Suppression of Mammary Tumorigenesis by

a Gemini Vitamin D Analog and a Synthetic Triterpenoid

By

JAE YOUNG SO

A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

And

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Joint Graduate Program of Cellular and Molecular Pharmacology

written under the direction of

Professor Nanjoo Suh

and approved by

_____

_____

_____

_____

_____

New Brunswick, New Jersey

January 2013

ABSTRACT OF THE DISSERTATION

Suppression of Mammary Tumorigenesis by

a Gemini Vitamin D Analog and a Synthetic Triterpenoid

By: Jae Young So

Dissertation Director: Professor Nanjoo Suh

Breast cancer is a heterogeneous disease categorized into multiple subtypes, including luminal, HER2-positive and basal-like subtypes, which exhibit distinct gene signatures and clinical outcomes. Basal-like breast cancer has the worst prognosis among these subtypes and has no clinically approved targeted therapy. While HER2-targeting therapy with a humanized HER2 monoclonal antibody markedly improved the prognosis of HER2-positive breast cancer, the *de novo* and acquired resistance against the antibody has emerged as a new challenge for patients with HER2-positive breast cancer.

MCF10 cell lines, a human breast cancer progression model representing the basal-like breast cancer subtype, were employed to identify key proteins involved in different stages of mammary tumorigenesis. Increased levels of IGF-IR, cyclin D1 and c-Myc were associated with HRAS-driven transformation. Higher levels of pErk, pAkt, STAT3 and Pak4 contribute to tumorigenicity *in vivo*, whereas CD44, HER2, COX-2 and Smad4 may be involved in the breast cancer progression.

The MCF10DCIS.com cells, one of the MCF10 cell lines, highly express a breast cancer stem cell marker, CD44. A Gemini vitamin D analog BXL0124 markedly repressed the CD44

protein level and the growth of MCF10DCIS.com xenograft tumors. CD44 overexpression was correlated with invasive phenotype in MCF10DCIS.com cells, and the repression of CD44 by BXL0124 contributed to the inhibition of cell invasion. STAT3, which interacts directly with CD44, was identified as a key downstream signaling molecule affected by BXL0124 in MCF10DCIS.com cells. The CD44 knockdown study supported the critical role of CD44-STAT3 signaling in the invasive potential of MCF10DCIS.com cells *in vitro* and *in vivo*.

The anti-cancer effects of BXL0124 and a synthetic triterpenoid CDDO-Im on HER2-positive breast cancer were tested in MMTV-HER2/neu transgenic mice. BXL0124, CDDO-Im and their combination delayed the development of mammary tumors and markedly inhibited the activation of HER2 and EGFR as well as their downstream molecules, such as Erk, Src and c-Myc in MMTV-HER2/neu mammary tumors.

In conclusion, we demonstrated therapeutic potential of Gemini vitamin D analog BXL0124 targeting CD44-STAT3 signaling in basal-like breast cancer. In addition, we found anti-cancer activities of BXL0124 and CDDO-Im in HER2-positive breast cancer and potentially additive effects of their combination.

ACKNOWLEDGMENT

First of all, I would like to express the deepest appreciation to my supervisor, Dr. Nanjoo Suh. She has been a great role model as a dedicated scientist with great ideas. She also has been a great mentor with timely guidance and support.

I would like to thank Dr. Audrey Minden, Dr. Fang Liu, Dr. Li Cai and Dr. George P. Studzinski for serving on my thesis committee and for their valuable advice through the preparation of my thesis.

I thank all the past and present members of Dr. Nanjoo Suh's laboratory. Especially, Dr. Hong Jin Lee taught all the experimental techniques and helped me develop ideas. Amanda Smolarek has been a great and pleasant partner to work with during my graduate study.

Also, I would like to thank all the members of the Susan Lehman Cullman Laboratory for Cancer Research. Especially the staffs in the office, Bobbie Busch, Erica DiPaola and Deborah Stalling, have been helpful for me to solve all the big and small problems in the laboratory.

I would like to thank all the friends whom I met during my graduate study. Especially, Dr. Jong Hun Lee has been my close friend over the past several years.

Last but not least, I would like to thank my parents, Myoung Hwan So and Young Sook Lee, and my brother, Jae Won So, in Korea. They have always been invaluable supporters of my life.

TABLE OF CONTENTS

Abstract of the Dissertation ii

Acknowledgment iv

Table of Contents v

List of Tables xi

List of Figures xii

List of Abbreviations xv

Chapter 1: Introduction 1

1.1 Breast cancer 1

1.1.1 Luminal breast cancer and therapies 1

1.1.2 HER2-positive breast cancer and therapies 3

1.1.3 Basal-like breast cancer and therapies 4

1.2. The preclinical models of breast cancer 5

1.2.1 Human breast cancer cell lines 6

1.2.2 Xenograft animal models 7

1.2.3 Carcinogen-induced mammary tumor models 7

1.2.4 Genetically engineered mouse (GEM) models 8

1.3 Molecular targets of breast cancer 10

1.3.1 Estrogen receptor (ER) signaling 10

1.3.2 Human epidermal growth factor receptor (HER) family receptors 10

1.3.3 BRCA1 and BRCA2 11

1.3.4 Phosphoinositide 3-kinase (PI3K) 11
1.3.5 Insulin-like growth factor-I receptor (IGF-IR) 12
1.3.6 Nuclear factor of κB (NF-κB) 13
1.3.7 Signal transducer and activator of transcription 3 (STAT3) 13
1.3.8 Vascular endothelial growth factor (VEGF) 14
1.4 Vitamin D 15
1.4.1 Biological actions of vitamin D 15
1.4.2 Vitamin D and breast cancer 17
1.4.3 *In vitro* and *in vivo* studies of vitamin D and vitamin D analogs in breast cancer 17
1.4.4 Clinical studies of vitamin D in breast cancer 20
1.5 Synthetic oleanane triterpenoids (SOs) 20
1.5.1 Biological actions of SOs 21
1.5.2 *In vitro* and *in vivo* studies of synthetic triterpenoids in breast cancer 23
1.5.3 Clinical studies of synthetic triterpenoids 24
Chapter 2: Differential expression of key signaling proteins in MCF10 cell lines, a human breast cancer progression model 37
2.1 Introduction 37
2.2 The MCF10 human breast cancer progression model 38
2.3 Materials and methods 39
2.3.1 Cell culture 39
2.3.2 Animal experiments in the xenograft model 39
2.3.3 Western blot analysis 40
2.4 Tumorigenicity of MCF10 cell lines in vivo 40
2.5 Key factors in the HRAS-driven initiation stage of breast cancer 41
2.6 Key signaling proteins in malignant transformation of the MCF10 model by spontaneous mutagenesis 42
2.7 Key proteins contributing to breast cancer progression in the MCF10 model 43

2.8 Breast cancer invasion and metastasis 45

2.9 Conclusion 46

Chapter 3: Repression of the expression of a stem cell marker CD44 by a novel Gemini vitamin D analog in basal-like breast cancer 51

3.1. Introduction 51

3.2. Material and methods 52

3.2.1. Reagents and cell culture 52

3.2.2. Quantitative real-time polymerase chain reaction (PCR) 53

3.2.3. Transient transfection of CD44 and promoter assay 53

3.2.4. Western blot analysis 53

3.2.5. Fluorescence microscopy 54

3.2.6. Animal experiments in the xenograft model 54

3.2.7 Analysis of serum calcium level 55

3.2.8 Immunohistochemistry and quantification 55

3.2.9 Flow cytometry 55

3.2.10 Knockdown of VDR by siRNA 56

3.2.11 Statistical analysis 56

3.3 Results 56

3.3.1 Inhibition of MCF10DCIS.com xenograft mammary tumor growth by Gemini vitamin D analog *in vivo*. 56

3.3.2 The expression of CD44 in MCF10DCIS.com xenograft mammary tumors during tumor growth. 57

3.3.3 Repression CD44 by BXL0124 in MCF10DCIS.com xenograft tumors *in vivo*. 58

3.3.4 Reduction of the level of CD44 protein by BXL0124 in MCF10DCIS.com cells in a VDR-dependent manner. 59

3.3.5 Suppression of CD44 mRNA with induction of osteopontin mRNA by BXL0124 in MCF10DCIS.com breast cancer cells. 60

3.3.6 Repression of the transactivation of CD44 promoter by BXL0124 in MCF10DCIS.com cells in a p53 dependent manner. 61

3.4 Discussion 61

3.5 Conclusion 63

Chapter 4: Inhibition of cell invasion with targeting CD44-STAT3 signaling by a Gemini vitamin D analog in basal-like breast cancer 77

4.1. Introduction 77

4.2. Materials and methods 78

4.2.1. Reagents and cell culture 78

4.2.2. [^{3}H] thymidine incorporation assay 79

4.2.3. MTT assay 79

4.2.3. Cancer cell invasion assays 79

4.2.4. Quantitative real-time PCR 80

4.2.5. Western blot analysis 81

4.2.6. Knockdown of VDR by siRNA 81

4.2.7. STAT3 DNA binding assay 81

4.2.8. Fluorescence microscopy 82

4.2.9. Immunoprecipitation 82

4.2.10. Xenograft tumor study 83

4.2.11. Statistical analysis 83

4.3. Results 83

4.3.1 Inhibition of cell proliferation, metabolic activity and invasion by 1α,25$(OH)_2D_3$ and Gemini vitamin D analog BXL0124 in MCF10DCIS.com cells. 83

4.3.2. Repression of the level of invasion markers and STAT3 signaling by in MCF10DCIS.com cells. 84

4.3.3. Inhibition of STAT3 signaling by reducing the complex formation of CD44, STAT3 and JAK2 with BXL0124. 85

4.3.4. Suppression of MMP-9, MMP-14 and uPA mRNA as well as invasion of MCF10DCIS.com cells by CD44 knockdown. 86

4.3.5. Inhibition of tumor growth and burden as well as invasion markers in MCF10DCIS.com xenograft tumors by CD44 knockdown. 87

4.3.6. Repression of CD44 and pSTAT3 in MCF10CA1a and MDA-MB-468 basal-like breast cancer cells. 88

4.4. Discussion 88

4.5 Conclusion 91

Chapter 5: Inhibition of tumorigenesis by a Gemini vitamin D analog and a synthetic triterpenoid in the MMTV-HER2/neu transgenic mouse model 107

5.1. Introduction 107

5.2 Materials and methods 108

5.2.1 Reagents 108

5.2.2 Transgenic mice and treatment 109

5.2.3 Western blot analysis 109

5.2.4 Fluorescence microscopy 110

5.2.5 Quantitative real-time polymerase chain reaction 110

5.2.6 Statistical analysis 110

5.3. Results 111

5.3.1. Delayed development of mammary tumors by BXL0124, CDDO-Im and the combination of BXL0124 and CDDO-Im in MMTV-HER2/neu transgenic mice. 111

5.3.2 Inhibition of the activation of the HER2/EGFR/Erk signaling pathway by BXL0124, CDDO-Im and the combination in mammary tumors of MMTV-HER2/neu transgenic mice. 112

5.3.3 Down-regulation of the downstream of the HER2 signaling pathway by BXL0124, CDDO-Im and the combination in mammary tumor of MMTV-HER2/neu transgenic mice. 112

5.3.4 Repression of HER2 activation by BXL0124, CDDO-Im and the combination at the invading edge of mammary tumors of MMTV-HER2/neu transgenic mice. 113

5.4 Discussion 114
5.5 Conclusion 115
Conclusion 124
References 125

List of Tables

Table 1.1 Key features in different subtypes of breast cancer 25

Table 1.2 *In vitro* studies of vitamin D and vitamin D analogs in breast cancer 26

Table 1.3 *In vivo* studies of vitamin D and vitamin D analogs in breast cancer 29

Table 1.4 Clinical trials of vitamin D and vitamin D analogs in breast cancer 30

Table 1.5 Case-control studies of vitamin D in breast cancer..... 31

Table 1.6 Nested case-control studies of vitamin D in breast cancer 32

Table 1.7 *In vitro* studies of synthetic triterpenoids in breast cancer..... 33

Table 1.8 *In vivo* studies of synthetic triterpenoids in breast cancer 34

Table 2.1 Protein and gene profiling studies using the MCF10 breast cancer progression model 48

List of Figures

Fig. 1.1 Structures of 1α,25$(OH)_2D_3$ and Gemini vitamin D analog BXL0124 35

Fig. 1.2 Structures of Oleanolic acid, CDDO and CDDO derivatives..... 36

Fig. 2.1 Comparison of xenograft tumor growth of MCF10 series of human breast cancer cells . 49

Fig. 2.2 Comparison of the expression level for key signaling molecules among MCF10A, MCF10AT1, MCF10DCIS.com and MCF10CA1a cell lines..... 50

Fig. 3.1 Repression of the growth of MCF10DCIS.com xenograft tumors by intraperitoneal injection of BXL0124 in SCID mice 65

Fig. 3.2 Repression of the growth of MCF10DCIS.com xenograft tumors by oral administration of BXL0124 in nu/nu mice 66

Fig. 3.3 Repression of the growth of MCF10DCIS.com xenograft tumors by oral administration of BXL0124 in SCID mice 67

Fig. 3.4 Expression of CD44 during the growth of MCF10DCIS.com xenograft tumors 68

Fig. 3.5 Effects of BXL0124 on the CD44 protein expression level in MCF10DCIS.com xenograft tumors *in vivo* 69

Fig. 3.6 Effects of BXL0124 on CD44 protein level in MCF10DCIS.com cells 70

Fig. 3.7 Effects of BXL0124 on the cell surface expression level of CD44 and proportion of subpopulation in MCF10DCIS.com cells..... 71

Fig. 3.8 VDR-dependent repression of CD44 by BXL0124 in MCF10DCIS.com cells 72

Fig. 3.9 Repression of mRNA level of CD44 by 1α,25$(OH)_2D_3$ and BXL0124 in MCF10DCIS.com cells..... 73

Fig. 3.10 Induction of mRNA level of osteopontin by 1α,25$(OH)_2D_3$ and BXL0124 in MCF10DCIS.com cells..... 74

Fig. 3.11 Repression of transactivation of CD44 promoter by 1α,25$(OH)_2D_3$ and BXL0124 in a p53 dependent manner in MCF10DCIS.com cells 75

Fig. 4.1 Repression of proliferation and metabolic activity of MCF10DCIS.com cells by $1\alpha,25(OH)_2D_3$ and BXL0124 92

Fig. 4.2 Repression of invasion of MCF10DCIS.com cells by $1\alpha,25(OH)_2D_3$ and BXL0124 93

Fig. 4.3 Down-regulation of invasion markers by BXL0124 in MCF10DCIS.com cells 94

Fig. 4.4 Repression of protein levels of CD44 and pSTAT3 by BXL0124 in MCF10DCIS.com cells 95

Fig. 4.5 Repression of protein levels of CD44 and pSTAT3 by BXL0124 in a VDR dependent manner in MCF10DCIS.com cells 96

Fig. 4.6 Inhibition of STAT4 activation by BXL0124 in MCF10DCIS.com cells 97

Fig. 4.7 Repression of CD44-STAT3 interaction by BXL0124 in MCF10DCIS.com cells 98

Fig. 4.8 Inhibition of cell proliferation and invasion of MCF10DCIS.com cells by CD44 knockdown 99

Fig. 4.9 Down-regulation of mRNA levels of MMP-9, MMP-14 and uPA by CD44 knockdown in MCF10DCIS.com cells 101

Fig. 4.10 Inhibition of the growth of MCF10DCIS.com xenograft tumors by CD44 knockdown 102

Fig. 4.11 Repression of expression levels of invasion markers by CD44 knockdown in MCF10DCIS.com xenograft tumors 103

Fig. 4.12 Repression of the CD44-STAT3 signaling in basal-like breast cancer cells 105

Fig. 4.13 A schematic diagram of proposed mechanism of action of BXL0124 on CD44-STAT3 signaling and breast cancer cell invasion in basal-like breast cancer 107

Fig. 5.1 Inhibitory effects of BXL0124, CDDO-Im and the combination on the mammary tumorigenesis in MMTV-HER2/neu transgenic mice 117

Fig. 5.2 Repression of HER2 and EGFR signaling pathway by BXL0124, CDDO-Im and the combination in MMTV-HER2/neu mammary tumors 119

Fig. 5.3 Effects of BXL0124, CDDO-Im and the combination on the activation of HER receptors and downstream molecules in MMTV-HER2/neu mammary tumors 120

Fig. 5.4 A schematic diagram of downstream signaling pathways of HER receptors regulated by BXL0124, CDDO-Im and the combination in mammary tumors of MMTV-HER2/neu transgenic mice..... 121

Fig. 5.5 Activation of HER2 at the invading edge of MMTV-HER2/neu mammary tumors and its repression by BXL0124, CDDO-Im and the combination 122

Fig. 5.6 Effects of BXL0124, CDDO-Im and the combination on the mRNA levels of HER receptor ligands in MMTV-HER2/neu mammary tumors 123

List of Abbreviations

15-PGDH	15-hydroxyprostaglandin dehydrogenase
1α,25$(OH)_2D_3$	1α,25-dihydroxyvitamin D_3
1α-OHase	25-hydroxyvitamind_3-1α-hydroxylase
25$(OH)D_3$	25-hydroxyvitamin D_3
25-OHase	25-hydroxylase
ADH	Atypical ductal hyperplasia
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CBP	CREB binding protein
CDDO	2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid
CDDO-EA	CDDO-ethyl amide
CDDO-Im	CDDO-imidazolide
CDDO-MA	CDDO-methyl amide
CDDO-Me	CDDO-methyl ester
COX-2	Cyclooxygenase-2
CREB	cAMP response element-binding
DAPI	4,6-diamidino-2-phenylindole
DCIS	Ductal carcinoma in situ
DMBA	7,12-Dimethyl benz-α-anthracene
DMSO	Dimethyl sulfoxide
DRIP	Vitamin D receptor interacting protein
DUSP10	Dual specificity phosphatase 10
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERE	Estrogen response element
GEM	Genetically engineered mouse
GPCR	G-protein-coupled receptor
H&E	Hematoxylin and eosin
HAS1	Hyaluronic acid synthase 1
HB-EGF	Heparin-binding epidermal growth factor
HER	Human epidermal growth factor receptor

HGF	Hepatocyte growth factor
HMEC	Human mammary epithelial cell
IDC	Invasive ductal carcinoma
IFNγ	Interferon γ
IGFBP-3	Insulin-like growth factor binding protein-3
IGF-IR	Insulin-like growth factor-I receptor
IL-6	Interleukin-6
iNOS	Cytokine-inducible Nitric oxide synthase
JAK2	Janus kinase 2
JNK	Jun-N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
mTOR	Mammalian target of rapamycin
NCoA62-SKIP	Nuclear coactivator 62 kDa-SKI-interacting protein
NF-κB	Nuclear factor of κB
NMU	N-nitroso-N-methylurea
Nrf2	Nuclear factor (erythroid-derived)-like 2
Nrg	Neuregulin
PAI-1	Plasminogen activator inhibitor-1
PARP	Poly ADP ribose polymerase
PCNA	Proliferation cell nuclear antigen
PG	Prostaglandin
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor γ
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PyMT	Polyoma middle T oncogene
RXR	Retinoic X receptor
SCID	Severe combined immunodeficiency
SDF-1	Stromal cell-derived factor-1

SERM	Selective estrogen receptor modulator
shRNA	Small hairpin RNA
SO	Synthetic oleanane triterpenoid
SRC	Steroid receptor coactivator
STAT3	Signal transducer and activator of transcription 3
TGF	Transforming growth factor
TGF-β-RI	Transforming growth factor β-receptor I
TNF α	Tumor necrosis factor α
tPA	Tissue plasminogen activator
TRAIL	TNF-related apoptosis-inducing ligand
TRPM-2	Testosterone repressed prostate message-2
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
VDR	Vitamin D receptor
VDRE	Vitamin D response element
VEGF	Vascular endothelial growth factor
WAP	Whey acidic protein

Chapter 1: Introduction

1.1 Breast cancer

Breast cancer is the most common cancer and the second leading cause of cancer-related death among women in America [1]. Approximately 1 in 8 women in America will develop invasive breast cancer over their lifetime [1]. At the pathological and molecular level, breast cancer has been recognized as strikingly heterogeneous diseases [2,3]. According to pathological evaluation, breast cancer has been divided into several types, invasive ductal carcinoma and invasive lobular carcinoma as well as inflammatory breast cancer in rare cases [2,4]. In addition to pathological classification, based on gene expression patterns, breast cancer has been classified into luminal A, luminal B, HER2-positive and basal-like subtypes [3,5,6]. Moreover, each subtype has distinct gene expression profiles and is associated with different clinical outcome and selective sensitivity to anti-cancer therapies [3,7,8].

1.1.1 Luminal breast cancer and therapies

Luminal breast cancers, which express estrogen receptor (ER) and luminal cytokeratin 8/18, are the most common subtype of breast cancer and account for 60-70% of invasive breast cancer cases [9,10,11]. Luminal breast cancers can be further divided into luminal A [ER^+ or progesterone receptor $(PR)^+$ and human epidermal growth factor receptor 2 $(HER2)^-$] and luminal B (ER^+ or PR^+ and $HER2^+$). Although both luminal A and luminal B breast cancers express ER, luminal B breast cancers display poor differentiation, higher histologic grades and higher expression of proliferative genes such as CCNB1 (encoding cyclin B1), MKI67 (encoding Ki-67) and MYBL2 (encoding Myb-related protein B) than luminal A breast cancers [5,12,13]. In

general, patients with luminal breast cancer show better prognosis than patients with all other subtypes of breast cancers [10]. Patients with luminal breast cancer typically receive endocrine therapies with selective estrogen receptor modulators (SERM) and aromatase inhibitors for premenopausal and postmenopausal women, respectively [14]. Among the two subtypes of luminal breast cancers, luminal B subtype has significantly poorer outcome and a higher chance of being resistant to endocrine therapy than luminal A subtype [15]. Moreover, the luminal B breast tumors with resistance to endocrine therapies are less responsive to chemotherapies in many cases, urging the development of new drugs targeting those endocrine therapy-refractory luminal B breast tumors [8,16].

The endocrine therapies have been important systemic therapies to treat the cancer patients with ER-positive tumors [17]. Tamoxifen, an antagonist of ER which binds to ER and blocks the function of ER, has been the first-line treatment for both early and advanced ER-positive breast cancer patients [18]. Moreover, several clinical trials found that tamoxifen reduced the incidence of ER-positive breast cancer, functioning as a preventive agent for women with high risk of breast cancer [19,20,21]. In the treatment for advanced breast cancer, half of patients exhibit *de novo* resistance to tamoxifen, but many patients who respond to tamoxifen eventually develop resistance [18]. Moreover, tamoxifen has ER-agonistic effects on other tissues including bone, liver and uterus, causing increased risk of side effects, such as endometrial cancer and thromboembolic disease [22]. Another treatment option for ER-positive breast cancers is the use of aromatase inhibitors, such as aminoglutethimide and 4-hydroxyandrostenedione, which repress the activity of cytochrome p450 enzyme aromatase to reduce circulating estradiol levels [22,23]. With a series of improvements, now the third-generation of aromatase inhibitors, such as anastrozole, letrozole and exemestane, show high selectivity to aromatase enzyme and significantly suppress plasma estradiol level by 85-92% [22,23]. However, in patients with advanced breast cancer, the response rates to aromatase inhibitors are just slightly higher than to

tamoxifen, and many patients also exhibit *de novo* and acquired resistance to aromatase inhibitors [22]. Particularly for luminal B subtype, the activation of HER2 signaling has been one of the mechanisms of resistance, and combination of HER2 targeted therapies has been utilized to overcome the endocrine therapy resistance [24]. A steroidal analogue of estradiol, fulvestrant, was developed to provide specific ER-antagonistic effects without agonistic effects and showed potent anti-cancer activity on ER-positive breast cancer in many preclinical studies [25,26,27,28]. However, in the clinical studies, fulvestrant showed extremely poor bioavailability, leading to a failure of clinical studies [29]. The development of many steroidal analogues of estradiol is undergoing to achieve better bioavailability [29].

1.1.2 HER2-positive breast cancer and therapies

HER2 (also known as ErbB-2) is a transmembrane receptor tyrosine kinase which has been shown to be overexpressed in 20-30% of invasive breast cancer cases [30]. The breast tumors with HER2 overexpression show high histologic grade and elevated growth rate [31]. The patients with HER2-overexpressing tumors develop early systemic metastasis and have decreased rates of disease-free and overall survival as compared to patients without HER2 overexpression [31]. The aberrant activation of Ras-MAPK and Akt-mTOR signaling has been identified as key biological features in HER2-driven breast tumor [32]. Currently, trastuzumab (Herceptin, a humanized monoclonal antibody binding to HER2) and lapatinib (Tykerb, a small-molecule inhibitor of HER2 tyrosine kinase activity) have been utilized as typical therapeutic options for the patient with HER2-positive breast tumors [33]. Since the therapeutics are selectively effective for the HER2 overexpressing breast tumors, diagnostic tests, such as immunohistochemistry to detect protein overexpression or in situ hybridization to detect gene amplification, are conducted to determine HER2 overexpression in patients before drug treatment [34].

For metastatic HER2-positive breast cancer, trastuzumab has been part of the standard of care, and the combination of trastuzumab with chemotherapies has significantly improved median overall survival of patients [35]. However, about half of patient with HER2-positive breast cancer did not exhibit objective response with trastuzumab, indicating *de novo* resistance to trastuzumab [35]. In addition, many patients who showed objective response acquired resistance over the trastuzumab treatment and developed disease progression [35]. Loss of function of the phosphatase and tensin homolog (PTEN) and activating mutations of PI3K which lead constitutive activation of PI3K signaling have been reported as the mechanisms of trastuzumab resistance [36]. Another mechanism of resistance to trastuzumab is the accumulation of a truncated form of HER2 receptor (p95-HER2), which is constitutively active [37]. Lapatinib, a reversible, ATP-competitive inhibitor of HER2 and EGFR tyrosine kinase, was developed to treat trastuzumab-resistant breast cancer patients [38]. However, treatment of lapatinib or trastuzumab-refractory patients also exhibited *de novo* and acquired resistance to lapatinib [39]. The combination treatment of trastuzumab with other HER2 targeting antibodies such as pertuzumab significantly improved complete response rate of HER2-positive patients than a single treatment of trastuzumab [40]. The combination of trastuzumab with downstream pathway inhibitors or inhibitors for other receptors, such as Met receptor and IGF1R, are ongoing investigations in preclinical studies as a new approach to overcome trastuzumab resistance [41,42,43].

1.1.3 Basal-like breast cancer and therapies

Basal-like breast cancer exhibits gene expression profile similar to basal/myoepithelial cells including cytokeratin 5, cytokeratin 17 and EGFR [3,12]. Most of the basal-like breast cancers are negative for ER and HER2, conferring *de novo* resistance to endocrine and HER2-targeted therapies [44]. Basal-like breast cancer comprises approximately 15% of all invasive

breast cancers, and majority of basal-like breast cancer has high histologic grade [44,45]. More than 75% of breast cancer patients carrying BRCA1 mutation have a basal-like phenotype, indicating BRCA1 as an important basal-like breast cancer susceptibility gene [44]. Mutations in TP53 are also frequent (44-82%), which lead to defect of cell cycle check points and genome instability in basal-like breast cancer [3,45,46]. Among different subtypes, basal-like subtype has the worst prognosis, and majority of basal-like breast cancer patients are at high risk for early relapse within first 2-5 years after treatment [47,48].

Without validated molecular targets, the conventional chemotherapies have been the only therapeutic option for patients with basal-like breast tumors, and no standard regimen has been established [8,45,49]. EGFR and c-kit, which are expressed in a high proportion of basal-like breast cancers, have been emerged as potential therapeutic targets [50,51]. For the basal-like breast cancers carrying BRCA1 mutation, platinum-based chemotherapies or poly ADP ribose polymerase (PARP) inhibitor have been investigated as targeted therapies [52]. In addition to the development of potential targeted therapies, recently there has been accumulating evidence that basal-like subtype might not be a single entity but rather groups of different breast cancers sharing basal-like gene signatures [48]. Therefore, identification of distinct subgroups within basal-like breast cancers might be one of the new directions of research for basal-like breast cancers [45,48].

1.2. The preclinical models of breast cancer

Preclinical studies have been invaluable for improvement of our understandings on cancer biology and development of anti-cancer therapeutics. With the complex and heterogeneous nature of breast cancers, variety of preclinical models have been developed, including *in vitro* human breast cancer cell lines, *in vivo* xenograft animal models, carcinogen-induced tumor

models and transgenic mouse models [53]. Although each of preclinical models has different limitations in terms of recapitulating human breast cancer, using multiple models may provide a powerful tool to investigate human breast cancer.

1.2.1 Human breast cancer cell lines

Breast cancer cell lines have been widely used in the preclinical studies to investigate the biology of breast cancer and to develop new therapeutics [53]. Breast cancer cell lines are inexpensive and easy to maintain, and the experimental condition is easy to control, yielding reproducible and quantifiable results [53]. There are wide ranges of well-defined assay tools for the biology of breast cancer cell lines [53]. The characteristics of each breast cancer cell line are well-established, providing selective options depending on the purpose of study [54,55]. Major limitation is the obvious difference between cell culture system and microenvironment of human breast tissue [56]. The apical-basal polarity of cells and the capability of cells to form functional structures *in vivo* cannot be replicated in the cell culture system [56]. In addition, establishing the model to elucidate complex multicellular interaction is practically difficult in the cell culture system [53]. The 3D culture system with a reconstituted basement membrane matrix and co-culture system with stromal or immune cells have been developed to make up the shortcoming of common cell culture system [53,56]. With the recent discovery of human breast cancer subtypes, the panels of human breast cancer cell lines that represent each subtype has been very useful to investigate the biological responses among different subtypes upon the anti-cancer drug treatments [54,55].

1.2.2 Xenograft animal models

In xenograft animal models, human breast cancer cells are subcutaneously or orthotopically transplanted into immunodeficient mice allowing the investigation of human breast cancer cell biology in the *in vivo* environment [57]. As preclinical models, the xenograft animal models have been extensively utilized to develop and screen anti-cancer drugs [57]. Although the xenograft animal models provide convenient *in vivo* tools, there are some limitations to elucidate the interactions between tumor cells and microenvironment happening in human breast cancer. In many cases of xenograft tumors, the stromal cells do not involve as much as in human breast cancer [58]. In addition, the different species origin of epithelial human cancer cells and mouse stromal cells may have significant impacts on the properties of xenograft tumors [58]. Interestingly, some breast cancer cell lines such as MCF10DCIS.com and SUM225 cells demonstrated significant infiltration of stromal cells and spontaneous progression of xenograft tumors from DCIS to invasive breast cancer, replicating the properties of human breast cancer [59,60,61]. Another limitation of the xenograft animal model is the lack of immune systems against the tumor cells which is a crucial component of human breast cancer development [58]. The co-transplantation of tumor-suppressing or tumor-promoting immune cells has been utilized to address the role of the immune system in breast cancer development [53]. With the recent identification of breast cancer stem cells (also known as tumor-initiating cells) in breast cancer, a serial transplantation of human breast cancer cells has been utilized to identify the tumorigenic breast cancer stem cell population in breast cancer [62].

1.2.3 Carcinogen-induced mammary tumor models

The chemical carcinogen-induced mammary tumor model has been served as a useful tool for preclinical studies with multiple advantages, such as easy tumor induction, short latency

period, and hormone responsiveness [63]. Not like most of xenograft tumors, which are developed from malignant breast cancer cells, carcinogen-induced mammary tumors can represent multiple stages from initiation, promotion and progression of cancer development as the tumors are developed from normal mammary epithelial cells [64]. In general, the chemical carcinogen-induced tumor model develops hormone-dependent tumors, therefore has been served as a conventional model to investigate ER-positive breast cancer and to screen ER-targeting pharmaceutical agents [64]. A polycyclic aromatic hydrocarbon DMBA and alkylating agent, N-methyl-N-nitrosourea (NMU) are the most commonly used chemical carcinogens to study mammary tumorigenesis in rats [65,66]. The tumors induced by those chemicals arise from the terminal end buds, which is also the proposed site of origin for human DCIS [64,67]. The histological similarities between carcinogen-induced mammary tumors and human breast cancers have been reported in many studies [63,68,69]. However, both DMBA- and NMU-induced mammary tumors rarely metastasize and do not have a p53 mutation which is common in human breast cancer [70]. With more aggressive histological phenotypes and estrogen dependency, the NMU-induced model has been considered as a better model to study ER-positive breast cancer than the DMBA-induced model [64]. As found in human breast cancers, an altered expression of TGF-α, HER2, cyclin D1 and gelsolin was found in NMU-induced mammary tumors [64]. Moreover, gene expression profile study demonstrated that the carcinogen-induced mammary tumors share molecular features with low-to-intermediate grade, ER-positive human breast cancer [70].

1.2.4 Genetically engineered mouse (GEM) models

The GEM model has been a useful tool to understand the specific functions of human breast cancer-associated genes in mammary tumorigenesis [71]. Tumor suppressor genes such as

p53 and BRCA1 or oncogenes such as c-Myc and HER2 have been genetically manipulated in GEM model, leading to development of mammary tumors. Several promoters, including mouse mammary tumor virus (MMTV) and whey acidic protein (WAP), have been utilized to have selective gene expression in mammary glands [53]. In addition to the conventional promoters, the advance of genetic engineering such as tetracyclin (tet)-regulated transgene and Cre/loxP recombinase-mediated gene regulation systems allowed more precise control over timing, tissue- and cell- selectivity in genetic alterations [53]. The precise spatial-temporal control demonstrated that the phenotypes of mammary tumors are determined in more complex manner by different origin of the target cell and timing of genetic alteration [53]. One major limitation of GEM tumors is that most of the GEM tumors are hormone-independent while more than half of human breast cancers are hormone dependent [72]. In addition, most GEM tumors metastasize predominantly to lung while human breast tumors metastasize to multiple tissues such as lymph nodes, lung, liver, and bone [72]. With numerous GEM models of different genetic manipulations, the gene expression profiles of about 100 mammary tumors from 13 different types of GEM models were compared to a large panel of human breast cancer to identify the association of each GEM models to the subtypes of breast cancers [73]. The different tumor samples from same GEM model showed significant similarities in gene expression profiles, confirming the molecular phenotypes of tumors in GRE models were determined by the genetic alteration [73]. In addition, the 13 GEM models were clustered into two groups, sharing features of luminal B or basal-like subtypes [73]. In particular, the gene expression profiles of two BRCA1 deficient GEM models showed significant overlap with the gene expression profile of basal-like human breast cancer, supporting the tumorigenic role of BRCA1 mutation in basal-like breast cancer [73]. Although no single GEM model exhibits all the features of given subtype, the various options of GEM models with distinct molecular signature to drive tumorigenesis may useful to dissect distinct signaling pathway for the investigation of targeted therapies.

1.3 Molecular targets of breast cancer

1.3.1 Estrogen receptor (ER) signaling

The persistently elevated level of estrogen has been closely associated with the risk of breast cancers, suggesting that the excessive exposure to endogenous estrogen may be key casual factor of breast cancer [74,75]. With binding of estrogen, ER undergoes a conformational change and binds to estrogen response element (ERE) to regulate ER-mediated genes, including c-Myc and cyclin D1, leading to dramatic stimulation of cell proliferation [76]. In addition to estrogen, growth factor-activated Akt or MAPK can also activate ER by phosphorylating ER, suggesting dynamic cross-talk between ER and other signaling pathways [76]. ERα and ERβ are the two members of ER family [77]. As the key transcriptional regulator associated cell proliferation and tumor malignancy, the tumorigenic function of ERα has been well established [77]. While ERβ appears to have opposing effect of ERα, the biological role of ERβ in mammary tumorigenesis is still controversial among different research groups [77].

1.3.2 Human epidermal growth factor receptor (HER) family receptors

The HER family (also known as ErbB receptor family) consists of four transmembrane receptor tyrosine kinases, HER1 (also known as EGFR), HER2, HER3 and HER4 [78]. The HER family receptors, expressed in various tissues, are activated by the EGF family of growth factors, such as EGF, transforming growth factors (TGF) and neuregulins, in a tightly controlled manner [78]. In breast cancer, EGFR and HER2 are frequently overexpressed, and the overexpression is associated with more aggressive clinical phenotype [31,79]. The HER family of receptors has complex signaling network by crosstalking with each other, for example HER2 without known

soluble ligand can heterodimerize with EGFR and HER3 amplifying signaling as co-receptor [32]. In addition, the transactivation of HER family receptors by G-protein-coupled receptors (GPCR) or seven-pass membrane receptor Frizzled (FZD)-induced activation of pro-EGFR ligands contributes the complex nature of HER receptor signaling network in breast cancer [80,81,82]. With the understanding of complexity of the HER receptor signaling pathway and the frequent resistance to drugs targeting single molecule, combination of multiple drugs or multi-functional agents targeting different factors among the HER receptor signaling network have been the new direction [83].

1.3.3 BRCA1 and BRCA2

BRCA1 and BRCA2 are the two major breast cancer susceptibility genes, playing critical roles for genomic stability through homologous recombination [84]. Germ line mutation of one copy of either gene results in hereditary breast and ovarian cancer syndrome which accounts for 5-7% of breast cancer cases. Interestingly, the breast cancers with BRCA1 mutation predominantly show phenotypic features of basal-like breast cancer, but the reason is still unknown [85]. The tumors with BRCA1 or BRCA2 mutation are shown to be intrinsically sensitive to PARP inhibitors [86]. In recent clinical trials, the combination of PARP inhibitor with chemotherapeutic drugs showed significant clinical response among triple negative breast cancer patients with BRCA1 and/or BRCA2 mutations [87,88].

1.3.4 Phosphoinositide 3-kinase (PI3K)

PI3K is involved in mediating growth and survival signaling from receptor tyrosine kinases, or RAS proteins to downstream effectors, such as Akt and mTOR complexes [89]. In

many cancers, several conditions such as persistent activation of upstream receptor tyrosine kinases, the genetic mutation of PI3K, loss of PTEN, or amplification of key components in the pathway lead to the abnormal activation of PI3K/Akt signaling pathway which promotes cancer cell growth and survival [90]. Recent studies with a large number of human breast cancer specimens reported PI3K mutation as one of the most frequently occurred mutations in human breast cancer along with P53 and GATA3 mutations [6,91]. While the frequency PI3K mutation varies among different subtypes of breast cancer, the alteration of genomic and proteomic signatures corresponding to PI3K/Akt signaling pathway is shown to occur in all subtypes of breast cancer [92]. Moreover, the amplification of PI3K or its pathway components have been demonstrated as a key mechanism for resistance to many receptor tyrosine kinase-targeted therapies, including HER2 [90].

1.3.5 Insulin-like growth factor-I receptor (IGF-IR)

IGF-IR is the primary receptor for IGF-I and IGF-II which are potent paracrine and autocrine mediators of cell proliferation [93]. The abnormal activation of IGF-IR signaling has been associated with malignant progression of cancer [94]. While the amplification or mutation in IGF-IR is rare, the abnormal expression of IGFs has been shown in many cancers [95,96]. In breast cancer, the elevated level of insulin and IGF-I was associated with the risk of incidence and recurrence [97,98]. In transgenic mice, overexpression of IGF-I or IGF-II increased the development of mammary tumors, while these tumors required additional oncogenic alteration, such as p53, to develop completely malignant phenotype [99,100]. In addition, IGF-IR signaling pathway has been identified as key signaling pathway that confers endocrine therapy-resistance to breast cancer [101]. Co-targeting of IGF-IR with ER or HER2 significantly enhanced the anti-cancer activity of ER or HER2 inhibitors in preclinical studies [102,103].

1.3.6 Nuclear factor of κB (NF-κB)

NF-κB is a sequence-specific transcription factor which plays a role in proliferation, anti-apoptosis, inflammation and innate immunity of cells [104]. The elevated or constitutive activity of NF-κB has been found in breast cancer cells and primary breast tumors [105,106]. In one animal study, the activation of NF-κB occurred before the malignant transformation of carcinogen-induced mammary tumors, indicating the association between the activated NF-κB and early stage of mammary tumorigenesis [107]. In addition, the inhibition of NF-κB activity delayed the development of mammary tumors in the polyoma middle T oncogene (PyMT) transgenic mouse model [108]. Although the precise mechanism of NF-κB in breast cancer progression is still unclear, anti-apoptotic activity of NF-κB by inhibiting p53 activity has been suggested as possible mechanisms [109]. Moreover, recent studies demonstrated that the NF-κB pathway played a major role in tumorigenesis of inflammatory breast cancer, exhibiting persistent activation of NF-κB with elevated expression of NF-κB target genes [110,111].

1.3.7 Signal transducer and activator of transcription 3 (STAT3)

STAT3 is a transcription factor that mediates the cellular response to various cytokines and growth factors, including interleukin-6, and EGF [112]. Upon activation, STAT3s are phosphorylated by intracellular kinases, including Janus kinase 2 (JAK2) and Src and translocated to nucleus to activate target genes [112,113]. While the activation of STAT3 occurs transiently in normal cells, constitutive activation of STAT3 is frequent in many cancer cells, including breast cancer [114]. Moreover, the constitutive activation of STAT3 is often associated with tumor progression [114,115]. Studies demonstrated that the constitutive activation of STAT3 was a crucial contributor to the growth, survival and invasion of breast cancer cells [116,117].

Moreover, overexpression of Bcl-2 or survivin by the constitutive activation of STAT3 has been found as one of the mechanisms for breast cancer cells to be resistant to chemotherapies [117,118]. Recent study reported that STAT3 is a key signaling to maintain the breast cancer stem cell population [119], and tumor-initiating breast cancer cells exhibited preferential activation of JAK2/STAT3 signaling [120]. On the other hand, inhibition of STAT3 signaling with STAT3 small hairpin RNA (shRNA) or use of STAT3 phosphorylation inhibitors repressed the formation and growth of xenograft tumors in mice as well as the invasive potential of breast cancer cells [121,122], demonstrating STAT3 as an important therapeutic target in breast cancer.

1.3.8 Vascular endothelial growth factor (VEGF)

VEGF is a potent inducer of vascular permeability as well as survival and proliferation of endothelial cells, involving in physiological neovascularization [123]. During the cancer progression, cancer exhibits the constitutively activated angiogenesis which is required to sustain neoplastic growth of tumor [124]. As the key inducer of angiogenesis, the overexpression of VEGF is common in various cancers, including breast cancer and is associated with disease progression and decreased survival rates in cancer patients [124,125]. Large number of preclinical studies demonstrated the anti-cancer effects of VEGF-targeting drugs, leading to the clinical trials of a humanized monoclonal antibody against VEGF (Bevacizumab). In the first trial, bevacizumab showed significant clinical benefit of overall survival in combination with chemotherapies, but the following trials failed to confirm the significant improvement of overall survival with bevacizumab in metastatic breast cancer patients [126,127,128]. However, all three trials showed the significantly improved progression-free survival by bevacizumab, supporting the potential of VEGF targeted therapies in advanced breast cancer [128,129].

1.4 Vitamin D

Vitamin D is derived from diets or synthesized by converting the precursor 7-dehydrocholesterol to vitamin D in the skin in response to ultraviolet B (sunlight) exposure [130]. In the liver, vitamin D is hydroxylated by 25-hydroxylase (25-OHase), producing 25-hydroxycholecalciferol ($25(OH)D_3$), and $25(OH)D_3$ is then converted into $1\alpha,25(OH)_2D_3$ (calcitriol) in the kidney by 25-hydroxyvitamin D3-1α-hydroxylase (1α-OHase, cyp27B) [131]. $1\alpha,25(OH)_2D_3$, the active form of vitamin D, is the major regulator of calcium and phosphorus homeostasis [130]. In addition to the systemic production, the enzymatic machinery to convert vitamin D into $1\alpha,25(OH)_2D_3$ has been also found in many other tissues, including breast. This local production of $1\alpha,25(OH)_2D_3$ has been demonstrated to induce various biological functions in a tissue-specific manner [130,132].

1.4.1 Biological actions of vitamin D

Many preclinical studies have demonstrated the important non-calcemic functions of vitamin D in association with various diseases, including cancer [132]. The major molecular mechanism of vitamin D is to regulate gene expression by activating vitamin D receptor (VDR), which is a member of the nuclear receptor transcription factors [133]. In addition to the genomic actions, recent studies have shown that vitamin D can exert rapid non-genomic action by interacting with membrane-bound VDR [133].

Genomic actions of vitamin D: Binding of vitamin D to VDR induces dimerization of VDR with retinoic X receptor alpha (RXRα) [134]. The activated VDR-RXR dimer binds to vitamin D response elements (VDRE) in the promoter or enhancer region of target genes to

regulate the transcription of the downstream target genes [134]. After binding to DNA, the VDR-RXR complex recruits co-factor proteins which determine the tissue- or gene-specific transcriptional regulations. For the activation of target genes, VDR-RXR complex D recruits steroid receptor coactivators (SRCs), nuclear coactivator 62 kDa-SKI-interacting protein (NCoA62-SKIP), CREB binding protein (CBP)-p300, and polybromo-and SWI-2-related gene 1 associated factor (PABF) to induce chromatin remodeling [135]. After chromatin unwinding, the mediator complexes (vitamin D receptor interacting protein coactivator complex, DRIP) are recruited to the region, which activate the basal transcription machinery [132,135,136]. For the repression of target genes, activated VDR-RXR dimer recruits co-repressors such as NCoR (nuclear receptor co-repressor) 1 and NCoR2/SMRT (silencing mediator of retinoid and thyroid receptors) [132,137]. Histone deacetylases and DNA methyltransferases are also recruited by VDR-RXR complex, leading to closed chromatin structure [135,137].

Non-genomic actions of vitamin D: Several studies have shown that vitamin D can exert rapid cell response (within 1~ 45 min) that does not depend on transcriptional regulation [138,139,140]. VDR, which is involved in the rapid response, was found to be localized in caveolae-enriched microdomain of plasma membrane [138]. The binding of vitamin D to the membrane-bound VDR activates the PKC signaling pathway, leading to rapid opening of voltage-gated Ca^{2+} channels and increase intracellular Ca^{2+} level [139,140]. The elevated Ca^{2+} level consequently activates the Raf/MAPK/ERK signaling pathway [138,139,140]. However, the reports on rapid response to vitamin D have been limited in many non-malignant cells, such as normal colon cells and skeletal muscle cells, and occurrence of rapid response to vitamin D in malignant cells is still unknown [141].

1.4.2 Vitamin D and breast cancer

Both normal and malignant breast epithelial cells express VDR, and an animal study with knockdown of VDR showed the important role of VDR in physiological mammary gland development [142]. Moreover, VDR-knockdown mice showed increased DMBA-induced preneoplastic mammary lesions, compared to wild-type mice [143]. In another study with the MMTV-HER2/neu transgenic mouse model, the loss of one or both copies of VDR gene by crossing with VDR-knockdown mice also resulted in the increased incidence of preneoplastic lesions and abnormal morphologic phenotype of duct [144], suggesting inhibitory function of vitamin D on mammary tumorigenesis. Many *in vitro* and *in vivo* studies demonstrated the potent anti-cancer activity of $1\alpha,25(OH)_2D_3$ and other vitamin D analogs [145,146,147,148,149]. However, in clinical studies, $1\alpha,25(OH)_2D_3$ induced hypercalcemic toxicity in the participants before reaching the dose comparable to the dose with anti-cancer activity in preclinical studies [150,151,152]. Various types of vitamin D analogs have been developed to obtain better anti-cancer activity with less toxicity than $1\alpha,25(OH)_2D_3$ [132,152].

1.4.3 *In vitro* and *in vivo* studies of vitamin D and vitamin D analogs in breast cancer

Anti-proliferation: Vitamin D and vitamin D analogs showed potent anti-proliferation activity on both ER-positive and ER-negative breast cancer cells *in vitro* and *in vivo* [132]. They inhibit cell proliferation by transcriptionally regulating expression levels of many cell cycle regulatory genes, such as CDKN1A (encoding p21), CDKN1B (encoding p27), CCND1 (encoding cyclin D1), CCND3 (encoding cyclin D3), CCNA1 (encoding cyclin A1) and CCNE1 (cyclin E1) [153,154,155]. The expression level of insulin-like growth factor binding protein-3 (IGFBP-3), which blocks the IGF-stimulated cell proliferation by sequestering IGF, was increased by vitamin D and vitamin D analogs in MCF-7 and T47D cells [156,157]. Functional

vitamin D response elements were later identified in IGFBP-3 promoter region [158], supporting the transcriptional regulation of IGFBP-3 by vitamin D. The TGF-β signaling pathway plays an important role for the growth regulation of cancer cells at the early stage of tumor progression [159]. In breast cancer cells, vitamin D and vitamin D analogs modulated the TGF-β signaling by inducing the mRNA expression level of TGF-β-RI [155,160]. Bone morphogenetic proteins (BMPs), another group of factors belonging to the TGF-β superfamily, are also regulated by vitamin D and vitamin D analogs in breast cancer cells [161,162]. Gemini vitamin D analogs activate the BMP signaling pathway by increasing mRNA level of BMP2 and BMP6, while decreasing mRNA level of Smad6, an inhibitor of TGF-β/Smad signaling [161]. Blocking of BMP signaling with chemical inhibitor partially repressed the anti-proliferation effect of Gemini vitamin D analogs [162], indicating the role of BMP signaling in the inhibitory effect of Gemini vitamin D analogs on cell proliferation.

Apoptosis: Vitamin D and vitamin D analogs have been reported to induce apoptosis in breast cancer cells by regulating the expression level of key apoptosis mediators [132]. The majority of apoptotic effects of vitamin D and its analogs have been demonstrated in ER-positive breast cancer model, such as MCF-7 cells or NMU-induced rat mammary tumor model [132]. Vitamin D and vitamin D analogs decreased Bcl-2 expression and increased testosterone repressed prostate message-2 (TRPM-2)/clusterin expression in MCF-7 cells, leading to apoptosis [163,164,165,166]. The increased release of intracellular free calcium and activation of calpain by vitamin D and vitamin D analogs have also been shown to induce apoptosis in MCF-7 cells [167]. In animal models, induction of the apoptotic markers, such as cleaved-PARP and cleaved-caspase-3 by vitamin D and vitamin D analogs in NMU-induced tumors and MCF-7 xenograft tumors have been reported [168,169,170,171].

Anti-invasion: Invasive growth is a critical property of malignant tumor progression for local invasion and metastasis [172]. Numerous studies have demonstrated that vitamin D

represses invasive potential of breast cancer cells [132,173,174]. Vitamin D and vitamin D analog decreased the secretion of urokinase-type plasminogen activator (uPA) and activity of tissue plasminogen activator (tPA) while increased secretion of plasminogen activator inhibitor-1 (PAI-1), resulting reduced total PA activity in highly invasive MDA-MB-231 cells [173]. The level of MMP-9 in MDA-MB-231 cells was decreased by vitamin D and vitamin D analogs [173]. cDNA microarray identified additional MMP-1, MMP-7, MMP-10 and MMP-12, which are down-regulated by vitamin D in MDA-MB-231 cells but not in non-invasive MCF-7 cells [155]. The repression of cancer cell invasion by vitamin D and vitamin D analogs were also shown in other ER-negative and highly invasive SUM-159PT cells [174]. Moreover, in a mouse model with intracardiac injection of MDA-MB-231 cells, the average area of osteolytic lesions and tumor burden within bone were significantly decreased by vitamin D analog, confirming the anti-invasion activity of vitamin D *in vivo* [175].

Anti-inflammation: Inflammation has been associated with reduced survival and increased recurrence among breast cancer patients [176,177]. Recent studies demonstrated that vitamin D regulates the prostaglandin (PG) pathway in both ER-positive (MCF-7, ZR-75-1 and T47-D cells) and ER-negative (MDA-MB-231 cells) by decreasing mRNA expression levels of COX-2 and increasing mRNA expression levels of 15-PGDH [178,179,180]. Gemini vitamin D analog significantly induced mRNA expression level of dual specificity phosphatase 10 (DUSP10, also known as MAP kinase phosphatase 5) which controls inflammatory responses by dephosphorylating p38 MAPK and the stress-activated protein kinase Jun-N-terminal kinase (JNK), leading to their inactivation [160,181].

The *in vitro* and *in vivo* studies of vitamin D and vitamin D analogs in breast cancer are summarized in Table 1.2 and Table 1.3, respectively.

1.4.4 Clinical studies of vitamin D in breast cancer

Two vitamin D analogs, EB1089 and paricalcitol, were tested in clinical studies for patients with solid tumors including breast cancer, colorectal cancer and prostate cancer [182,183,184,185]. However, the results of phase I and phase II trials with EB1089 and paricalcitol were less encouraging without significant anti-cancer effect [182,183,184,185]. In three clinical trials, vitamin D was used for patients with breast cancer, but the result of three trials also did not show significant association between vitamin D treatment and reduction of breast cancer incidence [186,187,188]. Many case-control studies have shown the significant inverse association between 25(OH)D, which is the commonly measured vitamin D metabolite in human, and risk of breast cancer [189,190,191,192,193,194,195]. However, in more defined nested case-control studies (listed in Table 1.5), most studies did not support the protective effects of vitamin D on breast cancer risk [196,197,198,199,200,201]. The clinical studies of vitamin D and vitamin D analogs in breast cancer are summarized in Table 1.4 to 1.6.

1.5 Synthetic oleanane triterpenoids (SOs)

Triterpenoids, synthesized from many plants by cyclization of squalene, are the largest group of phytochemicals with more than 20,000 triterpenoids occurring in nature [202]. The triterpenoids are further sub-classified into cucurbitanes, cycloartanes, dammaranes, euphanes, friedelanes, holostanes, hopanes, isomalabaticanes, lanostanes, limonoids, lupanes, oleananes, protostanes, squalenes, tirucallanes and ursanes [203,204]. Among those diverse natural triterpenoids, oleananes (also known as oleanolic acid, OA) has been shown to have anti-inflammation and anti-cancer effects *in vivo* [205]. However, the anti-inflammation and anti-cancer effects of naturally occurring OA were weak, therefore many SOs have been developed to improve the potency on anti-inflammation or anti-cancer activity [206,207]. With the screening of

over 300 new SOs for the anti-inflammation activity, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and its methyl ester (CDDO-Me) were found be most active ones [208,209,210,211]. Further modifications of CDDO produced CDDO-Im (imidazolide), CDDO-MA (methyl amide) and CDDO-EA (ethyl amide), exhibiting significantly increased bioactivity than CDDO [211,212].

1.5.1 Biological actions of SOs

Anti-inflammation: SOs effectively suppressed the *de novo* synthesis of cytokine-inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in primary culture of macrophages stimulated by wide ranges of pro-inflammatory molecules, such as interferon γ (IFNγ), tumor necrosis factor α (TNFα), interleukin 1β and lipopolysaccharide (LPS) both *in vivo* and *in vitro* [213,214,215,216]. The SOs also inhibited the production of inflammatory cytokines, including interleukin-6, from immune or cancer cells [217,218,219,220].

Cytoprotection: CDDO has been shown to interact with kelch-like ECH-associated protein 1 (Keap1) which induces ubiquitination and proteasomal degradation of nuclear factor (erythroid-derived)-like 2 (Nrf2). The interaction between CDDO or its derivatives with Keap1 dissociates Nrf2 from Keap1 and activates the phase 2 response [221,222]. The Nrf2-response genes, as an intrinsic mechanism to remove electrophilic or oxidative stress, include quinone reductase 1, γ-glutamylcysteine synthetase, thioredoxin, glutathione S-transferase, UDP-glucuronosyltransferase, epoxide hydrolase, superoxide dismutase and heme oxygenase 1 [223]. CDDO and its derivatives have been shown to be one of the most potent activator of the Nrf2 pathway *in vitro* and *in vivo* [224]. In addition, CDDO and its derivatives protected retinal pigment epithelial cells against photooxidative cytotoxicity induced by UVA radiation [224].

Anti-proliferation: The SOs have been shown to inhibit the proliferation of various malignant cells, such as breast cancer, colon cancer, melanoma, leukemia, and sarcomas [202,208]. CDDO, CDDO-Me and CDDO-Im modulated key regulatory molecules of cell proliferation, such as cyclin D1, p21, p27, PCNA, caveolin 1 and c-Myc in cancer cells [225,226,227]. In addition, the multiple signaling pathways that stimulate proliferation of cancer cells, including NF-κB, JAK/STAT3, PTEN/PI3K/Akt, mTOR and HER2, have been shown to be affected by CDDO, CDDO-Me and CDDO-Im [227,228,229,230,231,232,233].

Apoptosis: The SOs induced apoptosis in human cancer cells, including breast cancer [233]. Depending on the type of SOs and cells, the SOs induce apoptosis by different mechanisms. Studies demonstrated that CDDO and CDDO-Im induced apoptosis by activating extrinsic death-receptor-mediated pathway, whereas majority studies showed that CDDO-Me induced apoptosis through intrinsic mitochondria-mediated pathway [234,235,236,237,238,239,240]. Although there are some differences, overall studies demonstrated that cancer cells significantly increased ROS level with the treatment of CDDO and CDDO-Im but not of CDDO-me, contributing the induction of apoptosis [237,240,241,242]. Activation of JNK/p38, and inhibition of NF-κB and JAK/STAT signaling by SOs also have been shown to induce apoptosis in various cancer cells [131,228,243,244,245,246]. However, the first protein target triggering apoptosis by SOs has not been identified [247].

Differentiation: The SOs induced differentiation of human myeloid leukemia cells, neuronal differentiation of PC12 cells, adipocytic differentiation of 3T3L1 fibroblasts, osteoblastic differentiation of Saos-2 osteosarcoma cells, and megakaryocytic differentiation of normal hematopoietic progenitor cells [208,235,239,248,249]. In addition, the combination studies of synthetic triterpenoids with wide ranges of other molecules, including all-trans-retinoic acid, the RXR-specific ligand (LG100268), members of the transforming growth factor β family

and vitamin D analogs, demonstrated the additive or synergistic effects to induce terminal differentiation of leukemia cells [239,249,250].

1.5.2 *In vitro* and *in vivo* studies of synthetic triterpenoids in breast cancer

In the first screening for anti-cancer activity, CDDO demonstrated potent inhibitory effects on the proliferation of both ER-positive (MCF-7) and ER-negative (MDA-MB-231, MDA-MB-468, 21-MT-1, 21-MT-2, 21-NT and 21-PT) breast cancer cells [208]. Induction of p21 and repression of cyclin D1 causing the G1-S and G2-M cell cycle arrest have been identified key molecular mechanism of CDDO for anti-proliferation activity in MCF-7, MDA-MB-435 and MDA-MB-231 breast cancer cells [225]. The derivatives of CDDO, CDDO-Im and CDDO-Me were significantly more potent than CDDO for the inhibition of MCF-7 cell proliferation [216,251]. Moreover, the synthetic triterpenoids selectively sensitized breast cancer cells (T47D and MDA-MB-468) to TRAIL-induced apoptosis, whereas normal mammary epithelial cells (HMEC) were not sensitized by CDDO and CDDO-Im [252]. STAT3 was another key molecular target of synthetic triterpenoids in breast cancer cells, and the direct interaction between CDDO and STAT3 was demonstrated to cause the inhibition of STAT activation in MDA-MB-468 cells [228]. The proliferation of HER2-overexpressing mouse and human mammary tumor cells were repressed by CDDO-Me [253]. The tumorigenesis of MMTV-HER2/neu transgenic mice were also inhibited by CDDO-Me which showed synergistic inhibitory effects in combination with rexinoids, showing potent anti-cancer activity against HER2-overexpressing breast cancer [253]. CDDO-Me also delayed the mammary tumor development in the BRCA1 mutation mouse model and ER-negative PyMT mouse model [242,254]. The *in vitro* and *in vivo* studies of synthetic triterpenoids in breast cancer were summarized in Table 1.6 and Table 1.7, respectively.

1.5.3 Clinical studies of synthetic triterpenoids

In the phase I dose-escalation study, CDDO showed adverse thrombotic symptoms without significant anti-cancer activity, leading to early termination of the study [255]. In recent phase I study of patients with solid tumors and lymphomas, 40% of the patients achieved disease stabilization by CDDO-Me without significant adverse effect even in long-term treatment [256]. In addition to anti-cancer activity, recent clinical trials have shown that CDDO-Me also significantly improved kidney functions of patients in the clinical trials, suggesting CDDO-Me as a promising agent to treat chronic kidney disease [257,258]. World-wide phase 3 study is ongoing to evaluate the long-term clinical benefit of CDDO-Me in chronic kidney disease [247].

Table 1.1 Key features in different subtypes of breast cancer [3,6,12,45,259].

	Luminal (60-70%)		HER2-positive (20-30%)	Basal-like (15%)
	Luminal A	**Luminal B**		
ER	Positive	Positive	Positive in about half	Mostly negative
HER2	Usually negative	Positive	Positive	Mostly negative
Grade III	Usually not	About half	Mostly yes	Mostly yes
Prognosis in 5 year	Favorable	Relatively favorable	Generally adverse	Generally adverse
Current therapies	Endocrine	Endocrine/ HER2 targeted/ chemotherapy	HER2 targeted/ Chemotherapy	Chemotherapy
Potential therapies		PI3K-target drug, IGF-IR-target drug, FGF-target drug	PI3K-target drug, IGF-IR-target drug, multi-target kinase inhibitor, HER3-target drug, anti-angiogenesis drug	PARP inhibitor, EGFR-target drug, platinum salt, anti-angiogenesis drug
Additional properties	Good differentiation /Low Ki-67	Poor differentiation /High Ki-67		BRCA1 mutation is prevalent

Human epidermal growth factor receptor 2 (HER2); Estrogen receptor (ER); Phosphoinositide 3-kinase (PI3K); Poly (ADP-ribose) polymerase (PARP); Insulin-like growth factor-I receptor (IGF-IR); Epidermal growth factor receptor (EGFR); Fibroblast growth factor (FGF); Human epidermal growth factor receptor 3 (HER3)

Table 1.2 *In vitro* studies of vitamin D and vitamin D analogs in breast cancer

References	Compounds	Effects	Target molecules
Colston et al. (1992) [169]	1α,25(OH)$_2$D$_3$, /EB1089	• Inhibit proliferation of MCF-7 cells	
Simboli-Campbell et al. (1996)	1α,25(OH)$_2$D$_3$,	• Induced apoptosis in MCF-7 cells	• Protein: ↑TRPM-2/clusterin, ↑cathepsin B
James et al. (1996) [166]	1α,25(OH)$_2$D$_3$, /EB1089	• Induced apoptosis in MCF-7 cells	• Protein: ↓Bcl-2, ↑p53, ↑p21 • mRNA: ↑TRPM-2/clusterin
Simboli-Campbell et al. (1997) [164]	1α,25(OH)$_2$D$_3$, /EB1089	• Induced cell cycle arrest in G0/G1 phase and apoptosis in MCF-7 cells	• Protein: ↓Bcl-2, ↓ER
Wu et al. (1997) [260]	1α,25(OH)$_2$D$_3$ /EB1089	• Inhibited proliferation of MCF-7E (early passage), BT20, T47D and ZR75, not of MCF-7L (late passage)	• ↓Cdk2 kinases activity • Protein: ↑p21, ↑p27
Verlinden et al. (1998) [153]	1α,25(OH)$_2$D$_3$,	• Inhibited the proliferation of MCF-7 cells	• mRNA: ↓CycD1, ↑p21, ↑p27
Colston et al. (1998) [156]	EB1089 /CB1093	• Inhibited growth of MCF-7 and Hs578T cells	• Protein: ↑IGFBP3
Wu et al. (1998) [261]	1α,25(OH)$_2$D$_3$ /EB1089	• MCF-7E (sensitive, express TGF-β-RII) and MCF-7L (insensitive, no TGF-β-RII)	• mRNA: ↑TGF-β-RII in MCF-7E, -TGF-β-RII in MCF-7L
Mathiasen et al. (1999) [165]	1α,25(OH)$_2$D$_3$, /EB1089 /CB1093	• Induced apoptosis in MCF-7 and T47D cells	• Protein: ↓Bcl-2 • Caspase 3 and p53-independent apoptosis
Swami et al. (2000) [147]	1α,25(OH)$_2$D$_3$	• Inhibited basal and E_2-induced growth of MCF-7 cells	• ↓mRNA level of ER
Campbell et al. (2000) [262]	1α,25(OH)$_2$D$_3$	• Inhibited proliferation of MCF-7 cells	• ↑mRNA level of BRCA1
Koli et al. (2000) [173]	1α,25(OH)$_2$D$_3$ /Deltanoids	• Inhibited invasion of MDA-MB-231 cells	• ↓secretion of uPA and tPA, ↑secretion of PA inhibitor 1 • ↓MMP-9, ↑TIMP1 activity
Narvaez et al. (2001) [263]	1α,25(OH)$_2$D$_3$	• Induced apoptosis in MCF-7 cells	• Disruption of mitochondrial function: Bax translocation to mitochondria and cytochrome c release and production of ROS • Caspase-independent event

Table 1.2 Continued

Jensen et al. (2001) [154]	$1\alpha,25(OH)_2D_3$	• Inhibited proliferation of MCF-7 cells	• ↓cyclin D1/cdk4-associated kinase activity • Protein: ↑p21, ↓c-Myc
Mathiasen et al. (2002) [167]	$1\alpha,25(OH)_2D_3$ /EB1089	• Induced apoptosis in MCF-7 cells	• Up-regulate intracellular free calcium release and activation of calpain
Flanagan et al. (2003) [174]	$1\alpha,25(OH)_2D_3$ /EB1089	• Inhibited growth and invasion of SUM-159PT cells	• Protein: ↑p21, ↑p27, ↑cPARP, ↑released cytochrome c
Swami et al. (2003) [155]	$1\alpha,25(OH)_2D_3$	• Multiple gene regulation in MCF-7 and MDA-MB-231 cells	• MCF-7: ↑cell cycle and apoptosis (cyclin G1, cyclin I, PAK-1, p53 and IGFBP5); ↓ERα, growth factors, cytokines and kinases • MDA-MB-231: ↓genes for matrix metalloproteinases
McGaffin et al. (2004) [264]	$1\alpha,25(OH)_2D_3$	• Inhibited growth of MCF-7, T47D and BT474 cells, while did not affect growth of BT549 cells	• mRNA: ↓EGFR in MCF-7, T47D and BT549 cells, ↑EGFR in BT474 cells
Capiati et al. (2004) [265]	$1\alpha,25(OH)_2D_3$	• Inhibited serum-induced Erk1/2 in MCF-7 cells	• ↓Src tyrosine kinase activity • ↑VDR-Src association
Lee at el. (2006) [160]	Gemini vitamin D analogs	• Comparison of gene regulation in MCF10AT1 and MCF10CA1a cells	• Regulate many gene involved in cell proliferation, apoptosis, cell adhesion, invasion and angiogenesis as well as BMP and TGF-β signaling • More significant gene regulation in MCF10AT1 cells than MCF10CA1a cells
Lee et al. (2006) [161]	Gemini vitamin D analogs	• Activated BMP signaling in MCF10AT1 cells	• Protein: ↑pSmad1/5 • mRNA: ↑BMP2, ↑BMP6, ↓Smad6
O'Kelly et al. (2006) [266]	Gemini vitamin D analogs	• Inhibited proliferation of MCF-7 cells	• Protein: ↓Akt, ↓pFKHR, pmTOR, pS6K
Pendas-Franco et al. (2007) [267]	$1\alpha,25(OH)_2D_3$	• Regulated phenotype of MDA-MB-453 and MDA-MB-468 cells	• ↓Mesenchymal marker (N-cadherin) and ↓ myoepithelial markers (P-cadherin, α6-integrin, β4-intergrin and SMA)
Lee et al. (2007) [162]	Gemini vitamin D analogs	• Inhibited proliferation of MCF10AT1 cells in a BMP signaling-dependent manner	• Protein: ↑pSmad1/5 • mRNA: ↑BMP2, ↑BMP6 • ↑PKCα activity
Hussain-Hakimjee et al. (2009) [268]	$1\alpha,25(OH)_2D_3$	• Decreased mRNA level of ERα in BT-474 cells	• mRNA: ↓ERα

Testosterone repressed prostate message-2 (TRPM-2); Estrogen receptor (ER); Cyclin-dependent kinase 2 (Cdk2); Cyclin D1 (CycD1); Insulin –like growth factor binding protein-3 (IGFBP-3); Transforming growth factor β-receptor II (TGF-β-RII); urokinase-type Plasminogen activator (uPA); tissue Plasminogen activator (tPA); Plasminogen activator (PA); Matrix metalloprotease-9 (MMP-9); Tissue inhibitor of metalloprotease-1 (TIMP-1); Reactive oxygen species (ROS); Cyclin-dependent kinase 4 (Cdk4); cleaved Poly (ADP-ribose) polymerase (cPARP); Insulin-like growth factor binding protein-5 (IGFBP-5); Vitamin D receptor (VDR); Bone morphogenetic protein (BMP); phosphorylated Forkhead transcription factor (pFKHR); phosphorylated Mammalian target of rapamycin (p-mTOR); Smooth muscle actin (SMA); Protein kinase C alpha (PKCα)

Table 1.3 *In vivo* studies of vitamin D and vitamin D analogs in breast cancer

References	Compounds	Effects	Target molecules
Colston et al. (1992) [168,169]	EB1089 /MC903	• Repressed growth of NMU-induced mammary tumors	
Abe-Hashimoto (1993) [269]	22-oxa-calcitriol	• Suppressed the growth of MCF-7 and MX-1 xenograft tumors in immunodeficient mice	
Anzano et al. (1994) [270]	Ro24-5531	• Decreased incidence of NMU-induced mammary tumors • Enhanced tumor burden reduction in combination with tamoxifen	
Mehta et al. (1997) [148]	1α (OH)D_5	• Inhibited DMBA-induced preneoplastic lesions	• ↑VDR, ↑TGF-β1
Koshizuka et al. (1999) [271,272]	EB1089	• Showed additive effect with paclitaxel to inhibit growth of MCF-7 xenograft tumors in immunodeficient mice • Showed additive effect with all-trans-retinoic acid to inhibit growth of MCF-7 xenograft tumors in immunodeficient mice	
Mehta et al. (2000) [149]	1α (OH)D_5	• Decreased NMU-induced tumor incidence and multiplicity	
ElAbdaimi (2000) [175]	EB1089	• Inhibited the development of osteolytic bone metastasis in intracardiac injection model with MDA-MB-231 cells, and increased mice survival	
Flanagan et al. (2003) [174]	1α,25$(OH)_2D_3$ /EB1089	• Repressed growth and induced apoptosis of SUM-159PT xenograft tumors in immunodeficient mice	• IHC: ↓PCNA
Sundaram et al. (2003) [171]	EB1089	• Showed significantly higher rate of decline of tumor volume with ionizing radiation in MCF-7 xenograft tumors in immunodeficient mice	• IHC: ↓Ki-67
Mehta et al. (2004) [273]	1α (OH)D_5	• Showed selective inhibition of DMBA-induced mammary tumors during promotion or progression tumorigenesis	
Milliken et al. (2005) [274]	EB1089	• Suppressed the growth of mammary tumors in luteinizing hormone overexpressing transgenic mice, ↓BrdU	
Lee et al. (2008) [275]	Gemini vitamin D analogs	• Repressed tumorigenesis of NMU-induced rat mammary tumors	• Protein: ↑cPARP, ↑c-caspase-3, ↑p21, ↑IGFBP3
		• Inhibited growth of MCF10DCIS.com xenograft tumors in immunodeficient mice	• Protein: ↑IGFBP3, ↑p21, ↑pSmad1/5, ↓Apolipoprotein A-I
Lee et al. (2010) [276]	Gemini vitamin D analog	• Suppressed the growth of mammary tumors in MMTV-HER2/neu mice	• Protein: ↓pHER2, ↓pAkt, ↓pErk • IHC: ↓pHER2, ↓pAkt, ↓pErk

N-nitroso-N-methylurea (NMU); 7,12-dimethylbenz-α-anthracene (DMBA); Vitamin D receptor (VDR); Transforming growth factor β (TGF-β); Immunohistochemistry (IHC); Proliferation cell nuclear antigen (PCNA); cleaved Poly (ADP-ribose) polymerase (cPARP); Insulin-like growth factor binding protein-3 (IGFBP-3);Human epidermal growth factor receptor 2 (HER2); phosphorylated HER2 (pHER2), Bromodeoxyuridine (BrdU), Terminal deoxynucleotidyl transferase dUTP nick and labeling (TUNEL)

Table 1.4 Clinical trials of vitamin D and vitamin D analog in breast cancer

References	Participants	Design	Conclusion
Trivedi et al. (2003) [186]	649 postmenopausal women (65-85 years)	Vitamin D (100,000 IU), every 4 months for 5 years	Randomized trial: no significant anti-cancer effect
Lappe et al. (2007) [187]	1,179 postmenopausal women (>55 years)	Calcium (1,400-1,500 mg)+Vitamin D (1,100 IU), daily for 4 years	Randomized trial: fewer total cancer in calcium plus vitamin D supplement group ($p<0.05$) Not enough power for breast cancer alone
Chlebowski et al. (2008) [198]	36,282 postmenopausal women (50-79 years)	Calcium (1,000 mg) + Vitamin D (400 IU), daily for 7 years	Randomized trial: no significant anti-cancer effect
Amir et al. (2010) [188]	40 breast cancer patients with bone metastases	Vitamin D (10,000 IU), daily for 4 months	Phase II study: no significant changes in pain or bone turnover marker Indication of clinical benefits: Lower the number of sites of pain, Alleviation of hyperparathyroidism
Gulliford et al. (1998) [182]	25 advanced breast cancer patient	EB 1089 (0.15 to 17.0 μg/m^2), daily	Phase I dose escalation study: well tolerated around 7 μg/m^2·day No clinical response, 4/25 showed stabilization of disease for more than 3 months
Beer et al. (2001) [277]	15 patients with refractory malignancies, including 3 breast cancer patients	Vitamin D (0.06 to 2.8 μg/kg), once a week for 4 weeks orally	Phase I dose escalation study: 0.48 μg/kg (optimal dose), reaching serum vitamin D level associated with anti-cancer activity in preclinical studies. No anti-cancer activity was observed
Beer et al. (2007) [278]	37 patients with solid tumors	DN-101 (15 to 75 μg weekly oral administration	Phase I pharmacokinetic study: (MTD) 45 μg/week
Fakih et al. (2007) [279]	32 patients with advanced solid tumors, including 1 breast cancer patient	Vitamin D (10 to 96 μg weakly i.v. injection) + Gefitinib (250 mg/day)	Phase I dose escalation study: 74 μg/week (MTD), reaching serum vitamin D level associated with anti-cancer activity in preclinical studies. No anti-cancer activity was observed
Muindi et al. (2009) [280]	20 refractory solid tumor patients, including 1 breast cancer patient	Vitamin D (57 to 163 μg weekly i.v. injection) +Gefitinib (250 mg /day)+Dexamethasone (4 mg/day)	Phase I dose escalation study: 125 μg/week (MTD), reaching serum vitamin D level associated with anti-cancer activity in preclinical studies No anti-cancer activity was observed

International unit (IU); Maximum tolerated dose (MTD)

Table 1.5 Case-control studies of vitamin D in breast cancer

References	Cohort	Case / Control	Vitamin D cut-off	P_{trend}	Conclusion
Lowe et al. (2005) [189]	UK Caucasian population	179/179	Serum 25(OH)D level: 50 nM	<0.001	Significant association between low level of 25(OH)D and increased breast cancer risk in Caucasian women
Abbas et al. (2008) [190]	Germany	1,394/1,365	Serum 25(OH)D level: 50 nM	<0.0001	Significant association between low level of 25(OH)D and increased breast cancer risk in postmenopausal women
Crew et al. (2009) [191]	Long Island Breast Cancer Study Project	1,026/1,075	Daily intake: 40 ng/ml	0.0002	Significant association between low level of 25(OH)D and increased breast cancer risk in women
Rossi et al. (2009) [192]	Italy	2,569/2,588	Daily intake: 3.57 μg or 143 IU		Sufficient vitamin D intake has a protective effect against breast cancer in women
Anderson et al. (2010) [193]	Ontario Women's Diet and Health Study	3,101/3.471	Daily intake: 10 μg/d or 40 IU		Vitamin D intake is associated with reduced breast cancer risk in women
Kawase et al. (2010) [194]	Japan Aichi Cancer Center Hospital	1,803/3,606	Daily intake: 6.6 μg	<0.001	Significant inverse association between vitamin D intake and breast cancer risk in premenopausal women
Lee et al. (2011) [195]	Taiwan Taipei Hospital	200/200	Daily intake: 5 μg	0.02	Significant inverse association between vitamin D intake and breast cancer risk in premenopausal women

25-hydroxyvitamin D (25(OH)D); International unit (IU)

Table 1.6 Nested case-control studies of vitamin D in breast cancer

References	Cohort (n)	Case / Control	P_{trend}	Conclusion
Bertone-Johnson et al. (2005) [196]	Nurse's Health study (32,826)	701/724	0.06	High level of 25(OH)D may modestly associated with reduced risk of breast cancer
Freedman et al. (2008) [197]	Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (35,660)	1,005/1,005	0.81	No significant inverse association between 25(OH)D and breast cancer risk in postmenopausal women
Chlebowski et al. (2008) [198]	Women's Health Initiative (32,826)	895/898	0.20	No significant inverse association between 25(OH)D and breast cancer risk in postmenopausal women
McCullough et al. (2009) [199]	Cancer Prevention Study II Nutrition Cohort (21,965)	515/515	0.60	No significant inverse association between 25(OH)D and breast cancer risk in postmenopausal women
Almguist et al. (2010) [200]	Malmo Diet and Cancer Study (53,000)	764/764	NS	No significant inverse association between 25(OH)D and breast cancer risk
Engel et al. (2010) [201]	French E3N Cohort (17,391)	636/1,272	0.02	Significant association between decreased breast cancer risk and high 25(OH)D level in young women (<53 years old)

25-hydroxyvitamin D (25(OH)D); Not significant (NS)

Table 1.7 ***In vitro*** **studies of synthetic triterpenoids in breast cancer**

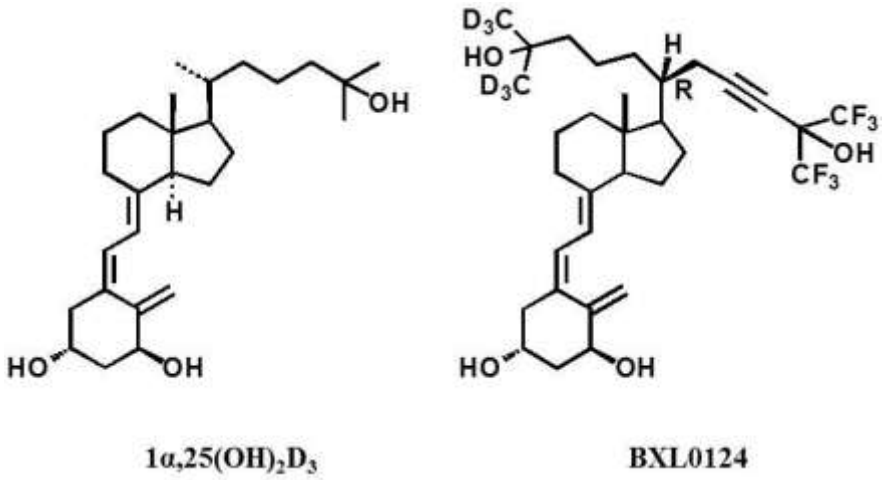
References	Compounds (Working Conc. Range)	Effects	Target molecules
Suh et al. (1999) [208]	CDDO (0.03-1 μM)	• Inhibit the proliferation of MCF-7, MDA-MB-231, 21-MT-1, 21-MT-2, 21-NT and 21-MT cells	
Lapillonne et al. (2003) [225]	CDDO (0.5-2 μM)	• Inhibit growth of MCF-7, MDA-MB-231 and MDA-MB-435 cells • ↓S phase, ↑G2-M, ↑apoptosis	• ↑PPARγ activity • mRNA: ↓CycD1, ↓Bcl2; ↑p21, ↑PPARγ
Place et al. (2003) [216]	CDDO (10-300 nM) and CDDO-Im (10-30 nM)	• Inhibit growth of MCF-7cells	
Honda et al. (2004) [251]	CDDO (0.16 μM) and CDDO-Me (0.05 μM)	• Inhibit the proliferation of MCF-7 cells	
Hyer et al. (2005) [252]	CDDO (0.5-2μM) and CDDO-Im (0.25-1μM)	• Sensitize T47D and MDA-MB-468 cells to TRAIL • Induce apoptosis	• Protein: ↑cPARP; ↓Caspase 8, ↓BID, ↓$FLIP_L$, DR5 • Flow cytometry: ↑DR4, ↑DR5
Konopleva et al. (2006) [227]	CDDO (1-5 μM)	• Inhibit growth of MCF-7/Neo, MCF-7/HER2, MDA-MB-435/Neo, MDA-MB-435/HER2	• Protein: ↓pHer2, ↓Her2, ↓CycD1
Ling et al. (2007) [229]	CDDO-Me (0.5 μM)	• Inhibit the proliferation of 4T1 cells • ↑G2-M phase, ↓cell invasion	• Protein: ↓pSTAT3, ↓pAkt, ↓pSrc, ↓c-Myc
Ahmad et al. (2008) [228]	CDDO-Me (1 μM)	• Inhibit the IL-6-iduced and constitutive JAK1 activation in MDMB-231 cells	• Protein: ↓pJAK1, ↓pSTAT3, ↓CycD1, ↓Survivin • CDDO-Me forms adducts with STAT3
Liby et al. (2008) [253]	CDDO-Me (0.3-1 μM)	• Inhibit the IL-6 induced STAT3 activation in E18-14C-27 and SK-BR-3 cells • Increased the TNF-α-repressed IκBα protein level in SK-BR-3 and MDA-MB-468 cells	
Kim et al. (2011) [242]	CDDO-Im and CDDO-Me (0.1-1 μM)	• Inhibit proliferation and induce apoptosis in BRCA1-mutated W780 and W0069 cells • DNA damage: ↑comet assay, ↑γH2AX	• Protein: ↑pChk1, ↑pChk2, ↑p21, ↓p-cdc2
Kim et al. (2012) [281]	CDDO-Me (0.1-1 μM)	• Inhibit the HER2 activation in BRCA1-mutated W780 cells	• Protein: ↓pHER2, ↓CycD1, ↑p21 • CDDO directly interact with HER2

Peroxisome proliferator-activated receptor γ (PPARγ); Cyclin D1 (CycD1); cleaved Poly (ADP-ribose) polymerase (cPARP); Death receptor (DR); Human epidermal growth factor receptor 2 (HER2); Signal transducer and activator of transcription 3 (STAT3); phosphorylated STAT3 (pSTAT3); Interleukin-6 (IL-6); Tumor necrosis factor-α (TNF-α); TNF-related apoptosis-inducing ligand (TRAIL); Janus kinase 1(JAK1); BH3 interacting-domain death agonist (BID); FLICE inhibitory protein (FLIP)

Table 1.8 ***In vivo*** **studies of synthetic triterpenoids in breast cancer**

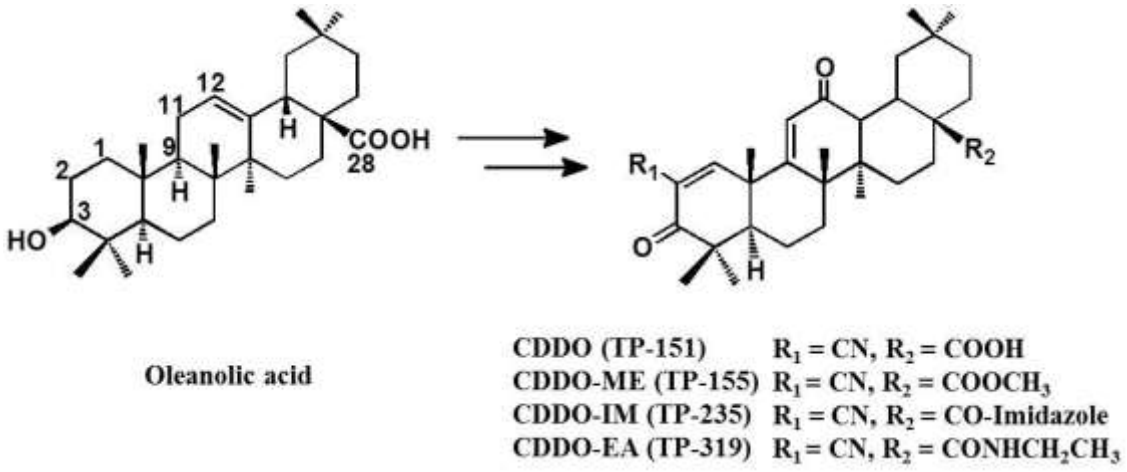
References	Compounds	Effects	Target molecules
Lapillonne et al. (2003) [225]	CDDO (i.v., 40 mg/kg/day, twice a week, 3 weeks)	• Repressed MDA-MB-435 xenograft tumor growth	
Konopleva et al. (2006) [227]	CDDO (i.v., 20 mg/kg/day, three times a week, 3weeks)	• Repressed the growth and burden of MCF-7/Neo or MCF-7/HER2 xenograft tumors	• IHC:↓ pHER2, ↓cycD1 • Tunnel assay: ↑apoptosis
Hyer et al. (2005) [252]	CDDO-Im (i.p., 5 mg/kg/day, 14 days)	• Repressed the growth of MDA-MB-468 xenograft tumor in combination with TRAIL	•
Ling et al. (2007) [229]	CDDO-Me (i.v., 200 μg/mouse; 5 times at 2-day intervals)	• Inhibit growth and lung metastasis of 4T1 xenograft tumors	• ↑Mature spleen dendritic cells
Liby et al. (2008) [253]	CDDO-Me (diet, 60-100 mg/kg, 4-45 weeks)	• Alone or combination with rexinoid delayed the development of mammary tumors in MMTV-HER2/neu transgenic mouse model	
Kim et al. (2012) [281]	CDDO-Me (diet, 50 mg/kg, 12-28 weeks)	• Delayed the mammary tumor development in Brca1-deficient mouse	• Protein: ↓pHER2, ↓HER2, ↓cycD1, ↓γH2AX

Intravenous (i.v.); Intraperitoneal (i.p.); Immunohistochemistry (IHC); Human epidermal growth factor receptor 2 (HER2); phosphorylated HER2 (pHER2); Cyclin D1 (cycD1); TNF-related apoptosis-inducing ligand (TRAIL); Mouse mammary tumor virus (MMTV)



1α,25(OH)$_2$D$_3$ BXL0124

Fig 1.1 Structures of 1α,25(OH)$_2$D$_3$ and Gemini vitamin D analog BXL0124.



Oleanolic acid

CDDO (TP-151) $R_1 = CN, R_2 = COOH$
CDDO-ME (TP-155) $R_1 = CN, R_2 = COOCH_3$
CDDO-IM (TP-235) $R_1 = CN, R_2 =$ CO-Imidazole
CDDO-EA (TP-319) $R_1 = CN, R_2 = CONHCH_2CH_3$

Fig. 1.2 Structures of Oleanolic acid, CDDO and CDDO derivatives.

Chapter 2: Differential expression of key signaling proteins in MCF10 cell lines, a human breast cancer progression model

2.1 Introduction

Human breast cancer is a heterogeneous disease which evolves through a multistep process of accumulating genetic changes such as gene mutations, rearrangements and copy number amplifications, loss of heterozygosity, and epigenetic alterations [282,283,284]. Breast cancer originates as benign hyperplasia of mammary duct epithelial cells, and progresses sequentially to atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC), which eventually metastasizes to distant organs [285]. Various stages commonly co-exist in a single tumor, suggesting that progression of breast cancer is not a linear process.

DCIS, which is non-invasive disease with neoplastic epithelial cells growing within the confined microenvironment of duct, has been demonstrated already equipped with invasive potential and the biological diversity found in IDC [286]. Although the precise initiating processes of mammary tumorigenesis is still poorly understood, DCIS has been generally recognized as the precursor of invasive breast cancers [287,288]. In the early progression from hyperplasia to DCIS, multiple pathological events are occurred, for example loss of heterozygosity has been observed in more than 70% of high-grade DCIS as compared to 35-40% in atypical hyperplasia [289,290]. However, along the progression from DCIS to IDC, most of genetic or molecular alterations are already present at DCIS stage [291,292]. Moreover, the diversity of genetic background in patients has been a limiting factor to identify DCIS with high risk to progress into IDC [292].

Recent studies demonstrated that the activation of autophagy is critical for DCIS cells to avoid hypoxia-induced cell death and progress to IDC since the growing DCIS cells are confined within the duct with limited blood supply [293,294]. In addition, other studies demonstrated that both cancer cells and the tumor-associated microenvironment, such as extracellular matrix and stromal cells, are critical contributors for the progression of DCIS to IDC [61,295,296]. Many of these features cannot be recapitulated in usual *in vitro* culture system with single breast cancer cell. Therefore, better *in vitro* and *in vivo* models should be developed and utilized to understanding the transition from DCIS to IDC.

2.2 The MCF10 human breast cancer progression model

The MCF10 model is a series of cell lines that originated from the human breast epithelial cells, MCF10A [297]. The MCF10 model, including MCF10A, MCF10AT1, MCF10DCIS.com and MCF10CA1a cells, shares the same genetic background and offer a unique model to study breast cancer progression in a cell culture system.

The MCF10A cells are spontaneously immortalized, non-malignant cells obtained from a patient with fibrocystic breast disease [297]. The MCF10A cell line is considered normal because it does not show any characteristics of invasiveness and does not form tumors when transplanted into immunodeficient mice [298]. MCF10AT1 is a pre-malignant cell line produced by transfection of MCF10A with constitutively active HRAS [297,299]; it forms simple ducts and lesions resembling human ADH and DCIS when transplanted into immunodeficient mice [300]. Approximately 25% of the MCF10AT1 cells transplanted into mice eventually produce IDC which indicates tumorigenic potential of MCF10AT1 with slow progression [297]. MCF10DCIS.com is a cell line cloned from cell culture of a MCF10AT1 xenograft lesion. The MCF10DCIS.com cells reproducibly form DCIS-like comedo lesions that spontaneously progress

to IDC as xenografts in immunodeficient mice [301]. MCF10CA1a is the most malignant and aggressive cell line from the MCF10 series; it was derived from the MCF10AT1 cells by multiple passages through immunodeficient mice. The MCF10CA1a cells rapidly generate large tumors without evidence of a precursor stage. In addition, intravenously injected MCF10CA1a cells readily produce tumors in the lungs of immunodeficient mice, indicating metastatic potential of the MCF10CA1a cells [302].

The MCF10 cell lines offer the opportunity to study genetic and molecular events during cancer progression from normal mammary epithelium to metastatic IDC within same genetic background [61,285,303,304,305,306]. Results from gene profiling studies of the MCF10 cell series are summarized in Table 2.1.

2.3 Materials and methods

2.3.1 Cell culture

MCF10A, MCF10AT1, MCF10DCIS.com and MCF10CA1a breast cancer cell lines were provided by Dr. Fred Miller at the Barbara Ann Karmanos Cancer Institute (Detroit, MI) [301]. MCF10A, MCF10AT1, MCF10DCIS.com and MCF10CA1a cells were maintained in DMEM/F12 medium supplemented with 5% horse serum, 1% penicillin/streptomycin, and 1% HEPES solution at 37^{o}C, 5% CO_2. For MCF10A and MCF10AT1 cells, 10 μg/ml insulin, 20 ng/ml EGF, 0.5 μg/ml hydrocortisone, and 100 ng/ml cholera toxin were additionally supplemented into culture medium.

2.3.2 Animal experiments in the xenograft model

MCF10AT1, MCF10DCIS.com and MCF10CA1a cells were trypsinized and prepared in Hanks' Balanced Salt Solution (Invitrogen, Carlsbad, CA). After the midline incision around

second teat in immunodeficient mice, 1×10^6 cells were injected in the mammary fat pad. The incision was closed by wound clips and the clips were removed after 4 days. Tumor size was measured twice a week.

2.3.3 Western blot analysis

The protein samples were separated on 4-15% SDS-PAGE gels (Biorad, Hercules, CA) followed by transfer to a polyvinylidene fluoride membrane (PALL, Ann Arbor, MI). The membranes were blocked with 5% milk in Tris buffer for 1 h and then incubated with the appropriate primary antibody solutions overnight at 4°C. The membranes were washed with Tris buffer, and incubated with horseradish peroxidase conjugated secondary antibody solutions for 1 h at room temperature. The protein bands were visualized using a chemiluminescence based kit from Amersham Biosciences (Buckinghamshire, UK). The primary antibodies, CD44, which recognizes all CD44 splicing variants, cyclin D1, c-Myc, Pak4, COX-2 were from Santa Cruz Biotechnology (Santa Cruz, CA); CD44 containing variant domain 3 (CD44v3) and CD44 containing variant domain 6 (CD44v6) was from R&D System (Minneapolis, MN); pErk, pAkt, STAT3 and Smad4 were from Cell Signaling Technology (Beverly, MA); β-actin was from Sigma (St. Louis, MO); and secondary antibodies were from Santa Cruz Biotechnology.

2.4 Tumorigenicity of MCF10 cell lines *in vivo*

Tumorigenicity and tumor growth rate of the MCF10AT1, MCF10DCIS.com and MCF10CA1a cells were examined *in vivo*. One million cells from each cell line except the MCF10A were injected into the mammary fat pad area of immunodeficient mice and the tumor volumes were measured. The MCF10AT1 cells did not form palpable tumors up to 60 days after

the injection (n=4) (Fig. 2.1). Compared to previous studies that reported sporadic incidence of IDC [297], we used fewer MCF10AT1 cells ($1x10^6$ cells per mouse) and a shorter experimental period which likely decreased the probability of producing IDC. In contrast, the MCF10DCIS.com cells formed tumors in all injected mice (n=9). The average tumor volume reached 1.03 ± 0.54 cm^3 at 45 days after the injection (Fig. 2.1). The MCF10CA1a cells also formed tumors in all mice (n=10), and the average tumor volume reached 1.79 ± 0.92 cm^3 at 21 days after the injection (Fig. 2.1) indicating a faster growth of the MCF10CA1a tumors compared to that of the MCF10DCIS.com tumors. These data confirm the different tumorigenic potential of MCF10 cell lines *in vivo*.

2.5 Key factors in the HRAS-driven initiation stage of breast cancer

Transformation of MCF10A cells with HRAS produced the MCF10AT1 cell line which showed markedly increased protein levels of c-Myc, cyclin D1 and IGF-IR (Fig. 2.2A). The other two cell lines derived from MCF10AT1 cells, MCF10DCIS.com and MCF10CA1a cells, also exhibit the increased protein levels for c-Myc, cyclin D1 and IGF-IR (Fig. 2.2A). c-Myc, a transcription factor and key regulator of cell proliferation known to contribute to breast cancer development and progression, has been found overexpressed in 45% of breast tumors [307]. Ras enhances the level of c-Myc by stabilizing the c-Myc protein [308]. Furthermore, a study which crossed MMTV/vHa-Ras and MMTV/c-myc transgenic mouse strains demonstrated a synergistic action of these two oncoproteins in accelerating mammary tumor formation [309]. Amplification of c-Myc in the MCF10A cell line has been recently reported [303,310], suggesting a collaborative nature of HRAS and c-Myc aberrant activity to initiate breast cancer. Cyclin D1 is a key regulator of cell cycle and one of the most frequently overexpressed oncoproteins in breast cancer [311]. The expression of cyclin D1 has been shown to be necessary for the transforming

activity of HRAS [312,313]. Moreover, cyclin D1-null mice showed remarkable resistance to mammary tumorigenesis driven by the NEU or RAS oncogene [314]. IGF-IR has critical roles in breast cancer growth, survival and transformation [315]. A systematic review of results from clinical studies revealed an association between high concentrations of circulating IGF-I and an increased risk of breast cancer in pre-menopausal women [316]. Our data suggest that c-Myc, cyclin D1 and IGF-IR contribute to HRAS-driven cancer initiation stage.

2.6 Key signaling proteins in malignant transformation of the MCF10 model by spontaneous mutagenesis

pErk, pAkt, Stat3 and Pak4 were highly expressed only in cell lines that form tumors quickly in immunodeficient mice–MCF10DCIS.com and MCF10CA1a (Fig. 2.2B). Importantly, their protein levels were markedly increased in the more aggressive MCF10DCIS.com and MCF10CA1a cells when compared with the MCF10AT1 cells (Fig. 2.2B). Erk and Akt are central protein kinases that mediate cellular responses to a diverse range of extracellular stimuli, including growth factors and cytokines, to regulate cell cycle progression and cell motility [317]. Although both the Erk pathway and the PI3-kinase activity can be stimulated by transfection of activated Ras [306], the high level of activated forms of Erk and Akt - pErk and pAkt - is found only in MCF10DCIS.com and MCF10CA1a cell lines, indicating that overactivation of Erk and Akt might be critical for developing malignant breast cancer. Moreover, the most common activating PIK3CA mutation in human cancers (H1047R) [318,319] has been detected in the MCF10CA1a cell line, but not in MCF10A and MCF10AT1 cells [303,306], suggesting the PIK3CA activating mutation as a critical genetic alteration of malignant phenotype in both human breast cancer and the MCF10 model. Activated Stat3 is found in approximately 70% of breast tumors and is often associated with invasive and metastatic phenotype of cancers [320].

Moreover, recent studies demonstrated that Stat3 has a crucial role in inducing and maintaining pro-carcinogenic inflammatory microenvironment during cancer progression [115]. Pak4, a serine/threonine kinase, was found to be highly expressed in human breast cancer and to be associated with a signaling pathway leading to malignant transformation [321,322,323]. A recent study demonstrated that overexpression of Pak4 in normal mammary epithelial cells disrupted the cell polarity and led to the formation of mammary tumors in immunodeficient mice [323].

2.7 Key proteins contributing to breast cancer progression in the MCF10 model

CD44v, CD44v3, CD44v6, HER2, COX-2 and Smad4 showed a gradual increase in protein expression from MCF10A to MCF10CA1a cells (Fig. 2.2C). We hypothesized that this group of proteins might be associated with breast cancer progression since their increased expression corresponds to increased cellular malignancy in the MCF10 model. CD44, encoded by a single gene, has multiple isoforms produced by alternative splicing of 10 variable exons [324]. CD44 is a cell-surface glycoprotein involved in cell-cell and cell-extracellular matrix interactions [325]. CD44 functions as a receptor for hyaluronan and other extracellular ligands such as osteopontin, collagens, and matrix metalloproteinases (MMPs) to mediate responses from the microenvironment, which lead to cancer cell survival and invasion [325]. Interestingly, CD44 has been used as a key cancer stem cell surface marker in various malignancies including breast cancer [325]. Recent studies demonstrated that activation of CD44 by high molecular weight hyaluronan stabilizes multidrug-resistant proteins in the cell membrane, suggesting a role of CD44 in drug resistance [326]. CD44 also has a role as a co-receptor for multiple receptor tyrosine kinases such as HER2, IGF-IR, epidermal growth factor receptor (EGFR) and Met receptor in cancer cells [327,328,329]. With the wide range of functions, many preclinical studies targeting CD44 with siRNA, antibodies, transcriptional inhibitors, and competitive antagonists

have shown inhibition of cancer cell growth, tumorigenesis, and metastasis [330,331,332,333,334]. Our results indicate that decreased expression of CD44s (the standard 85-kDa isoform) and increased expression of CD44v (the variants 100 to 250-kDa isoform) correlate with the increasing malignant potential of the MCF10 cell lines (Fig. 2.2C). A Western blot analysis with antibodies which specifically recognize CD44v3 and CD44v6 confirmed the increased expression of both of these isoforms in malignant MCF10 cells (Fig. 2.2C). Our findings suggest that CD44v, but not CD44s, may be associated with aggressive and invasive breast cancer. The CD44-hyaluronan interaction activates the HER signaling, induces the transcription of COX-2 [335], and also activates IGF-IR [328]. CD44, particularly the CD44v6 isoform, acts as a co-receptor for Met receptor through HGF binding, which in turn activates Stat3 and leads to tumor progression and invasion [336]. The CD44- phosphorylated ERM (ezrin-radixin-moesin) proteins initiate the activation of TGF-β-RII and downstream Smad signaling [337]. HER2 is overexpressed in approximately 20-30% of invasive breast cancer and is strongly associated with poor patient survival [8]. A recent study demonstrated that overexpression of HER2 in the MCF10A cell line induced epithelial-mesenchymal transition and cell invasion [338]. Our results show gradually increased expression of HER2 proteins from MCF10AT1 to MCF10CA1a cells (Fig. 2.2C), suggesting a critical role of HER2 in breast cancer progression and invasion. COX-2 is one of the downstream targets of HER2 signaling pathway; a strong correlation between COX-2 and HER2 expression has been revealed in a large clinical investigation [339,340]. Moreover, up-regulated COX-2 expression has been associated with aggressive DCIS phenotype in both ER-positive and ER-negative breast cancers [341,342]. Our results also show a corresponding pattern between COX-2 and HER2 protein expression and its correlation to malignant potential in the MCF10 cell model (Fig. 2.2C).

2.8 Breast cancer invasion and metastasis

Metastasized tumor growth at distant sites is the main cause of death from breast cancer [343]. Approximately 40% of early-stage breast cancer patients relapse and ultimately die of metastatic cancer, but accurate prediction of the risk of metastasis is still not possible [343]. Comprehensive molecular profiling of the transition from DCIS and IDC to metastatic cancer has not yet identified tumor stage-specific signatures [61]. Malignant precursor cells with metastatic capacity may already develop at early stages of tumorigenesis [293,344]. In addition, stromal cells in the environment surrounding the primary tumor are involved in facilitating metastasis [345]. Therefore, both tumor microenvironment and epithelial cells have to be considered in tumor invasion and metastasis. Among the panel of MCF10 cell lines, the MCF10DCIS.com cells are particularly interesting because they can form DCIS-like lesions which spontaneously progress into IDC in immunodeficient mice [60]. MCF10DCIS.com xenograft tumors showed increased expression of stromal cell-derived factor-1 (SDF-1) in stromal cells, which is known to be highly induced by tumor-associated fibroblasts, and increased expression of CXCR4, the receptor of SDF-1, in epithelial cancer cells during the DCIS to IDC transition [60]. Although the MCF10DCIS.com xenograft model mimicked some aspects of the dynamic interaction between epithelial cancer cells and stromal cells, the utility of such model might be limited because of human-mouse differences in the epithelial-stromal interaction. In the present study, we did not attempt to identify molecules associated with cancer cell invasion and metastasis because the influence of microenvironment cannot be fully reproduced in the cell culture system we used for our study.

2.9 Conclusion

Results of this study have identified differences in the expression level of several key signaling proteins among 4 cell lines of the MCF10 series, a model representing different stages of breast cancer. By linking the observed changes to capability of the cell lines to form xenograft tumors in immunodeficient mice – from immortalized but non-malignant to highly malignant and invasive – the analyzed proteins were grouped based on their potential roles in tumor development. We concluded that c-Myc, cyclin D1, and IGF-IR may have a role during the initiation stage of cancer development since their increased levels were found in all HRAS-transformed cells including those that did not form tumors in the present xenograft experiment. In contrast, high expression of pErk, pAkt, Stat3 and Pak4 was observed only in cell lines that form tumors in immunodeficient mice which suggests that these proteins are activated later and may be important for the maintenance of malignancy. CD44v, CD44v3, CD44v6, HER2, COX-2 and Smad4, which showed protein expression gradually increasing from non-malignant to highly malignant and invasive cells, may represent a group associated with breast cancer progression. CD44 is a particularly interesting protein whose multiple isoforms are likely involved in various stages of cancer development; our results showing an association between the increasing expression of CD44v and increasing malignant potential of the MCF10 cell lines suggest a benefit of high levels of CD44v for the cancer cell malignant progression possibly by activating multiple signaling pathways through receptor kinases. Mapping of the complex network of molecular interactions leading to the selection of increasingly more aggressive cancer cells during progression of breast cancer requires experimental models that can be relatively easily studied, such as the MCF10. Although development of malignancy is a continuous process of cellular selection driven by an increasing capacity to proliferate and manage resources, and a decreasing capacity to die and interact with the environment, it may be mediated by a relatively small

number of factors. Identification of these critical elements will ultimately lead to the design of more efficient therapeutics and better prognosis for cancer patients.

Table 2.1 Protein and gene profiling studies using the MCF10 breast cancer progression model [346].

Reference	Analysis	Differential expression of genes or proteins in the MCF10 breast cancer progression
Worsham et al. (2006) [310]	Multiplex ligation-dependent probe amplification assay (MLPA)	• MCF10A: Loss of one copy of CCND2 and IGF-IR / Homozygous loss of ERBB2, CDKN2A and CDKN2B gene / Gain of MYC • MCF10AT1: Restoration of ERBB2 and CCND2 / Gain of IL13, VEGF, HRAS, TRAF2 • MCF10CA1a: Gain of BCAS2, IL12A and MME / Restoration of IGF1R
Rhee et al. (2008) [304]	Comparative microarray analysis	• Down-regulation of TNFSF7, S100A4, S100A7, S100A8 and S100A9, and KLK5 and THBS1 were associated with transformation and progression of breast cancer in MCF10AT model • Demonstrated that the down-regulation of genes in malignant cell lines can be epigenetically reversed
Marella et al. (2009) [347]	Spectral karyotyping, Array comparative genomic hybridization (aCGH) and cDNA microarray	• Up-regulated genes in MCF10CA1a: SEPP1, DCN, FBN1, PTGER2, AOX1, MUC1, MMP2, FN1, RB1, CDKNB1, CCND3, IL7 and IL18 • Down-regulated genes in MCF10CA1a: CDH1, IL1B, S100A14, BDKRB2, VEGF, BRAF, ERBB2, EGFR, HRAS, MYC, PTEN, IL1A and IL1B
Kadota et al. (2010) [303]	Combination of high density SNP analysis and mutation analysis by sequencing	• MCF10A: CDKN2A deletion and MYC amplification • MCF10AT1: HRAS activation • MCF10CA1h: activating PIK3CA mutation / LRP1B, FHIT and CDH13 deletion • MCF10CA1a: activating PIK3CA mutation / LRP1B and RUNX1 deletion
Kim et al. (2009) [306]	IHC and western blot analysis	• Increased Ras, Rac, Rho and active forms of PDK1, eIF4E and 4E-BP1 protein level in malignant cells of MCF10AT model • Western blot of cell lines and immunohistochemistry of xenograft tumor demonstrated elevated expression of phospho-AKT and phospho-FOXO 1,3a and 4
Choong et al. (2010) [305]	Proteome-wide analysis	• The cancer progression of MCF10AT1 model is associated with a major-reprogramming in metabolism • MCF10CA cell lines: increased expression of AK1 and ATOX1 / decreased expression of HIST1H2BM
Mbeunkui et al. (2007) [348]	Analysis of conditioned medium proteome using LC-MS/MS	• Differential expression of secreted proteins in MCF10AT model • High secretion level of alpha-1-antichymotrypsin and galectin-3-binding protein in MCF10DCIS.com and MCF10CA cell lines • Galectin-3-binding protein has been associated with aggressiveness of other types of cancers
Chen et al. (2007) [349]	Combination of phosphotyrosyl affinity enrichment, iTRAQ and LC-MS/MS analysis	• TOLLIP, WBP2, NSFL1C, SLC4A7, CYFIP1 and RPS2 were detected as novel proteins that underwent differential phosphorylation during breast cancer progression in MCF10AT model
Wang et al. (2008) [350]	Combination of membrane extraction and lectin affinity methods with LC-MS/MS analysis	• Differential expression of membrane glycoprotein in MCF10AT model • CD44, Gamma-glutamyl hydrolase, Galectin-3-binding protein and Syndecan-1 were associated with malignant breast cancer cell lines

Single-nucleotide polymorphism (SNP); Immunohistochemistry (IHC); Isobaric tags for relative and absolute quantitation (iTRAQ); Liquid chromatography (LC); Mass spectrometry (MS)

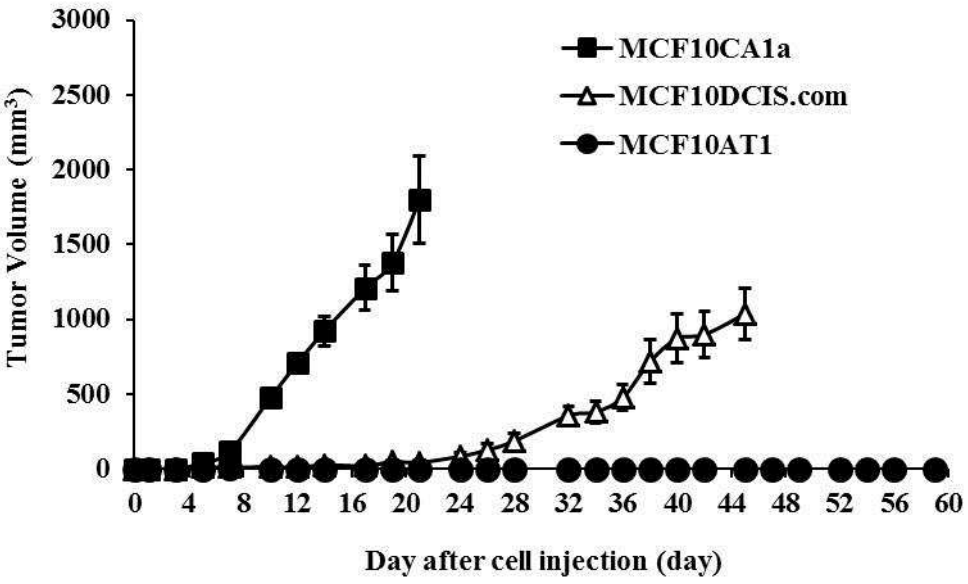


Fig. 2.1 Comparison of xenograft tumor growth of MCF10 series of human breast cancer cells. MCF10AT1 (n=4), MCF10DCIS.com (n=9) or MCF10CA1a (n=10) cells were injected into mammary fat pad area of immunodeficient nu/nu mice ($1x10^6$ cells per animal) as described previously [275].

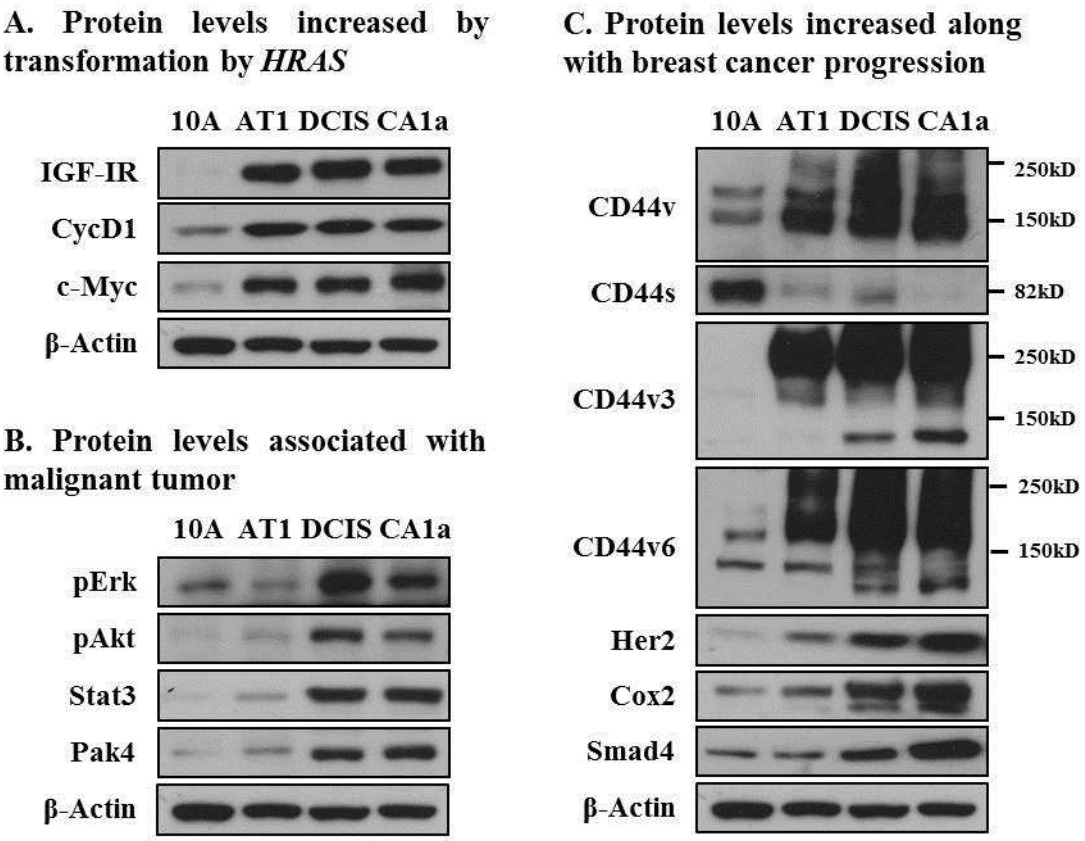


Fig. 2.2 Comparison of the protein levels for key signaling molecules among MCF10A, MCF10AT1, MCF10DCIS.com and MCF10CA1a cell lines. The four cell lines were incubated in the culture medium (DMEM/F12 supplemented with 5% horse serum, 1% penicillin/streptomycin, 10 μg/ml insulin, 20 ng/ml EGF, 0.5 μg/ hydrocortisone, and 100 ng/ml cholera toxin) at 37℃, 5% CO_2 for 2 days. The expression level of key signaling proteins was analyzed by western blot. **(A)** c-Myc, cyclin D1 and IGF-IR, whose protein levels are up-regulated in MCF10AT1, MCF10DCIS.com and MCF10CA1a cell lines, are categorized as a HRAS transformation-induced molecular event. **(B)** pErk, pAkt, Stat3, Pak4, whose protein levels are increased in MCF10DCIS.com and MCF10CA1a cell lines, are categorized as contributors of tumor malignancy. **(C)** CD44v, CD44v3, CD44v6, Her2, COX-2 and Smad4, whose protein level is gradually increased from MCF10A to MCF10CA1a cell lines, are categorized as markers of breast cancer progression.

Chapter 3: Repression of the expression of a stem cell marker CD44 by a novel Gemini vitamin D analog in basal-like breast cancer

3.1. Introduction

Conventional chemotherapies have been able to effectively shrink solid tumors, but in some cases the chemotherapies have not produced long-term clinical remissions without recurrence and metastasis [351]. Recently, it has been suggested that cancer stem cells, which represent a subset of tumor cells, are responsible for the origin and maintenance of tumors. Moreover, cancer stem cells are believed to be the main cause of metastasis and recurrences of cancer because of their strong tumor initiating abilities and resistance to conventional therapies [351,352,353].

A transmembrane glycoprotein, CD44, first known to be involved in cell-cell interaction and cell adhesion, has been identified as a key cell-surface marker for cancer stem cells in pancreas cancer, prostate cancer, head and neck squamous cell carcinoma and breast cancer [354,355,356,357]. Induction of CD44 expression in human breast cancer cell lines has been shown to enhance self-renewal, mammosphere growth, and drug resistance, demonstrating functional roles of CD44 in breast cancer stem cells [358]. CD44 is also known as an important mediator for the response of cells to their cellular microenvironment [325]. Al-Hajj *et al* first identified breast cancer stem cells from human breast cancer specimens, which are rich in $CD44^{+}/CD24^{-/low}$ cells, and showed that this distinct population of cells had exclusive ability to form tumors in mice [357]. A clinical study also indicated that $CD44^{+}/CD24^{-/low}$ cells were enriched in residual breast cancers after conventional therapies [359].

The MCF10DCIS.com cell line is one of the derivatives of the MCF10A series, which is a unique human model of breast tumor progression reflecting basal-like breast cancer [301,310].

Behbod *et al* showed that MCF10DCIS.com cells also contained $CD44^{+}/CD24^{-/low}$ subpopulations that formed a large number of DCIS-like lesions in xenografted mammary tumors [360]. Moreover, the bipotential progenitor properties of MCF10DCIS.com cells, which give rise to not only epithelial cells but also myoepithelial cells in mouse xenografts, demonstrate the ability of generating heterogeneous cell populations [61], suggesting that the MCF10DCIS.com cell line might be a useful model for studying the efficacy of preventive and therapeutic agents for inhibiting breast cancer stem cells.

In our previous study, novel Gemini vitamin D analogs inhibited the growth of MCF10DCIS.com cells *in vitro* and *in vivo*. [275]. Because of the importance of CD44 as a cancer stem cell marker and its suggested functional roles in breast cancer, we hypothesized that $1\alpha,25(OH)_2D_3$ or Gemini vitamin D analogs might regulate the proliferation of MCF10DCIS.com cells, which contains high proportion of $CD44^{+}$ breast cancer stem cell subpopulation, by targeting CD44. In this chapter, we examined the effects of Gemini vitamin D analog BXL0124 on regulation of CD44 in culture MCF10DCIS.com cells *in vitro* and in MCF10DCIS.com xenograft tumors *in vivo*.

3.2. Material and methods

3.2.1. Reagents and cell culture

$1\alpha,25(OH)_2D_3$ and Gemini vitamin D analog BXL0124 [1α,25-dihydroxy-20R-21(3-hydroxy-3-deuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-cholecalciferol, refer to 2S-1 in reference [361], >95% purity)] (Fig. 1A) were provided by BioXell, Inc. (Nutley, NJ) and dissolved in dimethyl sulfoxide (DMSO). For *in vivo* animal experiments, BXL0124 was diluted in cremophore/PBS (1:8, v/v) or in sesame oil (Sigma, MO) for i.p. injection or oral administration, respectively. The human MCF10DCIS.com breast cancer cell line was provided

by Dr. Fred Miller at the Barbara Ann Karmanos Cancer Institute (Detroit, MI) [301]. MCF10DCIS.com and MCF10CA1a cells were maintained in DMEM/F12 medium supplemented with 5% horse serum, 1% penicillin/streptomycin, and 1% HEPES solution at $37^{o}C$, 5% CO_2.

3.2.2. Quantitative real-time polymerase chain reaction (PCR)

The procedures have been previously reported [160]. Labeled primers, including glyceraldehyde-3-phosphate dehydrogenase, osteopontin and CD44 were obtained from Applied Biosystem (Carlsbad, CA).

3.2.3. Transient transfection of CD44 and promoter assay

The detailed procedures have been described previously [362]. pCMV-β-gal vector was provided by Dr. David Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX). CD44 promoter- and p53 binding site-mutated CD44 promoter-luciferase reporter vectors were provided by Dr. Robert A. Weinberg (Whitehead institute for Biomedical Research, Massachusetts Institute of Technology, Cambridge, MA). For the transient transfection, vectors were mixed with FuGene6 Transfection Reagent (Invitrogen, Carlsbad, CA), and MCF10DCIS.com cells were incubated with the mixtures for 6 h in serum free DMEM/F12 medium. Then, cells were treated with compounds for 24 h in 0.1% bovine serum albumin (BSA) containing DMEM/F12 medium. Luciferase activity was measured with a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) and normalized for β-galactosidase activity.

3.2.4. Western blot analysis

The procedure was described previously [362] and the primary antibodies, CD44, which recognizes all CD44 splicing variants was from Santa Cruz Biotechnology (Santa Cruz, CA);

CD44 containing variant domain 3 (CD44v3) and CD44 containing variant domain 6 (CD44v6) was from R&D System (Minneapolis, MN); vitamin D receptor (VDR) was from Thermo Scientific (Lafayette, CO); β-actin was from Sigma (St. Louis, MO); and secondary antibodies were from Santa Cruz Biotechnology.

3.2.5. Fluorescence microscopy

MCF10DCIS.com cells were incubated in glass bottom dishes (MatTek, Ashland, MA) with or without BXL0124 (10 nM). Cells were fixed with 4% paraformaldehyde, blocked with 10% BSA, and then incubated sequentially with CD44 primary antibody (Santa Cruz, CA) (1:100), fluorophore conjugated secondary antibody (Alexa Fluor® 488, Invitrogen, Carlsbad, CA) and 4,6-diamidino-2-phenylindole (DAPI). The cells were irradiated with green laser at 488 nm for detection of CD44 and with UV light at 364 nm for nuclear staining by DAPI.

3.2.6. Animal experiments in the xenograft model

The detailed procedure for the injection of MCF10DCIS.com cells was described previously [275]. To determine mammary tumor growth, MCF10DCIS.com cells were injected into the mammary fat pad area in severe combined immunodeficient (SCID) mice (4 per group), and mice were sacrificed at 1, 2, 3, 4 and 5 weeks after injection. Tumors were collected for further analysis. To test the anti-cancer activity of Gemini vitamin D analog BXL0124, MCF10DCIS.com cells were xenografted into the mammary fat pad area in nu/nu or SCID mice. Then, vehicle control (0.1 ml) or Gemini vitamin D analog BXL0124 (0.03 or 0.1 μg/kg body weight in 0.1 ml vehicle) was administered either intraperitoneally or orally 6 days a week from day 4 until the end of experiment. Body weight and tumor size were measured twice a week. Five

weeks after the injection of MCF10DCIS.com cells, mammary tumors were weighed and collected for further analysis. All animal studies were done in accordance with an institutionally approved protocol.

3.2.7 Analysis of serum calcium level

Calcium concentration in serum samples was determined with calcium reagent set (POINTE Scientific, INC. Canton, MI) by following the manufacturer's protocol. In brief, serum (4 μL) was mixed with the appropriately diluted reagent (200 μL) in a 96-well plate. After incubating for 1 min, the plate was read at 550 nm using a microplate reader (Tecan US, Inc., Morrisville, NC). The calcium concentration was calculated from calcium standards provided by the manufacturer.

3.2.8 Immunohistochemistry and quantification

The procedure for immunohistochemistry was described previously [275]. The slides were incubated overnight at -4 ℃ with CD44 primary antibody (1:50; Santa Cruz, CA) or PCNA primary antibody (1:1000; BD Pharmingen, San Diego, CA). The numbers of cells according to CD44 membrane staining intensity or PCNA nuclear staining intensity were quantified by using a Scan Scope program (Aperio, Vista, CA).

3.2.9 Flow cytometry

MCF10DCIS.com cells were incubated with DMSO, $1\alpha,25(OH)_2D_3$ or BXL0124 (10 nM) for 24 h. Cells were trypsinized into single cell suspension, counted, washed with phosphate-buffered saline (PBS), and stained with antibodies against CD44-APC and CD24-PE (BD Pharmingen, San Jose, CA). The cells (500,000 cells/well) were incubated with antibodies for 20 min on ice. Unbound antibodies were washed off and cells were analyzed on a BD FACSArray™ bioanalyzer (BD bioscience, San Jose, CA).

3.2.10 Knockdown of VDR by siRNA

The detailed procedure was previously described [363]. MCF10DCIS.com cells were incubated without siRNA or with 1 μM of negative control siRNA or each of two VDR siRNAs targeting different sequences in the VDR gene (Thermo scientific, Lafayette, CO) for 72 h in Accell siRNA delivery medium (Thermo Scientific). The cells were followed by incubation with DMSO or BXL0124 (10 nM) for 24 h in cell culture medium.

3.2.11 Statistical analysis

Statistical significance was evaluated using the Student's *t* test.

3.3 Results

3.3.1 Inhibition of MCF10DCIS.com xenograft mammary tumor growth by Gemini vitamin D analog *in vivo*.

We previously demonstrated that novel Gemini vitamin D analogs had better efficacy for growth inhibition of MCF10DCIS.com cells than $1\alpha,25(OH)_2D_3$ *in vitro* [275]. In our present study, we investigated the inhibitory effect of a potent Gemini vitamin D BXL0124 (Fig. 3.1, 3.2 and 3.3) on the growth of MCF10DCIS.com xenograft mammary tumors in SCID as well as in nu/nu mice. In SCID mice, when BXL0124 was given intraperitoneally at the dose of 0.1 μg/kg body weight, average tumor volume and weight were significantly reduced by 75% ($p<0.01$) and 66% ($p<0.01$), respectively (Fig. 3.1). In nu/nu mice, oral administration of BXL0124 at the dose of 0.1 μg/kg body weight suppressed tumor size and tumor weight by 47% ($p<0.05$) and 52% ($p<0.05$), respectively (Fig. 3.2). When BXL0124 was administered orally at the dose of 0.03 and 0.1 μg/kg bodyweight, tumor volume and weight were significantly reduced in MCF10DCIS.com

xenografted SCID mice (Fig. 3.3). Tumor volume was reduced by BXL0124 treatment at the dose of 0.03 and 0.1 μg/kg body weight by 36% (p<0.05) and 49% (p<0.05), respectively. Tumor weight was decreased by 37% (p<0.05) and 52% (p<0.05) with 0.03 and 0.1 μg/kg body weight of BXL0124 treatment, respectively (Fig. 3.3). In all animal studies, both intraperitoneal injection and oral administration of BXL0124 at the doses tested did not cause any significant changes in body weights and serum calcium levels, indicating no hypercalcemic toxicity at the given doses at the time period of this experiment (Fig. 3.1, 3.2 and 3.3).

3.3.2 The expression of CD44 in MCF10DCIS.com xenograft mammary tumors during tumor growth.

MCF10DCIS.com cells form DCIS like lesions which spontaneously progress to invasive tumors when they are xenografted into immunodeficient mice [301]. Hematoxylin and eosin (H&E) staining showed that MCF10DCIS.com cells xenografted into the mammary fat pad of SCID mice produced lesions which are histologically similar to DCIS of human breast cancers by week 2. By week 3, comedo DCIS-like lesions with apoptotic centers were formed and invasive tumor lesions were also detected. A majority of tumors became invasive by week 4 (Fig. 3.4A). Throughout the growth of tumors and transition from the DCIS-like lesions to invasive tumors, CD44 proteins were dominantly expressed in the membrane of epithelial cells in MCF10DCIS.com xenograft tumors (Fig. 3.4). Tumor growth from weeks 1 to 5 was measured, and the average tumor volumes for weeks 1, 2, 3, 4 and 5 were 0.02, 0.03, 0.05, 0.14 and 0.41 cm^3, respectively (Fig. 3.4B).

3.3.3 Repression CD44 by BXL0124 in MCF10DCIS.com xenograft tumors *in vivo*.

BXL0124 was given orally at the dose of 0.1 μg/kg body weight in nu/nu mice (Fig. 3.2). All tumors (one tumor for each mouse) were pooled for each group (n=5) and analyzed for CD44 protein expression level by Western blot analysis. The protein expression levels for both the standard form of CD44 (CD44s, the most widely expressed standard isoform of CD44, 85 kDa) and variant forms of CD44 (CD44v, 100 ~ 250 kDa) were markedly down-regulated by BXL0124. The repression of CD44v3 and CD44v6 variants expression by BXL0124 treatment was also shown by using antibodies that specifically recognized individual variant forms (Fig. 3.5A). The expression of proliferating cell nuclear antigen (PCNA), a marker for cell proliferation, was also significantly repressed by BXL0124 treatment (Fig. 3.5A). In the histological evaluation using H&E staining of mammary tumors, we confirmed that all mammary tumors from the control and BXL0124 treatment groups were determined to be adenocarcinomas (Fig. 3.5B). However, both CD44 and PCNA expression levels of MCF10DCIS.com xenograft tumors in nu/nu mice were significantly decreased in BXL0124-treated group when compared to the control (Fig. 3.5C). Three mammary tumors from each group were selected and three representative areas from each tumor were analyzed to quantify the staining intensity by using Scan Scope. The staining intensities of CD44 and PCNA were scored from 3+ (the strongest staining) to 0+ (negative staining) in each individual epithelial cell automatically. In both the control and BXL0124-treated groups, CD44 protein was localized exclusively on the plasma membrane of epithelial cells in mammary tumors. The distribution of cell fraction by CD44 staining intensity in the control group was 48% (3+), 32% (2+) and 20% (1+), whereas BXL0124-treated group showed 15% (3+), 62% (2+) and 23% (1+) of CD44 staining intensity (Fig. 3.5C). For PCNA staining, 82% of cells were PCNA-positive in control group whereas 62% of cells were PCNA-positive in BXL0124-treated group (Fig. 3.5C). Eighteen % of cells were

PCNA-negative in the control group, while 38% of cells were PCNA-negative in the BXL0124-treated group (p<0.01) (Fig. 3.5C).

3.3.4 Reduction of the level of CD44 protein by BXL0124 in MCF10DCIS.com cells in a VDR-dependent manner.

Next, we tested the effect of 1α,25$(OH)_2D_3$ or BXL0124 on the protein expression level of CD44 in MCF10DCIS.com cells *in vitro*. We found that 1α,25$(OH)_2D_3$ or BXL0124 down-regulated the protein expression of CD44s (85 kDa) and CD44v (100 ~ 250 kDa) in a dose-dependent manner. However, BXL0124 was more effective than 1α,25$(OH)_2D_3$ in MCF10DCIS.com cells (Fig. 3.6A). We have also tested the effect of BXL0124 on the CD44 marker in a different cell line, MCF10CA1a, which is known to be a highly aggressive cell line with metastatic capability among the MCF10 cell line series. BXL0124 also down-regulated the protein expression level of both CD44s and CD44v in MCF10CA1a cells (Fig. 3.6A). DAPI staining was used to recognize the nuclear morphology of cells. We found that the CD44 protein was localized specifically in the plasma membrane and the expression level was reduced by BXL0124 treatment without changes of any sub-cellular localization (Fig. 3.6B). MCF10DCIS.com cells were co-stained with CD44 and CD24 for flow cytometry. The significant reduction of CD44-conjugated fluorescent intensity was observed when only CD44 was detected with flow cytometry (p<0.01) (Fig. 3.7A). The fraction of $CD44^{+/hi}/CD24^{-/lo}$ cells, the sub population in which breast cancer stem cells are enriched, was significantly decreased by BXL0124 treatment (p<0.01), while the fraction of $CD44^{+/hi}/CD24^{+/hi}$ cells were increased by BXL0124 treatment (p<0.01) (Fig. 3.7B). Because the active form of vitamin D_3, 1α,25$(OH)_2D_3$, exerts the majority of its biological functions such as transcriptional activation or repression through binding to vitamin D receptor (VDR), we further tested whether the repression of CD44

is dependent on VDR. Knock-down of VDR by using two different VDR siRNAs blocked the repression of CD44 protein expression by BXL0124 in MCF10DCIS.com cells, indicating that the down-regulation of CD44 by BXL0124 is a VDR-dependent event (Fig. 3.8).

3.3.5 Suppression of CD44 mRNA with induction of osteopontin mRNA by BXL0124 in MCF10DCIS.com breast cancer cells.

The transcriptional regulation of CD44 and osteopontin expression was investigated in MCF10DCIS.com cells. The down-regulation of CD44 mRNA by 1α,25$(OH)_2D_3$ (10 nM) or BXL0124 (10 nM) in MCF10DCIS.com cells was determined at different time points, and it showed maximum inhibition at 4 h with both compounds (Fig. 3.9A). When MCF10DCIS.com cells were treated with 1α,25$(OH)_2D_3$ or BXL0124 for 4 h, CD44 mRNA expression was down-regulated in a dose-dependent manner, and BXL0124 showed stronger repressive effect than 1α,25$(OH)_2D_3$ at the same doses (Fig. 3.9B). Because osteopontin, which is one of the target genes regulated by vitamin D, is known to interact with CD44, we also investigated the regulation of osteopontin mRNA by BXL0124. Both 1α,25$(OH)_2D_3$ (10 nM) and BXL0124 (10 nM) induced the expression of osteopontin mRNA starting at 4 h, and the induction of osteopontin mRNA by BXL0124 was stronger than by 1α,25$(OH)_2D_3$ at 24 h (Fig. 3.10A). When MCF10DCIS.com cells were treated with 1α,25$(OH)_2D_3$ or BXL0124 for 4 h, osteopontin mRNA expression was induced in a dose-dependent manner (Fig. 3.10B).

3.3.6 Repression of the transactivation of CD44 promoter by BXL0124 in MCF10DCIS.com cells in a p53 dependent manner.

The transcriptional repression of CD44 by 1α,25$(OH)_2D_3$ or BXL0124 was shown by a CD44 promoter assay. Although both 1α,25$(OH)_2D_3$ and BXL0124 repressed transactivation of the CD44 promoter in a dose-dependent manner, BXL0124 showed a more potent inhibitory effect than 1α,25$(OH)_2D_3$ (Fig. 3.11A). Recently, p53 was reported to inhibit CD44 expression via binding to a p53-binding sequence on the CD44 promoter [364]. Therefore, we determined the involvement of p53 for the repression of CD44 by BXL0124. The repression of CD44 promoter transactivation by BXL0124 was abolished when the p53-binding site of the CD44 promoter was mutated, indicating p53 is necessary for the CD44 repression by BXL0124 (Fig. 3.11B).

3.4 Discussion

Accumulating evidence indicates that cancer stem cells are responsible for tumor-initiation, recurrence, metastasis, and the resistance to conventional chemotherapies [62,365]. Therefore, these cancer stem cells are becoming a critical target for cancer therapeutics. CD44 is a key cell-surface marker for cancer stem cells in pancreatic, prostate and breast cancer [355,356,357]. Recently, Godar *et al* demonstrated that CD44 suppression by CD44-specific shRNA infection not only inhibited tumor growth but also reduced the tumor-initiating ability of a human breast cancer cell xenograft [364]. Also, CD44 targeting by specific antibody treatment inhibited tumor recurrence after chemotherapy induced remission of tumors in human breast cancer xenografts [330]. These findings indicate that CD44 has a direct role in tumor initiation and recurrence in addition to serving as a useful marker for breast cancer stem cells.

CD44, a transmembrane glycoprotein encoded by a single gene with at least 19 exons, has multiple variants produced by alternative splicing [325]. In breast cancer, CD44 variants (CD44v, 100 ~ 250 kDa) rather than a standard CD44 (CD44s, 85 kDa) have been strongly associated with cancer metastasis and poor disease-free rate [324]. Among many different CD44 variants, CD44v3, which is typically modified by heparin-sulphate side chains, is known to recruit matrix metalloproteinases (MMPs) and induce cell invasion and survival signaling [325,366]. In several cell lines, CD44v6 forms complex with Met receptor and acts as co-receptor to promote cell growth [325,329]. In addition, CD44v6 has been shown to interact with osteopontin, one of target genes known to be regulated by vitamin D and its analogs [160,325,367]. The overexpression of endogenous osteopontin enhanced expression of CD44v6 protein, which may be essential for mediating osteopontin-induced tumor cell metastasis [368]. Because of the biological importance of CD44 variants, we investigated whether BXL0124 regulates the protein expression of CD44 variants, CD44v3 and CD44v6. MCF10DCIS.com xenograft mammary tumors expressed high level of CD44v3 and CD44v6, and the expression of these variants was markedly reduced by BXL0124 treatment, suggesting that repression of CD44 variants may contribute to the growth inhibitory effect of BXL0124.

In the previous study, we reported that a Gemini vitamin D analog significantly inhibited the growth of MCF10DCIS.com xenografted tumors in immunodeficient mice [275]. However, mechanistic studies including the upstream mediator regulated by the Gemini vitamin D analogs have not been determined in this model. Since CD44 is a main receptor for several key extracellular matrix proteins such as osteopontin and hyaluronan [325], the repression of CD44 by $1\alpha,25(OH)_2D_3$ and its analogs may contribute to their anti-cancer activities. A recent colon cancer study demonstrated that $1\alpha,25(OH)_2D_3$ or its analogs increased the expression of osteopontin, but they suppressed the expression of CD44 and enhanced the expression of E-cadherin which may contribute to their inhibitory effect on adenoma formation in $Apc^{Min/+}$ mice

[369]. In the present study, BXL0124 decreased the expression of CD44 mRNA and protein (Fig. 3.6A, 3.9A and 3.9B) while inducing the expression of osteopontin mRNA (Fig. 3.10A and 3.10B) in cultured MCF10DCIS.com cells. Although BXL0124 induced the expression of osteopontin mRNA, the repression of its receptor, CD44, may be the primary contributor to the growth inhibitory effect of BXL0124 on MCF10DCIS.com cells.

Godar *et al* found that the p53 tumor suppressor inhibits expression of CD44 via binding to a p53-binding sequence in the CD44 promoter in transformed human mammary epithelial cells, suggesting that CD44 repression by p53 is critical for the tumor suppressive action of p53 [364]. In the present study, repression of CD44 promoter transactivation by BXL0124 treatment was abolished when the p53-binding site in CD44 promoter was mutated. This observation indicates that p53 is crucially involved in the repression of CD44 by BXL0124, although exact molecular mechanisms of action involving p53 need to be further investigated. To determine how vitamin D and a Gemini vitamin D analog regulate the expression of CD44, we also investigated the involvement of the VDR. It is well known that the majority of the biological functions of vitamin D are exerted through binding to VDR [132]. We demonstrated that knock-down of VDR using siRNA resulted in reversing the inhibitory effect of BXL0124 on CD44 repression (Fig. 3.8). Furthermore, we found that putative VDRE sequences are present in the CD44 promoter region (personal communication with Dr. Fang Liu), suggesting that the VDR liganded by vitamin D or its analog may directly bind to CD44 promoter region to repress its expression.

3.5 Conclusion

MCF10DCIS.com cells highly expressed CD44, predominantly CD44v, *in vivo* and *in vitro*. In this chapter, we demonstrated the repression of CD44 expression by the Gemini vitamin D analog BXL0124 *in vivo* and *in vitro*, which is likely via VDR- and p53-dependent

mechanisms. Our study suggests novel Gemini vitamin D analogs as potentially useful agents for repressing CD44 expressing cancer stem cells in breast cancer.

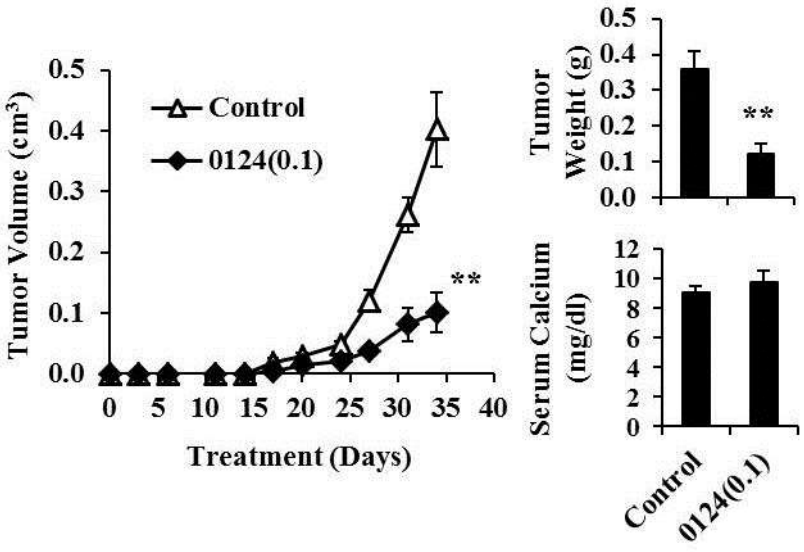


Fig. 3.1 Repression of the growth of MCF10DCIS.com xenograft tumors by intraperitoneal injection of BXL0124 in SCID mice. BXL0124 (0.1 μg/kg body weight) was given intraperitoneally in MCF10DCIS.com xenografted SCID mice 6 times a week (n=5). Tumor volume was measured twice a week. Final tumor weight was measured at necropsy. Serum was collected at necropsy and analyzed to determine serum calcium level. The data are presented as the mean ± standard error (statistical analysis, ** $p<0.01$).

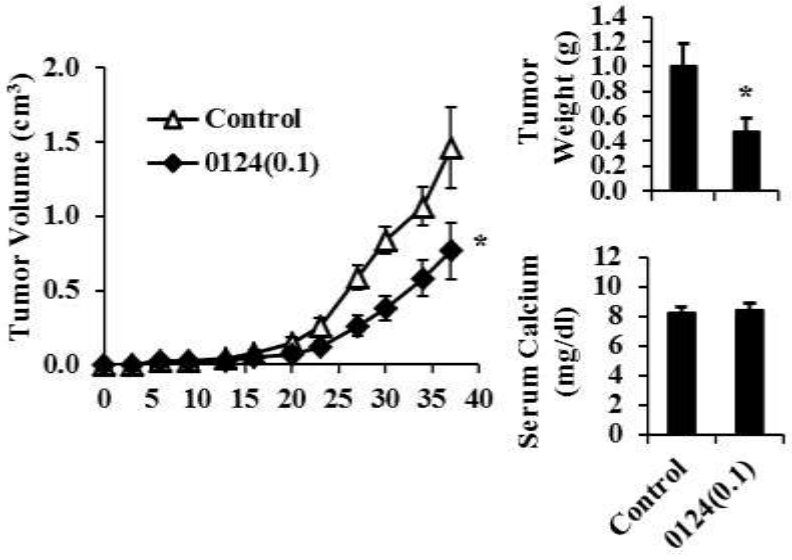


Fig. 3.2 Repression of the growth of MCF10DCIS.com xenograft tumors by oral administration of BXL0124 in nu/nu mice. BXL0124 (0.1 μg/kg body weight) was administered orally in MCF10DCIS.com xenografted nu/nu mice once a day 6 times a week (n=5). Tumor volume was measured twice a week. Final tumor weight was measured at necropsy. Serum was collected at necropsy and analyzed to determine serum calcium level. The data are presented as the mean ± standard error (statistical analysis, * $p<0.05$).

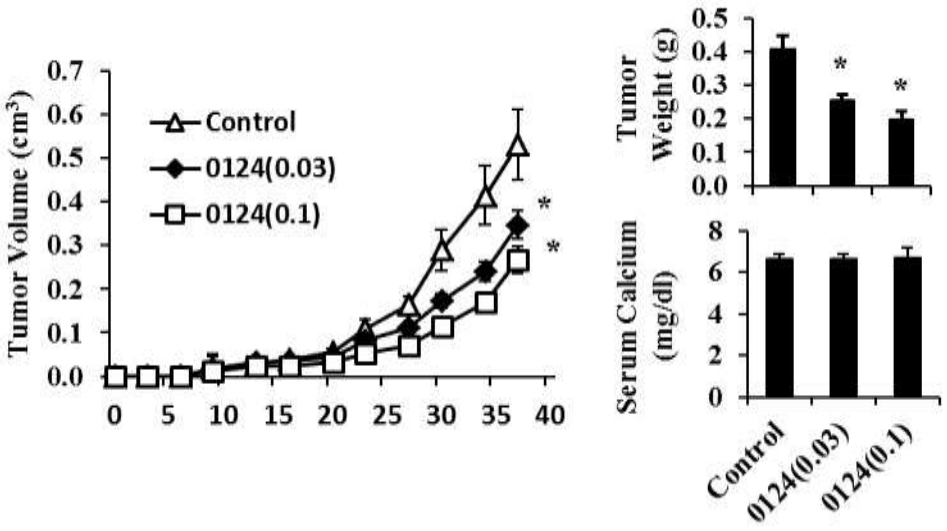


Fig. 3.3 Repression of growth of MCF10DCIS.com xenograft tumors by oral administration of BXL0124 in SCID mice. BXL0124 (0.03 or 0.1 μg/kg body weight) was administered orally in MCF10DCIS.com xenografted SCID mice 6 times per week (n=7). Final tumor weight was measured at necropsy. Serum was collected at necropsy and analyzed to determine serum calcium level. The data are presented as the mean ± standard error (statistical analysis, * $p<0.05$).

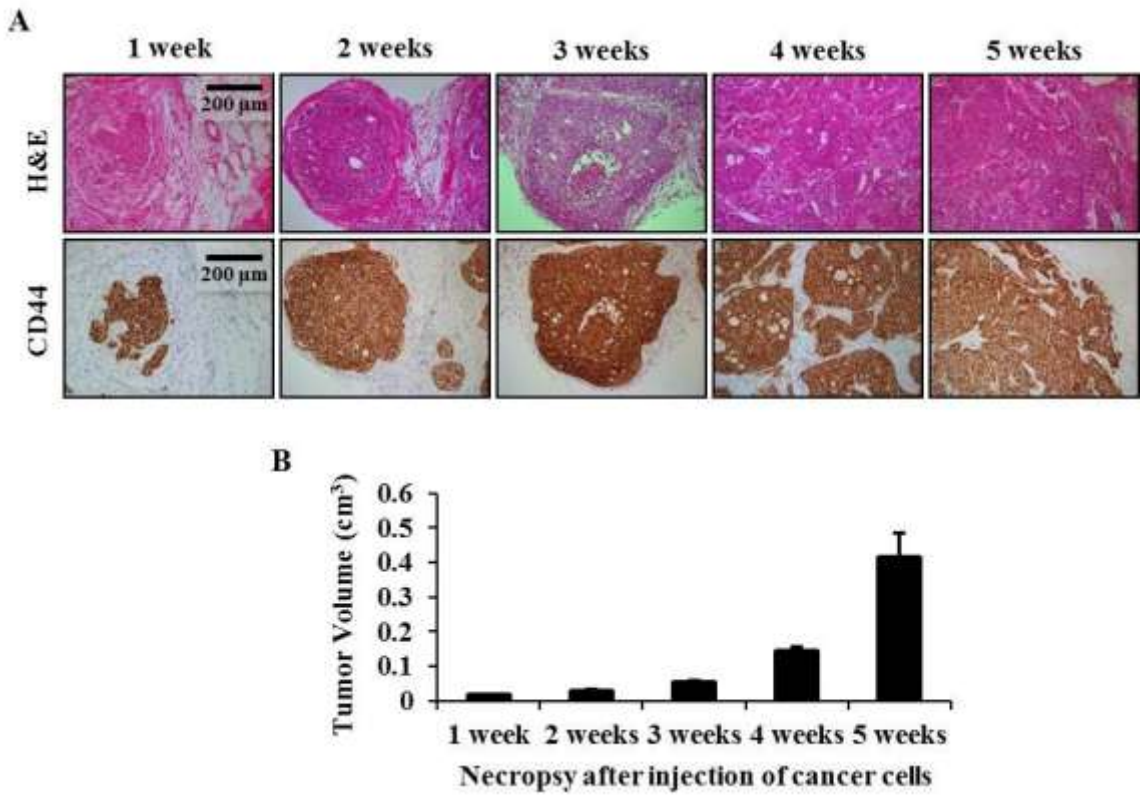


Fig 3.4 Expression of CD44 protein during the growth of MCF10DCIS.com xenograft tumors. (A) At day 0, MCF10DCIS.com cells ($1x10^6$ cells) were injected into the mammary fat pad area of SCID mice and divided into five groups. Mice (n=4) were sacrificed at 1, 2, 3, 4 and 5 weeks after injection. All tumors were collected and analyzed for H&E and immunohistochemical analysis, and a representative tumor staining for H&E and CD44 is shown for each week (200X). **(B)** Tumor volume was measured every week. The data are presented as the mean ± standard deviation (n=4).

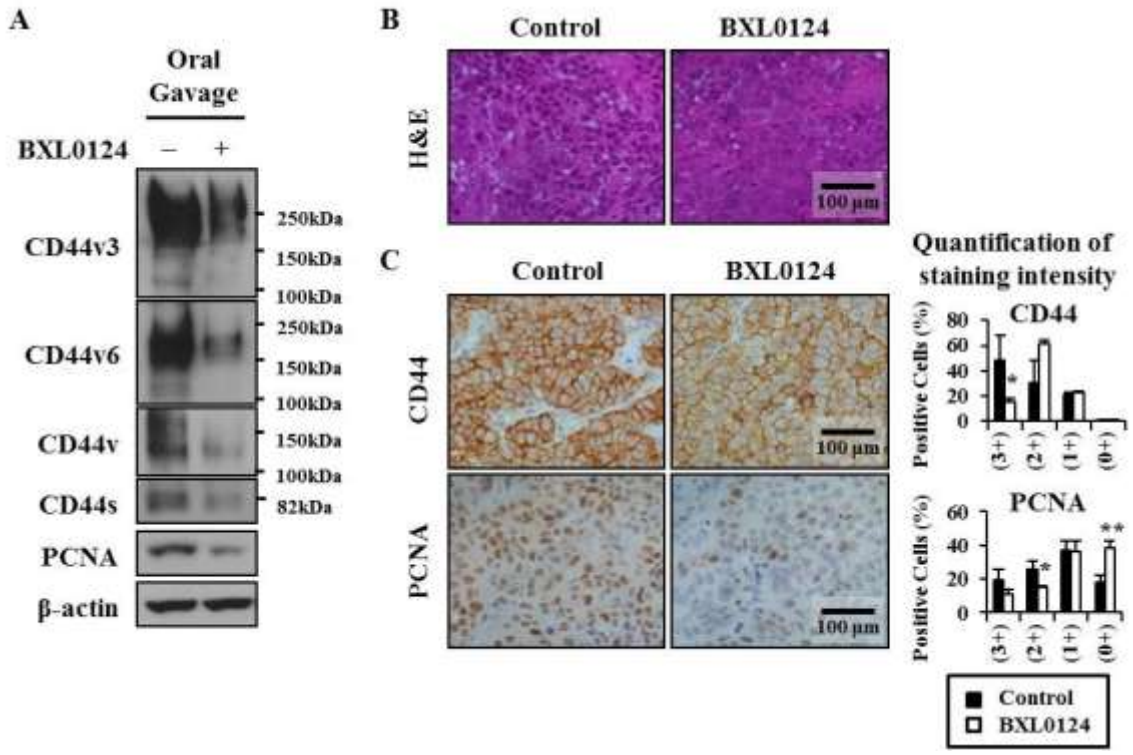


Fig. 3.5 Effects of BXL0124 on CD44 protein expression level in MCF10DCIS.com xenograft tumors *in vivo*. (A) MCF10DCIS.com xenografted nu/nu mice were treated with DMSO or BXL0124 (0.1 μg/kg body weight) orally, and mammary tumors were collected at necropsy. Mammary tumors (n=5) were pooled into either the control group or BXL0124-treated group for Western blot analysis against CD44, CD44v3, CD44v6, PCNA, and β-actin. **(B)** A representative H&E staining in mammary tumors from MCF10DCIS.com xenografted nu/nu mice is shown (400X). **(C)** A representative immunostaining against CD44 and PCNA in mammary tumors from MCF10DCIS.com xenografted nu/nu mice is shown (400X). Three mammary tumors from each group were selected and three representative areas from each tumor were analyzed for the expression of CD44 and PCNA. The mammary tumors immunostained against CD44 and PCNA were scored by four different levels of staining intensity and quantified by using Aperio® Scan Scope. The data are presented as the mean ± standard deviation (statistical analysis, * $p<0.05$, ** $p<0.01$).

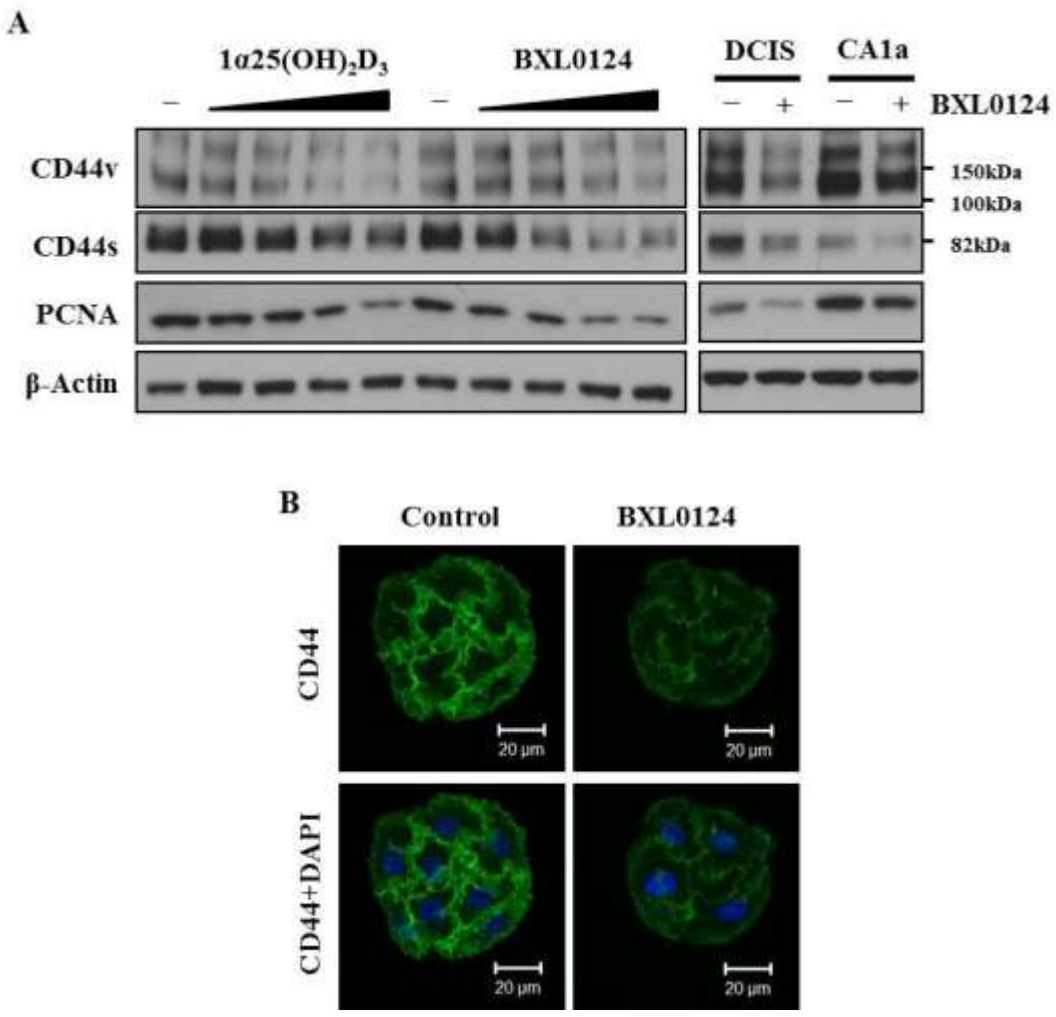
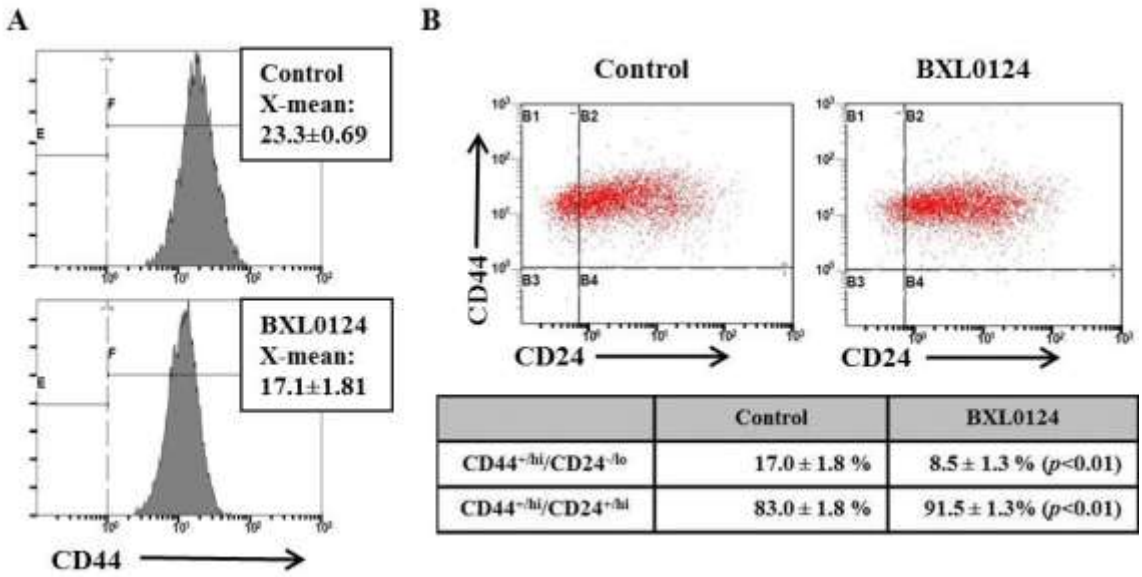


Fig 3.6 Effects of BXL0124 on CD44 protein level in MCF10DCIS.com and MCF10CA1a cells. (A) MCF10DCIS.com cells were treated with increasing doses of $1\alpha,25(OH)_2D_3$ or BXL0124 (0.01, 0.1, 1.0, and 10 nM) for 24 h and analyzed for CD44 and PCNA protein expression levels by Western blot analysis. MCF10DCIS.com and MCF10CA1a cells were treated with BXL0124 (10 nM) for 24 h and analyzed for CD44 and PCNA protein expression levels by Western blot analysis. All splicing isoforms of CD44 were recognized by a CD44 antibody, which recognizes both CD44 standard and variants. **(B)** MCF10DCIS.com cells were treated with DMSO or BXL0124 (10 nM) for 24 h and analyzed for CD44 expression level by confocal microscopy.



	Control	BXL0124
$CD44^{+/hi}/CD24^{-/lo}$	17.0 ± 1.8 %	8.5 ± 1.3 % (p<0.01)
$CD44^{+/hi}/CD24^{+/hi}$	83.0 ± 1.8 %	91.5 ± 1.3% (p<0.01)

Fig. 3.7 Effects of BXL0124 on the cell surface expression level of CD44 and proportion of subpopulation in MCF10DCIS.com cells. MCF10DCIS.com cells were treated with DMSO control or BXL0124 (10 nM) for 24 h. **(A)** CD44 cell surface expression level was determined by flow cytometry and shown as one parameter histograms. The average of CD44 expression level for sample was calculated as X-mean. **(B)** The percentage of cells, which were categorized by the combination of CD44 and CD24 cell surface expression, was determined by flow cytometry. The experiment was repeated three times, and the data are presented as the mean ± standard deviation.

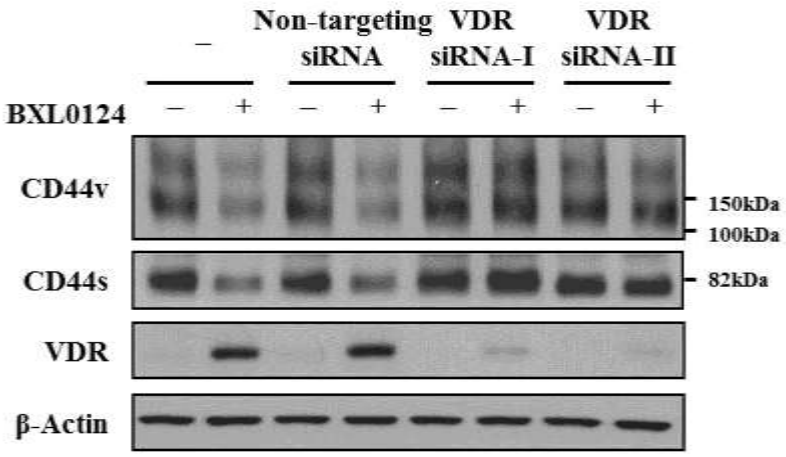


Fig.3.8 VDR-dependent repression of CD44 by BXL0124 in MCF10DCIS.com cells. MCF10DCIS.com cells were incubated without siRNA or with negative control siRNA or 1 μg of each of two VDR siRNAs targeting different sequences in the VDR gene in Accell siRNA delivery medium for 72 h and followed by treatment with DMSO or BXL0124 (10 nM) for 24 h. The levels of CD44 and VDR protein were determined by Western blot analysis.

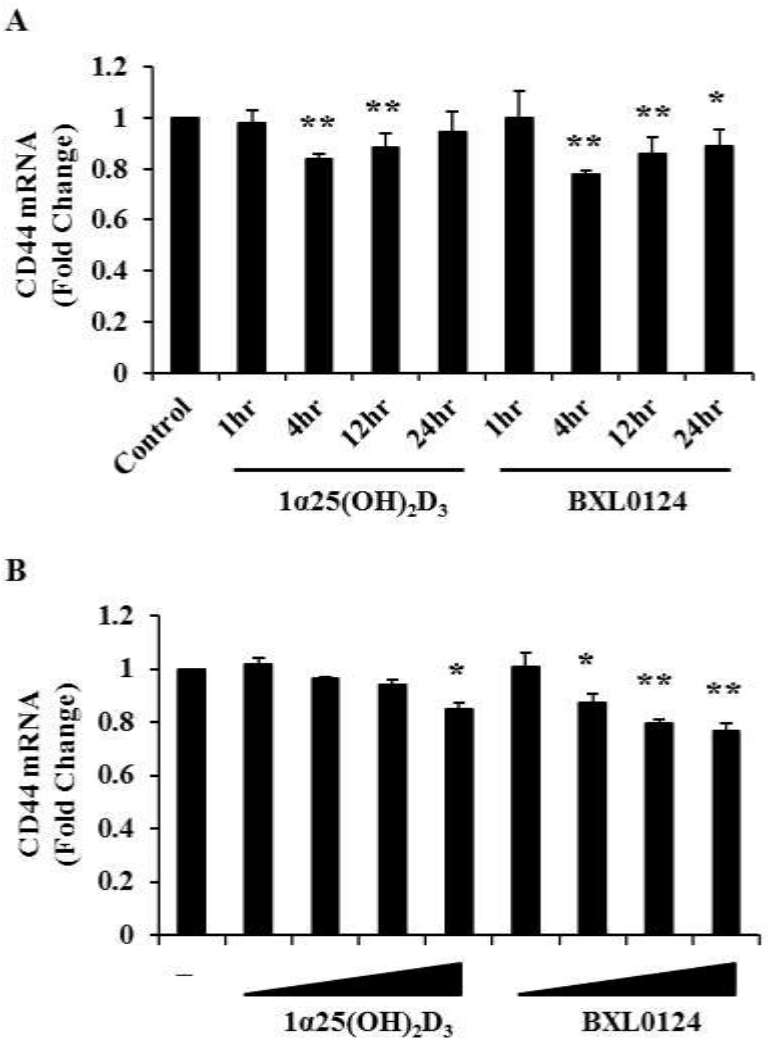


Fig. 3.9 Repression of mRNA level of CD44 by 1α,25(OH)$_2$D$_3$ and BXL0124 in MCF10DCIS.com cells. (**A)** MCF10DCIS.com cells were treated with 1α,25(OH)$_2$D$_3$ or BXL0124 (10 nM) for 1 h, 4 h, 12 h, and 24 h and analyzed for CD44 mRNA expression level by quantitative PCR (qPCR). (**B)** MCF10DCIS.com cells were treated with increasing doses of 1α,25(OH)$_2$D$_3$ or BXL0124 (0.01, 0.1, 1.0, and 10 nM) for 4 h and analyzed for CD44 mRNA expression level by qPCR. The data are presented as the mean ± standard deviation (statistical analysis, * p<0.05, ** p<0.01).

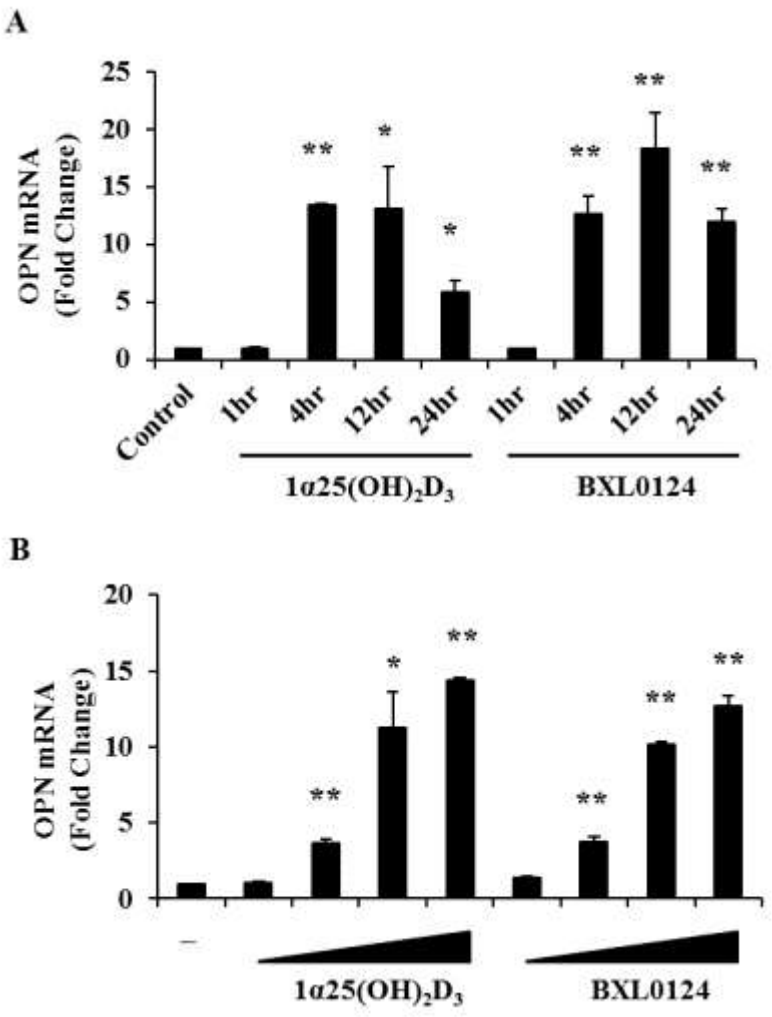


Fig. 3.10 Induction of mRNA level of osteopontin by 1α,25(OH)$_2$D$_3$ and BXL0124 in MCF10DCIS.com cells. (A) MCF10DCIS.com cells were treated with 1α,25(OH)$_2$D$_3$ or BXL0124 (10 nM) for 1 h, 4 h, 12 h, and 24 h and analyzed for osteopontin mRNA expression level by quantitative PCR (qPCR). **(B)** MCF10DCIS.com cells were treated with increasing doses of 1α,25(OH)$_2$D$_3$ or BXL0124 (0.01, 0.1, 1.0, and 10 nM) for 4 h and analyzed for osteopontin mRNA expression level by qPCR. The data are presented as the mean ± standard deviation (statistical analysis, * p<0.05, ** p<0.01).

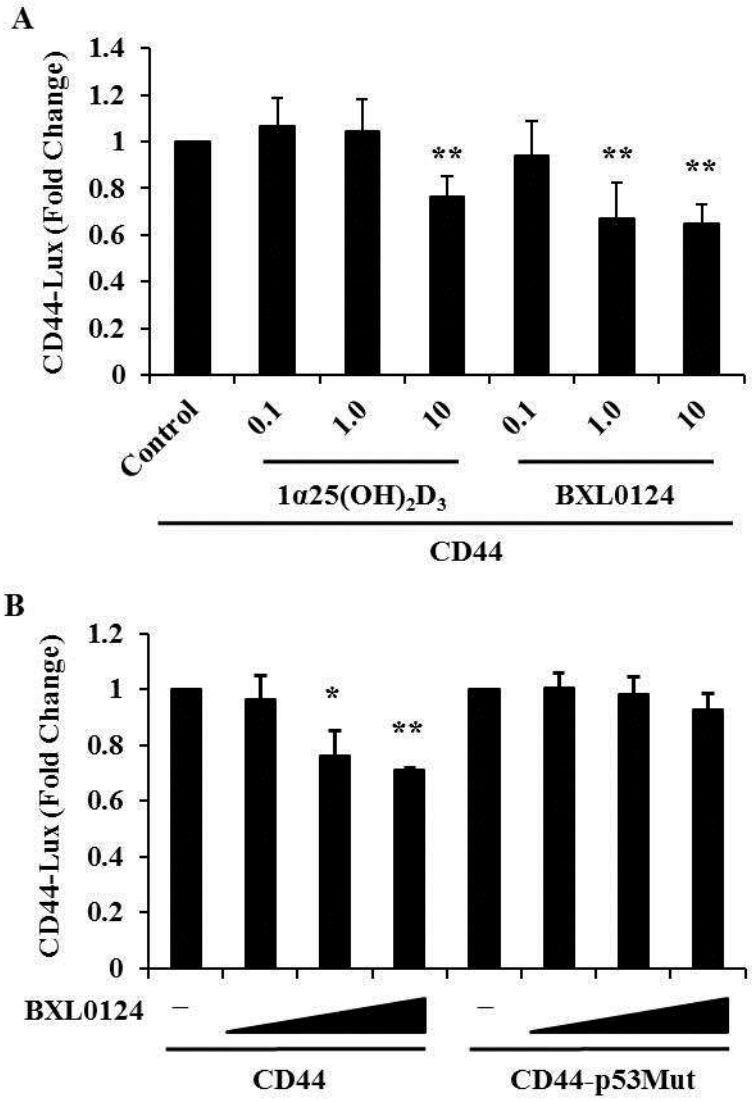


Fig. 3.11 Repression of transactivation of CD44 promoter by 1α,25(OH)$_2$D$_3$ and BXL0124 in a p53 dependent manner in MCF10DCIS.com cells. (A) MCF10DCIS.com cells were transfected with full length-CD44-Luc DNA vector (CD44) for 6 h and were treated with increasing doses of 1α,25(OH)$_2$D$_3$ or BXL0124 (0.1, 1.0, and 10 nM) for an additional 24 h. **(B)** MCF10DCIS.com cells were transfected with full length-CD44-Luc DNA vector (CD44) or p53 binding site mutated-CD44-Luc DNA vector (CD44-p53Mut) for 6 h and followed by treatment with BXL0124 (10 nM) for 24 h. Luciferase activity was measured with a luminometer and normalized by β-galactosidase activity. The experiments were repeated at least twice, with each

experiment done in duplicate. The data are presented as the mean ± standard deviation (statistical analysis, * $p<0.05$, ** $p<0.01$).

Chapter 4: Inhibition of cell invasion with targeting CD44-STAT3 signaling by a Gemini vitamin D analog in basal-like breast cancer

4.1. Introduction

Invasive growth is a physiological property of embryonic cells during development and epithelial cells during wound healing [370,371]. However, under pathological conditions, invasive growth of cancer cells is one of the hallmarks of malignancy progression evidenced by local invasion and distant metastasis [172]. In breast cancer, ductal carcinoma in situ (DCIS) has been recognized as a precursor of invasive ductal carcinoma (IDC) [372]. The acquisition of an invasive phenotype has been suggested to be a critical step in the transition from DCIS to IDC [61,372,373]. However, many studies failed to elucidate the complex nature of the DCIS to IDC transition [372]. Recent studies demonstrated that both cancer cells and the tumor-associated microenvironment, such as extracellular matrix and stromal cells, are critical contributors to cancer invasion [61,172,295]. These findings highlight the importance of molecules involved in microenvironment-epithelial interactions as potential therapeutic targets.

CD44 is one of the key molecules that regulate microenvironment-epithelial interactions by serving as a major receptor for several extracellular matrix proteins such as hyaluronan and osteopontin [325]. CD44 overexpression correlates with invasive and metastatic phenotype in breast cancer, and thus, is an indicator of poor prognosis [324,374]. Recently, CD44 has been recognized as one of the key cell surface markers for tumor-initiating cells in breast cancer [357,364]. Since CD44 does not have intrinsic kinase activity, it modulates intracellular signaling by interacting with other components of signaling transduction such as receptor tyrosine kinases or intracellular kinases [325,375]. The recruitment of signaling partners and resulting signaling by

CD44 depends on the types of microenvironment and tumors [375]. Therefore, identification of interacting molecules in a cell-type specific manner is important to understand the biological role of CD44 in human breast cancer.

Previously, it has been shown that a novel Gemini vitamin D analog, BXL0124, down-regulated CD44 expression in MCF10DCIS.com cells and inhibited tumor growth in a MCF10DCIS.com xenograft [334]. However, the biological role of CD44 repression by BXL0124 in breast cancer has not been fully explored. In this chapter, we investigated the effect of BXL0124 on key molecules in various signaling pathways and invasion of MCF10DCIS.com cells. We demonstrate that the repression of CD44 by BXL0124 contributes to the inhibition of STAT3 signaling and tumor invasion in MCF10DCIS.com cells.

4.2. Materials and methods

4.2.1. Reagents and cell culture

1α,25$(OH)_2D_3$ and Gemini vitamin D analog 1α,25-dihydroxy-20R-21(3-hydroxy-3-deuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluro-cholecalciferol (BXL0124, [361]) were provided by BioXell, Inc. (Nutley, NJ) and dissolved in dimethyl sulfoxide. The MCF10DCIS.com and MCF10CA1a human breast cancer cell lines were provided by Dr. Fred Miller at the Barbara Ann Karmanos Cancer Institute (Detroit, MI) [301,302]. The MCF10DCIS.com cell line was authenticated by short tandem repeat profiling at American Type Culture Collection (Manassas, VA). MCF10DCIS-shLuc and MCF10DCIS-shCD44 cells were generated by infecting the MCF10DCIS.com cells with lentivirus encoding shRNA to luciferase (shLuc) or shRNA to CD44 (shCD44) [364]. The infected cells were sorted by FACS through the green fluorescence protein (GFP) to obtain GFP-labeled DCIS-shLuc cells or GFP-labeled DCIS-shCD44 cells. Cells were maintained in DMEM/F-12 medium supplemented with 5% horse

serum, 1% penicillin/streptomycin, and 1% HEPES solution at 37 ℃ and 5% CO_2. MDA-MB-468 human breast cancer cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 ℃ and 5% CO_2.

4.2.2. [^{3}H] thymidine incorporation assay

The procedure was described previously [160]. In brief, MCF10DCIS.com cells (8,000 cells) were seeded onto each well of 24-well plate, and on the next day the cells were treated with given doses of 1α,25$(OH)_2D_3$ or BXL0124 for 72 h. DCIS-shLuc or DCIS-shCD44 cells (2,000 cells) were seeded and incubated for 48 h for the thymidine incorporation assay.

4.2.3. MTT assay

MCF10DCIS.com cells were seeded into each well of 96-well plate (1,000 cells/well), on the next day the cells were treated with 0,01, 0,1, 1, 10 or 100 nM of 1α,25$(OH)_2D_3$ or BXL0124 for given incubation times. At each time point, 10 μl of MTT-I solution (thiazolyl blue tetrazolium bromide, M2128, Sigma-Aldrich, St. Louis, MO) were added into each well and incubated for 5 h, followed by addition of 100 μl of MTT-II solution (distilled water with 10% SDS and 0.01 M HCl). The plate was then incubated overnight, and the absorbance was measured with a spectrophotometer (Tecan US, Durham NC) at 560 nm.

4.2.3. Cancer cell invasion assays

Three different cell invasion assays were used: 3D culture assay with Matrigel (BD Bioscience, Spark, MD), Cultrex®24 well basement membrane extract (BME) cell invasion assay

(Trevigen, Gaithersburg, MD), and Fluoroblok Biocoat cell invasion assay (BD Bioscience, Sparks, MD). For 3D culture, 4-well culture slides, coated with Matrigel, were prepared as previously described [376]. MCF10DCIS.com cells were seeded as single cells in M171 mammary epithelial medium (Invitrogen, Carlsbad, CA) supplemented with mammary epithelial growth supplement (Invitrogen, Carlsbad, CA). The cells were incubated for 10 days, and medium was replenished every 2 days. Cultrex®24 well BME cell invasion assay and Fluoroblok Biocoat cell invasion assay were performed as described in the manufacturers' protocols. In the Cultrex®24 well BME cell invasion assay, the cells that penetrated matrigel were dissociated from the bottom of chamber and stained with Calcein-AM as described in the manufacturer's protocol. The intensity of Calcein-AM fluorescence was measured by a fluorescent plate reader (Tecan US) and compared to a pre-measured standard curve to determine the number of cells per well. For quantitative evaluation of Fluoroblok Biocoat cell invasion assay, the green pixel counts per total pixel counts from 4 representative pictures per well were calculated using the Image J program (NIH, Bethesda, MD) (http://rsbweb.nih.gov/ij).

4.2.4. Quantitative real-time PCR

The procedure was described previously [160]; the labeled primers for CD44, matrix metalloproteinase (MMP)-2, MMP-9, MMP-13, MMP-14, MMP-15, MMP-16, tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, uPA, and glyceraldehyde 3-phosphate dehydrogenase were obtained from Applied Biosystems (Foster City, CA).

4.2.5. Western blot analysis

The detailed procedure was described previously [362]. The primary antibody against CD44 (sc-7298), which recognizes both CD44v and CD44s, was from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies recognizing pSTAT3 (9235), STAT3 (9139), pAkt (9271), Akt (2966), pErk (9101) and Erk (9109) were from Cell Signaling Technology (Beverly, MA); pNF-κB (sc-101749) and NF-κB (sc-372) were from Santa Cruz Biotechnology; MMP-9 (ab38898) was from Abcam (Cambridge, MA); vitamin D receptor (VDR) (GR37) was from Millipore (Billerica, MA); β-actin (A1978) was from Sigma-Aldrich (St. Louis, MO). Secondary antibodies were from Santa Cruz Biotechnology.

4.2.6. Knockdown of VDR by siRNA

The detailed procedure was described previously [334]. MCF10DCIS.com cells were incubated with 1 μM of non-targeting siRNA (D-001910-02-20, Thermo Fisher Scientific, Waltham, MA) or VDR siRNA (A-003448-13-0010, Thermo Fisher Scientific) for 72 h in Accell siRNA delivery medium (Thermo Fisher Scientific).

4.2.7. STAT3 DNA binding assay

Transfactor STAT3-specific chemiluminescent kit from Clontech (Mountain View, CA) was utilized according to the manufacturer's instructions. In brief, whole cell lysates of MCF10DCIS.com cells (40 μg) were incubated for 1 h in the Transfactor assay plates, which contained oligonucleotides with STAT3 binding sequences. STAT3 primary and secondary antibodies (provided with the kit) were incubated for 60 and 30 minutes, respectively. The mixture of chemiluminescent substrate A and B (1:1) was added, and chemiluminescent intensity

was measured by luminometer (Turner Biosystems, Sunnyvale, CA). The chemiluminescent intensity values of samples treated with BXL0124 were divided by the chemiluminescent intensity value of a control sample, and the fold changes were calculated.

4.2.8. Fluorescence microscopy

For *in vitro* samples, cells were fixed as previously described [334]. For *in vivo* samples, the tumors were embedded in paraffin (Electron Microscopy Sciences, Hatfield, PA) and then sectioned at 4 μm thickness. Both cell and tumor samples were incubated with PBS containing 10% goat serum to block non-specific binding. Fixed cells were incubated overnight at 4℃ with a primary antibody to pSTAT3 (Cell Signaling Technology, 1:500). Similarly, tumor samples were incubated with a combination of primary antibodies to pSTAT3 (Cell Signaling Technology, 1:100) and CD44 (Santa Cruz Biotechnology, 1:100). Fluorophore-conjugated secondary antibody (Alexa Fluor 488 or 546; Invitrogen, 1:200) and TO-PRO-3 iodide nuclear antibody (Invitrogen, 1 μM) were incubated at room temperature for 60 and 15 minutes, respectively. The images were taken using confocal microscope with laser at 488 nm (pSTAT3), 546 nm (CD44), and 633 nm (TO-PRO-3).

4.2.9. Immunoprecipitation

After 24 h incubation with or without BXL0124, MCF10DCIS.com cells were washed once with PBS and lysed in immunoprecipitation lysis buffer (Thermo Fisher Scientific). Antibodies to STAT3 or JAK2 (Cell Signaling Technology) were immobilized to protein G-conjugated Dynabeads (Invitrogen). The antibody-conjugated beads were washed by magnetic separation, and same amounts of protein samples were added. After a 10-minute incubation, the

Dynabead-antibody-protein complex was isolated by magnetic separation and washed three times. Immunoprecipitated proteins were then detected by Western blot analysis.

4.2.10. Xenograft tumor study

MCF10DCIS-shLuc or MCF10DCIS-shCD44 cells were injected into the mammary fat pad of immunodeficient nu/nu mice as described previously [275]. Tumor size was measured twice weekly. Five weeks after the cell injection, mice were sacrificed and xenograft tumors were weighed. The tumor samples were fixed in 10% formalin and transferred to 70% ethanol for immunofluorescent staining or flash frozen and stored in -80 ℃ for Western blot analysis or RNA analysis. All animal studies were conducted in accordance with an institutionally approved protocol. The protocol was approved by the Institutional Animal Care and Use Committee at Rutgers, the State University of New Jersey (Protocol Number: 04-001). All surgery was performed under ketamine anesthesia, and all efforts were made to minimize suffering.

4.2.11. Statistical analysis

Statistical significance was evaluated using the Student's *t* test.

4.3. Results

4.3.1 Inhibition of cell proliferation, metabolic activity and invasion by 1α,25(OH)$_2$D$_3$ and Gemini vitamin D analog BXL0124 in MCF10DCIS.com cells.

We investigated the potential inhibitory effects of 1α,25(OH)$_2$D$_3$ or BXL0124 on proliferation, metabolic activity and invasion of MCF10DCIS.com cells. MCF10DCIS.com cell

proliferation was significantly inhibited by 1α,25$(OH)_2D_3$ and BXL0124 (Fig. 4.1A). The metabolic activity of MCF10DCIS.com cells was also significantly repressed by 1α,25$(OH)_2D_3$ and BXL0124 (Fig. 4.1B). For both proliferation and metabolic activity in MCF10DCIS.com cells, BXL0124 was more potent than 1α,25$(OH)_2D_3$ (Fig. 4.1A and 4.1B). The number of MCF10DCIS.com cells that penetrated BME-coated layers was significantly decreased by 1α,25$(OH)_2D_3$ or BXL0124 treatment. However, BXL0124 was more effective than 1α,25$(OH)_2D_3$ to repress MCF10DCIS.com cell invasion (Fig. 4.2A). In the 3D culture, MCF10DCIS.com cells showed invasive outgrowth at Day 10 (Fig. 4.2.B, arrows), which was not detected in MCF10DCIS.com cells treated with BXL0124 (1 and 10 nM) or 1α,25$(OH)_2D_3$ (10 and 100 nM) (Fig. 4.2B).

4.3.2. Repression of the level of invasion markers and STAT3 signaling by in MCF10DCIS.com cells.

The mRNA expression levels of CD44, MMP-2, MMP-9, MMP-13, MMP-14, MMP-15, MMP-16, TIMP-1, TIMP-2 and uPA were investigated to identify the invasion markers regulated by BXL0124 in MCF10DCIS.com cells. The mRNA expression levels of CD44, MMP-2, MMP-9, and uPA were significantly decreased by BXL0124 treatment at 24 h and 48 h (Fig. 4.3); MMP-14 (Fig. 4.3) and other invasion markers (data not shown) did not show significant changes. To identify downstream signaling pathways that may be affected by BXL0124, the protein levels of CD44, as well as potential downstream signaling molecules (pAkt, pErk, pSTAT3 and NF-κB), were measured. The BXL0124 treatment decreased the protein levels of variant isoforms of CD44 (CD44v, 100~250 kDa), standard isoform of CD44 (CD44s, 85 kDa) and pSTAT3 in a dose-dependent manner, whereas the protein levels of pErk, pAkt and pNF-κB were not changed (Fig. 4.4A). Total protein levels of STAT3, Akt, Erk and NF-κB were not

affected by BXL0124 treatment (Fig. 4.4A). In a time-dependent study, the treatment with BXL0124 decreased the protein levels of CD44s and CD44v as well as pSTAT3 at 12 h and 24 h, while there was no change in the level of total STAT3 (Fig. 4.4B). The repression of CD44 and pSTAT3 protein levels shown by the treatment with BXL0124 was abolished by knockdown of VDR using VDR siRNA, indicating that the repression of CD44-STAT3 signaling by BXL0124 is a VDR-dependent event (Fig. 4.5).

4.3.3. Inhibition of STAT3 signaling by reducing the complex formation of CD44, STAT3 and JAK2 with BXL0124.

To determine STAT3 activity affected by BXL0124, nuclear localization and DNA binding activity of STAT3 were analyzed. Strong nuclear staining of pSTAT3 was evident in the control; it was reduced by treatment with BXL0124 (Fig. 4.6A). DNA binding of STAT3 was also significantly decreased in a dose-dependent manner by BXL0124 treatment for 24 h (Fig. 4.6B). Since BXL0124 decreased the protein levels of CD44 and inhibited activation of STAT3 signaling, we investigated whether CD44 activates STAT3 signaling by direct interaction. When MCF10DCIS.com cell lysates were immunoprecipitated with STAT3 antibody, the immunocomplex contained CD44s, CD44v and JAK2, and BXL0124 decreased the amounts of CD44v and CD44s proteins interacting with STAT3 (Fig. 4.7A). In addition, the protein level of pSTAT3, but not STAT3, in the complex was decreased by the treatment with BXL0124 (Fig. 4.7B). Since CD44 does not have kinase activity, JAK2 and Src were examined as the possible intracellular kinases required for the phosphorylation of STAT3 in the CD44-STAT3 complex. JAK2 was recruited by STAT3, and the amount of JAK2 proteins interacting with STAT3 was decreased with the BXL0124 treatment (Fig. 4.7A). Src was pulled down with STAT3, but the interaction was not changed by BXL0124 (Fig. 4.7A). When MCF10DCIS.com cell lysates were

immunoprecipitated with JAK2 antibody, significant amounts of CD44 and STAT3 were pulled down in the complex. This suggests that JAK2 forms a complex with CD44 and STAT3. The BXL0124 treatment decreased the amount of CD44v, CD44s, and pSTAT3 proteins interacting with JAK2 while the JAK2 level remained constant (Fig. 4.7B).

4.3.4. Suppression of MMP-9, MMP-14 and uPA mRNA as well as invasion of MCF10DCIS.com cells by CD44 knockdown.

To investigate the role of CD44 on DCIS invasion, we used CD44-knockdown MCF10DCIS.com cells transduced with shRNA for CD44 (DCIS-shCD44) or Luciferase (DCIS-shLuc) as a control. The decreased protein levels of CD44 and pSTAT3 in DCIS-shCD44 cells were detected with Western blot analysis (Fig. 4.8A). Knockdown of CD44 significantly decreased the proliferation of MCF10DCIS.com cells (Fig. 4.8B). As shown in Fig. 4C, the invasive potential of MCF10DCIS.com cells without lentivirus infection (DCIS) or DCIS-shLuc cells was not significantly different. However, the invasive potential of DCIS-shCD44 cells was significantly decreased, as shown by using the BME-coated chamber assay (Fig. 4.8C). To confirm the finding, we used Fluoroblok Biocoat cell invasion assay chambers with a fluorescence blocking bottom membrane that allows only cells that migrate through matrigel to be detected. Because DCIS-shLuc and DCIS-shCD44 cells were transduced with shRNA constructs containing green fluorescent protein (GFP), the green fluorescent cells that penetrated through matrigel were detected at the bottom of chamber and were quantified by counting green pixels (Fig. 4.8D). The knockdown of CD44 significantly inhibited invasive potential of MCF10DCIS.com cells (Fig. 4.8D). CD44 mRNA expression was significantly decreased in the DCIS-shCD44 cells at both 24 h and 48 h, confirming the knockdown of CD44 by shRNA (Fig. 4.9). We further determined the invasion markers that are changed by the knockdown of CD44.

The mRNA expression levels of MMP-2, MMP-9 MMP-13, MMP-14, MMP-15, MMP-16, and uPA at 24 h and 48 h in DCIS-shCD44 cells were compared to those in DCIS-shLuc cells. The mRNA expression of MMP-9, MMP-14 and uPA was significantly lower in the DCIS-shCD44 cells than in the DCIS-shLuc control cells at 48 h (Fig. 4.9); MMP-2 (Fig. 4.9) and other invasion markers (data not shown) did not show significant changes.

4.3.5. Inhibition of tumor growth and burden as well as invasion markers in MCF10DCIS.com xenograft tumors by CD44 knockdown.

To determine the role of CD44 *in vivo*, DCIS-shLuc and DCIS-shCD44 cells were injected into nu/nu mice, and tumor growth was compared. The growth rate of DCIS-shCD44 xenograft tumors was significantly slower than that of DCIS-shLuc control xenograft tumors (Fig. 4.10A). The average tumor weight from DCIS-shCD44 xenograft (560 ± 93 mg) was significantly lower than that from DCIS-shLuc xenograft (870 ± 150 mg) ($p<0.05$) (Fig. 4.10B). The levels of mRNA and protein of CD44 were significantly lower in the xenograft tumors from DCIS-shCD44 cells after 5 weeks of cell injection, indicating stable knockdown of CD44 (Fig. 4.11A and 4.11B). In addition, the mRNA expression levels of MMP-9 and uPA were significantly lower in DCIS-shCD44 xenograft tumors compared to those in DCIS-shLuc xenograft tumors (Fig. 4.11A). The protein levels of CD44v, CD44s, pSTAT3 and MMP-9 were markedly low in the DCIS-shCD44 xenograft tumors (Fig. 4.11B). Immunofluorescence staining confirmed the decreased levels of CD44 and pSTAT3 in DCIS-shCD44 xenograft tumors compared to DCIS-shLuc xenograft tumors (Fig. 4.11C).

4.3.6. Repression of CD44 and pSTAT3 in MCF10CA1a and MDA-MB-468 basal-like breast cancer cells.

To confirm the inhibitory effect of BXL0124 on CD44-STAT3 signaling in other basal-like breast cancer cells, MCF10CA1a and MDA-MB-468 cells were tested. Both MCF10CA1a and MDA-MB-468 cells showed markedly higher expression level of CD44v than CD44s, which is similar to CD44 expression pattern of MCF10DCIS.com cells (Fig. 4.12). The protein level of VDR was increased by BXL0124 treatment (Fig. 4.12). BXL0124 treatment decreased the protein levels of CD44v, CD44s and pSTAT3, whereas the protein level of total STAT3 was not affected (Fig. 4.12).

4.4. Discussion

MCF10DCIS.com cells form DCIS-like lesions which spontaneously progress to invasive ductal carcinoma (IDC) in immunodeficient mice [301]. The genetic alteration as well as expression patterns of molecular markers in the MCF10DCIS.com cells has been shown to highly resemble human DCIS [61]. In addition, with the unique bipotential progenitor property, MCF10DCIS.com cells give rise to not only epithelial cells but also myoepithelial cells which is critical component of DCIS to IDC transition [61,377]. Therefore, MCF10DCIS.com cells can serve as a unique tool to investigate preventive therapeutics to block or delay the progression from DCIS to IDC. Recently, Jedeszko *et al.* showed that the invasion by MCF10DCIS.com cells was significantly increased by recombinant hepatocyte growth factor (HGF), and identified increased expression of uPA and uPAR as critical cellular responses to HGF for the increased invasion [378]. Moreover, the coinjection of HGF-secreting fibroblasts increased the invasiveness of MCF10DCIS.com xenograft tumors, promoting the transition of DCIS to IDC in immunodeficient mice [378]. In the present study, BXL0124 treatment significantly decreased

proliferation and invasion markers in MCF10DCIS.com cells, suggesting BXL0124 as an important preventive agent to delay the transition of DCIS to IDC.

CD44 is overexpressed in many cancers and is involved in malignant tumor progression as well as metastasis [325]. A recent study by Montgomery *et al*. demonstrated that knockdown of CD44 repressed both basal and hyaluronan-induced invasion of basal-like breast cancer cells [379]. In the present study, we found that repression of CD44 by BXL0124 (Figs. 4.2 A and B) or CD44-shRNA (Figs. 4.8 C and D) significantly decreased the invasive potential of MCF10DCIS.com cells. Furthermore, we identified STAT3 as a downstream target of CD44 in MCF10DCIS.com cell invasion (Figs. 4.4 A and B). In mouse mammary tumor cells, knockdown of STAT3 strongly inhibits tumor invasion without affecting cell proliferation [121], supporting the notion of a specific role of CD44-STAT3 signaling in cancer cell invasion.

Hyaluronan stimulates the interaction between CD44 and Nanog, an embryonic stem cell transcription factor, leading to activation of STAT3, and knockdown of STAT3 by siRNA blocks hyaluronan-induced breast cancer cell growth [380]. In colon cancer cells, CD44 translocates into nucleus and directly interacts with STAT3 in response to osteopontin [381]. Moreover, ectopic expression of CD44 markedly increased STAT3 activation, indicating a direct regulation of STAT3 signaling by CD44 [381]. In our study, MCF10DCIS.com cells showed high CD44 protein level and constitutively activated STAT3 signal (Fig. 4.4A). In MCF10DCIS.com cells, CD44 interacts with STAT3 in the absence of exogenous ligands, suggesting that a constitutively high level of CD44 might be sufficient to activate STAT3 signaling for cell invasion. In addition, STAT3 and JAK2 interaction was decreased when protein level of CD44 was repressed by BXL0124 (Figs. 4.7 A and B), indicating that CD44 might function as a scaffold protein for the CD44-STAT3-JAK2 complex. The JAK2/STAT3 signaling pathway is preferentially activated in $CD44^{+}$ breast cancer stem cell population over other cell populations, and hyaluronic acid synthase 1 (HAS1) is a STAT3 signaling-related molecule in basal-like breast cancer [120]. In

addition, recent studies reported that STAT3 is one of the key signaling molecules that maintain breast cancer stem cell population [119], and that knockdown of STAT3 with shRNA markedly repressed mammary tumorigenesis in mice [121]. As summarized in Fig. 4.13, the direct interaction between CD44, STAT3 and JAK2 may be critical for activation of STAT3 in MCF10DCIS.com cells, and CD44 might function as a scaffold of the STAT3-JAK2 complex.

Gene regulation by STAT3 was mediated by binding of STAT3 onto the STAT-binding element with the consensus sequences [382]. STAT3 has been shown to regulate a wide range of genes, which are associated with cancer cell invasion and metastasis, and MMPs were one family of the critical STAT3 target genes [383,384]. MMP-2, which has biologically active STAT-binding sites with consensus sequences at the promoter region, has been identified as one of the key STAT3-regulated genes to promote tumor invasion and metastasis [385]. Potential STAT-binding sites with consensus sequences was also found in MMP-9 promoter, and transduction of constitutively activated STAT3 significantly increased mRNA level of MMP-9 and induced transformation of human epithelial cells [386]. In the present study, we demonstrated that BXL0124 treatment repressed mRNA expression levels of MMP-2, MMP-9 and MMP-14 as well as the binding of STAT3 onto the oligonucleotides which contain consensus sequence for STAT3 binding, suggesting possible STAT3-dependent MMPs regulation by BXL0124. In addition, mRNA levels of MMP-9 and MMP-14 as well as activation of STAT3 were significantly decreased by knockdown of CD44 in MCF10DCIS.com cells, indicating that MMPs might be downstream targets of CD44/STAT3 signaling in MCF10DCIS.com cells.

The expression level of MMP-9 has been correlated with the level of activated STAT3 in human breast cancer [386]. MMP-9 induces cancer cell invasion by degrading collagen type IV, the most abundant component of the basement membrane [387]. In breast cancer, high expression levels of MMP-9 have been associated with node metastasis and advanced tumor stage [388]. In addition, uPA is a critical enzyme for cancer cell invasion converting plasminogen into plasmin,

which degrades extracellular matrix and activates multiple MMPs, including MMP-9 [389]. Breast cancer patients with high levels of uPA activity showed a significantly shorter disease-free period [389]. In recent studies, expression of uPA was significantly elevated in a CD44 dependent manner in highly invasive basal-like breast cancer [379], and the protein microarray of primary breast cancer tissue found significant correlation between expression levels of uPA and STAT3 [390]. In addition, uPA was identified as a key molecule to be regulated by STAT3 in wound healing and cancer [391]. These reports support our results that MMP-9 and uPA via CD44-STAT3 signaling play a critical role in breast cancer invasion inhibited by BXL0124 in basal-like breast cancer.

4.5 Conclusion

CD44 plays an essential role in the modulation of STAT3 signaling by forming a complex with STAT3 and JAK2. Consequently, high expression levels of CD44 may lead to a constitutive activation of STAT3 signaling in basal-type breast cancer. The novel Gemini vitamin D analog BXL0124 represses the expression of CD44, which results in a decreased amount of the CD44-STAT3-JAK2 complex. Our study suggests that repression of STAT3 signaling by targeting CD44 may be a key molecular mechanism of BXL0124-induced inhibition of breast cancer invasion, a critical step in cancer progression.

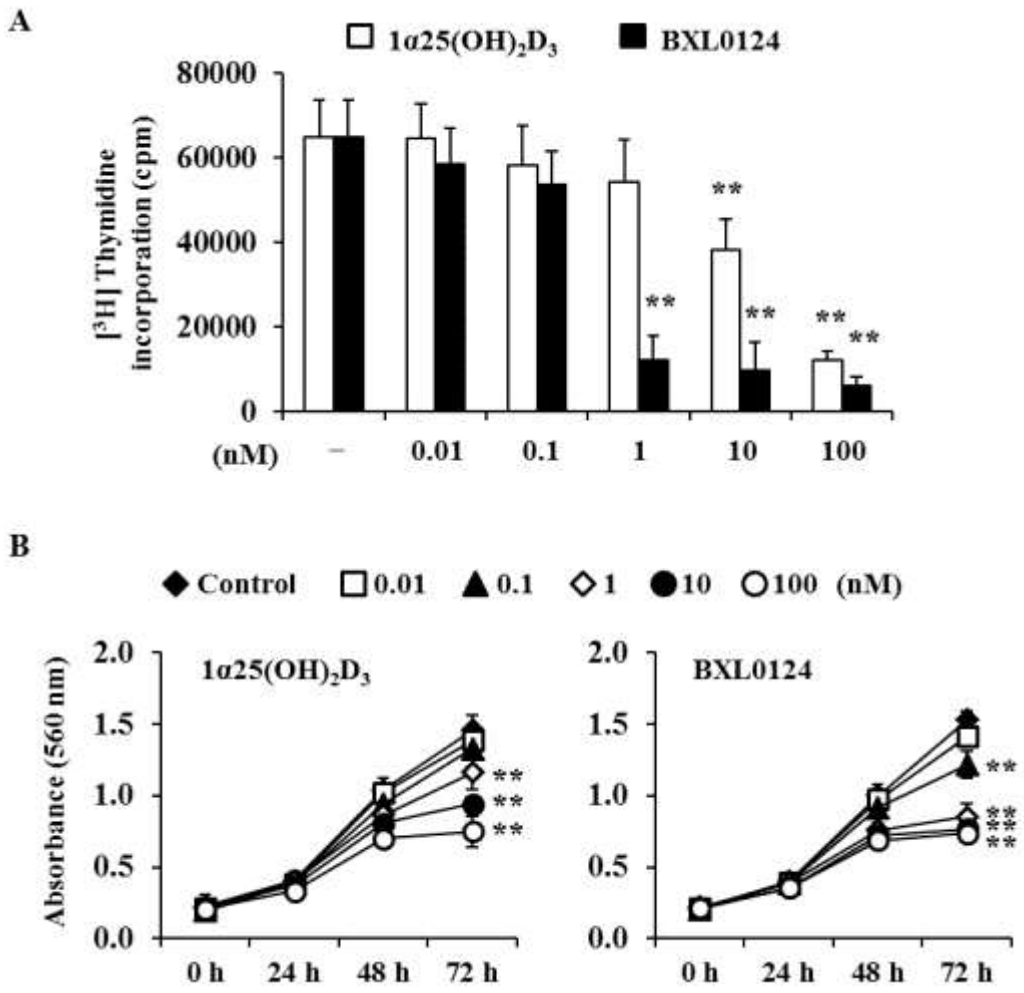


Fig. 4.1 Inhibition of proliferation and metabolic activity of MCF10DCIS.com cells by 1α,25(OH)$_2$D$_3$ and BXL0124. MCF10DCIS.com cells were incubated with 0.01, 0.1, 1, 10 or 100 nM of 1α,25(OH)$_2$D$_3$ or the Gemini vitamin D analog BXL0124 for 72 h. **(A)** The cell proliferation of MCF10DCIS.com cells was measured by thymidine incorporation rate. Two separate experiments with triplicates were conducted (**p < 0.01). **(B)** The metabolic activity of MCF10DCIS.com cells was determined by MTT assay. Two separate experiments with quadruplicates were conducted (**p < 0.01).

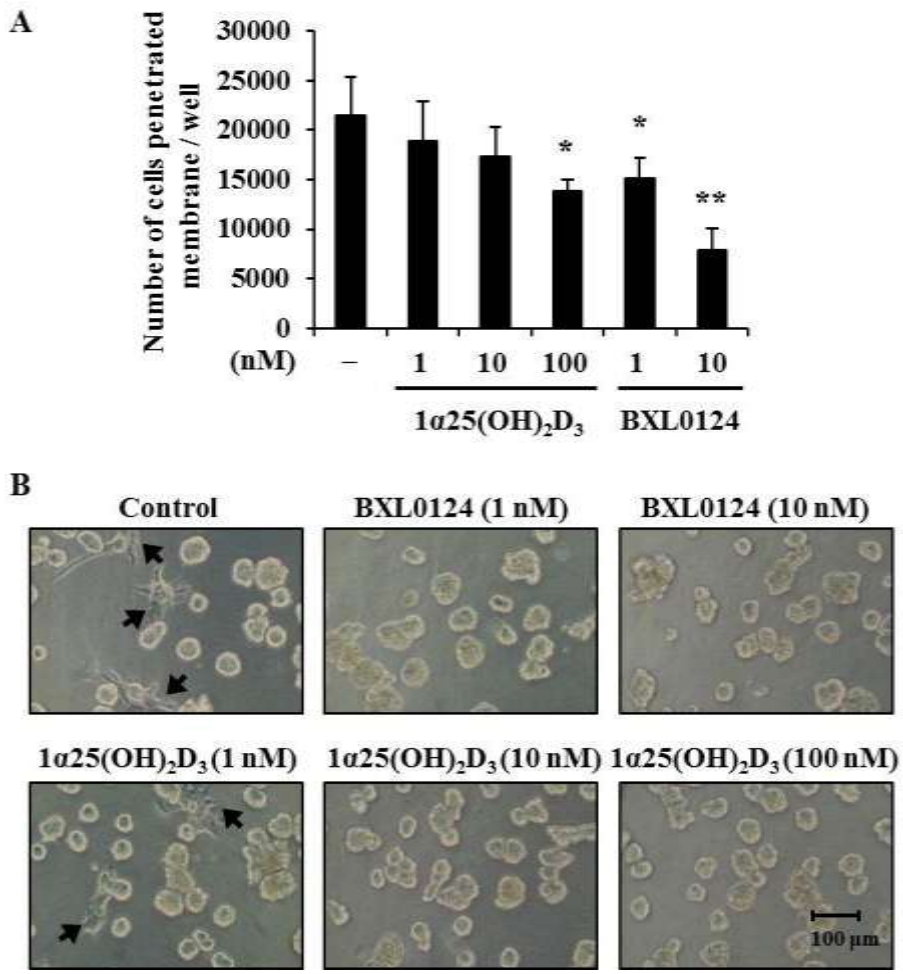


Fig. 4.2 Repression of invasion of MCF10DCIS.com cells by 1α,25(OH)$_2$D$_3$ and BXL0124. **(A)** MCF10DCIS.com cells were incubated in the basement membrane extract (BME)-coated invasion chambers in the presence or absence of 1α,25(OH)$_2$D$_3$ (1, 10 or 100 nM) or BXL0124 treatment (1 or 10 nM) for 48 h. The cells that penetrated through BME layer were detected from the bottom of chamber, and counted using Calcein-AM staining. Two separate experiments with triplicates were conducted (*$p < 0.05$, **$p < 0.01$). **(B)** MCF10DCIS.com cells were incubated in 3D culture with or without 1α,25(OH)$_2$D$_3$ (1, 10 or 100 nM) or BXL0124 (1 or 10 nM) for 10 days, with replenishing medium every 2 days. Representative images are shown, and the cells with invasive outgrowth are indicated with arrows.

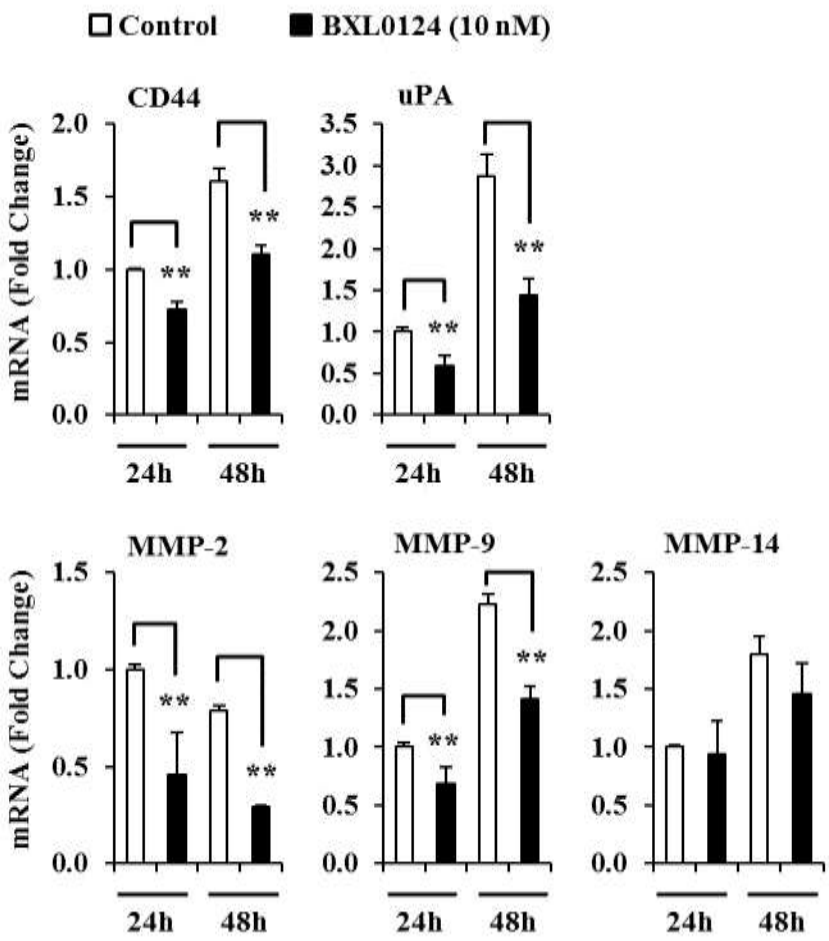


Fig. 4.3 Down-regulation of invasion markers by BXL0124 in MCF10DCIS.com cells. MCF10DCIS.com cells were treated with BXL0124 (10 nM) for 24 h and 48 h. The mRNA expression levels of CD44 (20, the approximate qPCR cycle number of 24 h control), uPA (21), MMP-2 (24), MMP-9 (29) and MMP-14 (23) were determined. Three separate experiments with duplicates were conducted (**$p < 0.01$).

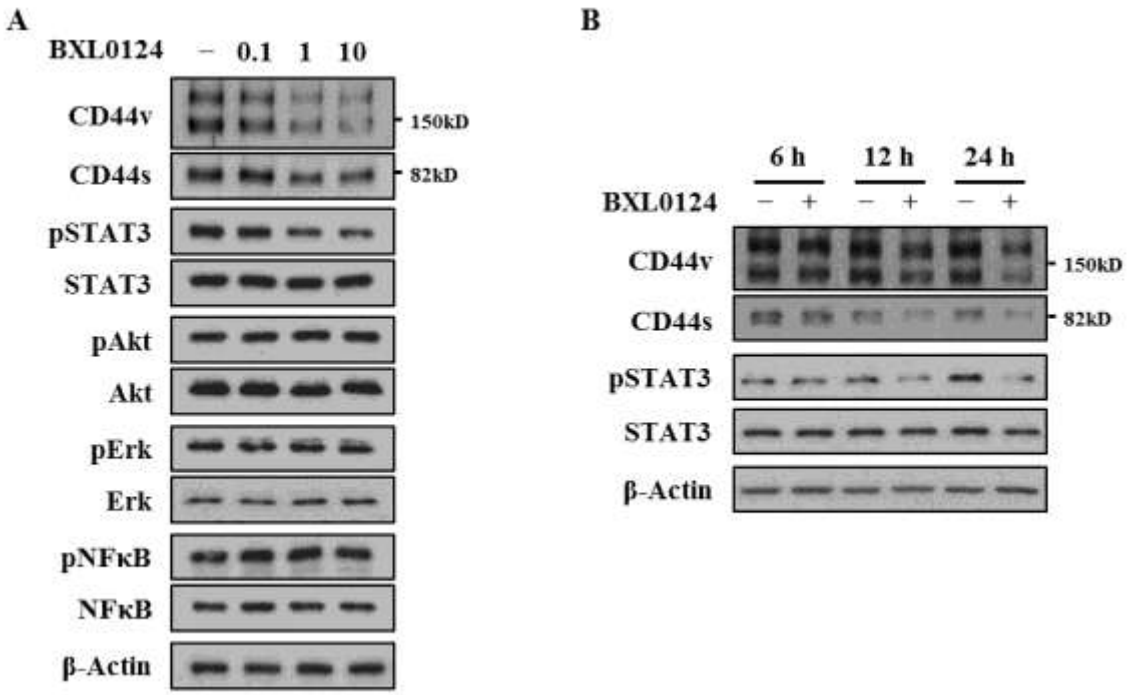


Fig. 4.4 Repression of protein levels of CD44 and pSTAT3 by BXL0124 in MCF10DCIS.com cells. (A) MCF10DCIS.com cells were treated with BX0124 (0.1, 1 or 10 nM) for 24 h. **(B)** MCF10DCIS.com cells were treated with BXL0124 (10 nM) for 6 h, 12 h and 24 h. The protein levels of indicated molecules were examined by Western blot analysis, and β-actin was used as a loading control.

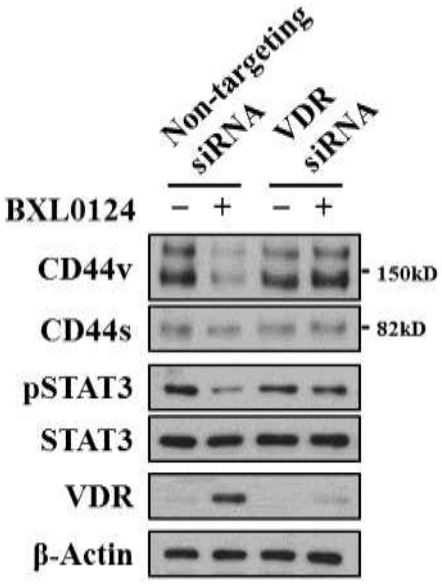


Fig. 4.5 Repression of the protein levels of CD44 and pSTAT by BXL0124 in a VDR-dependent manner in MCF10DCIS.com cells. MCF10DCIS.com cells were transfected with non-targeting siRNA or VDR siRNA and treated with BXL0124 (10 nM) for 24 h. The protein levels of indicated molecules were examined by Western blot analysis, and β-actin was used as a loading control.

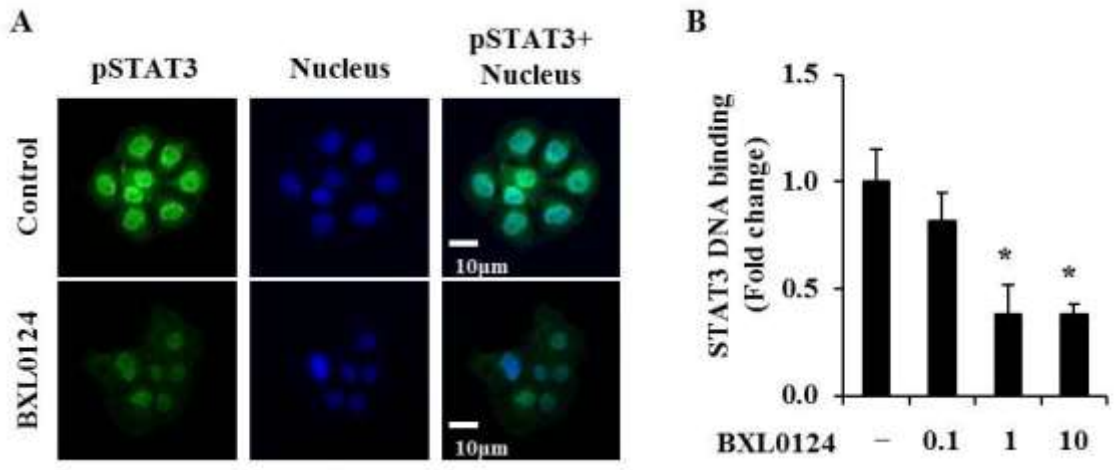


Fig. 4.6 Inhibition of STAT3 activation by BXL0124 in MCF10DCIS.com cells. (A) MCF10DCIS.com cells were treated with BXL0124 (10 nM) for 24 h. Cells were fixed using 4% paraformaldehyde and stained with antibody against pSTAT3 (green). Nuclei were stained with To-PRO-3 (blue). **(B)** MCF10DCIS.com cells were treated with BXL0124 (0.1, 1 or 10 nM) for 24 h. Each cell lysate was incubated with oligonucleotides containing STAT3 binding sequences. The amount of STAT3 bound to the oligonucleotides was measured as chemiluminescent intensity value by luminometer. The fold change of chemiluminescent intensity value in each sample from control was determined (*$p < 0.05$).

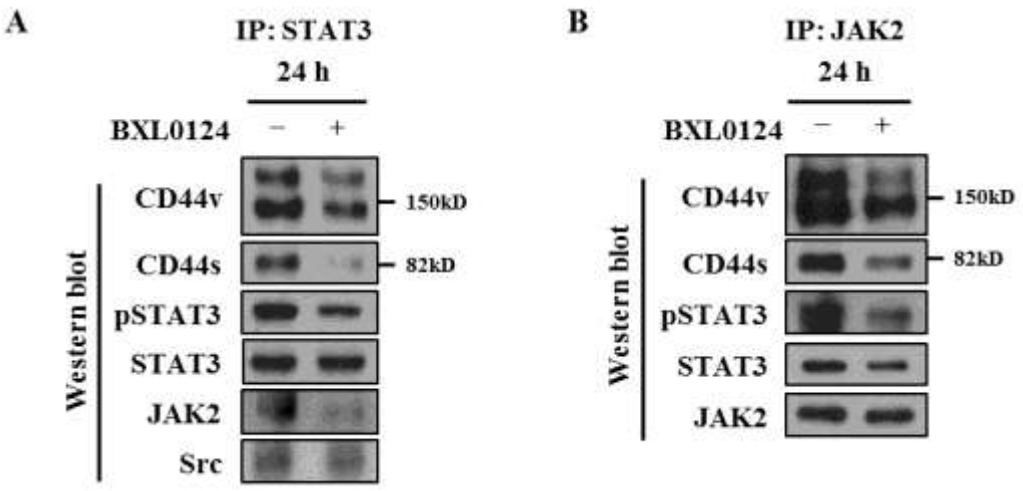


Fig. 4.7 Repression of CD44-STAT3 interaction by BXL0124 in MCF10DCIS.com cells. MCF10DCIS.com cells were treated with BXL0124 (10 nM) for 24 h, then the cell lysates were immunoprecipitated with **(A)** STAT3 or **(B)** JAK2 antibodies. The amounts of given proteins interacting with STAT3 or JAK2 were determined by Western blot analysis. STAT3 and JAK2 were used as loading control for each immunoprecipitation experiment, respectively.

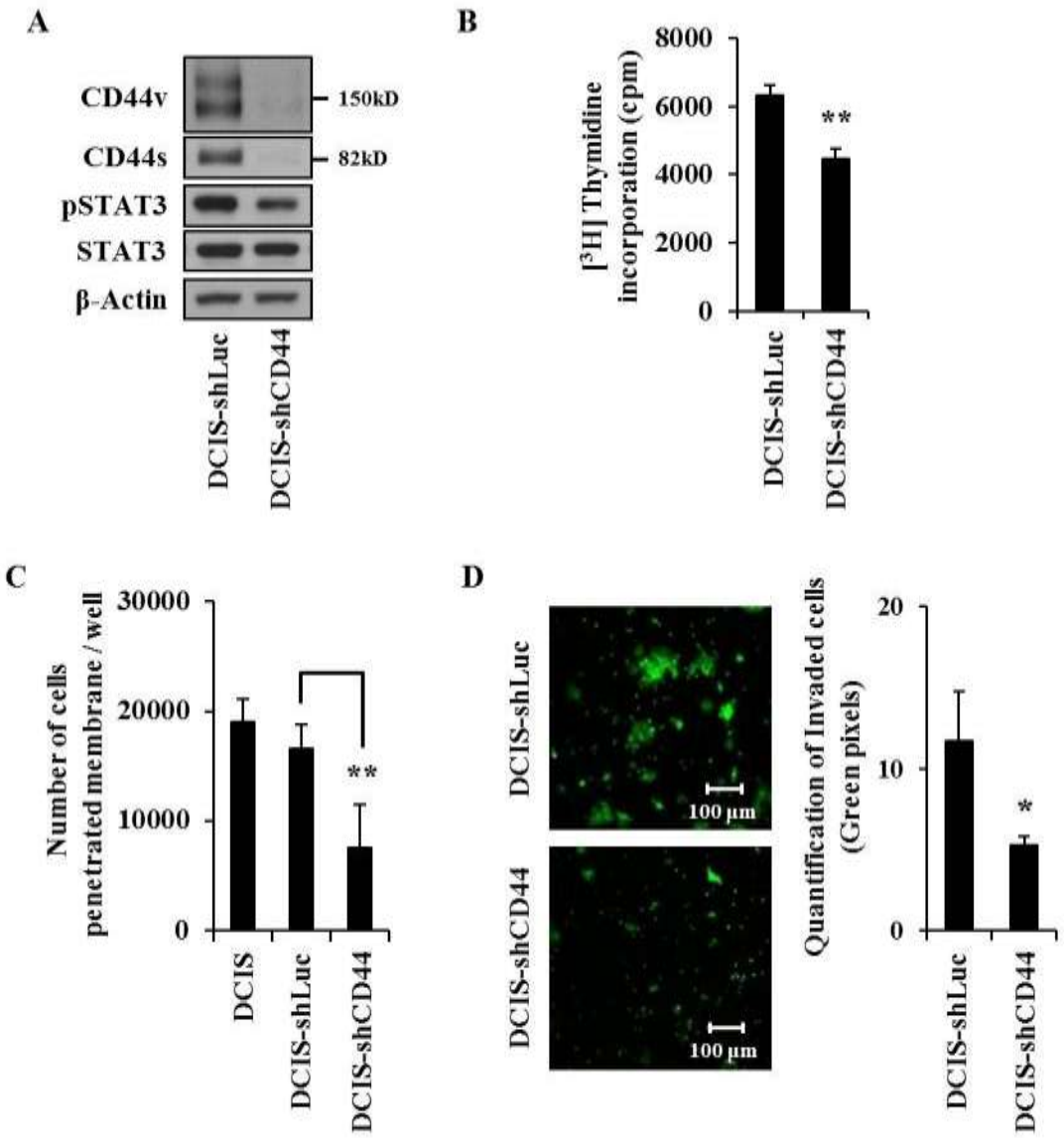


Fig. 4.8 Inhibition of cell proliferation and invasion of MCF10DCIS.com cells by CD44 knockdown. (A) The protein levels of CD44v, CD44s and pSTAT3 were markedly repressed in DCIS-shCD44 cells. **(B)** DCIS-shLuc or DCIS-shCD44 cells (2,000 cells/well) were incubated for 48 h and the cell proliferation was determined by thymidine incorporation. Two separate experiments with triplicates were conducted (** $p<0.01$). **(C)** MCF10DCIS.com (DCIS), DCIS-shLuc or DCIS-shCD44 cells were incubated for 48 h in BME-coated invasion assay chambers. The number of cells that penetrated the BME layer was counted by Calcein-AM staining. Two

separate experiments with triplicates were conducted (**$p < 0.01$). **(D)** DCIS-shLuc or DCIS-shCD44 cells were incubated for 48 h in Fluoroblok biocoat invasion assay chambers. Since both cells were labeled with green fluorescence, the cells that penetrated matrigel layer were shown as green pixels in the image. The green pixels were counted using Image-J program for quantitative evaluation. Two separate experiments with triplicates were conducted (*$p < 0.05$).

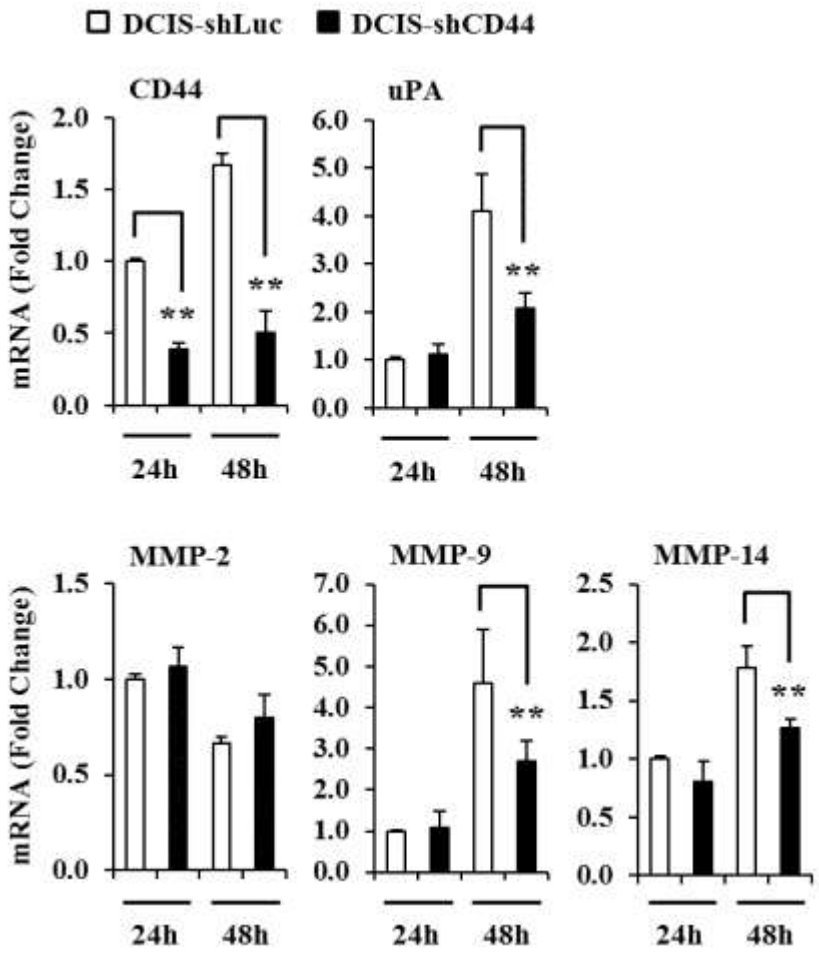


Fig. 4.9 Down-regulation of mRNA levels of MMP-9, MMP-14 and uPA by CD44 knockdown in MCF10DCIS.com cells. The mRNA expression levels of CD44 (20, the approximate qPCR cycle number of DCIS-shLuc cells at 24 h), MMP-2 (24), MMP-9 (29), MMP-14 (23) and uPA (21) in DCIS-shLuc and DCIS-shCD44 cells were determined after 24 h and 48 h of incubation. Three separate experiments with duplicates were conducted (**$p < 0.01$).

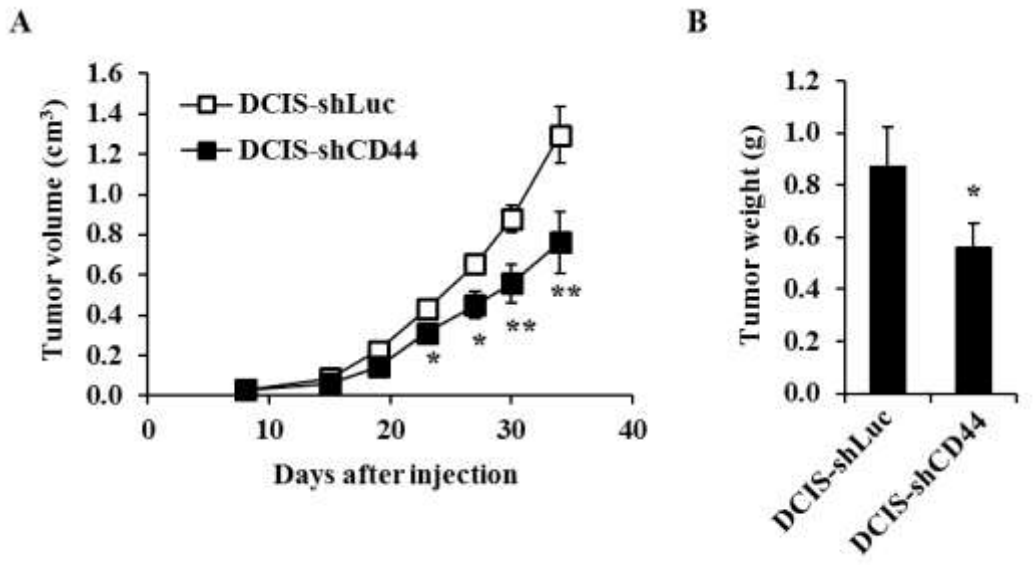


Fig. 4.10 Inhibition of the growth of MCF10DCIS.com xenograft tumors by CD44 knockdown. DCIS-shLuc or DCIS-shCD44 cells (1.0 X 10^6 cells) were injected into the mammary fat pad of nu/nu mice (n=5 per group), and mammary tumor size was measured twice a week. **(A)** The xenograft tumors from DCIS-shCD44 cells showed significantly slower growth rate than that of DCIS-shLuc xenograft tumors (*$p < 0.05$, **$p < 0.01$). **(B)** The average tumor weight from DCIS-shCD44 cells was significantly smaller than that from DCIS-shLuc cells (*$p < 0.05$).

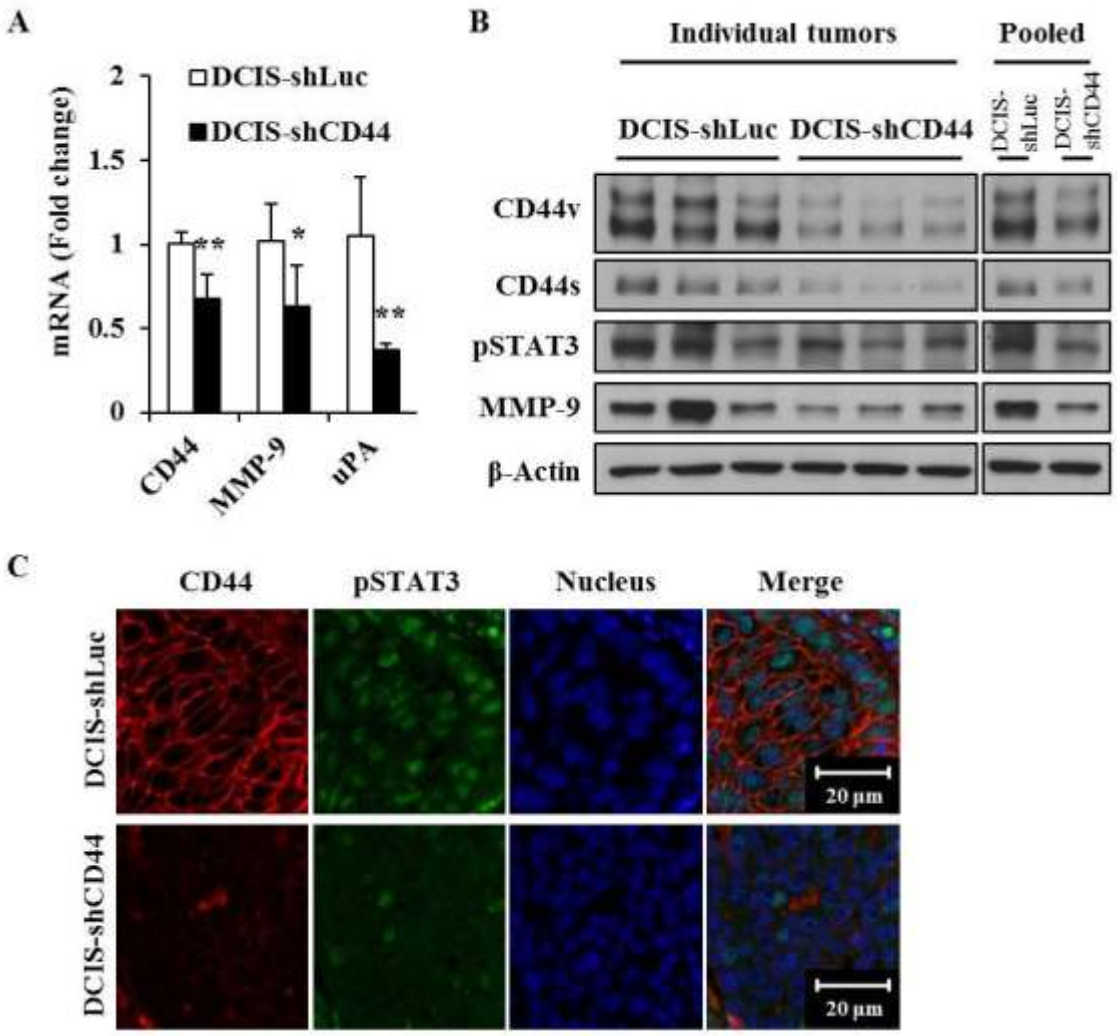


Fig. 4.11 Repression of expression levels of invasion markers by CD44 knockdown in MCF10DCIS.com xenograft tumors. (A) The mRNA expression levels of CD44 (21, the approximate qPCR cycle number of DCIS-shLuc tumors), MMP-9 (22) and uPA (22) were significantly down-regulated in DCIS-shCD44 xenograft tumors (n=5) (*$p < 0.05$, **$p < 0.01$). **(B)** The protein levels of CD44v, CD44s, pSTAT3, and MMP-9 were markedly decreased in DCIS-shCD44 xenograft tumors. Three xenograft tumors from each group were combined for pooled samples. β-Actin was used as a loading control. **(C)** The protein levels of CD44 and pSTAT3 in DCIS-shLuc and DCIS-shCD44 xenograft tumors were determined by

immunofluorescent staining of CD44 (red) and pSTAT3 (green). Nuclei were stained with TO-PRO-3 (blue).

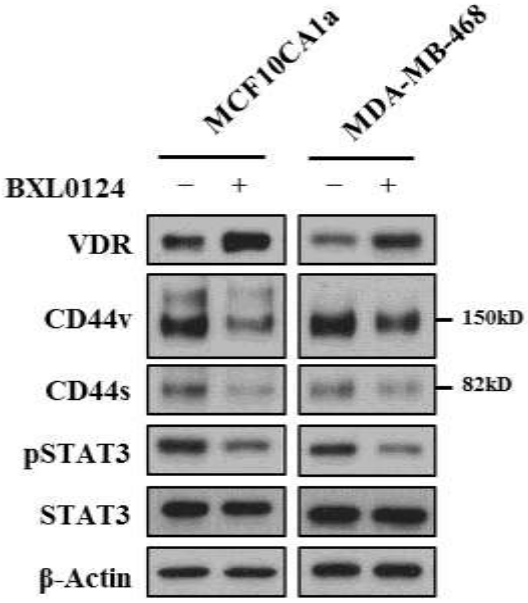


Fig. 4.12 Repression of the CD44-STAT3 signaling by BXL0124 in basal-like breast cancer cells. MCF10CA1a and MDA-MB-468 cells were incubated with BXL0124 (10 nM) for 24 h and the protein levels of VDR, CD44v, CD44s, pSTAT3 and STAT3 were determined by Western blot analysis. β-Actin was used as a loading control.

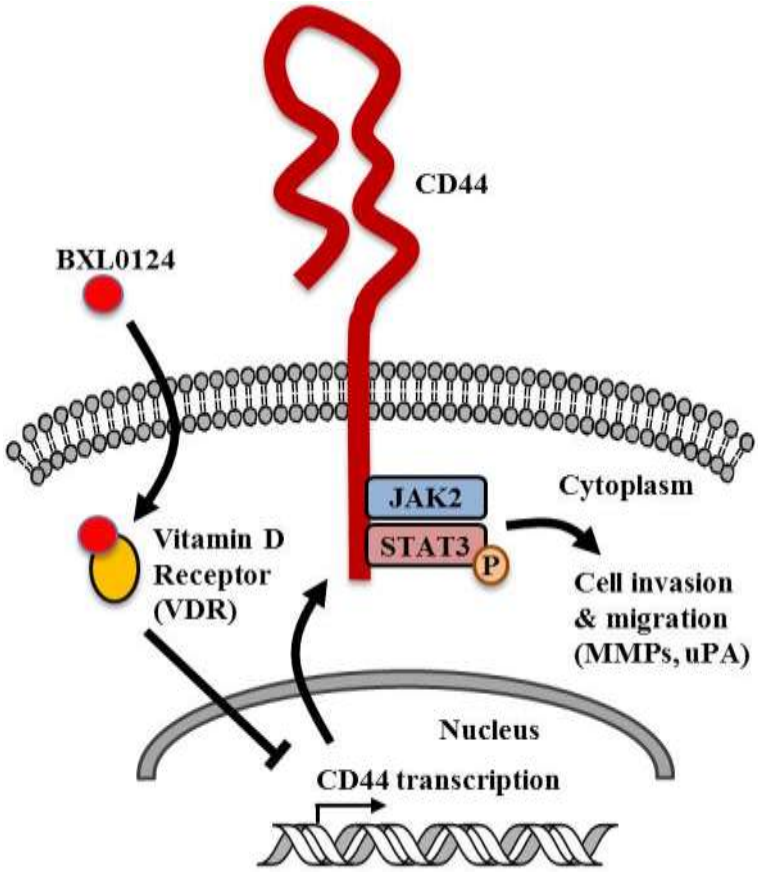


Fig 4.13 A schematic diagram of proposed mechanism of action of BXL0124 on CD44-STAT3 signaling and breast cancer cell invasion in basal-like breast cancer.

Chapter 5: Inhibition of tumorigenesis by a Gemini vitamin D analog and a synthetic triterpenoid in the MMTV-HER2/neu transgenic mouse model

5.1. Introduction

One of major genetic abnormalities occurring in invasive breast cancers is the amplification of the HER2 oncogene, representing about 20% of human breast cancer cases [83]. Overexpression of HER2 leads to constitutive activation of HER2 signaling, contributing aggressive phenotype of breast cancer [83]. While HER2 does not have specific ligand, it forms heterodimer with other HER receptors, EGFR (HER1), HER3 and HER4, and functions as signal amplifier [32]. The HER2-targeted therapies, such as trastuzumab and lapatinib, have been brought significant clinical benefits to the patients with HER-overexpressing tumors [33]. However, substantial proportion of HER2-positive patients has shown *de novo* or acquired resistance to the HER2-tageted therapies, and overcoming the resistance has emerged as new challenge [33].

$1\alpha,25(OH)_2D_3$ and its analogs have been shown to inhibit the growth of HER2-overexpressing breast cancer cells [392,393]. Moreover, loss of VDR by crossing with VDR knockdown mice significantly increased the incidence of preneoplastic lesions and promoted mammary tumor development in MMTV-HER2/neu transgenic mice [144]. In our previous study, the Gemini vitamin D analog have been shown to repress the growth of MMTV-HER2/neu mammary tumors by inhibiting the HER2/AKT/ERK signaling pathways [276].

Synthetic triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), showed potent anti-proliferative effects on various subtypes of breast cancer cells, including HER2-

overexpressing breast cancer cells [208]. CDDO also blocked the enhanced xenograft tumor growth of MCF-7 cells by overexpressing HER2 *in vivo* [227]. Recent study demonstrated that a derivative of CDDO, CDDO-methylester (CDDO-Me), significantly delayed the mammary tumor development in MMTV-HER2/neu transgenic mice model [253]. Moreover, the inhibitory effect of CDDO-Me on MMTV-HER2/neu mammary tumorigenesis was significantly enhanced in the combination with rexinoid (LG100268), a selective ligand to retinoid X receptors (RXR) [253].

CDDO and its derivatives have been shown to synergize with vitamin D or its analogs to induce differentiation in other cancers [216,249]. Therefore, in this chapter, I investigated the inhibitory effects of Gemini vitamin D analog BXL0124 and CDDO-Im, another potent derivative of CDDO, as well as the combination in mammary tumorigenesis of MMTV-HER2/neu transgenic mice. In addition, the MMTV-HER2/neu mammary tumors from each treatment group were analyzed for numbers of the HER2 signaling associated-molecules to extend our understanding on the molecular mechanisms of BXL0124 and CDDO-Im for their anti-cancer activities against HER2-positive breast cancer.

5.2 Materials and methods

5.2.1 Reagents

Gemini vitamin D analog 1α,25-dihydroxy-20R-21(3-hydroxy-3-deuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluro-cholecalciferol (BXL0124, [361]) was provided by BioXell, Inc. (Nutley, NJ) and dissolved in dimethyl sulfoxide (DMSO). 1-[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-imidazole (CDDO-Im) was provided by Dr. Michael Sporn at Dartmouth Medical School (Hanover, NH) and dissolved in DMSO. For *in vivo* animal experiment, BXL0124 and CDDO-Im were diluted in sesame oil (Sigma-Aldrich, St. Louis, MO) for oral administration.

5.2.2 Transgenic mice and treatment

MMTV-HER2/neu transgenic mice (6-7 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME). DMSO control, BXL0124 (0.3 μg/kg body weight), CDDO-Im (3 μmole/kg body weight) or the combination of BXL0124 and CDDO-Im was orally administered 3 times a week to the mice, beginning when they were 3 month old. The bodyweight and tumor size of each animal were measured weekly. The mice were sacrificed when they were 56 weeks old, and tumors were weighed at autopsy. The tumor samples were fixed in 10% formalin and transferred to 70% ethanol for immunofluorescent staining or flash frozen and stored in -80 ℃ for Western blot analysis or RNA analysis. All animal studies were conducted in accordance with an institutionally approved protocol. The protocol was approved by the Institutional Animal Care and Use Committee at Rutgers, the State University of New Jersey (Protocol Number: 04-001). All surgery was performed under ketamine anesthesia, and all efforts were made to minimize suffering.

5.2.3 Western blot analysis

The detailed procedure was described previously [362]. The primary antibodies recognizing pHer2-Tyr877, pHer2-Tyr1248, pHer2-Tyr1221/1222, Her2, pAkt, Akt, pErk1/2, Erk1/2, pMek1/2, c-Myc, pSTAT3, STAT3 and PPARγ were from Cell signaling technology (Danvers, MA); CD44, which recognizes both CD44v and CD44s, RXRα and PKCα were from Santa Cruz Biotechnology (Santa Cruz, CA); MMP-9 was from Abcam (Cambridge, MA); VDR was from Thermo scientific (Lafayette, CO); β-actin was from Sigma-Aldrich (St. Louis, MO). Secondary antibodies were from Santa Cruz Biotechnology.

5.2.4 Fluorescence microscopy

Tumors were embedded in paraffin (Electron Microscopy Sciences, Hatfield, PA) and then sectioned onto slides at 4 μm thickness. The sample slides were incubated with PBS containing 10% donkey serum to block non-specific binding. The sample slides were then incubated overnight at 4℃ with a combination of primary antibodies to pHer2 (1:100, Cell Signaling Technology) and Her2 (1:40, Cell Signaling Technology). Fluorophore-conjugated secondary antibodies (Alexa Fluor 488 and 546, 1:100; Jackson ImmunoResearch Laboratories Inc., West Grove) and TO-PRO-3 iodide nuclear antibody (Invitrogen, 1 μM) were incubated at room temperature for 60 and 15 minutes, respectively. The images were taken using confocal microscope with laser at 488 nm (pHer2), 546 nm (Her2), and 633 nm (TO-PRO-3).

5.2.5 Quantitative real-time polymerase chain reaction

The procedure was described previously [160]. The labeled primers for heparin-binding epidermal growth factor (HB-EGF), epidermal growth factor (EGF), neuregulin-2 (Nrg2), and glyceraldehyde 3-phosphate dehydrogenase were obtained from Life Technologies Corporation (Carlsbad, CA).

5.2.6 Statistical analysis

Data was analyzed using Graph Pad Prism 4.0 (GraphPad Software Inc.). Statistical significance was evaluated by using one-way analysis of variance (ANOVA) followed by

Dunnett's post-test. The data presented represents the mean ± S.E.M. P values <0.05 were considered significant.

5.3. Results

5.3.1. Delayed development of mammary tumors by BXL0124, CDDO-Im and the combination of BXL0124 and CDDO-Im in MMTV-HER2/neu transgenic mice.

To investigate the effects of BXL0124, CDDO-Im and the combination on HER2-overexpressing mammary tumorigenesis, I orally administered DMSO control, BXL0124, CDDO-Im or the combination to MMTV-HER2/neu transgenic mice beginning at 3 months of age. The incidence of mammary tumor was markedly delayed by the treatment of BXL0124, CDDO-Im and the combination (Fig. 5.1A). The control group reached 50% tumor incidence at week 45 (Fig. 5.1A). BXL0124 and CDDO-Im treated group showed delayed development of tumors with 50% tumor incidence by week 51 and 53, respectively (Fig. 5.1A). The combination group did not reach 50% tumor incidence until the mice were sacrificed at week 56 (Fig. 5.1A). The tumor multiplicities (the average number of tumors per mouse within a group) were significantly lower in the animals treated with CDDO-Im (0.65) and the combination (0.53) as compared to the animals from control group (1.11) at the end of experiment (Fig. 5.1B). The average tumor burden was decreased by the treatment with BXL0124, CDDO-Im and the combination, while the reduction was statistically significant only in the CDDO-Im and the combination groups (Fig. 5.1C). The average bodyweight and serum calcium levels were not affected by compounds treatment in all groups (Fig. 5.1D and E).

5.3.2 Inhibition of the activation of the HER2/EGFR/Erk signaling pathway by BXL0124, CDDO-Im and the combination in mammary tumors of MMTV-HER2/neu transgenic mice.

The effects of BXL0124, CDDO-Im and the combination on the activation of HER2 and EGFR in MMTV-HER2/neu mammary tumors were investigated. Oral administration of BXL0124, CDDO-Im and the combination markedly decreased the protein levels of pHER2 without significantly affecting the total protein levels of HER2 in MMTV-HER2/neu mammary tumors (Fig. 5.2). The protein levels of pEGFR were also markedly decreased by BXL0124, CDDO-Im and the combination (Fig. 5.2). The protein levels of EGFR were decreased only in CDDO-Im and the combination treatment groups (Fig. 5. 2). All three BXL0124, CDDO-Im and the combination treatment markedly decreased the protein levels of pErk1/2 and c-Myc (Fig. 5.2). The protein levels of pAkt were decreased only by the combination treatment, while BXL0124 and CDDO-Im treatments did not show significant changes of pAkt protein levels as compared to control group (Fig. 5.2).

5.3.3 Down-regulation of the downstream of the HER2 signaling pathway by BXL0124, CDDO-Im and the combination in mammary tumor of MMTV-HER2/neu transgenic mice.

The effects of BXL0124, CDDO-Im and the combination on HER receptors and their downstream pathway in HER2-overexpressing mammary tumor were investigated. The phosphorylation of HER2 at Tyr877 residue was markedly reduced by all three treatments, and the combination showed the strongest reduction (Fig. 5.3A). The phosphorylation of HER2 at Tyr1248, Tyr1221 and Tyr1222 residue was greatly repressed by CDDO-Im and the combination treatment, but the repression was less significant with BXL0124 treatment (Fig. 5.3A). The protein level of HER2 was not affected by all three treatments (Fig. 5.3A). The protein level of phosphorylated HER3 was decreased by CDDO-Im and the combination treatment, while the

protein level of HER3 was not affected (Fig. 5.3A). The protein levels of pJAK2 and pSrc were markedly decreased by all three treatments, showing most striking effect with the combination treatment (Fig. 5.3B). All three treatments did not have significant effect on the protein levels of JAK2 and Src (Fig. 5.3B). The protein levels of pMek1/2, pErk1/2, cyclin D1 and c-Myc were repressed by all three compound treatments, and BXL0124 and the combination treatment showed stronger repression than CDDO-Im treatment (Fig. 5.3B). The total protein level of Erk1/2 was not significantly changed by any compounds (Fig. 5.3B). The protein levels of VDR, PPARγ, RXRα and PCKα did not show noticeable changes by BXL0124, CDDO-Im and the combination (Fig. 5.3C). The downstream pathways of HER receptors regulated by BXL01242, CDDO-Im and their combination were shown as a schematic diagram (Fig. 5.4).

5.3.4 Repression of HER2 activation by BXL0124, CDDO-Im and the combination at the invading edge of mammary tumors of MMTV-HER2/neu transgenic mice.

The activation of HER2 predominantly occurs at the edge area of mammary tumor where tumor cells are invading into surrounding stromal cells (Fig. 5.5). BXL0124, CDDO-Im and the combination markedly decreased the activation of HER2 at the invading edge area of mammary tumors (Fig. 5.5).

5.3.5 The down-regulation of EGF mRNA by BXL0124 and HB-EGF mRNA by CDDO-Im and the combination in mammary tumors of MMTV-HER2/neu transgenic mice.

HER2 can be transactivated by other members of HER family receptors. Therefore, I investigated whether BXL0124, CDDO-Im and the combination regulate mRNA levels of ligands for EGFR and HER3, EGF, HB-EGF, neuregulin1 (Nrg1) and neuregulin2 (Nrg2) in mammary

tumors of MMTV-HER2/neu transgenic mice. The mammary tumors treated with BXL0124 showed significantly decreased mRNA level of EGF (Fig. 5.6). The mRNA level of HB-EGF was significantly lower in mammary tumors from CDDO-Im and the combination groups (Fig. 5.6). In the tumors from all groups, the mRNA expression level of Nrg1was too low (data not shown). The treatment with BXL0124, CDDO-Im and the combination did not show significant effects on the mRNA levels of Nrg2 (Fig. 5.6).

5.4 Discussion

The MMTV-HER2/neu transgenic mouse model has been a valuable tool to understand the HER2-driven mammary tumorigenesis and investigate HER2 targeting therapeutics [394]. In MMTV-HER2/neu mammary tumors, the overexpression of other two HER family members, EGFR and HER3, has been reported, which also observed in HER2-positive human breast tumors [395,396]. HER2 can form heterodimer with EGFR or HER3 resulting in the activation of downstream signaling pathways such as MAPK and PI3K/Akt signaling pathway [397]. In human breast cancer cells, persistent transactivation of EGFR and HER2 induced activation of MAPK signaling and facilitated cancer cell invasion [398]. In this chapter, the comparison of individual tumors demonstrated strong correlation of protein levels among pHER2, pEGFR and pErk1/2, suggesting that tumors with high protein level of pHER2 also exhibited high protein levels of pEGFR and pErk1/2 (Fig. 5.2). This result indicates the persistent transactivation between HER2 and EGFR in MMTV-HER2/neu mammary tumors, and the Erk signaling pathway as the key downstream signaling regulated by transactivation of HER2 and EGFR.

c-Myc is a key transcriptional regulator of cell proliferation, differentiation and apoptosis, and its amplification has been frequently found in human breast cancer [399]. In HER2-overexpressing breast cancer cells, c-Myc was demonstrated as one of key effectors of HER2

signaling to sustain the HER2-drvien cell proliferation [400]. Moreover, the strong correlation between c-Myc amplification and HER2 amplification was reported in human breast cancer [401]. Recent trastuzumab trial demonstrated that c-Myc alterations were associated with different clinical benefit with trastuzumab in patients with early stage HER2-positive breast cancer [402]. I found that c-Myc was highly expressed in MMTV-HER2/neu mammary tumors and identified c-Myc as the key signaling molecule to regulate further downstream effector molecules such as p21 and PCNA to inhibit cell proliferation (Fig 5.2 and Fig 5.3 A and B) supporting the association between c-Myc and HER2 signaling.

The stable expression of HER2 with activating mutation in non-invasive MCF10A mammary epithelial cells induced the invasive growth of MCF10A cells in 3D culture [403]. In addition, MCF10A cells infected with viral plasmid to overexpress wild-type HER2 induced invasive growth in 3D culture only with the treatment of EGF or neuregulin β-1 [404], suggesting the critical role of HER2 activation on the invasive potential of breast cancer. In this chapter, I showed the strong activation of the HER2 signaling in the invasive front edge area of MMTV-HER2/neu tumors (Fig. 5.5) where most abundant EGF secreted from surrounding stromal cells are available. Together with the evidence of previous *in vivo* studies [403,404], our data indicate that the ligand-dependent activation of HER2 signaling in MMTV-HER2/neu mammary tumors may increase the invasive potential of tumor cells, resulting in invading and expanding of tumors through surrounding microenvironment.

5.5 Conclusion

HER2 has been a critical target for breast cancer patients with HER2-positive tumors, and HER2-targeting therapies such as trastuzumab have been brought a huge impact on treating

HER2-positive breast cancer patients. With the complexity of inter-connected HER2 and other HER family receptors signaling pathways, drugs targeting multiple factors of the HER2 signaling pathway or the combination of drugs targeting different components of the HER2 signaling pathway might be promising therapeutics. In this chapter, I demonstrated BXL0124, CDDO-Im and the combination as potential therapeutics to target multiple components of the HER2 signaling pathway in HER2-overexpressing breast cancers.

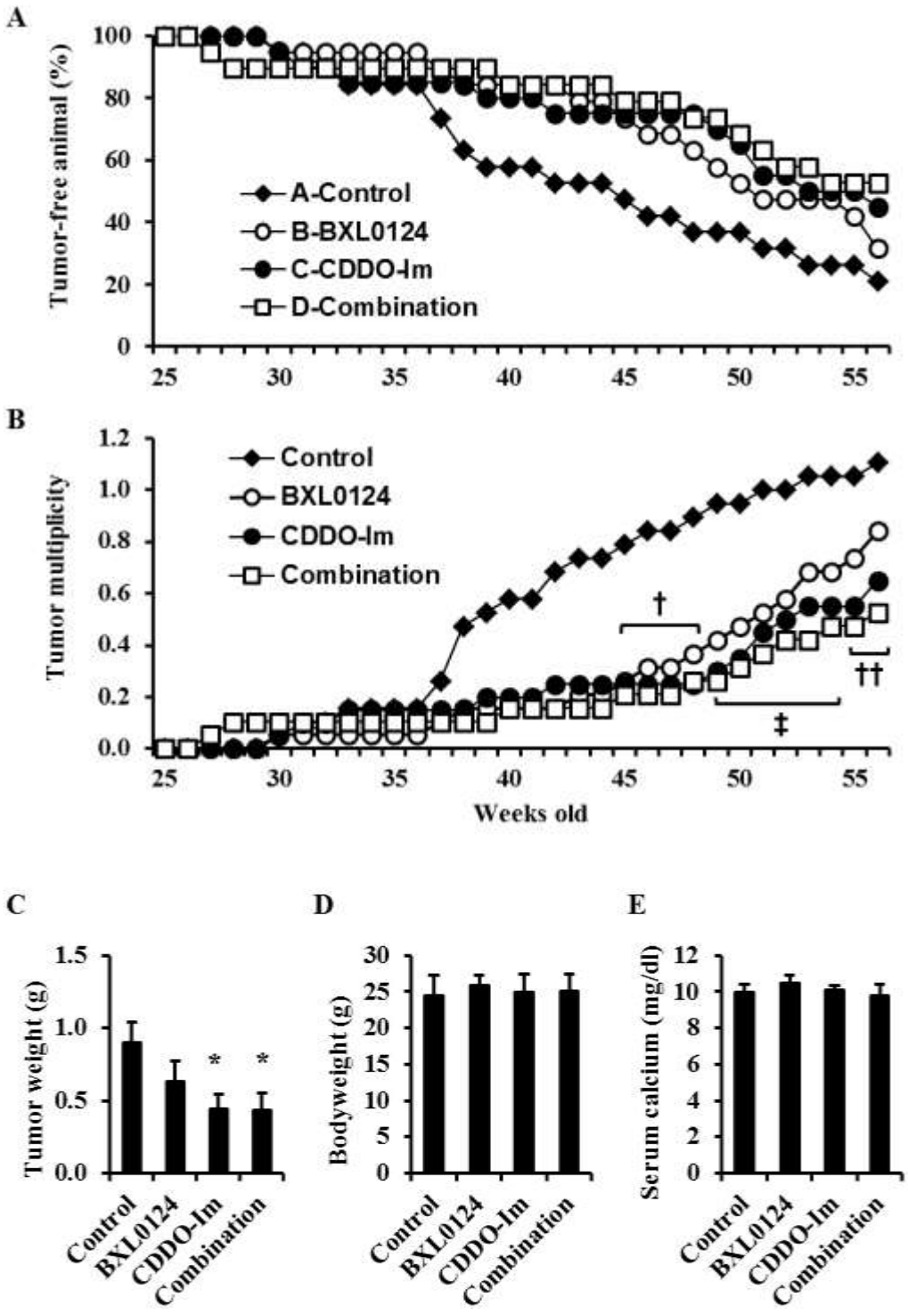

A
Tumor-free animal (%)
100
80
60
40
20
0
25
30
35
40
45
50
55
A-Control
B-BXL0124
C-CDDO-Im
D-Combination
B
Tumor multiplicity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
BXL0124
CDDO-Im
Combination
†
††
‡
Weeks old
C
Tumor weight (g)
1.5
1.0
0.5
0.0
*
*
Control
BXL0124
CDDO-Im
Combination
D
Bodyweight (g)
30
25
20
15
10
5
0
Control
BXL0124
CDDO-Im
Combination
E
Serum calcium (mg/dl)
12
10
8
6
4
2
0
Control
BXL0124
CDDO-Im
Combination

Fig. 5.1 Inhibitory effects of BXL0124, CDDO-Im and the combination on the mammary tumorigenesis in MMTV-HER2/neu transgenic mice. MMTV-HER2/neu transgenic mice were orally administered with vehicle control (DMSO, n=19), BXL0124 (0.3 μg/kg body weight, n=19), CDDO-Im (3 μmole/kg body weight, n=20) and the combination of BXL0124 and CDDO-Im (n=19) 3 times a week, beginning 3 month of age. **(A)** The tumor incidence was checked every week. **(B)** The number of tumors within a group was determined every week. Dunnett's multiple comparison tests were conducted to analyze statistical significance of each treatment group against control group. [† week 45-48: BXL0124, CDDO-Im and Combination ($p<0.05$), ‡ week 49-54: BXL0124 and CDDO-Im ($p<0.05$) / Combination ($p<0.01$), †† week 55-56: CDDO-Im and Combination ($p<0.05$)]. **(C)** Average tumor weight, **(D)** body weight and **(E)** serum calcium level of each group was determined at the end of experiment. Data are represented as mean ± S.E. Student t-tests were conducted to analyze statistical significance of each treatment group against control group (* $p<0.05$).

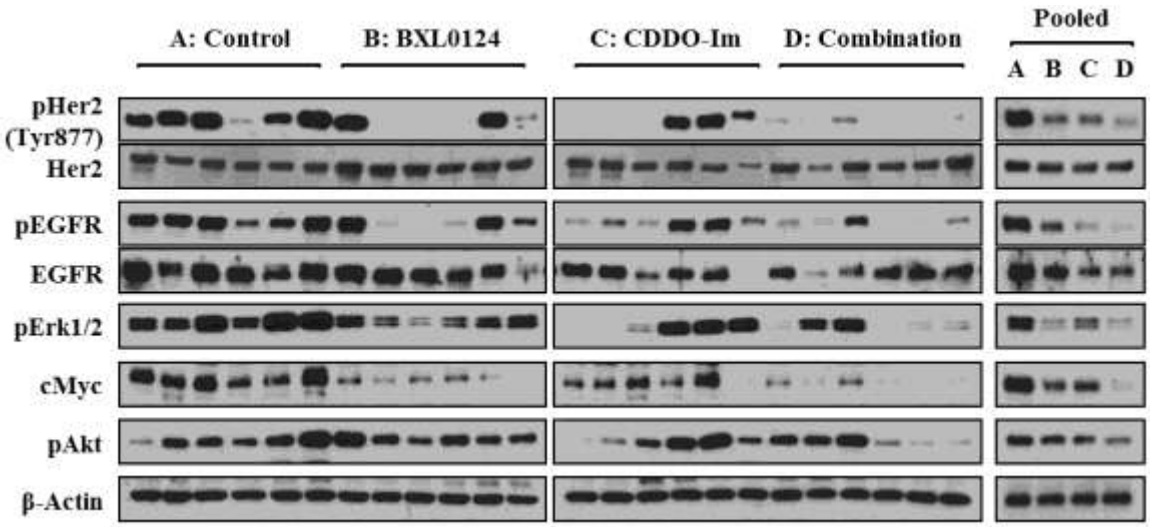


Fig. 5.2 Repression of the HER2 and EGFR signaling pathway by BXL0124, CDDO-Im and the combination on the mammary tumorigenesis in MMTV-HER2/neu transgenic mice. Six tumors from each group were analyzed as individual tumors (left panel). For the pooled sample representing each group, the six tumors from each group were combined (right panel). Protein levels of pHER2, HER2, pEGFR, EGFR, pErk1/2, c-Myc, pAkt were determined by Western blot analysis. β-Actin was used as loading control.

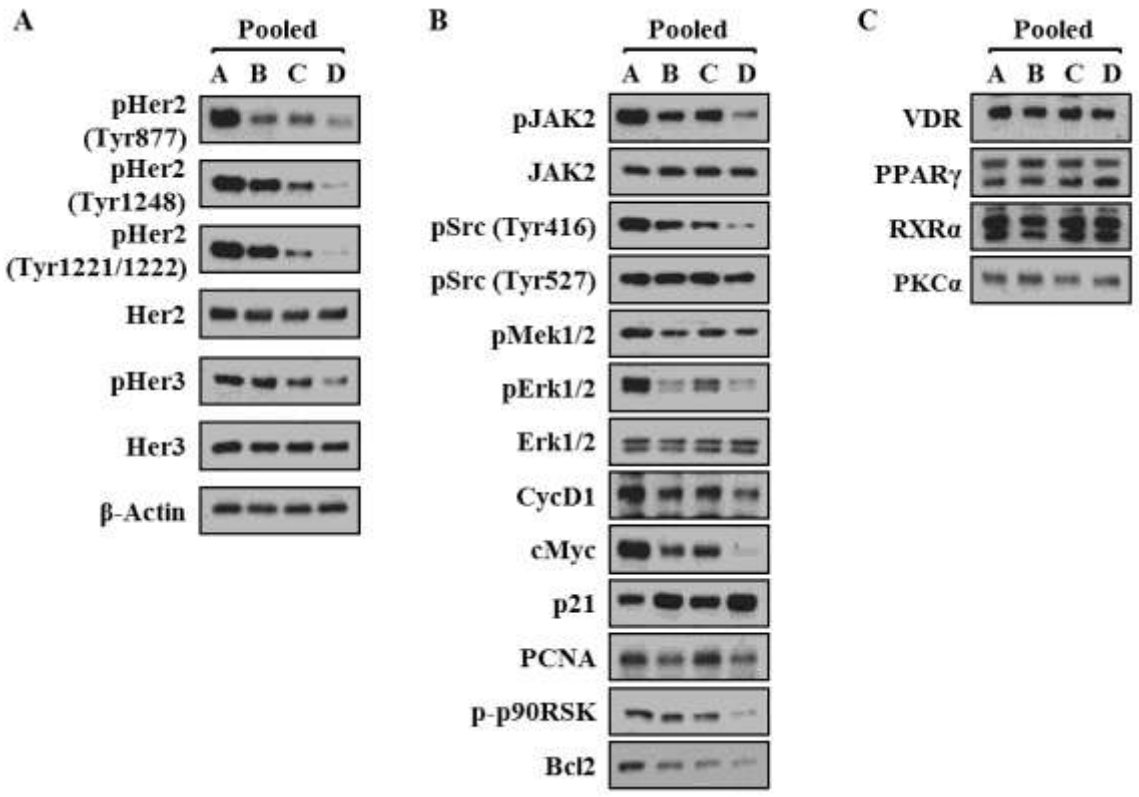


Fig. 5.3 Effects of BXL0124, CDDO-Im and the combination on the activation of HER receptors and downstream molecules in MMTV-HER2/neu mammary tumors. Six of individual tumors from each group were combined as pooled samples. **(A)** The regulation of HER2 and HER3 signaling by each compound was investigated by determining given protein levels with Western blot analysis. **(B)** The regulation of downstream signaling molecules of the HER signaling pathway by each compound was investigated by determining given protein levels with Western blot analysis. **(C)** The regulation of nuclear receptors and PKCα by each treatment was investigated by determining given protein levels with Western blot analysis. β-Actin was used as loading control.

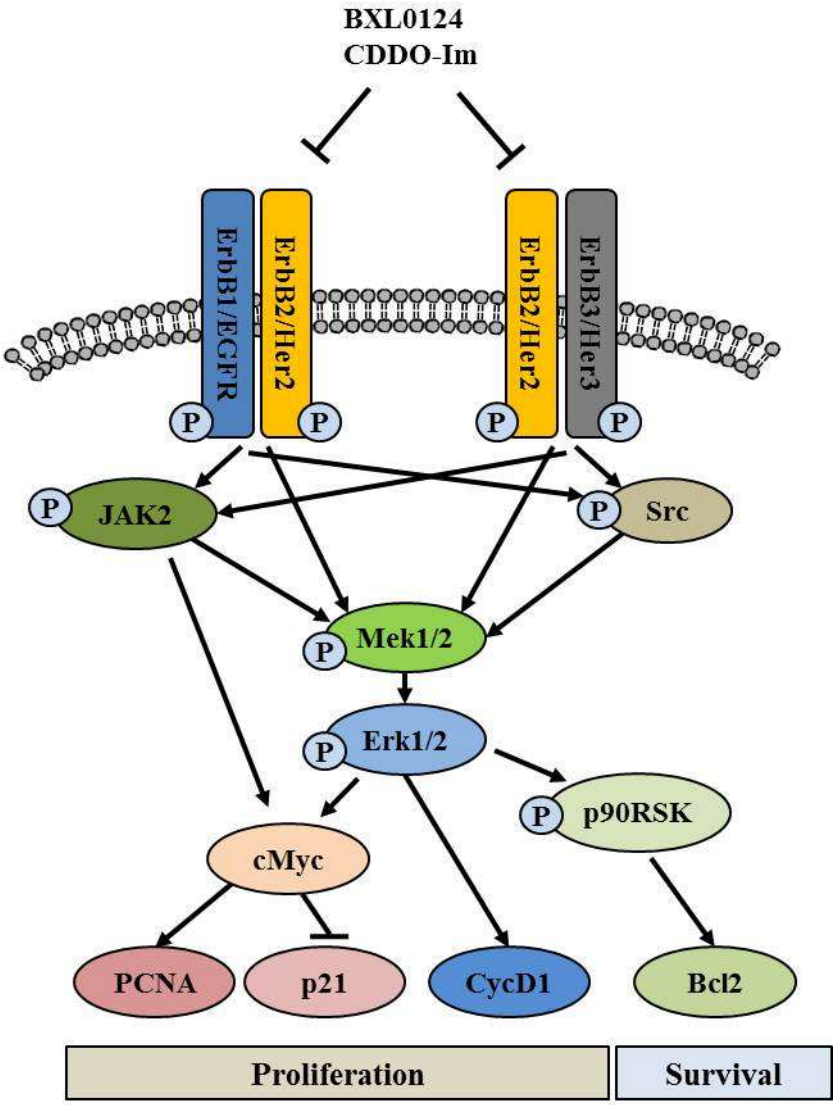


Fig. 5.4 A schematic diagram of downstream signaling pathways of HER receptors regulated by BXL0124, CDDO-Im and the combination in mammary tumors of MMTV-HER2/neu transgenic mice.

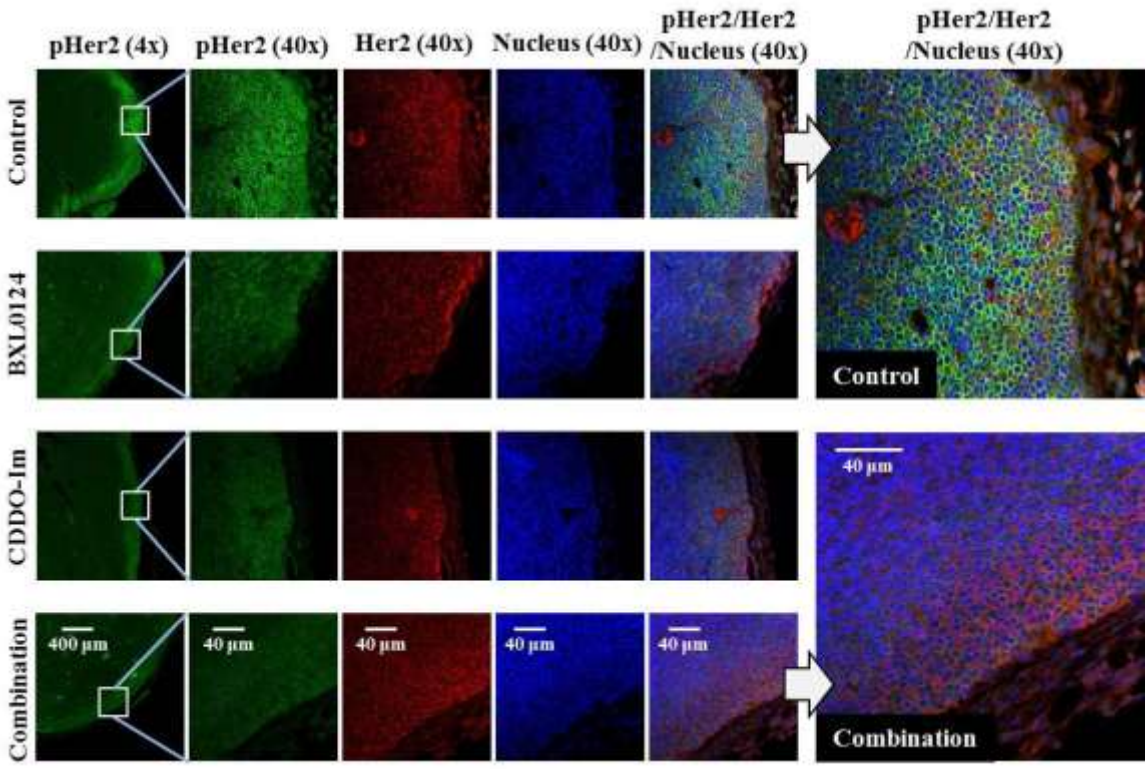


Fig. 5.5 Activation of HER2 at the invading edge of MMTV-HER2/neu mammary tumors and its repression by BXL0124, CDDO-Im and the combination. The protein expression levels of pHER2 (green) and HER2 (red) in HER2-overexpressing mammary tumors were determined by immunofluorescent staining. Nuclei were stained with TO-PRO-3 (blue).

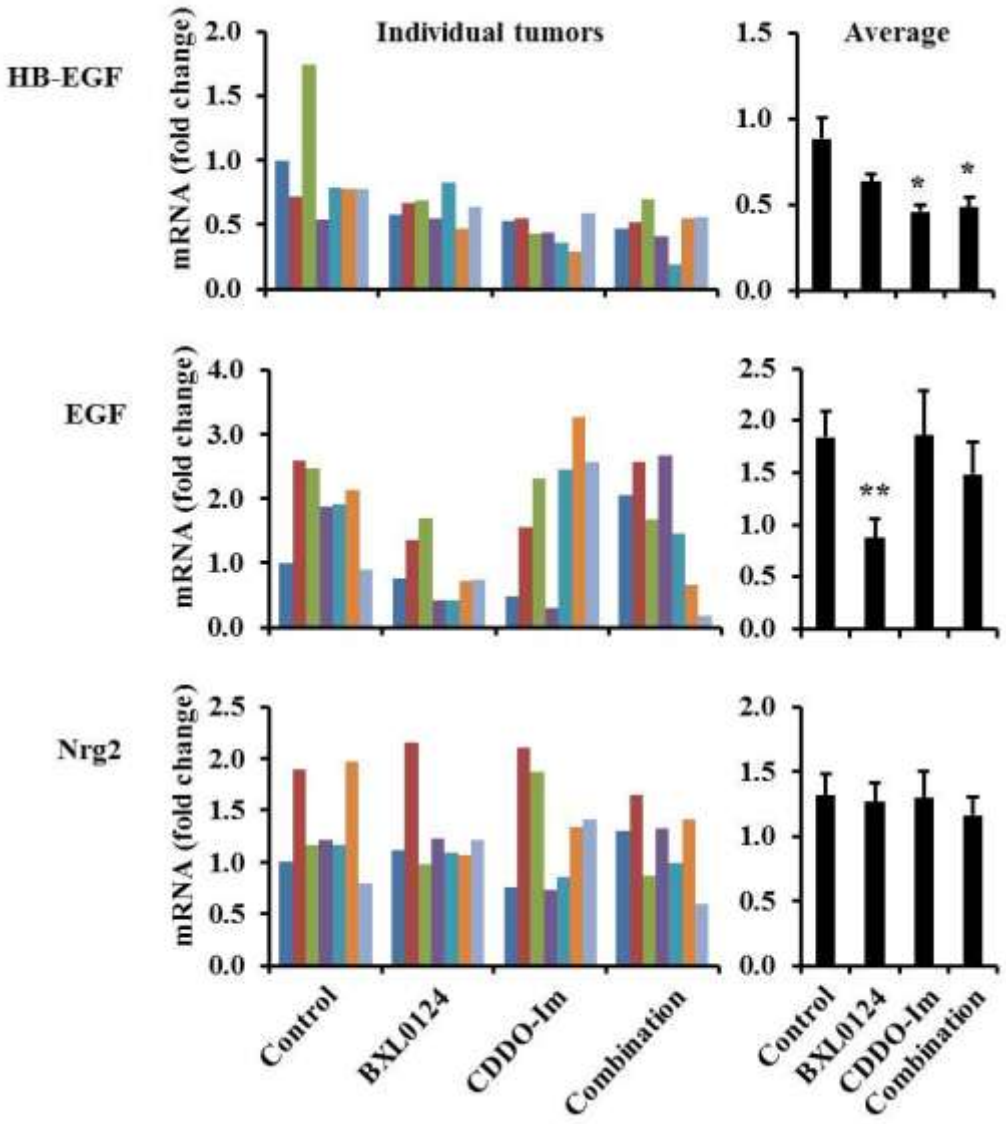


Fig. 5.6 Effects of BXL0124, CDDO-Im and the combination on the mRNA levels of HER receptor ligands in MMTV-HER2/neu mammary tumors. The mRNA expression levels of HB-EGF, EGF and Nrg2 in individual MMTV-HER2/neu mammary tumors were determined by qPCR (six samples per group, left panel). The mRNA expression levels of HB-EGF, EGF and Nrg2 of individual tumors from each group were averaged to represent each group (right panel). Data are presented as mean ± S.E. (* $p<0.05$, ** $p<0.01$).

Conclusion

Breast cancer is a major health problem among women in the world. In this project, the synthetic derivatives of natural compounds, a Gemini vitamin D analog and a synthetic triterpenoid, were investigated as therapeutic agents for different subtypes of breast cancer. In the study of a novel Gemini vitamin D analog BXL0124, BXL0124 inhibited the growth of MCF10DCIS.com cells, which belong to the basal-like subtype, *in vitro* and *in vivo*. In addition, the protein level of CD44, a key marker of breast cancer stem cells, was significantly decreased in MCF10DCIS.com cells by treatment with BXL0124 *in vitro* and *in vivo*. In a further study to identify the biological function of CD44 repression by BXL0124 in MCF10DCIS.com cells, STAT3 was identified as a key downstream signaling molecule of CD44 and demonstrated that CD44-STAT3 signaling is critical for MCF10DCIS.com cell invasion. In the investigation of HER2-overexpressing breast cancer, BXL0124, a synthetic triterpenoid CDDO-Im as well as their combination were tested for the anti-cancer activity using MMTV-HER2/neu transgenic mice. BXL0124, CDDO-Im and their combination delayed the development of MMTV-HER2/neu mammary tumors. Moreover, the activation of the HER2/EGFR/MAPK signaling pathway in MMTV-HER2/neu mammary tumors was markedly repressed by BXL0124 or CDDO-Im as well as their combination with an additive inhibitory effect. Overall, our results demonstrate diverse effects of BXL0124 on inhibition of basal-like and HER2-positive breast cancer, and inhibitory effects on the tumorigenesis of HER2-positive breast cancer in combination with CDDO-Im.

References

1. DeSantis C, Siegel R, Bandi P, Jemal A (2011) Breast cancer statistics, 2011. CA Cancer J Clin 61: 409-418.

2. Brunicardi FC, Andersen DK, Billiar TR, Dunn DL, Hunter JG, Matthews JB, Pollock RE (2010) Histopathology of Breast Cancer. Schwartz's Principles of Surgery, 9th Edition: McGraw-Hill. pp. 191-191.

3. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Lonning PE, Borresen-Dale AL (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 98: 10869-10874.

4. Sims AH, Howell A, Howell SJ, Clarke RB (2007) Origins of breast cancer subtypes and therapeutic implications. Nat Clin Pract Oncol 4: 516-525.

5. Brenton JD, Carey LA, Ahmed AA, Caldas C (2005) Molecular classification and molecular forecasting of breast cancer: ready for clinical application? J Clin Oncol 23: 7350-7360.

6. Network T (2012) Comprehensive molecular portraits of human breast tumors. Nature 490: 61-70.

7. Sørlie T, Tibshirani R, Parker J, Hastie T, Marron J, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 100: 8418-8423.

8. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, Hess KR, Stec J, Ayers M, Wagner P, Morandi P, Fan C, Rabiul I, Ross JS, Hortobagyi GN, Pusztai L (2005) Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clin Cancer Res 11: 5678-5685.

9. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. Nature 406: 747-752.

10. Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, Martiat P, Fox SB, Harris AL, Liu ET (2003) Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proc Natl Acad Sci U S A 100: 10393-10398.

11. Blows FM, Driver KE, Schmidt MK, Broeks A, van Leeuwen FE, Wesseling J, Cheang MC, Gelmon K, Nielsen TO, Blomqvist C, Heikkila P, Heikkinen T, Nevanlinna H, Akslen LA, Begin LR, Foulkes WD, Couch FJ, Wang X, Cafourek V, Olson JE, Baglietto L, Giles GG, Severi G, McLean CA, Southey MC, Rakha E, Green AR, Ellis IO, Sherman ME, Lissowska J, Anderson WF, Cox A, Cross SS, Reed MW, Provenzano E, Dawson SJ, Dunning AM, Humphreys M, Easton DF, Garcia-Closas M, Caldas C, Pharoah PD, Huntsman D (2010) Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. PLoS Med 7: e1000279.

12. Sotiriou C, Pusztai L (2009) Gene-expression signatures in breast cancer. N Engl J Med 360: 790-800.

13. Cheang MC, Chia SK, Voduc D, Gao D, Leung S, Snider J, Watson M, Davies S, Bernard PS, Parker JS, Perou CM, Ellis MJ, Nielsen TO (2009) Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Natl Cancer Inst 101: 736-750.

14. Winer EP, Hudis C, Burstein HJ, Wolff AC, Pritchard KI, Ingle JN, Chlebowski RT, Gelber R, Edge SB, Gralow J (2005) American Society of Clinical Oncology technology assessment on the use of aromatase inhibitors as adjuvant therapy for postmenopausal women with hormone receptor–positive breast cancer: status report 2004. J Clin Oncol 23: 619-629.

15. Tran B, Bedard PL (2011) Luminal-B breast cancer and novel therapeutic targets. Breast Cancer Res 13: 221.

16. de Ronde JJ, Hannemann J, Halfwerk H, Mulder L, Straver ME, Vrancken Peeters MJ, Wesseling J, van de Vijver M, Wessels LF, Rodenhuis S (2010) Concordance of clinical and molecular breast cancer subtyping in the context of preoperative chemotherapy response. Breast Cancer Res Treat 119: 119-126.

17. Harmsen H, Porsius A (1988) Endocrine therapy of breast cancer. Eur J Cancer Clin On 24: 1099-1116.

18. Arpino G, De Angelis C, Giuliano M, Giordano A, Falato C, De Laurentiis M, De Placido S (2009) Molecular mechanism and clinical implications of endocrine therapy resistance in breast cancer. Oncology 77 Suppl 1: 23-37.

19. Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90: 1371-1388.

20. Jordan VC. Tamoxifen for breast cancer prevention; 1995. Royal Society of Medicine. pp. 144-149.

21. Cuzick J, Powles T, Veronesi U, Forbes J, Edwards R, Ashley S, Boyle P (2003) Overview of the main outcomes in breast cancer prevention trials. Obstet Gynecol Surv 58: 463-465.

22. Nicholson RI, Johnston SR (2005) Endocrine therapy--current benefits and limitations. Breast Cancer Res Treat 93 Suppl 1: S3-10.

23. Wood AJJ, Smith IE, Dowsett M (2003) Aromatase inhibitors in breast cancer. New Engl J Med 348: 2431-2442.

24. Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, Schiff R (2004) Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2–positive breast cancer. J Natl Cancer Inst 96: 926-935.

25. Bundred NJ, Anderson E, Nicholson RI, Dowsett M, Dixon M, Robertson JF (2002) Fulvestrant, an estrogen receptor downregulator, reduces cell turnover index more effectively than tamoxifen. Anticancer Res 22: 2317.

26. Johnston S (2004) Fulvestrant and the sequential endocrine cascade for advanced breast cancer. Br J Cancer 90: S15-S18.

27. Jelovac D, Macedo L, Goloubeva OG, Handratta V, Brodie AMH (2005) Additive antitumor effect of aromatase inhibitor letrozole and antiestrogen fulvestrant in a postmenopausal breast cancer model. Cancer Res 65: 5439-5444.

28. Osborne CK, Wakeling A, Nicholson RI (2004) Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action. Br J Cancer 90 Suppl 1: S2-6.

29. McDonnell DP, Wardell SE (2010) The molecular mechanisms underlying the pharmacological actions of ER modulators: implications for new drug discovery in breast cancer. Curr Opin Pharmacol 10: 620-628.

30. Owens MA, Horten BC, Da Silva MM (2004) HER2 amplification ratios by fluorescence in situ hybridization and correlation with immunohistochemistry in a cohort of 6556 breast cancer tissues. Clin Breast Cancer 5: 63-69.

31. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235: 177-182.

32. Yarden Y, Sliwkowski MX (2001) Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2: 127-137.

33. Stern HM (2012) Improving treatment of HER2-positive cancers: opportunities and challenges. Sci Transl Med 4: 127rv122.

34. Bartlett J, Mallon E, Cooke T (2003) The clinical evaluation of HER-2 status: which test to use? J Pathol 199: 411-417.

35. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344: 783-792.

36. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB (2005) Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov 4: 988-1004.

37. Saez R, Molina MA, Ramsey EE, Rojo F, Keenan EJ, Albanell J, Lluch A, Garcia-Conde J, Baselga J, Clinton GM (2006) p95HER-2 predicts worse outcome in patients with HER-2-positive breast cancer. Clin Cancer Res 12: 424-431.

38. Konecny GE, Pegram MD, Venkatesan N, Finn R, Yang G, Rahmeh M, Untch M, Rusnak DW, Spehar G, Mullin RJ, Keith BR, Gilmer TM, Berger M, Podratz KC, Slamon DJ (2006) Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. Cancer Res 66: 1630-1639.

39. Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T, Jagiello-Gruszfeld A, Crown J, Chan A, Kaufman B, Skarlos D, Campone M, Davidson N, Berger M, Oliva C, Rubin

SD, Stein S, Cameron D (2006) Lapatinib plus capecitabine for HER2-positive advanced breast cancer. N Engl J Med 355: 2733-2743.

40. Gianni L, Pienkowski T, Im YH, Roman L, Tseng LM, Liu MC, Lluch A, Staroslawska E, de la Haba-Rodriguez J, Im SA, Pedrini JL, Poirier B, Morandi P, Semiglazov V, Srimuninnimit V, Bianchi G, Szado T, Ratnayake J, Ross G, Valagussa P (2012) Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. Lancet Oncol 13: 25-32.

41. Scaltriti M, Eichhorn PJ, Cortes J, Prudkin L, Aura C, Jimenez J, Chandarlapaty S, Serra V, Prat A, Ibrahim YH, Guzman M, Gili M, Rodriguez O, Rodriguez S, Perez J, Green SR, Mai S, Rosen N, Hudis C, Baselga J (2011) Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients. Proc Natl Acad Sci U S A 108: 3761-3766.

42. Shattuck DL, Miller JK, Carraway KL, 3rd, Sweeney C (2008) Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. Cancer Res 68: 1471-1477.

43. Browne BC, Crown J, Venkatesan N, Duffy MJ, Clynes M, Slamon D, O'Donovan N (2011) Inhibition of IGF1R activity enhances response to trastuzumab in HER-2-positive breast cancer cells. Ann Oncol 22: 68-73.

44. Rakha EA, Reis-Filho JS, Ellis IO (2008) Basal-like breast cancer: a critical review. J Clin Oncol 26: 2568-2581.

45. Foulkes WD, Smith IE, Reis-Filho JS (2010) Triple-negative breast cancer. N Engl J Med 363: 1938-1948.

46. Vousden KH, Lane DP (2007) p53 in health and disease. Nat Rev Mol Cell Biol 8: 275-283.

47. Liedtke C, Mazouni C, Hess KR, Andre F, Tordai A, Mejia JA, Symmans WF, Gonzalez-Angulo AM, Hennessy B, Green M, Cristofanilli M, Hortobagyi GN, Pusztai L (2008) Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol 26: 1275-1281.

48. Toft DJ, Cryns VL (2011) Minireview: Basal-like breast cancer: from molecular profiles to targeted therapies. Mol Endocrinol 25: 199-211.

49. Diallo-Danebrock R, Ting E, Gluz O, Herr A, Mohrmann S, Geddert H, Rody A, Schaefer KL, Baldus SE, Hartmann A, Wild PJ, Burson M, Gabbert HE, Nitz U, Poremba C (2007) Protein expression profiling in high-risk breast cancer patients treated with high-dose or conventional dose-dense chemotherapy. Clin Cancer Res 13: 488-497.

50. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, Hernandez-Boussard T, Livasy C, Cowan D, Dressler L, Akslen LA, Ragaz J, Gown AM, Gilks CB, van de Rijn M, Perou CM (2004) Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res 10: 5367-5374.

51. Livasy CA, Karaca G, Nanda R, Tretiakova MS, Olopade OI, Moore DT, Perou CM (2006) Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. Mod Pathol 19: 264-271.

52. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, Martin NM, Jackson SP, Smith GC, Ashworth A (2005) Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 434: 917-921.

53. Vargo-Gogola T, Rosen JM (2007) Modelling breast cancer: one size does not fit all. Nat Rev Cancer 7: 659-672.

54. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10: 515-527.

55. Kao J, Salari K, Bocanegra M, Choi YL, Girard L, Gandhi J, Kwei KA, Hernandez-Boussard T, Wang P, Gazdar AF (2009) Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. PLoS One 4: e6146.

56. Kenny PA, Lee GY, Myers CA, Neve RM, Semeiks JR, Spellman PT, Lorenz K, Lee EH, Barcellos-Hoff MH, Petersen OW (2007) The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. Mol Oncol 1: 84-96.

57. Heppner GH, Miller FR, Shekhar PVM (2000) Nontransgenic models of breast cancer. Breast Cancer Research 2: 331-334.

58. Hovey RC, Mcfadden TB, Akers RM (1999) Regulation of mammary gland growth and morphogenesis by the mammary fat pad: a species comparison. J Mammary Gland Biol 4: 53-68.

59. Gupta PB, Kuperwasser C (2004) Disease models of breast cancer. Drug Discov Today 1: 9-16.

60. Tait LR, Pauley RJ, Santner SJ, Heppner GH, Heng HH, Rak JW, Miller FR (2007) Dynamic stromal-epithelial interactions during progression of MCF10DCIS. com xenografts. Int J Cancer 120: 2127-2134.

61. Hu M, Yao J, Carroll DK, Weremowicz S, Chen H, Carrasco D, Richardson A, Violette S, Nikolskaya T, Nikolsky Y, Bauerlein EL, Hahn WC, Gelman RS, Allred C, Bissell MJ, Schnitt S, Polyak K (2008) Regulation of in situ to invasive breast carcinoma transition. Cancer Cell 13: 394-406.

62. Visvader JE, Lindeman GJ (2008) Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 8: 755-768.

63. Gould MN. Rodent models for the study of etiology, prevention and treatment of breast cancer; 1995. Elsevier. pp. 147-152.

64. Thompson HJ, Singh M (2000) Rat models of premalignant breast disease. J Mammary Gland Biol 5: 409-420.

65. Huggins C, Grand LC, Brillantes FP (1961) Mammary cancer induced by a single feeding of polynuclear hydrocarbons, and its suppression. Nature 189: 204-207.

66. Gullino P, Pettigrew HM, Grantham F (1975) N-nitrosomethylurea as mammary gland carcinogen in rats. J Natl Cancer Inst 54: 401-414.

67. Singh M, McGinley JN, Thompson HJ (2000) A comparison of the histopathology of premalignant and malignant mammary gland lesions induced in sexually immature rats with those occurring in the human. Lab Invest 80: 221-231.

68. Russo J, Russo IH (1996) Experimentally induced mammary tumors in rats. Breast Cancer Res Treat 39: 7.

69. Russo J, Russo IH (2000) Atlas and histologic classification of tumors of the rat mammary gland. J Mammary Gland Biol 5: 187-200.

70. Chan MM, Lu X, Merchant FM, Iglehart JD, Miron PL (2005) Gene expression profiling of NMU-induced rat mammary tumors: cross species comparison with human breast cancer. Carcinogenesis 26: 1343-1353.

71. Sharpless NE, DePinho RA (2006) The mighty mouse: genetically engineered mouse models in cancer drug development. Nat Rev Drug Discov 5: 741-754.

72. Cardiff RD, Anver MR, Gusterson BA, Hennighausen L, Jensen RA, Merino MJ, Rehm S, Russo J, Tavassoli FA, Wakefield LM (2000) The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting. Oncogene 19: 968-988.

73. Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, Rasmussen KE, Jones LP, Assefnia S, Chandrasekharan S (2007) Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biol 8: R76.

74. Toniolo PG, Levitz M, Zeleniuch-Jacquotte A, Banerjee S, Koenig KL, Shore RE, Strax P, Pasternack BS (1995) A prospective study of endogenous estrogens and breast cancer in postmenopausal women. J Natl Cancer Inst 87: 190-197.

75. Clemons M, Goss P (2001) Estrogen and the risk of breast cancer. N Engl J Med 344: 276-285.

76. Green KA, Carroll JS (2007) Oestrogen-receptor-mediated transcription and the influence of co-factors and chromatin state. Nat Rev Cancer 7: 713-722.

77. Thomas C, Gustafsson JÅ (2011) The different roles of ER subtypes in cancer biology and therapy. Nat Rev Cancer 11: 597-608.

78. Riese II DJ, Stern DF (1998) Specificity within the EGF family/ErbB receptor family signaling network. Bioessays 20: 41-48.

79. Harris AL, Nicholson S, Richard J, Sainsbury C, Farndon J, Wright C (1989) Epidermal growth factor receptors in breast cancer: Association with early relapse and death, poor response to hormones and interactions with *neu*. J Steroid Biochem 34: 123-131.

80. Prenzel N, Zwick E, Leserer M, Ullrich A (2000) Tyrosine kinase signalling in breast cancer: Epidermal growth factor receptor-convergence point for signal integration and diversification. Breast Cancer Research 2: 184.

81. Schlange T, Matsuda Y, Lienhard S, Huber A, Hynes NE (2007) Autocrine WNT signaling contributes to breast cancer cell proliferation via the canonical WNT pathway and EGFR transactivation. Breast Cancer Res 9: R63.

82. Bhola NE, Grandis JR (2008) Crosstalk between G-protein-coupled receptors and epidermal growth factor receptor in cancer. Front Biosci 13: 1857.

83. Hynes NE, Lane HA (2005) ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 5: 341-354.

84. Gene S (1994) Breast and Ovarian Cancer Susceptibility Gene BRCA1. Science 266: 7.

85. Foulkes WD, Stefansson IM, Chappuis PO, Bégin LR, Goffin JR, Wong N, Trudel M, Akslen LA (2003) Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. J Natl Cancer Inst 95: 1482-1485.

86. Turner NC, Lord CJ, Iorns E, Brough R, Swift S, Elliott R, Rayter S, Tutt AN, Ashworth A (2008) A synthetic lethal siRNA screen identifying genes mediating sensitivity to a PARP inhibitor. The EMBO journal 27: 1368-1377.

87. Fong PC, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M, Mortimer P, Swaisland H, Lau A, O'Connor MJ (2009) Inhibition of poly (ADP-ribose) polymerase in tumors from BRCA mutation carriers. New Engl J Med 361: 123-134.

88. O'Shaughnessy J, Osborne C, Pippen JE, Yoffe M, Patt D, Rocha C, Koo IC, Sherman BM, Bradley C (2011) Iniparib plus chemotherapy in metastatic triple-negative breast cancer. New Engl J Med 364: 205-214.

89. Katso R, Okkenhaug K, Ahmadi K, White S, Timms J, Waterfield MD (2001) Cellular function of phosphoinositide 3-kinases: implications for development, immunity, homeostasis, and cancer. Annu Rev Cell Dev Biol 17: 615-675.

90. Engelman JA (2009) Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer 9: 550-562.

91. Gray J, Druker B (2012) Genomics: The breast cancer landscape. Nature 486: 328-329.

92. Hernandez-Aya LF, Gonzalez-Angulo AM (2011) Targeting the phosphatidylinositol 3-kinase signaling pathway in breast cancer. The Oncologist 16: 404-414.

93. Adams T, Epa V, Garrett T, Ward C (2000) Structure and function of the type 1 insulin-like growth factor receptor. Cell Mol Life Sci 57: 1050.

94. Grimberg A, Cohen P (2000) Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. J Cell Physiol 183: 1-9.

95. Yee D, Paik S, Lebovic GS, Marcus RR, Favoni RE, Cullen KJ, Lippman ME, Rosen N (1989) Analysis of insulin-like growth factor I gene expression in malignancy: evidence for a paracrine role in human breast cancer. Mol Endocrinol 3: 509-517.

96. Osborne CK, Coronado EB, Kitten LJ, Arteaga CI, Fuqua SAW, Ramasharma K, Marshall M, Li CH (1989) Insulin-Like Growth Factor-ll (IGF-II): A Potential Autocrine/Paracrine Growth Factor for Human Breast Cancer Acting via the IGF-I Receptor. Mol Endocrinol 3: 1701-1709.

97. Gunter MJ, Hoover DR, Yu H, Wassertheil-Smoller S, Rohan TE, Manson JAE, Li J, Ho GYF, Xue X, Anderson GL (2009) Insulin, insulin-like growth factor-I, and risk of breast cancer in postmenopausal women. J Natl Cancer Inst 101: 48-60.

98. Al-Delaimy WK, Flatt SW, Natarajan L, Laughlin GA, Rock CL, Gold EB, Caan BJ, Parker BA, Pierce JP (2011) IGF1 and risk of additional breast cancer in the WHEL study. Endocr-Relat Cancer 18: 235-244.

99. Pravtcheva DD, Wise TL (1998) Metastasizing mammary carcinomas in H19 enhancers-Igf2 transgenic mice. J Exp Zool 281: 43-57.

100. Hadsell D, Murphy K, Bonnette S, Reece N, Laucirica R, Rosen J (2000) Cooperative interaction between mutant p53 and des (1-3) IGF-I accelerates mammary tumorigenesis. Oncogene 19: 889.

101. Fox EM, Miller TW, Balko JM, Kuba MG, Sánchez V, Smith RA, Liu S, González-Angulo AM, Mills GB, Ye F (2011) A kinome-wide screen identifies the insulin/IGF-I receptor pathway as a mechanism of escape from hormone dependence in breast cancer. Cancer Res 71: 6773-6784.

102. Chakraborty AK, Liang K, DiGiovanna MP (2008) Co-targeting insulin-like growth factor I receptor and HER2: dramatic effects of HER2 inhibitors on nonoverexpressing breast cancer. Cancer Res 68: 1538-1545.

103. Chakraborty AK, Welsh A, DiGiovanna MP (2010) Co-targeting the insulin-like growth factor I receptor enhances growth-inhibitory and pro-apoptotic effects of anti-estrogens in human breast cancer cell lines. Breast Cancer Res Treat 120: 327-335.

104. Karin M, Delhase M. The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling; 2000. pp. 85.

105. Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet R, Sledge G (1997) Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. Mol Cell Biol 17: 3629-3639.

106. Cogswell PC, Guttridge DC, Funkhouser WK, Baldwin Jr AS (2000) Selective activation of NF-kappa B subunits in human breast cancer: potential roles for NF-kappa B2/p52 and for Bcl-3. Oncogene 19: 1123.

107. Kim DW, Sovak MA, Zanieski G, Nonet G, Romieu-Mourez R, Lau AW, Hafer LJ, Yaswen P, Stampfer M, Rogers AE (2000) Activation of NF-κB/Rel occurs early during neoplastic transformation of mammary cells. Carcinogenesis 21: 871-879.

108. Connelly L, Barham W, Onishko HM, Sherrill T, Chodosh LA, Blackwell TS, Yull FE (2010) Inhibition of NF-kappa B activity in mammary epithelium increases tumor latency and decreases tumor burden. Oncogene 30: 1402-1412.

109. Ryan KM, Ernst MK, Rice NR, Vousden KH (2000) Role of NF-kB in p53-mediated programmed cell death. Nature 404: 892-896.

110. Van Laere S, Van der Auwera I, Van den Eynden G, van Dam P, Van Marck E, Vermeulen P, Dirix L (2007) NF-κB activation in inflammatory breast cancer is associated with oestrogen receptor downregulation, secondary to EGFR and/or ErbB2 overexpression and MAPK hyperactivation. Br J Cancer 97: 659-669.

111. Lerebours F, Vacher S, Andrieu C, Espie M, Marty M, Lidereau R, Bieche I (2008) NF-kappa B genes have a major role in inflammatory breast cancer. BMC cancer 8: 41.

112. Darnell Jr JE (1997) STATs and gene regulation. Science 277: 1630-1635.

113. Wen Z, Zhong Z, Darnell JE (1995) Maximal activation of transcription by Statl and Stat3 requires both tyrosine and serine phosphorylation. Cell 82: 241-250.

114. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE (1999) *Stat3* as an Oncogene. Cell 98: 295-303.

115. Yu H, Pardoll D, Jove R (2009) STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer 9: 798-809.

116. Garcia R, Bowman TL, Niu G, Yu H, Minton S, Muro-Cacho CA, Cox CE, Falcone R, Fairclough R, Parsons S (2001) Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. Oncogene 20: 2499-2513.

117. Gritsko T, Williams A, Turkson J, Kaneko S, Bowman T, Huang M, Nam S, Eweis I, Diaz N, Sullivan D (2006) Persistent activation of stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells. Clin Cancer Res 12: 11-19.

118. Real PJ, Sierra A, De Juan A, Segovia JC, Lopez-Vega JM, Fernandez-Luna JL (2002) Resistance to chemotherapy via Stat3-dependent overexpression of Bcl-2 in metastatic breast cancer cells. Oncogene 21: 7611.

119. Zhou J, Wulfkuhle J, Zhang H, Gu P, Yang Y, Deng J, Margolick JB, Liotta LA, Petricoin E, Zhang Y (2007) Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance. Proc Natl Acad Sci U S A 104: 16158-16163.

120. Marotta LLC, Almendro V, Marusyk A, Shipitsin M, Schemme J, Walker SR, Bloushtain-Qimron N, Kim JJ, Choudhury SA, Maruyama R (2011) The JAK2/STAT3 signaling pathway is required for growth of CD44+ CD24–stem cell–like breast cancer cells in human tumors. J Clin Invest 121: 2723.

121. Ling X, Arlinghaus RB (2005) Knockdown of STAT3 expression by RNA interference inhibits the induction of breast tumors in immunocompetent mice. Cancer Res 65: 2532-2536.

122. Lin L, Hutzen B, Zuo M, Ball S, Deangelis S, Foust E, Pandit B, Ihnat MA, Shenoy SS, Kulp S (2010) Novel STAT3 phosphorylation inhibitors exhibit potent growth-suppressive activity in pancreatic and breast cancer cells. Cancer Res 70: 2445-2454.

123. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM (1999) VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. The EMBO journal 18: 3964-3972.

124. Carmeliet P, Jain RK (2011) Molecular mechanisms and clinical applications of angiogenesis. Nature 473: 298-307.

125. Ferrara N (2005) VEGF as a therapeutic target in cancer. Oncology 69: 11-16.

126. Miller K, Wang M, Gralow J, Dickler M, Cobleigh M, Perez EA, Shenkier T, Cella D, Davidson NE (2007) Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. N Engl J Med 357: 2666-2676.

127. Miles DW, Chan A, Dirix LY, Cortés J, Pivot X, Tomczak P, Delozier T, Sohn JH, Provencher L, Puglisi F (2010) Phase III study of bevacizumab plus docetaxel compared with placebo plus docetaxel for the first-line treatment of human epidermal growth factor receptor 2–negative metastatic breast cancer. J Clin Oncol 28: 3239-3247.

128. Robert NJ, Diéras V, Glaspy J, Brufsky AM, Bondarenko I, Lipatov ON, Perez EA, Yardley DA, Chan SYT, Zhou X (2011) RIBBON-1: Randomized, double-blind, placebo-controlled, phase III trial of chemotherapy with or without bevacizumab for first-line treatment of human epidermal growth factor receptor 2–negative, locally recurrent or metastatic breast cancer. J Clin Oncol 29: 1252-1260.

129. Burstein HJ (2011) Bevacizumab for advanced breast cancer: all tied up with a RIBBON? J Clin Oncol 29: 1232-1235.

130. Holick MF (2011) Vitamin D: evolutionary, physiological and health perspectives. Curr Drug Targets 12: 4-18.

131. DEEB D, GAO X, DULCHAVSKY SA, GAUTAM SC (2007) CDDO-Me induces apoptosis and inhibits Akt, mTOR and NF-κB signaling proteins in prostate cancer cells. Anticancer Res 27: 3035-3044.

132. Deeb KK, Trump DL, Johnson CS (2007) Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nat Rev Cancer 7: 684-700.

133. Vuolo L, Di Somma C, Faggiano A, Colao A (2012) Vitamin D and cancer. Front Endocrinol 3.

134. Haussler MR, Whitfield GK, Haussler CA, Hsieh JC, Thompson PD, Selznick SH, Dominguez CE, Jurutka PW (1998) The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J Bone Miner Res 13: 325-349.

135. Pike JW, Meyer MB (2010) The vitamin D receptor: new paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D(3). Endocrinol Metab Clin North Am 39: 255-269, table of contents.

136. Rachez C, Lemon BD, Suldan Z, Bromleigh V, Gamble M, Näär AM, Erdjument-Bromage H, Tempst P, Freedman LP (1999) Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. Nature 398: 824-828.

137. Battaglia S, Maguire O, Campbell MJ (2010) Transcription factor co-repressors in cancer biology: roles and targeting. Int J Cancer 126: 2511-2519.

138. Haussler MR, Jurutka PW, Mizwicki M, Norman AW (2011) Vitamin D receptor (VDR)-mediated actions of 1alpha,25(OH)(2)vitamin D(3): genomic and non-genomic mechanisms. Best Pract Res Clin Endocrinol Metab 25: 543-559.

139. Wali RK, Baum CL, Sitrin MD, Brasitus TA (1990) 1, 25 (OH) 2 vitamin D3 stimulates membrane phosphoinositide turnover, activates protein kinase C, and increases cytosolic calcium in rat colonic epithelium. J Clin Invest 85: 1296.

140. Morelli S, Buitrago C, Boland R, de Boland AR (2001) The stimulation of MAP kinase by 1, 25 (OH)(2)-vitamin D (3) in skeletal muscle cells is mediated by protein kinase C and calcium. Mol Cell Endocrinol 173: 41.

141. Mizwicki MT, Norman AW (2009) The vitamin D sterol-vitamin D receptor ensemble model offers unique insights into both genomic and rapid-response signaling. Science Signalling 2: re4.

142. Zinser GM, Welsh J (2004) Effect of Vitamin D3 receptor ablation on murine mammary gland development and tumorigenesis. J Steroid Biochem Mol Biol 89-90: 433-436.

143. Zinser GM, McEleney K, Welsh J (2003) Characterization of mammary tumor cell lines from wild type and vitamin D3 receptor knockout mice. Mol Cell Endocrinol 200: 67-80.

144. Zinser GM, Welsh J (2004) Vitamin D receptor status alters mammary gland morphology and tumorigenesis in MMTV-neu mice. Carcinogenesis 25: 2361-2372.

145. Eisman J, Koga M, Sutherland R, Barkla D, Tutton P. 1, 25-Dihydroxyvitamin D3 and the regulation of human cancer cell replication; 1989. Royal Society of Medicine. pp. 221-226.

146. Cho YL, Christensen C, Saunders D, Lawrence W, Deppe G, Malviya V, Malone J (1991) Combined effects of 1, 25-dihydroxyvitamin D3 and platinum drugs on the growth of MCF-7 cells. Cancer Res 51: 2848-2853.

147. Swami S, Krishnan AV, Feldman D (2000) 1alpha,25-Dihydroxyvitamin D3 down-regulates estrogen receptor abundance and suppresses estrogen actions in MCF-7 human breast cancer cells. Clin Cancer Res 6: 3371-3379.

148. Mehta RG, Mehta RR, Lazzaro G, Constantinou A, Moriarty RM, Penmasta R, Guo L (1997) Prevention of preneoplastic mammary lesion development by a novel vitamin D analogue, 1α-hydroxyvitamin D5. J Natl Cancer Inst 89: 212-218.

149. Mehta R, Hawthorne M, Uselding L, Albinescu D, Moriarty R, Christov K (2000) Prevention of N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats by 1α-hydroxyvitamin D5. J Natl Cancer Inst 92: 1836-1840.

150. Gross C, Stamey T, Hancock S, Feldman D (1998) Treatment of early recurrent prostate cancer with 1, 25-dihydroxyvitamin D3 (calcitriol). J Urology 159: 2035-2040.

151. Smith DC, Johnson CS, Freeman CC, Muindi J, Wilson JW, Trump DL (1999) A Phase I trial of calcitriol (1, 25-dihydroxycholecalciferol) in patients with advanced malignancy. Clin Cancer Res 5: 1339-1345.

152. Trump DL, Hershberger PA, Bernardi RJ, Ahmed S, Muindi J, Fakih M, Yu WD, Johnson CS (2004) Anti-tumor activity of calcitriol: pre-clinical and clinical studies. J Steroid Biochem Mol Biol 89: 519.

153. Verlinden L, Verstuyf A, Convents R, Marcelis S, Van Camp M, Bouillon R (1998) Action of 1,25(OH)2D3 on the cell cycle genes, cyclin D1, p21 and p27 in MCF-7 cells. Mol Cell Endocrinol 142: 57-65.

154. Jensen SS, Madsen MW, Lukas J, Binderup L, Bartek J (2001) Inhibitory effects of 1α, 25-dihydroxyvitamin D3 on the G1–S phase-controlling machinery. Mol Endocrinol 15: 1370-1380.

155. Swami S, Raghavachari N, Muller UR, Bao YP, Feldman D (2003) Vitamin D growth inhibition of breast cancer cells: gene expression patterns assessed by cDNA microarray. Breast Cancer Res Treat 80: 49-62.

156. Colston K, Perks C, Xie S, Holly J (1998) Growth inhibition of both MCF-7 and Hs578T human breast cancer cell lines by vitamin D analogues is associated with increased expression of insulin-like growth factor binding protein-3. J Mol Endocrinol 20: 157-162.

157. Fanayan S, Firth SM, Butt AJ, Baxter RC (2000) Growth inhibition by insulin-like growth factor-binding protein-3 in T47D breast cancer cells requires transforming growth factor-beta (TGF-beta) and the type II TGF-beta receptor. J Biol Chem 275: 39146-39151.

158. Peng L, Malloy PJ, Feldman D (2004) Identification of a functional vitamin D response element in the human insulin-like growth factor binding protein-3 promoter. Mol Endocrinol 18: 1109-1119.

159. Massagué J (2008) TGFβ in cancer. Cell 134: 215-230.

160. Lee HJ, Liu H, Goodman C, Ji Y, Maehr H, Uskokovic M, Notterman D, Reiss M, Suh N (2006) Gene expression profiling changes induced by a novel Gemini Vitamin D derivative during the progression of breast cancer. Biochem Pharmacol 72: 332-343.

161. Lee HJ, Wislocki A, Goodman C, Ji Y, Ge R, Maehr H, Uskokovic M, Reiss M, Suh N (2006) A novel vitamin D derivative activates bone morphogenetic protein signaling in MCF10 breast epithelial cells. Mol Pharmacol 69: 1840-1848.

162. Lee HJ, Ji Y, Paul S, Maehr H, Uskokovic M, Suh N (2007) Activation of Bone Morphogenetic Protein Signaling by a Gemini Vitamin D3 Analogue Is Mediated by Ras/Protein Kinase Cα. Cancer Res 67: 11840-11847.

163. Simboli-Campbell M, Narvaez CJ, Tenniswood M, Welsh J (1996) 1,25-Dihydroxyvitamin D3 induces morphological and biochemical markers of apoptosis in MCF-7 breast cancer cells. J Steroid Biochem Mol Biol 58: 367-376.

164. Simboli-Campbell M, Narvaez CJ, VanWeelden K, Tenniswood M, Welsh JE (1997) Comparative effects of 1, 25 (OH) 2 D 3 and EB1089 on cell cycle kinetics and apoptosis in MCF-7 breast cancer cells. Breast Cancer Res Treat 42: 31-41.

165. Mathiasen IS, Lademann U, Jaattela M (1999) Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by Bcl-2 but does not involve known caspases or p53. Cancer Res 59: 4848-4856.

166. James SY, Mackay AG, Colston KW (1996) Effects of 1, 25 dihydroxyvitamin D_3 and its analogues on induction of apoptosis in breast cancer cells. J Steroid Biochem Mol Biol 58: 395-401.

167. Mathiasen IS, Sergeev IN, Bastholm L, Elling F, Norman AW, Jaattela M (2002) Calcium and calpain as key mediators of apoptosis-like death induced by vitamin D compounds in breast cancer cells. J Biol Chem 277: 30738-30745.

168. Colston KW, Chander SK, Mackay AG, Coombes RC (1992) Effects of synthetic vitamin D analogues on breast cancer cell proliferation in vivo and in vitro. Biochem Pharmacol 44: 693-702.

169. Colston KW, Mackay AG, James SY, Binderup L, Chander S, Coombes RC (1992) EB1089: A new vitamin D analogue that inhibits the growth of breast cancer cells *in vivo* and *in vitro*. Biochem Pharmacol 44: 2273-2280.

170. VanWeelden K, Flanagan L, Binderup L, Tenniswood M, Welsh JE (1998) Apoptotic regression of MCF-7 xenografts in nude mice treated with the vitamin D3 analog, EB1089. Endocrinology 139: 2102-2110.

171. Sundaram S, Sea A, Feldman S, Strawbridge R, Hoopes PJ, Demidenko E, Binderup L, Gewirtz DA (2003) The combination of a potent vitamin D3 analog, EB 1089, with ionizing radiation reduces tumor growth and induces apoptosis of MCF-7 breast tumor xenografts in nude mice. Clin Cancer Res 9: 2350-2356.

172. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 646-674.

173. Koli K, Keski-Oja J (2000) 1alpha,25-dihydroxyvitamin D3 and its analogues down-regulate cell invasion-associated proteases in cultured malignant cells. Cell Growth Differ 11: 221-229.

174. Flanagan L, Packman K, Juba B, O'Neill S, Tenniswood M, Welsh J (2003) Efficacy of Vitamin D compounds to modulate estrogen receptor negative breast cancer growth and invasion. J Steroid Biochem Mol Biol 84: 181-192.

175. El Abdaimi K, Dion N, Papavasiliou V, Cardinal PE, Binderup L, Goltzman D, Ste-Marie LG, Kremer R (2000) The vitamin D analogue EB 1089 prevents skeletal metastasis and prolongs survival time in nude mice transplanted with human breast cancer cells. Cancer Res 60: 4412-4418.

176. Pierce BL, Ballard-Barbash R, Bernstein L, Baumgartner RN, Neuhouser ML, Wener MH, Baumgartner KB, Gilliland FD, Sorensen BE, McTiernan A, Ulrich CM (2009) Elevated

biomarkers of inflammation are associated with reduced survival among breast cancer patients. J Clin Oncol 27: 3437-3444.

177. Cole SW (2009) Chronic inflammation and breast cancer recurrence. J Clin Oncol 27: 3418-3419.

178. Krishnan AV, Swami S, Peng L, Wang J, Moreno J, Feldman D (2010) Tissue-selective regulation of aromatase expression by calcitriol: implications for breast cancer therapy. Endocrinology 151: 32-42.

179. Krishnan AV, Swami S, Feldman D (2010) Vitamin D and breast cancer: inhibition of estrogen synthesis and signaling. J Steroid Biochem Mol Biol 121: 343-348.

180. THILL M, HOELLEN F, BECKER S, DITTMER C, FISCHER D, KÜMMEL S, SALEHIN D, FRIEDRICH M, KÖSTER F, DIEDRICH K (2012) Expression of Prostaglandin-and Vitamin D-metabolising Enzymes in Benign and Malignant Breast Cells. Anticancer Res 32: 367-372.

181. Lang R, Hammer M, Mages J (2006) DUSP meet immunology: dual specificity MAPK phosphatases in control of the inflammatory response. J Immunol 177: 7497-7504.

182. Gulliford T, English J, Colston K, Menday P, Moller S, Coombes R (1998) A phase I study of the vitamin D analogue EB 1089 in patients with advanced breast and colorectal cancer. Br J Cancer 78: 6.

183. Dalhoff K, Dancey J, Astrup L, Skovsgaard T, Hamberg K, Lofts F, Rosmorduc O, Erlinger S, Hansen JB, Steward W (2003) A phase II study of the vitamin D analogue Seocalcitol in patients with inoperable hepatocellular carcinoma. Br J Cancer 89: 252-257.

184. Evans T, Colston K, Lofts F, Cunningham D, Anthoney D, Gogas H, De Bono J, Hamberg K, Skov T, Mansi J (2002) A phase II trial of the vitamin D analogue Seocalcitol (EB1089) in patients with inoperable pancreatic cancer. Br J Cancer 86: 680-685.

185. Schwartz GG, Hall MC, Stindt D, Patton S, Lovato J, Torti FM (2005) Phase I/II study of 19-nor-1α-25-dihydroxyvitamin D2 (paricalcitol) in advanced, androgen-insensitive prostate cancer. Clin Cancer Res 11: 8680-8685.

186. Trivedi DP, Doll R, Khaw KT (2003) Effect of four monthly oral vitamin D3 (cholecalciferol) supplementation on fractures and mortality in men and women living in the community: randomised double blind controlled trial. Bmj 326: 469.

187. Lappe JM, Travers-Gustafson D, Davies KM, Recker RR, Heaney RP (2007) Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial. Am J Clin Nutr 85: 1586-1591.

188. Amir E, Simmons CE, Freedman OC, Dranitsaris G, Cole DEC, Vieth R, Ooi WS, Clemons M (2010) A phase 2 trial exploring the effects of high-dose (10,000 IU/day) vitamin D3 in breast cancer patients with bone metastases. Cancer 116: 284-291.

189. Lowe LC, Guy M, Mansi JL, Peckitt C, Bliss J, Wilson RG, Colston KW (2005) Plasma 25-hydroxy vitamin D concentrations, vitamin D receptor genotype and breast cancer risk in a UK Caucasian population. Eur J Cancer 41: 1164-1169.

190. Abbas S, Linseisen J, Slanger T, Kropp S, Mutschelknauss EJ, Flesch-Janys D, Chang-Claude J (2008) Serum 25-hydroxyvitamin D and risk of post-menopausal breast cancer—results of a large case–control study. Carcinogenesis 29: 93-99.

191. Crew KD, Gammon MD, Steck SE, Hershman DL, Cremers S, Dworakowski E, Shane E, Terry MB, Desai M, Teitelbaum SL (2009) Association between plasma 25-hydroxyvitamin D and breast cancer risk. Cancer Prev Res (Phila) 2: 598-604.

192. Rossi M, McLaughlin J, Lagiou P, Bosetti C, Talamini R, Lipworth L, Giacosa A, Montella M, Franceschi S, Negri E (2009) Vitamin D intake and breast cancer risk: a case–control study in Italy. Ann Oncol 20: 374-378.

193. Anderson LN, Cotterchio M, Vieth R, Knight JA (2010) Vitamin D and calcium intakes and breast cancer risk in pre-and postmenopausal women. Am J Clin Nutr 91: 1699-1707.

194. Kawase T, Matsuo K, Suzuki T, Hirose K, Hosono S, Watanabe M, Inagaki M, Iwata H, Tanaka H, Tajima K (2010) Association between vitamin D and calcium intake and breast cancer risk according to menopausal status and receptor status in Japan. Cancer science 101: 1234-1240.

195. Lee MS, Huang YC, Wahlqvist ML, Wu TY, Chou YC, Wu MH, Yu JC, Sun CA (2011) Vitamin D decreases risk of breast cancer in premenopausal women of normal weight in subtropical Taiwan. J Epidemiol 21: 87-94.

196. Bertone-Johnson ER, Chen WY, Holick MF, Hollis BW, Colditz GA, Willett WC, Hankinson SE (2005) Plasma 25-hydroxyvitamin D and 1, 25-dihydroxyvitamin D and risk of breast cancer. Cancer Epidem Biomar 14: 1991-1997.

197. Freedman DM, Chang SC, Falk RT, Purdue MP, Huang WY, McCarty CA, Hollis BW, Graubard BI, Berg CD, Ziegler RG (2008) Serum levels of vitamin D metabolites and breast cancer risk in the prostate, lung, colorectal, and ovarian cancer screening trial. CaCancer Epidem Biomar 17: 889-894.

198. Chlebowski RT, Johnson KC, Kooperberg C, Pettinger M, Wactawski-Wende J, Rohan T, Rossouw J, Lane D, O'Sullivan MJ, Yasmeen S (2008) Calcium plus vitamin D supplementation and the risk of breast cancer. J Natl Cancer Inst 100: 1581-1591.

199. McCullough ML, Stevens VL, Patel R, Jacobs EJ, Bain EB, Horst RL, Gapstur SM, Thun MJ, Calle EE (2009) Serum 25-hydroxyvitamin D concentrations and postmenopausal breast cancer risk: a nested case control study in the Cancer Prevention Study-II Nutrition Cohort. Breast Cancer Res 11: R64.

200. Almquist M, Bondeson AG, Bondeson L, Malm J, Manjer J (2010) Serum levels of vitamin D, PTH and calcium and breast cancer risk—a prospective nested case–control study. Int J Cancer 127: 2159-2168.

201. Engel P, Fagherazzi G, Boutten A, Dupré T, Mesrine S, Boutron-Ruault MC, Clavel-Chapelon F (2010) Serum 25 (OH) vitamin D and risk of breast cancer: a nested case-control study from the French E3N cohort. Cancer Epidem Biomar 19: 2341-2350.

202. Liby KT, Yore MM, Sporn MB (2007) Triterpenoids and rexinoids as multifunctional agents for the prevention and treatment of cancer. Nat Rev Cancer 7: 357-369.

203. Setzer WN, Setzer MC (2003) Plant-derived triterpenoids as potential antineoplastic agents. Mini Rev Med Chem 3: 540-556.

204. Ovesna Z, Vachalkova A, Horvathova K, Tothova D (2004) Pentacyclic triterpenoic acids: new chemoprotective compounds. Minireview. Neoplasma 51: 327-333.

205. Nishino H, Nishino A, Takayasu J, Hasegawa T, Iwashima A, Hirabayashi K, Iwata S, Shibata S (1988) Inhibition of the tumor-promoting action of 12-O-tetradecanoylphorbol-13-acetate by some oleanane-type triterpenoid compounds. Cancer Res 48: 5210-5215.

206. Connolly JD, Hill RA (2005) Triterpenoids. Nat Prod Rep 22: 230-248.

207. Dzubak P, Hajduch M, Vydra D, Hustova A, Kvasnica M, Biedermann D, Markova L, Urban M, Sarek J (2006) Pharmacological activities of natural triterpenoids and their therapeutic implications. Nat Prod Rep 23: 394-411.

208. Suh N, Wang Y, Honda T, Gribble GW, Dmitrovsky E, Hickey WF, Maue RA, Place AE, Porter DM, Spinella MJ, Williams CR, Wu G, Dannenberg AJ, Flanders KC, Letterio JJ, Mangelsdorf DJ, Nathan CF, Nguyen L, Porter WW, Ren RF, Roberts AB, Roche NS, Subbaramaiah K, Sporn MB (1999) A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, with potent differentiating, antiproliferative, and anti-inflammatory activity. Cancer Res 59: 336-341.

209. Honda T, Rounds BV, Bore L, Favaloro FG, Jr., Gribble GW, Suh N, Wang Y, Spor MB (1999) Novel synthetic oleanane triterpenoids: a series of highly active inhibitors of nitric oxide production in mouse macrophages. Bioorg Med Chem Lett 9: 3429-3434.

210. Honda T, Gribble GW, Suh N, Finlay HJ, Rounds BV, Bore L, Favaloro FG, Jr., Wang Y, Sporn MB (2000) Novel synthetic oleanane and ursane triterpenoids with various enone functionalities in ring A as inhibitors of nitric oxide production in mouse macrophages. J Med Chem 43: 1866-1877.

211. Honda T, Rounds BV, Bore L, Finlay HJ, Favaloro FG, Jr., Suh N, Wang Y, Sporn MB, Gribble GW (2000) Synthetic oleanane and ursane triterpenoids with modified rings A and C: a series of highly active inhibitors of nitric oxide production in mouse macrophages. J Med Chem 43: 4233-4246.

212. Honda T, Honda Y, Favaloro FG, Jr., Gribble GW, Suh N, Place AE, Rendi MH, Sporn MB (2002) A novel dicyanotriterpenoid, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile, active at picomolar concentrations for inhibition of nitric oxide production. Bioorg Med Chem Lett 12: 1027-1030.

213. Honda T, Rounds BV, Gribble GW, Suh N, Wang Y, Sporn MB (1998) Design and synthesis of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, a novel and highly active inhibitor of nitric oxide production in mouse macrophages. Bioorg Med Chem Lett 8: 2711-2714.

214. Suh N, Honda T, Finlay HJ, Barchowsky A, Williams C, Benoit NE, Xie QW, Nathan C, Gribble GW, Sporn MB (1998) Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. Cancer Res 58: 717-723.

215. Suh N, Roberts AB, Birkey Reffey S, Miyazono K, Itoh S, ten Dijke P, Heiss EH, Place AE, Risingsong R, Williams CR, Honda T, Gribble GW, Sporn MB (2003) Synthetic triterpenoids enhance transforming growth factor beta/Smad signaling. Cancer Res 63: 1371-1376.

216. Place AE, Suh N, Williams CR, Risingsong R, Honda T, Honda Y, Gribble GW, Leesnitzer LM, Stimmel JB, Willson TM, Rosen E, Sporn MB (2003) The novel synthetic triterpenoid, CDDO-imidazolide, inhibits inflammatory response and tumor growth in vivo. Clin Cancer Res 9: 2798-2806.

217. Chauhan D, Li G, Podar K, Hideshima T, Shringarpure R, Catley L, Mitsiades C, Munshi N, Tai YT, Suh N, Gribble GW, Honda T, Schlossman R, Richardson P, Sporn MB, Anderson KC (2004) The bortezomib/proteasome inhibitor PS-341 and triterpenoid CDDO-Im induce synergistic anti-multiple myeloma (MM) activity and overcome bortezomib resistance. Blood 103: 3158-3166.

218. Thimmulappa RK, Scollick C, Traore K, Yates M, Trush MA, Liby KT, Sporn MB, Yamamoto M, Kensler TW, Biswal S (2006) Nrf2-dependent protection from LPS induced inflammatory response and mortality by CDDO-Imidazolide. Biochem Biophys Res Commun 351: 883-889.

219. Nichols DP, Ziady AG, Shank SL, Eastman JF, Davis PB (2009) The triterpenoid CDDO limits inflammation in preclinical models of cystic fibrosis lung disease. Am J Physiol Lung Cell Mol Physiol 297: L828-836.

220. Liby KT, Royce DB, Risingsong R, Williams CR, Maitra A, Hruban RH, Sporn MB (2010) Synthetic triterpenoids prolong survival in a transgenic mouse model of pancreatic cancer. Cancer Prev Res (Phila) 3: 1427-1434.

221. Dinkova-Kostova AT, Massiah MA, Bozak RE, Hicks RJ, Talalay P (2001) Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. Proc Natl Acad Sci U S A 98: 3404-3409.

222. Talalay P, Dinkova-Kostova AT, Holtzclaw WD (2003) Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis. Adv Enzyme Regul 43: 121-134.

223. Itoh K, Mimura J, Yamamoto M (2010) Discovery of the negative regulator of Nrf2, Keap1: a historical overview. Antioxid Redox Signal 13: 1665-1678.

224. Dinkova-Kostova AT, Liby KT, Stephenson KK, Holtzclaw WD, Gao X, Suh N, Williams C, Risingsong R, Honda T, Gribble GW, Sporn MB, Talalay P (2005) Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress. Proc Natl Acad Sci U S A 102: 4584-4589.

225. Lapillonne H, Konopleva M, Tsao T, Gold D, McQueen T, Sutherland RL, Madden T, Andreeff M (2003) Activation of peroxisome proliferator-activated receptor gamma by a novel synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces growth arrest and apoptosis in breast cancer cells. Cancer Res 63: 5926-5939.

226. Chintharlapalli S, Papineni S, Konopleva M, Andreef M, Samudio I, Safe S (2005) 2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid and related compounds inhibit growth of colon cancer cells

through peroxisome proliferator-activated receptor gamma-dependent and -independent pathways. Mol Pharmacol 68: 119-128.

227. Konopleva M, Zhang W, Shi YX, McQueen T, Tsao T, Abdelrahim M, Munsell MF, Johansen M, Yu D, Madden T (2006) Synthetic triterpenoid 2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid induces growth arrest in HER2-overexpressing breast cancer cells. Mol Cancer Ther 5: 317-328.

228. Ahmad R, Raina D, Meyer C, Kufe D (2008) Triterpenoid CDDO-methyl ester inhibits the Janus-activated kinase-1 (JAK1)→ signal transducer and activator of transcription-3 (STAT3) pathway by direct inhibition of JAK1 and STAT3. Cancer Res 68: 2920-2926.

229. Ling X, Konopleva M, Zeng Z, Ruvolo V, Stephens LC, Schober W, McQueen T, Dietrich M, Madden TL, Andreeff M (2007) The novel triterpenoid C-28 methyl ester of 2-cyano-3, 12-dioxoolen-1, 9-dien-28-oic acid inhibits metastatic murine breast tumor growth through inactivation of STAT3 signaling. Cancer Res 67: 4210-4218.

230. Petronelli A, Saulle E, Pasquini L, Petrucci E, Mariani G, Biffoni M, Ferretti G, Scambia G, Benedetti-Panici P, Greggi S (2009) High sensitivity of ovarian cancer cells to the synthetic triterpenoid CDDO-Imidazolide. Cancer letters 282: 214-228.

231. Ahmad R, Raina D, Meyer C, Kharbanda S, Kufe D (2006) Triterpenoid CDDO-Me blocks the NF-κB pathway by direct inhibition of IKKβ on Cys-179. J Biol Chem 281: 35764-35769.

232. Deeb D, Gao X, Dulchavsky S, Gautam S (2008) CDDO-Me inhibits proliferation, induces apoptosis, down-regulates Akt, mTOR, NF-kappaB and NF-kappaB-regulated antiapoptotic and proangiogenic proteins in TRAMP prostate cancer cells. J Exp Ther Oncol 7: 31.

233. Gao X, Deeb D, Jiang H, Liu Y, Dulchavsky SA, Gautam SC (2007) Synthetic triterpenoids inhibit growth and induce apoptosis in human glioblastoma and neuroblastoma cells through inhibition of prosurvival Akt, NF-κB and Notch1 signaling. J Neuro-Oncol 84: 147-157.

234. Ito Y, Pandey P, Place A, Sporn MB, Gribble GW, Honda T, Kharbanda S, Kufe D (2000) The novel triterpenoid 2-cyano-3, 12-dioxoolean-1, 9-dien-28-oic acid induces apoptosis of human myeloid leukemia cells by a caspase-8-dependent mechanism. Cell Growth Differ 11: 261.

235. Ito Y, Pandey P, Sporn MB, Datta R, Kharbanda S, Kufe D (2001) The novel triterpenoid CDDO induces apoptosis and differentiation of human osteosarcoma cells by a caspase-8 dependent mechanism. Mol Pharmacol 59: 1094-1099.

236. Stadheim TA, Suh N, Ganju N, Sporn MB, Eastman A (2002) The novel triterpenoid 2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid (CDDO) potently enhances apoptosis induced by tumor necrosis factor in human leukemia cells. J Biol Chem 277: 16448-16455.

237. Ikeda T, Nakata Y, Kimura F, Sato K, Anderson K, Motoyoshi K, Sporn M, Kufe D (2004) Induction of redox imbalance and apoptosis in multiple myeloma cells by the novel triterpenoid 2-cyano-3, 12-dioxoolean-1, 9-dien-28-oic acid. Mol Cancer Ther 3: 39-45.

238. Kim KB, Lotan R, Yue P, Sporn MB, Suh N, Gribble GW, Honda T, Wu GS, Hong WK, Sun SY (2002) Identification of a Novel Synthetic Triterpenoid, Methyl-2-cyano-3, 12-

dioxooleana-1, 9-dien-28-oate, That Potently Induces Caspase-mediated Apoptosis in Human Lung Cancer Cells1. Mol Cancer Ther 1: 177-184.

239. Konopleva M, Tsao T, Ruvolo P, Stiouf I, Estrov Z, Leysath CE, Zhao S, Harris D, Chang S, Jackson CE (2002) Novel triterpenoid CDDO-Me is a potent inducer of apoptosis and differentiation in acute myelogenous leukemia. Blood 99: 326-335.

240. Samudio I, Konopleva M, Pelicano H, Huang P, Frolova O, Bornmann W, Ying Y, Evans R, Contractor R, Andreeff M (2006) A novel mechanism of action of methyl-2-cyano-3, 12 dioxoolean-1, 9 diene-28-oate: direct permeabilization of the inner mitochondrial membrane to inhibit electron transport and induce apoptosis. Mol Pharmacol 69: 1182-1193.

241. Brookes PS, Morse K, Ray D, Tompkins A, Young SM, Hilchey S, Salim S, Konopleva M, Andreeff M, Phipps R (2007) The triterpenoid 2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid and its derivatives elicit human lymphoid cell apoptosis through a novel pathway involving the unregulated mitochondrial permeability transition pore. Cancer Res 67: 1793-1802.

242. Kim EH, Deng CX, Sporn MB, Liby KT (2011) CDDO-imidazolide induces DNA damage, G2/M arrest and apoptosis in BRCA1-mutated breast cancer cells. Cancer Prev Res (Phila) 4: 425-434.

243. Konopleva M, Contractor R, Kurinna S, Chen W, Andreeff M, Ruvolo P (2005) The novel triterpenoid CDDO-Me suppresses MAPK pathways and promotes p38 activation in acute myeloid leukemia cells. Leukemia 19: 1350-1354.

244. Ikeda T, Sporn M, Honda T, Gribble GW, Kufe D (2003) The novel triterpenoid CDDO and its derivatives induce apoptosis by disruption of intracellular redox balance. Cancer Res 63: 5551-5558.

245. Shishodia S, Sethi G, Konopleva M, Andreeff M, Aggarwal BB (2006) A synthetic triterpenoid, CDDO-Me, inhibits IκBα kinase and enhances apoptosis induced by TNF and chemotherapeutic agents through down-regulation of expression of nuclear factor κB–regulated gene products in human leukemic cells. Clin Cancer Res 12: 1828-1838.

246. Liby K, Voong N, Williams CR, Risingsong R, Royce DB, Honda T, Gribble GW, Sporn MB, Letterio JJ (2006) The synthetic triterpenoid CDDO-Imidazolide suppresses STAT phosphorylation and induces apoptosis in myeloma and lung cancer cells. Clin Cancer Res 12: 4288-4293.

247. Liby KT, Sporn MB (2012) Synthetic oleanane triterpenoids: multifunctional drugs with a broad range of applications for prevention and treatment of chronic disease. Pharmacol Rev.

248. Wang Y, Porter WW, Suh N, Honda T, Gribble GW, Leesnitzer LM, Plunket KD, Mangelsdorf DJ, Blanchard SG, Willson TM, Sporn MB (2000) A synthetic triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), is a ligand for the peroxisome proliferator-activated receptor gamma. Mol Endocrinol 14: 1550-1556.

249. Ji Y, Lee HJ, Goodman C, Uskokovic M, Liby K, Sporn M, Suh N (2006) The synthetic triterpenoid CDDO-imidazolide induces monocytic differentiation by activating the Smad and ERK signaling pathways in HL60 leukemia cells. Mol Cancer Ther 5: 1452-1458.

250. Ikeda T, Kimura F, Nakata Y, Sato K, Ogura K, Motoyoshi K, Sporn M, Kufe D (2005) Triterpenoid CDDO-Im downregulates PML/RARalpha expression in acute promyelocytic leukemia cells. Cell Death Differ 12: 523-531.

251. Honda T, Janosik T, Honda Y, Han J, Liby KT, Williams CR, Couch RD, Anderson AC, Sporn MB, Gribble GW (2004) Design, synthesis, and biological evaluation of biotin conjugates of 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid for the isolation of the protein targets. J Med Chem 47: 4923-4932.

252. Hyer ML, Croxton R, Krajewska M, Krajewski S, Kress CL, Lu M, Suh N, Sporn MB, Cryns VL, Zapata JM (2005) Synthetic triterpenoids cooperate with tumor necrosis factor–related apoptosis-inducing ligand to induce apoptosis of breast cancer cells. Cancer Res 65: 4799-4808.

253. Liby K, Risingsong R, Royce DB, Williams CR, Yore MM, Honda T, Gribble GW, Lamph WW, Vannini N, Sogno I (2008) Prevention and Treatment of Experimental Estrogen Receptor–Negative Mammary Carcinogenesis by the Synthetic Triterpenoid CDDO-Methyl Ester and the Rexinoid LG100268. Clin Cancer Res 14: 4556-4563.

254. Tran K, Risingsong R, Royce D, Williams CR, Sporn MB, Liby K (2012) The Synthetic Triterpenoid CDDO-Methyl Ester Delays Estrogen Receptor–Negative Mammary Carcinogenesis in Polyoma Middle T Mice. Cancer Prev Res (Phila) 5: 726-734.

255. Speranza G, Gutierrez ME, Kummar S, Strong JM, Parker RJ, Collins J, Yu Y, Cao L, Murgo AJ, Doroshow JH (2012) Phase I study of the synthetic triterpenoid, 2-cyano-3, 12-dioxoolean-1, 9-dien-28-oic acid (CDDO), in advanced solid tumors. Cancer Chemoth Pharm 69: 431-438.

256. Hong DS, Kurzrock R, Supko JG, He X, Naing A, Wheler J, Lawrence D, Eder JP, Meyer CJ, Ferguson DA (2012) A Phase I First-in-Human Trial of Bardoxolone Methyl in Patients with Advanced Solid Tumors and Lymphomas. Clin Cancer Res 18: 3396-3406.

257. Pergola PE, Krauth M, Huff J, Ferguson D, Ruiz S, Meyer C, Warnock D (2011) Effect of Bardoxolone Methyl on Kidney Function in Patients with T2D and Stage 3b–4 CKD. Am J Nephrol 33: 469-476.

258. Pergola PE, Raskin P, Toto RD, Meyer CJ, Huff JW, Grossman EB, Krauth M, Ruiz S, Audhya P, Christ-Schmidt H (2011) Bardoxolone methyl and kidney function in CKD with type 2 diabetes. New Engl J Med 365: 327-336.

259. Ambrogi F, Biganzoli E, Querzoli P, Ferretti S, Boracchi P, Alberti S, Marubini E, Nenci I (2006) Molecular subtyping of breast cancer from traditional tumor marker profiles using parallel clustering methods. Clin Cancer Res 12: 781-790.

260. Wu G, Fan RS, Li W, Ko TC, Brattain MG (1997) Modulation of cell cycle control by vitamin D3 and its analogue, EB1089, in human breast cancer cells. Oncogene 15: 1555.

261. Wu G, Fan RS, Li W, Srinivas V, Brattain MG (1998) Regulation of Transforming Growth Factor-β Type II Receptor Expression in Human Breast Cancer MCF-7 Cells by Vitamin D3and Its Analogues. J Biol Chem 273: 7749-7756.

262. Campbell MJ, Gombart AF, Kwok SH, Park S, Koeffler HP (2000) The anti-proliferative effects of 1alpha, 25 (OH) 2D3 on breast and prostate cancer cells are associated with induction of BRCA1 gene expression. Oncogene 19: 5091.

263. Narvaez CJ, Welsh J (2001) Role of mitochondria and caspases in vitamin D-mediated apoptosis of MCF-7 breast cancer cells. J Biol Chem 276: 9101-9107.

264. McGaffin KR, Acktinson LE, Chrysogelos SA (2004) Growth and EGFR regulation in breast cancer cells by vitamin D and retinoid compounds. Breast Cancer Res Treat 86: 55-73.

265. Capiati DA, Rossi AM, Picotto G, Benassati S, Boland RL (2004) Inhibition of serum-stimulated mitogen activated protein kinase by 1alpha,25(OH)2-vitamin D3 in MCF-7 breast cancer cells. J Cell Biochem 93: 384-397.

266. O'Kelly J, Uskokovic M, Lemp N, Vadgama J, Koeffler HP (2006) Novel Gemini-vitamin D3 analog inhibits tumor cell growth and modulates the Akt/mTOR signaling pathway. J Steroid Biochem Mol Biol 100: 107-116.

267. Pendas-Franco N, Gonzalez-Sancho JM, Suarez Y, Aguilera O, Steinmeyer A, Gamallo C, Berciano MT, Lafarga M, Munoz A (2007) Vitamin D regulates the phenotype of human breast cancer cells. Differentiation 75: 193-207.

268. Hussain-Hakimjee EA, Mehta RG (2009) Regulation of steroid receptor expression by 1alpha-hydroxyvitamin D5 in hormone-responsive breast cancer cells. Anticancer Res 29: 3555-3561.

269. Abe-Hashimoto J, Kikuchi T, Matsumoto T, Nishii Y, Ogata E, Ikeda K (1993) Antitumor effect of 22-oxa-calcitriol, a noncalcemic analogue of calcitriol, in athymic mice implanted with human breast carcinoma and its synergism with tamoxifen. Cancer Res 53: 2534-2537.

270. Anzano MA, Smith JM, Uskoković MR, Peer CW, Mullen LT, Letterio JJ, Welsh MC, Shrader MW, Logsdon DL, Driver CL (1994) 1α, 25-Dihydroxy-16-ene-23-yne-26, 27-hexafluorocholecalciferol (Ro24-5531), a new deltanoid (vitamin D analogue) for prevention of breast cancer in the rat. Cancer Res 54: 1653-1656.

271. Koshizuka K, Koike M, Asou H, Cho SK, Stephen T, Rude RK, Binderup L, Uskokovic M, Phillip Koeffler H (1999) Combined effect of vitamin D3 analogs and paclitaxel on the growth of MCF-7 breast cancer cells in vivo. Breast Cancer Res Treat 53: 113-120.

272. Koshizuka K, Kubota T, Said J, Koike M, Binderup L, Uskokovic M, Koeffler H (1999) Combination therapy of a vitamin D3 analog and all-trans-retinoic acid: effect on human breast cancer in nude mice. Anticancer Res 19: 519.

273. Mehta RG (2004) Stage-specific inhibition of mammary carcinogenesis by 1alpha-hydroxyvitamin D5. Eur J Cancer 40: 2331.

274. Milliken EL, Zhang X, Flask C, Duerk JL, MacDonald PN, Keri RA (2005) EB1089, a vitamin D receptor agonist, reduces proliferation and decreases tumor growth rate in a mouse model of hormone-induced mammary cancer. Cancer letters 229: 205-215.

275. Lee HJ, Paul S, Atalla N, Thomas PE, Lin X, Yang I, Buckley B, Lu G, Zheng X, Lou YR, Conney AH, Maehr H, Adorini L, Uskokovic M, Suh N (2008) Gemini vitamin D analogues inhibit estrogen receptor-positive and estrogen receptor-negative mammary tumorigenesis without hypercalcemic toxicity. Cancer Prev Res (Phila) 1: 476-484.

276. Lee HJ, So JY, DeCastro A, Smolarek A, Paul S, Maehr H, Uskokovic M, Suh N (2010) Gemini vitamin D analog suppresses ErbB2-positive mammary tumor growth via inhibition of ErbB2/AKT/ERK signaling. J Steroid Biochem Mol Biol 121: 408.

277. Beer TM, Munar M, Henner WD (2001) A phase I trial of pulse calcitriol in patients with refractory malignancies. Cancer 91: 2431-2439.

278. Beer TM, Javle MM, Ryan CW, Garzotto M, Lam GN, Wong A, Henner WD, Johnson CS, Trump DL (2007) Phase I study of weekly DN-101, a new formulation of calcitriol, in patients with cancer. Cancer Chemoth Pharm 59: 581-587.

279. Fakih MG, Trump DL, Muindi JR, Black JD, Bernardi RJ, Creaven PJ, Schwartz J, Brattain MG, Hutson A, French R (2007) A phase I pharmacokinetic and pharmacodynamic study of intravenous calcitriol in combination with oral gefitinib in patients with advanced solid tumors. Clin Cancer Res 13: 1216-1223.

280. Muindi JR, Johnson CS, Trump DL, Christy R, Engler KL, Fakih MG (2009) A phase I and pharmacokinetics study of intravenous calcitriol in combination with oral dexamethasone and gefitinib in patients with advanced solid tumors. Cancer Chemoth Pharm 65: 33-40.

281. Kim EH, Deng C, Sporn MB, Royce DB, Risingsong R, Williams CR, Liby KT (2012) CDDO-Methyl ester delays breast cancer development in BRCA1-mutated mice. Cancer Prev Res (Phila) 5: 89-97.

282. Stephens PJ, McBride DJ, Lin ML, Varela I, Pleasance ED, Simpson JT, Stebbings LA, Leroy C, Edkins S, Mudie LJ (2009) Complex landscapes of somatic rearrangement in human breast cancer genomes. Nature 462: 1005-1010.

283. Albertson DG, Collins C, McCormick F, Gray JW (2003) Chromosome aberrations in solid tumors. Nat Genet 34: 369-376.

284. Jones PA. Overview of cancer epigenetics; 2005. Elsevier. pp. S3-S8.

285. Ma XJ, Salunga R, Tuggle JT, Gaudet J, Enright E, McQuary P, Payette T, Pistone M, Stecker K, Zhang BM (2003) Gene expression profiles of human breast cancer progression. Proc Natl Acad Sci U S A 100: 5974-5979.

286. Allred DC, Wu Y, Mao S, Nagtegaal ID, Lee S, Perou CM, Mohsin SK, O'Connell P, Tsimelzon A, Medina D (2008) Ductal carcinoma in situ and the emergence of diversity during breast cancer evolution. Clin Cancer Res 14: 370-378.

287. Gump F, Jicha D, Ozello L (1987) Ductal carcinoma in situ (DCIS): a revised concept. Surgery 102: 790.

288. Allred D, Mohsin S, Fuqua S (2001) Histological and biological evolution of human premalignant breast disease. Endocr-Relat Cancer 8: 47-61.

289. O'Connell P, Pekkel V, Allred DC, Fuqua SAW, Osborne CK, Clark GM (1998) Analysis of loss of heterozygosity in 399 premalignant breast lesions at 15 genetic loci. J Natl Cancer Inst 90: 697-703.

290. Aubele MM, Cummings MC, Mattis AE, Zitzelsberger HF, Walch AK, Kremer M, Höfler H, Werner M (2000) Accumulation of chromosomal imbalances from intraductal proliferative lesions to adjacent in situ and invasive ductal breast cancer. Diagn Mol Pathol 9: 14-19.

291. Chin K, de Solorzano CO, Knowles D, Jones A, Chou W, Rodriguez EG, Kuo WL, Ljung BM, Chew K, Myambo K (2004) In situ analyses of genome instability in breast cancer. Nat Genet 36: 984-988.

292. Castro NP, Osorio C, Torres C, Bastos EP, Mourão-Neto M, Soares FA, Brentani HP, Carraro DM (2008) Evidence that molecular changes in cells occur before morphological alterations during the progression of breast ductal carcinoma. Breast Cancer Res 10: R87.

293. Espina V, Mariani BD, Gallagher RI, Tran K, Banks S, Wiedemann J, Huryk H, Mueller C, Adamo L, Deng J (2010) Malignant precursor cells pre-exist in human breast DCIS and require autophagy for survival. PLoS One 5: e10240.

294. Yu KD, Shao ZM (2011) The two faces of autophagy and the pathological underestimation of DCIS. Nat Rev Cancer 11: 618-618.

295. Bhowmick NA, Neilson EG, Moses HL (2004) Stromal fibroblasts in cancer initiation and progression. Nature 432: 332-337.

296. Hanahan D, Coussens LM (2012) Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 21: 309-322.

297. Dawson PJ, Wolman SR, Tait L, Heppner GH, Miller FR (1996) MCF10AT: a model for the evolution of cancer from proliferative breast disease. Am J Pathol 148: 313.

298. Soule HD, Maloney TM, Wolman SR, Peterson WD, Brenz R, McGrath CM, Russo J, Pauley RJ, Jones RF, Brooks S (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 50: 6075-6086.

299. Miller FR, Soule HD, Tait L, Pauley RJ, Wolman SR, Dawson PJ, Heppner GH (1993) Xenograft model of progressive human proliferative breast disease. J Natl Cancer Inst 85: 1725-1732.

300. Miller FR (2000) Xenograft models of premalignant breast disease. J Mammary Gland Biol 5: 379-391.

301. Miller FR, Santner SJ, Tait L, Dawson PJ (2000) MCF10DCIS.com xenograft model of human comedo ductal carcinoma in situ. J Natl Cancer Inst 92: 1185-1186.

302. Santner SJ, Dawson PJ, Tait L, Soule HD, Eliason J, Mohamed AN, Wolman SR, Heppner GH, Miller FR (2001) Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. Breast Cancer Res Treat 65: 101-110.

303. Kadota M, Yang HH, Gomez B, Sato M, Clifford RJ, Meerzaman D, Dunn BK, Wakefield LM, Lee MP (2010) Delineating genetic alterations for tumor progression in the MCF10A series of breast cancer cell lines. PLoS One 5: e9201.

304. Rhee DK, Park SH, Jang YK (2008) Molecular signatures associated with transformation and progression to breast cancer in the isogenic MCF10 model. Genomics 92: 419-428.

305. Choong LY, Lim S, Chong PK, Wong CY, Shah N, Lim YP (2010) Proteome-wide profiling of the MCF10AT breast cancer progression model. PLoS One 5: e11030.

306. Kim SH, Miller FR, Tait L, Zheng J, Novak RF (2009) Proteomic and phosphoproteomic alterations in benign, premalignant and tumor human breast epithelial cells and xenograft lesions: biomarkers of progression. Int J Cancer 124: 2813-2828.

307. Xu J, Chen Y, Olopade OI (2010) MYC and breast cancer. Genes & cancer 1: 629-640.

308. Sears R, Nuckolls F, Haura E, Taya Y, Tamai K, Nevins JR (2000) Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. Genes Dev 14: 2501-2514.

309. Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P (1987) Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell 49: 465.

310. Worsham MJ, Pals G, Schouten JP, Miller F, Tiwari N, van Spaendonk R, Wolman SR (2006) High-resolution mapping of molecular events associated with immortalization, transformation, and progression to breast cancer in the MCF10 model. Breast Cancer Res Treat 96: 177-186.

311. Sutherland RL, Musgrove EA (2002) Cyclin D1 and mammary carcinoma: new insights from transgenic mouse models. Breast Cancer Research 4: 14-17.

312. Lovec H, Sewing A, Lucibello FC, Müller R, Mörröy T (1994) Oncogenic activity of cyclin D1 revealed through cooperation with Ha-ras: link between cell cycle control and malignant transformation. Oncogene 9: 323.

313. Liu JJ, Chao JR, Jiang MC, Ng SY, Yen J, Yang-Yen HF (1995) Ras transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH 3T3 cells. Mol Cell Biol 15: 3654-3663.

314. Yu Q, Geng Y, Sicinski P (2001) Specific protection against breast cancers by cyclin D1 ablation. Nature 411: 1017-1021.

315. Surmacz E (2000) Function of the IGF-I receptor in breast cancer. J Mammary Gland Biol 5: 95-105.

316. Renehan AG, Zwahlen M, Minder C, O'Dwyer ST, Shalet SM, Egger M (2004) Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. Lancet 363: 1346.

317. Sebolt-Leopold JS, Herrera R (2004) Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat Rev Cancer 4: 937-947.

318. Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, Riggins GJ (2004) High frequency of mutations of the PIK3CA gene in human cancers. Science 304: 554-554.

319. Wu G, Xing M, Mambo E, Huang X, Liu J, Guo Z, Chatterjee A, Goldenberg D, Gollin SM, Sukumar S (2005) Somatic mutation and gain of copy number of PIK3CA in human breast cancer. Breast Cancer Res 7: R609-616.

320. Walker SR, Nelson EA, Zou L, Chaudhury M, Signoretti S, Richardson A, Frank DA (2009) Reciprocal effects of STAT5 and STAT3 in breast cancer. Mol Cancer Res 7: 966-976.

321. Callow MG, Clairvoyant F, Zhu S, Schryver B, Whyte DB, Bischoff JR, Jallal B, Smeal T (2002) Requirement for PAK4 in the anchorage-independent growth of human cancer cell lines. J Biol Chem 277: 550-558.

322. Liu Y, Xiao H, Tian Y, Nekrasova T, Hao X, Lee HJ, Suh N, Yang CS, Minden A (2008) The pak4 protein kinase plays a key role in cell survival and tumorigenesis in athymic mice. Mol Cancer Res 6: 1215-1224.

323. Liu Y, Chen N, Cui X, Zheng X, Deng L, Price S, Karantza V, Minden A (2010) The protein kinase Pak4 disrupts mammary acinar architecture and promotes mammary tumorigenesis. Oncogene 29: 5883-5894.

324. Gotte M, Yip GW (2006) Heparanase, hyaluronan, and CD44 in cancers: a breast carcinoma perspective. Cancer Res 66: 10233-10237.

325. Ponta H, Sherman L, Herrlich PA (2003) CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 4: 33-45.

326. Zöller M (2011) CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? Nat Rev Cancer 11: 254-267.

327. Ghatak S, Misra S, Toole BP (2005) Hyaluronan constitutively regulates ErbB2 phosphorylation and signaling complex formation in carcinoma cells. J Biol Chem 280: 8875-8883.

328. Misra S, Toole BP, Ghatak S (2006) Hyaluronan constitutively regulates activation of multiple receptor tyrosine kinases in epithelial and carcinoma cells. J Biol Chem 281: 34936-34941.

329. Orian-Rousseau V, Chen L, Sleeman JP, Herrlich P, Ponta H (2002) CD44 is required for two consecutive steps in HGF/c-Met signaling. Genes Dev 16: 3074-3086.

330. Marangoni E, Lecomte N, Durand L, De Pinieux G, Decaudin D, Chomienne C, Smadja-Joffe F, Poupon M (2009) CD44 targeting reduces tumour growth and prevents post-chemotherapy relapse of human breast cancers xenografts. Br J Cancer 100: 918-922.

331. Tremmel M, Matzke A, Albrecht I, Laib AM, Olaku V, Ballmer-Hofer K, Christofori G, Héroult M, Augustin HG, Ponta H (2009) A CD44v6 peptide reveals a role of CD44 in VEGFR-2 signaling and angiogenesis. Blood 114: 5236-5244.

332. Slomiany MG, Dai L, Tolliver LB, Grass GD, Zeng Y, Toole BP (2009) Inhibition of functional hyaluronan-CD44 interactions in CD133-positive primary human ovarian carcinoma cells by small hyaluronan oligosaccharides. Clin Cancer Res 15: 7593-7601.

333. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, Lander ES (2009) Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell 138: 645-659.

334. So JY, Lee HJ, Smolarek AK, Paul S, Wang CX, Maehr H, Uskokovic M, Zheng X, Conney AH, Cai L, Liu F, Suh N (2011) A novel Gemini vitamin D analog represses the expression of a stem cell marker CD44 in breast cancer. Mol Pharmacol 79: 360-367.

335. Misra S, Hascall VC, Berger FG, Markwald RR, Ghatak S (2008) Hyaluronan, CD44, and cyclooxygenase-2 in colon cancer. Connect Tissue Res 49: 219-224.

336. Orian-Rousseau V, Morrison H, Matzke A, Kastilan T, Pace G, Herrlich P, Ponta H (2007) Hepatocyte growth factor-induced Ras activation requires ERM proteins linked to both CD44v6 and F-actin. Molecular biology of the cell 18: 76-83.

337. Takahashi E, Nagano O, Ishimoto T, Yae T, Suzuki Y, Shinoda T, Nakamura S, Niwa S, Ikeda S, Koga H (2010) Tumor necrosis factor-α regulates transforming growth factor-β-dependent epithelial-mesenchymal transition by promoting hyaluronan-CD44-moesin interaction. J Biol Chem 285: 4060-4073.

338. Kim IY, Yong HY, Kang KW, Moon A (2009) Overexpression of ErbB2 induces invasion of MCF10A human breast epithelial cells via MMP-9. Cancer letters 275: 227.

339. Wang SC, Lien HC, Xia W, Chen I, Lo HW, Wang Z, Ali-Seyed M, Lee DF, Bartholomeusz G, Ou-Yang F (2004) Binding at and transactivation of the *COX-2* promoter by nuclear tyrosine kinase receptor ErbB-2. Cancer Cell 6: 251-261.

340. Ristimäki A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H, Isola J (2002) Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer Res 62: 632-635.

341. Boland G, Butt I, Prasad R, Knox W, Bundred N (2004) COX-2 expression is associated with an aggressive phenotype in ductal carcinoma in situ. Br J Cancer 90: 423-429.

342. Barnes N, Haywood P, Flint P, Knox W, Bundred N (2006) Survivin expression in in situ and invasive breast cancer relates to COX-2 expression and DCIS recurrence. Br J Cancer 94: 253-258.

343. Weigelt B, Peterse JL, van't Veer LJ (2005) Breast cancer metastasis: markers and models. Nat Rev Cancer 5: 591-602.

344. Bernards R, Weinberg RA (2002) Metastasis genes: a progression puzzle. Nature 418: 823.

345. Joyce JA, Pollard JW (2008) Microenvironmental regulation of metastasis. Nat Rev Cancer 9: 239-252.

346. So JY, Lee HJ, Kramata P, Minden A, Suh N (2012) Differential expression of key signaling proteins in MCF10 cell lines, a human breast cancer progression model. Molecular and Cellular Pharmacology 4: 31-40.

347. Marella NV, Malyavantham KS, Wang J, Matsui S, Liang P, Berezney R (2009) Cytogenetic and cDNA microarray expression analysis of MCF10 human breast cancer progression cell lines. Cancer Res 69: 5946-5953.

348. Mbeunkui F, Metge BJ, Shevde LA, Pannell LK (2007) Identification of differentially secreted biomarkers using LC-MS/MS in isogenic cell lines representing a progression of breast cancer. J Proteome Res 6: 2993-3002.

349. Chen Y, Choong LY, Lin Q, Philp R, Wong CH, Ang BK, Tan YL, Hew CL, Shah N, Druker BJ (2007) Differential expression of novel tyrosine kinase substrates during breast cancer development. Mol Cell Proteomics 6: 2072-2087.

350. Wang Y, Ao X, Vuong H, Konanur M, Miller FR, Goodison S, Lubman DM (2008) Membrane glycoproteins associated with breast tumor cell progression identified by a lectin affinity approach. J Proteome Res 7: 4313-4325.

351. Morrison BJ, Schmidt CW, Lakhani SR, Reynolds BA, Lopez JA (2008) Breast cancer stem cells: implications for therapy of breast cancer. Breast Cancer Res 10: 210.

352. Al-Hajj M, Becker MW, Wicha M, Weissman I, Clarke MF (2004) Therapeutic implications of cancer stem cells. Curr Opin Genet Dev 14: 43-47.

353. Sheridan C, Kishimoto H, Fuchs RK, Mehrotra S, Bhat-Nakshatri P, Turner CH, Goulet R, Jr., Badve S, Nakshatri H (2006) CD44+/CD24- breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. Breast Cancer Res 8: R59.

354. Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, Weissman IL, Clarke MF, Ailles LE (2007) Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci U S A 104: 973-978.

355. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM (2007) Identification of pancreatic cancer stem cells. Cancer Res 67: 1030-1037.

356. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ (2005) Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 65: 10946-10951.

357. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100: 3983-3988.

358. To K, Fotovati A, Reipas KM, Law JH, Hu K, Wang J, Astanehe A, Davies AH, Lee L, Stratford AL (2010) Y-box binding protein-1 induces the expression of CD44 and CD49f leading to enhanced self-renewal, mammosphere growth, and drug resistance. Cancer Res 70: 2840-2851.

359. Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Sjolund A, Rimm DL, Wong H, Rodriguez A, Herschkowitz JI, Fan C, Zhang X, He X, Pavlick A, Gutierrez MC, Renshaw L, Larionov AA, Faratian D, Hilsenbeck SG, Perou CM, Lewis MT, Rosen JM, Chang JC (2009)

Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. Proc Natl Acad Sci U S A 106: 13820-13825.

360. Behbod F, Kittrell FS, LaMarca H, Edwards D, Kerbawy S, Heestand JC, Young E, Mukhopadhyay P, Yeh HW, Allred DC, Hu M, Polyak K, Rosen JM, Medina D (2009) An intraductal human-in-mouse transplantation model mimics the subtypes of ductal carcinoma in situ. Breast Cancer Res 11: R66.

361. Maehr H, Lee HJ, Perry B, Suh N, Uskokovic MR (2009) Calcitriol derivatives with two different side chains at C-20. V. Potent inhibitors of mammary carcinogenesis and inducers of leukemia differentiation. J Med Chem 52: 5505.

362. Lee HJ, Ju J, Paul S, So JY, DeCastro A, Smolarek A, Lee MJ, Yang CS, Newmark HL, Suh N (2009) Mixed tocopherols prevent mammary tumorigenesis by inhibiting estrogen action and activating PPAR-gamma. Clin Cancer Res 15: 4242-4249.

363. Paul S, Rimando AM, Lee HJ, Ji Y, Reddy BS, Suh N (2009) Anti-inflammatory action of pterostilbene is mediated through the p38 mitogen-activated protein kinase pathway in colon cancer cells. Cancer Prev Res (Phila) 2: 650-657.

364. Godar S, Ince TA, Bell GW, Feldser D, Donaher JL, Bergh J, Liu A, Miu K, Watnick RS, Reinhardt F, McAllister SS, Jacks T, Weinberg RA (2008) Growth-inhibitory and tumor-suppressive functions of p53 depend on its repression of CD44 expression. Cell 134: 62-73.

365. Dick JE (2008) Stem cell concepts renew cancer research. Blood 112: 4793-4807.

366. Yu WH, Woessner JF, Jr., McNeish JD, Stamenkovic I (2002) CD44 anchors the assembly of matrilysin/MMP-7 with heparin-binding epidermal growth factor precursor and ErbB4 and regulates female reproductive organ remodeling. Genes Dev 16: 307-323.

367. Lee JL, Wang MJ, Sudhir PR, Chen GD, Chi CW, Chen JY (2007) Osteopontin promotes integrin activation through outside-in and inside-out mechanisms: OPN-CD44V interaction enhances survival in gastrointestinal cancer cells. Cancer Res 67: 2089-2097.

368. Khan SA, Cook AC, Kappil M, Gunthert U, Chambers AF, Tuck AB, Denhardt DT (2005) Enhanced cell surface CD44 variant (v6, v9) expression by osteopontin in breast cancer epithelial cells facilitates tumor cell migration: novel post-transcriptional, post-translational regulation. Clin Exp Metastasis 22: 663-673.

369. Xu H, Posner GH, Stevenson M, Campbell FC (2010) Apc(MIN) modulation of vitamin D secosteroid growth control. Carcinogenesis 31: 1434-1441.

370. Trusolino L, Comoglio PM (2002) Scatter-factor and semaphorin receptors: cell signalling for invasive growth. Nat Rev Cancer 2: 289-300.

371. Boccaccio C, Comoglio PM (2006) Invasive growth: a MET-driven genetic programme for cancer and stem cells. Nat Rev Cancer 6: 637-645.

372. Espina V, Liotta LA (2010) What is the malignant nature of human ductal carcinoma in situ? Nat Rev Cancer 11: 68-75.

373. Hotary K, Li XY, Allen E, Stevens SL, Weiss SJ (2006) A cancer cell metalloprotease triad regulates the basement membrane transmigration program. Genes Dev 20: 2673-2686.

374. Kaufmann M, Heider KH, Sinn H, Von Minckwitz G, Ponta H, Herrlich P (1995) CD44 variant exon epitopes in primary breast cancer and length of survival. Lancet 345: 615-618.

375. Louderbough JMV, Schroeder JA (2011) Understanding the dual nature of CD44 in breast cancer progression. Mol Cancer Res 9: 1573-1586.

376. Lee GY, Kenny PA, Lee EH, Bissell MJ (2007) Three-dimensional culture models of normal and malignant breast epithelial cells. Nat Metohds 4: 359-365.

377. Adriance MC, Inman JL, Petersen OW, Bissell MJ (2005) Myoepithelial cells: good fences make good neighbors. Breast Cancer Res 7: 190-197.

378. Jedeszko C, Victor BC, Podgorski I, Sloane BF (2009) Fibroblast hepatocyte growth factor promotes invasion of human mammary ductal carcinoma in situ. Cancer Res 69: 9148-9155.

379. Montgomery N, Hill A, McFarlane S, Neisen J, O'Grady A, Conlon S, Jirstrom K, Kay EW, Waugh DJJ (2012) CD44 enhances invasion of basal-like breast cancer cells by upregulating serine protease and collagen-degrading enzymatic expression and activity. Breast Cancer Research 14: R84.

380. Bourguignon LYW, Peyrollier K, Xia W, Gilad E (2008) Hyaluronan-CD44 interaction activates stem cell marker Nanog, Stat-3-mediated MDR1 gene expression, and ankyrin-regulated multidrug efflux in breast and ovarian tumor cells. J Biol Chem 283: 17635-17651.

381. Lee JL, Wang MJ, Chen JY (2009) Acetylation and activation of STAT3 mediated by nuclear translocation of CD44. Science Signalling 185: 949.

382. Seidel HM, Milocco LH, Lamb P, Darnell Jr JE, Stein RB, Rosen J (1995) Spacing of palindromic half sites as a determinant of selective STAT (signal transducers and activators of transcription) DNA binding and transcriptional activity. Proc Natl Acad Sci U S A 92: 3041-3045.

383. Hsieh FC, Cheng G, Lin J (2005) Evaluation of potential Stat3-regulated genes in human breast cancer. Biochem Biophys Res Commun 335: 292-299.

384. Huang S (2007) Regulation of metastases by signal transducer and activator of transcription 3 signaling pathway: clinical implications. Clin Cancer Res 13: 1362-1366.

385. Xie T, Wei D, Liu M, Gao AC, Ali-Osman F, Sawaya R, Huang S (2004) Stat3 activation regulates the expression of matrix metalloproteinase-2 and tumor invasion and metastasis. Oncogene 23: 3550-3560.

386. Dechow TN, Pedranzini L, Leitch A, Leslie K, Gerald WL, Linkov I, Bromberg JF (2004) Requirement of matrix metalloproteinase-9 for the transformation of human mammary epithelial cells by Stat3-C. Proc Natl Acad Sci U S A 101: 10602-10607.

387. Duffy MJ, Maguire TM, Hill A, McDermott E, O'Higgins N (2000) Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. Breast Cancer Research 2: 252.

388. Wu ZS, Wu Q, Yang JH, Wang HQ, Ding XD, Yang F, Xu XC (2008) Prognostic significance of MMP-9 and TIMP-1 serum and tissue expression in breast cancer. Int J Cancer 122: 2050-2056.

389. Andreasen PA, Kjøller L, Christensen L, Duffy MJ (1997) The urokinase-type plasminogen activator system in cancer metastasis: A review. Int J Cancer 72: 1-22.

390. Wolff C, Malinowsky K, Berg D, Schragner K, Schuster T, Walch A, Bronger H, Höfler H, Becker KF (2011) Signalling networks associated with urokinase-type plasminogen activator (uPA) and its inhibitor PAI-1 in breast cancer tissues: new insights from protein microarray analysis. J Pathol 223: 54-63.

391. Dauer DJ, Ferraro B, Song L, Yu B, Mora L, Buettner R, Enkemann S, Jove R, Haura EB (2005) Stat3 regulates genes common to both wound healing and cancer. Oncogene 24: 3397-3408.

392. Elstner E, Linker-Israeli M, Said J, Umiel T, de Vos S, Shintaku IP, Heber D, Binderup L, Uskokovic M, Koeffler HP (1995) 20-epi-vitamin D3 analogues: a novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines. Cancer Res 55: 2822-2830.

393. Love-Schimenti CD, Gibson DFC, Ratnam AV, Bikle DD (1996) Antiestrogen potentiation of antiproliferative effects of vitamin D3 analogues in breast cancer cells. Cancer Res 56: 2789-2794.

394. Ursini-Siegel J, Schade B, Cardiff RD, Muller WJ (2007) Insights from transgenic mouse models of ERBB2-induced breast cancer. Nat Rev Cancer 7: 389-397.

395. Siegel PM, Ryan ED, Cardiff RD, Muller WJ (1999) Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. The EMBO journal 18: 2149-2164.

396. Witton CJ, Reeves JR, Going JJ, Cooke TG, Bartlett J (2003) Expression of the HER1–4 family of receptor tyrosine kinases in breast cancer. J Pathol 200: 290-297.

397. Graus-Porta D, Beerli RR, Daly JM, Hynes NE (1997) ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. The EMBO journal 16: 1647-1655.

398. Arora P, Cuevas B, Russo A, Johnson G, Trejo J (2008) Persistent transactivation of EGFR and ErbB2/HER2 by protease-activated receptor-1 promotes breast carcinoma cell invasion. Oncogene 27: 4434-4445.

399. Blancato J, Singh B, Liu A, Liao D, Dickson R (2004) Correlation of amplification and overexpression of the c-myc oncogene in high-grade breast cancer: FISH, in situ hybridisation and immunohistochemical analyses. Br J Cancer 90: 1612-1619.

400. Neve RM, Sutterlüty H, Pullen N, Lane HA, Daly JM, Krek W, Hynes NE (2000) Effects of oncogenic ErbB2 on G1 cell cycle regulators in breast tumour cells. Oncogene 19: 1647.

401. Park K, Kwak K, Kim J, Lim S, Han S (2005) c-*myc* amplification is associated with *HER2* amplification and closely linked with cell proliferation in tissue microarray of nonselected breast cancers. Hum Pathol 36: 634-639.

402. Perez EA, Jenkins RB, Dueck AC, Wiktor AE, Bedroske PP, Anderson SK, Ketterling RP, Sukov WR, Kanehira K, Chen B (2011) C-MYC Alterations and Association With Patient Outcome in Early-Stage HER2-Positive Breast Cancer From the North Central Cancer Treatment Group N9831 Adjuvant Trastuzumab Trial. J Clin Oncol 29: 651-659.

403. Wang SE, Narasanna A, Perez-Torres M, Xiang B, Wu FY, Yang S, Carpenter G, Gazdar AF, Muthuswamy SK, Arteaga CL (2006) HER2 kinase domain mutation results in constitutive phosphorylation and activation of HER2 and EGFR and resistance to EGFR tyrosine kinase inhibitors. Cancer Cell 10: 25-38.

404. Pradeep C, Zeisel A, Köstler W, Lauriola M, Jacob-Hirsch J, Haibe-Kains B, Amariglio N, Ben-Chetrit N, Emde A, Solomonov I (2011) Modeling invasive breast cancer: growth factors propel progression of HER2-positive premalignant lesions. Oncogene 31: 3569-3583.