Cell Proliferation

Cell Prolif. 2010, **43**, 67–76 doi: 10.1111/j.1365-2184.2009.00655.x

Involvement of fibroblast growth factor 18 in dedifferentiation of cultured human chondrocytes

H. Yamaoka*,†, S. Nishizawa*,‡, Y. Asawa*, Y. Fujihara*, T. Ogasawara*, K. Yamaoka‡, S. Nagata§, T. Takato¶ and K. Hoshi*

**Departments of Cartilage & Bone Regeneration (Fujisoft), Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, †Department of Plastic, Oral and Maxillofacial Surgery, Teikyo University School of Medicine, Tokyo, Japan, ‡Department of Pharmacy, Teikyo University Hospital, Tokyo, Japan, §Nagata Microtia and Reconstructive Plastic Surgery Clinic, Saitama, Japan, and ¶Departments of Sensory & Motor System Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*

Received 13 *November* 2008*; revision accepted* 11 *May* 2009

Abstract

Objective: Chondrocytes inevitably decrease production of cartilaginous matrices during long-term cultures with repeated passaging; this is termed dedifferentiation. To learn more concerning prevention of dedifferentiation, we have focused here on the fibroblast growth factor (FGF) family that influences chondrocyte proliferation or differentiation.

Materials and methods: We have compared gene expression between differentiated cells in passage 3 (P3) and dedifferentiated ones in P8 of human cultured chondrocytes. We also performed ligand administration of the responsive factor or its gene silencing, using small interfering RNA (siRNA).

Results: FGFs 1, 5, 10, 13 and 18 were higher at P8 compared to P3, while FGFs 9 and 14 were lower. Especially, FGF18 showed a 10-fold increase by P8. Ligand administration of FGF18 in the P3 cells, or its gene silencing using siRNA in the P8 cells, revealed dose-dependent increase and decrease respectively in type II collagen/type I collagen ratio. Exogenous FGF18 also upregulated expression of transforming growth factor beta (TGF-β), the anabolic factor of chondrocytes, in P3 chondrocytes, but P8 cells maintained a low level of TGF-β expression, suggesting a decrease in responsiveness of TGF-β to FGF18 stimulation in the dedifferentiated chondrocytes.

Conclusion: FGF18 seems to play a role in maintenance of chondrocyte properties, although its expression was rather high in dedifferentiated chondrocytes. Upregulation of FGF18 in dedifferentiated chondrocytes implied that it may be a marker of dedifferentiation.

Correspondence: K. Hoshi, Department of Cartilage & Bone Regeneration (Fujisoft), Graduate School of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8655, Japan. Tel.: +81-3-3815-5411; Fax: +81-3-5800-9891; E-mail: pochi-tky@umin.net

Introduction

Regenerative medicine using tissue engineering techniques aims to replace or support functions of defective or injured body parts using cells cultured in the laboratory. While researchers of various fields have focused on their interest in regenerative medicine/tissue engineering, cartilage regenerative medicine has also progressed well. It is already available for clinical use with full-thickness articular cartilage defects and repair of craniofacial or nose deformity, using the procedure with autologous chondrocyte implantation (ACI). Both articular and the auricular cartilage have been cell sources of chondrocytes used for these treatments (1–3). ACI involves three procedures, harvesting of a small amount of cartilage for biopsy, *in vitro* proliferation of isolated chondrocytes and subsequent transplantation of cultured chondrocytes into lesions. This kind of treatment reduces the number of invasive events for patients and has good functional biocompatibility, over long time periods *in vivo*.

During the ACI procedure, chondrocytes isolated from a patient's cartilage need to be expanded in long-term culture with repeated passaging to obtain sufficient cell numbers to develop a tissue-engineered prosthesis of a suitable size. However, chondrocytes isolated from native cartilage rapidly lose their chondrocytic properties during proliferation in culture (4). Such chondrocytes, morphologically, are rounded in shape and are embedded in type II collagen (COL2)/proteoglcan-based cartilaginous matrix. Once these chondrocytes are transferred to form a monolayer in culture, the typical round morphology changes to

fibroblast-like spindle shape, and cartilaginous protein synthesis decreases, a phenomenon which is termed dedifferentiation (4). Identifying a method to overcome dedifferentiation of cultured chondrocytes remains a significant hurdle in cartilage regenerative medicine, but little information on molecular mechanisms involved in chondrocyte dedifferentiation or its specific markers has yet been accumulated.

One of the candidate molecules which participates in chondrocyte dedifferentiation may belong to the fibroblast growth factor (FGF) family. The FGF family consists of 23 growth factors, many of which regulate chondrocyte proliferation and differentiation (5). Among the 23 FGFs identified so far, FGFs1, 2 and 18 seem to have significant involvement in cartilage development and regeneration (6). FGF1 (acidic) and FGF2 (basic) were originally isolated from brains and pituitary glands of freshly slaughtered cows and were found to promote the proliferation of cultured fibroblasts (7). Both factors stimulate proliferation of a wide variety of cells, including chondrocytes or other mesenchymal cell lineages and play important roles in angiogenesis and wound healing (8–10). Especially, FGF2 is highly expressed in chondrocytes and is also stored in the extracellular matrix of articular cartilage, suggesting that it has regulatory roles in cartilage matrix homeostasis and functions (11). FGF18 can stimulate repair of damaged cartilage; an intra-articular injection of FGF18 induces a dose-dependent increase in cartilage hypertrophy and overgrowth (12). Addition of FGF18 to cultured human articular chondrocytes also enhanced proliferation and matrix production, suggesting that it also appears to regulate cell proliferation and differentiation in chondrogenesis as well (13,14). Regarding FGF receptors, there are four members of the FGF receptor tyrosine kinases (FGFR1, FGFR2, FGFR3 and FGFrl1). Among them, FGFRs 1, 2 and 3 clearly contribute to development of the skeleton (15). Significance of the FGF signalling pathway became apparent after finding that several human skeletal dysplasias, including achondroplasia, occur as a result of specific mutations in FGFRs 1, 2 and 3 (5,16). Thus, it is possible that the FGF family and its signalling are associated with chondrocyte dedifferentiation.

Thus, ligands of the FGF family are potent mitogens and have been used practically as additive components in culture media for various kinds of human cells, such as endothelial cells (FGF1), embryonic stem cell and chondrocytes (FGF2), or keratinocytes (FGF7) (17–22). If the relationship of the FGF family with chondrocyte dedifferentiation is clearly understood, this information would directly provide improvement in culture systems for chondrocytes, as well as innovation in cartilage tissue engineering. To elucidate roles of the FGF family in chondrocyte dedifferentiation and to obtain useful information for the procedure of ACI, we have evaluated gene expression of the FGF family, in cultured human auricular chondrocytes, which are one of the major cell types in used in ACI. We have exhaustively examined changes in gene expression using microarray analysis relative to increase in passage number, and we have investigated the functions of responsive genes.

For comparison of the passages, we used our cells at P3 and P8. Ability to produce cartilage matrices is barely maintained in the primary chondrocyte culture, and rapidly decreases in repeated cell passaging. If possible, chondrocytes from primary cultures should be used for cartilage regenerative medicine. However, the volume of cartilage which can be biopsied from the patient for initial chondrocyte isolation is approximately 300–500 mg in practice, and hundreds of thousands of chondrocytes on average are harvested from the biopsied cartilage (23). According to our previous findings (24), 10^8 cells are needed for regenerating cartilage to 1 ml, the volume which we regard as optimal for the clinical use in cartilage regenerative medicine. This implies that cell replication with approximately a 1000-fold increase, corresponding to P3, should be achieved in the expansion culture. Thus, we have established a method to judge whether chondrocytes in P3 have exerted suitable proliferation, or else might have unexpectedly contained cells that profusely multiplied as do P8 cells (10^8-fold increase). Our previous report (24) showed that quality of cartilage regeneration in tissue-engineered constructs using human chondrocytes of P3 (10^3-fold increase) was acceptable, disclosing sufficient accumulation of cartilage matrices, although it became worse thereafter.

Materials and methods

Cell isolation and culture

Remnant auricular cartilage was obtained, with informed consent, from 10 patients who underwent microtia surgery at the University of Tokyo Hospital. Isolated cartilage was chopped into 1 mm^3 pieces and digested with 0.15% collagenase (Wako Pure Chemical Industries, Osaka, Japan) then incubated in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co. St. Louis, MO, USA) containing penicillin and streptomycin at 37 °C, for 24 h; 20 ml of the 0.15% collagenase solution was added to 0.3 g of the chopped cartilage. The digested suspension was filtered using a sterile 100 μm nylon cell strainer (BD Falcon, Bedford, MA, USA) and centrifuged at 430 ***g*** for 5 min. The resulting pellet of cells was washed twice in DMEM containing the antibiotics and then resuspended in the medium. The number of cells was calculated using a haemocytometer and their viability was determined using

the trypan blue vital dye exclusion technique. Isolated chondrocytes were seeded into a 100 mm plastic tissue culture dish containing bullet kit chondrocyte growth medium (CGM; Cambrex Bioscience Walkersville, Inc., Walkersville, MD, USA) at a density of 2500 cells/cm^2 and was incubated at 37 °C with 5% carbon dioxide (CO_2) incubator. The CGM contained 5% foetal bovine serum (FBS) with an undisclosed concentration of FGF2, insulin-like growth factor 1 (IGF-I) and insulin. We changed all volumes of medium in culture dishes three times a week. After approximately 1 week, when cell density had reached 4–6x10^4 cells/cm^2, cells were detached using 0.25% trypsin/1.0 mM EDTA (Sigma Chemical Co.) and re-plated at 2500 cells/cm^2. Passages were performed until P8. Cells were then cryopreserved using CELLBANKER (DIA-IATRON, Tokyo, Japan) according to the manufacturer's instructions at −80 °C, until used.

Total RNA extraction and conventional RT-PCR

The total RNA was extracted from the chondrocyte using the Isogen® solution (Nippon gene, Tokyo, Japan), according to the manufacturer's specifications, and stored frozen at −80 °C until needed. One microgram of the extracted total RNA in a 20-µl volume of each sample was reverse transcripted to obtain cDNA using the Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). Conventional PCR amplification was performed using a TaKaRa Ex Taq (Takara Shuzo, Shiga, Japan) according to the manufacturer's instructions. The primers used for the PCR are shown in Table 1. The PCR was carried out using a PROGRAM TEMP CONTROL SYSYTEM Models PC320 (ASTEC, Fukuoka, Japan) with pre-heating at 94 °C for 2 min followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s. The PCR products were run on 1.5% agarose gels with a GAPDH control. The gels were imaged using a BioDoc-It System (UVP, Upland, CA, USA).

Real time RT-PCR

For real time RT-PCR, ABI Prism Sequence Detection System 7700 was used. Aliquots of 1-µl cDNA template were amplified using a QuantiTect SYBER Green PCR Kit (Qiagen, Osaka, Japan) under the following conditions: initial denaturation for 10 min at 94 °C followed by 40 cycles consisting of 15 s at 94 °C and 1 min at 60 °C. Data on gene expression using real time RT-PCR consisted of a relative ratio, which was based on comparison of the copy units of the target gene mRNA in each total RNA. Copy units were calculated by threshold cycles at which increase in reporter fluorescence above baseline signal could be detected, with reference to a standard curve of three aliquots from one sample, and were divided (normalized) by the copy unit of GAPDH as internal control (25). To provide a standard curve, all positive control cDNAs, which determine gene expression, were synthesized according to the following method by Alaina *et al.* (26). Positive-control cDNAs were obtained using conventional RT-PCR. cDNA controls were cloned into pCR 2.1-TOPO Vector (Invitrogen); plasmid DNAs were purified using standard routine laboratory procedures. To construct standard curves, serial 10-fold dilutions of each plasmid containing 1 000 000 to 1000 copies were used as the starting template in real time PCR reactions. To be able to calculate relative ratio in a procedure with several groups, it is essential to first define the normalized copy unit of a certain group as the baseline (1.0), and divide those of other groups by value of the baseline.

Microarray gene expression profiling

Five micrograms of total RNA from chondrocyte culture in P3 and P8 were used to direct first-strand cDNA synthesis, using T7-oligo(dT)24 primer and PowerScript reverse transcriptase (Clontech, Palo Alto, CA, USA). After second-strand synthesis and clean-up with Qiaquick spin

Table 1. PCR primers sequence

Transcript name	Primer sequence (5′-3′)		Product size (bp)
	Sense	Antisense	
COL1A1	GTGCTAAAGGTGCCAATGGT	CTCCTCGCTTTCCTTCCTCT	228
COL2A1	GAGTCAAGGGTGATCGTGGT	CACCTTGGTCTCCAGAAGGA	300
FGF18	CCTGCACTTGCCTGTGTTTA	GGATGTGTTTCCCACTGGTC	180
FGFR1	TACCACCGACAAAGAGATGG	CTGGCTGTGGAAGTCACTCT	287
FGFR2	TGGAGCGATCGCCTCACCG	CTTCCAGGCGCTGGCAGAACTGT	352
FGFR3	AGTGGAGCCTGGTCATGGAA	GGATGCTGCCAAACTTGTTCTC	83
FGFrl1	CGGCTCCTACCTCAATAAGC	AACGAGGGAAGGTCCTTGT	359
TGF-β1	AAGTGGACATCAACGGGTTC	GTCCTTGCGGAAGTCAATGT	218
GAPDH	GAAGGTGAAGGTCGGAGTCA	GAAGATGGTGATGGGATTTC	226

GAPDH was used as the housekeeping gene.

column (Qiagen), the double-stranded cDNA was used in MEGAscript T7 RNA polymerase *in vitro* transcription reaction (Ambion, Austin, TX, USA), containing biotin-labelled ribonucleotides, CTP and UTP. Resulting labelled cRNAs were hybridized overnight in Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA), washed and scanned according to the manufacturer's instructions, using a GeneChip Scanner 3000 and GeneChip Operating Software (GCOS; Affymetrix).

FGF18 administration for functional analyses

Chondrocytes were cultured in a 100-mm plastic tissue culture dish at a density of 2500 cells/cm^2 with DMEM containing 10% FBS (Thermo Fisher Scientific, Kanagawa, Japan) supplemented with or without recombinant human FGF18 (Wako Pure Chemical Industries) at each final concentration; 1×10^{-8}, 1×10^{-9} and 1×10^{-10} M. After 7 days, total RNA was isolated from the cell culture plates, using Isogen® solution.

RNA interference

The small interfering RNAs (siRNAs) of FGF18 (Silencer® Pre-designed siRNA, siRNA ID#: #1 for 13979, #2 for 137291) and negative control siRNA, which consisted of a 19-bp scrambled sequence with 3-dT overhangs (Silencer ® Negative Control #1 siRNA for 4611), were purchased from Ambion, Inc. Sequences of siRNAs are described in Table 2.

Transfection of siRNA was performed into chondrocytes in P8 in 12-well plates. The cells were transferred on to a 12-well cell culture plate at 21 000 cells/cm^2 and cultured in DMEM supplemented with 10% FBS on the day before transfection. Transfections of siRNA were carried out using FuGENE6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. FuGENE6 reagent (3 μl) was incubated in 97 μl of DMEM at room temperature. After 5 min, FGF18 siRNA (#1 and #2) and negative control siRNA were added at concentration of 100 nM and incubated for 15 min at room temperature. FuGENE6-siRNA mixture (100 μl) was then added to the cells and incubated at 37 °C. After 72 h, total RNA was isolated from the 12-well cell culture plates using Isogen® solution.

Transfection rate of siRNA is crucial for its successful function as an inducer of the RNA interference process. We used Cy3-labelled siRNA to visualize transfection kinetics of siRNA. Cy3-labelled negative control for siRNA (*Silencer*® Cy3-labelled Negative Control #1 siRNA for AM4621) was purchased from Ambion, Inc. Cy3-labelled siRNA was transfected into chondrocytes using FuGENE6, as described above. For control experiments, Cy3-labelled siRNA was transfected without FuGENE6. At 24 hr post-treatment, Cy3 signals of siRNAs in living cells were monitored, using Leica confocal microscopy system TSC-SL (Leica, Wetzlar, Germany). Transfection rate was determined in chondrocytes 24 h after transfection, by counting the number of cells with Cy3-siRNA signal and total number of cells per microscope field, and calculating percentage of Cy3-positive cells relative to total cells. This process was repeated six times.

Statistical analysis

All data are expressed as mean ± SD. After homogeneity of the data (homoscedasticity) was verified using Bartlett's test, means of the groups were compared by analysis of variance. Significance of differences was determined using *post hoc* testing using Bonferroni's method. *P*-value of less than 0.05 was considered significant.

Results

Expression profile of FGF family genes in chondrocytes cultured to passage 3 and to passage 8

Human auricular chondrocytes rapidly lose their morphological phenotype when the cells are cultured in a monolayer. Significant increase in type I collagen αI chain (COL1A1) expression and downregulation of type II collagen αI chain (COL2A1) were found with in progression of passaging (Fig. 1). We examined expression patterns of 23 genes, known as the FGF family, in the human auricular chondrocytes of P3 and P8. When we compared expression intensity of the FGF family genes in chondrocytes originating from two donors, using microarray anal-

Table 2. siRNA primers sequence

Transcript name	Primer sequence (5′-3′)	
	Sense	Antisense
FGF18 #1	GGUUCUGGAGAACAACUACtt	GUAGUUGUUCUCCAGAACCtt
FGF18 #2	CGCAAAGGGACUGUAGUCAtt	UGACUACAGUCCCUUUGCGtg

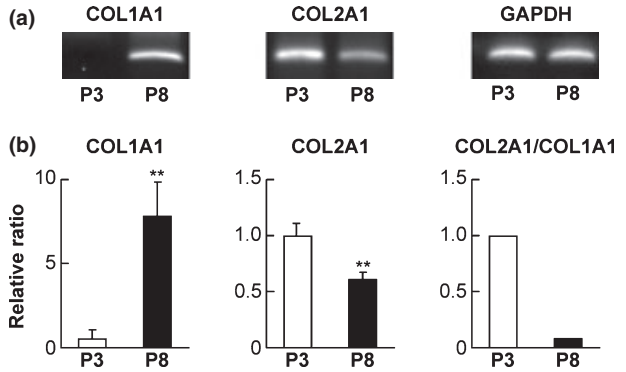

Figure 1. Gene expression of *COL1A1*, *COL2A1* and the *COL2A1/COL1A1* expression ratio in chondrocytes cultured to passages 3 (P3) and P8. (a) Agarose gels after migration of PCR amplification products from P3 chondrocytes (left) and P8 ones (right). GAPDH was used as internal positive control. (b) Gene expression of chondrocytes cultured to P3 and P8 were examined using real time RT-PCR. *COL1A1* expression in the P8 chondrocytes showed approximately 7-fold increase compared to that in P3 cells, while *COL2A1* expression in P8 cells diminished to nearly two-thirds of P3. Consequently, the *COL2A1/COL1A1* expression ratio in P8 cells was ten times less than that in P3 cells. $^{**}P < 0.01$ *versus* P3.

ysis, upregulation of FGFs 1, 5, 10, 13, and 18 was repeatedly observed in P8, compared to those of P3, although expression of FGFs 9 and 14 decreased (Fig. 2). Specially, gene expression of FGF18 showed more than a 10-fold increase in P8 chondrocytes, compared to those of P3 (Fig. 2). We confirmed that this FGF18 upregulation as a result of increase in passages was reproducible in six of eight donors, using real time RT-PCR (Fig. 3). Normalized copy units of FGF18 in P3 chondrocytes ranged between 0.0003 and 0.005, while many of those in P8 exceeded 0.005 and in some extreme cases were more than 0.01 (Fig. 3).

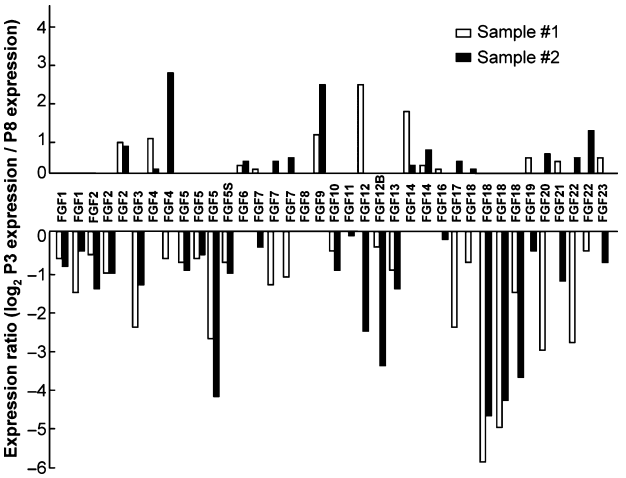

Figure 2. Expression profile of FGF family genes in chondrocytes cultured in passage 3 (P3) and P8, by microarray analysis. Gene expression in chondrocytes originating from two donors (samples #1 and #2) duplicated and showed downregulation of FGFs 1, 5, 10, 13, and 18, and upregulation of FGFs 9 and 14. Results from microarray analysis were expressed as log-transformed fold changes in P3 and P8 cells.

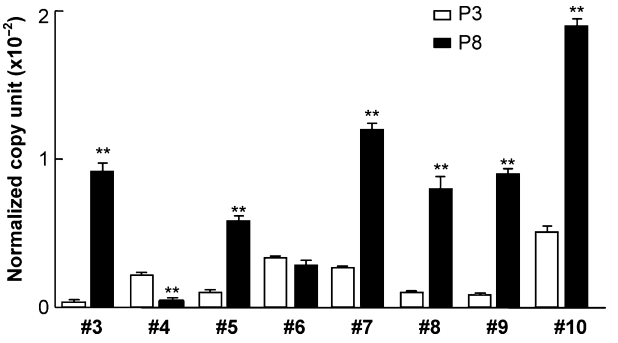

Figure 3. Gene expression of FGF18 in cultured chondrocytes originating from eight donors (Samples #3–10). Normalized copy units of FGF18 in the chondrocyte culture in passage 8 (P8) were compared to those in P3 cells, using real time RT-PCR, showing that 6 out of 8 samples significantly increased in FGF18 expression. $^{**}P < 0.01$ *versus* P3.

Administration of FGF18 ligands to chondrocytes and inhibition of FGF18 expression using siRNA

To elucidate effects of FGF18 on our cultured human chondrocytes, we administered FGF18 ligands to P3 cells. According to increase in doses of FGF18 ligands, expression level of *COL1A1* gene, significantly decreased (Fig. 4, *COL1A1*). Although *COL2A1* expression was also downregulated by stimulation of FGF18 ligands, dose dependency was not obvious and the *COL2A1/COL1A1* expression ratio increased, depending on doses of the FGF18 ligands (Fig. 4).

Next, we inhibited expression level of *FGF18* gene using siRNA in P8 chondrocytes. Transfection rate was calculated by quantification of percentage of Cy3-labelled siRNA positive cells relative to total cells, revealing the result of 77.4 ± 3.7% (Fig. 5a).

Two kinds of siRNA construct (#1 and #2 in Fig. 5b) effectively suppressed expression of FGF18. Although expression of *COL1A1* tended to increase in parallel with the FGF18 expression levels, *COL2A1* expression decreased in contrast, resulting in decreased *COL2A1/COL1A1* expression ratio, which was not different from the results of FGF18 administration (Fig. 4).

Molecular mechanisms of effects of FGF18 on chondrocytes

Although we measured expression level of FGF receptors, including FGFR1, FGFR2, FGFR3 and FGFrl1, no significant difference was observed between those at P3 or P8

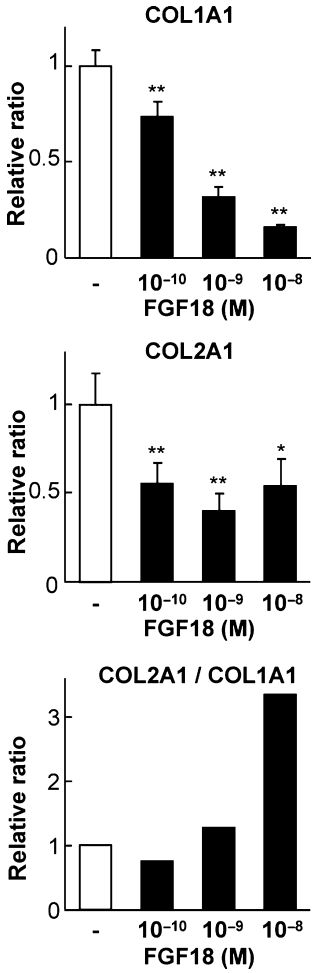

Figure 4. Gene expressions of *COL1A1*, *COL2A1* and *COL2A1/COL1A1* expression ratio in chondrocytes cultured to passage 3 with administration of FGF18 ligand to the culture medium. Gene expression was evaluated using real time RT-PCR. *COL1A1* expression significantly diminished due to doses of FGF18, while decrease in *COL2A1* expression was significant, but partial. Consequently, *COL2A1/COL1A1* expression ratio increased with doses of FGF18. $*P < 0.05$, $**P < 0.01$ *versus* control.

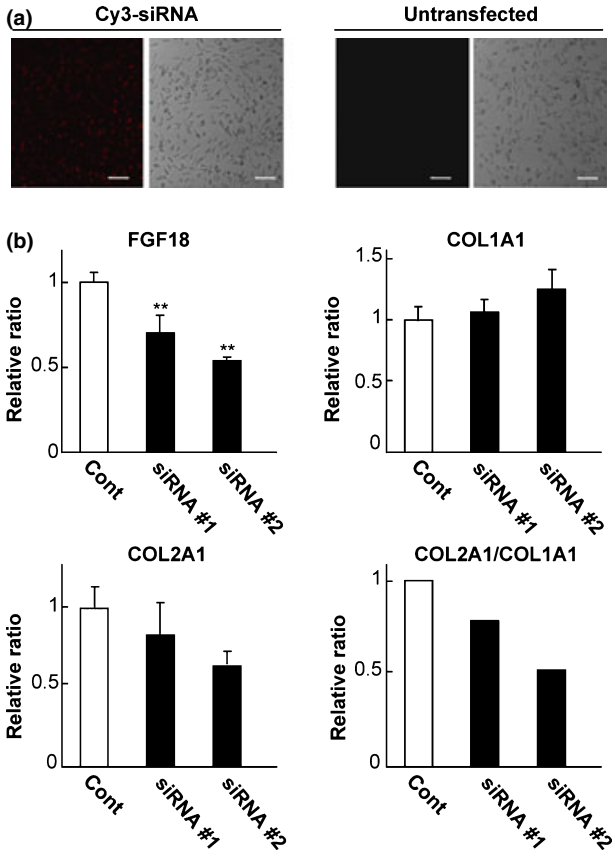

Figure 5. Gene expression of *COL1A1*, *COL2A1* and *COL2A1/COL1A1* expression ratio in chondrocytes cultured to passage 8, with expression level of *FGF18* gene suppressed using siRNA. (a) Cy3-labelled negative control of siRNA (Cy3-siRNA) was transfected well into chondrocytes at passage 8. Untransfected cells (Untransfected) at the same density and magnification were the control in this experiment. Left: fluorescence image, Right: phase contrast image. Bar = 100 μm. (b) Two siRNA constructs (#1 and #2) effectively suppressed expression of *FGF18*, compared to negative control of siRNA (cont). Gene expressions were evaluated using real time RT-PCR. *COL1A1* expression increased parallel to *FGF18* expression levels, although *COL2A1* expression diminished in contrast. Consequently, *COL2A1/COL1A1* expression ratio decreased according to *FGF18* expression level. $**P < 0.01$ *versus* control.

chondrocytes (Fig. 6). On the other hand, in cultured chondrocytes, administration of exogenous FGF18 increased gene expression level of transforming growth factor beta 1 (TGF-β1), which is one of the candidate factors to mutually cooperate with FGF18, and controls chondrocyte differentiation in a physiological situation (27) (Fig. 7). However, when we compared expression level of TGF-β1 in cultured chondrocytes at early and late passages (P3 and P8), chondrocytes of the late passages (P8) that were in the state of dedifferentiation showed a significant decrease in TGF-β1, in spite of upregulation of FGF18 expression (Figs 3 and 7), showing some discrepancy in the responses of TGF-β to increase of FGF18 in progression of passages and exogenous administration (Fig. 7) (28).

Discussion

As the exact mechanism of chondrocyte dedifferentiation has up to now been poorly understood, definition of gene expression markers would be a breakthrough leading to elucidation of mechanisms. However, in spite of several recent studies that have compared gene expression patterns between differentiated chondrocytes and dedifferentiated ones (29–32), these findings could not

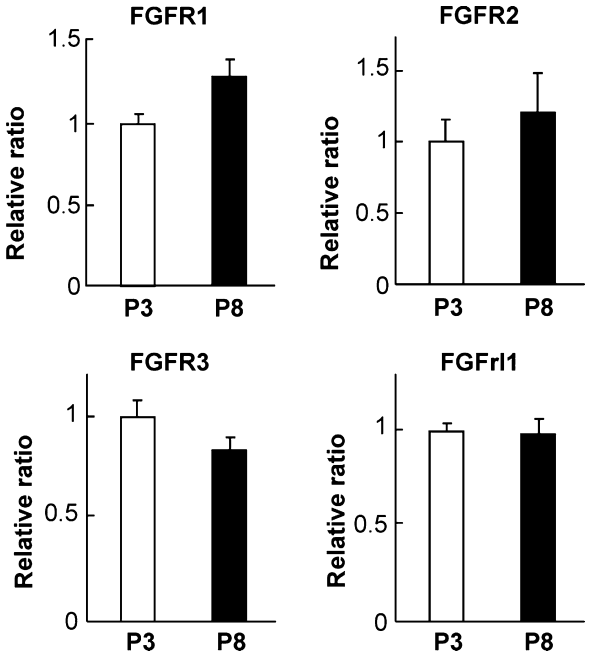

Figure 6. Gene expressionsof *FGFR1*, *FGFR2*, *FGFR3* and *FGFrl1* in chondrocytes cultured to passage 3 (P3) and P8. No significant differences in gene expressions were found using real time RT-PCR.

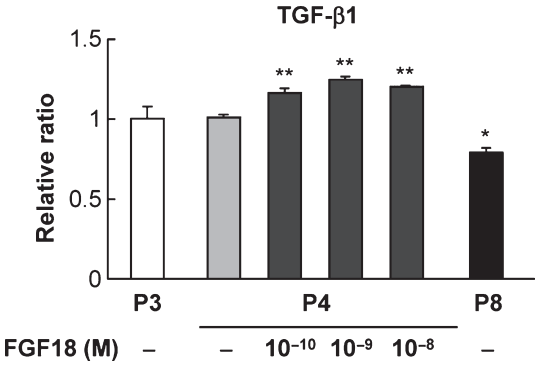

Figure 7. Gene expression of *TGF-β1* in chondrocytes cultured to passage (P3) and P8, and in which FGF18 ligands were administered to the culture medium in P4. Gene expressions were shown to be evaluated using real time RT-PCR. *TGF-β1* expression in chondrocytes at P8 significantly diminished when compared to those of P3. In contrast, it significantly increased as a result of doses of FGF18 in P4. $^{**}P < 0.01$ *versus* P4 without FGF18 administration, $^{*}P < 0.05$ *versus* P3.

converge into any common conclusions as different combinations were applied in the comparisons and hardly had any similar results except for significant downregulation of a typical cartilage matrix gene, for example, *COL2A1* or aggrecan in dedifferentiated chondrocytes (29–32).

Compared to the number of downregulated genes in chondrocyte dedifferentiation, that of upregulated ones were fewer, but have been reported to include growth factor/cytokine-related genes, IGF binding protein 3, IL-1β, TNF-α receptor 1, and others, such as cyclinD1, prothymosin alpha, TWIST, cadherin 11, fibronectin, link protein and TIMP-2/3 (33). Considering the clinical practice of autologous chondrocyte implantation, a method that can remove an exceptional case in which dedifferentiated chondrocytes excessively increase could be established. As we must determine increase in dedifferentiated chondrocytes that would usually consist of a minority in an entire population, increase in gene expression of a marker for minority cases rather than decrease in the majority may be significantly detectable by using RT-PCR. Thus, we focused on upregulated genes in dedifferentiated chondrocytes.

As a result, we found that FGF18 was significantly upregulated in chondrocyte dedifferentiation. FGF18 plays pivotal roles in bone and cartilage cell differentiation (14,34); it is expressed in joints of developing endochondral bones (14,34–36). In the present study, we administered ligands of FGF18 or siRNA to cultured chondrocytes to confirm the effect of FGF18. FGF18 has been regarded as a potent factor that can maintain differentiation properties in chondrocytes in monolayer culture, that has various effects on inhibition of progression of chondrocyte dedifferentiation (Figs 3 and 4). However, in a long-term culture with repeated passaging, by real time RT-PCR, we found that chondrocytes increasingly expressed FGF18 in six out of eight samples (Fig. 3).

To explain the reason why chondrocytes cultured with repeated passaging lose their chondrocyte properties, in spite of upregulation of the FGF18 expression, we considered two possibilities: (i) downregulation of FGF receptors, which cancels the effects of FGF18 overexpression and (ii) participation of a third factor that functions downstream of FGF18 for control of chondrocyte phenotype. The first possibility was speculated from well-known findings that ligands of the FGF family exert their function under control, with expression of their receptors, in skeletal cell growth and differentiation (37). To evaluate this, we examined expression of FGF receptors in the chondrocytes cultured to P3 and P8. As a result, neither FGF receptors including FGFR1, FGFR2, FGFR3 nor FGFrl1 (38,39) showed significant expressional changes (Fig. 5), which negatively supports suppression of FGF18 signalling by downregulation of FGF receptors.

Regarding participation of a third factor, the typical inhibitor of BMP signalling, noggin, had been regarded as a candidate for downstream molecules of FGF18 (40). Therefore, we postulated that noggin would also function as a factor for maintenance of chondrocyte differentiation and we attempted to detect changes in noggin expression

in dedifferentiation. However, no significant upregulation was noted in P8 cells, compared to those in P3 (data not shown).

Otherwise, growth factors, such as TGF-β1, IGF-I, and others have been reported to cooperate mutually with FGFs and control chondrocyte differentiation (27). Especially, under physiological conditions, FGF18 seemed to upregulate TGF-β expression that promotes matrix synthesis of chondrocytes (28). TGF-β has been found to regulate chondrocyte proliferation and hypertrophic differentiation in murine metatarsal organ cultures (41), while experiments using the FGF18 inhibitor, poly(4-styrenesulfonic acid), showed that effects of TGF-β1 could potentially be mediated by FGF signalling (27). These previous findings had suggested that TGF-β may also be associated with dedifferentiation of cultured chondrocytes, as downstream molecules of FGF18. Here, expression of *TGF-β1* gene actually increased in cultured chondrocytes because of the increase in administered doses of FGF18 ligands (Fig. 7). As TGF-β1 could also enhance chondrogenic differentiation in cultured chondrocytes, TGF-β1 may work as a downstream molecule on maintenance of the chondrocyte phenotype. However, expression of TGF-β1 decreased in chondrocytes in P8, which showed a relatively higher expression of FGF18. Responsiveness of TGF-β to FGF18 stimulation deteriorated in the long-term cultures, while expression of TGF-β1 was downregulated even as FGF18 expression was increased. This may be through mechanisms related to the long-term culture, and possibly an underlying cause of chondrocyte dedifferentiation.

Regarding mechanisms of FGF18 upregulation in dedifferentiated chondrocytes, cytoskeletal changes in chondrocytes may be related to repeated passaging. During long-term culture, chondrocytes begin to form large actin cables (F-action) within the cells and lose their specific ability to produce cartilaginous matrices (42). Formation of large actin fibres is related to cell adhesion and is promoted by connection with fibronectin and its receptors, such as integrins (42). Chondrocytes cultured on poly-(2-hydroxyethylmethacrylate)-coated plastic plates (which material disturbs cell adhesion, extension and probably formation of F-actin) can maintain a cartilage-specific phenotype, whereas phenotype of chondrocytes cultured on conventional plastic substrates leads to dedifferentiation (43). Otherwise, formation of a multiprotein complex containing S100A13 and Synaptotagmin (Syt) 1 has been reported to evoke release of FGF in mouse fibroblast-like cell strain NIH 3T3 (44). This complex formation was dependent on F-actin organization (44). Therefore, as seen in the present study, alterations of cytoskeletons, including F-actin (which is associated with progression of chondrocyte dedifferentiation), may affect secretion of FGF18, a member of the FGF family, during long-term culture with repeated passages.

In summary, although FGF18 seemed to play a role in maintenance of chondrocyte properties, its expression was much higher in dedifferentiated chondrocytes (P8) than in P3s. TGF-β, a downstream molecule of FGF18, was downregulated in cultured chondrocytes at P8, suggesting presence of complicated networks of growth factors in the molecular mechanisms of chondrocyte dedifferentiation. Further study is needed to determine the molecular mechanisms of FGF18 upregulation in dedifferentiated chondrocytes, but FGF18 expression may be useful in quality control of culture chondrocytes when used as a marker of dedifferentiation.

A quantitative method for comparisons of gene expression level of *FGF18* among separate experiments is needed for quality control of chondrocytes for practical application of cartilage regenerative medicine. For that, we have attempted to use normalized copy units (Fig. 3), with reference to previous reports (25). As a result, FGF18 upregulation was reproducibly observed with increase in passages. We noticed that normalized copy units of FGF18 in P3 chondrocytes ranged between 0.0003 and 0.005, while many of those in P8 exceeded 0.005 and some extreme ones were over 0.01 (Fig. 3). If we attempt to judge whether the chondrocyte population at P3 exerted suitable multiplication or unexpectedly contained cells that profusely multiplied like P8 cells, the threshold may be approximately 0.01 in normalized copy unit of *FGF18* gene expression by real time RT-PCR measurement. In the future, we should be able to establish a more precise method to quantitatively evaluate FGF18 expression in a chondrocyte culture which can be used for quality control in cartilage tissue engineering.

Acknowledgements

We thank Ms Miki Akisawa, Mr Motoki Yagi, Mr Tomoaki Sakamoto and Mr Makoto Watanabe for technical support. This study was supported by Grants-in-Aid for Establishment of evaluation method for tissue engineering, Japan Science and Technology Agency (JST) or Research and Development programs for Three-dimensional Complex Organ Structures from New Energy and Industrial Technology Development Organization (NEDO).

References

1 Marlovits S, Striessnig G, Kutscha-Lissberg F, Resinger C, Aldrian SM, Vecsei V *et al.* (2005) Early postoperative adherence of matrix-induced autologous chondrocyte implantation for the treatment of

full-thickness cartilage defects of the femoral condyle. *Knee Surg. Sports Traumatol. Arthrosc.* **13**, 451–457.
2 Yanaga H, Koga M, Imai K, Yanaga K (2004) Clinical application of biotechnically cultured autologous chondrocytes as novel graft material for nasal augmentation. *Aesthetic Plast. Surg.* **28**, 212–221.
3 Yanaga H, Yanaga K, Imai K, Koga M, Soejima C, Ohmori K (2006) Clinical application of cultured autologous human auricular chondrocytes with autologous serum for craniofacial or nasal augmentation and repair. *Plast. Reconstr. Surg.* **117**, 2019–2030; discussion 2031–2032.
4 von der Mark K, Gauss V, von der Mark H, Muller P (1977) Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. *Nature* **267**, 531–532.
5 Ornitz DM (2005) FGF signaling in the developing endochondral skeleton. *Cytokine Growth Factor Rev.* **16**, 205–213.
6 Marie PJ (2003) Fibroblast growth factor signaling controlling osteoblast differentiation. *Gene* **316**, 23–32.
7 Gospodarowicz D (1974) Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. *Nature* **249**, 123–127.
8 Bjornsson TD, Dryjski M, Tluczek J, Mennie R, Ronan J, Mellin TN *et al.* (1991) Acidic fibroblast growth factor promotes vascular repair. *Proc. Natl. Acad. Sci. USA* **88**, 8651–8655.
9 Friesel R, Maciag T (1999) Fibroblast growth factor prototype release and fibroblast growth factor receptor signaling. *Thromb. Haemost.* **82**, 748–754.
10 Dailey L, Ambrosetti D, Mansukhani A, Basilico C (2005) Mechanisms underlying differential responses to FGF signaling. *Cytokine Growth Factor Rev.* **16**, 233–247.
11 Vincent T, Hermansson M, Bolton M, Wait R, Saklatvala J (2002) Basic FGF mediates an immediate response of articular cartilage to mechanical injury. *Proc. Natl. Acad. Sci. USA* **99**, 8259–8264.
12 Moore EE, Bendele AM, Thompson DL, Littau A, Waggie KS, Reardon B *et al.* (2005) Fibroblast growth factor-18 stimulates chondrogenesis and cartilage repair in a rat model of injury-induced osteoarthritis. *Osteoarthritis Cartilage* **13**, 623–631.
13 Davidson D, Blanc A, Filion D, Wang H, Plut P, Pfeffer G *et al.* (2005) Fibroblast growth factor (FGF) 18 signals through FGF receptor 3 to promote chondrogenesis. *J. Biol. Chem.* **280**, 20509–20515.
14 Ohbayashi N, Shibayama M, Kurotaki Y, Imanishi M, Fujimori T, Itoh N *et al.* (2002) FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. *Genes Dev.* **16**, 870–879.
15 Ornitz DM, Marie PJ (2002) FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes Dev.* **16**, 1446–1465.
16 Chen L, Deng CX (2005) Roles of FGF signaling in skeletal development and human genetic diseases. *Front. Biosci.* **10**, 1961–1976.
17 Relou IA, Damen CA, van der Schaft DW, Groenewegen G, Griffioen AW (1998) Effect of culture conditions on endothelial cell growth and responsiveness. *Tissue Cell* **30**, 525–530.
18 Takahashi T, Ogasawara T, Kishimoto J, Liu G, Asato H, Nakatsuka T *et al.* (2005) Synergistic effects of FGF-2 with insulin or IGF-I on the proliferation of human auricular chondrocytes. *Cell Transplant.* **14**, 683–693.
19 Takahashi T, Ogasawara T, Asawa Y, Mori Y, Uchinuma E, Takato T *et al.* (2007) Three-dimensional microenvironments retain chondrocyte phenotypes during proliferation culture. *Tissue Eng.* **13**, 1583–1592.
20 Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M (2005) Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev.* **16**, 159–178.
21 Wang L, Li L, Menendez P, Cerdan C, Bhatia M (2005) Human embryonic stem cells maintained in the absence of mouse embryonic fibroblasts or conditioned media are capable of hematopoietic development. *Blood* **105**, 4598–4603.
22 Lee JG, Kay EP (2006) FGF-2-mediated signal transduction during endothelial mesenchymal transformation in corneal endothelial cells. *Exp. Eye Res.* **83**, 1309–1316.
23 Peterson L, Minas T, Brittberg M, Nilsson A, Sjogren-Jansson E, Lindahl A (2000) Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. *Clin. Orthop. Relat. Res.*, **374**, 212–234.
24 Yamaoka H, Tanaka Y, Nishizawa S, Asawa Y, Takato T, Hoshi K. The application of atelocollagen gel in combination with porous scaffolds for cartilage tissue engineering and its suitable conditions. *J. Biomed. Mater. Res. A*, in press.
25 Swiderski CE, Klei TR, Horohov DW (1999) Quantitative measurement of equine cytokine mRNA expression by polymerase chain reaction using target-specific standard curves. *J. Immunol. Methods* **222**, 155–169.
26 Arocho A, Chen B, Ladanyi M, Pan Q (2006) Validation of the 2-DeltaDeltaCt calculation as an alternate method of data analysis for quantitative PCR of BCR-ABL P210 transcripts. *Diagn. Mol. Pathol.* **15**, 56–61.
27 Mukherjee A, Dong SS, Clemens T, Alvarez J, Serra R (2005) Co-ordination of TGF-beta and FGF signaling pathways in bone organ cultures. *Mech. Dev.* **122**, 557–571.
28 Haque T, Nakada S, Hamdy RC (2007) A review of FGF18: Its expression, signaling pathways and possible functions during embryogenesis and post-natal development. *Histol. Histopathol.* **22**, 97–105.
29 Stokes DG, Liu G, Dharmavaram R, Hawkins D, Piera-Velazquez S, Jimenez SA (2001) Regulation of type-II collagen gene expression during human chondrocyte de-differentiation and recovery of chondrocyte-specific phenotype in culture involves Sry-type high-mobility-group box (SOX) transcription factors. *Biochem. J.* **360**, 461–470.
30 Schnabel M, Marlovits S, Eckhoff G, Fichtel I, Gotzen L, Vecsei V *et al.* (2002) Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. *Osteoarthritis Cartilage* **10**, 62–70.
31 Darling EM, Athanasiou KA (2005) Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *J. Orthop. Res.* **23**, 425–432.
32 Lin Z, Fitzgerald JB, Xu J, Willers C, Wood D, Grodzinsky AJ *et al.* (2008) Gene expression profiles of human chondrocytes during passaged monolayer cultivation. *J. Orthop. Res.* **26**, 1230–1237.
33 Stokes DG, Liu G, Coimbra IB, Piera-Velazquez S, Crowl RM, Jimenez SA (2002) Assessment of the gene expression profile of differentiated and dedifferentiated human fetal chondrocytes by microarray analysis. *Arthritis Rheum.* **46**, 404–419.
34 Liu Z, Xu J, Colvin JS, Ornitz DM (2002) Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. *Genes Dev.* **16**, 859–869.
35 Ohbayashi N, Hoshikawa M, Kimura S, Yamasaki M, Fukui S, Itoh N (1998) Structure and expression of the mRNA encoding a novel fibroblast growth factor, FGF-18. *J. Biol. Chem.* **273**, 18161–18164.
36 Hajihosseini MK, Heath JK (2002) Expression patterns of fibroblast growth factors-18 and -20 in mouse embryos is suggestive of novel roles in calvarial and limb development. *Mech. Dev.* **113**, 79–83.
37 Molteni A, Modrowski D, Hott M, Marie PJ (1999) Differential expression of fibroblast growth factor receptor-1, -2, and -3 and syndecan-1, -2, and -4 in neonatal rat mandibular condyle and calvaria during osteogenic differentiation in vitro. *Bone* **24**, 337–347.

38 Wiedemann M, Trueb B (2000) Characterization of a novel protein (FGFRL1) from human cartilage related to FGF receptors. *Genomics* **69**, 275–279.
39 Itoh N, Ornitz DM (2004) Evolution of the Fgf and Fgfr gene families. *Trends Genet.* **20**, 563–569.
40 Reinhold MI, Abe M, Kapadia RM, Liao Z, Naski MC (2004) FGF18 represses noggin expression and is induced by calcineurin. *J. Biol. Chem.* **279**, 38209–38219.
41 Alvarez J, Horton J, Sohn P, Serra R (2001) The perichondrium plays an important role in mediating the effects of TGF-beta1 on endochondral bone formation. *Dev. Dyn.* **221**, 311–321.
42 Mallein-Gerin F, Garrone R, van der Rest M (1991) Proteoglycan and collagen synthesis are correlated with actin organization in dedifferentiating chondrocytes. *Eur. J. Cell Biol.* **56**, 364–373.
43 Reginato AM, Iozzo RV, Jimenez SA (1994) Formation of nodular structures resembling mature articular cartilage in long-term primary cultures of human fetal epiphyseal chondrocytes on a hydrogel substrate. *Arthritis Rheum.* **37**, 1338–1349.
44 Prudovsky I, Bagala C, Tarantini F, Mandinova A, Soldi R, Bellum S *et al.* (2002) The intracellular translocation of the components of the fibroblast growth factor 1 release complex precedes their assembly prior to export. *J. Cell Biol.* **158**, 201–208.

