Biochemical and Biophysical Research Communications xxx (2018) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The high bone mass phenotype of *Lrp5*-mutant mice is not affected by megakaryocyte depletion

Timur Yorgan, Jean-Pierre David, Michael Amling, Thorsten Schinke*

Department of Osteology and Biomechanics, University Medical Center Hamburg Eppendorf, Hamburg 20246, Germany

ARTICLE INFO

Article history:
Received 12 February 2018
Accepted 14 February 2018
Available online xxx

Keywords:
Bone remodeling
Lrp5
Megakaryocyte
Mpl
High bone mass
Mouse model

ABSTRACT

Bone remodeling is a continuously ongoing process mediated by bone-resorbing osteoclasts and bone-forming osteoblasts. One key regulator of bone formation is the putative Wnt co-receptor Lrp5, where activating mutations in the extracellular domain cause increased bone formation in mice and humans. We have previously reported that megakaryocyte numbers are increased the bone marrow of mice carrying a high bone mass mutation (HBM) of Lrp5 ($Lrp5^{G170V}$). Since megakaryocytes can promote bone formation, we addressed the question, if the bone remodeling phenotype of $Lrp5^{G170V}$ mice is affected by megakaryocyte depletion. For that purpose we took advantage of a mouse model carrying a mutation of the *Mpl* gene, encoding the thrombopoietin receptor. These mice (Mpl^{hlb219}) were crossed with $Lrp5^{G170V}$ mice to generate animals carrying both mutations in a homozygous state. Using μCT, undecalcified histology and bone-specific histomorphometry of 12 weeks old littermates we observed that megakaryocyte number was remarkably decreased in $Mpl^{hlb219}/Lrp5^{G170V}$ mice, yet the high bone mass phenotype of $Lrp5^{G170V}$ mice was not significantly affected by the homozygous Mpl mutation. Finally, when we analyzed 24 weeks old wildtype and Mpl^{hlb219} mice we did not observe a statistically significant alteration of bone remodeling in the latter ones. Taken together, our results demonstrate that an increased number of bone marrow megakaryocytes does not contribute to the increased bone formation caused by Lrp5 activation.

1. Introduction

In order to maintain structural integrity and material quality, the skeleton is continuously remodeled. This process, which is facilitated by bone-resorbing osteoclasts and bone-forming osteoclasts, needs to be tightly controlled in order to maintain skeletal integrity. Disturbances of bone remodeling can lead to specific diseases, such as osteoporosis, a low bone mass disorder with high risk of skeletal fractures [1]. In recent years Wnt signaling has emerged as one of the key regulatory systems controlling the bone formation by osteoblasts [2,3]. Wnt signaling is mediated by soluble Wnt-ligands binding to membrane-bound receptors of the Frizzled family and is influenced by a vast number of co-receptors, inhibitors and modulators [4]. While it remains unclear, which ligand/receptor combination is most relevant for osteoblast activity, several other specific components of the receptor complex have proven to be of crucial importance in the context of bone remodeling, in particular the Wnt co-receptor Lrp5 and its antagonist Sclerostin.

Lrp5 is widely expressed in several cell types, including osteoblasts. Both, activating and inactivating mutations of Lrp5 have been found to severely influence bone mass [5]. More specifically, whereas inactivating *LRP5* mutations were identified in patients with osteoporosis pseudoglioma syndrome, activating *LRP5* mutations lead to osteosclerosis, a condition characterized by excessive bone formation and increased bone mass [6–8]. Meanwhile, there have been numerous reports of human skeletal disorders caused by alterations in Lrp5 and other Wnt-signaling components, firmly establishing the importance of the Wnt pathway for bone metabolism [2,5].

Since these conditions with altered bone mass are of central in the context of osteoanabolic osteoporosis treatment [9,10], mouse models harboring the murine equivalent of specific human mutations were developed. One of these mutations is $Lrp5^{G170V}$, equivalent to the human mutation $Lrp5^{G171V}$ [11,12]. Mice carrying this mutation display a marked increase in trabecular and cortical bone mass, presenting a phenocopy of the human disorder [12,13].

* Corresponding author. University Medical Center Hamburg Eppendorf, Martinistrasse 52, Hamburg 20246, Germany.
E-mail address: schinke@uke.uni-hamburg.de (T. Schinke).

https://doi.org/10.1016/j.bbrc.2018.02.127
0006-291X/

Interestingly, during our previous investigations utilizing this model, we observed an increased number of bone marrow megakaryocytes (MKs) in addition to the high bone mass phenotype [13,14]. Importantly, while the major function of this cell type is platelet production, MKs have been described to regulate skeletal remodeling, thereby affecting bone mass [15–26]. For example, it was reported that an increased number of bone marrow MKs can lead to increased osteoblast proliferation [15–22,24,25], an effect that was supported by coculture experiments [23]. Since MKs have also been described to produce specific ligands of the Wnt-family [27], we hypothesized that the high bone mass phenotype of $Lrp5^{G170V}$ mice is explained, at least in part, by an increased number of bone marrow MKs.

To address this question, we took advantage of the Mpl^{hlb219} mouse model that is characterized by a severely depleted bone marrow MK population due to a mutation in the *Mpl* gene encoding for the Thrombopoietin receptor [28].

These Mpl^{hlb21} mice were crossed with $Lrp5^{G170V}$ mice to obtain $Mpl^{hlb219/hlb219}/Lrp5^{G170V/G170V}$ animals that were analyzed by micro computed tomography, undecalcified histology and bone-specific histomorphometry.

2. Materials and methods

2.1. Animal husbandry and experiments

The mouse lines C57BL/6J-Mpl^{hlb219}/J (Mpl^{hlb219}) [28] and B6; 129-$Lrp5^{tm1.1Mawa}$/J ($Lrp5^{G170V}$) [12] were obtained from Jackson Laboratory (Maine, USA) and crossbred to generate mice carrying both mutations as well as control littermates. Genotyping of the *Mpl* allele was performed using the primers 5′-GCC AAG GTG AGG TGG ATA GA-3′ and 5′-TCC TCT GAT AGG GCC AAA GA-3′ to generate a PCR product that was subsequently analyzed by Sanger sequencing utilizing the forward primer to determine the presence of the mutation. Genotyping of the *Lrp5* allele was performed using primers (5′- AGT ACT GGC TGG CAC AGA-3′ and 5′- CAG GCT GCC CTT GCA GAT-3′) detecting a 246-bp wildtype or 400-bp $Lrp5^{G170V}$ allele. All mice were kept in a specific pathogen-free environment with a 12-h light/dark cycle, 45–65% relative humidity and 20–24 °C ambient temperature in open or individually ventilated cages with wood shavings bedding and nesting material in groups not surpassing 6 animals. The mice had access to tap water and standard rodent chow (1328P, Altromin Spezialfutter GmbH & Co. KG, Germany) *ad libitum*. After initiation of calcein labeling, the welfare of the mice was assessed daily based on overall appearance and body weight. All animal experiments were approved by the animal facility of the University Medical Center Hamburg-Eppendorf and by the "Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz" (G14/035 and Org529) in accordance with the local implementation of EU Directive 2010/63/EU for animal experiments.

2.2. Histology

To allow quantification of the bone formation rate, all animals received two doses of calcein (30 mg/kg i.p., Sigma–Aldrich Corp., USA) 10 and 3 days before sacrifice. The skeletons were fixed in 3.7% PBS-buffered formaldehyde for 24 h and subsequently stored in 80% ethanol. For bone histology, the lumbar vertebral bodies L1 to L4 and the right tibia of each mouse were dehydrated in ascending alcohol concentrations and then embedded in methylmetacrylate as described previously [29]. Sections of 4 μm thickness were cut in the sagittal plane on a Microtec rotation microtome (Techno-Med GmbH, Germany). These were stained by toluidine blue and von Kossa/van Gieson staining procedures as described [30]. Histomorphometry was performed according to the ASBMR guidelines [31] using the OsteoMeasure histomorphometry system (Osteometrics Inc., USA).

2.3. μCT analysis

For μCT analysis the right femur of each mouse was fixed and processed as described above. μCT scanning and analysis was performed with a voxel resolution of 10 μm as previously described using a μCT 40 desktop cone-beam microCT (Scanco Medical, Swizerland) [13] according to standard guidelines [32]. Trabecular bone was analyzed in the distal metaphysis in a volume situated 2500 μm–500 μm proximal of the distal growth plate. Cortical bone was analyzed in a 1000 μm long volume situated in the middle of the diaphysis. Cortical bone evaluation was performed with a threshold value of 300 and for trabecular bone a threshold value of 250 was implemented.

2.4. Statistical analysis

All data presented in the manuscript are presented as means ± standard deviations. Statistical analysis was performed using D'Agostino-Pearson omnibus normality test and unpaired, two-tailed Student's *t*-test or two-way ANOVA with Tukey's multiple comparison test using Prism (GraphPad Software Inc., USA). P-values below 0.05 were considered statistically significant. Sample size and power of analysis for animal experiments was determined by an *a priori t*-test with G*Power [33].

3. Results

Analysis of the femur from 12 weeks old female mice homozygous for the $Lrp5^{G170V}$ allele by μCT confirmed previously reported data [12,13]. They displayed a marked increase in trabecular bone mass in the distal metaphysis based on an increased trabecular thickness as well as an increase in the overall trabecular number (Fig. 1A and B). The cortical thickness at the femoral midshaft was significantly increased, while no change in the cortical porosity was observed. Importantly, introducing the homozygous Mpl^{hlb219} mutation into the $Lrp5^{G170V/G170V}$ background did not affect the high bone mass phenotype of $Lrp5^{G170V/G170V}$ mice, neither in the trabecular nor in the cortical bone compartment.

Additional histomorphometric analysis of the lumbar spine of these animals led to a similar result. While the $Lrp5^{G170V}$ mutation caused a significant and clear increase of the trabecular bone mass resulting from thicker and more abundant trabecular bone in comparison to wildtype littermates, the phenotype did not differ between $Mpl^{+/+}/Lrp5^{G170V/G170V}$ and $Mpl^{hlb219/hlb219}/Lrp5^{G170V/G170}$ mice (Fig. 2).

In-depth analysis by cellular histomorphometry on vertebral trabecular bone sections further revealed that while the number of MKs per marrow area was increased in $Mpl^{+/+}/Lrp5^{G170V/G170V}$ mice and, as expected, nearly completely depleted in $Mpl^{hlb219/hlb219}/Lrp5^{G170V/G170}$ mice (Fig. 3A and B), there were no other differences in the cellular parameters.

In fact, numbers of osteoblasts, osteocytes and osteoclasts did not differ between any of the three analyzed genotypes (Fig. 3 C). However, the bone formation rate was increased in $Mpl^{+/+}/Lrp5^{G170V/G170V}$ mice, and this aspect of the $Lrp5^{G170V}$ phenotype was not affected by the presence of the Mpl^{hlb219} mutation (Fig. 3 D). Of note, the only difference between $Mpl^{+/+}/Lrp5^{G170V/G170V}$ and $Mpl^{hlb219/hlb219}/Lrp5^{G170V/G170}$, apart from the number of MKs, was the mineralizing surface, which was significantly increased only in $Mpl^{hlb219/hlb219}/Lrp5^{G170V/G170}$ animals, when compared to wildtype controls.

Please cite this article in press as: T. Yorgan, et al., The high bone mass phenotype of *Lrp5*-mutant mice is not affected by megakaryocyte depletion, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.02.127