CEMIP (KIAA1199) induces a fibrosis-like process in osteoarthritic chondrocytes

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Abstract
CEMIP (for “Cell migration-inducing protein” also called KIAA1199 and Hybrid for “Hyaluronan-binding protein”) expression is increased in cancers and described as a regulator of cell survival, growth and invasion. In rheumatoid arthritis, CEMIP is referred to as an angiogenic marker and participates in hyaluronic acid degradation. In this study, CEMIP expression is investigated in healthy and osteoarthritis (OA) cartilage from human and mouse. Its role in OA physiopathology is deciphered, specifically in chondrocytes proliferation and dedifferentiation and in the extracellular matrix remodeling. To this end, CEMIP, αSMA and types I and III collagen expressions were assessed in human OA and non-OA cartilage. CEMIP expression was also investigated in a mouse OA model. CEMIP expression was studied in vitro using a chondrocyte dedifferentiation model. High-throughput RNA sequencing was performed on chondrocytes after CEMIP silencing. Results showed that CEMIP was overexpressed in human and murine OA cartilage and along chondrocytes dedifferentiation. Most of genes deregulated in CEMIP-depleted cells were involved in cartilage turnover (e.g., collagens), mesenchymal transition and fibrosis. CEMIP regulated β-catenin protein level. Moreover, CEMIP was essential for chondrocytes proliferation and promoted αSMA expression, a fibrosis marker, and TGFβ signaling towards the p-Smad2/3 (Alk5/PAI-1) pathway. Interestingly, CEMIP was induced by the pSmad1/5 (Alk1) pathway. αSMA and type III collagen expressions were overexpressed in human OA cartilage and along chondrocytes dedifferentiation. Finally, CEMIP was co-expressed in situ with αSMA in all OA cartilage layers. In conclusion, CEMIP was sharply overexpressed in human and mouse OA cartilage and along chondrocytes dedifferentiation. CEMIP-regulated transdifferentiation of chondrocytes into “chondro-myo-fibroblasts” expressing α-SMA and type III collagen, two fibrosis markers. Moreover, these “chondro-myo-fibroblasts” were found in OA cartilage but not in healthy cartilage.

Introduction
CEMIP for “Cell migration-inducing protein” (also called KIAA1199 and Hybrid), was originally detected in the inner ear and reported as the cause of nonsyndromic hearing loss.¹² The increase of CEMIP expression was also observed in various cancers,³⁴, and described as a key regulator of cell survival, growth and invasion.³⁶ Moreover, CEMIP expression was also enhanced in human papillomavirus (HPV) infection and characterized as an EGFR-binding protein that promotes EGF-mediated epithelial–mesenchymal transition (EMT).⁶ CEMIP is involved in the Wnt/β-catenin signaling pathway as well as in the enhanced degradation of hyaluronic acid (HA) in dermal fibroblast.⁸ Furthermore, CEMIP is increased in synovial fibroblasts from patients with osteoarthritis (OA) and rheumatoid arthritis (RA) and is detected in the synovium of RA patients and referred to as an angiogenic marker.⁸⁹ Recently, a role of CEMIP in endochondral ossification has been highlighted.¹⁰ Up to now, the role of CEMIP in OA chondrocytes remains unknown.

OA is a degenerative disease affecting the entire joint. It is mainly characterized by cartilage degradation, synovial...

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inflammation, subchondral bone erosion, and osteophyte formation. In OA, anabolic capacity of chondrocytes is largely decreased, thus impairing cartilage repair. In an advanced stage, chondrocytes dedifferentiate into fibrochondrocytes producing abnormal components such as fibronectin fragments. Ultimately, there is a reset of the cell cycle leading to chondrocyte proliferation, hypertrophy and finally cell death by apoptosis. The newly acquired proliferative activity of chondrocytes is often observed by clustering features in OA cartilage. In sum, a catabolic hyperactivity followed by a default of anabolic response and chondrocyte dedifferentiation/proliferation/apoptosis contributes to the degradation of the extracellular cartilage matrix in OA cartilage.

Several chondrocyte phenotypes can be depicted in cartilage according to their collagen expression profile and their localization inside cartilage. Activated chondrocytes synthesize collagen type II, IX, and XI, and are present in the middle zone of cartilage. Hypertrophic chondrocytes rather express collagen type X and are found in the deepest zones of cartilage. Chondrocytes expressing collagen type I and III are located in the upper middle zone of OA cartilage, and could be related to the so-called dedifferentiation process resulting from a modulation of the chondrocyte phenotype to a fibroblast-like phenotype. Recently, we highlighted that in vitro spontaneous dedifferentiated chondrocytes are able to express OA-related protein such as collagen type I, β-catenin, and leptin, in contrast to freshly isolated chondrocytes. Inversely, collagen type II and X and Sox-9 are expressed in freshly isolated chondrocytes but nearly not in dedifferentiated chondrocytes. In human normal cartilage, type II collagen is the main collagen type present while type X collagen is expressed by hypertrophic chondrocytes in OA cartilage.

In the present study, the expression of CEMIP is for the first time investigated in the cartilage of humans and mice. The role of CEMIP in cellular mechanisms leading to OA is also unraveled specifically in cell proliferation, in chondrocyte dedifferentiation and ECM remodeling but also in newly acquired functions as pro-fibrotic mediator.

Material and methods

Subject recruitment

Tissues collection was made in collaboration with the Musculoskeletal Surgery department (CHU Sart-Tilman, ULiege). OA cartilage was obtained from 64 patients (41 females and 23 males) undergoing knee (n = 49) or hip (n = 15) replacement surgery, mean age was 67 (range 22–91) years and mean BMI was 28.35 (range 11.97–41.4) kg/m². Healthy cartilage was obtained from 9 patients suffering from subcapital femoral neck fracture (5 females and 4 males). Mean age was 79 (range 65–90) years and mean BMI was 22.83 (range 17.99–25.71) kg/m².

The research ethics committee of CHU de Liege, Belgium, approved the study and verbal informed consent was obtained to allow research procedures on the tissues collected, as explained in the institutional information booklet written by the hospital and provided to each patient.

Animal experimentation procedures

All experiments were approved by the local ethical committee (#14–1721 University of Liege, Liege, Belgium). 10-weeks-old C57BL6 male mice were used for the study. The DMM and sham surgeries were performed on knees following the Glasson et al. protocol. Briefly, mice were anesthetized using isoflurane and knees were sanitized prior surgery. Surgery was performed using a surgical microscope. A longitudinal incision from the distal patella to the proximal tibial plateau was made. The joint capsule was incised from the distal patella to the proximal tibial plateau to expose the meniscotibial ligament of the medial meniscus. Sham surgery was stop at this point. For destabilization of the medial meniscus (DMM surgery) the medial meniscotibial ligament was sectioned using a fine steel surgical blades (SM65, Swann-Morton, Sheffield, United Kingdom). The joint capsule was closed with resorbable 10–0 VicrylTM (V971G, Ethicon, Somerville, New Jersey, USA) and the skin was closed with resorbable 8–0 VicrylTM (V400G, Ethicon).

All mice received a cocktail of Enrofloxacin (10 mg/kg) and Buprenorphin (0.09 mg/kg), preoperatively two times a day, 1 day before and 3 days post-surgery. Mice were sacrificed 4, 8, 12, 20, or 24 weeks after surgery for time-course analysis. Quantitative analyzes were performed on mice killed 4 and 8 weeks after surgery. OA score was quantified using the modified Pritzker OARSI score.

Immunohistochemistry analysis

IHC analyzes were performed on cartilage tissues obtained from human hip fractures, OA hips and OA knees and from sham and OA mice. Tissues were fixed in 4% paraformaldehyde for 24 h, decalcified in DC2 (Labonord, Templemars, France) for 24 h for human sample and in EDTA for 15 days for mouse samples, dipped in 70% (v/v) ethanol and embedded in paraffin. Immunohistochemistry was performed on cartilage tissue sections (5μm) after dewaxing and chondroitnase unmasking using a primary monoclonal antibody against CEMIP (Santa Cruz, Dallas, Texas, USA) for human cartilage and from Proteintech, Rosemont, Illinois, USA for mice sections), against αSMA (Agilent, Santa Clara, California, USA), against type I collagen (Abcam, Cambridge, Massachusetts, USA), against type III collagen (Abcam) or against type X collagen (Invitrogen, Carlsbad, California, USA) were used. Sections were then incubated
with Envision + System-HRP (Agilent, Santa Clara, California, USA). Peroxidase was detected with Liquid DAB + Substrate Chromogen System (Agilent). Sections were finally counterstained with Carazzi’s Hematoxylin (EMD Millipore, Billerica, Massachusetts, USA). Sections incubated without primary antibody served as controls. Staining was revealed with Nanozoomer Digital Pathology 2.0 HT scanner (Hamamatsu photonics, Hamamatsu, Japan). The number of CEMIP, αSMA, and type I and III collagen-positive cells was counted blindly by two different human operators with the cytamine software (http://www.cytomine.be/)

Cell culture and reagents

Human chondrocytes and synoviocytes were isolated from knee joint as previously described. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 1% l-glutamine (200 mM), 100 units/ml penicillin and 100 μg/ml streptomycin (BioWhittaker, Walkersville, Maryland, USA). Cells were maintained at 37 °C in a 5% CO2 atmosphere. For in vitro dedifferentiated model, freshly isolated chondrocytes were cultured in monolayer during 1, 4, or 14 days. TGFβ (Sigma-Aldrich, Saint Louis, Missouri, USA) 10 ng/ml and SB431542 (Sigma-Aldrich, Saint Louis, Missouri, USA) 10 ng/ml were employed on dedifferentiated chondrocytes.

Lentiviral cell infection

The lentiviral vectors (rLV) were generated with the help of the GIGA-Viral vector Plateform. Briefly, Lenti-X 293T cells (Clontech, Mountain view, California, USA, Plasmid #12260) and a VSV-G encoding vector. Pseudotype formation of murine leukemia virus with the G protein of vesicular stomatitis virus along with CEMIP shRNAs plasmids (Sigma-Aldrich, #1 TRCN0000118791, #2 TRCN0000118787), SMAD1 shRNA plasmid (Sigma-Aldrich, TRCN0000423087) or with a control sequence directed against eGFP (Sigma, Belgium, SHC005) encoding plasmid. 48 and 72h post transfection, viral supernatants were collected, filtrated and concentrated 100× by ultracentrifugation. The lentiviral vectors were then titrated with qPCR Lentivirus Titration (Titer) Kit (ABM, USA, LV900). After chondrocytes transduction with lentiviral vectors, supernatants were harvested and whole-cell lysates or RNA extractions were performed.

Total RNAs from articular chondrocytes were extracted using Nucleospin RNA kit (Macherey-Nagel, Düren, Germany). Reverse transcription was performed with RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Pittsburgh, Pennsylvania, USA) according to the manufacturer’s instructions. cDNA products were then amplified using Real-time reverse transcription quantitative PCR (RT-qPCR) with the KAPA SYBR FAST detection system (Sopachem, Eke, Belgium) and experiments were run on a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany). Data were analyzed using LC480 software release 1.5.0 SP4. For each gene, cDNA dilution curves were generated and used to calculate the individual real-time PCR efficiencies ($E = 10^{(-1/slope)}$). The $2^{-ΔΔCT}$ method was used to calculate the relative gene expression between freshly isolated (calibrator sample) and dedifferentiated chondrocytes or between shEGFP transduced chondrocytes (calibrator sample) and shCEMIP transduced chondrocytes. Input amounts were normalized with the GAPDH endogenous control gene. Primers were purchased from Eurogentec (Seraing, Belgium) or Integrated DNA Technologies (Coralville, Iowa, USA).

RNA-sequencing analysis

The Illumina Truseq stranded mRNA Sample Preparation kit was used to prepare libraries from 500 nanograms of total RNAs according to manufacturer protocol. RNA integrity was verified on the Bioanalyser 2100 with RNA 6000 Nano chips and RIN scores were >9 for all samples. Libraries were validated on the Bioanlyser DNA 1000 chip and quantified by qPCR using the KAPA library quantification kit. All libraries were sequenced on an Illumina NextSeq500 sequencer in a single run generating minimum 26 millions single-end 76 base reads per library. Library quality was successfully confirmed by FastQC and Picard analyses. Reads were aligned to UCSC hg19 human reference genome with Star aligner. Differential expression analysis was performed at the gene level with DESeq2 comparing samples corresponding to the same patient (paired analysis) between shEGFP and either shCEMIP#1 or #224. Genes significantly up or down-regulated (False discovery rate ≤ 10%) for both comparisons were selected for Gene set enrichment analysis (GSEA, http://www.broadinstitute.org/gsea).

Western blot analysis

Protein extracts were prepared by lysing the chondrocytes in Rippa buffer. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore). After blocking, membranes were incubated with anti-CEMIP (Phoenix Pharmaceutical, Burlingame, CA, USA), anti-β-catenin and HSP90 (Santa Cruz Technologies) and anti-β-TRCP, αSMA, SMAD1, p-Smad2, and pSmad1/5 (Cell Signaling, Beverly, Massachusetts, USA). Membranes were then incubated with 1:1000 diluted peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Cell Signaling). Reactions were revealed with the enhanced chemiluminescence detection reagent (ECL kit, Thermo Fisher Scientific, Pittsburgh, Pennsylvania, USA).
### Fig. 1 (See legend on next page.)

#### A

| Sham surgery | DMM surgery |
|--------------|-------------|
| 4 weeks      | 8 weeks     | 12 weeks | 20 weeks | 24 weeks |
| Negative control | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| IHC CEMIP    | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) | ![Image](image10.png) |

#### B

| Sham 4 weeks | DMM 4 weeks |
|--------------|-------------|
| Safranin / Fast Green | ![Image](image11.png) | ![Image](image12.png) |
| Negative control | ![Image](image13.png) | ![Image](image14.png) |
| IHC CEMIP | ![Image](image15.png) | ![Image](image16.png) |

#### C

| Sham 8 weeks | DMM 8 weeks |
|--------------|-------------|
| Safranin / Fast Green | ![Image](image17.png) | ![Image](image18.png) |
| Negative control | ![Image](image19.png) | ![Image](image20.png) |
| IHC CEMIP | ![Image](image21.png) | ![Image](image22.png) |

![Graph](image23.png)

![Graph](image24.png)
Fig. 1 CEMIP expression is increased in mouse OA cartilage. a Representative IHC picture of cartilage section from sham and DMM mice at 4, 8, 12, 20, and 24 weeks stained without (negative control) and with anti-CEMIP antibody. b Representative picture of cartilage section from sham and DMM mice at 4 weeks stained with SafraninO-FastGreen. OA score was significantly increased in DMM mice ($n = 10$) compared to Sham mice ($n = 7$) (up). Representative IHC picture of cartilage section stained without (negative control) and with an anti-CEMIP antibody. Analysis was done on several mice ($n = 9$ for each group): the percentage of CEMIP positive chondrocytes was significantly increased in DMM mice at 4 weeks compared to sham mice (bottom). (Mann–Whitney test: *$p < 0.05$ and **$p < 0.01$). c Representative picture of cartilage section from sham and DMM mice at 8 weeks stained with SafraninO-FastGreen. OA score was significantly increased in DMM mice ($n = 10$) compared to Sham mice ($n = 7$) (up). Representative IHC picture of cartilage section stained without (negative control) and with an anti-CEMIP antibody. Analysis was done on several mice ($n = 9$ for each group): the percentage of CEMIP positive chondrocytes was significantly increased in DMM mice at 8 weeks compared to sham mice (bottom). (Mann–Whitney test: *$p < 0.05$ and **$p < 0.01$)

Fig. 2 CEMIP expression is increased in human OA cartilage and in human dedifferentiated chondrocytes. a Representative IHC picture of cartilage section from hip fracture, OA hip and OA knee stained without (negative control) and with anti-CEMIP antibody (Left). Analysis was done on several patients: the percentage of CEMIP positive cells was significantly increased in OA hip ($n = 4$) and OA knee ($n = 5$) compared to hip fracture ($n = 3$) (right). (ANOVA tests: **$p < 0.01$). b Schematic representation of in vitro chondrocyte dedifferentiation (top left). CEMIP mRNA levels were significantly increased at D14 compared to D1 ($n = 16$) (error bar: SED, Wilcoxon-paired test: $p < 0.001$) (top right). Representative picture of western blot using CEMIP and HSP90-specific antibodies at D1, D4, and D14 is presented (bottom left). Analysis was done on several patients ($n = 4$): western blot quantification illustrating the significant increase of CEMIP/HSP90 expression at D14 compared to D1 and D4 and at D4 compared to D1 (bottom right). (ANOVA paired tests: *$p < 0.05$, **$p < 0.01$)
Fig. 3 (See legend on next page.)
CEMIP expression was also investigated in human cartilage. After quantification, CEMIP expression was significantly increased in OA human hip and knee compared to non-OA human hip cartilage (Fig. 2a). Representative images of IHC negative controls are shown in the figure.

Finally, CEMIP expression was studied, in vitro, in the dedifferentiation model of chondrocytes isolated from OA human knee cartilage. The dedifferentiation process was validated by assessing the increased expression of COL1A1 mRNA and β-catenin protein level as previously described\(^{17}\) (supplementary data 1). CEMIP mRNA and protein levels were detected in freshly isolated chondrocytes and were significantly increased at day 14 (D14) compared to day 1 (D1) (Fig. 2b).

Identification of CEMIP target genes in human dedifferentiated chondrocytes

To extensively identify all signaling pathways that rely on CEMIP expression, the transcriptome of human OA knee dedifferentiated chondrocytes was profiled in the presence or absence of CEMIP expression (using 2 specific shRNAs). 2675 genes were significantly down or up-regulated in CEMIP-depleted cells (with both shRNAs) compared to control cells (\(p < 0.001\)). Among them, 56 had a shrunk Log2 fold change higher than ±1 (Fig. 3a, left). The increased expression of three genes (TGFβ2, MSX2, and CAV2) and the decrease expression of two genes (ANKRD1 and DHC2R4) were confirmed with another set of patients by RT-qPCR (Fig. 3a, right). Among the 56 genes, several were involved in OA cartilage metabolism (Fig. 3b, left). Indeed, there was a decrease of MMP10, COL1A1, COL3A1, COL4A2, COL5A2 and MMP19 and an increase of COL2A1, COL6A1, COL9A1, COL9A3, and COL11A2 mRNA levels in CEMIP-depleted cells, compared to shEGFP control cells (supplementary data 2). On another set of patients, the increase of COL2A1 and COL6A1 and the decrease of MMP10 mRNA level upon CEMIP deficiency were confirmed by RT-qPCR (Fig. 3b, right). The decreased expression of COL1A1 and COL3A1 is further described below and illustrated in Fig. 5a.

**Statistical analysis**

For two groups comparisons, non-parametric Mann–Whitney test or non-parametric Wilcoxon-paired test were used. For multiple comparisons, a log-transform was applied to all variables to normalize their distribution. Data were analyzed by paired or unpaired ANOVA test, followed by Tukey post hoc test. Calculations and graphs (Mean with SEM) were done with GraphPad prism software (version 5.0, La Jolla, California, USA). Results were considered significant at the 5% critical level (\(p < 0.05, **p < 0.01, ***p < 0.001\)).

**Results**

**CEMIP expression in mouse and human OA cartilage and in human chondrocytes**

CEMIP immunohistochemistry (IHC) analysis was performed on the cartilage of sham and OA mice at different stages of the disease. After DMM surgery, mice were first killed at various intervals of time during 24 weeks and CEMIP expression was analyzed every 4 weeks. The expression of CEMIP was increased at 4 and 8 weeks post-surgery compared to sham mice but not in the late stages of the disease (at 12, 20, and 24 weeks post-surgery) (Fig. 1a). IHC quantification was then performed on tibia and femur of DMM mice after 4 and 8 weeks of surgery and CEMIP expression was significantly increased at both time in the cartilage of DMM mice compared to healthy cartilage of sham mice (Fig. 1b, c). SafraninO-FastGreen coloration, OA quantification (OARS1 score), and representative images of IHC negative controls are presented in the figure.
Fig. 4 (See legend on next page.)
These data suggest that CEMIP is involved in the cartilage turnover process.

CEMIP regulated a mesenchymal transition process

To figure out the role of CEMIP in OA chondrocyte regulation, we performed Gene Set Enrichment Analysis (GSEA). Both immune/inflammatory systems and the epithelial–mesenchymal transition (EMT) were impaired upon CEMIP depletion (Fig. 3c). Enrichment plots and heatmap of downregulated genes in dedifferentiated chondrocytes treated with shCEMIP #1 and #2 from the EMT dataset are shown on Fig. 4a, left. Decreased expression of mRNA levels of POSTN (coding for periostin), SERPINE1 (coding for PAI-1, a downstream target of TGF-β signaling), IL32, DKK1 and DKK2 (5 genes implicated in OA pathology)99–102 upon both CEMIP deficiency was confirmed on another set of patients by RT-qPCR (Fig. 4a, right). Moreover, at the protein level, the secretion of both periostin and DKK1 was also decreased in CEMIP-depleted chondrocytes compared to shEGFP control cells (Fig. 4b). As CEMIP regulates genes involved in EMT and participated to the expression of two Wnt/β-catenin inhibitors (DKK1 and DKK2), β-catenin expression, also known as an EMT regulator, was assessed. In the RNA-Seq analysis, no variation of CTNB1 (coding for β-catenin) expression was observed among the different experimental conditions. This was confirmed by RT-qPCR analysis. However, β-catenin protein levels were decreased in CEMIP-depleted cells (Fig. 4c). In parallel, the expression of β-TRCP at mRNA and protein level, a specific E3 ligase of β-catenin, was increased (Fig. 4c). However, there was no modification of Axin-2 expression, another regulator of β-catenin, in CEMIP-depleted cells (data not shown).

CEMIP as a pro-fibrotic mediator

Pro-fibrotic markers were also detected as downregulated genes from the EMT dataset of GSEA analysis after CEMIP depletion. The role of CEMIP in fibrosis was therefore studied accordingly. mRNA levels of fibrosis-related COL1A1, COL3A1, and ACTA2 (coding for the protein smooth muscle α–2 actin, “α-SMA”) were assessed by RT-qPCR. All levels were decreased in dedifferentiated chondrocytes treated with CEMIP shRNAs compared to control EGFP shRNA (Fig. 5a). Moreover, α-SMA expression was also decreased at the protein level in CEMIP-depleted cells (Fig. 5b). Recently, we highlighted that COL1A1 mRNA level was increased in dedifferentiated chondrocytes compared to freshly isolated chondrocytes17 (see also supplementary data 1C). Here, COL3A1 and ACTA2 mRNA as well as its protein α-SMA also increased along chondrocytes dedifferentiation (Fig. 5c). As TGFβ signaling is a well-known inducer of cell proliferation and articular fibrosis14, this pathway was investigated in the regulation of CEMIP. Upon TGFβ stimulation, CEMIP expression was decreased, α-SMA and p-Smad2 were increased (Fig. 5d), and pSmad1/5 was not modified (Supplementary data 3). Moreover, CEMIP deficiency interfered with the induction of α-SMA expression as well as with Smad2 activation upon stimulation with TGFβ but did not modified pSmad1/5 expression. Inhibition of p-Smad2/3 (Alk5/PAI-1) pathway of the TGFβ signaling by using the specific inhibitor SB431542 increased CEMIP expression and as expected, inactivated Smad2 in TGFβ-stimulated chondrocytes (Fig. 5e). Moreover, inhibition of pSmad1/5 (Alk1) pathway of the TGFβ signaling using shRNA directed against SMAD1 led to a decrease of CEMIP expression (Fig. 5f). Finally, the induction of dedifferentiated chondrocytes proliferation by TGFβ was severely impaired upon CEMIP deficiency (Fig. 5g). Of note, we did not detect any effect on dedifferentiated chondrocytes viability after CEMIP depletion (data not shown).

All these data unravel an important role for CEMIP in chondrocytes proliferation and as a pro-fibrotic mediator.

In situ expression of fibrosis markers

To see whether fibrosis could be observed in situ, α-SMA and type I and III collagen expressions in human cartilage were investigated by IHC. IHC quantification
Fig. 5 (See legend on next page.)
demonstrated that α-SMA and type III (but not type I) collagen expressions were significantly increased in OA human hip and knee compared to healthy human hip cartilage (Fig. 6). Representative images of IHC negative controls are shown in Supplementary data 4.

In situ characterization of CEMIP expressing chondrocytes

To better characterize chondrocytes expressing CEMIP in OA cartilage, immunodetection of CEMIP, αSMA (a marker of fibrosis), and type X collagen (an hypertrophic chondrocyte marker) were performed on serial tissue sections provided from the same OA hip or knee sample. CEMIP and αSMA were both expressed in the upper and the deep zone of OA cartilage section. By contrast, type X collagen was rather expressed in the deep and the calcified zone (Fig. 7). OA chondrocytes expressing CEMIP and αSMA in the upper zone appeared elongated while OA chondrocytes expressing CEMIP, αSMA, and type X collagen in the deep zone appeared round and in clusters.

Discussion

CEMIP is a protein of emerging interest. Many studies have shown that it plays a critical role in cancer cell survival, proliferation and migration, three critical processes involved in cancer development and dissemination. Interestingly, Yoshida and co-workers have highlighted that CEMIP was implicated in hyaluronic acid turnover and was increased in synovial fibroblasts from patients with OA and RA. Using KO mice, Shimoda et al. have shown that CEMIP controls the endochondral ossification.

In this work, we pointed out that CEMIP was overexpressed in human and mouse OA cartilage compared to healthy cartilage. Interestingly, in mouse OA cartilage, we observed that CEMIP overexpression decreased in the late stages of the disease. These data suggest that CEMIP could have a significant role in the molecular disturbance encountered at an early stage of the disease. In accordance, very recently Shimizu et al., showed that CEMIP expression correlated with Mankin score of OA patients.

Molecular mechanisms that lead to OA are still largely unknown. However, it is likely that aberrant activity of OA chondrocytes leads to cartilage ECM remodeling. Our results indicate that CEMIP induced a variation in the expression panel of collagen types. Indeed, human chondrocytes expressing CEMIP produced more type I, III, IV and V collagen mRNAs and less type II, VI, IX, and XI than chondrocytes where CEMIP expression is abrogated. Modulation of collagen expression pattern by chondrocytes occurred in line with a transition to a fibroblastic cell shape called “dedifferentiation”.

Recently, we highlighted that dedifferentiated chondrocytes were able to express type I collagen, and inversely that type II and X collagen were expressed by freshly isolated chondrocytes but nearly not by dedifferentiated chondrocytes. Here, we show that CEMIP is lightly expressed in freshly isolated OA chondrocytes expressing type X collagen, but is increased at an early stage of chondrocyte dedifferentiation (day 4) and before any other dedifferentiated chondrocytes markers (type I and III collagen, β-Catenin, and αSMA). All these data suggest...
that CEMIP could be an inducer of human chondrocyte dedifferentiation process leading to a fibroblastic-like phenotype.

This finding is supported by the role of CEMIP in the regulation of genes involved in mesenchymal transition process. Indeed, we showed that CEMIP depletion induced a modulation of genes belonging to the Hallmark_Epithelial_Mesenchymal_Transition pathway (GSEA analysis) with, among others, a decreased periostin secretion (and POSTN mRNA). As a ligand of αvβ3 and αvβ5 integrins, periostin fostered adhesion and migration of epithelial cells37. Moreover, periostin is upregulated in OA cartilage and intensifies inflammation and metalloproteinases production38,39. We also showed that CEMIP regulated SERPINE1 gene coding for PAI-1. PAI-1 is a downstream target of the TGFβ signaling (p-Smad2/3 (Alk5-PAI-1))30. It also has an important role in fibrinolysis and thrombosis40. Our results are in accordance with the literature in which the role of CEMIP in the EMT process is observed in different cell types6,41,42. We also found that CEMIP modulated the Wnt/β-catenin signaling, a well-known regulator of EMT, in human chondrocytes. The Wnt/β-catenin pathway contributes to OA pathogenesis43,44. We previously observed that β-catenin accumulated along chondrocytes dedifferentiation leading to leptin production17. Here, we detected that CEMIP expression also increased with chondrocytes dedifferentiation. Besides, we observed a decrease of β-catenin protein level (but not mRNA) in CEMIP-depleted chondrocytes. This could indicate that CEMIP prevented β-catenin degradation by reducing β-TRCP expression (a β-catenin-specific E3 ligase).
results are in line with findings on gastric and colon cancer cells in which CEMIP activate the Wnt/β-catenin pathway\textsuperscript{3,41}. Inversely, β-catenin silencing induces a decrease of CEMIP expression leading to a positive feedback loop in the regulation of β-catenin expression\textsuperscript{7}.

β-catenin pathway also appears to be implicated in fibrosis\textsuperscript{45,46}. Growing evidences suggest a cross-talk between Wnt/β-catenin and TGFβ pathway inducing a pro-fibrotic process\textsuperscript{45,47}. In this work, we observed that CEMIP induced a fibrosis-like process in OA chondrocyte. Indeed, additionally to its role in the Wnt/β-catenin pathway activation and in the induction of types I and III collagen expression, we showed that αSMA expression was sharply diminished with the decrease of CEMIP level even upon TGFβ induction. Furthermore, the abrogation of CEMIP expression led to the decrease of p-Smad2 expression induced by TGFβ and the decrease of SERPINE1 gene (coding for PAI-1) expression. This indicated that CEMIP promoted the TGFβ signaling towards its pro-fibrotic side (p-Smad2/3 (Alk5/PAI-1)). Moreover, TGFβ stimulation led to the activation of p-Smad2/3 (Alk5/PAI-1) pathway but not of pSmad1/5 (Alk1) pathway, and to the inhibition of CEMIP expression. Inactivation of p-Smad2/3 (Alk5/PAI-1) pathway by SB431542 revealed an increase of CEMIP level even after TGFβ stimulation. Additionally, the inhibition of SMAD1 leading to the inactivation of pSmad1/5 (Alk1) pathway induced a decrease of CEMIP expression. These data
Fig. 8 Schematic representation of CEMIP-, αSMA-, and collagen-type expression. CEMIP, αSMA, and type III collagen are overexpressed in OA cartilage compared to non-OA cartilage (upper panel). CEMIP, αSMA, and types I and III collagen are overexpressed in dedifferentiated chondrocytes compared to freshly isolated chondrocytes. All these decreased when CEMIP expression is abolished. Type II collagen expression decreased along chondrocytes dedifferentiation and increased while CEMIP expression is abolished (lower panel).
revealed that CEMIP is inhibited by the activation of p-Smad2/3 (Alk5/PAI-1) triggered by TGFβ whereas activated by the pSmad1/5 (Alk1) pathway. Interestingly, we previously demonstrated that pSmad1/5 (Alk1) pathway is activated in dedifferentiated chondrocytes, conversely to p-Smad2/3 (Alk5/PAI-1) which is activated in freshly isolated chondrocytes17. This suggests that the increase expression of CEMIP along chondrocytes dedifferentiation could be induced by pSmad1/5 (Alk1) pathway activation. Of note, the activation of fibrosis pathway, p-Smad2/3(Alk5/PAI-1), by CEMIP seemed to be independent of TGFβ. Indeed, CEMIP deficiency allowed the increase expression of TGFβ1 and 2 mRNA levels (RNA-Seq analysis) suggesting that CEMIP is a downregulator of TGFβ. Finally, we demonstrated that CEMIP is essential for chondrocytes proliferation (but not viability) triggered by TGFβ. This sustains the hypothesis that CEMIP is a cell proliferation inducer5,6.

In the same way as CEMIP, we observed that COL3A1 and ACTA2 (as well as αSMA protein) were not expressed in freshly isolated chondrocytes but largely expressed in dedifferentiated chondrocytes after 14 days in culture. This suggests that dedifferentiated chondrocytes shared expression pattern with activated myofibroblasts (namely “chondro-myo-fibroblasts”). Kinner et al.48 have also observed the lack of αSMA expression in freshly isolated chondrocytes and have observed an increase of expression with cell passage number. Moreover, like CEMIP, αSMA, and type III collagen were weakly expressed in healthy tissue and largely expressed in OA cartilage. Recently, Hosseininia and co-worker have underscored the increase of expression of type III collagen in OA hip cartilage compared to healthy hip cartilage50. Here, we validated that type III collagen was significantly increased in OA hip cartilage but also in OA knee cartilage compared to non-OA cartilage. Moreover, we showed that type I collagen is expressed in healthy cartilage and that its expression remained constant in OA hip and knee. This differs from the results of Aigner and colleagues who did not detect mRNA and a very low level of protein of type I collagen in the upper layer of cartilage50.

Both in vitro and in situ, CEMIP expression seems to be concomitantly expressed with the fibrosis marker αSMA. Indeed, in the same way than αSMA, CEMIP expression increased along chondrocyte dedifferentiation and in OA cartilage compared to healthy cartilage. However, in OA articular cartilage, chondrocytes are reported to express type X collagen, a marker of hypertrophic chondrocytes51. In situ characterization of CEMIP expressing chondrocytes showed that CEMIP was co-expressed with αSMA in all layers of OA cartilage and co-expressed with type X collagen only in the deep zone. This means that different chondrocyte phenotypes coexist in situ in osteoarthritic cartilage: on one hand, elongated chondrocytes expressing CEMIP and αSMA are in the upper part of OA cartilage, and on the other hand clusters of chondrocytes expressing CEMIP, αSMA, and type X collagen are in the lower part of OA cartilage. These results strengthen our postulate that CEMIP regulated chondro-myo-fibroblasts expressing αSMA. The role of CEMIP in hypertrophic chondrocytes is suggested by its co-expression with type X collagen. Nevertheless these hypertrophic chondrocytes expressed concomitantly αSMA. In addition, type X collagen was present in freshly isolated chondrocyte but not in dedifferentiated chondrocytes, conversely to CEMIP17.

Fibrosis is an unresolved wound-healing process characterized by excessive deposition of extracellular matrix components and cell proliferation leading to disrupted tissue function52. The unresolved wound healing is ensured by myofibroblasts expressing αSMA, which gives them contractility properties. In OA, it is well known that fibrosis occurs in synovium53. Moreover, macroscopic features of fibrosis have been identified in the cartilage of DMM mice54. We showed that αSMA, type III collagen (two fibrosis markers) and CEMIP (that we unraveled as a fibrosis regulator) were weakly expressed in healthy tissue and largely expressed in OA cartilage from hip and knee, which suggests that unresolved wound healing is occurring in OA cartilage and that chondrocytes have acquired new functions shared by activated myofibroblasts (“chondro-myo-fibroblasts”). It is likely that cartilage fibrosis occur in parallel with chondrocytes proliferation encountered in early stages of the pathology.

Our findings suggest that CEMIP is sharply increased in OA cartilage and along chondrocytes dedifferentiation. In vitro, CEMIP regulated proliferation and transdifferentiation of chondrocytes into “chondro-myo-fibroblasts” expressing α-SMA and COL3A1, two fibrosis markers. Moreover, these “chondro-myo-fibroblasts” (expressing CEMIP, α-SMA and type III collagen) are found in situ in OA cartilage but not in healthy cartilage revealing a fibrosis process during OA (Fig. 8). As CEMIP seems to be an early actor in the deregulation leading to OA, deciphering its precise role in cartilage remodeling using a KO mice model should be considered. Moreover, it could be interesting to investigate the role of CEMIP in other arthofibrosis conditions known and other pathologies for which, in the long term, fibrosis leads to the impairing of proper organ function.

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