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miR-4417 suppresses keloid fibrosis growth by inhibiting CyclinD1

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Mounting evidence has reported that microRNAs (miRNAs) play irreplaceable roles in the development of keloid fibrosis. miR-4417 has been reported to contribute to nickel chloride-promoted lung epithelial cell fibrogenesis and tumorigenesis. However, whether miR-4417 is involved in keloid fibrogenesis as well as its underlying mechanisms remain largely elusive. In this study, the expression levels of miR-4417 and CyclinD1 in keloid tissues and fibroblasts were examined by qRT-PCR. Cell proliferation was determined by CCK assay. Western blot and flow cytometry were performed to evaluate cell apoptosis. Cell migration and invasion were measured by Transwell assay. Luciferase reporter assay was used to confirm the relationship between miR-4417 and CyclinD1. As a result, we found that miR-4417 was significantly down-regulated in keloid tissues and fibroblasts. miR-4417 up-regulation led to the suppression of proliferation, migration, and invasion, while induced cell apoptosis in keloid fibroblasts. However, miR-4417 depletion exerted an opposite effect. CyclinD1 harbored the binding sites with miR-4417. Besides, the expression of CyclinD1 was evidently decreased in keloid tissues and fibroblasts. Meanwhile, miR-4417 was negatively correlated with CyclinD1 in keloid tissue. The effect of CyclinD1 knockdown on keloid fibroblasts was similar to that of miR-4417 overexpression. Furthermore, the elevated of CyclinD1 expression rescued the effect of miR-4417 up-regulation on keloid fibroblasts. miR-4417/CyclinD1 axis was required for cell proliferation, apoptosis, migration, and invasion in keloid fibroblasts. In conclusion, miR-4417 and CyclinD1 may be potential therapeutic targets for the treatment of keloid.

Keywords. Apoptosis; CyclinD1; fibrosis; keloid; miR-4417

1. Introduction

Keloid is a proliferative reaction or hyperplasia of skin scar tissue following trauma and cause dermatologic dysfunction and esthetic deformity by invading adjacent normal tissues (Huang *et al.* 2018; Li *et al.* 2018). More importantly, it usually leads to physical and psychological distress because of frequent episodes of itching and pain. To date, the pathogenesis of keloid remains dismal.

Increasing evidence has shown that microRNAs (miRNAs) can act as essential therapeutic targets for various diseases (O'Bryan *et al.* 2017; Yang *et al.* 2019). Recently, researchers have disclosed that differentially expressed miRNAs through a miRNA microarray between keloid tissues and normal skin tissues, raising the evidence that miRNAs may participate in the pathogenesis of keloid (Li *et al.* 2013; Liu *et al.* 2012). For example, miR-29 is significantly down-regulated in keloid and may play an important role in the fibrogenesis of keloid (Zhang *et al.* 2016). Previous study has shown that miR-141-3p suppresses the proliferation and migration of keloid fibroblasts (Feng *et al.* 2017). In addition, Yao et al. also reported the tumor suppressive role of miR-1224-5p in keloid proliferation, apoptosis and invasion (Yao *et al.* 2018). A study also revealed that miR-203 regulates keloid fibroblasts proliferation, invasion, and extracellular matrix expression (Shi *et al.* 2018). It has been reported that overexpression of miR-34a is involved in the apoptotic signaling pathway in keloid fibroblasts (De Felice *et al.* 2018). Those data suggested the involvement of miRNAs in the development of keloid fibrosis. Recently, miR-4417

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is reported to be decreased in keloid fibrosis *in vivo* (Yao *et al.* 2018). However, little is known about the function of miR-4417 in keloid fibrosis.

It is well accepted that miRNAs are responsible for diseases progression by repressing target gene (Feng *et al.* 2017; Shi *et al.* 2018). For instance, Xia *et al.* presented that miR-211 suppresses epithelial ovarian cancer proliferation and cell-cycle progression by targeting CyclinD1 (Xia *et al.* 2015). In human cervical cancer cells, miR-195 inhibits cell proliferation while induces cell apoptosis via targeting CyclinD1 (Li *et al.* 2016). Furthermore, CyclinD1 is widely associated with cellular biology processes such as proliferation and apoptosis in many diseases (Jiang *et al.* 2013; Li *et al.* 2015, 2016). Of note, CyclinD1 has been shown to promote cell proliferation and further lead to keloid formation (Liu *et al.* 2004). Although the role of CyclinD1 in various diseases has been revealed, whether CyclinD1 is associated with the regulation of miR-4417 in keloid fibrosis has not been investigated.

In this study, we sought to explore the functions of miR-4417 and CyclinD1 in keloid fibroblasts via evaluating cell proliferation, invasion, migration, and apoptosis.

2. Materials and methods

2.1 *Clinical samples and cell culture*

keloid tissues samples and keloid fibroblasts samples were taken from thirty patients who have experienced keloid excisional surgery without receiving any treatment before at Qilu Hospital of Shandong University. All patients have written informed consent and the study was approved by the Ethics Committee of Ethics Committee of Qilu Hospital of Shandong University. Keloid fibroblasts and normal human skin fibroblasts were isolated from keloid skin samples and normal skin samples, as described previously (Feng *et al.* 2017; Yao *et al.* 2018). Keloid fibroblasts and normal human skin fibroblasts were cultured in DMEM medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo Fisher Scientific) solution, in a humidified atmosphere containing 5% CO_2 at 37°C. The miR-4417 mimic (miR-4417), mimic negative control (NC), miR-4417 inhibitor (anti-miR-4417), inhibitor negative control (anti-NC), small interfering RNA for CyclinD1 (siRNA-CyclinD1), negative RNA control (siRNA-NC), CyclinD1 overexpression plasmid (CyclinD1), and pcDNA3.0 vector (pcDNA) were introduced into keloid fibroblasts using Liposome 3000 (Thermo Fisher Scientific). The transfected cells were used for following experiments.

2.2 *Luciferase reporter assay*

For the luciferase reporter assay, CyclinD1 3′UTR fragments containing the wild-type (CyclinD1-wt) or mutated (CyclinD1-mut) binding sites of miR-4417 were cloned into the p pRL-TK renilla plasmid (Promega, Madison, WI, USA). For the relationships between miR-4417 and CyclinD1, cells were co-transfected with miR-4417 mimic or miR-4417 inhibitor and CyclinD1-wt or CyclinD1-mut using Lipofectamine 3000. Dual-luciferase reporter assay system (Promega) was employed to detect the relative luciferase activity.

2.3 *qRT-PCR assay*

Total RNAs were extracted from tissues or cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into complementary DNA (cDNA) using the Primer Script RT reagent kit (TaKaRa, Dalian, China). Relative expressions of CyclinD1 and miR-4417 were determined by SYBR PrimeScript RT-PCR kit (Biosystems, Foster City, CA, USA) or TaqMan MicroRNA assay kit (TaKaRa), respectively. Relative expressions of mRNAs and miRNAs were calculated using $2^{-\Delta\Delta Ct}$ method, normalizing to β-actin or U6 small nuclear RNA, respectively. The primers were listed as follows: miR-4417 RT-Primer, 5′-GTCGTATCCAGTGCAGGGTCCG AGGTAT TCGCACTGGATACGACCC CTCC-3′; miR-4417 forward primer, 5′-CGCGGGT GGGCTTCCC-3′, and reverse primer, 5′-AGTGCA GGGTCCGAGGTATT-3′; CyclinD1 forward primer, 5′-CCCTCGGTGGGTCCTACTTCAA-3′, and reverse primer, 5′-TGGCATTTTGGAGAGGAAGT-3′; β-actin forward primer, 5′-GGACCTGACTGACTACCTC-3′, and reverse primer, 5′-TACTCCTGCTTGCTGAT-3′; U6 forward primer, 5′-GCTTCGGCAGCACATATA C-3′ and reverse primer, 5′- AACGCTTCACGAA TTTGCGT -3′.

2.4 *Western blot*

Cells were harvested and lysed with the RIPA buffer (Thermo Fisher Scientific) on ice for 30 min. BCA protein assay (Solarbio, Beijing, China) was performed to detect the protein concentration. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene

difluoride membranes (Millipore, Bedford, MA, USA). The primary antibodies were used in this study including anti-CyclinD1; anti-Bcl-2; anti-Bax; anti-ki-67; Abcam, Cambridge, UK). Bands were visualized using ECL kits (Thermo Fisher Scientific).

2.5 *Transwell assay and wound healing assay*

Transwell assay was employed to detect cell migration and invasion abilities. For cell invasion assay, the membranes of the upper compartments were matrigel pre-coated. And un-coated ones were used for cell migration assay. Cells (5×10^5/ml; 100 µl) seeded into the per transwell chamber (24-well plate; Corning, New York, USA) were cultured and cultured with FBS free medium. Complete growth medium with 10% FBS was added to the lower chamber. Then, the invaded and migrated cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet and counted in 5 randomly picked views under microscope (Leica, Wetzlar, Germany). For wound healing assay, we drew a horizontal line evenly, passing about every 0.5–1 cm across the hole. Pass at least 5 lines per well. Then we added 2×10^6/ml cells in each well. After 24 h, we used a sterile toothpick to scratch the cell layer along the line drawn on the back. Then cells were washed and added serum-free medium. At last, we put the plates in a 37°C incubator and took pictures at 24 h.

2.6 *CCK-8 assay*

Following transfection for 24, 48 and 72 h, cell proliferation was determined using a CCK-8 assay (MedChemExpress, Monmouth Junction, NJ, USA) at different times (0, 24, 48 and 72 h). The absorbance was measured at 490 nm as the reference wavelength.

2.7 *Cell apoptosis assay*

Cell apoptosis was evaluated with an Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocols. Apoptosis cells were determined using a FACS Calibur flow cytometer (BD Biosciences).

2.8 *Statistical analysis*

The results were presented as the mean ± standard deviation (SD) and processed using Graphpad 6.0 statistical software (Graphpad, San Diego, CA, USA). The differences were determined using Student's *t*-test or one-way ANOVA. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 *Enhanced expression of miR-4417 suppresses cell proliferation and metastasis, while promotes cell apoptosis in keloid fibroblasts*

Firstly, we examined the expression of miR-4417 in thirty pairs of normal skin tissues and keloid tissues. As displayed in figure 1A, miR-4417 was decreased in keloid tissues samples compared with that of normal skin tissues samples. Similarly, miR-4417 expression level was lower in keloid fibroblasts than that of normal human skin fibroblasts (figure 1B). Those findings suggested that miR-4417 may play an important role in the development of keloid fibrosis. To verify our hypothesis, we elevated or reduced the level of miR-4417 in keloid fibroblasts using miR-4417 mimic or miR-4417 inhibitor, respectively. The transfection efficiency revealed that the level of miR-4417 was significantly up-regulated in cells transfected with miR-4417 mimic, while it was evidently down-regulated in cells introduced with miR-4417 inhibitor (figure 2A), suggesting that transfection efficiency was sufficient for further analysis. As shown in figure e2B, our data demonstrated that cell viability of keloid fibroblasts was significantly inhibited after miR-4417 overexpression. However, miR-4417 depletion promoted cell viability of keloid fibroblasts. The results of flow cytometry further presented that delivery of miR-4417 mimic enhanced cell apoptosis, whereas silencing of miR-4417 induced the decrease of cell apoptotic rate (figure 2C). To expound the molecular basis of those processes, western blot was employed to detect the expressions of proliferation/apoptosis-related proteins (figure 2D). We disclosed that proliferation-related protein ki-67 and anti-apoptosis protein Bcl-2 were decreased in miR-4417 overexpression-mediated cells, while both of them were increased in miR-4417 ablation-mediated cells. On the contrary, the level of pro-apoptosis protein Bax was distinctly up-regulated in cells transfected with miR-4417 mimic. Interference of miR-4417 remarkably hampered the expression of Bax. Furthermore, ectopic expression of miR-4417 induced the inhibition of cell invasion and migration. Degradation of miR-4417 plays an opposite function on metastasis of keloid fibroblasts (figure 3A–C). Those

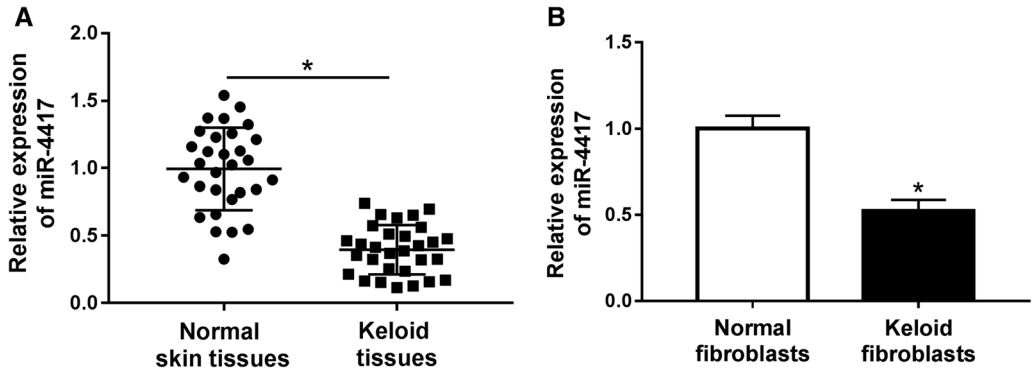

Figure 1. The expression of miR-4417 in keloid tissues and keloid fibroblasts. (**A**) qRT-PCR analysis of the expression of miR-4417 in 30 pairs of normal skin tissues and keloid tissues. (**B**) The expression level of miR-4417 in keloid normal fibroblasts and fibroblasts. *P<0.05.

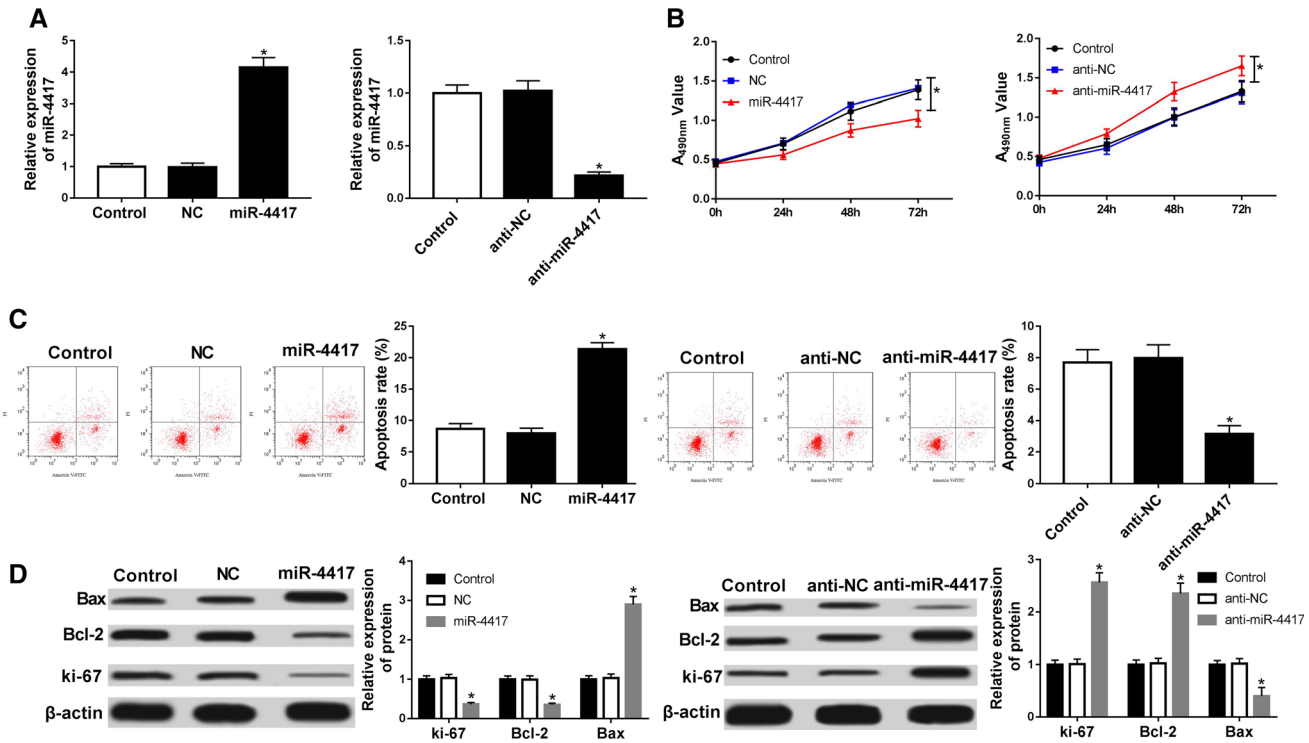

Figure 2. The effect of miR-4417 on cell proliferation and apoptosis in keloid fibroblasts. (**A**) Keloid fibroblasts were transfected with miR-4417 mimic or anti-miR-4417 and qRT-PCR assay was performed to examine the relative expression of miR-4417. (**B**) CCK-8 assay was used to evaluate cell viability. (**C**) Flow cytometry was employed to determine cell apoptosis. (**D**) Western blot was performed to examine the levels of proliferation or apoptosis-related proteins. *P<0.05.

data further confirmed that the miR-4417 may inhibit the progression of keloid fibrosis.

3.2 *miR-4417 negatively regulates the expression of CyclinD1*

Many researchers have reported that miRNAs could regulate cellular processes by repressing target genes in keloid fibrosis (Feng *et al*. 2017; Shi *et al*. 2018). To further determine the underlying molecular mechanisms of miR-4417-induced growth inhibition of keloid fibroblasts, the online tool Targetscan was used to search the potential target genes of miR-4417. The results presented that miR-4417 harbored binding sequences with CyclinD1 (figure 4A). Subsequently, we performed luciferase reporter assay to verify whether miR-4417 could directly bind to 3′UTR of CyclinD1 in keloid fibroblasts. As a result, luciferase activity was decreased in cells co-transfected with

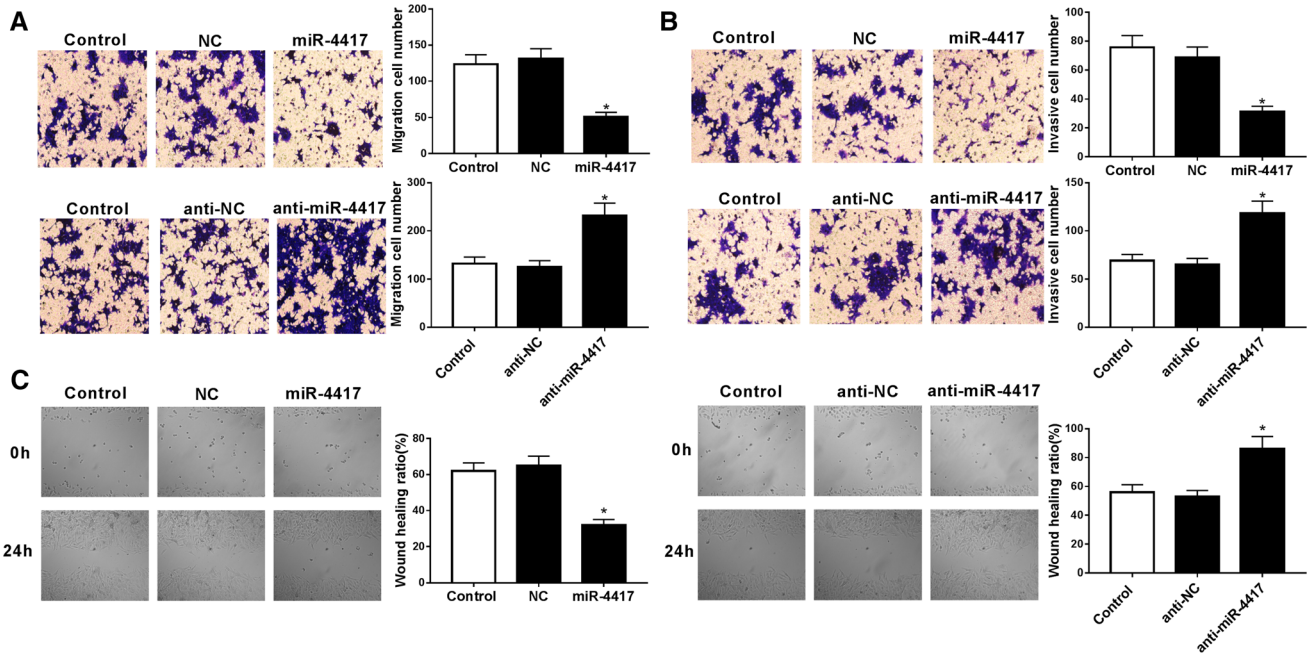

Figure 3. The effect of miR-4417 on cell metastasis of keloid fibroblasts. (**A** and **B**) Transwell assay was conducted to determine cell migration and invasion abilities in cells introduced with miR-4417 mimic or anti-miR-4417. (**C**) The data of wound healing assay in each group. *$P<0.05$.

CyclinD1-wt and miR-4417 mimic. By contrast, luciferase activity was increased in cells co-transfected with CyclinD1-wt and miR-4417 inhibitor. Interestingly, the activity was not affected in CyclinD1-mut group (figure 4B). As demonstrated in figure 4C, miR-4417 up-regulation-mediated cells had lower baseline CyclinD1 levels than that of NC group, while the absence of miR-4417 induced the increase of CyclinD1.

3.3 *CyclinD1 knockdown suppresses keloid fibroblasts growth in vitro*

Then, we examined the mRNA expression levels of CyclinD1 in keloid fibrosis *in vivo*. qRT-PCR analysis results revealed that CyclinD1 was dramatically enhanced in keloid tissues when compared to that of normal skin tissues (figure 5A). Consistently, it was observed that CyclinD1 was abnormally expression in keloid fibroblasts *in vitro* (figure 5B). Simultaneously, a significant negative correlation was further observed between miR-4417 expression and CyclinD1 mRNA levels (figure 5C). Moreover, loss-of-function experiment was also performed to explore the role of CyclinD1 in keloid fibrosis. As displayed in figure 5D, CyclinD1 protein expression level was decreased in keloid fibroblast using si-RNA-CyclinD1. The siRNA-mediated CyclinD1 down-regulation suppressed cell viability (figure 5E), induced apoptosis (figure 5F), while retarded cell migration (figure 5G) and invasion (figure 5H). All results from above indicated that CyclinD1 degradation curbed keloid fibrosis growth.

3.4 *Gain of CyclinD1 attenuates the effect of miR-4417 up-regulation on keloid fibroblasts*

At last, rescue-of-function experiment was carried out to confirm the involvement of miR-4417/CyclinD1 axis in the regulation of proliferation, metastasis, and apoptosis of keloid fibrosis. As shown in figure 6A, the protein level of CyclinD1 was decreased in miR-4417 overexpression-mediated cells, which was rescued by forced expression of CyclinD1. CCK-8 assay further indicated that elevated CyclinD1 expression regained miR-4417 up-regulation-induced inhibition of cell viability (figure 6B). When compared with miR-4417+pc-DNA group, cell apoptosis was reduced in miR-4417+CyclinD1 group (figure 6C). As expected, CyclinD1 overexpression also abolished the effect of miR-4417 up-regulation on cell migration and invasion (figure 6D and E). The results of western blot further noted that restoration of CyclinD1 mitigated the effect of miR-4417 overexpression on the protein levels of proliferation/apoptosis-related proteins (figure 6F). To sum up, we first raised the evidence that CyclinD1 overexpression plasmid transfection distinctly

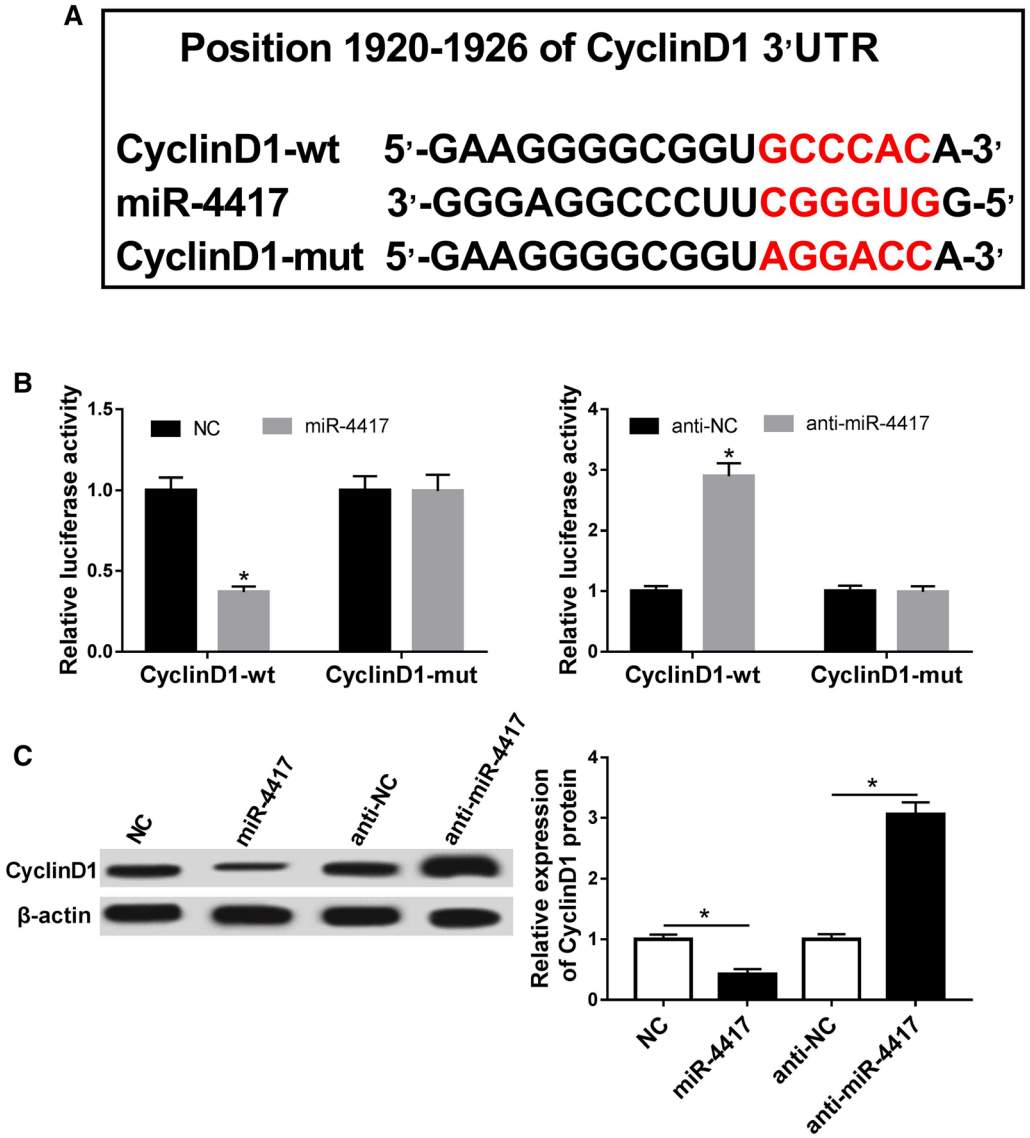

Figure 4. miR-4417 negatively regulated the expression of CyclinD1. (**A**) The binding sites between miR-4417 and CyclinD1 were predicted by Targetscan database. (**B**) miR-4417 or anti-miR-4417 and CyclinD1-mut or CyclinD1-wt were transfected into cells and then the luciferase activity was determined. (**C**) Western blot was performed to detect the protein level of CyclinD1 in cells introduced with miR-4417, anti-miR-4417, or their negative controls. *P<0.05.

overturned the impact of high expression of miR-4417 on cell proliferation, metastasis, and apoptosis.

4. Discussion

Keloids are pathological scars and results in cosmetic disfigurement and functional impairment, thus affecting the quality of life (Morelli Coppola *et al*. 2018; Zhu *et al*. 2016). Thus, it is urgent to understand the pathogenic mechanisms which drive keloid. miRNAs have been report to be associated with pathogenesis of keloid (An *et al*. 2017; Gras *et al*. 2015; Kashiyama *et al*. 2012; Zhang *et al*. 2016). In our research, we disclosed that miR-4417 expression was reduced in keloid tissues and keloid fibroblasts. Thus, we thought that miR-4417 may be a suppressor gene for the progression of keloid fibrosis.

Previous report has indicated that miR-4417 is increased in tumor of hepatocellular carcinoma (HCC) when compared with matched normal tissues (Park *et al*. 2015). Another study further demonstrated that miR-4417 knockdown suppresses proliferation and enhances apoptosis of HCC cells via upregulating TRIM35 (previous name is MAIR and Hls5) (Song *et al*. 2017). miR-4417 is being illustrated to contribute

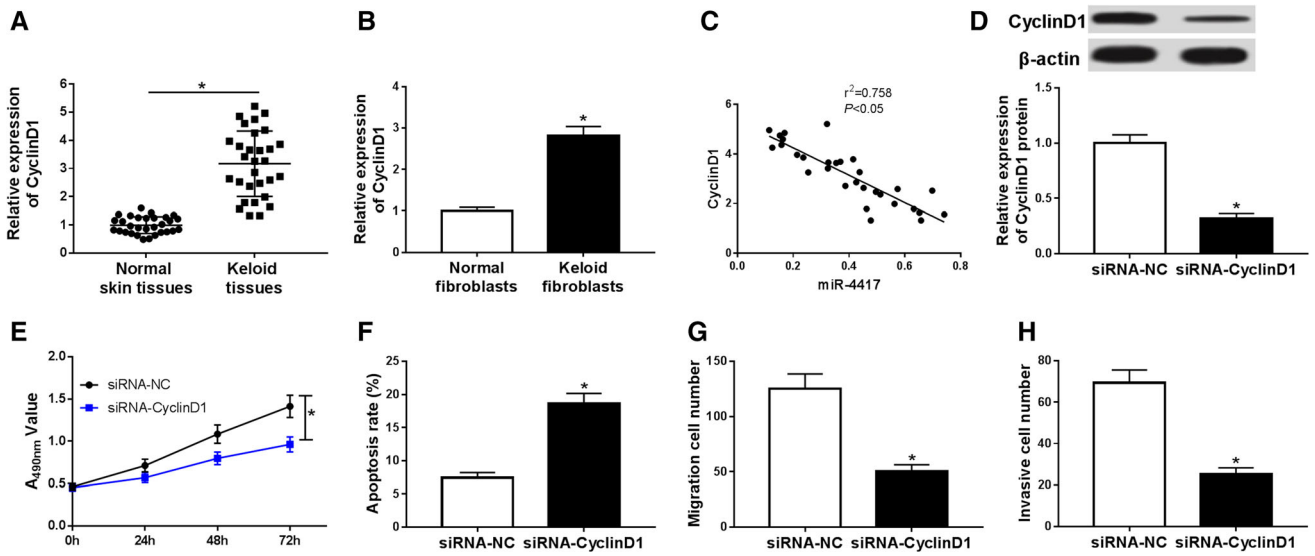

Figure 5. The effect of CyclinD1 down-regulation on keloid fibroblasts. (**A**) qRT-PCR analysis of the expression of CyclinD1 in normal skin tissues and keloid tissues. (**B**) The expression level of CyclinD1 in keloid normal fibroblasts and fibroblasts. (**C**) The expression correlation of CyclinD1 and miR-4417 in keloid tissues. (**D**) The protein level of CyclinD1 in cells transfected with siRNA-CyclinD1 or siRNA-NC. (**E** and **F**) Cell viability and apoptosis was measured. (**G** and **H**) Cell migration and invasion were detected by Transwell assay. **P*<0.05.

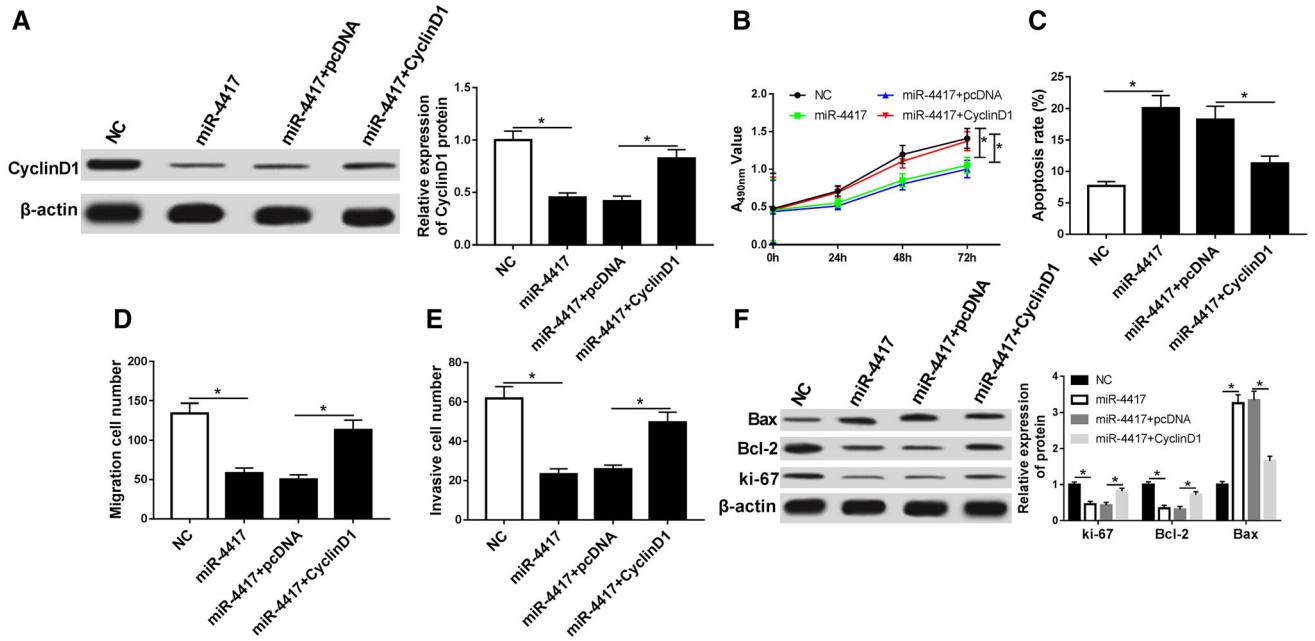

Figure 6. The effect of CyclinD1 overexpression on miR-4417 up-regulation-mediated function in keloid fibroblasts. (**A**) Western blot was conducted to evaluate the expression of CyclinD1 in cells co-transfected with miR-4417 mimic and CyclinD1 overexpression plasmid. (**B**–**E**) Cell viability, apoptosis, and metastasis were detected. (**F**) Western blot was performed to examine the protein levels of ki-67, Bcl-2, and Bax in keloid fibroblasts. **P*<0.05.

to nickel chloride-promoted lung epithelial cell fibrogenesis and tumorigenesis for that it mediates nickel-induced fibronectin expression (Wu *et al*. 2017). In the current study, we showed the low expression of miR-4417 in keloid fibrosis. Moreover, it was first screened out that overexpression of miR-4417 curbed cell proliferation, decreased cell migration and invasion, while promoted cell apoptosis in keloid fibroblasts. However, miR-4417 depletion showed the opposite effect on keloid fibroblasts. These data gave credence to our

hypothesis that miR-4417 plays an irreplaceable role in keloid formation.

CyclinD1 has attracted attention for that it is the marker of proliferation and survival (Dammann *et al*. 2018; Wu *et al*. 2016). Additionally, CyclinD1 could be the downstream target of many miRNAs and involved in the miRNA-mediated function in various diseases. In human colorectal cancer, miR-760 inhibits cancer progression via suppressing the expression of CyclinD1 (Cao *et al*.2018). In gastric cancer, Deng *et al.* revealed that miR-218 possesses the 3′-UTR regions of CyclinD1 and up-regulation of CyclinD1 abates miR-218-induced cell cycle arrest (Deng *et al*. 2017). In keloid, CyclinD1 degradation is also correlated with the mTOR-medaited anti-proliferative effect (Ong *et al*. 2007). Recently, miR-181a promotes human keloid fibroblast DNA synthesis and proliferation via increasing the expression levels of cell cycle-related proteins, including CyclinD1, CyclinE1 and CDK1 (Rang *et al*. 2016). Moreover, miR-637 also blocks cell proliferation through decreasing CyclinD1 expression in keloid (Zhang *et al*. 2018). In addition, a recent report also demonstrated that siRNA-mediated knockdown of CyclinD1 constrains cell cycle while promotes cell apoptosis, suggesting repression of CyclinD1 may be a potential therapy of keloid (Liang *et al*. 2008). Although many investigations have explored the functions of CyclinD1 in keloid, the involvement of CyclinD1 in miR-4417-mediated function in keloid is still far from clear. Interestingly, our report disclosed that miR-4417 contained the binding sites with CyclinD1. Meanwhile, CyclinD1 was negatively correlated with miR-4417 expression in keloid tissues. Loss of CyclinD1 exerted the same effect on keloid fibroblasts growth with that of miR-4417 up-regulation. In this scenario, we thought that miR-4417 may participate in keloid formation by inhibiting CyclinD1. In the following experiments, our findings showed that CyclinD1 overexpression could reverse the effect of miR-4417 on the proliferation, metastasis, and apoptosis of keloid fibroblasts. Taken together, our data highlighted the pivotal role of miR-4417 in the development of keloid fibrosis via modulating CyclinD1.

However, there are some limitations in this study. We only explored the role of miR-4417/CyclinD1 axis in the development of keloid fibrosis. The underlying signaling pathways of this process have not been completely understood. In addition, the function of miR-4417/CyclinD1 axis *in vivo* should be addressed in future. Therefore, further more comprehensive studies are needed.

Consequently, we found that miR-4417 level was descended, while CyclinD1 was highly expressed in keloid tissues and keloid fibroblasts. Meanwhile, miR-4417 may play a suppressor role in the regulation of the growth of keloid fibrosis. Our study for the first time provided the clue that miR-4417/CyclinD1 axis had an implication in the cell proliferation, apoptosis, migration, and invasion in keloid fibrosis. As a result, miR-4417 and CyclinD1 may be potential therapeutic targets for the treatment of keloid.

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