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SUMMARY

Objective: Transforming growth factor-β (TGFβ) is a major regulator of cartilage homeostasis and its deregulation has been associated with osteoarthritis (OA). Deregulation of the TGFβ pathway in mesenchymal stem cells (MSCs) has been proposed to be at the onset of OA. Using a secretome analysis, we identified a member of the TGFβ family, TGFβ-induced protein (TGFβi or βIGH3), expressed in MSCs and we investigated its function and regulation during OA.

Design: Cartilage, bone, synovium, infrapatellar fat pad and bone marrow-MSCs were isolated from patients with OA or healthy subjects. Chondrogenesis of BM-MSCs was induced by TGFβ3 in micropellet culture. Expression of TGFβi was quantified by RT-qPCR, ELISA or immunohistochemistry. Role of TGFβi was investigated in gain and loss of function experiments in BM-MSCs and chondrocytes.

Results: TGFβi was up-regulated in early stages of chondrogenesis and its knock-down in BM-MSCs resulted in the down-regulation of mature and hypertrophic chondrocyte markers. It likely occurred through the modulation of adhesion molecules including integrin (ITG)β1, ITGβ5 and N-cadherin. We also showed that TGFβi was upregulated *in vitro* in a model of OA chondrocytes, and its silencing enhanced the hypertrophic marker type X collagen. In addition, TGFβi was up-regulated in bone and cartilage from OA patients while its expression was reduced in BM-MSCs. Similar findings were observed in a murine model of OA.

Conclusions: Our results revealed a dual role of TGFβi during chondrogenesis and pointed its deregulation in OA joint tissues. Modulating TGFβi in BM-MSCs might be of interest in cartilage regenerative medicine.

Introduction

Osteoarthritis (OA) is the most common rheumatic disease affecting all joint tissues including cartilage, sub-chondral bone, synovium and fat pad[1]. It results in progressive destruction of articular cartilage and loss of joint function leading to morbidity and disability in adults. This induces a societal burden that will increase in the coming years due to the aging of population and increased prevalence of obesity and metabolic disorders[2]. Although the aetiology of OA is still imperfectly understood, a number of environmental and genetic factors contribute to the breakdown of cartilage homeostasis and OA initiation. Evidence from genome-wide association studies has emerged that growth factors belonging to the transforming growth factor-β (TGFβ) family play a role in the development of OA[3].

Members of the TGFβ superfamily can be divided into the TGFβ and activin group, the bone morphogenetic protein (BMP) group

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and the growth and differentiation factor (GDF) group[4]. The TGFβ signalling is essential for articular cartilage homeostasis. It is rapidly upregulated by mechanical loading, stimulates proteoglycan synthesis and blocks the expression of chondrocyte hypertrophic genes[5]. Indeed, under physiological conditions, TGFβ is expressed at basal levels in articular cartilage or upregulated by loading and prevents chondrocyte terminal differentiation and cartilage degeneration[6]. However, TGFβ signalling is deregulated in OA and high concentrations of TGFβ can be found in the synovial fluids of patients. Soluble TGFβ is produced by activated synovial cells and released from reservoirs in the cartilage matrix upon its degradation. High levels of TGFβ result in preferential activation of the SMAD1-5-8 pathways instead of the SMAD2-3 pathways and in upregulation of genes involved in fibrogenesis and hypertrophy leading to synovial fibrosis and osteophyte formation[7].

In OA, osteophytes arise from chondrocyte terminal differentiation of mesenchymal progenitors residing in the periosteum due to the increased expression of TGFβ. TGFβ expression is also increased in the subchondral bone where are located mesenchymal stem/stromal cells (MSCs). Demonstration that deregulation of TGFβ signalling in MSCs is involved in OA progression or initiation came from the study of Zhen and co-authors[8]. They showed that knock-down of TGFβ type II receptor in nestin-positive MSCs resulted in less severe cartilage lesions in mice whereas transgenic expression of TGFβ1 in osteoblasts induced OA. Indeed, excessive subchondral bone formation in OA is associated with enhanced TGFβ activity in osteoblasts and MSCs. Other studies have reported cartilage resident MSC-like progenitors with senescence features, which might be related to failure of repair[9] and the possible involvement of synovium-derived MSCs in cartilage repair following joint injury[10]. Our hypothesis was that dysregulation of TGFβ signalling in MSCs is responsible for lack of cartilage repair in OA. Based on a previous secretome analysis of MSCs derived from bone marrow and adipose tissue, we identified basal secretion of several TGFβ family members, including TGFβ-induced gene product-h3 (TGFβi/BIGH3/RGD-CAP)[11]. The aim of the present study was to determine the functional role of TGFβi in MSCs and to investigate its deregulation in OA.

Method

Cell culture

Human tissue specimens were recovered from post-mortem or amputated healthy subjects and patients with grade IV OA, aged 52.5 ± 8.5 years and 74.4 ± 5.1 years, respectively. The study was approved for OA specimen recovery by the French Ministry of Research and Innovation and the Personal data Protection ethics Committee (CPP) of Languedoc-Roussillon (approval DC-2010-1185) and CPP of Paris V, Ile de France and the Consultative Committee on the Treatment of Information in Research in Health field (CCTIRS) (approval 15-623-ter). Healthy tissues were recovered after approval by the Agence de la Biomédecine (authorization #: PFS16-006) for post-mortem subjects or for amputated patients (C17-53). Cartilage, bone, synovium and infrapatellar fat pad samples were recovered from the same patients, cut in small pieces and weighted. Supernatants were produced using 1 g tissue/6 mL IMDM medium containing 1% penicillin/streptomycin and 1% glutamine incubated at 37°C for 24 h. For RNA extraction, 300 mg of each tissue were snap frozen and stored at −80°C. MSCs isolated from bone marrow (BM-MSCs) or adipose tissue (ASCs) were characterized by phenotyping and trilineage differentiation potential as described[12]. They were cultured in αMEM containing 2 mmol/mL glutamine, 100 μg/mL penicillin/streptomycin, 10% foetal calf serum (FCS) and 1 ng/mL basic fibroblast growth factor (bFGF) (R&D Systems, Lille). Human chondrocytes were isolated from knee cartilage and cultured in DMEM supplemented with 2 mmol/mL glutamine, 100 μg/mL penicillin/streptomycin, 10% FCS, 5 μg/mL insulin, 5 ng/mL bFGF till passage 1[13].

Collagenase-induced osteoarthritis mouse model

The study was conducted in accordance with guidelines and regulations of the Ethical Committee for animal experimentation of the Languedoc-Roussillon (Approval 5349-2016050918198875). The experiment was performed after final approval given by the French Ministry for Education, Higher Education and Research. Collagenase-induced osteoarthritis (CIOA) model was performed as previously described[14]. They were euthanatized at day 7, 14, 42. Hind paws were collected and fixed in formaldehyde 3.7% for 2 days before histological processing.

MSC differentiation and chondrocyte culture

BM-MSCs were differentiated towards chondrocytes by culture in micropellet for 21 days. Briefly, 2.5×10^5 BM-MSCs were centrifuged in 15 mL conical tubes and cultured in DMEM high glucose (Lonza, Levallois) with 100 μg/mL penicillin/streptomycin, 0.35 mM proline, 0.1 μM dexamethasone, 0.17 mM ascorbic acid-2-phosphate, 1 mM pyruvate sodium, 1% insulin-transferrin-selenic acid (Lonza) and 10 ng/mL TGF-β3 or 100 ng/mL BMP-2 or IGF-1 (R&D Systems). BM-MSCs were differentiated towards osteoblasts or adipocytes by culture in inductive conditions for 21 days as described[15].

For the model of late passages, chondrocytes were maintained till reaching sub-confluency and replated at 6000 cells/cm^2 till passage 3. Otherwise, chondrocytes were used at Passage 1. After seeding, chondrocytes were cultured for 72 h before stimulation with 10 ng/mL IL-1β for 72 h or 10 ng/mL TGFβ3 for 24 h (R&D Systems). Media were replaced for 24 h before recovering supernatants and cells.

Cell transfection

BM-MSCs and chondrocytes were transfected at 60% confluence with 50 nM of control siRNA (si*CTRL*) or TGFβi siRNA (si*TGFβi*) (Ambion, ThermoFisher Scientific, Illkirsch) using Oligofectamine reagent (Life Technologies, Courtaboeuf). BM-MSCs transfection was done twice: at day 3 and 1 before pellet formation. Chondrocytes were harvested 1 week after transfection for RNA extraction.

Protein analysis

TGFβi was quantified in culture supernatants by ELISA (CliniSciences, Nanterre). Phospho-SMAD3 and 1 were quantified in cell extracts by InstantOne ELISA assays as indicated by supplier (eBioscience, Paris). For Western blotting, cells were incubated with RIPA (Sigma Aldrich, Saint-Quentin Fallavier) containing Halt protease inhibitor cocktail (150 μL/3 pellets) at 4°C for 30min. Cell lysates were incubated in Bolt LDS sample buffer and Bolt sample reducing agent at 70°C for 10min (ThermoFisher Scientific). Protein extracts (50 μg) were analyzed by SDS-PAGE on Bolt 4–12% Bis-Tris Plus resolving gels, followed by transfer to nitrocellulose membranes. Membranes were blocked in Tris Buffered Saline containing 5% milk for 2 h and incubated with anti-TGFβi (1:200, Proteintech, Manchester) and anti-βactin (1:5,000, Sigma) antibodies at 4°C overnight. Membranes were then incubated with Horseradish peroxidase-labeled anti-mouse IgG antibody (1:100,000, Sigma) for

2 h. Bands were scanned using ChemiDoc XRS + Imager (Bio Rad, Les Ulis).

Immunohistological analysis

Tissue samples from mice were fixed in 3.7% formaldehyde for 2 days and decalcified using 10% EDTA for 21 days before being processed. Pellets were fixed in 3.7% formaldehyde at room temperature for 1 h. Antigen retrieval was done on pellets with 1 mg/mL hyaluronidase (Sigma Aldrich) at 37°C for 1 h or on tissue sections with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) at 70°C overnight followed by 1 mg/mL hyaluronidase at 37°C for 15min and 0.1 U/mL chondroitinase ABC (Sigma Aldrich) at 37°C for 1 h. Immunolabelling was performed using anti-type II collagen (1:50, Acris, Interchim), anti-aggrecan (1:1000, Merck, Molsheim), anti-TGFβi (1:100, Proteintech) primary antibodies at 4°C overnight and UltraVision Detection System Anti-Polyvalent kit (Lab Vision, Interchim, Montluçon).

RNA extraction and RT-qPCR

RNA was extracted from pellets or cells using the RNeasy kit and from infrapatellar fat pad or synovium using the RNeasy Lipid Tissue Kit (Qiagen, Courtaboeuf). For cartilage and bone, RNA was isolated with 0.1 g/mL TRIzol reagent (ThermoFisher Scientific) followed by chloroform and phenol acid extraction. RNA (0.5 μg) was reverse transcribed using 100 units of the M-MLV reverse transcriptase (ThermoFisher Scientific, Illkirch) and PCR reactions were performed as described[16]. All details for primer sequences (SYBR Green technologies) are described in Table 1. All values were normalized to RPS9 housekeeping gene and expressed as relative expression or fold change using the respective formulae $2^{-\Delta CT}$ or $2^{-\Delta\Delta Ct}$.

Statistical analyses

Statistical analysis was performed with GraphPad Prism Software. Each sample/cell was independent and represented an experimental unit providing a single outcome. Normal distribution and variance homogeneity of values were determined with Shapiro–Wilk and Fisher (2 groups) or Bartlett (>2 groups) tests, respectively, followed by appropriate tests. Description of the tests used are detailed in each figure. Data are presented as the mean ± SD. *: P value < 0.05.

Table 1
List of primers and assays for PCR analysis

A– Primer sequences		
Gene	Sequence forward	Sequence reverse
ADAMTS5	CTCCACGCAGCCTTCACTGT	TGGGTGGCATCGTAGGTCTG
AGG	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA
AP	CCACGTCTTCACATTTGGTG	GCAGTGAAGGGCTTCTTGTC
COLIIB	CAGACGCTGGTGCTGCT	TCCTGGTTGCCGGACAT
COLX	GTGGACCAGGAGTACCTTGC	TGCTGCCACAAATACCCTTT
ITGαV	AGGAGAAGGTGCCTACGAAGCT	GCACAGGAAAGTCTTGCTAAGGC
ITGβ1	GGATTCTCCAGAAGGTGGTTTCG	TGCCACCAAGTTTCCCATCTCC
ITGβ3	CATGGATTCCAGCAATGTCCTCC	TTGAGGCAGGTGGCATTGAAGG
ITGβ5	GCCTTTCTGTGAGTGCGACAAC	CCGATGTAACCTGCATGGCACT
MMP13	TAAGGAGCATGGCGACTTCT	GTCTGGCGTTTTTGGATGTT
N-CAD	CCTCCAGAGTTTACTGCCATGAC	GTAGGATCTCCGCCACTGATTC
OC	GGCGCTACCTGTATCAATGG	TCAGCCAACTCGTCACAGTC
RUNX2	CGGAATGCCTCTGCTGTTAT	TTCCCGAGGTCCATCTACTG
TGFβ1	AAGAAGCGTGCTTTGGATGCGG	ATGCTCCAGCACAGAAGTTGGC
TGFβ2	AAGAAGCGTGCTTTGGATGCGG	ATGCTCCAGCACAGAAGTTGGC
TGFβ3	CTAAGCGGAATGAGCAGAGGATC	TCTCAACAGCCACTCACGCACA

B– TaqMan® Gene Expression Assay ID	
Gene	ID
RPS9	Hs02339424_m1
TGFβI	Hs00932747_m1

Results

TGFβi is expressed in MSCs from healthy individuals and down-regulated in MSCs from OA patients

From a previous proteomic study investigating the secretome of BM-MSCs and ASCs[11], we discovered that among members of the TGFβ family, latent binding protein (LTBP)1, LTBP2 and TGFβi were mutually expressed [Fig. 1(A)]. We selected TGFβi because its functional role in MSCs and cartilage homeostasis or physiopathology is still unclear. TGFβi expression was confirmed in BM-MSCs and ASCs at the mRNA and protein levels but at significantly higher levels in BM-MSCs [Fig. 1(B)–(C)]. In addition, TGFβi was expressed at significantly lower levels in cells belonging to other embryonic origin, such as endoderm-derived primary hepatocytes. Of interest, mRNA and protein expression of TGFβi was dramatically down-regulated in BM-MSCs from late stage OA patients, as compared to healthy subjects [Fig. 1(D)–(E)].

Expression of TGFβi is up-regulated in cartilage and bone from OA patients

Demonstration that TGFβi is down-regulated in OA BM-MSCs questioned on its expression in other joint tissues from OA patients. We therefore compared TGFβi expression in cartilage, bone, synovium and infrapatellar fat pad from healthy subjects and patients at latest stage of OA, who were undergoing surgery. Expression levels of TGFβi mRNA were significantly up-regulated in cartilage and bone from OA patients but unchanged in synovium or infrapatellar fat pad [Fig. 2(A)]. Similar results were observed at the protein level [Fig. 2(B)]. Immunohistological analysis revealed absence or low staining for TGFβi in cartilage from healthy subjects, predominantly found in the superficial layer of cartilage [Fig. 2(C)]. By contrast, intense staining for TGFβi was observed in the upper layers of cartilage from OA patients, in areas with signs of chondrocyte proliferation, of high erosion or in osteophytes (not shown). Similarly, in the CIOA mouse model, strong staining for TGFβi was visualized in the articular cartilage and particularly, in osteophytes (Supl. figure, panel (A)). In this model, we also revealed up-regulation of TGFβi mRNA at all time times during the course of the disease (Supl. figure, panel (B)). Similar findings were obtained in the aging and destabilization of the median meniscus (DMM) models (data not shown). Altogether, these data supported a dysregulation of TGFβi in OA.

TGFβi is up-regulated in IL1β-treated chondrocytes

Expression of TGFβi being dysregulated in OA samples, we wanted to determine its expression kinetics in an *in vitro* model of OA-like chondrocytes. We first used the model of freshly isolated chondrocytes induced to dedifferentiate by serial passages, in which expression of chondrocyte anabolic markers is lost, reproducing characteristics of OA cartilage[17]. As expected, we observed a down-regulation of type II collagen and aggrecan while type I collagen was up-regulated as soon as passage 1 [Fig. 3(A)]. However, concomitantly with decreased expression of anabolic markers, expression of TGFβi was down-regulated in dedifferentiated chondrocytes [Fig. 3(B)]. We therefore investigated the modulation of TGFβi expression in another *in vitro* model of OA: freshly isolated chondrocytes cultured with IL-1β. In this model, IL-

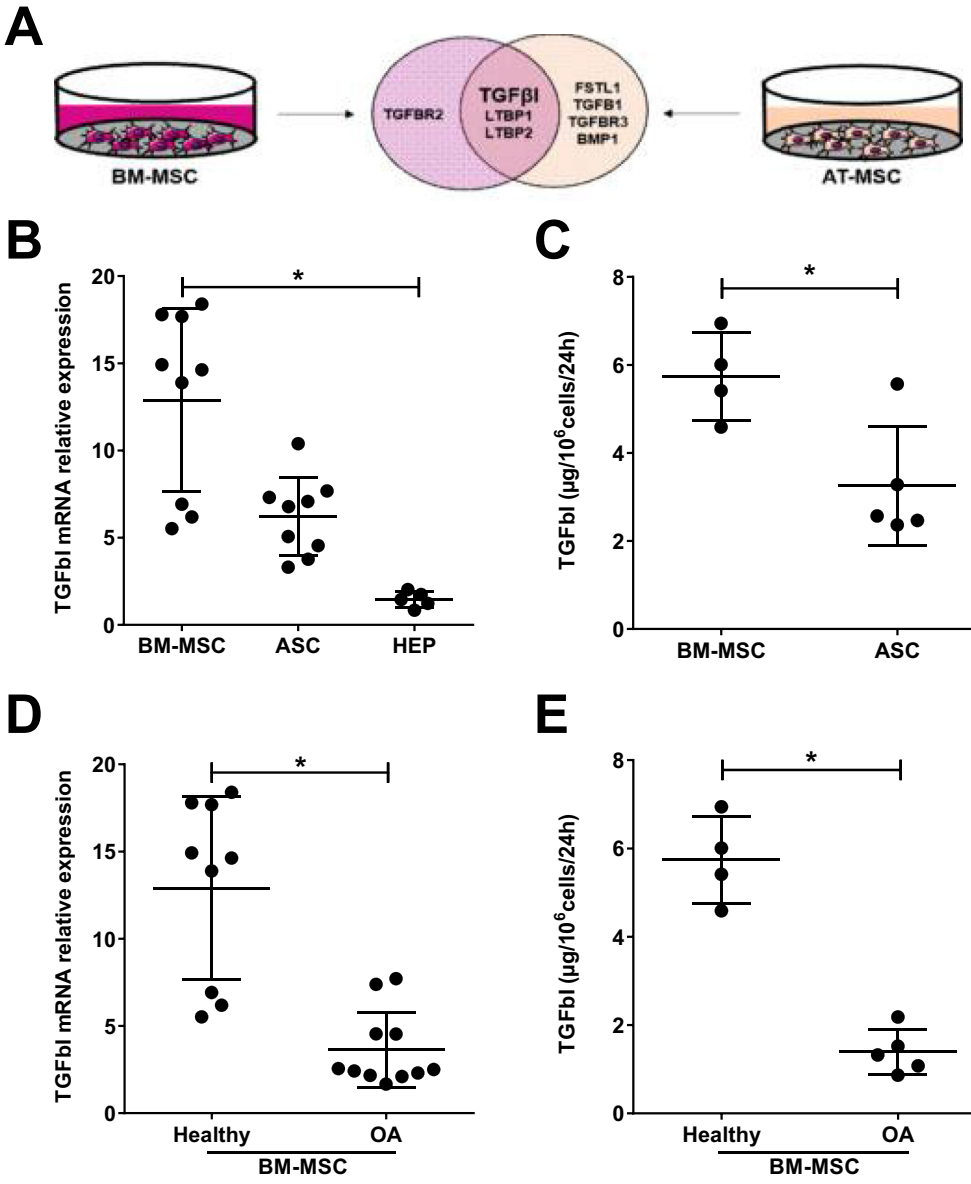

Fig. 1. TGFβi is downregulated in mesenchymal stem cells (MSCs) from OA patients. (A) Identification of members of the TGFβ family secreted by human MSCs from bone marrow (BM-MSC) and adipose tissue (ASC) by mass spectrometry. (B–C) Expression of TGFβi in BM-MSC, ASC and primary hepatocytes (Hep) from healthy subjects at the mRNA and protein level ($n = 4$–9 individuals). (D–E) Expression of TGFβi in BM-MSC from healthy subjects and late stage OA patients at the mRNA and protein level ($n = 4$–11 individuals). Results are expressed as mean ± SD. Statistical analysis used Kruskal–Wallis test followed by Dunn's multiple comparisons test in (B) or Mann–Whitney test in (C–E) (*$p < 0.05$).

1β down-regulated aggrecan, type II collagen and up-regulated MMP13, ADAMTS5 confirming the induction of OA phenotype [Fig. 3(C)]. In parallel, expression of TGFβi was increased at the mRNA and protein levels, as observed in OA chondrocytes [Fig. 3(D)–(E)]. Analysis of TGFβ isoforms modulated in IL1β-treated chondrocytes revealed down-regulation of TGFβ2 and up-regulation of TGFβ3 suggesting that TGFβ3 might be the inducer of TGFβi [Fig. 3(F)]. We validated that TGFβ3 can upregulate TGFβi mRNA and protein levels by a 7-fold factor in chondrocytes [Fig. 3(G)–(H)]. Finally, we investigated the effect of TGFβi silencing in chondrocytes. Both TGFβi mRNA and protein levels were down-regulated by a two-fold factor using siRNA interference approach, although statistically only for mRNA levels [Fig. 3I]. This down-regulation did not significantly modulate most of chondrocyte genes but significantly increased the expression of the hypertrophic chondrocyte marker, type X collagen [Fig. 3(J)].

Upregulation of TGFβi during the early stage of chondrogenic differentiation of BM-MSCs

With regards to the expression of type X collagen in late stage of chondrogenesis, we investigated the expression of TGFβi during the differentiation of BM-MSCs. Chondrogenic differentiation of BM-MSCs was induced by culture in micropellet in presence of TGFβ3 for 21 days. TGFβi expression peaked at days 1–3 and returned to basal levels as soon as day 7, till day 21 [Fig. 4(A)]. Up-regulation of TGFβi was also noticed in control conditions (MSCs cultured in aggregates in absence of TGFβ3) but the expression levels were not statistically different from day 0, except for day 3. Of interest, expression of TGFβi is low when the expression of the chondrocyte markers, Sox9, type IIB collagen, Aggrecan and type X collagen are high at day 21 (compared to day 0 or to micropellets cultured without TGFβ3) [Fig. 4(B)]. Protein levels of TGFβi tended to be

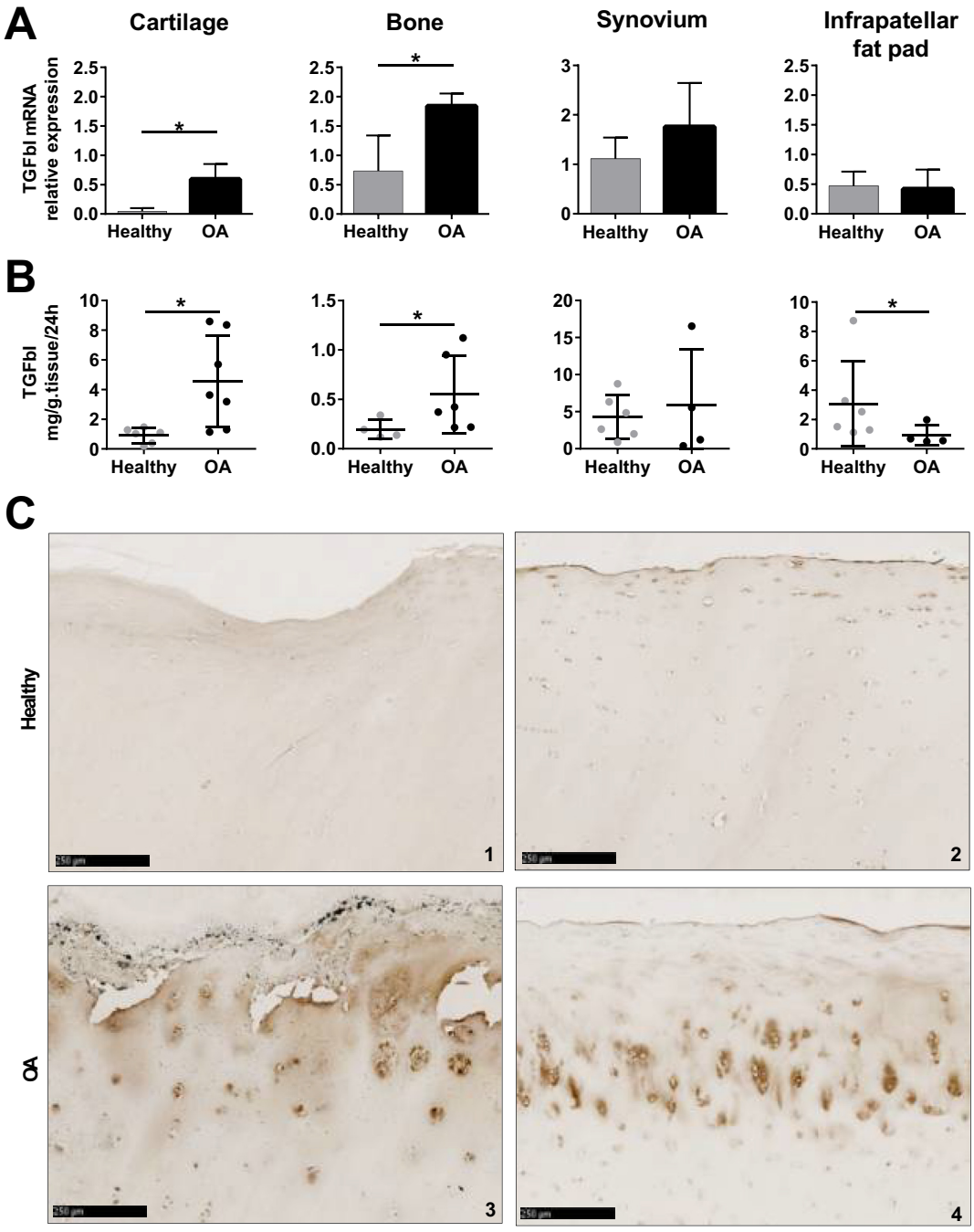

Fig. 2. TGFβi is upregulated in cartilage and bone from OA patients. (A) Expression of TGFβi mRNA in cartilage, bone, synovium and infrapatellar fat pad from healthy (grey) and OA (black) human individuals ($n = 4$). (B) Quantification of TGFβi protein in cartilage, bone, synovium and infrapatellar fat pad from healthy (grey; $n = 4$–6) and OA (black; $n = 4$–7) human individuals. (C) Immunohistological analysis of TGFβi in human cartilage from healthy and OA patients. Representative pictures of 2 healthy (#1-2) and 2 OA (#3-4) individuals. Note TGFβi expression in the superficial layer of healthy cartilage (#2) and upper layer of articular cartilage from OA samples (#3-4). Scale bar is 250 μm. Results are expressed as mean ± SD. Statistical analysis used Mann–Whitney test ($^*p < 0.05$).

increased in pellet culture supernatants of TGFβ3-induced pellets (2-fold factor at day 4 and 4.3-fold factor at day 21), as compared to un-induced pellets [Fig. 4(C)]. However, they were significantly lower at day 21 as compared to day 4. Lower amounts of TGFβi were observed in pellet supernatants than in MSC supernatants (day 0), which was likely due to retention of the protein in the pellet extracellular matrix. Immunohistochemical analysis detected TGFβi protein in TGFβ3-induced pellets at day 7 (not shown) and at day 14 while at day 21, the staining appeared less intense [Fig. 4(D)]. By contrast, chondrocyte markers, aggrecan and type II collagen, as well as proteoglycan secretion were slightly detected at day 14 but clearly distinguished at day 21. Interestingly, a significant positive correlation was found between TGFβi mRNA level at day 0 and mRNA levels of the 3 main chondrocyte markers (Sox9, type IIB collagen, aggrecan) at day 21 [Fig. 4(E)]. We also noticed that TGFβi was up-regulated by TGFβ3 from day 1 to day 3 of pellet culture but not by IGF1 or by another member of the TGFβ family (BMP2) [Fig. 4(F)]. Finally, TGFβi was not a marker of commitment of BM-MSCs to progenitor cells but was specific for chondrogenesis since a significant down-regulation of TGFβi was observed during

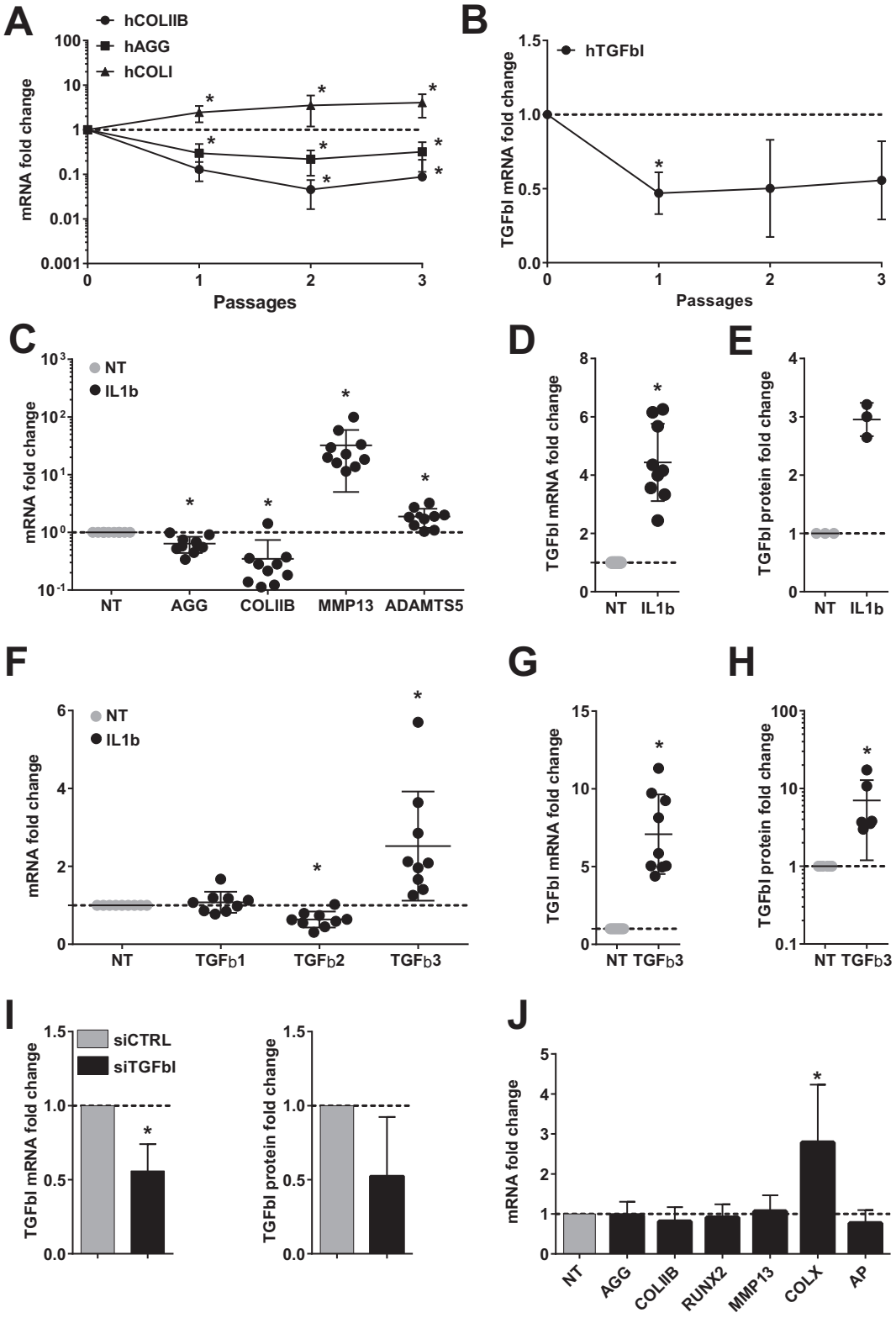

Fig. 3. TGFβi is downregulated during chondrocyte dedifferentiation and upregulated in OA chondrocytes. (A–B) Expression level of type I collagen, type IIB collagen, aggrecan and TGFβi mRNA with passages of primary human OA chondrocytes ($n = 4$ individuals). (C) Expression of chondrocyte markers in not treated (NT) or IL-1β-treated (IL-1β) primary human OA chondrocytes ($n = 9$ individuals). (D–E) Expression of TGFβi in NT or IL-1β-treated (IL-1β) primary human OA chondrocytes at the mRNA ($n = 9$ individuals) or protein ($n = 3$) levels. (F) Expression of TGFβ isoforms in NT or IL-1β-treated (IL-1β) primary human OA chondrocytes at the mRNA level ($n = 9$ individuals. (G–H) Expression of TGFβi in NT or TGFβ3-treated (TGFβ3) primary human OA chondrocytes at the mRNA ($n = 9$ individuals) and protein ($n = 6$) levels. (I) Expression of TGFβi mRNA ($n = 9$ individuals) at day 7 or TGFβi protein ($n = 4$) at day 4 after transfection of primary human OA chondrocytes with siCTRL (grey) or siTGFβi (black). (J) Expression of mature and hypertrophic chondrocyte markers at day 7 after transfection with siCTRL (grey) or siTGFβi (black) ($n = 9$ individuals). Results are expressed as mean ± SD. Statistical analysis used Kruskal–Wallis test followed by Dunn's multiple comparisons test in (A–B) or Wilcoxon signed rank test in (C, E, F, H) or One sample *t*-test in (D, G, I-J) (*$p < 0.05$).

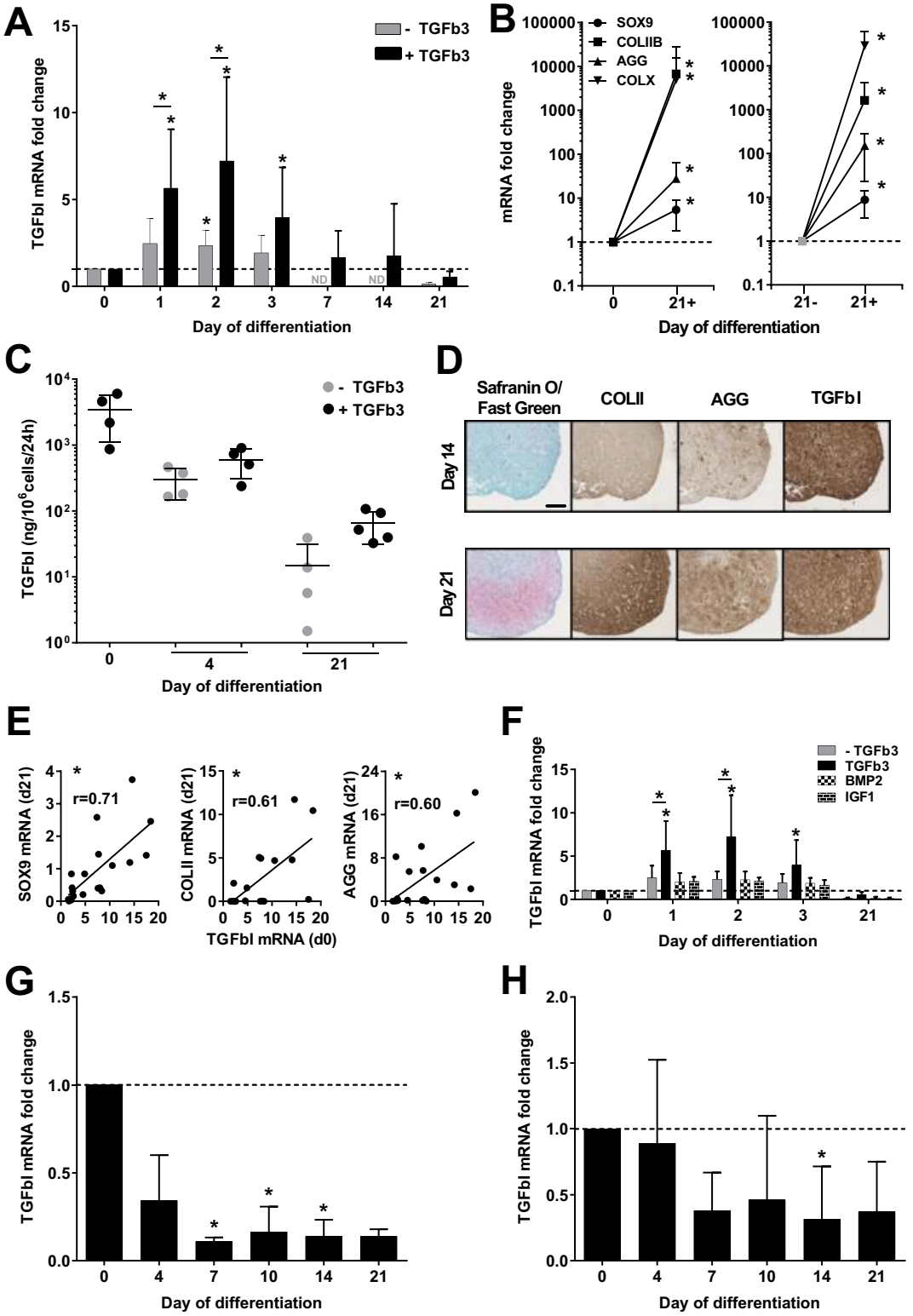

Fig. 4. TGFβi is upregulated during the early stages of chondrogenic differentiation of MSCs. (A) Expression of TGFβi at different time points during the chondrogenic differentiation of human BM-MSCs induced in pellets by TGFβ3 (black) or in absence of TGFβ3 (grey) at the mRNA levels ($n = 11–12$ individuals). (B) Expression of chondrocyte markers in human BM-MSCs induced in pellets by TGFβ3 (21+) in (A) and normalized to day 0 (left panel) or to pellets cultured in absence TGFβ3 (21-; right panel). (C) Quantification of TGFβi protein in supernatants from TGFβ3-induced BM-MSC pellets ($n = 4–5$). (D) Histological analysis of BM-MSC pellets at day 14 and day 21 after chondrogenesis induction. Safranin O Fast Green staining (left panel), type IIB Collagen and aggrecan immunostaining (middle panels, respectively), TGFβi immunostaining (right panel); scale bar is 100 μm. (E) Correlation analysis between the expression level of TGFβi mRNA at day 0 and those of SOX9, type IIB Collagen and Aggrecan at day 21. (F) Expression of TGFβi at different time points during the chondrogenic differentiation of human BM-MSCs induced in pellets by TGFβ3, bone morphogenetic protein (BMP)2 or IGF1 ($n = 3$ individuals). (G) Expression of TGFβi at different time points during the osteogenic differentiation of human BM-MSCs ($n = 4$ individuals). (H) Expression of TGFβi at different time points during the adipogenic differentiation of human BM-MSCs ($n = 6$ individuals). Results are expressed as mean ± SD. Statistical analysis used 2-way ANOVA test followed by Sidak's multiple comparisons test in (A, F) or One sample *t*-test in (B) or Pearson correlation test in (E) or Kruskal–Wallis test followed by Dunn's multiple comparisons test in (C, G, H) ($^*p < 0.05$).

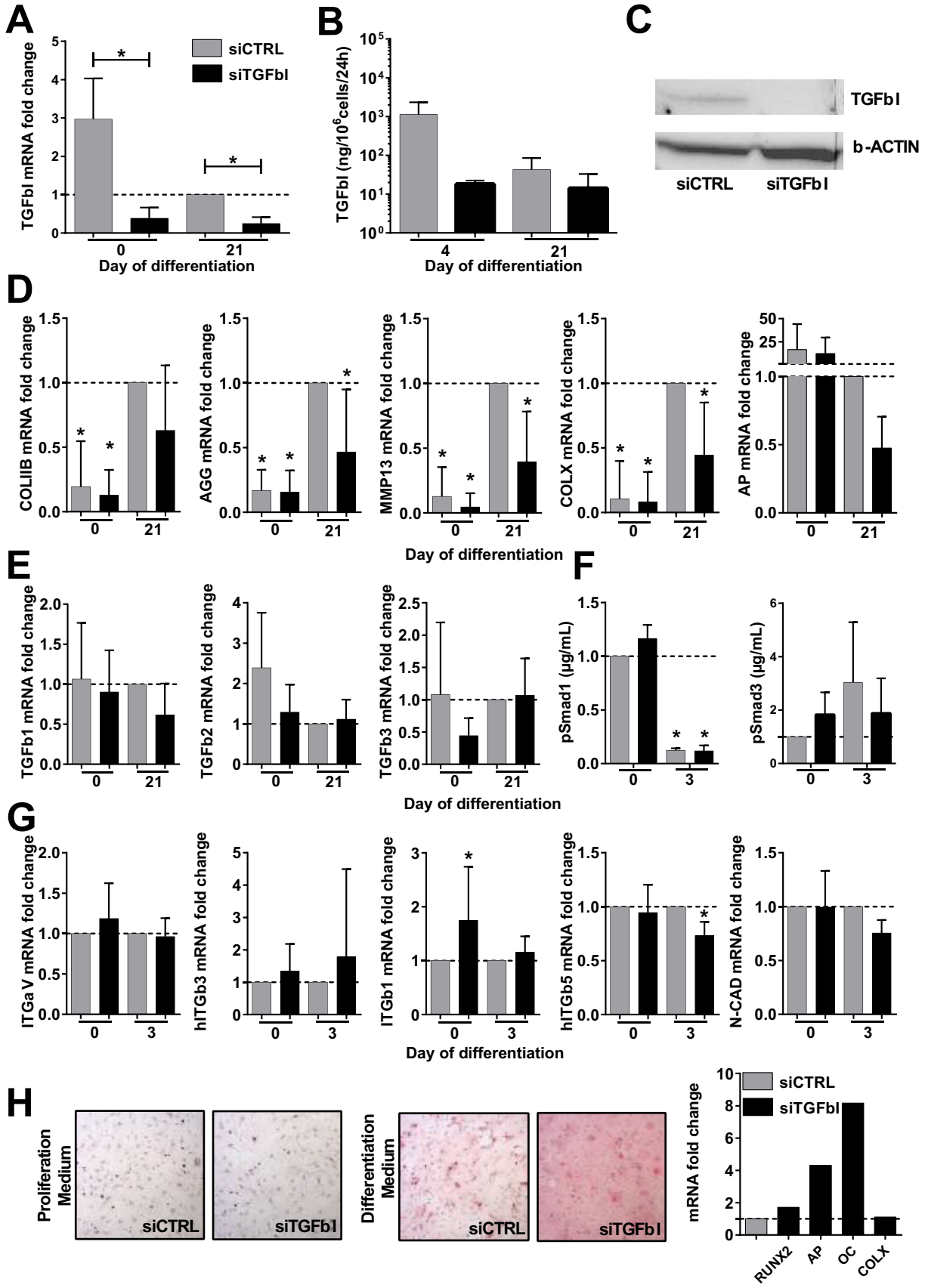

Fig. 5. Down regulation of TGFβi expression in MSCs impairs chondrogenesis. (A) Expression level of TGFβi at day 0 and 21 after specific siRNA transfection (siTGFβi) and chondrogenesis induction at the mRNA level ($n = 8$ individuals) as compared to control (siCTRL). (B) Expression level of TGFβi at day 4 and 21 after specific siRNA transfection (siTGFβi) and chondrogenesis induction at the protein level ($n = 3$). (C) Expression of TGFβi at the protein level at day 4 after chondrogenesis induction of BM-MSCs transfected with

osteoblastogenesis [Fig. 4(G)]. In addition, TGFβi expression remained stable or tended to decreased during adipogenesis [Fig. 4(H)]. Indeed, we demonstrated that TGFβi expression was specifically increased at early stages of chondrogenesis but was decreased upon chondrocyte maturation, indicating that the overall process was associated with a tight regulation of TGFβi.

Critical role of TGFβi in the chondrogenic differentiation of BM-MSCs

To determine whether TGFβi has a cell-autonomous function in chondrogenesis, we used a loss-of-function approach. TGFβi silencing resulted in a reduction of mRNA expression by 89% and 74% at day 0 and day 21, respectively [Fig. 5(A)]. TGFβi protein was reduced by 98% at day 4% and 66% at day 21 [Fig. 5(B)]. The TGFβi protein was undetectable by western blotting at day 4 after siRNA-mediated silencing [Fig. 5(C)]. Down-regulation of TGFβi reduced the expression of mature and hypertrophic chondrocyte markers, type IIB collagen, aggrecan, MMP13, type X collagen and alkaline phosphatase by day 21 [Fig. 5(D)]. The reduction was however not significant for type IIB collagen and alkaline phosphatase. To determine if the down-regulation of TGFβi alters TGFβ signalling, we quantified the expression of TGFβ isoforms or phosphorylated Smad proteins and found no significant change as compared to control MSCs [Fig. 5(E–F)]. However, at early stages, we noticed that siTGFβi-transfected pellets were flat and less dense than control pellets (not shown). We therefore investigated the effect of TGFβi silencing on adhesion molecules known to interact with TGFβi and/or to be important during initial stages of chondrogenesis. Of interest, down-regulation of TGFβi led to a low but significant increase of integrin (ITG)β1 at day 0 and a decrease of ITGβ5 and N-cadherin (N-CAD) at day 3 [Fig. 5(G)]. Finally, the down-regulation of TGFβi increased MSC differentiation towards osteoblasts as shown by increased mineralization and expression of osteoblast markers [Fig. 5(H)]. Altogether, these data indicated a key role of TGFβi at the early stage of chondrogenesis, possibly through the modulation of important adhesion molecules and, at the late stage of chondrocyte hypertrophy, when mineralization occurs. TGFβi may act as an anti-mineralizing factor whose expression is decreased during both osteoblastogenesis and chondrogenesis allowing mineralization to occur.

Discussion

Here, we show that TGFβi regulated the chondrogenic differentiation of adult BM-MSCs. TGFβi revealed a dual role stimulating the early phase of proliferation and differentiation while inhibiting the late phase of hypertrophy and mineralisation. For the first time, we further evidenced that TGFβi was upregulated in articular cartilage and bone of patients with OA suggesting a deregulated function in the disease.

Our study shows that TGFβi was up-regulated in early stages of chondrogenic differentiation of adult BM-MSCs and down-regulated in terminal stages. TGFβi upregulation in early stages is likely occurring upon TGFβ and Smad signaling pathway activation as proposed elsewhere[18]. In mouse, previous studies on embryonic and post-natal limb development reported that TGFβi was abundantly expressed in mesenchymal condensation areas, perichondrium, periosteum, prehypertrophic chondrocytes but absent in hypertrophic chondrocytes[19,20]. Increased expression of TGFβi was also observed at early stages of differentiation of the pre-chondrogenic cell line ATDC5 while it was decreased at late stages[19]. Other studies in chick embryos have shown that TGFβi was expressed in the prehypertrophic zone of vertebral cartilage but not in the proliferative zone. Moreover, addition of TGFβi on growth plate chondrocytes resulted in decreased mineralization[21]. These studies suggested an inhibitory role of TGFβi on mineralization and late chondrogenic differentiation. This is also supported by our data showing its downregulation during the osteoblastogenic differentiation of BM-MSCs. TGFβi was previously shown to inhibit the differentiation of the pre-osteoblastic cell line KS-483 and to be down-regulated in BM-MSCs induced to differentiate into osteoblasts[22,23]. In mature osteoblasts, TGFβi inhibited late differentiation and mineralization[24]. Here we confirmed these data using a RNA interference strategy. Our data and those from the literature indeed support a biphasic function of TGFβi during the chondrogenic differentiation of adult BM-MSCs. During the early stage of chondrogenesis, TGFβi likely regulates the condensation of BM-MSCs, a process involving cell proliferation and cell-to-cell contact, by bridging interactions between cells, collagens and proteoglycans. Our results revealed that TGFβi modulated expression of the adhesion molecules ITGβ1, ITGβ5, N-CAD, which are required at early stages of MSC condensation and chondrogenesis. It may also stimulate the proliferation of BM-MSCs and pre-chondrocytes at this early stage[21]. TGFβi expression is then down-regulated at the terminal stage of chondrogenesis, suggesting an inhibitory role on hypertrophy and mineralization. TGFβi contains gamma-carboxylated glutamic acid residues that are known to bind calcium with high affinity thereby inhibiting mineralization. However, whether TGFβi is subject to extensive gamma-carboxylation is still under debate[25]. In our study, silencing of TGFβi in BM-MSCs before inducing differentiation resulted in the down-regulation of both mature and hypertrophic chondrocyte markers. This evoked an impact primarily on the early condensation phase by reducing and/or delaying the overall differentiation process together with the inhibition of late differentiation.

Role of TGFβi has not been described in OA. Some transcriptomic studies have listed the upregulation of TGFβi at 2 weeks after OA induction in the DMM model or in damaged zones of cartilage from OA patients[26–28]. Proteomic studies have reported the up-regulation of TGFβi in the cartilage from OA patients[29,30]. However to our knowledge, a single study validated an increased expression of TGFβi transcripts in damaged vs non damaged regions from OA joints[27]. Nevertheless, cartilage matrix degradation was reported in TGFβi knock-out mice that was associated with reduced type II collagen levels and higher MMP13 expression in cartilage[31]. Although other features of OA were not investigated, these data suggested that TGFβi deficiency was associated with the degradation of cartilage matrix similar to what occurs in OA. Here, we demonstrate for the first time the up-regulation of TGFβi at the mRNA and protein levels in cartilage and bone from OA patients as compared to healthy subjects. We observed a preferential localization of TGFβi in osteophytes and upper layers of cartilage, in areas

siCTRL or siTGFβi by Western blotting. β-actin is used as loading control. (D) Expression of chondrocyte markers (type IIB collagen, aggrecan, MMP-13, type X collagen, Alkaline phosphatase) at day 0 and 21 of chondrogenesis after transfection of BM-MSCs with siCTRL (grey) or siTGFβi (black) ($n = 8$ individuals). (E) Expression of TGFβ isoforms after specific siRNA transfection (siTGFβi) at day 0 and chondrogenesis induction at day 21 at the mRNA level ($n = 3$). (F) Expression of phosphorylated Smad proteins after specific siRNA transfection (siTGFβi) at day 0 and chondrogenesis induction at day 3 ($n = 3$). (G) Expression of adhesion markers (integrin (ITG)-αV, ITG-β3, ITG-β1, ITG-β5, N-cadherin (CAD)) at day 0 and 3 of chondrogenesis after transfection of BM-MSCs with siCTRL (grey) or siTGFβi (black) ($n = 7$ individuals). (H) Mineralization assessment after 21 days of osteogenic differentiation of human BM-MSCs transfected with siTGFβi or siCTRL by alizarin red S staining. Proliferation medium was used as control (left panels). Expression of osteoblast markers (RUNX2, alkaline phosphatase, osteocalcin) and of type X collagen at day 21 of osteogenesis after transfection of BM-MSCs with siCTRL (grey) or siTGFβi (black) ($n = 1$). Results are expressed as mean ± SD. Statistical analysis used 2-way ANOVA test followed by Sidak's multiple comparisons test in (A-B, D-E) (*$p < 0.05$).

with clusters of chondrocytes, both in OA patients and in mice with OA. This likely indicates that TGFβi upregulation may be involved in the modulation of signalling pathways that are essential for the integrity and repair of cartilage. By reference to the role of TGFβi during chondrogenesis, it may reflect an attempt of chondroprogenitor cells to differentiate and secrete extracellular matrix components to induce cartilage regeneration or repair. This might occur at the early stages of the disease as shown in the DMM model where TGFβi expression was only detected till week 2 after disease induction[26] but could be not the case in samples at the latest stages as used here. This may also reveal an attempt to inhibit mineralisation that occurs in cartilage and sub-chondral bone as a result of TGFβ pathway activation at late stages of OA[32]. Of interest in the model of chondrocyte dedifferentiation, the expression of both TGFβi and other chondrocyte markers decreased while TGFβi silencing did not affect the expression of chondrocyte markers. Only type X collagen was increased after siTGFβi transfection. Expression of type X collagen was previously shown to precede alkaline phosphatase expression and mineralization in chondrocytes[33]. In the pellet model of BM-MSCs differentiated into chondrocytes, alkaline phosphatase secretion was detected at day 21 and type X collagen expression at day 14 but no mineralization was observed[34]. Indeed, our data indicated that TGFβi regulates type X collagen expression and hypertrophic chondrocyte phenotype, which could precede cartilage calcification. Alternatively, TGFβi up-regulation may reflect the deregulation of the TGFβ pathway in OA[3,7,35]. TGFβi up-regulation was seen both in IL1β-induced OA chondrocytes *in vitro* and in cartilage samples from OA patients. Interestingly, TGFβi was down-regulated in infrapatellar fat pad and BM-MSCs recovered from OA patients. This may indicate a lower capacity of BM-MSCs from OA patients to undergo a differentiation program and/or to counteract the inflammatory and pathological environment in OA. MSC-like chondroprogenitor cells with the capacity to migrate into and populate diseased cartilage tissue ex vivo has been reported[36]. Presence of native MSCs in the joint cavity and spontaneous MSC-mediated repair of cartilage has also been described in vivo (for review, see[10]). Evidence that multiple niches of MSCs exist in the joint cavity has put forward the possibility that MSCs with proliferative and unipotent differentiation potential may efficaciously repair microdefects in injured cartilage. Studies on exogenous MSC injection in the joint space have also reported neo-meniscus formation and integration into synovium confirming differentiation of MSCs into joint tissues[37]. Less is known about a deregulated function of MSCs in OA in situ but knocking-out the TGFβ pathway in nestin-positive MSCs in vivo was shown sufficient to attenuate OA[8]. Whether the down-regulation of TGFβi may be predictive of BM-MSCs efficiency to repair cartilage lesions in vivo warrants further investigation.

Altogether, our study identified TGFβi as a novel factor involved in the chondrogenic differentiation of BM-MSCs. Its expression is deregulated in OA joint tissues, suggesting a role in the formation of hypertrophic cartilage and mineralization. Modulating TGFβi expression in BM-MSCs might be of interest to enhance their therapeutic effect in cartilage regenerative medicine.

Author contributions

DN, CJ designed the experiments. Experimental work was performed by MR, MM, GF, YMP, RF, LD, CD, XH, FB, FR. MR, FB, FR, CJ, DN analyzed the data and prepared the manuscript. All authors have contributed to writing or revising the manuscript and final approval.

Conflict of interest

The authors disclose any financial or personal conflict of interest.

Role of the funding source

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Supplementary data

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