Original Article

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Heterozygous deletion of *LRP5* gene in mice alters profile of immune cells and modulates differentiation of osteoblasts

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Summary **Skeletal homeostasis is dynamically influenced by the immune system. Low density lipoprotein receptor-related protein-5 (*LRP5*) is a co-receptor of the Wnt signaling pathway, which modulates bone metabolism in humans and mice. Immune disorders can lead to abnormal bone metabolism. It is unclear whether and how LRP5 alters the balance of the immune system to modulate bone homeostasis. In this study, we used primary osteoblast to detect the differentiation of osteoblasts *in vitro*, the immune cells of spleen and bone marrow of 6-month old *LRP5* heterozygote (HZ) and wild-type (WT) mice were analyzed by Flow cytometry. We found that LRP5$^{+/-}$ could influence the differentiation of osteoblasts by decreasing the mRNA level of *Osterix*, and increasing the mRNA level of *Runx2* and the ratio of receptor activator for nuclear factor-κB ligand/osteoprotegerin (*RANKL/OPG*). In the *LRP5*$^{+/-}$ mice, percentages of NK cells, CD3e^{+} cells, and CD8a^{+} T cells were increased in both spleen and bone marrow, and percentages of CD106^{+} cells and CD11c^{+} cells were increased in spleen while decreased in bone marrow, conversely, CD62L^{+} cells were decreased in spleen while increased in bone marrow compared to the WT mice. Percentages of CD4^{+} cells, CD14^{+} cells, and CD254^{+} cells were increased in the spleen, and CTLA4^{+} cells were increased in the bone marrow of the *LRP5*$^{+/-}$ mice. The mRNA level of Wnt signaling molecules such as β-catenin, and c-myc were decreased and APC was increased in spleen lymphocytes and bone marrow lymphocytes, and the mRNA level of Wnt3a was decreased in spleen lymphocytes while no change in bone marrow lymphocytes was seen with silencing LRP5 by specific small interfering RNA. In conclusion, heterozygous deletion of the *LRP5* gene in mice could alter the profile of the immune cells, influence the balance of immune environment, and modulate bone homeostasis, which might present a potential mechanism to explore the Wnt signaling pathway in the modulation of the immune system.**

Keywords: Low density lipoprotein receptor-related protein-5, osteoblast, RANKL, OPG, T cells, NK cells, CD254

1. Introduction

Osteoporosis is a bone disorder which is characterized by reduced bone strength and increased bone fragility. Patients with osteoporosis usually show an increase in bone transformation, leading to imbalance of absorption and bone formation (*1,2*). The differentiation of osteoblasts from bone marrow stromal cells is under control of multiple factors, including Wnt family proteins (*3*). Wnt ligands play a central role in development and homeostasis of various organs and activate two signaling pathways, the β-catenin-dependent canonical and β-catenin-independent noncanonical pathways.

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Low density lipoprotein receptor-related protein-5 (LRP5) is a transmembrane low-density lipoprotein receptor that shares a similar structure with LRP6 and acts as a co-receptor with LRP6 and the Frizzled protein family members for transducing signals by Wnt proteins through the canonical Wnt pathway. In the activated canonical Wnt/β-catenin pathway, Wnt-protein ligands such as Wnt1 and Wnt 3a can bind to a Frizzled family receptor to dissociate the degradation complex consisting of Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β), then promote the accumulation of β-catenin and its nuclear translocation to regulate expression of various critical genes such as cyclin D1 and c-Myc (*4*). LRP5 is closely related to osteoblast differentiation, bone density and/or osteoporotic fracture, which plays a key role in skeletal homeostasis (*5*). In recent years, much attention has been paid to the role of the Wnt/β-catenin pathway and LRP protein in the pathomechanism of osteoporosis, indicating a possible contribution of polymorphic variants of the candidate LRP5 gene in disease development (*6,7*). The bone mass regulation mechanism of LRP5 provides new therapeutic targets for osteoporosis and other diseases. The finding that LRP5 mutations lead to abnormalities in bone growth in humans has led to attempts to generate animal models for bone defects (*8*).

The viewpoint of osteoimmunology is that the immune system and immune factors play an important role in the development of osteoporosis. In the bone-tumor micro-environment, immune cells such as dendritic cells, monocytes/macrophages, myeloid-derived suppressor cells, regulatory T cells, T helper cells, neutrophils, $CD4^+$ T lymphocytes, $CD8^+$ cytotoxic T lymphocytes, and natural killer cells, could cooperate with bone cells and tumor cells to enhance bone metastasis and tumor progression (*9-11*). The current hypothesis of the immune system regulating bone formation and bone resorption is linked to discovery of RANK and its ligand RANKL. These molecules were originally thought to be secreted by T lymphocytes and dendritic cells. T lymphocytes secrete inflammatory factors and Wnt ligands to promote bone formation and absorption (*12-14*).

The Wnt signaling cascade plays an important role in development and differentiation of immune cells, in the tumor microenvironment, loss of co-receptors LRP5 and LRP6 in dendritic cells results in reduced tumor growth with enhanced antitumor immune responses (*15*). Although the *LRP5* gene is well-recognized for regulating bone metabolism, its role on the immune system in the bone microenvironment has not been revealed. In this study, we examined the influence of $LRP5^{+/-}$ to explore whether immune cells participate in the effect of LRP5 on bone metabolism. The findings suggest that heterozygous deletion of the *LRP5* gene could inhibit the differentiation of osteoblasts and alter the balance of immune cells, indicating that LRP5 plays an important role in osteoimmunology influencing bone homeostasis.

2. Materials and Methods

2.1. *Animals*

8-week-old $LRP5^{+/-}$ mice with a body mass of between 20 and 30 g were purchased from the Jackson Laboratory. The genotype of the $LRP5^{+/-}$ mice was identified by the standard protocol provided by the Jackson Laboratory, and the identified primer sequences are listed in Table 1. The experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by the local ethics committee. Throughout the study period, the mice were housed in a temperature- (23 ± 0.5°C) and humidity-controlled (43 ± 8%) environment under a 12 h light-dark cycle with food and water ad libitum.

2.2. *Primary osteoblast isolation and induced differentiation culture*

Primary osteoblast cultures were prepared from post-natal hippocampi (P0-P1) mouse pups as previously described (*16*). Briefly, cells were collected from the calvaria of newborn HZ or WT mice and extracted and digested in α-MEM which contained 0.1% collagenase and 0.2% dispase. The osteoblasts of each mouse were seeded in 6-well culture plates at a density of 5×10^5 living cells into serum-free and phenol red-free α-MEM which contained 10 μg/mL streptomycin and 10 units/mL penicillin. The osteogenic differentiation medium, containing serum-free and phenol red-free α-MEM, 20 mM ascorbic acid, 1 M β-glycerophosphate disodium salt hydrate, and 1 mM dexamethasone, was used to induce the differentiation of osteoblasts until the cells reached 80% confluence.

2.3. *Lymphocyte culture, LRP5 siRNA transfection*

Spleens were harvested from 8-weeks-old WT mice, using aseptic scissors to cut into small pieces gently and then passed through cell strainers. Red blood cells were lysed to gain spleen lymphocytes. The femur and tibia were removed from mice and cleaned of muscle and

Table 1. Summary of oligonucleotide primers for $LRP5^{+/-}$ mice genotyping

Oligonucleotide	Sequence* (5'-3')
Common forward primer	CACTGCATGGATGCCAGTGAGGTG
Wild-type reverse primer	GCTGCCACTCATGGAGCCTTTATGC
Mutant reverse primer	CGCTACCGGTGGATGTGGAATGTGT

*The oligonucleotide sequences for genotyping were obtained from the Jackson Laboratory website: *https://www.jax.org/search?q=+005823*

Table 2. The primers used in the study

Gene	Forward Primer	Forward Primer
LRP5	CTGTGCTGATGGGTCTGATG	CTGTGCTGATGGGTCTGATG
Collagen1	TGACTGGAAGAGCGGAGAGTA	TGACTGGAAGAGCGGAGAGTA
Runx2	GACAGTCCCAACTTCCTGTG	GACAGTCCCAACTTCCTGTG
Osterix	GCTCGTAGATTTCTATCCTC	GCTCGTAGATTTCTATCCTC
OPG	CCTTGCCCTGACCACTCTTAT	CCTTGCCCTGACCACTCTTAT
RANKL	CAAGATGGCTTCTATTACCTGT	CAAGATGGCTTCTATTACCTGT
Wnt3a	CTCCTCTCGGATACCTCTTAGT	CTCCTCTCGGATACCTCTTAGT
β-cateinin	ATCACTGAGCCTGCCATCTG	ATCACTGAGCCTGCCATCTG
C -myc	CAGCTGCTTAGACGCTGGATT	CAGCTGCTTAGACGCTGGATT
APC	TTGTGGAATCTCTCAGCAAGAA	TTGTGGAATCTCTCAGCAAGAA
GAPDH	GTTGTCTCCTGCGACTTCA	GTTGTCTCCTGCGACTTCA

LRP5: low density lipoprotein receptor-related protein-5; *Runx2*: runt-related transcription factor 2; *OPG*: osteoprotegerin; *RANKL*: nuclear factor-κ B ligand; *APC*: *Adenomatous Polyposis Coli*.

connective tissue. The ends of the bones were cut, and bone marrow (BM) cells were flushed out with media using a needle attached to a syringe. Red blood cells were lysed to obtain BM lymphocytes. BM-derived lymphocytes were seeded in 6-well culture plates at a density of 10^6 cells/mL and cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1x penicillin/streptomycin/neomycin, and 10 mM HEPES buffer. A double-stranded small interfering RNA (siRNA) against the LRP5 transcript or the negative control (Shanghai GenePharma Co., Ltd., Shanghai, China) was transfected into lymphocytes in six-well cluster plates using EntransterTM-R4000 transfection reagent (Engreen Biosystem, Beijing, China) in accordance with the manufacturer's instructions. Transfected cells were harvested for extraction of mRNA after 24 h.

2.4. *RNA extraction, reverse transfection, and real-time RT-PCR*

For PCR analysis, total RNA was isolated with an RNA extraction Kit (Axygen, CA, USA) according to the manufacturer's protocol, and the concentration of total RNA was measured with a NanoDrop 2000c (Thermo, Fisher, MA, USA). RNA (1 μg) was converted into cDNA using reverse transcriptase (Promega, Madison, USA). RT-PCR was performed using SYBR Premix Ex Taq (Takara Bio, Tokyo, Japan). The cDNA levels were determined using the housekeeping gene GAPDH, which was amplified in parallel with the gene to be analyzed and used to normalize the results. The threshold cycle (Ct) values were calculated using software supplied by the Applied Biosystems 7900 Real-time PCR system. Primer sequences are listed in Table 2.

2.5. *Quantitative detection of alkaline phosphatase (ALP) activity*

5×10^5 primary osteoblasts were seeded into 6-well culture plates and cultured until they reached 90% confluence. The cells were digested with pancreatin and collected into a 1.5 mL EP tube. The ALP kit was used to detect the ALP activity with the supernatant from the cells handled by cell lysate according to the reagent instructions.

2.6. *Reagents*

Flow cytometry antibodies FITC-conjugated anti-pan-NK/CD103/CD106/TCRgd, APC-conjugated anti-cytotoxic T lymphocyte antigen-4 (CTLA-4)/CD62L, PE-conjugated anti-CD4/CD14/CD19/CD254, PEcy5.5-conjugated anti-CD3e/CD11c, APC-efluor780-conjugated anti-CD8a, and their corresponding isotypes were purchased from Biolegend (San Diego, CA, USA).

2.7. *Flow cytometry analysis*

Splenocytes and bone marrow cells were obtained and 1×10^6 cells of each sample were incubated with chosen antibodies for 30 minutes on ice, then washed and resuspended in PBS which contained 0.1% sodium azide and 1% bovine serum albumin. The non-specific signal was identified by rat FITC- and PE-conjugated IgG isotype controls. Approximately 50,000 stained cells in each sample were analyzed by a CyAN ADP flow cytometer (Beckman Coulter, Brea, CA, USA), and each sample was examined twice. Data are expressed as the percentage of positive cells.

2.8. *Statistical analysis*

All data are presented as the mean ± SEM, and the means were statistically analyzed using Student's *t*-test as appropriate. All assays were repeated at least three times. Significance levels were noted as follows: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

3. Results

3.1. *The identification of LRP5$^{+/-}$ mice*

The transgene identification of *LRP5*$^{+/-}$ mice was confirmed by the standard protocol provided by the

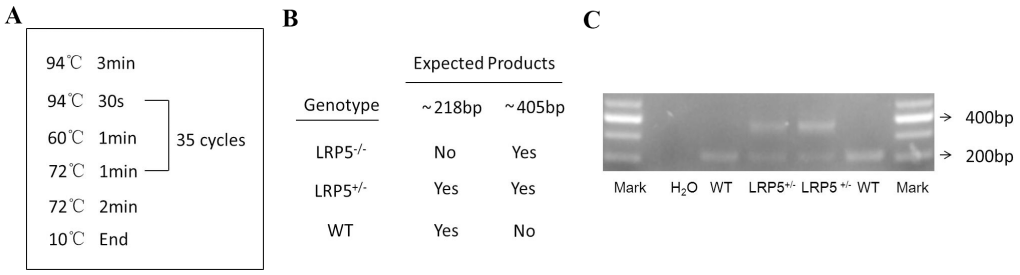

Figure 1. The gene identification of *LRP5*$^{+/-}$ mice. (A) The master protocol details provided by the Jackson Laboratory. **(B)** The expected results of mice. Mutant = 405 bp, Heterozygote = 218 bp and 405 bp, Wild type = 218 bp. **(C)** The gel image of PCR products.

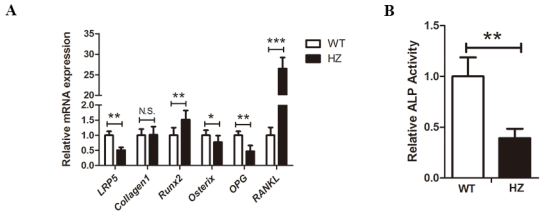

Figure 2. Heterozygous deletion of *LRP5* gene impaired the viability of osteoblasts and inhibited the differentiation of osteoblasts in vitro. (A) *LRP5*$^{+/-}$ affected mRNA expression of osteoblastogenesis-related genes, including *Runx2*, *Osterix*, *Collagen I*, *OPG*, and **RANKL**. **(B)** The ALP activity of osteoblasts was determined after 7 days of culture. *$p < 0.05$. All results are expressed as the mean ± SEM, and data are representative of at least three experiments.

Jackson Laboratory (Stock Number 005823, Strain Name: B6.129P2-Lrp5 tm1Dgen /J). The expected products' sizes of heterozygote were 218bp and 405bp (Figure 1). The mRNA expression level of LRP5 was lower in the HZ mice than in the WT mice (Figure 2A).

3.2. *Heterozygous deletion of LRP5 gene decreased ALP activity and inhibited the differentiation of osteoblasts in vitro*

WT and HZ mice were used and the mRNA expression level of *Runx2* and *Osterix* were detected as important transcriptional molecules in differentiation of osteoblasts. The mRNA levels of the *Osterix* gene was inhibited while the *Runx2* gene was increased in *LRP5*$^{+/-}$ mice (Figure 2A). Heterozygous deletion of the *LRP5* gene could increase the mRNA expression levels of *RANKL* and decrease the mRNA levels of *OPG*, while it showed no effect on the modulation of collagen1 (Figure 2A). After 7 days of cell differentiation culture to analyze vitality, the result showed that the ALP activity of HZ mice was decreased compared with WT mice (Figure 2B).

3.3. *Heterozygous deletion of LRP5 gene in mice modulated the innate immune cells*

Myeloid progenitors for osteoclasts can also differentiate into dendritic cells (DC) and macrophages, suggesting a close relationship of osteoclasts to cells of the innate immune system. Then we explored whether heterozygous deletion of *LRP5* gene could modulate the profile of innate immune cells in the spleen and bone marrow. Compared with WT mice, the percentage of NK cells increased in the HZ mice (Figure 3A and 3B), while the percentage of γδT cells showed no difference in the two groups in both spleen and bone marrow (Figure 3C). The proportion of $CD14^+$ cells increased in the spleen of HZ mice while there was no significant change in the bone marrow (Figure 3D and 3E). In HZ mice, we found that the $CD11c^+$ cells were increased in the spleen, while decreased in the bone marrow (Figure 3F and 3G).

3.4. *Heterozygous deletion of the LRP5 gene in mice regulated the balance of T cells while had no effect on B cells*

Skeletal homeostasis is dynamically influenced by the immune system. Bone destruction is attributable to excessive bone resorption by osteoclasts, the formation of which is directly and indirectly regulated by T cells. We found that heterozygous deletion of the *LRP5* gene could increase the percentage of $CD4^+$ T cells in the spleen, and increase $CD8a^+$ cells in both spleen and bone marrow (Figure 4A-4D). Further analysis of the immunophenotype of *LRP5*$^{+/-}$ mice showed that the $CD62L^+$ cells decreased in the spleen while increased in the bone marrow in HZ mice (Figure 4E-4G), and the $CD3e^+$ cells increased in both spleen and bone marrow in HZ mice compared to WT mice (Figure 4H, 4I). B cells are the main source of OPG in the bone marrow, but our result found no significant change in both spleen and bone marrow after heterozygous deletion of the *LRP5* gene (Figure 4J).

3.5. *Heterozygous deletion of LRP5 gene in mice regulated the functional immune cells to modulate osteoimmunology*

RANK and its ligand (RANKL; also known as CD254, OPGL, and TRANCE) are key regulators of bone

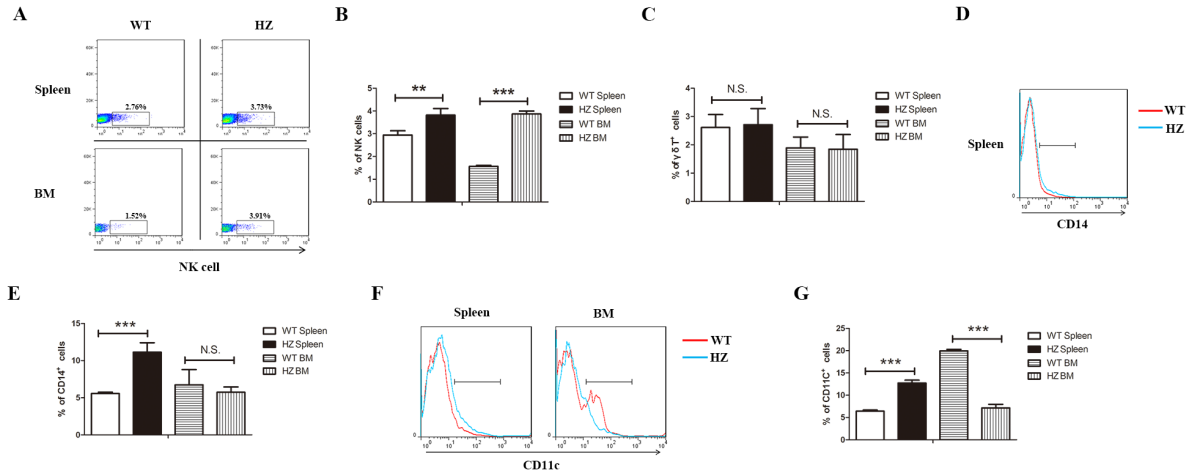

Figure 3. Proportion of innate immune cells in the spleen and bone marrow of *LRP5*$^{+/-}$ mice. (A) Flow cytometry of NK cells from each group. **(B)** Frequency of NK cells in mice as in A. **(C)** Frequency of γδT cells in each group. **(D)** Flow cytometry of CD14^{+} cells from spleen. **(E)** Frequency of CD14+ cells in each group. **(F)** Flow cytometry of CD11c^{+} cells from each group. **(G)** Frequency of CD11c^{+} cells in mice as in **F**. *$p < 0.05$. All results are expressed as the mean ± SEM, and data are representative of at least three experiments.

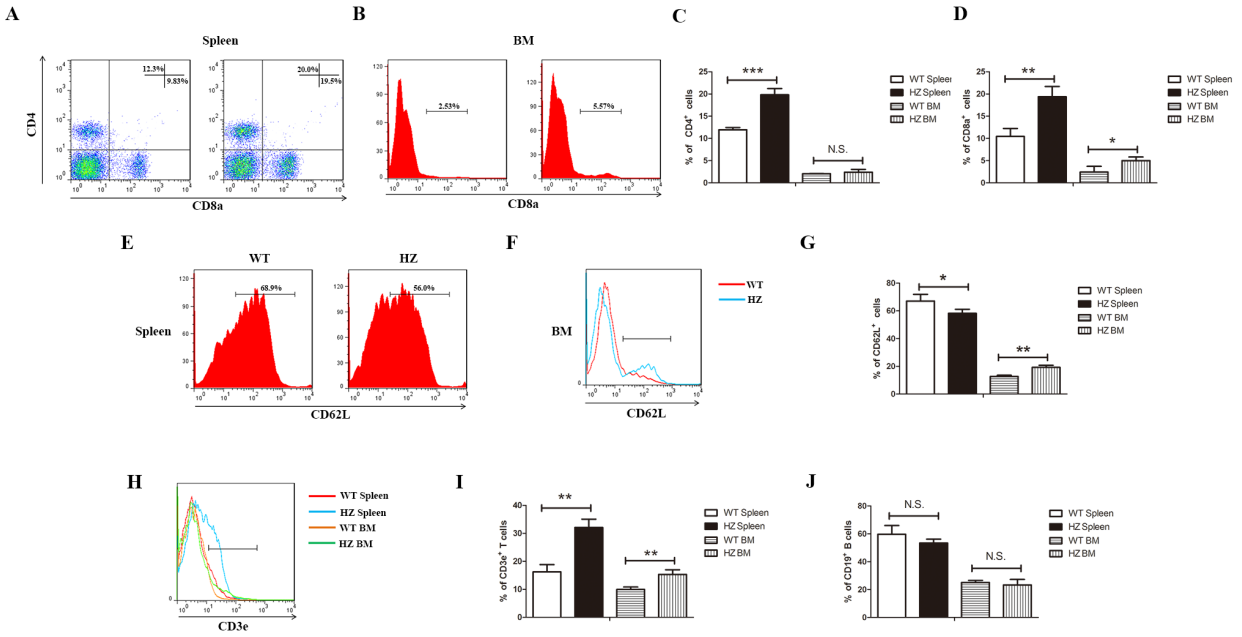

Figure 4. Heterozygous deletion of *LRP5* gene regulated the balance of T cells. (A) Flow cytometry of CD4^{+} and CD8^{+} T cells in spleen from HZ mice and WT mice. **(B)** Flow cytometry of CD8^{+} T cells in bone marrow from HZ mice and WT mice. **(C,D)** Frequency of CD4^{+} and CD8^{+} T cells in mice as in A and B. **(E,F)** Flow cytometry of CD62L^{+} cells from each group. **(G)** Frequency of CD62^{+} cells in mice as in E and F. **(H)** Flow cytometry of CD3e^{+} cells from each group. (I) Frequency of CD3e^{+} cells in mice as in H. **(J)** Frequency of CD19^{+} B cells from each group. *$p < 0.05$. All results are expressed as the mean ± SEM, and data are representative of at least three experiments.

remodeling, mammary gland formation, lymph node development, and T-cell/dendritic cell communication. Here we found that the percentage of CD254^{+} cells was increased in the spleen, while there was no significant change in the bone marrow in HZ mice (Figure 5A and 5B). As CD106 is a novel mediator of bone marrow mesenchymal stem cells *via* NF-κB in the bone marrow of acquired aplastic anemia, we also found the CD106^{+} cells increased in the spleen while they decreased in the bone marrow in HZ mice compared to WT mice (Figure 5C and 5D). The enhanced osteogenesis of mesenchymal stem cells (MSCs) modified by cytotoxic T lymphocyte-associated antigen 4 (CTLA4) has been shown in previous studies. The heterozygous deletion of the *LRP5* gene could evidently increase CTLA4^{+} cells in bone marrow (Figure 5E). CD103 is a representative molecular of dendritic cells as well as a hallmark of tumor-infiltrating regulatory T cells and modulates immune inflammatory disease. In this study we found there was no significant difference of the CD103^{+} cells

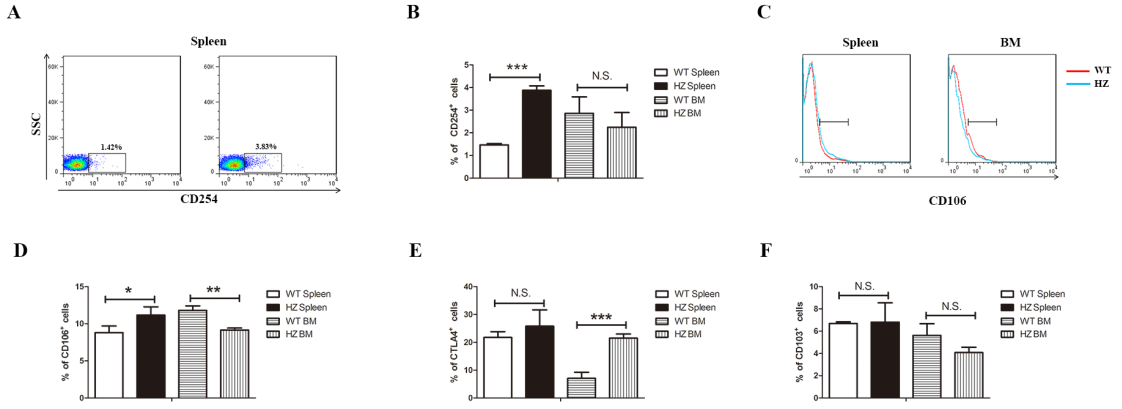

Figure 5. Heterozygous deletion of the *LRP5* gene regulated the functional immune cells. (A) Flow cytometry of $CD254^+$ T cells in the spleen from HZ mice and WT mice. **(B)** Frequency of $CD254^+$ from each group. **(C)** Flow cytometry of $CD106^+$ cells from each group. **(D)** Frequency of $CD254^+$ cells in mice as in C. **(E,F)** Frequency of $CTLA4^+$ cells and $CD103^+$ from each group. $*p < 0.05$. All results are expressed as the mean ± SEM, and data are representative of at least three experiments.

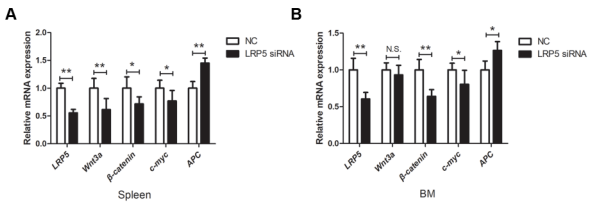

Figure 6. Silencing *LRP5* by specific small interfering RNA regulated Wnt signaling molecules of lymphocytes. The mRNA expression levels of Wnt3a, β-catenin, c-myc and APC in the WT spleen **(A)** and the bone marrow **(B)** lymphocytes treated with LRP5 siRNA or NC. $*p < 0.05$. All results are expressed as the mean ± SEM, and data are representative of at least three experiments.

in two groups of mice (Figure 5F).

3.6. *Wnt signaling pathway involved in the modulation of immune system with silencing of LRP5 by specific small interfering RNA*

As *LRP5* gene plays the part of co receptor in the Wnt signal, we examined whether the Wnt signaling pathway is involved in the regulation of immune system with low expression of LRP5. We found the mRNA levels of Wnt3a, β-catenin, and c-myc were decreased and the mRNA level of APC was increased in spleen lymphocytes treated with LRP5 siRNA compared with NC (Figure 6A). The mRNA levels of β-catenin, and c-myc were also decreased and the mRNA level of APC was increased in the bone marrow lymphocytes treated with LRP5 siRNA compared with NC, while the mRNA level of Wnt3a had no significant change (Figure 6B).

4. Discussion

The immune system and skeletal system share a variety of molecules, including cytokines, chemokines, hormones, receptors, and transcription factors. Bone cells interact with immune cells under physiological and pathological conditions (*17*). In the present study, we found *LRP5*$^{+/-}$ could inhibit the differentiation of osteoblasts and then investigated the immune cells in the spleen and bone marrow in *LRP5* HZ mice, the findings can be summarized as follows: heterozygous deletion of the *LRP5* gene can modulate the balance of T cells and innate immune cells in spleen and bone marrow congruously, such as increasing $CD8^+$ T cells, $CD4^+$ T cells, NK cells, total $CD3e^+$ cells, $CD14^+$ cells, $CD106^+$ cells, $CD11c^+$ cells and $CD254^+$ cells in spleen; and there are some other change trends in bone marrow, with increasing the percentage of NK cells and $CD3e^+$ cells, $CD8^+$ cells and $CD62L^+$ cells, while decreasing the percentage of $CD106^+$ cells, $CD11c^+$ cells; and there is no effect on the levels of B cells in both spleen and bone marrow.

LRP5 is the key mediator of β-catenin dependent Wnt signaling. However, very little is known about their specific roles in regulating bone-metabolism gene expression in osteoblasts. The OPG/RANKL/RANK system plays an important role in regulating the balance of osteoblast and osteoclast activity, preventing bone loss and ensuring normal bone regeneration (*18,19*). Both Runx2 and Osterix are important transcription factors in the process of osteoblast differentiation, Runx2 is expressed in the early stages of osteoblast differentiation, while Osterix is expressed in the late stage (*20*). The results that the mRNA expression of Runx2 was increased while Osterix was decreased in the *LRP5*$^{+/-}$ mice revealed that heterozygous deletion of the *LRP5* gene could induce the differentiation disorder in the late stage. The results that the activity of ALP in *LRP5*$^{+/-}$ mice was inhibited and the osteoblast differentiation related-genes were changed indicates that LRP5 participates in the modulation of Osteoporosis.

In previous studies, osteoimmunology was created as a new interdisciplinary field in large part to highlight

the shared molecules and reciprocal interactions between the two systems in both health and disease (*21*). The ratio of NK cells in *LRP5*$^{+/-}$ mice is higher suggesting that NK cells are in an activation form and have the ability to produce an immune response, while NK cells may play a role in bone formation and bone loss. Myeloid progenitors for osteoclasts can also differentiate into DCs and macrophages, suggesting a close relationship of osteoclasts to cells of the innate immune system (*22*). Previous studies have implicated that γδ T cells can be activated and inhibit osteoclast differentiation and absorptive activity in animal models of rheumatoid arthritis (RA). Interestingly, both human and mouse CD11c^{+} DC can differentiate into osteoclasts *in vitro* and mouse DC can contribute to osteoclast formation *in vivo* (*23,24*). As we found CD11c^{+} cells were increased in spleen and decreased in bone marrow, while the γδ T cells had no influence on the *LRP5*$^{+/-}$ mice, this indicated the *LPR5* gene may modulate osteoclast formation but have no effect on the differentiation of osteoblasts.

The stimulation and inhibition roles of T cells to osteoblasts and osteoclasts are closely related to the subsets of T cells, cytokines and local factors. The activation of CD8^{+} T cells in bone and the immune system plays a key role in maintaining the balance of osteoclasts-osteogenesis (*25,26*). Activation of CD4^{+} T cells can directly secrete soluble RANKL, TNF-α or IL-1, IL-6 and IL-17 to promote the formation of osteoclasts in inflammation diseases, such as rheumatoid arthritis, periodontitis, congenital adrenocortical hyperplasia and osteoporosis (*27,28*). Part of the TCR-CD3 complex present on the T-lymphocyte cell surface plays an essential role in the adaptive immune response (*29*). In our study, we found that *LRP5*$^{+/-}$ increased the proportion of CD4^{+} T cells, CD8^{+} T cells and CD3e^{+} cells, while there was no effect on B cells, this change in *LRP5*$^{+/-}$ mice may indicate that LRP5 could disturb the balance of T cells to promote the development of OP.

Neutrophilia is a normal response of an organism during inflammation. Various mechanisms are involved in the appearance of this response, including release of neutrophils from the bone marrow as well as demargination. Down-regulation of the CD62L antigen is regarded as a possible mechanism for neutrophilia during inflammation (*30,31*). We found the expression of CD62L was decreased in spleen and increased in bone marrow, indicating *LRP5*$^{+/-}$ might influence the inflammatory reaction to promote the OP.

The current hypothesis of the immune system regulating bone formation and bone resorption is linked to the discovery of RANK and its ligand RANKL (also known as CD254). RANKL and RANK increase the rolls of DCs, stimulate the proliferation of initial T cells, and improve the survival of DCs (*32,33*). We found the CD254^{+} cells were increased in spleen in

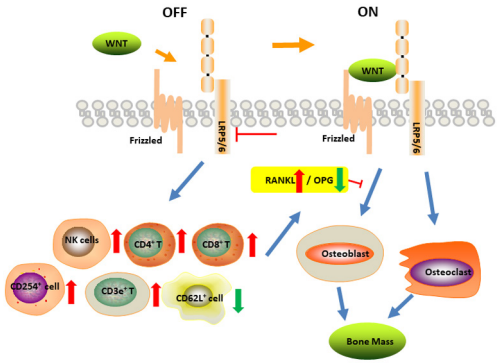

Figure 7. Schematic diagram of heterozygous deletion of the *LRP5* gene. Inhibition of the Wnt signal by heterozygous deletion of the *LRP5* gene influences the formation of bone mass through influencing the differentiation of osteoblasts, accompanied by disturbance of the balance of immune cells with an increased percentage of NK/CD3e^{+}/CD14^{+}/CD106^{+}/CD11c^{+}/CD4^{+}/CD254^{+}/CD8a^{+} cells and decreased CD62L^{+} cells in the spleen.

LRP5$^{+/-}$ mice, indicating that *LRP5*$^{+/-}$ could impact bone resorption. CD106 is a marker of placental chorionic mesenchymal stem cells with strong immune-modulatory function, and mesenchymal stem cells can differentiate into osteoblasts (*34*). It has been suggested that activation of the immune system by T cells has a negative impact on the reconstruction of bone within bone defects. As a vital co-inhibitory molecule expressed on activated T cells, CTLA4 can block the B7-CD28 co-stimulatory pathway to induce immune tolerance (*35*). CD103^{+} dendritic cells can combine with CD4^{+} T cells to initiate the classical RANKL/RANK osteoclast signaling pathway and participate in the formation of osteoporosis (*36*). In our study, *LRP5*$^{+/-}$ could disturb the percentage of CD106^{+} cells in spleen and bone marrow, and increase the CTLA4^{+} cells while having no effect on CD103^{+} cells. These functional immune cells may participate in osteoimmunology by modulated LRP5.

Increasing evidence indicates that Wnt/β-catenin signaling promotes some disease progression such as cancer and autoimmune disease by regulating dendritic cells, T cells, NK cells and tumor cells. Modulating the expression of LRP5 could influence the Wnt signal and the balance of immune cells directly. We propose that targeting Wnt/β-catenin signaling would potentially modulate the immune system to regulate bone metabolic disease.

In summary, we report in the present study that *LRP5*$^{+/-}$ can influence the differentiation of osteoblasts and disturb the balance of immune cells by modulating the Wnt signing pathway. This induction involves an increased percentage of NK/CD3e^{+}/CD14^{+}/CD106^{+}/CD11c^{+}/CD4^{+}/CD254^{+}/CD8a^{+} cells in the spleen of *LRP5*$^{+/-}$ mice (Figure 7). Thus, the role of LRP5 should be investigated further to examine the pathogenesis of osteoimmunology.

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