FULL LENGTH PAPER

STAT3 expression in patients with fragility fractures and its effect on the biological function of osteoblasts

Guojing Sun · Jingwei Lu · Zhenfang Wu · Xian Zhang · Yunfan Ti

Abstract To determine the expression of signal transducer and activator of transcription 3 (STAT3) in patients with fragility fractures (FFs) and its effect on the biological function of osteoblasts. The study included 32 patients with FFs who were diagnosed and treated in the research group and 30 concurrent healthy individuals in the control group. We observed STAT3 mRNA expression in the patients with FFs and controls and altered STAT3 mRNA to detect changes in the proliferation, invasion, and apoptosis of osteoblasts. The patients with FFs presented higher serum STAT3 mRNA expression than the controls ($P < 0.05$). We plotted receiver operating characteristic curves based on the STAT3 mRNA expression and found that the area under the curve for STAT3 mRNA was 0.856 ($P < 0.05$). Transfection of STAT3 mRNA mimics resulted in increased STAT3 mRNA expression, inhibited cell proliferation as detected by an MTT assay, and increased apoptosis rate, which was determined using flow cytometry with human fetal osteoblastic cell line 1.19 cells. STAT3 mRNA expression was elevated in the serum of patients with FFs and can be used as a biomarker for the diagnosis of the disease. Regulating STAT3 mRNA can inhibit the proliferation and induce the osteoblasts apoptosis.

Keywords Biological function · Fragility fracture · Osteoblast · Signal transducer and activator of transcription 3

Guojing Sun and Jingwei Lu contributed equally to this work and should be considered as equal first coauthors.

G. Sun · J. Lu · Z. Wu · X. Zhang · Y. Ti (✉)
Department of Orthopaedics, Jinling Hospital, Medicine College, Nanjing University, Nanjing 210002, Jiangsu, China
e-mail: yft1018@163.com

G. Sun
e-mail: sugine@126.com

J. Lu
e-mail: menghuan5642@163.com

Z. Wu
e-mail: zhenfang.wu@foxmail.com

X. Zhang
e-mail: 1874765347@qq.com

Introduction

A fragility fracture (FF) occurs in the absence of trauma and is mostly caused by osteoporosis, thereby frequently affecting elderly patients (Posen et al. 2013; World Health Organization 1998). Osteoporosis decreases bone mass and density; thus, fractures are more likely to occur in everyday life or during minor trauma (Haentjens et al. 2003). Compared with healthy individuals, patients with osteoporosis are at increased risk of fractures, which adversely affects public and individual health (Kanis et al. 2019). Therefore, it is essential to clarify the pathogenesis of FFs and find potential therapeutic targets.

MicroRNA (miR) is a highly conserved endogenous short-chain non-coding RNA that can regulate approximately one third of human genes (Li et al. 2016). Studies on miR function have revealed that they have obvious abnormal expression in various orthopedic diseases and can participate in disease progression. For example, miR-495 is upregulated in osteoarthritis and promotes the development of osteoarthritis by modulating AKT1 (Zhao et al. 2019), whereas miR-106b can inhibit osteoblast differentiation and bone formation by negatively regulating the expression of bone morphogenetic protein-2 (Liu et al. 2017). In addition, miR-142-5p promotes fracture healing in aged mice via the stimulation of osteoblast activity (Tu et al. 2017). Studies have reported the abnormal expression of signal transducer and activator of transcription 3 (STAT3) in osteosarcoma (Li et al. 2019); however, no studies have reported STAT3 expression in FFs. Osteoblasts are the primary cell type in bone formation and crucially contribute to the metabolic balance, growth, and injury repair of bone tissue (Li et al. 2015). Studies have found that miR can interfere with the biological behavior of osteoblasts by regulating the expression of their target genes, thereby participating in the pathogenesis of FFs (Yan et al. 2018; Zhang et al. 2017). We hypothesized that STAT3 could be similarly involved in the pathogenesis of FFs. Therefore, we explored the role and potential mechanisms of STAT3 in FFs by conducting a series of experiments, which might contribute to a better understanding of FF pathogenesis.

Materials and methods

General information

Totally, 32 patients with FFs who were diagnosed and treated in our hospital from March 2018 to November 2019 were included in the research group (RG) (21 men and 11 women; mean age, 50.8 ± 10.6 years). We also included 30 healthy individuals as the control group (CG) who underwent physical examinations at our hospital during the same period (18 men and 12 women; mean age, 51.2 ± 10.3 years). The study was approved by the Medical Ethics Committee of Jinling Hospital, Medicine College, Nanjing University.

Inclusion and exclusion criteria

Inclusion criteria: With complete case data and an informed consent form signed by the patient or their immediate family, all enrolled patients underwent treatment in our hospital after the diagnosis and cooperated with the hospital's medical staff.

Exclusion criteria: We excluded patients with vital organ damage, other cardiovascular and/or cerebrovascular diseases, physical disabilities, other autoimmune diseases, mental diseases, speech/language disorders, or any disease that might affect the study results. We also excluded referred patients and those with surgical contraindications.

Cell sources, reagents, and instruments

The following materials were used: human fetal osteoblastic cell line (hFOB) 1.19 cells (Beijing BeNa Culture Collection, Beijing, China, Resource No. BNCC344663); Lipofectamine™ 2000 (Shanghai Mituo Biotechnology Co., Ltd., Shanghai, China, Cat. No. 11668019); MMT kit and dimethyl sulfoxide reagent (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China, Cat. No. S30860, S24295); Roswell Park Memorial Institute-1640 (RPMI-1640; Shanghai Yanjin Biotechnology Co., Ltd., Shanghai, China, Cat. No. 31800-022) medium; phosphate buffer saline and fetal bovine serum (Shanghai Hengfei Biotechnology Co., Ltd., Shanghai, China, Cat. No. P1000, SA133); penicillin–streptomycin dual antibiotic solution (Beijing Biolab Technology Co., Ltd., Beijing, China, Cat. No. MT0104-SEY); TransScript II Green Two-Step qRT-PCR SuperMix (Beijing TransGen Biotech, Beijing, China, AQ301-01); Annexin V/PI apoptosis detection kit (Shanghai Hengfei Biotechnology Co., Ltd., Shanghai, China, Cat. No. CA1020); microplate reader (Beijing Image Trading Co., Ltd., Beijing, China, Cat. No. 21261000); polymerase chain reaction (PCR) instrument (Wuxi Microsep Biotechnology Co., Ltd., Wuxi, China, Cat. No. TC9639); and flow cytometer (Beijing Image Trading Co., Ltd., Beijing, China, Cat. No. AMG0002051). The primer sequence of STAT3 mRNA was designed and synthesized by Shanghai Sangon Bio-tech Co., Ltd. (Table 1).

Table 1 Primer sequence

	Upstream sequence	Downstream sequence
U6	5′-TCTCTGCTCCTCGTTCGA-3′	5′-GCGCCCATACGACCAAATC-3′
STAT3 mRNA	5′-TATCATGCCGATGAAGCATG-3′	5′-GTGGCATACCACCAGTGTTG-3′

Cell culture and transfection

We transferred the hFOB 1.19 cellsto RPMI-1640 medium (penicillin–streptomycin dual antibiotic solution, 10% fetal bovine serum) and cultured in an incubator at 37 °C with 5% carbon dioxide. Subsequently, we transfected the cells with STAT3 mRNA mimics and STAT3 mRNA inhibitor using the Lipofectamine™ 2000 kit strictly. All primers were transfected into the cells with the greatest difference in expression.

Quantitative reverse transcriptase (qRT)-PCR detection

Following the manufacturer's protocol, total RNA was extracted from the collected cells and serum using the TRIzol kit. We determined the purity, concentration, and integrity of the extracted total RNA using an ultraviolet spectrophotometer and agarose gel electrophoresis. We used the 5X TransScript ®II All-in-One SuperMix, TransScript ®miRNA RT Enzyme Mix, and $2\times$TS miRNA Reaction Mix kits for reverse transcription strictly according to the manufacturer's instructions. Next, we performed PCR amplification using a PCR reaction system consisting of 1 μL of cDNA, upstream and downstream primers (0.4 μL each), 10 μL of 2X TransScript® Tip Green qPCR SuperMix, 200 μL of passive reference dye (50×), and ultra-pure sterile water for a final volume of 20 μL. The PCR reaction conditions were as follows: 45 cycles comprising pre-denaturation at 94 °C for 30 s, denaturation at 94 °C for 5 s, and annealing and extension at 60 °C for 30 s. Three replicate wells were used for each sample, and the experiment was performed in triplicates. In this experiment, U6 was used as the internal reference and the delta-delta Ct method was used to analyze the data.

Detection of cell proliferation

We harvested the cells 24 h after transfection, adjusted the concentration to 3×10^4 cells/well, and inoculated the cells in 96-well plates for incubation at 37 °C for 24, 48, 72, and 96 h. We then added 20 μL of MTT solution (5 μg/mL) to each well at each of the aforementioned timepoints and incubated the culture at 37 °C for 4 h before adding 150 μL of dimethyl sulfoxide. We measured the optical density of the cells in each group using a microplate reader at 450 nm.

Detection of apoptosis

After 24 h of transfection, the cells were fully digested using 0.25% trypsin, washed twice with phosphate buffer saline, and added to 100 μL of binding buffer to prepare a suspension of 1×10^6 cells/mL. Next, we added AnnexinV-FITC and PI and incubated the cells for 5 min at room temperature in the dark. We detected apoptosis using the FC500MCL flow cytometry system and repeated the experiment 3 times to obtain the mean value.

Outcome measures

The primary outcome measures were STAT3 mRNA expression in the patients with FFs and healthy controls. We altered the STAT3 mRNA expression to detect the changes in proliferation ability, invasion ability, and apoptosis rate of osteoblasts. The secondary outcome was the diagnostic value of STAT3 mRNA in patients with FFs.

Statistical methods

We analyzed the collected data using SPSS 20.0 and visualized the results using GraphPad 7. We used the Kolmogorov–Smirnov test to analyze the dose data distribution, in which the normal distribution data were expressed as mean and standard deviation (mean ± SD). We used the independent sample and paired t-tests for intergroup and intragroup comparisons, respectively. The counting data are presented as percentages and were analyzed using the chi-squared test. We plotted the diagnostic value of STAT3 mRNA in FFs using a receiver operating characteristic curve

and the 5-year patient survival using a Kaplan–Meier survival curve, using the log-rank test for analysis. A p-value of < 0.05 was considered significant.

Results

Clinical data

There were no significant differences between the RG and CG with regard to age, sex, body mass index, marital status, ethnicity, residence, smoking, drinking history, and exercise, suggesting comparability ($P > 0.05$) (Table 2).

STAT3 mRNA expression in RG and CG

We used qRT-PCR to detect STAT3 mRNA in the serum of the RG and CG and found that STAT3 mRNA expression in the RG (4.32 ± 1.45) was higher than that in the CG (1.02 ± 0.34) ($P < 0.05$).

Diagnostic value of STAT3 mRNA in patients with FFs

The ROC curve revealed an area under the curve for STAT3 mRNA of 0.856 for diagnosing patients with FFs ($P < 0.05$) (Table 3, Fig. 1).

Effect of STAT3 mRNA on the biological function of osteoblasts

The transfection of STAT3 mRNA mimics increased STAT3 mRNA expression, inhibited cell proliferation, and increased the apoptosis rate in the hFOB 1.19 cells (Fig. 2).

Discussion

FF is the primary cause of high morbidity and mortality among older adults (Burge et al. 2007). Given the relationship between FF and aging, the number of patients with FFs is increasing with the increasing age

Table 2 Basic clinical data [n (%)]

Clinical data	Research group (n=32)	Control group (n=30)	χ^2 or t	P
Age	50.8 ± 10.6	51.2 ± 10.3	0.151	0.881
Sex			0.209	0.647
Male	21 (65.63)	18 (60.00)		
Female	11 (34.38)	12 (40.00)		
BMI (kg/m^2)	22.26 ± 0.37	22.21 ± 0.25	0.618	0.538
Marital status			0.242	0.623
Married	29 (90.63)	26 (86.67)		
Single	3 (9.38)	4 (13.33)		
Ethnicity			0.011	0.915
Han	22 (68.75)	21 (70.00)		
Ethnic minorities	10 (31.25)	9 (30.00)		
Residence			0.501	0.479
City	18 (56.25)	19 (63.33)		
Rural	14 (43.75)	11 (36.67)		
History of smoking			2.605	0.107
Yes	30 (93.75)	24 (80.00)		
No	2 (6.25)	6 (20.00)		
History of drinking			1.245	0.265
Yes	28 (87.50)	23 (76.67)		
No	4 (12.50)	7 (23.33)		
Exercise habits			0.047	0.829
Yes	13 (40.63)	13 (43.33)		
No	19 (59.38)	17 (56.67)		

Table 3 ROC curves

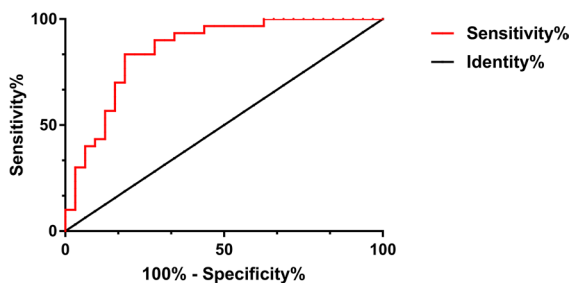|	STAT3 mRNA
AUC	0.856
SE	0.049
95% CI	0.761–0.952
P	0.001
Cut-off	4.041
Sensitivity (%)	81.25
Specificity (%)	83.33

Fig. 1 Diagnostic value of STAT3 mRNA. When the cut-off value was 4.041, the sensitivity and specificity of STAT3 mRNA were 81.25% and 83.33%, respectively

of the population, imposing a serious burden on individuals, families, and society (Varahra et al. 2018). Survivors of the first episode of FF are a well-defined high-risk group and are at significantly greater risk of experiencing a second fracture, with the highest incidence within the first 6 months of the first fracture (Munson et al. 2016). Therefore, the pathological processes of FFs are of great social and economic importance and must be explored.

With the expansion of research in recent years, the focus of investigators worldwide has shifted toward genetics. MiRNA can act as endogenous RNA interference to regulate the expression of target genes and participate in the regulation of various physiological and pathological functions (Vitsios et al. 2017). MiRNA can bind to 3′ UTR via base pairing to regulate gene expression (Rouleau et al. 2017) and shows significant expression profiles between different tissues and growth stages, indicating that miRNA has different spatial–temporal expression patterns (Li et al. 2017). Approximately 22 nucleotides in length (Takeda et al. 2015), miRNA can regulate posttranscriptional gene

expression by inducing mRNA degradation and inhibiting translation, degrade the target gene, and inhibit the translation of the target gene, thereby completing the posttranscriptional gene silencing (Oxnard et al. 2016). Studies have shown that miRNA can be used as a negative regulator of gene expression to regulate a series of biological functions, thus playing an essential role in various biological processes (Ganju et al. 2017; Rupaimoole et al. 2016).

In this study, we first detected STAT3 mRNA expression in patients using qRT-PCR and found that it was significantly higher in the RG than in the CG, suggesting that STAT3 mRNA may be involved in the pathogenesis of FFs. Patients with FFs have no obvious clinical manifestations before the occurrence of a fracture with no obvious cause and are therefore prone to missing the optimal treatment period (Yang et al. 2018). Studies have found that abnormal miR expression in serum can be used as a potential marker to diagnose various diseases, including FFs (Lin et al. 2019; Moya et al. 2019; Wang et al. 2019). Therefore, we analyzed the diagnostic value of serum STAT3 mRNA in FFs and found an area under the curve of 0.856 for STAT3 mRNA for diagnosing osteoporosis, indicating that STAT3 mRNA has potential as a biological indicator for diagnosing FFs. The decrease in osteoblast proliferation and increase in apoptosis are important factors in the occurrence and development of FFs (Wang et al. 2019). miR is known to regulate many cellular biological processes, such as proliferation, migration, apoptosis, differentiation, and metabolism (Lin et al. 2019). Therefore, in this study, we analyzed the role of STAT3 mRNA in FFs by observing changes in biological behaviors such as cell proliferation and apoptosis after an increase in STAT3 mRNA expression in osteoblasts. Using the MTT assay and flow cytometry, we found that after an increase in STAT3 mRNA expression in osteoblasts, the ability of the osteoblasts to proliferate was inhibited and the rate of apoptosis increased, indicating that STAT3 mRNA can affect the proliferation and apoptosis of osteoblasts and the occurrence and progression of FFs.

This study confirms the preliminary clinical value of STAT3; however, the study has certain limitations. First, the study did not include in vivo experiments, and second, the patients were not followed-up for the prognosis. Therefore, we aim to
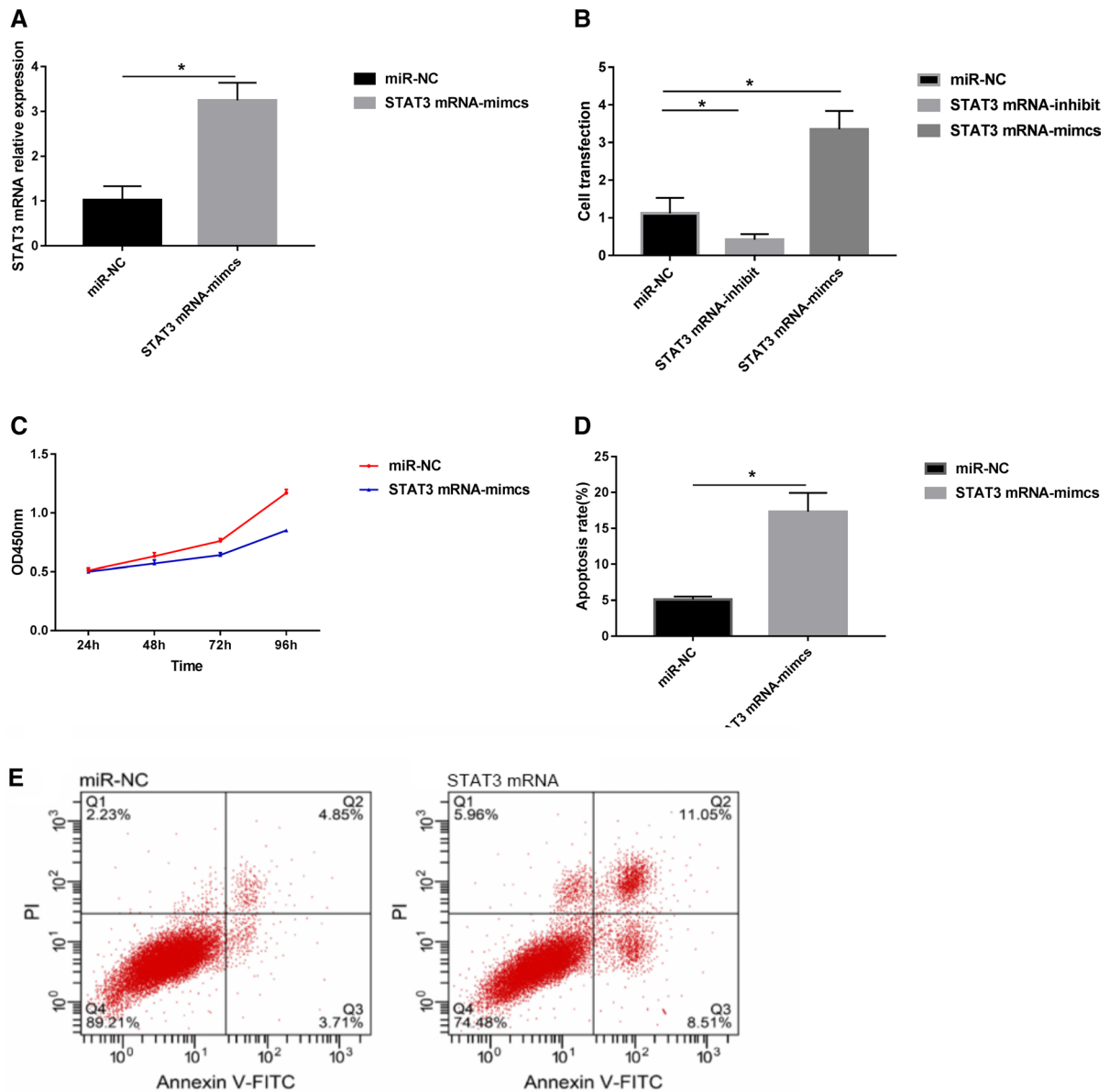
Fig. 2 Effect of elevated STAT3 mRNA expression on the proliferation, apoptosis and differentiation of human fetal osteoblastic cell line 1.19 cells. **A** STAT3 mRNA expression increased in hFOB 1.19 cells after transfection of STAT3 mRNA mimics. **B** STAT3 mRNA expression in hFOB 1.19 cells in the STAT3 mRNA mimics group and STAT3 mRNA inhibitors group was significantly higher than that in the miR-NC group after transfection of STAT3 mRNA mimics. **C** hFOB 1.19 cell proliferation was inhibited after transfection of STAT3 mRNA mimics. **D** The hFOB 1.19 cell apoptosis rate increased after transfection of STAT3 mRNA mimics. **E** Apoptosis diagram

support our research results by conducting in vivo experiments and prognostic follow-ups in future studies.

In conclusion, STAT3 mRNA expression was elevated in the serum of patients with FFs and can be used as a biomarker to diagnose the disease. STAT3 mRNA regulation can inhibit osteoblast proliferation and induce apoptosis.

Author contributions GS, JL and YT conceived and designed the research and interpreted the results of

experiments. GS, JL, ZW, XZ and YT performed experiments, analyzed data. GS and JL wrote the manuscript.

Availability of data and materials The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval The study was approved by the Medical Ethics Committee of Jinling Hospital, Medicine College, Nanjing University.

Consent to participate All patients and their families agreed to participate in the experiment and signed the informed consent form.

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