Original Article

DOI: 10.5582/irdr.2019.01025

A preliminary study on the mechanism of skeletal abnormalities in Turner syndrome using inducing pluripotent stem cells (iPS)-based disease models

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Summary **Osteoporosis represent one of main characteristics of Turner syndrome (TS), a rare diseases caused by aberrant deletion of X chromosomes, however, the underlying pathological mechanism remains unknown yet. In this study, we used pluripotent stem cells (iPSCs) derived from a Turner syndrome patient and a health control to induce functional osteoblasts and osteoclasts, in order to compare their difference in these two differentiation. We successfully produced functional osteoblasts and osteoclasts from iPSCs through embryoid bodies (EBs) and mesoderm stages, as demonstrated obvious mineralized nodules and multi-nuclear giant cells with positive tartrate-resistant acid phosphatase (TRAP) staining, and significant up-regulated differentiation marker genes. Interestingly, we found that there was no significant difference in phenotype and marker genes expression between osteoblasts from Turner syndrome and healthy control iPSCs. In contrast, Turner syndrome showed increased osteoclastogenesis compared to the healthy control indicating higher frequency of multi-nuclear TRAP staining cells and elevated osteoclast marker genes *TRAP*, *MMP9*, *CA2*, *OSCAR*. Therefore, our results suggest that the low bone density of Turner syndrome patients may be caused by aberrant osteoclast differentiation, and further investigation towards osteoclast function under Turner syndrome is deserved.**

Keywords: Turner syndrome, induced pluripotent stem cells, osteoblasts, osteoclasts

1. Introduction

Turner syndrome is a relatively common type of human chromosome aberration with an incidence rate of 1/2,500 (*1*). It is also called congenital ovarian dysplasia syndrome, which is caused by total or partial deletion of X chromosomes in all or part of the cells. Monosomy 45, X accounts for about 45% of TS, while the remaining patients exhibit multiple chimeras and structural abnormalities (*2*).

Released online in J-STAGE as advance publication May 14, 2019.

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Primary ovarian insufficiency and short stature are prominent clinical features of TS, osteoporosis and fractures are important consequences (*3*). The cause of skeletal fragility of TS may be multifactorial, for example chromosomal abnormalities, acquired osteoporosis and visual spatial cognitive dysfunction, and increased risk of fractures due to impaired balance (*4*). Several previous studies have found a significant reduction in bone density in TS using dual-energy X-ray absorptiometry (DXA) (*5*). Although recombinant growth hormone treatment has been given to TS patients to treat short stature in childhood, the effectiveness of this treatment for bone mineral density improvement remains controversial.

The underlying cellular and molecular mechanisms of osteoporosis, in particular, the role of two most important players, the functions of osteoblasts and osteoclasts are still unknown, as well as important. However, the unavailability of tissues from patients is

a main bottleneck in this field. In this study, we have used induced pluripotent stem cells to establish an osteoblast model and an osteoclast model to mimic the pathological process of bone remodeling *in vitro* to investigate the phenotype variation of these cells, and to explore their biological significance.

2. Materials and Methods

2.1. *TS and normal iPSCs lines*

One human iPSCs line derived from fibroblasts of a TS patient was obtained from Sidansai Biotechnology Company (Shanghai, China), an 8-year-old female with a 45,X karyotype. Cells derived from an iPSCs line from a healthy volunteer were established from a 25-year-old healthy woman. Undifferentiated iPSCs were seeded on six-well plates (Sorfa, Zhejiang, China) coated with Matrigel (Corning, Bedford, MA, USA) and cultured using mTesR medium (StemCell Technologies, Vancouver, BC, Canada). These two iPSCs were established with informed consent. The study procedures of this study were approved by the ethnic committee of Shandong Medical Biotechnological Center.

2.2. *Induction of osteoblasts from TS and normal iPSCs*

In order to induce differentiation, the two iPSCs lines were first cultured in six-well plates for 5-7 days, when the cells were overgrown, the embryoid bodies (EBs) were obtained by a mechanical method, and cultured in a low attachment six-well plate (Corning, Kennebunk, ME, USA) with added EBs medium (Osinglay, Guangzhou, China) (day 0). After 2 days culture, EB medium was changed with fresh EB medium, all-trans retinoic acid (RA) (MCE, Monmouth Junction, NJ, USA) was added after 48h to induce differentiation of EBs to mesoderm. At day 8, we digested EBs with BioC-PDE1 Digestive Enzyme (Osinglay, Guangzhou, China) allowing cells to grow adherently. The cells were further cultured 1 day, and the osteogenic medium was replaced (Cyagen, Guangzhou, China). The iPSCs-derived osteoblasts were induced for 7 days, 14 days, and 21 days, respectively.

2.3. *Induction of osteoclasts from TS and normal iPSCs*

The procedures for iPSCs and EBs culture were the same for induction of osteoblasts. For osteoclast differentiation, EBs were generated using 6-well low cluster plates in STEMDIFF APEL 2 medium (StemCell Technologies, Vancouver, BC, Canada) supplemented with 50 ug/mL ascorbic acid (Sigma, Louis, MO, USA), 4×10^{-4}M thioglycerol (Sigma, Louis, MO, USA), 2 mM glutamine (Sigma, Louis, MO, USA), 10 ng/mL human bone morphogenetic protein-4 (BMP4) (R&D Systems Inc., Minneapolis, MN, USA), then incubating for 24 hours in an environment of 37°C, 5% O_2, 5% CO_2, 90% N_2., 5 ng/mL human basic fibroblast growth factor (bFGF) (R&D Systems Inc., Minneapolis, MN, USA) was added for 3 days to induce mesoderm formation. Cells were cultured in STEMDIFF APEL 2 medium with 10 ng/mL human vascular endothelial growth factor (VEGF) (R&D Systems Inc., Minneapolis, MN, USA), 1 ng/mL bFGF, 10 ng/mL human interleukin-6(IL-6) (R&D Systems Inc., Minneapolis, MN, USA), 40 ng/mL human interleukin-3 (IL-3) (R&D Systems Inc., Minneapolis, MN, USA), 5 ng/mL human interleukin-11 (IL-11) (R&D Systems Inc., Minneapolis, MN, USA), 100 ng/mL human stem cell factor (HSCF) (R&D Systems Inc., Minneapolis, MN, USA) to promote hematopoietic cell growth. After 5 days culture, cells were moved to a 5% CO_2 environment and 10 ng/mL VEGF, 10 ng/mL IL-6, 40 ng/mL IL-3, 5 ng/mL IL-11, 100 ng/mL HSCF, 4 U/mL human erythropoietin (EPO) (R&D Systems Inc., Minneapolis, MN, USA), 50 ng/mL human thrombopoietin (TPO) (R&D Systems Inc., Minneapolis, MN, USA), 10% FCS (Gibco, Carlsbad, CA, USA) were added to STEMDIFF APEL 2 medium to culture for 10-14 days to promote hematopoietic cell maturation and myeloid cell expansion. Then we digested the cells and cultured on six-well plates in IMDM (Gibco, Carlsbad, CA, USA) containing 30 ng/mL M-CSF (R&D Systems Inc., Minneapolis, MN, USA) and 50 ng/mL RANKL (R&D Systems Inc., Minneapolis, MN, USA) and 10% FCS for 10 to 14 days.

2.4. *Real-time quantitative polymerase chain reaction (RT-qPCR)*

RT-qPCR was used to semi-quantitatively analyze the marker genes for osteoblasts (*ALP*, *RUNX2*, *COL1A1*, *OCN*), hematopoietic stem cells (*CD34*, *CD45*, *RUNX1*, *GATA2*), mononuclear macrophages (*TNFALP3*, *THBS1*, *RUNX1*) and osteoclasts (*MMP9*, *OSCAR*, *CATK*, *CTR*). The primers are listed in Table 1. Briefly, total RNA was extracted using Trizol reagent (Gibco, Carlsbad, CA, USA) and then we used the reverse transcription kit (Toyobo, Osaka, Japan) to synthesize cDNA. RT-qPCR reactions were performed according to the ratio of Sybr Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) : RNAase-free water (Tiangen, Beijing, China): forward: reverse: cDNA at 5:2:1:1:1. Each reaction was run on Light Cycler®480 (Roche Applied Science, Mannheim, Germany).

2.5. *Alizarin red staining*

Alizarin red staining was performed using STAINING Kit (GenMed Scientifics Inc., Arlington, MA, USA). After 50 days of induction from iPSCs, differentiated

osteoblasts were stained for mineralized nodules. Briefly, the cells in one well of a 6-well plate was washed twice with solution A, and then cell fixative B reagent was used to fix the cells for 10 minutes. After that, the well was washed twice using cell clearing solution A. Then, staining solution C was added to the culture plate and incubated for 20 minutes at room temperature.

Table1. List of primers RT-qPCR

Target gene		Primer sequence
ALP	Forward	CCGTGGCAACTCTATCTTGG
	Reverse	GCCATACAGGATGGCAGTGA
RUNX2	Forward	AGCAAGGTTCAACGATCTGAGAT
	Reverse	TTTGTGAAGACGGTTATGGTCAA
COL1A1	Forward	CCCTGGAAAGAATGGAGATGAT
	Reverse	ACTGAAACCTCTGTGTCCCTTCA
OCN	Forward	AAGAGACCCAGGCGCTACCT
	Reverse	AACTCGTCACAGTCCGGATTG
CD34	Forward	TGGACCGCGCTTTGCT
	Reverse	CCCTGGGTAGGTAACTCTGGG
CD45	Forward	TCAGCCTTGCACACCACAGCTC
	Reverse	AAATGACAGCGCTTCCAGAAGGGC
RUNX1	Forward	CCGAGAACCTCGAAGACATC
	Reverse	GTCTGACCCTCATGGCTGT
GATA2	Forward	GGGCTAGGGAACAGATCGACG
	Reverse	GCAGCAGTCAGGTGCGGAGG
TNFALP3	Forward	TCAACTGGTGTCGAGAAGTCC
	Reverse	CCAAGTCTGTGTCCTGAACG
THBS1	Forward	AAGCGTCTTCACCAGAGACC
	Reverse	TGGTGTGGTTCCAAAGACAA
RUNX1	Forward	CCGAGAACCTCGAAGACATC
	Reverse	GTCTGACCCTCATGGCTGT
Mmp9	Forward	GAACCAATCTCACCGACAGG
	Reverse	GCCACCCGAGTGTAACCATA
OSCAR	Forward	CCCAGCTTCATACCACCCTA
	Reverse	GAAGAGAAGGGGAGCGATCT
CATK	Forward	CAGTGAAGAGGTGGTTCAGA
	Reverse	AGAGTCTTGGGGCTCTACCTT
CTR	Forward	TCTCAGGAGTGAAAGCATTGCACATA
	Reverse	AATGCTATGACCGAATGCAGCAGTTA
GAPDH	Forward	GCACCGTCAAGGCTGAGAAC
	Reverse	ATGGTGGTGAAGACGCCAGT

2.6. *TRAP staining*

The osteoclast activity was evaluated by TRAP staining using a commercial kit (Sigma, Louis, MO, USA). The cells were washed twice with PBS (Gibco, Carlsbad, CA, USA), then fixed with paraformaldehyde (Solarbio, Beijing, China), and the chromogenic reagent was prepared according to the manufacturer's instructions and added to the well for 1 hour. Staining results were observed under the microscope.

2.7. *Statistics*

To determine the differences between groups, student's *t*-test was carried out. We used SPSS statistics 19 to calculate data, with significance accepted at $p < 0.05$. Mapping with Graphpad Prism 6.0.

3. Results

3.1. *Comparision of osteoblast differentiation of iPSCs from TS and normal control*

The procedure of osteoblast induction from iPSCs is shown in Figure 1A. Through EBs stage, typical osteoblasts with mineralizing ability could be established (Figure 1B). Marker genes at 7 days, 14 days and 21 days of osteogenesis induction, and mineralized nodule formation at 50 days from TS-iPSCs and normal-iPSCs were compared. As seen in Figure 2A, in all 3 stages, expression of osteogenesis marker genes seems to be higher in the TS-derived osteoblasts than the normal control. But there is no obvious difference in mineralization formation in

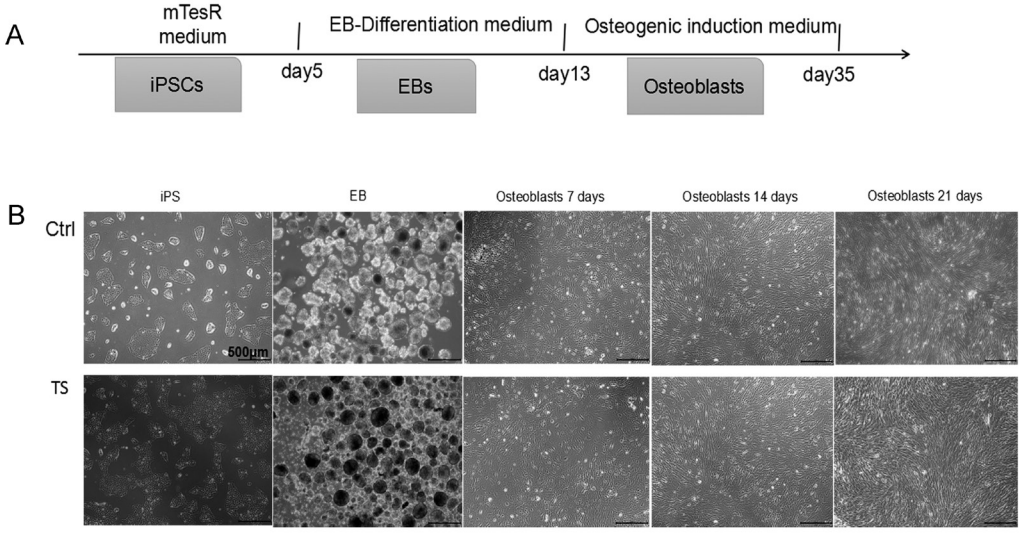

Figure 1. Main types of cell culture. (A) Time flow chart of osteoblast differentiation. **(B)** Cell picture of normal human and TS patients with iPSCs, EBs and osteoblasts differentiated for 7 days, 14 days, 21 days (Scale bar = 500 μm).

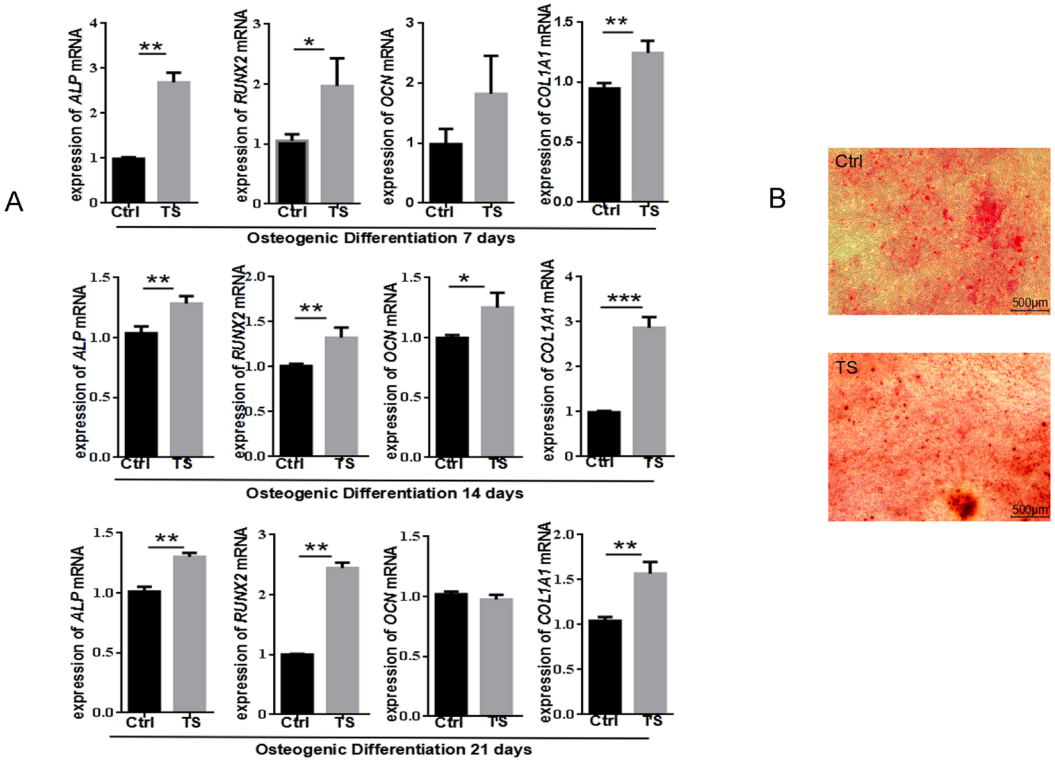

Figure 2. Gene and phenotype analysis of osteoblasts induced by iPSCs in normal and TS patients. (A) Detecting osteoblast marker genes *ALP*, *RUNX2*, *OCN*, *COL1A1* induced by normal and TS patients for 7 days, 14 days, and 21 days. There is no significant change in the TS group genes, and there is even a slight up-regulation. **(B)** A picture of 50-day alizarin red staining of normal human and TS patients with osteoblasts showed a large number of red nodules under the microscope, and there was no significant difference in the number of red nodules between the two groups (Scale bar = 500 μm).

osteoblasts at 50 days (Figure 2B).

3.2. *Comparision of osteoclast differentiation of iPSCs from TS and normal control*

Figure 3A demonstrated our stepwise procedure to induce TS- and normal- iPSCs to osteoclasts through hematopoietic cell and mononuclear macrophage stages. The induction at all stages was successful. As shown in Figure 3B and Figure 3C, after induction, hematopoietic cell-related genes *CD34*, *CD45*, *RUNX1*, *GATA2* and mononuclear macrophage-associated genes *PU.1*, *TNFALP3*, *THBS1* were significantly elevated. At the end of induction, significantly elevated osteoclast marker genes *TRAP*, *CA2*, *MMP9*, *OSCAR* were validated (Figure 3D).

When the extent of osteoclast differentiation of TS- and normal- iPSCs was compared, the TS-iPSCs derived osteoclasts indicated a stronger TRAP staining intensity and density than the normal iPSCs-derived control (Figures 4A and 4B). Consistent with the TRAP staining results, the expression marker genes of osteoclast differentiation were also significantly increased in the TS-iPSCs derived osteoclasts than that in the normal iPSCs-derived control (Figure 4C). These findings support that TS osteoclasts may have a higher osteolytic activity than normal osteoclasts.

4. Discussion

Abnormal skeletal differentiation is one of main characteristics of TS, which has been always neglected. It has been proposed that loss of function of some genes, in particular *SHOX*, may contribute to the pathology of TS. Ibarra-Ramírez *et al*. confirmed that *SHOX* and *VAMP7* showed the most obvious gene dose changes in the pseudo-autosomal region of TS (*6*). Regarding the short stature, it has been suggested that the homeobox gene *SHOX* in the pseudoautosomal region is a major player, and the haplotype deficiency of this gene leads to growth disorders in TS (*7,8*). In addition, Kosho *et al*. have reported that SHOX haploinsufficiency can lead to additional TS skeletal abnormalities such as short fourth metacarparls and cubitus valgus (*9*). Clement-Jones *et al*. showed that *SHOX* nonsense mutations can lead to rich internal phenotypic variability in some of these skeletal features (*10*).

Osteoporosis is another clinical manifestation, which makes TS patients more prone to fractures than the general population, but the etiology of decreased bone density has not been elucidated. The bone formation process can be influenced by several factors, such as genetic inheritance, gender, ethnicity and endocrine activity (endogenous factors), as well as nutrition and physical activity (exogenous factors) (*11*). About 60% of

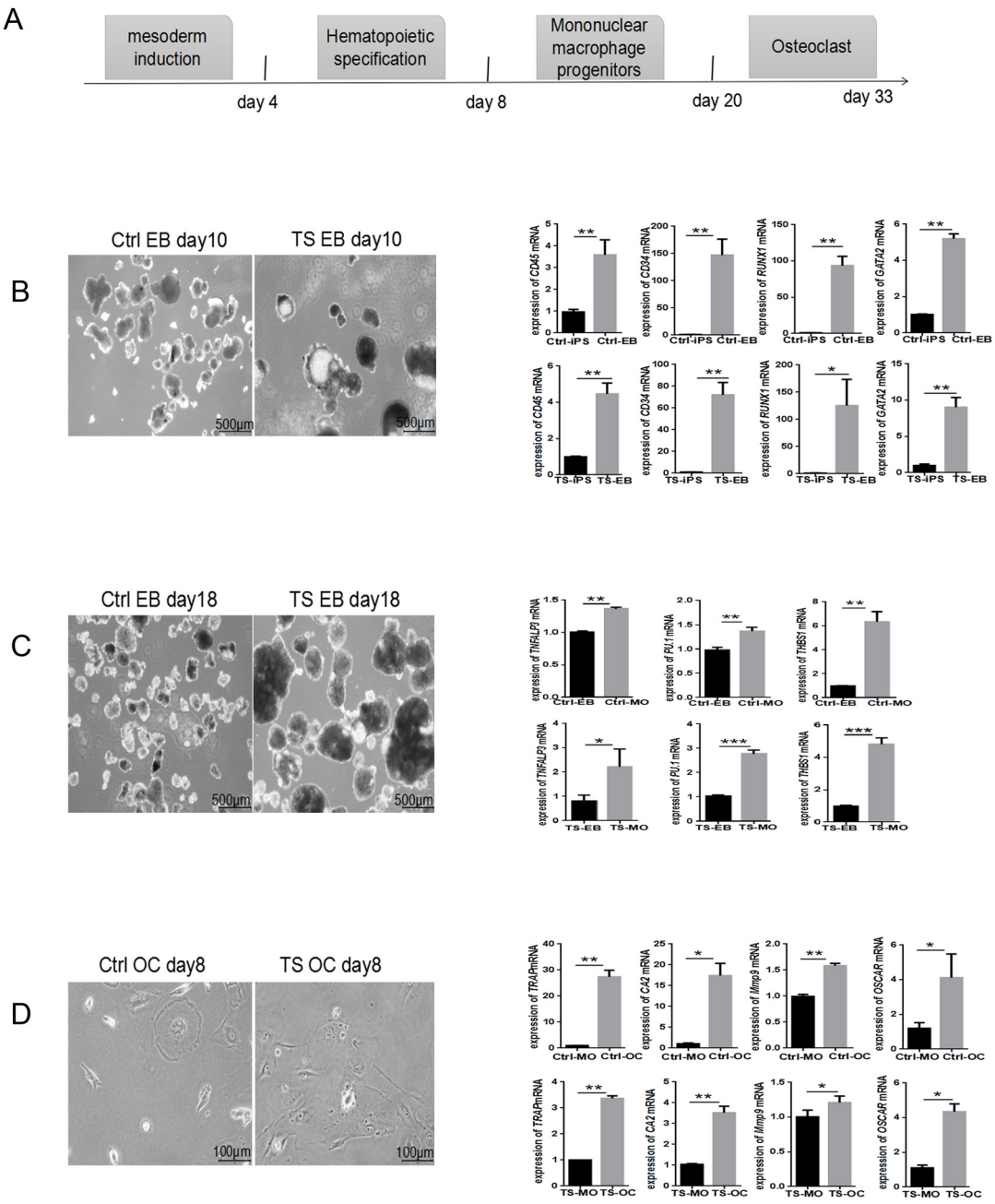

Figure 3. iPSCs induce osteoclast differentiation in normal and TS patients. (A) Used for schematic diagram of a stepwise protocol for differentiating normal and TS patient cells into osteoclast lineages. **(B)** In the process of inducing osteoclasts,cell morphology in the form of EBs in normal and TS patients after hematopoietic stem cell stage and RT-qPCR verified the marker genes *CD45*, *CD34*, *RUNX1*, *GATA2*, which are elevated (Scale bar = 500 μm). **(C)** In the process of inducing osteoclasts, cell morphology in the form of EBs in normal and TS patients after mononuclear macrophage and RT-qPCR verified the marker genes *TNFALP3*, *PU.1*, *THBS1*, which are elevated (Scale bar = 500 μm). **(D)** Inducing the final stage of osteoclasts, the osteoclast morphology induced by normal and TS patients and RT-qPCR verified the marker genes *Mmp9*, *OSCAR*, *CATK*, *CTR*, which were elevated (Scale bar = 100 μm).

the risk of osteoporosis may be due to damage to mineral bone at the beginning of adulthood (*12*). In healthy adults, bone remodeling is a highly coordinated process involving bone resorption and bone formation and is regulated by both osteoblasts and osteoclasts. Bone development and remodeling involves and depends on the interaction between bone cell precursors, bone cells, extracellular matrix molecules, growth factors, immune system and humoral factors (*13*).

For the scarcity of cases surgical procedures are rarely performed for TS patients, therefore, direct obtainment of bone tissues from patients is difficult. The application of iPSCs can provide a good model for rare diseases for they can be induced to form different disease-related cell types. In order to analyze the cause of abnormal bone density in TS patients, in this study,

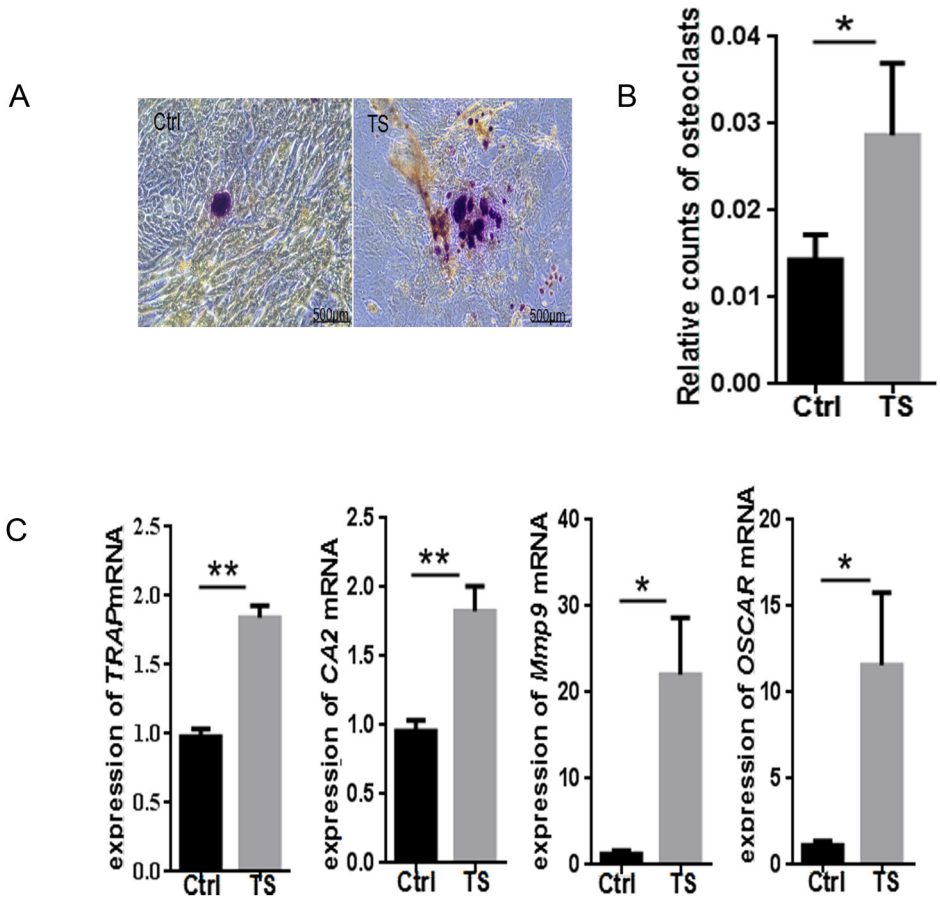

Figure 4. Comparison of iPSCs induced differentiation into osteoclasts in normal and TS patients. (A) Microscopic pictures of osteoclasts induced by iPSCs in normal and TS patients (Scale bar = 500 μm). (B) Count of osteoclasts induced by iPSCs in normal and TS patients, the number of osteoclasts in TS patients is slightly higher than normal control. **(C)** Compared with the RT-qPCR analysis of the osteoclast marker genes *Mmp9*, *OSCAR*, *CATK*, and CTR in normal and TS patients, TS patients were slightly higher than normal human osteoclast gene levels.

using fibroblasts and urine cell derived iPSCs from TS and healthy cases, we established two bone remodeling cell types, osteoblasts and osteoclasts, which were validated by functional analyses and differentiation marker detection.

For the first time, our results found that there is an obvious difference in osteoclast but not osteoblast differentiation between TS patients and healthy controls. Osteoclasts from TS iPSCs demonstrated a higher activity than normal iPSCs derived osteoclasts, which can result in unbalance in bone metabolism.

Lauren J. Massingham *et al*. found that *IGFBP5* overexpression in the Turner syndrome transcriptome (*14*), and its overexpression increases the formation of osteoclasts (*15*). Bisphosphonates are effective in preventing fragility fractures, and we can consider using it for the treatment of Turner syndrome (*16*). Together with the above findings, our data also supported that these patients also benefit from other osteoclast-targeted strategies.

In conclusion, this study revealed that osteoclasts but not osteoblasts from TS-derived iPSCs are abnormal which is helpful to explain the low bone density of TS patients. In future research, efforts to explore the role of genes and pathways related to aberrant osteoclast differentiation in TS will be investigated.

Acknowledgements

This study was supported by The Innovation Project of Shandong Academy of Medical Sciences.

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(Received March 6, 2019; Revised May 3, 2019; Accepted May 8, 2019)