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Sprifermin (rhFGF18) enables proliferation of chondrocytes producing a hyaline cartilage matrix

A. Gigout † *, H. Guehring †, D. Froemel ‡, A. Meurer ‡, C. Ladel †, D. Reker §, A.C. Bay-Jensen §, M.A. Karsdal §, S. Lindemann †

† *Osteoarthritis Research, Merck KGaA, Darmstadt, Germany*
‡ *Orthopaedic University Hospital Friedrichsheim, Frankfurt, Germany*
§ *Rheumatology, Biomarkers and Research, Nordic Bioscience, Herlev, Denmark*

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SUMMARY

Objective: Fibroblast growth factor (FGF) 18 has been shown to increase cartilage volume when injected intra-articularly in animal models of osteoarthritis (OA) and in patients with knee OA (during clinical development of the recombinant human FGF18, sprifermin). However, the exact nature of this effect is still unknown. In this study, we aimed to investigate the effects of sprifermin at the cellular level.
Design: A combination of different chondrocyte culture systems was used and the effects of sprifermin on proliferation, the phenotype and matrix production were evaluated. The involvement of MAPKs in sprifermin signalling was also studied.
Results: In monolayer, we observed that sprifermin promoted a round cell morphology and stimulated both cellular proliferation and Sox9 expression while strongly decreasing type I collagen expression. In 3D culture, sprifermin increased the number of matrix-producing chondrocytes, improved the type II:I collagen ratio and enabled human OA chondrocytes to produce a hyaline extracellular matrix (ECM). Furthermore, we found that sprifermin displayed a 'hit and run' mode of action, with intermittent exposure required for the compound to fully exert its anabolic effect. Finally, sprifermin appeared to signal through activation of ERK.
Conclusions: Our results indicate that intermittent exposure to sprifermin leads to expansion of hyaline cartilage-producing chondrocytes. These *in vitro* findings are consistent with the increased cartilage volume observed in the knees of OA patients after intra-articular injection with sprifermin in clinical studies.

Introduction

Osteoarthritis (OA) is characterised by cartilage degradation mediated by increased catabolic activity of chondrocytes with a concomitant inappropriate repair response. The associated symptoms include pain and limited functionality of the affected joint, resulting in considerably reduced quality of life. Current pharmacological treatment options – which consist primarily of the use of acetaminophen, non-steroidal anti-inflammatory drugs, cyclooxygenase 2 inhibitors or intra-articular corticosteroid injection[1] – are limited, focussing on symptom alleviation but not hindering disease progression. Consequently, there is a strong need for disease-modifying osteoarthritis drugs (DMOADs) that deliver both structural improvement and symptom relief.

DMOADs can either prevent cartilage breakdown during the course of the joint disorder (anti-catabolic approach), promote tissue regeneration (anabolic approach), or both. The anabolic approach consists of reversing the cellular events that occur during OA, thereby promoting repair of injured cartilage. Anabolic compounds under investigation mainly include growth factors involved in cartilage development and homeostasis. Among these, insulin

* Address correspondence and reprint requests to: A. Gigout, Osteoarthritis Research, Merck KGaA, Frankfurter Strasse 250, 64293 Darmstadt, Germany.
E-mail addresses: anne.gigout@merckgroup.com (A. Gigout), hans.guehring@merckgroup.com (H. Guehring), Dara.Froemel@friedrichsheim.de (D. Froemel), Andrea.Meurer@friedrichsheim.de (A. Meurer), christoph.ladel@merckgroup.com (C. Ladel), dre@Nordicbioscience.com (D. Reker), acbj@Nordicbioscience.com (A.C. Bay-Jensen), mk@NordicBio.com (M.A. Karsdal), Sven.Lindemann@merckgroup.com (S. Lindemann).

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growth factor (IGF), transforming growth factor-β (TGFβ), bone morphogenic protein (BMP)-2, BMP7, fibroblast growth factor (FGF) 2 and FGF18 have been evaluated for cartilage engineering or regeneration. They all exhibit anabolic effects and have demonstrated efficacy in preclinical models of OA or cartilage repair[2–4]. However, because of deleterious side effects (such as osteophyte formation, cartilage mineralisation, joint fibrosis or synovial inflammation) or insufficient efficacy, only BMP7 and FGF18 (under the international non-proprietary name, sprifermin) have progressed to clinical trials[2].

FGF18 specifically activates FGFR3 in cartilage and chondrocytes[5,6] and is involved in chondrogenesis[6] and skeletal development[7], and has been shown to stimulate proliferation and extracellular matrix (ECM) production by healthy chondrocytes in monolayer[5]. Furthermore, intra-articular injections of FGF18 have been shown to stimulate cartilage repair in a rat meniscal tear model, inducing a dose-dependent increase in cartilage thickness and significant reduction of degeneration scores[8,9]. Finally, sprifermin injected intra-articularly into the knee of patients with OA has resulted in a dose-dependent reduction of cartilage loss[10].

Published information concerning the biological effects of FGF18 on chondrocytes is very limited[5] and its effects on chondrocyte phenotype or on OA chondrocytes (i.e., chondrocytes from OA patients) are largely unknown. The aim of the present study was to elucidate FGF18's action at the cellular level, to better understand what is observed in *in vivo* preclinical models and in humans[8–10]. Several experiments in monolayer and in 3D culture were conducted using the pharmacological compound sprifermin to: 1) investigate its impact on chondrocyte proliferation and phenotype, 2) evaluate the ability of sprifermin to promote production of a hyaline-like ECM in mature and OA chondrocytes, 3) determine if permanent exposure is necessary for optimal pharmacological activity, and 4) study the signalling pathways involved in FGF18 signalling.

Methods

Sprifermin (recombinant human FGF18) production

Sprifermin, a recombinant human FGF18, was expressed in *Escherichia coli* and purified as previously described[5]. Sprifermin is a truncated, 170 amino acid form of FGF18 (molecular weight = 19.83 kDa), from which the signal sequence, and the 11 C-terminal amino acids, have been removed. Consequently, sprifermin starts with a methionine followed by amino acid 28 (Glu) and ends with amino acid 196 (Lys) of the wild-type human FGF18. The stock solution was in 4.7 mg/mL in 7 mM Na_2HPO_4, 1 mM KH_2PO_4, 2.7 mM KCL, pH 7.3 and was diluted directly in culture medium at wished working concentrations.

Porcine chondrocyte culture

Porcine chondrocytes were isolated from the femoral heads of pigs, approximately 1 year of age, obtained from a local slaughterhouse.

For the monolayer, cells were first cultured 1 week in monolayer in HAM's F12 supplemented with 10% foetal calf serum (FCS) (Promocell GmbH), 1% Penicillin/Streptomycin and 50 μg/mL ascorbate-2-phosphate (Sigma–Aldrich). Cells were then passaged and further cultured in the same medium with increasing sprifermin concentrations from 0.1 to 10,000 ng/mL. After 5 days, cells were stained for actin (see Supplementary Methods) and after 7 days cells were counted with a Vi-CELL™ Analyzer counter (Beckman Coulter Inc.) and glycosaminoglycan (GAG, see Supplementary Methods) concentration was measured in the medium. Gene expression analysis was performed by real-time polymerase chain reaction (PCR).

For the 3D culture, chondrocytes were cultured in suspension in DMEMHG, 10% serum (FCS from Promocell GmbH or foetal bovine serum from Merck Millipore), 50 μg/mL ascorbate-2-phosphate and 0.4 mM Proline. Cells were cultured for 1 week without treatment and then with 10 or 100 ng/mL sprifermin for 4 weeks according to the scheme Fig. 2(A). As a control, 3D constructs were also left untreated. At the end of the culture period, the 3D constructs were either used for biochemical analysis (deoxyribonucleic acid [DNA], GAG and hydroxyproline [HPro] content), gene expression (real-time PCR), or histological analysis. Prior to biochemical analysis the constructs were digested overnight at 60°C with Papain 0.125 mg/mL (Merck KGaA, Cat. No 1.07144.0025) in 0.1 M Na_2HPO_4, 0.01 M EDTA and 5 mM L-Cysteine.

Human chondrocyte 3D culture

For the culture of human cells, human material from patients who underwent total knee replacement was provided by the Universitätsmedizin Mannheim or the Orthopaedic University Hospital Friedrichsheim in Frankfurt, and obtained with full, ethical, written consent (for Frankfurt ethical approval No. 433/11; for Mannheim ethical approval No. 2013-576N-MA). For the 3D culture, the level of cartilage deterioration (chondromalacia) of the tibial plateau, the condyles and the patellofemoral groove was mapped according to the gross appearance of the cartilage, with Grade I describing the least and IV the most damaged tissue[11]. Cells from three different patients and different grades were isolated separately to obtain three different cell suspensions: two with chondrocytes isolated from Grade II cartilage (from patients 1 and 2), assigned 'Grade II-1' and 'Grade II-2', and one with chondrocytes isolated from Grade III–IV chondrocytes (pooled from patients 2 and 3), assigned 'Grade III–IV'.

Cells were first cultured for 4–5 days in monolayer, harvested and further cultured in 3D as previously described for the porcine chondrocytes or used for the mitogen-activated protein kinase (MAPK) array assay.

Analysis of signalling pathways

Human OA chondrocytes were cultured at 1.5×10^6 cell/T25 flask for 24 h to allow adherence to the flask, before the addition of medium containing 0.5% FCS with or without rhFGF18 100 ng/mL for 15 min. Afterwards, the cells were lysed and the analysis of phosphorylated kinases with the MAPK array was performed according to the recommendation of the manufacturer, 160 μg protein was loaded for each sample. Image acquisition was performed with a Chemidoc XRS+ (Biorad).

For the testing of the different MAPK inhibitors, porcine cells were cultured in monolayer as mentioned above with sprifermin 100 ng/mL in the presence of various inhibitors (see Supplementary Table I). After 5 days, cells were stained for actin (see Supplementary Methods) and after 7 days type I collagen expression was evaluated by real-time-PCR. All inhibition assays were performed at 3% O_2, a condition that was found to better preserve the cell phenotype in monolayer.

Real-time PCR

The cells were homogenised in RLT buffer (from the RNeasy Mini Kit, Qiagen, 3D construct were additionally treated with proteinase

K according to the recommendation of the manufacturer, Qiagen). Ribonucleic acid (RNA) isolation was performed with the RNeasy Mini Kit (Qiagen). mRNA concentration and quality were then analysed by an Agilent Bioanalyser with an Agilent RNA 6000 Nano Chip (Agilent Technologies Inc., Cat. No. G2938-80023).

The reverse transcription was realised with the SuperScript III First-Strand Synthesis SuperMix (Invitrogen Corp.) and followed by an RNAse H treatment. qPCR was performed with the SYBR-Green Jumpstart Taq Ready Mix (Sigma–Aldrich) with 200 nM of the reverse and forward primer (Eurofins MWG Operon; see Supplementary Table II) in the thermocycler Mx3000P (Agilent Technologies Inc). The PCR reaction was duplicated for each sample.

For each gene, the cycle threshold (Ct) was determined and the relative abundance was calculated according to the following formula:

$relative\_abundance = 2^{(ctHKG-ctGOI)} = \frac{2^{ctHKG}}{2^{ctGOI}}$ with house-keeping gene (HKG) = EF1α (for human cells), GAPDH or RLP13A (for porcine cells) and GOI = gene of interest.

Biochemical measurement

GAG, DNA and HPro were measured with the dimethylmethylene blue assay, the Picogreen assay or with high performance liquid chromatography, respectively (see Supplementary Methods for details).

Histology

Samples were embedded in paraffin and 5 μM slices cut with a rotary microtome. The standard staining was Safranin O 0.25% v/v with Fast Green 0.1% (v/v) as a counter stain. The immunohistochemical detection of type I and II collagens (rabbit anti-collagen II antibody, Abcam ab34712, 1:500 and mouse anti-collagen I antibody, Abcam, ab90395, 1:200) was realised using a fully automated immunohistochemistry stainer (Bond-III, Leica). To visualise the positive matrix, the Bond Polymer Refine Detection kit (Leica) was used with the "polymer refine red detection" for type I collagen in human and porcine samples and the "polymer refine detection" for type II collagen in porcine samples. To visualise the type II collagen in human sample the EnVision+ System-HRP (DAKO) was used.

Statistics

Statistical analysis consisted of a Kruskal–Wallis test corrected for multiple comparisons with a Dunn's *post hoc* test. For the results presented in Figs. 1–3, the different sprifermin conditions were compared to the control without sprifermin. In Fig. 4, for each

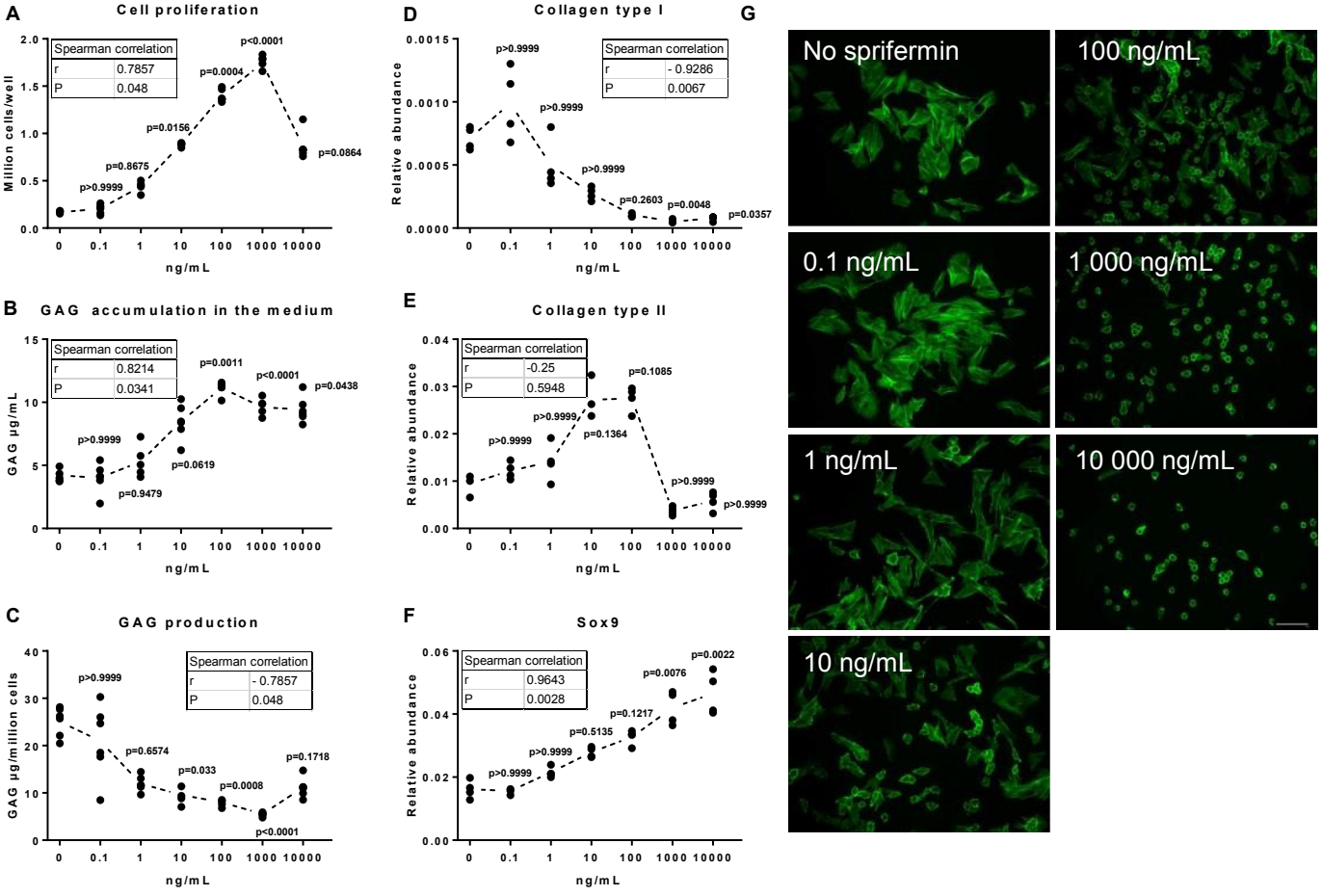

Fig. 1. Primary porcine chondrocytes in monolayer. Cells were cultured for 1 week in monolayer with ascending sprifermin concentrations. After 7 days of culture, the cell density (A) and the GAG content in the medium (B) were evaluated ($n = 6$), the GAG production in μg/million cells was also calculated (C) ($n = 6$). Expression of type I and II collagens, and Sox9 was assessed by real-time PCR (D–F; $n = 4$). *P* values on the graphs correspond to comparison to the control (0 ng/mL sprifermin). A Spearman correlation test was used to evaluate the dose dependency to sprifermin for each measured parameters; the resulting *P* and *r* values are tabulated on each graph. Actin staining with phalloidin-Alexa 488 was also performed after 5 days of culture (G). Scale bar = 100 μm.

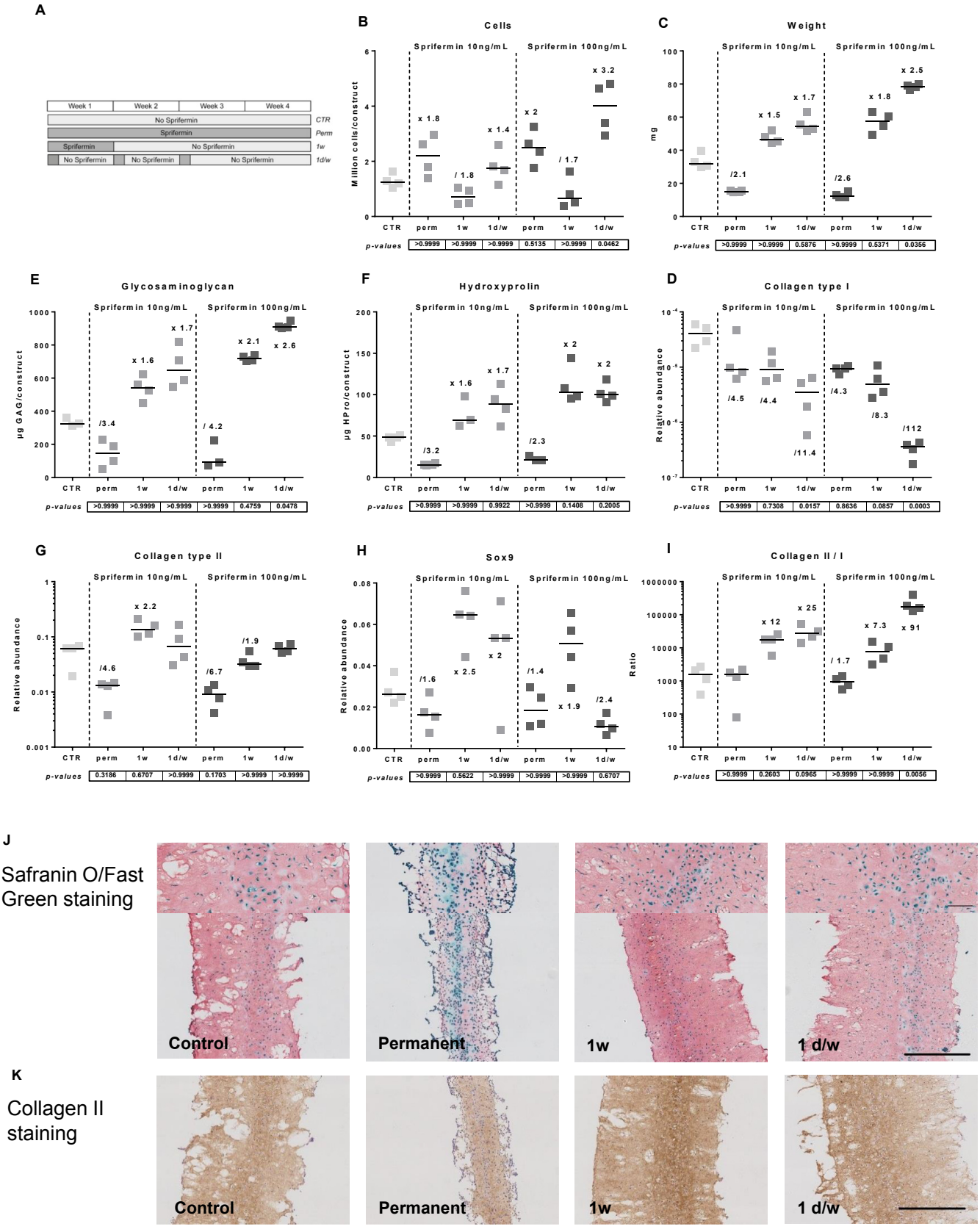

Fig. 2. Porcine chondrocytes in 3D, cell proliferation and matrix production. The cell constructs were cultured for 4 weeks with the permanent, 1d and 1 d/w sprifermin regimens (A; $n = 4$/regimen). The cell content (B), weight (C), GAG (D) and HPro (E), and gene expression (F–H) was examined. The type II:I collagen ratio was calculated (I). Median values (-) and individual data values (■) are shown in B–I. Additional constructs were processed for histology and stained with Safranin O/Fast Green (J) and Collagen II (K). Upper scale bar = 73 µm J), lower scale bar = 364 µm (J–K). The tabulated *P* values correspond to comparison to the control. The fold increase (×) and decrease (/) over control are indicated directly on the graphs. CTR = control; perm = permanent; 1 w = 1 week exposure followed by 3 weeks without sprifermin; 1 d/w = 1 day per week exposure for 3 weeks.

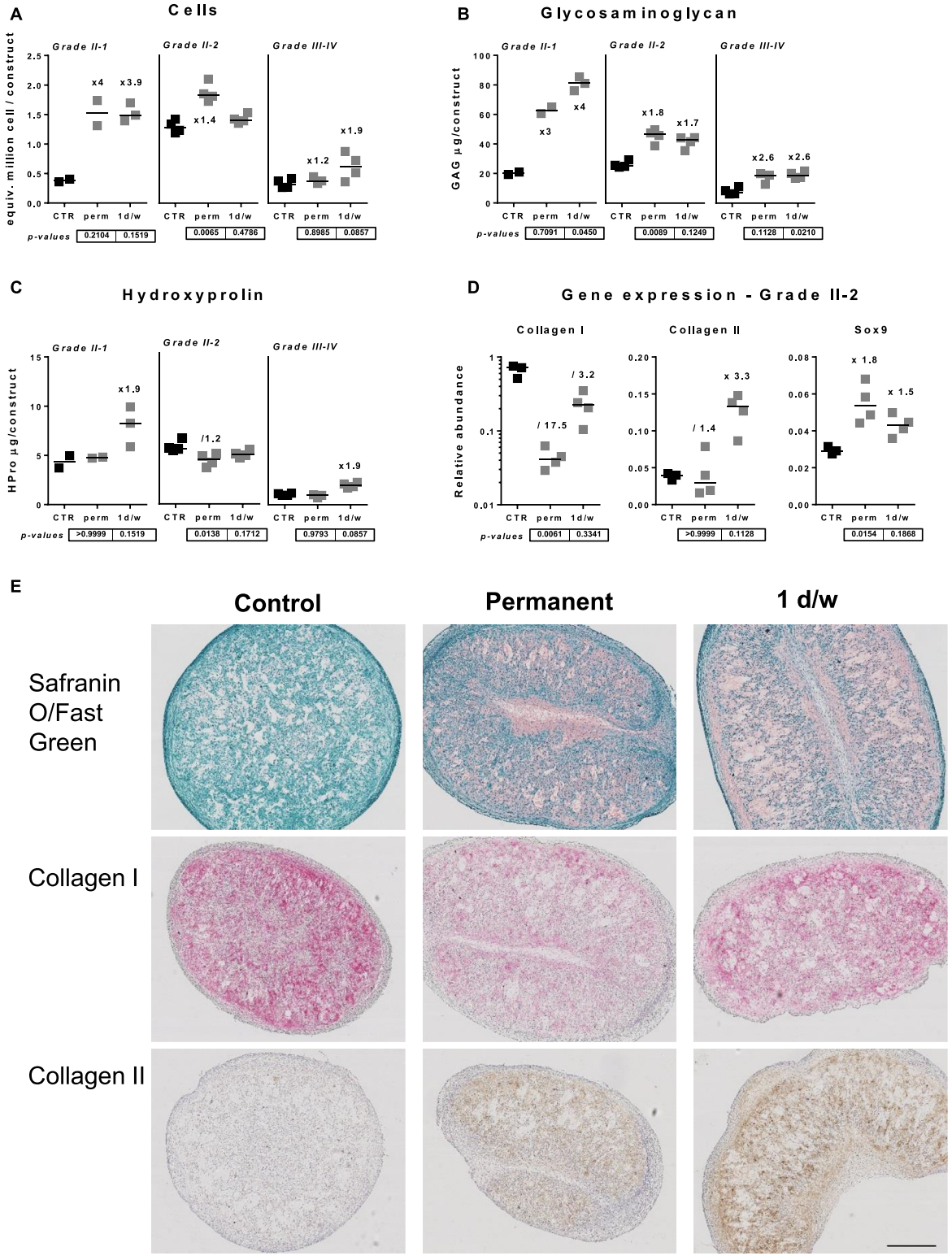

Fig. 3. Human OA chondrocytes in 3D culture. Chondrocytes from 3 OA patients with different disease grades (2 with Grade II and 1 with Grade III–IV) were cultivated in 3D with sprifermin 100 ng/mL permanently, 1 d/w, or left untreated ($n = 3$–4). The cell (A), GAG (B) and HPro (C) content were analysed. The gene expression of type I and II collagens and Sox9 was analysed for one culture only (Grade II-2) (D). Median values (-) and individual data values (■) are shown in A–D. Constructs were also stained with Safranin O and for type I and II collagens. Scale bar = 364 µm (E). Stainings are presented for Grade II-2 only. The tabulated *P* values correspond to comparison to the control. The fold increase (×) and decrease (/) over control are indicated directly on the graphs. CTR = control; perm = permanent; 1 w = 1 week; 1 d/w = 1 day per week for 3 weeks.

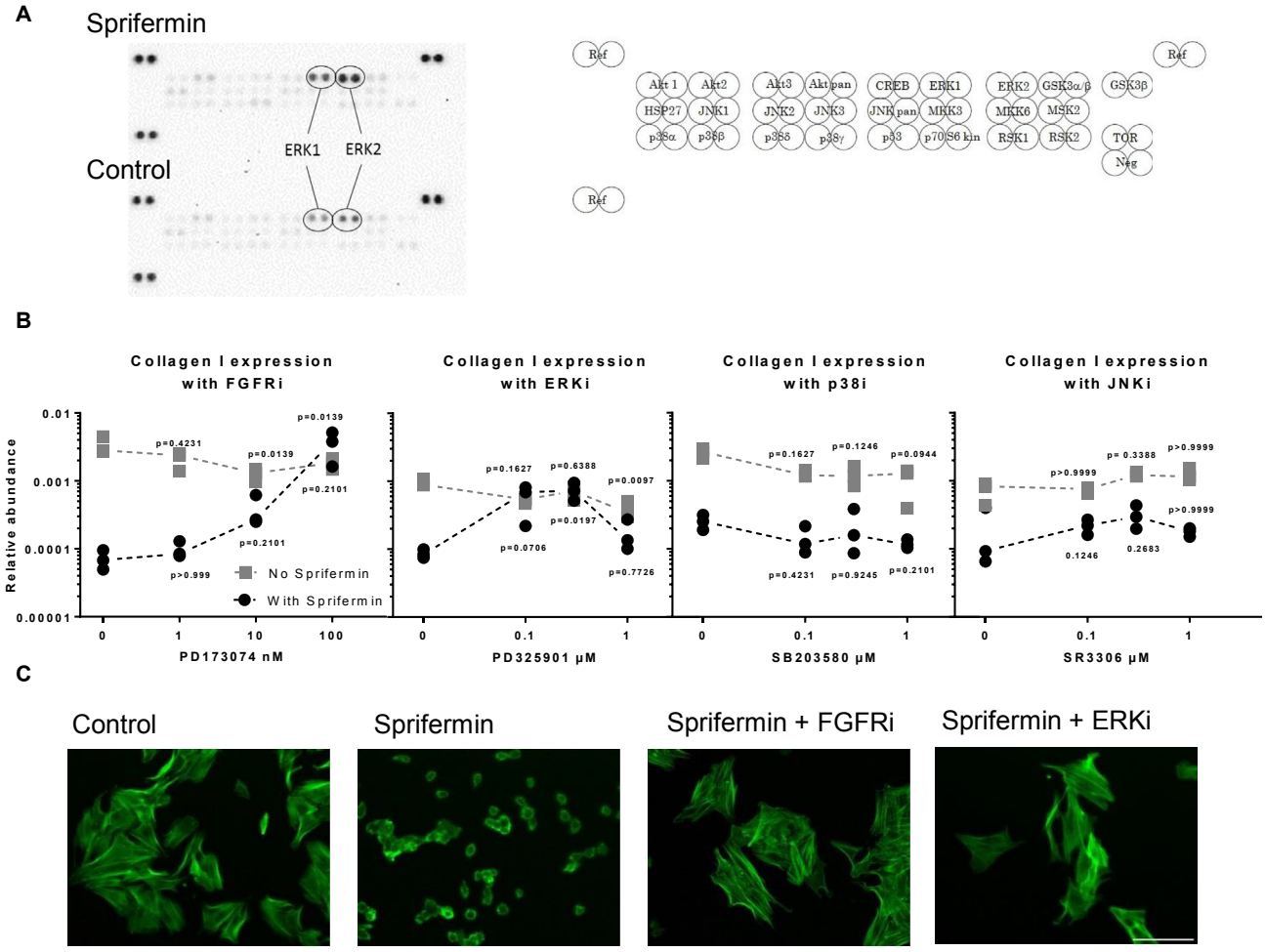

Fig. 4. Involvement of MAPKs in sprifermin signalling. Human OA chondrocytes were stimulated for 15 min with sprifermin 100 ng/mL or left untreated (control) and were analysed with a MAPK array kit. The experiment was repeated with six different donors, one representative result is shown (A). Porcine chondrocytes were cultured in monolayer with or without sprifermin 100 ng/mL and in the presence of FGFR inhibitor (PD173074), ERK inhibitor (PD325901), p38 inhibitor (SB203580) or JNK inhibitor (SR3306). Type I collagen expression was evaluated after 7 days of culture ($n = 3$). The *P*-values corresponds to the comparison for each condition (with or without sprifermin) to the control without inhibitor (0 µM) for the same condition (B). Cells were stained for actin with Phalloidin-Alexa 488 after 5 days of culture (C). Scale bar = 50 µm. *P* values correspond to comparison of data with and without sprifermin for the same inhibitor concentration.

condition (with or without sprifermin) the results obtained with each inhibitor concentration were compared to the control (no inhibitor) for the same condition. In addition, for the results presented in Fig. 1, a non-parametric Spearman correlation test was used to evaluate dose–response relationships. For the results presented in Fig. 5, a linear regression analysis was performed to evaluate the correlation between the two plotted parameters. Statistical analysis was performed with the software Graphpad Prism v6.05.

Results

In monolayer sprifermin strongly decreases type I collagen expression and promotes a round cell shape while stimulating cell proliferation

In both monolayer culture and OA cartilage, chondrocytes show an altered or dedifferentiated phenotype; they display a fibroblast-like elongated morphology[12,13], express mainly type I collagen[12,14] and have reduced Sox9 expression[13,15]. To evaluate the ability of sprifermin to prevent or reverse the alteration of the chondrocyte phenotype, porcine articular chondrocytes were cultured in monolayer with permanent exposure to sprifermin from 0.1 to 10,000 ng/mL.

Sprifermin strongly promoted cell proliferation in a dose-dependent manner [$P = 0.048$, Fig. 1(A)]. In parallel, total GAG accumulation increased in medium [$P = 0.0341$, Fig. 1(B)]. However, the normalised GAG production in µg/million cells decreased with sprifermin [$P = 0.048$, Fig. 1(C)]. Sprifermin also led to dose-dependent decrease in type I collagen expression [$P = 0.0067$, Fig. 1(D)]. Conversely, a dose-dependent increase in Sox9 expression was observed [$P = 0.0028$, Fig. 1(F)], while type II collagen expression increased until 100 ng/mL sprifermin and then was decreased [Fig. 1(E)].

In addition, sprifermin had an effect on cell morphology [Fig. 1(G)]; with increasing concentrations of sprifermin, the chondrocytes lost their elongated shape with numerous stress fibres and acquired a round morphology.

Interestingly, some effects of sprifermin, such as stimulation of chondrocyte proliferation or collagen II expression, dropped at high concentrations (1000 and/or 10,000 ng/mL). As it is known that FGF18 until 5 nM (≈90 ng/mL) does not activate FGFR1 and 2[16] and that chondrocytes poorly express FGFR4[17], we assume that until 100 ng/mL, the observed effects were FGFR3-driven effects.

However, at these very high concentrations, the other FGFRs might be activated resulting in a different cell response. It was also hypothesized that sprifermin could down-regulate FGFR3 expression at high concentrations. However, in porcine chondrocytes, no significant modulation of the FGFR3 expression was observed (Supplementary Material,Fig. 1).

In 3D culture, sprifermin exerts a strong anabolic effect on porcine chondrocytes when intermittently administered

The first set of experiments revealed that sprifermin over a 4-week culture period led to increased cell numbers, but a decrease in matrix molecule production (not shown). Because these results raised the possibility that sprifermin-induced proliferation may take place at the expense of matrix generation, we examined whether discontinuing sprifermin may allow ECM production to take place; tested exposures are illustrated in Fig. 2(A).

After 4 weeks of culture, the cell content was highest with intermittent 1 day/week (1 d/w) exposure with a 3.2 fold increase in the cell number with sprifermin 100 ng/mL in comparison to control [$P = 0.0462$, Fig. 2(B)]. Regarding ECM production [Fig. 2(C)–(E)], permanent exposure to sprifermin decreased the weight, GAG and HPro contents of the 3D constructs whereas they all increased under intermittent sprifermin exposure (by 2.5 fold with $P = 0.036$, 2.6 fold with $P = 0.0478$ and 2 fold with $P = 0.2005$, respectively for sprifermin 100 ng/mL in comparison to the control). When the GAG and HPro contents were normalised against the cell number, the matrix production/cell was down-regulated vs control with the permanent exposure regimen, was comparable to control with the 1 d/w regimen, and was strongly increased compared with control with the 1 w regimen (Table I).

Expression of type I collagen was reduced with all sprifermin regimens compared with controls [Fig. 2(F)] with the most important effect being observed with sprifermin 100 ng/mL 1 d/w (decreased by 112 fold with $P = 0.003$). Type II collagen expression was decreased with permanent exposure to sprifermin, but was mostly unchanged in the 1 w and 1 d/w regimens [Fig. 2(G)]. Similarly, Sox9 expression tended to decrease with permanent sprifermin, but increased with the 1 w and 1 d/w regimens [Fig. 2(H)]. Finally, as a consequence of reduced type I collagen expression, the type II:I collagen ratio was increased by 25-fold and 91-fold with sprifermin 1 d/w 10 and 100 ng/mL ($P = 0.0965$ and 0.0056, respectively), compared with control [Fig. 2(I)].

Histological examination revealed that, compared with control, permanent exposure to sprifermin yielded constructs that were smaller with less intense Safranin O staining, while the intermittent sprifermin regimens led to constructs that were larger [Fig. 2(J)]. All the constructs were strongly positive for type II collagen [Fig. 2(J)] and negative for type I collagen (not shown).

Sprifermin favours production of a hyaline cartilage-like ECM in human OA chondrocytes

The effect of sprifermin was also evaluated on chondrocytes derived from OA patients and the potential influence of sprifermin activity on disease stage was explored. To investigate this, chondrocytes were isolated from human cartilage areas with different damage severities (graded from I to IV). Three cell suspensions were thus generated: two containing 'Grade II' (Grades II-1 and II-2) chondrocytes and one containing 'Grade III–IV' chondrocytes (see Methods section). The OA chondrocytes were cultured for 4 weeks in 3D without or with sprifermin 100 ng/mL either permanently or 1 d/w.

The cell content increased with sprifermin in 4/6 cases [Fig. 3(A)]. The GAG content increased with all sprifermin regimens [Fig. 3(B)], with the largest effect being observed with sprifermin 1 d/w in the Grade II-1 culture (4-fold increase, $P = 0.045$). In addition, the HPro content was increased in the Grade II-1 and Grade III–IV cultures with sprifermin 1 d/w, but this increase was weak [Fig. 3(C)].

Gene expression analysis was only performed for the Grade II-2 chondrocytes. Here, permanent and 1 d/w sprifermin led to a decrease in type I collagen expression (by 17.5 and 3.2 fold with $P = 0.0061$ and 0.3341, respectively). Sprifermin 1 d/w but not permanent also led to a 3.3-fold increase in type II collagen expression ($P = 0.1128$). In addition, Sox9 expression was increased in the presence of permanent and 1 d/w sprifermin [by 1.8 and 1.5 fold with $P = 0.0154$ and $P = 0.1868$, respectively, Fig. 3(D)].

The histology results correspond well to the biochemical and gene expression results [Fig. 3(E); histology shown only for the Grade II-2 culture]. In the 3D constructs cultured in the absence of sprifermin, no Safranin O or type II collagen staining was visible, but the constructs were positive for type I collagen, illustrating that these chondrocytes were not able to produce a cartilage-like ECM. In the presence of both permanent and 1 d/w sprifermin, a Safranin O and type II collagen-positive matrix was observed, while type I collagen staining was less intense in comparison to the control. The cell constructs from Grade II-1 and Grade III–IV were also stained (not shown). The results are summarised in Table II.

Sprifermin signals through ERK1/2 but not p38 or JNK

To investigate which MAPKs may be involved in sprifermin signalling, we first determined which kinases were activated in response to sprifermin and then explored the functional implications of sprifermin-induced signalling by inhibition of the relevant pathways.

A kinase array was used to examine kinase activation in OA chondrocytes stimulated for 15 min with sprifermin. Signals corresponding to the phosphorylation of ERK1 and ERK2 were increased, but those corresponding to Akt, JNK and p38 were not activated, suggesting that sprifermin signals through ERK1 and ERK2 [Fig. 4(A)].

Down-regulation of type I collagen expression and induction of rounded cell morphology in porcine chondrocytes are considered relevant functional effects induced by sprifermin. In support, we demonstrated that an FGFR1–3 inhibitor, PD173074, suppressed

Table I
Matrix production by porcine chondrocytes in 3D: Chondrocytes were cultured for 4 weeks in 3D without sprifermin (CTR) or with sprifermin 10 or 100 ng/mL permanently, 1 w or 1 d/w. GAG production and HPro production per million cells were calculated (median and inter-quartile range in parentheses). CTR = control; 1 w = 1 week exposure followed by 3 weeks without sprifermin; 1 d/w = 1 day per week exposure for 3 weeks

CTR	Sprifermin 10 ng/mL			Sprifermin 100 ng/mL		
	Perm	1 w	1 d/w	Perm	1 w	1 d/w
GAG μg/million cells						
280 (237–296)	55 (36–86)	863 (567–1143)	326 (322–340)	52 (41–61)	1377 (1143–1645)	197 (196–232)
HPro μg/million cells						
40 (36–42)	8 (6–10)	75 (67–140)	41 (37–59)	9 (9–11)	184 (150–206)	27 (24–30)

Table II
Comparison of permanent and intermittent exposure in human OA chondrocytes: Cells were cultured for 4 weeks in 3D without sprifermin (CTR) or with sprifermin 100 ng/mL permanently or 1 d/w. The cell number, GAG and HPro content increase are expressed in x-fold over control. *Safranin O and type II collagen key*: (−) reduced staining vs CTR, (=) same staining vs CTR, (+) increased staining vs CTR, (++) much increased staining vs CTR. *Type I collagen key*: (+) reduced staining vs CTR, (++) much reduced staining vs CTR. The condition showing the most pronounced effect for each parameter appears in bold. CTR = controlperm = permanent; 1 w = 1 week; 1 d/w = 1 day per week for 3 weeks

	Grade II-1	Grade II-2	Grade III–IV
Cell number/construct	Perm × 4	**Perm × 1.7**	Perm × 1.2
	1 d/w × 4	1 d/w × 1.1	**1 d/w × 1.9**
GAG content/construct	Perm × 3.1	Perm × 1.7	Perm × 2.2
	1 d/w × 4	1 d/w × 1.6	1 d/w × 2.4
HPro content construct	Perm × 1.1	Perm × 0.7	Perm × 0.8
	1 d/w × 1.9	1 d/w × 0.9	**1 d/w × 1.9**
Safranin O staining	Perm:+	Perm:+	Perm:+
	1 d/w: +	1 d/w: +	1 d/w: +
Type II collagen staining	Perm: −	Perm: +	Perm: =
	1 d/w: =	**1 d/w: ++**	**1 d/w: +**
Type I collagen staining	All constructs negative	**Perm: ++**	**Perm: ++**
		1 d/w: +	1 d/w: +

the effect of sprifermin on cell morphology [Fig. 4(C) with FGFRi] and on type I collagen expression; indeed, type I collagen expression was significantly higher with FGFRi 100 nM ($P = 0.0139$) than in the control (0 nM) for the cells treated with sprifermin. Selective pathway inhibition was then studied using the ERK inhibitor, PD325901, which also completely inhibited the effect of sprifermin on cell morphology [Fig. 4(C) with ERKi]. Type I collagen was also significantly higher with 0.3 μM of ERK inhibitor ($P = 0.0197$) for the cells treated with sprifermin, indicating that its effect was inhibited. By contrast, neither SB203580 (p38 inhibitor) nor SR3306 (JNK inhibitor) showed an effect on either of these two parameters [Fig. 4(B); cell morphology not shown]. This confirmed the results obtained with the kinase array showing that ERK1 and 2 are involved in sprifermin signalling.

Discussion

During OA, chondrocytes display inappropriate activation of anabolic and catabolic activities, altered proliferation or apoptosis rates and failure to preserve tissue integrity. To investigate how sprifermin influences both chondrocyte proliferation and the ability of chondrocytes to (re)-express a hyaline ECM, several culture systems were used and proliferation, matrix production (via GAG and Hpro accumulation, representing proteoglycan and collagen production, respectively) and specific chondrocyte markers (higher type II:I collagen ratio and higher Sox9 expression are favourable) were studied. One effect was robustly reproducible among almost all the culture systems tested: sprifermin enabled chondrocyte proliferation concomitantly with promoting the chondrocyte phenotype.

In monolayer with porcine chondrocytes, sprifermin dose-dependently stimulated cell growth while strongly decreasing type I collagen expression, increasing chondrocyte marker expression (Sox9, type II collagen expression) and promoting a round cell morphology. According to our knowledge, this is the first time that both proliferation and a clear favourable influence on both chondrocyte phenotype and cell morphology has been reported. This relationship between chondrocyte shape and phenotype is well known; indeed, inhibition or alteration of stress fibre formation with cytochalasin D or RhoA inhibitors has been shown to stimulate Sox9, type II collagen expression and decrease type I collagen

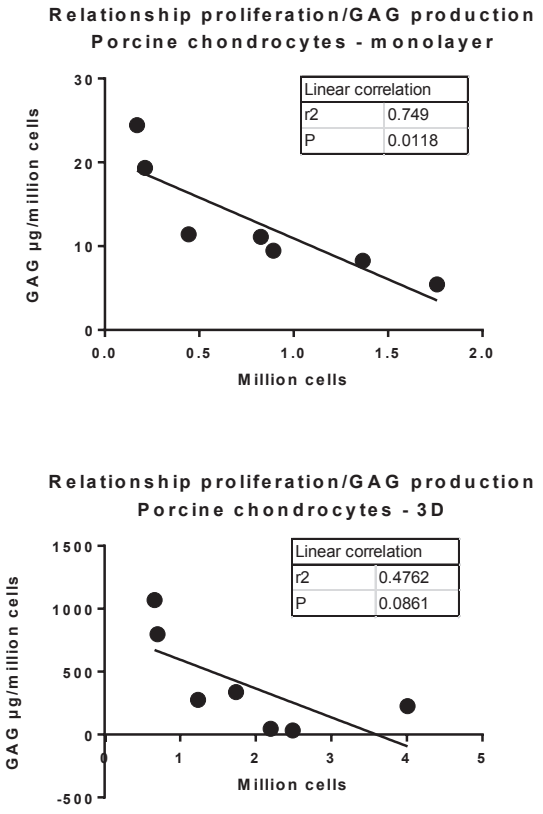

Fig. 5. Relationship between proliferation and matrix production. The cell concentration at the end of the culture in million cells was plotted against GAG production in μg/million cells. To evaluate the correlation between these two parameters, a linear regression was performed; the resulting *P* and *r*2 values are tabulated on each graph.

production[13,18–21]. However, forcing the cells to adopt a round morphology is usually also associated with reduced proliferation.

Others have also observed a change in chondrocyte morphology in the presence of growth factors such as FGF2[22], FGF9[23] or BMP2[24]. However, unlike sprifermin, they were not necessarily associated with phenotype maintenance or proliferation. Finally, the effect of sprifermin on human OA chondrocytes in monolayer was also evaluated, but was weaker or different compared to porcine chondrocytes (Supplementary Material, Fig. 2). The reason for these differences is unclear.

In monolayer culture it was also demonstrated that sprifermin activates ERK1/2 but not JNK, p38 or Akt. The blockade of ERK1/2 also abolished the effects of sprifermin on the chondrocyte morphology and type I collagen expression, confirming its role in sprifermin signalling.

In 3D cultures of both porcine and human chondrocytes, sprifermin promoted proliferation while decreasing type I collagen expression, and increasing type II collagen and Sox9 expression. This observation was supported by immunohistochemistry analysis, which showed that OA chondrocytes exposed to sprifermin produced a type II collagen-positive matrix with reduced type I collagen staining. While there have been other reports of anabolic effects with growth factors such as BMP7[25,26], BMP2[27,28] and FGF2[29], no effect on type I collagen was seen for BMP2 or FGF2 and for BMP7 it was not reported. It is possible, therefore, that this property may be specific to sprifermin.

The present study also revealed that sprifermin exerts a 'hit and run' mode of action, i.e., repeated exposures of short duration initiating a cascade of responses that finally induce an anabolic

effect. In porcine chondrocyte 3D cultures, the 1 d/w exposure to sprifermin over a 4-week period resulted in constructs that contained more GAG, HPro and cells than control constructs. Conversely, when constructs were permanently exposed to sprifermin, they contained less ECM than control constructs. This demonstrates that different exposures to a growth factor can generate very different – in this case opposite – effects. To explain these results we firstly hypothesise a clear dissociation between cell proliferation and matrix production. Indeed, when plotting the GAG production against the cell density at the end of the culture (Fig. 5) an inverse correlation became visible for both the monolayer and 3D cultures, indicating that when chondrocytes are proliferating they produce less ECM, and *vice versa*. In line with this, the 1 d/w exposure regimen (which delivered a higher cell number) was associated with lower matrix production/cell than the 1 w exposure regimen (Table I). Secondly, and more importantly, permanent exposure to sprifermin had a weaker effect on cell proliferation and type I collagen down-regulation than 1 d/w exposure, strongly suggesting that discontinuation after initial exposure was necessary for the full anabolic potential of sprifermin to be exerted. This suggests that intermittent exposure may transiently activate cellular mechanisms inducing an anabolic response, while permanent exposure may involve other signalling systems and a different response. A desensitisation phenomenon or a negative feedback loop could also occur upon prolonged exposure to sprifermin[23]. The expression of FGFR3 in human chondrocytes was evaluated and was not significantly influenced by sprifermin (Supplementary Material, Fig. 1). Interestingly, the parathyroid hormone (PTH) also exerts different effects with intermittent vs continuous exposure[30]: whereas one daily pharmacological injection of PTH results in more bone, continuous exposure (such as hyperparathyroidism[31]) results in loss of bone.

Finally, sprifermin had an anabolic effect on human OA chondrocytes. In 3D culture with human OA chondrocytes, sprifermin was able to stimulate cell proliferation and promote accumulation of a Safranin-O and a type II collagen-positive matrix accumulation. This anabolic effect was reproducible among cells derived from more than one patient and from cartilage areas with varying levels of damage. Overall, intermittent exposure showed a more robust effect than permanent exposure. Importantly, sprifermin did not lead to increased protease (MMP13, ADAMTS5) or hypertrophy marker (runx2, type X collagen) expression in any of the 3D cultures tested (in Supplementary Material, Fig. 3), confirming that neither catabolic events nor hypertrophy were induced. These results are in alignment with the positive data from clinical trials of sprifermin in OA patients. Indeed, in an initial safety study in which patients who were candidates for total knee replacement surgery received intra-articular sprifermin, cartilage samples obtained after total knee replacement revealed chondrocyte proliferation and an improved Mankin score in sprifermin-treated patients[32]. Reduced loss of total femorotibial cartilage thickness was also observed in sprifermin-treated patients in a separate safety and efficacy study[10]. In both studies, the multiple injection regimen was similar to the 1 d/w exposure in this preclinical study: patients were injected once weekly during 3 weeks (only one cycle for the first study and two cycles for the second).

In conclusion, the results of the present study clarified the mode of action of sprifermin on chondrocytes and demonstrated its DMOAD potential: sprifermin stimulates the proliferation of human OA chondrocytes while simultaneously inducing a more physiological chondrocyte phenotype. This expanded chondrocyte population is able to produce a hyaline ECM which has the potential to result in more cartilage tissue. Intermittent exposure 1 d/w enabled sprifermin to fully exert its anabolic effects, illustrating a 'hit and run' mode of action, which argue for the injection scheme used in prior and ongoing clinical trials with sprifermin in knee OA.

Author contributions

AG, CL, HG and SL designed the experiments. AG, SL and HG performed the experiments and acquired the data. AM and DF provided human material and a mapping of cartilage damage for this material. AG, CL, HG, SL, DR, ACBJ and MAK analysed the data and participated in the writing of the manuscript.

Conflicts of interest

AG, CL, HG and SL are all employees of Merck KGaA, Darmstadt, Germany. DR, MK and ACBJ are employees of Nordic Biosciences, Herlev, Denmark.

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Supplementary data

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