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Developmental regulation of the rat insulin-like growth factor I receptor gene

(rat insulin-like growth factor I receptor cDNAs/rat insulin-like growth factor I receptor mRNA/gene regulation/RNase protection assay)

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ABSTRACT **We have investigated the developmental regulation of the rat insulin-like growth factor I (IGF-I) receptor gene in various tissues using a sensitive and specific solution hybridization/RNase protection assay. For this purpose we characterized rat IGF-I receptor cDNAs that were cloned from a simian virus 40-transformed rat granulosa cell cDNA library. The specific cDNA clone used in these studies encoded the putative signal peptide and the first 53 amino acids of the α subunit and was ≈94% homologous to its human counterpart. IGF-I receptor gene expression was studied during the perinatal period and at various intervals until early adulthood. Overall, steady-state IGF-I receptor mRNA levels decreased dramatically during postnatal development; however, the extent of the decrease differed among the various tissues studied. In contrast to receptor mRNA levels, IGF-I mRNA levels increased in some of the same tissues. The molecular mechanisms underlying this apparent divergent transcriptional control of the IGF-I and IGF-I receptor genes warrant further study.**

The biological effects of insulin-like growth factor I (IGF-I)/somatomedin C are initiated by its binding to a membrane-associated glycoprotein receptor that is structurally related to the insulin receptor (1, 2). Molecular cloning of the human IGF-I receptor cDNA confirmed that the IGF-I receptor, like the insulin receptor, is synthesized as a single precursor polypeptide (M_r ≈180,000), which is subsequently glycosylated and cleaved into an α subunit (M_r ≈135,000) and a β subunit (M_r ≈95,000) (3). The mature receptor is composed of two α and two β subunits joined by disulfide linkages (4). Although the α subunit is totally extracellular and is involved mainly in ligand binding, the β subunit is a membrane-spanning protein that includes a tyrosine kinase domain in its cytoplasmic portion (5, 6). The overall similarity between the human insulin and IGF-I receptors approaches 84% in the tyrosine kinase domain, 64–67% in the regions flanking the cysteine-rich portion of the α subunit (the putative ligand-binding domain), and ≈44% in the α subunit C-terminus and β-subunit extracellular sequences (3). This relatively high degree of similarity may explain, at least partially, the ability of both insulin and IGF-I to interact with the other hormone's receptor at high concentrations (1).

The physiological roles of insulin and IGF-I were at one time thought to be quite different. While insulin was considered to be an anabolic hormone mainly involved in the regulation of short-term metabolic processes, IGF-I was believed to be a key factor primarily in the modulation of long-term, growth hormone-dependent processes (7, 8). However, it is now well established that both insulin and IGF-I have short-term metabolic (insulin-like) effects as well as more long-term, growth-promoting (IGF-I-like) actions (9, 10). Moreover, no significant differences have been demonstrated in the intrinsic capacities of the insulin and IGF-I receptors to mediate insulin and IGF-I effects (10). Therefore, both the short-term and long-term actions of IGF-I could involve either the IGF-I or the insulin receptor, depending upon which receptor predominates in a given specific tissue or developmental stage. This concept is further supported by the observations that the levels of insulin and IGF-I binding fluctuate during the *in vitro* differentiation of muscle cells as well as during the differentiation of preadipocytes to adipocytes (11, 12). These findings suggest that different receptors may be acting at diverse stages of differentiation and/or development.

To study the regulatory mechanisms involved in the expression of the IGF-I receptor gene, we have cloned, sequenced, and characterized rat IGF-I receptor cDNAs† and have used them to generate an IGF-I receptor-specific antisense RNA probe. This homologous probe was then used to analyze the developmental expression of the IGF-I receptor gene in various rat tissues by using a sensitive solution hybridization/RNase protection assay. For comparative purposes, we also determined the levels of the mRNAs encoding the IGF-I ligand itself at the same developmental stages.

We found that the IGF-I receptor gene is preferentially expressed perinatally and that the mRNA levels decrease subsequently. This finding is in contrast to the pattern of expression of the IGF-I gene in many of the tissues, as described (13). This complex developmental pattern raises a number of interesting issues concerning the relationship between the control of ligand vs. receptor gene expression.

MATERIALS AND METHODS

cDNA Cloning and Library Screening. Poly(A)$^+$ RNA was obtained from a simian virus 40-transformed rat granulosa cell line (14). Complementary DNA was synthesized by using a commercially available kit (Boehringer Mannheim) and random hexamer primers (Pharmacia); thereafter, *Eco*RI linkers (New England Biolabs) were added, and after size-selection the cDNA was ligated into λgt10 and packaged using the Gigapack Gold system (Stratagene).

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Abbreviations: E, embryonic day; P, postnatal day; IGF-I and -II, insulin-like growth factors I and II, respectively.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M27293).

The rat granulosa cell cDNA library (2×10^6 recombinant phage) was screened with a ^{32}P-labeled human IGF-I receptor cDNA probe, provided by A. Ullrich (3). Hybridization in 5× SSPE (1× SSPE is 180 mM sodium chloride/10 mM sodium phosphate, pH 7.7/1 mM EDTA)/0.1% Ficoll/0.1% bovine serum albumin/0.1% polyvinylpyrrolidone/1% $NaDodSO_4$/denatured salmon sperm DNA at 100 μg/ml was for 16 hr at 50°C. Filters were washed twice at 50°C in 2× SSPE with 0.2% $NaDodSO_4$ and then twice at 50°C in 0.1× SSPE. Positive plaques were isolated and purified by three rounds of screening. cDNA inserts were subsequently subcloned into pGEM vectors (Promega), and the double-stranded plasmid DNA was sequenced (15).

Animals. Sprague–Dawley rats were obtained from Zivic–Miller. They were fed ad libitum, and at the indicated developmental stages they were sacrificed by decapitation, and the following organs were removed and frozen in liquid nitrogen: brain, heart, lung, liver, stomach, kidney, and muscle. Tissues were pooled from entire litters for the embryonic day 20 (E20) and postnatal day 1 (P1) stages; tissues from three to eight littermates were pooled for P8–P32 stages, and tissues from individual animals were used at stage P50.

RNA Preparation. Total RNA was prepared using a modified guanidinium thiocyanate–lithium chloride technique (16). The RNA was quantitated by measuring its absorbance at 260 nm, and its integrity was confirmed by visualization of the ethidium bromide-stained 28S and 18S ribosomal RNA bands. Poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose chromatography as described (17).

Northern (RNA) Blots. Poly(A)$^+$ RNA was electrophoresed through a 1.2% agarose/2.2 M formaldehyde gel, as described (18). After electrophoresis, the RNA was transferred to a GeneScreen membrane (New England Nuclear) using an IBI electroblotting system and baked for 2 hr at 80°C *in vacuo*. Blots were hybridized with a 265-base-pair (bp) *Eco*RI–*Rsa* I rat IGF-I receptor cDNA fragment, which included 15 bases of 5′-untranslated sequence and the nucleotide sequence encoding the signal peptide and the first 53 amino acids of the α subunit. The probe was labeled with [α-^{32}P]dCTP (Amersham) by a modified random priming technique (16). Blots were hybridized and washed as described (18).

RNA Probe Construction. A 265-bp *Eco*RI–*Rsa* I fragment was isolated from one of the rat IGF-I receptor cDNA clones and subcloned into the plasmid vector pGEM-3 that had been digested with *Eco*RI and *Sma* I. The resulting construct was linearized with *Eco*RI, gel-purified, and subsequently used to generate a ^{32}P-labeled rat IGF-I receptor antisense RNA by using SP6 RNA polymerase and [α-^{32}P]UTP (Amersham). The antisense transcript contained 40 bases of vector sequence and 265 bases complementary to 15 bases of 5′-untranslated sequence as well as to the region encoding the signal peptide and the first 53 amino acids of the α subunit. Hybridization of this probe to rat IGF-I receptor mRNA, followed by RNase digestion, resulted in a protected band of 265 bases.

The RNA probe used to detect IGF-I transcripts has been described (19). This construct allows the simultaneous detection of the two IGF-I mRNA species that encode the IGF-Ia and IGF-Ib prohormones. The size of the protected band corresponding to IGF-Ia mRNA (which constitutes >90% of the total IGF-I message) was 224 bases.

The levels of β-actin mRNA were measured using a 650-bp *Eco*RI–*Hin*dIII fragment of a rat β-actin cDNA, provided by B. Patterson (National Institutes of Health). This cDNA fragment was cloned into *Eco*RI- and *Hin*dIII-digested pGEM-2 and, after linearization with *Eco*RI, was used to generate an antisense RNA probe that gave a β-actin mRNA-specific protected band of 650 bases.

Solution Hybridization/RNase Protection Assay. Solution hybridization/RNase protection assays were performed as described (20). Briefly, 20 μg of total RNA were hybridized with 2×10^5 dpm of each one of the following ^{32}P-labeled homologous antisense RNA probes: IGF-I receptor, IGF-I, and/or only 5×10^3 dpm of β-actin. (Because β-actin mRNA is highly abundant, we lowered the specific activity of the β-actin RNA probe by increasing the amount of unlabeled UTP in the transcription reaction; this modification enabled us to visualize the protected band together with the other transcripts in the same autoradiographic exposures.) Hybridization was performed in a buffer containing 75% formamide in sealed capillary glass tubes at 45°C for 16 hr. After hybridization, RNA samples were digested with RNase A at 40 μg/ml (Boehringer Mannheim) and RNase T1 at 2 μg/ml (Pharmacia) for 1 hr at 30°C. Protected hybrids were extracted with phenol/chloroform, ethanol-precipitated, and electrophoresed on an 8% polyacrylamide/8 M urea denaturing gel. Multiple autoradiographic exposures from each gel were quantified by scanning densitometry using a GS-300 scanner (Hoefer).

Statistical Analysis. Statistical analysis of the data was performed using one-way analysis of variance, and individual means were then compared using the Newman–Keuls test. Probability values <0.05 were considered to be statistically significant.

RESULTS

Cloning and Characterization of Rat IGF-I Receptor cDNAs. Rat IGF-I receptor cDNAs were isolated by screening a simian virus 40-transformed rat granulosa cell cDNA library with a ^{32}P-labeled human IGF-I receptor cDNA probe (IGF-I-R.8) (3). We had previously demonstrated that this cell line expresses abundant high-affinity IGF-I receptors; in addition, blots of Poly(A)$^+$ RNA prepared from these cells showed a major 11-kilobase (kb) band when hybridized to the human probe (14). Screening of 2×10^6 recombinant phage with the same human probe at progressively lower densities yielded five independent clones. The cDNA inserts were recovered by digestion of phage DNA with *Eco*RI, followed by agarose gel electrophoresis. The size of the inserts ranged from 0.6 to 1.6 kb; all of them hybridized to the human IGF-I receptor probe. Sequencing of the five cDNAs revealed that they were overlapping clones that spanned a region that

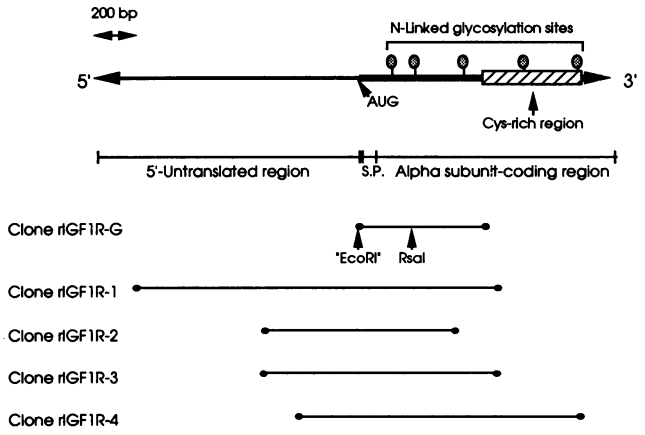

FIG. 1. Schematic representation of rat IGF-I receptor cDNA clones. Five overlapping partial IGF-I receptor cDNA clones were isolated, subcloned into pGEM plasmids, and sequenced. Potential N-linked glycosylation sites as well as the cysteine-rich region are indicated. Also indicated are the ≈1-kb 5′-untranslated region, signal peptide (S.P.), and the α-subunit coding region. Restriction sites for enzymes *Rsa* I and *Eco*RI (linker) are shown.

included ≈1 kb of 5′-untranslated region and sequences encoding the signal peptide and the first 333 amino acids of the α subunit (Fig. 1). Comparison of the published human sequence with the corresponding rat sequence showed ≈94% similarity at the nucleotide level and 97% similarity at the amino acid level. All of the cysteine residues are conserved, as well as all five of the N-glycosylation sites present in this region (3) (Fig. 2).

To demonstrate that the putative rat IGF-I receptor cDNA recognized the appropriate mRNA transcripts, we examined Poly(A)$^+$ RNA from various adult rat tissues using Northern (RNA) blot analysis. In all tissues examined, the size of the rat IGF-I receptor mRNA was ≈11 kb (data not shown), as previously demonstrated with a human cDNA probe (3). The same blot was subsequently hybridized with a ^{32}P-labeled human insulin receptor cDNA probe. A ≈9.5-kb and a ≈7.5-kb band, corresponding to the major rat insulin receptor mRNAs (21), were evident (data not shown). Thus, we conclude that the IGF-I receptor probe did not crosshybridize with insulin receptor mRNA.

Developmental Regulation of IGF-I Receptor Gene Expression. The ontogenic expression of the IGF-I receptor gene in different rat tissues was studied by means of a solution hybridization/RNase protection assay using total RNA obtained at various developmental stages and a specific ^{32}P-labeled rat IGF-I receptor RNA probe. The specificity of the developmental changes in the steady-state levels of the IGF-I receptor mRNA was evaluated by simultaneously measuring the levels of other transcripts. Specifically, ^{32}P-labeled IGF-I receptor, β-actin, and/or IGF-I RNA probes, each of unique size, were cohybridized to 20 μg of total RNA, followed by RNase digestion and PAGE. This multiple solution hybridization/RNase protection assay resulted in a collection of protected bands, whose intensities reflected the relative abundance of these transcripts at each stage. Because the probes differed in their specific activities, however, the relative levels of transcripts from different genes cannot be determined from this sort of data. Thus, it is possible to determine the changes in levels of several transcripts simultaneously as a function of the tissue or the developmental stage.

Scanning densitometry of the specific protected bands revealed that in liver and brain maximal expression of the IGF-I receptor gene occurred at E20, whereas in stomach maximal expression occurred between E20 and P8. Subsequently these levels decreased in a tissue-specific manner. Thus, in stomach and brain the levels of the message at P50 were ≈20% of those at E20, whereas in liver the levels at P50 were ≈6% of those seen at E20 (Figs. 3 and 4). These decreases were significant in liver and brain ($P < 0.05$). In muscle, IGF-I receptor mRNA levels decreased significantly between P1 and P50 ($P < 0.05$ for P50 vs. P8). IGF-I receptor mRNA levels in heart and kidney peaked at P1 and decreased to ≈17% and ≈30%, respectively, at P50 (Fig. 4). The decrease in heart was significant ($P < 0.05$). In lung, maximal levels of IGF-I receptor mRNA were seen at P8 and decreased to 14% at P50 ($P < 0.05$).

The levels of IGF-I receptor mRNA in liver and brain were compared to IGF-I mRNA levels at the same stages. The

FIG. 2. Sequence comparison of rat and human IGF-I receptor cDNA clones. (*A*) Rat (upper rows) and human (lower rows) sequences encoding 45 bases of 5′-untranslated region and 1090 bases encoding the signal peptide and 333 amino acids of the α subunit were compared. To compare rat and human sequences, nucleotide numeration of ref. 3 was followed. The translation initiation codon is boxed. (*B*) Derived partial amino acid sequence of rat (upper rows) and human (lower rows) IGF-I receptors. The sequence shown includes 30 amino acids of the putative signal peptide and 333 amino acids of the α subunit. Cysteine residues are underlined.

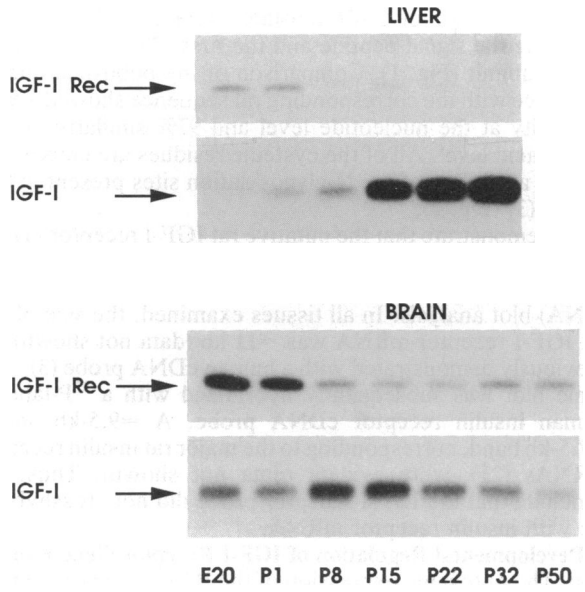

FIG. 3. Developmental regulation of IGF-I and IGF-I receptor (IGF-I Rec) mRNAs in brain and liver. A solution hybridization/RNase protection assay was done by using IGF-I and IGF-I receptor RNA probes, as described. The size of the IGF-I receptor mRNA-protected band was 265 bases. The IGF-I RNA probe gave two protected bands, corresponding to IGF-Ia mRNA (224 bases) and IGF-Ib mRNA (376 bases); only the 224-base-protected band, which constituted >90% of the total IGF-I mRNA, is shown. Because levels of transcript expression differed in each tissue, different exposures of the same gel were combined in this figure. In liver the IGF-I receptor band was a 3-day exposure, whereas the IGF-I band was a 5-hr exposure. In brain, the IGF-I receptor band was an 18-hr exposure, whereas the IGF-I band was a 3-day exposure. Therefore, no comparisons can be made between tissues or between the levels of the different transcripts in a single tissue.

present results confirmed previous findings (13) that maximal levels of IGF-I mRNA in brain were observed at P8, while in liver the peak values of IGF-I mRNA were demonstrated after 4 weeks (Fig. 3). Cohybridization with a β-actin RNA probe was used as a control for the specificity of the developmental changes in IGF-I receptor mRNA levels. In certain tissues, such as stomach and heart, no major changes occurred in the levels of β-actin mRNA throughout development, whereas in other tissues such as brain, liver, and muscle, the levels of this mRNA were modestly decreased (data not shown). These results confirm previous observations using Northern blot analysis (18). When we compared the relative abundance of IGF-I receptor mRNA transcripts in various tissues at a specific stage, we found that at E20 the highest levels were in the kidney, whereas at P50 the brain expressed the highest levels. When compared with other tissues, liver expressed very low levels at all stages.

DISCUSSION

Molecular cloning of partial rat IGF-I receptor cDNAs revealed a marked similarity to the human sequence (3). Particularly striking was the perfect conservation of all of the cysteine residues present in this region, both inside and outside the cysteine cluster. This finding suggests a fundamental role for these amino acid residues in the formation of a biologically active tetrameric protein, as well as in ligand binding (22).

The ontogenic expression of the IGF-I receptor gene was studied by using a solution hybridization/RNase protection assay with which we detected several transcripts simultaneously in a single sample. We report here that the steady-state

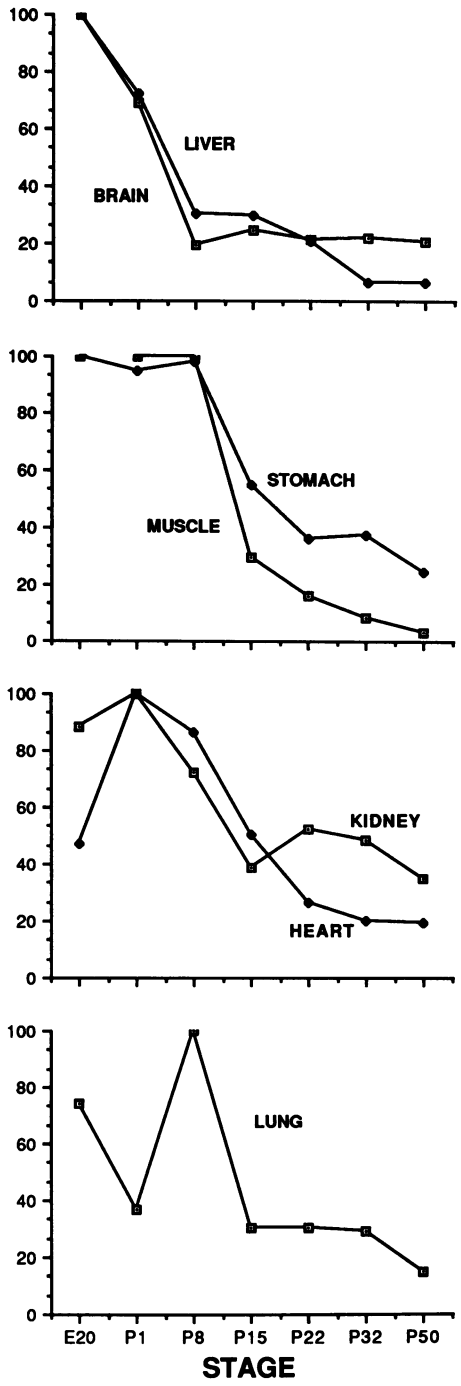

FIG. 4. Developmental regulation of IGF-I receptor gene expression in rat tissues. Total RNA was prepared from different rat tissues at various developmental stages, and the steady-state levels of IGF-I receptor mRNAs were measured by the solution hybridization/RNase protection assay, using a ^{32}P-labeled homologous IGF-I receptor antisense RNA probe. The autoradiograms were quantitated by densitometric scanning, and the results are expressed as percentage of relative arbitrary units of absorbance. For each tissue, the stage expressing the highest levels of the message was arbitrarily assigned a value of 100, and the *y* axis represents percentage of maximum. Each point represents the mean of two to three independent assays on each one of two RNA preparations. A single determination is presented for muscle at stage P1.

levels of IGF-I receptor mRNA are developmentally regulated. The general pattern of IGF-I receptor gene expression was an overall decrease in the levels of the message from perinatal stages throughout the postnatal stages of develop-

ment studied. However, the extent of mRNA decrease differed in the various tissues. Because the steady-state levels of mRNA depend upon both gene transcription and mRNA stability, further studies are required to determine the exact mechanism responsible for these developmental changes.

Developmental expression of the insulin and IGF-I receptors has been investigated in the human fetus (23, 24) and in different animal models (25, 26) by using radioreceptor methods. In rat brain (27) and skeletal muscle (28), IGF-I binding was greater than insulin binding at fetal and early postnatal stages; thereafter, binding of both ligands decreased to low levels in adults. The developmental changes in IGF-I receptor mRNA reported in this study are in accordance with these data on IGF-I binding. Thus, the overall decrease in IGF-I binding during development could be a consequence of altered gene expression.

Particularly interesting is the comparison of the developmental regulation of the IGF-I receptor and IGF-I genes. While IGF-I receptor mRNA levels decreased in all tissues studied, expression of the IGF-I gene in liver, heart, and kidney markedly increased during these same developmental stages (13). The high levels of IGF-I receptor mRNA and binding at perinatal stages strongly suggest that these receptors may be involved in both differentiation and growth of these organs. Because IGF-I gene expression is low at these stages, it is possible that a related ligand may be involved in this process. Indeed, IGF-II gene expression is maximal during fetal development (29), and this peptide can interact with IGF-I receptor (1), albeit with lower affinity than the IGF-I ligand. Moreover, in human fibroblasts, Furlanetto *et al.* (30) were able to inhibit IGF-II-mediated effects using a specific antibody (αIR3) directed towards the IGF-I receptor.

At later postnatal stages of development, when the process of differentiation is completed, IGF-I receptor gene expression and binding are reduced, while IGF-I gene expression in liver (13) and the resulting circulating levels of the protein are markedly increased (31). This decreased level of IGF-I receptor gene expression may be a direct result of the high IGF-I ligand levels. Nevertheless, these lowered levels of IGF-I receptor may still mediate IGF-I action because IGF-I mediates the effect of growth hormone on longitudinal growth by interacting with IGF-I receptors that are present in significant amounts in cartilage (8). That some of the effects of IGF-I at postnatal stages may be mediated through another receptor, such as the structurally related insulin receptor, is possible.

In summary, we have presented evidence that expression of the IGF-I receptor gene, like expression of the IGF-I ligand gene, is developmentally regulated. Intriguingly, these two genes are expressed in a diametrically opposed fashion during development. An understanding of the molecular mechanisms responsible for this complex developmental pattern will require characterization of the pertinent genetic regulatory regions and elucidation of the associated trans-acting factors that control expression of the IGF-I and IGF-I receptor genes.

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