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Runx2 induces bone osteolysis by transcriptional suppression of TSSC1

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ABSTRACT

Advanced breast cancers frequently metastasize to bone, resulting in osteolytic lesions, however, the underlying mechanisms are poorly understood. Runx2, a bone-specific transcriptional factor, is abnormally expressed in highly metastatic breast cancer cells. Here, we found that TSSC1 inhibits breast cancer cell invasion. Subsequently, TSSC1 is confirmed as a target of Runx2 and is negatively regulated by Runx2. Furthermore, overexpression of Runx2 induces bone osteolysis in a TSSC1-dependent manner. Our results may provide a strategy for the treatment of breast cancer and the prevention of skeletal metastasis.

1. Introduction

Breast cancer is the most common cancer affecting women in the world and the frequency of bone metastasis is much higher than that of lung and liver metastases. Breast cancer cells that establish themselves in the bone are thought to modulate the activity of osteogenic cells, resulting in local bone degradation [1]. What causes breast cancer cells to preferentially colonize the bone and influence osteogenic cell activity is a key question in breast cancer research and identification of the genes involved in this metastatic process should provide potential targets for therapeutic intervention.

The Runx transcription factors (Runx1, Runx2, and Runx3) are essential for organogenesis and regulate phenotypic genes through successive cell divisions determining cell cycle progression or exit in progeny cells [2]. Runx2 is a key regulator of bone development that is requisite for the maturation of hypertrophic chondrocytes and osteoblasts [3–5]. Runx2 promotes breast and prostate cancer progression and associated osteolytic lesions in the bone microenvironment, in part through direct transcriptional activation of genes that promote bone degradation, including matrixmetalloproteinase-9 (MMP-9) [3] and Matrixmetalloproteinase-13 [6]. Depletion of Runx2 expression in metastatic breast cancer MDA-MB-231 cells resulted in down-regulation of metastatic genes and reduced the invasive capacity of the cells [7]. Furthermore, Runx2 is highly expressed in MDA-MB-231 breast cancer cells that metastasize to bone and form osteolytic lesions [8]. The known pathways for which there is direct evidence of Runx involvement and which are relevant to the progression of breast and prostate tumor responses and associated metastatic bone disease include TGFβ/BMP [9], Wnt [10–12], PTHrP [9] and Src pathways [13]. In addition, several genes have been identified regulated by Runx2 including, MMP9, MMP13, vascular endothelial growth factor (VEGF) and bone sialoprotein (BSP), osteocalcin (OC) and osteopontin (OP) [14,15]. Thus, the identification of Runx2 target genes can provide insight into the mechanisms by which Runx2 functions in metastatic cancer cells.

In the present study, we identified a novel Runx2 target gene, tumor suppressing subtransferable candidate 1 (TSSC1), which is down-regulation in the bone metastatic breast cancer cell line (MDA-MB-231BO) compared to the primary human breast cancer cell line (MDA-MB-231) by microarray [16]. In addition, we demonstrate that TSSC1 suppresses breast cancer invasion. Furthermore, Runx2 induces bone osteolysis by transcriptional suppression of TSSC1. These findings suggest that the interaction between Runx2 and TSSC1 might represent a viable target for therapeutic intervention to inhibit bone metastasis.

2. Materials and methods

2.1. Cell culture

The MCF10A, MCF7, MDA-MB-435, and MDA-MB-231 cell lines were purchased from American Type Culture Collection (ATCC) and maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Plasmids and transfection

To construct the Runx2 and TSSC1 expression vector, the entire coding sequence of the Runx2 or TSSC1 was amplified by PCR using

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the pfu DNA polymerase (Thermo Scientific, Rockford, IL, USA). For transient transfection, 2×10^5 cells were plated into 6-well plates and kept in antibiotic-free medium for 24 h before transfection. The cells were then transfected with and then transfected with the plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For generation of stable cells, cells were transfected with the plasmid. Two days after transfection, cells were trypsinized, transferred to 10 cm cell culture dishes and selected by complete medium plus 1 mg/ml of G418 (Sigma–Aldrich, St. Louis, MO, USA) for about 2 weeks.

2.3. Reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was extracted with TRIZOL reagent according to the manufacturer's instructions. 5 μg of total RNA was used to perform reverse transcribed by using SuperScript II and oligo dT following the manufacturer's protocol (Invitrogen). The RT-qPCR analysis was performed using the Fast SYBR Green MasterMix System (Invitrogen) according to the manufacturer's protocol. The experiment was performed in triplicate.

2.4. Western blot

A quantity of 50 μg of whole cell lysates per sample was separated by SDS–PAGE using 10% polyacrylamide gels and transferred to PVDF membrane which was subsequently incubated with polyclonal rabbit anti-TSSC1, anti-Runx2 (Santa Cruz Biotech, Santa Cruz, CA, USA) and a second antibody (anti-rabbit IgG, Santa Cruz Biotechnology). The same membranes were stripped and blotted with an anti-GAPDH antibody (Santa Cruz Biotech) and used as loading controls. The probe proteins were detected using the ECL detection reagents (Millipore, Bedford, MA, USA).

2.5. Chromatin immunoprecipitation assay

ChIP was carried out using kit from Upstate Biotechnology according to manufacturer's protocol. The putative binding site of TSSC1 was amplified with the following primers: 5′-CCTTGCGATGCCATTTCTG-3′ and 5′-AATGGGAGTCGGAAGGGAA-3′. The PCR products were resolved electrophoretically on a 2% agarose gel.

2.6. Luciferase assay

The TSSC1 promoter (−800 to +1) was amplified by from genomic DNA of MDA-MB-231 cells and the fragment was cloned into the luciferase reporter plasmids pGL3-basic vector (Promega, Madison, WI, USA) (TSSC1p). 5×10^4 cells per well in 24-well plates were cultured without antibiotics overnight and then transfected the TSSC1p into the cells with or without pcDNA3.1-Runx2. After

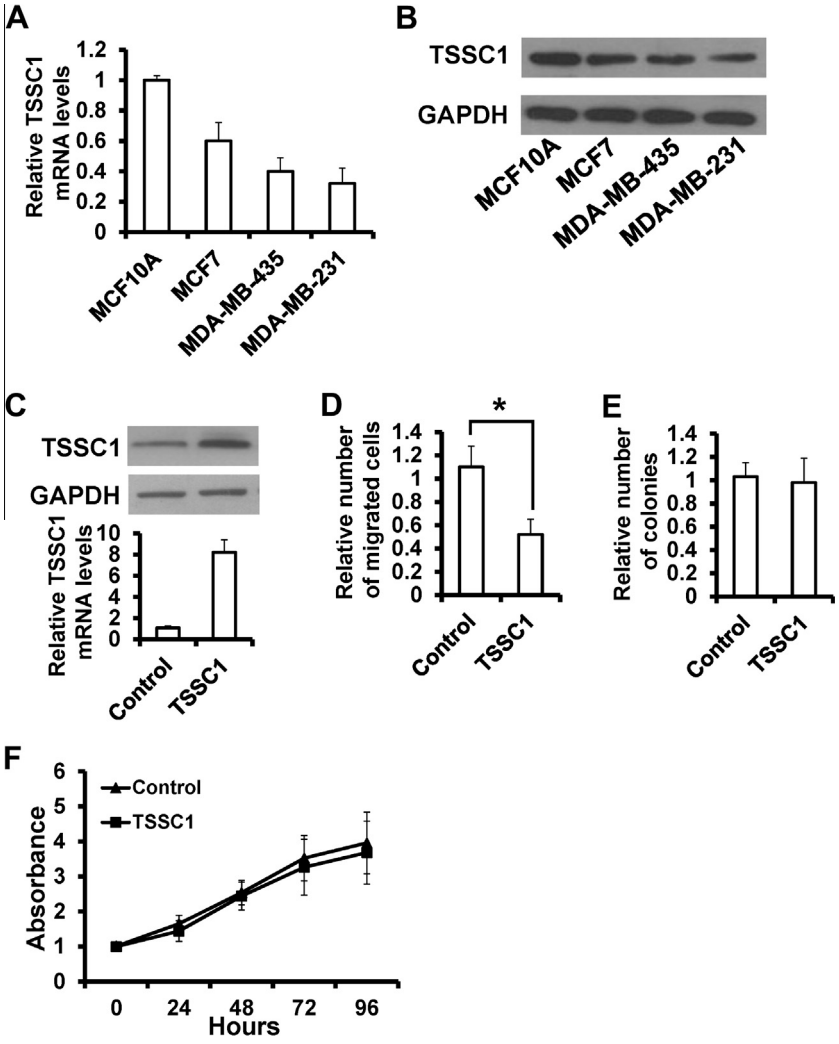

Fig. 1. TSSC1 inhibits breast cancer cell invasion. (A and B) TSSC1 expression in breast cancer cell lines by RT-qPCR (A) and western blot (B). (C) RT-qPCR and western blot analyses of TSSC1 expression in TSSC1-transfected and controls cells. (D) Transwell analysis of TSSC1-transfected and control cells. (E) Soft agar colony formation analysis of TSSC1-transfected and control cells. (F) MTT analysis of TSSC1-transfected and control cells. $^*P < 0.05$.

24 h, cells were washed with phosphate-buffered saline (PBS), subjected to lysis, and their luciferase activities measured by using a dual luciferase assay kit (Promega). The results were normalized against *Renella* luciferase. All transfections were performed in triplicate.

2.7. MTT assay

2×10^3 cells in 200 μl culture medium were plated into a well of 96-well plates. After culturing cell for an appropriate time, 10 μl of 5 mg/ml MTT was added into each well and cultured for 4 h. Then, the cell culture medium was replaced by 100 μl of dimethyl sulfoxide. Thirty minutes after dimethyl sulfoxide addition, the plates were placed on a microplate auto-reader (Thermo). Optical density was read at 570 nm wavelength and cell growth curves were determined according to the optical density value.

2.8. Soft agar colony formation assay

1×10^3 cell suspended in complete medium containing 0.33% agar were plated in a hard agar (0.6% agar in complete medium) coated well in 6 cm dishes. After 3 weeks, cells were stained by MTT to visualize colonies and then counted using an optical microscope.

2.9. Invasion assay

The invasion of cells *in vitro* was measured by the invasion of cells through Matrigel-coated transwell inserts (8 μm pore size, BD Biosciences, San Jose, CA, USA). 1×10^5 cells in 500 μl of serum-free medium were added to the upper chamber, Medium (DMEM/F12) containing 20% FBS were added into the lower chamber. After incubation for 16 h, cells that invaded the Matrigel were stained and counted under a microscope in six predetermined fields.

2.10. Xenograft assay

1×10^6 cells were injected tibiae of SCID mice ($n = 9$). 3 mice, per group were used as representatives for analysis. Bone lesions were analyzed by radiography using Faxitron MX-20 after 6 weeks (Faxitron X-ray, Wheeling, IL, USA).

2.11. Statistical analysis

Results of *in vitro* experiments were depicted as mean ± SD and Student's *t*-test (two-tailed) was used to compare values of test and control samples. All calculations were performed with the SPSS for Windows statistical software package (SPSS, Chicago, IL, USA). The level of significance was set to $P < 0.05$.

3. Results

3.1. TSSC1 expression in breast cancer cell lines regulates metastatic properties

Previously, the microarray demonstrated that down-regulation of the TSSC1 gene in the bone metastatic breast cancer cell line (MDA-MB-231BO) compared to the primary human breast cancer cell line (MDA-MB-231) [16]. To gain insight into the potential interaction between TSSC1 and breast cancer metastatic and tumor growth potential, we firstly detected the TSSC1 mRNA and protein in three widely studied breast cancer cell lines and a normal breast cells by RT-qPCR and Western blot. As shown in Fig. 1A and B, significantly lower TSSC1 expression was evident in highly metastatic MDA-MB-231 cells than MDA-MB-435, MCF7 breast cancer cell lines, as well as the normal breast cell line MCF10A. To further investigate the role of TSSC1 in breast cancer metastasis, we transfected pcDNA3.1-TSSC1 into MDA-MB-231 cells and high expression of TSSC1 was observed in TSSC1-transfected cells than the control cells (Fig. 1C). We further analyzed the potential role of TSSC1 in breast cancer cell metastasis. The results show that TSSC1 overexpression decreased invasion (Fig. 1D), although it did not

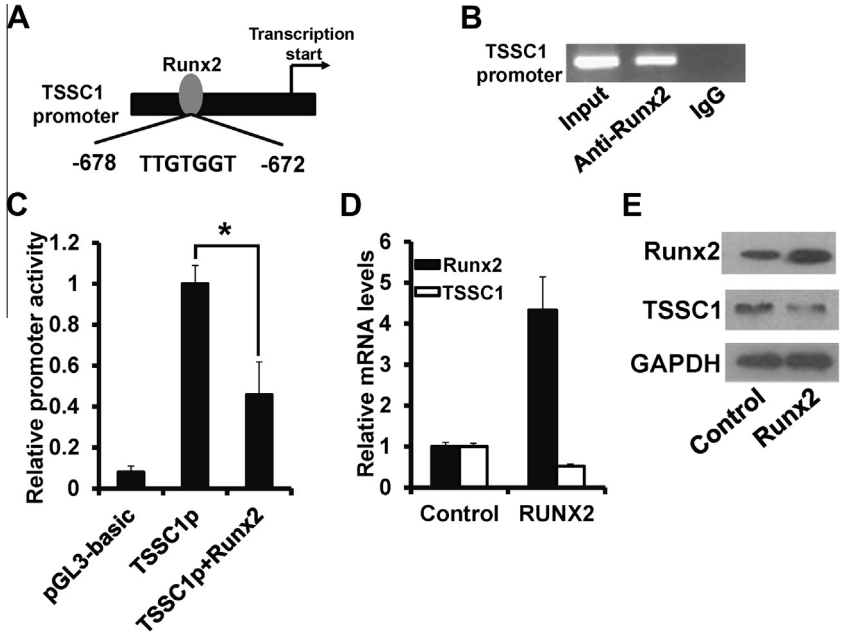

Fig. 2. Runx2 transcriptional down-regulated TSSC1 expression. (A) Schematic representation of Runx2 binding site in TSSC1 promoter. (B) ChIP analysis of the human TSSC1 promoter by anti-Runx2 antibody in MDA-MB-231 cells. (C) Luciferase activity of TSSC1 promoter in MDA-MB-231 cells. (D and E) RT-qPCR (D) and Western blot. (E) Analyses of TSSC1 expression in Runx2-transfected and control cells. $^*P < 0.05$.

significantly affect the proliferation *in vitro* (Fig. 1E and F). Taken together, these results suggest that TSSC1 suppresses cell invasion *in vitro*.

3.2. Runx2 transcriptionally down-regulated TSSC1 expression

Next, we found a Runx2 binding site on the TSSC1 promoter region (Fig. 2A). The chromatin immunoprecipitation (ChIP) assay was then performed to examine the association of Runx2 with the TSSC1 promoter *in vivo*. We observed recruitment of Runx2 to the TSSC1 promoter (Fig. 2B). To assess whether this Runx2 binding site contributes to TSSC1 transcription, the TSSC1-promoter (TSSC1p) was transient transfected into MDA-MB-231 cells with or without pcDNA3.1-Runx2. Our result indicates that Runx2 suppresses TSSC1p activity (Fig. 2C). To determine the effect of increased Runx2 expression on TSSC1 expression, we transfected MDA-MB-231 cells with pcDNA3.1-Runx2 as well as the vector control. We found that the Runx2-transfected cells exhibited significantly decreased TSSC1 mRNA and protein expression (Fig. 2D and E). Together, these results indicate that Runx2 transcriptionally suppresses TSSC1 expression.

3.3. Runx2 contributes to breast cancer metastasis in a TSSC1-dependent manner

TRunx2/TSSC1 interaction by assessing their roles in breast cancer cells, we stably overexpressed Runx2 in MDA-MB-231 cells (Runx2). Furthermore, we stable overexpressed TSSC1 expression in Runx2-overexpressed MDA-MB-231 cells. RT-qPCR and Western blot analyses showed that the Runx2 mRNA and protein levels were increased in Runx2-overexpressed cells, whereas the cells showed decreased TSSC1 mRNA and protein levels in Runx2-overexpressed cells (Fig. 3A and B). Furthermore, the TSSC1 expression was rescued in Runx2- and TSSC1-cooverexpressed cells (Fig. 3A and B). To observe the effects of Runx2/TSSC1 on the breast cancer cells, cell proliferation were evaluated by soft agar colony formation and MTT assays. The results show that Runx2/TSSC1 does not affect the proliferation in breast cancer cells (Fig. 3C and D). Furthermore, we performed transwell assay to assess the function role of Runx2/TSSC1 in breast cancer cell invasion. The rate of migrated cells was lower in Runx2- and TSSC1-cooverexpressedcells than the Runx2-overexpressedcells (Fig. 3E). Together, these results suggest that Runx2/TSSC1 interaction contributes to breast cancer metastasis but not proliferation.

3.4. Runx2/TSSC1 mediates breast cancer-induced osteolytic bone disease

Runx2 plays an important role in cancer metastasis and contributes to the osteolytic disease by regulating expression of a large spectrum of genes that are involved in vessel formation, invasion, extravasation, and colonization [15]. To identify the functional activities of Runx2/TSSC1 in MDA-MB-231 sublines *in vivo*, cells were inoculated into the intramedullary cavity of SCID mice. Osteolysis was evident in Runx2-overexpressed tumor by radiography at 6 weeks than the Runx2- and TSSC1-cooverexpressed tumor, as well as the control tumor. Furthermore, the bone resorbing factor IL11 and PTHrP mRNA expression were up-regulated in tumors from implanted Runx2-overexpressed cells (Fig. 4B). These results indicate that Runx2 mediates breast cancer-induced osteolytic bone disease by transcriptional regulation of TSSC1 expression.

4. Discussion

A number of studies have indicated that the osteogenic transcription factor Runx2, is principally linked to osteoblast proliferation and differentiation and is obligatory for regulation of skeletal genes, hypertrophic chondrocytes as well as end ochondral and intramembraneous bone formation and skeletal development [17]. Runx2 has been extensively studied in the context of osteoblasto genesis from mesenchymal progenitors, where as a master regulator it stimulates the expression of various bone matrix components such as osteocalcin and BSP [18,19]. Blocking Runx2 function either by expressing mutant Runx2 proteins or depletion of Runx2 by shRNA decreases expression of metastatic and osteolytic genes, *in vitro* invasive properties of MDA-MB-231 breast and PC3 prostate cancer cells and inhibits bone osteolytic properties *in vivo* [8,20,21]. In addition, overexpression of Runx2 promotes MDA-MB-231 cells invasion *in vitro* [22]. Our current findings are certainly consistent with prior work showing that Runx2 may promote the metastatic potential of breast cancer cells by modulating osteolytic properties [23,24].

Runx2 plays an important role in cancer metastasis by regulating expression of a large spectrum of genes that are involved in vessel formation, invasion, extravasation, and colonization. These genes include, but are not limited to, OP, IL8, VEGF, and MMPs [14]. Here, we showed that Runx2 binds to and inhibits the tran-

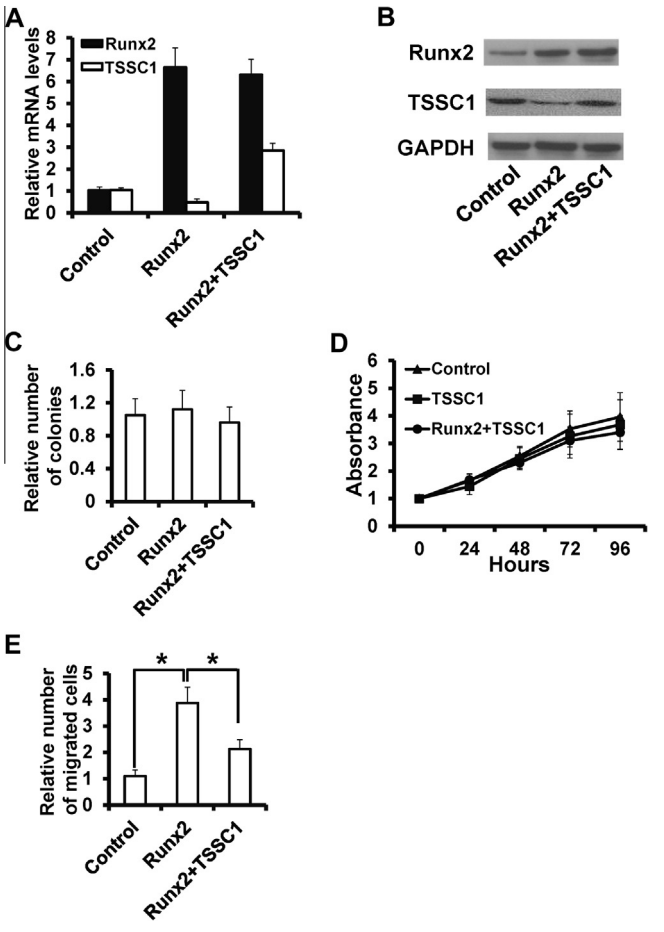

Fig. 3. Runx2 promotes breast cancer invasion by down-regulation of TSSC1. (A and B) RT-qPCR (A) and Western blot (B) analyses of TSSC1 expression in Runx2- or/and TSSC1 transfected and control cells. (C) Soft agar colony formation analysis of Runx2- or/and TSSC1-transfected and control cells. (D) MTT analysis of Runx2- or/and TSSC1-transfected and control cells. (E) Transwell analysis of Runx2- or/and TSSC1-transfected and control cells. $^{*}P < 0.05$.

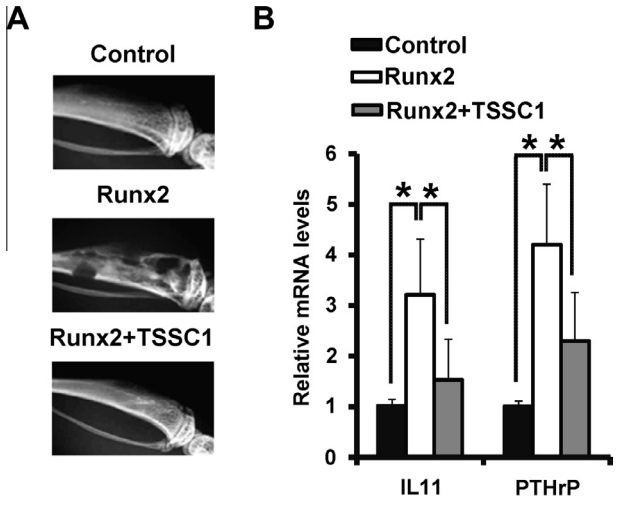

Fig. 4. Runx2 mediates breast cancer-induced osteolytic bone disease in a TSSC1-dependent manner. (A) Radiographs of tibiae six weeks post-injection of tumor cells show osteolytic lesions caused by Runx2- or/and TSSC1-transfected and control cells. ($n = 3$ per group). (B) RT-qPCR analysis of PTHrP and IL8 mRNS expression in tumors. $^{*}P < 0.05$.

scriptional activity of TSSC. TSSC1 has been reported to be a putative tumor suppressor [1], which is consistent with our work that overexpression of TSSC1 inhibits breast cancer cells invasion.

Growth of breast tumors in bone and the accompanying metastatic bone disease presents an imbalance in the normal process of bone remodeling (formation and resorption) as a result of factors secreted by the tumor cells, including parathyroid hormone related protein (PTHrP) [25] and interleukin 11 (IL11) [26] that promote bone resorption. In current study, we found the PTHrP and IL11 mRNA expression levels were up-regulation in Runx2-overexpressed tumor cells and rescued by overexpression of TSSC1.

In summary, we show here that the TSSC1 expression is negatively regulated by Runx2 in breast cancer cells. Our further analysis demonstrates that Runx2 induces bone osteolysis in a TSSC1-dependent manner. Thus, targeting TSSC1 for inhibition of Runx2-induced breast tumors is a potentially viable strategy for blocking breast cancer metastasis.

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