Vitamin D strongly influences skeletal metastasis development in breast cancer: comparison of systemic vitamin D deficiency versus local ablation of CYP27B1 in breast tumour cells

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Abstract

Vitamin D is very well known for its classical role in the maintenance of calcium and phosphorus homeostasis as well as in the prevention of rickets. More recent findings of its ability to inhibit cell proliferation, induce apoptosis, induce differentiation, inhibit angiogenesis, and modulate the immune system have made it a current topic of intense research, particularly in the field of cancer research. We used a murine model of breast cancer metastasis to bone to investigate the effect of vitamin D deficiency on the growth of breast cancer tumour cells within bone. We also established that these breast cancer tumour cells express the enzyme CYP27B1 (1α-hydroxylase) which is able to convert the inactive vitamin D precursor 25-hydroxyvitamin D (25(OH)D) to the active metabolite 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). We next examined the effect of the local activation of vitamin D by tumoral CYP27B1 on the growth of these tumour cells within bone. Although we did not see a significant difference in the growth of breast cancer tumour cells in the bones of vitamin D deficient mice as compared to vitamin D sufficient mice, we have demonstrated that breast cancer tumour cells that do not express CYP27B1 grow much more aggressively within bone than breast cancer tumour cells which express CYP27B1. This suggests a very important role for the local activation of vitamin D by extra-renal CYP27B1 on the growth of breast cancer tumour cells within the bone microenvironment. These findings suggest a potential use for 25(OH)D as a treatment for breast cancer metastasis to bone either alone or in combination.

Résumé

La vitamine D est bien connue pour son rôle dans le maintien des concentrations de calcium et du phosphore dans la circulation ainsi que dans la prévention du rachitisme. La découverte plus récente de sa capacité d'inhiber la prolifération cellulaire, induire leur différentiation ainsi que l'apoptose cellulaire, inhiber l'angiogenèse, et moduler le système immunitaire rend son étude un sujet de recherche très intéressant surtout dans le domaine de la recherche sur le cancer. Nous avons étudié l'effet de la carence en vitamine D sur la croissance tumorale dans un modèle murin de métastases osseuses du cancer du sein. Nous avons aussi établi que ces cellules expriment l'enzyme CYP27B1 (1α-hydroxylase) et sont donc capables d'activer la vitamine D en son métabolite actif la 1,25-dihydroxyvitamine D ($1,25(OH)_2D$) à partir du métabolite inactif, la 25-hydroxyvitamine D (25(OH)D). Nous avons ensuite examiné l'effet de l'activation locale de la vitamine D par les cellules tumorales dérivées du sein sur la croissance de ces cellules dans le microenvironnement osseux. Nous n'avons constaté aucune différence significative entre la croissance des cellules tumorales du cancer du sein dans l'os chez les souris carencées en vitamine D en comparaison aux souris non carencées en vitamine D. Cependant, nous avons démontré que les cellules tumorales du cancer du sein qui expriment le CYP27B1 croissent beaucoup moins vite dans l'os que les cellules tumorales qui n'expriment pas le CYP27B1. Ces résultats suggèrent un rôle très important de l'activation extra-rénale de la vitamine D par les cellules tumorales du cancer du sein pour inhiber la croissance de ces cellules dans l'os. En conclusion, ces travaux indiquent que le précurseur inactif 25(OH)D pourrait être utilisé seul ou en combinaison pour le traitement des métastases osseuses du cancer du sein.

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Abbreviations

$1,23,25(OH)_3$-25-oxo-D_3	1,23,25-trihydroxy-25-oxo-vitamin D_3
$1,24,25(OH)_3D_3$	1,24,25-trihydroxyvitamin D_3
$1,25(OH)_2$-24-oxo-D_3	1,25-dihydroxy-24-oxo-vitamin D_3
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
1α,$25D_3$-MARRS	1α,25-dihydroxyvitamin D_3-membrane-associated rapid-response steroid binding protein
$24,25(OH)_2D_3$	24,25-dihydroxyvitamin D_3
25(OH)D	25-hydroxyvitamin D
ADP	Adenosine diphosphate
AF-1	Activation function-1
AF-2	Activation function-2
Bcl-2	B-cell lymphoma 2
BMU	Basic multicellular unit
cAMP	Cyclic adenosine monophosphate
CASR	Calcium-sensing receptor
CDK	Cyclin-dependent kinase
DBP	Vitamin D binding protein
DMBA	7,12-dimethylbenzene(a)anthracene
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
EDTA	Ethlylenediaminetetraacetic acid
EGF	Epidermal growth factor
ER	Estrogen receptor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor

H&E	Hematoxylin and eosin
HIF1α	Hypoxia-inducible factor 1α
IGF	Insulin-like growth factor
IL	Interleukin
IU	International units
JNK	c-Jun N-terminal kinase
Kg	Kilogram
KO	Knockout
LBD	Ligand binding domain
LPA	Lysophosphatidic acid
MCP-1	Monocyte chemotactic protein-1
M-CSF	Monocyte/macrophage colony-stimulating factor
memVDR	Membrane vitamin D receptor
MIN	Mammary intraepithelial neoplasia
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumour virus
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
nM	Nanomolar
NMU	N-nitroso-N-methylurea
OPN	Osteopontin
OPG	Osteoprotegerin
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositide 3-kinase
PKC	Protein kinase C

PSA	Prostate-specific antigen
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
PyMT	Polyoma middle T oncoprotein
RANK	Receptor activator of NF-κB
RANKL	Receptor activator of NF-κB ligand
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute media
RXR	Retinoid X receptor
TNF	Tumour necrosis factor
TRPV6	Transient receptor potential cation channel, subfamily V, member 6
μCT	Micro-computed tomography
μL	Microliter
μPA	Serine protease urokinase
VDR	Vitamin D receptor
VDRE	Vitamin D response element
VEGF	Vascular endothelial growth factor
WT	Wild type

I. Literature Review

Introduction

In the 1600s it was observed that many children who lived in the industrialized cities of Europe developed rickets, which was characterized by severe bone deformity, growth retardation and weak muscles [1]. It was later recognized that exposure to sunlight could prevent and even cure rickets, and sunbaths were encouraged to prevent this condition [2]. In 1918 cod liver oil was used to prevent rickets in puppies and it was determined that there was a nutritional factor in the cod liver oil responsible for this effect [2]. This factor was later named vitamin D and has since been well characterized. Although vitamin D is now well known for its role in calcium and phosphorus homeostasis, it also has many other interesting functions which make it a topic of intense research.

Sources and metabolism of vitamin D

Vitamin D can be obtained by humans either from the diet in the form of vitamin D_2 or from exposure of the skin to ultraviolet light in the form of vitamin D_3. Certain foods such as salmon, shiitake mushrooms, and egg yolk naturally contain vitamin D, and other foods such as milk, butter, and yogurt are fortified with vitamin D in certain countries [3]. Vitamin D_2 and vitamin D_3 supplements are also widely available sources of vitamin D. Both vitamin D_2 and vitamin D_3 will be referred to as vitamin D unless otherwise specified. As vitamin D is fat-soluble, it is absorbed in the small intestine along with other dietary fats. Vitamin D is then incorporated into chylomicrons and is transported to the circulation via the lymphatic system [2].

The main natural source of vitamin D_3 for humans is through synthesis within the skin although vitamin D_3 supplements are now readily available. When exposed to ultraviolet B light with wavelengths between 270-300 nm, 7-dehydrocholesterol within the epidermis is converted to previtamin D_3 [4]. The molecular instability of previtamin D_3 leads to its conversion to vitamin D_3 in a heat dependent reaction [4]. Although UVB radiation is able to convert 7-dehydrocholesterol to previtamin D_3, there have been no reported cases of vitamin D intoxication due to excess sunlight exposure. This is because

previtamin D_3 formed in the skin can either isomerize to vitamin D_3 or can absorb UVB radiation to form the inactive isomers lumisterol and tachysterol [5]. Also, the vitamin D_3 produced in the skin can either be absorbed into the bloodstream or can absorb UVB radiation to form other isomers including suprasterol I, suprasterol II, and 5,6-transvitamin D_3 [6]. Therefore, sunlight itself is able to regulate the amount of active vitamin D_3 which is produced within the skin.

The vitamin D_3 synthesized in the skin leaves the keratinocyte and enters the bloodstream where it is bound to the vitamin D binding protein (DBP). Vitamin D is then either deposited within the adipose tissue where it can be stored for months, or it is transported to the liver for the first step of activation [7]. Within the liver, vitamin D is 25-hydroxylated by the enzyme 25-hydroxylase. Although the liver is the main site of 25-hydroxylation of vitamin D, other sites such as the intestine and kidneys have been shown to have 25-hydroxylase activity [8]. Although not the main active metabolite of vitamin D, 25-hydroxyvitamin D (25(OH)D) is the main circulating form of vitamin D and therefore plasma 25(OH)D levels are often used as an indicator of vitamin D status. Normal serum levels of 25(OH)D in humans can range from 25 to 200 nmol/L and this metabolite has a half-life of approximately 15 days [9]. 25(OH)D is also bound to DBP when in the circulation. In order to be activated, 25(OH)D requires one more hydroxylation reaction. The main site for this 1α-hydroxylation is in the kidneys [10], however many other tissues including the breast, prostate, colon, lung, among others have been shown to express 1α-hydroxylase activity [11]. In order to be activated within the kidneys, 25(OH)D must first be transported into the renal tubule cell by binding to megalin, a protein which facilitates endocytosis, on the plasma membrane of the cell [12]. Once in the cells of the proximal convoluted tubule, the enzyme 1α-hydroxylase hydroxylates 25(OH)D into the activate metabolite 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) [13]. This last activation step is tightly regulated within the kidneys. Normal serum levels of $1,25(OH)_2D$ fall between 75 and 200 pmol/L, and this metabolite has a half-life of 10-24 hours [14]. $1,25(OH)_2D$ is also bound to the DBP when in the circulation. The vitamin D binding protein has equal binding affinity for 25(OH)D and $24,25(OH)_2D$, has less affinity for $1,25(OH)_2D$, and has the least affinity for vitamin D [15].

The enzyme responsible for the inactivation of $1,25(OH)_2D$ is 24-hydroxylase which is found in vitamin D target tissues. 24-hydroxylase is able to catalyze a series of reactions at carbons 23 and 24, which lead to the inactivation of vitamin D and eventually lead to the formation of calcitroic acid which is excreted in the bile [16, 17].

25-hydroxylase enzymes

Researchers have sought to identify the main enzyme or enzymes responsible for 25-hydroxylation of vitamin D within the liver. Many enzymes have been discovered which are capable of 25-hydroxylation of vitamin D including CYP2R1, CYP27A1, CYP3A4, CYP2C11, CYP2J2, and CYP2J3. Although these cytochrome P-450s are all able to 25-hydroxylate vitamin D, some are better candidates than others for the main role of vitamin D-25-hydroxylase in humans.

i) CYP2R1 is the most likely candidate as the main 25-hydroxylase responsible for 25-hydroxylation of vitamin D in the human liver. CYP2R1 is a microsomal cytochrome P-450, with a size of 501 amino acids, found in humans as well as at least 47 other species. It is found mainly in the liver and testes [18], and is able to 25-hydroxylate both vitamin D_2 and vitamin D_3 equally [19]. It has been shown that CYP2R1 shows high regioselectivity to the C-25 position of vitamin D, and does not metabolize $25(OH)D_3$, 7-dehydrocholesterol, or cholesterol [20]. Furthermore, mutations in this gene have been found in an individual with low 25(OH)D levels and rickets [21]. All of this data combined provides strong support for CYP2R1 as the principal enzyme responsible for 25-hydroxylation of vitamin D at physiological conditions.

ii) CYP27A1 is a mitochondrial cytochrome P-450 found in the liver, with a size of 531 amino acids, which is found in humans as well as at least 56 other species. Although CYP27A1 was found to 25-hydroxylate vitamin D_3, this enzyme is not specific to vitamin D_3 25-hydroxylation [22]. It is thought to be a high capacity version of 25-hydroxylase with low affinity for vitamin D_3. Furthermore, CYP27A1 seems to be incapable of 25-hydroxylation of vitamin D_2 [22], and therefore cannot explain the metabolism of vitamin D_2 to

25(OH)D_2 within humans. When a CYP27A1 knockout mouse was generated, it was found that the mouse had disrupted bile acid synthesis, but normal serum levels of vitamin D metabolites [23]. Finally, individuals with mutations of the CYP27A1 gene have disrupted bile acid synthesis, but do not express rickets as would be expected with disrupted 25-hydroxylation of vitamin D [24]. Altogether this data suggests that although CYP27A1 is capable of 25-hydroxylation of vitamin D_3, it is not the main enzyme responsible for this step of vitamin D activation and rather plays a more important role in bile acid formation.

iii) CYP3A4 is a microsomal cytochrome P-450 found in human liver and intestinal tissue. Although it is capable of 25-hydroxylation of vitamin D_2, it is not able to 25-hydroxylate vitamin D_3 efficiently [25]. This inability to 25-hydroxylate vitamin D_3 suggests that this enzyme does not play a significant role in human vitamin D activation. This enzyme is of interest however because it has been shown to be induced by 1α,25(OH)$_2D_3$ in the intestine [26].

iv) CYP2C11 is a microsomal cytochrome P-450 which is capable of 25-hydroxylation of both vitamin D_2 and vitamin D_3, however it is only expressed in male rats [27], not in humans [28].

v) CYP2J2 is a human microsomal cytochrome P-450 which is found in the liver and is capable of 25-hydroxylating both vitamin D_2 and vitamin D_3, however it hydroxylates vitamin D_2 more efficiently than vitamin D_3 [29]. CYP2J3 is the equivalent cytochrome P-450 found in the rat and it hydroxylates vitamin D_3 more efficiently than vitamin D_2 [29].

1α-hydroxylase

The gene encoding the 1α-hydroxylase enzyme which is responsible for the activation of 25(OH)D to 1,25(OH)$_2$D is CYP27B1 [30]. Although the kidneys are the major site of activation of 1,25(OH)$_2$D [10], CYP27B1 has also been shown to be expressed in extra-renal tissues such as breast, colon, brain, skin, lung, and parathyroid cells [11, 31], suggesting that vitamin D may also have a paracrine or autocrine function within these

tissues. Of interest is the finding that extrarenal CYP27B1 expression is upregulated in certain cancers including breast cancer [32] and prostate cancer [33].

Human CYP27B1 is a 507 amino-acid protein which is able to 1α-hydroxylate both $25(OH)D_2$ and $25(OH)D_3$ efficiently. Individuals affected by vitamin D-dependent rickets type 1 have been found to have a mutation in the CYP27B1 gene [30]. This is an autosomal recessive condition, and therefore to be affected by the disease both alleles must be mutated or be compound heterozygotes [34]. Due to their inability to 1α-hydroxylate 25(OH)D, these patients have low or absent levels of $1,25(OH)_2D_3$ in the serum and therefore display symptoms of rickets despite the fact that they may have normal or elevated levels of 25(OH)D in the circulation. These patients respond well to treatment with $1,25(OH)_2D$ [35].

In CYP27B1 knockout mice, it has been found that they develop phenotypes very similar to individuals with vitamin D-dependent rickets type 1. These mice have low serum $1,25(OH)_2D$ and have elevated 25(OH)D levels, suggesting that CYP27B1 is indeed the enzyme responsible for the 1α-hydroxylation of 25(OH)D [36]. This rachitic phenotype of CYP27B1 knockout mice is rescued by feeding the mice either a high calcium diet or a diet containing $1,25(OH)_2D_3$ [37, 38].

1α-hydroxylase expression within the kidneys is tightly regulated to ensure that the proper amount of active $1,25(OH)_2D_3$ is maintained within the circulation in order to maintain stable serum calcium levels. There are many factors which regulate the expression of CYP27B1 within the kidneys. One factor that directly effects the renal expression of CYP27B1 is the parathyroid hormone (PTH). PTH is able to stimulate the activity of the 1α-hydroxylase enzyme [39] and transcription of the 1α-hydroxylase gene through cAMP by acting on its promoter [40]. Serum calcium levels also regulate the expression of CYP27B1 by the kidneys. Hypocalcemia can stimulate 1α-hydroxylase activity either indirectly by increasing PTH levels [41] or directly as has been shown in animals with parathyroidectomy on a hypocalcemic diet [42]. Conversely, hypercalcemia inhibits 1α-hydroxylase activity [43]. Serum phosphate levels also modulate 1α-hydroxylase expression indirectly through fibroblast growth factor 23 (FGF 23). FGF 23 acts to decrease phosphate levels and therefore inhibits CYP27B1 expression within the

kidneys [44]. Finally, $1,25(OH)_2D_3$ directly suppresses CYP27B1 expression [45] and also indirectly suppresses CYP27B1 expression due to its effect in increasing serum calcium and phosphate, as well as inhibiting PTH.

Although the renal CYP27B1 expression is tightly regulated, this is not necessarily the case for extra-renal 1α-hydroxylase expression. Extra-renal regulation of CYP27B1 expression in both normal and cancerous tissue is very different than the regulation in the kidneys and can vary depending on the tissue. For instance, it has been shown that in the prostate CYP27B1 expression is not regulated by PTH or calcium in contrast to the regulation in the kidneys [46]. Furthermore, CYP27B1 expression in extra-renal tissues is substrate dependent whereas in the kidneys this is not the case [47].

24-Hydroxylase

The gene encoding the 24-hydroxylase enzyme which is responsible for the catabolism of $1,25(OH)_2D$ and 25(OH)D is CYP24A1. CYP24A1 expression is upregulated by $1,25(OH)_2D$ as it contains two positive vitamin D response elements (VDREs) in its promoter region [48]. As CYP24A1 is expressed in the kidneys as well as in all vitamin D target cells, exposure of these cells to $1,25(OH)_2D_3$ leads to a 10- to 100-fold increase of the expression of CYP24A1 within these cells [49]. Furthermore, renal CYP24A1 expression is upregulated by phosphate [50] and downregulated by PTH [51].

CYP24A1 is responsible for the conversion of $1,25(OH)_2D_3$ to calcitroic acid for excretion in bile. This is a five step process in which $1,25(OH)_2D_3$ is first converted to $1,24,25(OH)_3D_3$ then to $1,25(OH)_2$-24-oxo-D_3, next to 25-oxo-$1,23,25(OH)_3D_3$ before finally being converted to calcitroic acid for excretion [7]. CYP24A1 knockout mice have reduced viability due to hypercalcemia and renal calcification, and elevated serum $1,25(OH)_2D_3$ levels due to their inability to inactivate this hormone [52].

Although all vitamin D target cells normally express CYP24A1, it has been shown to be upregulated or downregulated in certain cancerous cell types. CYP24A1 expression has been shown in some studies to be upregulated in certain cancers including colon cancer [53], ovarian cancer [53], breast cancer [54], and lung cancer [53]. However, other studies

have shown downregulation of CYP24A1 expression in certain human tumours including breast cancer [53]. The metabolism of vitamin D is summarized in Figure 1.

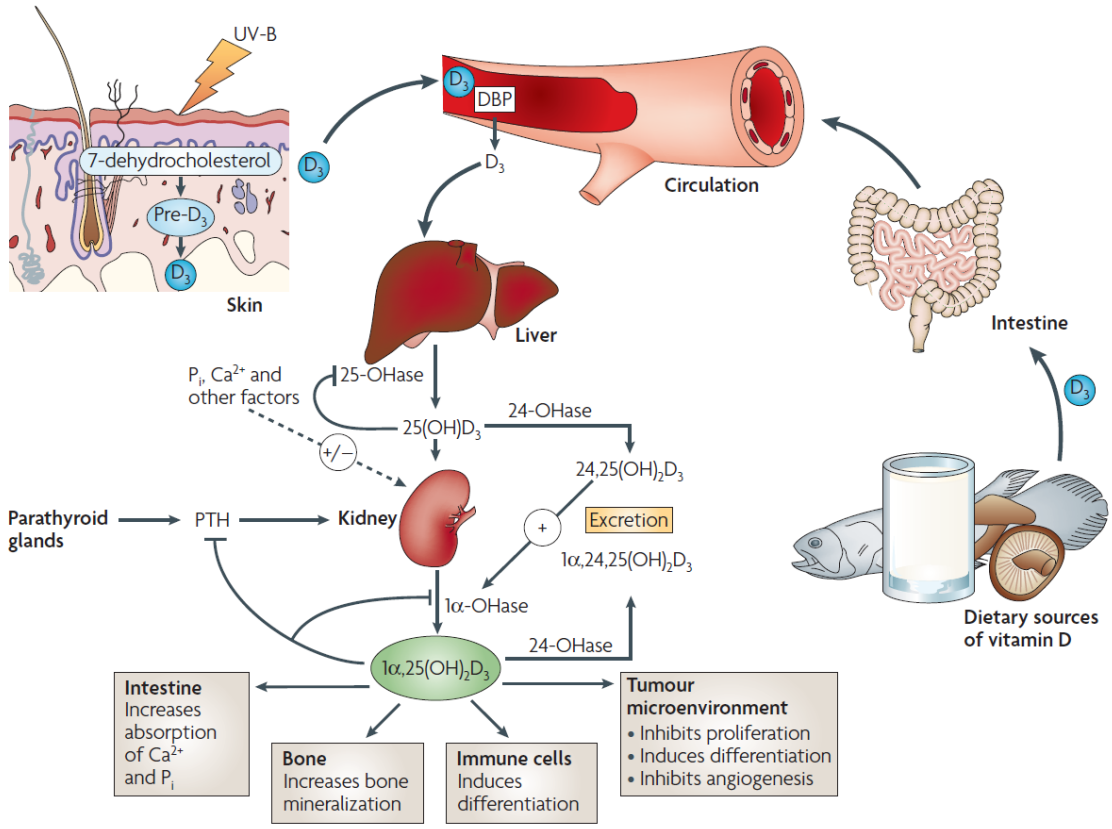

Figure 1 – Vitamin D metabolism. Vitamin D can be obtained either from the diet or from UVB conversion of 7-dehydrocholesterol to previtamin D_3 and vitamin D_3 in the skin. The vitamin D is then transported in the bloodstream bound to the vitamin D binding protein (DBP) to the liver where it is 25-hydroxylated by the enzyme 25-hydroxylase to form 25-hydroxyvitamin D (25(OH)D). The 25(OH)D then travels to the kidneys, once again bound to DBP where it is 1α-hydroxylated by the enzyme 1α-hydroxylase to the active vitamin D metabolite $1,25(OH)_2D$. The $1,25(OH)_2D$ is then transported to target tissues including the intestine, bone, immune cells, and the tumour microenvironment and can carry out its function. Within the target tissues, the 24-hydroxylase enzyme 24-hydroxylates $1,25(OH)_2D_3$ to an inactive excretion product. 24-hydroxylase can also hydroxylate 25(OH)D to 24,25(OH)2D. Factors regulating 1α-hydroxylase activity within the kidneys include phosphorus, calcium, parathyroid hormone (PTH), $1,25(OH)_2D$, and $24,25(OH)_2D_3$. 25(OH)D can inhibit the 25-hydroxylase enzyme.

Mechanism of genomic actions of vitamin D

In order to have an effect on the rate of transcription of specific target genes, $1,25(OH)_2D$ must first bind to the vitamin D receptor (VDR). The VDR belongs to the nuclear receptor superfamily, and is a ligand-induced transcription factor. The gene encoding the human vitamin D receptor is found on chromosome 12q. [55] The VDR protein can be divided by function into three regions. At the amino-terminal of the VDR there is a ligand-independent transactivation function called activation function-1 (AF-1). The DNA binding domain is found in the middle region of the VDR. The C-terminal region of the VDR contains the ligand binding domain (LBD), the RXR heterodimerization domain, and a ligand-dependent transactivation function, AF-2. [56]

When $1,25(OH)_2D$ binds to the VDR, the ligand-bound VDR heterodimerizes with the retinoid X receptor (RXR) [57] and this complex is translocated into the nucleus [58] where it can bind to the vitamin D response element (VDRE) in the promoter region of target genes [59]. Through the help of corepressors and coactivators, this complex is then able to alter the rate of transcription of target genes.

Nongenomic actions of vitamin D

Some actions of vitamin D occur much too rapidly to be a result of transcriptional regulation. One known nongenomic action of $1,25(OH)_2D_3$ is the fast intestinal absorption of calcium [60]. Although the exact mechanism of these non-genomic actions is not known, they are thought to begin with $1,25(OH)_2D_3$ binding to a membrane receptor (memVDR) [61] with the involvement of a $1\alpha,15(OH)_2D_3$-membrane-associated rapid-response steroid binding protein ($1\alpha,25D_3$-MARRS) [62].

Classical role of vitamin D in bone health

Vitamin D is most known for its classical role in the maintenance of calcium and phosphate homeostasis necessary for normal bone health, as seen in Figure 2.

Calcium homeostasis

The maintenance of normal serum calcium levels is necessary for many of the body's normal processes such as muscle contraction, neural transmission and the maintenance of healthy mineralized bone. In fact, calcium is very tightly regulated and maintained at serum levels of 2.17-2.52 mmol/L of total calcium or 1.12-1.32 mmol/L of ionized calcium [63]. In order to maintain the serum level of calcium $1,25(OH)_2D_3$ and PTH work together to maintain calcium homeostasis by acting on either bone, the intestines, or the kidneys.

When serum calcium levels drop, the calcium-sensing receptors of the parathyroid glands sense the decrease and cause the parathyroid glands to secrete PTH, an 84-amino acid hormone with a half-life of mere minutes [64]. PTH then acts on the osteoblasts and the proximal convoluted tubules of the kidneys. PTH induces the osteoblast to express receptor activator of NF-κB ligand (RANKL) [65]. RANKL on the osteoblast then binds either to osteoprotegerin (OPG), a decoy receptor, or to receptor activator of NF-κB (RANK) on preosteoclasts. When RANKL binds to RANK on the preosteoclasts, it triggers the activation of the κB nuclear factor which causes the maturation of the osteoclast [66]. These mature osteoclast are then able to resorb bone releasing the stored calcium and thereby increasing the calcium levels within the circulation. This mobilization of calcium from the bone requires both PTH and $1,25(OH)_2D$. PTH alone has a reduced ability to induce mobilization of calcium from calcium-abundant bone in vitamin D deficient animals as compared to vitamin D sufficient animals [67].

PTH is also able to act on the nephron of the kidney. Within the proximal convoluted tubules of the kidneys, PTH is able to induce the 1α-hydroxylase gene, which is responsible for the activation of 25(OH)D to $1,25(OH)_2D$, in a cAMP dependent mechanism [68]. As PTH activates 1α-hydroxylase within the kidney, it also inhibits 24-hydroxylase [69]. This leads to increased plasma levels of $1,25(OH)_2D$. PTH acts on the distal renal tubule to stimulate calcium reabsorption and $1,25(OH)_2D$ also acts to enhance calcium reabsorption in the kidneys[63].

$1,25(OH)_2D$ also regulates calcium homeostasis within the kidneys by regulating its own synthesis. As previously mentioned, $1,25(OH)_2D$ inhibits CYP27B1 within the kidneys and induces CYP24A1 thereby inhibiting its own synthesis and inducing its catabolism. $1,25(OH)_2D$ is also able to inhibit PTH synthesis and parathyroid cell growth [70]. Finally, $1,25(OH)_2D$ helps regulate calcium homeostasis by acting directly on the small intestine. When a person is vitamin D deficient, only 10 to 15% of dietary calcium is absorbed, however, with the presence of $1,25(OH)_2D$ and the VDR within the small intestine, this percentage increases to 30 to 40% [3]. $1,25(OH)_2D$ alone is capable of enhancing intestinal calcium absorption; PTH is not required. $1,25(OH)_2D$ is able to enhance intestinal calcium absorption by upregulating the expression of TRPV6, an epithelial calcium channel, and calbindin 9K, a calcium-binding protein [71].

Once the plasma calcium levels return to normal, the calcium-sensing receptor no longer induces the secretion of PTH by the parathyroid glands, leading to negative feedback regulation.

Phosphate homeostasis

Maintaining appropriate phosphate levels within plasma is necessary for proper mineralization of bone. $1,25(OH)_2D$ works in concert with fibroblast growth factor 23 (FGF23) and PTH to maintain serum phosphate levels within the normal range. When plasma phosphate levels are elevated, FGF23 is secreted mainly by the osteocytes in bone [72]. The secreted FGF23 has several effects that cause a lowering of serum phosphate levels. FGF23 has a direct effect on the proximal tubules of the kidneys by downregulating the expression of the sodium phosphate cotransporters, thereby decreasing the amount of phosphate which is reabsorbed by the kidneys [73]. FGF23 also inhibits the expression of CYP27B1 within the kidneys and induces the expression of CYP24A1 resulting in decreased circulating levels of $1,25(OH)_2D$ [44]. Furthermore, FGF23 inhibits PTH secretion by the parathyroid glands [74]. Overall, FGF23 acts to reduce circulating phosphate levels.

In contrast, $1,25(OH)_2D$ acts to increase circulating phosphate levels. $1,25(OH)_2D_3$ induces the expression of FGF23, and supresses PTH [73]. $1,25(OH)_2D$ directly increases

plasma phosphate levels by acting on osteoblasts, inducing them to express RANKL which binds to RANK on preosteoclasts inducing their maturation which leads to bone resorption and the release of phosphate stores from bone [73]. $1,25(OH)_2D$ also directly increases circulating phosphate levels by inducing the expression of the sodium phosphate cotransporters in the intestine which increases intestinal absorption of phosphate. [73]

PTH acts to lower circulating phosphate levels. PTH directly lowers circulating phosphate levels by supressing the sodium phosphate cotransporters in the proximal tubules thereby inhibiting phosphate reabsorption by the kidneys [73]. This hormone also induces CYP27B1 expression within the kidneys [68] and inhibits CYP24A1 expression [69] leading to an increase in $1,25(OH)_2D$ activation. This increase in $1,25(OH)_2D$ production indirectly leads to an increase in PTH levels.

PTH directly lowers circulating phosphate levels by supressing the sodium phosphate cotransporters in the proximal tubules thereby inhibiting phosphate reabsorption by the kidneys [73].

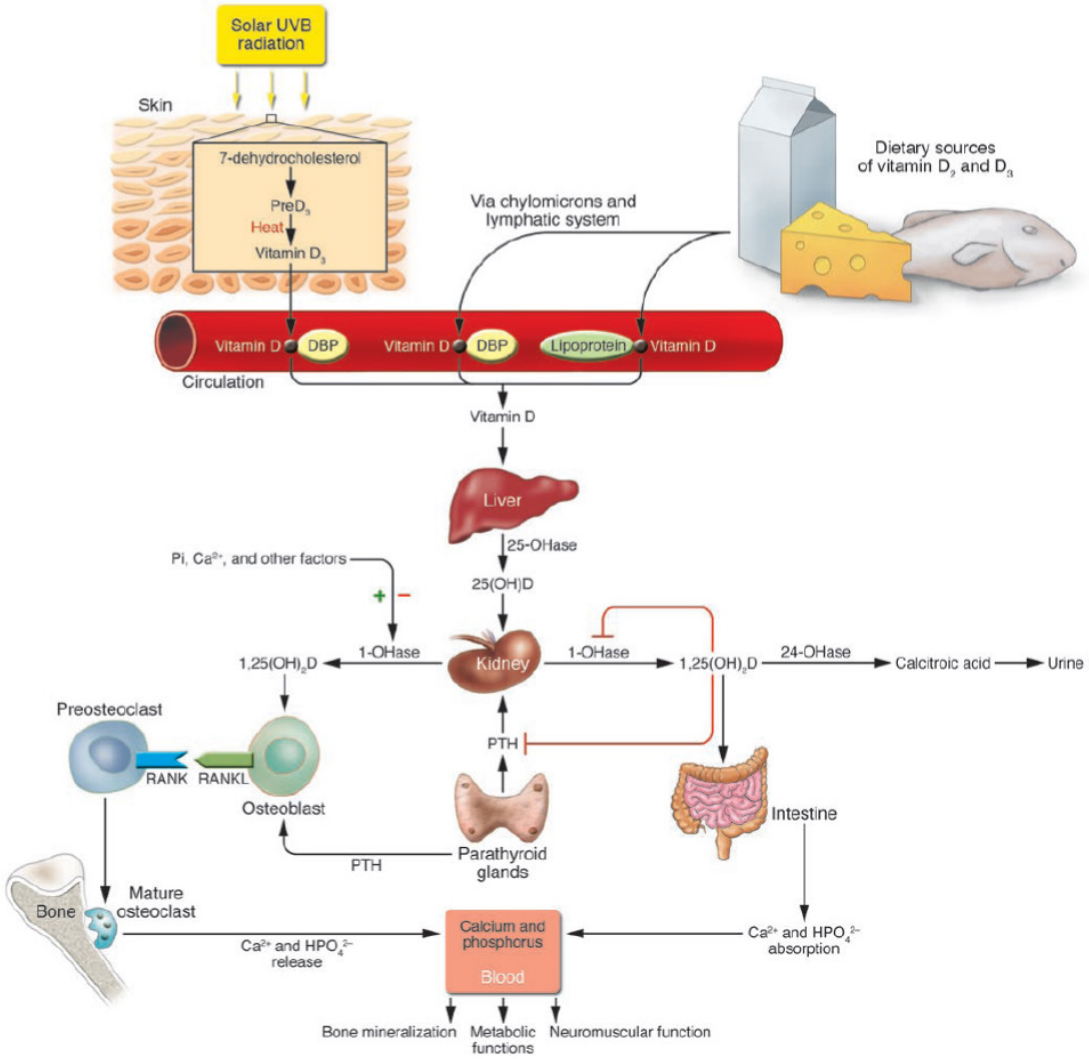

Figure 2 – Calcium and phosphorus homeostasis. After activation, $1,25(OH)_2D$ acts on osteoblasts, inducing them to express RANKL which binds to its receptor RANK on preosteoclasts. This induces them to mature to active osteoclasts which can then resorb bone releasing stored calcium and phosphorus. PTH also induces RANKL expression by osteoblasts. $1,25(OH)_2D$ can also act directly on the intestine to increase the absorption of calcium and phosphate. Both $1,25(OH)_2D$ and PTH can act together to increase the reabsorption of calcium and phosphorus from the kidneys although the action of PTH predominates in the kidneys. $1,25(OH)_2D$ inhibits 1α-hydroxylase and PTH. Within the kidneys, 1α-hydroxylase activity is regulated by PTH, calcium, and phosphorus.

Non-classical roles of vitamin D

Although vitamin D was first recognized for its role in maintaining proper bone health, it has also been shown to play a role in many other processes such as the control of cell proliferation, cell differentiation, apoptosis, angiogenesis, and modulation of the immune system.

i) Inhibition of cell proliferation

$1,25(OH)_2D$ has been shown to inhibit the proliferation of many normal and cancerous cell types through several mechanisms. In normal cells, $1,25(OH)_2D$ is able to regulate the cell cycle by increasing the expression of p21, p27, and p53 [59]. p21, p27, and p53 are cyclin-dependent kinase (CDK) inhibitors. CDKs are responsible to ensure the phosphorylation of proteins involved in the progression of the cell cycle. Therefore, by inducing the expression of CDK inhibitors, $1,25(OH)_2D$ is able to induce cell cycle arrest in normal cells.

In prostate cancer cells, $1,25(OH)_2D$ has been shown to inhibit proliferation through cell cycle arrest in the G_1/G_0 phase [75]. This mechanism is dependent on p53 [76] which ultimately causes hyperphosphorylation of the retinoblastoma protein [77], increases the expression of $p21^{Waf/Cip1}$ [78] and $p27^{Kip1}$ [75], and decreases the activity of cyclin-dependent kinase 2 (CDK2) [75].

Similarly, it has been shown in MCF-7 breast cancer cells that treatment with $1,25(OH)_2D$ increases the expression of p27 and inhibits cyclin D1 thereby reducing CDK activity and inducing hypophosphorylation of the retinoblastoma protein [79]. Furthermore, in MCF-7 cells, inhibition of cell proliferation by $1,25(OH)_2D$ was found to be associated to the ability of $1,25(OH)_2D$ to increase BRCA 1 gene expression [80].

In a model of colon cancer, it has been suggested that Wnt-ß-catenin-TCF4 may play a role in the antiproliferative role of vitamin D. $1,25(OH)_2D$ signalling has been shown to cause nuclear ß-catenin to translocate to the plasma membrane, which removes the ß-catenin-TCF4 transcription signal from the nucleus [81].

In bovine parathyroid cells in culture, $1,25(OH)_2D_3$ inhibits proliferation and the proto-oncogene c-myc [82]. Similarly, in normal and transformed keratinocytes,

$1,25(OH)_2D_3$ inhibits proliferation through cell cycle arrest in the G_1/G_0 phase while inhibiting c-myc [83, 84]. Although the antiproliferative effects of $1,25(OH)_2D_3$ have been studied in many types of tumour cells, the mechanism of action by which $1,25(OH)_2D$ regulates the cell cycle varies from one cell type to the other.

ii) Induction of cell differentiation

$1,25(OH)_2D$ is able to induce the differentiation of various normal and malignant cells. For instance, $1,25(OH)_2D$ induces the differentiation of various myeloid leukemia cell lines [78, 85]. This differentiation requires the formation of activated VDR and phosphatidylinositol 3-kinase complexes [86]. In HL-60 cells, a human promyelocytic leukemia cell line, $1,25(OH)_2D$ is able to stimulate their terminal differentiation to cells with characteristics of macrophages by inhibiting the expression of the *c-myc* oncogene [87]. In normal human keratinocytes, $1,25(OH)_2D_3$ stimulates differentiation through an induction of the proto-oncogene c-fos [83].

It has been shown in prostate cancer cells and colon cancer cells that $1,25(OH)_2D$ is able to induce the expression of genes that are associated with cell differentiation [88, 89]. In colon cancer cells, differentiation can be induced by $1,25(OH)_2D$ either by increasing PKC- and JNK-dependent JUN activation [90] or by regulating the expression of inhibitor of DNA binding 1 and 2 [91]. In colorectal cancer cells, $1,25(OH)_2D$ is able to induce differentiation by inducing the expression of the CDH1 gene which encodes E-cadherin [81].

iii) Induction and inhibition of apoptosis

Another non-classical role of vitamin D is its ability to induce or inhibit apoptosis in various cells. There are several mechanisms which have been identified by which $1,25(OH)_2D$ is able to induce apoptosis depending on the cell type. In MCF-7 breast cancer cells and in HL-60 leukemia cells, $1,25(OH)_2D$ has been shown to downregulate the expression of BCL2, which encodes Bcl-2, an anti-apoptotic pro-survival protein [92]. In carcinoma cells, prostate cancer cells and colorectal adenoma cells, $1,25(OH)_2D$ is able to upregulate the expression of BAX and BAK which are pro-apoptotic proteins [92]. In MCF-7 cells, $1,25(OH)_2D$

treatment leads to a significant reduction in Akt phosphorylation [93]. The PI3K/AKT/mTOR pathway is an important pathway for apoptosis with overactivation leading to a reduction of apoptosis which allows for cell proliferation [93].

In some cell types, $1,25(OH)_2D$ induction of apoptosis is caspase-dependent such as in non-malignant MCF-12A cells, whereas in malignant MCF-7 cells $1,25(OH)_2D$-induced apoptosis is not dependent on caspase activity [94]. Another mechanism by which $1,25(OH)_2D$ is able to promote apoptosis is by downregulating the mitogenic pathways by reducing IGF receptors and increasing IGF binding proteins [95]. $1,25(OH)_2D$ has also been shown to induce apoptosis by down-regulating telomerase activity which leads to telomere attrition in epithelial ovarian cancer cells [96].

In contrast, in normal human primary osteoblasts isolated from calvariae, $1,25(OH)_2D_3$ inhibits apoptosis through both the mitochondrial and Fas-related pathways [97]

iv) Inhibition of angiogenesis

Vitamin D also has an ability to inhibit angiogenesis by various mechanisms. $1,25(OH)_2D$ has been shown to counteract VEGF-induced endothelial cell proliferation both in vitro and in vivo [98]. $1,25(OH)_2D$ decreases the expression of VEGF, the most potent stimulator of angiogenesis, in several malignant cell lines by reducing the transcription of hypoxia-inducible factor 1 [99]. $1,25(OH)_2D$ is also able to directly inhibit the proliferation of endothelial cells [99].$1,25(OH)_2D$ can also increase mRNA levels of thrombospondin 1, a potent anti-angiogenic factor in human colon tumour cells [91]. $1,25(OH)_2D$ has also been shown to inhibit malignant cell-induced angiogenesis by suppressing the expression of IL-8, a proangiogenic factor, in a NF-κß-dependent mechanism [100].

v) Modulation of the immune system

Vitamin D effects many cells of the immune system. It has been shown to inhibit proliferation of B cells, block the differentiation of B cells, and block immunoglobulin secretion [101, 102]. Vitamin D inhibits the proliferation of T

cells [103], resulting in a shift from Th1 to Th2 cells [104]. This hormone also influences the maturation of T cells to facilitate the induction of T regulatory cells [105]. Vitamin D acts on monocytes to inhibit their production of the inflammatory cytokines IL-1, IL-6, IL-8, IL-12, and TNFα [106]. Lastly, vitamin D prevents the differentiation and maturation of dendritic cells, and preserves an immature phenotype [107].

Breast cancer

According to the Canadian Breast Cancer Foundation, breast cancer was the most common cancer in Canadian women over the age of 20 in 2012 and was the second leading cause of cancer-related death in Canadian women. They have estimated that in 2012 an average of 62 Canadian women were diagnosed with breast cancer every day. The risk of breast cancer increases with age, with 80 percent of the women diagnosed being over the age of 50. Although they have estimated that in 2012 the five-year relative survival rate of breast cancer was 88% for Canadian women, it was estimated that 5200 Canadian women died of the disease. Though advances in screening and early detection have helped to increase the survival rate of patients diagnosed with breast cancer, further research is needed to lower the incidence of breast cancer and to effectively treat the disease in order to eliminate breast cancer related deaths. [108]

Epidemiological link between vitamin D and breast cancer risk

A link between vitamin D status and breast cancer risk was first suggested by investigators looking at sun exposure and breast cancer incidence. Several researchers found an inverse relationship between sun exposure and breast cancer incidence or mortality. One study found that individuals who worked in professions with higher skin cancer rates had lower incidence of other cancers [109] suggesting that UVB exposure, the potential cause of skin cancer, might protect against other cancers. Another study showed that individuals who live farther from the equator and therefore receive less UVB exposure have higher rates of cancer deaths than people who live closer to the equator [110]. Within the United States it has been reported that breast cancer mortality rates are higher in the Northeast than in the South where UVB exposure is higher [111], suggesting

a protective role of solar radiation against breast cancer. It has also been reported that in areas of high solar radiation, women who have regular sunlight exposure and sufficient dietary vitamin D intake have a reduced risk of breast cancer by 25% to 65% [112].

Due to the suggested inverse relationship between solar radiation and breast cancer incidence and mortality, researchers began further investigating the relationship between vitamin D levels and breast cancer. A strong link was found between 25(OH)D levels in the blood and breast cancer risk in a meta-analysis of two correlative studies [113]. This meta-analysis found that 25(OH)D levels of greater than 52 ng/mL was estimated to decrease the risk of breast cancer by 50% as compared to 25(OH)D levels of less than 10 ng/mL [113]. An analysis of the Nurses Health Study reported an inverse relationship between the intake of dairy products, vitamin D and dairy calcium and breast cancer in premenopausal women but not in postmenopausal women [114]. This finding was also supported by a study which found an inverse relationship between vitamin D status and mammographic breast density, a predictor of breast cancer, which was found in premenopausal women but not in postmenopausal women [115]. A hospital-based case-control study which looked at the levels of $1,25(OH)_2D$ in the blood at the time of diagnosis with breast cancer found an inverse relationship between $1,25(OH)_2D$ levels and breast cancer [116].

Although many studies have shown an inverse relationship between breast cancer incidence and mortality and vitamin D levels, some studies fail to demonstrate this association. One study which looked at prediagnostic $1,25(OH)_2D$ levels in 96 postmenopausal women with breast cancer and 96 control postmenopausal women found no association between $1,25(OH)_2D$ levels and later diagnosis of breast cancer [117]. However in this study the levels of $1,25(OH)_2D$ were measured on average 15 years prior to the diagnosis of breast cancer and therefore does not necessarily reflect the $1,25(OH)_2D$ level at the time of diagnosis [117]. Another case control study which looked at vitamin D consumption and breast cancer risk in Canadian women found that women with higher vitamin D consumption were at greater risk of breast cancer than women with a lower vitamin D consumption [118]. A major limitation of this study was that the

investigators did not consider sunlight exposure and therefore did not consider vitamin D activation by UVB radiation.

Experimental evidence linking vitamin D to breast cancer prevention

As described in a previous section, vitamin D has been shown to have many properties which suggest that it could be useful in the prevention or treatment of breast cancer. Vitamin D is able to inhibit cell proliferation, induce differentiation, induce apoptosis, and inhibit angiogenesis. Based on these findings, many researchers have conducted *in vivo* experiments studying the effect of vitamin D on animal models of breast cancer. Many of these experiments have given promising results for the potential use of vitamin D as a treatment for breast cancer.

One mouse model which has been used to study the effect of vitamin D on breast cancer is vitamin D receptor knockout mice. It has been found in these mice that the lack of VDR causes excess branching and proliferation of the breast tissue as compared to wild type mice [119], and impaired apoptosis following lactation as compared to wild type mice with intact VDR [120]. This suggests that vitamin D plays a role in the regulation of normal breast development and that the VDR knockout mice may have increased susceptibility to breast cancer due to this loss of control of breast proliferation. It has been shown that when VDR knockout mice are treated with the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA), they develop more mammary hyperplasia and estrogen receptor (ER) negative tumours than wild type mice [121]. Mouse mammary tumour virus (MMTV)-neu transgenic mice which spontaneously develop breast tumours have also been used to look at the effect of vitamin D on breast cancer. When mice on the MMTV-neu background are heterozygous for VDR, they have been found to develop more tumours than wild type MMTV-neu mice [122].

The use of breast cancer xenograft models has also proven useful to study the effect of vitamin D on breast cancer tumour growth *in vivo*. It has been shown that when ER-positive human breast cancer cells are xenografted in immunocompromised mice, and the mice are treated with $1,25(OH)_2D_3$, the $1,25(OH)_2D_3$, is able to inhibit the growth of the tumours by disrupting estrogen-mediated survival signals and inducing growth arrest of

the cells [123]. When ER-negative human breast cancer cells are xenografted into immunocompromised mice and treated with analogs of $1,25(OH)_2D_3$, the tumour growth is inhibited as compared to control mice by inhibiting proliferation and inducing apoptosis [124]. Furthermore, combination therapy of $1,25(OH)_2D_3$ with aromatase inhibitors has been shown to inhibit the growth of ER positive breast cancer xenografts in mice to a greater extent than treatment with either agent alone [123]. Combination therapy with EB1089, an analog of $1,25(OH)_2D_3$, and all-trans-retinoic acid has also shown greater growth inhibition of human ER-positive breast cancer xenografts than either treatment alone in a mouse model [125]. An analog of $1,25(OH)_2D_3$ has been shown to sensitize xenografted human breast cancer cells in a mouse model to ionizing radiation [126]. An analog of $1,25(OH)_2D_3$ has also been shown to sensitize human breast cancer cells to paclitaxel, a chemotherapeutic agent, in a mouse xenograft model [127].

A potential serious complication of $1,25(OH)_2D_3$ treatment is hypercalcemia. Since both normal and cancerous breast tissue have been shown to express the gene CYP27B1 [32], which encodes the vitamin D activating enzyme 1α-hydroxylase, it has been suggested that treatment with vitamin D rather than $1,25(OH)_2D_3$ may be a better way to treat breast cancer. The rationale is that by increasing the circulating 25(OH)D levels by dietary vitamin D supplementation there would be increased substrate for the breast cancer cells or other target tissues to locally activate 25(OH)D to $1,25(OH)_2D$, which could then act on the cancer cells themselves in a paracrine or autocrine fashion. Since circulating $1,25(OH)_2D$ levels are tightly regulated by the kidneys where most of the activation of 25(OH)D takes place [10], increasing the circulating levels of 25(OH)D does not run as high a risk of inducing hypercalcemia as increasing the circulating levels of $1,25(OH)_2D$. It has been shown that in extra-renal CYP27B1 within cancer cells is not regulated by PTH [46] as it is in the kidneys and therefore the extra-renal activation of 25(OH)D may not be subject to normal feedback regulation.

Some researchers have performed experiments to study the effects of dietary vitamin D intake on breast cancer progression in mice. One study investigated the effect of a high vitamin D_3 diet of 5000 IU/Kg, or a normal vitamin D_3 diet of 1000 IU/Kg on the growth of MCF-7 human breast cancer xenografts in immunocompromised mice [128]. They

found that the mice on the 5000 IU/Kg diet had reduced tumour growth within one week on the diet, and by four weeks the tumours of the mice on the high vitamin D diet were 50% smaller than the mice on the normal vitamin D diet [128]. They also demonstrated that the growth inhibition of the tumours by the dietary vitamin D_3 was equivalent to the tumour cell growth inhibition in mice treated with $1,25(OH)_2D_3$ injections [128]. This study provides strong evidence for the potential use of dietary vitamin D_3 supplements for the treatment of breast cancer.

Clinical trials of vitamin D in breast cancer patients

Although there have been some clinical trials of vitamin D administration in breast cancer patients, they have not produced convincing results. One of the first clinical trials with vitamin D was using the analog calcipotriol [129]. 19 patients with advanced breast cancer participated in the study [129].The calcipotriol was applied topically at a dose of 100 micrograms once a day for 6 weeks [129]. Five of the patients were withdrawn from the trial prior to the end because of hypercalcemia [129]. Of the remaining 14 patients, three had a 50% reduction of the diameters of the lesions, one patient had a minimal response, five had no change and five had cancer progression [129].

A phase 1 clinical trial was conducted to determine the maximal tolerated dose of the $1,25(OH)_2D$ analog EB1089 in 36 patients with advanced breast or colorectal cancer [130]. The EB1089 was given to the 36 patients for five days and 21 patients continued taking the EB1089 as compassionate treatment [130]. All of the patients on the highest dose developed hypercalcemia [130]. Although the drug did not reduce the volume of the tumours in any patients, four of the patients had disease stabilization for over six months [130].

A Phase II clinical trial was conducted on women with metastatic breast cancer where they were given 10 000 IU of vitamin D_3 for four months [131]. They found a small increase in serum calcium and a decrease in PTH, however this high dose of vitamin D appears to be safe for four months [131]. Unfortunately they did not see a significant improvement on the disease progression [131].

To date there have been two randomized clinical trials of vitamin D supplementation and breast cancer risk in postmenopausal women. In the Women's Health Initiative, the participants were given either 400 IU of vitamin D and 1000 mg of calcium or a placebo [132]. This study showed no significant effect of vitamin D supplementation on the risk of breast cancer [132]. This may be because the dose of vitamin D given was too low to have a significant effect. Furthermore, the women were allowed to start taking vitamin D supplements of up to 1000 IU/day during the trial [132]. In the other study 1179 postmenopausal women were given either 1100 IU of vitamin D_3 and calcium, calcium alone, or a placebo [133]. After four years of supplementation this study found a 60% overall reduction in all cancers and a numerical decrease in breast cancer in the group which was given vitamin D_3 and calcium [133].

Overall, the clinical trials looking at vitamin D and breast cancer are inconclusive. They were either performed in the very late stage of the disease when vitamin D supplementation may be too late to be effective, or the dose given was low and serum levels of vitamin D may not have been elevated enough to see the benefits on breast cancer progression. Further studies need to be conducted in order to elucidate the potential therapeutic benefit of vitamin D.

Bone metastasis

A very serious complication of breast cancer as well as other cancers including prostate and lung cancer is metastasis to bone. 65-80% of patients diagnosed with metastatic breast or prostate cancer will develop bone metastases [134, 135]. Once a cancer has metastasized to bone, it is generally considered to be incurable. Although bone metastasis is rarely the cause of death in breast cancer patients, serious complications of bone metastasis include bone fracture, chronic bone pain, spinal cord compression, and hypercalcemia [136]. These complications can seriously affect the quality of life of breast cancer patients.

The process of metastasis inefficient, and requires many steps in order for the successful spread of cancer from the primary tumour to a distant site. As was first recognized by Stephen Paget in 1889, the process of metastasis depends on both the "seed" (tumour

cells) and the "soil" (host tissue) for the establishment of successful metastases [137]. The first step of bone metastasis involves the primary tumour cells invading the surrounding normal tissue by secreting proteolytic enzymes which allows the tumour cells to escape the primary tumour and enter the bloodstream either from small blood vessels in the normal tissue or from blood vessels which were induced to grow by the tumour itself [138]. Once they have entered the circulation, the cancer cells are transported through the bloodstream to various sites throughout the body. At this point, many of the cancer cells which have managed to enter the circulation will not survive due to the normal protective host-surveillance mechanisms [139, 140]. The tumour cells that do survive will have a tendency to lodge in certain tissues according to the blood flow through the target tissue, the distance from the primary tumour, and the properties of the vasculature [141]. Chemoattractant factors which can be released into the blood vessels by target tissues can help determine where a metastasis develops [142] as can the presence of adhesion molecules on the inside of the blood vessel surface [143]. In the case of bone, it is possible that increased bone resorption could increase the likelihood of developing bone metastases because increased bone resorption has been associated with angiogenesis, especially in the growth plate. Furthermore, the increased bone turnover causes the release of growth factors and calcium from bone, which could also enable the localization of tumour cells to bone. [141] In order to establish metastatic growth in bone, the cancer cells must next be able to extravasate out of the blood vessel by migrating across the sinusoidal wall, they must invade the marrow stroma, and finally induce angiogenesis in order to ensure adequate blood supply for tumour survival and growth [134].

Once tumour growth has been established within the bone environment, either osteolytic or osteoblastic lesions will develop. Osteolytic lesions are caused by osteoclast stimulation rather than by direct effects of the cancer cells on bone [144]. Osteoblastic lesions are caused by local stimulation of the osteoblasts which are next to the metastatic tumour cells [134]. In breast cancer metastasis, bone lesions tend to be primarily osteolytic, however with the use of bisphosphonates, breast cancer bone lesions are becoming more likely to have mixed osteolytic and osteoblastic phenotypes [145]. In prostate cancer, bone metastases tend to be mostly osteoblastic [146].

Normal bone remodeling

In order to understand the mechanism of bone metastasis, a basic understanding of normal bone remodeling is required (see Figure 3). Bone is a dynamic tissue which is constantly being remodelled. In adults, approximately 10% of the bone is renewed each year [147]. There are two main types of bone, trabecular and cortical. Cortical bone is mostly responsible for mechanical strength and protection whereas trabecular bone provides most of the metabolic function of bone and is the main site of bone remodeling [148].

The basic multicellular unit (BMU) is the anatomical and functional structure where bone remodeling takes place. Bone remodeling requires the coordinated action of four main types of bone cells within the BMU: bone lining cells, osteocytes, osteoclasts, and osteoblasts [149, 150].

1. Bone lining cells

   Bone lining cells originate from the osteoblast lineage and form a monolayer over the surface of the bone when it is in an inactive state [151].
2. Osteocytes

   Osteocytes also originate from the osteoblast lineage and are the most abundant bone cells [152]. These cells are embedded in the bone during bone development or remodeling [152]. It has been proposed that osteocytes could be the main mechanosensing cells within bone and therefore may be responsible for the initiation of bone remodeling [153].
3. Osteoclasts

   Osteoclasts are the cells responsible for bone resorption [154]. Osteoclasts are multinucleated giant cells which originate from the monocyte/macrophage hematopoietic lineage [155]. Mononuclear cells of the monocyte/macrophage lineage within the bone marrow or the circulation are first attracted to potential resorption sites and become attached to the bone matrix where they can differentiate into active osteoclasts in response to stimulation by two essential factors which are produced mostly by osteoblasts: the monocyte/macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor κß ligand (RANKL) [155].

4. Osteoblasts

   Osteoblasts are responsible for the deposition of new bone [156]. They are derived from mesenchymal stem cells which give rise to osteoprogenitors which differentiate into preosteoblasts and finally mature osteoblasts within the bone marrow [157]. Osteoblasts also produce RANKL and M-CSF which are responsible for the activation of preosteoclasts, as well as osteoprotegerin (OPG), a decoy receptor for RANKL [158]. This ratio of RANKL to OPG is important in the regulation of bone resorption [158].

For normal bone turnover, it has been suggested that the osteocytes within bone are able to detect microdamage or bone deformation and can signal to recruit osteoclast precursors to these damaged sites to initiate bone remodeling [159]. The osteoclast precursors are then able to bind to the bone matrix where they will differentiate to mature osteoclasts in response to elevated levels of M-CSF and RANKL. Resorption then predominates within this portion of the bone due to the activation of osteoclasts while osteoprogenitors and mesenchymal stem cells (MSCs) begin to be recruited to the site. These osteoprogenitors and MSCs will differentiate into mature osteoblasts which will begin to deposit osteoid where the osteoclasts have resorbed the bone. Osteoclast-mediated bone resorption will eventually cease while the osteoblasts continue to lay down the osteoid. Finally, the osteoid will be mineralized and the bone-remodeling cycle will be complete. [148]

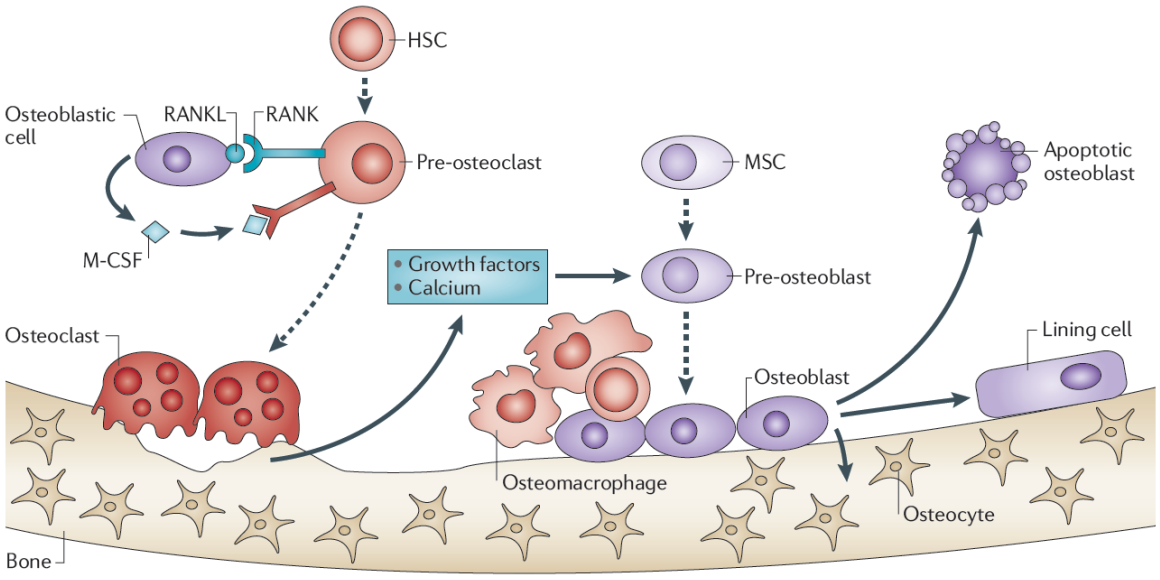

Figure 3 – Normal bone remodeling. Osteoclasts are derived from hematopoietic stem cells (HSC) which form pre-osteoclasts. Preosteoclasts bind to the bone matrix and form mature osteoclasts which are able to resorb bone, in response to elevated levels of RANKL and M-CSF. The bone resorption releases growth factors that act on pre-osteoblasts which are derived from mesenchymal stem cells (MSC). These factors induce preosteoblast maturation to active osteoblasts that are able to lay down new bone. After completing their function, osteoblasts can then either be embedded in bone as osteocytes, form lining cells or undergo apoptosis.

The vicious cycle of osteolytic bone metastasis

When breast cancer tumour cells invade bone, they secrete factors which can stimulate osteoclast activity. One factor secreted by breast tumour cells which has been identified as a main mediator of osteoclast activation is parathyroid hormone-related protein (PTHrP). It has been shown not only that breast cancer cells express PTHrP *in vivo*, but that the tumour cell expression of PHTrP is greater within the bone microenvironment than in the breast or soft-tissue sites [160, 161]. PTHrP secreted by tumour cells is able to activate osteoclasts by upregulating RANKL and downregulating OPG [162], which leads to increased osteolysis.

There are several other factors that are secreted by tumour cells which can lead to osteoclast activation. The cytokine interleukin 11 (IL-11) is normally produced by osteoblasts and bone marrow stromal cells, however breast cancer tumour cells can also secrete IL-11. This cytokine is a potent promoter of osteoclast formation by increasing RANKL expression in osteoblasts [158]. IL-11 expression has been shown to be increased in the presence of transforming growth factor beta (TGF-ß) [163]. Interleukin 8 (IL-8) is a pro-inflammatory cytokine which is normally secreted by monocytes, osteoblasts and endothelial cells but can also be secreted by breast tumour cells and is able to activate osteoclasts independent of RANKL [164]. Cancer cells can also activate osteolysis by inducing osteoblasts to increase the production of osteoclastogenic cytokines such as interleukin 6 (IL-6), IL-8, monocyte chemotactic protein-1 (MCP-1) and tumour necrosis factor (TNF) [165]. Breast cancer tumour cells can also secrete vascular endothelial growth factor (VEGF), which is able to induce osteoclast activity and can support angiogenesis [158]. Matrix metalloproteinases (MMPs) are also secreted by tumour cells within bone and can increase the ratio of RANKL to OPG thereby favouring osteoclastonegesis either directly by cleaving membrane-bound RANKL [166], or indirectly by cleaving epidermal growth factor (EGF)-like ligands [167]. Breast cancer cells can also induce osteolytic activity within bone by expressing Jagged 1 which activates the Notch pathway in bone cells [168]. This activation of the Notch pathway not only increases osteoclast activity, but can also increase the levels of IL-6 which provides a growth advantage to the tumour cells [168].

Certain transcription factors which are expressed by tumour cells have also been found to promote tumour osteolysis. RUNX2, an osteoblast transcription factor has been shown to inhibit proliferation and induce MMP2, MMP9, MMP13, p21, RANKL, VEGF, osteopontin (OPN), bone sialoprotein and PTHrP expression which results in increased bone turnover [169]. Disrupted nuclear trafficking of RUNX2 in breast cancer cells has been shown to inhibit osteolysis [170]. GLI2, a Hedgehog signalling molecule, can induce osteolysis by breast cancer cells by increasing the expression of PTHrP [171]. The expression of the transcription factor hypoxia-inducible factor 1α (HIF1α) in tumour cells is able to promote the differentiation of osteoclasts, and inhibit the differentiation of osteoblasts, thereby favouring osteolysis of bone [172]. Furthermore, it has been shown that tumour-associated hypoxia and HIF1α along with TGFß are able to increase the production of VEGF and CXCR4 by tumour cells which leads to increased angiogenesis and homing of tumour cells [173].

All of these factors from the cancer cells which induce osteoclast activity cause the bone matrix to be resorbed by the osteoclasts. As the bone is resorbed, certain factors are released from the bone matrix, some of which can affect the growth of tumour cells or can enhance the bone microenvironment to support tumour growth. An important growth factor which is released from the bone matrix is TGFß [168]. TGFß can induce pre-osteolytic factor production such as PTHrP by tumour cells by activating both Smad-dependent and Smad-independent signaling pathways [174]. As previously mentioned, TGFß also acts with HIF1A in a hypoxic state to increase the production of VEGF and CXCR4 by tumour cells [173]. Insulin-like growth factors (IGFs) are also released from the bone matrix due to osteolysis and can act on the tumour cells to stimulate proliferation [158]. This increased growth of the tumour induced by growth factors released from the bone matrix leads to a vicious cycle in which the tumour cells secrete more osteolytic factors and the osteolysis of the bone matrix releases more growth factors that induce proliferation of the tumour cells. Osteolysis of the bone also leads to the release of calcium from the bone matrix. Breast cancer cells, as well as other bone metastatic cancer cells express the calcium-sensing receptor (CASR) and can respond to this released calcium from bone [175]. This activation of the CASR in cancer cells can inhibit apoptosis and induce proliferation [176]. It has been shown that ionized calcium can lead

to increased secretion of PTHrP by tumour cells [177] and therefore perpetuates the vicious cycle of osteolytic bone metastasis [178]. Ionized calcium can act as a chemoattractant to breast cancer cells and therefore may draw cancer cells to the bone microenvironment [179].

It is of note that the immune system may play a role in the growth of tumour cells and possibly in bone metastasis. Lymphocytes and bone marrow-derived myeloid cells are recruited to tumours and to areas where there is angiogenesis and hypoxia where they can either enhance immune responses against the tumour or promote the growth of the tumour [180, 181]. T lymphocytes are also important in bone remodeling [182] and can influence tumour growth [183]. Many animal models of cancer metastasis to bone are conducted in immunocompromised mice which lack T cells and therefore the full response of the immune system is absent in these models.

Platelets can also affect tumour growth within bone by releasing factors such as the pro-angiogenic factor VEGF and the anti-angiogenic factor thrombospondin 1 [184]. Furthermore, platelet-derived factors such as lysophosphatidic acid (LPA) and adenosine diphosphate (ADP) are able to induce tumour growth and the release of IL-6 and IL-8 [185].

Overall the vicious cycle of osteolytic bone metastasis is a complex interplay between the tumour cells which induce osteolysis of bone by secreting factors that influence the activity of the bone cells and the growth factors released from bone which stimulate growth and survival of the tumour (summarized in Figure 4). Other factors such as the immune system can also influence the growth of the tumour cells within the bone microenvironment. This complex process is not completely understood and many areas remain to be explored.

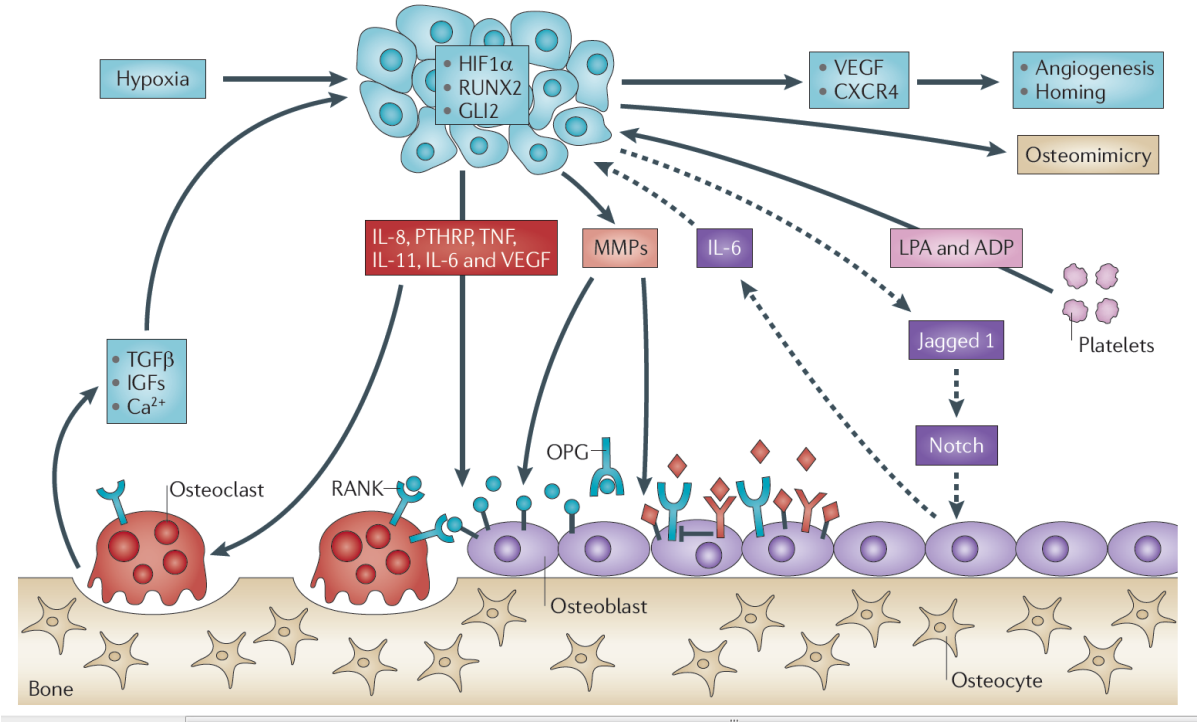

Figure 4 – The vicious cycle of bone metastasis. Tumour cells within bone secrete factors which can activate osteoclast activity either directly (solid arrows) or indirectly (dashed arrows) by increasing the ratio of RANKL to OPG. Bone resorption by osteoclast activation releases factors from the bone matrix which promote the growth and survival of the tumour cells. Hypoxia, hypoxia-inducible factor 1α (HIF1α) and TGFß can increase the production of VEGF and CXCR4 which induces angiogenesis and homing of tumour cells. MMPs can increase the ratio of RANKL to OPG by cleaving membrane-bound RANKL or ECF-like growth factors (red diamonds). LPA and ADP from platelets induce the growth of tumour cells and the release of IL-6 and IL-8.

Mechanisms of osteoblastic bone metastasis

The mechanism by which osteoblastic bone metastasis occurs is not fully understood however several possible mechanisms have been suggested. The growth factor endothelin-1 is able to stimulate osteoblast proliferation and bone formation, and circulating levels of endothelin-1 have been shown to be elevated in patients with osteoblastic bone metastases [186]. Endothelin-A receptor antagonists are able to inhibit osteoblast proliferation and bone metastasis *in vivo* [187], thereby suggesting that endothelin-1 plays an important role in osteoblastic bone metastasis.

TGF-ß has also been proposed to play a role in osteoblastic bone metastasis. TGF-ß2 stimulates the proliferation of osteoblasts and is a powerful inducer of bone formation [188]. The serine protease urokinase (uPA) may play a role in osteoblastic bone metastasis as uPA has been shown to have mitogenic activity on osteoblast-like cells [189]. Overexpression of uPA in prostate cancer cells was shown to induce osteoblastic bone metastasis in an *in vivo* rat model [190]. Another protease which may play a role in osteoblastic bone metastasis is prostate-specific antigen (PSA). PSA is overproduced in prostate cancer cells and is able to cleave PTHrP at the amino terminal [191].

Prostate cancer cells also express large amounts of fibroblast growth factors (FGFs) [192]. It is possible that FGFs could induce osteoblast proliferation in patients with prostate cancer metastasis, and FGFs have been shown to induce bone formation when administered to rats *in vivo* [193]. It was also shown in a human cell line that had been shown to cause osteoblastic lesions when injected in nude mice that FGF was able to activate osteoblasts *in vitro* and induce bone formation *in vivo* in these metastatic tumour cells [194].

Current pharmacological treatment strategies of breast cancer metastasis to bone

One form of treatment currently used to treat breast cancer metastasis to bone is bisphosphonate administration. Bisphosphonates inhibit bone turnover by binding to the hydroxyapatite of bone and blocking activation of the osteoclasts [195]. Bisphosphonates have been demonstrated to delay the onset of skeletal related events and reduce their occurrence [196]. They have also been shown to improve the quality of life of patients

affected by bone metastasis and decrease pain associated with bone metastasis [197]. Although effective in improving the quality of life of patients, bisphosphonates may have serious adverse events related to their use including osteonecrosis of the jaw, and renal toxicity [198].

A more recent treatment for bone metastasis is the use of Denusomab. Denusomab is a fully human monoclonal antibody against RANKL which inhibits osteoclast activation by inactivating RANKL [199]. Denusomab has been shown to be superior to bisphosphonates in the prevention of skeletal related events in breast cancer patients [200]. Although Denusomab use is not associated with renal toxicity, osteonecrosis of the jaw can occur [200].

Although there are treatment options for breast cancer metastasis to bone which can improve the quality of life of patients, bone metastasis is still considered to be incurable. Further research is therefore needed in order to find a cure for bone metastasis.

Vitamin D and the inhibition of breast cancer bone metastasis

Dr. Kremer's group first described the inhibition of bone metastases by vitamin D analogs in an animal model of breast cancer following intra-cardiac injection of MDA MB231 cells [201]. More recently another group of researchers has investigated the effect of dietary vitamin D on the growth of breast cancer metastasis within bone. They fed immunocompromised mice either a vitamin D-free diet or a 2000 IU/Kg normal vitamin D diet and injected the mice with human breast cancer cells [202, 203]. After monitoring the growth of the breast cancer cells within bone, they found that lesions appeared earlier in the vitamin D deficient group and grew larger as compared to the vitamin D sufficient group [202, 203]. They also inhibited bone turnover in a subset of the mice by administering OPG and found that there was a profound reduction of tumour growth within the bone, suggesting that the bone microenvironment plays a very important role in the growth of breast cancer cells within bone [203]. Although they did not see a difference between the size of the tumours between the group on the vitamin D deficient diet treated with OPG and the group on the vitamin D sufficient diet treated with OPG, they did notice that the vitamin D deficient mice had higher mitotic tumour activity,

higher levels of plasma PTH and bone turnover markers at sacrifice [203]. This suggests that vitamin D has additional effects on tumour growth which is independent of bone turnover [203].

II. Experimental Section

Objectives

Given that vitamin D is able to inhibit cell proliferation, induce cell differentiation, induce apoptosis, inhibit angiogenesis, and modulate the immune system, we chose to study the effect of vitamin D deficiency on the growth of breast cancer cells within bone. It has previously been shown that vitamin D administration in a mouse xenograft model of breast cancer was able to inhibit the growth of the breast cancer tumour as compared to control mice which were not administered vitamin D [123]. Based on these findings, we infer that vitamin D deficiency may promote the growth of breast cancer cells. It has been shown by Ooi *et al*. that indeed vitamin D deficiency leads to increased growth of human breast cancer tumour cells within the bone of immunocompromised mice as compared to vitamin D sufficient mice [202, 203]. Since this system implemented by Ooi *et al.* used immunocompromised mice, the full effect of the immune system on the growth of the tumour cells is not seen. We will use a mouse model in which mouse breast cancer tumour cells are injected into wild type FVB mice which have in intact immune system in order to study the effect of vitamin D deficiency on the growth of breast cancer tumour cells within bone. We hope to confirm findings by Ooi *et al*. that vitamin D deficiency leads to increased breast cancer growth within bone [202, 203].

Furthermore, we will look at the effect of the local activation of $25(OH)D_3$ within the breast cancer tumour cells that express the 1α-hydroxylase enzyme on the growth of the tumour cells within the bone microenvironment. An epidemiological link has been found between low circulating 25(OH)D levels and breast cancer risk [204]. This supports the hypothesis that local activation of vitamin D within breast cancer tumour cells inhibits the growth of these cells, as systemic vitamin D deficiency would reduce the amount of vitamin D available for local activation at the tumour site. It has also been shown by Dr. Kremer's lab that the local activation of vitamin D within the breast epithelial cells plays an important role in the inhibition of breast cancer tumour growth at the primary tumour site [205]. We therefore aim to study the effect of the local activation of vitamin D on the growth of breast cancer tumour cells once they have metastasized to bone.

Hypothesis

We hypothesize that vitamin D deficiency will lead to increased tumour growth of breast cancer cells within bone as compared to vitamin D sufficiency. Furthermore, we hypothesize that the local activation of vitamin D by extra-renal 1α-hydroxylase in the breast cancer tumour cells inhibits the growth of these cells within bone.

Introduction to the mouse models

MMTV PyMT 634 model of breast cancer

In order to study the effect of vitamin D on the growth of breast cancer tumour cells within bone, the MMTV PyMT 634 mouse model of breast cancer was used to obtain breast cancer cells. In this mouse model, the expression of the polyoma middle T oncoprotein (PyMT) is expressed under the control of the mouse mammary tumour virus (MMTV) long terminal repeat and therefore is expressed specifically in the breast epithelial cells [206]. Mice expressing the PyMT antigen develop spontaneous breast tumours which can be detected as early as four weeks of age [207]. The PyMT oncoprotein induces breast cancer by affecting several signal transduction pathways including phosphoinositide 3-kinase (PI3K), Src, and ras pathways which are also affected in human breast cancer [208]. An increase in c-myc is also seen with PYMT expression [207].

The breast cancer progression observed in this mouse model closely resembles breast cancer progression in breast cancer patients. By 4-6 weeks of age, hyperplasia can be detected; by 7-6 weeks adenoma/mammary intraepithelial neoplasia (MIN) is seen; at 10-11 weeks early carcinoma is reached, and by 12-14 weeks late carcinoma is seen with the development of lung metastases [209] (Figure 5). Furthermore, the breast cancer biomarkers in this mouse model mimic the biomarkers seen in the human progression of the disease [206]. For instance, loss of the estrogen receptor and progesterone receptor is seen [206]. The expression of cyclin D1 and Neu also increase, and integrin ß expression is lost [206]. At the cell morphology level, loss of the basement membrane and myoepothelial cells can also be seen along with the infiltration of leukocytes with the progression of breast cancer in this model [206]. The characteristics of breast cancer progression in the PYMT mouse model are depicted in Figure 5.

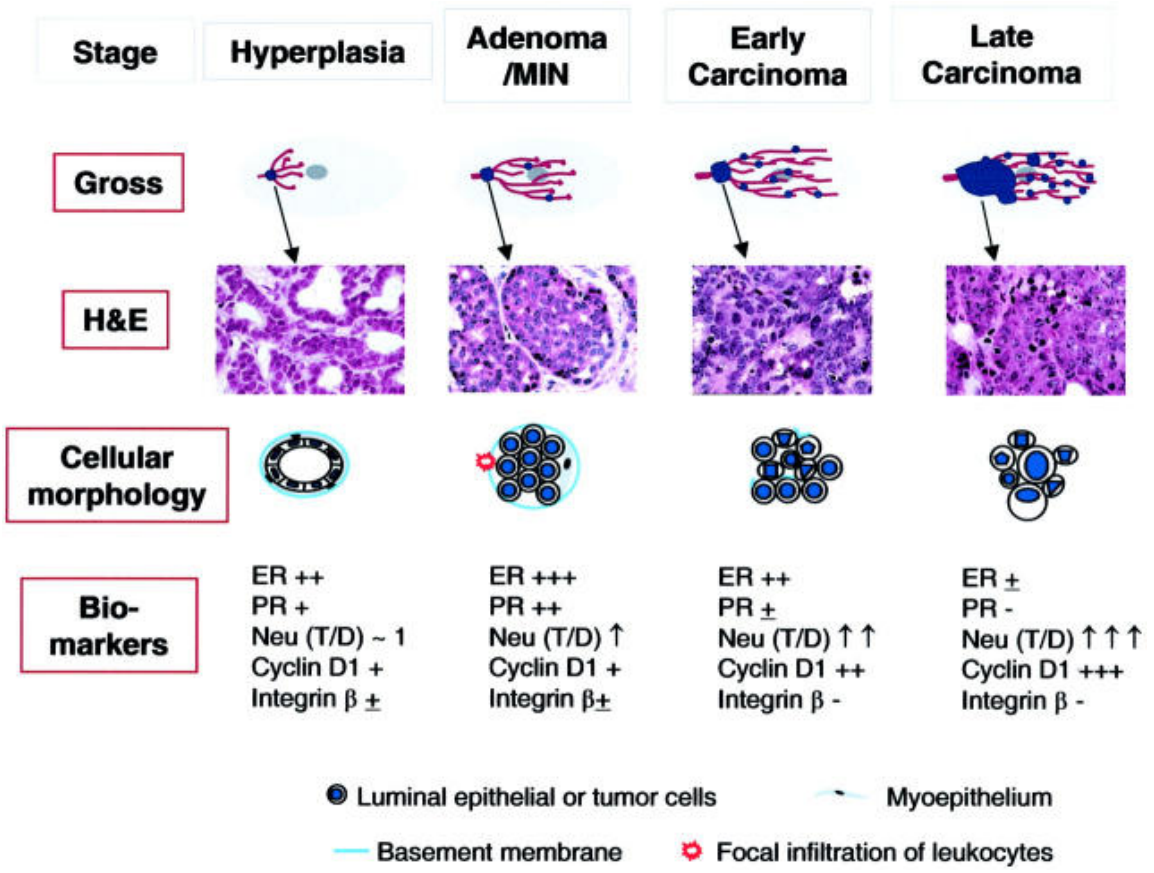

Figure 5 – Tumour progression in the MMTV PyMT 634 mouse model of breast cancer. Breast cancer progression in the MMTV PyMT 634 model of breast cancer closely resembles breast cancer progression seen in humans. It progresses from hyperplasia at 4-6 weeks of age to adenoma/mammary intraepithelial neoplasia (MIN) at 7-9 weeks of age, to early carcinoma at 10-11 weeks of age, and finally to late carcinoma at 12-14 weeks of age. Loss of the basement membrane and myoepithelial cells as well as infiltration of leukocytes can be seen with breast cancer progression in the mice. The breast cancer biomarkers also mimic what is seen in breast cancer patients with progression of the disease, including the loss of the estrogen receptor (ER), progesterone receptor (PR), increases in Neu and Cyclin D1, and loss of integrin ß.

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Tissue specific 1α-hydroxylase ablation in PyMT breast tumours

In order to investigate the effect of the local activation of vitamin D by 1α-hydroxylase expressed in PyMT breast cancer cells, 1α-hydroxylase ablated tumour cells were needed. The Cre-loxP system was used to remove the expression of *CYP27B1* specifically in the breast epithelial cells under the control of the mouse mammary tumour virus (MMTV). Cre is able to catalyze the deletion of the gene between two loxP sites which are in the same direction on a DNA molecule [210], and therefore the expression of two loxP sites on either side of the gene along with Cre recombinase in a mouse leads to deletion of the target gene. In order for the mice to develop tumours specifically in the breast which do not express 1α-hydroxylase, an MMTV PyMT 634 positive mouse is bred with a mouse which has loxP sites on either side of the *Cyp27B1* gene specifically in the breast epithelial cells under control of the MMTV. This mouse is then bred with a Cre recombinase positive mouse. If the offspring express PyMT, *Cyp27B1* flox/flox allele, and are Cre positive, they will develop tumours which do not express 1α-hydroxylase within the breast tissue. These tumour cells were used in our experiments as the 1α-hydroxylase ablated tumour cells.

Materials and Methods

In vitro studies

Extraction of tumour cells from PyMT breast tumours

Late stage breast tumours from wild type and CYP27B1 flox/flox cre+ PyMT mice were used to extract breast cancer cells which were put in culture. The mice were anesthetized with 0.2 to 0.3 ml of mouse cocktail (ketamine 100mg/kg (Bioniche, Pointe-Claire, QC), xylazine 10mg/kg (Bayer Heathcare, Montreal, QC), acepromazine 3mg/kg (Boehringer Ingelheim, Laval, QC)) and sacrificed by cervical dislocation. The primary breast tumour was then cut out using a scalpel and chopped into tiny pieces using a sterile razor blade in a laminar flow cabinet. The tumour cells were then disassociated from the extracted tissue using 20 mL of dispase II and collagenase for 2 hours in a 37 ˚C water bath. The cells were next washed twice with 50 mL of PBS (phosphate buffered saline) (Wisent, St-Bruno, QC) and centrifuged at 300g for 5 minutes at room temperature. 70 mL of Dulbecco's Modified Eagle Medium (DMEM) (Wisent, St-Bruno, QC) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen, Burlington, ON) and 1% penicillin streptomycin (Invitrogen, Burlington, ON) was used to resuspend the extracted cells, and 10 mL of the diluted cells was added to seven 10 mm culture plates. The cells were incubated at 37˚C with 5% carbon dioxide. The following three days the cells were washed twice with 10 mL of PBS. When confluent, the cells were passaged twice and then were frozen in liquid nitrogen until they were needed.

Cell culture

Breast tumour cells extracted from wild type MMTV PyMT 634 mice and Cyp27B1 flox/flox cre+ MMTV PyMT 634 mice (1α-hydroxylase ablated tumour cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Wisent, St-Bruno, QC) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen, Burlington, ON) and 1% penicillin streptomycin (Invitrogen, Burlington, ON). The cells were incubated at 37˚C with 5% carbon dioxide and passaged twice weekly.

Proliferation assays

Wild type PyMT and 1α-hydroxylase ablated PyMT tumour cells were seeded in 24-well plates with 5000 cells per well. 24 hours later the media was replaced with serum-free DMEM and the cells were serum-starved for 8 hours. The cells were then treated with either $1,25(OH)_2D_3$ or $25(OH)D_3$ (Sigma-Aldrich, Oakville, ON). Concentrations of $1,25(OH)_2D_3$ used were 0 M, 10^{-10} M, 10^{-9} M, 10^{-8} M, and 10^{-7} M added in a volume of 500 µl per well. Ethanol was added to each dilution in the appropriate volume so as to assure that each treatment had the same concentration of vehicle as the 10^{-7} M concentration. Concentrations of $25(OH)D_3$ used were 0 M 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M added in a volume of 500 µl per well. Ethanol was added as a vehicle to each dilution to assure that each dilution had the same concentration of vehicle as the 10^{-6} M concentration. One plate of treated cells was washed with PBS and then trypsinized (Invitrogen, Burlington, ON) and the cells for each treatment were counted using a Z1 Beckman Coulter Counter for time = 0 hours. The other plates were incubated at 37˚C with 5% CO_2 and one plate was counted at time = 24 hours, 48 hours, and 72 hours following treatment. Each treatment was done in triplicate. The total number of cells per well was calculated using the following formula based on the mean cell count from the Coulter Counter:

$$\text{Total \# Cells} = \frac{\text{Mean cell count X 40 X Total volume of cells in suspension}}{\text{Volume of suspended cells added to the isotonic fluid}}$$

Viability assays

Cells were seeded in 24-well plates with 2500 cells per well. 24 hours later the media was replaced with serum-free DMEM and the cells were serum-starved for 8 hours. The cells were then treated with either $1,25(OH)_2D_3$ or $25(OH)D_3$ (Sigma-Aldrich, Oakville, ON). Concentrations of $1,25(OH)_2D_3$ used were 0 M (ethanol control), 10^{-10} M, 10^{-9} M, 10^{-8} M, and 10^{-7} M added in a volume of 500 µl per well. Concentrations of $25(OH)D_3$ used were 0 M (ethanol control), 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M added in a volume of 500 µl per well. The media in one plate of treated cells was then replaced with RPMI (Roswell Park Memorial Institute media) (Wisent, St-Bruno, QC) supplemented with 5% FBS, 1%

pen/strep and with 10% Alamar Blue (Invitrogen, Burlington, ON). The plate was incubated with the Alamar Blue at 37˚C and 5% CO_2 for 75 minutes. Fluorescence readings were then taken with an excitation wavelength of 570 nm and an emission wavelength of 585 nm. The remaining plates for time = 24, 48, and 72 hours were incubated at 37˚C at 5% CO_2 until they were required. The procedure was repeated at each time point. Each treatment was done in triplicate.

In vivo studies

The MMTV PyMT murine model of breast cancer was used to investigate the role of locally produced $1,25(OH)_2D_3$ on the growth of breast cancer tumour cells within bone. A preliminary experiment was first conducted in order to optimize the conditions which would be used in our main in vivo experiment. The timeline for this experiment is depicted in Figure 6. This preliminary experiment had two different treatment groups of mice. Both groups were injected into the right tibia with wild type PyMT breast tumour cells however one group of mice was on a vitamin D deficient diet and the other group was on a normal vitamin D diet. The timeline for the main mouse experiment is depicted in Figure 7. Slight modifications in the timeline were made for the main experiment based on findings in the preliminary experiment. This experiment had four different groups of mice. One group was fed a normal vitamin D diet and injected with the wild type tumour cells. The second group was fed a normal vitamin D diet and injected with 1α-hydroxylase ablated tumour cells. The third group was fed a vitamin D deficient diet and was injected with wild type tumour cells, and the final group was fed a vitamin D deficient diet and injected with 1α-hydroxylase ablated tumour cells.

Preliminary Experiment:

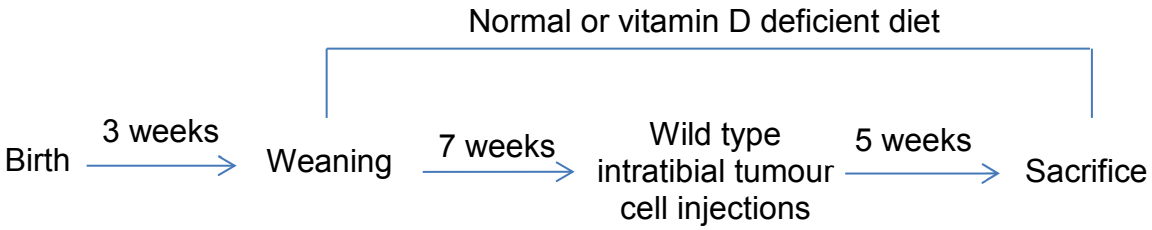


Figure 6 – Timeline for the preliminary mouse experiment.

Main Experiment:

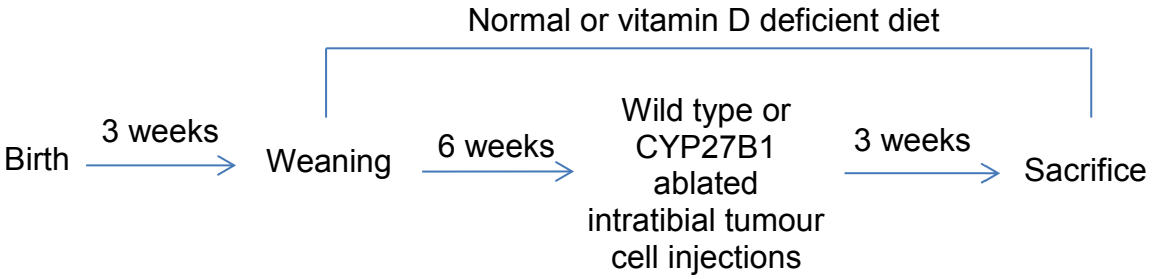


Figure 7 – Timeline for the main mouse experiment.

Establishment of vitamin D deficient and vitamin D sufficient mice

All in vivo experiments were approved by the McGill University Animal Committee prior to conduct of the study. All animals were housed at the Royal Victoria Hospital Institute for Animal Studies, McGill University. Wild type FVB mice were bred in a room without UVB lighting in order to minimize the endogenous production of vitamin D in the skin. Three weeks after birth, the female pups were weaned and fed *ad libitum* either a 25 IU/Kg vitamin D deficient diet (Harlan, Montreal, QC) or a 1000 IU/Kg normal vitamin D diet (Harlan, Montreal, QC). The mice were maintained on these specialized vitamin D diets throughout the experiment.

Preparation of tumour cells for intratibial injections

Wild type and 1α-hydroxylase ablated PyMT tumour cells were thawed, put in culture, and passaged three times prior to preparation for intratibial injections. On the day of the intratibial injections, the 90% confluent cells were washed once with PBS and then trypsinized for 4 minutes. Once the cells had detached from the culture dish, 8 ml of DMEM containing 10% FBS and 1% penicillin/streptomycin was added to the cells to neutralize the trypsin. The cells were then counted using a Z1 Coulter counter and were centrifuged at 300g for 5 minutes at room temperature. The media was then removed and the cell pellet was diluted to a concentration of $1.0x10^7$ cells/ml with sterile PBS. The cells were kept on ice until they were used for intratibial injections.

Intratibial injections

Six or seven weeks after weaning, the mice were injected with either wild type or 1α-hydroxylase ablated PyMT breast tumour cells. The mice were anesthetized with 0.20 to 0.25 ml of mouse cocktail (ketamine 100mg/kg (Bioniche, Pointe-Claire, QC), xylazine 10mg/kg (Bayer Heathcare, Montreal, QC), acepromazine 3mg/kg (Boehringer Ingelheim, Laval, QC)) as needed. A 25G X 5/8 needle was used to inject 50 µl of the PyMT breast tumour cells in suspension ($5.0x10^5$ cells) into the intra-medullary bone cavity of the right tibia of each mouse. The left tibia served as a control.

X-ray analysis

On the day of sacrifice, either 3 or 5 weeks after the intratibial tumour cell injections, the mice were anesthetized with 0.20 to 0.25 ml of mouse cocktail. X-rays of the hind limbs on the live mice were taken at the Center for Bone and Periodontal Research (Montreal, QC) at a magnification of 2X, and settings of 25 kV, and 750 uA.

The x-rays taken at sacrifice were scored according to the following scoring method: 0 (no lesion), 1 (minor changes), 2 (small lesions), 3 (significant lesions with minor peripheral margin breaks), 4 (significant lesions with major peripheral margin breaks. > 10% of bone surface disrupted). The x-rays were scored by two individuals independently and the scoring was performed in a blind fashion in which the scorer did not know to which group the mice belonged.

The NIH Image J program was then used to analyze the surface area of the osteolytic lesions by tracing each lesion in the tibia and adding the sum of the areas of lesion.

Blood collection and analysis

The anesthetized mice were sacrificed by cardiac puncture. A 25G X 5/8 needle was inserted into the heart under the rib cage and up to 1 ml of blood was collected into a syringe. The blood was then transferred to an eppendorf tube at kept at 4˚C overnight. The following day the blood was centrifuged at 13000g at 4˚C for 30 minutes. The serum was then transferred to another eppendorf tube and frozen at -80˚C until the time of analysis.

The serum collected at sacrifice was analyzed for 25(OH)D, calcium, phosphorus, albumin, creatinine, and alkaline phosphatase. Serum 25(OH)D concentration was measured using the LIAISON total 25(OH)D assay which equally detects $25(OH)D_2$ and $25(OH)D_3$ at McGill University in the School of Dietetics and Human Nutrition (Montreal, QC). Serum calcium, phosphorus, albumin, creatinine, and alkaline phosphatase were analyzed by the Diagnostic Lab of the Comparative Medicine Animal Resources Centre (Montreal, QC).

Collection and fixation of the tibiae

After the mice had been sacrificed by cardiac puncture, the legs were collected by cutting at the hip bone and removing the skin. The legs were fixed in 10% formalin solution (Sigma-Aldrich, Oakville, ON) for 24 hours, and were then washed with PBS and transferred to fresh PBS.

Decalcification of tibiae

Half of the tibiae that had been fixed in formalin were decalcified for paraffin embedding. They were decalcifified by first transferring the bones to 10% glycerol in PBS and placed on a rocker at 4ºC for 4 hours. The bones were then transferred to ethylenediaminetetraacetic acid (EDTA) (pH=7.30) and placed on a rocker at 4ºC for 14 days. The bones were then transferred to 7.5% glycerol in 15% sucrose in PBS for 2 hours, followed by 15% sucrose in PBS for 2 hours, and 7.5% sucrose in PBS for 2 hours on the rocker at 4ºC before finally being transferred to PBS and stored at 4ºC.

Preparation of slides of paraffin embedded tibiae

The decalcified tibiae were embedded in paraffin and cut into 6 µm sections using a Leica RM2165 microtome. The slides were stained with hematoxylin and eosin (H&E). This work was done by the Center for Bone and Periodontal Research (Montreal, QC).

Preparation of slides of plastic embedded tibiae

Half of the tibiae were not decalcified and these tibiae were embedded in methyl methacrylate. The methyl methacrylate embedded tibiae were then cut into 7 µm sections

using a Leica Ultracut Ultramicrotome, put on slides, and stained with Goldner Trichrome and H&E stain. This work was done by the Center for Bone and Periodontal Research (Montreal, QC).

Results

In vitro studies

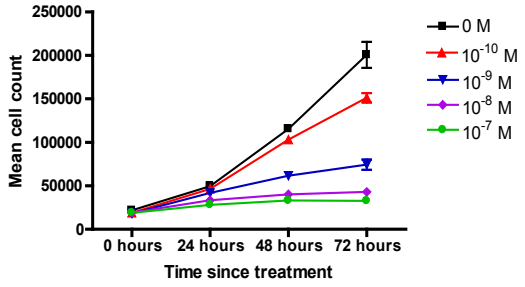
Proliferation and viability assays were performed on the wild type and 1α-hydroxylase ablated PyMT breast tumour cells in order to demonstrate that they respond to treatment with $1,25(OH)_2D_3$ and that the wild type tumour cells would respond to treatment with $25(OH)D_3$. Since the wild type cells express CYP27B1 they are able to activate $25(OH)D_3$ and should therefore respond to the treatment. Conversely, since the knock out cells do not express CYP27B1, they should be unable to activate $25(OH)D_3$ and therefore should not respond to the treatment.

The proliferation assays demonstrated that the wild type tumour cells treated with $1,25(OH)_2D_3$ showed a dose-dependent inhibition of cell proliferation when treated with concentrations ranging from 10^{-7} to 10^{-10} M $1,25(OH)_2D_3$ (Figure 8A). The 1α-hydroxylase ablated tumour cells treated with $1,25(OH)_2D_3$ also showed a dose-dependent inhibition of cell proliferation when treated with concentrations ranging from 10^{-7} to 10^{-9} M $1,25(OH)_2D_3$ (Figure 8C). When the wild type tumour cells were treated with $25(OH)D_3$, inhibition of cell proliferation was seen at concentrations from 10^{-6} to 10^{-9} M $25(OH)D_3$ as compared to control untreated cells (Figure 8B). Unexpectedly when the 1α-hydroxylase ablated tumour cells were treated with $25(OH)D_3$, inhibition of cell proliferation was seen at a concentration of 10^{-6} M $25(OH)D_3$ (Figure 8D). Unlike the wild type cells, 1α-hydroxylase ablated tumour cells did not respond to $25(OH)D_3$ at concentrations between 10^{-7} and 10^{-9} M as compared to the control untreated cells (Figure 8D).

The viability assays showed similar patterns for the wild type and 1α-hydroxylase ablated tumour cells treated with $1,25(OH)_2D_3$ and $25(OH)D_3$. The wild type tumour cells treated with $1,25(OH)_2D_3$ had a reduced number of metabolically active cells when treated with concentrations of 10^{-7} to 10^{-10} M $1,25(OH)_2D_3$ as compared to the vehicle treated control cells (Figure 9A). The 1α-hydroxylase ablated tumour cells treated with $1,25(OH)_2D_3$ also had reduced numbers of metabolically active cells when treated with concentrations of $1,25(OH)_2D_3$ ranging from 10^{-7} to 10^{-10} M as compared to the control cells (Figure 9B).

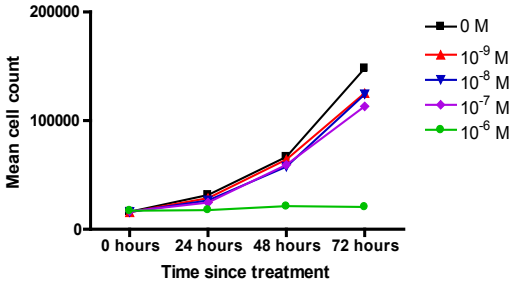
When the wild type tumour cells were treated with $25(OH)D_3$, the number of metabolically active cells was significantly reduced when treated with concentrations ranging from 10^{-6} to 10^{-9} M as compared to the vehicle treated control cells (Figure 9C). Finally, the 1α-hydroxylase ablated tumour cells treated with $25(OH)D_3$ did not have reduced numbers of metabolically active cells at concentrations ranging from 10^{-7} to 10^{-9} M as compared to the control cells, however at a concentration of 10^{-6} M $25(OH)D_3$, there was a significant decrease in the number of metabolically active cells (Figure 9D).

A)



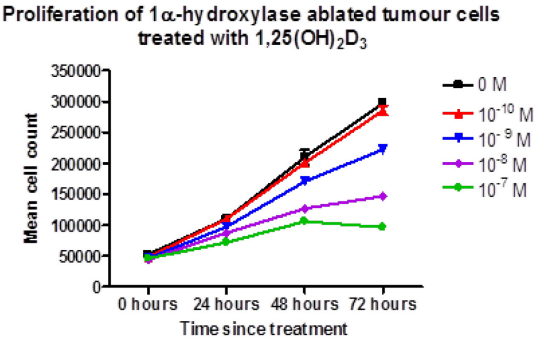
	0 hours	24 hours	48 hours	72 hours
10^{-10} M	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns	P<0.001 ***
10^{-9} M	P > 0.05 ns	P > 0.05 ns	P<0.001 ***	P<0.001 ***
10^{-8} M	P > 0.05 ns	P < 0.05 *	P<0.001 ***	P<0.001 ***
10^{-7} M	P > 0.05 ns	P<0.01 **	P<0.001 ***	P<0.001 ***

B)



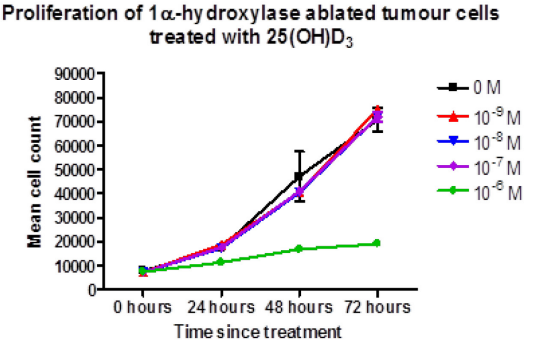
	0 hours	24 hours	48 hours	72 hours
10^{-9} M	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns	P<0.001 ***
10^{-8} M	P > 0.05 ns	P > 0.05 ns	P < 0.05 *	P<0.001 ***
10^{-7} M	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns	P<0.001 ***
10^{-6} M	P > 0.05 ns	P<0.01 **	P<0.001 ***	P<0.001 ***

C)



	0 hours	24 hours	48 hours	72 hours
10^{-10} M	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns
10^{-9} M	P > 0.05 ns	P > 0.05 ns	P<0.01 **	P<0.001 ***
10^{-8} M	P > 0.05 ns	P < 0.05 *	P<0.001 ***	P<0.001 ***
10^{-7} M	P > 0.05 ns	P<0.01 **	P<0.001 ***	P<0.001 ***

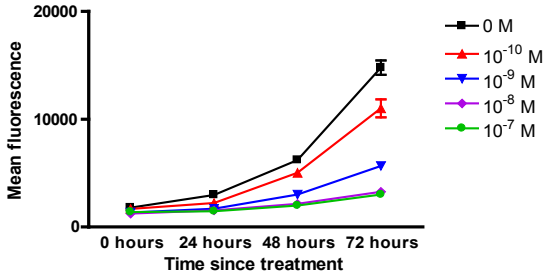
D)



	0 hours	24 hours	48 hours	72 hours
10^{-9} M	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns
10^{-8} M	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns
10^{-7} M	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns
10^{-6} M	P > 0.05 ns	P > 0.05 ns	P<0.001 ***	P<0.001 ***

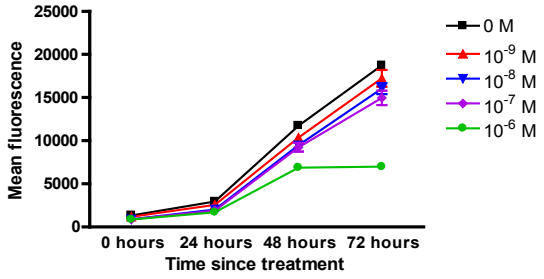
Figure 8 – Proliferation assays of wild type and 1α-hydroxylase ablated tumour cells treated with $1,25(OH)_2D_3$ or $25(OH)D_3$. A) Wild type tumour cells treated with $1,25(OH)_2D_3$. B) Wild type tumour cells treated with $25(OH)D_3$. C) 1α-hydroxylase ablated tumour cells treated with $1,25(OH)_2D_3$. D) 1α-hydroxylase ablated tumour cells treated with $25(OH)D_3$. P values are represented below the growth curves and $p<0.05$ was considered significant.

A) **Viability of wild type tumour cells treated with $1,25(OH)_2D_3$**



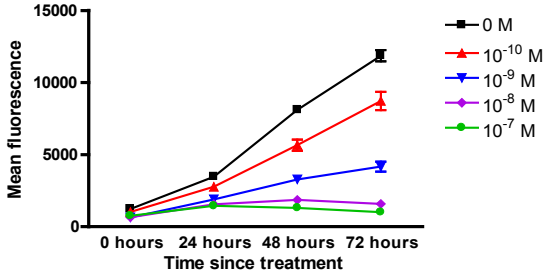
	0 hours	24 hours	48 hours	72 hours
10^{-10} M	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns	P<0.001 ***
10^{-9} M	P > 0.05 ns	P > 0.05 ns	P<0.01 **	P<0.001 ***
10^{-8} M	P > 0.05 ns	P > 0.05 ns	P<0.001 ***	P<0.001 ***
10^{-7} M	P > 0.05 ns	P > 0.05 ns	P<0.001 ***	P<0.001 ***

B) **Viability of wild type tumour cells treated with $25(OH)D_3$**



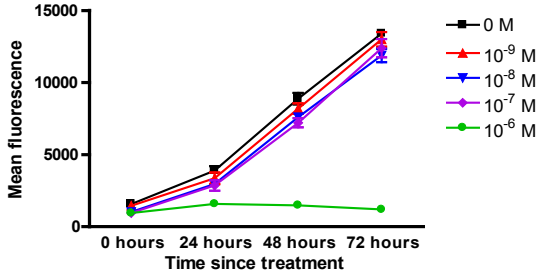
	0 hours	24 hours	48 hours	72 hours
10^{-9} M	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns	P<0.001 ***
10^{-8} M	P > 0.05 ns	P > 0.05 ns	P < 0.05 *	P<0.001 ***
10^{-7} M	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns	P<0.001 ***
10^{-6} M	P > 0.05 ns	P<0.01 **	P<0.001 ***	P<0.001 ***

C) **Viability of 1α-hydroxylase ablated tumour cells treated wtih $1,25(OH)_2D_3$**



	0 hours	24 hours	48 hours	72 hours
10^{-10} M	P > 0.05 ns	P > 0.05 ns	P < 0.05 *	P<0.01 **
10^{-9} M	P > 0.05 ns	P > 0.05 ns	P<0.001 ***	P<0.001 ***
10^{-8} M	P > 0.05 ns	P > 0.05 ns	P<0.001 ***	P<0.001 ***
10^{-7} M	P > 0.05 ns	P < 0.05 *	P<0.001 ***	P<0.001 ***

D) **Viability of 1α-hydroxylase ablated tumour cells treated with $25(OH)D_3$**



	0 hours	24 hours	48 hours	72 hours
10^{-9} M	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns
10^{-8} M	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns
10^{-7} M	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns	P > 0.05 ns
10^{-6} M	P > 0.05 ns	P > 0.05 ns	P<0.001 ***	P<0.001 ***

Figure 9 - Viability assays of wild type and 1α-hydroxylase ablated tumour cells treated with $1,25(OH)_2D_3$ or $25(OH)D_3$. A) Wild type tumour cells treated with $1,25(OH)_2D_3$. B) Wild type tumour cells treated with $25(OH)D_3$. C) 1α-hydroxylase ablated tumour cells treated with $1,25(OH)_2D_3$. D) 1α-hydroxylase ablated tumour cells treated with $25(OH)D_3$. P values are represented below the growth curves and $p<0.05$ was considered significant.

Preliminary in vivo experiment to determine the optimal parameters to assess the effect of vitamin D on breast cancer tumour growth within bone

We first began by performing a preliminary experiment in which we had two groups of mice who were fed either a normal vitamin D diet (1000 IU/Kg) or a vitamin D deficient diet (25 IU/Kg). We wanted to determine the optimal experimental parameters to use in order to assess the effect of vitamin D on the growth of breast cancer tumour cells within bone. The mice were fed these special vitamin D diets from the time of weaning and were injected into the right tibia with wild type PYMT breast cancer cells seven weeks later. Five weeks following tumour cell injections X-rays were taken of the hind limbs of the mice prior to sacrifice. The mice were then sacrificed by cardiac puncture, the blood was collected, and the legs were fixed in formalin.

Animal observation and mobility:

Seven weeks following the intratibial injections, very large tumours could clearly be seen in the right leg of both vitamin D deficient and vitamin D sufficient mice. The injected leg appeared to be at least five times the size of the control leg. Although they clearly had significant tumour growth within their right leg, the mice were still very mobile although they did have a tendency to drag the tumour bearing leg. In spite of having a large tumour, the mice did not seem to be in great distress.

Biochemical analysis:

Serum analysis was performed on the mouse serum collected at sacrifice. 25(OH)D, albumin, creatinine, alkaline phosphatase, calcium and phosphorus levels were measured. As expected there was a very statistically significant decrease in the serum levels of 25(OH)D in the mice on the 25 IU/Kg vitamin D diet as compared to the mice on the 1000 IU/Kg vitamin D diet (Figure 10). The serum levels of 25(OH)D for the group on the 1000 IU/Kg diet was 116.21 ± 15.72 nmol/L as compared to 16.67 ± 2.62 nmol/L for the group on the 25 IU/Kg diet ($P<0.0001$). There was also a significant increase in the serum levels of alkaline phosphatase in the vitamin D deficient group (Table 1). The group on the 1000 IU/Kg diet had alkaline phosphatase levels of 55.71 ± 17.72 U/L as compared to 83.00 ± 20.12 U/L for the group on the 25 IU/Kg diet ($P=0.0246$). This may

be an indicator of increased bone turnover. The other biochemical parameters did not differ significantly between the groups (P>0.05) (Table 1).

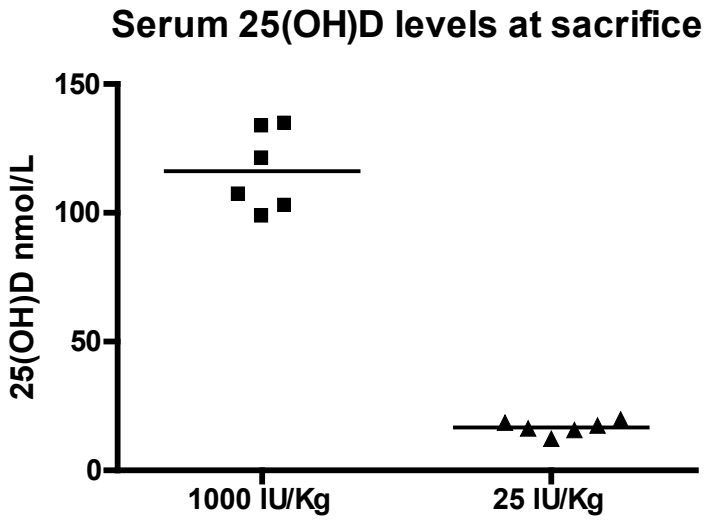


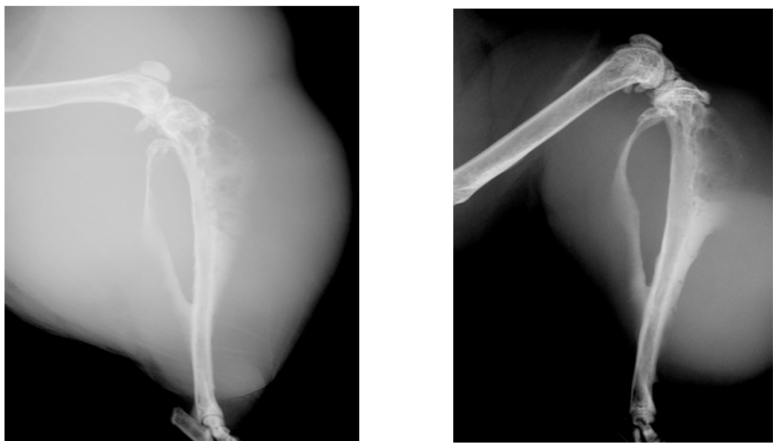
Figure 10 – Serum levels of 25(OH)D at sacrifice for the preliminary mouse experiment. Serum 25(OH)D concentration was measured using the LIAISON total 25(OH)D assay which equally detects 25(OH)D_2 and 25(OH)D_3. There was a significant difference in the levels of 25(OH)D between the group on the 1000 IU/Kg normal vitamin D diet (116.21 ± 15.72 nmol/L) and the group on the 25 IU/Kg vitamin D deficient diet (16.67 ± 2.62 nmol/L) (P<0.0001).

	25(OH)D (nmol/L)	Albumin (g/L)	Creatinine (µmol/L)	Alkaline Phosphatase (U/L)	Calcium (mmol/L)	Phosphorus (mmol/L)
Normal range	75-180	25–48	18–71	62-209	1.47–2.35	1.97–3.26
1000 IU/Kg diet, wild type cells	**116.21** ± 15.72 n=6	**19.57** ± 2.23 n=7	**14.57** ± 3.46 n=7	**55.71** ± 17.72 n=7	**2.31** ± 0.16 n=7	**3.00** ± 0.37 n=7
25 IU/Kg diet, wild type cells	**16.67 *** ± 2.62 n=6	**21.67** ± 1.21 n=6	**12.67** ± 1.21 n=6	**83.00 *** ± 20.12 n=6	**2.36** ± 0.10 n=6	**2.67** ± 0.20 n=6

Table 1 - Mean serum 25(OH)D, albumin, creatinine, alkaline phosphatase, calcium, and phosphorus at sacrifice for the preliminary mouse experiment. There was no significant difference between the groups for albumin, creatinine, calcium and phosphorus ($p > 0.05$), however there was a significant difference in the levels of alkaline phosphatase ($P=0.0246$) and 25(OH)D ($P<0.0001$).

X-ray analysis:

The x-rays taken of the hind limbs prior to sacrifice were next analyzed to assess the degree of osteolytic lesions within the bone (Figure 11). They were scored by two people independently in a blind manner and were scored according to the following scale: 0 (no lesion), 1 (minor changes), 2 (small lesions), 3 (significant lesions with minor peripheral margin breaks), 4 (significant lesions with major peripheral margin breaks. > 10% of bone surface disrupted). The x-ray score for the group on the 1000 IU/Kg normal vitamin D diet was 2.89 ± 0.98 and 3.167 ± 0.75 for the group on the 25 IU/Kg diet (Figure 12). There was no significant difference between the groups ($P=0.5430$). This is most likely because the tumour had grown so large that the bone was completely destroyed in both experimental groups and therefore the extent of tumour growth within the bone could not be compared.



1000 IU/Kg Diet 25 IU/Kg Diet

Figure 11 – Representative x-rays of the tibiae at sacrifice for the preliminary mouse experiment. The x-rays of the tibiae taken at sacrifice exhibited intense osteolysis of the bone caused by breast cancer tumour cell growth in both the group on the 1000 IU/Kg normal vitamin D diet and the group on the 25 IU/Kg vitamin D deficient diet. The tumour growth within the bone destroyed the cortex and the tumour spread to the surrounding tissue.

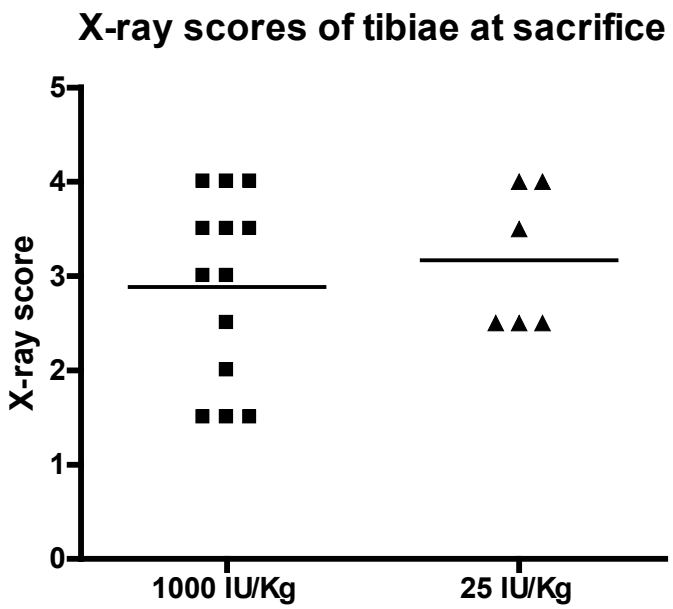


Figure 12 – X-ray scoring of the tumour-bearing tibiae at sacrifice for the preliminary mouse experiment. The X-rays taken at sacrifice were scored by two people independently in a blind manner and were scored according to the following scale: 0 (no lesion), 1 (minor changes), 2 (small lesions), 3 (significant lesions with minor peripheral margin breaks), 4 (significant lesions with major peripheral margin breaks. > 10% of bone surface disrupted). There was no significant difference between the groups (P=0.5430).

Effect of vitamin D diet and local 1α-hydroxylase inactivation on breast cancer tumour growth within bone

Since the tumours had grown so large within five weeks following tumour cell injections in the preliminary mouse experiment that a difference in the tumour growth within the bone could not be seen between the groups, the length of the experiment was modified in order to look at the tumour growth at an earlier time point. Furthermore, we extended the experiment to four treatment groups, some of which were injected with 1α-hydroxylase ablated tumour cells to investigate the effect of the local activation of vitamin D by breast tumour cells on the growth of the tumour cells within bone.

Animal weight and mobility:

The mice gained weight throughout the experiment from weaning to sacrifice and no significant difference was seen between the four groups (Figure 13). At the time of sacrifice, both groups of mice injected with 1α-hydroxylase ablated tumour cells had very large visible tumours in their right leg. The right legs of these mice appeared to be at least three times the size of the right legs of both groups of mice injected with wild type tumour cells which had barely visible tumours. Although the groups injected with 1α-hydroxylase ablated tumour cells clearly had larger tumours, this did not appear to affect their mobility. These mice would walk around the cage as much as the groups on injected with wild type cells however they did have a tendency to drag the tumour bearing leg and walk on the other three healthy legs. None of the mice appeared to be in great distress.

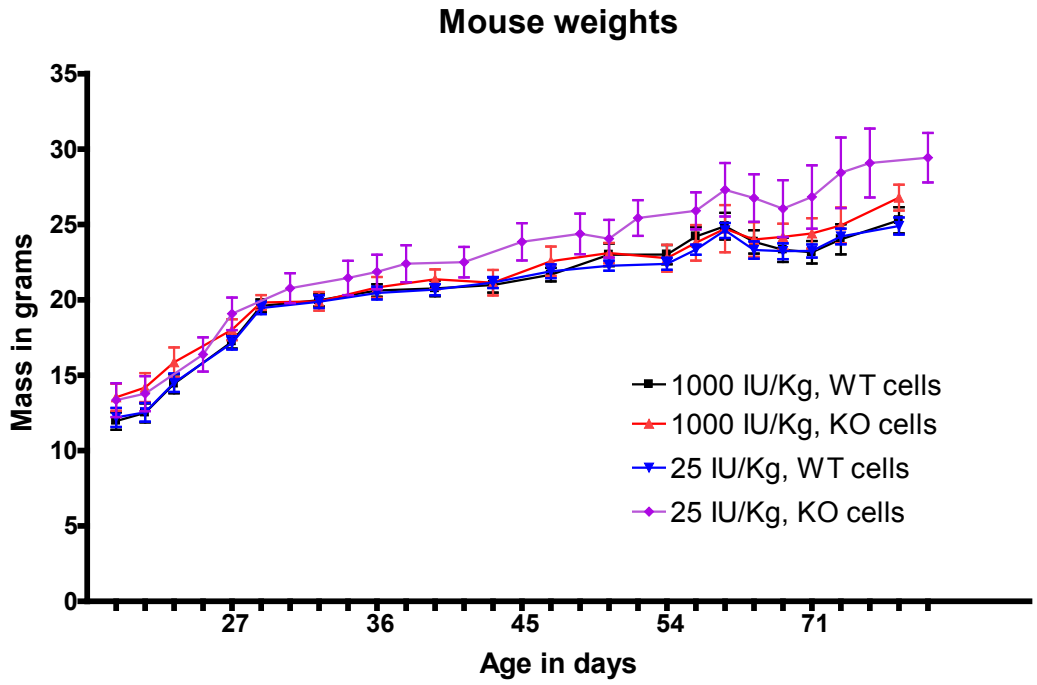


Figure 13 – Mean weights of the mice throughout the main mouse experiment. The mass of the mice throughout the experiment was not statistically different between groups ($p > 0.05$).

Biochemical analysis:

Serum analysis was performed on the mouse samples collected by cardiac puncture at sacrifice. 25(OH)D, albumin, creatinine, alkaline phosphatase, calcium and phosphorus levels were measured (Table 2). Serum 25(OH)D levels at sacrifice were 92.84 ± 14.82 nmol/L for the group fed the 1000 IU/Kg diet and injected with wild type tumour cells, 100.01 ± 15.75 nmol/L for the group fed the 1000 IU/Kg diet and injected with 1α-hydroxylase ablated tumour cells, and was significantly lower, 20.33 ± 2.02 nmol/L, for the group fed the 25 IU/Kg diet and injected with wild type cells ($p << 0.01$) (Figure 14). 25(OH)D levels for the group of mice on the 25 IU/Kg diet injected with 1α-hydroxylase ablated tumour cells are not available due to limited volumes of serum collected. Serum levels of albumin, alkaline phosphatase and calcium, did not differ significantly between the groups (Table 2). Serum levels of creatinine did not differ significantly between

groups except for the group on the 25 IU/Kg diet injected with 1α-hydroxylase ablated tumour cells which had an elevated creatinine level of 23.33 µmol/L as compared to the other groups (P<0.001) (Table 2). Serum phosphorus levels were also not significantly different between the groups except for the group fed the 25 IU/Kg diet injected with 1α-hydroxylase ablated tumour cells as compared to the group on the 1000 IU/Kg diet and injected with wild type tumour cells (p<0.05) (Table 2).

	25(OH)D (nmol/L)	Albumin (g/L)	Creatinine (µmol/L)	Alkaline Phosphatase (U/L)	Calcium (mmol/L)	Phosphorus (mmol/L)
Normal range	75-180	25–48	18–71	62-209	1.47–2.35	1.97–3.26
1000 IU/Kg diet, wild type cells	**92.97** ±15.71 n=9	**19.55** ±1.04 n=11	**10.91** ±2.51 n=11	**66.45** ±11.56 n=11	**2.25** ±0.15 n=11	**2.65** ±0.27 n=11
25 IU/Kg diet, wild type cells	**20.44 *** ±1.74 n=9	**18.29** ±1.11 n=7	**10** ±2.52 n=7	**48.14** ±17.15 n=7	**2.28** ±0.05 n=7	**2.98** ±0.23 n=7
1000 IU/Kg diet, 1α-hydroxylase ablated cells	**106.82** ±12.37 n=6	**18.71** ±0.76 n=7	**13** ±1.73 n=7	**68.29** ±17.00 n=7	**2.37** ±0.07 n=7	**2.89** ±0.26 n=7
25 IU/Kg diet, 1α-hydroxylase ablated cells	**N/A**	**20.33** ±1.53 n=3	**23.33 *** ± 5.86 n=3	**48.33** ± 7.09 n=3	**2.35** ±0.04 n=3	**3.37 *** ±0.69 n=3

Table 2 – Mean serum 25(OH)D, albumin, creatinine, alkaline phosphatase, calcium, and phosphorus at sacrifice for the main mouse experiment. Significance (*) was defined by P<0.05.

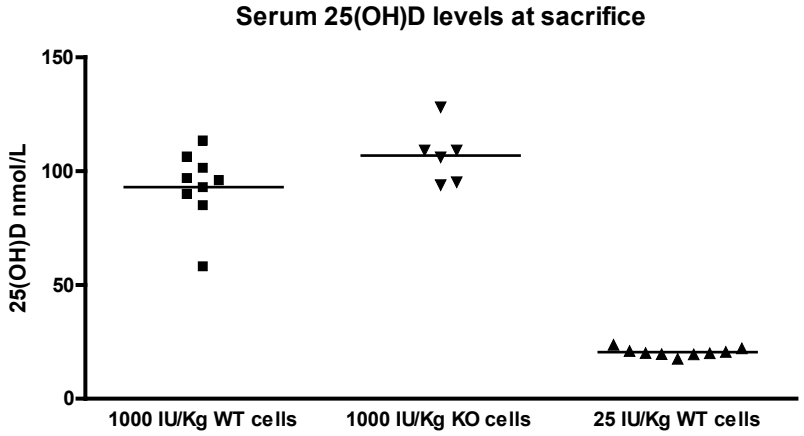


Figure 14 – Serum levels of 25(OH)D at sacrifice for the main mouse experiment. Serum 25(OH)D concentration was measured using the LIAISON total 25(OH)D assay which equally detects $25(OH)D_2$ and $25(OH)D_3$. The serum 25(OH)D levels were not significantly different between the group fed a 1000 IU/Kg diet and injected with wild type tumour cells and the group fed a 1000 IU/Kg diet and injected with 1α-hydroxylase ablated tumour cells, however, there was a significant difference between the mice fed a 25 IU/Kg diet and injected with wild type tumour cells and both groups on the 1000 (IU/Kg) diet ($p<<0.01$). Serum 25(OH)D levels for the mice fed a 25 IU/Kg diet and injected with 1α-hydroxylase ablated tumour cells are not available.

X-ray analysis:

The x-rays taken of the hind limbs at sacrifice were next analyzed to assess the degree of osteolysis within the bone (Figure 15). They were scored by two people independently in a blind manner and were scored according to the following scale: 0 (no lesion), 1 (minor changes), 2 (small lesions), 3 (significant lesions with minor peripheral margin breaks), 4 (significant lesions with major peripheral margin breaks. > 10% of bone surface disrupted). X-ray scoring of bone lesions in the tibiae at sacrifice were as follows: 1000 IU/Kg diet injected with wild type tumour cells = 1.469 ± 0.974, 25 IU/Kg diet injected with wild type tumour cells = 2.125 ± 0.991, 1000 IU/Kg diet injected with 1α-hydroxylase ablated tumour cells = 3.583 ± 0.764, and 25 IU/Kg diet injected with 1α-

hydroxylase ablated tumour cells = 3.875 ± 0.25. There is a statistically significant difference between both groups injected with 1α-hydroxylase ablated tumour cells and those injected with wild type tumour cells ($p<0.05$). Although not statistically significant, the x-ray scores for the groups on the 25 IU/Kg diet and injected with wild type tumour cells were numerically higher than those on the 1000 IU/Kg diet and injected with wild type tumour cells (Figure 15).

Image J was also used to calculate the surface area of the osteolytic lesions of each X-ray. The areas of osteolytic lesions in each group were as follows: 1000 IU/Kg diet injected with wild type tumour cells = 214.6 ± 137.9, 25 IU/Kg diet injected with wild type tumour cells = 1037 ± 551.0, 1000 IU/Kg diet injected with 1α-hydroxylase ablated tumour cells = 2116 ± 884.0, and 25 IU/Kg diet injected with 1α-hydroxylase ablated tumour cells = 2437 ± 386.3. By this more precise method of analyzing the X-rays, significant differences were seen between all groups ($p<0.05$) except for the group on the 1000 IU/Kg diet injected with 1α-hydroxylase ablated tumour cells as compared to the group on the 25 IU/Kg diet injected with 1α-hydroxylase ablated tumour cells (Figure 16). These findings by X-ray analysis suggest a very important role for the local activation of vitamin D in the inhibition of breast cancer tumour cell growth within bone. These results also demonstrate an importance in having sufficient serum vitamin D levels in order to inhibit the growth of breast cancer tumour cells within bone.

Preliminary bone histomorphometry:

Although the bone histomorphometry has not yet been completed, representative slides of the tumour-bearing tibiae can be seen in Figure 17. In general, the mice in the group on the 1000 IU/Kg diet and injected with wild type tumour cells have very little change in the bone structure. Small disruptions of the trabecular bone can be seen (Figure 17A) but the cortex appears normal. The slides of the mice on the 25 IU/Kg diet injected with wild type cells have a bit more destruction of the trabecular bone than the previously described group. This group also has some disturbance of the growth plate but the cortex still remains intact (Figure 17B). The group on the 1000 IU/Kg diet injected with 1α-hydroxylase ablated tumour cells had severe destruction of the trabecular bone, disruption of the growth plate and cortical bone. In this group, the tumour managed to destroy the

cortex and invade the surrounding soft tissue (Figure 17C). Finally the group of mice on the 25 IU/Kg diet and injected with 1α-hydroxylase ablated tumour cells had complete destruction of the trabecular bone structure, of the growth plate and the cortex and invasion of the tumour into the surrounding tissue (Figure 17D).

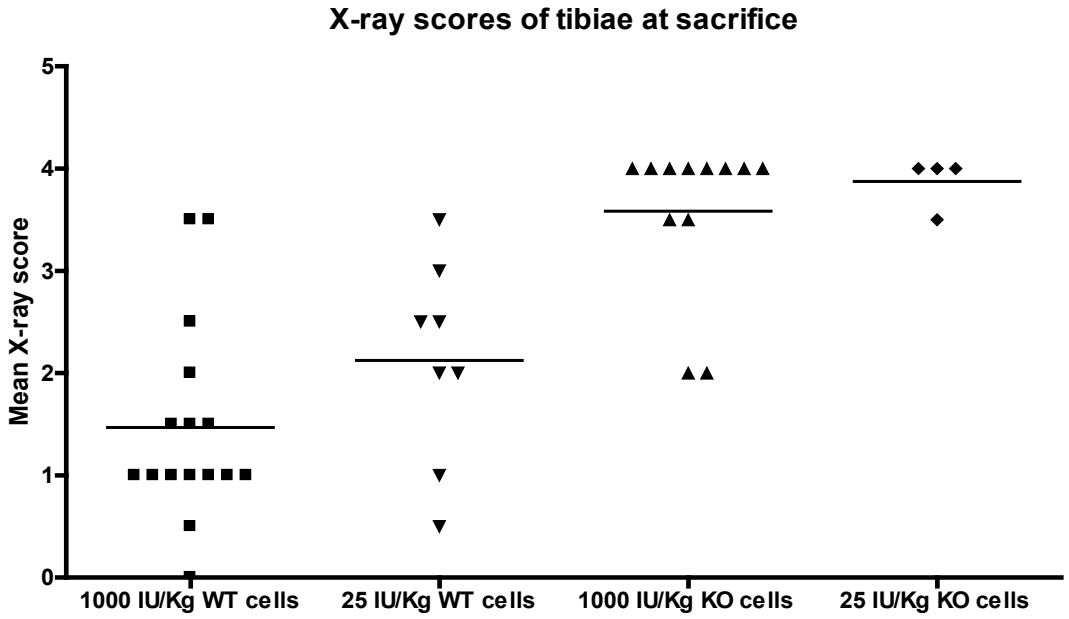


Figure 15 - X-ray scoring of the tumour-bearing tibiae at sacrifice for the main mouse experiment. The x-rays were scored according to the following scoring method: 0 (no lesion), 1 (minor changes), 2 (small lesions), 3 (significant lesions with minor peripheral margin breaks), 4 (significant lesions with major peripheral margin breaks. > 10% of bone surface disrupted). The x-rays were scored by two people independently and was performed in a blind fashion in which the scorer did not know to which group the mouse belonged. There is a statistically significant difference between both groups injected with 1α- hydroxylase ablated tumour cells and those injected with wild type tumour cells ($p<0.05$). Although not statistically significant, the x-ray scores for the groups on the 25 IU/Kg diet and injected with wild type tumour cells were numerically higher than those on the 1000 IU/Kg diet and injected with wild type tumour cells.

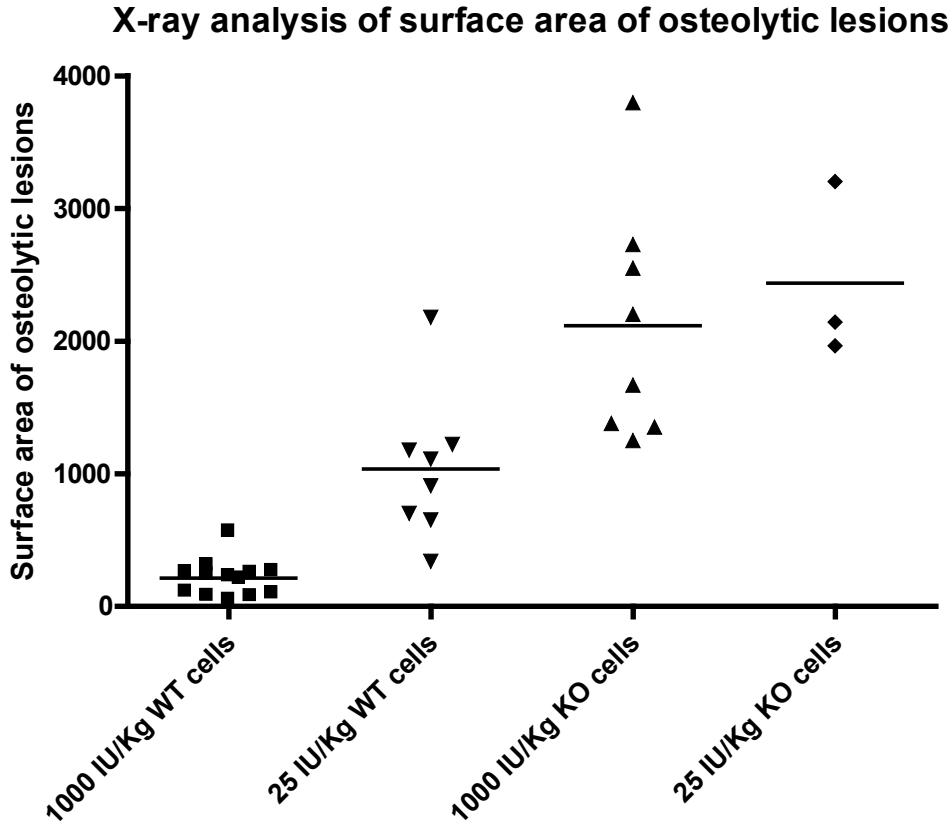


Figure 16 – Image J analysis of the surface area of the osteolytic lesions in the X-rays of the tibiae. Image J was used to trace the osteolytic lesions in each X-ray of the tibiae and the surface area was calculated. There was a significant difference in the osteolytic surface area between each group ($p<0.05$) except for the group on the 1000 IU/Kg diet injected with 1α-hydroxylase ablated tumour cells as compared to the group on the 25 IU/Kg diet injected with 1α-hydroxylase ablated tumour cells.

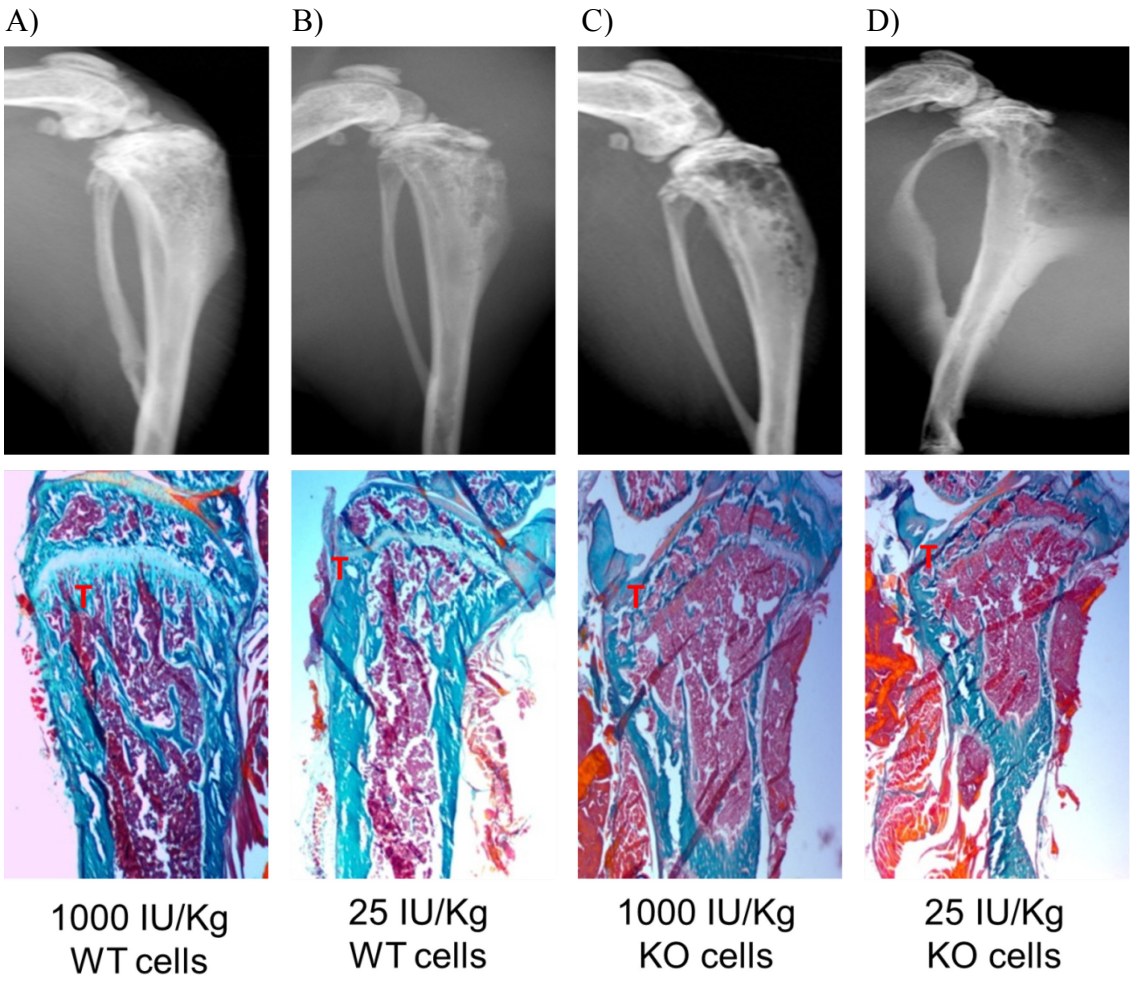


Figure 17 – Representative x-rays and histology of the tumour-bearing tibiae or the main mouse experiment. Mice on the 1000 IU/Kg diet injected with wild type tumour cells displayed minor osteolytic lesions in the x-ray and very little disturbance of the bone architecture can be seen in the histology. Mice on the 25 IU/Kg diet injected with wild type cells display more areas of osteolytic lesions in the x-ray, and more disturbance of the growth plate and trabecular bone caused by growth of the tumour within the tibia. The mice on the 1000 IU/Kg diet injected with 1α-hydroxylase ablated tumour cells have severe osteolysis of the tibia which can be seen in the x-ray. The histology shows disruption of the growth plate, trabecular bone, and cortical bone and growth of the tumour within the bone as well as outside into the surrounding tissue. Finally, the x-rays for the mice on the 25 IU/Kg diet injected with 1α-hydroxylase ablated tumour cells show complete destruction of the bone by the tumour and its spread to the surrounding tissue.

The histology shows complete disruption of the cortex on both sides of the tibia, destruction of the growth plate and trabecular bone, and growth of the tumour into the surrounding tissue.

Discussion

We chose to investigate the effect of vitamin D deficiency and the local activation of vitamin D by tumour cells on the growth of breast cancer tumour cells within bone. We began by treating wild type and 1α-hydroxylase ablated breast cancer tumour cells that were isolated from breast tumours on PyMT 634 mice and grown in culture with 1,25-dihydroxyviamin D_3 to demonstrate that both cell types would respond to 1,25-dihydroxyvitamin D_3. In addition, we treated both the wild type and 1α-hydroxylase ablated tumour cells with $25(OH)D_3$ in order to demonstrate that the wild type tumour cells would respond to 25-hydroxyvitamin D_3 whereas the 1α-hydroxylase ablated tumour cells would not as they are unable to activate the hormone. As expected, both the wild type and 1α-hydroxylase ablated tumour cells showed significant inhibition of cell proliferation and viability when treated with 1,25-dihydroxyvitamin D_3. We also saw significant inhibition of proliferation of wild type breast tumour cells when treated with 25-hydroxyvitamin D_3, suggesting that these cells which express 1α-hydroxylase are able to activate the 25-hydroxyvitamin D_3 to the active compound, 1,25-dihydroxyvitamin D_3 which can then act on the cells to inhibit proliferation. When the 1α-hydroxylase ablated tumour cells were treated with 25-hydroxyvitamin D_3, they did not respond to the treatment except at a concentration of 10^{-6} M. When treated with 10^{-6} M 25-hydroxyvitamin D_3, these cells had a drastic decrease in cell proliferation as compared to vehicle treated control cells. It is possible that this may be due to cytotoxic effects of the high concentration of $25(OH)D_3$ on the cells. This is also possible for the wild type cells as can be seen in Figures 6 and 7, since the wild type cells also displayed a drastic decrease in cell proliferation and viability with treatment of 10^{-6} M $25(OH)D_3$ as compared to the inhibition seen at concentrations ranging from 10^{-9} to 10^{-7} M. Another alternative explanation for the inhibition of cell proliferation seen in the 1α-hydroxylase ablated tumour cells could be that the 25-hydroxyvitamin D_3 can act as a vitamin D receptor ligand at the high concentration of 10^{-6} M. It has been shown by Lou *et al* that in human prostate cancer cells a concentration of 500 nM $25(OH)D_3$ can inhibit the growth of these cells by up to 60% [211]. It is possible that this is why we are seeing growth inhibition of the 1α-hydroxylase ablated tumour cells when treated with high concentrations of $25(OH)D_3$.

Once we had established that the wild type tumour cells were responsive to both $1,25(OH)_2D_3$ and $25(OH)D_3$, we went on to perform the preliminary mouse experiment in which one group of mice was fed a vitamin D deficient diet of 25 IU/Kg, and another group of mice was fed a normal vitamin D diet of 1000 IU/Kg. Both groups were injected into the right tibia with wild type PyMT breast cancer tumour cells after seven weeks on the special diets. Five weeks following tumour cell injections the mice were sacrificed, blood was collected, and x-ray scoring was assessed. As expected vitamin D deficiency was established in the mice on the vitamin D deficient diet as compared to those on the vitamin D sufficient diet.

We next analyzed the osteolytic lesions by X-ray scoring in order to assess tumour burden. We expected to see greater tumour growth in the vitamin D deficient mice due to the increased bone turnover that results from vitamin D deficiency and the loss of the direct growth inhibitory effect of vitamin D on the tumour cells. However, what we saw was no significant difference between the two groups. We let the tumours grow for five weeks following tumour cell injections before the mice were sacrificed and the tumours were analyzed. By this point the tumours had grown very large and broken through the cortical bone and had invading the surrounding tissue. We believe that the period of time between the intratibial injections the time of sacrifice exceeded the point at which we would be able to distinguish any differences between the groups. It is possible that the effect of vitamin D could be seen during the early stages of tumour development but by waiting five weeks to sacrifice the mice, we were unable to see an effect.

In a paper by Ooi *et al,* immunocompromised mice were put on either a vitamin D deficient diet or a normal vitamin D diet and were injected into the tibia with human breast cancer cells. They analyzed the difference between the growth of the tumours between the groups at two time points, 14 days and 28 days. They found that the mice on the vitamin D deficient diet developed tumours which could be seen radiographically earlier than the mice on the vitamin D sufficient diet. Furthermore, at 14 days after tumour cell injections they saw significantly greater lesion areas in the mice on the vitamin D deficient diet as compared to those on the vitamin D sufficient diet. However, they found no significant difference in the size of the lesion areas 28 days following

tumour cell injections. [202] This may explain why we did not see a significant difference in the x-ray scoring of the lesion areas between our groups since we waited 35 days to sacrifice the mice and analyze tumour burden. It is possible that by this time point in tumour development, the growing tumour has taken control of the bone microenvironment and the vicious cycle of osteolytic bone metastasis is prevailing, masking any effects of the vitamin D on the growth of the tumour. We would have liked to be able to monitor the tumour growth by X-rays at various time points after tumour cell injections however this was not possible due to animal facility policies.

Since we saw no significant difference between groups in the X-ray scoring for the preliminary mouse experiment, we decided to repeat the experiment with some modifications and to expand it to look at the effect of the local activation of vitamin D by tumour cells on the growth of these tumour cells within bone. This time the mice were on the special vitamin D diets for six weeks prior to tumour cell injections instead of seven weeks as in the first mouse experiment. We chose to wait six weeks prior to tumour cell injections this time since a similar experiment by Ooi *et al* determined that after four weeks on a vitamin D deficient diet, vitamin D deficiency was established in a mouse model [202]. Six weeks would assure appropriate vitamin D levels at the time of tumour cell injections. Also, in this experiment we did not wait as long after tumour cell injections to sacrifice the mice. Instead of allowing the tumours to grow within the bone for five weeks prior to sacrifice, we sacrificed the mice three weeks following tumour cell injections. We also added two more treatment groups of mice that were not present in the first mouse experiment. This time we included a group which was fed a normal vitamin D diet and injected with 1α-hydroxylase ablated tumour cells and another group on a vitamin D deficient diet and injected with 1α-hydroxylase ablated tumour cells. These groups were used in order to investigate the effect of the local activation of $25(OH)D_3$ by tumoral 1α-hydroxylase on the growth of breast cancer tumour cells within bone.

In this experiment, we saw significantly lower levels of 25(OH)D in the mice on the vitamin D deficient diet as compared to those on the normal vitamin D diet as expected. Although we saw some differences between the levels of creatinine and phosphorus in the group on the vitamin D deficient diet and injected with 1α-hydroxylase ablated tumour

cells as compared to the other groups, this is most likely due to the very small sample size. Unfortunately there were only four mice in this group and only three of these mice had sufficient amounts of serum extracted to be analyzed. In order to draw any real conclusions regarding serum levels, this experiment would have to be repeated with a greater number of mice in this group. The elevated creatinine and phosphorus could be an indicator of kidney failure in this group.

We next went on to analyze the x-rays taken at sacrifice to assess differences in the degree of osteolysis by X-ray scoring between groups, and obtained very interesting results. We found a significant difference in x-ray scoring of both groups injected with 1α-hydroxylase ablated tumour cells as compared to both groups injected with wild type tumour cells. The mice injected with 1α-hydroxylase ablated tumour cells had significantly greater x-ray scoring and therefore greater tumour osteolysis within bone than the mice injected with wild type cells. This suggests an important role for the local activation of vitamin D on the growth inhibition of breast cancer tumour cells within bone. Although we did not see statistically significant differences in the x-ray scoring between the two groups injected with wild type tumour cells, there was a small numerical difference in which the vitamin D deficient mice showed a trend towards worse osteolytic lesions than the mice on the vitamin D sufficient diet. As X-ray scoring is not the most precise way to analyze the degree of osteolysis within bone, image J was also used to calculate the surface area of the osteolytic lesions in the X-rays. This technique confirmed the results from the X-ray scoring with the same trends seen in each group, however with this more precise analytical method all groups had statistically significant differences between groups except for the group on the 1000 IU/Kg diet injected with 1α-hydroxylase-ablated tumour cells as compared to the group on the 25 IU/Kg diet injected with 1α-hydroxylase-ablated tumour cells. With this analysis there was a significant difference between the group on a 1000 IU/Kg diet injected with wild type tumour cells and the group on the 25 IU/Kg diet injected with wild type tumour cells which we did not see with simple X-ray scoring. Therefore, this suggests that vitamin D status is an important factor in breast cancer tumour cell growth within bone along with the local activation of vitamin D. Future hitstomorphometric analysis should further confirm the findings by X-ray analysis.

Future Directions

We plan on first completing the bone histomorphometric analysis of the tibiae collected from both mouse experiments in order to confirm the results found by x-ray scoring. We will assess the size of the tumours and on the cellular level, and examine the number of osteoclasts and osteoblasts to assess bone turnover. We may be able to see significant differences between the group on the 1000 IU/Kg diet injected with 1α-hydroxylase ablated tumour cells and the group on the 25 IU/Kg diet injected with 1α-hydroxylase ablated tumour.

We plan on performing μCT analysis of a representative sample of the tumour-bearing tibiae from each treatment group. This will help us quantify the areas of osteolytic lesions that we observed in the x-rays taken at sacrifice. It will also allow us to analyze the changes in bone structure and allow us to examine the bones in three dimensions.

We also plan on repeating the main mouse experiment using a greater number of mice in each group. In particular, the group on the low vitamin D diet and injected with 1α-hydroxylase ablated tumour cells consisted of only four mice and therefore a larger number is required to be able to confirm the findings from this experiment. We would also like to collect a sample of the tumours from the legs of the mice in order to extract the RNA to investigate differences in the expression of certain genes encoding proteins such as AKT, CXCR4, VEGF, and RANKL between the groups on the normal vitamin D diet and the groups on the vitamin D deficient diet. We would also like to compare the expression of these and other genes between the groups injected with wild type tumour cells and 1α-hydroxylase ablated tumour cells in order to have a better understanding of the mechanism responsible for the great differences we saw between these groups.

It has previously been shown in our laboratory that 1α-hydroxylase ablation within the breast epithelial cells leads to an increased growth rate and earlier development of breast tumours in CYP27B1 flox/flox cre+ PyMT mice than in wild type PyMT mouse primary breast tumours [205]. Although the effect seen in the bone microenvironment is similar, we see much greater tumour cell growth with 1α-hydroxylase ablation in the bone microenvironment than in the primary tumour site. We would like to explore what factors

in the bone microenvironment may be amplifying this effect and which signalling pathways may be differentially expressed in tumours in the bone microenvironment as compared to the primary tumour site. Some factors we are interested in exploring are AKT, RANKL, CXCR4, and VEGF, among others.

We also plan on analyzing the serum from each treatment group for osteocalcin and tartrate-resistant acid phosphatase 5b in order to look at bone turnover. We would like to confirm that there was increased bone turnover in the vitamin D deficient mice as compared to the vitamin D sufficient mice. We would also like to analyze the serum level of $1,25(OH)_2D_3$ between the groups. In a paper by Swami *et al* it was found that mice with a breast cancer xenograft fed a high vitamin D diet of 5000 IU/Kg had elevated levels of $1,25(OH)_2D_3$ in the circulation as compared to control mice which were fed the same 5000 IU/Kg vitamin D diet but were not tumour bearing [128]. They also found that the high vitamin D diet did not lead to an increase in renal CYP27B1 expression however the high vitamin D diet caused a significant increase in the tumoral CYP27B1 expression [128]. This data suggests that the elevated serum $1,25(OH)_2D_3$ levels seen in tumour bearing mice on a 5000 IU/Kg vitamin D diet as compared to non-tumour bearing mice on the same 5000 IU/Kg vitamin D diet is not due to increased renal activation of vitamin D but rather activation at another source, possibly at the tumour site. It would be interesting to investigate if the same phenomenon is seen in our mouse model of bone metastasis. Due to limited volumes of serum collected it was not possible to analyze $1,25(OH)_2D_3$ in our first two mouse experiments.

We are also interested in looking at the effect of vitamin D supplementation through the diet on the effect of breast cancer tumour growth within bone in combination with bisphosphonate treatment as this is the current treatment for bone metastasis in breast cancer patients. Since a bisphosphonate such as zoledronic acid inhibits bone turnover, it would be interesting to investigate whether locally produced $1,25(OH)_2D_3$ by tumour cells plays a role in inhibiting the growth of these tumour cells within bone, independent of bone turnover. It could also be interesting to study the effect of vitamin D deficiency on the growth of breast cancer cells within bone in ovariectomized mice. This would

mimic the hormonal situation seen in menopause, the time in life when women are most likely to develop breast cancer.

Overall more work needs to be conducted in order to determine by which mechanism or mechanisms locally activated vitamin D is able to inhibit tumour growth within bone and to determine how this information could be used to treat breast cancer patients most effectively.

Significance

Although there has been great progress in the early detection and treatment of breast cancer, bone metastasis continues to be a very serious and debilitating consequence of breast cancer progression. In fact, once it has metastasized to bone, it is generally considered to be incurable. Through our experiments using a mouse model of breast cancer metastasis to bone, we have demonstrated that the local activation of vitamin D by breast cancer cells plays a significant role in inhibiting the growth of these tumour cells within the bone microenvironment. This could lead to very useful clinical applications as it suggests that instead of treating breast cancer patients with the active metabolite, $1,25(OH)_2D_3$, it may be possible to elevate a patient's $25(OH)D_3$ levels with the same effect on inhibition of tumour cell growth without running the risk of hypercalcemia.

While we have demonstrated that local activation of vitamin D can significantly inhibit breast cancer cell growth within bone, it did not lead to complete cessation of tumour growth. Therefore, vitamin D supplementation would most likely prove useful as a combination therapy, administered in combination with the currently used bisphosphonates. Further research into combination therapies with vitamin D should be considered.

Conclusion

Although a link between vitamin D and breast cancer has been shown, very little is known about the importance of the local activation of vitamin D on the growth of breast cancer tumours. Using a murine model of breast cancer tumour growth within bone, we have demonstrated that the local activation of 25(OH)D by tumoral 1α-hydroxylase plays a very important role in the inhibition of breast cancer tumour cell growth in the bone microenvironment. This finding is very interesting as this would mean that instead of treating patients with $1,25(OH)_2D_3$ to treat breast cancer bone metastasis, it may be possible to treat patients with $25(OH)D_3$. Although the activation of vitamin D by renal 1α-hydroxylase is tightly regulated, this is not the case for tumoral 1α-hydroxylase and therefore it may be possible to increase the levels of $1,25(OH)_2D_3$ at the tumour site without running the risk of hypercalcemia which can occur with high circulating $1,25(OH)_2D_3$ levels.

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