016 on two different farms with different animals. Therefore, a farm-specific influence on the results comparably obtained in the two study trials can be excluded, thereby strengthening the results. In Study trial I, 4 pluriparous cows with fm were defined (3 between days 60 and 100 and 1 between days 42 and 60). In Study trial II, a higher number of animals ($n = 500$) was examined; therefore, 8 cows were found with late embryonic mortality (between 24–27 and 34–37), and 7 cows were found with late embryonic/early fm (between 34–37 and 54–57). The early fetal losses (between 42 and 60, $n = 1$) of Study trial I were most comparable to those of late embryonic/early fm (between 34–37 and 54–57) in Study trial II. The late losses in study I (>day 60) were not comparable to those in Study trial II, but the later fetal losses >day 42, which were not infectious abortions, might be due to placental dysfunction, fetal undernourishment or insufficient growth and, therefore,

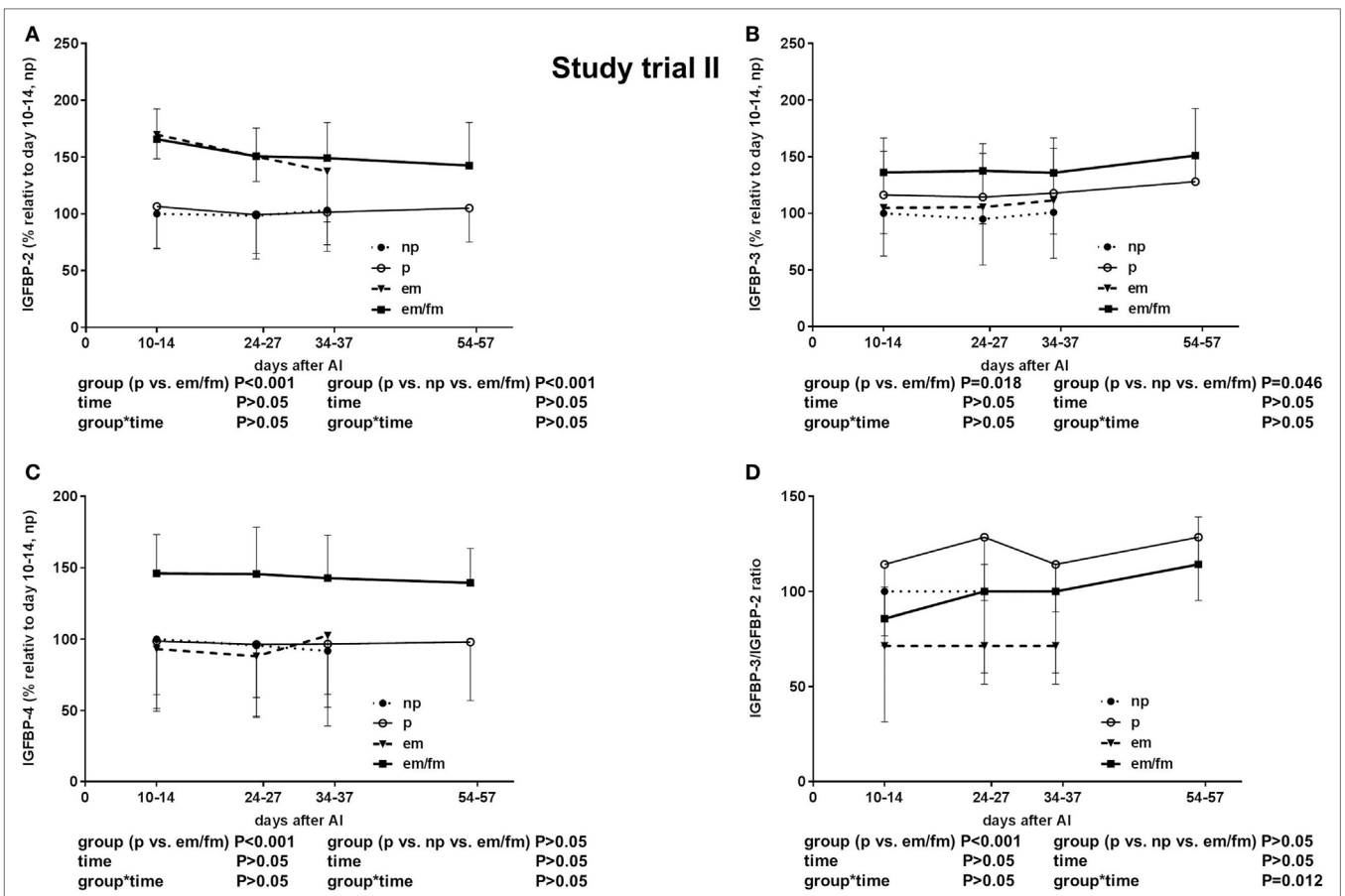

FIGURE 4 | Study trial II: insulin-like growth factor-binding protein (IGFBP) **(A)**: -2. **(B)**: -3. **(C)**: -4 and **(D)**: IGFBP3/IGFBO2 ratio in non pregnant (np, $n = 20$) and pregnant (p, $n = 30$) Holstein Friesian cows and cows with embryo mortality (em, $n = 7$) between days 24-27 and 34-37 or late embryo mortality/early fetal mortality (em/fm, $n = 8$) between days 24-27 and 54-57. Values are given in percentege relative to days 10–14 of non-pregnant cows. Values are given as mean ± SEM.

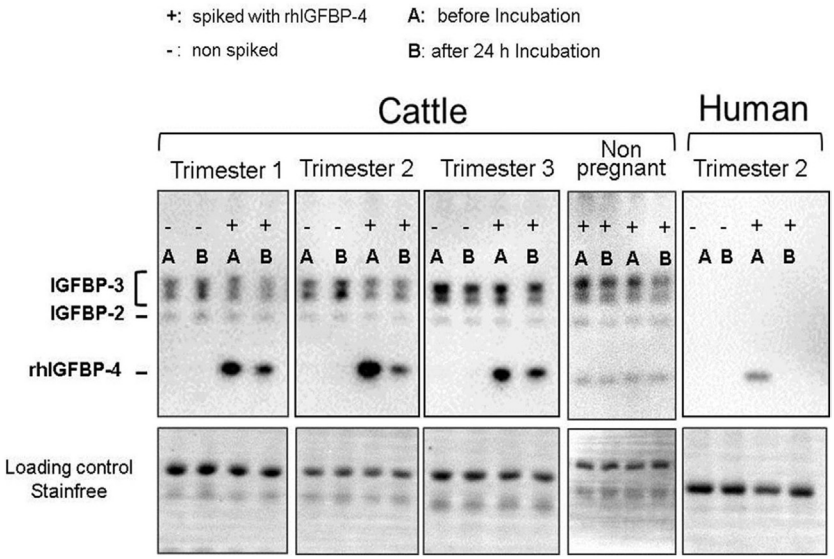

FIGURE 5 | Western ligand blots with serum samples of not-pregnant and pregnant cows before (–I) and after (+I) incubation (24 h, 39°C) with Insulin-like Growth factor 4 (IGFBP-4) and insulin-like growth factor 1 (IGF-1) spiked to the serum. As positive control serum of a pregnant woman was available for which proteolytic activity IGFBP-4 is clearly proven.

may be associated with IGF systems. However, no standardized microbiological or viral examination was done. Therefore, this assumption remains speculative.

In both studies, fetal loss was indicated not only by the empty uterus during ultrasound examination but also by declining PAG concentrations in the blood. PAGs are produced by binucleate cells of the placenta, indicating functional placentation (22, 23). Decreasing concentrations are associated with declining placenta function and pregnancy loss.

The local IGF system in the endometrium is crucial for embryonic development and is influenced by the cycle stage (24) but little is known about the role of the IGF-1 reservoir in blood in embryonic and fetal development or placental growth in cattle. IGF-1 is bound to specific binding proteins that indeed regulate either the transport of IGFs through the endothelium (IGFBP-2) or the retention in the blood stream (25). Specific proteolysis of the binding proteins in maternal blood can release IGF and may also enhance the free concentration at the tissue layer. Therefore, the aim of the present study was to characterize IGFBPs throughout early pregnancy in maternal blood circulation and compare those to either non-pregnant cows at the same cycle stage or to those suffering from embryonic or fm. Thus, embryonic mortality is defined as a pregnancy loss before day 42 of pregnancy in cows, and thereafter, the fetal period starts (26).

The total IGF-1 concentration in pregnant cows decreased from days 0 to 18 in the present study, typically due to sexual steroid hormone patterns during the cycle (19). This effect was previously observed by the application of estradiol and progesterone to ovariectomized heifers (27). In the first trimester of pregnancy (days 42–100), in Study trial I, a slight but steady increase in IGF-1 was measurable, whereas the concentrations remained relatively constant until day 60. However, the IGF-1 concentration was comparable between pregnant and non-pregnant cows and cows with embryonic/fm in both study trials. This finding is consistent with those of previous studies in Angus cattle, where no differences in pregnancy rates were found among animals selected for different IGF-1 concentrations (28). Consistently, in a recent study, the basal GH concentration was associated with the ability of cows to achieve pregnancy (29), but not total IGF-1 concentrations. In contrast to *in vivo* results, *in vitro* studies clearly demonstrated that IGF-I has a positive effect on embryo development (30–32). Indeed, *in vitro* embryos treated with IGF-1 during *in vitro* culture achieved a higher pregnancy rate after embryo transfer (28, 29). The conflicting *in vitro* and *in vivo* results might be due to IGFBPs, which on the one hand can be produced locally by the placenta but also the maternal IGFBPs might have an influence on the free IGF-1 concentration, which may have an influence on placental or fetal growth. The total IGF-1 concentration, also measured in the present study, does not reflect the bioavailability of IGF-1 on the local tissue level. The bioavailability differs with respect to which binding protein IGF is bound and the concentration of these single binding proteins. As in other species, distinct changes in IGFBP concentrations were previously measured throughout pregnancy, and their vital roles have been suggested (33). Interestingly, in women, there is a strong proteolysis of IGFBPs (IGFBP-2 and IGFBP5) modulated by the expression of PAPP-A in the placenta, resulting in high levels of circulating PAPP-A in pregnant women during the first trimester (10, 34). Moreover, a high level of PAPP-A could be detected in the human trophoblast. IGFBP-4 is described to be proteolysed as well by PAPP-A (35).

The present study examined the peripheral IGFBP-2, IGFBP-3, and IGFBP-4 concentrations by using a Western ligand blot technique, which enables the detection of functional binding

proteins in serum (20). In contrast, antibody based methods might also detect binding protein fragments and, therefore, might not necessarily correlate with the biological binding capacity of IGFBPs.

In both study trials, pregnancy in cows did not distinctly influence the IGFBP-2 concentration over time. Although, PAPP-A is described in bovine preovulatory follicles and there able to degrade IGFBP-2 (36). However, to the best of the authors' knowledge, there is no focused study on placental PAPP-A in bovine species. Interestingly, IGFBP-2 levels were higher in cows developing fm during the first 60 days of pregnancy than those in cows with physiological pregnancies. This fact was observed in both study trials consistently, specifically at days 0 and 18 in Study trial I and days 10–14 in Study trial II. IGFBP-2 is considered an activator and inhibitor of IGF action and is known as a main serum carrier of IGF-1 despite of IGFBP-3 (37). Therefore, increased concentrations may also inhibit IGF-1 action, and thus, influence fetal or placental growth. The association between fertility and this particular binding protein has previously been demonstrated by Wathes (38), who examined SNPs in the IGFBP-2 gene associated with a lower rate of pregnancy in cows with a specific SNP in this gene. Moreover, hepatic IGFBP-2 production was also directly controlled by growth hormone (37), which might explain the results of the above-mentioned study, where GH, but not IGF-1, is directly linked to pregnancy success. Under conditions of elevated, IGFBP-2 expression inhibitory effects have been provided both on growth and reproductive development (39). In women, the IGFBP-2 concentration declined after conception and began to steadily increase thereafter (33). Interestingly, elevated IGFBP-2 in the maternal blood circulation has been associated with growth restriction due to placental dysfunction in human medicine (38). In the present study, a comparable pathomechanism might be involved, as the pregnancies were lost after conception and placentation, suggesting that placental dysfunction and fetal growth retardation might be involved in fm. In the present study, ultrasound was conducted to detect pregnancy, but in a routine manner. Thus, there is no information on fetal length available, but this measurement should be considered in further studies. On the other hand, also the metabolic adaption throughout early lactation may lead to the peripheral IGFBP-2 concentrations as it was indicated that during early lactation IGFBP-2 was higher and decreases over the lactation period (2).

In human pregnancies, the complete proteolysis of IGFBP-3 was previously observed (40), and it was also observed in the human control serum in the present *in vitro* trial. It has to be mentioned that the human control was from a twin pregnancy and, therefore, likely have a higher proteolytic potential than in singleton human pregnancies (41). However, in cows, there was no drastic decline in IGFBP-3 throughout pregnancy until day 100, but rather an increase was measured until day 100 of pregnancy. Also *in vitro* there is still the IGFBP-3 band detectable.

In addition to the elevated IGFBP-2, IGFBP-3 was also higher in cows developing fm; this finding was also observed already during early embryonic development around days 10–18 after insemination. IGFBP-3 is the most abundant binding protein, and 80–90% of IGFs are bound to IGFBP-3 (38); in human pregnancy, the proteolysis of binding proteins, particularly IGFBP-3 (42), shifts the control of IGF action to IGFBP1, which offers the highest affinity to IGF-1 (43). The present study (Trial I) indicated that in cows, IGFBP-3 concentrations increased toward day 100 of pregnancy. Western ligand blotting only detects functional binding proteins; therefore, it is unlikely that single proteolytic fragments individually bind IGF-1, resulting in increased concentrations. Therefore, the mechanism controlling fetal and placental growth by the maternal somatotropic axis appears to be different between bovine and human. Increased concentrations of IGFBP-3 may indicate that less IGF-1 is free for local action. In future studies, the additional measurement of free IGF-1 (in blood and local) should be contemplated in order to answer this question.

One of the main goals of the present study was to measure the concentration particularly of IGFBP-4, as inhibitory binding proteins previously associated with fetal growth restriction. In early physiological pregnancies in women, an increase in IGFBP-4 was measured, followed by lower concentrations (15). We previously indicated a decrease in IGFBP-4, also measured by Western Ligand blot (13). In contrast to these results, no specific decrease in IGFBP-4 was found in cows in the present study in pluriparous cows. One difference between these both studies was that Meyerholz et al. (13) used heifers exclusively whereas in the present study mainly pluriparous, meaning lactating animals, are examined. This could be the underlying reason for differences in peripheral IGFBP pattern.

Oxvig (12) summarizes the importance of PAPP-A within the IGF-1 system and concluded that PAPP-A is an important component of the IGF system and describes this part of the IGF system as the "PAPP-A→IGFBP-4→IGF axis." In cattle, PAPP-A has also been described in the context of follicular growth as already mentioned above, where preovulatory ovarian follicles are characterized by a high proteolytic cleavage of IGFBP-4 through PAPP-A (44). Moreover, variants in the PAPP-A gene were associated with "daughter pregnancy rate" but no detection during gestation in cattle was reported so far. In the present study, in pregnant cattle, *in vivo* data suggest that no distinct cleavage of IGFBP-4 occurs in bovine pregnancies as the concentrations stay comparable. But, *in vitro* a degradation of IGFBP-4 could be verified, however, no proof of the responsible enzyme e.g., PAPP-A was intended and should be the focus of further studies. Interestingly, in both study trials, IGFBP-4 was clearly lower in physiological pregnancies than in those with fm. Thus, we speculate that a high concentration of an inhibitory binding protein in maternal blood may be associated also with lower free IGF levels, which might have an important influence on fetal growth or placentation or that this high concentrations just mirrors a sub function of local proteases also released in the maternal blood circulation. Moreover, also the metabolic adaption of the dairy cow with regard to parallel lactation maybe lead to higher IGFBP-4 concentrations (2). Therefore, further studies are warranted to elucidate the underling pathomechanism for higher IGFBP-2- and IGFBP-4 concentrations in cows with pregnancy loss.

In conclusion, distinct differences in the peripheral IGFBP pattern could be associated with late embryonic and early fetal losses in cattle. Specific IGFBP-4 proteolysis could be identified in

cattle but less intense than in human as peripheral concentrations were comparable between pregnant and not pregnant animals. Therefore, circulating concentrations of IGFBP-2, IGFBP-3, and IGFBP-4 in the mother may have a biomarker potential for maintenance of pregnancy in dairy cows.

ETHICS STATEMENT

The study (Trial I) was performed in accordance with the German legislation on animal welfare (Lower Saxony Federal State Office for Consumer Protection 279 and Food Safety, AZ 33.9-42502-05-14A487). The study (Trial II) was performed in accordance with the German legislation on animal welfare (state office of Brandenburg for work protection, consumer protection, and physical health; AZ 2347-29-2014). The human serum was available from one of the researchers, therefore no ethical approval was necessary according to the ethics committee of the Hanover medical school.

AUTHOR CONTRIBUTIONS

MK: organization Study trial II, sampling and examinations for Study trial II and manuscript proof reading. H-DJ: organization and sampling for Study trial I. WE and BY: *in vitro* tests and writing of material and methods as well as results for the *in vitro* test. BR: statistical analyses. PS: sampling and examination for Study trial II and manuscript proof reading. JM: organization of Study trial II and manuscript proof reading. HC: western ligand blotting. HA: interpretation of data and manuscript proof reading. SM: coordination of the study, writing the manuscript, and interpretation of data.

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Conflict of Interest Statement: HC and HA are related to Ligandis. All other authors state that there are no conflicts of interest to declare.

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Effects of Transport Duration and Environmental Conditions in Winter or Summer on the Concentrations of Insulin-Like Growth Factors and Insulin-Like Growth Factor-Binding Proteins in the Plasma of Market-Weight Pigs

Elisa Wirthgen[1†], Sébastien Goumon[2†], Martin Kunze[1], Christina Walz[1], Marion Spitschak[1], Armin Tuchscherer[3], Jennifer Brown[4], Christine Höflich[5], Luigi Faucitano[6] and Andreas Hoeflich[1]**

[1] Institute of Genome Biology, Leibniz-Institute for Farm Animal Biology (FBN), Dummerstorf, Germany, [2] Department of Ethology, Institute of Animal Science, Prague, Czechia, [3] Institute of Genetics and Biometry, Dummerstorf, Leibniz-Institute for Farm Animal Biology (FBN), Dummerstorf, Germany, [4] Prairie Swine Centre, Saskatoon, SK, Canada, [5] Ligandis UG, Gülzow-Prüzen, Germany, [6] Sherbrooke Research and Development Centre, Sherbrooke, QC, Canada

In previous work using market-weight pigs, we had demonstrated that insulin-like growth factors (IGFs) and insulin-like growth factor binding proteins (IGFBPs) are regulated during shipment characterized by changing conditions of stress due to loading or unloading, transportation, lairage, and slaughter. In addition, we found in a previous study that IGFBP-2 concentrations were lower in pigs transported for longer periods of time. Therefore, we performed a more detailed study on the effects of transport duration and season on the plasma concentrations of IGFs and IGFBPs in adult pigs. For the study, exsanguination blood was collected from 240 market-weight barrows that were transported for 6, 12, or 18 h in January or July. IGF-I and -II were detected using commercial ELISAs whereas IGFBPs were quantified by quantitative Western ligand blotting. In addition, established markers of stress and metabolism were studied in the animals. The results show that plasma concentrations of IGFBP-3 were significantly reduced after 18 h of transport compared to shorter transport durations (6 and 12 h; $p < 0.05$). The concentrations of IGF-I in plasma were higher ($p < 0.001$) in pigs transported 12 h compared to shorter or longer durations. Season influenced plasma concentrations of IGFBP-3 and IGF-II ($p < 0.05$ and $p < 0.01$, respectively). Neither transport duration nor differential environmental conditions of winter or summer had an effect on glucocorticoids, albumin, triglycerides, or glucose concentrations ($p > 0.05$). However, low-density lipoprotein concentrations decreased after 18 h compared to 6 h of transport ($p < 0.05$), whereas high-density lipoprotein concentrations were higher ($p < 0.05$) in pigs transported for 12 or 18 h compared to those transported for only 6 h. Our findings indicate differential regulation of IGF-compounds in response to longer transport duration or seasonal changes and support current evidence of IGFs and IGFBPs as innovative animal-based indicators of psycho-social or metabolic stress in pigs.

Keywords: animal welfare, stress hormones, insulin-like growth factor, pig shipment, metabolism, biomarker

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****Correspondence:***
Luigi Faucitano
luigi.faucitano@agr.gc.ca;
Andreas Hoeflich
hoeflich@fbn-dummerstorf.de

[†]These authors have contributed equally to this work.

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INTRODUCTION

Due to concentration of the slaughter process, more pigs are being transported to less and bigger slaughter plants resulting in increasingly longer transport distances and lengths (1). Longer transport distance or duration is associated with increased loss of live-weight (2), mortality (3), serum concentrations of acute phase proteins (4), creatine kinase (CK) (5, 6), glucose, lactate, and hematocrit levels (7, 8). The collection of exsanguination blood directly after the slaughtering of pigs is a suitable, non-invasive technique for the evaluation of the physiological response to different transport durations and preslaughter conditions. As described in preliminary studies (9), cortisol concentrations, measured in exsanguination blood, were not affected after prolonged duration of shipment. Though, insulin-like growth factor-binding protein (IGFBP)-2 concentrations decreased over time due to the length of shipment indicating biomarker potential for components of insulin-like growth factor (IGF) system. Furthermore, IGFBP-3/-2 ratio was increased in pigs, which were repeatedly stressed in the period of 24 h before slaughter. In humans, parameters of the growth hormone (GH) axis may have biomarker potential for acute or prolonged illness (10, 11). In mammals, it is assumed that acute physical stress, energy restriction, or acute phase of severe illness induce an amplification of GH secretion and increased levels of GH (10, 12, 13). GH affects body growth and metabolism directly and indirectly *via* control of IGF-I production in the liver or in other tissues (14). In blood, IGF is bound to IGFBPs that control the availability of IGF, but also have IGF-independent functions (15). As IGFBPs are sensitive markers to detect changes of the GH-dependent growth (16, 17), they play a central role in linking nutritional intake with somatic growth (18–20). Furthermore, it is known that glucocorticoids influence the levels of IGF-I and IGFBPs (21–23) suggesting an interference of acute or prolonged stress with the IGF system. Accordingly, in the present study, effects of transport duration under different seasonal conditions on the IGF-system were discussed in conjunction with established stress markers.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the current guidelines of the Canadian Council on Animal Care (CCAC, 2009).

Animals and Preslaughter Conditions

Details about the experimental conditions applied in this experiment were previously described by Goumon et al. (24). Briefly, this experiment was part of a larger study involving 5,040 crossbred pigs (*Sus scrofa*, Landrace × Large White; mean body weight = 120.8 ± 0.4 kg) transported over 6, 12, or 18 h (average loading density of 0.37–0.38 m^2/pig) to a slaughterhouse located in western Canada. This study was conducted with subset of 240 male pigs (barrows, 120.8 ± 0.4 kg) at the age about 24 weeks and includes two trials. One trial was conducted in January to February with a temperature range between −28.8 and 1.9°C during the transport. The second trial was conducted in July and the temperature ranged between 12.5 and 40.1°C. Food was removed from pigs transported for 6 and 12 or 18 h for 20 and 24 h, respectively. As previously described, fasting did not affect the circulating levels of IGF-1 and IGFBPs within the time frame of this experiment (9). The pigs had no access to water on the truck, but water was available in lairage. After unloading, pigs were held in a lairage for approximately 150 min.

Blood Collection

At exsanguination, 2 ml of blood were collected from a subsample of 240 barrows (40 barrows/transport duration/season) and EDTA-plasma was extracted (centrifugation at 1,400 × *g* at 4°C for 12 min) (24). Plasma samples were stored at −80°C before shipment to the laboratory of the Institute of Genome Biology at the Leibniz Institute for Farm Animal Biology (FBN) in Dummerstorf (Germany) for further analyses.

Plasma Analyses

Insulin-Like Growth Factor Binding Proteins

Quantitative Western ligand blotting was applied for the assessment of IGFBP-3, -2 and -5 concentrations as described previously (25). Briefly, plasma samples were denatured for 5 min in sample buffer (312.5 mM Tris (pH 6.8), 50% (w/v) glycerol, 5 mM EDTA, 1% (w/v) SDS, and 0.02% bromophenol blue). After separation by 12% SDS-PAGE, proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA), blocked and incubated with biotin labeled human IGF-II (1:500; BioIGF2-10; ibt-systems, Binzwangen, Germany). IGFBPs were detected by enhanced chemiluminescence using LuminataTM Forte (Millipore, Bedford, MA, USA). The signal intensities were corrected for background using the Gelanalyzer2010a software. On each blot, serial dilutions of recombinant human IGFBP-2 to -5 standards (R & D Systems, Wiesbaden, Germany) in artificial serum matrix (Biopanda, County Down, UK) were used as calibrators enabling signal quantification. Each signal was corrected for unspecific background Curve fitting was achieved by non-linear regression of each separate IGFBP. Due to low abundance, IGFBP-4 was detected but not quantified in the porcine plasma. The analytical range for each plasma IGFBP was 150–15,000 ng/ml. Inter-assay coefficients of variation (CV) were determined by *in study* validation according to recommendations of EMA guideline (26) using a random selected pig plasma sample of the investigated study samples. The inter-assay CVs ($n = 10$) for IGFBP-3 (mean: 4850 ng/ml), -2 (mean: 2324 ng/ml), and -5 (679 ng/ml) were 12.8, 15.1, and 20.1%, respectively.

IGF-I and IGF-II

Plasma concentrations of IGF-I and IGF-II ($n = 90$) were analyzed by Ligandis GbR using commercially available ELISA Kits E20 and E30 according to manufacturer's instruction (Mediagnost, Reutlingen, Germany). For IGF-I, the analytical range was 21–1,050 ng/ml and the inter- and intra-assay CVs were less than 6.8 and 6.7%, respectively. For IGF-II, the analytical range was from 120 to 2,400 ng/ml.

Corticosterone

Plasma concentrations of corticosterone ($n = 90$) were analyzed using LC-MS technique as already described (27). In brief, after protein precipitation, the supernatant was dried and stored at −20°C. For LC–MS/MS analysis, samples were dissolved in MeOH/H_2O (50/50) adding DXM (100 ng/ml), vortexed for 30 s, sonicated for 2 min, and centrifuged at 4°C for 2 min at 14,000 rpm. Subsequently, samples were transferred to mass spectrometry analysis using an Accela HPLC/autosampler system (Thermo Fisher Scientific) coupled to the LTQ Orbitrap high-resolution hybrid mass spectrometer (Thermo Fisher Scientific, Dreieich, Germany). At various concentrations between 5 and 500 ng/ml, the intra-assay CVs were 13.05–4.64%. The inter-assay CV for 100 ng/ml ($n = 20$) was 5.57%.

Metabolites

Triglycerides (TG), cholesterol, glucose, high-density lipoprotein cholesterol (HDL-C), and albumin ($n = 90$) were analyzed in plasma using commercial enzymatic colored kits according to the manufacturer's instructions (TG: No. LT-TR 1002, total cholesterol: No. LT-CH 0503, glucose: LT-GL 0251, HDL: LT-HD 0053, albumin LT-AB 0103; Labor & Technik Eberhard Lehmann Berlin, Germany, respectively) as previously described (28). The intra-assay CV for TG, total cholesterol, glucose, HDL and albumin were 1.60, 1.32, 1.80, 2.44, and 1.66%, respectively. The inter-assay CV% for TG, total cholesterol, glucose, HDL, and albumin were 4.30, 1.98, 2.40, 2.50, and 1.11%, respectively. Low-density lipoprotein cholesterol (LDL-C) was calculated according to the Friedewald Formula = [total cholesterol] − [HDL-C] − ([TG]/5) (29, 30).

Statistics

Statistical analyses were performed using SAS software version 9.3 (SAS, Cary, NC, USA). The data of all blood parameters were evaluated by ANOVA using the MIXED procedure. The ANOVA model included transport duration (6, 12, and 18 h), environment (January/February versus July), week within environment (subset of weeks 1, 2, 3, and 4 in January/February, subset of weeks 1, 2, 3, and 4 in July) and the two-way interaction duration × environment as fixed effects. For all data, the least squares means and their SE were calculated and tested for each fixed effect in the model using the Tukey–Kramer procedure for all pair-wise multiple comparisons. Effects and differences were considered significant if $p < 0.05$.

RESULTS

Effect of Transport Duration

An overview of all calculated *F*-values, degrees of freedom, and probability *F*-values of each fixed effect and their interaction is provided by **Table 1**. Quantitative Western ligand blot analyses of IGFBPs revealed IGFBP-3 as being the most abundant plasma IGFBP in pigs followed by IGFBP-2 and IGFBP-5 (**Figures 1A–D**) and having similar molecular weights as human recombinant reference standards. Plasma concentrations of IGFBP-3 and IGFBP-2 were lower in pigs transported for 18 h compared to pigs transported for 6 h ($p < 0.001$ and $p < 0.001$, respectively; **Figures 1A,C**). However, the concentrations of both IGFBP did not differ in pigs transported for 6 and 12 h ($p > 0.05$). There was no significant effect of transport duration on plasma concentrations of IGFBP-5 either ($p > 0.05$; **Figure 1D**). Concentrations of IGF-I were higher ($p < 0.001$) in pigs transported for 12 h than in those transported for 6 or 18 h (**Figure 1E**). By contrast, plasma concentrations of IGF-II were not significantly affected by transport duration ($p > 0.05$; **Figure 1F**). The total amount of quantified IGFBPs, which is an indicator for IGF-binding capacity, was lower in pigs transported for 18 h compared to pigs transported for 6 and 12 h ($p < 0.001$ and $p < 0.05$, respectively; **Figure 2A**). The ratio of IGF-I to total IGFBPs, calculated as an indicator for IGF-I bioavailability in circulation, was greater ($p < 0.001$) in pigs transported for 12 h compared to those transported for 6 h (**Figure 2B**). As shown in **Figure 2C**, the ratio of IGFBP-3 to -2, used as a marker for somatic growth and metabolic homeostasis, was greater in pigs transported for 18 h compared to pigs transported for 6 or 12 h ($p < 0.001$ and $p < 0.05$, respectively).

Plasma concentrations of corticosterone, glucose, and albumin were not significantly affected by transport duration ($p > 0.05$; **Figures 3A–C**). Plasma concentrations of total cholesterol were lower ($p < 0.01$) in pigs transported for 18 h compared to those transported for 12 h (**Figure 3D**). When compared to 6 h transports, HDL-cholesterol levels were greater after 12 h ($p < 0.01$) and 18 h ($p < 0.001$) transports (**Figure 3E**). By contrast, LDL-cholesterol concentrations were lower in pigs transported for 18 h compared to pigs transported for 6 or 12 h ($p < 0.05$ and $p < 0.01$, respectively; **Figure 3F**). An effect of transport duration on plasma TG was only detected in summer. Thereby, the concentrations of TG were increased after 12 and 18 h transport compared to 6 h ($p < 0.001$ and $p < 0.05$, respectively, **Figure 3G**).

Effect of Differential Environment in Winter or Summer

The environmental conditions had an effect on some plasma parameters in this study, with concentrations of IGFBP-3 being higher ($p < 0.05$) in January/February than in July and IGF-II concentrations being lower ($p < 0.01$) in January/February than in July (**Table 2**). Plasma IGFBP-2, IGFBP-5, IGF-I, cortisol, corticosterone, albumin, and glucose concentrations were not affected by the differential environmental conditions in this study ($p > 0.05$).

DISCUSSION

The results of this study clearly demonstrate that transport duration and differential environmental conditions in winter or summer contribute to the variation of IGFs and IGFBPs in blood collected from pigs at slaughter. Indeed, the concentrations of IGFBP-3 were lower after 18 h transport, contributing also to reduction of total IGFBPs levels in the circulation. Due to a stronger reduction of IGFBP-2, the ratio of IGFBP-3/IGFBP-2 increased after 18 h of transportation. An increase of IGFBP-3/IGFBP-2 ratio was also reported in plasma of pigs subjected to repeated restraint stress in the preslaughter period (9) indicating that this ratio might

TABLE 1 | *F*-test for the fixed effects season, week within season, duration, and the interaction of environment × duration.

Parameter	Effect	NumDF	DenDF	*F*-value	ProbF
IGFBP-3	Environment	1	228	5.50	**0.020**
	Week (environment)	6	228	1.61	0.147
	Duration	2	228	8.01	**<0.001**
	Environment × duration	2	228	0.26	0.768
IGFBP-2	Environment	1	225	0.02	0.892
	Week (environment)	6	225	1.62	0.143
	Duration	2	225	11.30	**0.001**
	Environment × duration	2	225	2.05	0.131
IGFBP-5	Environment	1	154	0.46	0.500
	Week (environment)	6	154	1.18	0.322
	Duration	2	154	0.79	0.455
	Environment × duration	2	154	0.48	0.622
IGF-I	Environment	1	81	0.55	0.460
	Week (environment)	3	81	2.84	**0.043**
	Duration	2	81	15.51	**<0.001**
	Environment × duration	2	81	2.22	0.115
IGF-II	Environment	1	81	7.82	**0.006**
	Week (environment)	3	81	1.31	0.277
	Duration	2	81	0.04	0.962
	Environment × duration	2	81	1.86	0.163
total IGFBPs	Environment	1	228	3.22	0.074
	Week (environment)	6	228	1.59	0.151
	Duration	2	228	11.50	**<0.001**
	Environment × duration	2	228	0.41	0.664
IGF-I/total GFBPs	Environment	1	81	0.30	0.585
	Week (environment)	3	81	1.29	0.284
	Duration	2	81	10.22	**<0.001**
	Environment × duration	2	81	2.13	0.126
IGFBP-3/IGFBP-2	Environment	1	225	1.67	0.198
	Week (environment)	6	225	0.50	0.809
	Duration	2	225	6.66	**0.001**
	Environment × duration	2	225	1.87	0.157
Corticosterone	Environment	1	81	0.19	0.663
	Week (environment)	3	81	1.17	0.325
	Duration	2	81	0.09	0.915
	Environment × duration	2	81	1.78	0.176
Glucose	Environment	1	74	0.94	0.334
	Week (environment)	3	74	2.14	0.103
	Duration	2	74	0.26	0.772
	Environment × duration	2	74	0.13	0.880
Albumin	Environment	1	75	2.64	0.108
	Week (environment)	3	75	1.67	0.180
	Duration	2	75	2.13	0.126
	Environment × duration	2	75	0.79	0.456
Triglycerides	Environment	1	81	0.00	0.951
	Week (environment)	3	81	1.55	0.208
	Duration	2	81	8.38	**<0.001**
	Environment × duration	2	81	4.88	**0.010**
Cholesterol	Environment	1	81	3.35	0.071
	Week (environment)	3	81	0.45	0.719
	Duration	2	81	5.16	**0.008**
	Environment × duration	2	81	1.87	0.160
HDL-C	Environment	1	81	1.05	0.308
	Week (environment)	3	81	0.17	0.915
	Duration	2	81	11.67	**<0.001**
	Environment × duration	2	81	1.71	0.188

(*Continued*)

TABLE 1 | Continued

Parameter	Effect	NumDF	DenDF	*F*-value	ProbF
LDL-C	Environment	1	81	2.67	0.106
	Week (environment)	3	81	0.64	0.588
	Duration	2	81	6.06	**0.003**
	Environment × duration	2	81	0.89	0.415

(NumDF, numerator degrees of freedom; DenDF, denominator degrees of freedom; ProbF, significance probability corresponding to the F-value). IGFBP, insulin-like growth factor-binding protein; IGF, insulin-like growth factor; HDL, high-density lipoprotein; LDL, low-density lipoprotein. ProbF < 0.05 were considered significant and marked with bold font.

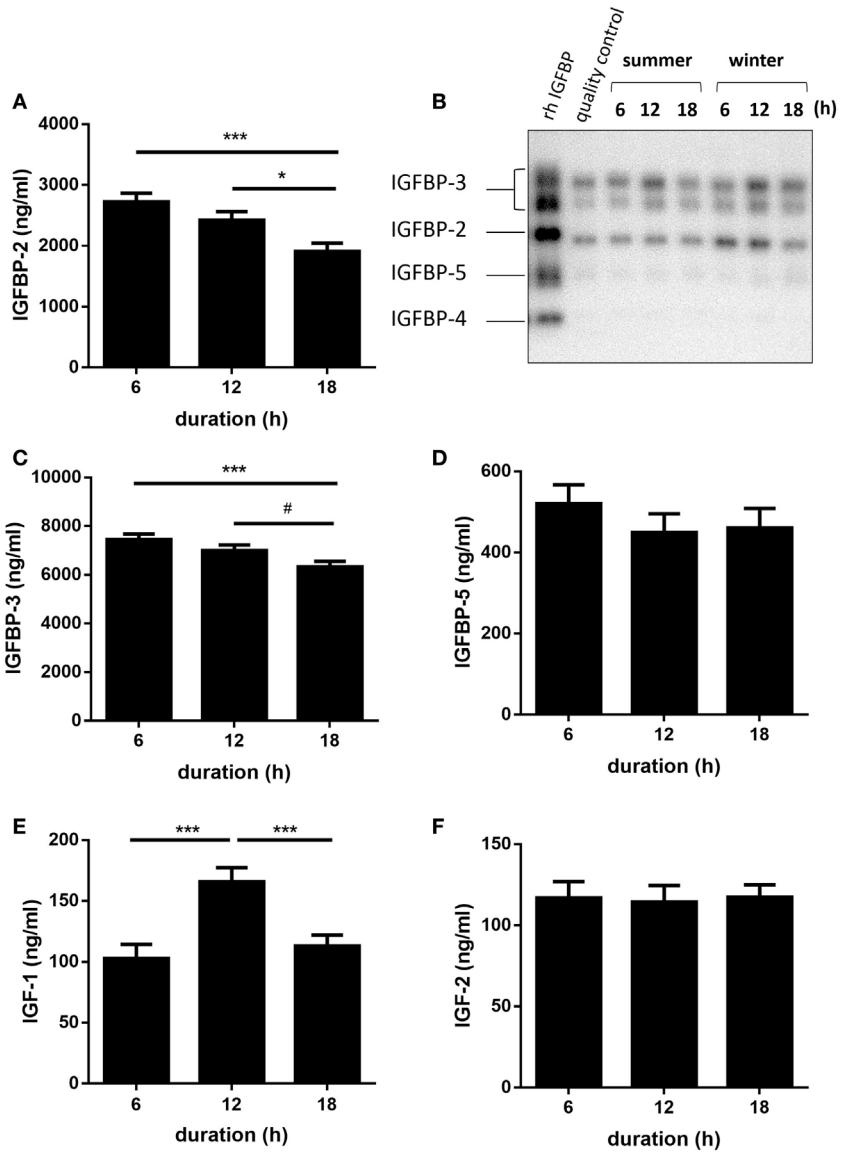

FIGURE 1 | Effect of transport duration on insulin-like growth factor (IGF)-axis: insulin-like growth factor-binding protein (IGFBP)-2 **(A)**, with kind permission from Nature Publishing Group as previously published in Ref. (9), IGFBP-profile **(B)**, IGFBP-3 **(C)**, IGFBP-5 **(D)**, IGF-I **(E)**, and IGF-II **(F)**. Quantitative data are presented as LS-Means + SE and include the summarized data of the trials in January/February and July due to the absence of the significant interaction duration × environment (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, #$p < 0.1$, rh: recombinant human, quality control: pig plasma sample). IGFBP-2, -3, -5: $n = 80$ per duration; IGF-I and IGF-II: $n = 30$ per duration.

reflect increased stress conditions before slaughtering. In fact, in a previous study (24), 18 h of transportation were considered as a more stressful condition because the animals were characterized by higher body temperatures or greater drinking and differential resting behavior, compared to 6 or 12 h transports. In the present study, pigs were fasted either 20 h (6 and 12 h transport) or 24 h

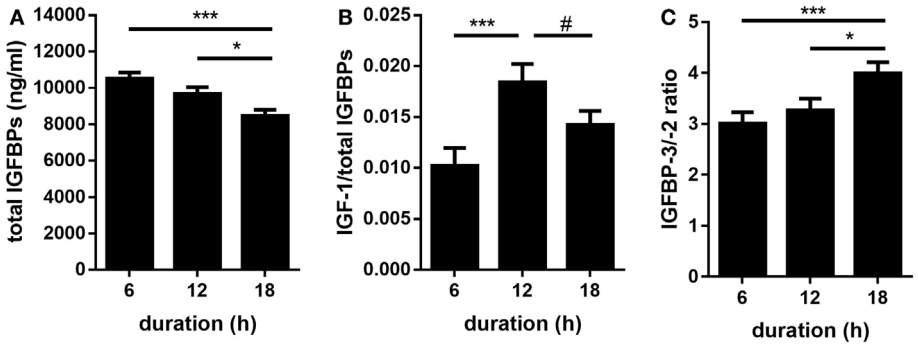

FIGURE 2 | Effect of transport duration on total amount of total insulin-like growth factor-binding proteins [IGFBPs; **(A)**] as a marker for IGF-binding capacity and on the ratio of IGF-I/total IGFBPs **(B)** as indicator for IGF-I bioavailability. IGFBP-3/-2 ratio **(C)** was detected as a marker of growth hormone action. Data are presented as LS-Means + SE. Total **(A,B)**: $n = 30$ per duration, **(C)** $n = 80$ per duration. $^{*}p < 0.05$, $^{***}p < 0.001$, $\#p < 0.1$.

(18 h transport). Because it has been shown, that fasting of 70 h reduced serum levels of IGF-I and IGFBP-3 (31) in sheep, it might be that the IGF-compounds, at least in theory, were coregulated by metabolic stress in pigs. However, preliminary studies revealed that 19 h of fasting were not sufficient to affect IGFBP-2, -3, or -5 concentrations also in market-weight pigs (9). Furthermore, circadian effects as described for IGFBP-3 (9) are no explanation for the decrease of IGFBP-3 due to the same time of arrival of animals at the slaughterhouse. Therefore, together with the findings from preliminary studies, the reduction of plasma IGFBP-2 and IGFBP-3 levels in two independent conditions of physiological and psycho-social stress in pigs, such as restraint and long transport, provides consistent evidence for the potential of both IGFBPs as stress biomarker in pigs.

Interestingly, in the present study, IGF-I concentrations and IGF-I bioavailability were increased after 12-h transport followed by a decrease after 18 h to levels similar to those after 6 h. Since the amount of IGFBPs present in the circulation was not significantly different after 6 and 12 h of transport, it is difficult to conclude whether the IGFBPs are responsible for the increased IGF-I plasma concentrations after 12 h transport. Nevertheless, altered biosynthesis of IGF-I might be assessed by follow-up studies in hepatic and non-hepatic tissues. Without excluding a possibly reduced expression of IGF-I during 18 h transport, the reduced levels of IGFBPs may explain the reduction of IGF-I concentrations in pig plasma after long-term transport due to reduced half-life of free IGF-I.

In the present study, IGFBP-3 concentrations were higher in pigs transported in January/February, while IGF-II concentrations were higher in pigs transported in July. This might be an effect of environmental conditions such as different temperatures during the transport or husbandry. To our knowledge, there is little evidence regarding the effect of ambient conditions on the IGF-system in farm animals and, before this study, there was none for pigs. In fish, it has been shown that plasma and mRNA levels of IGF-I increase with ambient water temperature, while IGF-II concentrations decrease (32). In the Gabillard et al. (2003) study, ambient temperature variation appears to promote fish growth through IGF-I secretion by the liver following GH stimulation. However, this effect was biased by the fish nutritional condition. By contrast, plasma IGF-II was not affected by the growth-promoting effect of temperature, but appeared to be more related to the metabolic status of the fish. A positive correlation of circulating levels of IGF-1 with increased water temperatures was also described in other fish species (33–36). Differently from the findings in fish, no significant changes in IGF-I levels were found in this study. A possible explanation for this lack of effect may be that IGF-I action was modulated by the increased levels of IGFBP-3, which was by the way the most abundant IGFBP in pig serum in this study, at the conditions of lower temperatures. In addition, regarding the thermoregulation, most fish species are poikilothermic ectotherms. This means, the body temperature is not constant but varies with water temperature which may have direct effects on metabolic and endocrine parameters in contrast to endothermic mammals. Contrary to the results of the fish study (32), IGF-II level variation was not inversely proportional to that in ambient temperature in the present study, probably due to the potential negative effect of heat stress on the expression of growth-related genes (37). It has been also suggested that potential environmental effects on IGF-system in market pigs are strongly dependent on the farm management system. This hypothesis also needs to be assessed in future studies.

Longer transport duration and/or extreme ambient temperatures are known to decrease the welfare of pigs on the truck (3, 24, 38–40). However, the transport times either applied during differential conditions in winter or summer in this study did not result in any variation in the concentrations of corticosterone, glucose, and albumin in exsanguination blood. This is in accordance to previous findings that cortisol was affected by the transport duration (6, 9). The lack of variation in these blood metabolites at slaughter may indicate that lairage conditions and time applied in this study were sufficient to help pigs recover from transport stress, regardless of travel time. A number of studies (41–43) actually reported that during two or 3 h in lairage, levels of blood cortisol are normalizing.

Higher plasma total protein and albumin concentrations in blood of pigs at exsanguination are associated with the dehydration rate at the time of slaughter (44, 45). It is sensible that pigs are getting dehydrated during extended periods of shipment, which is in line with greater drinking behavior in pigs transported

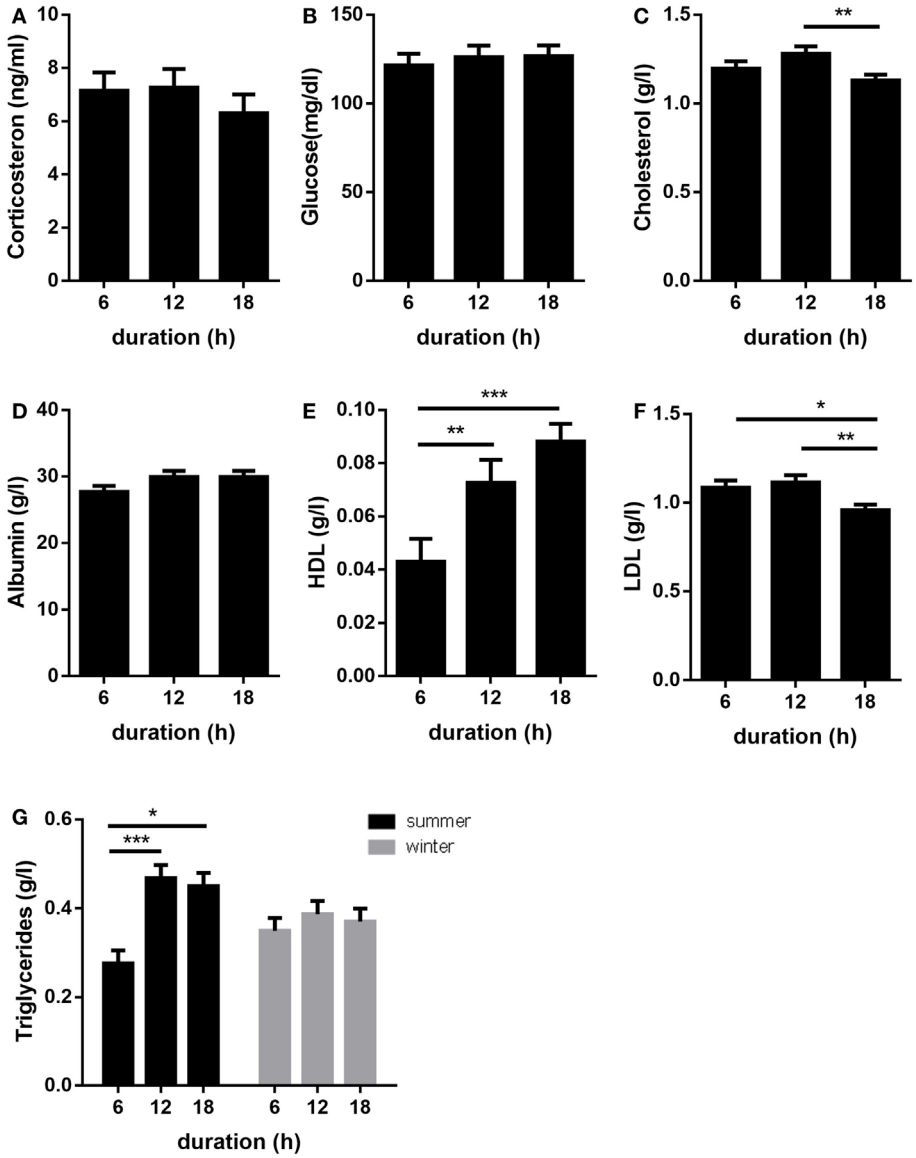

FIGURE 3 | Effects of transport duration on plasma levels of corticosterone **(A)**, glucose **(B)**, albumin **(C)**, cholesterol **(D)**, HDL **(E)**, LDL **(F)**, and triglycerides **(G)** (n = 28–30 per duration for all parameters). Data except triglycerides include the summarized data of the trials in January/February and July due to the absence of the significant interaction duration × environment. A significant interaction of duration × environment ($p < 0.05$) was calculated for TG. All data are presented as LS-Means + SE $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; LDL = low-density lipoprotein, HDL = high-density lipoprotein.

longer versus shorter periods of time (41, 46). However, in the present study, no effects of transport duration or environment were observed on blood albumin levels at slaughter. The increased drinking behavior observed in lairage in pigs after a transport period of 18 h in a previous study (24) may have helped circulating levels of albumin to return to the rest ones.

Differently from glucocorticoids and albumin, the indicators of lipid metabolism in the present study were affected by transport duration and/or environment. Plasma lipid levels may reflect constitutional or nutritional status of an animal, but there is also some evidence that plasma lipid concentrations can also be affected by short-term emotional arousal (47), acute stress (48), or activation of inflammatory pathways (49). In the present study, total cholesterol was reduced after 18 h of transport and this reduction was due to the simultaneous reduction of LDL-C concentrations. Conversely, HDL-C concentrations increased in pigs transported for 12 and 18 h. Transport longer than 6 h also increased blood TG levels, but only in summer. These results may be explained by the concurrent effects of longer feed deprivation (24 h) and transport (18 h) on increased lipolysis or even protein degradation for maintenance of metabolic homeostasis as showed by the reduced levels of cholesterol and LDL-C in this study, and the variation of body temperature and post-transport resting behavior reported in a companion study (24). However,

TABLE 2 | Effect of different environmental conditions in January/February and July on plasma parameters.

	January/February	July	n_{total}	*p*-Value
IGFBP-3 (ng/ml)	7,295 ± 246	6,559 ± 194	240	**0.02**
IGFBP-2 (ng/ml)	2,364 ± 150	2,338 ± 122	240	0.89
IGFBP-5 (ng/ml)	461 ± 53.0	414 ± 44.0	240	0.50
IGF-1 (ng/ml)	133 ± 13	121 ± 8.91	90	0.46
IGF-2 (ng/ml)	97.1 ± 11.3	135 ± 7.72	90	**0.01**
Corticosterone (ng/ml)	6.3 ± 1.07	6.8 ± 0.73	90	0.66
Albumin (g/dl)	3.1 ± 0.10	2.9 ± 0.11	84	0.11
Glucose (mg/dl)	129 ± 6.02	120 ± 6.71	84	0.33
Total IGFBPs	9,981 ± 374	9,125 ± 295	240	0.07
IGF-1/total IGFBP	0.01 ± 0.002	0.01 ± 0.001	90	0.58
IGFBP-3/IGFBP-2	3.6 ± 0.24	3.22 ± 0.20	240	0.20
Total cholesterol (g/l)	1.15 ± 0.04	1.25 ± 0.03	90	0.07
HDL (g/l)	0.1 ± 0.01	0.07 ± 0.01	90	0.31
LDL (g/l)	1.0 ± 0.04	1.09 ± 0.03	90	0.11

For all presented parameters the interaction duration × environment was not significant. The data are summarized over the transport duration (6, 12, and 18 h) and are presented as LS-means ± SE. The Tukey–Kramer procedure was used for pair-wise comparisons.

ProbF < 0.05 were considered significant and marked with bold font.

IGFBP, insulin-like growth factor-binding protein; IGF, insulin-like growth factor; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

as in 24 h fasted pigs, live-weight reductions are possibly related to excretion (50, 51) and considering that the preslaughter fasting intervals between transport groups were almost identical (i.e., 20 h fasting in the 6 and 12 h groups, and 24 h of fasting in the 18 h group), preslaughter lipid metabolism may have been affected by the length of transport rather than fasting time in this study. Warriss, in fact, considered the possibility that shipment might have a stronger effect on reductions of live-weight than fasting alone (52).

To summarize and conclude, in this study the IGF-system was regulated under conditions of different transport durations. With prolonged transport, plasma IGFBP-2 and IGFBP-3 levels were reduced, whereas IGF-I concentrations were dynamically regulated with increased concentrations after intermediate transport duration (12 h) compared to shorter or extended periods of time (6 or 18 h). By contrast, based on the results of this study, glucocorticoids, glucose, or albumin cannot be considered as useful indicators of transport stress in pigs. The metabolic stress condition of pigs being transported for 18 h was also indicated by the reduced levels of cholesterol and LDL-C and was associated with a decline of plasma IGFBPs concentrations. Further studies on the differential effects of psycho-social or metabolic stress on IGFs, IGFBPs, and complex traits of IGF-related signatures in pigs might identify specific biomarker potential for compounds from the IGF-system for animal welfare.

ETHICS STATEMENT

All experimental procedures were approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the current guidelines of the Canadian Council on Animal Care (CCAC, 2009).

AUTHOR CONTRIBUTIONS

All authors designed the experiments and wrote the manuscript. SG, JB, and LF performed the animal experiment. EW, MK, CW, MS, and CH carried out wet lab experiments. EW, MK, CW, MS, AT, CH, and AH analyzed data.

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Insulin-Like Growth Factor Binding Proteins and IGFBP Proteases: A Dynamic System Regulating the Ovarian Folliculogenesis

Sabine Mazerbourg[1] and Philippe Monget[2]**

[1] Université de Lorraine, CNRS, CRAN, Nancy, France, [2] INRA, Physiologie de la Reproduction et des Comportements, UMR 6078 INRA-CNRS-Université de Tours, Nouzilly, France

The aim of the present article is to update our understanding of the expression of the insulin-like growth factor binding proteins (IGFBPs), IGFBP proteases and their implication in the different processes of ovarian folliculogenesis in mammals. In the studied species, IGFs and several small-molecular weight IGFBPs (in particular IGFBP-2 and IGFBP-4) are considered, respectively, as stimulators and inhibitors of follicular growth and maturation. IGFs play a key role in sensitizing ovarian granulosa cells to FSH action during terminal follicular growth. Concentrations of IGFBP-2 and IGFBP-4 in follicular fluid strongly decrease during follicular growth, leading to an increase in IGF bioavailability. Inversely, atresia is characterized by an increase of IGFBP-2 and IGFBP-4 levels, leading to a decrease in IGF bioavailability. Changes in intrafollicular IGFBPs content are due to variations in mRNA expression and/or proteolytic degradation by the pregnancy-associated plasma protein-A (PAPP-A), and likely participates in the selection of dominant follicles. The identification of PAPP-A2, as an IGFBP-3 and -5 protease, and stanniocalcins (STCs) as inhibitors of PAPP-A activity extends the IGF system. Studies on their implication in folliculogenesis in mammals are still in the early stages.

Keywords: insulin-like growth factor, insulin-like growth factor binding protein, ovary, folliculogenesis, pregnancy-associated plasma protein-A, stanniocalcins

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****Correspondence:***
Sabine Mazerbourg
sabine.mazerbourg@univ-lorraine.fr;
Philippe Monget
philippe.monget@inra.fr

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Until the 2000s, involvement of insulin-like growth factors (IGFs) and IGF-binding proteins (IGFBPs) in ovarian folliculogenesis has been extensively described in several mammalian species (1). Comparative analysis reveals some species differences concerning the role of IGFs on the different stages of folliculogenesis, and on the changes of levels and expression of the different elements of IGF system during follicular growth and atresia. In all these species, IGFs and several small-molecular weight IGFBPs (in particular IGFBP-2 and IGFBP-4) are considered, respectively, as stimulators and inhibitors of follicular growth and maturation. Based on our complete review of the IGF system in the mammalian ovary (1), we will present an update on IGFBPs expression and IGFBP proteolytic degradation in the ovary, with a focus on the protease, pregnancy-associated plasma protein-A (PAPP-A).

THE IGF SYSTEM

The IGF system is composed of different elements (2–4):

- Two ligands, IGF-I and IGF-II.
- Two receptors: the type I receptor mediates most of the somatomedin-like actions of both IGF-I and -II. The type II receptor, or IGF-II/Mannose-6-Phosphate (IGF-II/M6P) receptor, binds

IGF-II but not insulin, and binds IGF-I with very low affinity, and can be considered as an inhibitor of IGF-II action.

– Six IGFBPs, which bind IGF-I and -II with high affinity. IGFBPs are present in all biological fluids. They can be arbitrarily classified in two groups: (1) the small molecular weight IGFBPs, or IGFBPs <40 kDa including IGFBP-1, -2, -4, -5, and -6 that are present in the serum and in other fluids in a so called "small complex." When visualized by western-ligand blotting (WLB), their apparent molecular weights range between 24 and 35 kDa. In serum, their levels are either negatively regulated (IGFBP-1 and -2) or unaffected by growth hormone (GH); (2) IGFBP-3, which is the predominant IGFBP in serum. In this fluid, it is mostly present in a 150 kDa form ("large complex"), composed of IGF-I or IGF-II, and an acid-labile 85 kDa subunit (ALS). Native IGFBP-3 is visualized as a 44–42 kDa doublet by WLB. The concentration of IGFBP-3 is positively regulated by GH and IGF-I.

Insulin-like growth factor binding proteins increase IGFs half-life and constitute a large pool of IGFs in all the compartments of the organism (5, 6). Furthermore, IGFBPs can both inhibit and potentiate IGFs action at the level of target cells. They can indeed inhibit IGFs action by sequestration, since the affinity of IGFBPs for IGF-I and -II is of the same order of magnitude as the affinity of type I receptors. However, the affinity of IGFBPs for IGF-I and -II can be modulated by post-translational changes. Particularly, the affinity of IGFBP-1 for IGFs can be increased by phosphorylation as reported in human amniotic fluid at the end of pregnancy (2, 7, 8). Moreover, the affinity of IGFBPs for IGFs can be reduced when IGFBPs are bound to ECM (IGFBP-5), or when they are proteolyzed (IGFBP-2, IGFBP-3, IGFBP-5, and IGFBP-4) (3, 8). Such proteolysis has been first described in the serum of female rodents and humans during pregnancy, after nutritional fasting, during severe illness or following extensive trauma. Limited proteolysis of IGFBPs has also been described in the serum and lymph of normal human adults, and in culture media of osteoblastic cells, granulosa cells, and tumor cells (9). It is, therefore, likely that such a phenomenon exists *in vivo* in most tissues. Moreover, it is important to note that this proteolysis can occur at the level of the cell membrane (10). Such decreases in the affinity of IGFBPs for IGFs, hereby increasing bioavailability of IGFs, can lead to a potentiation rather than an inhibition of action of the ligands.

IGFs IN THE OVARY

There are many *in vitro* and *in vivo* evidence that IGF-I and IGF-II are stimulators of ovarian follicular development (1, 11, 12). IGF-I stimulates either proliferation, or differentiation of granulosa cells depending on the stage of development of the follicle, and plays a key role in the responsiveness of the ovary to FSH action. Moreover, the increase in expression and/or bioavailability of IGFs in large preantral follicles results in an increase in the number of functional FSH receptors, leading to an increase in type I IGF receptors. This positive feedback loop might partly be responsible for the amplification of FSH action and the expression of aromatase and LH receptors in fully mature follicles. In contrast to adult rodents that have trace amounts of IGF-II in serum, adult humans (as well as adult sheep, cattle, and pigs), contain twofold to threefold more IGF-II than IGF-I in serum, the former being less dependent on GH than the latter. In cattle and mice, IGF-I seems to play a key role in increasing the sensitivity of small antral follicles to gonadotropin action, and plays a key role in their transition to the gonadotropin-dependent follicular stage. In human, circulating IGF-I does not seem to be essential for the development or maturation of ovarian follicles (13–15). In this case, it is possible that the low (likely GH-independent) expression of IGF-I in small growing follicles and of IGF-II in large dominant follicles are able to replace the contribution of serum GH-dependent IGF-I.

There is some heterogeneity in the localization of IGFs expression in the ovary of different species (1), but several arguments play in favor of a main seric origin of IGFs (16). Except in human, it is likely that small changes in local expression of IGFs would not have any significant consequence on their intrafollicular concentration, considering the high levels present in serum. Moreover in the dog, Reynaud et al. show that the wide span in body height among dogs with different breeds is associated with dramatic differences in IGF-I and IGFBP-3 levels in both plasma and follicular fluid from preovulatory follicles (17). These differences of levels impact follicular development: large dogs have a higher number of preovulatory follicles than small dogs, these follicles being 70% larger in the largest dog than in the smallest dog (17). These differences are not associated with differences in estradiol serum levels, suggesting an uncoupling between the number and the size of preovulatory follicles in this species, and steroidogenesis.

Actually, IGFs bioavailability, rather than IGFs concentration, dramatically changes during growth and atresia of ovarian follicles (see below).

IGFBPs IN THE OVARY

The intrafollicular IGFBP content fluctuation is a very conserved phenomenon among mammalian species: the disappearance of IGFBPs < 40 kDa (IGFBP-2, IGFBP-4 as well as IGFBP-5 in ruminants follicles) characterizes the preovulatory follicles and the increase in their levels is observed in atretic follicles. All these changes are due to two processes: changes in mRNA expression, and changes in proteolytic degradation (**Table 1**).

CHANGES IN mRNA EXPRESSION

IGFBP-1

Most of the studies report the absence of significant expression of IGFBP-1 in mammalian ovaries (18–22). However, in human ovaries, some studies have shown IGFBP-1 mRNA expression in granulosa cells of mature follicles (23, 24). Overall, it could be postulated that in most mammalian species, intrafollicular IGFBP-1 derives mainly from serum, rather than *de novo* synthesis.

IGFBP-2

In the ewe, the sow, the cow, and the mare, intrafollicular levels of IGFBP-2 strongly decrease from 1 to 2 mm diameter follicles

TABLE 1 | General overview of IGFBP contents variations during follicular growth and atresia in mammalian ovary.

	Growing follicles			Atretic follicles	
	mRNA	**Protein**	**Proteolysis**	**Protein**	**Proteolysis**
IGFBP-1		Mainly from serum	nd	nd	nd
IGFBP-2	↔/↓*	↓	↑	↑	0
IGFBP-3	↔/0*	↔ or mainly from serum*	nd	↔	nd
IGFBP-4	↓/↔*	↓	↑	↑	0
IGFBP-5	↓/↔/0*	↓/0*	↑	↑/↔/0*	0
IGFBP-6	↔/↑*	nd	nd	nd	nd

This table summarizes global changes in follicular mRNA and protein expression levels of IGFBP-1 to -6 and variations in intrafollicular IGFBP proteolytic activity observed in most mammalian species. Species specificities are reported in the text.
**, depending on the species; ↓, decrease; ↑, increase; ↔, no change; 0, absence; nd, not determined.*

to preovulatory follicles. By contrast, its intrafollicular level strongly increases in atretic follicles (1, 25–28). *In vivo*, a strong decrease in IGFBP-2 mRNA expression has been observed in granulosa cells during follicular growth in ovine (29), porcine (30, 31), bovine (32), brushtail possum (33), but not in primate and human ovaries (24, 34). By contrast, IGFBP-2 mRNA expression has been shown to increase in the same compartment and in theca cells of atretic follicles in sheep (29). In the brushtail possum, IGFBP-2 mRNA is also expressed in granulosa and theca cells of atretic antral follicles (33). So in several species, the decrease and the increase in IGFBP-2 mRNA expression during follicular growth and atresia, respectively, partly explains changes in intrafollicular levels of the protein. *In vitro*, FSH and/or cAMP strongly inhibit IGFBP-2 expression by granulosa cells in pigs (35, 36), in cattle (32), and humans (37), suggesting that the diminution of levels of this IGFBP in preovulatory follicles is FSH dependent (32). In the pig, the decrease in IGFBP-2 protein level is not associated with a decrease in mRNA levels (35, 36), suggesting that FSH could act through an increase in IGFBP-2 degradation *in vitro*.

IGFBP-3

IGFBP-3 levels do not seem to change during folliculogenesis in any species. In ovarian follicles, expression of IGFBP-3 is low and poorly or not associated with growth or atresia (24, 38). *In vitro*, IGFBP-3 mRNA expression was positively regulated by FSH in small bovine granulosa cells and downregulated by estradiol in theca cells of large bovine follicles (39). Inversely, in cultured pig and human granulosa cells, FSH inhibited IGFBP-3 production (40, 41).

IGFBP-4

Changes in IGFBP-4 intrafollicular levels are similar to those observed with IGFBP-2 with a decrease in growing follicles and an augmentation in atretic ones (1, 25–27). In contrast to IGFBP-2, expression of IGFBP-4 mRNA differs between species. In particular, IGFBP-4 mRNA levels are low in both granulosa and thecal cells of ovine and bovine species, and exhibit only slight changes during follicular growth and atresia (32, 42). In the particular case of cystic ovarian disease, one of the main causes of infertility in dairy cattle characterized by the persistence of large follicular structures, IGFBP4 expression *in situ* is higher in granulosa cells in persistent follicles than in control follicles (43). In human ovaries, IGFBP-4 mRNA content is high in immature follicles and dramatically decreases in mature follicles (24). In the brushtail possum, IGFBP-4 mRNA is limited to theca cells of large preantral and antral follicles (33). In the rat, IGFBP-4 mRNA expression strongly increases in granulosa cells of atretic follicles (44). This upregulation of IGFBP-4 mRNA in atretic rat follicles is likely due to their loss of FSH sensitivity. Indeed, *in vitro*, FSH was shown to strongly decrease expression of IGFBP-4 by rat granulosa cells (45). In sharp contrast, IGFBP-4 mRNA expression increases in thecal cells during follicular growth in the sow and the monkey (31, 46). In the latter species, 7 days treatment of hCG-induced a marked increase in IGFBP-4 mRNA expression detected by *in situ* hybridization in thecal cells *in vivo* (46). However, a more recent study reported a reduction of IGFBP-4 mRNA levels in monkey granulosa cells 24 h after injection of hCG (47).

In vivo, GnRHa injection in sheep, disrupting FSH and LH regulation, induces a significant decrease in IGFBP-4 mRNA expression after 36 h from start of treatment in large but not medium and small follicles (48). Interestingly, LH stimulates rather than inhibits IGFBP-4 expression by bovine thecal cells *in vitro* (32). These results are concordant with *in vivo* effects of hCG on IGFBP-4 expression in rhesus monkeys described by Zhou et al., as well as with data on the increase in IGFBP-4 mRNA expression in large preovulatory porcine follicles (31, 46). However, these observations clearly contrast with the fact that in all these species, IGFBP-4 protein levels are undetectable in the follicular fluid of these follicles. This is due to its proteolysis by the PAPP-A protease (see below).

IGFBP-5

Ovarian expression of IGFBP-5 mRNA also strongly differs between species. This expression slightly decreases during follicular growth in ovine thecal cells, but dramatically increases in granulosa cells from ovine and rat atretic follicles (42, 44, 48). In bovine follicles, high levels of IGFBP-5 mRNA and protein are detected in subordinate follicles compared to dominant follicles (49). In sharp contrast, in the mouse, IGFBP-5 transcript levels are elevated in granulosa cells of healthy primary and secondary follicles, and decrease in subsequent follicular stages and in atretic follicles (50). In rhesus monkeys, IGFBP-5 mRNA is selectively expressed in thecal and granulosa cells of dominant but not immature follicles (34). In the pig, expression of IGFBP-5 mRNA is concentrated on the surface of the germinal epithelium and in capillary endothelium (20). Finally, in the brushtail possum, IGFBP-5 mRNA expression is limited to granulosa cells of primordial, primary, and some secondary follicles. Strong expression is also observed in the theca of secondary and antral follicles (33). As for IGFBP-4, FSH strongly inhibits IGFBP-5 mRNA expression by rat granulosa cells *in vitro* (45). By using a model of serum withdrawal-induced programmed cell death, we have

shown that IGFBP-5 mRNA expression is enhanced in apoptotic ovine granulosa cells *in vitro*, suggesting that the expression of this IGFBP is associated with cell viability in this species (28). *In vivo*, in sheep, Hastie et al. disrupted FSH and LH regulation with a GnRHa injection, leading to a transient increase in IGFBP-5 mRNA expression after 12 h from start of treatment. These data suggest that gonadotrophins modulate IGFBP-5 expression (48).

IGFBP-6

To our knowledge, IGFBP-6 intrafollicular content has never been documented. In monkey ovaries, IGFBP-6 mRNA was present at low levels in the interstitium and theca externa and was more abundant in the ovary surface epithelium (34). In bovine follicles, IGFBP-6 mRNA has been detected only in theca cells with a higher expression during the final follicular growth (21). In cycling ewes, IGFBP-6 mRNA expression does not change in small follicles, no matter their health status. However, its expression significantly decreases in large atretic follicles compared to large healthy follicles (22).

CHANGES IN INTRAFOLLICULAR PROTEOLYTIC DEGRADATION

Changes in IGFBP levels during folliculogenesis can partly be explained by changes in intrafollicular proteolytic activity. Indeed, Chandrasekher et al. have shown the presence of a proteolytic activity degrading IGFBP-4 in follicular fluid from human dominant estrogenic but not atretic follicles (51). Proteolytic degradation of IGFBP-4 and -5 was also maximal in ovine, bovine, porcine, as well as equine preovulatory follicles (42, 52–54). IGFBP-2 proteolysis was also detected in follicular fluid of preovulatory follicles of the different mammalian species (42, 52, 53, 55, 56). Nevertheless, *in vivo*, proteolytic degradation of IGFBP-2 likely occurs only in preovulatory follicles that exhibit a high IGF bioavailability, whereas IGFBP-4 is already degraded in healthy growing follicles. Interestingly, in the ewe, native IGFBP-4, assessed by WLB, was undetectable in follicles that contained more than 10 ng/ml estradiol. By contrast, native IGFBP-2 was undetectable only in follicles that contained more than 100 ng/ml estradiol, suggesting that the disappearance of IGFBP-2 during follicular growth occurs later than that of IGFBP-4 (1, 42). Besides, cleavage of IGFBP-2 was also observed after incubation with cultured mural granulosa cells from antral bovine follicles (57). No proteolysis was detected after incubation with denuded oocyte or oocyte cumulus complexes, suggesting that the bovine mural granulosa cells are the major source of the soluble protease (57). In these different conditions of culture, the addition of recombinant IGF-I or FSH had no effect on IGFBP-2 degradation rate (57).

IGFBP PROTEASES IN THE OVARY

Pregnancy-Associated Plasma Protein-A (PAPP-A)

Pregnancy-associated plasma protein-A is a member of the pappalysin family of metzincin metallo-proteinases (58). PAPP-A is a large dimeric glycoprotein of 400 kDa present in increasing concentrations in serum during human pregnancy. It circulates as a 2:2 disulfide bound complex of 500 kDa with the proform of eosinophil major basic protein (proMBP), denoted PAPP-A/proMBP (59, 60). No physiological function has been attributed to this circulating protein until Lawrence et al. showed that PAPP-A was the protease responsible for the proteolytic degradation of IGFBP-4 in human fibroblasts and osteoblasts cells-conditioned media (61). Then, PAPP-A was identified as the protease targeting IGFBP-4 in human, ovine, bovine, equine, and porcine follicular fluid (62–64). Thereafter, it was shown that IGFBP-2 was a substrate of PAPP-A in bovine, porcine, and equine preovulatory follicles (55, 56). In the mare, intrafollicular injection of PAPP-A into the second largest follicle (F2) decreases the concentration of IGFBP-2 to a level similar to the concentration in the largest follicle F1 (65). Finally, Rivera and Fortune have reported that IGFBP-5 is degraded by PAPP-A in bovine preovulatory follicles but not in subordinate follicles of the same cohort (64). Overall, these results suggest that degradation of IGFBP-2, IGFBP-4, and IGFBP-5 by PAPP-A in preovulatory follicles is a well-conserved mechanism in mammalian species. In bovine and equine ovary, PAPP-A, and the associated decrease in intrafollicular IGFBP-4 level, could be a key factor in the mechanism leading to selection of dominant follicles (56, 64). In female mice, the absence of PAPP-A results in altered fertility, associated with reduced estradiol levels and reduced ovulation (66). The ovaries exhibit a small size but all follicular stages are present. Follicular fluid from *Pappa* knockout (KO) mice is totally deprived of IGFBP-4 proteolytic activity, strongly suggesting that the altered reproductive capacity of these female mice is a consequence of reduced IGF bioavailability in the follicular compartment (66). Interestingly, this study highlights that the PAPP-A is the exclusive intraovarian IGFBP-4 protease in the mouse. Since PAPP-A tethers to the surface of cells by binding to surface glycosaminoglycans, this protease is believed to regulate IGF signaling locally in tissues by increasing the pericellular level of bioactive IGF (67, 68).

PAPP-A Expression Has Been Characterized in the Mammalian Ovary

Several studies analyzed PAPP-A mRNA expression in the mammalian ovary. In the bovine and porcine ovary, a first study reports that PAPP-A mRNA expression in granulosa cells was maximal in preovulatory follicles and positively correlated with expression of both aromatase and LH receptors (63). In a second study in bovine ovary, Sudo et al. did not observe a significant difference of PAPP-A mRNA expression levels between follicles at all development stage, but the expression showed a tendency to increase with follicular growth (69). In a third study, Santiago et al. reported neither a significant differential expression of PAPP-A mRNA in granulosa cells of dominant and subordinate bovine follicles, nor a correlation between levels of PAPP-A mRNA and estradiol levels in follicular fluid (49). In the rodent ovary, ovarian cells expressing detectable PAPP-A mRNA are the centrifugally located mural granulosa cells of healthy growing antral, as well as preovulatory follicles, and the lutein cells of the corpus luteum. Both PMSG and hCG strongly stimulate PAPP-A expression *in vivo* (66, 70, 71). By *in situ* hybridization in the human ovary, Hourvitz et al. have observed PAPP-A expression

in preovulatory but not immature follicles, as well as in the corpus luteum (72). Recently, an immunohistochemical study on human ovaries revealed that PAPP-A expression is primarily observed in the theca cells of small antral follicles, then in both theca and (slightly) granulosa cells of antral follicles, and finally in granulosa cells of preovulatory follicles (73). In that case, PAPP-A is co-expressed with aromatase in granulosa cells of antral and preovulatory follicles. Furthermore, the intrafollicular PAPP-A concentration is strongly positively correlated with estrogen and progesterone secretion and inversely correlated with testosterone and androstenedione levels (73). To support these expression data, Jepsen et al. have shown that intrafollicular concentration of PAPP-A increases in human antral follicles after administration of hCG (74). Similarly, in the rhesus monkey, PAPP-A mRNA expression shows a peak of induction 3 and 6 h after hCG induction leading to an increase in intrafollicular PAPP-A protein concentration 24 h later (47). By contrast, Zhou et al. have shown by *in situ* hybridization that PAPP-A is expressed in granulosa cells of antral follicles of all sizes, but did not observe any correlation with LH-receptor expression (46). Most of these results suggest a role for gonadotropin-stimulated PAPP-A gene expression in the shift from an androgenic to an estrogenic environment, characteristic of the follicular selection, as well as in the ovulation and luteogenesis processes in the mammalian ovary. However, we have to emphasize that ovarian PAPP-A protein level may not be systematically linked to IGFBP proteolytic activity. Indeed, in the human ovary, PAPP-A activity dramatically decreases in follicular fluid post-hCG treatment even though the intrafollicular concentration of PAPP-A was still maintained at a high level (74).

In vitro, Liu et al. and Resnick et al. have shown that FSH is able to induce the degradation of both IGFBP-4 and IGFBP-5 by rat granulosa cells-derived proteases (45, 75). Later on, PAPP-A was identified as the protease responsible for degradation of IGFBP-4 in FSH-primed rat granulosa conditioned media (71). Interestingly, the oocyte-derived bone morphogenetic protein-15 inhibits FSH-induced PAPP-A gene expression in rat mural and cumulus granulosa cell cultures. This result is likely to explain the absence of PAPP-A mRNA expression in the cumulus granulosa cells *in vivo* (see above) (71). In bovine granulosa cell cultures, Sudo et al. further report that PAPP-A mRNA is induced by FSH, and estradiol amplifies this hormonal stimulation (69). In humans, Conover et al. have shown that PAPP-A is secreted *in vitro* by granulosa cells from estrogen- but not androgen-dominant follicles (76). However, they did not observe any effect of hCG on PAPP-A levels in cell-culture medium.

Overall, these data suggest that PAPP-A is a granulosa cells-derived protease in the ovary and a marker of follicle selection and corpus luteum formation. The regulation of its expression highly depends on gonadotropins FSH or hCG/LH in most mammalian species. The involvement of intra-ovarian factors remains to be elucidated.

Intrafollicular PAPP-A Proteolytic Activity Is Modulated by IGFs

In ovine, bovine, and equine preovulatory follicular fluid, cleavage of IGFBP-4 and IGFBP-2 is enhanced in the presence of IGF-I and IGF-II (55, 56, 63). However, one can note that, in contrast to IGFBP-4, bovine and porcine preovulatory follicular fluids only induce a partial proteolytic degradation of exogenous IGFBP-2 in the absence of exogenous IGFs with this degradation being clearly enhanced in the presence of exogenous IGFs (55). The enhancing effect of IGF is confirmed by different biochemical analyses, suggesting that it is due to a conformational change of IGFBP-4 and -2 after IGF binding, and not to the binding of IGF to PAPP-A (77–80). The cleavage of IGFBP-5 by PAPP-A does not require the presence of IGF, but is slightly inhibited by IGF (78, 79). Thus, the concentration of IGF is likely to control the dynamics of proteolysis of IGFBP-2, -4, and -5 by PAPP-A.

Intrafollicular PAPP-A Proteolytic Activity Is Not Modulated by Pro-MBP but by Stanniocalcins (STCs)

As stated above, PAPP-A circulates in pregnancy serum as a disulfide-bound complex with proMBP that is able to strongly inhibit PAPP-A activity. *In vitro*, Conover et al. failed to detect any proMBP in human granulosa cell-conditioned media (76). Since then, STCs 1 and 2 (STC1 and STC2) have been presented as regulators of PAPP-A activity (81, 82). STC1 binds PAPP-A non-covalently but with high affinity, whereas STC2 requires covalent binding between the two proteins (81, 82). The stable covalent complex STC2/PAPP-A inhibits PAPP-A proteolytic activity toward IGFBP-4. In mice, transgenic overexpression of STC2, but not of the mutated proteolytically inactive STC2, induces a reduction in size of up to 45% (81, 83). A similar phenotype is observed in transgenic mice overexpressing STC1 as well as in *Pappa* and *Igf2* KO mice (84–86). The excess of STCs could neutralize PAPP-A activity leading to IGF sequestration by IGFBP-4 and a reduced local IGF bioavailability. Before the discovery of their new function, STC1 and STC2 expression had been described in some mammalian species. In the porcine ovary, STC1 protein was detected in theca and granulosa cells of growing follicles, in the oocyte and in luteal cells (87). A similar pattern of expression of STC1 protein has been observed in the mouse, but with the absence of expression in granulosa cells (88, 89). In this species, STC1 mRNA expression increases significantly after hCG treatment (89). In the rat, STC1 transcripts are localized in theca cells and are downregulated after PMSG treatment. After hCG induction, their levels remain low in the theca cells and in the corpora lutea, which contrasts with data in the mouse (89, 90). Conversely, STC2 mRNA expression is limited to theca cell layer of rat antral and preovulatory follicles after PMSG stimulation. Its level keeps decreasing after hCG treatment (91). In cultured early antral follicles, STC2 transcripts are induced by estrogen (91). In granulosa cell cultures, STC1 and STC2 were shown to function as paracrine factors suppressing FSH-induced progesterone production (90, 91). Overall, in the rat, due to the low expression of STC1 in the PMSG-primed follicles, STC2 is probably the STC isoform playing a role in the inhibition of luteinization of preovulatory follicles (91). In humans, in contrast to other species in which the STCs are expressed mainly in the theca cells, STC1 and STC2 are coexpressed in the granulosa cells and/or the theca-interstitial cells with variable intensity depending on the follicles (74). These authors showed a similar profile of expression with PAPP-A in primordial, late primary, antral follicles, and in the oocyte. The three proteins are consistently co-expressed

in the same cell type during the process of follicle maturation, suggesting that they are able to form functional complexes (74). Indeed, PAPP-A:STCs complexes were immunoprecipitated in human follicular fluid, showing for the first time the physiological existence of these complexes (74). The presence of STCs could explain the decrease of PAPP-A activity observed in follicular fluid post-hCG treatment although the intrafollicular concentration of PAPP-A was still maintained at a high level (74).

Several points remain to be answered: (1) STCs are co-expressed with PAPP-A in human growing follicles while PAPP-A activity is high. Could STCs function/activity be controlled by another partner? (2) current studies suggest that the intra-ovarian STCs expression is not well conserved among species. More studies need to be done on the STCs protein expression rather than mRNA expression. Indeed, Varghese et al. have reported a different profile of expression in the mouse ovary between *in situ* hybridization and immunohistochemistry (88); (3) how is regulated the formation of the intrafollicular complexes PAPP-A:STCs? One could suggest that the expression level of each partner could influence their association rate; and (4) more studies are needed to define whether STC1 and/or STC2 are the physiological regulators of the PAPP-A activity. STC1 and STC2 belong to the same family but their protein sequences show only 30% identity (91). Due to the presence of additional disulfide bridges, STC2 could have a different tertiary structure compared to STC1 (91). However, both recombinant proteins STC1 and STC2 form disulfide-linked homodimer or an inhibitory complex with recombinant PAPP-A (74, 82, 91). Moreover, both STC1 and STC2 are expressed in human developing follicles, and are present in complexes with PAPP-A in follicular fluid (74, 82). At this time, it is impossible to delineate the relative importance of STC1 compared to STC2 in PAPP-A inhibition; (5) The exact role of STCs in the ovary has to be characterized. STC1 and STC2 have been presented as homodimeric ligands inhibiting FSH stimulation of rat granulosa cells differentiation through activation of specific receptors (90, 91), but also as the modulators of the IGF system (74). In this context, studies on mice with ovarian invalidation of *Stc1* and/or *Stc2* genes would be very informative.

Pregnancy-Associated Plasma Protein-A2 (PAPP-A2)

Pregnancy-associated plasma protein-A2, a paralog of PAPP-A, is responsible for the IGF-independent proteolytic degradation of IGFBP-5 and IGFBP-3 (92, 93). Both STC1 and STC2 can inhibit the activity of PAPP-A2, blocking the release of IGFs from IGFBP-3 and IGFBP-5 (82, 94). Homozygous loss-of-function mutations in PAPP-A2 result in a novel syndrome of growth retardation with markedly elevated circulating IGF-I and IGF-II, but a decrease of bioactivity due to an increase in

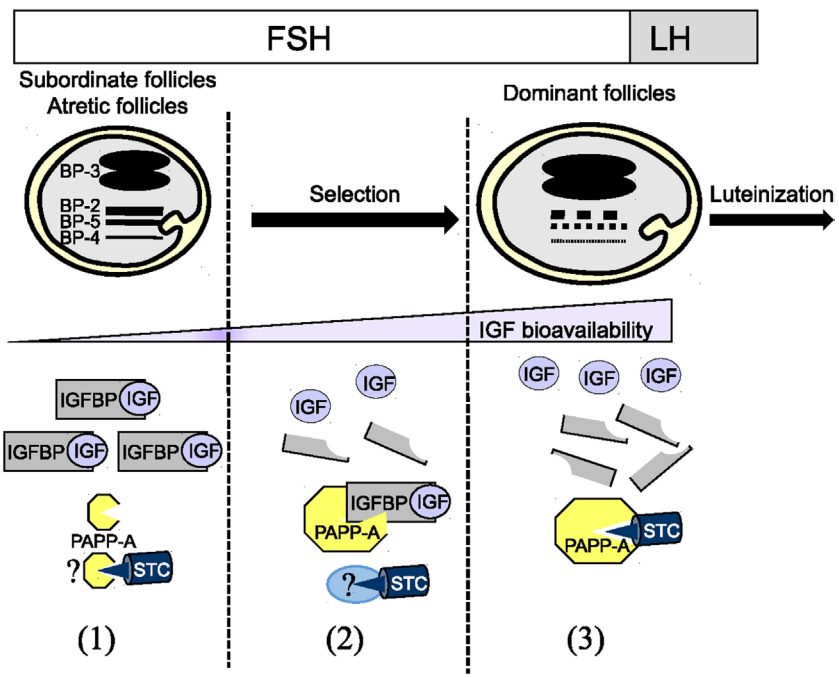

FIGURE 1 | Dynamic of the insulin-like growth factor binding protein (IGFBP) system during folliculogenesis (1) High concentrations of IGFBP-2, IGFBP-4, and IGFBP-5 in follicular fluid of subordinate and atretic follicles lead to a low IGF bioavailability. The absence of IGFBP proteolytic degradation is due to the low expression level of PAPP-A and potentially to its association with the inhibitor STCs STC1/2. (2) During terminal follicular growth under FSH control, the decrease in IGFBP-2, IGFBP-4, and IGFBP-5 is due to a decrease in IGFBP mRNA expression and an increase in the proteolytic degradation by PAPP-A in follicular fluid. This decrease might participate in the selection of dominant follicles. At this stage, PAPP-A expression and activity are maximal. Surprisingly, the inhibitor STC1/2 is coexpressed with PAPP-A suggesting that STC1/2 activity could be neutralized by a partner to allow PAPP-A full activity. (3) Just before ovulation, after LH stimulation, PAPP-A activity is reduced likely through its association with STC1/2.

serum concentrations of IGFBP-3 and -5 sequestrating IGFs (93). In *Pappa2* KO mice, ovarian IGFBP-5 expression increases, but *Pappa2* deletion does not affect female reproduction (95). This phenotype does not mimic the complex phenotype of *Igfbp-5* overexpressing transgenic mice showing reduced female fertility (96). The expression profile and the local action of ovarian PAPP-A2 during folliculogenesis remain to be evaluated.

CONCLUSION

In the last two decades, studies on IGFBPs and folliculogenesis have been revisited with the identification of IGFBP proteases, PAPP-A and PAPP-A2, and more recently by the PAPP-A inhibitors STCs. The dynamics of ovarian IGFBP and PAPP-A concentrations has been linked to the selection of dominant follicles. However, much is yet to be learned on the role of STCs in this process (**Figure 1**).

Most of the studies on ovarian folliculogenesis attribute an inhibitory function to IGFBPs by sequestrating IGFs. However, it is well documented that IGFBPs could favor a local action of IGFs by concentrating the ligand in the vicinity of the IGF receptor (97, 98). Indeed, deletion of IGFBP-4 in *Igfbp-4* null mice results in growth retardation, suggesting that loss of IGFBP-4 leads to the loss of its pericellular reservoir function (99). A similar phenotype is observed in *Pappa* null mice with accumulation of IGFBP-4. In this case, IGFBP-4 is likely to sequester IGF-II, prevent its release, and abolish most of IGF-II-stimulated growth. Then, PAPP-A could potentiate IGF action through its binding to the cell surface to target IGF-II/IGFBP-4 complex to the vicinity of the IGF receptor. Thus, these models of null mice highlight that IGFBP-4 can have both stimulatory and inhibitory effects on growth and point out the absence of any redundancy between IGFBPs in this context (99). What about in the ovary? KO mice lacking either IGFBP-2, -3, -4, or -5 and the triple KO IGFBP-3/-4/-5 mice are still fertile suggesting redundancy between IGFBPs and compensatory mechanisms (100, 101). Indeed, in *Igfbp-2* null mice, seric IGFBP-1, IGFBP-3, and IGFBP-4 levels are increased relative to wild-type mice (100). To our knowledge, no data are available on changes of intrafollicular IGFBP protein content. Complementary data come from the study on *Pappa* KO model showing a lower fecundity (reduction of the number of ovulated oocytes and the litter size), with no alteration of follicular growth (normal histology) (66). In this context, follicular fluid is deprived of IGFBP-4 proteolytic activity suggesting the accumulation of IGFBP-4. It would be of interest to know whether high intrafollicular IGFBP-2 and -5 levels are also maintained in the absence of PAPP-A. Nevertheless, this study reveals that limiting intrafollicular IGFBP proteolysis may decrease IGF bioavailability in growing follicles, but not enough to block folliculogenesis as observed in *Igf1* null mice (11). Potential compensatory mechanisms could be initiated such as reduction of expression of IGFBP-2, -3, -4 or activation of other IGFBP-2 and -5 proteases (66). Overall, in the mouse ovary, these results strengthen the inhibitory role of intrafollicular IGFBPs.

Insulin-like growth factor (IGF)-independent functions of IGFBPs have not been really tackled in the ovarian follicle context. In cancer cells, the interaction of IGFBPs with non-IGF ligands, the intracytoplasmic and nuclear action of IGFBPs have been reported (97, 98, 102). The contribution of the non-canonical function of IGFBPs remained to be investigated on ovarian cells.

AUTHOR CONTRIBUTIONS

SM and PM contributed to the redaction of the review.

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Potential Functions of IGFBP-2 for Ovarian Folliculogenesis and Steroidogenesis

*Marion Spitschak and Andreas Hoeflich**

Institute of Genome Biology, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany

Ovarian follicles, as transient structural and functional complexes with the oocyte and the associated cells, determine the female reproductive cycle and thus fertility. Ovarian function is subject to the strict control of hormones and growth factors and thus regulated by auto-, para-, and endocrine mechanisms but influenced also by endogenous factors. During the waves of follicular growth and development, one follicle (monoovulatory) or a limited number of them (polyovulatory) are selected under hypothalamic–gonadal control for maturation until ovulation, resulting in the fertile oocyte. Subordinate follicles inevitably enter different stages of atresia. A number of studies have observed species-specific alterations of IGFBP-2 levels during the phases of growth and development or selection and atresia of follicles. IGFBP-2 is thus probably involved in the process of follicle growth, differentiation, and degeneration. This may occur on the levels of IGF-dependent and -independent growth control but also due to the control of steroidogenesis, e.g., *via* induction of aromatase expression. In mice, IGFBP-2 delayed reproductive development most probably by IGF-independent mechanisms. Because reproductive development is closely linked to the control of life- or health-span and energy metabolism, we feel that the time is right now to resume research on the effects of IGFBP-2 in the ovarian follicular compartment.

Keywords: IGFBP-2, ovary, follicle, folliculogenesis, steroidogenesis, aromatase

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***Correspondence:**
Andreas Hoeflich
hoeflich@fbn-dummerstorf.de

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INTRODUCTION

Mammalian germ cell development is a continuous process under the strict control of hormones and growth factors that can also be affected by environmental factors. Ovarian follicles are transient functional complexes of the oocyte and associated somatic cells at different stages of development or atresia (**Figure 1**). Already during the prenatal phase, the proliferation and partial maturation of a species-specific number of primordial follicles take place within the stroma. By the fifth day after birth, a pool of about 8,000 oocytes within a mouse ovary (1) created in the prophase of the first meiotic maturation division (GV I) are effectively arrested under the influence of meiosis arresting factor (2, 3). However, 2 days later, the number of oocytes in mice is reduced by 60% as a result of apoptosis (1). The initiated follicle development is characterized by the appearance of a high proportion of secondary follicles around the 12th day of life (1, 4). Under the control of the hypothalamic–pituitary–gonadal axis, tertiary follicles develop with the formation of a large antrum, and their increasing 17beta-estradiol (E2) secretion finally induces the onset of puberty (5). The timing of follicular development not only depends on species or genetic background but also is under epigenetic control and can be regulated by nutrients (6, 7). However, less than 1% of primordial follicles in

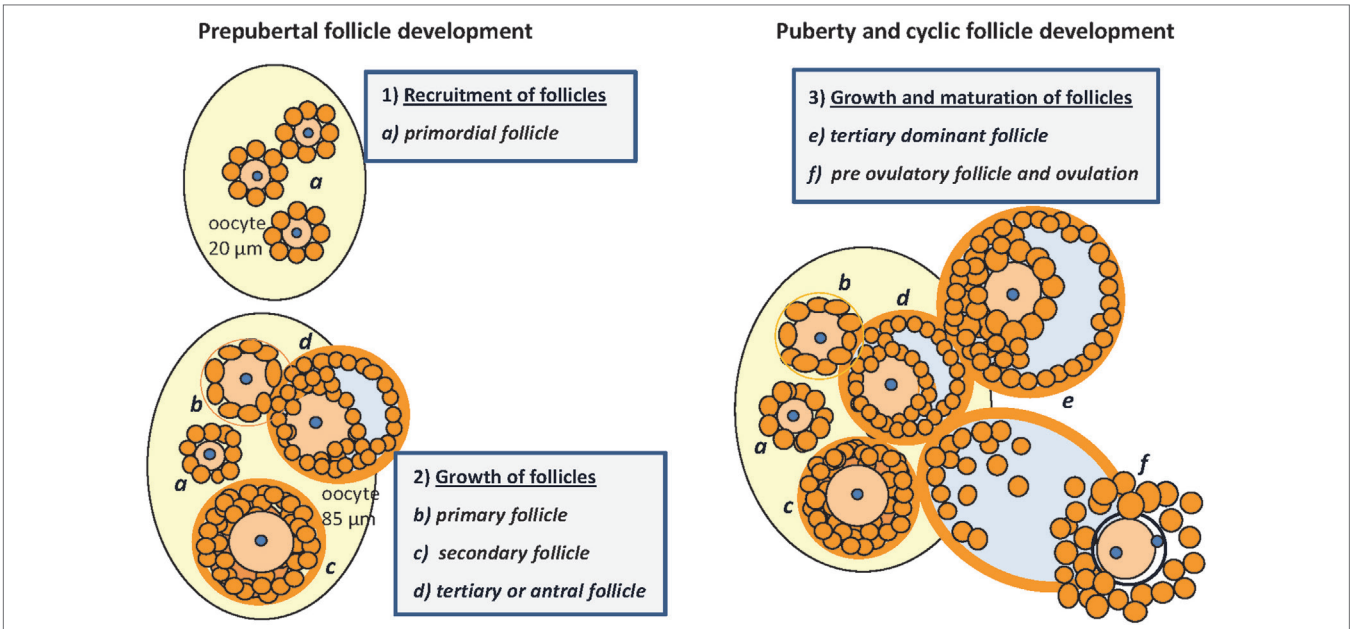

FIGURE 1 | Folliculogenesis in mice: prepubertal follicular development (1) and (2): (a) pool of primordial follicles within the stroma of the neonatal ovary, the oocyte is surrounded by one layer of GC, no TC; nucleus arrested in GV I of meiosis; (b) primary follicle with one layer of cuboidal granulosa cells, signaling the recruitment of TC from the stroma; (c) secondary or preantral follicle with more than one layer of GC, recruitment of TC, and differentiation with low production of androgens; (d) tertiary follicle with formation of the antrum, proliferation of GC, maturation of TC that become steroidogenic under control of LH, increasing androgen production and E2 synthesis in GC under the control of FSH, growing to become the dominant follicle. Puberty and cyclic follicle development (3): (e) tertiary dominant follicle growth with increasing antral volume, mitosis of GC and E2 synthesis → puberty; (f) preovulatory follicle, oocyte with one layer of expanded GC and resumption of meiosis after the LH surge, increasing progesterone production → ovulation [E2, 17beta-estradiol; GC, granulosa cells; TC, thecal cells; GV, germinal vesicle; LH, luteinizing hormone; FSH, follicle-stimulating hormone; follicle classification according to (1, 4, 10, 17)].

fact succeed to enter the cycle of follicle maturation and ovulation (8). A limited number of small tertiary follicles are responsive to increased follicle-stimulating hormone (FSH) secretion from the pituitary gland. This selection is characterized by further mitosis and maturation of the FSH-receptor expressing granulosa cells, with increasing proteoglycan synthesis, and requires the strict coordination of follicular cells and oocyte. The luteinizing hormone (LH) surge stimulates further maturation and ovulation (9). In response to LH, the resumption of meiosis is promoted by glycosaminoglycans (GAGs), secreted by granulosa cells which now also express the LH receptor and which are known to inhibit FSH (2). Oocyte maturation continues, with the production and release at ovulation of a fertilizable egg, with a haploid set of chromosomes and a separated polar body, surrounded by follicular granulosa cells. The remaining theca and granulosa cells then differentiate into the progesterone-secreting cells of the corpus luteum. Follicular atresia can be found in all stages of development, but the proportion is greatest in tertiary follicles (10). In postnatal and cyclic folliculogenesis, the total number of healthy follicles remains constant with an alternating population of secondary and tertiary follicles of between 4 and 15%, presumably due to new formation of primordial follicles (1). Puberty and cycle length or estrus intensity and duration vary line specifically and are subject to environmental factors. Ovarian function can be influenced by systemic effects, among others the body fat distribution or nutrient intake (11–13). An active contribution of IGFBP-2 or other IGFBPs for development and atresia was already postulated by Cataldo and Giudice in 1992 (14). The last 10 years have witnessed a "relative paucity" of studies on the role of IGFBPs in general (15) also including their effects on reproductive performance; the last review on the functions of IGFBPs for folliculogenesis goes back to 2002 (16), warranting an update now. The present review summarizes evidence for the interactive regulation of different ovarian developmental stages by IGFBP-2 and addresses a particular role of IGFBP-2 for the control of steroidogenesis in the maturing follicle.

FOLLICULOGENESIS AND THE CONTROL OF THE IGF-SYSTEM

Insulin-like growth factor-1 (IGF-I) is produced already in granulosa cells of murine primary follicles and with a maximum in late preantral and early antral follicles, where it is associated with antrum remodeling and the growth of healthy follicles (18). In the brain, and depending on the concentrations of E2, IGF-I was demonstrated to control the hypothalamic release of LH and reproductive development in female rats (5). In human granulosa cells, IGF-I receptor signaling (19) is permissive for the positive effect of FSH on the expression of aromatase (CYP19A1) mediated by AKT signaling (**Figure 2**). The differentiation from the preantral to the large antral follicle requires IGF-IR activity with subsequent AKT activation for FSH-induced steroidogenic gene expression, which in turn is maintained *via* synergistic effects

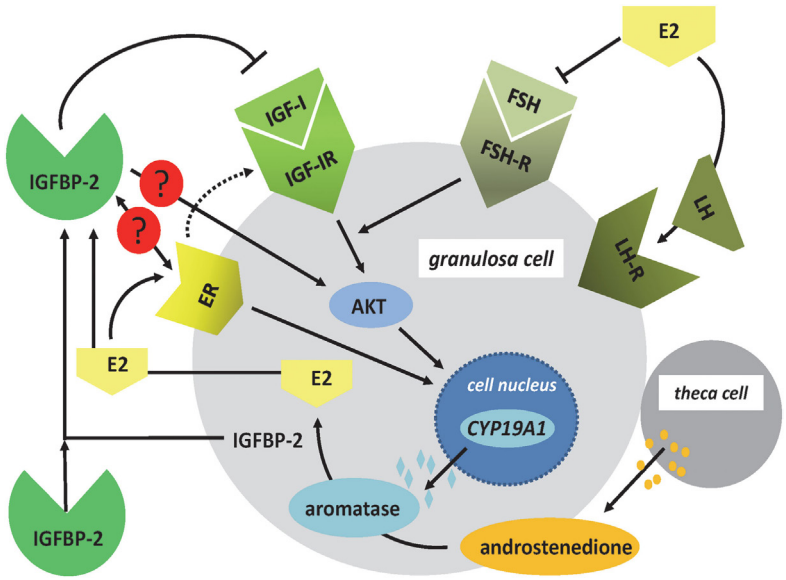

FIGURE 2 | Regulation of E2 synthesis in the ovarian follicle. In granulosa cells, nuclear expression of *CYP19A1* is regulated by FSH- and ER signaling. The effects of FSH on the expression of *CYP19A1* gene are mediated by IGF-IR signaling and AKT. ER signaling can also include the IGF-IR pathways. In addition, the ER has direct effects on *CYP19A1* gene expression within the cell nucleus. Androstenedione, produced by theca cells, is used as a substrate for aromatase to produce E2. In granulosa cells, IGFBP-2 is regulated by E2, and IGFBP-2 has been shown to block FSH-dependent E2 production. Increased levels of E2 block the expression of FSH and induce the LH surge. Open questions in granulosa cells include the function of IGFBP-2 for ER- and IGF-independent effects of IGFBP-2 for E2 production (AKT, protein kinase B; *CYP19A1*: aromatase; E2, 17beta-estradiol; ER, estrogen receptor; FSH, follicle-stimulating hormone; IGF-IR, IGF-I receptor; LH, luteinizing hormone).

with local IGF-II (19, 20). Mice lacking the IGF-I receptor in ovarian granulosa cells are devoid of antral follicles, show high rates of apoptosis, low AKT activation, low levels of serum E2, and are infertile (21). Synergistically with FSH, IGF-I stimulates activation of AKT-dependent aromatase expression, cell proliferation, and expression of apoptosis-regulating genes in the granulosa cells (22–24). The positive effect of FSH on expression of aromatase was blocked by the addition of IGFBP-2, and an excess of IGF-I was able to abolish the inhibitory effect of IGFBP-2 on FSH-dependent aromatase expression (22). Accordingly, the negative effect of IGFBP-2 on steroidogenesis was IGF dependent in granulosa cells. IGF-I is also one of the stimulators of androgen synthesis in the theca cells under LH control (17). Thus, IGF-I, under the influence of gonadotropic hormones, is an essential regulatory component for the growth of antral follicles and their increasing E2 biosynthesis in humans, mice, and rats. Furthermore, IGF-I expression during folliculogenesis in mice and rats is controlled by estrogen receptors α and β. E2 has an autocrine dose-dependent stimulatory or inhibitory effect on the IGF-I-IGF-IR pathway (25–27).

FOLLICULOGENESIS AND THE CONTROL OF IGFBP-2

IGFBP-2 is present in follicular fluid and subject to dynamic changes during follicle growth and maturation. Accordingly in sows, IGFBP-2 was reduced during follicular development (28). Within the mouse ovary, IGFBP-2 can be localized in discrete regions characterized by altered follicular growth, developmental stage, and atresia and thus accordingly was discussed in a functional context of folliculogenesis (18). Equine growing follicles exclusively produced IGFBP-2, and dominant follicles had lower concentrations of IGFBP-2 (29). In this experimental setting, E2 increased expression of IGFBP-2 and FSH increased expression of IGFBP-2 *in vitro* (29). During selection to a healthy dominant follicle in heifers, the capacity of the granulosa cells to enhance steroid synthesis consistently correlated with low concentrations of IGFBP-2 (30). The dominance of follicles was associated with lower amounts of IGFBP-2 and markedly higher E2 contents (30). This observation is in line with substantial increases in IGFBP-2 in follicular fluids of subordinate follicles derived also from heifers (31, 32). There were transient increases in LH-induced differentiation with enhanced IGF-I and E2, but decreased IGFBP-2 (33). Interestingly, the levels of IGFBP-2 were in a positive correlation when compared to caspase-3 activity (31, 32). IGFBP-2 expression was reduced in granulosa cells simultaneously with increased expression of IGF-1 and IGF-1R as also the steroidogenic genes responsible for synthesis from cholesterol to E2 and progesterone (34). From the dynamic changes in IGFBP-2 expression/concentration during folliculogenesis or because of the correlations of IGFBP-2 with reproductive hormones, an active contribution of IGFBP-2 during the maturation of follicles has been assumed with an effect also on the expression of aromatase in growing bovine follicles (35).

The reduction of local IGFBP-2 or other IGFBPs (36) in the follicular compartment can also be a result of active proteolysis. In dominant follicles, proteolytic degradation of IGFBP-4 and

-5 and lower concentrations of IGFBP-2 were discussed in the context of increased levels of free IGF-I, and a separate review was dedicated to the control of IGFBPs during follicle selection (37). In bovine follicles, it was demonstrated that IGFBP-2 proteolytic activity originates from granulosa cells but not from the oocyte, and a self-regulatory mechanism of IGF-I activation in granulosa cells was discussed by the authors (38). For further reading on the effect of PAPP-A-dependent IGFBP-proteolysis on the selection of dominant follicles, we would like to refer to the actual discussion of Monget and Mazerbourg (39).

FOLLICULAR ATRESIA AND THE CONTROL OF IGFBP-2

In the ovaries from polyovulatory as well as in monoovulatory females, permanent follicle selection with development and atresia is taking place. This process is subject to hypothalamo-pituitary control in interaction with intra-ovarian control. Distinct characteristics of follicular atresia are present at the level of morphology and apoptosis, but also lower E2 concentrations can indicate atretic degeneration of follicles. When compared to healthy or atretic follicles from human donors, IGFBP-2 concentrations were increased in human atretic follicular fluid (40). IGFBP-2 was also increased in atretic follicles from pigs after estrus (41). At the same time, E2 was decreased, whereas apoptosis was increased in follicles from pigs (41) and humans (40), and therefore, the authors discussed control of IGFBP-2 concentrations by E2. As reviewed before, expression of IGFBP-2 by steroids is observed in multiple tissues including various tissues from the female reproductive system (42). Notably, the vast majority of studies identified positive effects of exogenous steroids on the expression of IGFBP-2 (42). In mice, higher expression of IGFBP-2 mRNA was associated with late but not with earlier stages of atresia (18). The potential effects of IGFBP-2 on follicular atresia could be mediated by IGF-dependent or IGF-independent mechanisms (43). Interestingly, IGFBP-2 was able to inhibit FSH-dependent induction of aromatase and cholesterol side-chain cleavage enzyme (CYP11A1) expression (22). The inhibitory effect of IGFBP-2 was compensated by the addition of excess IGF-I, and the contribution of IGFBP-2 in the control of steroidogenesis thus cannot be excluded (22).

EFFECTS OF IGFBP-2 ON REPRODUCTIVE PERFORMANCE

In granulosa cells from polycystic follicles isolated from dairy cows, reduced mRNA expression of IGFBP-2 was found when compared to granulosa cells from normal follicles (44). Also in human granulosa cells isolated from polycystic ovaries, IGFBP-2 expression was reduced when compared to controls (45). Therefore, an active contribution of IGFBP-2 on reproductive performance might be indicated. In fact, single nucleotide polymorphisms (SNP) in the *IGFBP2* gene locus were identified as candidate markers for reproduction traits or litter size in different pig populations (46, 47). In dairy cows, reproductive development (e.g., age of first conception or calving) was correlated with a number of distinct SNPs on the *IGFBP2* gene (48). In fact, forced expression of IGFBP-2 delayed reproductive development in female transgenic mice (49). In this model, the expression of wildtype but not mutated IGFBP-2 delayed the onset of first estrus and hence ovarian cycle activity (49). Mutated IGFBP-2 lacked the integrin binding sequence and was thus discussed in a functional context in regard to altered reproductive performance; the negative effect of IGFBP-2 on reproductive development appears to be IGF independent. Regulation of IGFBP-2 expression by steroid hormones is observed in different vertebrate species in multiple cells and tissues, including the follicle (42). In addition, a mutual relationship was observed between expression of IGFBP-2 and estrogen receptors in breast epithelial cells (50). Notably, the presence of the RGD motif was also required for the effects on ER expression as demonstrated by Foulstone et al. (50). However, the relationship between estrogen receptor expression and IGFBP-2 remains to be assessed in ovarian follicles.

SUMMARY AND CONCLUSION

IGFBP-2 is present in high abundance in follicular fluid and a number of studies identified IGFBP-2 by Western ligand blotting. Accordingly, it is unclear why mainly only descriptive studies are available on the functions of IGFBP-2 in regard to folliculogenesis. Studies describing altered expression of IGFBP-2 in growing versus atretic, in dominant versus subordinate, or in earlier versus later stages of follicles are available. The majority of studies reported lower levels of IGFBP-2 in healthy, larger, or later developmental stages of follicles or higher expression of IGFBP-2 in atretic follicles.

In follicles, IGFBP-2 is regulated by steroids, FSH, and LH, and there is experimental evidence that also steroidogenesis is negatively coregulated by IGFBP-2. The effect of IGFBP-2 on steroidogenic gene expression, including aromatase in the dominant follicles, could be exerted by IGF-dependent or IGF-independent mechanisms. In fact, in IGFBP-2 transgenic mice, the negative effects on reproductive development have been suggested to be IGF independent. In ovarian follicles, so far only IGF-dependent effects of IGFBP-2 on steroidogenesis have been provided. Since AKT is regulated both in an IGF-dependent and -independent fashion, e.g., by integrins or proteoglycans in various cell types and required for steroidogenesis in follicles, AKT appears as an attractive target for future research also on IGF-independent effects of IGFBP-2 during folliculogenesis and steroidogenesis.

AUTHOR CONTRIBUTIONS

MS and AH have written the manuscript. MS has developed the figures.

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Conflict of Interest Statement: AH is related to Ligandis UG.

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Follicle-Stimulating Hormone Regulates *igfbp* Gene Expression Directly or *via* Downstream Effectors to Modulate Igf3 Effects on Zebrafish Spermatogenesis

*Diego Safian[1], Henk J. G. van der Kant[1], Diego Crespo[1], Jan Bogerd[1] and Rüdiger W. Schulz[1,2]**

[1] Reproductive Biology Group, Division Developmental Biology, Institute of Biodynamics and Biocomplexity, Department of Biology, Faculty of Science, University of Utrecht, Utrecht, Netherlands, [2] Institute of Marine Research, Bergen, Norway

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Andreas Hoeflich,
Leibniz Institute for Farm Animal Biology (LG), Germany

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Toshio Sekiguchi,
Kanazawa University, Japan
Takashi Yazawa,
Asahikawa Medical College, Japan

***Correspondence:**
Rüdiger W. Schulz
r.w.schulz@uu.nl

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Previous work showed that pharmacological inactivation of Igf-binding proteins (Igfbps), modulators of Igf activity, resulted in an excessive differentiation of type A undifferentiated (A_{und}) spermatogonia in zebrafish testis in tissue culture when Fsh was present in the incubation medium. Using this testis tissue culture system, we studied here the regulation of *igfbp* transcript levels by Fsh and two of its downstream effectors, Igf3 and 11-ketotestosterone (11-KT). We also explored how Fsh-modulated *igfbp* expression affected spermatogonial proliferation by adding or removing the Igfbp inhibitor NBI-31772 at different times. Fsh (100 ng/mL) decreased the transcript levels of *igfbp1a*, *-3*, and *-6a* after 1 or 3 days, while increasing *igfbp2a* and *-5b* expression, but only after 5 days of incubation. Igf3 down-regulated the same *igfbp* transcripts as Fsh but with a delay of at least 4 days. 11-KT increased the transcripts (*igfbp2a* and *5b*) that were elevated by Fsh and decreased those of *igfbp6a*, as did Fsh, while 11-KT did not change *igfbp1a* or *-3* transcript levels. To evaluate Igfbps effects on spermatogenesis, we quantified under different conditions the mitotic indices and relative section areas occupied by the different spermatogonial generations (type A_{und}, type A differentiating (A_{diff}), or type B (B) spermatogonia). Igf3 (100 ng/mL) increased the area occupied by A_{diff} and B while decreasing the one for A_{und}. Interestingly, a concentration of Igf3 that was inactive by itself (25 ng/mL) became active in the presence of the Igfbp inhibitor NBI-31772 and mimicked the effect of 100 ng/mL Igf3 on spermatogonia. Studies exploiting the different dynamics of *igfbp* expression in response to Fsh and adding or removing NBI-31772 at different times showed that the quick downregulation of three *igfbp* as well as the delayed upregulated of two *igfbps* all support Igf3 bioactivity, namely the stimulation of spermatogonial differentiation. We conclude that Fsh modulates, directly or *via* androgens and Igf3, *igfbp* gene expression, supporting Igf3 bioactivity either by decreasing *igfbp1a*, *-3*, *-6a* or by increasing *igfbp2a* and *-5b* gene expression.

Keywords: follicle-stimulating hormone, Igf3, Igf-binding proteins, spermatogonia, differentiation

INTRODUCTION

In vertebrates, the brain–pituitary system is the major regulator of spermatogenesis and coordinates the activities of somatic cell types in the testis. These activities include the modulation of spermatogonial stem cell (SSC) fate (1–3). The SSCs can self-renew or differentiate, depending on the signaling environment produced by Sertoli cells (SCs) and other somatic cell types (4, 5). Follicle-stimulating hormone (Fsh) regulates the activity of SCs, which then communicate with germ cells *via* short-range signaling. In fish, the *fshr* is expressed not only by SCs but also by Leydig cells (LCs), thus stimulating for example androgen and insulin-like peptide 3 (Insl3) production (6, 7). In eel and zebrafish, recombinant Fsh-induced spermatogonial proliferation and differentiation by stimulating androgen production (8, 9). In zebrafish, Fsh also promoted spermatogenesis in an androgen-independent manner by promoting Igf3 (9) and Insl3 (7), by suppressing anti-Müllerian hormone signaling (10), and by modulating the Notch, Wnt, and Hedgehog signaling systems (11).

IGF signaling promotes proliferation and differentiation of many different cell types across animal species. In most vertebrates, the IGF signaling system is composed of two ligands (IGF1 and 2), two IGF1 receptors (IGF1R1 and 2), and six IGF-binding proteins (IGFBP1-6) (12). Systemic IGFs are mainly secreted by the liver, controlled by growth hormone (GH), but IGFs are also produced locally in many tissues (13).

IGF signaling modulates spermatogenesis in a wide range of animals. Insulin/IGF signaling regulates early stages of male germ cell development (14–16). In mice, the combined knockout of insulin and IGF1 receptors strongly reduced testis size as a consequence of decreased SC proliferation and the resulting reduction of the germ cell supporting capacity (17). A more recent report indicated that blocking the IGF1 receptor in primary cultures of mouse SSCs reduced their proliferation and decreased their colonization capacity when injected in busulfan-treated recipients (18). In rainbow trout, *igf1* and *igf1r* expression was found in cell fractions enriched in SCs but also in spermatogonia and primary spermatocytes (19), while *igf1* expression was restricted to cysts containing spermatogonia in sea bass (20). Furthermore, primary tissue culture studies using prepubertal eel testis showed that IGF was required as permissive factor for the androgen-stimulated differentiating proliferation of spermatogonia (21).

An additional ligand member of the Igf family, Igf3, has been identified in fish gonads (22). In zebrafish testis, Fsh increased *igf3* but not *igf1*, *2a*, or *2b* transcript levels, and recombinant zebrafish Igf3 increased the proliferation activity of A_{und} and A_{diff} spermatogonia and upregulated the expression of *dazl*, a marker for type B spermatogonia and spermatocytes (9). Also, Igfbps appear to be relevant for testis function in zebrafish (23). Igfbps bind Igfs with high affinity, thereby inhibiting or potentiating Igf actions (12, 24). Similar to *igfs*, the *igfbp*s are expressed in several tissues, suggesting that local Igfbps can modulate systemic but in particular locally produced Igfs (12). Fsh and triiodothyronine (T_3), another regulator of *igf3* expression (25), modulated the expression of selected *igfbp*s, and interestingly, adding an Igfbp inhibitor further shifted spermatogonial development toward differentiation at the expense of A_{und} spermatogonia (23). This observation suggested that Igfbps play important roles in modulating spermatogonial proliferation and differentiation behavior.

Here, we studied the transcriptional regulation of the nine zebrafish *igfbps* that are all expressed in testis tissue, by examining the effects of Fsh and of two downstream mediators of Fsh action in the testis, Igf3, and 11-KT. We also report the effects of Igf3 on spermatogonial proliferation and the area occupied by spermatogonia in zebrafish testis, under basal conditions or in the presence of an Igfbp inhibitor. Finally, we have started exploring a potential, functional differentiation among the Igfbps in the zebrafish testis.

MATERIALS AND METHODS

Animals

Adult male zebrafish between 4 and 12 months of age were used in this study. Six to eight animals were used per experiment. All experiments carried out in this study followed the Dutch National regulations for animal care and use in experimentation, and the experimental protocols have been submitted to, and were approved by, the Utrecht University Experimental Animal Committee (2015.I.857.013 and AVD108002015333).

Tissue Culture

To study the regulation of *igfbp* transcript levels, adult zebrafish testes were dissected for tissue culture experiments using a previously described system (26), in which one testis was incubated under control conditions, the other testis under experimental conditions.

Zebrafish testes were incubated for 5 days under basal conditions or in the presence of recombinant zebrafish Fsh (25, 50, 100, or 1,000 ng/mL) (6). In a second series of experiments, zebrafish testes were incubated under basal conditions or in the presence of Fsh (100 ng/mL) for 1, 3, 5, or 7 days.

To study the effect of Igf3 on *igfbp* expression, zebrafish testes were incubated in the absence or presence of recombinant zebrafish Igf3 (100 ng/mL) (9) for 3 or 7 days. Based on the slow effect of Igf3 on *igfbp* expression, testes were incubated for 5 or 7 days in the presence of Fsh (100 ng/mL) with or without NVP-AEW541 [10 μM; Selleckchem (25)], an inhibitor of Igf1 receptors; incubation media for the control and experimental groups contained the same final concentration of dimethyl sulfoxide (0.1%).

In a different set of experiments, zebrafish testes were incubated under basal conditions or in the presence of 11-KT [200 nM in ethanol (0.01%); Sigma] for 3 or 7 days (10), or in the presence of 11-KT (200 nM) with or without 10 μM NVP-AEW541 for 7 days. The reason to carry out this experiment was the previously reported, slight stimulatory effect of 11-KT on *igf3* transcript levels (9). At the end of the incubation period, testis tissue was snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction.

We have reported previously that 100 ng/mL Igf3 stimulated the proliferation of type A spermatogonia and increased the

transcript levels of marker genes associated with spermatogonial differentiation (9). To further study Igf3 effects and the role of Igfbps on zebrafish spermatogenesis, we incubated testes under basal conditions or in the presence of 25 or 100 ng/mL Igf3 for 3 days. The lower dose was expected to have no/little effect on its own, based on a previous dose-response study (9). In addition, zebrafish testes were incubated for 3 days in the presence of 25 ng/mL Igf3 with or without NBI-31772 (10 μM; Sigma-Aldrich), an Igfbp inhibitor (27, 28); NBI-31772 alone has no effects on spermatogenesis or expression of germ cells markers (23).

The Fsh time-course experiment showed that two *igfbp* mRNAs were upregulated with a delay of at least 3 days, so that two experiments were designed to preferentially study these two *igfbps* upregulated by Fsh. To this end, zebrafish testes were incubated for 7 days with 100 ng Fsh/mL in both control and experimental groups. During the last 4 days, the experimental group was in addition exposed to 10 μM NBI-31772. In the second experiment, testes of the control group were incubated in the presence of 100 ng/mL Fsh and 10 μM NBI-31772 for 7 days, whereas in the experimental group, testes were incubated for the first 3 days under the same conditions but for the remaining 4 days, the medium contained only Fsh but no NBI-31772. At the end of the 7 days long incubation period, testis tissue was fixed for morphological analyses.

The production of biologically active steroids was blocked by including trilostane (25 μg/mL; Chemos), an inhibitor of 3β-hydroxysteroid dehydrogenase activity, in all experiment with Fsh, a potent steroidogenic hormone in fish (6).

Gene Expression

The relative transcript levels of *igfbp*s, germ cells markers, and other genes of interest (**Table 1**) were analyzed by real-time, quantitative polymerase chain reaction (qPCR) assays. The expression of *igfbp3* was analyzed using a commercial available TaqMan gene expression assay (Applied Biosystems, Cat# 4351372).

Total RNA was isolated from the tissue using an RNAqueous Micro kit (Ambion), according to the manufacturer's protocol. cDNA synthesis from total RNA and quantification of transcript levels were carried out as described previously (31). In brief, 2 μg of total RNA were reverse transcribed using 250 U of Supercript II RNase-reverse transcriptase (Life Technologies). qPCR were performed by using 2× SYBER Green assay mix (Applied Biosystems), specific qPCR primers (900 nM) and 5 μL of cDNA in a total volume of 20 μL. The quantification cycle (Cq) values were determined in a Step One Plus Real-Time PCR System (Applied Biosystems) using default settings. The relative amounts of mRNA in the cDNA samples were calculated using the arithmetic comparative method

TABLE 1 | Primers used for gene expression studies.

Target genes	Primers name	Sequence (5′–3′)	Gene information
igfbp1a	4194 (Fw)	GAGCCCCGAGCCTAACCA	Safian et al. (23)
	4196 (Rv)	TCTCATAACGGGCCGACG	
igfbp1b	4199 (Fw)	GTGGAGCACCACCCTACTGAAG	Safian et al. (23)
	4200 (Rv)	TGCATCACCTGCTGAGCC	
igfbp2a	4206 (Fw)	GACCCTAAAGCACCACATGCTAA	Safian et al. (23)
	4207 (Rv)	TTGACCAGGTGCTGGAAAGG	
igfbp2b	4211 (Fw)	GCCCACCATGACCAACCA	Safian et al. (23)
	4213 (Rv)	GAAGTAAATGGCACGCGGTC	
igfbp5a	4226 (Fw)	CTCCCCTTCCCATCGACAA	Safian et al. (23)
	4227 (Rv)	CAGAAGGAAGCTGGACGGAAT	
igfbp5b	4333 (Fw)	CGCAAACATGTAAGCCCTCTAG	Safian et al. (23)
	4334 (Rv)	ATGGAGTTCAAATGCCGGG	
igfbp6a	4955 (Fw)	CCTCTGGTGGCGACAAATATG	Safian et al. (23)
	4956 (Rv)	TGCATCAACTGCCAGAACTCTAA	
igfbp6b	4928 (Fw)	TGACATCTACATCCCAAACTGTGA	Safian et al. (23)
	4929 (Rv)	GGAAAAAGCAGTGTCGGTCC	
foxa2	5741 (Fw)	GTCAAAATGGAGGGACACGAAC	Potential marker for type A undifferentiated spermatogonia
	5743 (Rv)	CATGTTGCTGACCGAGGTGTAA	
piwil2	2994 (Fw)	TGATACCAGCAAGAAGAGCAGATCT	Expressed in all germ cell type except type A_{und} spermatogonia and spermatozoa (29)
	2995 (Rv)	ATTTGGAAGGTCACCCTGGAGTA	
dazl	3104 (Fw)	AGTGCAGACTTTGCTAACCCTTATGTA	Expressed mainly in type B spermatogonia and primary spermatocytes (30)
	3105 (Rv)	GTCCACTGCTCCAAGTTGCTCT	
igf1ra	2362 (Fw)	TACATCGCTGGCAACAAGCA	Igf1 receptor a (30)
	2363 (Rv)	TCATTGAAACTGGTCCTTATGCAAT	
igf1rb	2595 (Fw)	GTGCTGGTCCTCTCCACACTCT	Igf1 receptor b (30)
	2596 (Rv)	TTACCGATGTCGTTGCCAATATC	

Fw, forward; Rv, reverse.

(ΔΔCt method), according to Bogerd et al. (31). Expression of the *ribosomal RNA 18S* (*18S*) transcript was stable (Figure S1 in Supplementary Material). *18S* expression served as reference transcript and was analyzed using a commercially available TaqMan gene expression assay (Applied Biosystems). All results were expressed as fold change with respect to the control group.

Morphological Analysis

To quantify the proliferation activity of A_{und}, A_{diff}, and B spermatogonia, 100 µg/mL of the proliferation marker 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) was added to the tissue culture medium during the last 6 h of the incubation period. After fixation in methacarn (60% [v/v] absolute ethanol, 30% chloroform, and 10% acetic acid), the samples were dehydrated in graded ethanol (70, 96, and 100%), embedded in Technovit 7100 (Heraeus Kulzer) and sectioned at a thickness of 4 µm. To determine the proliferation activity, one set of sections was used to localize BrdU as described previously (26). The mitotic index was determined by analyzing 100 spermatogenic cysts (A_{diff} and B spermatogonia) or 100 A_{und} cells, discriminating between BrdU positive and negative cysts/cells, respectively.

To quantify the proportion of section area occupied by the different spermatogonial cell types, another set of sections was stained with toluidine blue and 10 randomly chosen, non-overlapping fields were photographed at ×400 magnification with a digital camera. The images were analyzed quantitatively based on the number of points counted over the germ cell types investigated (A_{und}, A_{diff}, and B spermatogonia), using the ImageJ freeware (National Institutes of Health, Bethesda, MD, USA, http://rsbweb.nih.gov/ij) with a 540-point grid.

Statistical Analysis

Statistical analyses were carried out using the GraphPad Prism 5 software package (San Diego, CA, USA). Since our tissue culture system compares the two testes of a given fish incubated under control versus experimental conditions, we applied Student's *t*-test for paired observation to estimate statistical significance. All data are presented as fold of basal (mean ± SEM). The individual data before normalization are provided as supplemental figures (Figure S2 in Supplementary Material). To achieve homogeneity of variance, data were log transformed when appropriate.

RESULTS

Fsh and Downstream Mediators Modulate igfbp Expression

To study the regulation of *igfbp* transcript levels by Fsh, dose-response and time-course experiments were carried out. *igfbp1b*, *2b*, *5a*, and *6b* transcript levels were not regulated by Fsh at any concentration or time evaluated in the present study (data not shown). From the five remaining transcripts, three (*igfbp1a*, *3*, and *6a*) were down- and two (*igfbp2a* and *5b*) were upregulated by Fsh. The Fsh dose-response experiment was carried out using 5 days of incubation. The downregulated *igfbp1a*, *3*, and *6a* responded to the two higher concentrations of 100 and 1,000 ng/mL Fsh (**Figures 1A–C**). This was also the case as regards the upregulated *igfbp5b*, while the second upregulated *igfbp2a* responded to all Fsh concentrations used (**Figures 1D,E**).

Based on these data, we used 100 ng Fsh/mL in the time-course experiment. We found that *igfbp1a* expression was quickly downregulated after 1 day, while *igfbp3* and *6a* transcript levels had decreased significantly after 3 days of incubation (**Figures 2A–C**). Upregulation of *igfbp2a* and *5b* required more time and became significant after 5 days of tissue culture (**Figures 2D,E**).

Since Fsh increased Igf3 release, we studied if (some of) the changes induced by Fsh are mediated by Igf3. As shown in **Figure 2**, Fsh modulated the transcript levels of some *igfbps* after a short (1 or 3 days for *igfbp1a*, *igfbp3*, and *igfbp6a*) and others after a longer (5 or 7 days for *igfbp2a* and *igfbp5b*) period of incubation. Therefore, testes were incubated in the presence of recombinant zebrafish Igf3 (100 ng/mL) for 3 or 7 days. Effects of Igf3 on *igfbp* expression were evident after 7 days of incubation only, when we found significantly decreased transcript levels of *igfbp1a*, *3*, and *6a* (**Figure 3A**); *igfbp2a* and *igfbp5b* expression did not change in response to Igf3 after 3 or 7 days of incubation (data not shown). To directly examine if the slow Igf3 effects on *igfbp* transcript levels are downstream of Fsh, we incubated testis tissue for 5 or 7 days with Fsh in the absence or presence of a pharmacological Igf receptor inhibitor. While *igfbp* transcript levels did not change after 5 days (data not shown), all three transcripts (*igfbp1a*, *igfbp3*, and *igfbp6a*) increased in response to the Igf receptor inhibitor after 7 days of incubation (**Figure 3B**). This data shows that the late decrease of *igfbp1a*, *-3*, and *-6a* transcripts specifically depends on Fsh-triggered, Igf3-dependent signaling.

In fish, other mediators of Fsh effects are androgens, considering the strong steroidogenic potency of zebrafish Fsh, for example (6). While steroid-mediated effects were neutralized by including trilostane in the incubation medium in experiments with Fsh, the next set of experiments aimed at investigating potential androgen effects. 11-KT (200 nM) upregulated the expression of *igfbp2a* and *igfbp5b*, while *igfbp6a* was downregulated after 7 days but not after 3 days of incubation (**Figure 3C**). *Igfbp1a*, *3*, and *6a* transcript levels did not response to 11-KT after 3 or 7 days of incubation (data not shown). Since *igf3* expression also responds to 11-KT (9) and since *igfbp* transcript levels responded to 11-KT after 7 days, the experiment was repeated in the presence of the Igf receptor inhibitor for 7 days. However, the effects of 11-KT on the transcript levels of *igfbp*s did not change in the additional presence of the Igf receptor inhibitor (data not shown), suggesting that the 11-KT effects were not mediated by Igf3.

Igf3 Effects on Spermatogonial Development

Exposure to 25 ng/mL Igf3 did not modulate the mitotic index and proportion of spermatogonia after 3 days of incubation (**Figures 4A,C,D**), different from a higher concentration of Igf3 (100 ng/mL) that increased the mitotic indices of all spermatogonia (**Figures 4A,E**). The proportion of area occupied by

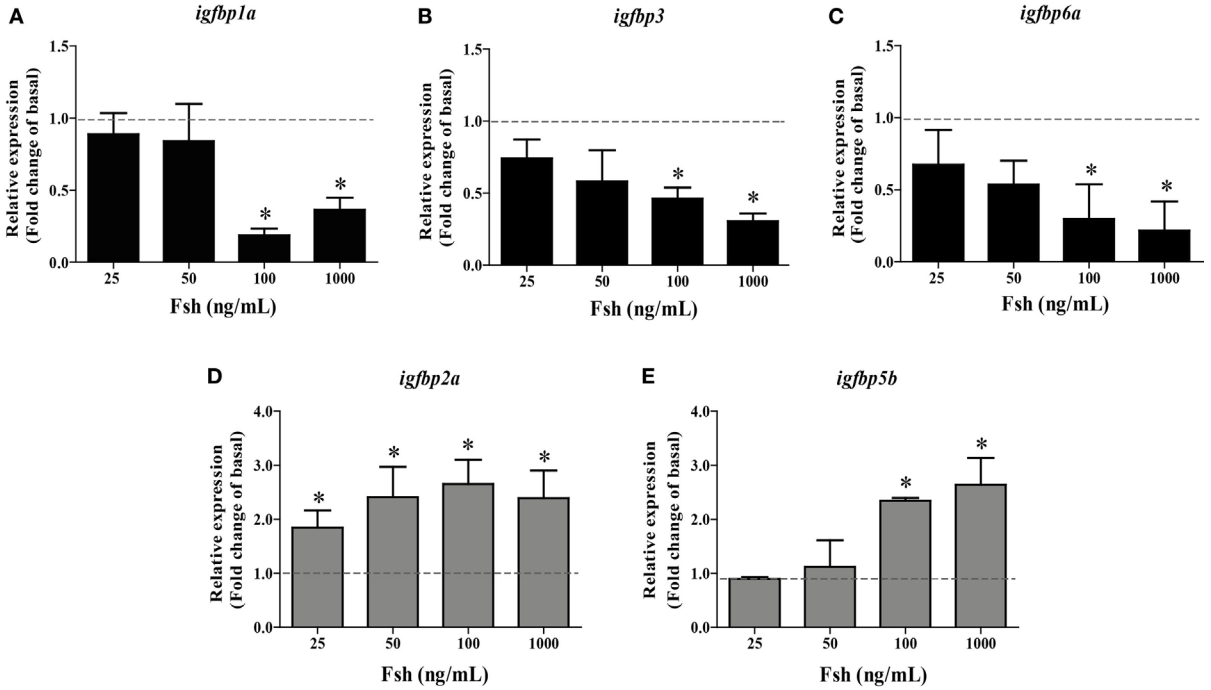

FIGURE 1 | Follicle-stimulating hormone (Fsh) dose-response study on *igfbp* transcript levels in adult zebrafish testis after 5 days of primary organ culture. Fold-change of gene expression of *igfbp1a* **(A)**, *igfbp3* **(B)**, *igfbp6a* **(C)**, *igfbp2a* **(D)**, and *igfbp5b* **(E)** under basal conditions (dotted line) or in the presence of 25, 50, 100, or 1,000 ng recombinant zebrafish Fsh per mL ($n = 6$ for all concentrations) (bars). Asterisks indicate significant differences (*$P < 0.05$) compared to the respective control group.

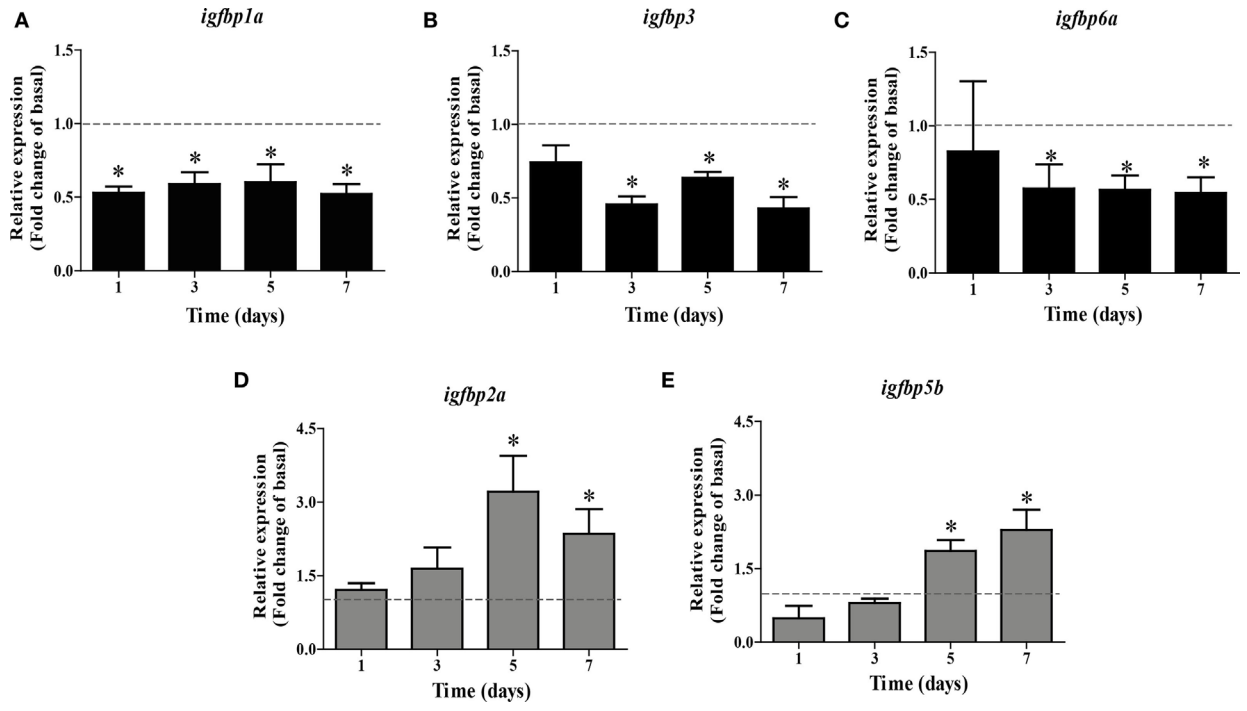

FIGURE 2 | Follicle-stimulating hormone (Fsh) time-course study on *igfbp* transcript levels in adult zebrafish testis after 1–7 days of primary organ culture. Transcript levels of *igfbp1a* **(A)**, *igfbp3* **(B)**, *igfbp6a* **(C)**, *igfbp2a* **(D)**, and *igfbp5b* **(E)** are expressed as fold-change compared to basal conditions (absence of Fsh; represented by a dotted line), as induced by recombinant zebrafish Fsh (100 ng/mL) (bars). The duration of the *ex vivo* culture varied between 1 and 7 days ($n = 5$–7). Asterisks indicate significant differences (*$P < 0.05$) compared to the respective control group.

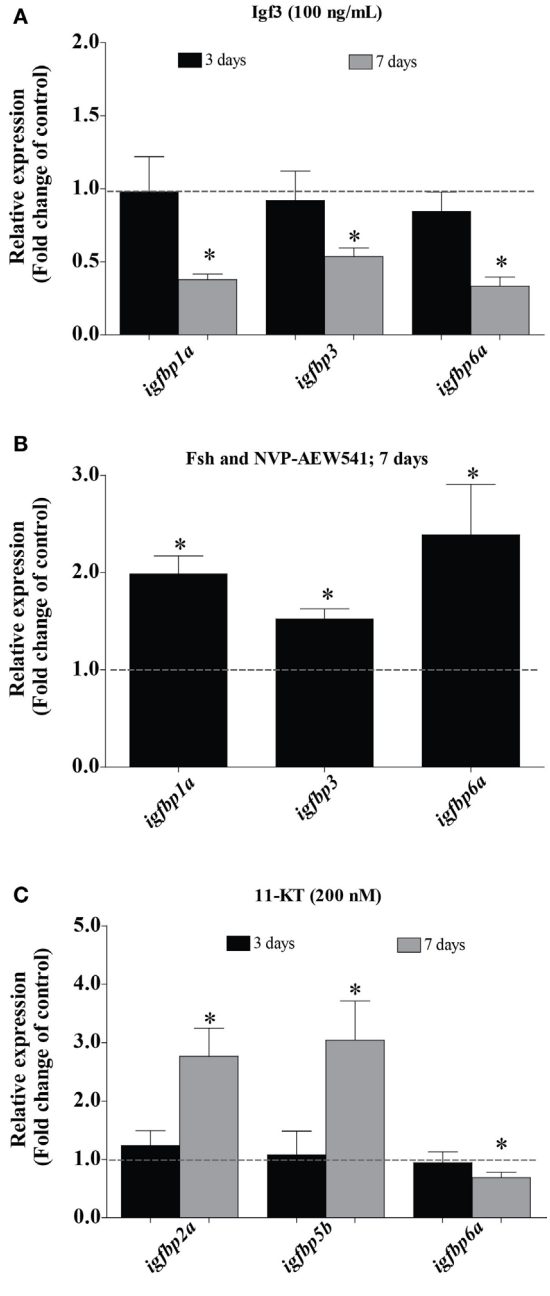

FIGURE 3 | Effect of Igf3 and 11-KT on *igfbp* transcript levels in adult zebrafish testis. **(A)** Transcript levels of *igfbp*s in the presence of Igf3 (100 ng/mL) after 3 or 7 days of incubation ($n = 8$). **(B)** *Igfbp* expression in response to follicle-stimulating hormone (Fsh; 100 ng/mL) in the absence or presence of Igf receptor inhibitor NVP-AEW541 (10 μM) after 7 days of incubation ($n = 8$). **(C)** Transcript levels of *igfbp*s in basal conditions or in the presence of 11-KT (200 nM) after 3 ($n = 7$) or 7 ($n = 6$) days of incubation. Transcript levels of *igfbp*s are expressed as fold-change compared to the respective control condition (basal or 100 ng/mL Fsh) represented by a dotted line. Asterisks indicate significant differences (*$P < 0.05$) compared to the respective basal group.

A_{und} was reduced, while the one for A_{diff} and B spermatogonia increased in the presence of 100 ng/mL of Igf3 for 3 days (**Figure 4B**).

A Subthreshold Dose of Igf3 Becomes Active in the Presence of an Igfbp Inhibitor

In order to better understand Igf3 signaling and its modulation by Igfbps in regulating spermatogenesis, we used NBI-31772, an inhibitor of Igf-Igfbp interaction. We asked if a low concentration of Igf3 not eliciting effects by itself (25 ng/mL; see **Figure 4**), does modulate BrdU incorporation and the proportion of section area occupied by spermatogonia when NBI-31772 was present as well. Indeed, the mitotic indices of all types of spermatogonia increased after 3 days of incubation in response to Igf3 and NBI-31772 (**Figures 5A,C**); also, the proportion of section surface area occupied by type A_{diff} and B spermatogonia increased, while the one for A_{und} decreased (**Figure 5B**). In parallel experiments, we quantified the transcript levels of selected genes to complement morphological with molecular data. Considering germ cell marker transcripts, *foxa2* (a potential marker for undifferentiated spermatogonia) transcript levels decreased, whereas *dazl* [expressed by B spermatogonia and primary spermatocytes (30)] and *piwil2* [expressed by all germ cells except A_{und} and spermatozoa (29)] expression was upregulated in the presence of Igf3 and NBI-31772 (**Figure 5D**), suggesting that the total number of germ cells has increased, associated with a shift from undifferentiated spermatogonia to B spermatogonia and spermatocytes. Transcript levels of *igf1rb* were also upregulated significantly (**Figure 5D**).

Igfbps Upregulated by Hormones Support Spermatogonial Differentiation

Previous work has shown that blocking Igf binding to Igfbps by NBI-31772 during 4 days of incubation with Fsh resulted in a strong pro-differentiation signal for spermatogonia and depleted undifferentiated spermatogonia (23), suggesting that Igfbps mainly restricted Igf3 bioactivity. However, the present time course and dose response experiments also showed that Fsh and 11-KT, two hormones promoting germ cell differentiation, can upregulate two *igfbp* transcripts with a delay of at least 3 days. It therefore seems possible that these Igfbps can support Igf3 bioactivity and contribute to the pro-differentiation signaling of Fsh and 11-KT. When the Igfbp inhibitor NBI-31772 was present only during the last 4 days of incubation (**Figures 6A,B**), when *igfbp2a* and *-5b* transcripts were upregulated by Fsh (**Figures 2D,E**) or 11-KT (**Figure 3**), the mitotic indices of A_{und} and A_{diff} did not change, whereas the one for type B decreased in response to Fsh (**Figure 6A**). The section surface area occupied by A_{und} increased, while the one for type B spermatogonia decreased in the presence of Fsh in combination with NBI-31772 during the last 4 days (**Figure 6B**). These observations suggest that blocking the "late rising" Igfbps partially inhibited spermatogonial differentiation. Inversing the experimental setting (i.e., NBI-31772 was only absent during the last 4 days of incubation) showed that the mitotic index and proportion of surface area of type B spermatogonia increased (**Figures 6C,D**). Under these conditions, the "early decreasing" Igfbps were blocked from the start of Fsh exposure, and the "late rising" Igfbps were allowed to bind Igf ligands. This resulted in a stronger pro-differentiation effect of Fsh, in particular for the type B spermatogonia (**Figure 6D**).

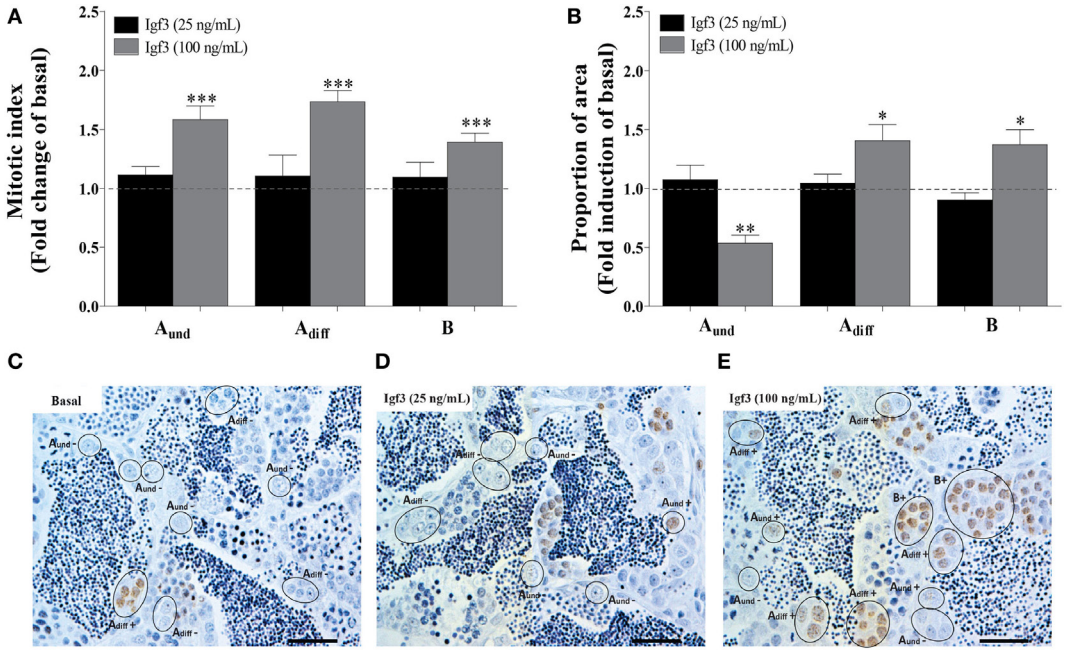

FIGURE 4 | Effect of 25 or 100 ng/mL Igf3 on spermatogonial proliferation and proportion of area after 3 days of primary testis tissue culture. **(A)** Mitotic index of type A_{und}, type A_{diff}, and type B spermatogonia in the presence of 25 ng/mL (black bars) ($n = 6$) or 100 ng/mL Igf3 (gray bars) ($n = 7$). **(B)** Proportion of section surface area occupied by cysts containing type A_{und}, type A_{diff}, or type B spermatogonia, following exposure to 25 ng/mL (black bars) ($n = 6$) or 100 ng/mL Igf3 (gray bars) ($n = 7$). **(C–E)** Immunocytochemical detection of BrdU in sections of zebrafish testis incubated under basal conditions **(C)** or in the presence of 25 ng/mL **(D)** or 100 ng/mL Igf3 **(E)** for 3 days, showing BrdU positive (+) and negative (–) A_{und}, A_{diff}, and B spermatogonia. Bars, 25 µm. Dotted lines in A and B represent the mean values of the control groups (absence of Igf3). Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) compared to the respective control group.

DISCUSSION

Regulation of the igfbp Expression in Zebrafish Testis by Fsh and Downstream Mediators

More than 90% of the circulating IGF is bound to IGFBPs (32); hence, locally produced IGFBPs seem primarily involved in modulating locally produced IGF bioactivity. The recently discovered Igf3 is prominently [e.g., zebrafish (33)], in certain species preferentially (22), expressed in gonadal tissue of adult fish (34–37). Previous studies showed that one possibility for Fsh to stimulate the differentiating proliferation of type A spermatogonia in an androgen-independent manner is to release Igf3 (9). This Fsh effect was strengthened, leading to a partial depletion of type A_{und} spermatogonia, by blocking Igfbps during a 4-day culture period, suggesting that Igfbps protected A_{und} from excessive differentiation *via* Fsh-stimulated Igf3 release (23). These recent studies highlight the importance of the Igf signaling system in modulating zebrafish spermatogenesis. Here, we report that Fsh, next to regulating *igf3* and *igfbp1a* expression, modulated the expression of four other *igfbp*s. While *igfbp1a*, *igfbp3*, and *igfbp6a* transcript levels were downregulated quickly by Fsh, or more slowly by Igf3 or 11-KT, the expression of *igfbp2a* and *igfbp5b* increased with a delay of at least 3 days in response to Fsh or 11-KT. Information on the regulation of *igfbp* transcript levels is scarce, and few studies have addressed *igfbp* expression in gonads. In rat, *Igfbp2*, *3*, and *4* transcripts have been detected in LCs and seminiferous tubules (38) and FSH reduced *Igfbp3* transcript levels in hypophysectomized rats (39). *Igfbp2-6* were found in sheep testis in association with high *Igf1* levels (40). In rainbow trout testis, the expression of *igfbp6* was upregulated by Fsh and its levels slightly decreased in the additional presence of trilostane, suggesting that both Fsh and androgens increased *igfbp6* expression in this species (41). To our knowledge, our study is the first to investigate dose and time effects of Fsh, revealing a dynamic modulation of *igfbp* transcript levels that is apparently relevant for modulating Igf3 bioactivity in zebrafish testis.

Igf and steroid hormones modulated *igfbp* expression in non-gonadal tissues in fish (42, 43). We examined if Igf3 or 11-KT, both mediators of Fsh bioactivity, were involved in the regulation of the Fsh-modulated testicular *igfbp*s. The transcript levels of *igfbp1a*, *-3*, and *-6a* were modulated in the presence of Igf3 or in response to Fsh and an Igf1r inhibitor after 7 days of incubation, suggesting that Igf3 is a downstream mediator of Fsh on *igfbp* expression and that the faster response induced by Fsh used a different mechanism than the delayed response mediated by Igf3.

However, since Fsh increases Igf3 release (9), we can expect a fast drop of *igfbp* transcript levels induced by Fsh, and a continued suppression of transcript levels mediated by Igf3. The androgen 11-KT, on the other hand, selectively increased *igfbp2a* and *-5b*

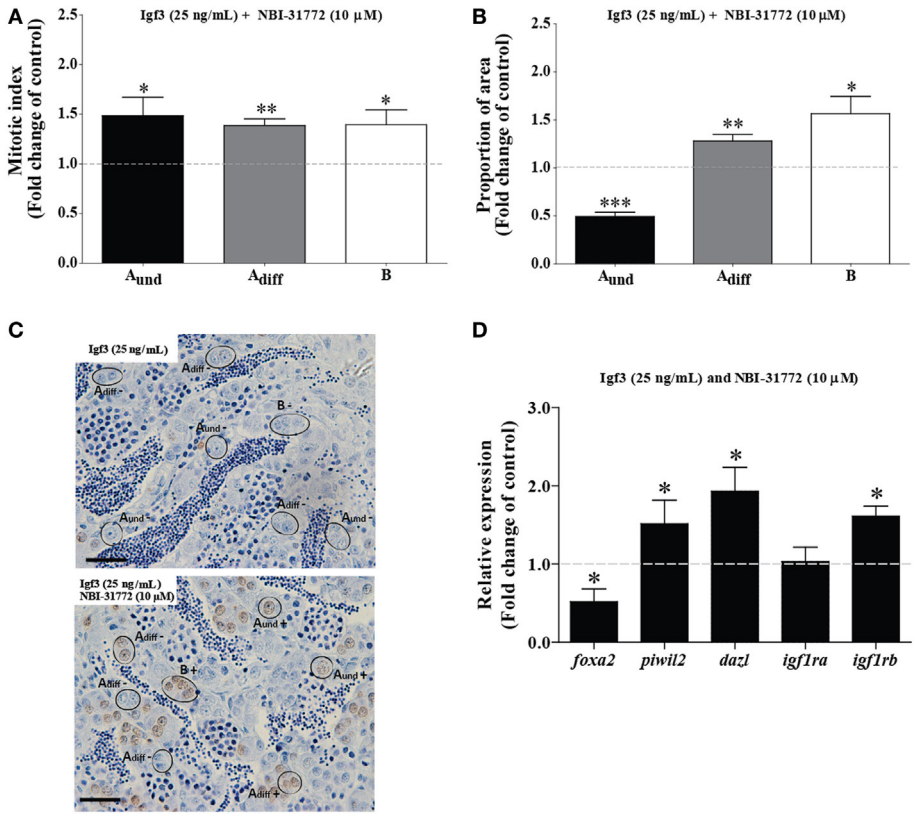

FIGURE 5 | Effect of 25 ng/mL Igf3 in the presence of an IGF-binding protein inhibitor on spermatogonial proliferation and proportion of area after 3 days of primary testis tissue culture. **(A)** Mitotic indices of type A_{und}, type A_{diff}, and type B spermatogonia in the presence of Igf3 alone (25 ng/mL) (dotted line; control condition) or in combination with 10 µM NBI-31772 (bars) ($n = 6$). **(B)** Proportion of section surface area occupied by cysts containing type A_{und}, type A_{diff}, or type B spermatogonia in the presence of Igf3 alone (25 ng/mL) (dotted line; control condition) or in combination with 10 µM NBI-31772 (bars) ($n = 6$). **(C)** Immunocytochemical detection of BrdU in sections of zebrafish testis incubated with 25 ng/mL alone (upper panel; control condition) or in combination with 10 µM NBI-31772 (lower panel) for 3 days showing BrdU positive (+) and negative (–) A_{und}, A_{diff}, and B spermatogonia. Bars, 25 µm. **(D)** Gene expression analysis in adult zebrafish testis after 3 days of tissue culture in the presence of Igf3 (25 ng/mL) (represented by a dotted line) or in combination with 10 µM NBI-31772 (bars) ($n = 7$). Results are presented as fold changes with respect to the control group (25 ng/mL Igf3). Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) between groups.

but reduced *igfbp6a* transcript levels after 7 days of incubation. Based on these results, the *igfbp*s produced in the testis can be grouped in three categories: (1) non-responding to Fsh, Igf3, or 11-KT, (2) downregulated by Fsh, Igf3 or 11-KT, and (3) upregulated by Fsh and 11-KT but not by Igf3 (**Figure 7**).

The present data not only show that *igfbp* transcript levels respond to Fsh and downstream mediators but also open the possibility that Igfbps exert differential effects on testicular Igfs, potentially restricting or supporting Igf signaling. Still, the *igfbp*s not modulated by Fsh, Igf3 or 11-KT should not be disregarded. In addition, Igfbps can act in an Igf-independent manner in mammals (44). Also in zebrafish, Igfbp3 blocked bone morphogenetic protein (Bmp) signaling by binding Bmp2a during embryonic development (45). Zebrafish Igfbp3, -5a, and -5b were localized also in the nucleus of U2O2 and HEK 293 cells (45, 46). Igfbp5a and -5b differ considering that Igfbp5b, but not -5a, shows transactivation activity in zebrafish (46). Since *igfbp5b* transcript levels are ~250-fold higher than those of *igfbp5a*, the latter also not being regulated by Fsh or 11-KT, it seems possible that Igfbp5b might be the more relevant form for potential nuclear functions in the testis. However, in general, the functional significances of nuclear Igfbps are still not well understood.

Fsh-Modulated igfbps Can Support or Inhibit Spermatogonial Differentiation in Zebrafish Testis

Previous studies have suggested that Igfbp can inhibit or enhance Igf action. While IGFBP1 and -6 generally inhibited IGF actions, IGFBP2-5 can inhibit or potentiate the IGF action, depending on the cell or tissue type, or on the physiological or experimental context (24). Due to the important role of Igf in muscle, many studies on extrahepatic IGFBP function addressed this tissue. In vascular smooth muscle cells, IGFBP2 and IGFBP4 exert an inhibitory effect on IGF1-induced DNA synthesis, while IGFBP5 potentiates the mitogenic effect of IGF1 (47, 48). Knock down of IGFBP5 impairs myogenesis and downregulated *IGF2* expression in cultured myoblast cells (49). Administration of IGFBP5 in combination with a low concentration of IGF2 restored *IGF2* expression and myogenic differentiation, whereas a

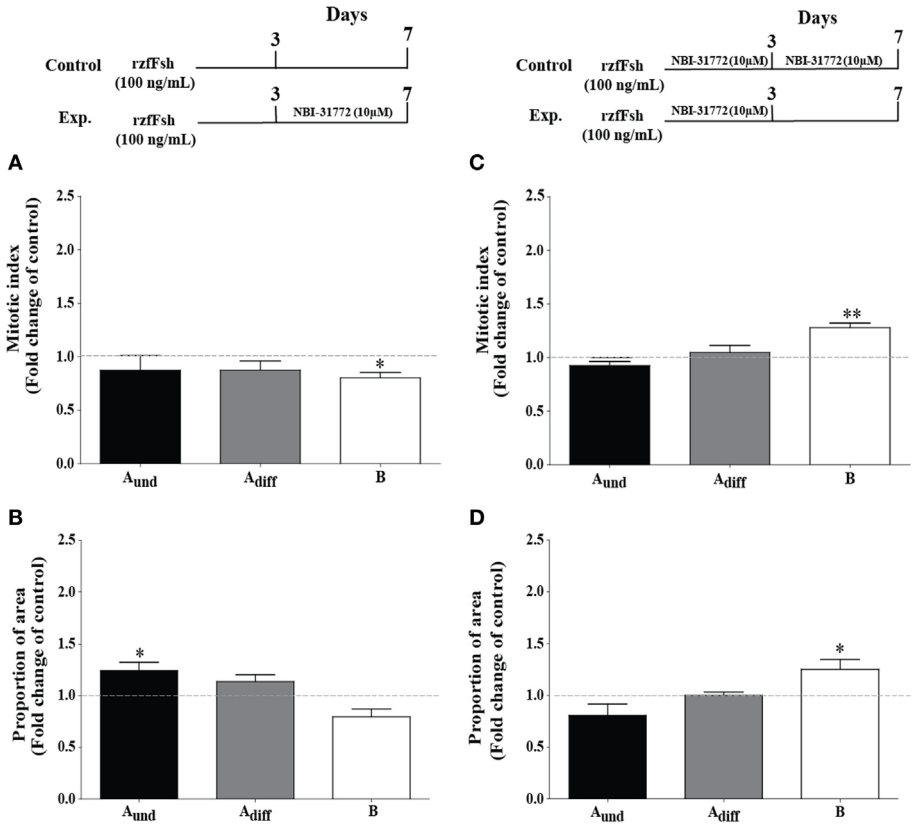

FIGURE 6 | Effect of the presence (left panel) or absence (right panel) of an IGF binding protein inhibitor on follicle-stimulating hormone (Fsh)-stimulated spermatogonial proliferation and proportion of area after 7 days of primary testis tissue culture. **(A)** Mitotic indices and **(B)** proportion of area occupied by cysts containing type A_{und}, type A_{diff}, and type B spermatogonia in the presence of Fsh (100 ng/mL) for 7 days (control group, represented by stippled line) or 3 days in the presence of Fsh (100 ng/mL) and the remaining 4 more days in the additional presence of 10 μM NBI-31772 (experimental group); a schematic representation of the experimental setup is shown in the upper left panel ($n = 6$). **(C)** Mitotic indices and **(D)** proportion of area occupied by cysts containing type A_{und}, type A_{diff}, and type B spermatogonia in the presence of Fsh (100 ng/mL) and 10 μM NBI-31772 for 7 days (control group, represented by stippled line) or 3 days under the same conditions and the remaining 4 more days in the presence of Fsh (100 ng/mL) only (experimental group); a schematic representation of the experimental setup is shown on the upper right panel ($n = 7$). The production of biologically active steroids by Fsh was blocked by trilostane (25 μg/mL) in all cases. Results are presented as fold changes with respect to the control group. Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$) between groups.

non-functional IGFBP-5 or an IGF analog that activates the IGF1R but cannot bind IGFBPs, had no or a limited effect, respectively (49). These differential Igfbp effects seem to be conserved in fish. The transition from zero growth (achieved by food restriction) to fast growth involves upregulation of *igf1*, *igfbp4*, and *-5b* in Atlantic salmon skeletal muscle (50). Moreover, primary cultures of Atlantic salmon myogenic satellite cells (stem cells in muscle) respond to amino acids and/or Igf1 by expressing high levels of *igf1* and *igf2*, and *igfbp4*, *-5a*, and *-5b* (42). Similarly, upregulation of *igfbp2*, *igfbp4*, *igfbp5*, and *igf1* was recorded during muscle growth recovery after the end of a starvation period in rainbow trout (51). A dual role for the Igfbps has also been suggested in the regulation of zebrafish muscle growth and differentiation (12). Here, we have started exploring the potentially dual role of Fsh-regulated *igfbps* on zebrafish spermatogenesis.

Stimulatory effects of Igf3 on spermatogonial proliferation have been reported previously (9). The latter study did neither examine potential effects on type B spermatogonia nor on the volume fractions occupied by the different spermatogonia. We report here that Igf3 (100 ng/mL) promoted the proliferation of all spermatogonial cell types and also increased the areas occupied by A_{diff} and B spermatogonia while reducing the one occupied by A_{und} spermatogonia. This suggests that Fsh-stimulated Igf3 release promotes differentiation of A_{und} into A_{diff} and further into B spermatogonia. Our study also reports several findings as regards Igfbp functions in testis physiology, based on examining the effects of NBI-31772 on Igf3 activity. For example, blocking Igfbps increased the biological activity of a sub-threshold dose of Igf3, and Igf3 release stimulated by either Fsh or thyroid hormone preferentially promoted differentiation of A_{und} spermatogonia when Igfbps were blocked (23). These findings suggest that Igfbps protect the pool of A_{und} spermatogonia against excessive differentiation driven by high levels of Igf3. Our data moreover indicate that this protective effect may be mediated by the three *igfbp*s rapidly down regulated by Fsh. Interestingly, the response to blocking Igfbps included upregulation of the expression of *igf1rb*. As mentioned above, zebrafish testis tissue expresses both *igf1 receptor* genes and the expression of *igf1rb* was previously

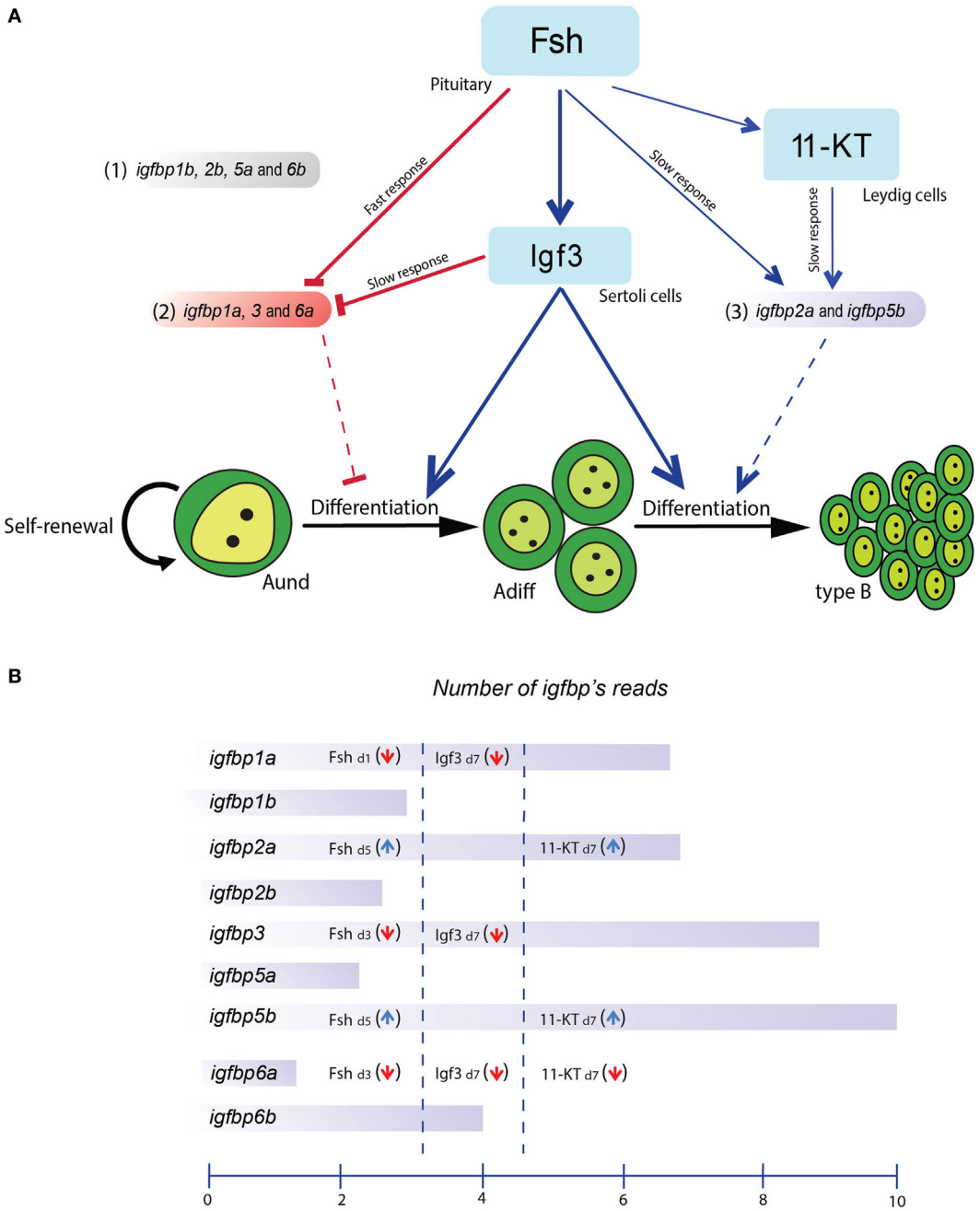

FIGURE 7 | Schematic representation of the effects of follicle-stimulating hormone (Fsh) and two downstream mediators, Igf3 and 11-KT, on IGF-binding protein (*igfbp*) transcript levels and potential roles of Igfbps in adult zebrafish testis. **(A)** Fsh rapidly reduced transcript levels of subgroup 2 (consisting of *igfbp1a*, *-3*, and *-6a*) and increased the expression/release of Igf3 and 11-ketotestosterone (11-KT). Igf3 also reduced the transcript levels of subgroup 2 members whereas those of subgroup 3 (consisting of *igfbp1b*, *-2b*, *-5a*, and *-6b*) were increased by Fsh and 11-KT. The transcript levels of subgroup 1 (consisting of *igfbp2a* and *-5b*) were not modulated in the present experiments. **(B)** Schematic representation of the *igfbp* transcript levels and their hormonal regulation in zebrafish testis tissue. The representation of the *igfbp* transcript amounts reflects read numbers from RNAseq data (Crespo, Bogerd, and Schulz, unpublished data) from 5 testes of normal adult males. The mean read numbers were transformed using the logarithm to the base 2, i.e., the scale covers a 1024-fold (2^{10}) difference in average read numbers.

upregulated under experimental conditions promoting spermatogonial proliferation (30). Altogether these results suggest that Igf3-mediated stimulation of spermatogonial proliferation and differentiation that is enhanced by blocking inhibitory Igfbps may involve upregulation of *igf1 receptor* expression.

In addition to the three *igfbp* transcripts being downregulated by Fsh, Igf3, or 11-KT, two other family members (*igfbp2a* and *-5b*) were upregulated in a delayed manner by Fsh or 11-KT. Blocking and de-blocking experiments suggested that the "late-rising" binding proteins facilitate pro-differentiation effects of Igf.

Therefore, we propose that the concept of specific Igfbps either limiting or supporting Igf bioactivity, is also valid for testis tissue, where Fsh, but also downstream mediators (Igf3 and androgens), modulate *igfbp* gene expression. While Fsh and Igf3 or Fsh and 11-KT have similar effects as regards the direction of change, they exert their effects on *igfbp* transcript levels with differences in the time course, suggesting the use of different mechanisms to modulate *igfbp* gene expression. This may allow more sustained effects. Both, the acute as well as the delayed effects of Fsh *via* the Igf signaling system affected all spermatogonial cell types (A_{und}, A_{diff}, and B), suggesting that Fsh promotes spermatogonial development in a broad sense, making use of the Igf signaling system to generate different signals over time to different germ cell generations.

Several other signaling systems are also modulated by Fsh, next to the Igf/Igfbp system (11). It will be interesting to address in future studies the differentiation of the response to Fsh in space, e.g., by examining if spermatogenic cysts containing germ cells in different stages of development respond differently to a given Fsh challenge with respect to the expression of different *igfbp* transcripts or transcript amounts. Also in context with our previous observations (23), we propose that Igfbps negatively modulate the activity of Igf3 in the presence of a comparatively weak stimulator of Igf3 release, T_3, whereas when Fsh is present, Igfbps restricting Igf3 action (Igfbp1a, Igfbp3, and Igfbp6a) are rapidly suppressed, while the availability of Igfbps supporting Igf3 action (Igfbp2a and Igfbp5b) increases after a lag phase of 3–5 days, when suppression of the inhibitory Igfbps is also supported by downstream effectors of Fsh, such as Igf3 and androgens (**Figure 7**).

In conclusion, we have shown that of the nine *igfbp*s expressed in zebrafish testis tissue, five are selectively modulated by Fsh and two Fsh downstream mediators (Igf3 and 11-KT) to promote spermatogonial differentiation. We also report that the pro-differentiation effect of Igf3 is reinforced by blocking the binding of Igf to the rapidly downregulated Igfbps, supporting the role for certain Igfbp as protecting A_{und} from excessive differentiation in response to Igf3.

ETHICS STATEMENT

All experiments carried out in this study followed the Dutch National regulations for animal care and use in experimentation, and the experimental protocols have been submitted to, and were approved by, the Utrecht University Experimental Animal Committee (2015.I.857.013 and AVD108002015333).

AUTHOR CONTRIBUTIONS

DS, HK, and DC conducted all the experiments and analyzed the data. DS, JB, and RS designed the experiments and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fendo.2017.00328/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Nuclear Insulin-Like Growth Factor Binding Protein-3 As a Biomarker in Triple-Negative Breast Cancer Xenograft Tumors: Effect of Targeted Therapy and Comparison With Chemotherapy

*Sohel M. Julovi, Janet L. Martin and Robert C. Baxter**

Kolling Institute, University of Sydney, Royal North Shore Hospital, St. Leonards, Sydney, NSW, Australia

Triple-negative breast cancer (TNBC) typically has a worse outcome than other breast cancer subtypes, in part owing to a lack of approved therapeutic targets or prognostic markers. We have previously described an oncogenic pathway in basal-like TNBC cells, initiated by insulin-like growth factor binding protein-3 (IGFBP-3), in which the epidermal growth factor receptor (EGFR) is transactivated by sphingosine-1-phosphate (S1P) resulting from sphingosine kinase (SphK)-1 activation. Oncogenic IGFBP-3 signaling can be targeted by combination treatment with the S1P receptor modulator and SphK inhibitor, fingolimod, and the EGFR kinase inhibitor, gefitinib (F + G). However, the interaction of this treatment with chemotherapy has not been documented. Since we observed nuclear localization of IGFBP-3 in some TNBC tumors, this study aimed to evaluate the prognostic significance of nuclear IGFBP-3 in pre-clinical models of basal-like TNBC treated with F + G and doxorubicin. Orthotopic xenograft tumors were grown in nude mice from the human basal-like TNBC cell lines MDA-MB-468 and HCC1806, and were treated with gefitinib, 25 mg/Kg, plus fingolimod, 5 mg/Kg, 3-times weekly. In some studies, doxorubicin was also administered once weekly for 6 weeks. Tumor tissue proteins were quantitated by immunohistochemistry (IHC). Interaction between doxorubicin and F + G was also studied in proliferation assays *in vitro*. In both tumor models, tissue staining for IGFBP-3 was predominantly nuclear. Combination of F + G significantly enhanced mouse survival, decreased nuclear IGFBP-3 and Ki67 staining, and increased apoptosis (cleaved caspase-3) staining. Kaplan–Meier survival analysis showed that a high tumor IGFBP-3 IHC score (>median), like a high Ki67 score, was significantly associated with shorter survival time, whereas a high apoptosis score was associated with prolonged survival. Studied *in vitro* in both cell lines, low-dose doxorubicin that had little effect alone, strongly enhanced the cytostatic effect of low-dose F + G combination. However, in both *in vivo* models, doxorubicin at maximum-tolerated dose neither inhibited tumor growth when administered alone, nor enhanced the significant inhibitory effect of F + G. We conclude that doxorubicin may not add benefit to the inhibitory effect of F + G unless its dose-limiting toxicity can be overcome. Nuclear IGFBP-3 appears to have potential as a prognostic marker in TNBC and could be evaluated for clinical utility.

Keywords: insulin-like growth factor binding protein-3, basal-like, triple-negative, breast cancer, targeted therapy, chemotherapy

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Briony Forbes,
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Leibniz-Institut für Nutztierbiologie (FBN), Germany
Wendie Cohick,
Rutgers University, The State University of New Jersey, United States

***Correspondence:**
Robert C. Baxter
robert.baxter@sydney.edu.au

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INTRODUCTION

Breast cancer is recognized as a heterogeneous disease, commonly classified into subtypes on the basis of phenotypic and/or molecular characteristics (1, 2). Of the breast cancers with absent or very low estrogen receptor (ER) and progesterone receptor (PR), and without overexpression of the human epidermal growth factor receptor-2, known as triple-negative breast cancers (TNBC), about 80% have a basal-like profile, in terms of both gene expression profile (3) and the display of established basal histology markers, such as cytokeratin 5/6 and the epidermal growth factor receptor (EGFR) (4). Triple-negative breast cell lines that display a basal-like phenotype and have high EGFR expression (5) include MDA-MB-468 (classified as basal-like 1) and HCC1806 (classified as basal-like 2) (3).

There are no established molecular targets or prognostic markers for TNBC, which typically has a worse outcome than other breast cancer subtypes (6). Immunotherapeutic approaches have recently shown some durable responses, and the development of tumor-specific neoantigens and other new therapeutic targets offers hope for the future (7). Our recent pre-clinical studies have revealed an unexpected immune modulator in a murine TNBC-like model, with the demonstration that mammary tumors in mice null for insulin-like growth factor binding protein-3 (IGFBP-3) grow 50% smaller than those in wild-type mice and show increased accumulation of $CD8^+$ lymphocytes (8). In wild-type mice, tumor IGFBP-3 gene expression was positively associated with tumor weight (8), consistent with some clinical studies showing that high IGFBP-3 abundance in breast tumor tissue is associated with high tumor grade (9) and poor prognosis (10, 11).

Insulin-like growth factor binding protein-3 is also known to drive an oncogenic pathway in human TNBC cell lines involving activation of the receptor tyrosine kinase, EGFR, and the lipid kinase, sphingosine kinase (SphK) (12, 13). We have shown that inhibitors of these two IGFBP-3-activated kinases, administered in combination, act synergistically to significantly inhibit TNBC cell growth *in vitro* and in xenograft tumors (5, 13). However, the importance of high tumor IGFBP-3 levels in the progression of many cancer types remains unclear because there are some cancers in which *IGFBP3* appears to act as a tumor suppressor gene, with *low* IGFBP-3 levels associated with poor patient outcome (14).

Insulin-like growth factor binding protein-3, a secreted glycoprotein found in both the circulation and the pericellular/intracellular environment, is known to translocate to the cell nucleus in some conditions, and its interaction with nuclear ligands, influencing both gene transcription and DNA damage repair, has been documented (15). Intriguingly, while nuclear interactions of IGFBP-3 have been associated with its induction of apoptotic death in prostate cancer cell lines (16, 17), a clinical study showed that high nuclear staining of IGFBP-3 in prostate cancer tissue was prognostic for earlier disease recurrence (18).

In breast cancer, the significance of nuclear IGFBP-3, both functionally and as a biomarker, is not fully understood. The primary goal of this study was to evaluate the prognostic significance of nuclear IGFBP-3 using pre-clinical models of basal-like TNBC treated with EGFR and SphK inhibitors. We also examined the relationship between nuclear IGFBP-3 and indicators of tumor proliferation and apoptosis. Our secondary goal was to compare combination kinase inhibition with the chemotherapeutic agent doxorubicin *in vitro* and *in vivo*, and to evaluate the effect of co-administering the targeted drugs with chemotherapy.

MATERIALS AND METHODS

Reagents

The EGFR kinase inhibitor gefitinib (ZD1838, Iressa), the SphK inhibitor and sphingosine-1-phosphate receptor modulator, fingolimod (FTY720, Gilenya), and the chemotherapy drug doxorubicin (adriamycin), were purchased from MedChem Express, Princeton, NJ USA.

Human TNBC Cell Lines

The human basal-like TNBC cell lines, MDA-MB-468 and HCC1806, were obtained from ATCC, Manassas, VA, USA and maintained under standard conditions in RPMI 1640 medium containing 5% FBS and 10 μg/mL bovine insulin. Stocks of these cells were frozen within 1 month of purchase, and fresh cultures for experimental use were established every 2–3 months. All cell lines were negative on mycoplasma testing.

Measurement of Cell Proliferation

Cells (4×10^3/well for HCC1806; 8×10^3/well for MDA-MB-468) were dispensed into 96-well plates in complete medium and incubated overnight before changing to fresh medium containing 5% FBS and inhibitors. The inhibitors tested were the targeted therapies fingolimod (1 μM) plus gefitinib (1 μM), doxorubicin (10 nM), or the targeted therapies plus doxorubicin. Plates were transferred to the IncuCyte live-cell imager (Essen BioScience, Ann Arbor, MI, USA), and incubated for 120 h, with images collected every 3 h.

Murine Models of Human TNBC

All animal procedures were approved by the institutional Animal Ethics Committee (Protocols RESP/14/280 and RESP/15/103), and followed recently described protocols (5). In brief, xenograft tumors were grown from human basal-like TNBC cell lines, implanted into the fourth left mammary fat pad of 8-week-old female BALB/c nude (immune-deficient) mice. For both MDA-MB-468 and HCC1806 tumors, 5×10^6 cells were implanted in a volume of 150 μL, which included 50 μL of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Tumors were allowed to develop until they reached a volume of 100 mm^3, as determined by Vernier caliper measurement. For each cell type, randomized groups of tumor-bearing mice were then started on 3-times weekly i.p. injections of a combination of fingolimod (5 mg/Kg) plus gefitinib (25 mg/Kg), or vehicle, for up to 12 weeks. We previously showed that this drug combination substantially increased survival time in mice bearing both of these tumor types (5). For both tumor models, some studies included two additional groups of mice bearing ~100 mm^3 tumors, treated with doxorubicin, 2 mg/Kg i.p., once weekly for 6 weeks, either alone or together with the fingolimod-gefitinib combination as described above (3-times weekly for up to 12 weeks). A higher doxorubicin dose (4 mg/Kg), or administration for more than 6 weeks, both

caused toxicity (weight loss and/or death) that exceeded our ethical guidelines. Each mouse was terminated when its tumor reached the ethically approved endpoint of 1,000 mm^3, otherwise 12 weeks after cell implantation.

Immunohistochemistry (IHC) and Western Blotting

Immunohistochemistry staining for IGFBP-3, the proliferation marker Ki67, and the apoptosis marker cleaved caspase-3 (CCasp-3), was carried out as described previously (5). In brief, 4 µm sections of formalin-fixed paraffin-embedded tumor samples were incubated with antibodies against Ki67 (ab66155, 1:600, Abcam, Melbourne, VIC, Australia), CCasp-3 (Asp175) (#9661, 1:200, Cell Signaling), IGFBP-3 (in-house antiserum R-100, 1:2,000), and isotyped-matched IgG antibodies or rabbit serum alone, with nuclear counterstaining using Mayer's hematoxylin and Scott's bluing solution. For all three markers, the percentage of positive cells was scored for each tumor following recommendations for Ki67 analysis (19), calculated from the five highest staining areas at 10× magnification for each slide. Nuclear IGFBP-3 immunoreactivity was detected by western blotting of solubilized nuclei isolated from cell cultures, after immunoprecipitation using R-100 anti-IGFBP-3 Fab fragment conjugated to agarose beads, as previously described (20).

Statistical Analysis

Kaplan–Meier survival analyses were undertaken using SPSS v.22 for Mac (IBM Corp., Armonk, New York, NY, USA). IHC scores were coded as low (≤the median value) or high (>the median value). Mice with MDA-MB-468 tumors below the ethical endpoint of 1,000 mm^3 at day 82 after cell implantation were regarded as survivors. For HCC1806 tumors, all mice had reached the ethical endpoint before 12 weeks (i.e., there were no survivors). Linear and non-linear (exponential) correlations between IHC scores were fitted using Deltagraph v.7 (Red Rock Software, Salt Lake City, UT, USA) with *P* values calculated by SPSS.

RESULTS

Nuclear IGFBP-3 Is Associated With Poor Outcome in TNBC Xenografts

As recently reported (5), the combination of SphK inhibition with fingolimod and EGFR kinase inhibition with gefitinib (F + G) was significantly inhibitory to the proliferation of both HCC1806 and MDA-MB-468 basal-like TNBC tumors. For HCC1806 tumors, mean mouse survival (measured for ethical reasons as the time for tumors to reach 1,000 mm^3) was increased 87% by treatment compared to untreated controls, from 18.5 ± 2.8 to 34.6 ± 3.5 days (mean ± SEM, $P = 0.001$), while for MDA-MB-468 tumors, no tumors in treated mice had reached 1,000 mm^3 after 82 days, compared to 8/10 tumors in control mice ($P < 0.001$). **Figures 1A,B** shows Kaplan–Meier survival curves for combination-treated vs. control-treated mice for HCC1806 and MDA-MB-468 tumors, respectively.

To evaluate the relationship between tumor IGFBP-3 immunohistochemical staining and mouse survival, tumor tissues were stained for IGFBP-3 as well as the proliferation marker, Ki67 and the apoptosis marker, CCasp-3. Representative examples of staining patterns have been reported previously (5). Notably, IGFBP-3 staining, using a high-titer, primate-specific antiserum that does not detect murine IGFBP-3, was predominantly nuclear in both tumor types, as shown in **Figures 2A,B**. For both cell types,

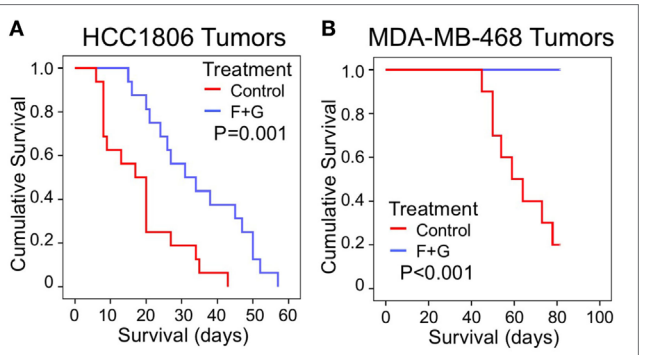

FIGURE 1 | The effect of combination (fingolimod + gefitinib) treatment on the survival of nude mice bearing human xenograft TNBC tumors. **(A,B)** Kaplan–Meier survival curves compare the effect of control (no treatment) or combination fingolimod + gefitinib (F + G) therapy on survival of mice bearing HCC1806 **(A)** or MDA-MB-468 **(B)** xenograft tumors. For ethical reasons mouse survival is defined as tumor size below 1,000 mm^3. For HCC1806, control: $n = 16$; combination: $n = 16$. For MDA-MB-468, control: $n = 10$; combination: $n = 10$.

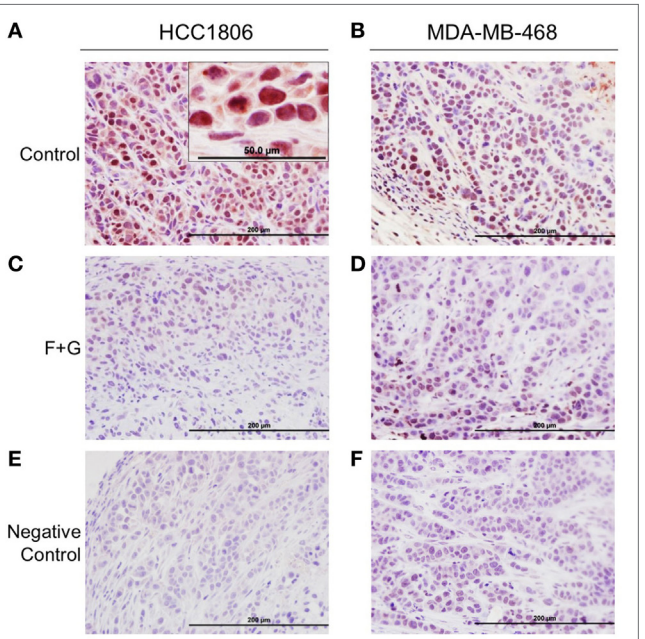

FIGURE 2 | Insulin-like growth factor binding protein-3 (IGFBP-3) immunohistochemistry of HCC1806 and MDA-MB-468 tumors. **(A,B)** Representative IGFBP-3 staining of each tumor type from control mice, showing strong nuclear localization. **(C,D)** Representative IGFBP-3 staining of each tumor type from mice treated with fingolimod + gefitinib (F + G). **(E,F)** Negative control staining—non-immune rabbit serum at the same dilution as IGFBP-3 antiserum. Bar = 200 µm. Inset: higher magnification of HCC1806 section from control tumor. Bar = 50 µm.

nuclear IGFBP-3 IHC staining in tumor sections was greatly decreased in combination-treated mice compared to controls (**Figures 2C,D**). **Figures 2E,F** shows negative staining controls for both tumor types.

Nuclear IGFBP-3, examined by western blotting of nuclear extracts from both HCC1806 and MDA-MB-468 cell lines, typically showed diffuse bands of approximately 40 kDa as well as immunoreactivity around 35 kDa which may represent partially proteolyzed or underglycosylated IGFBP-3. The origin of diffuse IGFBP-3 immunoreactivity around 55 kDa is unknown (**Figures 3A,B**; left panels). **Figures 3A,B** (right panels) show mean IHC scores for IGFBP-3 from control and combination-treated mice. For both HCC1806 (A) and MDA-MB-468 (B) tumors, IGFBP-3 staining was significantly decreased. Kaplan–Meier survival analysis indicated a strong association between nuclear IGFBP-3 and mouse survival, IGFBP-3 IHC scores above the median value being associated with poor survival for both HCC1806 tumors ($P = 0.009$; **Figure 3C**) and MDA-MB-468 tumors ($P = 0.001$; **Figure 3D**).

To assess whether decreased nuclear IGFBP-3 was linked to changes in proliferation or apoptosis, we examined the effect of combination F + G treatment on Ki67 and CCasp-3. As previously reported (5), the combination treatment significantly decreased cell proliferation, as indicated by Ki67 staining (**Figures 4A–D**). Nuclear IGFBP-3 staining was positively correlated with nuclear Ki67, more strongly in HCC1806 tumors (**Figure 4E**) than MDA-MB-468 tumors (**Figure 4F**). This was reflected in the Kaplan–Meier survival curves, in which high Ki67 levels (above the median) were strongly associated with poor survival for HCC1806 tumors (**Figure 4G**), but not with MDA-MB-468 tumors (**Figure 4H**).

Apoptosis, as indicated by caspase-3 cleavage, was also strongly induced by combination F + G treatment (**Figures 5A–D**). The

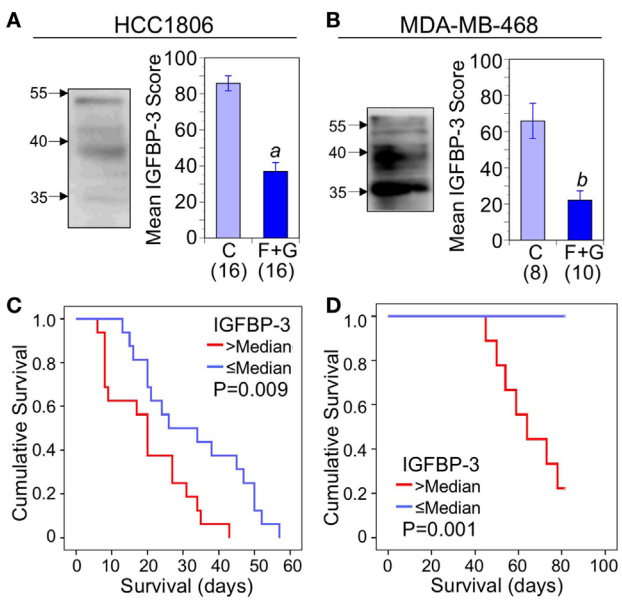

FIGURE 3 | The relationship between tumor nuclear insulin-like growth factor binding protein-3 (IGFBP-3) and mouse survival. **(A,B)** Left: western blot of immunoreactive IGFBP-3 after immunoprecipitation from solubilized nuclei isolated from cultured cells; molecular weight markers in kDa are indicated by arrows. Right: quantitation of immunohistochemistry (IHC) scores for nuclear IGFBP-3 in control mice and mice treated with fingolimod + gefitinib (F + G). For HCC1806 **(A)** and MDA-MB-468 **(B)** tumors, mean scores ± SEM are shown, numbers of mice in parentheses. Comparison with controls (2-sided *t*-test): (*a*) $P < 0.001$; (*b*) $P = 0.001$. **(C,D)** Kaplan–Meier survival curves compare the effect of tumor nuclear IGFBP-3 IHC scores > or ≤ the median value on survival of mice bearing HCC1806 **(C)** or MDA-MB-468 **(D)** xenograft tumors. For HCC1806, *n* (>median) = 16; *n* (≤median) = 16. For MDA-MB-468, *n* (>median) = 9; *n* (≤median) = 9.

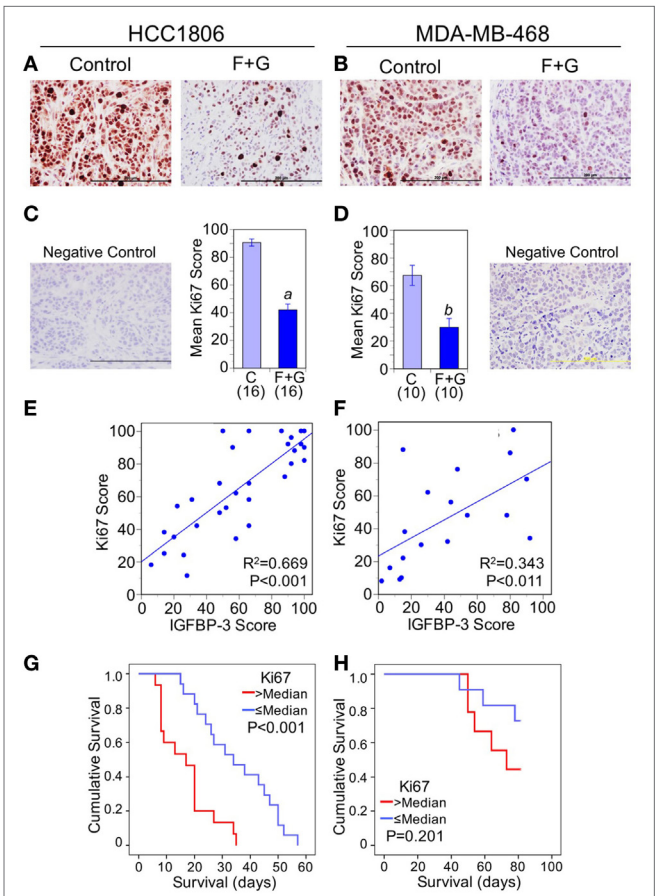

FIGURE 4 | The relationship between tumor Ki67 and mouse survival. **(A,B)** Representative Ki67 staining of each tumor type from control mice and mice treated with fingolimod + gefitinib (F + G). **(C,D)** Quantitation of immunohistochemistry (IHC) scores for Ki67 in control and combination-treated mice. For HCC1806 **(C)** and MDA-MB-468 **(D)** tumors, mean scores ± SEM are shown, numbers of mice in parentheses. Comparison with controls (2-sided *t*-test): (*a*) $P < 0.001$; (*b*) $P = 0.001$. Also shown are IgG isotype negative staining controls for both tumor types. **(E,F)** Nuclear insulin-like growth factor binding protein-3 scores are positively correlated with Ki67 scores for both HCC1806 **(E)** and MDA-MB-468 **(F)** tumors. Linear regression lines are shown with R^2 and *P* values indicated. **(G,H)** Kaplan–Meier survival curves compare the effect of tumor Ki67 IHC scores > or ≤ the median value on survival of mice bearing HCC1806 **(G)** or MDA-MB-468 **(H)** xenograft tumors. For ethical reasons mouse survival is defined as tumor size below 1,000 mm^3. For HCC1806, *n* (>median) = 15; *n* (≤median) = 17. For MDA-MB-468, *n* (>median) = 9; *n* (≤median) = 11.

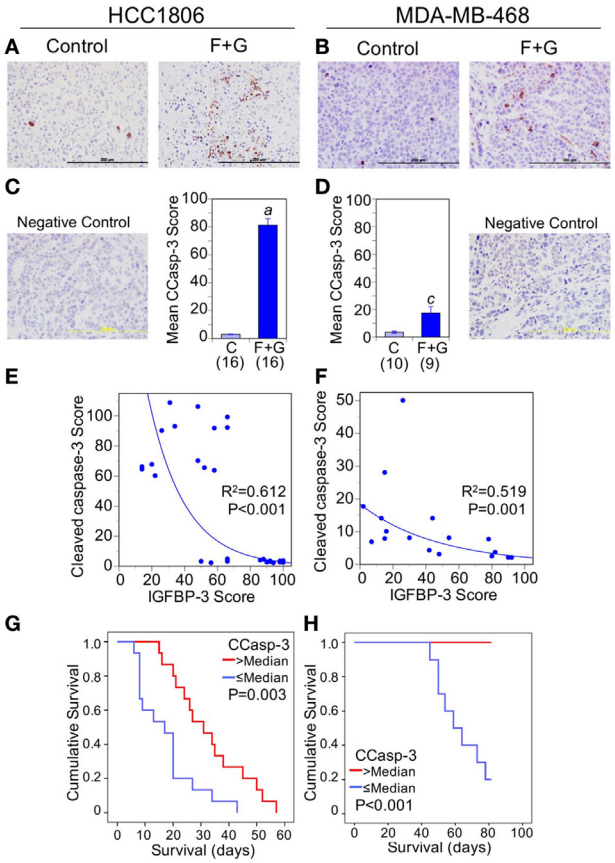

FIGURE 5 | The relationship between tumor cleaved caspase-3 (CCasp-3) and mouse survival. **(A,B)** Representative CCasp-3 staining of each tumor type from control mice and mice treated with fingolimod + gefitinib (F + G). **(C,D)** Quantitation of immunohistochemistry (IHC) scores for CCasp-3 in control and combination-treated mice. For HCC1806 **(C)** and MDA-MB-468 **(D)** tumors, mean scores ± SEM are shown, numbers of mice in parentheses. Comparison with controls (2-sided *t*-test): (*a*) $P < 0.001$; (*c*) $P = 0.006$. Also shown are IgG isotype negative staining controls for both tumor types. **(E,F)** Nuclear insulin-like growth factor binding protein-3 scores are negatively correlated with CCasp-3 scores for both HCC1806 **(E)** and MDA-MB-468 **(F)** tumors. Exponential regression lines are shown with R^2 and P values indicated. **(G,H)** Kaplan–Meier survival curves compare the effect of tumor CCasp-3 IHC scores > or ≤ the median value on survival of mice bearing HCC1806 **(G)** or MDA-MB-468 **(H)** xenograft tumors. For ethical reasons mouse survival is defined as tumor size below 1,000 mm^3. For HCC1806, *n* (>median) = 15; *n* (≤median) = 15. For MDA-MB-468, *n* (>median) = 9; *n* (≤median) = 10.

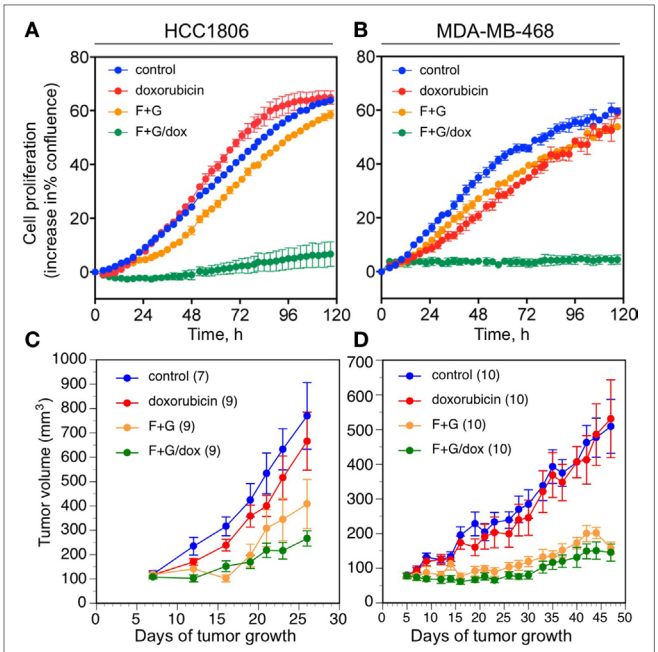

FIGURE 6 | The effect of kinase inhibitors and doxorubicin on the proliferation of basal-like triple-negative breast cancer cells. **(A,B)** Cell culture studies using the IncuCyte live-cell imager. **(A)** HCC1806 cells; **(B)** MDA-MB-468 cells. Data are expressed as the change in percent confluence, corrected for confluence at time zero; mean values ± SEM at each time point, from triplicate wells for each treatment. Similar results were obtained in three replicate experiments for each cell line. Treatments are: control (blue); doxorubicin, 10 nM (red); fingolimod, 1 μM + gefitinib, 1 μM (F + G; orange); and F + G plus doxorubicin (green). **(C,D)** *In vivo* studies of orthotopic xenograft tumor growth in nude mice; mean values ± SEM, numbers in parentheses. **(C)** HCC1806 tumors. Repeated measures ANOVA up to day 26: overall effect of treatment, $P = 0.003$. *Post hoc* Tukey's test: F + G vs. control, $P = 0.036$; F + G/dox vs. control, $P = 0.004$; F + G/dox vs. dox, $P = 0.045$. **(D)** MDA-MB-468 tumors. Repeated measures ANOVA up to day 47: overall effect of treatment, $P < 0.001$. *Post hoc* Tukey's test: F + G vs. control, $P = 0.001$; F + G/dox vs. control, $P < 0.001$; F + G/dox vs. dox, $P = 0.001$. Treatments are: control (blue); doxorubicin, 2 mg/Kg (red); fingolimod, 5 mg/Kg + gefitinib, 25 mg/Kg (F + G; orange); and F + G plus doxorubicin (green). See Section "Materials and Methods" for further details.

increased apoptosis was particularly notable in HCC1806 tumors, in which CCasp-3 staining increased over 25-fold. Nuclear IGFBP-3 was inversely correlated with CCasp-3 staining for both tumor types (**Figures 5E,F**), and survival analysis showed that CCasp-3 staining above the median level was significantly associated with improved mouse survival (**Figures 5G,H**). Collectively these data suggest that, in these xenograft models of human basal-like TNBC, a high nuclear IGFBP-3 level may be a poor prognostic feature, associated with high tumor proliferation and decreased apoptosis.

Comparison Between Combination Targeted Therapy and Chemotherapy

In the absence of any approved targeted therapies for TNBC, cytotoxic chemotherapy is regarded as the front-line treatment (6). We, therefore, evaluated the efficacy of F + G therapy in combination with the widely used anthracycline chemotherapy drug, doxorubicin. We previously established concentrations of the two kinase inhibitors that showed a strongly synergistic cytostatic effect *in vitro* in various TNBC cell lines (5). In this study, their concentrations were lowered to ensure that the drug combination, when used alone, would have minimal cytostatic effect, in order to examine the additional effect of doxorubicin. **Figures 6A,B** shows that 1 μM fingolimod + 1 μM gefitinib had little cytostatic effect, measured by IncuCyte real-time imaging over 120 h, in either HCC1806 or MDA-MB-468 cell

cultures. Similarly, doxorubicin was minimally cytostatic at 10 nM. However, treatment with doxorubicin plus the targeted drug combination resulted in almost complete inhibition of cell proliferation in both cell lines, suggesting a synergistic effect between the kinase inhibitors with the chemotherapeutic agent (**Figures 6A,B**).

To evaluate this effect *in vivo*, the two xenograft models of TNBC were treated with the combination F + G therapy plus doxorubicin. Doxorubicin was administered at the maximum tolerated dose (MTD) of 2 mg/Kg i.p. once weekly for 6 weeks, since a higher dose (4 mg/Kg) or longer duration resulted in drug-related toxicity in our BALB/c nude mouse model (data not shown). As shown in **Figures 6C,D**, doxorubicin at the highest tolerable dose of 2 mg/Kg had only a small inhibitory effect on the growth of either tumor type *in vivo*, not statistically significant, whereas the targeted combination was significantly inhibitory in both models as previously reported (5). Notably, and in contrast to the *in vitro* studies, treatment with both doxorubicin and the F + G combination did not significantly slow down the tumor growth rate in either model, beyond the effect seen with F + G alone. Immunohistochemical analysis of tumors for IGFBP-3, Ki67, and CCasp-3 showed that, at the doses tested in these experiments, doxorubicin had no significant effect either administered alone or in combination with F + G treatment, in either HCC1806 (**Figures 7A–C**) or MDA-MB-468 (**Figures 7D–F**) tumors.

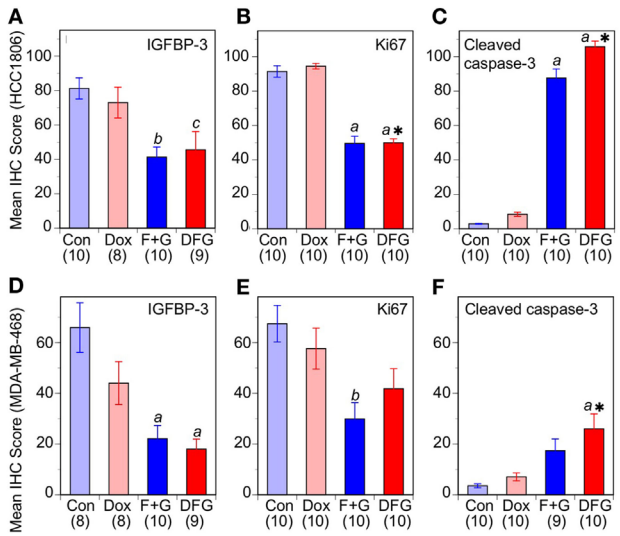

FIGURE 7 | Response of basal-like triple-negative breast cancer tumors to doxorubicin and combination therapy. Immunohistochemistry scores for nuclear insulin-like growth factor binding protein-3, Ki67, and cleaved caspase-3 in mice with HCC1806 tumors **(A–C)** or MDA-MB-468 tumors **(D–F)** treated with vehicle alone (Con), doxorubicin (Dox), fingolimod + gefitinib (F + G), or Dox plus F + G (DFG). For both tumor types, data are mean values ± SEM, numbers in parentheses. Comparison with controls (one-way ANOVA, *post hoc* Tukey's test): (*a*) $p < 0.001$; (*b*) $p < 0.005$; (*c*) $p < 0.02$. Comparison with Dox: *, $P < 0.01$.

DISCUSSION

IGFBP-3 As a Therapeutic Target and Biomarker

The first goal of this study was to evaluate tumor IGFBP-3 abundance for potential value as a prognostic indicator in basal-like triple-negative breast tumors, using as model systems, two basal-like TNBC cell lines grown as orthotopic xenograft tumors in nude mice. Our studies over several years have shown that IGFBP-3 stimulates an oncogenic pathway in which the activation of SphK 1, leading to increased sphingosine-1-phosphate generation, results in the transactivation of EGFR which drives tumor proliferation (12, 13). The importance of SphK/sphingosine-1-phosphate in oncogenesis, and its potential as a therapeutic target in breast and other cancers, has been studied extensively (21, 22), and a recent study in a metastatic TNBC cell model (23) has confirmed our earlier observations that SphK inhibition decreases EGFR-dependent cell proliferation and survival.

Although IGFBP-3 itself, which is abundant in the circulation (14) and in ER-negative breast cancer tissue (24), is a challenging therapeutic target, we showed that IGFBP-3-dependent oncogenic signaling can be successfully targeted by dual therapy with an EGFR kinase inhibitor and a SphK inhibitor, giving a highly synergistic inhibitory effect (5, 13). This may offer benefits over EGFR-directed therapies used alone, since both EGFR kinase inhibitors and monoclonal antibodies have failed to demonstrate consistent results in the treatment of TNBC (25). Since we found, using cell models, that IGFBP-3 downregulation attenuated the synergism between the EGFR and SphK inhibitors, we proposed that tumor IGFBP-3 levels might act as a biomarker for the efficacy of the combination therapy (5).

As a key regulator of the bioavailability of circulating IGF-1 and IGF-2, IGFBP-3 has major effects outside the cell (14), but its intracellular (including intranuclear) actions are also well documented (15, 26). IGFBP-3 has been identified in the cell nucleus in a variety of healthy and cancerous human tissues, including non-malignant colon (27), lung (28), articular cartilage (29), and bone (30), as well as malignant colon (27), liver (31), Barrett's tissue associated with esophageal cancer (32), and prostate (18) [including reactive stroma (33)] tissue.

Nuclear and Cytoplasmic IGFBP-3 in Cancer

Although *IGFBP3* is epigenetically suppressed in some cancers, with an apparent tumor suppressor role (34, 35), in other cancer types it shows high expression in association with high tumor grade and/or poor patient survival, although often the IGFBP-3 protein is located predominantly in the cytoplasm. For example, IGFBP-3 is highly overexpressed in the cytoplasm of high-grade clear cell renal cell carcinomas compared to low-grade or normal kidney (36). A similar finding was reported in brain tumors, with highest IGFBP-3 levels by both gene expression and IHC found in glioblastoma (grade IV) compared to normal brain and astrocytomas of lower grade (37). High IGFBP-3 staining in glioblastoma patients was significantly associated

with shorter survival, and was described as "mostly confined to the cytoplasm" although some positive nuclei were also evident (37). In pancreatic endocrine (i.e., islet cell) neoplasms (38) and melanoma (39), high tumor IGFBP-3 levels by IHC are associated with increased metastasis, but again the staining is predominantly cytoplasmic. In head and neck squamous cell carcinoma, high IGFBP-3 staining was associated with higher clinical stage (40) and decreased time to progression (41), and was similarly mainly seen in the cytoplasm. In an IHC study of breast cancer tissue sections, in which high IGFBP-3 staining showed a trend (non-significant) of association with worse patient survival, "no clear evidence" of nuclear IGFBP-3 staining was reported (42).

In contrast, in a study of hepatocellular carcinoma Yan et al. (31) scored nuclear IGFBP-3 staining, finding an association between *low* nuclear IGFBP-3 and several markers of poor prognosis. Finally, Seligson et al. (18) observed increased IGFBP-3 staining in both the cytoplasm and nucleus of prostate cancers compared to benign tissue, but only the nuclear staining was significantly prognostic for cancer recurrence, being more highly predictive than baseline PSA or any other pathological marker. Therefore, both the subcellular localization and prognostic value of tumor IGFBP-3 staining appears to be highly dependent on tumor type.

We recently reported that human basal-like TNBC cells growing as xenograft tumors showed predominantly nuclear IGFBP-3 staining (5). We have now extended this observation by showing in two xenograft models that high levels of nuclear IGFBP-3 (i.e., above the median value), measured by IHC, are significantly associated with shorter mouse survival time. Mature human IGFBP-3 is a glycoprotein of 264 amino acids with a core molecular weight of 28.7 kDa, increased to approximately 40 kDa by *N*-glycosylation on residues 89, 109, and 172. Variable glycosylation leads to the appearance of diffuse bands around 40 kDa when analyzed by SDS-PAGE (43–45). The IGFBP-3 structure may be viewed as three domains of approximately equal size: cysteine-rich amino- and carboxyterminal domains that bind IGF-1 and IGF-2 cooperatively (46), and a central or linker domain that is the major site for posttranslational modification. Limited proteolysis in the central domain may be involved in the release of IGFs from the high-affinity binding pocket, and accounts for frequently observed IGFBP-3 fragments in the circulation and tissues (26, 45).

Although nuclei were not isolated from xenograft tumors in this study, nuclear extracts of the corresponding cell lines showed IGFBP-3 immunoreactive bands of approximately 40 kDa that may represent glycosylated isoforms of the full-length protein, as well as larger (~55 kDa) and smaller (~35 kDa) bands that may represent glycosylation and/or proteolysis variants. Our studies do not indicate which of these bands are downregulated by drug treatment, or whether the size distribution of the variants changes on treatment. Nuclear IGFBP-3 IHC staining correlated positively with nuclear Ki67 and inversely with CCasp-3, consistent with the role of IGFBP-3 in promoting proliferation and survival in basal-like TNBC cells. If extrapolated to TNBC tumors in patients, our findings suggest that a high nuclear IGFBP-3 score by IHC, complementing a high Ki67 score and a low CCasp-3 score, may be prognostic for poor patient survival in women with TNBC. Further refinement of such a prognostic test might be possible with more detailed analysis of the structure and size distribution of IGFBP-3 variants in tumors. Since treatment with combination fingolimod and gefitinib significantly prolonged survival in our mouse models, this combination therapy might be considered for evaluation in clinical trials.

Effect of Doxorubicin in TNBC Models

Anthracycline drugs (e.g., doxorubicin and epirubicin) are a standard component of adjuvant chemotherapy for women with TNBC (47). The second goal of this study was to evaluate whether the SphK/EGFR inhibitor combination (F + G) might act cooperatively with doxorubicin as an effective treatment for TNBC. Since doxorubicin has well-documented toxicity toward the brain, liver, kidney, and notably the heart (48), lowering the dose by the addition of a targeted therapy might be clinically advantageous. In this pre-clinical study, the dose of doxorubicin, 2 mg/Kg i.p. weekly for 6 weeks was used to avoid toxicity seen at higher or more prolonged dosing schedules in the BALB/c nude mice. In the initial cell growth studies *in vitro*, a strong combination effect between F + G and doxorubicin was observed, in which sub-cytostatic doses of each caused almost complete cytostatsis when combined. However, this trend was not recapitulated in two xenograft tumor models in which doxorubicin, administered alone or in combination with the kinase inhibitors, showed no significant effect on tumor volume or on cellular markers of proliferation and apoptosis. Similarly, IGFBP-3 staining did not show a differential response to doxorubicin in our models. Although *IGFBP3* is activated by wild-type p53 (49), and, therefore, expected to be induced by chemotherapy, we have previously shown that in some TNBC cell lines with gain-of-function p53 mutations, IGFBP-3 is paradoxically downregulated by DNA-damaging chemotherapeutic drugs (50). Effective doxorubicin activity might, therefore, be expected to downregulate IGFBP-3 (as seen for the effective combination kinase inhibitor therapy), but this was not observed in our study.

While it is unclear why the *in vivo* experiments failed to show any significant beneficial effect of doxorubicin in these models, a likely limiting factor was the MTD of 2 mg/Kg i.p. weekly for 6 weeks in our BALB/c nude mouse model. A similar doxorubicin dose for 3 weeks gave only a "minimum to partial response" in patient-derived TNBC xenograft tumors in NOD-SCID mice (51), whereas 1.5 mg/Kg weekly as an i.v. bolus significantly reduced the volume of MDA-MB-231 TNBC xenograft tumors in nude mice (52). In our study, the very small and non-significant effect of doxorubicin provided an opportunity to observe a cooperative inhibitory effect with F + G treatment, but no such effect was observed in the tumor models used. It is possible that by increasing doxorubicin tolerability through the use of liposomes or other nano-delivery systems (48), a tolerable dose may have been achieved at which doxorubicin would complement the effects of the kinase inhibitors. However, our study reinforces the conclusion (5) that, since both fingolimod (53) and gefitinib (54) are FDA-approved drugs (though not for breast cancer), inhibiting IGFBP-3-dependent oncogenic signaling by F + G therapy is a plausible approach to targeting basal-like TNBC tumors.

CONCLUSION

Insulin-like growth factor binding protein-3-dependent signaling may be viewed as a controversial target for cancer therapy, since in some cancers *IGFBP3* appears to act as a tumor suppressor gene, but our data suggest the plausibility of this approach for women with TNBC. IGFBP-3 is highly expressed and a poor prognostic feature in ER-negative breast cancers, and initiates an oncogenic signaling cascade in both *in vitro* and *in vivo* pre-clinical models of basal-like TNBC. Combined EGFR and SphK inhibition, to block IGFBP-3-dependent signaling, synergistically inhibits cell proliferation and this effect is attenuated by IGFBP-3 downregulation (5). This pre-clinical study has shown that combination of fingolimod + gefitinib-targeted therapy is more effective than doxorubicin at its MTD, in inhibiting the growth of basal-like TNBC xenograft tumors. IGFBP-3, detected predominantly in the cell nucleus in these tumors, is less abundant in more slowly growing tumors, and its high nuclear staining is similar to high Ki67 staining as a marker of decreased mouse survival. We, therefore, conclude that fingolimod + gefitinib combination therapy for basal-like TNBC tumors with high IGFBP-3 might be suitable for evaluation in a neoadjuvant setting as an effective alternative to cytotoxic chemotherapy.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of, and with the approval of, the Northern Sydney Local Health District Animal Ethics Committee (Protocols RESP/14/280 and RESP/15/103).

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the study. SJ and JM contributed to the acquisition, analysis, and interpretation of data. RB contributed to data analysis and interpretation and wrote the manuscript. All authors contributed to its critical revision and approved the final version for publication.

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Hyperglycemia Promotes TMPRSS2-ERG Gene Fusion in Prostate Cancer Cells *via* Upregulating Insulin-Like Growth Factor-Binding Protein-2

Jeff M. P. Holly[1], *Jessica Broadhurst*[1], *Rehanna Mansor*[1], *Amit Bahl*[2] *and Claire M. Perks*[1]*

[1] *IGFs & Metabolic Endocrinology Group, School of Clinical Sciences, Southmead Hospital, Bristol, United Kingdom,* [2] *Department of Clinical Oncology, Bristol Haematology and Oncology Centre, University Hospitals Bristol, Bristol, United Kingdom*

Background: Epidemiologic evidence shows that obesity is associated with a greater risk of aggressive prostate cancer (PCa) and PCa-specific mortality and this is observed mainly in men with the *TMPRSS2-ERG* gene fusion. Obesity is often associated with comorbid conditions such as type 2 diabetes and hyperglycemia: we investigated whether some of the exposures associated with disturbed metabolism can also affect the frequency of this gene fusion.

Methods: Fusion was induced in LNCaP PCa cells in normal or high levels of glucose, with or without insulin-like growth factor binding protein-2 (IGFBP-2) silenced or the presence of insulin-like growth factor-1 (IGF-I), insulin, or epidermal growth factor (EGF). RNA was extracted for analysis by nested PCR. Abundance of IGFBP-2, γH2AX, DNA-dependent protein kinase catalytic subunit (DNAPKcs), and β-actin were analyzed by Western immunoblotting.

Results: Our data suggest that hyperglycemia-induced IGFBP-2 increased the frequency of the gene fusion that was accompanied by decreased levels of DNAPKcs implying that they were mediated by alterations in the rate of repair of double-strand breaks. In contrast insulin, IGF-I and EGF all decreased gene fusion events.

Conclusion: These novel observations may represent a further mechanism by which obesity can exert an effect aggravating PCa progression.

Keywords: prostate cancer, insulin-like growth factor-binding protein-2, TMPRSS2-ERG, hyperglycemia, type II diabetes

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Andrea Morrione,
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Maximilian Bielohuby,
Sanofi, France

***Correspondence:**
Claire M. Perks
claire.m.perks@bristol.ac.uk

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INTRODUCTION

The *TMPRSS2-ERG* fusion oncogene is thought to be important during tumor progression and development as it is found in approximately half of all prostate cancer (PCa) biopsies and also in metastases (1–3).

Joining of the 5′-untranslated region of *TMPRSS2* with the oncogenic *ETS* transcription factor, ERG culminates in the *TMPRSS2-ERG* gene fusion. TMPRSS2 possesses androgen-responsive elements and so in response to androgens *TMPRSS2* drives *ERG* overexpression. Antiandrogens can decrease *ERG* in patients carrying *TMPRSS2-ERG* through its ability to reduce the levels of androgen. In contrast, for patients whose PCa progresses and becomes resistant to antihormone therapy, the fusion oncogene*TMPRSS2-ERG* can be reactivated and could thus contribute to tumor progression (4).

We are currently facing a global obesity epidemic that has been associated with a negative impact on PCa. There is strong epidemiologic evidence that obesity is associated with a greater risk of aggressive PCa and increased PCa-specific mortality (5–7). Furthermore, the negative impact of obesity on PCa prognosis has mainly been observed in men with the *TMPRSS2-ERG* gene fusion (8) implying an interaction.

Obesity is often associated with comorbid conditions such as insulin resistance, hyperglycemia, and type 2 diabetes. We have shown previously that hyperglycemia-induced chemoresistance in PCa cells and that this was mediated by an epigenetic upregulation of insulin-like growth factor binding protein-2 (IGFBP-2) (9). IGFBP-2 is one of the six high-affinity IGF-binding proteins, which bind to IGFs, acting as a carrier and protecting them from clearance, increasing their half-lives, and modulating their availability and activity. These IGFBPs, including IGFBP-2 can also regulate cell function independently of the insulin-like growth factor-1 (IGF-I) receptor (10, 11). IGFBP-2 is considered to be a key player in PCa progression (12) with IGFBP-2 levels being raised in the serum and in the tumors of patients with PCa (13, 14).

PTEN is a phosphoprotein that exhibits both protein- and lipid-phosphatase activity that inhibits the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase signaling pathways (15–17), thereby acting in an opposite manner to growth factors, which promote cell growth and survival. We identified that IGFBP-2 inhibited PTEN function in PCa cells by increasing its phosphorylation (18) and global expression profiling indicated that IGFBP-2 was the most important biomarker to indicate the status of PTEN in tumors (19). When PTEN is silenced, mice develop high grade prostatic intraepithelial neoplasia, but do not progress to develop cancer (20). 93% of ERG rearrangement positive samples showed either absent or reduced PTEN (21) and tumors lacking functional PTEN express higher levels of ERG rearrangement (22). IGFBP-2 has also been shown to translocate to the nucleus in neuroblastoma cells, *via* its nuclear localization sequence, where it directly associates with DNA and functions as a transcription factor, modulating specific tumorigenic genes (23, 24).

The high frequency of the TMPRSS2-ERG gene fusion in PCa is not due to random translocations but is promoted by the androgen receptor inducing changes in chromosomal architecture leading to the proximity of the TMPRSS2 and ERG genes that are then fused following double-strand breaks (DSB) and repair *via* the non-homologous end joining (NHEJ) pathway (25). There have been several studies examining the effects of androgen exposure on the formation of fusion products (26, 27), but little work examining potential effects of other exposures. In this study, we examined the effect of some of the exposures associated with disturbed metabolism on *TMPRSS2-ERG* gene fusion, in particular, hyperglycemia and the potential role of IGFBP-2 in the latter.

MATERIALS AND METHODS

Materials

All chemicals, unless otherwise stated, were purchased from Sigma (Poole, UK). LNCaP cells were bought from ATCC and cultured as described previously (9). All cell lines tested negative for mycoplasma.

Fusion Induction

LNCaP cells were seeded in 6-well plates in DMEM growth media (Basel, Switzerland, GM: 5 mM glucose) with or without IGFBP-2 silenced for 24 h, serum starved for 24 h in the presence of aphidicolin (2 μg/ml) in DMEM and HAM'S Nutrient Mix F12 media containing charcoal-stripped serum (Invitrogen, Paisley, UK, SFM: 25 mM) and then dosed with dihydrotestosterone (DHT:0.1 μM) in the presence or absence of IGF-I, Gropep, Adelaide, SA, Australia (100 ng/ml), insulin, Novo Nordisk, West Sussex, UK (100 ng/ml), or epidermal growth factor (EGF), Merck, Hertfordshire, UK (20 ng/ml) for 2 h in fresh charcoal-stripped serum based media followed by the addition of etoposide (60 μM) for 1 h. We confirmed that dosing with etoposide at this dose for 1 h did not induce any consequent cell death (data not shown). Before assessing whether IGF-I, insulin, or EGF affected the rate of fusion in LNCaP cells, we initially assessed how responsive these cells were to the factors in relation to DNA proliferation. On performing dose responses, we found that 20 ng/ml EGF and 100 ng/ml IGF-I and insulin gave the greatest response in terms of growth and so used these doses for the fusion experiments. Optimum doses of DHT and etoposide that were used were selected from previous dose response curves (data not shown). Cells were incubated in fresh charcoal-stripped serum based media for a further 24 h prior to the extraction of RNA using Trizol reagent from Invitrogen (Carlsbad, CA, USA) according to manufacturer's instructions and conversion to cDNA using a kit from Invitrogen (SuperScript III First-Strand Synthesis System). IGFBP-2 was silenced, parallel to non-silencing controls, using siRNA (100 nM) and a second siRNA for IGFBP-2 was also used to exclude off-target responses: sequences of siRNAs and methodology described previously (9, 28).

Quantitative Nested PCR

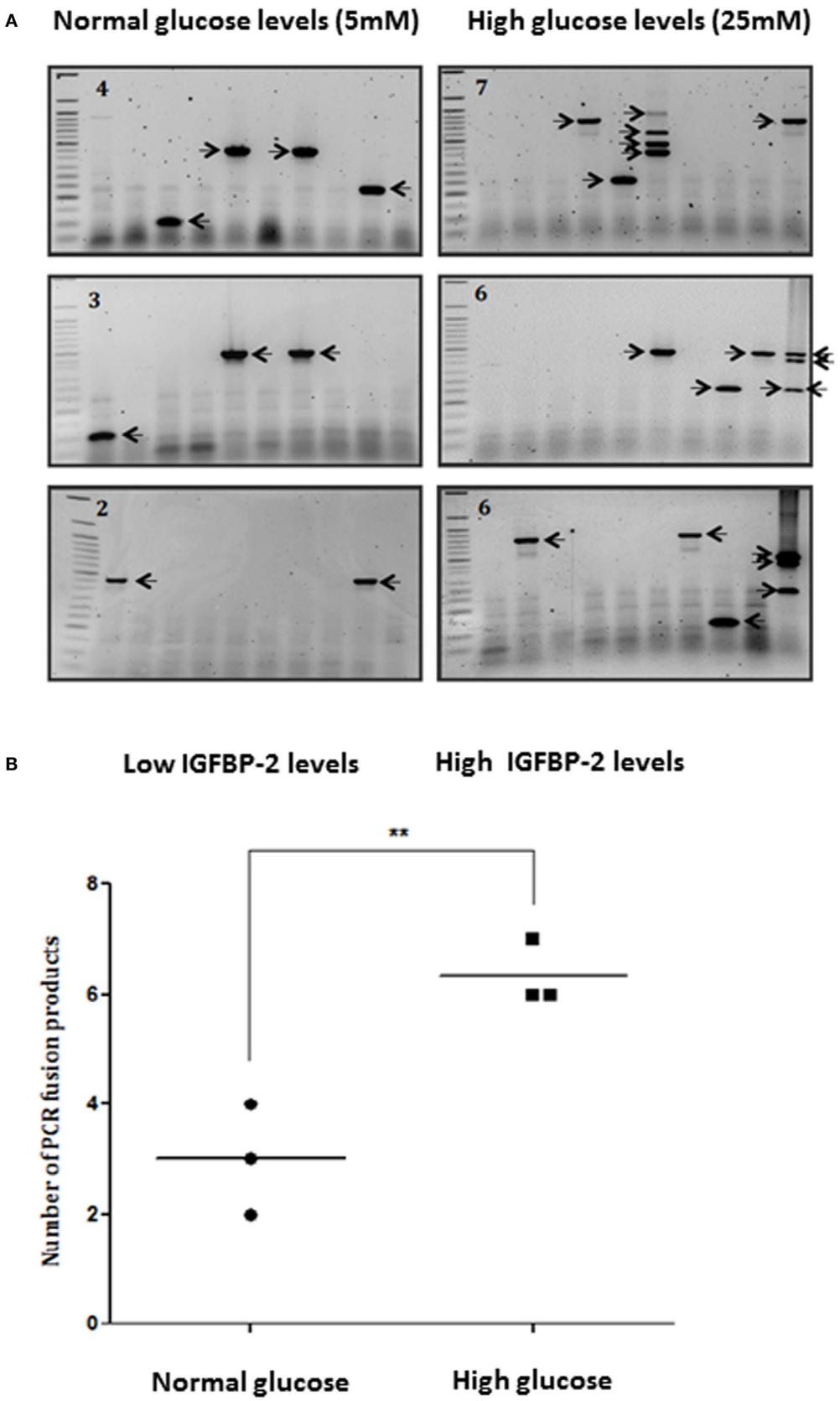
Each tube of cDNA was separated into 10 × 2 μl aliquots. These were used in 10 separate nested PCR reactions amplified using primer pair 1 (TMPRSS2 forward CAGGAGGCGGAGGCGGA: ERG reverse GGCGTTGTAGCTGGGGGTGAG). 2 μl of this PCR product was then taken and used to initiate the second round of PCR amplified using primer pair 2 (TMPRSS2 forward GGAGCGCCGCCTGGAG: ERG reverse CCATATTC TTTCACCGCCCACTCC) in a further 10 reactions as described previously (29). Each PCR product was run on a 1.7% agarose gel and the total number of PCR products from these 10 reactions counted and compared. This process was repeated for each treatment in triplicate.

Western Immunoblotting

Insulin-like growth factor binding protein-2, γH2AX, DNA-dependent protein kinase (DNAPK)cs, and β-actin were analyzed by Western immunoblotting as described previously (9).

Statistical Analysis

Data were analyzed with SPSS 12.0.1 for Windows using one-way ANOVA followed by least significant difference *post hoc* test. A statistically significant difference was present at $^*p < 0.05$.



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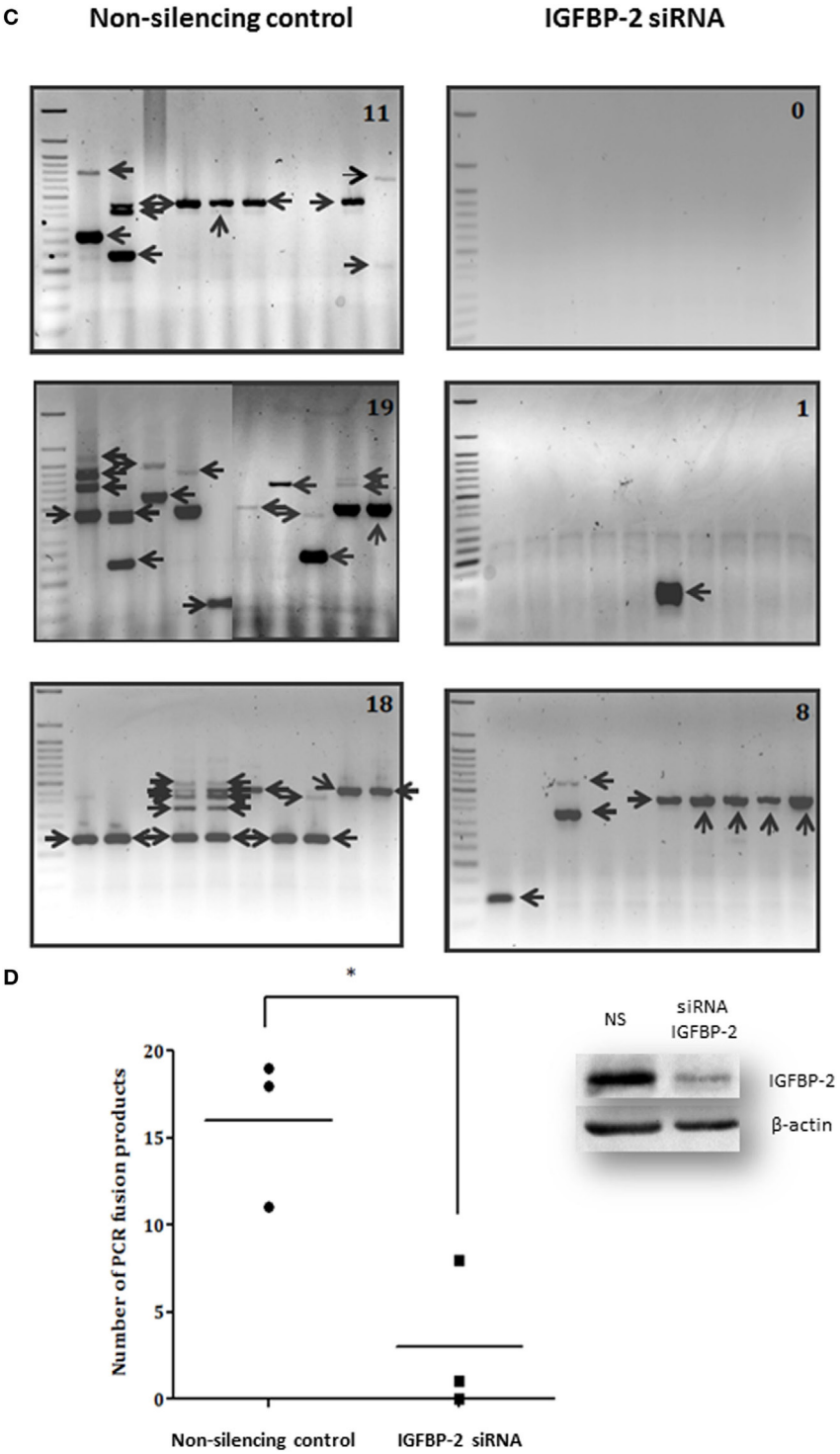
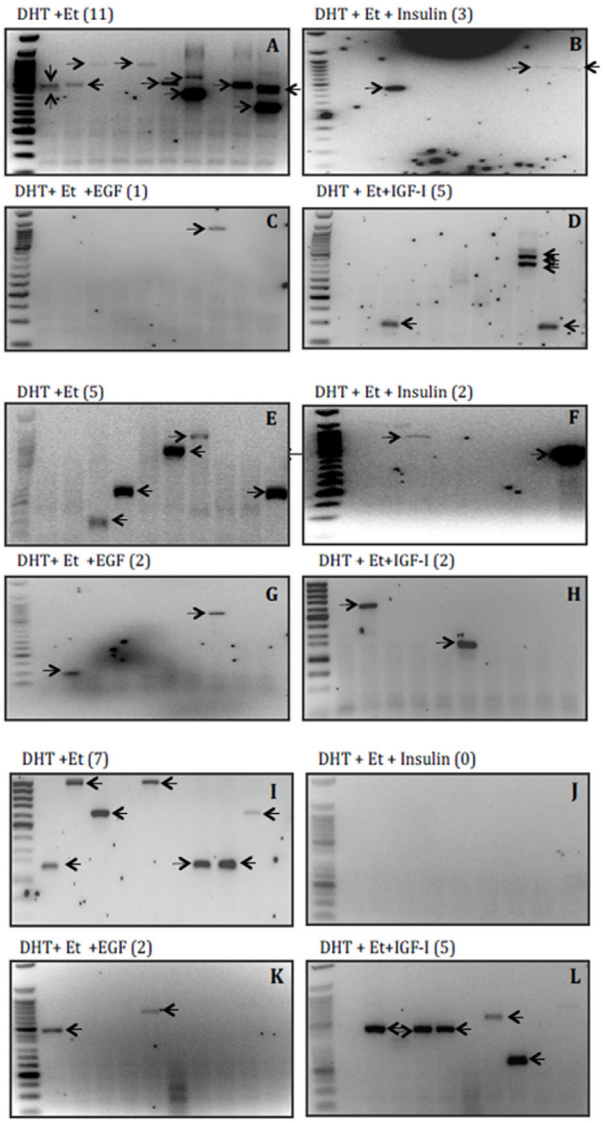


FIGURE 1 | Effects of hyperglycemia on TMPRSS2:ERG fusion induction in LNCaP prostate cancer cells. LNCaP cells were seeded (0.7×10^6 cells/well) in 6-well plates in normal glucose-containing DMEM growth media (GM: 5 mM glucose) for 24 h, serum starved for 24 h in the presence of aphidicolin (2 μg/ml) in either normal or high glucose-containing DMEM and HAM'S Nutrient Mix F12 media containing charcoal-stripped serum (SFM: 25 mM), and then dosed with DHT (0.1 μM) for 2 h in fresh charcoal-stripped serum based media followed by the addition of etoposide (60 μM) for 1 h. Cells were incubated in fresh charcoal-stripped serum based media for a further 24 h. LNCaP cells were also seeded (0.7×10^6 cells/well) in high glucose-containing GM and insulin-like growth factor binding protein-2 (IGFBP-2) was silenced parallel to non-silencing controls using siRNA (100 nM). After 16 h cells were serum starved in high glucose-containing SFM as above for a further 24 h and treated with 0.1 μM DHT for 2 h and 60 μM etoposide for a further 1 h. Cells were incubated in fresh SFM for a further 24 h. Cells were extracted in trizol for performing nested PCR. **(A,C)** Illustrate three repeats of the gels showing PCR products (each indicated by an arrow) and number of PCR products in top corner of each blot **(B,D)** is the quantification of the PCR products. Insert to **(D)** is a representative western immunoblot for IGFBP-2 and β-actin (NS, non-silencing siRNA).



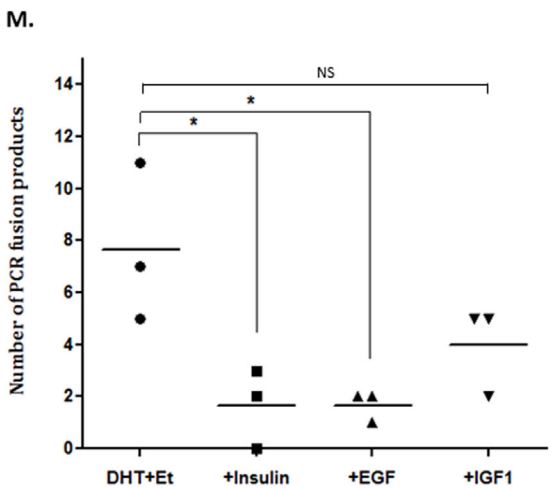


FIGURE 2 | Continued

FIGURE 2 | Continued
Effects of insulin, epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-I) on TMPRSS2:ERG fusion induction in LNCaP prostate cancer cells. LNCaP cells (0.7×10^6 cells/well) were seeded in 6-well plates and serum starved as described in figure legend 1 for a further 24 h and treated with 0.1 μM DHT for 2 h in the presence or absence of IGF-I (100 ng/ml), insulin (100 ng/ml), or EGF (20 ng/ml) followed by 60 μM etoposide for a further hour. A final 24 h incubation with fresh SFM was followed by RNA extraction in Trizol for analysis by nested PCR. **(A–L)** Illustrates three repeats of the gels showing PCR products with the number of PCR products in brackets after the title of each blot and **(M)** is the quantification of the PCR products.

RESULTS

Effect of Glucose on the Number of TMPRSS2:ERG Fusion Products: A Role for IGFBP-2

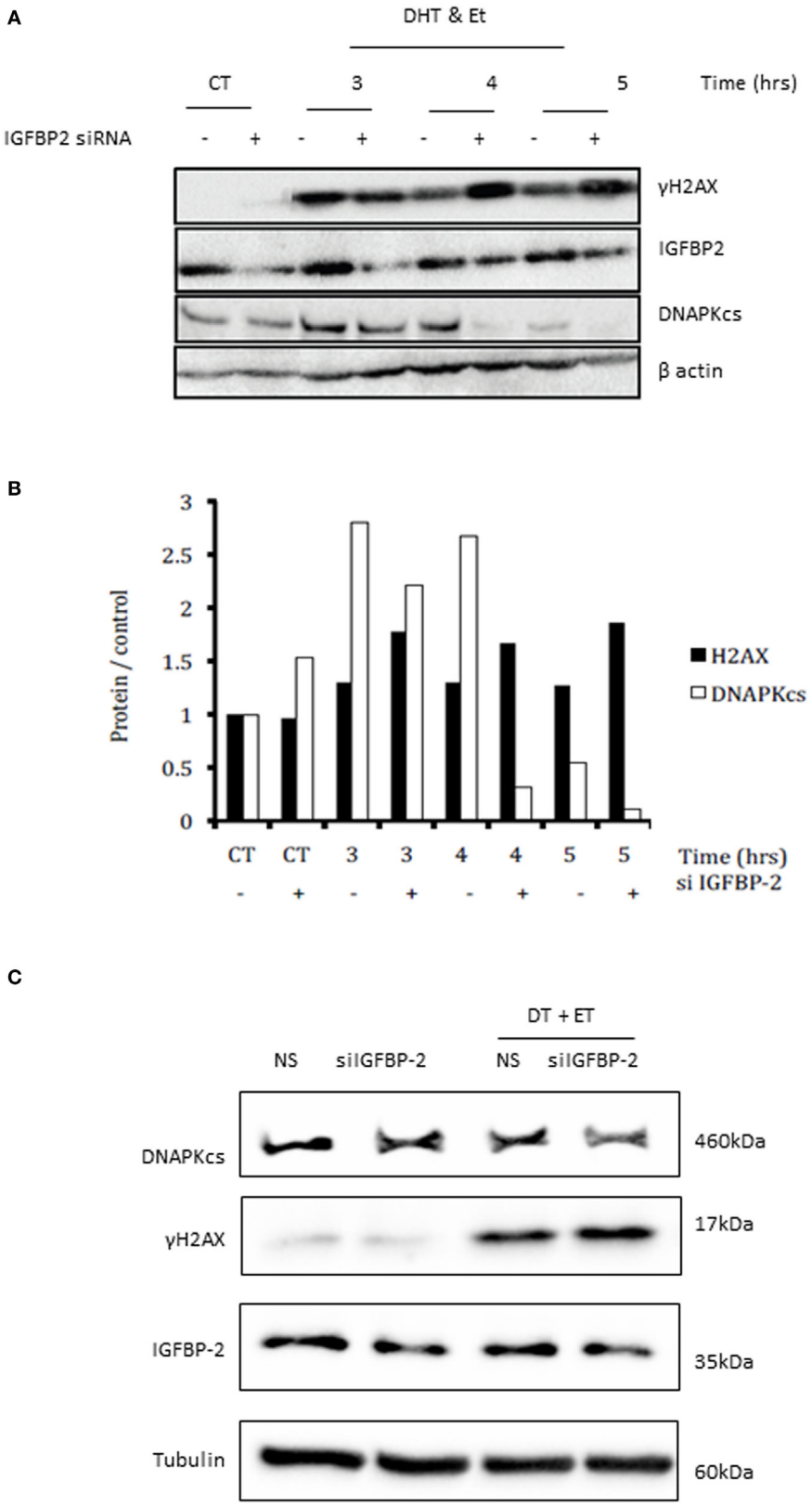
Figures 1A,B show that the average number of TMPRSS2:ERG fusion products was higher in 25 mM glucose (6.3) compared to 5 mM glucose (3), with the average rate over 2.1-fold higher at 25 mM than at 5 mM ($p < 0.01$). As we have shown previously, using ELISA and western blotting that high glucose increases the abundance of IGFBP-2 compared with levels observed in 5 mM glucose by 1.8-fold ($p < 0.01$) (9), these data suggested that the glucose-induced increase in IGFBP-2 may be related to the increase in TMPRSS2:ERG fusion products. Therefore, to examine this more specifically, we silenced IGFBP-2 using siRNA in high glucose conditions and observed a significant decrease in TMPRSS2:ERG fusion products ($p < 0.05$): effective silencing of IGFBP-2 is indicated by a western blot (**Figures 1C,D**).

Effect of Insulin, EGF, and IGF-I on the Number of TMPRSS2-ERG Fusion Products

The average rate of fusion induction was decreased 3.5-fold by insulin (that does not bind IGFBPs) (**Figures 2A,B,E,F,I,J**) and over 2.5-fold by IGF-I (**Figures 2A,D,E,H,I,L**). We also observed a 3.5-fold decrease by an alternative growth factor, EGF (**Figures 2A,C,E,G,I,K**). While the decrease in fusion induction with insulin and EGF treatment were statistically significant ($p < 0.05$), the decrease observed in the presence of IGF-I was not statistically significant (**Figure 2M**).

Effects of Silencing IGFBP-2 or Adding IGF-I on Levels of γH2AX and DNA-Dependent Protein Kinase Catalytic Subunit (DNAPKcs)

Figure 3A shows an increase in γH2AX after etoposide treatment, corresponding to a dramatic increase in DSBs. At 3, 4, and 5 h the bands depicting the levels of γH2AX were substantially higher in cells in which IGFBP-2 had been knocked down compared to non-silencing controls. A decrease in the levels of DNAPKcs after IGFBP-2 knockdown was observed at 3, 4, and 5 h after etoposide and DHT dosing compared to non-silencing treated cells. This



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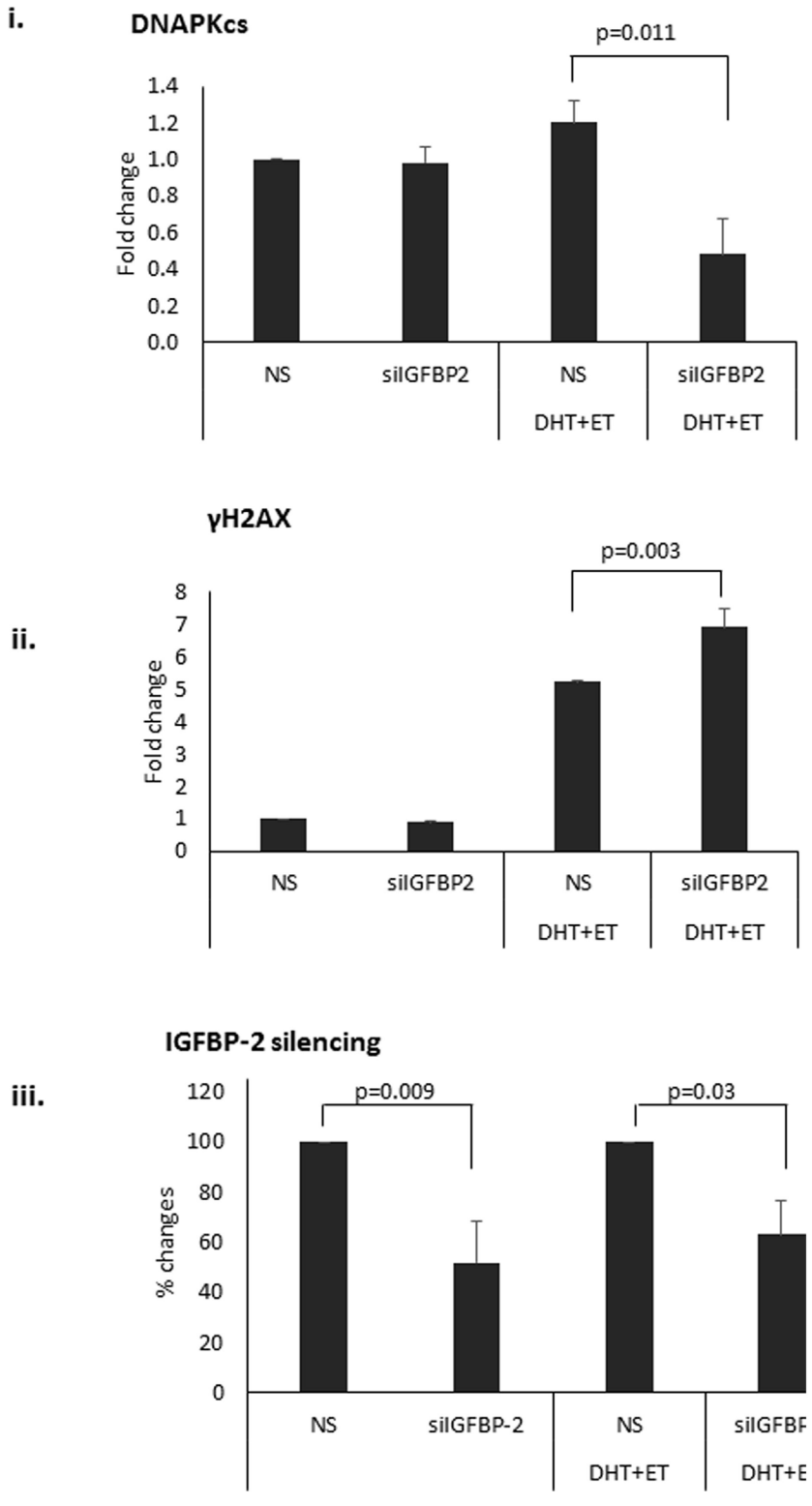

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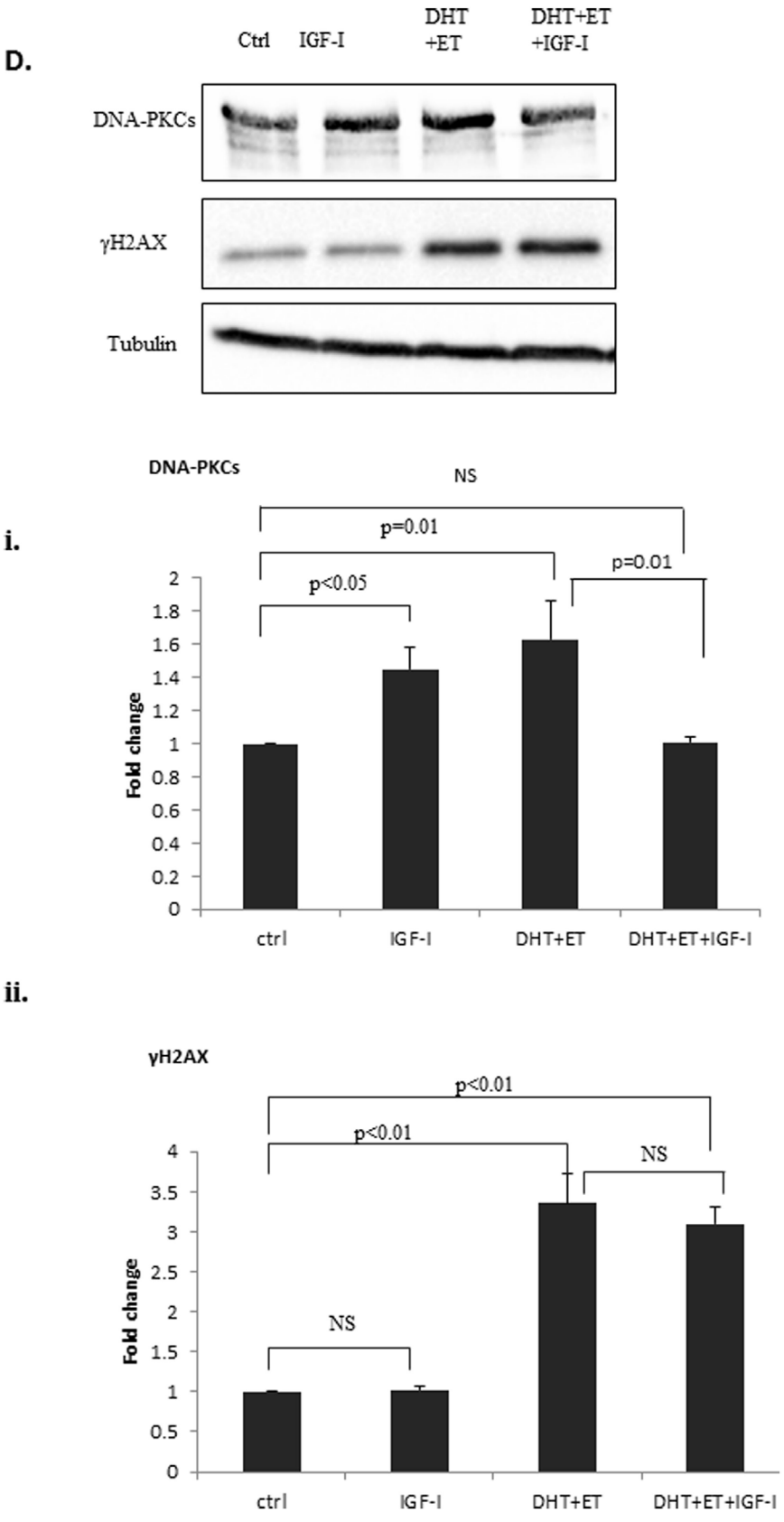

FIGURE 3 | Continued

FIGURE 3 | Continued
Effects of silencing insulin-like growth factor binding protein-2 (IGFBP-2) or adding insulin-like growth factor-1 (IGF-I) on levels of γH2AX and DNA-dependent protein kinase catalytic subunit (DNAPKcs) in LNCaP prostate cancer cells. Cells were seeded and dosed as in the legend for **Figure 1** and following etoposide treatment were incubated in fresh SFM and lysed every 1 h from 3 h in SFM. **(A)** Shows a representative blot of levels of IGFBP-2, γH2AX, DNAPKcs, and β-actin analyzed by Western immunoblotting as described previously (9). **(B)** Densitometry of the western blot shown in **(A)** indicating levels of γH2AX, DNAPKcs normalized to β-actin levels relative to the non-silencing untreated control. Following etoposide, treatment cells were lysed after 4 h in fresh SFM and **(C)** shows a representative western blot that has been repeated three times showing of levels of IGFBP-2, γH2AX, DNAPKcs normalized to tubulin levels. [**(C)**i,ii,iii] densitometry showing the mean changes of three experiments showing levels ofγH2AX and DNAPKcs, respectively, normalized to tubulin levels relative to the non-silencing untreated control. Cells were seeded and dosed with IGF-I (100 ng/ml) as in the legend for **Figure 2** and following etoposide treatment cells were lysed after 4 h in fresh SFM. **(D)** Shows a representative western blot that has been repeated three times showing of levels of γH2AX and DNAPKcs normalized to tubulin levels and [**(D)**i,ii] show densitometry of the mean changes of three experiments indicating levels of DNAPKcs and γH2AX, respectively, normalized to tubulin levels.

was verified by densitometry shown in **Figure 3B**. We repeated the experiment at the 4 h time point to confirm that levels of γH2AX were significantly increased ($p = 0.003$) and those of DNAPKcs were significantly ($p = 0.011$) decreased with IGFBP-2 silenced ($p = 0.009$) (**Figure 3C**i,ii,iii). **Figure 3D**i,ii show that DHT and etoposide treatment alone increased levels of DNAPKcs and of γH2AX ($p = 0.01$ and $p < 0.01$, respectively). Although IGF-I alone increased DNAPKcs ($p < 0.05$), in the presence of DHT and etoposide, IGF-I acted in an opposite way and reduced DNAPKcs ($p = 0.01$). IGF-I, however, had no effect on γH2AX either alone or in combination with DHT and etoposide.

DISCUSSION

Knowing that high glucose increases the abundance of IGFBP-2 in PCa cells (9), we used this model to assess whether increasing levels of glucose altered the number of TMPRSS2-ERG fusion products induced by exposure to DHT and etoposide and if this was mediated by IGFBP-2. Our data suggest that high glucose increases the number of TMPRSS2:ERG fusion products, and this was not seen when the accompanying increase in IGFBP-2 was prevented, consistent with IGFBP-2 playing a role in this effect. It would be interesting to investigate whether other inducers of IGFBP-2 also elicit such an effect and indeed whether adding exogenous IGFBP-2 to the cells in normo-glycemic conditions also increased the number of TMPRSS2-ERG fusion products induced by exposure to DHT and etoposide, as this would infer a more general role for IGFBP-2. The NHEJ pathway is a process that repairs DSB in DNA (30). Silencing components involved in the process of NHEJ prevents TMPRSS2:ERG gene fusions (25) indicating that NHEJ is a major method for generating fusions. DNAPK is a large protein complex that plays an important role in NHEJ in DNA-DSB repair and possesses a catalytic subunit called DNAPKcs. DNAPK is critical for controlling progression through the cell cycle and maintaining genomic stability (31). As well as DNAPK being modulated through its interactions with DNA, its activity can also be regulated by a variety of other mechanisms, including modulation of DNAPKcs. A study in HeLa cells (32) concluded that DNAPKcs plays an important role in the regulation of γH2AX phosphorylation in response to DNA damage. Phosphorylation of γH2AX is essential to mark the DSB allowing the DNA repair machinery to identify its location (33). Our data suggest that IGFBP-2 has a role in increasing the rate of repair of DSBs by increasing levels of DNAPKcs and this culminates in increased TMPRSS2:ERG gene fusion. An effect of IGFBP-2 on DNAPK has previously been observed: treatment of astrocytes with IGFBP-2 resulted in a direct induction of DNAPKcs (34). Additional studies provide further support suggesting a role of IGFBP-2 in facilitating DNA repair: in glioblastoma studies, IGFBP-2 alters the expression of the following DNA repair genes: X-ray repair complementing defective repair 2, cyclin-dependent kinase inhibitor 1A, and CDC28 protein kinase 2 (35). In addition, a large-scale study, also in glioblastomas, showed that both the DNA-DSB repair pathway and the homologous recombination pathway are associated with IGFBP-2 expression, altering a broad range of proteins including p53, GADD45, TOP2A, and BRCA1 (36). Of all the six similar IGFBPs, IGFBP-2 has most frequently been reported to be overexpressed in a range of human cancers and only IGFBP-2 has been linked to the DNA-DSB repair pathway. It would, however, be interesting to investigate whether other IGFBPs could have similar actions. In our model showing that adding or silencing any of the other IGFBPs had no effect on fusion induction would imply that this was a specific effect of IGFBP-2.

It has become increasingly clear that IGFBPs, including IGFBP-2, can exert effects that are both dependent and independent of its interactions with IGFs (11). To investigate if the effects of IGFBP-2 in promoting TMPRSS2:ERG gene fusions through facilitating DNA repair were dependent on IGF-I, we exposed LNCaP cells to IGF-I alone or following treatment with DHT and etoposide and compared this to the effects of insulin (that does not bind IGFBPs) and EGF (an alternative growth factor). The effects of IGF-I on DNAPKcs might suggest the effects of IGFBP-2 on DNAPKcs were dependent on binding to IGF-I and negating its effect. Further work is required to confirm the IGF-dependency of IGFBP-2 in DSB repair and the induction of fusion; as we did not observe any effect of IGF-I on γH2AX at this time point, although IGF-I induced a reduction in the frequency of fusion products, it was not statistically significant. It is possible that a potential reduction in fusion products induced by IGF-I was not due to an effect on DNA repair but could have been an effect on chromosomal architecture. It has been observed that, in LNCaP-LN3 cells (a derivative of the LNCaP cells that were used in our study), blocking the IGF-I receptor had no effect on γH2AX focus formation, suggesting that activation of the IGF-IR in this cell line has no effect on DSB repair (37).

In summary, our data suggest that exposure to insulin and potentially IGF-I reduced the frequency of formation of fusion products whereas both hyperglycemia and IGFBP-2 increased the number of TMPRSS2-ERG gene fusions and these factors

could contribute to the negative impact that obesity has on PCa progression.

AUTHOR CONTRIBUTIONS

JH and CP made substantial contributions to the design and with JB, RM, AB were responsible for the work, interpretation, and analysis of the data. All authors contributed to drafting and revising the manuscript and all approved the final version with an agreement of accountability for the work presented.

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REVIEW
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IGF Binding Protein-5 Induces Cell Senescence

Fumihiro Sanada[1], Yoshiaki Taniyama[1,2], Jun Muratsu[1,2], Rei Otsu[1], Hideo Shimizu[1], Hiromi Rakugi[2] and Ryuichi Morishita[1]**

[1] Department of Clinical Gene Therapy, Osaka University Graduate School of Medicine, Suita, Osaka, Japan, [2] Department of Geriatric and General Medicine, Osaka University Graduate School of Medicine, Suita, Osaka, Japan

Cellular senescence is the complex process of deterioration that drives the aging of an organism, resulting in the progressive loss of organ function and eventually phenotypic aging. Senescent cells undergo irreversible growth arrest, usually by inducing telomere shortening. Alternatively, senescence may also occur prematurely in response to various stress stimuli, such as oxidative stress, DNA damage, or activated oncogenes. Recently, it has been shown that IGF binding protein-5 (IGFBP-5) with the induction of the tumor suppressor p53 is upregulated during cellular senescence. This mechanism mediates interleukin-6/gp130-induced premature senescence in human fibroblasts, irradiation-induced premature senescence in human endothelial cells (ECs), and replicative senescence in human ECs independent of insulin-like growth factor I (IGF-I) and IGF-II. Additionally, a link between IGFBP-5, hyper-coagulation, and inflammation, which occur with age, has been implicated. Thus, IGFBP-5 seems to play decisive roles in controlling cell senescence and cell inflammation. In this review, we describe the accumulating evidence for this role of IGFBP-5 including our new finding.

Keywords: IGF binding protein-5, cell senescence, inflammation, coagulation system, age-related disease

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Fumihiko Hakuno,
The University of Tokyo, Japan

****Correspondence:***
Fumihiro Sanada
sanada@cgt.med.osaka-u.ac.jp;
Ryuichi Morishita
morishit@cgt.med.osaka-u.ac.jp

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INTRODUCTION

Insulin-like growth factor I (IGF-I) and II (IGF-II) are insulin superfamily members and are ubiquitously distributed in several organs (1, 2). Six high-affinity IGF binding proteins (IGFBPs) interact with IGFs, regulating IGF-I/II bioavailability, distribution, and signaling. IGFBPs are secreted and bind to IGFs in the circulation and extracellular environment (3). In addition to IGF-dependent action, IGF-independent functions of IGFBPs, many of which occur intracellularly, have recently been reported (4, 5). For example, IGFBP-1 and -2 are associated with cancer cell proliferation, adhesion, and migration through the specific binding of IGFBP-1 and -2 to alpha 5 beta1 integrin, followed by alterations in the phosphorylation status of downstream signaling molecules (6, 7). By regulating enzymes involved in sphingolipid metabolism, IGFBP-3 and -5 affect the balance between growth inhibitory lipids and growth stimulatory lipids (8, 9). Additional evidence has implicated that IGF binding protein-5 (IGFBP-5) is upregulation in the irradiation-induced premature senescence and replicative senescence of umbilical vein endothelial cells (HUVECs) (10). Knockdown of IGFBP-5 in aged HUVECs partially reversed the process of senescence, whereas the application of exogenous IGFBP-5 or IGFBP-5 overexpression induced premature senescence in HUVECs *in vitro* (11), indicating a decisive role for IGFBP-5 in controlling cell senescence and proliferation. The insulin/IGF signaling pathway has been implicated in the aging of many organisms, ranging from nematodes to mammals. The observation that IGFBPs modulate the availability or the distribution of

IGF-1 adds further support to the hypothesis that IGFBPs have a vital role in the aging process (12). Many changes in the immune system, hemostasis, and vasculature, including alterations in inflammation, coagulation, and vascular senescence, occur during aging. However, its mechanism is not fully understood.

In this review, we first overview the mechanism of chronic inflammation during aging and later possible mechanism linking between cell senescence and senescence-inducing stimuli *via* IGFBP-5 is discussed.

MECHANISM OF CHRONIC INFLAMMATION IN AGING

Inflammation is required for an acute, transient immune response to invading pathogens or acute traumatic injury. This process is essential for facilitating the tissue repair by increasing division and migration of cells. In contrast, chronic inflammation causes low-grade and persistent inflammation leading to tissue degeneration rather than the solution to infection, injury, or disease (13). Many aged tissues are chronically inflamed, which is the common pathological mechanism for age-associated diseases, such as cardiovascular disease, diabetes, cancer, and Alzheimer's disease (14). Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) counteracts anabolic signaling, including insulin and erythropoietin cascades. Thus, chronic low-grade inflammation is now recognized as an important causative factor for insulin resistance and sarcopenia (15). Several sources of chronic inflammation during aging termed "inflammaging" have been described (**Figure 1**) (14). (i) Debris from cells or immunoglobulin accumulation due to increased cell death or inappropriate cell elimination systems in aging activates the innate immune system, resulting in chronic inflammation in aged organs (16). According to Zhang et al. (17), circulating mitochondrial damage-associated molecular patterns cause inflammation in response to injury. Mitochondrial damage-associated molecular patterns released from damaged cells are evolutionally conserved with bacterial pathogen-associated

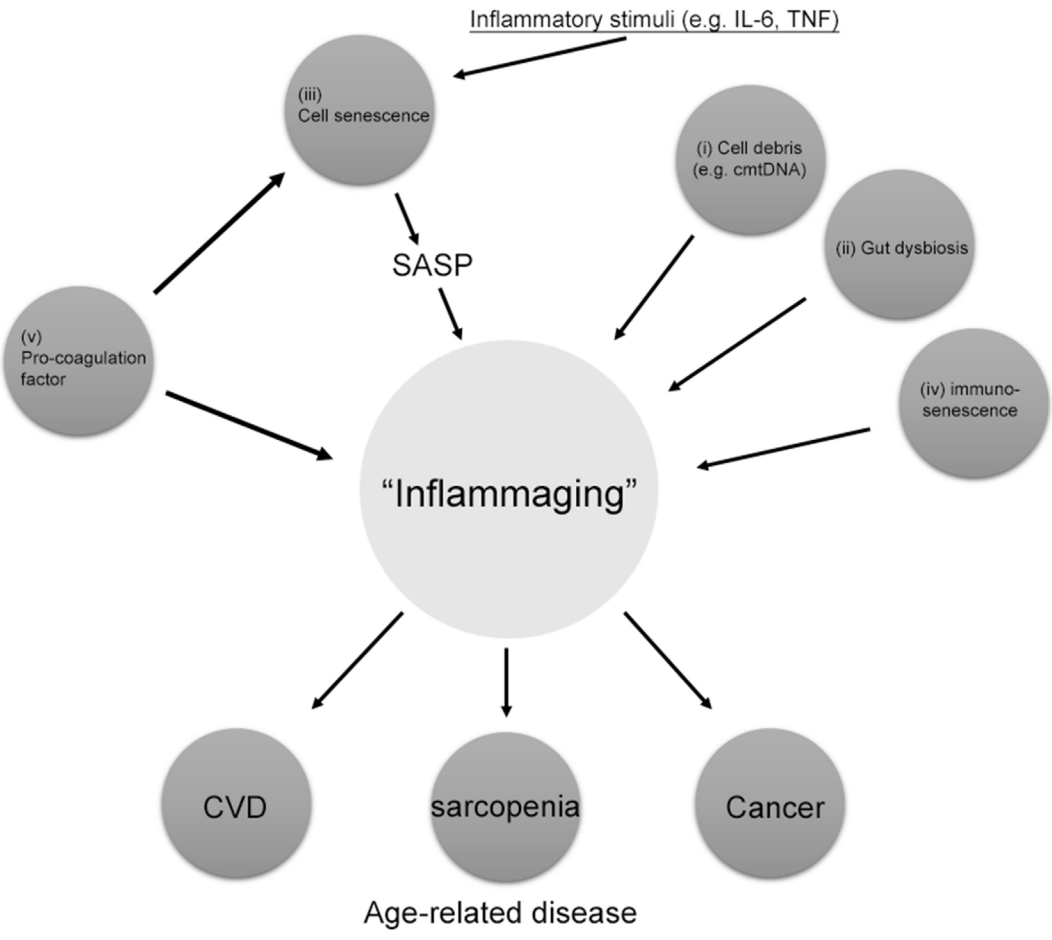

FIGURE 1 | Source of "inflammaging." Among the main causes of inflammaging, the accumulation of pro-coagulation factors, cell senescence, cell debris such as circulating mitochondrial DNA (cmtDNA), gut dysbiosis, and immune senescence are the main causes of inflammaging. Pro-coagulation factors also cause cell senescence. Inflammaging can also be influenced by many other factors, including age, reactive oxygen species, and those not directly related to inflammation, such as microRNAs (miRs) and agalactosylated *N*-glycans. SASP, senescence-associated secretory phenotype.

molecular patterns activating innate immunity (18). Thus, age-related failing of mitochondria quality control is associated with inflammaging. (ii) The ability of the oral and gut mucosa to protect against bacterial invasion declines with age, leading to persist low-grade inflammation (19). Periodontal disease has been also demonstrated to increase the inflammatory response with age (20). Additionally, the abundance of anti-inflammatory microbiota decreases with age and is inversely correlated with serum level of inflammatory cytokines, such as TNF-α and IL-1β (21). (iii) The increased number of senescent cells in tissue secretes various inflammatory cytokines, which play a causal role in age-related diseases. Senescent cells have been identified in age-related diseases including atherosclerosis, cancer, and diabetes (22–24). Senescence-associated secretory phenotype (SASP) is considered to be the main mechanism by which persistent prolonged inflammation occurs even after the initial stimulus has been removed. (iv) Age-related changes in the immune system termed "immunosenescence" increase the susceptibility to infections, malignancy, and autoimmunity, decrease the response to vaccinations, and impair wound healing (25, 26). These changes in the innate and adaptive immune responses associated with increasing age cause inappropriate inflammation. (v) Increased activity of the coagulation and fibrinolysis systems during aging has recently been reported to increase inflammation through the protease-activated receptors (PARs) (27, 28). The plasma concentrations of coagulation factors V, VII, VIII, and IX increase in healthy humans in parallel with the physiological processes of aging. In addition, fibrinogen levels, which have emerged in several trials as a primary risk factor for thrombotic disorders, have been shown to increase with advancing age (29). Thus, uncontrolled coagulation activity and the subsequent activation of the fibrinolysis system contribute to the pathophysiology of aging and several age-related chronic inflammatory diseases, such as atherosclerosis and lung fibrosis (30).

IGFBP-5 AND CELL SENESCENCE

Increasing evidence has implied that the clearance of senescent cells in animal models attenuates the progression of age-related disorders, including osteoarthritis and atherosclerosis (31–33). This evidence strongly supports the hypothesis that senescent cell clearance or the modulation of pro-inflammatory pathways related to the acquisition of SASP might be potential therapeutic strategies for combating age-related diseases and expanding the health span of humans. The IGF/IGFBPs system has been implicated to be a potential target of age-related disease. Of the six IGFBPs, IGFBP-5 plays a critical role in the process of replicative and premature cell senescence (10, 11). PAR-1/2 signaling induced by coagulation factor Xa (FXa) and the fibrinolytic factor plasmin has been shown to increase IGFBP-5 expression in endothelial cells (ECs) and smooth muscle cells (SMCs) (34–36). FXa stimulation of ECs and SMCs increased inflammatory cytokine secretion *via* enhancing cellular senescence through the early growth response 1–IGFBP-5–p53 pathway (34, 37). Interestingly, the FXa- and IGFBP-5-positive areas within the atherosclerotic plaques of human were similarly distributed (37). Kojima et al. have demonstrated that IGFBP-5, as a major signal transducer and activator of transcription 3 mediator, regulates IL-6-induced reactive oxygen species (ROS) production, as well as the subsequent DNA damage response and senescence of TIG3 fibroblast cells (38). They also discovered that IGFBP-5 itself had senescence-inducing activity in TIG cells with increased ROS production. Knocking down of IGFBP-5 significantly reduced IL-6/IL-6R-induced ROS increase and premature senescence. Together, all of these data support the hypothesis that IGFBP-5, which is produced in p53-dependent manner, plays an important role in FXa- or IL-6-induced premature senescence of ECs, SMCs, and fibroblast. IGFBP-5 plays a multifunctional role, possessing growth inhibitory and growth promoting functions (39). IGFBP-5 in breast cancer cells enhances cell proliferation (40). In contrast, IGFBP-5 transgenic mice show retarded growth and reduced litter size (41). Additionally, IGFBP-5 directly regulates apoptosis by interfering with the IGF signaling cascade (42). Moreover, cytoplasmic accumulation of IGFBP-5 in breast cancer cells interacted with sphingosine kinase and protein kinase C, stimulating antiapoptotic effects (9, 43). Thus, IGFBP-5 seems to exert its multifunction depending on cell type, pattern of its distribution in cells and tissue, and IGF-I/II bioavailability.

IGFBP-3, -4, and -6 are also associated with the process of cell senescence. Through Akt, p53, FOXO3a, IGFBP-3 promotes ECs and fibroblast senescence (44, 45). Senescent mesenchymal stem cells secrete IGFBP-4, and it promotes their senescence (39).

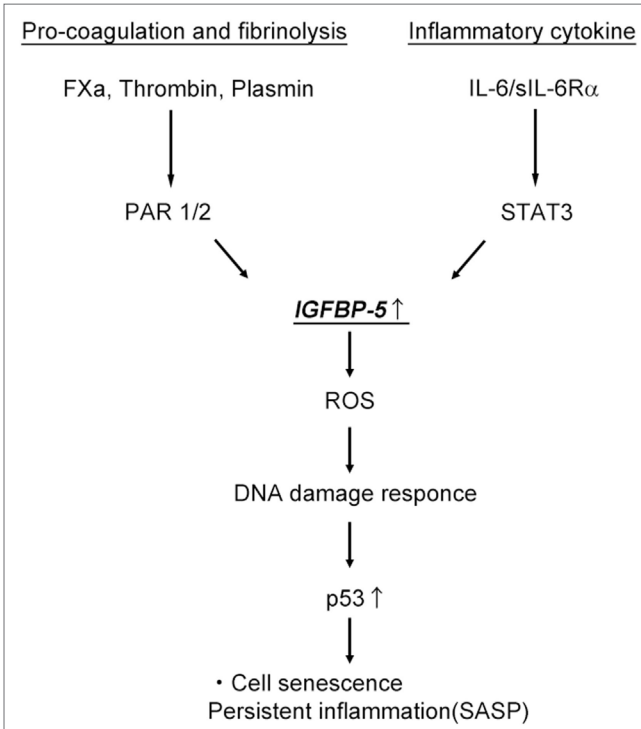

FIGURE 2 | Activation of the pro-coagulation cascade and interleukin-6 (IL-6)/signal transducer and activator of transcription (STAT3) pathway induce cell senescence and persistent inflammation through IGF binding protein-5 (IGFBP-5). ROS, reactive oxygen species; SASP, senescence-associated secretory phenotype.

Senescence induced by pro-oxidative stimuli increases IGFBP-6 levels and IGFBP-6 enhances cell senescence in fibroblast (46, 47), although some experiments demonstrated contradictory results (48). Structurally, six IGFBPs have highly conserved N- and C-terminal domains (49) and different protein sequence in the linker domains (3). Considering their tissue distribution pattern (50), the six IGFBPs might have similar effect on cell senescence in different tissues. Alternatively diverse posttranslational modification in the linker domain of IGFBPs during aging might modify their function on cell growth and senescence. Additional work is required for the elucidation of their function in cell senescence.

CONCLUSION

IGF binding protein-5 has decisive roles in controlling cell senescence and subsequent cell inflammation independent of IGF-I and -II. IGFBP-5 expression was recently shown to be increased following stimulation with coagulation fXa, plasmin, IL-6, and irradiation, leading to cell senescence (**Figure 2**). Additionally, IGFBP-5 induces fibroblast activation and the inflammatory response, contributing to tissue fibrosis. Currently, information on the roles of IGFBPs in the aging of different cells and tissues and the molecules related to IGFBPs signaling is limited. Therefore, the molecular mechanisms underlying the effect of the IGFBP system on aging requires further research. Therapies targeting the coagulation and fibrinolysis cascades might represent new options for the treatment of chronic inflammatory diseases.

AUTHOR CONTRIBUTIONS

FS and YT organized, performed experiment, and wrote manuscript. JM, RO, and HS collected data. HR and RM supervised experiment.

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IGFBP-5 Promotes Fibrosis via Increasing Its Own Expression and That of Other Pro-fibrotic Mediators

*Xinh-Xinh Nguyen[1], Lutfiyya Muhammad[2], Paul J. Nietert[2] and Carol Feghali-Bostwick[1]**

[1] Division of Rheumatology and Immunology, Department of Medicine, Medical University of South Carolina, Charleston, SC, United States, [2] Department of Public Health Sciences, Medical University of South Carolina, Charleston, SC, United States

Pulmonary fibrosis is a hallmark of diseases such as systemic sclerosis (SSc, scleroderma) and idiopathic pulmonary fibrosis (IPF). To date, the therapeutic options for patients with pulmonary fibrosis are limited, and organ transplantation remains the most effective option. Insulin-like growth factor-binding protein 5 (IGFBP-5) is a conserved member of the IGFBP family of proteins that is overexpressed in SSc and IPF. In this study, we demonstrate that both exogenous and adenovirally expressed IGFBP-5 promote fibrosis by increasing the production of extracellular matrix (ECM) genes and the expression of pro-fibrotic genes in primary human lung fibroblasts. IGFBP-5 increased expression of the pro-fibrotic growth factor CTGF and levels of the matrix crosslinking enzyme lysyl oxidase (LOX). Silencing of IGFBP-5 had different effects in lung fibroblasts from normal donors and patients with SSc or IPF. Moreover, we show that IGFBP-5 increases expression of ECM genes, CTGF, and LOX in human lung tissues maintained in organ culture. Together, our data extend our previous findings and demonstrate that IGFBP-5 exerts its pro-fibrotic activity by directly inducing expression of ECM and pro-fibrotic genes. Further, IGFBP-5 promotes its own expression, generating a positive feedback loop. This suggests that IGFBP-5 likely acts in concert with other growth factors to drive fibrosis and tissue remodeling.

Keywords: fibrosis, insulin-like growth factor binding protein-5 (IGFBP-5), systemic sclerosis (SSc), idiopathic pulmonary fibrosis (IPF), extracellular matrix (ECM)

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Briony Forbes,
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Shioko Kimura,
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***Correspondence:**
Carol Feghali-Bostwick
feghalib@musc.edu

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INTRODUCTION

Pulmonary fibrosis is a complication of several different diseases such as systemic sclerosis (SSc, scleroderma) and idiopathic pulmonary fibrosis (IPF). SSc is a complex autoimmune disease characterized by progressive fibrosis of the skin and multiple visceral organs (1, 2). Despite active research, the etiology of this connective tissue disease, which causes high morbidity and mortality in the patients, is still unknown. In recent years, SSc-associated lung disease has become the leading cause of death in scleroderma patients (2, 3). Lung fibrosis is also the hallmark of IPF. In fact, IPF and SSc, while being different diseases, show some similarities (4, 5). Pulmonary fibrosis in both of these diseases is characterized by the overproduction of extracellular matrix (ECM) components in the lung. To date, the therapeutic options for patients with pulmonary fibrosis are limited, and lung transplantation remains the most effective treatment (2). Therefore, identifying novel therapeutic targets would significantly advance the treatment of IPF and SSc-associated lung disease.

Insulin-like growth factor-binding proteins (IGFBPs) comprise a family of six secreted proteins that interact with insulin-like growth factors (IGF)-I and II to modulate their bioavailability (6). Although IGFBPs can regulate IGF activity, they also have IGF-independent effects (7). IGFBP-5 is the most conserved member of the IGFBP family and binds IGF-1 with high affinity (7, 8). Similar to other IGFBPs, IGFBP-5 also exerts both IGF-dependent and -independent effects (7, 9, 10).

We previously demonstrated increased expression of IGFBP-5 in skin and lung tissues of patients with SSc and lung tissues of patients with IPF (7, 11, 12). We further showed that IGFBP-5 induced a fibrotic phenotype *in vitro* in primary human pulmonary fibroblasts, *in vivo* in mouse skin and lung, and *ex vivo* in human skin maintained in organ culture (7, 10–15). Furthermore, the expression of IGFBP-5 is increased in bleomycin-induced pulmonary fibrosis in rats (8). Taken together, these findings suggest that IGFBP-5 levels are elevated in the setting of tissue fibrosis and that IGFBP-5 can promote the development of fibrosis.

Multiple growth factors have been implicated in the development and progression of pulmonary fibrosis. Although some of the mechanisms mediating the effects of IGFBP-5 and downstream signaling pathways have been identified, the effect of IGFBP-5 on other growth factors and proteins known to promote fibrosis has not been previously examined. Our goal was to determine whether IGFBP-5 can modulate the levels of known pro-fibrotic factors. Our findings demonstrate that IGFBP-5 increases expression of pro-fibrotic factors, creating a positive feedback loop that may explain how IGFBP-5 triggers fibrosis and perpetuates it.

MATERIALS AND METHODS

Lung Tissues

Lung tissues were obtained from patients with SSc and IPF undergoing lung transplantation at the Unviersity of Pittsburgh Medical Center under a protocol approved by the Institutional Review Board of the University of Pittsburgh and following written informed consent. Lung tissues were also obtained from organ donors (normal lung; NL) whose lungs were not used for transplantation under a protocol approved by the Institutional Review Board of the University of Pittsburgh.

Ex vivo Human Lung Culture and Stimulation

Human normal lung tissues were cut into approximately 3 mm^2 pieces, and 6 pieces of tissue were placed in each well of a 6-well plate in serum-free Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Inc., Manassas, VA, USA) supplemented with penicillin, streptomycin, and antimycotic agent (Invitrogen, Carlsbad, CA, USA). Lung tissue cores were treated with 500 ng/ml recombinant IGFBP-5 (rBP5) (GroPep Bioreagents Pty Ltd, Adelaide BC, Australia), a concentration within the physiological range found in the serum of healthy donors (16–19). 10 mM HCl was used as a vehicle control. RNA was extracted from lung tissues after 16 and 30 h of incubation.

Primary Human Lung Fibroblast Culture

Primary human lung fibroblasts were cultured from lung tissues of patients with SSc and IPF undergoing lung transplantation following written consent as previously described (12) under a protocol approved by the University of Pittsburgh Institutional Review Board. Primary fibroblasts were also cultured from the lung tissues of normal donors whose lungs were not used for transplantation (12). Briefly, $\sim$ 2–3 mm^2 pieces of tissue were minced and fibroblasts were cultured and maintained in DMEM (Mediatech, Inc., Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), penicillin, streptomycin, and antimycotic agent (Invitrogen, Carlsbad, CA, USA). Cells were used in passages 2–7.

In vitro Fibroblast Stimulation

Actively growing primary human lung fibroblasts were stimulated as previously described with some modifications (12). Briefly, 2.0×10^5 primary fibroblasts were plated in 35 mm tissue culture plates in 10% FBS-containing DMEM. After 24 h, the cells were serum-starved in DMEM for 24 h prior to stimulation with 500 ng/ml recombinant IGFBP-5 (GroPep Bioreagents Pty Ltd, Adelaide BC, Australia) or vehicle (10 mM HCl) and harvested after 1 and 3 h for RNA extraction. In addition, primary human lung fibroblasts were infected with adenovirus expressing human IGFBP-5 or a control adenovirus at a multiplicity of infection (MOI) of 50 as we previously described (12).

Adenovirus Construct Preparation

The full-length cDNA of human IGFBP-5 was generated as previously described (7, 12), cloned into the shuttle vector pAdlox with a C-terminal triplicate (3x) Flag tag, and used for the generation of replication-deficient adenovirus expressing IGFBP-5-3xFlag in the Vector Core facility at the University of Pittsburgh. Adenovirus expressing 3x Flag tag alone (AdCN-Flag) was used as a control (7, 14).

Small Interfering RNA (siRNA) Transfection

Primary human lung fibroblasts were seeded in 35 mm plates 24–48 h prior to transfection with siRNA. Insulin-like growth factor binding protein-5 (IGFBP-5) sequence-specific siRNA and negative control scrambled siRNA were purchased from Dharmacon™ (Lafayette, CO, USA). Transfection was done using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and 100 nM siRNA diluted in Opti-MEM I Reduced-Serum Medium (Life Technologies, Carlsbad, CA, USA) following the manufacturer's recommendation. Fibroblasts were harvested at 48 h.

Quantitative PCR

Total RNA was extracted from primary human lung fibroblasts using the RNeasy® mini kit (Qiagen Inc., Valencia, CA, USA). First-strand cDNA was reverse-transcribed with an oligo (dT)12-15 primer (Invitrogen, Carlsbad, CA, USA) and SuperScript IV Reverse Transcriptase (Invitrogen). Gene mRNA expression levels were evaluated by quantitative PCR using the TaqMan® real-time PCR system (Applied Biosystems, Foster

City, CA, USA) according to the manufacturer's protocol. Gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative expression levels of fibroblasts were compared to RNA levels using the comparative CT method formula $2^{-\Delta\Delta Ct}$. Specific primers and probes for amplifying genes encoding human collagen 1A1 (Col) (Hs00164004_m1), human fibronectin (FN) (Hs00365052_m1), human lysyl oxidase (LOX) (Hs00184700_m1), human IGFBP-5 (Hs00181213_M1), human connective tissue growth factor (CTGF) (Hs01026927_g1), and human GAPDH (Hs02758991_g1) were purchased from Applied Biosystems. Human B2M (Hs00187842_m1) was also used to confirm results obtained with GAPDH with no notable differences (data not shown).

Western Blot Analysis

Western blot analysis of fibroblast extracellular matrix fractions was done as previously described (12). The following antibodies were used: fibronectin (FN) monoclonal antibody (clone EP5), collagen type I (COL) polyclonal antibody, GAPDH monoclonal antibody (Santa Cruz, Dallas, TX, USA), vitronectin (VN) polyclonal antibody (Biogenesis, Poole, UK), and horseradish peroxidase-labeled secondary antibody (Santa Cruz, Dallas, TX, USA). Signals were detected using chemiluminescence (ProteinSimple, San Jose, CA, USA).

Statistical Analysis

For graphical purposes, fold-change estimates were calculated and displayed. For **Figure 1**, the data was analyzed using the Mann-Whitney U-test with 2-sided *p*-values. For the rest of the figures, due to a lack of normality of the underlying expression level fold-change estimate, comparisons between treatments (i.e., vehicle vs. IGFBP-5, control adenovirus vs. adenovirus expressing IGFBP-5-Flag, scramble siRNA vs. small interfering RNA targeting IGFBP-5) at each time point were conducted using Wilcoxon signed rank tests, which account for the fact that the data are paired within cell lines. Since pro-fibrotic effects of IGFBP-5 have been demonstrated in the past, one-sided hypothesis testing was used for these analyses. *P*-values < 0.05 were considered statistically significant, and no adjustment was made for multiple comparisons. SAS v9.4 (SAS Institute, Cary, NC, USA) or GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla, California, USA) were used for all analyses.

RESULTS

IGFBP-5 Expression Is Increased in Lung Tissues and Primary Fibroblasts From Patients With SSc

We previously reported that IGFBP-5 levels are increased in lung tissues of patients with IPF and primary fibroblasts derived from those lung tissues (12). We also reported that IGFBP-5 expression is increased in dermal fibroblasts from patients with SSc and dermal fibrosis (11). We now show that IGFBP-5 expression is also significantly elevated in lung tissues (**Figure 1A**) and matching primary fibroblasts (**Figure 1B**) from patients with SSc-associated lung disease. These and our previous findings suggest that IGFBP-5 is increased in different fibrotic tissues, skin and lung, and across two diseases, IPF and SSc.

IGFBP-5 Induces Extracellular Matrix and Pro-Fibrotic Gene Expression in Primary Human Lung Fibroblasts

We previously showed that IGFBP-5 promotes deposition of collagen and fibronectin in the extracellular matrix fraction of fibroblasts (12, 20). However, we had not examined whether the regulation of ECM production also occurred at the transcriptional level. To determine if exogenous recombinant IGFBP-5 contributes to the development of fibrosis by increasing expression of ECM genes, human primary lung fibroblasts from normal donors were treated with recombinant IGFBP-5 (rBP5) for 1 and 3 h and gene expression was measured using quantitative PCR. rBP5 treatment significantly increased expression of the ECM gene collagen 1A1 (Col) and showed an increasing trend in fibronectin (FN) (**Figures 2A,B**). Since several pro-fibrotic factors have been implicated in fibrosis, we also examined the effect of IGFBP-5 on CTGF, the ECM-crosslinking enzyme lysyl oxidase (LOX), and IGFBP-5 itself. IGFBP-5 resulted in an increased trend in expression of CTGF (**Figure 2C**), and significantly increased its own expression (**Figure 2D**) and the expression of LOX (**Figure 2E**) within 1 h of stimulation. Expression levels of all genes examined were comparable in IGFBP-5 and vehicle-treated fibroblasts 3 h post-treatment, suggesting that they are immediate early genes downstream of IGFBP-5 and may respond to IGFBP-5 stimulation in a transient manner. The increased production of collagen 1α1 (Col1α1) and fibronectin (FN) was also confirmed at the protein level in fibroblast ECM fractions (**Figure 2F** and **Supplemental Figure 1**). Our findings show that increased protein levels can be detected at earlier time points, and the protein response is sustained as we previously reported (10, 12, 20).

To further confirm the ECM-promoting effect of exogenous IGFBP-5 and compare it to adenovirally-mediated overexpression of the protein, IGFBP-5 was expressed in normal human primary lung fibroblasts using replication-deficient adenovirus as we previously described (7, 12). Primary lung fibroblasts were infected with adenovirus expressing full length human IGFBP-5 (AdIGFBP-5-3xFlag) or a control adenovirus (AdCN-3xFlag) for 16 h after which the media was changed and incubation was continued for an additional 6 or 12 h, corresponding to 22 and 28 h of total infection time, respectively. These time points were selected as they represent the earliest time points when adenovirally encoded IGFBP-5 protein is detected in the supernatants of primary lung fibroblasts (data not shown). We first confirmed expression of human IGFBP-5 in fibroblasts infected with AdIGFBP-5-3xFlag. Significantly increased IGFBP-5 expression levels were noted at both 22 and 28 h (**Figure 3A**). Adenovirally-mediated expression of IGFBP-5 was on average 259- and 463-fold higher than control virus-infected cells at 22 and 28 h, respectively. Collagen 1A1 expression showed a trend toward an increase at 28 h (**Figure 3B**), although the difference did not reach statistical significance, unlike the response to rBP5

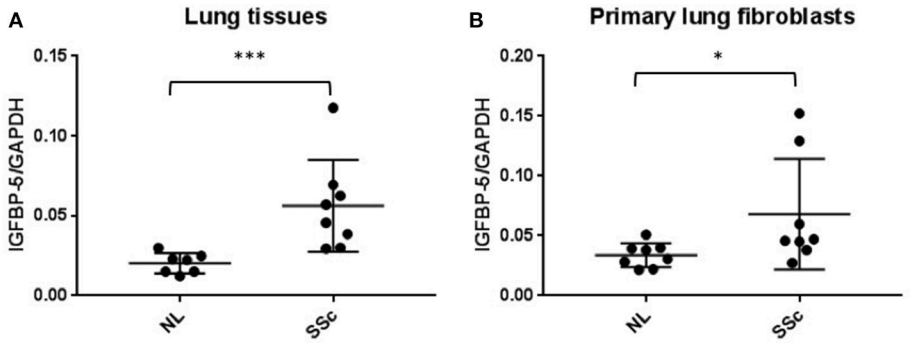

FIGURE 1 | IGFBP-5 mRNA levels are increased in lung tissues and primary fibroblasts of SSc patients. **(A)** Expression of IGFBP-5 was evaluated in lung tissues from 7 normal controls (NL) and 8 patients with SSc pulmonary fibrosis using real-time PCR. **(B)** Expression of IGFBP-5 was evaluated in human primary lung fibroblasts from 8 normal controls (NL) and 8 patients with SSc pulmonary fibrosis using real-time PCR. Each dot represents one individual sample. Graphical presentation of the data analyzed by Mann-Whitney U-test. Values represent mean ± standard deviation. $^{*}P < 0.05$, $^{***}P < 0.001$.

treatment. IGFBP-5 expression significantly increased mRNA levels of FN, CTGF, and LOX at 22 h (**Figures 3C–E**). Although AdIGFBP-5 infection significantly increased levels of CTGF at 22 h, it reduced its expression levels 28 h post-infection. Thus, both exogenously added recombinant IGFBP-5 and adenovirally expressed IGFBP-5 induce the expression of ECM and pro-fibrotic factors in primary human lung fibroblasts. We further validated increased ECM protein levels in the extracellular matrix fraction of fibroblasts using a representative donor fibroblast strain that had shown increased ECM gene transcription in response to IGFBP-5 (**Figure 3F**). Interestingly, increased collagen 1α1 (Col1α1) was noted in the ECM fraction of fibroblasts expressing IGFBP-5 for 24 h, although mRNA levels were not significantly increased. This could in part be due to the fact that IGFBP-5 acts by protecting ECM protein from degradation (20). The increased deposition of ECM proteins in the matrix of fibroblasts confirms our previously reported findings (12).

Silencing of IGFBP-5 Has Different Effects on the Expression of Extracellular Matrix and Pro-Fibrotic Genes in Fibroblasts From Normal and Fibrotic Lung Tissues

We previously reported increased IGFBP-5 production by fibroblasts from fibrotic skin and lung tissues (11, 12). To further understand the role of IGFBP-5 in the development of fibrosis, we silenced endogenous IGFBP-5 in primary human lung fibroblasts derived from lung tissues of normal donors (NL) and patients with IPF or SSc using a sequence-specific siRNA (siBP-5). Scrambled siRNA that in our experience parallels gene expression levels in untreated fibroblasts was used as a control (siCN). We first confirmed efficient silencing of IGFBP-5 expression using siBP5 (**Figure 4A**). siBP-5 resulted on average in 57, 72, and 69% reduction in IGFBP-5 mRNA levels in NL, IPF, and SSc primary human lung fibroblasts, respectively. Decreased IGFBP-5 expression was significant in IPF fibroblasts ($P = 0.03$) and trended toward significance in NL and SSc fibroblasts ($P = 0.06$). As shown in **Figure 4B**, silencing of IGFBP-5 in IPF fibroblasts significantly reduced Col1A1 expression, but had no significant effects on the expression levels of FN (**Figure 4C**). For the pro-fibrotic factors, silencing IGFBP-5 reduced CTGF levels in IPF fibroblasts (**Figure 4D**), whereas silencing endogenous IGFBP-5 increased LOX expression (**Figure 4E**) at the examined time point of 48 h. Similar to what we observed in IPF fibroblasts, silencing endogenous IGFBP-5 in NL fibroblasts also increased LOX expression (**Figure 4E**). Increases in LOX expression did not reach statistical significance as our hypothesis was one-sided. In NL and SSc fibroblasts, silencing IGFBP-5 had no effect on the other genes examined, although CTGF levels showed a trend toward decrease in SSc fibroblasts ($P = 0.06$). Thus, the effect of silencing endogenous IGFBP-5 in healthy and diseased lung tissue fibroblasts had different effects on the expression of ECM and pro-fibrotic genes. The difference in response of cells from different donors and diseases may be due to the different extent of silencing of IGFBP-5, with more efficient silencing of endogenous IGFBP-5 noted in IPF fibroblasts.

IGFBP-5 Induces Expression of ECM and Pro-Fibrotic Factors *ex vivo*

To extend our findings from the *in vitro* studies using primary fibroblasts, we examined the effect of rBP5 *ex vivo* in human normal donor lung tissues maintained in organ culture. Lung tissue cores were treated with rBP5 (500 ng/ml) or 10 mM HCl as a vehicle control for 16 and 30 h. Recombinant IGFBP-5 increased expression of the ECM genes Col1A1 and FN (**Figures 5A,B**), although the increase in Col1A1 did not reach statistical significance whereas that of FN did. IGFBP-5 also significantly increased expression of CTGF in human lung tissues (**Figure 5C**). Furthermore, IGFBP-5 significantly increased LOX expression (**Figures 5D,E**). Although the increase in ECM and CTGF occurred at 16 h and was reduced by 30 h (data not shown), the induction of LOX was sustained through 30 h. This suggests that the increase in ECM occurs early while the increase in the levels of LOX is sustained, thus providing longer ECM crosslinking activity. Thus, IGFBP-5 can promote expression of

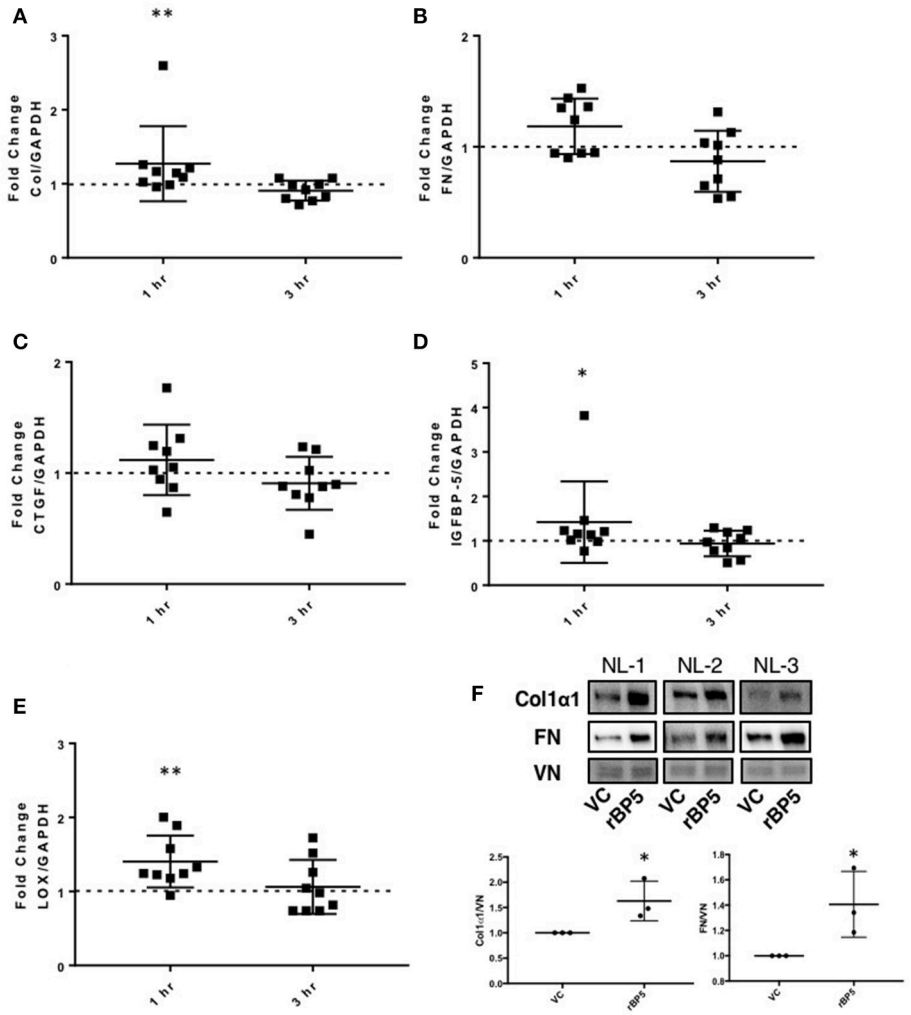

FIGURE 2 | IGFBP-5 exogenously promotes extracellular matrix (ECM) and pro-fibrotic factor production *in vitro*. Primary human lung fibroblasts were treated with vehicle control or recombinant protein IGFBP-5 (rBP5). Samples were harvested after 1 and 3 h of stimulation. Gene expression levels were quantified using qPCR, and fold-change estimates were calculated to compare rBP5 to vehicle. The following genes were analyzed: **(A)** Collagen 1A1. **(B)** FN. **(C)** CTGF. **(D)** IGFBP-5. **(E)** LOX. The data were obtained from 9 different experiments using fibroblasts from lung tissues of 9 different individual normal donors. Graphical presentation of the data analyzed by one-sided Wilcoxon signed rank tests. A dotted line at a fold-change of 1.0 (i.e., which would represent no change) is provided in each graph for reference. **(F)** Immunoblotting results of extracellular matrix fractions of primary human lung fibroblasts from three different donors treated with vehicle or rBP5 for 1 h. Upper images: Collagen1α1 (COL) and Fibronectin (FN) in ECM fractions from an equivalent number of fibroblasts were detected by immunoblotting and signals were normalized to vitronectin (VN). Lower graphs: Graphical presentation of the data analyzed by one-sided paired *t*-test. Values represent mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$.

ECM and fibrotic genes *ex vivo* in human lung tissues, validating and extending our *in vitro* findings.

DISCUSSION

Pulmonary fibrosis is a manifestation of diseases such as SSc and IPF. Elevated levels of IGFBP-5 have been detected in primary fibroblasts from both of those diseases and *in vivo* in fibrotic lung tissues (12). Further, IGFBP-5 expression is increased in liver fibrosis *in vivo* and during hepatic stellate cell (HSC) transdifferentiation *in vitro* (21, 22). IGFBP-5 has also been implicated in different types of cancers such as breast, ovarian, and colorectal cancer as well as in wound healing and tissue regeneration (6, 23–26). The effects of IGFBP-5 are known to be cell type- and tissue-specific.

Fibroblasts are essential effector cells responsible for the increased production of ECM and thus fibrosis in different organs (27). As a result, examining the response of fibroblasts to both pro- and anti-fibrotic factors is essential for elucidating mechanisms that regulate these cells in the setting of fibrosis. In fact, fibroblasts have been widely used for assessing the effect of pro-fibrotic factors and for testing the efficacy of potential anti-fibrotic therapies (27). To complement findings using pulmonary

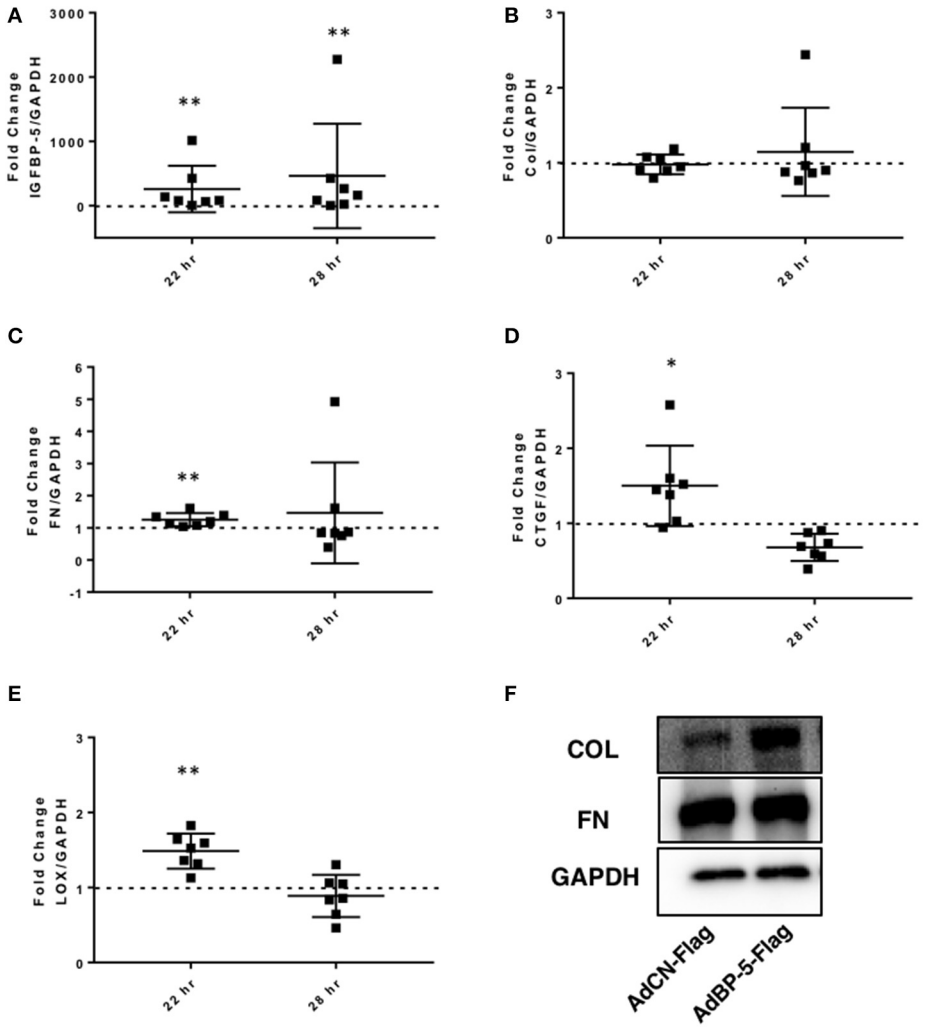

FIGURE 3 | IGFBP-5 expression promotes extracellular matrix (ECM) and pro-fibrotic factor expression *in vitro*. Primary human lung fibroblasts were infected with control adenovirus AdCN-Flag or adenovirus expressing IGFBP-5-Flag (AdBP-5-Flag). Samples were harvested after 22 and 28 h of infection. Levels of expression were quantified using qPCR at 22 and 28 h, and post-infection fold-change estimates were calculated to compare AdBP-5-Flag to control. The following genes were analyzed: **(A)** IGFBP-5. **(B)** Collagen 1A1. **(C)** FN. **(D)** CTGF. **(E)** LOX. The data are obtained from 7 different experiments using fibroblasts from lung tissues of 7 different normal donors. Graphical presentation of the data analyzed by one-sided Wilcoxon signed rank tests. Values represent mean ± standard deviation. A dotted line at a fold-change of 1.0 (i.e., which would represent no change) is provided in each graph for reference. **(F)** Representative protein levels of Collagen 1A1 (Col) and Fibronectin (FN) in the ECM fraction from an equivalent number of fibroblasts infected with AdCN-Flag or adenovirus expressing IGFBP-5-Flag (AdBP-5-Flag) for 24 hr and analyzed by immunoblotting. GAPDH was detected in the corresponding lysates using immunoblotting. $^{*}P < 0.05$, $^{**}P < 0.01$.

fibroblasts, lung tissue slices have been used to extend *in vitro* findings and establish their relevance in a human tissue. We have used a similar approach with our own modifications using lung tissue cores rather than slices for assessing the effects of IGFBP-5 in human lung tissues. This allows us to extend our findings from isolated cells in culture and use a model that is more comparable to the *in vivo* milieu.

In this study, we investigated the effect of endogenous and exogenous IGFBP-5 on fibroblast production of ECM components and factors involved in the promotion of fibrosis in different organs. Our results demonstrate that the increase in IGFBP-5 levels is a primary and early event in pulmonary fibrosis since IGFBP-5 induces expression of ECM and pro-fibrotic genes as early as 1 h post-stimulation. The rationale for examining both exogenously added recombinant IGFBP-5 and the adenovirally expressed form is that these forms of IGFBPs may exert different effects (28, 29). As we previously reported (12), both adenovirally-expressed and exogenous IGFBP-5 promote ECM deposition in primary human lung fibroblasts (12). In contrast, in osteosarcoma cells, endogenous and exogenous IGFBP-5 have been shown to exert opposite effects (29). Further, Yamaguchi et al. (20) demonstrated a role for IGFBP-5 trafficking into fibroblasts and ECM-protective effects of extracellular IGFBP-5 (20). Our findings suggest that endogenous and exogenous

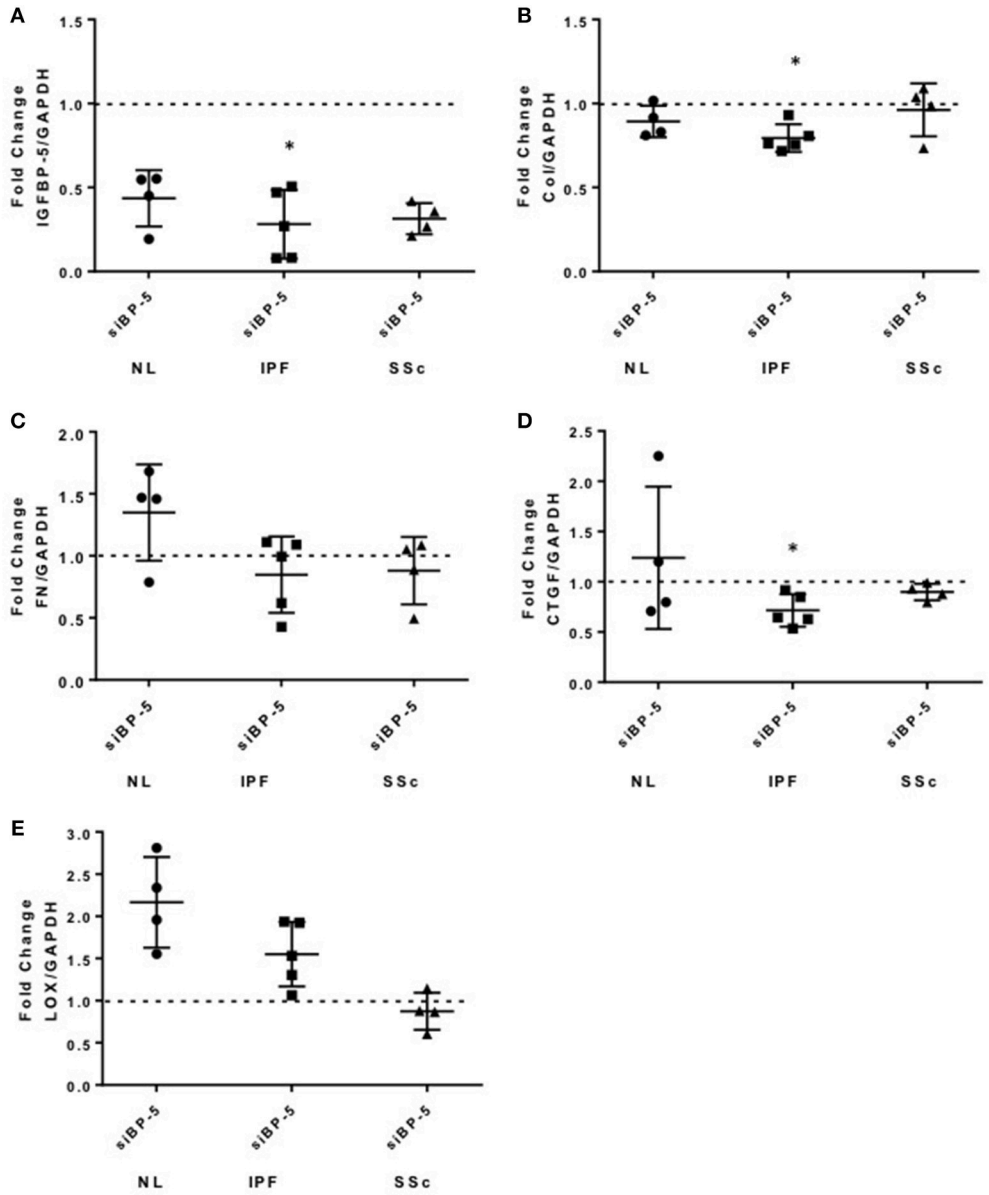

FIGURE 4 | Knockdown of IGFBP-5 shows variable effects in primary pulmonary fibroblasts from different donors. IGFBP-5 was silenced in primary human lung fibroblasts from normal lung (NL, circles) and lung tissues of patients with IPF (squares) and SSc (triangles) using small interfering IGFBP-5 (siBP-5) or scramble siRNA as a negative control (siCN) for 48 h. Levels of expression were quantified using qPCR, and fold-change estimates were calculated to compare siBP-5 to siCN. The following genes were analyzed: **(A)** IGFBP-5. **(B)** Collagen 1A1. **(C)** FN. **(D)** CTGF. **(E)** LOX. The data shown are obtained using fibroblasts from 4 different normal donors (NL), 5 patients with IPF and 4 patients with SSc. Graphical presentation of the data analyzed by one-sided Wilcoxon signed rank tests. A dotted line at a fold-change of 1.0 (i.e., which would represent no change) is provided in each graph for reference. Values represent mean ± standard deviation. $^*P < 0.05$.

IGFBP-5 may exert similar effects on the expression of certain genes such as ECM components and CTGF which were induced by rBP5 and reduced by silencing endogenous IGFBP-5. In contrast, our data show that endogenous and exogenous IGFBP-5 may exert opposite effects on other genes examined in the same cells. This is the case for LOX expression which was induced by recombinant IGFBP-5 and silencing of endogenous IGFBP-5. It is possible that endogenous IGFBP-5 may provide an inhibitory effect and silencing endogenous expression would support the pro-fibrotic effects of recombinant exogenous IGFBP-5. Thus, understanding the effect of both endogenous and exogenous IGFBP-5 will lead to a better understanding of the role that compartmentalization of this protein plays in fibrosis. Our findings support the concept that endogenous and exogenous IGFBP-5 might exert different effects in primary human lung fibroblasts from healthy and diseased tissues as well. Since

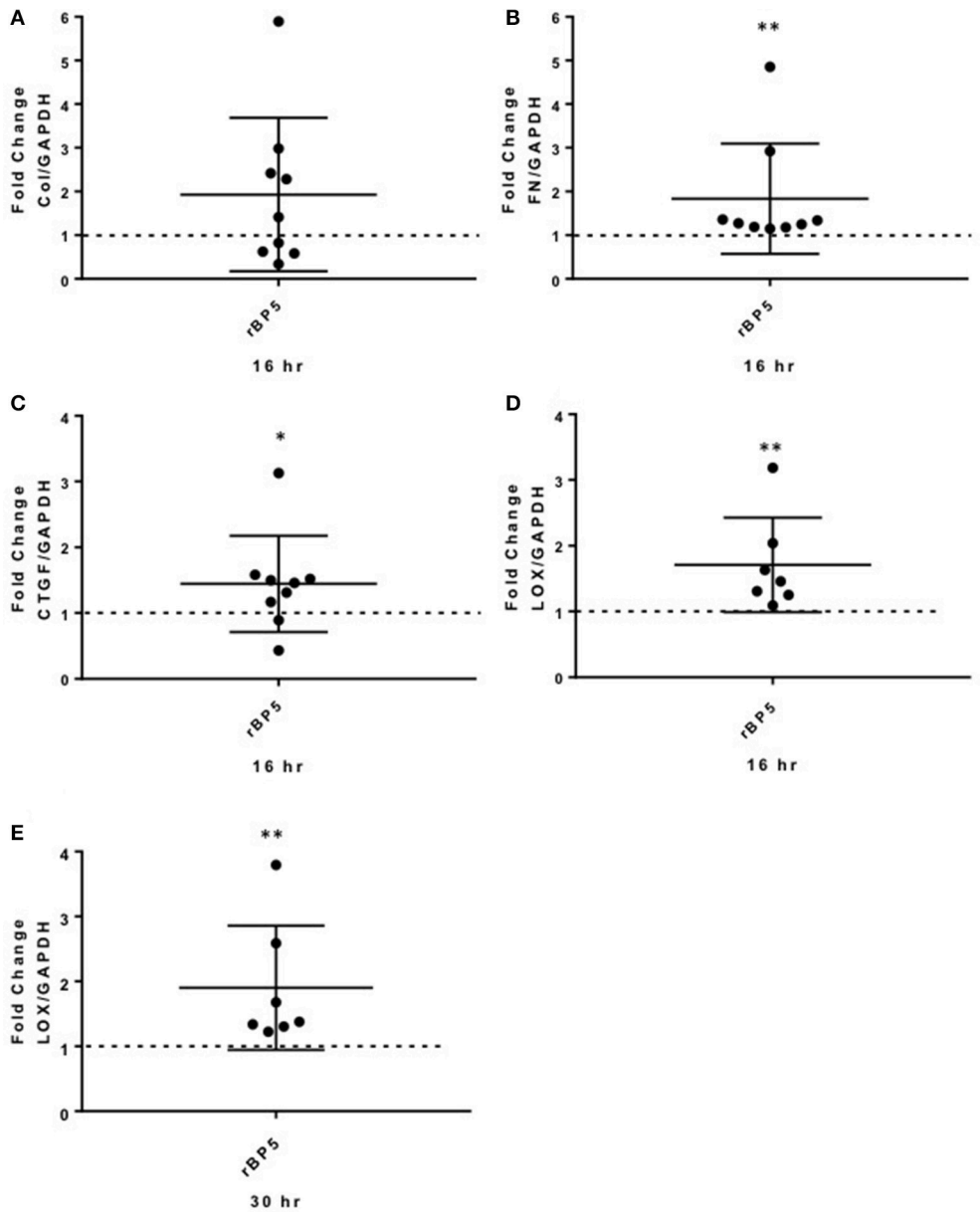

FIGURE 5 | IGFBP-5 promotes ECM and pro-fibrotic factor production *ex vivo* in human lung tissues. Human lung tissue cores were treated with vehicle control (VC, 10 mM HCl) or recombinant IGFBP-5 (rBP5, 500 ng/ml) for 16 and 30 h. Gene expression levels were measured using qPCR at the indicated time points, and fold-change estimates were calculated to compare IGFBP-5 to VC. The following genes were analyzed: **(A)** Collagen 1A1 mRNA levels at 16 h. **(B)** FN mRNA levels at 16 h. **(C)** CTGF mRNA levels at 16 h. **(D)**. LOX mRNA levels at 16 h. **(E)** LOX mRNA levels at 30 h. The data shown are obtained from lung tissues of 7–9 different donors. Graphical presentation of the data analyzed by one-sided Wilcoxon signed rank tests. A dotted line at a fold-change of 1.0 (i.e., which would represent no change) is provided in each graph for reference. Values represent mean ± standard deviation. $^{*}P < 0.05$; $^{**}P < 0.01$.

IGFBP-5 is a secreted protein, this suggests that localization of IGFBP-5 intracellularly or extracellularly may dictate its effects on cell function.

We had previously shown that IGFBP-5 can trigger a fibrotic phenotype *in vitro* in primary human fibroblasts, *in vivo* in mouse skin and lung, and *ex vivo* in human skin in organ culture (12–15). However, the question as to whether the promotion of fibrosis was directly mediated by IGFBP-5 or via other pro-fibrotic factors that may be downstream of IGFBP-5 had remained unanswered. In fact, our data show that IGFBP-5 not only directly induced the expression of ECM genes such as collagen I and fibronectin, but it also increased the expression of pro-fibrotic genes such as CTGF and IGFBP-5 itself. Further, IGFBP-5 increased the expression of LOX, an enzyme responsible for the covalent crosslinking of extracellular matrix proteins such as collagen and elastin (30, 31). Elevated expression of cytokines

and growth factors with pro-fibrotic activity such as CTGF have been reported in SSc and related diseases. For example, the pro-fibrotic activity of CTGF is well documented. Mori et al. (32) reported that subcutaneous injection of TGF-β and CTGF promoted dermal fibrosis. The investigators demonstrated that persistent fibrosis required both CTGF and TGF-β stimulation and that CTGF alone caused little granulation (32). In contrast, others have shown that CTGF is required for bleomycin-induced skin fibrosis in mice (33), and transgenic targeted expression of CTGF alone in fibroblasts promotes fibrosis in different organs including skin, lung, kidney, and vasculature (34). The critical role of CTGF in fibrosis has been the focus of ongoing research and the development of potential therapies targeting this growth factor (35). For example, a recent study by Makino et al. (36) examined the therapeutic effect of CTGF inhibition using anti-CTGF monoclonal antibody (36). CTGF inhibition reduced inflammation and vascular damage in a murine model of fibrosis (36). We propose that IGFBP-5 promotes pulmonary fibrosis by directly inducing expression of ECM genes and by increasing levels of other pro-fibrotic proteins such as CTGF, resulting in further increase of ECM production. Thus, IGFBP-5 is likely to promote fibrosis by working in concert with other pro-fibrotic factors, such as CTGF, which then potentiate the anti-fibrotic activity of IGFBP-5.

In addition to regulating ECM and growth factor genes, IGFBP-5 also increased expression of LOX, an enzyme responsible for cross-linking the matrix. Thus, in addition to increasing expression of ECM components, IGFBP-5 also promotes their cross-linking via increasing LOX levels, thus altering tissue structure and function. The critical role of LOX in fibrosis has been demonstrated in several studies. For example, targeting LOX has been shown to reduce peritoneal fibrosis (37) cardiac fibrosis (38) and pulmonary fibrosis (39–41). LOX has even been proposed as a biomarker of fibrosis in patients with SSc (42). Thus, induction of LOX by IGFBP-5 further potentiates the pro-fibrotic effects of IGFBP-5 by increasing the enzymatic crosslinking of collagen and other matrix components, rendering the ECM more resistant to proteolytic degradation.

The induction of pro-fibrotic genes by IGFBP-5 showed an immediate early response pattern. Such a pattern has been reported for other pro-fibrotic factors such as TGF-β which increases the expression of genes such as early growth response (Egr-1) (43), inhibitor of differentiation 1 (Id1) (44, 45) and CTGF (46) early while it induces collagen levels in a delayed manner. Growth factors typically induce an immediate early transcriptional response that is independent of protein synthesis and a more delayed response that requires proteins. Often, these immediate early genes are transcription factors. The immediate early response to IGFBP-5 stimulation of fibroblasts follows the pattern typical of growth factors in the kinetics of its effects, although its immediate early gene response includes ECM components and growth factors. We recently showed that IGFBP-5 with a mutated NLS that abrogates its nuclear localization retains the ability to induce dermal fibrosis when expressed in primary human fibroblasts or in human skin in organ culture using an adenoviral vector (7). This may reflect (a) the ability of secreted IGFBP-5 to bind ECM components and protect them from degradation, thus promoting ECM accumulation and fibrosis, and/or (b) the possibility that cytoplasmic IGFBP-5 can promote the translocation of a "partner" factor/protein to the nucleus, thus exerting transactivating effects, which might be further facilitated by target genes being in a "transcriptionally poised" state—a chromatin state that allows for rapid gene activation—thus allowing for an "immediate early" gene response.

Growth factors mediating their effects via other cytokines or pro-fibrotic factors has also been previously noted in the scientific literature. For example, polypeptide growth factors such as the platelet-derived growth factor (PDGF) (47) family and the epidermal growth factor (EGF) (48) family are also known to mediate pro-fibrotic effects by inducing expression of extracellular matrix genes directly or as mediators of the effects of TGF-β (47, 48). Further, the pro-fibrotic factor, IL-6 is known to induce collagen directly, and to also regulate IGFBP-5 (49, 50). O'Reilly et al. (50) examined the effects of IL-6 *trans* signaling in mediating fibrosis. Their study showed that IL-6 mediated fibrosis through enhanced TGF-β signaling which was due to Gremlin-1, a bone morphogenetic protein antagonist and a member of TGF-β family (50). We now add IGFBP-5 as an upstream regulator of pro-fibrotic growth factor gene expression, suggesting that IGFBP-5 regulation of genes such as CTGF may be one of the mechanisms that sustain its fibrotic effects.

To further delineate the role of endogenous IGFBP-5 in primary fibroblasts, we silenced IGFBP-5 expression in fibroblasts from normal donors and patients with IPF and SSc. Knock down of IGFBP-5 showed that NL, IPF, and SSc fibroblasts respond differently to a reduction in endogenous IGFBP-5 expression. Since silencing does not result in a complete loss of IGFBP-5, it is plausible that the variable effects are due to the fact that fibroblasts from different diseases and different individuals vary in their sensitivity to IGFBP-5 and that some retain their phenotype with residual low levels of expression of IGFBP-5. Use of tissue-derived primary fibroblasts from different donors has inherent challenges with respect to variability of respone to stimuli (which is noted even in response to potent pro-fibrotic factors such as TGF-β), however it provides greater relevance of findings to human disease than immortalized cell lines. Several factors may contribute to the variability of the response, including different kinetics in fibroblasts from different donors, variable levels of the IGFBP-5 "receptor," dissimilar levels of secreted proteases that target IGFBP-5, as well as differing propensities of individuals to develop fibrosis, to name a few. In contrast to the variability we see with primary fibroblasts, IGFBP-5 silencing was shown to consistently affect the survival of hepatic stellate cells in liver fibrosis due to increased cell apoptosis (22). Our findings show that reducing endogenously expressed IGFBP-5 does not necessarily impair the fibrotic phenotype in all fibroblasts examined, at least within the duration of transient *in vitro* silencing and to the extent of silencing observed in fibroblasts from different donors, and confirm the diverse functions of IGFBP-5 in different cells. They further suggest that targeting extracellular IGFBP-5 in diseases such as SSc or IPF where IGFBP-5 levels are elevated may be a more appropriate strategy for ameliorating fibrosis.

In summary, our data builds on our previous findings and provides new compelling evidence that IGFBP-5 is directly involved in the pathogenesis of pulmonary fibrosis by increasing production of extracellular matrix proteins and indirectly by inducing expression of growth factors that promote and sustain fibrosis. IGFBP-5 also functions in an autocrine manner to increase its own expression, further potentiating the fibrotic effect. Our current study establishes the role of IGFBP-5 as an important mediator in fibrosis that is upstream of known pro-fibrotic factors, suggesting that strategies to inhibit IGFBP-5 function might be effective for the amelioration of fibrosis.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Medical University of South Carolina and University of Pittsburgh Institutional Review Boards (IRB) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the IRB of our institution where tissues were obtained.

AUTHOR CONTRIBUTIONS

X-XN performed the research, collected data, analyzed and interpreted data, and wrote the manuscript. LM and PN analyzed the data. CF-B designed the research, interpreted data, supervised and organized the study, wrote and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2018.00601/full#supplementary-material

Supplemental Figure 1 | Original uncropped images for **Figure 2F** showing that IGFBP-5 increases collagen and fibronectin in the ECM fraction.

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