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# p16<sup>INK4a</sup> and its regulator miR-24 link senescence and chondrocyte terminal differentiation-associated matrix remodeling in osteoarthritis

Didier Philipot<sup>1,2</sup>, David Guérit<sup>1,2</sup>, Daniela Platano<sup>3</sup>, Paul Chuchana<sup>1,2</sup>, Eleonora Olivotto<sup>3</sup>, Francisco Espinoza<sup>1,2</sup>, Anne Dorandeu<sup>4</sup>, Yves-Marie Pers<sup>1,2,5</sup>, Jacques Piette<sup>6</sup>, Rosa Maria Borzi<sup>3†</sup>, Christian Jorgensen<sup>1,2,5†</sup>, Danièle Noel<sup>1,2†</sup> and Jean-Marc Brondello<sup>1,2\*†</sup>

## Abstract

**Introduction:** Recent evidence suggests that tissue accumulation of senescent p16<sup>INK4a</sup>-positive cells during the life span would be deleterious for tissue functions and could be the consequence of inherent age-associated disorders. Osteoarthritis (OA) is characterized by the accumulation of chondrocytes expressing p16<sup>INK4a</sup> and markers of the senescence-associated secretory phenotype (SASP), including the matrix remodeling metalloproteases MMP1/MMP13 and pro-inflammatory cytokines interleukin-8 (IL-8) and IL-6. Here, we evaluated the role of p16<sup>INK4a</sup> in the OA-induced SASP and its regulation by microRNAs (miRs).

**Methods:** We used IL-1-beta-treated primary OA chondrocytes cultured in three-dimensional setting or mesenchymal stem cells differentiated into chondrocyte to follow p16<sup>INK4a</sup> expression. By transient transfection experiments and the use of knockout mice, we validate p16<sup>INK4a</sup> function in chondrocytes and its regulation by one miR identified by means of a genome-wide miR-array analysis.

**Results:** p16<sup>INK4a</sup> is induced upon IL-1-beta treatment and also during *in vitro* chondrogenesis. In the mouse model, *Ink4a* locus favors *in vivo* the proportion of terminally differentiated chondrocytes. When overexpressed in chondrocytes, p16<sup>INK4a</sup> is sufficient to induce the production of the two matrix remodeling enzymes, MMP1 and MMP13, thus linking senescence with OA pathogenesis and bone development. We identified miR-24 as a negative regulator of p16<sup>INK4a</sup>. Accordingly, p16<sup>INK4a</sup> expression increased while miR-24 level was repressed upon IL-1-beta addition, in OA cartilage and during *in vitro* terminal chondrogenesis.

**Conclusions:** We disclosed herein a new role of the senescence marker p16<sup>INK4a</sup> and its regulation by miR-24 during OA and terminal chondrogenesis.

## Introduction

Tissue loss of function and integrity are inherent to aging and age-related disease onset. Because senescent p16<sup>INK4a</sup>-positive cells accumulate within numerous tissues throughout life [1], recent strong evidence suggested that these cells contribute to tissue degeneration by sustaining chronic inflammation and extracellular matrix remodeling [2]. Indeed, p16<sup>INK4a</sup>-positive cells exhibit a specific secretome

called SASP (senescence-associated secretory phenotype) including pro-inflammatory cytokines (such as interleukin-6 (IL-6), IL-8, and IL-1 $\beta$ ) and matrix remodeling regulatory metalloproteases (such as MMP1 and MMP13) [2]. Remarkably, specific conditional elimination of these cells in a premature aging murine model has revealed their essential role in the onset of several age-related diseases [3]. Interestingly, *Ink4a*, which encodes an archetypical cyclin-dependent inhibitor (CKI) associated with senescence, is also known to participate in terminal differentiation onset of several cellular lineages [4,5].

Osteoarthritis (OA) is a chronic degenerative disease characterized by progressive cartilage erosion and lesions

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in subchondral bone as well as in other joint tissues [6]. The anabolic function of chondrocytes, the major cellular component of articular cartilage, decreases with disease progression. This loss of function is associated mainly with an accumulation of p16<sup>INK4a</sup>-positive articular chondrocytes [7] harboring short telomeres [8] but also features of hypertrophic/terminally differentiated cells [9,10]. The latter is normally associated with endochondral ossification process during bone development [9,10]. Although OA regulatory mechanisms remain under investigation, it's now believed that articular mature chondrocytes in response to either inflammatory cytokines or aberrant developmental signals exemplified by Notch activation [10] are producing matrix remodeling enzymes (MMP1 and MMP13) and inflammatory cytokines (IL-8 and IL-6) [11,12]. All of these factors are deleterious for cartilage integrity. Therefore, OA is a multi-factorial complex disease in which articular chondrocytes exhibit characteristics of senescent-like and hypertrophic-like cells secreting SASP factors leading to impaired anabolic capacities [7]. Moreover, a reduction of p16<sup>INK4a</sup> expression by RNA interference in OA chondrocytes was shown to lead to their functional rescue [13]. These results demonstrate a deleterious role for this senescence-associated CKI on articular chondrocytes. It remains to be understood how p16<sup>INK4a</sup> increased expression occurs and could contribute to OA progression.

MicroRNAs (miRs) are small non-coding RNAs that are part of the miRNA-induced silencing complex (RISC) [14] and are involved in the regulation of gene expression. MiRs are key regulators of numerous physiological processes that are deregulated in pathological conditions [15], in particular OA [16,17]. Among miRs identified in OA, miR-22 targets BMP7, a factor inducing chondrocyte terminal differentiation [18]; miR-140 targets HDAC4, a histone deacetylase inducer of chondrocyte terminal differentiation [19,20]; and miR-27b targets MMP13, a key remodeling enzyme in hypertrophic terminally differentiated chondrocyte [21]. So far, none of these miRs has been found to be regulators of p16<sup>INK4a</sup>-associated senescent phenotypes during OA progression.

In this study, we demonstrate that p16<sup>INK4a</sup> accumulates not only in response to inflammatory stimuli but also during chondrogenesis. *Ink4a* participates in cell cycle exit required for chondrocyte terminal differentiation onset during endochondral ossification. Moreover, p16<sup>INK4a</sup> overexpression is sufficient to trigger MMP1 and MMP13 production in mature chondrocytes. By genome-wide microRNA array, we identify miR-24 as a regulator of p16<sup>INK4a</sup> in chondrocytes. As expected, miR-24 is repressed in IL-1 $\beta$ -treated chondrocytes, in cartilages of patients with OA but also at the end of chondrogenesis while p16<sup>INK4a</sup> accumulates. Finally, downregulation of miR-24 by an antagomir approach in primary chondrocytes leads to an increase in p16<sup>INK4a</sup> expression and

MMP1 secretion. Taken together, these data reveal for the first time that the senescent marker p16<sup>INK4a</sup> and its epigenetic regulator miR-24 are reciprocally involved in both OA and bone developmental-associated matrix remodeling secretomes.

## Materials and methods

### Cell culture, chondrocytes, mesenchymal stem cells, cartilage samples, and mouse models

Primary human chondrocytes were isolated from cartilage of 11 OA patients (mean age of 62 years) undergoing knee arthroplasty after informed written consent from patients and approval by the local and national ethics committee ('Cellule de bioéthique de la direction générale pour la recherche et innovation, Ministère de l'Enseignement Supérieur et de la Recherche'; registration number DC-2009-1052) were obtained, as described previously [22]. Cartilages from six healthy adult subjects (mean age of 53 years) were forensic waste from legal medicine with no need of informed consent after consultation with the national ethics committee and in strict agreement with French legislation. OA primary chondrocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum as described [23]. Primary OA chondrocytes ( $2.5 \times 10^5$  cells) were pelleted by centrifugation in 15-mL conical tubes, placed in three-dimensional (3D) setting for 7 days in chondrogenic medium—DMEM supplemented with 0.1  $\mu$ M dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 1 mM pyruvate sodium (Invitrogen, Paisley, UK), 0.17 mM ascorbic acid (Sigma-Aldrich), 0.35 mM Proline (Sigma-Aldrich), 1% Insulin Transferin Selenium (Lonza, Basel, Switzerland), 2 mM L-glutamine (Lonza), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (Lonza)—supplemented with transforming growth factor-beta 3 (TGF- $\beta$ 3) at 10 ng/mL (R&D Systems, Minneapolis, MN, USA). Treatment with recombinant human IL-1 $\beta$  at 10 ng/mL (R&D Systems) was applied for the first 5 days. Wild-type or *ink4a* knockout mice (1 month old) were obtained as reported [24]. Mice were housed and cared for in accordance with the laboratory animal care guidelines. Approval was obtained from the regional ethics committee on animal experimentation before initiation of the study (approval CEEA-LR-10042). Experiments were performed in accordance with the regional ethics committee on animal research and care.

### MicroRNA array analysis

Total RNA was extracted from chondrocytes in micropellet treated (or not) with IL-1 $\beta$  by using a miRvana isolation kit (Ambion, Carlsbad, CA, USA). MiRNA expression profiling was performed by using Miltenyi (Bergisch Gladbach, Germany) microarray facilities. Labeling and hybridization were performed in accordance with the protocol of the manufacturer. Raw data were normalized and additional

data analysis was performed as described previously [25]. Microarray data are available in the ArrayExpress database [26] under accession number E-MTAB-2229.

#### **Reverse transcription, microRNA reverse transcription, and quantitative polymerase chain reaction**

One microgram of Trizol-extracted total RNAs including microRNAs from the different samples were poly(A)-tailed with poly(A) polymerase (NEB M0276L). Then the polyadenylated RNA samples were reverse-transcribed as previously described using 50 units M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and either random primers or dTmiR adapter [27]. For microRNA and mRNA quantitative analysis, cDNA was mixed with Sybr Green Master Mix (Roche Diagnostics, Indianapolis, IN, USA) in 96-well plates containing specific primers for hsa-miR-24 (universal reverse + specific primer), interest genes or the ribosomal subunit protein-9, housekeeping gene (*hRSP9*). Quantitative polymerase chain reaction (qPCR) conditions as described [27] used the following primer-probe combinations: for *hRSP9* sense 5'-GATTACATCCTGGGCCTGAA antisense 5'-ATGAAGGACGGGATGTTTAC; for Aggrecan (*hACAN*) sense 5'-TCGAGGACAGCGAGGCC anti-sense 5'-TCGAGGGTGTAGCGTGTAGAGA; for *hCOL2A1*, variant 2 (*hCol2A1*) sense 5'-CAGACGCTGGTGCTGCT anti-sense 5'-TCCTGGTTGCCGGA CAT; For *hMMP13* sense 5'-TAAGGAGCATGGCGACTTCT anti-sense 5'-GTCTGGCGTTTTTGGATGTT; for *hp16<sup>INK4a</sup>* sense 5'-GAAGGTCCCTCAGACATCCCC anti-sense 5'-CCCTGTAGGACCTTCGGTGAC; for hsa-miR-24 sense 5'-TGGCTCAGTTCAGCAGGAACAG Universal Reverse 5'-GCGAGCACAGAATTATACGACT.

#### **cDNA constructs and luciferase reporter assay**

Plasmids encoding for miR-24-2 promoter (-2041 base-pair) Luciferase and CMV  $\beta$ -galactosidase were provided by Charles Lecellier [28]. Empty vector or *p16<sup>INK4a</sup>* encoding vector were purchased from Addgene [29]. For promoter activity assay, OA human primary chondrocytes were transfected at day 0, placed in pellet culture conditions and treated with IL-1 $\beta$  during 48 hours. Cells were then lysed according to the dual luciferase/ $\beta$ gal kit (Promega, Charbonnières-les-Bains, France). Firefly Luciferase and  $\beta$ -galactosidase activities were detected using specific substrates with MultiScan FC (Thermo Scientific, Loughborough, UK). Luciferase activity was normalized to  $\beta$ -galactosidase activity.

#### **In vitro differentiation of human bone marrow-mesenchymal stromal cells to chondrocytes**

Human bone marrow-mesenchymal stromal cell (hBM-MSC) culture were established from bone marrow of patients undergoing Hip replacement surgery, after patient informed written consent and approval by the local and

national ethics committee ("Cellule de bioéthique de la direction générale pour la recherche et innovation, Ministère de l'Enseignement supérieur et de la Recherche"; registration number DC-2009-1052). Human mesenchymal stromal cells (hMSCs) were isolated and amplified by using a complete alpha-minimum essential medium supplemented with 10% fetal bovine serum + 1 ng/mL of basic fibroblast growth factor. hBM-MSCs were positive for CD44, CD73, CD90, and CD105 but negative for CD14, CD34, and CD45. Chondrogenic differentiation of BM-MSCs was induced by 21-day culture in micropellet [30]. Chondrogenesis was monitored by measuring the expression of chondrocyte-specific markers by reverse transcription-qPCR (RT-qPCR) as described [30].

#### **Transfections**

Human chondrocytes ( $75 \times 10^4$ ) were transfected with 15  $\mu$ g of plasmid for 24 hours by using Transit-LT1 Reagent (Euromedex, Souffelweyersheim, France). Chondrocytes were transfected with 100 nM of AntagomiR control or AntagomiR-24 (purchased from Ambion) by using oligofectamine (Invitrogen, USA). After transfection, cells were trypsinized and pelleted in chondrogenic medium and cultured for 7 days.

#### **Western Blot and enzyme-linked immunosorbent assay**

For Western blotting, chondrocytes in pellet cultures were lysed in RIPA-Benzodase buffer [22]. After the addition of the lysis buffer, the samples were left on ice for 15 minutes with vortexing every 5 minutes for 10 seconds. Lysate protein samples were then sonicated for 5 minutes, followed by centrifugation at 7000 *g* for 15 minutes. The protein quantity loaded on Western blot gel corresponded to  $25 \times 10^4$  cells. Primary antibodies and dilutions were anti-CDKN2A/p16INK4a (ab54210; Abcam; 1:1,000) and anti- $\beta$  actin (Sigma-Aldrich A228; 1:8,000). Secondary antibody used for Western blot analysis was goat anti-mouse IgG HRP conjugate (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; 115-035-003; 1:80,000). Enzyme-linked immunosorbent assays (ELISAs) were performed by using kits (IL-6, IL-8, pro-MMP13, and MMP1) from R&D Systems on supernatants stored at -20°C until analysis. Data were normalized and expressed as picograms per milliliter.

#### **Immunohistochemistry and staining**

Samples were fixed in 3.7% paraformaldehyde for 24 hours, washed in phosphate-buffered saline (PBS), and processed for routine histology. Paraffin-embedded sample sections (5  $\mu$ m) were rehydrated through a gradient of xylene and ethanol. Samples were first incubated for 20 minutes at room temperature with pepsin solution (EmergoEurope, The Hague, The Netherlands) for antigen retrieval. Endogenous peroxidase blocking was done

with 1% H<sub>2</sub>O<sub>2</sub> for 20 minutes at room temperature. Samples were pre-incubated with blocking solution (PBS + 10% goat serum + 0.1% Triton) for 30 minutes at room temperature. Endogenous biotins were blocked by using a Streptavidin/Biotin blocking kit (Vector SP-2002) for 30 minutes. Primary antibody anti-CDKN2A/p16<sup>INK4a</sup> monoclonal mouse antibody (1:200; Abcam ab54210) was incubated for 72 hours at 4°C. Incubation with Biotin-coupled secondary antibody, IgG (1:200; ABC kit Vector PK6100) was done for 1 hour at room temperature. Incubation with Avidin/Biotin complex (ABC kit Vector PK6100) was done for 30 minutes at room temperature. Immunolocalized antigens were detected by means of a DAB revelation kit (Sigma-Aldrich). Safranin-O staining was performed as described [31]. Quantification of proliferating cell nuclear antigen (PCNA)-positive or PCNA-negative hypertrophic chondrocytes was performed on four different sections of long bones of four mice of each genotype by using ImageJ software (D-0426) in accordance with the instructions of the manufacturer.

#### Statistical analysis

Experiments were performed with at least three independent individual chondrocyte or MSC samples. Comparisons of two conditions were done by using a paired Student *t* test. Unpaired Mann-Whitney test was applied for cartilage samples by using GraphPad Prism Software (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered significant when *P* values were less than 0.05.

## Results and discussion

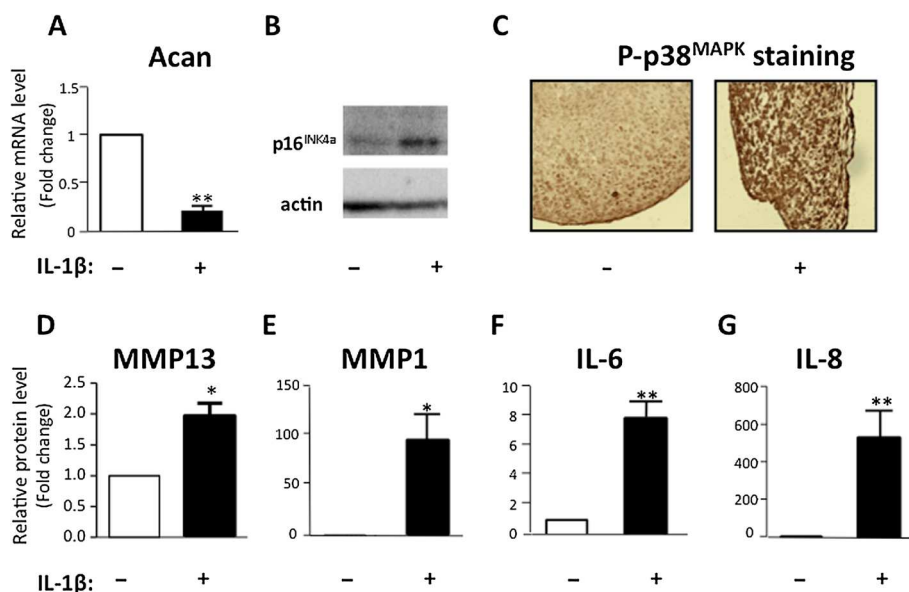
### p16<sup>INK4a</sup> accumulates with other senescence-associated secretory phenotype factors in interleukin-1-beta-treated mature chondrocytes

We first wanted to establish an *in vitro* model based on mature chondrocytes, mimicking the senescence-like phenotypes found in OA cartilage. This model should associate a 3D setting reproducing that of chondrocytes within the tissue, expression of senescence markers such as p16<sup>INK4a</sup>, and production of SASP factors. We therefore isolated primary chondrocytes from OA cartilage patients and that were placed in pellet culture conditions to maintain/reinduce the chondrocyte phenotype. We used IL-1 $\beta$  as one of the major cytokine inducers of cartilage degradation in OA [32] to induce senescence-associated phenotype. We observed that, in response to chronic IL-1 $\beta$  treatment for 5 days, mature chondrocytes lose (as expected) the expression of specific differentiation markers such as Aggrecan (Figure 1A) [33], concomitantly with p16<sup>INK4a</sup> accumulation (Figure 1B), phospho-active form of p38<sup>MAPK</sup> (Figure 1C), and production of reactive oxygen species (data not shown), hallmarks of senescence-associated signaling pathways [34]. Furthermore, as expected, IL-1 $\beta$ -treated mature chondrocytes significantly produce senescence-associated secretory factors such as

MMP1 and MMP13, two markers normally associated with chondrocyte terminal differentiation onset and IL-6 and IL-8, two pro-inflammatory cytokines (Figure 1D-G). Therefore, this 3D *in vitro* model recapitulates the p16<sup>INK4a</sup>-associated secretory phenotype characterizing senescent-like chondrocytes found in OA cartilage.

### The senescence marker p16<sup>INK4a</sup> is expressed during *in vitro* chondrogenesis and participates in the terminal differentiation-dependent cell cycle exit during endochondral ossification

Besides inflammatory cytokines, aberrant chondrogenic differentiation signals can also promote OA features. For instance, Notch activation and HIF-2 $\alpha$  transcription factor, both inducers of OA, are also central in controlling chondrocyte terminal differentiation onset during *in vitro* chondrogenesis and *in vivo* endochondral ossification [9,10]. Thus, we next wanted to determine whether p16<sup>INK4a</sup> could also be part of a normal *in vitro* and *in vivo* chondrogenic differentiation process as has been reported for Notch or HIF-2 $\alpha$ . We therefore evaluated the p16<sup>INK4a</sup> expression profile in human MSCs from three different donors undergoing TGF- $\beta$ 3-induced chondrogenic differentiation to recapitulate *in vitro* all stages of chondrogenesis. Surprisingly, p16<sup>INK4a</sup> expression increases during chondrogenesis in parallel with a chondrocyte differentiation marker such as collagen IIB at days 7 to 14 and in a concomitant manner with MMP13, a chondrocyte terminal differentiation marker, at day 21 (Figure 2A-D). Of note, during chondrogenesis, p14<sup>ARF</sup> mRNA, an alternative splicing form of p16<sup>INK4a</sup>, was not detected at any time (data not shown). Thus, the senescence marker p16<sup>INK4a</sup>, which is known to be required for astrocyte [4] or epidermal cell [5] differentiation, seems also to play a role during chondrogenesis. To dissect its *in vivo* function during chondrogenic differentiation, we compared the endochondral ossification process of transgenic mice deficient in *ink4a* locus with that of wild-type mice. PCNA and Safranin-O staining were used to quantify proliferative chondrocytes versus non-proliferative chondrocytes within the growth plate of 1-month-old mice. Without affecting the total number of chondrocytes, *ink4a* ablation reduces the height of hypertrophic terminally differentiated non-proliferative chondrocytes by 48%  $\pm$  2.2 (Figure 2E-G), demonstrating a role for *ink4a* locus in chondrocyte cell fate decision to engage in terminal differentiation. A main function of p16<sup>INK4a</sup> is to specifically inhibit cell cycle progression by targeting G1 CDK4/6 activities [35] and therefore maintain retinoblastoma (pRb), p107, and p130 under their active unphosphorylated forms [36]. These three pocket proteins are known to control chondrocyte cell fate decision during bone growth [37-39]. Interestingly, two other CKIs—p27<sup>KIP1</sup> and p57<sup>KIP2</sup>—are also part of the cell cycle regulation during terminal differentiation through CDK inhibition



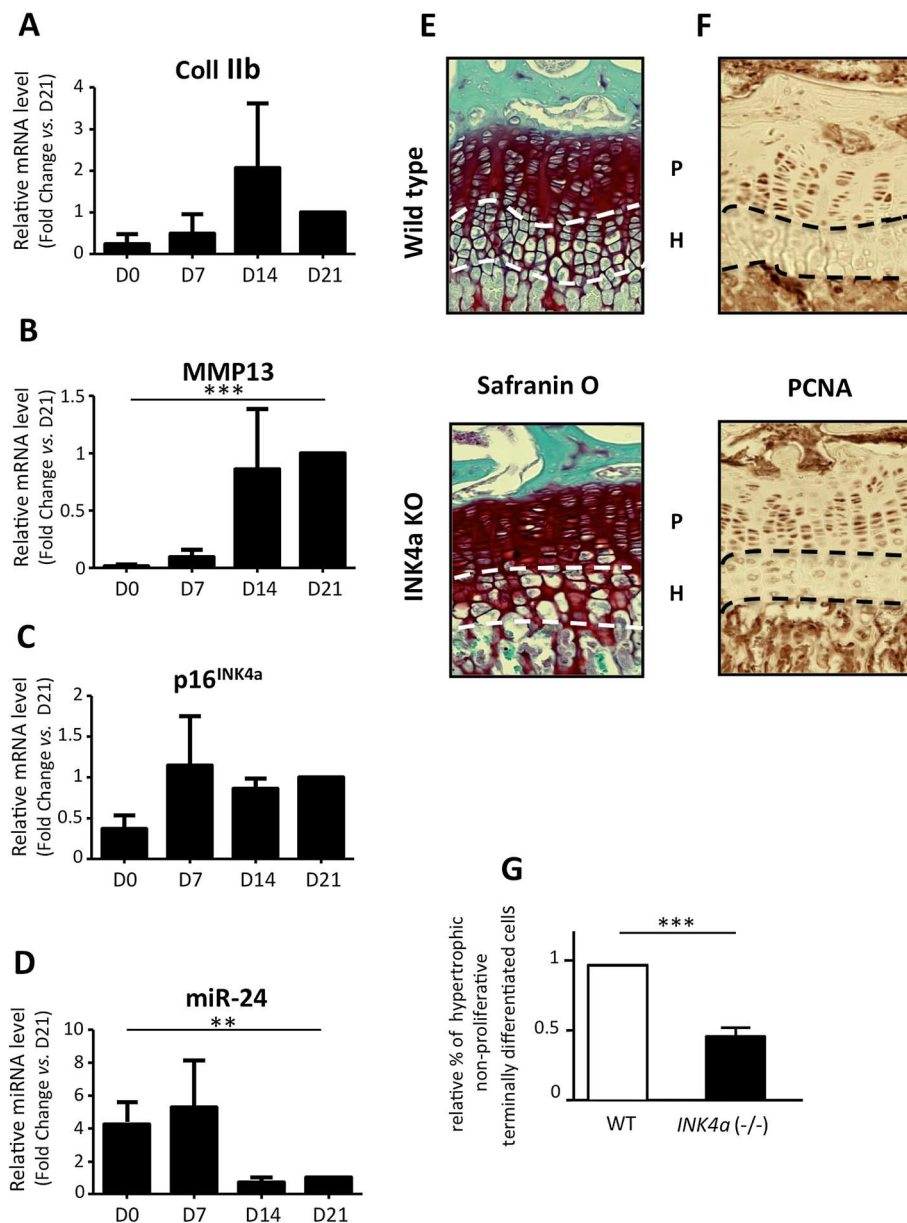
**Figure 1** Interleukin-1-beta (IL-1 $\beta$ ) induces both p16<sup>INK4a</sup> expression and a senescence-associated secretory phenotype (SASP) in mature chondrocytes. Osteoarthritis (OA) human primary chondrocytes were placed in pellet culture and treated with IL-1 $\beta$  (10 ng/mL) for 5 days. **(A)** Aggrecan mRNA (Acan) expression level was evaluated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (n = 3). **(B)** p16<sup>INK4a</sup> protein expression level was measured by Western blotting. **(C)** P-p38<sup>MAPK</sup> protein level was detected by immunohistochemistry (IHC) on a section of paraffin-embedded pellets. Images were taken with a  $\times 20$  objective. **(D-G)** Matrix metalloproteinase 1 (MMP1), MMP13, IL-6, and IL-8 secretion were measured by enzyme-linked immunosorbent assay (ELISA) (n = 4). Data are shown as mean  $\pm$  standard deviation (SD) of fold changes compared with control. \* $P < 0.05$ , \*\* $P < 0.01$ .

leading to pocket proteins activation [40,41]. But only p16<sup>INK4a</sup> plays a major role in both cellular senescence and differentiation onset as revealed in *ink4a* knockout mice studies. Taken together, these findings point at the multiple roles of several CKIs and their indirect targets—pRb, p107, and p130—as orchestrators of the differentiation program and, in particular, in chondrocyte terminal differentiation during bone development.

#### Expression of p16<sup>INK4a</sup> is sufficient for MMP1 and MMP13 secretion by mature chondrocytes

To determine how *Ink4a* could participate in both OA initiation and chondrocyte terminal differentiation, we assessed whether p16<sup>INK4a</sup> expression impacts the establishment of matrix remodeling secretome common in both events. We thus transiently transfected a p16<sup>INK4a</sup>-encoding vector in human chondrocytes, before initiating the pellet culture for 7 days and in the absence of IL-1 $\beta$ . Overexpression of p16<sup>INK4a</sup> was checked at the mRNA level (Figure 3A) and protein level (Figure 3B). Compared with the control, p16<sup>INK4a</sup>-overexpressing mature chondrocytes produced significantly higher levels of MMP1 and MMP13 but did not modulate IL-6 and IL-8. Of note, our findings confirm recent published data showing that p16<sup>INK4a</sup> is dispensable for the establishment of inflammatory secretome associated with senescent fibroblasts [42].

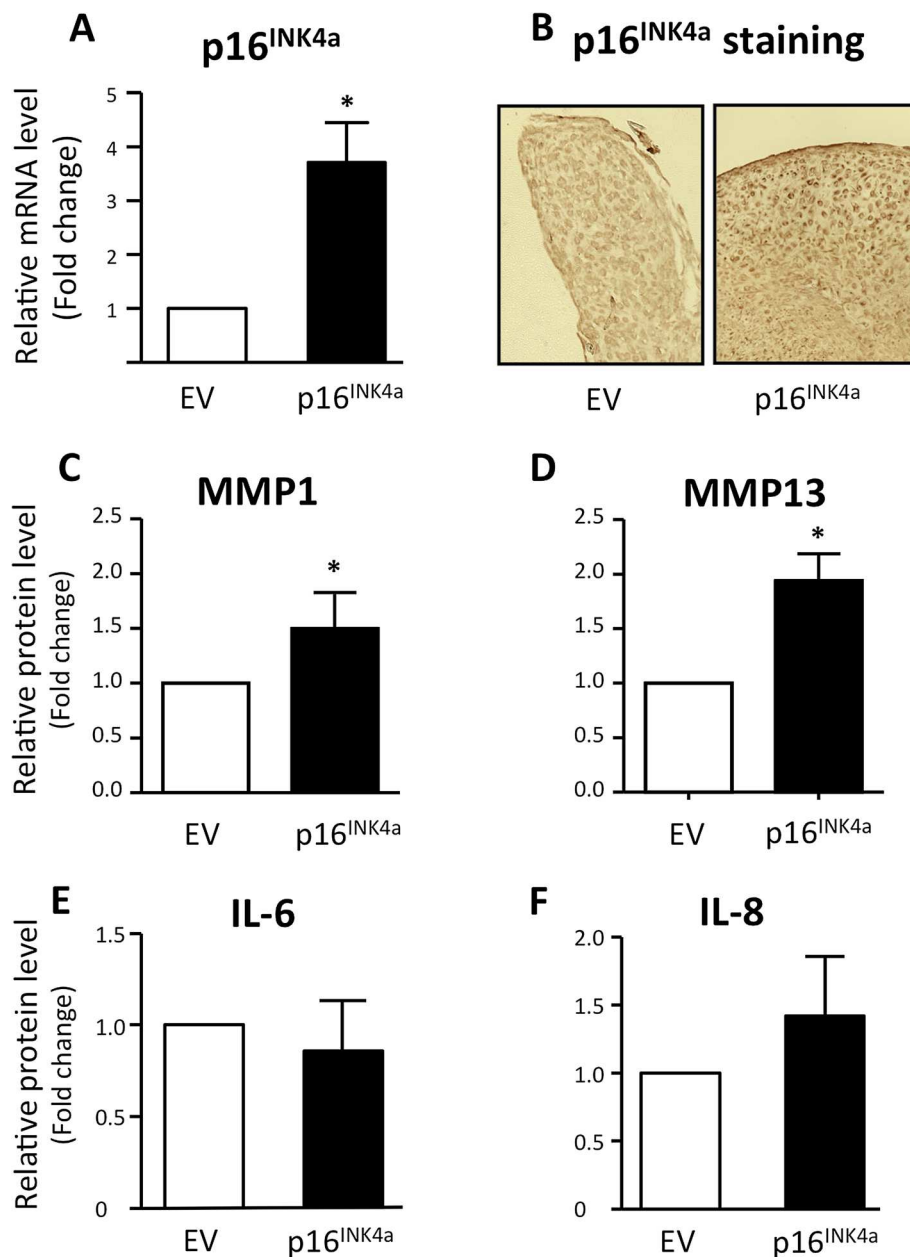
Taken together, our results revealed that the senescence-associated CKI, p16<sup>INK4a</sup>, triggers the secretion of both MMP1 and MMP13 in mature chondrocytes. These two metalloproteases are matrix remodeling enzyme family members playing a central role in physiological and pathological processes occurring in cartilage [33,43,44]. Indeed, both are expressed during chondrocyte terminal differentiation within the growth plate to coordinate matrix remodeling that promotes bone growth [12] but also in OA articular cartilage during disease progression [45]. How could p16<sup>INK4a</sup> control MMPs production? Firstly, *ink4a* locus could directly regulate MMP1 transcriptional activation through its described physical interaction with APA1/ZNF410 transcription factor which is bound to MMP1 promoter [46]. Secondly, by activating pRb-, p130-, and p107-dependent terminal differentiation programs through CDK inhibition, p16<sup>INK4a</sup> accumulation could also contribute indirectly to MMPs production in OA and during endochondral ossification. Indeed, these three pocket proteins can modulate gene expression and cell fate decision through their interactions with chromatin-modifying enzymes [47]. One found, among these enzymes, histone deacetylase (HDAC) family members, which are controlling chondrocyte pre-hypertrophy/hypertrophy transition [16,17]. Remarkably, recent work from Culley and colleagues [48] reveals that pharmacological



**Figure 2** MiR-24 and p16<sup>INK4a</sup> expressions during chondrogenesis and role of p16<sup>INK4a</sup> in chondrocyte cell cycle arrest. Human primary mesenchymal stromal cells (MSCs) were placed in three-dimensional (3D) culture conditions for 21 days. RNAs were harvested at indicated time points. **(A-D)** Gene expression analysis was performed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for Collagen 2b, matrix metalloprotease 13 (MMP13), p16<sup>INK4a</sup>, and miR-24. Data are shown as mean ± standard deviation (SD) (n = 3) normalized to D21. Immunohistochemistry (IHC) was performed on sections of formalin fixed paraffin-embedded long bones of transgenic mice deficient in p16<sup>INK4a</sup> or wild-type at the age of 1 month. **(E,F)** Growth plate was marked by Safranin-O staining and proliferating cell nuclear antigen (PCNA) (P, proliferative zone; H, pre-hypertrophic/hypertrophic zone). Images were taken with ×20 objective. **(G)** Quantification of the percentage of hypertrophic non-proliferative terminally differentiated cells on total cells within the growth plate in transgenic and wild-type mice (n = 4) was carried out by using ImageJ software. Data were normalized to 1 for wild-type and are shown as mean ± SD. \*\*P <0.01, \*\*\*P <0.001.

inhibition of HDACs prevents the expression of metalloproteases such as MMP13 by OA chondrocytes. Thus, p16<sup>INK4a</sup> contributes to MMP secretion by activating the pocket protein-HDAC axis in pathological and physiological conditions.

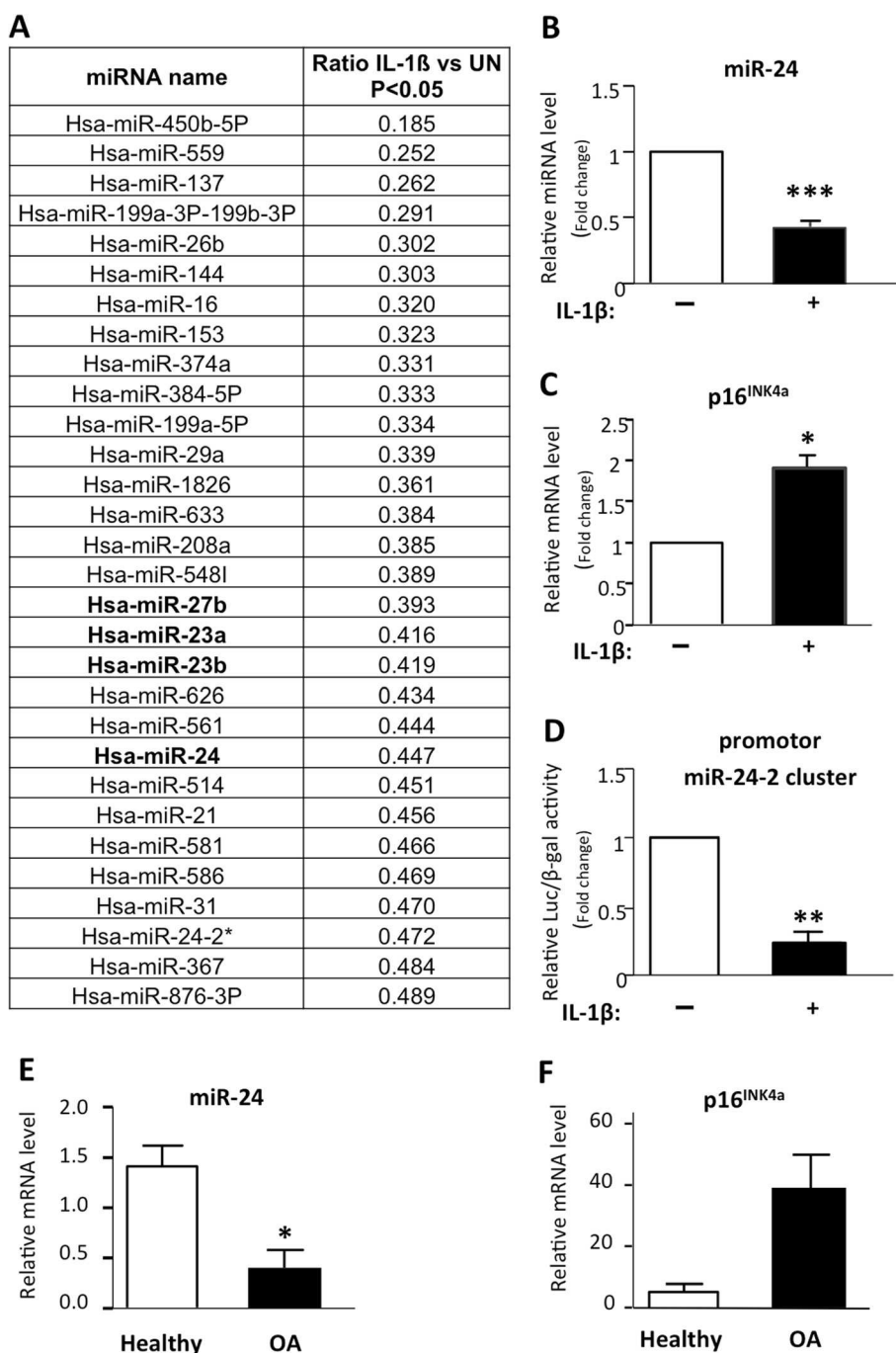
**p16<sup>INK4a</sup> induction correlates with miR-24 repression in interleukin-1-beta-treated chondrocytes, osteoarthritic cartilage, and the end of an *in vitro* chondrogenesis**  
 We next wanted to identify putative regulators of p16<sup>INK4a</sup> in mature chondrocytes. Since miRs have been shown to



**Figure 3** Cyclin-dependent kinase inhibitor p16<sup>INK4a</sup> participates in matrix metalloproteinase 1 (MMP1) and MMP13 expression in mature chondrocytes. Osteoarthritis (OA) human primary chondrocytes were transfected with vector encoding for p16<sup>INK4a</sup> or empty vector (EV) for 24 hours and placed in pellet for 7 days. **(A)** Gene expression analysis was performed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for p16<sup>INK4a</sup> (n = 5). **(B)** p16<sup>INK4a</sup> protein expression was detected by immunohistochemistry (IHC) on pellet paraffin sections. Images were taken with a  $\times 20$  objective. **(C-F)** MMP1, MMP13, interleukin-6 (IL-6), and IL-8 secretions were measured by enzyme-linked immunosorbent assay (ELISA) (n = 5). Data are shown as mean  $\pm$  standard deviation (SD). \* $P < 0.05$ .

play an important role in cartilage physio-pathology [16], we asked whether some miRs could regulate p16<sup>INK4a</sup> expression in IL1 $\beta$ -treated chondrocytes. miR-array analysis was performed on small RNAs extracted from chondrocytes that were from three different donors and that were cultured in pellets in the presence or absence of IL-1 $\beta$  (Figure 4). Bioinformatic analysis revealed that 179

miRs (128 up and 51 down) are differentially expressed in response to IL-1 $\beta$  compared with untreated cells. These deregulated miRs have at least a 1.4-fold change and a  $P$  value of less than 0.05 (Figure 4A and data not shown). Interestingly, we found among them several previously OA-associated miRs such as miR-27b, miR-199, miR-29a, miR-26, and miR-365 [16,17].



**Figure 4 MiR-24 repression correlates with p16<sup>INK4a</sup> induction in interleukin-1-beta (IL-1 $\beta$ )-treated chondrocytes and osteoarthritis (OA) cartilage.** OA human primary chondrocytes are placed in pellet and treated with IL-1 $\beta$  at 10 ng/mL for 5 days. **(A)** List of microRNAs downregulated by IL-1 $\beta$ . **(B,C)** Gene expression analysis was performed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for miR-24 and p16<sup>INK4a</sup> (n = 3). **(D)** Luciferase/ $\beta$ -gal activities of promoter miR-24-2 cluster after IL-1 $\beta$  treatment (n = 3). Data are shown as mean  $\pm$  standard deviation (SD). MiR-24 repression in OA cartilage samples is shown. **(E,F)** MiR-24 repression in OA cartilage: gene expression for miR-24 and p16<sup>INK4a</sup> on OA (n = 5) and healthy (n = 6) cartilage samples. \*P <0.05, \*\*P <0.01, \*\*\*P <0.001.

Since inhibition of miR-processing enzymes such as Dicer induces senescence-associated phenotypes in primary cells [49] and promotes chondrocyte terminal differentiation in animal models [50], we focused our attention

on miRs that were repressed by IL-1 $\beta$  (Figure 4A). We found miR-24, a known negative regulator of p16<sup>INK4a</sup>, through the presence of two binding sites for this miR within its encoding and 3' untranslated region (UTR) [51].

We next confirmed, on three independent chondrocyte samples placed in 3D, that IL-1 $\beta$  significantly repressed miR-24 expression (Figure 4B) with a concomitant expected induction of p16<sup>INK4a</sup> mRNA (Figure 4C).

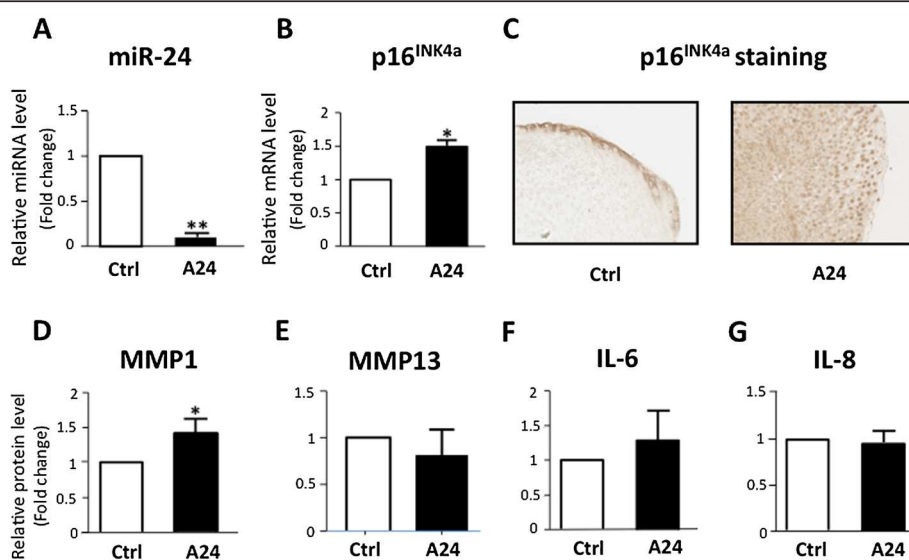
miR-24 is encoded by two genes: miR-24-1 and miR-24-2 [52]. These genes are organized in a cluster including three different miRs (miR-23a or b/27a or b/24). Each cluster is regulated by one promoter common for the three miRs of the cluster [52]. Our array analysis revealed that, upon IL-1 $\beta$  stimulation, chondrocytes show a reduced expression of several members of these two clusters (Figure 4A), suggesting a global repression of the transcription of the clusters. In keeping with this hypothesis, we confirmed the transcriptional repression of miR-24-2 promoter, upon IL-1 $\beta$  addition (Figure 4D), by using a reporter luciferase assay (Figure 4D) previously described [28].

An increase in expression of p16<sup>INK4a</sup> has been demonstrated in cartilage from patients with OA [13]. We then checked whether miR-24 expression could be reversely correlated with that of p16<sup>INK4a</sup> in OA cartilage compared with healthy cartilage. By RT-qPCR on mRNA from OA (n = 5) versus healthy (n = 6) human cartilage samples, we revealed a significant miR-24 downregulation in OA cartilage (Figure 4E) while p16<sup>INK4a</sup> is increased (Figure 4F). These results were confirmed at the protein level on serial sections of OA cartilage samples by using p16<sup>INK4a</sup> immunohistochemistry and miR-24 *in situ* hybridization (Additional file 1).

Figure 2 shows that p16<sup>INK4a</sup> mRNA accumulates throughout the time course of an *in vitro* chondrogenesis from days 7 to 21 (Figure 2C). We therefore evaluated, during chondrogenesis, whether the expression of miR-24 could also be reciprocal to that of p16<sup>INK4a</sup> expression. By RT-qPCR, we revealed that, compared with days 0 to 7, miR-24 level is decreased at day 14 and significantly at day 21 (Figure 2D) in parallel with an increase in expression of the terminal differentiation marker, MMP13 (Figure 2B), while p16<sup>INK4a</sup> remains elevated (Figure 2C). Taken together, these results demonstrate that the expressions of Ink4a and its epigenetic regulator are mutually exclusive in both *in vitro* and *in vivo* OA models and during the end of the chondrogenesis. Moreover, miR-24 downregulation seems to follow and sustain a high level of p16<sup>INK4a</sup> rather than initiate p16<sup>INK4a</sup> accumulation.

#### MiR-24 downregulation is sufficient to trigger p16<sup>INK4a</sup> expression and MMP1 production in mature chondrocytes

Finally, we aimed at determining whether modulation of miR-24 could be sufficient to promote p16<sup>INK4a</sup> accumulation and p16<sup>INK4a</sup>-dependent matrix remodeling secretome by using our 3D chondrocyte model. Because miR-24 overexpression has been reported to induce apoptosis by repressing DNA damage response pathways [53], we relied on a loss-of-function experiment based on transfection of chondrocytes by either a specific inhibitor of miR-24 (antagomiR-24) or an irrelevant antagomiR as control. As expected, transfection of antagomiR-24 led to a dramatic



**Figure 5** MiR-24 downregulation is sufficient to trigger p16<sup>INK4a</sup> expression and matrix metalloproteinase 1 (MMP1) production in mature chondrocytes. Osteoarthritis (OA) human primary chondrocytes were transfected with irrelevant antagomiR (Ctrl) and antagomiR-24 (A24) at a concentration of 100 nM. They were placed in pellet culture conditions for 7 days. (A,B) Gene expression analysis was performed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for miR-24 and p16<sup>INK4a</sup> (n = 4). (C) p16<sup>INK4a</sup> protein expression by immunohistochemistry (IHC) on pellet paraffin-embedded sections. Images were taken with a  $\times 20$  objective. (D-G) MMP1, MMP13, interleukin-6 (IL-6), and IL-8 secretion were measured by enzyme-linked immunosorbent assay (ELISA) (n = 4). Data are shown as mean  $\pm$  standard deviation (SD). \* $P < 0.05$ .

downregulation of miR-24 level (Figure 5A) and the concomitant significant upregulation of p16<sup>INK4a</sup> at both mRNA (Figure 5B) and protein levels as shown by immunostaining with p16<sup>INK4a</sup> antibodies (Figure 5C). Furthermore, we showed that miR-24 downregulation is sufficient to promote a marked increase in MMP1 secretion (Figure 5D) but has no significant effect on MMP13, IL-6, or IL-8 secretion (Figure 5E-G), suggesting the existence of a direct axis miR-24-p16<sup>INK4a</sup>-MMP1. The discrepancy between this result and p16<sup>INK4a</sup> overexpression alone could be explained by mutual redundancy and interference between the signaling pathways. Nevertheless, miR-24 is part of a cluster containing two other miRs and regulated by the same promoter (Figure 4D). Therefore, miR-24 repression is *in vivo* always accompanied by that of miR27a/b and miR23a/b (Figure 4A and D). Remarkably, miR27b was recently shown to inhibit MMP13 expression in IL-1 $\beta$ -treated chondrocytes [21] and miR23a/b could negatively regulate Runx2, a transcription factor involved in chondrocyte terminal differentiation, OA, and osteoblastogenesis [54]. Thus, repression of these clusters during OA progression within articular cartilage would promote the appearance of several OA-induced features, including p16<sup>INK4a</sup>, MMP1, MMP13, but also Runx2. These findings propose that miR-24-1/miR-24-2 clusters, together with the recently identified miR-140, which targets ADAMTS5 and HDAC4, two hypertrophic inducers [19,20], are crucial in preventing chondrocyte terminal differentiation in OA.

## Conclusions

Determining the role and the regulatory pathways controlling p16<sup>INK4a</sup> expression in chondrocytes during OA progression is essential for future innovative long-term therapeutic approaches. In the present work, we demonstrated that the senescence CKI, p16<sup>INK4A</sup>, is also associated with chondrocyte terminal differentiation and can regulate the expression of matrix remodeling metalloproteases MMP1 and MMP13. We further showed that miR-24 expression plays a role as a negative regulator of the p16<sup>INK4a</sup>/MMP1 axis.

We propose that—during OA progression, in response to IL-1 $\beta$ , or during endochondral-induced terminal chondrogenesis—a repression of miR-24- and miR-24-encoding clusters takes place. This is likely to trigger p16<sup>INK4a</sup>, MMP1, MMP13, and Runx2 expression, thereby pushing chondrocytes toward a senescent-like phenotype resembling that of terminally differentiated chondrocytes [7]. Accumulation of p16<sup>INK4a</sup>-positive chondrocytes within articular cartilage could thus be deleterious not only for tissue regeneration by blocking cell proliferation and replacement but also for tissue integrity through MMPs secretion [7,55-57]. On the other hand, p16<sup>INK4a</sup> accumulation within the growth plate will favor bone

development. One therapeutic strategy for OA treatment could be to restore/maintain the expression level of miR-24-encoding clusters in order to prevent p16<sup>INK4a</sup>-dependent pathways in articular chondrocytes.

## Additional file

**Additional file 1: p16<sup>INK4a</sup> and miR-24 are reversely correlated in osteoarthritis (OA) articular cartilage.** (A) p16<sup>INK4a</sup> immunohistochemistry (IHC) on a cryosection of OA cartilage comprising superficial and intermediate layer. (B) miR-24 *in situ* hybridization on an adjacent section of the same OA cartilage sample. Results are representative of experiments on two OA cartilage samples. Chondrocytes expressing either p16<sup>INK4a</sup> or miR-24 are marked by arrows showing mutual exclusion. OA cartilage samples were fixed with 4% paraformaldehyde during 3 hours at 4°C. After fixation, samples were placed in PBS with sucrose 30% during 24 hours at 4°C. The next day, PBS-sucrose 30% was replaced with Tek OCT solution and the samples were stored at -80°C. OA cartilage samples were sectioned at 13  $\mu$ m and collected on Superfrost PLUS slides. *In situ* hybridization experiment was performed as described [58]. LNA DIG-hsa-miR-24 probe and DIG-hsa-miR-141c (as negative control) were purchased from Exiqon (Copenhagen, Denmark) and diluted at 1 $\mu$ M. Alkaline phosphatase conjugated anti-DIG- antibody was diluted at 1:2,000 in blocking solution. This file can be viewed with Acrobat Reader.

## Abbreviations

3D: three-dimensional; BM-MSC: bone marrow-mesenchymal stromal cell; CDK: cyclin-dependent kinase; CKI: cyclin-dependent kinase inhibitor; DMEM: Dulbecco's modified Eagle's medium; hBM-MSC: human bone marrow-mesenchymal stromal cell; HDAC: histone deacetylase; HIF-2 $\alpha$ : hypoxia-inducible factor-2; IL: interleukin; miR: microRNA; MMP: matrix metalloproteinase; OA: osteoarthritis; PBS: phosphate-buffered saline; PCNA: proliferating cell nuclear antigen; pRB: retinoblastoma protein; RT-qPCR: reverse transcription-quantitative polymerase chain reaction; SASP: senescence-associated secretory phenotype; TGF- $\beta$ 3: transforming growth factor-beta 3.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

DPh participated in immunochemistry, immunoassays, gene expression, statistical analysis and manuscript drafting. DG participated in MSC isolation, chondrogenesis experiments, and statistical analysis and critically revised the manuscript. DPI and EO participated in chondrocyte isolation, Western blot experiments, and manuscript drafting. FE participated in transfection experiments and immunoassay experiments and critically revised the manuscript. AD collected post-mortem human cartilages, participated in microRNA isolation, and critically revised the manuscript. Y-MP collected human OA cartilage, acquired clinical data, obtained patient written consents, and critically revised the manuscript. PC performed microarray analysis and participated in data interpretation, manuscript drafting, and critical reading. JP participated in mice INK4a KO design experiments and immunostaining knee analysis and critically revised the manuscript. CJ initiated the project; participated in experimental designs, microarray and data interpretation, and manuscript drafting; and critically revised the manuscript. DN participated in experimental conception, cell banking, data interpretation, and manuscript drafting and critical reading. RMB participated in experimental designs, microRNA isolation, Western blot analysis, data interpretation, and manuscript drafting and critical reading. J-MB participated in immunoassay experiments, collecting data, statistical analysis, manuscript drafting, and correspondence to editor. All authors read and approved the final manuscript.

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