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Original Article

Mesenchymal stem cell extracellular vesicles-derived microRNA-194-5p delays the development of intervertebral disc degeneration by targeting TRAF6

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ABSTRACT

Objective: Mesenchymal stem cells-derived extracellular vesicles (MSCs-EVs) can improve intervertebral disc degeneration (IDD). Considering that, their concrete mechanisms from microRNA-194-5p/tumor receptor-associated factor 6 (miR-194-5p/TRAF6) axis in IDD ask for disclosure in a scientific way.
Methods: Nucleus pulposus (NP) cells and MSCs were obtained. EVs were isolated from the obtained MSCs and identified. miR-194-5p expression in MSC-EVs was altered by sequence transfection. Subsequently, MSCs-EVs were co-cultured with NP cells intervened by tumor necrosis factor α (TNF-α). NP cell proliferation and apoptosis, along with their osteogenic differentiation ability were evaluated. miR-194-5p and TRAF6 expression and their interaction were determined.
Results: In TNF-α-intervened NP cells, miR-194-5p was down-regulated and TRAF6 was up-regulated. Restoring miR-194-5p effectively enhanced proliferation and osteogenic differentiation, and reduced apoptosis of TNF-α-intervened NP cells. miR-194-5p-enriched MSCs-EVs protected TNF-α-intervened NP cells. miR-194-5p targeted TRAF6, TRAF6 overexpression exerted negatively for the growth of TNF-α-intervened NP cells, and could reduce the protective effects of miR-194-5p on TNF-α-intervened NP cells.
Conclusion: It is elucidated that miR-194-5p derived from MSCs-EVs protects TNF-α-intervened NP cells through restricting TRAF6, replenishing a potential target for IDD treatment.

1. Introduction

Intervertebral disc (IVD) degeneration (IDD) refers to an abnormal molecular degeneration process, which is mediated by cells, age and genetic molecules [1], and critically results in low back pain [2]. In the process of IDD, loss of collagen II, increased proteolytic degradation of the extracellular matrix, and proteoglycan in the nucleus pulposus cells (NP) are the main traits [3]. The regeneration capacity of adult degenerated IVD is very limited, blaming on the limitations of intrinsic progenitor cells and the avascular nature of IVD [4]. Though with non-surgical therapies (physical therapy and spinal injections of steroids) and surgical treatments (spinal fusion and joint replacement) to relieve pains, no method has been validated to prevent and reverse IDD [5]. Considering the embarrassments in IDD treatment, novel agents are required for development.

Bone marrow mesenchymal stem cells (BMSCs)-derived exosomes are able to facilitate NP cell proliferation and healthy extracellular matrix generation in degenerated IVD [6]. MSCs-derived exosomes have developed as feasible nanovehicles to treat IDD [7]. For example, MSCs-derived exosomes are considered to disrupt NP cell apoptosis to attenuate IDD, which is at least assisted by microRNA (miR)-21 delivery [8]. Moreover, BMSCs-secreted exosomes protect NP cells against fibrosis deposition, extracellular matrix degradation and apoptosis by transporting

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miR-532-5p [9]. Abnormally expressed miR-194-5p have been clarified in IDD, suggesting its attended role in the pathogenesis of IDD [10]. Additionally, miR-194 has the capacity to modify extracellular matrix production and inflammation in IDD through targeting tumor receptor-associated factor 6 (TRAF6) [11]. TRAF6 has been discussed to involve in the deterioration of IDD, as mirrored in the fields of NP cell growth, cell cycle entry, apoptosis and osteoblast differentiation [12]. Recognized in an innovative study, TRAF6 suppression is dedicated to repressed inflammation of NP cells [13]. However, the loop feedback of MSCs-derived extracellular vesicles (EVs), miR-194-5p and TRAF6 in NP cell apoptosis, proliferation and osteoblast differentiation in IDD requires thorough explanation. Taken the aforementioned facts together, it is reasonably hypothesized that miR-194-5p may be delivered by exosomes to reach NP cells, thereafter alleviating cell senescence, which is potentially tied up with its target TRAF6.

2. Methods and materials

2.1. Ethics statement

The experiment approval was obtained from the Ethics Committee of our Hospital. The written informed consent of the patients was obtained.

2.2. Isolation and identification of NP cells and MSCs

Bone marrow biopsies and NP tissue samples were obtained from 5 patients (28 ± 6.4 years old) who had undergone spinal surgery because of thoracolumbar fractures. The NP tissues were successively treated with 0.25% pronase (Sigma–Aldrich, Louis, MO, USA) and 0.2% type II collagenase (Invitrogen, CA, USA) and filtered through a 70-μm filter. After that, cells were cultivated in Dulbecco's modified Eagle medium of 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin-streptomycin (Gibco, NY, USA), 2 mM glutamine (Sigma–Aldrich) and 50 μg/mL L-ascorbic acid (Sigma–Aldrich). With the medium replaced 3 times a week, cells were growing to 80% confluence and subcultured at 1:3 [8]. Then, NP cells were trypsinized and fixed in 2% paraformaldehyde, after which incubation with antibodies to CD34 (1:200, Abcam, MA, USA), CD24 (1:100, Invitrogen), and CD29 (1:100, BD Biosciences, NJ, USA) was performed. Flow cytometry was aimed to identify cells [14,15].

Isolation of MSCs was completed by density gradient centrifugation and adherence to tissue culture plastic. MSCs were cultured in α-minimum essential medium (α-MEM, Gibco) of 10% FBS and 1% penicillin-streptomycin, and subcultured to passage 3 or 4. Followed by detachment and fixation in 2% paraformaldehyde, the MSCs were probed with the antibodies to CD29 (1:100), CD45 (1:200) and CD90 (1:100, all from BD Biosciences), and then determined by flow cytometry [14].

2.3. Isolation and identification of MSCs-EVs

EVs were isolated from MSCs by ultracentrifugation. When MSCs confluence reached 90% or more, the serum-free culture medium was collected and stored at −4°C, and then EVs were isolated within 24 h, MSCs were processed through differential centrifugation to collect cell supernatant and remove cell debris and large vesicles (300 ×*g* for 10 min, 2000 ×*g* for 20 min, and 10,000 ×*g* for 40 min). Then, through ultracentrifugation at 110,000 ×g for 90 min, the pellets were resuspended. All centrifugation steps were performed at 4 °C, and the protein concentration was determined by bicinchoninic acid. The shape and size of EVs were evaluated by a transmission electron microscope (TEM). CD81 (1:1000, sc-166,029), and CD63 (1:1000, sc-5275, Santa Cruz Biotechnology, CA, USA) on EVs were checked by western blot assay [14].

2.4. Co-culture of MSCs-EVs and NP cells

The purified MSCs-EVs were incubated with PKH26 (Sigma–Aldrich) for 5 min, after which they were centrifuged at 120,000 g for 90 min. The labeled MSCs-EVs suspended in a basal medium, were incubated with NP cells for 12 h and stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich). The stained cells were observed under a fluorescence microscope (DMI3000B; Leica Microsystems, Inc., Germany) [8].

2.5. Cell transfection and EVs treatment

Referring to the known miR-194-5p and TRAF6 sequences in NCBI, the construction of required oligonucleotides and plasmids was commissioned to Sangon Biotech Co., Ltd. (Shanghai, China). Next, the plasmids were transfected into MSCs and NP cells by oligonucleotides or Lipofectamine 3000 (Invitrogen). MSCs were transfected with negative control (NC)-mimic or miR-194-5p-mimic, while NP cells with NC-mimic, miR-194-5p-mimic, NC-inhibitor, miR-194-5p-inhibitor, oe-NC, overexpression (oe)-TRAF6, and miR-194-5p-mimic + oe-TRAF6, respectively. NP cells were co-cultured with MSCs-EVs (NC-mimic- or miR-194-5p mimic-transfected MSCs-EVs), namely EVs, EVs–NC–mimic and EVs-miR-194-5p-mimic groups [17]. The transfected or co-cultured NP cells were incubated for 36 h and intervened by TNF-α (5 ng/mL) for 12 h [8]. The normally cultured NP cells were employed as the control.

2.6. Cell counting kit (CCK)-8 assay

Cell proliferation was detected on Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Cells were seeded on 96-well plates (1×10^3 cells/well). An ELx 800 microplate reader (Bio-Tek Instruments Inc., VT, USA) was applied to test absorbance at 450 nm at the first, second, third and forth day, respectively [12].

2.7. Flow cytometry

An Annexin V/propidium iodide (PI) apoptosis detection kit (BD Biosciences) was included in determination of apoptosis rate of NP cells as detailed previously [18].

2.8. Alkaline phosphatase (ALP) staining and alizarin red staining

Osteogenic differentiation of NP cells was induced by an osteogenic-inducing medium (Biowit Technologies, Shenzhen, China) [19]. At 7 days post osteogenic differentiation, ALP activity was evaluated by an ALP staining kit (Beyotime) and the mineralized matrix in NP cells was measured by alizarin red staining (Sigma–Aldrich). Calcium nodules were imaged under an optical microscope, and the absorbance was measured at 562 nm by a microplate reader (Bio-Tek) [12].

2.9. Dual luciferase reporter gene assay

The wild-type (WT) and mutant type (MUT) of TRAF6 3′ untranslated region (UTR) with the binding site of miR-194-5p were generated and inserted into pmirGLO luciferase vector (Promega, Madison, MI, USA). NP cells, seeded in 96-well plates at 8×10^3 cells per well, were transfected with WT/MUT plasmids and miR-194-5p-mimic or NC-mimic through Lipofectamine 3000. A dual luciferase

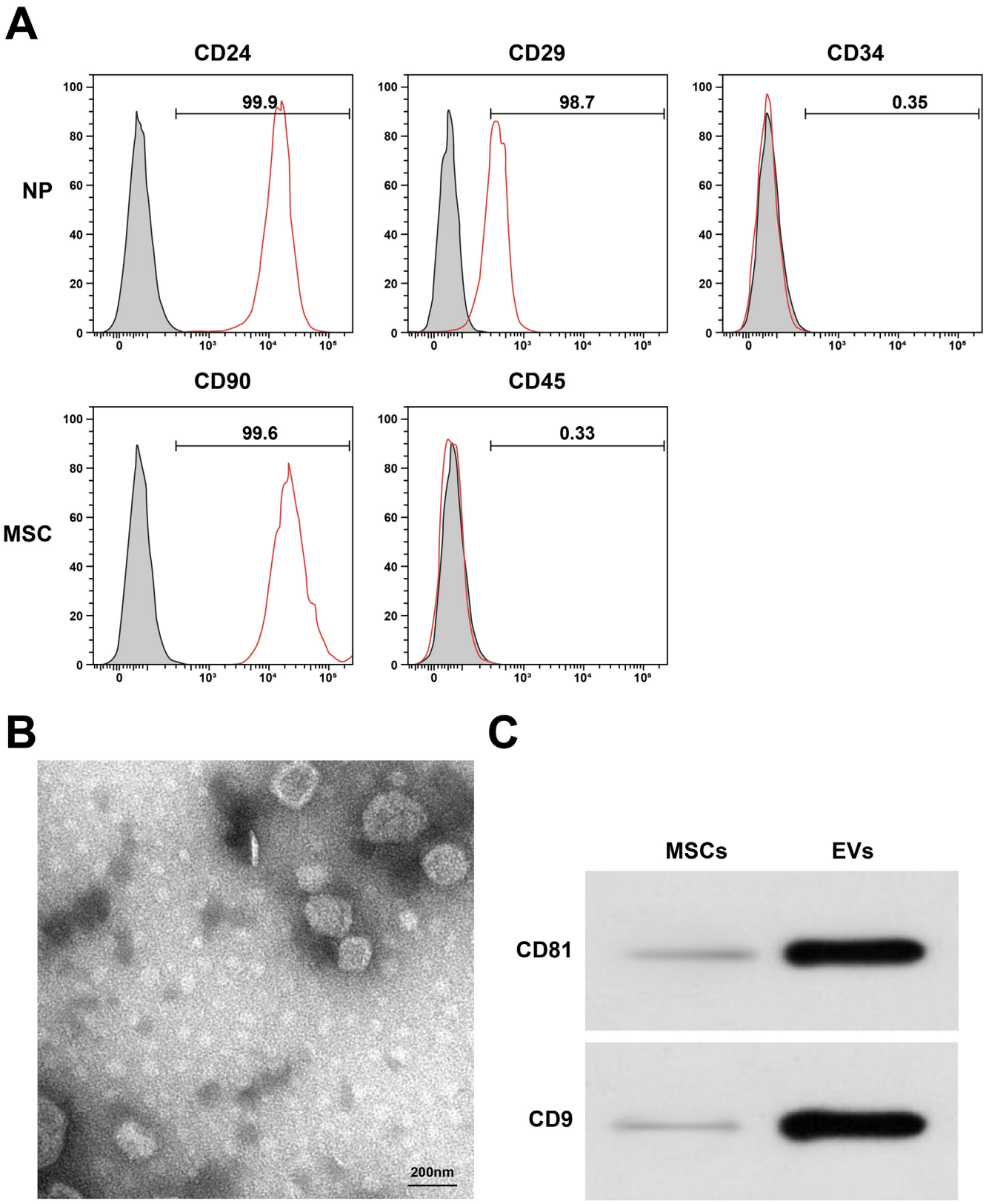

Fig. 1. Identification of NP cells, MSCs and MSCs-EVs. A. Flow cytometry identified NP cell surface antigens (CD24, CD29 and CD34), and MSC surface antigens (CD90 and CD45); B. TEM observed MSCs-EVs; C. Western blot assay detected surface markers (CD81 and CD9) of MSCs-EVs.

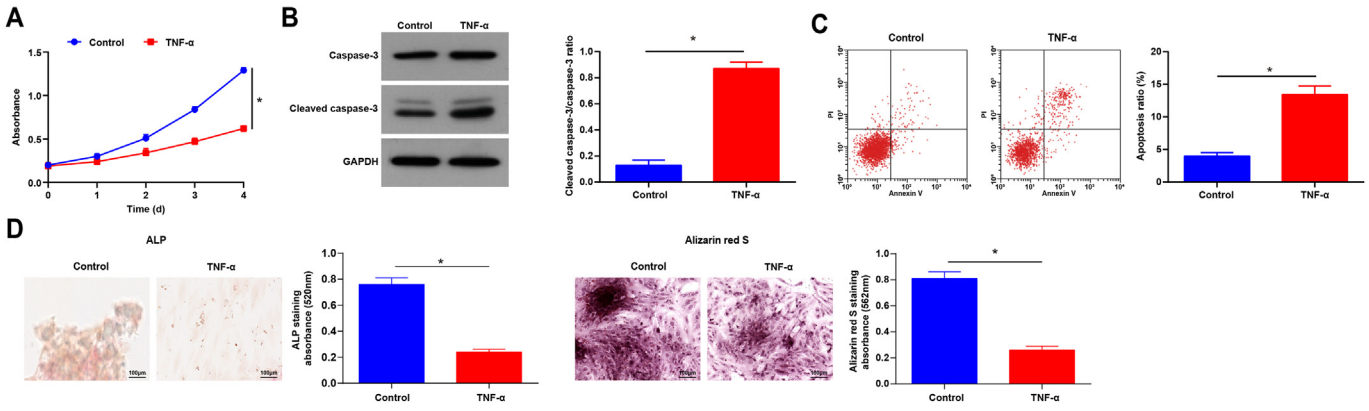

Fig. 2. TNF-α intervenes NP cell proliferation and enhances apoptosis, and restricts osteogenic differentiation. A. CCK-8 assay detected the effects of TNF-α on NP cell proliferation; B. Western blot assay detected the effects of TNF-α on Cleaved Caspase-3 and Caspase-3 expression in NP cells; C. Flow cytometry detected the effects of TNF-α on apoptosis of NP cells; D. ALP staining and alizarin red staining detected the effects of TNF-α on osteogenic differentiation of NP cells. Data were expressed as mean ± standard deviation (N = 3). *, $p < 0.05$.

reporter gene analysis system (Promega) was employed to detect luciferase activity at 48 h post transfection. Optical density values were measured by a SpectraMax i3x multi-plate reader (Molecular Instruments, Sunnyvale, CA, USA). The ratio of luciferase to Renilla luciferase activity was calculated [8].

2.10. RNA immunoprecipitation (RIP) assay

An EZ-Magna RIP kit (Millipore, MA, USA) was subjected to RIP analysis. NP cells were lysed with NP-40 lysis buffer of 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1% protease inhibitor cocktail (Sigma–Aldrich) and RNase inhibitor (Invitrogen). Then, cells were reacted with RIP buffer containing A + G magnetic beads conjugated with anti-Argonaute 2 (Ago2, 1:200, Abcam), immunoglobulin G (Millipore, 1:300, Abcam). The obtained protein samples were detached with proteinase K buffer, and the immunoprecipitated RNA was isolated and analyzed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) [20].

2.11. RT-qPCR

Total RNA was extracted by Trizol reagent [21]. Complementary DNA (cDNA) was synthesized by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) or miRNA 1st Strand cDNA Synthesis Kit (Vazyme). With SYBR Green Master Mix and C1000TM Thermal Cycler, RT-qPCR was launched. U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were the internal controls for genes, whose expression would be calculated by $2^{-\triangle\triangle Ct}$ method. Primers were displayed in Supplementary Table 1.

2.12. Western blot assay

The lysates of NP cells and MSCs-EVs made by radioimmunoprecipitation assay buffer (Beyotime) were detected for determining protein concentration with a bicinchoninic acid protein analysis kit II (BIO-RAD, Hercules, CA, USA). Protein samples were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes (Invitrogen). Reacted with the CD9 (1:1000, Santa Cruz Biotechnology), CD81 (1:1000, Santa Cruz Biotechnology), Caspase-3 (1:1000, Cell Signaling Technology), Cleaved Caspase-3 (1:1000, Cell Signaling Technology), TRAF6 (1:1000, Abcam) and GAPDH (1:1000, Cell Signaling Technology) overnight, the protein samples were probed with horseradish peroxidase-conjugated secondary antibodies. The signals on the membrane were observed with

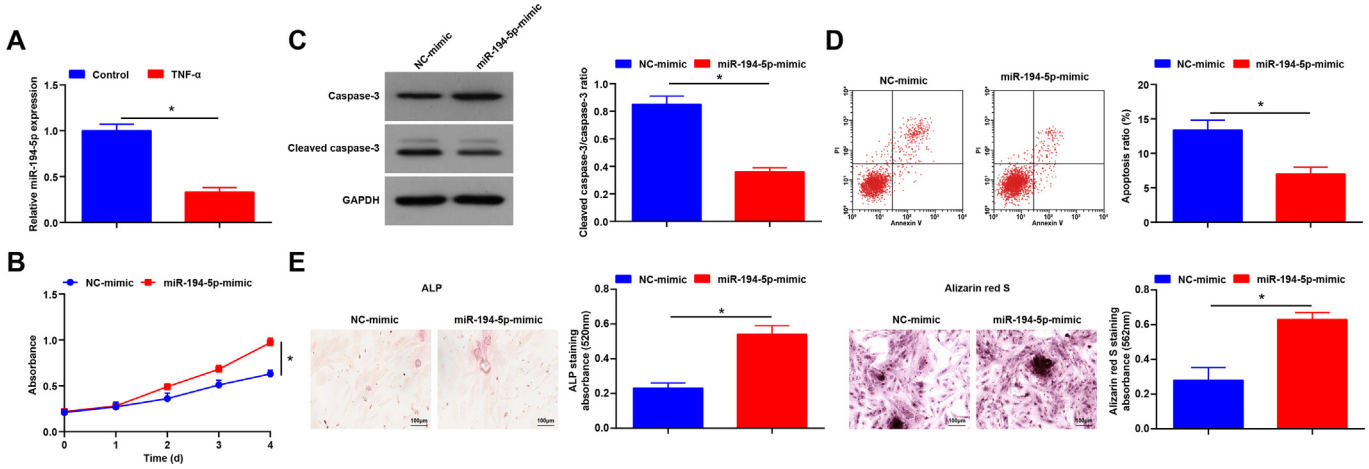

Fig. 3. miR-194-5p is down-regulated in IDD; Restoring miR-194-5p alleviates IDD. A. RT-qPCR detected miR-194-5p expression in TNF-α-intervened NP cells; B. CCK-8 assay detected the effect of miR-194-5p overexpression on TNF-α-intervened NP cell proliferation; C. Western blot assay detected the effect of miR-194-5p overexpression on Cleaved Caspase-3 and Caspase-3 protein expression in TNF-α-intervened NP cells; D. Flow cytometry detected the effect of miR-194-5p overexpression on TNF-α-intervened NP cell apoptosis; E. ALP staining and alizarin red staining detected the effect of miR-194-5p overexpression on osteogenic differentiation of TNF-α-intervened NP cells. Data were expressed as mean ± standard deviation (N = 3). *, $p < 0.05$.

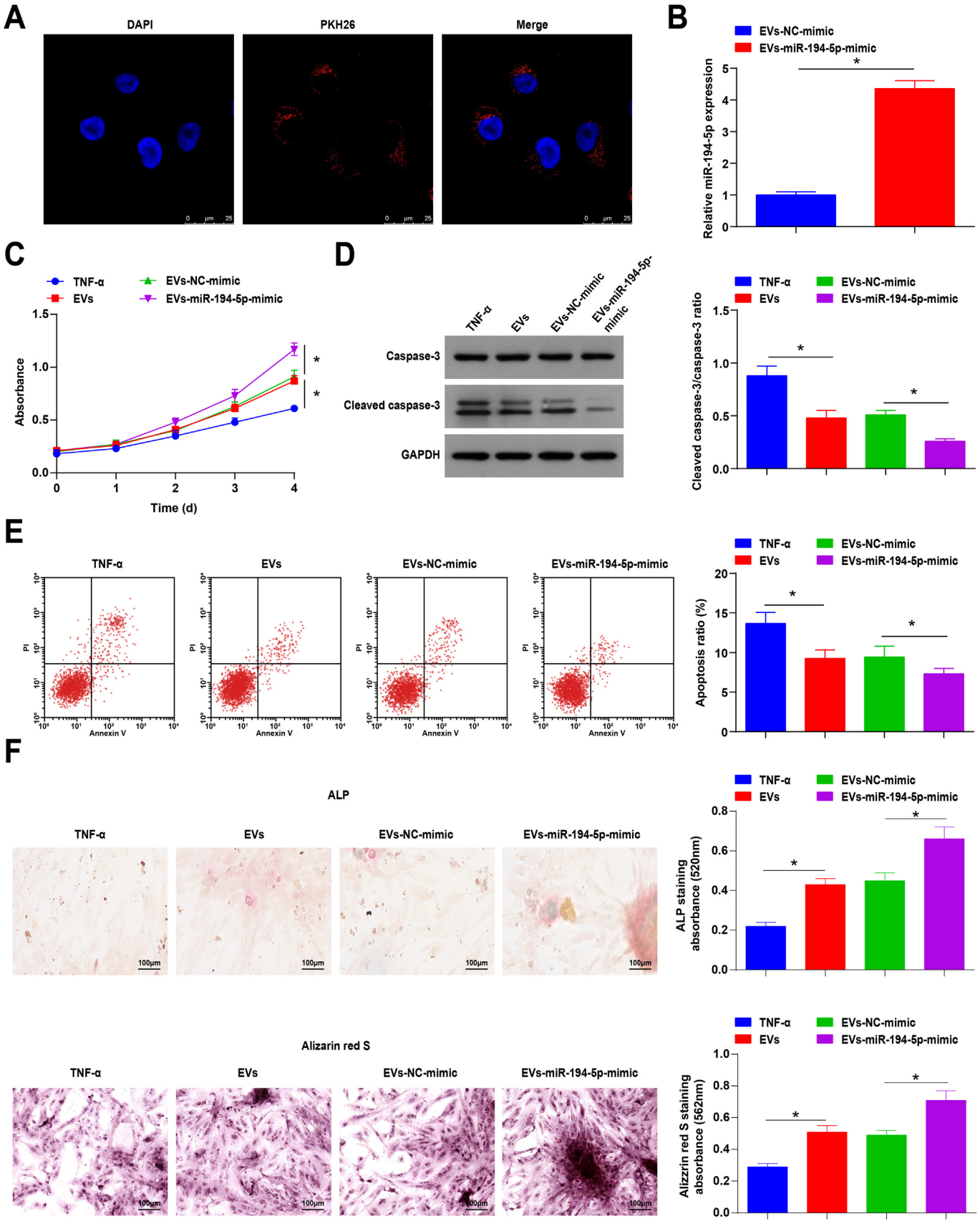

Fig. 4. miR-194-5p-enriched MSCs-EVs ameliorate IDD. A. MSC-EVs uptake by NP cells; B. RT-qPCR detected miR-194-5p expression; C. CCK-8 assay detected the effect of MSCs-EVs overexpressing miR-194-5p on TNF-α-intervened NP cell proliferation; D. Western blot assay detected the effect of MSCs-EVs overexpressing miR-194-5p on Cleaved Caspase-3 and Caspase-3 protein expression in TNF-α-intervened NP cells; E. Flow cytometry detected the effect of MSCs-EVs overexpressing miR-194-5p on TNF-α-intervened NP cell apoptosis; F. ALP staining and alizarin red staining detected the effect of MSCs-EVs overexpressing miR-194-5p on osteogenic differentiation of NP cells. Data were expressed as mean ± standard deviation (N = 3). *, $p < 0.05$.

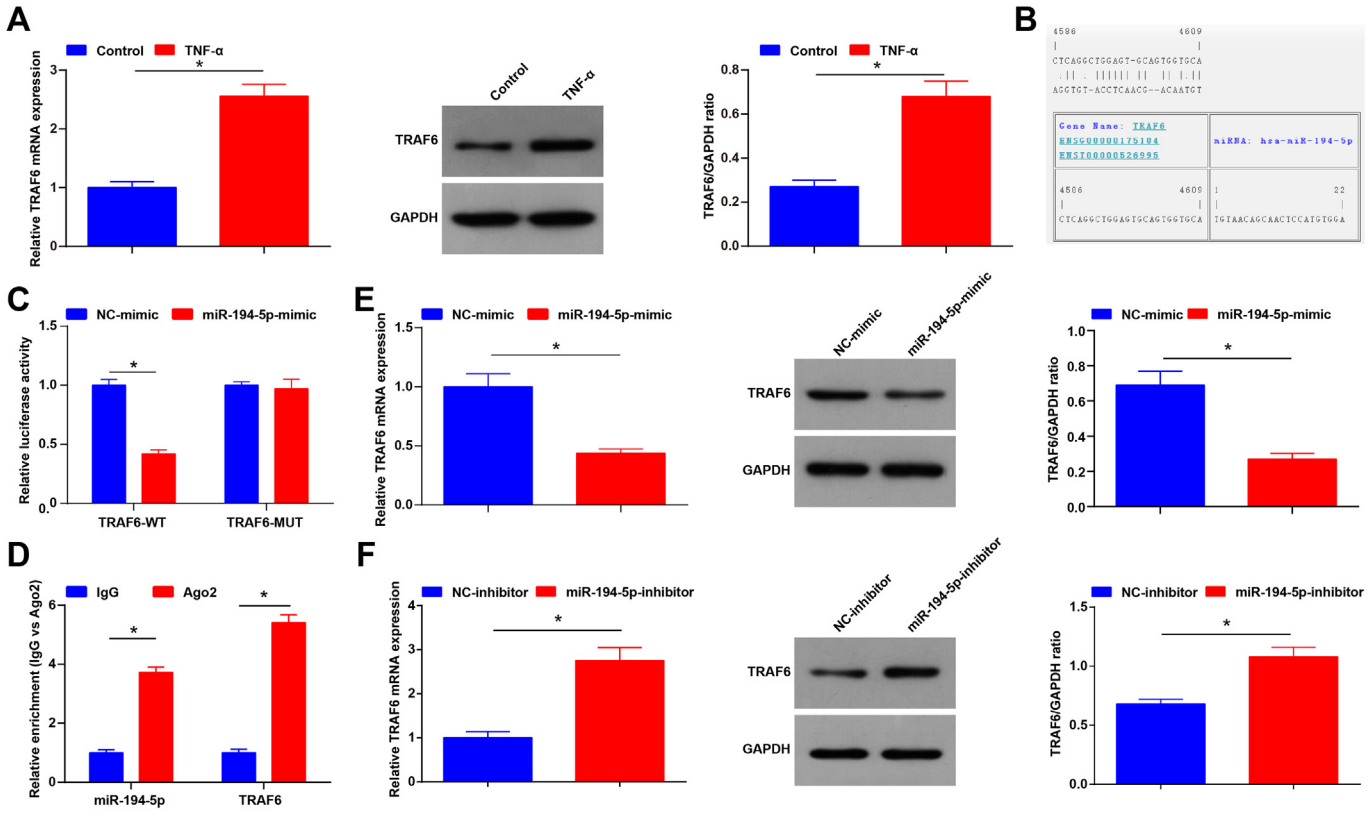

Fig. 5. TRAF6 is targeted by miR-194-5p. A. RT-qPCR and western blot assay detected TRAF6 expression; B. Bioinformatics website predicted the binding sequence of miR-194-5p and mutant sequence of TRAF6; C. Dual luciferase reporter gene assay verified the interaction between TRAF6 and miR-194-5p; D. RIP assay detected the binding of TRAF6 and miR-194-5p to Ago2; E. RT-qPCR and western blot assay detected TRAF6 expression after miR-194-5p up-regulation; F. RT-qPCR and western blot assay detected TRAF6 expression after miR-194-5p down-regulation. Data were expressed as mean ± standard deviation (N = 3). *, $p < 0.05$.

enhanced chemiluminescence reagent (Millipore) and detected by Amersham™ Imager 680 (GE Healthcare Life Sciences) [22].

2.13. Statistical analysis

Analysis of variance (ANOVA) ort test was utilized to evaluate the disparities of data, followed by Tukey's test. All statistical analyses were conducted with software GraphPad Prism 7 (GraphPad Software, Inc.). Data were expressed as mean ± standard deviation. $P < 0.05$ was of statistical significance.

3. Results

3.1. Identification of NP cells, MSCs and MSCs-EVs

NP cells, the main cell type in NP, were responsible for the synthesis and maintenance of gelatinous extracellular matrix, and IDD was characterized by the absence of NP cells and extracellular matrix [23]. MSCs could reduce apoptosis of NP cells in IDD, which may be attributed to EVs (rich in nucleic acids, proteins and miRNA) secreted by MSCs [24,25]. The potential mechanism of MSCs-EVs in relieving IDD was translated in this work. NP cells and MSCs were obtained from patients with thoracolumbar fractures, and identified through determining their surface antigens by flow cytometry. CD24 and CD29 were positively expressed while CD34 was negatively expressed in NP cells. CD90 was positively expressed while CD45 was negatively expressed on the surface of MSCs (Fig. 1A). Subsequently, MSCs-EVs were isolated from MSCs by differential centrifugation and observed by TEM (Fig. 1B). It was noticeable that MSCs-EVs exhibited a typical circular shape with a diameter of 60–200 nm. Also, CD81 and CD9, the markers of MSCs-EVs were determined by western blot assay (Fig. 1C).

3.2. TNF-α intervenes NP cell proliferation and enhances apoptosis, and restricts osteogenic differentiation

Based on the application of TNF-α in IDD induction [26], the IDD model in this work was established by 5 ng/mL TNF-α to intervene NP cells [8]. According to CCK-8 assay results, it was found that TNF-α intervention inhibited the proliferation of NP cells (Fig. 2A). As shown in Western blot test (Fig. 2B), TNF-α intervention increased the ratio of Cleaved Caspase-3/Caspase-3expression. Flow cytometry demonstrated that TNF-α promoted cell apoptosis (Fig. 2C). Considering the link between IDD and calcification process, ALP staining and alizarin red staining were applied to clarify the effects of TNF-α on osteogenic differentiation of NP cells. It was demonstrated that TNF-α inhibited osteogenic differentiation of NP cells (Fig. 2D).

3.3. miR-194-5p is down-regulated in IDD; Restoring miR-194-5p alleviates IDD

Previously evidenced, miR-194-5p was down-regulated in IDD and affected IDD by regulating target genes [10]. Enlightened by that, it was speculated that the beneficial effects of MSCs-EVs on NP cells might depend on miR-194-5p to exert biological effects. RT-qPCR displayed that miR-194-5p expression in TNF-α intervened NP cells was degraded (Fig. 3A), but elevated by miR-194-5p mimic (Fig. 3B). Then, miR-194-5p overexpression could enhance proliferation (Fig. 3C), reduce the ratio of Cleaved Caspase-3/Caspase-3

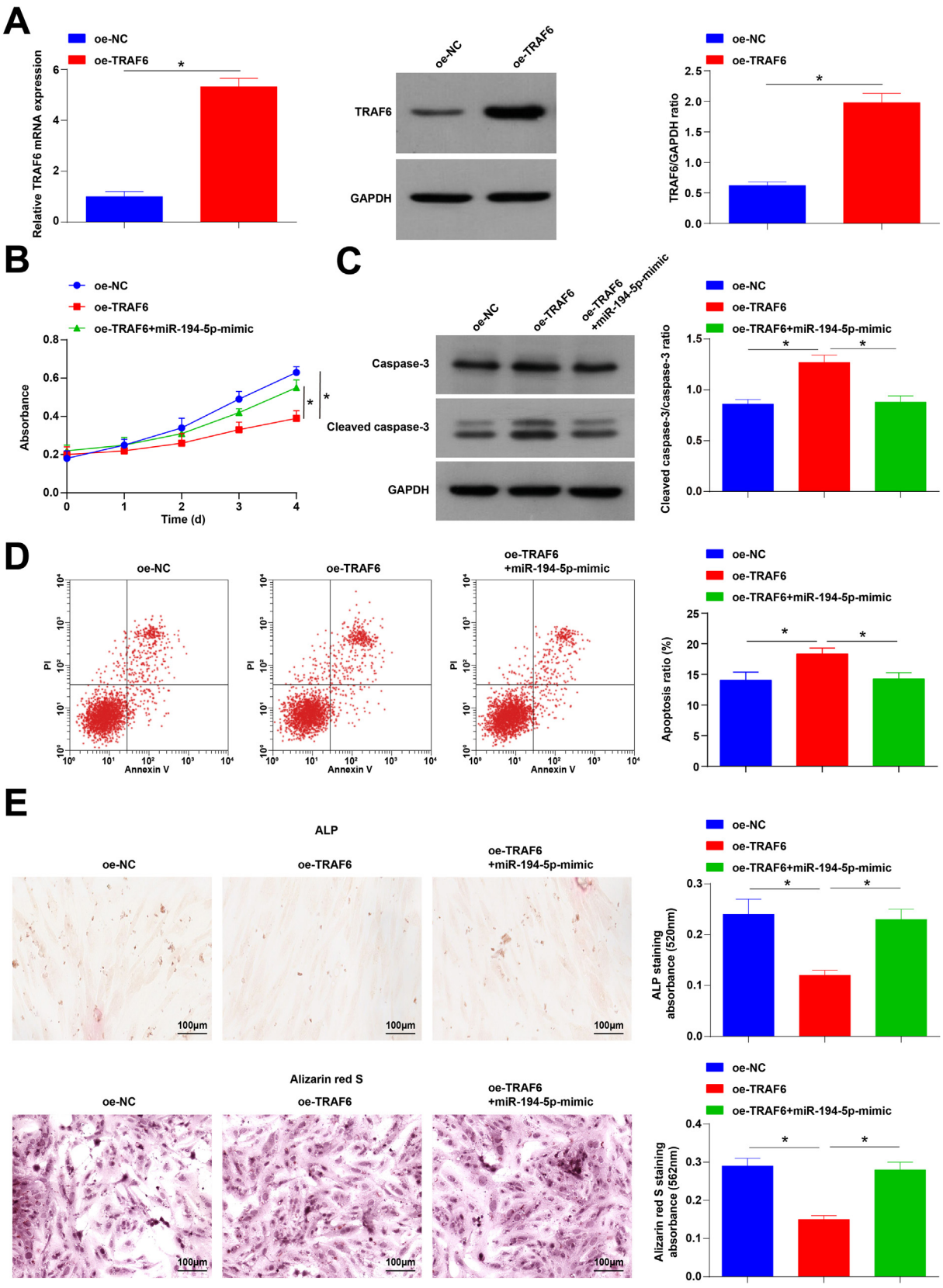

Fig. 6. MSCs-EVs-derived miR-194-5p mediates TRAF6 to relieve IDD. A. RT-qPCR and western blot assay detected TRAF6 expression; B. CCK-8 assay detected NP cell proliferation; C. Western blot assay detected Cleaved Caspase-3 and Caspase-3 protein expression; D. Flow cytometry detected NP cell apoptosis; E. ALP staining and alizarin red staining detected osteogenic differentiation of NP cells. Data were expressed as mean ± standard deviation (N = 3). *, $p < 0.05$

expression (Fig. 3C) and apoptosis (Fig. 3D), as well as induce osteogenic differentiation of TNF-α intervened NP cells (Fig. 3E).

3.4. miR-194-5p-enriched MSCs-EVs ameliorate IDD

The impacts of MSCs-EVs on TNF-α-intervened NP cells were studied after co-culture. The uptake of MSCs-EVs by NP cells was observed (Fig. 4A). NC-mimic or miR-194-5p-mimic-modified MSCs-EVs co-cultured with NP cells. miR-194-5p expression was raised in NP cells after co-culture with miR-194-5p-mimic-modified MSCs-EVs (Fig. 4B). It was obvious that co-culture with MSCs-EVs induced proliferation (Fig. 4C), suppressed the ratio of Cleaved Caspase-3/Caspase-3 expression (Fig. 4D) and apoptosis (Fig. 4E), and facilitated osteogenic differentiation of TNF-α-intervened NP cells (Fig. 4F). MSC-EVs-miR-194-5p-mimic had a strengthened effects than MSCs-EVs on NP cells (Fig. 4B–F).

3.5. TRAF6 is targeted by miR-194-5p

miRNAs usually bind to and silence target genes to exert biological effects [27]. TRAF was a binding protein of TNF superfamily and toll/IL-1 receptor (TIR) superfamily, that involved in innate immunity and acquired immunity. TRAF6 was reported to connect with the occurrence of IDD [12,28]. In TNF-α-intervened NP cells, TRAF6 expression was increased (Fig. 5A). Therefore, TRAF6 was speculated to be a potential target gene of miR-194-5p. Predicted by the RNA22 bioinformatics website, TRAF6 might be the potential target of miR-194-5p (Fig. 5B). Subsequently, dual luciferase reporter gene assay verified that (Fig. 5C) TRAF6-WT and miR-194-5p mimic co-transfection undermined NP cell luciferase activity, while TRAF6-MUT and miR-194-5p mimic co-transfection imposed no impact on the luciferase activity of NP cells. Further validated by RIP assay (Fig. 5D), miR-194-5p and TRAF6 were enriched in Ago2. Next, RT-qPCR and western blot assay detected that TRAF6 expression was suppressed by miR-194-5p up-regulation (Fig. 5E) while it was promoted by miR-194-5p inhibition in NP cells (Fig. 5F). All the experimental outcomes were evident that TRAF6 was a potential target gene for miR-194-5p.

3.6. MSCs-EVs-derived miR-194-5p mediates TRAF6 to relieve IDD

To investigate whether miR-194-5p from MSCs-EVs functioned through regulating TRAF6 in IDD, NP cells were transfected with oe-TRAF6 plasmid and intervened with TNF-α. It was demonstrated that oe-TRAF6 plasmid raised TRAF6 mRNA and protein expression (Fig. 6A). The functional results presented that TRAF6 overexpression diminished TNF-α-intervened NP cell proliferation (Fig. 6B), elevated the ratio of Cleaved Caspase-3/Caspase-3 expression (Fig. 6C) and apoptosis of NP cells (Fig. 6D), and reduced osteogenic differentiation (Fig. 6E). Then, TNF-α-induced NP cells transfected with miR-194-5p mimic and oe-TRAF6 plasmids were similar to TNF-α-induced NP cells transfected with oe-NC (Fig. 6A–E).

4. Discussion

IDD is a degenerative disease that causes NP cell collapse and herniation, thereby developing into radiculopathy [29]. In this work, the significance of miR-194-5p from MSCs-EVs in IDD has been evidently proved. Firstly, it is elucidated that MSCs-EVs alone induce NP cell proliferation and restrict apoptosis, and enhance osteoblast differentiation in IDD. Then, miR-194-5p is down-regulated by TNF-α in NP cells, and miR-194-5p overexpression further enhances the positive effects of MSCs-EVs in IDD. It is also illustrated that miR-194-5p up-regulation inhibits TRAF6, thereafter to alleviate IDD.

mscMSCs-derived exosomes delay the progressive degeneration of IVD by mediating the oxidative stress- and inflammation-related indicators [30]. More recently, the disrupted NP cell apoptosis and attenuated IDD progression are profoundly achieved by treatment of MSCs-derived exosomes [31]. Moreover, in rats with IDD, the apoptotic NP cells and degenerated IVD are relieved upon rats being intradiscally injected with MSCs-derived exosomes [8]. Besides, BMSCs-derived exosomes could elevate the proliferation rate of NP cells in IDD [6]. In osteoporosis, BMSCs-derived exosomes are able to facilitate osteoblast differentiation [32]. Noticeably, exosomes from BMSCs-derived induce osteoblast differentiation by elevating the early osteoblast parameter ALP [33]. MSCs-derived exosomes are verified to collaborate with miRNAs to function in IDD. For example, human placental MSCs-derived exosomes exert attenuated effects on IDD, such as inhibition of apoptosis and NP cell damage by delivering miR-4450 inhibitor [7]. Exactly, the therapeutic efficacy of BMSCs-derived exosomes in IDD has been testified by the suppressed apoptosis of NP cells via the transportation of miR-532-5p [9]. Given the reciprocal between MSCs-EVs and miRNAs, MSCs-EVs are subjected to transfection with miR-194-5p mimic and co-culture with NP cells in this article.

Discussed independently, miR-194-5p expression is decreased dependent on inflammation (IL-6 or TNF-α stimulation) in IDD samples [10]. Also, miR-194 expression is lower than the basic line in IDD and miR-194 overexpression attenuates the inflammation in NP cells, and it is participated in the mediation of TRAF6 [11]. miR-194-5p-oriented studies ate mainly focused on cancers. For instance, miR-194-5p is down-regulated in clear cell renal cell carcinoma, leading to enhanced proliferation and destructed apoptosis of the malignant cells [34]. In acute myeloid leukemia, miR-194-5p expression declines, and restoration of miR-194-5p retards proliferation and triggers cell apoptosis [35]. In addition, a decrease is presented in miR-194-5p expression in laryngeal cancer and miR-194-5p restoration results in inhibited proliferation and accelerated apoptosis [36]. miR-194 expression is induced to elevate by osteoblast differentiation induction and raising miR-194 encourages osteoblast differentiation of BMSCs [37]. Additionally, enhanced osteoblast differentiation in osteogenesis is attained by elevated miR-194 [38].

TRAF6 has been validated to involve in IDD in this work. In fact, long non-coding RNA HCG18 is witnessed to deteriorate IDD by inhibiting miR-146a-5p and elevating TRAF6 expression, while up-regulating TRAF6 interferes NP cell growth through arresting cells in the S phase and stimulating cell apoptosis [12]. In addition, miR-146a expression is reduced upon the initiation of IDD, and miR-146a up-regulation decreases TRAF6 expression to elicit attenuation IDD [13]. Similarly, miR-146a restoration in lung adenocarcinoma cells raises the manifestation of TRAF6, thereby delaying cell proliferation and speeding up cell apoptosis [39]. Furthermore, knocking down lncRNA sex-determining region Y-box 2 overlapping transcript results in increased miR-455-3p and decreased TRAF6 to improve proliferation but obstruct apoptosis in ischemic heart failure [40]. Experimentally, carboxyl terminus of Hsp70-interacting protein reinforces osteoblast differentiation and destroys osteoclast formation to promote bone remodeling partly through impairing TRAF6 activity [41]. Lnsoprazole increases polyubiquitination of TRAF6 to give an impetus for osteoblast differentiation [42].

All in all, this work has figured out that MSCs-EVs over-expressing miR-194-5p ameliorate the pathology of IDD via repression of TRAF6. Due to the limitations of the research scale, the findings in the present work are subjected to more explorations in a larger cohort for further consolidation and advancement.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2021.12.001.

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