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Insulin-like growth factor– oestradiol crosstalk and mammary gland tumourigenesis

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Abstract

Development and differentiation of the mammary gland is dependent on the appropriate temporal expression of both systemically acting hormones and locally produced growth factors. A large body of evidence suggests that molecular crosstalk between these hormonal and growth factor axes are crucial for appropriate cell and tissue function. Two of the most important trophic factors involved in this process are the oestrogen (E) and insulin-like growth factor (IGF) molecular axes. The reciprocal crosstalk that exists between these pathways occurs at transcriptional/post-transcriptional and translational/post-translational levels regulates the expression and activity of genes involved in this process .In a clinical context an important consequence of such crosstalk in the mammary gland is the role which it may play in the aetiology, maintenance and development of breast tumours. Although oestradiol (E_2) acting through oestrogen receptors α and β (ERα/β) is important for normal mammary gland function it can also provide a mitogenic drive to ER+ breast tumours. Therefore over several years anti-oestrogen therapeutic regimens in the form of selective oestrogen receptor modulators (SERMs – e.g. tamoxifen), aromatase inhibitors (AI e.g. anastrozole) or selective oestrogen receptor down regulators (SERDs – e.g. fulvestrant) have been used in an adjuvant setting to control tumour growth. Although initial response is usually encouraging, large cohorts of patients eventually develop resistance to these treatments leading to

tumour recurrence and poor prognosis .Although there are potentially many routes by which breast cancer (BC) cells could escape anti-oestrogen based therapeutic strategies one of the most studied is the putative growth factor mediated activation of ER(s). Such E-independent activation of ER could provide a route of escape from classical anti-oestrogen based therapeutic strategies. Because of this growth factor modulation of ER activity has been an intensively studied route of molecular crosstalk in the mammary gland. The insulin-like growth factors (IGF-1 and -2) are amongst the most potent mitogens for mammary epithelial cells and there is accumulating evidence that they interact with the E_2 axis to regulate mitogenesis, apoptosis, adhesion, migration and differentiation of mammary epithelial cells. Such interactions are also bi-directional and E_2 has been shown to regulate the expression and activity of IGF axis genes with the general effect of sensitising breast epithelial cells to the actions of IGFs and insulin. In this short review we discuss the evidence for the involvement of crosstalk between the insulin-like growth factor (IGF) and oestrogen axes in the mammary gland and comment on the relevance of such studies in the aetiology and treatment of BC.

Keywords: insulin-like growth factor; oestradiol; breast cancer; endocrine resistance

1.1 Introduction

In the mammary gland IGF activity is regulated as part of a multi-component molecular axis comprising the growth factors themselves IGF-1 and IGF-2; the cell surface IGF-1 and IGF-2 receptors: IGF-IR and IGF-2R; and six well characterised soluble IGF binding proteins: IGFBP 1-6 [1, 2]. Ancillary members of the axis include IGFBP related proteins IGFBPrP-1 and 2 – sometimes called IGFBP-7 and -8 [3, 4], specific and non-specific IGFBP proteases [5] and an acid labile subunit (ALS) protein which complexes with IGFBP-3 and IGFBP-5 in serum and interstitial fluids [6]. IGF-1 and -2 are small (~7.5kDa) polypeptides which show structural and functional homology to insulin [7] although unlike insulin IGFs are expressed by several tissues and cell types. The IGF-1R also shows a high degree of homology with the insulin receptor (IR) and hybrid IGF-1R/insulin receptor species have been reported for both the A and B isoforms of the insulin receptor (IR-A / IR-B)

comprising an IGF-1R αβ dimer combined with an αβ dimer of the IR-A or IR-B isoform. Although the physiological significance of hybrid receptor expression remains unknown they have been reported in BC cells [8]. The IGF-2R has no structural homology to either IGF-1R or IR and is a single chain transmembrane protein identical to the cation-independent mannose-6-phosphate receptor [9].

This partial structural homology amongst the ligands and receptors in the IGF and insulin family leads to a degree of promiscuity in ligand binding and also results in some functional redundancy between IGF-1, IGF-2 and insulin polypeptides [10] . Binding of IGF-I to IGF-1R leads to recruitment of adaptor proteins belonging to the insulin receptor substrate (IRS) family of proteins to autophosphorylated regions within the cytosolic domain of IGF-1R with subsequent activation of downstream signalling molecules including Shc/Grb2, Ras/Raf-1, MAPK/Erk and PI3K/Akt. Ultimately such signalling pathways control various cellular functions including mitogenesis, apoptosis, adhesion, migration and differentiation [11].

IGF activity is modified by soluble high affinity IGFBPs which are present ubiquitously in biological fluids. IGFBPs can exert enhancing or inhibiting effects on IGF action [12, 13] or display IGF independent effects [14, 15]. IGFBPs are secreted by several cell types and show tissue specific expression profiles. They are expressed and secreted by BC cell lines and primary breast tissue cultures and the role of these proteins in the regulation of normal and abnormal breast epithelial cell function has been intensively studied. IGFBPs are substrates for IGFBP proteases which act to release IGFs from IGFBPs allowing access of growth factors to cell surface receptors. BC cell lines and primary cultures also express and secrete such proteases [16, 17] and it is suggested that the activity of these enzymes may regulate access of growth factors to malignant breast epithelial cells [18]. It is clear therefore that the intuitive view of the sequestration and thus inhibition of IGF activity by IGFBPs is an over simplification of the biological reality in those tissues such as the breast where multiple components of the IGF axis are expressed. Therefore the potential role of the IGF axis in the functioning of normal mammary gland and possible role in BC is predicated on a comprehensive understanding of the expression and activity of the axis in normal and tumourigenic breast tissue. A diagrammatic representation of the principle genes and components of the IGF axis is outlined in Fig 1.

1.2 IGF axis expression and function in mammary gland

In vitro cell culture models (mainly BC lines) have been used extensively to examine IGF axis effects on various biological end points including cell mitogenesis, migration, adhesion, apoptosis and less commonly differentiation [1, 12, 14, 15]. Although the data generated is on occasion contradictory, in some instances findings reported in vitro have been confirmed in vivo by the use of conditional knock out or tissue specific over expression of IGF axis genes in the mammary gland.

Initially the role of IGFs in the function of normal and malignant breast tissue was the subject of some controversy. This was mainly due to reports of low or absent IGF expression in cultures of mammary epithelial cell lines or primary breast epithelial cell cultures. Although early reports described the accumulation of IGF-1 in medium conditioned by BC lines [19, 20] this activity was subsequently shown to be associated with incomplete extraction of IGFBPs from media samples leading to an over estimation of IGF activity by radio-immunoassay procedures in use at that time [21, 22]. mRNA analysis confirmed very low levels of IGF-1 and -2 expression in BC epithelial cells [23] and although experiments with an inhibitory monoclonal antibody(Mab) specific for the IGF-1R suggested an autocrine role for secreted IGF-1 in BC epithelial cell proliferation [24] such experiments were often confounded by the use of inappropriate basal culture conditions used to examine the effects of added growth factors on cell division [25]. Further experiments led to the suggestion that *in vivo* in breast tissue IGFs are secreted mainly by stromal cells to act in a paracrine manner on closely associated epithelial cells [26, 27]. Consistent with this hypothesis, the expression of IGF-1R and IGF-2R in BC cell lines and primary mammary epithelial cell cultures is well established and following the initial demonstration of IGF-1 stimulation of thymidine incorporation in MCF-7 and T47-D BC lines [28] several groups have confirmed that BC cell lines and primary cultures are exquisitely sensitive to mitogenic stimulation by both IGF-1 and IGF -2 [25, 29-32]. IGF-1 also displays well characterised anti-apoptotic effects in MCF-7 cells, a feature which may be associated with a permissive role for the growth factor in the establishment of tumour cells [33]. The use of antibodies which specifically block IGF-1R together with specific knockdown of IGF-1R expression suggest that the mitogenic action of both IGF-1 and -2 are probably mediated by the IGF-1R [24, 31, 34-39]. However IGF-2 may signal separate defined biological end points through its

own cognate receptor in BC cells and binding of both IGF-2 and insulin to cognate receptors in BC cells has been confirmed [40, 41].

IGFBP expression in mammary tissue and BC cells is well documented and it was suggested that IGFBP sequestration of growth factor inhibited IGF action in BC cells [42, 43]. Accordingly agents which increased pericellular IGFBP, including vitamin D analogues and anti-oestrogens (see below) attenuated the mitogenic activity of IGFs in BC cultures. However, a major confounding factor is the many observations which report an enhancing action of IGFBPs on IGF activity in BC cells [12-14]. Although the mechanisms of these enhancing effects are not fully elucidated amongst the theories postulated include the establishment of a low affinity “sink” formed by ECM bound IGFBP-IGF complexes (see Fig 1) or an increased half-life of IGFBP bound IGF compared with free IGF. It has also been suggested that IGFBPs may inhibit the IGF-1 induced desensitisation of IGF-1R and prolong the activity of growth factor [13] and some reports also indicate that IGFBPs may have a direct IGF independent activity in breast tissues [44]. These findings introduce a further layer of complexity to studies which examine the activity of the IGF axis in BC cell physiology and are especially important for a full understanding of potential mechanisms involved in cross talk between IGF axis and other hormones/growth factors in normal mammary gland and BCs.

In BC cells the profile of IGFBP expression is cell line specific and correlates with oestrogen receptor (ER) status. ER+ MCF-7 cells express IGFBP-2, -4 and -5 at moderate to high levels [45-47] while under basal conditions the expression of IGFBP-1, -3 and -6 is very low. This particular pattern of IGFBP secretion is by and large replicated in other ER+ BC cells [31, 48, 49] although there is some inconsistency in reports of IGFBP-3 expression in MCF-7 cells [50-53]. It is difficult to rationalise these inconsistencies although one explanation may be associated with the degree of cell confluence under which studies are conducted. IGFBP-3 expression is reported to be up regulated in senescent cells [54-56] and it may be that in confluent cultures of MCF-7 cells this gene is expressed. At least one study demonstrating IGFBP-3 expression used confluent cultures of cells [51] and our studies which reported low IGFBP-3 levels used cultures which were 70-80% confluent (data not shown). Alternatively IGFBP-3 expression may vary with cell passage number. IGFBP-3 expression was also increased by treatment with anti-

oestrogens or retinoic acid derivatives. Although the significance of these studies remains open to interpretation the hypothesis has been advanced that increased IGFBP-3 expression mediated by such agents is mechanistically associated with their growth inhibitory properties and may have significance in vivo [51, 53, 57-60] .However the mechanism by which such effects occur is not fully established and regulation of IGFBP-3 concentration was often evident at the post transcriptional level and in some instances was not associated with concomitant increases in IGFBP concentrations in conditioned medium [58, 59]. Such disjunction between gene expression and protein secretion may be due to proteolysis of IGFBPs and proteases which act on binding proteins are expressed in BC cells [16, 17]. This further highlights some of the difficulties in interpretation of IGF axis effects on mammary epithelial cells and in the potential disruption of the axis which may occur in BC cells.

1.3 IGF – E crosstalk: general considerations

The observation that IGF and oestrogen axes interact within the mammary gland to regulate mammary cell physiology is well established and is an active research area. It is postulated that a greater understanding of such crosstalk mechanisms may shed light on potential involvement of the IGF axis on the escape of ER+ mammary tumours from anti-oestrogen therapies (see Abstract). Although the discussion which follows separates these directions of cross talk between the E and IGF axes it is important to appreciate that this is somewhat artificial and that cross talk between IGF and E_2 signalling pathways is bi-directional. This was exemplified in early studies in uterine cell cultures demonstrating reciprocal phosphorylation of ERα and IGF-1R following treatment of cell cultures with IGF-1 or E_2 respectively [61, 62]. The direction of E_2 crosstalk to the IGF axis displays an extra level of complexity derived from the multi-component nature of the IGF axis and E_2 affects the expression and activity many of these components (IGFs, IGFRs and IGFBPs). A review of the published data in this area highlights some inconsistencies usually related to the non-uniform use of standardised tissue culture methods. The most meaningful experiments have been conducted on cells usually maintained in the absence of Phenol Red (an oestrogenic stimulant) and under serum free or in the presence of dextran-charcoal stripped serum preparations. Ultimately examination of added

tropic factors against a completely E_2 and serum free background gives the most reliable and robust data.

Although crosstalk between IGF and E is important in mammary gland function it is also important to appreciate that IGF and E independently regulate an overlapping set of genes in mammary tissue and that this may have consequences for the development of BC treatments. An elegant microarray based study in MCF-7 cells demonstrated over 450 genes which were co-regulated by both IGF-1 and E_2 [63]. Interestingly this pool was enriched in genes involved in aspects of DNA replication and metabolism. Similarly co-repressed genes included known or putative tumour suppressors. The clinical significance of these findings were underscored in an analysis of ERα +ve breast tumour samples where an "activated IGF-E_2 signature" was associated with a shorter time to metastasis in these patients.

The ultimate goal of cell line work is to eventually translate this information into the clinic. A good example of this is selective estrogen receptor down-regulator Fulvestrant (Faslodex), developed as a therapy from initial experiments showing that anti-estrogens regulated the growth of tamoxifen-stimulated MCF-7 cells [64]**.** In a similar vein the role of the IGF1R signaling pathway in clinical breast cancer is now beginning to be explored in trials. It is known that diabetic women who are treated with metformin have a lower risk of breast cancer and results from a retrospective cohort study showed that diabetic breast cancer patients receiving metformin had a better response rate with neoadjuvant chemotherapy than patients who received other forms of diabetic medication [65]. As a result, a trial is underway examining the effects of 5 years of adjuvant metformin versus placebo in pre- and postmenopausal women with either ER-positive or ER-negative breast cancer post-surgery and chemotherapy [66] One of the biological end points is the effect of metformin on plasma insulin levels. However data from the MA.17 trial was less promising showing no benefit and even some adverse effects of octreotide, one of the first IGF-1 targeting agents available in the clinic, plus 5 years of tamoxifen on disease-free or overall survival [67] While it would appear there is still some way to go before the IGF-1 pathway can be effectively targeted in humans, cell line work can be a very effect vehicle to identify potential pathways and targets for therapeutic development. Further studies into the molecular details associated with co-regulation of mammary

gland gene expression and the potential significance for BC treatment is fully warranted.

1.4 IGF axis effects on ER function

Since early studies using subcutaneous implants of des (1-3) IGF-1 (an IGF-1 analogue with reduced affinity for IGFBPs but native affinity for IGF-1R) demonstrated an enhanced effect of E_2 on growth factor stimulated terminal end bud formation and alveolar development in mammary gland [68] there has been a great deal of interest in the mechanistic aspects of this hormone-growth factor interaction. As discussed above, the main route described for IGF cross talk to oestrogen signalling mechanisms is through the regulation of ERα activity by IGF-1. In vitro this has usually been reported as an alteration in phosphorylation status of ERα following IGF-1 treatment. Although ERα is subject to extensive post translational modifications [69] the growth factor mediated phosphorylation of serine residues S118 and S167 in the AF-1 domain of human ERα have been the most intensively studied and there is some evidence that this route of E_2 independent activation of ERα may be partly involved in the development and maintenance of tumourigenesis in breast tissue [1, 70, 71]. In this fashion, IGF-1 (and other GFs) may allow the escape of ERα+ breast tumours from anti-oestrogen therapeutic regimens [72, 73]. It is important to note however that this hypothesis is challenged by clinical data which relate the phosphorylation status of ERα with such parameters as disease free survival (DFS) in various cohorts (see below).

IGF-1 stimulates serine phosphorylation of ERα at S118 in an Akt dependent manner [74] and the receptor is subsequently translocated to the cell membrane with resulting activation of the MAPK cascade [61, 75]. It is known that IGF-1 acts via MAPK to phosphorylate ERα at S118 [76] and this suggest that both Akt and MAPK signalling pathways converge at S118. The significance of these findings is not fully understood but different pathways of IGF-1 signalling may show cell specificity in activation profiles. IGF-1 also acts via PI3K/Akt to phosphorylate at S167 within the AF-1 domain of ERα [77] and phosphorylated ERα subsequently associates with many components of the IGF signalling pathway including IRS proteins [78], Shc [79] PI3K [80] and IGF-1R itself [81]. In this manner ERα may regulate the

expression, stability and intracellular localisation of these signalling intermediates [75, 82, 83]. Recent studies suggest that mTor/S6K1 lies down stream of Akt in the pathway of S167 phosphorylation although somewhat unusually this study did not demonstrate simultaneous S118 phosphorylation of ERα after IGF-1 treatment of MCF-7 cells [84]. The ERα protein is in fact a somewhat promiscuous substrate and is phosphorylated at S118 by several different kinases [85-87]. Whether there is a cell or tissue type specificity associated with individual kinase activity on ERα remains largely unknown but further elucidation of specificities associated with kinase modification of ERα activity is of obvious physiological significance.

There may also be important developmental and temporal aspects associated with IGF-E axis interaction in breast tissue. An elegant study using transgenic mice expressing IGF-1 in the mammary gland from the bovine keratin 5 (BK5) promoter demonstrated developmentally regulated control of IGF-E crosstalk. In pre-pubertal animals IGF-1 signalling was dominated by the PI3K/Akt pathway and the presence of an IRS-1/ERα complex was clearly identified. In post pubertal animals with lower levels of ERα expression no such complex was observed and the main route for signalling proceeded via the Ras/Raf MAPK pathway [88]. Such differences in signalling patterns may differentially affect tumourigenesis during different developmental stages and indicate a potential role for ERα in routing IGF signalling pathways. IGF-1 may phosphorylate ERα at sites other than S118/S167. Studies with S305A mutant ERα suggest S305 as important IGF-1/IRS-1/Akt mediated phosphorylation site [89] and this modification may regulate acetylation at the nearby K303 residue regulating sensitivity of ERα to E_2 activation [89, 90]. Two excellent reviews on the characterisation and physiological significance of phosphorylated ERα isoforms are available [91, 92] and Fig 2 shows some of the IGF dependent pathways of ERα serine phosphorylation.

There is limited data on the interaction of other IGF axis genes and ER function. A recent report indicates that silencing IGFBP-2 expression leads to a loss of ERα expression in MCF-7 and T47D cell lines and that expression can be up regulated in wild type (wt) cells by the addition of exogenous IGFBP-2 [93].and the authors provide evidence for an anti-apoptotic role of IGFBP-2 by a mechanism which is dependent on ERα expression. Conversely, IGFBP-4 and -5 have been reported to inhibit E_2 induced ERα activity in MCF-7 cells possibly via a PI3-K/Akt dependent

pathway resulting in an inhibition of E_2 stimulated cell growth [94]. However in this study E_2 treatment did not stimulate IGF-1R phosphorylation nor did IGF-1 addition stimulate ERα phosphorylation. Both of these observations run counter to the current weight of evidence. Also in this study neither IGFBP-4 nor -5 were detectable by Western blot of non-transfected MCF-7 cell conditioned medium despite the fact that the expression and secretion of these proteins by ER+ BC cell lines has been widely reported in the literature [95]. Despite these caveats the area of IGFBP crosstalk with ERs is very new and no doubt will provide much interesting data in the near future.

It is interesting to position studies of IGF-ER crosstalk within a clinical setting. Phosphorylation of ERα is generally viewed as an activating post translational modification (PTM) [96, 97] and is postulated as a route of escape of ER+ BC cells from SERM therapeutic strategies – see above. However, some evidence from immunohistochemical (IHC) screening of archived BC tissues using a well characterised anti-pS118-ERα antibody suggests that phosphorylation of ERα at S118 is associated with longer relapse free survival and longer time to progression in tamoxifen treated cohorts [98, 99]. While this does not necessarily negate a role for IGF-1 stimulated phosphorylation at S118 as an activating PTM, at the moment it is difficult to reconcile these observations although other factors such as precise patterns of PTMs of ERα, temporal aspects related to PTMs or intracellular location of ERs may prove to be significant.

Much less data is available on the IGF-1 mediated regulation of ERβ. No effect of IGF-1 on ERβ expression in the MDA-MB-231 cell line was reported [100] although pro- IGF-2 may stimulate translocation of ERβ to intracellular organelles in the Hs578T and basal-like BC line CRL-2335 [101]. This latter observation may have some clinical relevance as high localised levels of mature IGF-2 have been reported in early stage BC [27]. In prostate cancer cells ERβ (and ERα) can associate with Src and the p85 subunit of PI3K in an E_2 dependent fashion and up regulate IGF-1R expression [102]. Knockdown of IGF-1R in MCF-7 cells using siRNA increases ERβ expression and the subsequently altered ERβ: ERα ratio may increase apoptosis via activation of tumour suppressor p53 [103]. The phosphorylation of the ERβ isoform has also been described [104] although whether such modification is regulated by IGF axis activity is currently unknown. However the mouse ERβ is phosphorylated at S106 and S124 via the MAPK pathway [105] and as the former residue is conserved

as S118 in human ERα it raises the possibility that ERβ may be subject to similar IGF-1 mediated PTMs as ERα. In addition ERβ has at least 5 alternatively spliced variants (ERβ1-5) which are expressed in a cell and tissue specific manner and which may display differential functions [106]. A novel route for growth factor modulation of ERβ function may be through the regulation of the differential expression of receptor isoforms. To date there is little information in this area although it is a subject of ongoing research in our laboratories.

The G protein coupled oestrogen receptor (GPER30) is the most recently described ER [107]. This cell membrane associated protein is believed to be partly responsible for the rapid non-genomic effects of E_2 – reviewed in [108]. Very little data is available on crosstalk between GPER and the IGF axis although a recent report indicates that IGF-1 up regulates GPER expression in MCF-7 cells in a PKCδ/ERK dependent fashion [109]. Interestingly this effect was dependent on ERα activity suggesting a close relationship between these two ERs. In addition IGF-1R associates with the G protein linked chemokine receptor CXCR4 in the MDA-MB-231 cells leading to an IGF-1 mediated dissociation of Gα2 and Gβ subunits from the complex with associated stimulation of chemotaxis [110]. Whether such a mechanism(s) exists for the GPER receptor is currently unknown.

1.5 ER crosstalk to IGF axis

As indicated above the evidence for IGF -1 and/or IGF-2 expression in BC cell lines and in primary tumour epithelial cells is not convincing however mammary stromal cells express IGF-1 and -2 and this population may provide a source of local growth factor [111].With this in mind an intriguing hypothesis suggests that E_2 increases vascular permeability via a VEGF dependent mechanism and thus allows access of circulating growth factors (including IGFs) to cell surface receptors [112]. The extent to which this mechanism may operate in the mammary gland requires further investigation. Irrespective of whether such a mechanism operates there is compelling data that E_2 can increase the expression of IGF-1R on the surface of BC epithelial cells [113-116]. Up regulation of IGF-1R expression may occur through interaction between liganded ERα and the trans-acting zinc finger transcription factor Sp1 at the IGF-1R promoter [117]. More recent evidence indicates that in the PC3 prostate cancer cell line E_2 also up regulates IGF-1R expression via a c-Src/ERK1/2 mediated activation of the cyclic AMP response element binding (CREB) protein

which subsequently acts as a trans activating factor within a defined enhancer region in the IGF-1R promoter [118] see Fig 3. The resulting increase in IGF-1R density in breast epithelial cell membranes may account for the sensitisation of cells to the mitogenic effects of paracrine IGF-1 following E_2 exposure [119]. Some findings also suggest that E_2 decreases IGF-2R expression in both ERα+ and ERα- BC cell lines thus routing the mitogenic action of IGF-2 via the IGF-1R and possibly the A isoform of the insulin receptor (IR-A) [120, 121].

As well as influencing IGF-1R expression, E_2 treatment stimulates phosphorylation of IGF-1R. In MCF-7 cells E_2 stimulated tyrosine phosphorylation of IGF-1R is dependent on ERα expression and Shc activity [75] and these latter two proteins physically associate through interaction between the AF-1 domain of ERα and the phosphotyrosine binding SH2 domain of Shc [79]. E_2 induced activation of IGF-1R leads to downstream MAPK signalling [122]. An important upstream element in the activation of MAPK by this route may be the matrix metalloproteinase -2/9 (MMP-2/9) mediated release of heparin bound EGF (HB-EGF) and subsequent activation of EGF receptor signalling [123] and in this sense E_2 mediated IGF-1R activation can be viewed as lying up stream of EGF-R. Such mechanisms may provide alternative routes for ER+ tumour escape from SERM based chemotherapy. The effects of E_2 on IGF-1R function discussed above are commonly referred to as " non-genomic" ERα activity [124] and are associated with a localisation of ERα out with the nucleus - typically at the plasma membrane where it is in juxtaposition with growth factor receptors and associated signalling complexes. Changes in signalling associated with ERs complexed with such membrane and cytosolic signalling complexes are temporally acute and are differentiated from the more chronic alterations in gene expression associated with the classical nuclear action of ERs as direct trans acting transcription factors at oestrogen response elements (EREs) or as accessory transcription factors at other non-ERE cis acting promoters. However changes in plasma membrane and cytosolic signalling events often ultimately lead to changes in gene expression and as such the commonly used term "non-genomic" may be somewhat misleading.

E_2 also influences the expression and activity of other IGF signalling components. Perhaps one of the most important of these is the insulin receptor substrate (IRS) family of signalling molecules [117]. These adaptor proteins directly bind to activated

IGF-1R in a phosphotyrosine (pY) dependent manner and following their own receptor catalysed tyrosine phosphorylation provide docking sites for other proteins involved in IGF-1 signalling including the p85 subunit of PI3K, Grb2 and Jak1. As such the role of IRS family proteins in BC is a subject of intense study – reviewed in [125]. In essence E_2 acts as a positive factor in IRS function. Thus, as for IGF-1R, E_2 can enhance IRS-1 expression via activation of transcription factor Sp1 [126]. In addition, ERα is reported to bind and stabilise IRS-1 protein [78] leading to increased signalling through the IGF-1 pathway [127]. The sum effect of ERα action on IRS-1 is to reinforce the stimulatory action on IGF-1R function and provide a mitogenic drive toward cell division. In this context, it is important to appreciate that the ultimate molecular effect of IGF-E_2 cross talk is to enhance the transition of cells through the cell cycle process and involves the regulation of G1-S phase cell cycle progression through the regulation of key regulators such as cyclinD1 or c-myc [73]. Limitations of space prevent a detailed discussion of this area but the E_2-IGF control of cell cycle regulation is an important area of research with respect to BC progression and the interplay between the axis in the control of this process is an area of active study [101, 128].

There is much less evidence for a direct route of functional cross talk between E_2 and IGFBPs. Most studies have been concerned with profiling IGFBP expression in ER+ and ER- cells and with investigation of the effects of E_2 on IGFBP expression. – (see above) One study reported that E_2 stimulated secretion IGFBP-2, -4 and -5 in MCF-7 cells [129]. Although the contribution of such studies to any causality in E_2 – IGF axis crosstalk has not been determined such findings suggest that E_2 stimulation of IGFBP expression does not inhibit mitogenic activity through any autocrine mechanisms as ER+ remain mitogenically sensitive to added E_2 under these conditions. Such arguments also indicate prima facia that under basal conditions of cell growth the mitogenic effects of E_2 are unlikely to be mediated by the very low levels of IGFs reported in most BC cell culture models in turn suggesting that autocrine regulation of BC cell growth by IGF-1 is not a tenable hypothesis (see above). Although IGFBPs may act in an IGF independent manner to stimulate BC cell mitogenesis [1] whether this is a potential mechanism of action for the mitogenic action of E_2 requires rigorous experimental verification and in general terms the role of IGFBPs in BC cell physiology requires further investigation and clarification.

1.6 Clinical implications

The development of resistance to SERM treatment in ER+ breast tumours presents a major therapeutic challenge in the treatment and management of this cancer. The molecular phenotypes associated with such escape from SERM control are the subject of intense study through such techniques as gene array and extensive proteomic analyses. The design of studies aimed at investigating this phenomenon have been predicated on the recruitment of alternative mitogenic signalling pathways by cancer cells resulting in continued tumour cell growth and metastasis. It is in this context that studies of signalling cross talk discussed in this paper may have some clinical relevance. In vitro, BC cell lines can acquire tamoxifen resistance through growth in the presence of drug. Although caution must be exercised in the interpretation of findings using such cells, especially with regard to their clinical relevance, this model is useful for the examination of signalling pathways which may be activated in tamoxifen resistant breast tumours. The MCF-7 cell line is most commonly used in this research and there is a small literature which describes perturbations in IGF axis expression and function in such cells following the acquisition of tamoxifen resistance. For example, increased IGF-1R expression in tamoxifen resistant cells may enhance E_2 independent growth of the cells and an early study in wt and TamR cells suggested that this may indeed occur [130]. However subsequent literature in this area is contradictory with reports describing both the up regulation [131-133] and down regulation [134-136] of IGF-1R expression in TamR cells and the significance of altered IGF-1R activity in the development of TamR remains under investigation. Nonetheless, there is evidence that in TamR cells which have also developed resistance to the EGF-R inhibitor AG 1478 subsequent cell growth is largely dependent on activation of the IGF-1R [137]. This may provide a mechanism of escape of BC cells from both anti-oestrogen and anti-EGF-R (Herceptin) based therapy.

Considerations such as these have led to the investigation of anti-IGF directed therapeutic strategies aimed at regulation BC progression. Essentially these strategies involve three different classes of compound – 1. Anti-IGF-1R monoclonal

antibodies (Mabs); 2- less commonly Mabs directed towards IGF-1/IGF-2; 3 – tyrosine kinase inhibitors (TKIs) designed to inhibit of IGF-1R signalling activity. Such reagents have been used to target many solid and blood based tumours although we restrict ourselves to a discussion of the use of these strategies in BC. In the adjuvant setting, anti-IGF directed strategies may be used as mono-therapy or more commonly in association with anti-oestrogen directed therapy (SERM, SERD or AI). Although such protocols are currently the subject of numerous clinical trials it is fair to say that results to date have not been encouraging with the latest report of lack of efficacy of the humanised anti-IGF-1R Ganitumab in combination with either exemestane (AI) or fulvestrant (SERD) in increasing progression free survival compared to placebo with anti-oestrogen therapy [138] one of several negative studies which has encouraged the pharmaceutical industry to withdraw from this area. However, some consideration of possible reasons for the failure of such approaches may lead to the development of improved strategies [139]. A recent study indicated that the acquisition of tamoxifen resistance in MCF-7 cells was associated with decreased IGF-1R expression and subsequent impaired activation of Akt activation in response to IGF-1. The subsequent lack of effect of anti-IGF1R antibodies demonstrated in vitro may provide a rationale for the general lack of clinical utility demonstrated by these reagents in clinical trials and indicate that alternative strategies may be required for treatment of TamR tumours[140]. For those reagents developed as Mabs against the IGF-1R concern has been expressed that increased activation of the insulin receptor could arise due to increased level of serum insulin. Such an effect may occur secondarily to increases in pituitary growth hormone (GH) secretion due to incomplete feedback by IGF-1 subsequent to IGF-1R blockade. Typically anti-IGF-1R Mabs are not effective at the IR and thus under these circumstances increased signalling may occur through this IR. Prophylactic treatment with metformin has been investigated in attempt to deal with the hyperinsulinemia which is evident in these circumstances [135]. Alternatively, elevated IGF-1 levels subsequent to GH elevation may activate IGF-1R if anti-IGF-1R directed Mabs are not able to completely inhibit ligand binding. In addition to this a heterogeneous array of IR/IGF-1R is often displayed on the surface of tumour cells which comprise alternatively spliced forms of the IR and IGF-1R present either as holo-receptors or hybrid receptors comprising the αβ subunits of IR in association with the αβ subunits of IGF-1R. Such variation in receptor presentation may call for a

more sophisticated approach to the development of Mabs with appropriate blocking activities at individual receptors. Such observations also argue for the careful analysis of appropriate clinical specimens prior to decisions on potential therapies and informs in the general area of patient selection based on appropriate biomarker expression. Due to some of these findings an alternative route of developing small molecule inhibitors of receptor tyrosine kinase activity (TKIs) has been pursued [18] and several of these compounds are currently under investigation in clinical trials. Indeed a combination of Mab/TKI directed therapy is clearly feasible and may have advantages over treatment with either agent alone. More recently the development of specific kinase inhibitors has allowed the combination of anti-IGF directed therapies with inhibitors of the mTOR serine/threonine kinase in treatment of solid tumours and have generated some encouraging preliminary data [141, 142].

Due to some of the difficulties associated with manipulation of IGF receptor and ligand activity outlined above there has been a level of ongoing interest in the third main component of the IGF axis -the IGFBPs- and their possible role in the development of BC. .Some early studies reported altered IGFBP profiles on the acquisition of tamoxifen resistance by the MCF-7 and ZR-75-1 cell lines. However the species of BP reported in this study were not identified immunologically but only by molecular weight on ligand blot [143]. Subsequently the same group identified IGFBP-2 as down regulated in TamR MCF-7 cell lines [144] although dexamethasone – a known regulator of IGFBP expression in mammary epithelial cell lines [145] - was present in these cultures. We have shown that IGFBP-5 is down regulated at both mRNA and protein levels in TamR MCF-7 cells (unpublished observations). This may have biological significance as this binding protein has been shown to inhibit growth of BC cells [15] and IGFBP-5 treatment of BC tumour cells in a xenograft mouse model has been shown to restore tamoxifen sensitivity [146]. Similarly, the mechanism of action of the SERD fulvestrant may be associated with increased expression of IGFBP-5 in BCs [115]. Fulvestrant treatment of MCF-7 cells enhances ERα association with IGF-1R in a Src dependent fashion and is associated with ERα translocation to the plasma membrane, phosphorylation of IGF-1R and MAPK activation.[124]. These findings are unexpected for a drug whose principle use is in the inhibition of BC cell mitogenesis by enhancing ERα degradation and the findings of this study are important enough to require

independent confirmation. As for studies with wt BC cells any causality between alterations IGFBP expression and acquisition of TamR remains to be established. Although we and others have clearly demonstrated IGFBP-5 down regulation in these circumstances our preliminary observations suggest that IGFBP-2 is up regulated in TamR cells. Given the functional redundancy that exists in the IGFBP family with respect to high affinity binding of IGFs this argues that any effects of locally altered IGFBP concentrations in a TamR environment most likely would occur in an IGF independent manner. However, there are studies which report distinct, differential and IGF-independent effects of IGFBPs in BC cells. For example IGFBP-3 was reported to enhance ceramide or paclitaxel induced apoptosis in the human Hs578T BC cell line whereas IGFBP-4 and -5 inhibited ceramide induced apoptosis [147, 148]. In an extension of these studies the direct IGF-independent effects of IGFBP-3 and -5 on cell survival, attachment and apoptosis were found to be regulated by the presence of fibronectin in the ECM [12]. As perturbation in ECM composition and structure are a common feature in many tumours, including breast, such observations may have important clinical consequences. A further refinement of the action of IGFBP-3 was reported from the same group who demonstrated cell line specific effects of IGFBP-3 on cell survival which was also dependent on the liganded status of the β1 integrin receptor [149]. Clearly there are many factors which influence potential activities of IGFBP in the pericellular environment of breast tissue including the local concentrations of different IGFBP species. Further extensive experimentation is required to outline the detail of IGFBP action in mammary gland and possible role(s) in BC and how such activity may be related to the development of resistance to SERM or AI treatment.

1.7 Concluding remarks

Although this review has focussed on crosstalk between the E and IGF axes we would highlight to the reader that E also interacts with other growth factor axes. Understanding the biology behind steroid-growth factor crosstalk has recently been emphasised as a 'knowledge gap' in a gap analysis conducted by a panel of breast cancer experts http://breast-cancer-research.com/content/15/5/R92. In the context of the mammary gland and BC perhaps the most significant of these is the crosstalk that exists between the ERα and members of the EGF family [150, 151]. As for IGF-1 molecular crosstalk between EGF receptors and ERα is bi-directional and such

mechanisms may be associated with promotion of tumour cell growth. This area has been expertly reviewed [152]. In conclusion the interaction of E and IGF axes in the mammary gland is important not only for normal tissue function but may also be involved in breast tumour aetiology and progression. A further understanding of the mechanisms involved in this crosstalk will mostly likely impact on the future treatment of BCs which develop resistance to anti-oestrogen therapy.

Figure Legends

Fig 1

The IGF axis comprises six soluble high affinity IGF binding proteins (IGFBP1-6 – yellow) together with the cell surface IGF-1 receptor (IGF-1R - blue) and IGF-2 receptor (IGF-2R - green). The insulin receptor (IR - red) and hybrid IR/IGF-1R are also able to bind IGFs although with lower affinity than cognate receptors. Similarly insulin and IGF-2 are able to bind to IGF-1R but with lower affinity than IGF-1 itself. Most IGFBPs can associate with various extra-cellular matrix (ECM) structures - shown is IGFBP-5- and can be hydrolysed are by various IGFBP proteases. Both of these features are used to regulate the access of pericellular IGFs to cell surface receptors. Recent evidence also suggests that some IGFBPs (shown is IGFBP-3) display IGF-independent effects by association with specific IGFBP receptors. In serum and other biological fluids IGFBP-3 and -5 can associate with an acid labile subunit (ALS).

Fig 2

Dual regulation of IGF-1 mediated phosphorylation of ERα. S118 in the AF-1 domain of ERα is phosphorylated by both PI3K and MAPK pathways. S167 and S305 are phosphorylated by PI3K mediated S6K1 activation. ERα is phosphorylated at several other S, T and Y residues by a range of cellular kinases (see text for further details).

Fig 3

E_2 acting via ERα regulates IGF1IR expression via Src/Erk1/2 mediated activation of CREB at a distal enhancer region within the IGF-1R gene. Gene expression is also stimulated by an E_2/ERα dependent activation of Sp1 at a more proximal promoter site within the 5’ flanking region of the IGF-1R gene. For further details see text.

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