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Requirement of miR-144 in CsA Induced Proliferation and Invasion of Human Trophoblast Cells by Targeting Titin

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ABSTRACT

MicroRNAs (miRNAs) are endogenous 19–25 nucleotide noncoding single-stranded RNAs that regulate gene expression by blocking the translation or decreasing the stability of mRNAs. In this study, with the treatment of CsA (Cyclosporin A), we showed that miR144 expression levels were decreased while titin mRNA expression levels were increased in human trophoblast (HT) cells, and identified titin as a novel direct target of miR-144. Overexpression of miR-144 suppressed titin and its downstream signaling molecule such as p-ERK1/2 and MMP2/9 expression, and attenuated cell proliferation and invasion. Forced expression of titin can partly rescue the inhibitory effect of miR-144 in the cells. Taken together, these findings will shed light to the role and mechanism of miR-144 in regulating HT cells proliferation and invasion via miR-144/titin axis, and miR-144 may serve as a potential therapeutic target in HT in the future. J. Cell. Biochem. 115: 690–696, 2014.

KEY WORDS: CsA; miR-144; TITIN; HUMAN TROPHOBLAST

Cyclosporin A (CsA) is a powerful immunosuppressive drug that has been widely used to prevent organ rejection and to treat certain autoimmune disease. CsA has revolutionized organ and tissue transplantation [O'Keefe et al., 1992; Won et al., 1994]. It was found that treatment with CsA can promote proliferation and invasion of human trophoblast cells (HT cells) and in mice, increase fetal viability in abortion-prone matings to levels observed with normal pregnant matings [Yan et al., 2002; Du et al., 2007b]. However, the mechanism by which CsA regulates the HT cells functions is still unclear.

MicroRNAs (miRNAs) are small regulatory RNAs, which control a large number of gene expressions by translational suppression and destabilization of target mRNAs [Ambros, 2004; Bartel, 2004]. Currently, the number of validated mature human miRNAs approaches 1,000. Individual miRNAs can repress multiple genes [Lim et al., 2005; Baek et al., 2008], which implies a broad regulatory potential. Not surprisingly, miRNAs have emerged as important regulators of virtually every biological process associated with tissue development, differentiation, cellular proliferation, cell type-specific function, and homeostasis. Consequently, dysregulation of miRNAs has been implicated in dysfunction of genetic regulatory networks. Indeed, several pathological conditions have been linked to altered expression of miRNA [Croce and Calin, 2005; Esquela-Kerscher and Slack, 2006; Latronico and Condorelli, 2009; Pandey et al., 2009].

Titin is a giant sarcomeric protein responsible for the elasticity of striated muscle [Machado and Andrew, 2000; Granzier and Labeit, 2007]. It was reported that titin is expressed in the terminal web region of the brush border array of microvilli of intestinal epithelial cells, where it is thought to mediate the association of myosin II and alpha-actinin with the cytoskeleton [Eilertsen and Keller, 1992]. Thus it is likely that titin expression in the fetus may be involved in the process of placentation and embryo development. On the other hand, it was reported that CsA increased the titin expression to induce proliferation and invasion of HT cells through activation of the ERK1/2 pathway [Du et al., 2007b]. Thus it was proposed that the

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CsA-induced titin expression might be involved in placentation and embryonic development. The action of CsA on HT cells may be mediated through multiple signals and molecules rather than a certain specific molecule. The penetrative ability of HT cells was facilitated by degradation of the ECM of the decidua via various proteinases, among which the matrix metalloproteinases (MMPs), such as MMP2 and MMP9, played an important role. Human first-trimester HT cells had been shown to secrete MMP2 and MMP9, which could help to degrade the ECM and participate in HT cells invasion [Zhou et al., 2007].

In this study, we investigated the effect of CsA on miRNAs predicted to target titin and in vitro in HT cells. We also determined the role of miR-144 in regulating titin and its downstream molecules such as ERK1/2, MMP2, and MMP9 expression. Then we examined the role of miR-144 in CsA induced proliferation and invasion of HT cells. MiR-144 was found to play several roles in HT funtions like behavior, and also shown to bind to specific complementary sites within 3′ untranslated regions (3′UTR) of titin mRNA. It appears that miR-144 may play a role in the control of cell proliferation and migration by targeting titin through MAPK and MMPs pathways.

MATERIALS AND METHODS

CELLS AND CULTURE

The Human trophoblast (HT) cells were isolated by trypsin-DNase I digestion, and discontinuous Percoll gradient centrifugation, as described by the previous study [Wu et al., 2004]. This assay supplies a 95% purity of HT cells. The isolated HT cells were cultured in DMEM supplemented with 2 mM glutamine, 10% heat inactivated FCS, 25 mM HEPES, 100 UI/ml penicillin and 100 mg/ml streptomycin at 37°C in 95% air and 5% CO_2. Human embryonic kidney cell line 293T was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen).

For treatment of HT cells with CsA, HT cells were seeded at a density of 5×10^5 on the 25 mm round glass coverslips in six-well plates overnight (37°C, 5% CO_2), and then treated with a series of doses of CsA (0, 0.01, 0.1, and 1 μmol/L; Millipore, Billerica, MA) for 48 h.

CELL TRANSFECTION

Ectopic expression of miR-144 in cells was achieved by transfection with miR-144 mimics (Genepharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, CA, USA). Overexpression of titin was performed using titin ORF expression clone (OriGene, Beijing, China). Cells were plated in 6-well clusters or 96-well plates and transfected for 24 or 48 h. Transfected cells were used in further assays or RNA/protein extraction.

RNA EXTRACTION AND SYBR GREEN QUANTITATIVE PCR ANALYSIS

Total RNA was extracted from cells using Trizol reagent (Invitrogen). Mature miR-144 expressions in cells were detected using a Hairpin-it TM miRNAs qPCR kit (Genepharma, Shanghai, China). Expression of RNU6B was used as an endogenous control. titin expression was measured by SYBR green qPCR assay (Takara, Dalian, China). Data were processed using $2^{-\Delta\Delta CT}$ method.

WESTERN BLOT ANALYSIS

The HT cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 10 mM NaF, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, and phosphatase inhibitors; Roche), and the extracts were incubated for 20 min on ice and cleared by centrifugation. The cell lysates were assayed for protein contents using the Bradford protein assay. The samples were separated by 10% (50 μg/lane) and transferred onto PVDF membranes. Membranes were blocked in Tris–buffered saline, pH 7.4, 0.1% Tween-20 with 5% non-fat milk/blotto, incubated overnight at 4°C with primary antibody ERK1/2, p-ERK1/2, MMP2, and MMP9 (Abcam, Cambridge, UK; 1:1,000 dilution) in blocking buffer, washed with Tris–buffered saline, pH 7.4, 0.1% Tween-20 and incubated with horseradish peroxidase secondary antibodies. The bands were visualized with the enhanced chemiluminescence detection kit (ECL, Amersham Pharmacia Biotech). β-actin was used as an endogenous protein for normalization. The experiments were carried out in triplicate, and repeated three times.

LUCIFERASE REPORTER ASSAY

UTR luciferase reporter assays were performed in HT cells. Vectors based on pMIR-REPORT harboring the wild-type (WT) 350 bp fragment of the titin 3′UTR (GeneCopoeia, Guangzhou, China), or the same fragment in which the miR-144 binding site was mutated (MUT), were inserted downstream of the luciferase reporter gene stop codon in pMIR-REPORT using *Hin*dIII and *Spe*I. The cells were cotransfected with (1) miR-144 mimics or miR-SCR mimics (50 nM; Genepharma, Shanghai, China), (2) pMIR-REPORT vectors containing the WT or MUT miR-144 binding sites (400 ng), and (3) pRL-SV40 (Promega, Sunnyvale, CA) expressing Renilla luciferase (400 ng) for normalization of transfection efficiency. Cells were grown in high-glucose DMEM supplemented with 10% fetal bovine serum, and luciferase activities were measured at 48 h post-transfection using the Dual-Luciferase Reporter Assay System (Promega).

MTT ASSAY

Cell viability was evaluated using a modified MTT assay. The viability of HT cells transfected with miR-SCR + Vector, miR-SCR + titin, miR-144 + Vector, miR-144 + titin were assessed at five time points (on day 1, 2, 3, 4, and 5) after seeding 2×10^3 transfected cells/well into 96-well culture plates. Briefly, quantification of mitochondrial dehydrogenase activity was achieved via the enzymatic conversion of MTT [3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma–Aldrich, MO, USA] to a colored formazan product. MTT (10 μl, 10 mg/ml) was added to the cells, incubated for 4 h, and the reaction was terminated by removal of the supernatant and addition of 100 μl DMSO to dissolve the formazan product. After 0.5 h, the optical density (OD) of each well was measured at 570 nm using a plate reader (ELx808 Bio-Tek Instruments, City, ST, USA).

BrdU INCORPORATION ASSAY

DNA synthesis in proliferating cells was determined by measuring 5-Bromo-2-deoxyUridine (BrdU) incorporation. BrdU assays were performed at 24 and 48 h after transfecting HT cells with miR-SCR + Vector, miR-SCR + titin, miR-144 + Vector, miR-144 + titin. The transfected cells were seeded in 96-well culture plates at a density

of 2×10^3 cells/well, cultured for 24 or 48 h, and incubated with a final concentration of 10 μM BrdU (BD Pharmingen, San Diego, CA, USA) for 2–24 h. At the end of the incubation period, the medium was removed, the cells were fixed for 30 min at RT, incubated with peroxidase-coupled anti-BrdU-antibody (Sigma–Aldrich) for 60 min at RT, washed three times with PBS, incubated with peroxidase substrate (tetramethylbenzidine) for 30 min, and the absorbance values were measured at 490 nm. Background BrdU immunofluorescence was determined in cells not exposed to BrdU but stained with the BrdU antibody.

CELL INVASION ASSAY

The invasive and migratory potential of cells was evaluated using transwell inserts with 8 μm pores (Coring, NY, USA). For invasion assay, at 24 h after transfection, 3.0×10^5 cells in serum free medium were added to each upper insert pre-coated with matrigel matrix (BD, NJ, USA). Five hundred microliter 10% FBS medium was added to the matched lower chamber. After 48 h incubation, noninvaded cells were removed from the upper surface of the transwell membrane with a cotton swab, and invaded cells on the lower membrane surface were fixedin methanol, stained with 0.1% crystal violet, photographed, and counted. For migration assay, the procedures were similar, except that 2×10^5 cells were added into the inserts without matrix gel pre-coated. Six random fields at 100× magnification for each insert were counted. Inserts were conducted in triplicate in three separate experiments.

STATISTICAL ANALYSIS

All data from three independent experiments were expressed as mean ± SD and processed using SPSS17.0 statistical software. The difference among the groups in these assays was estimated by Student's *t*-test or one-way ANOVA. A *P*-value of <0.05 was considered to be statistically significant.

RESULTS

THE EXPRESSION OF miR-144 IS FREQUENTLY DOWNREGULATED IN CsA TREATMENT HT CELLS IN REVERSE CORRELATION OF TITIN mRNA AND PROTEIN EXPRESSION

It was reported that titin expression in the fetus was involved in the process of placentation and embryo development, and CsA increased the titin expression to induce the proliferation and invasion of HT cells [Yan et al., 2002; Du et al., 2007b; Zhou et al., 2007]. We started the study with titin as the focus molecule, and Targetscan, microRNA.org and Pictar [Lewis et al., 2005; Esquela-Kerscher and Slack, 2006; Hwang and Mendel, 2006] predicted that miR-24, miR-101, miR-451, and miR-144 could bind to and target the titin 3′UTR. As the results showed that miR-24 levels increased while miR-101, miR-451, and miR-144 levels decreased in HT cells following CsA treatment. The kinetics of these changes was examined by Quantitative RT-PCR analysis of microRNA levels. There was a modest increase in miR-24 following CsA treatment, while there were modest decreases in miR-101 and miR-451 following CsA treatment (Fig. 1A–C). But in miR-144, the expression level significantly decreased as compared with control following no CsA treatment (Fig. 1D). The minimal miR-144 expression levels at 0.1 or 1 μmol/L CsA treatment were approximately 0.7 times those of control basal. On the other hand, the kinetics of these changes was examined by Quantitative RT-PCR and Western blot analysis of titin levels. The maximal titin mRNA expression levels at 1 μmol/L CsA treatment were approximately 1.6 times those of control basal (Fig. 1E). These results indicated that lower expression levels of miR-144 were significantly associated with higher levels of titin mRNA expression in the same set of CsA treatment HTcells.

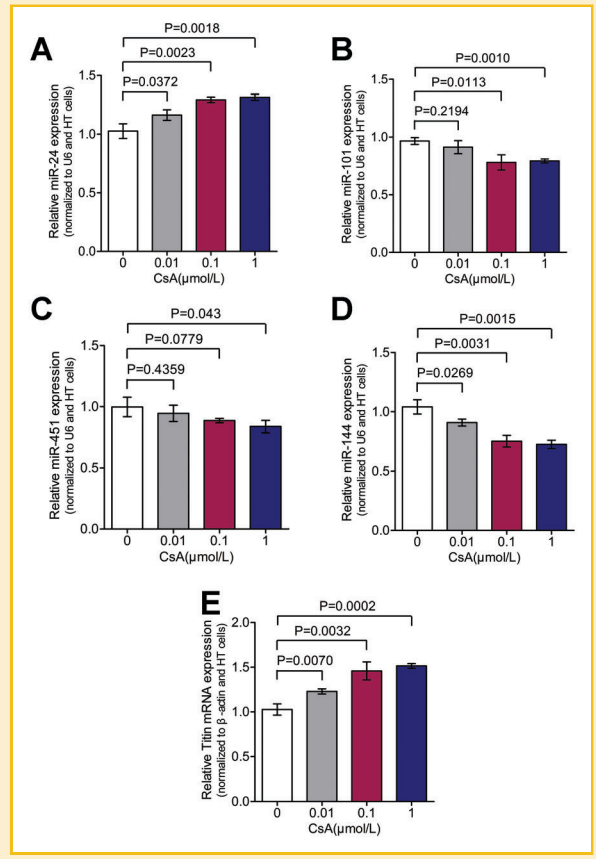

Fig. 1. The Titin mRNA and protein levels were negatively correlated with miR-144 in HT cells incubated with CsA. HT cells were incubated with CsA (0, 0.01, 0.1, 1 μmol/L) for the indicated times and the cells were collected for Quantitative RT-PCR. (A) The expression of miR-24 was increased in the cells incubated with CsA as compared with untreated cells; miR-101 (B), miR-451 (C) and miR-144 (D) were significantly decreased in the HT cells inbubated with CsA as compared with the untreated cells. (E) Increased expression of Titin was tested by Quantitative RT-PCR in HT cell lines treated with CsA in comparison with untreated HT cell line. Figure is representative of three experiments with similar results.

miR-144 DIRECTLY TARGETS TITIN

To further study the relationship of miR-144 and titin in HT cells, we transfected HT cells with miR-SCR and miR-144 mimics. Quantitative RT-PCR showed that, at 48 h after transfection, the expression of miR-144 was significantly up-regulated as compared with miR-SCR (Fig. 2A). Moreover, we observed the enhanced miR-144 in HT cells significantly repressed titin mRNA expression compared to cells transfected with scramble control by Quantitative RT-PCR (Fig. 2B).

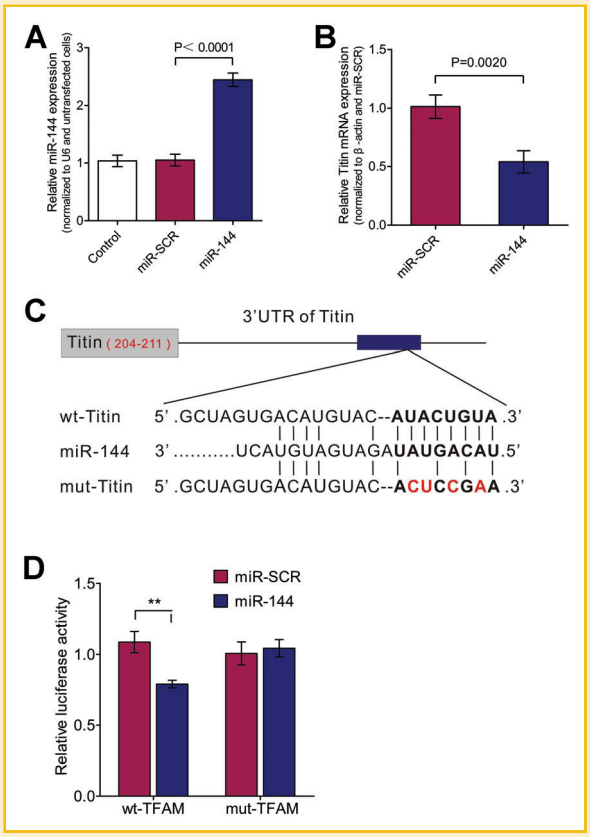

Fig. 2. miR-144 directly targets Titin by binding to its 3′UTR. (A) Up-regulated expression of miR-144 was tested by Quantitative RT-PCR in HT cell lines transfected with miR-144 mimics in comparison with the HT cell lines transfected with miR-SCR mimics. (B) Down-regulated expression of Titin was tested by Quantitative RT-PCR in HT cell lines transfected with miR-144 mimics in comparison with the HT cell lines transfected with miR-SCR mimics. (D) The predicted miR-144 binding site within Titin 3′UTR and its mutated version by site mutagenesis are as shown. (E) Relative luciferase activities of Titin wild type (WT) and mutant (Mut) 3′-prime UTR regions were obtained by cotransfection of scrambled control miRNA or miR-144 precursor, and psiCHECK-2 plasmid; and calculated as the ratio of firefly/renilla activities in the cells and normalized to those of the control. The results were presented as mean ± SD from three independent experiments with each experiment in triplicate. Double asterisks indicate significant difference when compared to control (** $P < 0.01$). Figure is representative of three experiments with similar results.

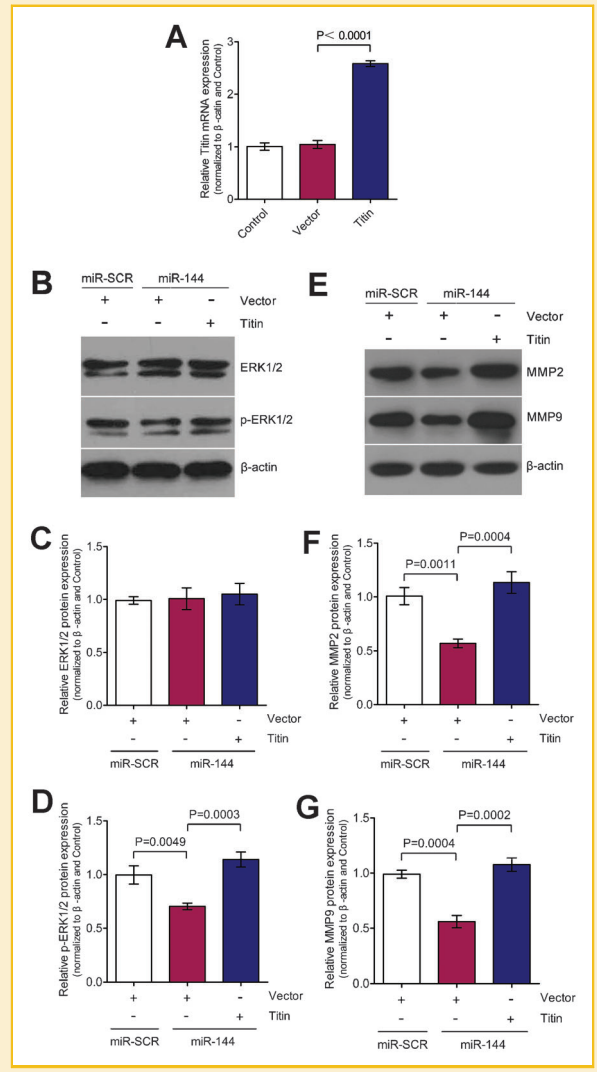

Fig. 3. Over-expression of Titin restored the inhibitory effects of miR-144 in MAPK and MMPs pathway. HT cells stably expressing miR-SCR or miR-144 were transfected with Titin ORF plasmid at 2 μg per well. After transfection for 48 h, the levels of specific proteins were analyzed by immunoblotting. Band intensities were quantitated by Image-Pro Plus. (A) Overexpression of Titin increased levels of Titin mRNA expression tested by Quantitative RT-PCR. (B–D) Overexpression of Titin did not change the level of ERK1/2 protein expression but restore the p-ERK1/2 protein expression inhibited by miR-144. (E–G) Forced expression of Titin restored the MMP2 and MMP9 protein expression inhibited by miR-144. Figure is representative of three experiments with similar results.

Additionally, To determine whether the 3′-UTR of titin mRNA is a functional target of miR-144 in HT cells, we cloned the 3′UTR of titin downstream to a luciferase reporter gene (wt-titin), its mutant version (mut-titin) by the binding site mutagenesis was also constructed (Fig. 2C). We co-transfected wt-titin vector and miR-144 mimics or scramble control into HT cells. The luciferase activity of miR-144 transfected cells was significantly reduced compared to scramble control cells (Fig. 2D). Moreover, miR-144-mediated repression of luciferase activity was abolished by the mutant putative binding site (Fig. 2D).

FORCED EXPRESSION OF TITIN RESTORED THE EFFECTS OF miR-144 INHIBITED THE MAPK AND MMPs PATHWAY

As we showed above, titin is a direct target of miR-144. Therefore, we wondered whether forced expression of titin is enough to reverse the expression of miR-144-inhibited downstream molecules. Once titin expression was effectively upregulated by titin ORF clone (Fig. 3A), titin ORF plasmid was transfected into miR-144- or miR-SCR-expressing cells. As shown in Figure 3B–D, Overexpression of titin did not change the level of ERK1/2 protein expression but restore the p-ERK1/2 protein expression inhibited by miR-144. Similarly, ectopic expression of titin also restored miR-144-inhibited MMP2 and MMP9 expression in HT cells (Fig. 3E–G). This results indicated that miR-144 inhibits MAPK and MMPs signaling pathway by targeting titin.

FORCED EXPRESSION OF TITIN RESTORED THE EFFECTS OF miR-144 INHIBITED HT CELLS PROLIFERATION AND INVASION

Taken together, it was reported that CsA up-regulated titin expression to induce proliferation and invasion of HT cells through ERK/MMPs pathway [Du et al., 2007a; Zhou et al., 2007]. And our results above showed that CsA induced up-regulation of Ttin and down-regulation of miR-144 that targeted titin in suppression of titin protein in HT cells in vitro. To validate if miR-144 regulates HT cells proliferation and invasion, we performed MTT, BrdU and invasion assays by transfecting miR-144 mimics or scramble control into HT cells. It showed that the increased expression of miR-144 induced significant inhibition on cell proliferation (Fig. 4A,B). As shown in Figure 4C–D, compared to the scramble control, miR-144 mimics transfected HT cells exhibited significant impairment of invasion ability. The corresponding effect on invasive ability was also observed in parallel invasion assay. To further confirm the potential relationship between miR-144 and the downstream gene titin, we tested cell proliferation and invasion under the condition of overexpression of titin. Once titin expression was effectively up-regulated by titin ORF clone, transfected HT cells exhibited increased cell proliferation (Fig. 4A,B), up-regulated cell invasion ability (Fig. 4C,D), which was in contrast with the effects induced by up-regulation of miR-144. These results indicated that miR-144 functions as a potent tumor suppressor through regulating titin expression.

DISCUSSION

CsA is a widely used immunosuppressant agent to prevent from organ rejection and to treat certain autoimmune diseases. It has been reported that CsA treatment of adenocarcinoma cells results in striking morphological alterations, including membrane ruffling and numerous pseudopodial protrusions, increased cell motility, and anchorage-independent growth. However, this effect was independent of the immunosuppression of CsA [Hojo et al., 1999]. Similarly, Han et al. [2010] have shown that CsA increases primary skin tumor growth in immunodeficient mice and promotes keratinocytes growth in vitro. These findings imply that CsA modulates the biological behavior in various cells which does not concern its immunosuppressive action. Previous reports have demonstrated that CsA promotes the proliferation and invasion of HT cells, and improves the outcome of pregnancy [Du et al., 2007a, 2008; Zhou et al., 2007]. Further study also reported that CsA may promote the invasion through decreasing E-cadherin expression and increasing the

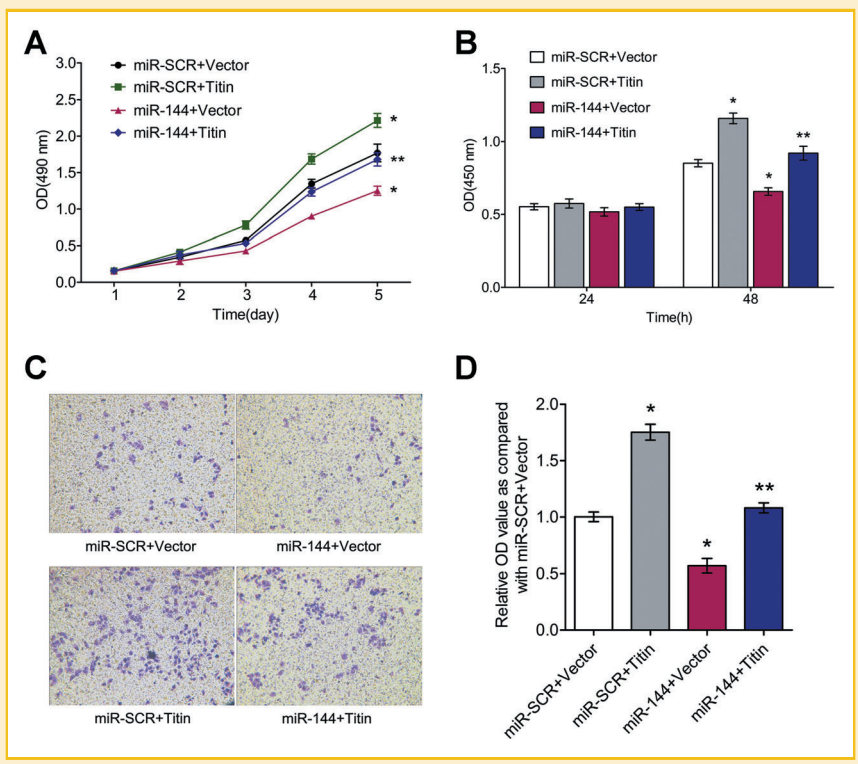

Fig. 4. Over-expression of Titin restored the inhibitory effects of miR-144 in proliferation and invasion of HT cell lines. (A) Over-expression of Titin by transfecting Titin ORF clone significantly restored proliferation of HT cells, in comparison with miR-144. (B) Cell viability, as determined by the BrdU incorporation assay. (C) Over-expression of Titin notably restored cell invasion of HT cells (100× magnification). (D) The inhibition rate was tested by MTT assay. The asterisk indicates significant difference when Titin or miR-144 compared with control; while two asterisks indicate significant difference when miR-144 + Titin compared with Titin or miR-144. (** $P < 0.01$) figure is representative of three experiments with similar results.

expression of titin, MMP-2, and MMP-9 [Du et al., 2007a; Zhou et al., 2007; Zhao et al., 2010].

The CsA-induced titin expression may contribute to HT cells proliferation and invasion through altering elasticity and extensibility, and remodeling the maternal vascular system, thus opening the placental blood supply. It has been reported that titin, a component of the contractile apparatus of the sarcomere, or a titin-like protein may reside in the nucleus of mammalian cells, and by analogy to its function in muscle, may play a critical role in the functional organization of the nucleus. These results were coincident with the study that the titin performs a significant non-sarcomeric, nuclear function, that is, participating in and possibly mediating chromosome condensation. In this study, the results showed that the CsA-induced titin expression was involved in contributing to the proliferation and invasion of HT cells and consistent with Du et al. [2007b] report.

MicroRNAs are newly identified modulators of many complicated signaling pathways involved in various human diseases. In preeclamptic placentas, several reports demonstrated differential expressed microRNAs compared with normal pregnant ones [Zhu et al., 2009; Enquobahrie et al., 2011]. MiR-210 and miR-155 have been subjected to further functional studies, and data revealed that they are related to angiogenesis and HTcells invasion/migration [Zhang et al., 2010, 2012; Dai et al., 2011]. Fu et al. [2013] reported that miR-376c expression had a significant effect on HT cells growth in vitro. miR-376c inhibits both ALK5 and ALK7 expression to impair transforming growth factor-β/Nodal signaling, leading to increases in cell proliferation and invasion. By far, it remains to be clarified as to how the deregulated miRNAs participate in the HT cells proliferation and invasion.

In this study, we raised the hypothesis that miR-144 might be involved in CsA-induced titin expression regulated HT cells proliferation and invasion process. Finally, we confirmed the link between CsA and miR-144, miR-144, and titin, and found that CsA down-regulated miR-144 inhibited HT cell proliferation and invasion via targeting titin. Our results obtained from quantitative RT-PCR validated that miR-144, one of the potential microRNAs target the titin 3′UTR, was commonly down-regulated in HT cell lines following CsA treatment. We forced miR-144 expression in HT cells and found that miR-144 inhibited cell proliferation and invasion. These findings suggested that miR-144 was involved in the processes of HT cells proliferation and invasion. Interestingly, the levels of miR-144 were negatively correlated with p-ERK1/2, MMP2and MMP9 protein levels in HT cells. It was reported that CsA-induced titin expression contributed to HT cells proliferation and invasion through MAPK pathway. Interestingly, U0126 completely abolished the expression of titin, while it only partially suppressed the proliferation and invasiveness of the HT cells up-regulated by CsA [Zhou et al., 2007], suggesting that other molecules or signal pathways may be involved in the CsA-promoted proliferation and invasion of HT cells. Also, it was coincident with evidence that MMP-9 and MMP-2 played an important role in the CsA-enhanced invasiveness of the HT cells [Zhou et al., 2007]. Thereby, the action of CsA on HT cells may be mediated through multiple signals and molecules rather than a certain specific molecule.

Our results confirmed a vital molecular relationship among miR-144, titin, ERK1/2, MMP2, and MMP9. We showed that, at both mRNA and protein level, up-regulation of miR-144 expression in HT cells effectively suppressed titin and p-ERK1/2, MMP2, and MMP9 expression, and forced expression of titin could reverse the expression of p-ERK1/2, MMP2, and MMP9. It suggested a potential inverse relevance of miR-144 and titin in HT and the main effect of titin of the cells is autocrine affect, because the miR-144 down-regulated the level of the cellular titin mRNA and protein. Additionally, these results suggested miR-144 might inhibit p-ERK1/2, MMP2 and MMP9 expression via titin signaling. Furthermore, by luciferase reporter assay study, we verified that miR-144 directly target titin gene through binding to specific complementary site within its 3′UTR. These results verified that miR-144 inhibited the expression of p-ERK1/2, MMP2, and MMP9 via titin signaling. A model to account for the role of miR-144 as a regulator of HT cells proliferation and invasion is shown in Figure 5. Taken together, these findings sufficiently consolidated that miR-144 played a suppressive role in cellular proliferation, migration and invasion, at least, in part due to directly inhibiting titin expression. Activation of MAPK and MMPs signaling pathway leads to multiple malignant processes, including cell growth, angiogenesis, cell motility, invasiveness, and metastasis [Lee et al., 2013; Park, 2013; Wei et al., 2013; Zhu et al., 2013].

In conclusion, we newly described miR-144/titin/MAPK/MMPs link and provided a potential mechanism for CsA deregulation and contribution to HT cells proliferation and invasion. As a result, restoration of miR-144 expression could have an important implication for the clinical management of HT cells and enhance the CsA treatment.

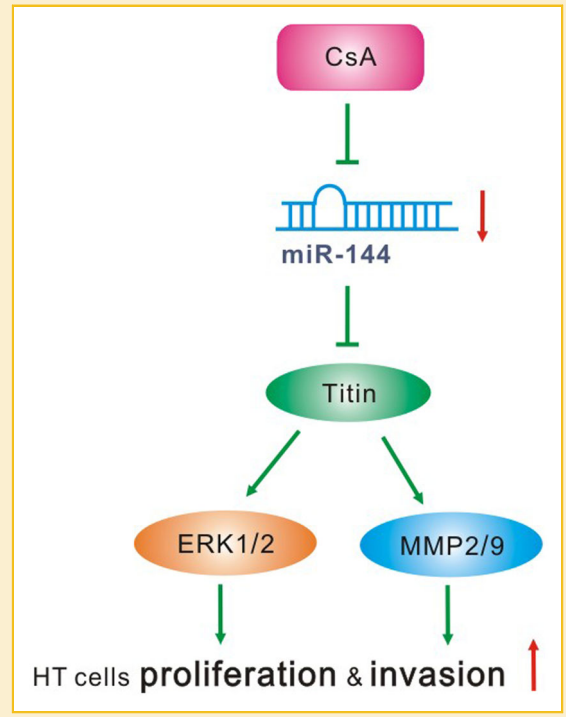

Fig. 5. A model for the role of miR-144 in HT cells. Decreased expression of miR-144 and increased expression of Titin in HT cells. MiR-144 inhibited the expression of Titin in HT cells. Moreover, miR-144 increased the HT cells proliferation and invasion through inhibiting the Titin-MAPK and Titin-MMPs pathway. The process plays an important role in the proliferation of HT cells.

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