Spatiotemporal expression of alternatively spliced IGF-I mRNA in the rat costochondral growth plate

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Abstract

IGF-I acts as a local proliferation and maturation factor for chondrocytes in the growth plate. However, the expression of different alternative IGF-I mRNA classes in the growth plate has not been characterized. Using quantitative reverse transcription PCR, the abundance of each alternative IGF-I mRNA class in resting, proliferative and hypertrophic chondrocytes was measured in rat costochondral growth plates. Class 1Ea mRNA was the most abundant IGF-I transcript overall and was highly expressed in proliferative chondrocytes at 2 and 4 weeks of age; by 6 weeks, the majority of 1Ea mRNA expression had shifted to hypertrophic chondrocytes. Class 1Eb mRNA was the second most abundant transcript and its

distribution was uniform across all the cell types at 2 weeks of age. The expression pattern changed with increasing age such that at 6 weeks a gradient existed with hypertrophic chondrocytes expressing higher levels of 1Eb than resting chondrocytes. Class 2Ea mRNA was constitutively expressed at low levels across the growth plate at all ages, while class 2Eb mRNA expression was negligible. The distribution of total IGF-I mRNA also shifted across growth plate cell types as the animals aged from 2 to 6 weeks. These findings suggest that IGF-I class 1 mRNA plays the predominant role in the maturation of the growth plate.

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Introduction

The growth of long bones is driven by the cellular activity of chondrocytes within the growth plate (Isaksson *et al.* 1987, Kember 1993). Small germinal chondrocytes in the resting zone enter the cell cycle and undergo successive mitotic divisions to form columns of chondrocytes in the proliferative zone. After leaving the cell cycle, differentiated chondrocytes hypertrophy and are gradually replaced by bone matrix. Therefore the rate of bone elongation is determined by chondrocyte performance during the progression from resting to proliferative to hypertrophic stages (Hunziker & Schenk 1989).

Insulin-like growth factor-I (IGF-I) mediates the stimulatory effect of growth hormone (GH) on longitudinal bone growth chondrocytes (Daughaday & Rotwein 1989) by increasing the proliferation rate, matrix production and cell hypertrophy of growth plate chondrocytes (Isaksson *et al.* 1990, Hunziker *et al.* 1994). The source of IGF-I that regulates the chondrocyte performance is the circulating IGF-I produced in liver and the autocrine/paracrine IGF-I synthesized from the growth plate (Daughaday & Rotwein 1989, Ohlsson *et al.* 1993). Although IGF-I mRNA has been detected in the proliferative and hypertrophic zones of rat growth plate by *in situ* hybridization (Nilsson *et al.* 1990), the method can neither quantify the abundance of

IGF-I mRNA nor distinguish among different classes of alternative IGF-I mRNA.

Rat tissue IGF-I mRNA is a heterogeneous array of transcripts resulting from different transcription start sites, alternative splicing and various polyadenylation sites (Bucci *et al.* 1989, Adamo *et al.* 1991, Hall *et al.* 1992). Transcription can start at either exon 1 or exon 2, which are exclusively spliced to exon 3. Exon 3, 4 and 6 are present in all transcripts, while exon 5 is alternatively spliced to exon 6. According to the presence of exon 1 (class 1 transcripts) or exon 2 (class 2 transcripts) and the presence (Eb form) or absence (Ea form) of exon 5 in the mRNA, the IGF-I transcripts are classified into class 1Ea, 1Eb, 2Ea and 2Eb mRNA (Fig. 1A) (Holthuisen *et al.* 1991). While these four classes of IGF-I mRNA display variations in developmental regulation, tissue specificity and GH responsiveness (Lowe *et al.* 1987, Adamo *et al.* 1989, Arkins *et al.* 1993), their relative abundance and developmental expression in the growth plate have not been characterized.

In this study, we investigated the distributional patterns of these alternative IGF-I mRNA transcripts in the costochondral growth plates of rats during postnatal development. We used percoll density fractionation to collect growth plate chondrocytes at various stages of differentiation (Oberbauer & Peng 1995) and the

Figure 1 Detection of alternative IGF-I mRNA in the costochondral growth plate. (A) The four classes of IGF-I mRNA and locations of primers for RT-PCR amplification; exons are not drawn to scale and PCR primer sites are indicated by arrows. (B) Electrophoresis of amplified RT-PCR products from growth plate RNA. Lane 1, 100 bp DNA marker; lanes 2–4, 412, 446 and 393 bp DNA amplified from 1Ea, 1Eb and 2Ea mRNA respectively; lane 5, no detected DNA fragment amplified using the primer pair specific for 2Eb mRNA.

abundance of each class of alternative IGF-I mRNA was measured by quantitative reverse transcription (RT) PCR (RT-PCR).

Materials and Methods

Growth plate chondrocyte fractionation

Growth plate chondrocytes at various stages of differentiation were fractionated by discontinuous percoll gradient centrifugation using a protocol modified from that previously described (Oberbauer & Peng 1995). Costochondral growth plates from six male Sprague–Dawley rats were dissected free of adherent tissue, cut to 2–3 mm, pooled and predigested by incubation in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco BRL, Grand Island, NY, USA) containing 0.05% trypsin (JRH Biosciences, Lenexa, KS, USA), 0.5% hyaluronidase (Worthington Biochemical, Freehold, NJ, USA) and 2% fetal bovine serum (FBS) (Gibco) at 37 °C for 45 min. The growth plates were then digested in DMEM/F12 containing 0.3% collagenase type II (Worthington) and 2% FBS at 37 °C for 5 h. After the first 30 min of collagenase digestion, the media with released tissue debris and freed cells were discarded and fresh reagents added. Following the 5 h enzymatic digestion of the extracellular matrix, the freed cells were separated from tissue debris by filtration through a 70 µm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA) and collected from the filtrate by centrifugation at 200 g for 5 min, washed in DMEM/F12 twice and resuspended in 1 ml DMEM/F12 medium. Growth plate cells were layered on a discontinuous isotonic percoll (Pharmacia,

Piscataway, NJ, USA) gradient prepared by weight (densities of 1.0250, 1.0340, 1.0402, 1.0436 and 1.0526 g/ml) and centrifuged at 1875 g for 15 min in a swinging bucket rotor. Five cell fractions were formed after centrifugation: fraction 1 enriched with 90% hypertrophic cells and 10% proliferative cells; fraction 2 consisted of 30% hypertrophic cells and 70% proliferative cells; fraction 3 consisted of 5% hypertrophic cells and 95% proliferative cells; fraction 4 consisted of 75% proliferative cells and 25% resting cells; fraction 5 consisted of 5% proliferative cells and 95% resting cells (Oberbauer & Peng 1995). Each of the five cell fractions was collected, washed with 10 ml DMEM/F12 medium and centrifuged at 500 g for 10 min. Total RNA from the resulting cell pellets was then prepared.

RNA isolation

For RNA isolation from the entire growth plate, costochondral growth plates of five 4-week-old male Sprague–Dawley rats were dissected free of adherent tissue and homogenized in 5 M guanidinium thiocyanate, 17 mM n-lauryl sarcosine, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol (Sigma Chemical Co., St Louis, MO, USA) (Chomczynski & Sacchi 1987). For RNA isolation from fractionated chondrocytes, the cells from each of the five fractions were lysed in the guanidinium solution. Total RNA was then isolated either from the tissue homogenate or fractionated cell lysate (Chomczynski & Sacchi 1987). Total RNA was quantified by spectrophotometric measurement of the absorbance at 260 nm. The integrity of RNA was assessed by electrophoresis in a formaldehyde denaturing 1% agarose gel.

Figure 2 (A) Structure of IGF-I cRNA standard and (B) standard curves for the quantification of three classes of alternative IGF-I mRNA derived from amplification of the varying quantities of input cRNA.

Quantitative RT-PCR analysis

Synthesis of IGF-I cRNA standard To measure the absolute abundance of each class of IGF-I mRNA, an IGF-I cRNA (Fig. 2A) was synthesized as previously described (Lin & Oberbauer 1998). This cRNA contains the primer sequence for each class of IGF-I mRNA (see below) and a polyA tail, therefore it can be reverse transcribed with oligo(dT)₁₂₋₁₈ and PCR amplified with the same primer pair as endogenous IGF-I mRNA. Briefly, a 418 bp IGF-I DNA fragment was synthesized by oligonucleotide overlap extension PCR (Horton *et al.* 1989, Wang *et al.* 1989). This IGF-I synthetic DNA contained the T7 promoter sequence, primer 1F sequence, 2F sequence at the 5' end, (dA)₂₀, primer 5R sequence, 4/6R sequence at the 3' end and an 80 bp-deleted IGF-I exon 3 and exon 4 sequence in the middle. A 395 base cRNA was then synthesized by *in vitro* transcription of the 418 bp IGF-I synthetic DNA with T7 RNA polymerase (MEGAscript *in vitro* transcription kit, Ambion, Austin, TX, USA) (Heuvel *et al.* 1993). Because this IGF-I cRNA carried an 80 bp deletion in the exon 3 sequence, the RT-PCR fragment amplified from this cRNA could be distinguished from the RT-PCR fragment amplified from endogenous IGF-I mRNA on a 2% agarose gel based on the size difference.

PCR primers and amplification of alternative IGF-I mRNA Amplification of each alternative IGF-I mRNA class was done using oligonucleotide primers designed from the published rat IGF-I cDNA sequence (Shimatsu &

Rotwein 1987, Bucci *et al.* 1989, Hall *et al.* 1992) and as described (Lin & Oberbauer 1998). The locations of the primers are shown in Fig. 1A. The exon 1 forward primer (1F) was paired with a reverse primer spanning the junction of exon 4 and 6 (4/6R) to amplify IGF-I class 1Ea, and class 1Eb was amplified with primer 1F in conjunction with a reverse primer specific for exon 5 (5R). IGF-I class 2Ea was amplified using a forward primer specific for exon 2 (2F) and the 4/6R primer, while class 2Eb was amplified using the primer pair 2F and 5R.

To determine what alternative forms of IGF-I were expressed in the growth plate, total RNA isolated from the entire growth plate was amplified at high cycle numbers. Briefly, 1 µg total growth plate RNA was reverse transcribed into first strand cDNA in a 50 µl reaction containing 1 µg oligo(dT)₁₂₋₁₈, Moloney murine leukemia virus reverse transcriptase (MMLVRT) 1 × buffer, 1 mM dNTPs, 40 units RNasin and 300 units MMLVRTase (Promega, Madison, WI, USA) at 39 °C for 60 min, heated to 95 °C for 10 min and quenched on ice. PCR amplification was assembled in a 50 µl reaction containing 2 µl RT reaction product, 30 pmol each of forward and reverse primer, 1 × Thermo buffer, 0.2 mM dNTPs, 2.5 mM MgCl₂ and 1.25 units Taq DNA polymerase (Promega). The reaction was cycled 38 times at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. Twelve microliters of PCR product were electrophoresed on a 2% agarose gel to check the specificity and size of the RT-PCR product.

To quantitatively assess the abundance of each of the alternate IGF-I mRNA forms for each growth plate cellular fraction, the amplification was done at low cycle number to ensure the amplification was in the exponential phase. For these analyses 100 ng samples of total RNA or serial dilutions (10⁻⁶–10⁻² pg) of the 395 base IGF-I cRNA standard were reverse transcribed into first strand cDNA by incubation in a 20 µl reaction containing MMLVRT 1 × buffer, 0.5 mM dNTPs, 0.2 µg oligo(dT)₁₂₋₁₈, 6 units of RNasin and 100 units of MMLVRTase (Gibco) at 39 °C for 60 min, heated to 95 °C for 10 min and quenched on ice. Sixty picomoles of forward primer were end-labeled with [γ-³²P]ATP (6000 Ci/mmol) (New England Nuclear, Boston, MA, USA) by T4 polynucleotide kinase (Pharmacia) and purified with a Sephadex G-25 (Sigma) spin column to remove unincorporated nucleotides. PCR was performed in a 15 µl reaction containing 1.2 µl RT reaction product, 4.8 pmol forward primer, 3 µl (2–3 × 10⁶ c.p.m.) ³²P end-labeled forward primer, 6 pmol reverse primer, 1 × Thermo buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs and 0.55 unit of Taq DNA polymerase (Promega). The first strand cDNA was amplified in the exponential phase of the PCR reaction for 24 or 26 cycles (94 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min). All chondrocyte RNA samples and cRNA standards were reverse transcribed and PCR amplified in parallel tubes at the same

time using the same master mix of reagents. To help visualize the specific PCR product, 6 µl of a nonradioactive PCR product generated from a 35 cycle amplification were added to 6 µl of the radioactive PCR product; this was then electrophoresed in a 2% agarose gel in the presence of ethidium bromide. Gel bands were cut out and incorporated radioactivity determined.

Data analysis

The c.p.m. in the excised gel bands from the amplification of cRNA standard were plotted against the input cRNA standard concentration by log-log transformation. Simple regression analysis was used to determine the linear equation ($\log \text{ c.p.m.} = a + b \times \log \text{ concentration}$) of the standard curve for each class of IGF-I mRNA. The abundance of each class of alternative IGF-I mRNA in the sample was calculated by extrapolating against the standard curve generated in that particular PCR run. The data were expressed as an absolute amount of IGF-I mRNA class (in fg) as a proportion of total RNA (µg) for each cell fraction. The abundance of total IGF-I mRNA was the sum of class 1Ea, 1Eb and 2Ea mRNA abundance. Results were expressed as the means $\pm$ S.E.M. from three replicate chondrocyte fractionation experiments for each age group. Significance of difference was determined by Fisher's least significant difference test using SAS version 6.11 (SAS Institute Inc., Cary, NC, USA).

Results

Detection of alternative IGF-I mRNA in the growth plate

Initial RT-PCR amplification using a high number of amplification cycles was carried out to determine whether the primer pairs could specifically detect the alternative IGF-I mRNA classes. The RT-PCR amplification from the growth plate RNA produced cDNA fragments of expected sizes for class 1Ea, 1Eb and 2Ea mRNA, but not for class 2Eb mRNA (Fig. 1B). This result verified the specificity of the primer pairs and demonstrated that the growth plate expressed 1Ea, 1Eb and 2Ea IGF-I mRNA. The absence of 2Eb mRNA in the growth plate did not reflect a defect in the primer pair because the 2Eb form was detected in mouse hepatic tissue (Lin & Oberbauer 1998).

Standard curves

RT-PCR amplification of increasing amounts of IGF-I cRNA (10^{-4} – 10^2 pg) for 24 or 26 cycles directly correlated with increasing amounts of RT-PCR products as measured by the radioactivity of cut gel bands (c.p.m.). As shown in Fig. 2B, the relationship between log pg cRNA input and log c.p.m. RT-PCR output was linear for the

Figure 3 Total IGF-I in growth plate chondrocytes representing various stages of differentiation was calculated as a sum of class 1Ea, 1Eb and 2Eb mRNA as measured by quantitative RT-PCR. F1–F5 represent the fractions of chondrocytes from percoll separation. Two-week-old resting cells contained greater total IGF-I than resting cells at 4 or 6 weeks of age ($P < 0.05$) while hypertrophic cells of 6-week-old rats had greater IGF-I levels than those for 2- or 4-week-olds ($P < 0.05$).

analysis of class 1Ea, 1Eb or 2Ea mRNA. The expression of 2Eb mRNA in growth plate chondrocytes was much lower than the linear sensitivity of the RT-PCR assay; therefore the abundance of 2Eb mRNA was not analyzed.

Distribution patterns of alternative IGF-I mRNA in the growth plate

To study the developmental expression of alternative IGF-I mRNA in particular regions of the growth plate, the levels of each class of IGF-I mRNA were measured in percoll-fractionated chondrocytes from 2-, 4- and 6-week-old rats. Percoll fractions 5, 3 and 1 represented relatively pure populations of resting, proliferative and hypertrophic chondrocytes respectively (Oberbauer & Peng 1995).

The expression pattern of total IGF-I mRNA (the sum of levels of IGF-I mRNA classes detected) changed from 2 to 6 weeks of age (Fig. 3). At 2 weeks, the levels of total IGF-I mRNA in resting, proliferative and hypertrophic chondrocytes were not significantly different ($P > 0.05$). At 4 weeks, the expression of total IGF-I mRNA peaked in the proliferative chondrocytes and the abundance (23.18 ± 4.67 fg/µg total RNA) was significantly higher than in resting and hypertrophic chondrocytes (1.98 ± 0.76 and 6.46 ± 1.47 fg/µg total RNA, $P < 0.05$). At 6 weeks, the peak expression of total IGF-I mRNA shifted from proliferative chondrocytes (15.38 ± 1.83 fg/µg total RNA) to hypertrophic (26.25 ± 5.66 fg/µg total RNA, $P < 0.05$). The resting chondrocytes expressed higher levels of total IGF-I mRNA at 2 weeks of age (8.23 ± 2.91 fg/µg total RNA) than at 4 or 6 weeks (1.98 ± 0.76 and 2.91 ± 0.56 fg/µg total RNA respectively, $P < 0.05$). The levels of total IGF-I mRNA in proliferative chondrocytes were not significantly different during the postnatal period from 2 to 6 weeks ($P > 0.05$),

while the hypertrophic chondrocytes expressed higher levels of total IGF-I mRNA at 6 weeks than at 2 or 4 weeks ($P<0.05$).

At all ages studied, 1Ea mRNA was the most abundant class expressed in the costochondral growth plate (Fig. 4). At 2 weeks of age, proliferative chondrocytes expressed significantly higher levels of 1Ea mRNA (11.55 ± 2.14 fg/ μ g total RNA) than the hypertrophic (4.50 ± 1.60 fg/ μ g total RNA) ($P<0.05$). At 4 weeks, this was still true: 19.55 ± 3.90 , 0.99 ± 0.41 , 1.83 ± 0.40 fg/ μ g total RNA for proliferative, resting and hypertrophic chondrocytes respectively ($P<0.05$). At 6 weeks, the abundance of 1Ea mRNA in proliferative chondrocytes (12.47 ± 1.10 fg/ μ g total RNA) was significantly lower than that of hypertrophic chondrocytes (21.24 ± 3.34 fg/ μ g total RNA), but higher than that of resting chondrocytes (1.96 ± 0.45 fg/ μ g total RNA) ($P<0.05$).

Class 1Eb mRNA was the second most abundant class expressed in growth plate chondrocytes (Fig. 4). In 2-week-old rats, the growth plate chondrocytes expressed similar levels of 1Eb mRNA across the growth plate (ranging from 2.59 to 3.72 fg/ μ g total RNA). In 4-week-old rats, the level of 1Eb mRNA was low in resting chondrocytes (0.67 ± 0.28 fg/ μ g total RNA) and significantly higher ($P<0.05$) in hypertrophic chondrocytes (3.70 ± 0.82 fg/ μ g total RNA). In 6-week-old rats, 1Eb mRNA abundance was significantly higher in hypertrophic chondrocytes (3.96 ± 2.01 fg/ μ g total RNA) relative to that detected for resting chondrocytes (0.74 ± 0.07 fg/ μ g total RNA) ($P<0.05$).

Class 2Ea mRNA was expressed at very low levels in growth plate chondrocytes (ranging from 0.20 to 1.34 fg/ μ g total RNA). The levels were not significantly different across the growth plate in 2- and 6-week-old rats ($P>0.05$), although in 4-week-old rats, the levels of 2Ea mRNA in proliferative and hypertrophic chondrocytes were higher than those determined for resting chondrocytes ($P<0.05$).

Discussion

We have previously described a density separation method to generate cell fractions representing growth plate chondrocytes at various stages of differentiation with each fraction exhibiting morphology, proliferative rates and marker mRNA expression consistent with *in vivo* positional phenotypes (Oberbauer & Peng 1995). In the present study, by applying quantitative RT-PCR analysis to fractionated chondrocytes, we showed that total IGF-I mRNA was differentially expressed in the rat growth plate.

The total IGF-I mRNA distribution within the rat growth plate was altered during the 2 to 6 week growth period. The 2-week-old rat resting chondrocytes expressed significantly higher levels of IGF-I mRNA than

Figure 4 Expression of alternative IGF-I mRNA classes in percoll-fractionated growth plate chondrocytes representing various stages of differentiation; mRNA abundance was determined by quantitative RT-PCR. F1–F5 represent fractions 1–5 from percoll separation. (A) Cells isolated from 2-week-old rats, * designates that the 1Ea form was significantly elevated in proliferative fraction 3 ($P<0.05$) relative to the hypertrophic fraction 1. (B) Cells isolated from 4-week-old rats, * designates that the 1Ea form was significantly ($P<0.05$) elevated in proliferative fraction 3 relative to all other fractions while # designates significantly higher ($P<0.05$) levels of the 1Eb form relative to that of fractions 4 and 5, resting cells. (C) Cells isolated from 6-week-old rats; * designates levels of 1Ea and 1Eb forms significantly elevated relative to levels in the resting cells ($P<0.05$).

resting cells from 4- and 6-week-old rats suggesting that IGF-I may play a role in stimulating resting chondrocytes to enter the cell cycle during early postnatal development. This idea is supported by the *in vivo* observation that IGF-I

treatment shortens cell cycle times in resting chondrocytes (Hunziker *et al.* 1994). In the 4-week-old rat, the predominant expression of IGF-I mRNA in the proliferative chondrocytes correlates with the important role of IGF-I in promoting clonal expansion (Nilsson *et al.* 1986, Lindahl *et al.* 1987), shortening cycle time and increasing cell volume of chondrocytes in the proliferative zone (Hunziker *et al.* 1994). Our finding that at 4 weeks of age proliferative chondrocytes expressed much higher levels of total IGF-I mRNA than resting and hypertrophic chondrocytes is in accordance with the immunolocalization of IGF-I to the proliferative zone reported by Nilsson *et al.* (1986). The increased total IGF-I mRNA expression in 6-week-old hypertrophic chondrocytes supports a role for IGF-I modulation of cellular hypertrophy by increasing cell height and cell volume at later ages (Hunziker *et al.* 1994). Taken together, the changes of IGF-I mRNA distribution within the growth plate confirm that IGF-I plays an important role in chondrocyte function at all stages of differentiation. Additionally, its primary site of action may shift from resting chondrocytes to proliferative chondrocytes and, further, to hypertrophic chondrocytes during the postnatal period from 2 to 6 weeks of age.

Specific IGF-I mRNA classes also exhibited temporal and spatial regulation within distinct zones of the growth plate during postnatal development. Growth plate chondrocytes expressed high levels of class 1Ea and 1Eb mRNA, indicating that the transcription start sites on leader exon 1 were more frequently used than those on leader exon 2 in the growth plate. While the 1Ea class was the most prevalent, the relative abundance of 1Eb mRNA in resting, proliferative or hypertrophic chondrocytes of the present study (25–30%, 12–22% and 15–60% of total IGF-I mRNA respectively) was much higher than that reported for extrahepatic tissues (~2–5% of total IGF-I mRNA) and the same or higher than that for liver (13% of total IGF-I mRNA as reported by Lowe *et al.* (1988)). As GH is known to regulate total IGF-I mRNA in the rat growth plate (Isgaard *et al.* 1988, Nilsson *et al.* 1990) and can increase both class 1 and class 2 mRNA abundance (Lowe *et al.* 1987, Lin & Oberbauer 1998) while promoting the inclusion of exon 5 (Lowe *et al.* 1988), the abundance of the exon 1-containing transcripts and the significant expression of 1Eb mRNA in growth plate chondrocytes may represent preferential upregulation of exon 1- and exon 5-containing IGF-I mRNA transcripts in growth plate chondrocytes by GH.

In the growth plate, as in bone (West *et al.* 1996), class 1 transcripts were the major contributors to total IGF-I mRNA. The mechanism by which the distribution pattern of alternative IGF-I mRNA is regulated during this postnatal period is not clear. It is known, however, that GH increases total IGF-I mRNA and immunoreactivity in the growth plate of the hypophysectomized rat (Nilsson *et al.* 1986, Isgaard *et al.* 1988) and regulates the choice of IGF-I transcription initiation (Foyt *et al.*

1992). In the present study, we observed that hypertrophic chondrocytes expressed higher levels of total IGF-I mRNA in later development (4- to 6-week-old rats). At these ages, GH receptor mRNA is more abundant in mature hypertrophic chondrocytes (Oberbauer & Peng 1995). We suggest that the shift of IGF-I mRNA expression from proliferative to hypertrophic chondrocytes from 4 to 6 weeks of age is mediated by GH to increase the degree of chondrocyte hypertrophy and facilitate bone formation.

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