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# Chondrocyte mTORC1 activation stimulates miR-483-5p via HDAC4 in osteoarthritis progression

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The hyperactivation of the mechanistic target of rapamycin complex 1 (mTORC1) in chondrocytes has been shown to accelerate the severity of destabilization of the medial meniscus-induced and age-related osteoarthritis (OA) phenotypes with aberrant chondrocyte hypertrophy and angiogenesis. Meanwhile, we previously reported that miR-483-5p is essential for the initiation and development of OA by stimulating chondrocyte hypertrophy and angiogenesis. The connection between mTORC1 and miR-483-5p activation in OA progression, however, remains unclear. In this study, we elucidated their relationship and identified the underlying mechanisms. The expression of miR-483-5p in the articular cartilage of cartilage-specific TSC1 knockout mice was assessed compared with control mice using the Agilent Mouse miRNA (8*60K) V19.0 array and real-time polymerase chain reaction (RT-PCR). The functional effects of the stimulation of miR-483-5p via histone deacetylase 4 (HDAC4) by mTORC1 in OA development, subsequently modulating its downstream targets matrilin 3 and tissue inhibitor of metalloproteinase 2, were examined by immunostaining, western blotting, and real-time PCR. This study revealed that miR-483-5p was responsible for mTORC1 activation-stimulated OA. Mechanistically, mTORC1 controls the HDAC4-dependent expression of miR-483-5p to stimulate chondrocyte hypertrophy, extracellular matrix degradation, and subchondral bone angiogenesis, and it consequently initiates and accelerates the development of OA. Our findings revealed a novel mTORC1-HDAC4-miR-483-5p pathway that is critical for OA development.

**KEYWORDS**
chondrocyte, histone deacetylase 4 (HDAC4), miR-483-5p, mechanistic target of rapamycin complex 1 (mTORC1), osteoarthritis (OA)

## 1 | INTRODUCTION

Osteoarthritis (OA) is a chronic degenerative joint disease, affecting an estimated 10% of men and 20% of women over 60 years of age (Hiligsmann et al., 2013). The progressive loss of articular cartilage, remodeling of the subchondral bone, and synovial inflammation are common characteristics, making OA a major source of disability, pain, and socioeconomic burden worldwide (Glyn-Jones et al., 2015). Nevertheless, there is limited understanding of its pathogenesis and no effective medical therapy is available for clinical treatment.

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The mechanistic target of rapamycin (mTOR) is a serine/threonine protein kinase that functions as a master regulator of critical cellular processes such as growth, proliferation, and stress. Mechanistic target of rapamycin complex (mTORC) 1 and 2 are two distinct functional complexes formed by mTOR (Laplante & Sabatini, 2009; Zoncu, Efeyan, & Sabatini, 2011). mTORC1 is inhibited by a functional complex, tuberous sclerosis complex (TSC) 1 and 2, and rapamycin (Inoki et al., 2006; Inoki, Corradetti, & Guan, 2005). Chondrocytes, the one cell type present in articular cartilage (Kronenberg, 2003), responds to mTORC1 signaling to participate both in endochondral bone development and OA pathophysiology. Our previous research demonstrated that dynamically controlled mTORC1 activity is crucial for the coordination of chondrocyte proliferation and differentiation during endochondral bone development, partially through the regulation of parathyroid hormone-related peptide (Yan et al., 2016). Furthermore, we generated cartilage-specific TSC1 knockout (Col2a1TSC1KO) and inducible TSC1 KO mice. The literature shows that mTORC1 activation initiates OA progression by stimulating articular chondrocyte proliferation and differentiation, in part through downregulating fibroblast growth factor receptor (FGFR3) and PTH-related protein (PTHrP) receptor (PPR; Zhang et al., 2017). Because mTORC1 is a complex mediator, it may also affect other regulatory processes independently of the aberrant protein expression in articular cartilage. For example, mTORC1 plays a role in modulating the levels of microRNAs (miRNAs).

miRNAs, a class of 18–22 nucleotide noncoding RNAs, regulates gene expression at the posttranscriptional level by inhibiting both the translation and stability of specific mRNAs, therefore affecting the cell fate during proliferation and differentiation (Bartel, 2004). Accumulating evidence has suggested that miRNAs play an important role in cartilage homeostasis, mechanotransduction, and the pathogenesis of OA (Cheleschi et al., 2017; De Palma et al., 2017; Wu et al., 2014). The miR-483 gene is located in the second intron of insulin-like growth factor 2 (IGF 2) and can generate two mature isoforms: miR- 483-5p and miR-483-3p (Veronese et al., 2010). Several studies have shown that miR-483-5p is relevant in the progression of various cancers (Fan et al., 2015; Loo et al., 2015; Song et al., 2014). Other researchers have reported that it is upregulated in chondrocytes from patients with OA and mice compared with the controls (Díaz-Prado et al., 2012; Qi et al., 2013; Yang, Zhang, & Gibson, 2015). Furthermore, our previous research demonstrated that intra-articular delivery of antago-miR-483-5p inhibited OA by modulating matrilin 3 (Matn3) and tissue inhibitor of metalloproteinase 2 (TIMP2; Wang et al., 2017). Notably, in this study, the miR-483 transgenic (TG483) mice was present with a similar phenotype as Col2a1TSC1KO mice, with effects such as increased chondrocyte hypertrophy and subchondral bone angiogenesis. However, the relationship between mTORC1 and miR-483-5p remains to be elucidated.

In this study, we screened the mTORC1-regulated miRNA profile and analyzed transgenic mice, discovering that miR-483-5p mediated mTORC1 activation-stimulated OA, at least in part. Furthermore, we demonstrated that mTORC1 controls miR-483-5p via histone deacetylase 4 (HDAC4). Our findings revealed a novel mTORC1-HDAC4-miR-483-5p pathway that was shown to be critical for OA development.

## 2 | MATERIALS AND METHODS

### 2.1 | Establishment of chondrocyte-specific TSC1 deletion mice

Mice with the chondrocyte-specific deletion of TSC1 (TSC1f/f; Col2a1-Cre) were backcrossed against 129 inbred mice for nine generations. Both Col2a1-Cre mice and TSC1 loxP mice were obtained from the Jackson Laboratory (Jax no. 003554 and 005680, respectively). Male homozygous TSC1 loxP mice were mated with female Col2a1-Cre mice to yield mice heterozygous for loxP TSC1 and heterozygous for Col2a1-Cre. These mice were then bred with mice homozygous for loxP TSC1, to obtain mice homozygous for loxP TSC1 and heterozygous for Col2a1-Cre. These mice carried the Col2a1-specific deletion of TSC1 and were termed as Col2a1TSC1KO mice, and the mice homozygous for loxP TSC1 without Col2a1-Cre from the same litter were used as controls (TSC1loxp/loxp; $n = 5$/group).

### 2.2 | Generation of TG483 transgenic mice

The detailed methods used for the generation of TG483 mice were previously reported by our lab. In brief, the expression of miR-483 is time-specific and regulated by the tet-on system. From 6 weeks old, TG483 and control mice were treated with 2 mg/ml doxycycline drinking water.

Genotyping of Col2a1TSC1KO was performed according to the Jackson Laboratory's instructions and TG483 mice according to the Cyagen Biosciences's instructions (primers are listed in Supplementary Information Table 1).

### 2.3 | Destabilization of the medial meniscus (DMM) OA mouse model

Experimental OA was surgically induced in 8- to 10-week-old male C57Bl/6J mice. The protocols for DMM surgery and histological staining have been published by our lab previously (Huang et al., 2014). These mice were purchased from the Laboratory Animal Centre of the Southern Medical University.

### 2.4 | Isolation of articular cartilage, primary chondrocytes, and cell culture

Knee articular cartilage was obtained from 8-week-old Col2a1TSC1KO mice. Primary chondrocytes were isolated from the rib cartilage of newborn mice. All procedures followed previously described protocols. Human vascular endothelial cells (HVECs) were cultured in Dulbecco's modified eagle medium (DMEM) with high glucose, supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY) and were

maintained under standard cell culture conditions of 5% $CO_2$ and 95% humidity. Cells were treated with rapamycin (50 nM) or Actinomycin D (10 μg/ml) for 24 hr after mRNA or protein extraction. Constructed luciferase plasmid, miRNA, or siRNA oligonucleotides (GenePharma, Suzhou, China, Supporting Information Table 3) were transfected with Lipofectamine 2000 following the manufacturer's instructions (Invitrogen).

## 2.5 | Intra-articular injection

Lentivirus-mediated miR-483-5p mimics (LV3-miR-483-5p, GenePharma, Suzhou, China) or nitrocellulose (NC; LV3-NC, $1 \times 10^9$ TU/ml) was injected into DMM OA mice knee joints using a 33-gauge needle and a microsyringe (Hamilton Co.; Zhu et al., 2015). All experimental mice were injected with lentivirus on Days 7 and 14 after surgery. The sequence of intra-articular injection is shown in Supporting Information Table 2. Knee joints were harvested 5 weeks later.

## 2.6 | Immunohistochemistry (IHC) and immunofluorescence

At the end of treatment, the knee joints from male mice were fixed with 4% paraformaldehyde for 48 hr and decalcified with 0.5 M ethylenediaminetetraacetic acid (EDTA) at pH 7.4 for 3 weeks. The specimens were then routinely embedded in paraffin, and 2 μm serial sections were obtained from the sagittal sections through the medial side of the knee. For the IHC analysis, we used the following primary antibodies: rat anti-Matn3 (1:500; Abcam, Cambridge, MA), rat anti-HDAC4 (1:500; ABclonal, Wuhan, China), and goat anti-CD31 (1:100; R&D, Minneapolis, MN). Sections were then stained with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). For immunofluorescence, the primary antibodies were anti-TIMP2 (1:100; ABclonal, Wuhan, China) and Alexa 488 dye-labeled secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The sections were mounted with medium containing 4′,6-diamidino-2-phenylindole (DAPI) and images were obtained using a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan).

## 2.7 | RNA isolation and real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from cell pellets or cartilage using Trizol reagent (Invitrogen, Carlsbad, CA). We used the Mmu-miR-483-5p hairpin-it RT-PCR kit to analyze the miR-483-5p levels. Meanwhile, the *U6* gene was used as an endogenous control and was checked using the *U6* snRNA RT-PCR normalization kit (GenePharma, Suzhou, China). To analyze IGF2, Col2a1 and indian hedgehog (IHH) levels, Takara reverse transcription reagents, and RT-PCR Mix (Takara, Tokyo, Japan) were used and checked on the light cycler (Roche, Basel, Switzerland). The cDNA was amplified using sequence-specific primers (Supporting Information Table 4), and the *GAPDH* gene was used as an endogenous control to normalize for differences in the amount of total RNA.

## 2.8 | Tube formation assays

We used 20 μl of growth factor-reduced Matrigel (BD Biosciences, San Jose, CA) per well to coat 96-well culture plates. The culture plates were then incubated at 37°C for 30 min until solidification. HVECs in the logarithmic growth phase were trypsinized and resuspended in supernatant from cells transfected with siTIMP2 or/and treated with Rapa at a density of $5 \times 10^4$/ml, whereupon 100 μl of the cell suspension was added to each well. Tube formation was allowed at 37°C for 12 hr. Tube length was measured using the NIH Image J 1.31v Program (NIH, Bethesda, MD).

## 2.9 | Western blot analysis

After treatment, the cells were harvested on ice in buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 50 mM dithiothreitol, and 0.01% bromophenol blue) and immediately lysed for 5 min at 95°C. TSC2, Matn3, HDAC4, and TIMP2 proteins were analyzed by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE) and transferred to an NC membrane (Bio-Rad Corp., Hercules, CA). The immunoreactive proteins were revealed using an enhanced chemiluminescence kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

## 2.10 | Luciferase assay

To investigate the transcription regulation of miR-483, an approximately −2000 bp promoter sequence was amplified by PCR from mouse genomic DNA, as follows: forward primer: 5′-CCGCTCGAGTGCTGTA GCTATAGCCTGC-3′; reverse primer: 5′-CCCAAGCTTGTAGTTGTTT CCAGAGCAG-3′. The PCR product was then subcloned into the *Xho*I and *Hin*dIII (Thermo, Waltham, MA) cloning sites of the PGL4.15 empty vector (Promega, Madison, WI). Meanwhile, pRL-TK was used as the control plasmid. The cells were cotransfected with the luciferase constructs with or without rapamycin treatment and were lysed. Luciferase assays were then performed with the dual-luciferase reporter assay system (Promega, Madison, WI) following the manufacturer's instructions. Luminescent signals were quantified with a luminometer (Glomax, Promega).

## 2.11 | Accession number

The original microarray data were deposited in the NCBI's Gene Expression Omnibus (GSE71484).

## 2.12 | Study approval

All mice were housed in a pathogen-free animal facility at the Southern Medical University (Guangzhou, China). In addition, all animal experiments were approved by the Southern Medical University Committee on the Use and Care of Animals and were performed in accordance with the committee's guidelines.

## 2.13 | Statistics

Statistical analysis was performed using GraphPad Prism 5.01 software (GraphPad Software, Inc., La Jolla, CA). All data are presented as mean ± 95% confident interval. To compare three groups, the one-way analysis of variance was used. Differences between two groups were detected by the Student $t$-test. $p < 0.05$ was considered statistically significant.

## 3 | RESULTS

### 3.1 | mTORC1 upregulates miR-483-5p in chondrocytes

As we have previously reported, adult Col2a1TSC1KO mice exhibited significant morphologic changes that paralleled with the aged TG483 mice. Safranin O and fast green staining (Supporting Information Figure 1a,b) showed accelerated destruction of cartilage and enhanced chondrocyte hypertrophic differentiation in the tibial cartilage of the genetically modified mice. Accordingly, the Osteoarthritis Research Society International (OARSI) scores based on the staining increased significantly in these mice. Additionally, toluidine blue staining (Figure 1a,b, upper) was destaining or uneven in the tibial cartilage of adult Col2a1TSC1KO mice and aged TG483 mice. These results demonstrate a similar loss of cartilage in the tibial cartilage of the modified mice.

Furthermore, as compared with control mice, the expression of Runt-related transcription factor 2 (Runx2), an essential transcription factor for chondrocyte hypertrophy, was markedly enhanced in the tibial cartilage of adult Col2a1TSC1KO mice and aged TG483 mice (Figure 1a,b, middle). Meanwhile, we detected CD31 (endothelial progenitor, angiogenesis marker) in the subchondral bone of these mice. Interestingly, the number of blood vessels in the subchondral bone was shown to have increased significantly (Figure 1a,b, bottom). Taken together, these results confirm that Col2a1TSC1KO mice developed a typical OA pathological progression that paralleled with that of the aged TG483 mice, such as loss of proteoglycan, degradation of the cartilage matrix, hypertrophy of chondrocytes, and enhanced angiogenesis.

Consequently, our aim was to elucidate the relationship between mTORC1 and miR-483-5p during OA development. At first, knee articular cartilage from 8-week-old Col2a1TSC1KO ($n = 3$) and control ($n = 3$) mice was microdissected and an mTORC1-regulated miRNA profile screen (GSE71484) was performed (Figure 1c). After excluding all miRNAs expressed at extremely low levels or those that were statistically nonsignificant (<2-fold), we identified 133 differentially expressed and 70 upregulated miRNAs in the articular cartilage of the Col2a1TSC1KO mice compared with the control mice. We found miR-483-5p (about 5.47-fold) among the upregulated miRNAs. RT-PCR analysis showed enhanced expression of miR-483-5p in the articular cartilage of Col2a1TSC1KO mice when compared with the control mice (Figure 1d), suggesting that mTORC1 upregulates miR-483-5p in chondrocytes.

### 3.2 | mTORC1 stimulates OA, partly through the upregulation of miR-483-5p

To confirm the regulation of miR-483-5p by mTORC1, the primary chondrocytes were either treated with rapamycin (mTORC1 inhibition) or transfected with TSC2 siRNA (mTORC1 activation). Accordingly, miR-483-5p was upregulated in chondrocytes transfected with TSC2 siRNA (Figure 2a,b) and downregulated in cells treated with rapamycin (Figure 2c). To determine whether miR-483-5p was functionally upregulated by mTORC1 during OA development, primary chondrocytes were transfected with miR-483-5p mimics or with TSC2 siRNA. As expected, miR-483-5p can both reduce Col2a1 and enhance IHH (a prehypertrophic chondrocyte-produced cytokine important for OA initiation) expression in chondrocytes (Figure 2d), similar to the activation of mTORC1 using TSC2 siRNA (Figure 2e). Furthermore, primary chondrocytes were isolated and treated with Rapa alone or Rapa plus miR-483-5p mimics. We found that Col2a1 increased, whereas IHH mRNA levels were markedly reduced in cells treated with Rapa compared with those treated with the dimethyl sulfoxide (DMSO) control. Importantly, both Col2a1 and IHH mRNA expression were rescued to normal levels by transfection with miR-483-5p mimics (Figure 2f). Together, these findings clearly demonstrate that mTORC1 stimulates OA through the upregulation of miR-483-5p in vitro.

To investigate the mTORC1 stimulation of OA through the upregulation of miR-483-5p in vivo, an experimental model of OA was induced in 8- to 10-week-old male C57BL/6J mice by surgical DMM. Mice with DMM surgery underwent the daily intragastric administration of rapamycin (2 mg/kg body weight/day) for 5 weeks and/or an intra-articular injection of LV3-miR-483-5p lentivirus ($n = 5$) on Days 7 and 14 after surgery. As expected, the rapamycin treatment decreased the level of miR-483-5p and the severity of DMM-induced OA. Importantly, LV3-miR-483-5p reversed the effect of rapamycin on DMM-induced OA (Figure 2g). The OARSI scores based on the results of the safranin O and fast green staining reincreased (Figure 2h). In addition, miR-483-5p was upregulated in the knee joint cartilage of Col2a1TSC1KO mice and rapamycin treatment was shown to reduce miR-483-5p expression in chondrocytes. Similarly, when Col2a1TSC1KO and control mice were intragastrically administered a daily dose of rapamycin daily for 5 weeks starting at 4 weeks of age, the rapamycin eliminated the mTORC1 activation and decreased the severity of OA in Col2a1TSC1KO mice (Supporting Information Figure 2a), including decreased destruction of cartilage and loss of proteoglycan (safranin O and fast green staining and toluidine blue staining) and limited expression of Runx2 and CD31. Taken together, these data indicate that mTORC1 partly stimulates OA through the upregulation of miR-483-5p.

### 3.3 | mTORC1 downregulates Matn3 through miR-483-5p in chondrocytes

Matn3 has been proven to be a functional target of miR-483-5p in OA progression. To determine the role of Matn3 in mTORC1-stimulated OA development, we examined the levels of Matn3 in
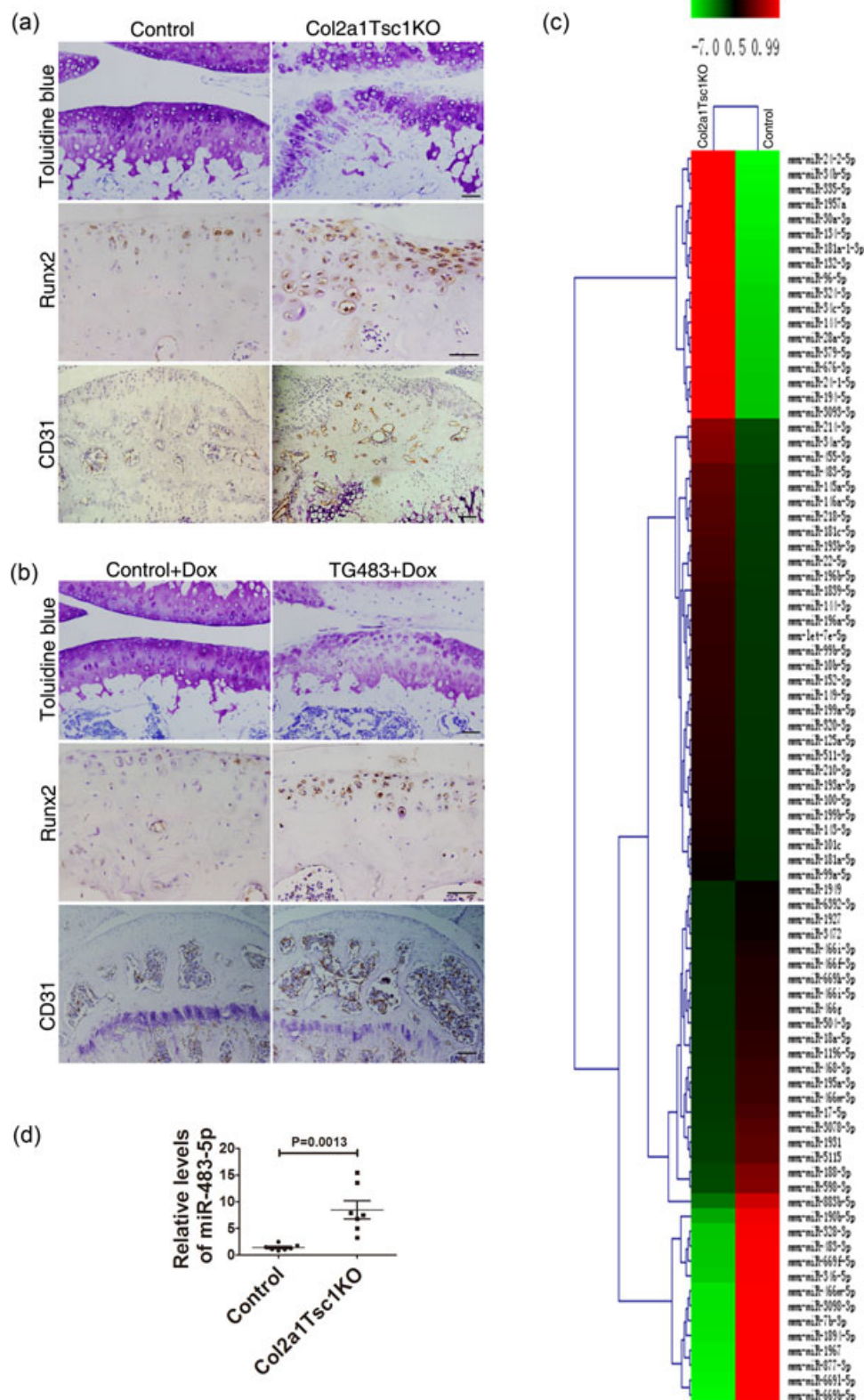
**FIGURE 1** mTORC1 upregulates miR-483-5p in chondrocytes. (a) Toluidine blue staining (bar = 50 μm) and immunohistochemical analysis of *Runx2* (hypertrophic chondrocyte marker, bar = 40 μm) or CD31 (angiogenesis marker, bar = 100 μm) expression levels in mouse knee joints from 16-week-old Col2a1TSC1KO and control mice (*n* = 5). (b) Toluidine blue staining and immunohistochemical analysis of *Runx2* or CD31 expression levels in mouse knee joints from old TG483 and control mice (*n* = 5, 13.5-month-old). (c) Cluster tree showing miRNA gene expression in knee joint cartilage from 8-week-old Col2a1TSC1KO and control mice. (d) RT-PCR analysis of miR-483-5p levels in knee joint cartilage from Col2a1TSC1KO and control mice (*n* = 7, 8-week-old). Dox: doxycycline; Col2a1TSC1KO: cartilage-specific TSC1 knockout; miRNA: microRNA; mTORC1: mechanistic target of rapamycin complex 1; RT-PCR: real time polymerase chain reaction; *Runx2*: Runt-related transcription factor [Color figure can be viewed at wileyonlinelibrary.com]
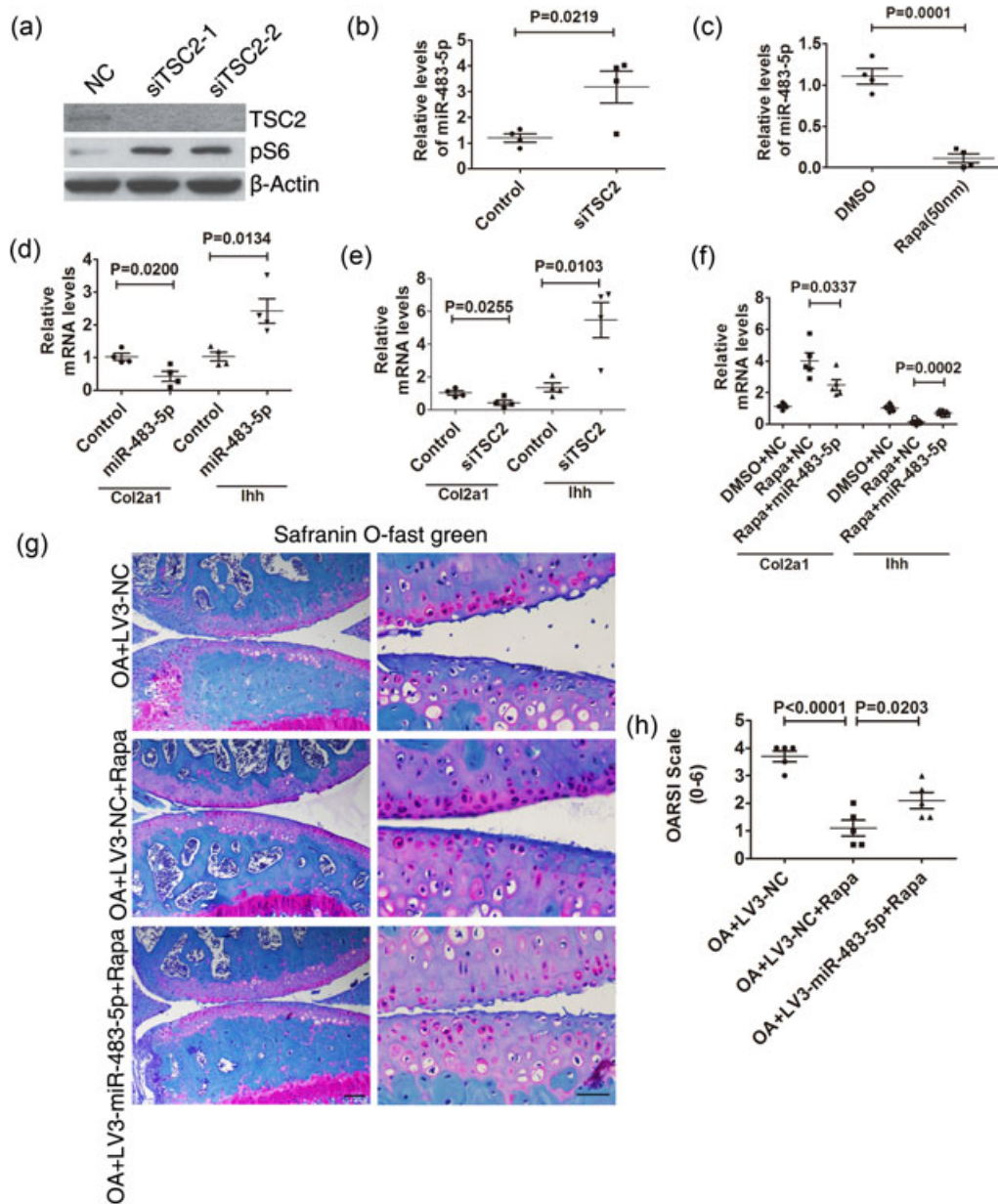
**FIGURE 2** mTORC1 stimulates OA through upregulation of miR-483-5p in part. (a) Western blot analysis of TSC2 and pS6 in primary chondrocytes transfected with TSC2 siRNA for 60 hr. (b,c) Real-time PCR analysis of miR-483-5p levels in primary chondrocytes (b) transfected with TSC2 siNA for 60 hr or (c) treated with 50 nM rapamycin for 24 hr. $n = 4$. (d,e) Real-time PCR analysis of Col2a1 and IHH mRNA levels in primary chondrocytes transfected with miR-483-5p mimics (d) or TSC2 siRNA (e). $n = 4$. (f) Real-time PCR analysis of Col2a1 and IHH mRNA levels in primary chondrocytes treated with NC, Rapa, or miR-483-5p mimics plus Rapa for 48 hr. n = 5. (g) Safranin O-fast green staining of knee joints from DMM OA mice treated with rapamycin (2 mg/kg/day) only and with rapamycin plus LV3-miR-483-5p for 5 weeks ($n = 5$, 13- to 15-week-old). Left, bar = 100 μm and right, bar = 40 μm. (h) The OARSI scores based on the staining results of (g). IHH: indian hedgehog; mRNA: messenger RNA; mTORC1: mechanistic target of rapamycin complex 1; NC: nitrocellulose; OA: osteoarthritis; OARSI: osteoarthritis research society international; PCR: polymerase chain reaction; TSC2: tuberous sclerosis complex 2 [Color figure can be viewed at wileyonlinelibrary.com]

Col2a1TSC1KO mice. Matn3 expression was markedly reduced in the cartilage of Col2a1TSC1KO mice compared with their littermate controls, and rapamycin treatment was shown to restore the Matn3 level (Figure 3a). In other words, mTORC1 activation restricted Matn3 during OA development.

Furthermore, we investigated whether or not the regulation of Matn3 by mTORC1 was mediated by miR-483-5p. The primary

chondrocytes were isolated and transfected with *siTSC2* alone or with *siTSC2* plus miR-483-5p inhibitor. Interestingly, TSC2 knockdown resulted in the activation of mTORC1 and substantially decreased Matn3 in chondrocytes. The TSC2 knockdown-decreased Matn3 was then restored by miR-483-5p inhibitors (Figure 3b,c). Similarly, rapamycin treatment inhibited mTORC1 and upregulated Matn3 expression. As expected, the increased level of Matn3 induced by rapamycin was subsequently
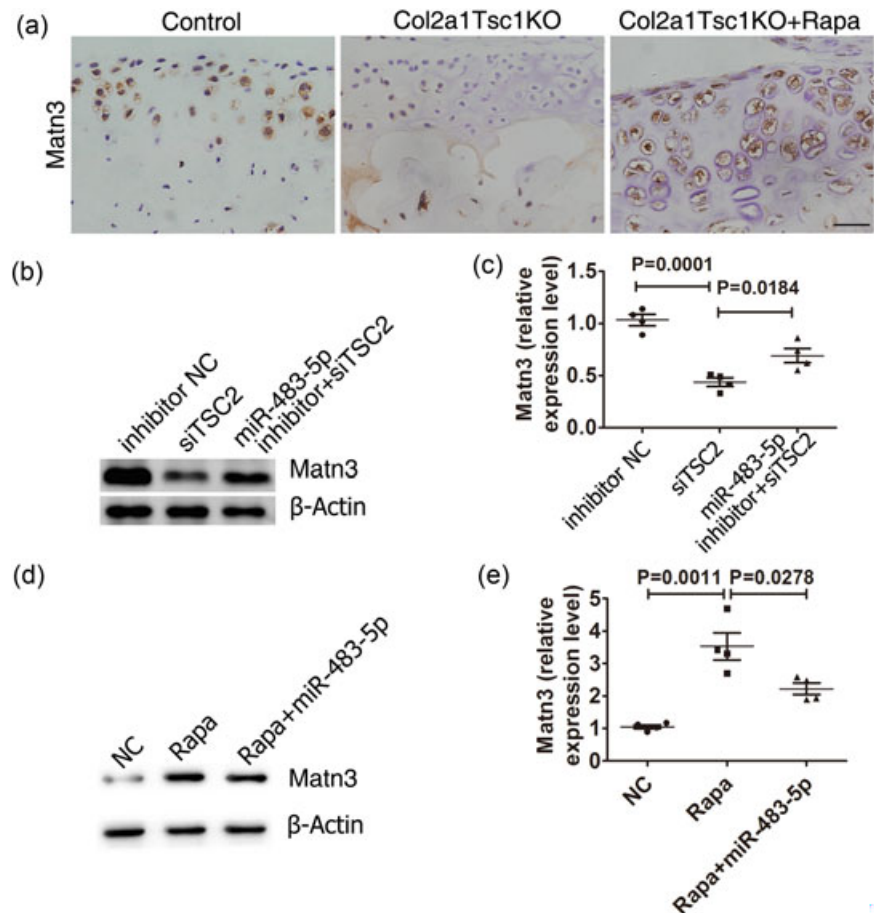
**FIGURE 3** mTORC1 downregulates Matn3 through miR-483-5p in chondrocytes. (a) Immunohistochemical analysis (bar = 40 μm) of Matn3 in the knee joints from Col2a1TSC1KO mice, Col2a1TSC1KO mice treated with Rapa and control mice (*n* = 5, 12-week-old). (b,c) Western blot analyzed and quantified the expression of Matn3 in primary chondrocytes transfected with TSC2 siRNA alone or TSC2 siRNA plus miR-483-5p inhibitor, *n* = 4. (d,e) Western blot analyzed and quantified the expression of Matn3 in primary chondrocytes treated with Rapa alone or Rapa plus miR-483-5p mimics. *n* = 4. Col2a1TSC1KO: cartilage-specific TSC1 knockout; Matn 3: matrilin 3; NC: nitrocellulose; TSC 2: tuberous sclerosis complex 2

reduced by the overexpression of miR-483-5p (Figure 3d,e). Taken together, these data demonstrate that mTORC1 downregulates Matn3 through miR-483-5p in chondrocytes during OA development.

## 3.4 | mTORC1 downregulates TIMP2 through miR-483-5p in chondrocytes to stimulate angiogenesis

To determine whether or not TIMP2 is regulated by mTORC1 in chondrocytes, we treated 8-week-old Col2a1TSC1KO mice with rapamycin for 5 weeks. mTORC1 activation in Col2a1TSC1KO mice downregulated cartilage TIMP2. In contrast, treatment with rapamycin reinduced TIMP2 expression in chondrocytes (Figure 4a). Thus, mTORC1 activation restricted TIMP2 during OA development.

We then sought to determine whether mTORC1 regulates TIMP2 through miR-483-5p, so mice with DMM surgery received daily intragastric administration of rapamycin (2 mg/kg body weight/day) for 5 weeks and/or an intra-articular injection of LV3-miR-483-5p lentivirus (*n* = 5) on Days 7 and 14 after surgery. As expected, rapamycin treatment decreased the level of miR-483-5p and increased the proportion of cells positive for TIMP2 in the tibial plateau. Importantly, the increased level of TIMP2 induced by rapamycin was then reduced by the overexpression of miR-483-5p in chondrocytes (Figure 4b,c). Our results suggest that mTORC1 regulated chondrocyte TIMP2 expression through miR-483-5p during OA development.

Considering that miR-483-5p targets TIMP2 to stimulate subchondral bone angiogenesis, we next examined the role of TIMP2 in chondrocyte mTORC1 activation-induced angiogenesis. Primary chondrocytes were isolated and treated with Rapa alone or with Rapa plus siTIMP2. The culture supernatant was then harvested 48 hr after transfection to simulate human vascular endothelial cells (Zhang, Cao, & Rao, 2005) tube formation in vitro. Interestingly, rapamycin reduced the ability of chondrocytes to stimulate HVEC tube formation, whereas siTIMP2 reinduced the ability of rapamycin-treated chondrocytes to stimulate HVEC tube formation (Figure 4d,e). This evidence indicates that the chondrocyte mTORC1 regulated TIMP2 to control angiogenesis.

Taken together, these findings suggest that mTORC1 downregulates TIMP2 through miR-483-5p in chondrocytes to stimulate subchondral bone angiogenesis.

## 3.5 | mTORC1 regulates miR-483-5p expression via HDAC4 partly

Now that the essential role of miR-483-5p in mTORC1-stimulated OA has been established, the immediate next step is the determination of how mTORC1 controls miR-483-5p. miR-483 is located within intron 2 of the IGF2 gene. Therefore, we first determined whether miR-483 is coexpressed with its host gene IGF2 in response to mTORC1 signals in chondrocytes. Rapamycin was found to decrease miR-483-5p, but not the IGF2 mRNA level (Figure 5a), indicating that mTORC1 controls miR-483-5p expression independently of IGF2 in chondrocytes.
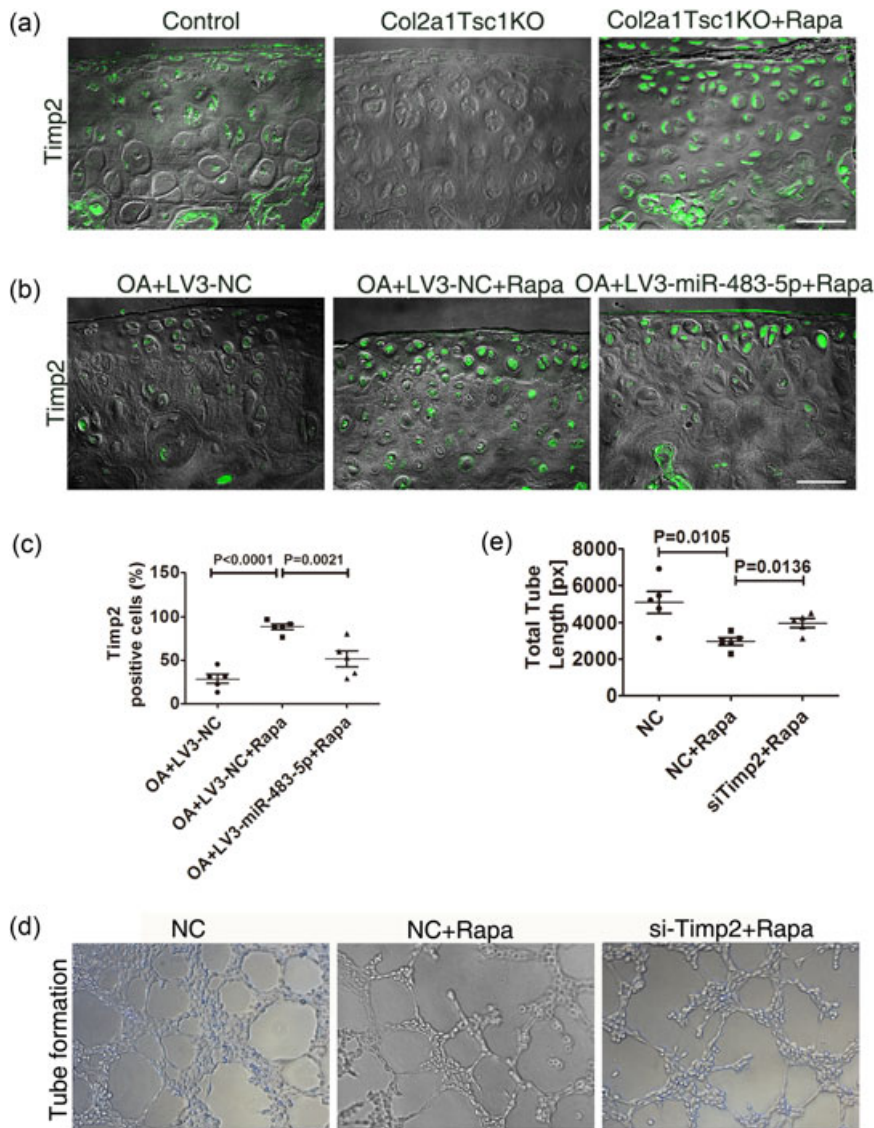
**FIGURE 4** mTORC1 downregulates TIMP2 through miR-483-5p in chondrocytes to stimulate angiogenesis. (a) Immunofluorescence analysis of TIMP2 in Col2a1TSC1KO and control mice treated with or without rapamycin ($n = 5$, 12-week-old), bar = 40 μm. (b) Immunofluorescence analysis of TIMP2 in DMM OA mice treated with rapamycin and injected with or without LV3-miR-483-5p ($n = 5$, 13- to 15-week-old). (c) Statistical analysis of TIMP2-positive cells based on the staining results of (b), $n = 5$. (d,e) HVECs were cocultured with the supernatant of primary chondrocytes treated with rapamycin (50 nM) and infected with or without siTIMP2 for 12 hr, and tube length was measured, $n = 5$. Col2a1TSC1KO: cartilage-specific TSC1 knockout; HVECs: human vascular endothelial cells; mTORC1: mechanistic target of rapamycin complex 1; NC: nitrocellulose; OA: osteoarthritis; TIMP2: tissue inhibitor of metalloproteinase 2; siTIMP2: silencing tissue inhibitor of metalloproteinase 2 [Color figure can be viewed at wileyonlinelibrary.com]

Next, we determined whether mTORC1 affects miR-483-5p at the transcriptional or posttranscriptional level. Rapamycin failed to decrease the level of mature miR-483-5p in primary chondrocytes treated with the transcription inhibitor actinomycin D (Hwang et al., 2017), indicating that rapamycin is unlikely to block the processing of posttranscriptional of miR-483 (Figure 5b). Thus, mTORC1 may regulate transcription rather than the maturation of miR-483-5p.

To further investigate the possibility that mTORC1 signaling may regulate miR-483-5p transcription, we examined luciferase reporters for the miR-483 promoter in primary chondrocytes. As shown in Figure 5c, the miR-483 promoter reporter activity increased compared with the control plasmid, indicating that the promoter regulates miR-483 function. However, rapamycin treatment did not abolish this increased promoter activity (Figure 5c). These observations show that mTORC1 may regulate miR-483 expression through alternative mechanisms, perhaps through epigenetics.

Most work in the OA field has implicated the role of histone deacetylases (HDAC) in chondrocytes (Barter, Bui, & Young, 2012). HDAC4 has also been reported to be associated with chondrocyte hypertrophy (Vega et al., 2004) and OA cartilage degeneration (Kun Cao et al., 2014). Therefore, we analyzed HDAC4 expression in the knee joint cartilage of patients with OA and found that it decreased in human OA cartilage (Figure 5d,e). Interestingly, mTORC1 activation reduced cartilage HDAC4 levels and rapamycin treatment restored the HDAC4 expression in Col2a1TSC1KO mice (Figure 5f). Similar results were observed in DMM OA mice treated with rapamycin (Figure 5g,h). These data demonstrate that mTORC1 downregulates HDAC4 in chondrocytes during OA development.

To investigate the relationship between HDAC4 and miR-483-5p in chondrocytes, HDAC4 was silenced with silencing histone deacetylase 4 (*siHDAC4*). Surprisingly, the downregulation of HDAC4 contributed to an increased miR-483-5p level in primary chondrocytes (Figure 6a). Next, to investigate whether the regulation of miR-483-5p by mTORC1 is mediated by HDAC4, cells were treated with Rapa only or Rapa plus *siHDAC4*. miR-483-5p was downregulated in cells treated with rapamycin, and notably, *siHDAC4* restored rapamycin-downregulated miR-483-5p (Figure 6b) in primary chondrocytes. Considering the role of miR-483-5p in mTORC1-stimulated OA via targeting Matn3 and TIMP2,
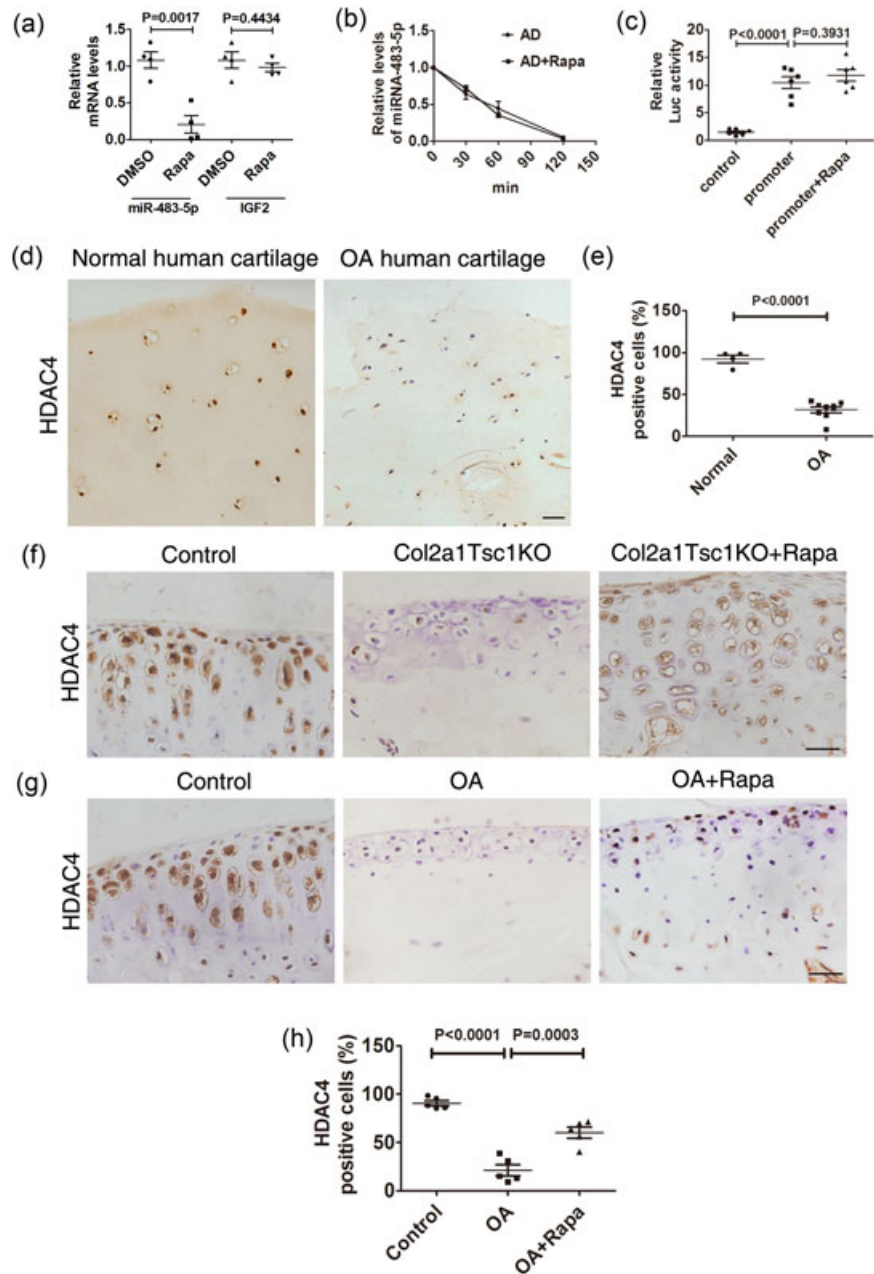
**FIGURE 5** mTORC1 downregulates HDAC4 in chondrocytes during OA development. (a) RT-PCR analysis of miR-483-5p and IGF2 mRNA in primary chondrocytes treated with 50 nM rapamycin for 24 hr, $n = 4$. (b) RT-PCR analysis of the miR-483-5p level in primary chondrocytes incubated with actinomycin D and/or rapamycin, $n = 5$. (c) miR-483 promoter luciferase assay in primary chondrocytes treated with rapamycin for 24 hr, $n = 6$. (d) Immunohistochemical analysis of HDAC4 in human normal ($n = 4$) and OA cartilage (n-9), bar = 50 μm. (e) Statistical analysis of HDAC4-positive cells based on the staining results of (d). (f) HDAC4 immunohistochemical analysis of the knee joints from Col2a1TSC1KO mice treated with or without rapamycin (2 mg/kg/day) for 5 weeks ($n = 5$, 12-week-old), bar = 40 μm. (g) HDAC4 immunohistochemical analysis of knee joints from DMM OA mice treated with or without rapamycin ($n = 5$, 13- to 15-week-old), bar = 40 μm. (h) Statistical analysis of HDAC4-positive cells based on the results of (g). Col2a1TSC1KO: cartilage-specific TSC1 knockout; HDAC 4: histone deacetylase 4; IGF2: insulin-like growth factor 2; mRNA: messenger RNA; mTORC1: mechanistic target of rapamycin complex 1; OA: osteoarthritis; RT-PCR: real time polymerase chain reaction [Color figure can be viewed at wileyonlinelibrary.com]

we detected Matn3 and TIMP2 levels in primary chondrocytes transfected with *siHDAC4* by western blot. As expected, reduced Matn3 and TIMP2 expressions were observed in the cells (Figure 6c). Taken together, our data suggest that mTORC1 downregulates HDAC4 to stimulate miR-483-5p expression in chondrocytes.

## 4 | DISCUSSION

The results of this study demonstrate that articular chondrocyte mTORC1 activation upregulates miR-483-5p during the OA pathologic process. We propose a pathway in which mTORC1 activation partially downregulates HDAC4 in chondrocytes, which subsequently stimulates miR-483-5p to induce cartilage and ECM degradation, chondrocyte hypertrophy, and subchondral bone angiogenesis. This

is a novel mTORC1-HDAC4-miR-483-5p pathway and is critical for OA development.

Under different physiologic and pathologic conditions, mTORC1 responds to many upstream signals, such as insulin, growth factors, amino acids, oxygen, and energy levels to be a central regulator of cell growth, proliferation, motility, survival, protein synthesis, autophagy, and transcription. An important role of activated mTORC1 in OA has been previously reported. Recent studies have established the activation of mTORC1 promotes articular chondrocyte apoptosis via inhibiting autophagy during OA development (Carames et al., 2012; Chagin, 2016; Huang et al., 2014). Our lab also reported mTORC1 activation, partially through the downregulation of FGFR3 and PPR in resting articular chondrocyte to induce proliferation and prehypertrophy. Considering the multiple functions and multiple targets of mTORC1, it may play different roles via different mechanisms at
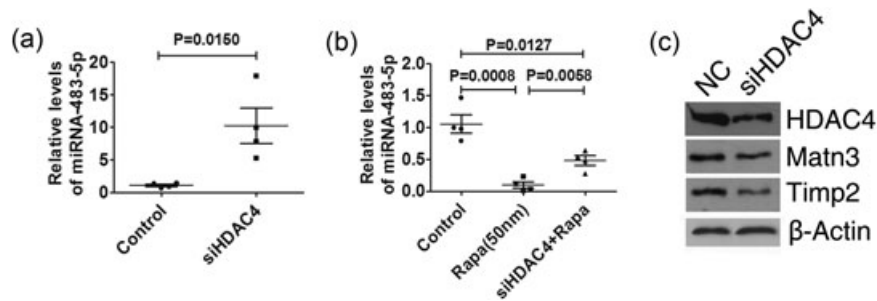
**FIGURE 6** mTORC1 regulates miR-483-5p expression via HDAC4 partly. (a) RT-PCR analysis of miR-483-5p expression in primary chondrocytes transfected with *siHDAC4* for 60 hr, n = 4. (b) RT-PCR analysis of the miR-483-5p level in primary chondrocytes treated with rapamycin and/or *siHDAC4*, n = 4. (c) Western blot analysis of Matn3 and TIMP2 in primary chondrocytes transfected with *siHDAC4*. HDAC4: histone deacetylase 4; Matn3: matrilin 3; mTORC1: mechanistic target of rapamycin complex 1; NC: nitrocellulose; RT-PCR: real time polymerase chain reaction; TIMP2: tissue inhibitor of metalloproteinase 2; *siHDAC4*: silencing histone deacetylase 4

different stages of OA development. Therefore, in this study, we demonstrated a novel mTORC1-HDAC4-miR-483-5p pathway that modulates chondrocyte hypertrophy and subchondral bone angiogenesis in OA development.

We established the regulation of miR-483-5p by mTORC1 via HDAC4 (Figures 5 and 6). HDACs modulate cell growth and differentiation through governing chromatin structure and repressing the activity of specific transcription factors. HDAC4 belongs to the class II HDACs (Christina & Grozinger, 2002), and is expressed in prehypertrophic chondrocytes, regulating chondrocyte hypertrophy, and endochondral ossification through interacting with and inhibit the activity of *Runx2*. Hypertrophic chondrocytes are known to play a transient role in skeletal development. In contrast, articular chondrocytes and the articular cartilage produced by these chondrocytes ideally persist for the lifetime of the individual and must continually avoid entering the hypertrophic pathway. OA development resembles the steps driving the remodeling of the growth plate during the cartilage to bone transition. In OA development, HDAC4 levels decrease and play a similar role as in endochondral ossification. In fact, several studies have implicated the induction of hypertrophy in articular chondrocytes as an OA-promoting factor (Dreier, 2010; Pitsillides & Beier, 2011; van der Kraan & van den Berg, 2012). In our study, we found chondrocyte hypertrophy with decreased levels of HDAC4 in adult Col2a1TSC1KO mice and DMM OA mice. We also found chondrocyte hypertrophy in older TG483 mice. Therefore, the mTORC1 signaling control of HDAC4 and miR-483-5p may be an essential regulator of articular cartilage metabolism, as well as a potential pathway for early intervention in OA.

In conclusion, our results collectively indicate that hyperactivated mTORC1 in articular chondrocytes stimulates miR-483-5p during OA progression, by downregulating HDAC4. Our findings facilitate the understanding of the mechanism of mTORC1 in OA initiation and development. Meanwhile, our study also demonstrates that aberrant levels of histone acetylation in articular chondrocytes regulate the expression of miR-483-5p. Nevertheless, some study limitations need to be mentioned. First, the molecular mechanism of the regulatory between mTORC1 and HDAC4 is still unknown. In future studies,

HDAC4 interaction protein will be screened in the articular cartilage of Col2a1TSC1KO mice compared with control mice using immunoprecipitation mass spectrometry. Further bioinformatics and proteomics will be used to clarify the unknown interaction between mTORC1 and HDAC4. In addition, we need to know how HDAC4 is regulating miR-483-5p. Vega et al. (2004) had reported that HDAC4 reduced acetylation of histone H3 around the *Runx2* promoter, thereby suppressing transcriptional expression and inhibiting chondrocyte hypertrophy. In this study, we will try to examine the effect of HDAC4 on miR-483-5p association with the miR-483-5p promoter and the acetylation of histone H3 on the promoter by chromatin immunoprecipitation in mouse primary chondrocytes.

## CONFLICTS OF INTEREST

The authors declared no conflicts of interest.

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## SUPPORTING INFORMATION

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