

Role of Zip1 in the regulation of NPY expression by MLT to promote fracture healing in rats

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ABSTRACT

Our previous study documented that melatonin (MLT) induced the osteogenic differentiation of mesenchymal stem cells (MSCs) and promoted the healing of femoral fractures in rats *via* the neuropeptide Y (NPY)/neuropeptide Y1 receptor (NPY1R) signaling pathway. MLT treatment upregulated the expression of the zinc uptake transporter zinc transporter 1 (Zip1) in nerve cells. Prior research demonstrated that oral zinc upregulated NPY expression. MSCs were isolated from rat bone marrow and identified using flow cytometry in our study. The results showed that MLT treatment upregulated NPY and NPY1R levels in MSCs with osteogenic differentiation, which was accompanied by upregulated Zip1 expression. However, the MLT-induced osteogenic differentiation of MSCs was reversed after interference of Zip1 expression. It was confirmed by the decreased alkaline phosphatase (ALP) level; downregulated activities of type I collagen $\alpha 1$ chain (COL1A1), osteocalcin (OCN), runt-related transcription factor 2 (Runx2) and ALP; and reduced mineralized nodule formation. MLT promoted fracture healing in rats with femoral fracture, which was accompanied by increased expression of NPY and NPY1R and significantly increased expression of Zip1. In contrast, the silencing of Zip1 expression reversed MLT-mediated fracture healing. In summary, Zip1 participated in the regulation of the NPY/NPY1R signaling pathway *via* MLT to promote the osteogenic differentiation of MSCs and fracture healing.

Key words: Zip1 protein; lentivirus; melatonin; neuropeptide Y; neuropeptide Y receptor Y1; mesenchymal stem cells; fracture healing.

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Introduction

There has been a remarkable increase in the incidence of trauma-induced fracture with the rapid development of society. Because of the influence of age and disease factors, patients frequently suffer difficulty in healing and subsequent complications, such as limb disuse atrophy. Approximately 5% to 10% of all fractures cannot be completely healed, which imposes a heavy physiological and economic burden on individuals.¹ Therefore, it is of great economic and social significance to study the mechanism of fracture healing in clinical practice and experimental technology and examine the key targets and effective therapeutic approaches to promote fracture healing.

Zinc is an essential trace element for human growth. It is widely recognized that zinc plays a role in bone formation, bone reconstruction and fracture healing. Recent studies showed that zinc levels affected spinal fusion in rats.² Zinc transporter 1 (Zip1) is a major zinc uptake transporter that is intimately correlated with the accumulation of zinc *in vivo*.^{3,4} The overexpression of Zip1 induced osteogenic differentiation in mesenchymal stem cells (MSCs).⁵ Bone morphogenetic protein 2 (BMP2) may regulate the osteogenic differentiation of MSCs *via* Zip1 upregulation.⁶ Therefore, Zip1 may be involved in fracture healing by affecting the osteogenic differentiation of MSCs, but its mechanism is not clear. Our previous study found that melatonin (MLT) treatment upregulated the expression of Zip1 in N2a cells under hypoxia treatment,⁷ which suggests a regulatory role of MLT in Zip1 expression. Prior research⁸ demonstrated that oral zinc upregulated neuropeptide Y (NPY) expression, and our study hypothesized that MLT would affect the level of NPY and its receptor *via* the upregulation of Zip1 expression and the osteogenic differentiation of MSCs. However, the role of Zip1 in the regulation of MLT on NPY level is not known. Our research group previously demonstrated increased NPY and NPY1R expression in the healing bone tissue and muscles around a fracture after MLT treatment in a rat model of femoral fracture, and an NPY1R inhibitor (BIBP3226) reversed the role of MLT in accelerating fracture healing.⁹ The present study isolated MSCs from rat bone marrow and measured the levels of Zip1, NPY and NPY1R during osteogenic differentiation. The role of Zip1 in MLT regulation of NPY and NPY1R expression was studied in the process of osteogenic differentiation of MSCs and the rat model of femoral fracture.

Materials and Methods

Animals and reagents

Male Sprague-Dawley rats were purchased from Liaoning Changsheng Technology Co., Ltd., China. All animals were housed in a room with an alternating 12-hour dark-light cycle with free access to food and water. The Second Military Medical University approved the study, which was completed in accordance with ethical guidelines stated in the Guide for the Care and Use of Laboratory Animals. MLT was obtained from SeraCare Life Sciences Inc, Shanghai, China (BBI solutions). BIBP3226 was purchased from Tocris, Shanghai, China. MLT was dissolved in or 1% alcohol, and BIBP3226 was dissolved in normal saline (NS).

Construction and identification of Zip1 interfering lentivirus vector

Lentivirus vectors interfering with Zip1 and its negative control were constructed according to the sequence of the Zip1 gene in rats (accession No. NM_001134577.1). After 72 h, Zip1

expression was identified using real-time quantitative PCR and Western blotting.

Isolation and identification of MSCs

MSCs were isolated from the bone marrow of 4-week-old rats. The experimental rats were euthanized *via* an intraperitoneal injection of sodium pentobarbital, and the femur and tibia were separated under sterile conditions.¹⁰ The marrow cavity was washed repeatedly with Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Grand Island, NY, USA). The cell suspension was centrifuged at 350 g for 10 min, and the supernatant was discarded. The precipitate was mixed with erythrolysis buffer (Solarbio, Beijing, China) for 3 min. Cells were subjected to centrifugation and washing, followed by culture in DMEM supplemented with 10% fetal bovine serum.

The specific antibody markers of MSCs were detected using flow cytometry. Positive antibody markers included CD90-FITC (eBioscience, San Diego, CA, USA) and CD105-FITC (Abcam, Cambridge, UK). Negative markers were CD34 (Abcam) combined with goat anti-rabbit IgG (Abcam) labeled with phycoerythrin and FITC-CD45. Cells stained with an isotype control (IgG-FITC) were used as the negative control.

Transfection of MSCs with Zip1 interfering lentivirus vector and culture

MSCs at P5 and the logarithmic growth phase in good condition were digested with trypsin, followed by the adjustment of cell concentration to 1×10^5 cells/9.6 cm² and inoculation into a 6-well plate.¹¹ When the cells reached 30%-50% cell fusion, Zip1 interfering lentivirus vector was used to interfere with the cells. The cells were supplemented with 10 mg/L Polybrene and incubated overnight in a 5% CO₂ incubator at 37°C. Cells were selected for subsequent experiments after drug addition and screening.

Four groups were established in this experiment: a control group, an MLT group, and two groups of MSCs transfected with Zip1 interfering lentivirus vector. Cells were cultured in osteogenic induction medium (OIM) containing DMEM, 10% fetal rat serum, 100 nmol/L dexamethasone, 50 μmol/L ascorbic acid and 10 mmol/L sodium β-glycerophosphate. No treatment was applied to cells in the control group, and cells in the MLT group received 2 mmol/L MLT. The other two groups received 2 mmol/L MLT and 2 mmol/L MLT+0.1 nmol/L BIBP3226 to study the role of Zip1 in MLT regulation of NPY in the process of the osteogenic differentiation of MSCs.

Western blotting

Total protein was extracted from bone using RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China) containing 1% phenylmethylsulphonyl fluoride. A bicinchoninic acid (BCA) assay kit (Beyotime Institute of Biotechnology) was used to determine the protein concentration. The sample was resolved using 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and blocked with 5% skim milk. The membrane was incubated with primary antibodies against Zip1 (1: 100; Santa Cruz Biotechnology, Santa Cruz, California, USA), NPY (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), NPY1R (1:500; Abcam), type I collagen α1 chain (COL1A1; 1:300; CST, Boston, MA, USA), runt-related transcription factor 2 (Runx2; 1:1000; Bioss, Beijing, China) and GAPDH (1:1000; Key-GEN, Nanjing, China) overnight at 4°C. Membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies, and the proteins were visualized using enhanced chemiluminescence (Beyotime Institute of Biotechnology). The expression of target genes was evaluated using gray value analysis, which was normalized to the *GAPDH* reference gene.

Real-time PCR

Total RNAs were separated from cells, and bones using TRIpure reagent (BioTeke, Beijing, China) according to the manufacturer's instructions. cDNA was obtained *via* reverse transcription with a super M-MLV reverse transcriptase (BioTeke). The following primers were used for real-time PCR:

ALP forward, 5'-CATCGGACCCTGCCTTAC-3'
reverse, 5'- GGAGACGCCCATACCATC -3';
COL1A1 forward, 5'- CGATTCACCTACAGCAGCCTTG -3'
reverse, 5'- TGGGATGGAGGGAGTTTACACG -3';
OCN forward, 5'- TAAGGTGGTGAATAGACTCCG-3'
reverse, 5'- TAAACGGTGGTGCCATAGAT-3';
Runx2 forward, 5'-CCATAACGGTCTTCACAAATC -3'
reverse, 5'- GAGGCGGTCCAGAGAACAAACT-3';
Zip1 forward, 5'-GAGCAGTCCAGTCCACCACA-3'
reverse, 5'-GACCAGTACACAGGCACGCA-3';
NPY forward, 5'- CTATCCCTGCTCGTGTGTTT-3'
reverse, 5'- TGAGATTGATGTAGTGTGCG-3';
NPY1R forward, 5'- GATGGACAAAATCAGGGACAG -3'
reverse, 5'- AGAGCAGGAACAGCAGATTG -3';
GAPDH forward, 5'- CGGCAAGTTCAACGGCACAG -3'
reverse, 5'- CGCCAGTAGACTCCACGACAT-3';
The relative gene expression was calculated according to the $2^{-\Delta\Delta CT}$ method.

Alizarin red staining

Mineralized nodule formation was observed using alizarin red staining. MSCs transfected with control lentivirus or Zip1 interfering lentivirus vector were inoculated in 12-well plates, cultured in OIM, and treated with DMSO, MLT or MLT + BIBP3226. After 21 d of culture, cells were fixed with 4% paraformaldehyde for 30 min, followed by the addition of alizarin red staining solution at room temperature for 10 min. Photography was performed using a Motic microscope (100 \times). The absorbance of the release of alizarin red was determined using an automatic quantitative microplate reader (BioTek) at the wavelength of 570 nm.

Detection of alkaline phosphatase

The activity of alkaline phosphatase (ALP) in cell supernatants was determined using a commercial kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

Establishment of femoral fracture model in rats

Thirty 12-week-old rats were randomly divided into five groups: the model group, MLT group, MLT+Zip1 interference group, MLT+BIBP3226+Zip1 interference group, and control group, with 6 rats in each group. The femoral fracture model in rats was established according to previous research.^{12,13} Briefly, rats were anesthetized *via* an intraperitoneal injection of sodium pentobarbital, and the right femoral shaft was exposed *via* a lateral incision. A transverse osteotomy was made with a needle file in the proximal-middle segment of the femur, followed by wound suture layer by layer. Rats in the MLT group received an intravenous injection of MLT solution at a dose of 30 mg/kg postoperatively. Rats in the MLT+Zip1 interference group received a postoperative intravenous injection of 30 mg/kg/day MLT, followed by a single injection of 100 μ L 3×10^7 of virus and matrix glue mixture at the fracture site on the 4th day after surgery.¹⁴ Rats in the MLT+BIBP3226+Zip1 interference group were injected intravenously with 20 μ g/rat/2 days BIBP3226 and 30 mg/kg/day MLT postoperatively and received a single injection of 100 μ L 3×10^7 of virus and matrix glue mixture at the fracture site on the 4th day after surgery. Rats in the NC group had no treatment for the fracture. Rats were euthanized 8 weeks after treatment. The bone tissue samples of the injection site were extracted and fixed with a 10% colorless formaldehyde solution. The healing tissue at the fracture site was immediately frozen in liquid nitrogen in a -80 $^{\circ}$ C freezer for subsequent use.

Histological staining

The fixed specimens were embedded in paraffin and cut into serial sections at a thickness of 5 μ m. The sections were evaluated using hematoxylin-eosin (H&E) and Masson staining. The histological staining was evaluated using a commercial kit (Sigma-Aldrich, St. Louis, MO, USA). Photography was performed using an Olympus microscope (40 \times and 100 \times).

Statistical analysis

Each experiment was repeated three times, and the results are expressed as the means \pm SD. One-way or two-way analysis of variance was used for statistical analysis, followed by *post-hoc* analysis using GraphPad Prism 7 software. $p < 0.05$ indicated that the difference was statistically significant. There was no indication in the assessment of the normal distribution of data to justify the use of conventional ANOVA.

Table 1. General observation results of each group.

No.	Mental state	Temperature	Diet and drinking	Response to external stimuli
Blank control group	Good	Normal	Normal	Responsive
Model group	Obvious mental fatigue	Increase	Obvious decrease	Obviously slow response
MLT group	Passable	Basically normal	Basically normal	Faster
MLT+Zip1 interference group	Mild mental fatigue	Slight increase	Slight decrease	Slightly slow response
MLT+BIBP3226+Zip1 interference group	Mental fatigue	Increase	Obvious decrease	Slow response

Results

Identification of Zip1 interfering lentivirus vector

Three days after transfection, Western blotting and real-time PCR showed that the inhibition rate using Zip1 interfering lentivirus vector exceeded 70% (Figure 1 a,b).

Identification of MSCs

MSCs were isolated from rat bone marrow, and the cell phenotype was evaluated using flow cytometry. As shown in Figure 2, the cells were CD34 and CD45 negative and CD90 and CD105 positive, which is consistent with the reported phenotype of MSCs.

Role of Zip1 in MLT regulation of NPY and NPY1R in the promotion of the osteogenic differentiation of MSC

The role of Zip1 in MLT regulation of NPY and NPY1R in the promotion of osteogenic differentiation of MSCs was investigated *in vitro*. DMSO, 2 mmol/L MLT or 2 mmol/L MLT + 0.1 nmol/L BIBP3226 were used to treat the cells in osteogenic differentiation medium, and cells were transfected with control lentivirus or Zip1 interfering lentivirus vector.

The results of Western blotting showed that MLT upregulated the protein levels of Zip1, NPY, NPY1R, COL1A1 and Runx2 in MSCs, and interference with the expression of Zip1 partially inhibited

these effects. The inhibitory effect was more obvious with interference of the expression of Zip1 and NPY (Figure 3a). Real-time PCR results also detected that interference with Zip1 expression inhibited the MLT-mediated increase in ALP, COL1A1, OCN, and Runx2 mRNA, and interfering with Zip1 and NPY expression increased the inhibitory effect (Figure 3b). Real-time PCR results also detected that interference with Zip1 expression inhibited the MLT-mediated increase in ALP, COL1A1, OCN, and Runx2 mRNA, and interference with Zip1 and NPY expression increased the inhibitory effect (Figure 3c). Alizarin red staining results indicated that MLT accelerated the osteogenic differentiation of MSCs, which was inhibited by interfering with the expression of Zip1. Suppression of the expression of Zip1 and NPY further inhibited the osteogenic differentiation of MSCs (Figure 3d). Role of Zip1 in the regulation of NPY and NPY1R by MLT to promote fracture healing in a rat model. A rat model of femoral fracture was established for further examination. General observation results are shown in Table 1. Western blotting analysis showed that the levels of Zip1, NPY, and NPY1R in bones increased during MLT treatment, and the function of interfering with Zip1 expression was inhibited. Interfering with the expression of Zip1 and NPY increased the inhibitory effect (Figure 4a). Real-time PCR analysis also demonstrated that the levels of Zip1, NPY and NPY1R in bones increased during MLT treatment, and the function of interfering with Zip1 expression was inhibited. Interfering with the

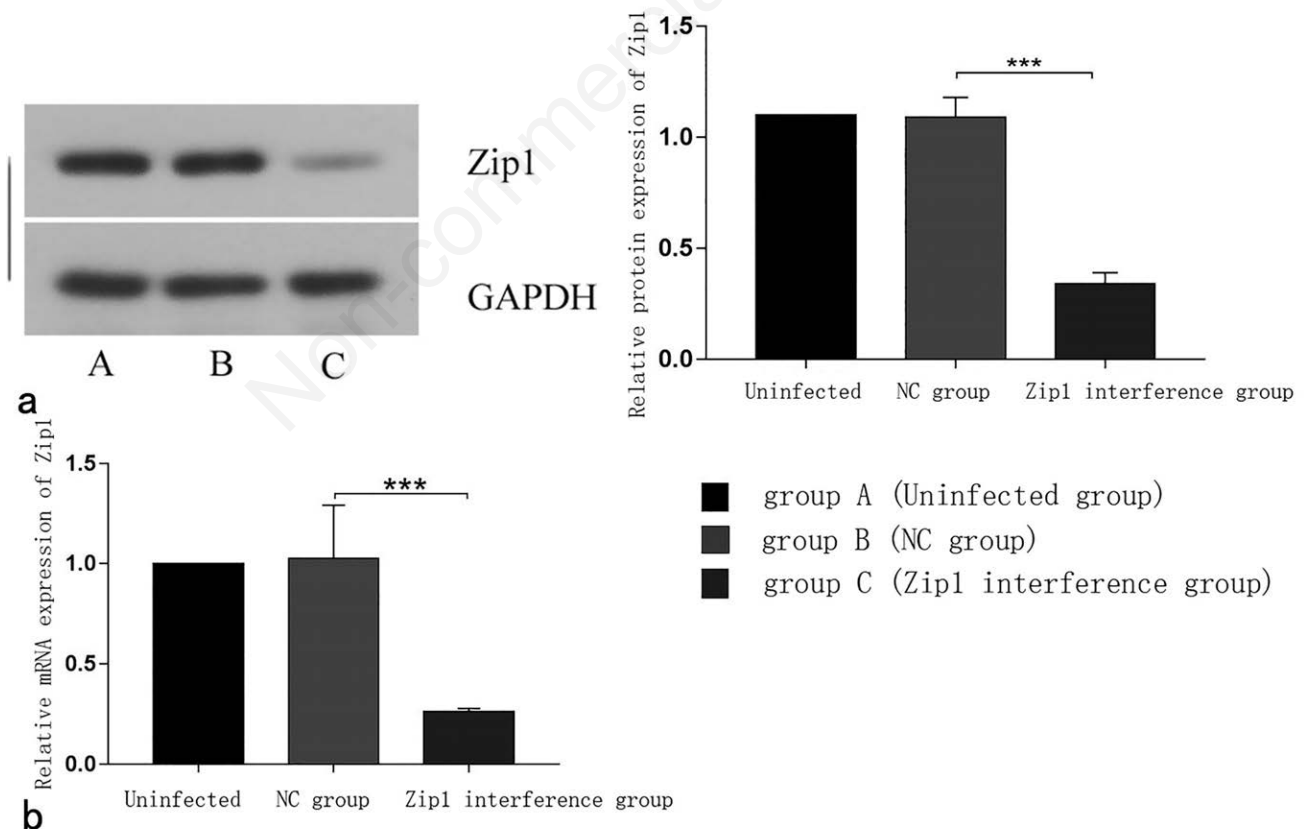


Figure 1. a) Zip1 expression level 3 days after transfection using Western blotting; b) Zip1 expression level 3 days after transfection using real-time PCR.

expression of Zip1 and NPY increased the inhibitory effect (Figure 4b). H&E staining results showed that the blank control group had normal bone morphology and obvious bone marrow cavity structure. The model group showed large-area granulation tissue hyperplasia and filling, a small amount of inflammatory cell infiltration, and a large number of osteoblasts. The basic bone structure was in the MLT group, and granulation tissue is formed in the local area. Compared with the model group, the number of bone cells increased, and more mature bone trabeculae were observed. Compared with the MLT group, the number of osteoclasts in the MLT+Zip1 interference group increased, and the bone tissue area decreased. The MLT+BIBP3226+Zip1 interference group had granulation tissue formation, but it was less than the model group (Figure 5a).

Masson staining results showed that there was no obvious collagen fiber formation in the blank control group. The model group showed a large area of collagen fibers, a large amount of hyperemia in the granulation formation, and osteoblasts in some areas. The formation of collagen fibers in the MLT group was less than the model group. The tissue structure showed that the collagen fiber area of the MLT+Zip1 interference group was higher than the MLT group. The MLT+BIBP3226+Zip1 interference group had a large number of osteoclasts, and the collagen area gradually increased (Figure 5b). The results of fracture healing area showed that MLT promoted fracture healing, and interference with Zip1 expression inhibited the effect of MLT on promoting fracture healing. Interfering with the expression of Zip1 and NPY further reduced the fracture healing area (Figure 5c).

Discussion

The present study demonstrated the role of Zip1 in the regulation of the NPY/NPY1R signaling pathway by MLT to promote

fracture healing. Our results suggested that MLT accelerated the osteogenic differentiation of MSCs and increased the levels of Zip1, NPY and NPY1R. In contrast, inhibition of Zip1 expression reversed MLT-mediated osteogenic differentiation, which was accompanied by decreased expression of NPY and NPY1R. MLT treatment in the established rat model of femoral fracture increased the expression of NPY, NPY1R and Zip1 in the callus. The inhibition of Zip1 expression reversed the role of MLT in promoting the expression of NPY and NPY1R in the callus and inhibited fracture healing. These results suggest that Zip1 mediates MLT regulation of NPY and NPY1R to promote fracture healing. The simultaneous inhibition of the expression of Zip1 and NPY further inhibited the osteogenic differentiation of MSCs and fracture healing, and MLT may play a role *via* receptor-dependent and -independent mechanisms.^{15,16} MLT may regulate NPY and NPY1R expression *via* the MLT receptor. Viruses are potent pathogens that have highly evolved to effectively deliver genetic material to susceptible host cells. The virus is incorporated into the host cell genome and produces relatively long-term expression of genetic information, or it exists in a free state outside the genome and avoids the risk of insertional mutagenesis.^{17,18} Adenovirus vector has been used in a preclinical bone repair model for a long time, and it exhibits good transduction efficiency.^{19,20} However, the immune response produced certain risks in an *in vivo* experiment.²¹ A lentivirus vector enables long-term protein expression with minimal immunogenicity,^{25,26} and it is commonly used in research.^{12,22-24} Therefore, the current experiment used lentivirus to interfere with the expression of Zip1. Fracture healing is a complex process in which the integration of cells, growth factors and extracellular matrix play crucial roles.²⁷ Thirty-six hours after fracture, periosteal cells proliferate in the injured area that is adjacent to the fracture site.²⁸ These cells may be susceptible to retrovirus infection, and a good result was obtained after the injection of a virus vector directly into the fracture site.^{14,29} Therefore, direct injection of a virus vector may be an effective method.

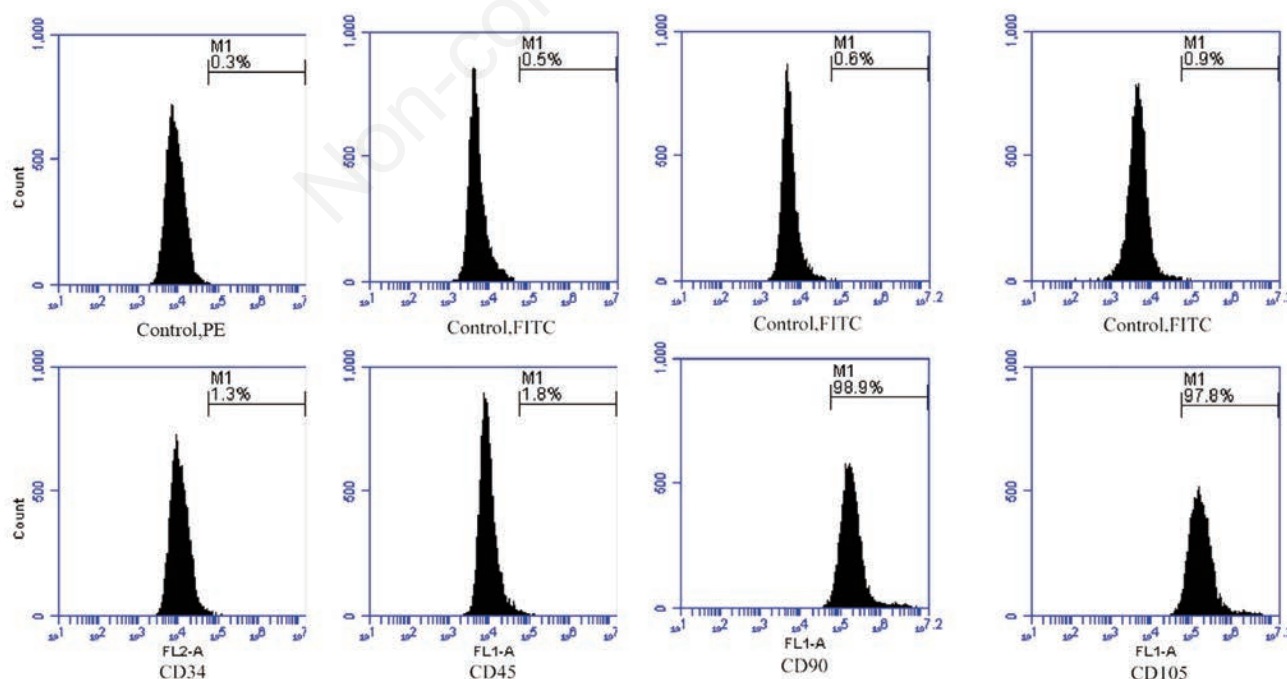


Figure 2. Percentages of CD34+, CD45+, CD90+ and CD105+ cells in MSCs using flow cytometry.

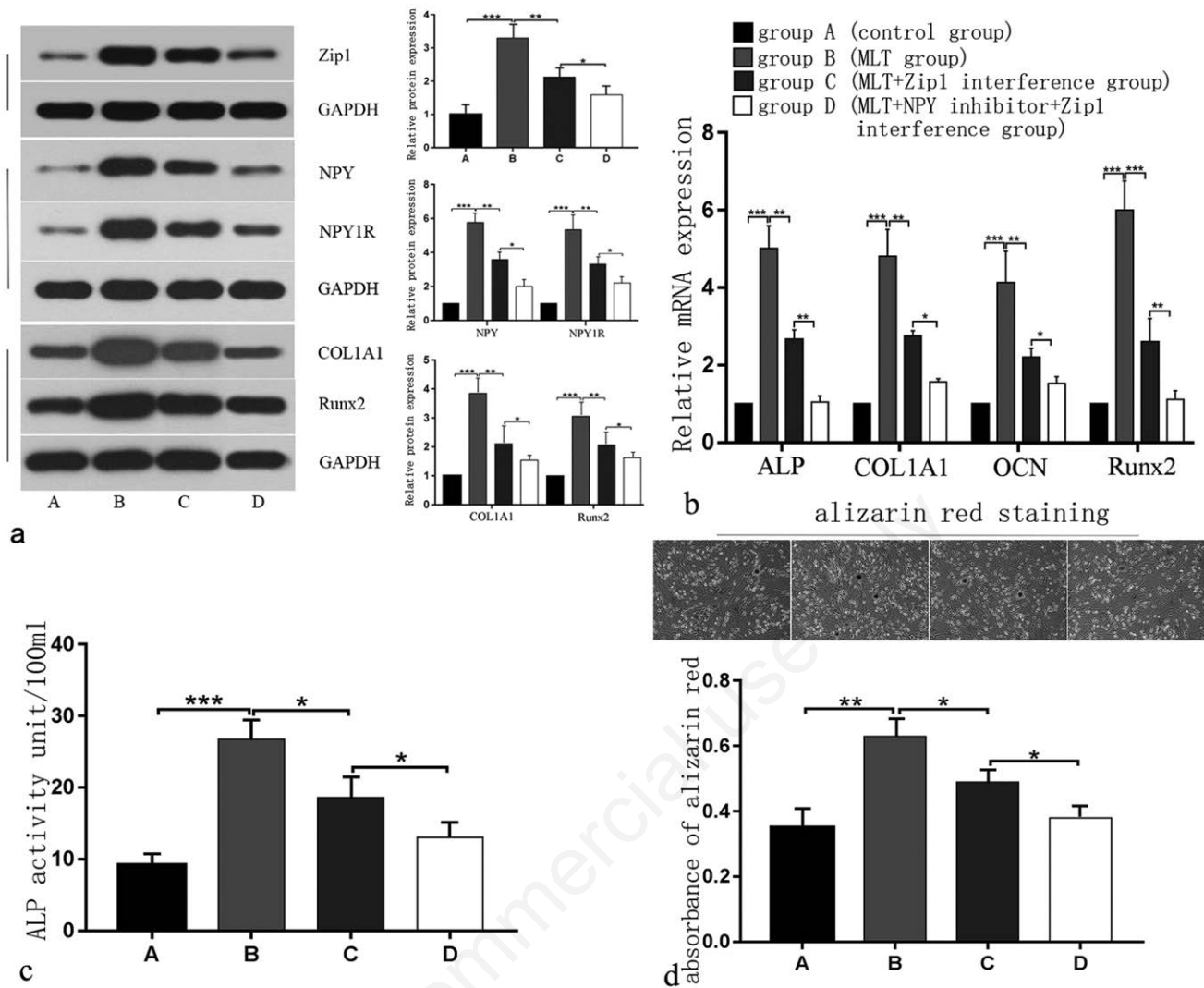


Figure 3. Induction of osteogenic differentiation from bone marrow MSCs and treatment with DMSO, 2 mmol/LMLT or 2 mmol/L MLT + 0.1 nmol/L BIBP3226. a) COL1A1 and Runx2 levels on the 7th day, and Zip1, NPY and NPY1R expression levels using Western blotting on the 21st day. b) ALP, COL1A1, OCN and Runx2 expression levels using real-time PCR on the 7th day after treatment. c) The activity of ALP in supernatant using a commercial kit on the 21st day. d) The osteogenic differentiation of MSCs using alizarin red staining on the 21st day. The data are shown as mean±SD. ALP, alkaline phosphatase; COL1A1, collagen type I α 1 chain; MLT, melatonin; BIBP3226, NPY1R inhibitor; OCN, osteocalcin; Runx2, runt-related transcription factor 2; *p<0.05; **p<0.01; ***p<0.001. Control groups: A and B; B and C; C and D.

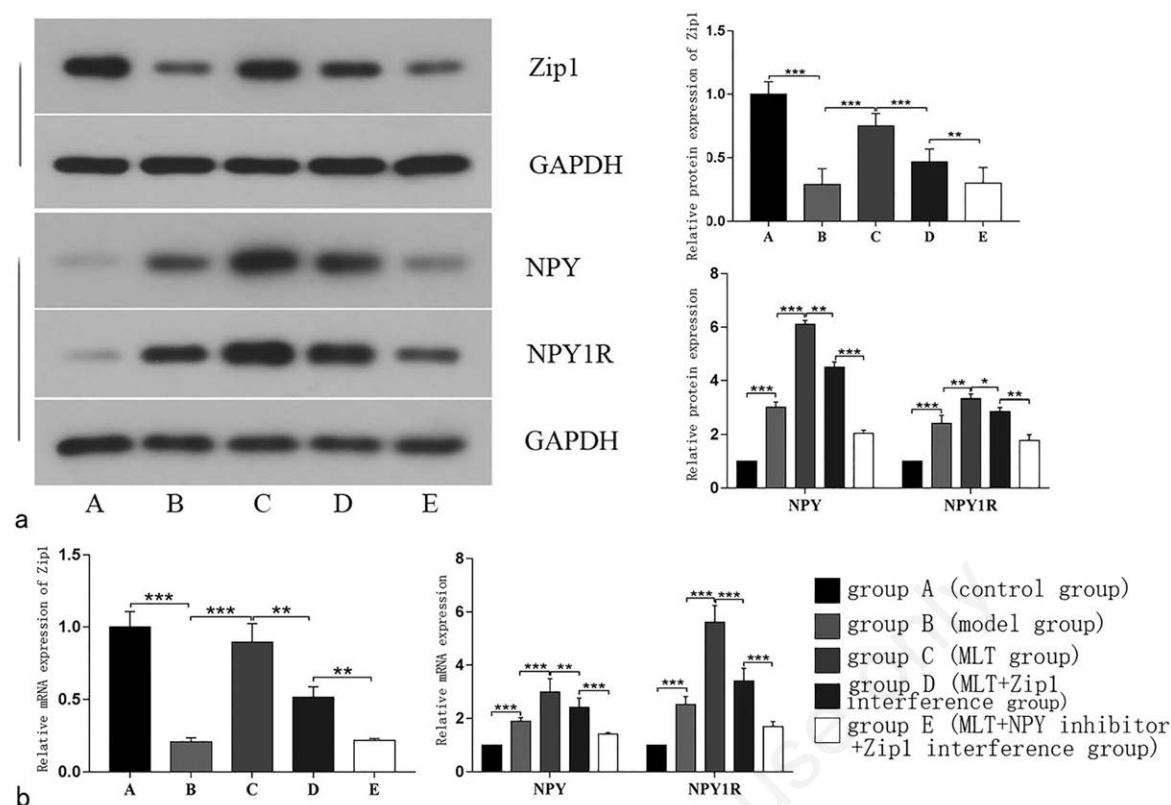


Figure 4. Rat model of femoral shaft fracture in each group (n=6) treated with 1% alcohol+NS (model group), MLT, MLT+Zip1 interfering lentivirus vector or MLT+BIBP3226+Zip1 interfering lentivirus vector for 8 weeks. a) Western blotting detection of the expression of Zip1, NPY and NPY1R in callus. b) Real-time PCR detection of the expression of Zip1, NPY and NPY1R in callus. Data are shown as mean±SD in panels a and b. MLT, melatonin; BIBP3226, NPY1R inhibitor; *p<0.05; **p<0.01; ***p<0.001. Control groups: A and B; B and C; C and D; D and E.

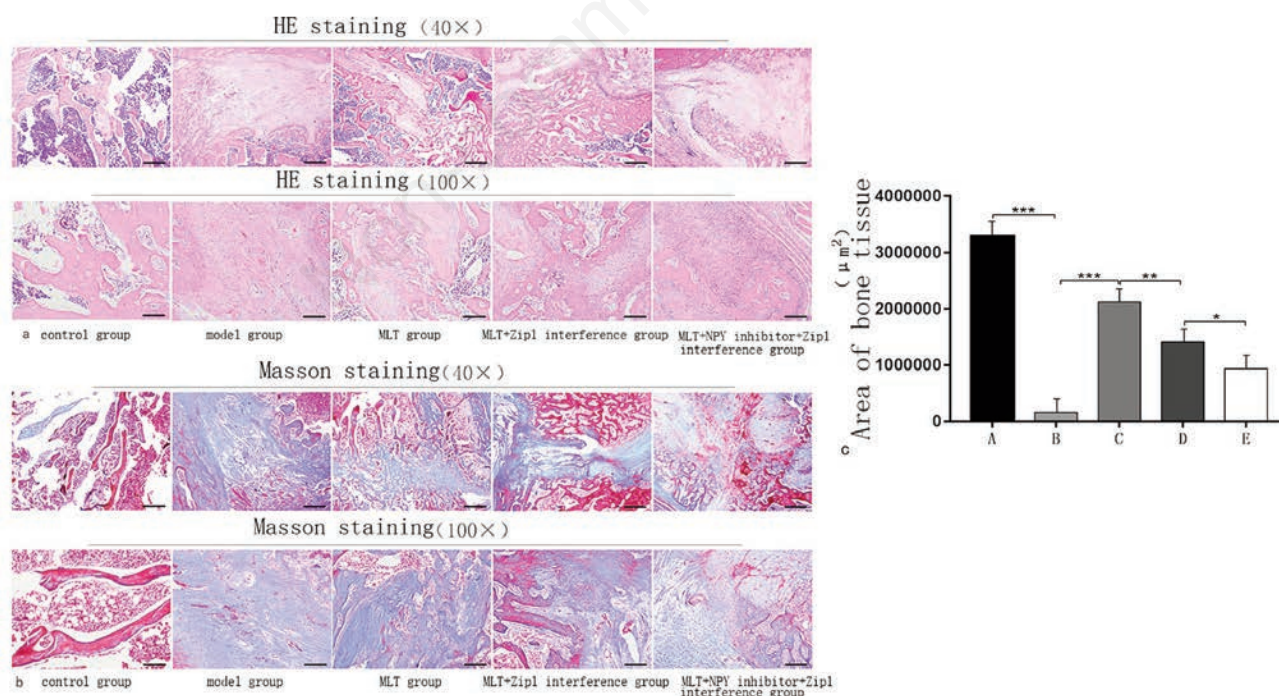


Figure 5. Rat model of femoral shaft fracture in each group (n=6) treated with 1% alcohol+NS (model group), MLT, MLT+Zip1 interfering lentivirus vector or MLT+BIBP3226+Zip1 interfering lentivirus vector for 8 weeks. a) H&E staining. b) Masson staining. c) Healing area of bone tissue for the evaluation of osteogenic differentiation. Data are shown as mean±SD in panel c. MLT, melatonin; BIBP3226, NPY1R inhibitor; *p<0.05; **p<0.01; ***p<0.001. Control groups: A and B; B and C; C and D; D and E.

Zinc is an essential element that plays an important role in bone growth and development, which has been gradually recognized in recent decades. Balanced zinc nutrition promotes the healthy development of bone, and zinc deficiency may inhibit the growth of bone and reduce bone density.^{30,31} Therefore, the homeostasis of zinc metabolism is of great significance in the process of fracture repair. Our previous study revealed that MLT treatment increased NPY levels.⁸ MLT exerts a protective role in N2a cells *via* the upregulation of the zinc transporter Zip1 under hypoxia. Zip1 functions as the major endogenous zinc uptake transporter, which transports zinc across the cell membrane.⁷ Increased Zip1 expression induced the osteogenic differentiation of MSCs.⁵ The overexpression of Zip1 may mediate the role of MLT in the promotion of the osteogenic differentiation of MSCs *via* regulation of NPY and NPY1R levels. Our further research will investigate whether Zip1 is also involved in the process of MLT promotion of MSCs proliferation and migration.

The present study suggests that Zip1 participates in MLT regulation of NPY and NPY1R to promote the osteogenic differentiation of MSCs and fracture healing. The findings in our study provide a reference to understand the mechanism of MLT and clarify the relationship between MLT and Zip1 in the promotion of fracture healing. The results provide further evidence for application in clinical settings.

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