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Laccase Domain-Containing Protein 1 Promoted Inflammation of Osteoporosis Model via NOD2 Ubiquitination by RIP2

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KEYWORDS Laccase Domain-Containing Protein 1. Osteoporosis. Receptor Interacting Protein 2. Nucleotide-Binding Oligomerization Domain 2

ABSTRACT The purpose of this experiment was to elucidate the latent influence of Laccase Domain-Containing Protein 1 (LACC1) regulatory loop in the progression of osteoporosis. Serum samples of volunteers were obtained from the researchers' hospital. Mice were induced by an osteoporosis model using ovariectomy. LACC1 mRNA expression levels were up-regulated in patients with osteoporosis. LACC1 protein and mRNA expression levels in mice with osteoporosis were measured. LACC1 promoted inflammation of osteoporosis in the in vitro model. LACC1 promoted osteoporosis in the mice model. LACC1 protein interlinked nucleotide-binding oligomerisation domain 2 (NOD2)/receptor interacting protein 2 (RIP2) protein to reduce ubiquitination of NOD2. NOD2/RIP2 participated in the effects of LACC1 in the model of osteoporosis. These findings suggest that LACC1 promoted inflammation of the osteoporosis model via the induction of NOD2-RIP2 signalling by the inhibition of NOD2 ubiquitination. The inhibition of LACC1 regulatory loop provides new targets for treatment of osteoporosis.

INTRODUCTION

With the aggravation of the population ageing trend, the morbidity of osteoporosis (OP) shows an apparently increasing trend (Babiuch et al. 2021). The OP-induced complications such as ostealgia, humpback and thoracolumbar compression fracture have severely affected the quality of life of OP patients, which not only pose a severe threat to human health, but also bring tremendous burdens on both the family and the society (Kendler et al. 2018; Campisi et al. 2021). According to statistics, there are over 0.2 billion patients with OP in the world, while in China, over 1/3 of the elderly aged over 50 years are affected by OP, and OP has become an increasingly severe public health problem (Cheon et al. 2021).

Inflammation-related OP refers to the osteopenia and bone destruction induced by inflammation, especially systemic inflammation (Al-Daghri et al. 2017). Experiments discovered that in patients with rheumatoid arthritis (RA), the cortical bone of metacarpal shaft experiences an early-phase rapid bone loss stage, followed by a gradually slowing bone loss stage, and systemic inflammation is an independent risk factor for OP (Guo et al. 2019; Fang et al. 2020).

NOD-like receptors (NLRs) have been verified to be the major mediators of inflammatory response and immunity (Ke et al. 2017; Ding et al. 2022; Liu et al. 2022). NOD2 is one of the major members of the NLRs family, which extensively participates in the recognition of immune cell pathogens and the induction of inflammatory response as an important intracellular pattern recognition receptor (PRR) (Posovszky et al. 2013). It has been proved that NOD2 is involved in inflammatory response, and excessive inflammatory response will aggravate injury (Hoffmann et al. 2021). The activated NOD2 receptor can recruit RIP2 molecules, and excessive expression of RIP2 can activate the transcriptional factor NF-κB, which thus triggers the NF-κB-dependent target gene transcription and secretes inflammatory factors (Pu et al. 2020a; Takada et al. 2021).

Laccase Domain-Containing Protein 1 (LACC1) is the amino acid sequence deduced by the laccase annotation gene *Lacc1*, which belongs to the multicopper oxidase (MCO). LACC1 is also known as multicopper oxidoreductases. LACC1 was required for MAPK and NFκB pathway activation and cytokine secretion in primary human monocyte-derived macrophages (MDMs).

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Objective of the Study

The purpose of this experiment was to elucidate the latent influence of LACC1 regulatory loop in the progression of osteoporosis.

MATERIAL AND METHODS

Clinical Samples

Serum samples of volunteers were obtained from the researchers' hospital from May 2020 to November 2020. The number of patients with osteoporosis was 24 and the number of normal healthy volunteers was 24. This study was approved by the ethics committee of the researchers' Hospital. The written consent was received from all participants.

Microarray Analysis, PCR and ELISA Kit for Inflammation

After extracting total RNA from serum samples, use NanoDrop 1000 to quantify the amount of RNA. Microarray Analysis were executed as per literature (Pu et al. 2020b). Total cellular RNA was extracted from serum or cell samples using Trizol (Thermo Fisher Scientific). By using Moloney mouse leukemia virus reverse transcriptase, RNA was reverse transcribed into cDNA . By the Real-Time PCR System, the mRNA expressions were quantified.

According to the manufacturer's instructions, Inflammatory factor levels in cultured supernatants were then quantified using an ELISA kit .

Animal Experiments

In this study, we used C57BL/6 mice (eleven-week-old). Standard guidelines (humidity: 60±5%; 23±2°C) were used for maintaining the animals with a 12-hour light and dark cycle. Mice were induced by intraperitoneal (IP) injection of pentobarbital sodium at the dosage of 50 mg/kg and induced osteoporosis model using ovariectomy.

Cell Culture and Transfection

Bone marrow macrophages (BMMs) were isolated from female C57BL/6J mice. BMMs were cultured in α-MEM medium (GE Healthcare, Pittsburgh, USA) containing ten percent FBS (Gibco, Rockville, USA) and 50 ng/mL M-CSF for 24 hours. Transfection vectors and si-mimics were provided by GenePharma (Shanghai, China). BMMs were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Western Blot

Cells or bone tissue samples were collected and lysed in a lysis buffer on ice. Total proteins were separated by ten percent SDS-PAGE and blotted on the PVDF membrane. At 4°C overnight, membranes were blocked with ten percent non-fat milk powder and incubated with primary antibodies of anti-β-Actin body (3700, 1:5000, Cell Signaling Technology, Inc.), anti-RIP2 body (ab8428, 1:1000, Abcam), anti-NOD2 body (ab188646, 1:1000, Abcam) and anti-LACC1 body (ab108597, 1:1000, Abcam). Bound antibodies were detected using a Chemiluminescence Kit and horseradish peroxidase-labelled secondary IgG (dilution 1:1000).

Statistical Analysis

Data were presented as mean ± standard deviation (SD) using SPSS version 21.0 software. Difference was considered significant at $P < 0.05$. Student's t-test and multiple comparison were used to compare the two groups.

RESULTS

Expression Levels of LACC1 in Patients or Mice Model of Osteoporosis

To confirm the expression levels of LACC1 in the model of osteoporosis, real-time quantitative RT-PCR were executed to measure LACC1 expression levels.

Real-time quantitative RT-PCR in patients with osteoporosis was performed to explore the expression levels of LACC1 in the model of osteoporosis. LACC1 mRNA expression levels were up-regulated in patients with osteoporosis (1.078±0.368 vs 6.302±1.628, Fig. 1A). LACC1 protein and mRNA expression levels in mice with osteoporosis (1.039± 0.074 vs 3.510±0.486, Fig. 1B-1D).

LACC1 Promoted Inflammation of Osteoporosis In Vitro Model

To explore the possible pro-inflammation of LACC1 in inflammation of osteoporosis, the researchers analysed the pro-inflammation effects of LACC1 in vitro model of osteoporosis. LACC1

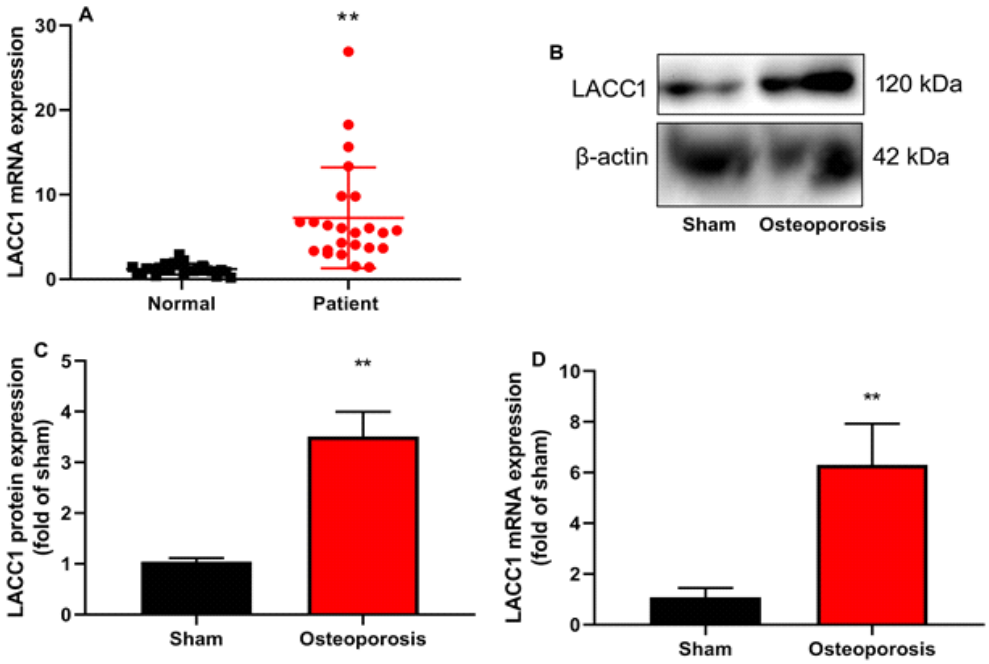

Fig. 1. LACC1 promoted inflammation of osteoporosis in vitro model
LACC1 mRNA expression (A) in patients with depression-like behaviors;
LACC1 protein expression (B) and mRNA expression (C and D) in mice with osteoporosis
****p<0.01 compared with normal or sham group**

plasmid increased the expression of LACC1 mRNA (1.120±0.562 vs 6.038±1.068), and promoted INF-γ (56.67±9.39 vs 123.67±12.28 pg/ml), IL-1β (442.5± 48.99 vs 1005±69.55 pg/ml), IL-6 (171.33±17.15 vs 243.33±23.79 pg/ml), TNF-α (541.67±159.23 vs 1355±139.28 pg/ml) and IL-18 (723.33±4.19 vs 1640± 30.54 pg/ml) levels in vitro model (Fig. 2A-2F).

Si-LACC1 reduced LACC1 mRNA expression, and inhibited INF-γ (54.33±3.86 vs 14±2.16 pg/ml), IL-1β (442.5±16.20 vs 190±36.91 pg/ml), IL-6 (79.33± 7.41 vs 22±2.94 pg/ml), TNF-α (601.67± 254.2090129 vs 183.3333333±87.31 pg/ml) and IL-18 (786.67± 1.699 vs 256.67±2.86 pg/ml) levels in the in vitro model (Fig. 2G-2L).

LACC1 Promoted Osteoporosis in Mice Model

Subsequently, the researchers explained the effects of LACC1 in the mice model of osteoporosis. LACC1 recombination protein reduced connectivity density of bone (39.40±1.90 vs 26.13±1.92 1/mm3), trabecular number (0.55±0.06 vs 0.21±0.02 %) and ratio of bone volume to total volume (5.67±0.66 vs 3.67±0.33 1/mm2), expanded osteoporosis in mice model (Fig. 3A-3D). LACC1 recombination protein promoted INF-γ(59.33±1.25 vs 163±9.09 pg/ml), IL-1β (427.5±40.16 vs 937.5±69.01 pg/ml), IL-6 (148±8.64 vs 328.67±20.67 pg/ml), TNF-α (575±123.29 vs 1100± 174.55 pg/ml) and IL-18 (541.67±26.25 vs 1128.33±94.19 pg/ml) in bone tissue of mice model of osteoporosis (Fig. 3E-3I).

LACC1 Regulated NOD2/RIP2 in Model of Osteoporosis

The researchers next analysed the mechanism of LACC1 on osteoporosis using the gene chip assay (Fig. 4A-4B). LACC1 protein induced NOD2 (0.868±0.108 vs 2.745±0.339) and RIP2 (1.172±0.133 vs 2.695±0.471) protein expression levels in mice model of osteoporosis (Fig. 4C-4D). Next, in the in vitro model, over-expression of LACC1 induced NOD2 (1.098±0.427 vs 7.057±1.433) and RIP2 (1.067±

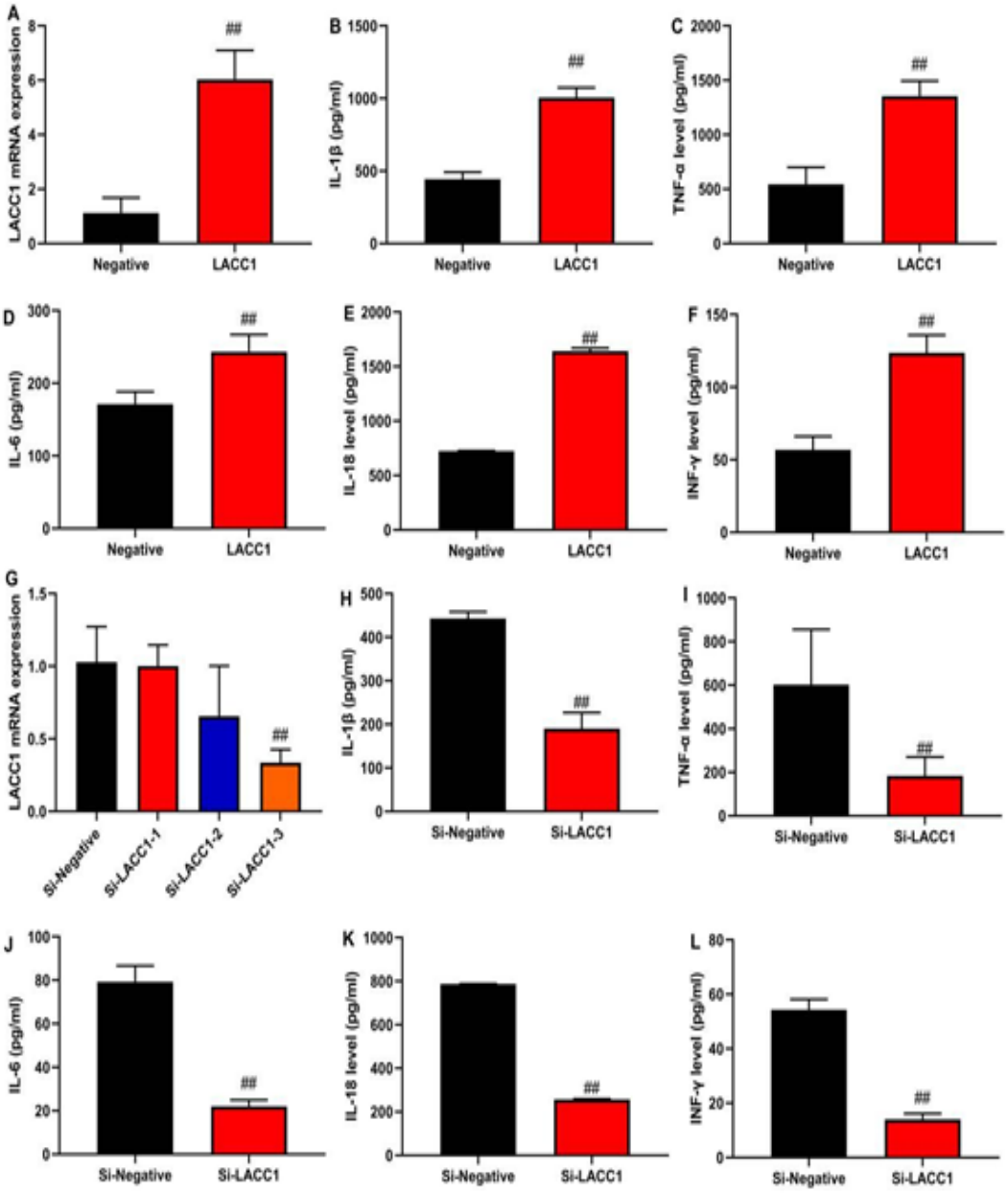

Fig. 2. LACC1 promoted inflammation of osteoporosis in vitro model
LACC1mRNA expression (A), IL-1β (B), TNF-α (C), IL-6 (D), IL-18 (E), INF-γ (F) in vitro model of LACC1; LACC1 mRNA expression (G), IL-1β (H), TNF-α (I), IL-6 (J), IL-18 (K), INF-γ (L) in vitro model of si-LACC1 ##p<0.01 compared with control negative group

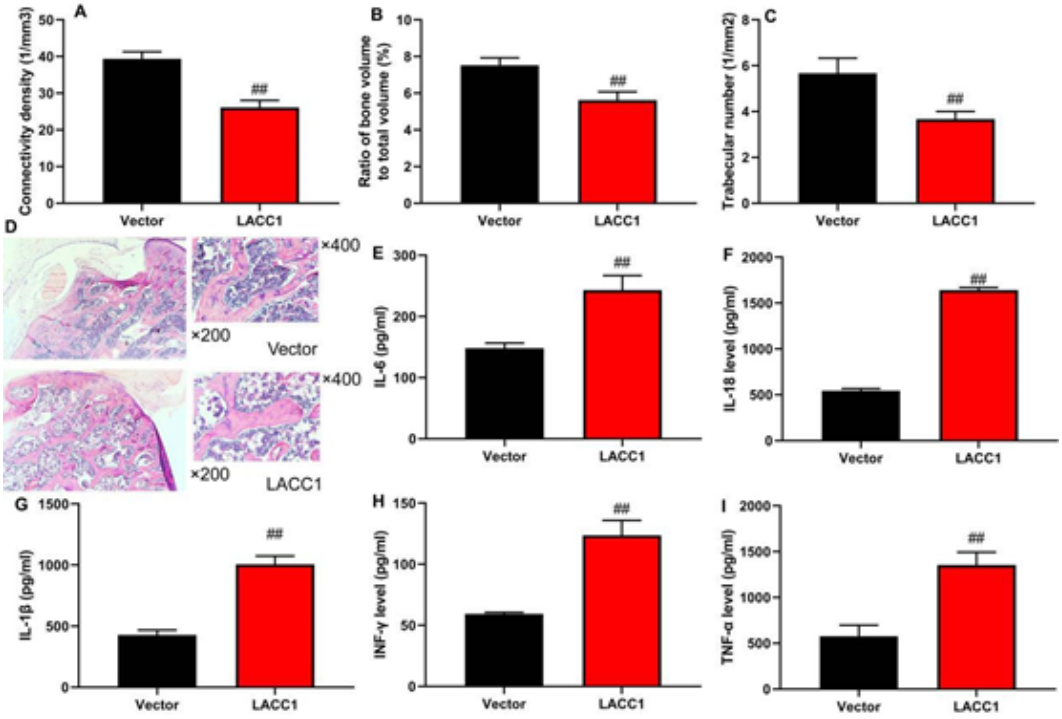

Fig. 3. LACC1 promoted osteoporosis in mice model
Connectivity density of bone (A), ratio of bone surface to bone volume (B), trabecular number (C), osteoporosis (HE staining, D), IL-6 (H), IL-18 (J), IL-1β (G), INF-γ (H), TNF-α (I)
Vector, mice with osteoporosis; LACC1, mice with osteoporosis by LACC1 recombination protein
##p<0.01 compared with mice with osteoporosis group

0.397 vs 4.844±1.428) mRNA expression levels (Fig. 4E-4F). Down-regulation of LACC1 suppressed NOD2 (1.086±0.39 vs 0.31±0.186) and RIP2 (1.021± 0.195 vs 0.243±0.028) mRNA expression levels (Fig. 4G-4H).

LACC1 Protein Interlinked NOD2/RIP2 Protein to Reduce Ubiquitination of NOD2

Furthermore, immunofluorescence indicated that LACC1 increased LACC1 and NOD2 expression in vitro model (Fig. 5A). LACC1 induced LACC1 (1.076±0.056 vs 3.187±0.382), NOD2 (1.153± 0.139 vs 0.391±0.077) and RIP2 (1.143±0.102 vs 0.279± 0.042) protein expression levels in vitro model of osteoporosis (Fig. 5B-5D). LACC1 down-regulation suppressed LACC1 (1.167±0.119 vs 0.284± 0.029), NOD2 (1.153±0.139 vs 0.391±0.077) and RIP2 (1.143±0.102 vs 0.279±0.042) protein expression levels in vitro model of osteoporosis (Fig. 5E-5G).

LACC1 protein could interlink the protein of NOD2 and NOD2 protein also interlinked the protein of NOD2 (Fig.6A-6B). Down-regulation of LACC1 increased Ubiquitination of NOD2 and Over-expression of LACC1 reduced Ubiquitination of NOD2 in vitro model (Fig. 6C).

NOD2/RIP2 Participated in the Pro-inflammation Effects of LACC1 In Vitro Model of Osteoporosis

The researchers then asked whether NOD2/ RIP2 participated in the pro-inflammation effects of LACC1 in vitro model of osteoporosis. Si-NOD2

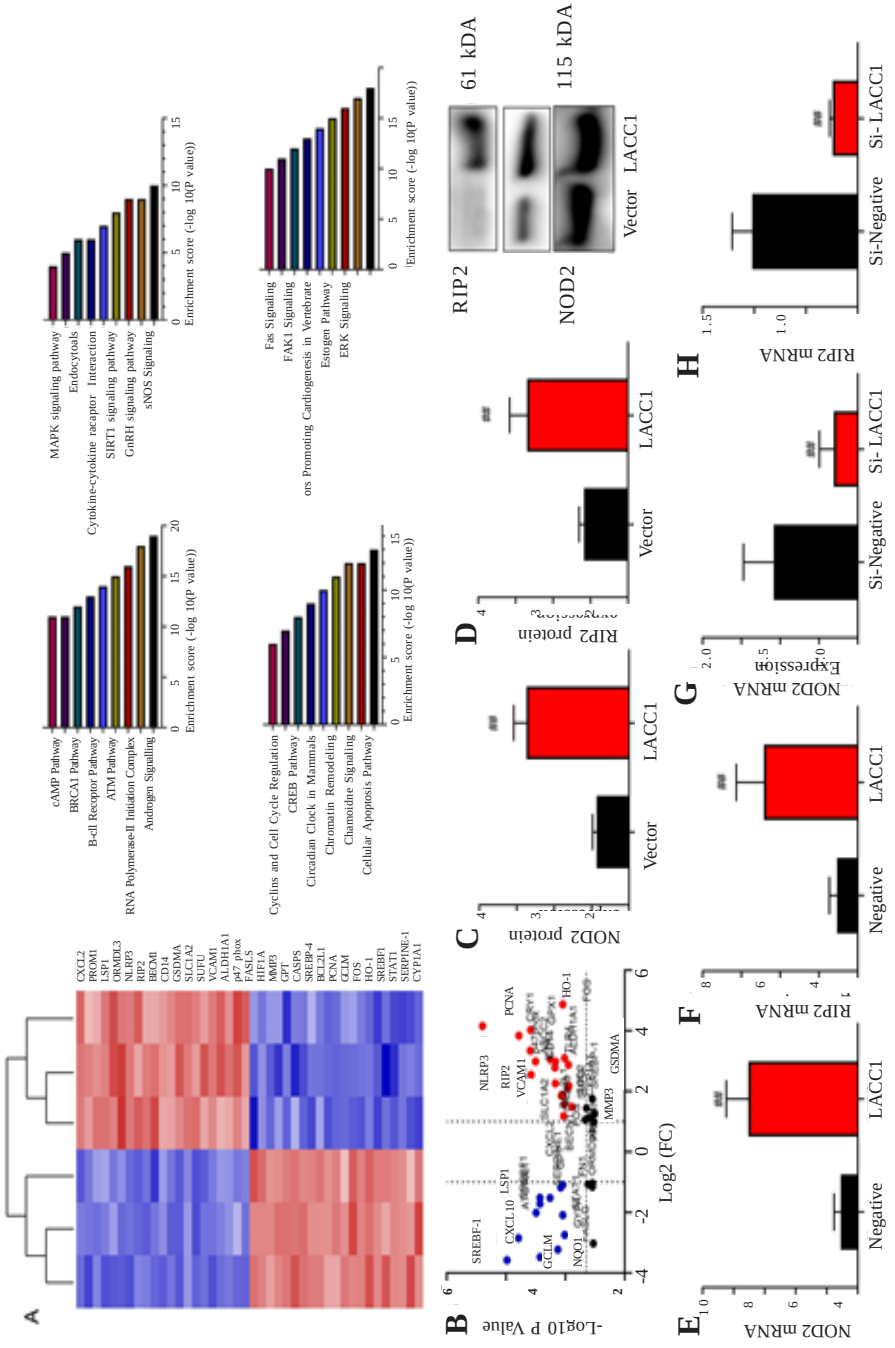

Fig. 4. LACC1 regulated NOD2/RIP2 in model of osteoporosis
Heat map, signaling pathway and analysis results (A), Volcano map (B), NOD2 and RIP2 protein expression (C and D); NOD2 and RIP2 mRNA expression (E and F) in vitro model by over-expression of LACC1; NOD2 and RIP2 mRNA expression (G and H) in vitro model by down-regulation of LACC1
Vector, mice with osteoporosis; LACC1, mice with osteoporosis by LACC1 recombination protein
Negative, control negative group; LACC1, over-expression of LACC1 group; Si-negative, control negative group; Si- LACC1, down-regulation of LACC1 group
##p<0.01 compared with mice with osteoporosis or control negative group

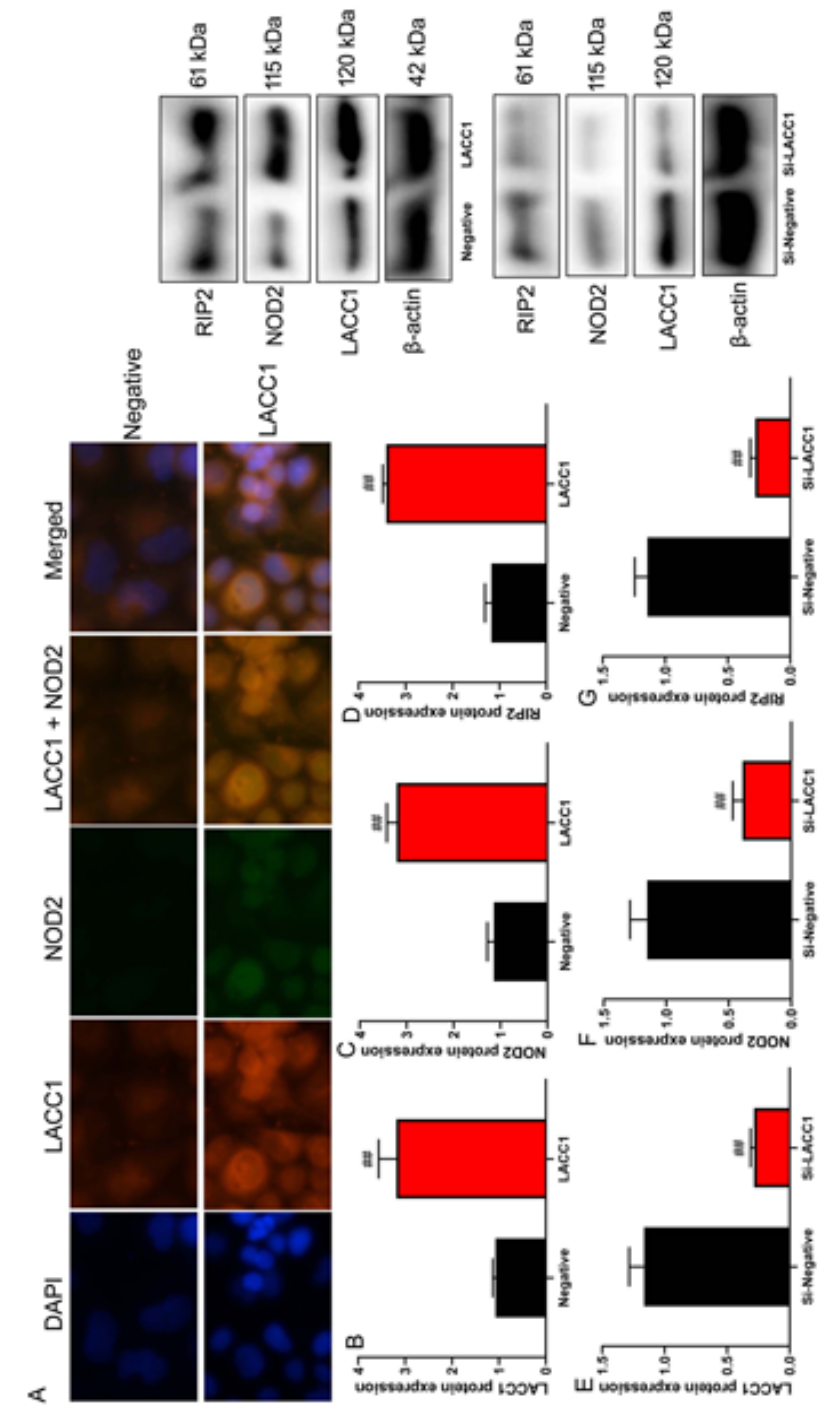

Fig. 5. LACC1 regulated NOD2/RIP2 in vitro model of osteoporosis
LACC1 and NOD2 expression (Immunofluorescence, A), LACC1, NOD2 and RIP2 protein expression in vitro model of LACC1 (B, C and D); LACC1, NOD2 and RIP2 protein expression in vitro model of si-LACC1 (E, F and G)
$^{##}p<0.01$ compared with control negative group

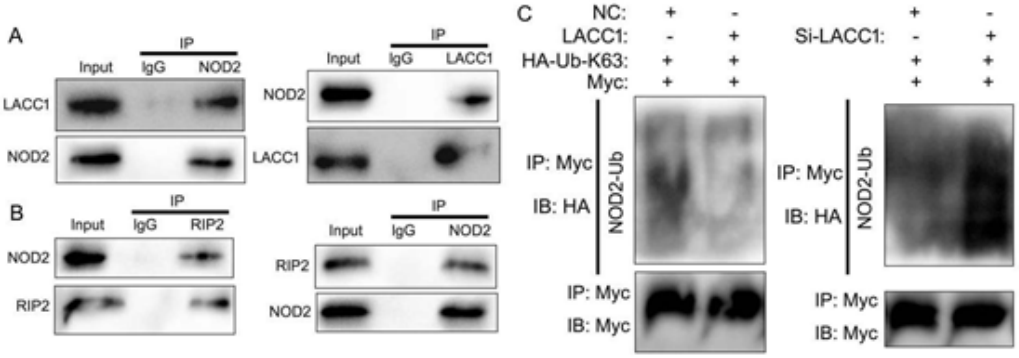

Fig. 6. LACC1 protein interlinked NOD2/RIP2 protein to reduce ubiquitination of NOD2
LACC1 protein interlinked NOD2 (A), NOD2 protein interlinked RIP2 (B), ubiquitination of NOD2 (C)

suppressed NOD2 (1.003±0.023 vs 3.572±0.161 vs 1.669±0.091) and RIP2 (1.119±0.089 vs 3.034±0.192 vs 1.462±0.097) protein expression levels, and reduced TNF-α (598.33±122.23 vs 1375±213.54 vs 771.67±82.19 pg/ml), INF-α (78.33±8.055364 vs 172.6667±4.50vs 90±2.16 pg/ml), IL-1β (505±33.72 vs 1205±134.49 vs 622.5±67.18 pg/ml), IL-18 (820±3.74 vs 2096.67±30.55 vs 1186.67±27.86 pg/ml) and IL-6 (121.33±19.06 vs 356.67±33.36 vs 214±20.06 pg/ml) levels in vitro model by over-expression of LACC1, comparison with over-expression of LACC1 group (Fig. 7A-7G). NOD2 induced NOD2 (0.953±0.036 vs 0.269±0.067 vs 0.648±0.079 pg/ml) and RIP2 (1.091± 0.105 vs 0.289±0.059 vs 0.684±0.132 pg/ml) protein expression levels, and promoted TNF-α (5536.67± 49.21608 vs 166.67±41.10 vs 378.33±55.57 pg/ml), INF-α (59±2.16 vs 14.33±1.70 vs 50±5.10 pg/ml), IL-1β (525±46.23 vs 135±18.37 vs 382.5±123.90 pg/ml), IL-18 (753.33±10.78 vs 196.67±2.62 vs 596.6666667± 4.784233365) and IL-6 (170.67±21.06 vs 54±6.53 vs 118.67±2.49 pg/ml) levels in vitro model by LACC1 down-regulation, comparison with LACC1down-regulation group (Fig. 7H-7N).

DISCUSSION

OP is a common metabolic bone disease, and the OP-induced fracture and other complications have severely affected the quality of life of patients, and brought tremendous burdens on both the family and the society (Ding and Overgaard 2022). OP is listed as one of the "three major killers" that damage health in middle-aged and elderly people (Chung et al. 2016; Kim et al. 2022). At present, China is the country with the greatest number of OP patients in the world, with about 70 million patients in 2006 (Tao et al. 2021). Skon-Hegg et al. (2019) hypothesised that in models of arthritis, LACC1 induced the activity of TNF and IL-17 levels.

The researchers' studies revealed that LACC1 mRNA expression levels were up-regulated in patients with osteoporosis. LACC1 protein and mRNA expression levels in mice with osteoporosis were induced. These results showed that LACC1 may be a promising candidate for osteoporosis.

With the development of research, more and more evidence suggests that inflammation is also an important factor that results in bone turnover rate change, osteoclast activation and bone loss (Ilich et al. 2020). In some post-menopausal OP patients, the levels of inflammatory factors increase, especially for IL-6 and TNF-α (Laudisio et al. 2018). According to reports, the inflammatory factor level in the body is one of the causes of hip bone density decline in post-menopausal women (Lerner 2006). After oral administration of vitamin C to reduce the inflammatory factors, the hip bone density increases by two percent. It remains to be further investigated whether inflammatory factor is the independent factor of post-menopausal OP, or the secondary consequence induced by estrogen deficiency (Lerner 2006). Yet, it can be certain that inflammatory factors participate in the pathophysiological processes of post-menopausal OP (Nordqvist et al. 2020). Kang et al. (2020) showed that LACC1 was used to control intestinal inflammation.

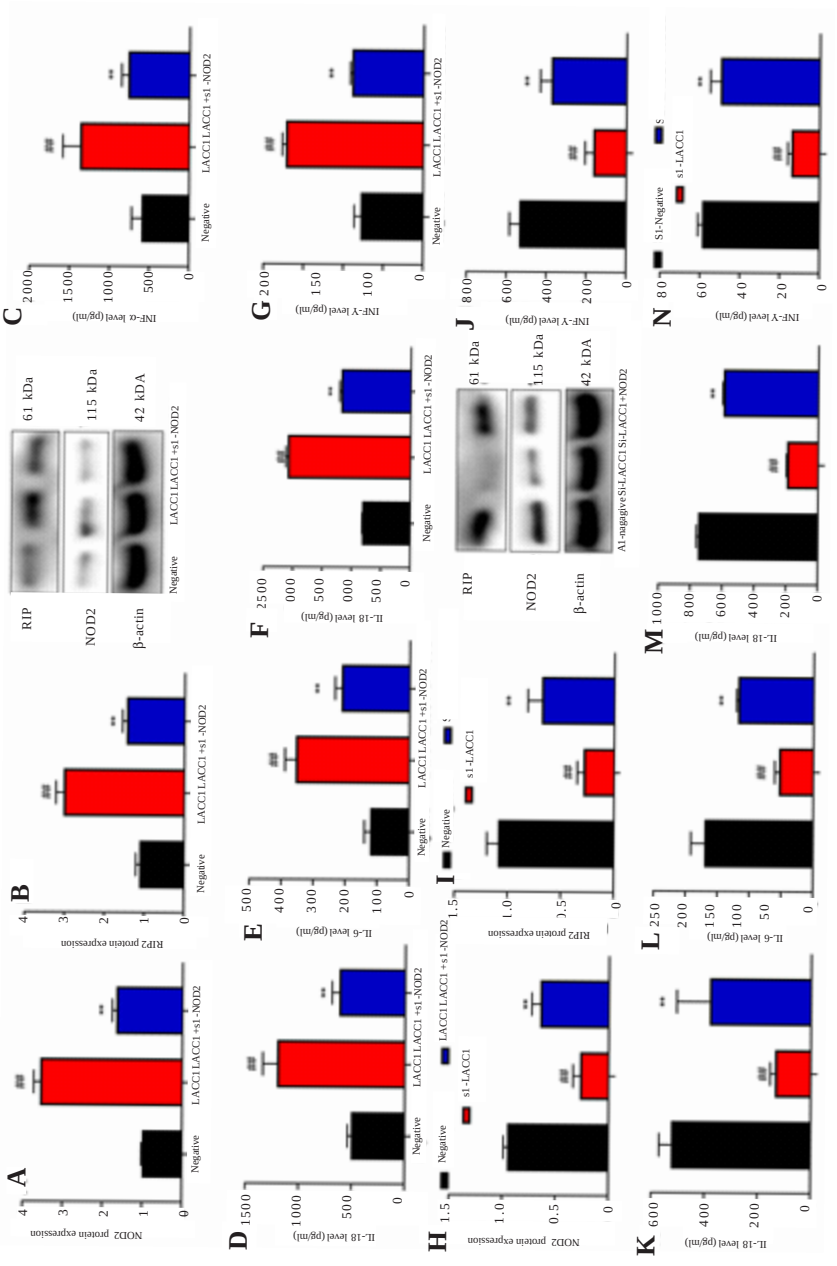

Fig. 7. NOD2/RIP2 participated in the pro-inflammation effects of LACC1 in vitro model of osteoporosis
NOD2 and RIP2 protein expression (A and B), TNF-γ (C), IL-1β (D), IL-6 (E), IL-18 (F), INF-α (G) in vitro model of osteoporosis by LACC1 and si-NOD2;
NOD2 and RIP2 protein expression (H and I), TNF-γ (J), IL-1β (K), IL-6 (L), IL-18 (M), INF-α (N) in vitro model of osteoporosis by si- LACC1 and NOD2.
##p<0.01 compared with control negative group; **p<0.01 compared with LACC1 group or si-LACC1 group

LACC1 plasmid increased LACC1 mRNA expression, and promoted inflammatory factor levels in vitro models. Si-LACC1 reduced LACC1 mRNA expression, and inhibited inflammatory factor levels in vitro models. LACC1 recombination protein reduced connectivity density of bone, trabecular number and ratio of bone volume to total volume, expanded osteoporosis in mice model. LACC1 recombination protein promoted inflammatory factor levels in bone tissue of mice model of osteoporosis. These results suggested that the function of LACC1 may promote inflammation of osteoporosis, which warrants further investigation.

NOD2–RIP2 signalling is associated with non-hereditary inflammatory diseases and some hereditary diseases that lack specific and effective treatment (Pellegrini et al. 2018; Soyocak et al. 2020). NOD2–RIP2 signalling may also be involved in inflammatory arthritis, breast cancer, colorectal cancer and asthma (Goncharov et al. 2018; Yao et al. 2019; Ma et al. 2020; Zhu et al. 2022). In this study, LACC1 protein interlinked NOD2/RIP2 protein to reduce ubiquitination of NOD2. Huang et al. (2019) identified that NOD2 induces LACC1 required for human macrophage mediated innate immune outcomes.

Over-expression of LACC1 induced LACC1, NOD2 and RIP2 protein expression levels in vitro model of osteoporosis. Down-regulation of LACC1 suppressed LACC1, NOD2 and RIP2 protein expression levels in vitro model of osteoporosis. LACC1 protein could interlink the protein of NOD2 and NOD2 protein also interlinked the protein of NOD2. Over-expression of LACC1 reduced Ubiquitination of NOD2 and down-regulation of LACC1 increased Ubiquitination of NOD2 in vitro model. Si-NOD2 suppressed NOD2 and RIP2 protein expression levels, and reduced inflammatory factor levels in vitro model by over-expression of LACC1, compared with over-expression of LACC1 group. NOD2 induced NOD2 and RIP2 protein expression levels, and promoted inflammatory factor levels in vitro model by down-regulation of LACC1, comparison with own-regulation of LACC1 group. This study suggests that LACC1 can be used as a therapeutic agent for osteoporosis via the induction of NOD2-RIP2 signalling by the inhibition of NOD2 ubiquitination.

CONCLUSION

In conclusion, this study shows that LACC1 promoted inflammation of the osteoporosis model via the induction of NOD2-RIP2 signalling by the inhibition of NOD2 ubiquitination. The LACC1 regulatory loop provides new targets for treatment of osteoporosis.

RECOMMENDATIONS

In order to prevent and treat osteoporosis, early control of inflammation should be actively recommended to prevent and treat osteoporosis, especially orthopaedic diseases.

CONFLICT OF INTEREST

The authors state that there are no financial, personal, or professional conflicts of interests that may hinder this work.

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