Cellular Signalling 63 (2019) 109379

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Long non-coding RNA MEG3 inhibits chondrogenic differentiation of synovium-derived mesenchymal stem cells by epigenetically inhibiting TRIB2 via methyltransferase EZH2

Di You, Cheng Yang, Jun Huang, Haoli Gong, Mingming Yan, Jiangdong Ni*

Department of Orthopedics, The Second Xiangya Hospital, Central South University, Changsha, Hunan, PR China

ARTICLE INFO

Keywords:
Osteoarthritis
Synovium-derived mesenchymal stem cells
MEG3
TRIB2
EZH2
Chondrogenic differentiation

ABSTRACT

Osteoarthritis (OA) is a highly prevalent skeletal disease. Mesenchymal stem cell-derived cartilage tissue engineering is a clinical method used for OA treatment. Investigations on the molecular regulatory mechanisms of the chondrogenic differentiation of synovium-derived mesenchymal stem cells(SMSCs) will help promote its clinical applications. In this study, bioinformatics analysis from three different databases indicated that the long non-coding RNA (lncRNA) MEG3 may regulate the chondrogenic differentiation of SMSCs by targeting TRIB2. We then performed assays and found that both knockdown of MEG3 or overexpression of TRIB2 can stimulate the chondrogenic differentiation of SMSCs and increase Col2A1 and aggrecan expression. Knockdown of MEG3 can induce the expression of TRIB2; conversely, overexpression of MEG3 can inhibit the expression of TRIB2. Futhermore, knockdown of the TRIB2 can rescue the MEG3 silencing-mediated promotion of chondrogenic differentiation. Moreover, RNA immunoprecipitation(RIP) and RNA pull-down assays demonstrated that MEG3 can interact with EZH2, thus recruiting it to induce H3K27me3, which promotes the methylation of TRIB2 by binding with the promoter of TRIB2 in SMSCs. Additionally, EZH2 silencing significantly rescued the MEG3 overexpression-mediated inhibition of TRIB2 expression and chondrogenic differentiation of SMSCs. Taken together, these data indicated that MEG3 regulates chondrogenic differentiation by inhibiting TRIB2 expression through EZH2-mediated H3K27me3.

1. Introduction

Osteoarthritis (OA) is a highly prevalent age-related skeletal disease [1]. A recent epidemiological study has shown that an estimated 10% of males and 13% of females over 60 years old suffer from OA [2]. Despite this, no efficient clinical therapeutic agent or method has been established to cure OA [3]. The secretion of inflammatory factors (IL1-b and TNF-a) from synovial membranes and cartilage causes the production and secretion of matrix degradation enzymes, which degrade the cartilage matrix, and impair articular cartilage [4]. Therefore, the treatment of OA must involve the repair of articular cartilage.

Mesenchymal stem cells (MSCs) are found in the bone marrow, adipose tissue, synovial membranes and synovial fluid. MSCs possess multipotent and the ability to differentiate into osteoblasts, chondrocytes, adipocytes, and myoblasts [5,6]. The application of MSCs to repair articular cartilage provides a potential approach for the treatment of OA [7]. Synovium-derived mesenchymal stem cells (SMSCs) possess the full multipotent of MSCs and are involved in the regeneration of osteoarthris cartilage in vivo [8]. Furthermore, SMSCs have been shown to have the greatest chondrogenesis potential, compared with MSCs derived from other tissues [9]. Therefore, SMSCs are considered to be ideal stem cells for cartilage repair in OA therapy [10]. However, the molecular regulation of chondrogenesis by MSCs requires further elucidation.

Long non-coding RNAs (lncRNAs) are RNA sequences of > 200 nucleotides (nt) in length and which are not translated into proteins [11]. They are known to regulate cellular activities, such as proliferation [12–14], differentiation [15] and apoptosis [16,17]. Recently, increasing evidence has revealed that lncRNAs regulate MSC differentiation [15,18–20]. LncRNA MEG3 (maternally expressed 3) is a maternally expressed lncRNA located on the human chromosome 14 [21]. Early studies have shown that lncRNA MEG3 can activate *p53*,

* Corresponding author at: Department of Orthopedics, The Second Xiangya Hospital, Central South University, No. 139 Renminzhong Road, 41000 Changsha, Hunan, PR China.

E-mail addresses: youdi1988@csu.edu.cn (D. You), doctoryang@csu.edu.cn (C. Yang), 505447@csu.edu.cn (J. Huang), gonghaoli@csu.edu.cn (H. Gong), yaomnike@csu.edu.cn (M. Yan), nijiangdong@csu.edu.cn (J. Ni).

https://doi.org/10.1016/j.cellsig.2019.109379
Received 30 May 2019; Received in revised form 26 July 2019; Accepted 27 July 2019
Available online 31 July 2019

thus acting as a tumor suppressor [22]. In addition, MEG3 was downregulated in OA tissues [23] (PMID: 26090403) and MEG3 knockdown might lead to the progression of OA [24](PMID: 29255591); also, upregulation of lncRNA MEG3 was found to enhance the osteogenic differentiation of MSCs [25]. Therefore, we hypothesized that MEG3 may participate in the chondrogenic differentiation of SMSCs.

Biochemical investigations have indicated that lncRNA MEG3 regulates gene expression by association with chromatin, and exert an effect via epigenetic mechanisms [26,27] Polycomb Repressive Complex 2 (PRC2) is a large molecular machine which catalyzes the methylation of histone H3 at Lys(H3K27). It is evolutionarily conserved in Drosophila, mammals, and plants [28,29] (PMID: 31032401/PMID: 30967505). EZH2 protein, the catalytic component of PRC2, was found to bind with lncRNA MEG3 directly [26]. In this manner, MEG3 recruits PRC2 to specific gene targets in chromatin, and PRC2 exerts its function for modulating chromatin, subsequently leading to the regulation of specific gene expression.

Tribbles homolog 2 (TRIB2), a member of the tribbles family (TRIB1, TRIB2, TRIB3), is first identified in Drosophila as mitosis blocker that regulates embryo and germ cell development [30] (PMID:10850494). TRIB2 plays a crucial role in regulating human diseases, such as acute myeloid leukemia [31] (PMID:17097562), liver cancer [32] (PMID: 2376967), colorectal cancer [33] (PMID: 30541550). Also, TRIB2 appears to play roles in inflammatory diseases [34] (PMID:26517922), however, the biological role of TRIB2 in OA is not fully understood.

In our study, we focused on the regulatory molecular mechanisms of chondrogenic differentiation of SMSCs. We revealed that TRIB2 was a target of MEG3 and MEG3 can inhibit TRIB2 expression. Also, the inhibition of MEG3 or upregulation of TRIB2 can promote the chondrogenic differentiation of SMSCs. Furthermore, we demonstrated that MEG3 can suppress TRIB2 by interacting with EZH2 in SMSCs. Taken together these data suggest that MEG3 is a promising target for therapeutic interventions in patients with OA.

2. Materials and methods

2.1. Bioinformatics analysis

Differentially expressed genes in GSE114007 and GSE31980 fom the GEO database were analyzed using bioinformatics analysis. Limma-Microarray/Counts were used to calculate gene expression with $|\log2FC| > 1$, with FDR < 0.05 as a screening threshold. Differentially expressed genes were analyzed using the Pearson's correlation coefficient method. The GSE114007 dataset comprises ten samples of human healthy knee chondrocytes and ten samples of osteoarthritis articular cartilage tissue, and the GSE31980 dataset comprises three samples of synovial stem cell monolayer cultured samples and synovial stem cell aggregation (cartilage tissue repair) cultured samples. Simultaneously, bioinformatics was used to analyze ChIP-seq data [26] to obtain differentially expressed genes following MEG3 knockdown. The STRING database was used for analysis of the TRIB2 action network diagram, and the Cyctoscape 3.6 software was used to draw the network diagram.

2.2. Isolation and characterization of synovial MSC

The human synovium was harvested from the knees of ten donors with osteoarthritis during total knee arthroplasty. Cells were isolated from the synovium tissue of extracted as previously described [35]. P4 cells at confluence in 25 cm^2 flasks were collected and stained for 30 min at 4 °C with antibodies against human CD44 (338803, Biolegend), CD90 (328107, Biolegend), CD14 (367103, Biolegend) and CD45 (368509, Biolegend), as well as anti-IgG antibody (400109, Biolegend). Subsequently, cells were analyzed using a FACS Calibur flow cytometer and Cell Quest software (BD Biosciences). The study was approved by the Ethics Committee of the Second Xiangya Hospital of Central South University. Written informed consent was obtained from all donors.

2.3. Multilineage differentiation of SMSCs

SMSCs were cultured in Human Mesenchymal Stem Cell Growth Medium (Cyagen Biosciences, Inc., Guangzhou, China) supplemented with 10% FBS, 10 mM glutamine and 100 U/L penicillin-streptomycin at 37 °C with 5% CO_2. Cells were then treated with osteogenic, chondrogenic and adipogenic induction medium for 2–3 weeks as previously reported [36,37]. Trilineage differentiation of human synovial MSCs was evaluated with alizarin red, alcian blue and oil red O staining using a Human Mesenchymal Stem Cell Differentiation kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

2.4. Cell transfection

For cell transfection, SMSCs (1.5×10^5 cells/well) were cultured in 6-well plates overnight. SiMEG3, siTRIB2, pcDNA-TRIB2 or corresponding negative controls were transfected into SMSCs using Lipofectamine-2000 (Invitrogen) according to the manufacturer's protocol. Following transfection for 48 h, the transfection efficiency was measured by quantitative reverse transcription PCR (qRT-PCR) and western blotting analysis. MEG3 small interfering RNA (siRNA), TRIB2 siRNA, EZH2 siRNA and pcDNA-TRIB2 were purchased from GenePharma (Shanghai, China).

2.5. RNA isolation and quantification

Total RNA was extracted using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. A Primer-Script One Step RT-PCR Kit (TaKaRa, China) was used for reverse transcription. An SYBR Premix DimerEraser Kit (TaKaRa, China) was used for real-time RT-PCR. Primers were designed by Shanghai Sangon Biotech Co., Ltd., and their sequences were as follows: MEG3 (GenBank Accession Number NR_046465); forward 5′-AGACAACAGGCCGTCCAGGAG-3′, reverse 5′-GAAGAGCGAGTCAGGAAGCAGTG-3′; TRIB2 (GenBank Accession Number NM_021643); forward 5′-TGTTCTACCAGATTGCCTCGG-3′, reverse 5′-GCTTGTCGGAGAGGGAATCAT-3′; and β-actin (GenBank Accession Number JN038572); forward 5′-AGGGGCCGGACTCGTCAT ACT-3′, reverse 5′-GGCGGCACCACCATGTACCCT-3′. β-actin expression was used for normalization. The $2^{-\triangle\triangle Ct}$ method was used to calculate the relative expression changes in RNAs.

2.6. Nuclear-cytoplasmic fractionation

The nucleus and cytoplasm were separated of SMSCs according to the manufacturer's instructions of PARIS™ Kit (Life Technologies). The expression of MEG3, GAPDH and U6 in nucleus and cytoplasm were detected through RT-qPCR. GAPDH was used as a cytoplasm control and U6 was used as a nuclear control.

2.7. Western blotting

Western blotting was performed as previously described [38]. The blot was probed using the following antibodies: anti-TRIB2 (ab117981, Abcam), anti-EZH2 (ab191250, Abcam), anti-Col2A1 (YT1022, Immunoway), anti-aggrecan (ab3778, Abcam), anti-H3K27me3 (ab6147, Abcam), anti-β-actin (#4970, Cell Signaling Technology) and anti-GAPDH(#5174, Cell Signaling Technology). Secondary antibodies were sourced from Beijing Zhong Shan Biotechnology Co.Ltd. (Beijing, China).

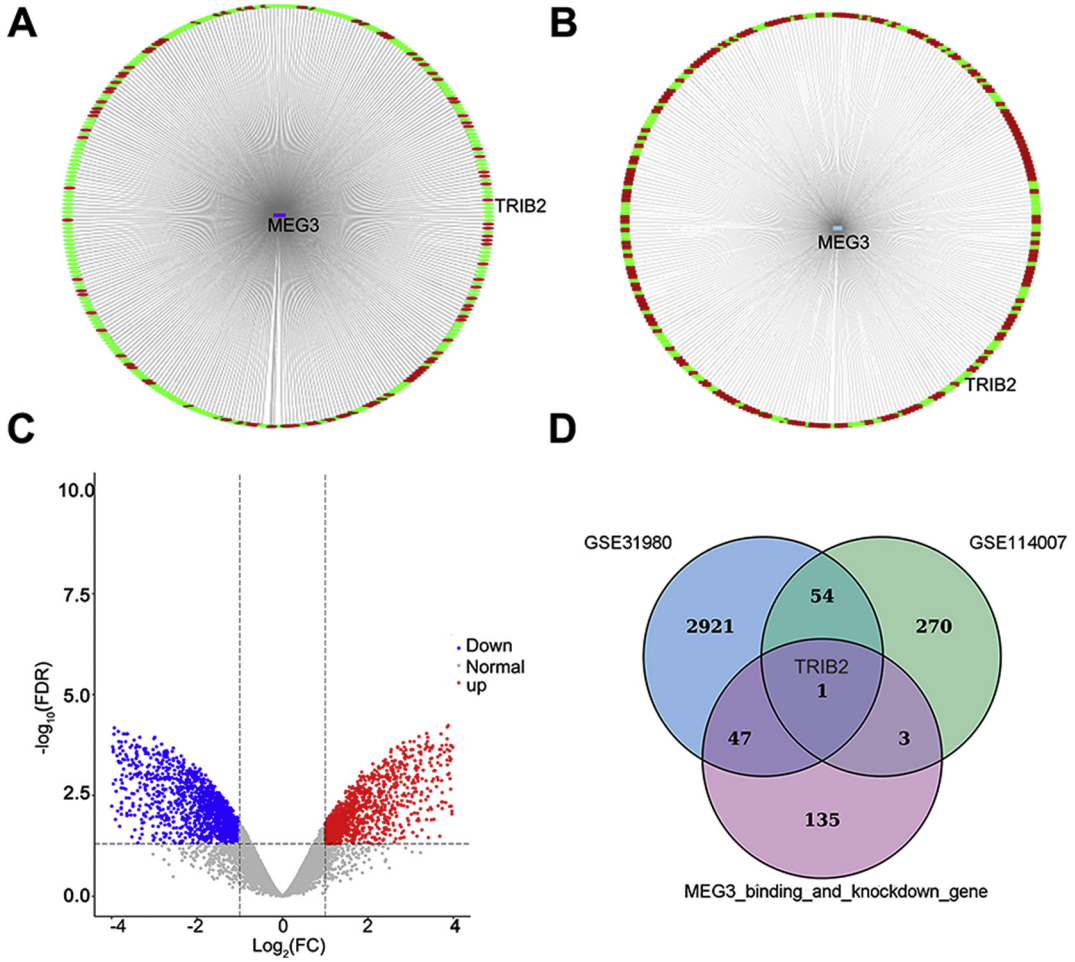

Fig. 1. Bioinformatics analysis. A Differentially expressed genes in the GSE114007 dataset co-expressed with MEG3 (osteoarthritis vs healthy). B Effects of MEG3 knockdown on differentially expressed genes (knockdown vs control). C Differentially expressed genes (SMSCs vs SMSC-aggregates) in GSE31980 dataset. D Differentially expressed genes in the GSE114007, MEG3 knockdown and GSE31980 shown as a Wayne diagram.

2.8. Immunofluorescence assay

Cells were inoculated on coverslips in a 6-well plate overnight. Subsequently, they were fixed using 4% paraformaldehyde, permeabilized using PBS solution and 0.5% Triton X-100, followed by blocking. Primary antibodies (anti-Col2A1 (YT1022, Immunoway), anti-aggrecan (ab3778, Abcam)) were added to the cells and they were then incubated for 30–45 min at 37 °C followed by incubation with secondary antibodies for another 30–45 min at 37 °C. Finally, the cells were mounted onto slides with a mounting solution, and 0.2 mg/mL DAPI was added, cover slips were placed over the cells and sealed with nail polish. Slides were stored in the dark at 4 °C until they could be examined under a fluorescence microscope.

2.9. RNA pull-down assay

RNA pull-down assay was performed to identify the interactions between MEG3 and EZH2 proteins using an RNA-Protein Pull-Down Kit (Thermo Scientific), according to the manufacturer's instructions. MEG3 was labeled with biotin using Biotin RNA Labeling Mix (Roche) according to the manufacturer's instructions. Biotinylated RNAs were then incubated with streptavidin magnetic beads (Invitrogen) and the cell lysates were incubated for 1 h. The RNA-protein complexes were used to conduct western blotting analysis of EZH2 and H3K27me3. The input sample was used as a control to verify if the western blot was functioning correctly.

2.10. RNA-binding protein immunoprecipitation (RIP)-PCR assay

An RNA-binding protein immunoprecipitation (RIP) assay was performed using anti-EZH2 antibodies to determine whether MEG3 can be enriched in the complex that was immunoprecipitated by anti-EZH2 antibodies. The assay was performed using an RIP Kit (BersinBio, Guangzhou, China) with EZH2 (Abcam, ab191250) antibodies according to the manufacturer's instructions. qRT-PCR was performed as described above to measure the levels of MEG3 associated with EZH2. The results were normalized relative to the input control.

2.11. Chromatin immunoprecipitation-PCR

The EZ-ChIP KIT (Millipore, USA) was used for ChIP assays according to the manufacturer's protocol. Normal IgG antibodies served as negtive control (NC). Specific primers used for the TRIB2 promoter were as follows: forward; 5′-AGGAGGATGTCGGAGAGAT-3′, reverse; 5′-CATGTTCCTGGCGCTTTTCCC-3′. The results were calculated as a percentage relative to the input DNA level.

2.12. Statistical analysis

The data are presented as mean ± SD. Statistical significance ($p < .05$), which is indicated in the figures, was determined using two-tailed paired Student's *t*-test or analysis of variance (ANOVA) for the comparison of more than two groups comparison (GraphPad Prism 5, San Diego, California, USA). TheImage-Pro-Plus 6.0 software (Media

Cybernetics, Rockville, USA) was used to analysis fluorescence quantitative.

3. Results

3.1. TRIB2 as a candidate gene based on bioinformatics analysis

To explore how MEG3 can regulate the chondrogenic differentiation of SMSCs (a potential target for cartilage repair in OA therapy), we analyzed three datasets using bioinformatics approach. The GSE114007 dataset, comprises ten samples of human healthy knee chondrocytes and ten samples of osteoarthritis articular cartilage tissue from patients with osteoarthritis. The results of the GSE114007 dataset analysis showed that there were 3200 differentially expressed genes in human healthy knee chondrocytes and osteoarthritis chondrocytes, among which 328 were co-expressed with MEG3 (Fig. 1A). To further analyze the target of MEG3, we analyzed ChIP-seq data from previous study [26], the results of which showed that the expression of 300 genes changed following MEG3 knockdown. Among these genes, 118 genes were negatively correlated with MEG3 expression (Fig. 1B). Furthermore, we also analyzed the GSE31980 dataset that comprises three samples of synovial stem cell monolayer cultured samples and synovial stem cell aggregation (cartilage tissue repair) [39]. The results showed that there were 3024 differentially expressed genes in the monolayer culture compared with the aggregation culture of synovial stem cells (cartilage repair) (Fig. 1C); therefore, we speculated 3024 differentially expressed genes may be involved in the chondrogenic differentiation of SMSCs. Next, we determined that TRIB2 was a candidate gene to cross the intersection of GSE114007, GSE31980 and MEG3 knockdown data (Fig. 1D). These data showed that TRIB2 expression was lower in osteoarthritis chondrocytes than in healthy chondrocytes, and that it is co-expressed with MEG3(GSE114007); Furthermore, TRIB2 was negatively correlated with MEG3 expression and TRIB2 was highly expressed in the chondrogenic differentiation of SMSCs. Therefore, we speculated that MEG3 affects the differentiation of SMSCs into cartilage by regulating TRIB2.

3.2. Culture and identification of SMSCs

To confirm the previous hypothesis, we isolated SMSCs from synovium tissue, and analyzed the markers using flow cytometry. We found that SMSCs can express the mesenchymal stem cell markers CD44 and CD90, whereas the hematopoietic cell markers CD45 and CD14 were negatively expressed (Fig. 2A). The positive and negative immunophenotypic characteristics of the isolated SMSCs were consistent with those observed in previous studies [40,41]. We also detected multipotency of human SMSCs. And found separated SMSCs long spindles (Fig. 2B). SMSCs cultured in an osteogenic medium showed a high osteogenic differentiation ability when analyzed using alizarin red staining (Fig. 2C). Additionally, oil red O staining showed that SMSCs possessed adipogenic differentiation ability (Fig. 2D), whereas alcian blue staining demonstrated their chondrogenic differentiation ability (Fig. 2E).

3.3. Knockdown of MEG3 expression or overexpression of TRIB2 promotes chondrogenesis in SMSCs

To identify the role of MEG3 and TRIB2 in the chondrogenic differentiation of SMSCs, the expression of MEG3 and TRIB2 following SMSC-induced chondrogenic differentiation was investigated. MEG3 expression decreased following the induction of chondrogenic differentiation (Fig. 3A), whereas TRIB2 expression increased (Fig. 3B & C) compared with control cells. To further study the effect of MEG3 on chondrogenic differentiation of SMSCs, a loss-of-function study using siRNAs was performed. Si-MEG3 was transfected into SMSCs to reduce the expression of MEG3 (Fig. 3D). Alcian blue staining showed that interfering with MEG3 in SMSCs can induce SMSCs chondrogenic differentiation (Fig. 3E), and that downregulation of MEG3 can promote the expression of aggrecan and Col2A1 as determined by western blotting and immunofluorescence analysis (Fig. 4A & B). Bioinformatics analysis showed that TRIB2 may be a target of MEG3 and may also be negatively regulated by MEG3. Therefore, we explored the role of TRIB2 in the chondrogenic differentiation of SMSCs. SMSCs were transfected with OE-TRIB2 to increase TRIB2 expression (Fig. 5A & B). Surprisingly, the overexpression of TRIB2 resulted in a phenomenon similar to that resulting from the downregulation of MEG3. Briefly, TRIB2 induced the chondrogenic differentiation of SMSCs (Fig. 5C) and also promoted the expression of aggrecan and Col2A1, which are cartilage markers (Fig. 5D & E).

3.4. MEG3 influences differentiation of MSC into chondrocytes via regulating of TRIB2

According to the above results, both MEG3 and TRIB2 affect the chondrogenic differentiation of SMSCs. Simultaneously, bioinformatics analysis showed that the knockdown of MEG3 upregulate the expression of TRIB2. To further verify whether MEG3 regulates TRIB2, we examined the effect of MEG3 interference on TRIB2. As shown in Fig. 6A, B & C, the overexpression of MEG3 inhibited TRIB2 expression, whereas TRIB2 expression increased after MEG3 interference. These results suggest that MEG3 plays a role in the expression of TRIB2. To further investigate the relationships among MEG3, TRIB2 and the chondrogenic differentiation of SMSCs, we performed rescue assays by either transfection of si-MEG3, or si-TRIB2, or co-transfection with si-MEG3 and si-TRIB2 into SMSCs. The results showed that the inhibition of TRIB2 expression can inhibit the expression of AGGRECAN and Col2A1. Conversely, the inhibition of MGE3 expression can promote the expression of AGGRECAN and Col2A1. Meanwhile, si-TRIB2 can significantly reverse the effects of si-MEG3 (Fig. 6D & E). Taken together, the results of our study indicated that TRIB2 is a downstream target of MEG3 in chondrogenic differentiation of SMSCs.

3.5. MEG3 binds to EZH2 and the epigenetic silencing of TRIB2 expression in SMSCs

To further elucidate the underlying mechanism of how MEG3 regulates TRIB2, the major locus of MEG3 in SMSCs was investigated. The results showed that MEG3 is mainly located in the nucleus (Fig. 7A), which is consistent with that reported in previous reports [42], suggesting that MEG3 exerts its regulatory function at the level of gene transcription. A previous study has shown that MEG3 plays a role in gene expression by recruiting PRC2 and acting as the corresponding target gene site of chromatin via an epigenetic mechanism [43],furthermore, MEG3 was found to be directly associated with EZH2, the catalytic subunit of PRC2 [26]. Other studies have also shown the function of some lncRNAs to be dependent on EZH2 [44–46]. An RNA immunoprecipitation assay using EZH2 antibodies verified that MEG3 can bind to EZH2 in SMSCs(Fig. 7B) and an RNA pull-down assay showed similar results, that MEG3 can bind with EZH2 and H3k7me3 (Fig. 7C). Moreover, EZH2 and H3K27me3 which bind to MEG3 were significantly reduced when MEG3 was knocked down in SMSCs (Fig. 7D). Furthermore, we silenced EZH2 expression using siRNAs and promoted EZH2 expression using pCDNA3.1-EZH2. As expected, the results showed that silencing EZH2 promoted TRIB2 expression, whereas the overexpression of EZH2 inhibited TRIB2 expression (Fig. 8A & B). OE-MEG3(pcDNA3.1-MEG3) significantly inhibited the expression of TRIB2, but promoted the expression of H3K27me3, while co-transfection with si-EZH2 and OE-MEG3 partially rescued the OE-MEG3-inbited expression of TRIB2 and OE-MEG3-promoted expression of H3K27me3 (Fig. 8C). Therefore, we hypothesized that MEG3 may repress TRIB2 by binding to EZH2 and H3K27me3-trimethylation. To investigate our hypothesis, we performed ChIP-qPCR and rescue assays.

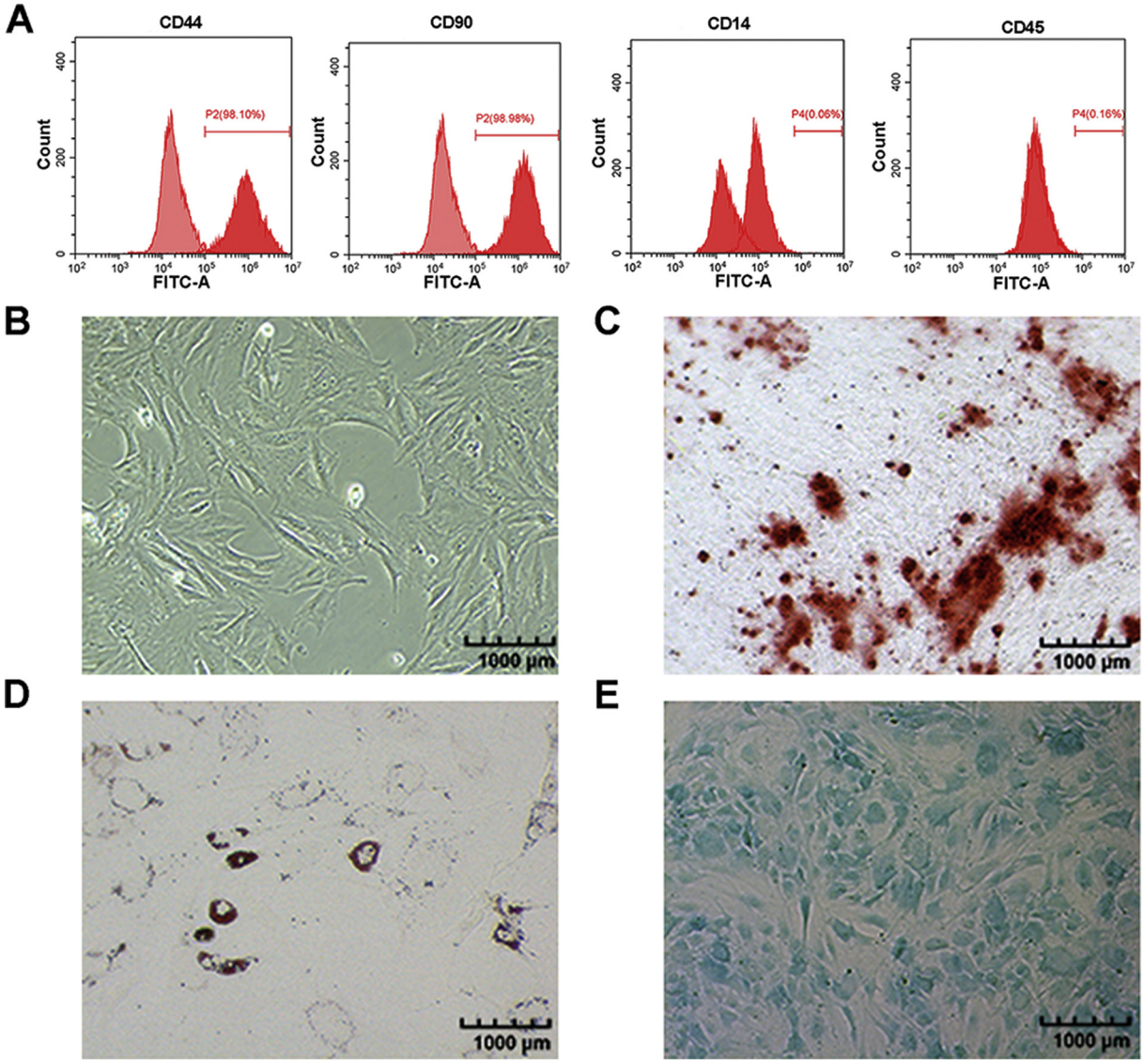

Fig. 2. Isolation and identification of SMSCs. A Molecular markers on cell surfaces were identified using flow cytometry (CD44 and CD90 were positive markers, CD45 and CD14 were negative markers). B SMSCs under white light-microscopy (×100). C Alizarin red staining after SMSCs were induced (×100). D Oil red O staining after SMSCs were induced (×100). E Alcian blue staining after SMSCs were induced (×100). Note: The scale bar represents 1000 μm(×100). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The ChIP-qPCR results showed that while EZH2 and H3K27me3 can bind to the promoter of TRIB2 and H3K27me3 binding in the promoter regions of TRIB2 was significantly reduced when MEG3 was knocked down in SMSCs(Fig. 8D). Furthermore, the rescue assay showed that OE-MEG3 can inhibit the expression of aggrecan and Col2A1, whereas co-transfection with si-EZH2 and OE-MEG3 partially rescued the OE-MEG3-inbited expression of AGGRECAN and Col2A1 (Fig. 8E). Thus, these data indicated that MEG3 can act as a co-inhibitor with EZH2 to inhibit TRIB2 expression and subsequently inhibit cartilage formation.

4. Discussion

As a highly prevalent skeletal disease, osteoarthritis(OA) is a major threat to public health and is also associated with a high economic burden [47]. Unfortunately, at present there are no efficient therapeutic agents or surgical methods with which to cure OA, therefore, the investigations on OA pathogenesis and the development of new therapeutic methods are of critical importance. According to previous reports, lncRNA MEG3 can regulate the development of OA. Su W. et al. have revealed that lncRNA MEG3 was downregulated and negatively associated with VEGF levels in OA tissue [23]. Recent studies have also indicated that the downregulation of lncRNA MEG3 can led to OA progression [24]. In our study, we used bioinformatics analysis of OA databases in order to determine if there are other mechanisms in OA pathogenesis that involve MEG3. After analyzing the OA database GSE114007, we found that 328 OA-related genes were correlated with the expression of MEG3. Insights from the ChIP-sequence database indicated that the expression of 118 genes has a negative correlation with MEG3. Given that synovial MSCs possess the greatest potency for chondrogenesis, they may also have the highest potential for cartilage repair in the treatment of OA. We attempted to analyze the gene expression profile of synovial MSCs during chondrogenesis from another database GSE31980. We found that the TRIB2 gene is correlated with MEG3 in all the three databases mentioned above. Therefore, TRIB2 may be a regulatory target for MEG3, as predicted by bioinformatics analysis. TRIB2, which belongs to the Tribbles pseudokinase group, is a critical mediator for various cell signaling activities and is essential for different types of cellular processes, such as differentiation, proliferation and survival [48]. Our bioinformatics analysis results implicated that MEG3 and TRIB2 may both play a role in SMSCs.

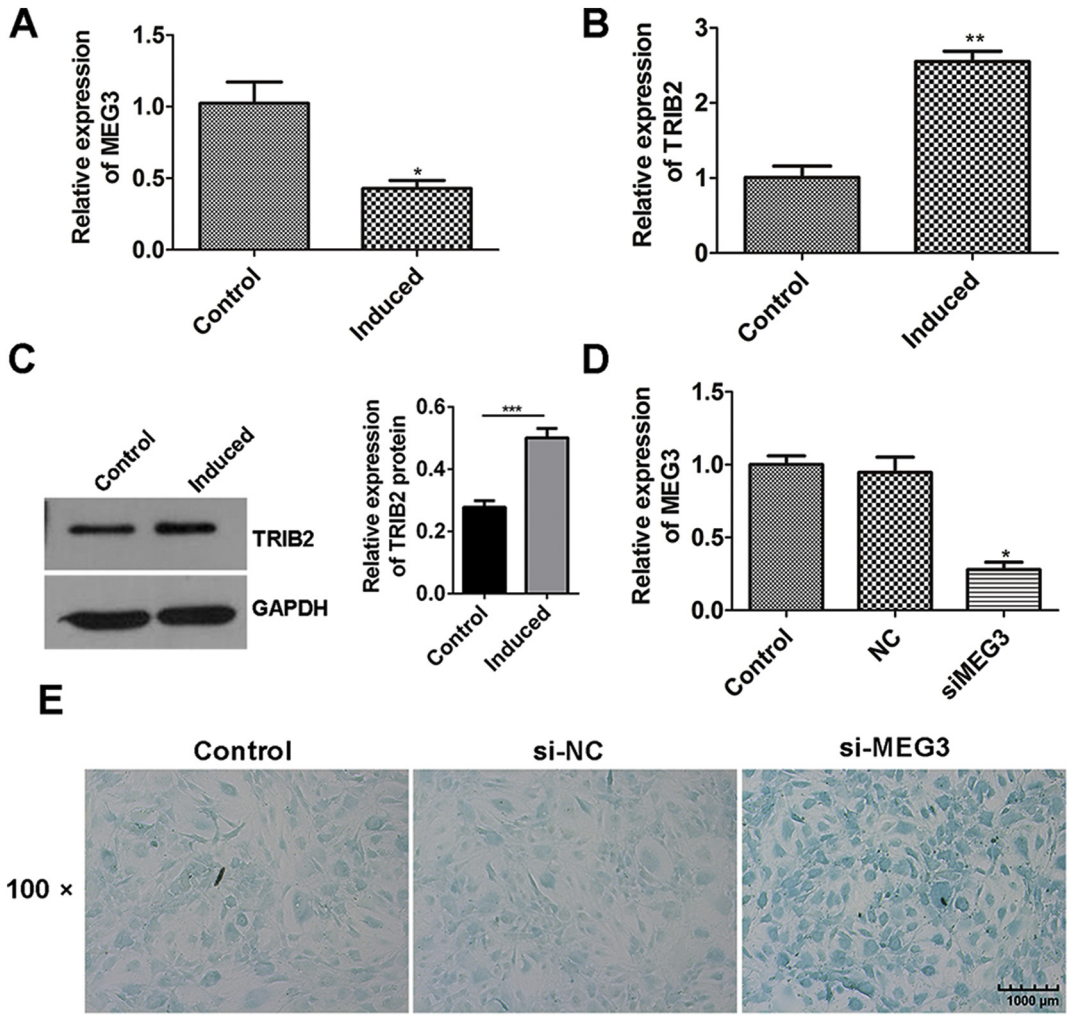

Fig. 3. MEG3 knockdown promotes chondrogenesis in SMSCs. A Expression of MEG3 detected by qPCR after SMSCs were induced. B Expression of TRIB2 detected by qPCR after SMSCs were induced. C Expression of TRIB2 detected by western blotting after SMSCs were induced. D Expression of MEG3 detected by qPCR after transfection with si-MEG3 for 48 h. E Chondrocytes were detected by alcian blue staining after transfection with si-MEG3 for 48 h. Notes: Control, cells without treatment; Induced, cells with induction medium added; si-NC, cells transfected with si-RNA as a negative control(si-NC); si-MEG3, cells transfected with si-MEG3; The scale bar represents 1000 μm(100 ×). All data are presented as mean ± SD, n = 3. *p < .05, **p < .01, ***p < .001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To investigate our hypothesis, we isolated SMSCs, which were confirmed by the assay of the positive surface markers CD44 and CD90 [49], as well as the negative markers CD45 and CD14. In addition, SMSCs were confirmed by testing different types of differentiation potency, including osteogenesis, chondrogenesis and adipogenesis. We found that the expression of MEG3 was downregulated; whereas, TRIB2 expression was stimulated following chondrocyte differentiation. Therefore, we hypothesized that MEG3 exerts a negative effect on chondrogenesis, but TRIB2 potentiates chondrogenesis in SMSCs. This hypothesis was confirmed by the knockdown of MEG3, or the overexpression of TRIB2 in SMSCs. Each of these assays showed that chondrogenesis of SMSCs was promoted by the knockdown of MEG3 or the promotion of TRIB2. Therefore, MEG3 inhibits the chondrogenic differentiation of SMSCs, whereas TRIB2 promotes the chondrogenic differentiation.

Our study showed that the knockdown of MEG3 can promote the expression of TRIB2, conversely, the overexpression of MEG3 can inhibit TRIB2 expression. Furthermore, si-MEG3 stimulated chondrogenic differentiation can be reversed by the knockdown of TRIB2. Therefore, it is reasonable to conclude that MEG3 suppresses the chondrogenic differentiation of SMSCs by inhibiting the expression of TRIB2. The molecular mechanisms of the interactions between MEG3 and TRIB2 in SMSCs remained unclear, therefore we next aim to gain a deeper insight into the biochemical details of how MEG3 suppresses the expression of TRIB2. Previous studies have shown that lncRNAs can activate or inactivate gene expression via DNA methylation, chromosome reprogramming or histone protein modification through their interactions with RNA binding proteinsc [44,46]. More than 20% of lncRNAs are bound by the epigenetic functional protein complex PRC2 to silence the downstream target genes [50]. EZH2 is a core subunit of the PRC2 complex that can catayze the trimethylation of lysine residue 27 of histone 3(H3K27me3) which is overexpressed in many cancers [51]. According to previous studies, MEG3 recruits PRC2 to chromatin and binds to specific DNA regions of target genes [52]. Here, the RNA-protein interaction between MEG3 and EZH2, the main component of PRC2, was examined. The RIP and RNA-pull-down experiments revealed that MEG3 binds to EZH2 and H3K27me3, and that the knockdown of MEG3 can reduce the binding between MEG3 and EZH2 or H3K27me. Furthermore, the knockdown of EZH2 promoted the expression of TRIB2 and H3K27me3; conversely, the overexpression of EZH2 inhibited the expression of TRIB2 and H3K27me3. The overexpression of MEG3 can reverse the effect of si-EZH2 on TRIB2 and H3K27me3. ChIP-qPCR showed that knockdown of MEG3 resulted in EZH2 and H3K27me3 losing their ability to bind to the promoter of

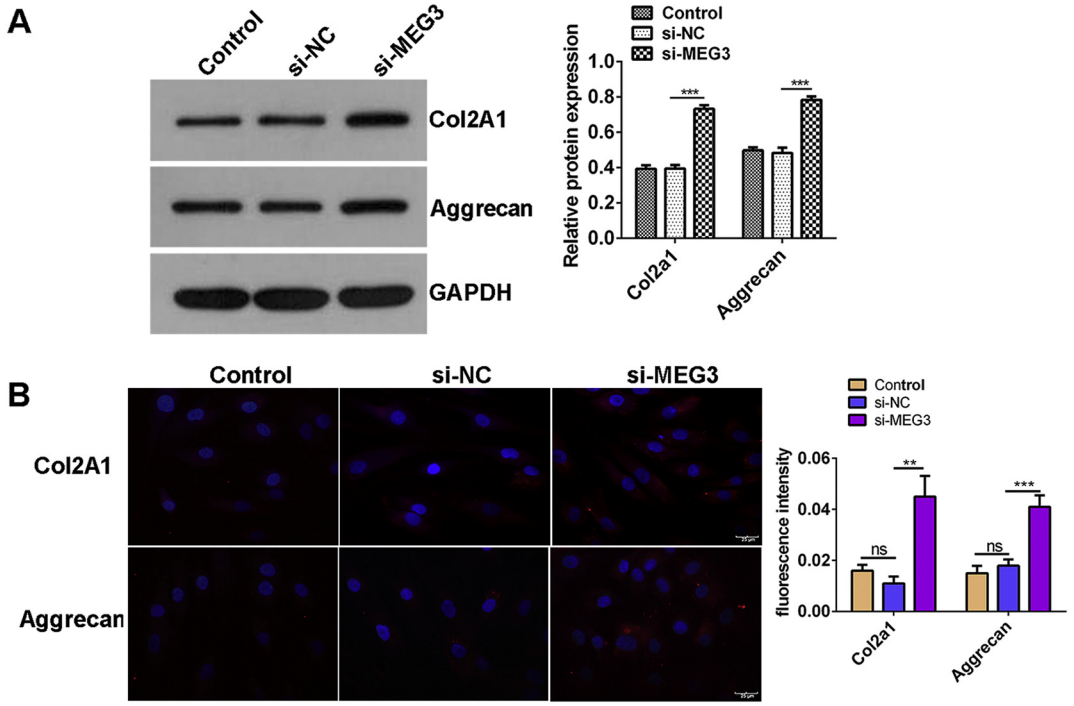

Fig. 4. MEG3 knockdown promotes aggrecan and Col2A1 in SMSCs. A Expression of aggrecan and Col2A1 detected by western blot after transfection with si-MEG3 for 48 h. B Expression of aggrecan and Col2A1 detected by immunofluorescence after transfection with si-MEG3 for 48 h. Notes: Control, cells without treatment; si-NC, cells transfected with siRNA as a negative control(si-NC); si-MEG3, cells transfected with si-MEG3 which is a small interfering RNA targeting MEG3; The scale bar represents 1000 μm. All data mean ± SD, n = 3. *p < .05, **p < .01, ***p < .001.

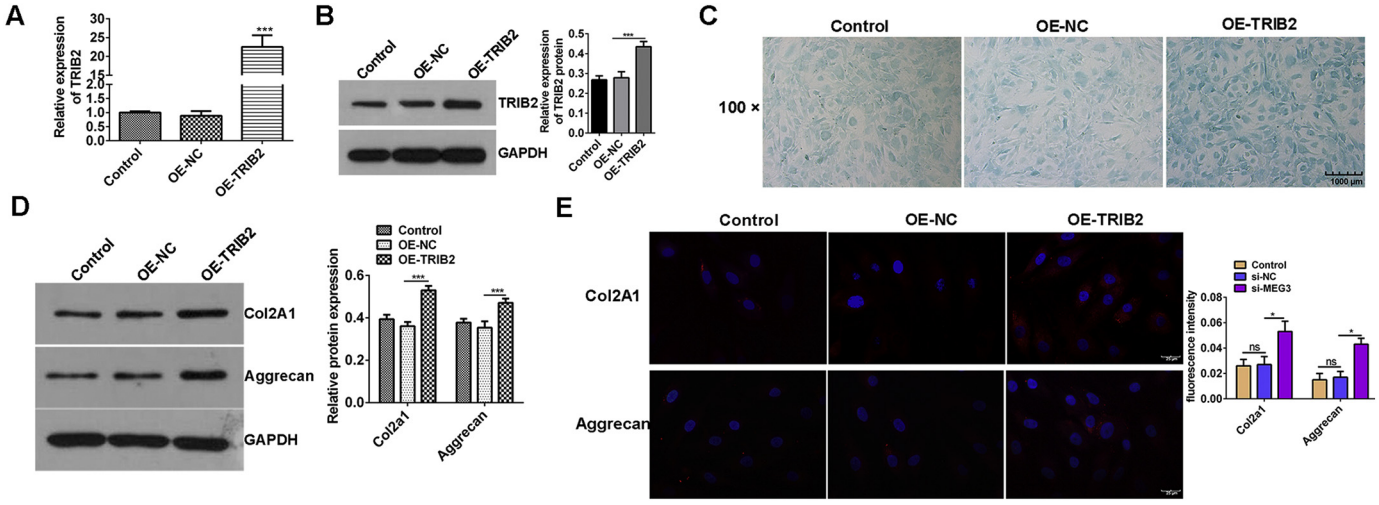

Fig. 5. Overexpression of TRIB2 promotes chondrogenesis in SMSCs. A Expression of TRIB2 detected by qPCR. B Expression of TRIB2 detected by western blotting after transfection with OE-TRIB2 for 48 h. C Chondrocytes detected by alcian blue staining after transfection with OE-TRIB2 for 48 h. D Expression of aggrecan and Col2A1 detected by western blotting after transfection with OE-TRIB2 for 48 h. E Expression of aggrecan and Col2A1 detected by immunofluorescence after transfection with OE-TRIB2 for 48 h. Notes: Control, cells without treatment; Induced, cells with induction medium added; OE-NC, cells transfected with OE-NC which was an empty vector(pCDNA3.1); OE-MEG3, cells transfected with OE-MEG3 which was a vector that overexpression MEG3(pCDNA3.1-MEG3); The scale bar represents 25 μm(400 ×). All data are presented as mean ± SD, n = 3. *p < .05, **p < .01, ***p < .001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

TRIB2, These results confirm that TRIB2 is a downstream target of MEG3. Importantly, knockdown of EZH2 can promote the expression of aggrecan and Col2A1, whereas co-transfection with si-EZH2 and OE-MEG3 partially mitigates this phenomenon. Taken together, our findings show that inhibition of MEG3 promotes the chondrogenic differentiation of SMSCs by binding to EZH2/PRC2 and epigenetically repressing TRIB2 expression in SMSCs.

In summary, bioinformatics analysis of osteoarthritis and SMSC databases led to the discovery that lncRNA MEG3 plays a role in the chondrogenic differentiation of SMSCs, and that TRIB2 may be its target gene. Experimental studies demonstrated that the down-regulation of lncRNA MEG3 promotes the chondrogenic differentiation of SMSCs by alleviating the suppression of TRIB2 expression, thus stimulating the TRIB2 level. This lncRNA MEG3-EZH2-TRIB2 axis is a newly discovered regulatory mechanism for the chondrogenic differentiation of SMSCs. This discovery may provide new information for the potential improvement of SMSC-derived cartilage tissue engineered for the treatment of osteoarthritis.

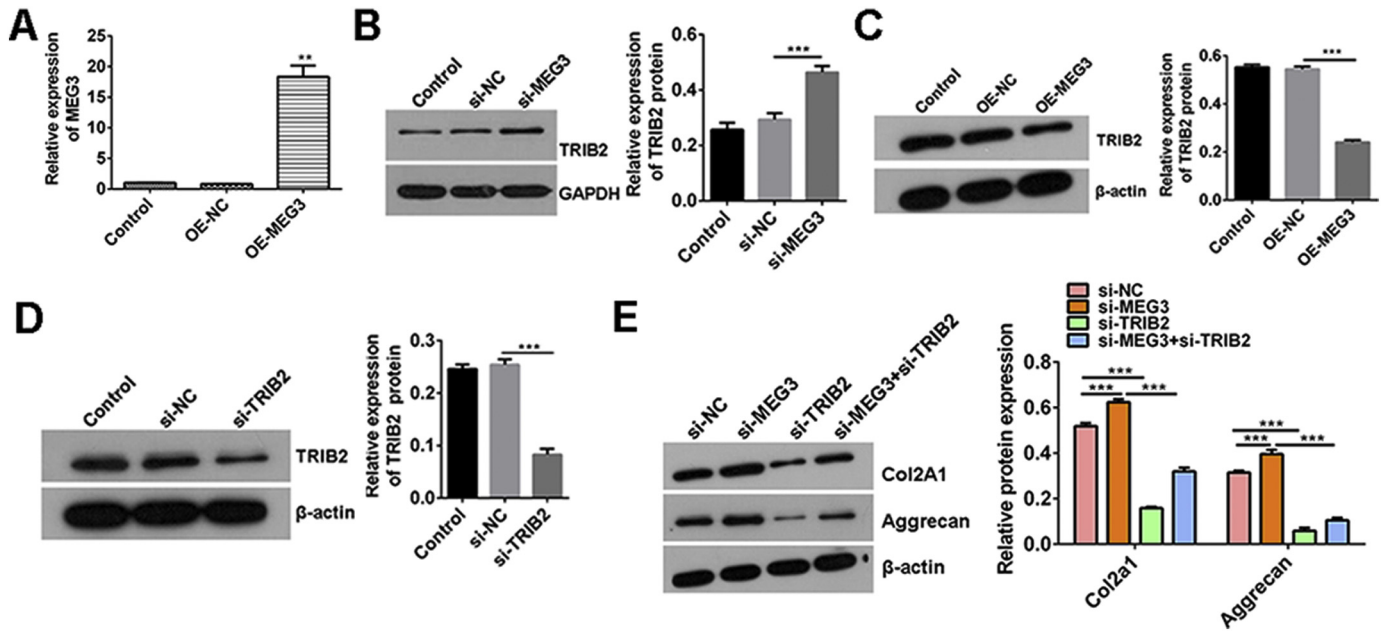

Fig. 6. MEG3 regulates differentiation of mesenchymal stem cells into chondrocytes via the regulation of TRIB2. A Expression of MEG3 detected by qPCR after transfection with si-MEG3 for 48 h. B Expression of TRIB2 detected by western blotting after transfection with si-MEG3 for 48 h. C Expression of TRIB2 detected by western blotting after transfection with OE-MEG3 for 48 h. D Expression of TRIB2 detected by western blotting after transfection with si-TRIB2 for 48 h. E Expression of aggrecan and Col2A1 detected by western blot. Notes: si-NC, cells transfected with siRNA as a negative control(si-NC); si-MEG3, cells transfected with si-MEG3 which is a small interfering RNA targeting MEG3; si-TRIB2, cells transfected with si-TRIB2 which is a small interfering RNA targeting TRIB2; si-MEG3 + si-TRIB2, cells transfected with si-MEG3 and si-TRIB2 which are two small interfering RNAs targeting MEG3 and TRIB2. All data are presented as mean ± SD, n = 3. All data are mean ± SD, n = 3. *p < .05, **p < .01, ***p < .001.

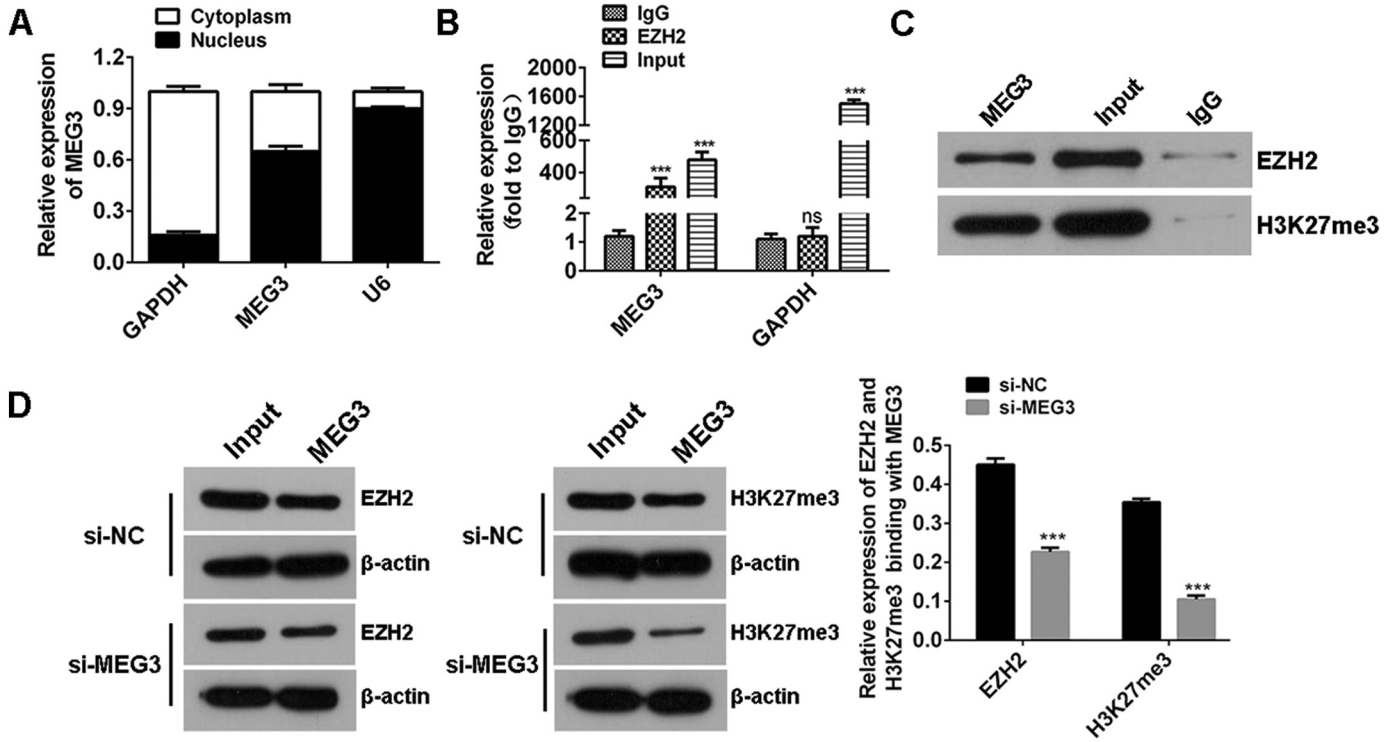

Fig. 7. MEG3 can directly bind to EZH2. A qPCR was used to detect the expression of MEG3 in the cytoplasm and nuclei. U6 was used as a nucleus marker, and β-actin was used as a cytosol marker. B qRT-PCR on EZH2 RIP from SMSCs. The fold enrichment of Meg3 in EZH2 RIP was relative to its matching IgG control RIP. β-actin was used as a control and the RIP was performed with EZH2 antibody. C RNA pull-down assays were performed with biotin-labeled MEG3 in vitro. After capture using beads, EZH2 protein in the samples was detected using western blotting. Input, the lysate sample; MEG3, the sample pulled-down by biotin-labeled MEG3; IgG, the sample pulled down by the negative control of MEG3. D RNA pull-down assays were performed using biotin-labeled MEG3 in vitro after transfection with si-MEG3. Input, the lysate sample; MEG3, the sample pulled down by the biotin-labeled MEG3.

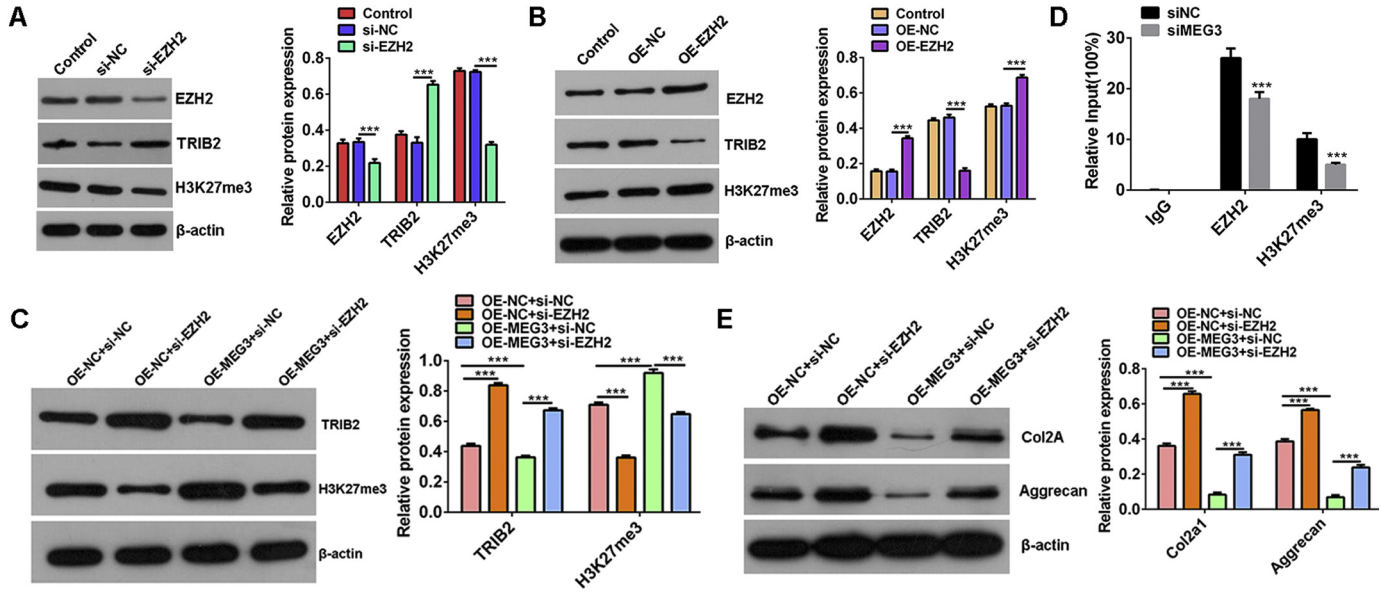

Fig. 8. MEG3 binds to EZH2 and epigenetic silencing of TRIB2 expression in SMSCs. A Western blotting of TRIB2 and H3K27me3 protein levels in SMSCs after transfection with si-EZH2. B Western blotting of TRIB2 and H3K27me3 protein levels in SMSCs after transfection with OE-EZH2. C Western blotting of TRIB2 and H3K27me3 protein levels in SMSCs after transfection with OE-MEG3 and si-EZH2. D ChIP-qPCR assays were performed with EZH2 or H3K27me3 antibodies, qPCR detected expression of the TRIB2 promoter. E Western blotting of Col2A1 and Aggercan protein level in SMSCs after transfection with OE-MEG3 and si-EZH2. Notes: All data are presented as mean ± SD, n = 3. *p < .05, **p < .01, ***p < .001.

5. Conclusions

MEG3 inhibits the chondrogenic differentiation of SMSCs by epigenetically inhibiting TRIB2 via methyltransferase EZH2.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Contributors

Jiandong Ni designed the work and offered funds. Di You designed the work, performed the experiment and submitted the manuscript. Cheng Yang, Jun Huang and Haoli Gong performed the experiment and collected the data. Mingming Yan analysized data and modified the manuscript.

Declaration of Competing Interest

None.

References

[1] A. Bortoluzzi, F. Furini, C.A. Scire, Osteoarthritis and its management - epidemiology, nutritional aspects and environmental factors, Autoimmun. Rev. 17 (2018) 1097–1104.
[2] Y. Zhang, J.M. Jordan, Epidemiology of osteoarthritis, Clin. Geriatr. Med. 26 (2010) 355–369.
[3] Y. Wu, E.L. Goh, D. Wang, S. Ma, Novel treatments for osteoarthritis: an update, Open Access Rheumatol. 10 (2018) 135–140.
[4] C.M. Chan, C.D. Macdonald, G.J. Litherland, D.J. Wilkinson, A. Skelton, G.N. Europe-Finner, A.D. Rowan, Cytokine-induced MMP13 expression in human chondrocytes is dependent on activating transcription factor 3 (ATF3) regulation, J. Biol. Chem. 292 (2017) 1625–1636.
[5] N.K. Dubey, V.K. Mishra, R. Dubey, S. Syed-Abdul, J.R. Wang, P.D. Wang, W.P. Deng, Combating osteoarthritis through stem cell therapies by rejuvenating cartilage: a review, Stem Cells Int. 2018 (2018) 5421019.
[6] H. Long, B. Sun, L. Cheng, S. Zhao, Y. Zhu, R. Zhao, J. Zhu, miR-139-5p represses BMSC osteogenesis via targeting Wnt/beta-catenin signaling pathway, DNA Cell Biol. 36 (2017) 715–724.
[7] C. De Bari, A.J. Roelofs, Stem cell-based therapeutic strategies for cartilage defects and osteoarthritis, Curr. Opin. Pharmacol. 40 (2018) 74–80.
[8] G.I. Im, Endogenous cartilage repair by recruitment of stem cells, Tissue Eng. Part B Rev. 22 (2016) 160–171.
[9] E.B. de Sousa, P.L. Casado, V. Moura Neto, M.E. Duarte, D.P. Aguiar, Synovial fluid and synovial membrane mesenchymal stem cells: latest discoveries and therapeutic perspectives, Stem Cell Res Ther 5 (2014) 112.
[10] E.J. Kubosch, G. Lang, D. Furst, D. Kubosch, K. Izadpanah, B. Rolauffs, N.P. Sudkamp, H. Schmal, The potential for synovium-derived stem cells in cartilage repair, Curr. Stem Cell Res. Ther. 13 (2018) 174–184.
[11] N.P. Huynh, B.A. Anderson, F. Guilak, A. McAlinden, Emerging roles for long noncoding RNAs in skeletal biology and disease, Connect. Tissue Res. 58 (2017) 116–141.
[12] J.B. Chen, Y.W. Zhu, X. Guo, C. Yu, P.H. Liu, C. Li, J. Hu, H.H. Li, L.F. Liu, M.F. Chen, H.Q. Chen, Z. Xiong-Bing, Microarray expression profiles analysis revealed lncRNA OXCT1-AS1 promoted bladder cancer cell aggressiveness via miR-455-5p/JAK1 signaling, J. Cell. Physiol. 234 (2019) 13592–13601.
[13] M. Deng, H. Yuan, S. Liu, Z. Hu, H. Xiao, Exosome-transmitted LINC00461 promotes multiple myeloma cell proliferation and suppresses apoptosis by modulating microRNA/BCL-2 expression, Cytotherapy 21 (2019) 96–106.
[14] A. Sanchez Calle, Y. Kawamura, Y. Yamamoto, F. Takeshita, T. Ochiya, Emerging roles of long non-coding RNA in cancer, Cancer Sci. 109 (2018) 2093–2100.
[15] S. Huo, Y. Zhou, X. He, M. Wan, W. Du, X. Xu, L. Ye, X. Zhou, L. Zheng, Insight into the role of Long non-coding RNAs during osteogenesis in mesenchymal stem cells, Curr. Stem Cell Res. Ther. 13 (2018) 52–59.
[16] B. De Paepe, S. Lefever, P. Mestdagh, How long noncoding RNAs enforce their will on mitochondrial activity: regulation of mitochondrial respiration, reactive oxygen species production, apoptosis, and metabolic reprogramming in cancer, Curr. Genet. 64 (2018) 163–172.
[17] L. Li, G. Lv, B. Wang, L. Kuang, The role of lncRNA XIST/miR-211 axis in modulating the proliferation and apoptosis of osteoarthritis chondrocytes through CXCR4 and MAPK signaling, Biochem. Biophys. Res. Commun. 503 (2018) 2555–2562.
[18] X. Feng, T. Lin, X. Liu, C. Yang, S. Yang, D. Fu, Long non-coding RNA BDNF-AS modulates osteogenic differentiation of bone marrow-derived mesenchymal stem cells, Mol. Cell. Biochem. 445 (2018) 59–65.
[19] S. Peng, L. Cao, S. He, Y. Zhong, H. Ma, Y. Zhang, C. Shuai, An overview of Long noncoding RNAs involved in bone regeneration from mesenchymal stem cells, Stem Cells Int. 2018 (2018) 8273648.
[20] L. Zhang, C. Yang, S. Chen, G. Wang, B. Shi, X. Tao, L. Zhou, J. Zhao, Long non-coding RNA DANCR is a positive regulator of proliferation and chondrogenic differentiation in human synovium-derived stem cells, DNA Cell Biol. 36 (2017) 136–142.
[21] V. Balik, J. Srovnal, I. Sulla, O. Kalita, T. Foltanova, M. Vaverka, L. Hrabalek, M. Hajduch, MEG3: a novel long noncoding potentially tumour-suppressing RNA in meningiomas, J. Neuro-Oncol. 112 (2013) 1–8.
[22] Y. Zhou, X. Zhang, A. Klibanski, MEG3 noncoding RNA: a tumor suppressor, J. Mol. Endocrinol. 48 (2012) R45–R53.
[23] W. Su, W. Xie, Q. Shang, B. Su, The long noncoding RNA MEG3 is downregulated and inversely associated with VEGF levels in osteoarthritis, Biomed. Res. Int. 2015 (2015) 356893.

[24] J. Xu, Y. Xu, The lncRNA MEG3 downregulation leads to osteoarthritis progression via miR-16/SMAD7 axis, Cell Biosci. 7 (2017) 69.
[25] W. Zhuang, X. Ge, S. Yang, M. Huang, W. Zhuang, P. Chen, X. Zhang, J. Fu, J. Qu, B. Li, Upregulation of lncRNA MEG3 promotes osteogenic differentiation of mesenchymal stem cells from multiple myeloma patients by targeting BMP4 transcription, Stem Cells 33 (2015) 1985–1997.
[26] T. Mondal, S. Subhash, R. Vaid, S. Enroth, S. Uday, B. Reinius, S. Mitra, A. Mohammed, A.R. James, E. Hoberg, A. Moustakas, U. Gyllensten, S.J. Jones, C.M. Gustafsson, A.H. Sims, F. Westerlund, E. Gorab, C. Kanduri, MEG3 long noncoding RNA regulates the TGF-beta pathway genes through formation of RNA-DNA triplex structures, Nat. Commun. 6 (2015) 7743.
[27] M. Terashima, S. Tange, A. Ishimura, T. Suzuki, MEG3 Long noncoding RNA contributes to the epigenetic regulation of epithelial-mesenchymal transition in lung cancer cell lines, J. Biol. Chem. 292 (2017) 82–99.
[28] K.S. Jani, S.U. Jain, E.J. Ge, K.L. Diehl, S.M. Lundgren, M.M. Muller, P.W. Lewis, T.W. Muir, Histone H3 tail binds a unique sensing pocket in EZH2 to activate the PRC2 methyltransferase, Proc. Natl. Acad. Sci. U. S. A. 116 (2019) 8295–8300.
[29] Y. Tian, H. Zheng, F. Zhang, S. Wang, X. Ji, C. Xu, Y. He, Y. Ding, PRC2 recruitment and H3K27me3 deposition at FLC require FCA binding of COOLAIR, Sci. Adv. 5 (2019) eaau7246.
[30] J. Grosshans, E. Wieschaus, A genetic link between morphogenesis and cell division during formation of the ventral furrow in *Drosophila*, Cell 101 (2000) 523–531.
[31] K. Keeshan, Y. He, B.J. Wouters, O. Shestova, L. Xu, H. Sai, C.G. Rodriguez, I. Maillard, J.W. Tobias, P. Valk, M. Carroll, J.C. Aster, R. Delwel, W.S. Pear, Tribbles homolog 2 inactivates C/EBPalpha and causes acute myelogenous leukemia, Cancer Cell 10 (2006) 401–411.
[32] M. Becker, The Canton Zurich is coordinating personnel recruitment for health professions: propaganda offensive for nursing professions, Krankenpfl Soins. Infirm. 83 (1990) 75.
[33] Z. Hou, K. Guo, X. Sun, F. Hu, Q. Chen, X. Luo, G. Wang, J. Hu, L. Sun, TRIB2 functions as novel oncogene in colorectal cancer by blocking cellular senescence through AP4/p21 signaling, Mol. Cancer 17 (2018) 172.
[34] A.D. Rowan, G.J. Litherland, Tribbles and arthritis: what are the links? Biochem. Soc. Trans. 43 (2015) 1051–1056.
[35] M. Mizuno, H. Katano, Y. Mabuchi, Y. Ogata, S. Ichinose, S. Fujii, K. Otabe, K. Komori, N. Ozeki, H. Koga, K. Tsuji, C. Akazawa, T. Muneta, I. Sekiya, Specific markers and properties of synovial mesenchymal stem cells in the surface, stromal, and perivascular regions, Stem Cell Res Ther 9 (2018) 123.
[36] Y. Kohno, M. Mizuno, N. Ozeki, H. Katano, K. Komori, S. Fujii, K. Otabe, M. Horie, H. Koga, K. Tsuji, M. Matsumoto, H. Kaneko, Y. Takazawa, T. Muneta, I. Sekiya, Yields and chondrogenic potential of primary synovial mesenchymal stem cells are comparable between rheumatoid arthritis and osteoarthritis patients, Stem Cell Res Ther 8 (2017) 115.
[37] M. Lei, K. Li, B. Li, L.N. Gao, F.M. Chen, Y. Jin, Mesenchymal stem cell characteristics of dental pulp and periodontal ligament stem cells after in vivo transplantation, Biomaterials 35 (2014) 6332–6343.
[38] L. Jin, Q. Cai, S. Wang, S. Wang, T. Mondal, J. Wang, Z. Quan, Long noncoding RNA MEG3 regulates LATS2 by promoting the ubiquitination of EZH2 and inhibits proliferation and invasion in gallbladder cancer, Cell Death Dis. 9 (2018) 1017.
[39] S. Suzuki, T. Muneta, K. Tsuji, S. Ichinose, H. Makino, A. Umezawa, I. Sekiya, Properties and usefulness of aggregates of synovial mesenchymal stem cells as a source for cartilage regeneration, Arthritis Res. Ther. 14 (2012) R136.
[40] Y.S. Kim, H.J. Lee, J.E. Yeo, Y.I. Kim, Y.J. Choi, Y.G. Koh, Isolation and characterization of human mesenchymal stem cells derived from synovial fluid in patients with osteochondral lesion of the talus, Am. J. Sports Med. 43 (2015) 399–406.
[41] Y. Liang, E. Idrees, A.R.A. Szojka, S.H.J. Andrews, M. Kunze, A. Mulet-Sierra, N.M. Jomha, A.B. Adesida, Chondrogenic differentiation of synovial fluid mesenchymal stem cells on human meniscus-derived decellularized matrix requires exogenous growth factors, Acta Biomater. 80 (2018) 131–143.
[42] N. Wang, Y. Zhu, M. Xie, L. Wang, F. Jin, Y. Li, Q. Yuan, W. De, Long noncoding RNA Meg3 regulates Mafa expression in mouse beta cells by inactivating Rad21, Smc3 or Sin3alpha, Cell. Physiol. Biochem. 45 (2018) 2031–2043.
[43] L. Deng, H. Hong, X. Zhang, D. Chen, Z. Chen, J. Ling, L. Wu, Down-regulated lncRNA MEG3 promotes osteogenic differentiation of human dental follicle stem cells by epigenetically regulating Wnt pathway, Biochem. Biophys. Res. Commun. 503 (2018) 2061–2067.
[44] W. Huang, G. Su, X. Huang, A. Zou, J. Wu, Y. Yang, Y. Zhu, S. Liang, D. Li, F. Ma, L. Guo, Long noncoding RNA PCAT6 inhibits colon cancer cell apoptosis by regulating anti-apoptotic protein ARC expression via EZH2, Cell Cycle 18 (2019) 69–83.
[45] M. Su, Y. Xiao, J. Tang, J. Wu, J. Ma, B. Tian, Y. Zhou, H. Wang, D. Yang, Q.J. Liao, W. Wang, Role of lncRNA and EZH2 interaction/regulatory network in lung cancer, J. Cancer 9 (2018) 4156–4165.
[46] L. Wang, D. Meng, Y. Wang, J. Hu, Long non-coding RNA LINC01296 promotes esophageal squamous cell carcinoma cell proliferation and invasion by epigenetic suppression of KLF2, Am. J. Cancer Res. 8 (2018) 2020–2029.
[47] B. Attardi, H.K. Happe, Modulation of the estradiol-induced luteinizing hormone surge by progesterone or antiestrogens: effects on pituitary gonadotropin-releasing hormone receptors, Endocrinology 119 (1986) 274–283.
[48] P.A. Eyers, K. Keeshan, N. Kannan, Tribbles in the 21st century: the evolving roles of tribbles pseudokinases in biology and disease, Trends Cell Biol. 27 (2017) 284–298.
[49] T. Hermida-Gomez, I. Fuentes-Boquete, M.J. Gimeno-Longas, E. Muinos-Lopez, S. Diaz-Prado, F.J. de Toro, F.J. Blanco, Quantification of cells expressing mesenchymal stem cell markers in healthy and osteoarthritic synovial membranes, J. Rheumatol. 38 (2011) 339–349.
[50] D. Yin, X. Lu, J. Su, X. He, W. De, J. Yang, W. Li, L. Han, E. Zhang, Long noncoding RNA AFAP1-AS1 predicts a poor prognosis and regulates non-small cell lung cancer cell proliferation by epigenetically repressing p21 expression, Mol. Cancer 17 (2018) 92.
[51] C.M. Dougherty, J.D. Burrowes, R.K. Hand, Why registered dietitian nutritionists are not doing research-perceptions, barriers, and participation in research from the Academy's dietetics practice-based research network needs assessment survey, J. Acad. Nutr. Diet. 115 (2015) 1001–1007.
[52] Y. He, X.M. Meng, C. Huang, B.M. Wu, L. Zhang, X.W. Lv, J. Li, Long noncoding RNAs: novel insights into hepatocelluar carcinoma, Cancer Lett. 344 (2014) 20–27.