Tripterine emerges as a potential anti-scarring agent in NIH/3T3 cells by repressing ANRIL

Lei Jiang*, Junjun Sun* and Peng Wang

Department of Aesthetic, Plastic, and Burn Surgery, Yuhuangding Hospital Affiliated Hospital of Qingdao University, Yantai City, Shandong Province, China

Abstract. Extensive scarring normally causes hypertrophic or keloid scars. This intuitively results in psychosocial distress and reduction in life quality. Tripterine is a bioactive pentacyclic triterpenoid compound while it is still poorly understood whether it possesses an anti-scarring function. NIH/3T3 cells were administrated with tripterine at increased concentrations (0–10 μM). Antisense RNA to INK4 locus (ANRIL) was transformed into NIH/3T3 cells, and the cells transfected with empty vector (mock transfection) were used as negative control. Then, cell viability and migration were profiled by cell counting kit-8 (CCK-8) and 24-Transwell assay. Protein expression was analyzed by Western blot assay. ANRIL was quantified by quantitative reverse transcription-PCR (qRT-PCR). Tripterine administration induced the growth inhibition of NIH/3T3 cells indicated by a trend toward the decreased expression of matrix metallopeptidase (MMPs), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). This process was accompanied by the decreased phosphorylation of p65, inhibitor of nuclear factor kappa-B alpha (IκBα) and the downregulation of β-catenin. Moreover, ANRIL expression was notably repressed by tripterine. By contrast, in ANRIL-transfected cells, the effect of tripterine was abolished. Tripterine exhibited an anti-scarring bioactivity in NIH/3T3 cells by inhibiting ANRIL, and this process was accompanied by the blockade of nuclear factor-kappa B (NF-κB) and β-catenin cascades.

Key words: Antisense RNA to INK4 locus — NF-κB — β-catenin — Scarring

Introduction

Scar tissue is a type of cellular fibrotic matrix, which contributes to the manifestation and progression of a myriad of diseases when it occurs in heart (Furtado et al. 2016) and kidney (Salvatore et al. 2013), although it aids regeneration in some contexts, such as central nervous system axon regeneration (Anderson et al. 2016). Mostly, injury to skin generates a wound, which is normally irreversible and eventually evokes the formation of scar, a physical and chemical barrier for regeneration, with a disfiguring condition. For patients, these visible scars definitely and intuitively cause psychosocial distress and sacrifice quality of life (Verma et al. 2014; Finnerty et al. 2016). As a consequence, better approaches are currently required to reduce scarring, which are supposed to have substantial clinical efficacy.

During wound repairing progress, fibroblasts are recruited into extracellular matrix (ECM), proliferate in response to cytokines and synthesize ECM molecules which are implicated in restoration of structure and function. Mostly, severe scarring, such as hypertrophic or keloid scars, is characterized by high proliferation of fibroblasts and imbalance between collagen synthesis and degradation (Keane et al. 2018). Particularly, matrix metalloproteinases (MMPs) are described to be essential for wound healing because of the function in re-epithelialization, scarring and clearance (Rohani et al. 2015). Correspondingly, it has been proposed that the cellular therapy, based on matrix control, might assist in reducing scarring (Sidgwick et al. 2012).

Antisense RNA to INK4 locus is identified as long non-coding RNA and generally marked as ANRIL. It has been

* These authors contributed equally to this work.
Correspondence to: Peng Wang, Department of Aesthetic, Plastic, and Burn Surgery, Yuhuangding Hospital Affiliated Hospital of Qingdao University, No. 20 Yuhuangding East Road, Yantai City, Shandong Province, China
E-mail: wang71wp@sina.com
associated with atherosclerosis (Holdt et al. 2016), cancers (Naemura et al. 2015) and diabetic retinopathy (Thomas et al. 2017). Intriguingly, a recent study found the enhancement of ECM proteins and vasoactive factors is suppressed in ANRIL-knockout diabetic animals (Thomas et al. 2018). It is well-known that scarring presents perturbation between spatiotemporal accumulation and degradation of ECM (Keane et al. 2018). Particularly, Zhou et al. (2016) found tumor necrosis factor alpha (TNF-α) induced the abundance of ANRIL by activating nuclear factor-kappa B (NF-κB) of which the key subunit p65 increasingly binds to the promoter of ANRIL; subsequently, ANRIL mediates the expression of inflammatory genes downstream of TNF-α by interacting with transcriptional factor which is guided onto the corresponding promoter sites.

Tripterine (its chemical structure as shown in Fig. 1), known as celastrol, is a bioactive pentacyclic triterpenoid compound which had been detected in traditional Chinese medicinal herb Tripterygium wilfordii, Hook F. Clinically, Tripterygium wilfordii, Hook F is approved to treating rheumatoid arthritis, and the monotherapy shows a good efficacy according to the results from follow-up visit (Lv et al. 2015; Zhou et al. 2018). It is a potent antioxidant evidenced by inhibiting lipid peroxidation (Bian et al. 2016) and an anti-inflammatory agent by balancing pathogenic and regulatory T cells (Astry et al. 2015). What’s more, it exhibits an anti-cancer capacity evidenced by anti-angiogenic proper ties (Gao et al. 2019). However, there are few reports that tripterine has shown clinical activity in scarring formation. Besides, a further proof-of-principle study is performed in our study.

To further evaluate the potential function of tripterine as an anti-scarring agent, we examined the cellular and ECM alteration in NIH/3T3 cells. Moreover, we applied the ANRIL-transfected NIH/3T3 system to investigate the molecular underpinnings.

Materials and Methods

Cell culture and administration

Fibroblast NIH/3T3 cells (Product No., CRL-1658™) (American Type Culture Collection) (ATCC) (Rockville, MD, USA) were grown in Dulbecco’s modified Eagle’s medium (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and were maintained in a humidified incubator containing 95% air and 5% CO₂ at 37°C. According to the information from the supplier, NIH/3T3 cells were derived from the embryo of NIH/Swiss mouse. Tripterine (Product No., C0869, Sigma-Aldrich), with a purity more than 98% at high performance liquid chromatography (HPLC) grade, was dissolved and stocked in dimethylsulfoxide (DMSO) (Sigma-Aldrich) at a concentration of 1 mM and then diluted into a series of concentration at 2, 4, 6, 8 and 10 μM. NIH/3T3 cells were co-incubated with tripterine for 4 h.

Cell viability analysis

Cell viability was evaluated by using a convenient and commercial kit, cell counting kit-8 (CCK-8) (APExBIO, Houston, TX, USA). It is based on a mechanism, that is, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt is reduced by dehydrogenases in viable cells into formazan, which can be detected by a Microplate reader at 450 nm, in the presence of electron mediator. Briefly, NIH/3T3 cell suspension (100 μl) was inoculated in 96-well plates in a density of 5,000 cells per well. After pre-incubation for 24 h, NIH/3T3 cells were co-incubated with tripterine at a concentration of 2–10 μM for 4 h with a control group in which NIH/3T3 cells were administrated with culture medium containing an equivalent concentration of DMSO (less than 1%). Each well was added with 10 μl of CCK-8 solution and continually incubated for 1 h. Finally, the absorbance was read using a Varioskan LUX Multimode Microplate Reader under 450 nm (Thermo Fisher Scientific, Waltham, MA, USA). Cell viability was depicted as the percentage of the control group.

Transfection

To enforce the up-regulation of ANRIL (GenBank No., AB548314.1) in NIH/3T3 cells, MIH/3T3 cells were transfected with ANRIL. Shortly, the full sequence of ANRIL (the sequence information was available on https://www.ncbi.nlm.nih.gov/nuccore/AB548314.1) was ligated into pcDNA3.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA, USA). Plasmids transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen). Then, the cells were incubated in the culture medium in the
presence of G418 (0.5 mg/ml; Invitrogen). The transfection efficiency was finally identified using quantitative reverse transcription-PCR (qRT-PCR).

**Migration analysis**

To assay migration behaviors of NIH/3T3 cells, a transwell chamber (BD Biosciences, Bedford, USA) was applied in our study. In short, NIH/3T3 cell were inoculated onto the upper compartment at density of 30,000 cells per filter after administration. The bottom wells were filled with medium. After 24 h of culture, the non-migrated cells in the upper surface of the filter were gently scraped with a cotton swab. Meanwhile, the cells on the lower surface were stained with crystal violet. Then, the absorbance was detected using a Microplate reader at 540 nm. Data was presented as the percentage of migration relative to the control.

**qRT-PCR assay**

Total RNA was isolated from NIH/3T3 cells using RNasy Plus Micro kit (QIAGEN, Hilden, Germany). To obtain cDNA, reverse transcription was conducted with Oligo(dT)20 primers (Invitrogen). Next, PCR was carried out using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with specific primers of ANRIL, forward 5'-TGC TCT ATC CGC CAA TCA GG-3'; reverse 5'-GGG CCT CAG TGG CAC ATA CC-3'. Reaction was conducted triply on a QuantStudio 3 Real-Time PCR system (Applied Biosystems). The expression of ANRIL was normalized to GAPDH with 2^{-ΔΔCT} method.

**Western blotting assay**

Protein expression was assayed by Western blotting assay. Firstly, total proteins were extracted from the cells using RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with protease and phosphatase inhibitors (Roche Applied Science, Indianapolis, USA). Then, the supernatant was obtained after centrifugation (15,000 rpm, 20 min) and protein concentration was evaluated using BCA™ protein assay kit (Pierce, Appleton, WI, USA). Next, protein separation and transference were achieved using a Bio-Rad V3 Western Blot Workflow (Bio-Rad, Hercules, CA, USA). The polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) carrying protein imprints were blocked with bovine serum albumin (BSA) (Thermo Fisher Scientific) for 1 h at room temperature, followed by co-incubation with primary antibodies against cyclin D1 (ab166663; Abcam, Cambridge, MA, USA; 1:100), MMP-2 (87809; Cell Signaling Technology, CST, Danvers, MA, USA; 1:1,000), MMP-9 (ab38898; Abcam; 1:1,000), vascular endothelial growth factor (VEGF) (MAB4931; R&D Systems, Abingdon, UK; 1 μg/ml), basic fibroblast growth factor (bFGF) (NBP1-18579; Novus Biologicals, Colombia, USA; 1:1,000), β-actin (4967; CST; 1:1,000), total-p65 (8242; CST; 1:1,000), phospho (p) Ser536-p65 (3033; CST; 1:1,000), total-inhibitor of nuclear factor kappa-B alpha (IκBα) (4812; CST; 1:1,000), pSer32-IκBα (2859; CST; 1:1,000). Then the primary antibodies were probed by secondary antibodies, goat anti-rabbit (7074; CST; 1:5,000) and anti-rat (ab97057; CST; 1:5,000) IgG conjugated by horseradish peroxidase. Finally, an enhanced chemiluminescence agent was exploited for the visualization of protein bands on ChemiDoc™ MP Imaging System (Bio-Rad) with a quantification using Image Lab™ software (Bio-Rad).

**Statistical analysis**

Each experiment was carried out in triplicate using three independent performances. All data was presented as the mean ± standard deviation (SD). p-values were calculated by two-tailed Student’s t-test and one-way analysis of variance (ANOVA) followed by Bonferroni’s test using GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA). The significance was statistically considered when p-values were less than 0.05.

**Results**

**Tripterine weakened cell viability and migration with the decline of cyclin D1, MMPs, VEGF and bFGF**

To confirm the effect of tripterine on NIH/3T3 cells, we incubated the cells with tripterine at different concentrations (2–10 μM). Apparently, tripterine reduced (p < 0.05) cell viability with a dose-response manner (4–10 μM) while a not significant alteration of cell viability was observed at a concentration of 2 μM (p > 0.05) as shown in Fig. 2A. After 4 h of treatment with 6 μM tripterine, around 50% of NIH/3T3 cells remained (p < 0.01) obviously viable compared to un-treated cells (Fig. 2A). Consequently, further studies were carried out with NIH/3T3 cells administrated by 6 μM tripterine. Our results showed that NIH/3T3 cells treated with tripterine showed the notable blockade of (p < 0.01) migration (Fig. 2B) and significant reduced expression of (p < 0.01) cyclin D1 (Fig. 2C), (both p < 0.05) MMPs (MMP-2 and MMP-9) (Fig. 2D), (p < 0.01) VEGF and (p < 0.01) bFGF (Fig. 2E). Collectively, tripterine exhibited a potential suppressive role in scar formation.

**Tripterine decreased expression of ANRIL**

In the present study, NIH/3T3 cells were pre-incubated with 6 μM tripterine for 4 h and then examined for ANRIL
mRNA level. The results showed that after administration, NIH/3T3 cells showed (p < 0.01) a remarked increase in ANRIL expression (Fig. 3). This raised a hypothesis whether tripterine could repress cell viability and migration as well as attenuate the accumulation of cyclin D1, MMPs, VEGF and bFGF by down-regulating ANRIL.

**ANRIL served as a negative mediator for tripterine during repressing the growth of NIH/3T3 cells**

NIH/3T3 cells were stably transfected with human ANRIL gene and selected in G418-supplemented medium. Obviously, a notable overexpression (p < 0.01) of ANRIL was observed in ANRIL-transfected cells (Fig. 4A). As indicated, evaluation of ANRIL-overexpressed NIH/3T3 cells treated with tripterine exhibited a trend toward visibly (p < 0.05) enhanced cell viability (Fig. 4B) and (p < 0.05) increased expression of cyclin D1 (Fig. 4C). In addition, the migration behavior was (p < 0.05) evidently fortified (Fig. 4D). What’s more, ANRIL-transfected NIH/3T3 cells showed a prominent accumulation of (p < 0.01) MMP-2, (p < 0.01) MMP-9 (Fig. 4E) though the cells were pre-incubated with tripterine. Consistently, the overexpression of VEGF (p < 0.01) and bFGF (p < 0.01) was detected in ANRIL-overexpressed NIH/3T3 cells in spite of tripterine treatment (Fig. 4F). All these results illuminated that tripterine might block the growth of fibroblast NIH/3T3 cells by down-regulating ANRIL.
Tripterine blunted NF-κB and β-catenin signaling pathways by down-regulating ANRIL

To get a better picture of its effects on associated signaling transduction cascades, we evaluated NF-κB and β-catenin signaling pathways. As shown in Fig. 5A, tripterine resulted in a down-regulation in phosphorylated expression of (p < 0.05) p65 and (p < 0.05) IκBα in NIH/3T3 cells, whereas ANRIL overexpression contributed to the phosphorylation of (p < 0.01) p65 and (p < 0.05) IκBα, suggesting that tripterine might blunt NF-κB cascade by repressing ANRIL. Moreover, our results indicated that tripterine, which (p < 0.05) broadly down-regulated β-catenin expression, was actually (p < 0.01) up-regulated its level in ANRIL-overexpressed cells (Fig. 5B). In fact, this up-regulation might be ascribed to ANRIL over-expression. Taken together, tripterine might blunt NF-κB and β-catenin transduction cascades via down-regulating ANRIL.

Discussion

A recent study showed an efficient inhibitory role of tripterine in the formation of erythema and scaling by decreasing the level of cytokines in psoriasis mouse models (Meng et al. 2017). Moreover, the down-regulation of β-catenin expression might be ascribed to ANRIL overexpression, which further supports the role of tripterine in regulating the expression of cytokines and inhibiting the formation of erythema and scaling.

Figure 4. Effect of tripterine on the growth of ANRIL-overexpressed NIH/3T3 cells. NIH/3T3 cells were transfected with or without ANRIL, empty vector as a negative control, and then treated with 6 μM tripterine for 4 h. A. ANRIL level assayed by qRT-PCR. B. Cell viability evaluated by cell counting kit-8 assay. C. Cyclin D1 protein expression quantified by Western blot method. D. Migration behaviors assessed by 24-Transwell assay. MMPs (MMP-2 and MMP-9; E) as well as VEGF and bFGF (F) examined by Western blot analysis. Data represented means ± SD. * p < 0.05, ** p < 0.01. MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor. For more abbreviations, see Fig. 3.
The results of our study revealed that tripterine administration has an evident effect on ANRIL known to participate in modulating production of ECM molecules (Thomas et al. 2018) which are crucial for scarring (Keane et al. 2018). Moreover, tripterine does suppress cell viability, block migration and blocking inflammatory pathways. The overall results suggested a striking potent of tripterine in repressing scarring.

As proved in traumatic spinal cord injury, cell cycle-related proteins are chronically enhanced and result in astroglial scar formation with chronic inflammation and even tissue loss (Wu et al. 2012). Tripterine notably repressed the accumulation of cyclin D1. Considering that cyclin D1 is a crucial activator of cell cycle activation and its ablation confers neuroprotective effects by attenuating hippocampal neuronal cell loss (Kabadi et al. 2012), we considered that cyclin D1 served as an important mediator of tripterine to play roles. Besides, we hypothesized that tripterine administration caused direct decrease of cell viability; proliferation and migration were associated with a sharp reduction in MMPs, VEGF, and bFGF. However, the anti-cell viability and anti-proliferation effects of tripterine were retarded in ANRIL-overexpressed cells. It might be acknowledged that ANRIL modulates target-genes in trans, which leads to increased cell viability (Holdt et al. 2013).

Moreover, the increase in MMP expression is associated with impaired wound healing and fibrosis, and MMP inhibition has been represented as a potential therapeutic strategy to improve tissue repair outcomes (Caley et al. 2015). Interestingly, tripterine attenuates intrahepatic cholestasis of pregnancy symptoms via inhibiting MMP-2 and MMP-9 (Guo et al. 2018). Similarly, we found tripterine down-regulated MMP-2 and MMP-9 levels in fibroblasts. However, ANRIL overexpression abrogated the down-regulatory role of tripterine. A recent study found that ANRIL functions in tissue remodeling by modulating the expression of genes which participate in mediating ECM remodeling (Congrains et al. 2012). These results substantiated that ANRIL might be a mediator of tripterine in modulating MMPs expression.

VEGF-mediated angiogenic response is one of the key causative factors on hypertrophic scarring (Kwak et al. 2016). Tripterine has been identified to attenuate the secretion of VEGF and consequently suppress vascular tube formation (Huang et al. 2012). Consistently, we observed that tripterine decreased the expression of VEGF. Additionally, we found ANRIL overexpression contributed to the abundance of VEGF. Of particular, ANRIL regulates VEGF expression in mediation of specific miRs in diabetic retinopathy (Thomas et al. 2017). In this regard, it seems that through repressing ANRIL and subsequently mediated suppression of VEGF, tripterine favored an anti-angiogenic profile.

Besides, tripterine obviously impeded the accumulation of bFGF, which has been recognized as a critical inducer of proliferation (Choi et al. 2010) and migration (Kanazawa et al. 2010). Despite the fact that most studies confirmed that bFGF facilitates wound healing and reduces scarring (Eto et al. 2012; Figure 5. Effect of tripterine-mediated ANRIL on NF-κB and β-catenin transduction cascades. NIH/3T3 cells were transfected with or without ANRIL, empty vector as a negative control, and then treated with 6 μM tripterine for 4 h. Cell lysates analyzed by Western blotting with anti-p65 antibody, pSer536-p65 antibody, 1xBa antibody, pSer32-1xBa antibody (A) and β-catenin antibody (B). Data represented means ± SD after normalization with β-actin. *p < 0.05, **p < 0.01. p, phospho; t, total; 1xBa, inhibitor of nuclear factor kappa-B alpha; NF-xB, nuclear factor-kappa B. For more abbreviations, see Fig. 3.
Shi et al. 2013), abnormal overexpression of bFGF from fibroblasts might be implicated in the pathological fibrotic progress in hypertrophic scars (Akimoto et al. 1999), which might be ascribed to the type of fibroblasts (Song et al. 2011). What’s more, bFGF might mainly function as a strong activator of proliferation and migration instead of an anti-scarring mediator. In addition, bFGF was found to be reversely up-regulated in ANRIL-transfected cells treated by tripterine, suggesting that tripterine weakened proliferation and migration by repressing bFGF which might be a downstream target of ANRIL.

Signaling transduction by NF-κB pathway is crucial for wound healing process since it mediates the expression of proteins including fibronectin, cyclin D1 and vascular endothelial growth factor (Park et al. 2018). The crucial anti-inflammatory effects of tripterine have been elucidated through NF-κB pathway (Tozawa et al. 2011). Our results on the dephosphorylation of p65 and IκBα pointed toward a blockade of NF-κB pathway by tripterine, and ANRIL overexpression caused the abundance of phosphorylated forms. In fibrotic skin diseases, β-catenin activation can substantially exhibit a pro-fibrotic role because it mediates the expression of target genes implicated in the pro-fibrotic effects (Hamburg et al. 2012). In spinal cord injury, the glial scarring is reduced by abrogating the signaling transduction of β-catenin pathway since the bluntness of β-catenin improves the environment which is permissive to axonal regeneration (Rodriguez et al. 2014). We found tripterine caused the inactivation of β-catenin pathway by down-regulating ANRIL, implying that the anti-scarring potential of tripterine might be attributed to the bluntness of β-catenin.

Ultimately, we concluded that tripterine holds important implication in anti-scarring by blunting proliferation and migration with down-regulating cyclin D1, MMPs, VEGF and bFGF. Furthermore, we consolidated that tripterine might function as an anti-scarring agent by down-regulating ANRIL which can block NF-κB and β-catenin pathways.

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Conflict of interest. The authors declare that there are no conflicts of interest.

Availability of data and materials. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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