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Abstract

Title of Thesis: The Role of Semaphorin 4D (Sema4D) in Bone Metastasis.

Asma Buhamrah, Master of Science, 2014

Thesis Directed by: Dr. John Basile, Assistant Professor, Department of Oncology and Diagnostic Sciences at University of Maryland School of Dentistry.

Background: Bone metastasis is a catastrophic endpoint of many neoplastic diseases, but especially for patients with advanced breast cancer. Despite the continuous advances in pharmacological and cancer research, bone loss and subsequent bone complications are seen in 70% of females diagnosed with breast cancer. Semaphorin 4D (Sema4D), a protein originally described to regulate the immune response, is now known to have a novel role in bone regulation. Sema4D also found to be highly expressed by many tumor cells including those of breast cancer. In this study we focus on the role of Sema4D produced by tumor cells on their ability to metastasize to bone.

Materials and methods: The osteoblast cell line MC3T3 was treated under different osteogenic conditions to examine the effects of Sema4D on bone differentiation *in vitro.* We also used tumor cells with silenced Sema4D to investigate the effects of tumor-derived Sema4D on their ability to metastasize to bone *in vivo.*

Results: Sema4D produced by the breast cancer cell line MDA-231 inhibited bone matrix formation and mineralization *in vitro*. *In vivo,* however, MDA-231 cells tend to spread to bone only when Sema4D was highly expressed by these cells and not when it was silenced.

Conclusion: Over-expression of Sema4D by breast cancer cells inhibits bone formation *in vitro* and tends to increase the ability of these cells to metastasize to bone

in vivo and establish osteolytic lesions characterized by this tumor type. Our findings may serve as a solid starting point to investigate the role of anti-Sema4D therapy in tumor metastasis. Further *in vivo* studies are strongly encouraged to clinically determine their effects.

The Role of Semaphorin 4D (Sema4D) in Bone Metastasis

By
Asma Buhamrah

Thesis submitted to the faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
Of the requirements for the degree of
Master of Science
2014

To my parents, thank you for your unconditional love, support and continuous prayers. No words or deeds can express my appreciation and my love to you. I wish one day I can offer my children part of what you offered me.

To my eldest sisters, Eman and Dia. Your wisdom and realism have always encouraged me to do better and see the bright side of life. Thank you for being great role models. To my younger brothers. Ahmad, Abdulmalik and Abdullah. Thank you for your silent support. You are my pride and my backbone.

To my husband, Dr. Hussein Irhama. Thank you for always being there for me loving, supporting and encouraging me through this rocky yet beautiful journey in our career and I hope one day it will be an inspiring experience for our children.

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Chapter (1)

Introduction

Cancer presents a major public health issue in both the United States and worldwide. 1 in 4 deaths in the United States is due to cancer. [1] Every year, the American Cancer Society publishes a report of the estimated number of new cancer cases and deaths expected in the United States. The report shows the most recent data on the incidence, mortality and survival rate based on information obtained from the National Cancer Institute, The National Center of Health Statistics and The Center of Disease Control and Prevention. According to the 2013 Cancer Statistics, Breast cancer continues to be a leading cause of the new estimated cancer cases and the second most common cause of cancer-related mortality, resulting in approximately 39,620 deaths per year and thereby comprising 14% of the cancer-related death in females. [1]

Mortality due to cancer is usually related to distant metastasis. The five-year survival rates for breast cancer significantly drop from 98% when diagnosed in the primary site to 24% in distant metastasis [1]. Bone seems to be the preferred site for several cancer types [2] but especially in patients with advanced breast and prostate cancer. Bone is a dynamic vital tissue. It constantly changes and adapts throughout life. The interaction between osteoclasts and osteoblasts through the process of bone remodeling is fundamental to maintain the structural integrity of the skeleton and allow for the maintenance of bone volume, bone repair and mineral homeostasis. Early work by Harold Forest in the 1960's helped us establish the sequential cellular activities in bone remodeling. He showed that the process takes place in "bone

multicellular units" (BMUs) asynchronously throughout the skeleton [3-5]. Two main phases occur:

1- Resorption phase, consisting of the removal of old damaged bone by osteoclasts.

2- Formation phase, where new bone is formed by osteoblasts to replace the old bone.

Although the BMU concept implied that a communication between the two phases existed, it was only a decade later when the idea of investigating a possible cell-to-cell signaling pathway became possible.

We now know that systemic and local factors can directly or indirectly target and affect the bone remodeling process. This makes the interaction of BMUs with their environment very complex. Figure 1 below shows a summary of the BMU remodeling sequence.

Briefly, when various inputs such as fractures, mechanical loading or factors released in the bone microenvironment including Tumor necrosis factor α (TNF-α), interlukin-6 (IL-6), Insulin growth factor-I (IGF-I) and parathyroid hormone (PTH) activates the osteoblasts lining the BMUs to increase their expression of the protein RANKL (receptor activator of nuclear κB ligand). RANKL binds to its receptor RANK (receptor activator of nuclear κB) located on the pre-osteoclasts. RANKL/RANK interaction stimulates pre-Osteoclasts to differentiate into multinucleated Osteoclasts that have the ability to adhere to the bone surface and secrete acids and enzymes like cathepins k and MMP9 to dissolve both inorganic and organic bone components, respectively. Like cells then start to remove debris generated by matrix degeneration while osteoclasts undergo apoptosis to prevent further resorption.

Bone matrix resorption also leads to the release of several growth factors stored within, including Transforming Growth Factor β (TGF-β), bone morphogenic proteins (BMPs), and fibroblast growth factors (FGFs), which are likely responsible for stimulating osteoblasts to produce new bone matrix and initiate mineralization, thus completing the remodeling process [6].

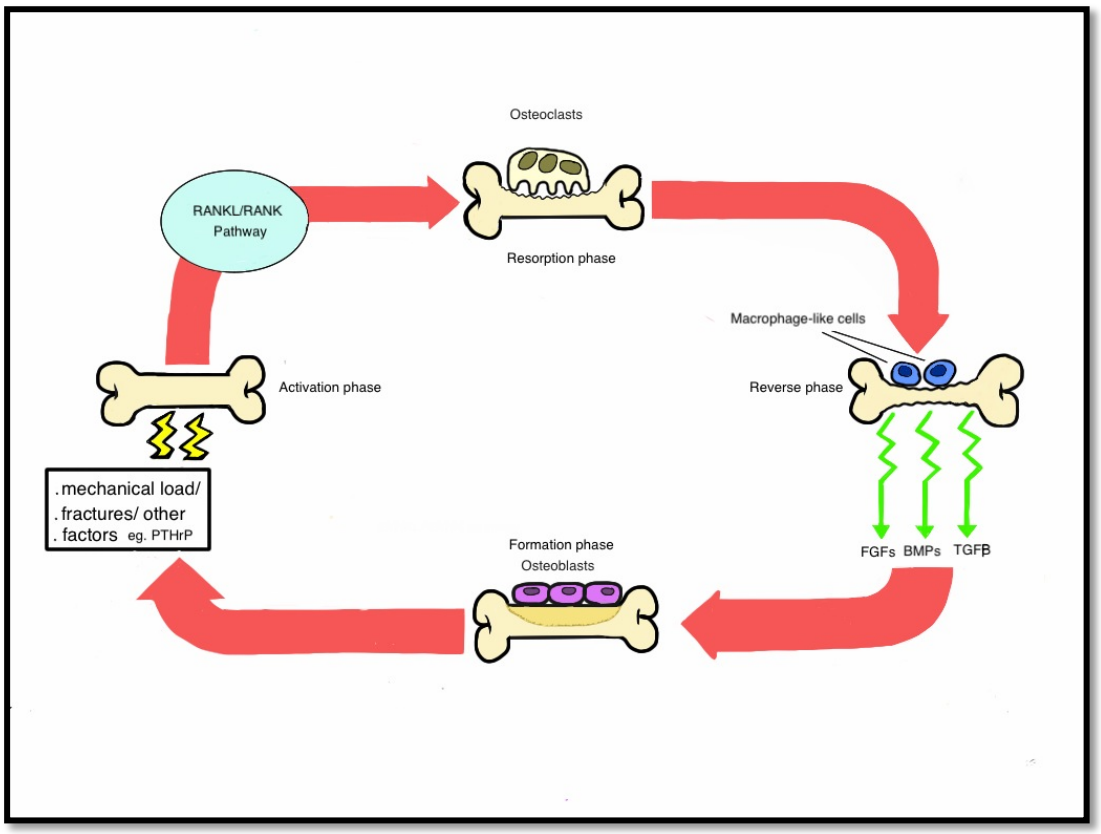


Figure 1: Bone Remodeling Cycle – systemic and local factors activate bone-lining cells to express RANKL, which binds to RANK on pre-osteoclasts and forms osteoclasts. These resorb bone and as a result growth factors are released to induce the osteoblasts to form bone matrix. The reverse phase is an intermediate phase where osteoclasts undergo apoptosis and like cells remove debris.

Recently, Semaphorin 4D (Sema4D) protein was identified as a new signaling factor in bone remodeling process. [7-9]. Sema4D, also known as CD100, belongs to the large family of Semaphorins. Semaphorins are secreted and membrane bound proteins originally identified as axon guidance mediators and later shown to be key regulators in many biological process including cardiogenesis [10,11], angiogenesis [12],

vasculogenesis [13], oncogenesis [14] and regulation of immune responses [15,16]. Of the class IV semaphorins, Sema4D, as shown in figure 2, is a membrane bound protein. It contains an amino terminal signal sequence followed by sema domain Ig-like domain, lysine-rich stretch, a hydrophobic transmembrane region and a cytoplasmic tail [17].

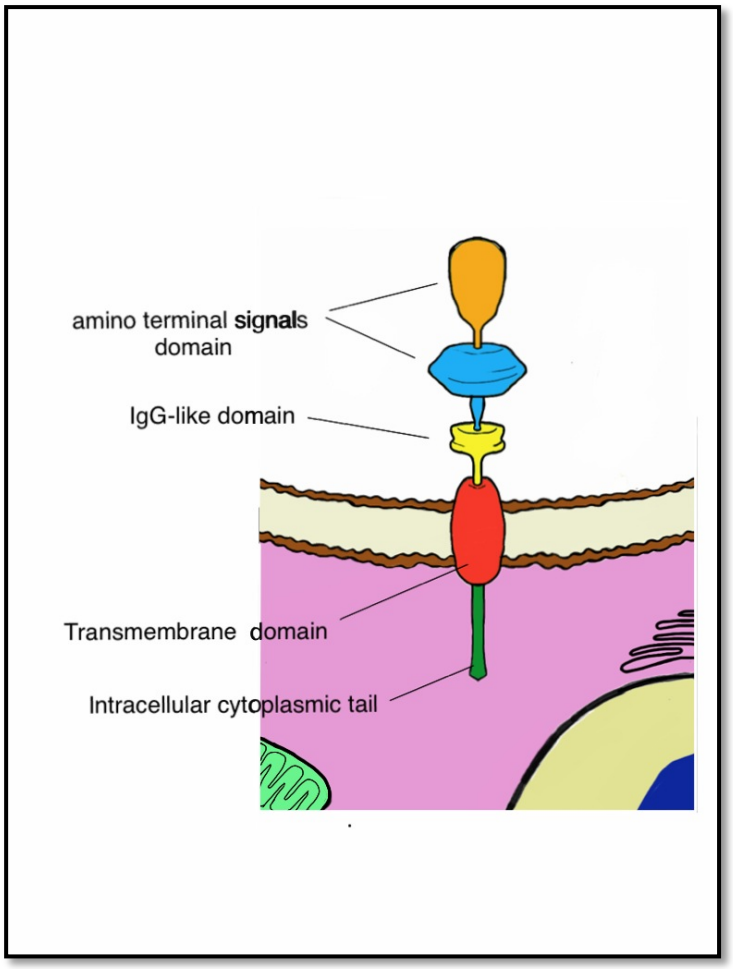


Figure2:Sema4D structure-A diagram showing the main components of Sema4D structure

When Sema4D binds to its receptor, Plexin-B1, the protein exerts an effect on angiogenesis, endothelial cell migration and bone cell communication [8,18,19].

The Semaphorins have been previously recognized to be involved in bone function. Earlier findings of Takegahara et al [20] showed that mice deficient of Sema 6D

receptor, Plexin-A1, had impaired osteoclast formation and showed osteopetrotic characteristics. On the other hand, Hayashi et al. [21] found that Sema 3A produced by osteoblasts act on both osteoclasts and osteoblasts to alter their formation and function. Deficiency in Sema 3E and/or its receptor Plexin-D1 also exhibited bone abnormalities especially in axial skeleton [22,23]. The work of Negishi-Koga et al [8] led to the focus on Sema4D role in bone biology.

Negishi-Koga's group investigated the role of Sema4D in bone remodeling. By using expression analysis in osteoclasts and osteoblasts in mice, they showed that Sema4D is highly expressed in osteoclasts but not in osteoblasts. They also showed that Sema4D expression is further increased during RANKL induced osteoclasogenesis. The authors then examined the function of Sema4D in the skeletal system of three mice models: Sema4D-/-, Plexin-B1-/- and mice expressing a dominant negative RhoA specifically in osteoblasts. The results showed that by blocking these factors, an increase in osteoblast rate of bone formation was observed as well as an increase in the mass and quality of that bone formed. This lead them to the conclusion that Sema4D expressed on osteoclasts binds to its receptor Plexin-B1 on osteoblasts leading to the activation of the small GTPase RhoA, which inhibits bone formation by suppressing insulin like growth factor-1 (IGF-1) signals and by altering osteoblast motility.

Negishi-Koga et al. have also investigated the possible therapeutic effect of Sema4D antibody in an animal model of osteoporosis (a bone disease characterized by decrease in bone mass) and found that it was effective in increasing bone formation. They concluded that their findings could hold a promise to new therapeutic approaches to bone disease.

Dacquin et al. [9] study examined the role of Sema4D in osteoporosis. By using a loss of function approach, they addressed whether Sema4D played role in bone resorption. They showed similar results of the previous study in that Sema4D is expressed in osteoclasts and not osteoblasts and that *in vitro* Sema4D was a novel factor controlling bone resorption. However, when the authors utilized a mouse model, they noticed that Sema4D was a bone regulator in mature females and not in males or even sexually immature female mice. They concluded that Sema4D in their study served as a direct bone regulator only *in vitro* and as an indirect regulator of bone resorption through its effect on the reproductive system in females.

Interestingly, Dr. Basile and his group have been working on the role of Sema4D in tumor cells. Basile et al. [24] have shown using tumor tissue arrays that Sema4D is highly expressed in prostate and breast cancer. They also observed that Sema4D could be shed from tumor cells, which may mediate or alter other cellular activities throughout the body. In their study, they were investigating the role of Sema4D in tumor-induced angiogenesis. However, such findings encourage us to think of the potential effects of Sema4D overexpression in tumor cells on the communication between osteoblasts and osteoclasts and on the mechanism of these tumors in metastasis to the skeleton.

Indeed, bone metastasis is a drastic consequence of cancer. It's even more prevalent than primary tumors of the bone [25] 70-90% of patients who die from breast cancer have bone metastasis [25,26] Clinically and histopathologically, bone metastasis can be seen as a spectrum between osteolytic, osteoblastic or mixed lesions. In breast cancer, bone metastasis is predominantly osteolytic and characterized by an increase in osteoclast activity. According to one model, the mechanism was explained by the

over-expression of the parathyroid hormone related protein (PTHrP) in the metastatic breast cancer cells.

It is thought that PTHrP stimulates the RANKL/RANK pathway to activate osteoclasts to resorb bone matrix and release TGF-β, which positively feeds back and stimulates breast cancer tumor cells to produce more PTHrP and cause further bone resorption, eventually producing multiple radiolucencies evident on radiographs that is characteristic of this type of bone destruction [27].

Another model explaining the mechanism of osteolytic metastasis is as a result of over-expression of another protein, interlukin-8 (IL-8), in breast cancer cells. This model also relates the osteolytic lesions to the continuous positive feedback loop of PTHrP, but in a path independent of the RANKL/RANK interaction. [28-30]

Regardless of which pattern is seen, osteoclast activation and bone resorption is a pre-requisite for subsequent bone formation. It is thought that the environment of the bone provides a fertile ground to the growth and survival of the tumor cells that reach it. This concept is known as the "seed and soil," where the tumor cell is the seed and the bone is the soil, and was first proposed more than hundred years ago by Stephen Paget.

The concept served as a general tool to think of the possible causes of bone metastasis but the actual mechanism of cancer spreading to bone is still under investigation.

Researchers described a "vicious cycle" of bone metastasis (figure 3). Once tumor cells invade the bone, a series of factors such as PTHrP, IL-6, TNF-α, macrophage-colony stimulating factor (M-CSF), IL-8 and prostaglandin E2 (PGE2) is secreted triggering the formation of osteoclasts with a subsequent increase of bone resorption.

The resorbed matrix is rich in pro-tumor factors, which get released in the BMU and further stimulate cancer proliferation and attract tumor cells to bone. This “fatal attraction” between breast and prostate cancer and bone is usually silent resulting in severe late stage complications such as intractable bone pain, pathological fractures, leukoerythroblastic anemia, cord compression, bone deformity and an overall reduced quality of life [31, 32].

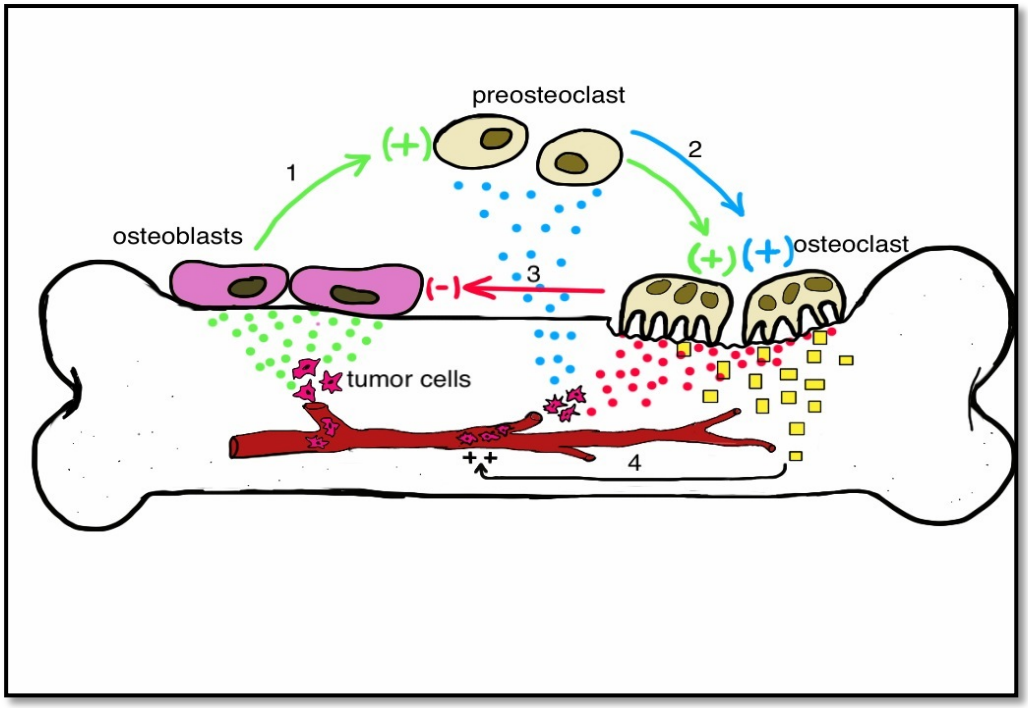


Figure 3: "Vicious Cycle" of bone metastasis. PTHrP (1), IL-8 (2), Sema4D (3) and other factors released by tumor cells invading the bone stimulate osteoclasts to continuously resorb the bone. The growth factors released as a result of bone matrix resorption (4) provide a fertile environment for tumor cells to grow and survive.

Therapeutic agents currently in the market are predominantly antiresorptive medications used for the management of bone disease such as osteoporosis and osteopenia as well as bone cancer. FDA approved treatments include Bisphosphonates and Denosumab (Prolia®, XGVA®) [33, 34].

Bisphosphonates (BPs) have high affinity to bind to bone minerals due their chemical structure. All BPs contain two phosphate groups linked to a central carbon (P-C-P) and a variable R' chain which determine their side effects and relative potency. Nitrogen-containing BPs such as Zoledronic acid (Zometa®) is the most potent. Following administration, usually via intravenous route, Zometa® binds strongly to exposed bone minerals in the resorbed site at high concentrations. It then inhibits osteoclast mediated resorption by binding its Nitrogen group with fernesyl pyrophosphate synthaze (FPPs) thereby blocking the activity of small GTPases important for osteoclast cell function and survival [35-37]. Denosumab (Prolia®, XGVA®) is human monoclonal antibody to RANKL. When the drug is administered intravenously, it acts on the RANKL/RANK pathway leading to the inhibition of osteoclast formation and bone resorption.

The clinical benefits of the drugs in cancer treatment are evident despite inevitable side effects seen. One of these unfortunate side effects is the development of osteonecrosis of the jaw (ONJ), a well-known clinical complication of poor wound healing and subsequent necrosis of the underlying exposed bone [38-40]. New therapies in stage II or stage III trials include inhibitors of Cathepsin K (an osteoclast-secreted protease), Src enzyme, and TGFβ. In addition, inhibitors of Endothelin1 in osteoblastic metastases target osteoblasts. [2]. Most of these medications inhibit osteoclastic bone resorption, while only few target bone formation. A better understanding of the bone biology and its cellular structure will give better insight and lead to the development of new therapeutic means to prevent the most catastrophic complications in cancer patients.

The main objective of our study is to determine the effects of production of Sema4D by tumor cells to on their ability to establish metastatic deposits in bone.

We hypothesize that inhibition of Sema4D in tumor cells will inhibit osteoclast formation and encourage mineralization by osteoblasts. If successful, this could present a new therapeutic approach to prevent bone metastasis if used alone or combined with other therapeutic agents.

Chapter (2)

Materials and Methods

Cell culture

MDA-MB-231 (MDA-231), an estrogen independent human breast cancer cell line and MC3T3 cells, a murine osteoblast precursor cell line, were purchased from the American Type Culture Collection (ATCC, Manassas, VA). MDA-231 were cultured in Dulbecco's Modified Eagle's Medium (DMEM 1X, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and 100 units/ml penicillin/ streptomycin/ amphotericin B. MC3T3 cells were first cultured in Alpha Minimum Essential Medium (alpha MEM, GIBCO, Life Technologies) with 10% fetal bovine serum and ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine and 1 mM sodium pyruvate, but without ascorbic acid. Both cell lines were kept at 37°C in a 5% CO_2 atmosphere until 80% confluent and sub-cultured using a disaggregation assay with Trypsin according to manufacturer's instructions. Briefly original medium was discarded and the cell layer was briefly rinsed with 0.25% (w/v) Trypsin - 0.53 mM Ethylenediamintetraacetic acid (EDTA) solution to remove all traces of serum, which contains trypsin inhibitor. 2.0 to 3.0 mL of Trypsin-EDTA solution (pH7.5) was added to the flask and incubated for 10 min. before transfer to a new flask where the medium was changed every 3-4 days in order to maintain cell vitality.

Soluble Sema4D preparation

Sema4D was produced and purified as described previously [18] Briefly, the extracellular portion of mouse Sema4D was subjected to PCR, and the resulting product was cloned into the plasmid pSecTag2B (Invitrogen, Carlsbad, CA). This construct was transfected into 293T cells growing in serum free media using the calcium chloride (Fluka Chemika; Sigma Aldrich, St. Louis, MO)-*N*, *N*′-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid-buffered saline (Fluka Chemika) method [41]. Media containing soluble Sema4D were collected 1 and 2 days post transfection and purified with TALON metal affinity resin (Clontech Laboratories, Palo Alto, CA) according to manufacturer's instructions. Concentration and purity of the TALON eluates were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with silver stain (Amersham Life Science, Piscataway, NJ) and the Bio-Rad assay (Bio-Rad, Hercules, CA). In all cases, media collected from parallel transfectants using the empty pSecTag2B vector were used as controls.

***In vitro* mineralization assay**

Previously cultured MC3T3 cells were divided into two groups:

1- Plexin-B1 short hairpin (sh) RNA, where the cells were infected with lentivirus (as described in *Lentivirus infections*, below) to genetically silence Plexin-B1.
2- MC3T3 control group, where the receptor (Plexin-B1) is expressed

Plexin-B1 levels in both groups were confirmed using a western blot (data not shown). Cells were transferred in 2 x 6-well plates (10 cm^2/well) containing an osteogenic medium (50μM ascorbic acid, 10nM dexamethasone and 10mM β-glycerophosphate) and incubated at 37°C in a 5% CO_2 air atmosphere for future treatment with phosphate buffered saline (PBS), soluble Sema4D, media conditioned by MDA-231 cells, and anti-Sema4D antibody (obtained from Vaccinex, Inc., Rochester, NY) as shown in figure 4.

Previously cultured MDA-231 cells were divided into two groups:

1- Sema4D shRNA (S4D shRNA), where Sema4D protein was genetically silenced by infection with shRNA expressing lentiviruses
2- Control group, where Sema4D is expressed

Levels of Sema4D were confirmed with Western Blot (data not shown). Both groups were cultured separately as described above and their growth media was collected treatment of MC3T3 cells.

Control MC3T3 and those infected with Plexin-B1 shRNA expressing lentivirus were grown in 2 6-well plates with either 1ml of osteogenic media along with 4μl of PBS or soluble Sema4D protein, or in 300μl of osteogenic media along with 700μl media conditioned by MDA-231 and MDA-231, Sema4D shRNA cells (breast cancer cells expressing high and undetectable levels of Sema4D, respectively). Into one of these wells 5μl/ml of anti-Sema4D antibody was added. The media for all plates were changed twice a week for 21 days and differentiation determined by staining to look for deposition of mineralized matrix.

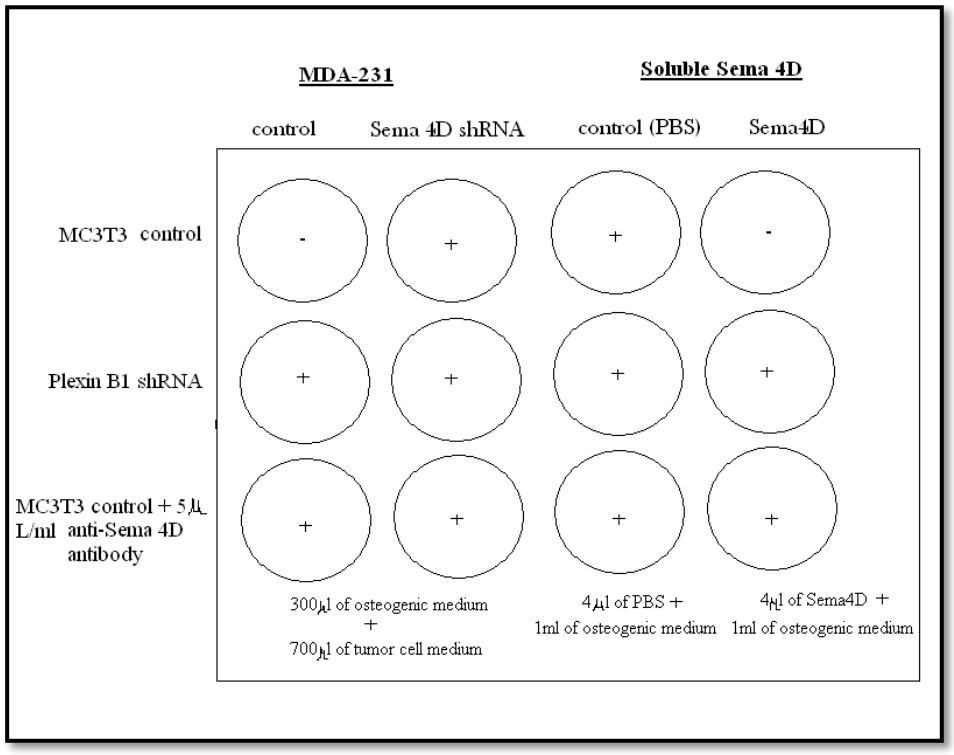


Figure 4: Summary of *in vitro* mineralization assay. (+) And (-) indicate wells likely to exhibit differentiation or not, respectively.

Following MC3T3 treatment (at day 21), detection of mineralization was carried out using an Alizarin red-based assay of mineralization as described before [42]. Briefly, a monolayer growing in 6-well plates (10 cm^2/well) were washed with PBS and fixed in 10% (v/v) formaldehyde (Sigma–Aldrich) at room temperature for 15min. The monolayers were then washed twice with excess distilled water (dH2O) prior to addition of 1mL of 40mM Alizarin red-based stain (ARS, pH 4.1) per well. The plates were incubated at room temperature for 20 min. with gentle shaking. After aspiration of the unincorporated dye, the wells were washed four times with 4mL dH2O while shaking for 5 min. The plates were then left at an angle for 2 min. to facilitate removal of excess water, re-aspirated, and stored at 20 °C prior to visualization.

Lentivirus

The shRNA sequences for human Sema4D were obtained from Cold Spring Harbor Laboratory's RNAi library (RNAi codex;http/katahdin.cshl.org:9331/homepage/portal/script/main2.pl) [44,45]. Oligonucleotides (Invitrogen) based on the following sequence worked best to knock down Sema4D protein levels: 5′-GGCCTGAGGACCTTGCAGAAGA-3′. Oligonucleotides were digested with XhoI/EcoRI and cloned into pSHAG MAGIC2 [46,47]. Where indicated, pSHAG MAGIC2 or pSHAG MAGIC2 Sema4D shRNA was transfected into MDA-231/luc+ cells by using Lipofectamine Plus (Invitrogen) supplemented with CombiMag transfection agent (Oz Biosciences, Marseille, France) to achieve high transfection efficiency. Because the pSHAG MAGIC2 vector is Gateway (Invitrogen) compatible, an LR reaction was performed according to the supplier's instructions to transfer the Sema4D shRNA insert into pWPI GW, a Gateway-compatible CSCG-based lentiviral destination vector. Viral stocks were prepared and infections were performed as reported in refs. 18 and 48

***In vivo* tumor metastasis**

A highly bioluminescent clone was developed from the previously cultured MDA-231 cells after transfection with Lenti-Fire™ (In vivo Imaging Solutions, Cheyenne WY), a luciferase-expressing pLazarus retroviral construct, according to the manufacturer instructions. The cells were then divided into 2 groups, one of which Sema4D proteins was genetically silenced using shRNA as previously described [24]. 6 week-old female athymic nude mice were utilized for this experiment. All animal studies were approved by the University of Maryland Office of Animal Welfare, Institutional animal care and use committee and maintained in accordance with the NIH Guide for

the care and use of Laboratory Animals. For surgical manipulation, mice were anesthetized using 2.5% veterinarian grade isoflurane administered continuously for the entire procedure by the dedicated XG18 gas inhalation anesthesia apparatus on the XENOGEN IVIS-200 IMAGER (xenogeny corp, Alamedo, CA) provided by the animal facility at the University of Maryland and supervised by Dr. Stewart Martin lab. The depth of anesthesia was verified by loss of mouse's pedal withdrawal reflex prior to start of the surgical procedure and an ophthalmic ointment was applied to both eyes of each animal to prevent corneal desiccation. All mice were monitored throughout the experiment for signs of distress. At the end of the experiment, or in case of distress, the animals were euthanized using 30% CO2 asphyxiation followed by cervical dislocation. The lower limbs of each animal were harvested for further tumor analysis. Figure 6 below shows an illustrated summary of the *in vivo* experiment.

Previously cultured MDA-231/luc+ and MDA-231/luc+ (Sema4D knockdown) cells were prepared at the same day and shortly before their intra-cardiac inoculation in experimental mice. After removing the media from the cultured plate, the cells were washed twice with PBS 0.05% Trypsin –EDTA was added and incubated for 3-5 minutes to detach cells from the plate. Trypsin –EDTA solution was then neutralized with media and cells were transferred to 50ml conical polypropylene tube (to prevent clumping) and centrifuged at 800-900 rpm for 10 min (eppendorf centrifuge 5702 R, Germany). This process was then repeated twice using PBS to reach a final cell concentration of $2x10^5$ ml. A suspension of $2x10^5$ cell in 100 μl Dulbecco's PBS was prepared for both MDA-231/luc+ and MDA-231/luc+ (Sema4D knockdown) and kept in ice for immediate inoculation in experimental mice [49,50]. For inoculation, the mouse was placed on its back with arms and legs extended. It was then firmly secured

in place on the working surface with a piece of paper tape across the abdomen and on upper extremities. Caution was made not to place the tape on the abdomen too tight to help the mouse breath properly. It is imperative that the mouse is symmetrically positioned. Once positioned, the mouse's chest was wiped with 70% ethanol to help visualize the landmarks as well as clean the inoculation site. A permanent marker was used to locate the left ventricle (midway between 2 points, the junction of the left clavicle to the sternum and xyploid process to the sternum, illustrated in figure 5). After marking left ventricle, the cell suspension was gently mixed and drawn using tuberculin syringe maintaining an air space near hub of needle to allow spontaneous entrance of blood when left ventricle is entered. A new needle was used for each animal. To enter the ventricle, the needle was inserted perpendicularly to the chest at the left ventricle mark at approximately 6-8mm depth and a rapid blood entrance was observed. The syringe was held steady with one hand and a 100 μl of the cell suspension slowly injected. The needle was then withdrawn quickly from the chest to prevent seeding of tumor cells into the heart and lungs. Pressure was placed on chest with sterile gauze for about 30 seconds. To ensure chances of survival after inoculation, the mouse was kept warm by holding it between the hands (heating pad is preferred) to accelerate recovery. The process was repeated for the rest of the animals. Each experimental group was kept in one case and the case was labeled for identification.

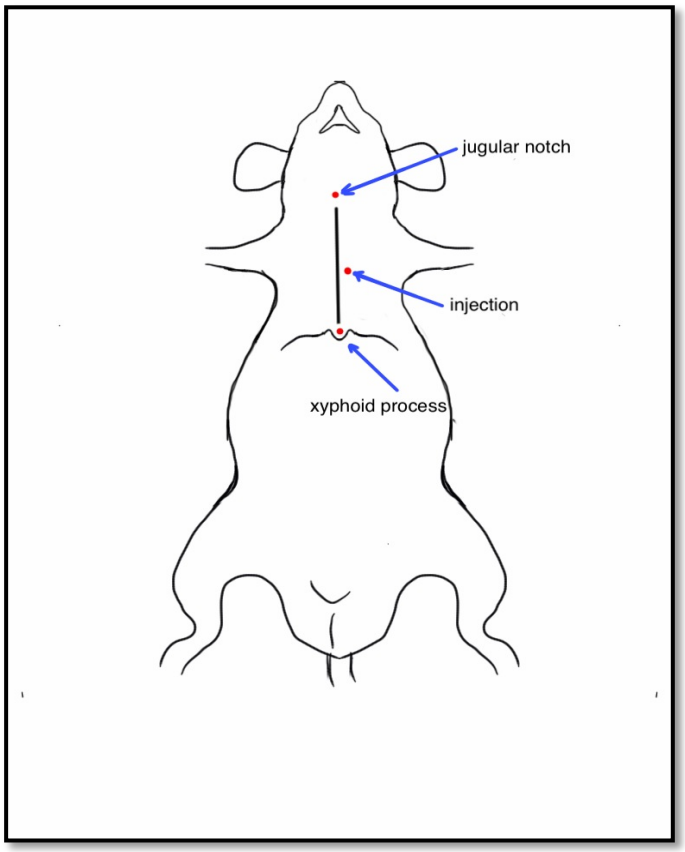


Figure 5: Intra-cardiac injection land marks. The left ventricle is located slightly to the left of a midway point between a line drawn from the jugular notch to the xyphoid process.

In vivo bioluminescent imaging (BLI)

Once a week for 5 weeks, the 2 groups of mice were separately imaged for possible bone metastasis of previously injected cells. The in vivo BLI was carried out at the university of Maryland Marlene and Stewart Greenbaum cancer center using the Xenogen IVIS®200 series and living image®software (Xenogen Crop, Alameda, CA). This is a light-tight imaging chamber which is coupled with a highly sensitive CCD camera system cooled to - 95°C. The camera system is capable of quantitating single photo signal originating within the tissue of living mice. For the detection of luciferase-expressing cells, the mice were injected intraperitonealy (IP) with 100 µl of 40mg/ml luciferin dissolved in PBS (previously prepared and sterile filtered) and then anaesthetized as described above using the XGI-8 gas inhalation apparatus. Ventral

images were required 10-15 min. after injection with the IVIS®200 machine and analyzed in the LIVING IMAGE software to determine the location and the relative intensity of luciferase expression as a measurement of tumor deposits. At week 5, the mice were radiographed using a micro radiography apparatus provided in the animal facility at the University of Maryland then euthanized as described above for histopathological analysis.

Histology

Following euthenization, hind limbs were dissected and fixed in 10% formalin for 24-48 hours at 4°C. All specimens were decalcified in 10% EDTA (7.4) for 10 days and embedded in paraffin the specimens were sent for sectioning and staining with hematoxylin/eosin and returned for evaluation for bone metastasis. Stained sections were scanned in ScanScope FL (eAperio®, Vista, CA) and additional sections were left unstained to perform a Tartrate-resistant acid phosphatase (TRAP) staining to detect the presence of osteoclast cells.

Interlukin-8 enzyme-linked immunosorbent assay (IL-8 ELISA)

Confluent human osteoblasts were serum starved for 4h, then cultured in serum free medium without or with 100ng/ml or 400ng/ml Sema4D or 400ng/ml Sema4D with 10μM BAY 11-7085 or C3, where indicated, for 12h. The culture supernatant was collected and used to analyze IL-8 by ELIZA (Cytokine Core Facility, University of Maryland School of Medicine). The results are expressed as the average of three independent experiments.

Tartrate-resistant acid phosphatase (TRAP) staining

Acid phosphatase, leukocytes kit was used according to manufacturer instruction (sigma –Aldrich, Inc St Louise, MO). Briefly, the unstained bone sections were prepared by first dissolving the paraffin layer on the slide using xylene and alcohol. The slides were then left to dry for 1 hour before they were fixed using a solution of 25 ml citrate solution, 65ml acetone and 8 ml of 37% formaldehyde for 30 sec. The slides were then rinsed thoroughly in deionized water and placed in a coplin jar containing a solution of 45ml deionized water pre-warmed to 37°C, 1.0 ml deionized fast Garnet GBC solution, 0.5 ml Naphthol AS-B1 phosphate solution, 2.0ml acetate solution and 1.0 ml tartrate solution. The coplin jar was incubated in a light tight 37°C water bath for 1 hour and later rinsed thoroughly with deionized water then counterstained 2 minutes in Hematoxylin solution, Gill No.3 provided in the kit. Finally, the slides were rinsed several minutes in alkaline tap water until blue nuclei seen, air dried and evaluated microscopically. Specimens were scanned in ScanScope FL (eAperio®, Vista, CA)

Statistical analysis

Student's paired *t* tests were performed on means, and *p* values calculated: *, $p < 0.05$, **, $p < 0.01$ using the GraphPad software program.

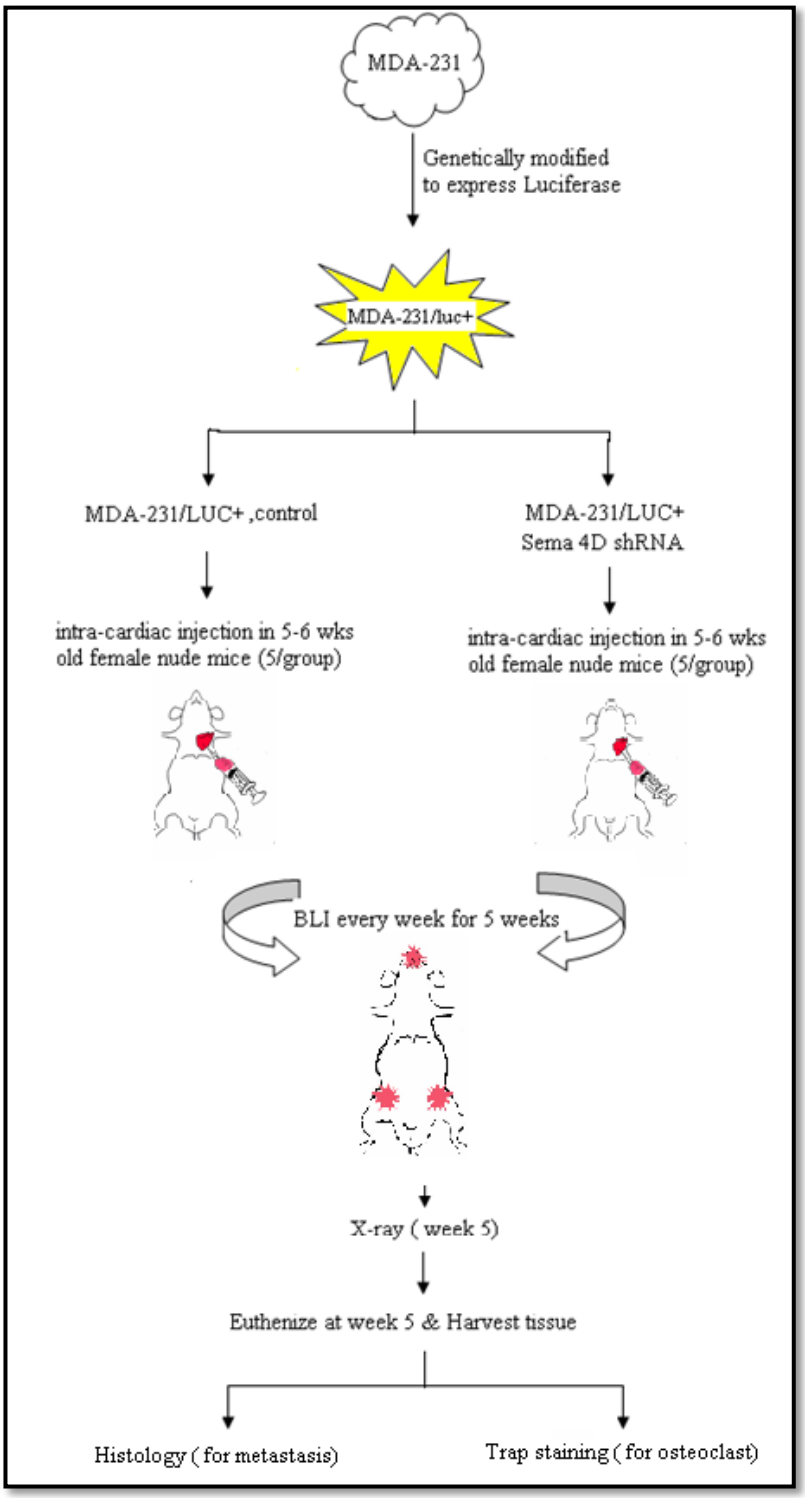


Figure 6: Summary of *in vivo* experiment. MDA-231^{Luc} cell line was divided into two groups, a wild group expressing Sema4D and Sema4D knockdown group. These cells were inoculated in the left ventricle of young mice and were weekly BLI to detect bone metastasis. At week 5, the mice were euthanized and the tissues were collected for further analysis.

Chapter (3)

Results

Sema4D is highly expressed in the MDA-231 breast cancer cell line

Despite constant medical developments in cancer treatment, breast cancer still holds second place in estimated cancer-related death among females in the United States. 1 in 8 women will be diagnosed with breast cancer throughout their life [1]. Cancer cells produce many factors that promote their survival in the host environment. Sema4D is one of them and our group has previously shown that Sema4D is expressed at high levels in many cancer cells [24].

In order to determine the amount of Sema4D in different breast cancer cell lines, we performed an immunoblot for Sema4D in breast cancer cell lines (fig 7).

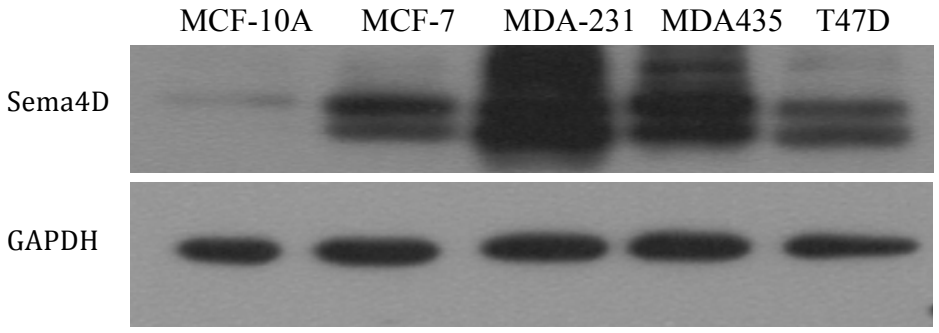


Figure 7: Expression of Sema4D by Breast Cancer Cell Lines- MDA-231 shows high expression of Sema4D, GAPDH was used as a loading control (lower panel)

MDA-231 cells, compared to the other cell lines, showed the highest expression of Sema4D. MDA-231 is a well-known breast cancer cell line that has been shown to stimulate bone resorption in humans [51]. For these purposes it was utilized in our experiments.

Sema4D suppresses bone formation *in vitro*

Genetic evidence showed that osteoclasts highly express Sema4D during their differentiation while osteoblasts possess Plexin-B1, the receptor that recognizes Sema4D and binds to it to activate a series of molecular reactions that leads to inhibition of bone formation [8]. We examined the effect of different concentrations of Sema4D on the cultured osteoblast cell line, MC3T3, to determine which concentration of Sema4D can inhibits bone formation. We found that at a concentration of 400 ng/ml, Sema4D significantly ($p<0.01$) inhibited mineralization matrix deposition in the cultured cells (fig 8.A)

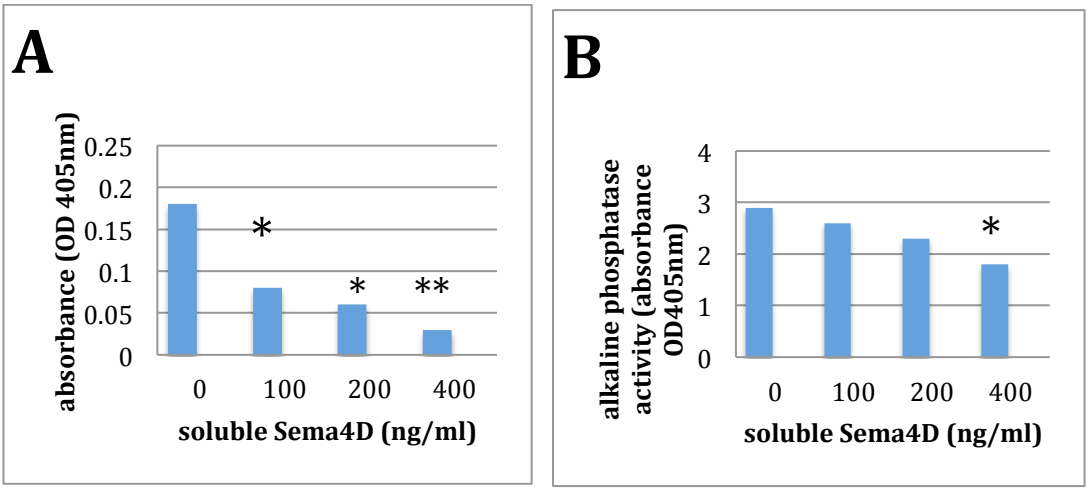
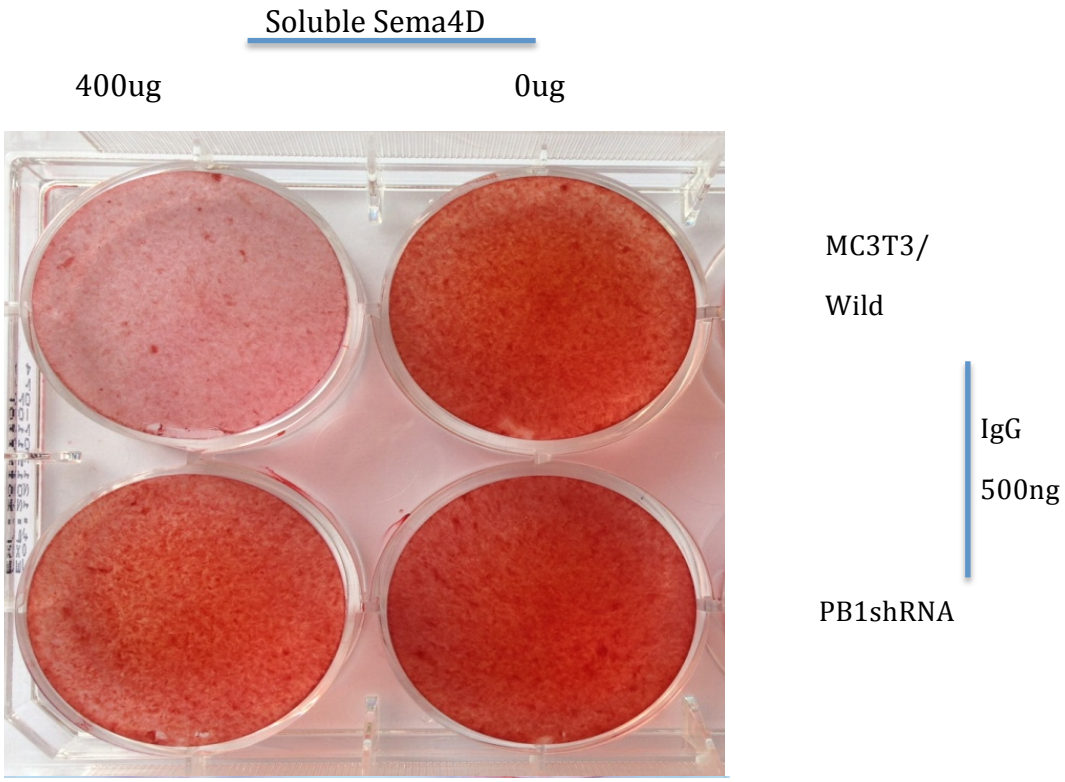


Figure 8: A) Optical density absorption of Sema4D. B) Alkaline Phosphatase absorption.

We also observed a significant ($p<0.05$) effect on inhibition of mineralization at both 100 and 200 ng/ml concentrations. However, a decreased activity in Alkaline Phosphatase, an enzyme implicated in bone formation and mineralization, was only significant ($p<0.05$) when osteoblast cell lines were treated with 400 ng/ml Sema4D (Fig. 8B).

We used this concentration to demonstrate the effect of Sema4D on bone by testing soluble Sema4D (mimicking Sema4D secreted by osteoclasts) under different conditions (Fig. 9).

When Sema4D was added to MC3T3 cultured cells under osteogenic conditions but was lacking the expression of Plexin-B1 (Fig. 9, bottom row) we observed that the bone differentiation process was unaffected. This was also true for the MC3T3/wild groups which were just treated with Phosphate Buffered Saline (PBS; no Sema4D) also showed normal differentiation and matrix deposition. However, when Sema4D was added to the MC3T3/wild group, bone differentiation was significantly inhibited ($p< 0.05$). This confirms the previous findings of Koga's group [8].



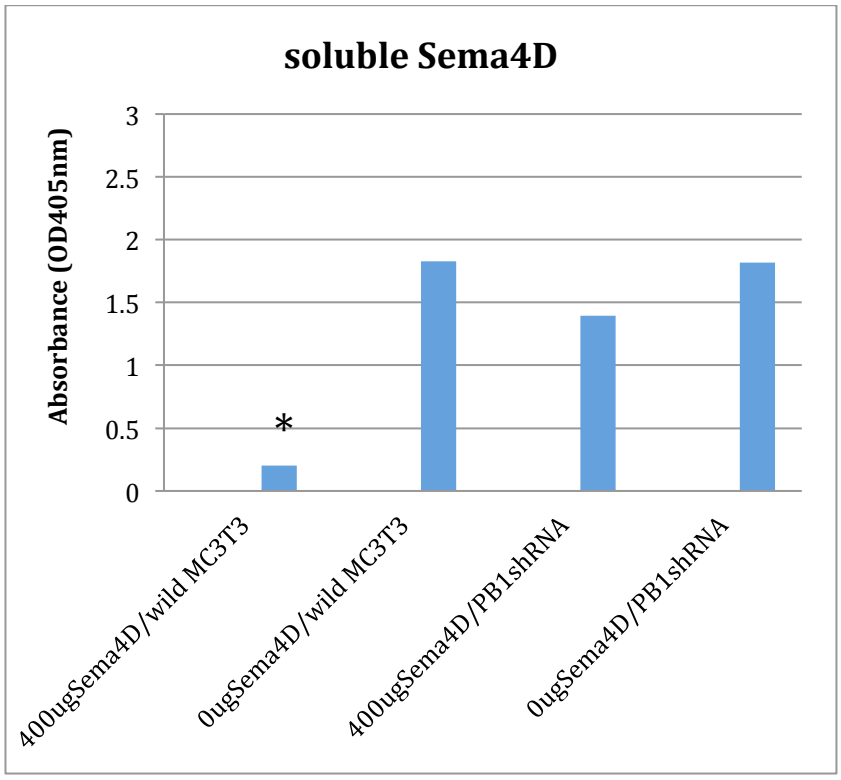


Figure 9: ***In vitro*** **mineralization assay for soluble Sema4D**

Sema4D produced by tumor cells inhibit bone formation *in vitro*

To test our hypothesis that Sema4D produced by tumor cells has an effect on bone formation, we collected Sema4D from the breast cancer cell line MDA-231 media and used a wild group where Sema4D was present and the other where we genetically silenced the protein (with Sema4D shRNA). When the first group was cultured with untreated MC3T3 cells (MC3T3 wild) we observed that Sema4D suppressed bone formation and matrix deposition significantly ($p< 0.05$). But when the same group was added to the treated MC3T3 cells (MC3T3 Plexin-B1 shRNA) bone formation was unaffected and matrix deposition was observed (Fig. 10, first columns). This later observation was also true for the second group (Sema4D shRNA). However, we noticed no effect on bone matrix deposition when both MC3T3 cells groups were treated by them (Fig. 10, second columns).

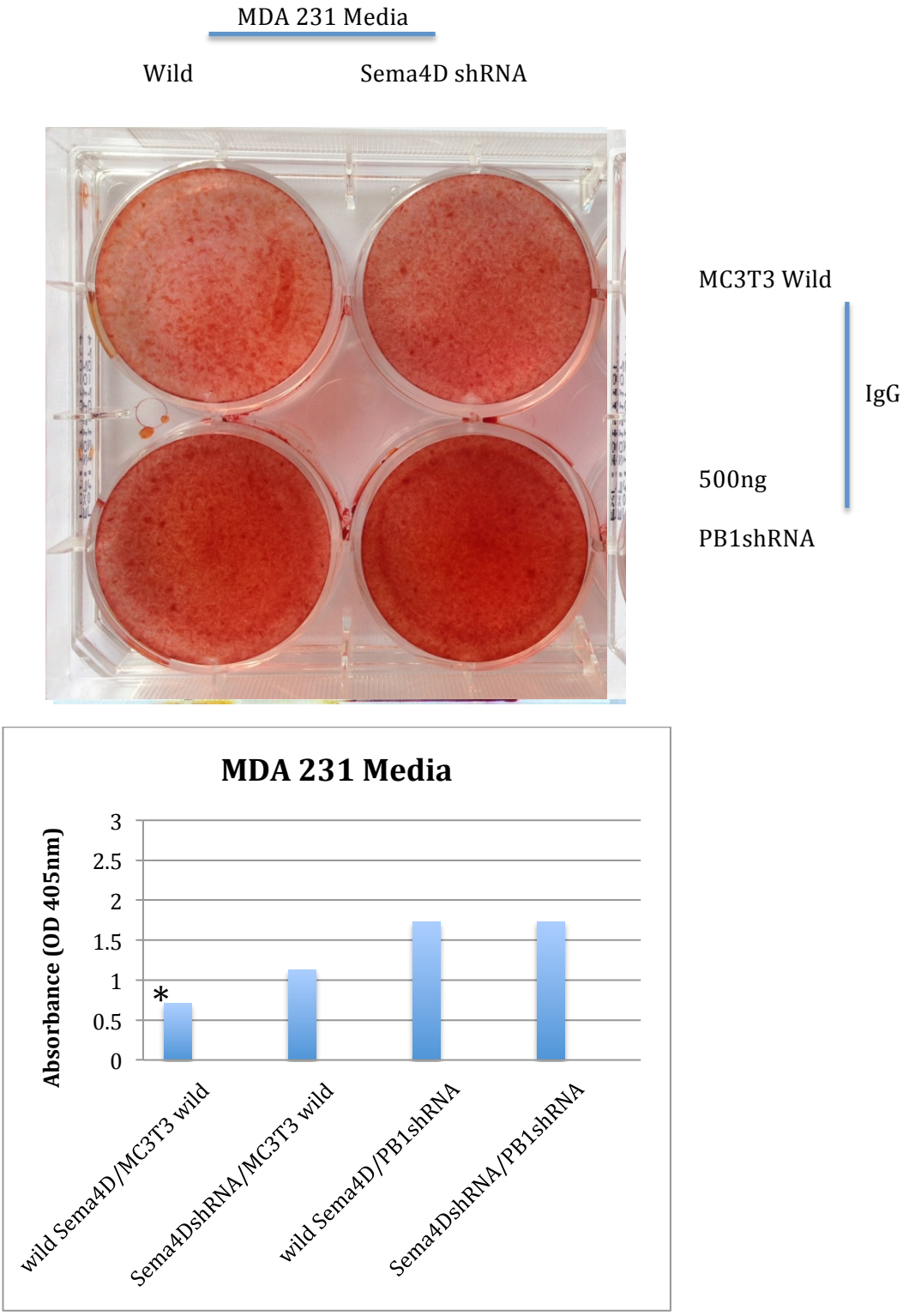


Figure 10: *In vitro* mineralization assay for Sema4D produced by MDA 231

Sema4D produced by MDA-231 cells causes bone metastasis *in vivo* and promotes osteoclast activity both *in vitro* and *in vivo*

We also tested our hypothesis in an animal model of metastasis using MDA231 cells with silenced Sema4D. At week five, our BLI results showed that Sema4D knockdown cells (Sema4D-/-) had few to no bone metastasis when compared to the wild type mice (Fig.11). After week 5, the mice were euthanized and the tissues were collected for histological exam.

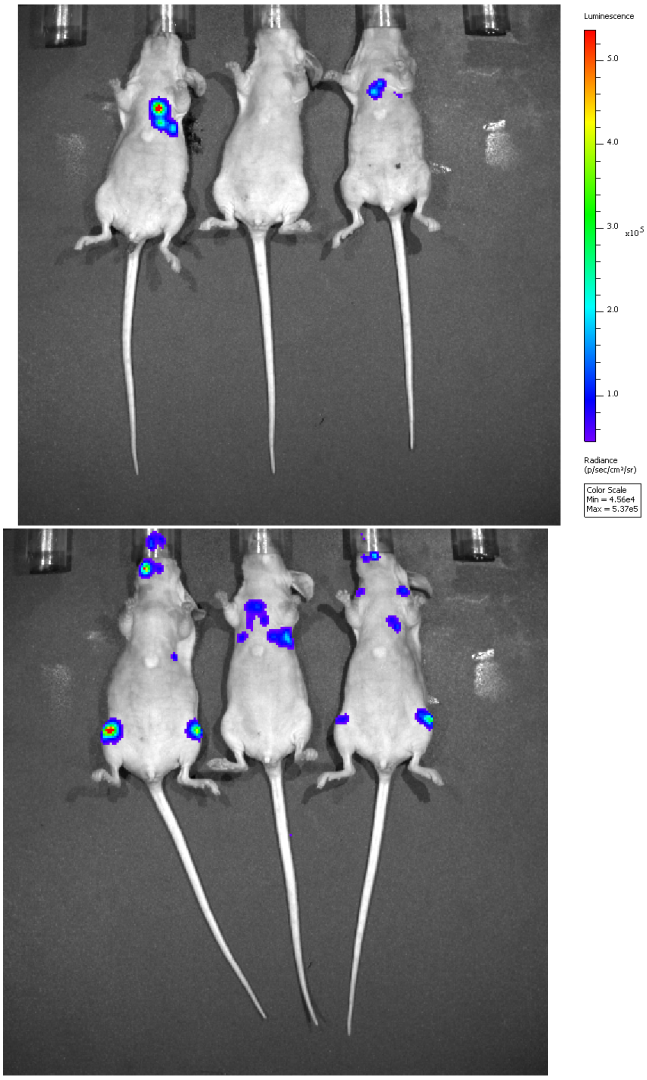


Figure 11: *In vivo* metastasis model of knockdown Sema4D mice (top) and wild mice (bottom). At week 5 after the intercardiac inoculation of MDA-231^{Luc} cells, positive BLI signals were observed in the hind limbs and craniofacial region of the wild mice group.

We performed an ELISA on media of human osteoblasts (HOB) conditioned by Sema4D looking for the presence of IL-8, a target of NFκB known for activation of osteoclasts and bone resorption [68], human osteoblast treated with increasing concentrations of soluble Sema4D produced increasing amount of IL-8 and a similar (unaffected by concentration) amount of RANKL (data not shown).

IL-8 is produced by breast cancer cells to mediate their metastatic behavior [68]. To determine the effect of Sema4D produced by our experimental cells on IL-8 production, we added the pre-determined concentration (400ng/ml) from our wild group and Sema4D knockdown group to a culture of human osteoblasts (Fig.12). We noticed a robust stimulation of IL-8 in the wild group compared to the other group, unless when co-treated with the NFκB inhibitory compound BAY or the RhoA inhibitory compound C3 (Fig. 13) the production of IL-8 became attenuated.

These results may explain our later observations of the increased osteoclastic activity in the wild group in both histological and TRAP staining results.

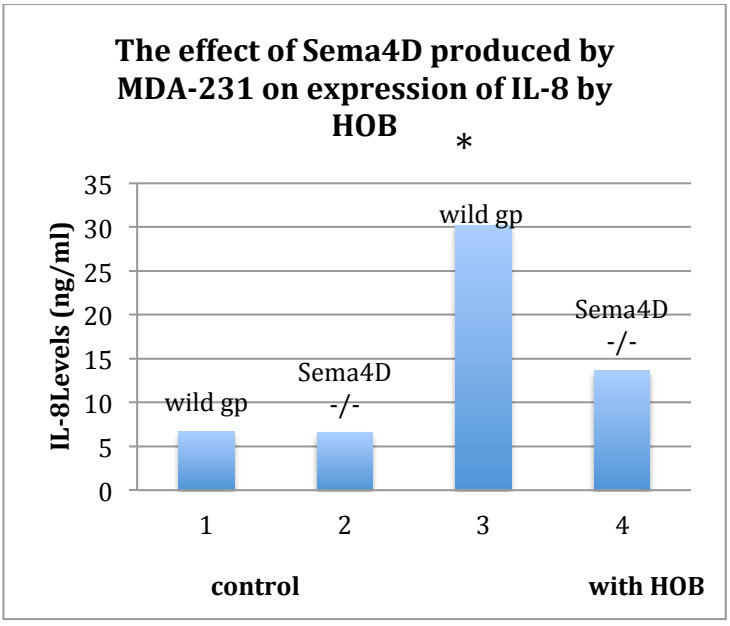


Figure 12: The effect of Sema4D produced by MDA-231 on expression of IL-8 by human osteoblasts. The presence of Sema4D in the wild group caused a robust stimulation of IL-8. IL-8 is a chemokine reported to stimulate osteoclasts activity and bone resorption.

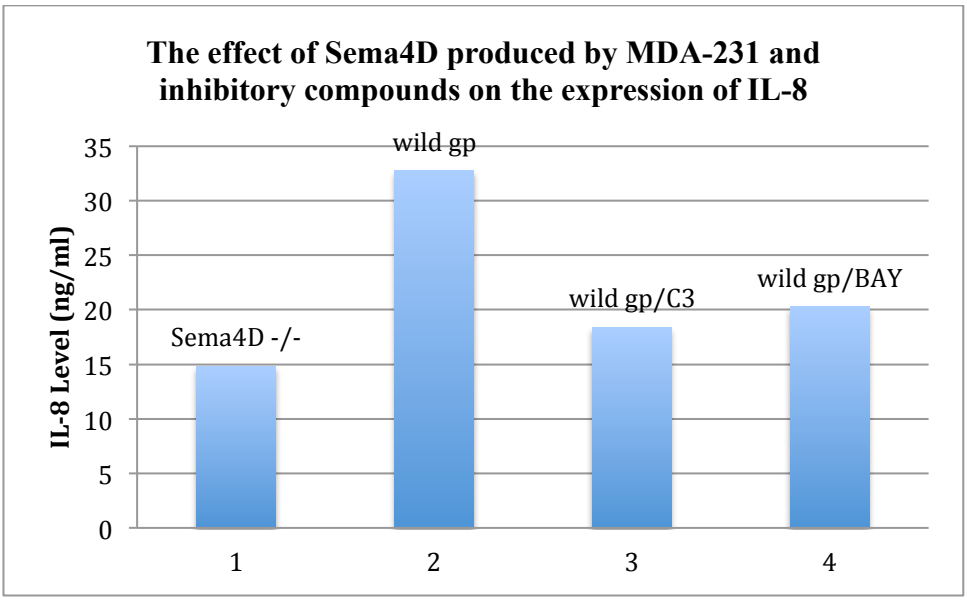


Figure 13: The effect of Sema4D produced by MDA-231 and inhibitory compounds on the expression of IL-8. Sema4D activates NFκB via Plexin-B1 to produce IL-8. Co-treatment with BAY or C3 (inhibitors of NFκB and RhoA respectively) interrupts this pathway and attenuates the expression of IL-8.

Fig.14 present normal bone structure of a mouse limb showing intact cortical, trabecular bone and bone marrow. Fig. 15 and fig. 16 shows the tissue collected from the experimental wild mice and Sema4D knockdown mice respectively. Although both groups showed evidence of metastatic activities, in the wild type (fig 15) we can clearly see osteoclastic activities (black arrows) that were difficult to appreciate in the Sema4D knockdown group (fig.16).

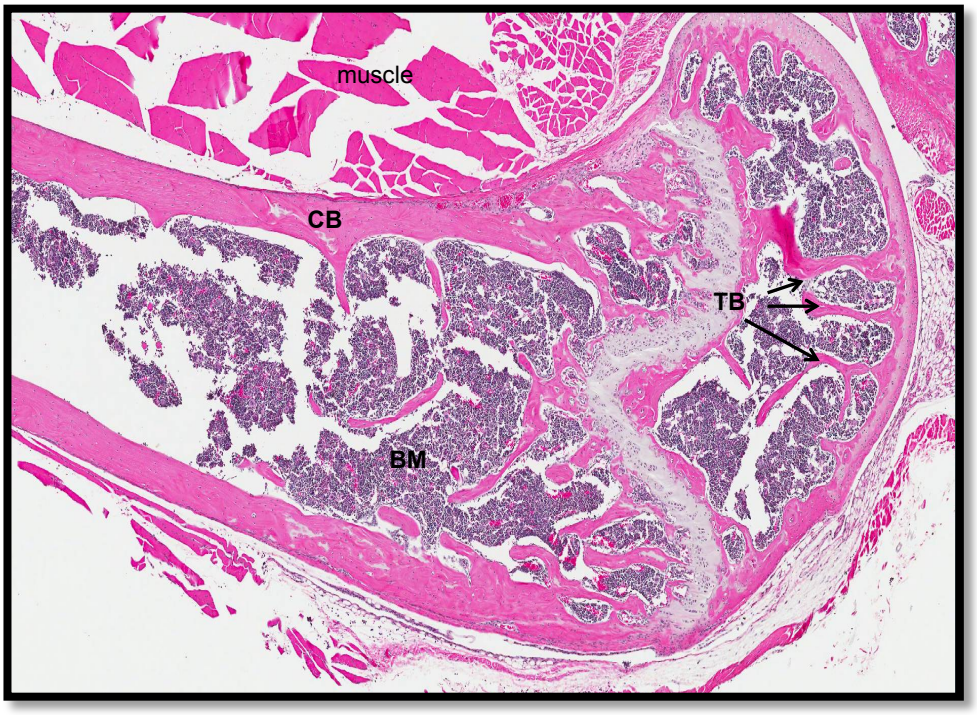
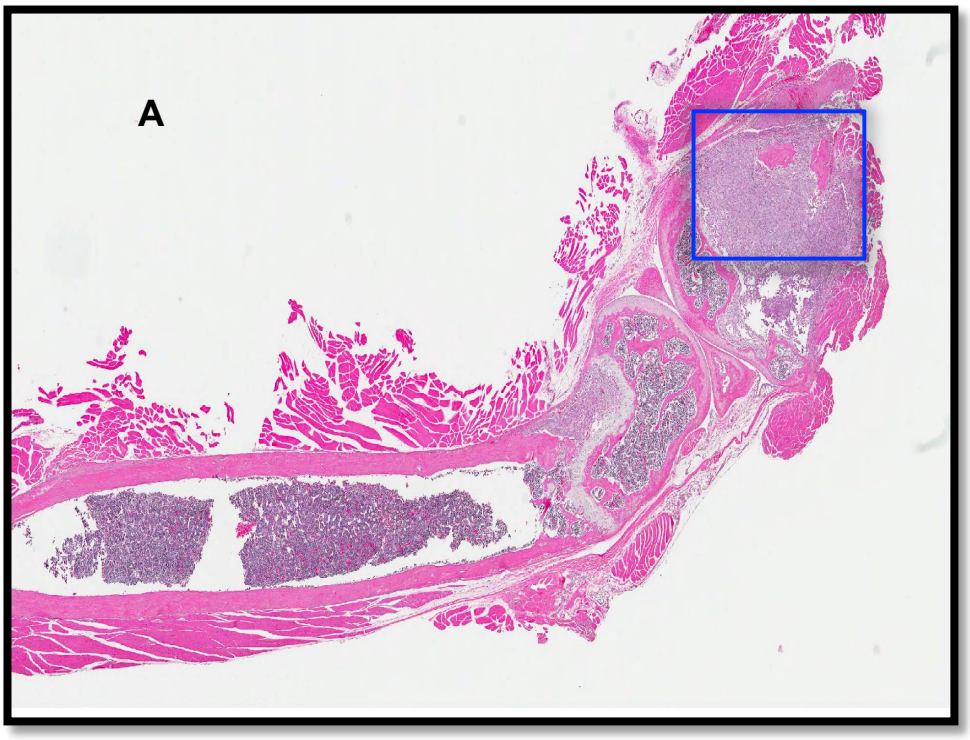
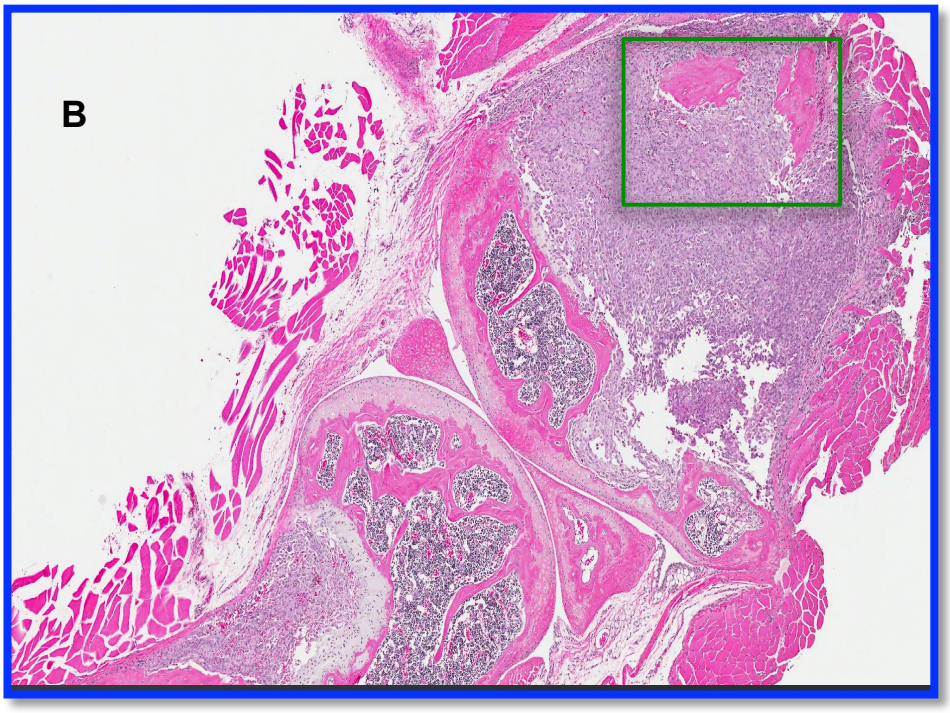


Figure 14: H & E stain of *in vivo* normal bone. A lower limb of (control) mouse limb showing intact cortical (CB) and trabecular (TB) bone as well as an intact bone marrow (BM)





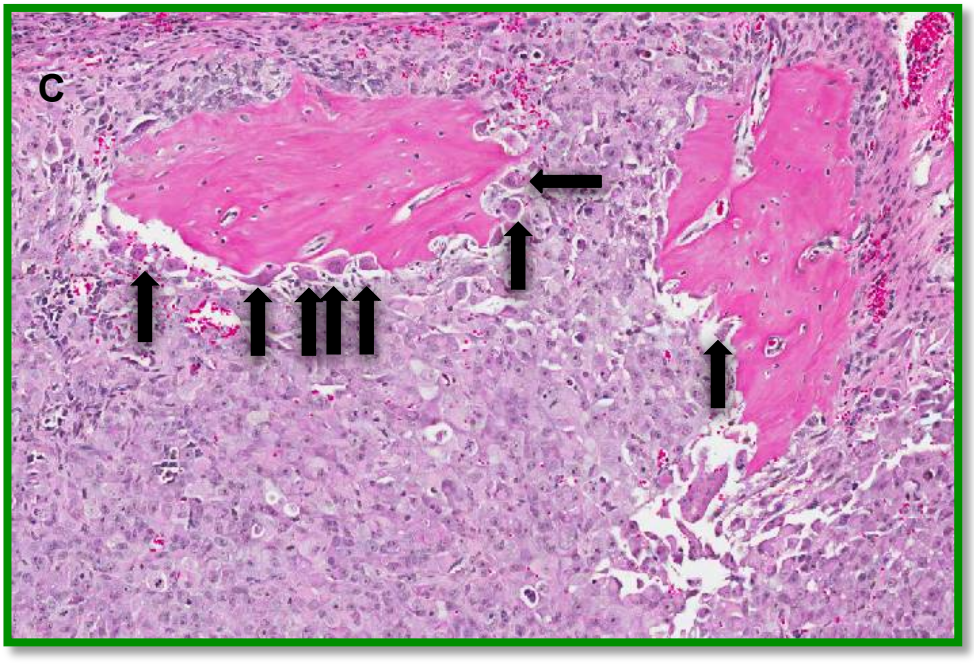


Figure 15: magnified H&E images of *in vivo* wild mice group. A, section of a hind limb of mice inoculated with MDA-231 wild type. B, magnified section demonstrates metastatic activity and tumor cells invading the bone marrow compared to the normal (control) specimen (Fig.14). C, higher magnification of box depicted in B demonstrates the presence of active multinucleated osteoclastic cells inside resorptive lacunae (black arrows).

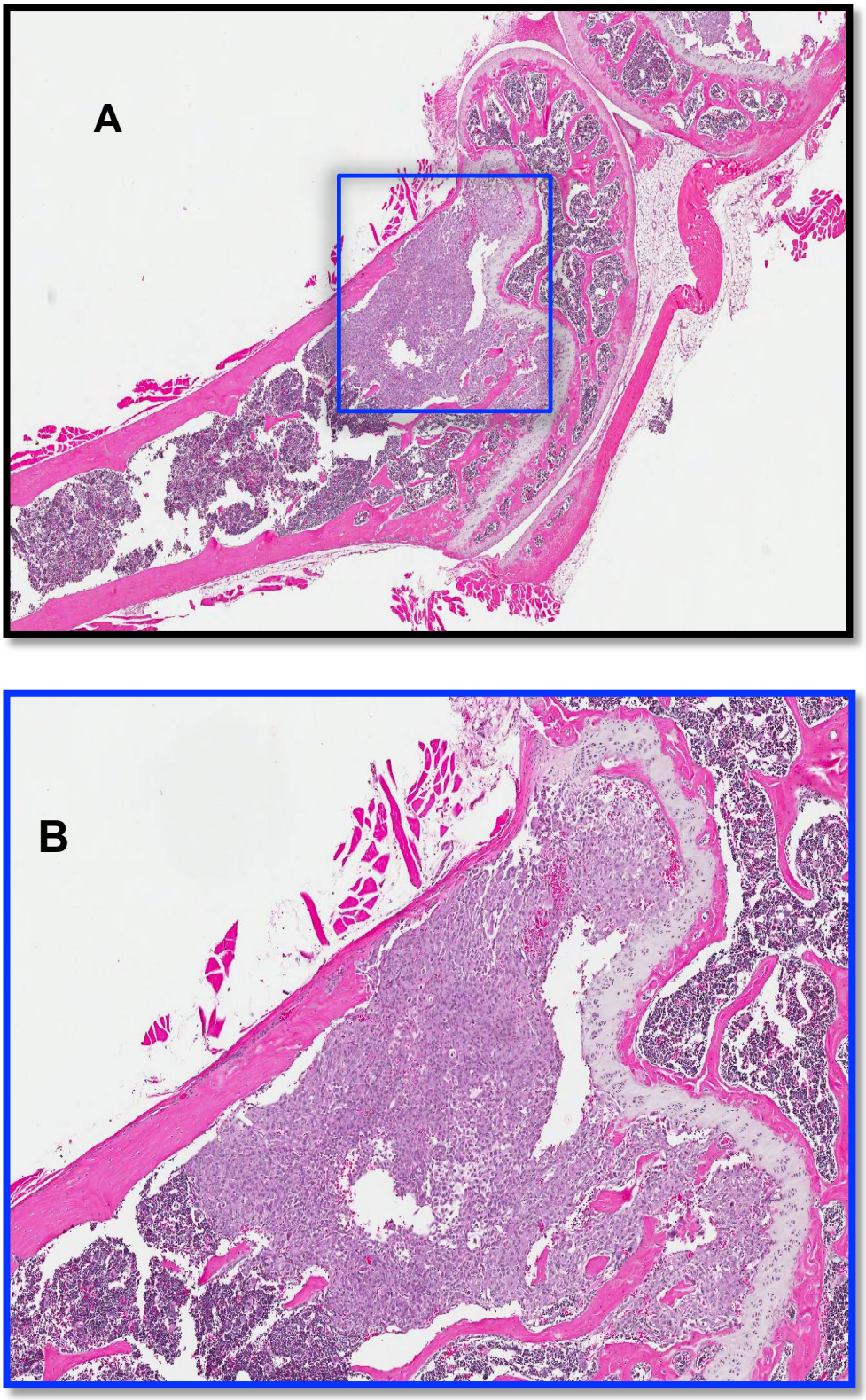


Figure 16: magnified H&E images of *in vivo* Sema4D knockdown mice. A section of hind limb of mice inoculated with MDA-231 Sema4D knockdown cells. B, magnified box depicted in A. despite the presence of metastatic lesion, it is difficult to see osteoclastic activity compared to the wild type.

Our TRAP stain (Fig. 17) also confirmed the osteoclastic activity. The wild-type tumors showed increased number of osteoclasts (yellow arrows), which may suggest that Sema4D production by tumor cells can activates NFκB dependent IL-8 production and promotes osteoclastic activity hence increases the tumor cells ability to metastasis to bone.

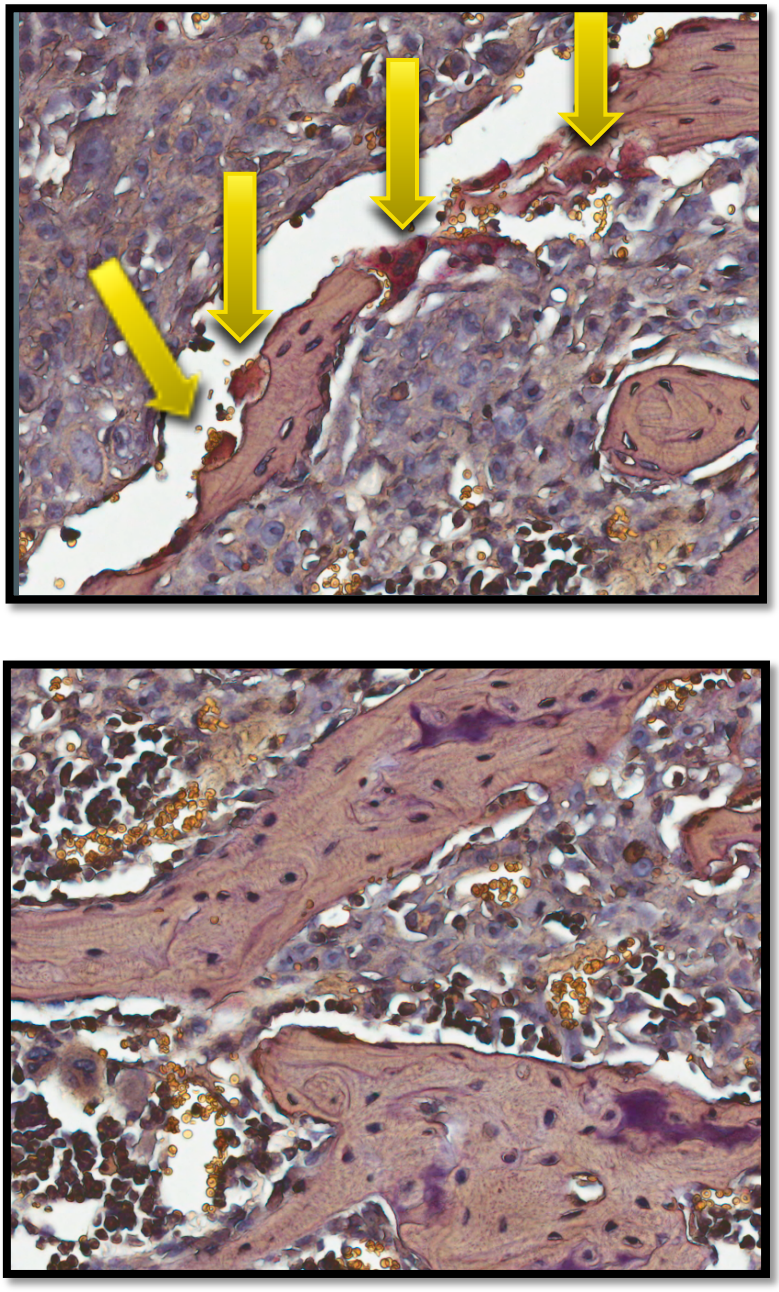


Figure 17: Sema4D promotes osteoclast activity *in vivo*. In this TRAP staining the yellow arrows in the wild group (top), show increased numbers of active osteoclast inside resorptive lacunae which could not be detected in the MDA-231 Sema4D knockdown group (bottom).

Anti-Sema4D therapy promotes bone formation *in vitro*

Interfering with Sema4D function, either through shRNA knockdown of Plexin-B1 receptor or Sema4D itself, could promote bone matrix deposition. We wanted to look at the effect of Anti-Sema4D antibody treatment on bone mineralization. We added 5µl/ml anti-Sema4D antibody to four cultured plates of MC3T3 cells treated in the same manner as in Fig. 9 and 10. We observed that anti-Sema4D antibody had favorable results (Fig. 18) on bone matrix formation. A full summary of the *in vitro* experiments is shown in figure 19 below.

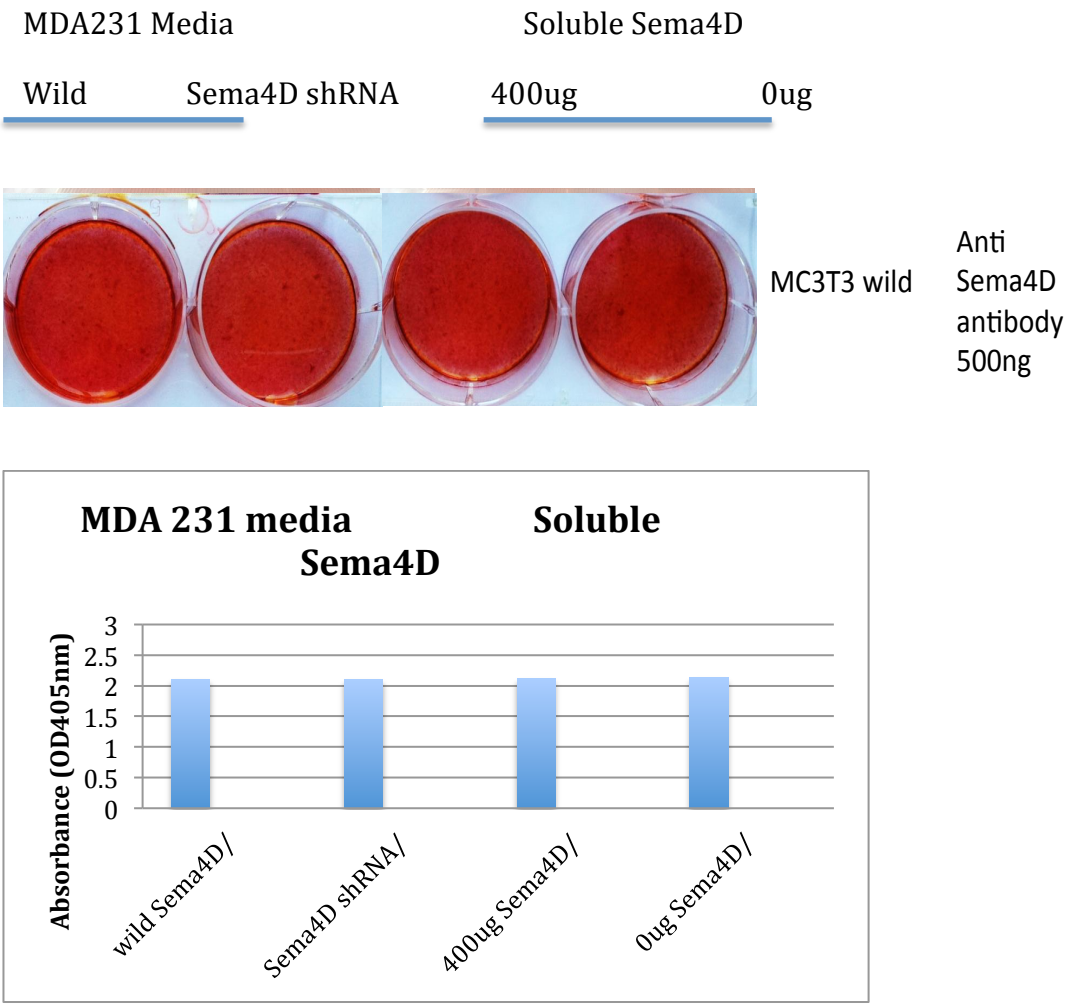
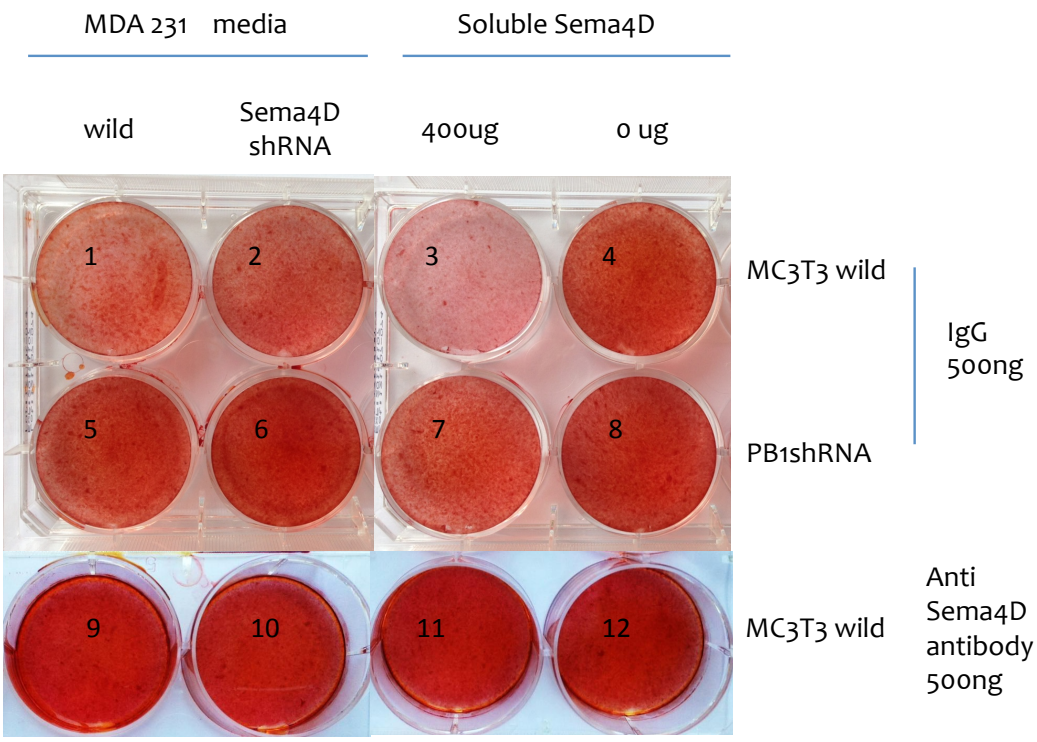


Figure 18: *In vitro* mineralization assay for Anti-Sema4D antibody



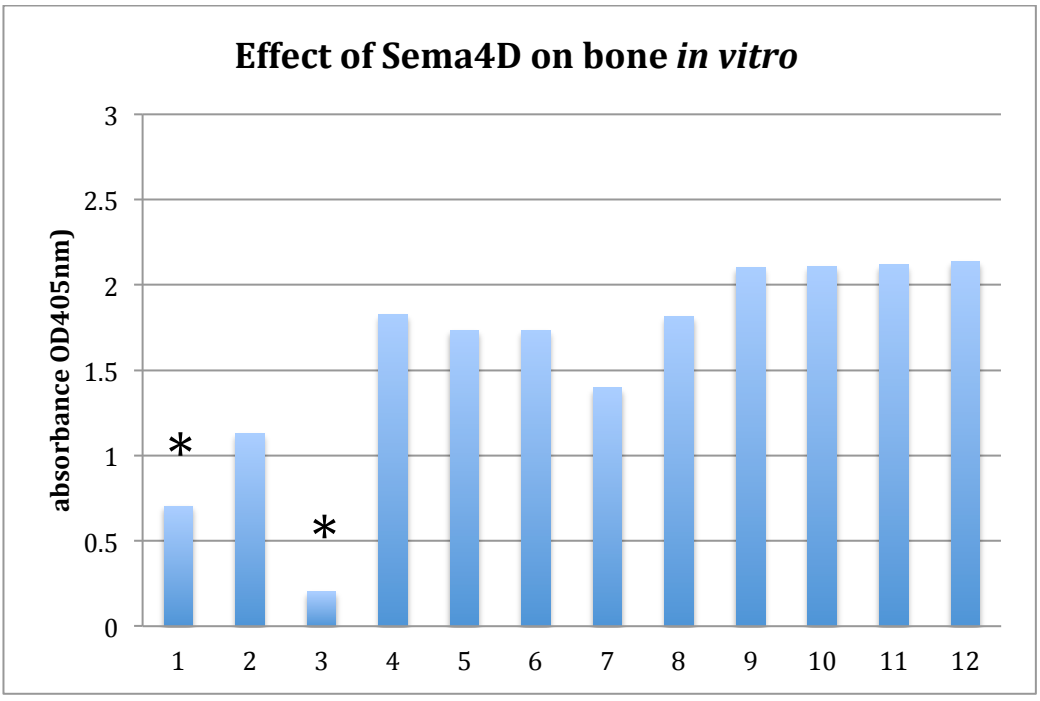


Figure 19: summary of the *in vitro* experiments. Soluble Sema4D and Sema4D produced by MDA-231 cell line can only inhibit the formation of bone significantly when both the protein itself and its receptor Plexin-B1 are present and active. The effect of bone inhibition can be reversed by using anti-Sema4D antibody that have been shown *in vitro* to tip the balance in favor of bone formation and deposition.

Chapter (4)

Discussion

The semaphorins and plexins are a large family of proteins controlling a variety of biological processes. It has been suggested that semaphorins have a role in bone remodeling [8,52-56].

Sema4D, was the first known semaphorin involved in the immune system. It is highly expressed in both lymphoid and non-lymphoid tissues [57,58] and we now know that Sema4D is also involved in the regulation of many biological events such as vascularization, organogenesis and tumor progression when it's coupled with its receptor, Plexin-B1 [8]. Emerging studies have provided genetic evidence that Sema4D have a role in bone biology [59,60].

Bone remodeling is a physiological bone renewal process finely regulated by hormonal or molecular factors to control communication among bone cells, namely osteoclasts and osteoblasts, in order to maintain skeletal integrity throughout life. [61-63]. Sema4D is expressed by osteoclasts during their differentiation while osteoblasts express its receptor Plexin-B1 [8]. When Plexin-B1 recognizes Sema4D and binds to it, a series of molecular reactions occur and a small GTPase protein called Ras homolog gene family member A (RhoA) gets activated. RhoA is well known for its ability to regulate and time cell division.

Eventually, the Sema4D-Plexin-B1-RhoA signaling axis inhibits the formation of osteoblasts and suppresses the process of bone formation.

In our study, we confirmed this finding *in vitro* by using a previously determined concentration of Sema4D (Fig. 8) to treat cultured cells of the osteoblast cell line MC3T3 under osteogenic conditions. Sema4D significantly inhibited the formation of bone only when Plexin-B1 was active in the cultured cells (Fig. 9). Our findings coincided with those shown by Negishi-Koga et al [8].

Sema4D is highly expressed in certain cancers and was found to play a role in tumor behavior and ability to survive in a host environment. Beside their known role in suppressing or promoting tumor growth [64], it has been shown again that when Sema4D binds to Plexin-B1 many pathological events may occur such as tumor-induced angiogenesis and bone disease [65].

We showed that Sema4D produced by tumor cells has the ability to suppress bone formation significantly (Fig. 10). This finding may provide another key in understanding the mechanism of the ability of some cancers to metastasize to bone. Bone metastasis is a tragic endpoint of breast and prostate cancers and a leading cause of cancer –related deaths in the United States and worldwide. It not only reduces the quality of a patient's life, but also reduces their survival rate significantly [66,67]. The mechanism of how neoplastic cells invade bone has been extensively investigated but only partially explained.

The role of Sema4D in bone metastasis may be fundamental. We studied the role of Sema4D in the breast cancer cell line MDA-231, examining its ability to metastasis to bone in a mouse model (Fig.11).

We noticed a trend in suppressed bone metastasis in Sema4D-/- knockdown tumor cells when compared to the wild type (Fig.11). The bioluminescent imaging (BLI) at 5 weeks showed less to no lesions in the Sema4D knockdown mice in comparison to the wild type mice.

On histological slides (Figs 14-16) both groups showed signs of metastatic tumor cells. One reason why the Sema4D knockdown group had metastasis may be due to the protein knockdown process that may have left active Sema4D protein. Another reason may be related to other tumor cell factors inducing the metastasis process.

The role of IL-8 in stimulating osteoclasts activity and bone resorption is well documented in the literature [68-70]. Different studies have shown the correlation between the overexpression of IL-8 in breast cancer cells and their increased ability to establish their osteolytic bone lesions [71,72]. Although a detailed insight about this topic is out the scoop of this project, Dr. Basile group showed previously that Sema4D activates NFκB, the main transcriptor for stimulating IL-8, via Blexin-B1 [73].

In this study, we have shown that Sema4D produced by tumor cells can lead to the overexpression of IL-8 *in vitro* and hence increase in osteoclast activity (Fig. 12,13).

In vivo, this was also observed on histological slides where only the wild mice group showed an osteoclastic activities and increase number of osteoclast which may suggest that silence Sema4D may help reduce pathological osteoclast activity. This was also observed in our TRAP staining of these specimens (Fig.15)

Unfortunately, our *in vivo* findings couldn't be analyzed statistically due to the small sample size and thus results are not significant. But a trend can be seen and future study with larger samples is highly recommended.

The reason we ended up with a small sample is due to the complexity of the tumor inoculation process. The intracardial injection is technique sensitive and requires experienced hands. This may led to the death of many of our experimental mice due to internal bleeding or the inoculation of tumor cells into the heart wall instead of the blood vessels.

It is also important to carry out this experiment in relatively young mice (5-6weeks old) due to their high bone turnover which sometimes become a challenge by uncontrolled factors.

Bisphosphanates, predominantly Zometa (zoledronic acid) and Denosumab, are the only approved FDA drugs to treat skeletal-related events (SRE's) in advanced cancer patients. Zometa is thought to exert its effect on the inhibition of bone resorption. Although the mechanism of action is not completely understood, *in vitro* studies showed that Zometa inhibits osteoclastic activity and induces osteoclast apoptosis. It also blocks the osteoclastic resorption of mineralized matrix by binding to bone.

Denosumab on the other hands works on a cellular level. It prevents RANKL from osteoblasts from binding to its receptor RANK on osteoclasts thus inhibiting the development, activation and survival of osteoclasts [35-37]

Both drugs have been used successfully with inherited side effects. The most serious being osteonecrosis of the jaw (ONJ).

Up to date no treatment actually provided a bone formation solution. We have confirmed that anti-Sema4D antibody can rescue bone formation and differentiation *in vitro* (Fig.16). These findings encourage us to provide a future *in vivo* model to further investigate the potential therapeutic properties of anti-Sema4D antibodies and may hold the promise for a new drug to replace and increase the quality of the destroyed bone due to cancer.

Conclusion

There are great advantages to improving our understanding of the underlying molecular mechanisms involved in the ability of certain cancer cells to metastasis to bone to develop new therapeutic approaches to bone disease including neoplastic bone tumors. Our present findings supports the emerging concept that Sema4D may paly a key role in normal bone remodeling and tumor induced bone metastasis *in vivo* and thus could presents a new therapeutic solution in treating cancer patients. Further *in vivo* studies are needed and are highly encouraged to significantly investigate the role of anti-Sema4D therapy on their ability to prevent pathological bone loss.

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