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Fibroblast growth factor-18 is a trophic factor for mature chondrocytes and their progenitors

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Summary

Objective: The aim of this study was to examine the effects of recombinant human *Fgf18* on chondrocyte proliferation and matrix production *in vivo* and *in vitro*. In addition, the expressions of *Fgf18* and *Fgf* receptors (*Fgfr*) in adult human articular cartilage were examined.

Methods: Adenovirus-mediated transfer of *Fgf18* into murine pinnae and addition of FGF18 to primary cultures of adult articular chondrocytes were used to assess the effects of FGF18 on chondrocytes. *In situ* hybridization was used to examine the expression of *Fgf18* and *Fgfr*s in adult human articular cartilage.

Results: Expression of *Fgf18* by adenovirus-mediated gene transfer in murine pinnae resulted in a significant increase in chondrocyte number. Chondrocytes were identified by staining with toluidine blue and a monoclonal antibody directed against type II collagen. *Fgf18*, *Fgfr 2-(IIIc)*, *Fgfr 3-(IIIc)*, and *Fgfr 4* mRNAs were detected within these cells by *in situ* hybridization. The nuclei of the chondrocytes stained with antibodies to PCNA and FGF receptor (FGFR) 2. Addition of FGF18 to the culture media of primary articular chondrocytes increased the proliferation of these cells and increased their production of extracellular matrix. To assess the receptor selectivity of FGF18, BaF3 cells stably expressing the genes for the major splice variants of *Fgfr 1–3* were used. Proliferation of cells expressing *Fgfr 3-(IIIc)* or *Fgfr 2-(IIIc)* was increased by incubation with FGF18. Using FGFR–Fc fusion proteins and BaF3 cells expressing *Fgfr 3-(IIIc),* only FGFR 3-(IIIc)-Fc, FGFR 2-(IIIc)-Fc or FGFR 4-Fc reduced FGF18-mediated cell proliferation. Expression of *Fgf18, Fgfr 3-(IIIc) and Fgfr 2-(IIIc)* mRNAs was localized to chondrocytes of human articular cartilage by *in situ* hybridization.

Conclusion: These data demonstrate that *Fgf18* can act as a trophic factor for elastic chondrocytes and their progenitors *in vivo* and articular chondrocytes cultured *in vitro*. Expression of *Fgf18* and the genes for two of its receptors in chondrocytes suggests that *Fgf18* may play an autocrine role in the biology of normal articular cartilage.

Key words: Cartilage, FGF, FGF receptor, Chondrocyte, Collagen.

Introduction

Cartilages are specialized connective tissues that dissipate loads as the bearing surfaces in joints (hyaline cartilages and fibrocartilages), interface between tendons, ligaments, and bone (fibrocartilage), and provide a flexible support for soft tissues (elastic cartilages, e.g. pinna, and hyaline cartilages, e.g. trachea). All cartilage subtypes are composed of chondrocytes embedded in a complex extracellular matrix containing collagens, proteoglycans, and non-collagenous proteins. The amount of extracellular matrix and its composition most obviously differentiate between cartilage types. For example, hyaline and elastic cartilages contain predominately type II collagen, whereas fibrocartilages contain predominately type I collagen[1,2].

Trauma and disease of the synovial joint frequently cause structural damage to the articular cartilage. Changes include age-related fibrillation, cartilage degeneration due to osteoarthritis (OA), and focal chondral and osteochondral defects[2]. In most other tissues, such lesions would be rapidly repaired. Adult articular cartilage, however, has only a very limited capacity to heal[2]. Efforts to stimulate cartilage healing have focussed on two general areas: surgical procedures[2], designed to stimulate the endogenous repair response, and biological repair, designed to stimulate chondrocyte proliferation and/or matrix production by the application of peptide growth factors 3 or by cell transplantation[4].

As part of a bioinformatics-driven search for novel genes with potential clinical utility, a novel member of the fibroblast growth factor (FGF) family of genes was identified[5]. This gene and its encoded protein were subsequently given the designation of *Fgf18* by the Human Genome Organization. It has been mapped to chromosome 5q34[5] and is a member of *Fgf8* and *Fgf17* subfamily of FGFs[6]. As part of a screen for factors that regulate angiogenesis, *Fgf18* was expressed by adenovirus-mediated gene transfer in the pinnae of nude mice. Although an angiogenic response was not observed, a surprising phenotype developed: compared to pinnae that received null adenovirus, those that received adenovirus-expressing *Fgf18* became visibly thicker. The increase in thickness was largely due to an

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Fgf18-mediated increase in chondrocyte proliferation, type-II collagen synthesis, and extracellular matrix production. Similar effects were seen in subsequent studies of primary cultures of porcine and adult human articular chondrocytes incubated with highly-purified FGF18 protein. That *Fgf18* might play a role in the biology of normal cartilage was suggested by our observations that *Fgf18* mRNA and mRNA for two of its receptors, *Fgfr 3-(IIIc)* and *Fgfr 2-(IIIc),* were localized within chondrocytes of human articular cartilage.

Materials and methods

FGF-MEDIATED PROLIFERATION OF FGFR-BAF3 CELLS

Cultures were maintained in growth media containing RPMI 1640 media (Life Technologies, Rockville, MD, U.S.A.) containing 10% fetal bovine serum (FBS), L-glutamine, 600 μg G418/ml, and 2.0 ng/ml murine IL-3 (R&D Systems, Minneapolis, MN, U.S.A.) in a 37°C humidified atmosphere of 95% air and 5% CO_2. For mitogenic assays, cells were washed three times with assay media containing RPMI 1640, 10% FBS, L-glutamine, 600 μg G418/ml, 2.0 μg/ml porcine intestinal heparin (Sigma Chemical Company (St Louis, MO, U.S.A.) and no IL-3. Cells were plated at 10,000 cells per well in 96-well microtiter plates in 200 μl of assay medium containing various concentrations of growth factors. In some experiments, soluble FGF receptor fusion proteins, containing the ligand-binding domain of each receptor fused to the carboxyl terminal Fc region of human IgG1 (R&D Systems, Minneapolis, MN, U.S.A.) were added to the assay media in the amounts indicated in the figure legends. After 72 h at 37°C, a 20-μl sample of Alamar BlueTM (AccuMed International, Westlake, OH, U.S.A.) was added to each well and the cells were returned to the incubator. After 20 h, the fluorescence was monitored at excitation and emission wavelengths of 544 nm and 590 nm, respectively. The background signal intensity (200–400 fluorescent units) from wells without growth factor was subtracted from the values obtained in its presence.

ISOLATION AND CULTURE OF CHONDROCYTES FROM HUMAN AND PORCINE ARTICULAR CARTILAGE

Adult human talus joints were obtained from the Northwest Tissue Center (Seattle, WA, U.S.A.). Full thickness articular cartilage slices were aseptically collected and placed in Dulbecco's Modified Eagles Medium (DMEM, Hyclone Laboratories, Logan, UT, U.S.A.). Chondrocytes were isolated using a modification of the method reported by Kim and Conrad[7]. The slices were incubated with 0.5-mg trypsin/ml in DMEM for 30 min at 37°C. The supernatant was removed and the slices further incubated at 37°C for 18 h in DMEM containing 10% FBS (Hyclone Laboratories, Logan, UT, U.S.A.), 1-mg collagenase-P/ml (Boehringer-Mannheim, Indianapolis, IN, U.S.A.), 1.0-mg porcine hyaluronidase/ml (Sigma Chemical Company, St Louis, MO, U.S.A.). The supernatant was passed through a 70 μm cell strainer to remove cell debris and undigested cartilage. The isolated chondrocytes were counted with the aid of a Neubauer haemocytometer and were plated in DMEM containing 10% FBS in 24-well tissue culture plates at a high density of 2.6×10^5 cells per well and returned to the incubator. After 24 h, the cell layers were rinsed, and cultured in serum-free DMEM without additional supplements. A week later, fresh serum-free DMEM was added with or without 100 ng FGF18 protein/ml. The appropriate media were replaced twice per week and cell numbers were determined weekly. At various times, cells were extracted with 0.4 M NaCl, 50 mM Tris, pH 7.4 containing 0.5 M EDTA and 0.1% Triton (50 μl/well). 30 μl aliquots were acidified with 70 μl 0.5 M acetic acid, and treated with 10-μg pepsin/ml at 4°C for 6 h. The reaction was stopped with pepstastin (1:20 w/w) and the samples dried. All samples were brought up in 30 μl Laemmli sample buffer and the proteins in 15 μl aliquots were separated by SDS-PAGE. Type II collagen was detected by chemiluminisence (NEN Life Sciences Products, Boston, MA, U.S.A.), after transblotting onto PVDF using the antibody 1C10 (1.0 μg/ml) that specifically detects an epitope in denatured α1(II) CB9, 7. It detects type II collagen chains on Western blots (D. Eyre, unpublished data). Pepsin treated human fetal type II collagen was used as a control.

Porcine articular cartilage slices were obtained from 3–5-month-old pigs and digested at 37°C overnight with 1 mg/ml collagenase (Worthington Biochemicals, NJ) in RPMI medium (Life Technologies). After passing through a 100 μm Falcon cell strainer , the cells were washed, and plated directly into 48-well plates to monitor either cell growth (4000 cells per well) or proteoglycan synthesis (30,000 cells per well). The assay medium was DMEM/F12 supplemented with 2 μM L-glutamine, 1 mM Na pyruvate, 20 μg/ml bovine transferrin, 3 ng/ml Na selenite, 10 μg/ml fetuin, 25 mM Hepes (pH 7.2), and 100 μg/ml L-ascorbic acid 2- phosphate. FGF18 was added 1–2 days after plating and fresh factor was added 3 days later. The experiments were terminated at 6–7 days. Cell proliferation was measured using Alamar Blue™ as described above. Proteoglycan synthesis was measured by labeling the cells during the last 16 h of culture with 5-μCi/ml [^{35}S]-Sulfur (1325 Ci/mmole, NEN, Boston, MA) in the presence of 80-μg/ml B-aminopropionitrile. Glycosaminoglycans were precipitated with 1% cetylpyridinium chloride using 1 μg/ml chondroitin sulfate as carrier. Pellets were washed three times with PBS, solubilized with 0.5 ml of methanol, and counted as described above.

ADENOVIRUS-MEDIATED GENE TRANSFER

The cDNA for *Fgf18* was cloned into pACCMV (Microbix Biosystems, Inc., Ontario, Canada) and co-transfected into 293 cells (ATCC No. CRL-1573) with the plasmid pJM17[8] containing an E1 deleted adenovirus genome. Recombination between the two plasmids results in viral plaque formation. The recombinant adenovirus was plaque purified, amplified, and purified as previously described[9]. Virus particle number was determined by spectrophotometry and infectious particle number was determined by a plaque forming unit assay. All adenovirus preparations were demonstrated to be free of wild-type virus. The virus preps were diluted to a working concentration of 5×10^{10} pfu/ml in 5% glycerol and a final concentration of 1 mg/ml trypan blue. Prior to injection of adenovirus, female BALB/C nu/nu mice (JAX, Bar Harbor, ME, U.S.A.), 7–9 weeks of age, were anesthetized using a single dose of ketamine (40 mg/kg)+xylazine (8 mg/kg). Each pinna was held loosely across the center with forceps and the adenovirus was delivered using a 30 ga needle that was inserted subdermally 2–3 mm from the tip of the pinna. Approximately

20 μl of adenovirus solution (containing 10^9 pfu virus) was then delivered slowly through the needle, creating a small pocket under the skin. Six mice were injected in the left pinna with adeno-*Fgf18* and in the right pinna with adeno-null. After recovery from anesthesia, mice were returned to their cages and were monitored daily with minimal manipulation of the pinnae. Two mice were sacrificed 5 days, 11 days, and 17 days following injection of the virus. Pinnae were removed and fixed immediately in 10% neutral buffered formalin and transferred to 70% ethanol 24 h later. Each pinna was routinely processed, embedded in paraffin, sectioned at 5 μm and stained with hematoxalin/eosin or toluidine blue. Elastin was stained in some sections by the method of Verhoeff[10].

DETECTION OF MRNA BY *IN SITU* HYBRIDIZATION

In situ hybridization probes were designed against the human *Fgf18*, human *Fgfr 2-(IIIc)*, human *Fgfr 3-(IIIc)*, human *Fgfr 4*, mouse *Fgf18* and rat *Fgfr 4* sequences. A full-length cDNA plasmid, containing 0.6 kb of the coding region, 0.4 kb of the 5′UTR and 0.3 kb of the 3′UTR, was used as a template to generate the human *Fgf18* ribonucleotide probe. For the human *Fgfr 2-(IIIc)*, human *Fgfr 3-(IIIc),* and human *Fgfr 4*, PCR amplified products were used as templates for probe synthesis. The working primer sequences for human *Fgfr 2-(IIIc)*, human *Fgfr 3-(IIIc)* and human *Fgfr 4* were GGCTGCCCTACCTCAAG (upper) and AGCCGAAACTGTTACCTGTC (lower); TGACGCACAGC CCCACATCCAGT (upper) and GCAGGCGGCAGAGCGT CACAG (lower); and TGACACAGTGCTCGACCTTGATAG (upper) and ACGCCATTTGCTCCTGTTTTC (lower) respectively. A sequence of the T7 RNA polymerase promoter was linked to all lower (antisense) primers. For the mouse *Fgf18* probe, a plasmid containing 0.3 kb of the coding region and 0.7 kb of the 3′UTR was used as the template for riboprobe synthesis. A plasmid containing 0.4 kb of the 3′UTR and the entire coding region was used to generate the rat *Fgfr 4* probe. Probes for human collagen type II and type I were used as positive and negative controls, respectively in studies of human talus cartilage. The type II collagen probe is 0.4 kb located in the 3′UTR of human alpha1 (II) and the collagen type I probe covered exons 1-9 of human alpha1 (I). Antisense probes were synthesized using RNA polymerases following procedures described in the Riboprobe *in vitro* Transcription System (Promega Corp., Madison, WI, U.S.A.). The DIG RNA Labeling Mix was used for the digoxigenin labeled ribonucleotide probes (Boehringer Mannheim Corp. Indianapolis, IN, U.S.A.). Purification with a Centriflex Gel Filtration Cartridge (Edge BioSystems, Gaithersburg, MD, U.S.A.) was performed to eliminate non-incorporated nucleotides. The probe was evaluated by direct immunological detection using antibodies against DIG to determine labeling efficiency, and further analysed by electrophoresis to confirm the transcript size.

Human talus joint articular cartilage slices and mouse pinnal tissues were rinsed with PBS and fixed in 10% buffered formalin, embedded in paraffin and sectioned at 5 μm. After removal of the paraffin, tissues were digested with 50 μg Proteinase K/ml for 5 min at 37°C, briefly fixed again in 4% paraformaldehyde and acetylated. Human *Fgf18*, human *Fgfr 2-(IIIc)*, human *Fgfr 3-(IIIc)* and human *Fgfr 4* probes were used on all human talus joint tissue samples. For mouse pinnal tissues, mouse *Fgf18*, human *Fgfr 2-(IIIc)* and human *Fgfr 3-(IIIc),* and rat *Fgfr 4* probes were utilized. Homologies in the probe sequences between the mouse and human *Fgfr*s is 92–94%, and between mouse and rat *Fgfr 4* is 87%. Stringent reaction conditions were selected to ensure specific annealing of the probes. Hybridization was performed with DIG labeled probes at 1–5 pmol/ml in solution containing 50% formamide and 2×SSC for 12 to 16 h at 55–60°C. The slides were subsequently washed in 2×SSC and 0.1×SSC at 50–55°C. The signals were amplified with two to three rounds of tyramide signal amplification (TSA *in situ* indirect kit, NEN Life Science Products, Boston, MA, U.S.A.) and visualized with Vector Red substrate kit (Vector Laboratories, Burlingame, CA, U.S.A.). Slides of human articular cartilage were counterstained with hematoxylin and imaged under bright field. For mouse pinnal tissue sections, DAPI was used as the counterstain and the images were taken under fluorescence.

IMMUNOCYTOCHEMISTRY

Fixed pinnal tissues were processed in a Tissue-Tek VIP 2000, embedded in paraplast X-tra, and sectioned at 5 μm. Pinnal sections were deparaffinized, hydrated with water, and subjected to epitope retrieval with pepsin (NeoMarkers, Fremont, CA, U.S.A.) for 20 min at room temperature. The localization of type II collagen, FGF18, and FGFR 1–4 was determined using an immunoperoxidase (IP) immuno-histochemical protocol with avidin-biotin-complex detection system (Vantana Biotek Systems, Tucson, AZ, U.S.A.). Sections were preblocked with 5% normal goat serum (NGS) (Vector Laboratories, Burlingame, CA, U.S.A.) and were incubated with a primary antibody against type II collagen (mouse anti-collagen type II Ab-2, clone 2B1.5, 200 μg/ml, 1:100 dilution, NeoMarkers) or a primary rabbit anti-human FGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.): FGF R1, Flg (C-15): sc-121; FGF R2, Bek (C-17): sc-122; FGF R3, (C-15):sc123; or FGF R4 (C-16): sc-124 each tested at working dilutions of 1:200, 1:400, and 1:800. The FGFR 1–3 antibodies are directed against a common domain in the carboxy terminal region and thus do not distinguish the ‘b’ and ‘c’ receptor splice variants. Detection of proliferating cell nuclear antigen (PCNA) was by direct immunohistochemical staining with a biotinylated mouse anti-PCNA antibody (0.2 mg/ml, 1:100 dilution, Zymed, South San Franscisco, CA, U.S.A.). Biotinylated goat antimouse IgG (1.5 mg/ml, 1:200 dilution, Vector Laboratories) plus 2% NGS in PBS was used as the secondary-linking antibody for detection of type II collagen and FGF18. For FGFR staining, a biotinylated goat anti-rabbit IgG (1:200 dilution in PBS containing 2% NGS, Vector Laboratories) was used. Antibody specificity was confirmed using the appropriate non-immune primary sera and known positive tissue sections. Immunoperoxidase labeling was performed with a Vantana peroxidase DAB kit (Vantana Biotek Systems) using diaminobenzidine precipitation. Tissues were counterstained with methyl green. Slides were observed using a Nikon microscope and images were recorded with a CoolSnap (RS Photometrics) digital camera system.

EXPRESSION AND PURIFICATION OF RECOMBINANT HUMAN FGF18

Fgf18 was expressed in *E. coli* using the MBP (maltose binding protein) fusion system (New England Biolabs). In this system, the mature *Fgf18* cDNA was attached to the 3′

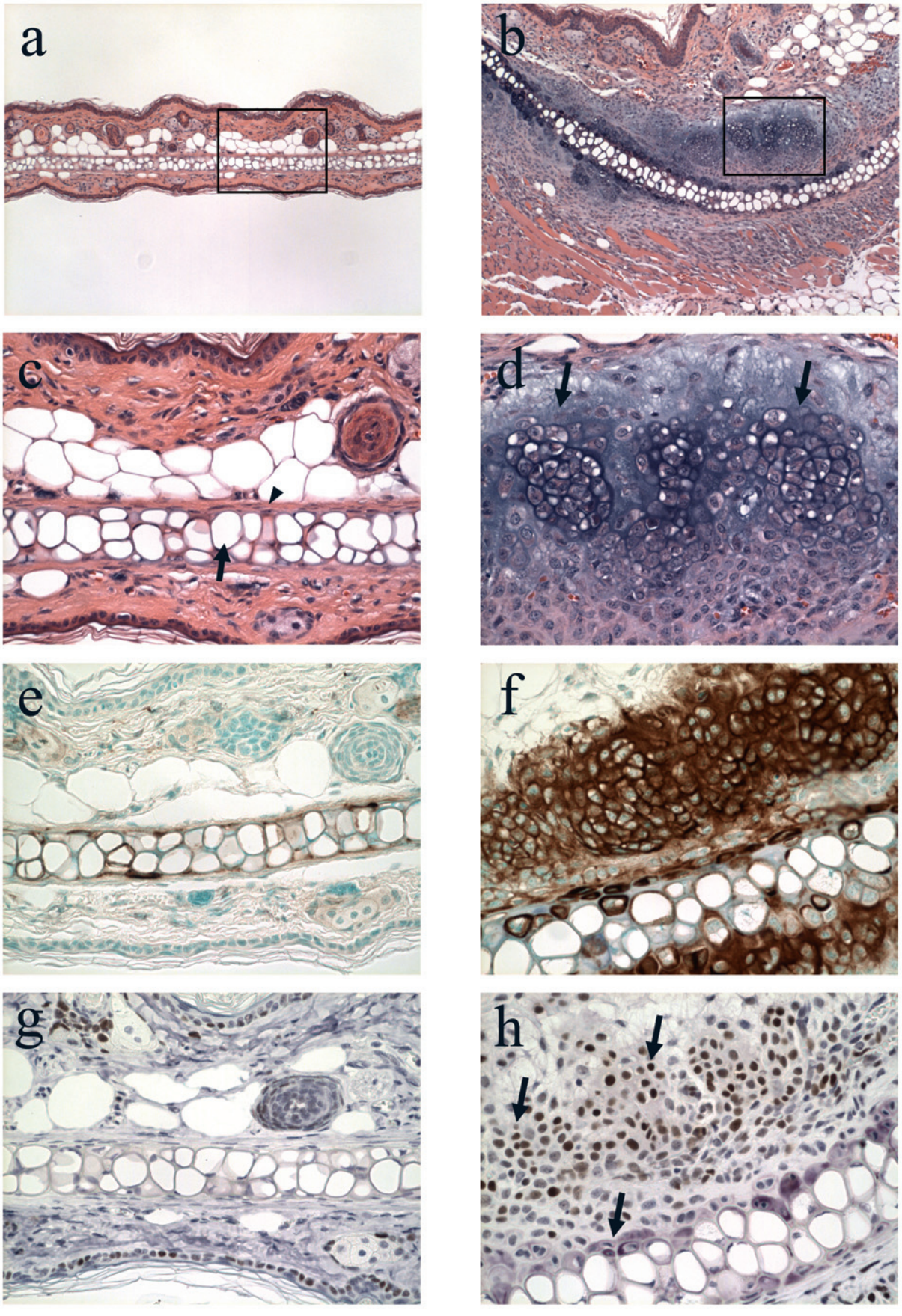


Fig. 1. (a–h).

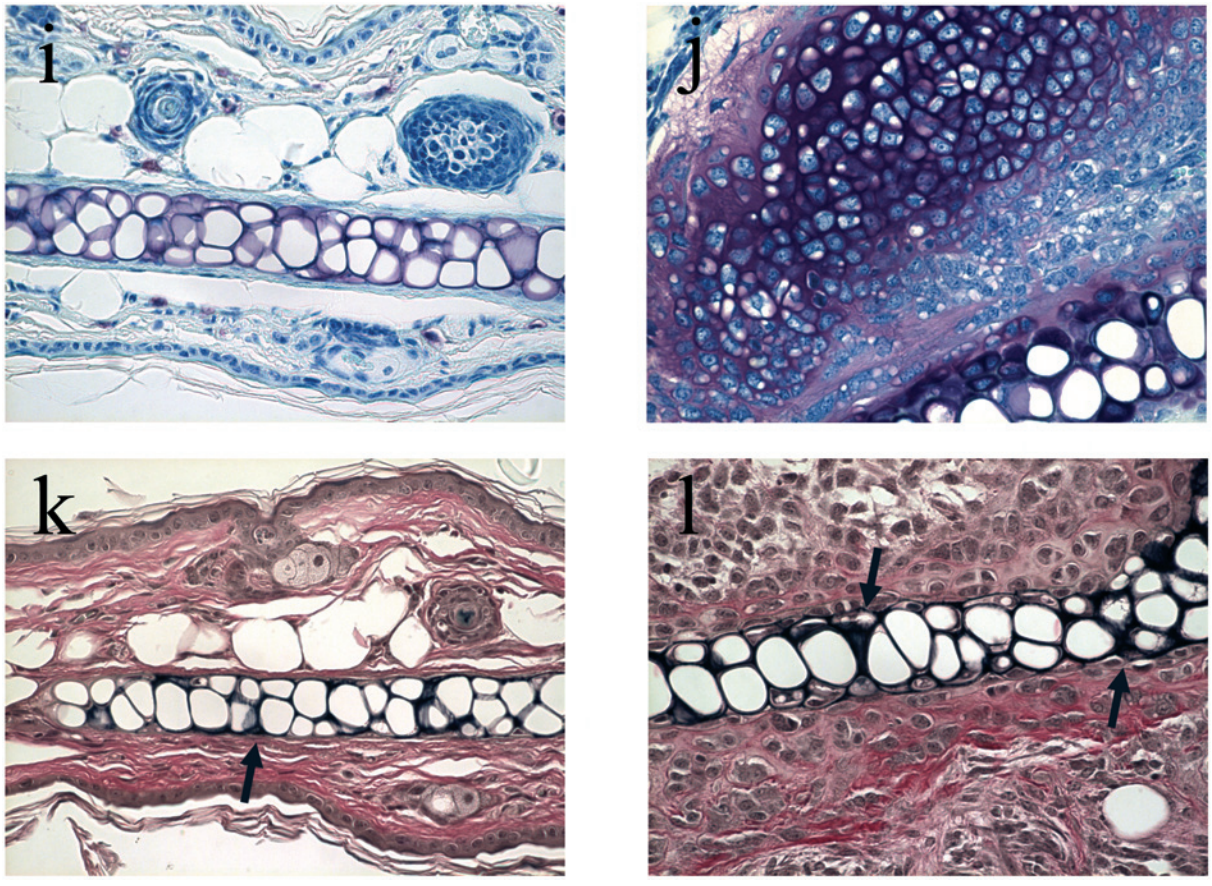


Fig. 1. (i–l)

Fig. 1. Histology of mouse pinnae transduced with adenovirus expressing *Fgf18*. Pinnae were harvested on day 5 after infection with either a control adenovirus (left panels) or adenovirus expressing *Fgf18* (right panels). Panels a,b: hematoxylin and eosin [10×, boxed outlines magnified areas in panels (c) and (d)]; panels (c), (d): hematoxylin and eosin, 20×, panel (c) arrow, lipochondrocyte, panel (c) arrowhead, perichondrial cells; panel (d) arrows, newly formed 'chondrocyte-like' cells; panels (e), (f): immunoperoxidase, type II collagen, 10×; panels (g), (h): immunoperoxidase, proliferating cell nuclear antigen, 10×, panel (h) arrow demonstrates PCNA positive chondrocytes; panels (i), (j): toluidine blue, 10×; panels (k), (l): Verhoeff's stain for elastin, 10×, arrows illustrate elastin deposition.

end of the malE gene to form an MBP-FGF18 fusion protein. The construct was built as in-frame fusions with MBP in accordance with the Multiple Cloning Site (MCS) of the pMAL-c2 vector (New England Biolabs) according to the manufacturer's instructions. *E. coli* fermentation medium containing MBP-FGF18 was solubilized in a cell disrupter, using a lysis buffer containing 50 mM phosphate, pH 7.2, 0.4 M NaCl, 10 mM EDTA, 0.4 mM phenylmethylsulfonylfluoride, and 1 μM each of leupeptin and pepstatin. The lysate was centrifuged and the resulting supernatant was batch loaded on AF-Heparin-650M resin (TosoHaas, Montgomeryville, PA, U.S.A.) in roller bottles overnight at 4°C. The resin was washed and then incubated in roller bottles at room temperature for 3 h in a modified lysis buffer containing 15 mg/l thrombin without EDTA or protease inhibitors. The resin was then washed with 50 mM phosphate, pH 7.2, 0.4 M NaCl, 10 mM EDTA and the cleaved product FGF18 was step-eluted with wash buffer containing 1.75 M NaCl. The pooled AF-Heparin fractions were diluted to ~46 mS/cm conductivity using 50 mM sodium phosphate, pH 7.8, and passed over a bed of Benzamidine Sepharose (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, U.S.A.) to remove residual thrombin. The unbound fractions were collected together and were diluted five-fold with 50 mM phosphate, pH 7.5, and loaded on

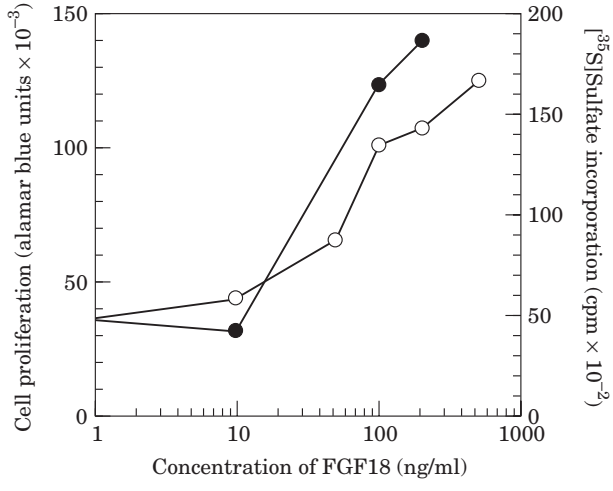


Fig. 2. Effect of FGF18 on DNA and proteoglycan synthesis in primary cultures of porcine articular chondrocytes. Primary cultures of porcine articular chondrocytes were incubated in increasing amounts of FGF18 for 5–6 days. Cell proliferation (open symbols) was monitored by AlamarBlue™ and proteoglycan synthesis (closed symbols) was measured by [^{35}S] sulfate incorporation as described under Materials and methods.

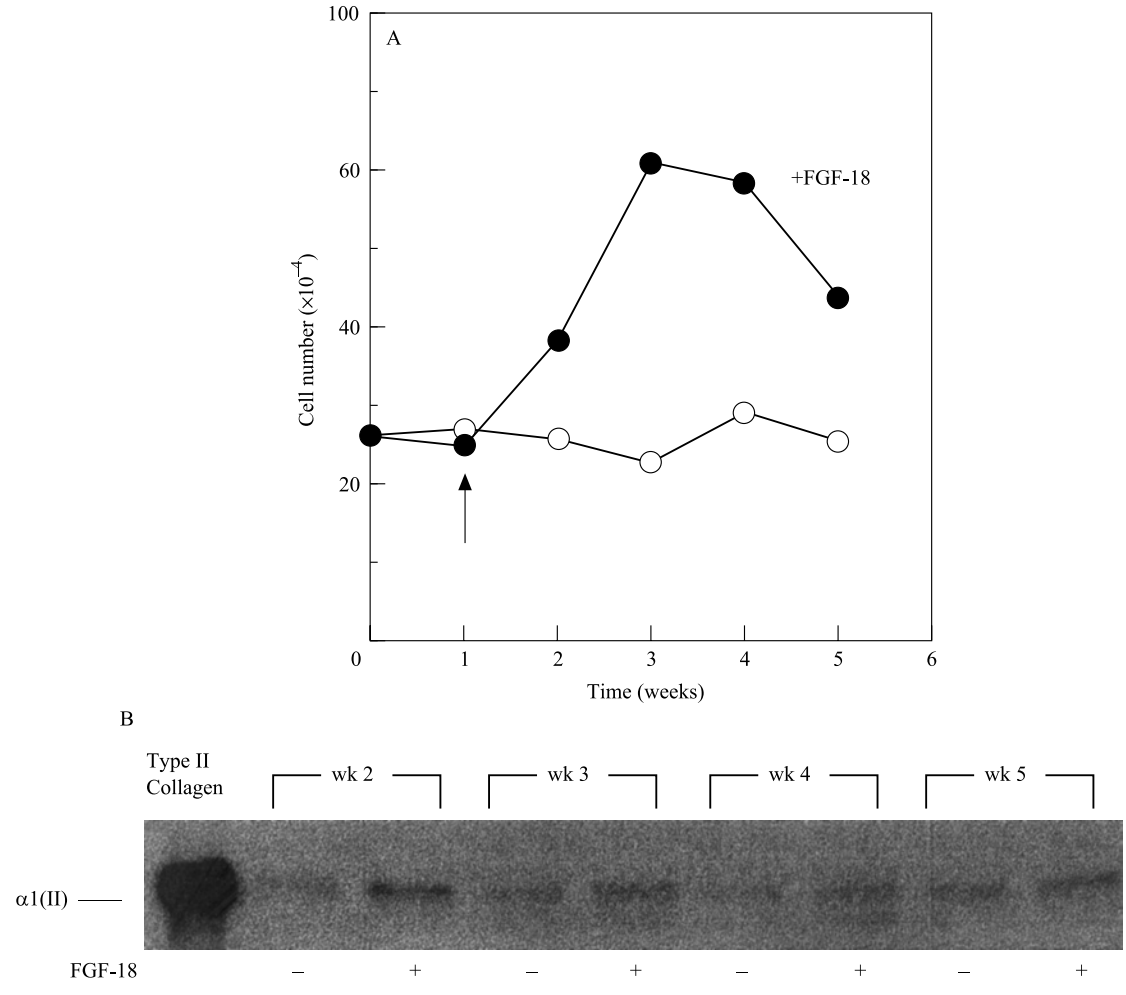


Fig. 3. Effect of FGF18 on cell proliferation and synthesis of type II collagen in primary cultures of adult human articular chondrocytes. Chondrocytes from the human talus joint were isolated from cartilage slices and cultured in the absence of FGF18 for one week as described under Methods. Vehicle alone or vehicle containing FGF18 (100 ng/ml final concentration) was added (arrow) and the cells were cultured for the indicated time as described under Methods. Cell proliferation [panel (A)] was measured weekly by direct cell counts. Type II collagen deposited in the extracellular matrix (panel B) was assessed by Western blotting as described under Methods. The antibody 1C10 detected the α1(II) collagen chains of type II collagen. **The faint band migrating below the α1(II) collagen chain is a pepsin over-cleavage product of type II collagen[33,34]. Wk=weeks.

a Poros 20 HS cation exchange column (Perceptive Biosystems, Framingham, MA, U.S.A.) before eluting with a gradient (0–2.0 M) of NaCl. Fractions containing FGF18 were collected together and disulfide formation in FGF18 was then carried to completion by oxidation with CuSO4 at a final concentration of 0.5 mM. Finally, aggregates and residual impurities were removed with a Sephacryl S-100 size exclusion column (Amersham Pharmacia Biotech, Inc.) operated with formulation buffer containing 50 mM Na phosphate, pH 7.2, 1 M NaCl, and 10 mM EDTA. The purified FGF18 showed a single species at about 20 kDa on reducing SDS-PAGE analysis. The homogeneity of the FGF18 preparation was confirmed by N-terminal sequencing and mass spectrometry analyses. The FGF18 was dialysed into 1.0 M NaCl, 0.01 M EDTA, 0.05 M Na-phosphate, and pH 7.2 and was stored at −80°C until use.

Results

The angiogenic potential of *Fgf18* was assessed by adenovirus-mediated transfer of *Fgf18* into the pinnae of nude mice. While no apparent angiogenic response was observed, pinnae that received adeno-*Fgf18* became visibly thicker and developed a reddish-brown coloration. Tissue thickening was observed within 4 days of inoculation and remained localized around the injection site for up to 17 days. Pinnae that received adeno-null contained a narrow band of chondrocytes within the central zone of elastic cartilage [Fig. 1(a), (c)]. The vacuolated cells in the center of this region [Fig. 1(c), arrow] are 'lipochondrocytes' whose cytoplasmic spaces are swollen with a large cytoplasmic lipid droplet[11]. Few chondrocytes were observed outside of this region, the central zone lipochondrocytes were bordered by flattened perichondrial cells without obvious lipid inclusions or chondrocyte morphologies [Fig. 1(c), arrowhead]. Infection with adeno-*Fgf18* greatly expanded the number of basophilic 'chondrocyte-like' cells observed around the site of inoculation [Fig. 1(b), (d)] and extending outwards from the auricular cartilage zone into the skeletal muscle and subcutis. These cells were strongly stained with an antibody to PCNA as were hair follicles and epithelium [Fig. 1(g), (h)]. Chondrocytes were identified by

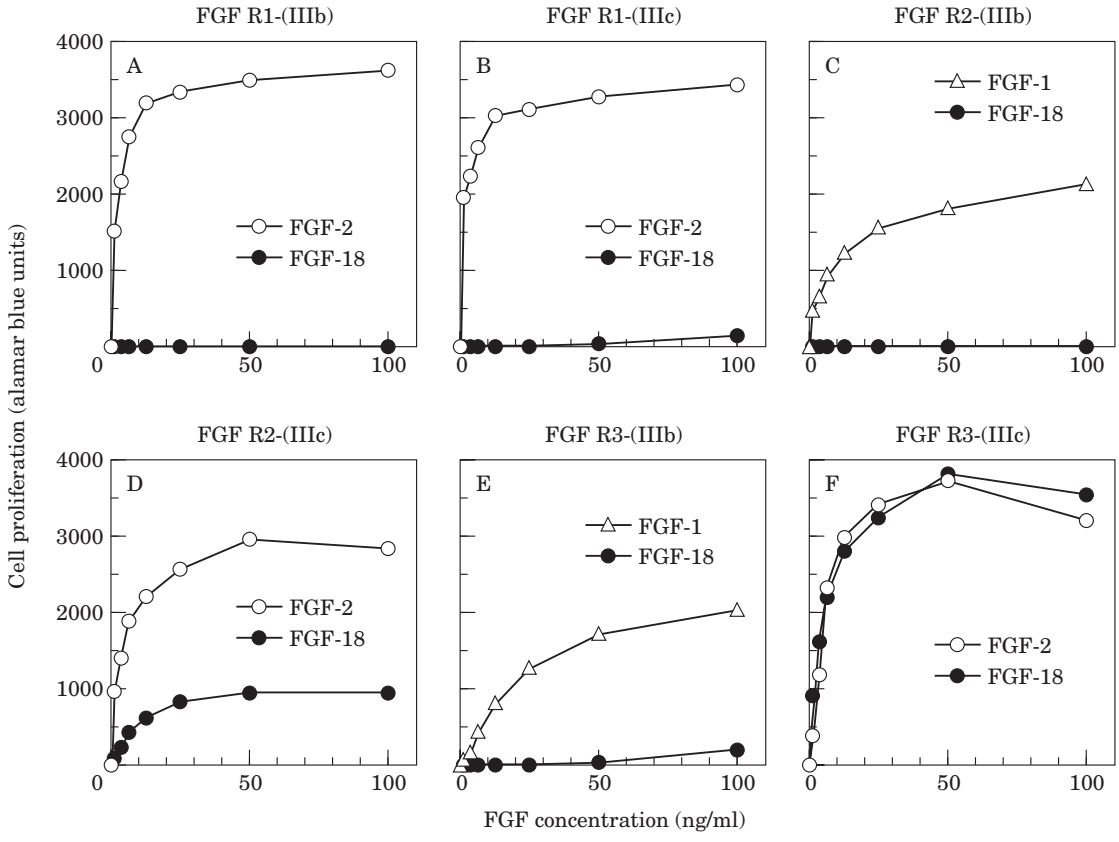


Fig. 4. Effects of FGF18 on the proliferation of BaF3 cells expressing FGF receptor splice variants. BaF3 cells expressing the 'b' and 'c' splice variants of FGF receptors 1-3 were incubated in the presence and absence of the indicated concentration of FGF-1, FGF-2 or FGF18 and cell proliferation was measured as described under Methods. The following BaF3 cell lines were used: panel (A), FGF R1α-(IIIb) cells; panel (B), FGF R1α-(IIIc) cells; panel (C), FGF R2α-(IIIb) cells; panel (D), FGF R2α-(IIIc) cells; panel (E), FGF R3α-(IIIb) cells; and panel F, FGF R3α-(IIIc) cells. Each point represents the average of duplicate wells of cells from two separate experiments.

staining of adjacent sections with a type II collagen antibody. Relative to pinnae infected with adeno-null [Fig. 1(e)], type II collagen immunoreactivity was greatly increased by infection with adeno-*Fgf18* [Fig. 1(f)]. Enhanced staining for type II collagen was evident by day 5 and was maintained near this level for up to 17 days. No staining was detected with a mouse isotype-specific control antibody (data not shown). The matrix surrounding the chondrocytes also showed increased staining with toluidine blue [Fig. 1(i), (j)]. In contrast, adeno-*Fgf18* had little or no effect on elastin (19) deposition in mouse pinnal tissue [Fig. 1(k), (l)]. Thus, adenovirus-mediated transfer of *Fgf18* into mouse pinnae increased the proliferation of pinnal chondrocytes and greatly increased the production of type II collagen and extracellular matrix by these cells.

In short-term (≤1-week) cultures of primary porcine chondrocytes, addition of FGF18 to the cell media produced an increase in cell proliferation that was paralleled by an increase in [^{35}S]-sulfate incorporation into proteoglycans (Fig. 2). The proliferation of primary adult human articular chondrocytes in long-term (≤5 weeks) culture was also increased by the addition of 100 ng FGF18/ml [Fig. 3(A)]. In these experiments, chondrocytes were cultured for 1 week in the absence of FGF18 and then in its presence for up to 4 weeks. Increased chondrocyte cell numbers were observed within 1 week after the addition of FGF18. Chondrocyte proliferation was maintained for three weeks of culture in the presence of FGF18 and then declined [Fig. 3(A)]. The reason for the reduction in chondrocyte cell number after 5 weeks of culture is not clear but could be due to a loss of sensitivity to FGF18 or to a more generalized reduction in proliferative activity after long-term culture. Western blot analysis of collagen deposited in the extracellular matrix showed an apparent increase in type II collagen accumulation within one week after the addition of FGF18 [Fig. 3(B)]. Type II collagen accumulated in the matrix throughout the 5-week duration of the study [Fig. 3(B)]. Similar observations were made with adult human talus chondrocytes maintained as high-density micromass cultures: chondrocyte cell numbers, staining of chondrocyte nuclei with antibodies to PCNA, type II collagen accumulation, and staining of proteoglycans with safranin-O were increased by incubation with media containing 100 ng FGF18/ml for 2 weeks (data not shown). Taken together, these data demonstrate that FGF18 stimulated the proliferation of porcine and adult human articular chondrocytes in primary cell culture. In addition, these data show that the differentiation state of the chondrocytes, as determined by type II collagen and proteoglycan production, was maintained by FGF18 under these conditions.

BaF3 cells have been used extensively to study the activities of receptor tyrosine kinases, including receptors of the FGF family[12]. To evaluate the receptor selectivity of FGF18 in this system, BaF3 cells expressing the major splice variants of the *Fgfr* genes were incubated with increasing amounts of FGF18. In a separate series of wells, either FGF1 or FGF2 was added in place of FGF18 as a positive control. Addition of FGF18 had little or no effect on

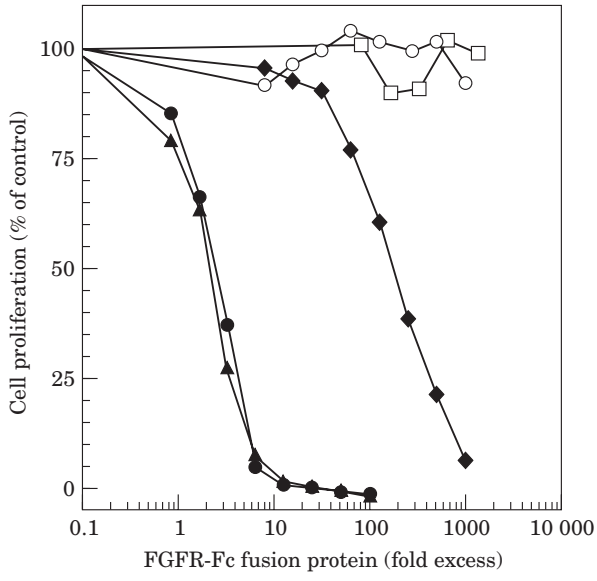


Fig. 5. Competition for FGF18-mediated proliferation of BaF3 cells by soluble FGF receptor-Fc fusion proteins. BaF3 cells expressing FGF R3α-(IIIc) were incubated in the presence and absence of 0.8 ng FGF18/ml and the indicated fold-excess (based on protein mass) of the various FGF R-Fc fusion proteins. Cell proliferation was measured as described under Methods. The 100% value of cell proliferation measured in the absence of FGFR-Fc was about 1000 Alamar blue units. Each point represents the average of duplicate dishes of cells from two separate experiments. FGFR1c-Fc, —○—; FGFR2b-Fc, —□—; FGFR2c-Fc, —◆—; FGFR3c-Fc, —●—; FGFR4-Fc, —▲—.

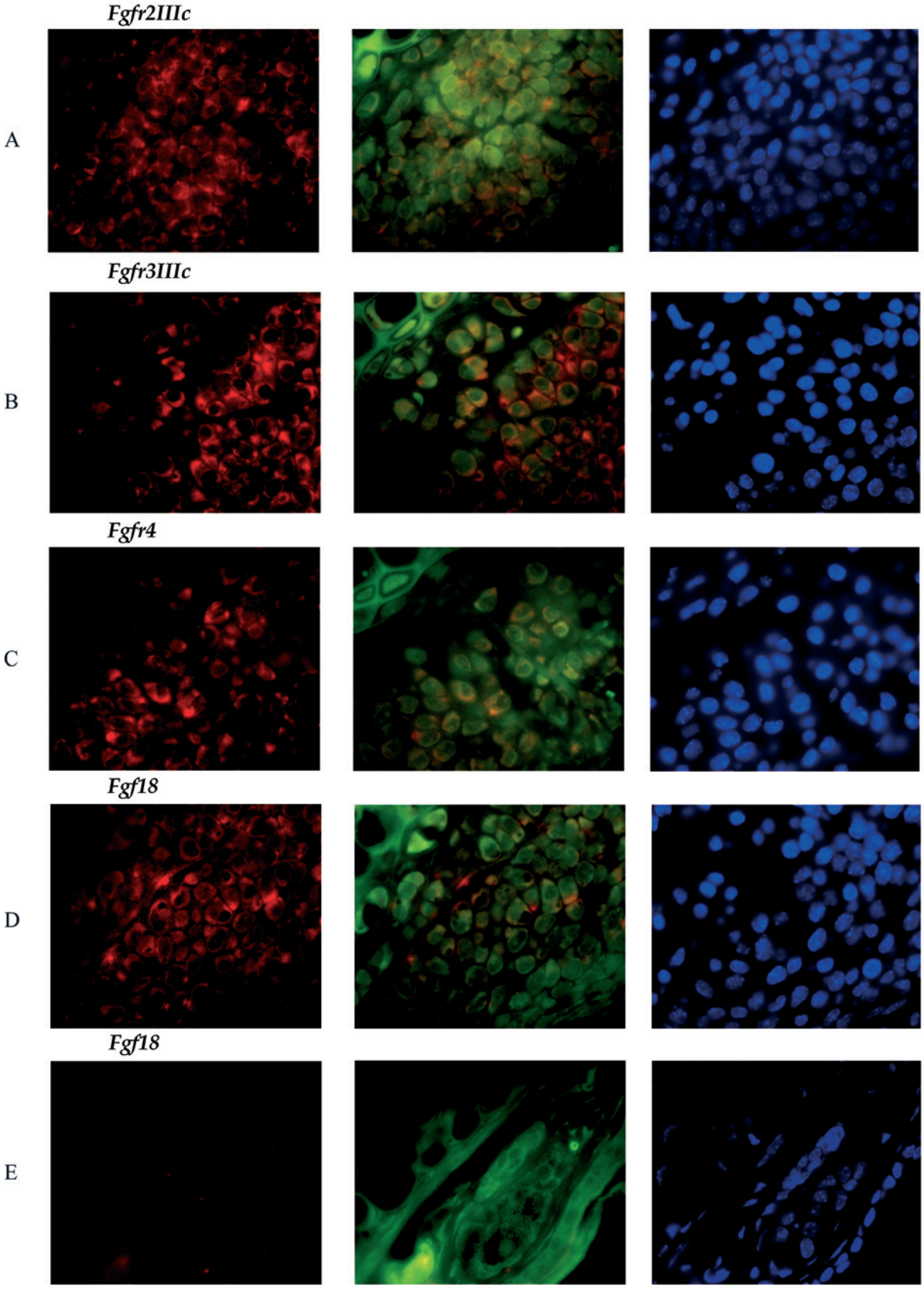
the proliferation of cells expressing the *Fgfr 1-(IIIb), Fgfr 1-(IIIc), Fgfr 2-(IIIb),* or *Fgfr 3-(IIIb)* genes [Fig. 4(A)–(C),(E)]. In parallel cultures, addition of either FGF2 [Fig. 4(A),(B)] or FGF1 [Fig. 4(C),(E)] increased cell proliferation, as reported previously[12]. BaF3 cells expressing either *Fgfr 2-(IIIc)* [Fig. 4(D)] or *Fgfr 3-(IIIc)* [Fig. 4(F)] responded to FGF18 with a dose-dependent increase in cell proliferation. The apparent EC_{50}s for these effects in the *Fgfr 3-(IIIc)* and *Fgfr 2-(IIIc)* expressing cells were approximately 2 and 12 ng FGF18/ml, respectively. Heparin was absolutely required for the induction of cell proliferation, as under identical conditions in the absence of heparin, FGF18 was without effect (data not shown). A similar pattern of receptor selectivity in the BaF3 cells was observed with FGF18 produced in Chinese hamster ovary cells (data not shown).

To further assess the relative receptor selectivity of FGF18, a series of soluble human FGF receptor-Fc fusion proteins were used in competition studies with BaF3 cells expressing *Fgfr 3-(IIIc)*. The fusion proteins used consisted of the extracellular domain of each FGFR fused to the carboxy-terminal Fc region of human IgG_1. Addition of either FGFR 3-(IIIc)-Fc or FGFR 4-Fc produced a dose-dependent inhibition of FGF18-mediated cell proliferation (Fig. 5). Competition was also observed with FGFR 2-(IIIc)-Fc, however, the apparent IC_{50} was about 100-fold higher than that found with FGFR 3-(IIIc)-Fc or FGFR 4-Fc (Fig. 5). Over this concentration range, no competition was observed with FGFR 1-(IIIc)-Fc or with FGFR 2-(IIIb)-Fc (Fig. 5). Taken together, these data demonstrate that FGF18, in the presence of heparin, can bind and activate FGFR 4, FGFR 3-(IIIc) and FGFR 2-(IIIc). FGF18 appeared, moreover, to bind neither the FGFR 'b' splice variants nor did it bind and activate FGFR 1 under these conditions. These data are consistent with FGF18 receptor specificity reported recently by Xu *et al*.[13]

To assess the expression of *Fgf18* and *Fgfr*s within chondrocytes of murine pinnae, paraffin embedded pinnal tissue from adeno-*Fgf18* treated mice was subjected to *in situ* hybridization with specific riboprobes for *Fgf18* and its receptors. For clarity, images of the perichondrial zone of proliferating chondrocytes adjacent to the central zone lipochondrocytes are shown. A portion of the central zone is shown in the upper left hand corner of each image. Expressions of *Fgf18*, *Fgfr 2-(IIIc)*, *Fgfr 3-(IIIc)*, and *Fgfr 4* were detected within the expanded chondrocyte zone in the pinnae [Fig. 6(A),(D), left panels]. Little or no expression of these genes was detected, in contrast, within the mature lipochondrocytes [Fig. 6(A),(D), upper left corner of each image] of the pinnal tissue. Similarly, little or no *Fgf18* mRNA was detected in resting pinnal chondrocytes in tissue infected with adeno-null [Fig. 6(E), left panel]. Relative to pinnae treated with adeno-null [Fig. 6(E)], an increased number of cells within this zone was clearly evident from images of tissue autofluorescence [Fig. 6(A)–(E), middle panels] or from nuclear staining with DAPI (Fig. 6, right panels). Thus, the chondrocytes of murine pinnae transduced with adeno-*Fgf18* express *Fgf18*, *Fgfr 4* and the (IIIc) splice variants of *Fgfr 2* and *Fgfr 3*.

To evaluate expression of *Fgfr* in more detail, pinnae infected with adeno-null or adeno-*Fgf18* were immunostained with antibodies directed against the carboxyterminal domains of FGFRs 1–4. A high level of background staining was observed with the antibodies directed against FGFR 1, 3, and 4 in virtually all cell types within the tissue. Antibodies to FGFR 2, in contrast, displayed minimal background and intensely stained the cells of the expanded chondrocyte population in pinnae treated with adeno-*Fgf18* [Fig. 7(a)]. Staining was specific for FGFR 2 as a non-immune isotype control antibody failed to stain the tissue [Fig. 7(b)]. On higher magnification, most of the staining with the FGFR 2 antibody was observed within nuclei of the proliferating chondrocytes [Fig. 7(c),(d)]. In tissue treated with adeno-null, a few chondrocyte nuclei in the perichondrial zone were stained with the FGFR 2 antibody [Fig. 7(c)]. With adeno-*Fgf18*, the number of anti-FGFR 2 stained chondrocyte nuclei was greatly increased [Fig. 7(d)]. The highest level of expression of FGFR 2 within pinnal chondrocytes was seen on day 5 through day 11 post-infection. Anti-FGFR 2 staining decreased in these

Fig. 6. Detection of *Fgf18*, *Fgfr 2-(IIIc)*, *Fgfr 3-(IIIc)*, and *Fgfr 4* mRNAs in murine pinna by *in situ* hybridization. The hybridization signals were visualized with Vector Red substrate and imaged with a FITC/Texas Red™ filter (left panels). Tissue intrinsic fluorescence (green) was imaged with a Blue filter to provide tissue orientation (middle panels). Due to broad excitation and emission wavelength of the Vector Red dye, a fraction of the Vector Red staining can also be visualized with this filter. Nucleic counter staining was achieved by DAPI and imaged with a DAPI filter (right panels). Panels (A)–(D) are images of adeno-*Fgf18* infected pinnae. Panel (E) is an image of adeno-null infected pinna. All images are orientated with central zone lipochondrocytes in the upper left corner. (A): *Fgfr-2(IIIc)*; (B): *Fgfr-(3IIIc);* (C): *Fgfr4;* (D) & (E): *Fgf18.*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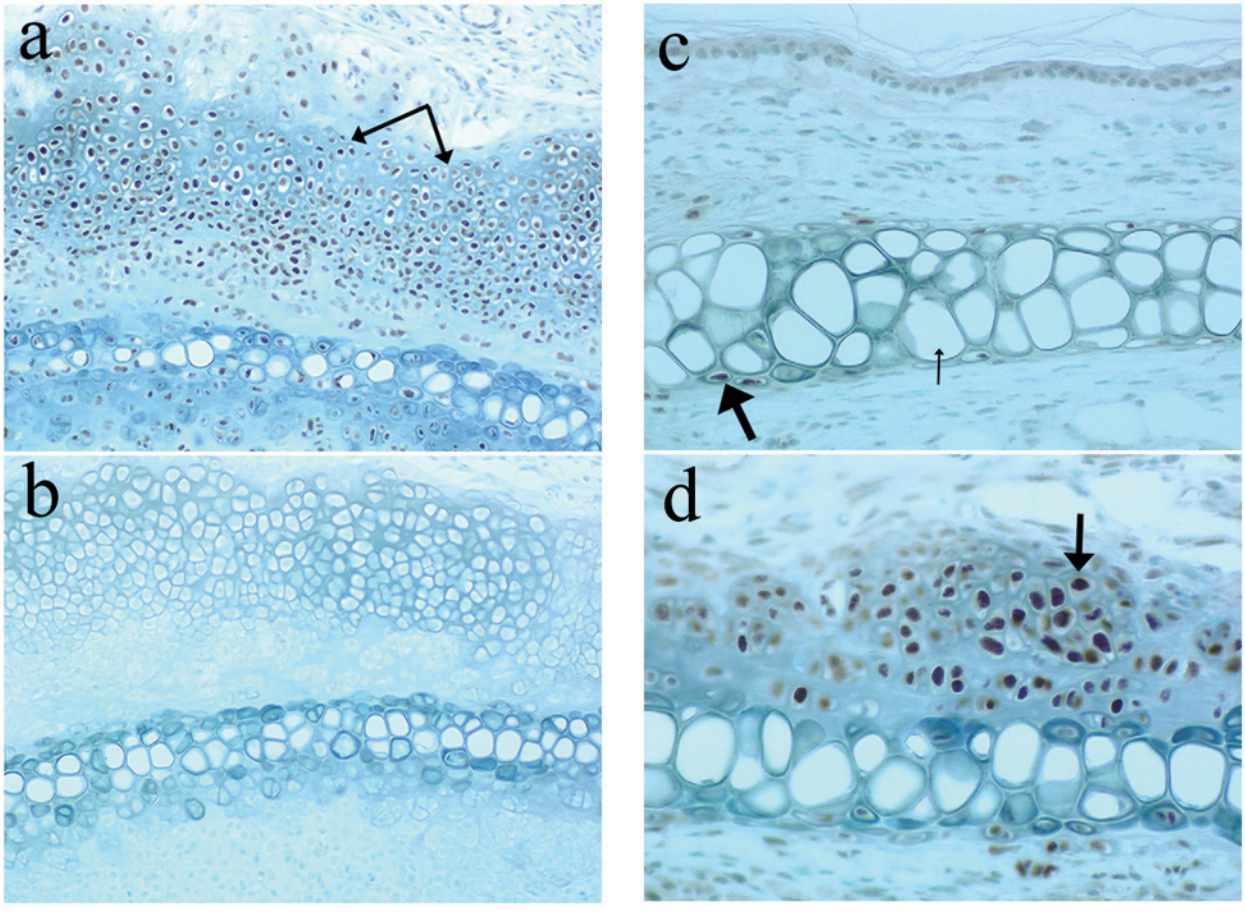

Fig. 7. Expression of FGF R2 within chondrocytes of murine pinna transduced with adeno-*Fgf18*. Mouse pinna were harvested on day 5 after infection with either adeno-null (panel c) or adeno-*Fgf18* [panels (a), (b), (d)]. Immunoperoxidase staining using antibodies to FGF R2 [panels (a), (c), (d)] or a non-immune isotype control antibody [panel (b)]. Sections were imaged at either 20× [panels (a), (b)] or 40× [panels (c), (d)]. Large arrows: chondrocytes expressing FGF R2; small arrow: lipochondrocyte.

cells by post-infection day 17. Staining with the FGFR 2 antibody was also observed within blood vessels and epidermis of pinna treated with either adeno-null or adeno-*Fgf18* (data not shown). As described above, FGF18 can bind and activate the 'IIIc' but not the 'IIIb' form of FGFR 2 suggesting that FGFR 2-(IIIc) may mediate, at least in part, chondrocyte proliferation in response to infection of pinnae with adeno-*Fgf18*.

The data described above suggested that FGF18 could act as a trophic factor for auricular and articular chondrocytes. To evaluate whether the *Fgf18* is expressed in normal human articular cartilage, cartilage slices from the adult human talus were fixed and processed for mRNA localization by *in situ* hybridization. *Fgf18* mRNA was specifically localized within the cytoplasm of the chondrocytes in these tissues [Fig. 8(a)]. On consecutive sections, these cells also stained with a riboprobe specific for type II collagen [Fig. 8(d)] but not with a probe for type I collagen [Fig. 8(e)]. Using splice variant specific riboprobes for two of the high affinity FGF18 receptors, *Fgfr 3-(IIIc)* [Fig. 8(b)] and *Fgfr 2-(IIIc)* [Fig. 8(c)] mRNAs were also consistently detected within chondrocytes in these tissues. In contrast, little or no consistent expression of *Fgfr* 4 was detected (data not shown). Similar results were obtained using RT-PCR to examine expression of these genes in fresh samples of adult human articular cartilage and in primary chondrocytes isolated and cultured from these tissues (data not shown). These data demonstrate that *Fgf18*, *Fgfr 2-(IIIc) and Fgfr 3-(IIIc)* are expressed in chondrocytes of normal human articular cartilage.

Discussion

The results reported here demonstrate that FGF18 has significant anabolic effects on chondrocytes *in vitro* and *in vivo*. The effects included both increased deposition of matrix proteoglycans and type II collagen and stimulated cell proliferation. The cellular source of the proliferating chondrocytes in adeno-*Fgf18* treated pinnae is not entirely clear. The cartilage plate of pinnal tissue consists of a perichondrium, a boundary zone and a central zone[11]. The perichondrial cells are flattened and are surrounded by and contain abundant type II collagen, while boundary zone cells have an abundant cytoplasm rich in organelles and a matrix containing type II collagen, proteoglycan and elastin[11]. The central zone contains what appear to be fully differentiated chondrocytes swollen by cytoplasmic lipid inclusion bodies and abundant pericellular elastin fibers[11]. Boundary or perichondrial cells are thought to respond to cartilage plate injury[11], and so are candidate progenitors. This region expands in the adeno-*Fgf18* treated pinnae; with numerous PCNA positive cells and cells staining for proteoglycan and type II collagen. Alternatively, the

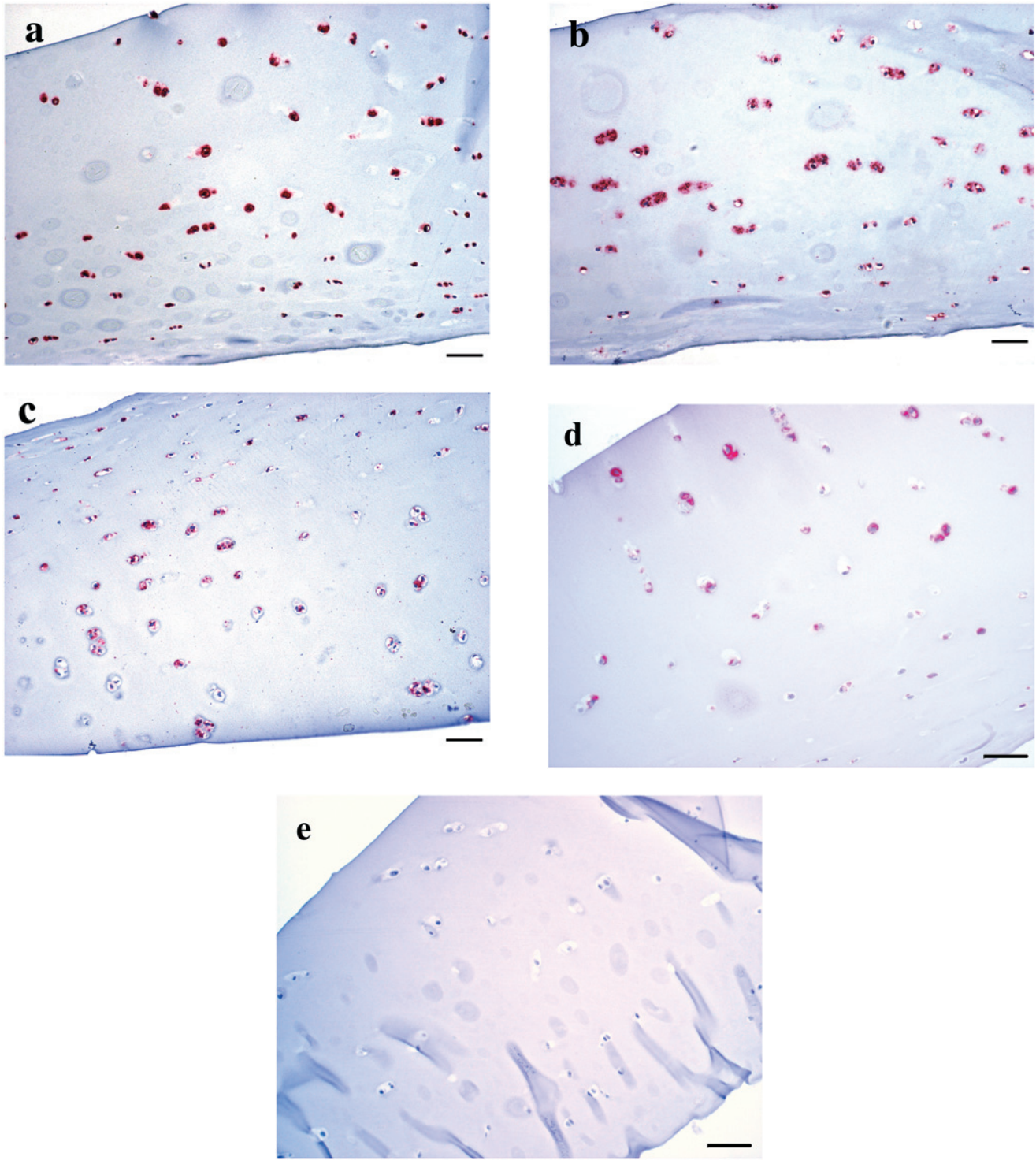

Fig. 8. Localization of *Fgf18, Fgfr 2-(IIIc)*, and *Fgfr 3-(IIIc)* mRNAs in human articular cartilage by *in situ* hybridization. Articular cartilage from the human talus joint was isolated and prepared for *in situ* hybridization with specific ribonucleotide probes for either human *Fgf18* [panel (a)], *Fgfr 3-(IIIc)* [panel (b)], or *Fgfr 2-(IIIc*) [panel (c)] as described under Methods. Alpha1(II) [panel (d)] and alpha1(I) [panel (e)] collagen probes were used as positive and negative controls, respectively. Detection of a specific mRNA is indicated by red staining. Similar results were obtained with articular cartilage from three separate individuals. Each bar represents 20 nm.

chondrocytes may have formed from mesenchymal stem cells in this region. It is notable that in punch wounds in the rabbit ear; mesenchymal cells appeared to grow out of the dermis and perichondrium to form a chondrocyte cell condensation during the later stages of the repair response[14].

Little or no effect of adeno-*Fgf18* was seen on the lipochondrocyte cell population or the matrix of the central zone in the mouse pinnae. This is consistent with the lack of expression of *Fgfr*s in these cells observed in the present study and their lack of proliferative activity by post-natal day

12 of development[11]. It is possible that the newly formed chondrocytes would have differentiated into mature lipochondrocytes over a longer observation period than 17 days. Pinnal perichondrial cells and chondrocytes arise from the pre-cartilaginous mass in the developing ear[15]. The effects of FGF18 on auricular cartilage thus appear to be specific to inducing a population of chondrocytes that synthesize and secrete type II collagen and proteoglycans, and not the characteristic resident elastic cartilage cells.

Another member of the FGF family, FGF2, appears to be a key regulator of bone growth and development. Disruption of *Fgf2* in mice results in decreased osteoblast replication and bone formation[16], while overexpression of *Fgf2* in transgenic mice produced chondrodysplasia and major defects in the long bones and bones of the skull[17,18]. It was proposed[17,18] that the latter activity was mediated by the inhibitory actions of FGF2 on chondrocyte terminal differentiation. This hypothesis is supported by other studies that have demonstrated a negative role of FGF2 on the growth and differentiation of growth plate chondrocytes[18,19]. However, *in vitro* studies with FGF2 and chondrocytes from a variety of sources have yielded inconsistent results both with regards to growth and production of extracellular matrix[20–24]. In this study, we show that auricular and articular chondrocytes appear to respond to FGF18 similarly. In addition to the trophic effects of adeno-*Fgf18* on auricular chondrocytes *in vivo*, we found that FGF18 stimulated the proliferation of porcine articular chondrocytes *in vitro*, and that this proliferation was accompanied by a parallel increase in proteoglycan synthesis. With adult human articular chondrocytes, moreover, the synthesis and accumulation of type II collagen increased after one week of incubation with FGF18. The differentiated phenotype of the proliferating chondrocytes, therefore, appears to be maintained in the presence of FGF18. Whether FGF18 is required for the formation or maintenance of cartilages *in vivo* must await the production and study of models of FGF18 gene disruption.

Transduction of signals from extracellular FGF is mediated by members of the FGF receptor gene family, FGFR 1–4[25]. In combination with a sulfated proteoglycan, FGFs bind and activate receptor tyrosine kinase activity, a process believed to be regulated by receptor dimerization[25]. Using FGF receptor-bearing BaF3 cells, highly purified FGFs, and soluble FGFR-Fc fusion proteins, FGF18 was shown in the present report to bind and activate FGFR 4 and the IIIc splice variants of FGFR 2 and FGFR 3. Consistent with studies with other FGFs[25], receptor activation by FGF18 was entirely dependent on exogenous heparin. Although FGF18 appears to be more selective in receptor specificity than either FGF-1 or FGF-2 in the BaF3 cell system[12], how these data translate to receptor selectivity in intact tissues is not clear. Chang *et al*.[26] have recently reported that tissue-specific heparan sulfate molecules can differentially affect FGF and FGF receptor binding *in situ*.

It is not yet understood which FGF receptor(s) mediates the effect of FGF18 on chondrocytes. In the mouse pinnae transduced with adeno-*Fgf18*, *Fgfr 2-(IIIc)*, *Fgfr 3-(IIIc)*, and *Fgfr 4* mRNAs were detected within proliferating chondrocytes in the expanded perichondrial zone. In addition, the nuclei of the proliferating chondrocytes were intensely stained with antibodies to FGFR 2. The nuclear localization is consistent with previous observations that other FGFRs traffic from the plasma membrane to the nucleus in response to FGF1[27]. Hence the nuclear localization of FGFR 2 in the auricular chondrocytes suggests that this receptor is involved in the proliferation of these cells in response to FGF18. Unfortunately, a high level of background staining prevented us from determining whether other FGFR proteins can also be localized to the nuclei of auricular chondrocytes. Analysis of adult human articular cartilage by *in situ* hybridization demonstrated the presence of *Fgfr 3-(IIIc)* as well as *Fgfr 2-(IIIc)* mRNAs, thus opening the possibility that *Fgfr 3-(IIIc)* may also be involved in the response to FGF18. In fact, FGFR3 is known to play a significant role in cartilage biology particularly in the growth plate. Many of the common forms of dwarfism in humans are caused by activating mutations in *Fgfr 3*[28] and targeted expression of an activated form of *Fgfr 3* to the growth plate of mice produced dwarf mice[29]. These data suggest that in the growth plate of long bones, *Fgfr 3* is a negative regulator of chondrocyte proliferation. However, Iwata *et al*.[30] have reported that signaling through FGFR 3 can both promote and inhibit chondrocyte proliferation depending on the stage of development. Although FGFR 3 signaling appears to be a negative regulator in long bones, excessive growth of the skull is seen in human chondrodysplasia caused by activating mutations in *Fgfr 3*, implying differing responses to FGFR 3 signaling in the skull and long bones[31,32]. Thus, the relative role of FGFR 2 and FGFR 3 in the response of articular chondrocytes to FGF18 will be important to elucidate.

Lastly, we found that FGF18 mRNA is expressed in normal human articular chondrocytes, suggesting that FGF18 plays a physiological role in cartilage biology. Since articular cartilage is avascular, alymphatic and populated by a single cell type of relatively immobile chondrocytes, an autocrine mode of action of FGF18 on cell proliferation and extracellular matrix production seems most likely. The anabolic effects of FGF18 on auricular and articular chondrocytes that we have described here suggest that FGF18 could potentially be useful in promoting repair of damaged cartilages. Clearly, much more work is required to determine its range of activity and mechanisms of actions.

Acknowledgments

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