Control of Bone Resorption by Semaphorin 4D Is Dependent on Ovarian Function

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Abstract
Osteoporosis is one of the most common bone pathologies, which are characterized by a decrease in bone mass. It is well established that bone mass, which results from a balanced bone formation and bone resorption, is regulated by many hormonal, environmental and genetic factors. Here we report that the immune semaphorin 4D (Sema4D) is a novel factor controlling bone resorption. Sema4D-deficient primary osteoclasts showed impaired spreading, adhesion, migration and resorption due to altered β3 integrin sub-unit downstream signaling. In apparent accordance with these in vitro results, Sema4D deletion in sexually mature female mice led to a high bone mass phenotype due to defective bone resorption by osteoclasts. Male mutants, however, displayed normal bone mass and the female osteopetrotic phenotype was only detected at the onset of sexual maturity, indicating that, in vivo, this intrinsic osteoclast defect might be overcome in these mice. Using bone marrow cross transplantation, we confirmed that Sema4D controls bone resorption through an indirect mechanism. In addition, we show that Sema4D /−− mice were less fertile than their WT littermates. A decrease in Gnrh1 hypothalamic expression and a reduced number of ovarian follicles can explain this attenuated fertility. Interestingly, ovariectomy abrogated the bone resorption phenotype in Sema4D /−− mice, providing the evidence that the observed high bone mass phenotype is strictly dependent on ovarian function. Altogether, this study reveals that, in vivo, Sema4D is an indirect regulator of bone resorption, which acts via its effect on reproductive function.

Introduction

Osteoporosis is one of the most common bone pathology, which is characterized by a decrease in bone mass, leading to an increased fracture risk. Therefore, slowing the osteoporosis progression has become a major health issue in the aging population of the northern hemisphere. Reproductive hormones fluctuations have a well-established role in osteoporosis. In early menopause, the acute phase of estrogen deficiency is associated with an increased bone loss [1]. However, clinical studies, have provided evidence that the etiology of this disease is more complex than just a question of estrogen withdrawal and that overall bone mass is influenced by many environmental, but also genetic factors [2]. Among those factors, several studies have suggested a role for semaphorin family members in the control of bone mass. Sema3B expression in osteoblasts is induced by vitamin D3 and its deficiency leads to osteopenia, secondary to increased osteoclastogenesis [3]. Sema7A is also expressed in bone cells [4]. Sema7A as well as Plexin A2 (Sema 3A receptor) polymorphisms have been associated with decreased bone mineral density and increased fracture risk in postmenopausal Korean woman [5,6]. Deficiency in Plexin A1, the Sema6D receptor, leads to increased bone mass due to a defect in osteoclast differentiation [7].

Sema4D is a membrane-bound class 4 semaphorin that was first identified in the immune synapse where it mediates cell-to-cell interactions and regulates the immune response [8]. Sema4D is in fact expressed in other tissues including platelets, ovaries and the nervous system, where it has several functions [9]. Sema4D stimulates both in vitro and in vivo angiogenesis [10,11]. In the nervous system, Sema4D is involved in neuronal migration and neurite outgrowth. For example, Sema4D has been shown to regulate gonadotropin hormone-releasing hormone-1 (Gnrh1) positive neuron migration through the PlexinB1-Met complex [12,13]. Sema4D acts on another level of the hypothalamic-hypophyseal-ovarian axis, as ex vivo data has shown that Sema4D is involved in mouse ovary follicle maturation [14]. Interestingly, structural studies have revealed strong similarities between the Sema4D homodimer and the αVβ3 integrin heterodimer, which is the most abundant integrin receptor expressed in bone resorbing osteoclasts [15]. αVβ3 integrin plays a major role in osteoclast function, both in vitro and in vivo. For instance, integrin β3 /−− mice become progressively osteosclerotic with age due to dysfunctional osteoclasts that fail to polarize correctly and develop abnormal ruffled membranes [16]. In the present study, using the loss of function approach, we addressed the question whether Sema4D could also play a role in bone resorption. We first show that Sema4D is expressed in osteoclasts and that like β3 integrin-deficient osteoclasts, Sema4D /−− osteoclasts exhibit a resorption defect consecutive to reduced migration and spreading. We then provide
evidence that Sema4D−/− females displayed a high bone mass phenotype associated to a bone resorption defect. Interestingly this high bone mass phenotype was not found in males and in sexually immature female. Besides this phenotype, we showed that Sema4D−/− females exhibited a reproductive defect. Finally, we demonstrate that the bone resorption phenotype does not result from an osteoclast-specific defect but that it is strictly dependent on Sema4D action on ovarian function. Thus Sema4D is an indirect regulator of bone resorption that acts through its effect on the reproductive system in females.

Results

Sema4D expressed in osteoclasts, delays osteoclasts differentiation and affects osteoclast function

The most abundant integrin in osteoclasts is αVβ3 integrin, which plays a major role in regulating the bone resorption process [17]. Because Sema4D homodimers share many structural similarities with the αVβ3 integrin heterodimer [15], we hypothesized that Sema4D could have similar functions. We found that Sema4D mRNA, as well as the protein, is expressed in pre- and in mature primary osteoclasts (Figs. 1A–B) [18]. In contrast, Sema4D was not detected in primary osteoclasts derived from mouse calvaria (Fig. 1B). Moreover, FACS analysis showed that Sema4D is expressed at the osteoclast cell membrane. We further localized Sema4D within cytoskeletal structures by specific β3 integrin co-immunolabeling in mature osteoclasts (Figs. 1C–D). As expected, anti-β3 integrin labeled the podosome belt at the periphery of mature osteoclasts and Sema4D partially colocalized with β3 integrin. These observations were compatible with a role for Sema4D in osteoclast mediated bone resorption. To explore the potential effect of Sema4D on osteoclast differentiation, we first carried out classical in vitro primary osteoclast differentiation assays in the presence of M-CSF and RANKL. Osteoclast differentiation assays of WT or Sema4D-deficient bone marrow isolated precursors revealed that differentiation was reduced in the absence of Sema4D, as the number of osteoclasts measured in WT cultures was higher that in Sema4D−/− on days 4 and 5 (Fig. 1E). In order to assess if the reduced number of osteoclasts obtained in Sema4D−/− cultures was due to a proliferation or a fusion defect in progenitor cells, we first performed a proliferation test on osteoclast precursors isolated from Sema4D−/− or WT bone marrow in the presence of M-CSF. Crystal violet proliferation tests revealed that bone-marrow derived precursors isolated from Sema4D-deficient mice exhibited a small but statistically significant proliferation delay at early time points, which was abrogated after 5 days in culture (Fig. 1F). This later result strongly suggested that the fusion process had decreased, as a consequence of a slightly lower proliferation of Sema4D−/− precursors. To definitively prove that the reduced number of Sema4D osteoclasts was due to an intrinsic fusion defect, but to proliferation, we plated 20% more Sema4D−/− precursors than WT in the presence of M-CSF and RANKL, to overcome their slower proliferation rate. Under such plating conditions, the same number of mature osteoclasts was obtained. Indeed, they contained the same number of nuclei as WT, showing that under such conditions the process of differentiation was not affected by Sema4D deficiency (Fig. 1G). These plating conditions allowed us to really compare Sema4D−/− and WT osteoclasts from the same differentiation stage, and have been used all along this study.

To then test whether osteoclast function could be affected in the absence of Sema4D, we performed resorption tests on an apatite collagen complex (ACC) and showed that in the absence of Sema4D, the percentage of resorbed area per osteoclast was significantly reduced, by two fold, in the Sema4D-deficient osteoclasts compared to WT (Fig. 2A). As resorption by osteoclasts is a multistep process that requires adhesion, spreading, resorption per se, and migration [19], we wanted to determine which of these steps was affected in the absence of Sema4D. Measurements by time-lapse video microscopy of WT and Sema4D−/− mature osteoclasts (n=50) revealed that mature Sema4D-deficient osteoclasts migrated more slowly than WT osteoclasts (Fig. 2B). Cell spreading was delayed in the absence of Sema4D−/− as cell area was reduced during the early stages of adhesion on vitronectin. This difference was not observed at the latest time point, indicating that Sema4D−/− osteoclasts were able to make up the delay (Fig. 2C). Altogether, these data indicate that Sema4D-deficient osteoclasts migrate more slowly than WT and subsequently resorb mineralized matrix less efficiently than WT osteoclasts.

Altered αVβ3 integrin mediated adhesion dependent signaling in Sema4D−/− osteoclasts

Osteoclast migration, adhesion and resorption are highly linked processes. They are all promoted by dynamic cytoskeletal reorganization, which involves αVβ3 integrin-dependent signaling. Having shown that spreading, migration and resorption are affected in Sema4D−/− osteoclasts, we asked whether downstream signaling in response to αVβ3 integrin activation could normally occur in osteoclasts lacking Sema4D. To address this question, we analyzed c-Src and Pyk2 phosphorylation, both molecular components of the adhesion complex triggered by αVβ3 integrin engagement. Osteoclasts cultured with M-CSF and RANKL for four days were detached and either maintained in suspension or layered on vitronectin coated plates for 30 min. Hyper-phosphorylation of both kinases was observed in the Sema4D−/− osteoclasts, either maintained in suspension or adherent as if they were constitutively active in the mutant cells (Fig. 2E). We sought to determine whether further αVβ3 downstream signaling was also perturbed. Rac1-GTPase regulates cellular actin-dynamics, and it is the downstream target of αVβ3 and Vß3 integrin activation could normally occur in osteoclasts lacking Sema4D. In accordance with c-Src and Pyk2 hyper-phosphorylation, we found that the Rac1-GTP content was higher in Sema4D−/− osteoclasts, regardless of the substratum (Fig. 2F), whereas the GTP-bound Rac1 level in early-state osteoclasts was similarly low in both WT and Sema4D−/−. In summary, a Sema4D deficiency in primary osteoclasts led to reduced resorption activity, in part due to dysfunction of the adhesion properties and an unbalanced regulation of β3 integrin sub-unit signaling.

Sema4D deficiency leads to high bone mass phenotype due to decreased bone resorption in sexually mature female mice

To determine if Sema4D has a function in bone, we first analyzed Sema4D-deficient mice using histomorphometry. In 3-month-old female vertebrae, quantification of the trabecular bone volume revealed a 33% increased bone mass in Sema4D-deficient mice compared to WT (Fig. 3A). Bone structure was also modified, the thickness of the trabeculae being unaffected, while the number of nodes and the trabecular spacing were significantly lower in Sema4D-deficient mice compared to WT, indicating that the number, but not the size, of the trabeculae was modified in the Sema4D-deficient mice (Fig. 3B). We then tested resorption parameters in vivo. Deoxypyridinoline crosslinks (DPD) were decreased in the Sema4D-deficient mice when compared to their WT littermates, showing that the increased bone mass resulted, at least in part, from decreased bone resorption (Fig. 3D). Neither the
Figure 1. Sema4D is expressed in osteoclasts and its deletion delays osteoclasts progenitors proliferation and differentiation in vitro. A–D) Sema4D expression and localization. A/ QPCR analysis showing an increase of Sema4D expression along the osteoclast differentiation pathway. B/ Total cell extract, either from primary calvaria derived osteoblast cultures or from osteoclasts isolated from WT or Sema4D-deficient mice, were immunoblotted using an anti-Sema4D specific antibody. Sema4D is present in WT osteoclasts. ß-tubulin was used as loading control. C/ FACS analysis on non-permeabilized pre-osteoclasts shows that Sema4D is expressed at the WT cell surface but not in Sema4D−/− cells. D/ Mature osteoclasts were fixed and immunolabeled with anti-Sema4D or anti-ß3 integrin chain antibodies. Merge picture and inset show that Sema4D partially colocalized with the ß3 integrin chain (bar: 10 μm). E–G) Sema4D−/− osteoclast progenitors differentiation and proliferation rate. E/ Bone marrow precursor cultured for either 4 or 5 days in the presence of MCSF and RANKL. Cultures were then stained for TRAP activity and TRAP positive multinucleated cells formed were counted. This showed that the formation of TRAP positive multinucleated osteoclasts from Sema4D−/− precursors was reduced compared with WT. F/ Bone marrow precursors were cultured in the presence of MCSF to assess proliferation of progenitor cells. At each time point, the cells were fixed and stained with crystal and the 590 nm OD measured, reflecting the cell number. This shows that Sema4D−/− precursors proliferate more slowly than their WT counterparts during the growth phase. G/ To confirm that the delay in Sema4D−/− osteoclast formation could be due to their impaired proliferation rate, 20% more Sema4D−/− bone marrow precursors (375 cells/mm²) were seeded than WT (312 cells/mm²) in the presence of M-CSF and RANKL. Mature multinucleated TRAP positive osteoclasts were counted on day 5. Similar osteoclast counts were obtained in both cultures and they displayed the same degree of multinucleation. Error bars represent SEM, *** indicate a p value=0.001. doi:10.1371/journal.pone.0026627.g001
number nor the size of osteoclasts had been affected in the Sema4D-deficient mice, showing that the decreased bone resorption observed in Sema4D-deficient mice was due to a functional defect in the osteoclasts (Fig. 3C). On the other hand, the measured cellular and dynamic bone formation parameters were similar in Sema4D-deficient and WT mice (Fig. S1A–B). Altogether these data show that Sema4D is indeed a physiological regulator of bone resorption, without affecting bone formation.
The bone resorption phenotype of Sema4D-deficient mice is gender dependent and is not osteoclast autonomous

We further analyzed the bone phenotypes of WT and Sema4D-deficient females at earlier and later stages (1 and 6 months). These analyses revealed that the bone resorption phenotype was only present in sexually mature females, since 6-month-old females had increased bone mass similar to the 3-month-old mice, while 1-month-old Sema4D-deficient females had normal bone mass (Fig. 4A). On the other hand, 3-month-old Sema4D-deficient males had similar bone mass than their WT littermates (Fig. 4A). These observations questioned the assumption that the bone phenotype observed in Sema4D−/− animals relied on an osteoclast-related intrinsic defect. To answer this question, we performed Sema4D−/− or WT bone marrow cross transplants in WT and Sema4D-deficient mice, following lethal doses of gamma radiation leading to hematopoietic lineage depletion, including that of osteoclasts. The results of these bone marrow cross transplants revealed that the bone phenotype of the transplanted mice did not depend on the donor genotype but on genetic background of the transplanted mice. Indeed, Sema4D-deficient mice had a higher BV/TV than the WT mice, whether they have been grafted with Sema4D-deficient or WT osteoclasts (Fig. 4B). Similarly, urinary DPD levels were higher in Sema4D−/− recipient mice than in the WT mice regardless of the graft genotype (Fig. S2). Thus, this unexpected result showed that the Sema4D−/− bone resorption phenotype is not secondary to an osteoclast-specific defect. In other words, the functional defects of Sema4D−/− osteoclasts that we observed in vivo are either too mild to be detected in vivo or are compensated for in vivo. Defective activities of osteoclasts lacking Sema4D have revealed an altered β3 integrin sub-unit down-stream signaling, suggesting a common pathway for both proteins and a functional cross talk. To test this latter hypothesis, we transduced BMMs derived either from WT or Sema4D−/− mice, with empty retroviruses (PMX) or retroviruses expressing the full-length β3 cDNA. After antibiotic selection, mature osteoclasts were differentiated and their ability to resorb mineralized matrix assessed. Over expression of the integrin β3 chain in Sema4D−/− osteoclasts rescued their deficiencies. Indeed, integrin β3 transduced Sema4D−/− osteoclasts were able to resorb mineral matrix to the same extent as WT osteoclasts (Fig. 4C). These results show that the integrin β3 chain over expression can compensate for Sema4D−/− osteoclast resorption defects at least in vitro and indicate that Sema4D regulates bone resorption by an indirect mechanism in vivo.

Reproductive defect in Sema4D-deficient mice

While characterizing the origin of the Sema4D−/− mice high bone mass phenotype, we noticed that these mice produced fewer pups than WT, suggesting that Sema4D−/− mice were less fertile. Quantification of both the number of pups per brood and the number of litters per breeding period confirmed the decreased reproductive ability of Sema4D-deficient mice (Fig. 5A-D). We then asked how the Sema4D deficiency influenced the decreased fertility observed in these mice. It has been reported that deletion of Sema4D receptor plexinB1 (PlexB1) leads to a reduced number of GnRHI secreting neurons in adult mice hypothalamus. We speculated though that Sema4D-deficient mice might present a
similar phenotype [12]. Indeed, quantitative PCR revealed a 40% reduction in the Gnrh1 transcript level in the hypothalamus of Sema4D −/− mice, whereas no variation was observed in frontal brain used as a tissue control (Fig. 5D). As gonadotropin-hormone releasing hormone-1 (Gnrh1) is involved in the hypothalamic-pituitary-ovarian axis, we then wondered if the decreased expression of Gnrh1 could result in impaired ovarian function, which could explain the decreased fertility observed in Sema4D −/− mice. We compared the morphological follicles organization and counted the number of classified follicles in the ovaries of mutant versus WT mice. Analysis of histological ovary sections showed a significant reduced number of secondary follicles in the Sema4D −/− mice (Fig. 5E). On the other hand, ovary and uterus weights were similar in three-month-old Sema4D −/− females, compared to their WT counterparts (Fig. S3A-B). Accordingly, we did not find any significant variations in the basal levels of estradiol (E2) (Fig. S3B). Altogether our results show that Sema4D deficiency is required for proper female reproductive function.
Sema4D regulation of bone resorption is dependent on female reproductive function

The bone marrow transplant experiment demonstrated that the bone resorption defect observed in Sema4D−/− mice did not result in an intrinsic osteoclast defect (Fig. 4B). Moreover this phenotype was observed only in sexually mature females and the Sema4D−/− reproductive function was impaired (Fig. 4A and 5A–C). Altogether these results strongly suggested that the Sema4D bone resorption defect was dependent on ovarian function. To confirm this assertion, we ovariectomized 2-month-old Sema4D-deficient females and analyzed their bone phenotype one month later. As expected, WT ovariectomized mice had a lower uterine weight and lost about 20% of their tibial trabecular bone mass as a result of an increased bone resorption (Fig. 6A–B and S2A). On the other hand, following ovariectomy, Sema4D−/− mice showed a 45% increase in bone resorption than WT mice (Fig. 6B). As a result, Sema4D−/− ovariectomized females had similar bone volumes to those of the WT ovariectomized females (Fig. 6A). The observation that the phenotype is osteoclast independent but sex dependent, together with impaired reproductive function, strongly suggests that the Sema4D−/− resorption phenotype is directly linked to ovarian function.

Discussion

Although semaphorins were first identified as axon guidance cues, they have since been shown to be important regulators of a wide variety of biological functions. The present study has uncovered new functions for semaphorin 4D as a regulator of bone resorption both in vitro and in vivo.

In vitro, Sema4D−/− osteoclasts are unable to resorb mineralized matrix properly and this defect is, at least in part, due to altered adhesion ability and subsequently to a migration defect. Remarkably these features are similar to those observed in integrin β3−/− osteoclasts, such as defective adhesion, spreading and bone resorption [16]. Together with those similarities, we found that Sema4D deficiency in osteoclasts leads to sustain phosphorylation of components of the adhesion complex (Pyk2 and c-Src) (Fig. 2E). This also affects the activity of the downstream effector Rac-1 (Fig. 2E). Moreover, Sema4D−/− defective resorption is rescued by integrin β3 chain overexpression. Thus our data show for the first time that Sema4D can act as a modulator of αVβ3 integrin downstream signaling in osteoclasts and suggest functional cross-talk between β3 integrin and Sema4D (Fig. 4C). This might be a general property of Sema4D. Notably, Sema4D and β3 integrin are co-expressed in platelets and it is conceivable that the functional crosstalk between β3 integrin and
Sema4D also occurs platelets [9]. Strengthening this idea a very recent study reported that such a crosstalk between Sema4D and α1β3 integrin takes place for thrombus formation by platelets [21].

In apparent agreement with the in vitro data showing functional defects in osteoclasts, we report that Sema4D-deficient females present a high bone mass phenotype linked to defective bone resorption. Histomorphometrical data show a functional rather than a differentiation defect of the osteoclasts in Sema4D-deficient females, since their osteoclast numbers and sizes did not differ from WT (Fig. 3B-C). However, this phenotype was gender specific, and only observed in sexually mature females. In addition, bone marrow cross transplant experiments confirmed that the decreased bone resorption in vivo was not due to an intrinsic defect in the osteoclasts (Fig. 4A-B). The discrepancy between our in vitro and in vivo data indicates that the functional defect of Sema4D −/− osteoclasts might be compensated for in vivo and that, unexpectedly, Sema4D controls bone mostly through an indirect pathway. Two non-exclusive mechanisms could explain these differences. First, our data suggest a functional cross-talk between β3 integrin and Sema4D as it modulates β3 integrin intracellular signaling, although they do not interact directly (data not shown). It is thus conceivable, that the Sema4D osteoclast-specific effect could be compensated, at least partially, in vivo, by β3 integrin subunit. Second, in vivo Sema4D −/− high bone mass phenotype is dependent on mice hormonal status that dominates over the osteoclast intrinsic resorption defect. Therefore, the bone resorption defect observed in Sema4D-deficient mice is the result of an indirect mechanism.

In addition to the bone phenotype, we observed that reproduction is impaired in Sema4D-deficient mice, revealing a new physiological function of Sema4D. Our data showed that the Sema4D −/− defective gonadal function could, at least in part, be explained by a significant decrease in Gnrh1 hypothalamic expression and reduced ovarian follicle maturation (Fig. 5D–E). These latter results obtained from the analysis of Sema4D −/− mice abnormalities strengthen two recent reports showing that Sema4D is indeed involved in hypothalamic-hypophysal-ovarian axis development. First, ex vivo, Sema4D is implicated in mouse ovary follicular development [14]. Second, analysis of Sema4D receptor PlexinB1-deficient mice phenotype revealed that PlexinB1 depletion results in a reduction of Gnrh1 secreting neuron in mice adult brain due to a defective Sema4D induced neuron migration [12].

On the other hand no change was detected in Sema4D −/− mice body or uterine weights, in accordance with the fact that these mice have similar E2 blood basal levels as WT mice (Fig. S3A-B). Although no hypogonadism was detected in Sema4D −/− mice, they presented a decreased fertility. This observation reminds of clinical cases in women, in which primary ovarian deficiencies were observed along with normal E2 blood levels [22,23]. Although we measured normal basal E2 blood level found in Sema4D −/− mice, we cannot also exclude a fine deregulation of E2 cycles. Alternatively, it is also possible that other hormones from the ovarian axis might explain the reproductive function defects and the secondary bone phenotype. Indeed, contribution of other than E2 hypothalamic-hypophysal-ovarian axis hormones to bone mass regulation has largely been established [1,24,25]. For instance, it has been demonstrated that FSH directly regulate bone mass independently of E2 by activating bone resorption and therefore it might be a potential factor involved in Sema4D −/− bone resorption phenotype [24]. In the same way, we did not completely rule out the possibility that Gnrh1 could act directly on osteoclasts, to promote bone resorption, although it has been shown that Gnrh1 have no effect on osteoclastogenesis [24] and that hypogonadism is currently associated with bone loss [26]. In addition to the FSH direct effect on bone, recent studies have reported that elevated FSH

Figure 6. Increased bone mass in Sema4D −/− mice depends on ovarian function. A/ Bone volume quantification of 3-month-old Sema4D −/− and WT mice vertebrae one month after ovariectomy or sham surgery. B/ Quantification of urinary deoxypyridinoline residues from 3-month-old Sema4D −/− and WT mice, one month after ovariectomy or sham surgery show greater increases in bone resorption in Sema4D −/− than in WT, (n=8). Error bar represents SEM; * p<0.01; ** p<0.001; *** p<0.0001. doi:10.1371/journal.pone.0026627.g006
levels increase bone formation, independently of E2, and that this increased bone formation is correlated to the FSH induced ovarian secretion of inhibin and androgens [27]. There are accumulating evidences showing that gonadal factors, such as inhibins, would regulate bone remodeling either acting on bone formation if their mode of exposure is continuous or bone resorption if it secreted in a cycling mode [28]. This complex endocrine regulation of bone mass by the inhibins can also be observed in woman prior to menopause [29,30]. Thus inhibins might also be good candidates to play a role in the indirect regulation of bone mass by Sema4D.

Nevertheless, we have shown the dependence of the Sema4D+/− mice bone resorption phenotype on ovarian functions. Beside the gender specificity of the bone resorption phenotype, we found that the ovariection of sexually mature Sema4D+/− females abrogates the bone phenotype. This result clearly demonstrates the absolute requirement of mature ovaries to observe the bone phenotype in Sema4D-deficient female mice (Fig. 6A–B). In summary, our present study demonstrates first that in vitro the defective resorption activity of Sema4D+/− osteoclast is due to dysfunction of adhesion and migration properties reflected by an hyperphosphorylation of ß3integrin sub-units downstream effectors. Secondly, we provide evidence that the observed high-bone mass phenotype of Sema4D+/−/− females is due to a functional defective resorption. We show that this in vivo effect of Sema4D on bone resorption does not result from an osteoclast-specific defect but is rather dependent on the ovarian function by an as-yet-undefined pathway. Thus Sema4D is an indirect regulator of bone resorption that acts through its effect on the reproductive system in females. In conclusion, Sema4D is a factor involved in the cross talk between bone remodeling and gonadal function.

Materials and Methods

Kits and Reagents

LSM (Lymphocyte Separation Media) and RNA-Now were purchased from Eurobio. z-MEM and fetal calf serum were from Invitrogen and BioWest, respectively. Ascorbic acid, sodium b-glycerophosphate, culture cell BSA and Leukocyte acid phosphatase kit for TRAP staining were from SIGMA. Vitronectin was purchased from BD Biosciences. RNeasy Kit, Quantitec primer assay and Mix-Quantitect SYBR Green were from Qiagen. I-ScripTM cDNA synthesis Kit was from BioRad. Antibodies: anti-Sema4D antibodies were BMA12, Abm30 clones and CD16/32 monoclonal antibodies were BMA12, Abm30 clones and CD16/32. Sema4D cDNA synthesis Kit was from BioRad. Pyrlinkins-D immunoassay and creatinin kits were used according to the manufacturer Quidel Corporation’s protocols. E2 levels we measured by ELISA from 2 consecutive days collected blood samples (Cayman, Chemical). Secondary antibodies, mouse and rat IgGs were from Jackson.

The RNeasy Kit, Quantitec primer assay (QT 01062600) and Mix-Quantitect SYBR Green were from Qiagen. I-ScripTM cDNA synthesis Kit was from BioRad. Pyrlinkins-D immunoassay and creatinin kits were used according to the manufacturer Quidel Corporation’s protocols. E2 levels we measured by ELISA from 2 consecutive days collected blood samples (Cayman, Chemical). Statistical differences between groups were assessed using t-tests.

Primary osteoclast cultures

Bone marrow mononuclear cells from 8-week-old WT or Sema4D+−/− mice were cultured at 500cells/mm² for 5 to 6 days in the presence of a-MEM containing 10% (v/v) fetal calf serum, M-CSF (20 ng/ml) and GST-RANKL (30 ng/ml) [31]. We designated as pre-osteoclasts, cells containing 2 to 3 nuclei, obtained after 3 days of culture, whereas osteoclasts were multinucleated cells showing 5 to 10 nuclei and mature osteoclasts were large multinucleated cells with a high number of nuclei (n≥10). TRAP activity in cells was assessed after staining with leukocyte acid phosphatase kit (Sigma).

Quantification of proliferation

At each time point cells seeded on multi-well plates are fixed in 1,1% glutaraldehyde for 15 min and washed twice in water and air dried. Prior lyse in acetic acid 10%, cells are stained in crystal violet 0,1% for 20 min, washed and air dried, plates are read at OD 590 nm for quantification.

Adhesion and spreading assay

For in vitro spreading assays, osteoclasts were detached with 0.02% EDTA in PBS and washed twice with a-MEM containing 10 mM Heps and 0.1% culture cell BSA. They were kept in suspension for 2 hours at 37°C and then either maintained in suspension or plated on 5 μg/ml vitronectin-coated dishes, according to the manufacturer’s instructions. At the stated time, cells were lysed, and an equal amount of protein was analyzed by Western blotting.

For the spreading assay, osteoclasts were seeded on 5 μg/ml vitronectin pre-coated cover slips for 5, 10, 15 or 30 min and fixed with 4% PFA, permeabilized and F-actin labeled with 485-Alexa Fluor phallolidin. Cells were imaged with a planNeofluar 20× objective (NA 0.5) using a Zeiss axioplan microscope. The surface area was automatically measured with ImageJ software, using an ImageJ plug-in developed by our imaging facility (Platim IFR128).

BMMs retroviral infection

PMX derived retroviral particles were produced in Plat-E retroviral packaging cells (Cell Biolabs). In parallel PMX-IRES-GFP retrovirus (from Dr. T. Kitamura –Tokyo) was produced using the same conditions to be able to visualize and follow transfection and infection under an inverted microscope. Briefly, on day 1, PMX DNA constructs (PMX empty, or expressing human integrin ß3 chain) were transfected in Plat-E cells with Fugen 6 (Roche), on day 2 fresh media was added. The media was collected on day 4 and centrifuged to remove the cell debris. M-CSF (20 ng/ml) and 8 μg/ml of polybrene (Sigma) were added to the 110 tested markers were specific of the C57Bl6 background. Approval was given by “the comité regional d’éthique pour l’expérimentation animale” on May 06 2000 with the register approval number (265). For the induced sex hormone deficiency, 8-week-old female mice underwent ovariecotomies (ovx), whereas the controls consisted of sham-operated mice (sham).

Statistical differences between groups were assessed using t-tests.
supernatant, which was used to infect overnight bone marrow mononuclear cells prepared on day 3 in presence of MEM 10% FCS and 25 ng/ml of M-CSF. The following day, the inoculum was removed and the transduced cells were subjected to blasticidin selection (1 μg/ml) for 2 days in presence of MEM 10% FCS and 20 ng/ml of M-CSF. Then medium was replaced by classical osteoclast differentiation medium (MEM 10% FCS 20 ng/ml of M-CSF and 30 ng/ml of RANK-L) for an additional 3 days before lifting and seeding the mature osteoclasts on mineral for 24 h [32].

Cell transfer experiments
Two-month-old wild-type or Sema4D−/− mice were irradiated with lethal doses of gamma radiation (10 Gray) and reconstituted with a retro-orbital injection of 1 million bone marrow cells derived from wild-type or Sema4D−/− mice, un-transplanted mice died after 10 days. One month after grafting, the mice were sacrificed for bone analysis.

Histology and histomorphometric analyses
Non-decalcified bones were embedded in methyl methacrylate, before being stained, TRAP staining or histological analysis. Mice were injected with 100 μl of a 5 mg/ml of a calcein solution, 6 and 2 days before sacrifice and were processed for plastic embedding, as previously described [33]. Measurements were performed with the OsteoMeasure Analysis System (Osteometrics) using a 3CCD color video DXC-390camera (Sony) coupled to a Leica microscope, according to standard procedure [34].

Morphological classification of follicles
Three-month-old females were sacrificed. Frozen ovaries were sectioned and hematoxylin stained. Results were obtained through averaging all serial sections (200 μM). Follicles were classified according to Myers [35].

RNA extraction and RT-PCR
Real-time RT-PCR was performed using the MXP-3000P PCR-system (Stratagene). Samples were run in triplicate and the ΔΔCt method. Expression was normalized using the mouse ribosomal gene (Rps17).

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Statistical differences were analyzed with Student’s t-test and error bars represent SEM. Values of P<0.01 were considered significant. * p<0.01; ** p<0.001; *** p<0.0001 when not otherwise specified.

Supporting Information
Figure S1 A/ Cellular and B/ Dynamic bone formation parameter measurements using alcinan blue and double calcine labeling, respectively, show an absence of bone formation defects in 3-month-old Sema4D−/− females compared to their WT littermates. [N.Oh./B.Pm., number of Ob per bone perimeter; Ob.S./B.S, Ob surface per bone surface; BFR, bone formation rate; MAR, mineral apposition rate; M.S./B.S., mineralized surface per bone surface]. Error bar represents SEM, *** indicate a p value≤0.001 between two groups (n = 12).

Figure S2 Urinary DPD measurement of 3-month-old Sema4D−/− and WT irradiated mice one month after transplantation (n = 10 for WT and n = 12 for Sema4D−/− respectively).

Figure S3 A/ Uterine weight of 3-month-old Sema4D−/− and WT mice: after ovariectomy or sham surgery (n = 7 and 8 respectively). B-C/ No significant differences were observed in ovary weight and estradiol levels in 3 month-old females (n = 8 and n = 20 respectively). Error bar represents SEM, *** p<0.005.

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Author Contributions
Conceived and designed the experiments: RD IM. Performed the experiments: RD IM. CD. Analyzed the data: RD PJ IM. Contributed reagents/materials/analysis tools: AK HK. Wrote the paper: RD PJ IM.
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