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Diversity of insulin and IGF signaling in breast cancer: implications for therapy

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Abstract

This review highlights the significance of the insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF-1R) signaling pathway in cancer and assesses its potential as a therapeutic target. Our emphasis is on breast cancer, but this pathway is central to the behavior of many cancers. An understanding of how IR/IGF-1R signaling contributes to the function of the normal mammary gland provides a foundation for understanding its aberrations in breast cancer. Specifically, dysregulation of the expression and function of ligands (insulin, IGF-1 and IGF-2), receptors and their downstream signaling effectors drive breast cancer initiation and progression, often in a subtype-dependent manner. Efforts to target this pathway for the treatment of cancer have been hindered by several factors including a lack of biomarkers to select patients that could respond to targeted therapy and adverse effects on normal metabolism. To this end, we discuss ongoing efforts aimed at overcoming such obstacles.

Keywords

Insulin; Insulin-like Growth Factor (IGF); insulin receptor; IGF-1R; Breast Cancer; Signal Transduction; Therapy

A number of comprehensive reviews have been written about the insulin and IGF signaling pathway and its role in cancer (Belfiore et al., 2009; Gallagher and LeRoith, 2020; Pollak, 2008), and especially in breast cancer (Farabaugh et al., 2015), and they have elaborated the pre-clinical evidence to support the involvement of this signaling pathway in both cancer risk and progression. Unfortunately, efforts to inhibit this pathway in the clinic have been unsuccessful to date, in part due to adverse effects of therapies on normal physiology

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Competing Interests

The authors declare that they have no competing interests.

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(Pollak, 2012; Yang and Yee, 2012). In this review we will provide an overview of the IR/IGF-1R signaling pathway and its importance for breast cancer and we will highlight the more recent discoveries that provide continued optimism for targeting insulin and IGF signaling for the treatment of breast cancer patients. The lessons learned at the bedside when targeting the IR/IGF-1R pathway need to be brought back to the bench for further basic research. A more rigorous understanding of the molecular mechanisms by which this pathway signals to regulate breast tumor progression is needed to facilitate the development of more selective and effective therapies.

The IR/IGF-1R signaling pathway in normal physiology

Before discussing the IR/IGF-1R signaling pathway in breast cancer, it is necessary to understand the players involved and their role in normal organismal growth and metabolism, because it is the dysregulation of these normal functions that contribute to breast cancer development and progression. In addition, issues that arise in response to targeting this pathway for cancer treatment are related to adverse effects of disrupting these normal functions.

Receptors and ligands

The insulin and insulin-like growth factor (IGF) signaling pathway is comprised of a family of cell surface receptor tyrosine kinases (RTKs) that arose from a common ancestral gene and share a high degree of homology (reviewed in (Barbieri et al., 2003)). Each receptor contains subunits of di-sulfide bonded extracellular α and transmembrane β chains that dimerize to form holoreceptors (Figure 1). The IR has two splice variants, IR-A and IR-B, that differ by the inclusion of exon 11 in IR-B that encodes a 12 amino acid region in the α chain (Frasca, 1999). In addition to forming IR and IGF-1R homodimers, the IR and IGF-1R can also heterodimerize to form hybrid receptors, increasing the complexity of this signaling pathway. The ligands for the IR and IGF-1R signaling pathway include insulin, IGF-1 and IGF-2, which differ in their affinity for each receptor dimer (Figure 1). At physiological levels, insulin binds primarily to the IR-A and IR-B and IR-A/IR-B hybrid receptors, and IGF-1 to the IGF-1R and IR/IGF-1R hybrids (Belfiore et al., 2009). IGF-2 binds to the IGF-1R and also the IR-A, as well as hybrid IGF1R/IR-A receptors (Frasca, 1999). The 12 amino acid region encoded by exon 11 reduces the affinity of IR-B for IGF2, making this ligand primarily selective for the IR-A isoform (Frasca et al., 1999). IGF-2 also binds to a separate receptor, the IGF-2R, that functions as a decoy receptor to inhibit IGF-2-dependent signaling through the IR/IGF-1R pathway (Brown et al., 2009; Kornfeld, 1992).

Normal Function of the IR/IGF-1R pathway: Metabolism vs. Mitogenesis

In normal physiological contexts, insulin stimulation of the IR regulates metabolic signaling, whereas IGF-1 and IGF-2 stimulation of the IGF-1R regulates mitogenic signaling (Figure 2). In adults, insulin-responsive tissues such as the liver, muscle and adipose tissue express primarily the IR-B isoform and the primary role of this signaling axis is to regulate glucose uptake to control organismal glucose homeostasis (Belfiore et al., 2017). Upon feeding, increased glucose levels stimulate insulin expression and secretion from the pancreas into the circulation. Insulin binds to IRs on cells in the target tissues, primarily muscle and

adipose, stimulating glucose uptake and storage as glycogen and triglycerides, respectively, for later energetic needs. Transport of glucose is rate limiting and insulin signaling promotes glucose transporter-4 (GLUT-4) trafficking to the cell surface for glucose uptake (Klip et al., 2019). Gluconeogenesis is concurrently suppressed by insulin stimulation in the liver, which is the major site of insulin clearance.

IGF-1 and IGF-2 are expressed predominantly in the liver under control of growth hormone (GH) and released into the circulation where they travel to target tissues to bind IGF-1R for their growth regulatory action (Kleinberg et al., 2009). The importance of IGF-1 for normal growth regulation is demonstrated by individuals with Laron dwarfism, a condition that is the result of low IGF-1 expression (Laron and Werner, 2020). *Igf1^{-/-}* and *Igf1r^{-/-}* mice are small in body size as well (Baker et al., 1993; Liu et al., 1993; Powell-Braxton et al., 1993). IGF-2-dependent embryonic growth has also been attributed in part to stimulation of IR-A, which is the predominant IR isoform expressed in fetal tissues and regulates mitogenic signaling (Frasca et al., 1999). The bioavailability of circulating IGF ligands is regulated by IGF-binding proteins (IGFBPs), which stabilize IGF expression and restrict binding to receptors to prevent signaling (Baxter, 2014). In contrast with insulin, which acts exclusively as a systemic hormone, the IGFs can also be produced locally and act in an autocrine or paracrine manner within tissues (Figure 2) (Kleinberg et al., 2009; Livingstone, 2013).

Upon stimulation by ligands, IR/IGF-1R receptors undergo a conformational change that activates intracellular tyrosine kinases in the β -chains, resulting in receptor trans-autophosphorylation (De Meyts and Whittaker, 2002). These phosphorylation events lead to the recruitment of effectors that are phosphorylated by the receptors, activating signaling pathways to modulate cellular functions that regulate organismal growth and metabolic homeostasis. Major signaling adaptors recruited to the IR and IGF-1R are the insulin receptor substrate (IRS) and Shc proteins, which activate the PI3K and MAPK signaling pathways, respectively (Figure 3) (Belfiore et al., 2009). As most cells express both IR and IGF-1R, the added complexity of hybrid receptor formation has made it difficult to decipher the specific signaling functions of each individual receptor. However, studies on chimeric receptors in which the extracellular domains of each receptor were swapped with the transmembrane and intracellular domains of the other receptor, and expressed in double *IR^{-/-}*, *IGF1R^{-/-}* knockout cells, provide evidence for unique effector interactions and signaling dynamics that may contribute to the distinct metabolic and mitogenic activities of these receptors (Cai et al., 2017).

The signaling outcomes of the IR and IGF-1R are influenced by the IRS proteins that play an essential role in determining the diversity of cellular responses to IR/IGF-1R stimulation (Mardilovich et al., 2009). IRS-1 and IRS-2 are ubiquitously expressed and are the primary mediators of insulin and IGF-dependent regulation of glucose metabolism and mitogenesis in most cell types (reviewed in (White, 2002)). Humans also express IRS-4, which has restricted expression primarily in the kidney, thymus and liver (Lavan et al., 1997a). An additional IRS protein, Irs-3, was identified in rodents, but it is not expressed in humans (Bjornholm et al., 2002; Lavan et al., 1997b). The IRS proteins share their highest degree of homology in their N-termini, which contain pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains that mediate their recruitment to the IR and IGF-1R (Mardilovich et

al., 2009). The IRS proteins are phosphorylated by the receptors on tyrosine residues in their C-termini, generating binding sites that recruit downstream effectors which include PI3K, Grb-2, SHP-2, Fyn, c-Crk, CrkII and Nck (Mardilovich et al., 2009). An additional motif in IRS-2 that contributes to receptor engagement, the kinase regulatory loop binding (KRLB) domain, may contribute to differential signaling downstream of distinct receptor dimers as it differentially interacts with the IR and IGF-1R and limits IRS-2 tyrosine phosphorylation by the IR (Sawka-Verhelle et al., 1996).

Despite sharing significant homology and activating many similar downstream pathways, the functional outcomes of signaling via IRS-1 or IRS-2 are diverse and non-redundant, as highlighted by knockout mouse studies. *Irs1*^{-/-} mice are born small and remain runted throughout their lives, implicating a primary role for this IRS protein in somatic growth regulation (Araki et al., 1994; Tamemoto et al., 1994). *Irs2*^{-/-} mice are born normal in size but have small brains and are infertile (Schubert et al., 2003; Withers et al., 1998). With regard to metabolic regulation, mice lacking either *Irs1* or *Irs2* develop insulin resistance, but only *Irs2*^{-/-} mice develop early-onset diabetes due to a loss of pancreatic β -cell function (Withers et al., 1999; Withers et al., 1998). *Irs4*^{-/-} mice are phenotypically normal, with only mild growth, reproductive and insulin sensitivity defects (Fantin et al., 2000), supporting a more restricted role for this family member. These differences in knockout phenotype indicate unique roles for the IRS proteins in IR/IGF-1R signaling in normal development and physiology, a finding that is also evident in cancer.

Regulation of IR/IGF-1R pathway function

The IR/IGF-1R signaling pathway is tightly regulated in normal physiology to maintain growth control and metabolic homeostasis. Upon stimulation by ligands, receptors are internalized from the cell surface and their signaling longevity is controlled by endocytic trafficking either to the late endosome and lysosome for receptor/ligand degradation or to the recycling endosome for trafficking back to the cell surface where a subsequent round of signaling may occur (Romanelli et al., 2007). The metabolic and mitogenic signaling differences of the IR and IGF-1R, respectively, may be explained in part by differential trafficking of each receptor and the longevity of their signals (Chow et al., 1998; Zapf et al., 1994). Negative feedback regulation of the IRS proteins also controls insulin sensitivity and glucose homeostasis by limiting the magnitude and duration of signaling responses (Gual et al., 2005). Activation of downstream signaling pathways feedback to phosphorylate the IRS proteins on serine residues, causing either inhibition of interactions with upstream receptors and/or downstream effectors, or degradation via ubiquitin-mediated targeting to the proteasome (Lee et al., 2000; O'Reilly et al., 2006; Rui et al., 2001). The end result is a cessation of signaling. If sustained, this negative feedback leads to insulin resistance. Differential sensitivity of IRS-1 and IRS-2 to the effects of negative feedback regulation likely explains some of the distinct functional outcomes elicited by each IRS protein in response to insulin and IGF-1 stimulation.

IR/IGF-1R pathway function in the normal breast

In addition to systemic regulation of organismal metabolism and growth, the insulin and IGF-1 signaling pathway also plays a functional role locally in the normal breast, both

during development and in the mature adult gland. Knockout and expression studies in mice reveal that Igf-1 ligand and the Igf-1r are required for terminal end bud (TEB) development and ductal morphogenesis during mammary gland development, and they are also required for alveolar differentiation during pregnancy and lactation (Bonnette and Hadsell, 2001; Ruan and Kleinberg, 1999; Sun et al., 2011). The Irs proteins have unique expression patterns in the developing and adult mammary gland, suggesting unique signaling functions (Lee et al., 2003). Irs-1 is expressed in a subset of luminal mammary epithelial cells in ducts and also in the body of the TEB of the developing gland. In contrast, Irs-2 is expressed homogeneously in the developing ductal luminal epithelial cells and also throughout the TEB, including the cap cell layer, which is highly proliferative. Proliferation is decreased in the cap cells of TEBs in *Igf1r*^{-/-} mice, suggesting a selective role for Igf-1r/Irs-2 signaling in extension of the developing ductal structures (Sun et al., 2011). Interestingly, the cap cell layer also contains a population of multipotent mammary stem cells that contribute to both the luminal and basal lineages of the gland (Ruan and Kleinberg, 1999). In this regard, a recent study examining overexpression of a constitutively active IGF-1R in the mammary gland provided evidence that signaling by this receptor regulates luminal cell fate through the maintenance of luminal progenitor cell populations (Farabaugh et al., 2020). This regulation could underlie the aberrant development and defective adult glands in the *Igf1*^{-/-} and *Igf1r*^{-/-} mice. The involvement of the IR/IGF-1R signaling pathway in stem cell regulation in the normal mammary gland foreshadows a role for this pathway in breast cancer stem cells.

The IR/IGF-1R signaling pathway in breast cancer

IR/IGF-1R pathway function is tightly regulated to maintain normal organismal growth and metabolism. Dysregulation of this pathway leads to pathophysiological conditions including diabetes and cancer. With regard to the latter, aberrant IR/IGF-1R signaling has been implicated in the initiation and progression of many types of cancer, including breast cancer (reviewed in (Gallagher and LeRoith, 2020; Pollak, 2008)). Alterations in expression and function of ligands, receptors and signaling effectors in breast tumors drive enhanced activity of this signaling pathway and impact tumor behavior, often in a subtype-dependent manner (Figure 3). The significant associations of the IR/IGF-1R pathway with breast cancer risk and outcomes underscore the importance of understanding mechanistically how this pathway functions to regulate tumor growth and progression. In this section, we will discuss the progress that has been made in deciphering how IR/IGF-1R signaling contributes to breast tumor biology and the tumor-specific functions of this pathway that may be exploited for future therapeutic benefit.

The IR/IGF-1R pathway in breast cancer: links to obesity and cancer disparities

A significant amount of evidence to support the importance of the IR/IGF-1R signaling pathway in cancer comes from the connection between obesity and cancer. Obesity is linked to both the risk of developing cancer and reduced overall survival of cancer patients (Poloz and Stambolic, 2015). In breast cancer, obesity is associated with a 2-fold increased relative risk of death (Calle, 2003). Although there are many factors that contribute to breast cancer development and progression in obese states, including increased sex hormone production,

altered adipokine expression and enhanced inflammation, a major consequence of obesity is insulin resistance, which results in systemic hyperinsulinemia (Gallagher and LeRoith, 2020; Lorincz and Sukumar, 2006). The fact that high insulin levels are an independent risk factor for breast cancer initiation and recurrence and are significantly associated with poor outcomes of breast cancer patients highlights the important role of the IR/IGF-1R pathway in the context of obesity driven breast cancer (Ferroni et al., 2016; Formica et al., 2012; Goodwin et al., 2002).

The ability of hyperinsulinemia to promote mammary tumor progression has been demonstrated experimentally using mouse models. Transgenic mice that express a kinase-dead IGF-1R in the skeletal muscle are hyperinsulinemic, but otherwise exhibit normal weight and lack other obesity characteristics (Fernandez et al., 2001). Mammary tumor growth and metastatic progression are enhanced in both spontaneous (*MMTV-PyMT*) and orthotopic tumor models in these mice, and this increased tumor progression is dependent upon IR function in the tumors (Novosyadlyy et al., 2010; Rostoker et al., 2015). The fractions of free IGF-1 and IGF-2 also increase in response to hyperinsulinemia due to suppression of systemic IGFBP expression, which enhances the bioavailability of these ligands to activate their receptors (Figure 2) (Clemmons and Underwood, 1991). The association of IGF-1 with breast cancer risk is indicated by a reduced overall cancer incidence in patients with Laron dwarfism (Laron and Werner, 2020). Moreover, free IGF-1 is elevated in the serum of breast cancer patients and higher overall IGF-1 levels are associated with an increased risk of developing estrogen receptor positive (ER+) breast cancer (Murphy et al., 2020; Qian and Huo, 2020).

It should be noted that hyperinsulinemia, which results from obesity and other factors, not only drives systemic increases in free IGF-1 and IGF-2, but it also promotes their expression locally in the tumor microenvironment (Figure 2). This elevated expression impacts autocrine tumor cell signaling and paracrine cross-talk with stromal cells to promote tumor growth and progression (De Vincenzo et al., 2019; Gui et al., 2019; Sciacca et al., 1999). *IGF2* transcription and IGF-2 protein expression are also commonly increased locally in tumors through loss of imprinting of the *IGF2* locus as a result of demethylation of the differentially methylated region (DMR) of the maternal allele (DeChiara et al., 1991; Kaneda et al., 2007; Wu et al., 1997). Evidence that dysregulation of IGF-2 can promote breast cancer initiation comes from transgenic mouse studies in which overexpression of IGF-2 in the mammary epithelium drives tumor formation, and these tumors are suppressed by concurrent expression of the inhibitory IGF-2R (Bates et al., 1995; Wise and Pravtcheva, 2006). In summary, all three ligands of the IR/IGF-1R pathway are upregulated in obese/hyperinsulinemic states and activate their cognate receptors to drive breast tumor growth and progression.

Obesity and Type-2 Diabetes (T2D) share the common feature of insulin resistance and hyperinsulinemia and they link the IR/IGF-1R signaling pathway to health disparities in breast cancer (Gallagher et al., 2016). African American women have a higher incidence of obesity and T2D than Caucasian women (Calle et al., 2003; Peairs et al., 2011). Although their incidence of breast cancer development is similar, African American women are more likely to develop aggressive triple negative breast cancer (TNBC) and do so at a younger age

than Caucasian women and this is associated with a higher mortality rate (Scott et al., 2019). Insulin resistance is positively associated with poor prognosis in African American breast cancer patients (Gallagher et al., 2020). Tumors in these patients exhibit higher IR expression and a higher ratio of IR:IGF-1R than in tumors from Caucasian women, supporting a role for IR signaling in disparities of breast cancer outcomes. IGF-2 is also associated with disparities in breast cancer risk and survival outcomes in African American women. IGF-2 expression is elevated in the normal breast tissue of African American women when compared with Caucasian women, and this higher level of IGF-2 expression persists in aggressive breast tumors in younger African American women (Kalla Singh et al., 2010).

IR and IGF-1R function: redundant or diverse roles in breast cancer?

The specific role of each receptor/ligand pair in driving breast cancer initiation and progression is difficult to determine due to common expression of both IR and IGF-1R in tumors and the resultant complexity of hybrid receptor formation, as well as the promiscuous binding of each ligand to multiple receptor dimers (Pandini et al., 1999). Moreover, currently available antibody reagents do not discriminate between phosphorylated receptor forms, preventing the determination of the individual activity of each receptor dimer within a tumor. However, some common themes emerge from the studies that have evaluated receptor expression and activity in breast tumors. The IGF-1R is frequently over-expressed in breast tumors, with higher expression in luminal A and B tumors and lower expression in HER2+ and basal/triple negative breast cancer (TNBC) (Shimizu et al., 2004). Early studies revealed the contribution of IGF-1R to tumor growth through the use of inhibitory antibodies that blocked ligand binding to the receptor and reduced mitogenic responses (Cullen et al., 1990). However, the expression of IGF-1R is not associated with prognosis for most breast cancer subtypes, with the exception of luminal B tumors in which IGF-1R expression correlates with better outcomes (Yerushalmi et al., 2012). Although expression levels are a poor predictor of prognosis, an IGF-1 gene signature is associated with poor disease outcomes, indicating that IGF-1R pathway activity is a more accurate measure of function in tumors than relative expression (Creighton et al., 2008; Litzenburger et al., 2011). However, IGF-1R signaling may inhibit tumor progression in some contexts, specifically in Wnt1-driven TNBC (Rota et al., 2014).

IR expression is also altered in breast tumors, although studies have been conflicting on its association with prognosis. The impact of the IR on tumor growth and progression may be more related to isoform ratios than total expression levels, with a higher ratio of IR-A:IR-B most commonly observed in tumors, but this varies with molecular subtype (Harrington et al., 2012). Luminal breast tumors express higher overall IR levels and a greater IR-A:IR-B ratio than HER2+ and basal/TNBC tumors due to the elevated luminal expression of the CUGBP1 splicing factor that favors IR-A splicing (Huang et al., 2020). A reduction in IR-B expression in tumors may also drive the elevated IR-A:IR-B ratio (Huang et al., 2011). Of clinical relevance, higher IR-A:IR-B ratios correlate positively with OncotypeDX proliferation scores (Huang et al., 2011), supporting the contribution of IR-A to tumor growth.

Although the total level of IR and IGF-1R expression varies across breast cancer subtypes, phosphorylated IR/IGF-1R is observed in luminal, HER2 and basal/TNBC tumors and this phosphorylation event is associated with poor patient survival (Law et al., 2008). The staining pattern of strong IGF-1R, moderate/strong IR and pIR/pIGF-1R positivity together is associated with poor breast cancer patient outcomes, highlighting that it may be the coincident expression of IR and IGF-1R, resulting in hybrid receptor formation, and not individual homodimer expression, that determines the prognostic outcomes of this pathway (Bjorner et al., 2017). This has significant implications for targeting this pathway in cancer as it suggests that both IR and IGF-1R signaling drive tumor progression and need to be co-inhibited for therapeutic benefit.

The IRS proteins and breast cancer

As described for normal breast function, the IRS proteins also play essential roles in regulating the response of tumors to IR and IGF-1R signaling in breast cancer, with impact on patient outcomes. IRS-1 is regulated by ER- α and it is expressed at moderate to strong levels in more well-differentiated, ER-positive (ER+), luminal cell lines and tumors where it is localized diffusely in the cytoplasm or in the nucleus (Schnarr et al., 2000; Sisci et al., 2007). In the nucleus, IRS-1 interacts with ER- α to modulate gene expression (Morelli et al., 2004). The nuclear localization of IRS-1 is associated with positive tamoxifen response and improved patient survival, likely because expression of IRS-1 in the nucleus is a biomarker for active ER signaling, which renders tumors more sensitive to a selective estrogen receptor modulator (SERM) such as tamoxifen (Migliaccio et al., 2010). IRS-1 expression decreases in more poorly differentiated, higher-grade tumors that lack ER expression (Schnarr et al., 2000).

In contrast with IRS-1, IRS-2 is expressed at higher levels in ER negative (ER-) basal/TNBC cells and tumors (Porter et al., 2013). IRS-2 is excluded from the nucleus and is instead localized either in the cytoplasm or at the plasma membrane (Clark et al., 2011). IRS-2 membrane staining is significantly associated with decreased overall survival of breast cancer patients, in particular in patients with progesterone receptor (PR) negative tumors (Clark et al., 2011). Although IRS-2 expression is positively regulated by the PR (Cui et al., 2003; Vassen et al., 1999), alternative mechanisms of regulation drive expression in these tumors that lack functional PR. Epidermal growth factor receptor (EGFR) signaling promotes IRS-2 expression in a JNK-dependent manner through the AP-1 transcription factor (Cui et al., 2006). IRS-2 expression is also positively regulated by hypoxia in the tumor microenvironment, and this transcriptional regulation is dependent upon hypoxia-inducible factor-1 (HIF-1) and HIF-2 (Figure 2) (Mardilovich and Shaw, 2009).

Genetic modifications of the IR/IGF-1R pathway in breast cancer

In addition to expression changes of components of the IR/IGF-1R signaling pathway in breast cancer, genetic alterations may also contribute to the enhanced activity of this pathway. Analysis of somatic mutation data from a genome sequencing study of a rare but aggressive form of lobular breast cancer, pleomorphic invasive lobular carcinoma (PILC), identified frequent mutations in IR/IGF1R/IRS2 signaling pathway components (Zhu et al., 2018). Top hits for this sequencing analysis were 'IRS-related events triggered by IGF1R'

and ‘Insulin receptor signaling cascade’. When downstream PI3K effectors were also included in the analysis, components of the IR/IGF1R/IRS2/PI3K signaling pathway were mutated in 15/17 (88%) of the PILC tumors that were sequenced. Patients with PILC tend to have poor clinical outcomes with short relapse times, a high risk of recurrence and decreased overall survival, supporting the contribution of the IR/IGF-1R pathway to this aggressive behavior.

Relationship of IR/IGF-1R signaling and the ER in breast cancer

There is significant cross-talk between the ER and the IR/IGF-1R pathway in breast cancer (Figure 2). As mentioned previously, IGF-1, IGF-1R and IRS-1 are all ER- α target genes and their expression is upregulated by estrogen in ER+, luminal breast cancers (Huynh et al., 1996; Lee et al., 1999). Enhanced IR/IGF-1R signaling and promotion of proliferation are observed in ER+ cells treated with both estrogen and insulin/IGF-1, but cooperation is not observed in ER- cells (Hamelers et al., 2002). In turn, IGF-1 and insulin signaling can regulate ER activity through estrogen-dependent and independent mechanisms. Stimulation of IR/IGF-1R signaling phosphorylates ER in the presence or absence of estrogen, and this phosphorylation event is dependent upon downstream PI3K activation (Martin et al., 2000). This phosphorylation (Ser167) enhances ER transcriptional activity. An additional cross-talk of these pathways occurs in an estrogen-dependent manner. Elevated circulating levels of insulin and IGF-1 suppress the expression of sex-hormone-binding globulin (SHBG), which binds to testosterone and estrogen with high affinity (Figure 2) (Pugeat et al., 1991). As a result, the bioavailability of estrogen is increased and can stimulate ER+ breast tumor growth. In post-menopausal women, levels of SHBG are inversely correlated with breast cancer risk, supporting the important contribution of this cross-talk to breast cancer promotion, especially in the obese setting (Zeleniuch-Jacquotte et al., 2004).

Functional outcomes of IR/IGF-1R signaling in breast cancer

The IR/IGF-1R signaling pathway promotes breast cancer initiation and progression by regulating many of the hallmarks of cancer including proliferation, survival, metabolism and migration/invasion (Hanahan and Weinberg, 2011). Although some of these functional responses to pathway stimulation are observed across all breast tumor subtypes, other outputs occur more selectively. Some of this specificity is contributed by differential receptor function, such as the preferential regulation of proliferation by the IGF-1R and IR-A receptors and metabolic pathways by the IR-B receptor. As a result, extrinsic factors that impact the relative expression levels of each receptor, for example, ER- α regulation of IGF-1R expression or splicing factors that alter IR-A:IR-B ratios, will determine the outcomes of stimulation of this pathway and confer subtype-specific differences in tumor behavior.

As discussed previously, IGF-1R signaling maintains normal mammary luminal progenitor cells to regulate mammary gland development and function of the mature gland. In breast cancer, the IR/IGF-1R signaling pathway has also been implicated in cancer stem cell (CSC) regulation. CSCs are a subpopulation of tumor cells with the properties of self-renewal and pluripotency, the ability to differentiate and repopulate the heterogeneity of a tumor (Brooks et al., 2015). CSCs contribute to primary tumor initiation, metastatic colonization and drug

resistance (Lawson et al., 2015; Lorincz and Sukumar, 2006). Pre-clinical studies support a role for IR/IGF-1R signaling in breast CSC self-renewal and both receptors have been implicated in maintaining CSC populations (Chang et al., 2013; Rostoker et al., 2015; Rostoker et al., 2016). Analysis of published breast CSC gene expression datasets reveals decreased IGF-1R expression in some contexts (Dox-resistant MCF-7 cells), but no significant change for IGF-1R or IR in other datasets (Calcagno et al., 2010). It should be noted, however, that the IR-A and IR-B isoforms are not distinguished by total mRNA analysis and the ratio of IR-A:IR-B may be more important than total expression for CSC regulation. IGF-2, which can stimulate both the IGF-1R and IR-A, is elevated in Dox-MCF-7 and CD44⁺,CD24⁻ CSC datasets and has been implicated in the regulation of breast CSCs, suggesting that the activity of these receptors, rather than their expression, may be altered in CSCs (Liu et al., 2007; Pandini et al., 1999; Sciacca et al., 1999; Tominaga et al., 2017). The fact that IGF-2/IR-A are both associated with more aggressive TNBC tumors in African American women may reflect their role in CSC regulation.

Role of the IRS proteins in the tumor response to IR/IGF-1R signaling

As key signaling nodes downstream of the IR/IGF-1R, the IRS proteins play a major role in determining the functional outputs of these receptors in tumors (Gibson et al., 2007). Although some functions are commonly regulated by IRS-1 and IRS-2, such as the promotion of IR/IGF-1R-dependent tumor cell survival (Cesarone et al., 2006; Nagle et al., 2004), other functions are uniquely regulated by each adaptor protein (Figure 4). In ER⁺, luminal breast carcinoma cells, IRS-1 is the predominant IRS family member that is expressed and activated by IGF-1 stimulation and this signaling adaptor mediates IGF-1-dependent proliferation in these cells (Byron et al., 2006; Jackson et al., 1998). In contrast, ER⁻ breast carcinoma cells that express predominantly IRS-2 do not proliferate when stimulated with IGF-1 (Byron et al., 2006). Instead, ER⁻ tumor cells exhibit enhanced migration and invasion in response to IGF-1 stimulation (Jackson et al., 2001; Mercado-Matos et al., 2018; Nagle et al., 2004; Zhang et al., 2004). Therefore, similar to IR-A:IR-B ratios influencing the outcomes of insulin stimulation, the ratio of IRS-1:IRS-2 expression modifies the response to receptor stimulation, and in so doing significantly impacts tumor outcomes. As a result, autocrine and paracrine factors in the tumor microenvironment that impact the relative expression of each adaptor protein influence the functional outcome of signaling when the IR/IGF-1R pathway is stimulated.

As breast tumors lose ER expression and function and become more poorly differentiated and invasive, IRS-1 expression decreases and IRS-2 expression increases, changing the balance to favor IRS-2 as the dominant adaptor protein in these tumors. Disruption of IGF-1R function in ER⁻ breast carcinoma cells does not inhibit tumor growth, as it does in ER⁺ tumors, but it prevents metastasis of these cells in mouse xenograft models (Dunn et al., 1998; Sachdev et al., 2004; Sachdev et al., 2010). Mammary tumor metastasis is also significantly diminished in *PyMT:Irs2*^{-/-} mice, whereas *PyMT:Irs1*^{-/-} tumors that express elevated levels of active (i.e. tyrosine phosphorylated) Irs-2 have enhanced metastatic rates (Ma et al., 2006; Nagle et al., 2004), supporting that IRS-2 plays an essential role in metastatic progression. The ability of IRS-2 to promote invasion, an early pre-requisite step for the dissemination of metastatic cells to secondary organs, is one mechanism by which it

contributes to tumor progression (Mercado-Matos et al., 2018; Nagle et al., 2004). Moreover, the selective regulation of aerobic glycolysis by IRS-2, via maintenance of the GLUT-1 glucose transporter on the tumor cell surface and increased glucose uptake, is required for its ability to promote invasion (Figure 4) (Landis and Shaw, 2014; Pankratz et al., 2008).

The differential functional outcomes of signaling through IRS-1 and IRS-2 raises the question of how these similar proteins regulate divergent tumor cell responses. The localization of IRS-1 and IRS-2 within distinct intracellular compartments likely plays a role in their distinct functions. The targeting of IRS-1 and IRS-2 to unique intracellular compartments would facilitate their access to distinct subsets of downstream effectors, altering their functional output. For example, in the nucleus, IRS-1 interacts with transcription factors such as ER- α , β -catenin, the androgen receptor, and upstream binding factor-1 to positively regulate target gene expression (Chen et al., 2005; Lanzino et al., 2009; Morelli et al., 2004). IRS-1-dependent regulation of Cyclin D1 and c-Myc expression is likely to play a role in the stimulation of tumor proliferation (Wu et al., 2007). In contrast, IRS-2 is excluded from the nucleus and instead this adaptor is localized in the cytoplasm or at the cell membrane, which puts IRS-2 in proximity to downstream effectors that are involved in regulating dynamic adhesive and cytoskeletal rearrangements that are required for cell migration and invasion (Clark et al., 2011). IRS-2 interacts with microtubules and the ability of IRS-2, but not IRS-1, to activate AKT is dependent upon an intact microtubule cytoskeleton, suggesting that this interaction controls access of IRS-2 to its downstream effectors (Mercado-Matos et al., 2017). Membrane recruitment of IRS-2 would also localize its signaling to regulate the surface expression of glucose transporter 1 (GLUT1) to promote aerobic glycolysis (Landis and Shaw, 2014; Pankratz et al., 2008). The fact that both IRS-1 and IRS-2 localization are associated with clinical outcomes of breast cancer patients underscores the importance of intracellular localization to their activities (Clark et al., 2011; Migliaccio et al., 2010).

IRS-1 and IRS-2 share approximately 35% identity in their C-termini where they are phosphorylated by the IR/IGF-1R and recruit downstream effectors to initiate their signaling cascades (Mardilovich et al., 2009). Many phosphotyrosine motifs are conserved between IRS-1 and IRS-2 and they activate some common signaling pathways including PI3K and the Erk1/2 kinases in a variety of cancer model systems. However, the fact that PI3K has been implicated in the regulation of both proliferation and invasion by IRS-1 and IRS-2, respectively, underscores that unique pathways that either modify the outcomes of PI3K signaling or cooperate with this signaling pathway must be regulated by each adaptor protein to promote divergent functions (Mercado-Matos et al., 2018; Myers et al., 1996). To date, relatively few unique interacting proteins have been described that would explain the distinctive downstream intracellular compartmentalization or functional outcomes of IRS-1 and IRS-2. Proteomic analysis has identified potential interacting partners of IRS-1 and IRS-2, but these effectors have not been validated by functional studies (Hanke and Mann, 2009). Finally, IRS-1 and IRS-2 are differentially regulated by post-translational modifications such as serine phosphorylation, ubiquitination and acetylation which control effector interactions and signaling duration and could contribute to their distinct downstream functions (Fukushima et al., 2015; Gual et al., 2005; Kaiser and James, 2004; Rui et al.,

2001; Yoshihara et al., 2012; Zhang, 2007). Additional study of the signaling mechanisms of the IRS proteins is needed to understand fully their divergent mechanisms of action, and importantly to elucidate their potential as therapeutic targets to inhibit pathway signaling.

Targeting the IR/IGF1R signaling pathway in breast cancer

The strong association of the IR/IGF-1R pathway with clinical outcomes in cancer and the extensive pre-clinical data supporting the functional role of this pathway in cancer progression have led to efforts to develop targeted drugs to inhibit this signaling axis in tumors (Figure 2) (King et al., 2014).

IGF-1R pathway inhibition

Given the importance of the IR for normal metabolic homeostasis and concern that disrupting normal insulin signaling would lead to adverse metabolic outcomes, initial efforts to inhibit the IR/IGF-1R pathway in tumors focused on targeting IGF-1R function selectively. The first drugs to be developed were inhibitory antibodies specific for the IGF-1R (Figure 2; IGF-1Ri). These antibodies not only block receptor ligand binding, but also cause internalization to deplete receptors from the cell surface (Sachdev et al., 2003). Neutralizing antibodies that target IGF-1 and IGF-2 to block receptor interactions have also been developed and showed promise in pre-clinical studies (Figure 2; IGFi) (Haluska et al., 2006). However, few positive responses to these targeted agents have been observed in clinical trials and outcomes for breast cancer and most other cancers have been disappointing.

A major reason for the poor response to selective IGF-1R targeting agents may be compensation by the IR when IGF-1R function is inhibited. IR signaling in tumors sustains growth promoting signals and promotes tumor progression when IGF-1R expression or function are selectively targeted in pre-clinical studies. Evidence of this IR compensation was also observed in one phase II human study. Breast cancer patients with low total expression of IR had significantly longer progression free and overall survival when treated with the anti-IGF-1R drug Cixutumumab (Gradishar et al., 2016). To address this compensation by IR signaling, small molecule inhibitors that dually target both IR and IGF-1R have been developed (Figure 2; RTKi). These drugs inhibit the tyrosine kinase activity of both receptors and they have been shown in many pre-clinical studies to inhibit tumor growth and viability with greater efficacy than targeting IGF-1R function alone (Haluska et al., 2006; Hou et al., 2011). As an example of one such pre-clinical mouse study, a small molecule inhibitor of IR/IGF-1R activity was more effective at inhibiting tumor growth in endocrine resistant tumors than agents that targeted IGF-1R alone, supporting an important role for IR in this therapeutic context (Kruger et al., 2020). Unfortunately, as anticipated, adverse systemic metabolic issues resulting from the inhibition of normal insulin receptor function have limited the efficacy of these small molecular inhibitors in human clinical trials. The poor outcomes of IR/IGF-1R targeted therapies in human trials emphasizes the need to develop alternative approaches to inhibit IR/IGF-1R signaling in tumors, while preserving normal systemic metabolic functions of this pathway.

Impact of diet on the tumor response to IR/IGF-1R pathway inhibition

One recent approach that has been investigated to improve the systemic response to IR/IGF-1R inhibition is the use of diet to suppress the feedback upregulation of hyperglycemia and hyperinsulinemia (Nencioni et al., 2018). As discussed previously, normal insulin signaling is tightly regulated to control the uptake of glucose from the circulation and maintain glucose homeostasis. When insulin signaling is disrupted in mice by therapies that target the pathway, glucose levels rise and stimulate insulin secretion to compensate, resulting in hyperinsulinemia and consequent elevation of IGF expression. Basal glucose levels can be reduced by altering diet, either through complete fasting or a fasting mimicking diet (FMD) that is low in carbohydrate (Nencioni et al., 2018). Lower basal glucose levels suppress the spike in insulin and IGF-1 expression that occurs when the pathway is disrupted and prevent hyperstimulation of the pathway in tumors.

The efficacy of this dietetic approach for cancer treatment has been demonstrated in several pre-clinical studies in mice, providing hope that this approach could improve clinical outcomes in tumors that are responsive to the IR/IGF-1 pathway. For example, PI3K is a frequently mutated gene in cancer and activity of this kinase is also upregulated by additional molecular alterations in tumors such as loss of the tumor suppressor PTEN or activation of growth factor receptors including IR/IGF-1R (Thorpe et al., 2015). For this reason, considerable effort has gone into developing PI3K targeted inhibitors for cancer treatment (Figure 2; PI3Ki). However, inhibition of PI3K prevents insulin-dependent glucose uptake, which causes systemic hyperglycemia and subsequent hyperinsulinemia. As a result, IR/IGF-1R signaling is hyperactivated in tumors and their growth is not diminished by these inhibitors (Hopkins et al., 2018). The hyperglycemic response to PI3K inhibition is suppressed in mice fed a ketogenic diet that is low in carbohydrates. Breast tumor growth becomes sensitive to inhibition of PI3K in mice fed this diet (Hopkins et al., 2018). A fasting or a fasting mimicking diet (FMD) also improved the inhibition of ER+ breast tumor growth in mice by ER-targeting drugs (Caffa et al., 2020). When fulvestrant was combined with the CDK4/6 inhibitor Palbociclib and mice were fed in periodic cycles of FMD, tumor regression was observed and acquired resistance to treatment was prevented.

In light of these positive pre-clinical results, alternative diets have started to be evaluated in humans undergoing breast cancer treatment. In a small cohort of patients with ER+ breast cancer receiving endocrine therapy, cycles of a FMD led to reduction in circulating levels of insulin, IGF-1 and leptin, mimicking the metabolic effects observed in the pre-clinical mouse studies (Caffa et al., 2020). Although this cohort was limited in size, clinical outcomes were promising with several patients showing clinically controlled disease and prolonged progression free survival. Similarly, a recent Phase II clinical trial evaluated the impact of a FMD on response to neoadjuvant chemotherapy in HER2- stage II/III breast cancer. This study reported that a radiological complete or partial response occurred more often in patients on the FMD diet (de Groot et al., 2020). Together, these studies provide encouraging evidence that restrictive diets may be effective as an approach to suppress IR/IGF-1R signaling in breast tumors and improve responses in a variety of therapeutic contexts. However, compliance of patients with fasting and/or strict dietary restrictions is a

concern, and for this reason there remains a need for the development of novel targeted drugs to disrupt IR/IGF-1R signaling in a tumor-specific manner.

The IRS proteins as novel therapeutic targets

One potential approach for selectively inhibiting tumor-specific functions of the IR/IGF-1R pathway is through targeting the IRS proteins. As key determinants of IR/IGF-1R signaling outcomes, the IRS proteins represent nodes of the pathway that could be targeted to inhibit tumor functions while maintaining essential normal physiological functions of the pathway. A small molecule inhibitor that indirectly targets both IRS-1 and IRS-2 for degradation has been described, but the dual suppression of both adaptors would likely also impact normal metabolic signaling (Reuveni et al., 2013). The IRS proteins share their highest degree of homology in their structured N-terminal domains (PH and PTB) that are required for recruitment to upstream receptors, followed by long predominantly disordered tails which are less conserved (Mardilovich et al., 2009). In a recent study to identify unique structural features of IRS-2 that are required for the ability to promote invasion, deletion mutants were generated that preserved the ability of IRS2 to be recruited to upstream receptors and activate PI3K, functions that are shared by IRS-1 and are required for normal glucose regulation (Mercado-Matos et al., 2018). A region in the IRS2 C-terminal tail was identified that is necessary and sufficient for promoting invasion but is not required for the IRS2-dependent regulation of glucose uptake. The results of this study provide proof of concept that a targeting strategy to disrupt tumor-specific functions of the IR/IGF-1R pathway while maintaining essential metabolic functions in normal cells is feasible.

Future Directions

The evidence to support an important role for the IR/IGF-1R signaling pathway in breast cancer promotion and progression is compelling. Unfortunately, early excitement about the potential for targeting the IR/IGF-1R signaling axis as a therapeutic approach in breast cancer has been diminished by disappointing results from clinical trials, and many drug programs have been halted. One lesson that has been learned from efforts to target the IGF-1R pathway in cancer is that improved biomarkers that can predict response to disruption of the pathway are needed. Unlike other oncogenic receptors such as HER2 or ER, where expression correlates with response to targeted therapies, IGF-1R expression alone has not been predictive as a biomarker for response to targeted inhibitors. The IR and the IRS proteins impact tumor response to pathway stimulation and their expression should be explored further as predictive biomarkers for efficacy of pathway inhibition. In this regard, it should be noted that tumor growth is most often the endpoint monitored to determine successful outcomes in clinical trials. However, disruption of IR/IGF-1R signaling in ER– breast tumors that express primarily IRS-2 does not inhibit tumor growth, but instead inhibits metastasis (Dunn et al., 1998; Sachdev et al., 2004; Sachdev et al., 2010). Therefore, consideration for alternative endpoints to monitor outcomes of these targeted therapies is warranted.

The IRS proteins represent an important, but relatively unexplored area with regard to development of targeted therapies to inhibit IR/IGF-1R signaling in a tumor-specific manner.

Relatively little is known regarding the structure of the IRS proteins and how they mediate their distinct signaling functions. Structural studies of both IRS-1 and IRS-2 are necessary to understand their unique mechanisms of action, a pre-requisite to selectively targeting their tumor-promoting functions for therapeutic benefit. Further identification and interrogation of IRS effectors that play tumor-selective roles in IR/IGF-1R signaling will also open new avenues for novel drug development. In this regard, bone morphogenic protein-2-inducible kinase (BMP2K) binds to the invasion-promoting region in IRS-2 and it contributes to IRS2-dependent invasion (Mercado-Matos et al., 2018). The potential of targeting this kinase or other effectors to selectively prevent tumor functions of IR/IGF-1R signaling requires additional investigation. As continued research illuminates a greater understanding of the molecular mechanisms of insulin receptor (IR) and IGF-1 receptor (IGF-1R) function and signaling, novel possibilities for targeting this pathway will be revealed, with the hope of improving outcomes for breast cancer patients.

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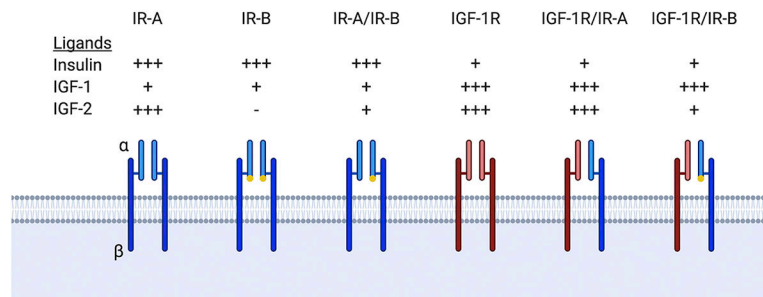


Figure 1: Schematic of the insulin/IGF signaling pathway receptors and ligands.

The insulin receptor (IR) is expressed as two splice isoforms (IR-A and IR-B) and the insulin-like growth factor-1 (IGF-1) receptor (IGF-1R) has one form. The IR-B differs from the IR-A receptor by the inclusion of a 12 amino acid region in the α chain. Each half receptor, which are comprised of disulfide bonded α and β chains, can form homodimers or heterodimers, resulting in 6 different receptors. Each receptor has differing affinities for the insulin, IGF-1 and IGF-2 ligands. – no binding, + low binding, ++ moderate binding, +++ high binding.

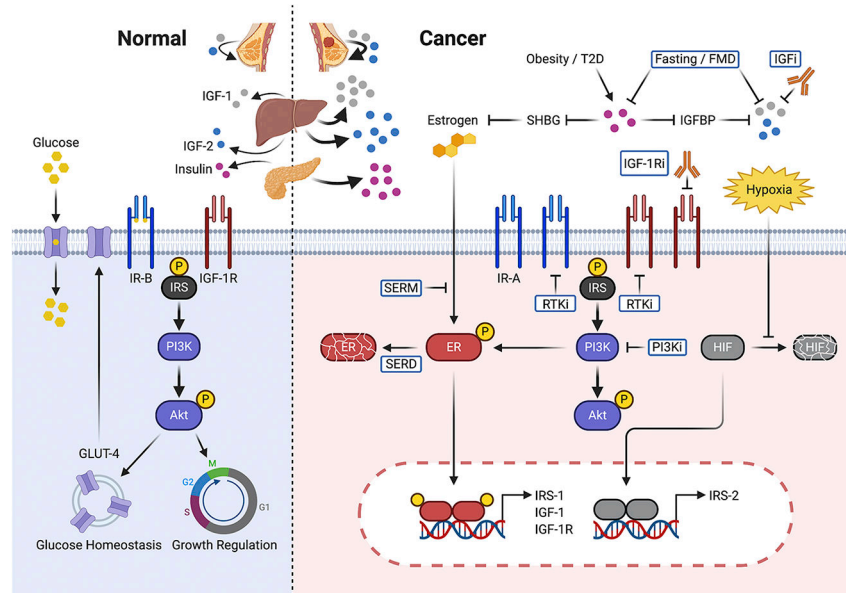


Figure 2: Functional outcomes of the IR/IGF-1R/IRS pathway in normal and breast cancer settings.

Ligands are expressed both systemically (from the liver and pancreas) and locally in the breast in both normal and cancer and elevated ligand expression is observed in breast cancer. In normal tissues (on left), stimulation of the IR-B and IGF-1R regulates glucose homeostasis and growth, respectively. In breast cancer (on right), expression of IR-A and IGF-1R is increased. Systemic factors such as obesity and type 2 diabetes (T2D) promote pathway activity by upregulating ligand and receptor expression. Moreover, hypoxic tumor microenvironments enhance IRS-2 expression. The IR/IGF-1R pathway cross-talks with the estrogen receptor (ER) pathway to enhance signaling and promote tumor growth and progression. Targeted inhibitors are shown that disrupt pathway function (white boxes): FMD, fasting mimicking diet; IGFi, inhibitory IGF-1 and IGF-2 antibodies; IGF-1Ri, inhibitory IGF-1R antibodies; RTKi, IR/IGF-1R tyrosine kinase inhibitors; PI3Ki, PI3K inhibitors; SERM, selective estrogen receptor modulators; SERD, selective estrogen receptor degraders. T2D, Type 2 Diabetes; SHBG, sex-hormone binding globulin; IGFBP, insulin-like growth factor binding protein

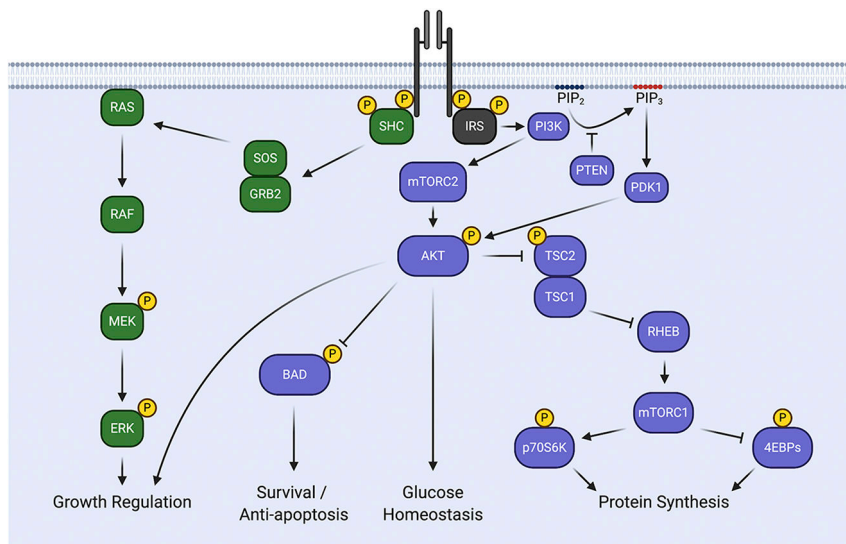


Figure 3: Signaling via the IR and IGF-1R.

Upon stimulation with ligand, receptors undergo trans-autophosphorylation and recruit the downstream signaling adaptors SHC and the IRS proteins. Once bound, these adaptors are phosphorylated on tyrosine residues, creating docking sites for the recruitment of signaling effectors. Recruitment of SHC activates the RAS/MEK/ERK signaling pathway to stimulate growth. Recruitment of the IRS proteins activates PI3K and the downstream signaling effectors AKT and mTOR to regulate survival, glucose metabolism and protein synthesis.

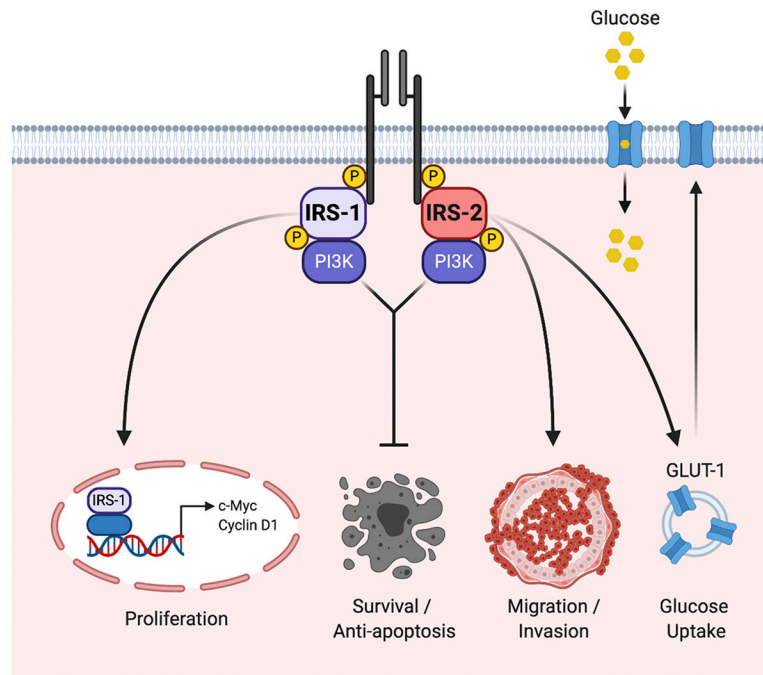


Figure 4: The IRS proteins determine IR/IGF-1R signaling outcomes.

The IRS proteins are essential signaling intermediates of the IR and IGF-1R and they influence the functional response to pathway stimulation. Both IRS-1 and IRS-2 promote tumor cell survival by activating anti-apoptotic pathways in response to IR/IGF-1R stimulation. However, IRS-1 and IRS-2 also regulate distinct functions and their relative expression determines these outcomes. Breast carcinoma cells that express predominantly IRS-1 proliferate in response to IR/IGF-1R stimulation. IRS-1 localizes to the nucleus where it promotes the transcription of cell cycle regulatory genes. Breast carcinoma cells that express predominantly IRS-2 migrate and invade in response to IR/IGF-1R stimulation. IRS-2 signaling also regulates glucose uptake through GLUT-1, which enhances invasion.