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Review

Essential roles of IGFBP-3 and IGFBP-rP1 in breast cancer

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Abstract

Insulin and insulin-like growth factors (IGFs) have critical functions in growth regulatory signalling pathways. They are part of a tightly controlled network of ligands, receptors, binding proteins and their proteases. However, the system becomes uncontrolled in neoplasia. The insulin-like growth factor binding protein 3 (IGFBP-3) and the insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) have unique properties among the sixteen known members of the IGFBP superfamily. IGFBP-3 has very high affinity for IGFs ($k_d \sim 10^{-10}$ M), it transports > 75% of serum IGF-I and -II, whereas it's affinity for insulin is very low. On the other hand, IGFBP-rP1 binds insulin with very high affinity (500-fold higher compared to other IGFBPs), but has low affinity for IGF-I and -II proteins ($k_d = 3 \times 10^{-8}$ M). In this review, we have examined the roles of IGFBP-3 and IGFBP-rP1 in breast cancer, and discuss the potential impact of these two proteins in mammary carcinoma risk assessment and the development of treatments for breast cancer.

Keywords: Insulin-like growth factor-binding protein 3; IGFBP-related protein 1; Breast cancer; Therapeutic possibilities

1. Introduction

The insulin-like growth factor (IGF) signalling pathway plays a key role in regulating proliferation, differentiation and apoptosis in mammalian organisms. Energy metabolism, body size, ageing and cancer risk are influenced by insulin and IGFs [1,2].

The IGF system involves the complex coordination of growth factors (IGF-I and IGF-II), and the cell surface receptors (IGF-IR, IGF-IIR and the insulin receptor, IR), high-affinity binding proteins (IGFBP-1 to 6), IGFBP proteases, as well as several low affinity IGFBP-related proteins (IGFBP-rP1 to 10). In addition to endocrine effects exerted by circulating IGFs that are mainly produced in the liver, locally produced IGFs exert paracrine as well as autocrine effects on cell proliferation (Fig. 1) (reviewed in [1,3]).

IGF signalling, mediated by interaction with IGF-Rs is modulated by IGFBPs and their regulators, which influence IGF bioavailability [3,4] (Fig. 1). IGF bioactivity is not only dependent on interaction with IGF-Rs, but is also influenced by the multifunctional family of IGFBPs. When bound to IGFs, IGFBPs function by regulating their transport between intra- and extravascular spaces and by interaction with their receptors [5], prolonging IGF-I/II half-life [6] and precluding their

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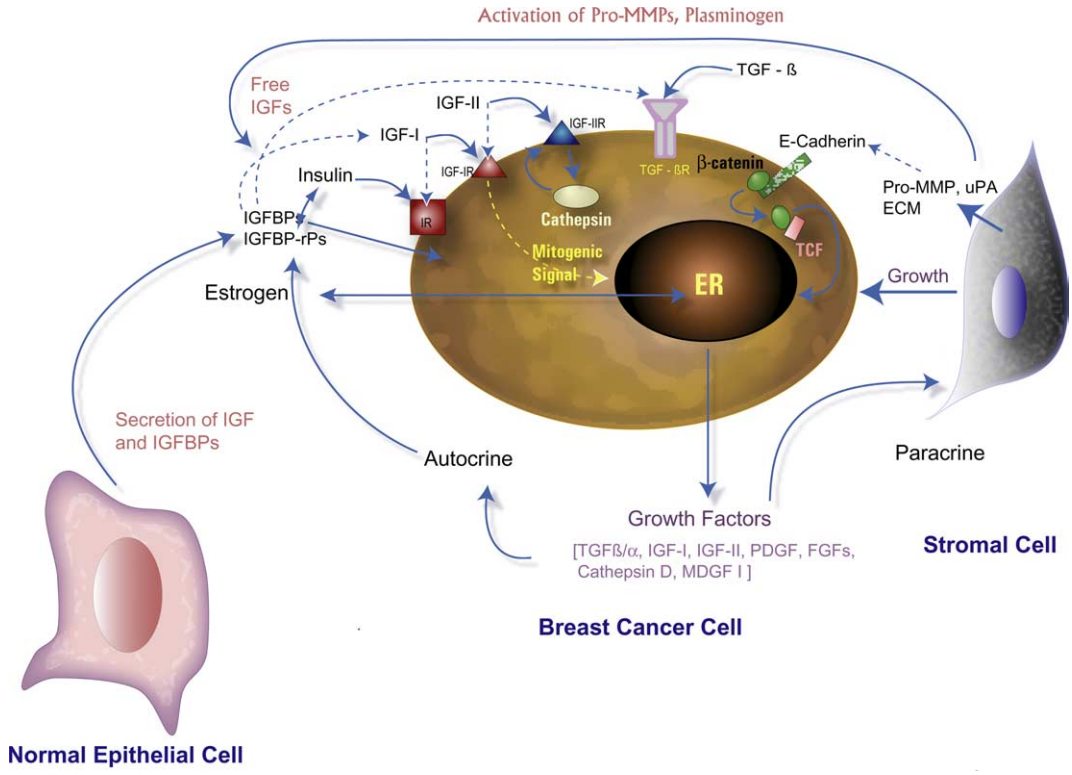

Fig. 1. Overview of the IGF-signalling pathway and interactions with other important growth regulators in breast cancer. The IGFs interact with specific cell surface receptors designated IGF-IR IGF-IIR, and can also interact with the insulin receptor (IR). The mitogenic effects of IGFs are mainly mediated through interactions with the type I IGF receptor, which like the insulin receptor is a receptor with tyrosine kinase activity. The type II IGF receptor is structurally distinct and binds primarily IGF-II. Insulin-like growth factor-binding proteins (IGFBPs) and related proteins (IGFBP-rPs) interact with the receptor ligands, but can also have direct effects on the cancer cell. Some IGFBPs are thought to have a surface receptor and the latter is believed to be the TGF-β receptor. Paracrine and autocrine loops exist through which growth factors such as TGF-β, oestrogen and components of the extracellular matrix (ECM) can influence the IGF system. ER, oestrogen receptor; MMP, matrix metalloproteinase; uPA, urokinase-type plasminogen activator; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; MDG I, mammary-derived growth factor-1; TCF, T cell factor (reviewed in [3] and [8,37,75,92,96]).

mitogenic activity (Fig. 1) [7]. Other related IGFBP activities include cell motility and adhesion, as well as regulation of TGF-β and nuclear signalling (Fig. 1) [8].

This review focuses on IGFBP-3 and the related protein IGFBP-rP1, which have indispensable roles in breast cancer.

1.1. The IGFBP superfamily

The members of the IGFBP superfamily are all secreted, cysteine-rich factors that share a common conserved N-terminal cysteine-rich domain termed IGFBP motif (GCGCCXXC). The IGFBP motif is encoded by a single exon and has overall similar topology and is only present in vertebrates. These members include six proteins (IGFBP-1 to IGFBP-6) that bind IGFs with high affinity and a group of ten IGFBP-related proteins (IGFBP-rPs 1-10) that bind IGFs with low affinity [9–12]. The 100-times less affinity of IGFBP-rPs for IGFs is believed to result from loss of the C-terminal domain and both C- and N-termini seem to be required for high affinity binding [3]. The IGFBPs have greater affinity for IGFs than the IGF receptors: IGFBP-1 to 6 have a k_d for IGF-I/-II of $\sim 10^{-10}$ M, whereas the affinity of IGF I/II for type I IGF receptors, $k_d = 10^{-8}$ to 10^{-9} M, is considerably lower [3]. IGFBPs exert endocrine, paracrine and autocrine effects, but can also have activities independent of IGF action (Fig. 1).

IGFBP activity is controlled by IGFBP-degrading proteases, which include several matrix metalloproteinases (MMPs) such as MMP-7 and MMP-9 (Figs. 1–3) [13–15]. Post-translational modifications including glycosylation and phosphorylation can also influence IGFBP activities. IGFBPs might be differentially targeted to tissues depending upon both their primary structure and their posttranslational modifications: glycosylation affecting cell interactions, phosphorylation affecting IGF-binding affinity and susceptibility to proteases, and proteolysis affecting both IGF-dependent and IGF-independent actions [8].

2. Insulin-like growth factor-binding protein-3

IGFBP-3 was initially described as one of three secreted distinct polypeptide growth factors with high

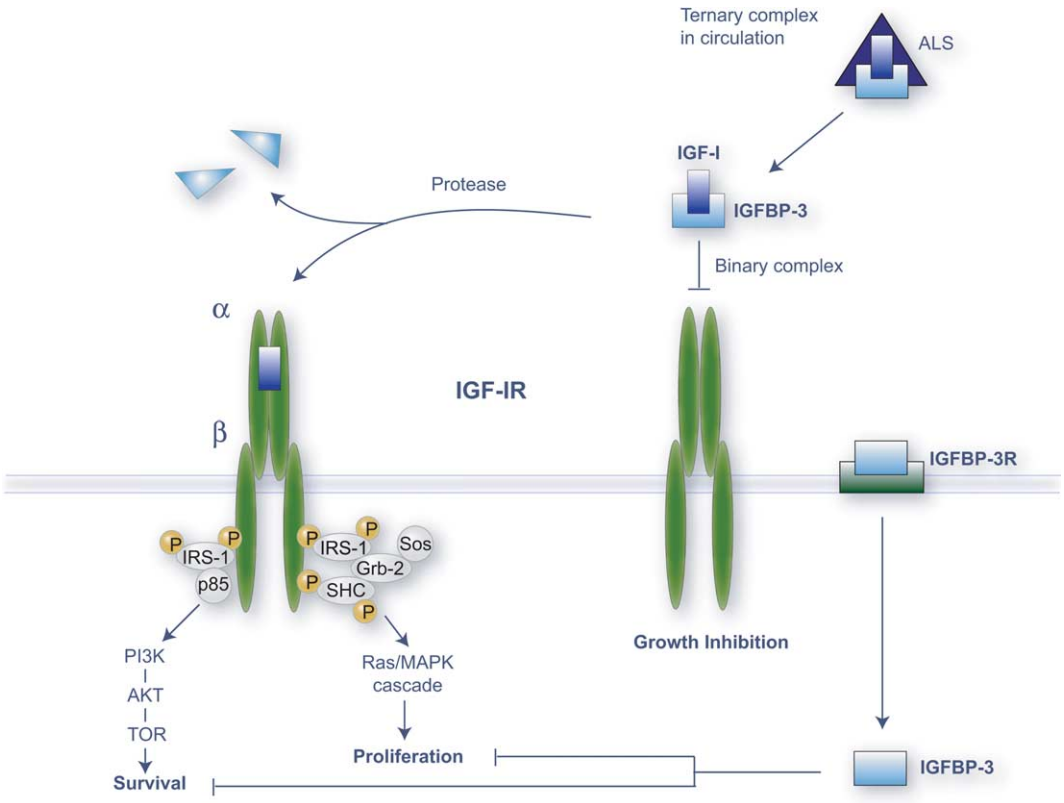

Fig. 2. IGFBP-3 and signal transduction pathways activated by the IGF-IR. IGF bioavailability is determined by sequestration in ternary complexes with IGFBP-3 and ALS in the circulation, or in binary complexes with IGFBP-3 in the cellular environment. Modification of IGFBP-3 (e.g., proteolysis, phosphorylation, glycosylation, interaction with the cell surface or extracellular matrix) liberates IGF-I, enabling interaction with the IGF-IR. Ligand binding activates the tyrosine kinase activity of the IGF-IR, which phosphorylates itself and several downstream targets, including the adaptor proteins IRS-1 and SHC, which link the IGF-IR to the PI3K and Ras-MAPK signalling pathways leading to cell survival and proliferation, respectively. IGFBP-3 also has IGF-independent antiproliferative and proapoptotic effects, which are thought to be mediated by its association with incompletely characterised cell surface proteins or receptors, the TGF-β receptor has been implicated as an IGFBP-3 cell surface binding protein. ALS, acid-labile subunit; P, indicates phosphorylation; PI3K, phosphatidylinositol-3 kinase; p85, PI3 kinase subunit P85; IRS-1, insulin receptor substrate-1; MAPK, mitogen activated protein kinase (reviewed in [1]).

affinity IGF binding noted in conditioned media of breast cancer cells [16]. The IGFBP-3 gene was cloned as part of a cluster of six different insulin-like growth factor-binding proteins (IGFBPs) from rat and human tissues. Shimasaki and Ling found that rat and human IGFBP-3 possess multiple N-linked glycosylation sites [17].

2.1. IGFBP-3 binding partners

IGFBP-3 is the largest and the most abundant circulating IGFBP. It carries 75% or more of serum IGF-I and IGF-II in binary or ternary complexes that also contain ALS (acid-labile subunit), a liver-derived growth hormone-regulated glycoprotein (Fig. 2) [8,18]. Approximately 90% of IGFBP-3 circulates in these complexes in healthy adults. The IGFBP-3 C-terminal domain, notwithstanding its ability to bind IGF, has also been shown to be essential for interactions with the acid-labile subunit through the basic region Lys 228–Arg 232 [3].

Further, related to its central role in regulating IGF bioavailability *via* sequestration in circulating ternary complexes, IGFBP-3 also competitively inhibits IGF action at the cellular level. This characteristic has been demonstrated by a number of experiments in various cell types, using an IGF-I analogue des-(1-3)-IGF-I, that binds IGF-IR and stimulates DNA synthesis but cannot bind IGFBP-3. In these studies IGFBP-3 inhibited cellular proliferation and oestradiol production induced by IGF-I or IGF-I and follicle-stimulating hormone, respectively, but not by des-(1-3)-IGF-I. [19,20].

2.2. IGF-independent cellular actions of IGFBP-3

IGFBP-3 is believed to act in an IGF-independent manner by initiating intracellular signalling from a cell surface receptor and/or by direct nuclear action (Fig. 2). IGFBP-3 can translocate into the nucleus from the extracellular compartment in rapidly dividing human breast cancer cells even if bound to IGFs [21]. IGFBP-3 is active in the cellular environment as a potent antiproliferative agent where it functions by cell cycle blockade and induction of apoptosis, independent of IGF-binding (Fig. 2).

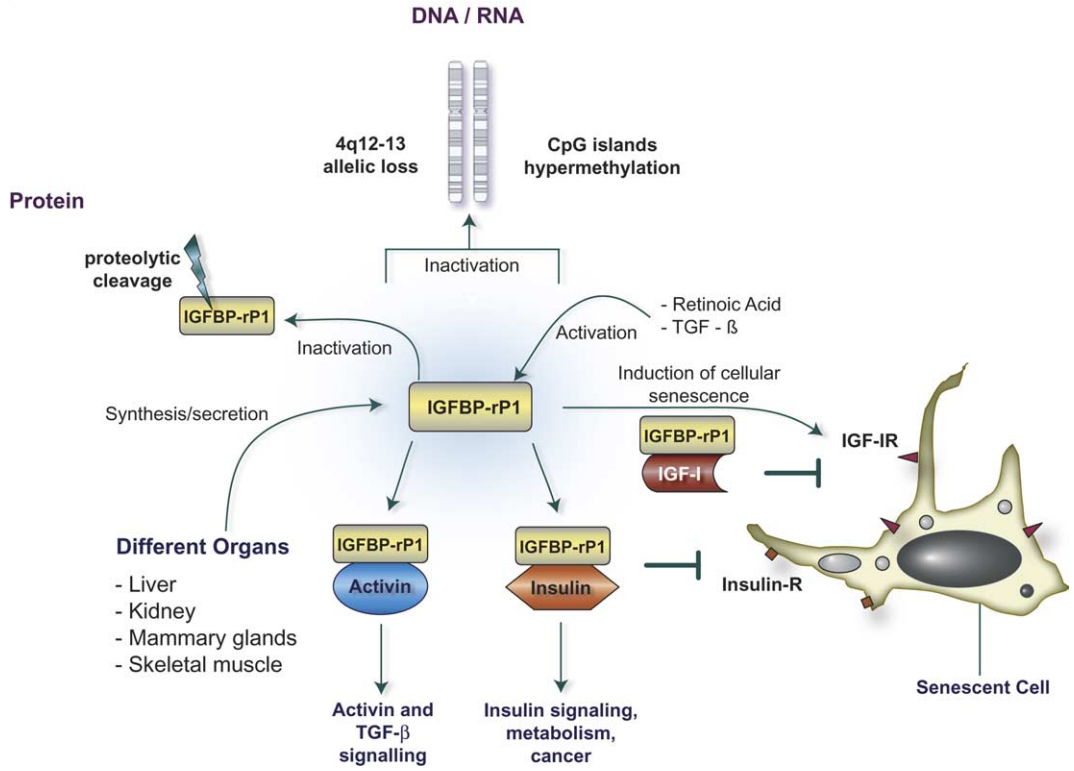

Fig. 3. IGFBP-rP1 actions and regulation. IGFBP-rP1 is expressed at the mRNA and protein level in most normal tissues including liver, kidney, mammary glands and skeletal muscle and is secreted into circulation. It can be found in many body fluids. Major IGFBP-rP1 binding partners are insulin, IGF-I and activin, not shown is type IV collagen. If bound to the ligands for insulin and IGF-I receptors, IGFBP-rP1 neutralises mitogenic signalling and induces senescence in breast and prostate cancer cells. IGFBP-rP1 levels can be regulated by retinoic acid and TGF-β, which induce IGFBP-rP1. The protein is inactivated by proteolytic cleavage involving a trypsin-like serine proteinase. In breast cancers, allelic loss at the IGFBP-rP1 locus, can lead to its down-regulation as well as hypermethylation of CpG islands.

A direct role for IGFBP-3 in transcriptional regulation has been proposed based on its localisation in the nucleus in several cell types [22–24]. It was later shown that IGFBP-3 has a C-terminal nuclear localisation signal [21], which allows its translocation to the nucleus by β-importin [25]. Subsequently, Liu et al. [26] demonstrated an essential role for the nuclear retinoid X receptor α (RXR-α) in mediating IGFBP-3-induced apoptosis, which is stimulated by RXR ligands.

Besides retinoic acids, cytokines such as TGF-β have been described to influence the levels and cellular actions of IGFBP-3. An increase in IGFBP-3 expression was observed followed by a decrease in the activity of the protein kinase B after exposure of mammary epithelial cells to TGF-β1 [27]. Interestingly, in breast cancer cells, the epithelial growth-inhibitory actions of IGFBP-3 are mediated through specific binding of IGFBP-3 to cell surface molecules that are not type I IGF receptors. A specific IGFBP-3 receptor however has not been cloned, but there is some evidence that the type V transforming growth factor-β receptor, a membrane glycoprotein that mediates TGF-β–induced growth inhibition in selected cells, could be the putative IGFBP-3 receptor that might also bind other IGFBPs [28,29] (Figs. 1 and 2). This evidence is largely based on affinity labelling of TGF-β receptors with ^{125}I-labeled TGF-β1, which showed that IGFBP-3 can displace binding to TGF-β RII and TGF-βRV in a concentration-dependent fashion [30].

IGF-independent effects may be mediated by IGFBP-3 association with specific cell surface proteins or receptors such as TGF-βRV [31–34]. However, characterisation of a mutant form of IGFBP-3 (mutBP-3) revealed that cell surface binding and nuclear translocation are not required for IGFBP-3-mediated growth inhibition and apoptosis in T47D human breast cancer cells [35]. These results suggest the existence of multiple pathways by which IGFBP-3 elicits its antiproliferative and proapoptotic effects.

2.3. IGFBP-3 regulation and posttranslational modifications

The IGF-dependent and IGF-independent activity of IGFBP-3 is regulated by deactivation *via* several proteases (Fig. 2). IGFBP-3 specific proteases seem to vary depending on the tissue type. For example, MMP-19 is a likely candidate for being the major IGFBP-3 degrading matrix metalloproteinase (MMP) in the quiescent epidermis [36], whereas matrix metalloproteinase-7 appears to cleave IGFBP-3 in tumour tissues, facilitating

insulin-like growth factor bioavailability. Co-incubation of the IGF-I/IGFBP-3 complex with MMP-7 restored IGF-I-mediated IGF-IR phosphorylation and activation of AKT in cancer cell lines. These results indicate that MMP-7 proteolysis of IGFBP-3 plays a crucial role in regulating IGF-I bioavailability, thereby promoting cell survival [37].

Related to direct IGFBP-3 proteolysis by MMP-7, is an indirect MMP-7 proteolysis *via* processed ADAM28s. ProADAM28s has to be processed with active MMP-7 into specific fragments including ADAM28s without pro-peptide. Processed ADAM28s is able to digest IGFBP-3 in both free and complex forms with IGF-I or IGF-II [38].

Phosphorylation and glycosylation of IGFBP-3 are additional avenues available to regulate its extracellular levels. Once phosphorylated, the ability of IGFBP-3 to bind IGF-I is enhanced [39]. Phosphorylation of IGFBP-3 also increases both the nuclear import of IGFBP-3 and the binding of IGFBP-3 to components within the nucleus [40].

IGFBP-3 exists as two glycoforms, which reduce to a single form upon enzymatic deglycosylation. While the carbohydrate units appear to be non-essential to ALS or IGF binding (Fig. 2), cell-surface association experiments indicate that glycosylation may influence the partitioning of IGFBP-3 between the extracellular milieu and the cell surface [41].

3. IGFBP-rP1

IGFBP-rP1 was originally identified as a cDNA, termed mac25 that was overexpressed in normal leptomeningeal cells compared to meningiomas [42]. It is the first of the IGFBP-related proteins found to bind to IGF-I and to IGF-II, and was therefore initially named IGFBP-7 [43]. Other nomenclature for IGFBP-rP1 includes TAF (tumour adhesion factor) related to its isolation as a major secretory protein with cell-adhesion activity from culture medium conditioned by human bladder carcinoma; and AGM (angiomodulin), which was suggested as a functionally appropriate designation after finding that AGM is accumulated in small blood vessels of tumour tissues [44–46]. IGFBP-rP1 was also identified as a factor that stimulated prostacyclin (PGI2) production using conditioned medium from cultured human diploid fibroblast cells and therefore termed PSF (prostacyclin stimulating factor) [47]. Our laboratory has isolated IGFBP-rP1 as T1A12 cDNA by differential hybridisation cloning from the Hs578T breast cancer cell line *versus* its normal counterpart Hs578Bst [48]. Based on its phylogeny in the IGFBP superfamily and its low affinity for IGF, mac25/T1A12/IGFBP-7/TAF/AGM is now appropriately termed IGFBP-rP1 [3]. IGFBP-rP1 is distinct from other IGFBP-rPs in that it can bind strongly to insulin [49].

3.1. IGFBP-rP1 binding partners

The ability of IGFBP-rP1 to bind insulin is unique among other family members in that this protein has a putative insulin-binding site at the amino terminal region, which is exposed for high affinity insulin binding due to the lack of conserved cysteine residues at the carboxyl terminus. The latter residues are important for IGF binding by IGFBPs [8,49]. For a long period of time no insulin-binding partners were known, and it was believed that none of the IGFBPs could bind insulin with any degree of significance. However, recently due to improvements in assay technology, low affinity binding to insulin was demonstrated for IGFBPs 1–6 [49]. An existing model proposes that the N- and C-terminus of IGFBPs form a tertiary structure that would accommodate IGF binding. The variations of the C-terminus (i.e., loss of 5/6 cysteines) would expose the insulin-binding site [49]. This was shown for recombinant N-terminal fragment of IGFBP-3 and is seen in the case of IGFBP-rP1 [34], both proteins have less affinity for IGFs: IGFBP-rP1 binds IGF-I and -II with 100-fold lower affinity ($k_d = 3 \times 10^{-8}$ M) than the IGFBPs ($k_d \sim 10^{-10}$ M), respectively [3]. Although the affinity of IGFBP-rP1 to IGFs and insulin is similar (IC_{50} = 20–34 nM), its insulin:IGF binding ratio is 500-fold higher than that of IGFBP-1 to 6 [49]. IGFBP-rP1 can compete with insulin receptors for binding of insulin, whereas IGFBP-3 cannot. For example, IGFBP-rP1 inhibits insulin binding to placental membranes at 100–300 pmol, concentrations at which IGFBP-3 showed no effect [49].

Besides the obvious binding partners for IGFBP-rP1, like most of the members of the IGFBP superfamily, IGFBP-rP1 also has IGF-independent actions [3]. IGFBP-rP1, which possesses a "follistatin module" in its protein sequence, has been shown to bind to activin, a member of the TGF-β superfamily of growth factors (Fig. 3) [50]. Activins were first identified by virtue of their ability to regulate follicle-stimulating hormone (FSH) secretion from the anterior pituitary. Activins are also powerful regulators of gonadal functions. However, the physiological functions of activins are not restricted to reproductive tissues [51]. Activin is associated with growth modulation in glandular organs and its receptors. These signalling proteins are also present and regulated during postnatal mammary gland development, primarily during the lactational phase [52]. Interestingly, activin is also necessary for normal mammary cell function and when activin signalling is disrupted or lost, malignant progression is potentiated as demonstrated by a global decrease in the abundance of activin and its receptors in high grade breast cancer [52].

Another IGF-independent binding partner of IGFBP-rP1 was identified in type IV collagen. Based on immunostaining evidence, IGFBP-rP1 was found to co-localise with type IV collagen in the vascular basement membrane. Subsequent *in vitro* experiments demonstrated that IGFBP-rP1 preferentially bound to type IV collagen among various extracellular matrix components tested, and promoted adhesion of human umbilical vein endothelial cells to type IV collagen substrate as well as induced their morphological changes [46]. Furthermore, when the endothelial cells were induced to form capillary tube-like structures by type I collagen, IGFBP-rP1 and type IV collagen were detected exclusively on these tubular structures. Based on the finding from that study, IGFBP-rP1 was termed TAF (tumour adhesion factor) [45].

Correspondingly, St. Croix and co-workers discovered a function for IGFBP-rP1 in endothelial cells in concert with type IV collagen. They showed that IGFBP-rP1 is specifically elevated in tumour-associated endothelium relative to normal endothelial cells. IGFBP-rP1 was the principal gene identified by SAGE (serial analysis of gene expression) as a potential tumour endothelial cell marker and one that had twice the expression level of normal endothelial cells. Type IV collagen was number 9 of the top 10 genes and 4 times more elevated in tumour *versus* normal endothelial cells, suggesting IGFBP-rP1 and type IV collagen are co-expressed and could interact with each other [53].

Together, the binding partners of IGFBP-rP1 indicate its unique role in the IGF signalling network and strongly suggest a function in the TGF-β signalling pathway, which plays an important role in breast cancers as well as in the organisation of tumour endothelial cells.

3.2. IGFBP-rP1 regulation

As described above, proteolytic cleavage is a common means by which IGFBPs are regulated [13,54] and it is thought to be proteolytically cleaved into a two-chain form. The cleavage site sequence suggested that a trypsin-like serine proteinase could be responsible for this processing. The cleavage of IGFBP-rP1 led to an almost complete loss of both insulin/IGF-1-binding activity and insulin/IGF-1-dependent growth-stimulatory activity suggesting that proteolytic cleavage leads to inactivation of IGFBP-rP1 (Fig. 3). However, the cell attachment activity of IGFBP-rP1 was markedly increased by the proteolytic processing, and this observation indicates that the cleaved fragments also possess biological activity [55].

Compared to other individual IGFBPs such as IGFBP-3 and -5, IGFBP-rP1 seems not to be affected by glycosylation or phosphorylation. Besides proteolytic processing, which can regulate IGFBP-rP1 expression at the protein level (Fig. 3), IGFBP-rP1 is distinguished from other IGFBPs through regulatory mechanisms at the DNA and RNA level (Fig. 3). Methylation has been reported to lead to the down-regulation of IGFBP-rP1 in liver cancers [56]. Regional DNA hypermethylation of CpG islands is believed to contribute to tumour progression by silencing of tumour suppressor genes such as E-cadherin, p16/Ink4a and VHL [56]. By screening for alterations in DNA methylation associated with hepatocarcinogenesis in transgenic mice, IGFBP-rP1 was identified as a putative tumour suppressor gene that is silenced by promoter hypermethylation [56]. The 1 kb region that contains the promoter and exon 1 of the IGFBP-rP1 gene in these murine liver tumours, was reported to be located in a typical CpG island and methylation of this CpG island could therefore be directly associated with the reduced expression of the IGFBP-rP1 gene and subsequent tumourigenesis [57].

In line with this observation, when studying the role of IGFBP-rP1 in breast cancer, our laboratory found that loss of heterozygosity (LOH) occurs on chromosome 4q in 50% of informative invasive breast cancers when matched to their adjacent normal tissues. Allelic loss is a hallmark of tumour suppressor genes and a regulatory element of gene expression at the DNA level (Fig. 3) [58]. IGFBP-rP1 is located on 4q12-13; 4q is also a suspected tumour suppressor locus in hepatocellular, bladder, cervical, lung, esophageal, papillary thyroid, head and neck squamous cell carcinoma, as well as leukaemia [50,58,59]. Our initial study of IGFBP-rP1 protein expression in 60 primary breast cancers using immunohistochemistry revealed that 12 normal and benign breast tissues had strong IGFBP-rP1 expression, 16 ductal carcinomas *in situ* showed weak IGFBP-rP1 levels and the invasive carcinomas were negative for IGFBP-rP1, all consistent with its role as a tumour suppressor [58].

IGFBP-rP1 can be activated by retinoic acid and TGF-β [3]. IGFBP-rP1 has been found to accumulate in senescent cells and to be up-regulated in normal, growing mammary epithelial cells by all-*trans*-retinoic acid or the synthetic retinoid fenretinide [59]. IGFBP-rP1 showed also marked response to retinoic acid and modest response to TGF-β in human prostate epithelial cells (Fig. 3) [60]. TGF-β response supports the finding that IGFBP-rP1 might be involved into TGF-β signalling pathways *via* activin binding [50].

3.3. Physiological roles of IGFBP-rP1

IGFBP-rP1 regulates cellular proliferation, adhesion, angiogenesis and stimulates prostacyclin synthesis. Monoclonal antibodies have been used to study the distribution of IGFBP-rP1 in human biological fluids and tissues. IGFBP-rP1 was readily detectable in major human body fluids such as serum, urine, amniotic fluid,

and cerebrospinal fluid. In random normal human adult sera ($n = 37$), the median IGFBP-rP1 level was 21.0 μg/liter, and this value did not correlate with levels of IGF-I, IGF-II, or IGFBP-3 [61]. Immunohistochemistry performed on normal human tissues showed a ubiquitous intense staining of peripheral nerves, smooth muscle cells, including those from blood vessel walls, gut, bladder, breast and prostate. Cilia from the respiratory system, epididymis, and fallopian tube also showed intense immunoreactivity. Most endothelial cells were seen to be positive, whereas fat cells, plasma cells, and lymphocytes were negative. Specific IGFBP-rP1 expression was limited to certain cell types in the kidney, adrenal gland, and skeletal muscle, indicating a possible specialised function in these organs [62].

Evidence is accumulating that IGFB-rP1 plays an important physiological role in the female reproductive system. Studies that have examined gene expression in relation to aspects of female fertility have found IGFBP-rP1 as one of the major genes implicated in human endometrial receptivity, folliculogenesis as well as growth development and regression of the *corpus luteum* in higher mammals [63–65].

Follicular and luteal development involves cell proliferation, terminal differentiation, and angiogenesis. Hence IGFBP-rP1 RNA expression was found to be strongly induced in terminally differentiated granulosa cells of preovulatory follicles in pigs. Transcripts, which were also abundant in ovarian blood vessels, increased in the *theca interna* with follicular development, suggesting that IGFBP-rP1 may promote terminal differentiation of granulosa cells in preovulatory follicles consistent with its tumour suppressor function [64]. Other investigators showed that IGFBP-rP1 mRNA remained constant during the oestrous cycle and was markedly up-regulated in late luteal bovine tissue implying a role in *corpus luteum* regression [65].

Corpus luteum function and progesterone production is linked to endometrial receptivity. A genomic approach comparing receptive *versus* pre-receptive human endometria revealed that IGFBP-rP1 had a 35-fold increase in expression during the receptive phase compared with the pre-receptive phase while in the late luteal phase, IGFBP-rP1 levels sharply rose again. IGFBP-rP1 protein was localised at the apical part of the luminal and glandular epithelium, as well as in stromal and endothelial cells. These data suggest a role for IGFBP-rP1 in endometrial physiology, one that seems related to endometrial receptivity [63]. In this context supporting a normal function in the endometrium, IGFBPrP1 mRNA expression was found to be significantly lower in large uterine leiomyomas than in adjacent myometrium from untreated patients [66].

In order to study the normal function of IGFBP-rP1, we have created the first knockout mouse of IGFBP-rP1 [67]. The first- and second-generation mice are viable. In mice 5–8 months old, we observed significant changes in the histology of the ovaries, muscle tissue and liver of these mice. In particular, we have seen gross abnormalities in the *corpus luteum.* This latter observation is in agreement with an essential role of IGFBP-rP1 in female reproductive organs.

4. The IGFBP/IGFBP-rP system and breast cancer

Breast cancer cells respond to extracellular signals that are necessary for their unregulated proliferation, but are in some cases also required for the growth of the normal mammary gland. A well known example are oestrogen and the oestrogen receptor, but various other signalling stimuli such as the network of insulin-like growth factors, their binding proteins and receptors, cytokines, cadherin-dependent signalling and extracellular matrix proteins (ECM) are also essential for breast cancer cells to proliferate and metastasise (Fig. 1) [68].

IGFs are potent mitogens for breast cancer cells and act synergistically with oestrogen to stimulate cell growth, interdiction of their signalling action(s) results in tumour growth inhibition. Moreover, transgenic mouse models, which overexpress IGF-I or -II targeted to the mammary gland, display specific alterations in mammary gland development and an increased incidence of mammary tumours [68,69]. In line with this are epidemiological studies that suggest that individuals with circulating levels of insulin-like growth factor-1 (IGF-I) at the highest quartiles of the normal range, are at increased risk for several common cancers including breast cancer [1,2,70].

4.1. IGFBP-3 and breast cancer

High levels of IGFBP-3 in the serum have been reported to reduce the relative risk of developing breast cancer predicted by high IGF-I levels in premenopausal women in some studies [71], while other reports have indicated the opposite [72]. A recent systematic review and meta-regression analysis of 21 studies on IGF-I and IGFBP-3 concentrations in serum or plasma that incorporated a total of 3609 cases and 7137 controls of the four most common cancer types (including breast), concluded that high concentrations of IGF-I were associated with an increased risk of premenopausal breast cancer. High IGFBP-3 levels were also associated with an increased risk of premenopausal breast cancer. This report further suggested that effects of circulating IGF-I and IGFBP-3 levels on cancer risk are however modest, vary with sites and that additional prospective studies with standardised laboratory methods would be required in order to use these parameters for assessment of risk and prevention of cancer [70].

The antiproliferative and proapoptotic activities of IGFBP-3 seen in breast cancer cells *in vitro* seem to contradict the epidemiological association of high IGFBP-3 levels with increased cancer risk in premenopausal women [70]. The actions of IGFBP-3 could therefore be two-fold towards tumours. Dependent on the cellular environments, IGFBP-3 might be antiapoptotic or proapoptotic. Alternatively, the activity of IGFBP-3 found to confer high or low risk in certain cancer types such as breast cancer might be governed by its activation state. Future studies should determine whether circulating IGFBP-3 is intact or fragmented (i.e., lacking its C-terminal domain), or posttranslationally modified.

4.2. IGFBP-rP1 and breast cancer

Swisshelm et al. [59] identified IGBP-rP1 as one of the genes overexpressed in senescent human mammary epithelial cells (HMEC) and which was up-regulated in normal mammary epithelial cells by all-*trans*-retinoic acid. The secreted IGFBP-rP1 protein was found in the conditioned media of Hs578T breast cancer cells, in normal human urine, cerebrospinal fluid and amniotic fluid [73]. Subsequently we cloned the gene by subtractive hybridisation from the Hs578T breast cancer cell line and found IGFBP-rP1 to be down-regulated in primary breast cancer tissues. Loss of IGFBP-rP1 could be associated with disease progression in that normal breast tissues had very high protein levels, which gradually decreased in hyperproliferative and *in situ* disease. High grade invasive breast cancers lacked IGFBP-rP1 expression [58,74,75]. In addition, we found loss of heterozygosity at the IGFBP-rP1 locus on the long arm of chromosome 4q in 50% of informative, matched normal *versus* invasive breast cancers suggesting that IGFBP-rP1 has tumour suppressor function [58]. This observation was later confirmed in a larger cohort of tumour samples where we found that down-regulation of IGFBP-rP1 in human breast cancers is also associated with the inactivation of the retinoblastoma protein, cyclin E overexpression and increased proliferation in oestrogen receptor negative tumours. Breast cancers patients with low IGFP-rP1 levels had also a significantly reduced survival compared to those with higher levels. Of all breast cancer cases examined, 15% lacked IGFBP-rP1 staining completely, 20% had weak staining, 32% intermediate and 33% showed strong staining [76].

Our data obtained from clinical samples together with the findings that IGFBP-rP1 can induce senescence of tumour cells, and that it accumulates in senescent mammary epithelium, support a tumour suppressive role for IGFBP-rP1 in breast cancer [59].

Our IGFBP-rP1 knockout mouse will be instrumental in studying loss of IGFBP-rP1 tumour suppressor effects on mammary gland development. Moreover, it is likely that inactivation of this gene in combination with overexpression of other genes such as Her2/neu or cyclin E would likely result in the formation of more frequent and aggressive tumours in crossbred animals. Their availability would enable us to generate new, more relevant mouse models of human breast cancer.

5. Therapeutic possibilities

IGFs and IGF-R have been clearly shown to have a role in malignant transformation of normal breast epithelial cells; they are potent mitogens and have been seen to be involved in growth and progression of breast cancer [68,77,78]. The signalling system is delicately balanced by the interaction with IGFBPs (Figs. 1–3). The latter factors stabilise IGFs and make them available to their receptors. Targeted disruption of the IGF-IR genes resulted in mice with reduced birth weight indicating the involvement of IGF-IR with normal growth. However, the inhibition of growth was not complete, but limited to an extend of 45–60% of normal growth [79]. This reduction suggests that inhibition of IGF-1R might lead to retardation of tumour growth and thus, IGF-R1 would be a good anticancer target. Indeed it was shown that blockage of IGF-1R by antibodies inhibited the growth of human breast cancer cell lines that had formed tumours in mice [80].

The dominant action of IGFBPs is to compete with receptors for ligands. Under circumstances in which IGFBP levels are low, IGF's and insulin's growth stimulatory activities would be expected to be high [1]. A possible therapeutic intervention would therefore involve the stabilisation of IGFBP-3 or IGFBP-rP1, their chemical induction and/or the systemic administration of recombinant proteins.

Proteases present in the tumour microenvironment digest IGFBPs and release free ligands (Fig. 1). Production of proteases by a cancer might therefore increase IGF-1R signalling. Conversely, inhibitors of these proteases should increase IGFBP levels, and additionally anti-oestrogens, TGF-β and retinoic acid have been documented to increase IGFBP and IGFBP-rP expression (Figs. 1–3) [3].

5.1. Recombinant IGFBP-3

Stable transfection of a construct expressing human IGFBP-3 has been shown to induce growth inhibition and apoptosis in breast cancer cells [81,82].

Recombinant human insulin-like growth factor binding protein-3 (rhIGFBP-3), which has been developed by Insmed Incorporated [83], is currently under advanced preclinical investigations in our laboratory and is being assessed for its usefulness as an anticancer therapy. So far, we have studied the *in vivo* activity of rhIGFBP-3 in murine and human cancer models

including breast cancer xenografts. MCF-7 xenografts were treated with doses of 10 mg/kg, rhIGFBP-3 was given intraperitoneally for 21 days, either alone or in combination with taxol. No single agent effect was seen in this *in vivo* model however, rhIGFBP-3 markedly enhanced the effect of taxol on inhibiting net tumour growth. The anti-tumour activity of rhIGFBP-3 against ER+ MCF-7 human breast cancer xenografts was compared to effects in ER- MDA-MB-231 xenografts [84]. Mice with established MDA-MB-231 tumours were treated for three weeks with rhIGFBP-3, taxol or a combination of the two agents. In contrast to the effects noted in MCF-7, rhIGFBP-3 inhibited tumour growth in MDA-MB-231 by up to 40% of control as a single agent, but failed to show synergy with taxol. Furthermore, Western blot analysis showed that rhIGFBP-3 treatment had differential effects on PI3K-AKT and MAPK signalling in MCF-7 and MDA-MB-231 cells [84].

Recombinant human IGFBP-3 was also examined for activity against herceptin-resistant breast cancer sublines (MCF-7/HER2-18, SKBR3/IGF-IR and BT474/HerR) with elevated IGF-IR levels. Treatment with rhIGFBP-3 resulted in dose-dependent growth inhibition of breast cancer cells. Moreover, the rhIGFBP-3 elicited a strong dose-dependent increase in herceptin sensitivity of SKBR3/IGF-IR and BT474/HerR, but showed a less marked effect with MCF-7/HER2-18 *in vitro* [85]. Subsequently, a related *in vivo* study was undertaken to evaluate the anti-tumour activity of rhIGFBP-3 in a xenograft model of herceptin-resistant breast cancer. Mice bearing HER-2-transfected (MCF-7/HER2-18) human breast carcinomas were treated for 21 days with either herceptin, rhIGFBP-3 or a combination of the two agents. rhIGFBP-3 displayed potent single-agent activity superior to that of herceptin alone and modest combinatorial activity with it [86]. Our data strongly indicate that IGFBP-3 exerts antiproliferative and proapoptotic effects as demonstrated with *in vitro* and *in vivo* models of human breast cancer and that its therapeutic efficacy should be tested in clinical trials.

5.2. Recombinant IGFBP-rP1

IGFBP-rP1 has been studied for antiproliferative activity in tumour cells *in vitro* either by induction with retinoic acid, exogenously expressed in viral vectors, or by using the recombinant protein [50,59,87]. After 1 week of treatment, where IGFBP-rP1 cDNA is expressed by retroviral vector in the IGFBP-rP1-deficient MCF-7 breast cancer cell line, cell numbers were reduced by 39% and 74% for IGFBP-rP1-transduced polyclonal or clonal cell-cultures, respectively. In addition, medium conditioned by IGFBP-rP1-producing cultures reduced cumulative MCF-7 cell numbers by 20% compared with medium from cultures of a control vector-transduced MCF-7 cell line [87]. Addition of 1 nM recombinant mac25/IGFBP-rP1 protein into the culture medium of human cervical carcinoma cells (HeLa) and osteosarcoma cells (Saos-2) induced significant suppression of the growth [50]. This *in vitro* growth inhibition was associated with increased cells in G1 and decreased cells in G2/M phase of the cell cycle. Alteration of cell cycle kinetics upon overexpression of IGFBP-rP1 was also noted in a prostate cancer cell line, in which the cells were delayed in the G1 phase of the cell cycle [88]. In all of the *in vitro* studies, IGFBP-rP1 effects were consistently associated with the occurrence of a senescent cell phenotype as supported by positivity for the senescence-associated β-galactosidase expression [59,87,88]. Together these findings suggest that IGFBP-rP1 is an inhibitor of cancer cell proliferation and may act *via* a cellular senescence-like mechanism, which could be therapeutically exploited *in vivo*.

5.3. Small molecules

Retinoids have been described to induce and/or upregulate both IGFBP-3 and IGFBP-rP1 expression [59,89,90]. Interestingly, the synthetic retinoid fenretinide showed a beneficial effect on second breast cancers in premenopausal women in a Phase III trial and thus, long-term effects on IGF-I levels were studied in this cohort. Fenretinide was found to change the IGF-I:IGFBP-3 molar ratio and as a result induced a moderate decline in IGF-I levels in women $\leqslant$50 years of age [91]. These findings indicate that the effects of retinoids should be examined in more depth, specifically in relation to its modulation of IGFBP levels in breast cancer patients, and preclinical and clinical studies should be conducted to define optimal dosage and administration schedules. Other possibilities for therapeutic intervention with small molecules can be deduced from existing targets in the IGFBP-3/-rP1 regulatory pathways (Figs. 2 and 3). For example, as suggested by Pollak et al. [1,2], IGFBPs could be stabilised by inhibiting proteases responsible for their cleavage and inactivation. Thus, it might be worthwhile to revisit the arsenal of existing MMP inhibitors for their specificity to inhibit IGFBP-3 and IGFBP-rP1 degrading proteases.

Inhibitors of PI3 kinase, TOR and AKT (Fig. 2) such as rapamycin or geldanamycin analogues, might also lead to increased levels of IGFBPs and thus, change the IGFBP-3/IGF-I serum ratio, by inactivating IGF-RI signalling. This approach would impair IGF-dependent activities of the IGFBPs and should direct them towards the IGF-independent pathways. In fact, a recent study of IGF-IR expression in breast cancers found that 17-AAG (17-allylaminogeldanamycin), an investigational new drug in advanced clinical development, induced degradation of IGF-IR *in vitro* and inhibited cellular signalling through AKT and MAPK. These

effects were similar to that seen in our studies with recombinant IGFBP-3 (Fig. 2) [84,92,93].

Thus, ongoing clinical trials with rapamycin and geldanamycin analogues, should consider measuring levels of circulating IGFs, insulin and their partner IGFBPs to help elucidate their pharmacodynamic actions.

6. Conclusions and future directions

IGFBP-3 and IGFBP-rP1 are unique members of the IGF-R signalling axis as IGFBP-3 is a major regulator of bound and circulating IGF and IGFBP-rP1 is a high affinity insulin-binding protein. Both proteins however, also have IGF-independent actions that may be physiologically important.

Despite current knowledge of the existence of 16 members of the IGFBP superfamily, which might argue for redundancy of function and for compensatory overlapping IGFBP or -rP function, this effect appears not to be relevant for IGFBP-3 and IGFBP-rP1. IGFBP-3 is one of the few high affinity IGF binders with a clear hazard significance associated with it in breast cancer risk assessment. Also IGFBP-rP1 appears to be essential in regulating important processes occurring during the female reproductive cycle and to have a tumour suppressor function associated with it in breast and other cancers. The strong breast cancer link and the secretory nature of these two IGF family proteins, suggest that they may not only be good prognostic indicators for malignant disease progression, but also useful surrogate markers for monitoring therapeutic responses in the treatment of breast cancer.

Conflicting literature, particularly in relation to high IGFBP-3 levels being a risk factor for breast cancer or its having an inverse relationship, might be due to lack of standardised sample collection and assay procedures. Measurement of proteolytic fragments and phosphorylated products as well as investigating their function should lead to better understanding and a more defined assessment regarding the risks associated with levels of circulating IGFBPs.

Transgenic and knockout mouse models have provided us with valuable insights into the functions of many genes. Hence, a number of transgenic and knockout models for the ligands, receptors and binding proteins of the IGF system were developed over the past decade [94]. For IGFBP-3, only transgenic mouse models were developed. The phenotype of IGFBP-3 transgenic animals was disappointing, overexpression of IGFBP-3 led only to pre- and postnatal growth retardation, even targeted expression to the mammary gland did not result in tumour development [reviewed [94,95]]. Instead, a moderate retarded mammary tissue remodelling and reduced size of alveoli was seen [95]. This transgenic work suggests that an increase in circulating IGFBP-3 might not increase cancer risk. However, the signalling pathways in this transgenic animal model may not reflect that of humans with increased IGFBP-3, and thus knockout animals should be developed to study a possible protective role and/or proapoptotic role of IGFBP-3.

We have developed a knockout mouse for IGFBP-rP1 to study its function as a tumour suppressor and the role of IGFBP-rP1 in growth and senescence. Our IGFBP-rP1 knockout mouse will be a valuable resource to elucidate the normal function of the gene. It will also be useful in determining the conditions under which tumours may arise, i.e., by chemical carcinogenesis or crossing the mice with existing transgenic models of oncogenes or knockout models of tumour suppressor genes. Animal models created for biological studies would help to define IGFBP-3 and IGFBP-rP1 functionally better and thus be highly relevant to breast cancer.

Conflict of interest statement

None declared.

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