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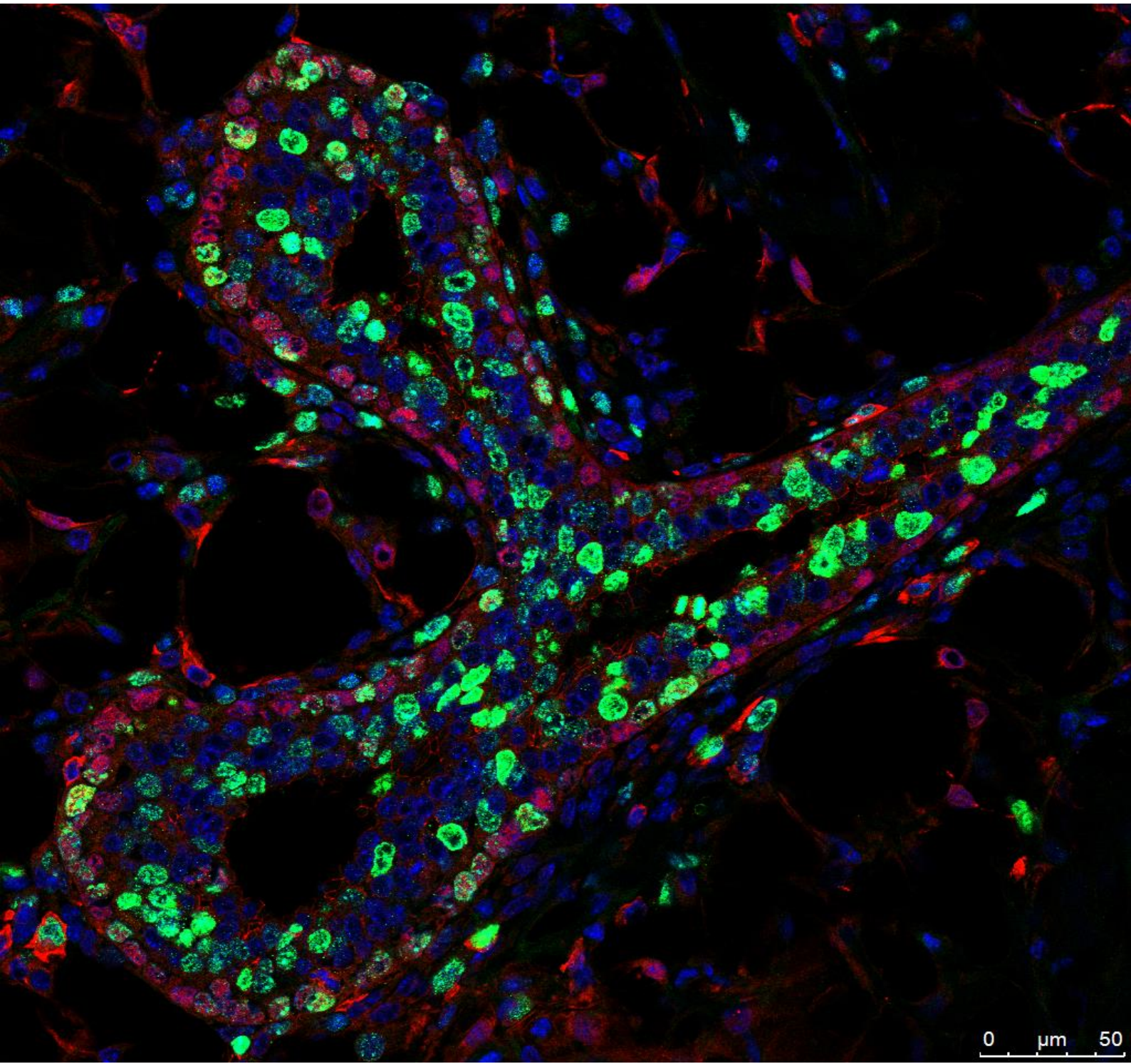
VITAMIN D SIGNALING PATHWAY AND BREAST CANCER

Doctoral Thesis by

Lei Sheng



Vitamin D Signaling Pathway and Breast Cancer

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This thesis is submitted in fulfilment of the requirements for the

Doctor of Philosophy

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Overview

Although the mortality rate of breast cancer has continued to decrease over the past several decades, it remains the most frequently diagnosed cancer and the leading cause of cancer death in women worldwide. Accumulating epidemiological and clinical evidence suggest that vitamin D insufficiency is associated with increased breast cancer incidence and poor clinical outcomes in patients with breast cancer, which makes vitamin D supplementation a potential preventive or therapeutic option for the management of breast cancer. However, the detailed mechanisms on how vitamin D protects against breast cancer remains largely unknown.

This thesis mainly investigates the role of vitamin D signaling pathway in the prevention of breast cancer. There are two major themes. The first theme focuses on identifying target genes and molecular pathways of vitamin D in human breast cancer, while the second theme aims to characterize the role of the vitamin D inactivating enzyme Cyp24a1 in the development of mouse mammary gland morphogenesis. This thesis is in the publication format with Chapter III as a published article, Chapter IV, V, and VI as manuscripts in preparation, while the remaining parts of this thesis (Chapter I and II) are regarded as an introduction.

Theme 1: Identification of vitamin D3 target genes in human breast cancer tissue

Chapter I summarizes the basic knowledge of vitamin D metabolism in breast tissue, our current understandings of the anti-tumor effects of vitamin D, and the role of vitamin D in breast cancer prevention based on epidemiological and clinical studies. Target genes of the vitamin D signaling pathway mainly generated from investigation of breast cancer cell lines are also reviewed.

Chapter II presents the evidence from randomized clinical trials that vitamin D, plus or minus calcium supplementation, has the potential to prevent breast cancer. Our analyses indicate that the available data are insufficient to confirm any protective effect of vitamin D supplementation, with or without calcium, on the risk of breast cancer. More participants in future trials are required to make a reliable and conclusive assessment.

Chapter III, published as an original research article in the Journal of Steroid Biochemistry and Molecular Biology, determines the target genes and molecular pathways influenced by $1,25(OH)_2D$ in normal human breast and cancer tissues in an *ex vivo* explant system. RNA-Seq revealed 523 genes that were differentially expressed in breast cancer tissues in response to $1,25(OH)_2D$ treatment, and 127 genes with altered expression in normal breast tissues. The major finding of the present study is that exposure of both normal and malignant breast tissue to $1,25(OH)_2D$ results in changes in cellular adhesion, metabolic pathways and tumor suppressor-like

pathways, which support epidemiological data suggesting that adequate vitamin D3 levels may improve breast cancer outcome.

Theme 2: Characterization of the vitamin D-inactivating enzyme Cyp24a1 in pubertal mouse mammary gland morphogenesis.

Chapter IV, presented as a manuscript in preparation, summarizes our current understanding from transgenic mouse models of the vitamin D receptor (Vdr) and vitamin D associated metabolic enzymes in mammary gland development, cancer initiation, and progression and discusses the implications of these findings for human breast cancer. An improved understanding of the mechanisms of action of vitamin D signaling pathway derived from these mouse models provides support that activation of vitamin D signaling pathway is a potential approach for human breast cancer prevention.

Chapter V, also presented as a manuscript in preparation, characterizes the role of vitamin D-inactivating enzyme Cyp24a1 in mammary gland development. A novel mouse model was available with conditional knockout of the *Cyp24a1* gene specifically in the mammary epithelium (MMTV-Cre x Cyp24a1$^{lox/lox}$). Our major finding is that mammary glands from virgin *Cyp24a1* knockout females display impaired ductal morphogenesis compared with age- and weight-matched wild-type mice, which is due

to reduced proliferation of mammary epithelial cells with the ablation of Cyp24a1 activity.

The final chapter (VI), presented as a manuscript in preparation, explores the regulatory network of RANKL expression in human breast cells. Although *RANKL* is well characterized as a target gene of vitamin D in bone tissue, it becomes a different story when it comes to breast tissue.

Taken together, although the detailed mechanisms of how women with adequate levels of vitamin D have a lower incidence of breast cancer are yet to be defined, our findings definitely contribute to a better understanding of the role of vitamin D signaling pathway in the prevention of breast cancer. Our work focuses on analysing data from human breast cancer tissue and transgenic mouse models, which could facilitate translation from the bench to the bedside and ultimately benefit the welfare of people in general.

Publications

Original Research Paper and published abstract

1. Lei Sheng, Paul H. Anderson, Andrew G. Turner, Kathleen I. Pishas, Deepak J. Dhatrak, Peter G. Gill, Howard A. Morris, David F. Callen. Identification of vitamin D_3 target genes in human breast cancer tissue. Journal of Steroid Biochemistry and Molecular Biology. 164 (2016)90-97.
2. Lei Sheng, Andrew G. Turner, Gerard A. Tarulli, Kate Barratt, Richard Kremer, Howard A. Morris, David F. Callen, Paul H. Anderson. Conditional inactivation of the 25-hydroxyvitamin D-24-hydroxylase (Cyp24a1) in the mouse mammary epithelium alters mammary gland development [abstract]. In: Proceedings of the Thirty-Ninth Annual CTRC-AACR San Antonio Breast Cancer Symposium; 2016 Dec 6-10; San Antonio, TX. Philadelphia (PA): AACR; Cancer Res 2017; 77(4 Suppl): Abstract nr P4-05-02.

Collaborative Cancer Research Publications

3. Ya'nan Yang*, Xue Yin*, Lei Sheng*, Shan Xu, Lingling Dong, and Lian Liu. Perioperative chemotherapy more of a benefit for overall survival than adjuvant chemotherapy for operable gastric cancer: an updated Meta-analysis. Scientific Reports. 5, 12850; doi: 10.1038/srep12850 (2015) (*Contribute equally to this work).

4. Yan Li, Xin-Yue Liang, Yi-Qi Yue, Lei Sheng, Ji-Kai Liu, Zhan-Yu Wang, Gang Chen. The addition of drugs targeting the vascular endothelial growth factor pathway to first-line chemotherapy increase complete response? A meta-analysis of randomized clinical trials. Tumor Biol. (2016) 37: 6297. doi:10.1007/s13277-015-4493-9.
5. Reshma Shakya, Gerard Tarulli, Lei Sheng, Noor Alia Lokman, Carmela Ricciardelli, Kathleen Irene Pishas, Christina I Selinger, Maija RJ Kohonen-Corish, Wendy A Cooper, Andrew Grant Turner, Paul Matthew Neilsen, David Frederick Callen. Mutant p53 upregulates Alpha-1 Antitrypsin (A1AT) expression and promotes invasion in lung cancer. Accepted by the Journal Oncogene on 7th February 2017.

Poster Presentations

1. Conference poster presentation: 2016 San Antonio Breast Cancer Symposium, San Antonio, TX, USA, Dec 2016.
2. Conference poster presentation: 2016 Florey International Postgraduate Research Conference, National Wine Center, Adelaide, SA, Australia, Oct 2016.
3. Conference poster presentation: 27th Lorne Cancer Conference, Lorne VIC, Australia, Feb 2015.
4. Conference poster presentation: 2014 Florey International Postgraduate Research Conference, National Wine Center, Adelaide, SA, Australia, Sep 2014.

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Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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CHAPTER I

INTRODUCTION: VITAMIN D SIGNALING PATHWAY AND BREAST CANCER

1.1 Introduction

Although the mortality rate of breast cancer has continued to decrease over the past several decades, it remains the most frequently diagnosed cancer and the leading cause of cancer death in women worldwide, accounting for 23% of all new cancer cases and 14% of the total cancer deaths estimated in 2008 [1]. Because of the application of mammography screening programs and decline in the use of hormonal replacement therapy, the incidence of advanced breast cancer at diagnosis is decreasing in the USA [2]. However, there is a lack of any preventive strategies that can reduce the overall incidence of breast cancer in women. Therefore, it is imperative to discover effective approaches that may reduce risk of breast cancer.

Epidemiological data suggest that the incidence and severity of many types of cancer, including breast cancer, inversely correlates with serum 25-hydroxyvitamin D (25(OH)D) concentration [3], indicating vitamin D as a potential nutrient for breast cancer prevention. This review summarises our current understandings of vitamin D metabolism in breast tissue, the anti-tumor effects of vitamin D and the role of vitamin D in breast cancer prevention.

1.2 Vitamin D metabolism

1.2.1 The endocrine paradigm of vitamin D metabolism

The major source of vitamin D in the circulating system is obtained when 7-dehydrocholesterol in the skin is exposed to the ultraviolet B (UVB) radiation. An

alternative source is from dietary consumption [4] (Fig 1). Vitamin D is transported in the circulation mainly by binding to the vitamin D binding protein (DBP). There are several *in vivo* steps in the metabolism of vitamin D. First, vitamin D is hydroxylated and converted to the prohormone 25(OH)D by 25-hydroxylase (CYP2R1) in the liver [4]. 25(OH)D is the circulating form of vitamin D and the best indicator of the body's vitamin D status. A second hydroxylation by 1-α-hydroxylase (CYP27B1) in the kidney generates 1,25-dihydroxyvitamin D ($1,25(OH)_2D$, also known as calcitriol), the biologically active form of vitamin D [4]. Both 25(OH)D and $1,25(OH)_2D$ are finally degraded to less active vitamin D metabolites $24,25-(OH)_2D$ and $1,24,25-(OH)_3D$ respectively by the enzyme 24-hydroxylase (CYP24A1). Serum $1,25(OH)_2D$ concentrations are feedback regulated. While *CYP27B1* expression is induced by parathyroid hormone (PTH) and repressed by $1,25(OH)_2D$ [5], *CYP24A1* expression is strongly induced by $1,25(OH)_2D$ [6]. So the blood level of $1,25(OH)_2D$ is determined by the balance of the CYP24A1 and CYP27B1 enzyme activities, which are inversely regulated by $1,25(OH)_2D$.

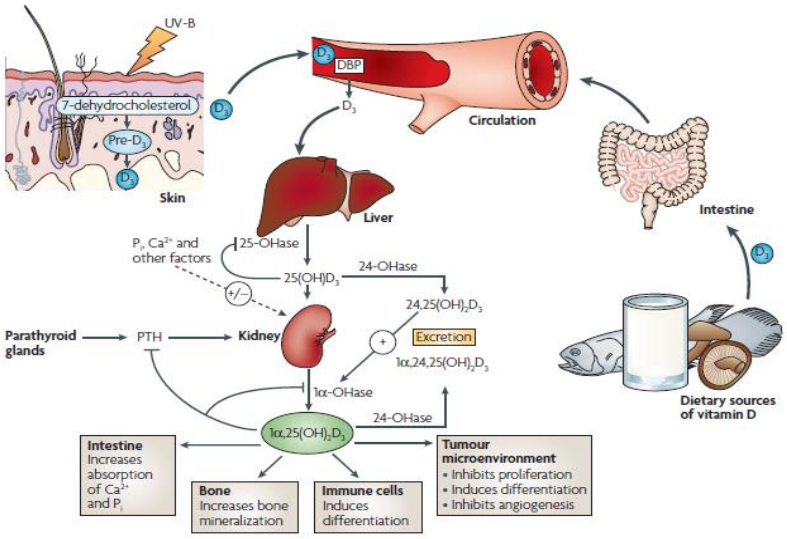

Fig 1. Vitamin D metabolism. (*Deeb, 2007*)

1.2.2 The paracrine/autocrine paradigm of vitamin D metabolism

$1,25(OH)_2D$ functions in an autocrine and paracrine manner since the vitamin D metabolizing enzyme CYP27B1 is expressed in a variety of extra-renal sites, such as skin, immune system, brain, placenta, pancreas and breast tissues [7, 8]. The circulating 25(OH)D can be locally absorbed and converted to $1,25(OH)_2D$ by the enzyme CYP27B1 expressed in the above mentioned tissues. The locally generated $1,25(OH)_2D$ influences cell proliferation, apoptosis and/or modulation of the immune system [9, 10]. The presence of $1,25(OH)_2D$ strongly up-regulates *CYP24A1* gene expression [6, 7], which may offset any anti-tumor effects of $1,25(OH)_2D$, by degrading $1,25(OH)_2D$ to less active metabolites. The levels of biologically active $1,25(OH)_2D$ in the surrounding tissue are critically dependent on the balance of localised activation of 25(OH)D by CYP27B1 enzyme and the subsequent degradation through the action of the CYP24A1 enzyme. In bone tissues, unlike in the kidney, the genetic expression of *CYP27B1* and *CYP24A1* are positively correlated, but independent of the circulating $1,25(OH)_2D$ levels [11], which supports the notion that vitamin D metabolism has both autocrine and paracrine function. Normally the levels of *CYP24A1* expression are suppressed through the action of the unliganded VDR (vitamin D receptor, VDR) [12], but are rapidly and highly induced in the presence of $1,25(OH)_2D$. Since $1,25(OH)_2D$ is toxic to cells, this provides a negative

regulatory mechanism for initiating intracellular autocrine signaling, and minimises any damaging cell toxicity that could result from elevated levels of 1,25$(OH)_2$D.

1.2.3 The paracrine/autocrine paradigm of vitamin D metabolism in the breast

Mammary epithelial cells express three genes (*VDR*, *CYP27B1* and *CYP24A1*) required to metabolise vitamin D and activate vitamin D signaling pathways. The VDR is expressed and functional in non-transformed human mammary epithelial cells (HMECs) [13]. In mice, *Vdr* expression increases 100-fold during the course of pregnancy and lactation while *Vdr* knockout mice exhibit excess mammary gland proliferation and branching with impaired apoptosis during the reproductive cycle [14, 15]. These data suggest that the vitamin D signaling pathway participates in a growth inhibitory network during normal mammary gland development. Studies with cultured non-transformed HMECs have demonstrated the conversion of 25(OH)D to the active form 1,25$(OH)_2$D through the expression of CYP27B1 [13]. In *CYP27B1* knockout HMECs, 1,25$(OH)_2$D clearly induced *CYP24A1*, the endogenous target gene of 1,25$(OH)_2$D, but 25(OH)D did not [13]. Furthermore, these HMECs co-express megalin and cubilin, which contribute to the endocytic uptake of the circulating 25(OH)D-DBP complex. Collectively, these data strongly indicate that mammary cells are capable of taking up the circulating 25(OH)D, transactivating 25(OH)D to 1,25$(OH)_2$D by the enzyme CYP27B1, which in turn initiates biological activities such as growth arrest through activating VDR in an autocrine/paracrine manner.

Analysis of benign and malignant breast lesions has confirmed the expression of VDR and two metabolising enzymes CYP27B1 and CYP24A1, which are required to regulate the vitamin D activity [8, 16]. In our previous study, the *CYP24A1* expression was upregulated when the explanted non-malignant and malignant breast tissues were treated either with 25(OH)D or 1,25$(OH)_2$D [17] (Fig 2), which suggests that the breast tissues have the metabolic potential to locally convert 25(OH)D into 1,25$(OH)_2$D. Taken together, the breast tissues contain all the necessary components of a vitamin D signaling axis that mediates metabolism of the circulating prohormone 25(OH)D, local synthesis and inactivation of 1,25$(OH)_2$D.

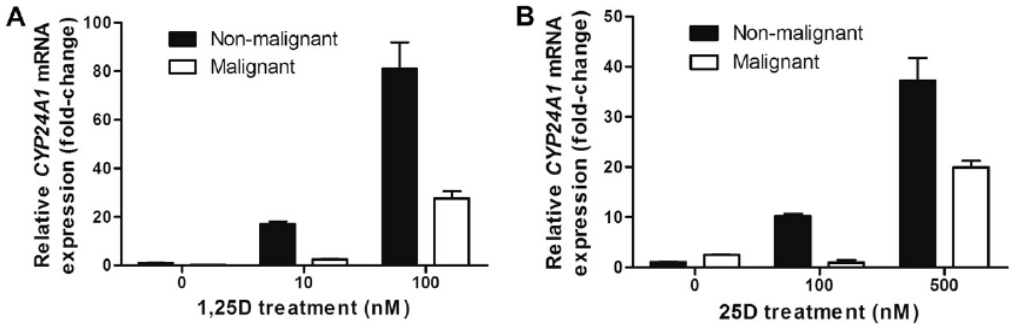

Fig 2. Induction of *CYP24A1* expression by vitamin D metabolites in paired non-malignant and malignant breast tissue. (*Suetani, 2013*)

1.3 Biological function of Vitamin D

The biologically active hormone, 1, 25$(OH)_2$D, has a wide range of biological functions through interacting with vitamin D receptor (VDR), a member of the steroid/thyroid hormone receptor family [18]. The major target tissues of 1, 25$(OH)_2$D are the intestine and bone, where it promotes intestinal calcium absorption and mobilizes osteoclastic activity [19]. In other tissues, such as the colon, prostate and

breast, 1, 25$(OH)_2$D is thought to be involved in controlling cell growth, cellular differentiation, inducing apoptosis and decreasing angiogenesis [20, 21]. Furthermore, 1, 25$(OH)_2$D exerts an immunomodulatory activity on immune cells, in particular on antigen presenting cells (APCs), such as macrophages [22] and dendritic cells [23, 24], T cells [25], as well as B lymphocytes [26] . Of great current interest is the role of vitamin D in decreasing the risk of many chronic diseases, such as cancers, immune diseases, infectious diseases and cardiovascular diseases [4]. This review focuses on discussing the role of vitamin D in bone health, normal mammary gland development and cancer, in particular breast cancer.

1.3.1 The effect of vitamin D in bone

The main effect of 1, 25$(OH)_2$D is to stimulate the absorption of calcium and phosphorus from the gut [27, 28]. 1, 25$(OH)_2$D, as well as calcium, is an essential element for bone mineralization and calcium homeostasis [29]. Although there is no consensus on optimal circulating 25(OH)D levels, vitamin D deficiency is defined by most experts as a serum 25(OH)D level of less than 50 nmol/L [30-32]. In cases of vitamin D deficiency, the low circulating levels of vitamin D cause less absorbed calcium available for bone mineralization. Lower levels of serum calcium stimulate the secretion of parathyroid hormone (PTH), which maintains serum calcium levels at the expense of increased bone turnover, bone loss and increased risk of fractures [33] (Fig 3). Epidemiological studies show positive correlations between vitamin D deficiency and lower bone mineral density [34], higher bone turnover and higher

fracture incidence [35, 36]. For example, the serum 25(OH)D levels below or equal to 30 nmol/L were associated with an almost 3-fold increase in fracture risk in persons aged 65-75 years old [36]. Vitamin D supplementation studies have demonstrated an increase of bone mineral density, a decrease of bone turnover, as well as fracture incidence. A meta-analysis of randomized controlled trials suggests oral vitamin D supplementation between 700 to 800 IU/d appears to reduce the risk of fracture in elderly persons. But vitamin D dose of 400 IU/d is insufficient for fracture prevention [37].

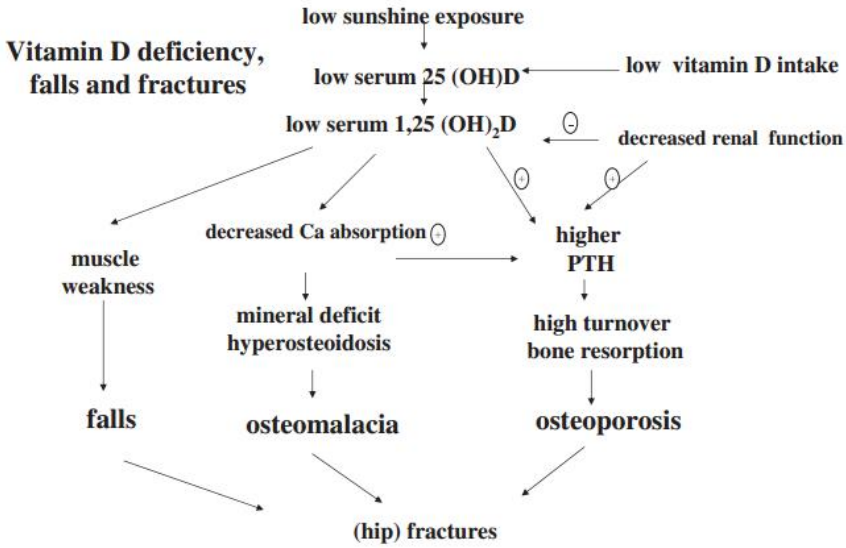

Fig 3. The pathophysiological pathways from vitamin D deficiency to osteoporosis, osteomalacia, falls and fracture (*Lips, 2011*).

1.3.2 The effect of vitamin D in the murine mammary gland

It is well known that estrogen, progesterone and prolactin are the master regulators of postnatal mammary gland development. Estrogen was shown to promote ductal elongation during puberty [38], whereas progesterone is required for side branching

[39]. Prolactin signaling is required in mammary epithelium for lobuloalveolar development and differentiation into milk-producing cells during pregnancy [40]. However, additional regulators, such as growth factors (e.g. TGF β), transcriptional factor (e.g. VDR), and epidermal growth factor (EGF) family of receptors are also involved in mammary gland development [41, 42].

Vitamin D receptor (VDR) is widely expressed in stromal cells, epithelial cells and immune cells in the mammary gland. Mammary glands from vitamin D deficient mice display decreased production of milk protein compared to glands from vitamin D sufficient mice [43]. The presence of external 1, 25$(OH)_2$D in mammary gland explants enhanced calcium uptake [44]. However, these observations were not confirmed in an another *Vdr* null mice model where it shows that *Vdr* ablation in mammary gland did not affect casein production or calcium transport into milk, although there is an increase of milk production in response to exogenous oxytocin in *Vdr* knockout mice compared with wild-type mice [15].

Apart from its role in maintenance of calcium homeostasis, 1, 25$(OH)_2$D regulates epithelial cell proliferation, differentiation and apoptosis. 1, 25$(OH)_2$D induces differentiation of normal and transformed mammary epithelial cells *in vitro* [45, 46]. During pubertal development, VDR expression is inversely correlated with proliferation, being highest in differentiated ductal epithelial cells and lowest in rapidly proliferating cap cells of terminal end bud [14]. Mouse on a western diet containing reduced calcium and vitamin D was associated with increased proliferation rate of epithelial cells in the interlobular, intralobular ducts, as well as in the terminal

end ducts of the mammary gland [15]. Furthermore, knockout mice studies further confirm that vitamin D status has a direct impact on normal mammary gland development [14, 15]. Comparison of mammary whole mounts from wild-type and *Vdr* global knockout mice particularly at 6 weeks of age revealed difference in the extent of ductal differentiation and elongation [14]. Mammary glands from virgin *Vdr* knockout mice exhibited enhanced ductal morphogenesis, as compared with glands from age- and weight-matched wild-type mice. The number of terminal end buds structure was higher in mammary glands from *Vdr* knockout mice than in mammary glands from wild-type mice. During pregnancy, lactation, and involution, *Vdr* knockout mice exhibit precocious alveolar development, increased milk release, and delayed postlactational apoptotic regression [15]. Collectively, the vitamin D signaling pathway takes part in negative growth regulation of the mammary gland during stages of puberty, pregnancy, lactating, and involution.

1.3.3 The effect of vitamin D in cancer, particularly in breast cancer

In general, the effects of vitamin D on diverse cancer cell lines are similar: growth arrest at either G0/G1 or G1/M, induction of differentiation, activation of cell death via apoptosis and autophagy, inhibition of angiogenesis and/or inhibition of invasion and metastasis [47] (Fig 4). Of note, studies on cells from *Vdr* null mice have established that VDR is required in mediating the anti-proliferative effects of $1,25(OH)_2D$ [48, 49], indicating that functional VDR is the major determinant of cancer cell sensitivity to $1,25(OH)_2D$. Additionally, *Vdr* knockout mice are more

susceptible to dimethylbenzanthracence (DMBA)-induced carcinogenesis in mammary gland than the wild type, providing the support that VDR signaling pathway might serve to suppress tumorigenesis [50].

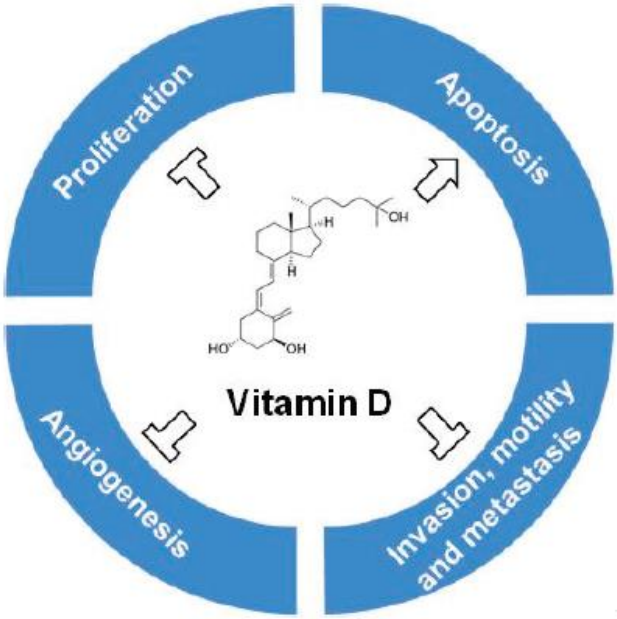

Fig 4. Schematic view of vitamin D effects in cancer. (*Lopes, 2012*)

1.3.3.1 Anti-proliferative effects of vitamin D

Progression of the cell cycle is controlled by the concerted action of cyclins, and their association between CDKs (cyclin-dependent kinases) and CDKIs (cyclin-dependent kinase inhibitors). Many studies have consistently shown that $1,25(OH)_2D$ is able to induce G0/G1 cell cycle arrest by increasing the expression of CDKIs, such as p21 and p27 and leading to inhibition of CDKs activity and the activation of hypophosphorylated form of the RB (retinoblastoma protein) [51-53]. $1,25(OH)_2D$ has also been shown to down-regulate oncogenes, such as *c-myc* [51] and *c-fos* [54],

further contributing to inhibition of cell proliferation. Other potential mechanisms of $1,25(OH)_2D$ mediated antiproliferative effects have also been reported.

Additionally, the CCAAT enhancer-binding protein α (C/EBPα), a potential tumor suppressor in breast cancer, was found to mediate the anti-proliferative effects of $1,25(OH)_2D$ in breast cancer cells. Antiproliferative effects of $1,25(OH)_2D$ were observed in C/EBPα-negative MDA-MB-231 cells transfected with C/EBPα, while knockdown of C/EBPα suppressed antiproliferative effects of $1,25(OH)_2D$ in MCF-7 cells [55], thus providing evidence for C/EBPα as a mediator of $1,25(OH)_2D$ to inhibit growth of breast cancer cells. Another tumor suppressor in breast cancer, TCF-4, has been identified to be up-regulated by $1,25(OH)_2D$ and VDR [56], further providing evidence for its anti-tumor role in breast cancer. Furthermore, the antiproliferative effects of $1,25(OH)_2D$ on breast cancer lines are associated with induction of *BRCA1* gene expression [57].

It is of particular interest that breast cancer cell lines display a varied antiproliferative sensitivity to $1,25(OH)_2D$. For example, the ER α and β positive cell line MCF-7 displays an antiproliferative sensitivity to $1,25(OH)_2D$, while the more aggressive cell type MDA-MB-231, which is ER α and β negative, is essentially insensitive to the antiproliferative effects of $1,25(OH)_2D$ [57]. Several studies have shown that $1,25(OH)_2D$ down-regulates ERα expression in breast cancer cells [58, 59]. The mechanism of ERα down-regulation appears to be a direct transcriptional repression of the ERα gene through the binding of $1,25(OH)_2D$ to negative vitamin D response elements (nVDREs) in the ERα promoter [59, 60]. Thus, the down-regulation of ERα

by 1,25$(OH)_2$D partly contributes to anti-proliferative effects of 1,25$(OH)_2$D on ER+ breast cancer cells.

Addition of histone deacetylase (HDAC) inhibitor trichostatin A (TSA) enhanced the anti-proliferative action of 1,25$(OH)_2$D in the MDA-MB-231 breast cancer cells [61, 62], indicating that epigenetic mechanisms may play a role in mediating the anti-proliferative effects of 1,25$(OH)_2$D in breast cancer. Normally, in the absence of ligand, the VDR associates with large complexes containing HDAC enzymes, co-repressors such as the nuclear receptor co-repressor 1 (NCoR1) or silencing mediator of retinoic acid and thyroid hormone receptors (SMRT), that together maintain chromatin in a transcriptionally repressed state [63, 64]. For example, cells of the non-malignant breast cell line MCF12A are highly sensitive to 1,25$(OH)_2$D, but display increased resistance to 1,25$(OH)_2$D when transiently transfected with *NCoR1* compared with both mock and non-transfected controls, supporting the notion that elevated co-repressors such as NCoR1 attenuate the capacity of VDR as a transcriptional factor for initiating anti-proliferative target genes [65].

In summary, 1,25$(OH)_2$D exhibits anti-proliferative effects in breast cancer cells through a variety of mechanisms, which support the idea of using vitamin D for the prevention and/or treatment of breast cancer.

1.3.3.2 Apoptosis induced by vitamin D

Apart from its anti-proliferative effects on breast cancer cells, 1,25$(OH)_2$D has a role in induction of apoptosis, since characteristic apoptotic features, including

cytoplasmic condensation, pyknotic nuclei, condensed chromatin and/or nuclear matrix re-organisation, were observed in various cancer cells treated with $1,25(OH)_2D$ [66-68]. The most plausible mechanism of $1,25(OH)_2D$-mediated apoptosis is through down-regulation of the antiapoptotic protein Bcl-2 and up-regulation of the proapoptotic protein Bax [68, 69], since Bcl-2 and Bax act antagonistically in the regulation of apoptosis. Additionally, $1,25(OH)_2D$ potentiates the tumor necrosis factor (TNF)-α induced cytotoxicity in both caspase-dependent and -independent pathways [70]. In support of caspase-independent $1,25(OH)_2D$-mediated apoptosis, it was shown that induction of apoptosis in MCF-7 was through disruption of mitochondrial function, which was associated with Bax translocation to mitochondria, cytochrome c release, and production of reactive oxygen species (ROS). Furthermore, these mitochondrial effects were not blocked by a specific caspase inhibitor [69], and were independent of the mutant status of the tumor suppressor gene *TP53* [70]. A recent study also suggests a role of insulin-like growth factor binding protein-3 (IGFBP-3) in $1,25(OH)_2D$ signaling, since $1,25(OH)_2D$ increases the intracellular IGFBP-3 protein in MCF-7, which alone produces cleavage of caspase 7,8 and 9 and PARP-1 and is effective in inhibiting IGF-1/Akt pathways [71].

1.3.3.3 Anti-angiogenesis of vitamin D

Angiogenesis, the formation of new blood vessels from an existing vascular bed, is of critical importance in tumor growth and metastasis [72]. In the chick embryo chorioallantoic membrane essay, $1,25(OH)_2D$ has been shown to inhibit angiogenesis

[73]. These studies are now supported by both *in vitro* and *in vivo* studies, demonstrating that anti-angiogenic properties of $1,25(OH)_2D$ are, at least in part, owing to inhibition of endothelial cell proliferation and induction of apoptosis by $1,25(OH)_2D$ [21, 74]. Studies on tumor-derived endothelial cells (TDEC) from VDR knockout mice showed that $1,25(OH)_2D$-mediated growth inhibition on TDEC is VDR-dependent [74]. Using nude mice xenografted with the breast cancer cell line MCF-7 overexpressing vascular endothelial growth factor (VEGF), it was demonstrated that $1,25(OH)_2D$ reduced tumor vascularisation [21].

However, the role of VDR in mediating the effects of $1,25(OH)_2D$ on the angiogenic signaling pathways is still not well characterised. In various cancer cells, $1,25(OH)_2D$ inhibits hypoxia-inducible factor-1 (HIF-1) transcriptional activity and its target genes, including *VEGF, ET-1, and GLUT-1* [75]. HIF-1 is considered as the key contributor to human angiogenesis in response to hypoxia [76]. HIF-1 loss-of-function has shown to inhibit angiogenesis in nude mice [77], whereas its gain-of-function has the opposite impact [78]. In another study, *VEGF, ANGPT1, and PDGF-BB* expression were down-regulated in TDEC cells treated with $1,25(OH)_2D$. A vitamin D receptor response element has been identified in the promoter of the rat *VEGF* gene [79], suggesting that $1,25(OH)_2D$ is likely to modulate *VEGF* expression at a transcriptional level. But the mechanisms of how other above-mentioned genes are regulated by $1,25(OH)_2D$ are yet to be delineated.

1.3.3.4 Invasion and metastasis

The metastatic process comprises several sequential steps, including escape of the cancer cells from primary tumor, transport in the circulation, arrest in a distant organ, extravasation, and growth of cells in the new sites [80]. The production of proteolytic enzymes, namely the serine proteases, the matrix metalloproteinases (MMPs), and the cysteine proteases, is a critical event in the sequential steps of metastasis [81, 82]. Laboratory studies show that $1,25(OH)_2D$ plays an important role in modulation of invasion and metastasis of cancer. It has been shown that $1,25(OH)_2D$ inhibited migration and invasion of the ER-negative metastatic breast cancer cell line MD-MBA-231 *in vitro*, which was not due to the anti-proliferative effects of $1,25(OH)_2D$, since no growth reduction was observed [83]. The reduction of invasive capability of breast cancer cells by vitamin D was associated with down-regulation of MMP-9 and serine protease uPA activity, simultaneously with increased activity of their corresponding inhibitors, tissue inhibitors of metalloproteinase (TIMPs) and PA inhibitor 1 (PAI-1) [84]. In another similar study, down-regulation of MMP13 in MCF-7 by $1,25(OH)_2D$ or its more potent analogue MART-10, was observed [85]. Furthermore, $1,25(OH)_2D$ and MART-10 may prevent metastasis of MCF-7 in an *in vitro* model by inhibiting its ability of invasion and migration, likely through their effects on inhibiting epithelial-mesenchymal transition (EMT), since E-cadherin was found to be up-regulated, while Snail/Snug and Twist was significantly down-regulated by $1,25(OH)_2D$ or by MART-10 [85]. Additionally, *Vanoirbeek et al* [86] recently proposed an alternative mechanism of $1,25(OH)_2D$ suppression of breast

cancer cell invasion and migration. PDZ-LIM domain-containing protein 2 (PDLIM2) is an adaptor molecule that links different components of cytoskeleton [87], and was shown to be up-regulated by 1,25$(OH)_2$D in a VDR-dependent manner, leading to the enhanced cell adhesion [86], further providing evidence for a vitamin D role in the modulation of invasion and migration.

Taken together, these *in vitro* studies show a potential role of vitamin D in the modulation of cancer cell invasion and metastasis.

Several *in vivo* studies have also been conducted to explore the role of vitamin D in modulating invasion and migration. A Ron-driven murine model of breast tumorigenesis was crossed with VDR knockout mice [88]. Ron overexpression drives mammary tumorigenesis through direct β-catenin activation and augments tumor cell proliferation and metastasis to the liver and lung. Loss of *Vdr* significantly increased the number of liver and lung metastases in MMTV-Ron mice compared with controls, which is associated with increased active β-catenin. Similar results were obtained from the MMTV-PyMT transgenic mouse model [89]. In MMTV-PyMT mice, microscopic lung metastases spontaneously develop in animals by 12 to 13 weeks of age [90]. 25(OH)D and 1,25$(OH)_2$D perfusion significantly decreases the number of lung metastases per mouse by 27% and 40% respectively. 25(OH)D and 1,25$(OH)_2$D perfusion raise local levels of 1,25$(OH)_2$D in mammary tumors, resulting in reduced expression of the cell proliferation markers Ki-67, ErbB2, and cell-cycle progression marker cyclin D1. These *in vivo* data suggest a protective role for vitamin D signaling in mammary tumor metastasis.

Collectively, both *in vitro* and *in vivo* evidence from preclinical models suggest $1,25(OH)_2D$ plays a critical role in inhibiting invasion and migration of breast cancer. However, it is still unknown whether it could be translated into an improved clinical outcome for patients with breast cancer.

1.4 Vitamin D and breast cancer risk

A series of epidemiological studies show that sun exposure, serum 25(OH)D levels and vitamin D intake are inversely associated with incidence and severity of breast cancer [91-93], potentially via regulation of cell growth, differentiation, apoptosis and a wide range of cellular mechanisms central to the development of cancer [94].

Several epidemiological studies have shown that increasing UVB exposure, and as a consequence of higher vitamin D levels, reduces the risk of breast cancer [95-97]. However, this inverse association can be attenuated as many other contributing factors affecting vitamin D status may vary both between and within subjects over time. While serum 25(OH)D, the indictor of body vitamin D status, is mainly derived via sun exposure and dietary intake, other factors also affect the circulating level of 25(OH)D. While significant negative correlates include body mass index, winter and spring blood draw, history of diabetes, sedentary behaviour, black pigmented skin and smoking; positive ones are physical activity and summer sample [98]. Serum 25(OH)D level is the combined result of multiple indices known to influence vitamin D status [99], and its half-life is far greater than that of vitamin D or $1,25(OH)_2D$

[100]. Therefore, serum 25(OH)D level is the generally accepted clinical measure of vitamin D status.

A recent nested case-control study demonstrated that the risk of breast cancer was 48% less in women with the highest tertile of pre-diagnostic circulating 25(OH)D levels (> 84 nmol/L) compared with those with the lowest tertile (< 60 nmol/L) [99]. An inverse association was also found in a previous case-control study nested within the Nurses' Health Study cohort, comparing 701 cases and 724 controls [101]. This study reported a 27% borderline significant reduction of breast cancer in women with the highest quintile of 25(OH)D (>100 nmol/L) compared with those with the lowest quintile (<50 nmol/L).

There are, however, apparent contradictions to these epidemiological findings by other studies where high vitamin D levels were not protective for breast cancer [102, 103]. For example, the Women's Health Initiative found no beneficial effect of vitamin D and calcium supplementation on the risk of benign proliferative breast disease in postmenopausal women [104], which was supported by a meta-analysis [105]. The lack of observed effect of vitamin D supplementation on reducing risk of breast cancer might be at least partly due to the dose investigated (400 IU per day), as recent findings suggest that a vitamin D intake of 2000 IU to 4000 IU per day is necessary in order to maintain circulating 25(OH) levels above 100 nmol/L [106]. Nevertheless, a recent dose-response meta-analysis [107], after pooling 9 prospective studies compromising 5,206 cases and 6,450 controls, showed an observed inverse association in postmenopausal women with plasma 25(OH)D concentrations ranging

from 68 nmol/L to 88 nmol/L, while no association was found in premenopausal women. In this study, a 12.5 nmol/L increase in serum 25(OH)D concentration was associated with a 12% lower risk of breast cancer (Fig 5).

Taken together, women, especially those in postmenopausal phase, with high levels of serum 25(OH)D have a reduced risk of breast cancer. The benefit of vitamin D supplementation for postmenopausal women need to be validated in larger clinical trials. Future studies are awaited to determine the optimal dose of vitamin D supplementation sufficient to modify the levels of circulating 25(OH)D in women.

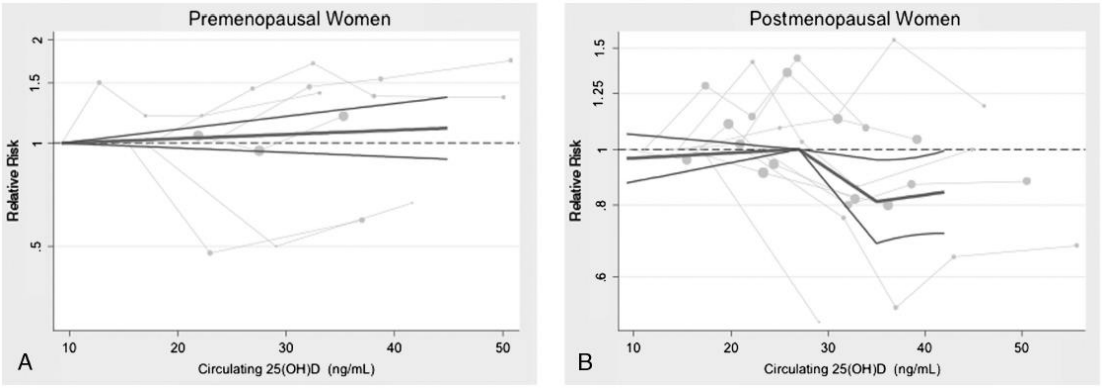

Fig 5. Pooled dose-response relationship between circulating 25(OH)D levels and breast cancer risk stratified by menopausal status. (*Bauer, 2013*)

1.5 Vitamin D and the clinical outcome of breast cancer

The association between serum vitamin D levels and prognostic factors (histological characteristics, pathological stages, estrogen [ER] and progesterone [PR] receptor status, human epidermal growth factor 2 [HER2/neu]) has been investigated by several studies. Breast cancer patients had significantly lower serum 25(OH)D levels

than disease free controls [108]. Among these cases, those who have more aggressive molecular phenotype (basal-like) and worse prognostic indicators (triple negative) had even lower serum 25(OH)D levels. Decreased serum 25(OH)D levels have been linked to higher histological grade and advanced breast cancer stage [109-111]. Lower 25(OH)D levels have also been associated with ER negative status in some, but not all, clinical research studies [109, 110, 112, 113]. The association between low levels of serum 25(OH)D and poor prognostic factors may explain the higher risk of distant recurrence and of death among the vitamin D deficient breast cancer patients observed by Goodwin *et al* [109]. This association, however, was attenuated in multivariate analyses [109]. In two similar studies, no association between serum 25(OH)D levels and subsequent breast cancer recurrence was observed [110, 111]. Several meta-analyses, however, have consistently shown that high vitamin D status is strongly associated with improved breast cancer survival [114-116]. Collectively, accumulating evidence suggests that higher 25(OH)D levels are associated with improved breast cancer survival.

1.6 Target genes of VDR signaling pathway

The biologically active form of vitamin D, $1,25(OH)_2D$ exerts its transcriptional activation and repression of target genes by binding to its intracellular receptor, vitamin D receptor (VDR). Binding of $1,25(OH)_2D$ to VDR promotes association of VDR with RXR (retinoid X receptor) to form a heterodimer, which is essential for VDR transcriptional activity. The activated $1,25(OH)_2D$-VDR-RXR complex specifically binds to vitamin D response elements (VDREs), which consist of two

hexanucleotide repeats interspaced by varying numbers of nucleotides, in the promoter regions of target genes [117]. For transcriptional activation, VDR occupies the 3' half-site whereas RXR binds the 5' half-site of VDRE [118]. This complex further recruits co-regulators, either co-activators or co-suppressors, to initiate or suppress target genes expression. However, the target genes and biological mechanisms by which 1, 25(OH)2D exerts its anti-proliferative effects in intact human breast tissues, are still not clear. Despite multiple studies [119-121] that have explored the expression changes of genes in animal models or human breast cell lines treated with vitamin D or its derivatives, it is unclear if these targets are relevant to the intact human breast tissue. Moreover, the identified target genes modulated by vitamin D signaling pathway vary considerably across different studies. Microarray data generated from vitamin D treated breast cancer cell lines by Swami *et al* [120] suggest that several functional categories, such as genes associated with cell cycle regulation and DNA repair, might be involved in the anti-tumor actions of 1, $25(OH)_2D$. However, vitamin D transduction pathways from studies of MCF-7 which is ER^+, and MDA MB 231 which is ER^-, are quite different with only a few overlapping genes. Milani *et al* [122] recently have tried to explore the candidate genes regulated by 1, $25(OH)_2D$ with physiological supra-physiological concentrations in human breast slices, but the major drawback of this study was the small number of specimens in RNA-seq experiments. Despite the disparities in terms of target tissues used for sequencing, and vitamin D treatment concentration and duration, some genes (*CYP24A1*, *CLMN*, and *SERPINB1*) are consistently up-

regulated (over 1.5-fold) by 1, 25$(OH)_2$D. However, the majority of genes reported to regulated by 1, 25$(OH)_2$D differ in the varying tissues and cell lines that have been used (Table 1.). The detailed mechanisms of how CLMN or SERPINB1 medicate the anti-tumor effects of 1, 25$(OH)_2$D are yet to be defined. The vitamin D target genes currently identified were all gained from experiments from short-term duration of vitamin D treatment. Little is known how these genes would be changed with the extended time, likely to be years, of vitamin D supplementation that would be expected to influence breast cancer risk [123, 124].

1.7 Conclusion

Vitamin D plays a critical role in many physiologic processes. Vitamin D exhibits various anti-tumor effects in *in vitro* studies and animal models, including anti-proliferation, anti-angiogenesis, anti-invasion and/or anti-migration. Though accumulating epidemiologic evidence suggest vitamin D deficiency as a risk factor for breast cancer, the underlying mechanism of the protective effect of vitamin D remains unclear. In the coming chapters, target genes of vitamin D and potential molecular pathways in breast cancer will be explored, which will ultimately facilitate the personalised prevention and/or treatment of breast cancer. Moreover, the CYP24A1, the inactivating enzyme of vitamin D, is always highly induced in the presence of 1, 25$(OH)_2$D, potentially abrogating the anti-tumor effects of 1, 25$(OH)_2$D. In this thesis we will use a novel transgenic mouse model with conditional knockout of *Cyp24a1* in mammary epithelial cells to explore its role in mammary gland morphogenesis. This has possible implications for breast cancer prevention

and/or treatment by manipulating the enzymatic activity of CYP24A1 in breast epithelium.

Table 1. Gene signatures of 1,25(OH)2 exposure in human breast-derived cell lines (hTERT immortalised normal epithelial cells, the breast cancer cell lines MCF7 and SKBR3) and in breast tumor slices.

Study	Simmons et al 2015	Goeman et al 2014	Milani et al 2013
Tissue or cell line	hTERT-HME cells and MCF7	SKBr3	Breast Tumor Slices
Treatment	100 nM 1,25(OH)2D for 24 hours	100 nM 1,25(OH)2D for 6 hours	100 nM 1,25(OH)2D for 24 hours
1.5-fold (up)	**CYP24A1**	**CYP24A1**	**CYP24A1**
	SERPINB1	KRT6A	IL1RL1
	IGFBP3	IL1RL1	CILP
	G6PD	TLX1	KCNK3
	P2RY2	AMZ1	PI15
	RASA4	FAM131B	TMEM37
	EFTUD1	IFLTD1	SHE
	CAMP	SERPINB9	CA2
	CLMN	SLC2A4	DPP4
	CNTNAP3	BDKRB1	G0S2
	TMEM194B	KRT40	FBP1
	VLDLR	SLC16A5	FOXF1
	SLC4A7	C1orf38	TKTL1
	TIMP3	PNMT	CD300LF
	CD97	ADRB2	THBD
	BHLHE40	PADI3	BMP6
	CSF1	VIT	CHN2
	AKR1C3	RNF222	CD14
	DENND1B	LYPD5	SULT1C2

	RGNEF	KRT78	CPM
	TRPV6	HBEGF	CD226
		TRPV6	P2RY8
		MERTK	**SERPINB1**
		IRX6	GRK5
		SERPINB1	SOX7
		FFAR2	SEMA6D
		FOS	FAM20A
		TIMP3	EFTUD1
		SEMA3B	DHRS9
		CLCF1	PALMD
		EDARADD	OSM
		SMOX	LOC339524
		KAZN	CD28
		EPGN	SCARA5
		AHRR	ACVRL1
		BDKRB2	FRAS1
		ZNF703	VMO1
		PLCL2	APBB1IP
		TSKU	**CLMN**
		TMC5	GIMAP1
		ARMC7	KIAA0500
		AREG	FGL2
		TMEM37	ALDH1A2
		FANCE	CD1D
		ITGAL	TRIM35
		SLC26A2	GIMAP4
		TMPRSS2	ADAMDEC1
		CLMN	CYP19A1

		ABHD8	FAM124A
		CSF3R	STEAP3
			GIMAP6
			CLIC6
			TIMP1
			COQ2
			ZNF709
			GPR171
			RCSD1
			LXN
			PTEN
			DRAM
			LOC153346
			PROCR
			FAM20C
			DOK5
			PPP1R16B
			CARD6
			ABCB4
			LOC134466
1.5-fold (down)	RNF144B	FIBIN	TLR2
	KLF6		NPTX1
	CTGF		CDCP1
	RGS2		ZMYM2
			YME1L1
			CCNT1
			LOC153222
			PTK2

			PIK3C2A
			SOD2
			GNA13
			SFRS11
			VIM
			IFIH1
			CTTN
			MYH10
			TRIO
			PER3
			HES4
			ZBTB11
			PGK1
			SNRPA1
			NUPL1
			NR4A3
			SQLE
			ALCAM
			RTN4
			P2RY1
			BCOR

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CHAPTER II

THE EFFECT OF VITAMIN D SUPPLEMENTATION ON THE RISK OF BREAST CANCER: A TRIAL SEQUENTIAL META-ANALYSIS

2.1 Prelude

This chapter presents the evidence from randomized clinical trials that vitamin D, plus or minus calcium supplementation, has the potential to prevent breast cancer. Our analyses indicate that the available data are insufficient to confirm any protective effect of vitamin D supplementation, with or without calcium, on the risk of breast cancer. More participants in future trials are required to make a reliable and conclusive assessment.

2.2 Abstract

Background and objective

Epidemiological studies suggest an inverse association between vitamin D levels and risk of breast cancer. However, the effect of vitamin D supplementation on the risk of breast cancer remains controversial based on the results of current randomized controlled trials (RCTs). The aim of this study was to conduct a systematic review of published RCTs, to explore the effect of vitamin D supplementation on breast cancer risk.

Methods

PubMed, Embase, and Cochrane library were searched from inception to March 2015. We undertook a trial sequential meta-analysis of RCTs reporting the effect of vitamin D with or without calcium on the risk of breast cancer. The effect of vitamin D supplementation with or without calcium on the risk of breast cancer was estimated using a relative risk reduction threshold of 20%.

Results

Six trials comprising 44,049 participants were included in this meta-analysis. This meta-analysis yielded a relative risk of 1.12 (95% CI, 0.76-1.64, *P*= 0.57) for the effect of vitamin D supplementation (7,459 participants), and 0.96 (95% CI, 0.67-1.38, *P*=0.84) for co-administration of vitamin D and calcium (39,654 participants). Importantly, the required information size was 55,386 participants for vitamin D supplementation and 463,775 participants for co-administration of vitamin D and calcium. The Z-curve did not cross the O'Brien-Fleming α-spending boundary or reach futility, indicating that the non-significant result was probably due to a lack of statistical power. Dose-response analyses suggest a strong linear inverse correlation between risk of breast cancer and post-intervention circulating 25-hydroxyvitamin D levels within a range from 61 to 96 nmol/L.

Conclusion

Our analyses indicate that the available data are insufficient to confirm any protective effect of vitamin D supplementation, with or without calcium, on the risk of breast cancer. More participants in future trials are required to make a reliable and conclusive assessment.

Keywords: Vitamin D, Breast cancer, Prevention, Randomized controlled trials, Meta-analysis

2.3 Introduction

Vitamin D insufficiency, as determined by the serum level of the prohormone 25-hydroxyvitamin D (25(OH)D), is a prevalent issue worldwide that has traditionally been linked to poor bone and musculoskeletal health. Increasingly, inadequate vitamin D levels have also been associated with cardiovascular disease, autoimmune disease and cancer [1]. Ecological studies have consistently found strong inverse correlations between solar ultraviolet-B (UVB) exposure and various types of cancer, supporting the UVB-vitamin D-cancer hypothesis [2]. For example, women in the United States with high exposure to sunlight were reported to have a 25-65% reduced risk of breast cancer [3]. Consistent with ecological and epidemiological studies, a meta-analysis of five case-control studies reported that increasing serum 25(OH)D by 20 ng/ml (50 nmol/L) was associated with a 41% lower risk of breast cancer [4]. However, the most recent meta-analysis on 30 prospective studies only suggested a weak association between high serum 25(OH)D levels and reduced risk of breast cancer (RR 0.92, 95% CI 0.83-1.02) [5]. As all the studies included in this meta-analysis were observational, randomized controlled trials (RCTs) using vitamin D supplementation are warranted to further investigate the relationship between vitamin D status and breast cancer risk. However, no benefit of vitamin D supplementation in reducing breast cancer risk was found in an early meta-analysis that included two RCTs [6], largely due to the limited number of participants included, dose inadequacy and insufficient study length.

We aimed to perform a trial sequential meta-analysis (TSA) of RCTs to evaluate the value of vitamin D supplementation on breast cancer prevention. This approach reduces the risk of random errors due to repetitive testing on accumulative data in the conventional meta-analysis [7]. An added advantage of TSA is to determine how many more participants are

needed to undergo further randomization before the meta-analysis can be deemed to be conclusive and reliable.

2.4 Methods

2.4.1 Search strategy and eligibility criteria

We searched PubMed, Embase, and the Cochrane library using the terms "vitamin D" and "cancer" with search filters for RCTs. We included any RCTs that evaluated the effectiveness of vitamin D supplementation, with or without calcium, versus placebo to decrease the incidence of breast cancer. Where results were reported or updated in more than one publication, only the most recent publication was used.

2.4.2 Data collection

One reviewer (L.S.) extracted data using a standardized pre-designed data extraction form and this was crosschecked by the second reviewer (A.T.). Reviewers collected the information about: the first author's surname to represent the corresponding study, year of publication, trial sample size, mean age of participants, intervention regimes, intervention duration, post-intervention follow-up, breast cancer cases, and circulating 25(OH)D levels at pre- and post-intervention where available.

2.4.3 Data analysis

For all analyses, we assessed the effects of vitamin D with or without calcium separately. RCTs in which calcium supplements were provided to both treatment groups, so that the groups only differed in terms of vitamin D treatment were included in the vitamin D analysis (vitamin D). Trials comparing co-administered calcium and vitamin D versus placebo were included in the vitamin D plus calcium analysis (CaD). Initially, we undertook a conventional meta-analysis in which data were pooled as relative risk (RR), using the DerSimonian-Laird random-effects models. The I^2 statistic ($I^2 > 50\%$) was calculated to assess the heterogeneity between results of the subgroup of trials of vitamin D and the subgroup of CaD trials. Egger's

regression test was used to assess publication bias. We then performed cumulative meta-analyses, in which we added the results of each trial sequentially by date to calculate updated pooled effect estimates. Finally, we calculated a pooled estimate of the dose-response relationship between post-interventional circulating 25(OH)D levels and breast cancer risk. All statistical analyses were performed with the Stata statistical software package (release 12.0; Stata Corporation, College Station, TX, USA). All tests were two-tailed and $P < 0.05$ was regarded as significant.

2.4.4 Trial sequential analysis

Cumulative meta-analyses are at risk of false-positive results because of repetitive statistical testing [7]. Thus we employed TSA to maintain the overall risk of type-I error at 5%. TSA provides estimates of treatment effects, and thresholds for statistical significance and futility. For our analysis, we calculated the information size (optimum sample size) required to detect a 20% relative risk reduction in breast cancer incidence based on a value for alpha of 5%, and beta equal to 20% (80% power). Statistical monitoring boundaries and futility boundaries were subsequently constructed according to the set error levels and the required information size. These analyses were conducted using Trial Sequential Analysis (version 0.9 beta).

2.5 Results

2.5.1 Results of database search

The trial selection process is illustrated in Figure 1. In our initial review, we identified 711 articles that potentially fit our study eligibility criteria. 165 duplicate citations were identified and removed through Endnote X6. After title and abstracts were screened, 526 citations not meeting inclusion criteria were excluded. Among the remaining 20 unique citations, 14 were excluded for the following reasons: 1. Nine did not report on breast cancer outcomes [8-16]; 2. One was not an RCT [17]; 3. One is an ongoing trial [18]; 4. Three were not the most recent publications [19-21]. Finally, six studies were included for further meta-analysis [22-27].

Table 1 lists the characteristics of the six studies included. All studies were RCTs published between 2003 and 2013. A total of 44,049 participants were included with 1-7 years of active intervention. Two studies were multiple-arm trials to assess both the interventional effect of vitamin D and CaD [25, 26]. Therefore, five studies had data available to assess the effect of vitamin D [23-27], while three studies compared the effect of co-administration of vitamin D and calcium with placebo [22, 25, 26].

2.5.2 Quality assessment of included trials

According to the Cochrane Handbook for Systematic Review of Interventions 5.1.0, the methodological quality for each included study was assessed based on the following six requirements: randomization method, allocation concealment, blindness, incomplete outcome data, selective reporting results, and other potential bias. The overall results of risk of bias assessment are shown in Table 2, with four studies graded A (low risk of bias) and two graded B (moderate risk of bias). None of them were graded as C (high risk of bias).

2.5.3 Effects of intervention

We included six trials in our meta-analysis, comprising a total of 44,029 participants (Table 1). Figure 1 shows the results of conventional meta-analyses of the effects of vitamin D with or without calcium on the incidence of breast cancer. We found no significant heterogeneity between the results of trials of vitamin D (I^2 = 0.0%) and the results of trials of CaD (I^2 = 35.5%). The pooled RR for the effect of vitamin D supplementation was 1.12 (95% CI, 0.76-1.64, P= 0.57). For comparing CaD with placebo, the pooled RR is 0.96 (95% CI, 0.67-1.38, P= 0.84).

We next determined the correlation between circulating 25(OH)D concentrations after vitamin D treatment and incidence of breast cancer . There was a strong linear, inverse dose-response relationship between post-interventional circulating 25(OH)D levels and relative risk of breast cancer within the concentration range from 61 to 96 nM after the intervention with vitamin D (R^2=0.94) and vitamin D plus calcium (R^2=0.89) (Figure 3). It is noteworthy that two studies were excluded in this dose-response analysis because of short-term follow-up and small sample size [23,24].

2.5.4 Trial sequential analysis

Figure 2 shows cumulative meta-analysis and trial sequential analyses for the effects of vitamin D with or without calcium on breast cancer incidence. We estimated that a meta-analysis information size (optimum size) of 55,386 participants was required to yield statistical significance or futility for the effect of vitamin D supplementation, and a meta-analysis information size of 463,755 participants for CaD intervention. For both vitamin D and CaD interventions, there was insufficient information to calculate futility boundaries. From our current analysis, the cumulative Z-statistic did not cross TSA monitoring

boundaries (Figure 4). This suggests the pooled meta-analytic evidence of the effectiveness of vitamin D or CaD on the incidence of breast cancer is inconclusive.

We further investigated possible protective effects of vitamin D by repeating the trial sequential analyses using a lower threshold of 15% relative risk reduction. The optimum information size increased to 98,439 participants for vitamin D supplementation, but could not be quantified for CaD supplementation. There was insufficient information to calculate futility boundaries. The effect estimate did not cross the O'Brien-Fleming α-spending boundary, indicating uncertainty as to whether vitamin D alone or CaD decreases the relative risk of breast cancer by 15% or more.

2.5.5 Publication bias

Publication bias was assessed by using Egger's regression test (Figure 5). The standard normal deviate (SND) was regressed against the estimates precision. The intercept of the regression line is -0.12 (95% CI: -1.70 to 1.50, $P = 0.83$), indicating no statistically significant publication bias.

2.6 Discussion

We conducted a trial sequential meta-analysis of RCTs focused on vitamin D supplementation with or without calcium in breast cancer prevention. Vitamin D with or without calcium had no effect on altering the risk of breast cancer in traditional meta-analysis. However, trial sequential analysis suggested that there is insufficient evidence to confidently ascertain whether vitamin D with or without calcium decreases the incidence of breast cancer. Notably, there is a strong inverse dose-response relationship between circulating 25(OH)D levels and risk of breast cancer when circulating 25(OH)D levels are within the range from 61 to 96 nmol/L. This result is consistent with a previous dose-response meta-analysis of nine prospective studies that indicates a nonlinear reduction of breast cancer risk with increasing 25(OH)D levels between 67.5 and 87.5 nmol/L in postmenopausal women [28].

Strength of this trial sequential meta-analysis is that the included studies are RCTs, most of which have a low risk of bias. Publication bias could not be detected based on Egger's regression test, and due to a restricted number of trials cannot be assessed by funnel plot. The use of TSA allows assessment of whether evidence is sufficient to draw a conclusive result.

Despite epidemiological studies suggesting that a higher vitamin D status is associated with a lower incidence of breast cancer, current meta-analysis of RCTs has failed to validate a protective effect of vitamin D. The reasons for the failure of RCTs may be multi-faceted, including inadequate dose of vitamin D, short-term follow-up, population selection or other, as yet unknown, factors.

A meta-analysis of 14 prospective cohort studies suggested the optimal concentration range to reduce mortality risk in the general population is from 75 to 87.5 nmol/L [29]. Similarly, another meta-analysis reported that breast cancer patients with a higher serum concentration

of 25(OH)D (greater than 75 nmol/L) had substantially lower fatality rates [30]. A dose-response meta-analysis of nine prospective studies further suggested that circulating 25(OH)D concentration ranging from 67.5 to 87.5 nmol/L was associated with breast cancer risk reduction in postmenopausal women [28]. Taken together, a circulating 25(OH)D level of 75 nmol/L might be a critical threshold, below which people could benefit from vitamin D supplementation. However, vitamin D intakes in most included RCTs (4 out of 6) have been insufficient to bring the circulating 25(OH)D levels up to at least 75 nmol/L [22, 23, 25, 27]. According to a previous analysis [31], mean serum 25(OH)D levels of about 75 to 100 nmol/L provide optimal benefits for all investigated endpoints (including risk of fall, fracture, cardiovascular, and colorectal cancer incidence) without increasing health risks (e.g. hypercalcemia and nephrolithiasis). These levels can be reached with oral doses of 1,800 to 4,000 IU vitamin D per day [31]. Unfortunately, only one of the included studies in this paper supplemented vitamin D within that range [24]. Therefore, the neutral effect of vitamin D on the risk of breast cancer might partly be due to inadequate vitamin D supplementation.

Another possible explanation for the null findings is that the baseline vitamin D levels of participants recruited in RCTs were not low enough to benefit. Vitamin D insufficiency is normally defined as a circulating 25(OH)D level of less than 50 nmol/L, whereas a level of 50 to 75 nmol/L is considered as a relative insufficiency of vitamin D [1]. It is possible that a population with a higher prevalence of vitamin D deficiency could benefit more when circulating 25(OH)D levels can be restored to adequate, as indicated in a previous meta-analysis [32]. Pooled results of two observational studies show that a serum 25(OH)D level of 130 nmol/L was associated with a 50% lower incidence of breast cancer, compared to a baseline of < 30 nmol/L. The baseline 25(OH)D levels reported were below 50 nmol/L in four out of the six studies involved in this meta-analysis [22, 23, 25, 27], while in the remaining two studies baseline 25(OH)D levels were near-normal [24, 26]. But no study

reported mean baseline values less than 30 nmol/L, considered as vitamin D deficiency. On the other hand, it would not be surprising that those with sufficient or near-normal baseline levels of circulating 25(OH)D achieved limited or even null benefit from vitamin D supplementation.

Moreover, given the latency of breast cancer, the total duration of supplementation, including active intervention and post-intervention period, may also be relevant. Only two out of the six studies [22, 25] reported post-intervention follow-up after active intervention of vitamin D with or without calcium supplementation. The median total duration of these six studies was 3.5 years. Only one study had a total duration longer than 10 years [22], while duration in the remaining five studies was less than five years, which might be insufficient to detect any changes in the incidence of breast cancer.

2.7 Conclusions

Our study could not identify any protective effect of vitamin D supplementation with or without calcium on reducing the risk of breast cancer. However, studies are impacted by limited numbers of participants with high prevalence of vitamin D insufficiency, relatively short period of post-intervention follow-up, and dose inadequacy, thus more RCTs are required to make a conclusive and reliable conclusion. It is preferable that future RCTs would recruit people with higher prevalence of vitamin D deficiency and prescribe them with an adequate dosage of vitamin D.

List of abbreviations

RCTs Randomized Controlled Trials

25(OH)D 25-hydroxyvitamin D

TSA Trial Sequential Meta-Analysis

CaD Vitamin D and Calcium

RR Relative Risk

SND Standard Normal Deviate

Table 1. Study characteristics

Study	Age (mean, years)	Participants (vitamin D / no vitamin D)	Intervention duration / post-intervention follow-up (years)	Treatment groups	Dose (vitamin D or CaD)	Vitamin D group	Control group
						Circulating 25(OH)D levels (pre- / post-intervention, nM)	Circulating 25(OH)D levels (pre- / post-intervention, nM)
Trivedi, 2003	74	326/323	5 / 0	vitamin D and placebo	100,000 IU every 4 months	NA / 72 (n=59)	NA / 45 (n=57)
Lappe, 2007	67	466/733	4 / 0	CaD, calcium, placebo	1100 IU per day + 1.4-1.5 g per day / 1.4-1.5 g per day	72 (all) / 96 (all)	72 (all) / 71 (all)
Avenell, 2012	77	2649/2643	2-5.2 / 3	2*2 factorial €: CaD, vitamin D, calcium, control	800 IU per day, 1000 mg per day	38 (n=60) / 62	38 (n=60) / 42 (calcium group); 46 (placebo group)
Murdoch, 2012	47	161/161	1.5 / 0	vitamin D and placebo	200,000 IU for the first month, then 100,000 IU monthly	73 (n=161) / 120 (n=150)	70 (n=161) / 57 (n=154)
Wood, 2012	64	203/102	1 / 0	vitamin D and placebo	400 or 1,000 IU per day	32 (all) / 65 (n= 97; 400 IU daily group); 75 (n= 95; 1000 IU daily group)	36 (all) / 29 (n= 100)
Cauley, 2013	62	18176/18106	7 / 4.9	CaD and control	400 IU per day, 1000 mg per day	48 (n=357) / 28% higher than control group	48 (n=357) / 28% lower than vitamin D group

25(OH)D: 25-hydroxyvitamin D; CaD: vitamin D plus calcium; NA: not available; €factorial study with all possible combinations of the interventions.

Table 2. Risk of bias assessment of included studies

Study	Sequence generation	Allocation concealment	Blinding	Incomplete outcome data	Selective outcome reporting	other bias	Overall grade
Trivedi, 2003	Unclear	Unclear	Low risk	Low risk	Low risk	Low risk	B
Lappe, 2007	Low risk	Low risk	Unclear	Unclear	Low risk	Low risk	B
Murdoch, 2012	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	A
Wood, 2012	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	A
Avenell, 2012	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	A
Cauley, 2013	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	A

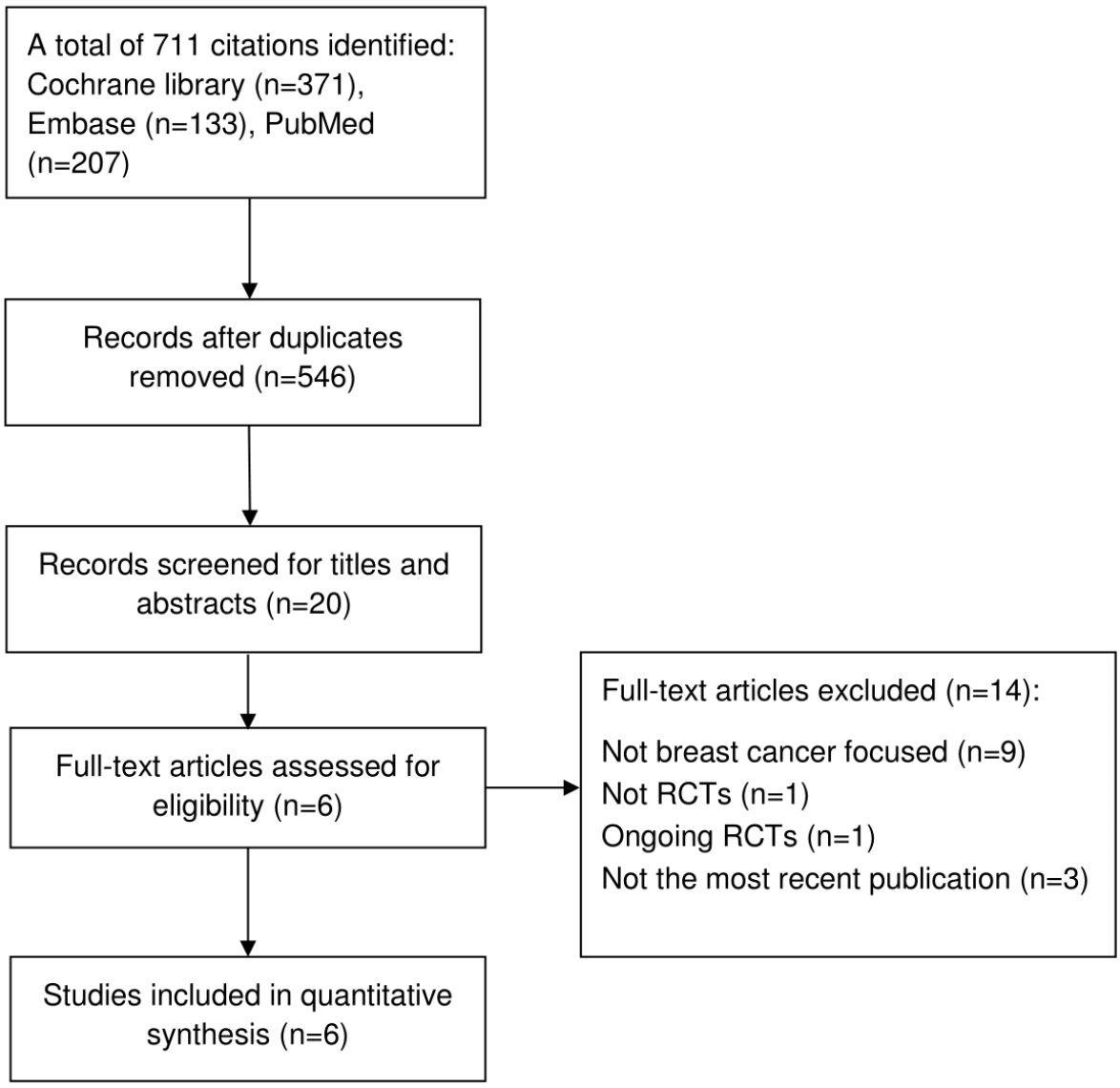

Figure 1. Flowchart of study selection.

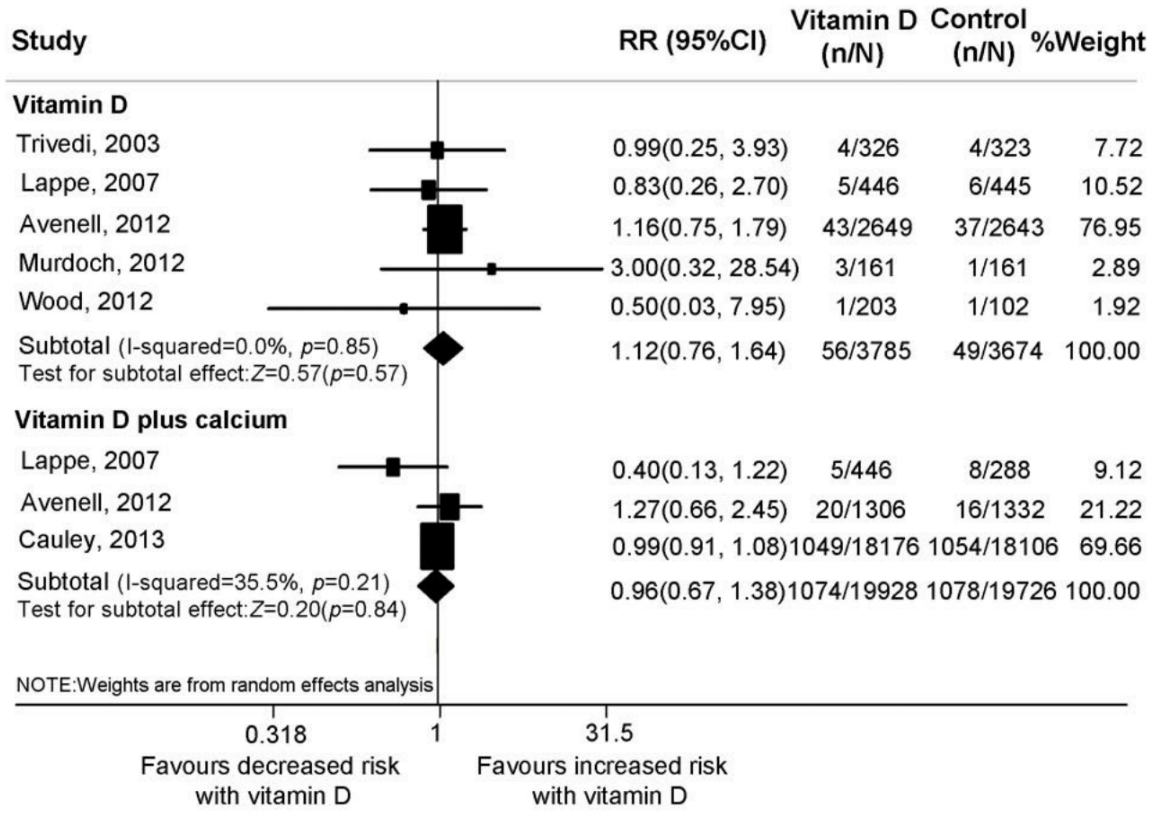

Figure 2. Random effects meta-analyses of vitamin D with or without calcium on the incidence of breast cancer. Relative risk (RR) is determined either by comparing two groups with the only difference being vitamin D or by comparing vitamin D plus calcium to placebo. Breast cancer incidence is presented as a ratio of number of breast cancer cases (n) to overall included paritcipants in that arm (N).

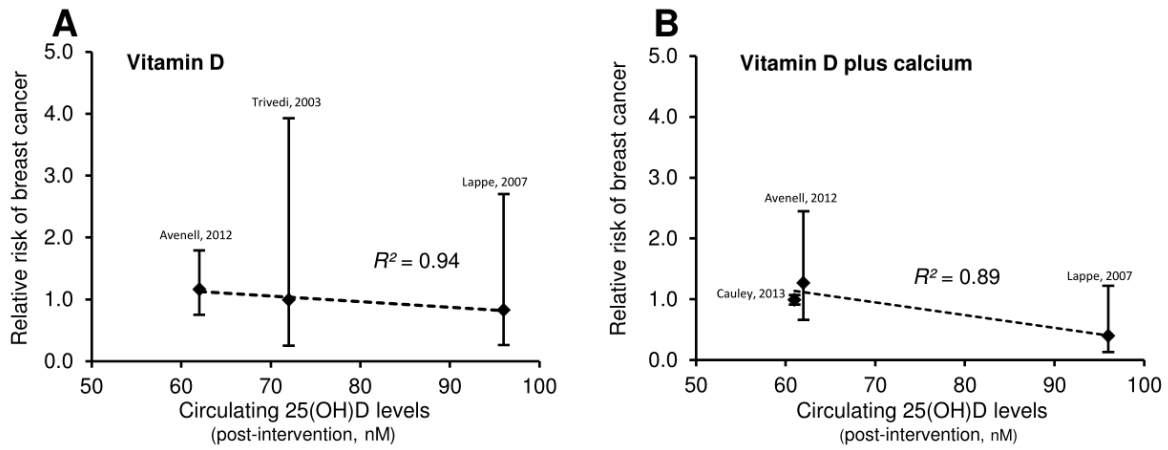

Figure 3. Overall dose-response relationship between post-interventional circulating 25(OH)D levels and relative risk of breast cancer, with coefficient determination. Relative risk is determined either by comparing two groups with the only difference being vitamin D (A) or by comparing vitamin D plus calcium to placebo (B). R^2 is determined for the correlation from the linear regression.

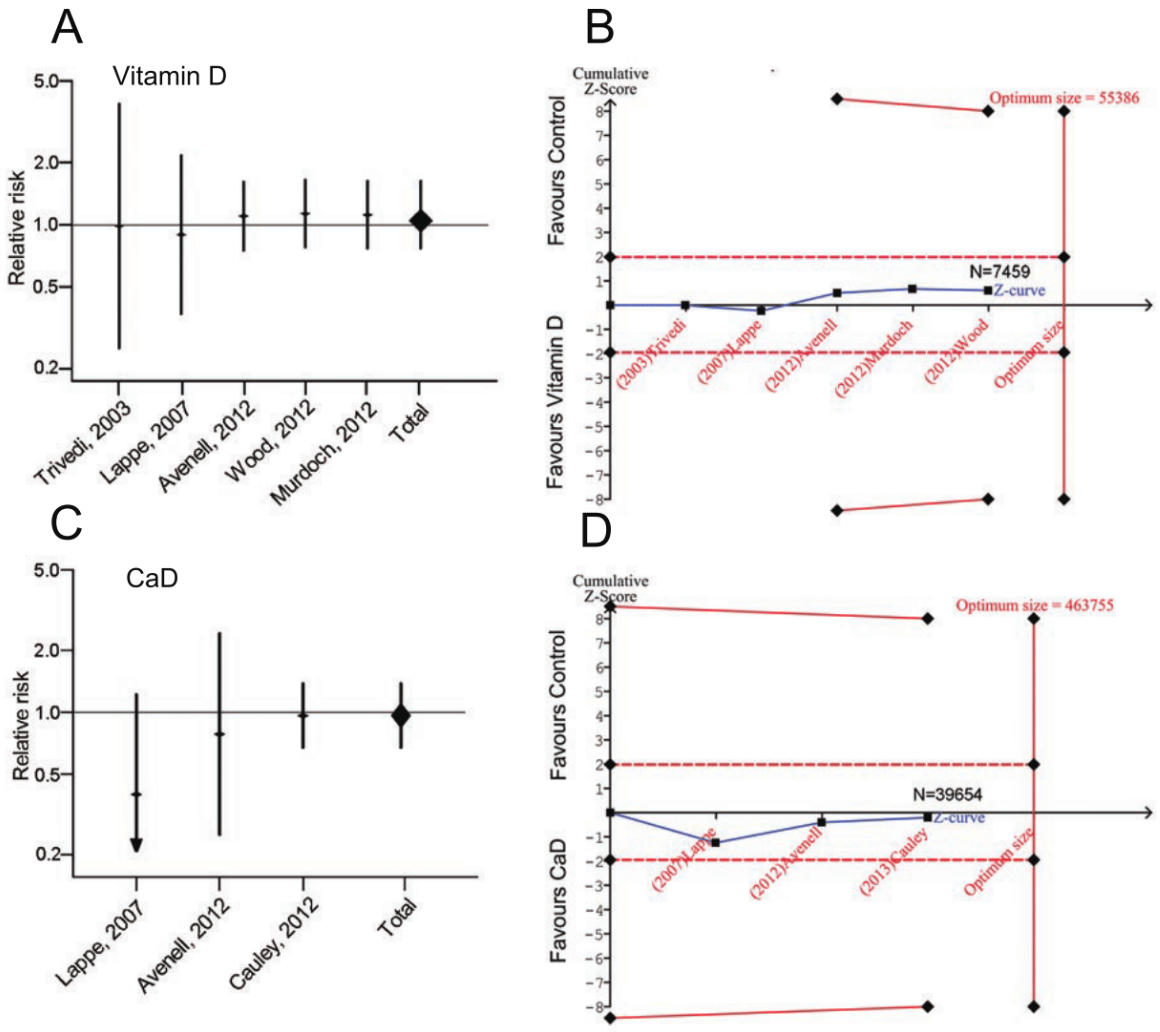

Figure 4. Cumulative random effects meta-analyses and trial sequential analyses of vitamin D with or without calcium on the risk of breast cancer

Cumulative random effects meta-analyses and trial sequential analysis of vitamin D (A, B) and combination of vitamin D and calcium (CaD) (C, D) on the risk of breast cancer. For trial sequential analyses, the Z curve is a measure of treatment effect, and the boundaries are thresholds for statistical significance adjusted for heterogeneity of trial results and multiple statistical testing. A treatment effect outside the significance boundary (red solid line) indicates there is reliable evidence of a treatment effect. Optimum size indicates the calculated optimum

sample size for statistical inference and N indicates the number of participants in the meta-analysis.

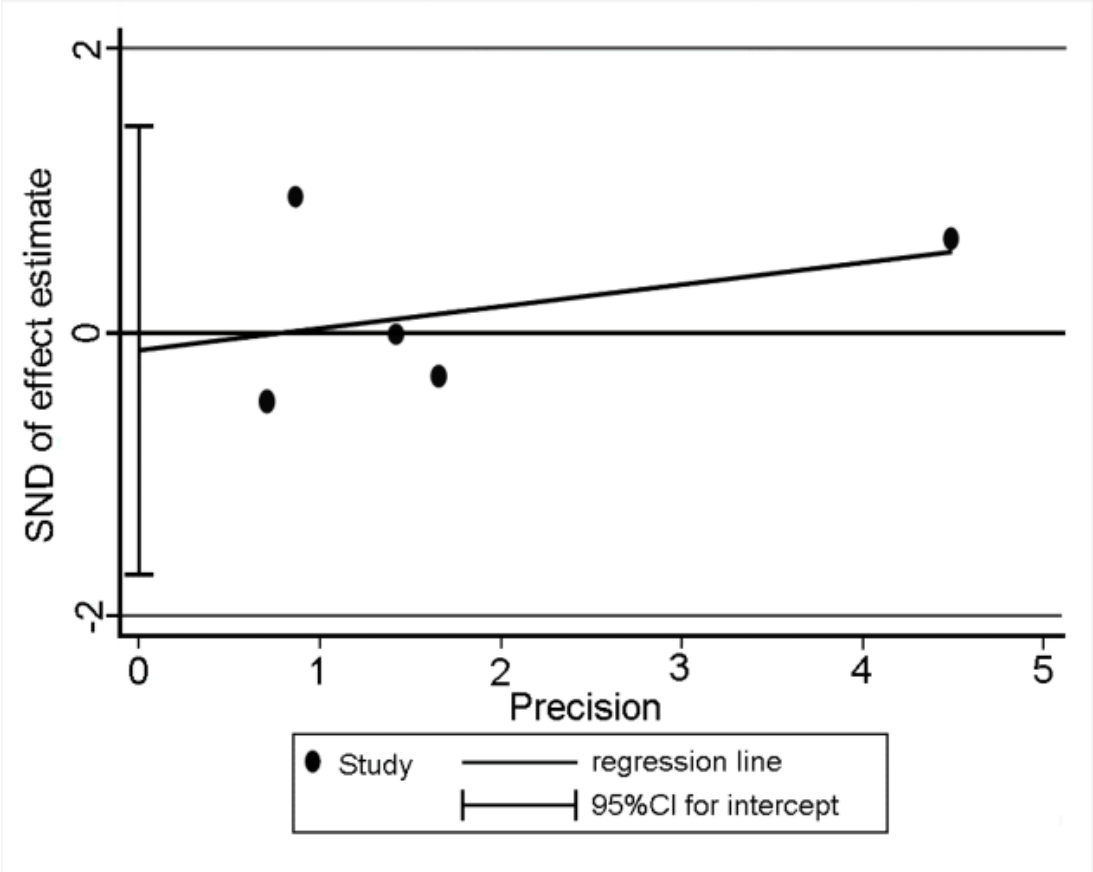

Figure 5. Publication bias was assessed using Egger's regression test. The standard normal deviate (SND) is regressed against the estimates precision. The intercept of the regression line is -0.12 (95% CI: -1.70 - 1.50, *P* = 0.83), indicating no statistically significant publication bias.

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CHAPTER III

IDENTIFICATION OF VITAMIN D_3 TARGET GENES IN HUMAN BREAST CANCER TISSUE

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3.1 Prelude

This study is the first to use RNA-Seq analyses to investigate vitamin D target gene expression and molecular pathways in *ex vivo* explants of human breast tissue, which was treated with vehicle control or $1,25(OH)_2D$. The current study further confirms that *CLMN*, *SERPINB1*, *EFTUD1*, and *KLK6* are direct up-regulated targets of $1,25(OH)_2D$, which have reported functions consistent with roles as tumor suppressors. These tumor suppressor roles are further supported by analysis of publically available breast cancer cohorts where high expression levels, particularly of *CLMN*, *SERPINB1*, and *KLK6* are associated with prolonged relapse-free survival for breast cancer patients.

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Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Lei Sheng
Contribution to the Paper	Conducted experiments, interpreted data, and wrote manuscript.
Overall percentage (%)	60%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 10.03.2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	10/3/2017

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Contribution to the Paper	Assisted in data interpretation and revised manuscript.		
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Signature		Date	6/3/17

3.2 Abstract

Multiple epidemiological studies have shown that high vitamin D_3 status is strongly associated with improved breast cancer survival. To determine the molecular pathways influenced by 1 alpha, 25-dihydroxyvitamin D_3 (1,25D) in breast epithelial cells we isolated RNA from normal human breast and cancer tissues treated with 1,25D in an *ex vivo* explant system. RNA-Seq revealed 523 genes that were differentially expressed in breast cancer tissues in response to 1,25D treatment, and 127 genes with altered expression in normal breast tissues. GoSeq KEGG pathway analysis revealed 1,25D down-regulated cellular metabolic pathways and enriched pathways involved with intercellular adhesion. The highly 1,25D up-regulated target genes *CLMN*, *SERPINB1*, *EFTUD1*, and *KLK6* were selected for further analysis and up-regulation by 1,25D was confirmed by qRT-PCR analysis in breast cancer cell lines and in a subset of human clinical samples from normal and cancer breast tissues. Ketoconazole potentiated 1,25D-mediated induction of *CLMN*, *SERPINB1*, and *KLK6* mRNA through inhibition of 24-hydroxylase (CYP24A1) activity. Elevated expression levels of *CLMN*, *SERPINB1*, and *KLK6* are associated with prolonged relapse-free survival for breast cancer patients. The major finding of the present study is that exposure of both normal and malignant breast tissue to 1,25D results in changes in cellular adhesion, metabolic pathways and tumor suppressor-like pathways, which support epidemiological data suggesting that adequate vitamin D_3 levels may improve breast cancer outcome.

Keywords: Vitamin D_3; RNA-Seq; Mammary tissue; Genomics; Breast cancer

3.3 Introduction

Epidemiological studies have related high levels of blood 25-hydroxyvitaminD_3 (25D) to a lower risk of various types of cancer, particularly colorectal cancer [1]. Defining the nature of this relationship in breast cancer has been controversial. Two recent large meta-analyses provide the most definitive insights [2, 3]. One study suggests that postmenopausal breast cancer risk decreases with 25D levels 27-<35 ng/mL while the second study shows a weak association between high vitamin D_3 status and low breast cancer risk (relative risk=0.92, 95% confidence interval (CI): 0.83-1.02). Several meta-analyses, however, have consistently shown that high vitamin D_3 status is strongly associated with improved breast cancer survival [3-5]. The availability and economy of vitamin D_3 supplementation make this an attractive potential option for breast cancer prevention and/or treatment. Several randomized controlled trials have examined the broad health benefits of vitamin D_3 supplements, including the potential to lower the risk of cancer. However, these trials, which have often included breast cancer as a secondary outcome, are yet to deliver a clear result and have been criticized for not adequately raising participant 25D levels [6]. Therefore the question of what 25D level should be recommended to lower breast cancer risk remains unanswered. This is perhaps in part due to an incomplete knowledge of the biological activities of vitamin D_3 within normal breast epithelial and cancer cells.

In its biologically active form, 1 alpha, 25-dihydroxyvitamin D_3 (1,25D) binds to the vitamin D receptor (VDR) to exert a variety of biological effects. Consistent with a possible role in cancer prevention, 1,25D inhibits cell proliferation via G1/S arrest [7], induces differentiation, activates cell death through apoptosis, inhibits angiogenesis and/or invasion and metastasis [8-12]. Attempts to profile the biological response to 1,25D within breast cancer cell lines and epithelium have yielded variable data. Only 11 genes were regulated in the same direction by comparing three datasets from MCF-7, hTERT-HME, and SKBr3 cells treated with 1,25D [13,

14]. In SKBr3 breast cancer cells, 318 genes were identified by RNA-Seq to be differentially expressed following treatment with 1,25D [14], but only 14 of these genes were regulated in common with another micro-array study using 1,25D-treated human breast cancer slices [15]. These data demonstrate not only varied responses to 1,25D between breast cancer cell lines, but divergence from intact breast cancer tissue.

We previously have shown that human breast tissue remains viable and retains an intact VDR signaling pathway during short-term *ex vivo* incubation [16]. Using *ex vivo* cultured human breast tissues, this study employed RNA-Seq technology to identify VDR regulated target genes and molecular pathways that may mediate the anti-tumor effect of 1,25D.

3.4 Material and Methods

3.4.1 Patient explant tissue culture

Breast mastectomy specimens were obtained from patients undergoing treatment for invasive breast cancer at the Royal Adelaide Hospital from 2012 to 2014. The clinical characteristics of these patients with cancer tissue available (n=7) are presented in Supplementary Table 1. Adjacent non-malignant tissue (n=8) was collected where available. Non-malignant samples were also obtained from patients undergoing cosmetic reduction mammoplasty (n=3). The *ex vivo* breast tissue explant system was conducted as previously described [16]. Briefly, fresh human tissue was collected immediately following surgical resection, dissected into small pieces and explanted on gelatin sponges. Following overnight equilibration, fresh media containing 10% charcoal-stripped fetal calf serum (FCS) was added with either 100 nM 1,25D (Wako Pure Chemical Industries, Osaka, Japan) or vehicle control (0.1% ethanol). Following 24 hours of treatment, tissues were collected with total RNA extracted using the RNeasy mini kit (Qiagen). Informed written consent was obtained from all participants. This study was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital and the procedures used were in accordance with institutional and NHMRC guidelines.

3.4.2 RNA-Seq

A genome-wide search for genes that were regulated by exogenous 1,25D was performed by RNA-Seq analysis using non-malignant (n=3) and malignant human breast tissue (n=3). RNA-Seq cDNA libraries for each sample were constructed from 1 μg of total RNA using poly A selection. The pooled libraries were sequenced on the Illumina HiSeq sequencing platform using single-end 50 bp reads at the Australian Cancer Research Foundation Cancer Genomics Facility. Single end reads were aligned to the hg19 human reference genome with TopHat 2.

Gene level counts were generated using HTSeq. 1,25D and vehicle treated samples were treated as paired and differential expression was determined with count-based negative binomial generalized linear models (GLMs) implemented in the R package "edgeR", in which a normalization factor was calculated by the trimmed mean of M values (TMM) method [17]. This normalization constant was incorporated into the models to account for varying library sizes. We applied the Cox-Reid profile-adjusted likelihood method to estimate dispersion and GLMs likelihood ratio test for differentially detected genes (false discovery rates, abbreviated FDR, of <5% and fold change ≥ 1.5).

3.4.3 Pathway analysis and statistics

Genes with differential expression were used in pathway analysis. The Bioconductor R software (version 3.1.2) package GoSeq (version 1.18.0) was used to identify pathways enriched with differentially expressed genes [18]. Pathways were considered significantly enriched if they had an over represented adjusted $p<0.05$. To identify significantly enriched KEGG (Kyoto Encyclopedia of Genes and Genomics) pathways in GoSeq, the gene length bias was first quantified by calculating the Probability Weighting Function (PWF) to determine the probability that a gene will be differentially expressed based only on its length. The *p* values for over-represented and under-represented pathways were calculated using the GoSeq default method "Wallenius" by the Wallenius non-central hypergeometric distribution.

3.4.4 Cell culture

Several malignant (MDA-MB-468, MCF-7, and T47D) and non-malignant (MCF10A) breast cell lines, obtained from the American Tissue Culture Collection (ATCC; Manassas), were treated with 100 nM 1,25D or ethanol control for 24 hours. MCF10A and MDA-MB-468 cells

were further treated with the cytochrome P450 inhibitor ketoconazole (Cayman Chemical, Ann Arbor, U.S.A.) for 24 hours at a 10 μM. MDA-MB-468 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, Saint Louis, U.S.A) supplemented with 10% charcoal-stripped FCS and penicillin streptomycin L-glutamine (PSG; 0.2 U/mL penicillin, 200 μg/mL streptomycin and 5.8 μg/mL L-glutamine). MCF10A cells were maintained in DMEM/F12 (Sigma-Aldrich, Saint Louis, U.S.A) supplemented with 10% charcoal-stripped horse serum, 20 ng/mL epidermal growth factor, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin and PSG. MCF-7 and T47D breast cancer cells were cultured in RPMI supplemented with 10% charcoal-stripped FCS, PSG, and 10 μg/mL insulin.

3.4.5 Quantitative RT-PCR analysis

RNA extracted from cell lines (1 μg) or explants (100 ng) was reverse transcribed to cDNA using MMLV-RT (Promega) and random hexamers (Promega). Quantitative RT-PCR (qRT-PCR) was carried out on a CFX Connect™ real-time instrument using the iQ5 SYBR Green detection system (Bio-Rad). Primer sequences and amplification conditions are shown in Supplementary Table 2. Transcript expression was compared between treatment groups and control and normalized to Peptidylpolylisomerase G (*PPIG*). All measurements were performed in triplicate. The Kaplan-Meier plotter (www.kmplot.com) was used to assess the effect of gene expression on breast cancer patient survival by downloading the Kaplan-Meier curves, hazard ratios (HRs), and log-rank *p* values of gene expression [19].

3.4.6 Data analysis and statistical methods

Statistical significance was determined by unpaired Student's *t*-test or one-way ANOVA followed by Tukey's post-hoc multiple comparison tests or two-way ANOVA as appropriate. A p value < 0.05 was considered significant. All statistical calculations were performed using GraphPad Prism software (San Diego, CA).

3.5 Results

3.5.1 Genome-wide transcriptional profiling of 1,25D regulated genes in human breast explants

Six human breast tissue samples (3 malignant, 2 adjacent normal, and 1 cosmetic reduction) were treated with 1,25D (100 nM) or vehicle control for 24 hours and were subjected to RNA-Seq analysis. The average number of raw reads across all samples was 76.36 million. For individual explants, 38%-42% of total reads were mapped to known mRNA sequences. To compare gene regulation following 1,25D treatment between breast cancer and non-malignant tissue (including adjacent normal and cosmetic reduction samples), RNA-Seq data were examined separately for each tissue type. The well-characterized VDR target gene *CYP24A1* expression was induced on average 243-fold by 1,25D versus vehicle in breast cancer and 156-fold in non-malignant tissue (Supplementary Table 3), indicating the efficacy of treatment within our explant model. A higher percentage of genes were differentially regulated by 1,25D in breast cancer explants compared to non-malignant tissues (523 versus 127 genes) (Supplementary Table 3). A total of 51 genes (35 up, 16 down) were commonly responsive to 1,25D in both normal and breast cancer explants, while three genes (*LAMA3*, *KRT15*, and *LBP*) displayed divergent responses.

Approximately two-thirds of 1,25D responsive transcripts in both normal and malignant breast tissue showed expression differences less than two-fold (64% and 60% respectively). A total of 207 genes were regulated ≥ 2 fold by 1,25D in cancer samples, whereas only 46 genes were regulated ≥ 2 fold in non-malignant tissue (Supplementary Table 3). 16 of these genes were regulated ≥ 2 fold in the same direction (13 up, 3 down) in both cancer samples and non-malignant breast tissue after 1,25D treatment.

3.5.2 GoSeq KEGG pathway and ontology analysis

To further investigate the molecular pathways involved in mediating the anti-tumor activities of 1,25D in breast cancer, we performed GoSeq KEGG pathway and ontology analysis on genes that were regulated ≥ 2 fold. Analyses revealed 15 KEGG pathways that were over-represented and 1 KEGG pathway that was under-represented (Supplementary Table 4).

The enriched vitamin D_3 responsive pathways included cell adhesion molecules, ECM-receptor interaction, tight junction, and focal adhesion while gene ontology (GO) analysis revealed extracellular matrix organization, tissue development, biological adhesion, and cell adhesion were over-represented (Supplementary Table 5). E-cadherin (*CDH1*) was found to be up-regulated in breast cancer explants following treatment with 1,25D. These findings are in accordance with the role of 1,25D to modulate the phenotype of breast, colon, and other types of cancer via regulation of cell adhesion molecules [20, 21]. Previous studies have shown that 1,25D treatment of cancer cells lines results in cells displaying a more adhesive phenotype, which could be attributed to up-regulation of E-cadherin [22, 23].

Conversely, our data demonstrate a number of metabolic pathways that were significantly under-represented in breast cancer tissue in response to 1,25D (Supplementary Table 4). Consistent with these observations, ontology analysis indicated that multiple cell metabolism pathways were significantly under-represented in 1,25D-treated breast cancer compared to

vehicle control (Supplementary Table 5). These metabolic processes included cellular nitrogen compound metabolism, nucleic acid metabolism, cellular macromolecule metabolism, organic cyclic compound metabolism as well as transferase activity and ATP binding.

3.5.3 Confirmation of differentially expressed genes by qRT-PCR

To focus our investigations, we next examined the genes that were most highly regulated by 1,25D in RNA-Seq analyses, and selected candidates that had a known or predicted function consistent with a role to reduce tumorigenesis. Recently, integration of four datasets from 1,25D treated hTERT-HME, MCF-7 and SKBr3 cells, and human breast tumor explants revealed four 1,25D responsive genes (*CYP24A1, CLMN, SERPINB1,* and *EFTUD1*) in common [13]. These four genes were also present in the list of 1,25D target genes with expression >=2-fold in common between cancer and non-malignant breast tissue in our study (Supplementary Table 3). For further characterization of 1,25D gene targets, *CLMN, SERPINB1,* and *EFTUD1* and in addition *KLK6,* the second most highly regulated gene in 1,25D treated breast cancer explants, were chosen. To confirm the RNA-Seq results, qRT-PCR analysis of *CLMN, SERPINB1, EFTUD1,* and *KLK6* was undertaken in an additional 12 breast explants treated with vehicle or 100 nM 1,25D for 24 hours (Fig. 1). All samples displayed an increase in *CYP24A1* expression, indicating the presence of a functional VDR signaling pathway in each of these tissue samples. Significant induction of *CLMN, EFTUD1*, and *KLK6* mRNA was observed in breast tissue explants following treatment with 1,25D, while there was a trend towards increased level of *SERPINB1* mRNA (p=0.08). It is of note that the basal expression levels of *SERPINB1* and *KLK6* were on average significantly higher in adjacent normal tissue than in malignant tissue. Basal *SERPINB1* expression levels varied considerably with high values observed in the adjacent normal samples, half of which (3/6) show no further increase or even decrease in *SERPINB1* expression upon 1,25D treatment. Similarly, one

adjacent normal with high basal level of *KLK6* shows a reduction of *KLK6* expression following 1,25D treatment, but was still higher than in all malignant samples assessed in the present study. It is possible that variations in stromal composition may contribute to this observed variation. Collectively, qRT-PCR analysis confirmed up-regulation of *CLMN*, *EFTUD1*, and *KLK6* mRNA in breast explants after 1,25D treatment, although regulation of *SERPINB1* expression by 1,25D was variable in adjacent normal tissue.

The induction of *CYP24A1*, *CLMN*, *SERPINB1*, *EFTUD1*, and *KLK6* mRNA by 1,25D was further investigated in human non-malignant breast epithelial, MCF10A, and breast cancer cell lines T47D, MCF-7, and MDA-MB-468. In all cases there was significant induction of the expression of all these five genes following 24-hour treatment with 100 nM 1,25D, although expression of *SERPINB1* mRNA was relatively low in T47D and MCF-7 (Fig. 2).

3.5.4 Ketoconazole potentiates the effect of 1,25D

The cytochrome P450 inhibitor ketoconazole (KTZ) was used to block catabolism of 1,25D to less active metabolites by the 24-hydroxylase (encoded by the gene *CYP24A1*). We investigated the possibility that KTZ could augment the response of cancer cells to 1,25D. MCF10A and MDA-MB-468 cells were treated with vehicle control, 1,25D (100 nM), KTZ (10 μM) or the combination of 1,25D and KTZ for 24 hours. Consistent with the previous experiments, 100 nM 1,25D alone caused a significant increase in the level of *CLMN*, *SERPINB1*, *EFTUD1*, and *KLK6* mRNA in MCF10A and MDA-MB-468 cells (Fig. 3). In MCF10A cells, KTZ alone (10 μM) did not affect the expression of *CLMN*, *SERPINB1*, *EFTUD1* or *KLK6* mRNA. However, KTZ significantly potentiated 1,25D mediated induction of *SERPINB1* and *KLK6* mRNA in MCF10A cells. Similarly, induction of *CLMN*, *SERPINB1,* and *KLK6* mRNA expression by 1,25D was further enhanced by KTZ in MDA-MB-468 breast

cancer cells (Fig. 3). No significant increase in *EFTUD1* mRNA levels was observed with the addition of KTZ to 1,25D-treated MCF10A and MDA-MB-468 cells.

3.5. Elevated *CLMN*, *SERPINB1*, *EFTUD1*, and *KLK6* expression levels predict prolonged breast cancer survival

To further extend the significance of these findings, KM-plotter was used to determine any influence of the expression levels of *CLMN*, *SERPINB1*, *EFTUD1*, and *KLK6* on breast cancer patient outcome. High expression levels of *CLMN*, *SERPINB1*, and *KLK6* were significantly associated with a more favorable relapse-free survival probability, while the influence of high *EFTUD1* expression level was marginal (Fig. 4).

3.6 Discussion

While *in vitro* studies suggest that 1,25D may exert anti-tumor effects, including anti-proliferation, anti-invasion, and anti-migration [7, 12], these observations have not yet translated to strategies that prevent or treat breast cancer. Here, we have attempted to elucidate the molecular pathways by which 1,25D acts in human breast cancer. Previous attempts to identify 1,25D target genes within breast cancer have largely focused on cancer cell lines, where VDR target gene regulation is highly heterogeneous [24]. We have investigated VDR target genes and molecular pathways within human breast tissue explants in which tissue architecture and multiplicity of cell types are maintained. Using RNA-Seq, 523 genes were identified to be differentially regulated by 1,25D in human breast cancer explants and 127 genes were differentially expressed in non-malignant breast tissue.

A greater number of differentially regulated genes were identified in malignant breast tissue compared to normal, which might be partly due to different basal expression levels of some

genes in two different types of tissue, as we have observed in additional breast tissue samples using qRT-PCR. It is of note that all three tumor samples subjected to RNA-Seq analysis in the present study are estrogen receptor (ER) positive (Supplementary Table 1). Further studies recruiting other subtypes of breast cancer are needed as gene expression profiling might be distinctive among various breast cancer subtypes following 1,25D treatment.

GoSeq KEGG pathway and gene ontology analyses were used to explore the possible functions of the 1,25D regulated genes. Cellular metabolic pathways that were significantly down-regulated in 1,25D-treated breast cancer explants included cellular nitrogen compound metabolism, transferase activity, ATP binding, nucleic acid metabolism, and cellular macromolecule metabolism. Taken together, these pathway analyses suggest a decreased rate of nucleotide metabolism and RNA production, which is consistent with reports that 1,25D inhibits breast cancer cell proliferation. Pathways that were enriched within 1,25D-treated breast cancer explants included cell adhesion molecules (CAMs), ECM-receptor interaction, focal adhesion, and tight junction, suggesting that intercellular and epithelial-stromal contacts became tighter following 1,25D treatment. For instance, expression of E-cadherin (*CDH1*) and Claudin 7 (*CDH7*) were up-regulated by 1,25D and have previously been associated with reduced progression and invasiveness of tumors, as well as breast cancer metastasis [25, 26]. The enriched pathways identified here using intact tissue differ somewhat from pathways altered by 1,25D in MCF-7, hTERT-HME, and SKBr3 cells [13, 14]. This may be partly due to the heterogeneous nature of tumor samples which include cancer cells, stromal cells, as well as extracellular molecules.

Within our explant model, it is important to consider that stromal cells may contribute to the overall response to 1,25D. Previously Campos *et al* [27] identified 25 genes that were commonly regulated between 1,25D treated cancer associated fibroblasts (CAFs) and breast

cancer slices, indicating that 1,25D may exert anti-tumor actions not only by targeting cancer cells but also their surrounding stromal compartment.

In total, 32 differentially expressed genes (fold change ≥ 1.5) in our explant models were also reported to be regulated by 1,25D in a recent RNA-Seq analysis of 1,25D-treated SKBr3 breast cancer cells [14]. Of these genes, the expression of *CYP24A1*, *CLMN*, *EFTUD1*, *RGNEF*, *SERPINB1*, and *TMEM37* were also regulated by 1,25D in a micro-array analysis of human breast cancer slices [15]. Most recently Simmons *et al* reported differential gene regulation in hTERT-HME and MCF-7 breast cancer cells treated with 1,25D [13]. By comparison, 7 genes (*CYP24A1*, *CLMN*, *SERPINB1*, *EFTUD1*, *RGNEF*, *G6PD*, and *SLC4A7*) were commonly up-regulated by 1,25D in our tumor explant model and in hTERT-HME and MCF-7 cells. We then focused further investigations on *SERPINB1*, *EFTUD1*, and *CLMN* that were common between these published studies and our explant system, and *KLK6* that showed the highest response to 1,25D treatment (other than *CYP24A1*) in our study. Induction of *CLMN*, *SERPINB1*, *EFTUD1*, and *KLK6* gene expression by 1,25D was confirmed by qRT-PCR analysis in a subset of breast tissue explants and breast cancer cell lines (Fig. 1 and Fig. 2).

A recent meta-analysis of epidemiological studies showed that higher 25D levels were significantly associated with improved overall survival (hazard ratio [HR]=0.63, 95% CI (0.51-0.77)) and disease-free survival (HR=0.42, 95% CI (0.29-0.62)) for patients with breast cancer [5]. Previous *in vitro* studies also suggest that high levels of 1,25D inhibit cancer cell invasion and migration [11, 28]. Therefore it is speculated that the targets of 1,25D in the breast will have a tumour suppressor type role. SERPINB1, a member of the SERPINB family, was up-regulated in breast cancer explants following 1,25D treatment. SERPINB1 was reported to be down-regulated in lung and liver cancer biopsies compared to normal matched tissues [29, 30]. Overexpression of *SERPINB1* was previously shown to be associated with significantly suppressed invasion and migration of MDA-MB-231 breast cancer cells, presumably by

blocking neutrophil elastase/proteinase-3/cathepsin G-mediated activation of MMP-2 [29]. KLK6, a member of the kallikrein family, was previously reported to be suppressed in breast cancer [31]. Its re-expression was shown to reverse the malignant phenotype of MDA-MB-231 cells, evidenced by decreased cell proliferation, inhibition of anchorage-independent growth, reduced invasion and down-regulation of the epithelial-to-mesenchymal transition marker vimentin [32]. CLMN function has not been previously investigated in breast tissue, however its overexpression causes inhibition of neuroblastoma cell proliferation via G1/S arrest [33]. The gene *EFTUD1* codes for domain one of EF-TU (elongation factor thermo unstable, also known as eEF1A). The function of EF-TU is to deliver amino acyl-tRNAs to acceptor sites of ribosomes during protein synthesis [34]. Overexpression of eEF1A is known to be involved in oncogenic transformation, cell proliferation and metastasis in several cancers [35, 36]. Under stress conditions, however, its overexpression can accelerate apoptosis [37, 38]. Based on these observations, we speculate that EFTUD1 might play an important role to mediate the pro-apoptotic effect of 1,25D.

Using an *ex vivo* explants model, we identified 1,25D induced expression of *CLMN*, *SERPINB1*, *EFTUD1*, and *KLK6* mRNA in a subset of malignant breast, adjacent normal, and cosmetic reduction breast tissue and available functional data are consistent with a role in the suppression of tumorigenesis. Analysis of publically available breast cancer gene expression data linked to patient clinical outcomes [19] showed high levels of *CLMN*, *SERPINB1*, and *KLK6* were significantly associated with prolonged relapse-free survival while the association with *EFTUD1* expression was marginal. Therefore, we propose that CLMN, SERPINB1, and KLK6 function as vitamin D_3 driven tumour suppressors in breast epithelium.

Two early studies reported that CYP24A1 inhibition by KTZ can enhance the anti-tumor activity of 1,25D in human prostate cancer PC3 cells and breast cancer MCF-7 cells [39, 40]. In the present study, KTZ was able to potentiate the effect of 1,25D to induce *CLMN*,

SERPINB1, and *KLK6* mRNA expression. Taken together, these findings demonstrate the potential of pharmacological agents to support the beneficial activities of 1,25D in cancer. The development of more specific and potent CYP24A1 inhibitors may provide a valuable adjunct therapy to maintain higher cellular levels of 1,25D within the breast tissue.

Although *ex vivo* culture of human breast tissue potentially provides a more clinically relevant model than cell lines, some limitations remain. For example, the explants lack a functional vasculature and systemic hormonal regulation and feedback loops with other organs, and therefore may not completely reflect dynamic signaling pathway changes responding to 1,25D under *in vivo* conditions. As well, the limited number of breast cancer samples yielded variable data, and the effects of a relatively high concentration of 1,25D were assessed at a single time-point. Future studies are required to confirm that the observed gene expression changes are reflected in altered protein levels under *in vivo* conditions.

3.7 Conclusions

This study is the first to use RNA-Seq analyses to investigate 1,25D target gene expression in *ex vivo* explants of human breast tissue, which maintain higher order structures and multiple cell types. The major finding of the present study is that, in this *ex vivo* explants system, exposure of both normal and malignant breast tissue to 1,25D results in changes in cellular adhesion, metabolic pathways and tumor suppressor-like pathways, which support epidemiological data suggesting that adequate circulating 25D levels may improve breast cancer outcome. The current study confirmed that *CLMN*, *SERPINB1*, *EFTUD1,* and *KLK6* are direct up-regulated targets of 1,25D, which have reported functions consistent with roles as tumor suppressors. These tumor suppressor roles are further supported by analysis of publically available breast cancer cohorts where high expression levels, particularly, of *CLMN*,

SERPINB1, and *KLK6* are associated with prolonged relapse-free survival for breast cancer patients. Further investigation is needed to determine whether adequate circulating 25D levels are able to alter the expression of these genes under *in vivo* conditions.

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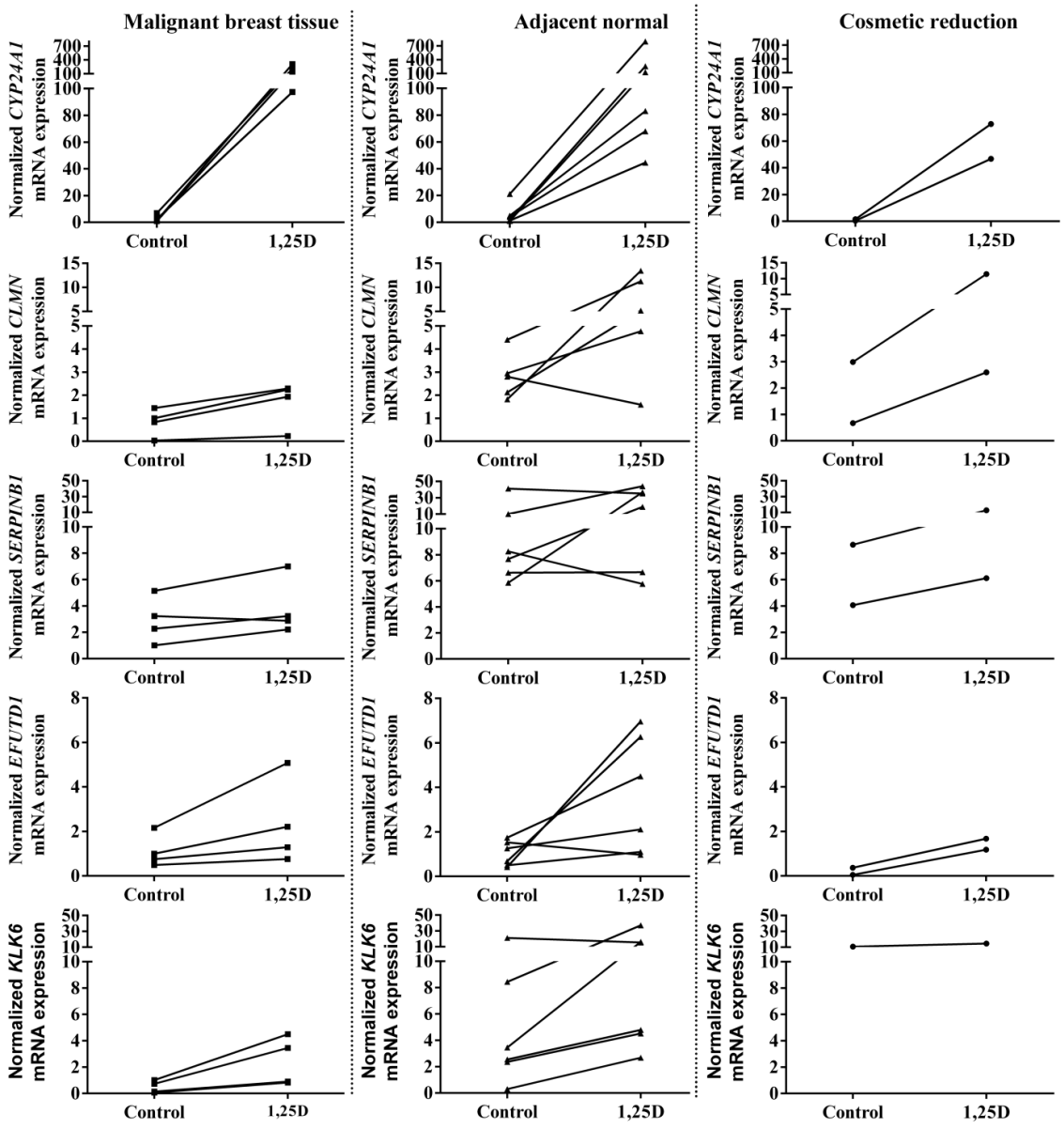

Fig. 1. *CYP24A1*, *CLMN*, *SERPINB1*, *EFTUD1*, and *KLK6* expression levels following treatment with 1,25D in malignant breast tissue, adjacent normal and cosmetic reduction mammoplasties. Tissues were treated with 100 nM 1,25D or ethanol vehicle (Control) for 24 hours. Tissues were collected and qRT-PCR measurements of expression levels of *CYP24A1*, *CLMN*, *SERPINB1*, *EFTUD1*, and *KLK6* were made. Expression data were normalized against the housekeeping gene *PPIG* and expressed relative to values for the vehicle treated tumor explant (Case 1, Supplementary Table 1). Each line of 2 connected points represents one independent sample. Data were analyzed by two-way ANOVA to determine the effect of 1,25D treatment for each gene. Two-way ANOVA

indicated no interaction between tissue type and treatment for CLMN, SERPINB1, EFTUD1, and KLK6.

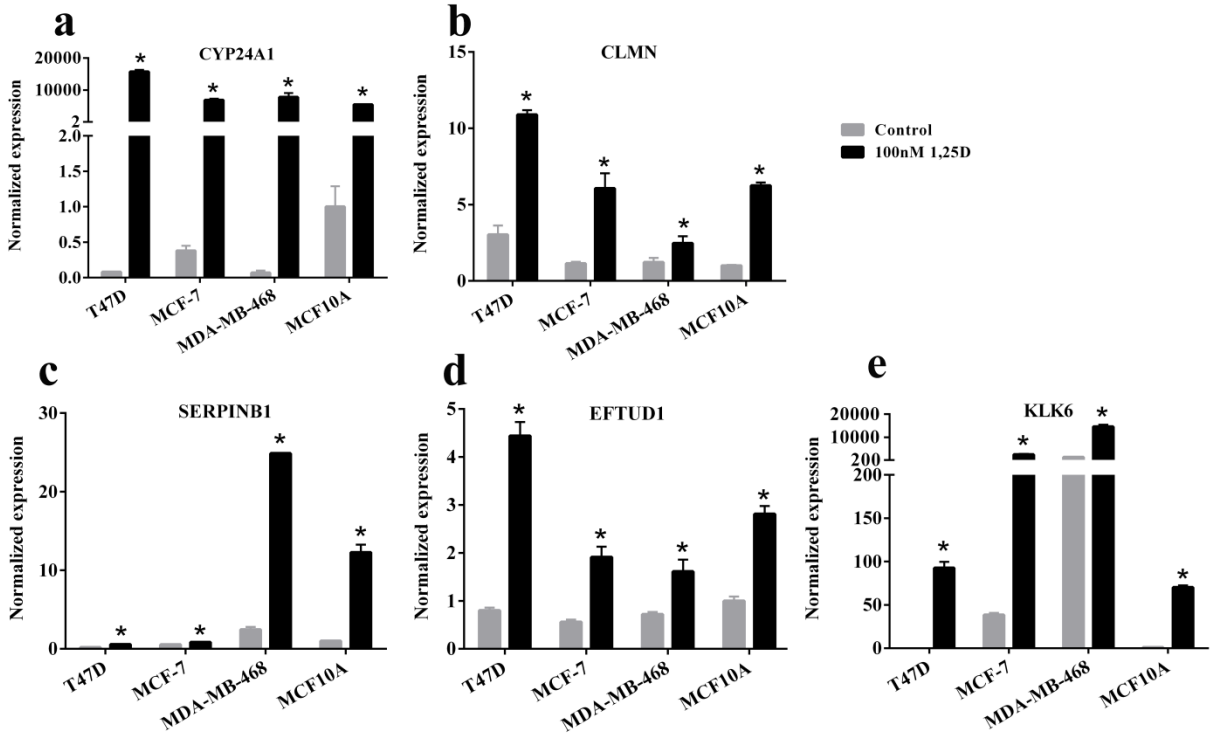

Fig. 2. *CYP24A1* (**a**), *CLMN* (**b**), *SERPINB1* (**c**), *EFTUD1* (**d**), and *KLK6* (**e**) mRNA expression levels following treatment with 1,25D in non-malignant and malignant breast cell lines. qRT-PCR analyses of *CYP24A1*, *CLMN*, *SERPINB1*, *EFTUD1*, and *KLK6* mRNA expression were made in T47D, MCF-7, MDA-MB-468, and MCF10A treated with 100 nM 1,25D or vehicle for 24 hours. Data were normalized against *PPIG* and expressed relative to values for the vehicle treated MCF10A cells which was set to 1. Each bar represents mean ± standard deviation of three independent samples. *p-value < 0.05 for the effect of 1,25D within each cell line was assessed by unpaired t-test.

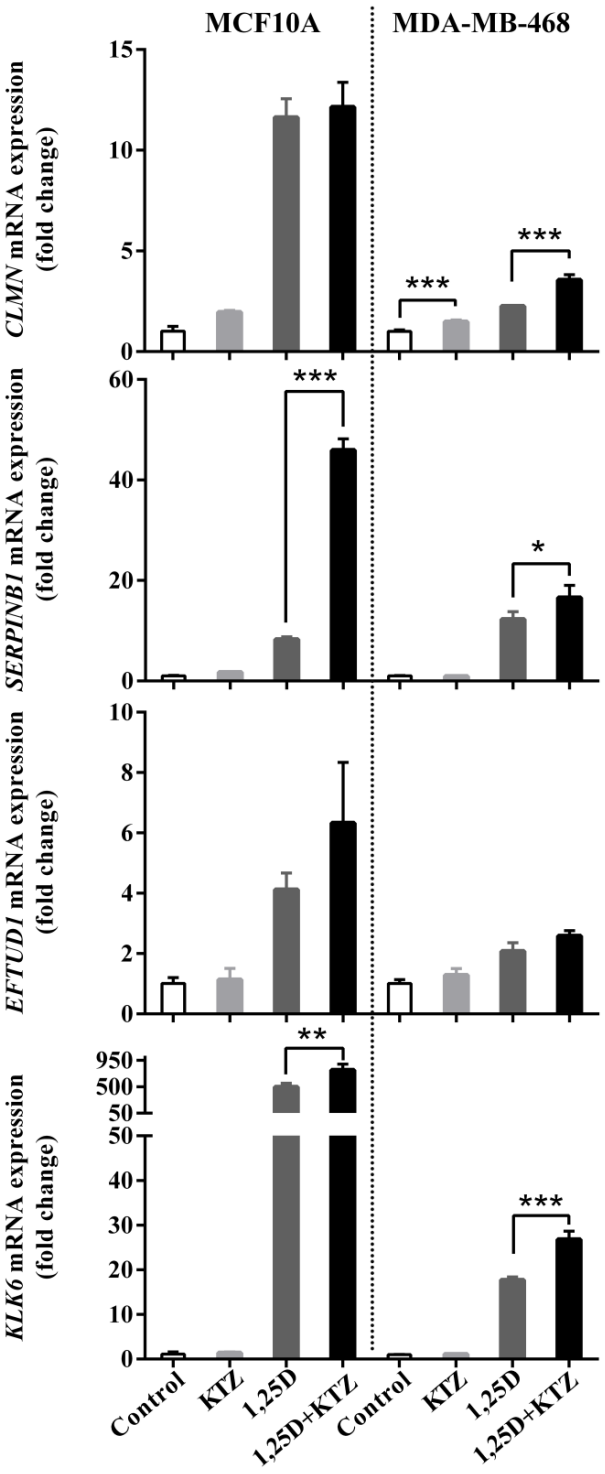

Fig. 3. Ketoconazole (KTZ) inhibits the catabolism of 1,25D through inhibition of CYP24A1, and potentiates 1,25D mediated induction of *CLMN*, *SERPINB1*, and *KLK6* mRNA, but not *EFTUD1* mRNA. The expression levels of *CLMN*, *SERPINB1*, *EFTUD1*, and *KLK6* mRNA were determined in MCF10A and MDA-MB-468 cells following treatment with vehicle, KTZ (10 μM), 1,25D (100 nM) alone, or in combination with KTZ for 24 hours. Mean values ± SD of triplicates are shown to represent the fold change of gene expression across various treatments. Statistical significance was assessed by one-way ANOVA followed by Tukey's multiple comparison tests ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

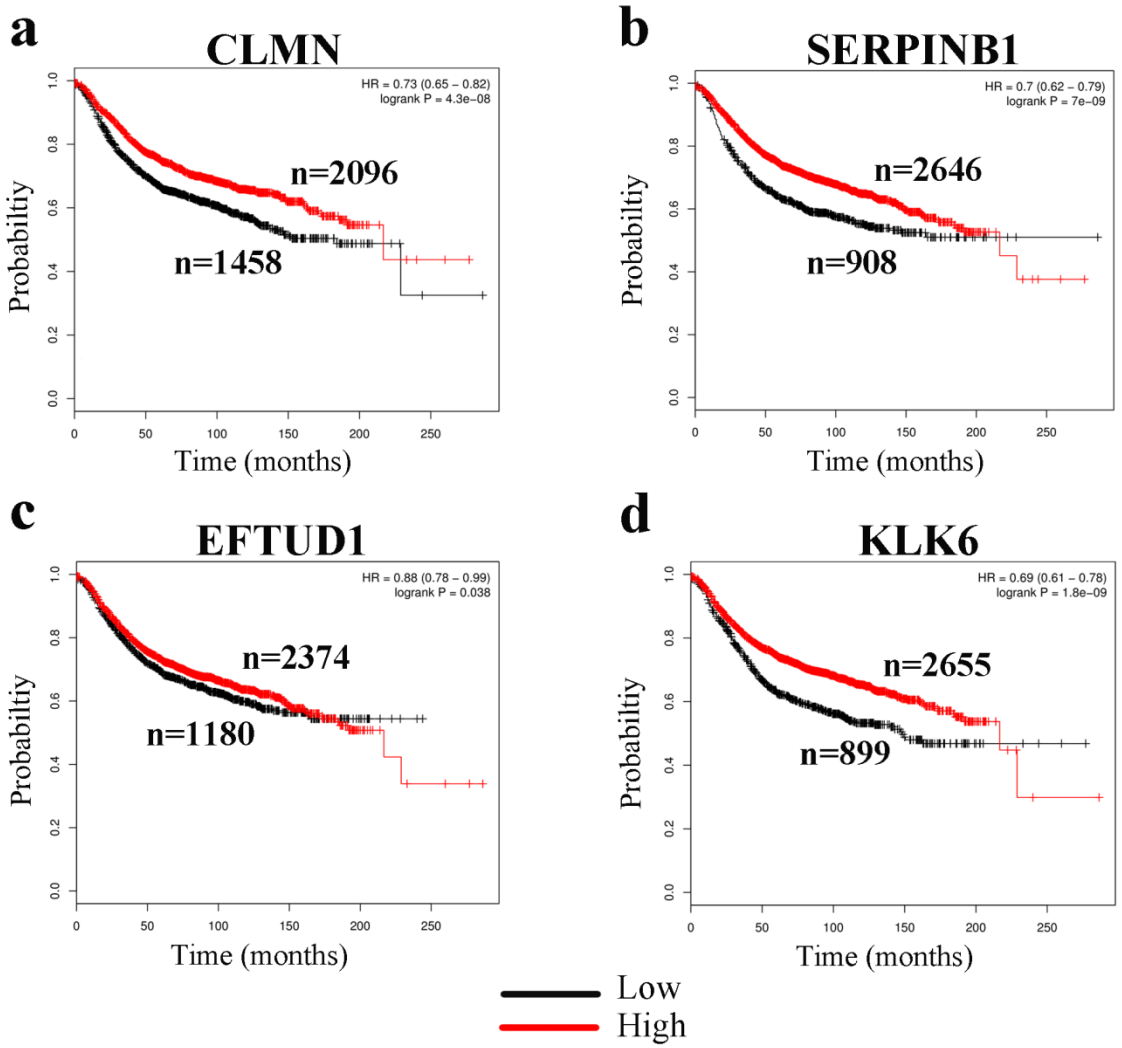

Fig. 4. Correlation of *CLMN* (a), *SERPINB1* (b), *EFTUD1* (c), and *KLK6* (d) expression with relapse-free survival for breast cancer patients. Data were analyzed with the KM-plotter by allowing the software to select the best cut-off and only JetSet best probe set [19]. Red and black lines indicate patients with higher and lower *CLMN*, *SERPINB1*, *EFTUD1*, and *KLK6* expression respectively. The total number of patients in the two categories for each gene is shown. Hazard ratios (HRs) and *p* values (log rank *p*) are shown at the right top of the panel.

Supplementary Table 1

Characteristics of patients with breast cancer subjected to RNA-Seq analysis and qRT-PCR analysis

Cases	Age (year)	Histological type	ER status	PR status	HER2 status	Ki-67 positive cells (%)	Grade	AJCC pTNM stage (7th edition)
Tumor samples for RNA-Seq analysis								
Case A	34	IDC	(+)	(+)	(+)	40	III	pT2N1bM0
Case B	34	IDC	(+)	(+)	(-)	65	III	pT1cN0M0
Case C	54	IDC	(+)	(+)	(-)	25	I	pT2N0M0
Tumor samples for qRT-PCR analysis								
Case 1	74	ILC	(+)	(+)	(-)	20	II	pT2N1miM0
Case 2	71	IDC	(-)	(-)	(-)	72	III	pT3N3aM0
Case 3	59	IDC	(-)	(-)	(-)	70	III	pT3N1aM0
Case 4	73	IDC	(+)	(+)	(-)	55	III	pT2N0M0

IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma; ER: estrogen receptor immunoexpression; PR: progesterone receptor immunoexpression; ER/PR/HER2 status: ER/PR positivity and HER2 amplification are indicated ('-': negative or not amplified ; '+': positive or amplified)

Supplementary Table 2

Primers for qRT-PCR

Gene symbols	Primers	Annealing Temperature	PCR products(bp)
PPIG	Forward: 5'-CAGATGCAGCTAGCAAACCGTTTG-3' Reverse: 5'-CTCTTCAGTAGCACTTTCGGAATCAGAGG-3'	60℃	200
CYP24A1	Forward: 5'-CCTGAGAATCAGGTGCCACGGGCA-3' Reverse: 5'-CTTCACTGGATCCCAACACCTGGGT-3'	60℃	199
CLMN	Forward: 5'-GTGAAAGACCAGAGGAAGGCTA-3' Reverse: 5'-TGATGCGAACAAAAGTGGAT-3'	60℃	397
SERPINB1	Forward: 5'-TCAGCTTGCCCAGGTTCAAACTG-3' Reverse: 5'-GGATGCTACCTGAGGAATTATGC-3'	60℃	300
EFTUD1	Forward: 5'-GCCGCCTAGCAGGCAAGTTA-3' Reverse: 5'-AGCCAAGCTTGTCGCAGAACT-3'	60℃	265
KLK6	Forward: 5'-GCCCAGCCAAACTCTCTG-3' Reverse: 5'-TGTTACCCCATGACACAAGG-3'	60℃	306

Supplementary Table 3_1

Vitamin D target genes identified in human breast cancer

Gene	Regulation	FDR	Fold Change	Gene Description
CYP24A1	up	1.45E-139	243.00	cytochrome P450, family 24, subfamily A, polypeptide 1
KLK6	up	1.59E-22	7.27	kallikrein-related peptidase 6
CASP14	up	3.62E-08	5.82	caspase 14, apoptosis-related cysteine peptidase
MMP8	up	4.72E-14	4.80	matrix metallopeptidase 8 (neutrophil collagenase)
KRT16	up	1.76E-08	4.25	keratin 16; keratin type 16-like
CILP	up	3.44E-04	4.21	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase
CYP2B7P1	up	1.22E-10	4.11	cytochrome P450, family 2, subfamily B, polypeptide 7 pseudogene 1
CALML3	up	5.75E-07	3.71	calmodulin-like 3
CYP4F11	up	3.35E-10	3.60	cytochrome P450, family 4, subfamily F, polypeptide 11
SERPINB1	up	6.83E-11	3.59	serpin peptidase inhibitor, clade B (ovalbumin), member 1
TMPRSS2	up	1.41E-06	3.40	transmembrane protease, serine 2
CYP26B1	up	6.11E-17	3.23	cytochrome P450, family 26, subfamily B, polypeptide 1
SEMA3B	up	2.19E-11	3.20	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B
CA2	up	5.39E-08	3.18	carbonic anhydrase II
S100A7	up	2.03E-06	3.17	S100 calcium binding protein A7
PRSS22	up	3.10E-04	3.00	protease, serine, 22

SLC34A2	up	1.88E-07	2.99	solute carrier family 34 (sodium phosphate), member 2
CD14	up	5.30E-05	2.99	CD14 molecule
FBP1	up	5.92E-03	2.93	fructose-1,6-bisphosphatase 1
KLK10	up	1.37E-05	2.92	kallikrein-related peptidase 10
PDZK1IP1	up	6.04E-05	2.92	PDZK1 interacting protein 1
CEACAM1	up	2.01E-06	2.90	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)
CYP1A1	up	8.11E-08	2.88	cytochrome P450, family 1, subfamily A, polypeptide 1
CLDN4	up	2.80E-04	2.86	claudin 4
TNNT1	up	5.17E-07	2.85	troponin T type 1 (skeletal, slow)
CYP4F22	up	3.62E-08	2.85	cytochrome P450, family 4, subfamily F, polypeptide 22
CDH3	up	4.41E-07	2.84	cadherin 3, type 1, P-cadherin (placental)
SULT2B1	up	1.06E-04	2.80	sulfotransferase family, cytosolic, 2B, member 1
COL17A1	up	2.92E-05	2.80	collagen, type XVII, alpha 1
KLK7	up	1.16E-05	2.79	kallikrein-related peptidase 7
DSP	up	2.52E-06	2.69	desmoplakin
COL4A5	up	1.11E-05	2.69	collagen, type IV, alpha 5
KRT6B	up	4.75E-10	2.67	keratin 6B
ARHGEF37	up	3.71E-06	2.67	Rho guanine nucleotide exchange factor (GEF) 37
KCNK3	up	5.90E-06	2.64	potassium channel, subfamily K, member 3
RARRES1	up	1.15E-06	2.59	retinoic acid receptor responder (tazarotene induced) 1

ERBB3	up	2.72E-05	2.59	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
SLPI	up	3.12E-03	2.58	secretory leukocyte peptidase inhibitor
PRLR	up	6.95E-09	2.58	prolactin receptor
CGN	up	4.04E-04	2.56	cingulin
PAQR5	up	2.36E-05	2.56	progestin and adipoQ receptor family member V
G0S2	up	3.49E-03	2.52	G0/G1switch 2
HSPB8	up	1.01E-10	2.50	heat shock 22kDa protein 8
TNS4	up	7.73E-06	2.49	tensin 4
SCNN1B	up	2.65E-06	2.49	sodium channel, nonvoltage-gated 1, beta
EFTUD1	up	1.69E-10	2.49	elongation factor Tu GTP binding domain containing 1
CEACAM6	up	5.07E-03	2.48	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)
CADM3	up	1.48E-05	2.47	cell adhesion molecule 3
MAPK8IP2	up	1.52E-06	2.46	mitogen-activated protein kinase 8 interacting protein 2
DSG3	up	5.07E-05	2.46	desmoglein 3 (pemphigus vulgaris antigen)
SERPINB5	up	7.54E-09	2.45	serpin peptidase inhibitor, clade B (ovalbumin), member 5
TSPAN1	up	1.13E-04	2.44	tetraspanin 1
MAPT	up	1.15E-06	2.44	microtubule-associated protein tau
CXCL17	up	9.93E-06	2.43	chemokine (C-X-C motif) ligand 17
CELSR2	up	7.33E-10	2.42	cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)

CLDN7	up	2.10E-04	2.42	claudin 7
PLEKHG6	up	9.05E-04	2.42	pleckstrin homology domain containing, family G (with RhoGef domain) member 6
SCNN1A	up	5.15E-05	2.42	sodium channel, nonvoltage-gated 1 alpha
FAM110C	up	6.84E-03	2.41	family with sequence similarity 110, member C
RND1	up	4.26E-02	2.41	Rho family GTPase 1
IGSF9	up	8.08E-04	2.41	immunoglobulin superfamily, member 9
IRF6	up	8.06E-06	2.40	interferon regulatory factor 6
SOX10	up	2.65E-03	2.39	SRY (sex determining region Y)-box 10
CLMN	up	2.06E-10	2.37	calmin (calponin-like, transmembrane)
CDH1	up	2.13E-04	2.36	cadherin 1, type 1, E-cadherin (epithelial)
SHE	up	3.56E-05	2.36	Src homology 2 domain containing E
TACSTD2	up	1.53E-03	2.36	tumor-associated calcium signal transducer 2
RAB11FIP1	up	1.75E-03	2.35	RAB11 family interacting protein 1 (class I)
PPL	up	2.57E-08	2.34	periplakin
TP63	up	8.11E-06	2.30	tumor protein p63
SERINC2	up	3.03E-04	2.29	serine incorporator 2
CAMSAP3	up	8.77E-03	2.28	calmodulin regulated spectrin-associated protein family, member 3
LAMB3	up	1.80E-05	2.28	laminin, beta 3
MUC1	up	3.34E-04	2.27	mucin 1, cell surface associated
TRIM29	up	2.71E-07	2.24	tripartite motif-containing 29

LTF	up	4.13E-02	2.23	lactotransferrin
EVPL	up	1.30E-03	2.23	envoplakin
TMC4	up	4.01E-04	2.22	transmembrane channel-like 4
PTPRF	up	8.39E-05	2.21	protein tyrosine phosphatase, receptor type, F
ELF3	up	2.61E-02	2.21	E74-like factor 3 (ets domain transcription factor, epithelial-specific)
PKIB	up	5.42E-04	2.21	protein kinase (cAMP-dependent, catalytic) inhibitor beta
MYH14	up	7.62E-04	2.20	myosin, heavy chain 14
SBK1	up	1.73E-02	2.20	SH3-binding domain kinase 1
CLDN11	up	2.72E-05	2.18	claudin 11
RGNEF	up	8.72E-06	2.18	Rho-guanine nucleotide exchange factor
FBXO2	up	7.54E-09	2.16	F-box protein 2
WWC1	up	7.54E-09	2.16	WW and C2 domain containing 1
TGM2	up	1.19E-02	2.16	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)
ALS2CL	up	7.23E-07	2.16	ALS2 C-terminal like
CDS1	up	3.26E-03	2.15	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 1
CP	up	3.17E-06	2.15	ceruloplasmin (ferroxidase)
ST6GALNAC2	up	1.23E-02	2.15	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2
TMEM37	up	1.18E-04	2.15	transmembrane protein 37
FXYD3	up	5.02E-04	2.14	FXYD domain containing ion transport regulator 3

PGBD5	up	9.36E-03	2.14	piggyBac transposable element derived 5
KRT15	up	6.96E-05	2.14	keratin 15
RIPK4	up	7.26E-05	2.14	receptor-interacting serine-threonine kinase 4
CASZ1	up	3.84E-04	2.14	castor zinc finger 1
ITGB4	up	1.21E-04	2.13	integrin, beta 4
CHMP4C	up	4.24E-03	2.13	chromatin modifying protein 4C
EPB41L4B	up	1.66E-03	2.13	erythrocyte membrane protein band 4.1 like 4B
RHPN2	up	8.00E-04	2.12	rhophilin, Rho GTPase binding protein 2; similar to rhophilin, Rho GTPase binding protein 2
KLHDC7B	up	1.44E-04	2.12	kelch domain containing 7B
CACNG4	up	5.93E-03	2.11	calcium channel, voltage-dependent, gamma subunit 4
WFDC2	up	1.46E-04	2.11	WAP four-disulfide core domain 2
FBXL16	up	6.27E-04	2.11	F-box and leucine-rich repeat protein 16
PROM2	up	1.29E-04	2.10	prominin 2
LBP	up	3.17E-02	2.10	lipopolysaccharide binding protein
GABRP	up	2.28E-03	2.10	gamma-aminobutyric acid (GABA) A receptor, pi
KRT19	up	2.28E-02	2.10	keratin 19
ITGA2	up	1.88E-06	2.10	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)
OCLN	up	2.81E-03	2.08	occludin pseudogene; occludin
KRT23	up	2.41E-03	2.07	keratin 23 (histone deacetylase inducible)
C1orf106	up	4.71E-02	2.07	chromosome 1 open reading frame 106

MREG	up	7.56E-05	2.07	melanoregulin
ARHGEF16	up	2.84E-02	2.07	Rho guanine exchange factor (GEF) 16
GRHL1	up	1.02E-03	2.07	grainyhead-like 1 (Drosophila)
KIAA1467	up	4.88E-03	2.06	KIAA1467
C1orf116	up	1.21E-03	2.06	chromosome 1 open reading frame 116
DHCR24	up	5.28E-05	2.06	24-dehydrocholesterol reductase
THSD4	up	1.15E-05	2.06	thrombospondin, type I, domain containing 4
DSC3	up	1.23E-03	2.05	desmocollin 3
RORC	up	5.80E-03	2.05	RAR-related orphan receptor C
TREM1	up	2.95E-03	2.05	triggering receptor expressed on myeloid cells 1
MYO5B	up	9.69E-04	2.05	similar to acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase); similar to KIAA1119 protein; myosin VB
PLEKHA6	up	5.37E-04	2.05	pleckstrin homology domain containing, family A member 6
GATA3	up	6.69E-07	2.05	GATA binding protein 3
NRARP	up	1.02E-02	2.04	NOTCH-regulated ankyrin repeat protein
ESRP1	up	1.11E-03	2.04	epithelial splicing regulatory protein 1
TFAP2A	up	2.10E-06	2.04	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)
CLDN3	up	3.38E-02	2.04	claudin 3
GRHL2	up	1.07E-03	2.02	grainyhead-like 2 (Drosophila)
TM4SF1	up	2.42E-03	2.01	transmembrane 4 L six family member 1

STEAP3	up	8.00E-07	2.01	STEAP family member 3
CHDH	up	1.47E-03	1.99	choline dehydrogenase
HES2	up	4.34E-02	1.99	hairy and enhancer of split 2 (Drosophila)
KIAA1244	up	4.26E-02	1.99	KIAA1244
TJP3	up	1.27E-02	1.99	tight junction protein 3 (zona occludens 3)
G6PD	up	2.91E-04	1.99	glucose-6-phosphate dehydrogenase
LYZ	up	6.61E-03	1.99	lysozyme (renal amyloidosis)
SNCG	up	6.54E-03	1.99	synuclein, gamma (breast cancer-specific protein 1)
ZNF185	up	1.64E-02	1.98	zinc finger protein 185 (LIM domain)
DPP4	up	1.16E-05	1.98	dipeptidyl-peptidase 4
KRTCAP3	up	7.75E-03	1.98	keratinocyte associated protein 3
CD24	up	1.68E-02	1.97	CD24 molecule; CD24 molecule-like 4
KRT5	up	9.15E-03	1.97	keratin 5
SERPINA5	up	1.92E-03	1.95	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5
CELSR1	up	1.46E-05	1.94	cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, Drosophila)
DSC2	up	1.01E-03	1.94	desmocollin 2
THBD	up	3.30E-05	1.94	thrombomodulin
KRT18	up	2.29E-02	1.93	keratin 18; keratin 18 pseudogene 26; keratin 18 pseudogene 19
BSPRY	up	2.41E-03	1.93	B-box and SPRY domain containing
C8orf85	up	1.60E-02	1.93	chromosome 8 open reading frame 85

HR	up	3.40E-04	1.93	hairless homolog (mouse)
DMKN	up	6.18E-04	1.92	dermokine
SERPINA3	up	1.30E-02	1.92	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3
TFF1	up	2.07E-02	1.92	trefoil factor 1
EPPK1	up	3.61E-03	1.92	epiplakin 1
KLF5	up	1.92E-03	1.92	Kruppel-like factor 5 (intestinal)
DEFB1	up	5.08E-03	1.92	defensin, beta 1
HOOK1	up	2.80E-02	1.91	hook homolog 1 (Drosophila)
PVRL4	up	4.10E-02	1.91	poliovirus receptor-related 4
CNKSR1	up	6.61E-03	1.91	connector enhancer of kinase suppressor of Ras 1
RBM24	up	2.23E-02	1.91	RNA binding motif protein 24
SUSD3	up	1.91E-02	1.90	sushi domain containing 3
MAPK13	up	4.23E-06	1.90	mitogen-activated protein kinase 13
RBP7	up	1.02E-03	1.89	retinol binding protein 7, cellular
LYPD3	up	3.75E-02	1.89	LY6/PLAUR domain containing 3
SFN	up	1.22E-05	1.89	stratifin
AP1M2	up	1.25E-03	1.89	adaptor-related protein complex 1, mu 2 subunit
KIF5C	up	2.42E-03	1.89	kinesin family member 5C
SERPINA1	up	4.52E-02	1.88	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
PKP3	up	6.97E-03	1.88	plakophilin 3

TNK1	up	5.93E-03	1.88	tyrosine kinase, non-receptor, 1
SEZ6L2	up	1.48E-03	1.87	seizure related 6 homolog (mouse)-like 2
KIAA1324	up	8.20E-04	1.87	KIAA1324
LGALS9	up	3.51E-04	1.87	lectin, galactoside-binding, soluble, 9
LOC100130899	up	1.31E-02	1.87	hypothetical protein LOC100130899
KLK5	up	1.38E-03	1.87	kallikrein-related peptidase 5
TTC9	up	1.23E-02	1.86	tetratricopeptide repeat domain 9
JUP	up	9.50E-04	1.86	junction plakoglobin
KRT6A	up	2.63E-02	1.86	keratin 6A
PPM1H	up	9.59E-05	1.86	protein phosphatase 1H (PP2C domain containing)
FAM83H	up	3.83E-05	1.85	family with sequence similarity 83, member H
SLC4A7	up	3.56E-05	1.85	solute carrier family 4, sodium bicarbonate cotransporter, member 7
RAB17	up	2.89E-02	1.85	RAB17, member RAS oncogene family
ITGA3	up	3.20E-03	1.84	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)
FAM110A	up	2.64E-02	1.84	family with sequence similarity 110, member A
ITPR1	up	9.36E-06	1.84	inositol 1,4,5-triphosphate receptor, type 1
ENTPD2	up	2.74E-02	1.83	ectonucleoside triphosphate diphosphohydrolase 2
SPINT2	up	3.23E-02	1.83	serine peptidase inhibitor, Kunitz type, 2
LSR	up	3.29E-03	1.83	lipolysis stimulated lipoprotein receptor

CRLF1	up	5.58E-03	1.82	cytokine receptor-like factor 1
MANEAL	up	2.04E-02	1.82	mannosidase, endo-alpha-like
ZNF107	up	3.40E-03	1.82	zinc finger protein 107
LMX1B	up	7.13E-03	1.82	LIM homeobox transcription factor 1, beta
TTC39A	up	2.42E-03	1.82	tetratricopeptide repeat domain 39A
NFASC	up	1.26E-02	1.82	neurofascin homolog (chicken)
ITGB8	up	7.65E-05	1.81	integrin, beta 8
DDR1	up	5.08E-03	1.81	discoidin domain receptor tyrosine kinase 1
S100A2	up	1.39E-02	1.81	S100 calcium binding protein A2
MMP7	up	7.61E-03	1.81	matrix metallopeptidase 7 (matrilysin, uterine)
GPR56	up	8.00E-04	1.80	G protein-coupled receptor 56
JAG2	up	3.87E-03	1.80	jagged 2
SYT12	up	2.92E-02	1.80	synaptotagmin XII
ESR1	up	2.59E-02	1.80	estrogen receptor 1
PPP1R14C	up	1.07E-02	1.80	protein phosphatase 1, regulatory (inhibitor) subunit 14C
ZNF462	up	1.69E-02	1.80	zinc finger protein 462
N4BP3	up	6.27E-04	1.80	Nedd4 binding protein 3
MAP7	up	4.49E-03	1.79	microtubule-associated protein 7
FLJ23867	up	1.06E-02	1.79	hypothetical protein FLJ23867
IL34	up	1.94E-02	1.79	interleukin 34

TFCP2L1	up	2.38E-03	1.79	transcription factor CP2-like 1
MFI2	up	1.38E-02	1.79	antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5
C17orf28	up	6.02E-03	1.79	chromosome 17 open reading frame 28
AFF3	up	4.54E-02	1.79	AF4/FMR2 family, member 3
MAOB	up	3.93E-03	1.79	monoamine oxidase B
MPP7	up	2.15E-02	1.79	membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7)
LCN2	up	3.30E-03	1.78	lipocalin 2
OBSCN	up	1.08E-03	1.77	obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF
DSG2	up	8.95E-03	1.77	desmoglein 2
RPS6KA1	up	2.47E-05	1.77	ribosomal protein S6 kinase, 90kDa, polypeptide 1
ACTG2	up	1.18E-04	1.77	actin, gamma 2, smooth muscle, enteric
CDCP1	up	5.01E-03	1.76	CUB domain containing protein 1
LAMA3	up	3.12E-03	1.76	laminin, alpha 3
DBNDD1	up	2.07E-02	1.76	dysbindin (dystrobrevin binding protein 1) domain containing 1
EHF	up	1.23E-03	1.75	ets homologous factor
INPP4B	up	2.09E-02	1.75	inositol polyphosphate-4-phosphatase, type II, 105kDa
NGFR	up	8.25E-03	1.75	nerve growth factor receptor (TNFR superfamily, member 16)
FAM46B	up	3.09E-02	1.75	family with sequence similarity 46, member B
TPD52	up	4.31E-02	1.75	tumor protein D52
TMEM79	up	1.44E-02	1.74	transmembrane protein 79

SHROOM3	up	5.58E-03	1.74	shroom family member 3
MAP3K1	up	5.19E-04	1.73	mitogen-activated protein kinase kinase kinase 1
SEMA4B	up	7.84E-03	1.72	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4B
ALDH3B2	up	2.07E-02	1.72	aldehyde dehydrogenase 3 family, member B2
KRT7	up	4.14E-02	1.71	keratin 7
IL24	up	6.22E-04	1.70	interleukin 24
GPT2	up	8.85E-03	1.70	glutamic pyruvate transaminase (alanine aminotransferase) 2
MFSD6	up	2.28E-03	1.70	major facilitator superfamily domain containing 6
ACADSB	up	4.88E-03	1.68	acyl-Coenzyme A dehydrogenase, short/branched chain
ANGPT2	up	3.91E-04	1.68	angiopoietin 2
PLXNB3	up	4.36E-02	1.68	plexin B3
HOMER2	up	4.26E-02	1.68	homer homolog 2 (Drosophila)
LINGO1	up	2.85E-02	1.68	leucine rich repeat and Ig domain containing 1
FAM84A	up	2.39E-02	1.67	hypothetical LOC653602; family with sequence similarity 84, member A
PTPLB	up	1.43E-02	1.67	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member b
BCAM	up	3.12E-03	1.67	basal cell adhesion molecule (Lutheran blood group)
AGL	up	1.91E-02	1.67	amylo-1, 6-glucosidase, 4-alpha-glucanotransferase
GJA4	up	5.29E-03	1.67	gap junction protein, alpha 4, 37kDa
SYNM	up	2.97E-02	1.66	synemin, intermediate filament protein

ADAMTS9	up	3.69E-02	1.66	ADAM metallopeptidase with thrombospondin type 1 motif, 9
DHCR7	up	1.58E-03	1.65	7-dehydrocholesterol reductase
IL33	up	1.31E-03	1.65	interleukin 33
PTPN3	up	1.52E-02	1.65	protein tyrosine phosphatase, non-receptor type 3
TPD52L1	up	4.54E-02	1.65	tumor protein D52-like 1
GUCY1A3	up	2.98E-03	1.65	guanylate cyclase 1, soluble, alpha 3
KIAA1522	up	5.70E-03	1.65	KIAA1522
VTCN1	up	4.57E-02	1.64	V-set domain containing T cell activation inhibitor 1
IL1RAP	up	8.41E-03	1.64	interleukin 1 receptor accessory protein
DTNB	up	1.19E-02	1.64	dystrobrevin, beta
TMEM30B	up	4.60E-02	1.64	transmembrane protein 30B
PCDH1	up	6.37E-03	1.64	protocadherin 1
ABCA3	up	2.80E-02	1.63	ATP-binding cassette, sub-family A (ABC1), member 3
LDLR	up	1.37E-03	1.63	low density lipoprotein receptor
ERMP1	up	2.19E-02	1.63	endoplasmic reticulum metallopeptidase 1
IRS1	up	1.11E-03	1.63	insulin receptor substrate 1
PDE4B	up	1.60E-03	1.63	phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)
GALNT6	up	8.53E-03	1.62	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 6 (GalNAc-T6)
PPAP2C	up	4.86E-02	1.62	phosphatidic acid phosphatase type 2C

CARD10	up	3.78E-03	1.62	caspase recruitment domain family, member 10
KIAA1671	up	1.94E-03	1.62	KIAA1671 protein
AOC3	up	2.31E-03	1.61	amine oxidase, copper containing 3 (vascular adhesion protein 1)
CLCF1	up	1.54E-03	1.60	cardiotrophin-like cytokine factor 1
FLT1	up	1.56E-02	1.60	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
IGSF3	up	1.60E-02	1.60	immunoglobulin superfamily, member 3
PPIP5K1	up	2.67E-02	1.60	histidine acid phosphatase domain containing 2A
PIK3R3	up	2.87E-03	1.60	phosphoinositide-3-kinase, regulatory subunit 3 (gamma)
KCNC4	up	4.86E-02	1.59	potassium voltage-gated channel, Shaw-related subfamily, member 4
PER2	up	2.96E-02	1.59	period homolog 2 (Drosophila)
MST4	up	3.23E-02	1.58	serine/threonine protein kinase MST4
VWF	up	1.67E-02	1.57	von Willebrand factor
TJP2	up	2.86E-02	1.57	tight junction protein 2 (zona occludens 2)
MSMO1	up	4.14E-02	1.56	methylsterol monooxygenase 1
AZGP1	up	6.54E-03	1.56	alpha-2-glycoprotein 1, zinc-binding pseudogene 1; alpha-2-glycoprotein 1, zinc-binding
SLC46A1	up	4.70E-03	1.56	solute carrier family 46 (folate transporter), member 1
VWA1	up	4.31E-02	1.55	von Willebrand factor A domain containing 1
USP53	up	2.19E-02	1.55	ubiquitin specific peptidase 53
EFNA1	up	2.34E-02	1.55	ephrin-A1

C15orf52	up	1.12E-02	1.55	chromosome 15 open reading frame 52
MICAL3	up	3.08E-02	1.55	microtubule associated monoxygenase, calponin and LIM domain containing 3
ST14	up	4.54E-02	1.55	suppression of tumorigenicity 14 (colon carcinoma)
EFNA5	up	3.91E-02	1.55	ephrin-A5
FAM84B	up	1.62E-02	1.54	family with sequence similarity 84, member B
MLLT4	up	1.23E-02	1.54	similar to Afadin (Protein AF-6); myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 4
LAMA5	up	2.56E-02	1.54	laminin, alpha 5
INADL	up	1.60E-02	1.54	InaD-like (Drosophila)
DYSF	up	1.60E-02	1.53	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)
FOSL2	up	2.91E-02	1.53	FOS-like antigen 2
SPTBN2	up	3.75E-02	1.53	spectrin, beta, non-erythrocytic 2
SEMA3C	up	3.42E-02	1.52	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C
RMND5B	up	3.69E-02	1.50	required for meiotic nuclear division 5 homolog B (S. cerevisiae)
RAP1GAP2	up	2.94E-02	1.50	GTPase activating Rap/RanGAP domain-like 4
PIP4K2C	up	2.11E-02	1.50	phosphatidylinositol-5-phosphate 4-kinase, type II, gamma
STON1	down	3.76E-02	-1.50	stonin 1; STON1-GTF2A1L readthrough transcript; general transcription factor IIA, 1-like
FILIP1L	down	2.96E-02	-1.50	filamin A interacting protein 1-like
TMEM200B	down	3.75E-02	-1.51	transmembrane protein 200B
ATF5	down	7.69E-03	-1.51	activating transcription factor 5

GPX8	down	1.27E-02	-1.52	glutathione peroxidase 8 (putative)
EFEMP2	down	8.95E-03	-1.52	EGF-containing fibulin-like extracellular matrix protein 2
PPM1M	down	4.71E-02	-1.53	protein phosphatase 1M (PP2C domain containing)
FLNC	down	2.11E-02	-1.53	filamin C, gamma (actin binding protein 280)
PARP8	down	2.13E-02	-1.54	poly (ADP-ribose) polymerase family, member 8
ST3GAL4	down	1.76E-02	-1.54	ST3 beta-galactoside alpha-2,3-sialyltransferase 4
LIMD2	down	1.56E-02	-1.55	LIM domain containing 2
GPX7	down	4.37E-02	-1.55	glutathione peroxidase 7
MMP19	down	1.01E-02	-1.55	matrix metallopeptidase 19
CAPG	down	1.13E-02	-1.55	capping protein (actin filament), gelsolin-like
KIAA0513	down	3.57E-02	-1.55	KIAA0513
PSMB9	down	4.68E-02	-1.56	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)
MXRA5	down	6.43E-03	-1.56	matrix-remodelling associated 5
SMPD1	down	6.37E-03	-1.56	sphingomyelin phosphodiesterase 1, acid lysosomal
EMP3	down	5.04E-03	-1.56	epithelial membrane protein 3
NEXN	down	3.11E-02	-1.56	nexilin (F actin binding protein)
ZNF469	down	6.97E-03	-1.58	zinc finger protein 469
FGFR1	down	2.61E-02	-1.58	fibroblast growth factor receptor 1
SH3PXD2A	down	2.41E-02	-1.58	SH3 and PX domains 2A

BTN3A3	down	3.76E-02	-1.59	butyrophilin, subfamily 3, member A3
CLTCL1	down	3.41E-02	-1.60	clathrin, heavy chain-like 1
ALDH1B1	down	1.63E-03	-1.60	aldehyde dehydrogenase 1 family, member B1
ECM1	down	2.41E-03	-1.61	extracellular matrix protein 1
C1S	down	2.56E-02	-1.61	complement component 1, s subcomponent
CPXM2	down	3.25E-02	-1.61	carboxypeptidase X (M14 family), member 2
ATP10A	down	3.18E-02	-1.61	ATPase, class V, type 10A
IFI27	down	2.26E-02	-1.62	interferon, alpha-inducible protein 27
TMEM176A	down	8.73E-03	-1.62	transmembrane protein 176A
SHOX2	down	8.84E-03	-1.62	short stature homeobox 2
RNF144A	down	6.38E-03	-1.62	ring finger protein 144A
APCDD1L	down	1.67E-02	-1.62	adenomatosis polyposis coli down-regulated 1-like
OLFML2B	down	3.67E-02	-1.62	olfactomedin-like 2B
TRAF1	down	3.26E-02	-1.63	TNF receptor-associated factor 1
ARL4C	down	1.91E-02	-1.63	ADP-ribosylation factor-like 4C
COL1A2	down	2.34E-02	-1.63	collagen, type I, alpha 2
RGS16	down	1.67E-02	-1.64	regulator of G-protein signaling 16
SSC5D	down	3.70E-02	-1.64	hypothetical LOC284297
LOC100505678	down	1.66E-02	-1.65	STARD4 antisense RNA 1

APBA2	down	2.15E-02	-1.65	amyloid beta (A4) precursor protein-binding, family A, member 2
THBS2	down	2.07E-02	-1.65	thrombospondin 2
SLC7A7	down	7.61E-03	-1.65	solute carrier family 7 (cationic amino acid transporter, y+ system), member 7
DNM1	down	2.04E-02	-1.66	dynamin 1
FMNL1	down	1.11E-03	-1.66	formin-like 1
WIPF1	down	2.91E-03	-1.66	WAS/WASL interacting protein family, member 1
ADCY7	down	2.28E-03	-1.66	adenylate cyclase 7
GLT8D2	down	1.70E-02	-1.66	glycosyltransferase 8 domain containing 2
LPAR1	down	5.61E-03	-1.66	lysophosphatidic acid receptor 1
BAG2	down	3.79E-03	-1.66	BCL2-associated athanogene 2
ITGA10	down	5.79E-03	-1.66	integrin, alpha 10
PLXDC2	down	2.63E-04	-1.67	plexin domain containing 2
ZFHX4	down	4.53E-02	-1.67	zinc finger homeobox 4
SSPN	down	4.31E-03	-1.68	sarcospan (Kras oncogene-associated gene)
SLA	down	4.17E-02	-1.68	Src-like-adaptor
ST3GAL5	down	2.19E-02	-1.69	ST3 beta-galactoside alpha-2,3-sialyltransferase 5
C2	down	3.62E-02	-1.69	complement component 2
FUT8	down	6.81E-04	-1.69	fucosyltransferase 8 (alpha (1,6) fucosyltransferase)
HAVCR2	down	1.56E-02	-1.69	hepatitis A virus cellular receptor 2
ARHGAP4	down	2.34E-02	-1.69	Rho GTPase activating protein 4

HLA-DQA1	down	2.90E-02	-1.70	similar to hCG2042724; similar to HLA class II histocompatibility antigen, DQ(1) alpha chain precursor (DC-4 alpha chain); major histocompatibility complex, class II, DQ alpha 1
RAB23	down	5.25E-04	-1.70	RAB23, member RAS oncogene family
EPSTI1	down	1.27E-02	-1.70	epithelial stromal interaction 1 (breast)
CLEC11A	down	6.95E-04	-1.70	C-type lectin domain family 11, member A
MRVI1	down	6.54E-03	-1.70	murine retrovirus integration site 1 homolog
IFFO1	down	7.27E-04	-1.71	intermediate filament family orphan 1
KIF26B	down	1.68E-02	-1.71	kinesin family member 26B
TSC22D3	down	2.51E-02	-1.71	TSC22 domain family, member 3; GRAM domain containing 4
MIR100HG	down	9.79E-04	-1.71	mir-100-let-7a-2 cluster host gene
DERL3	down	2.38E-02	-1.72	Der1-like domain family, member 3
CREB3L1	down	1.41E-04	-1.72	cAMP responsive element binding protein 3-like 1
FAM65C	down	9.15E-03	-1.72	family with sequence similarity 65, member C
TMEM200A	down	8.17E-04	-1.72	transmembrane protein 200A
ID2	down	5.84E-05	-1.73	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein
FOXO1	down	3.03E-04	-1.73	forkhead box O1
CMTM3	down	4.36E-02	-1.73	CKLF-like MARVEL transmembrane domain containing 3
RFX8	down	2.02E-02	-1.74	hypothetical protein LOC731220
KIAA0226L	down	2.34E-02	-1.74	KIAA0226-like
SCARF2	down	1.06E-04	-1.75	scavenger receptor class F, member 2

GALM	down	8.39E-03	-1.75	galactose mutarotase (aldose 1-epimerase)
TMEM176B	down	1.31E-03	-1.75	transmembrane protein 176B
GAS7	down	3.50E-03	-1.75	growth arrest-specific 7
PTPRCAP	down	4.36E-02	-1.76	protein tyrosine phosphatase, receptor type, C-associated protein
S100A4	down	1.52E-02	-1.76	S100 calcium binding protein A4
IKZF1	down	4.36E-02	-1.76	IKAROS family zinc finger 1 (Ikaros)
CD53	down	2.09E-02	-1.77	CD53 molecule
LSP1	down	4.89E-04	-1.77	lymphocyte-specific protein 1
SERPINF1	down	3.09E-03	-1.77	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1
IL7R	down	1.16E-02	-1.77	interleukin 7 receptor
RUNX2	down	7.37E-05	-1.78	runt-related transcription factor 2
LAMA2	down	1.87E-03	-1.78	laminin, alpha 2
BNC2	down	9.69E-04	-1.78	basonuclin 2
PRR16	down	8.38E-03	-1.78	proline rich 16
ARHGAP30	down	1.71E-02	-1.78	Rho GTPase activating protein 30
MSR1	down	7.63E-03	-1.78	macrophage scavenger receptor 1
C1QTNF6	down	1.62E-04	-1.78	C1q and tumor necrosis factor related protein 6
SLC7A8	down	2.80E-04	-1.78	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8
FERMT3	down	6.37E-03	-1.79	fermitin family homolog 3 (Drosophila)

FAM176A	down	6.08E-03	-1.79	family with sequence similarity 176, member A
VSTM4	down	6.88E-05	-1.79	V-set and transmembrane domain containing 4
PTGDS	down	7.65E-03	-1.80	prostaglandin D2 synthase, hematopoietic; prostaglandin D2 synthase 21kDa (brain)
FOXF2	down	5.28E-03	-1.80	forkhead box F2
PTPRC	down	1.51E-02	-1.80	protein tyrosine phosphatase, receptor type, C
DACT3	down	8.91E-03	-1.81	dapper, antagonist of beta-catenin, homolog 3 (Xenopus laevis)
CRISPLD2	down	7.27E-03	-1.81	cysteine-rich secretory protein LCCL domain containing 2
COL3A1	down	2.84E-03	-1.82	collagen, type III, alpha 1
TNFRSF19	down	4.57E-02	-1.82	tumor necrosis factor receptor superfamily, member 19
CPZ	down	5.93E-03	-1.82	carboxypeptidase Z
SYTL3	down	3.97E-02	-1.82	synaptotagmin-like 3
PTPRD	down	9.11E-03	-1.82	protein tyrosine phosphatase, receptor type, D
ALOX5AP	down	9.05E-05	-1.82	arachidonate 5-lipoxygenase-activating protein
THY1	down	1.45E-03	-1.83	Thy-1 cell surface antigen
FAP	down	4.31E-03	-1.83	fibroblast activation protein, alpha
P4HA3	down	6.27E-04	-1.84	prolyl 4-hydroxylase, alpha polypeptide III
CTHRC1	down	6.61E-03	-1.84	collagen triple helix repeat containing 1
ITGBL1	down	2.44E-05	-1.84	integrin, beta-like 1 (with EGF-like repeat domains)
IL16	down	9.79E-04	-1.85	interleukin 16 (lymphocyte chemoattractant factor)
MS4A7	down	3.30E-03	-1.85	membrane-spanning 4-domains, subfamily A, member 7

CTSK	down	1.32E-02	-1.86	cathepsin K
ABI3BP	down	1.38E-02	-1.86	ABI family, member 3 (NESH) binding protein
TYROBP	down	1.87E-03	-1.86	TYRO protein tyrosine kinase binding protein
MAFB	down	2.59E-03	-1.87	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)
AMPD3	down	5.84E-03	-1.87	adenosine monophosphate deaminase (isoform E)
VSIG4	down	1.91E-02	-1.87	V-set and immunoglobulin domain containing 4
MXRA8	down	2.13E-03	-1.87	matrix-remodelling associated 8
RNASE1	down	2.13E-03	-1.87	ribonuclease, RNase A family, 1 (pancreatic)
COPZ2	down	2.16E-03	-1.88	coatomer protein complex, subunit zeta 2
HLA-DMB	down	3.40E-03	-1.90	major histocompatibility complex, class II, DM beta
LOXL1	down	3.54E-06	-1.90	lysyl oxidase-like 1
DOCK2	down	1.78E-02	-1.91	dedicator of cytokinesis 2
STAB1	down	3.04E-02	-1.91	stabilin 1
C1QC	down	3.75E-02	-1.91	complement component 1, q subcomponent, C chain
CLEC2D	down	2.28E-03	-1.91	C-type lectin domain family 2, member D
PTPRN	down	2.44E-05	-1.91	protein tyrosine phosphatase, receptor type, N
MEG3	down	7.96E-04	-1.94	maternally expressed 3 (non-protein coding)
KIAA1199	down	1.97E-03	-1.94	KIAA1199
ODZ3	down	7.35E-04	-1.95	odz, odd Oz/ten-m homolog 3 (Drosophila)
MMP2	down	1.17E-03	-1.96	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)

METTL7A	down	4.13E-02	-1.97	methyltransferase like 7A
C3AR1	down	1.94E-02	-1.98	complement component 3a receptor 1
ITGAX	down	2.45E-03	-1.98	integrin, alpha X (complement component 3 receptor 4 subunit)
PTGFR	down	3.23E-02	-1.98	prostaglandin F receptor (FP)
SELPLG	down	2.26E-04	-1.99	selectin P ligand
GFPT2	down	2.78E-04	-1.99	glutamine-fructose-6-phosphate transaminase 2
EVI2A	down	3.50E-04	-2.00	ecotropic viral integration site 2A
VCAN	down	5.40E-05	-2.01	versican
ADAM33	down	2.80E-02	-2.02	ADAM metallopeptidase domain 33
C2CD4A	down	1.21E-02	-2.02	family with sequence similarity 148, member A
MFAP2	down	2.47E-05	-2.04	microfibrillar-associated protein 2
FBLN1	down	1.01E-03	-2.06	fibulin 1
COL8A1	down	7.55E-05	-2.08	collagen, type VIII, alpha 1
RASL11B	down	1.91E-04	-2.08	RAS-like, family 11, member B
PDGFRL	down	7.92E-05	-2.09	platelet-derived growth factor receptor-like
OLFML3	down	7.31E-07	-2.09	olfactomedin-like 3
ITGA11	down	2.91E-04	-2.10	integrin, alpha 11
F2RL2	down	1.07E-05	-2.11	coagulation factor II (thrombin) receptor-like 2
HCK	down	2.96E-02	-2.11	hemopoietic cell kinase
PIK3R5	down	2.49E-03	-2.12	phosphoinositide-3-kinase, regulatory subunit 5

SAMSN1	down	2.45E-03	-2.13	SAM domain, SH3 domain and nuclear localization signals 1
FCGR3A	down	1.22E-05	-2.13	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)
GREM1	down	5.06E-03	-2.14	gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)
NEGR1	down	1.18E-04	-2.14	neuronal growth regulator 1
C1QB	down	8.95E-03	-2.16	complement component 1, q subcomponent, B chain
GAS1	down	2.92E-05	-2.16	growth arrest-specific 1
CCDC80	down	8.72E-06	-2.17	coiled-coil domain containing 80
CPA3	down	5.97E-03	-2.18	carboxypeptidase A3 (mast cell)
OLFML1	down	2.72E-04	-2.19	olfactomedin-like 1
NKD2	down	1.21E-03	-2.21	naked cuticle homolog 2 (Drosophila)
GAL3ST4	down	2.47E-04	-2.21	galactose-3-O-sulfotransferase 4
PLXNC1	down	9.05E-05	-2.22	plexin C1
ARHGEF19	down	5.25E-09	-2.25	Rho guanine nucleotide exchange factor (GEF) 19
MIAT	down	1.97E-06	-2.25	myocardial infarction associated transcript (non-protein coding)
WISP1	down	1.35E-05	-2.26	WNT1 inducible signaling pathway protein 1
FCER1G	down	5.15E-05	-2.28	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide
FPR3	down	1.98E-04	-2.28	formyl peptide receptor 3
PLA2G7	down	1.25E-04	-2.29	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)
CORO1A	down	4.83E-06	-2.30	coronin, actin binding protein, 1A
PTH1R	down	6.09E-05	-2.30	parathyroid hormone 1 receptor

MS4A4A	down	1.29E-03	-2.31	membrane-spanning 4-domains, subfamily A, member 4
SASH3	down	5.07E-05	-2.32	SAM and SH3 domain containing 3
DNM3OS	down	1.52E-05	-2.33	DNM3 opposite strand/antisense RNA
ADAM8	down	1.87E-04	-2.37	ADAM metallopeptidase domain 8
MFAP4	down	2.43E-09	-2.37	microfibrillar-associated protein 4
SLAMF8	down	7.37E-05	-2.40	SLAM family member 8
LUM	down	1.56E-04	-2.43	lumican
DCN	down	7.23E-07	-2.43	decorin
PARVG	down	5.84E-05	-2.44	parvin, gamma
PODN	down	2.46E-07	-2.45	podocan
SDS	down	1.28E-06	-2.45	serine dehydratase
RGS1	down	6.00E-05	-2.46	regulator of G-protein signaling 1
SLC24A2	down	6.27E-04	-2.47	solute carrier family 24 (sodium/potassium/calcium exchanger), member 2
MFAP5	down	5.93E-07	-2.55	microfibrillar associated protein 5
COL11A1	down	7.56E-04	-2.63	collagen, type XI, alpha 1
ELK2AP	down	1.37E-08	-2.66	ELK2, member of ETS oncogene family, pseudogene 1
FIBIN	down	9.34E-12	-2.66	fin bud initiation factor homolog (zebrafish)
SFRP4	down	1.56E-02	-2.67	secreted frizzled-related protein 4
SLCO2B1	down	5.06E-05	-2.67	solute carrier organic anion transporter family, member 2B1
CD300A	down	6.05E-06	-2.77	CD300a molecule

TWIST1	down	5.99E-07	-2.79	twist homolog 1 (Drosophila)
DPT	down	1.77E-12	-2.80	dermatopontin
GJB2	down	8.84E-03	-2.84	gap junction protein, beta 2, 26kDa
SPON2	down	1.48E-06	-2.84	spondin 2, extracellular matrix protein
CD163	down	2.89E-04	-2.87	CD163 molecule
C1orf162	down	1.96E-05	-2.91	chromosome 1 open reading frame 162
SGCD	down	1.81E-07	-2.91	sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)
LRRC15	down	2.74E-07	-2.93	leucine rich repeat containing 15
PRELP	down	3.92E-09	-2.94	proline/arginine-rich end leucine-rich repeat protein
FPR1	down	1.23E-05	-3.02	formyl peptide receptor 1
MPEG1	down	4.70E-04	-3.06	macrophage expressed 1
IL1R2	down	1.31E-03	-3.34	interleukin 1 receptor, type II
PPAPDC1A	down	7.54E-09	-3.39	phosphatidic acid phosphatase type 2 domain containing 1A
ELN	down	7.99E-07	-3.53	elastin
COMP	down	2.65E-16	-3.90	cartilage oligomeric matrix protein
FCGR2A	down	1.14E-10	-3.91	Fc fragment of IgG, low affinity IIa, receptor (CD32)
ASPN	down	5.58E-06	-3.94	asporin
MMP13	down	1.25E-03	-4.03	matrix metallopeptidase 13 (collagenase 3)
ADORA3	down	1.37E-08	-4.35	adenosine A3 receptor

Supplementary Table 3_2

Vitamin D target genes identified in human non-malignant breast tissue

Gene	Regulation	FDR	Fold Change	Gene Description
CYP24A1	up	1.06E-116	155.97	cytochrome P450, family 24, subfamily A, polypeptide 1
TRPV6	up	5.92E-71	10.10	transient receptor potential cation channel, subfamily V, member 6
LOXL4	up	3.50E-54	6.86	lysyl oxidase-like 4
UCA1	up	1.77E-22	6.63	urothelial cancer associated 1
KLK6	up	1.01E-17	5.11	kallikrein-related peptidase 6
SERPINB1	up	5.92E-29	4.77	serpin peptidase inhibitor, clade B (ovalbumin), member 1
SYT8	up	1.18E-11	4.59	synaptotagmin VIII
DOK5	up	8.66E-26	4.55	docking protein 5
CD14	up	8.66E-26	4.05	CD14 molecule
TMEM37	up	6.24E-15	4.00	transmembrane protein 37
LOC100127888	up	2.16E-14	3.38	hypothetical protein LOC100127888
CA9	up	1.84E-02	3.29	carbonic anhydrase IX
CYP26B1	up	3.77E-11	3.18	cytochrome P450, family 26, subfamily B, polypeptide 1
KCNK3	up	5.04E-15	3.15	potassium channel, subfamily K, member 3
C7orf29	up	6.65E-12	3.06	chromosome 7 open reading frame 29
G0S2	up	2.36E-07	2.93	G0/G1switch 2

CCL20	up	2.42E-06	2.83	chemokine (C-C motif) ligand 20
EFTUD1	up	2.27E-19	2.76	elongation factor Tu GTP binding domain containing 1
ATP8A1	up	1.05E-10	2.56	ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1
SPTSSB	up	4.01E-08	2.49	serine palmitoyltransferase, small subunit B
SPP1	up	9.73E-03	2.47	secreted phosphoprotein 1
TMEM91	up	1.17E-06	2.47	transmembrane protein 91
CRLF1	up	3.86E-11	2.45	cytokine receptor-like factor 1
KRT16	up	8.48E-06	2.35	keratin 16; keratin type 16-like
COL13A1	up	1.12E-06	2.33	collagen, type XIII, alpha 1
LILRB4	up	2.27E-06	2.32	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 4
S100B	up	1.34E-03	2.30	S100 calcium binding protein B
CALML3	up	5.04E-09	2.29	calmodulin-like 3
IFITM10	up	1.71E-03	2.29	interferon induced transmembrane protein 10
CEACAM6	up	6.77E-05	2.22	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)
LGALS9	up	2.26E-06	2.19	lectin, galactoside-binding, soluble, 9
CLMN	up	1.84E-08	2.13	calmin (calponin-like, transmembrane)
COL16A1	up	3.42E-09	2.11	collagen, type XVI, alpha 1
MSI1	up	1.18E-03	2.07	musashi homolog 1 (Drosophila)
PYROXD2	up	1.28E-02	2.02	pyridine nucleotide-disulphide oxidoreductase domain 2

STAC	up	5.19E-03	1.98	SH3 and cysteine rich domain
DBC1	up	8.61E-04	1.94	deleted in bladder cancer 1
CA2	up	2.36E-07	1.93	carbonic anhydrase II
G6PD	up	3.46E-08	1.91	glucose-6-phosphate dehydrogenase
HAUS7	up	2.00E-05	1.88	three prime repair exonuclease 2; HAUS augmin-like complex, subunit 7
RPS6KA1	up	2.60E-07	1.87	ribosomal protein S6 kinase, 90kDa, polypeptide 1
RGNEF	up	7.81E-06	1.87	Rho-guanine nucleotide exchange factor
IGFLR1	up	7.28E-03	1.87	IGF-like family receptor 1
HPN	up	2.76E-03	1.87	hepsin
MFSD2A	up	8.38E-04	1.85	major facilitator superfamily domain containing 2
KLK7	up	5.76E-04	1.85	kallikrein-related peptidase 7
LYZ	up	1.24E-04	1.84	lysozyme (renal amyloidosis)
PPP1R14C	up	3.56E-02	1.84	protein phosphatase 1, regulatory (inhibitor) subunit 14C
TIMP3	up	1.96E-03	1.84	TIMP metallopeptidase inhibitor 3
PDE4B	up	1.61E-07	1.83	phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)
HR	up	2.76E-03	1.82	hairless homolog (mouse)
LAMB3	up	2.88E-04	1.81	laminin, beta 3
AIG1	up	1.30E-05	1.80	androgen-induced 1
ARHGEF6	up	2.40E-05	1.78	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6

PLLP	up	1.65E-02	1.77	plasma membrane proteolipid (plasmolipin)
CYP7B1	up	1.04E-02	1.76	cytochrome P450, family 7, subfamily B, polypeptide 1
HRCT1	up	5.75E-03	1.76	histidine rich carboxyl terminus 1
TGFB2	up	5.00E-02	1.74	transforming growth factor, beta 2
PRSS22	up	2.93E-02	1.74	protease, serine, 22
KLK8	up	3.77E-04	1.74	kallikrein-related peptidase 8
SULT2B1	up	2.58E-02	1.73	sulfotransferase family, cytosolic, 2B, member 1
CSF3R	up	4.36E-03	1.72	colony stimulating factor 3 receptor (granulocyte)
CXCL3	up	1.92E-03	1.71	chemokine (C-X-C motif) ligand 3
CHI3L1	up	3.56E-02	1.71	chitinase 3-like 1 (cartilage glycoprotein-39)
CLCF1	up	6.77E-04	1.71	cardiotrophin-like cytokine factor 1
TIAM1	up	8.95E-03	1.68	T-cell lymphoma invasion and metastasis 1
PRLR	up	1.81E-02	1.68	prolactin receptor
EPB41L4B	up	5.62E-03	1.64	erythrocyte membrane protein band 4.1 like 4B
PDZRN3	up	3.47E-04	1.63	PDZ domain containing ring finger 3
IL1RL1	up	1.55E-02	1.63	interleukin 1 receptor-like 1
PAPPA	up	4.40E-02	1.63	PAPPA antisense RNA (non-protein coding); pregnancy-associated plasma protein A, pappalysin 1
SHE	up	1.10E-02	1.62	Src homology 2 domain containing E
TSPAN1	up	4.95E-02	1.62	tetraspanin 1

AGL	up	3.34E-03	1.61	amylo-1, 6-glucosidase, 4-alpha-glucanotransferase
SNTB1	up	1.16E-02	1.59	syntrophin, beta 1 (dystrophin-associated protein A1, 59kDa, basic component 1)
CYP1A1	up	1.95E-02	1.59	cytochrome P450, family 1, subfamily A, polypeptide 1
FRAS1	up	3.43E-02	1.58	Fraser syndrome 1
PLCB4	up	2.47E-02	1.58	phospholipase C, beta 4
IL8	up	6.77E-04	1.58	interleukin 8
PMEPA1	up	6.77E-04	1.57	prostate transmembrane protein, androgen induced 1
SDC1	up	9.59E-03	1.54	syndecan 1
SLC46A1	up	4.06E-03	1.53	solute carrier family 46 (folate transporter), member 1
ISM1	up	2.65E-02	1.50	isthmin 1 homolog (zebrafish)
LTBP3	down	1.77E-03	-1.51	latent transforming growth factor beta binding protein 3
IFITM1	down	4.35E-02	-1.51	interferon induced transmembrane protein 1 (9-27)
CCL2	down	2.42E-03	-1.53	chemokine (C-C motif) ligand 2
SLCO2B1	down	2.53E-02	-1.54	solute carrier organic anion transporter family, member 2B1
SERPINF1	down	3.44E-02	-1.54	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1
LAMA3	down	9.40E-03	-1.55	laminin, alpha 3
PLTP	down	1.16E-02	-1.55	phospholipid transfer protein
ATP2A3	down	2.58E-02	-1.58	ATPase, Ca++ transporting, ubiquitous
CFI	down	4.31E-02	-1.60	complement factor I

CYP27A1	down	2.90E-02	-1.61	cytochrome P450, family 27, subfamily A, polypeptide 1
RNASE1	down	6.54E-03	-1.62	ribonuclease, RNase A family, 1 (pancreatic)
METTL7A	down	4.35E-02	-1.62	methyltransferase like 7A
LTB4R	down	2.18E-03	-1.64	leukotriene B4 receptor
FMO2	down	2.60E-05	-1.64	flavin containing monooxygenase 2 (non-functional)
CFD	down	4.22E-02	-1.66	complement factor D (adipsin)
LOXL1	down	4.23E-02	-1.66	lysyl oxidase-like 1
ADAM33	down	4.95E-02	-1.73	ADAM metallopeptidase domain 33
ELK2AP	down	3.50E-03	-1.76	ELK2, member of ETS oncogene family, pseudogene 1
SOD3	down	1.12E-03	-1.77	superoxide dismutase 3, extracellular
AK5	down	2.47E-02	-1.78	adenylate kinase 5
S100A4	down	3.05E-02	-1.79	S100 calcium binding protein A4
APCDD1L	down	2.04E-02	-1.79	adenomatosis polyposis coli down-regulated 1-like
FCGR2A	down	2.94E-03	-1.79	Fc fragment of IgG, low affinity IIa, receptor (CD32)
GRIK3	down	4.88E-03	-1.81	glutamate receptor, ionotropic, kainate 3
KRT15	down	1.15E-03	-1.82	keratin 15
PAMR1	down	1.32E-04	-1.83	peptidase domain containing associated with muscle regeneration 1
CD163	down	2.88E-04	-1.83	CD163 molecule
OXTR	down	1.53E-06	-1.84	oxytocin receptor
MFAP4	down	3.81E-02	-1.85	microfibrillar-associated protein 4

AQP5	down	1.05E-02	-1.87	aquaporin 5
WDR86	down	8.13E-03	-1.89	WD repeat domain 86
LRP4	down	2.93E-02	-1.90	low density lipoprotein receptor-related protein 4
GAS1	down	8.24E-08	-1.91	growth arrest-specific 1
TWIST1	down	4.53E-04	-2.00	twist homolog 1 (Drosophila)
S100A8	down	5.00E-02	-2.03	S100 calcium binding protein A8
SPON2	down	7.79E-04	-2.08	spondin 2, extracellular matrix protein
LBP	down	6.77E-04	-2.13	lipopolysaccharide binding protein
CRABP1	down	1.84E-02	-2.29	cellular retinoic acid binding protein 1
IFI6	down	4.43E-04	-2.44	interferon, alpha-inducible protein 6
PTGIS	down	9.97E-03	-2.77	prostaglandin I2 (prostacyclin) synthase
IL1R2	down	8.24E-08	-2.79	interleukin 1 receptor, type II
PTPRZ1	down	5.49E-10	-2.83	protein tyrosine phosphatase, receptor-type, Z polypeptide 1
MMP9	down	1.30E-05	-3.53	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
F13A1	down	5.82E-06	-4.21	coagulation factor XIII, A1 polypeptide

Supplementary Table 3_3

Vitamin D target genes commonly regulated in human breast cancer and non-malignant breast tissue

Gene	Regulation	Fold change in non-malignant	Fold change in tumor	Gene Description
CYP24A1	up	155.97	243.00	cytochrome P450, family 24, subfamily A, polypeptide 1
KLK6	up	5.11	7.27	kallikrein-related peptidase 6
SERPINB1	up	4.77	3.59	serpin peptidase inhibitor, clade B (ovalbumin), member 1
CD14	up	4.05	2.99	CD14 molecule
TMEM37	up	4.00	2.15	transmembrane protein 37
CYP26B1	up	3.18	3.23	cytochrome P450, family 26, subfamily B, polypeptide 1
KCNK3	up	3.15	2.64	potassium channel, subfamily K, member 3
G0S2	up	2.93	2.52	G0/G1switch 2
EFTUD1	up	2.76	2.49	elongation factor Tu GTP binding domain containing 1
CRLF1	up	2.45	1.82	cytokine receptor-like factor 1
KRT16	up	2.35	4.25	keratin 16; keratin type 16-like
CALML3	up	2.29	3.71	calmodulin-like 3
CEACAM6	up	2.22	2.48	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)
LGALS9	up	2.19	1.87	lectin, galactoside-binding, soluble, 9
CLMN	up	2.13	2.37	calmin (calponin-like, transmembrane)

CA2	up	1.93	3.18	carbonic anhydrase II
G6PD	up	1.91	1.99	glucose-6-phosphate dehydrogenase
RPS6KA1	up	1.87	1.77	ribosomal protein S6 kinase, 90kDa, polypeptide 1
RGNEF	up	1.87	2.18	Rho-guanine nucleotide exchange factor
KLK7	up	1.85	2.79	kallikrein-related peptidase 7
LYZ	up	1.84	1.99	lysozyme (renal amyloidosis)
PPP1R14C	up	1.84	1.80	protein phosphatase 1, regulatory (inhibitor) subunit 14C
PDE4B	up	1.83	1.63	phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)
HR	up	1.82	1.93	hairless homolog (mouse)
LAMB3	up	1.81	2.28	laminin, beta 3
PRSS22	up	1.74	3.00	protease, serine, 22
SULT2B1	up	1.73	2.80	sulfotransferase family, cytosolic, 2B, member 1
CLCF1	up	1.71	1.60	cardiotrophin-like cytokine factor 1
PRLR	up	1.68	2.58	prolactin receptor
EPB41L4B	up	1.64	2.13	erythrocyte membrane protein band 4.1 like 4B
SHE	up	1.62	2.36	Src homology 2 domain containing E
TSPAN1	up	1.62	2.44	tetraspanin 1
AGL	up	1.61	1.67	amylo-1, 6-glucosidase, 4-alpha-glucanotransferase
CYP1A1	up	1.59	2.88	cytochrome P450, family 1, subfamily A, polypeptide 1
SLC46A1	up	1.53	1.56	solute carrier family 46 (folate transporter), member 1

SLCO2B1	down	-1.54	-2.67	solute carrier organic anion transporter family, member 2B1
SERPINF1	down	-1.54	-1.77	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1
RNASE1	down	-1.62	-1.87	ribonuclease, RNase A family, 1 (pancreatic)
METTL7A	down	-1.62	-1.97	methyltransferase like 7A
LOXL1	down	-1.66	-1.90	lysyl oxidase-like 1
ADAM33	down	-1.73	-2.02	ADAM metallopeptidase domain 33
ELK2AP	down	-1.76	-2.66	ELK2, member of ETS oncogene family, pseudogene 1
S100A4	down	-1.79	-1.76	S100 calcium binding protein A4
APCDD1L	down	-1.79	-1.62	adenomatosis polyposis coli down-regulated 1-like
FCGR2A	down	-1.79	-3.91	Fc fragment of IgG, low affinity IIa, receptor (CD32)
CD163	down	-1.83	-2.87	CD163 molecule
MFAP4	down	-1.85	-2.37	microfibrillar-associated protein 4
GAS1	down	-1.91	-2.16	growth arrest-specific 1
TWIST1	down	-2.00	-2.79	twist homolog 1 (Drosophila)
SPON2	down	-2.08	-2.84	spondin 2, extracellular matrix protein
IL1R2	down	-2.79	-3.34	interleukin 1 receptor, type II

Supplementary Table 4 KEGG pathways significantly altered by 1,25D treatment in human breast cancer

KEGG pathway	Gene symbols	Gene count	*p*-value
Over-represented			
Cell adhesion molecules (CAMs)	*CLDN3*,*CLDN4*,*CLDN7*,***NEGR1***,*PTPRF*,*CADM3*,*CDH1*,***VCAN***,*CLDN11*,*CDH3*	10	0.00001
ECM-receptor interaction	*COL4A5*,***COL11A1***,*LAMB3, ITGA2*,*TSPAN1*,***ITGA11***,*ITGB4*	7	0.00029
Staphylococcus aureus infection	***C1QB***,***FCGR2A***,***FCGR3A***,***FPR1***,***FPR3***	5	0.00071
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	*ITGA2*,***ITGA11***,*ITGB4*,*CACNG4*,*DSP*,***SGCD***	6	0.00085
Amoebiasis	*COL4A5*,***COL11A1***,*LAMB3*,***IL1R2***,*CD14*,*SERPINB1*,***PIK3R5***	7	0.00089
Focal adhesion	*COL4A5*,***COL11A1***,*LAMB3*,***COMP***,***PIK3R5***,*ITGA2*,***ITGA11***,*ITGB4*,***PARVG***	9	0.00314
Protein digestion and absorption	***CPA3***,***ELN***,*COL4A5*,***COL11A1***,*COL17A1*	5	0.00582
Hypertrophic cardiomyopathy (HCM)	*ITGA2*,***ITGA11***,*ITGB4*,***SGCD***,*CACNG4*	5	0.00759
Dilated cardiomyopathy	*ITGA2*,***ITGA11***,*ITGB4*,***SGCD***,*CACNG4*	5	0.01055
Tight junction	*CLDN3*,*CLDN4*,*CLDN7*,*CLDN11*,*CGN*,*MYH14*	6	0.01190
Phagosome	***CORO1A***,***FCGR2A***,***FCGR3A***,*ITGA2*,***COMP***,*CD14*	6	0.01670
Leukocyte transendothelial migration	*CLDN3*,*CLDN4*,*CLDN7*,*CLDN11*,*OCLN*	5	0.02392
Aldosterone-regulated sodium reabsorption	*SCNN1A*,*SCNN1B*,***PIK3R5***	3	0.02541
Hepatitis C	*CLDN3*,*CLDN4*,*CLDN7*,*CLDN11*,***PIK3R5***	5	0.03685
Small cell lung cancer	*COL4A5*,*LAMB3*,***PIK3R5***,*ITGA2*	4	0.04096
Under-represented			
Metabolic pathways	*FBP1*,*CDS1*,*CYP26B1*,*CYP1A1*,*CYP24A1*,*DHCR24*,***SDS***	7	0.00917

KEGG pathways significantly enriched for differentially expressed genes (FDR < 0.05,|log2 Fold Change|>1) in human breast cancer after 24 hours treatment with 100 nM 1,25D. Gene symbols marked as bold were down-regulated by 1,25D, while the remaining genes were up-regulated.

Supplementary Table 5

Overview of the top 40 most significantly over- and under-represented Gene Ontology (GO) terms associated with differentially expressed genes

GO-term	Number of genes	*p*-value	Ontology
Over-represented GO terms			
GO: extracellular region	102	2.31E-23	CC
GO: extracellular region part	87	2.73E-19	CC
GO: extracellular matrix organization	29	2.41E-17	BP
GO: extracellular structure organization	29	2.62E-17	BP
GO: vesicle	78	7.20E-15	CC
GO: extracellular organelle	67	8.34E-15	CC
GO: extracellular membrane-bounded organelle	67	8.34E-15	CC
GO: extracellular vesicular exosome	67	8.34E-15	CC
GO: proteinaceous extracellular matrix	25	9.78E-15	CC
GO: membrane-bounded vesicle	76	1.68E-14	CC
GO: biological adhesion	42	3.87E-14	BP
GO: tissue development	52	4.42E-14	BP
GO: extracellular matrix	26	9.38E-14	CC
GO: single-organism process	169	2.10E-13	BP
GO: cell adhesion	40	7.12E-13	BP
GO: single-organism cellular process	155	1.18E-11	BP
GO: skin development	21	1.29E-11	BP
GO: single-multicellular organism process	104	1.44E-11	BP
GO: multicellular organismal process	106	1.98E-11	BP
GO: locomotion	43	5.28E-11	BP
Under-represented GO terms			
GO: nuclear part	7	1.54E-05	CC
GO: nuclear lumen	6	8.98E-05	CC
GO: nucleic acid metabolic process	25	2.33E-04	BP
GO: nucleus	38	2.41E-04	CC
GO: cellular nitrogen compound metabolic process	40	3.02E-04	BP
GO: heterocycle metabolic process	40	9.04E-04	BP

GO: nucleic acid binding	20	1.09E-03	MF
GO: nucleobase-containing compound metabolic process	39	1.14E-03	BP
GO: nucleoplasm	4	1.24E-03	CC
GO: nitrogen compound metabolic process	46	1.42E-03	BP
GO: cellular aromatic compound metabolic process	41	1.47E-03	BP
GO: cellular macromolecule metabolic process	51	2.65E-03	BP
GO: organic cyclic compound metabolic process	44	2.83E-03	BP
GO: RNA metabolic process	25	3.57E-03	BP
GO: protein modification by small protein conjugation or removal	1	3.69E-03	BP
GO: heterocyclic compound binding	38	4.06E-03	MF
GO: ATP binding	6	6.59E-03	MF
GO: DNA metabolic process	2	6.77E-03	BP
GO: transferase activity	10	7.36E-03	MF
GO: intracellular organelle lumen	15	7.86E-03	CC

BP, biological process; CC, cellular component; MF, molecular function.

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CHAPTER IV

VITAMIN D_3 SIGNALING AND MAMMARY CANCER: INSIGHTS FROM TRANSGENIC MOUSE MODELS

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4.1 Prelude

This review summarizes our current understanding from transgenic mouse models of the vitamin D_3 receptor (Vdr) and vitamin D_3 associated metabolic enzymes in mammary gland development, cancer initiation, and progression and discussed the implications of these findings for human breast cancer. This review provides the background for the subsequent chapter V which is a study of a transgenic mouse with knockout of the gene *Cyp24a1*.

This chapter is the unpublished version of the manuscript.

Contribution of the PhD candidate: Searched literature and wrote manuscript.

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Lei Sheng
Contribution to the Paper	Searched literature and wrote manuscript.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Andrew G. Turner
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Signature	Date 6/3/17

4.2 Abstract

The biologically active form of vitamin D_3 (1, 25$(OH)_2$D) regulates proliferation, differentiation, and apoptosis in diverse cell types through binding to the vitamin D_3 receptor (VDR). Accumulating epidemiological and clinical evidence suggest that vitamin D_3 insufficiency is associated with increased breast cancer incidence and poor clinical outcomes in patients with breast cancer, which makes vitamin D_3 supplementation as a potential preventive or therapeutic option for the management of breast cancer. Studies have reported local dysregulations of critical vitamin D_3 metabolic components in patients with breast cancer, implicating that disruption of VDR signaling pathway may contribute to the development of breast cancer in an autocrine or paracrine manner. Vitamin D_3 metabolic enzymes Cyp2r1, Cyp27b1, Cyp24a1, and its receptor Vdr, the key components of the vitamin D_3 signaling pathway, are expressed in the normal mammary glands of mice. In mice, a reduction of the Vdr or Cyp27b1 in mammary cancer resulted in reduced response to vitamin D_3 effects, while elevation of Cyp24a1 levels increased ability to degrade 1,25$(OH)_2$D. Numerous transgenic murine models of individual metabolic enzyme or *Vdr* knockout have been generated to investigate their unique role in mammary gland and cancer development. This review discusses the recent transgenic mouse models of vitamin D_3 metabolism, Vdr signaling network, and how this contributes to mammary gland development, cancer tumorigenesis, and progression. An improved understanding of the mechanisms of action of vitamin D_3 signaling pathway derived from these mouse models provides support for activation of vitamin D_3 signaling pathway as a potential method for human breast cancer prevention.

4.3 Introduction

Although vitamin D_3 plays a central role in calcium and bone homeostasis, multiple lines of evidence suggest that vitamin D_3 deficiency also may contribute to a number of disease states including cancer [1]. Epidemiological studies have been more consistent, suggesting that increased sunlight exposure, dietary vitamin D_3 intake, and circulating levels of the pro-hormone 25-hydroxyvitamin D_3 (25(OH)D) are associated with a reduced risk of breast cancer [2-4]. Several clinical trials have examined the relationship between vitamin D_3 supplementation and breast cancer incidence and mortality, but the potential benefit remains controversial [5]. Mouse models where the vitamin D_3 pathway has been genetically modified provide the most unequivocal and consistent data to support the hypothesis that high levels of vitamin D_3 are protective against mammary gland tumor formation. This review summarizes our current understanding from transgenic mouse models of the vitamin D_3 receptor (Vdr) and vitamin D_3 associated metabolic enzymes in mammary gland development, cancer initiation, and progression and discussed the implications of these findings for human breast cancer.

4.4 Metabolism of vitamin D3

The major source of vitamin D_3 in the circulating system is synthesized from its precursor 7-dehydrocholesterol through skin exposure to ultraviolet-B (UVB) light, with additional amounts obtained from dietary consumption [1]. The bioactivation of vitamin D_3 requires hydroxylation by Cyp2r1 (also known as 25-hydroxylase) predominantly expressed in the liver to produce 25(OH)D. A second hydroxylation step catalysed by Cyp27b1 (also known as 1α-hydroxylase) mainly in the kidney to generate the active hormone metabolite 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$). 88% of the serum prohormone 25(OH)D and 85% of

$1,25(OH)_2D$ are primarily bound to the vitamin D_3 binding protein (DBP) (Figure 1.). The concentration of circulating 25(OH)D has been regarded as one of the most reliable biomarkers of vitamin D_3 status. $1,25(OH)_2D$, the active form of vitamin D_3, induces expression of Cyp24a1, which converts 25(OH)D and $1,25(OH)_2D$ to less active metabolites, initiating their degradation (Figure 1.) [6]. The expression of Cyp24a1 in extra-renal sites is usually negligible but strongly and transiently induced following exposure to $1,25(OH)_2D$. Intriguingly, Cyp27b1 is also expressed in a variety of extra-renal sites, such as skin, the immune system, brain, placenta, pancreas, and mammary tissue, indicating that vitamin D_3 can be locally activated and function in an autocrine or paracrine manner (Figure 1.) [7].

4.5 Vitamin D3 and breast cancer

Vitamin D_3 insufficiency, as determined by the serum level of the prohormone 25(OH)D, is a prevalent issue worldwide that has traditionally been linked to poor bone and musculoskeletal health. Increasingly, inadequate vitamin D_3 levels have also been associated with cardiovascular disease, autoimmune disease and cancer [1]. Ecological studies have consistently found strong inverse correlations between solar UVB exposure and various types of cancer, supporting the UVB-vitamin D_3-cancer hypothesis [8]. For example, women in the United States with high exposure to sunlight were reported to have a 25-65% reduced risk of breast cancer [9]. Consistent with ecological and epidemiological studies, a meta-analysis of five case-control studies reported that increasing serum 25(OH)D by 20 ng/ml (50 nmol/L) was associated with a 41% lower risk of breast cancer [2]. The most recent meta-analysis on 30 prospective studies suggested association between high serum 25(OH)D levels and reduced risk of breast cancer (RR 0.92, 95% CI 0.83-1.02) [4]. Moreover, a large, well-characterized prospective cohort study involving 1,666 cases of breast cancer found that

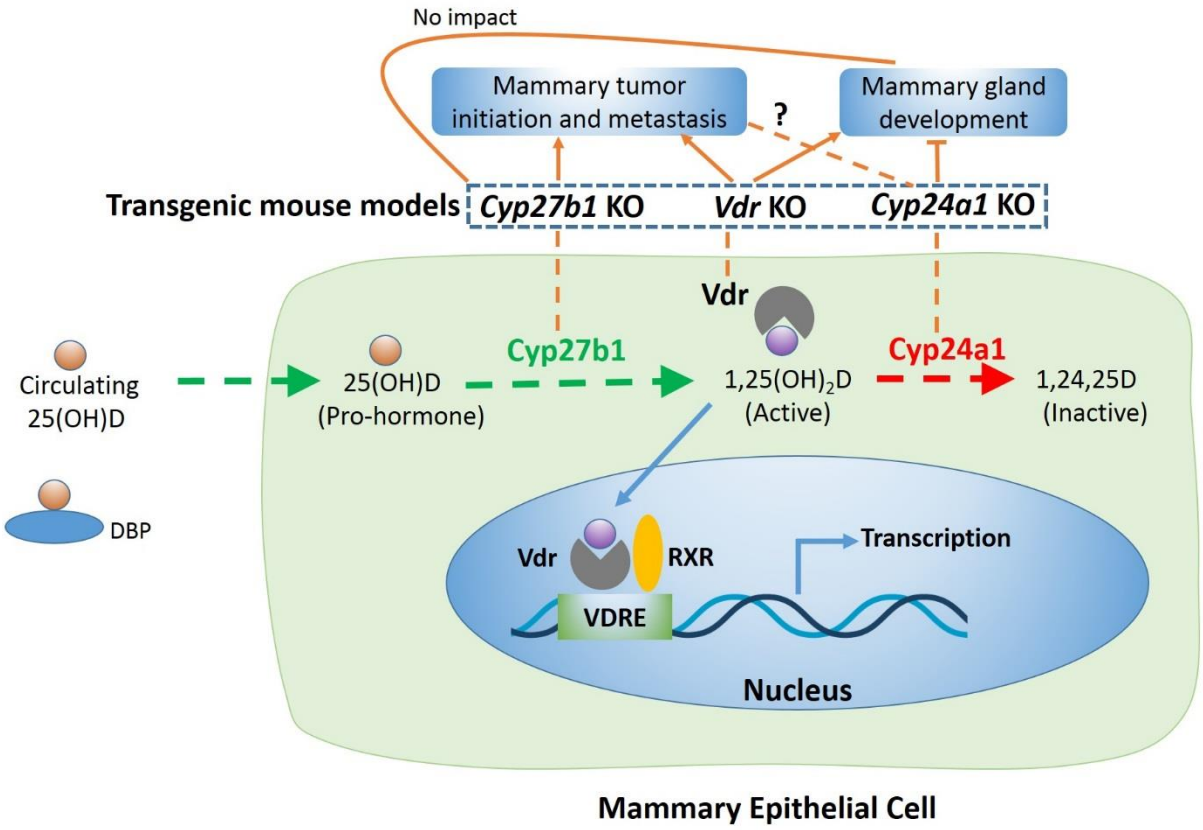

Figure 1. The majority of pro-hormone 25-hydroxyvitamin D_3 (25(OH)D) are bound to vitamin D_3 binding protein (DBP), while the free forms of 25(OH)D diffuse across the plasma membrane of mammary epithelial cells. The mammary epithelial cells express vitamin D_3 metabolic enzymes (Cyp27b1 and Cyp24a1), which are capable of activating or inactivating vitamin D_3. The genomic mechanism of 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$) action involves direct binding of the $1,25(OH)_2D$ activated Vdr/retinoic X receptor (Vdr/RXR) heterodimeric complex to specific DNA binding sites, inducing transcriptional activation or repression of target genes. The impact of several transgenic mouse models on mammary gland development and mammary tumor initiation and metastasis is marked on the figure. Abbreviations: VDRE, vitamin D_3 response element; 1,24,25D, 1,24,25-trihydroxyvitamin D_3; DBP, vitamin D_3 binding protein.

higher serum 25(OH)D levels were significantly associated with superior prognosis for women with breast cancer (overall survival: hazard ratio, 0.45; 95% CI, 0.21-0.96) [10]. Collectively, epidemiological data have shown that sufficient vitamin D_3 is not only associated with decreased risk of breast cancer, but also is significantly associated with improved clinical outcome for breast cancer patients. However, large randomized clinical trials are required to confirm the impact of vitamin D_3 supplementation on breast cancer risk.

4.6 Vitamin D3 binding protein knockout mice

Normally, only 0.04% of circulating 25(OH)D and 0.4% of $1,25(OH)_2D$ are unbound, the remainder being bound to either DBP (85%–88%; high affinity) or albumin (12%–15%; low affinity) [11, 12]. A strain of mice deficient in DBP was generated by targeted mutagenesis [13]. Fed with vitamin D_3-replete diet, $DBP^{-/-}$ mice had significantly lower total serum 25(OH)D and $1,25(OH)_2D$ levels than $DBP^{+/+}$ but serum calcium, phosphorus, and parathyroid hormone (PTH) were normal, indicating that there might be an adequate concentration of intracellular $1,25(OH)_2D$ levels. A further study confirms this hypothesis as the concentration of $1,25(OH)_2D$ in intestinal tissues is not reduced in DBP-null mice, indicating that the tissues of DBP-null mice are capable of accumulating significant concentrations of $1,25(OH)_2D$ despite low total serum $1,25(OH)_2D$ levels [14]. This also suggested total $1,25(OH)_2D$ was not the key determinant of access of $1,25(OH)_2D$ to the target tissues. A small-scale human study shows that free 25(OH)D levels, but not total 25(OH)D levels, correlate well with bone mineral density as determined by dual-energy X-ray absorptiometry [15]. Collectively, these data suggest that serum free 25(OH)D or $1,25(OH)_2D$ levels, rather than total levels, may be a better biomarker to predict the association between the vitamin D_3 status and target organ disease. Studies of DBP-null mice on a depleted vitamin D_3 diet indicated that DBP has a protective role against dietary-induced vitamin D_3 deficiency through prolonging the serum half-life of 25(OH)D and retarding its entry into the liver. However, from the view of side effects, the

removal of DBP can also be beneficial, as DBP-null mice display increased resistance to vitamin D_3 toxicity and reduced susceptibility to hypercalcemia [13].

In humans, a meta-analysis of 28 independent studies found there is a borderline decrease in all-type cancer risk for subjects with high compared with low levels of DBP [16], suggesting a possible role of DBP in cancer etiology. However, the association between DBP serologic levels and breast cancer risk remains undetermined due to the limited number of published studies. Thus further studies are required to not only determine total serum 25(OH)D, but also freely available serum 25(OH)D and DBP levels, and to explore their relationship with breast cancer risk.

4.7 Cyp2r1 enzyme activity and mammary cancer

Cyp2r1 is a member of the cytochrome P450 family and is primarily expressed in the liver [17]. Cyp2r1 is a major, but not exclusive, contributor to circulating 25(OH)D production *in vivo* [18]. A reduction of more than 50% of serum 25(OH)D level was observed in *Cyp2r1*$^{-/-}$ mice compared with that of the wild-type. Cyp2r1 is also found in extrahepatic sites, including testis and mammary gland [18, 19]. Cyp2r1 is located within the *SuperMam1* locus, a mammary tumor susceptibility locus identified in the BALB/c- Trp53+/- mouse model of spontaneous mammary cancer. The expression of Cyp2r1 mRNA levels are significantly lower in mammary glands from mice containing the *SuperMam1* locus than glands from wild-type mice, suggesting that local dysregulation of Cyp2r1 may contribute to mammary cancer development [19].

Although single nuclear polymorphisms (SNPs) of *CYP2R1* are associated with circulating 25(OH)D concentrations [20-22], there is no significant association between an individual SNP of *CYP2R1* (rs10741657) and risk of human breast cancer as reported in a large pooled study of 9,456 cases and 10,816 controls from six cohorts [23]. Since there are large cohorts

of breast cancer gene expression data and patient survival data, we used Kaplan-Meier analysis to determine if *CYP2R1* expression was related to clinical outcome for human breast cancers. A total of 1,764 of breast cancer patients, 883 with low expression and 881 with high expression, were included in this analysis. High expression levels of *CYP2R1* were significantly associated with a more favourable relapse-free survival (Hazard ratio=0.78, 95% CI 0.67-0.91) (Figure 2.)[24]. This suggests that in addition to the local metabolism of 25(OH)D to the active hormone 1,25$(OH)_2$D in the breast (Figure 1.), CYP2R1 is expressed in the breast and could be involved with generation of local 25(OH)D. However, the role of CYP2R1 in the vitamin D_3 status of the human breast has not been explored in detail and additional studies are required.

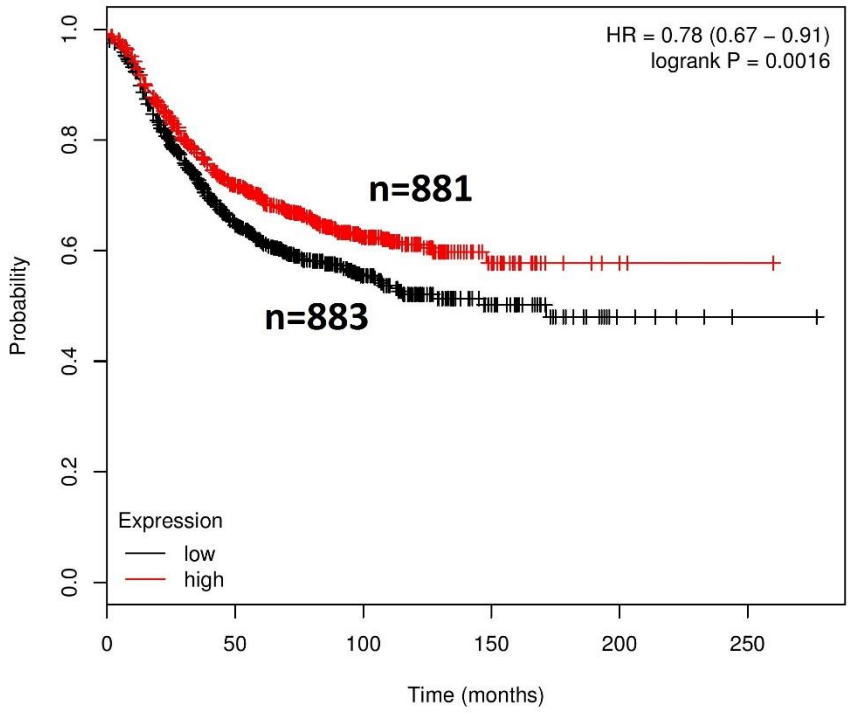

Figure 2. Correlation of *CYP2R1* expression with relapse-free survival for breast cancer patients. Data were analyzed with the KM-plotter by allowing the software to select the best cut-off and only JetSet best probe set. Red and black lines indicate patients with higher and lower *CYP2R1* expression respectively. The total number of patients in the two categories for the gene *CYP2R1* is shown. Hazard ratios (HR) and *P* values (log rank *P*) are shown at the right top of the panel.

4.8 Vdr knockout mice and mammary gland development

The Vdr is a member of the steroid hormone receptor superfamily and regulates gene expression in a ligand-dependent manner [25]. To examine the effects of disruption of the vitamin D_3 signaling pathway on mammary gland development, global *Vdr* knockout mice were generated [26]. Compared with glands from wild-type mice, glands from *Vdr* knockout mice were significantly heavier and showed accelerated pubertal ductal growth and branching morphogenesis. In addition, mammary epithelial cells from *Vdr* knockout mice were sensitized to exogenous estrogen and progesterone, as their growth was enhanced compared with wild-type epithelial cells. This is further confirmed in a subsequent study which used novel mouse models with mammary epithelial- or adipocyte-specific loss of *Vdr* [27]. It was found that loss of *Vdr* in either cellular compartment accelerated ductal morphogenesis with increased epithelial cell proliferation and decreased apoptosis within the mammary gland terminal end buds. This study showed that the Vdr-regulated cross-talk between epithelial and adipocyte cells is partly through the vitamin D_3-dependent production of the cytokine IL-6.

During pregnancy, glands from *Vdr* knockout mice exhibited accelerated lobuloalveolar development and delayed postlactational involution compared with glands from wild-type mice [28]. In vitro, when human VDR is stably expressed in murine *Vdr*-null cells, vitamin D_3 target gene expression is restored, together with the growth inhibition in response to $1,25(OH)_2D$ [29]. Taken together, these data suggest that Vdr signaling pathway plays a significant role in mammary ductal morphogenesis and differentiation.

4.9 Vdr knockout mice and mammary cancer

Several transgenic mice models have been used to determine whether ablation of *Vdr* would alter sensitivity of mammary epithelial cells to tumorigenesis. Wild-type and *Vdr* knockout mice were exposed to the chemical carcinogen dimethylbenzanthracene (DMBA). Glands from *Vdr* knockout mice develop an increased percent of DMBA-induced preneoplastic mammary lesions compared with glands from wild-type mice [30], supporting the notion that *Vdr* ablation enhances sensitivity of mammary epithelial cells to tumorigenesis. Cell lines established from DMBA-induced mammary tumors derived from *Vdr* knockout mice and wild-type mice were further characterized [31]. Mammary epithelial cells from wild-type mice were highly sensitive to $1,25(OH)_2D$ with concentrations as low as 1 nM inducing G_0/G_1 growth arrest and apoptosis. In contrast, *Vdr* knockout mammary epithelial cells are resistant to $1,25(OH)_2D$ mediated growth arrest and apoptosis over the range of 0.01 - 100 nM. These data suggest that the anti-tumor effects of Vdr are driven directly through the vitamin D_3 signaling pathway.

Mouse mammary tumor virus (MMTV)-*neu* transgenic mouse model of mammary cancer was also used to determine whether *Vdr* ablation would enhance sensitivity of mammary gland to transformation [28]. MMTV-*neu* mice were crossed with *Vdr* knockout mice to generate mice with *neu* expression and altered *Vdr* status. Loss of one or two copies of *Vdr* results in increased incidence of preneoplastic lesions and abnormal ductal morphogenesis compared with *neu*/$Vdr^{+/+}$ mice. Moreover, inactivation of one allele of the *Vdr* gene is sufficient to accelerate onset of *neu*-driven mammary tumors. Similar results were obtained when MMTV-Ron transgenic mice were crossed with *Vdr* knockout mice to explore the role of Vdr signaling in the development of Ron-mediated mammary tumorigenesis [32]. Overexpression of Ron, a member of the Met receptor tyrosine kinase proto-oncogene family,

is sufficient to induce mammary transformation in all transgenic mice and is associated with a high degree of metastasis to the liver and lung [33]. Loss of either one or two copies of *Vdr* accelerates Ron-mediated mammary gland hyperplasia, mammary tumor onset, and increases metastasis to the lungs and liver. Tumor from $Vdr^{-/-}$/Ron mice expresses higher levels of *β*-catenin, indicating that upon *Vdr* deficiency 1,25$(OH)_2$D is no longer able to relocate *β*-catenin from the nucleus to the plasma membrane and to suppress transcription of *β*-catenin target genes.

Collectively, these data suggest that the vitamin D_3 signaling pathway mediated through the Vdr has a role in normal mammary gland development and morphogenesis and in addition has a tumor suppressor role in mammary cancer tumorigenesis and progression.

As determined by immunohistochemical staining, the VDR protein is expressed in epithelial cells of normal human breast tissue (100%) and benign lesions (93.5%) while its levels of expression were diminished in carcinoma *in situ* (47%) and in invasive breast cancer (56%) [34], indicating that disruption of vitamin D_3 signaling pathway is frequently associated with breast tumor development. This result is consistent with the most recent VDR expression analysis in 1,114 breast cancers with only 58% of sections showed moderate or strong VDR expression [35]. A small study with a cohort of 82 breast cancer patients suggests that enhanced VDR expression levels in breast cancer are associated with a better prognosis and prolonged overall survival [36].

In summary, data from *Vdr* knockout mice indicate that an intact and functional vitamin D_3 signaling pathway plays a crucial role in delaying mammary cancer initiation and progression. These findings are consistent with observations from human breast cancer which suggest disruption of vitamin D_3 signaling pathway may contribute to breast cancer development. These data suggest that VDR expression profiling may serve as a potential

biomarker to predict the prognosis for patients with breast cancer. In addition, it is possible that activation of the VDR signaling pathway by its ligand 1,25$(OH)_2$D, or analogues, may provide a preventive or therapeutic option for the management of human breast cancer.

4.10 Cyp27b1 knockout mice and mammary gland development

Cyp27b1 (also known as 1α-hydroxylase) is mainly expressed in the kidney and acts to maintain adequate circulating 1,25$(OH)_2$D levels for normal calcium homeostasis and bone metabolism [37]. Studies also have shown that Cyp27b1 is expressed in a variety of extrarenal sites, including mammary gland tissue and malignant mammary lesions [38], indicating that there may be a local synthesis of 1,25$(OH)_2$D. Global *Cyp27b1* knockout mice have been generated to address the role of Cyp27b1 activity in mammary gland development. Mammary gland morphogenesis is similar between glands from wild-type and *Cyp27b1* knockout mice [39], suggesting that 25(OH)D or other Vdr ligands may substitute for 1,25$(OH)_2$D in control of mammary gland development in *vivo*. Similar results were obtained in transgenic mouse model with conditional ablation of the *Cyp27b1* gene in mouse mammary epithelial cells [40]. The *Cyp27b1*-ablated mice presented normal mammary gland development compared with non-ablated polyoma middle T antigen-mouse mammary tumor virus (PyMT-MMTV) controls.

4.11 Cyp27b1 knockout mice and mammary cancer

A study using a mouse mammary gland organ culture model shows that administration of 25(OH)D, the precursor of active vitamin D_3 1,25$(OH)_2$D, suppressed mammary precancerous lesions, particularly in the promotion stage. This suggests a critical role of local Cyp27b1 activity in activating 25(OH)D, thus preventing mammary cancer development

[41]. This is further supported by the PyMT-MMTV mammary cancer model that closely recapitulates the main features of aggressive human disease including distal metastasis. Systemic perfusion with 25(OH)D caused a 50% increase in the tumor $1,25(OH)_2D$ levels [42]. Furthermore, an *in vivo* study also demonstrates that systemic perfusion of MMTV-PyMT mice with 25(OH)D delayed the onset of mammary tumor and significantly decreased lung metastasis.

To more clearly define the role of Cyp27b1 activity in modulating mammary cancer initiation and progression, *Cyp27b1* was specifically deleted in the mammary epithelium of PyMT-MMTV mammary cancer model [40]. This study shows that conditional ablation of *Cyp27b1* resulted in the decrease of locally synthesized $1,25(OH)_2D$ by two thirds, while the expression level of Cyp24a1, the enzyme which degrades biologically active $1,25(OH)_2D$, was unaltered. Furthermore, ablation of *Cyp27b1* was associated with significant acceleration of the early stages of neoplasia and was accompanied by a decrease in apoptotic cell numbers in the mammary tumor and a general acceleration of cell proliferation and angiogenesis capacity, suggesting Cyp27b1 is involved in delaying mammary tumor initiation and progression, at least in the MMTV-PyMT mouse as locally produced $1,25(OH)_2D$ in the mammary gland inhibits tumor growth in an autocrine or paracrine fashion.

In humans, a meta-analysis of epidemiological studies shows that higher circulating 25(OH)D levels are significantly associated with improved long-term disease-free survival [43]. This is consistent with the information from the mice model of localized knockout of Cyp27b1 in the mammary gland, where the reduced $1,25(OH)_2D$ levels accelerate mammary tumour initiation and progression. This implies that local CYP27B1-mediated production of $1,25(OH)_2D$ in the breast influences the clinical outcome for human breast cancer patients.

One approach to achieve higher levels of $1,25(OH)_2D$ in the breast is to maintain a high circulating level of 25(OH)D. Since this can be achieved by dietary supplementation, this provides a low-cost preventive or therapeutic option for human breast cancer, without increasing the risk of hypercalcemia.

Although accumulating evidence points to a link between the lack of CYP27B1 activity in tumors and progression of various human cancers including pancreatic cancer, human colon cancer, and skin lesions [44-46], the molecular pathways involved are poorly characterized. In breast only two studies have investigated gene expression profiles in malignant and adjacent normal breast tissue and the results are conflicting in regard to CYP27B1 mRNA expression levels [47, 48]. However, in both studies the mRNA expression of CYP24A1 was significantly upregulated in the tumor tissues as compared with adjacent normal tissues, implicating dysregulated expression of CYP24A1 (24-hydroxylase) abrogate the effects of local $1,25(OH)_2D$ production regardless of CYP27B1 activity in the tumors. Thus, CYP24A1 probably has its unique role in the context of initiation and progression of breast cancer, which will be further discussed below.

4.12 Cyp24a1 knockout mice and mammary gland development

Cyp24a1 is strongly induced by $1,25(OH)_2D$ and its primary function is to prevent the accumulation of toxic levels of $1,25(OH)_2D$ and 25(OH)D. Global *Cyp24a1* knockout mice fail to thrive and 50% of homozygous mutant mice die before 3 weeks of age. This is most likely due to severe hypercalcemia caused by sustained elevated levels of $1,25(OH)_2D$ in the absence of Cyp24a1 [49]. To examine the role of Cyp24a1 activity in mammary gland development, a novel mouse model with conditional ablation of the *Cyp24a1* gene in mammary epithelium driven by *MMTV-Cre* was generated [50]. Conditional ablation of

Cyp24a1 activity within the mammary epithelium does not adversely impact on reproduction, pup survival, and normal growth of the fat pad (unpublished data). Moreover, circulating $1,25(OH)_2D$ levels were comparable between wild-type and Cyp24a1 knockout mice (unpublished data), indicating localized ablation of Cyp24a1 does not affect systemic vitamin D_3 status. However, mammary glands from conditional *Cyp24a1* knockout mice displayed decreased ductal elongation, impaired side branching, and reduced number of undifferentiated terminal end buds in comparison with glands from wild-type mice [50]. In addition, luminal cells derived from glands with *Cyp24a1* ablation show increased susceptibility to exogenous $1,25(OH)_2D$ treatment, particularly at lower levels (unpublished data). Together, Cyp24a1 activity within mammary epithelium contributes to enhancing ductal elongation and branching morphogenesis during the pubertal development by abrogating the inhibitory effects of the vitamin D_3 signaling pathway. However, the role of Cyp24a1 activity in mammary tumor tumorigenesis and progression has yet to be defined. Generating and characterizing conditional *Cyp24a1* knockout in epithelial cells to delineate its role in mammary cancer development is in progress.

4.13 CYP24A1 and human breast cancer

The *CYP24A1* gene is located on chromosome 20q13.2., a region of the genome that is amplified in human breast cancer [51]. Numerous human studies have consistently shown elevated expression of CYP24A1 mRNA and protein levels in tumor tissue compared with normal breast [34, 47, 48], indicating its candidacy as a putative oncogene. In agreement with these findings, analysis of the datasets from The Cancer Genome Atlas shows 10-13% of human breast cancers exhibit alterations in the *CYP24A1* gene, mostly due to amplifications and increased transcriptional levels of the *CYP24A1* [52]. It has been reported that CYP24A1 overexpression is associated with poorer survival in several cancers, including lung

adenocarcinoma, esophageal cancer, and colorectal cancer, independent of other clinical and pathological prognostic parameters of survival, suggesting it as a potential prognostic biomarker for various types of cancer [51, 53, 54]. The most likely underlying mechanism is that CYP24A1 overexpression facilitates cancer growth by abrogating the effects of locally produced $1,25(OH)_2D$. Thus, inhibition of CYP24A1 activity by selective and potent CYP24A1 inhibitors may provide a new therapeutic option for breast cancer patients, particularly those cancers with high levels of CYP24A1.

Recently, a study used the malignant human breast cancer cell line MDA-MB-231 with stable knockdown of CYP24A1 [55]. Suppression of constitutive CYP24A1 expression resulted in increased susceptibility of these cancer cells to $1,25(OH)_2D$-induced apoptosis and inhibited anchorage-independent growth. Moreover, suppression of vitamin D_3 metabolism following knockdown of CYP24A1, significantly reduced MDA-MB-231 tumor growth in a xenograft model [55]. This is consistent with similar studies using cancer cell lines from different types of cancer, which show that suppression of CYP24A1 activity potentiates antiproliferative effects of $1,25(OH)_2D$ in human prostate cancer PC3 cells or human colorectal cancer Caco-2 cells, and promotes the activation of $1,25(OH)_2D$-mediated apoptosis [56, 57]. According to a previous study, suppression of CYP24A1 enhances the anti-tumor effects of $1,25(OH)_2D$, which is at least partly due to enhanced $1,25(OH)_2D$-mediated induction of target genes, such as *CLMN*, *EFTUD1*, *SERPINB1*, and *KLK6* [58]. However, the vitamin D_3 mediated molecular pathways that modulate breast oncogenesis are yet to be elucidated in detail.

In summary, in preclinical models, inhibition of CYP24A1 activity potentiates the anti-tumor effects of $1,25(OH)_2D$. While there are CYP24A1 inhibitors available [56, 59] , these are not clinically validated and the development of highly selective and potent CYP24A1 inhibitor is warranted. Vitamin D3 or 25(OH)D intake may be ineffective in microenvironment where high levels of CYP24A1 quickly inactivate their biological active form $1,25(OH)_2D$. Thus,

CYP24A1 inhibitors have potential clinical utility for breast cancer patients, particularly where cancers have high expression levels of CYP24A1.

4.14 Conclusion

Mice studies have demonstrated that decreasing the activity of the vitamin D_3 pathway accelerates mammary gland development, while increasing the activity of vitamin D_3 pathway by conditional knockout of Cyp24a1 shows impaired ductal morphogenesis, suggesting that maintaining functional vitamin D_3 signaling pathway is critical in the normal mammary gland development. Vdr or Cyp27b1 acts as a tumor suppressor in the mammary cancer development, while Cyp24a1 has oncogenic properties that may contribute to initiation and progression of mammary tumor which requires further investigation. Data regarding the role of Cyp2r1 and DBP in mammary cancer development are limited. Thus further studies are required to establish the relationship between these two components and mammary cancer. Based on clinical observations and *in vitro* studies on human and mice cell lines, elevated CYP24A1 or Cyp24a1 activity is associated with breast or mammary cancer development through abrogating the inhibitory effects of locally synthesized $1,25(OH)_2D$. Ablation of CYP24A1 or Cyp24a1 activity potentiates the anti-tumor effects of $1,25(OH)_2D$. This suggests there may be of clinical benefits from using a CYP24A1 inhibitor. An alternative to providing vitamin D_3 in high amount, the avenue of combining CYP24A1 inhibitor and the pro-hormone 25(OH)D is interesting since it enhances activity of vitamin D_3 signaling pathway without increasing vitamin D_3-associated side effects (e.g. hypercalcemia). In summary, evidence from transgenic mouse models is consistent with epidemiological data suggesting that disruption of vitamin D_3 signaling pathway contributes to the initiation and progression of breast cancer.

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CHAPTER V

CONDITIONAL INACTIVATION OF THE 25-HYDROXYVITAMIN D-24-HYDROXYLASE (CYP24A1) IN THE MOUSE MAMMARY EPITHELIUM ALTERS MAMMARY GLAND DEVELOPMENT

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5.1 Prelude

This chapter characterized the role of vitamin D-inactivating enzyme Cyp24a1 in mammary gland development. A novel mouse model was generated with conditional knockout of the *Cyp24a1* gene specifically in the mammary epithelium (MMTV-Cre x Cyp24a1$^{lox/lox}$). Our major finding is that mammary glands from virgin *Cyp24a1* knockout females display impaired ductal morphogenesis compared with age- and weight-matched wild-type mice, which is due to increased sensitivity of mammary epithelial cells to 1,25$(OH)_2$D with the ablation of Cyp24a1 activity.

This chapter is the unpublished version of the manuscript.

Contribution of the PhD candidate: Designed and performed experiments, interpreted data, and wrote manuscript.

The transgenic mouse model was generated by Paul Anderson's group. The contribution of other co-authors was stated in Statement of Authorship in the coming pages.

Statement of Authorship

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Name of Principal Author (Candidate)	Lei SHENG
Contribution to the Paper	Conducted experiments, interpreted data, and wrote manuscript.
Overall percentage (%)	60%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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5.2 Abstract

Degradation of $1,25(OH)_2D$, the biologically active form of vitamin D, is driven by the enzyme Cyp24a1 in target tissues, providing critical local control of $1,25(OH)_2D$ bioactivity. *In vitro*, blockade of Cyp24a1 activity potentiates the anti-proliferative effects of $1,25(OH)_2D$. However, the extent to which endogenous Cyp24a1 activity within the mammary epithelium regulates local $1,25(OH)_2D$ levels to modulate normal mammary gland development, with possible implications for cancer, has not been investigated. We generated a novel mouse model with conditional knockout of the *Cyp24a1* gene specifically in the mammary epithelium (MMTV-Cre x Cyp24a1$^{lox/lox}$). Ablation of Cyp24a1 activity in the mammary epithelium does not alter reproduction, peri-natal survival, serum $1,25(OH)_2D$ levels, mammary gland or body weight. Analyses of mammary gland whole mounts indicate that virgin *Cyp24a1* knockout mice form fewer terminal end buds and reduced ductal elongation compared to glands from wild-type weight-matched littermates. Moreover, the width of the ducts proximal to the central lymph node of knockout mice was significantly less than that of wild-type mice at 4, 6, and 10 weeks of age. In addition, the number of secondary and tertiary branching points is significantly reduced in mammary glands from *Cyp24a1* knockout mice at 6 weeks of age. We confirmed that impaired mammary gland morphogenesis in *Cyp24a1* knockout mice is at least partly due to reduced proliferation of mammary epithelial cells with the ablation of Cyp24a1 activity. In summary, our findings suggest that Cyp24a1 activity within epithelial cells plays a crucial role to modulate postnatal mammary gland development, presumably by limiting the local accumulation of $1,25(OH)_2D$.

5.3 Introduction

Mammary gland morphogenesis is driven by complex signaling networks during pubertal development [1]. The major driving factors are estrogen receptor, which promotes ductal elongation and progesterone, which stimulates side branching [2]. Accumulative evidence suggest vitamin D signaling pathways can also modulate postnatal mammary gland development [3, 4]. For example, conditional ablation of *Vdr* in mammary epithelial cells accelerates ductal morphogenesis via increased cell proliferation and decreased apoptosis within the ductal terminal end buds.

Vdr, Cyp24a1, and Cyp27b1 are the key components of the vitamin D metabolism and signaling network. $1,25(OH)_2D$, the active form of vitamin D, exerts its various biological functions through binding to its receptor VDR. Bioavailability of vitamin D within mammary epithelium is regulated by a coordinated balance between $1,25(OH)_2D$ biosynthesis mediated by Cyp27b1 and catabolism to inactive metabolites by Cyp24a1. A previous study showed that Cyp24a1 mRNA expression levels and inactive 1,24,25 trihydroxyvitamin D metabolites are significantly higher in breast cancer than in normal tissue, implicating *Cyp24a1* as a potential oncogene [5]. A recent study characterised MDA-MBA-231 breast cancer cells with constitutive knockdown of *Cyp24a1* expression [6]. Suppression of *Cyp24a1* expression resulted in increased apoptosis, inhibited anchorage-independent growth, and reduced MDA-MBA-231 tumor growth in a mouse xenograft model as well, consistent with function of *Cyp24a1* as an oncogene. However, the specific role of *Cyp24a1 in vivo* in mammary epithelial growth during mammary gland development remains speculative. The viability of mice with global ablation of *Cyp24a1* was poor most likely due to hypercalcemia and nephrocalcinosis during the neonatal period [7]. Here, we investigated whether *MMTV-Cre* driven conditional ablation of *Cyp24a1* within mammary epithelial cells modulates mammary gland development.

5.4 Methods

5.4.1 Mice

To selectively eliminate *Cyp24a1* from the mammary epithelium, FVB mice containing the *MMTV-Cre* transgene (gift from Kay-Uwe Wagner, University of Nebraska Medical Center) were bred with homozygous *Cyp24a1* floxed (*Cyp24a1*$^{lox/lox}$) mice on a C57BL/6 background. F1 mice heterozygous for the floxed *Cyp24a1* gene and positively expressing the *Cre* transgene, were back-crossed with *Cyp24a1*$^{lox/lox}$ mice to generate mice containing the cre-recombinase transgene and homozygous for the floxed *Cyp24a1* allele. The recombinant *Cyp24a1* null allele was detected by PCR using primer p1 (5'-GCGCATAACGATACCACGAT-3') in combination with primer p2 (5'-CCAGCCCCAGGTTTTAATGT-3'). The location of both primers is illustrated in Figure 1a, and the resulting PCR amplicon is 369 bp after deletion of the exon 5 of the *Cyp24a1* gene. Mammary gland development was compared between *Cyp24a1*$^{-/-}$ mice and WT (their littermate controls with genotypes *Cyp24a1*$^{lox/lox}$ lacking cre-recombinase) following at least F6 backcrosses to C57BL/6 mice. Mammary glands from nulliparous female mice were harvested at 4, 6, and 10 weeks of age. Blood was removed by cardiac puncture for measurement of 1,25$(OH)_2$D levels. To assess whether the presence of the Cyp24a1 exerted a gross effect on mammary gland development, wet weights of surgically removed inguinal mammary fat pats were obtained at sacrifice between 4 and 10 weeks of age for calculation of organ weight to body weight ratio (expressed as mg/g). All conditional transgenic mice and control animals were fed with a standard rodent chow diet. All animal procedures were approved by the University of Adelaide Animal Ethic Committee and the University of South Australia Animal Ethics Committee.

5.4.2 Whole mount preparation

Inguinal mammary glands from 4, 6, and 10 week-old virgin female *Cyp24a1*$^{lox/lox}$ and *Cyp24a1*$^{-/-}$ mice were harvested. Glands were spread on glass slides and fixed for 2 to 4 hours in Carnoy's Fixative, rinsed in 70% ethanol, and transferred into Carmine Alum stain overnight. Glands were rinsed in graded series of ethanol. Image of whole mounts were taken using an Olympus DP20 digital camera (Olympus, Tokyo, Japan). Ductal outgrowth was measured on the whole mounts as the distance from middle of the central lymph node (except for 4-week-old gland where the distance was measured from the nipple) to the leading edge of the ductal mass using ImageJ software. The number of secondary and tertiary branches and terminal end bud (bulb-like structures with area greater than 0.03 mm^2) number were also evaluated from the whole mounts. A minimal of six mice per genotype at 4, 6, and 10 weeks of age was used for quantification.

5.4.3 Indirect immunofluorescence

Preparation of formalin-fixed, paraffin embedded tissue sections for immunofluorescence was done as described previously [8]. Formalin-fixed, paraffin-embedded right inguinal mammary glands from 6-week-old mice were cut into 5 µm tissue sections. Sections were adhered to Superfrost UltraPLUS slides (Thermo-Fisher Scientific #1014356190) overnight at 37 °C. Slides were dewaxed in xylene (3 × 5 minutes) followed by 100% EtOH (10 dips) and then PBS (2 × 5 minutes). Antigen retrieval was performed in 10 mM citric acid buffer (pH 6.5) within a decloaking chamber (Biocare Medical #DC2012), for 5 min at 120 °C, and then cooled at room temperature for 1 hour. Slides were washed in PBS for 5 minutes. Sections were encircled with a wax pen and primary antibody diluted in PBS + 10% goat serum, was applied and incubated at 4 °C overnight. Sections were washed in PBS (2 × 5 minutes) before the addition of secondary antibody for 30 mins at room temperature. Sections

were washed in PBS (2 × 5 minutes) before the addition of DAPI (200 ng/ml) for 2 minutes at room temperature. Primary antibodies used for immunofluorescence are the following: p63 (Poly6190, rabbit, 1:100; Biolegend Inc., San Diego, CA), Ki-67 (16A8, rat, 1:100; Biolegend Inc., San Diego, CA). Secondary antibodies used at 1:300 dilution are from Invitrogen Corporation: Alexa568-coupled goat anti-rabbit (A11036) and Alexa488-coupled goat anti-rat (A11006). Sections were mounted under DAKO fluorescent mounting media (S3023) and each fluorescent channel captured separately using a Zeiss LSM700 confocal microscope. For cell enumeration, at least seven fields were randomly selected, and at least 1,000 cells were countered per animal.

5.4.4 Isolation of primary mammary epithelial cells

Isolation of primary mammary epithelial cells, fluorescence activated cell sorting, and direct qPCR with limited number of cells were described previously [9]. The number four and five mammary glands were excised after removal of mammary lymph nodes. Glands were chopped 3 times by using a McIlwain tissue chopper (Mickle Laboratory Engineering, Guildford, UK) on the finest setting, with a 90-degree rotation of the base plate between each round of chopping. Chopped glands from one animal were then place in 10 ml digestion mix containing 3 mg/ml of collagenase A (Roche 11088793001, Mannheim Germany) and 0.67 mg/ml trypsin (Becton Dickinson (BD) 215240, Sparks, MD, USA) at 37 °C for 45 minutes with agitation every 15 minutes. Digested glands were subsequently centrifuged at 1,300 rpm for 6 minutes at 4 °C, and the fat layer supernatant discarded. The pellet containing mammary epithelial organoids were resuspended in 10 ml of L15 media (Sigma L1518, St. Louis, MO, USA) containing 6% fetal calf serum (Gibco 12662029, Grand Island, NY, USA) (L15+) and centrifuged at 1,500 rpm at room temperature. Supernatant was removed, and the pellet was resuspended in 5 ml of red blood cell lysis buffer (Sigma R7757, St. Louis, MO, USA) and incubated at room temperature for 5 minutes before centrifugation at 1,500 rpm for 5 minutes

at 4 °C. From this point, all centrifugation steps were performed at 1,500 rpm for 5 minutes at 4 °C. Pellet was then resuspended in DMEM +10% FCS and incubated for 30 minutes at 37 °C in a T75 flask to allow the selective adherence of fibroblasts. Media containing organoids were collected and centrifuged. Supernatant was removed, and organoids were resuspended in L15+ and kept overnight at 4 °C. The next day, organoids were pelleted, washed twice in Ca^{2+} /Mg^{2+}- free PBS/0.02% wt/vol EDTA and incubated in 2 ml of Joklik MEM (Sigma M8028, St. Louis, MO, USA) for 15 minutes at 37 °C. Organoids were centrifuged and resuspended in 2 ml of 0.25% trypsin-0.04% EDTA solution (Gibco 25200, Grand Island, NY, USA) and placed at 37 °C for 2 minutes to generate single cells. Next, 5 ml of 5 µg/ml DNase I (type II) in serum-free L15 was added for a further 5 minutes at 37 °C to disperse cellular clumps. Then, 7 ml of L15+ was added and the cell solution was passed through a 40-µm cell strainer (BD 352340, Sparks, MD, USA). The resultant single cells were pelleted, resuspended in L15+, and counted by using trypan blue and a hemocytometer. Cell was brought to a concentration of 1×10^6 /ml and kept on ice.

5.4.5 Cell labelling, flow-cytometric analysis, and fluorescence activated cell sorting

Fluorochrome-conjugated antibodies were titrated on primary mammary epithelial cells to ensure maximal positive-to-background fluorescence ratio (see Supplementary file 1). Anti-rat compensation beads (BD 552845) were used for single-stain antibody controls. Compensation controls also included two cellular samples: unstained cells and cells with DAPI (Sigma D8417, St. Louis, MO, USA). Cells were incubated with antibodies on ice for 45 minutes with agitation each 15 minutes. Samples were then washed with twice the sample volume and resuspended in L15+ containing 200 ng/ml of DAPI, except non DAPI compensation controls. All multiple-labeled samples were gated on FSC-A versus SSC-A and doublet discrimination (FSC-H versus FSC-W and SSC-H versus SSC-W) and DAPI negativity (see Supplementary file 2). Samples contained anti-CD45 to exclude lymphocytes

from analysis. Cells were analyzed and sorted on a BD FACS-Aria II containing 355 nm UV, 488 nm blue, 561 nm yellow-green, and 633 nm red lasers. Sorting for culture was performed into L15+.

5.4.6 Generation of cDNA by direct reverse transcription and qPCR analysis

For analysis of transcript levels by quantitative polymerase chain reaction (qPCR), cells were sorted directly into lysis buffer (10 IU RNase inhibitor (Invitrogen 10777, Carlsbad, CA, USA), 2 mM DTT, 0.15% Tween-20 (Biorad) in 12 µl of nuclease-free water) in PCR tubes. Then 500 cells were sorted into each tube (making approximately 14 µl total volume). Reverse transcription was performed by using Superscript VILO (Invitrogen 11754, Carlsbad, CA, USA), as per manufacturer's protocol. Primers were designed that span introns to exclude the detection of genomic DNA and selected for optimal melt curve and amplification profiles (for primer sequences (see Supplementary file 3). qPCR was performed by using iQ^{TM} SYBR Green Supermix (Biorad 1708880, Hercules, CA, USA) as per manufacturer's protocol. Per subpopulation, two to three tubes were assayed, normalized with *PPIA,* averaged, and compared with matched WT samples according to the delta-delta c(t) method. The relative values from three to five sets of mice were assessed with paired *t* test for statistical significance.

5.4.7 In vitro culture and cell viability assay

Sorted primary mouse mammary luminal cells were cultured at 37 °C in a 5% vol/vol CO2/5% vol/vol O_2 atmosphere in 6-well plate in 1:1 DMEM/F12 (Sigma, NSW, Australia) with 6% FCS, 5 µg/ml insulin (Sigma), 10 ng/ml epidermal growth factor (Sigma), and 5 ng/ml cholera toxin (Sigma). After reaching confluency, cells were passaged and plated at 2,000 cells per well in 96-well plates and allowed to incubate overnight. Cells were then treated with either vehicle control ethanol or 20 or 100 nM 1,25$(OH)_2$D for 4 days. Cell

viability was assessed at day 1, day 2, day 3, and day 4 using Cell Titer-Glo assay (Promega) for ATP metabolism. Cell viability assays were performed in triplicate in at least 3 independent mice. The remaining passaged cells were plated at 100,000 cells per well of a 12-well plate and allowed to incubate overnight. Cells were treated with either vehicle control ethanol or 20 or 100 nM $1,25(OH)_2D$. Following 24 hours treatment, cells were harvested and total RNA was extracted for subsequent qRT-PCR analysis.

5.4.8 Statistical analysis

Statistical analysis was conducted using GraphPad Prism software (La Jolla, CA). Data are analyzed by Student's *t*-test and expressed as means ± SD. A *P* value of < 0.05 was regarded as significant.

5.5 Results

5.5.1 Conditional deletion of the Cyp24a1 gene in mouse mammary epithelium

To directly investigate whether loss of *Cyp24a1 in vivo* alters postnatal mammary gland development, we generated a new model of epithelial cell specific *Cyp24a1* deletion in the mammary gland using Cre/loxp-mediated recombination (Figure 1a). The *Cyp24a1* gene was specifically inactivated in the mammary epithelium, by intercrossing the floxed *Cyp24a1* mice with the MMTV-Cre mice. To assess the efficiency of the *Cyp24a1* knockout, we performed PCR analysis of genomic DNA, which revealed ablation of exon 5 of the *Cyp24a1* gene (Figure 1b). PCR amplicon is 1131 bp in wild-type mice while PCR amplicon is reduced to 369 bp after deletion of exon 5 of *Cyp24a1*. After deletion, there is a new stop codon located upstream of exons 9 and 10, which encode the Heme-binding domain [10]. Consistent with these results, real time qPCR analysis revealed a significant decrease (~88 - 100%) in Cyp24a1 mRNA levels in luminal and basal epithelial cells (Figure 1c). Relative Cyp24a1 mRNA expression level was higher in wild-type luminal cells than in basal cells, which is consistent with an early RNAseq study showing Cyp24a1 mRNA is mainly expressed in luminal progenitor cells [11].

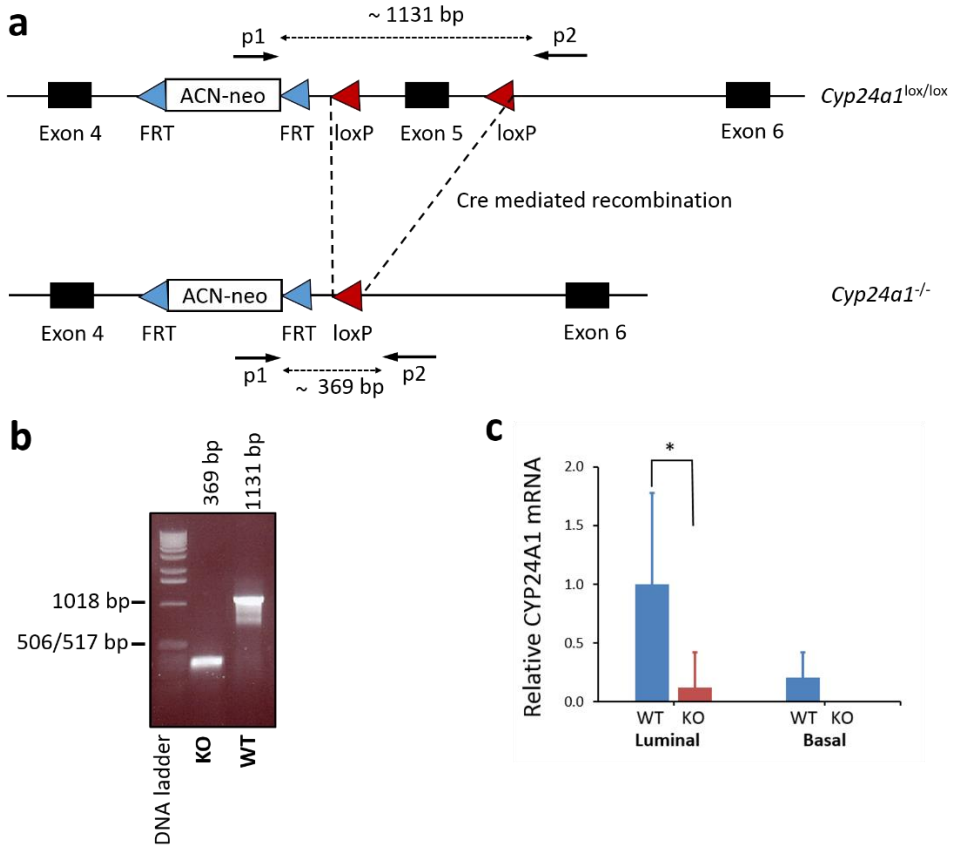


Figure 1. Conditional deletion of *Cyp24a1* in the mammary epithelium using the Cre-Lox system. **a**: schematic outline of the Cre-mediated, conditional excision of exon 5 of the *Cyp24a1* gene. Targeting strategy to flank the fifth coding exon of *Cyp24a1* with *loxP* sites. An ACN-neomycin (ACN-neo) selectable marker was placed upstream of exon 5. The ACN-neo cassette is surrounded by FRT sites and can be excised using FLP recombinase. **b**: PCR analysis using primer set p1 and p2 illustrated in panel **a**. to determine the conversion of the *Cyp24a1* floxed allele (*Cyp24a1*$^{lox/lox}$) into recombinant knockout of *Cyp24a1* (*Cyp24a1*$^{-/-}$). **c**: relative expression of Cyp24a1 at the mRNA level as assessed by qRT-PCR within luminal and basal epithelial cells from 6-week-old mice (n=6 mice per genotype).

5.5.2 Cyp24a1 ablation does not alter normal growth of mammary gland fat pad, body weight, pup survival, and circulating 1,25(OH)2D levels

Previous data have indicated that global *Cyp24a1* knockout mice fail to thrive and 50 % of homozygous mutant animals died before 3 weeks of age due to hypercalcemia caused by sustained elevated levels of $1,25(OH)_2D$ [12]. To determine whether localized ablation of *Cyp24a1* retards the normal growth and reproduction in the *Cyp24a1* knockout mice, breeding pairs of wild-type and *Cyp24a1* knockout mice were continuously maintained on a standard diet, and litter size and pup survival were monitored. Body weight and mammary gland weight were also monitored during pubertal stage. Data from 10 litters indicated that each litter contains approximately 3 pups of wild-type and *Cyp24a1* knockout mice and no statistical significance was detected between these two genotypes (Figure 2a). Pup survival to weaning was 95% for both *Cyp24a1* knockout and wild-type mice with no significant differences due to genotype (Figure 2b). Localized ablation of Cyp24a1 activity does not affect systemic levels of $1,25(OH)_2D$, as there is no statistical difference of circulating $1,25(OH)_2D$ levels measured at 6 weeks of age between Cyp24a1 knockout and wild-type mice (Figure 2c). The body weights of mice through puberty were similar for wild-type and *Cyp24a1* knockout mice (Figure 2d). Cyp24a1 ablation did not significantly alter fat pad growth as normalized to body weight, shown as ratio of gland weight to body weight (Figure 2e). These data indicate that conditional ablation of *Cyp24a1* in mammary epithelial cells does not adversely affect reproduction, peri-natal survival, mammary gland fat pad, body weight, or circulating $1,25(OH)_2D$ levels even with a standard diet.

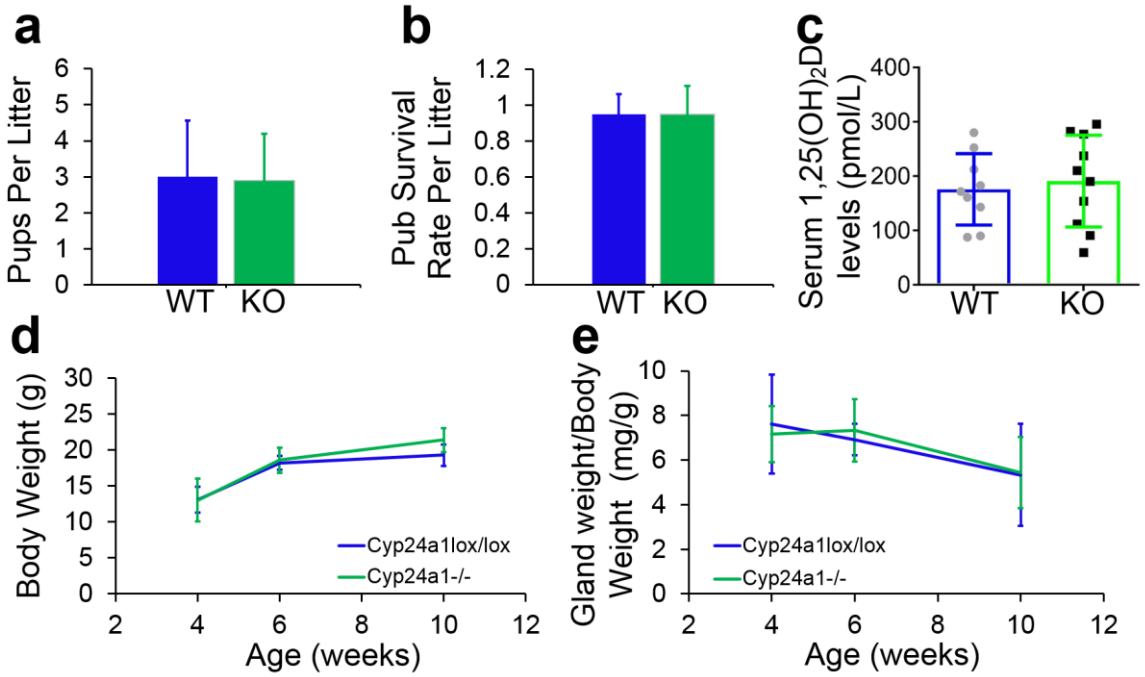


Figure 2. Fertility and growth of wild-type and *Cyp24a1* knockout mice. Litter size (**a**) and pup survival to weaning (**b**) were monitored for both male and female offspring. Circulating 1,25(OH)$_2$D levels were measured in both wild-type and *Cyp24a1* knockout mice at 6 weeks of age (**c**). Body weight (**d**) and ratio of mammary gland weight to body weight (**e**) were monitored for female offspring. Results are mean ± SD (n=5-10); no statistically significant differences were observed between the genotypes.

5.5.3 Cyp24a1 knockout female displays impaired mammary gland ductal morphogenesis

To assess the ductal development of the pubertal mammary glands, whole mounts of inguinal mammary glands were prepared from virgin wild-type and *Cyp24a1* knockout mice sacrificed from 4, 6, and 10 weeks of age. Representative whole mounts of inguinal mammary glands from 6-week-old mice are shown in Figure 3a and Figure 4a. In comparison with glands from wild-type mice, ductal morphogenesis was delayed in glands from *Cyp24a1* knockout mice, as evidenced by reduced ductal elongation with the ablation of *Cyp24a1* (Figure 3b). The number of terminal end buds (defined as bulb-like structures with an area greater than 0.03 mm^2) was counted manually over the pubertal developmental time course. As presented in Figure 3c, the number of terminal ends was significantly reduced in glands from *Cyp24a1* knockout mice than in glands from wild-type mice at 4 and 6 weeks of age ($P < 0.05$). Further analysis of ductal morphogenesis was conducted on inguinal glands obtained from 6-week-old wild-type and *Cyp24a1* knockout mice, as this was the time point at which the difference in the ductal elongation and terminal end bud was most evident. To determine whether ablation of Cyp24a1 impacted on ductal branching, the number of secondary and tertiary branching points were counted in wild-type and *Cyp24a1* knockout mice. A significant decrease in the number of both secondary and tertiary branching points was detected in 6-week-old *Cyp24a1* knockout mice as shown in Figure 4b ($P<0.01$ and $P<0.05$ respectively). Furthermore, ductal width was measured in wild-type and *Cyp24a1* knockout mice at 4, 6, and 10 weeks of age. A significant reduction of ductal width was detected in *Cyp24a1* knockout mice during the 4-10 week course of pubertal development (Figure 4c).

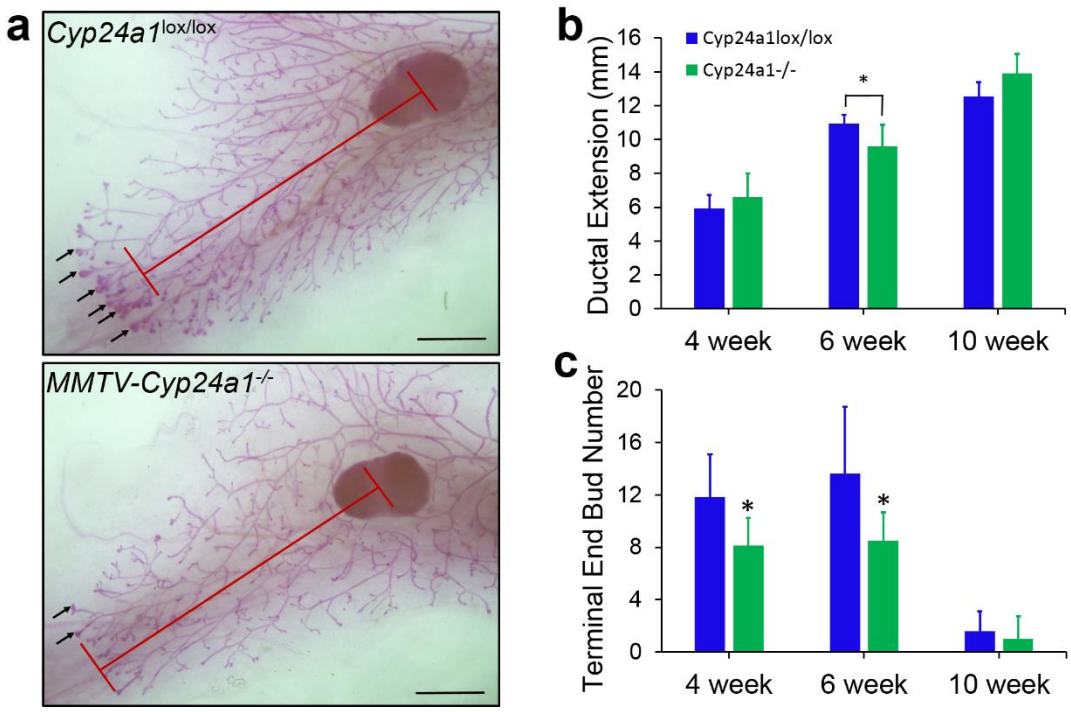


Figure 3. Reduced mammary ductal extension and terminal end bud number with Cyp24a1 ablation in the mammary epithelium. **a**: representative whole mounts of inguinal mammary glands from 6-week-old *Cyp24a1*$^{-/-}$ transgenic mice showing impaired ductal outgrowth compared with age-matched controls. The darkly stained round object within the ducts is the inguinal lymph node. Scale bar, 2 mm. Arrows indicate terminal end buds. **b**: average ductal extension in mammary glands from 4-, 6-, and 10-week-old transgenic mice compared with wild-type controls. **c**: The number of terminal end buds per mammary gland was counted in whole mounts of inguinal glands removed from weight-matched wild-type and *Cyp24a1* knockout mice from 4, 6, and 10 weeks of age. Data in b-c represents mean ± SD of at least five animals per genotype per time point. *Statistically significant, wild-type versus *Cyp24a1* knockout ($P < 0.05$).

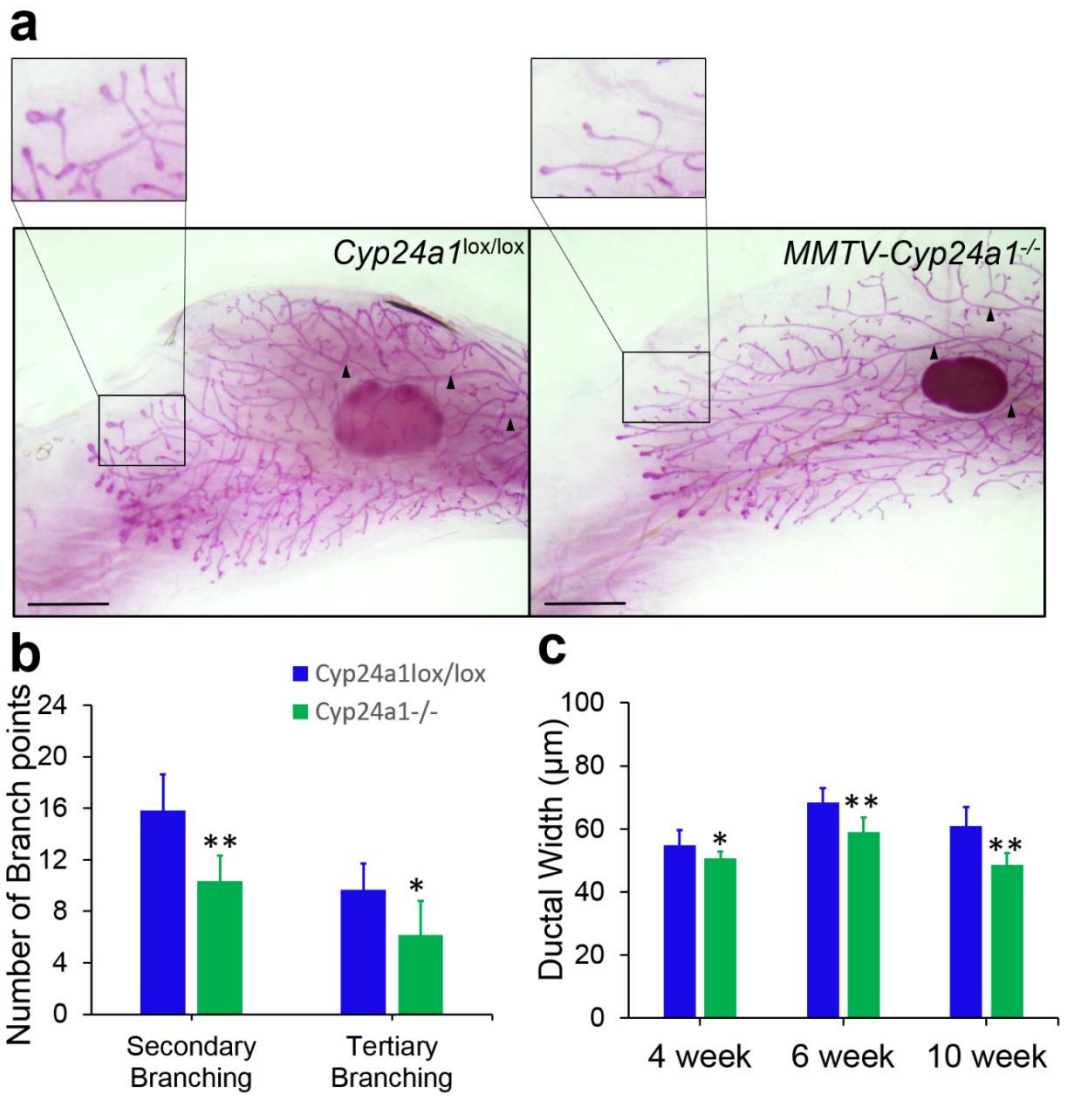


Figure 4. Quantitative analysis of mammary gland branching and ductal width in virgin wild-type and *Cyp24a1* knockout mice. **a**: representative whole mounts of inguinal mammary glands from 6-week-old *Cyp24a1*$^{-/-}$ and *Cyp24a1*$^{lox/lox}$ transgenic mice. Arrowheads indicate ductal width. Black scale bar = 2 mm. **b**: number of secondary and tertiary branching arising from three of 2 mm primary branches at 6 week of age were counted (n=6 to 7 for each group). **c**: ductal width was measured at 4, 6, and 10 week of age (n=5 to 8 for each group). Data in **b-c** represents mean ± SD. *$P < 0.05$ and **$P < 0.01$ vs. *Cyp24a1*$^{lox/lox}$ mice.

5.5.4 The ablation of Cyp24a1 activity reduces proliferation of mammary epithelial cells

As $1,25(OH)_2D$ was implicated in the regulation of cell proliferation, we determined whether the ablation of its inactivating enzyme Cyp24a1 was associated with a disruption in the control of proliferation of mammary epithelial cells. The mammary glands were harvested from wild-type and *Cyp24a1* knockout mice at 6 weeks of age and processed for immunofluorescence staining of the proliferation cell marker Ki-67 (green) (Figure 5a and 5b). p63, a myoepithelial marker, was stained by anti-p63 (red) to distinguish myoepithelial cells (or basal epithelial cells) from luminal epithelial cells (Figure 5a and 5b). Quantitative analysis of Ki-67 positive cells indicated that there were significant reductions in proliferation of ductal luminal (from 10.8% to 5.9%) and basal epithelial cells (from 13.3% to 4.2%) from *Cyp24a1* knockout mice as compared with wild-type mice (Figure 5a and 5c). Similarly, *Cyp24a1* ablation reduces proliferation of both body cells (luminal cells) and cap cells (basal cells) by 16.8% and 21.4% respectively in the terminal end buds (TEBs) (Figure 5b and 5d). At the same time, TUNEL assay did not detect any increase in the apoptosis rates in the mutants (Supplementary file 4). Thus, the ablation of *Cyp24a1* causes a net decrease in the proliferation of mammary epithelial cells, which underlies, at least in part, the impaired ductal morphogenesis described earlier.

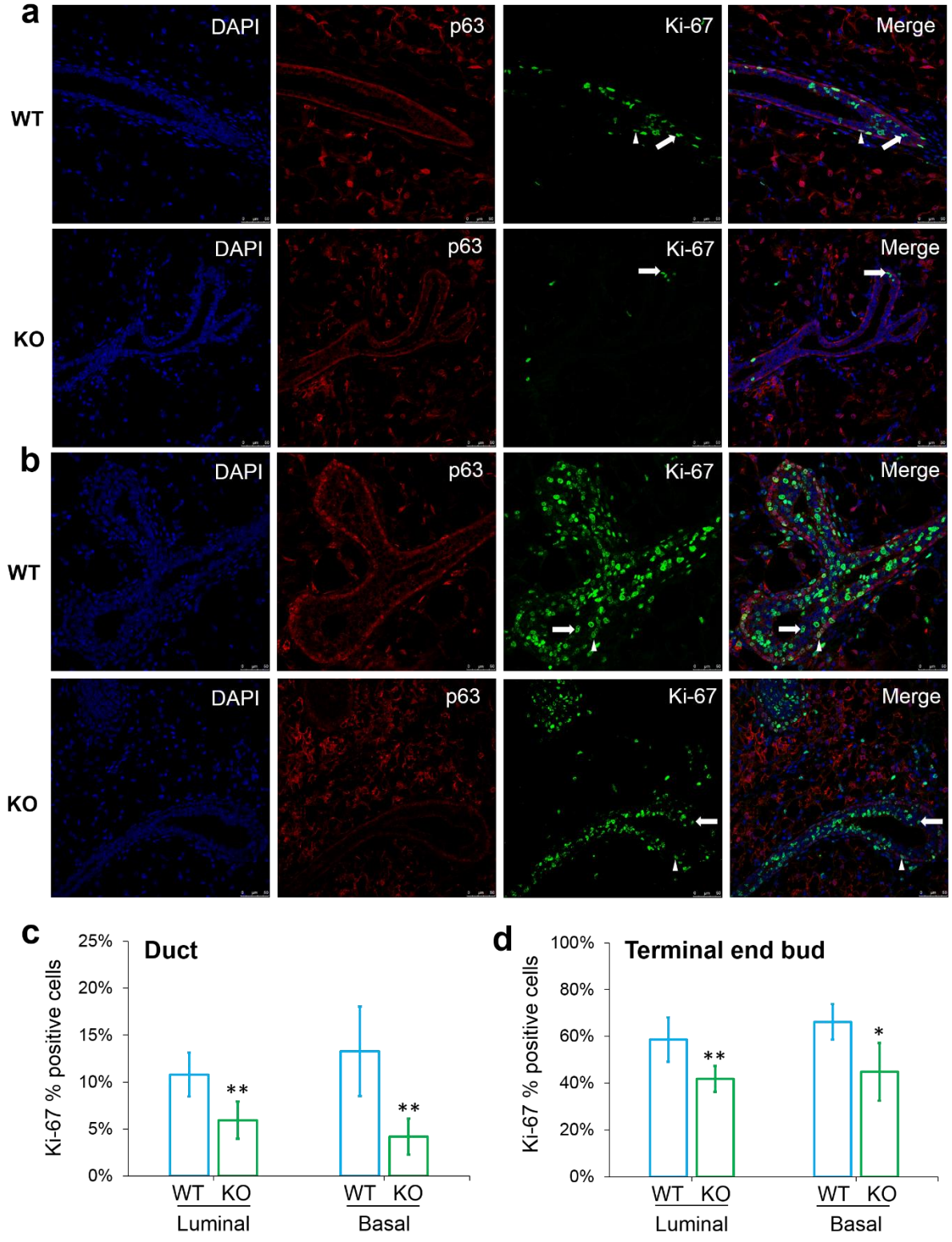


Figure 5. The loss of *Cyp24a1* inhibits proliferation of mammary epithelial cells.

Representatives of immunofluorescence labelling of duct (**a**) or terminal end bud (**b**) from 6-week-old wild-type (WT) or *Cyp24a1* knockout (KO) mice mammary glands with ant-Ki-67

(green) and anti-p63 (red). Basal cell layers are positive for p63, whereas luminal cells are negative. Cell nuclei were stained with DAPI (blue). Arrows indicate Ki-67 positive luminal cells. Arrowheads indicate Ki-67 positive basal cells. Scale bar = 50 μm. Quantification of Ki-67 % positive cells of luminal or basal cells in the ducts (**c**) and terminal end buds (**d**) of mouse mammary glands from WT or *Cyp24a1* KO mice. Student *t* tests were performed to determine statistical difference (*$P < 0.05$ and **$P < 0.01$).

5.5.5 Ablation of Cyp24a1 activity sensitizes luminal cells to low level of exogenous 1,25(OH)2D treatment

As shown by immunofluorescence staining, ablation of *Cyp24a1* reduces proliferation of mammary epithelial cells. We assume the ablation of *Cyp24a1* leads to increased local $1,25(OH)_2D$ levels, which cannot be confirmed directly due to technical issues. As an alternative approach, mammary luminal cells from wild-type or *Cyp24a1* knockout mice were sorted by fluorescence-activated cell sorting (FACS) for *in vitro* culture. These were exposed to the same concentration of $1,25(OH)_2D$ to determine whether ablation of *Cyp24a1* could sensitize luminal cells to exogenous $1,25(OH)_2D$ treatment. Luminal and basal subsets were identified by using CD24 and CD49f respectively after exclusion for debris, doublets, dead cells, and lymphocytes, as outlined in Supplementary file 2. Subpopulations of epithelial cells were validated based on the expression of hormone-sensing cell markers (estrogen receptor: ER and progesterone receptor: PR) (Figure 6a and 6b) by using a direct qPCR protocol developed for the convenient interrogation of gene expression in small numbers of cells. For each population, two to three independent tubes of 500 sorted cells were assayed per animal (n=9).

VDR and Cyp27b1 mRNA expression levels within luminal cells are comparable between *Cyp24a1* knockout mice and age-matched wild-type mice (Figure 6c and 6d). Sorted luminal cells were further subjected to proliferation assay. Cells from wild-type or *Cyp24a1* knockout mice were treated with vehicle control ethanol, 20 nM or 100 nM $1,25(OH)_2D$ for 4 days. Cell viability was analyzed in a time-dependent manner. Luminal cells from both wild-type and *Cyp24a1* knockout mice were significantly inhibited by 96 hours of 100 nM $1,25(OH)_2D$ compared with vehicle control (Figure 6e). The inhibitory effect was comparable between these two groups, probably because the high levels of $1,25(OH)_2D$ exceeded the maximum catabolic activity of the Cyp24a1 enzyme in wild-type luminal cells. When the concentration

of $1,25(OH)_2D$ was reduced to 20 nM, luminal cells from wild-type mice were resistant to growth inhibition while luminal cells from *Cyp24a1* knockout mice were still significantly inhibited compared with vehicle control (Figure 6e). These data support the notion that the ablation of Cyp24a1 sensitizes luminal epithelial cells to exogenous $1,25(OH)_2D$, presumably by the continued maintenance of $1,25(OH)_2D$ levels in the absence of metabolism by Cyp24a1. Cyp24a1 mRNA expression levels were also determined in luminal cells following vehicle control ethanol, 20 nM or 100 nM $1,25(OH)_2D$ treatment for 24 hours. Cyp24a1 mRNA levels in wild-type luminal cells were highly induced by $1,25(OH)_2D$ in a concentration dependent manner, while in *Cyp24a1* knockout luminal cells Cyp24a1 mRNA remain at low levels (Figure 6f). The *Cyp24a1* knockdown efficiency of knockout mice versus control is 96%, 87%, and 92% respectively across control, 20 nM, and 100 nM $1,25(OH)_2D$ treatment groups, which are consistent with data obtained from the original mammary epithelial cells as shown in Figure 1c. These data indicate that the induction of *Cyp24a1* expression following exposure of mammary luminal cells to $1,25(OH)_2D$ alleviates the vitamin D driven growth inhibition.

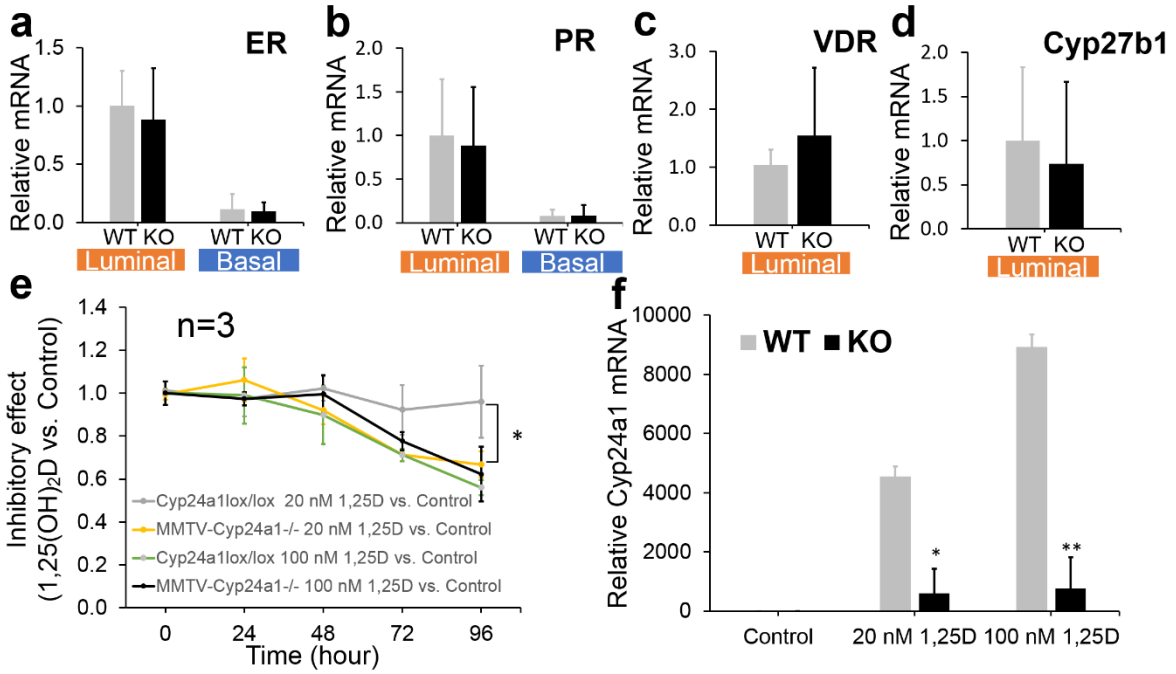


Figure 6. Ablation of Cyp24a1 activity sensitizes luminal cells to low level of $1,25(OH)_2D$ treatment. Quantitative polymerase chain reaction (qPCR) analysis of estrogen receptor (ER; **a**), progesterone receptor (PR; **b**), vitamin D receptor (VDR; **c**), and Cyp27b1 (**d**) transcription in wild-type (WT, grey bars) and *Cyp24a1* knockout (KO, black bars) luminal cells (orange) and basal cells (blue) obtained from 6-week-old *Cyp24a1*$^{-/-}$ and *Cyp24a1*$^{lox/lox}$ transgenic mice. Data are presented as mean ± SD of nine separate sets of WT and *Cyp24a1* KO animals in duplicate qPCR experiments. **e**: sorted luminal cells from wild-type or *Cyp24a1* knockout mice were exposed to vehicle control ethanol or 20 nM or 100 nM $1,25(OH)_2D$ for 4 days. Data are presented as ratio of luminescence from $1,25(OH)_2D$ treated cells to ethanol treated controls. qPCR analysis of Cyp24a1 transcription was made in luminal cells following 24 hours treatment (**f**). Data are presented as mean ± SD of two separate sets of WT and *Cyp24a1* KO animals in duplicate qPCR experiments. **P*< 0.05 and ***P*< 0.01 vs. *Cyp24a1*$^{lox/lox}$ mice.

5.6 Discussion

In this study, we have used mice with a conditional ablation of the *Cyp24a1* gene to provide evidence of its role in mammary gland morphogenesis. Our major finding is that mammary glands from virgin *Cyp24a1* knockout females display impaired ductal morphogenesis compared with age- and weight-matched wild-type mice, suggesting that Cyp24a1 promotes signaling network during mammary gland pubertal development. Specifically, glands from *Cyp24a1* knockout mice display decreased branching, shorter ductal extension and lower numbers of undifferentiated terminal end bud structures compared with glands from wild-type animals. It has been shown that highly-proliferative terminal end buds are the targets for chemical carcinogens in the mammary gland, and increased number is positively associated with increased risk of breast cancer [13]. Thus the decreased terminal end bud number in the glands of *Cyp24a1* knockout mice may translate to reduced susceptibility to chemically induced mammary tumorigenesis. This concept is consistent with a previous report that Cyp24a1 downregulation in MDA-MB-231 cells inhibits tumorigenic potency *in vitro* and *in vivo* [14].

Our data shows that conditional *Cyp24a1* knockout in mammary epithelial cells does not influence the growth of the mouse or of the mammary gland, which mainly consists of fat pad tissue. An early study has shown that disrupted vitamin D3 signaling pathway has an impact on growth of the gross mammary gland [3]. Mammary glands from virgin global *Vdr* knockout mice are much heavier compared with glands from age- and weight-matched wild-type mice. Also, loss of VDR in either mammary epithelial cells or adipocyte tissue accelerated ductal morphogenesis [4]. However, in our case, ablation of *Cyp24a1* driven by *MMTV-Cre* is confined to mammary epithelial cells while vitamin D3 signaling and metabolic networks remain intact within fat pad tissue. All data support the notion that there are independent roles for VDR signaling pathway in mammary adipocytes and epithelial cells

in controlling postnatal mammary gland development. Thus, it is not surprising that conditional ablation of Cyp24a1 only alters ductal morphogenesis without affecting adipocyte tissue.

Cyp24a1 is strongly induced by $1,25(OH)_2D$ and its primary function is to prevent the localised accumulation of toxic levels of $1,25(OH)_2D$ and 25(OH)D. Global *Cyp24a1* knockout mice fail to thrive and 50% of homozygous mutant mice die before 3 weeks of age. This is most likely due to severe hypercalcemia caused by sustained elevated levels of $1,25(OH)_2D$ in the absence of global Cyp24a1 activity [12]. However, conditional ablation of Cyp24a1 activity within the mammary epithelium does not alter circulating $1,25(OH)_2D$ levels, productivity, and peri-natal pup survival, and therefore is an ideal model to explore the role of Cyp24a1 activity in mammary gland development. Thus any changes of ductal morphogenesis occur within the mammary gland will be attributed to localized changes of active $1,25(OH)_2D$ levels due to the ablation of Cyp24a1 activity.

Our data revealed that *Cyp24a1* knockout inhibited proliferation of mammary epithelial cells from both mammary duct and terminal end buds. This inhibitory effect was further confirmed by *in vitro* assay where isolated luminal epithelial cells showed increased susceptibility to exogenous $1,25(OH)_2D$ treatment, particularly at lower levels. Immunohistochemistry staining of normal human breast demonstrated that VDR positive cells were localised exclusively in the luminal layer of human breast epithelium [15]. However, localization of VDR protein in mouse mammary epithelium is slightly different from that of human breast. Although VDR-positive epithelial cells are mainly found in the luminal layer of mammary epithelium from 6-week-old virgin female mice, cap cells (basal cells of TEBs) were infrequently positive for VDR. As expected, the VDR-positive epithelial cells, including majority of luminal cells and scarce basal cells, showed increased sensitivity to localized accumulation of $1,25(OH)_2D$ in the absence of Cyp24a1, its inactivating enzyme. However,

the myoepithelial layer, where the majority of basal or cap cells are VDR negative, also displayed a reduction of proliferation rate in *Cyp24a1* knockout epithelial cells. A possible explanation for this phenomenon is that inhibited proliferation of luminal epithelial cells from *Cyp24a1* knockout mammary gland diminished the production of paracrine factors (e.g. WNT4), which contribute to the growth and differentiation of basal epithelial or cap cells [16].

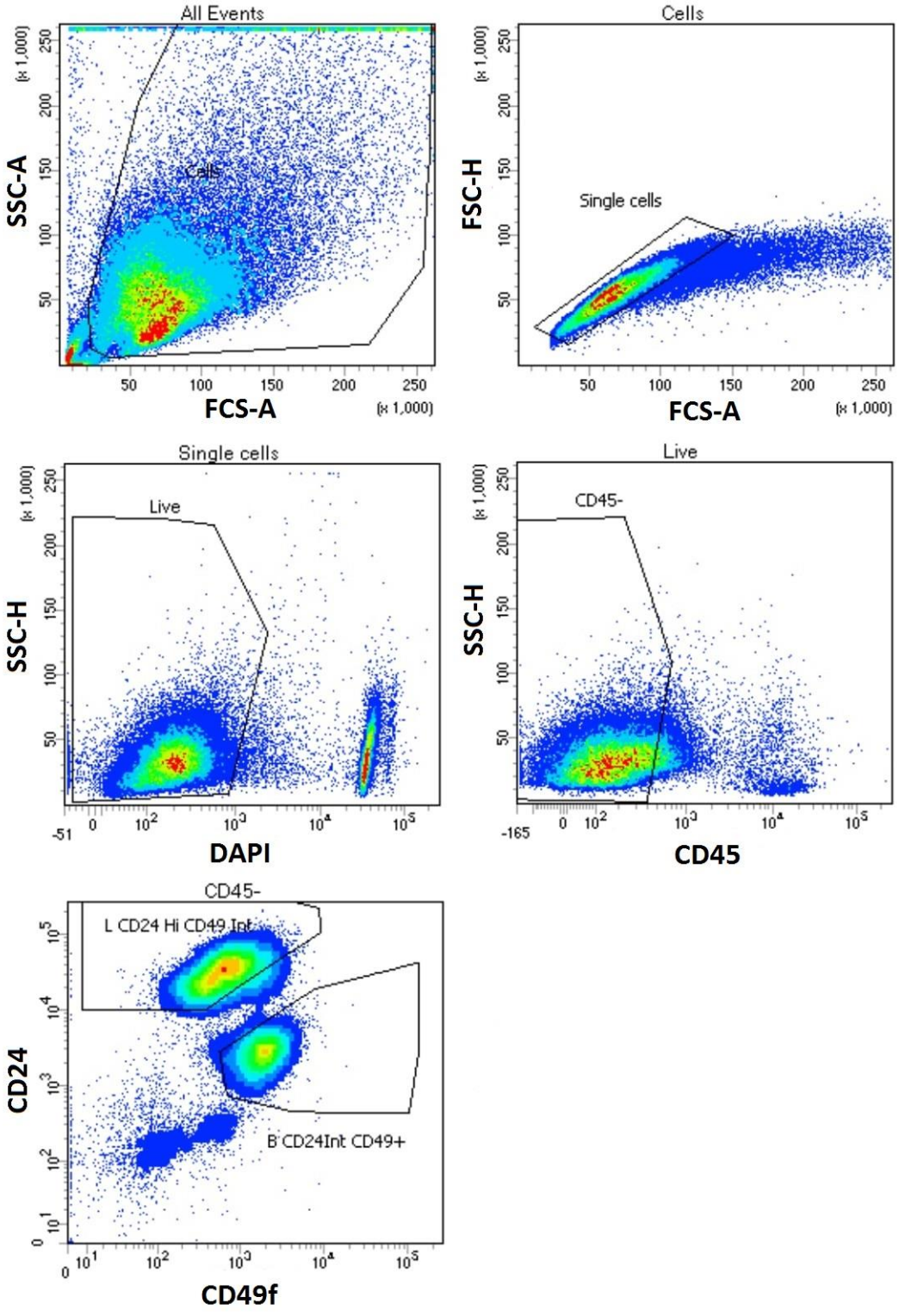
We conclude that in the absence of Cyp24a1 metabolic activity, the inhibited proliferation of mammary epithelial cells a responce to local accumulation of $1,25(OH)_2D$, although due to technical reasons no direct $1,25(OH)_2D$ levels were measured within cells. However, it remains unknown whether Cyp24a1 has gain-of-function effects independent of vitamin D to promote mammary ductal morphogenesis. Further studies are needed to address this question. Human trials have shown that increased vitamin D intake, particularly high levels, reduces the risk of breast cancer [17]. However, excessive vitamin D intake is associated with increased occurrence of side effects, such as hypercalcemia [18]. Specific inhibitors of CYP24A1 are in the process of clinical development [19, 20]. Such an inhibitor will provide a new strategy to maintain and increase the tumor suppressor function of breast epithelial cells where circulating vitamin D levels are normal to low in the absence of vitamin D-associated side effects. However, any systemic use of CYP24A1 inhibitors may negatively impact endocrine metabolism. Thus a new formulation of CYP24A1 inhibitors with selectively targeting specific tissue should be taken into consideration.

In summary, this study has used the localised *Cyp24a1* knockout in the mammary gland of mice to demonstrate a role for the Cyp24a1 in enhancing elongation and branching morphogenesis during pubertal development of mammary gland ducts. Our data provide the first *in vivo* evidence that Cyp24a1 impacts on mammary gland development.

Supplementary File 1-Specifications for antibodies used in confocal immunofluorescence and FACS analysis

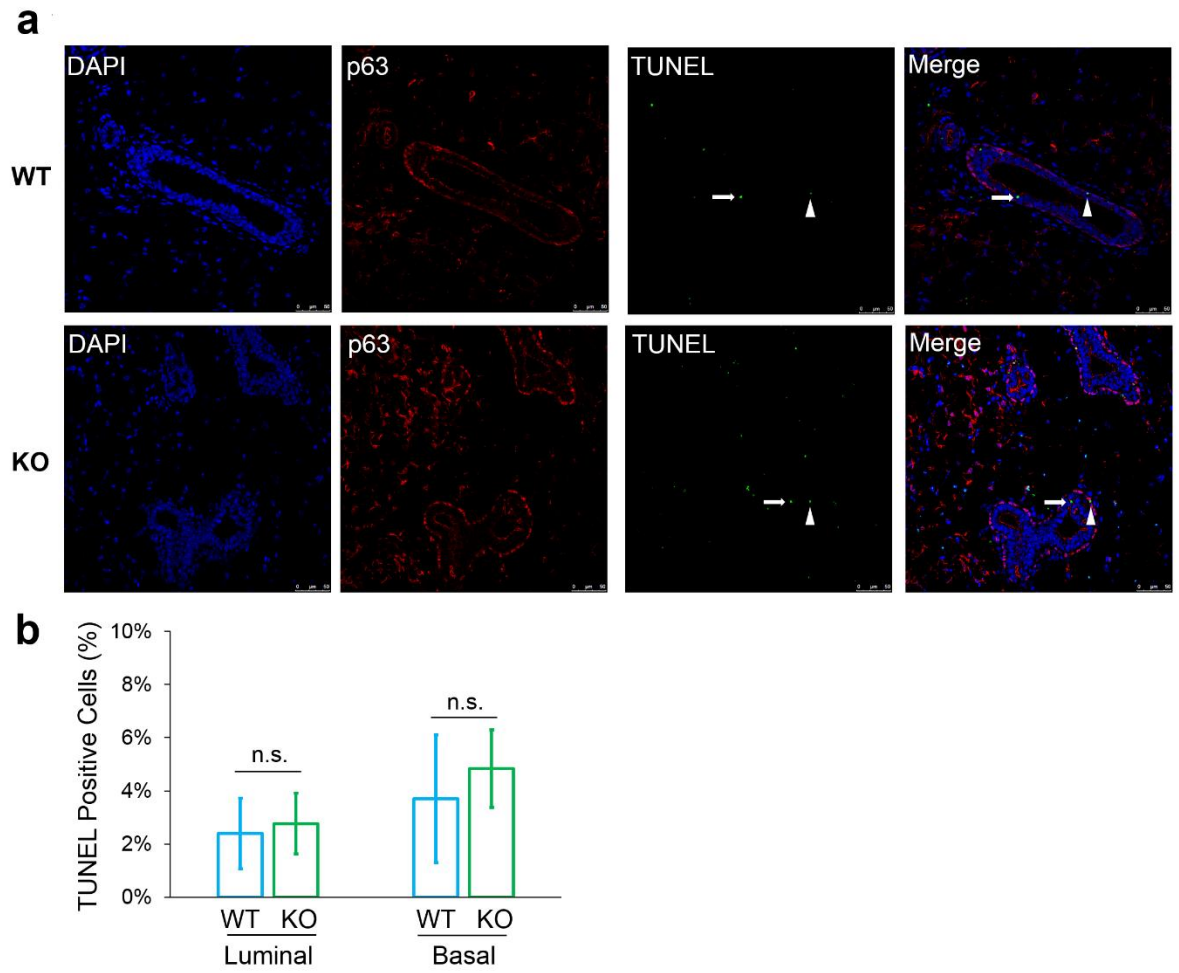
Antigen	Species	Dilution	Supplier	Cat#	Conjugate
CD45	Rat	1:250	Becton Dickinson	550994	PerCP-Cy5.5
CD24	Rat	1:250	Becton Dickinson	553261	FITC
CD49f	Rat	1:500	Becton Dickinson	555736	PE-Cy5
Sca 1	Rat	1:125	Becton Dickinson	558162	PE-Cy7

Supplementary file 2: Gating strategy used in all FACS analysis and sorting experiments



Supplementary File 3- Nucleic acid sequence for primers used in qPCR experiments

Mouse Gene ID	Accession Number	Sequence
PPIA (F)	NM_008907.1	TGTGCCAGGGTGGTGACTT
PPIA (R)		CGTTTGTGTTTGGTCCAGCAT
Estrogen Receptor (F)	NM_007956.5	GGCTGCAAGGCTTTCTTTAAGA
Estrogen Receptor (R)		GCCTTTCATCATGCCCACTT
Progesterone Receptor (F)	NM_008829.2	GGATGAAGCATCTGGCTG
Progesterone Receptor (R)		GCTGGAAGTGTCAGGCTTTGTGT
VDR (F)	NM_009504.4	GAAGCGCAAGGCCCTGTT
VDR (R)		CGCTGCACCTCCTCATCTGT
Cyp24a1 (F)	NM_009996.4	TTGAAAGCATCTGCCTTGTGT
Cyp24a1 (R)		GTCACCATCATCTTCCCAAAT
Cyp27b1 (F)	NM_010009.2	GACCTTGTGCGACGACTAA
Cyp27b1 (R)		TCTGTGTCAGGAGGGACTTCA



Supplementary file 4: The loss of *Cyp24a1* does not cause an increase in the rate of apoptotic cell death in the mammary gland. Representatives of immunofluorescence labelling of duct (**a**) from 6-week-old wild-type (WT) or *Cyp24a1* knockout (KO) mice mammary glands with TUNEL (green) and anti-p63 (red). Basal cell layers are positive for p63, whereas luminal cells are negative. Cell nuclei were stained with DAPI (blue). Arrows indicate TUNEL positive luminal cells. Arrowheads indicate TUNEL positive basal cells. Scale bar = 50 μm. Quantification of TUNEL positive cells (%) of luminal or basal cells in the ducts (**b**) of mouse mammary glands from WT or *Cyp24a1* KO mice. Student *t* tests were performed to determine statistical difference. n.s., no significance.

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CHAPTER VI

EGF-INDUCED CONFLUENCE-DEPENDENT EXPRESSION OF RANKL IN MCF10A CELLS IS ASSOCIATED WITH EPITHELIAL-MESENCHYMAL TRANSITION

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6.1 Prelude

This chapter explored the regulatory network of RANKL expression in human breast cells. Although *RANKL* is well characterized as a target gene of vitamin D in bone tissue, it becomes a different story when it comes to breast tissue. In this study, we show that majority of breast cells don't expression RANKL. Surprisingly, RANKL expression was restored in hormone receptor negative MCF10A cells after reaching confluence and was induced even higher at day 1 and day 2 post confluence. Then several potential mechanisms responsible for this change were explored.

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ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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6.2 Abstract

RANKL (ligand to receptor activator of NFκB ligand) and its receptor RANK are the key molecules in regulating osteoclast activation and differentiation. RANK/RANKL system has also been shown to play a crucial role in mammary gland development, mammary tumorigenesis and bone metastasis of various cancers, including breast cancer. However, the regulatory network in controlling expression of RANKL in breast cancer remains largely unknown. The aim of this study is to explore RANKL expression in a panel of malignant and non-malignant breast cells and unravel potential mechanisms driving RANKL expression. In this study, we show that majority of breast cells (T47D, MCF-7, MDA-MB-468, and MCF10A) do not express RANKL mRNA *in vitro* except MDA-MB-231 and MCF12A cells express certain levels of RANKL mRNA. Surprisingly, RANKL expression was restored in hormone receptor negative MCF10A cells after reaching confluence and was induced even higher at day 1 and day 2 post confluence. Moreover, confluence-dependent expression of RANKL is associated with induction of two RANKL cleavage enzymes (MMP14 and ADAM17) and suppression of RUNX2 and OPG mRNA levels. In addition, EGF-induced confluence-dependent expression of RANKL in MCF10A cells is not via the COX-2/PGE2 pathway. Rather, EGF-induced confluence-dependent expression of RANKL in MCF10A cells is associated with epithelial-mesenchymal transition (EMT), as evidenced by induction of N-cadherin, vimentin, Snail, and suppression of E-cadherin. Higher levels of RANKL mRNA and increased ratio of RANKL/OPG mRNA were found in human breast cancer samples in comparison with their adjacent normal breast tissues. Together, our findings show that EGF induces confluence-dependent expression of RANKL in MCF10A cells, which is associated with EMT. These results suggest that RANKL overexpression may contribute to initiation and progression of breast cancer.

6.3 Introduction

RANKL (ligand to receptor activator of NFκB ligand) and its receptor RANK are the key molecules in regulating osteoclast activation and differentiation [1]. Increased activation of the RANK/RANKL contributes to increased bone loss and osteoporosis, particularly in postmenopausal women. In contrast, osteoprotegerin (OPG), a decoy receptor for RANKL, functions to inhibit osteoclast activation and prevent bone loss. RANK/RANKL have also been shown to play crucial roles in mammary gland development and mammary tumorigenesis [2, 3]. Deletion of *RANK* or *RANKL* in mice results in a complete block of the development of lobuloalveolar mammary structures during pregnancy because of defective proliferation and increased apoptosis of the mammary epithelium [4]. Local rescue with recombinant RANKL can restore a functional lactating mammary gland in *RANKL* deficient females [4]. Both progesterone and prolactin are also essential for the development of a functional lactating mammary gland during pregnancy, since progesterone receptor B or prolactin receptor knockout mice not fail to develop milk secreting lobuloalveolar structures during pregnancy. These mice also exhibit decreased RANKL expression [5, 6]. A recent study shows that progesterone drives mammary secretory differentiation via RANKL-mediated induction of the transcriptional factor Elf5 [7]. Although the detailed mechanism remains largely unknown, it has become clear that RANKL is the crucial downstream molecule influencing mammary secretory differentiation in progesterone receptor signaling.

In two separate studies, to investigate whether RANK/RANKL could play a role in the development of mammary cancer, transgenic mouse model with deletion or overexpression of RANK was generated in mammary epithelial cells [3, 8]. Inactivation of RANK within the mammary epithelium from mouse mammary tumour virus (MMTV)-Cre $rank^{flox/\Delta}$ mice results in a markedly decreased incidence and delayed onset of mammary cancer in mice following treatment with the synthetic progestin medroxyprogesterone acetate (MPA).

Moreover, deletion of RANK sensitizes the mammary epithelial cells to DNA-damage-induced cell death after γ-irradiation. In contrast, transgenic overexpression of RANK driven by MMTV in the mammary gland accelerates pre-neoplasia and significantly increases mammary tumor formation in mice after the treatment with carcinogen and MPA. Taken together, it is evident that the RANK/RANKL system plays a critical role in mammary gland development and in regulating the development of carcinogen- or hormone-induced mammary cancer. Thus, blocking the RANK/RANKL signaling pathway may provide a potential therapeutic option to prevent the onset of mammary cancer.

In addition to a role in breast cancer development, RANK and RANKL are critical regulators of breast cancer metastasis to bone. An *in vitro* study shows that the immortalised non-malignant mammary epithelial MCF10A with overexpression of RANK exhibits epithelial-mesenchymal transition (EMT), increased migration, and anchorage-independent growth [9]. *In vivo* xenograft study shows RANK overexpression in the MDA-MB-231 breast cancer cell line enhances metastatic cell growth in the bone [10]. In addition, recombinant RANKL triggered migration of three stable RANK-overexpressing breast cancer cell lines (MDA-MB-231, MCF-7 and Hs578T), and the non-transformed mammary epithelial cell line MCF10A, through inducing actin polymerization [2]. More importantly, aggressive and metastatic human breast cancers express higher levels of RANKL and RANK [9]. In a recent prospective, randomised, double-blind, placebo controlled study involving 3,425 postmenopausal patients, adjuvant denosumab, a monoclonal fully human RANKL-blocking antibody, reduces the recurrence of breast cancer [11], which supports the notion that RANKL blockade could be used as a potential way to prevent breast cancer.

In this study, we analyzed RANKL expression in a panel of normal and malignant human breast cells and explored potential regulatory networks in driving RANKL expression.

RANKL, RANK, and OPG mRNA levels were also measured from human breast cancer samples (n=9) and adjacent normal tissues (n=9) to explore its clinical relevance.

6.4 Methods

6.4.1 Cell culture

Non-malignant epithelial cell lines (MCF10A and MCF12A) and breast cancer cell lines (T47D, MCF-7, MDA-MB-468, and MDA-MB-231) were obtained from the American Type Culture Collection (ATCC; Manassas). MCF10A and MCF12A cells were maintained in DMEM/F12 (Sigma–Aldrich, Saint Louis, U.S.A.) supplemented with 10% horse serum, 20 ng/mL epidermal growth factor (EGF), 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin and penicillin streptomycin l-glutamine (PSG; 0.2 U/mL penicillin, 200 μg/mL streptomycin and 5.8 μg/mL l-glutamine). MDA-MB-468 and MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma–Aldrich, Saint Louis, U.S.A.) supplemented with 10% fetal calf serum (FCS) and PSG. MCF-7 and T47D breast cancer cells were cultured in RPMI-1640 (Sigma–Aldrich, Saint Louis, U.S.A.) supplemented with 10% FCS, PSG, and 10 μg/mL insulin. MCF10A cells were treated with 10% horse serum, 10% charcoal-stripped horse serum or 10% FCS and collected at sub-confluence (Sub-con; less than 100% confluence), 100% confluence (Con), and day 1 (Post-d1) and day 2 (Post-d2) after reaching confluence. MCF10A cells were treated with vehicle control (CTRL), 20 ng/mL or 100 ng/mL of EGF and collected at Post-d1.

6.4.2 Patient samples

Breast mastectomy specimens (n=9) and adjacent normal tissue (n=9) were obtained from patients undergoing treatment for invasive breast cancer at the Royal Adelaide Hospital from 2013 to 2015. This study was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital and the procedures used were in accordance with institutional and NHMRC guidelines.

6.4.3 Quantitative RT-PCR analysis

Cells or tissues were collected with total RNA extracted using the RNeasy mini kit (Qiagen). RNA extracted from cell lines (1 μg) or breast samples (100 ng) was reverse transcribed to cDNA using MMLV-RT (Promega) and random hexamers (Promega). Quantitative RT-PCR (qRT-PCR) was carried out on a CFX Connect™ real-time instrument using the iQ5 SYBR Green detection system (Bio-Rad). Primer sequences and amplification conditions are shown in **Table 1**. Transcript expression was compared between different groups and normalized to Peptidylpolylisomerase G (*PPIG*). All measurements were performed in triplicate.

6.4.4 Western blot

Western blot analysis was performed as previously described [12]. Whole protein lysates (20 μg) were resolved using SDS PAGE electrophoresis, and probed overnight at 4 °C with the following primary antibodies RANKL (1:200; 70525, Sapphire Bioscience) and β-actin (1:1000, AC-15, Sigma).

6.4.5 Data analysis and statistical methods

Statistical significance was determined by unpaired or paired Student's *t*-test, Wilcoxon signed-rank tests, or one-way ANOVA followed by Tukey's post-hoc multiple comparison tests as appropriate. A P value < 0.05 was considered significant. All statistical calculations were performed using GraphPad Prism software (San Diego, CA).

6.5 Results

6.5.1 Increased RANKL mRNA levels and RANKL/OPG mRNA ratio in breast cancer as compared with matched adjacent normal tissue

To investigate the potential clinical relevance of the RANKL/RANK/OPG system in human breast cancer, we analyzed RANKL, RANK, and OPG mRNA expression levels in tissue biopsies of human breast cancer (n=9) and the matched adjacent normal breast tissue (n=9). RANKL mRNA levels are significantly increased in breast cancer as compared with the matched adjacent normal tissue (7-fold) (**Figure 1A**), while RANK mRNA levels are unchanged (**Figure 1B**). Moreover, RANKL/OPG mRNA ratio is also significantly elevated in human breast cancer as compared to the matched adjacent normal tissue (4.5-fold) (**Figure 1C**). These data suggest that disruption of RANKL/RANK/OPG system may contribute to breast cancer development.

6.5.2 Restoration of RANKL protein expression in hormone receptor negative MCF10A cells

Basal RANKL mRNA expression levels were investigated in a panel of malignant and non-malignant breast cell lines. T47D, MCF-7, MDA-MB-468, and MCF10A cells did not express detectable RANKL mRNA, while RANKL mRNA was detectable in MDA-MB-231 and MCF12A cells at sub-confluent states (**Figure 2A**). It has been shown that RANKL is the downstream target of progesterone and mediates progesterone-driven proliferation of mammary epithelium in the human breast [8]. However, treatment of PR^+ breast cancer cell lines T47D and MCF-7 with synthetic progestin R5020 did not induce RANKL protein expression, indicating that genes modulated by hormone signaling in these cell lines differ from target genes identified in mice *in vivo* [13]. Surprisingly, expression of RANKL mRNA (**Figure 2B**) and protein (**Figure 2C**) can be induced in the non-malignant epithelia MCF10A cells grown to confluence. The levels of RANKL protein continued to increase at day 1 (Post-

d1) and day 2 (Post-d2) post confluence. We speculated that tight cell contact may contribute to the expression of RANKL in MCF10A. Since MCF10A cells do not express hormone receptors, either ER or PR, other factors within media, rather than progesterone, may involve in regulating the expression of RANKL. Interestingly, culture media with horse serum or charcoal-stripped horse serum, but not with fetal calf serum induce the RANKL expression in MCF10A cells following confluence (**Figure 2D**). But other breast cancer cells like T47D, MCF-7, MDA-MB-468, and MDA-MB-231 did not grow well in culture media containing horse serum, making it difficult to study RANKL expression profile in these cell lines at confluence.

6.5.3 RANKL expression is associated with induction of two RANKL cleavage enzymes and suppression of OPG and RUNX2 mRNA

There are two forms of RANKL, transmembraneous and soluble. The extracellular portion of transmembraneous RANKL can be cleaved by MMP14 and ADAM17 to produce soluble RANKL [14]. MMP14 and ADAM17 mRNA levels were increased following confluence of MCF10A cells (**Figure 3A and 3B**), which is positively correlated with changes of RANKL levels, indicating that soluble RANKL levels may be also increased in MCF10A cells at confluence and over-confluent states. OPG is a decoy receptor for RANKL. A decrease in OPG levels can result in increased RANKL activity. OPG mRNA expression levels were markedly decreased in MCF10A cells at over-confluent states (Post-d1 and Post-d2) (**Figure 3C**), where RANKL levels were significantly increased.

Previous studies have shown that siRNA-mediated knockdown of RUNX2 expression increases the steady-state RANKL gene expression in osteoblastic cells [15, 16]. Consistent with these results, RUNX2 mRNA expression levels were reduced in MCF10A cells after reaching confluence, which may partly contribute to increased RANKL levels (**Figure 3D**). It

is possible media influence RUNX2 expression, but the precise mechanisms of RUNX2-mediated regulation of RANKL in MCF10A cells are yet to be defined.

6.5.4 EGF-induced confluence-dependent expression of RANKL in MCF10A was associated with epithelial-mesenchymal transition (EMT)

EGF is a major factor driving the continuous growth of MCF10A cells. To test whether EGF could influence RANKL expression, MCF10A cells were cultured with media containing vehicle control (CTRL), 20 ng/mL or 100 ng/mL of EGF and collected at day 1 after confluence (Post-d1). RANKL mRNA levels were higher when MCF10A cells were cultured with 100 ng/mL of EGF as compared to 20 ng/mL (**Figure 4A**). EGF is known to mainly regulate OPG, but not RANKL, expression in osteoblastic cells [18], which is consistent with our finding that RANKL is not induced in MCF10A cells following the treatment with EGF when cells are at a sub-confluent state (**Figure 4B**). Thus, the increased RANKL mRNA levels may be primarily due to EGF driving overgrowth of cells, causing tighter cell contacts.

Previous studies show that the COX-2/PGE2 pathway is involved in driving RANKL expression in human osteoblasts [17, 18]. We tested whether EGF induced confluence-dependent expression of RANKL in MCF10A cells was also through the COX-2/PGE2 pathway. COX-2 mRNA levels remain unchanged in MCF10A cells following treatment with various levels of EGF, indicating that the COX-2/PGE2 pathway is unlikely to mediate the EGF-induced confluence-dependent expression of RANKL in MCF10A cells (**Figure 4B**).

It has been established that high levels of RANK induces gene expression changes typical of epithelial-mesenchymal transition (EMT) [9]. We further tested whether EGF induced confluence-dependent expression of RANKL is associated with changes of EMT markers. E-cadherin, N-cadherin, vimentin, and Snail mRNA levels were compared following treatment with control, 20 ng/mL, or 100 ng/mL of EGF. EGF-induced confluence-dependent

expression of RANKL was associated with suppression of E-cadherin by 64% and induction of N-cadherin (2-fold), vimentin (3-fold) and Snail (18-fold) with 20 ng/mL of EGF compared to control (**Figure 4C**). Snail mRNA expression levels were even higher (24-fold) when over-confluent MCF10A cells were treated with 100 ng/mL of EGF (**Figure 4C**).

6.6 Discussion

In the present study, we demonstrated that RANKL expression is increased in MCF10A cells grown to confluence. In particular, we found that the mRNA levels of two RANKL cleavage enzymes MMP14 and ADAM17 were positively correlated with RANKL levels, indicating that cleavage of transmembranous RANKL was likely to release the soluble form. In contrast, RUNX2 and OPG expression levels decreased significantly with confluence, which may contribute to increased RANKL levels. In addition, we demonstrated that EGF is not able to induce RANKL expression in MCF10A cells at sub-confluent states, but increased RANKL expression in a concentration-dependent manner at confluence, indicating EGF is partly contributing to increased expression of RANKL. Furthermore, EGF-induced confluence-dependent expression of RANKL is associated with EMT, as evidenced by induction of N-cadherin, vimentin, Snail, and suppression of E-cadherin.

A previous study has suggested that cell-to-cell contact between MDA-MB-231 cells and osteoblasts induces the expression of RANKL on the surface of osteoblasts, indicating tight contact between cells can cause the expression of RANKL [19]. RANKL mRNA levels were undetectable in MCF10A cells at sub-confluence, but significantly induced following confluence, suggesting cell-to-cell contact may contribute to the expression RANKL in MCF10A cells. We further explored whether other factors within the culture media, other than cell-to-cell contacts, contribute to expression of RANKL. Fetal calf serum and horse serum differ in terms of total proteins, IgG, and endotoxin levels [20]. We found that there was no induction on RANKL expression in media with fetal calf serum even at confluence, indicating that fetal calf serum may contain some inhibitory factors, or lack promoting factors, in driving RANKL expression. The nature of these features is currently unknown.

EGF is a major factor driving the continuous growth of MCF10A cells. We further found that EGF induces RANKL expression in a concentration-dependent manner in confluent MCF10A cells. The induced RANKL mRNA levels may be partly due to overgrowth of cells driven by EGF, causing tighter contact across cells. We further explored other potential pathways that mediate EGF-induced RANKL expression. It has been reported that activation of EGFR signaling by EGF leads to induction of COX-2 transcription, which further results in enhanced production of Prostaglandin E2 (PGE2) [21]. Another study shows that PGE2 could induce RANKL in osteoblasts [17]. Thus, we hypothesize that EGF could induce the RANKL via the COX-2/PGE2 pathway in confluent MCF10A cells. However, COX-2 mRNA levels exhibit no change with various concentrations of EGF, indicating it is likely that the regulatory network in driving RANKL expression is different between MCF10A cells and osteoblasts. Therefore, further studies are required to explore other potential pathways that mediate EGF-induced RANKL expression.

It has been reported that EGF could upregulate the expression of Snail, vimentin, and fibronectin, while suppressing E-cadherin expression in MCF-7 cells via the phospho-Smad2/3 signaling pathway [22]. Here we show that RANKL expression induced by EGF in confluent MCF10A cells is also associated with induction of N-cadherin, vimentin, and Snail, and suppression of E-cadherin. An association between RANK expression and EMT has been previously reported in MCF10A cells [9]. Collectively, these data support a potential alternative pathway where EGF induces EMT through the RANKL/RANK system, which requires further investigation.

We would also like to emphasize that our observations are based on the specific cells and proteins elevated in our experimental conditions, and are not necessarily applicable to other cell types and proteins. In addition, tight junctions between cells growing at post-confluence may prevent binding of inhibitory factors to the cell surface, resulting in the upregulation of

RANKL. However, the potential contributors secreted from cells at post-confluence cannot be excluded [23]. Thus EGF is the only one factor, which has been identified to contribute to enhanced RANKL expression when MCF10A cells are at confluent or over-confluent states.

Our study demonstrates that primary breast cancer express higher RANKL mRNA levels than adjacent normal tissue and ratio of RANKL to OPG was increased in tumor, which is consistent with a previous study showing high RANKL expression levels are found in aggressive and metastatic breast adenocarcinomas [9]. It has been described that RANKL/OPG ratio was significantly increased in bone metastasis from other primary origins and correlated with markers of bone resorption, osteolytic lesions, and markers of disease activity [24]. These data provide a rationale for further investigation of anti-RANKL antibody such as denosumab for breast cancer prevention and progression.

In summary, we show that RANKL can be restored in the immortalised human mammary epithelial MCF10A cells with negative hormone receptor at confluent state. EGF in serum contributes to confluence-dependent expression of RANKL, which is associated with EMT. Increased levels of RANKL in tumor samples suggest that RANKL may promote tumor initiation, progression, and metastasis in breast cancer.

Table 1

Primers for qRT-PCR

Gene symbols	Primers	Accession Number	Annealing Temperature	Products(bp)
PPIG	Forward: 5'-CAGATGCAGCTAGCAAACCGTTTG-3'	NM_004792.2	60℃	200
	Reverse: 5'-CTCTTCAGTAGCACTTTCGGAATCAGAGG-3'			
RANKL	Forward: 5'-TCAGCCTTTTGCTCATCTCACTAT-3'	NM_003701.3	60℃	94
	Reverse: 5'-CCAACCCCGATCATGGT-3'			
MMP14	Forward: 5'-CAACACTGCCTACGAGAGGA-3'	NM_004995.3	60℃	380
	Reverse: 5'-GTTCTACCTTCAGCTTCTGG-3'			
ADAM17	Forward: 5'-GCACAGGTAATAGCAGTGAGTGC-3'	NM_003183.5	60℃	440
	Reverse: 5'-CACACAATGGACAAGAATGCTG-3'			
RUNX2	Forward: 5'-CGGCCCTCCCTGAACTCT-3'	NM_001024630.3	60℃	75
	Reverse: 5'-TGCCTGCCTGGGGTCTGTA-3'			
OPG	Forward: 5'-TTCCGGAAACAGTGAATCAA-3'	NM_002546.3	57℃	287
	Reverse: 5'-CGCTGTTTTCACAGAGGTCA-3'			
RANK	Forward: 5'-ATGCGGTTTGCAGTTCTTCTC-3'	NM_003839.3	57℃	216
	Reverse: 5'-ACTCCTTATCTCCACTTAGG-3'			
E-CADHERIN	Forward: 5'-GGTGCTCTTCCAGGAACCTC-3'	NM_004360.4	61℃	195
	Reverse: 5'-GAAACTCTCTCGGTCCAGCC-3'			
N-CADHERIN	Forward: 5'-CAACTTGCCAGAAAACTCCAGG-3'	NM_001792.4	61℃	205
	Reverse: 5'- ATGAAACCGGGCTATCTGCTC-3'			
VIMENTIN	Forward: 5'-CTCCGGGAGAAATTGCAGGA-3'	NM_003380.3	61℃	111
	Reverse: 5'-TTCAAGGTCAAGACGTGCCA-3'			
SNAIL	Forward: 5'-CGCGCTCTTTCCTCGTCAG-3'	NM_005985.3	61℃	181
	Reverse: 5'-TCCCAGATGAGCATTGGCAG-3'			
COX-2	Forward: 5'-GCTTTATGCTGAAGCCCTATGA-3'	NM_000963.3	60℃	70
	Reverse: 5'-TCCAACTCTGCAGACATTTCC-3'			

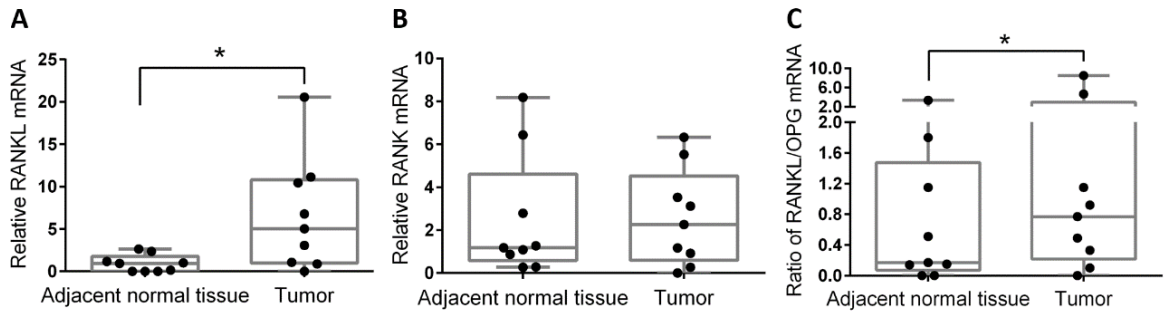

Figure 1. RANKL, RANK mRNA levels, and RANKL/OPG mRNA ratio in human breast cancer and matched adjacent normal tissue. qRT-PCR measurements of expression levels of RANKL (**A**), RANK (**B**) and OPG mRNA were made in human breast cancer (n=9) and matched adjacent normal breast tissue (n=9). Expression data were normalized against the housekeeping gene *PPIG*. Ratio of RANKL to OPG was calculated in each sample (**C**). Data were analyzed by paired Student's *t*-test or two-tailed Wilcoxon rank sum test (**P* <0.05).

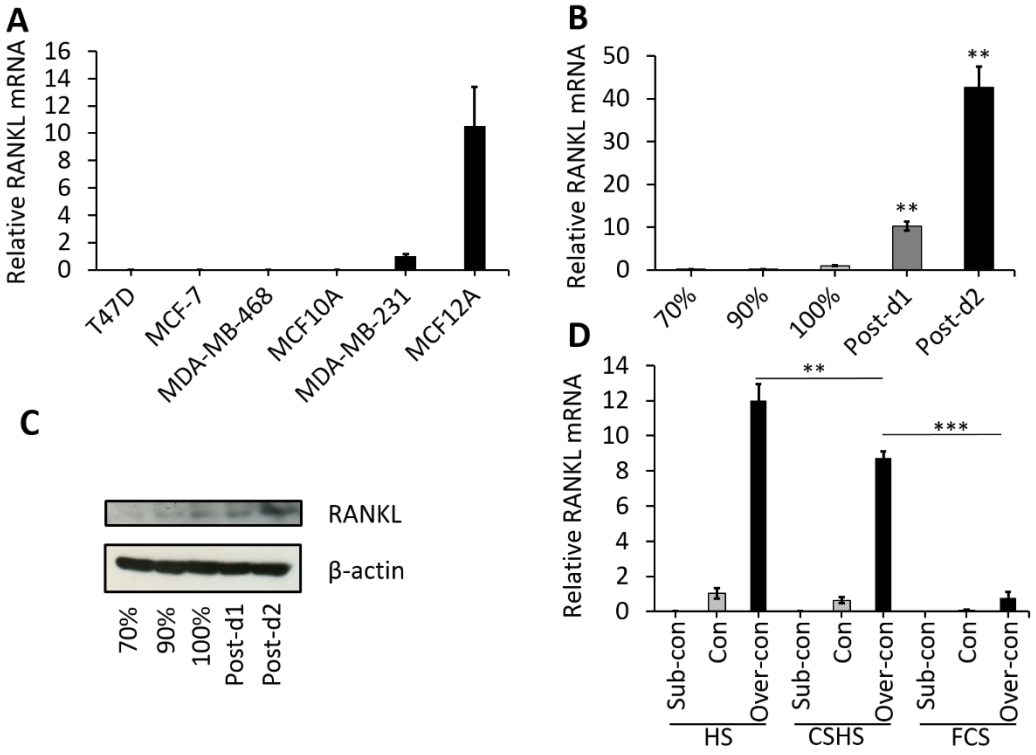

Figure 2. Confluence-dependent expression of RANKL in MCF10A cells. **A**: basal expression of RANKL mRNA in malignant breast cells (T47D, MCF-7, MDA-MB-468, and MDA-MB-231) and non-malignant breast cells (MCF10A and MCF12A) at sub-confluent state. RANKL mRNA (**B**) and protein (**C**) levels were measured at 70%, 90%, 100% confluence, and day 1 (Post-d1) and day 2 (Post-d2) after 100% confluence of MCF10A cells. **D**: MCF10A cells were cultured with 10% horse serum (HS), 10% charcoal-stripped horse serum (CSHS) or 10% fetal calf serum (FCS). MCF10A cells were then collected and RANKL mRNA levels were measured at sub-confluent (Sub-con), confluent (Con), and over-confluent (Over-con) states. Data were normalized against *PPIG*. Each bar represents mean ± standard deviation of three independent samples. Statistical significance was assessed by one-way ANOVA followed by Tukey's multiple comparison tests (**P-value < 0.01 and ***P-value < 0.001).

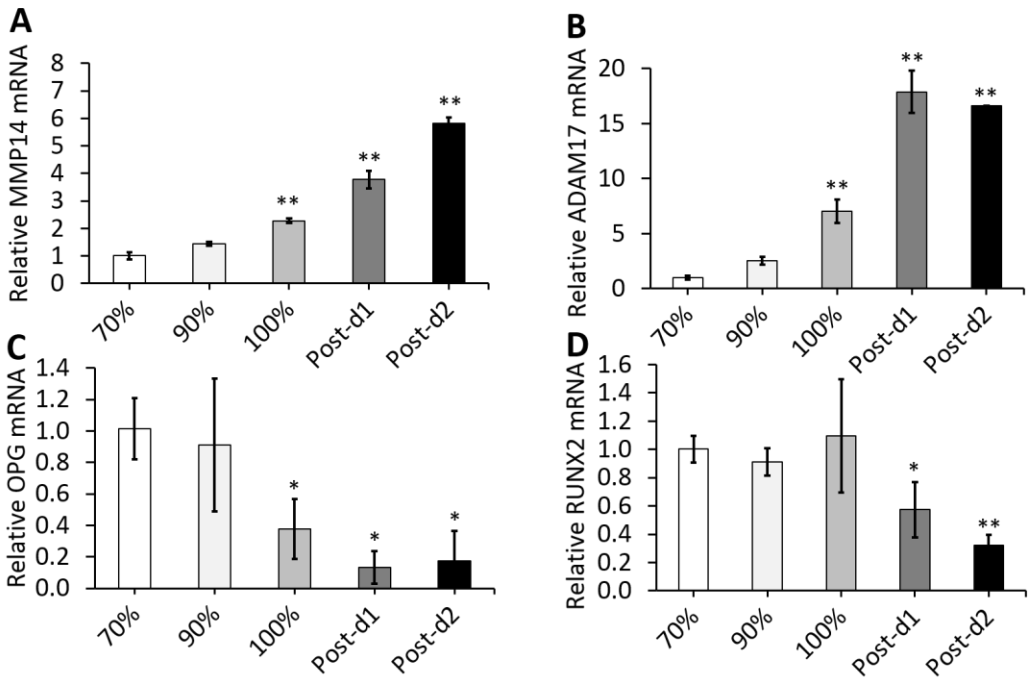

Figure 3. MMP14 (**A**), ADAM17 (**B**), OPG (**C**), and RUNX2 (**D**) mRNA levels in MCF10A at different states of confluence. MCF10A cells were collected at 70%, 90%, 100%, day 1 (Post-d1), and day 2 (Post-d2) after confluence. Data were normalized against *PPIG* and expressed relative to values for MCF10A cells at 70% confluence which was set to 1. Mean values ± SD of triplicates are shown to represent the fold change of gene expression across various confluent states. Statistical significance was assessed by student *t*-tests (*$P < 0.05$ and **$P < 0.01$).

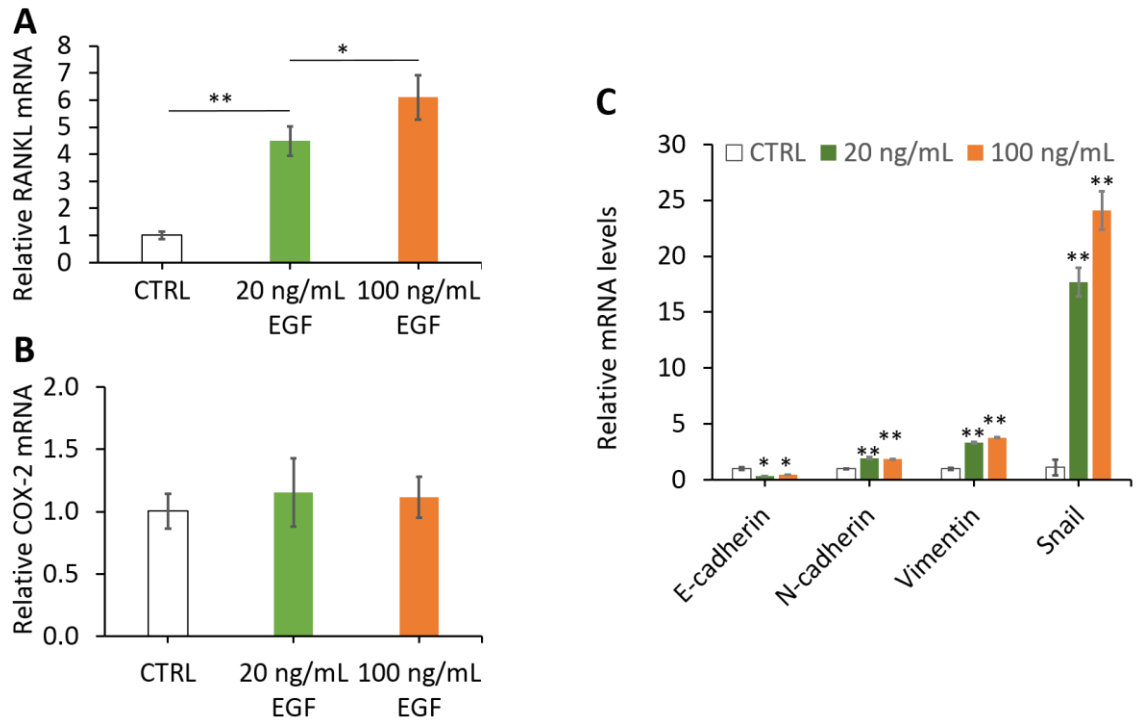

Figure 4. EGF-induced confluence-dependent expression of RANKL in MCF10A was associated with epithelial-mesenchymal transition (EMT). qRT-PCR analyses of RANKL (A), COX-2(B), and EMT markers(C)(E-cadherin, N-cadherin, Vimentin, and Snail) mRNA expression were made in MCF10A cells treated with control (CTRL), 20 ng/mL or 100 ng/mL epidermal growth factor (EGF) and collected at the post day 1 of confluence. Mean values ± SD of triplicates are shown to represent the fold change of gene expression across various treatments. Statistical significance was assessed by one-way ANOVA followed by Tukey's multiple comparison tests (*$P < 0.05$ and **$P < 0.01$).

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FINAL CONCLUSION

Accumulating epidemiological and clinical evidence suggest that sufficient vitamin D is associated with decreased breast cancer incidence and better clinical outcomes in patients with breast cancer. However, the underlying mechanism of how vitamin D functions as a tumor suppressor role is largely unknown. In this thesis, two experimental models, namely an *ex vivo* explant system and a transgenic mouse model, were used to explore the underlying mechanism and molecular pathways of vitamin D in the prevention of breast cancer.

The first half of the thesis investigated vitamin D target genes and molecular pathways in *ex vivo* explants of human breast tissue. Of particular interest, *CLMN*, *SERPINB1*, *EFTUD1*, and *KLK6* were found as direct up-regulated targets of 1,25D, and these genes have reported functions consistent with roles as tumor suppressors. These tumor suppressor roles are further supported by analysis of publically available breast cancer cohorts where high expression levels, particularly, of *CLMN*, *SERPINB1*, and *KLK6*, are associated with prolonged relapse-free survival for breast cancer patients.

Moreover, evidence from randomized clinical trials was summarized to investigate whether vitamin D, plus or minus calcium supplementation, has the potential to reduce the incidence of breast cancer. Our analyses indicate that the available data are insufficient to confirm any protective effect of vitamin D supplementation, with or without calcium, on the risk of breast cancer. These clinical studies are impacted by limited numbers of participants with high prevalence of vitamin D insufficiency, relatively short period of post-intervention follow-up, and dose inadequacy, thus more RCTs are required to make a conclusive and reliable conclusion. It is preferable that future RCTs would recruit people with higher prevalence of vitamin D deficiency and prescribe them with an adequate dosage of vitamin D.

The second part of thesis mainly focused on characterizing the role of vitamin D-inactivating enzyme Cyp24a1 in mammary gland development and discussed its potential role in breast cancer development. A novel mouse model was available with conditional knockout of the *Cyp24a1* gene specifically in the mammary epithelium (MMTV-Cre x Cyp24a1$^{lox/lox}$). Our major finding is that mammary glands from virgin *Cyp24a1* knockout females display impaired ductal morphogenesis compared with age- and weight-matched wild-type mice, and this is due to reduced proliferation of mammary epithelial cells in the absence of Cyp24a1.

Additionally, the final chapter explored the regulatory network of RANKL expression in human breast cells. We show that majority of breast cells don't expression RANKL. Surprisingly, RANKL expression is restored in hormone receptor negative MCF10A cells after reaching confluence. EGF was identified as a factor contributing to confluence-dependent expression of RANKL in MCF10A cells.

In conclusion, our findings definitely contribute to a better understanding of the role of vitamin D signaling pathway in the prevention of breast cancer. Moreover, our study identifies Cyp24a1 as a potential preventative and/or therapeutic target for the management of breast cancer.