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Original Research

Luteolin affects keloid fibroblast proliferation and apoptosis by regulating FRAT1 gene expression

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Abstract: The current experiment was performed to investigate whether luteolin affects the proliferation and apoptosis of keloid fibroblasts by regulating the expression of FRAT1 gene. Keloid fibroblasts were treated with luteolin at different concentrations. MTT method, western blot, flow cytometry, and real-time quantitative PCR (qPCR) were used to detect cell proliferation, cyclin D1 (CyclinD1), p21, B-cell lymphoma / leukemia-2 (Bcl-2), Bcl-2 related X protein (Bax), FRAT1 protein expression, apoptosis and ARHI mRNA expression. Keloid fibroblasts were transfected with si-FRAT1, or pcDNA-FRAT1 and treated with luteolin to observe their roles in cell proliferation and apoptosis. Compared with the control group, luteolin significantly reduced the keloid fibroblast activity, CyclinD1, Bcl-2, and FRAT1 protein levels, and obviously improved the cell apoptosis rate, p21 and Bax protein expression ($P<0.05$). The expression of FRAT1 mRNA and protein in keloid fibroblasts was greatly increased ($P<0.05$). Inhibition of FRAT1 expression evidently decreased cell viability at 24 h, 48 h, and 72 h, CyclinD1, and Bcl-2 protein expression of keloid fibroblasts, while-dramatically enhanced cell apoptosis, p21, and Bax protein levels ($P<0.05$). FRAT1 overexpression reversed the inhibitory effect of luteolin on keloid fibroblast activity, FRAT1, CyclinD1, and Bcl-2 protein expression, and promotion of apoptosis, p21 and Bax protein expression. Luteolin can inhibit the proliferation and induce apoptosis of keloid fibroblasts by regulating the expression of FRAT1 gene.

Key words: Luteolin; Keloid fibroblasts; FRAT1; Proliferation; Apoptosis.

Introduction

Keloids often occur in wound healing after burn treatment or trauma surgery, which affects over 0.3-16% of the world's population (1). Some methods have been developed to treat keloids, including surgical resection, steroids, 5-fluorouracil injection, and radiation and stress therapy (2). However, due to its high recurrence rate, keloid still has an unsatisfactory treatment effect (3). Hence, it is important to study the mechanism of fibroblasts in keloids and explore new treatments for keloids. Luteolin is a natural flavonoid mainly extracted from vegetables and fruits, which displays great antitumor potential in many malignant cancers (4). Studies show that luteolin can inhibit proliferation, migration and invasion of lung cancer A549 cells, and induce its apoptosis (5); inhibit T lymphocyte proliferation and induce T lymphocyte apoptosis (6); inhibit the proliferation of hepatocellular carcinoma cells HepG2 in vitro and its angiogenic activity (7). FRAT1 (frequently rearranged in advanced T-cell lymphomas 1) gene has up-regulated expression in non-small cell lung cancer tissues. Silencing its expression will inhibit the proliferation of non-small cell lung cancer A549 cells and promote cell apoptosis (8). High FRAT1 expression may be closely related to the occurrence of human brain gliocytoma (9). Silencing FRAT1 gene expression can effectively induce colon cancer cell apoptosis, block the cell cycle and inhibit cell proliferation (10). Nevertheless, it is yet unknown regarding the effect of luteolin on proliferation and apoptosis of keloid fibroblasts, FRAT1 expression in keloid fibroblasts and its effect on the proliferation and apoptosis of keloid fibroblasts, and whether luteolin affects proliferation and apoptosis of keloid fibroblasts by regulating FRAT1 expression. Based on this, this study examines the role of luteolin in keloid fibroblast proliferation and apoptosis and investigates its potential mechanism of action based on the FRAT1 gene.

Materials and Methods

Cells and reagents

Cell separation

Separation of keloid fibroblasts (KFB) and normal skin fibroblasts (NFB) was performed according to the report of Yang Li et al. (11). That is, 4 fresh specimens of keloids surgically excised in our hospital and normal skin tissues were collected, soaked in 5% Chlorhexidine and fully washed with normal saline, cut into pieces of about 1 mm^3, added with DMEM medium (Dulbecco's modified Eagle's medium) containing 20% fetal bovine serum (FBS), incubated in 5% CO_2 and 37°C incubator. Change the culture solution 2-3 times a week. Passage

the cells when about 80% confluence was reached.

Main reagents

Luteolin was purchased from National Institutes for Food and Drug Control, FBS and DMEM were purchased from Gibco, USA, methyl thiazolyl tetrazolium (MTT) was purchased from Sigma, USA. Bicinchoninic acid protein assay kit, Annexin-V-Propidium iodide (PI) apoptosis kit were purchased from Shanghai Beyotime Institute of Biotechnology, PrimeScript RT Master Mix and SYBR Premix Ex Taq kit were purchased from TaKaRa, Japan.

Methods

Cell culture and liposome transfection

The isolated keloid fibroblasts and normal skin fibroblasts were incubated in a DMEM medium containing 10% FBS, incubated in a 5% CO_2, 37°C incubator. Cells of 4-8th generations were used for subsequent experiments. Transfected si-NC, si-FRAT1, pcDNA, and pcDNA-FRAT1 plasmids were purchased from Shanghai GenePharma. At cell transfection, keloid fibroblasts were seeded into a 6-well plate. When the cell confluence reached about 70%, the above plasmid was transfected into keloid fibroblasts using Lipofectamine 2000 in strict accordance with the instructions. 4-6 h after the transfection, the medium was replaced with a fresh one for further culture.

Cell grouping

Before transfection, keloid fibroblasts were divided into control group (Control group: normal cultured cells), low concentration luteolin group (luteolin-L group: 20 μmol/L luteolin-treated cells), medium concentration luteolin group (luteolin-M group: 40 μmol/L luteolin-treated cells), high concentration luteolin group (luteolin-H group: 60 μmol/L luteolin-treated cells). The action time was 48 h [5].

After transfection, keloid fibroblasts were divided into the si-NC group (cells transfected with si-FRAT1 as negative control si-NC), si-FRAT1 group (cells transfected with si-FRAT1), luteolin + pcDNA group (cells treated with 60 μmol/L luteol and transfected with pcDNA-FRAT1 as negative control pcDNA), luteolin+ pcDNA-FRAT1 group (cells treated with 60 μmol/L luteolin and transfected with pcDNA-FRAT1). 48 h after treatment, cell proliferation, apoptosis and related protein expression were analyzed.

MTT detection of cell proliferation

Keloid fibroblasts were seeded in a 96-well plate at a density of 3×10^5 cells/well, incubated at 37°C and 5% CO_2 for 24 h, 48 h and 72 h, respectively. 5 mg/mL MTT reagent was added to each well for 4 h reaction. Then add 150 μL dimethyl sulfoxide, shake vigorously to dissolve the crystals, use a microplate reader to measure the absorbance OD value at 490 nm.

Western blot detection of CyclinD1, p21, B cell lymphoma/leukemia-2 (Bcl-2), Bcl-2 associated X protein (Bax), FRAT1 expressions

RIPA buffer was added to keloid fibroblasts or normal skin fibroblasts to extract protein from the cells, and then the protein concentration was measured by a bicinchoninic acid kit. A 30 μg protein sample was separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% skim milk at room temperature for 2 h. Afterward, the membrane was incubated with anti-CyclinD1, p21, Bcl-2, Bax, FRAT1 (with dilutions at 1: 1,000) and control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (with dilution at 1: 2,000) antibodies overnight at 4°C. It was then incubated with horseradish peroxidase-labeled secondary antibody (with dilution 1: 5,000) for 2 h at room temperature. ELC chemiluminescence reagent was used for coloration and development, and CyclinD1, p21, Bcl-2, Bax, and FRAT1 protein bands were analyzed by Image J software.

Apoptosis detection by flow cytometry

Keloid fibroblasts were seeded in a 96-well plate at a density of 1×10^6 cells/well. After different treatments, the cells were suspended in 500 L Annexin V binding buffer, added with 5 L Annexin V-FITC and mixed thoroughly. Then, add 5L PI, mix well and react at room temperature. After 10 minutes, detect apoptosis by flow cytometry.

FRAT1 mRNA expression detection by quantitative real-time PCR (qPCR)

Total RNA of keloid fibroblasts and normal skin fibroblasts was extracted with Trizol solution. According to the kit instructions, cDNA synthesis and real-time quantitative PCR were performed respectively using the PrimeScript RT Master Mix kit and SYBR Premix Ex Taq kit, and the reactions were performed in an ABI 7500 PCR instrument. The primer sequences used are: FRAT1: forward 5'-GCCCTGTCTAAAGTGTATTTTCAG-3',reverse 5'-CGCTTGAGTAGGACTGCAGAG-3'; internal reference GAPDH: forward 5'-GAAGTGAAGGTCGGAGTCA-3', reverse 5'-TTCACACCCATGACGAACAT-3'. FRAT1 mRNA level was determined by formula $2^{-\Delta\Delta Ct}$.

Statistical analysis

Data were statistically processed using SPSS 22.0 software, and the result was expressed as the mean ± standard deviation ($\bar{x}\pm s$). t-test was used for data comparison between the two groups. One-way analysis of variance was used for data comparison among multiple groups. SNK-q test was used for multiple comparisons within the group. $P<0.05$ suggests a statically significant difference.

Results

Effect of luteolin on the proliferation of keloid fibroblasts

Detection results in Table 1 and Figure 1 show that compared with the control group, low concentration luteolin group at 24 h has no obvious change in cell viability, while low concentration luteolin group at 48 h, 72 h and medium and high concentration luteolin group at 24 h, 48 h and 72 h have significantly reduced cell viability, significantly reduced CyclinD1 protein expression and significantly increased p21 protein level in keloid fibroblasts ($P<0.05$). Compared with the low concentration

Table 1. Effect of luteolin on the proliferation of keloid fibroblasts (x±s, n=9).

Groups	OD490nm			CyclinD1 protein	p21protein
	24h	48h	72h		
Control	0.44±0.04	0.85±0.08	1.14±0.09	0.65±0.06	0.34±0.03
luteolin-L	0.41±0.03	0.72±0.06*	0.91±0.08*	0.52±0.05*	0.48±0.04*
luteolin-M	0.37±0.03*#	0.60±0.05*#	0.79±0.07*#	0.39±0.03*#	0.61±0.06*#
luteolin-H	0.33±0.03*#&	0.43±0.04*#&	0.58±0.05*#&	0.24±0.03*#&	0.75±0.05*#&
F	19.186	81.532	89.918	140.658	129.070
P	0.000	0.000	0.000	0.000	0.000

Note: compared with the Control group, *P<0.05; compared with luteolin-L group, #P<0.05, compared with the luteolin-M group, &P<0.05.

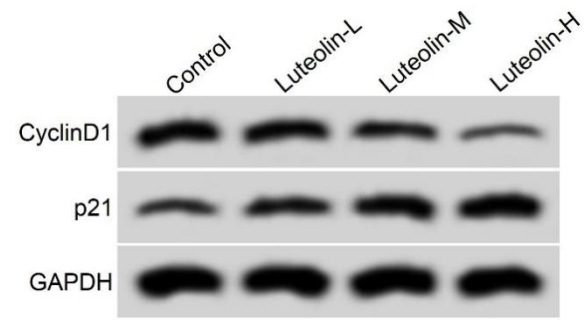

Figure 1. Proliferation-related protein expression.

luteolin group, medium and high concentration luteolin groups have significantly reduced cell viability at 24 h, 48 h, and 72 h, significantly reduced CyclinD1 protein expression and significantly increased p21 protein level in keloid fibroblasts (P <0.05). Compared with the medium concentration luteolin group, the high concentration luteolin group has significantly reduced cell viability at 24 h, 48 h and 72 h, significantly reduced CyclinD1 protein expression and significantly increased p21 protein level in keloid fibroblasts (P <0.05).

Effect of luteolin on apoptosis of keloid fibroblasts

Detection results in Table 2 and Figure 2 show that compared with the control group, low, medium, and high concentration luteolin groups have significantly increased apoptosis rate of keloid fibroblasts, significantly reduced Bcl-2 protein expression and significantly increased Bax protein level (P <0.05); compared with the low concentration luteolin group, medium and high concentration luteolin groups have significantly increased apoptosis rate of keloid fibroblasts, significantly decreased Bcl-2 protein expression, significantly increased Bax protein level (P <0.05); compared with medium concentration luteolin group, high concentration luteolin group has significantly increased apoptosis rate of keloid fibroblasts, significantly reduced Bcl-2 protein expression and significantly increased Bax protein level (P <0.05).

FRAT1 expression in keloid fibroblasts

Detection results in Table 3 and Figure 3 reveal that compared with normal skin fibroblasts (NFB), keloid fibroblasts (KFB) have significantly increased FRAT1 mRNA and protein expressions (P <0.05).

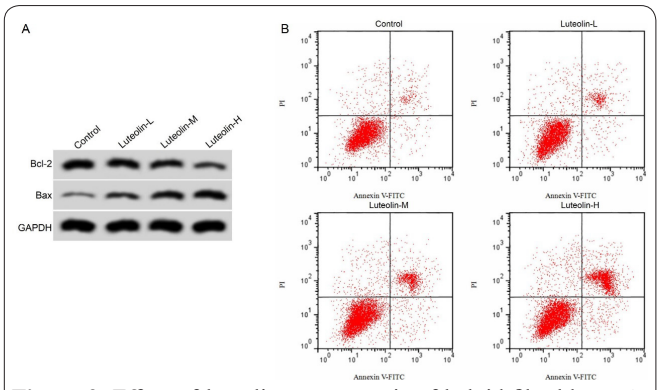

Figure 2. Effect of luteolin on apoptosis of keloid fibroblasts. A: Apoptosis-related protein expression; B: flow cytometry of apoptosis.

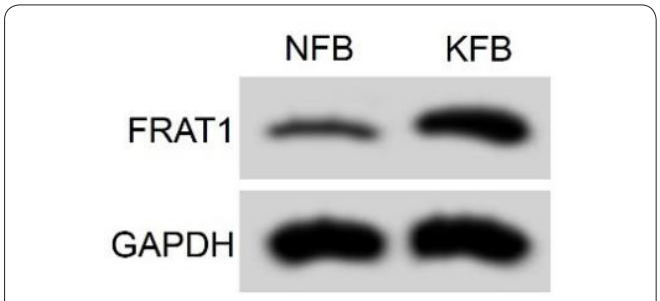

Figure 3. FRAT1 protein expression.

Table 3. FRAT1 expression in keloid fibroblasts (x±s, n=9).

Groups	FRAT1 mRNA	FRAT1 protein
NFB	1.01±0.07	0.26±0.03
KFB	2.74±0.26*	0.67±0.06*
t	19.275	18.336
P	0.000	0.000

Note: compared with NFB group, *P<0.05.

Table 2. Effect of luteolin on apoptosis of keloid fibroblasts (x±s,n=9).

Groups	Apoptosis rate (%)	Bcl-2 protein	Bax protein
Control	6.58±0.66	0.71±0.07	0.21±0.02
luteolin-L	11.42±1.13*	0.58±0.05*	0.33±0.03*
luteolin-M	19.84±1.25*#	0.43±0.04*#	0.46±0.04*#
luteolin-H	28.14±2.34*#&	0.29±0.03*#&	0.63±0.06*#&
F	371.437	120.576	179.585
P	0.000	0.000	0.000

Note:compared with the Control group, *P<0.05;compared with the luteolin-L group, #P<0.05;compared with the luteolin-M group, &P<0.05.

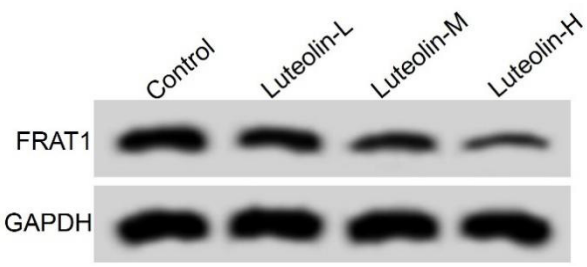

Figure 4. FRAT1 protein expression.

Table 4. Effect of luteolin on FRAT1 protein expression in keloid fibroblasts (x±s, n=9).

Groups	FRAT1 protein
Control	0.68±0.06
luteolin-L	0.56±0.05*
luteolin-M	0.41±0.04*#
luteolin-H	0.27±0.03*#&
F	133.116
P	0.000

Note: compared with the Control group, *P<0.05; compared with luteolin-L group, #P<0.05; compared with luteolin-M group, &P<0.05.

Effect of luteolin on FRAT1 protein expression in keloid fibroblasts

Detection results in Table 4 and Figure 4 show that compared with the control group, low, medium, and high concentration luteolin groups have significantly reduced FRAT1 protein expression in keloid fibroblasts (P <0.05); compared with low concentration luteolin group, medium and high concentration luteolin groups have significantly reduced FRAT1 protein level in keloid fibroblasts (P <0.05); compared with medium concentration luteolin group, high concentration luteolin group has significantly reduced FRAT1 protein expression level in keloid fibroblasts (P <0.05).

Effect of FRAT1 expression inhibition on proliferation and apoptosis of keloid fibroblasts

Detection results in Table 5 and Figure 5 show that compared with the si-NC group, FRAT1 expression inhibition significantly reduces FRAT1 protein expression, cell viability at 24 h, 48 h, and 72 h, CyclinD1 and Bcl-2 protein expression level in keloid fibroblasts, thus significantly increasing apoptosis rate, p21 and Bax protein levels of the cells (P<0.05).

FRAT1 overexpression reverses the effect of luteolin (60 μmol/L) on proliferation and apoptosis of keloid fibroblasts

Detection results in Table 6 and Figure 6 reveal that compared with the control group, luteolin (60 μmol/L) group significantly affects FRAT1 protein expression, cell viability at 24 h, 48 h and 72 h, apoptosis rate, CyclinD1, p21, Bcl-2 and Bax protein levels in keloid fibroblasts (P <0.05). The results are the same as 2.1, 2.2 and 2.4. Compared with luteolin + pcDNA group, luteolin (60 μmol/L) + pcDNA-FRAT1 group have significantly increased FRAT1 protein expression, cell activity at 24 h, 48 h, and 72 h, CyclinD1 and Bcl-2 pro-

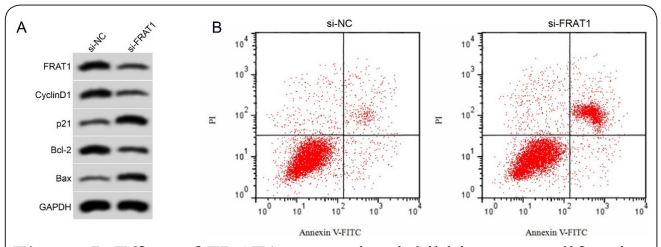

Figure 5. Effect of FRAT1 expression inhibition on proliferation and apoptosis of keloid fibroblast. A: FRAT1 and proliferation and apoptosis-related protein expression; B: flow cytometry of apoptosis .

Table 5. Effect of FRAT1 expression inhibition on the proliferation and apoptosis of keloid fibroblasts (x±s, n=9).

Groups	FRAT1 protein	OD490nm			Apoptosis rate (%)	CyclinD1 protein	p21protein	Bcl-2 protein	Bax protein
		24h	48h	72h					
si-NC	0.66±0.06	0.43±0.04	0.82±0.08	1.12±0.09	7.25±0.71	0.63±0.06	0.33±0.03	0.73±0.07	0.23±0.03
si-FRAT1	0.32±0.03*	0.36±0.03*	0.51±0.05*	0.69±0.06*	21.36±2.11*	0.29±0.03*	0.71±0.07*	0.36±0.03*	0.58±0.05*
t	15.205	4.200	9.858	11.926	19.014	15.205	14.969	14.575	18.007
P	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Note: compared with si-NC group, *P<0.05.

Table 6. FRAT1 overexpression reverses the effect of luteolin on proliferation and apoptosis of keloid fibroblasts (x±s, n=9).

Groups	FRAT1 protein	OD490nm			Apoptosis rate (%)	CyclinD1 protein	p21protein	Bcl-2 protein	Bax protein
		24h	48h	72h					
Con	0.69±0.06	0.45±0.04	0.86±0.07	1.17±0.11	8.21±0.81	0.64±0.05	0.32±0.03	0.72±0.06	0.22±0.02
luteolin	0.28±0.03*	0.35±0.03*	0.48±0.04*	0.62±0.06*	27.36±2.71*	0.25±0.03*	0.74±0.07*	0.31±0.03*	0.60±0.06*
luteolin+ pcDNA	0.27±0.03	0.34±0.03	0.45±0.04	0.57±0.05	29.58±2.84	0.23±0.03	0.76±0.06	0.29±0.03	0.62±0.05
luteolin+ pcDNA-FRAT1	0.58±0.05#	0.42±0.03#	0.75±0.06#	0.93±0.08#	12.43±1.24#	0.53±0.04#	0.41±0.04#	0.61±0.05#	0.34±0.03#
F	206.127	24.000	125.231	115.646	232.313	254.797	166.336	211.861	188.595
P	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Note: compared with Con group, *P<0.05; compared with luteolin+pcDNA group, #P<0.05.

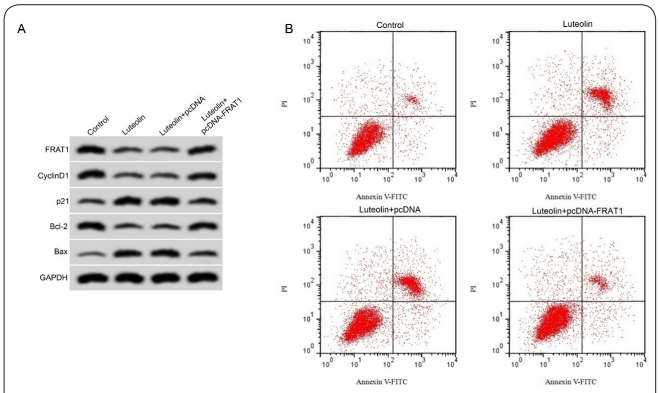

Figure 4. FRAT1 overexpression reverses the effect of luteolin on proliferation and apoptosis of keloid fibroblasts. A: FRAT1 and proliferation and apoptosis-related protein expression; B: flow cytometry of apoptosis.

tein expression in Keloid fibroblasts, while cell apoptosis rate, p21 and Bax protein levels are significantly reduced (P<0.05).

Discussion

Keloids are benign skin proliferative tumors, which are the result of the abnormal wound healing process after skin injury (12). As a common disease in dermatology and plastic surgery practice, keloids are characterized by pathologically excessive skin fibrosis, accumulation of extracellular matrix and infiltration of inflammatory cells (13). A number of studies have shown that proliferation imbalance and insufficient apoptosis of fibroblast- the main component of keloids, greatly accelerates the development of keloids (14). This study describes the effect of luteolin on keloid fibroblasts under FRAT1 gene regulation, finding that luteolin can inhibit the proliferation activity of keloid fibroblasts and promote apoptosis in a mechanism closely related with regulation of FRAT1 gene expression, which will provide a way to develop new keloid treatment strategies.

Luteolin is a natural flavonoid with strong antioxidant properties, which has anti-cancer effects on a variety of malignancies, such as bladder cancer (15), pancreatic cancer (16), colon cancer (17) and squamous cell carcinoma (18). According to reports, luteolin reduces cell viability by inducing p21 in hepatocellular carcinoma Hep3B cells and induces apoptosis in Hep3B cells in a non-p53 manner (19). In vitro, luteolin significantly inhibits the proliferation, migration and invasion of human melanoma cells A375 in a concentration-dependent manner, and induces apoptosis of A375 cells. In vivo, luteolin significantly inhibits tumor growth of A375 cells in the xenograft mouse model (20). Luteolin effectively inhibits the proliferation of breast cancer cell line MDA-MB-231 in a dose-dependent manner. In addition, luteolin was found to increase the apoptosis rate of breast cancer cells (21). Nevertheless, the function and mechanism of luteolin in keloids remain unknown. This experiment found that luteolin significantly reduced keloid fibroblasts activity, CyclinD1 and the protein level of anti-apoptotic protein Bcl-2, while cell apoptosis rate, protein expression of p21 and pro-apoptotic protein Bax were significantly increased, indicating that luteolin inhibits proliferation of keloid fibroblasts on the one hand, and promotes cell apoptosis, on the other hand, thereby slowing the growth of keloid fibroblasts and inhibiting keloids, which is consistent with its anti-tumor function.

In this study, it was detected that compared with normal skin fibroblasts, keloid fibroblasts had significantly up-regulated FRAT1 mRNA and protein expression, suggesting that FRAT1 gene may promote keloid progress, which is similar to previous reports (8). FRAT1 is a proto-oncogene that has been verified to be highly expressed in a variety of tumors in a way associated with tumorigenesis (22). For example, in colorectal cancer tissues, FRAT1 has significantly higher expression compared to adjacent tissues, whose expression is significantly positively correlated with the degree of tumor malignancy. After the shRNA transfection of colorectal cancer HT-29 cells to reduce FRAT1 expression, HT-29 cells showed reduced proliferation and migration (23). FRAT1 is highly expressed in non-small cell lung cancer tissues and cells, while down-regulation of FRAT1 inhibits the proliferation of non-small cell lung cancer cells, promotes apoptosis and hinders its migration and invasion (24). FRAT1 is overexpressed in prostate cancer, which may activate Wnt/β-catenin signaling pathway and promote cell growth by blocking glycogen synthase kinase 3β (GSK3β) -mediated inhibition of β-catenin/TCF (T-cell factor) viability, thus showing oncogenic properties in prostate cancer (25). However, the role of FRAT1 gene in keloids remains unknown. The results of functional experiments in this study showed that inhibition of FRAT1 expression significantly reduced cell viability of keloid fibroblasts and the expression of CyclinD1 and Bcl-2 proteins, while significantly increasing the cell apoptosis rate, p21 and Bax protein levels, indicating that FRAT1 promotes the formation of keloids. In addition, luteolin at different concentrations significantly reduced the protein level of FRAT1 in keloid fibroblasts. It is speculated that luteolin's inhibition of keloid occurrence and development is related to the regulation of FRAT1 gene expression. Further experimental results showed that FRAT1 overexpression reversed the inhibitory effect of luteolin on keloid fibroblast activity, FRAT1, CyclinD1 and Bcl-2 protein expression, and reversed the promoting effect of luteolin on keloid fibroblast apoptosis, p21 and Bax protein expressions. These results indicate that luteolin inhibits keloid fibroblast proliferation and induces its apoptosis by down-regulating FRAT1 expression in keloid fibroblasts. Using genome editing techniques can overcome many biological problems (26).

In summary, luteolin at 20, 40 and 60 μmol/L can effectively inhibit the proliferation of keloid fibroblasts and promote its apoptosis. Moreover, luteolin's mechanism of slowing the occurrence and development of keloids is related to the regulation of FRAT1 gene expression, which confirms the potential of luteolin in treating keloids.

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