IGFBP-2 and –5: important regulators of normal and neoplastic mammary gland physiology

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Abstract The insulin-like growth factor (IGF) axis plays an important role in mammary gland physiology. In addition, dysregulation of this molecular axis may have a causal role in the aetiology and development of breast cancer (BC). This report discusses the IGF axis in normal and neoplastic mammary gland with special reference to IGF binding proteins (IGFBPs) -2 and -5. We describe how these high affinity binders of IGF-1 and IGF-2 may regulate local actions of growth factors in an autocrine and/or paracrine manner and how they also have IGF-independent effects in mammary gland. We discuss clinical studies which investigate both the prognostic value of IGFBP-2 and -5 expression in BC and possible involvement of these genes in the development of resistance to adjuvant endocrine therapies.

Keywords IGFBP · Mammary gland · Tumourigenesis

Introduction

The insulin-like growth factor (IGF) axis comprises cell surface receptors (IGF1R and IGF2R), polypeptide growth factors (IGF-1 and IGF-2) and six soluble high affinity IGF binding proteins (IGFBP1-6) together with a group of ancillary proteins—acid labile subunit (ALS), IGFBP proteases and IGFBP related proteins (IGFBP-RPs). In many cell types IGF1R can form functional heterodimeric receptors with both the A and B isoforms of the insulin receptor (IR-A and IR-B) and these should also be considered as ancillary members of the IGF axis in this context. The IGF axis regulates the function of almost all mammalian cells including processes such as

mitogenesis, differentiation, adhesion, migration and apoptosis. Dysfunction or deregulation of the IGF axis can have profound effects on tissue homeostasis and is often associated with neoplastic disease. This is particularly evident in tissues such as the mammary gland which is subject to repeated cycles of growth, differentiation and apoptosis during reproductive life and has profound consequences for the biology of epithelial, myoepithelial and stromal cells which constitute the gland. As well as a role in normal mammary gland biology evidence suggests that deregulation of the IGF axis may be associated with neoplastic disease and many anti-IGF based therapeutic strategies have been investigated in breast cancer (BC). This review discusses briefly the role of the IGF axis during normal mammary gland function followed by a more detailed discussion of the relationship between IGF axis dysfunction and the aetiology of BC. In both of these sections we focus on the role of IGFBP-2 and -5 although at appropriate locations we discuss the role of other IGF axis components especially with respect to development of endocrine and chemotherapeutic resistance in BC. Finally we appraise the future prospects for novel anti-IGF based strategies in combating BC.

IGF axis and mammary gland function

Initially IGF-1 was demonstrated to have a galactopoetic effect in bovines (Hadsell et al. 1993; McGrath et al. 1991) and this was quickly followed by reports of IGF axis gene expression in different mammary cell types. Although initial studies suggested that IGF-1 and -2 were secreted by mammary epithelial cells (MECs) and BC cell lines (Huff et al. 1986; Minuto et al. 1987) this was soon shown to be an artefact caused by incomplete extraction of IGFBPs prior to assay (Ahmed et al. 1990; De Leon et al. 1988). There followed confirmation that IGF-1 and -2 mRNA levels are very low

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in MECs (De Leon et al. 1990) and the currently established paradigm is that IGFs are secreted by stromal cells in the mammary gland to act in a paracrine fashion on nearby MECs (Yee et al. 1988, 1989). In accordance with this MECs express both IGF1R and IGF2R and are very sensitive to mitogenic stimulation by both growth factors (Osborne et al. 1990; Pollak 1998). Anti-receptor and gene knock out studies suggest that IGF1R probably mediates the mitogenic effects of both growth factors (Arteaga et al. 1989; Cullen et al. 1992) although separate IGF-2 mediated actions through its cognate receptor cannot be ruled out. In addition both growth factors can signal via heterodimeric IGF1R/IR which are present on the surface of BC cells (Belfiore and Frasca 2008).

Although IGFs are the main drivers of MEC mitogenesis and differentiation, the expression and role of IGFBPs in both normal mammary tissue and in BC has been investigated. IGFBP-2 and -5 in particular have pleiotropic effects in normal and neoplastic tissue and this is discussed in some detail below. However other IGFBPs (particularly IGFBP-3) have important functions and when these impact on the molecular physiology of the mammary gland this will be indicated. Prior to this however it is important to clearly outline the theoretical mechanisms by which IGFBPs may act. This is an area of some confusion and controversy although most of these mechanisms are supported by experimental evidence - reviewed in (Beattie et al. 2006)

- a. IGFBPs may bind IGFs with high affinity to sequester and remove them from the vicinity of cell surface receptors. This is an inhibitory activity and is IGF dependent.
- b. IGFBPs may act as a “sink” for IGFs releasing growth factor slowly to increase serum half-life and prolong action. This is an enhancing activity and is IGF dependent.
- c. IGFBPs may localise growth factors in the extracellular matrix (ECM) thus removing them or presenting IGFs to their site(s) of action. This can be either an inhibitory or enhancing activity and may show tissue dependent or ECM dependent specificity. This activity is IGF dependent.
- d. Unliganded IGFBPs may show direct effects of either an inhibitory or stimulatory nature in a cell/tissue or ECM dependent manner. Mechanisms associated with some of these effects are now being elucidated. These activities are IGF independent.

In addition, IGFBP activity is influenced by post-translational modifications including glycosylation, phosphorylation and (importantly) proteolysis. IGFBP activity is also influenced by the presence of other IGFBPs in the pericellular environment and there is evidence for functional redundancy amongst these proteins. Finally IGFBP action is also influenced by the presence of both intra- and extra-

cellular binding partners. A disparate group of IGFBP binders have been described including components of the ECM, transcription factors and signalling complexes (Amaar et al. 2002, 2005; Arai et al. 1994).

Our group first reported that IGFBP-5 expression is massively up regulated during involution of the mammary gland coinciding with the early stages of MEC apoptosis (Tonner et al. 1997, 2000) and tissue specific overexpression of IGFBP-5 in the mammary gland is associated with reduced cell proliferation and increased apoptosis (Tonner et al. 2002). MECs are the main source of tissue IGFBP-5 (Marshman et al. 2003) and the current paradigm suggests that IGFBP-5 gains access to the extracellular space to neutralise stromal derived IGFs to blunt the anabolic function(s) of the growth factors. In support of this mechanism R3-IGF-1, a non-IGFBP binding analogue, partially restores lactation and direct addition of IGFBP-5 to primary cultures of mouse mammary epithelial cells inhibits IGF-1 activation of insulin receptor substrate-1 (IRS-1), an early signalling event in IGF-1 action. However, as is often the case in IGFBP biology, the action of IGFBP-5 in mammary gland is not straightforward. Therefore IGFBP-5 mRNA is also present in MECs at mid- to late-pregnancy when cells are undergoing differentiation prior to parturition (Wood et al. 2000). IGFBP-5 secretion is stimulated in a differentiating mouse MEC line and in primary cultures of mouse MECs grown on an appropriate ECM background (Lochrie et al. 2006; Phillips et al. 2003) and microarray analysis shows that IGFBP-5 expression is elevated both at mid-pregnancy and during involution (Clarkson and Watson 2003; Rudolph et al. 2003). IGFBP-5 has differentiating activity in other cell types (Cheng et al. 1999; Rotwein et al. 1995) and evidence suggests it may have stage specific differentiating and apoptotic roles in the mammary gland which may be differentially regulated by factors such as local IGF: IGFBP ratios and proteolysis of IGFBPs. These actions of IGFBP-5 may occur in either an IGF-dependent or IGF-independent manner. IGFBP-5 is also expressed in BC tissues and primary cultures of BC cells (Huynh 1998; Manni et al. 1994; McGuire et al. 1994; Pekonen et al. 1992). Pekonen et al. used in situ hybridisation to show that IGFBP-5 mRNA was present in 47 breast tumour samples and that tumour tissue showed higher levels of IGFBP-5 than surrounding normal tissue (Pekonen et al. 1992). Thus although the role of the IGF axis in normal mammary gland function remains an active research area, most investigations are now concerned with IGF axis function in neoplastic mammary tissue. We discuss such studies in the following section especially with respect to IGFBP-2 and IGFBP-5.

IGF axis and breast cancer

Sheikh et al. were the first to report IGFBP-5 mRNA and protein secretion in oestrogen receptor-positive (ER+) BC

lines (MCF-7, T47D, ZR75 and BT474) (Sheikh et al. 1992) and this pattern of IGFBP expression with moderate to high expression of IGFBP-2, -4 and -5 in ER+ BC cell lines has been confirmed by others (De Leon et al. 1989; McGuire et al. 1992). The observation that both IGF-1 and oestradiol (E2), which act synergistically to increase MCF-7 cell proliferation, also increased IGFBP-5 secretion argued that the binding protein was not by itself inhibitory to cell proliferation (McGuire et al. 1992). Although this somewhat counters the pro-apoptotic action of IGFBP-5 in normal mammary gland, in common with most studies examining IGFBP action in BC cell lines neither the concentration nor extent of IGFBP proteolysis was routinely reported in these early studies. Notwithstanding these observations, the hypothesis that IGFBP-5 plays a causal role in the aetiology and metastasis of BC was examined further. In support of a tumour promoting role, an invasive sub-clone of MCF-7 expressed high levels of IGFBP-5 whereas a non-invasive cell line expressed very low levels of the protein (Dubois et al. 1995). However experiments to investigate this hypothesis further (e.g., KO or over-expression of IGFBP-5) were not conducted and it is unusual that the non-invasive clone of MCF-7 did not express IGFBP-5 as this gene has almost universally been shown to be expressed in MCF-7 cells. In Hs578T cells IGFBP-5 inhibited ceramide or RGD induced apoptosis through sphingosine kinase and PKC mediated survival signals (McCaig et al. 2002; Perks et al. 1999) although these effects of IGFBP-5 show cell line specificity. Thus both wt and a non-IGF binding mutant of IGFBP-5 inhibit ceramide induced apoptosis in Hs578T cells but only the wt IGFBP-5 was effective in MCF-7 cells. In addition mutant IGFBP-5 ablated the pro-survival effects of IGF-1 in MCF-7 cells (an IGF responsive cell line) whereas the wt protein enhanced IGF-1 survival properties (Perks et al. 2002).

Such exquisite regulation of IGF and IGFBP-5 activity has obvious significance in a tissue such as the mammary gland where IGF-IGFBP affinity can be regulated by mechanisms such as IGFBP-ECM association and post-translational modification of IGFBP (see above). When experiments designed to manipulate IGFBP-5 concentrations in BC cell lines were eventually undertaken they provided conflicting data. Treatment of the T47D BC cell line with the anti-oestrogen RU486 was reported to inhibit cell growth and decrease IGFBP-5 concentrations leading to the suggestion that IGFBP-5 acted as a growth promoter (Coutts et al. 1994). However other studies have shown that anti-oestrogen treatment of MCF-7 cells increased IGFBP-5 mRNA and protein expression and caused inhibition of cell growth (Huynh et al. 1996; Parisot et al. 1999a; Rozen and Pollak 1999). Experiments in the ER-ve and IGF1R-ve Hs578T cell line suggested that IGFBP-5 could act as an anti-apoptotic factor in an IGF-independent fashion (Perks et al. 1999) and that this may occur via a PKC – dependent mechanism. However once again the mechanisms by which IGFBP-5 exerts this effect may be cell line

dependent. In contrast to the above reports, stable or adenovirus based transfection of MDA-MB-231 and Hs578T cells inhibited cell growth with cells arrested at the G₂/M transition and activation of the caspase-8/9 signalling pathway. This same study showed that formation and growth of tumours derived from MDA-MB-231 cells over expressing IGFBP-5 was inhibited when the cells were transplanted in nude mice (Butt et al. 2003). No effect was seen following addition of exogenous IGFBP-5 to cells arguing for an intracellular mechanism of action for IGFBP-5. Further studies confirmed that the intracellular location of IGFBP-5 can differentially regulate the activity of the protein in BC cells. For example in MDA-MB-435 BC cells nuclear location of IGFBP-5 is associated with a growth inhibitory action whereas accumulation of IGFBP-5 in the cytoplasm is associated with a growth stimulatory activity and is also a poor prognostic factor for BC (Akkiprik et al. 2009). Accordingly IGFBP-5 contains a nuclear localisation signal (NLS) in the C-terminal domain and in many instances nuclear transport of the protein is required for activity (Butt et al. 2005; Schedlich et al. 1998). As well as growth inhibition, IGFBP-5 has also reduces MCF-7 cell migration but induces cell adhesion (Sureshbabu et al. 2012; Vijayan et al. 2013) and although the data is still somewhat conflicted a consensus appears to be emerging that IGFBP-5 may act as a tumour suppressor in BC inhibiting both mitogenesis and metastasis.

For IGFBP-2 the emerging paradigm suggests an opposite effect from IGFBP-5 with the binding protein stimulating BC cell mitogenesis and progression and acting as a tumour promoter. IGFBP-2 acts via an integrin based mechanism to suppress PTEN in MCF-7 cells thus prolonging PI3K activity to provide a pro-tumourigenic signal (Perks et al. 2007). Further studies suggested a pro-survival action of IGFBP-2 through an ER α dependent mechanism and interestingly knock down of IGFBP-2 ablated ER α expression- an effect reversed by addition of exogenous IGFBP-2 (Foulstone et al. 2013). A related study from this group confirmed decreased ER α expression when IGFBP-2 secretion was inhibited by the flavin EGCG in MCF-7 cells. Under these conditions cell growth was inhibited and the expression of the p53 and p21 tumour suppressors was enhanced (Zeng et al. 2014) and analysis of shRNA based IGFBP-2 knockdown in the BC cell line BT474 indicates regulation of numerous pro-tumourigenic pathways.

However there are also reports which suggest that IGFBP-2 may inhibit tumourigenesis in vitro and in vivo. In MCF-7 cells over expressing integrin β 3, IGFBP-2 associates with the α v β 3 complex and inhibits IGF-1 or -2 mediated cell migration. Reduced tumour growth in these transfected cells is associated with integrin mediated localisation of IGFBP-2 to the cell surface (Pereira et al. 2004). In the BC cell line Hs578T, although IGFBP-2 promotes de-adhesion of cells it also inhibits proliferation through an α 5 β 1 integrin binding

mechanism (Schutt et al. 2004). As this cell line lacks a functional IGF1R such effects were postulated to occur in an IGF-1 independent fashion and indeed subsequent studies using microarray analysis in this cell line demonstrated that exogenous IGFBP-2 regulated the expression of several genes associated with cell proliferation, adhesion and apoptosis (Frommer et al. 2006). Finally, an engineered protease resistant IGFBP-2 inhibits MCF-7 tumour cell growth as a xenograft in a female nude Balb/c mouse model illustrating the importance of post-translational modification on the activity of IGFBPs. (Soh et al. 2014). Therefore as for IGFBP-5, data with respect to IGFBP-2 is somewhat conflicted and further experiments are required to definitively identify functions for these proteins in BC. With this in mind the next section focuses on some clinical aspects of IGF axis action in BC, particularly with activation of the axis as a route for escape from adjuvant endocrine or chemotherapeutic therapy. Again we mainly discuss the role of IGFBP-2 and -5 although where appropriate we will allude to other members of the axis. Figure 1 depicts mechanisms by which the IGF axis may influence growth and metastasis of tumour cells.

IGF axis and BC: clinical aspects

Of serious concern in BC is the development of resistance to anti-endocrine and chemotherapeutic strategies. This is an area of intense research activity using advanced genomic, epigenomic and proteomic technologies. Underpinning many

of these investigations is the concept that resistance to traditional adjuvant therapies is associated with inappropriate activation of alternative growth factor pathways which subsequently drive mitogenic and metastatic processes in BC cells. Two of the most intensively studied are the epidermal growth factor (EGF) and IGF signalling pathways. The IGF axis has been studied particularly in relation to development of resistance to tamoxifen – a selective oestrogen receptor modulator (SERM). Initial reports suggested that increased IGF1R expression in tamoxifen resistant (TamR) cells was associated with enhanced growth responses to E2 (Wiseman et al. 1993). However subsequent evidence from several studies has been contradictory with reports of both up and down regulation of IGF1R expression in TamR cells (Boylan et al. 1998; Knowlden et al. 2005; Massarweh et al. 2006; Parisot et al. 1999b; van den Berg et al. 1996) and the significance of altered expression and activity of IGF1R in TamR cells is still under investigation. Because of these observations, and due to the generally disappointing trial results of anti-IGF and anti-IGF1R based strategies in treatment of endocrine resistant BC, effort has been directed towards the third arm of the IGF axis – the IGFBPs. Some rationale is provided for this in the discussions above where evidence is presented that IGFBP-5 may act as a tumour suppressor and IGFBP-2 as a tumour promoter. McCotter et al. provided the earliest report of altered IGFBP profile in tamoxifen resistant MCF-7 and ZR-75-1 cells although the BP species were not definitively identified in this initial study (McCotter et al. 1996). The same group subsequently reported down regulation of IGFBP-2 in

Fig. 1 Stromal, tumour or systemically derived IGF-1 can cause growth and metastasis of tumour cells by several mechanisms. These include direct mitogenic stimulation of tumour cells via cell surface IGF1R; stimulation of tumour cells migration and metastasis and regulation of endothelial cell barrier through action on extracellular matrix (ECM) integrity.

All of these actions of IGFs may be affected through interaction with circulating and tissue derived IGFBPs which may have inhibitory or enhancing effects on IGF-1 action. In addition IGFBPs may display IGF independent actions of either a stimulatory or inhibitory nature—see text. Adapted from reference (Clemmons 2007)

tamoxifen resistant MCF-7 cells (Maxwell and van den Berg 1999) although changes in IGFBP-2 concentrations may have been influenced by the presence of dexamethasone in these cell cultures (Phillips et al. 2003). However Juncker-Jensen et al. (Juncker-Jensen et al. 2006) demonstrated IGFBP-2 up regulation in tamoxifen resistant MCF-7 cells as well as cells resistant to the selective oestrogen receptor down regulator (SERD) fulvestrant and the pure anti-oestrogen R58 668 and these findings agree with our observations in tamoxifen resistant MCF-7 cells. However Juncker-Jensen et al. also demonstrated that IGFBP-2 knock down using antisense oligonucleotides or siRNA did not affect the growth of resistant cells and concluded that IGFBP-2 may be a marker for resistance but had no causal role in this respect. Whether this is indeed the case is under further investigation in our laboratory. Expression of IGFBP-2 in MCF-7 cells is regulated through the PI3K/Akt/mTor activation of Sp1 on the IGFBP-2 promoter (Martin and Baxter 2007; Mireuta et al. 2010). We did not examine whether this pathway was up regulated in TamR cells although such studies would clearly be of merit and experiments in our laboratory continue in this area. Interestingly over expression of IGFBP-2 in the MDA-MB-231 cell line was associated with increased chemotherapeutic resistance in vitro and in vivo and this effect was ablated by down regulation of IGFBP-2 expression using antisense directed oligonucleotides (So et al. 2008). For IGFBP-5, Ahn et al. used an RNA interference based screening methodology to identify this gene as a determinant of tamoxifen sensitivity in MCF-7 cells (Ahn et al. 2010). They also demonstrated that shRNA based knockdown of IGFBP-5 expression conferred tamoxifen resistance in these cells perhaps related to a concomitant loss of ER α expression. TamR cells showed decreased IGFBP-5 expression and addition of exogenous IGFBP-5 partly restored sensitivity to tamoxifen. Although such data suggests a role for IGFBP-5 in TamR further independent experimental confirmation is required. This reciprocal regulation of IGFBP-2 and -5 expression in TamR cells is interesting given that in the human genome IGFBP-2 and -5 are located very close together in a 3' to 3' configuration on chromosome 2 separated by only 30 kbs of genomic sequence (Allander et al. 1994). Such architecture suggests IGFBP-2 and -5 may have arisen via a gene duplication event and that a common cis or trans acting regulatory mechanism of reciprocal gene expression may exist. Clearly further experimentation is required to test this hypothesis but such a co-ordinate and reciprocal regulation would provide a novel mechanism of gene regulation in the IGFBP family and may shed light on genetic mechanisms involved in BC (see below).

In a clinical context, IGFBP-2 and/or IGFBP-5 may have predictive or prognostic value in BC (Taylor et al. 2010) and may also be used to predict responses to different therapeutic regimes including SERMs, SERDs and aromatase inhibitors

(AIs) (Huynh et al. 1996; Ahn et al. 2010; Yamashita et al. 2009). In general terms increased expression of IGFBP-2 in BC tissues is associated with poorer survival rates agreeing with some of the evidence discussed above of a tumour promoter activity. An IHC based study in Norwegian women using 120 breast resections reported a gradual increase in IGFBP-2 expression from atypical hyperplasia through to carcinoma in situ and invasive carcinoma. (Busund et al. 2005). Similarly a tissue microarray (TMA) analysis of >4000 primary invasive BCs identified over expression of IGFBP-2 in tumour tissue and an adverse survival outcome correlated with IGFBP-2 expression in ER α negative cancers (So et al. 2008). Expression of IGFBP-2 in association with the cell adhesion protein β -catenin is correlated with lymph node metastasis of BCs (Sehgal et al. 2013) and high levels of IGFBP-2 expression together with loss of PTEN expression were associated in triple negative (TN) BC along with poorer survival rates (Dean et al. 2014). IGFBP-2 is up regulated in an in vitro model of trastuzumab resistant breast cancer (SKBR3 cells) (Dokmanovic et al. 2011) and may act via an ErbB2 signalling mechanism to provide a route of escape from anti-HER2 based therapeutic strategies. Some interesting recent reports have shown that IGFBP-2 may be a target for an immune based route for BC treatment and multiple antigenic peptides (MAPs) containing IGFBP-2 epitopes have been used to block tumour development in a transgenic mouse model (Disis et al. 2013; Park et al. 2008).

For IGFBP-5 there is less evidence for a role in tumorigenesis. IGFBP-5 mRNA was up regulated in breast cancer tissue relative to normal gland although there was no correlation between tumour grade and IGFBP-5 expression (Li et al. 2007). Similarly there is evidence that IGFBP-5 is either elevated (Hao et al. 2004; Wang et al. 2008) or decreased in lymph nodes metastases (Li et al. 2007). An analysis of 116 patient samples identified that a high IGFBP-5/IGFBP-4mRNA ratio was related to poorer prognosis and a decreased period of disease free survival (Mita et al. 2007) and therefore high expression of IGFBP-5 mRNA was defined as a poor prognostic factor in BC. Similarly, an IHC based study of 76 BC samples indicated IGFBP-5 expression in invasive BC tissue. This study also independently reported that an increased IGFBP-5: IGFBP-4 expression ratio was negatively associated with recurrence free survival (RFS) and disease free survival (DFS) (Becker et al. 2012). However a more recent tissue microarray analysis (TMA) of 153 BC biopsies from tamoxifen treated patients suggested that high expression of IGFBP-5 was associated increased overall survival (Ahn et al. 2010) and a very recent IHC based study reported reduced IGFBP-5 protein in the stroma surrounding aggressive metastatic BC tissues (Plant et al. 2014) although the source of stromal associated IGFBP-5 was not clear. Interestingly single nucleotide polymorphisms (SNPs) in the 3' end of both *IGFBP2* and *IGFBP5* genes were associated with an

increased risk of BC in a cohort of African-American women age <40 and similar findings were confirmed in a population of Nigerian women (Gamer et al. 2008). These observations have been developed by the publication of an extensive sequence analysis of the IGFBP-5 gene (at 2q35 in humans) which describes an SNP close to an enhancer region of the IGFBP-5 promoter. This allele was associated with down regulation of IGFBP-5 and increases risk of ER+ breast cancers (Ghoussaini et al. 2014). Whether this SNP is associated with TamR BC is unknown but the association of TamR with decreased IGFBP-5 expression suggests that this is worthy of further investigation.

Conclusions

Recent and ongoing studies suggest a role for the IGF axis in the aetiology and metastasis of some BCs. Although most clinical trials have involved anti-receptor based strategies (either humanised monoclonal antibodies directed towards the extracellular domain of the IGF1R or small molecule inhibitors of receptor tyrosine kinase activity) there has been a growing interest in the IGFBPs as possible therapeutic targets. As more knowledge becomes available on the role of this protein family in normal and neoplastic mammary gland then the prospect of developing anti-neoplastic reagents directed at the IGFBP family may become more realistic. This strategy may be particularly useful in those situations where resistance to more traditional forms of endocrine (SERM, SERD, and AI) or chemotherapeutic treatment has resulted in disease relapse.

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