Osteoclasts are multinucleated giant cells derived from myeloid progenitors. Excessive bone resorption by osteoclasts can result in serious clinical outcomes for which better treatment options are needed. Here, we identified fibronectin leucine-rich transmembrane protein 2 (Flrt2), a ligand of the Unc5 receptor family for neurons, as a novel target associated with osteoclast differentiation. Flt2 expression is induced by stimulation with receptor activator of nuclear factor-kB ligand (RANKL). Flt2 deficiency in osteoclasts results in reduced hyper-multinucleation, which could be restored by RNAi-mediated knockdown of Unc5b. Treatment with Netrin1, another ligand of Unc5b which negatively controls osteoclast multinucleation through down regulation of RANKL-induced Rac1 activation, showed no inhibitory effects on Flt2-deficient cells. In addition, RANKL-induced Rac1 activation was attenuated in Flt2-deficient cells. Taken together, these results suggest that Flt2 regulates osteoclast multinucleation by interfering with Netrin 1-Unc5b interaction and may be a suitable therapeutic target for diseases associated with bone remodeling. [BMB Reports 2019; 52(8): 514-519]

INTRODUCTION

Skeletal homeostasis is maintained by balancing the bone forming function of osteoblasts and bone resorbing function of osteoclasts. Functional imbalance of osteoblasts and osteoclasts results in abnormal bone homeostasis (1). Osteoclasts are specialized multinucleated giant cells derived from monocyte/macrophage lineage bone marrow precursors (2). Osteoclast differentiation is initiated and sustained primarily by the osteoclast differentiation factor RANKL, which is mainly produced by osteoblasts and osteocytes (3, 4). Under pathogenic inflammatory conditions, excessive bone resorption by osteoclasts is often observed (5, 6). Currently, bisphosphonate and anti-RANKL antibody (Denosumab), which target early osteoclast commitment and/or osteoclast viability, are used for treatment (7). Although these treatments are efficiently anti-resorptive, long-term use compromises bone strength due to unintended inhibition of coupled bone formation. A better treatment strategy may be to target only late-stage osteoclast biological processes (i.e., osteoclast “maturation”), while preserving early osteoclast differentiation necessary for coupled bone formation (8). To do so in a manner that could lead to novel effective therapeutics to target bone remodeling diseases will require further investigation of the molecular mechanisms that control osteoclast maturation.

A hallmark of osteoclast maturation is multinucleation (9). Multinucleation is required for physiological bone resorbing activities (10), but hyper-multinucleation (defined by osteoclasts with > 100 micron diameter) often results in pathological bone loss (5, 6, 11). To identify gene targets associated with the late/maturation stage of osteoclast differentiation, we have previously performed comparative gene expression profiling by using multinucleation as a functional readout (8). As a result, we identified the gene Flt2 which encodes the protein fibronectin leucine-rich transmembrane protein 2 (Fht2) (Supplementary Fig. 1). Flt2 is a member of the fibronectin leucine-rich transmembrane family of proteins (Flrts: Flrt1-3) (12), which are classified as cell adhesion molecules (CAMs) (13), and are also reported to interact with heterophilic receptors, functioning as guidance factors in vascular, neural, and early embryonic development (14-21). Flt2 has been reported to function as a chemorepellent in axon guidance and cell migration through interactions with uncoordinated-5 (Unc5) receptors (19), and also to function in cell adhesion through an interaction with Latrophilins (22).

In this study, we identified Flt2, which has no previously reported function in bone, as a regulator of osteoclast multinucleation. We report here that gene deletion of Flt2 in osteoclasts resulted in reduced osteoclast hyper-multinucleation. Treatment with

Keywords: FLRT2, Hyper-multinucleation, Netrin 1, Osteoclast, Rac1, UNC5b
Netrin1, an alternative ligand of Unc5b which has been shown to inhibit osteoclast multinucleation through negative regulation of RANKL-induced Rac1 activation (23), showed no inhibitory effects on Flrt2-deficient cultures. Additionally, RANKL-induced Rac1 activation was impaired in Flrt2-deficient osteoclasts. These results suggest the possibility that Flrt2 and Netrin1 compete for Unc5b binding during osteoclast multinucleation, as osteoclast hyper-multinucleation is inhibited in Flrt2-deficient cells. Collectively, our results reveal a novel role for Flrt2 in fine-tuning of osteoclast multinucleation.

RESULTS

Flrt2 is involved in in vitro osteoclast differentiation

In order to understand the role of Flrt2 in osteoclasts, we first examined Flrt2 gene expression dynamics during osteoclast differentiation. We generated osteoclasts in vitro from mouse bone marrow-derived monocytes (BMMs) treated with M-CSF + RANKL for up to three days, and performed temporal qPCR expression analysis and western blots using whole cell lysates pulled down with a lectin that recognize glycoproteins including Flrt2. Expression of Flrt2 was induced, and peaked on day one after RANKL stimulation (Fig. 1A). We next generated Flrt2 conditional knockout mice by crossing floxed Flrt2 mice (Flrt2fl/fl) with Mx1Cre mice, which express inducible Cre recombinase, as osteoclast deletion of Flrt2 expression in mice leads to embryonic lethality (17). In Flrt2fl/flMx1Cre mice, the Flrt2 gene is deleted upon polyinosinic-polycytidylic acid (pol I:C) treatment in osteoclast precursors, enabling us to examine the effect of Flrt2 deletion on osteoclast differentiation. We generated BMMs from Flrt2fl/fl, Flrt2fl/flMx1Cre, and Flrt2fl/flMx1Cre mice, and confirmed Flrt2 gene deletion in genomic DNA from Flrt2fl/flMx1Cre BMMs using PCR (Fig. 1B). Consistent with this, Flrt2 gene expression was completely abolished in Flrt2fl/flMx1Cre BMMs (Fig. 1B). We then examined whether Flrt2 gene deletion affects osteoclast formation. We prepared Flrt2fl/fl and Flrt2fl/flMx1Cre BMMs and cultured with M-CSF + RANKL to generate osteoclasts. We found reductions in total tartrate-resistant acidic phosphatase (TRAP) activity and in frequency of multinucleated TRAP+ cells (i.e., mature osteoclasts) in Flrt2fl/flMx1Cre cultures compared to Flrt2fl/fl cultures (Fig. 1C). Reductions in hyper-multinucleated osteoclasts (> 100 μm) were particularly dramatic (Fig. 1C). Message levels for the osteoclast differentiation and maturation markers Nfatc1, CtsK, and Atp6v0d2 showed small but still significant differences in Flrt2fl/flMx1Cre cultures on Day 3, while Destamp showed no differences (Fig. 1D). Retroviral transduction of Flrt2Mx1Cre BMMs with full length Flrt2 completely restored TRAP+ hyper-multinucleated cells in Flrt2fl/flMx1Cre cultures (Fig. 1E), suggesting that the phenotypes observed in Flrt2fl/flMx1Cre cultures can be attributed to deletion of the Flrt2 gene. These results suggest a role for osteoclast-derived Flrt2 in osteoclast differentiation, and more specifically in osteoclast hyper-multinucleation.

Osteoclast-derived Flrt2 is required for bone homeostasis

To examine whether osteoclast-derived Flrt2 is required for bone development and homeostasis, we performed representative 3D reconstructions of trabecular bone from the femurs of gender- and age-matched Flrt2fl/fl and Flrt2fl/flMx1Cre mice. Bone microstructure imaging by high resolution microcomputed tomography (μCT) of Flrt2fl/flMx1Cre mice revealed slight but significant increases in bone mass, characterized by...
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Flrt2 is required for bone homeostasis.

To understand the molecular mechanism that regulates Flrt2-mediated osteoclast hyper-multinucleation, we examined RANKL-dependent signaling pathways, including ERK, p38, JNK, and NF-kB, which are activated via the RANK-TRAF6 axis. We found no significant differences in activation of these signaling molecules between Flrt2fl/fl and Flrt2fl/flMx1Cre cultures (data not shown), suggesting that osteoclast-expressing Flrt2 is not involved in regulation of the RANK-TRAF6 signaling axis.

Flrt2, Netrin 1 and Unc5b regulation of osteoclast multinucleation

Flrts have been reported to act as CAMs and also as ligands of repulsive receptors (21, 24). Flrts have also been reported to be proteolytically cleaved near the plasma membrane in neurons (19). To determine whether Flrt2 functions as a CAM or as a ligand in osteoclasts, we examined cleavage of Flrt2 during osteoclast differentiation. We prepared whole cell lysates and supernatants from Flrt2fl/fl and Flrt2fl/flMx1Cre cultures, pulled down with a lectin, and performed western blotting using Flrt2-specific antibody. Membrane-bound Flrt2, which ranges in size from 80 to 100 kDa, was detected in whole cell lysate of Flrt2fl/fl cultures one day after RANKL stimulation (Fig. 3A). Expression of membrane-bound Flrt2 gradually decreased during osteoclast differentiation, and disappeared almost completely in mature osteoclast cultures. By contrast, the cleaved form of Flrt2, which ranges in size from 60 to 80 kDa, was detected in supernatants from two days after RANKL stimulation (Fig. 3A). The cleaved form of Flrt2 was decreased after treatment with Brefeldin A, a trafficking inhibitor, suggesting that trafficking of membrane-bound Flrt2 was inhibited, and consequently, that presence of the cleaved form was decreased (Supplementary Fig. 2).

Neither membrane-bound nor cleaved forms of Flrt2 were detected in Flrt2fl/flMx1Cre cultures (Fig. 3A). The cleaved form of Flrt2 was detected in Flrt2fl/flMx1Cre cultures after retroviral transduction of Flrt2 (Fig. 1E). These results suggest that osteoclast-expressed Flrt2 is cleaved during osteoclast differentiation, and further suggest that Flrt2 might predo-

Fig. 1E

Fig. 2. Osteoclast-derived Flrt2 is required for bone homeostasis. μCT images of femurs from Flrt2fl/fl and Flrt2fl/flMx1Cre mice. BV/TV, Tb.Th, Tb.N, Tb.Sp, and Ct.Th are shown. Scale bars represent 0.5 mm. Data are shown as mean ± S.D. *P < 0.05, **P < 0.01.
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Flr2 has been shown to interact with Unc5b, Unc5d and/or Latrophilin3 (18-20, 22). Unc5b has been demonstrated as the predominant receptor for Flr2 in endothelial cells during vascular development (20). To gain insight into the predominant receptor for Flr2 in osteoclast formation, we performed qPCR expression analysis. We detected expression of Unc5b (Fig. 3B) but not Unc5d or Latrophilin3 (data not shown). The expression levels of Unc5b were comparable between Flr2\textsuperscript{fl/fl}Mx1Cre and Flr2\textsuperscript{fl/fl} cultures, and decreased in osteoclasts (Fig. 3B). To identify the involvement of Unc5b in Flr2-mediated regulation of osteoclast multinucleation, we performed RNAi experiments using retrovirus encoding shRNA for Unc5b. RNAi-mediated knockdown of Unc5b in Flr2\textsuperscript{fl/fl}Mx1Cre BM\textsuperscript{M}Vs restored osteoclast hyper-multinucleation, and furthermore, knockdown of Unc5b in Flr2\textsuperscript{fl/fl} cultures resulted in slightly increased numbers of TRAP\textsuperscript{+} hyper-multinucleated osteoclasts (Fig. 3B).

Unc5 receptors are known as classical signaling receptors for secreted Netrin ligands in the nervous system (24). In the bone system, Netrin 1 is produced by various bone cells, including osteoclasts (23, 25), and Netrin 1-Unc5b interactions negatively regulate osteoclast multinucleation (23). Interestingly, Netrin 1 has been shown to compete with Flr2 for Unc5d (19). We found comparable message levels of Netrin 1 in both Flr2\textsuperscript{fl/fl} and Flr2\textsuperscript{fl/fl}Mx1Cre BM\textsuperscript{M}Vs and osteoclasts (Fig. 3C). We examined whether Netrin 1-Unc5b interactions mediated inhibitory effects on Flr2-deficient osteoclast maturation. We prepared BM\textsuperscript{M}Vs from Flr2\textsuperscript{fl/fl} and Flr2\textsuperscript{fl/fl}Mx1Cre mice and treated them with M-CSF and RANKL in the presence or absence recombinant Netrin 1. Formation of hyper-multinucleated osteoclasts was significantly inhibited in Flr2\textsuperscript{fl/fl} cultures, consistent with a previous report (Fig. 3C) (23). By contrast, an inhibitory effect of recombinant Netrin 1 was not observed in Flr2fl/flMx1Cre cultures, consistent with a previous report (Fig. 3D) (23). The expression levels of Rac1 and Rac proteins were normal after RNAi-mediated knockdown of Unc5b (Fig. 3C). These results suggested that Flr2 might compete with Netrin 1 for Unc5b, such that Unc5b might be saturated with osteoclast-derived Netrin 1. In the absence of Flr2, and consequently, no additional inhibition was observed in Flr2\textsuperscript{fl/fl}Mx1Cre cultures by adding recombinant Netrin 1.

We here showed that osteoclast-expressed Flr2 is cleaved during osteoclast differentiation, suggesting that osteoclast-derived Flr2 might function as a ligand, but not as a CAM. A previous report showed that Flr2 cleavage requires metalloproteinase (MMP) activity (19). In osteoclasts, MMP-9 is the most abundant MMP (27), suggesting the possibility that MMP-9 is involved in Flr2 cleavage in osteoclasts. Although we showed here that most osteoclast-expressed Flr2 was cleaved in mature osteoclasts, we cannot conclude that membrane-bound Flr2 does not play a role in osteoclasts, and further studies will be required to address this issue.

We sought to identify the molecular mechanisms by which Flr2 regulates osteoclast multinucleation, and found that RNAi-mediated knockdown of Unc5b, the predominant receptor for Flr2 in endothelial cells and also a receptor for Netrin 1 in osteoclasts, restored hyper-multinucleation in Flr2\textsuperscript{fl/fl}Mx1Cre cultures. These findings suggest that Unc5b is involved in Flr2-mediated regulation of osteoclast hyper-multinucleation. Given that Netrin 1 competes with Flr2 for Unc5d (19), we hypothesized that Flr2 could also compete with Netrin 1 for Unc5b, thus interfering with the Netrin 1-Unc5b-mediated negative regulation on osteoclast multinucleation (Fig. 3E). Indeed, we showed that Flr2 competes with Unc5b for Unc5b binding, and contributes to fine-tuning of osteoclast multinucleation.

**DISCUSSION**

Osteoclast maturation involves multinucleation, which occurs via incomplete cytokinesis and/or cell fusion (26). The importance of multinucleation in osteoclast formation is demonstrated by the impaired bone-resorbing activity of osteoclasts that cannot become multinuclear cells (10). Although multinucleation is important for osteoclast function, hyper-multinucleation is often observed in pathogenic inflammatory conditions where it contributes to excessive bone resorption. However, the molecular mechanisms underlying regulation of osteoclast hyper-multinucleation are not well understood. In this study, we identified Flr2, which has previously been functionally characterized in systems other than bone, as a regulator for osteoclast multinucleation. Using conditional knockout mice, we revealed an osteoclast-intrinsic role of Flr2 in multinucleation. Additionally, we showed involvement of Unc5b in Flr2-mediated regulation of osteoclast multinucleation. Our results reveal Flr2 as a novel regulator of osteoclast multinucleation.
and 2) RANKL-induced Rac1 activation was inhibited in Flrt2\textsuperscript{-/-}x1Cre cultures. Since message levels of Netrin 1 were not affected by the absence of Flrt2, it is plausible that reduced hyper-multinucleation in the absence of Flrt2 is due in some part to negative regulation of Rac1 activation by Netrin 1-Unc5b interactions. However, there is a difference between Flrt2\textsuperscript{-/-} and Flrt2\textsuperscript{-/-}x1Cre cultures in terms of frequency of hyper-multinucleated osteoclasts after RNAi-mediated knockdown of Unc5b (Fig. 3B), suggesting the involvement of a Unc5b-independent mechanism. Further studies will be required to determine the molecular mechanism underlying regulation of osteoclast multinucleation through Flrt2.

Considering the severity of the in vitro phenotype of Flrt2\textsuperscript{-/-}x1Cre osteoclast differentiation, the Flrt2\textsuperscript{-/-}x1Cre bone phenotype seems modest. We found expression of another Flrt family member, Flt3 (but not Flt1), was induced in both Flrt2\textsuperscript{-/-} and Flrt2\textsuperscript{-/-}x1Cre cultures (Supplementary Fig. 3, and data not shown). Flt3 has been demonstrated to act as a ligand of Unc5b in the nervous system. Indeed, RNAi-mediated knockdown of Flt3 in Flrt2\textsuperscript{-/-}x1Cre BMMs showed an additive inhibitory effect on osteoclast hyper-multinucleation (Supplementary Fig. 3), raising the possibility of involvement of Flt3 in osteoclast differentiation and bone homeostasis in vivo. Future characterization of Flt2 and Flt3 double conditional knockout mice may be useful for addressing this issue. In addition, Flts are broadly expressed proteins. Given that Flts are cleaved, cleaved Flts derived from other tissues might compensate the lack of osteoclast-derived Flt2 in vivo. Further studies will be required to clarify the impact of Flts in bone homeostasis.

Taken together, although a full understanding of how Flt2 contributes to osteoclast multinucleation and its impact on bone homeostasis remain unclear, we provide evidence of a fine-tuning function of the chemorepellent Flt2 in osteoclast multinucleation. With additional characterization, Flt2 may be a good candidate for a therapeutic target for bone diseases that are accompanied by bone destruction.

MATERIALS AND METHODS

**Mice**

Flt2\textsuperscript{-/-} mice were generated as described previously (20). To obtain Flt2 conditional knock-out mice (Flt2\textsuperscript{-/-}x1Cre) in hematopoietic cells, homozygous Flt2\textsuperscript{-/-} mice were crossed with x1Cre transgenic mice [Tg(x1Cre)1Cgn] purchased from Jackson Laboratory. For induction of Cre recombinase expression in x1Cre mouse, mice were injected intraperitoneally with 250 μg PolyI:C (SIGMA) 10 days after birth three times every other day. Mice were used for in vitro and in vivo experiments 6 weeks after the first injection. All mice were maintained and used in accordance with guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

**Reverse transcription and real-time PCR (qPCR)**

Total RNAs were extracted from cells using Trizol reagent (Invitrogen), and reverse transcription was performed with High capacity cDNA kit (Thermo Fisher Scientific). qPCR was performed using Quant Studio3 (Applied Biosystems) with the following TaqMan probes: Flt2 (Mm03809571_m1), Flt3 (Mm01328142_m1), Unc5b (Mm0050454_m1), Dcstatc (Mm04209236_m1), CtsK (Mm00484039_m1), Nfatc1 (Mm00479445_m1), ATP6v0d2 (Mm01222963_m1), 18s (Hs99999901_s1).

**In vitro osteoclast differentiation and tartrate-resistant acid phosphatase (TRAP) staining**

BMMs and osteoclasts were prepared as described previously (8). Cells were fixed with 3.7% formalin and stained using the Acid Phosphatase, Leukocyte (tartrate-resistant acid phosphatase kit (387A-1KT, Sigma) according to the manufacturer's instructions. TRAP positive multi-nuclear cells were counted. Recombinant mouse Netrin1 (1109-N1) was purchased from R&D. Brefeldin A was purchased from Thermo Fisher Scientific.

**Retrovirus preparation and transduction**

To prepare retroviral particles, Plat-E packaging cells were transfected with pMX vectors encoding C-terminally FLAG-tagged Flt2, pSuper vectors encoding siRNA targets for Unc5b (5'-GCCACGCAGATCTACTTCAAGTGTA-3'), and Flt3 (5'-GA GCACCATCATGATTAGACCAGCA-3') using PElmax (Poly-sciences). Empty pMX vector and pSuper vector were used as negative controls. After 3 days, medium containing retrovirus was harvested and passed through a syringe filter (0.45 μm pore diameter). BMMs were transduced with retroviruses for 16 h with hexadimethrine bromide (8 μg/ml) in the presence of M-CSF (60 ng/ml). Infected cells were selected by culturing for 2 days in the presence of puromycin (2 μg/ml) with M-CSF (60 ng/ml). Puromycin-resistant BMMs were used for experiments.

**Immunoblot analysis**

Cells were lysed with sample buffer (0.5% Triton-X 100, 20nM Tris-HCl, pH 7.5, 150 mM NaCl). Glycoprotein were pulled down from total cell lysates or culture supernatants using lectin (SIGMA: L1882) to enrich Flt2. Protein concentrations were measured from total cell lysates or culture supernatants using BCA assay (Thermo Fisher Scientific). Western blotting was performed with the following antibodies: anti-Flt2: AF2877 (R&D), and anti-Actin: sc-47778 (Santacruz). Rac1 activity was measured using Rac1 activation detection kit (Thermo scientific).

**Micro-computed tomography**

Femurs from Flt2\textsuperscript{-/-} and Flt2\textsuperscript{-/-}x1Cre mice were harvested and scanned by μCT (μCT35, SCANCO Medical AG, Brüttisellen, Switzerland). For trabecular bone analysis, scans were performed at the distal femoral metaphysis 0.4 mm
proximal to the growth plate. For cortical bone, the mid-diaphysis of femurs was scanned. All scans were performed at a resolution of 6 µm per slice using an X-ray energy of 55 kvp and an integration time of 300 ms. A total of 200 slices for trabecular bone and 50 slices for cortical bone were analyzed using the instrument’s software.

Statistical analysis
All experiments were analyzed using two-way ANOVA or unpaired Student’s t-test by Prism 6.0 (GraphPad). P < 0.05 was considered statistically significant.

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CONFLICTS OF INTEREST
The authors have no conflicting interests.

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