THEMATIC REVIEW

40 YEARS OF IGF1

IGF1 receptor signaling pathways

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This paper forms part of a special section on 40 years of IGF1. The guest editors for this section were Derek LeRoith and Emily Gallagher.

Abstract

Insulin-like growth factors (IGFs) bind specifically to the IGF1 receptor on the cell surface of targeted tissues. Ligand binding to the α subunit of the receptor leads to a conformational change in the β subunit, resulting in the activation of receptor tyrosine kinase activity. Activated receptor phosphorylates several substrates, including insulin receptor substrates (IRSs) and Src homology collagen (SHC). Phosphotyrosine residues in these substrates are recognized by certain Src homology 2 (SH2) domain-containing signaling molecules. These include, for example, an 85 kDa regulatory subunit (p85) of phosphatidylinositol 3-kinase (PI 3-kinase), growth factor receptor-bound 2 (GRB2) and SH2-containing protein tyrosine phosphatase 2 (SHP2/Syp). These bindings lead to the activation of downstream signaling pathways, PI 3-kinase pathway and Ras-mitogen-activated protein kinase (MAP kinase) pathway. Activation of these signaling pathways is known to be required for the induction of various bioactivities of IGFs, including cell proliferation, cell differentiation and cell survival. In this review, the well-established IGF1 receptor signaling pathways required for the induction of various bioactivities of IGFs are introduced. In addition, we will discuss how IGF signals are modulated by the other extracellular stimuli or by themselves based on our studies.

Key Words

- ▶ insulin-like growth factor (IGF)
- ▶ insulin-like growth factor (IGF)-I receptor
- ▶ signal transduction
- ▶ insulin receptor substrate (IRS)
- ▶ PI 3-kinase
- ▶ MAP kinase

*Journal of Molecular
Endocrinology*
(2018) **61**, T69–T86

Introduction

In many cell types, insulin-like growth factors (IGFs; IGF1 and IGF2) have been shown to possess a variety of bioactivities, such as induction of growth or differentiation of target cells, cell survival and maintenance of cell function. On the other hand, insulin, whose structure is similar to IGFs, mediates anabolic biological activities, including increases in glucose and amino acid transport, induction of glycogen, lipid and protein syntheses and inhibition of gluconeogenesis, lipolysis and protein degradation. IGFs predominantly mediate long-term action to determine the cell fates, whereas insulin mainly possesses metabolic activity.

Despite the profuseness and diversity of these effects of IGFs, the *in vitro* biological effects of IGFs are relatively

weak and often are not demonstrable except in the presence of other hormones or growth factors (Table 1). These findings suggest that IGFs act as permissive factors to augment the signals of other factors. This mechanism is very important in order that IGF induces specific bioactivities in the right tissues at the right times. Accordingly, to elucidate how IGF action is potentiated by other intercellular signaling molecules is essential for revealing IGF significance in target tissues.

In contrast, insulin resistance is an important contributor to diabetes mellitus and IGF resistance is also observed under various physiological and pathological conditions, such as malnutrition and inflammation (White 2002) (Table 2). There are accumulated reports

Table 1 Modulation of IGF signaling by other factors.

Factors	Target tissues and cells	Activities/actions
Growth factors PDGF, FGF, EGF	Fibroblasts	DNA synthesis
Tropic hormones ACTH	Adrenal	Corticosterone production
FSH	Ovary (granulosa cell) Ovary (theca-interstitial cell)	Aromatase activity and progesterone production Androgen production and proliferation
LH	Testis (leyding cell)	Androsterone and testosterone production
TSH	Thyroid	Proliferation
GH	Pre-adipocyte	Differentiation
Steroid hormones Estrogens	Cartilage Uterus Breast cancer cell	Proliferation Proliferation Proliferation
Androgens	Prostate	Proliferation
Glucocorticoid/cAMP	Pre-adipocyte	Differentiation
Erythropoietin Erythropoietin	Liver (embryo), bone marrow	Erythropoiesis
Extracellular matrix Vitronectin–integrin	Smooth muscle cell	Proliferation and cell migration

showing that various intercellular signaling molecules impair insulin signaling as well as IGF signaling, leading to insulin resistance or IGF resistance. However, the precise mechanisms of action of each factor are not yet clear. Elucidation of each mechanism is very important for the development of specific treatments.

In general, binding of IGF1 or insulin to its specific receptor on a target cell membrane is followed by activation of tyrosine kinase in the β -subunit of the cognate receptor (Fig. 1). The activated IGF1 receptor or insulin receptor in turn phosphorylates specific substrates, in particular insulin receptor substrate (IRS)1, IRS2 and Shc. Recently, it has become clear that phosphorylated tyrosine residues of IRS-1, IRS-2 and Shc are recognized by various signaling molecules that contain a Src homology 2 (SH2) domain. These include Grb2 and the 85kDa regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase), suggesting that the binding of tyrosine phosphorylated substrates to these SH2 domain-containing proteins mediates insulin-like

bioactivities. These associations are thought to stimulate the MAP kinase cascade or the PI 3-kinase cascade, well-known signaling pathways, which mediate many significant actions of growth factors (Jones & Clemmons 1995) (Fig. 1). Potentiation or repression of IGF signals occurs at certain steps in these signaling pathways (Hakuno *et al.* 2015).

Clearly, novel signaling pathways must exist to mediate specific biological activities. In addition, the researchers in this field like to know how the signaling of IGFs differ from insulin, because IGFs and insulin are shown to share common signaling pathways, but IGFs predominantly mediate long-term action to determine the cell fates and insulin mainly possesses metabolic activity.

IGF signaling molecules

IGF1 receptor

The existence of the IGF1 receptor in a variety of cells and tissues has been demonstrated by many researchers;

Table 2 Examples of insulin resistance and IGF resistance.

Insulin resistance	IGF resistance
Mutation of insulin receptor	Mutation of IGF1 receptor
Production of anti-insulin receptor antibody	Production of anti-IGF1 receptor antibody
Obesity	Malnutrition
Aging	Catabolic condition
Diabetes	Excess cytokine
Inflammation	Kidney failure
Excess growth hormone	
Excess glucocorticoid	
Oxidative stress	
Uremia

Figure 1

Commonly accepted intracellular signaling mechanisms of IGF/insulin. A detailed explanation is given in the text.

however, purification and characterization of this protein needed enormous efforts because of limited quantities of purified IGFs and difficulty in distinguishing IGF1 receptor from insulin receptor. In 1986, Ullrich *et al.* first published the cloning of IGF1 receptor cDNAs (Ullrich *et al.* 1986). This ground-breaking work followed determination of the partial amino acid sequence of purified IGF1 receptor using wheat germ agglutinin-sepharose and affinity column chromatography using anti-human IGF1 receptor monoclonal antibody, α IR3 (Van Wyk *et al.* 1985). This study showed that the structure of IGF1 receptor is highly homologous to insulin receptor. IGF1 receptor is a disulfide bond-conjugated tetramer, which consists of two α subunits that are extracellular proteins binding to specific ligands and two β subunits that are transmembrane proteins possessing tyrosine kinase activity (Steele-Perkins

Figure 2

Schematic illustration of IGF1 receptor/insulin receptor. A detailed explanation is given in the text.

et al. 1988, Werner *et al.* 1989). When IGF1 receptor and insulin receptor are produced in the same cells, some of them form a hybrid receptor comprised of an insulin receptor α - β -'hemireceptor' and an IGF1 receptor α - β -hemireceptor (Moxham *et al.* 1989, LeRoith *et al.* 1995) (Fig. 2).

IGF1 receptor is shown to possess a high affinity for IGF1 (IC_{50} : 0.2–0.8 nM) and IGF2 (IC_{50} : 0.5–4.4 nM), but it can also bind to insulin with 50- to 100-fold (IC_{50} : over 30 nM) lower affinity. On the other hand, insulin receptor B-type, which is an alternative splicing variant of insulin receptor RNA, binds to insulin with high affinity (IC_{50} : 0.5–1.6 nM) and binds to IGFs with low affinity (IC_{50} : over 30 nM for IGF1 and over 10 nM for IGF2). This difference plays important roles in mediation of distinct bioactivities of IGFs and insulin (Hintz *et al.* 1972, Marshall *et al.* 1974, Megyesi *et al.* 1974, Rechler *et al.* 1980, Bayne *et al.* 1989). On the other hand, another insulin receptor RNA splicing variant, insulin receptor A-type has a high affinity for not only insulin (IC_{50} : 0.2–0.9 nM) but also IGF2 (IC_{50} : around 2.5 nM but over 30 nM for IGF1), suggesting that alternative splicing of insulin receptor RNA causes changes in cellular responsiveness to insulin-like peptides. In addition, interestingly, a hybrid of IGF1 receptor and insulin receptor binds to IGFs with higher affinity than insulin (Belfiore *et al.* 2009).

Because insulin receptor and IGF1 receptor are highly homologous and the research on insulin receptor was initiated first, the studies on IGF1 receptor have followed those on insulin receptor. Binding of IGFs to the α subunit of IGF1 receptor leads to the conformational change in the β subunit, resulting in the activation of receptor tyrosine kinase activity (lysine 1003 is an ATP-binding site) (Hanks *et al.* 1988). The β subunit of the receptor possesses many tyrosine residues which are possibly phosphorylated. Tyrosine phosphorylation of the β subunit is shown to be caused by tyrosine kinase of another β subunit of the tetrameric IGF1 receptor, which we call autophosphorylation. Autophosphorylation further activates tyrosine kinase of the β subunit. When the tyrosine 950 residue of the NPXY motif in the juxta membrane domain is phosphorylated, docking proteins, such as IRSs which possess the phosphotyrosine binding (PTB) domain can recognize this motif and bind to the β subunit, and docking proteins are phosphorylated by the receptors (Craparo *et al.* 1995, Tartare-Deckert *et al.* 1995, Dey *et al.* 1996, Xu *et al.* 1999, Hu & Hu 2017). Phosphorylation of other tyrosine residues leads to different outcomes. For example, autophosphorylation of tyrosine 1131, 1135, 1136 and 1221 activates receptor kinase

followed by transformation and migration (Li *et al.* 1994, Hernandez-Sanchez *et al.* 1995, Stannard *et al.* 1995, Jiang *et al.* 1996, O'Connor *et al.* 1997) and phosphorylation of tyrosine 1250 and 1251 plays important roles in receptor internalization and degradation (Miura *et al.* 1995, Girnita *et al.* 2014). In addition, we found that prolonged association of PI 3-kinase with IGF1 receptor was induced by IGF1 stimulation. Furthermore, we demonstrated that Tyr1316-X-X-Met of IGF1 receptor functioned as a PI 3-kinase-binding sequence when this tyrosine is phosphorylated. Based on other results, we concluded that PI 3-kinase activity bound to IGF1 receptor, which is continuously sustained by IGF1 stimulation, is required for IGF1-induced cell proliferation (Fukushima *et al.* 2012).

Because IGFs predominantly mediate long-term action to determine cell fates and insulin mainly possesses metabolic activity, it is believed that the signaling of IGFs differs from insulin even though IGFs and insulin are shown to share common signaling pathways. Although structurally, the IGF1 receptor and the insulin receptor are highly homologous, homology of their C-terminal regions is relatively low (LeRoith *et al.* 1995). There are reports indicating that tumorigenesis is abolished by either a 108 or 44 amino acid C-terminal deletion of IGF1 receptor β subunit (Liu *et al.* 1993a, Prager *et al.* 1994). In addition, when the C-terminal 121 amino acids of the IGF1 receptor are replaced by the corresponding insulin receptor domain, such a chimeric IGF1 receptor increased IGF1-induced glycogen synthesis (Faria *et al.* 1994, Tartare *et al.* 1994). These results suggested that the IGF1 receptor and insulin receptor are not interchangeable, and that within the C-terminus, specific regions have importance in control of various functions (LeRoith *et al.* 1995). The C-terminal region of IGF1 receptor is shown to associate with various proteins such as Grb10, which may affect specificity (Dey *et al.* 1996, Laviola *et al.* 1997, Wang *et al.* 1999).

Multiple modifications of IGF1 receptor are also reported (Girnita *et al.* 2014). Two lysine residues in the IGF-IR activation loop (Lys-1138 and Lys-1141) contribute to the promotion of internalization and downregulation of the receptor (Mao *et al.* 2011). Various serine/threonine residues of IGF1 receptor are phosphorylated in IGF-dependent or -independent manner. Some of these phosphorylations are critical for binding of signaling molecules to IGF1 receptor. For example, phosphorylation of serine 1291 induces β -arrestin 1 binding to IGF1 receptor, resulting in degradation of the receptors as well as activation of the downstream pathway, such as the MAP kinase pathway (Girnita *et al.* 2007, Zheng *et al.*

2012). SUMOylation of IGF1 receptor is reported to cause nuclear transport of IGF1 receptor leading to tyrosine phosphorylation of histone (Sehat *et al.* 2010). Especially, C-terminal region of IGF1 receptor is shown to associate with various proteins such as Receptor for Activated C Kinase 1 (RACK1; (Hermanto *et al.* 2002, Kiely *et al.* 2002), Vav (Uddin *et al.* 1996), Focal Adhesion Kinase (FAK (Baron *et al.* 1998), Jun N-terminal Kinase (JNK; (O'Connor 2003), Tissue Inhibitor of Metalloproteinase 2 (TIMP2; (Fernandez *et al.* 2010), Janus Kinase 1/2 (JAK1/2; (Gual *et al.* 1998) and Suppressor Of Cytokine Signaling 1/2/3 (SOCS1/2/3; (Dey *et al.* 1998, 2000), which may mediate or modulate IGF1 receptor signaling (Girnita *et al.* 2014).

However, we have to emphasize that the differences between IGF and insulin activities must first reflect expression of each receptor and/or hybrid receptor in each cell and tissue. In general, during prenatal development, IGF1 receptor is expressed at high level but after birth, expression level of IGF1 receptor is low. In liver, muscle and adipose tissues, high expression of insulin receptor is observed instead. IGFs produced from liver and adipose tissues function in an endocrine manner and from other tissues work in a paracrine/autocrine manner (LeRoith *et al.* 1995). On the other hand, secretion of insulin is transient after feeding but IGF secretion is constant, which may explain the observations that IGFs predominantly mediate long-term action to determine the cell fates and insulin mainly possesses metabolic activity.

Docking proteins: receptor tyrosine kinase substrates

Insulin receptor substrates (IRSs)

In vertebrates, three to four types of genes encoding structurally related IRS proteins (IRS1, -2, -3 and -4) have been found in many species (White *et al.* 1985, Sun *et al.* 1991, 1995, Lavan *et al.* 1997a,b, White 2006). Human genome harbors IRS-1, -2 and -4 genes, but it apparently lost the IRS3 gene during its evolutionary process though the mouse genome contains all four *Irs* genes (Bjornholm *et al.* 2002). Common structural features of the IRS proteins are the pleckstrin homology (PH) and PTB domains in the amino-terminal region. Since IRS lacks any enzymatic activity in their primary sequence, they are regarded as docking proteins. The IRSs interact with tyrosine phosphorylated IGF1 receptor or insulin receptor through the PTB domains. IRSs possess multiple tyrosine residues, which are subsequently phosphorylated by the receptor tyrosine kinases (White 1998). IRS-2 has a unique structural feature called the

KRLB region that also interacts with the tyrosine kinase domain of insulin receptor (Sawka-Verhelle *et al.* 1997). The tyrosine phosphorylated IRS recruits major insulin-like signaling molecules to the membrane through its PH domain, which leads to the activation of a Ras-MAPK signaling pathway or a PI 3-kinase pathway as described later (Fig. 3).

For IGF/insulin-dependent activation of PI 3-kinase pathway, several specific tyrosine residues in the binding motif of SH2 domains of class-I, a p85 regulatory subunit of PI 3-kinase (YXXM) are typically important (Copps & White 2012). The phosphotyrosine residues at 608, 628 and 658 in IRS-1, and at 649, 671, 734 and 814 in IRS-2, were shown to be involved in a p85 regulatory subunit of PI 3-kinase. The tyrosine 891 in IRS-1 is known to be the Grb2-binding site phosphorylated by IGF/insulin-stimuli, which is important for Ras-MAPK cascade activation (Copps & White 2012). In addition, the serine/threonine phosphorylation and the posttranslational modifications of lysine residues in IRS also play major roles for fine-tuning the insulin-like signaling. mTORC1 or S6 kinase phosphorylates serine/threonine residues of IRS, which in turn leads to the degradation of IRS protein and attenuation of signals through IRSs. The S6 kinase-dependent negative regulation of IRS is involved in insulin insensitivity in obesity. In contrast, a recent study shows IRS-1 serine 307 promotes insulin sensitivity. The serine/threonine phosphorylation of IRSs could be complex but an important clue for deciphering the pathology of insulin/IGF signal. The ubiquitination on lysine residues in IRS causes degradation of the IRSs. Also, the IRS-2 acetylation on its lysine residue decreases the IGF-induced MAPK signaling in neurons that protects neurons from

oxidative damage-induced cell death. Therefore, the IRS-associating molecules that regulate these posttranslational modifications have recently gained increasing attention (Copps & White 2012).

Recently, we and others suggested IRS-1 and IRS-2 are functionally different. Actually, IRS-1 knockdown or overexpression did not affect the IGF signals but IRS-2 knockdown impaired the IGF1 signals and IGF bioactivities (Fukushima *et al.* 2011b, 2015, Hakuno *et al.* 2011). As described before, IRS-1 and IRS-2 shared the binding partners as docking proteins. However, we have shown that IRS-1 and IRS-2 interact with many proteins without hormone stimulation in a phosphotyrosine-independent manner (Fukushima *et al.* 2011a, Hakuno *et al.* 2015). Isolation of IRS-associated proteins revealed that IRS-1- and IRS-2-associated protein complexes contained quite different proteins (Hakuno *et al.* 2007, Fukushima *et al.* 2011a,b, 2015, 2017, Yoshihara *et al.* 2012, Yoneyama *et al.* 2013, Ando *et al.* 2015, Liu *et al.* 2016). IRS-1 and IRS-2 have a unique sequence in the C-terminal domain except for the putative tyrosine residues, which are phosphorylated by active receptor. Therefore, IRS-associated proteins interact with IRSs through such non-homologous regions. It is possible that functional differences are due to the different partners in a phosphotyrosine-independent manner. Nedd4 was isolated as an IRS-2-associated protein and hardly interacts with IRS-1. In addition, the effect of Nedd4 overexpression is specific on IRS-2 tyrosine phosphorylation (Fukushima *et al.* 2015, 2017). On the other hand, DGK ζ was isolated as an IRS-1-binding protein. The interaction was dissociated by insulin stimulation, but the interaction of DGK ζ with IRS-2 was not observed (Liu *et al.* 2016). These observations might be clues to evaluate the functional differences between IRS-1 and IRS-2.

Docking proteins

Adaptor proteins

Figure 3

Schematic illustration of docking proteins and adaptor proteins of IGF/insulin signaling pathways. A detailed explanation is given in the text.

Other substrates including Shc

Shc has been shown to have three protein isoforms, with molecular masses 46, 52 and 66kDa respectively (Pelicci *et al.* 1992, Pronk *et al.* 1993, Sasaoka *et al.* 1994). These isoforms are synthesized by using different translation starting sites from two mRNAs produced by alternative splicing, one of which encodes 46kDa and 52kDa Shc, and the other of which encodes 46, 52 and 66kDa Shc. Shc possesses the PTB domain and IGFs stimulated phosphorylation of tyrosine 317 (in the CH1 domain) of 46, 52 and 66kDa Shc. The expression and tyrosine phosphorylation of these three Shc isoforms in response to growth factors has been reported to be

regulated differently in various cell types. It has been reported that unlike the other two isoforms, 66 kDa Shc has a collagen-homology 2 domain (CH2 domain). Studies on the physiological roles of the CH2 domain are clearly needed in the light of our findings and the intriguing findings of Pellich *et al.* that the CH2 domain is a negative regulator of the c-fos promoter (Pellicci *et al.* 1992, Migliaccio *et al.* 1997).

There are reports showing that Gab1, Crks and STAT function as substrates of IGF1 receptor even though these proteins do not possess the PTB domain.

Adaptor proteins: signaling molecules containing SH2 domain

Tyrosine phosphorylated substrates (pIRS-1, pIRS-2, pShc and pGab1) were recognized by some SH-2 domain-containing molecules, including Grb2, a p85 regulatory subunit of PI 3-kinase and SHP2/Syp. Amino acid motif sequences, which form the preferred binding site to each SH-2 domain-containing protein were determined (Backer *et al.* 1992, Shoelson 1997) (Fig. 3).

GRB2-SOS

Growth factor receptor-bound 2 (GRB2) was identified as a binding protein of the activated growth factor receptor (e.g. EGF receptor, HER-2). GRB2 consists of a Src homology 2 (SH2) domain flanked by N- and C-terminal SH3 domains (Giubellino *et al.* 2008). GRB2 binds through its SH2 domain to phosphorylated tyrosines and their surrounding sequences (i.e. pTyr-x-N-x) in the IGF-IR substrate proteins (IRS-1, IRS-2 and Shc). On the other hand, GRB2 binds through its SH3 domain to a proline-rich sequence in Son of Sevenless (SOS; GEF, Guanine nucleotide exchange factor for Ras). Since blocking the interaction between GRB2 and the growth factor receptor by phosphopeptides or mimetics, inhibits activation of Ras and the downstream MEK pathway (Burke 2006), it is believed that Ras was activated when GRB2-SOS complex was recognized by phosphorylated IGF-IR substrates. However, the data showing that knockdown of GRB2 suppressed IGF-induced Ras-MAPK pathway were not observed. Thus, it is not established whether binding of GRB2 with phosphorylated IGF1 receptor substrates is required for IGF1-induced Ras-MAPK pathway activation. Recently, some researchers argued that GRB2 binds through its C-SH3 domain to a proline-rich sequence at the extreme C-terminal tail of dimerized fibroblast growth factor receptor 2 (FGFR2). This intracellular GRB2-mediated receptor dimerization results in basal

transphosphorylation of the kinase domain of FGFR2 (Lin *et al.* 2012).

PI 3-kinase

PI 3-kinase is composed of a p85 regulatory subunit and a p110 catalytic subunit. The p85 regulatory subunit of PI 3-kinase binds to the phosphorylated pYxxM motif via their SH2 domains, resulting in PI 3-kinase membrane recruitment and activation of the p110 catalytic subunit. Activated PI 3-kinase generates the second messenger phosphatidylinositol 3,4,5 triphosphate (PI3,4,5P₃) by phosphorylating phosphatidylinositol 4,5 diphosphates (PI4,5P₂). The interaction between p85 and pYxxM motifs might be required for the activation of p110 PI 3-kinase catalytic domain since tyrosine-phosphorylated peptides themselves possess the ability to activate PI 3-kinase.

Other proteins including SHP-2/Syp2

SH2 domain-containing protein tyrosine phosphatase (SHP)-2/Syp2 was known to interact with tyrosine-phosphorylated IGF-IR substrates. Recently, some reported that the interaction between IRSs and SHP-2 was important for the cross-talk between IGF1 signals and the integrin pathway (Maile & Clemmons 2002, Ling *et al.* 2003, 2005).

Downstream signaling molecules

Components of a MAP kinase pathway

The interaction between GRB2 and IGF1 receptor substrates leads to the activation of GRB2-associated SOS guanine nucleotide exchange activity, resulting in the activation of Ras small GTPase. Activated Ras interacts with and activates Raf/MAPKKK/MEKK, which in turn phosphorylates and activates MAPKK/MEK, then activates MAPK. This kinase cascade is called a Ras-MAPK pathway. Activated MAPK phosphorylates transcriptional activators, resulting in induction of various IGF bioactivities. Necessity of the MAPK pathway activation for various IGF1 signals has been demonstrated by adding the MAPKK/MEK-specific inhibitor, PD98059 or U0126 as discussed later. From these experiments, MAPK activation is shown to be required for the IGF-induced DNA synthesis and cell survival in various cell types.

Components of a PI 3-kinase pathway

The interaction between p85 and IGF-IR substrates leads to the activation of PI 3-kinase. Activated PI 3-kinase phosphorylates PIP₂ to produce PIP₃. PIP₃ in turn activates

the PDK1. PDK1 phosphorylates threonine 308 residue of Akt kinase and mTORC2 phosphorylates serine 473 residue of Akt kinase, resulting in full activation of Akt/PKB kinase. Activated Akt phosphorylates several Akt substrates including AS160, Bad, forkhead box-containing protein, O sub-family (FoxO1), Tsc1, glycogen synthase kinase 3 β (GSK3 β), etc. Since an antibody against the phosphorylated Akt motifs was developed, many reports identified novel Akt substrates (Brazil & Hemmings 2001). For example, FoxO family proteins, transcriptional activators were phosphorylated by active Akt and excluded from the nuclei (Brunet *et al.* 1999, Alvarez *et al.* 2001, Brownawell *et al.* 2001). Thus, FoxO family proteins were inactivated by IGF1 stimulation. GSK3 β was also phosphorylated and inactivated by active Akt kinase. GSK3 β continuously targets cyclin D1 to the proteasome. GSK3 β is phosphorylated by Akt, which leads to a rise in cyclin D1 (Vivanco & Sawyers 2002). Pro-apoptotic activity of Bad was regulated by Akt, and this regulation mediates the IGF1-induced cell survival activity (Datta *et al.* 1997, del Peso *et al.* 1997). Akt inhibits the tuberous sclerosis complex, which is composed of Tsc1 and Tsc2 and its non-phosphorylated form is a GTPase-activating protein (GAP) for Rheb small G protein (Inoki *et al.* 2002, Dibble *et al.* 2012). Since Rheb activation leads to mTORC1 activity, Akt activation is required for the mTORC1 activation. AS160 was phosphorylated by active Akt and its GAP activity was inactivated, resulting in activation of Rab10. Rab10 activation leads to GLUT4 translocation to plasma membrane (Miinea *et al.* 2005, Sano *et al.* 2007, Yuasa *et al.* 2009). By phosphorylating various Akt substrates, Akt regulates substrate activity or localization, resulting in the regulation of various IGF bioactivities (Brazil & Hemmings 2001).

Necessity of the PI 3-kinase pathway activation was demonstrated by adding PI 3-kinase inhibitor, LY294002 or Wortmannin (Wymann *et al.* 2003, Simon 2006). From these experiments, PI 3-kinase activation is shown to be required for the IGF-induced DNA synthesis, cell survival and cell differentiation in various cell types. Specific inhibitors against Akt, GSK3 β and mTORC1 have been already developed. Requirement or sufficiency of Akt substrate phosphorylation can be also elucidated by just adding these inhibitors.

Components of other pathways including PLC pathway

It is known that inositide-specific phospholipase C (PI-PLC) β 1 is activated by IGF1 stimulation. The conventional view of PI-PLC β 1 activation occurred at the plasma membrane,

and it has been suggested that G α q/ α 11 and G β γ subunits can activate PI-PLC β 1 (Rhee 2001). However, recently PI-PLC β isoforms were activated in the nuclei in a MAPK-dependent manner. PI-PLC β 1 activation in the nuclei is important for the cell proliferation and differentiation (Faenza *et al.* 2005, Manzoli *et al.* 2005). U73122, an inhibitor of PLC, is utilized to evaluate the contribution of PLC pathway.

Small G protein, Rac1, Rho1 and CDC42 protein had also been reported to be activated by IGF stimulation. This activation is important for the IGF1-induced actin remodeling as shown by the experiments adding the Rho family protein-specific inhibitor, Toxin B. However, the detailed mechanisms by which Rho family small G protein was activated remain unknown.

Evidences of significance of each signaling molecule in IGF signaling

As described earlier, IGF stimulation activates several known downstream pathways including the PI 3-kinase pathway, MAPK pathway and PLC pathway. However, it is not necessarily true that the activated signaling pathways are required for induction of various bioactivities of IGFs. To evaluate requirement or sufficiency of each pathway for IGF bioactivities, specific inhibitors for enzymes on each pathway are often utilized as good tools. Recently genetic knockdown using siRNA techniques or genetic knockout by a conventional methods or the CRISPR-Cas9 system have become strong methods to evaluate the necessity for the pathway activation. In this section, we will introduce the necessity for pathways or molecules for the IGF1 bioactivities using some techniques or tools in cell model systems, *in vivo* mice or human clinical model.

Cultured cell models

Necessity of signaling pathways

In many types of IGF target cells, both PI 3-kinase pathway and MAPK pathway are activated by IGF stimulation. Until 1990s, only inhibitors of each pathway were used to elucidate the requirement for IGF bioactivities because siRNA techniques and CRISPR-Cas9 system were not yet established. LY294002 and Wortmannin are inhibitors of PI 3-kinase by which serine/threonine kinase Akt activation is blocked and these inhibitors are used for the inhibitor of a PI 3-kinase pathway. On the other hand, PD98059 or U0126 is an inhibitor of MAPKK (MEK) by which Erk activation was blocked and these inhibitors are used for the inhibitor of a MAPK pathway. Combinational

usage of these inhibitors demonstrated the necessity of two pathways in various IGF bioactivities in various cells.

In a thyroid follicular cell line, FRTL-5, IGF1 stimulates cell growth, and this was synergistically enhanced by thyrotropin (TSH) pretreatment. IGF1-dependent cell proliferation was impaired just by blocking one pathway, PI 3-kinase pathway or MAPK pathway, indicating that activation of two pathways is required for the IGF1-induced cell proliferation (Ariga *et al.* 2000, Nedachi *et al.* 2000, Fukushima *et al.* 2008). In neuroblastoma cells, SH-SY5Y cells, IGF1 stimulation induced cell proliferation and neuronal differentiation, which was evaluated by neurite outgrowth. Addition of both LY294002 and PD98059 partially blocked IGF1-induced cell growth and neurite outgrowth, suggesting that both pathways equally contributed to mediate the IGF1 bioactivities (Kurihara *et al.* 2000). It is established that IGF1 is required for the myogenic differentiation using L6 or C2C12 myoblast cells. By adding LY294002, myogenic differentiation was completely blocked, but adding PD98059 enhanced myogenic differentiation. This observation indicated that IGF1 signaling pathway activation was tightly regulated in the same cells during the myogenic differentiation.

Necessity of signaling molecules

IGF signaling consists of many IGF signaling proteins such as receptors, receptor substrates, SH2-domain-containing molecules, PI 3-kinase, Akt, Akt substrates, Ras and MAP kinase. Most of these proteins and enzymes except for the receptors and receptor substrates are shared by many other hormone signals. Therefore, to elucidate the necessity of signaling molecules in the IGF signaling activation or IGF bioactivities, it is a good strategy to elucidate the function of receptors and receptor substrates. Recently, a receptor inhibitor was developed in some agencies, and it is demonstrated that the IGF receptor tyrosine kinase activities are required for almost all of the IGF-induced bioactivities. However, necessity of the receptor substrates, in particular, IRS was not well studied because the concept of IRS function had been already established when siRNA techniques had not yet been developed. To evaluate the IRS function in IGF signal activation or the IGF bioactivities, we need to deplete the signaling molecules by siRNA knockdown or CRISPR-Cas9 knockout. Recently, we have reported that IRS-1 knockdown neither affected insulin-induced Akt activation nor insulin-induced glucose uptake in 3T3-L1 adipocytes (Sasaki-Suzuki *et al.* 2009). In addition,

IRS-1 overexpression in L6 myoblast cells did neither enhance the IGF1-induced Akt activation nor IGF1-induced DNA synthesis (Hakuno *et al.* 2011). These data strongly suggested that IRS-1 is not required for the IGF1 signal activation. On the other hand, IRS-2 could be a mediator of insulin/IGF1 signal. In FRTL-5 cells, IRS-2 knockdown suppressed IGF1-induced Erk activation and IGF1-induced DNA synthesis (Fukushima *et al.* 2008, 2011b, 2012, 2015). In 3T3-L1 adipocytes, IRS-2 knockdown suppressed insulin-induced Akt activation and insulin-induced glucose uptake (Sasaki-Suzuki *et al.* 2009). Whether each IGF signaling molecule is really required for the IGF1 bioactivities should be examined using siRNA and CRISPR-Cas9 systems.

Animal models

Methods to evaluate the necessity of IGF signaling for IGF bioactivities in mice are phenotypic analysis of established knockout mice or administration of specific inhibitors. However, administration of inhibitor is expected to have side effects, and we cannot rule out the possibility that an inhibitor may affect different sites *in vivo*. It is not the best way to study the signaling molecules in the mouse model.

IGF1 receptor

IGF1 receptor-knockout mice were born weighing only 45% of normal mice. Further, these mice died soon after birth from respiratory failure, caused by impaired development of the diaphragm and intercostal muscles, and they have multiple abnormalities, including morphological deviation of the central nervous system, muscular hypoplasia, delayed ossification, thin epidermis with decreased cell numbers and profoundly affected embryonic bone development (Liu *et al.* 1993b). In addition, heterozygous IGF1 receptor-knockout mice displayed low birth weight and postnatal growth deficits, which are consistent with the phenotype of short stature born with small for gestational age (SGA), indicating that IGF1 receptor is critical for the prenatal and postnatal development and growth in mice.

IRSs

Mice with targeted disruption of IRS-1 had a 50% reduction in intrauterine growth and impaired glucose tolerance. In addition, heterozygous IRS-1-knockout mice showed 30% reduction in growth and no difference in insulin sensitivity, suggesting that IRS-1 has more

function in IGF1 signaling than the insulin signaling. On the other hand, IRS-2-knockout mice showed progressive deterioration of glucose homeostasis because of insulin resistance in the liver and skeletal muscle and a lack of β -cell compensation for this insulin resistance. Thus, ablation of IRS-2 impairs both peripheral insulin signaling and pancreatic β -cell function. IRS-2-knockout mice did not exhibit retarded growth, suggesting that functional difference in IRS-1 and IRS-2 in mice (Araki *et al.* 1994, Withers *et al.* 1998, 1999).

The physiological roles of IRSs are not limited to the glucose metabolism and growth (Yamauchi *et al.* 1996, Abe *et al.* 1998, Kulkarni *et al.* 1999, Ogata *et al.* 2000, Miki *et al.* 2001, Akune *et al.* 2002). For instance, *Irs1* deficiency induced higher blood pressure and plasma triglyceride levels and lower vascular relaxation and lipoprotein lipase activity suggesting the importance of IRS-1 in maintenance of vascular health. IRS-1 and IRS-2 governed osteoblast behavior to coordinate bone turnover and metabolism. IRS-1 and IRS-2 also regulated the adipocyte differentiation (Yamauchi *et al.* 1996, Miki *et al.* 2001). Protein malnutrition enhanced hepatic insulin sensitivity accompanying with increase in IRS-2 protein level (Toyoshima *et al.* 2014). It is also noteworthy that loss of *Irs* gene in both rodent and fly models result in extended lifespan (Clancy *et al.* 2001, Tatar *et al.* 2001, Tu *et al.* 2002, Kurosu *et al.* 2005). Another report suggests a link between cancer risk and IRS-4 expression level. Moreover, IRS-3 was found to act as a transcriptional regulator to protect the cell from TNF α -induced cell death, which is totally distinct from the canonical IRS function (Kabuta *et al.* 2002, 2008, 2010). Intriguingly, it has been recently reported that IRS-1 interacts with RNA molecules (Ozoe *et al.* 2013, 2014). These facts underscore the significance of IRS in various pathophysiological contexts, and further research would open a new avenue for the better understanding of the roles of this multifunctional protein.

Shc

Although it was shown that Shc proteins are possible candidate substrates of IGF1 receptor/insulin receptor, p66Shc is reported to function as a redox enzyme when Ser36 in the CH2 domain is phosphorylated in response to insulin, H₂O₂ or other stress signals. Recent studies on p66Shc-knockout mice have indicated that this gene is a crucial regulator of reactive oxygen species (ROS) levels and is involved in age-related dysfunction (Berry & Cirulli 2013).

Patients who have mutation in the gene of a signaling molecule

As a result of the mouse model analyses, it is established that IGF signal molecules have important roles in normal fetal and postnatal growth and development. Therefore, it is assumed that human patients who show growth retardation including intrauterine growth retardation (IUGR) have some mutations in the IGF signaling molecules. Actually, mutations in IGF1 have been implicated in intrauterine and postnatal growth retardation (Woods *et al.* 1996). It was also reported that an IGF2 mutation was found in a multigenerational family with four members who have growth restriction (Begemann *et al.* 2015). Moreover, heterozygous IGF-IR mutations causing intrauterine and postnatal growth retardation were observed in some families (Abuzzahab *et al.* 2003, Kawashima *et al.* 2005). However, IRS mutations have not yet been found in the patients who showed growth retardation.

Modulation of IGF signaling

Amplification of IGF signaling

The *in vitro* biological effects of IGFs are potentiated in the presence of other hormones or growth factors (Table 1), and these phenomena are also observed *in vivo*. In the beginning stages of studies on the biological activities of IGFs using fibroblasts, platelet-derived growth factor (PDGF), epidermal growth factor (EGF) or fibroblast growth factor (FGF) were shown to prime the cells to IGFs for cell proliferation (Stiles *et al.* 1979, Qureshi *et al.* 1997). These growth factors induce activation of protein kinase (PKC) or expression of proto-oncogenes, such as *c-fos* and *c-jun*, preparing the cell to be ready for cell cycle progression in response to IGFs. Some reports proposed the restriction points from the G1 phase to S phase regarding responsiveness to IGFs. Recently, these signal interactions play important roles to regulate chondrocyte growth. In endocrine cells, effects of IGFs on growth or hormone production are augmented by tropic hormones, which activate the cAMP-dependent pathways (Adashi *et al.* 1985, Tramontano *et al.* 1988, Meroni *et al.* 2004). Chronic cAMP stimulus is shown to potentiate IGF-dependent cell growth through amplification of IGF signaling as explained below in detail. In contrast, chronic IGF stimulus increases Gs protein levels, resulting in elevations of cAMP concentrations and upregulation of cAMP-dependent phenomenon in granulosa cells stimulated with FSH (Adashi *et al.* 1985, 1988a,b). These

interactions between trophic hormones and IGFs are believed to be essential for normal growth and function of endocrine cells. Interaction of steroid hormones and IGFs are reported in mammary gland, uterus and prostates (Stewart *et al.* 1990, Itagane *et al.* 1991). Especially, effects of IGFs on cell growth of breast cancer and prostate cancer are potentiated by estrogen and androgen. These steroid hormones increase IGF production as well as enhance IGF signals. Recently interactions of estrogens and IGF in the brain may function for neuroprotection (Chowen *et al.* 1992, Pons & Torres-Aleman 1993, Duenas *et al.* 1994). On the other hand, interaction between cAMP signals through adrenaline and glucocorticoid stimulated by fasting or stress and IGF signals is important for induction of adipogenesis. cAMP and glucocorticoid signals actually need to decrease IGF signals, which is essential for differentiation (Hasegawa, personal communication). We have obtained similar signal changes for myogenesis (Hakuno *et al.* 2011). It is shown that cell migration of skeletal muscle cells is induced by IGFs and integrin signals triggered by extracellular matrix. In this case, interaction of IGF1 receptor with integrin receptor complex may possess some roles. As we explained earlier, the mechanism of IGF potentiation by other factors is very important in order that IGF induce specific bioactivities in the right tissues at the right times. Accordingly, to elucidate how IGF action is potentiated by other intercellular signaling molecules is essential for revealing IGF significance in target tissues.

We have shown that in FRTL-5, a thyroid follicular cell line, TSH and IGF1 stimulate cell growth synergistically and TSH pretreatment through activation of cAMP signaling pathway is essential for the potentiation of IGF1-dependent DNA synthesis (Takahashi *et al.* 1990, 1991). We are utilizing this model to elucidate the mechanisms by which various factors potentiate IGF bioactivities (Fig. 4). In FRTL-5 cells, cAMP pretreatment caused an increase in tyrosine kinase activity and tyrosine phosphorylation of intracellular proteins such as a 125-kDa protein (p125), which was well correlated with a cAMP-priming effect on potentiation of DNA synthesis induced by IGF1 (Nedachi *et al.* 2000). We found that the phosphotyrosyl p125 bound to a PI 3-kinase p85 regulatory subunit, and a PI-3 kinase inhibitor blocked the cAMP-priming effect. Recently, we succeeded in identifying p125 (we named it PI3KAP/XB130), and it is a novel protein possessing two PH domains and a p85 PI 3-kinase recognition motif containing tyrosine residues that may be phosphorylated (Yamanaka *et al.* 2012). On the other hand, we demonstrated that cAMP pretreatment potentiated IRS-2 tyrosine phosphorylation

Figure 4

Working model showing how trophic hormone enhances IGF-dependent proliferation. A detailed explanation is given in the text.

induced by IGF1, although pretreatment with cAMP did not affect autophosphorylation of the IGF1 receptor, suggesting that cAMP stimulus amplifies the IGF1 signals through cAMP-dependent potentiation of tyrosine phosphorylation of IGF1 receptor substrates (Ariga *et al.* 2000). In addition, we found that availability of IRS-2 to the IGF1 receptor has been increased via serine/threonine phosphorylation followed by association of other proteins with the complex. Recently, our study demonstrated that TSH induces association between IRS-2 and Nedd4, one of the E3 ubiquitin ligases and IRS-2 monoubiquitination in cultured thyroid FRTL-5 cells, thereby enhancing IGF signaling and mitogenic activity (Fukushima *et al.* 2015). We demonstrated that Nedd4-dependent IRS-2 monoubiquitination induces the interaction of IRS-2 with an ubiquitin-binding protein Epsin1. Epsin1 knockdown decreased IGF1-induced IRS-2 tyrosine phosphorylation and cell proliferation, indicating a role as a positive regulator of IRS-2-mediated signaling (Fukushima *et al.* 2015). Epsin1 is known to be localized to the plasma membrane and to promote the formation of clathrin-coated invaginations. Monoubiquitinated IRS-2 by Nedd4 is shown to be recruited to the plasma membrane through the association with Epsin1, where IGF1 receptor is co-localized and effectively phosphorylates IRS-2. In addition, USP15, a deubiquitinating enzyme, which is bound to IRS-2, possibly decreases IRS-2 monoubiquitination and counteracts Nedd4 (Fukushima *et al.* 2017). Thus, it is also important to identify and characterize the counteracting deubiquitinating enzyme. HSP90 may play some roles to keep serine/threonine phosphorylation of IRS-2 resulting in stabilizing formation of signaling complex including

IRS-2 (Fukushima *et al.* 2011a). Lastly, we are elucidating how cAMP and/or IGF1 stimuli regulate the G1 cyclins, CDKs-inhibitors system and determining the roles of PI 3-kinase activation by cAMP or IGF1 stimulus in this system. We found that cAMP pretreatment enhanced IGF1-dependent increases in cyclin D1, due to synergistic increases in mRNA and elevation of translation rates (Fukushima *et al.* 2008). Furthermore, cAMP pretreatment enhanced protein degradation of CDK inhibitor, p27^{Kip1} induced by IGF1. These changes induced an increase in cyclin E leading to marked activation of G1 cyclin-dependent kinases followed by Rb phosphorylation. Our results using a PI 3-kinase inhibitor showed that cAMP-dependent PI 3-kinase activation plays an important role in an increase in cyclin D1 translation through PI3KAP. In contrast, IGF1-dependent PI 3-kinase activation was required for synergistic increase in cyclin D1 mRNA levels and degradation of p27^{Kip1}. Furthermore, we found that PI 3-kinase bound to IGF1 receptor in response to IGF1 stimulus was important for stabilization of cyclin D1. Taken together, we conclude that PI 3-kinase controlled by cAMP or IGF1 stimulus plays different roles for synergistic changes in cyclin D1 and p27^{Kip1}, leading to cAMP-dependent potentiation of CDK activation and DNA synthesis induced by IGF1 (Fukushima *et al.* 2008). This 'cross-talk' mechanism may mediate the synergistic effects of tropic hormones and IGF in endocrine cells.

We also investigated roles of Nedd4-IRS-2 association in IGF action in cancer cells; Nedd4 is required for the maximal IRS-2 tyrosine phosphorylation and cell proliferation induced by IGF-1 in prostate cancer PC-3 cells. Nedd4 is known to be overexpressed in several cancer types including prostate cancer and to function as an oncogenic protein. Thus, we speculate that Nedd4 may promote cancer development partly through IRS-2 monoubiquitination. We also revealed roles of Nedd4-IRS-2 association *in vivo*, using zebrafish embryos. Nedd4 overexpression increased body length of the embryos, and IRS-2 knockdown suppressed this effect, suggesting that Nedd4 accelerates zebrafish embryonic growth through IRS-2 (Fukushima *et al.* 2015).

Taking these results together, we showed the significance of the IRSome in IGF action in normal/cancer cells as well as in somatic growth (Fukushima *et al.* 2011a, Hakuno *et al.* 2015).

IGF signal resistance

In contrast, IGF resistance is also observed under various physiological and pathological conditions, such as

malnutrition and inflammation (Jain *et al.* 1998) (Table 2). When IGF resistance is observed, anabolic effects of IGFs should be impaired since nutrients are not sufficient, nutrients or energy should be utilized to survive or excess IGF signals are transmitted. From this point of view, IGF resistance may function as a safety valve to inhibit anabolic activities to sustain life. However, the precise mechanisms of action of each factor are not yet clear. Elucidation of each mechanism is very important for the development of specific treatments.

Insulin resistance is defined as the phenomenon that insulin signaling is impaired even though insulin is present. Therefore, it is necessary to measure the IGF signals to demonstrate occurrence of the IGF resistance in the animal models. However, it is not easy to measure the IGF signal activation in an animal model because IGF signals are not synchronized due to the stable secretion of plasma IGF1. On the other hand, IGF signal activation was easily measured in the cell model. Therefore, the mechanism of IGF resistance was well studied in some cell models.

Cross-talk of IGF signals with amino acid signals

Recently, we have reported that protein malnutrition during the growth phase inhibited animal growth accompanied by reduced circulating IGF1 following reduced hepatic IGF1 synthesis. These animals developed marked accumulation of hepatic triglycerides (Toyoshima *et al.* 2010, 2014). Such a phenotype is typically observed in kwashiorkor, a form of severe protein malnutrition in infants and children. Detailed analysis in this animal model revealed that insulin sensitivity was enhanced in liver and muscle accompanied by a decrease in plasma insulin level (Toyoshima *et al.* 2010, 2014, Ozaki *et al.* 2014). In contrast, IGF1 signaling should be suppressed because growth was retarded in these animals. IGF1 injection into these animal models failed to restore normal growth, indicating that IGF1 resistance occurred. As a mechanism of IGF1 resistance, we could speculate two possibilities. One is that active plasma IGF1 level was not increased even though IGF1 was injected. The other possibility is that IGF1 signal was impaired by the unknown mechanisms. Since IGF1 signal activation is difficult to measure *in vivo*, no one knows whether IGF1 stimulated signaling was impaired or not. However, it is easily speculated that there was the involvement of an mTORC1 activation mechanism in this IGF1 resistance. As described before, mTORC1 is activated

by IGF1 stimulation through PI 3-kinase-Akt-Tsc1/2-Rheb pathway. Amino acids promote the interaction of mTORC1 with the Rag GTPases and localize to the lysosomal surface where Rheb directly stimulates the kinase activity of mTORC1. The Rheb and Rag GTPases form two arms of a co-incident detector that ensures that mTORC1 activation occurs only when nutrients and growth factors are both present (Martin & Hall 2005). In these protein malnutrition animal models, it is possible that mTORC1 was not fully activated due to the lack of nutrition signals through Rag GTPase activation.

Negative feedback loop of IGF signals through mTORC1 pathway

It is well known that growth factor signaling is desensitized by prolonged stimulation. Insulin and IGF1 signals were also downregulated by chronic stimulation through a negative feedback loop. For example, chronic insulin stimulation impaired the insulin-induced signal activation and glucose uptake in 3T3-L1 adipocytes. Thus, hyperinsulinemia possibly caused peripheral insulin resistance. On the other hand, because of constant secretion of plasma IGF1, the activity of IGF1 signals might be always downregulated. Because IGF1 signaling oscillates but is not synchronized in each cell, it is difficult to measure the IGF1 signal activity in an animal body. But, in the cell systems, IGF1 signal desensitization could be observed. Chronic IGF1 stimulation induced both the degradation of IRS proteins and internalization of IGF1 receptor into the lysosome, thereby Akt activation was downregulated. Since addition of PI 3-kinase inhibitor or mTORC1 inhibitor significantly suppressed chronic IGF1-induced IRS-1 degradation, indicating that IGF1 signaling was desensitized by negative feedback loop through mTORC1 activation (Haruta *et al.* 2000, Lee *et al.* 2000, Berg *et al.* 2002, Hartley & Cooper 2002, Hakuno *et al.* 2011, Kim *et al.* 2012). Taken together with our previous data that, in L6 myoblast cells, IRS-1 overexpression sustained the signal activation for longer time without affecting the intensity of Akt activation (Hakuno *et al.* 2011), the degradation of IRS-1 by prolonged IGF1 stimulation might be a key event for the IGF1 signal downregulation.

In some cancer cells, enhancement of PI 3-kinase-Akt-mTOR pathway was observed. Thus, inhibitors of mTOR activity have shown preclinical potential as drugs for cancer treatment including myeloma. mTOR inhibition leads to suppression of cell cycle proteins, resulting in

G2 arrest and prevention of cell proliferation. However, inhibition of mTOR also suppressed the negative feedback loop, thereby increasing the IRS-1 protein and enhancing PI 3-kinase pathway activation (Pene *et al.* 2002, Shi *et al.* 2005, Frost *et al.* 2007, Hu & Hu 2017). This may be a problem because enhancement of IGF1-induced activation of PI 3-kinase is an important anti-apoptotic cascade.

As described before, IGF1 mediates long-term action to determine the cell fates, including cell proliferation and differentiation, and we have reported that IGF1-induced DNA synthesis requires sustained IGF1 signals (Fukushima *et al.* 2008, 2011b, 2012). It is reasonable that IGF1 bioactivity, which mediates long-term action requires the sustained activation. To express such long-term action, the negative feedback loop of IGF1 signals should be tightly regulated. For example, IRS-1 overexpression that sustained IGF1 signaling, completely inhibited myogenic differentiation in L6 myoblasts (Hakuno *et al.* 2011), indicating that IGF1 signals must be suppressed during the myogenic differentiation. Thus, to evaluate the regulatory mechanism of the negative feedback of IGF signaling is indispensable for full understanding of IGF long-term action.

Conclusion

Both IGFs and insulin exert a variety of bioactivities that mainly increase anabolism and decrease catabolism. IGFs promote chronic effects that determine cell fates, such as induction of proliferation, inhibition of cell apoptosis and induction of differentiation. In contrast, insulin shows short-term effects that regulate metabolism, such as increase glucose and amino acid transport, induce glycolysis, glycogenesis, lipogenesis and protein synthesis and inhibit gluconeogenesis, lipolysis and protein degradation. They share the common signaling pathway, which is distinct from other tyrosine receptor kinase pathways since insulin-like signaling is mediated by specific docking proteins, IRSs. The common signaling pathways mediate appropriate bioactivities in each cell and tissue under various physiological conditions, which may be reflected in the duration of IGF or insulin stimulation (secretion of IGFs are constitutive but insulin is transient and a half-life of IGFs is over 15h and insulin is around 10min) and expression of IGF1 receptor/insulin receptor (IGF1 receptor is expressed highly in embryo and in various tissues except liver and adipocytes of adults and insulin receptor is expressed in liver, adipocytes and muscle). IRS-1 and IRS-2 work in

a complementary manner but clearly possess distinct physiological roles. Our results suggest that IRS-1 mainly functions as a scaffold protein forming high molecular signaling complexes containing various proteins and RNAs, which mediate/modulate signaling and bioactivities of IGFs and insulin. IRS-2 may function as a signal transducer through its tyrosine phosphorylation by IGF1 receptor/insulin receptor.

When dysregulation of insulin-like activities is induced, growth disorders and many types of age-related diseases including diabetes, cancer, neurodegenerative disease, arteriosclerosis and osteoporosis develop. For these reasons, the levels of insulin, IGF1, IGF2 and their binding proteins and receptors possess the clinical significance as biomarkers. Recently, we discovered that IRSs form high-molecular-mass complexes (IRSomes) even without IGF/insulin stimulation. These complexes contain proteins, which modulate tyrosine phosphorylation of IRSs by receptor kinases, control IRS stability and determine intracellular localization of IRSs. We found that under the pathological status, binding of IRSs with their associated proteins must also be changed. In some cancer cells, we have observed that proteins in the IRSome are changed, resulting in maintenance of high IRS levels and availability of IRSs is increased by the tyrosine kinase of the IGF1 receptor/insulin receptor. Excessive potentiation of insulin-like signals through IRSs is shown to maintain cancer phenotypes, suggesting that the IRSome is a novel cause of diseases and a new target for treatment of cancer. In contrast, repression of insulin-like signals by IRSomes is shown to induce diabetes.

In conclusion, linking growth disorder and various age-related diseases is the aberration of modulation of insulin-like activities and is possibly explained by dysregulation of IGF signaling through IRSs. Because IGF signaling is believed to be an important new target for treatment of these diseases by controlling insulin-like activities, anti-IGF1 receptor antibodies, IGF1 receptor tyrosine kinase inhibitors, various activators and inhibitors of IGF signaling molecules have been developed as candidates of therapeutic agents. However, practical application has been slow to develop since the side effects or resistance is observed. These facts mean that insulin-like bioactivities are essential for quality of life and the feedback loop is important for regulation of normal insulin-like bioactivities. Through tight international collaboration, it is an urgent research task to elucidate detailed molecular mechanisms underlying how particular insulin-like activities are regulated in specific tissues at certain stages of life.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

This work was supported in part by Grant-in-Aid for Scientific Research (B) #15H04583 from the Japan Society for the Promotion of Science (JSPS) to F H; Grant-in-Aid for Scientific Research (S) #25221204 and Core-to-core program A. A. Advanced Research Networks from JSPS and R&D matching funds on the field for Knowledge Integration and innovation from Bio-oriented Technology Research Advancement Institution, NARO, to S-I T.

Acknowledgments

The authors thank members of the Takahashi lab for valuable support and discussion, and Dr Susan Hall (University of North Carolina, NC, USA) and Dr Asako Takenaka (Meiji University, Kawasaki, Japan) for critically reading the manuscript.

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Received in final form 3 March 2018

Accepted 12 March 2018