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Effect of Fibroblast Growth Factors 1, 2, 4, 5, 6, 7, 8, 9, and 10 on Avian Chondrocyte Proliferation

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Abstract It has been demonstrated that fibroblast growth factor receptors are key regulators of endochondral bone growth. However, it has not been determined what fibroblast growth factor ligand(s) (FGFs) are important in this process. This study sought to determine whether FGFs 1, 2, 4, 5, 6, 7, 8, 9, and 10 were capable of stimulating avian chondrocyte proliferation in vitro. We have found that FGFs 2, 4, and 9 strongly stimulate avian chondrocyte proliferation while FGFs 6 and 8 stimulate proliferation to a lesser extent. RT-PCR indicates that FGF-2 and FGF-4 are expressed in the postnatal avian epiphyseal growth plate (EGP) while FGF-8 and FGF-9 are not. Thus, FGF-2 and FGF-4 stimulate chondrocyte proliferation and are both present in the EGP. This suggests that FGF-2 and FGF-4 may be important ligands, in vivo, for the regulation of endochondral bone growth. These observations coupled with our observation that multiple avian FGF receptors (Cek1, Cek2, Cek3, and FREK) are expressed in proliferative chondrocytes highlights the complexity of FGF signaling pathways in postnatal endochondral bone growth. J. Cell. Biochem. 84: 359–366, 2002. © 2001 Wiley-Liss, Inc.

Key words: fibroblast growth factor; endochondral bone growth; cartilage; chondrocyte

Fibroblast growth factor signaling pathways are essential for normal endochondral bone growth. Thanatophoric dysplasia (TD), a lethal skeletal disorder, achondroplasia (ACH), the most common form of human dwarfism, and hypochondroplasia, a less severe form of dwarfism are all caused by different mutations in fibroblast growth factor receptor 3 (FGFR3) [Rousseau et al., 1994; Shiang et al., 1994; Prinos et al., 1995; Rousseau et al., 1995; Tavormina et al., 1995]. The mutations associated with ACH and TD cause constitutive activation of the FGFR3 receptor [Naski et al., 1996].

Studies using transgenic mice have shown that overexpression of FGF-2 or FGF-9 causes inhibition of chondrocyte hypertrophy and subsequent endochondral bone growth, while knockout of the FGFR3 gene causes an enlargement of the hypertrophic zone and enhanced endochondral bone growth [Coffin et al., 1995; Colvin et al., 1996; Deng et al., 1996; Garofalo et al., 1999]. In addition, a number of in vitro studies have demonstrated that FGF-2 is capable of inhibiting chondrocyte differentiation [Kato and Iwamoto, 1990; Suzuki, 1992; Trippel et al., 1993]. Thus, one role for the FGF signaling pathway in the epiphyseal growth plate (EGP) is the inhibition of hypertrophy.

FGFs affect chondrocyte proliferation as well as differentiation but the nature of that effect is in dispute. Knockout of FGFR3 results in increased chondrocyte proliferation [Deng et al., 1996], while the constitutive activating mutation of FGFR3 that causes ACH leads to an inhibition of chondrocyte proliferation in transgenic mice [Segev et al., 2000]. FGF-9 over-expression also inhibits chondrocyte proliferation [Garofalo et al., 1999]. However, these studies are in conflict with the observations that over-expression of FGF-2 results in an expansion of the proliferative zone of the growth plate and a neonatal lethal mutation that causes constitutive activation of FGFR3 enhances chondrocyte proliferation in transgenic mice [Coffin et al., 1995; Iwata et al., 2000].

Our laboratory has demonstrated that FGF-2 is a potent permissive mitogen which greatly enhances avian chondrocyte proliferation in a serum-free culture system [Rosselot et al., 1994; Luan et al., 1996]. Other laboratories have made similar observations on the in vitro effects

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of FGF-2 on the proliferation of chicken, as well as rat, rabbit, and bovine growth plate chondrocytes [Hiraki et al., 1988; Trippel et al., 1993; O'Keefe et al., 1994; Wroblewski and Edwall-Arvidsson, 1995]. More recently, Weksler et al. [1999] have shown that a rat chondrogenic cell line responds to FGF-9 as well as FGF-2. In contrast, Sahni et al. [1999] have observed that the proliferation of primary murine chondrocytes is inhibited by FGF-1. Finally, Nagai et al. [1995] have demonstrated that administration of low doses of FGF-2 to rats enhances bone formation while higher doses inhibit bone growth. All these studies demonstrate some effect of FGFs or FGFRs on chondrocyte proliferation, whether that effect is inhibitory or stimulatory seems to vary with the system under study.

It is clear that FGF signaling plays an important role in the regulation of endochondral bone growth and that FGFR3 is a key receptor in this pathway. However, it has not been determined what FGF is the important ligand that regulates endochondral bone growth. The FGF family of ligands has at least 21 members, and many of these have been studied utilizing knockout mice [reviewed by Zhou et al., 1998; Xu et al., 1999; Montero et al., 2000; Moon et al., 2000; Sun et al., 2000]. Studies of single knockouts of FGFs 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 have not revealed which FGF ligand is essential for postnatal endochondral bone growth. The ligand important in regulating endochondral bone formation could be any one of the remaining unstudied members of the FGF family or another member of the FGF family could compensate for the knockout of a single FGF. This question has begun to be addressed by the experiments of Miller et al. [2000] who have shown that both single and double knockouts of FGF-1 and FGF-2 have no effect on endochondral bone growth.

We have taken a pragmatic approach in attempting to elucidate the FGFs' importance in endochondral bone growth. We have examined the effects of FGFs 1, 2, 4, 5, 6, 7, 8, 9, and 10 on the proliferation of cultured chondrocytes. These studies in conjunction with the use of RT-PCR show that FGFs 2 and 4 strongly stimulate proliferation and are present in the growth plate and thus may be important to endochondral bone growth in vivo. In addition, our observations that four different avian FGF receptors (Cek1, Cek2, Cek3, FREK) are expressed by proliferative chondrocytes demonstrates the complexity of the FGF signaling pathway in the growth plate.

MATERIALS AND METHODS

Chondrocyte Isolation

Articular cartilage chondrocytes as well as proliferative and hypertrophic zone growth plate chondrocytes were isolated from the proximal end of the tibiotarsi as previously described [Rosselot et al., 1992; Rousche et al., 2001]. Male Avian × Avian chicks (Longnecker's Hatchery, Elizabethtown, PA) were raised on commercial broiler feed at The Pennsylvania State University Poultry Education and Research Center. At 4 weeks of age, the chicks were sacrificed by cervical dislocation and their tibiotarsi removed using a protocol approved by IACUC (# 99R024-0). Under aseptic conditions, the outer surface of the proximal articular cartilage was removed and discarded. Thin slices of inner articular cartilage tissue were collected and minced in Ham's F12 nutrient media (F12). The remaining articular cartilage was then discarded and the exposed growth plate was scraped gently with a scalpel blade to collect the proliferative cell layer. The transitional or pre-hypertrophic zone was then discarded and thin slices of the hypertrophic cell layer were collected and minced in F12. Articular cartilage, proliferative, and hypertrophic tissues were incubated separately at 37°C for 15 min in F12 containing 0.083% trypsin (Worthington Biochemical Corp., Freehold, NJ) and 0.017% hyaluronidase (Sigma, St. Louis, MO). Following two rinses with F12, proliferative and hypertrophic tissues were incubated separately in F12 containing 0.10% type 1A collagenase (Sigma) for 3 h at 37°C. Articular cartilage tissue was also rinsed twice with F12 and then incubated in F12 containing 0.15% type 1A collagenase for 4.5 h at 37°C. All digested tissues were passed through a 149 μm nylon mesh, rinsed twice with F12, and then resuspended in F12.

Proliferation Assay

Proliferative zone chondrocytes were plated in 96-well, tissue culture treated plates at a density of 2.2×10^5 cells per cm^2. The plating media was modified Webbers media containing 5% newborn calf serum (Sigma) [Rosselot et al., 1992]. After approximately 18 h of culture at

37°C, the media was discarded and the cells were rinsed briefly using Dulbecco's PBS with 0.001% calcium chloride. Cells were then incubated in a growth factor mixture consisting of 25 ng/ml recombinant human insulin-like growth factor-1 (IGF-1) (Bachem, King of Prussia, PA), 1 ng/ml porcine transforming growth factor-β (TGF-β) (R&D Systems, Minneapolis, MN) and varying concentrations of one of the FGFs listed below. FGF-1, FGF-2, FGF-9, and FGF-10 are recombinant human growth factors that were obtained from R&D Systems. Recombinant human FGF-4, FGF-5, FGF-6, and FGF-7 as well as recombinant mouse FGF-8b were purchased from Sigma. Forty-eight hours after the cells were initially plated, the growth factor mixture was supplemented with 1 μCi per well of methyl-^{3}H-thymidine (ICN, Irvine, CA). Approximately 65 h after the cells were initially plated they were detached using 0.4 mg per well of protease in 0.15 M Tris-HCl and 0.1 M EDTA (pH 8.0) and transferred onto filter disks (Skatron Instruments, Sterling, VA). The disks were then immersed in liquid scintillation fluid and counted.

RT-PCR

Total RNA was isolated from 4-day-old chicken embryos and from total growth plate tissue taken from the proximal tibiotarsi of 4-week-old chickens using TRIzol reagent (Life Technologies Inc., Rockville, MD) according to the manufacturer's directions.

Contaminating DNA was removed from isolated total RNA using DNA-free according to the manufacturer's instructions (Ambion, Austin, TX). Total RNA was reverse transcribed with random-priming using standard conditions (Promega, Madison, WI).

The PCR primers used are listed in Table I. The primer sequences of FGF-2, FGF-4, and FGF-8 are based on the published chicken sequences and have been used previously to amplify these sequences from chicken cDNA [Luan et al., 1996; Mitchell et al., 1999]. The primers for FGF-9 were based on conserved sequences found in the published sequences of the human, mouse, rat, and Xenopus FGF-9 genes [Miyamoto et al., 1993; Santos-Ocampo et al., 1996; Song and Slack, 1996]. The PCR reaction mixtures contained: 200 ng of reverse transcribed RNA, 0.4 μM of each primer, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.65 mM $MgCl_2$, 0.1% Triton X-100, 200 μM each of dATP, dCTP, dGTP, dTTP (Life Technologies), and 2.5 U of Taq polymerase (Promega). Samples were subjected to 35 cycles at 95°C for 1 min, 55°C for 2 min, and 72°C for 1 min in a Stratagene robocycler gradient 40 (Stratagene, La Jolla, CA). Following amplification PCR products were electrophoresed in a 1.7% agarose gel along with a 100 base pair DNA ladder (Life Technologies). The identities of the PCR products were confirmed by their size as well as by their digestion with a diagnostic restriction enzyme (Table I). The identity of FGF-9 was confirmed by direct sequencing of the product [unpublished data].

TABLE I. Primers, Product Size, and Diagnostic Restriction Enzymes Used in the PCR Analysis of FGF Expression

	Forward/reverse primers	Product size/ diagnostic restriction enyme
FGF-2	acggcgtccgcgagaaga	279bp
	gtccaggtccagtttttg	Taq I
FGF-4	gtctctattgcaacgtgg	276 bp
	tcccgggataaatcctgg	BamH I
FGF-8	agcacgtgcagatcttgg	335 bp
	aagtgcacctcgcgttgg	Pst I
FGF-9	tgggagctatttcggtgtgc	529 bp
	tttctggtgccgtttagtcc	Directly sequenced

Northern Analysis of FGF Receptor Expression

Articular, proliferative, and hypertrophic chondrocytes were obtained as described above. Chondrocytes (1×10^7 cells of each type) were lysed in 1 ml of TRIzol reagent and RNA was isolated according to the manufacturer's instructions. Twenty micrograms of total RNA from each chondrocyte type was electrophoresed, blotted, and probed according to standard protocols [Ausubel et al., 1989]. The cDNAs of chicken FGF receptors Cek1 (homologous to mammalian FGFR1), Cek2 (homologous to mammalian FGFR3), and Cek3 (homologous to mammalian FGFR2) were used as probes and were kindly provided by Dr. Elena Pasquale (The Burnham Institute, La Jolla, CA) [Pasquale and Singer, 1989; Pasquale, 1990]. The quail FGF receptor FREK (homologous to Z-FGFR4 from the zebrafish [Thisse et al., 1995] and to PFR4 from the amphibian Pleurodeles; and similar to FGFR4 from mammals) cDNA was also used as a probe and was kindly provided by Dr. Christophe Marcelle

(Division of Biology, Beckman Institute, Pasadena, CA) [Marcelle et al., 1994].

RESULTS

The effect of various concentrations of FGFs 1, 2, 4, 5, 6, 7, 8, 9, and 10 on avian chondrocyte proliferation was tested in vitro, in the presence of IGF-1 and TGF-β but in the absence of serum (Fig. 1). Chondrocyte proliferation was strongly stimulated by FGF-2, FGF-4, and FGF-9.

At 0.61 nM FGF-2, FGF-4, or FGF-9 there is a very significant ($P < 0.01$) stimulation of chondrocyte proliferation (Fig. 2). At this concentration, there was a much smaller, yet statistically significant ($P < 0.05$), stimulation of chondrocyte proliferation by FGF-6 and FGF-8. Even at levels as high as 6.1 nM, there was no enhancement of proliferation by FGF-5, FGF-7, or FGF-10 (data not shown). However, FGF-1 does stimulate proliferation at this concentration, albeit to a level not equal to that of 0.061 nM FGF-2, FGF-4, or FGF-9 (data not shown).

RT-PCR of RNA isolated from avian growth plates demonstrates that FGF-2 and FGF-4 were present while FGF-8 and FGF-9 could not be detected (Fig. 3). All four FGFs could be easily detected in RNA isolated from 4-day-old chicken embryos.

Northern blot hybridization was performed to determine what avian FGF receptors were expressed in articular, proliferative, and hypertrophic chondrocytes (Fig. 4). In proliferative chondrocytes, Cek2 and FREK were easily detected while Cek1 and Cek3 were weakly expressed. The levels of Cek1, Cek2, and FREK all declined in hypertrophic chondrocytes while Cek3 expression was increased in comparison to proliferative chondrocytes.

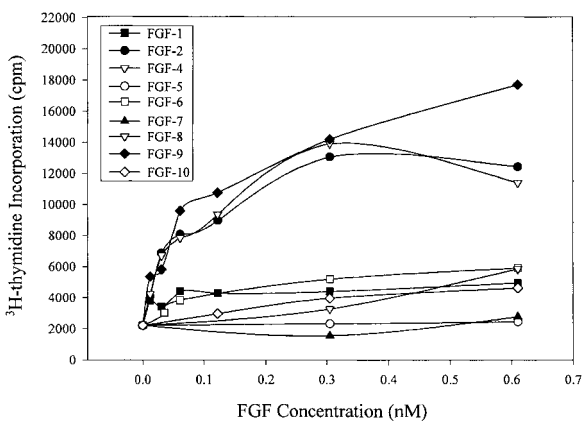

Fig. 1. The effects of FGFs 1, 2, 4, 5, 6, 7, 8, 9, and 10 on chondrocyte proliferation. Proliferative zone chondrocytes were treated with various doses of FGF 1, 2, 4, 5, 6, 7, 8, 9, or 10 in serum-free media containing 25 ng/ml IGF-1 and 1 ng/ml TGF-β. ^{3}H-thymidine incorporation was measured to determine cell proliferation.

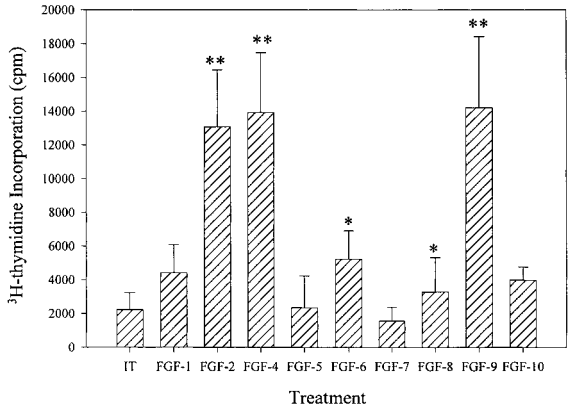

Fig. 2. The effect of 0.61 nM FGF 1, 2, 4, 5, 6, 7, 8, 9, and 10 on chondrocyte proliferation. Proliferative zone chondrocytes were incubated with 0.61 nM FGF 1, 2, 4, 5, 6, 7, 8, 9, or 10 in serum-free media containing 25 ng/ml IGF-1 and 1 ng/ml TGF-β. Control chondrocytes were incubated in serum-free media containing 25 ng/ml IGF-1 and 1 ng/ml TGF-β alone (IT). ^{3}H-thymidine incorporation was measured to determine cell proliferation. *Indicates treatment is significantly different than control ($P < 0.05$).**Indicates treatment is significantly different than control ($P < 0.01$).

DISCUSSION

Previous studies from our laboratory indicated that FGF-2 is a powerful permissive stimulator of avian epiphyseal chondrocyte proliferation [Rosselot et al., 1994; Luan et al., 1996]. This work supports those observations and adds FGF-4 and FGF-9 to the list of FGFs that are capable of strongly stimulating chondrocyte proliferation in vitro. FGFs 6 and 8 are also mitogenic but are less active on a molar basis. FGFs 5, 7, and 10 show no significant stimulation of proliferation at any level. Our observations of the positive effect that some FGFs have on chondrocyte proliferation in vitro are supported by a number of other studies that have demonstrated the stimulatory effects of FGF-2 [Hiraki et al., 1988; Trippel et al., 1993; O'Keefe et al., 1994] and FGF-9 [Weksler et al., 1999] in vitro.

Although our culture system is subject to many of the drawbacks of other in vitro culture systems, we believe it is superior because it employs primary cells in a serum-free media. It should be noted that in our system, serum, IGF,

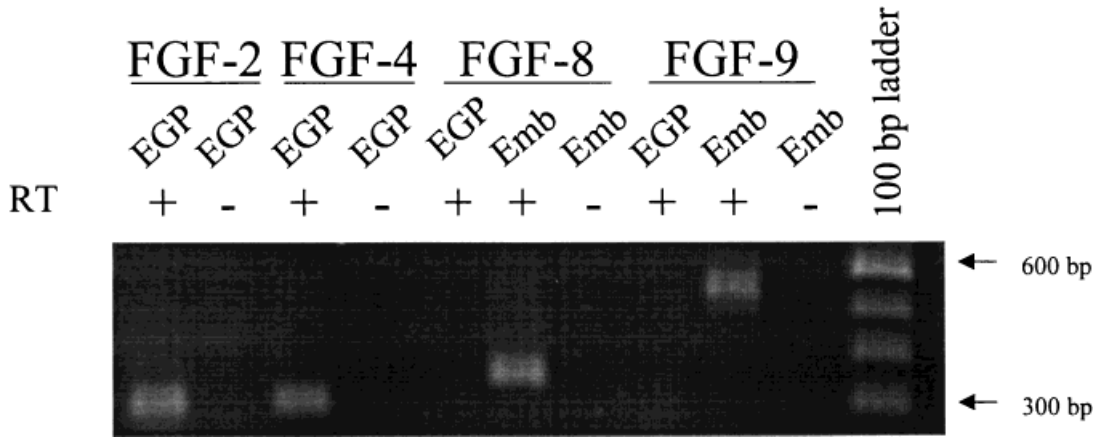

Fig. 3. The expression of FGFs 2, 4, 8, and 9 in the epiphyseal growth plate (EGP). Total RNA was isolated from the EGP and subjected to RT-PCR. Negative controls were not reverse transcribed prior to PCR. Total RNA isolated from chick embryos (Emb) was used as a positive control.

TGF-β, or FGFs alone have little or no mitogenic effect on proliferative chondrocytes. The FGFs are permissive mitogens since they significantly stimulate proliferation only in the presence of IGF-1 and TGF-β, or serum [Rosselot et al., 1994]. While this study utilized our serum-free culture system, preliminary data indicates that all of the responses seen above are the same if the FGFs are assayed in 5% serum (data not shown).

In our assays, FGF-1 had little effect on chondrocyte proliferation, and it is questionable if stimulation by FGF-1 at 6.1 nM is physiologically relevant. In agreement with our observations is Weksler et al. [1999] who observed that FGF-1 (0.61 nM) had no effect on the proliferation of a rat chondrogenic cell line. These observations are surprising in light of the work of Ornitz et al. [1996], who assayed the ability of FGFs to stimulate individual FGFRs and their splice variants in vitro. Their system utilized BaF3 cells, which lack FGFR expression. Using this cell line they expressed FGFRs one at a time and tested the effects of FGFs 1–9. They found that FGF-1 was a universal ligand in the sense that it was capable of maximally stimulating all FGFR splice variants.

We have detected the expression of avian FGF receptors Cek1, Cek2, Cek3, and FREK in proliferative chondrocytes. Our observations generally agree with other studies that have shown a broad distribution of the mammalian FGF receptors FGFR1, FGFR2, and FGFR3 in the growth plate [Peters et al., 1993; Gonzalez et al., 1996; Delezoide et al., 1998; Hamada et al., 1999]. The fact that we observed several FGFs capable of stimulating proliferation while FGF-1 is ineffective implies that when multiple FGF receptor types are present, the interactions between ligand and receptors may produce results not predicted by assay systems that lack such complexity.

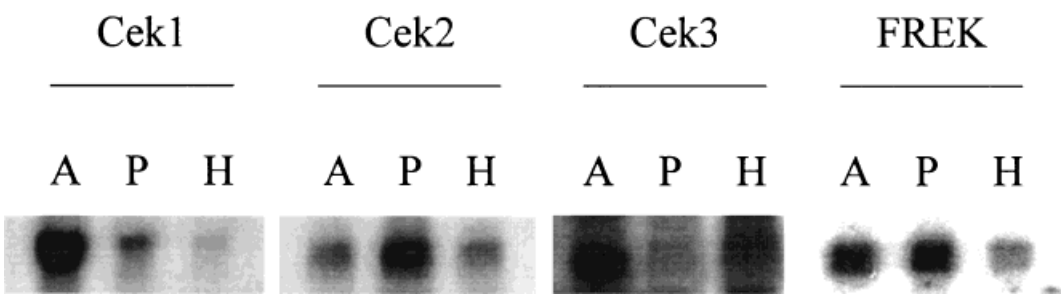

Fig. 4. The expression of Cek1, Cek2, Cek3, and FREK in articular, proliferative, and hypertrophic chondrocytes. Total RNA was isolated from articular (A), proliferative (P), and hypertrophic (H) chondrocytes and analyzed for the presence of the chicken FGF receptors Cek1, Cek2, Cek3, and FREK by Northern blotting.

Studies examining the role of FGF signaling in vivo are divided as to the effect of FGFs on proliferation. Over-expression of FGF-2 and constitutive activation of FGFR3 are reported to stimulate chondrocyte proliferation [Coffin et al., 1995; Iwata et al., 2000]. Many other in vivo studies conclude that FGF signaling inhibits chondrocyte proliferation. Knockout of FGFR3 stimulates chondrocyte proliferation [Deng et al., 1996] while over-expression of FGF-9 [Garofalo et al., 1999], or in some cases constitutive activation of FGFR3, inhibits chondrocyte proliferation [Segev et al., 2000]. However, an in vivo study examining the FGFR3 mutation that causes TD demonstrated no difference in the proliferative response of wild type and TD chondrocytes when stimulated by FGF-2 or FGF-9 [Legeai-Mallet et al., 1998].

These conflicting observations on the effect of FGF on chondrocyte proliferation can perhaps be explained by the work of Liu et al. [1998], who demonstrated that FGF-2 could have either stimulatory or inhibitory effects on the proliferation of cells depending on the activation state of the mitogen-activated protein (MAP) kinase pathway. They observed that FGF-2 signaling inhibited proliferation when MAP kinase was chronically stimulated, while FGF-2 enhanced proliferation when MAP kinase was transiently stimulated. Lending some additional support to this idea is the work of Henderson et al. [2000], who found that FGF-1 could stimulate the proliferation of a chondrocytic cell line while a constitutively activating FGFR3 mutation inhibited the proliferation of the same cell line. Constitutively activating mutations of FGFR3 or over-expression of FGFs could cause an inhibition of chondrocyte proliferation, while under normal conditions chondrocyte proliferation is stimulated by FGF.

Naski et al. [1998] have suggested that FGF signaling is also responsible for inhibiting the transition from resting to proliferation. This theory is supported by the work of Chen et al. [1999], who examined the growth plates of mice expressing an FGFR3 ACH mutation and noted both a reduced zone of proliferation and an enlarged resting zone. These effects may be confounding the interpretation of the role that FGFs have on chondrocyte proliferation.

This study is the first examination of FGFs 4, 8, and 9 expression in the postembryonic EGP and illustrates the complexity of the FGF signaling system. We have previously observed FGF-2 in the EGP by immunohistochemistry, Western blotting, and PCR [Twal et al., 1994; Luan et al., 1996], and FGFs 4, 8, and 9 have all been detected during embryonic limb development or during embryonic intramembranous bone growth [Crossley et al., 1996; Kim et al., 1998]. While proliferative chondrocytes express the FGF receptors Cek1, Cek2, Cek3, and FREK, and can be stimulated to proliferate by FGFs 2, 4, 8, and 9, RT-PCR shows that only FGF-2 and FGF-4 are expressed in the avian growth plate. Therefore, we believe FGF-2 and FGF-4 merit further study to determine if either or both are the key FGF ligand for endochondral bone growth.

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