# <u>Modulation of Murine Bone Marrow-derived CFU-F and CFU-OB by *in vivo* <u>Bisphosphonate and Fluoride Treatment</u></u>

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#### ABSTRACT

MING-YI CHOU: Modulation of Murine Bone Marrow-derived CFU-F and CFU-OB by in vivo Bisphosphonate and Fluoride Treatment (Under the direction of Eric T. Everett, Ph.D.)

Bisphosphonates (BPN) are potent inhibitors of bone resorption with suggested dose dependent effects on osteoblasts. Fluoride (F) is an anabolic agent capable of increasing bone mass and, depending upon genetic background, stimulates osteoclastogenesis. This study aimed to investigate BPN and F's *in vivo* effects on bone marrow derived osteoprogenitor cells.

Mice from the C3H/HeJ (C3H), C57BL/6J (B6), FVB/NJ (FVB) and BALB/cByJ (BALB-c) strains were treated for 3-weeks with 0, 3, 30, or 150 mcg/kg/week alendronate (ALN) administered s.c. alone or in combination with 50ppm fluoride (F). Bone marrow cells were harvested and subjected to *in vitro* CFU-F and CFU-OB assays.

Results showed strain dependent baseline differences in CFU-F, CFU-OB/ALP+, and CFU-OB/total. ALN and F treatments resulted in strain specific responses for CFU-F, CFU-OB/ALP+, and CFU-OB/total. No dose responses to ALN were observed. Genetic factors appear to play a role in ALN's effects on CFU-F and CFU-OB/total, but not on CFU-OB/ALP+.

I dedicate this thesis to my family, especially Ray. Without their patience, understanding, support, and most of all love, the completion of this work would not have been possible.

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### ABBREVIATIONS

ALN	Alendronate
ALP+	Alkaline phosphatase positive
BMC	Bone marrow cell
BMD	Bone mineral density
BPN	Bisphosphonates
CFU-F	Colony Forming Unit-Fibroblasts
CFU-OB	Colony Forming Unit-Osteoblasts
CFU-OB/ALP+	Alkaline phosphatase positive CFU-OB
CFU-OB/total	Total count of CFU-OB
F	Fluoride
FPPS	Farnesyl pyrophosphate synthase
HAP	Hydroxyapatite
MSC	Mesenchymal stem cell
NaOH	Sodium hydroxide
PBS	Phosphate buffered saline
S.C.	Subcutaneous
w/v	Weight/Volume percent

#### CHAPTER 1

#### INTRODUCTION

#### **Bisphosphonates**

Bisphosphonates (BPNs) are a major class of compounds used for the treatment of bone diseases such as Paget's disease of bone, multiple myeloma, bone metastases, osteoporosis (adults), and pediatric bone diseases (i.e. osteogenesis All BPNs contain two phosphonate groups attached to a single imperfecta). carbon atom, forming a "P-C-P" structure. The presence of the two phosphate groups is essential for targeting the compound to bone and for its molecular mechanism of action. They are stable analogues of naturally occurring inorganic pyrophosphate, resistant to enzymatic and chemical breakdown. BPNs have high affinity for hydroxyapatite (HAP) crystals. BPNs have multiple direct effects on HAP and can inhibit calcification, crystal growth, and crystal dissolution. BPNs have selective localization and retention in bone. highly They preferentially compartmentalize to bone and can achieve high local levels with prolonged deposition.(1-3)

Bisphosphonates have two side groups:  $R^1$  and  $R^2$ . (Figure 1) When  $R^1$  is an –OH group, binding to calcium, and therefore bone, is enhanced.  $R^2$  group determines the compound's anti-resorptive potency biochemically, including effects on binding to HAP.(3)

Figure 1. The generic structure of bisphosphonate and its functional domains,  $R^1$  and  $R^2$ .



Bisphosphonates can be classified into two groups based on the molecular modes of action. All BPNs bind to bone mineral. The simpler non-nitrogen containing BPNs, such as etidronate and clodronate, (Figure 2) can be metabolically incorporated into non-hydrolyzable analogues of adenosine triphosphate (ATP) that may inhibit ATP-dependent intracellular enzymes. The earliest clinical applications of BPNs included use of etidronate as an inhibitor of calcification in fibrodysphasia ossificans progressive and in patients who had undergone total hip replacement surgery to prevent subsequent heterotropic ossification and improve mobility.(3) Simple BPNs are also extremely useful as agents for bone imaging, in detecting bone metastases and bone lesions, due to their high affinity to bone mineral.(4)

Figure 2. Etidronate—a simple non-nitrogen containing bisphosphonate



The most impressive clinical application of bisphosphonates involves the use of the more potent nitrogen-containing BPNs, as inhibitors of bone resorption, especially for diseases in which no effective treatment existed previously. BPNs can inhibit bone resorption indirectly through impairment of osteoclast function. The nitrogen-containing BPNs, such as alendronate, risedronate, ibandronate, and zoledronic acid, (Figure 3) are not metabolized, but can inhibit a key enzyme, farnesyl pyrophosphate synthase (FPPS) within the mevalonate pathway. FPPS is crucial for the biosynthesis of isoprenoid lipids involved in the post-translational modification, prenylation, of small GTP-binding proteins, which are also GTPases, such as Ras, cdc42, Rab, Rho, and Rac. Small GTPases are important signaling proteins that regulate a variety of cell processes including cell morphology, cytoskeletal arrangement, membrane ruffling, trafficking of vesicles, and apoptosis, all of which are essential for osteoclast formation, function and survival. The inhibition of protein

prenylation and the disruption of the function of these key regulatory proteins result in the loss of osteoclastic activity.(1-3)

Figure 3. Alendronate—a nitrogen-containing bisphosphonate



Bisphosphonates are potent inhibitors of bone resorption. Alendronate, a BPN characterized by a free amino group in its side chain, is approved as therapy and widely used for prevention of postmenopausal osteoporosis.(1-3,5) BPNs disrupt osteoclast cellular metabolism and induce apoptosis in osteoclasts.(6-9) BPNs inhibit osteoclast differentiation(10) and prevent fusion of osteoclast precursors.(11,12) BPNs also indirectly suppress bone resorption by stimulating the osteoblasts to produce an osteoclast-inhibitory factor.(13-15) The effects BPNs have on osteoclasts are much better understood than their effects on osteoblasts.

Recent studies demonstrate a role of bisphosphonates on osteoblast function. BPNs exhibit biphasic effects on osteoblastic cell precursors *in vitro*—stimulatory at low doses and inhibitory at higher doses.(16,17) At a lower dose/exposure, ALN inhibits osteoblast apoptosis.(18,19) Several investigators also noted that low

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dose/exposure of BPNs stimulates osteoblast proliferation and differentiation *in vitro*.(17,20,21) On the other hand, at a higher dose/exposure, BPNs have been shown to exhibit no stimulatory effect on osteoblasts(22,23) and suppress bone formation independently of bone resorption.(24) Effects of bisphosphonates, such as ALN, have been observed to be dose dependent and animal model dependent.(25) However, no study has investigated the dose-dependent modulation of formation of early and late osteoblastic cell precursors by *in vivo* ALN treatments with genetic background as a factor.

Bisphosphonates also have actions on angiogenesis. Clodronate, risedronate, ibandronate, pamidronate and zoledronic acid have anti-angiogenic actions.(26-28) Bisphosphonates can inhibit *in vitro* proliferation, chemotaxis, circulation, and capillary formation of bone marrow endothelial cells via VEGF and VEFG receptors.(29-31) Furthermore, bisphosphonates can lead to transient reduction in circulating levels of VEFG, bFGF, and Mmp2 following zolendronate infusion.(32)

#### Fluoride

Fluoride is an important micronutrient which, similar to bisphosphonates, preferentially compartmentalizes to bone and accumulates with deposition.(33,34)

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Fluoride is known for its anabolic effects on bone and its use as a therapeutic agent for postmenopausal osteoporosis has been investigated with mixed results.(35,36) Fluoride can affect osteoblasts anabolically in vivo(37) and in vitro(33) through undetermined mechanism and result in increase in osteoblast proliferation and activities, and increased bone formation.(38) It has been demonstrated using inbred mouse strains that genetic factors play a role in the effects of fluoride both in dental fluorosis(39) and in variation in bone properties in response to fluoride exposure(40). Past bone histomorphometric studies have suggested that it is the increase in bone formation, and not the reduction in bone resorption, that is responsible for the increase in bone mass by fluoride treatment.(41,42) Nevertheless, Yan et al.(43) used B6 and C3H inbred strains of mice to show that generic background influences fluoride's effect on osteoclastogenesis. Since both fluoride and bisphosphonate target the same physiological compartment, it is possible that bisphosphonate will modulate fluoride's effects on the bone-formation process with genetic background as a factor.

#### Bone marrow derived MSCs and osteogenic potential

In 1970s, Friedenstein et al. first showed that bone marrow stroma contains cells,

referred to as marrow stromal cells or mesenchymal stem cells (MSC), with the capacity to form bone when transplanted in vivo.(44,45) These precursors of non-hematopoietec cells have also been referred to as plastic-adherent cells because they readily adhere to plastic culture dishes and form fibroblast-like colonies.(44,46) Friedenstein et al. initially exploited the ability of these cells to adhere to the plastic of the tissue-culture dishes as a means of isolating them from the bone marrow. A standard liquid culture system was developed for isolation of the MSCs. Friedenstein et al. also noted that under these conditions, the MSCs grew as foci with fibroblast-like morphology and appeared to be clonal in nature. By using the nomenclature used in colony assay for hematopoietic precursors, these foci of cells derived from clonal expansion of single MSC precursors were termed—colony-forming-unit fibroblasts (CFU-F). CFU-F is recognized as the early osteoblastic cell precursors. By counting the number of CFU-Fs, the clonogenic precursors, the CFU-F assay is routinely used to quantitate the relative abundance of MSCs in bone marrow.(44,46-48)

Friedenstein *et al.* later reported that these MSCs, from the bone marrow, possess the potential to differentiate along multiple, non-hematopoietic, mesenchymal cell lineages, including osteocytes, chondrocytes, and adipocytes.(44,47,48) Since the

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CFU-F's are a heterogeneous population of stem and progenitor cells, their differentiation *in vitro* can be modified at the colony level. With the addition of ascorbic acid and dexamethasone, the differentiation of the plastic adherent cells can be modified *in vitro* to give rise to cells capable of forming mineralized nodules—colony-forming-unit osteoblasts (CFU-OB).(46)

#### Animal model—inbred mouse strains

Using inbred strains of mice, various studies have shown that genetic differences play a role in bone properties and bone cell biology, such as bone-regenerative capacity. Beamer *et al.* reported genetic variability in adult bone density among 11 inbred strains of mice.(49) Comparison of bone parameters, such as total and cortical density, mineral and volume, between the 11 strains revealed that the strain C3H/HeJ (C3H) had the highest value for any given bone parameter, and values from the C57BL/6J (B6) strain were absolutely and statistically the lowest. Akhter *et al.* assessed breed-related differences in bone histomorphometry, bone biomechanics and serum biochemistry in three mouse strains, C3H/HeJ, C57BL/6J, and DBA/2J, shown to differ in bone mineral density.(50) Similar to other reports, of the strains tested, C3H mice had higher bone mineral density, greater bone volume and biomechanical strength. A variation in distribution of cortical bone in the three mouse strains was also noted, and was suggested to represent a difference in adaptive response to mechanical loading and a result of strain-specific genetically regulated cellular mechanisms. Genetic variations in bone properties and bone metabolism have been extensively investigated, often using the two genetically distinct inbred strains of mice, C3H and B6.(51-53) Compared to B6 mice, C3H mice have higher peak bone density(37,52), higher serum alkaline phosphatase (ALP) activity(51), higher rate of bone formation(54), lower rate of bone resorption(51,52), higher osteoblastic activity(53,55), and lower number of osteoclasts(52). Differences in peak bone mass between these two inbred strains have been identified as the result of genetic differences, and thus they are commonly chosen as model to investigate mechanisms controlling bone density and bone biology.

The use of CFU-F and CFU-OB assays provides an opportunity for the assessment of the effects of *in vivo* ALN treatment, alone and with fluoride, on early and late osteoblastic cell precursors of different inbred strains of mice. We also had the opportunity to investigate the inter-strain responses to ALN treatment (+/-fluoride). Four strains of mice, C57BL/6J (B6), BALB/cJ (BALB-c), C3H/HeJ (C3H), and FVB/NJ (FVB) were selected based on differences in genetics, bone biology and

wound healing characteristics.(39,56,57) The current study operates under the hypotheses that systemic exposure of ALN will affect the bone marrow pool of mesenchymal stem cells (MSCs) capable of forming early (CFU-F) or late (CFU-OB) osteoprogenitor colonies in a strain-specific manner; and that fluoride will modulate ALN's effects on the MSC pool in the bone marrow when genetic background is a factor.

#### CHAPTER 2

#### MATERIALS AND METHODS

#### Animals

Male mice of the inbred strains C57BL/6J (B6), BALB/cJ (BALB-c), C3H/HeJ (C3H), and FVB/NJ (FVB) were obtained from the Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age. The mice were acclimated for 1 week in the UNC Dental Research Center Bioresearch Facility. Food and water were provided ad libitum. A laboratory rodent diet LabDiet® 5001 (PMI® Nutrition International) was provided and contained 0.95% calcium, 0.67% phosphorous, 4.5 IU/gm vitamin D3, and an average [F] of 6.56±0.28 µg/gm. Mice from each strain were caged in trios and housed in the Division of Lab Animal Medicine facility within the Dental Research Center, a fully AAALAC accredited unit, at an ambient temperature of 21°C and maintained on a 12:12 h light/dark cycle. All experimental procedures were approved by the IACUC at the University of North Carolina at Chapel Hill (UNC-IACUC approved protocol #06-118).

In vivo ALN and F treatments

A total of 64 mice per strain were used for this study. After 1 week of acclimation, 8 mice per strain were randomly assigned to one of 8 treatment groups (Group 1 = ALN 3  $\mu$ g/kg/week, F 0 ppm; Group 2 = ALN 3  $\mu$ g/kg/week, F 50 ppm; Group 3 = ALN 30  $\mu$ g/kg/week, F 0 ppm; Group 4 = ALN 30  $\mu$ g/kg/week, F 50 ppm; Group 5 = ALN 150  $\mu$ g/kg/week, F 0 ppm; Group 6 = 150  $\mu$ g/kg/week, F 50 ppm; Group 7 = ALN 0  $\mu$ g/kg/week, F 0 ppm; and Group 8 = ALN 0  $\mu$ g/kg/week, F 50 ppm).

Fluoride ion (0 or 50ppm) was provided as NaF in drinking water. Alendronate (ALN) (alendronate sodium, a gift from Merck Research Laboratories, Rahway, NJ) was prepared in 0.9% w/v NaCl and administered subcutaneously (s.c.). The four different levels of ALN treatments were as follows:

<u>0 ALN (vehicle control)</u>: For the control groups, 0.9% Nal was administered s.c. weekly (once on Mondays).

Low dose of ALN: 3 µg/kg/week as a single weekly dose s.c. (once on Mondays). This dose has previously been shown to significantly increased the number of CFU-F colonies in the bone marrow from young and old animals and better permit assessment of bone-forming effects of low dose ALN in osteoporosis.(17) This suggests that bisphosphonates, *in vivo*, may have a potentially relevant influence on

cells of the osteoblastic lineage, distinct from their inhibitory action on osteoclasts.

<u>Intermediate dose of ALN</u>: 30 µg/kg/week administered 2 times/week at 15µg/kg s.c. (Monday and Friday). This dose was shown to stop bone loss in ovariectomized rats.(58)

<u>High dose of ALN:</u> 150µg/kg/week administered 2 times/week at 75µg/kg s.c. (Monday and Friday). It was anticipated that mice required 5 times the dose as rats. This was the equivalent dose for mice which may totally prevent bone loss without toxic effect.

After the treatment period of 3 weeks, the mice were euthanized. Bone marrow cells were flushed and collected from the tibia and femur from one hind leg of each animal.

#### Colony forming unit-fibroblast (CFU-F) assays

The bone marrow cells obtained from the mice were plated in triplicate cultures (six-well plates) at 2 different densities (5.0 x 10<sup>5</sup>cells/well and 1.0 x 10<sup>6</sup>cells/well) using complete media prepared with MesenCult<sup>™</sup> Basal Medium and Mesenchymal stem cell Stimulatory Supplements (StemCell Technologies; Vancouver, BC, Canada). The bone marrow cells were plated according to the protocol provided by the

company. The formation of CFU-F was evaluated after 14 days of culture in a humidified 5% CO<sub>2</sub>/37°C environment. Cultures were washed with calcium and magnesium free Dulbecco's phosphate buffered saline (PBS) twice and then fixed with cold ethanol. CFU-Fs were stained with Giemsa stain and then colonies containing >50 cells counted using light microscopy.

#### Colony forming unit-osteoblast (CFU-OB) assays

Bone marrow cells collected from mice were plated in triplicate cultures (six-well plates) at 2 different densities (5.0 x 10<sup>5</sup>cells/well and 1.0 x 10<sup>6</sup>cells/well) as described above. The complete media used was prepared from MesenCult<sup>™</sup> Basal Medium and Mesenchymal stem cell Stimulatory Supplements (StemCell Technologies; Vancouver, BC, Canada) with the addition of 50µg/mL of ascorbic acid and 10<sup>-8</sup>M dexamethasone. After the incubation period of 14 days, the cultures were terminated by washing with PBS twice and then fixed with cold ethanol. Formation of osteoblast progenitors was detected using an alkaline phosphatase assay (86-R, Sigma). The alkaline phosphatase positive (ALP+) colonies with >50 cells were counted using light microscopy. Afterwards, the plates were washed with borate buffer (10mM boric acid, adjusted pH to 8.8 with NaOH) and stained with

borate buffer containing 1% w/v methylene blue for total colonies. The colonies with >50 cells were counted using light microscopy to obtain a total count of colonies present.

#### Statistics

Results of the CFU-F and CFU-OB assays were reported as mean  $\pm$  SD of the triplicate cultures. For each variable, effects across treatment groups were compared using one-way ANOVA. For comparison of treatment groups in pairs, Student's t-test was used. Adjusted p-values  $\leq$  0.05 were considered significant.

#### CHAPTER 3

#### RESULTS

#### Isolation of stromal cells from bone marrow

As stated above, mesenchymal stem cells (MSCs) adhere to the plastic of the tissue culture plates and thus allow for their isolation from the bone marrow cells (BMCs). MSCs proliferate into colonies and represent a unique cell population capable of differentiating along multiple mesenchymal cell lineages. Microscopic examination showed that plastic adherent cell cultures derived from BMCs consisted of a heterogeneous collection of morphologically distinctive cell types. (48,59) An example of a typical plastic adherent cell colony, taken from a B6 culture, viewed using light microscopy under various magnifications (40X, 100X, 200X and 400X) was shown in Figure 4. Higher magnifications of the colony showed that it contained fibroblastoid-shaped cells, some round cells and also stellate cells. The nature of the various cell types observed had not been well characterized or understood.(59,60) Although observations from our present study agreed with that from Phinney et al. (59) suggesting that the fibroblastoid-shaped cells displayed higher levels of ALP

expression, we did not investigate the specific cell types related to ALP expression and thus no conclusion can be drawn as to the nature of the different cell types present.

Figure 4. Plastic adherent colony stained with Giemsa stain viewed under light microscope at magnifications of 40X, 100X, 200X and 400X.







#### CFU-F & CFU-OB assays

The results from the CFU-F and CFU-OB assays following alendronate (ALN) and fluoride (F) treatment are presented in Tables 1 and 2. As shown, at the density of 5x10<sup>5</sup> BMCs/well, a large number of treatment groups (from B6, FVB and C3H strains of mice) had mean CFU-OB, both alkaline phosphatase positive (ALP+) and total count, of less than one colony per well. This means that at this density, so few precursors for CFU-OB were present that the colonies failed to thrive, and that the comparison of treatment groups using statistical analysis of the data obtained from

this plating density would have been misleading. This finding was in agreement with that of Phinney et al. (59) They investigated the frequency of CFU-F of four different strains of mice, one of which is B6. The protocol and size of tissue culture plates used in their study were different from our study. Also, only one plating density was However, they reported that at the plating density used in their study, the yield used. of plastic adherent cells is so low from B6 that the cultures failed to thrive in vitro by day 15. Our data shows that, when the plating density was doubled at  $1 \times 10^6$ BMCs/well, for all treatment groups, the mean CFU-F increased about two-folds, reflective of the increase in plating density; the mean CFU-OB, on the other hand, increased much more than two-fold because the CFU-OB populations were growing successfully at the higher plating density, compared to the few colonies present at the lower plating density. At 1x10<sup>6</sup> BMCs/well, the mean CFU-Fs of all four strains are comparable to the corresponding mean CFU-OBs. The data obtained from this higher plating density better reflected the differences in colony forming unit potentials of the BMCs from the various strains mice. Giuliani et al. also used the same plating density of 1x10<sup>6</sup> BMCs/well when they investigated the effect of BPN on formation of osteoblast precursors for murine BMCs.(17) The CFU-F protocol used in their study was similar to that of ours, with some differences. A lower plating density was not used in their study. Statistical analysis for the study was performed using only data

obtained from BMCs plated at 1x10<sup>6</sup> BMCs/well.

Treatment	CFU-F @ [5>	(10 <sup>5</sup> BMCs/well]	CFU-F @ [1:	<10 <sup>6</sup> BMCs/well]	
Group*	Mean	SD	Mean	SD	
B6 -1	2.56	1.74	9.89	4.01	
B6 -2	2.11	1.45	6.67	5.57	
B6 -3	3.11	4.23	9.56	9.58	
B6 -4	3.89	4.78	7.33	6.08	
B6 -5	2.89	2.37	7.44	6.31	
B6 -6	1.33	1.22	6.00	3.57	
B6 -7	4.00	3.57	13.00	6.14	
B6 -8	2.11	2.03	7.89	2.71	
BALB-c -1	17.89	6.85	35.00	14.02	
BALB-c -2	16.89	6.86	34.44	12.74	
BALB-c -3	17.44	5.05	34.78	4.79	
BALB-c -4	17.89	4.88	32.78	6.04	
BALB-c -5	14.56	4.03	29.44	3.75	
BALB-c -6	13.33	3.24	29.44	3.78	
BALB-c -7	14.00	5.24	28.22	7.12	
BALB-c -8	13.67	3.54	29.89	6.17	
C3H -1	10.22	5.42	16.56	5.50	
C3H -2	7.89	3.48	15.78	2.77	
C3H -3	9.67	5.96	17.22	7.36	
C3H -4	8.56	4.61	15.33	5.34	
C3H -5	4.89	2.42	11.22	2.44	
C3H -6	5.56	2.46	12.56	1.51	
C3H -7	11.00	4.12	20.33	5.66	
C3H -8	7.22	3.56	13.11	5.23	
FVB -1	3.78	2.22	11.33	3.84	

Table 1. Mean colony forming unit-fibroblast (CFU-F) following ALN and F treatment plated as triplicate cultures at  $[5x10^5 \text{ BMCs/well}]$  and  $[1x10^6 \text{ BMCs/well}]$ 

FVB -2	2.67	2.40	11.44	5.20
FVB -3	6.33	4.66	13.56	8.85
FVB -4	5.44	3.17	16.11	5.11
FVB -5	3.56	1.94	10.78	4.06
FVB -6	2.56	2.19	10.11	6.43
FVB -7	6.89	2.85	10.56	4.82
FVB -8	3.44	3.21	9.11	5.73

\*Group 1 = ALN 3 μg/kg/week; F 0 ppm; Group 2 = ALN 3 μg/kg/week; F 50 ppm; Group 3 = ALN 30 μg/kg/week; F 0 ppm; Group 4 = ALN 30μg/kg/week; F 50 ppm; Group 5 = ALN 150 μg/kg/week; F 0 ppm; Group 6 = ALN 150 μg/kg/week; F 50 ppm; Group 7 = ALN 0 μg/kg/week; F 0 ppm; and Group 8 = ALN 0 μg/kg/week; F 50 ppm

Table	2.	Mean	colony	forming	unit-os	steoblas	t (CFU-O	B) fc	ollowing	ALN	and	F
treatm	ent	plated	as tripl	licate cultu	res at	[5x10 <sup>5</sup> E	MCs/well]	and	[1x10 <sup>6</sup> B	MCs/v	well]	

Treatment	CFU-OB @ [5x10 <sup>5</sup> BMCs/well]				CFU-OB @ [1x10 <sup>6</sup> BMCs/well]			
Group*	ALF	° +	Tota	Total A		ALP+		otal
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
B6 -1	0.22	0.44	1.00	1.12	2.78	2.39	6.89	3.02
B6 -2	0.11	0.33	0.78	1.30	1.44	1.24	4.67	2.40
B6 -3	0.56	1.01	1.00	1.58	4.22	4.87	9.33	8.69
B6 -4	0.67	1.12	1.22	1.64	2.89	2.93	8.00	5.48
B6 -5	0.33	0.5	1.00	1.12	3.56	4.25	7.67	6.60
B6 -6	0.11	0.33	0.44	0.88	1.00	1.12	3.33	3.08
B6 -7	3.00	4.33	4.33	6.42	3.56	4.00	8.33	4.42
B6 -8	0.44	0.88	0.56	0.88	3.22	2.73	7.56	5.13
BALB-c -1	6.89	4.34	10.67	6.20	20.33	9.11	31.89	12.59
BALB-c -2	5.00	2.78	8.22	4.17	18.78	6.85	31.22	10.50
BALB-c -3	6.11	3.18	9.44	4.28	18.44	3.81	31.89	3.95
BALB-c -4	4.00	1.66	6.56	2.30	18.56	2.70	31.00	3.00
BALB-c -5	4.22	1.48	6.44	2.79	17.89	2.71	28.22	4.18
BALB-c -6	3.56	3.05	5.33	3.64	17.67	3.32	29.56	2.96
BALB-c -7	5.11	3.69	7.44	5.17	15.78	5.93	26.00	7.70
BALB-c -8	4.22	1.48	7.67	3.08	16.78	2.95	26.44	3.47

C3H -1	1.00	0.87	3.22	1.09	8.89	5.13	17.11	8.71	
C3H -2	1.11	1.05	3.22	3.77	5.67	2.24	14.44	3.54	
C3H -3	2.00	1.73	5.67	4.61	8.56	6.29	18.11	11.44	
C3H -4	1.11	1.76	3.44	3.84	6.22	5.33	13.89	10.95	
C3H -5	1.22	1.20	3.67	2.35	5.11	1.96	10.33	4.06	
C3H -6	0.22	0.44	1.78	1.56	5.67	2.55	11.56	5.34	
C3H -7	2.22	2.28	6.11	4.43	7.22	3.70	19.56	8.05	
C3H -8	0.89	0.78	3.33	2.55	2.89	1.36	9.00	4.53	
FVB -1	0.44	0.73	0.78	1.20	4.78	3.99	9.11	6.75	
FVB -2	0.78	1.30	1.00	1.80	5.00	4.44	6.78	5.87	
FVB -3	1.78	2.17	2.89	3.14	6.56	5.83	12.44	10.85	
FVB -4	2.33	2.40	2.78	2.86	5.33	3.67	9.33	6.63	
FVB -5	1.11	1.05	2.00	1.58	4.33	2.65	6.89	3.72	
FVB -6	1.78	2.28	2.33	2.78	5.56	4.59	8.33	6.86	
FVB -7	1.00	1.32	2.22	1.79	7.33	5.48	12.22	8.53	
FVB -8	1.00	1.32	2.00	2.55	5.89	6.11	10.33	9.35	

\*Group 1 = ALN 3 μg/kg/week; F 0 ppm; Group 2 = ALN 3 μg/kg/week; F 50 ppm;

Group 3 = ALN 30 µg/kg/week; F 0 ppm; Group 4 = ALN 30µg/kg/week; F 50 ppm;

Group 5 = ALN 150 µg/kg/week; F 0 ppm; Group 6 = ALN 150 µg/kg/week; F 50 ppm;

Group 7 = ALN 0 µg/kg/week; F 0 ppm; and Group 8 = ALN 0 µg/kg/week; F 50 ppm

#### Colony forming unit-fibroblast (CFU-F)

The intrinsic differences between strains were observed with the comparisons of mean CFU-F from control groups of the four strains tested (Table 3, Figure 5). BALB-c mice demonstrated a significantly higher mean CFU-F, of 28.22  $\pm$  7.12, compared to B6 (13.00  $\pm$  6.14; p<0.0001), FVB (10.56  $\pm$  4.82; p<0.0001), and C3H (20.33  $\pm$  5.66; p=0.0088). Of the four strains, FVB had the fewest mean CFU-F and this difference was significant when compared to C3H (p=0.0016) and BALB-c

### (p<0.0001), but not B6.

Table 3. Colony forming units (CFUs) fibroblast and osteoblast following ALN and F treatment from triplicate cultures at  $[1x10^6 \text{ BMCs/well}]$ 

Treatment	CFU-F <sup>#</sup>		CFU-OB/A	CFU-OB/ALP+		CFU-OB/total	
Group*	Mean	SD	Mean	SD	Mean	SD	
B6-1	9.89	4.01	2.78	2.39	6.89	3.02	
B6-2	6.67	5.57	1.44	1.24	4.67	2.40	
B6-3	9.56	9.58	4.22	4.87	9.33	8.69	
B6-4	7.33	6.08	2.89	2.93	8.00	5.48	
B6-5	7.44	6.31	3.56	4.25	7.67	6.60	
B6-6	6.00	3.57	1.00	1.12	3.33	3.08	
B6-7	13.00	6.14	3.56	4.00	8.33	4.42	
B6-8	7.89	2.71	3.22	2.73	7.56	5.13	
BALB-c -1	35.00	14.02	20.33	9.11	31.89	12.59	
BALB-c -2	34.44	12.74	18.78	6.85	31.22	10.50	
BALB-c -3	34.78	4.79	18.44	3.81	31.89	3.95	
BALB-c -4	32.78	6.04	18.56	2.70	31.00	3.00	
BALB-c -5	29.44	3.75	17.89	2.71	28.22	4.18	
BALB-c -6	29.44	3.78	17.67	3.32	29.56	2.96	
BALB-c -7	28.22	7.12	15.78	5.93	26.00	7.70	
BALB-c -8	29.89	6.17	16.78	2.95	26.44	3.47	
C3H-1	16.56	5.50	8.89	5.13	17.11	8.71	
C3H-2	15.78	2.77	5.67	2.24	14.44	3.54	
C3H-3	17.22	7.36	8.56	6.29	18.11	11.44	
C3H-4	15.33	5.34	6.22	5.33	13.89	10.95	
C3H-5	11.22	2.44	5.11	1.96	10.33	4.06	
C3H-6	12.56	1.51	5.67	2.55	11.56	5.34	
C3H-7	20.33	5.66	7.22	3.70	19.56	8.05	
C3H-8	13.11	5.23	2.89	1.36	9.00	4.53	
FVB-1	11.33	3.84	4.78	3.99	9.11	6.75	
FVB -2	11.44	5.20	5.00	4.44	6.78	5.87	
FVB -3	13.56	8.85	6.56	5.83	12.44	10.85	
FVB -4	16.11	5.11	5.33	3.67	9.33	6.63	
--------	-------	------	------	------	-------	------	
FVB -5	10.78	4.06	4.33	2.65	6.89	3.72	
FVB -6	10.11	6.43	5.56	4.59	8.33	6.86	
FVB -7	10.56	4.82	7.33	5.48	12.22	8.53	
FVB -8	9.11	5.73	5.89	6.11	10.33	9.35	

\*Group 1 = ALN 3 µg/kg/week; F 0 ppm; Group 2 = ALN 3 µg/kg/week; F 50 ppm;

Group 3 = ALN 30 µg/kg/week; F 0 ppm; Group 4 = ALN 30µg/kg/week; F 50 ppm;

Group 5 = ALN 150 µg/kg/week; F 0 ppm; Group 6 = ALN 150 µg/kg/week; F 50 ppm;

Group 7= ALN 0µg/kg/week; F 0 ppm; and Group 8 = ALN 0 µg/kg/week; F 50 ppm

<sup>#</sup>mean CFU colonies / 1x10<sup>6</sup> BMCs from triplicate cultures

Figure 5. Comparison of mean CFU-F of control groups from 4 mice strains (F=0ppm) plated at [1x10<sup>6</sup> BMCs/well]



The different mice strains also displayed intrinsic differences in the rate of expansion of the plastic adherent colonies. Figure 6 displayed the tissue culture plates from the CFU-F assays of the control groups of the four mice strains. One can appreciate that the plates from the different strains did not only differ in the number of colonies present, but also in the size of the colonies. B6 strain displayed the least number of colonies at the lower plating density of [5x10<sup>5</sup>BMCs/well], with no colonies at all in some of the wells. The colonies present for B6, however, are slightly larger in size than the colonies present for C3H and BALB-c strains. Both C3H and BALB-c strains displayed higher number colonies that were smaller in size, with BALB-c strain showing the highest number of colonies present than all other three strains. FVB strain displayed fewest colonies, but the sizes of the colonies present were larger than those of the other strains.

Figure 6. CFU-F plates of control groups of the 4 mice strains at [1]= $[5x10^5BMCs/well]$ and [2]=  $[1x10^6BMCs/well]$ 



Comparisons were made for the mean CFU-F between groups of mice, within each strain, treated with different levels of ALN, with the absence and presence of F in drinking water (F=0ppm and F=50ppm). As seen in figures 7-10, for the B6, BALB-c and FVB strains, no significant differences were noted between the mean CFU-F of groups, within the same strain, receiving the same fluoride dose but varying ALN doses. However, in the C3H strain, in the absence of F, the group that received a high dose of ALN (150 $\mu$ g/kg/week) displayed significantly fewer number of CFU-F (11.22 ± 2.44) compared to the control (20.33 ± 5.66; p=0.0014). Nevertheless, for all four strains tested, no dose response to ALN was observed.

Figure 7. Comparison of mean CFU-F from B6 mice treated with different levels of ALN plated at  $[1x10^{6}/well]$ 





F=50ppm

Figure 8. Comparison of mean CFU-F from BALB-c mice treated with different levels of ALN plated at  $[1x10^{6}/well]$ 



Figure 9. Comparison of mean CFU-F from C3H mice treated with different levels of ALN plated at  $[1x10^{6}/well]$ 



F=50ppm



Figure 10. Comparison of mean CFU-F from FVB mice treated with different levels of ALN plated at  $[1x10^{6}/well]$ 

F=0ppm

F=50ppm



Comparisons were made between treatment groups, within each strain of mice, treated with the same dose of ALN, but received different dose of F in drinking water (0ppm or 50ppm). (Figure 11-14) In the absence of ALN treatment (ALN=0), the presence of 50ppm of fluoride in drinking water did not significantly alter the frequency of CFU-F's for BALB-c and FVB mice; Significantly fewer mean CFU-Fs were observed in groups of B6 mice treated with fluoride (7.89 ± 2.71) compared control (13.00  $\pm$  6.14; p=0.036) and C3H mice treated with fluoride (13.11  $\pm$  5.23) compared to control (20.33  $\pm$  5.66; p=0.013). When comparisons were made between groups receiving the same levels of ALN, no significant difference was noted between the mean CFU-F of groups treated with and without fluoride. This was true for all four strains of mice. In the context of both ALN and fluoride treatment, within all four strains, there was no significant difference between the mean CFU-F of groups receiving the same ALN dose but different fluoride dose.





Figure 12. Comparison of mean CFU-F from BALB-c mice treated with & without fluoride plated at  $[1x10^{6}/well]$ 



Figure 13. Comparison of mean CFU-F from C3H mice treated with & without fluoride plated at  $[1x10^{6}/well]$ 



\*=Significant difference between F=0 and F=50ppm (p<0.05)

Figure 14. Comparison of mean CFU-F from FVB mice treated with & without fluoride plated at  $[1x10^{6}/well]$ 



## Colony forming unit-osteoblast (CFU-OB)

The normal differences noted between the mean CFU-F of different strains were also observed with the CFU-OB assay. A comparison of the number of alkaline phosphatase positive CFU-OB (CFU-OB/ALP+) from the control groups of the four strains (Figure 15) revealed that BABL-c (15.78  $\pm$  5.93) displayed a significantly higher number of CFU-OB/ALP+ than B6 (3.56  $\pm$  4.00; p<0.0001), C3H (7.22  $\pm$  3.70; p=0.0008) and FVB (7.33 ± 5.48; p=0.0009). When the total numbers of CFU-OB (CFU-OB/total) were counted, a greater difference between the strains was noted. As displayed in Figure 16, BALB-c ( $26.00 \pm 7.70$ ) had significantly higher number of CFU-OB/total compared to B6 (8.33 ± 4.42; p<0.0001) and FVB (12.22 ± 8.53; 0.0004); C3H (19.56 ± 8.05) also displayed significantly higher number of CFU-OB compared to B6 (p=0.0028) and FVB (p=0.0422). Intrinsic differences were again observed in the ratios of the total CFU-OB that were ALP+ in the control groups of the individual strain. As shown in Figure 17, both BALB-c and FVB showed higher ALP+/total CFU-OB ratios, at 0.60 ± 0.10 and 0.50 ± 0.20 respectively, than B6 (0.32 ± 0.31; BALB-c, p=0.004; FVB, p=0.006) and C3H (0.36 ± 0.07; BALB-c, p=0.011; FVB, p=0.017).

Figure 15. Comparison of mean CFU-OB/ALP+ of control groups from 4 mice strains (F=0ppm) plated at  $[1x10^{6}/well]$ 







Figure 17. Comparison of mean CFU-OB (ALP+/total) ratio of control groups from 4 mice strains (F=0ppm) plated at  $[1x10^{6}/well]$ 



<sup>\*=</sup>Significantly different from Balb-c (p<0.05) •=Significantly different from FVB (p<0.05)

An analysis of the results from groups within the B6 strain (Figures 18-20) demonstrated that when F level remained 0ppm, the increase in ALN dose did not result in significant changes in the frequency of CFU-OB/ALP+, CFU-OB/total, or the CFU-OB (ALP+/total) ratio. However, when the mice received 50ppm of F in the drinking water, there were significant differences in the number of CFU-OB/ALP+ between the group receiving no ALN  $(3.22 \pm 2.73)$  and the group receiving  $150\mu g/kg/week$  of ALN (1.00 ± 1.12; p=0.0373); significant differences were also noted in the mean CFU-OB/total between the group treated with no ALN (7.56  $\pm$  5.13) and the group treated with 150µg/kg/week of ALN (3.33 ± 3.08; p=0.0420), and between the group treated with  $30\mu g/kg/week$  of ALN (8.00 ± 5.48) and the group treated with 150µg/kg/week of ALN (p=0.0256). No significant difference was observed in the changes of mean CFU-OB (ALP+/total) ratios in response to increasing levels of ALN for the groups treated with 50ppm of F.

Figure 18. Comparison of mean CFU-OB/ALP+ from B6 mice treated with different levels of ALN plated at  $[1x10^{6}/well]$ 



Figure 19. Comparison of mean CFU-OB/total from B6 mice treated with different levels of ALN plated at  $[1x10^{6}/well]$ 



Figure 20. Comparison of mean CFU-OB (ALP+/Total) ratio from B6 mice treated with different levels of ALN plated at  $[1x10^{6}/well]$ 



Comparisons were also made between groups of B6 mice receiving 0 ppm F and 50ppm of F in drinking water, but treated with the same ALN doses. When the dose of ALN remained constant, no significant difference was noted, between groups treated with and without fluoride, in the mean CFU-OB/ALP+ (Figure 21), the mean CFU-OB/total (Figure 22), and the mean CFU-OB (ALP+/total) ratio (Figure 23).

Figure 21. Comparison of mean CFU-OB/ALP+ from B6 mice treated with & without fluoride plated at  $[1x10^{6}/well]$ 



Figure 22. Comparison of mean CFU-OB/total from B6 mice treated with & without fluoride plated at  $[1x10^{6}/well]$ 



Figure 23. Comparison of mean CFU-OB (ALP+/Total) ratio from B6 mice treated with & without fluoride plated at  $[1x10^{6}/well]$ 



Analysis of the data from BALB-c mice revealed that as the level of F remained constant, either at 0ppm or 50ppm, the increase in ALN dose did not have a significant effect on the frequencies of mean CFU-OB/ALP+ (Figure 24), mean CFU-OB/total (Figure 25), and the mean CFU-OB (ALP+/total) ratio (Figure 26). There was also no significant difference in the mean CFU-OB/ALP+ (Figure 27), the mean CFU-OB/total (Figure 28), and the mean CFU-OB (ALP+/total) ratio (Figure 29) between groups receiving the same ALN dose, but different doses of F (0ppm or 50ppm).

Figure 24. Comparison of mean CFU-OB/ALP+ from BALB-c mice treated with different levels of ALN plated at [1x10<sup>6</sup>/well]



F=50ppm



Figure 25. Comparison of mean CFU-OB/total from BALB-c mice treated with different levels of ALN plated at [1x10<sup>6</sup>/well]



Figure 26. Comparison of mean CFU-OB (ALP+/total) ratio from BALB-c mice treated with different levels of ALN plated at  $[1x10^{6}/well]$ 



Figure 27. Comparison of mean CFU-OB/ALP+ from BALB-c mice treated with & without fluoride plated at  $[1x10^{6}/well]$ 



Figure 28. Comparison of mean CFU-OB/total from BALB-c mice treated with & without fluoride plated at  $[1x10^{6}/well]$ 



Figure 29. Comparison of mean CFU-OB (ALP+/total) ratio from BALB-c mice treated with & without fluoride plated at [1x10<sup>6</sup>/well]



The results from C3H mice showed some interesting relationships between treatment groups. When level of F remained at 0ppm, no significant difference was observed in the mean CFU-OB/ALP+ between treatment groups receiving different doses of ALN (Figure 30); However, a significant decrease in the mean CFU-OB/total was noted in the group receiving  $150\mu g/kg/week$  of ALN ( $10.33 \pm 4.06$ ) compared to the control (ALN=0) ( $19.56 \pm 8.05$ ; p=0.028) (Figure 31); There were also significant increases in the mean CFU-OB (ALP+/total) ratio in response to increased levels of ALN reflected by the comparisons made between the control group (ALN=0) ( $0.36 \pm 0.07$ ) and the groups receiving  $3\mu g/kg/week$  of ALN ( $0.49 \pm 0.14$ ; p=0.0116), and

150µg/kg/week of ALN (0.51  $\pm$  0.11; p=0.004) (Figure 32). On the other hand, when level of F remained constant at 50ppm, a significant increase in the mean CFU-OB/ALP+ was noted in the group treated with 30µg/kg/week of ALN (6.22  $\pm$  5.33) compared to the control (2.89  $\pm$  1.36; p=0.0361) (Figure 30); However, the changes in the mean CFU-OB/total (Figure 31) and the mean CFU-OB (ALP+/total) ratio (Figure 32), in response to increasing levels of ALN, were not significant.

Figure 30. Comparison of mean CFU-OB/ALP+ from C3H mice treated with different levels of ALN plated at  $[1x10^{6}/well]$ 



Figure 31. Comparison of mean CFU-OB/total from C3H mice treated with different levels of ALN plated at [1x10<sup>6</sup>/well]



<sup>\*=</sup>Significantly different from control (ALN=0) (p<0.05)

Figure 32. Comparison of mean CFU-OB (ALP+/Total) ratio from C3H mice treated with different levels of ALN plated at  $[1x10^{6}/well]$ 



Comparisons were then made between C3H groups receiving the same levels of ALN, but different levels of F (0ppm or 50ppm). At ALN dose of 0, significantly fewer mean numbers CFU-OB/ALP+ and CFU-OB/total were present in the group treated with 50ppm F (ALP+  $2.89 \pm 1.36$ ; total  $9.00 \pm 2.18$ ) compared to the group not treated with F (ALP+  $7.22 \pm 0.93$ ; p=0.005; total  $19.56 \pm 2.18$ ; p=0.003). At higher doses of ALN, no difference was observed in the mean number of CFU-OB/ALP+ or the mean CFU-OB/total between groups treated with and without F. (Figure 33, 34) The differences in mean CFU-OB (ALP+/total) ratio between groups treated with and without F, at the various levels of ALN, were not significant (Figure 35).

Figure 33. Comparison of mean CFU-OB/ALP+ from C3H mice treated with & without fluoride plated at  $[1 \times 10^{6}/well]$ 



Figure 34. Comparison of mean CFU-OB/total from C3H mice treated with & without fluoride plated at  $[1x10^{6}/well]$ 



<sup>\*=</sup>Significantly different from group F=0 (p<0.05)

Figure 35. Comparison of mean CFU-OB (ALP+/Total) ratio from C3H mice treated with & without fluoride plated at  $[1 \times 10^{6}/well]$ 



The same analyses were performed for results from the FVB strain. As displayed in Figures 36-38, as the level of F remained constant, no significant change in the mean frequency of CFU-OB/ALP+, CFU-OB/total and CFU-OB (ALP+/total) ratio was noted in response to increasing doses of ALN. When comparisons were made between groups treated with the same levels of ALN, but different levels of fluoride (0ppm or 50ppm), there were no significant differences in the mean CFU-OB/ALP+ (Figure 39), mean CFU-OB/total (Figure 40) and mean CFU-OB (ALP+/total) ratio (Figure 41).

Figure 36. Comparison of mean CFU-OB/ALP+ from FVB mice treated with different levels of ALN plated at  $[1x10^{6}/well]$ 



Figure 37. Comparison of mean CFU-OB/total from FVB mice treated with different levels of ALN plated at  $[1x10^{6}/well]$ 



Figure 38. Comparison of mean CFU-OB (ALP+/Total) ratio from FVB mice treated with different levels of ALN plated at  $[1x10^{6}/well]$ 



Figure 39. Comparison of mean CFU-OB/ALP+ from FVB mice treated with & without fluoride plated at [1x10<sup>6</sup>/well]



Figure 40. Comparison of mean CFU-OB/total from FVB mice treated with & without fluoride plated at  $[1x10^{6}/well]$ 



Figure 41. Comparison of mean CFU-OB (ALP+/Total) ratio from FVB mice treated with & without fluoride plated at [1x10<sup>6</sup>/well]



## Summary of effects of F and ALN

As shown above, the intrinsic strain dependent differences in CFU-F, CFU-OB/ALP+ and CFU-OB/total were observed at baseline. In summary, fluoride alone did not significantly alter the frequencies of CFU-F, CFU-OB/ALP+, or CFU-OB/total for BALB-c and FVB mice. Significant decreases in mean CFU-F were found in B6 (p=0.036) and C3H mice (p=0.013) treated with 50ppm fluoride in drinking water. Only the C3H strain demonstrated significant reductions in CFU-OB/ALP+ (p=0.005) and CFU-OB/total (p=0.003) with fluoride treatment.

Systemic alendronate had no significant effect on the frequencies of CFU-F,

CFU-OB/ALP+, or CFU-OB/total for the BALB-c, B6, and FVB strains. ALN treatment alone significantly reduced the frequencies of CFU-F and CFU-OB/total in C3H mice in a dose dependent manner, which is greatest between the control and the highest dose (150µg/kg/week) (CFU-F p=0.0014; CFU-OB/total p=0.028). In the C3H strain, ALN alone had no significant effect on the frequency of CFU-OB/ALP+.

## CHAPTER 4

## DISCUSSION

In our review of the literature, we found that the majority of studies have focused upon bisphosphonates' actions on osteoclasts and osteoblasts. We chose to investigate potential effects of the nitrogen-containing bisphosphonate, alendronate, in the context of another bone seeking agent, fluoride, on the bone marrow mesenchymal stem cell (MSC) pool. This study investigated the inter-strain responses to ALN treatment (+/- fluoride). Four strains of mice, C57BL/6J (B6), BALB/cByJ (BALB-c), C3H/HeJ (C3H), and FVB/NJ (FVB) were selected based on differences in genetics, bone biology and wound healing characteristics(39,40,43,56,57,61). This study tested the hypothesis that systemic ALN and F would interact and affect the bone marrow pool of MSCs capable of forming early (CFU-F) or late (CFU-OB) osteoprogenitor colonies in a strain-specific manner.

As shown above, colonies formed from murine BMCs under CFU culture condition consisted of a variety of morphologically distinct cells. Some cells were

fibroblastoid-shaped, some were round, and some were stellate shaped. Although observations from our present study agreed with that of Phinney *et al.*(59) suggesting that the fibroblastoid-shaped cells displayed higher levels of ALP expression, we did not investigate the specific cell types related to ALP expression and thus no conclusion can be drawn as to the nature of the different cell types present.

The intrinsic differences between the isolated bone marrow cells from different strains of inbred mice have been investigated extensively. As reported by Phinney et al.(59), MSCs from inbred strains of mice vary greatly in yield, growth and differentiation in CFU assays. As a result, the optimal plating densities vary between strains. Phinney et al. provided direct comparison of frequency of CFU-Fs in commonly used inbred strains of mice. They estimated the frequency of MSCs per 10<sup>6</sup> bone marrow cells as 0.3±0.02 for B6 mice, and 3±0.22 for BALB-c mice. The differences in frequencies of CFU-F from the various strains of mice supported the findings in our study. However, the frequencies reported did not match our data This difference in reported frequencies may have resulted from a difference exactly. in culture conditions. We chose to not include the data obtained from BMCs plated at 5x10<sup>5</sup> cells/well in the analyses, as the yield of CFUs was less than 1 for many of the groups and may have resulted in misleading conclusions. We noted that plating

density of 5x10<sup>5</sup> BMCs/well may have been ideal for some strains of mice, but not for others which may have required higher plating density to acquire identifiable colonies. This finding was supported by those of Peister *et al.*(60).

Peister et al. (60)demonstrated that MSCs from different strains of inbred mice vary in their media requirements and plating density for optimal growth. In a controlled environment, the MSCs from various strains of mice vary in their rates and potential of proliferation. Their study showed that both BALB-c and B6 MSCs expanded more rapidly than FVB. However, the rate and proliferative potential in their study were measured by comparison of cell numbers. One must note that the size of each CFU-F/CFU-OB, thus, the total cell number, vary greatly between strains. We did observe significantly higher CFU-F and CFU-OB counts for BALB-c strain compared to others. B6 strain exhibited low counts of CFU-F and CFU-OB. Our findings contradicted that of Peister's. However, it was noted during our study that B6 MSCs, when successfully adhere to plastic tissue culture plates, form few, but, large colonies when plated for CFU-F and CFU-OB assays. This may account for the discrepancy in number of colonies and cell numbers. Interestingly, Phinney et al. also looked at the growth kinetics of plastic adherent cell cultures of the various strains of mice.(59) They also observed high proliferation rate of BALB-c BMCs,

about 1.5 – to 4-fold greater than that calculated for FVB BMCs. Similar to our findings, they noted that the growth rate of plastic adherent cultures from B6 BMCs were so low that the cultures failed to thrive in vitro. Interestingly, in our study, even though FVB strain had the fewest mean number of CFU-F, its colonies were also larger in size than those of the B6 strain. This was in agreement with the findings from the Phinney et al. study showing that B6 plastic adherent cells had the slowest A possible explanation for the difference observed between the growth rate. findings from the various studies is that Phinney et al., like our present study, observed the differentiation of BMCs plated right after extraction from the bone marrow, while Peister et al. studied the rate of proliferation of passage 7 cells. Plastic adherent cells from various strains of mice differ in composition of morphologically distinct cells and differentiation potential. It was noted that at passage 7, cells from all strains of mice had similar morphologies. The difference in composition of whole marrow cells and passage 7 cells likely contributed to the difference in the expansion potential observed for the B6 mice. Neither groups included C3H strain in their studies. Nevertheless, from our observation of the number and size of colonies present, we suspect that the growth rate of the C3H plastic adherent cells may be close to that of the BABL-c cells. However, we could

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only speculate the growth rates of the cells since no quantitative measures were taken in our study to calculate the total number of cells over time.

The mismatch between the osteoblastic potential of the various strains, measured by ALP+/total CFU-OB, the number of osteoprogenitor precursors present in the bone marrow, measured by mean CFU-F and CFU-OB, and the reported bone mineral density suggest that there are many factors involved in bone metabolism in determining the amount of bone present, the mineral content of the bone, and the turnover rate of the bone. It has been shown that the cortical densities and mineral contents in C3H femurs were significantly higher than in B6 and BALB-c femurs. (49) Data from the Mouse Phenome Database showed that the whole body bone mineral density (BMD) (without head) for 8 week old male mice of the four mice strains used in our study, arranged from the greatest to the least, were as follows: C3H > FVB > BALB-c > B6(62). The difference in BMD between C3H and B6 has been well documented and investigated. C3H mice, compared to B6 mice, have higher BMD, higher serum ALP level, higher bone healing potential and higher number of osteoblast precursors in terms of ALP+ colony-forming units in bone marrow cells cultures(51,53). We found that C3H mice, compared to B6 mice, did have significantly higher number of CFU-F, ALP+ CFU-OB and total number of CFU-OB.

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Interestingly, when we compared the ratios of ALP+/total CFU-OB between the two strains, no significant difference was found. It is likely that MSCs from both C3H and B6 strains have similar potential in differentiation into osteoblasts despite the significantly higher number of early osteoprogenitors found in the C3H strain. A study by Amblard *et al.* showed that BMD did not depend only on the factors listed above, but also on the bone cellular activities.(63) Bone formation activity, and histomorphometric indices of bone formation in the femoral metaphysis and cortical tibia were shown to be lower in C3H than B6 mice. The lower bone cellular activities, together with high number of osteoprogenitors, all contributed to the higher bone mineral density in C3H mice.

We also found that MSCs from BALB-c and FVB display similar, and significantly higher osteoblast differentiation potential than that of C3H and B6 strains, as shown by their ratios of ALP+/total CFU-OB. This finding is again interesting since BALB-c mice, as mentioned earlier, displayed significantly higher counts of CFU-F and CFU-OB compared to all three other strains, and FVB mice consistently displayed significantly lower counts of CFU-F and CFU-OB compared to that of BALB-c and C3H. One must also note that the whole body BMD of BALB-c is low compared to both C3H and FVB mice, despite the higher CFU-F, CFU-OB/ALP+ and CFU-OB/total

in BALB-c than the other three strains of mice. This again suggested that BMD did not depend only on the number of osteoprogenitors and their osteoblastic differentiation potential. Similar to the example shown above for C3H and B6 mice, other factors such as rate of bone cellular metabolism, rate of bone resorption, osteoclastic potentials, etc. may have contributed to the low BMD in BALB-c mice. Unfortunately, at this point in time, we can only speculate on the reason for the low BMD in BALB-c mice. The BALB-c mice, unlike C3H or B6, are not typically used for bone studies, and thus no data exists, to date, involving other aspects of bone metabolism.

While the four strains differed in baseline frequencies of CFU-F, CFU-OB/ALP+, and CFU-OB/total, their responses to alendronate or fluoride were limited. Our data showed no significant change in frequency of CFU-F in response to increasing doses of ALN, without the influence of fluoride, in B6, BALB-c and FVB strains. This finding is different from that of the work of Giuliani *et al.*(17) and Still *et al.*(16). Both groups of authors reported an increase in colony formation as a result of ALN treatment at a low concentration. However, in both those studies, rats were used as the animal model, and ALN was administered *in vitro*, as part of the medium, for the plated MSCs. In our study, the ALN was administered to the mice *in vivo*, and then, the MSCs were collected and then plated for CFU-F assay. This difference in method of ALN treatment, together with the species difference, may have resulted in the contrasting findings. Although Still et al. suggested through their experience that agents that can stimulate colony formation in CFU-F assay may also be anabolic in vivo, this was not observed in our study. It is also possible that murine CFU-F assays produce few colonies at the density and size of tissue culture plate used, unlike the hundreds of colonies reported in rat CFU-F studies, and it is difficult for us to detect mild effects of the treatments. Both Giuliani et al. and Still et al. have also noted that ALN inhibited colony formation when administered at a high dose. We did not see a significant decrease in number of CFU-F in response to high ALN dose in B6, BALB-c or FVB strains of mice. However, in the C3H strain, although no significant change in frequency of CFU-F was observed with low and intermediate doses of ALN, high dose of ALN at 150µg/kg/week did result in a significant decrease in the number of CFU-F compared to the control. However, since C3H is the only strain of mice with this finding, we are not able to conclude that high dose ALN inhibits colony formation of CFU-F in mice.

Giuliani *et al.* did include in their study an experiment involving *in vivo* ALN treatment on female Swiss/Weber mice at three different levels ALN treatment.

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They noted that *in vivo* ALN treatment, like *in vitro* treatment, resulted in increase of CFU-F formation at low ALN dose, and inhibited CFU-F formation at high ALN dose. However, their low and intermediate doses were 0.3µg/kg/week and 3µg/kg/week respectively, which were much lower than our doses at 3µg/kg/week and 30µg/kg/week. Their high dose of ALN, was 30µg/kg/week, which is the intermediate dose used in our study. This variation in dose and in strains of mice used between our study and theirs may have contributed to the difference in results.

The effect of ALN treatment on CFU-OB was also investigated by Giuliani *et al.*(17) They noticed that ALN had a biphasic effect on formation of CFU-OB, defined by this group as colonies with formation of bone-like nodules. They believed that ALN had a stimulatory effect at low doses and inhibitory effect at high doses. The range of the "effective dose" would depend on the bisphosphonate used and the species. The results from our CFU-OB assay did not agree with the observations made by Giuliani *et al.* Our results from B6, BALB-c and FVB mice failed to show significant changes in frequency of ALP+ CFU-OB, total number of CFU-OB, and ratio of ALP+/total CFU-OB in response to ALN treatments. However, the results from C3H mice showed significant decrease in number of total CFU-OB in response to high dose ALN (150µg/kg/week), yet, the ratio of ALP+/total colonies significantly

increased in CFU-OB from groups treated with ALN, compared to control. This suggests that ALN, despite having an inhibitory effect on the number of osteoprogenitors available in the bone marrow of C3H mice, it does have stimulatory effect on osteoblast differentiation in the available osteoprogenitors. One must note again the differences in our protocol for CFU-OB compared to that of the Giuliani group, which may have contributed to the differences in our results. In their study ALN was administered in vitro, and CFU-OB formation was determined by counting mineralized nodules positive for Alizarin Red S staining after 21 days of incubation using the same growth medium as their CFU-F assay. In our study, the ALN was given to the animal and CFU-OB was determined after 14 days of incubation, using growth medium containing dexamethasone and ascorbic acid to promote the differentiation of osteoblast precursors, and staining for both ALP+ colonies and total colonies.

Fluoride, known for its anabolic actions on bone, has been suggested by many to have anabolic effects on osteoblasts through undetermined mechanism.(33,37,38) Fluoride dose-dependent increases in serum ALP activity, in B6 mice, were observed by Yan *et al.*(43). It was suggested that genetic background may have played a role in fluoride's effects on bone biology. Fluoride treatment of 50ppm in the drinking

water would raise the serum F concentration to a physiologically relevant level of approximately 6-10µM/L(43,64). Our results failed to show fluoride's anabolic effect on the osteoprogenitors in the bone marrow. In fact, without the influence of ALN treatment, no significant change was observed in the number of CFU-F between groups treated with and without fluoride for BALB-c and FVB mice. There was also no difference in number of CFU-OB/ALP+, CFU-OB/total and ratio of ALP+/total CFU-OB in B6, BALB-c and FVB mice treated with and without fluoride. However, 50ppm of fluoride had the most pronounced effect on the C3H strain by reducing the frequencies of CFU-F, CFU-OB/ALP+, and CFU-OB/total; despite the lack of change in the ratio of ALP+/total CFU-OB compared to the control. B6 was the only other strain that responded to F with a significant reduction in CFU-F. This variation in responses from the different strains of mice suggests that genetic background influences fluoride's effect on osteoblast differentiation. Our result also suggest that fluoride's anabolic effect on osteoblast does not involve increasing the number of osteoprogenitor cells in the bone marrow, nor does fluoride, under our experimental conditions, promote osteoblast differentiation.

As noted above, only few strains having significant difference between treatment and control group when treated with only one reagent, bisphosphonate or fluoride

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alone. Since both bisphosphonate and fluoride act on the same target site in the body, we were interested in seeing whether or not there is a combined effect on the The results from CFU-F assays showed that when [F] osteoblast precursors. remained constant at 50ppm, no significant change was observed in the colony number between groups receiving different levels of ALN. Similarly, comparisons between groups receiving the same ALN dose, except ALN=0, treated with and without fluoride, no significant difference in CFU-F was noted. Similar results were observed with the CFU-OB assays. No significant difference in ALP+ CFU-OB, total number of CFU-OB, or ratio of ALP+/total CFU-OB was observed between groups receiving the same ALN dose, not including the groups receiving no ALN, but treated with and without fluoride; the same goes for groups treated with 50ppm of fluoride, but receiving different doses of ALN. The only exceptions being B6 mice, which showed a significant decrease in number of ALP+ CFU-OB and total CFU-OB when treated with a combination of 50ppm of fluoride and 150µg/kg/week of ALN. However, for this group of mice, the ratio of ALP+/total CFU-OB was not significantly different from the control. Another exception is the C3H mice, which exhibited significantly higher number of ALP+ CFU-OB when treated with 50ppm of fluoride and 30µg/kg/week of ALN. Nevertheless, this combination of treatments did not change the number of total CFU-OB or the ratio of ALP+/total CFU-OB significantly. Therefore, in general, no significant difference in CFU-F, ALP+ CFU-OB, total number of CFU-OB, or ratio of ALP+/total CFU-OB was noted between treatment groups and control when a combination of the two reagents are administered.

In summary, intrinsic differences between the strains were observed. When ALN or fluoride was administered alone, significant changes in colony counts CFU-F or CFU-OB were observed only in few of the treatment groups. This suggests that these reagents have strain-specific effects in mice. No dose-dependent effect of systemic ALN was observed on the murine bone marrow-derived CFU-F and CFU-OB. Given the relatively small number of strains investigated, it would appear that the systemic ALN has minimal effects on bone marrow MSC pool and no effect on the frequency of osteoblast precursors potential. Furthermore, fluoride does not appear to modulate the effect of ALN on the MSC pool when genetic background is a factor.

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