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# Fibroblast Growth Factor (FGF) 18 Signals through FGF Receptor 3 to Promote Chondrogenesis\*S

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Signaling by fibroblast growth factor (FGF) 18 and FGF receptor 3 (FGFR3) have been shown to regulate proliferation, differentiation, and matrix production of articular and growth plate chondrocytes in vivo and in vitro. Notably, the congenital absence of either FGF18 or FGFR3 resulted in similar expansion of the growth plates of fetal mice and the addition of FGF18 to human articular chondrocytes in culture enhanced proliferation and matrix production. Based on these and other experiments it has been proposed that FGF18 signals through FGFR3 to promote cartilage production by chondrocytes. Its role in chondrogenesis remains to be defined. In the current work we used the limb buds of FGFR3<sup>+/+</sup> and FGFR3<sup>-/-</sup> embryonic mice as a source of mesenchymal cells to determine how FGF18 signaling affects chondrogenesis. Confocal laser-scanning microscopy demonstrated impaired cartilage nodule formation in the FGFR3<sup>-/-</sup> cultures. Potential contributing factors to the phenotype were identified as impaired mitogenic response to FGF18, decreased production of type II collagen and proteoglycan in response to FGF18 stimulation, impaired interactions with the extracellular matrix resulting from altered integrin receptor expression. and altered expression of FGFR1 and FGFR2. The data identified FGF18 as a selective ligand for FGFR3 in limb bud mesenchymal cells, which suppressed proliferation and promoted their differentiation and production of cartilage matrix. This work, thus, identifies FGF18 and FGFR3 as potential molecular targets for intervention in tissue engineering aimed at cartilage repair and regeneration of damaged cartilage.

Articular cartilage has a very limited capacity for regeneration and repair. Irreparable structural damage can, therefore,

S The on-line version of this article (available at http://www.jbc.org) contains Supplemental Figs. S1 and S2.

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occur during the progression of degenerative disorders like osteoarthritis, in which the metabolic activity of articular chondrocytes is altered to favor catabolic over anabolic function (1, 2). When the articular surface is denuded, the underlying bone and marrow is exposed, and spontaneous repair mechanisms result in the formation of tissue that resembles fibro-cartilage. This tissue has neither the structural nor functional properties that are required to withstand the excessive biomechanical forces that it is subjected to and rapidly deteriorates. The focus of tissue engineering for cartilage repair is, therefore, aimed at developing strategies that are modeled after spontaneous repair mechanisms but which result in the generation of durable hyaline cartilage. This will be critically dependent on identification and manipulation of the signaling pathways that will selectively promote chondrogenesis and cartilage production by the multifunctional and pleiotropic growth factors present in the repair microenvironment.

Growth factors that have been implicated in articular cartilage repair include fibroblast growth factors (FGFs),<sup>1</sup> plateletderived growth factor, bone morphogenetic proteins, and transforming growth factor  $\beta$  (3–5). FGF2 has long been recognized as a mitogen for cells of the chondrogenic lineage (6, 7). More recently, FGF18 was shown to act as a trophic factor for articular chondrocytes (8). FGFs mediate their effects on chondrocytes by binding to three related trans-membrane receptors that are linked to multiple signal transduction pathways and which network with other cell surface-binding proteins for growth factors and for matrix molecules (9). In the context of tissue engineering for cartilage repair it is, therefore, critical to determine the ligand-receptor interactions that are primarily responsible for proliferation and those that are primarily responsible for differentiation and cartilage production.

In the current work we have used a three-dimensional cell culture model to define the role of FGF signaling through FGFR3 in the commitment of pre-chondrogenic mesenchymal cells to chondrogenesis and to cartilage production. This mouse limb bud micro-mass system has been used for more than two decades as a biologically relevant assay to examine the roles of growth factors, cytoskeletal proteins, teratogens such as thalidomide and retinoids, and gene mutations on cartilage development (10–17). Wide-field and confocal laser scanning fluorescence microscopy were used in conjunction with recognized molecular probes to capture the differentiation status of chondrogenic cells and the architecture of the cartilage nodules that they produced. Comparisons were made between cultures of cells derived from genetically modified embryonic mice lacking

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<sup>&</sup>lt;sup>1</sup> The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor; FBS, fetal bovine serum; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

expression of fibroblast growth factor receptor 3 (FGFR3) and their wild type littermates. Whereas both  $FGFR3^{+/+}$  and  $FGFR3^{-/-}$  cells were responsive to FGF2, only the  $FGFR3^{+/+}$ cells responded to FGF18 stimulation. We conclude that FGF18promotes differentiation and cartilage production by an FGFR3-mediated pathway in cells with chondrogenic potential. This knowledge can be exploited for the differential manipulation of FGF signaling pathways to optimize tissue engineering for cartilage repair and regeneration.

#### MATERIALS AND METHODS

*Mice*—All mouse procedures were performed in accordance with McGill University guidelines, which are set by the Canadian Council on Animal Care. E11.5 embryos were removed from timed pregnant FGFR3<sup>+/-</sup> mice that had been mated with FGFR3<sup>+/-</sup> male mice. The FGFR3 colony was maintained on a C3H background as described (18) and genotyped by PCR analysis of tail DNA using the following primers: 5'-GGGCTCCTTATTGGACTCGC-3', 5'-AGGTATAGTTGCCACGATC-GGAGGG-3', and 5'-TGCTAAAGCGCATGCTCCAGACTGC-3'. Embryos were genotyped with the same PCR protocol but using yolk sac and liver as a source of DNA. After decapitation the fore and hind limb buds were removed from embryonic mice under sterile conditions and digested with 1 mg/ml dispase (Sigma) to release mesenchymal, prechondrogenic cells for micro-mass culture.

Preparation of Micro-mass Cultures and in Situ Analyses—Cells released from E11.5 limb buds were plated in 20-µl spots containing  $1 \times 10^7$  cells/ml on 60-mm dishes and left for 1 h at 37 °C before adding 4 ml of Dulbecco's modified Eagle's medium, F-12 (1:1; Invitrogen) containing 10% FBS (Wisent, St. Bruno, Quebec, Canada) and incubating overnight. Cultures were then rinsed once with serum-free medium before adding medium supplemented with 2% FBS or 2% FBS with  $10^{-9}$ M FGF18 (Peprotech, Ottawa, Ontario) and incubating for 2, 4, or 8 days with medium changes every second day. At the indicated times dishes were rinsed 3 times with PBS (pH 7.4) before fixing for 1 h in 4% paraformaldehyde in PBS. Fixed cultures were rinsed 3 times for 5 min with PBS and stored in PBS at 4 °C according to the protocol of Weston *et al.* (19).

In situ staining for proteoglycan and alkaline phosphatase was performed as described previously (20). Type II collagen was localized immunohistochemically with the avidin-biotin-peroxide method as described (21) and by immunofluorescence confocal laser scanning microscopy (see next paragraph) using a monoclonal antibody from the Developmental Studies Hybridoma Bank (Ohio State University). Localization of type X collagen mRNA by *in situ* hybridization was performed as described by Miao *et al.* (22) using a digoxigenin-labeled probe, a kind gift from Benoit St. Jacques, Shriner's Hospital for Children, Montréal, Québec. Stained dishes were overlaid with 1 ml of 75% glycerol and imaged using a  $2.5 \times$  magnification Zeiss Plan-Neofluar objective on a Zeiss Axioskop 40 microscope fitted with an AxioCam MR digital camera. Adobe Photoshop image analysis software was used to quantify the integrated optical density of representative dishes from at least three independent experiments for each treatment group.

Immunofluorescent Staining of Limb Bud Cultures-At the indicated times cultures were rinsed with PBS and fixed with 4% paraformaldehyde in PBS (pH 7.3) for 1 h. After washing with PBS to remove residual paraformaldehyde, cultures were incubated overnight in a humidified chamber at 37 °C with PBS containing 400 milliunits/ml chondroitinase and 800 milliunits/ml and keratanase (Sigma), rinsed with PBS, permeabilized by treating with 0.1% Triton X-100 in PBS for 30 min, and then placed on ice and treated with 5 mg/ml NaBH<sub>4</sub> ( $2 \times 30$ min) to reduce background fluorescence. Cultures were blocked for 2 h with 20% normal goat serum before applying anti-type II collagen antibody (Developmental Studies Hybridoma Bank) or anti-a1 integrin antiserum (Santa Cruz, San Diego, CA) diluted 1:200 with PBS containing 10% normal goat serum. Incubations were carried out overnight at 4 °C before washing in several changes of PBS and incubating overnight at 4 °C in goat anti-mouse secondary antibody conjugated to Alexa-488 (Molecular Probes, Eugene, OR) at a dilution of 1:500. Cultures were then washed thoroughly and counter-stained for 2 h at room temperature with a mixture of 2  $\mu$ g/ml TRITC-phalloidin and 2  $\mu$ g/ml Hoechst 33258 to identify actin filaments and DNA, respectively. Permeabilization and actin staining were not performed on cultures in which cell-surface  $\alpha 1$  integrin was localized. The images are representative of at least three sets of specimens from independent cultures.

Confocal Laser Scanning Microscopy—Fluorescence imaging was performed using a LSM 510 META confocal laser scanning microscope

(Zeiss, Germany). The system consisted of a laser scanning module that was mounted on an Axioplan 2 microscope (Zeiss), an argon laser (458, 488, and 514 nm), two helium-neon lasers (543 and 633 nm), and a pulsed titanium sapphire laser for multiphoton excitation (VerdiV10/ Mira 900 from Coherent). Imaging of stained limb bud cultures was performed using Achroplan 20×/0.5 and Achroplan 63×/0.9 water immersion objectives. The Meta function was used to select filter and dichroic mirror (beam splitter) configurations that minimized any overlap from different fluorochromes. Images were recorded at an excitation wavelength of 488 nm and a BP 510/520 infrared band pass filter for Alexa 488, at an excitation wavelength of 543 nm and a BP 565-615 infrared pass filter for TRITC, and a 2-photon excitation wavelength of 780 nm and a BP 390-465 infrared band pass filter for Hoechst 33258. The pinhole size was adjusted to obtain the optimal spatial resolution of the confocal laser scanning microscope system using the diameter of the inner diffraction ring of the light spot calculated by the software.

Stack images were recorded with a z-step of 0.94  $\mu$ m and x/y resolution of 0.4  $\mu$ m with Zeiss LSM 510 META software (Version 3.0). High magnification images were recorded with a z-step of 0.45  $\mu$ m and x/y resolution of 0.08  $\mu$ m. For each set of samples the laser intensity and detector sensitivity were set for the most intensely stained specimen, and all other samples were imaged at the same intensity. The images are representative of at least three sets of specimens from independent cultures.

*Mitogenic Assay*—Primary limb-bud cells prepared as described were plated at a density of  $5 \times 10^4$  cells/well on 24-well cluster plates (Corning, NY) and grown for 24 h in medium containing 10% FBS. Cells were washed once with serum-free medium and cultured in medium containing 2% FBS, 2% FBS with  $10^{-9}$  M FGF2, or 2% FBS with  $10^{-9}$  M FGF18. Four replicate wells for each treatment were trypsinized at 2, 4, and 8 days post-inoculation and counted using a hemacytometer, and statistical comparisons were made using the Student's *t* test. The results are representative of two independent experiments.

Reverse Transcription-PCR Analysis of Gene Expression Profiles-RNA was harvested at 2 and 8 days using Trizol reagent (Invitrogen) according to the manufacturer's instructions. 1 ml of Trizol reagent was added to each 60-mm plate, and the suspension was aspirated 10-15 times through an 18-gauge needle. The RNA pellet was re-suspended in 50  $\mu$ l of diethyl pyrocarbonate-treated water, and 1  $\mu$ l was run on a 1% agarose gel to evaluate the quality and quantity of extracted RNA. Reverse transcription and PCR were performed as described, and the conditions were optimized for control levels of expression in wild type mice by generating linear curves from products removed at 26-40 cycles (18). The plateau effect was determined for each set of primers using different annealing temperatures, and test reactions were run with the primers and conditions shown in Table I. PCR products were run on 1% agarose gels with a GAPDH control, and the results for each gene of interest were normalized to the GAPDH product. Gels were imaged using a Bio-Rad Gel Doc system, and the amplification products were quantified using Adobe Photoshop image analysis software. Results are representative of three independent experiments for each time point and treatment condition.

Transfection of Limb Bud Cells—Freshly isolated limb bud cells were transfected in suspension with the previously described plasmids (20) according to the protocol of Weston *et al.* (19). In brief, the plasmids were pCDNA3 vector or pCDNA3-carrying cDNAs encoding wild type FGFR3iiic or FGFR3iiic with the achondroplasia (FGFR3<sup>G380R</sup>) or thanatophoric dysplasia mutation (FGFR3<sup>K650E</sup>). The plasmids, which were kind gifts from David M. Ornitz, were mixed 2:1 with FuGENE 6 (Roche Applied Science) and transfected according to the manufacturer's directions into limb bud cells isolated from E11.5 embryos of FGFR3<sup>+/-</sup> matings. 20-µl spots were plated in 60-mm dishes and cultured for 8 days as described before harvesting RNA for reverse transcription-PCR analysis from cells identified as FGFR3<sup>-/-</sup>. The results are representative of at least two independent experiments.

#### RESULTS

Expression of Molecular Markers of Chondrocyte Differentiation in Mesenchymal Cell Cultures—Condensations of mesenchymal cells released from the limb buds of wild type E11.5 mice plated at high density first appeared at day 2 and increased in size and density up to day 8. Clearly circumscribed nodules stained intensely for proteoglycan and for type II collagen on days 4 and 8 and for alkaline phosphatase and type X collagen on day 8 of culture (Supplemental Fig. S1). Images of nodules stained with TRITC-phalloidin (orange) for actin, with FGF18 and FGFR3 in Cartilage Repair



FIG. 1. Defective cartilage nodule formation in limb bud cells from FGFR3<sup>-/-</sup> mice. Mesenchymal cells harvested from the limb buds of E11.5 progeny of FGFR3<sup>+/-</sup> mating were seeded in micro-mass cultures and maintained for 8 days in medium containing 2% serum (basal). Cells from FGFR3<sup>+/+</sup> and FGFR3<sup>-/-</sup> mice, which were identified by genotyping of soft tissues, were immuno-stained with type II collagen antiserum (A–D) or probed with a type X collagen antisense probe (*E–F*). Scale bars represent 2 mm (A–B) and 100  $\mu$ m (*C–F*).

Hoechst nuclear stain (*blue*), and with a monoclonal antibody for type II collagen (*green*) were captured in three planes using confocal laser scanning microscopy. The small, shallow condensations seen on day 2 differentiated into nodules of rounded chondrocytes surrounded by flattened un-differentiated cells by day 4 and stained intensely for type II collagen by day 8 (Supplemental Fig. S2). The pattern of expression of these molecular markers recapitulated the *in vivo* differentiation program of cells of the chondrogenic lineage and validated the model system for examination of the roles of FGF2, FGF18, and FGFR3 in cartilage production *in vitro*.

Defective Cartilage Nodule Formation in  $FGFR3^{-/-}$  Limb Bud Cell Cultures—To determine how the absence of signaling through FGFR3 influenced this differentiation program, we established cultures from the limb buds of the E11.5 progeny of mating FGFR3<sup>+/-</sup> mice (Fig. 1). FGFR3<sup>+/+</sup> and FGFR3<sup>-/-</sup> cultures were identified by genotyping the yolk sac and liver DNA of the embryos (data not shown). Cultures were maintained to day 8 under basal conditions (2% serum) and immuno-stained for type II collagen (Fig. 1, *A*–*D*) or hybridized with a probe for type X collagen mRNA (Fig. 1, *E*–*F*). In both cases the signals obtained for wild type cells were far more



FIG. 2. Quantification of FGF-stimulated proliferation and differentiation. Mesenchymal cells harvested from the limb buds of E11.5 offspring of FGFR3<sup>+/-</sup> mating were plated at a density of  $5 \times 10^4$ cells/ml and maintained for 2, 4, or 8 days in the presence of 2% serum, 2% serum supplemented with  $10^{-9}$  M FGF2 or 2% serum with  $10^{-9}$  M FGF18 before counting cell numbers (A). Differentiation was evaluated on cells plated at a high density and maintained for 8 days in the presence of 2% serum (*Basal*) or 2% serum with  $10^{-9}$  M FGF18. The surface area and intensity of Alcian blue-stained nodules was quantified, and the results are expressed as the integrated optical density (*IOD*). Solid boxes, 2% serum; hatched boxes,  $10^{-9}$  M FGF18. The surface area,  $10^{-9}$  M FGF18. Results represent the mean  $\pm$  S.D. of 16 wells, significantly different from FGFR3<sup>+/+</sup>; \*, p < 0.01.

intense, and the nodules were much deeper and well formed than those for  $FGFR3^{-/-}$  cells. This data suggested that signaling through FGFR3 promoted chondrogenic cell differentiation and cartilage matrix production.

Quantification of Cell Proliferation and Nodule Formation in Response to FGF Ligands—The reduction in type II and type X collagen positive reactivity seen in Fig. 1 could have resulted from a reduction in the number of cells that survived under basal conditions to differentiate into matrix-producing chondrocytes or from defective differentiation. Mesenchymal cells from the limb buds of FGFR3<sup>+/+</sup> and FGFR3<sup>-/-</sup> embryonic mice were plated at low density and grown in the presence of 2% serum (basal), 2% serum with  $10^{-9}$  M FGF2, or 2% serum with 10<sup>-9</sup> M FGF18 (Fig. 2A). FGFR3<sup>+/+</sup> cells continued to proliferate under basal conditions, whereas FGFR3<sup>-/-</sup> cells did not. The addition of FGF2 stimulated the proliferation of both FGFR3<sup>+/+</sup> and FGFR3<sup>-/-</sup> cells, whereas FGF18 inhibited the mitogenic response to 2% serum of FGFR3<sup>+/+</sup> cells and had no effect in the FGFR3<sup>-/-</sup> cultures. A similar lack of response to FGF18 was seen in FGFR3<sup>-/-</sup> cells plated at high density and stained at day 8 with Alcian blue to identify proteoglycan (Fig. 2B). This was in contrast to the increased staining intensity, quantified as integrated optical density, seen in the FGFR3<sup>+/+</sup> cultures. This set of experiments revealed a differential response of FGFR3<sup>+/+</sup> and FGFR3<sup>-/-</sup> limb bud mesenchymal cells to FGF18 and indicated that FGF18 was a more selective ligand than FGF2 for FGFR3.



FIG. 3. Reduced type II collagen and  $\alpha$ 1 integrin expression in FGFR<sup>-/-</sup> cultures. Cultures of FGFR3<sup>+/+</sup> and FGFR3<sup>-/-</sup> cells were grown at high density for 8 days in the absence (A-B and E-F) or presence (C-D and G-H) of 10<sup>-9</sup> M FGF18, fixed with 4% paraformal-dehyde, and treated with proteases and Triton X to permeabilize. Cultures were stained with Hoechst 33258 (*blue*), TRITC-phalloidin (*orange*), and type II collagen antibody (*green*) (A-D) or with Hoechst 33258 (*blue*) and  $\alpha$ 1 integrin antiserum (*green*) (E-H). The high magnification inset panels show punctate  $\alpha$ 1 integrin staining. The scale bar represents 50 (A-H) and 5  $\mu$ m (inset, E-H).

Reduction in Type II Collagen and  $\alpha$ 1 Integrin in Response to FGF18 in FGFR3<sup>-/-</sup> Cultures—In previous work we showed that expression of FGFR3 carrying the activating G380R achondroplasia mutation (FGFR3<sup>Ach</sup>) in CFK2 chondrogenic cells stimulated expression of  $\alpha 1$  integrin, which effectively favored binding of the cells to type II collagen rather than fibronectin (20). FGFR3<sup>+/+</sup> and FGFR3<sup>-/-</sup> limb bud mesenchymal cells were grown to day 8 in basal medium or medium supplemented with 10<sup>-9</sup> M FGF18 and immuno-stained for type II collagen or  $\alpha 1$  integrin (Fig. 3). Both basal and FGF18stimulated levels of type II collagen (Fig. 3, A-D) and  $\alpha 1$  integrin (Fig. 3, E-H) were significantly reduced in FGFR3<sup>-/-</sup> cultures. These experiments confirmed the hypothesis that FGF18 signals through FGFR3 to promote cartilage matrix production and corroborated previous work demonstrating a relationship between FGF signaling and integrin expression in chondrogenic cells.

Gene Expression in FGF18-treated Cells and Those Expressing Constitutively Active FGFR3—The differentiation of mesenchymal cells to cartilage-producing chondrocytes is accompanied by concomitant changes in the expression of FGF receptors, integrin receptors, and components of the extracel-



FIG. 4. Gene expression profiles in FGF18 stimulated FGFR3<sup>+/+</sup> and FGFR3<sup>-/-</sup> cultures. Total RNA was harvested from FGFR3<sup>+/+</sup> and FGFR3<sup>-/-</sup> cells cultured for 2 or 8 days in the absence or presence of  $10^{-9}$  M FGF18 and subjected to reverse transcription (*RT*)-PCR using the primers and conditions shown in Table I. Semiquantitative data were obtained by normalizing the signal for the genes of interest (*A*) to that for GAPDH in the same sample and expressing the result as a ratio (*B*). Data are significantly different from FGFR3<sup>+/+</sup> \*, p < 0.01.

lular matrix. To determine how altered FGFR3 signaling influenced these expression patterns, RNA was harvested from FGFR3<sup>+/+</sup> and FGFR3<sup>-/-</sup> limb bud cells after 2 or 8 days in basal medium (2% serum) or basal medium supplemented with  $10^{-9}$  M FGF18 (Fig. 4). Treatment of FGFR3<sup>+/+</sup> cells with FGF18 increased expression of FGFR1 and FGFR2 to similar levels as seen in the FGFR3<sup>-/-</sup> cells by day 8.  $\alpha$ 1 integrin, type II collagen, and type X collagen expression were also increased in FGFR3<sup>+/+</sup> cells in response to FGF18, although there was no response to this ligand in FGFR3<sup>-/-</sup> cells (Fig. 4A). Densitometric analysis of expression levels at day 8, normalized to GAPDH (Fig. 4B), showed that in the FGFR3<sup>-/-</sup> cells  $\alpha$ 1 integrin and type X collagen were un-detectable and that type II

TABLE I Primer sequence and reference information

r, lorward; h, reverse	e.							
Gene		Sequence $5'$ to	3′		Size	Anneal temperature	Cycles	Reference
						$^{\circ}C$		
FGFR1	F	tgg agt tca tgt gca ag	ıg tg		856	58	35	18
	R	ata gag agg acc atc ct	g tg					
FGFR2	$\mathbf{F}$	aaa tac caa atc tcc ca	la CC		373	58	35	18
	R	Gcc gct tct cca tct to	:t					
FGFR3	$\mathbf{F}$	aga agg ctg ctt tgg ad	a		331	58	35	18
	R	tga gct gtt cct ctg go	a					
$\alpha 1$ Integrin	$\mathbf{F}$	cct gta ctg tac cca at	t gga tg	ıg	241	55	35	20
	R	gtg ctc tta tga aag to	g gtt to	C				
$\alpha 5$ Integrin	$\mathbf{F}$	ctg cag ctg cat ttc co	ja gtc tg	ıg	275	55	30	20
	R	gaa gcc gag ctt gta ga	ıg gac gt	a				
$\beta$ 1 Integrin	$\mathbf{F}$	tgt gtt cag tgc aga go	C.		258	55	30	20
	R	ttg gga tga tgt cgg ga	ιC					
Fibronectin	F	tat gct ctc aag gac ad	a		597	55	30	49
	R	ctg tct tct tcc tcc ca	ιa					
Type II collagen	$\mathbf{F}$	cca gac tgc ctc aac co	c gag a		489	58	35	50
	R	aga gac acc agg ctc go	c agg t					
Type X collagen	$\mathbf{F}$	tgg gta ggc ctg tat aa	la gaa cg	ıg	209	58	40	50
	R	cat ggg agc cac tag ga	a tcc tg	ra ga				
GAPDH	F	ggt gaa ggt cgg tgt ca	ia cg		496	55	30	50
	R	caa agt tgt cat gga to	ja cc					

collagen was poorly expressed using the PCR conditions outlined in Table I. Expression patterns for the same panel of marker genes was also determined after transfecting FGFR3<sup>+/+</sup> and FGFR3<sup>-/-</sup> cells with plasmids encoding wild type or mutant FGFR3 (Fig. 5). Expression of the constitutively active FGFR3<sup>G380R</sup> in FGFR3<sup>+/+</sup> cells elicited a similar pattern of increased gene expression as seen in wild type cells stimulated with FGF18 (Fig. 4). Similar results were seen in FGRR3<sup>-/-</sup> cells expressing FGFR3<sup>K650E</sup> receptors.

#### DISCUSSION

Although FGF18 shares up to 80% amino acid identity and similar receptor binding properties with its two closest relatives, FGF8 and FGF17, its biological function appears to be unique. This is due in part to its restricted pattern of expression, to the time- and tissue-specific expression of FGF receptor isoforms, and also to the presence of specific heparan sulfate proteoglycans (9, 23, 24). In mouse models of human chondrodysplasia, FGF2 and FGF18 have been identified as potential ligands for FGFR1 in hypertrophic chondrocytes, for FGFR3 in resting and proliferating chondrocytes, and for FGFR2 in the perichondrium and periosteum of developing long bones (25). In this study we explored the hypothesis that FGF18 signals selectively through FGFR3 to promote the differentiation of pre-chondrogenic mesenchymal cells to cartilage-producing chondrocytes.

Mitogenic Response of Pre-chondrogenic Cells to FGF2 and FGF18 through FGFR3—Although FGFR3 was identified in the cartilage primordia of developing mouse long bones more than a decade ago, its role in cartilage development remains poorly understood (26). We show here that the absence of FGFR3 in mesenchymal cells released from the limb buds of FGFR3<sup>-/-</sup> embryonic mice resulted in failure of the dispersed cells to proliferate under low serum conditions. This may have been due to the concomitant reduction in expression of FGFR1 (Fig. 4), which would otherwise mediate a mitogenic response to residual FGF2 present in the medium containing 2% serum. This hypothesis was supported by a robust mitogenic response to the addition of  $10^{-9}$  M FGF2, which has equivalent affinity for FGFR1, FGFR2, and FGFR3 (27) to FGFR3<sup>-/-</sup> cultures. The addition of FGF18 at the same concentration had no effect on FGFR3<sup>-/-</sup> cultures and elicited mild suppression of proliferation stimulated by 2% serum in the FGFR3<sup>+/+</sup> cultures.





FIG. 5. Gene expression profiles in FGFR3<sup>-/-</sup> cells transfected with FGFR3 constructs. Total RNA was harvested from FGFR3<sup>+/+</sup> cells expressing pcDNA vector as control (V), cDNA encoding wild type FGFR3iiic ( $R3^{iiic}$ ), or FGFR3iiic with the G380R achondroplasia mutation ( $FGFR3^{G380R}$ ). In an independent experiment, FGFR3<sup>-/-</sup> cells were transfected with vector, cDNA encoding wild type receptor or the receptor<sub>K650</sub><sup>K0</sup>. All cultures were maintained for 8 days in the presence of 2% serum, except one set of dishes containing FGFR3<sup>-/-</sup> cells with the wild type FGFRiiic receptor, which were stimulated with  $10^{-9}$  M FGF18. Semiquantitative data were obtained by normalizing the signal for the genes of interest (A) to that for GAPDH in the same sample and expressing the result as a ratio (B). RT, reverse transcription.

This observation supported *in vivo* data attributing a weak inhibitory role to FGFR3 signaling in proliferating growth plate chondroblasts and chondrocytes. This was shown in mice homozygous for targeted disruption of FGFR3 that exhibited elongation of the zones of resting, proliferating, and hypertrophic cells in their growth plates (28, 29). The phenotype was subsequently attributed to the combined effects of increased proliferation and to defective vascular invasion and endochondral ossification, which resulted from decreased vascular endothelial growth factor signaling.

The congenital absence of FGF18 resulted in a similar elongation of the developing growth plates (30, 31). The similarity between the phenotypes and the localization of FGF18 to perichondrial cells, adjacent to FGFR3-positive cells in the growth plate, suggested that FGF18 activation of FGFR3 limited proliferation of chondroblasts and chondrocytes. An inhibitory role for FGFR3 signaling in growth plate chondrocytes was also supported by transgenic expression in chondrogenic cells of a constitutively active receptor carrying the achondroplasia mutation (FGR3<sup>Ach</sup>) in vivo (32) and in vitro (20). Expression of the chimeric receptor FGFR3/1 with the FGFR3<sup>Ach</sup> extracellular domain and FGFR1 intracellular domain also suppressed proliferation of growth plate chondrocytes (33). However, the same chimeric receptor promoted proliferation and chondrogenic differentiation of cells in the presumptive joint spaces, suggesting a critical role for FGF signaling in chondrogenic differentiation. Signaling through FGFR3 has, thus, been assigned both mitogenic and anti-mitogenic activity in cells of the chondrogenic lineage, where the effect appears to be largely contextdependent.

Regulation of Chondrogenesis and Matrix Production by FGF18—The limb bud micro-mass culture system has been used extensively as an *ex vivo* tool to dissect the molecular pathways that regulate the orderly progression of cells through stages of chondrogenic differentiation, matrix production, and hypertrophy. In vivo and in vitro, immature, proliferating chondrocytes secrete an extensive matrix rich in type II collagen and proteoglycan, whereas fully differentiated, hypertrophic cells express high levels of alkaline phosphatase and type X collagen. In the current studies FGFR3<sup>+/+</sup> cells faithfully recapitulated these events in vitro to form multilayered cartilage nodules, whereas FGFR3<sup>-/-</sup> cells did not (Figs. 1 and 3). It is unlikely that this was due exclusively to the lack of a mitogenic response of these cells to the 2% serum supplemented medium. This could have been a contributing factor as it is well known that a critical density of pre-chondrogenic cells is required for condensation and differentiation to occur. However, it is evident from Figs. 1 and 2 that numerous small, un-differentiated colonies were present in the FGFR3<sup>-/-</sup> cultures, suggesting that sufficient numbers of cells were present but that they did not produce significant quantities of cartilage matrix. Taken together with the evidence in Figs. 4 and 5 that type II collagen mRNA was reduced compared with wild type cultures, the data suggest that FGFR3 signaling acted primarily to promote differentiation and cartilage production. These observations support those from in vivo studies of the growth plates of human thanatophoric dwarfs (34) and in vitro studies of human articular chondrocytes (8) as well as those of transgenic mice expressing constitutively active FGFR3 in the articular joints (33) and those examining mouse limb explants (35). In total, these studies support a role for FGFR3 signaling in promoting chondrocyte differentiation. They do, however, contradict work in which excessive FGFR3 signaling was claimed to inhibit chondrocyte differentiation and matrix production (32, 36, 37). The apparent discrepancies most probably arise from a combination of the different experimental models employed, the use of different surrogate markers for proliferation and cartilage synthesis, and also from the lack of discrimination between the stages of differentiation under investigation. The focus of the current work has been on the early differentiation of cells with chondrogenic potential, such as those present in condensing mesenchyme during development and those in the subperiosteal cambium of adult bone (38). The *in vivo* studies, on the other hand, have focused primarily on the differentiation of growth plate chondrocytes from a proliferative to a hypertrophic phenotype.

Regulation of Integrin Receptor Expression by FGF18—The activity of chondrogenic cells is critically dependent not only on signals received from soluble factors like FGF but also on those emanating from the cartilage matrix that surrounds them (39– 42). This has been well illustrated in mice expressing the dominant negative mutation in the col2 $\alpha$ 1 pro-collagen gene that results in lethal chondrodysplasia in humans (43). The primary defect in collagen synthesis and assembly was correlated with decreased expression of FGFR3, Ihh, and type X collagen and with impaired chondrocyte differentiation. A similar pattern of reduced collagen expression in association with impaired cell differentiation was seen during fracture healing in  $\alpha$ 1 integrin-deficient mice, implying that adequate expression of  $\alpha$ 1 was required for chondrocyte differentiation and cartilage matrix production (44).

The heterodimeric, cell surface integrin receptors that receive and transduce signals from an immature, type II collagen-rich cartilage matrix are necessarily different from those that effectively recognize fibronectin or type X collagen. In our model system we demonstrated concomitant increases in  $\alpha 1$ integrin and type II collagen protein (Fig. 3) and mRNA (Figs. 4 and 5) over time and in response to FGF18 stimulation in FGFR3<sup>+/+</sup> cultures. A similar increase in the  $\alpha$ 1 integrin subunit was seen previously in CFK2 cells expressing FGFR3<sup>Ach</sup>, which was accompanied by increased spreading and formation of focal adhesions on a type II collagen substrate (20). In contrast to the situation in wild type cells,  $\alpha 1$  integrin and type II collagen mRNA and protein were drastically reduced in the FGFR3<sup>-/-</sup> cultures. This situation was rectified by transfection of wild type FGFR3iiic and treatment with FGF18 or by transfection of FGFR3<sup>K650E</sup> with the activating thanatophoric dysplasia mutation. Taken together, the data indicate that FGF18 signals through FGFR3 to increase expression of type II collagen and its  $\alpha 1\beta 1$  receptor in chondrogenic cells. The observed impairment in cartilage nodule formation in FGFR3<sup>-/-</sup> cultures could, thus, have been the combined result of decreased proliferation of chondrogenic cells, decreased production of type II collagen, and decreased "outside-in" signaling through the  $\alpha 1\beta 1$  type II collagen receptor.

Implications for Cartilage Tissue Engineering—Focal lesions in articular cartilage generally do not heal adequately in the absence of assisted regeneration or repair and are believed to progress to severe osteoarthritis (1). There is consequently great interest in developing improved tissue engineering methods for articular cartilage repair. One approach is based on the conjecture that articular cartilage (45), the periosteum (38, 46) bone marrow (47), and peripheral blood (47) all represent sources of prechondrogenic mesenchymal cells. However, these cells are very limited in number, and their availability is believed to decrease significantly with advancing age. Under the influence of the appropriate combination of signals from soluble factors and the surrounding matrix, these cells can replicate and undergo chondrogenic differentiation and cartilage production *in situ*.

FGF2 has been identified as a critical mitogen for expansion of chondrocytes for cartilage tissue engineering, although it appears to inhibit their differentiation (48). In the current work we showed that FGF18 had little effect on the proliferation of chondrogenic cells. It did, however, promote their differentiation and cartilage production in association with changes in integrin expression that favored adhesion to collagen type II. FGF18 has been localized to the periosteum during embryonic bone development and is, therefore, a candidate endogenous factor for cartilage repair that resides in the cambial layer of adult bones. Controlled delivery of FGF2 in the area of a focal defect could, therefore, support the rapid expansion of prechondrogenic cells, and subsequent delivery of FGF18 would promote their differentiation and production of cartilage matrix. In view of evidence that bone spurs and osteophytes develop at sites of cartilage repair, the relative value of using FGF18 rather than transforming growth factor  $\beta$  or bone morphogenetic proteins to promote chondrogenic differentiation merits further investigation. As an alternative strategy, FGF2 and FGF18 could be used in conjunction with a type II collagenbased scaffold for ex vivo expansion and differentiation before transplantation using arthroscopic procedures. This type of tissue engineering approach shows great promise for the treatment of focal lesions in articular cartilage by delaying or even circumventing the need for total joint replacement.

In this study we have used a genetic-based model to demonstrate that FGF18 signals through FGFR3 to promote cartilage formation. Given the presence of FGF18 ligand and FGFR3positive cells with chondrogenic potential in the periosteum, the work identifies the FGF18/FGFR3 axis as a potential target for manipulation in tissue engineering approaches to the regeneration and repair of articular cartilage in degenerative disease such as osteoarthritis.

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