Arch Biol Sci. 2020;72(3):373-378 https://doi.org/10.2298/ABS200507031L

The scinderin gene (*SCIN*) is the direct target of miR3085-3p in chondrocytes

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Received: May 7, 2020; **Revised**: July 15, 2020; **Accepted**: August 1, 2020; **Published online**: August 24, 2020

Abstract: MiR-3085-3p was shown to play a crucial role in cartilage biology, with potential impacts in osteoarthritis (OA). Insight into this miRNA function could be of practical importance for future miRNA-based therapy, however, little is known regarding the biological roles of this miRNA. The physiologic function of an individual miRNA is dictated through its mRNA targets, and as *SCIN* (scinderin, also known as adseverin) was reported to be involved in chondrocyte differentiation, maturation, and phenotype maintenance, this study aimed to prove *SCIN* is a direct target of miRNA-3085-3p. Bioinformatics algorithms were utilized for predicting their interacting sites. Gain- and loss-of-function experiments with miRNA-3085-3p were performed and *SCIN* expression was measured by real-time RT-PCR. *SCIN* 3'UTR regions harboring either the miR-3085-3p seed site or its mutant version were cloned into pmirGLO downstream of a reporter firefly luciferase encoding gene. The effect of miR-3085-3p on this region was determined by the luciferase assay. Four binding sites of miR-3085-3p in *SCIN* 3'UTR were identified. *SCIN* expression level was found to be inversely correlated with the level of miRNA-3085-3p. MiR3085-3p directly binds to its binding sites in *SCIN* 3' UTR. These data suggest that *SCIN* is the direct target of miR-3085-3p in chondrocyte cells.

Keywords: chondrocyte; miR-3085-3p; osteoarthritis; *SCIN*

INTRODUCTION

MicroRNAs (miRNAs) are short (20-25 nucleotides in length) non-coding RNAs that play an important role in the regulation of gene expression at the posttranscriptional level [1]. miRNAs control gene expression by binding to a specific sequence, called the seed site, in the target messenger RNA (mRNA), which leads to repression of mRNA translation or mRNA degradation [2]. There are 1917 precursor miRNAs and 2654 mature miRNAs known in humans according to the miRBase databases (http://www.mirbase.org/, release 22.1). Each miRNA can directly affect hundreds of target genes. Bioinformatics programs predict about 30% of protein-coding genes that are regulated by miRNAs [3]. Identifying the direct target genes of a miRNA will help to understand its function.

Various bioinformatics algorithms and experimental approaches have been employed to determine the putative targets of a given miRNA. The base-pairing interaction between the miRNA seed site and its corresponding complementary target sites harboring in the 3'UTR of the putative target mRNA is one of the general parameters of many computational algorithms [4,5]. Besides perfect base pairing, evolutionary conservation of the seed site or the accessibility of seed sites in mRNAs are additional parameters included in some prediction programs [4-6]. Hence, there are large discrepancies together with some overlap in results obtained from different bioinformatics algorithms. Of note, a given bioinformatics miRNA/mRNA interaction may not necessarily be a firm confirmation of an authentic target for a miRNA of interest since many putative targets have been disproved by experiment [7,8]. Therefore, for determining miRNA targets, bioinformatics algorithms are utilized first as the driving force for identifying potential targets. The biological targets subsequently need to be verified experimentally.

Human miR-3085-3p was identified [9]. It is localized in an intron region of cartilage acidic protein 1

(*CRTAC1*). miR-3085-3p is highly expressed in cartilage and chondrocyte differentiation [9]. The expression level of miR-3085-3p in cartilage tissue of osteoarthritis (OA) patients is higher than in normal counterparts [9]. These results suggest that miR-3085-3p may play a role in OA pathogenesis. Gain- and loss-of-function of miR-3085-3p experiments were performed, followed by a whole genome array to identify the function of miR-3085-3p. *SCIN* (the gene encoding for scinderin, also known as adseverin) was one of the genes whose expression increased when miR-3085-3p decreased, and conversely decreased when miR-3085-3p increased. This result suggests that *SCIN* is a potential target of miR-3085-3p.

SCIN is located on chromosome 7 at 7p21.3. SCIN is a $Ca^{(2+)}$-dependent actin-severing/-capping protein, which contributes to the regulation of osteoclast structure [10], the articular chondrocyte phenotype [11,12], osteoclast and chondrocyte differentiation [10-12]. SCIN was significantly increased during chondrocyte maturation [12]. Moreover, *SCIN* was identified as one of the top 15 genes that is downregulated in injured human meniscus at the age of >40 years as compared to that at ≤ 40 years [13].

The main aim of this study was to examine whether *SCIN* is a direct target of miR-3085-3p and if miR-3085-3p suppresses *SCIN* expression by binding directly to the 3'UTR region of *SCIN*.

MATERIALS AND METHODS

Bioinformatics prediction

The mature hsa-miR-3085-3p sequence (miR-3085-3p) was retrieved from miRBase (http://www.mirbase.org/). The *SCIN* 3'UTR sequence was downloaded from Ensembl Genome Browser (https://asia.ensembl.org/index.html). miR-3085-3p potential target genes were computationally predicted by several bioinformatics programs, TargetScanHuman (http://www.targetscan.org/vert_72/), miRDB (http://mirdb.org/) and miRanda (http://www.microrna.org/microrna/getGeneForm.do). Three different seed sites of miR-3085-3p, "AGCCAG" (6 mer-seed site), "AGCCAGA" (7 mer-seed site), and "CAGCCAGA" (8 mer-seed site) were searched in *SCIN* 3'UTR

Gain-and loss-of-function experiments

Human primary chondrocytes were isolated from human OA knee cartilage. The cartilage chips were incubated with collagenase for 18 h (Sigma, Singapore), followed by washing with Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher, USA) to isolate primary chondrocytes. The cells were grown in DMEM (Thermo Fisher, USA) supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher, USA), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Thermo Fisher, USA) at 37°C and 5% (v/v) CO_2 to reach about 80% confluence. The cells were then plated at $2x10^5$ cells/mL on a 6-well plate to 80% confluence. They were then transfected using Lipofectamine 2000 (Thermo Fisher, USA) with 50 nM miR-3085-3p mimic or inhibitor (Qiagen, Holland) or non-targeting control (Qiagen, Holland) for 24 h according to the manufacturer's instructions.

RNA isolation and real-time RT-PCR

RNA from cultured cells was isolated using TRIzol™ Reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA concentration was measured by Nanodrop. The purity of RNA was determined according to the ratio of A260/280 and A260/230. SuperScript II RT (Thermo Fisher, USA) was used to convert mRNA to cDNA according to the manufacturer's instructions. Expression of *SCIN* mRNA was determined using SYBR® Green I dye, Kappa Fast Universal qPCR Master Mix (2X) (Kappa Biosystems, USA) with forward primer 5'-GAGTGGTGAGAGCCACAGAAG-3' and reverse primer 5'-GTTGCACGAGGAACCACAC-3'. 18S rRNA was used as the endogenous control. *SCIN* relative quantification was calculated using the ΔΔCT method. The real-time PCR reaction was performed on LightCycler® 96 System (Roche, USA).

Luciferase assay

SW1353 chondrosarcoma cells were obtained from the American Type Culture Collection. Cells were cultured in DMEM (Thermo Fisher, USA) supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher, USA), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Thermo Fisher, USA) at 37°C and 5% (v/v) CO_2. The cells were grown in a monolayer to

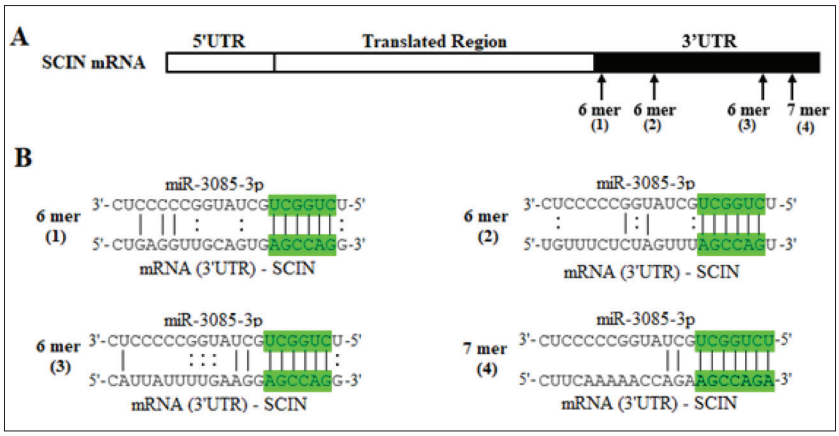

Fig. 1. MiR-3085 seed sites are harbored in *SCIN* 3'UTR. The *SCIN* 3'UTR sequence was downloaded from Ensemble Genome Browser; 3 different seed sites of miR-3085-3p, "AGCCAG" (6 mer-seed site), "AGCCAGA" (7 mer-seed site), and "CAGCCAGA" (8 mer-seed site) were searched in *SCIN* 3'UTR. **A** – Location of seed sites of miR-3085 in the 3'UTR region of *SCIN* mRNA. **B** – Base pairing between miR-3085 seed sites and their complementary target sites in *SCIN* 3'UTR. The perfect match of 6 mer-, 7 mer-seed sites is highlighted in green. The vertical sign indicates complementary base pairing. The colon indicates G:U base-pairs.

about 80% confluence before use. 3'UTR wild type of human *SCIN* containing the predicted binding site of miR-3085-3p was subcloned into pmirGLO (Promega, USA) using In-Fusion HD Cloning plus (Takara, Japan), with the forward primer 5'-GCTCGCTAGCCTCGAAATGAAACAAGAAAGGGAGGC-3', reverse primer 5'-CGACTCTAGACTCGACAGGCATCACCAGTTAAGGG-3'. QuikChange II XL site-directed mutagenesis kit (Agilent, UK) was used to introduce mutants. For site 1, the forward primer 5'-TACAACCAGGTTCCCCAAGCACAGCTCTC CTTCAAAATAATGGAGATAATCAAACA-3' and reverse primer 5'-TGTTTGATTATCTCCATTATTTTGAAGG GAGCTGTGCTTGGGGAACCTGGTTGTA-3' were used; For site 2, the forward 5'-CTTTCATGGACAAATCTAGCTACC TGATTTCATATCTCTGGTTTTTGAAGGTGCTGTTCAAATACAG-3' and reverse primers 5'-CTGTATTTGAACAGCACCTTCAAAAACCAGAGATATGAAATCAGGTA GCTAGATTTGTCCATGAAAG-3' were used. Constructs were sequenced using a 3500 Genetic Analyzer (Applied Biosystems, USA). SW1353 cells were seeded into a 96-well plate at $5x10^4$ cells/mL in 100 µL medium overnight and transfected with 100 ng reporter plasmid, 50 nM miR-3085-3p mimic or non-targeting control using lipofectamine 2000 (Thermo Fisher, USA) for 24 h according to the manufacturer's instructions. The luciferase assay was performed using the dual-luciferase Reporter Assay Kit (Promega, USA) and read with EnVision 2103 Multilabel plate reader (Perkin Elmer, UK). Relative luciferase was calculated by the ratio of firefly luciferase to *Renilla* luciferase activity.

Statistical analysis

Data were analyzed using unpaired Student's t-test for 2 sample comparisons using GraphPad Prism version 6.

RESULTS

The binding site of miR-3085-3p harboring in the 3'UTR region of *SCIN*

We first utilized bioinformatics algorithms to identify potential targets of miR-3085-3p. However, the results obtained from these predictive tools were not as expected since none of the computer programs predicted *SCIN* as a direct target for miRNA-3085-3p. As computational algorithms failed to identify *SCIN* as a potential target, we utilized base pairing between the miRNA seed site and its complementary element in mRNA 3'UTR as an alternative parameter. Thus, we looked for the seed sites of miR-3085-3p in the *SCIN* 3'UTR. We found that there were four binding sites of miR-3085-3p in the *SCIN* 3'UTR (Fig.1A). Of those, three 6 mer-seed sites and one 7 mer-seed site were identified. The base pairing between miR-3085-3p seed sites and their corresponding target sites in *SCIN* 3'UTR is shown in Fig. 1B. There is good probability that the miR-3085/*SCIN* interaction is authentic. However, whether it was in the physiological context required experimental verification.

miR-3085-3p negatively regulates *SCIN* expression

To validate the miR-3085-3p/*SCIN* interaction, a typical gain-of-function experiment was performed. A miR-3085-3p mimic or non-targeting control was transiently transfected into human primary chondrocytes, and the relative expression level of *SCIN* was subsequently determined. The results presented in Fig. 2A show that after 24 h of transfection, the *SCIN* expression level significantly decreased in cells transfected with miR-3085-3p mimic as compared with the non-targeting control. These data show that *SCIN* is modulated by miR-3075, but this may not be a direct effect. A complementary approach in which the endogenous miRNA was inhibited by miRNA inhibitor was also employed. In this loss-of-function study, the miR-3085-3p inhibitor or an inhibitor control were transiently transfected into the human primary chondrocyte for 24 h and *SCIN* expression was quantified. The data presented in Fig. 2B showed that when endogenous miR-3085-3p was inhibited, the *SCIN* level was slightly increased (but not statistically significantly) as compared to the inhibitor control. These gain-of-function and loss-of-function studies demonstrated that the change in *SCIN* expression level was the consequence of miR-3085-3p modulation, thus supporting the conclusion that miR-3085-3p suppressed *SCIN* expression.

MiR-3085-3p directly binds to its seed sites in *SCIN* 3'UTR

The *SCIN* 3'UTR harboring 4 miR-3085-3p binding sites were cloned downstream of the firefly luciferase encoding gene in pmirGLO vector. The pmirGLO-*SCIN* 3'UTR recombinant vector and miR-3085-3p mimic or a control, non-targeting mimic, were transiently transfected into chondrosarcoma SW1353 for 24 h, and luminescence was measured (Fig. 3A). The effect

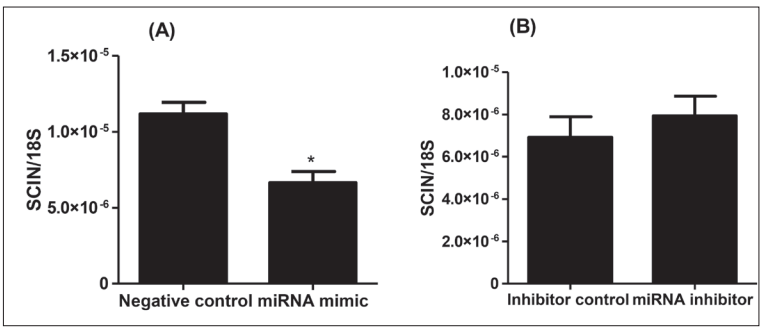

Fig. 2. Expression level of *SCIN* was suppressed by miR-3085. **A** – Human primary chondrocytes were transfected with miR-3085-3p mimic or negative control for 24 h. Expression of *SCIN* was determined by real-time RT-PCR. 18S rRNA was the endogenous control. **B** – Human primary chondrocytes were transfected with miR-3085-3p inhibitor or inhibitor control and the relative expression of *SCIN* was measured. Means±standard errors are presented. The difference between various conditions was analyzed by unpaired two-tailed t test, **P*<0.05, n=3.

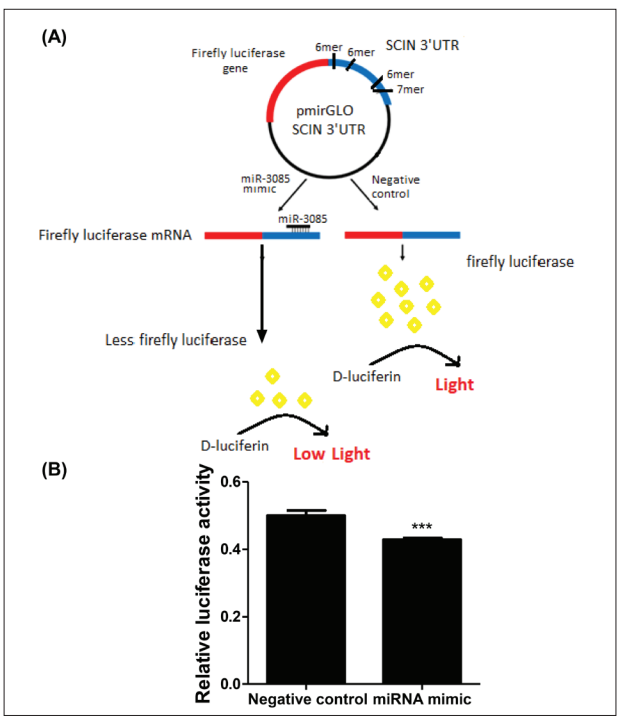

Fig. 3. miR-3085 suppresses the luciferase activity of a *SCIN* 3'UTR reporter construct. **A** – Principle of reporter assay employed to test the probability of miR-3085 directly binding to *SCIN* 3'UTR. The *SCIN* 3'UTR harboring the miR-3085 binding sites was cloned downstream of the firefly luciferase encoding gene in pmirGLO vector. The pmirGLO-*SCIN* 3'UTR along with either miR-3085 mimic or negative control were transiently transfected into SW1353 chondrosarcoma cells. In this scenario, the miR-3085 mimic would bind to its binding site available in 3'UTR, which resulted in firefly luciferase translation inhibition or mRNA cleavage. The luciferase activity was detected by measuring the luminescence. **B** – MicroRNA 3085 mimic significantly reduced luciferase activity of the *SCIN*-3'UTR reporter. A luciferase assay was performed after transient co-transfection of the pmirGLO-*SCIN* 3'UTR and a miR-3085 mimic and negative control. *Renilla* luciferase activity was used as the endogenous control. Means±standard errors are presented. The difference between various conditions was analyzed by unpaired two-tailed t test, ****P*<0.001, n=3.

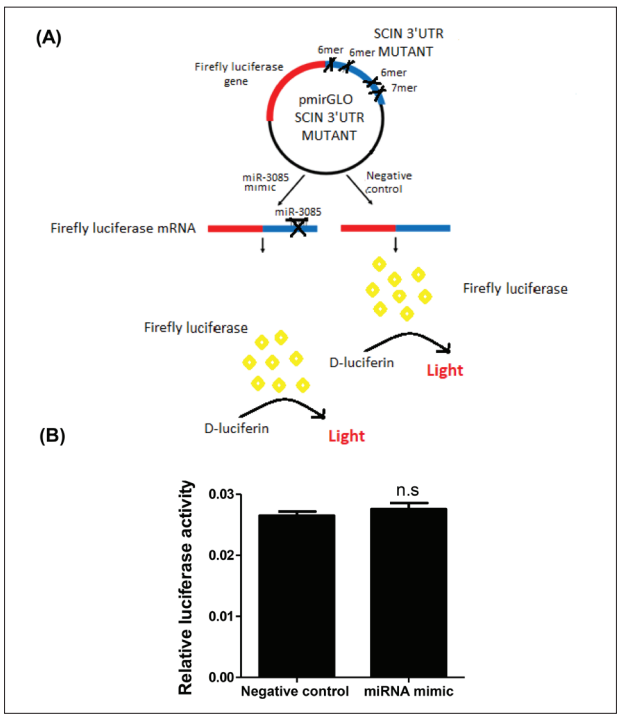

Fig. 4. miR-3085 has no clear effect on luciferase activity of *SCIN* 3'UTR-mutant reporter (**A**) principle of the assay. The four binding sites of miR-3085 in pmirGLO-*SCIN* 3'UTR were all mutated to obtain the *SCIN* 3'UTR-mutant reporter construct. This reporter no longer harbored the seed sites for the miRNA, therefore, the firefly luciferase would not be suppressed in the presence of miR-3085 mimic, which did not exert its effect on luciferase activity of the *SCIN* 3'UTR mutant reporter (**B**). The mutant reporter was transiently co-transfected with either miR-3085 mimic or negative control for 24 h and luciferase activity was subsequently measured. *Renilla* luciferase activity also served as the endogenous control. Means±standard errors are presented. The difference between different conditions were analyzed by unpaired two-tailed t test, n=3.

that the miR-3085-3p mimic exerted on luciferase activity is presented in Fig. 3B, which shows that after 24 h of transfection, cells transfected with miR-3085-3p mimic exhibited significantly reduced luciferase activity of pmirGLO-*SCIN* 3'UTR as compared to the negative control.

Further support for the authenticity of the miR-3085-3p/*SCIN* interaction was provided by another complementary approach, which utilized the reporter carrying a mutant version of the miR-3085-3p seed sites (Fig. 4A). The pmirGLO-*SCIN* 3'UTR mutant, along with either miR-3085-3p mimic or the negative control, was transiently transfected in SW1353 for 24 h. Subsequently, luciferase activity was measured by detecting the level of luminescence. The results presented in Fig. 4B showed that there was no significant difference in relative luciferase activity in cells with a mutant construct and cotransfected with miR-3085-3p mimic as compared to the negative control. Together, the reporter and its complementary mutant assay showed that miR-3085-3p could directly bind to its seed sites in the *SCIN* 3'UTR.

DISCUSSION

The fact that computational algorithms could not identify *SCIN* as a putative target of miR-3085-3p might not necessarily mean the miR-3085-3p/*SCIN* interaction is non-functional. miR-3085-3p has been recently annotated and most of bioinformatics algorithms do not include this miRNA in their database. The ability of a miRNA to suppress gene expression is highly dependent on the number of the seed sites in the mRNA [3, 14]. In the 3'UTR region of SCIN, there are 4 seed sites of miR-3085-3p. Crucially, the 7 mer-seed site does not contain G:U base-pairs which could potentially reduce the efficiency of regulation by the seed site [14]. Thus, there is good probability that the miR-3085/SCIN interaction is authentic.

We experimentally proved that miR-3085-3p directly binds to these seed sites. Moreover, modulation of miR-3085-3p resulted in an inverse change in *SCIN* level. These experiments demonstrated that *SCIN* is a direct target of miR-3085-3p, thus supporting our conclusion that in the physiologic context, miR-3085-3p inhibits *SCIN* expression at least in part through direct binding to the *SCIN* 3'UTR.

It is well established that miRNAs are important regulators of gene expression [15,16]. The aberrant expression of miRNAs has been linked to a variety of human disorders [17-21]. Contrary to the number of novel miRNAs being annotated, their biological functions have yet to be completely unveiled. Successfully discovering individual biological functions of miRNAs is potentially useful for future therapeutic treatment. The biological role of a given miRNA will be dictated by the mRNAs it regulates. Thus, miRNA target identification and verification is a fundamental requirement for determining its clinical function.

Aberrant expression of miR-3085-3p was shown in human OA [9]; however, the consequence of this

miRNA modulation in OA remains unclear: this would be technically feasible if its direct targets were thoroughly explored. Herein we showed that *SCIN* is a miR-3085-3p direct target. Therefore, *SCIN* expression is expected to be modulated and to inversely correlate with the miR-3085-3p level. Crucially, *SCIN* has been shown to have a vital role in cartilage homeostasis in which it exerts an effect on the chondrocyte phenotype. Knockdown of *SCIN* promoted features of dedifferentiation such as an increase in cell size, elongated shape, a decreased G-/F-actin ratio and an increase in the number of actin-free barbed ends. Overexpression of *SCIN* reversed all these changes in chondrocytes [11]. Therefore, the modulation of SCIN may affect the normal development process of chondrocytes, as well as in disease. Taken together, the implication is that miR-3085-3p may partly perform its function in OA through direct targeting of *SCIN*.

Funding: This study was supported by a grant from Ho Chi Minh City Open University (Code: E.2018.01.1).

Author contributions: IC, LL designed the experiment; LL, BP performed all the experiments; LL, BP, IC interpreted the data; ICLL, BP, IC wrote the manuscript.

Conflict of interest disclosure: There are no potential conflicts of interest.

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