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Estrogen Receptor- β and the Insulin-Like Growth Factor Axis as Potential Therapeutic Targets for Triple-Negative Breast Cancer

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Abstract

Triple-negative breast cancers (TNBCs) lack estrogen receptor- α (ER α), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) amplification and account for almost half of all breast cancer deaths. This breast cancer subtype largely affects women who are premenopausal, African-American, or have BRCA1/2 mutations. Women with TNBC are plagued with higher rates of distant metastasis that significantly diminish their overall survival and quality of life. Due to their poor response to chemotherapy, patients with TNBC would significantly benefit from development of new targeted therapeutics. Research suggests that the insulin-like growth factor (IGF) family and estrogen receptor beta-1 (ER β 1), due to their roles in metabolism and cellular regulation, might be attractive targets to pursue for TNBC management. Here, we review the current state of the science addressing the roles of ER β 1 and the IGF family in TNBC. Further, the potential benefit of metformin treatment in patients with TNBC as well as areas of therapeutic potential in the IGF-ER β 1 pathway are highlighted.

Keywords

insulin-like growth factors; estrogen receptors; estrogen receptor-beta; basal-like breast cancer; estrogen receptor-beta isoforms; metabolism

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I. INTRODUCTION

Breast cancer (BC) is the second most common malignancy in women. Of the estimated 256,000 women diagnosed with breast cancer annually in the United States, about 10–15% of all breast cancer cases are identified as triple-negative breast cancer (TNBC).^{1–3} However, this BC subtype accounts for almost half of all breast cancer deaths. Triple-negative breast cancers lack estrogen receptor-alpha (ER α) and progesterone receptor (PR) expression as well as human epidermal growth factor receptor-2 (HER2) amplification. TNBC tumors are generally larger in size, are initially of higher grade, exhibit lymph node involvement at diagnosis, and are biologically more aggressive than other breast cancer subtypes. Exploring novel therapeutic approaches for TNBC is critical because the median survival for women with metastatic TNBC is less than 12 months, and virtually all women with metastatic TNBC ultimately die of their disease despite systemic therapy.⁴ Currently, no targeted therapies are approved for treatment of TNBC, and cytotoxic chemotherapy is the mainstay of systemic treatment.⁵ Although TNBC patients tend to have higher clinical response rates to chemotherapy, they also have higher rates of distant recurrence and a worse prognosis than women with other breast cancer subtypes.^{2,6–8} TNBC tumors occur frequently in premenopausal women, in women of African-American descent,⁹ and in patients with BRCA1 or BRCA2 mutations.^{1,8,10} Unlike breast cancers that express ER α and/or PR, patients with TNBC are not generally considered to be candidates for current targeted endocrine therapies. Further, HER-2-targeted treatments are not reported to be useful for clinical management of TNBC. This report will consider new therapeutic targets and alternative treatment strategies that may hopefully stop TNBC progression and prolong patient survival.

II. BACKGROUND

Treatment of patients with TNBC is challenging due to the heterogeneity of the disease and the absence of well-defined molecular targets.^{2,6,11} One of the first molecular insights into TNBCs was the report that these tumors are likely to arise in *BRCA1* mutation carriers and often have gene expression patterns similar to those of BRCA1-deficient tumors.⁸ BRCA1 plays an important role in DNA double-strand break repair, contributing to maintenance of DNA stability. Poly ADP-ribose polymerase (PARP) enzymes are critical for the processing and repair of DNA breaks.³ Tumor cell lines lacking functional BRCA1 or BRCA2 are sensitive to PARP inhibitors in preclinical studies.^{12–14} Clinical trials using both PARP inhibitors and DNA-damaging agents (such as cisplatin) in TNBC are currently underway in *BRCA1/2*-mutant tumors.¹⁵ Independent studies identifying other molecular markers associated with TNBC, such as VEGF,¹⁶ EGFR,¹⁷ Src,¹⁸ and mTOR,¹⁹ have been important for the design of clinical trials investigating targeted treatments. Other reports indicate that a TNBC subgroup predicted to have a relatively favorable prognosis is characterized by high expression of “luminal-like” genes such as androgen-receptor (AR), whereas TNBC subgroups with worse prognosis are characterized by expression of cancer stem-cell-like markers.²⁰ Clearly, there is a major need to better understand the molecular basis of TNBC and to develop effective treatments for this aggressive and deadly type of breast cancer. More extensive genomic, molecular, pathologic, and immunologic analyses of TNBCs are

required to understand the complexity of the disease and to identify potential molecular “drivers” that can be therapeutically targeted in the clinic.

III. ESTROGEN RECEPTOR- β IN TRIPLE-NEGATIVE BREAST CANCER

Although the classical estrogen receptor form ER α is not expressed in TNBC, a number of reports have investigated expression of a more recently discovered member of the nuclear transcription factor superfamily termed ER β .²¹ The two forms of the estrogen receptor are encoded by different genes, *ESR1* and *ESR2*, on the sixth and fourteenth chromosome (6q25.1 and 14q23.2), respectively. Although *ESR2* is located on a different chromosome, the DNA-binding domain of the ER β protein product shares 96% homology with ER α and 60% homology at the ligand-binding domain, suggesting that the receptors are capable of binding similar DNA sites, with both similar and distinct ligand preferences. Five ER β variants that are mostly generated by alternative splicing have been described and include ER β 1, ER β 2, ER β 3, ER β 4, and ER β 5.^{22–24} Multiple ER β variants can occur in normal breast tissue and in breast tumors, thus presenting a challenge to understand their specific biologic functions. Importantly, only ER β 1, generally considered to be the wild-type form of ER β , retains an intact ligand-binding domain (LBD) to interact with specific ligands, thus making ER β 1 an attractive option for potential systemic drug therapy.^{23,25} Notably, ER β 2 and ER β 5 are reported to form dimers and partner with ER β 1. Neither ER β 2 nor ER β 5 have known ligands, and their biologic activity is presumed to be dependent on ER β 1.^{23,26} The only known function of ER β 5 is to modulate or interfere with ER β 1 signaling. The potential role of other variants in malignancy remains to be clarified.

Immunohistochemical (IHC) detection of ER α in tumors is a standard assay in the clinic to plan patient management.²⁷ In spite of the considerable structural homology shared by ER α and ER β , ER β is not identified in standard IHC clinical assays for ER α , nor is ER β considered at this time in patient management decisions in the clinic. It is critical to decipher the role of ER β in TNBC as earlier studies suggested that ER β levels are higher in breast tumors of African-American as compared to Caucasian women, and that ER β may play a critical role in TNBC progression.²⁸

A major problem in detecting ER β in clinical specimens using IHC is the lack of well-validated and specific antibodies. Currently, there is little to no consensus on the best laboratory protocols for ER β detection. As a consequence, reports on ER β expression in breast cancer tissues, particularly in TNBC and ER α -negative specimens, exhibit significant variability in findings and in correlates with clinical outcomes.^{23,26,29,30} Several available monoclonal and polyclonal antibodies directed to ER β and used with different tissue preparative and staining techniques have been tested in comprehensive studies.^{23,31–36} From this work, two antibodies have been recommended for immunohistochemical investigation of breast and other tissue specimens—namely, PPG5/10 specific for ER β 1^{26,35,36} and 14C8 directed to the N-terminus of ER β that detects most isoforms of the protein.^{34–38} Figure 1 shows specific IHC staining of ER β 1 in TNBC specimens from the clinic using validated antibody PPG5/10 as reported before.³⁹ Specific nuclear staining of ER β is shown on a representative patient specimen [Figs. 1(a)–1(c)]. Extranuclear staining of ER β 1 was also present in a majority of TNBC cases examined previously (unpublished data).³⁹ As

described before, nuclear and extranuclear staining of ER β 1 and variants is often present in TNBC tumor specimens from patients^{23,26,34–36} and similar data are reported for non-small cell lung cancers.^{40–42}

An overview of currently available evidence indicates that ER β expression may be decreased in pre-invasive breast cancer as compared with equivalent normal tissue⁴³ and the ER β /ER α ratio in breast cancer subtypes other than TNBC declines significantly as disease progresses from normal epithelium to DCIS and invasive ductal carcinoma.^{23,43,44} Collectively, these findings led to the hypothesis that ER β counteracts the effects of ER α when both receptors are expressed together in breast cancer cells,^{23,45–47} with ER β thereby acting as a tumor suppressor. In contrast, other reports indicate a significant association between ER β with proliferative biomarkers (such as Ki-67) in clinical specimens, and ER β in node-negative breast cancer is reported to associate with a positive response to tamoxifen therapy and longer patient survival.^{23,30,33,48} In TNBC, nuclear expression of ER β associates with significantly reduced overall patient survival (Fig. 2) in some^{23,25,39} but not in all studies,²⁶ while ER β 5 expression appears to be a prognostic marker of worse prognosis in TNBC subsets in another recent investigation.²⁶ As reported before,³⁹ the overall survival of a small cohort of patients with advanced TNBC was worse for those who expressed high nuclear ER β 1 (positive) than for those with low ER β 1 expression (negative). Of note, a significant number of African-American patients were included in this sample and all of these patients were positive for high nuclear ER β 1 expression.³⁹ Furthermore, high nuclear ER β 2 is an independent marker of early relapse in ER α -negative breast cancer and especially TNBC.⁴⁹ Since ER β 2 can form heterodimers with ER β 1 and modulate its transactivation in a ligand-dependent manner to modulate gene expression by ER β 1,²² it will be important to assess the impact of ER β 2 on correlates with clinical outcome in future studies. It is possible that ER β 1 and ER β 2 or ER β 5 are necessary partners in promoting aggressive TNBC. Indeed, the ratio of ER β 1: ER β 2 expression in TNBC specimens may be critical in predicting clinical outcome.^{23,25}

In pursuing new therapeutic approaches for TNBC, it is informative to consider results of additional studies on both TNBC and related ER α -negative disease. Hence, ER β 1 is reported to be a biomarker for improved survival in TNBC patients when treated with tamoxifen.^{23,25,30} Although previous work suggested that tamoxifen use only reduces the risk of ER α -positive breast cancer, Yan et al.²⁵ report that both ER β 1 and ER co-regulator SRAP are predictive biomarkers of tamoxifen response/benefit in women with ER α -negative breast cancer. In ER α -negative tumors, ER β 1 expression correlates with Ki-67 proliferation marker, suggesting that ER β 1 may have a role in driving proliferation, and that antiestrogen treatment, by inhibiting ER β 1, could slow tumor progression.^{23,25} With the discovery of ER β expression alone in TNBC, this presents the possibility that, in this ER α -negative cohort, tamoxifen or alternative antiestrogen strategies may mediate activity via ER β 1, the full-length ligand-binding receptor isoform. Further, among hereditary breast cancer cases, the majority of BCs arising in *BRCA2* mutation carriers are ER α -positive, whereas most BCs arising in *BRCA1* mutation carriers are ER α -negative at diagnosis including a significant proportion that are TNBC.^{20,23} Nevertheless, estrogen may be important in pathogenesis of breast cancers in *BRCA1* mutation carriers, particularly given reports that premenopausal bilateral oophorectomy is associated with reduced breast cancer risk for

BRCA1 mutation carriers. Furthermore, ER β is commonly expressed in breast cancers of patients with *BRCA1* mutation and is postulated to be a target for tamoxifen. Thus there are plausible mechanisms by which tamoxifen may prevent breast cancer for *BRCA1* mutation carriers. The recent work of Phillips et al.⁵⁰ provides further evidence that, for *BRCA1* mutation carriers, tamoxifen use for breast cancer might reduce the risk of contralateral breast cancer. Of note, Shazer et al.⁵¹ report that the antiestrogen raloxifene, used as an ER β -targeted therapy, inhibits growth of ER β -positive, androgen-independent prostate cancer.

Although earlier work confirmed that ER α -positive breast cancer cells are more sensitive than ER α -negative breast cells to growth-inhibitory effects of tamoxifen, moderate anti-proliferative responses to tamoxifen and to ICI-164,384 were found in ER α -negative cells including TNBC cells.⁵² These effects may potentially be modulated in part by a second unique binding site identified for hydroxytamoxifen in the coactivator-binding groove of ER β that may disrupt ER β -coactivator interactions.⁵³ Collectively, these reports have important implications since approved breast cancer treatments (such as tamoxifen, raloxifene) may be other alternatives for ER β -positive TNBC patients with generally few options other than cytotoxic chemotherapy.^{1,25} Of note, there is an ongoing clinical trial to investigate the effectiveness of adjuvant endocrine therapy for operable ER β -positive, TNBC patients (<http://clinicaltrials.gov/show/NCT02089854>). This trial includes two adjuvant endocrine therapies, either toremifene for premenopausal and perimenopausal patients or anastrozole for postmenopausal patients. The primary target of toremifene is hypothesized to be ER β .

Although ER β activity is generally considered antagonistic to ER α when both receptors occur together in a cell, the role of ER β forms in isolation is not well documented. Molecular studies show that when both ER α and ER β are present together in tumor cells, each ER restricts the binding site occupancy of the other, with ER α generally being dominant to ER β . It is clear that ER β binding and actions in gene regulation are different in the absence of ER α expression in BC cells.^{47,54} Correlation of ER β with high proliferative biomarkers is reported in ER α -negative tumors but not in those with ER α expression.^{25,34} Among the first studies on stable transfection of ER β in TNBC MDA-MB-231 cells, it was determined that the proliferation rate of the tumor cells positively correlates with the level of ER β expression.⁵⁵ The results using stable ER β clones demonstrated that proliferation is increased as ER β expression is increased in ER α -negative tumor cells. A confirmation of these experiments was published later when ER β was transfected in MDA-MB-435 cells leading to significant stimulation of tumor progression as well as metastasis *in vivo*.⁵⁶ These reports on introduction of ER β in ER α -negative BC cells showing stimulation of tumor proliferation^{55,56} are consistent with more recent data using a different strategy, namely, using shRNA to silence ER- β expression, resulting in significant suppression of TNBC cell proliferation.³⁹ In contrast, other studies on transfection of ER β in ER α -low or -negative breast cancer cells indicate that ER β inhibits cell proliferation.^{23,57} Several reasons may explain such contrasting results. For example, transfection of ER α led to the paradoxical finding that ER α was a growth inhibitor in breast cancer, a result that is clearly inconsistent with established clinical findings.⁵⁸ This unexpected result and similar ER β transfection data may be due in part to excessive levels of ER β expression in the model systems used and

other complicating factors.²³ Furthermore, conflicting data may also be due to differences in expression and/or activity of ER β 2 or ER β 5 isoforms.^{25,26} High nuclear ER β 2 is an independent marker of early relapse in ER α -negative breast cancer and especially TNBC.^{23,36} Since ER β 2 can form heterodimers with ER β 1 and modulate its transactivation in a ligand-dependent manner to modulate gene expression by ER β 1,⁴⁹ it will be important to assess the impact of ER β 2 in TNBC.

It is important to note that there are also conflicting reports on activity of ER β -specific ligands in TNBC as well as in other tumor types.^{59,60} ER β 1 expression correlates with tumor proliferation and progression in lung cancer,^{41,48,59,61} but not in colon cancer.⁶² A new report shows that under basal conditions ER β agonists induce apoptosis in breast cancer cells. However, when extracellular signal-regulated kinase 1 and 2 (ERK 1/2) was activated by co-incubation with epidermal growth factor (EGF), ER β agonist DPN induced proliferation in breast cancer cells.⁶³ Hence, ER β agonist DPN induces proliferation in breast cancer cells when EGFR and downstream ERK 1/2 signaling pathways are activated (see Fig. 3).⁶³ The results indicate that cellular context such as EGFR-induced signaling activity, which is known to be frequently overexpressed and active in TNBCs, modulates ER β growth-promoting effects.^{64,65}

Based on current data, estrogen receptors are known to regulate gene expression by both genomic and non-genomic inputs.^{66,67} Genomic signals involve direct action of nuclear-localized estrogen receptors as an estradiol-regulated transcription factor or co-regulator. By contrast, non-genomic signaling involves extranuclear events mediated by extranuclear estrogen receptors often in cooperation with co-activator or adaptor proteins;²⁵ these then impact gene expression indirectly by stimulating signaling cascades such as MAPK and PI3K/AKT to activate co-regulators or other transcription factors.^{66–68} In target cells, extranuclear ER α forms derive from the same transcript as nuclear ER α , but minor extranuclear ER α splice variants occur.^{66,67} Notably, several reports note that ER β may localize in tumor cell nuclei as well as in extranuclear sites.^{39,69} Work in our laboratory indicates that ER β expression is reduced in the nuclei of metastatic TNBCs from the clinic, but levels of ER β localized in extranuclear sites of the metastatic TNBC specimens are either maintained or increased relative to that found in paired primary TNBC specimens (unpublished observations).³⁹

Recent studies indicate that the tumor microenvironment also has a significant impact on tumor progression, particularly on tumor metastasis, which is a critical factor in TNBC. The tumor microenvironment includes surrounding supporting cells and fibroblasts, blood endothelial vessels, immune cells, secreted molecules, and extracellular matrix. Notably, tumor infiltrating leucocytes (TILs) that play a role in immune recognition of tumor cells express estrogen receptors that are known to be involved in the regulation of immune functions and inflammation.^{70–72} A recent report shows that about 61% of TILs express ER β , but ER α is not expressed,⁴⁴ thus raising questions on the role of ER β in immune modulation in breast cancer. Further, growth-stimulating effects of ER β in TNBC may be due, in part, to downstream actions to promote production and secretion of other growth factors such as vascular endothelial growth factor (VEGF), amphiregulin, and Wnt-10b,

which can act in turn to activate specific receptor signaling pathways known to be associated with TNBC progression.³⁹

IV. IGF AXIS AND TRIPLE-NEGATIVE BREAST CANCER

In the twenty-first century, the obesity rate among men (> 20 years) increased from 20.2% to 31.1% while the rate among women increased from 25.4% to 33.2% (Healthy People 2010; www.healthypeople.gov). Obesity is an established risk factor for breast cancer. In fact, obese patients with a high waist-to-hip ratio (WHR) have more frequent tumor recurrence, metastasis, and worse outcomes than nonobese patients.^{73,74} Obesity and lack of physical activity have been associated with hyperinsulinemia and insulin resistance, and obesity often influences the amount of free insulin-like growth factor (IGF) available to cells.⁷⁵ The insulin family of proteins have critical pleiotropic effects on metabolism and growth. A large body of evidence indicates the insulin/insulin-like growth factor (IGF-1, IGF-2) pathway plays a role in breast cancer progression.^{31,75–78} The insulin-like growth factor (IGF) pathways mediate metabolism, cell proliferation, differentiation, migration, invasion, and survival.^{79–81} The tyrosine kinase receptors, insulin-like growth factor-1 receptor (IGF-1R), and the insulin receptor isoform A (IR-A) are able to form homo- or heterodimers when bound by their ligands insulin, insulin-like growth factor-1 (IGF-1), or insulin-like growth factor-2 (IGF-2). Ligand binding then activates the downstream mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) pathways.⁸² Although early phase clinical trials raised hope for the use of IGF1R-specific antibodies for cancer therapy, initial phase III results in unselected patients have not been effective. Further clinical studies may benefit from use of predictive biomarkers to identify probable responders, the use of rational combination therapies, and consideration of alternative targeting.⁸³

Studies in ER α -positive breast tumor cell lines confirm a relationship between ER α and IGF-1R.^{84,85} Since increased expression of IGF-1 and IGF-2 are associated with aggressive tumor types as well as metastatic phenotypes,^{86–89} the IGF axis is now being explored in TNBC. The gene for IGF-2 (*Igf2*) is transcribed from the paternal allele and lies tangential to H19.^{90,91} The expression of *Igf2* and *H19* are regulated by a differentially methylated region (DMR) that contains estrogen response elements (ERE).^{92,93} In murine and rat testis, Pathak et al. demonstrate a direct association between ER β and *Igf2* methylation.^{93,94} Preclinical studies show that ER β activation and localization in TNBC cell lines may be initiated by IGF-2, acting via IGF-1R and IR (Fig. 3).⁷⁷ Expression of ER β in TNBC cell lines is due, in part, to the ability of IGF-2 to modulate ER β transcription.^{39,77} Furthermore, assessment of archival breast tissue obtained from patients with TNBC reveal high levels of both IGF-2 and ER β 1 as compared to controls.³⁹ Further investigation of the cross talk between IGF-2 and ER β may improve our understanding of TNBC progression as well as present potentially unique opportunities for treatment and screening.

A. IGF Binding Proteins

Even though IGF-2^{39,79} is implicated as a potential biomarker for TNBC development, it is important to consider that this growth hormone is a single component of a superfamily. The

bioavailability of both IGF-1 and IGF-2 are modulated by IGF binding proteins (IGFBPs). Six IGFBPs have been identified and studied.^{82,95,96} Circulating IGFBPs 1–5 have equal binding affinity for IGF-1 and IGF-2, whereas IGFBP-6 has a significant preference for IGF-2.^{96,97} Complexes formed between IGFBPs and IGFs, accompanied with an acid-labile subunit (ALS), increase the half-life of unbound IGFs and prevent their interaction with IGF-1R. Their sequestration of IGFs suggests IGFBPs act as tumor suppressors. Investigating the viability and activity of IGFBPs in the absence of ALS and/or IGFs, as well as their ability to form associations with other growth hormones, may help to improve our understanding of these binding proteins and clarify their potential therapeutic roles.

1. IGFBP-1—TNBCs are highly proliferative, and an IGFBP significantly associated with cell proliferation and motility is IGFBP-1. This ~30 kDa non-glycosylated protein contains an integrin recognition sequence, Arg-Gly-Asp, allowing IGFBP-1 to bind the $\alpha 5 \beta 1$ integrin (fibronectin) receptor.⁹⁸ As shown by Zhang and Yee, inhibition of IGF-1R and integrin motif of IGFBP-1 prevented migration of bone-seeking metastatic TNBC MDA-MB-231 (MDA-231BO) cells.⁹⁹ In TNBC/basal-like murine tumors lacking matrix metalloproteinase-9 (MMP9), increased levels of IGFBP-1 were detected resulting in slower tumor growth and reduced IGF-1R phosphorylation.¹⁰⁰ Consequently, increasing IGFBP-1 levels, whether endogenously or exogenously, could conceivably lead in part to improved TNBC patient outcomes.

2. IGFBP-2—IGFBP-2, the second most abundant IGFBP in the circulation, is a non-glycosylated ~36 kDa protein whose proliferative effects are well documented.^{96,101,102} Like IGFBP-1, IGFBP-2 contains an integrin motif and plays a role in cell mobility.^{95,103} This RGD sequence enables IGFBP-2 to enhance cell adhesion and facilitate neovascularization.¹⁰⁴ It is also an adipocyte-secreted factor that increases migration of tumor cells.¹⁰⁵ In fact, secreted IGFBP-2 enables migration and invasion of MCF-7 cells, an ER α -positive cancer cell line with low metastatic potential.¹⁰⁵ Furthermore, adipocytes cocultured with tumor cells have a greater than tenfold increase of IGFBP-2 mRNA expression.¹⁰⁵ These data underscore the effect of stroma on tumor invasiveness. Given that obesity is risk factor for breast cancer, Catsburg et al. assessed the relationship between atypical hyperplasia and IGFBP-2.¹⁰⁶ Their study suggested that in postmenopausal, nondiabetic women, an inverse relationship exists between serum IGFBP-2 and patient BMI.¹⁰⁶ In this population, high circulating IGFBP-2 might protect against the development of atypical hyperplasia, but clearly further studies are needed. Of note, IGFBP-2 contains a nuclear localization signal and via importin- α is transported to the nucleus.¹⁰⁷ Target genes of IGFBP-2 include those involved in focal adhesion, MAPK and Wnt signaling.¹⁰⁸ Tumor metastasis is also reported to be regulated in part by IGFBP-2. Knockdown of *IGFBP-2* decreases levels of β -catenin, a marker of metastasis, through the IGF-1R or integrin signaling pathways.¹⁰⁸ Assessment of lymph node metastasis reveals a positive correlation between IGFBP-2 and β -catenin.¹⁰⁸ Although little data currently exists on the role of IGFBP-2 in TNBC, assessment of IGFBP-2 expression may be helpful in predicting the progression of this cancer subtype since TNBC exhibits a high proliferative index and a propensity to widely metastasize.

3. IGFBP-3—IGFBP-3, the most abundant of all the circulating IGFBPs, forms a 150 kDa ternary complex with IGF-1 and an acid-labile subunit (ALS).⁹⁵ Through processes not completely defined, nuclear IGFBP-3 in combination with other regulatory complexes may either stimulate or inhibit cellular processes and tends to support genomic integrity.^{96,109} In invasive tumors, expression of IGFBP-3 tends to associate with tumor proliferation, ER α negativity, and HER-2 overexpression, while in ductal carcinomas *in situ*, a significant association exists between IGFBP-3 and ER α negativity.¹¹⁰ In early stages of breast cancer, IGFBP-3 behaves as a tumor suppressor while in a more advanced stages, IGFBP-3 may interact with the tumor microenvironment to promote tumor progression.^{111–113} Again, little data is currently available in understanding the role of IGFBP-3 specifically in TNBC.

4. IGFBP-4 and IGFBP-5—In the circulation IGFBP-4 exists as a 24 kDa doublet,⁹⁵ and as other members of this family, IGFBP-4 limits the bioavailability of IGF-1 and IGF-2. When IGFBP-4 is cleaved by pregnancy-associated plasma protein-a (PAPP-A), or prostatic kallikreins, hK2 and hK3, IGF-1 is released.^{114,115} In a murine model, PAPP-A-resistant IGFBP-4 reduced mammary tumor growth and increased cell apoptosis.¹¹⁶ Another binding protein, IGFBP-5, affects cell proliferation and migration via IGF-dependent and -independent modes.^{117–123} Based on *in vitro* experiments, IGFBP-5 confers resistance to BMS-536924, an IGF-1/IR inhibitor and it is significantly expressed in invasive tumors and the adjacent normal tissue.¹²⁴ Independently, the prognostic potential of IGFBP-5 or IGFBP-4 in breast malignancy appears limited.

5. IGFBP-6—Unlike other IGFBPs, the expression of IGFBP-6 is low in the liver.^{103,125} A unique characteristic of this IGFBP is its preferential binding affinity for IGF-2. IGFBP-6, along with IGF-2R, has the principle role in limiting IGF-2 bioavailability.⁹⁷ Oncogenic action of IGFBP-6 has been described¹²⁶ as well as an ability to inhibit tumor cell migration and tumor-associated angiogenesis.^{127,128} Conversely, IGFBP-6 promotes cell migration in rhabdomyosarcoma and colon cancer cells independent of IGF-2, an action mediated by p38, pERK, JNK1 signaling pathways.^{129–131} Evaluation of the role of IGFBP-6 in ovarian cancer decreased migration of HEY cells, an aggressive ovarian cancer cell line, whereas in less aggressive cultures such as SKOV3, IGFBP-6 stimulated cell migration.¹³² Although IGFBP-6 induced phosphorylation of ERK and JNK in both cell lines, exposure to inhibitors of ERK and JNK only reduced the migration of HEY cells.¹³² This suggests that IGFBP-6 has the potential to reduce metastasis independent of IGF-2.^{128–131} Under hypoxic conditions, IGFBP-6 also inhibits angiogenesis independent of IGF-2 *in vitro* and *in vivo*.¹²⁸ Concurrently, IGFBP-6 has the ability to induce cell migration by binding directly to prohibitin-2 (PHB2), a single-span membrane protein.^{129–131} What makes IGFBP-6 an attractive therapeutic target is its preference for partnering with IGF-2, which is highly expressed in invasive ductal carcinoma and TNBC,^{39,133} as well as its IGF-independent activity.

The IGF superfamily offers a plethora of potential therapeutic targets against TNBC. The ability of family members to impact cell proliferation, migration, angiogenesis, and adhesion, critical factors that affect patient outcome, underscores the need for further research in the role of the IGF family in TNBC development.

B. Metformin and TNBC

As noted above, obesity and the metabolic syndrome are associated with multiple factors that may cause an increased risk for breast cancer and breast cancer-related mortality.³¹ A known link between obesity and breast cancer risk is insulin resistance, a condition commonly treated with metformin, an orally bioavailable biguanide for diabetes mellitus type 2. Metformin decreases hepatic glucose production and intestinal glucose absorption. While increasing insulin sensitivity, metformin decreases circulating levels of insulin and insulin-like growth factors. Investigations of the therapeutic potential of metformin reveals an ability to inhibit TNBC proliferation and reduce TNBC tumor growth *in vitro* and *in vivo*.^{134,135} Moreover, survival outcomes among women with TNBC taking metformin demonstrate reduced distant metastasis even among postmenopausal, black, and obese women.¹³⁶

Metformin is an attractive potential therapeutic for TNBC due to its availability and impact on tumor growth and metastasis. This oral, antidiabetic medication reduces circulating glucose creating a beneficial environment for TNBC treatment. At physiologic or low levels of glucose, the ability of metformin to inhibit cell proliferation and tumor formation, and induce apoptosis is optimized.^{137,138} Metformin induces apoptosis of TNBC. Although not completely understood, metformin mediates this process by activating miR-193b, an inhibitor of fatty acid synthase, and AMP-activated protein kinase (AMPK), which inhibits signaling by mammalian target of rapamycin (mTOR) and signal transducer and activator of transcription-3 (STAT-3).^{138–141} *In vitro* metformin reduces mRNA levels of *vimentin* and *snail*, markers of epithelial-mesenchymal transition (EMT), while increasing *E-cadherin* highlighting a preventive action against TNBC metastasis.¹⁴¹ In endometrial cells, metformin inhibits cell proliferation stimulated by IGF-2 and mTOR signaling while increasing the expression of the progesterone receptor (PR) and sensitivity to medroxyprogesterone acetate (MPA).¹⁴² In these same cells, metformin upregulated IGFBP-1 mRNA and protein, reduced IGF-1R mRNA, and decreased proliferation, an anti-proliferative effect enhanced with PPP, an IGF-1R inhibitor.¹⁴³ In addition to the IGF-1R promoter, metformin significantly reduces promoter activity of IR,¹⁴⁴ accentuating its putative effect on the IGF/IR receptor axis. In lung tumorigenesis, metformin reduces circulating levels of insulin and IGF-1, and inhibits AMPK-independent receptor tyrosine kinase (RTK) signaling pathways (e.g., EGFR, cMET, VEGFR, FGFR;).¹⁴⁵ However, Capp et al. did not detect a significant change in IGF-1R or IGF-2R in the presence of metformin using primary cultures.¹⁴⁶ Another study evaluating patients >50 years with endometrial cancer and type 2 diabetes mellitus (DM2) did not detect a significant difference in IGF-1R among patients receiving metformin, insulin, or sulfonylurea derivatives.¹⁴⁷ To develop a better understanding of the impact of metformin on the IGF axis, further studies are clearly needed.

V. FUTURE DIRECTIONS FOR TNBC TREATMENT

Unfortunately, patients afflicted with TNBC often respond poorly to chemotherapy, and currently targeted therapies for TNBC are not available. However, preclinical *in vitro* and *in vivo* studies utilizing multiple therapies provide hope for improved outcomes for those with

TNBC. Lau et al. found that basal breast cancer responds well to metformin and erlotinib.¹³⁷ A combination of gefitinib, an epidermal growth factor receptor (EGFR) inhibitor, and inhibition of activation of sphingosine kinase-1 (SphK1)/sphingosine 1-phosphate (S1P) system by IGFBP-3 prevented TNBC tumor growth.¹⁴⁸ To prevent metastasis, Mancini et al.⁷⁹ investigated the efficacy of co-targeting IGF1 and IGF-2, and their receptors. In this study NVP-AEW541, an antagonist IGF-1R, was sufficient to inhibit IGF-1 initiated migration of MDA-MB-231 cells, whereas NVP-AEW541 complemented with MAB292, an IGF-2 monoclonal antibody, were necessary to inhibit IGF-2 mediated migration.⁷⁹ This highlights the benefit in determining levels of IGF-1 and IGF-2 in TNBC tumors in investigations to reduce metastasis. Another study assessed the response of TNBC cultures to BMS-754807, an anti-IGF-1R/IR inhibitor, and found significant reduction in cell proliferation. Furthermore, xenografts underwent complete regression when treated with BMS-754807 and docetaxel.¹⁴⁹ In addition, development of novel delivery methods to introduce IGFBP-1, IGFBP-2, IGFBP-6, or protease-resistant IGFBP-4 could promote improved methods to elicit tumor regression including TNBC (Fig. 3). Finally, as detailed in recent reports, cooperative interactions between IGF and ER β signaling pathways in TNBC and potential racial/ethnic differences in the biology of TNBC may lead to additional therapeutic strategies in this deadly disease.^{28,39}

VI. CONCLUSION

Targeted and more effective therapies for TNBC are urgently needed. It is clear that TNBC is a molecularly, pathologically, and clinically diverse disease that will likely require more targeted therapeutic approaches.^{1-4,6} Standard chemotherapy has some efficacy in a subset of patients with TNBC, and better strategies to identify these patients, as well as those less likely to respond prior to treatment, could have a significant impact on clinical management and patient outcomes. The success of clinical trials going forward in patients with TNBC may require stratification of tumors by molecular subtypes, specific genomic alterations, and/or pathologic prescreening for “driver” signal transduction pathways in TNBC specimens. For example, some novel therapeutic strategies are currently being tested in phase I–III trials, including antiestrogens and aromatase inhibitors as adjuvant therapies in TNBC, antiandrogens (such as enzalutamide) for androgen receptor-positive, and potentially other TNBC subtypes that have androgen receptor dependence,^{150,151} and poly(ADP-ribose) polymerase inhibitors for BRCA-mutated TNBC[142]. Treatment of TNBC based on other actionable targets such as IGF-2 also deserve consideration for future clinical translation. The discovery of ER β and its expression in TNBC initially raised hope that targeting ER β might offer new treatment options for TNBC patients, but several caveats, as discussed above, still remain. To accomplish the goal of discovering new targeted treatments for TNBC, further preclinical investigation utilizing a wide array of established TNBC models will help to develop more effective therapeutics and guide strategies for clinical intervention.

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ABBREVIATIONS

AR	androgen receptor
BC	breast cancer
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ERα	estrogen receptor-alpha
ERβ	estrogen receptor-beta
IGF-2	insulin-like growth factor-2
IGFBP	IGF binding protein
IHC	immunohistochemistry
PR	progesterone receptor
TNBC	triple-negative breast cancer
VEGF	vascular endothelial growth factor

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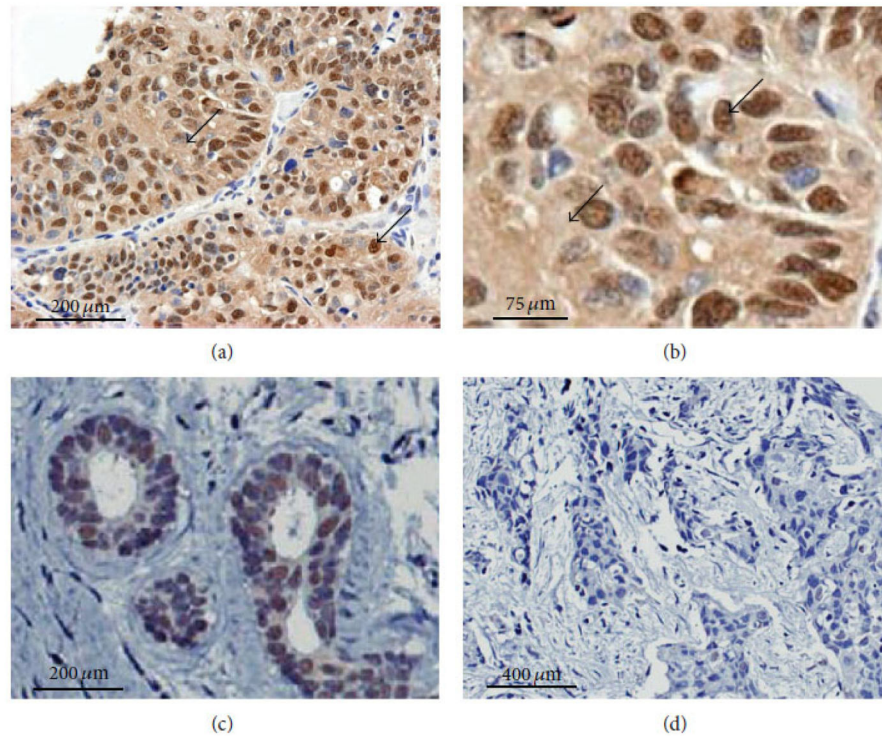


FIG. 1. ER β 1 expression in archival TNBC specimens detected by immunohistochemistry using anti-ER β 1 antibody (PPG5/10, AbDSerotec). A representative example of ER β 1 immunostaining is shown using tumor and neighboring, nonmalignant tissue from the same patient. (a) Nuclear and cytoplasmic staining are shown on a representative specimen of TNBC at low magnification. (b) The same specimen from the previous panel shows nuclear and cytoplasmic staining at higher magnification as indicated by arrows. (c) Expression of nuclear ER β 1 is also present in neighboring normal tissue of the same patient tumor tissue displayed in panels (a) and (b). (d) A different TNBC tumor specimen that does not express ER β 1 is shown for comparison. Antibody binding was detected by using diaminobenzidine (DAKO). Sections were counterstained with Harris hematoxylin. (Reprinted with permission from the Hindawi Publishing Corporation, Copyright 2015).³⁹

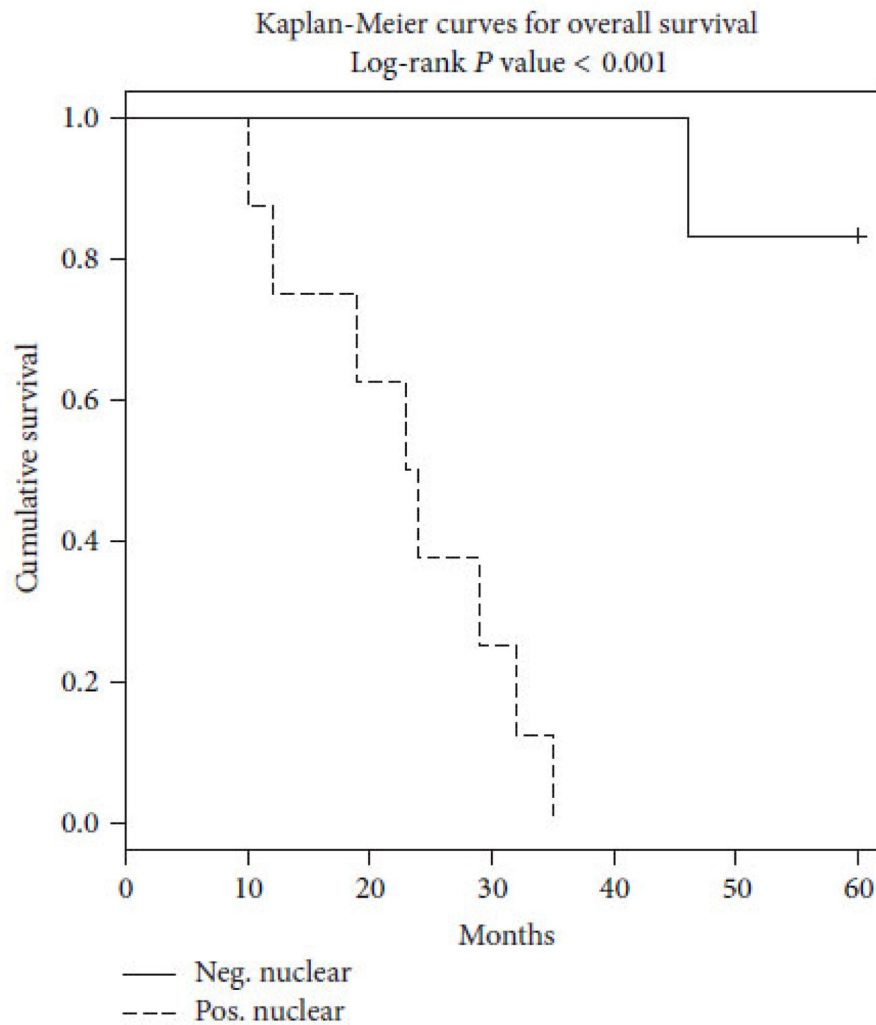


FIG. 2. Tumor expression of ER β 1 associates with reduced overall survival (OS) in TNBC specimens from the clinic. TNBCs from three African-American and 11 Caucasian women were scored for nuclear (using IHC with validated antibody anti-ER β 1 antibody PPG5/10 (AbDSerotec). Allred scores >2 are denoted as positive. In this group of patients with advanced TNBC, overall survival (OS) was significantly worse for TNBC patients with high nuclear ER β 1 (positive) as compared to those with low (negative) ER β 1 ($p < 0.001$). We note that TNBCs from all three African-American women were high ER β 1-positive. (Reprinted with permission from the Hindawi Publishing Corporation, Copyright 2015).³⁹

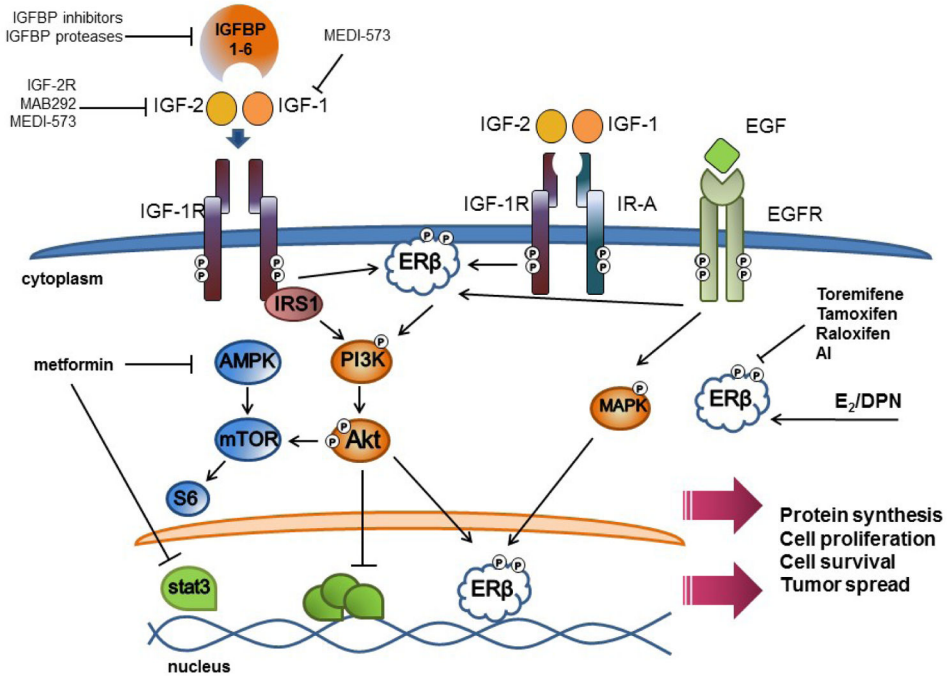


FIG. 3. Interactions of IGF and ERβ signaling pathways in malignancy. TNBC proliferation is regulated closely by a complex network of growth factor signaling pathways. It is well known that obesity and diabetes can increase insulin and insulin-like growth factor signaling, which in turn may stimulate specific receptors to drive the activation of downstream signaling pathways in TNBC. IGF-1 and IGF-2 bind receptors activating PI3K and Akt signaling and downstream mTOR activation that promotes protein synthesis, cell proliferation, and inhibition of apoptosis. In addition, EGFR is frequently overexpressed in TNBC and is stimulated by binding with epidermal growth factor (EGF). Cross-communication of EGFR signaling with ERβ may induce downstream signaling that contributes to tumor cell survival. ERβ is activated by estrogen (E2) and by synthetic estrogen receptor-selective ligands such as diarylpropionitrile (DPN) and can reportedly be modulated in part by breast cancer therapies such as toremifene, tamoxifen, and raloxifen. IGF-2 signaling may also promote acute phosphorylation of ERβ and late induction of ERβ transcription. Metformin, a common therapeutic used to manage diabetes mellitus type 2, has been demonstrated to be effective in partial suppression of TNBC by activating AMPK (which in turn inhibits mTOR downstream signaling) and/or by suppressing systemic insulin-like growth factor levels *in vivo*. Arrows represent a pathway of activation and solid lines represent inhibition. See text for details.

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