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Fibroblast growth factor expression in the postnatal growth plate

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Abstract

Fibroblast growth factor (FGF) signaling is essential for endochondral bone formation. Mutations cause skeletal dysplasias including achondroplasia, the most common human skeletal dysplasia. Most previous work in this area has focused on embryonic chondrogenesis. To explore the role of FGF signaling in the postnatal growth plate, we quantitated expression of FGFs and FGF receptors (FGFRs) and examined both their spatial and temporal regulation.

Toward this aim, rat proximal tibial growth plates and surrounding tissues were microdissected, and specific mRNAs were quantitated by real-time RT-PCR. To assess the FGF system without bias, we first screened for expression of all known FGFs and major FGFR isoforms. Perichondrium expressed FGFs 1, 2, 6, 7, 9, and 18 and, at lower levels, FGFs 21 and 22. Growth plate expressed FGFs 2, 7, 18, and 22. Perichondrial expression was generally greater than growth plate expression, supporting the concept that perichondrial FGFs regulate growth plate chondrogenesis. Nevertheless, FGFs synthesized by growth plate chondrocytes may be physiologically important because of their proximity to target receptors. In growth plate, we found expression of FGFRs 1, 2, and 3, primarily, but not exclusively, the c isoforms. FGFRs 1 and 3, thought to negatively regulate chondrogenesis, were expressed at greater levels and at later stages of chondrocyte differentiation, with FGFR1 upregulated in the hypertrophic zone and FGFR3 upregulated in both proliferative and hypertrophic zones. In contrast, FGFRs 2 and 4, putative positive regulators, were expressed at earlier stages of differentiation, with FGFR2 upregulated in the resting zone and FGFR4 in the resting and proliferative zones. FGFRL1, a presumed decoy receptor, was expressed in the resting zone.

With increasing age and decreasing growth velocity, FGFR2 and 4 expression was downregulated in proliferative zone. Perichondrial FGF1, FGF7, FGF18, and FGF22 were upregulated.

In summary, we have analyzed the expression of all known FGFs and FGFRs in the postnatal growth plate using a method that is quantitative and highly sensitive. This approach identified ligands and receptors not previously known to be expressed in growth plate and revealed a complex pattern of spatial regulation of FGFs and FGFRs in the different zones of the growth plate. We also found temporal changes in FGF and FGFR expression which may contribute to growth plate senescence and thus help determine the size of the adult skeleton.

Keywords: FGF; FGFR; Growth plate; Senescence; Chondrocyte

Introduction

Long bones form initially from mesenchymal cells that subsequently differentiate into chondrocytes. Within these cartilage structures, ossification centers arise and expand, converting most of the cartilage into bone tissue. However, a thin band of cartilage located near the end of the long bone persists well into postnatal life. This cartilaginous structure, the growth plate, is responsible for longitudinal bone growth [1].

The growth plate is composed of three principal zones: the resting zone, the proliferative zone, and the hypertrophic zone. Resting zone chondrocytes appear to serve as a pool of stem-like cells that can generate new clones of chondrocytes, which are arranged in columns aligned parallel to the long axis of the bone [2]. Within the columns, which are located in the proliferative zone, the cells proliferate rapidly, but then undergo

Abbreviations: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor.

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terminal differentiation and enlarge, forming the hypertrophic zone. Hypertrophic chondrocytes finally undergo apoptosis, and the hypertrophic zone is invaded by blood vessels, osteoclasts, and differentiating osteoblasts, which remodel the newly formed cartilage into bone tissue [3,4]. This combined process of chondrogenesis and ossification leads to progressive lengthening of the bone.

During postnatal life, the rate of chondrocyte proliferation and hence the rate of longitudinal bone growth slows with age and eventually approach zero as the animal approaches its adult size. This functional change in the growth plate is accompanied by structural changes; with age, the number of resting, proliferative, and hypertrophic chondrocytes decreases as does the size of the individual hypertrophic cells. The chondrocyte columns also become more widely spaced. This decline in growth plate function and cellularity is termed growth plate senescence [5].

This process of endochondral ossification is coordinated by multiple extracellular signaling molecules including the fibroblast growth factors (FGFs) [1]. FGFs comprise a family of secreted proteins that form a trimolecular complex by binding with differing affinities to one of four high-affinity fibroblast growth factor receptors (FGFRs) [6,7] and heparan sulfate proteoglycans [8,9]. FGF signaling was definitively implicated in endochondral ossification with the discovery that activating mutations in FGFR3 cause achondroplasia, the most common human short-limbed skeletal dysplasia [10,11], as well as thanatophoric dysplasia [12] and hypochondroplasia [13].

Mouse models recapitulating those activating mutations show similar impairment in longitudinal bone growth [14–16]. Conversely, mice with FGFR3 inactivating mutations display skeletal overgrowth, further implicating FGFR3 as a negative regulator of longitudinal bone growth [17,18]. FGFRs 1 and 2, in addition to their roles in intramembranous ossification [19,20], also have effects on endochondral bone formation. In mice, targeted ablation of FGFR2 impairs postnatal long bone growth, suggesting that FGFR2 acts as a positive regulator of endochondral bone formation at the growth plate [21,22]. In contrast, activating mutations in FGFR1 cause osteoglophonic dysplasia, another short-limbed skeletal dysplasia in humans, raising the possibility that FGFR1 acts as a negative regulator of skeletal growth [23]. Recent studies have also demonstrated that mice conditionally deleted for FGFR1 in osteo-chondroprogenitor cells display an increased hypertrophic zone size, probably due to a decrease in the rate of cartilage resorption and ossification [24]. Finally, although ablation of FGFR4 alone produces no apparent growth plate phenotype, double FGFR3/FGFR4-null mice show impaired long bone growth, implicating FGFR4 as another potential positive regulator of growth in cooperation with other FGFRs [25]. Whether a given receptor acts as a positive or negative regulator of bone growth may depend on its localization in the growth plate and the availability of ligand partners [26].

Parallel research has sought to identify ligand partners for these receptors [19,27,28]. Early reports that demonstrated accelerated endochondral ossification upon exogenous application of FGF2 [29,30] and that documented dwarfism in mice overexpressing FGF2 [31] suggested a role for that ligand at the growth plate. However, FGF2-null mice display major defects primarily in osteoblastic function with only minor perturbations in chondrogenesis [32]. More recent evidence supports important roles for FGF9 and FGF18 in growth plate signaling. Mice overexpressing FGF9 develop a skeletal dysplasia involving proximal long bones [33] while FGF9-null mice display disproportionately short proximal skeletal elements [28]. Ectopic expression of FGF9 in cranial bones induces *de novo* endochondral ossification [34]. FGF18-null mice display long bone phenotypes similar to but even more severe than FGFR3 knockouts [35,36].

Expression of these and other FGFs has been previously detected in the perichondrium, a dense collagenous and chondrogenic structure that surrounds the growth plate. These and other data have led to the hypothesis [28,35] that FGFs from the perichondrium may act on FGFRs on growth plate chondrocytes to regulate endochondral bone growth.

Although these data have begun to clarify the roles of FGF signaling in the embryonic skeleton, much less is known about their influence on postnatal bone growth, the period when the majority of increase in length takes place. In particular, it is unknown whether FGFs may help mediate the process of growth plate senescence including the growth deceleration that occurs as an animal approaches adulthood. To investigate these questions, we undertook an unbiased, comprehensive, and quantitative analysis of FGF and FGFR expression in the postnatal growth plate. We analyzed both the spatial and temporal expression of FGF signaling molecules, investigating changes in the perichondrium as well as between different growth plate zones (representing different stages of chondrocyte differentiation) as well as changes in gene expression with increasing age. We surveyed all major receptor isoforms and their interacting ligands. The results implicate new members of the FGF family in the orchestration of endochondral bone formation in the postnatal growth plate and suggest that changes in FGF signaling may contribute to the process of growth plate senescence.

Materials and methods

Animal procedures and tissue processing

Sprague–Dawley rats (Harlan, Indianapolis, IN) were maintained and used in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council 2003). To obviate the effects of sex steroids on the growth plate, castrated males were used in our study. 1-, 3-, 6-, and 9-week rats were sacrificed by carbon dioxide inhalation, and proximal tibial epiphyses were rapidly excised. Most of the epiphyseal and metaphyseal bone was removed by dissection, and the remaining growth plate, adjacent metaphyseal bone, and perichondrium were embedded in Tissue-Tek O.C.T. Compound (Electron Microscopy Sciences, Hatfield, PA) and stored at −80°C for subsequent processing.

Growth plate microdissection and RNA isolation

Frozen longitudinal sections 60 μm thick were obtained from the epiphyses and mounted on Superfrost Plus slides (Fisher Scientific, Chicago, IL). Slides were thawed, fixed in methanol, stained in eosin, dehydrated in graded ethanol, and dissected under xylene. For the initial screen, samples ($n=3$ animals) from

the whole growth plate, perichondrium, and metaphyseal bone were collected from these sections using an inverted microscope, razor blades, and hypodermic needles. To assess spatial regulation of gene expression in 1-week-old rats, additional longitudinal sections of growth plate were microdissected into resting zone, proliferative zone, and hypertrophic zone ($n=5$ animals). To avoid cross-contamination, the uppermost part of the proliferative columns and the lowest part of resting zone were discarded. Proliferative–hypertrophic transition zone cartilage was also discarded to prevent cross-contamination (Fig. 1). Due to decreasing growth plate height, only proliferative zone and perichondrium were collected from 3-, 6-, and 9-week-old animals ($n=5$ for growth plate, $n=6$ for perichondrium). For each zone, tissue from 15 to 35 sections from a single animal was pooled prior to RNA isolation. RNA isolation was performed as previously described except that one tenth of described volumes was used [37]. The final pellet was suspended in 9 μl DEPC-treated water. Approximately 30–200 ng of total RNA was extracted from each growth plate zone in individual 1-week-old animals and at least 200 ng from proliferative zone of 3-, 6-, and 9-week-old animals. The 28S/18S ratio was typically between 1.7 and 2.0 as assessed by the Bioanalyzer 2100 using RNA Pico Chips and version A.02.12 of the Bio Sizing software according to manufacturer's instructions (Agilent Biotechnologies, Inc., Palo Alto, CA).

As a positive control for the real-time RT-PCR assays, RNA was extracted from whole E18 rat embryos using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA) on ice and further purified using RNeasy Mini Kits (Qiagen, Valencia, CA) per manufacturers' directions.

Quantitative real-time RT-PCR

For PCR, 30–200 ng total RNA was reverse transcribed using 200 U Superscript II Reverse Transcriptase (Invitrogen) according to manufacturer's instruction. Resulting cDNA was diluted 10–20 times and stored at −20°C. Quantitative real-time RT-PCR was performed using the following assays containing primers and specific intron-spanning FAM-labeled TaqMan probes (Applied Biosystems, Foster City, CA): FGFR2b, Rn01275520_m1; FGFR2c, Rn01506944_m1; FGFR4, Rn01441815_m1; FGFRL1, Rn01531250_g1; FGF1, Rn00563362_m1; FGF2, Rn00570809_m1; FGF3, Rn00590754_m1; FGF4, Rn00709728_m1; FGF5, Rn00573575_m1; FGF6, Rn00590927_m1; FGF7, Rn00573319_m1; FGF8, Rn00590996_m1; FGF9, Rn00564116_m1; FGF10, Rn00564115_m1; FGF15, Rn00590708_m1; FGF16, Rn00573201_m1; FGF17, Rn00569970_m1; FGF18, Rn00433286_m1; FGF20, Rn00576686_m1; FGF21, Rn00590706_m1; FGF22, Rn00445750_g1; FGF23, Rn00590709_m1; and β-glucuronidase, Rn00566655_m1. In addition, intron-spanning primers with the following sequences were designed using Primer Express 2.0 (Applied Biosystems): collagen type X forward, GCAGCAGCCAGAATCCATTT; collagen type X reverse, AAGTGCGCTCTTCACAACCTGT; FGFR1b forward, CCAAAAGCCCTGGAAGAGAGAC; FGFR1b reverse, CCCACCATACAGGAGATCAGGA; FGFR1c forward, GCATGGTTGACCGTTCTGGAA; FGFR1c reverse, AGCCCACCATACAGGAGATCAG; FGFR3b forward, CTGAAGCACGTGGAGGTGAA; FGFR3b reverse, TCTGCCTCCACATTCTCACTGA; FGFR3c forward, AAGCACGTGGAGGTGAATGG; FGFR3c reverse, TGTCGGTGGTGTTAGCTCCT. To differentiate between FGFR splice variants, primers were designed to anneal to exons exclusive to the isoform of interest. These primers and SYBR Green PCR Master Mix (Applied Biosystems) were used for the PCR reactions. These assays were validated by demonstrating generation of a single PCR product of expected size using gel electrophoresis and by dissociation curve analysis. Reactions were performed in triplicate using cDNA, TaqMan Universal PCR Master Mix (Applied Biosystems) or SYBR Green PCR Master Mix, primers, and probes (Applied Biosystems), using the ABI Prism 7000 or 7300 Sequence Detection System (Applied Biosystems) according to manufacturer's instructions using the following thermal cycling conditions: 1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The quantity of each transcript was calculated relative to the amount of starting cDNA using the formula: relative expression$_i$=$(2)^{CTr}/(2)^{CTi}$ where r represents β-glucuronidase (a housekeeping gene with desirable normalization properties [38]), i represents the gene of interest, and CT represents the threshold cycle. For convenience, relative expression values were multiplied by 10^9. Serial 10-fold dilutions of embryonic cDNA were used to confirm near-theoretical efficiencies of assays.

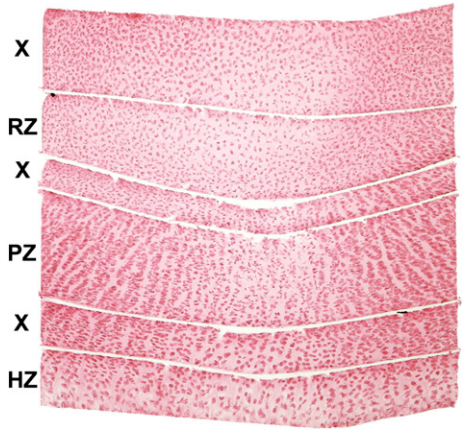

Fig. 1. Growth plate microdissection. Representative photomicrograph of microdissected proximal tibial epiphysis from 1-week-old rats. The 60-μm-thick longitudinal frozen sections were cut into resting zone (RZ), proliferative zone (PZ), and hypertrophic zone (HZ). Boundary regions (X) were discarded to minimize cross-contamination. The surrounding perichondrium was also isolated (not shown). In the section depicted, cuts were made, but the cartilage was left in place on the microscope slide.

Table 1
Normalized expression in screened tissues

	GP	PC	MB	Emb
FGFR1b	2.6 (1.6)	11.5 (5.6)	2.2 (0.6)	17.2 (3.5)
FGFR1c	340.3 (127.9)	1428.9 (253.7)	361.9 (55.2)	617.1 (163.5)
FGFR2b	ND*	0.5 (0.2)	0.2 (0.2)	34.7 (5.9)
FGFR2c	24.0 (5.8)	40.8 (8.0)	13.2 (2.3)	20 (3.8)
FGFR3b	472.0 (156.4)	106.3 (14.1)	54.3 (9.5)	608.1 (101.0)
FGFR3c	2560.2 (1103.7)	94.4 (9.8)	51.0 (4.7)	135. 7 (22.5)
FGFR4	6.6 (2.1)	27.3 (17.1)	0.1 (0.0)	23.5 (3.9)
FGFRL1	107.5 (67.1)	306.0 (140.9)	4.8 (1.0)	47.9 (8.9)
FGF1	ND	35.4 (20.8)	2.7 (0.9)	3.1 (0.6)
FGF2	3.8 (1.1)	20.9 (6.2)	0.2 (0.1)	7.5 (1.0)
FGF3	ND	ND	ND	0.3 (0.0)
FGF4	ND	ND	ND	0.1 (0.1)
FGF5	ND	0.3 (0.1)	ND	0.2 (0.0)
FGF6	ND	22.6 (15.8)	ND	4.3 (0.8)
FGF7	0.6 (0.3)	17.5 (5.8)	1.6 (0.5)	4.7 (0.7)
FGF8	ND	ND	ND	0.2 (0.0)
FGF9	ND	8.4 (2.5)	0.3 (0.1)	2.9 (0.5)
FGF10	ND	0.9 (0.3)	ND	1.6 (0.2)
FGF15	ND	ND	ND	2 (0.2)
FGF16	ND	0.7 (0.1)	ND	0.2 (0.0)
FGF17	ND	ND	ND	0.2 (0.0)
FGF18	3.0 (1.6)	84.4 (18.0)	0.9 (0.2)	26.6 (3.2)
FGF20	ND	ND	ND	0.2 (0.0)
FGF21	ND	1.1 (0.4)	0.1 (0.0)	0.8 (90.1)
FGF22	0.4 (0.2)	0.9 (0.6)	0.0 (0.0)	3.9 (0.4)
FGF23	ND	ND	0.6 (0.4)	0.6 (0.1)
ColX	187.6 (124.2)	9.5 (7.0)	8.1 (1.7)	20.1 (3.0)

Mean (SEM). GP=growth plate, PC=perichondrium, MB=metaphyseal bone, Emb=embryo. ND=not detected.

Statistical analysis

Data are presented as mean±SEM. All data were log-transformed to generate a normal distribution before statistical analysis. Comparison of mRNA levels between adjacent growth plate zones was made by paired t-test, correcting

for multiple comparisons using the Holm method. Comparison of mRNA levels between different ages in the proliferative zone and perichondrium was performed using one-way ANOVA using age as the independent variable. Post hoc pair-wise comparisons were made using the Holm method to correct for multiple comparisons. FGFs or FGFRs were identified as present if detected in the majority of animals.

Results

Initial detection screen

To identify ligands and receptors important to growth plate FGF signaling in an unbiased manner, we assessed expression of all known FGFs and FGFRs. We included the recently discovered FGFRL1, a putative decoy receptor [39–41], but not the fibroblast homologous factors (FGFs 11–14), which do not participate in classical FGF signaling [42]. FGF19 is not present in the rat genome [43].

For this screen, we microdissected whole growth plates, perichondrium, and metaphyseal bone individually from proximal tibiae of 1-week rats ($n=3$). RNA was isolated and analyzed by real-time RT-PCR.

mRNA for all FGFRs was present in perichondrium, whereas, in growth plate, mRNA for all FGFRs except FGFR2b was detected (Table 1). c isoforms appear to be the predominant splice variant for all alternatively spliced receptors, although appreciable quantities of b isoform mRNA were also detectable (Table 1).

In perichondrium, we detected expression of FGFs 1, 2, 6, 7, 9, and 18, and also, at low levels, FGFs 5, 10, 16, 21, and 22 (Table 1). In whole growth plate, FGFs 2, 7, 18, and 22 were

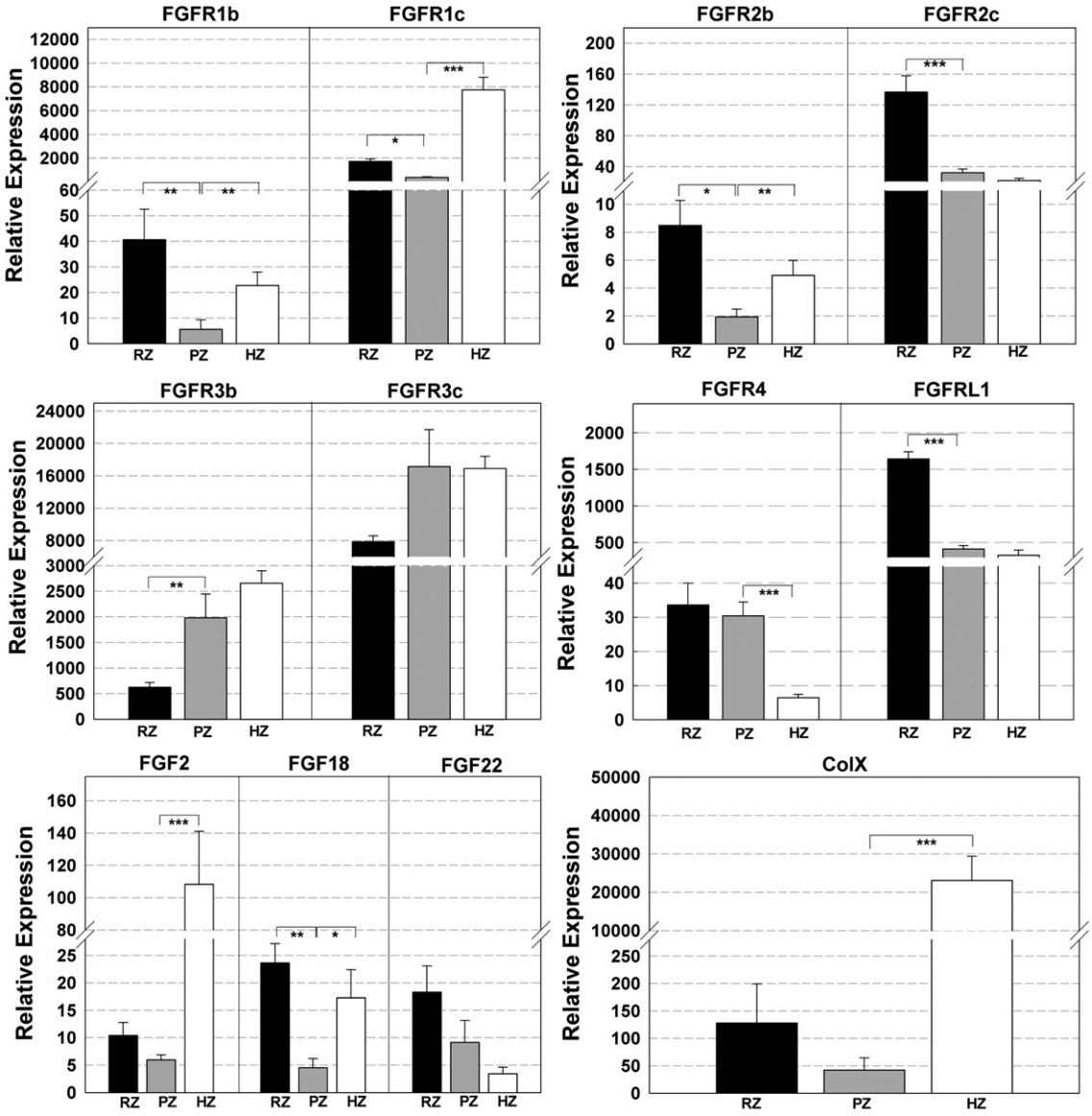

Fig. 2. mRNA expression of FGFs and FGFRs in growth plate zones from 1-week-old rats ($n=6$). After microdissection of zones from proximal rat epiphyses, quantitative real-time RT-PCR was performed on samples from individual animals ($n=6$) and normalized to mRNA levels of β-glucuronidase, and, for convenience, multiplied by 10^9. Type X collagen (ColX) was also assayed to assess the accuracy of the microdissection. Pair-wise comparisons of adjacent zones were performed. *$P<0.05$, **$P<0.01$, ***$P<0.001$. RZ=resting zone, PZ=proliferative zone, HZ=hypertrophic zone.

detected at low levels, and in metaphyseal bone, FGFs 1, 2, 7, 9, 18, 21, 22, and 23 were detected at low levels (Table 1). No ligands were detected in growth plate that were not also present in perichondrium, and all overlapping ligands showed similar or greater expression in perichondrium (Table 1).

As a positive control, cDNA samples from whole E18 mouse fetuses were assayed. All assays yielded a detectable value (Table 1).

Spatial regulation within growth plate

To assess regulation of FGF ligands and receptors in different regions of the growth plate, we next microdissected and isolated individual zones of proximal tibial growth plates of 1-week-old rats ($n=6$, Fig. 1). Accuracy of the dissection was confirmed by quantitation of a well-characterized marker of the hypertrophic zone, type X collagen (ColX) [1]. We found that expression was approximately 500-fold higher in hypertrophic zone compared to proliferative zone ($P<0.001$, Fig. 2).

FGF ligands and receptors that had been detected in whole growth plate in the initial screen were next assayed in individual growth plate zones. FGFR1c showed a higher level of expression in hypertrophic zone (23-fold higher than in proliferative zone, $P<0.001$, Fig. 2). FGFR3c mRNA showed a similar trend in the hypertrophic zone but also high expression in the proliferative zone (Fig. 2). FGFR2 and FGFRL1 mRNAs were also expressed in all three zones, but in contrast, showed highest expression in resting zone with 5-fold greater expression compared to proliferative zone for FGFR2b ($P<0.05$, Fig. 2) and more than 4-fold greater expression for FGFR2c and FGFRL1 (both $P<0.001$, Fig. 2). FGFR4 mRNA levels were more than 4-fold greater in resting zone and proliferative zone compared to hypertrophic zone ($P<0.001$, Fig. 2).

FGF2 mRNA levels were highest in hypertrophic zone with levels more than 18-fold greater than in proliferative zone ($P<0.001$, Fig. 2). In contrast, FGF18 mRNA showed relatively higher levels in both resting zone (nearly 5-fold, $P<0.01$) and hypertrophic zone (more than 3-fold, $P<0.05$) compared to

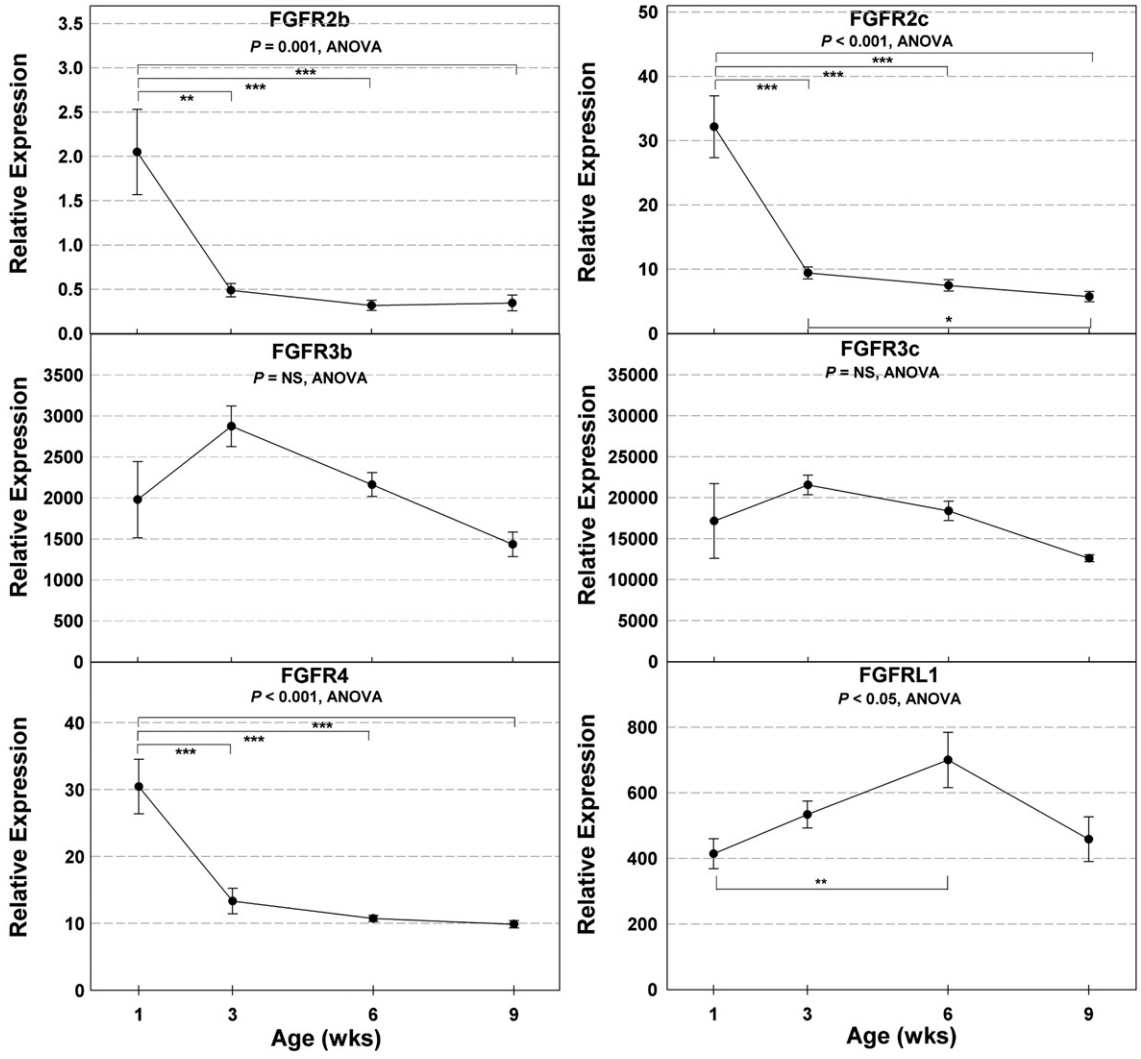

Fig. 3. Effect of age on FGFR expression in growth plate proliferative zone from 1-, 3-, 6-, and 9-week-old rats ($n=5$). After microdissection from proximal rat epiphyses, quantitative real-time RT-PCR was performed and normalized to β-glucuronidase, and, for convenience, multiplied by 10^9. One-way ANOVA and pairwise comparisons of all time points were performed. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

proliferative zone (Fig. 2). FGF22 varied significantly across the growth plate and was present in greatest amounts in the resting zone, though not significantly more than the proliferative zone (Fig. 2). FGF7, present in the initial screen at a very low level, was undetectable in the majority of growth plate zone samples (data not shown).

Temporal regulation in growth plate chondrocytes

To investigate temporal changes in FGF signaling, we assayed expression of FGFs and FGFRs in the proliferative zone of 3-, 6-, and 9-week-old rats ($n=6$). Neither isoform of either FGFRs 1 (data not shown) or 3 (Fig. 3) showed significant changes with age. In contrast, significant age-dependent decreases in growth plate expression were detected for FGFRs 2b, 2c, and 4 (Fig. 3). For example, FGFR2c mRNA decreased more than 5-fold by week 9 ($P<0.001$). FGFR2b mRNA levels mirrored this decline. FGFR4 mRNA levels decreased more than 3-fold with age ($P<0.001$).

FGF2 expression in proliferative zone chondrocytes remained constant with age (data not shown) while both FGF18 and FGF22 mRNA levels declined (Fig. 4). FGF18 mRNA dropped approximately 14-fold by week 9 ($P<0.001$). By week 9, FGF22 expression dropped approximately 6-fold ($P<0.001$).

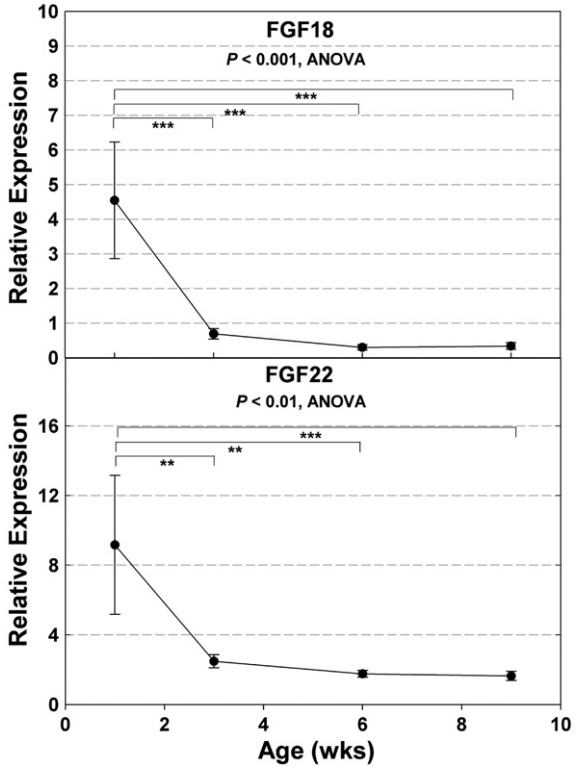

Fig. 4. Effect of age on FGF expression in growth plate proliferative zone from 1-, 3-, 6-, and 9-week-old rats ($n=5$). After microdissection from proximal rat epiphyses, quantitative real-time RT-PCR was performed and normalized to β-glucuronidase, and, for convenience, multiplied by 10^9. One-way ANOVA and pair-wise comparisons of all time points were performed. $^{**}P<0.01$, $^{***}P<0.001$.

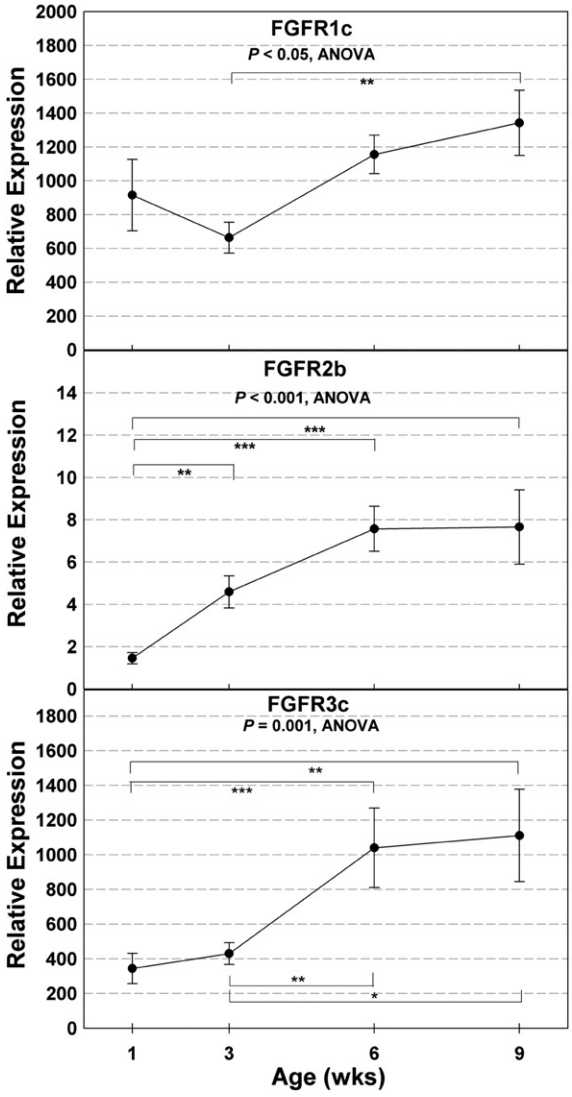

Fig. 5. Effect of age on FGFR expression in perichondrium from 1-, 3-, 6-, and 9-week-old rats ($n=6$). After microdissection from proximal rat epiphyses, quantitative real-time RT-PCR was performed and normalized to β-glucuronidase, and, for convenience, multiplied by 10^9. One-way ANOVA and pair-wise comparisons of all time points were performed. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Temporal regulation in perichondrium

In perichondrium, expression of FGFRs 1b, 2c, 3b, 4, and L1 did not change significantly with age (data not shown). In contrast, FGFR1c, FGFR2b, and FGFR3c mRNA levels all increased in expression with age (Fig. 5) with FGFR1c doubling between week 3 and 9 ($P<0.01$). By week 9, expression of FGFR2b had increased more than 5-fold ($P<0.001$). FGFR3c showed a similar pattern, tripling by week 9 ($P<0.01$).

Several FGF mRNAs showed significant increases in expression with increasing age. FGFs 1 and 7 increased more than 3- and 10-fold respectively (both $P<0.01$, Fig. 6). After a 4-fold drop from week 1 to week 3 ($P<0.01$), FGF18 mRNA increased more than 9-fold between week 3 and 9 ($P<0.001$,

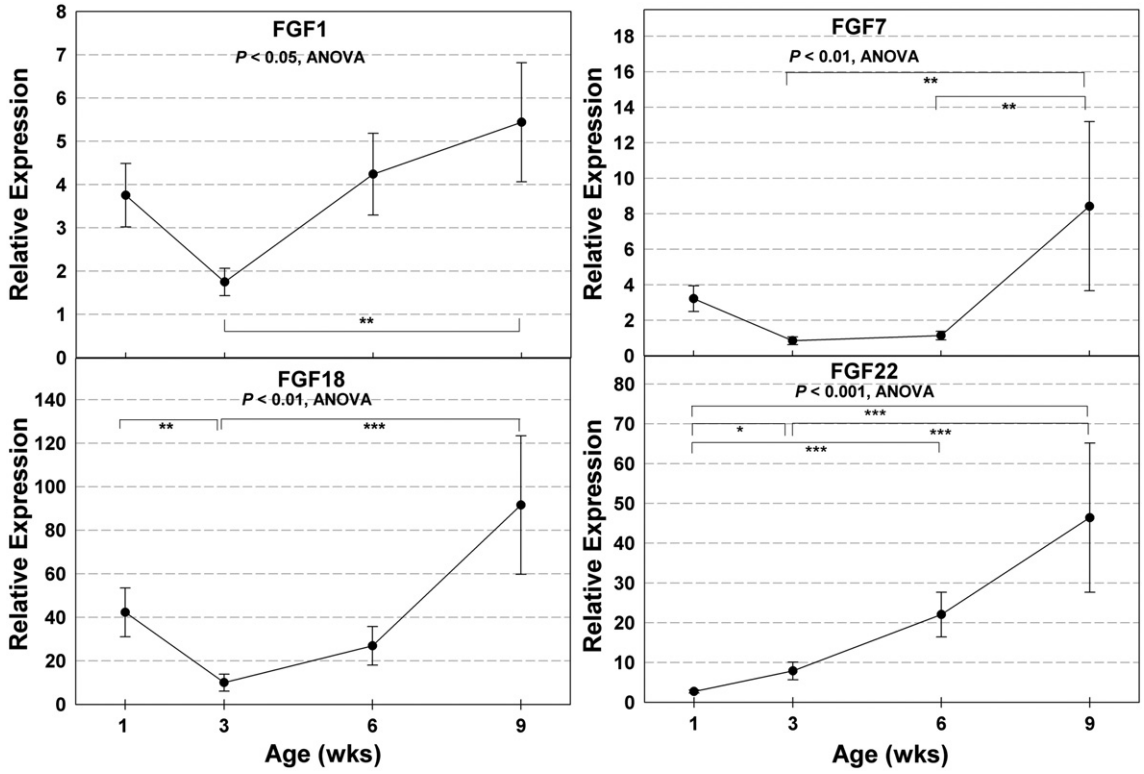

Fig. 6. Effect of age on FGF expression in perichondrium from 1-, 3-, 6-, and 9-week-old rats ($n=6$). After microdissection from proximal rat epiphyses, quantitative real-time RT-PCR was performed and normalized to β-glucuronidase, and, for convenience, multiplied by 10^9. One-way ANOVA and pair-wise comparisons of all time points were performed. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Fig. 6). FGF22 also showed a progressive increase in expression, finally increasing by more than 17-fold ($P<0.001$, Fig. 6) by week 9. Expression of FGFs 9 and 21 did not change with age while FGF2 varied with age by ANOVA but was not significantly different between any two points (data not shown). FGFs 5 and 16, which had shown minimal expression in the initial screen, were undetectable in the majority of animals. FGF6 and FGF10 were detectable at 1 week but were not detectable at later time points (data not shown).

Discussion

Identification of FGF ligands and receptors expressed in the growth plate and perichondrium

The initial screen revealed that, in general, FGF ligands are expressed in greater number and at higher levels in the postnatal perichondrium than in the growth plate. This greater level of ligand expression is broadly consistent with the proposed concept that the FGFs that regulate growth plate chondrocytes derive in large part from the adjacent perichondrium [28,35]. However, our findings also indicate that FGF ligands are expressed by growth plate chondrocytes as well, raising the possibility that local production may also regulate growth plate function. Low-level expression by growth plate chondrocytes themselves might have a disproportionate autocrine or paracrine effect because the FGFs would be secreted at closer proximity to the target receptors than would FGF produced in the perichondrium. Local production of FGFs in the growth plate may become increasingly important in the postnatal animal where the distance between the perichondrium and the interior of the growth plate increases.

In addition to FGFs 2, 7, 9, and 18, which have previously been localized to perichondrium in the embryonic skeleton [28,35,44], we have also identified mRNA for FGFs 1 and 22 in the postnatal perichondrium. Previous gene ablation studies have shown that mice deficient in FGFs 9 or 18 display growth plate phenotypes, implicating those molecules as crucial factors in the growth plate [28,35,36]. Mice lacking FGFs 1, 2, or 7 do not display a striking growth plate phenotype, but these ligands may still have an important but redundant role [45,46]. FGF22 is a relatively newly characterized ligand, and its role in the growth plate is unexplored.

We have found that FGFRs 1, 2, 3, and 4 and FGFRL1 all expressed in the growth plate. Although FGFRL1 was known to be expressed in cartilage [41], to our knowledge, ours is the first study identifying it in the growth plate. For FGFRs 1, 2, and 3, alternative splicing can generate two major isoforms with different receptor specificity [28]. In growth plate and perichondrium, c isoforms were expressed at higher levels, as has traditionally been observed in mesoderm-derived tissues. However, because of the sensitivity of our technique, we were able to detect appreciable levels of b isoform receptors, especially for FGFR3.

Spatial regulation within growth plate

In the 1-week growth plate, FGFRs showed a complex spatial pattern of expression. FGFRs 1 and 3, thought to negatively regulate chondrogenesis, were expressed late in chondrocyte differentiation, with FGFR1 upregulated in the hypertrophic zone and FGFR3 upregulated in both the proliferative and hypertrophic zones. Although FGFR1 has consistently been shown to be upregulated in the hypertrophic zone in embryonic bone, FGFR3 localization has been more controversial. Some studies show it to be predominantly expressed in the proliferative zone [17] while others have also demonstrated significant levels of expression in the prehypertrophic zone [44]. Our findings are more consistent with the latter conclusion and also consistent with previous studies in mice lacking FGFR1 [24] or FGFR3 [47] suggesting that both receptors promote vascular invasion and ossification of the hypertrophic zone.

FGFRs 2 and 4, putative positive regulators, were expressed at lower levels and, in contrast to FGFRs 1 and 3, primarily in the resting and/or proliferative zones. This pattern of FGFR4 expression agrees well with *in situ* hybridization data from the embryonic mouse [44]. FGFR2c has previously been found by *in situ* hybridization to be expressed primarily in embryonic metaphyseal bone compared to growth plate [22]. Using a more sensitive technique, we found that it is expressed in growth plate, which may explain the growth defect of postnatal onset in FGFR2-null mice [21]. In addition, differences in animal model and animal age may also explain differences between the current findings and those from previous studies.

FGFRL1, a recently characterized FGFR which lacks a tyrosine kinase domain and thus may not transduce a signal, has previously been shown to be present in high amounts in cartilage [41]. Here we identified it for the first time in the growth plate and showed that its expression is enriched in the resting zone. By sequestering FGFs from interaction with kinase-containing FGFRs, FGFRL1 may contribute to the resting zone's relatively quiescent state in postnatal life.

FGF2 is upregulated nearly 20-fold in the hypertrophic zone compared to the proliferative zone. The role of FGF2 in the hypertrophic zone is not clear. Previous studies suggest that high concentrations of FGF2 inhibit chondrocyte hypertrophy [30,31] however FGF2-deficient mice display only a minor hypertrophic phenotype but do have decreased bone mass and defective mineralization [32] raising the possibility that FGF2 might act as a paracrine signal to metaphyseal bone.

Temporal regulation in growth plate chondrocytes

As a mammal ages, its growth plate undergoes senescence (a general term for the reduced proliferation rate and cellularity that eventually leads to the cessation of longitudinal bone growth). Although this senescent decline is thought to be due to mechanisms intrinsic to the growth plate, little is understood about the specific molecular mechanisms that cause the rate of longitudinal bone growth to drop precipitously with age [2,5,48,49].

We identified several changes in FGF and FGFR expression that may contribute to growth plate senescence. In the growth plate, FGFRs 2 and 4, both implicated as positive regulators of growth [22,25], undergo a drop in expression with age. Mice lacking FGFR2 in growth plate and bone show severe postnatal growth retardation, indicating that FGFR2 is critical for postnatal growth plate function. Thus, our data raise the possibility that declining FGFR2 expression may contribute to growth plate senescence [22]. The declines in FGFs 18 and 22 in the growth plate might also contribute to growth plate senescence.

Temporal regulation in perichondrium

In the perichondrium, we observed increases in FGFs 1, 7, 18, and 22 mRNA with age. Increasing levels of these ligands, interacting with constant levels of FGFR3 in growth plate might contribute to growth plate senescence. In addition to increases in ligand expression, FGFR1c and FGFR3c also undergo significant upregulation in perichondrium with advancing age. Because the perichondrium is chondrogenic itself, an increase in these receptors may coordinate with a local increase in FGF18 and/or FGF1 to affect chondrogenesis, perhaps particularly involving lateral enlargement of the growth plate. FGF18 and FGFR3 are also expressed in osteoblasts and may regulate bone formation [50], and thus the observed temporal expression changes might be related to the osteogenic potential of the perichondrium.

Summary

In summary, we have performed a comprehensive analysis of FGF and FGFR expression in the postnatal growth plate using a quantitative and highly sensitive technique. We found that perichondrium expresses FGFs 1, 2, 6, 7, and 18 and, at lower levels, FGFs 21 and 22. Growth plate chondrocytes express FGFs 2, 7, 18, and 22. Expression in perichondrium is generally greater than in growth plate, supporting the concept that FGFs from the perichondrium regulate growth plate chondrocytes. However, growth plate FGFs might be physiologically important because of their greater proximity to target receptors. In growth plate, we found expression of FGFRs 1, 2, and 3 involving primarily, but not exclusively, the c isoforms. FGFRs 1 and 3, thought to negatively regulate chondrogenesis, are expressed at greater levels later in chondrocyte differentiation, whereas FGFRs 2 and 4, putative positive regulators, are expressed primarily at earlier stages of growth plate chondrocyte differentiation. We have also found that FGFRL1, a presumed decoy receptor not previously implicated in the growth plate, is expressed in the resting zone. With increasing age, FGFR2 and 4 expression is downregulated in proliferative zone, whereas FGFR1 and FGFR3 expression increases in perichondrium. Finally, with age, FGFs 1, 7, 18, and 22 are coordinately upregulated in perichondrium. Further experiments will be necessary to determine if the combined effects of this regulation act to promote growth plate senescence, thus contributing to the age-

dependent decline in growth rate and ultimately determining the size of the adult skeleton.

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