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Distinct and Overlapping Functions of Insulin and IGF-I Receptors

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Targeted gene mutations have established distinct, yet overlapping, developmental roles for receptors of the insulin/IGF family. IGF-I receptor mediates IGF-I and IGF-II action on prenatal growth and IGF-I action on postnatal growth. Insulin receptor mediates prenatal growth in response to IGF-II and postnatal metabolism in response to insulin. In rodents, unlike humans, insulin does not participate in embryonic growth until late gestation. The ability of the insulin receptor to act as a bona fide IGF-II-dependent growth promoter is underscored by its rescue of double knockout *Igf1r/Igf2r* mice. Thus, IGF-II is a true bifunctional ligand that is able to stimulate both insulin and IGF-I receptor signaling, although with different potencies. In contrast, the IGF-II/cation-independent mannose-6-phosphate receptor regulates IGF-II clearance. The growth retardation of mice lacking IGF-I and/or insulin receptors is due to reduced cell number, resulting from decreased proliferation. Evidence from genetically engineered mice does not support the view that insulin and IGF receptors promote cellular differentiation *in vivo* or that they are required for early embryonic development. The phenotypes of insulin receptor gene mutations in humans and in mice indicate important differences between the developmental roles of insulin and its receptor in the two species. (*Endocrine Reviews* 22: 818–835, 2001)

I. Introduction

THE EASE WITH which the murine embryo lends itself to genetic tampering has resulted in rapid progress in elucidating the physiological role of gene products through targeted mutagenesis in embryonic stem cells. During the past decade, the joint efforts of several laboratories have firmly established physiological functions for various components of the insulin/IGF system. At the same time, naturally occurring mutations of the homologous human genes have revealed similarities and differences between the roles of these peptides in the two species. Since murine and human embryonic development differ in substantial ways, it is not surprising that the phenotypes associated with mutations in similar genes may differ. Within the purview of insulin and IGF action, it is indeed remarkable how conserved the functions of the various genes are. Without the functional insight afforded by gene knockouts, cross-species comparisons can be seriously misleading. For example, in mice both IGF-I and IGF-II contribute to prenatal growth (1, 2), but only IGF-I is required for postnatal growth (1–3), and *Igf2* is not expressed after birth (4, 5). In contrast, in humans *IGF2* is expressed

Abbreviations: DIR, *Drosophila* IR; Gk, glucokinase; Hgf, hepatic growth factor; Igf1r, IGF receptor; Ins, insulin; Insl, insulin-like; Ir, insulin receptor; Irs, insulin receptor substrate; IUGR, intrauterine growth retardation; SMAD, similar to *Drosophila melanogaster* Mad proteins.

Mouse genetic loci are in lowercase italics, human genetic loci are in uppercase italics, and protein products are in uppercase Roman.

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throughout life. Nevertheless, the phenotype of a single individual carrying a functional *IGF1* knockout is remarkably consistent with the null *Igf1* phenotype in mice, suggesting that *IGF2* expression cannot compensate for lack of IGF1 in human postnatal growth. Moreover, different developmental timing in the two species results in a delayed onset of insulin action on fuel metabolism in rodents. With these caveats in mind, it will be easier to appreciate the lessons of mouse knockouts affecting insulin and IGF signaling.

A. The growing family of insulin-like peptides and their receptors

The insulin/Igf family of ligands and receptors controls key aspects of mammalian life, including growth, metabolism, and reproduction (6–8). In the past decade, the daunting complexity of these functions has become apparent as more insulin-like peptides have been cloned. There are at least nine different genes encoding insulin-like peptides: the two nonallelic *Insulin* genes (in rodents), *Igf1, Igf2, Relaxin,* and four insulin-like peptides: *Insl3, 4, 5,* and *6* (9–13).

There are at least three separate receptors that interact with this host of ligands: insulin receptor (14, 15), Igf1 receptor (16), and Igf2 receptor (17). A fourth member of the family, Ir-related receptor (18), is as yet orphaned, although its ability to bind all the various insulin-like peptides has not been extensively tested. Three of the four receptors (IR, IGF1R, and IRR) belong to the family of ligand-activated receptor kinases. Indeed, unlike other receptor tyrosine kinases, these receptors exist at the cell surface as homodimers composed of two identical α/β-monomers, or as heterodimers composed of two different receptor monomers (*e.g.*, $IR_{\alpha\beta}$/$IGF1R_{\alpha\beta}$, or $IR_{\alpha\beta}$/$IRR_{\alpha\beta}$). Upon ligand binding, they undergo a conformational change, which enables them to bind ATP and become autophosphorylated (19, 20). Autophosphorylation increases the kinase activity of IR-type receptors by 3 orders of magnitude, enabling them to phosphorylate a number of substrate proteins and engender growth or metabolic responses (21). It is likely that this receptor family contains additional members: there is evidence for a separate IGF-II receptor that regulates placental growth (1, 3, 6, 22), and for an insulin-like peptide receptor (23).

Unlike IR, IGF1R, and IRR, the product of *Igf2r* is not a tyrosine kinase. Instead, it is a monomeric receptor with a large extracellular domain made up of 15 repeat sequences and a small region homologous to the collagen-binding domain of fibronectin. IGF2R functions also as the cation-independent mannose-6-phosphate receptor (17). IGF2R does not have a signaling domain and is thought to be recycled between the plasma membrane and intracellular compartments. Interestingly, in adipose cells, IGF2R colocalizes with the insulin-sensitive compartment known as GLUT4 vesicles (24). Based on the *in vivo* mutagenesis experiments described below, it is now clear that IGF-II binding to IGF2R serves as a mechanism to clear circulating IGF-II, rather than as a signaling mechanism.

Finally, there are at least six different circulating IGF-binding proteins (IGFBPs), which regulate IGF bioavailability. The interaction between IGFBPs and IGFs is controlled by two different mechanisms: 1) proteolytic cleavage by a family of specific serine proteases, which decreases IGF binding affinity; and 2) binding to the extracellular matrix, which has been shown to potentiate IGF actions (25, 26). In addition, there is limited evidence that the cell surface proteoglycan Glypican-3, mutations of which cause the overgrowth syndrome known as Simpson-Golabi-Behmel type I ("bulldog" syndrome, OMIM 312870), also binds IGF-II and may modulate its function (27).

II. Null Mutations of *Insulin1*, *Insulin2*, and *Insulin Receptor (IR)*

The existence of a specific receptor for insulin was first proposed by Roth and co-workers (28), based on the identification of saturable, inhibitable insulin binding to liver plasma membranes. Biochemical studies in the following decade culminated in the identification of the receptor's tyrosine kinase activity (29). Cloning of the receptor cDNA (14, 15) and gene (30) ushered in molecular investigations of insulin action, with the identification of insulin receptor mutations in humans with extreme insulin resistance (reviewed in Ref. 31), the determination of the crystal structure of the receptor kinase (20, 32), and the development of pharmacological agents that enhance receptor signaling to treat diabetes (33).

The generation of mice bearing insulin receptor mutations has been instrumental in dissecting the pathogenesis of insulin resistance, diabetes, and obesity (34–43). The metabolic phenotypes of these mice have been reviewed elsewhere (8). Mice lacking IR are born at term with slight growth retardation (~10%) (22). With the exception of a marked hypotrophy of sc adipose tissue (44), their embryonic development is unimpaired. After birth, metabolic control rapidly deteriorates: glucose levels increase upon feeding, despite insulin levels approximately 100- to 1,000-fold higher than in normal littermates (Fig. 1A). β-Cell failure occurs within a few days, characterized by the disappearance of insulin storage granules within the β-cell cytoplasm (Fig. 1B) and followed by death of the animals in diabetic ketoacidosis. This experiment indicates that *Ir* is necessary for postnatal, but not for prenatal, fuel homeostasis.

These findings are confirmed by studies of mice lacking both nonallelic insulin genes (*Ins1* and *Ins2*). There are two insulin genes in rodents; *Ins1* represents a functional retroposon (45). In adult mice, insulin is synthesized from transcripts of both genes, but *Ins2* mRNA appears to be translated more efficiently than *Ins1* mRNA (46). However, ablation of either gene is without consequences, suggesting that reciprocal compensation can occur. In contrast, after inactivation of both genes, mice develop diabetic ketoacidosis and die within days of birth. Inactivation of the two insulin genes results in a slight impairment of embryonic growth, with a 15–20% decrease of birth weight (47). These findings suggest that insulin signals exclusively through IR, since the phenotypes of the two gene ablations are indistinguishable. However, the definitive experiment of generating knockout mice lacking *Ins1*, *Ins2,* and *Ir* has not yet been reported.

The development of diabetes in *Ins* or *Ir* null mice in the early postnatal phase is consistent with the notion that the

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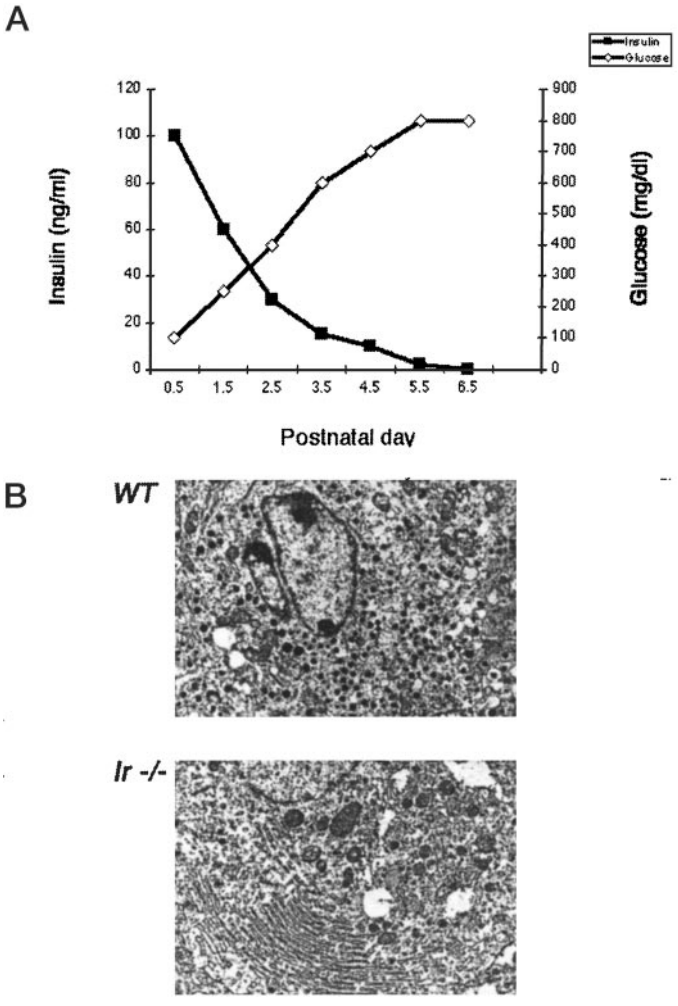

FIG. 1. A, Insulin and glucose levels in mice lacking IR. Plasma insulin and glucose levels in newborn mice lacking IR are plotted as a function of age. Mice are born with normal metabolic values. However, as they begin suckling, insulin and glucose levels increase rapidly. During the first 3 postnatal days, insulin secretion remains elevated. Death occurs when insulin levels drop, between postnatal d 2.5 (P2.5) and P4.5, depending on the genetic background. B, Electron microscopy of pancreatic β-cells in newborn mice. This electron micrograph shows the ultrastructure of a normal pancreatic β-cell from a P4.5 mouse (*top*) and an *Ir*−/− litter mate (*bottom*). In a normal β-cell, insulin secretory granules at various stages of maturation can be seen in the cytoplasm. In contrast, in the β-cell from *Ir*−/− mice, there are virtually no insulin secretory granules left. Moreover, the prominent Golgi stacks indicate that the cell is in an active secretion mode. Note also the swollen and disorganized mitochondria, suggestive of impaired oxidative phosphorylation.

functional maturation of a fuel-sensing mechanism in rodents occurs in the perinatal period. This represents an important developmental difference with humans, in whom insulin responsiveness is established during the last trimester of gestation (48). For example, in rodents, enzymes required for glucose storage and release, as well as lipid synthesis and oxidation, are induced at birth (49–53). Similar to *Ins* and *Ir* knockouts, mutations in other key genes required for glucose metabolism give rise to early neonatal diabetes, for example *Glucokinase* (54–56), *Glut2* (57), and *Pepck* (58), as well as genes encoding transcription factors required for insulin gene transcription and/or pancreatic β-cell development (reviewed in Ref. 8).

The growth impairment observed in mice lacking *Ins1* and *Ins2* indicates that the effect of insulin to promote mouse embryo growth is paltry compared with that of IGF-I and IGF-II. This is hardly surprising, as significant insulin secretion in rodents does not begin until late gestation (59, 60). In fact, while preproinsulin mRNA can be detected by RT-PCR at a premorphogenetic stage [embryonic d 9 (E9.0)] (61), the first insulin-producing cells appear at E12.5 (62), and islets do not become organized until E18.5 (63–65). Insulin secretion rises about 3-fold between E18.5 and birth (48, 66, 67). It should be noted that the embryonic patterns of insulin gene expression are drastically different in humans. During embryonic development, *INS* transcripts can be first detected at 8 wk of gestation (68, 69). Clusters of β-cells can be observed at 12–16 wk (70, 71) and become organized into functioning islets by 25 wk, after which plasma insulin concentrations increase substantially (72).

The lack of significant growth retardation in *Ir*-deficient mice is more surprising, since *Ir* mediates IGF-II signaling during gestation (see below). This discrepancy appears to be due to two major factors: a difference in developmental timing between humans and rodents, and a 2-fold increase in *Igf1r* expression in IR-deficient mice, which enables *Igf1r* to partially compensate for lack of IR (22).

A. Developmental phenotype of humans lacking IR

Mutations of *IR* in humans are phenotypically heterogeneous: the severity of the syndrome runs the gamut from mild insulin resistance (73, 74) to leprechaunism (Refs. 75–82; reviewed in Ref. 31) (OMIM no. 147670). The latter represents the severest form of insulin resistance due to *IR* mutations, and, in four separate cases, has been shown to be caused by functional *IR* knockouts (78, 79, 81, 82). As in mice, lack of *IR* in humans is compatible with embryonic development and term birth. However, the similarities between the two species are limited (83, 84).

B. Growth retardation is associated with IR mutations in humans

Most strikingly, humans lacking *IR* are severely growth retarded at birth and gain little if any weight thereafter (75–82, 85). The onset of growth retardation is unclear, but in one case in which the patient was delivered by cesarean section at 35 wk gestation, growth retardation was already severe: the patient weighed 940 g, *i.e.*, less than the expected weight of a 27-wk fetus (86).

The likeliest explanation of the difference between *Ir*-deficient mice and children with leprechaunism is that embryonic growth of humans and rodents follows different patterns. Rodents are born comparatively earlier than humans, at a stage corresponding to 26 wk of human gestation. Not only are rodents born developmentally "earlier" than humans, their body composition at birth is quite different (87). During the last trimester of human gestation, corresponding to the first weeks of postnatal life in mice, there is a sizable increase in adipose mass, which coincides with an

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increase in insulin production (72). As a result, lipid content is significantly higher at birth in humans (16% of total body wt) compared with rodents (2% of total body wt) (87). The adipose "organ" is exquisitely sensitive to insulin, as demonstrated by the excessive adiposity of fetuses exposed to high insulin concentrations *in utero* as a result of maternal diabetes (88, 89), Beckwith-Wiedemann syndrome (90), erythroblastosis fetalis (91, 92), or persistent hypoglycemic hyperinsulinism of infancy (nesidioblastosis) (93). These data indicate that insulin exerts growth-promoting effects on the human adipose "organ" during the third trimester of gestation. Because the increase in the insulin-sensitive adipose compartment occurs postnatally in rodents, the growth retardation defect in *Ins-* or *Ir*-knockout mice is not as severe as the growth retardation of children with leprechaunism at birth. Interestingly, IR-deficient mice present with a similar phenotype of undernourished adipose tissue as children with leprechaunism (44), suggesting that both lack the trophic actions of insulin on adipose tissue.

Thus, in contrast to mice, insulin is a fetal growth factor in humans. There have been no reports of null mutations of the human insulin gene. However, the developmental role of insulin can be gleaned from conditions of relative hypoinsulinemia, *e.g.*, mutations of the *glucokinase* (94), and *PDX1* genes (95), as well as rare cases of transient neonatal diabetes (96). Mutations of the glucokinase gene provide an especially intriguing paradigm to gauge the effects of insulin on fetal growth. Glucokinase is the low-Michaelis-Menten constant (K_m) (7–9 mM) enzyme that phosphorylates glucose in liver and β-cells. Because it is active at physiological glucose concentrations (~5 mM), it acts as a enzymatic link between plasma glucose levels and insulin secretion. Thus, an increase in glucose concentrations will result in increased glucose phosphorylation, a fall of the intracellular ATP:ADP ratio, closure of ATP-sensitive K channels, Ca^{++} influx, and insulin release from storage granules (97). Heterozygous *GK* mutations result in haploinsufficiency, with a higher threshold for glucose-dependent insulin release and mild hyperglycemia. Children heterozygous for a loss-of-function *GK* allele are approximately 0.5 kg smaller than unaffected siblings at birth, suggesting that the decrease in insulin levels caused by the *GK* mutation impairs fetal growth (98). Moreover, when the mother carries a *GK* mutation and has hyperglycemia during pregnancy, children who do not inherit the mutation are moderately macrosomic, as expected in light of the maternal diabetes, whereas children who inherit the mutation are of normal size. These findings suggest that the detrimental effect of the maternal mutation was balanced out by the inability of the fetus to properly sense glucose variations and increase insulin secretion accordingly (98). Similar data were obtained in mice with a heterozygous *Gk* mutation (99).

In a similar vein, null mutations of the insulin gene transcription factor *PDX1* cause pancreatic agenesis (OMIM no. 260370) and result in severe intrauterine growth retardation (IUGR) (95, 100). Congenital diabetes, either permanent (OMIM no. 304790) (101) or transient (OMIM no. 601410) (96, 102), is also associated with severe IUGR. Thus, fetal hypoinsulinemia is associated with IUGR in humans.

C. Metabolic abnormalities in humans lacking IR

Another important and seemingly paradoxical difference between IR-deficient mice and humans is that mice are steadily hyperglycemic, whereas humans develop alternating postprandial hyperglycemia and fasting hypoglycemia. However, this is an instance in which the human phenotype is harder to explain than the murine phenotype. It is not clear why children with leprechaunism develop fasting hypoglycemia. The expectation would be that insulin resistance would cause unrestrained glucose production with fasting hyperglycemia, but in small children with limited glycogen stores, the liver's ability to generate glucose may be intrinsically poor (75, 77, 103, 104). The murine phenotype of uncontrolled hyperglycemia is easier to explain, because newborn mice do not fast. Indeed, the presence of "milk spots" in the stomach is a hallmark of neonatal well-being. Under these circumstances, there is a constant flow of nutrients, and glucose concentrations in the bloodstream steadily rise.

A second reason for the absence of hypoglycemia in mice is that the β-cell compensatory ability in the face of extreme insulin resistance is greater in humans than in mice, and the increase in insulin levels may cause hypoglycemia through insulin binding to IGF1R. Thus, whereas the murine pancreas becomes functionally exhausted within 3–7 d of birth in mice lacking IR (Fig. 1B), high insulin levels persist in children with extreme insulin resistance for months or years (reviewed in Refs. 31 and 83). The different β-cell compensatory response in humans and mice is likely to reflect the limited development of the endocrine pancreas at birth in rodents (63–65). To support this hypothesis, it should be noted that children with Rabson-Mendenhall syndrome, a milder variant of insulin-resistance syndromes due to *IR* mutations (OMIM no. 262190) (104–107), generally experience an improvement of hypoglycemia in infancy, in association with declining plasma insulin values (106, 108).

Finally, the absence of hypoglycemia in mice could be due to species-specific differences in the role of different tissues in metabolic control. In rodents, liver accounts for a greater fraction of glucose uptake and storage than in humans. In contrast, skeletal muscle plays a more important role in glucose homeostasis in humans. In both species, muscle expresses a sizable amount of IGF1R, while liver is virtually devoid of it. Thus, if insulin at high concentrations binds to muscle IGF1R and promotes glucose uptake, there is a potential for greater glycemic control in humans than there is in rodents. Experimental evidence provides support for this hypothesis. In leprechauns, there is some evidence that IGF-I can ameliorate glucose homeostasis (109), although other studies failed to demonstrate an effect (103). IGF-I treatment of mice lacking IR results in a rapid decrease of glucose levels, suggesting that IGF-I can indeed stimulate muscle glucose uptake through its receptor. However, this decrease is not sufficient to rescue mice from death (110), presumably because of incomplete rescue by IGF-I of hepatic insulin action (111–113).

We had originally ascribed the lack of hypoglycemia in *Ir* knockout mice to relatively lower insulin levels in newborn mice compared with humans (34). However, based on a

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much more extensive data set, and based on insulin measurements in 0.5- to 1.5-d-old pups, we now recognize that insulin levels can indeed be as high in newborn *Ir* knockout mice as they are in children with leprechaunism (Fig. 1A). Thus, this explanation is no longer tenable.

III. Null Mutations of *Igf1* and *Igf1r*

Lack of *Igf1* or *Igf1r* results in intrauterine growth retardation. Nullizygous animals are born with Mendelian frequency, suggesting that *Igf1* and *Igf1r* are not required for successful completion of gestation. The birth weight of *Igf1* null mice is 60% of normal; that of *Igf1r* nulls is 45% (1, 3, 114). Survival of *Igf1* null mice is strain dependent and is associated with postnatal growth retardation, so that, by 2 months of age, the size of *Igf1* knockout mice is only 30% of normal (1, 3, 114). Prenatally, IGF-I mediates growth independently of GH; postnatally, GH is required for hepatic IGF-I synthesis and mediates approximately 50% of IGF-I action on growth (see below) (115). Postnatal development of surviving *Igf1* knockout mice has been analyzed in detail. At 2 months of age, IGF-I-deficient mice show extensive reductions of brain size and preserved brain morphology, consistent with a role of IGF-I in axon growth and central nervous system myelination (116). Different cell types within the brain are differentially affected by the lack of IGF-I. While axons and oligodendrocytes are greatly reduced in number, dopaminergic, striatal, and motor neurons are unaffected, as are cerebellar neurons and cholinergic neurons of the forebrain (116). Interestingly, the latter express high levels of *Irr* mRNA, the orphan receptor of the insulin receptor family (117, 118). These cell-specific differences within the brain are at odds with observations in other organs, where the decrease in size associated with ablation of *Igf1* appears to be due to a generalized decrease in cell number, supporting the notion that IGF-I acts as a general growth promoter by favoring cell division (6).

Morphological and morphometric analyses of long bones in mice lacking *Igf1* indicate that IGF-I promotes bone development by increasing cellular proliferation, without affecting differentiation. Long bones are reduced in size because of a reduction in cell number due to decreased proliferation, as indicated by bromodeoxyuridine labeling indexes. The growth plates are uniformly affected, with reductions in the resting, proliferative, and hypertrophic chondroyctes. As a result, the formation of secondary ossification centers is delayed (115). By combining the *Igf1* mutation with a null *Ghr* mutation, Lupu and colleagues (115) have been able to analyze the relative contributions of IGF-I and GH to bone formation (119). Bone growth is equally affected in *Igf1* and *Ghr* mutants, while combined mutations do not add significantly (~5%) to the growth impairment caused by single mutations. These data indicate that the actions of GH to promote osteogenesis depend on the presence of IGF-I (115), and that the IGF-I-independent contribution of GH to bone formation is minimal. The observation that IGF-I plays a critical role in osteogenesis is supported by studies of a patient lacking IGF-I, who showed a severe reduction in bone mineral density that was moderately increased upon recombinant human IGF-I administration (120).

In contrast to *Igf1* mutants, *Igf1r*-deficient mice invariably die within minutes of birth, probably as a result of respiratory failure caused by impaired development of the diaphragm and intercostal muscles. Mice are born with multiple abnormalities, including muscular hypoplasia, delayed ossification, and thin epidermis (3). Muscle hypoplasia results from decreased cell number. It is unclear whether muscle hypoplasia is isometric (proportionate to the generalized organ hypoplasia) or anisometric (disproportionate to overall size decrease). Embryonic bone development is also profoundly affected by the lack of IGF1R, as expected based on the findings in IGF-I-deficient mice. The appearance of ossification centers is delayed by 2 embryonic days in cranial and facial bones, and between 1–2 d in long bones and trunk. Skin thickness is reduced as a consequence of a thinned stratum spinosum and results in a translucent skin in mutant embryos. These abnormalities are opposite to those observed in skin of patients with insulin resistance (increased skin thickness and pigmentation, *i.e.*, acanthosis nigricans), consistent with the hypothesis that increased insulin levels in these patients lead to insulin binding to IGF1R, thus stimulating keratinocyte proliferation (75, 77, 121). *Igf1r* knockout mice also show a significant increase in cell density in the central nervous system, which is thought to result from a depletion of intercellular matrix and crowding of neural cells in the spinal cord and brain stem (3).

Igf1r null mice have also been reported to develop metabolic abnormalities. These include mild hyperglycemia (~250 mg/dl) and decreased β-cell mass (122), although the latter was reportedly normal in other studies (123). Since IGF1R shares many signaling properties with IR (124), these findings are not altogether surprising. It should be noted, however, that the hyperglycemia reported by Withers *et al.* (122) is unlikely to be a contributory cause of death in *Igf1r* null mice, since *Ir* null mice survive longer with considerably higher glucose levels (34, 35, 110).

A. IR can substitute for IGF1R to mediate growth

Targeted gene knockouts in mice have been especially useful to address the vexing question of whether the functions of IR and IGF1R are distinct or overlapping. The phenotypes of *Ir* and *Igf1r* knockouts are very similar to those of *Ins* and *Igf1* knockouts, respectively. Moreover, combined ablation of *Igf1* and *Igf1r* results in the same phenotype as lack of *Igf1r* (45% of normal birth weight), suggesting that IGF-I signals exclusively through IGF1R (3). These data indicate that the ability of the two receptors to compensate for each other is limited. A notable exception to this paradigm is the phenotype of mice lacking both *Igf1r* and *Igf2r*, which provides evidence for the ability of IGF-I to signal through IR (125). It has been shown that mice lacking IGF2R are rescued from perinatal lethality and undergo near-normal postnatal development when they carry null mutations of IGF1R. Genetic evidence indicates that the receptor supporting the growth of *Igf1r/Igf2r* double mutants is IR, since mice lacking all three genes (*Ir, Igf1r, Igf2r*) are nonviable 30% dwarfs (22). Thus, embryonic growth of *Igf1r/Igf2r* knockout mice must be sustained through IGF-II binding to IR (Fig. 2), since this is an existing embryonic growth pathway. The

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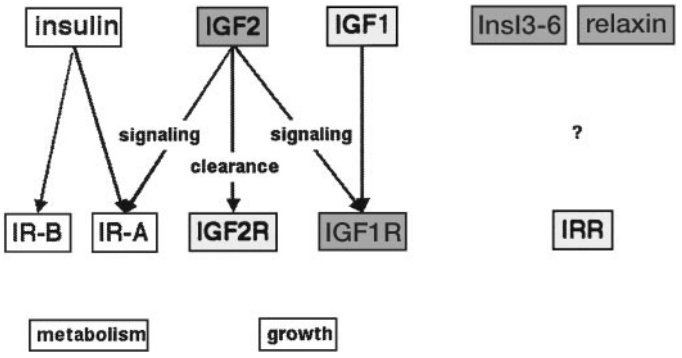

FIG. 2. Interactions among ligands and receptors of the insulin/IGF family. In this scheme, the ligand/receptor interactions deduced from single and combined gene knockouts are illustrated. Unlike insulin and IGF-I, which bind with high affinity (in the low nanomolar range) to their own receptors and with low affinity (in the high nanomolar range) to the cognate receptor, IGF-II has the ability to bind to both receptors with comparably high affinities. It is thought that alternative splicing of exon 11 confers onto IR the ability to bind IGF-II with high affinity. Receptors for insulin-like peptides have not yet been identified. IRR ligand(s) are similarly unknown.

impaired IGF-II clearance caused by the *Igf2r* mutation causes a rise in IGF-II levels, which likely accounts for the normal embryonic growth of *Igf1r/Igf2r* mutant mice. However, after birth, *Igf2* expression is supposedly extinct (126). Thus, the survival and postnatal growth of these mice can only be accounted for by IGF-I signaling through IR, although the possibility of persistent postnatal expression of *Igf2* has not been formally ruled out. In one sense, the ability of IGF-I to activate IR should not be considered surprising, since circulating IGF-I levels are approximately 1,000-fold higher than insulin and would theoretically allow for low-affinity IGF-I binding to IR (127). However, since IGF-I mostly circulates in a protein-bound form and there are significant differences in tissue distribution of *Ir* and *Igf1r* transcripts, the rescue of *Igf1r/Igf2r* knockout mice remains unexplained.

B. Embryonic growth and heterodimeric ("hybrid") insulin/IGF-I receptors

Unlike other receptor tyrosine kinases, which are activated through a process of ligand-induced dimerization (21), receptors of the IR subfamily exist as dimers in the unliganded state and are activated by their respective ligands through a conformational change that enables the β-subunits to bind ATP (20, 32, 128). In addition to forming homodimers, IR, IGF1R, and IRR can engage in the formation of heterodimers with each other (129–131). It is unclear whether these "hybrid" receptors, as they are mostly—if somewhat inappropriately—referred to, subserve specific functions, *e.g.*, by recruiting different substrates.

A discussion of the potential role of heterodimeric receptors is beyond the scope of this review. However, a critical review of mice with targeted null mutations provides some clues on this issue. It is fair to assume that, if heterodimeric receptors were required for a specific developmental function, the latter should be reflected in an overlapping phenotype in mice with a single knockout of either *Ir*, *Igf1r*, or *Irr*. Nevertheless, the phenotypes of the various receptor knockouts could hardly be more distinct, with diabetes in *Ir* knockouts, dwarfism in *Igf1r* knockouts, and no phenotype in *Irr* knockouts. Thus, circumstantial evidence suggests that heterodimeric receptors do not have a specific developmental role. Indeed, the only available experimental evidence speaks against a function of heterodimeric receptors. Expression of a kinase-inactive *Ir* transgene in *Ir* knockout mice (132) leads to heterodimer formation between IR encoded by the mutant transgene and endogenous IGF1R, but does not impair growth of the resulting transgenic/knockout mice above and beyond the growth retardation induced by the *Ir* knockout (Table 1). Thus, it is unlikely that hybrid receptors are specifically required for the growth-promoting actions of either IR or IGF1R in embryos. The question of whether heterodimeric receptors play a metabolic role in the adult animal remains open. There have been numerous reports suggesting that the ratio of homodimers to heterodimers is altered in conditions of insulin resistance (133), although a consensus is yet to emerge (134).

C. Endocrine vs. autocrine/paracrine actions of IGF-I

The central tenet of the somatomedin hypothesis is that IGF-I is produced by the liver in response to GH and mediates GH actions in peripheral tissues (135). Over the years, various observations have suggested that this concept represented an oversimplification of a complex biological problem, since 1) GH has direct growth effects of its own (136–139); and 2) IGF-I is produced by multiple tissues and has the theoretical capability of acting in an autocrine/paracrine fashion (reviewed in Ref. 140). To address this issue in a conclusive manner, Lupu *et al.* (115) have generated mice lacking both IGF-I and GHR. Double knockout mice are more growth retarded (17% of normal) than mice lacking either gene alone (1, 3, 114, 141), indicating that the two genes act both independently and synergistically to promote growth (115). While IGF-I promotes both prenatal and postnatal growth, GH appears to be required exclusively for postnatal growth, since the growth defect in *Ghr*-deficient mice only becomes apparent after postnatal d 20 (115, 141). Based on the growth curves of the various mutant mice, the partition of

TABLE 1. Embryonic growth in mice expressing heterodimeric IR/IGF1R: a kinase-inactive IR transgene does not impair growth of *Ir* knockout mice

	Genotype			
	WT	*Igf1r*−/−	*Ir*−/−	*Ir*−/−, K1030M
Birth wt (g)	1.2 ± 0.1	0.5 ± 0.1 ($P < 0.05$)	1.1 ± 0.1	1.1 ± 0.1

The birth weights of mutant mice lacking *Ir* and *Igf1r* were compared to those of transgenic knockout mice expressing a kinase-inactive IR transgene (K1030M) in the genetic background of *Ir*-deficient mice (*Ir*−/−, K1030M). If the kinase-inactive transgene interfered with the endogenous IGF1R by way of hybrid receptors, the expectation would have been that *Ir*−/−, K1030M transgenic knockout mice would be more growth-impaired than *Ir*−/− mice. The failure to see more severe growth retardation than that caused by the *Ir* mutation is indirect evidence that hybrid receptors do not play a major physiological role to promote embryonic growth. Hybrid receptor formation was demonstrated in several tissues, albeit as a minor fraction (~10–30%, depending on the tissue) of total receptor number.

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growth effects appears as follows: IGF-I-dependent, about 35%; GH-dependent, about 14%; combined GH/IGF-I-dependent, about 34%; while growth that occurs independently of either GH and IGF-I is about 17% (115) (Fig. 3). Ablation of *Ghr* impairs hepatic IGF-I synthesis by about 98%, resulting in undetectable serum IGF-I. Synthesis of IGF-I in other tissues is largely unaffected, suggesting that GH controls primarily hepatic IGF-I production (115). Conditional *Igf1* ablations in liver support the conclusion that circulating ("endocrine") IGF-I is hepatic in origin (142, 143). The conclusion of these experiments is that the endocrine component of IGF-I action is GH dependent and accounts for about 50% of total IGF-I-dependent growth, whereas the autocrine component of IGF-I action is GH independent and accounts for the remaining approximately 50% of IGF-I action (115). These data are in apparent contrast to data showing normal growth in mice lacking hepatic IGF-I as a result of conditional mutagenesis (142, 143). However, since it is not simple to measure the biologically active component of circulating IGF-I, it is still possible that residual IGF-I expression in these mice is sufficient to support growth. The only generalization possible from these studies is that conditional knockouts have as many drawbacks as constitutive knockouts, due, for example, to the patterns of Cre expression or the efficiency of recombination (144).

D. Developmental phenotypes of humans lacking IGF-I or IGF1R

The *IGF1* locus has been extensively analyzed in several groups of children with "idiopathic" congenital growth retardation; however, no mutations have been identified, leading to the suggestion that IGF-I mutations are not a common cause of growth retardation in humans (145–147). The debate has been rekindled by the identification of a single case of human *IGF1* knockout due to a partial deletion of *IGF1*. This patient strikingly resembles the phenotype of *Igf1*-deficient mice, with severe prenatal and postnatal growth failure (148). The offspring of consanguineous parents, the patient was delivered by cesarean section because of poor fetal growth at 37 wk gestation. At that time, the patient weighed 1.4 kg. He continued to grow poorly throughout infancy and childhood, and reached a height of 120 cm and a weight of 23 kg at age 15, more than 6 SD below the mean. In addition, the patient presented with sensorineural deafness and mental retardation. Unlike GH-insensitive ("Laron") dwarfs, the IGF-I-deficient patient had normal insulin sensitivity without hypoglycemia (149). Thus, the main finding of *Igf1* knockout mice, namely prenatal and postnatal growth retardation, is borne out. There are, however, areas of divergence. For example, the patient appeared to undergo normal—if somewhat delayed—sexual development, and placental growth was moderately impaired, in contrast to *Igf1*-deficient mice (3, 114, 150). It bears emphasizing, however, that some of these differences may reflect the inbred genetic make-up of this individual, who is expected to be homozygous by descent at about 6% of the genome, based on the degree of consanguinity between the parents. In this case, both parents and their siblings had short stature. This finding was interpreted to suggest that heterozygosity for loss-of-function alleles of IGF-I results in haploinsufficiency and impairs growth (148), as has been suggested by Powell-Braxton and colleagues (114)of the null *Igf1* allele in mice. This hypothesis awaits further experimental confirmation.

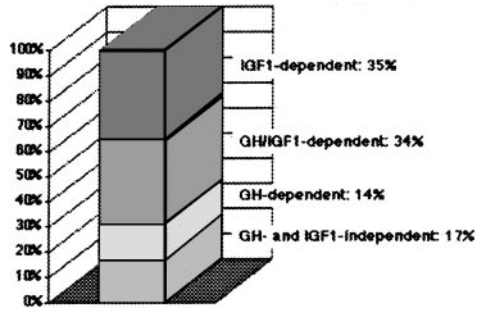

FIG. 3. Interactions between GH and IGF-I. The development of mice with combined *Igf1* and *Ghr* mutations has led to a redefinition of the "somatomedin hypothesis." Before birth, IGF-I expression is independent of GH. Subsequently, hepatic IGF-I synthesis becomes GH dependent, an event associated with loss of hepatic IGF-I receptors (139, 140). Tissue synthesis of IGF-I remains mostly GH independent. Postnatally, IGF-I-dependent growth accounts for about 35% of total, GH-dependent for about 14%, combined GH/IGF-I-dependent for about 34%, while the remaining 17% occurs independently of both GH and IGF-I (115).

E. IGF1R mutations in humans with IUGR

There have been sporadic reports of *IGF1R* mutations in humans. These mutations appear to be associated with considerable phenotypic heterogeneity. A deletion encompassing *IGF1R* has been identified in an 11-yr-old girl with a clinical diagnosis of Silver-Russell syndrome. The patient presented with prenatal and postnatal growth deficiency associated with multiple dysmorphic abnormalities, including a characteristic facies, bilateral clinodactyly, cafe-au-lait spots, and mental retardation (151). Molecular analyses of *IGF1R* have suggested that mutations of this gene are not a common cause of IUGR. In a single case, a heterozygous deletion of chromosome 15q26.1-qter was associated with monozygosity for *IGF1R*. The patient presented with IUGR, microcephaly, micrognathia, renal and pulmonary abnormalities, and postnatal growth failure (152). Recently, molecular scanning of *IGF1R* in a larger series of IUGR patients has been reported in a preliminary form. Of 74 *IGF1R* alleles analyzed, two missense mutations have been identified in four chromosomes, two in a compound heterozygote. The two mutations are expected to affect the function of IGF1R, since they localize to the receptor's amino-terminal domain, a region in which numerous mutations have been identified in the cognate IR (153–155). Because these observations are derived from a limited analysis of *IGF1R*, it is possible that the actual prevalence of IGF1R mutations in IUGR is higher than the reported 5% (156).

IV. Opposing Effects of *Igf2* and *Igf2r* Mutations

A. Igf2 and Igf2r are reciprocally imprinted

In mice, *Igf2* and *Igf2r* are parentally imprinted, *i.e.*, they are expressed only from one of the two alleles: *Igf2* is ex-

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pressed only from the paternal allele, whereas *Igf2r* is expressed only from the maternal allele. Accordingly, when mice inherit an *Igf2* mutation from the sire (*Igf2*+/p), they are indistinguishable from a homozygous null mutant (*Igf2*−/−) (2, 157). Likewise, mice that inherit a maternal *Igf2r* mutation (*Igf2r*+/m) are the functional equivalent of a complete knockout (*Igf2r*−/−) (125). The *H19* gene is located downstream of *Igf2* and is imprinted in an opposite fashion (*i.e.*, it is maternally expressed) (158). A deletion of this gene is associated with relaxation of imprinting and increased IGF-II levels (158, 159). The role of imprinting in the function of these genes remains unclear. In humans, loss of *IGF2* imprinting is seen in sporadic cases of Beckwith-Wiedemann, a genetically heterogeneous overgrowth syndrome resulting from modification of a cluster of imprinted genes on chromosome 11p15.5 (OMIM no. 130650) (160). This region also contains the *INS* gene. Both in humans and in mice, there is evidence for parental imprinting of *INS* (in mice, *Ins2*), along with *Igf2* and *H19,* in the yolk sac (161, 162). It is unclear whether imprinting of *INS* accounts for differential expression of insulin mRNA in extrapancreatic tissues, which may trigger autoimmunity in type 1 diabetes (163). Parental imprinting of *INS* has also been linked to parent-of-origin differences in the transmission of type 1 diabetes (164), although other factors probably contribute to this effect (165).

B. Phenotypic consequences of Igf2 and Igf2r ablations

Igf2 mutants are approximately 60% of normal size at birth. However, their postnatal growth is unaffected, consistent with a role of *Igf2* in embryonic growth and with the lack of *Igf2* expression in adult mice (126, 157, 166). This is in contrast to *Igf1* mutants, postnatal growth of which is as impaired as their prenatal growth. However, some tissues continue to express *Igf2* after birth, *e.g.*, the choroid plexus. Moreover, there have been scattered reports of *Igf2* mRNA expression and secretion of mature IGF-II peptide from pancreatic β-cells (167–171). Since *Igf2* is located near *Ins2*, it is possible that active *Ins2* transcription would alter the chromatin structure around the *Igf2* promoter and cause *Igf2* transcription. Secreted IGF-II could potentially activate β-cell proliferation through IGF1R, as recently proposed (122, 172). This mechanism could play an important role in the response to insulin resistance.

The phenotype of *Igf2* mutant mice is in stark contrast with that of *Igf2r* mutants (Table 2). When mice inherit the *Igf2r* null allele through the maternal route, they show increased serum and tissue levels of IGF-II, associated with an approximately 40% increase in size by weight and generalized organomegaly with heart abnormalities, kinky tails, postaxial polydactyly, and edema (173, 174). A similar phenotype is observed in true homozygous knockouts (125). *Igf2r*-deficient mice usually die perinatally and rarely survive to adulthood. The elevation of IGF-II levels in these mice suggests that *Igf2r* is important for IGF-II clearance, and that failure to remove IGF-II from the circulation results in developmental abnormalities (125, 173, 174). Indirectly, a similar effect is associated with deletions of the *H19* gene, which cause a relaxation of imprinting at the *Igf2* locus and a secondary increase in IGF-II levels (158, 159).

As described above, the lethal phenotype due to IGF-II-induced overgrowth can be rescued by a homozygous null mutation of *Igf1r* (125). This experiment indicates that IGF-II signaling through IGF1R is responsible for the developmental abnormalities found in *Igf2r* or *H19* mutants. In contrast, in *Igf1r/Igf2r* mutant mice there are no developmental abnormalities. This finding indicates that IGF-II signaling through IR is sufficient to engender growth, but insufficient to induce lethal embryonic abnormalities (125).

TABLE 2. Growth retardation phenotypes in mice with null mutations of the insulin/IGF system

Genotype	Growth (% of WT birth weight)	Phenotype	Reference
Ins1 + *Ins2*	80–85	Diabetes	(47)
Igf1	60	Prenatal and postnatal growth retardation, infertility	(3, 114)
Igf2	60	IUGR	(157)
Ir	90	Diabetes	(34, 35)
Igf1r	45	IUGR	(3)
Igf2r	140	Perinatal death, organ abnormalities	(173, 174)
Irs1	60–80	Prenatal and postnatal growth retardation, insulin resistance	(172, 181, 182)
Irs2	100	Insulin resistance, β-cell failure, infertility	(172, 183)
Irs3	100	Normal	(186)
Irs4	80	Prenatal and postnatal growth retardation, insulin resistance	(190)
Igf1 + *Igf2*	30	IUGR	(1)
Igf1 + *Igf1r*	45	IUGR	(3)
Igf1r + *Igf2r*	100	Normal growth	(125)
Igf2 + *Igf2r*	65–75	IUGR	(279)
Igf2 + *Igf1r*	30	IUGR	(1)
Igf2 + *Igf1r* + *Igf2r*	30	IUGR	(22)
Ir + *Igf1r*	30	IUGR	(22)
Igf2 + *Ir* + *Igf1r*	30	IUGR	(22)
Igf1 + *Ghr*	17	Prenatal and postnatal growth retardation	(115)

These data are compiled from all available publications describing the various mutant mice. WT, wild type.

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V. Ablation of Insulin Receptor Substrates (IRS)

IRS proteins act as mediators of insulin, IGF, and cytokine signaling in a variety of cell types. The IRS family comprises five members, including IRS1, -2, -3, -4, and Gab1 (175–179). The general structure of these proteins consists of two protein-protein interaction domains, the pleckstrin-homology and phosphotyrosine-binding domains, and several tyrosine residues within YXXM motifs that are phosphorylated by growth factor receptors. Phosphorylation increases the affinity with which these domains bind to other adaptor molecules, such as the various regulatory subunits of PI3K, grb-2, syp, nck, crk, 14.3.3, and fyn (180).

Absence of *Irs1* in mice gives rise to prenatal and postnatal growth retardation and insulin resistance. The onset of growth retardation occurs on about E15.5, and mice are born at 80% of normal in one report (181), and 40–60% of normal in another report (182), suggesting that there might be strain-specific differences in the growth-promoting role of IRS1. The pattern of growth retardation of IRS1-deficient mice is comparable to that seen in IGF1-deficient mice (*i.e.*, both prenatal and postnatal), consistent with a model in which IRS1 mediates the growth-promoting actions of IGF1R, in addition to some of the metabolic actions of IR (181, 182).

Mice that lack IRS2 are of normal size but develop hyperglycemia as a result of impaired β-cell growth. The extent of β-cell growth impairment is strain dependent: in one knockout strain it results in death from diabetes in male animals (172), whereas in another strain it results in mild hyperglycemia (183). In contrast to the normal size of IRS2-deficient mice, mice with combined heterozygous *Ir* and *Irs2* mutations are slightly growth retarded, indicating that IRS2 may mediate postnatal growth in response to IR (37).

IRS3 is the smallest IRS protein and is expressed at high levels in adipose tissue, where it represents the most abundant IRS isoform (177, 184, 185). However, lack of IRS-3 has no apparent effect on adipose cell function or metabolism and growth (186). This finding should not be construed as suggesting that IRS3 has no role in insulin action. In fact, combined *Irs1* and *Irs3* mutations give rise to severe impairment of insulin-dependent glucose uptake in adipose cells, suggesting that the two proteins can substitute for each other in this cell type (187). Alternatively, it has been proposed that IRS3 and IRS4 may act as negative modulators of IRS1 and IRS2 function (188). IRS4 was originally cloned from human kidney cells but is expressed in several tissues, including pancreatic β-cells (189). Ablation of *Irs4* results in modest growth retardation and glucose intolerance (190). In contrast, ablation of *Gab1* results in an embryonic lethal phenotype (191) that is inconsistent with a role in insulin/IGF signaling, since none of these gene ablations is embryonic lethal. This developmental defect would rather suggest a role for Gab1 in hepatic growth factor (HGF) signaling, since null mutations of *Hgfr* are associated with a similar phenotype (192, 193).

VI. Interactions Among Ligands and Receptors of the Insulin/IGF Family

To understand the functional correlation among *Ins1*, *Ins2*, *Igf1*, *Igf2* and their receptors, we must once again turn to the phenotypes of mice with combined gene ablations (Table 2) (6). As stated earlier, insulin exerts a modest effect on murine prenatal growth, beginning in late gestation (~E18.5) (22, 47). In contrast, a combined knockout of *Igf1* and *Igf2* results in nonviable 30% dwarfs, consistent with an additive effect of the two mutations. The "30% phenotype" as Efstratiadis (6) originally termed it, indicates that the contribution of IGF to growth is about 70% of total body size, so that additional growth factors presumably sustain the residual 30%. A more severe growth retardation (17% of normal) is found in mice lacking both IGF1 and GHR, suggesting that a significant component of IGF-independent growth is mediated directly by GH postnatally (see above) (115). The IGF-deficient phenotype is first apparent at about 11.5 in *Igf2* knockout mice (1, 157), and at about E13.5 in *Igf1* knockout mice (1, 3, 114), indicating that IGFs (and insulin) do not contribute to early embryogenesis in mice, despite numerous suggestions to the contrary (reviewed in Ref. 194). Indeed, those suggestions were based on indirect evidence showing that IR and IGF1R are expressed in preimplantation embryos (195, 196), but it is possible that they are either inactive or not indispensable at that stage. These data also indicate that the onset of IGF-II action precedes that of IGF-I. The size reduction of IGF-less mice results from a reduced cell number and, in a few instances, reduced cell size (1, 197, 198). It should be emphasized that findings in mice with targeted IGF mutations thus far do not support a direct role of IGFs in cellular differentiation. This is in contrast with *in vitro* experiments with cultured cell lines, in which IGF-I has been shown to promote differentiation of diverse cell types, including preadipocytes (199), myoblasts (200, 201), and lymphoblasts (202).

The growth retardation of double *Igf1/Igf2* knockouts (30%) is more severe than that of double *Igf1/Igf1r* knockouts (45%), but identical to that of *Igf2/Igf1r* doubles, *Ir/Igf1r* doubles, and *Igf2/Ir/Igf1r* triple mutants (1, 22) (Table 2). This genetic evidence indicates that IGF-I signals only through IGF1R, while IGF-II signals through both IR and IGF1R. The relative contribution of *Ir* and *Igf1r* to IGF-II-mediated growth change during embryogenesis. At E15.5, IGF-II binding to IGF1R accounts for approximately 90% of IGF-II action. By E18.5, this contribution has decreased to 60%. Contrariwise, the contribution of IR to IGF-II signaling increases from 10 to 40% (22). It is conceivable, although unproven, that this change correlates with changes in expression of the two receptors (203). The fact that IGF1R bears the brunt of IGF-II-dependent growth in midgestation provides a potential explanation of why embryos overexpressing IGF-II (*e.g.*, *Igf2r* knockouts) can be rescued by ablation of *Igf1r* (125). In fact, the most serious abnormalities in these mice occur in heart morphogenesis at midgestation (158, 159). Conceivably, if the main IGF-II signaling receptor (IGF1R) is lacking, the deleterious effects of IGF-II cannot take place through IR.

A. Alternative splicing of exon 11 modulates the affinity of IGF-II binding to IR

It is known that IGF-II binds with comparable affinities to both IR and IGF1R (204). However, recent data have contributed to unravel the molecular determinants of IGF-II binding to IR. The *Ir* is expressed as two variably spliced

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isoforms (IR-A and IR-B), which differ by the presence or absence of a 12-amino acid peptide at the carboxyl terminus of the extracellular α-subunit encoded by *Ir* exon 11 (14, 15, 205–209). Frasca *et al.* (210) reported that IGF-II binds IR-A, but not IR-B, with similar affinity to that of insulin. Moreover, IGF-II acts as bifunctional ligand, binding IR-A and IGF1R with comparable affinities. IR-A is primarily expressed in fetal cells, with lower expression in metabolically active adult tissues such as muscle, liver, and adipose (210), consistent with a primary role in embryonic growth. These data are supported by the observation that IGF-II can rescue the growth of embryonic fibroblasts derived from IGF1R-deficient mice through IR (211), and that IGF-II-dependent growth is impaired in hepatocytes lacking IR (113). These data indicate that IR is a physiological mediator of IGF-II action in cultured cells. In summary, converging genetic, cellular, and molecular evidence indicate that IR serves as a fetal receptor for IGF-II. The function of IGF-II binding to IR in the adult organism is unclear. In humans, for example, IGF-II continues to be produced at high levels after birth. There have been scattered reports that the alternatively spliced IR-A occurs more frequently in various disease conditions, including cancer (212) and diabetes (206, 213, 214), although the latter findings remain controversial (215–219).

B. Odd man out: Irr

IRR is the only known orphan receptor of the *Ir* family (18). Despite extensive investigations, its ligand remains unknown (220–223). It is unclear whether IRR functions as an independent homodimeric receptor or whether it functions primarily by engaging in heterodimer formation with IR and IGF1R (222, 224), similarly to ErbB-2 in the epidermal growth factor receptor family (225, 226). *Irr* transcripts are predominantly found in kidney, neural tissues, stomach, and pancreatic β-cells (117, 227–233).

Mice lacking IRR are phenotypically normal; double knockouts of *Irr* and *Ir* are phenotypically identical to *Ir* knockouts (234). Thus, the function of IRR remains unclear. It appears that the plot either thickens or thins out, depending on one's taste for orphan receptors.

VII. Reproductive Phenotypes of Mutations in Insulin-Like Peptides and Their Signaling Pathways

There exists a close connection between growth, metabolism, and reproduction. Targeted gene mutations in mice have confirmed this correlation and revealed unsuspected roles in the regulation of reproductive behavior by peptides of the insulin family and their receptors. In an excellent article, Nef and Parada (7) recently reviewed the role of insulin-like peptides in reproduction. Some aspects related more specifically to insulin and IGFs are summarized here.

A. Igf1 mutants

Lack of IGF-I leads to infertility in both males and females. In males, testosterone (T) levels are reduced to 18% of normal and are associated with reduced size of testis, epididymus, and distal regions of the spermatic duct. Infertility appears to be due to impaired mating behavior, since the ability of capacitated spermatozoa to fertilize eggs *in vitro* is normal. Females show hypoplastic uterus and anovulation, which cannot be corrected by exogenous gonadotropins (150). Since the general paradigm is that IGF-I-stimulated growth occurs through IGF1R, the expectation would be that mice lacking IGF1R are as infertile as mice lacking IGF-I. Contrary to this prediction, however, *Igf1r*-deficient mice (in the *Igf1r/Igf2r* double-knockout background) are fertile (125), suggesting that IGF-I signaling through IR is sufficient to restore reproductive function. These data are consistent with the notion that IR, rather than IGF1R, mediates the reproductive functions of IGF-I. Indeed, it is well established that subfertility is a common occurrence in insulin-resistant women (235, 236), and that mutations of *IR* are associated with anovulation and hyperandrogenism (polycystic ovaries), although the mechanistic basis for this association remains elusive (84, 153, 237).

B. Brain-specific ablation of Ir impairs LH production

Bruning and colleagues (43) have reported that ablation of *Ir* in neurons using a nestin promoter-driven Cre recombinase impairs fertility by decreasing spermatogenesis in males and ovarian follicle maturation in females. They attributed these changes to hypothalamic dysregulation of LH production, suggesting that hypothalamic IR regulates gonadotropin synthesis.

C. Irs2 and Irs4 mutants

Infertility and subfertility have also been observed in female mice lacking IRS2 and IRS4, respectively. Lack of IRS2 is associated with hypogonadotrophic hypogonadism, anovulation, and small ovaries. It is unclear whether, in addition to a reduced number of gonadotrophs in the pituitary, the *Irs2* mutation also causes intrinsic changes in the ovary (238). It should be emphasized, however, that *Irs2* knockout mice generated in a different laboratory do not have reproductive abnormalities, suggesting that the effect of the *Irs2* mutation is modified by the genetic background (183). In contrast to the mouse data, an increase in *IRS2* expression has been reported in ovarian specimens from women with insulin resistance (239).

Irs4 ablation is associated with a reduced number of litters and reduced litter survival, although the significance of the latter observation remains unclear (190). Since these abnormalities are not observed when *Irs4* null males are bred with heterozygous females, it is likely that the *Irs4* null females are subfertile (190). Interestingly, *Irs4* mRNA has been detected in the hypothalamus, consistent with a role of IRS4 in gonadotropin production (240).

D. Insl3 mutations cause cryptorchidism

The insulin-like peptide-3 (*Insl3*) is expressed in Leydig cells of the testis (241) and theca cells of the ovary (242). Its expression increases during puberty (242). Homozygous null *Insl3* mice develop bilateral cryptorchidism as a result of abnormal development of the gubernaculum testis (243, 244). This abnormality appears to be a primary defect rather than

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secondary to defective androgen production. Interestingly, prenatal exposure to estrogens inhibits *Insl3* expression in embryonic Leydig cells, thus providing an explanation for the effect of synthetic estrogens like diethylstilbestrol to cause cryptorchidism (7). The peculiar phenotype of *Insl3* mutant mice has rekindled interest in the identification of a specific receptor for insulin-like peptides. Preliminary studies have led to the identification of a single subunit receptor (23). Its structure has not been determined.

VIII. Insulin-Like Signaling in *Caenorhabditis elegans*

The identification of an insulin-like signaling cascade in the nematode *C. elegans* has provided novel insight into mechanisms governing insulin action in mammals (245). Mutations of the insulin/IGF receptor ortholog Daf-2 give rise to *dauer* larvae, characterized by increased life span and reduced metabolic activity (246). In addition to Daf-2 mutations, a similar phenotype is brought about by mutations of the genes encoding the PI3K, Akt (245, 247–249), and SMAD protein orthologs (250). Other mutations suppress, to varying degrees, the effect of Daf-2 mutations: these genes presumably counteract the effect of insulin signaling and are therefore of considerable interest for mammalian growth and metabolism. Two of these genes, Daf-16 and Daf-18, have been implicated in PI3K signaling (249, 251, 252).

Daf-16 mutations completely suppress the *dauer* phenotype due to Daf-2 mutations (251). The product of the Daf-16 gene is homologous to the mammalian FOXO forkhead transcription factors (253–257). Work in several laboratories has indicated that FOXO1 is a transcriptional promoter, and that its activity is inhibited by Akt and other phosphoinositol-tris-phosphate-dependent kinases through phosphorylation and nuclear exclusion (258–263). FOXO1 has been proposed to induce apoptosis (261), inhibit entry into the cell cycle (264), and stimulate glucose production (265). The *dauer* phenotype can also be caused by mutations in SMAD proteins, which are part of the TGFβ signaling cascade (250). Interestingly, SMAD proteins have recently been shown to potentiate apolipoprotein CIII promoter activity in a HNF4α-dependent fashion (266). Since apolipoprotein CIII is a candidate FOXO1 target gene, it is possible that SMAD proteins interact with FOXO1, providing a potential mechanistic link between the TGFβ and insulin/IGF signaling pathways in both *C. elegans* and mammals.

Daf-18 encodes a phosphoinositide phosphatase with homology to the mammalian PTEN tumor suppressor gene (267, 268). The mammalian ortholog of Daf-18 has been shown to dephosphorylate PI3K-generated phosphoinositol (269), providing a potential mechanism to terminate insulin signaling. Indeed, null mutations of the related gene SHIP-2 in mice cause increased insulin sensitivity and hypoglycemia (270). Daf-18 rescues the *dauer* phenotype due to Daf-2 mutations with less efficiency than Daf-16 (268), suggesting that, in *C. elegans*, PI3K is but one of the mediators of insulin/IGF signals, and that these signals converge on Daf-16. Consistent with these findings, the mammalian ortholog of Daf-16, FOXO1, is regulated by several related kinases (260, 261, 271).

IX. Insulin Receptor Signaling in *Drosophila melanogaster*

The *Drosophila* insulin receptor homolog (DIR) encodes a protein of 2,148 amino acids, larger than the human insulin receptor due to amino- and carboxyl-terminal extensions. The overall level of identity between DIR and human IR and IGF1R is 32.5 and 33.3%, respectively. DIR contains a 400-amino acid carboxyl-terminal extension with four YXXM or YXXL motifs. The presence of multiple putative SH2 domain-binding sites in DIR represents a significant difference from its mammalian homologs and suggests that, unlike vertebrate IR and IGF1R, DIR forms stable complexes with signaling molecules as part of its signal transduction mechanism (272–275).

Chen *et al.* (276) used chemical mutagenesis to induce mutations that lead to a loss of expression or function of DIR. These mutations cause recessive embryonic, or early larval, death. Some alleles exhibit heteroallelic complementation to yield a phenotype of developmental delay, growth retardation, and infertility. The growth deficiency appears to be due to a reduction in cell number, suggesting a role for DIR in regulation of cell proliferation during development (276). This interesting conclusion is borne out by studies of CHICO, a *Drosophila* homolog of vertebrate IRSs (277). CHICO mutants are less than 50% of the size of wild-type flies, due to a reduction of both cell size and number (278). In mosaic animals, CHICO-deficient cells grow more slowly than normal cells and give rise to smaller organs. CHICO mutants also show a 2-fold increase in lipid levels. The findings in *Drosophila* and *C. elegans* suggest that insulin-like signaling plays a highly conserved role in evolution to regulate cell growth and metabolism.

X. Conclusions

Over the past decade, numerous physiological functions of the insulin/IGF system have been analyzed using genetic tools. In addition to the wealth of information derived from gene-targeted mice, chemical mutagenesis in *Drosophila* and *dauer* mutations in *C. elegans*, the characterization of naturally occurring human mutations has enabled investigators to use cross-species comparisons to identify elements in insulin/IGF signaling. As outlined in this review, there remain gray areas, especially with respect to the functional overlap between insulin and IGF signaling and the role of insulin-like peptides. Thanks in no small measure to the technical advances in gene manipulation, we are positioned to continue to make progress.

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