Original Article

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Tenascin C affects mineralization of SaOS2 osteoblast-like cells through matrix vesicles

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Summary **Tenascin C (TNC) is an extracellular matrix glycoprotein involved in osteogenesis and bone mineralization. In a previous study, we identified TNC protein located in the matrix vesicles (MVs) of osteoblasts. MVs are determinant in the mineralization formation. Therefore, we hypothesize whether TNC can modulate osteoblast mineralization *via* MVs. In this study, we demonstrated that the expression level of TNC was increased with osteoblast differentiation of osteoblast-like SaOS2 cells, and down-regulation of TNC expression by siRNA could significantly inhibit SaOS2 differentiation toward osteoblasts and mineralization as evidenced by decreases in ALP activity, mineralized nodule formation, calcium deposition, and down-regulation of osteogenic marker genes *ALP*, and *COL1A1*. Furthermore, we validated that TNC located in the MVs of mineralized SaOS2 cells, and that down-regulation of TNC could decrease MVs mineralization ability *in vitro*, and the decrease of MVs mineralization ability was not associated with annexins. In conclusion, in this study, we extended the role of TNC during osteogenesis previous progresses, and that supported TNC as an important functional MVs component in modulating osteoblast mineralization.**

Keywords: Matrix vesicles, tenascin, mineralization

1. Introduction

Elucidation of novel cellular and molecular mechanisms in moderating osteoblast mineralization is of great significance in osteogenesis mechanism research and bone tissue engineering. During the process of osteoblast mineralization, matrix vesicles (MVs) function as an initial or primary nucleation site that may be the prerequisite to subsequent secondary mineralization (*1,2*). Aberrant function of MVs have been observed in multiple mineralization-defective diseases (*3*). MVs are highly enriched in proteins and lipids (*4*). Until now, about 2,000 proteins have been identified to be located in MVs. However, only a small set of MVs proteins such as annexins, had been demonstrated to be involved in osteoblast differentiation. The roles of most of the MVs proteins during mineralization remain unclear. Therefore, we hypothesized that these MVs-enriched proteins could be specific sources to identify novel regulators for the physiological and pathological mineralization process.

Tenascin C (TNC) is an extracellular matrix glycoprotein synthesized by osteoblasts during bone growth and morphogenesis (*5,6*). It has been established that TNC was implicated in osteoblastic differentiation and mineralization within bone. In the mechanism, TNC may act as a mediator of TGF-β-induced new bone formation (*7,8*). Additionally, bone morphogenetic protein (BMP) and Wnt growth factors, or mechanical loading and stress can also increase TNC expression in osteoblasts, but *via* distinct signaling pathways (*9,10*). However, little is known about the role of TNC in

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the regulation of osteogenesis and mineralization. In a previous study, we identified TNC as a novel MVs protein, and raised our speculation that TNC might influence the mineralization process during osteoblast differentiation.

Therefore, in this study, we investigated the effect of TNC on mineralization formation and MVs activity in an osteosarcoma-derived osteoblast-like cell line SaOS2. Our findings suggest that endogenous TNC helps to maintain the mineralization activity and MVs function in SaOS2 cells as a novel MVs protein.

2. Materials and Methods

2.1. *Cell culture and osteoblast differentiation induction*

Human osteosarcoma-derived osteoblast-like cell line SaOS2 was acquired from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and was routinely maintained in McCoy's 5A medium (Gibco, Carlsbad, CA, USA), supplemented with 15% fetal bovine serum (Gibco, Carlsbad, CA, USA), 50 U/mL penicillin, and 50 μg/mL streptomycin (both from Invitrogen, Carlsbad, CA, USA) at 37°C under 5% CO_2 in a humidified atmosphere. To induce osteoblast differentiation, SaOS2 cells were treated with 10 mM β-glycerophosphate (β-GP) and 50 μg/mL L-ascorbic acid-2-phosphate as described previously (*9*).

2.2. *siRNA transfection*

TNC targeted siRNA and negative control siRNA were purchased from Guangzhou RiboBio Co., LTD (RiboBio Co., Ltd., Guangzhou, China). The target gene and siRNA sequences are shown in Table 1. Fifty nM of TNC siRNA or negative control siRNA were transfected using Ribo FECTTM CP Transfection Kit (RiboBio Co., Ltd., Guangzhou, China) according to the manufacturer's protocol. After 3 days of transfection, cells were collected and subjected to the subsequent analysis.

2.3. *Isolation of MVs*

MVs were harvested from SaOS2 cells with and without osteoblast induction, and also from cells with siRNA transfection and osteoblast induction. MVs were isolated using a strategy as described previously (*11,12*). Briefly, SaOS2 cells were digested with collagenase Type IA (1 mg/mL, Sigma, St. Louis, MO, USA) at 37°C for 3 h. Cells were then pelleted by centrifugation at 3,000 g for 30 min. The supernatant was concentrated by ultrafiltration using a 100 KD Amicon Ultra filter (Millipore Corporation, Billerica, MA, USA), and then mixed with ExoQuick precipitation reagent (System Biosciences, Mountain View, CA, USA). After incubation overnight, the mixture was centrifuged at 1,500 g for 30 min to collect the MVs precipitation.

2.4. *Alkaline phosphatase (ALP) activity, AlizarinRed staining, and measurement of matrix-deposited calcium*

After treatment with siRNA transfection and osteoblast induction, ALP expression was assayed by means of an ALP activity staining kit (GenMed Scientifics Inc., USA), and the activity was measured as described previously (*13*). Briefly, lysis buffer (25 mM Tris-HCL, 0.5% TritonX-100) was added to precipitated SaOS2 cells and MVs to release ALP at 4°C for 1 h respectively, and then after incubating with *p*-nitrophenyl phosphate (*p*-NPP) substrate at 37°C for 20 min, the absorbance of the mixture at 405 nm was measured using a micro plate reader.

After 6 days of osteoblast differentiation, cells were fixed with ice-cold 70% ethanol and stained with Alizarin Red to detect mineralization using a staining kit according to manufacturer's instruction (GenMed Scientifics Inc., USA) (*14*).

Matrix-deposited calcium was quantitatively measured as described previously (*15*). Briefly, SaOS2 cells were decalcified with 0.6 M HCl at 37°C for 12 h. The calcium content of supernatants was determined using a Calcium Colorimetric Assay Kit (Bioassay Systems, Carlsbad, CA, USA), and then the absorbance of the mixture was measured at 560 nm. After decalcification, cells were solubilized with 0.1 M NaOH/0.1% SDS at 4°C for 1 h. The protein content was measured with a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA), and the calcium content of the cell layer was normalized by protein content.

2.5. *Quantitative real-time polymerase chain reaction (RT-qPCR)*

Total RNA was extracted using Trizol reagent (Gibco, Carlsbad, CA, USA) and the purified total RNA was used for cDNA synthesis with a first-strand cDNA synthesis kit (Toyobo, Osaka, Japan). After the reverse transcription reaction, cDNA was used as the template for RT-qPCR of *ALP*, *COL1A1*, and *TNC*. RT-qPCR was

Table 1. The target sequences and the sequences of siRNA

Items	siRNA-1	siRNA-2
Target 5'→3'	CCTGGCCTATAAGCACTTT	CCAGTTTGCTGAGATGAAA
Forward 5'→3'	CCUGGCCUAUAAGCACUUUdTdT	CCAGUUUGCUGAGAUGAAAdTdT
Reverse 3'→5'	dTdTGGACCGGAUAUUCGUGAAA	dTdTGGUCAAACGACUCUACUUU

Table 2. Quantitative real-time PCR primer sequences

Gene	Sequences5'-3'
GAPDH	
forward	GCA CCG TCA AGG CTG AGA AC
reverse	ATG GTG GTG AAG ACG CCA GT
ALP	
forward	CCGTGGCAACTCTATCTTGG
reverse	GCC ATA CAG GAT GGC AGT GA
COL1A1	
forward	CCC TGG AAA GAA TGG AGA TGAT
reverse	ACT GAA ACC TCT GTG TCC CTT CA
TNC	
forward	GTG CAG AAC TCT CCT GTC CAA AT
reverse	ATC TTT GCT CCT TGC AGT CTT TG

carried out using a SYBR Green qPCR Kit (Toyobo, Osaka, Japan) by LightCycler 480 thermocycler (Roche Applied Science, Mannheim, Germany). The primer sequences of target genes that were used in this study are shown in Table 2.

2.6. *Western-Blot*

From each sample, 40 μg aliquots of total protein was subjected to 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to a polyvinylidene fluoride (PVDF) membrane. After 2 h of blocking with 5% low fat milk in TBST (10 mM Tris, 100 mM NaCl, and 0.05% Tween-20), the membrane was incubated overnight at 4°C with specific antibodies against TNC (1:500, Cell Signaling Technology, Danvers, MA, USA), annexin 2 (AnxA2, 1:500, Proteintech Group, Wuhan, China), annexin 5 (AnxA5, 1:500, Proteintech Group, Wuhan, China), annexin 6 (AnxA6, 1:500, Proteintech Group, Wuhan, China) and GAPDH (1:1,000, Santa Cruz Biotechnology, Carlsbad, CA, USA).

2.7. *MV-collagen calcification assay*

MVs-collagen calcification assay was used to evaluate the mineralization induction activity of MVs as described previously by Chen *et al*. with minor modifications *in vitro* (*16,17*). Briefly, isolated MVs were re-suspended in TBS (pH = 7.6) with 0.25 M sucrose and then added to type I collagen-coated glass cover slips, and incubated for 12 h. Then calcification media (DMEM medium with 15% FBS and 10 mM β-glycerophosphate) were added, and further incubated at 37°C for 72 h. To determine the magnitude of calcification, the MVs-collagen complex was incubated with 0.6 M HCl for 12 h, and the calcium content of HCL supernatants was determined as described above.

2.8. *Statistics*

Measurements in each experiment were run in triplicate. For quantitative data, results are reported as the mean ± S.D. To determine the differences between groups, one-way analysis of variance (ANOVA) was carried out using SPSS software (version 17.0), with significance accepted at $p < 0.05$.

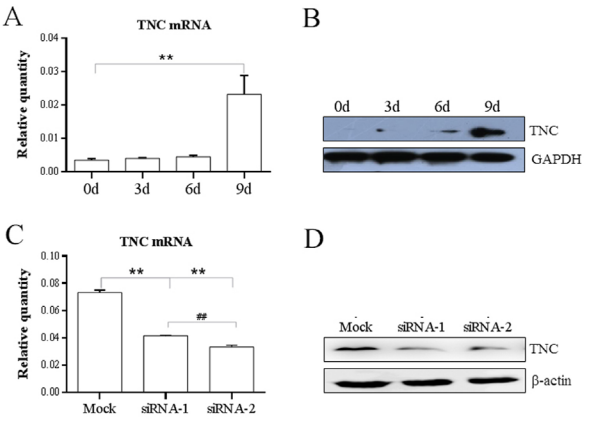

Figure 1. Expression of TNC during SaOS2 cells osteogenesis induction and expression after siRNA transfection. Quantitative real-time PCR analyses of TNC expression in mRNA level **(A)** and Western blotting analyses of TNC expression in protein level **(B)** during SaOS2 cells osteogenesis induction; expression of TNC in mRNA **(C)** and protein **(D)** level after siRNA transfection 3 days and 6 days respectively. Bars are shown as the mean ± S.D., $n = 3$. $^{**}p < 0.01$ *vs*. group 0 d and mock respectively.

3. Results

3.1. *Expression of TNC during SaOS2 cells osteogenesis induction*

First, we observed the expression pattern of TNC during osteogenesis at mRNA and protein levels. As seen in Figures 1A and 1B, TNC expression was elevated during SaOS2 osteoblast differentiation in a time-dependent manner and reached the highest level at 9 days after induction, suggesting that TNC may play an important role at the later mineralization stage.

3.2. *Effect of TNC siRNA on osteoblast differentiation of SaOS2 cells*

After siRNA transfection, the expression of TNC was significantly decreased (60%-70%) at both mRNA and protein levels as measured by RT-qPCR and Western-blot analyses (Figures 1C and 1D). We further observed that down-regulation of TNC by siRNA had a significant inhibitory effect on SaOS2 cell osteogenic differentiation, as manifested by down-regulated expression of osteogenic marker genes *COL1A1*, and *ALP*, and decreased ALP activity and matrix mineralization (Figure 2).

3.3. *Effect of TNC siRNA on the function of MVs*

We confirmed that TNC protein located in the

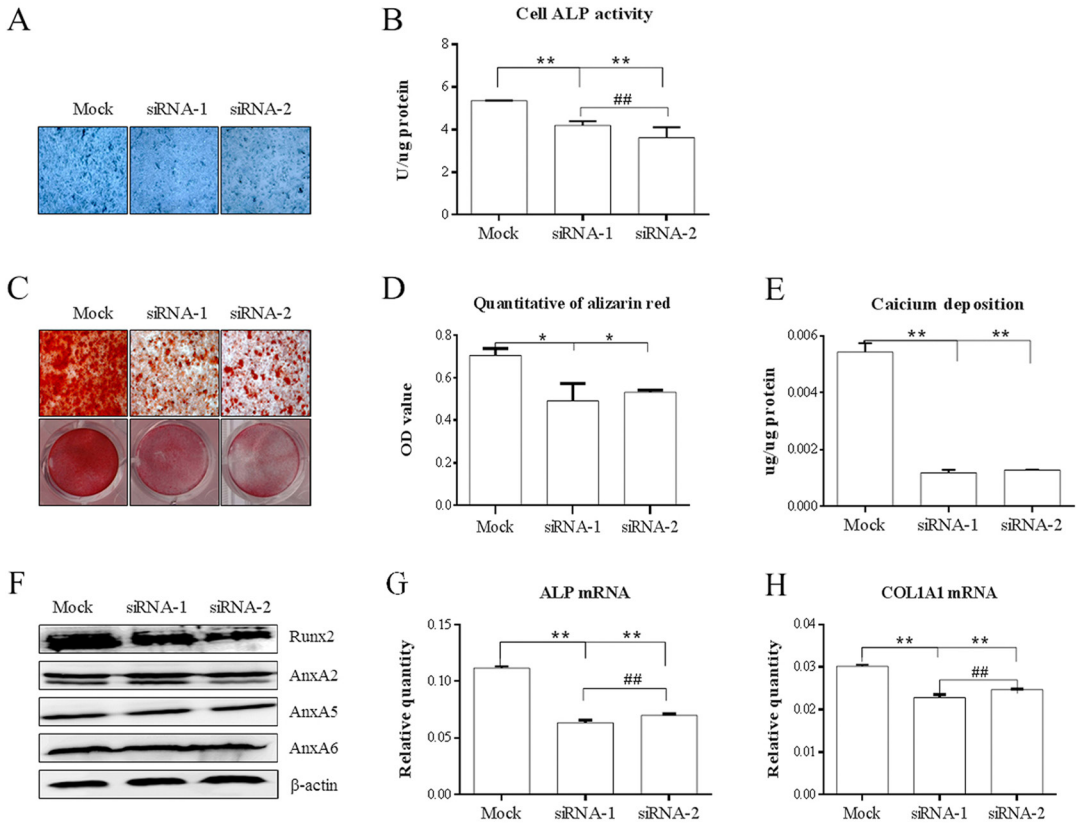

Figure 2. Effect of TNC siRNA on osteoblast differentiation of SaOS2 cells. ALP activity was assayed by means of an ALP expression staining kit **(A)** and the activity was measured by *p*-NPP **(B)**; the calcium deposits in the mineralized matrix were analyzed by Alizarin Red staining **(C)** which was quantified at 562 nm **(D)** and Calcium Assay Kit **(E)**; Western blot analyses of expression of Runx2, AnxA2, AnxA5, and AnxA6 in siRNA transfected and osteoplastic inducted SaOS2 cells **(F)**; quantitative real-time PCR analyses of expression of osteoplastic marker genes: *ALP* **(G)**, *COL1A1* **(H)**. Bars are shown as the mean ± S.D., $n = 3$. $^{*}p < 0.05$ *vs*. mock, $^{**}p < 0.01$ *vs*. mock, $^{\#\#}p < 0.01$, siRNA-1 *vs*. siRNA-2.

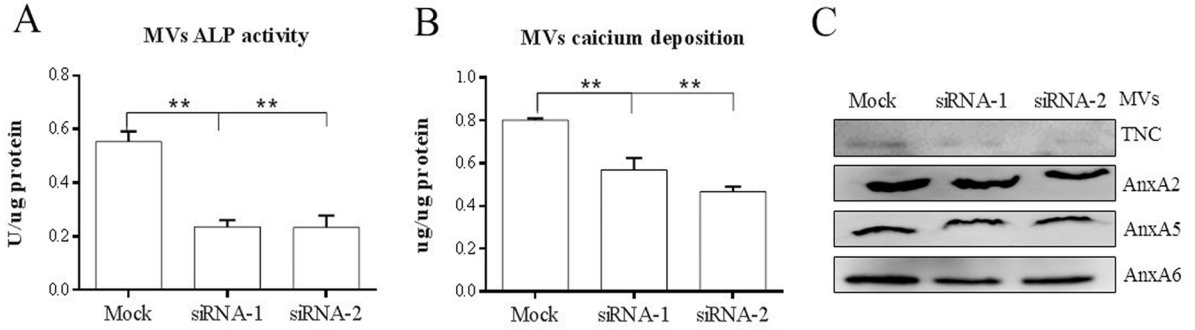

Figure 3. Effect of TNC siRNA on the function of MVs. The activity of MVs was measured by *p*-NPP **(A)**, and the calcium deposits caused by MVs in vitro were analyzed by Calcium Assay Kit **(B)**; Western blot analyses of expression of TNC, AnxA2, AnxA5, and AnxA6 in MVs after siRNA transfection **(C)**. Bars are shown as the mean ± S.D., $n = 3$. $^{**}p < 0.01$ *vs*. mock.

mineralized SaOS2 cell derived MVs, which was consistent with our previous finding (Figure 3C). Furthermore, accompanying the decrease of TNC expression in SaOS2 cells, siRNA also significantly inhibited TNC expression in MVs derived from mineralized SaOS2 cells (Figure 3C). Moreover, we also observed that ALP activity of MVs was markedly inhibited after TNC siRNA transfection (Figure 3A). Meanwhile, MVs induced calcium deposition was significantly decreased by TNC down-regulation (Figure 3B). Therefore, our above findings suggest that TNC expression is necessary for MVs in directly inducing mineralization *in vitro*.

3.4. *Effects of TNC siRNA on annexins expression in SaOS2 cells and MVs*

Annexin proteins are confirmed to be functional MVs components participating in mineralization. Therefore, we next explored whether there was some association

between TNC and annexins. We observed the expression of three annexin members, AnxA2, AnxA5, and AnxA6 in SaOS2 cells and their released MVs after TNC siRNA transfection, and found that TNC siRNA exerted no effect on the expression of three annexins on both cell and MVs levels (Figure 2F, Figure 3C), indicating that the influence of TNC on MVs function seems not to be associated with annexins.

4. Discussion

TNC is an osteoblast-secreted extracellular matrix protein which is able to stimulate osteoblastic differentiation and help to maintain the functional state of cultured osteoblast-like cells including SaOS2 cells (*18,19*). Meaningfully, TNC has been indicated to be associated with chronic kidney disease mineral and bone disorder, suggesting TNC may act as a key mediator and target for osteogenesis, especially for mineralization formation (*20*).

Most of the previous studies focused on the role of TNC on the early events of osteogenesis (*21*). In this present study, we identified that TNC expression level reached a peak at the late mineralization stage. Inhibiting TNC expression can decrease the early marker genes for osteoblast differentiation such as *COL1A1* and *ALP*, which was consistent with previous studies. Moreover, we also observed that TNC down-regulation could significantly inhibit late mineralization as manifested by decreased matrix calcium deposition and lower *COL1A1* gene expression. These findings are helpful to explain the close association between TNC and pathologic ossification, and suggest TNC may play an important role in mediating mineralization.

It has been believed that tenascin is absent from mineralized bone matrix (*22*). Different from the previous study, in this study, we confirmed that TNC was located in the MVs released from the mineralized matrix. Matrix vesicles (MVs) are specific membranous nanovesicles released by cells capable of mineralization such as chondrocytes, osteoblasts or odontoblasts (*23*). It has been shown that MVs play a critical role in modulating mineralization through modulating the local Pi/PPi ratio in the mineralizing matrix or providing initial mineral nucleation sites (*24*). In this study, for the first time, we found that the presence of TNC in MVs is necessary for the normal mineralization function of MVs.

Currently, over 2,000 proteins have been identified in MVs. Among them, annexin family transport proteins, especially, annexin 1, annexin 5, and annexin 6, are key components which have been shown to be linked to the two key functions of MVs in modulating mineralization (*16,25*). However, our results indicate that TNC cannot affect the expression of annexins in both cell and MVs levels. This finding suggests that the role of TNC involving MVs function seems not to be associated with annexins. TNC may participate in the mineralization process directly. Considering a calcium-binding domain existing at the C-terminal end of TNC, we speculate that TNC may regulate the MVs activity to initiate mineralization by combining Ca^{2+} and influx into MVs, but the exact mechanism needs further investigation.

In conclusion, our results indicate that TNC plays an important role during early and late osteoblast differentiation. For the first time we linked the effect of TNC on osteoblast mineralization with its regulatory function on MVs activity. We conclude that TNC is an important and functional component of MVs that might participate in physiological and pathological mineralization.

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