Biochemistry and Biophysics Reports 29 (2022) 101216

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

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

miR-29b inhibits TGF-β1-induced cell proliferation in articular chondrocytes

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ARTICLE INFO

Keywords:
Chondrocyte
TGF-β1
microRNA-29b-3p
Osteoarthritis
Cell proliferation

ABSTRACT

Transforming growth factor β1 (TGF-β1) is a known regulator of chondrocyte proliferation and promotes cartilage repair in osteoarthritis (OA). microRNA-29b-3p (miR-29b-3p) is downregulated by TGF-β1 and overexpressed in OA cartilage. However, the ability of miR-29b-3p to mediate the chondrocyte pro-proliferative effects of TGF-β1 is not yet understood. This current study aimed to investigate the effect of miR-29b-3p on TGF-β1-induced cell proliferation in murine articular chondrocytes. The stimulation of chondrocytes by TGF-β1 for 24 h resulted in the downregulation of miR-29b-3p expression. The ratio of G0/G1 phase cells decreased in response to TGF-β1 whereas the ratio of S phase cells was increased. Consistent with this observation, miR-29b-3p overexpression inhibited TGF-β1's ability to promote the ratio of S phase cells and downregulate the ratio of G0/G1 phase cells. These findings suggest that the downregulation of miR-29b-3p is a likely requirement for TGF-β1-mediated proliferation of murine articular chondrocytes. Furthermore, implying that miR-29b-3p expression may be involved in reduced chondrocyte proliferation in OA.

1. Introduction

Chondrocytes are the dominant cells within articular cartilage and through the synthesis and secretion of a collagen and proteoglycan rich extracellular matrix, they are responsible for the formation of smooth articulating joint cartilage which is required for pain free movement [1,2]. The proliferation and apoptosis of chondrocytes are in dynamic balance, and maintaining the number of articular chondrocytes is crucial to proper function [3,4]. In osteoarthritis (OA), cartilage homeostasis is disrupted by increased chondrocyte apoptosis and the loss of type II collagen and proteoglycans within the extracellular matrix, together these lead to a progressive loss of joint function [5]. Transforming growth factor β1 (TGF-β1) has been implicated in both chondrocyte proliferation and the repair of cartilage in OA but as the precise cellular mechanisms involved are unclear we are not yet in a position to utilize this knowledge to facilitate therapeutic options for the treatment of OA [6].

MicroRNAs (miRNAs) are endogenous small non-coding RNAs with a length of approximately 22 nucleotides and regulate gene expression via binding to their target sites in the 3'-untranslated region of messenger RNA (mRNA) [7,8]. Specifically, members of the microRNA-29 (miR-29) family have been reported to contribute to the cell and matrix changes characteristic of OA [9]. The miR-29 family consists of miR-29a, miR-29b, and miR-29c and the expression of miR-29b-3p is upregulated in mouse and human OA cartilage [10–13]. Whilst TGF-β1 induces cell proliferation and accelerates the transition from G1 to S phase in the cell cycle of deer antler chondrocytes, miR-29b-3p inhibits cell proliferation and induces cell cycle arrest in rat articular chondrocytes [12,14]. Although miR-29b-3p has been identified as a downstream target of TGF-β1 in human OA chondrocytes, it is unknown whether miR-29b-3p can mediate TGF-β1's ability to promote chondrocyte proliferation [10]. Therefore, the aim of this current research was to investigate the functional role of miR-29b-3p during TGF-β1-induced cell proliferation in murine articular chondrocytes.

2. Materials and methods

2.1. Isolation and culture of murine articular chondrocytes

All animal experiments were approved by the Roslin Institute's named veterinary surgeon and named animal care and welfare officer (NACWO), with animals maintained in accordance with the Home Office

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https://doi.org/10.1016/j.bbrep.2022.101216
Received 23 November 2021; Received in revised form 12 January 2022; Accepted 20 January 2022

code of practice (for the housing and care of animals bred, supplied, or used for scientific purposes). Pregnant C57BL/6J mice were purchased from Charles River (Margate, UK). Articular cartilage was obtained by dissection of knee joints and femoral heads of 5–6 day old pups of either sex as previously described [15,16]. Chondrocytes were obtained after digestion with 3 mg/ml collagenase D (2 × 45min) and overnight with 0.5 mg/ml collagenase D. The cell suspension was filtered through a 70 μm cell strainer and the filtrate centrifuged for 10 min at 400×*g* at room temperature. The cell pellet was washed with phosphate buffered saline (PBS) and resuspended in DMEM (Sigma Aldrich, Gillingham, UK) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Paisley, UK), 4 mmol/l L-glutamine (Sigma Aldrich), and 0.5% 10 mg/ml gentamicin (Life Technologies). The chondrocytic phenotype of cultured cells was confirmed by expression of type II collagen alpha 1 (Col2a1) and aggrecan by quantitative real time PCR (qRT-PCR) and western blotting. The expression of both were decreased in passage 2 (P2) cells compared with P0 and P1 cells and therefore P1 chondrocytes were used in all experiments (Supplementary Figs. 1A–D). Chondrocytes were plated at 7.7×10^4 cells/well in each well of a 6-well plate and maintained in a humidified incubator at 37 °C in 5% CO_2 for all experiments.

2.2. Cytokine (TGF-β1, IL-4, and IL-1β) stimulation of murine articular chondrocytes

Cells were grown to 70% confluence and then serum starved in DMEM containing 1% FBS for 24 h. Finally, the cells were stimulated with recombinant human TGF-β1, IL-4, and IL-1β (R&D system, Oxfordshire, UK) at 10 ng/ml for 24 h.

2.3. miR-29b-3p overexpression in murine articular chondrocytes

When the cells reached 70% confluence, the medium was replaced with DMEM containing 1% FBS and cells were transfected for 24 h with either non-targeting Allstars Negative Control siRNA (SI03650318, QIAGEN, Manchester, UK) or *syn*-mmu-miR-29b-3p miScript mimic (MSY0000127, QIAGEN, Manchester, UK) using Lipofectamine® RNAiMAX (Thermo Fisher Scientific, Paisley, UK) according to the manufacturer's instructions. The sequence of the miRNA-29b-3p mimic (miR-29b) was 5 '-UAGCACCAUUUGAAAUCAGUGUU-3'.

Briefly, control siRNA or miR-29b and Lipofectamine® RNAiMAX complexes were prepared as follows: 25 pmol of diluted control siRNA or miR-29b in 125 μl Opti-MEM® medium (Thermo Fisher Scientific, Paisley, UK) and 7.5 μl of Lipofectamine® RNAiMAX in 125 μl Opti-MEM® medium were mixed and incubated for 5 min at room temperature. The 250 μl complexes were added to each well of a 6-well plate containing 1750 μl of DMEM with 1% FBS, resulting in a 12.5 nM final concentration. This mixture without control siRNA or miR-29b was used as mock. 24 h after transfection, cells were stimulated with recombinant human TGF-β1 (R&D system) at 10 ng/ml for 24 h without medium change.

2.4. ADAM12 siRNA transfection in murine articular chondrocytes

siRNA transfection for a disintegrin and metallopeptidase domain 12 (ADAM12) was performed with siRNA reagent system (sc-45064, Santa Cruz Biotechnology, Dallas, TX, USA) following the manufacturer's instructions. Briefly, cells were transfected at 70% confluency, either with ADAM12 siRNA (sc-41415, Santa Cruz Biotechnology) or unconjugated control siRNA-A (sc-37007, Santa Cruz Biotechnology) in transfection medium (sc-36868, Santa Cruz Biotechnology) and transfection reagent (sc-29528, Santa Cruz Biotechnology). This mixture, without siRNA or siRNA-A was used as control. Cells were incubated for 8 h before addition of medium supplemented with 20% FBS with 2% antibiotics for another 24 h. Cells were then incubated in DMEM including 1% FBS for 24 h and stimulated with recombinant human TGF-β1 (R&D system) at 10 ng/ml for a further 24 h.

2.5. RNA extraction and qRT-PCR

The cells were washed three times in PBS, scraped in Qiazol (Qiagen, Crawley, UK) and stored at −70 °C prior to processing. Total RNA was extracted using miRNeasy kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. RNA concentration was measured using nanodrop spectrophotometry (Thermo Fisher Scientific, Loughborough, UK), and the purity of the RNA was assessed using the 260:280-nm wavelength ratio. RNA was reverse transcribed using SuperScript II (Thermo Fisher Scientific, Paisley, UK) according to the manufacturer's instructions and the cDNA was mixed with gene specific primers and qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems, London, UK). The primer sequences were: glyceraldehyde 3-phosphate dehydrogenase (Gapdh), forward 5′-ATACGGCTACAGCAACAGGG-3′; reverse 5′-GCCTCTCTTGCTCAGTGTCC-3′; Col2a1, forward 5′- CACACTGG-TAAGTGGGGCAAGACCG-3′; reverse 5′- GGATTGTGTTGTTT-CAGGGTTCGGG-3′; aggrecan, forward 5′-GTTGGTTACTTCGCCTCCAG-3′; reverse 5′-GTCCTCCAAGCTCTGTGACC-3′; Adam12, forward 5′-CACGAATCGCTGCTGTAACGCTA-3′; reverse 5′- CTCTCAGCTCA-CATTTGGCGAAGGC-3′. qRT-PCR reactions were conducted at 95 °C for 2min followed by 40 cycles of 95 °C for 5s and 60–65 °C for 30s using a Stratagene Mx3005P PCR machine (Agilent Technologies, Cheadle, UK). The levels of mRNA were normalized to Gapdh.

To examine the expression of miRNA, total RNA was used to synthesize cDNA using the miScript II RT kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Qiagen miScript primers were used to detect mature miR-29a-3p (MS00001372, Qiagen, Manchester, UK), miR-29b-3p (MS00005936, Qiagen, Manchester, UK), and miR-29c-3p (MS00001379, Qiagen, Manchester, UK). Each miRNA primer was mixed with the cDNA, universal primer, and miScript SYBR Green solution provided in miScript SYBR Green PCR kit (Qiagen, Crawley, UK). qPCR reactions were conducted at 95 °C for 15min followed by 40 cycles of 94 °C for 15s, 55 °C for 30s, and 70 °C for 30s using a Stratagene Mx3005P PCR machine. The small nuclear RNA U6 (RNU6) (MS00033740, Qiagen, Manchester, UK) was used as an endogenous control for miRNA detection. Both mRNA and miRNA relative quantifications were calculated using the $\Delta\Delta C_T$ method [17].

2.6. Western blotting

Proteins were isolated with RIPA-buffer (Thermo Fisher Scientific, Paisley, UK) and protein concentrations were measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, Paisley, UK). Equal amounts of total protein were applied to polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE). After electrophoresis, proteins were blotted to PVDF membranes and blocked in TBS-T (Tris Buffered Saline containing 0.1% Tween-20) supplemented with 5% skimmed milk powder. The membranes were then probed overnight at 4 °C with a goat polyclonal antibody against COL2A1 (1:1000 dilution; sc-7764, Santa Cruz Biotechnology), a mouse monoclonal antibody against aggrecan (1:1000 dilution; sc-166951, Santa Cruz Biotechnology), and a rabbit monoclonal antibody against β-actin (1:1000 dilution; 4970, Cell Signaling Technology, Beverly, MA, USA). Membranes were washed four times with TBS-T and probed with the appropriate horseradish-peroxidase-conjugated (HRP) secondary antibody at room temperature for 50min (polyclonal rabbit anti goat IgG HRP, 1:1000, polyclonal goat anti mouse IgG HRP, 1:1000 or polyclonal goat anti rabbit IgG HRP, 1:1000, All Dako, Glostrup, Denmark). The specific protein was detected by Super Signal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Paisley, UK) according to the manufacturer's instructions. Quantification of Western blot images was performed with ImageJ software and expression levels were normalized to those of β-actin as a loading control.

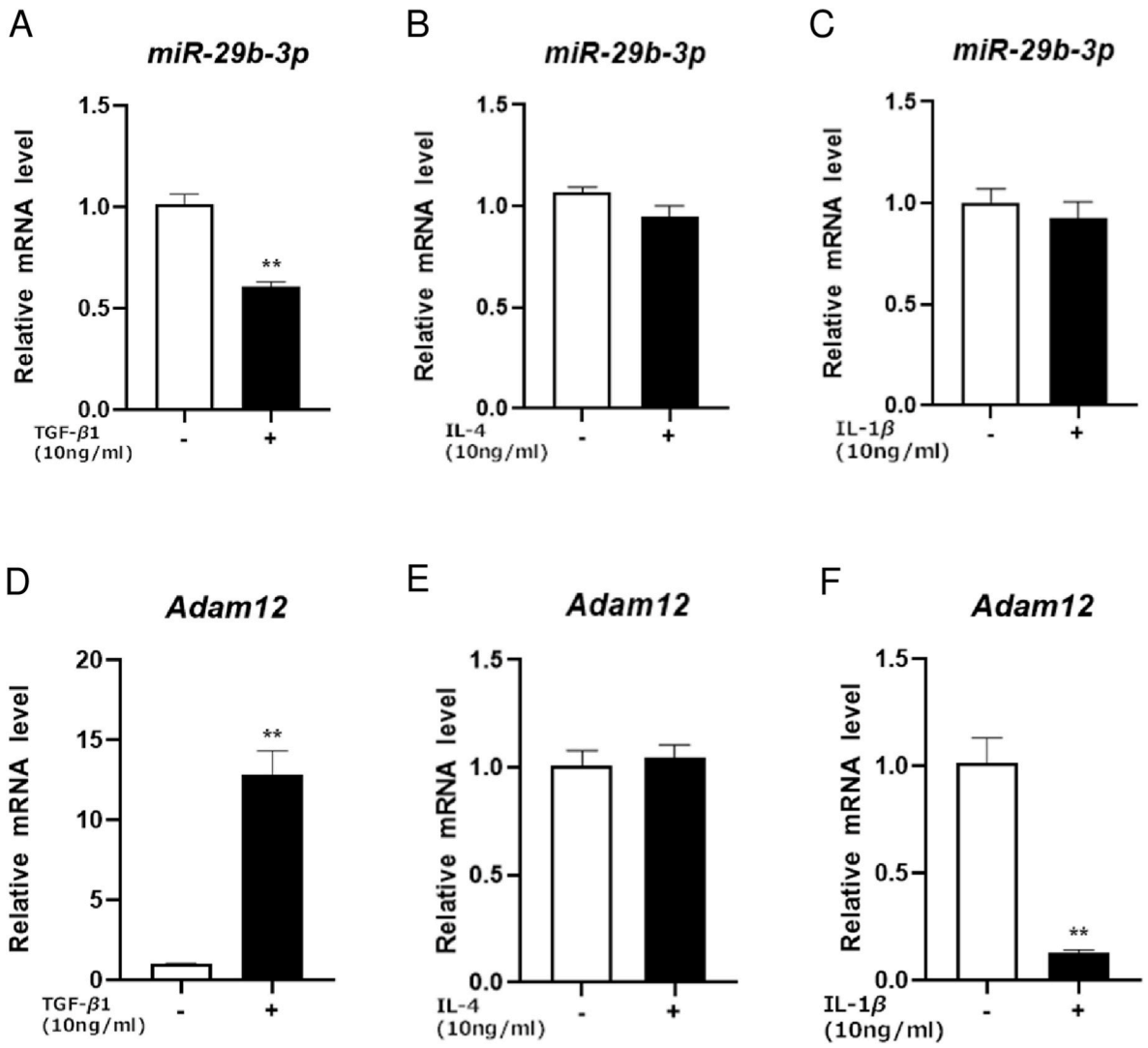

Fig. 1. miR-29b-3p expression is reduced and *Adam12* expression is increased by TGF-β1 stimulation. Results of qRT-PCR showing (A–C) miR-29b-3p and (D–F) *Adam12* expression after 24 h of stimulation with TGF-β1, IL-4 and IL-1β at 10 ng/ml in murine articular chondrocytes. Following 24 h of TGF-β1 stimulation, the expression levels of miR-29b-3p was reduced by 0.6-fold whilst *Adam12* expression was increased by approximately 12-fold. The stimulation by IL-4 didn't alter miR-29b-3p or *Adam12* expression. The stimulation by IL-1β did not alter miR-29b-3p expression but decreased *Adam12* expression. Data represent the mean ± SEM of triplicate determinations. $^{**}P < 0.01$ compared with 24 h of non-stimulation.

2.7. BrdU incorporation and cell cycle analysis

To evaluate the cell cycle, cells were labelled with BrdU, *in vitro* using a FITC BrdU flow kit (BD Pharmingen™, San Diego, CA, USA) according to the manufacturer's instructions. BrdU solution was added directly to culture medium at a concentration of 10 μM and incubated for 45min. After BrdU labeling, cells were harvested by trypsinization and staining with fluorescent *anti*-BrdU antibody was carried out at room temperature for 20 min. Cells were then stained for cell cycle analysis using 7-amino-actinomycin D (7-AAD) (BD Pharmingen™) and analyzed by flow cytometry using the LSR Fortessa/X20 flow cytometer (BD Biosciences). The acquired multiparameter data were analyzed using FlowJo 10 (BD Biosciences, Heidlberg, Germany). The combination of BrdU and 7-AAD, flow-cytometric analysis permits the enumeration and characterization of cells that are actively synthesizing DNA (BrdU incorporation) in terms of their cell cycle position (i.e., G0/1, S, or G2/M phases defined by 7-AAD staining intensities).

2.8. Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). All *in vitro* studies were performed in triplicate and repeated on three independent occasions. Statistical analyses were performed using GraphPad Prism v8 (GraphPad Software, CA, USA). The student's *t*-test was used to compare two groups of data. Differences among more than two groups were compared using ANOVA with post hoc Tukey's test. Significance was defined by a p value of <0.05.

3. Results and discussion

The etiology of OA remains unclear but a number of studies have implicated a role for TGF-β1 and miR-29 family members in chondrocyte proliferation, differentiation and OA progression [10]. A key observation is the upregulation of miR-29b-3p in OA cartilage [10–13]. By directly targeting the 3'-UTR of Col2a1, miR-29b-3p may disturb matrix production and the development of the mature chondrocyte phenotype [18]. In mouse OA models, miR-29b-3p expression is highest a few days after destabilization of the medial meniscus surgery with expression

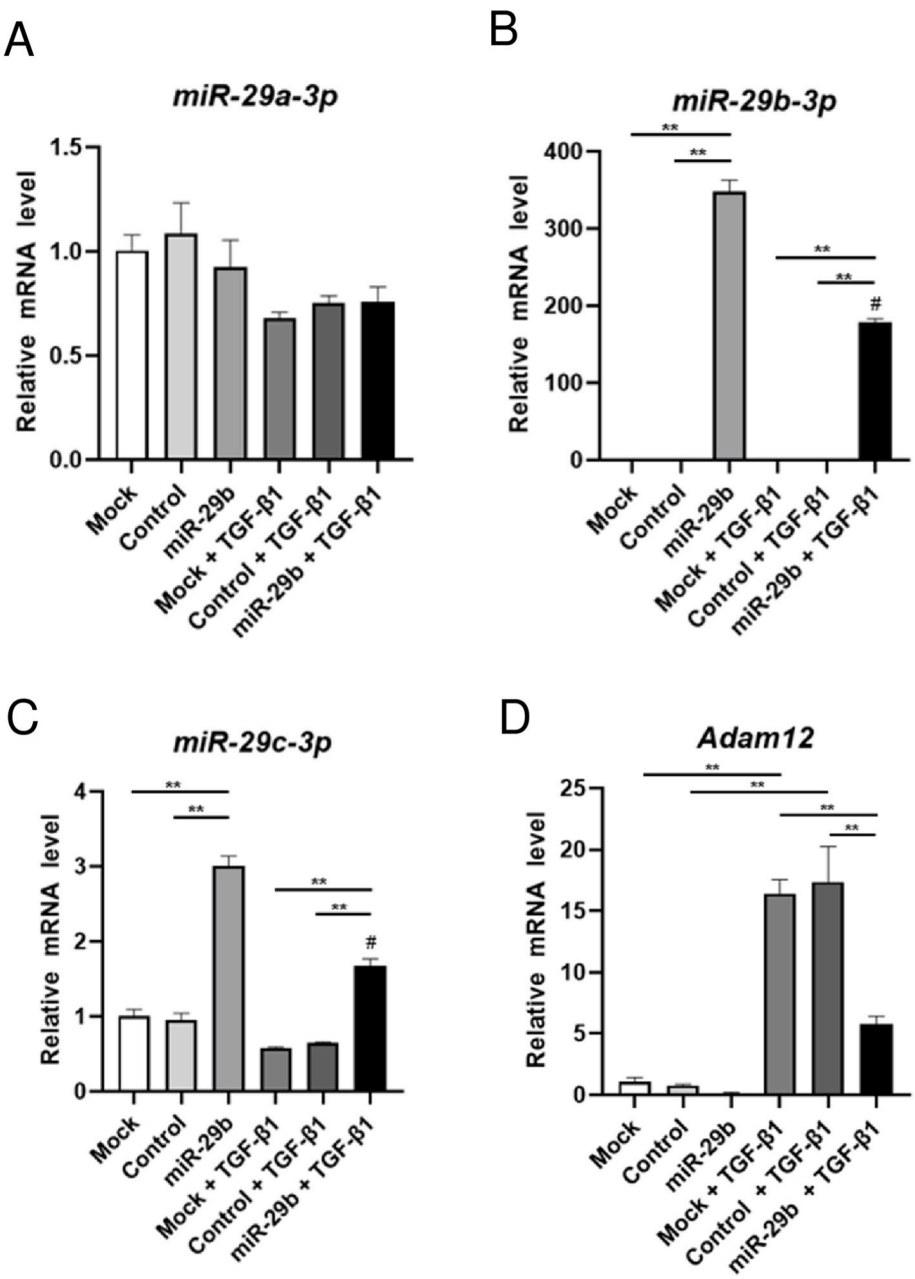

Fig. 2. miR-29b-induced expression changes are inhibited by TGF-β1. Results of qRT-PCR showing (A) miR-29a-3p, (B) miR-29b-3p, (C) miR-29c-3p, and (D) *Adam12* expression after miR-29b-3p mimic (miR-29b) transfection in murine articular chondrocytes. The transfection of miR-29b did not alter miR-29a-3p expression and TGF-β1 also had no additional effect. The expression levels of miR-29b-3p and miR-29c-3p was elevated 350-fold and 3-fold, respectively in miR-29b transfected chondrocytes, whilst TGF-β1 stimulation decreased expression levels of both miR-29b-3p and miR-29c-3p by approximately 50%. miR-29b transfection decreased TGF-β1-induced *Adam12* expression by approximately 70%. Data represent the mean ± SEM of triplicate determinations. $^{**}P < 0.01$, $^{\#}P < 0.01$ compared with miR-29b group.

levels normalising with advancing OA [10,12]. Limited evidence has also disclosed that in human OA chondrocytes, the expression of miR-29 family members are decreased by TGF-β1 and its downstream phosphorylation products, Smads 2 and 3 (Smad2/3); an observation consistent with findings that suggest TGF-β1/miR-29b-3p axis may be involved in OA cartilage repair [6,10].

ADAM12 has also been reported to be involved in OA chondrocyte proliferation and upregulated by TGF-β/Smad2/3 signaling [19,20]. In addition, ADAM12 may be a direct target gene of miR-29b in human breast cancer cells and miR-29b is downregulated ADAM12 expression in rat renal cells [20,21]. The data from the present study confirmed that TGF-β1 downregulates the expression of miR-29b-3p and upregulates *Adam12* expression after 24 h of treatment (Fig. 1A,D). IL-4 and IL-1β are known not to stimulate chondrocyte proliferation and were used as negative controls. These cytokines were unable to alter the expression of miR-29b-3p and *Adam12* in a similar manner to that of TGF-β1 (Fig. 1B, C,E,F). In addition, TGF-β1 was able to temper the increased expression of miR-29b-3p and miR-29c-3p seen in chondrocytes transfected with miR-29b compared with non-stimulated cells. Specifically, the expression of miR-29b-3p and miR-29c-3p was elevated 350-fold and 3-fold, respectively in miR-29b transfected chondrocytes, whilst TGF-β1 stimulation decreased expression levels of both miR-29b-3p and miR-29c-3p by approximately 50%. It should be noted that due to the similarity between the miR-29b and miR-29c sequences we cannot rule out the possibility that the small increase in miR-29c is due to cross reactivity of the primers used. The transfection of miR-29b did not alter miR-29a-3p expression and TGF-β1 also had no additional effect (Fig. 2A–C). Interestingly miR-29b overexpression inhibited TGF-β1-induced *Adam12* expression in murine articular chondrocytes (Fig. 2D). These results suggest that a target for miR-29b-3p is ADAM12 which mediates the effect of TGF-β1 on cell proliferation.

In addition to altering the chondrocyte phenotype and matrix production, miR-29b-3p may also interfere with TGF-β1 function within cartilage [10]. TGF-β1 promotes chondrocyte proliferation and is essential for maintaining cartilage homeostasis and joint health [6, 22–25]. Furthermore, mice with reduced TGF-β signaling have

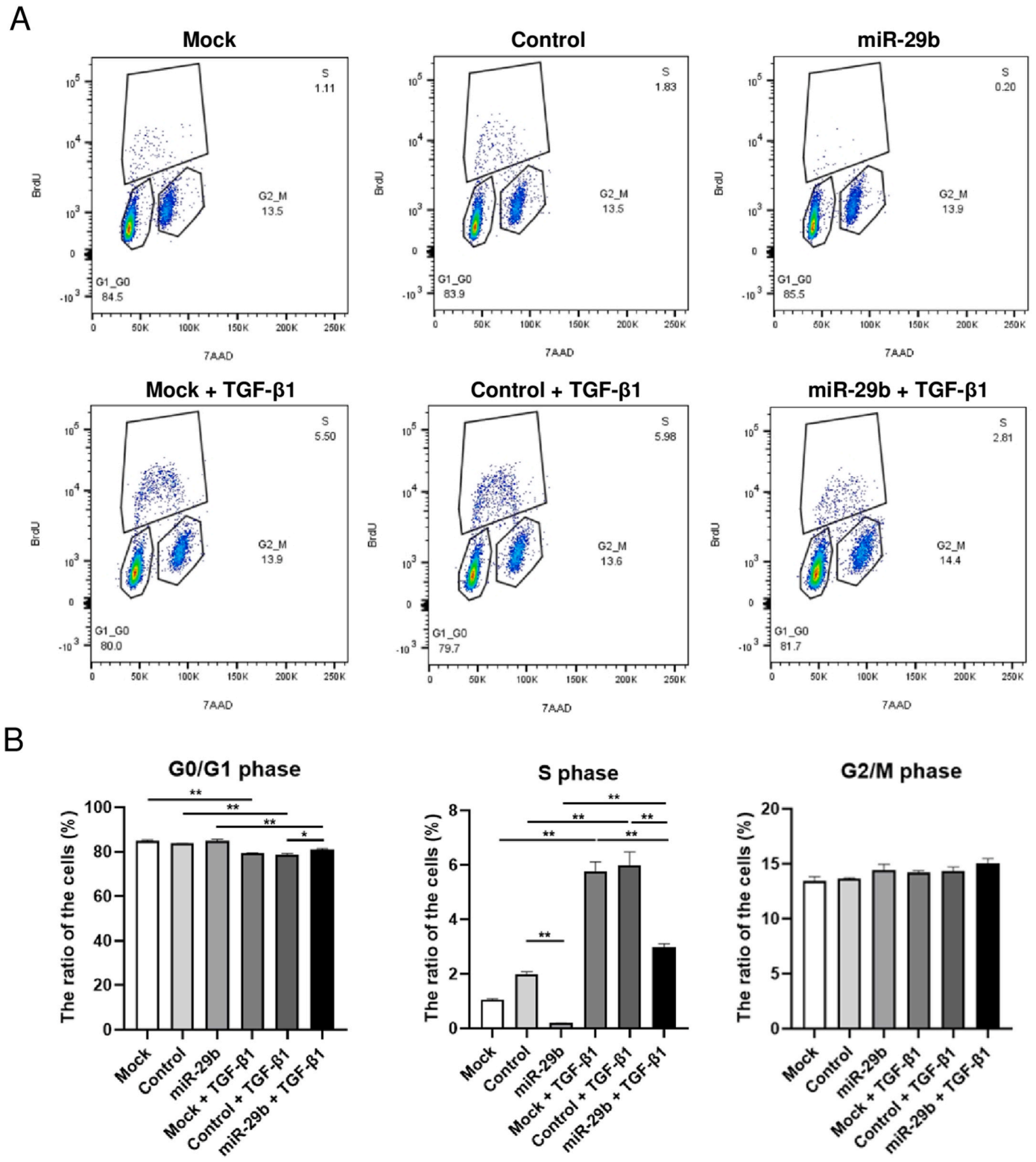

Fig. 3. miR-29b-3p overexpression inhibited TGF-β1-induced cell proliferation in murine articular chondrocytes.
(A) Representative images of the flow-cytometric analysis of cells labelled with BrdU in non-stimulated and 10 ng/ml TGF-β1-stimulated cells with the transfection of Mock, control, and miR-29b-3p mimic (miR-29b). The cells labelled with BrdU were indicated they progressed through S phase of the cell cycle. (B) Quantitative analysis of cell cycle phase of each group. Data represent the mean ± SEM of triplicate determinations. $*P < 0.05$ and $**P < 0.01$.

accelerated articular chondrocyte maturation, leading to defective cartilage matrix and cartilage erosion [26,27]. Therefore, the finding that overexpression of miR-29b-3p inhibits TGFβ1-induced Smad signaling is consistent with the concept that the higher miR-29b-3p present in OA cartilage is capable of disrupting TGF-β1 signaling and promoting the OA phenotype [10].

Previous studies have reported that miR-29b-3p can inhibit cell proliferation in various cells types including rat articular chondrocytes,

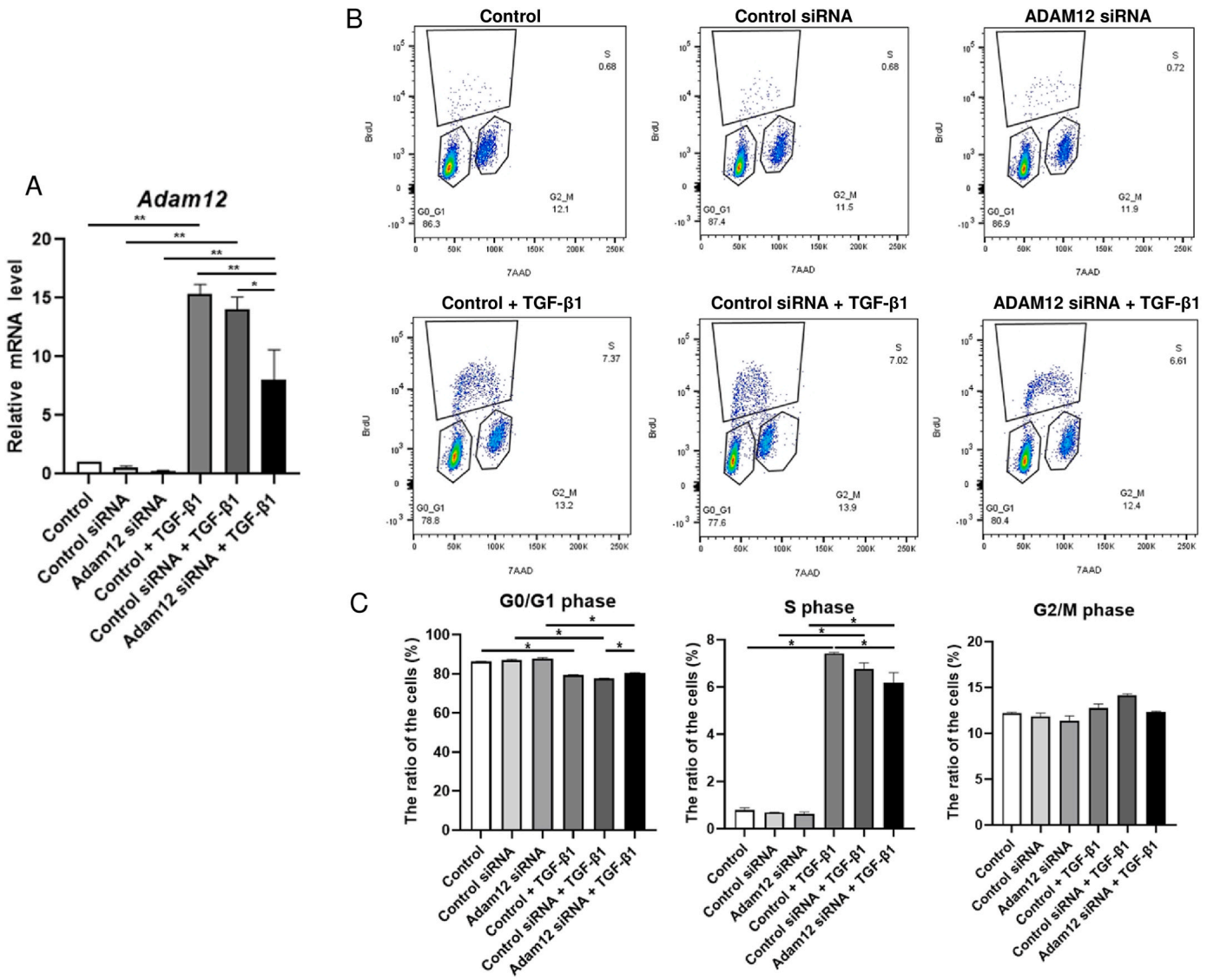

Fig. 4. ADAM12 downregulation did not inhibit TGF-β1-induced cell proliferation in murine articular chondrocytes.
(A) Results of qRT-PCR showing *Adam12* expression after ADAM12 siRNA transfection in murine articular chondrocytes. ADAM12 siRNA transfection decreased TGF-β1-induced *Adam12* expression by approximately 50% (B) Representative images of the flow-cytometric analysis of cells labelled with BrdU in non-stimulated and 10 ng/ml TGF-β1-stimulated cells with the transfection of control, control siRNA, and ADAM12 siRNA. (C) Quantitative analysis of cell cycle phase of each group. Data represent the mean ± SEM of triplicate determinations. $^{*}P < 0.05$ and $^{**}P < 0.01$.

SW-1353 human chondrosarcoma cells, human renal cell carcinoma cell lines, and human gastric cancer cell lines [12,28,29]. Therefore, to investigate the potential of miR-29b-3p to perturb TGF-β1's ability to stimulate chondrocyte proliferation we labelled chondrocytes with BrdU and monitored their progression through the various phases of the cell cycle, by flow cytometry. TGF-β1 stimulated cell proliferation was evidenced by a significant decrease in the ratio of G0/G1 phase cells transfected with Mock, control, and miR-29b (from 84.8% to 79.3%, 83.8%–78.7%, and 85.1%–81.0%, respectively) and a significant increase in the ratio of S phase cells (from 1.1% to 5.4%, 1.9%–6.4%, and 0.2%–3.0%, respectively). This promotion of cell proliferation by TGF-β1 was significantly repressed by miR-29b transfection compared with control transfection in both non-stimulated and TGF-β1-stimulated cells (from 1.9% to 0.2% and 6.4%–3.0%, respectively). On the other hand, the ratio of G0/G1 phase cells in TGF-β1-stimulated cells was significantly increased by miR-29b transfection compared with control transfection (from 78.7% to 81.0%). The ratio of G2/M phase cells was not significantly altered by miR-29b transfection nor treatment with TGF-β1 (Fig. 3A and B). Whilst these results suggest that miR-29b-3p overexpression inhibits TGF-β1-induced cell proliferation, a role for miR-29c-3p cannot be discounted as transfection of miR-29b also upregulated this miR-29 family member (Fig. 2C). The mechanisms by which miR-29c-3p is able to dampen TGF-β1's ability to promote chondrocyte proliferation are not explored in this study, and requires further investigation, but it is likely that miR-29b-3p regulates gene targets to provide a permissive environment for TGF-β to have its pro-mitotic effect.

Previous studies have reported that the downregulation of ADAM12 could inhibit TGF-β1-induced cell proliferation in various cells types including human OA chondrocytes, murine stomal cells, and mouse pituitary adenoma cells [19,30,31]. In addition, a recent study indicated that ADAM12 overexpression can stimulate IGF-1 expression which is a major mitogen [32]. These data suggest that ADAM12 may mediate the pro-proliferation actions of TGF-β1. In the current study, ADAM12 siRNA was used to evaluate the effect of ADAM12 on TGF-β1-induced chondrocyte proliferation. ADAM12 siRNA transfection inhibited the TGF-β1-induced *Adam12* expression (Fig. 4A). TGF-β1 stimulated cell proliferation was evidenced by a significant decrease in the ratio of

G0/G1 phase cells transfected with control, control siRNA, and ADAM12 siRNA (from 86.4% to 79.2%, 87.0%–77.7%, and 87.6%–80.6%, respectively) and a significant increase in the ratio of S phase cells (from 0.8% to 7.4%, 0.7%–6.8%, and 0.6%–6.2%, respectively) (Fig. 4B and C). TGF-β1-induced chondrocyte proliferation was significantly inhibited by ADAM12 siRNA transfection compared with control (from 7.4% to 6.2%), but there was no significant difference in the % of S cells between TGF-β1-stimulated control siRNA and ADAM12 siRNA transfected cells. On the other hand, the ratio of G0/G1 phase cells in TGF-β1-stimulated cells was significantly increased by ADAM12 siRNA transfection compared with control siRNA transfection (from 77.7% to 80.6%) (Fig. 4B and C). These results suggest that ADAM12 may not be involved in mediating the effects of TGF-β1 on murine chondrocytes proliferation. The other specific target possibilities of miR-29b-3p include CDK6, IGF-1, and AKT3 which are known to influence cell proliferation [33–35].

In conclusion, we have demonstrated that miR-29b-3p overexpression inhibited TGF-β1-induced cell proliferation in murine articular chondrocytes, *in vitro*. We also showed that TGF-β1 stimulation downregulated the expression of miR-29b-3p in the cells. These findings suggest that downregulation of miR-29b-3p could be required for TGF-β1 promotion of chondrocyte proliferation and maintenance of cartilage homeostasis. In future studies it will be profitable to identify potential target genes of miR-29b-3p, which may improve our understanding of miRNA modulation during TGF-β1-induced cell proliferation and OA progression.

Author contributions

MH, CF and LAS designed the study. MH performed the experiments. MH, S–NH, and AR analyzed the data. MH wrote and CF and LAS edited the manuscript. All authors interpreted the data and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgement

MH was supported by the Teraoka Scholarship Foundation. S-NH was supported by the Tri-Service General Hospital (TSGH), National Defense Medical Center (NDMC), Taiwan. CF and LAS were supported by the Biotechnology and Biological Sciences Research Council (BBSRC) through an Institute Strategic Programme Grant Funding (BB/J004316/1).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101216.

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