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Antibody-based inhibition of circulating DLK1 protects from estrogen deficiency-induced bone loss in mice

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Short title: DLK1 antibody protects from bone loss in mice
Abstract

Soluble delta-like 1 homolog (DLK1) is a circulating protein that belongs to the Notch/Serrate/delta family, which regulates many differentiation processes including osteogenesis and adipogenesis. We have previously demonstrated an inhibitory effect of DLK1 on bone mass via stimulation of bone resorption and inhibition of bone formation. Further, serum DLK1 levels are elevated and positively correlated to bone turnover markers in estrogen (E)-deficient rodents and women. In this report, we examined whether inhibition of serum DLK1 activity using a neutralizing monoclonal antibody protects from E deficiency-associated bone loss in mice. Thus, we generated mouse monoclonal anti-mouse DLK1 antibodies (MAb DLK1) that enabled us to reduce and also quantitate the levels of bioavailable serum DLK1 in vivo. Ovariectomized (ovx) mice were injected intraperitoneally twice weekly with MAb DLK1 over a period of one month. DEXA -, microCT scanning, and bone histomorphometric analyses were performed. Compared to controls, MAb DLK1 treated ovx mice were protected against ovx-induced bone loss, as revealed by significantly increased total bone mass (BMD) due to increased trabecular bone volume fraction (BV/TV) and inhibition of bone resorption. No significant changes were observed in total fat mass or in the number of bone marrow adipocytes. These results support the potential use of anti-DLK1 antibody therapy as a novel intervention to protect from E deficiency associated bone loss.

Keywords: Dlk1, Pref-1, Monoclonal antibody, Osteoporosis, Osteoblast, Osteoclast

Highlights:

- Generation of neutralizing mouse monoclonal antibody against circulating DLK1
- DLK1 antibody injection in mice inhibits sDLK1
- Antibody-based inhibition of sDLK1 protects from estrogen deficiency induced bone loss.
1.0 Introduction

Osteoporosis is a metabolic bone disorder characterized by reduced bone mass, strength, and microarchitecture deterioration leading to increased risk of fracture [1]. Postmenopausal estrogen (E) deficiency is a major risk factor for bone loss and osteoporotic fractures [2]. Recently, a number of antibody-based biological therapies have been employed for treatment of osteoporosis [3] e.g. neutralizing monoclonal antibodies against receptor activator of NF-κB ligand (RANK) (Denosumab®) [4] sclerostin (Romosozumab ®) [5] or Dickkopf-related protein 1 (DKK1) [6]. Clinical outcome measures showed that biological therapy is a feasible strategy for the prevention and treatment of osteoporosis.

Delta-like 1 homolog (DLK1) is a membrane-bound protein that is proteolytically cleaved by the ADAM17/TACE enzyme to form the active soluble circulating protein (sDLK1) which have six epidermal growth factor (EGF)-like repeats and belongs to the Notch/Serrate/delta family, (for review, see [7]). The function of membrane bound DLK1 versus the soluble form remains to be determined definitively. However, DLK1 was shown to function mainly as a circulating active protein to regulate a variety of physiological processes including adipogenesis [8, 9], osteoblastogenesis [7] and hematopoiesis [10]. Several signaling pathways have been reported to mediate the biological function of DLK1 in these differentiation processes, including a non-canonical interaction between DLK1 and Notch family receptors [11]; an interaction between DLK1 and insulin growth factor binding protein 1 (IGFBP-1) [12]; an interaction between DLK1 and fibronectin that lead to an activation of integrin and MEK/ERK signaling [13] and blocking of Akt signal activation [14]. We have identified soluble DLK1 as a novel factor that functions in a paracrine/endocrine fashion to inhibit osteoblast differentiation and bone formation [15] and stimulate bone resorption [16]. Furthermore, we identified soluble DLK1 as a possible mediator of E-deficiency associated-bone loss as its serum levels are significantly elevated following E-deficiency in both rodents [16] and humans [17] and these levels were positively associated with biochemical markers of bone turnover under E-deficiency condition [16]. In addition, the elevated serum levels of DLK1 in postmenopausal women were normalized upon E-replacement therapy [17]. In this study, we aimed to examine whether the inhibition of DLK1 using a neutralizing monoclonal antibody is an effective strategy to protect from E deficiency-induced bone loss in mice. For this purpose, we generated a highly specific mouse monoclonal anti-mouse DLK1 antibody (MAb DLK1) that neutralized the inhibitory effects of serum
DLK1 on osteogenesis. In vivo injection of MAb DLK1 in ovx mice inhibited bone resorption and maintained bone mass.

2.0 Materials and Methods

2.1 Generation of monoclonal DLK1 antibody

Cell cultures and reagents
The mouse NIH3T3 fibroblast-, 3T3-L1 preadipocyte, C2C12 myoblast- and human HT1080 epithelial cell lines were obtained from the American Type Culture Collection. Mouse stromal cells ST2 were obtained from Leibniz Institute DSMZ (ACC 333, Braunschweig, Germany). Primary osteoprogenitor cells (OB) were isolated from the calvarias of neonatal (3-4 days old) pups and subjected to sequential collagenase II digestion at 37°C as described [18]. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Carlsbad, CA, USA) with 10-20% fetal bovine serum (FBS) or 10% calf serum (3T3-L1 cells) and 100 mg/mL of streptomycin (Gibco) and 100 U/mL of penicillin (Gibco). For osteoblast differentiation, the same culture medium was supplemented with 50 mg/mL of vitamin C (Sigma-Aldrich ApS, Brondby, Denmark), and 10 mM β-glycerol-phosphate (Sigma), and medium was changed every three days.

Western blot analysis
Twenty µg of protein were separated on 8% to 12% NuPAGE® Novex® Bis-Tris gel systems (Invitrogen, Taastrup, Denmark) and transferred to a PVDF membrane (Millipore, USA). The membrane was blocked and probed with MAb DLK1 (2 µg/mL) and incubated with peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Aarhus, Denmark). Proteins were visualized by ECL chemiluminescence (Thermo Fisher Scientific, Roskilde, Denmark).

Immunohisto-/cytochemistry
For immunohistochemistry, E12.5 mouse embryos were snap-frozen in Tissue Tek. Four to 5 µm cryosections were then fixed in 4% NBF prior to blocking in 2% BSA/TBS. Sections were incubated with MAbs diluted to 2 µg/mL followed by Alexa 488-conjugated donkey anti-IgG (1:200, Molecular Probes). Nuclei were stained with DAPI (Vectashield, Vector Labs). Images were captured using a Leica DMI4000B Cool Fluo Package instrument equipped with a Leica DFC340 FX Digital Camera. Exposure (camera settings) and picture processing (minor adjustments of contrast/brightness in Adobe
Photoshop) were applied equally to sample sections and controls. For immunofluorescent staining, cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS and stained with MAb DLK1 using Alexa Fluor® 488-conjugated mouse anti-mouse IgG (H+L) as secondary antibody (Cell signaling). Fluorescent images were analyzed by Operetta high content imaging system (Perkin Elmer, Germany) at 20x magnification.

**Alkaline phosphatase (ALP) activity assay**

Each sample was measured in 6 replicates in 96-well plates. Cells induced with osteogenic medium were stained with FITC fluorescent ALP live stain dye according to the manufacturer’s instructions (ThermoFisher Scientific, Denmark) and counter stained with DAPI (Sigma-Aldrich ApS) for nuclear staining. Fluorescent images were analyzed by Operetta high content imaging system (Perkin Elmer, Germany) using Harmony® Software (Perkin Elmer) and ALP activity was represented after normalization to the cell count.

**Purification and biotinylation of mouse DLK1**

Soluble mouse DLK1 (mDLK1) was purified from mouse amniotic fluid or from conditioned 3T3-L1 medium by immuno-specific affinity chromatography (monospecific rabbit anti-mDLK1 coupled sepharose) and further concentrated using cation exchange (Resource Q column, GE Healthcare). For immunizations, mDLK1 was dialyzed against PBS and sterile filtered through a low-protein binding 0.22µm filter. For the inhibition/epitope specificity assay, mDLK1 was biotinylated by addition of 1/6 (w/w) biotinsuccimid ester (BNHS; 10 mg/mL in dimethylsulfoxide). Unreacted BNHS was removed by buffer exchange to PBS.

**Immunization of mice.**

Three adult female Dlk1−/− mice were each injected subcutaneously with 25µg purified mouse DLK1 mixed with Al(OH)₃ and incomplete Freund’s adjuvant (IFA). DLK1-deficient (Dlk1−/−) mice were obtained from J. Laborda (University of Castilla–La Mancha, Ciudad Real, Spain) [19]. The immunizations were repeated further two times, 14 days apart and by replacing IFA with PBS for intraperitoneal injections. One week after the third immunization, a blood sample from each mouse was tested for circulating antibodies against mDLK1 using direct- (antigen) ELISA, cyto-ELISA and flow cytometry (see below). Normal mouse serum (Chemicon) was used as a non-immune reference serum.
in all screening assays. Two mice that revealed a positive polyclonal signal in all three assays were selected for a final boost i.p. with 25 µg mDLK1 in PBS, three days prior to fusion.

**Fusion and cloning**

Using PEG 4000, spleen cells from the responding mice were fused with the myeloma cell line SP2/0-Ag14 as described by Köhler and Milstein (1975)[20] and modified by Reading (1982)[21]. Hybridoma supernatants from 96-well plates were screened using cyto-/antigen ELISAs and later in the cloning process, also by flow cytometry. Selected hybridomas were cloned 3-5 times using the standard limiting dilution procedure [21]. In total, 19 clones were further propagated in RPMI/10%FCS/1%PS and stored frozen at -80°C in FCS- containing 10% DMSO while the antibody containing supernatants were kept at 4°C until further analyses.

**Screening of hybridoma supernatants**

Hybridoma supernatants were screened throughout the cloning process using direct (antigen, Ag) ELISA and cyto-ELISAs (positive screening on 3T3-L1 cells and negative on C2C12 cells). An intermediate screen was also performed on live C2C12 and 3T3L1 cells by flow cytometry (see below). For direct ELISAs, purified mouse DLK1 (0.5-1.0 µg/mL) was coated onto 96-well Maxisorp flat bottom microtiter plates. For cyto-ELISAs, 3T3-L1 and C2C12 cells were cultured in 96-well plates and fixed with 4% NBF at sub-confluence, as previously described [22]. Hybridoma supernatants were then transferred to the wells and plates incubated overnight. After an additional incubation with horseradish peroxidase conjugated rabbit-anti mouse immunoglobulins (P260, Dako Denmark), plates were developed using O-phenylenediamine (Kem-En-Tec Diagnostics A/S) /H2O2 and read at 490 nm on a Labsystems iEMS Reader.

**Epitope specificity of MAbs**

Based on a final screen by flow cytometry, seven MAbs were selected for epitope specificity analysis by a previously described inhibition assay [23]. Briefly, MAbs (one MAb for each of the rows A-G, 5 µg/100 µL/well) were coupled indirectly via rabbit anti-mouse immunoglobulin (Z109, Dako Denmark, 0.2 µg/100 µL/well) to a Maxisorp flat bottom microtiter plate to ensure correct presentation of the antigen binding sites. Residual mouse immunoglobulin binding sites were blocked with normal mouse serum. In parallel, in a non-absorbing round bottom microtiter plate the MAbs (one MAb for each of the columns 1-7, 5 µg/100 µL/well) were allowed to react with biotinylated mDLK1
(1:2000/100 μL/well). To determine the maximal signal and background signals of each MAb respectively, one column was incubated with buffer and biotinylated mDLK1 and another with buffer alone. Following incubation of the 2 plates, 100 μL from each well in the round bottom plate (liquid phase) was transferred to the flat bottom plate (solid phase) that was incubated for 2hr at room temperature before being washed and developed as described below for the mDLK1 ELISAs.

Large-scale production of serum free MAbs for in vivo use.

The three clones CC-5, CC-11 (both anti-mDLK1) and DJ3 (isotype control immunoglobulin) all IgG1, kappa, were cultured in RPMI 1640 (Sigma) free of serum and antibiotics using CELLine Bioreactors (CL1000, Argos Technologies). MAbs were purified from culture supernatants as described below.

IgG purification and isotype determination

Protein G affinity chromatography was performed according to the manufacturers recommendations (Amersham Biosciences, UK) using 5 mL HiTrap MabSelect SuRe columns attached to an Äkta Fast Performance Liquid Chromatography system (Amersham Biosciences, UK). The isotypes of the generated antibodies were determined using an Amersham Kit as recommended by the manufacturer. All MAb preparations were dialyzed against PBS and for in vivo studies they were also sterile filtered by passing through a 0.22 μm filter.

mDLK1 ELISAs

A previously described mouse DLK1 ELISA based on polyclonal rabbit anti-mouse DLK1 antibodies [24, 25] was used for initial mDLK1 quantifications and as “golden standard” during the development of an ELISA based on the two monoclonal antibodies, MAb CC-5 and CC-11. CC-5 and CC-11 were used as catcher/detector antibodies respectively. For this purpose CC-11 was biotinylated as described above for mDLK1, and HRP-conjugated streptavidin and O-phenylenediamine were used to develop the reaction. As for the polyclonal ELISA, mouse amniotic fluid was used as a native mDLK1 source for generating calibrators (mDLK range: 0.24-3.78 ng/mL) and quality controls (Q1: 0.76 ng/mL and Q2: 1.74 ng/mL).

Flow cytometry

For each reaction, 2x10^5 proliferating C2C12 or 3T3-L1 cells were incubated with 100 μL hybridoma supernatant (diluted 1:2-1:10), purified MAbs/isotype control Ig (2-10 μg/mL) or dilution buffer
(HBSS with 5% FCS). Secondary antibody was Alexa 488-Donkey anti-mouse (1:200; Molecular Probes). Sample acquisition was done using a FACScan or FACSCalibur instrument (Becton Dickinson) while the FlowJo v10 software was used for analyses.

2.2 Animal treatment
All experimental procedures were approved by the Danish Animal Ethical committee. All mice were bred and housed under standard conditions (21°C, 55% relative humidity) on a 12-h light, 12-h dark cycle. Ad libitum food (Altromin® or a special diet without phytoestrogen (Diet R70 LABFOR) after ovariectomy) and water were provided.

Bone mass measurement
Bone (total, femur, and spine) mineral density (BMD) (g/cm^2), total body fat and lean percentage were measured using dual-energy X-ray absorptiometry (DEXA) by using a PIXImus2® (version 1.44; Lunar Cooperation, USA.). DEXA scans were acquired after sedating mice with isoflurane every two weeks.

Micro-computer tomography scanning (μCT)
At baseline and after one month of treatment, tibiae were scanned with a high-resolution μCT system (vivaCT40; Scanco Medical, Bassersdorf, Switzerland), resulting in three-dimensional reconstruction of cubic voxel sizes 10.5 x 10.5 x 10.5 μm^3. Seventy image slices were analyzed at the proximal metaphysis and the diaphysis for cancellous and cortical bone, respectively. A detailed description for the quantification of 3D microarchitecture of trabecular and cortical bone has been presented previously [26].

Ovariectomy and injection of MAb DLK1.
Ovariectomy (ovx) was performed in 10-weeks-old female C57BL/6 mice. Mice were divided into 4 groups (n=8/group); sham control group, ovx control group, ovx group injected with isotype IgG and ovx group injected with MAb DLK1 (CC-5). Ovaries were removed through the dorsal side, and sham-operated mice had both ovaries externalized briefly and then gently reinserted. Ovx-induced bone loss was assessed after one month in ovx-operated mice using micro-CT analysis. One day after operation, mice were intra-peritoneally injected with 100 µl of saline solution containing 10 mg/kg of either MAb DLK1 or a mouse IgG antibody control (Abmart, NJ, USA) twice a week over a period of 4 weeks.
Histomorphometric analysis

All histomorphometric analyses were performed according to a protocol described previously [27]. In a defined area located just under the growth plate, parameters of histomorphometry for osteoblast surface (as Ob.S per trabecular surface in the defined area) and number (as Ob.N per trabecular surface in the defined area), eroded surface (as ES per trabecular surface in the defined area), and marrow adiposity (as ratio of area occupied by adipocytes per marrow area in the defined region) were quantified on H&E –stained decalcified proximal tibia sections of 5 µm thickness. Osteoclast surface and number was quantified on Tartrate resistant acid phosphatase (TRACP) stained sections. For each animal and staining, 4 serial sections (every 100 µm) were analyzed. Measurements were performed using open source ImageJ software.

RNA extraction and Real time qRT-PCR

RNA was extracted using TRIzol and reverse-transcribed using a RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative real-time PCR was performed with an Applied Biosystems 7500 Real-Time PCR System using Fast SYBR Green Master Mix (Applied Biosystems) with specific primers (Supplemental Table 8). Normalization of gene expression was done with 36B4 as a housekeeping gene.

Bone turnover markers

Mouse serum CrossLaps ® (CTX-I) Elisa Immunoassay for the quantification of the degradation products of C-terminal telopeptides of Type I collagen in mouse serum (Immunodiagnostic Systems Nordic, Herlev, Denmark) and Rat/Mouse P1NP EIA for the determination of the N-terminal propeptide of type I procollagen (P1NP) for bone formation (Immunodiagnostic Systems Nordic, Herlev, Denmark) were measured according to manufacturer recommendations.

2.3 Statistical analysis

All values are expressed as mean ± SD (standard deviation), of at least 3 independent experiments with at least 3 replicates for each biological replicate. P values were calculated by unpaired Student’s T-test comparing two groups or appropriate ANOVA followed by appropriate post hoc test if comparing more than two groups (accordingly, 1-way ANOVA with Sidak’s post hoc test in Figure 2 or with Tukey’s
post hoc test in Figure 3A and Figure 4 and 2-way ANOVA followed by a Tukey’s post hoc test for Figures 3B-D or by Sidak’s or/and Tukey’s post hoc test for Figures 3F-J. P < 0.05 was considered statistically significant.

3.0 Results

3.1 Generation and characterization of monoclonal DLK1 antibodies

We aimed to generate a highly specific neutralizing mouse monoclonal anti-mouse DLK1 (mDLK1) antibody that could be used directly for injections into immunocompetent mice, and without any need for immunosuppressive agents. To elicit an immune response against mDLK1 in mice, we injected Dlk1−/− mice with purified native mDLK1, ensuring presentation of an antigen with normal post-translational modifications and -configuration. Two of the three animals revealed a strong polyclonal response as judged by direct antigen- and cyto-ELISAs as well as flow cytometry on 3T3-L1 cells, a positive cell line for mDLK1 expression (Supplemental Figures 1A-C). These two animals were used for hybridoma production and 19 clones were selected for further characterization based on positive/negative signals in 3T3-L1/C2C12 cyto-ELISAs respectively, and positive staining of E12.5 embryos (Supplemental Table 1). We screened these hybridomas for producing highly specific monoclonal antibodies against mDLK1 using immunofluorescent staining, Western blotting (of cell lysates and conditioned medium) and flow cytometry techniques. In all assays, positive (3T3-L1 or NIH3T3) and negative (ST2 or C2C12) control cells were tested and for Western blotting, a human MSC cell line overexpressing human DLK1 [28] was included to determine any cross reaction (Supplemental Table 1). The seven best candidate hybridomas were evaluated for their epitope specificity in an inhibition assay [23] (Supplemental Table 2) and two clones, CC-5 and CC-11, recognizing distinct epitopes, were selected for further analyses and assay development. Both monoclonal antibodies (MAbs) stained mDLK1 expressing cells (Figure 1A) and tissues specifically (Figure 1B). As seen in Figure 1C, CC-5 and CC-11 each demonstrated a high degree of specificity to the large soluble form of mDLK1 (50 kDa) produced and secreted by NIH3T3 cells. Similarly, the MAbs reacted specifically with mDLK1-positive 3T3-L1 but not -negative C2C12 cells by flow cytometry (Figure 1D). Finally, CC-5 and CC-11 were tested for their ability to neutralize the inhibitory effects of soluble mDLK1 on osteoblast differentiation of primary mouse osteoblast culture
by quantitative ALP activity measurement and staining (Figure 1E). A significant neutralizing effect (p< 0.005) was only observed for CC-5, which was therefore chosen as the MAb for in vivo injections.

3.2 Assay development

Prior to initiating the in vivo studies, it was necessary to develop an assay that would allow us to quantitate changes in bioavailable serum mDLK1 upon CC-5 administration. For this purpose we aimed for a sandwich ELISA setup using CC-5 as catcher and CC-11 as detector antibodies respectively. Theoretically, any mDLK1 bound to CC-5 in liquid phase would not be able to react with the solid phase CC-5 of the ELISA and therefore lead to a reduced or completely negative signal in the ELISA. The ELISA was constructed using mouse amniotic fluid as mDLK1 source for the assay calibrator/quality controls and other mDLK1 sources shown to dilute in parallel with the calibrator (Supplemental Figures 2A-B). Intra- and inter-assay coefficients of variation were below 5% and the assay was thoroughly validated with respect to other parameters as well (e.g. recovery, comparison of serum/plasma ± hemolysis, repeated freeze-thaw cycles, species cross reactions; data not show) before further use. A final ex vivo neutralization test revealed that addition of CC-5 to normal mouse serum was able to effectively reduce ELISA signals to below the assay detection limit (Supplemental Figure 2C).

3.3 In vivo pilot studies/validation of concept

As CC-5 (from now on referred to as MAb DLK1) was found to neutralize serum mDLK1 ex vivo, we next proceeded to in vivo administration in ovx-mice. Based on considerations of mouse total extracellular fluid volume, DLK1 concentrations, half-life in circulation etc, we initially calculated that two weekly injections with MAb DLK1 doses of 1-3 µg/g would be more than sufficient to neutralize all soluble mDLK1. However, as seen in Figure 2A, ovx mice injected with both MAb DLK1 concentrations developed highly elevated serum mDLK1 levels compared to isotype- and normal controls. We speculated that this increase in serum mDLK1 could be a result of an unknown compensatory feedback mechanism and next performed a dose-response experiment in normal animals (Figure 2B). Clearly, while doses of 0.1 and 1 µg/g triggered an increase in systemic DLK1, a dose of 10 µg/g was efficient to reduce serum mDLK1 well below normal values. qPCR on endocrine and other tissues from animals receiving a suboptimal dose of MAb DLK1 did not reveal any conclusive data on the origin of the excess mDLK1 (data not shown). A final test of the optimal MAb DLK1 doses
was performed in ovx-mice showing a significant reduction (77 and 86%) of serum DLK1 that lasted for at least 3 days (p=0.0002, 1 way ANOVA, Sidak’s post-test) (Figure 2C), confirming neutralization in the ovx model.

3.4 MAb DLK1 injection protects from estrogen deficiency-associated bone loss in mice

MAb DLK1 was twice weekly injected in ovx-mice over a period of one month and animals were analyzed. The concentration of DLK1 in the serum of the animals from all groups was evaluated at the end of the experiment by ELISA (Figure 3A). As shown in Figure 3B, no changes in body weight, lean mass, or fat mass percentage (data not shown) were observed following MAb DLK1 treatment. Ovx-mice treated with MAb DLK1 showed significantly increased total and spine BMD compared to ovx-antibody control treated mice after 2 and 4 weeks treatment while femur BMD was unchanged in between the ovx groups (Figure 3C-D). μCT scanning of trabecular bone after 4 weeks of treatment, revealed significant increased trabecular BV/TV and trabecular thickness while the trabecular number was unchanged between the ovx-mice (Figures 3E-H). No significant changes were observed in the cortical thickness following MAb DLK1 treatment (Figure 3I) while the cortical area per total area was unchanged after treatment (Figure 3J). Additional information regarding the statistical analysis is provided in the supplemental tables 3-7. The measurement of the bone turnover markers CTX and P1NP for bone resorption and formation, respectively, showed no significant change between the groups (Supplemental Figures 3A-B). However, histomorphometric analysis of proximal tibia showed reduced osteoclastic bone surface by 37 % compared to the other ovx-mice while there was no significant variation of the number of the osteoclasts (p<0.05) (Figure 4A). The evaluation of the eroded surface per bone surface showed a tendency to decreased bone resorption in the group treated with MAb DLK1 compared to Ab control (Figure 4B). While the effect of MAb-Dlk1 on reducing the number of osteoblasts in ovx mice was not significant, the MAb effect in Fig. 4C showed a clear tendency toward reducing the Ob.S/BS by 15.9% and 25.1% as compared to control ovx and antibody control injected ovx mice respectively. The effect of Mab DLK1 on the expression of genes related to bone formation, bone resorption, Wnt signaling regulators and Notch signaling have been evaluated by qPCR on bone samples. No significant variation between the ovx groups were observed, probably due to the fact that our bone samples were not pure but with marrow cells as well (Supplemental Figure 4). Finally, the marrow adipocytes were comparable between the ovx-mice groups (Figure 4D).
4.0 Discussion

In this study we confirmed that serum DLK1 is a mediator of E-deficiency associated bone loss. Our data demonstrated that antibody-based inhibition of serum DLK1 is an efficient strategy to protect against ovx-induced bone loss and thus provides DLK1 as a possible therapeutic target for prevention of bone loss in estrogen deficiency states.

Using a novel approach of immunizing DLK1 deficient mice with the native soluble form of mDLK1 protein, we successfully generated a range of high-affinity monoclonal anti-mDLK1 antibodies and selected one for neutralization in vivo, and two with different epitope specificities for assay development. Given our rigorous screening strategy and end-point criteria, the number of neutralizing MAbs obtained was not high and it may not be surprising that no commercially available antibodies managed to fulfill our criteria. Also, it seems likely that the region(s) responsible for DLK1’s modulatory effect on bone formation may be very similar across species. If so, immunization of one species with DLK1 originating from another species would fail to elicit a strong immune response and generate high-affinity antibodies recognizing the involved epitope(s). This problem was overcome herein by using the Dlk1 null mouse as host. Another aim of generating a neutralizing antibody in mouse was to avoid the need for immunosuppressive agents or use of an immunocompromised mouse strain when looking at the effects of antibody administration in our ovx-model. In this regard, MAb DLK1 did not show any toxicity effects upon its injection into ovx mice over a period of 2 months and was concluded to be a safe treatment in these animals. Surprisingly, normal and ovx females as well as normal males (data not show) revealed an increase in circulating bioactive DLK1 when injected with low concentrations of MAb DLK1. This phenomenon has been described for proteins like IGF-1 and IL-6 as well [29, 30] and could in theory be due to the formation of antibody-antigen (Ab-Ag) complexes that are eliminated from circulation at a lower rate than un-complexed DLK1. These Ab-Ag complexes would then diminish at excess Ab levels. Alternatively, the antibody could block an epitope involved in a negative feedback loop by DLK1 itself. Using qPCR we were however unable to pinpoint an organ in which an increase in dkl1 transcript levels could explain the highly elevated serum levels.

Estrogen plays an important role in the regulation of bone remodeling by inhibiting osteoclast differentiation and survival, while promoting survival and differentiation of osteoblastic cells, as well as inhibiting their apoptosis. Estrogen deficiency induces bone loss in rodents and humans by increased
osteoclastic bone resorption [31, 32]. Estrogen acts directly on osteoclastic cells by inhibiting their apoptosis [33] and by suppressing RANK ligand-induced osteoclast differentiation [34].

Several pre-clinical and clinical studies have reported the successful use of antibody-based biological therapies for treatment of bone loss in osteoporotic conditions. These include antibodies that neutralize RANKL [35], Sclerostin [5] and more recently DKK1 [6]. Administration of selected MAb DLK1 to ovx mice resulted in increased total BMD and trabecular BV/TV. Similarly, the administration of anti-RANKL or anti-DKK1 to ovx mice showed significantly increased femur and lumbar spine BMD [36, 37]. Treatment of ovx mice with anti-RANKL antibody resulted in decreased TRAP staining of the distal femur [37]. Our histomorphometric data demonstrate that the mode of action of MAb DLK1 in protecting bone loss is primarily mediated by reducing bone resorption as shown by significant reduction in the OC number in the proximal tibia of ovx mice injected with MAb DLK1. Thus, MAb DLK1 can be classified as an anti-resorptive drug. In support to this notion, we have previously demonstrated the stimulatory effect of sDLK1 protein on osteoclastogenesis via an indirect mechanism [16]. In this mechanism, sDLK1 showed to activate the NF-κB signaling pathway by osteoprogenitor cells and to increase the production of pro-inflammatory osteoclast-activating cytokines i.e. IL-7, TNFα and CCL3 [16, 28]. Taken together, our data from this first pre-clinical trial, demonstrated the potential therapeutic effect of MAb DLK1 [16, 17, 28] in antagonizing the E deficiency-mediated bone loss. However, further mechanistic studies are needed to define the exact mechanism of action of MAb DLK1 in bone.

Our data demonstrated a preferential effect of MAb DLK1 on cancellous bone versus cortical bone. In agreement with this finding, we have previously demonstrated that the inhibitory effect of sDLK1 on bone mass (in mice systemically overexpressing DLK1) is mediated via its effect on trabecular bone micro-architecture parameters including trabecular thickness and number without affecting cortical bone parameters [15]. In addition, serum levels of DLK1 were inversely correlated with the trabecular BV/TV in these Mab DLK1 injected mice [15].

Despite the established inhibitory effect of DLK1 on the differentiation of the skeletal stem cell (BMSCs) into osteoblast and adipocyte lineages [7, 38], our data demonstrated that short-term inhibition of serum DLK1 in ovx mice did affect neither osteoblasts nor adipocytes in bone marrow. This could be explained by the existence of a compensatory mechanism that acts to compensate for the
reduced serum levels of DLK1 by MAb DLK1. In support of this notion, adult mice lacking DLK1 expression were shown to display a normal bone mass phenotype (with normal osteoblastic bone formation rate and mineral apposition rate) [39]. Furthermore, it should be noted, that our ovx mice were treated with MAb-DLK1 for only 1 month and therefore, it is plausible that long term inhibition of sDLK1 by MAb-DLK1 might be effective to show stimulatory effect on the number of osteoblasts and adipocytes in the bone marrow of ovx mice.

In our study, we demonstrated a decrease in bone resorption after ovariectomy as shown by the measurement of the Oc.S/BS, while the CTX concentration was unchanged. Despite it has been reported that the reduced cancellous bone in post-menopausal women is due to an increase in bone turnover with enhanced bone resorption compared to bone formation [40, 41], this phenomenon was less consistent in ovariectomized mice. In agreement with our data, a previous study by Iwaniec et al, comparing the response of cancellous bone to estrogen deficiency in different strains of mice, demonstrated no significant change in the osteoclast surface at 1 and 3 months in ovariectomized C57BL/6 mice, while the cancellous bone loss was effective [42].

Furthermore the use of 3-month-old mice, where the bone growth at this young age is still going on [43] is a limitation in our study. Thus, we cannot ignore that the observed significantly increased BV/TV in MAb-DLK1 treated mice compared to control ovx mice, as well as the decreased bone resorption (Oc.S/BS) observed, may partially be explained by the developing skeleton in these young mice. However, we still can demonstrate a significant increase in BV/TV in the MAb-DLK1 treated group over both the antibody control-treated group and the non-treated group of ovx mice and our treatment still induces a significant decrease of the Oc.S/BS compared to the ovx Ab control groups.

5.0 Conclusion

In summary, targeting the inhibition of DLK1 using our generated neutralizing monoclonal antibody is protective against E deficiency induced bone loss. Our studies propose DLK1 as a possible therapeutic target for prevention of bone loss in E-deficiency states.
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The authors declare that they have no conflicts of interest with the contents of this article.

Authors’ contributions: BMA, CHJ and MK conceived the project. BMA, FF, CANN, ND, DCA, CHJ, KS performed the in vitro and in vivo experiments. FF, ND, BMA, CHJ, SPS, and DCA collected and analyzed data. FF, DCA, CHJ and BMA designed and supervised the study and wrote the manuscript, revised and approved the manuscript.

References


Figure legends

Figure 1: Generation of mouse monoclonal mDLK1 antibody. The specificity of the selected mDLK1 antibody has been examined by (A) immunofluorescent staining for mDLK1 expression using mDLK1-Ab in cultured NIH3T3 cells (+ve control for mDLK1 expression). (B) Immunostaining of mouse embryonic E12.5 bone anlage using MAbs CC-5 and CC-11 (both at 2 µg/mL) alone, or in combination. Nuclei were stained with DAPI (magnification x200). (C) Western blot analysis of the large soluble form of mDLK1 expression and secretion in NIH3T3 cells lysate and collected conditioned medium (CM), versus stromal ST2 cells lysate (negative control of mDLK1 expression) and ST2 CM. (D) Flow cytometry on 3T3-L1 (DLK1 positive control) and C2C12 (DLK1 negative control) cells with CC-5, CC-11 antibodies and a control (Ctrl; cells without primary Ab). (E) Effect of the selected mDLK1-Ab (at 5 µg/mL) on neutralizing the inhibitory effect of mDLK1 on osteogenesis assessed by quantitative alkaline phosphatase (ALP) fluorescent staining measurements using Operetta® high content imaging system. Primary mouse calvarial osteoprogenitor cells were cultured and induced to osteoblast differentiation in mDLK1-containing CM in the presence or absence of mDLK1-Ab. ALP activity was measured after 6 days of induction as described in M&M. Fluorescent images of ALP staining are shown.

Figure 2: In vivo neutralization of mDLK1. (A) Initially, ovx mice were injected with MAb DLK1 (CC-5) or isotype control at a concentration of 1 or 3µg/g body weight (n=5 in each group) at days 4, 7, 10, and 13 after OVX and serum samples were measured in the mDLK1 ELISA at days 0 (baseline), 5, 7, 10, and 16. A non-treated group was included (n=3). (B) A dose-response experiment was performed
in normal female mice receiving 0, 0.01, 0.1, 1, or 10 µg MAb DLK1/g body weight (n=3 in each group) via IP injections at days 0 and 3. Serum samples from days 1, 3, 4, and 6 were quantified in the ELISA. (C) Effective inhibition of bioavailable mDLK1 in ovx mice was accomplished using an optimal dose of 10 µg MAb DLK1/g after one injection, with the effect sustaining for 3 days (one-way ANOVA with Sidak’s post-test).

**Figure 3: µCT evaluation of the protective effect from estrogen deficiency induced bone loss of the mDLK1-antibody treatment.** Ten weeks female mice were divided into 4 groups: sham control group (SHAM CTL), ovx control group (OVX CTL), ovx injected with antibody control (OVX Ab CTL) and ovx injected with 10mg/kg mDLK1-Ab (OVX mDLK1-Ab) (n=8/group). (A) Level of DLK1 in serum at the end of the experiment (one-way ANOVA with Tukey’s post-test). All mice were followed by DEXA scan every 2 weeks post-surgery for evaluating (B) body weight, (C) total bone mineral density (BMD) and femur (D, left panel) and spine (D, right panel) BMD (two-way ANOVA with Tukey multiple test). BMD was represented as percent change from the baseline. (**p<0.01; ***p<0.001, compared to SHAM CTL; $p<0.01$ compared to OVX Ab CTL). µCT analysis of trabecular bone measured in proximal tibia after 1 month of treatment. (E) µCT 3D reconstruction representative images are shown. Bone parameters: (F) trabecular bone volume per total volume (BV/TV), (G) trabecular thickness (Tb.Th), (H) trabecular number (Tb.N), (I) Cortical thickness (Co.Th) and (J) Cortical area per total area (CoA/TAr). (*p<0.05; **p<0.01; ***p<0.001, compared to SHAM CTL; ##p<0.01; ###p<0.001, compared to OVX CTL and $p<0.05; $p<0.01; $$$$p<0.001$ compared to OVX Ab CTL) (only two-way ANOVA repeated measure with Sidak’s post hoc test are represented on the graphs F-J, more statistical analyses are in supplement tables 3-7). Values are mean ± SD (n=8).

**Figure 4: Histomorphometric analysis of the protective effect from estrogen deficiency induced bone loss of the mDLK1-antibody treatment.** Histomorphometric analysis on tibia sections from SHAM CTL, OVX CTL, OVX Ab CTL, and OVX mDLK1-Ab groups. (A) Osteoclast surface per trabecular bone surface (Oc.S/BS) (left panel) and Osteoclast number per trabecular bone surface (Oc.N/BS) (middle panel) were quantified in Tartrate resistant acid phosphatase (TRAP) stained bone sections. Representative TRAP stained of tibia sections from SHAM CTL, OVX CTL, OVX Ab CTL and OVX mDLK1-Ab groups (right panel). Scale bar: 100µm. (B) Eroded surface per trabecular bone surface (ES/BS). (C) Osteoblast surface per trabecular bone surface (Ob.S/BS) (left panel) and Ob.N/BS (right panel). (D) Marrow adiposity as percentage of adipocyte surface per marrow surface (left panel). Representative sections for adiposity evaluation (right panel). Scale bar: 100µm. (*p<0.05; **p<0.01; ***p<0.001, compared to SHAM CTL; and $p<0.05$ compared to OVX Ab CTL; not significant are not represented for more clarity) (one-way ANOVA with Tukey post hoc test). Values are mean ± SD (n=8).
Figure 2

A

![Graph showing serum DLK1 levels over time for different groups.]

- Base line
- Day 5
- Day 7
- Day 10
- Day 16

<table>
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B

![Graph showing serum DLK1 levels at different doses of MAb DLK1.]

- Day 1
- Day 3
- Day 4
- Day 6

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C

![Bar graph showing serum DLK1 levels on Day 1 and Day 3.]

- Vehicle
- MAb DLK1

Statistical significance:

- p = 0.0002
- p = 0.0002

Figure 2
Figure 3
Figure 4