THESIS

DEVELOPMENT OF AN INSTURMAL METHOD TO EVALUATE INSULIN RECEPTORS AND INSULIN-LIKE GROWTH FACTOR-1 RECEPTORS HOMODIMERS AND HYBRID RECEPTORS IN BREAST CANCER

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ABSTRACT

DEVELOPMENT OF AN INSTURMAL METHOD TO EVALUATE INSULIN RECEPTORS AND INSULIN-LIKE GROWTH FACTOR-1 RECEPTORS HOMODIMERS AND HYBRID RECEPTORS IN BREAST CANCER

Breast cancer is a major public health problem in the United States and many other parts of the world. It is the second most common cancer and second leading cause of cancer death among women in the US. Insulin receptors (IR) and insulin-like growth factor-1 receptors (IGF1R) are found in normal mammary gland cells where they are involved in normal development and differentiation of these cells. There is substantial clinical, epidemiological, and experimental evidence indicating that these receptors are also involved in initiation and progression of neoplasia of mammary gland cells. IR and IGF1R numbers are increased in breast cancers and raise the possibility that IR and IGF1R may have roles in the biology of these tumors. In this project, we have developed new methods to evaluate the presence of IR homodimers and hybrid receptors formed from IR and IGF1R monomers. In initial experiments we have used three cell lines of CHO that stably express IR-GFP. Using flow cytometry, we found that low expressing cells had ~ 62,000receptors/cell, moderately expressing cells had ~ 130,000receptors/cell, and high expressing cells had ~ 205,000 receptors/cell. We then used homo-transfer fluorescence resonance energy transfer (homo-FRET) methods to evaluate possible interactions between IR monomers in the IR homodimer or IR and IGF1R subunits in hybrid receptors. Our results suggest that IR exists as an apparent monomer in cells expressing low numbers of IR-GFP. In cell lines highly expressing IR-GFP, there are minimally dimers and

higher order receptor oligomers present on the plasma membrane. These results suggest that when IR is highly expressed, as in the case in many breast cancers, the receptor is likely to be found as a IR homodimer rather than as a hybrid IR-IGF1R receptor. Further studies beyond the scope of this project will evaluate the effects of IGF1R expression on the formation of IR homodimers.

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BACKGROUND

Breast cancer forms in the cells of the breasts. It can occur in both men and women, but it is far more common in women. Breast cancer is a major public health problem in the United States and many other parts of the world. It is the second most common cancer and second leading cause of cancer death among women in the US. An estimated 230,000 new cases of invasive breast cancer will be diagnosed in 2016 and approximately 40,000 women are expected to die from breast cancer (1). Insulin receptors (IR) and insulin-like growth factor-1 receptors (IGF1R) are found in normal mammary gland cells where they are involved in normal development and differentiation of these cells. There is substantial clinical, epidemiological, and experimental evidence indicating that these receptors are also involved in initiation and progression of neoplasia of mammary gland cells (2). IR and IGF1R numbers are increased in breast cancer and raise the possibility that IR and IGF1R may have roles in the biology of these tumors (3,4). Thus methods for evaluating the numbers of these receptors on tumor cells and evaluating receptor-receptor interactions would be clinically useful.

Insulin receptor (IR)

The insulin receptor (IR) is a transmembrane receptor that belongs to the receptor tyrosine kinase (RTK) superfamily. It is located in the plasma membrane of insulin-sensitive cells, e.g., adipocytes, myocytes, and hepatocytes. It mediates the effect of insulin on specific cellular responses including glucose transport, glycogen synthesis, lipid synthesis, and protein synthesis. The receptor homodimer is a heterotetrameric protein consisting of two extracellular α-subunits with a molecular weight of 135 kDa and two membrane spanning β-subunits with a molecular

weight 95 kDa (Figure 1). These subunits are covalently linked by disulfide bonds to form a α2β2-structure. The receptor is encoded by a single gene which is located on human chromosome 19 and consists of 22 exons where exons 1–11 make up the α subunit and exons 12–22 make up the β subunit. The single chain polypeptide precursor is post translationally cleaved in the endoplasmic reticulum into α and β subunits which subsequently dimerize. Both subunits are glycosylated in the Golgi apparatus. Alternative splicing of exon 11 generates two isoforms of α subunit. Exon 11 is absent in IR-A isoform, but is present in the IR-B isoform which has a 12-amino acid peptide located at the carboxyl-terminal end of the receptor's α subunit. The two receptor isoforms are also different in their C-terminus and in their affinity to bind insulin, IGF-1 or IGF-2 factors (5). In addition to insulin, insulin receptors can bind IGF-1 and IGF-2 but with different affinity. Insulin receptor has an affinity for IGF-1that is 100-1000 fold less than for insulin. However, the A-isoform of insulin receptor binds IGF-1 and IGF-2 with same affinity as insulin (6).

The α subunit of IR has a molecular weight 135 kDa, and it consists of 723 amino acid residues. It is glycosylated and located entirely extracellularly. Each α subunit is linked to one β subunit of same monomer by a class II disulfide bond. The two α subunits in the α-β dimer of the insulin receptor are connected by interchain disulfide bond which is formed by cysteine 524 and cysteine 682 in the cysteine-rich domain of the α subunit (7). The α subunit of IR contains the ligand binding sites in the cysteine-rich domain. Besides the role of the cysteine-rich region of the α subunit in formation of disulfide bonds with the β subunit, it is possible that this region is involved in the binding of insulin. Also, this region shows one of the lowest levels of sequence identity between IR and IGF1R, and this could cause the differences in the binding specificity of these receptors for insulin, IGF-1 and IGF-2 (8).

In absence of insulin, the tyrosine kinase domain is inactive, but when the insulin binds to the ligand binding site on the α subunit, it brings the two α subunits closer together. This conformational change causes ATP binding to the intracellular domain of β subunits which in turn leads to trans-autophosphorylation and subsequent receptor activation. Therefore, binding of insulin to α subunits turns on the receptor kinase activity. As a result, the α subunit is considered to be the regulatory subunit of insulin receptor (6).

The β subunit (95 kDa) is composed of 620 amino acids and consists of both an intracellular and an extracellular domain that are anchored via a transmembrane domain which consists of 23 amino acids. The extracellular domain has O- and N-glycosylation sites. The intracellular portion of the β subunit contains the kinase catalytic domain (980-1255 amino acids) flanked by two regulatory regions, a juxtamembrane region involved in docking of insulin receptor substrate (IRS) as well as functioning in receptor internalization. A C-terminal tail contains two phosphotyrosine binding sites, tyrosine 1328 and tyrosine 1334 (9).

Activation of the tyrosine kinase domain of IR is essential for the receptor function. The tyrosine kinase domain of IR is localized in the cytoplasmic region of the β subunit and contains many tyrosine residues. The three main tyrosine residues that have been recognized to play a functional role are tyrosine 1158, tyrosine 1160, and tyrosine 1162. While several phosphorylated tyrosine residues in the catalytic domain are essential for receptor kinase activity, phosphorylation of the tyrosine residue 972 in the juxtamembrane domain and tyrosine 1328 and tyrosine 1334 in the carboxyl-terminal domain are also important. The juxtamembrane autophosphorylation site is essential for the interaction of the receptor with intracellular substrates and provides a docking site that increases the stability of receptor/substrate complex. Tyrosine phosphorylation in the carboxyl terminal domain has a functional role in mitogenic activity (6,10).

IGF1R structure

Although IGF-1 binds weakly to IR, it has its own high affinity receptor, IGF1R. IGF1R is transmembrane protein that belongs to tyrosine kinase superfamily of receptors. It is widely expressed in many cell types and different human tissues (11). IGF1R binds both IGF-1and IGF-2 with high affinity but binds insulin with very low affinity (12,13).

IGF1R has important physiological functions in regulation of cell growth and development and in protection from apoptosis. Disturbance of IGF1R function leads to serious complications in mice including abnormalities in fetal growth, and in bone, skin, and the central nervous system (11). The level of IGF1R is increased in many human cancers like breast carcinoma, and interference in the function of IGF1R may induce malignant transformation of normal cells in many cancer types (11). Therefore, IGF1R is considered a therapeutic target for many human cancers.

IGF1R, like IR, is synthesized as a single polypeptide chain that is processed to form a glycopeptide with a molecular weight of 180 kDa (14). The precursor protein contains polar residues in the terminal domain that function as a signal peptide facilitating the transfer of the nascent protein to the endoplasmic reticulum. Then the precursor protein is partially processed to form the disulfide linked pro-receptor dimers which in turn are proteolytic cleaved at the basic tetrapeptide sequence (Arg-Lys-Arg-Arg). The mature heterodimers have β-α-α-β conformation. IGF1R is a membrane glycoprotein (300-350 kDa) which consists of two extracellular ligand binding α subunits, each with a molecular weight of 135 kDa, and two transmembrane catalytic β subunits, each with a molecular weight of 90 kDa, connected together by disulfide bonds to form a functional heterotetramer (11). The α subunit consists of a cysteine-rich region and several N-linked glycosylation sites (Asn-X-Ser/Thr motifs). The cysteine-rich region is important because

it is used for high affinity binding of the IGF-1. IGF1R is different from IR in that the N- and C-terminal domains are both important for insulin binding. The β subunit has a hydrophobic sequence that forms the transmembrane domain. The cytoplasmic part of β subunit consists of juxtamembrane domain, a tyrosine kinase domain and the C-terminus region. The juxtamembrane domain has an Asn-Pro-X-Tyr motif which is important in receptor internalization and the biological function of the receptor. The tyrosine kinase domain is the catalytic part of the receptor that contains a glycine-rich motif (Gly-X-Gly-X-X-Gly) which participates in transferring a phosphate group from ATP to specific intracellular substrates that initiate the signaling transduction after ligand binding to receptor (15). The tyrosine kinase domains in IR and IGF1R are highly homologous. While the homology between the two receptors reaches 65-80% in the extracellular domains homology ranges from 45 to 65% (Figure 2) (12).

Although, the two receptors have a high degree of homology, experimental evidence suggests that each receptor has specific biological functions. IR is a key regulator in glucose metabolism and synthesis of glycogen and fat. IGF1R has a specific role in regulation of cell growth and development (11). The specificity of function for each receptor is due the difference in the relative expression of these receptors in the tissues. For example, IRs are highly expressed in liver and fat cells where they have metabolic functions after ligand binding. On the other hand, tissues that express more IGF1Rs than IRs, produce a response to IGF1R stimulation rather than IR (13).

Structure of the IR/IGF1R hybrid receptor

Both IR and IGF1R are highly homologous tetrameric complexes that are overexpressed in most human breast carcinoma cells. Hybrid receptors are assembled randomly from one IR α and one β subunit hemicomplex and one IGF1R α and one β subunit hemicomplex. IGF-1 binds to hybrid receptors with same affinity as to IGF1R, but it binds IR with very low affinity. Therefore, the hybrid receptors behave like IGF1Rs rather than IRs. In breast carcinoma cells, IGF-1 is locally secreted by the stromal fibroblast of breast cancer cells, but no insulin is secreted locally. The hybrid receptors become autophosphorylated in breast cancer cells when exposed to IGF-1rather than to insulin. Collectively, hybrid receptors will be affected more by IGF-1 than insulin (2).

Signal transduction by insulin receptors, IGF1 R and hybrid receptors

The IRs, IGF1Rs, and hybrid receptors bind to the same ligands (insulin, IGF-1, and IGF-2), but with different affinities (Figure 5). When the ligand binds to the α subunit of these receptors, a conformational change occurs that brings the two α subunits closer to each other, and this will induce the trans-autophosphorylation of one β subunit by another via specific tyrosine residues in the catalytic domain. Activation of tyrosine kinase domain in the β subunit of both IR and IGF1R catalyze the phosphorylation of other tyrosine residues of insulin receptor substrate family of proteins (IRS-1,-2,-3,-4), Shc, APS, adaptor protein containing a PH domain and an SH2 domain, and Gab1 (Grb2-associated binder 1).

Among these various substrates, the IRS proteins are the most extensively characterized proteins. They act as intermediates between activated IRs and IGF1Rs and the subsequent

intracellular signaling cascade. They have no kinase activity but, instead, serve as scaffolds for complexes to initiate the intracellular signaling cascades. There are four groups of IRS. IRS-1 plays an important role in the biological function of metabolic and mitogenic pathway. It may also play a role in cancer development; transgenic mice overexpressing IRS-1 develop breast cancer (16). IRS-2 is important primary mediator of insulin-dependent mitogenesis and regulation of glucose metabolism in most cell types. In addition it enhances survival and growth of insulin-producing β cells in the pancreas (17). IRS-3 is only expressed in rodents where it is most abundant in adipocytes. It is tyrosine-phosphorylated in response to insulin in these cells (5). IRS-4 is found predominantly in brain, liver, thymus, and kidney. The phosphorylated tyrosine residues of IRS proteins recruit other intracellular molecules that contain the Src-homology-2 (SH2) domain, including the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K), the SH2-containing protein tyrosine phosphatase (SHP2) and the adaptor proteins GRP2 (growth factor receptor binding protein 2) (5). This assembly of intracellular proteins with IRS will initiate subsequent downstream cascade of protein-protein interactions due to binding of phosphorylated tyrosine residues of IRS to the Src-homology-2 (SH2) domain of signaling molecules like PI3K, growth factor receptor binding protein 2 (GRP2), and SH2 domain of tyrosine phosphatase 2.

PI3K consists of two subunits, a 110-kDa catalytic subunit (p110) and the 85-kDa regulatory subunit (p85) which has two SH2 domains (Figure 3) (10). When the phosphorylated tyrosine of IRS binds to the SH2 domain of the regulatory subunit p85 of PI3K, they form a complex that facilitates the binding of the catalytic subunit of PI3K to the phospholipids in the plasma membrane. This complex is consists of the receptor, IRS, and PI3K. The activation of PI3K catalyzes the addition of phosphate on the D3 position of the inositol ring of phosphatidylinositol 4,5-bisphosphate (PIP2) to produce phosphatidylinositol (3,4,5)-triphosphate

(PIP3) in the cell membrane which acts as intracellular messenger that activates the PI-dependent kinases, stimulates growth, and changes the intracellular trafficking. The activated phosphoinositide-dependent kinases (PDK1 and PDK2) are subsequently phosphorylated and activate the serine/threonine kinase Akt (protein kinase B, PKB). Activated Akt has important metabolic functions as well as growth mediating functions (5). Upon receptor activation, Akt, together with other atypical protein kinase C (PKC), stimulates the translocation of glucose transporter 4 (GLUT 4) from intracellular part to the cell membrane where it mediates glucose internalization. Therefore, PI3K is essential for insulin-stimulated glucose transport (6). In addition, PI-3K stimulates glycogen storage by inactivating glycogen synthase kinase 3 (GSK-3). Subsequently, glycogen synthase enzyme will be active, increasing the rate of glycogen synthesis (18).

Akt is able to phosphorylate the Forkhead box protein O1 (FOXO1), previously known as forkhead in rhabdomyosarcoma (FKHR), a transcriptional enhancer that responds to insulin and other growth factors. It is a nuclear protein that regulates the genes controlling apoptosis, glucose production and cell cycling. When the PI3K pathway is active, FOXO1 is phosphorylated by Akt which then moves from the nucleus to cytoplasm leading to inhibition of gene expression (6).

IR and IGF1R also affect protein synthesis and degradation by activation of the target of rapamycin (mTOR) pathway. mTOR is a serine/threonine protein kinase encoded by mTOR gene in human. It has important functions in protein synthesis, transcription, translation, cell growth, and autophagy. Upon PI3k/Akt pathway activation, mTOR is activated and directly phosphorylates the p70 ribosomal S6 (S6K) kinase which is a regulatory enzyme for translation. Also, it phosphorylates the eukaryotic initiation factor binding protein (4EBP1) which bound to S6K, and subsequently it causes dissociation of these two proteins (S6K and 4EBP1) from other eukaryotic

initiation factor 3 (eIF3) translation initiation complex leading to increase the translation of mRNA (19).

The PI3K/Akt pathway is inhibited by other phosphatase enzyme which is PTEN, phosphatase and tensin homology. PTEN is tumor suppressor protein encoded in human by PTEN gene that dephosphorylates the second messenger of PI3K which is phosphatidylinositol (3, 4, 5)-trisphosphate (PtdIns (3, 4, 5) P3). In this way it inactivates the Akt pathway and inhibits the cell cycle and survival (Figure 4) (20).

The second major signaling pathway for IR and IGF1R is the mitogen-activated protein kinase (MAPK) pathway which plays a critical role in the regulation of cellular growth, survival, and gene expression. Abnormal MAPK signaling may cause uncontrolled cell proliferation and resistance to apoptosis. The phosphorylation of tyrosine residues of IRS proteins and/or Shc cause interactions between IRS and Grb2 adaptor protein which in turn recruits another exchange protein called Son-Of-Sevenless (SOS). This event causes translocation of SOS to the plasma membrane for activation of RAS, a small GTPase. RAS is usually in its inactive state bound to GDP (guanosine diphosphate), and it is transiently activated when SOS displaces the GDP from Raf which allows GTP to bind. The activated RAS acts as adaptor protein and binds to Raf kinases (Rapidly accelerated fibrosarcoma kinases) with high affinity causing their translocation to the cell membrane and activation. Therefore RAS activates Raf which is known generally as mitogen-activated protein kinase (MAPKKK) and which phosphorylates the second protein kinase in this cascade, MAPKK (MEK). The MAPKK activates the final kinase enzyme in this pathway which is the MAPK by phosphorylation of tyrosine residue and threonine residue. Then MAPK becomes active and translocated to nucleus where it activates transcription factors and regulates gene expression (21).

Although, the IRS and Shc pathways are common signaling pathways for both the IR and IGF1R, a specific interaction of IR and IGF1R with IRS-1 and IRS-2 may be the key to the distinct functions of each receptor. For example, the proto-oncogene c-Crk or p38 is widely expressed pro-oncogene that associates with IRS-1 and IRS-2 and interacts directly with the IGF1R via the SH2 domain but not with IR. On the other hand, IR downstream signaling causes dephosphorylation of tyrosine residues on protein tyrosine kinase 2, also called Focal Adhesion Kinase FAK, which is a focal adhesion-associated protein kinase involved in cell adhesion by actin and cellular movement. When FAK is blocked, breast cancer cells became less metastatic due to decreased mobility (22).

Do IRs and IGF1Rs in breast cancer use the same physiological signaling system?

Although, the IR and IGF1R are highly homologous, the normal biological function for each receptor is distinct. Whereas IRs have important roles in the regulation of many metabolic processes via PI3K signaling system, IGF1Rs regulate growth and development via the MAPK pathways**.** However, in breast cancer cells, the signaling specificity of these receptors is lost. Both the PI3K and MAPK intracellular signaling pathways are activated. Various mechanisms are responsible for disturbance of the physiological functions of IRs and IGF1Rs. One of these mechanisms is the overexpression of both receptors and hybrid receptors. In addition, the local production of IGFs factors from tumor cells occurs in autocrine-paracrine manner, and these factors bind to hybrid receptors with high affinity. Finally, there is a chronic increase in insulin levels in blood due to insulin resistance. This causes activation of IR signaling through mitogenic pathways and increases the bioavailability of IGF-1 (5).

The role of IR and IGF1R in breast cancer

Although insulin and insulin-like growth factor 1 share extensive sequence homology and share downstream signaling pathways, IGF1R exhibits stronger mitogenic and anti-apoptotic effects. IGF1R is a potent mitogen for both normal and transformed breast epithelial cells and increased serum IGF-1 levels are associated with the development of mammary gland hyperplasia and cancer (4). In addition, the presence of the IGF1R is required for development of a neoplasia. With a few exceptions cells lacking IGF1R do not undergo malignant transformation (3).

Studies performed by specific ELISAs have indicated that approximately 80% of breast cancers have IR content higher than the mean value found in normal breast and approximately 20% of cancers have IR at least 10-fold higher than normal breast tissue (23). Functional studies indicate that IR expressed in breast cancer is more sensitive to insulin than is normal breast tissue, and patients whose tumors have high IR content have a lower 5-year disease-free survival than patients with tumors with moderate IR content (24). Analysis of these data to establish prognostic factors for breast cancer have confirmed that IR content is the strongest independent predictive factor for disease free survival. However, IR overexpression is not specific to breast cancer but seems to be a common phenomenon in cancer, IR overexpression is not occurs only in breast cancer, but also in colon, lung, ovary, and thyroid cancer (5).

The possible mechanism for IR overexpression in breast cancer cells is the inactivation of P53 (tumor suppressor protein) and activation of HMGA1 (High-Mobility Group A1) proteins. This finding explains at least in part why IGF1R are overexpressed in most human cancer (23). P53 has many anticancer functions and plays a role in apoptosis, genomic stability, and inhibition of angiogenesis. HMGA1 belongs to the family of non-histone chromatin proteins that in humans

are encoded by the HMGA1 genes. They are involved in many cellular processes including gene transcription, DNA replication, and metastatic progression of cancer cells. In addition, they have the ability to bind to DNA forming multi-complexes that regulate gene transcription. The expression of HMGA1 is directly correlated with transformation and metastasis of tumors and also participates in the regulation of IR transcription (25). Some studies indicate that HMGA1 inhibits P53, a tumor suppressor protein, so it may be involved in up-regulation of IR in cancer cells by inactivating p53 factor. Thus, these data suggest that IR overexpression is driven by multiple mechanisms that are activated during development of cancer (24). Some of these mechanisms also cause IGF1R overexpression. Like other growth factors, insulin is able to stimulate cell motility toward a ligand gradient (26). This effect requires IR autophosphorylation and activation of the same signaling pathways involved in mitogenesis. Furthermore, IR enhances the tumor cells metastasis, so the breast cancer cells overexpressing IRs may have an increased metastatic potential (24).

On the other hand, IGF-1 is a hormone related to insulin that is also considered to be an endocrine risk factor for breast cancer (4). In recent years it has become clear that IGF-1 has a significant role in cancer development and progression (27). Some studies show that IGF1Rs in breast cancer are more critical than IRs as IGF-1 together with IGF1R has more potent transformation effects on breast cells (24). Clinical evidence suggests that there is a link between IGF1R and breast epithelial cell transformation because the expression level of IGF1Rs is higher in breast cancer tissue than adjacent normal tissue. However, IGF1R and IR can form hybrid receptors by heterodimerization and these hybrid receptors have a high affinity for IGF-1 and IGF-2and lower affinity for insulin. Hybrid receptors behave functionally like IGF1Rs rather than IRs. Recent evidence supports the idea that IGF1R/IR hybrid receptors may also be involved in biology

of breast cancer by increasing the rate of tumor proliferation and metastasis potential. Because IR also activates signaling pathways similar to IGF1R in breast cancer cells, agents targeting both receptors may be necessary to disrupt the malignant phenotype regulated by this growth factor system (28).

Methods used for counting IR and IGF1R

Counting receptor numbers is an important first step in determining the role of IR and IGF1R in breast cancer development and progression. A number of methods have been developed that can accomplish this. Most of these methods have major limitations and are not likely to be useful in a clinical setting. These methods are discussed below.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis is a method to separate proteins and macromolecules. It has been used to count receptors like IR and IGF1R during the 1980s. It separates molecules according to their molecular weight and charges. It is usually done using binding of photo-labeled insulin or IGF-1 to cells or membranes that express IR, IGF1R, or both receptors. The proteins labeled with ^{125}I-insulin or ^{125}I-IGF-1 are identified by electrophoresis and autoradiography and they appear as radiolabeled bands. The intensity of these bands reflects the expression rate of these receptors. However, this method cannot measure exactly the molecular weight of IR or IGF1R; it gives an estimate of numbers because the glycosylated proteins have very slow mobility on polyacrylamide gel. In addition, proteins may migrate with abnormal behavior due to interactions

between insulin binding components and other membrane constituents with detergents and gel buffers. This could be a major source of result errors (29).

Selective immunoadsorption of radioligand or radio-labeled receptor complexes and immunoblotting

This method is used to detect the fraction of IR and IGF1R that form hybrid receptors in different tissues that express both receptors. In this method, receptors are isolated from membranes of tissues with a specific detergent, and then incubated with ^{125}I-IGF-1. After selective immunoadsorption of radioligand receptor complexes, immunoblots are prepared and probed using specific IR and IGF1R antibodies to the receptors. Therefore, IR and IGF1R levels are estimated by blotting. The level of IR and IGF1R does not reflect the level of hybrid receptors because of heterogeneity of receptor expression between different tissues (30).

Fluorescence Correlation Spectroscopy (FCS)

This technique was developed by Elliot Elson in the 1970s. It measures the fluctuation of fluorescence intensity in a sub-femtoliter volume**,** a tiny volume excited by a laser source. This method is used to detect some dynamic biological processes like concentration localization of proteins and lipids within the cells, interactions between ligand and receptor on cell membrane, and rate of diffusion of some molecules. Some studies have used FCS to measure the density and thermodynamics of insulin and IGF1 receptors (31). IR and IGF1R are labeled with specific antibodies against the α or β subunit of each receptor and, as a result, the number of IRs and IGF1Rs can be estimated. On the other hand, when IR and IGF1R are labeled with fluorescein

isothiocyanate-insulin (FITC-insulin), the total number of IRs is slightly lower than estimated using specific antibodies. However, FCS measurement of IRs and IGF1Rs using a specific antibodies or fluorophore-conjugated ligands such as FITC-insulin is only estimation and not accurate because it measures all binding of fluorescent ligands to receptors including IR/IGF1R hybrid receptors (32).

Methods for evaluating IR or IGF-1R organization as homodimers or hybrid receptors

Single Molecule Localization Microscopy (SMLM)

Previous methods that studied interactions and organization of molecules in the cell membranes were dependent on the classical biochemical measurements and assays. Using new methods that depend on super resolution imaging like single molecule localization microscopy, biologists can assess the organization of molecules in the cell membrane directly. SMLM can overcome limitations of standard fluorescence microscopy that identify protein co-localization within 250 nm. Due to the high resolution obtained by SMLM, two proteins can be co-localize to around 25 nm making SMLM useful for the detection of detect receptor clustering, aggregation, and signaling. SMLM is done on IR and IGF1R in presence and absence of insulin in cell medium and data shows clustering of both receptors. In addition, they can measure the accurate numbers of each receptor in cluster exactly, distance between clusters, and distance between receptors. On the other hand, single molecule imaging is more complicated than standard fluorescence microscopy because it is computational and requires the use of different fluorophores, some of which are not well characterized. Also, it is important to use very densely labeled samples because

it affects the sharpness of constructed images and facilitates observation of fine details within the image, so SMLM is time consuming and technically demanding (32).

Homo-transfer or Hetero-transfer fluorescence resonance energy transfer (FRET)

These are techniques that provide measurements of interactions between partner molecules in live cells (33). They are based on energy transfer between two molecules, an energy donor and an energy acceptor. FRET depends on the distance between the donor and acceptor. Energy transfer occurs when the intermolecular distances are within 1-10nm. When the two fluorophores are the same, the technique is called homo-transfer FRET. If the fluorophores are different, the technique is called hetero-transfer FRET. In both types of FRET, there must be sufficient overlap between excitation and emission spectra of donor and acceptor fluorophores.

Hetero-FRET has some limitations and complication because it occurs between two different fluorophores. One of these complications is difficulties in interpreting results when the level of expression for fluorophore-tagged proteins is different. In addition, when the receptors organized in higher oligomers than dimers especially in regions that are crowded with protein clusters like the cell membrane, hetero-transfer FRET cannot distinguish dimers from other clusters. However, homo-transfer FRET is simpler than hetero-transfer FRET because it uses only a single fluorophore. Also, it is highly sensitive to oligomerization of proteins between the fluorophores because it proportional to the six power of distance between the donor and acceptor molecules in nanometer scale.

In these studies, polarization homo-transfer FRET methods were used to evaluate interactions between IR-GFP monomers. Energy transfer between two or more GFP molecules

occurs only when the donor GFP and acceptor GFP molecules are within 2 -10 nm distance of one another (34). Homo-FRET id highly sensitive to distance between donor and acceptor because the FRET efficiency is inversely proportional to the six power of distance (r) between the two molecules (39).

$$E=1/ (1+r/R_0^6)$$

Where E is FRET efficiency, r is distance between donor and acceptor, and R_0 is the Forster distance between donor and acceptor at which 50% of energy transfer occurs.

Homo-transfer FRET data is collected from two detectors in a Zeiss Axiovert 200M microscope using an Andor Du897E EMCCD camera. Images were acquired using an arc lamp for fluorescence excitation together with a polarized excitation filter, a GFP emission filter and a 63x water objective for observing cells. The two detectors collect vertical and horizontal emission images that represent fluorescence parallel to polarization of excitation light, and anisotropy of the emitted light can be measured from the following equation:

$$r = (I_{vv} - GI_{vh})/ (I_{vv}+2GI_{vh})$$

r is the anisotropy of emitted light, I_{vv} is the fluorescence intensity of emission that is vertical to excitation light, I_{vh} is fluorescence intensity of emission that is horizontal to excitation light. G is G factor that is used as correction factor for different sensitivities in detection of vertical and horizontal fluorescent intensities (39).

Image J software is then used to obtain the vertical and horizontal fluorescence intensities from cell measurements and for calculation of the G factor as discussed in "Results and Discussion". These studies assume that the fundamental anisotropy of the GFP fluorophore is 0.38 (33) which represents an immobile IR-GFP monomer.

The goal of these studies was to use homo-transfer FRET methods to determine whether there were differences in the organization of IRs when receptors numbers per cell are comparatively low or significantly higher as may occur in more aggressive forms of breast cancer. We hypothesize that in cells that highly expressing IR-GFP, IR monomers appear apparently as IR homodimers, and in cells expressing low numbers of IR-GFP, most IR appear as monomers and may exist as hybrid receptors.

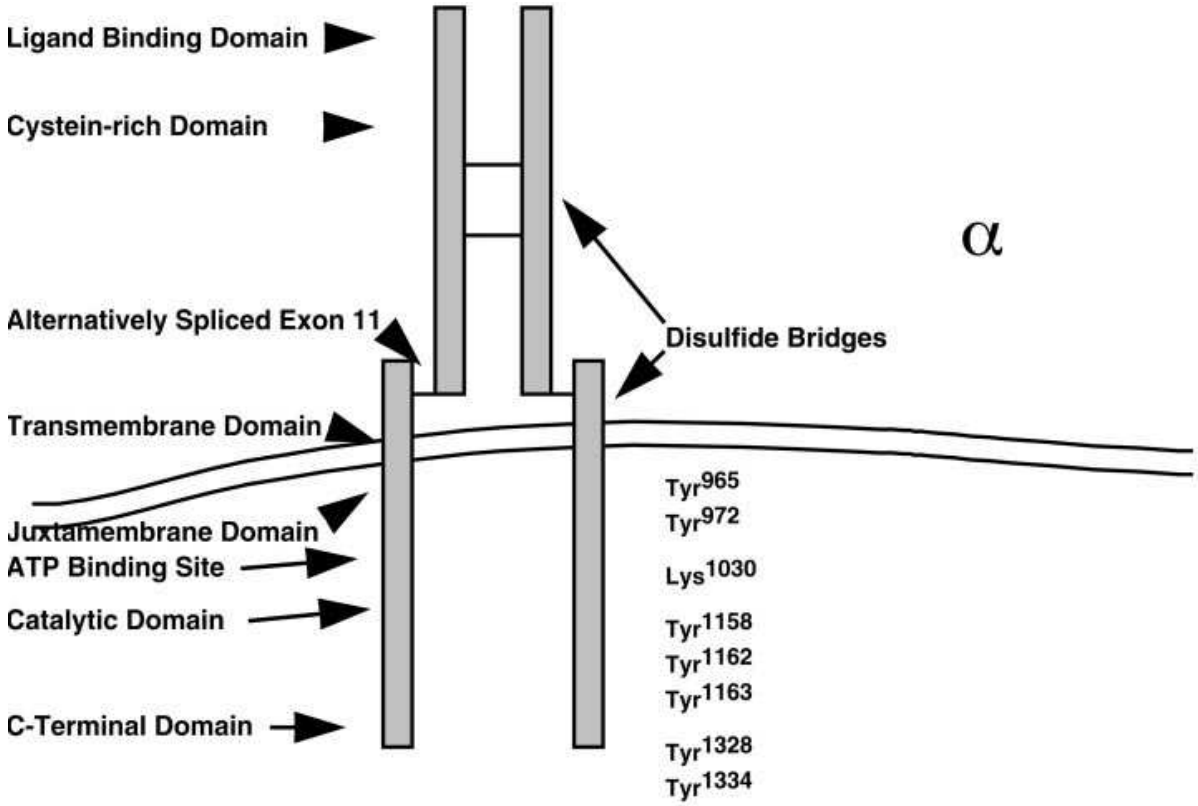

Figure 1. Subunit structure of the insulin receptor. Schematic diagram of the IR subunit organization and major structural features. The insulin-binding domain is localized to the N-terminus of the α subunit. The β subunit intracellular domain contains the tyrosine-specific protein Kinase activity. Several tyrosine residues are phosphorylated in the receptor's juxtamembrane domain (tyrosine 965 and 972), catalytic loop (tyrosine 1158, 1162, and 1163), and carboxyl-terminal domain (tyrosine 1328 and 1334). The variably spliced exon 11 is indicated at the COOH-terminus of α subunit (6).

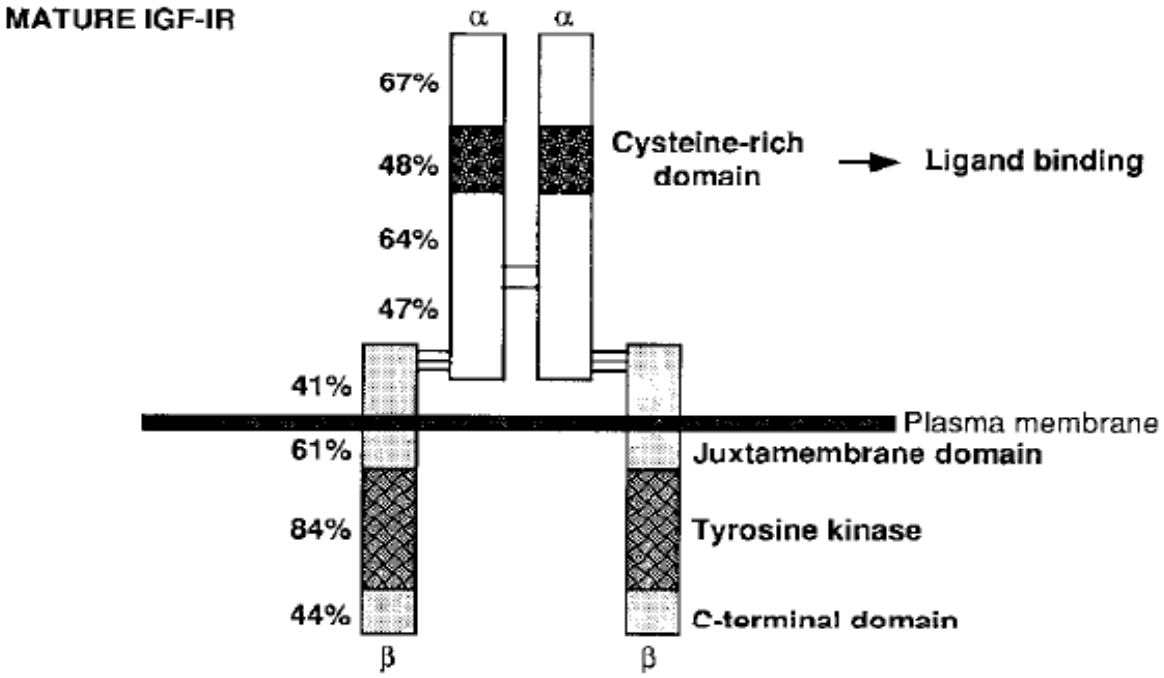

Figure 2. Structure of IGF1R. IGF1R consists of α–β subunit dimers. The percentage of homology between IGF1R and IR is indicated to the left of the receptor (12).

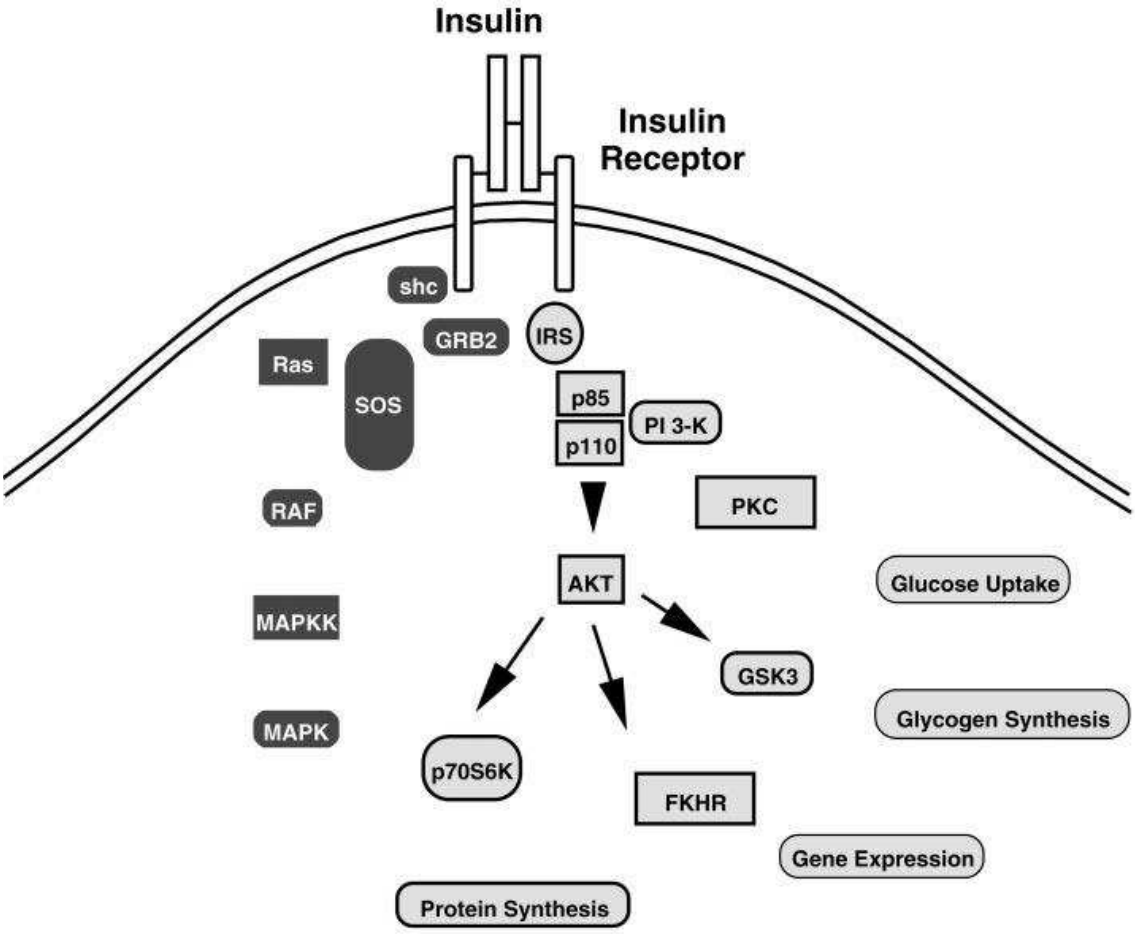

Figure 3. Insulin signaling pathways. SOS, son-of-sevenless; GAP, GTPase-associated protein; PDK, PI-dependent protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; GSK3, glycogen synthase kinase 3.

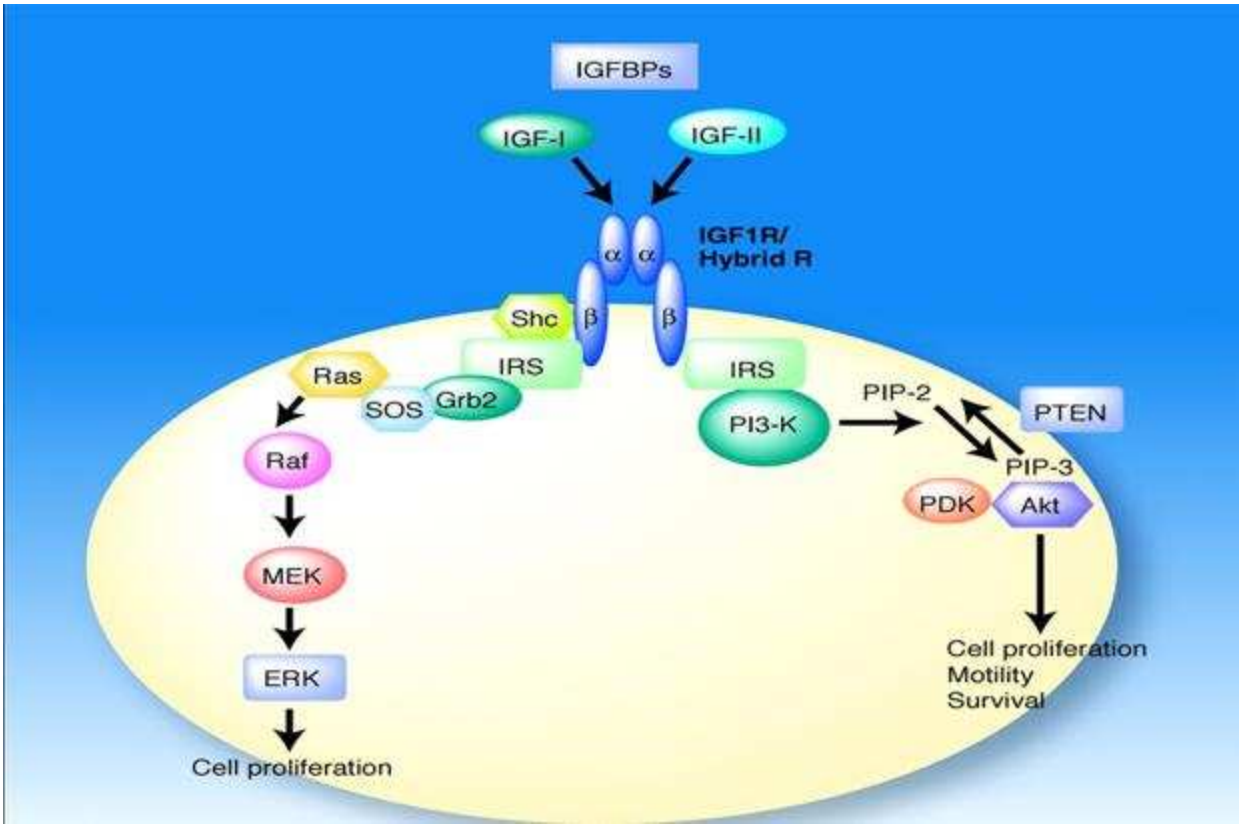

Figure 4. IGF1R signaling cascades. IGF1R or hybrid receptor can be activated by their ligands IGF-1 and IGF-2, whose bioavailability is regulated by IGF-binding proteins (IGFBP). Insulin receptor substrate (IRS) proteins or Shc couple activated IR to Grb2/SOS, leading to the activation of Ras/Raf/mitogen-activated protein kinase (MAPK) kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway. Activated extracellular signal-regulated kinase promotes cell proliferation. Insulin receptor substrate proteins also couple the receptor to phosphatidylinositol 3-kinase (PI3-K) activation. Activated phosphatidylinositol 3-kinase phosphorylates PIP-2 to PIP-3, which recruits Akt to the plasma membrane. Akt is activated by phosphoinositide-dependent kinase (PDK). The lipid phosphatase PTEN can dephosphorylate PIP-3 to inhibit the activation of phosphatidylinositol 3-kinase signaling (35).

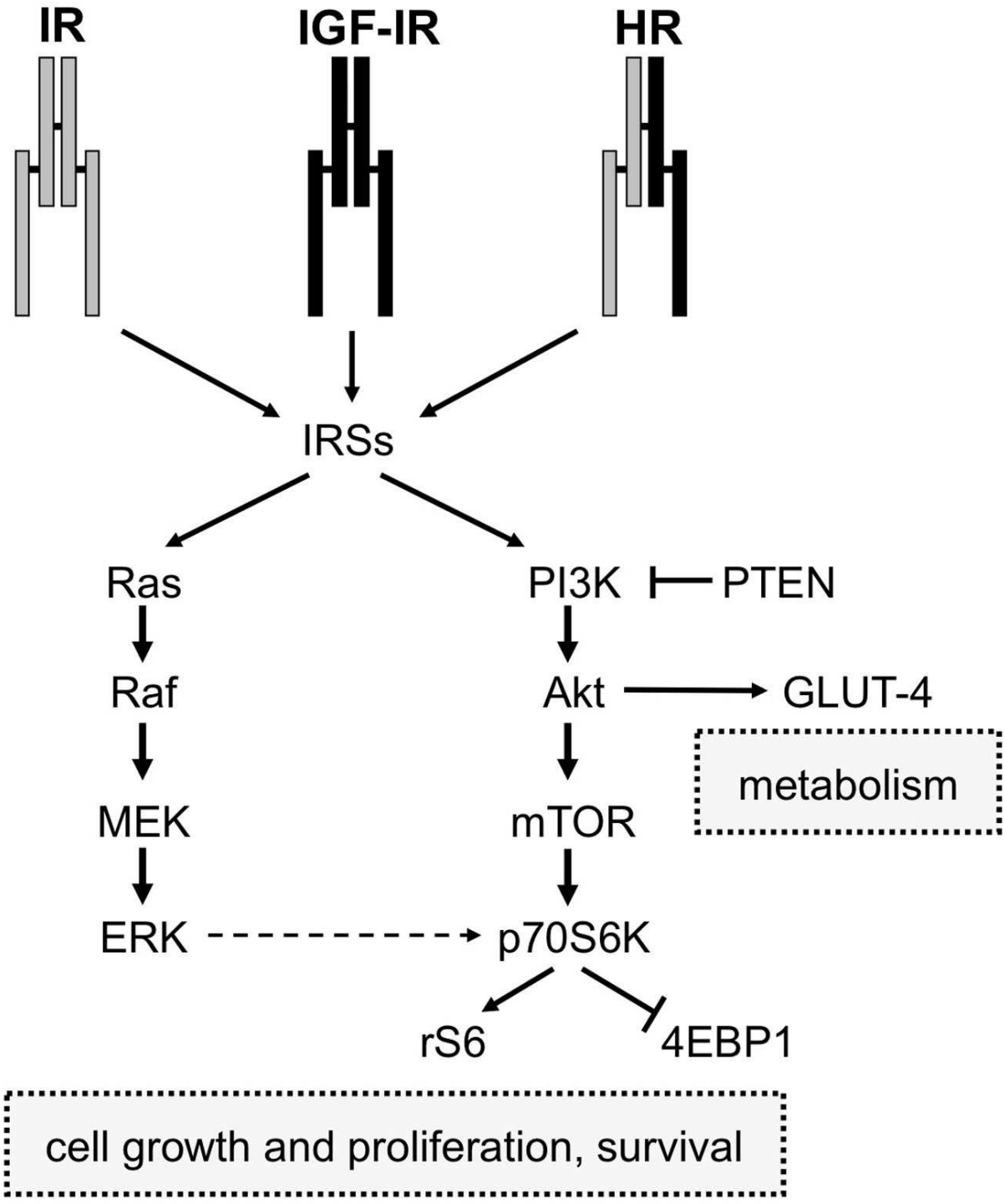

Figure 5. Schematic diagram of IR, IGF1R, and hybrid receptor signaling. IR, IGF1R and hybrid receptors induce the phosphorylation of IRSs proteins which trigger activation of two signaling pathways, Ras/Erk and the PI3K pathway.

Materials and Methods

Materials

Chinese Hamster Ovary cells (CHO) were purchased from ATCC (American Type Culture Collection), (Manassas, VA). Dulbecco's Modified Eagle medium (DMEM) to maintain CHO cells were purchased from Corning Cellgro (Visalia, CA). Geneticin (G418 sulfate) added to DMED was also purchased from Cellgro. Fetal bovine serum (FBS) was purchased from Atlas Biologicals (Fort Collins, CO). L-glutamine and penicillin/Streptomycin solutions were purchased form Gemini Bio-products (West Sacramento, CA). MEM non-essential amino acids and EDTA (ethylene diamine tetraacetic acid) were purchased from Sigma Aldrich (St. Louis, MO). Rhodamine 6G (R6G) was purchased from Allied Chemical (Vadodara, Gujarat. India). Glass bottom cell culture dishes (WillCO) with 35 mm diameter and 14 mm diameter glass bottoms were purchased from In Vitro Scientific (Sunnyvale, CA).

Cell culture and cell lines

We used three cell lines of CHO cells that stably express insulin receptors tagged with green fluorescent protein (GFP) to the C-terminus of the insulin receptor. The first cell line expressed low numbers of insulin receptors (IR ~62,000 receptors/cell). The second cell line expresses moderate numbers of insulin receptors (IR ~ 130,000 receptors/ cell). The third cell line expresses high number of insulin receptor (IR ~205,000 receptors/cell). CHO IR-GFP cells are maintained in MEM medium supplemented with 10% FBS, geneticin, L-glutamine, and

penicillin/streptomycin solution. All cells are grown in plastic 25 cm^2 culture flask in incubator at 37℃ with humidified environment and 5% CO_2.

Preparation of cell samples for homo-transfer FRET studies

CHO stably expressing IR tagged with GFP were maintained in culture flasks in an incubator with MEM medium. Then the medium was removed and 5 ml of EDTA was added to the culture flask and incubated for 5 minutes. Cells were then diluted with medium and platted in 35mm bottom glass dishes and incubated from 1-2 days until they occupied at least 70% of the plate surface. At time of the experiment, cells are washed twice by 1 X PBS (phosphate buffered saline) PH 7 and resuspended in 1-2ml PBS. After that, images were taken by Zeiss Axiovert 200 M inverted microscope in a dark room with all light sources turned off except for arc lamp illumination.

Use of flow cytometry to determine receptor numbers

Flow cytometry is a techniqnique that is used to measure different physical and chemical characterestics of cells such as cell size and shape, cell surface markers, DNA content, location of cell organelles and viability. We used flow cytometry to measure the numbers of IR in the three CHO cell lines. CHO cells experssing IRs which are labeled with GFP were put in a microfluid system that directs the cells in a single stream with a laser is focused on each single cell. Then, the flow cytometer captures the scattered light and emitted fluorescence at different angles and different wave lengths for each cell. Signals are collected and analysed electronicly to provide the numbers of IRs in each cell. In our experiment, we have three cell lines of CHO that expressing

IR-GFP. Using flow cytometry, we found that low expreesing cells had~ 62,000 receptors/cell, moderately expressing cells had ~130,000 receptors/cell, and high expressing cells had ~ 205,000 receptors/cell.

Results and Discussion

Experimental Determination of the G factor

The G factor is the correction factor for different detection sensitivities of vertical and horizontal polarized fluorescence in our instrumental setup. It acts as a normalization factor to compensate for optical properties such as reflections from mirrors that may change the polarization angle of excitation light. The G factor can be measured using aqueous solutions that have anisotropy values near zero such as Rhodamine 6G, which has an anisotropy value of about 0.012 (36,37). The G factor is then calculated using the following equation described by Dix and Verkman (36).

$$G = (1/F)\ (1-R)/\ (1+2R)$$

Where F is the difference in sensitivity of detection system that is used to detect the fluorescence vertical and horizontal intensities of emitted light of Rhodamine 6G subtracted from that for H_2O as in following equation:

$$F = (I_{vv}\ R6G - I_{vv}\ H_2O) / (I_{vh}\ R6G - I_{vh}\ H_2O)$$

Where I_{vv} is fluorescence intensity that is parallel directed to vertical polarization light of R6G and H_2O. I_{vh} is fluorescence intensity that has perpendicular direction to vertical polarization light of R6G and H_2O (36). Then, G factor is used to calculate the fluorescence anisotropy r, and

the range values measured for our samples were from 1.5 to 1.7. These values indicate that the sensitivity of detection in our Zeiss Axiovert 200M microscope is higher for vertical polarization compared to horizontal polarization.

Establishing Parameters for Photobleaching of IR-GFP

We next established the time required for photobleaching of IR-GFP in cell lines expressing low, moderate and high numbers of IR-GFP. Representative experiments shown in (Figures 6) demonstrate that >90% photobleaching of GFP was achieved in approximately 20 minutes for each of the cell lines used. In subsequent experiments, anisotropy data were collected for 12 minutes at 20 seconds per point.

Polarized homo-transfer FRET measurements of IR-GFP

Polarized homo-transfer FRET measurements were made using three cell lines that stably express IR-GFP where GFP is coupled covalently to the C-terminus of IR. The first cell line expresses low numbers of IR (~ 62,000 receptors/cell); the second cell line expresses moderate numbers of IR (~ 130,000 receptors/ cell); and, the third cell line expresses high numbers of IR (~ 205,000 receptors/cell). These receptor numbers were determined by flow cytometry. The mean, standard deviation and standard error of the mean for each data point obtained in homo-transfer FRET measurements were calculated for 10,9,8 cells, respectively. Anisotropies were corrected so that the final anisotropy after >90% photobleaching of GFP was 0.38 (33).

In cells expressing low numbers of IR, the initial anisotropy was approximately 0.33 compared to a predicted value of 0.38 for receptor monomers (Figure 7). This small difference between the observed and predicted value for a GFP monomer suggests that, when levels of IR expression are low, IR exists predominantly as a monomer. This is an interesting result given the factor that IR monomers are typically organized as homo-dimers or are half of an IR-IGF1R hybrid receptor. Thus low expressing receptors in these experiments may exist as true monomers or are part of IR-IGF1R hybrid receptors that appear, in these studies to be indistinguishable from IR monomers.

As receptor numbers per cell increase in cell lines expressing moderate or higher numbers of IR, the initial anisotropies decreased to 0.29 and 0.27, respectively (Figure 7). These results suggest that, with increasing levels of IR-GFP expression, more receptors are present as either IR homodimers or as receptor clusters containing either IR homodimers or hybrid receptors. A comparison of initial and final anisotropies (Table 1) for the three cell lines suggest that cluster sizes increase with increased expression of IR per cell. In addition, there is inverse relationship between the fluorescence intensity and anisotropy for the three cell lines because when the fluorescence intensity is high, the anisotropy is at it is lower value. While cells lose it is fluorescence intensities upon progressive photobleaching, the anisotropy is increases until it reaches maximum value (Figure 8)

In conclusion, polarized homo-transfer FRET permits us to monitor the oligomerization state of IR when receptors are expressed at various densities. These are the first studies to demonstrate a relationship between cluster formation and IR receptor number/density although similar results have been demonstrated for two G protein-coupled receptors, the serotonin 1A receptor (33) and the LH receptor (38). It is important to note that, particularly at lower IR

numbers, the likelihood of molecular crowding is reduced by the use of stable cell lines that are unlikely to exhibit large cell-to-cell variations in receptor number as is the case for transiently transfected cells (38).

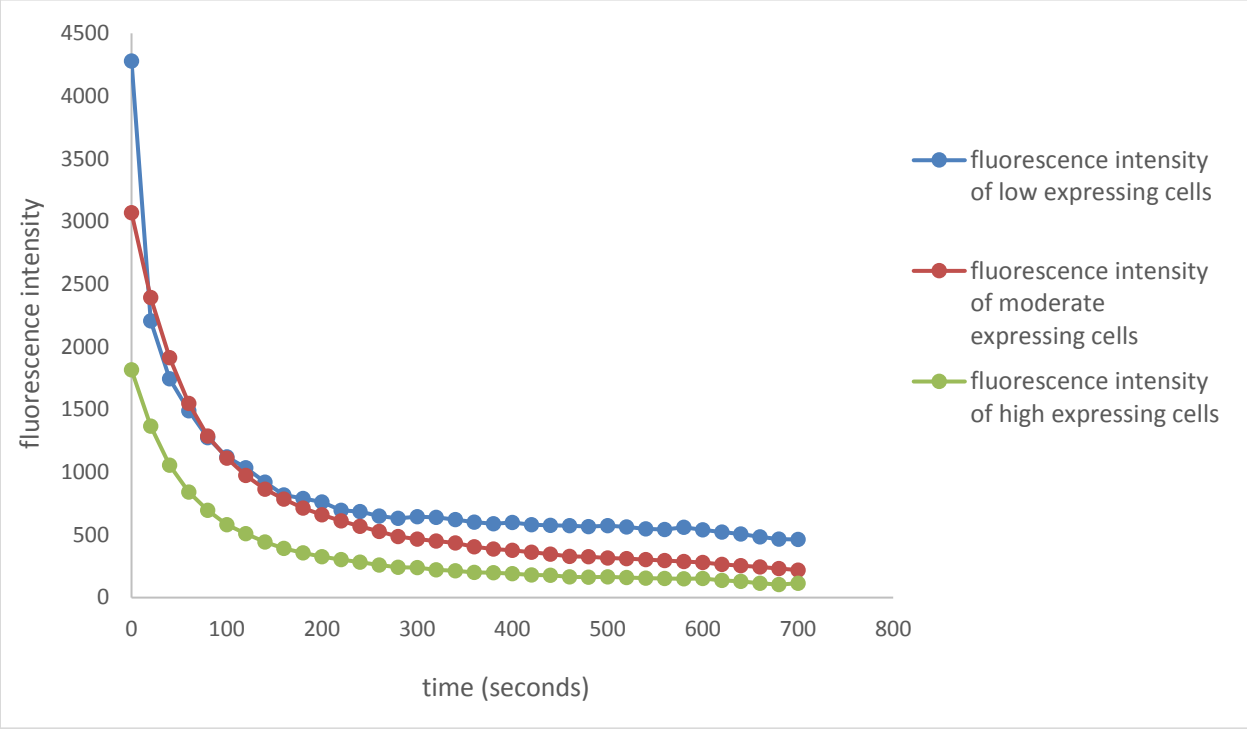

Figure 6. Intensity of the three cell lines (low, moderate, and high expressing CHO IR-GFP upon progressive photobleaching for 700 seconds. Images are taken every 20 seconds, and the cells are photobleached >90%.

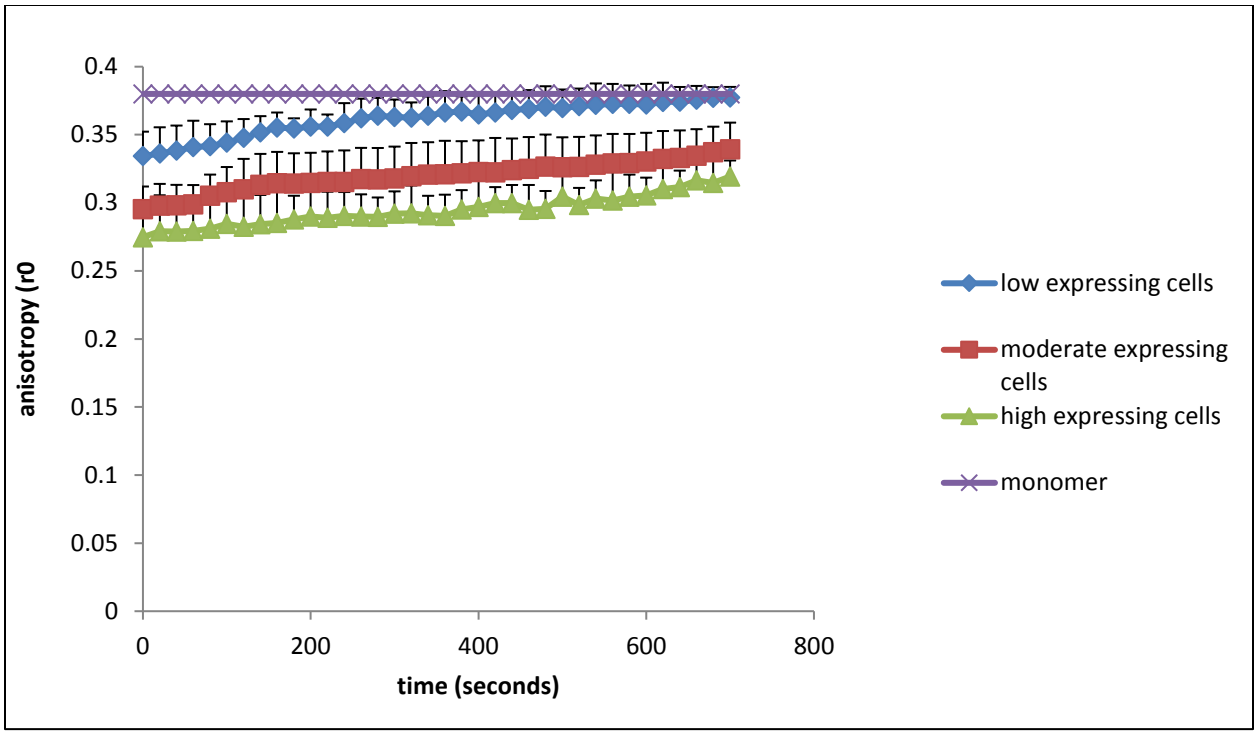

Figure 7. Fluorescence anisotropy for low, moderate, and high expressing CHO-IR-GFP cells upon progressive photobleaching for 700 seconds.

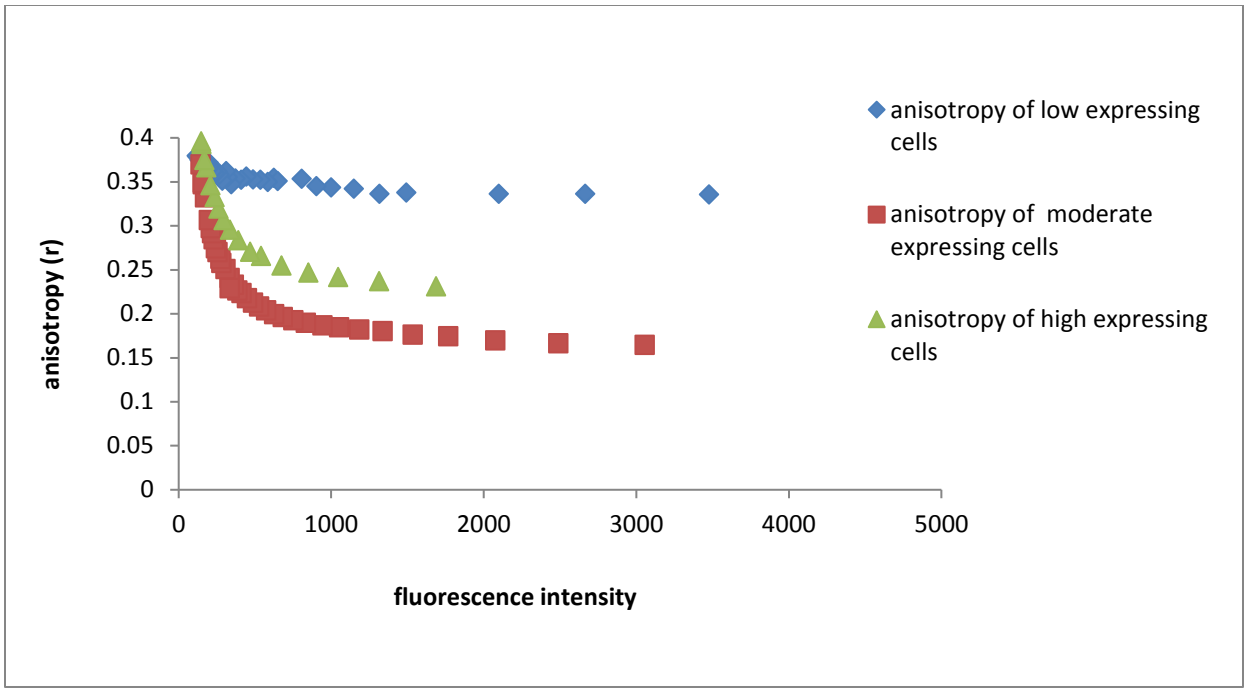

Figure 8. Increase in anisotropy with progressive loss of fluorescence intensity in the three cell lines.

Table 1: Difference between initial fluorescence anisotropy and final fluorescence anisotropy for three cell line expressing IR-GFP

Cell line	Initial anisotropy	Final anisotropy
Low expressing cells (62,000 receptors/cell)	0.334284	0.377466
Moderate expressing cells (130,000 receptors/cell)	0.295226	0.339361
High expressing cells (205,000 receptors/cell)	0.274763	0.319285

Conclusions and Future Directions

These studies desmonstrate that increased IR-GFP numbers per cell are likely to result in the formation of either IR homodimeric receptors or clusters of IR homodimers and hybrid receptors that exhibit FRET. There are, however, serious limitations to these studies which should be addressed.

First, it is not clear whether IR exists as a true receptor monomer when receptor numbers are low. It is possible that under these conditions, IR is participating in hybrid receptors formed from IR and IGF1R monomers. To determine whether this is the case, future studies will use cell lines that express various numbers of IGF1R-mCherry together with IR-GFP. Fluorescence correlation methods will then be used to distinquish IR monomers which will travel independently from IGF1R-mCherry from hybrid receptors where motions of both GFP and mCherry are correlated to one another.

Second, when IR receptor numbers are high, the formation of IR homodimers cannot be distinguished readily from larger receptor clusters that contain either IR homodimers or mixtures of IR homodimers and hybrid receptors. Again, fluorescence correlation spectroscopy will address question in cells that are stably expressing both larger numbers of IR-GFP and IGF1R-mCherry. We believe that as the relative numbers of IGF1R-mCherry increase, the likelihood of IGF1R-mCherry homodimers will also increase and the number of hybrid receptors will decrease.

Finally, the overall goal of this project is to assess numbers of IR and IGF1R on breast cancer cells. Although homo-transfer FRET is a relatively straightforward techniques, this method is not likely to be clinically useful until we have a better understanding of IR and IGF1R

expression levels on normal and breast cancer cells. To achieve this, the first step will be to evaluate IR and IGF1R numbers using both homo-transfer FRET methods as described in this thesis together with fluorescence correlation spectroscopy methods. There are a number of breast cancer cell lines where receptor levels have been characterized and these will be used in initial work. Ultimately, a more robust method is desirable that evaluates homotransfer-FRET using a single fluorophore and then calculates the relative numbers of IR and IGF1R homodimers and hybrid receptors from apparent numbers of receptor monomers (present in hybrid receptors) and receptor dimers. Such a method will only be possible if IR and IGF1R homodimers and hybrid receptors assemble randomly based on the availability of IR and IGF1R monomers.

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LIST OF ABBREVIATIONS

IR: insulin receptor

IGF1R: insulin-like growth factor one receptor

IGF-1: insulin-like growth factor 1

IGF-2: insulin-like growth factor 2

GFP: green fluorescent protein

Homo-FRET: homo-transfer fluorescent resonance energy transfer

Hetero-FRET: hetero-transfer fluorescent resonance energy transfer

α: alpha

β: beta

ATP: adenosine triphosphate

IRS: insulin receptor substrate

PI3K: phosphatidylinositol 3-kinase

GRP2: growth factor receptor binding protein

SH2: 2 Src-homology-2 domain

Gab: Grb2-associated binder 1

PKC: protein kinase C

PDK1 and PDK2: phosphoinositide-dependent kinases 1 and 2

PIP3: phosphatidylinositol (3, 4, 5)-triphosphate

PIP2: phosphatidylinositol 4, 5-bisphosphate

GSK-3: glycogen synthase kinase 3

FOXO1: Forkhead box protein O1

mTOR: target of rapamycin

GLUT 4: glucose transporter 4

MAPK: mitogen-activated protein kinase

SOS: Son-Of-Sevenless

HMGA1: High-Mobility Group A1 proteins

P53: tumor suppressor protein

GDP: guanosine diphosphate

CHO: Chinese hamster ovary cells

DMEM: Dulbecco's modified minimum essential medium

EDTA: ethylenediaminetetraacetic acid

FBS: fetal bovine serum

G418: genticin

PBS: phosphate buffered saline

FCS: Fluorescence Correlation Spectroscopy

FITC-insulin: fluorescein isothiocyanate-insulin

SMLM: Single Molecule Localization Microscopy