The *miR-7* Identified from Collagen Biomaterial-Based Three-Dimensional Cultured Cells Regulates Neural Stem Cell Differentiation

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Increasing evidence suggests that three-dimensional (3D) cultures provide more appropriate microenvironments to control stem cell response compared with traditional two-dimensional (2D) cultures. However, the molecular mechanism involved in 3D cultured stem cells is not well known. Several microRNAs whose target genes involved in the regulation of self-renewal and differentiation of stem cells were found to be down-regulated in 3D cultured PA-1 cells. Among them, *miR-7* was predicted to target Kruppel-like factor 4 (*Klf4*), a key gene for self-renewal of neural stem cells (NSCs). We showed that the differentiation of NSCs was inhibited in 3D collagen scaffolds compared with 2D cultured cells. The quantitative real-time PCR (qPCR) analysis indicated that the expression of *miR-7* and *Klf4* changed significantly in 2D cultures, whereas the expression stability of *miR-7* and *Klf4* was detected in 3D cultures. Using luciferase assay and western blot, *Klf4* was identified as a target of *miR-7* indicating that *miR-7* plays a critical role in maintaining the self-renewal capacity through a *Klf4*-dependent mechanism in 3D cultured cells. Thus, the collagen scaffold-based 3D cell cultures may provide a platform to reveal the regulatory mechanism of cell regulators, which are difficult to find in traditional 2D cell cultures.

Introduction

STEM CELLS CULTURED in vitro can provide access to study the molecular mechanisms that control cellular development in a manner pertinent to human embryogenesis. Current understanding of many biological processes is largely based on the studies of homogenous populations of cells cultured on the two-dimensional (2D) culture systems. The biomaterial-based three-dimensional (3D) culture system provides more closely a representative of in vivo conditions compared with the conventional 2D cell culture system [1,2]. In recent years, a variety of biomaterials have been used for stem cell cultures, primarily to provide an appropriate microenvironment for their growth and differentiation and to ultimately mimic the stem cell niche [3]. Despite the vast interest by scientists, it is

still a challenging task to understand the mechanisms that drive the differential responses of cells in 2D and 3D cultures. It is crucial to define exactly which factors are operative in 3D cultured stem cells.

MicroRNAs (miRNAs) are small (~20–25 nucleotides), noncoding RNAs that are expressed in a tissue-specific and developmentally regulated manner and function as negative regulators of gene expression in a variety of eukaryotic organisms [4,5]. The emerging concept is that miRNAs play a central role in controlling stem cell self-renewal and fate determination by regulating the expression of stem cell regulators. Given that the changes in the expression of miRNAs may directly impact cell function, we profiled the specific miRNA expression patterns and constructed the miRNA-target gene network in 3D cultured PA-1 cells. PA-1 can

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express many stemness-related genes such as *Oct4*, *Sox2*, *Nanog*, and Kruppel-like factor 4 (*Klf4*), but not differentiate during long-term culture. Therefore, it is a good cell model to study the molecular mechanism regulating self-renewal of stem cells. The network analysis clarified the relationship between differentially expressed (DE) miRNAs and self-renewal related genes. Among the network, the *miR-7* was predicted to regulate the *Klf4* gene, which had been reported to play a pivotal role in neural differentiation during brain development [6]. We therefore hypothesized that *miR-7* may be involved in regulating the self-renewal and differentiation of neural stem cells (NSCs) in 3D cell culture.

Our previous studies have suggested that the 3D collagen culture system may help to maintain the self-renewal status of NSCs [7]. Nevertheless, the molecular mechanisms underlying self-renewal and differentiation of NSCs in 3D collagen cultures are not fully understood. In our study, the quantitative real-time PCR (qPCR) results indicated that the expression of *miR-7* was gradually increased in a time-dependent manner in 2D cultured NSCs. However, the expression of *miR-7* was relatively stable in 3D cultured NSCs. Both overexpression and knockdown experiments demonstrated the role of *miR-7* in regulating *Klf4* expression through its 3'-UTR. Furthermore, we demonstrated that overexpression of *miR-7* promotes neural differentiation in 3D cultured NSCs. Therefore, the differential expression pattern of *miR-7* may contribute to explain why the neural differentiation was attenuated in the 3D culture system. In this study, our results indicated that *miR-7* was involved in maintaining the undifferentiated cell state of NSCs in a *Klf4*-dependent way in 3D culture systems. Our study not only provided new insights in understanding the roles of miRNAs in regulating the self-renewal and differentiation of NSCs, but also opened the way to use the collagen material-based 3D cell cultures as an ideal platform to investigate stem cell functions.

Materials and Methods

Collagen scaffold preparation

The 3D collagen sponge scaffolds were fabricated according to the published protocol [8]. The surface morphology of the collagen scaffold was observed by scanning electron microscopy (SEM).

Cell culture

Primary neural progenitors were prepared by a published protocol [7]. All animal procedures were performed in accordance with the Chinese Ministry of Public Health Guide for the care and use of laboratory animals. The neurospheres were digested with 0.25% trypsin into single cell suspensions and seeded at the density of 3×10^5 cells per well in poly-D-lysine-coated six-well plates (Corning Costar). For 3D culture, NSCs (1×10^6) were added on one collagen scaffold. After 24 h of adhesion, the adhesion medium was changed by a differentiation medium. PA-1 cells were cultured in the Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. For 3D culture, PA-1 (1×10^5) cells were seeded onto one collagen sponge scaffold. The cells were harvested for microarray and qPCR analysis after 7 days of culture.

SEM characterization

The morphology of cells seeded in 3D collagen scaffolds was shown by SEM (S-3000N; Hitachi) analysis. Cells were fixed in phosphate-buffered saline (PBS) containing 1% glutaraldehyde and 4% formaldehyde (pH 7.2), dehydrated with ethanol, and dried in a critical point dryer. Then, the samples were coated with gold using a sputter. The images were captured by HITACHI S-3000N SEM (Hitachi).

Microarray hybridization and data analysis

Total RNA was extracted from PA-1 cells using the miRNAeasy Mini Kit (Qiagen GmbH). The miRNA microarray assay was performed by a service provider (LC Sciences). The processed data were got by first subtracting the background noise of raw data from hybridization images and then normalizing using the LOWESS filter (locally weighted regression) [9]. Differentially expressed miRNAs were identified by a cutoff of fold change ≥ 1.5 and P value < 0.01 in the Student's t -test. MiRNA targets were predicted by TargetScan (release 6.2). The miRNA gene network was constructed using Cytoscape (version 3.0.1). The node size correlated with Indegree computed by Cytoscape. Edges in the type of solid line, dashed line, and dotted line represented high to low conservation of binding sites.

Construction of reporter plasmids and luciferase assay

The full length 3'-UTR of the human *Klf4* gene and the fragment containing the putative *miR-7* binding site were amplified from human cDNA and individually cloned into a modified pGL3-promoter vector (Promega). The pGL3 luciferase reporter vector was modified according to previous reports [10]. The mutated 3'-UTR of *Klf4* was generated using a site-directed mutagenesis kit (TransGen Biotech). The primers are listed in Table 1. All PCR products were digested at the *SpeI* and *SphI* sites before cloning into the pGL3-promoter vector. Hela cells were seeded in 24-well plates and then transfected with 500 ng of wild-type or mutant reporter plasmid, 25 ng of Renilla luciferase expression vector, *miR-7* mimics (10 nM) or *miR-7* inhibitors (10 nM) or equal amounts of negative control (NC), inhibitor NC (INNC) (GenePharma Co., Ltd.) using the Lipofectamine 2000™ reagent (Invitrogen). After 48 h, the cell extract was obtained, and firefly and Renilla luciferase activities were measured with the dual-luciferase reporter system (Promega).

Transient transfections and cell treatments

Twenty-four hours before transfection, 3×10^5 cells/well were seeded in six-well plates (Corning Costar). When cells reached 80%–90% confluency, 10 nM of appropriate miRNA mimics, miRNA inhibitors, NC, and INNC were transfected using the X-tremeGENE siRNA Transfection reagent (Roche). In RNAi experiments, 1 μ M of siRNA solution containing a pool of two different siRNA duplexes (GenePharma Co., Ltd.) were used for each well. The sequence of siRNA duplexes was synthesized according to previous reports [11]. In the rescue experiment, NSCs were co-transfected with the *Klf4* siRNA and *miR-7* inhibitors at the same time.

TABLE 1. PRIMERS USED TO CONSTRUCT LUCIFERASE REPORTER PLASMIDS OF *Klf4*

Gene name	Primer sequence	Product size (bp)
<i>Klf4</i> 3'-UTR (<i>miR-7</i> sense)	Forward/Spel: 5'-TCACTAGT TTTACTTTTCACACTGCTCTCC-3' Reverse/SphI: 5'-ATAGCATGCCGGAAGACAAATATAGAT-3'	531
<i>Klf4</i> 3'-UTR (<i>miR-7</i> mutated putative binding region)	Forward/Spel: 5'-CTTTTCACACT ATATTAACGATGAGGG-3' Reverse/SphI: 5'-TTAATAT AGTGTGAAAAGTAAAAAAT-3'	531

Quantitative real-time PCR

Total RNA was extracted with TRIzol reagent (Invitrogen) and cDNA was synthesized with reverse transcription performed using the SuperScript[™] III First-Strand Synthesis System (Invitrogen). The primer sequences used in the experiment are listed in Table 2. QPCR was performed using the SYBR[®] Green PCR Master Mix (Roche) on the CFX96 Real-Time PCR Detection System (Biorad). The results were calculated using the $2^{-\Delta\Delta CT}$ method [12].

MiRNA extraction and TaqMan assay

MiRNAs were extracted using the miRvana extraction kit (Ambion). For quantification of miRNA, 2 μ g of total RNA was reverse transcribed and amplified by using the miRNA reverse transcription and detection kit (Applied Biosystems, Inc.). All results were normalized to U6 levels that were detected by using the ABI miRNA U6 assay kit (Applied Biosystems, Inc.).

Flow cytometry

Cell cycle distribution and measurement were performed by flow cytometry. Cell cycle analysis was performed according to previous reports [13]. Briefly, the cells were harvested and then fixed with ice-cold 70% (v/v) ethanol for 12 h. The samples were concentrated by removing the ethanol and resuspending the cells at a concentration of 10^6 cells/mL in a PBS solution containing propidium iodide (50 μ g/mL) and RNaseA (25 μ g/mL; Sigma Chemical Co.). Cell cycle analysis was performed using FACSaria II flow cytometer (BD Biosciences).

Western blotting

For western blotting of transfected NSC samples, equal amounts of protein were loaded on sodium dodecyl sulfate–10% polyacrylamide gelelectrophoresis under reducing conditions and transferred to polyvinylidene fluoride membranes (Amersham, GE) by electrophoresis. Primary antibodies were incubated overnight at 4°C, and primary antibodies included anti-*Klf4* (SC-20691; Santa Cruz Biotechnology, Inc.), anti-p53 (sc-1311; Santa Cruz Biotechnology, Inc.), and anti- β -Actin (TA-09, ZSGB-BIO). The horseradish peroxidase-labeled secondary antibody (PIERCE) was used for 2 h at room temperature at a dilution of 1:5,000.

Immunofluorescence

The transfected NSC samples were fixed in cold 4% paraformaldehyde for 30 min, and then blocked with 0.2% Triton X-100 and 3% goat serum in PBS. The primary antibodies used in our study included *Nestin* (1:400; MAB353, Millipore), *Tuj1* (1:500, 05-549, Upstate), *Sox2* (1:100; 481400, Life Technologies), *Map-2* (1:400; M1406, Sigma), and *Vimentin* (1:100; V6630, Sigma). The secondary antibodies were the anti-mouse IgG FITC antibody and anti-rabbit IgG FITC antibody (all from Sigma). Nuclei were counterstained with Hoechst 33342 (1:500; 94403, Sigma). The fluorescent images of 2D cultured cells were visualized on the Zeiss 200 inverted fluorescent microscope (Carl Zeiss). The number of immunostained cells was counted in each of the three random fields per well and the fluorescence images were selected randomly. Quantification of the immunofluorescence signal was performed by measuring the average intensity in individual cells according to the ImageJ software manual. The

TABLE 2. PRIMERS USED IN QUANTITATIVE REVERSE TRANSCRIPTION-PCR

Gene symbol	Primer sequence (5'-3')	Product size (bp)
<i>P53</i>	Forward: TCCGACTATACCACTATCCACTAC Reverse: GCACAAACACGAACCTCAAAG	148
<i>Klf4</i>	Forward: ACTTGTGACTATGCAGGCTG Reverse: ACAGTGGTAAGGTTTCTCGC	95
<i>Tuj1</i>	Forward: AGCAGATGCTGGCCATTCAGAGTA Reverse: TAAACTGCTCGGAGATGCGCTTGA	174
<i>Nestin</i>	Forward: AGAGAAGCGCTGGAACAGAG Reverse: AGGTGTCTGCAACCGAGAGT	234
<i>Sox2</i>	Forward: AAAGGAGAGAAGTTTGGAGCCCCGA Reverse: GGGCGAAGTGCAATTGGGATGAAA	113
<i>Vimentin</i>	Forward: AGGTGGATCAGCTACCAATGACA Reverse: TCAAGGTCAAGACGTGCCAGAGAA	184
<i>Map2</i>	Forward: GCAGCGCCAATGGATTTCATACA Reverse: TCCGTTGATCCCGTCTCTTTGGT	104
<i>Gapdh</i>	Forward: AAGGGCTCATGACCACAGTC Reverse: GTGAGCTTCCCATTACAGCTC	169

fluorescent images of 3D cultured cells were taken with a scanning laser confocal fluorescence microscope Leica TCS SP5 (Leica Microsystems, Inc.).

Statistics

Each experiment was carried out in triplicate at least three times, and data are expressed as mean $\pm$ SD. Statistical analysis was performed using the Student's *t*-test. $P < 0.05$ was considered as significantly different.

Results

Three-dimensional culture system promotes stemness of cells partly through affecting miRNA expression

As embryonal carcinoma cells, the human ovarian teratocarcinoma cell line PA-1 stably expressed stemness-related genes like *Oct4*, *Nanog*, *Sox2*, and *Klf4*, but does not undergo differentiation during long-term culture. Thus, it may be used as a good model to clarify the underlying molecular mechanism of self-renewal ability of 3D cultured stem cells. As shown in Fig. 1A, the collagen sponge scaffolds have an irregular multiporous structure with a mean pore size of 60 μ m in diameter and a flat form structure. Cell attachment and morphology were observed using SEM. The SEM analysis showed that the seeded PA-1 cells were well attached to the scaffold with cytoplasmic extensions and lamellipodia. The excellent morphological characteristics of PA-1 cells demonstrated that collagen sponge scaffolds have a good biocompatibility with cells (Fig. 1B). The global expression patterns of miRNAs were studied using microarray technologies, 326 DE miRNAs were screened between 3D and 2D cultured PA-1 cells (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd). Using the miRNA target gene pairs predicted by TargetScan, we constructed the regulatory network between DE miRNAs and self-renewal related key genes; 30 miRNAs downregulated in 3D cultures were related with the four

self-renewal key factors *Oct4*, *Sox2*, *Nanog*, and *Klf4* (Fig. 2A). The altered expression of miRNAs may contribute to explain the regulatory mechanism of maintaining self-renewal ability in 3D cultured stem cells. To validate the microarray analysis results, five selected miRNAs (*miR-335*, *miR-339*, *miR-361-3p*, *miR-145*, and *miR-7*) were analyzed by qPCR analysis (Fig. 2B) and the expression variation tendency of all tested miRNAs was coincident with the microarray results.

Differentiation of NSCs grown in 2D culture system and 3D culture system

SEM indicated that NSCs cultured on 3D collagen sponge scaffolds showed significant differences in appearance. The cells assembled in the pores of collagen and tended to clump together as a ball (Fig. 3A). To evaluate the ability of neural differentiation of NSCs in 3D cultures, the NSC marker and neural differentiation-related genes, including *Sox2*, *Nestin*, *Vimentin*, *Tuj1*, and *Map2*, were examined by qPCR analysis. After 7 days in differentiation conditions, the expression level of neural differentiation-related genes *Tuj1* and *Map2* was lower in 3D cultured NSCs than in 2D cultured cells (Fig. 3B). The results indicated that the neural differentiation of NSCs was inhibited in 3D collagen sponge scaffolds.

During the differentiation process of 2D cultured NSCs, the expression of *miR-7* was upregulated in a time-dependent manner, while its predicted target gene, *Klf4*, was downregulated at the same time (Fig. 4D). However, the expression of *miR-7* and *Klf4* in 3D cultured NSCs was relatively stable when cultured in the same differentiation conditions (Fig. 4A, B). Previous studies have shown that *Klf4* may serve as a repressor of *p53* [14]. Consistent with that, the expression level of *p53* was higher in 2D cultured NSCs than in 3D cultured cells (Fig. 4C). The results indicated that the neural differentiation of NSCs was attenuated in 3D cultures along with stably expressed *Klf4* and low expression of *p53*.

FIG. 1. Morphological character of the collagen sponge scaffold materials and PA-1 cells cultured in the two-dimensional (2D) culture and three-dimensional (3D) culture system. **(A)** Left: macroscopic view of collagen scaffolds; Right: scanning electron microscopy (SEM) image of the collagen scaffold; **(B)** Left: phase-contrast image of PA-1 cells in 2D cultures; Right: SEM image of PA-1 cells cultured on collagen sponge scaffolds for 7 days.

FIG. 2. Microarray analysis of PA-1 cells cultured in 2D culture and 3D culture system. **(A)** The MicroRNA (miRNA) gene network of these self-renewal related differential expressed miRNAs down-regulated in 3D cultures. Node size correlates with Indegree computed by Cytoscape. Edges in type of solid line, dashed line, and dotted line represent high to low conservation of binding sites. The color of edges is heatmapped with the fold change value of miRNAs. **(B)** Differentially expressed mRNAs detected by microarrays were validated by quantitative real-time PCR (qPCR) in PA-1 cells. Data represent mean $\pm$ SD of three different experiments analyzed in triplicate. $**P < 0.01$, $n = 3$.

FIG. 3. Morphological character of the neural stem cells (NSCs) cultured in 2D culture and 3D culture system for 7 days in the differentiation medium. **(A)** Left: Phase-contrast image of NSCs seeded on poly-D-lysine-coating culture plate. Right: SEM image of the NSCs cultured in 3D cultures. **(B)** qPCR analysis of *Nestin*, *Sox2*, *Vimentin*, *Tuj1*, and *Map2* expression in NSCs cultured in 2D and 3D cultures. Data represent mean $\pm$ SD of three different experiments analyzed in triplicate. $**P < 0.01$, $n = 3$.

FIG. 4. The relative expression of *Klf4*, *miR-7*, and *P53* in NSCs in 2D culture and 3D culture for 7 days. **(A)** The relative expression of *miR-7* in NSCs cultured in 3D cultures compared with 2D cultures. **(B)** The relative expression of *Klf4* in NSCs cultured in 3D cultures compared with 2D cultures. **(C)** The relative expression of *P53* in NSCs cultured in 3D cultures compared with 2D cultures. **(D)** Expression pattern of *miR-7* and *Klf4* in NSCs during neural differentiation.

miR-7 inhibits KLF4 expression by binding to its 3'-UTR

Using the miRanda algorithm, *miR-7* was identified to have a conserved target site in the 3'-UTR of *Klf4* gene. To test whether the predicted *miR-7* target site in *Klf4* 3'-UTR is critical for repression of *Klf4* expression by *miR-7*, point mutations disrupting the base pairing in the predicted *miR-7* target site were introduced into *Klf4* 3'-UTR in the luciferase reporter construct. Mutation of the *miR-7* 3'-UTR target site abolished the repression by *miR-7* (Fig. 5). The results suggested that *miR-7* represses *Klf4* expression through the predicted target site in *Klf4* 3'-UTR.

Next, we tested whether endogenous *miR-7* directly targeted *Klf4* in NSCs. To check the transfection efficiency, we detected the relative expression of *miR-7* in NSCs after transfection with *miR-7* mimics or *miR-7* inhibitors. The intracellular level of *miR-7* in NSCs was elevated when transfected with *miR-7* mimics, and was decreased by the transfection of *miR-7* inhibitors, showing that the cellular level of *miR-7* could be controlled by the transfection of *miR-7* mimics and *miR-7* inhibitors (Fig. 5D). The western blot results indicated that the protein level of *Klf4* was down-regulated when the expression of *miR-7* was elevated (Fig. 5E). These results together demonstrated that endogenous *miR-7* can directly repress the expression of *Klf4*.

Biological effects of miR-7 during proliferation and neural differentiation of NSCs

Cell proliferation is regulated by the cell cycle, which is divided into a series of phases. To determine whether *miR-7* regulates NSC proliferation through regulating the cell cycle,

we analyzed the cell cycle in NSCs transfected with *miR-7* mimics, *miR-7* inhibitors, NC, and INNC. The results indicated that overexpression of *miR-7* induced G1/S arrest. In contrast, knockdown of *miR-7* promoted G1/S transition of cell cycle following transfection of *miR-7* inhibitors. The results further supported the notion that *miR-7* negatively regulated NSC proliferation through induction of G1/S cell cycle arrest (Fig. 6).

After transfected with *miR-7* mimics, *miR-7* inhibitors, NC, and INNC, NSCs were cultured in differentiation conditions for 72 h. The percentages of *Tuj1*-positive cells and *Map2*-positive cells in NSCs transfected with *miR-7* mimics were significantly higher than the control group (Fig. 7D, E). On the other hand, a significant decrease in the number of *Nestin*-positive cells and *Vimentin*-positive cells was found in NSCs transfected with *miR-7* mimics compared with the control group (Fig. 7A, C). These results together indicated that *miR-7* promotes neuron differentiation. The results of qPCR assays also supported this view. Overexpression of *miR-7* increased the expression of neural markers *Tuj1* and *Map2*, while decreased the expression of *Nestin* and *Vimentin* (Fig. 7F). However, staining for *Sox2*, a marker for NSCs, did not show a remarkable difference among these groups (Fig. 7B) suggesting that overexpression of *miR-7* did not alter NSC identity [15]. In addition, a single miRNA can target many genes; *miR-7* may affect its expression through regulating other *Sox2*-related genes, which need to be further elucidated in future research.

miR-7 regulates NSC activities by targeting Klf4

As mentioned above, we demonstrated that *Klf4* expression decreased during neural differentiation in a time-dependent

FIG. 5. Identification of a *miR-7* target site in the 3'-UTR of *Klf4* mRNA. **(A)** Putative binding sites of *miR-7* in the *Klf4* 3'-UTR predicted by Microcosm Targets in rat and human. The nucleotide pairing of *miR-7* and *Klf4* 3'-UTR. The vertical lines indicate perfect pairing, while ":" indicates G-U wobble. **(B)** Schematic representation of the constructs used in the luciferase reporter assay. The *Klf4* 3'-UTR sequences containing the putative binding sites of *miR-7* were inserted into the pGL3 luciferase reporter vector. Site-directed mutagenesis was performed to mutate base pairs in the predicted seed region targeted by *miR-7* in the *Klf4* 3'-UTR. The mutant binding site is underlined. **(C)** Luciferase assay was performed 72 h after transfection using the Dual Luciferase. *miR-7* downregulated luciferase activities controlled by wild-type *Klf4* 3'-UTR, but did not affect the luciferase activity controlled by mutant *Klf4* 3'-UTR. The firefly luciferase activity was standardized to renilla luciferase control. $^{**}P < 0.01$ versus WT/NC (wild type *Klf4* 3'-UTR + negative control). The results are mean of three independent experiments $\pm$ SD. **(D)** qPCR was used to assess the efficiency of *miR-7* transfection at 72 h after differential transfection. *U6* was included as a loading control. **(E)** *Left*: The expression level of *Klf4* was analyzed by western blot in NSCs after transfection with *miR-7* mimics, inhibitors, negative control (NC), and inhibitor NC (INNC). *Right*: Quantification of relative expression level of *Klf4*. $n = 3$. Bars show mean $\pm$ SD. $^{*}P < 0.05$ versus ctr, $^{**}P < 0.01$ versus ctr.

manner (Fig. 4C). To verify whether knockdown of *Klf4* expression by siRNA could have a similar effect on NSC activities, we determined the cell proliferation and differentiation of NSCs following RNAi knockdown of *Klf4* (Figs. 6 and 7). The results indicated that knockdown of *Klf4* reduced cell proliferation, but promoted neural differentiation of NSCs. The siRNA transfected NSCs showed a similar biological behavior as observed in the cells transfected with *miR-7* mimics. Our data suggested that *miR-7* regulates NSC

proliferation, differentiation, and apoptosis through targeting *Klf4* expression. Furthermore, we determined to test whether depletion of *Klf4* may weaken the role of *miR-7* inhibitors in NSCs. The stimulus effect on cell proliferation and the inhibitory effect on differentiation, which *miR-7* inhibitors bring about, were weakened when *Klf4* was knocked down by siRNA. Our data supported the idea that *miR-7* regulated the proliferation and differentiation of NSCs in a *Klf4*-dependent way.

FIG. 6. The effect of *miR-7* on cell cycle of NSCs. **(A)** Cell cycle distribution of NSCs after transfection with *miR-7* mimics, inhibitors, NC, and INNC. **(B)** The relative percentage of cell cycle analysis indicated that *miR-7* causes cell cycle arrest at the G1/S stage. * $P < 0.05$ versus ctr. **(C)** Up: The expression level of *P53* was analyzed by western blot in NSCs after transfection with *miR-7* mimics, inhibitors, NC, and INNC. Down: Quantification of relative expression level of *P53*. $n = 3$. Bars show mean $\pm$ SD. ** $P < 0.01$ versus siRNA.

The role of *miR-7* in 3D cultured NSCs

Although we have verified the role of *miR-7* in neural differentiation by targeting *Klf4* in 2D cultured NSCs, we need further studies to examine if the role of *miR-7* is involved in maintaining the self-renewal ability of 3D cultured NSCs. To verify our hypothesis, we transfected *miR-7* mimics, *miR-7* inhibitor, and *Klf4* siRNA in 3D cultured NSCs for 4 days. Using immunofluorescence assay, we detected that the expression of neuron marker *Tuj-1* was increased in the *miR-7* mimics and *Klf4* siRNA transfected groups, whereas the expression of *Tuj-1* was decreased in the *miR-7* inhibitor transfected group (Fig. 8). The results demonstrated that overexpression of *miR-7* enhanced neural differentiation of 3D cultured NSCs obviously. Therefore, we may draw a conclusion that the stable expression of *miR-7* is required for preserving the undifferentiated state of NSCs in 3D culture systems.

Discussion

Microenvironmental factors are critical for maintaining self-renewal of stem cells or guiding stem cell differentiation in neural tissue engineering. In a living organism, cells are surrounded by other cells and embedded in an extracellular matrix that defines the architecture, signaling, and biomechanics of the cellular microenvironment. The utilization of 3D cultures enables more rigorous experimentation under conditions that are tightly controlled and closer to those present in vivo [16–19]. Biomaterial-based scaffolds have been the most important tool in providing a 3D environment to cells in culture. A wide variety of materials with a range of properties have been designed and utilized for interactions with stem cells, including natural materials and synthetic

materials such as collagen, fibrinogen, hyaluronic acid, and copolymer polylactide-co-glycolide [3]. Of the many scaffold materials being investigated, collagen sponge has been shown to have many advantageous features to culture cells in the 3D culture system. The highly porous collagen sponges comprised a web or lattice of interconnected fibers that support cell distribution and matrix accumulation, which have been used to support short- and long-term growth of various cell types. Lacking the physical and chemical cues defining their natural in vivo microenvironment, cells in 2D cultures differ substantially in their shape, organization, physiology, and metabolism from cells observed in more physiologically relevant 3D environments. In this study, we focused to investigate the molecular mechanism of the influence of 3D collagen sponge microenvironments on stem cell function.

Given the pivotal role of miRNAs in regulatory circuitries that control self-renewal and pluripotency of stem cells, we determined to have a thorough understanding of the differential miRNA expression pattern of stem cells between 3D and 2D cultures. The microarray analyses revealed a pronounced global change in the miRNA expression pattern between 3D and 2D cultured PA-1 cells. Functional analysis of verified targets and predicted targets demonstrates that these downregulated miRNAs may contribute to maintain the self-renewal ability of stem cells in the 3D culture systems. Specially, *miR-7*, which is predicted to regulate *Klf4* triggered our interest. The nucleotide sequence of *miR-7* is conserved between humans and invertebrates, and it shows conserved expression in neural tissues even if its functions differ from one species to another. As previous studies reported, *miR-7* appears to be present predominantly in neural and brain tissues [20]. Both in human and mouse the biogenesis of *miR-7* was inhibited in nonbrain cells.

FIG. 7. *miR-7* accelerates neural differentiation. The expression of the NSC progenitor markers *Nestin* (A), *Sox2* (B), *Vimentin* (C), and the neural marker *Tuj1* (D), *Map2* (E) were detected by immunofluorescence 72 h after transfection. Nuclei were counterstained using Hoechst 33342. Images were acquired by a microscope using a 20 $\times$ objective lens. (F) qPCR analysis of *Nestin*, *Sox2*, *Vimentin*, *Tuj1*, and *Map2* expression 72 h after transfection. $n=3$. Bars show mean $\pm$ SD. * $P<0.05$ versus ctr. ** $P<0.01$ versus ctr.

(Continued)

FIG. 7. (Continued)

FIG. 7. (Continued).

Post-transcriptional regulation plays a pivotal role in controlling its abundance in a tissue-specific manner. Many important factors are responsible for the post-transcriptional regulation of *miR-7* biogenesis such as HuR and MSI2 [21]. Accumulating evidence has indicated that *miR-7* played an important role in neuron differentiation. It has been reported

that *miR-7* promotes photoreceptor neuron differentiation through modulating components in the epidermal growth factor receptor signaling pathway in *Drosophila* [22]. In addition, it has been demonstrated that the induction of *miR-7* contributes to the extensive neurite outgrowth observed in differentiating NSCs [23]. Previous studies suggested that

FIG. 8. Effect of *miR-7* on neural differentiation in 3D cultures. The expression of *Tuj1* in 3D cultured neural stem cells was detected by immunofluorescent staining 96 h after transfection with *miR-7* mimics, inhibitors, and *Klf4* siRNA. Nuclei were counterstained using Hoechst 33342. Images were acquired by a confocal microscopy using a 20× objective lens (scale bar 50 μm).

brain-enriched *miR-7* showed poor expression in neural progenitors, but highly expressed in neurons. It is likely that the distinct temporal expression patterns of *miR-7* reflect its specific roles in the self-renewal process of NSCs [24,25], but to date the precious roles of *miR-7* in NSCs remain unknown.

As a subset of undifferentiated precursors, NSCs retain the ability to self-renew and have the capacity to give rise to neural and glial lineages in the nervous system [26]. Recent publication has demonstrated that the NSC fate decisions are controlled by a molecular network of transcription factors and miRNAs. By performing a high-throughput mRNA and miRNA profiling, the researchers revealed that neuronal differentiation of NSCs induces drastic changes in the transcriptomic profile. These changes include the activation of numerous miRNAs [27]. QPCR results indicated that 3D culture environments contribute to maintain the self-renewal ability of NSCs. In the meantime, we detected that the expression of *miR-7* is much lower in 3D cultures than 2D cultures when NSCs were cultured for 7 days in the differentiation medium without growth factors. The apparent differences in the expression of *miR-7*, at least in part, indicated that it may participate in regulating the differentiation of 3D collagen scaffold cultured NSCs. Furthermore, we verified the role of *miR-7* in promoting the neural differentiation by detecting the staining of neural markers in NSCs transfected by exogenous *miR-7*. Overexpression of *miR-7* resulted in the percentage of *Tuj1*-positive neural cells increased both in 2D cultured NSCs and in 3D cultured NSCs. Flow cytometry results indicated that *miR-7* regulates NSC proliferation by reducing cell cycle progression leading to cell cycle arrest in the G1/S transition. Taken together, our results indicated that overexpression of *miR-7* in NSCs resulted in attenuated proliferation capacity, but increased neural differentiation, whereas antisense knockdown of *miR-7* resulted in increased proliferation of NSCs and inhibited NSC differentiation.

Using luciferase assay, we showed that *miR-7* directly targeted 3'-UTR of *Klf4*. The western blot results indicated that overexpression of *miR-7* decreased *Klf4* protein expression, and inhibition of *miR-7* led to elevated *Klf4* protein levels. As a member of the KLF family of transcription factors, *Klf4* is expressed in NSCs, but is drastically down-regulated in differentiated neurons. Emerging evidence implicates *Klf4* as an important regulator of neurogenesis [6, 28]. In this study, we demonstrated that the *Klf4* siRNA transfected NSCs showed a similar biological behavior as observed in the cells transfected with *miR-7* mimics. To support the hypothesis that the effect of *miR-7* on cellular process is mediated largely through *Klf4*, NSCs were co-transfected with *miR-7* inhibitors and *Klf4* siRNA, the co-transfected results indicated that *miR-7* inhibitor had little effect on cell functions when NSCs were transfected with *Klf4* siRNA simultaneously. The results suggested that *Klf4* was identified as a target of *miR-7*, which mediated the effect of *miR-7* on the activities of NSCs.

Recent studies have shown that expression of *Klf4* was also induced by DNA damage and that such induction is dependent on *p53* [14]. Then, we examined whether the expression level of *p53* was affected by *miR-7*. As we expected, overexpression of *miR-7* elevated the expression of *p53*, on the contrary, knockdown of *miR-7* suppressed the expression

of *p53*. This may contribute to explain the G1 arrest in NSCs, which *miR-7* induces appears to be dependent on *p53* function. Next, we examined the expression pattern of *Klf4* and *p53* in 2D and 3D cultured NSCs. In 2D cultured NSCs, the real-time PCR results indicated that the expression of *Klf4* was gradually decreased in a time-dependent manner, whereas the expression of *p53* was gradually increased in a time-dependent manner. Compared with 2D cultured NSCs, the expression of *Klf4* and *p53* was relatively stable in 3D cultured NSCs. Taken together, our findings suggest that *miR-7* is involved in maintaining the self-renewal ability of NSCs in 3D cultures through the *Klf4*-mediated *p53*-dependent response.

There is a wealth of information on how cells confined in conventional 2D monolayer cultures differ substantially in their properties from cells cultured in a 3D configuration [29–31]. The discovery of miRNAs opens new possibilities in terms of modulation of stem cell lineage commitment and differentiation. Our study provides the first molecular insight into understanding the miRNAs' function in a 3D collagen culture system, which will contribute to move toward more physiological culture systems for studying fundamental cellular processes.

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Author Disclosure Statement

The authors indicate no potential conflicts of interest.

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