

Minireview: Tissue-Specific Versus Generalized Gene Targeting of the *igf1* and *igf1r* Genes and Their Roles in Insulin-Like Growth Factor Physiology

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ABSTRACT

The insulin-like growth factors (IGF-I and IGF-II) and the IGF-I receptor are critically important for normal growth and development of the organism. Gene-deletion of these elements has demonstrated that IGF-I is important for both prenatal and postnatal development, whereas IGF-II is important during prenatal stages only. The IGF-I receptor gene-deleted mouse dies at birth apparently as a result of poor muscular development. Utilizing the conditional gene-deletion

approach, we have demonstrated that mice lacking the liver IGF-I gene have an approximately 80% reduction in circulating total IGF-I levels. Despite this marked reduction, postnatal growth and development was normal, suggesting that liver IGF-I is not essential for this function. Local tissue IGF-I production was unaffected and may compensate for the lack of the liver IGF-I. Further studies are ongoing to establish the role of the endocrine *vs.* the autocrine/paracrine IGF-I. (*Endocrinology* **142**: 1685–1688, 2001)

OF ALL growth factor families studied, one that will most surely benefit from the application of the new gene targeting technologies allowing temporal and cell-specific control of gene expression are the insulin-like growth factors. The expression of the various components of the insulin-like growth factor system of ligands (IGF-I/II), receptors (IGF-IR, IGF2R), and soluble binding proteins (IGFBP1–6) is ubiquitous throughout intrauterine and postnatal development (1, 2). The original knockout studies confirmed that the development of most, if not all, tissues and organs is regulated to some degree by the IGF system (3, 4). While the original knockout studies were excellent experiments in their own right, the interpretation of subsequent studies designed to distinguish the role of the IGF system in any one cell-type or organ system is complicated by the presence of severe developmental and endocrine abnormalities. This review will focus on recent attempts to study cell and organ-specific functions of the IGF system using a combination of molecular tools, specifically the use of cell-specific promoters and the Cre-loxP system. While technically challenging, these approaches are beginning to yield some surprising results.

The IGF family of ligands and receptors is homologous to the insulin/insulin receptor (IR) combination, suggesting a common evolutionary heritage. The IGF-IR is activated by IGF-I and IGF-II, and like the IR has an $\alpha_2\beta_2$ heterotetrameric structure (1). The receptors are coupled to several common intracellular second messenger pathways, for example the tyrosine kinase of both receptors target the insulin-receptor substrate (IRS) family of docking proteins (1). However, there is increasing evidence for receptor specificity in second

messenger systems. Still, one of the questions that remains to be definitively answered is how such similar receptors possess distinct biological actions. The IGF2R (also known as the mannose-6 phosphate receptor) is structurally distinct, and binds IGF-II but not IGF-I with high affinity. The IGF2R appears to lack any of the major intracellular domains associated with signal transduction. Its primary function appears to be inhibition of the actions of IGF-II *in utero*, controlling interstitial IGF-II concentrations by endocytosis and targeting to lysosomes for degradation (5).

The first papers from Argiris Efstratiadis and colleagues at Columbia University (New York, NY) describing the phenotype of *igf1*^{-/-}, *igf2*^{-/-}, *igf1r*^{-/-}, *igf2r*^{-/-}, and the various crosses were published almost a decade ago (3, 4, 6, 7). These papers revealed for the first time the essential role of the IGFs in promoting growth *in* and *ex utero*. While this is perhaps not surprising given their ubiquitous nature, it is easy to forget today that these original studies yielded some surprising insights into the role of IGF-I, IGF-II, IGF-IR, and the IGF2R. Mice lacking either the *igf1* or *igf2* genes exhibited intrauterine growth retardation, with weights approximately 60% that of wild-type littermates. Mice lacking a functional *igf1r* gene were born weighing only 45% of normal and died soon after birth from respiratory failure. While exhibiting intrauterine growth retardation, the postnatal growth in *igf2* null mice was found to be essentially normal. In distinct contrast, *igf2r* null mice exhibit a fetal overgrowth syndrome that is lethal. The lethal phenotype associated with the inactivation of the *igf2r* gene could be partially rescued by crossing onto a *igf2*^{-/-} background, indicating that excess IGF-II is detrimental to fetal development and that the IGF2R functions to prevent this from occurring (5).

The studies of DeChiara and colleagues also made a seminal contribution to our understanding of the parental imprinting of genes (7). The discovery of the reciprocal parental imprinting of the *igf2* and *igf2r* genes was an extremely im-

Received December 15, 2000.

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portant insight, both in terms of understanding the conflicting paternal and maternal priorities for controlling fetal growth and for discovering an important mechanism underlying carcinogenesis (8, 9). The information gathered from the crosses of the respective lines was also important, giving critical insights into how the components of the system interact.

The results derived from experiments where the various knockouts were crossed also provided genetic evidence of a role for a further receptor, recently identified as the IR, in mediating IGF-II's effects on fetal growth. The dwarf phenotype of *igf1/igf2* double knockouts was more severe than that associated with either single deletion (*i.e.* *igf1*^{-/-} or *igf2*^{-/-}) or the *igf1r* knockout, resulting in pups only 30% normal weight. The phenotype of the *igf1/igf1r* double knockout appeared identical to that of the *igf1r* knockout, suggesting that IGF-I was acting exclusively through the IGF-IR. In contrast, the *igf2/igf1r* double knockout had a growth retardation more severe than the *igf1r* knockout, suggesting that IGF-II acts through another receptor in addition to the IGF-IR. The unknown receptor mediating some of the effect of IGF-II on fetal growth was recently identified by genetic methods as being the IR (10). An independent study has revealed the existence of a splice-variant of the IR which has a high affinity for IGF-II and is highly expressed in fetal and neoplastic tissues (11). Thus the IGFs act to stimulate fetal growth through both the IGF-IR and the IR, whereas the IGF-II/mannose-6-phosphate receptor appears to inhibit the effects of IGF-II on fetal growth.

Despite the obvious success of these experiments, questions remain to be answered about the role of the IGF system in the more specialized tissues of the adult. The original knockout studies painted a picture of the somatotrophic axis using a necessarily broad brush. However, the fragility of the offspring meant that investigating the organ-specific functions of the IGF system in the adult was extremely difficult. For example, what role do the IGFs have in regulating ovarian function? Is the regulation of adrenal steroidogenesis by IGF physiologically relevant? Does the IGF-IR have insulin-like functions in muscle, and what is the function of liver IGF-I? Due to the severity of the growth retardation, not to mention significant pup mortality, answering these questions is difficult, if not impossible, using the *igf1* and *igf1r* knockouts first described.

The discovery by Brian Sauer that the Cre recombinase of bacteriophage P1 could be used to rearrange the eukaryote genome suggested a mechanism that would remove the restriction against introducing a mutation specifically into somatic cells (12). The first report of the successful application of this approach, inactivating the DNA polymerase β gene in T cells, was published in 1994 (13). Before this, gene targeting by necessity meant knocking out a gene in stem cells, and introducing this null allele into the germline. The study of genes in mature mice was only possible if these genes did not also possess a significant function during embryonic and fetal growth. Two lines of mice are required for the Cre-loxP approach to work. In one line, the targeted allele is flanked by two loxP sequences, which target the flanked region for excision by the Cre recombinase. In another line, the expression of Cre is controlled by a cell-specific promoter. Crossing

the two lines of mice leads to an animal lacking the gene only in cells that express Cre, while leaving the gene functional in all other tissues. The advantages of such a system are obvious: the function of genes necessary for fetal development can be preserved, allowing the function(s) of the gene to be examined during adulthood.

Mice in which exons of the *igf1* or *igf1r* genes have been flanked by loxP sites have been generated (14–16). One of the two *igf1r/lox* mouse lines has been reported as a model of partial IGF-IR deficiency, in that the neomycin resistance gene interferes with the processing of the nascent IGF-IR transcript (14). However, so far there has been no information reported regarding tissue-specific knockouts using either line. Of the three lines generated, only the *igf1* gene has been successfully targeted in a truly tissue-specific manner (17). To control the function of the *igf1* gene, loxP sites were inserted by homologous recombination 5' and 3' to exon 4 (16). Mice homozygous for the loxP-flanked allele displayed a minor (less than 10%) reduction in size, but were otherwise normal. Analysis of the offspring of mice hemizygous for the loxP-flanked allele and expressing Cre under the control of the EIIa promoter proved that the loxP sites were functional. The EIIa promoter used to drive Cre expression in this study is active during early embryogenesis, and perhaps during preimplantation development. The use of the EIIa promoter resulted in offspring with variable levels of recombination of the loxP-flanked allele, with a significant correlation observed between the degree of recombination and the severity of growth retardation. In the most severe cases of recombination (90–100%), a phenotype similar to that reported for the classical *igf1* knockout was observed, with a 30% reduction in body weight and perinatal mortality.

Having established that the *igf1/lox* allele was functional, we next set out to answer perhaps one of the most intriguing and long-standing of questions concerning the somatotrophic axis: what is the function of IGF-I produced in the liver? Whether the liver is the primary source of endocrine IGF-I had been in question for nearly two decades, since the widespread expression of the IGFs was first recognized. At the time, the liver was believed to be the primary source of IGF-I in the circulation. IGF-I mRNA levels in the liver are expressed at a level 10- to 100-fold greater than in the majority of other tissues, with the exception being adipose tissue. Hepatic IGF-I mRNA expression increases dramatically in the liver postnatally, coincident with a dramatic increase in serum IGF-I levels; thus, the function of hepatic derived IGF-I appears to become more important with age. The coincidence of the increase in serum IGF-I and hepatic IGF-I mRNA with the onset of GH-dependent growth is provocative, suggesting the liver-derived IGF-I might also play a role in the "GH-dependent" phase of growth. Liver IGF-I mRNA expression also exhibits by far the greatest response to manipulation of GH levels. Finally, hepatocytes do not express the IGF-IR; thus, IGF-I produced by these cells appears destined for export and not for local action. Taken together, the data suggested that circulating IGF-I played an integral role in the actions of GH.

By crossing the *igf1/lox* mice with a transgenic line expressing Cre under the control of the albumin promoter (Alb-Cre), we were able to generate mice with essentially no

IGF-I mRNA expression in the liver (17). Surprisingly, there appeared to be little effect on growth postnatally, despite a marked (75%) reduction in the concentration of total IGF-I in serum. This study, along with the results of further experiments on these mice, have confirmed that hepatocytes are indeed the primary source of IGF-I in the circulation. In a more recent study, it was shown that while GH treatment of female hepatocyte-specific *igf1*^{-/-} mice increased growth rate and IGF-I mRNA expression in brown and white fat; this occurred without significant changes in serum IGF-I levels (18). Both observations are consistent with the liver representing the primary source of serum IGF-I, and that the increase in IGF-I in the circulation following GH treatment is most likely hepatic in origin. The data derived from the experiments described in Yakar *et al.* were confirmed independently by a group using the same *lox-igf1* line of mice in conjunction with a line of mice expressing Cre under the control of an interferon-inducible promoter (Mx-Cre) (19). Treatment of the *lox-igf1*. Mx-Cre mice with interferon causes almost complete recombination in liver, and partial recombination in spleen, leading to a marked reduction in serum IGF-I without affecting growth rate.

Interestingly, the phenotype of the acid-labile subunit (ALS) knockout mice bears a striking resemblance to that of the hepatic-specific *igf1* knockouts (20). ALS mRNA expression is restricted to hepatocytes (21). By forming a ternary complex with an IGF and either IGFBP-3 or IGFBP-5, ALS stabilizes and thereby prolongs the half life of IGF in the circulatory system. Targeted deletion of the murine ALS gene was associated with a 60% reduction in circulating IGF-I levels, and an even more dramatic 90% reduction in IGFBP-3 levels. Yet despite the reduction in serum IGF-I, as observed in the studies using hepatic-specific deletion of the *igf1* gene, there was not a major effect on growth.

While answering one question (*i.e.* increasing IGF-I in the circulation is not necessary for GH-dependent growth), the initial results of these studies did not really provide a firm answer as to what the function of circulating IGF-I is. One observation suggests that circulating IGF-I has an important feedback function within the somatotrophic axis: serum GH levels are increased in both models of hepatic IGF-I deficiency, consistent with a reduction in the inhibitory effect of circulating IGF-I on GH release from the somatotroph. This result, while not novel in that the feedback effect of IGF-I on pituitary GH release has been known of for almost two decades, might be an important clue as to what the primary function of liver-derived IGF-I is. The increase in serum GH might also explain the lack of growth retardation. The expression of IGF-I mRNA in nonhepatic tissues was normal, as was the concentration of "free" IGF-I in the circulation. Thus, perhaps by a combination of increased GH and by the normal expression of IGF-I mRNA in extra-hepatic tissues, growth is not affected in the absence of hepatic-derived IGF-I.

One question remained, however, and that is whether a persistent mild hypersomatotropism would ultimately have pathological effects in the adult. In this respect, it is interesting to note that there is a deficit associated with the loss of hepatic-derived IGF-I (22). Mice in which the hepatocyte *igf1* allele has been deleted develop insulin resistance. This

effect is tissue-specific: IR and IRS-1 phosphorylation in response to insulin treatment is markedly inhibited in muscle but not in liver. Whether the insulin-insensitivity is due to the chronic elevation of GH, or perhaps reflects a direct "sensitizing" effect of IGF-I remains to be determined. In any event, this data clearly shows that while not necessary for growth *per se* a possible physiological role for hepatic-derived IGF-I is to act as a restraint GH secretion and regulate glucose homeostasis, perhaps at a postreceptor level. It will be interesting to determine whether there are other pathological consequences associated with chronic hypersomatotropism and hyperinsulinemia (*e.g.* hypertension, atherosclerosis, glomerular sclerosis, obesity).

There are still uncertainties to the model of endocrine IGF-I function presented in this text. For example, the ALS knockout mouse does not exhibit a defect in glucose homeostasis (20). This discrepancy might be due to the different mouse strains employed, Ueki *et al.* (20) crossed the chimeric mice with BALB/c females, whereas Yakar *et al.* (22) used the C57BL/6J strain. It is also important to note that the results of these studies do not rule out a role of circulating IGF-I in mediating the effects of GH on somatic growth, since the hypersomatotropism itself may mask any effects. "Free" IGF-I levels in the circulation of hepatic *igf1*^{-/-} mice are also normal, suggesting that there has not been a reduction in biologically active IGF-I. If this is indeed the case, why the increase in serum GH? Clearly, much work remains to be done characterizing the deficits associated with the loss of hepatocyte IGF-I mRNA expression.

The overexpression of IGF-I and IGF-II using cell-type specific promoters has also provided some valuable insights and remains a valid method for investigating IGF function. This approach has been successfully used to examine cell-type specific functions of IGF *in vivo*. For example, overexpression of IGF-I in osteoblasts leads to an increase rate of bone formation and a significant increase in bone mineral density (23). Surprisingly, the increase in bone density associated with IGF-I expression in osteoblasts occurs without changes in the number of osteoblasts or osteoclasts.

Loss of function experiments, in which a transgene controlled by a cell-type specific promoter expresses a dominant negative protein, have also been successfully used to examine the role of the IR in liver and muscle in glucose homeostasis by using kinase-deficient receptors (24). This laboratory has successfully targeted the expression of a dominant negative IGF-IR to muscle, using a muscle creatine kinase promoter (25). These mice exhibit a transient postnatal growth retardation, with a reduction in weight in the first 4 to 6 weeks postpartum. Interestingly, these mice also exhibit insulin resistance, perhaps due to the formation of hybrids composed of the wild-type IR and the kinase-deficient IGF-IR. The *in vivo* approach may ultimately prove to be a valuable adjunct to cell culture studies that have examined the function of the distinct C-terminal regions of the IGF-IR in controlling cell division and migration.

Both the Cre-lox and transgenic approaches can provide important information about the functions of the IGF system. Mice in which the *igf1* and the *igf1r* contain loxP sites flanking important coding regions have been generated and successfully manipulated by crossing with transgenic mice express-

ing Cre controlled by a variety of promoters. The *igf1* gene has been successfully deleted in hepatocytes; future efforts will include the use of muscle-, bone-, neural-, hematopoietic, and adipocyte-specific Cre transgenes. Despite the technical challenges in successfully employing this technology, new insights into the IGF system have never been closer.

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