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# Ectodermal FGFs Induce Perinodular Inhibition of Limb Chondrogenesis *in Vitro* and *in Vivo* via FGF Receptor 2

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**The formation of cartilage elements in the developing vertebrate limb, where they serve as primordia for the appendicular skeleton, is preceded by the appearance of discrete cellular condensations. Control of the size and spacing of these condensations is a key aspect of skeletal pattern formation. Limb bud cell cultures grown in the absence of ectoderm formed continuous sheet-like masses of cartilage. With the inclusion of ectoderm, these cultures produced one or more cartilage nodules surrounded by zones of noncartilaginous mesenchyme. Ectodermal fibroblast growth factors (FGF2 and FGF8), but not a mesodermal FGF (FGF7), substituted for ectoderm in inhibiting chondrogenic gene expression, with some combinations of the two ectodermal factors leading to well-spaced cartilage nodules of relatively uniform size. Treatment of cultures with SU5402, an inhibitor FGF receptor tyrosine kinase activity, rendered FGFs ineffective in inducing perinodular inhibition. Inhibition of production of FGF receptor 2 (FGFR2) by transfection of wing and leg cell cultures with antisense oligodeoxynucleotides blocked appearance of ectoderm- or FGF-induced zones of perinodular inhibition of chondrogenesis and, when introduced into the limb buds of developing embryos, led to shorter, thicker, and fused cartilage elements. Because FGFR2 is expressed mainly at sites of precartilage condensation during limb development *in vivo* and *in vitro*, these results suggest that activation of FGFR2 by FGFs during development elicits a lateral inhibitor of chondrogenesis that limits the expansion of developing skeletal elements.** 

***Key Words:*** **limb development; pattern formation; fibroblast growth factor; FGF receptor; lateral inhibition.**

## INTRODUCTION

The formation of cartilage elements both in the developing vertebrate limb, where they serve as primordia for the appendicular skeleton, and in high-density cultures of limb bud mesenchyme, a well-studied *in vitro* model for this process, is preceded by discrete cellular condensations (Hall and Miyake, 1995, 2000; Newman and Tomasek, 1996). Control of the size and spacing of these condensations is a key aspect of skeletal pattern formation. In particular, the tandem and parallel arrangements of individual elements that characterize all vertebrate limbs depend on most precartilage condensations remaining discrete, although fusion of certain condensations also plays an important part in limb development in some species (Shubin, 1991). Cultures of chicken precartilage mesenchyme can be used to study both modes of condensation development. When grown in a serum-free medium, wing bud cells obtained from Hamburger–Hamilton stages 24–26 embryos form condensations that expand and eventually fuse, differentiating into continuous sheets of cartilage. In contrast, the corresponding leg bud cells form condensations that remain isolated, each surrounded by cells that fail to condense or chondrify (Downie and Newman, 1994, 1995).

Condensation of precartilage mesenchyme results from altered adhesive interactions (reviewed in Newman and Tomasek, 1996). Fibronectin (Frenz *et al.,* 1989; Kosher *et al.,* 1982; Tomasek *et al.,* 1982), tenascin (Mackie *et al.,*

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1987), NCAM (Widelitz *et al.*, 1993), and N-cadherin (Oberlender and Tuan, 1994) are all elevated in foci of condensation in the developing limb and in high-density cultures of limb bud mesenchyme. Spatiotemporal regulation of condensation formation depends, in part, on the activity of one or more diffusible molecules of the TGF-$\beta$ family (Downie and Newman, 1994, 1995; Leonard *et al.*, 1991; Merino *et al.*, 1998; Miura and Shiota, 2000), which stimulate the production of extracellular matrix (ECM) and cell-adhesive proteins. Clearly, the domain of action of any initiator of condensation formation must also be limited if condensations, and the cartilage elements that ultimately arise from them, are to remain discrete (Newman, 1996).

In previous studies, we found that chicken wing and leg bud precartilage mesenchymal cells exhibited responses to various factors, such as TGF-$\beta$, retinoic acid, and serum, that affected the pattern of cartilage that formed (Downie and Newman, 1994, 1995). In the present work, we have studied these mesenchymal cell populations in serum-containing culture, in which they are particularly sensitive to inhibitory effects of ectoderm and fibroblast growth factors (FGFs). Our objective was to gain insight into the factors and interactions responsible for the organized patterns of chondrogenesis seen *in vivo* and, under certain conditions, *in vitro*. We have found that both limb ectoderm and FGF2 and -8, independently and in combination, induce peripheral and perinodular inhibition of chondrogenesis in wing and leg cell cultures, resulting in the conversion of a continuous sheet of cartilage into an array of individual nodules of cartilage surrounded by noncartilaginous mesenchyme.

The inhibitory effect of the FGFs did not occur in the presence of the FGF receptor family-specific tyrosine kinase inhibitor SU5402 (Mohammadi *et al.*, 1997). The specific receptor responsible for this effect was identified as FGF receptor 2 (FGFR2). This receptor was present at sites of incipient condensation, and both the ectoderm- and FGF-induced perinodular inhibition of chondrogenesis failed to occur under conditions in which the synthesis of the receptor was blocked by transfection with an antisense oligodeoxynucleotide (ODN) or a Morpholino oligonucleotide (MO) directed against this receptor. Transfection of chicken embryos *in ovo* with FGFR2 antisense oligonucleotides also produced abnormally short and thick cartilaginous limb skeletal primordia. These results suggest that ectodermal FGFs act on incipient precartilage condensations to limit their expansion by means of a laterally acting inhibitor of chondrogenesis.

## MATERIALS AND METHODS

### *Cell Culture*

Fertile White Leghorn eggs were obtained from Avian Services, Inc. (Frenchtown, NJ). Primary cultures were prepared by separately pooling mesenchyme from the myoblast-free distal 0.3 mm (Brand *et al.*, 1985; Newman *et al.*, 1981) of stage 24 (Hamburger and Hamilton, 1951) wing and leg buds. Cells were dissociated in 1% trypsin–EDTA (Sigma), filtered through Nytex 20-$\mu$m monofilament nylon mesh (Tetco, Briarcliff Manor, NY), and washed with and resuspended in medium for plating at $2.5 \times 10^5$ (unless otherwise specified) cells per 10-$\mu$l spot.

For some experiments, ectoderm was removed from isolated limb bud distal tips. Limb bud tips were incubated at 38°C for 10 min in calcium- and magnesium-free Earle's balanced salt solution (CMF EBSS; GIBCO) containing 2 mM EDTA. The tips were then transferred to cold EBSS containing 2% fetal bovine serum (FBS; Hyclone), and the ectoderm was released by gentle pipetting. The mesoblasts were manually isolated and rinsed in EBSS before dissociation and resuspension for plating as described above.

Cell spots were deposited in Costar 24-well tissue culture plates and allowed to attach for 45 min before wells were flooded with 1 ml of medium [55% Ham's F12, 35% Dulbecco's modified Earle's Medium (DMEM)] containing 10% FBS unless otherwise indicated. Some cultures were grown in serum-free defined medium [DM; Paulsen and Solursh, 1988): 60% Ham's F12, 40% DMEM, 5 $\mu$g/ml insulin (Sigma), 10 nM hydrocortisone (Sigma), 50 $\mu$g/ml L-ascorbic acid, 5 $\mu$g/ml chicken transferrin (Sigma)]. Media were changed every other day. Recombinant human FGF7, FGF2 (Isacchi *et al.*, 1991), mouse FGF8b (R&D Systems, Minneapolis, MN), or combinations of the latter two factors, at concentrations of 10–100 ng/ml, were added to some cultures on the day of plating and with the first change of medium on day 2. In some cultures, the indolinone tyrosine kinase inhibitor SU5402 (Mohammadi *et al.*, 1997) (Calbiochem) was added at 2 $\mu$M on day 2 of the culture period. Cultures were maintained for 6 days. Hoffman Modulation Contrast images of precartilage condensations in living cultures were obtained with a Zeiss IM 35 inverted microscope using a 4× objective (Frenz *et al.*, 1989).

### *Transfection of Precartilage Mesenchyme*

Isolated stage 24–25 limb bud tips were suspended in 1 ml PBS containing 100–300 $\mu$g 15-mer antisense or control phosphorothioate ODN (GeneLink, Inc., Hawthorne, NY) in a 4-mm gap BTX electroporation cuvette. These explants were subjected to three 50-ms 2-V pulses in a BTX Electro Square Porator T820 equipped with an Enhancer 400 oscilloscope (Genetronics, Inc., San Diego, CA). They were then treated with trypsin and dissociated as described above, with or without removal of ectoderm, as indicated in the figure legends. The conditions for electroporation were optimized by a series of transfections using a cytomegalovirus–green fluorescent protein promoter–reporter construct (Clontech Inc., Palo Alto, CA). The ODNs used were chicken FGFR2 antisense (AN), 5′-ATCCCAGCTGACCAT-3′, and scrambled (SC), 5′-TACGACGTCACCCTA-3′. Some transfection experiments were also performed using 25-mer Morpholino oligonucleotides (MO; Genetools, Covallis, OR) at 10–60 $\mu$M, employing the same electroporation protocol described above. The Morpholino sequences used were chicken FGFR2 antisense (AMO), 5′-GCTGACCATCTTTTTAGAGTAAGTT-3′, and a standard control (CMO), 5′-CCTCTTACCTCAGTTACAATTTATA-3′.

### *Transfection of Chicken Embryos in Ovo*

*In ovo* electroporation of 5-day chick embryos with Morpholino oligoribonucleotides was performed according to the method of Momose *et al.* (1999) by using the BTX T820 electroporator equipped with platinum and tungsten microelectrodes as the anode

and cathode, respectively (kind gifts of Dr. Kunio Yasuda, Nara Institute, Japan). Eggs were incubated horizontally in a humidified atmosphere at 38.5°C for 2–3 h, at which time, 4 ml of albumin was removed. The eggs were windowed on day 3 of incubation, sealed with Parafilm and permitted to develop for an additional 2 days before treatment. On day 5, 2 $\mu$l of antisense or control MO (60 $\mu$M in PBS) were injected into the embryos by using a 25-$\mu$l Hamilton syringe with the electrodes placed on either side of the embryo, flanking the dorsoventral axis. Three 50-ms pulses of 7 V were then administered. The eggs were resealed and permitted to develop for 2–3 additional days before fixing and staining.

## Alcian Blue Staining of Cultures and Embryos

Cultures were fixed in 10% formalin, 0.5% cetylpyridinium chloride, for 5 min, washed with 3% acetic acid, pH 1.0, for 1 min, and then stained overnight with Alcian blue 8GS (Electron Microscopy Sciences; 0.5% in 3% acetic acid) at pH 1.0 (Lev and Spicer, 1964). Cartilage patterns were visualized under a binocular dissecting microscope after washing stained culture spots with 3% acetic acid, pH 1.0, to remove unbound stain. Content of highly sulfated proteoglycan was quantitatively determined by extraction of the bound dye with 8 M guanidinium chloride followed by spectrophotometry using an EIA reader equipped with a 600-nm filter (Hassell and Horigan, 1982; Leonard *et al.*, 1989). Whole embryos were fixed in 5% trichloroacetic acid overnight, stained overnight with 0.087% Alcian blue in acid alcohol, destained in acid alcohol overnight, dehydrated in an ascending series of ethyl alcohol, and cleared in methyl salicylate (Saunders, 1996).

## DNA Determination

The DNA content of 6-day cell cultures was measured fluorometrically in the presence of the dye Hoechst 33258 (Labarca and Paigen, 1980). Cultures were harvested in 50-$\mu$l/well 0.05 M $NaH_2PO4$, 2.0 M NaCl, 2 mM EDTA, pH 7.4, and disrupted by sonication. Measurements were made in the same buffer containing 1 $\mu$g/ml dye in a Hoefer TKO 100 Mini Fluorometer. Each sample consisted of homogenate pooled from three culture wells.

## Cell Proliferation Assay

Ectoderm-free leg cell cultures were plated at $1.6 \times 10^5$ cells per spot in the presence of 75 ng/ml FGF2. At specified times after plating, cells were exposed to BrdU Photolyte for 40 min, followed by a 20-min treatment with 2% DMSO and Photolyte Enhancer (Absolute-S SBIP Cell Proliferation Assay Kit; Molecular Probes, Eugene, OR). Wells were washed and flooded with 70% ethanol, and the plates stored at −20°C for a minimum of 72 h. DNA breaks were introduced by photolysis with UV light (254 nm; UV Stratalinker 1800) for 10 min. Broken strands were labeled overnight with rocking, by bromodeoxyuridine triphosphate (BrdUTP) using terminal deoxytransferase (TdT) (TUNEL reaction) according to the kit manufacturer's instructions. Labeled DNA was detected by using Alexa Fluor 488 dye-labeled anti- bromodeoxyuridine (BrdU) antibody (room temperature, 30 min). In some cases, antibody treatment was followed by incubation with propidium iodide/RNase A. Cultures were viewed on an inverted microscope by using a fluoroscein-GFP filter set (Omega Optics) for BrdU labeling and a rhodamine filter set (Carl Zeiss, Inc.) for propidium iodide labeling. Images were captured with an Optronics three-chip color imaging system. Proliferation-independent DNA end labeling (e.g., apoptosis) was determined in cultures not treated with BrdU Photolyte but exposed to UV light and subjected to TUNEL. No such labeling was detected in these cells.

## Quantitative Analysis of Gene Expression

Expression of the gene for the cartilage-specific proteoglycan core protein aggrecan was quantitatively analyzed by using reverse transcription followed by real-time polymerase chain reaction with the LightCycler System (Roche Laboratories). The SYBR Green-I dye binding method was used, followed by melting peak analysis of specific product accumulated after 40 cycles, which was within the exponential phase of amplification of the aggrecan product. Actin expression was used as an internal standard. Total RNA was extracted from untreated (82 wells) and FGF2 and -8-treated (65 wells) micromass cultures prepared from 5-day leg bud precartilage mesenchyme and grown for 3 days. Treated cultures contained 80 ng/ml FGF2 and 40 ng/ml FGF8 throughout the culture period. Primers used for reverse transcription and amplification were chicken aggregan (Chandrasekaran and Tanzer, 1993; Accession No. M88101), forward primer: 5′-TCTGATCACAGGCGTTTCAG-3′; reverse primer: 5′-AGTAGCAGTGGCTGGCAGAT-3′; chicken $\beta$-actin (Wang and Morais; Accession No. L08165), forward primer: 5′-AGGCTGTGCTGTCCCTGTAT-3′; reverse primer: 5′-GCTGTGGTGGTGAAGCTGTA-3′.

## Immunochemical Detection of FGFR2

Leg cell cultures incubated for various times in the presence of 80 ng/ml FGF2 and 40 ng/ml FGF8 were fixed in 2% formaldehyde/PBS for 20 min, washed in PBS, and incubated in 1% Tergitol NP-40 (Sigma) in PBS for 10 min (Downie and Newman, 1995). They were then blocked for 2–3 h (2% nonfat dry milk, 5% normal rabbit serum in PBS), followed by an overnight incubation at 4°C with a 1:50 dilution in blocking solution of a goat anti-FGFR2/bek polyclonal antibody with broad species reactivity but nonreactivity with either FGFR1 or FGFR3 (FGFR2abG; Research Diagnostics Inc.). Cultures were then incubated in CY3-conjuagated rabbit anti-goat secondary antibody (Jackson Laboratories; 1:200) for 2 h at room temperature in blocking solution (2% dry milk, in PBS), washed three times in PBS, and visualized by using a rhodamine filter set. For immunoblot analysis, cell cultures transfected with antisense or control ODN or MO were solubilized in 2× sample buffer [50 mM Tris, pH 6.8, 1% sodium dodecyl sulfate (SDS), 20% glycerol, 15 mM dithiothreitol, and 7.5 $\mu$g/ml each of leupeptin and chymostatin], at a ratio of three cultures per 50 $\mu$l buffer. The tissue was disrupted by sonication and heated at 100°C for 2 min. Samples were loaded into 12% SDS–polyacrylamide gels (Novex/Invitrogen, Carlsbad, CA) and electrophoresed for 1.5 h. Proteins were transferred to PVDF membrane (Millipore) by electroblotting for 1 h in 10 mM Tris–glycine, pH 11.0, 10% MetOH. Blots were blocked for 1 h at 37°C in blocking buffer (33 mM Tris base, 137 mM NaCl, 5 mM KCl, 5% dry milk) then incubated overnight with rocking at 4°C using goat anti-FGFR2 polyclonal antibody diluted 1:1000 in blocking buffer. Blots were washed with TTBS (Tris-buffered saline: 33 mM Tris base, 500 mM NaCl, 0.01% Tween), incubated for 1 h with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch, West Grove, PA; 1:25,000) in TTBS containing 1% gelatin, and washed 5–10 times with TTBS. Stained bands were visualized by using the ECL Plus detection kit (Amersham–Pharmacia) according to the manufacturer's instructions.

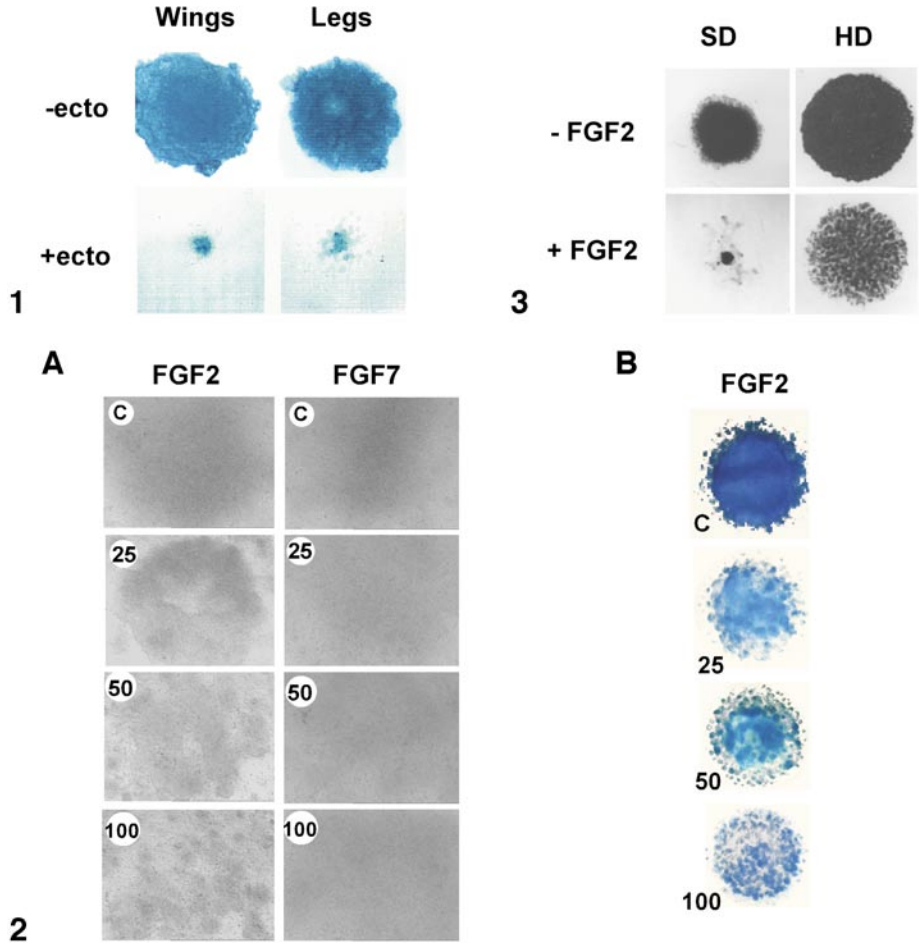



**FIG. 1.** Effect of ectoderm on cartilage pattern *in vitro*. (Upper left) Cultures established from wing bud mesenchyme from which ectoderm was removed, grown in serum-supplemented medium, form a sheet-like mass of cartilage. (Lower left) In the presence of ectoderm, a central mass of cartilage forms which is surrounded by a large zone of nonchondrogenic cells. (Upper right) Leg cell cultures devoid of ectoderm, grown in serum-supplemented medium, also form a sheet-like mass of cartilage, whereas (Lower right) the presence of ectoderm leads to the formation of one or more central nodules surrounded by zones of nonchondrogenic tissue, similar to the effect seen in wing cell cultures. Alcian blue-stained cultures photographed through a dissecting microscope. Culture diameters are approximately 5 mm.

**FIG. 2.** (A) Effect of increasing concentrations of FGFs on the formation of precartilage condensations by limb bud precartilage cells *in vitro*. Leg cell cultures were grown for 3 days in the presence of the indicated amounts (ng/ml) of FGF2 and FGF7. Living cultures were visualized by Hoffman Modulation contrast microscopy (4× objective) (Frenz *et al.*, 1989). FGF2-treated, but not FGF7-treated, cultures exhibit a nodular appearance. (B) Effect of FGF2 on cartilage pattern in serum-containing cultures of limb bud mesenchyme. Leg cell cultures were grown in the absence of ectoderm. A continuous sheet of cartilage formed in the absence of FGF2 (control, C). Increasingly larger regions of noncartilaginous tissue appear within the cartilage mass with increasing concentrations of FGF2 (25, 50, and 100 ng/ml). Staining and photography as in Fig. 1.

**FIG. 3.** Induction of perinodular inhibition in limb cell cultures is independent of cell density. Cultures plated at $2.5 \times 10^5$ (standard density, SD) or $5.0 \times 10^5$ (high density, HD) cells per 10-$\mu$l spot were grown in the absence or presence of 25 ng/ml FGF2. Staining and photography as in Fig. 1.

## RESULTS

### *Ectoderm Induces Perinodular Inhibition of Chondrogenesis of Limb Bud Mesenchyme in Vitro*

In previous studies, stage 24–26 wing and leg bud precartilage mesenchymal cells were shown to exhibit different patterns of chondrogenesis in serum-free culture as well as distinct responses to the addition of serum to the culture medium (Downie and Newman, 1994, 1995). We subsequently found that the response of these mesenchymal populations to serum was influenced by whether or not limb ectoderm was present in the cultures. We therefore decided to study the effects of ectoderm in serum-

**TABLE 1**

Effect of FGF2 on Accumulation of Cartilage Proteoglycan in Cultures of Wing Bud Precartilage Mesenchyme

| Treatment | Proteoglycan |
|---|---|
| Control | 100[a] |
| 1[b] | 88.0 ± 2.9 |
| 5 | 81.9 ± 2.6 |
| 15 | 72.9 ± 4.9 |
| 25 | 67.9 ± 4.2 |
| 50 | 51.1 ± 4.7 |
| 100 | 44.7 ± 2.1 |

*Note.* Cultures were grown for 6 days in serum-containing medium in the presence of the designated concentrations of FGF2, added on the day after cultures were established.

[a] Data represent percentage of control values of $OD_{600}$ of GuHCl-extracted Alcian blue stain from three to five stage 24 wing tip cell cultures.

[b] ng/ml FGF2.

containing cultures. Alcian blue staining at pH 1.0, a quantitative index of cartilage development in these cultures (Leonard *et al.*, 1989), was used to monitor the pattern of chondrogenesis. We found that serum-containing wing cell cultures grown in the absence of ectoderm formed a continuous, sheet-like mass of cartilage by 6 days of culture (Fig. 1, upper left). If limb ectoderm was included in the cultures, however, the extent of the cartilaginous mass was severely reduced (Fig. 1, lower left). In this case, a single large nodule was surrounded by a peripheral halo of nonchondrified connective tissue (see below).

For serum-containing leg cell cultures, the response to ectoderm was equally dramatic: in the absence of ectoderm, these cultures formed a sheet of cartilage like that produced by wing cells grown under the same conditions (Fig. 1, upper right). But when leg cultures were grown in the presence of ectoderm, they formed one or more nodules of cartilage surrounded by cells that failed to undergo chondrogenesis (Fig. 1, lower right). The effects of ectoderm in inducing perinodular inhibition were seen both in cultures in which limb bud distal tips were dissociated without prior removal of ectoderm, and in cultures in which the ectoderm had been removed before dissociation and added back once the mesenchymal micromasses were established (not shown).

### *FGF2 and -8 Inhibit Chondrogenesis of Limb Mesenchyme in Vitro and Cooperatively Substitute for Ectoderm in Mediating Perinodular Inhibition*

To determine whether the effects of ectoderm on the pattern of cartilage differentiation in serum-containing leg mesenchymal cell cultures were due to known limb ectodermal products, we cultured ectoderm-free leg cells in the presence of serum and various concentrations of FGF2 and FGF8.

At 3 days of culture, when control leg cells were first massing into a large condensation, progressively higher concentrations of FGF2 caused the single condensation to break up into separate condensing masses. In contrast, the same concentrations of the mesodermally produced factor FGF7 had no such effect (Fig. 2A). FGF2-treated cultures showed a pattern of Alcian blue-positive cartilage formation by 6 days that was comparable to that in cultures grown in the presence of ectoderm. In particular, the sheet-like mass of cartilage seen in ectoderm-free controls was constricted and/or interrupted. Moreover, the amount of cartilage formed was reduced, in a fashion that reflected

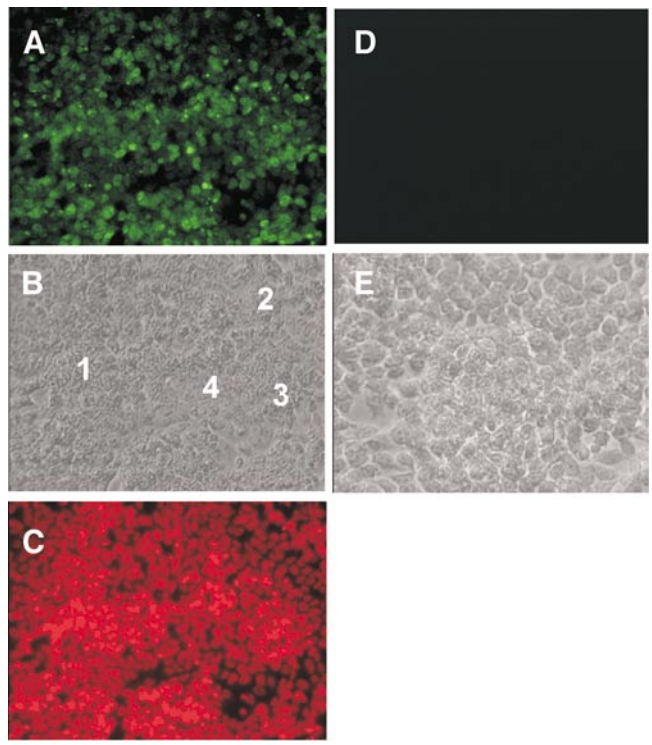



**FIG. 4.** Cell proliferation in 1-day leg cell cultures. Cells grown in the presence of 75 ng/ml FGF2 were pulse labeled for 40 min with BrdU after 23 h in culture. (a) BrdU label (green); (b) phase contrast image of same field; (c) propidium iodide label (red) of same field; (d) Lack of detection of BrdU label in cells not exposed to BrdU in culture, but exposed to UV light and assayed for DNA breaks by TUNEL-dependent BrdU incorporation (see Materials and Methods); (e) phase contrast image of field in (d). The control represented by (d) and (e) indicates that these cells are not apoptotic and that labeling in (a) is DNA synthesis-dependent. Numbers on (b) indicate (1, 3) regions of incipient condensation with, respectively, low and high BrdU labeling, and (2, 4) noncondensing regions with, respectively, low and high BrdU labeling. (a–c) Photographed using a 16× and (d, e) using a 32× objective. Examination of >15 FGF2-treated cultures at 23–24 h and 13 h (not shown) disclosed no relationship between condensation and proliferation rate.

the presence of nonchondrogenic tissue surrounding the cartilaginous nodules (Fig. 2B; leg cultures shown; similar results were also obtained with wing cultures).

After 6 days of culture, wing bud cells that had been exposed to 25 ng/ml of FGF2 contained one major and a few scattered peripheral nodules of cartilage instead of the uniform sheet of cartilage formed by control cultures (Fig. 3, lower left). In some cultures, we increased the initial plating density of cells from 2.5 to 5.0 $\times$ $10^5$ per 10-$\mu$l spot to determine whether enhanced cell contact could overcome the inhibitory effect. However, the addition of FGF2 still led to a final chondrogenic pattern in which nodules of cartilage were separated by domains in which chondrogenesis had failed to progress (Fig. 3, lower right).

The effect of FGF2 on cartilage differentiation, while dose-dependent, exhibited a plateau. The extent to which chondrogenesis was inhibited by FGF2 was quantitated spectrophotometrically. Corresponding to the formation of regions of perinodular inhibition, there was a dose-dependent decline in cartilage formation. At 50 ng/ml FGF2, only half the control amount of stainable matrix was produced by wing cell cultures. This level of inhibition was only slightly increased by further raising the FGF2 concentration to 100 ng/ml (Table 1).

The observed perinodular inhibition was an effect on cartilage differentiation, not cell proliferation. For both wing and leg cells, mesenchymal condensations in 3-day ectoderm-containing or FGF2-treated (Fig. 2A) cultures were surrounded by a layer of less condensed mesenchyme, and Alcian blue-stained nodules at 6 days (Figs. 1 and 2B) were surrounded by dense, but unstained, tissue. The DNA content of wing and leg cultures grown in the presence of ectoderm was no different from cultures grown in its absence. The presence of 50 ng/ml FGF2 led to a slightly increased DNA content relative to ectoderm-lacking controls (Table 2). On the cellular level, there was no strict relationship between proliferation rate and condensation formation: FGF2-treated (75 ng/ml) cultures exposed to the thymidine analogue BrdU for 40 min at 23 h of culture, when incipient condensations were first apparent, contained areas of high and low DNA synthesis-dependent labeling in both condensing and noncondensing regions (Fig. 4). The suppression of chondrogenesis in the zones peripheral to the condensations in FGF2-treated cultures was therefore not due to differential proliferation of condensing and noncondensing mesenchyme.

Addition of the ectodermally produced factor FGF8 to wing and leg cultures also had an inhibitory effect on cartilage formation (Fig. 5). Limb bud ectoderm is a source of several FGFs, and FGF2 and FGF8 are the predominant ones supplied by the apical ectodermal ridge (AER) during the formation of digits from the distal tip of the 5-day chicken limb bud (Martin, 1998). We therefore studied the effect of combinations of the two factors on the cartilage pattern generated by the mesenchymal cells. Compared with 50 or 100 ng/ml of either factor, 50 ng each of both factors together produced an array of nodules that were relatively uniform in size, well spaced, and similar in scale to cartilaginous skeletal primordia in the developing limb *in vivo* (Fig. 5). As with FGF2 alone, combinations of FGF2 and FGF8 led to modest increases in cell proliferation, despite decreases in Alcian blue staining (Table 2).

### *FGF2 and -8 Inhibit Chondrogenesis at the Level of Gene Expression*

Ectoderm and FGFs could potentially inhibit the formation of cartilage at several different levels. The most relevant level of regulation from the standpoint of skeletal pattern formation would be that of cartilage-specific gene expression. We used quantitative real-time PCR to determine the effects of exposure to a combination of FGF2 and -8 on expression of the gene for the cartilage-specific proteoglycan core protein, aggregan (Upholt and Olsen, 1991; Chandrasekaran and Tanzer, 1993). We found that exposure of leg cell cultures to FGF2 and -8 caused a nearly 50% reduction in the amount of aggrecan RNA in these cultures (Table 3). This indicates that the inhibition of Alcian blue staining by FGF (Figs. 2B and 3; Table 1) reflected inhibition of cartilage-specific gene expression.

### *Ectoderm and FGFs Induce Perinodular Inhibition via FGF Receptor 2*

Signaling by FGFs is mediated by at least four FGF receptors (Givol and Yayon, 1992), three of which, FGFR1, FGFR2, and FGFR3, are expressed during limb development (Xu *et al.*, 1999). The indolinone tyrosine kinase inhibitor SU5402 is specific for the FGFR family of receptors, contacting a peptide motif in the hinge region between the two kinase lobes (Mohammadi *et al.*, 1997), which is conserved in chicken FGFR1, -2, and -3 (Pasquale, 1990). Limb cell cultures grown in the presence of a combination of FGF2 and -8 formed a solid sheet of cartilage over the majority of the culture area when treated with SU5402 (Fig. 6), indicating involvement of standard FGF receptors in the induction of perinodular inhibition of chondrogenesis by the FGFs.

In the developing limb, FGFR1 mRNA is widely dispersed throughout the precartilage mesenchyme prior to the formation of condensations (Delezoide *et al.*, 1998; Peters *et al.*, 1992; Szebenyi *et al.*, 1995). FGFR3 mRNA, in contrast, does not appear until cartilage has differentiated (Delezoide *et al.*, 1998; Szebenyi *et al.*, 1995). FGFR2 mRNA first appears at significant levels in precartilage condensations (Peters *et al.*, 1992; Szebenyi *et al.*, 1995; Delezoide *et al.*, 1998). We assayed for the presence of FGFR2 protein in FGF2- and -8-treated leg cell cultures by indirect immunofluorescence (Fig. 7). During the course of development the first FGFR2, immunoreactive cells appear around the same time the first evidence of cell clustering can be observed (17 h; Fig. 7, upper pair of panels). These rounded cells were not always part of multicell clusters. By 24 h, confined groups of intensely immunoreactive cells coincided with expanding condensations, but isolated cells

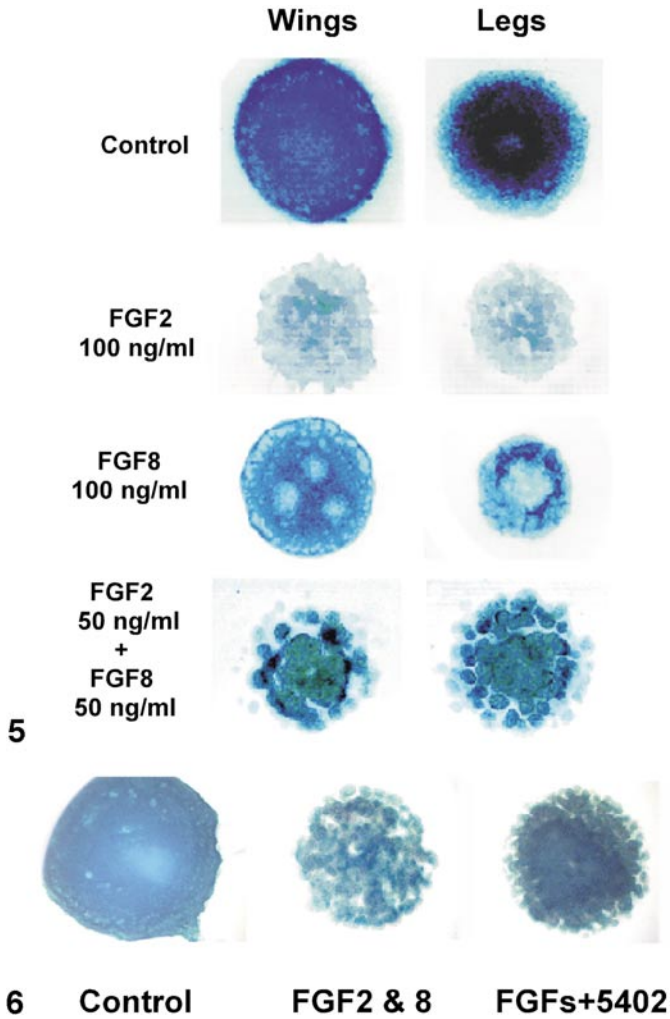






**FIG. 5.** Synergistic effect of FGF2 and FGF8 on the induction of the perinodular inhibition of chondrogenesis in ectoderm-free wing cell (left panels) and leg cell (right panels) cultures. (Top row) control cultures without FGFs; (Second through fourth rows) cultures grown in the presence of 100 ng/ml FGF2, 100 ng/ml FGF8, and 50 ng/ml of each of the factors, respectively. Staining and photography as in Fig. 1.

**FIG. 6.** Suppression of perinodular inhibition of chondrogenesis by a FGF receptor tyrosine kinase inhibitor. Ectoderm-free leg cell cultures produced a continuous sheet of cartilage (left). In the presence of 80 ng/ml FGF2 and 20 ng/ml FGF8, the cartilaginous sheet of cells was transformed into a nodular pattern with zones of perinodular inhibition (middle). Addition of the inhibitor SU5402 (2 $\mu$M) to FGF-treated cultures suppressed the formation of nodules and led to the replacement of zones of inhibition by cartilage (right).

continued to appear as well (Fig. 7, second and third pairs of panels). By 48 h, all immunoreactivity was gone in the maturing condensations (Fig. 7, fourth pair of panels). FGFR2 is therefore present in incipient condensations just at the time at which limb mesenchyme responds to FGF2 and -8 by forming regions of perinodular inhibition.

Transfection of wing or leg mesenchyme with an antisense ODN directed against FGFR2 completely blocked the ability of ectoderm (Figs. 8A and 8B), or a combination of FGF2 and -8 (Fig. 8C), to induce perinodular inhibition. Immunoblot analysis using a FGFR2-specific polyclonal antibody shows that the antisense treatment is effective at reducing receptor levels (Fig. 8D).

Because antisense ODN treatment acts by inducing RNase H and can thereby have nonspecific effects, we also designed an antisense MO spanning the translational start site of chicken FGFR2. Antisense MOs act by specifically blocking translational initiation and are thus less prone than ODN to spurious effects (Heasman *et al.*, 2000; Summerton, 1999). Similarly to the antisense phosphorothioate ODN, the antisense MO suppressed the formation of FGF2- and -8-stimulated zones of perinodular inhibition in leg (Fig. 9A) and wing cultures (not shown). When the antisense MO was introduced by electroporation into living embryos at 5 days of development when midlimb and digital patterning is occurring, it induced abnormally short and thick zeugopodal and autopodal elements in the developing leg (Fig. 9B). Transfection with control MO did not lead to skeletal defects.

## DISCUSSION

We have shown that FGFs produced by the ectoderm of the developing avian limb bud affect chondrogenic pattern formation by inducing the formation of zones of perinodular inhibition around developing precartilage condensations. Previous studies reported that partial removal of the dorsal limb bud ectoderm in developing chicken limbs *in ovo* leads, in many instances, to thickening and fusions of

**TABLE 2**

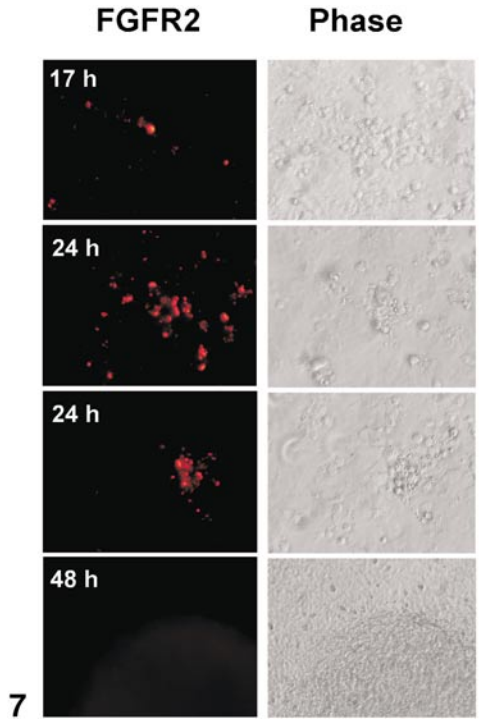
DNA Content of 6-Day Limb Precartilage Mesenchymal Cultures Grown in the Absence and Presence of Ectoderm and FGFs

| | Treatment | | |
|---|---|---|---|
| Experiment | −Ecto − FGFs | +Ecto | −Ecto + FGFs[a] |
| 1. Legs | 2.5 | 2.5 | 3.3* |
| 2. Wings | 4.1 | 3.6 | 5.2* |
| 3. Legs | 3.9 | 3.6 | 4.1 |
| 4. Wings | 2.5 | 2.4 | 3.2 |

*Note.* Values are expressed as mean $\mu$g DNA per culture well. Each data point represents measurements of three to seven samples consisting of combined extracts from three culture wells. Standard errors of mean were <15% of mean.

[a] FGFs for Experiments were as follows: Expt. 1, 50 ng FGF2/ml; Expts. 2–4, 60 ng FGF2 + 40 ng FGF8 per ml.

*, Significantly different from −Ecto − FGFs.



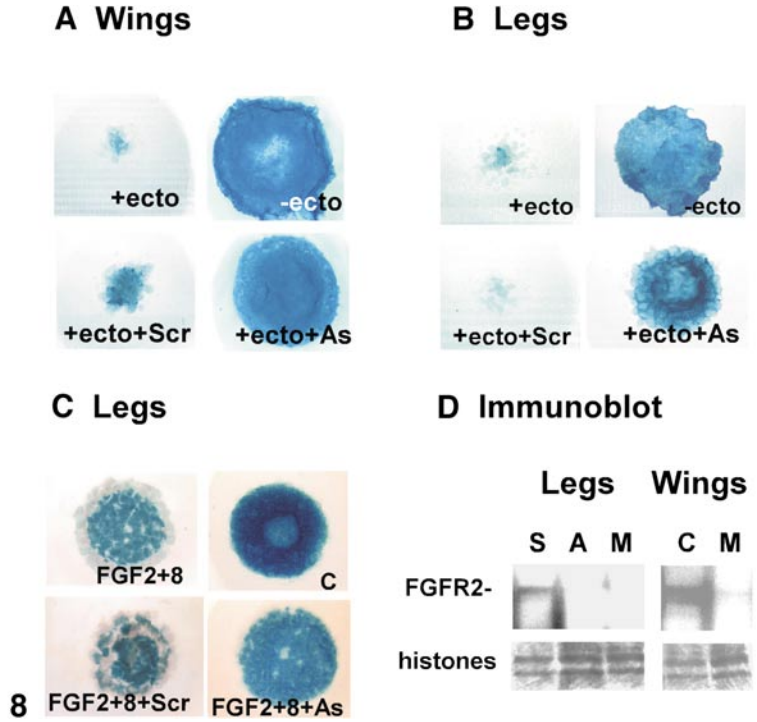




**FIG. 7.** Spatiotemporal expression of FGFR2 protein in FGF2-, -8-treated leg cell cultures. Each pair of panels shows an immunostained (left) and phase contrast (right) image of the same microscopic field. Top row, a culture at 17 h; second and third rows, cultures at 24 h; fourth row, a culture at 48 h. Objective magnification, 25× in all cases.

**FIG. 8.** Ectoderm- and FGF-induced perinodular inhibition of chondrogenesis is mediated by FGFR2. Electroporation of (A) wing or (B) leg cell cultures with an antisense ODN directed against FGFR2 (As) caused the disappearance of the zone of perinodular inhibition of chondrogenesis induced by the presence of ectoderm (Top left), transforming it into cartilage (Bottom right). The cultures thereby assume the same morphology as those grown in the absence of ectoderm (Top right). Transfection with a scrambled ODN (Scr) caused virtually no change in the pattern of ectoderm-containing cultures (Bottom left). One or a few central cartilaginous nodules surrounded by zones of noncartilaginous tissue still formed. (C) Leg cultures grown in serum-supplemented medium without ectoderm (Top right), when treated with 50 ng/ml each of FGF2 and FGF8 formed a nodular pattern of cartilage (Top left). Cartilage formation was enhanced and nodularity was suppressed in cultures electroporated with antisense ODN directed against FGFR2 (Bottom right). Electroporation with a scrambled ODN had no effect on the generation of zones of internodular inhibition (Bottom left). (D) Immunoblot analysis of FGFR2 expression shows that a band of ~90 kDa detected by an anti-FGFR2 antibody is greatly reduced in cultures electroporated with antisense (A) but not scrambled (S) ODN, or antisense (M) but not control (C) MO. Cultures for the ODN experiments were grown in the presence of 50 ng/ml each of FGF2 and FGF8. Cultures for the MO experiments were grown in the presence of 80 ng/ml FGF2 and 20 ng/ml FGF8. Staining and photography as in Fig. 1.

skeletal elements (Martin and Lewis, 1986). *In vitro* analysis showed that inclusion of the AER, or dorsal and ventral limb bud ectoderm, suppressed chondrogenesis in limb cell cultures (Solursh *et al.*, 1981). Those investigators suggested that the ectodermal product responsible for the suppression of chondrogenesis was hyaluronan (Solursh and Reiter, 1988; Solursh *et al.*, 1981). But because all regions of the chicken limb bud ectoderm produce FGF2 (Savage *et al.*, 1993), and during the digit-forming stage of development, the AER produces FGF8 (Martin, 1998), the earlier findings are likely to have been attributable, in part, to the effect of ectodermal FGFs.

An inhibitory effect of limb bud ectoderm on chondrogenesis was also suggested by studies in which removal of portions of the AER or dorsal ectoderm from the developing limb bud led to interdigital chondrogenesis (Hurle and Gañan, 1986). Because the presence of an intact ectoderm during development does not suppress digital (or other normal) chondrogenesis, these earlier experiments suggested that ectoderm is an indirect, rather than direct, inhibitor of chondrogenesis.

In the present studies, FGF2 was unable to suppress more than about 55% of the production of Alcian blue-staining matrix in wing cultures (Table 1). Moreover, neither ectoderm (Fig. 1) nor the combination of FGF2 and -8 (Fig. 5) suppressed chondrogenesis in a spatially uniform fashion, but rather allowed the formation of a cartilaginous mass surrounded by a set of relatively regularly spaced nodules. Together, these results provide additional evidence that ectoderm and its FGFs are indirect inhibitors of chondro-

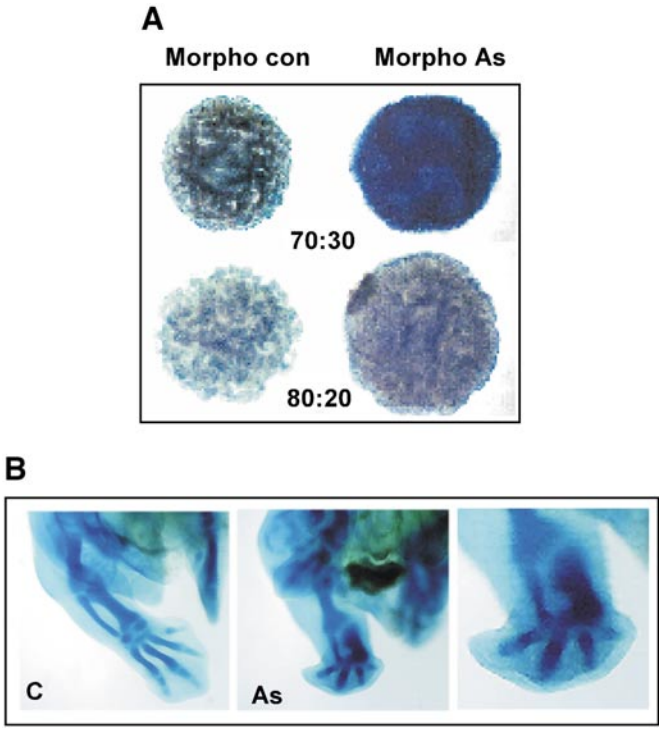



**FIG. 9.** Suppression of perinodular inhibition of chondrogenesis by antisense MO. (A) Ectoderm-free leg cell cultures grown in the presence of exogenous FGF2 and FGF8 exhibited nodularity when electroporated with control MO. In cultures transfected with FGFR2 antisense MO, zones of perinodular inhibition did not appear. Similar results obtained in leg cultures with different combinations of the FGFs (70 ng/ml FGF2 + 30 ng/ml FGF8 and 80 ng/ml FGF2 + 20 ng/ml FGF8) and in wing cultures (not shown) confirmed the robustness of the effect. (C) Cartilage patterns in legs of stained and cleared 7-day chicken embryos. The embryo on the right was electroporated with FGFR2 antisense MO. Note that the elements of zeugopod and autopod are abnormally shortened and broadened and that the tarsal elements are fused in the antisense-transfected embryo (detail, right panel).

genesis. In addition, the mediation of both ectodermal and FGF inhibition of chondrogenesis by FGFR2, which is localized to incipient centers of chondrogenesis *in vivo* (Peters *et al.*, 1992; Szebenyi *et al.*, 1995; Delezoide *et al.*, 1998) and *in vitro* (Szebenyi *et al.*, 1995), and Fig. 7, argues strongly for an indirect mechanism. Taken together, these results suggest that the inhibitory activity elicited by ectoderm, or the combination of FGF2 and -8, spares certain centers of condensation (perhaps those that are initiated earliest), but prevents their expansion by affecting cells that would otherwise be recruited into condensations later during development. In keeping with this are studies that indicate that developing chicken and mouse digits are themselves sources of anti-chondrogenic activity (Gañan *et al.*, 1994; Lee *et al.*, 1994).

Given the commonality of their receptors, the finding that FGF2 and FGF8 have distinct effects on an apparently uniform cell type is unexpected, but not unprecedented (Hajihosseini and Dickson, 1999). Indeed, the factors have different patterning effects when applied separately to developing limbs *in vivo* (Akiba *et al.*, 2001), and it is therefore reasonable to expect that their effects together would be different from each separately. Our *in vitro* evidence that they are required in combination in order to produce a pattern of regular cartilage nodules in distal tip mesenchyme suggests a reason for observed transitions in the complex of FGFs produced by the limb bud ectoderm at successive stages of development (Martin, 1998).

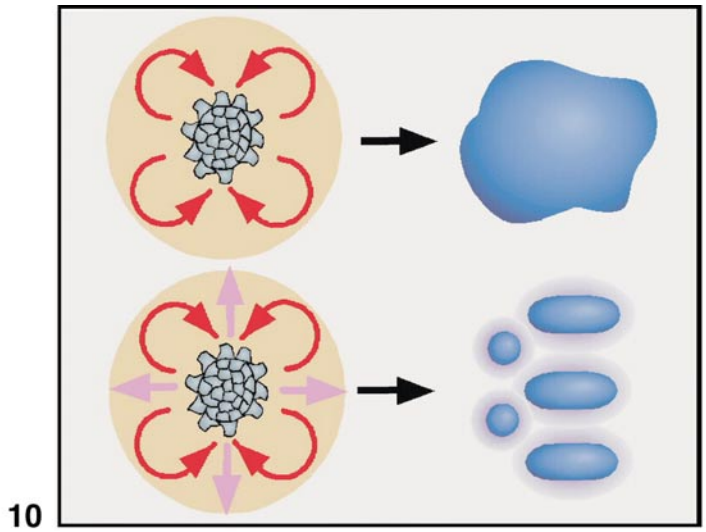
Previous work has provided evidence that initiation and expansion of precartilage condensations in the developing limb are regulated by one or more members of the TGF-$\beta$ family (Downie and Newman, 1994, 1995; Leonard *et al.*, 1991; Miura and Shiota, 2000). Because TGF-$\beta$ is positively autoregulatory (Van Obberghen-Schilling *et al.*, 1988), if unchecked it would lead to the formation of condensations and resulting cartilage elements of indefinite size. Prepatterns of TGF-$\beta$ immunoreactivity precede overt condensation of limb bud mesenchyme by at least half a day (Leonard *et al.*, 1991); it is at this early stage that the effects of FGFs in restricting the expansion of precartilage condensations must be exerted. The release of a diffusible or otherwise laterally transmissible inhibitor of condensation from the centers of incipient condensation (Fig. 10) could perform this function. Our data demonstrate that blocking an endogenous FGF signaling pathway enhances the amount of cartilage formed (Figs. 6 and 8). Furthermore, FGF2 does not act by differentially enhancing or suppressing proliferation of condensing or noncondensing mesenchyme (Fig. 4). Therefore, the production of a lateral inhibitor at the sites of incipient condensation is the most likely mechanism for generation of the nodular pattern. Although our evidence for an inhibitor is indirect, it is notable that, in zebrafish embryos, FGF3 induces the expression of chordino, a se-

**TABLE 3**

Quantitation of Aggrecan Gene Expression in 3-Day Limb Precartilage Mesenchymal Cultures Grown in the Presence of FGF2 and −8

| Sample | Primers | Peak area | $T_m$ | Aggrecan/Actin |
|---|---|---|---|---|
| Untreated | Aggrecan | 7.44 | 87.8 | |
| Untreated | Actin | 5.30 | 89.1 | 1.4 |
| FGF2-, 8-treated | Aggrecan | 4.46 | 87.8 | |
| FGF2-, 8-treated | Actin | 5.93 | 89.2 | 0.75 |

*Note.* Each sample was derived from the pooled total RNA from multiple 3-day leg bud micromass cultures grown in the absence and presence of 80 ng FGF2 + 40 ng FGF8. Equal amounts of RNA were used for each reverse transcription reaction and corresponding gene-specific sequences were quantitated by using the LightCycler with the SYBR Green-I-dye binding method (see Materials and Methods).



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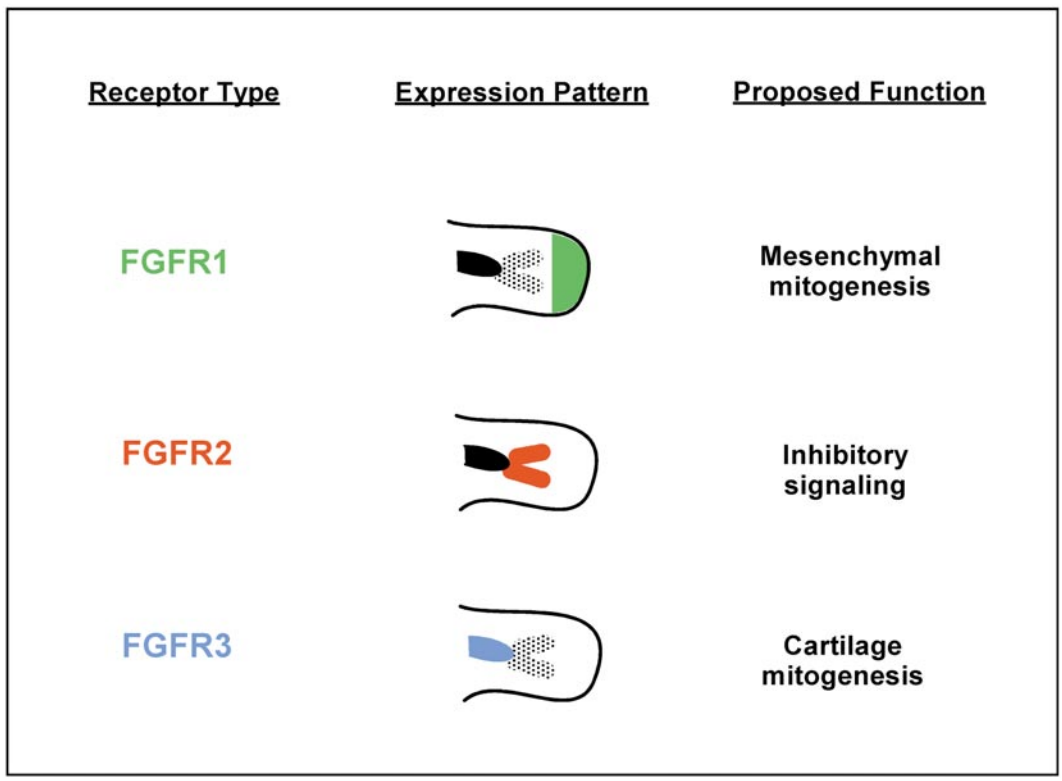


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**FIG. 10.** Schematic model for formation of discrete cartilaginous skeletal elements by a self-organizing biochemical network. (Top) Positively autoregulatory activator of chondrogenesis (red arrows), such as TGF-$\beta$, stimulates production of ECM and cell adhesion molecules, and itself, leading to expanding condensation and unpatterned mass of cartilage. (Bottom) Production from the sites of activation of diffusible inhibitor (pink arrows) of the activator, or of its downstream effectors, limits expansion of condensations and produces pattern of well-spaced cartilage elements.

**FIG. 11.** Spatiotemporal expression of FGF receptors in the developing chicken limb. FGFR1 is expressed in precartilage mesenchyme, FGFR2 in condensing mesenchyme, and FGFR3 in cartilage. Based on Szebenyi *et al.* (1995). A similar developmental pattern of FGFR expression occurs in the mouse (Peters *et al.*, 1992, 1993) and the human (Delezoide *et al.*, 1998).

creted antagonist of members of the TGF-$\beta$ superfamily (Koshida *et al.*, 2002).

FGF2 is known to promote outgrowth and distal progression of cartilage development in limb bud mesenchyme, and indeed can substitute for the corresponding functions of the AER (Fallon *et al.*, 1994). It is important to recognize, however, that signaling by FGFs is transduced by three different receptors in the developing limb that appear in distinct spatiotemporal patterns (Fig. 11); activation of each of these receptors may have distinct effects on cellular function. FGFR1 is widely dispersed throughout the precartilage mesenchyme prior to the formation of condensations (Fallon *et al.*, 1994; Patstone *et al.*, 1993; Peters *et al.*, 1992; Szebenyi *et al.*, 1995) and is therefore a likely mediator of the mitogenic effect of the growth factor during the outgrowth phase. FGFR3, in contrast, does not appear until cartilage has differentiated (Peters *et al.*, 1993; Szebenyi *et al.*, 1995), and its activation appears to transduce a negative effect on cartilage growth, particularly in the epiphyseal plates (Deng *et al.*, 1996; Shiang *et al.*, 1994; Webster and Donoghue, 1996). Most significantly for the present study, FGFR2 first appears in the mesoblast of the developing limb in the condensing precartilage mesenchyme (Delezoide *et al.*, 1998; Peters *et al.*, 1992; Szebenyi *et al.*, 1995), and is thus ideally situated to mediate the production of a laterally acting anti-chondrogenic factor.

Apert syndrome in the human, the characteristic phenotype of which includes synostoses and severe syndactyly, is associated with a set of mutations in FGFR2 (Oldridge *et al.*, 1999; Wilkie *et al.*, 1995). Because certain Apert syndrome FGFR2s have abnormally high affinity for a subset of normal ligands (Anderson *et al.*, 1998) or exhibit reduced specificity of ligand-binding (Oldridge *et al.*, 1999; Yu *et al.*, 2000), it has been suggested that the range of phenotypic effects results from FGFR2 gain-of-function (Hajihosseini *et al.*, 2001; Yu and Ornitz, 2001). The specific function gained under these circumstances, however, is not known, and the relationship between these biochemically defined activities and developmental function is unclear. Our *in vitro* and *in vivo* results suggest that one normal function of mesenchymal FGFR2 is to mediate perinodular inhibition of chondrogenesis. Further studies may determine whether hyperactivation of FGFR2 may, by exceeding a threshold, abrogate this function, thereby bringing about the syndactyly seen in Apert patients.

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