UNDERSTANDING THE ROLE OF CANCER STEMNESS IN BREAST TUMORIGENESIS

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ABSTRACT OF THE DISSERTATION

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Breast cancer is a heterogeneous disease; its high degrees of intra- and inter-tumoral diversity are attributable to the presence of a subset of tumor-initiating cells called breast cancer stem cells (BCSCs). BCSCs are endowed with self-renewal and differentiation potential, ultimately culminating in a hierarchically organized tumor with distinct degrees of differentiation and functions. Accumulating evidence suggests BCSCs play roles in tumor initiation, progression, metastasis, chemoresistance, and relapse. Thus, targeting BCSCs can be a promising therapeutic strategy for eradicating breast cancer.

The goal of this study is to examine the significance of cancer stem cells in breast tumorigenesis and understand the mechanism of vitamin D compounds targeting cancer stem cell signaling pathways. Specifically, we seek to investigate (1) the effects of vitamin D-mediated inhibition of BCSCs in triple negative breast cancer (TNBC), (2) transcription factor-mediated regulation of breast cancer stemness, and (3) the transcriptomic signature regulated by vitamin D compounds in early breast cancer. In the pursuit of these goals, we

utilize BCSC-enriching mammosphere culture, genetic modulation, and next-generation sequencing technologies in two experimental models of breast cancer: TNBC cells (SUM159), which are reported to harbor enriched cancer stem cell populations, and pre-invasive ductal carcinoma in situ cells (MCF10DCIS). In SUM159 cells, we observed vitamin D compounds (1,25-dihydroxyvitamin D_3 and the Gemini vitamin D analog BXL0124) to inhibit mammosphere forming efficiency and self-renewal by downregulating markers of pluripotency and cancer stemness pathways. To understand the mechanism by which transcription factors control cancer stemness, we further studied octamer-binding transcription factor 4 (Oct4) as a potential factor in reprogramming BCSCs. We observed Oct4 overexpression to upregulate the $CD44^+/CD24^-$ BCSC subpopulation, increasing expression of CD44 and the activated form of NFκB, but with no significant changes in epithelial-mesenchymal transition markers and tumor formation *in vivo*.

To understand whether breast cancer stem cells promote the progression of ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC), along with vitamin D-mediated inhibition of this transition, we used RNA and DNA sequencing technologies to identify transcriptomic signatures in MCF10DCIS mammospheres. Our study revealed a global view of genes differentially regulated by vitamin D compounds, along with genes potentially involved in breast cancer stemness and progression. Ingenuity Pathway Analysis identified the TP63-VDR axis as a plausible target that can be explored for inhibition of DCIS transition to IDC. Collectively, these findings suggest a role for vitamin D as a potential preventive agent in targeting cancer stemness and breast cancer progression.

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1.1 Breast cancer

After lung cancer, breast cancer is the leading cause of cancer and the second most common cause of cancer-related deaths in females. According to the American Cancer Society's *Cancer Statistics 2020*, an estimated 276,480 new cases of invasive breast cancer and 48,530 cases of ductal carcinoma in situ (DCIS) will be diagnosed in US women in 2020; an estimated 42,170 women will die from breast cancer in the same year. For the last decade, the incidence of invasive breast cancer in females has increased by 0.3% every year [1]. Risk factors for breast cancer include older age, female gender, family history of breast or ovarian cancer, history of atypical hyperplasia, DCIS or lobular carcinoma in situ, obesity, hormone replacement therapy, alcohol consumption, smoking, physical inactivity, and reproductive factors such as oral contraceptive use, early menarche, and late menopause [2]. Depending on the clinico-pathological staging at the time of diagnosis, breast cancer is treated by multiple modalities—surgery, chemotherapy, radiation, hormonal therapy, targeted therapy, and immunotherapy [3].

1.2 Molecular heterogeneity of breast cancer

Breast cancer is a phenotypically diverse cancer with a large degree of inter- and

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[1]Part of this chapter is under preparation for publication.
[2]**Keywords**: breast cancer stem cells; self-renewal; OCT4; tumor microenvironment; epithelial-mesenchymal transition; differentiation, vitamin D
[3]**Abbreviations**: CSCs, cancer stem cells; BCSCs, breast cancer stem cells; TNBC, triple negative breast cancer; EMT, epithelial mesenchymal transition; TME, tumor microenvironment

intra-tumoral genetic and epigenetic heterogeneity. Intertumoral heterogeneity exists between tumors of the same origin among different patients, whereas intra-tumoral heterogeneity occurs within a given tumor. Intertumoral heterogeneity is best distinguished by clinical staging and histopathologic classification. Heterogeneous tumors feature different hormonal receptor expression profiles, biomarkers, genetic profiles, and clinical outcomes [4]. Breast tumors are divided into subtypes based on hormonal receptor status—specifically concerning the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). ER and PR are expressed either alone (ER+/PR- or ER-/PR+) or together (ER+/PR+) in a majority of breast carcinomas, and as biomarkers, are used as prognostic factors as well as in guidance for clinical management [5]. ER+ breast cancers are well differentiated and less aggressive relative to PR^+ breast cancers. Co-expression of both ER and PR receptors carries better prognosis when compared to ER+/PR- or ER-/PR+ cases [6]. Meanwhile, HER2+ carcinomas feature the most aggressive phenotype among invasive breast cancers comprising about 25% of all breast cancer cases [7]. However, a pathological complete response can be achieved from HER2-targeted therapy along with conventional chemotherapy [8]. Breast carcinomas that do not express ER, PR, or HER2 are referred to as triple negative breast cancer (TNBC) and constitute about 15-20% of breast cancer cases. These are a group of genetically and phenotypically heterogenous tumors having poor prognosis and variable responsiveness to treatment [9]. Additional functional biomarkers have been investigated for potential implications in diagnosis, treatment, and predictions of drug resistance and prognosis; these include antigen Ki-67 (KI-67; cell proliferation), programmed death-ligand 1 (PD-L1;

immune response), HER2Δ16 (drug resistance), and matrix metallopeptidase 9 (MMP-9; invasion and metastasis) [10].

Using gene expression patterns, breast cancer can be divided into four main molecular intrinsic subtypes (luminal A, luminal B, HER2-enriched, and basal like) and a Normal Breast-like group [11]. Each subtype has distinct molecular and pathological characteristics that govern incidence, survival, and therapeutic response [12]. Luminal A and luminal B subtypes respectively account for 30-40% and 20-30% of invasive breast cancers and are characterized by specific genetic profiles that illustrate tumor heterogeneity. Specifically, luminal A tumors are enriched for stem-like signature and immune check-point genes [13], while luminal B tumors are less well differentiated and carry worse prognosis, manifested by an increased level of genetic mutations implicated in tumor suppression and cell proliferation [14]. The HER2-enriched subtype has high expression of erb-b2 receptor tyrosine kinase 2 (*ERBB2*) and low to intermediate expression of the luminal genes [15]. Finally, the basal-like subtype, which accounts for 60-90% of TNBC cases, is enriched with basal markers (keratins 5, 6, 14, and 17) and genes relating to cell proliferation [16, 17]. In terms of subtype-based prognostic significance, the basal-like subtype carries the worst prognosis [18].

Breast cancers with intra-tumoral heterogeneity are characterized by diversity in morphology, biomarkers, circulating tumor cells, and genetic and epigenetic modifications. Moreover, tumor-specific cellular plasticity, genetic evolution, and microenvironment impart intra-tumoral diversity [19]. Morphologic heterogeneity can be observed tissue histopathology based on the expression of clinical biomarkers, hormone receptor staining, and different morphological structures [4]. Breast cancer also displays extensive intra-

tumoral heterogeneity in terms of genetic and chromosomal variation. Comparative genomic hybridization and genomic fingerprinting can be utilized to investigate heterogeneity at the genomic level, namely chromosomal aberrations and copy number variations [20]. Bulk sequencing and single-cell sequencing are also exploited to illustrate intra-tumoral heterogeneity for distinct clonality of gene expression, driver mutations, functional heterogeneity, and gene expression profiles of treatment resistance and metastasis [21]. In breast cancer progression, intra-tumoral heterogeneity is reflected in epigenetic features such as histone modifications, miRNA networks, and DNA methylation. For example, there is significant variation in tumor-related gene methylation patterns pertinent to the ER and HER2 status of breast tumors [22]. Several studies, including The Cancer Genome Atlas (TCGA) Network, have used integrative multi-omic analyses on data from primary breast cancer patients to identify genomic heterogeneity, including subtype-specific mutations, through integrating platforms including DNA methylation, genomic DNA copy number arrays, exome sequencing, and mRNA and microRNA arrays [23].

1.3 Cancer stem cells

Cancer stem cells (CSCs) are proposed to play roles in tumor initiation, maintenance of heterogeneity, tumor growth, recurrence, therapeutic resistance, and evasion of immunological surveillance [24]. Two models have been proposed to explain the evolution of CSCs [25]. According to the clonal evolution model, genetic mechanisms are the culprits underlying clonal expansions, with the stepwise acquisition of mutations in single clones culminating in tumor progression. This is followed by selection of more

aggressive dominant subclones having survival advantage and tumorigenic potential [26]. Meanwhile, the CSC model hypothesized a role for nongenetic mechanisms as the source of intra-tumoral heterogeneity. In this model, cancers originate from a small subpopulation of tumor cells that can initiate tumorigenesis. CSCs were first identified in acute myeloid leukemia, when a $CD34^{+}/CD38^{-}$ subpopulation of human leukemia cells transplanted into immunocompromised (NOD/SCID) mice underwent leukemic transformation and differentiation *in vivo* [27]. Subsequently, CSCs have been identified in a variety of cancer types, including breast cancer, colon cancer, melanoma, prostate cancer, lung cancer, and glioblastoma [28]. Since "cancer stem cell" do not always originate from normal stem cells but rather from mature tissue cells, the term tumor-initiating cells (TICs) is interchangeably used [29].

CSCs are similar to normal stem or progenitor cells in their ability to self-renew and recapitulate heterogeneity [26]. Self-renewal is a hallmark of stem cells, in which a stem cell produces two daughter cells with stem cell properties (symmetric division) or one daughter cell with stem cell properties and a second that undergoes differentiation (asymmetric division) [30]. CSCs express transcription factors (OCT4, NANOG homeobox [NANOG], and SRY-box transcription factor 2 [SOX2]) that are found in early embryonic stem cells. The core stem cell factors regulate pluripotency and self-renewal, and their overexpression is associated with signaling pathways related to malignant transformation, tumorigenicity, tumor progression, relapse, and inhibition of apoptosis [31]. OCT4, NANOG and SOX2 markers are induced in many cancer types, including breast, prostate, lung, colorectal, and gastrointestinal cancers [32]. Likewise, normal stem cells and CSCs share common self-renewal signaling pathways including the Notch,

Hedgehog, STAT3, and Wnt/beta-catenin pathways; all of these are documented as being important signaling cascades in embryonic development and have been shown to contribute to tumorigenesis in multiple types of tumors [33]. The plausibility of the CSC theory in breast cancer, which hypothesizes BCSCs to derive from normal progenitor/stem cells, is supported by phenotypic features similar to their lineage-specific normal stem cell counterparts [34].

CSCs arise from deregulation of the self-renewal program in stem cells, giving rise to their malignant transformation, or from the dedifferentiation of committed mature cells to acquire CSC-like properties [35]. In addition to self-renewal, CSCs also display quiescence in response to environmental cues. Thus, while anti-mitotic chemotherapeutic agents have been developed to target proliferating CSCs, the resident quiescent CSCs remain resistant to chemo- and radio-therapies even at high doses and so are the major cause of relapse—the living evidence of CSC plasticity and the supreme challenge faced by current therapies [36].

Ultimately, numerous intrinsic and extrinsic factors regulate CSC traits, including developmental pathways, epigenetics, stem cell transcription factors, epithelial mesenchymal transition (EMT) factors, cell cycle regulation mechanisms, apoptosis pathways, and the tumor microenvironment. All of these factors interact constantly and dynamically regulate CSC survival, proliferation, and metastasis [37]. As a consequence, CSCs exhibit a spectrum of functional and phenotypic heterogeneity, confirmed by *in vitro* clonogenic and anchorage-independent growth assays (tumor sphere assays) as well as *in vivo* limiting dilution xenotransplantation assays [38]. CSCs constitute only a small proportion (0.01-2%) of the tumor cells in a tumor mass, and isolating and identifying a

pure CSC population remains challenging [39]. The identification of stem cell surface markers that can be used to isolate BCSCs will provide key insights into BCSC biology along with opportunities to develop therapeutic targets. To date, CSCs have been identified by using one or multiple cell surface markers in fluorescence-activated cell sorting (FACS); measuring functional markers such as aldehyde dehydrogenase 1 (ALDH1) enzyme activity and ATP-binding cassette (ABC) transporter expression; single-cell DNA detection; and screening side population (SP) cells with the Hoechst-33342 dye exclusion technique [28]. Identifying, isolating, and characterizing the BCSC populations has so far primarily utilized cell surface markers. In particular, the CD44, CD24, and ALDH1(+) markers have become increasingly used to isolate BCSCs and as prognosis markers for patients [40]. **Table 1.1** summarizes the BCSC markers, their functions, target genes and tumorigenesis.

CD44, a non-kinase single-span transmembrane glycoprotein that binds hyaluronan, is involved in cell proliferation, survival, and differentiation; it likewise regulates CSC properties including self-renewal, tumor initiation, metastasis, and radio- and chemo-resistance. Alternatively-spliced variants of CD44 play roles in tumor development and progression. CD44 expression is high in BCSCs; its downregulation induces differentiation and sensitizes the cells to chemotherapy [41, 42].

CD24 is a glycosylphosphatidylinositol-linked cell surface glycoprotein that has been implicated in immunological functions, tumorigenesis, chemoresistance, and metastasis. CD24 expression is low or absent in BCSCs, and its upregulation is associated with poor prognosis in the luminal A and TNBC subtypes [43].

ALDH1 is a member of the aldehyde dehydrogenase (ALDH) family, a group of enzymes that oxidize intracellular aldehydes to carboxylic acids. Its activity is measured by the ALDEFLUOR assay, which assesses nine active isoforms of ALDH; in breast cancer, high ALDH1 activity is associated with stem-like features and chemoresistance. $ALDH1^+$ breast cancers are also characterized by being ER-, $EGFRII^+$ and $Ki\text{-}67^{hi}$ [44]. Suppression of ALDH1 decreases tumorigenicity and cell migration [45].

BCSCs were first isolated from immunocompromised xenografts using a combination of cell surface markers: $CD44^+/CD24^{-/low}$ Lin^-. The cells with this phenotype are tumorigenic at a number as low as 100 cells; in contrast, those with different phenotypes failed to form tumors even at tens of thousands of cells [46]. A high CD44/CD24 ratio is directly correlated to cell proliferation and tumorigenesis, as indicated by increased formation of mammospheres *in vitro* and xenograft tumors [45]. In addition, $CD44^+/CD24^-$ breast cancer cells are enriched for EMT-associated traits, including expression of matrix metalloproteinase 1 (MMP-1), vimentin, and zinc finger E-box binding homeobox 1 (ZEB1); this is suggestive of interplay between EMT and CSC status [47]. These cells also demonstrate increased expression of the molecular chaperones glucose-regulated protein 78 (GRP78) and 94 (GRP94), which regulate endoplasmic reticulum homeostasis in stem cell development and in invasion of cancer [48]. Furthermore, the cells exhibit dysregulation of major signaling pathways otherwise involved in the regulation of normal mammary stem cells, such as the Notch, Hedgehog, and Wnt/beta-catenin pathways; blockage of these pathways by chemotherapeutic agents inhibits the CSC-like phenotype and tumorigenesis [49]. In mice, breast cancer cells derived from BRCA1-deficient mammary tumors show increased numbers of $CD44^+/CD24^-$ and $CD133^+$ cells and

increased expression of stem cell-associated genes including *Oct4*, *Notch1*, *Aldh1*, *Fgfr1*, and *Sox1* [50]. In the clinical context, the $CD44^+/CD24^-$ phenotype is associated with resistance to cytostatic agents, degree of malignancy, and patient survival [51]. Furthermore, $CD44^+/CD24^-$ BCSCs are resistant to radiation treatment and demonstrate increased expression of Jagged-1, Notch-1, and p-S6K1 (a major downstream regulator of the mTOR pathway) [52]. The phenotypic radioresistance in these cells is mediated through upregulation of the checkpoint kinase pathway (CHK), with application of the CHK inhibitor debromohymenialdisine effectively overcoming the resistance [53].

Regarding ALDH as a population marker, Ginestier et al. found that ALDH1 enzymatic activity is high in a subpopulation of breast carcinomas having tumorigenic and self-renewal abilities both *in vivo* and *in vitro* [54]. $ALDH^{hi}CD44^+$ subpopulations of BCSCs are resistant to chemotherapy and radiotherapy and feature increased expression of glutathione-S-transferase pi, p-glycoprotein, and checkpoint kinase 1 (CHK1). Pretreatment of these cell populations with all-trans retinoic acid or the ALDH inhibitor diethylaminobenzaldehyde (DEAB) significantly sensitizes the stem-like breast cancer cells and reduces resistance [55]. In MCF-7 xenograft tumors, ALDH1A1 (an isoform of ALDH1) promotes tumor angiogenesis by upregulating the retinoic acid/HIF-1α/VEGF signaling pathway, thereby affecting breast cancer progression [56]. In $ALDH1^+$ BCSCs, the Wnt/β-catenin signaling pathway, known to regulate stem cell niche during development, is dysregulated; downregulation of Wnt expression inhibits the CSC phenotype and suppresses breast cancer metastasis [57]. In ductal carcinoma in situ (DCIS), expression of ALDH1 along with enhancer of zeste 2 polycomb repressive

complex 2 subunit (EZH2), a marker implicated in stem cell maintenance and renewal, is associated with tumor recurrence and progression to invasive breast cancer [58].

Studies of invasive breast carcinomas and breast cancer cell lines have shown basal-like (TNBC) tumors to be enriched with $CD44^{+}/CD24^{-}$ and $ALDH1^{+}$ phenotypes [59]. Quiescent mesenchymal-like BCSCs are $CD44^{+}/CD24^{-}$ and localize to the tumor front, whereas proliferative epithelial-like BCSCs are ALDH1(+) and localize in the center [60].

In addition to cell surface markers, various functional assays are employed in the study of BCSCs; these include the mammosphere forming assay in serum free medium, which enriches for BCSCs, and the *in vivo* injection of FACS-sorted cells by limiting dilutions into immunocompromised mice, with consequent initiation of tumor growth [61]. Despite the multiplicity of BCSC markers and assays available, universal putative markers have yet to be resolved that can identify specific subpopulations having the most tumorigenic potential in each breast cancer case. Identification of those subpopulations is essential for the development of CSC-targeted therapy and overcoming resistance to chemo-radio treatments.

1.4 Major self-renewal pathways in BCSCs

CSC populations are maintained by their self-renewal capacity. The current notion of CSCs states that the self-renewal signaling and transcription factors which regulate growth and maintenance in normal stem cells are dysregulated in BCSCs **(Figure 1.1)** [62]. The following section will discuss the major self-renewal pathways in BCSCs.

1.4.1 Notch signaling pathway

Notch is a family of four transmembrane receptors (NOTCH 1-4) that interacts with five ligands: the jagged proteins (JAG1 and JAG2) and the delta-like ligands (DLL1, DLL3, and DLL4) [63]. Two forms of Notch signaling are known, canonical and non-canonical. While canonical Notch signaling is involved in multiple cellular processes, including embryonic development, stem cell fate determination, apoptosis, cell cycle progression, self-renewal and lineage specific differentiation, non-canonical Notch signaling is associated with immune activation and breast tumorigenesis [64]. The canonical Notch signaling pathway is triggered by the interaction between a transmembrane ligand and a receptor in adjacent cells, resulting in successive proteolytic cleavage by ADAM metallopeptidase 10 (ADAM10) or ADAM17/TACE and γ-secretase to release the Notch intracellular domain (NICD). The NICD in turn translocates to the nucleus, binds to the CSL/RBPJ transcription complex, and activates Notch target genes, including the HES and HEY families of transcription factors and other genes associated with tumorigenesis [65]. In contrast, the activation of non-canonical Notch signaling is independent of RBPJκ and CSL signaling. Upon binding of the ligand to the receptor, NICD is released and enters the nucleus directly [64]. The pleiotropic nature of Notch signaling is attributed to the presence of structurally diverse non-canonical ligands including integral membrane-tethered, GPI-linked and secreted proteins [66]. Non-canonical Notch4 signaling is implicated in mammary gland tumorigenesis in *RBPJκ* conditional knockout mice [67]. In breast tumor cells, non-canonical Notch signaling activates IL-6/JAK/STAT signaling which further escalates the oncogenic potential of

tumor cells. The upregulation of IL-6 was regulated by IKKα and IKKβ proteins of NF-κB signaling pathway [68].

Notch signaling is documented to regulate mammary gland development via NOTCH4, in conjunction with WNT family member 1 (WNT-1) [69]. In MMTV/Notch1(intra) and MMTV/Notch3(intra) transgenic mice, increased expression of NOTCH1 and NOTCH3 impairs ductal and lobulo-alveolar mammary gland development and induces mammary gland tumors [70]. Oncogenic RAS activates NOTCH1 and upregulates the Notch ligand DLL1 along with presenilin-1 through a p38-mediated pathway. There is a correlation between Ras overexpression and upregulation of NOTCH1 in breast carcinomas [71]. Relatedly, aberrant activation of Notch signaling has been reported in invasive breast cancer and is associated with poor survival in patients [72]. In clinical breast cancer samples, Notch signaling is found to promote BCSCs by inducing expression of sirtuin 2 (SIRT2), leading to deacetylation and activation of ALDH1A1 [73]. Notch1 and Notch4 signaling are higher in $ESA^{+}/CD44^{+}/CD24^{low}$ enriched BCSCs. NOTCH1 overexpression in MCF7 and MCF10A breast cancer cells increased the abundance of the BCSC $CD44^{+}/CD24^{low}$ subpopulation, along with increasing tumor cell invasion and migration. Increased NOTCH1 expression also promotes the EMT phenotype and tumor growth *in vivo* through crosstalk with STAT3 signaling [74].

Notch signaling and expressions of its target genes are also elevated in mammosphere-derived stem-like cells. Inhibition of Notch signaling by a γ-secretase inhibitor significantly reduces sphere formation, proliferation and colony formation, and also induces apoptosis [75]. Likewise, pharmacologic and genetic inhibitions reduce stem cell activity in *in vitro* and tumor formation *in vivo* [76]. In $CD44^{+}/CD24^{-}$ TNBC

mammospheres, the breast tumor suppressor signal peptide, CUB domain and EGF like domain containing 2 (SCUBE2) is overexpressed, with concomitant overexpression of SOX2, OCT4, and NANOG. Ectopic expression of SCUBE2 in adherent cells promotes EMT and metastasis by activating Notch signaling and its components [77].

In addition to the EMT process, another contributor to the generation of CSCs is the tumor microenvironment. In the hypoxic microenvironment of breast cancer tumors, Notch signaling is hyperactivated, and this aberrant activation of JAG2 and Notch signaling upregulates EMT, cell survival, and the metastasis and growth of cancer stem-like cells [78]. In TNBC, cellular communication network factor 6 (CCN6), a secreted matrix-associated family and negative regulator of breast cancer progression, regulates Slug and Notch1 signaling by inducing mesenchymal-epithelial transition (MET) and reducing TICs. In human invasive breast carcinomas, CCN6 expression is inversely correlated with NICD1 expression [79]. Finally, in a xenograft model using 231-BR brain metastatic breast cancer cells, inhibition of Notch1 by a γ-secretase inhibitor (DAPT) reduced the $CD44^{+}/CD24^{low}$ subpopulation, tumor invasion, and brain metastasis, indicating a role of Notch signaling in distant metastasis [80].

Notch4 expression is high in TNBC patients and has a significant negative correlation with overall survival in breast cancer patients [81]. $Notch4^{+}$ BCSCs are characterized by increased expression of stemness factors (OCT4, SOX2, NANOG), mammosphere formation *in vitro*, and tumorigenicity in a serial dilution tumor transplantation xenograft model [82]. In addition, Notch signaling mediates drug resistance in breast cancer. PI3K/mTOR signaling is dysregulated in 30% of TNBC cases [83]. Treating TNBC cells with mTOR inhibitors leads to increased stemness features and

greater *in vivo* tumor initiating capacity. The intrinsic resistance of these cells from TORC1/2 inhibition is driven by their activated Notch1 and FGF1 pathways in association with increased mitochondrial metabolism and FGFR1 signaling. Notably, abrogation of the FGFR-mitochondrial metabolism-Notch1 axis overcomes resistance to TORC1/2 inhibitors by eliminating drug-resistant CSCs [84]. Meanwhile, JAG1-NOTCH4 receptor activation increases BCSC activity and induces tamoxifen resistance in both patient-derived tumors and xenograft models. Targeting Notch4 reverses the increase in Notch, reducing BCSC activity and improving the tamoxifen resistance [85]. Thus, in combination with other modalities, targeting the Notch pathway could be a promising strategy for enhancing the effectiveness and sensitivity of breast cancer treatment while essentially eradicating BCSCs.

1.4.2 Wnt signaling in BCSCs

The Wnt/Frizzled/β-catenin pathway is an evolutionarily conserved signaling pathway that plays significant roles in embryonic development and tissue homeostasis [86]. There are 19 Wnt glycoproteins that serve as ligands for the receptors Frizzled (FZD) and LDL receptor related protein 5/6 (LRP5/6) [87]. Binding of Wnt ligands to the receptors can trigger either canonical or non-canonical signaling. Canonical signaling activated by Wnt binding to FZD or LRP5/6 receptors regulates the stability of β-catenin and controls its expression. In the absence of Wnt ligands, β-catenin undergoes phosphorylation and proteasomal degradation via the destruction complex; but when Wnt is present and binds to its cognate receptor, the destruction complex is degraded, thus releasing β-catenin to translocate into the nucleus and form a complex with TCF/LEF family of proteins to

regulate downstream Wnt target genes [88]. Meanwhile, the non-canonical Wnt pathway is independent of β-catenin, instead being mediated through the FZD receptor (along with other co-receptors such as receptor like tyrosine kinase [RYK]) to transduce the signal to Dishevelled family proteins, leading to their activation [89].

In normal breast tissues, Wnt signaling is critical for mammary stem homeostasis due to maintaining the undifferentiated state of stem cells; it is therefore vital for breast development and tissue remodeling during pregnancy and lactation. Aberrant Wnt signaling is implicated in breast cancers [90]. For one, Wnt signaling is constitutively activated in basal breast cancer cells, affecting their self-renewal and differentiation [91]. Canonical Wnt signal transduction is frequently activated in TNBC, and intracellular accumulation of β-catenin is associated with poor survival in breast cancer patients [92]. Regulators of the Wnt signaling pathway, such as lymphoid enhancer-binding factor 1 (LEF1), cyclin D1, β-catenin, and TCF-4 are upregulated in $ALDH^+$ BCSCs. Treating 4T1 BCSCs with Wnt3a ligand induced Wnt/β-catenin signaling and transcriptional activity, while Wnt1 silencing decreased tumor sphere formation and the $CD44^+/CD24^-$ population *in vitro*, along with decreasing tumorigenesis and metastasis in xenografts [57]. Thyroid hormone receptor interactor 6 (TRIP6), an adapter protein involved in regulating the functions of CSCs, enhances stemness in breast cancer cells through activation of the Wnt/β-catenin pathway [93]. On the converse side, β-catenin silencing has been shown to reduce tumorigenesis *in vivo* and to suppress cancer stemness *in vitro* by decreasing the abundance of $ALDH^+$ breast cancer cells and the expression of stemness-related genes, including B lymphoma Mo-MLV insertion region 1 homolog (*BMI-1*) and MYC proto-oncogene, bHLH transcription factor (*c-Myc*). In TNBC cells, such silencing also impaired

formation of anchorage-independent colonies in soft agar assay and improved chemoresistance [94]. Finally, treatment of TNBC cells with WNT-targeting pharmacological agents modulates the expression of PD-L1, a ligand for the inhibitory immune checkpoint receptor PD-1, which is highly expressed in the stem cell compartment ($ALDH^+$ or CD44v6-positive) alongside WNT signaling-related genes. This indicates a role of Wnt signaling in TNBC-related immune escape [95].

There is increasing evidence showing that long non-coding RNAs (lncRNAs), a diverse class of transcribed RNA molecules that regulate gene expression at the post-transcriptional level, modulate CSCs and promote tumor progression. For example, the expression of *LncCCAT1* is highly upregulated in BCSCs and associated with poor patient outcomes. In MCF-7 and MDA-MB-231 breast cancer cells, this lncRNA enhances breast cancer cell proliferation, stemness, migration, and invasion by upregulating TCF4 through mechanisms associated with miR-204/211, miR-148/152, and Annexin A2, leading to activation of Wnt signaling; this finding suggests a complex crosstalk of Wnt signaling with microRNAs and long non-coding RNAs involved in regulating BCSCs [96]. The pleiotropic effects of Wnt signaling and its components in breast cancer initiation, progression, and the maintenance of different cancer subtypes remain to be elucidated, and deeper understanding of them is essential for developing BCSC-targeted therapies.

1.4.3 Hedgehog signaling

The Hedgehog (Hh) signaling pathway is involved in animal development and tissue homeostasis, and is associated with many solid tumors including pancreatic cancer, lung cancer, breast cancer, basal cell carcinoma, and hematological malignancies. Hh

family members include Sonic hedgehog (SHH), Indian hedgehog (IHH), and Desert hedgehog (DHH). Hh binds to the receptor Patched (PTCH1), a transmembrane protein that constitutively represses Hh signaling, to release its repression on the G protein-coupled receptor Smoothened (SMO). This derepression further activates transcriptional mediators of Hh responses, the GLI family proteins (GLI1/2/3). Regulation of the processing of these factors and their nuclear translocation plays a key role in activating Hh target genes such as cyclin D and E [97].

In cancer, this pathway plays roles in malignant transformation, proliferation, drug resistance, metastasis, and the expansion of cancer stem cells [98]. Hh signaling is known to drive oncogenesis, specifically resulting from mutations in components of Hh pathway, over-expression of ligands of the Hh pathway, and maintenance of CSC phenotype through regulation of stemness-related genes [99]. The pathway is significantly upregulated in luminal B and TNBC breast cancer subtypes [100]. An earlier study in mice showed that overexpression of *Gli1* under the MMTV promoter is sufficient to promote development of breast tumors expressing progenitor cell markers [101]. In addition, there is emerging evidence demonstrating the significance of Hh signaling in TNBC. In TNBC cells, overexpression of *Gli1* enhances tumor migration, invasion, vascularization, and metastasis by upregulating *MMP-11* and cysteine-rich angiogenic inducer 61 (*CYR61*) [102]. In mammospheres, PTCH, SMO, GLI1 and GLI2 are highly expressed, becoming downregulated upon differentiation. Activation of Hh signaling increases mammosphere forming efficiency (MFE) and size, effects mediated by the polycomb gene BMI-1. Hh signaling is also hyperactivated in the $CD44^{+}/CD24^{-}/Lin^{-}$ BCSC population [103]. In mammospheres of estrogen receptor-positive MCF7 breast cancer cells, components of the

Hh pathway (PTCH, SMO, GLI1 and GLI2) are highly expressed relative to monolayer cells; treatment with salinomycin, which targets CSCs, induced apoptosis and downregulated target genes of the Hh pathway (*c-Myc, Bcl-2, and Snail*) *in vitro* and reduced the tumor growth and expression of PTCH, SMO, GLI1 and GLI2 in xenograft tumors [104]. In basal-like breast cancer, increased expression of forkhead box C1 (FOXC1), an EMT-associated transcription factor, acts via activation of SMO-independent Hh signaling mediated by GLI2 to enrich CSC properties of the cancer, including $ALDH^+$ cell populations and mammosphere growth. Furthermore, expression of FOXC1 in TNBC cells confers resistance to anti-Hh drugs [105]. LncRNAs were demonstrated to regulate EMT-associated BCSC stemness through the growth arrest specific 1 (GAS1)-activated lncRNA-Hh pathway. The upregulated Hh signaling increased GLI1, SOX2, and OCT4 expression and MFE *in vitro* and tumorigenicity *in vivo*. Silencing lncRNA-Hh reversed these findings [106]. Hh signaling is also associated with chemoresistance in TNBC. Chemotherapy-induced drug resistance is mediated by GLI1 via upregulation of multidrug resistance protein 1 (MDR1) and breast cancer resistance protein (BCRP) [107]. Ultimately, activation of the Hh signaling pathway is well-documented as a poor prognostic indicator in both hormone receptor (+) breast cancer and TNBC. However, there are limited Hh-targeted therapies available. Selective inhibition of GLI and other targets might represent an effective strategy for impeding breast cancer development and the activity of cancer stem cells.

1.4.4 TGF-β signaling

Members of the transforming growth factor β (TGF-β) superfamily are implicated in regulating cellular behaviors encompassing cellular growth, maturation, hormonal and immune responses, differentiation, migration, cell death and fate determination [108]. This superfamily consists of 42 ligands including TGF-β, activins, Nodal, inhibins, bone morphogenic proteins (BMPs), and growth differentiation factors (GDFs) [108]. TGF-β itself has three mammalian isoforms, TGF-β 1-3. TGF-β signaling exhibits tissue specificity and distinctive affinities for TGF-β receptors (TβR1, TβR2, and TβR3). It is transduced through (1) the canonical SMAD pathway, which activates SMAD2 and SMAD3 to regulate TGF-β1–dependent genes, and (2) the non-canonical pathway, inducing ERK1/2 and PI3K/Akt signaling [109].

In cancer, TGF-β displays context-dependent dichotomous behaviors, being a tumor suppressor that inhibits cell cycle progression and promotes apoptosis or a tumor promoter that induces EMT and invasion [108]. Differential expression of TGF-β isoforms in breast cancer dictates molecular subtypes, progression, and patient outcome. Levels of the *TGF-β1* and *TGF-β3* isoforms are increased in breast cancer tissues and metastatic lymph nodes, with particular predisposition in ER+ and PR+ tumors, whereas the *TGF-β1* and *TGF-β2* isoforms are highly expressed in TNBC. Expression of TGF-β isoforms correlates with expression of TGF-β receptors [110]. Consistent with its tumor suppressor role, constitutive expression of *TGF-β1* in mammary epithelial cells of xenografts increased latency of tumor growth and decreased mammary cancer risk [111]. Similarly, TGF-β reduces the BCSC population and induces luminal differentiation [112]. Loss of

TGF-β-mediated tumor suppression in breast cancer is associated with downregulation of luminal markers and upregulation of basal markers [112]. In another example, transgenic expression of MMV-driven dominant-negative TβR2 (*DNIIR*) in female mice decreased tumor latency and induced spontaneous tumor formation and invasion [113]. In contrast, mammary epithelial cell-specific expression of TGF-β ligands or TβRs in xenograft tumors promotes lung metastasis, while attenuation of TGF-β signaling decreases metastasis [114]. These findings suggest a paradoxical role of TGF-β signaling in inhibiting tumor initiation while promoting metastasis.

During breast cancer development, activation of HER2/EGFR is reported to induce phosphorylation of SMAD3 through AKT, with its subsequent nuclear localization and the activation of genes implicated in EMT and cell migration. Inhibiting HER2/EGFR activity or SMAD3 disrupts TGF-β signaling-mediated EMT and migration but augments anti-proliferative activity [115]. Notably, TGF-β signaling is involved in the crosstalk between tumor cells and lymphatic endothelial cells during the process of dissemination in the lymphatic system. Expression of C-C motif chemokine receptor 7 (CCR7), which promotes lymph node metastasis, is increased via p38 MAPK signaling in transformed mammary tumor cells that have undergone TGF-β1–induced EMT. Simultaneously, TGF-β upregulated the production of C-C motif chemokine ligand 21 (CCL21) by lymphatic endothelial cells to promote the chemotactic migration of EMT cells expressing CCR7 in a paracrine manner. Pharmacological inhibition of CCR7 or p38 MAP kinases may be a plausible target for inhibiting TGF-β1-induced EMT and lymphatic spread [116]. In immortalized human mammary epithelial cells, TGF-β1-induced EMT generates stem cell-like cells that express EMT markers and have increased ability to form mammospheres,

colonies in soft agar, and xenograft tumors [117]. Meanwhile, $CD44^{+}/CD24^{-}$ BCSCs generated by TGF-β1-induced EMT are more resistant to radiation compared to their parental cells, mediated by upregulating antioxidant-related genes and reducing activation of death receptor pathways [118].

Accumulating evidence has implicated the epigenetic regulation of TGF-β signaling in breast cancer progression [119]. In TNBC, TGF-β1 inhibits miR-196a-3p and activates its downstream target gene neuropilin-2 to promote metastasis [120]. Meanwhile, miR-133b and miR-190 have been shown to inhibit TGF-β-induced EMT and metastasis by targeting *SMAD2*, indicating their roles as tumor suppressors and potential diagnostic biomarkers of breast cancer [121]. In mouse epithelial NMuMG cells, lncRNA-HIT mediates TGF-β-induced EMT and invasion by targeting E-cadherin; this long noncoding RNA is conserved in humans and elevated in invasive breast cancer. Attenuation of lncRNA-HIT resulted in decreased invasion, migration, and tumor growth [122]. Overall, due to the complexity of functional switches in TGF-β signaling, specific drugs targeting downstream signaling would be preferable as therapeutics, as they can be utilized without compromising other physiological functions of TGF-β.

1.4.5 STAT3 signaling

The transcription factor signal transducer and activator of transcription 3 (STAT3) is known to contribute to tumor cell proliferation, progression, metastasis, immune suppression, and stem cell self-renewal and maintenance [123]. The STAT family consists of seven highly conserved members, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6; all share structural and functional similarities. Each STAT-family protein

contains the following functional domains: an N-terminal domain (NH_2), a coiled-coil domain for binding with interactive co-regulators and transcription factors, a DNA binding domain specific for the interferon-gamma activated sequence in the promoter regions of specific genes, a linker domain, a Src homology-2 domain for dimerization and phosphorylation, and the C-terminal transcription activation domain that confers structural diversity among STAT family members. [124]. STAT3 is activated through binding of cytokines (IL-6 and IL-10) and growth factors (IGF, EGF, and FGF) to their corresponding receptors. Once the receptors are bound by the ligands, they undergo dimerization and activate Janus kinases (JAKs), which in turn phosphorylate tyrosine residues on the cytoplasmic tail of the cognate receptor; those residues interact with the SH2 domain of STAT3, resulting in STAT3 phosphorylation. Phosphorylated STAT3 translocates to the nucleus and regulates target gene transcription with the help of coactivators [125]. STAT3 overexpression is found in more than 40% of breast cancers, mainly in the TNBC subtype. Aberrant activation of STAT3 promotes breast cancer development by deregulating genes implicated in proliferation, angiogenesis, and EMT [126]. In TNBC, hypoxia induces an increase in the $CD44^{high}/CD24^{low}$ BCSC population and in chemoresistance by activating STAT3 signaling. Genetic knockdown of *STAT3* reverses the acquisition of stem-like features, which suggests a significant role of STAT3 in promoting the induction of cancer stemness by hypoxia [127].

Cytokines are known risk factors that induce inflammation and promote breast cancer progression. Oncostatin M (OSM), a member of the gp130 family of cytokines, has been implicated in inflammatory functions driving tumor aggressiveness and in increased STAT3 phosphorylation and STAT3-dependent IL-6 production, which promote breast

cancer progression. High expression of OSM correlates with poor breast cancer patient survival [128]. High levels of another cytokine, IL-35, are associated with poor prognosis in patients. Breast cancer cell-derived IL-35 inhibits conventional T (Tconv) cell proliferation and induces the cells to transform into IL-35-producing induced regulatory T (iTr35) cells by activating STAT1/STAT3, thereby promoting breast cancer progression [129].

Cancer-associated fibroblasts (CAFs) also play crucial roles in every stage of breast tumorigenesis and metastasis by inducing inflammation and repressing the antitumor immune response [130]. Histone deacetylase 6 (HDAC6), a member of the histone deacetylase family whose expression is elevated in CAFs of breast tumors, promotes an immunosuppressive microenvironment by upregulating *STAT3* and targeting prostaglandin E2/cyclooxygenase-2. Inhibition of HDAC6 in CAFs slows tumor growth and facilitates antitumor immunity [131].

MiR-124, a tumor suppressor that modulates breast cancer cell proliferation and invasion, is downregulated in breast cancer cells. Overexpression of miR-124 in TNBC decreased STAT3 and suppressed cell proliferation and invasion. Restoration of STAT3 expression reversed miR-124-mediated tumor cell invasion [132]. Similarly, miR-7 was demonstrated to act as a tumor suppressor by inhibiting breast cancer cell invasion and metastasis, decreasing BCSC populations, and reversing EMT in MCF-7 and MDA-MB231 cell lines. These miR-7-mediated effects occurred through targeting the oncogene *SETDB1*, which led to suppression of the downstream target *STAT3* as SETDB1 binds to its promoter and regulates its expression [133]. All told, STAT3 signaling is not simply

limited to a role in tumorigenesis but is also important in invoking the immune cell response. STAT3 will be a promising target for breast cancer prevention and therapy.

1.4.6 Other signaling in the regulation of BCSCs

Breast tumorigenesis is driven by aberrant regulation of cell signal transduction pathways owing to the accumulation of genetic and epigenetic changes over time. Apart from the aforementioned pathways, other significant signaling involved in BCSC enrichment and maintenance includes the Hippo, PI3K/Akt/mTOR and BMI-1 pathways [40]. Dysregulation of any of these individual pathways or of the interplay between them poses a risk of developing breast cancer. In addition, the receptor tyrosine kinase (RTK) class of specialized cell surface receptors respond to environmental cues by relaying appropriate signals in the tumor cell; these include epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), and AXL receptor tyrosine kinase (AXL). RTKs play a multifaceted role in breast cancer development, sharing common downstream pathways such as MAPK, NFκB, PI3K/Akt, and JAK/STAT signaling; these crosstalk with other key signaling pathways relevant to the regulation of angiogenesis, metastasis, and maintenance of BCSCs. Mutation in or overexpression of RTKs has been observed in different stages of breast cancer to lead to constitutive activation of various signal transductions that promote BCSCs and chemoresistance [134].

1.5 Signature of cancer stem cell transcription factors in breast cancer

Pluripotency in embryonic stem cells (ESCs) is regulated by a well-characterized core transcriptional network. The circuitry of this network constitutes major transcription

factors of pluripotency, signal transduction machinery, and epigenetic regulators. In human embryonic stem cells, OCT4, NANOG, and SOX2 function as master regulators of pluripotency and self-renewal properties while inhibiting differentiation to control cell fate [135]. Pluripotency can be induced in adult somatic cells, as evidenced by reprogramming of adult fibroblast cells into pluripotent stem cells with characteristic features of ESCs using the OSKM transcription factors (OCT3/4, SOX2, c-Myc, and Kruppel-like factor 4 [KLF4]) [136]. Astrocytes transduced with the H-ras oncogene or with OSKM factors undergo reprogramming into progenitor cells, resulting in tumorsphere formation. When these tumorspheres are transplanted as xenografts, they form heterogeneous tumors, suggesting an interplay between tumorigenicity and pluripotency [35]. It can be assumed that CSCs share characteristics with ESCs. The pluripotency transcription factors *OCT4, NANOG, and SOX2* are upregulated in human cancers, including breast cancer, glioma, melanoma, and prostate cancer, and their overexpression in tumors is associated with poor differentiation, stem-like phenotype, and inhibition of apoptosis [31].

1.5.1 OCT4

OCT4, a homeodomain transcription factor of the Pit-Oct-Unc family, is one of the most important transcription factors governing pluripotency [137]. The human *OCT4* gene has three transcript variants (*OCT4A*, *OCT4B*, and *OCT4B1*) and four protein isoforms (OCT4, OCT4B-190, OCT4B-265, and OCT4B-164). Each alternative transcript variant and isoform demonstrates diverse expression patterns and functions. Among them, OCT4A is responsible for maintenance of stemness in pluripotent embryonic stem cells [138]. The OCT4 protein consists of three domains: the central POU domain for DNA binding, the N-

terminal transactivation domain, and a cell type-specific transactivation C-terminal domain. OCT4 controls the expression of target genes by recognizing and binding to DNA regulatory regions through an octamer motif (AGTCAAAT) or by recruiting other transcription factors to regulate a specific set of genes [139]. Analysis of 319 cases of invasive breast cancer revealed that OCT4 expression is directly correlated with ALDH1 expression, but not with EMT markers. In hormone receptor-positive breast cancer, OCT4 can be used a prognosis indicator for poor clinical outcome and tamoxifen resistance [140].

PD-L1, a T-cell inhibitory molecule with immunomodulatory function, regulates breast cancer stemness via modulating OCT4 and NANOG. In breast cancer, its expression is associated with EMT, chemoresistance, and maintenance of stemness. *PD-L1* knockdown inhibits AKT phosphorylation and mTOR activity, with downstream reduction of OCT4 phosphorylation at T235 and therefore of OCT4 activity [141]. Another regulator of OCT4 is the E3 ubiquitin ligase carboxy terminus of HSP70-interacting protein (CHIP), which was demonstrated to mediate its proteasomal ubiquitination at lysine 284 through microarray analysis of mammospheres derived from MDA-MB231 and MCF7 cells. *CHIP* overexpression decreased OCT4 stability and BCSC populations, while *CHIP* depletion promoted breast tumor and lung metastasis in xenografts. This finding suggests that CHIP-induced post-translational modification of OCT4 is important in maintenance of BCSCs [142].

Although OCT4 is well studied in the context of stemness maintenance, its role in metastasis still remains controversial. Overexpression of *OCT4* in MDA-MB231 and 4T1 breast cancer cell lines induced E-cadherin while suppressed cell migration and invasion *in vitro* and lung metastasis *in vivo* [143]. The inhibitory effect of OCT4 on metastasis is

mediated through downregulation of Rho family GTPase 1 (*RND1*) by binding to its promoter region [143]. In contrast, a previous study from the same group showed downregulation of *OCT4* in MCF-7 cells to promote cell migration and invasion by inducing EMT (decreased E-cadherin expression and increased alpha-smooth muscle actin expression) [144].

1.5.2 SOX2

SOX2 is a member of the Sox (SRY-related HMG box) family member of transcription factors with a single high-mobility group DNA-binding domain. It is recognized as a key player in the regulation of early embryonic development, maintenance of undifferentiated ESCs, and cell fate determination, and its expression is dysregulated in several cancer types, including breast, prostate, brain, and lung cancers. SOX2 is additionally involved in tumorigenesis, drug resistance, poor prognosis, and metastasis, indicating a major role in cancer and positioning it as an attractive therapeutic target [145]. Overexpression of *SOX2* in breast cancer cells increased mammosphere formation, while its knockdown suppressed mammosphere formation and also delayed tumor formation in xenograft tumor initiation models. Mechanistically, *SOX2* overexpression was induced through the activation of a distal enhancer of *SOX2* promoter, the same element that natively regulates *SOX2* transcription in pluripotent stem cells [146]. In ER-positive breast cancer patients, *SOX2* expression is associated with poor prognosis and endocrine treatment failure, and SOX2 promotes tamoxifen resistance via activation of Wnt signaling [147]. It also targets SOX9 to regulate luminal progenitor cells and Wnt signaling activity [148]. In TNBC cases, SOX2 is implicated in BCSC chemoresistance through modulation

of *TWIST1*. Silencing *SOX2* increased paclitaxel sensitivity and diminished stemness and *TWIST1* expression. This illustrates the significance of SOX2 as a connector between pluripotency, chemoresistance, and the EMT axis [149]. Likewise, *SOX2* knockdown in MCF-7 cells decreased mammosphere formation, $CD44^{+}/CD24^{-}$ subpopulation, ALDH+ population, viability *in vitro*, and tumorigenicity *in vivo* [147].

1.5.3 NANOG

NANOG is a homeodomain protein found in undifferentiated mammalian ESCs and pluripotent cells. Endogenous Nanog drives ESC self-renewal by maintaining the level of OCT4, which is integral to ESC function. Although Nanog is absent in differentiated cells, its abnormal expression is reported in human cancers including prostate cancer, hepatocellular carcinoma, glioblastoma, colon cancer, and breast cancer. Expression of Nanog is associated with stemness, self-renewal, and tumorigenesis [150]. When coexpressed with Wnt-1 in the mouse mammary gland, Nanog promotes mammary tumorigenesis and metastasis. Ectopic expression of Nanog in MCF7 cells enhances colony formation, migration, and invasion *in vitro* and tumor growth *in vivo* [151]. Meanwhile, silencing Nanog reduces colony formation, cell proliferation, and invasion; it furthermore downregulates the cell cycle regulators cyclin D1 and c-Myc, leading to cell cycle arrest at G0/G1 [152]. In BCSCs, Nanog and OCT4 modulate TGF-β-mediated EMT; their induction promoted invasion while knockdown of both inhibited CSC migration in vitro [153]. In addition, Nanog confers drug resistance in MCF-7 breast cancer cells through STAT3-mediated activation of *MDR1* [154], and in breast ductal carcinoma, its expression has statistically significant relationship with tumor grade, lymph node metastasis, and

disease staging [155]. Tissue microarray analysis revealed that breast cancer patients with strong Nanog expression have significantly lower disease-free survival and overall survival rates than those with weak expression [156].

1.5.4 KLF4

KLF4 is a member of the highly conserved Kruppel-like zinc finger transcription factor family, and is one of the four major transcription factors of pluripotency. It plays diverse roles in physiology and disease, with functions in cell cycle regulation, proliferation, apoptosis, differentiation, somatic cell reprogramming, and pluripotency [157]. KLF4 is differentially expressed in human cancers, and furthermore is bifunctional; it can act as either tumor suppressor or oncogene depending on the tissue, tumor type, and staging [157]. In breast cancer tissues, its protein expression is correlated with pathological type, histological grade, and lymph node involvement; low-level expression is found in normal breast epithelium, while increased expression is detected in neoplastic cells and prior to invasion [158]. In estrogen-dependent breast cancer, KLF4 acts as a tumor suppressor by regulating the transcriptional activity of ERα, specifically binding to its DNA-binding region and preventing it from binding to estrogen response elements in promoter regions [159]. It is also self-regulating, in that the isoform KLF4α antagonizes the function of KLF4 and stimulates breast cancer cell proliferation by binding and retaining KLF4 in the cytoplasm, opposing its regulatory activities in the nucleus [160]. KLF4 is highly expressed in BCSCs from primary mammary tumor and breast cancer cell lines. In the MCF7 and MDA-MB-231 cell lines, *KLF4* knockdown decreased the population of $ALDH1^+$ progenitor cells; it furthermore suppressed cell migration, invasion,

and mammosphere formation *in vitro* and tumorigenesis *in vivo* [161]. In BCSCs, KLF4 and the androgen receptor have been demonstrated to mediate stem cell phenotype; this effect is negatively regulated by dual specificity tyrosine phosphorylation regulated kinase 2 (DYRK2), a protein kinase that controls EMT via Snail degradation. Downregulation of *DYRK2* promotes KLF4 expression and cancer stem-like properties [162].

1.5.5 MYC

MYC is a dimeric transcription factor of the basic helix-loop-helix (bHLH) DNA-binding protein superfamily that regulates a broad range of biological processes such as cell proliferation, differentiation, growth, and apoptosis; it is also implicated in embryonic stem cell self-renewal and pluripotency [163]. The MYC promoter is a downstream effector target of self-renewal pathways such as the Notch, Wnt, NF-κB and TGF-β signaling pathways [164]. Of the three MYC family members l-MYC, c-MYC, and n-MYC, the latter two play crucial roles in the maintenance of pluripotency. Co-deletion of both transcription factors in ESCs and in induced pluripotent stem cells (iPSCs) led to destabilization of pluripotency and spontaneous differentiation into primitive endoderm [165].

As an important transcription regulator in ESCs, MYC also displays similar regulatory role in CSCs [166]. In fact, MYC was first recognized as one of the most potent oncogenes, inducing neoplastic transformation of target cells and a wide variety of tumors [167]. Transient overexpression of *MYC* in Rat1A cells evoked genomic instability and increased tumorigenicity [168]. In breast cancer, MYC amplification is associated with disease progression; additionally, its expression is higher in TNBC than in other subtypes.

MYC overexpression in the BRCA1-deficient TNBC subtype is associated with poor prognosis [169]. Meanwhile, targeting MYC in TNBC with triptolide (C1572), a small-molecule natural product, depletes cancer-stem like cells via a proteasome-dependent mechanism [170]. In combination with MCL1 apoptosis regulator, BCL2 family member (MCL1), MYC promotes chemoresistance of CSCs in TNBC by increasing mitochondrial oxidative phosphorylation and the generation of reactive oxygen species [171]. Additionally, *c-MYC* is the effector target of the tumor suppressor gene p53 in mammary stem cells; loss of p53 function is implicated in the development of cancers. In breast tumors, p53 mutation activates *c-MYC*, leading to maintenance of cancer stemness features and expression of a mitotic gene signature, which correlates with breast cancer aggressiveness and poor prognosis [172]. Transducing *MYC* in human mammary epithelial cells induces luminal epithelial morphology changes, spheroid formation, and dedifferentiation into progenitor-like states. MYC-driven epigenetic changes are mediated through suppression of lineage-specific transcription factors and activation of *de novo* enhancers, determined by hyperactivation of the Wnt pathway, which further drives transcriptional activation of oncogenic pathways [173].

1.6 Role of tumor microenvironment in BCSCs

The mammary gland is a network of interconnected ductal and alveolar structures composed of mature luminal and myoepithelial cells. The regenerative bi-potential property of the mammary gland is a function of mammary stem cells (MaSCs), which are characterized by the self-renewal and differentiation capacity necessary to maintain the structural integrity of the breast [174]. In the normal mammary epithelial differentiation

hierarchy, MaSCs are located at the apex, responsible for maintaining long-term expansion of stem cell pool; accordingly, they are susceptible to neoplastic transformation as a consequence of the accumulation of genetic and epigenetic alterations over time [175]. Similar to stem cells in other tissues, MaSCs reside in a microenvironmental stroma comprised of cellular components such as fibroblasts, immune cells, adipocytes, epithelial and endothelial cells, and non-cellular components including extracellular matrix, soluble growth factors, and cytokines. MaSC function is regulated by the epithelial-stromal interactions initiated by growth factors and cytokines through autocrine and paracrine signaling [176].

Studies have suggested that breast cancer is comprised not only of neoplastic cells but also of the surrounding tumor microenvironment (TME). The TME plays a significant role in driving malignant phenotype and is implicated in promoting breast cancer stemness and tumor progression [177]. Within the TME, breast cancer cells are influenced by an array of cytokines (such as IL-6, IL-8, tumor necrosis factor α [TNF-α], and oncostatin-M), cell types (mesenchymal stem cells, cancer-associated fibroblasts, tumor-associated leukocytes, tumor-associated macrophages, and adipocytes), subcellular elements (exosomes and microvesicles), and chemical and physical factors (such as hypoxia, pH, and nutrient availability) [178]. MaSCs and BCSCs share common molecular traits in terms of cellular plasticity and self-renewal. Both populations share common markers and marker combinations such as CD44, ALDH, leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), protein C receptor (PROCR), $Lin^{-}CD24^{+}CD29^{h}$, $Lin^{-}CD24^{+}CD49f^{h}$, $EpCAM^{-/low}/CD49f^{h}$, $CD24^{h}/CD49f^{h}/DNER^{h}/DLL1^{h}$; these have been identified as promoting tissue homeostasis, BCSC maintenance, and breast cancer progression and serve

as predictors of poor clinical outcome in breast cancer patients [179]. Both MaSCs and BCSCs rely upon conserved signaling pathways such as Notch and Wnt for mammary development, homeostasis, and maintenance of stem cell phenotype [180]. In addition, transcription factors and pluripotency factors known to regulate MaSCs are implicated in the regulation of BCSC activities [179]. These findings suggest that BCSCs may have transformed from MaSCs; however, direct experimental evidence for this relationship remains to be elucidated.

Within the TME, inflammatory cytokines secreted by various cell types can create an inflammatory niche that favors the self-renewal and survival of BCSCs, thereby mediating chemotherapeutic resistance [181]. IL-6, a multifunctional cytokine, is linked to signaling pathways implicated in BCSC survival and proliferation [182]. In breast cancer cells, IL-6 secreted from adipocytes activates IL-6/STAT3 signaling to induce EMT. Inhibiting IL-6 signaling blocks proliferation, migration, and invasion, and furthermore changes the expression of EMT-regulating genes [183]. In an experimental model using MDA-MB-231 and MDA-MB-453 breast cancer cells and control cells, IL-6 secreted from non-stem cells converted non-CSCs to CSCs by activating the JAK1/STAT3/OCT4 signal transduction pathway and consequently produced mammospheres and xenograft tumors [184]. Moreover, IL-6 and hypoxia in the mammary TME induce CCAAT enhancer binding protein delta (C/EBPδ), a pro-tumorigenic and pro-metastatic transcription factor, which in turn activates HIF-1α and IL-6 signaling in a positive feedback loop. C/EBPδ engagement with both hypoxia and IL-6 synergizes TME factors promoting CSC-associated phenotypes in breast cancer The IL-8 receptor C-X-C motif chemokine receptor 1 (CXCR1 or IL8RA) is consistently expressed in $ALDH^+$ BCSCs, and treatment with

recombinant IL-8 increased cancer stem cell self-renewal, mammosphere formation, CSC invasion, and metastasis [185]. In TNBC, IL-8 enhances mesenchymal features, cancer stemness, chemoresistance, and recruitment of immune-suppressive myeloid-derived suppressor cells, while its neutralization reverts these features and helps with immune-mediated tumor eradication [186]. Similarly, other cytokines—TGF-β and TNF-α—regulate BCSC activity in the TME by upregulating various transcriptional factors and BCSC markers [187].

The TME also constitutes several tumor-associated immune cell populations, such as tumor-associated macrophages (TAMs), T and B lymphocytes, neutrophils, and dendritic cells. These immune cells communicate with nearby cells through secreted cytokines and growth factors that permeate the CSC niche to promote self-renewal of stem cells and development of cancer [188]. Cytokines and chemokines secreted from tumor cells and immune cells recruit monocytes from the mononuclear phagocytic immune system in the blood. Notably, macrophages show remarkable plasticity and assume multiple phenotypes in response to environmental cues—mainly the classically-activated macrophage M1 (pro-neoplastic) and alternatively-activated M2 (anti-neoplastic/immune-suppressing) types [189]. When recruited to a TME and exposed to conditions such as inflammation, local anoxia, and increased lactic acid, monocytes undergo differentiation to become TAMs. TAMs have been demonstrated to comprise a majority of the cell population in the TME and to play significant functional roles in cancer progression, regulation of angiogenesis, and immune evasion [188]. In breast cancer, incubating MCF-7 cells with supernatant from M1 macrophages induced in a paracrine manner EMT and CSC phenotypes such as increased expansion of $CD44^{+}/CD24^{-}$ or $ALDH1^{+}$

subpopulations, chemoresistance, mammosphere formation, and tumor growth. This process is triggered by cytokine signaling in the TME and mediated through STAT3/NF-kB pathways or the LIN-28B-LET-7-HMGA2 axis. These findings underscore the significance of TAM mediation of the EMT-induced CSC state [190]. Similarly, inflammatory breast cancer releases monocyte recruitment and macrophage polarizing factors that convert monocytes to M2 macrophages. These M2 macrophages in turn secrete IL-8 and GRO chemokines to activate STAT3 signaling, promoting EMT and CSC-like states in this highly aggressive breast cancer type [191].

Another study demonstrated that the interactions between CSCs and EMT induced membrane proteins of surrounding cells—in particular the Thy-1 cell surface antigen (CD90 or THY1) and EPH receptor A4 (EPHA4), which serve as anchors and mediate the interactions of TAMs and CSCs through their corresponding counter-receptors in breast cancer cells. This juxtacrine signaling facilitates tumor outgrowth and activates EPHA4 receptors on the tumor cells, which further stimulates SRC and NF-kB pathways, resulting in cytokine production that maintains stem-cell state, ultimately suggesting a complex interaction between CSCs and the TME to sustain tumor survival [192].

Hypoxia is a hallmark of the TME niche. Recent evidence has shown that a hypoxic microenvironment maintains the stem cell phenotype of normal stem cells as well as of CSCs. Impaired oxygen in the TME additionally enhances malignant progression and is associated with poor patient survival [193, 194]. Hypoxic tumor cells harvested from xenografts are enriched with a $CD44^{+}CD24^{-}$ CSC subpopulation associated with self-renewal and tumorigenic potential, and hypoxia-induced phosphorylation in the PI3K-AKT pathway maintains CSC phenotype [193]. Mechanistically, the cellular

transcriptional response to hypoxia is mediated by hypoxia-inducible factors (HIFs). In basal-like breast cancers, hypoxia induces HIF-1-dependent expression of the adenosine A2B receptor (*A2BR*). *A2BR* expression is likewise increased in mammospheres and $ALDH^+$ breast cancer cells. The A2BR protein mediates BCSC enrichment through the A2BR-PKCδ-STAT3 signaling pathway, which leads to transcriptional activation of *IL-6* and Nanog [195]. Similarly, chronic hypoxia in breast cancer cells increased *HIF-2α* expression, rendering the cells resistant to Paclitaxel. *HIF-2α* overexpression likewise promoted stem-cell associated phenotype, increased expression of cancer stem cell markers, and activation of the Notch and Wnt pathways [196]. Given that BCSCs are maintained and enriched in a hypoxic TME, it is plausible to develop therapeutics that inhibit breast cancer progression through targeting HIFs and reversing hypoxia.

1.7 Epithelial-mesenchymal transition and BCSCs

In normal embryonic development, conversion from epithelial to mesenchymal phenotype is driven by the developmental differentiation program called the epithelial-mesenchymal transition (EMT). In addition to a role in embryological processes, mounting evidence suggests that EMT transdifferentiation processes are hijacked in pathological conditions such as cancer and metastasis [197]. EMT is a dynamic process initiated by several intracellular signaling pathways, including TGF-β, Notch, Wnt, and Hedgehog; these are activated upon the binding of ligands and mitogenic growth factors (EGF, FGF, HGF, and PDGF) secreted from the tumor microenvironment to their cognate receptors, resulting in activation of receptor tyrosine kinases [198]. These signaling events further activate EMT-inducing embryonic transcription factors (EMT-TFs) to initiate EMT.

Notable EMT-TFs include the Snail, Slug, Zeb, Twist, Gata3, Foxc2, GSC, and Prrx families [199]. Hallmarks of EMT are loss of epithelial polarity; loss of expression of epithelial markers such as E-cadherin; gain of expression of mesenchymal markers such as vimentin, fibronectin, and α-smooth muscle actin; and increased activity of matrix metalloproteinases [200].

During developmental EMT, epithelial cells lose their polarity and undergo cytoskeletal rearrangement to convert to mesenchymal phenotype. Molecular and biochemical changes that underlie this phenotypic conversion enable the polarized cells to migrate away from their epithelial layer of origin to distant organs [201]. In the context of cancer, cells with mesenchymal traits can permeate the endothelial barrier to enter the bloodstream and lymphatic system, either collectively or as individual cells. Once they reach remote organs, those cells undergo mesenchymal-epithelial transition (MET) to invert to epithelial phenotype and establish a metastasis. It is worth noting that cancer cells displaying heterogeneous phenotypes and malignant potential lose most of their epithelial traits during progression [202]. In an animal model of breast cancer metastasis, EMT lineage tracing revealed that pre-EMT cancer cells expressed EMT-related phenotypes and played a predominant role in the establishment of lung metastases, while post-EMT cells were capable of MET inversion and supporting invasion and angiogenesis [203]. Recent studies have demonstrated that cells in a hybrid epithelial/mesenchymal state are more tumorigenic and apoptosis-resistant compared to those of either distinct epithelial or mesenchymal phenotype [199].

EMT-TFs are mediators of cellular plasticity in development and cancer. The EMT-TF ZEB1 and miR-200 family members participate in a reciprocal feedback loop to

regulate cellular plasticity in cancer progression by inducing stem-cell properties [204]. EMT-TFs such as SNAIL and SLUG bind to the E-box sequences located in the promoter region of E-cadherin, thereby regulating its transcription. Loss of E-cadherin in tumors induces multiple EMT-TFs, including TWIST and ZEB1, to promote invasion and metastasis [205]. Notably, activation or suppression of a single EMT-TF in cancer cells is sufficient to induce partial EMT, metastasis, and chemoresistance [206]. Additionally, EMT-TFs modulate the cytokinome and inflammasome of cancer cells undergoing EMT to trigger the recruitment of tumor-associated immune cells and thereby remodel the tumor microenvironment [207, 208].

In hormone-dependent breast cancer, the estrogen receptor plays a crucial role in mediating EMT. Silencing *ER-α* induces EMT and promotes cancer cell migration and invasion, and loss of *ER-α* abolishes the gene and protein expression of EGFR and HER2 receptor tyrosine kinases and matrix macromolecules [209]. Twist 1 expression in breast cancer has been demonstrated to induce other EMT-TFs and partial EMT, and is required for basal-like tumor progression and metastasis [210]. Notably, basal-like breast cancer is the most aggressive and poorly differentiated of breast cancer subtypes, and has high metastatic ability. Immunohistochemistry studies revealed that basal-like breast cancer epithelial cells attain mesenchymal characteristics in the process of de-differentiation and express mesenchymal markers, indicating a role of EMT in breast cancer progression [211]. In clinical metaplastic breast cancer and ductal carcinoma of the breast, transcriptional profiling showed that in the metaplastic subtype, which lacks *ER-α* and *HER-2/neu* expression, genes functionally related to EMT and the extracellular matrix

were upregulated and those encoding proteins associated with maintaining epithelial phenotype were downregulated [212].

Induction of EMT in cancer cells by EMT-TFs promotes cancer stemness, chemoresistance and immune evasion [213]. Ectopic expression of either EMT-inducing transcription factor (TF) Twist or Snail in immortalized human mammary epithelial cells conferred mesenchymal traits and stem-like features, including mammosphere formation [117]. Similarly, co-expression of Slug and *SOX9* in nonmetastatic MCF7ras breast cancer cells induced partial EMT and promoted tumorigenesis and metastasis *in vivo* [214]. ShRNA-mediated inhibition of E-cadherin in transformed HMLER breast cancer cells activates EMT and prompts an increase in the $CD44^{high}/CD24^{low}$ cell population [215]. As previously mentioned, $CD44^{+}/CD24^{-}$ identifies mesenchymal-like BCSCs, and $ALDH^{+}$ identifies epithelial-like BCSC populations. The $CD44^{+}/CD24^{-}$ subpopulation is enriched with EMT-related genes—vimentin, *ZEB1*, *ZEB2*, β-catenin, and *MMP9*. In contrast, the $ALDH^{+}$ subpopulation is enriched with cadherin, occludin, claudins, and desmoplakin. However, cellular plasticity exists between the EMT- and MET-like CSC states in breast cancer cell lines, producing heterogeneous populations in culture [60].

It is reported that the $CD44^{+}/CD24^{-}$ BCSC population in TNBC is comprised of two distinct epithelial and mesenchymal subpopulations distinguished by differential expression of two α6-integrin isoforms: *α6A* and *α6B*. The α6Bβ1 splice form is regulated by epithelial splicing regulatory protein 1 (ESRP1), an RNA-splicing factor that also controls the splicing of CD44 isoforms, promotes BCSC function, promotes mammosphere, formation and initiates tumor formation in non-obese diabetic mice [216]. A number of other regulators have been studied in the context of TNBC. Expression of the

EMT activator *ZEB1* is high in mesenchymal TNBC cells. ZEB1 represses stemness-inhibiting microRNAs (miR200 family members), resulting in activation of stem cell factors and promoting migration of cancer stem cells [217]. It also suppresses the expression of *TAp63α*, an epithelial transcription factor, which suppression promotes expression of integrin-β4 (*ITGB4*). ITGβ4 is associated with poor relapse-free survival in TNBC patients who received chemotherapy [218]. In basal-like breast cancer, Twist 2 upregulates the expression of aldo-keto reductase family 1 member B (*AKR1B1*), an arachidonic acid-related enzyme, to activate NF-kB. This in turn upregulates Twist 2 in a positive feedback loop to induce EMT and stem cell-like properties; thus, *AKR1B1* expression promotes breast tumor progression and metastasis [219]. FOXC2, a mesenchymal transcription factor, is highly expressed in basal-like breast cancer, and is known to participate in tumor progression by directly inducing EMT and through cross-talk with other EMT pathways. Overexpression of *FOXC2* in transformed human mammary epithelial cells induces CSC properties and promotes EMT and metastasis [220]. *FOXC2* is in turn transcriptionally regulated by FOXF2, both of which serve as prognosis predictors in TNBC [221].

The interplay between pluripotency transcription factors and EMT/MET in breast cancer has become increasingly recognized over the years. Simultaneous overexpression of *OCT4* and Nanog in $CD44^{+}/CD24^{-}$ BCSCs increased expression of vimentin, Slug, Snail, and N-cadherin and reduced expression of E-cadherin and CK-18. Perturbation of *OCT4* and Nanog expression modulates TGF-β induced EMT gene expression and inhibits the invasiveness of CSC *in vitro* [153]. Aurora B, a serine/threonine kinase highly expressed in basal-like breast cancer, induces EMT to promote breast cancer metastasis by

phosphorylating OCT4, which subsequently mediates Oct4/AKT/GSK3β/Snail1 signaling. Knockdown of Aurora B suppresses this signaling pathway and reverses EMT and metastasis [222]. In MCF-7 cells, SOX2 suppresses invasiveness by binding to the promoter region of Twist 1. However, this mechanism depends on the transcriptional status of *SOX2* [223]. Similarly, KLF4 maintains epithelial phenotype in mammary epithelial cells, and its silencing induces EMT, resulting in loss of E-cadherin mRNA and protein. Overexpression of *KLF4* in MDA-MB-231 breast cancer cells restored E-cadherin expression and inhibited migration and invasion [224]. Mechanistically, KLF4 binds to the promoter region of E-cadherin to regulate its expression, and does so in direct competition with ZEB2 [225]. Ultimately, the multitude of EMT functions linked to cancer stemness, metastasis, and chemoresistance underscores the importance of developing EMT-targeted therapies to enhance the efficacy of other therapeutic modalities.

1.8 Epigenetic regulation of BCSCs

Cellular plasticity is a signature of stem cells on account of their sustained self-renewal and differentiation. In the case of EMT, the exhibited plasticity is maintained by reversible epigenetic mechanisms including DNA methylation, histone modifications, and microRNAs; these factors are critical for transcriptional regulation during progenitor differentiation. Dysregulation of epigenetic modifications induced by the tumor microenvironment can influence gene transcription and support oncogenic reprogramming in cancer cells [226].

In DNA methylation, addition of a methyl group at position C-5 of a cytosine regulates gene expression by engaging with protein complexes that repress gene expression

or hinder the binding of transcription factors to DNA [227]. Human mammary epithelium exhibits unique gene profiles in terms of both histone modifications and DNA methylation, demonstrating the epigenetic function of DNA methylation in lineage commitment [228]. Furthermore, promoter methylation that acts to suppress genes implicated in pluripotency and self-renewal is indispensable for mammary epithelial differentiation. For example, genes related to stemness are hypermethylated in differentiated luminal $CD24^+$ human mammary epithelial cells, but hypomethylated in progenitor $CD44^+$ cells, which express transcription factors implicated in stem cell function [229]. In tumorigenesis, aberrant *de novo* DNA methylation of CpG island promoters catalyzed by DNA methyltransferases (DNMT3A and DNMT3B) is associated with repression of tumor suppressor genes. In a meta-analysis of breast cancer patients, promoter methylation of *BRCA1*, a tumor suppressor gene that accounts for 30-40% of hereditary breast cancer, was associated with breast cancer lymph node metastasis and triple negative phenotype [230]. Similarly, bisulfite sequencing analysis of sporadic breast cancer cells and peripheral blood lymphocytes demonstrated aberrant cytosine methylation of the *BRCA1* promoter CpG island when compared to normal breast cells [230]. Overall, dysregulation of DNA methylation patterns can help predict responsiveness to endocrine therapy in breast cancer patients [231]. Methylation patterns can also distinguish stem cells, as differentially methylated regions (DMRs) in BCSCs are more hypomethylated than in non-BCSCs. This hypomethylated DMR signature is enriched in genes related to TGF-β signaling and confers worse prognosis than in cases featuring a non-BCSC signature [232]. BCSCs are also characterized by downregulation of homeobox C8 (*HOXC8*), a regulator of cell fate during development; this altered expression is associated with DNA methylation of the

gene's promoter. Upregulation of *HOXC8* prompts a reduction in the $CD44^+/CD24^+$ population and in chemoresistance along with an increase in $CD24^+$ differentiated cells, suggesting *HOXC8* as a possible suppressor of stemness and a regulator of phenotype transformation [233]. DNA methylation is additionally implicated in aspects of the antigen processing machinery. The immune evasion of Aldefluor+ BCSCs is epigenetically regulated through DNA methylation in the promoter of transporter associated with antigen processing (*TAP*). *TAP* is downregulated in BCSCs, and treatment with the DNA demethylation agent decitabine reverses this altered expression [234]. Finally, in addition to local promoter methylation, global hypomethylation in the early stage of tumorigenesis contributes to genomic instability and malignant transformation. In breast cancer, this hypomethylation is associated with gene repression and repressive chromatin formation [235].

Histones are core components of nucleosomal subunits, around which is wrapped a 147-base-pair segment of genomic DNA. Histones are rich in arginine and lysine and are subject to post-translational modifications such as acetylation, methylation, and phosphorylation. These modifiers induce chromatin remodeling that regulates gene transcription programs implicated in normal development and tumor differentiation. In most species, methylation and acetylation of lysine and arginine residues in the tails of histones H3 and H4 are well-characterized and have been linked to gene activation, gene repression, and DNA damage response. These modifications are respectively regulated by methyltransferase "writers"/demethylase "erasers" and histone acetyltransferases (HATs)/histone deacetylases (HDACs) [236]. In BCSCs, overexpression of epigenetic modifiers is involved in promoting the expression of genes facilitating plasticity. In

particular, HDAC1 and HDAC7 are overexpressed in BCSCs and necessary to maintain CSC phenotype. HDAC7 targets histone 3 lysine 27 (H3K27ac) at transcription start sites and super-enhancers (SEs) and represses the transcriptional activity of SE-associated oncogenes including *c-MYC*, *CD44*, Slug, cyclin dependent kinase inhibitor 1B (*CDKN1B*), vitamin D receptor (*VDR*), SMAD3, and X-box binding protein 1 (*XBP1*) [237]. Members of the histone lysine demethylase KDM4 subfamily (A, B, C and D) are deregulated in cancer and play roles in controlling chromatin structure, cellular transformation, and gene expression. *KDM4-A* and *-D* are overexpressed in basal-like breast cancer, while *KDM4B* is amplified in ER^+ luminal B breast cancer. In basal breast cancer, targeting KDM4 demethylases with the small-molecule inhibitor NCDM-32B affected cell growth pathways, evidenced by inhibiting cell viability and anchorage-independent growth in soft agar [238]. Similarly, KDM7A is essential in BCSC maintenance due to upregulating the stemness-associated factors *KLF4* and *c-MYC*. Silencing *KDM7A* reduced the BCSC population *in vivo* and *in vitro* and promoted apoptosis in breast cancer cells [239]. Also relevant to stemness maintenance is protein arginine methyltransferase 5 (*PRMT5*), which catalyzes demethylation of arginine residues and upregulates *NANOG* and *OCT4* expression in embryonic stem cells. In breast cancer, increased *PRMT5* expression is associated with poor clinical outcome. Depletion of *PRMT5* reduced BCSC populations and xenograft tumor growth, suggesting a role of PRMT5 in epigenetic control of BCSC maintenance [240].

MicroRNAs (miRNAs) are small non-coding RNA molecules of less than 25 nucleotides in length that regulate post-translational gene expression via mRNA degradation or translational repression [241]. They regulate differentiation and can act as

oncogenes or tumor suppressors depending on their targets. Dysregulation of miRNAs is observed in human cancers, with effects on cell growth, proliferation, apoptosis resistance, and metastasis. [242].

1.9 BCSCs and drug resistance

Tumor relapse in breast cancer has been attributed to drug-resistant CSCs, and the persistence of CSCs after chemotherapy pinpoints this population as an 'ultimate target' that must be eliminated to eradicate cancer. Deciphering the mechanisms by which these cells resist conventional chemotherapies, interact with the TME, escape immune response, and undergo metabolic adaptation is of paramount importance for developing BCSC-targeted therapies. BCSCs share many features of normal stem cells and modulate a multitude of drug resistance mechanisms, including overexpression of drug efflux pumps (e.g. ATP-binding cassette family members ABCG2, P-gp, ABCC1, ABCB5, etc.), enhanced DNA repair activity, increased scavenging of reactive oxygen species, activation of anti-apoptotic proteins, and induction of dormancy [243]. Notably, ABCG2 is a potential marker for tumor-initiating cells as well as a marker of chemoresistance in cancer stem cells [244].

ATP-binding cassette (ABC) transporters are widely studied for their diverse roles in cancers, including their contributions to multidrug resistance. ABC transporters are membrane-associated ATPases that utilize the energy of ATP binding and hydrolysis to translocate substrates across biological membranes, and their expression is known to be regulated by oncogenic transcription factors or signaling pathways implicated in the regulation of CSCs, such as PI3/AKT and MAPK/ERK. Targeted agents that inhibit these

pathways can downregulate ABC transporters and sensitize the cancer cells to chemotherapeutic agents [245]. Likewise, EMT transcription factors mediate chemoresistance in BCSCs by regulating ABC transporters, and a relationship between pluripotency and chemoresistance was demonstrated through silencing of *SOX2* in mammospheres, which led to downregulation of *OCT4*, *NANOG*, *ALDH1A1*, *ABCG2*, and *TWIST*. This downregulation reduced the stemness and chemoresistance of BCSCs and improved sensitivity to paclitaxel treatment. This work elucidated the pluripotency-chemoresistance-EMT axis and paved a comprehensive strategy for future BCSC-based therapy [149].

Other main treatment modalities are chemotherapeutic agents and radiation, which induce DNA damage. BCSCs exhibit DNA damage repair mechanisms that render them chemo- and radiation-resistant, thus targeting DNA repair pathways is another plausible approach for BCSC-directed therapy [246]. In radiation treatment of mammospheres, a lower level of ROS increase was obtained relative to treated monolayer cultures, and it was followed by an increase in expression of Jagged-1 and Notch1 [247]. Broadly, BCSCs trigger increased expression of free radical scavenging systems at lower ROS levels than do other cells, protecting them from anti-cancer agents. Doxorubicin-dependent $CD44^{+}/CD24^{-}$ BCSCs in MCF-7 cells demonstrate upregulated levels of nuclear factor, erythroid 2 like 2 (*NRF2*), a key transcription factor that regulates cellular responses to oxidative damage. Specifically, CD44 regulates NRF2 level through p62 expression, and NRF2 activation endows the BCSCs with aggressive phenotype and chemoresistance [248].

Drug-resistant CSCs also activate anti-apoptotic proteins that can withstand cytotoxic agents. Inhibiting these anti-apoptotic proteins (such as Bcl-2) can be a potential therapeutic avenue against chemo-resistant BCSCs [249]. Recently, evidence has accumulated for a role of the pro-survival autophagic pathway in BCSC survival and maintenance. Autophagy flux is high in the $ALDH^+$ BCSC population and is essential for tumorigenicity [250]. Autophagic BCSCs show chemoresistance that is enhanced by hypoxia, but the inhibition of autophagic CSCs in TNBC can overcome chemoresistance [251].

Last but not least, tumor dormancy is implicated in chemotherapy resistance and metastasis. Dormant cancer cells can survive an unfavorable microenvironment and undergo reversible growth arrest; furthermore, while in a dormant state, committed tumor cells de-differentiate to become stem-like cells [252]. Tumor dormancy is characterized by upregulation of autophagic signaling (which maintains the metabolic homeostasis of dormant cancer cells), epigenetic features, stress-lenient signaling, and microenvironmental cues [253]. In BCSCs, autophagy maintains low-level expression of the glycolysis mediator 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) to maintain cellular dormancy. Inactivation of autophagy signaling components re-establishes normal-level PFKFB3 expression, culminating in the reactivation of BCSC self-renewal, tumor aggressiveness, and metastatic outgrowth [254].

1.10 Differentiation Therapy and BCSCs

Loss of differentiation coupled with uncontrolled proliferation is a hallmark of malignant neoplasms. Differentiation therapy is a therapeutic strategy that re-instates

endogenous differentiation programs to induce maturation in tumor cells. Upon differentiation, tumor cells revert back to a non-malignant phenotype, culminating in reduction of proliferation and metastatic potentials and upregulation of differentiating markers [255]. An early success story of differentiation therapy was the use of all-trans retinoic acid (ATRA) in acute promyelocytic leukemia (APML); treating APML patients with ATRA in combination with chemotherapy can induce complete remission. ATRA targets an APML fusion protein (PML-RARα), which ultimately leads to degradation of the PML-RARα/HDAC complex, permitting epigenetic modifications and further differentiation of leukemic cells. Although the remission in AML patients treated with ATRA is quite sustainable, emergence of secondary resistance later challenges ATRA treatment alone, thus it is combined with chemotherapy for a more complete cure [256].

Since chemotherapies target only rapidly-proliferating tumor cells and spare the slowly-dividing population of CSCs, relapse is common. The presence of dedifferentiated CSCs in solid tumors gives rise to their heterogeneous nature with regard to proliferation, metastasis, and relapse after radio- or chemotherapy. A prospective alternative CSC-targeted therapy is to use differentiation-inducing agents to target CSCs and self-renewal signaling, influence the functional hierarchy between tumor cells, and thereby reduce their chemo- and radioresistance [257]. In fact, ATRA-induced differentiation in glioma stem-like cells reduced CSC motility, sensitized tumor cells to chemotherapy, and inhibited tumorigenicity [258]. In a similar fashion, ATRA treatment induced differentiation of CSC-enriched radioresistant MCF-7 breast tumor cells, reducing tumor invasiveness and migration and increasing sensitivity to chemotherapy [259]. In a TNBC xenograft model, combined treatment of ATRA with the epigenetic and chemotherapeutic agents entinostat

and doxorubicin targets CSCs and induces differentiation by activating ETS transcription factor 1 [260].

Genistein, a phytoestrogen, was shown to induce differentiation in BCSCs by a paracrine mechanism, affecting differentiation- and stem cell-associated genes and inhibiting mammosphere formation [261]. Acetaminophen, an anti-inflammatory drug, was evaluated for its effect on differentiation and tumorigenicity in breast cancer. Treatment of MDA-MB-231 cells with acetaminophen induced morphological changes, decreased $CD44^{+}/CD24^{-}$ and $ALDH^{+}$ subpopulations, altered markers for differentiation and stemness, and inhibited tumorigenicity. It also increased susceptibility to anti-tumor drugs through suppressing the expression of multidrug efflux pumps. The differentiation-inducing effect of acetaminophen is mediated through the Wnt/β-catenin signaling pathway [262]. Another study showed that knockdown of *CD44*, a BCSC marker involved in the differentiation, adhesion, and metastasis of cancer cells, sensitized breast cancer cells to doxorubicin or radiation. Its depletion BCSCs to differentiate into non-stem-like cells, targeting drug resistance, metastasis, and stem cell-related genes [263]. These findings indicate that targeting BCSC markers can modulate differentiation and inhibit breast tumorigenicity at the same time. **Table 1.2** summarizes differentiation-promoting natural products and synthetic chemicals that have been indicated to target breast cancer stemness signaling.

When it comes to development and stem cell differentiation, it is well-established that epigenetic regulation plays a significant role. Aberrant epigenetic modifications (including microRNAs and histone modifications) have been implicated in differentiation programs in cancer [264]; of these, microRNAs provide an appealing target for

differentiation therapy. Petrelli *et al.* showed that miR-100 promotes differentiation in basal-like BCSCs, transforming the basal-like phenotype to luminal type. In basal-like breast cancer, miR-100 inhibits maintenance of BCSCs by targeting the Wnt signaling pathway and polo like kinase 1 (*PLK1*); conversely, its inhibition induces a stem-like phenotype [265]. Also of interest in breast cancer is the potential role of histone deacetylase inhibitors (HDACi) as avenues for differentiation therapy [266]. A low dose of the HDACi abexinostat induces BCSC differentiation in sensitive breast cancer cells, with treated cells exhibiting high expression of luminal and epithelial markers and low expression of mesenchymal markers. Furthermore, abexinostat reduces the BCSC population in patient-derived xenografts expressing low levels of the lncRNA *Xist* [267].

Collectively, the above studies illustrate the promise of differentiation therapy either as a stand-alone therapy or as part of a combinatorial regimen targeting BCSCs.

1.11 Vitamin D and breast cancer

Vitamin D belongs to a group of fat-soluble secosteroids produced as a result of skin exposure to UV light or obtained from dietary sources such as plants and fish [268]. It is hydroxylated in the liver by the 25-hyroxylase enzyme encoded by cytochrome P450 family 2 subfamily R member 1 (*CYP2R1*), producing 25D3 (25-hydroxyvitamin D). It is then further metabolized in the kidneys to its final active form, $1\alpha,25(OH)_2D_3$ (1,25-hydroxyvitamin D, **Figure 1.2**) mediated by 1α-hydroxylase enzyme encoded by *CYP27B1*. Both 25D3 and 1,25D3 are catabolized by *CYP24A1* [269]. The function of the active form of vitamin D (1,25D3) is mediated through the vitamin D receptor (*VDR*), which is a nuclear receptor protein that binds to vitamin D response elements and

modulates transcription of target genes. *VDR* is expressed in tissues involved in calcium and phosphate homeostasis, such as bone and kidney, as well as in other non-calcium regulating tissues [270]. Human *VDR* gene is located on the long arm of chromosome 12 (12q13.11) constituting three regions: one coding region and non-coding regions. It spans 75 kb long and encompasses 11 exons [271]. Exons 1A, 1B and 1C comprises the 5'-noncoding sequence and exons 2-9 encode the coding region of the *VDR* gene. The promoter region of *VDR* gene serves as the putative binding site for multiple transcription factors that mediate extracellular signal transduction. The structural portion of the VDR gene contains translation start codons and ligand binding site [271]. In the mammary gland, it is involved in development and homeostasis during pregnancy, lactation, and involution, and furthermore regulates calcium transport during lactation. *Vdr* null mice showed accelerated lobuloalveolar development during pregnancy and delayed post-lactational involution [272].

Notably, there is mounting evidence documenting an inverse relationship between vitamin D level and breast cancer. Serum 25D3 deficiency is highly prevalent in breast cancer patients; conversely, increased serum vitamin D level is associated with reduced risk of breast cancer [273, 274]. A recent meta-analysis of sixty-eight studies published between 1998 and 2018 showed a protective relationship between 25D3 and breast cancer in premenopausal women [275]. In addition to serum vitamin D level, VDR expression may be a prognostic factor. High VDR expression in invasive breast cancer is associated with favorable tumor characteristics such as lower grade, estrogen receptor and progesterone receptor positivity, and lower breast cancer mortality [276]. However, there is some evidence indicating *VDR* gene polymorphisms to be associated with breast cancer,

including *Bsm1, Poly*(A), *Fok1, Cdx2 and Taq1* [277]. *Bsm1* gene polymorphism is located in the 3' end of the VDR gene. Individuals with bb genotype has higher incidence of developing breast cancer compared to BB or Bb genotypes [278]. Similarly, *Poly*(A), a biallelic polymorphism, is in linkage disequilibrium with Bsm1 polymorphism in 3' UTR region, with characteristic variable number of tandem repeats. Individuals with long poly(A) stretches variant (LL) is associated with breast cancer risk in contrast to the ones with short poly(A) stretches (SS) that carry less risk of breast cancer. The 5' *Fok1* gene polymorphism augmented the breast cancer risk associated with bb/LL genotype [278]. Furthermore, *Fok1* gene polymorphism has a significant interaction with the ER status in breast cancer [279]. The Cdx2 polymorphism is a guanine (G) to adenine (A) sequence alteration, located in the promoter region of VDR gene. A meta-analysis of case control studies showed *Cdx2* polymorphism is significantly associated with breast cancer susceptibility in Africans but not Caucasians [280]. *Taq1* polymorphism increases the risk of breast cancer development in Caucasians although no association was found among Asians [281]. It is associated with a significant risk of ER+ tumors for t allele carriers compared to non-carriers (T allele: absence of Taq restriction site; t allele: presence of Taq1 restriction site) [282].

Binding of active metabolites of vitamin D to VDR regulates the transcription of genes implicated in pro-apoptotic, pro-differentiating, anti-proliferative, and anti-metastatic effects on cancer cells [283]. Prior studies have demonstrated that vitamin D and its analogs inhibit breast tumorigenesis *in vivo* and trigger apoptotic and autophagic cell death *in vitro* [284-286]. Compared to 1,25D3, Gemini vitamin D analogs exert more potent anti-proliferative and tumor-inhibiting effects in xenografts without causing

hypercalcemic toxicity, which was detected in 20-30% of patients treated with 1,25D3 [287-289]. In addition to its effect on primary breast tumors, vitamin D has demonstrated inhibitory effects on metastasis, achieved through inhibiting EMT [290]. In breast tumor cells, vitamin D and its analogs induced epithelial markers such as E-cadherin, but repressed the expression of mesenchymal markers such as N-cadherin and α-SMA, culminating in epithelial differentiation and reduction in tumor progression [291, 292]. Solid tumor progression can also be effectively prevented through targeting angiogenesis in the tumor microenvironment. 1,25D3 inhibits VEGF-driven endothelial cell sprouting and elongation in three-dimensional gels *in vitro*, the effects mediated through apoptosis. Likewise, treatment of VEGF-overexpressed xenografts with vitamin D reduced tumor vascularity *in vivo* [293]. An immunomodulatory action of vitamin D has also been shown in xenograft tumors, promoting $CD8^+$ T cell tumor infiltration and reducing tumor weight [294].

The mechanism by which vitamin D exerts its tumor-inhibiting effect has been examined in MMTV-Wnt1 mammary tumors. Dietary vitamin D and injection calcitriol delayed *in vivo* tumor growth in limiting dilution analyses and inhibited self-renewal in spheroid culture, mediated through the Wnt/β-catenin pathway [295]. In MCF10DCIS.com xenograft tumors, the Gemini vitamin D analog BXL0124 has been shown to inhibit ductal carcinoma in situ (DCIS) progression to invasive ductal carcinoma (IDC) by maintaining the myoepithelial cell layer and basement membrane [296]. BXL0124 inhibited the expression of CD44 protein and suppressed the mammary tumor growth in MCF10DCIS xenografts. It repressed the expression of CSC marker CD44 at both mRNA and protein levels in MCF10DCIS.com cells via *VDR*-dependent mechanism [297]. Knockdown of

CD44 in MCF10DCIS.com cells decreased STAT3 signaling and inhibited invasion and proliferation by downregulating the expression levels of invasion markers – matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA) [298]. Administration of BXL0124, the synthetic triterpenoid CDDO-Im and the combination delayed the development of MMTV-ErbB2/neu mammary tumors by downregulating ErbB2 signaling pathway including activated-Erk1/2, activated-Akt, c-Myc, CycD1 and Bcl2 [299]. In MCF10DCIS.com cells, vitamin D and BXL0124 inhibit BCSCs by reducing both $CD44^{+}/CD24^{-/low}$ subpopulations and mammosphere forming efficiency. Treatment of mammospheres with vitamin D compounds targets stem cell phenotype markers (including CD44, CD49f, pNFkB, and c-Notch1) and pluripotency markers (such as OCT4 and KLF4) [300]. However, the effect of vitamin D on BCSCs is dependent on VDR expression; the receptor is significantly downregulated in mammospheres and $ALDH^{+}$ breast cancer cells. Overexpression of *VDR* in mammospheres led to upregulated E-cadherin expression and downregulated Snail expression, resulting in impaired mammosphere formation [301]. Therefore, in at least some contexts, vitamin D compounds can be used as an effective differentiation-promoting therapy, targeting BCSCs and EMT to prevent breast cancer progression and metastasis.

1.12 Summary

Breast cancer is one of the most commonly diagnosed cancers in US women. It is a heterogeneous disease with a high degree of intra- and inter-tumoral diversity predisposed by multiple genetic and non-genetic risk factors. Accumulating evidence has indicated cancer stem cells as responsible for the origin of breast cancer, along with tumor

maintenance, progression, and relapse. BCSCs are TICs mainly characterized by self-renewal, high proliferative capacity, the ability to generate daughter cells with differentiation potential, and chemo- and radio-resistance. BCSC populations can be identified by select cellular markers, including $CD44^+/CD24^-$ and ALDH enzymatic assay. The self-renewal and proliferative potentials of BCSCs are studied using mammosphere formation assays and limiting dilution xenograft studies. A hallmark of BCSC is the deregulation of signal transduction pathways involved in the regulation of normal mammary stem cells, endowing them with the characteristic features of cancer stemness. Transcription factors typical of embryonic stem cells are expressed in breast cancer cells; their expression is associated with stem-like phenotypes, EMT, and tumor aggressiveness, suggesting a significant role of transcription factor-mediated stemness and tumorigenesis in breast cancer. In addition, the tumor microenvironment and epigenetic regulation have been revealed as critical aspects of BCSC biology in which further study is needed to advance our understanding of BCSCs and their contributions to cancer progression, relapse, and resistance.

With poor differentiation being a hallmark of malignant cells, differentiation therapy has recently emerged as an alternative strategy for inducing differentiation in cancer cells and thereby reducing malignancy. The application of all-*trans* retinoic acid as differentiation therapy in acute myeloid leukemia has proved to be safe and effective in targeting leukemic stem cells. Vitamin D and its analogs have been studied as potential chemopreventive agents to induce differentiation in several malignancies, including myeloid leukemia, breast, colon, and breast cancers. These compounds have been demonstrated to target breast cancer stem cells and progression in both *in vitro* and *in vivo*

studies. Building upon this literature, we will in the following chapter identify the effects of vitamin D compounds in targeting BCSCs in TNBC. We will also investigate the role of transcription factors(s) modulating BCSCs in breast tumorigenesis.

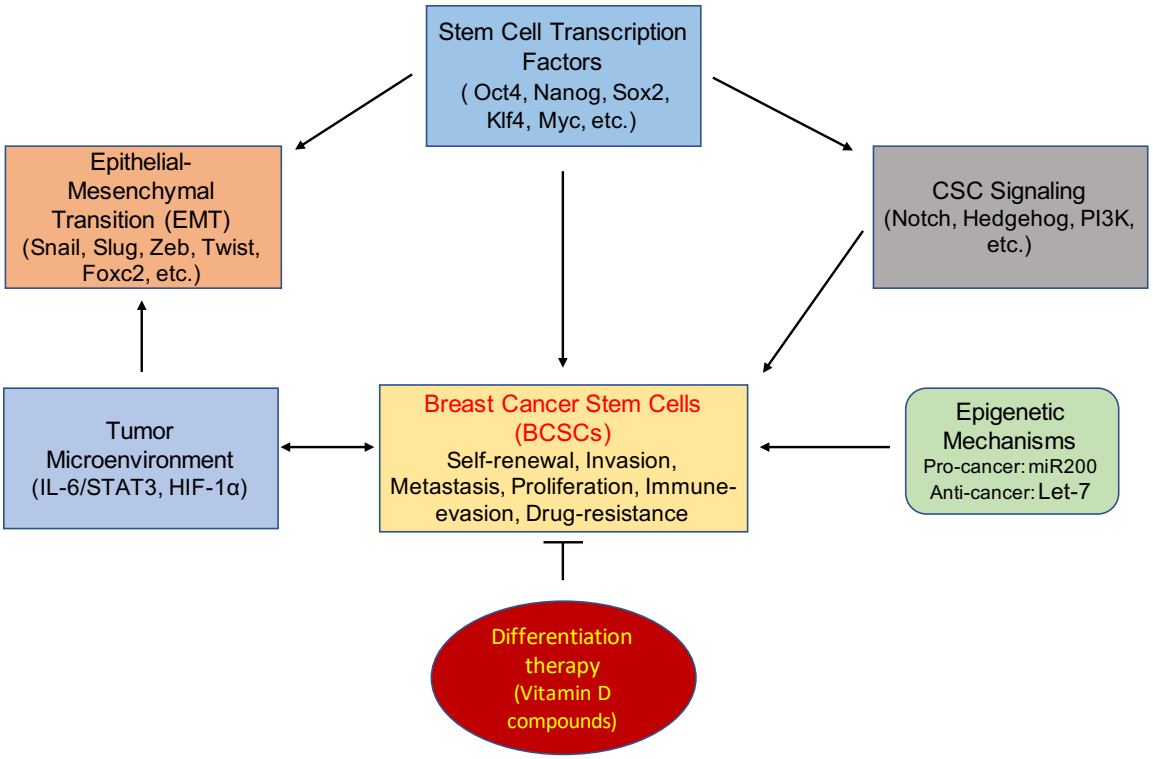
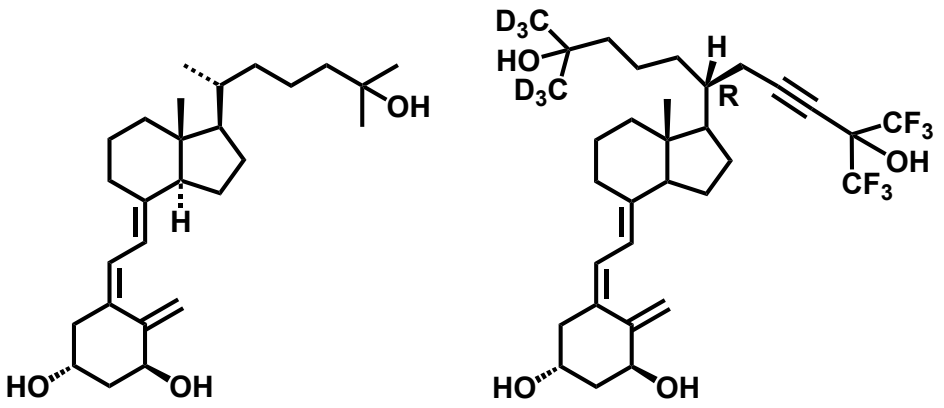


Figure 1.1 Diagram of self-renewal pathways and associated transcription factors regulating breast cancer stem cells



1a,25(OH)$_2$D$_3$ BXL0124

Figure 1.2 The structures of 1α,25(OH)2D3 and BXL0124

Table 1.1 Breast cancer stem cell markers, their functions, target genes and tumorigenesis

Cancer cell surface marker(s) /Transcription factor	Function in stem cell	Experimental system	Target gene/marker studied	TS/ CFA[4]	In vivo	Ref.
$ABCG2^{+}$ (CD338)	Tumor initiation	HCC1937	CD326/EpCAM, CD49f/α6-integrin, CD24, CD10, CD133	+	Injection of unsorted cells ($4x10^5$ and $2x10^6$ cells)	[302]
$ALDH^{+/hi}$ $CD44^{+}$	Chemo-radio resistance	MDA-MB231 and MDA-MB-468	GSTP1, P-gp, CHK1, CK8/18/19	+	NA	[303]
$ALDH1^{+}$	Self-renewal; tumor initiation	Human breast cancer cells	NA	+	Injection of sorted cells (500 ~ 50,000 cells)	[54]
C/EBPδ	Tumor initiation; EMT; metastasis	• SUM159 • MDA-MB-231, MDA-MB-468 • MCF-7 • T47D	• IL-6, HIF-1α, CD44, N-cadherin, Vimentin, Twist and STAT3 • CD44, Myc, Nanog, and KLF4 • CD44, Vimentin, OCT4, SOX2, Nanog, KLF4, CDH1, FBXW7, HIF-1α, E-cadherin, IL6, NOTCH and NICD • Vimentin, E-cadherin	+	Injection of unsorted cells ($3x10^6$ or $0.3x10^6$ cells)	[304]
CCR5	Self-renewal; DNA repair	SUM159, SUM149 and FC-IBC-02	DNA damage repair genes: FANCB, LIG3, POLE, CRY1; PI3/Akt signaling; cell survival signaling	+	Injection of sorted cells (4000 cells)	[305]

[4]TS/CFA: Tumor Spheroid or Colony formation assay
NA: Not Available

$CD133^+$	Tumor initiation; self-renewal	Cell lines developed from *Brca1*Δ^{exon11}/*p53*$^{+/-}$ mouse mammary tumors	Expression of stem cell associated genes: OCT4, NOTCH1, ALDH1, FGFR1 and SOX1	+	Injection of sorted cells (50 ~ 5000 cells)	[50]
$CD24^+CD29^+/CD49f^+$	Metastasis; EMT	BRCA-1 mutant mouse derived CSCs	E-cadherin	+	Injection of sorted cells ($2x10^5$ cells)	[306]
$CD24^+Thy1^+$	Tumor initiation	MMTV-Wnt-1 mouse	CK5, CK14, CK17, NOTCH4, BCL6B	+	Injection of sorted cells (50 ~ 2,000 cells)	[307]
$CD29^{lo}CD24^+CD61^+$	Tumor initiation	MMTV-Wnt-1, MMTV-neu and p53 mutant mice	CK14, CK8	+	NA	[308]
$CD44^+/CD24^{-/low}Lineage^-$	Tumor initiation	Human breast tumor-derived tumor cells	NA	-	Injection of sorted cells; Limiting dilution (100 ~ 5×10^5 cells)	[46]
$CD44^+/CD24^{-/low}/EpCAM^+$	Tumor initiation; self-renewal	Human mammary epithelial cells, MDA-MB-231, MCF7, MCF10A, SUM149, SUM159, SUM1315 and SUM225	NA	+	Injection of sorted cells; Limiting dilution (100 ~ 1×10^6 cells)	[309]
$CD44^+/CD49f^{hi}/CD133/2^{hi}$	Tumor initiation, self-renewal	Human breast cancer tissues	Sox2, Bmi-1, Nanog	+	Injection of sorted cells; Limiting dilution (50 ~ 2500 cells)	[310]
$CD44^+CD24^{+/lo}SSEA\text{-}3^+$ or $ESA^{hi}PROCR^{hi}SSEA\text{-}3^+$	Tumor initiation	MCF-7 and MDA-MB-231	SSEA-3, Caspase-3, capase-8, caspase-9, caspase-12 SSEA-4, globo-H, β3GalT5	+	Injection of sorted cells (10 ~ 2500 cells)	[311]
$CD49F^+/DLL1^{hi}/DNER^{hi}$	Tumor initiation; self-renewal	Human normal mammary stem cells and breast tumors	SERPINB5, TOP2A, CK5, TP63, SOX4, CD24, ADRM1, DNER, DLL1, and JAG1	+	Injection of sorted cells (500 ~ 1000 cells)	[312]

$CD49f^{+}CD61^{+}$	Tumor initiation; self-renewal	MMTV-Her2/neu-induced primary mammary gland tumor	CSC markers: Abcg2, Aldh1, CD133, Gli1 and Tp63; differentiation marker genes: CK5, CK6, CK14, CK18; TGFβ signaling: Pai, Il6, Igfbp3, Foxc2; EMT genes: CDH2, SMA, SNAIL, TWIST1 and ZEB1	+	Injection of sorted cells (5000 cells)	[313]
CD61(Integrin αvβ3)	Tumor initiation; self-renewal	BT-20, MDA-MB-231and MDA-MB-468	Slug	+	Injection of unsorted cells; Limiting dilution (100 ~ $1x10^5$ cells)	[314]
$CD70^{+}$	Self-renewal; EMT; lung-specific metastasis; differentiation	MCF-7, MDA-MB-231 and CN34	E-cadherin and Vimentin	+	Injection of sorted cells; limiting dilution	[315]
Cx26	Self-renewal; tumor initiation	Triple negative breast cancer samples; MDA-MB-231 and HCC70	NANOG, FAK, OCT4, SOX2	+	Serial dilution injections (8,000 ~ 800,000 cells)	[316]
CXCR2	Chemo-radio resistance; tumor initiation	Human breast cancer tissues; 4T1	ALDH, ABCG2, NOTCH1, SOX2, and NANOG	+	Injection of sorted cells (200 ~ 20,000 cells)	[317]
$GD2^{+}$	Self-renewal; tumor initiation; EMT	HMLER and MDA-MB-231	GD3S; MMPs: MMP2, MMP7 and MMP19; EMT markers: N-cadherin, Vimentin, E-cadherin; stemness markers: CD44, CD24	+	Injection of sorted cells (1 ~ 10,000 cells)	[318]
Glyoxalase 1 (GLO1)	Self-renewal	MDA-MB-157 and -468	ALDH1	+	NA	[319]
HIF-2α	Self-renewal; chemoresistance	MCF-7 and MDA-MB-231	Stem cell markers: C-MYC, OCT4, NANOG; Notch pathway related proteins: $NOTCH^{NICD}$ and HEY2; Wnt-pathway related proteins: β-catenin, Axin2 and Survivin	+	Injection of unsorted cells (3×10^6 or 1×10^6 cells)	[196]

$Lgr5^{hi}$	Self-renewal; tumor initiation; EMT	MCF-7, MDA-MB-231	EMT markers: E-cadherin, β-catenin, Vimentin, Fibronectin, Snail, slug; Cyclin D1, C-myc, CK14, and CK18, CD44, CD24	+	Injection of sorted cells (200 ~ 20,000 cells)	[320]
miR-1	Negative regulator of breast cancer stem cells and EMT	Human breast cancer tissues; MDA-MB-231	EVI-1; EMT markers: E-cadherin, N-cadherin	-	Injection of sorted cells ($5x10^6$ cells)	[321]
miR-221	Self-renewal	Human breast cancer tissues; T47D and MCF-7	Stemness genes: NANOG, OCT3/4; β-Catenin, DNMT3b, CD44, CD24, Numb, p53	+	NA	[322]
$MUC1^+$	Tumor initiation; self-renewal	MCF-7	ABCG2, CK18, CK19, EpCAM, CD49f	+	NA	[323]
Nectin-4^+	EMT; metastasis; self-renewal	MDA-MB-231	CD44, CD133, PI3K, Akt, β-catenin, E-cadherin, Vimentin	+	Injection of unsorted cells ($1x10^7$ cells)	[324]
$PROCR^+/ESA^+$	Self-renewal; tumor initiation; EMT	MDA-MB 231	EMT markers: VIM, E-cadherin, SLUG, FOXC2; stem cell markers: ALDH, CD44, CD24, ESA, CD133, CXCR4, ABCG2	+	Injection of sorted cells; Limiting dilution (100 ~ 2500 cells)	[325]
RUNX1	Negative regulator of self-renewal and CSC	MCF10AT1, MCF10A, MCF10CA1 and MCF7	E-cadherin, Vimentin, FN1, VEGF, MMP13, MMP9, CXCR4, CLCX12, CSC markers: Zeb1, Twist1, CD44, CD24	+	Injection of unsorted cells ($1x10^6$ cells)	[326]
$Sca1^+$	Self-renewal; tumor initiation; chemoresistance	BALB-neuT mouse	Stem cell markers: Oct-4, CD44, CD29, CD24; differentiation markers: CK14, CK18, CK19, α–SMA	+	Injection of unsorted cells (100 ~ 10,000: cells)	[327]

Syndecan-1	Self-renewal	SUM149 and SKBR3	CSC markers: CD44, CD24, ALDH; Notch signaling: NOTCH-1, -3, -4, HEY-1; Gli-1, IL-6, IL-8, gp130, STAT3, NFκB, CCL20, EGFR	+	NA	[328]
tDR-000620	Predictor of TNBC recurrence	Human patients- derived TNBC samples; MCF-7 and MDA-MB-231,	SOX2, OCT4, ALDH1, CD44, CD24	+	NA	[329]

Table 1.2 Potential compounds regulating cancer stem cell markers, differentiation and EMT in breast cancer

Agent	Classification	Experimental Model	Significance	Ref
Acetaminophen	Synthetic chemical	In vitro, MDA-MB-231	mRNA level: ↑: KRT19, AKT2, CD24, and TIMP1 ↓: MMP2, ALDH1, MMP9, TWIST, NOTCH1, and AKT1 Protein level: Vimentin↓/E-cadherin↑, Twist↓ Cell surface marker: CD44hi/CD24low↓, CD44+/CD24+↑ Differentiation induction of CSC: Twist↓, Vimentin↓/E-cadherin↑	[330]
AF38469	Synthetic chemical	In vivo, MDA-MB-231 Xenograft mouse	Sortilin inhibition Decreased mammosphere formation Reduced EMT	[331]
AHCC (Active Hexose Correlated Compound)	Natural product	In vitro, MCF-7	Decreased mammosphere formation (AHCC alone and in combination with Wasabi) Observation of monocyte-to-macrophage differentiation	[332]
All trans Retinoic Acid	Natural product	In vitro, MDA-MB-231, T47D, ZR75-1, BT549 and MCF7	RARβ-TET2 complex recruitment, leading to activation of miR-200c Inhibition of RARβ-TET2-miR-200c-PKCζ↓ CHIP-seq with RARβ-TET2: co-occupancy with *RUNX1, BMP6, IKZF1, CAV1* decreased T47D CSC in 3D gel. Polarized expression of α6-integrin and E-cadherin differentiation (Increase symmetric commitment and repress asymmetric division in CSC)↑ tamoxifen sensitization	[333]
All trans Retinoic Acid	Natural product	In vitro, MCF7	Cell marker: CD44+/CD24- cell↓, NANOG↓, Oct3/4↓ Differentiation induction of CSC: marker involucrin↑, syndecan↑ Reduced invasiveness, migration Epirubicin sensitization	[259]
Arsenite	Synthetic chemical	In vitro, MDA-MB-231	Cell surface marker: ICAM-1 expression induction (in combination with Tetrandine)	[334]
Atorvastatin	Synthetic chemical	In vitro, MDA-MB-468, MDA-B-231	Gene expression: Hippo, Notch, Wnt↓ Protein level: Yap/Taz protein↓, vimentin↓/E-cadherin↑ Differentiation induction of CSC: CD24+↑ Reduced EMT	[335]
β-lapachone	Natural product	In vitro, MDA-MB-231	NQO1 induction Gene expression: CD44, ALDH1A1, DLGAP5↓ Decreased mammosphere formation	[336]

Benzo(a)pyrene	Synthetic chemical	In vitro, MCF10A	TDLU assay: Organized ducts and lobules in BMP2 presence cells with BaP treatment BMP mediated progenitor response shown: Myoepithelial dif. (SMA, KRT14) and luminal dif. (KRT18, EpCAM) with BMP4 treatment	[337]
BEZ235	Synthetic chemical	In vitro, HCC1143, SUM149	PI3K/mTOR inhibition K19, K14, VIM expression in HCC1143 and SUM149 cell line → K19hi/K14hi/VIMlo Drug Tolerant Progenitor (DTP) cells enrichment RNA seq: cytokeratin upregulation	[338]
		In vitro, HCC1143	Induction of K19hi/VIMlow/K14low enrichment in HCC1143 cell line. De-enrichment of VIMhi/K14low population	[338]
Bisphenol A	Synthetic chemical	In vitro, MCF10A	TDLU assay: Organized ducts and lobules in BMP2 presence cells with BaP treatment BMP mediated progenitor response shown: Myoepithelial dif. (SMA, KRT14) and luminal dif. (KRT18, EpCAM) with BMP4 treatment	[337]
BXL0124	Synthetic chemical	In vitro, SUM159	mRNA level: OCT4, CD44, LAMA5, NOTCH↓ Differentiation marker: cytokeratin14, SMA↑, cytokeratin18, cytokeratin5↓ Decreased mammosphere formation Differentiation induction of CSC	[339]
CHM-09	Synthetic chemical	In vitro, MDA-MB-231	EGFR Tyrosine kinase inhibitors Differentiation marker: N-cadherin↓, E-cadherin↑ Induction of CSC apoptosis and cell cycle arrest Increased MET	[340]
Cholera toxin	Natural product	In vitro, HMLE, NAMEC8, MCF10A Xenograft NOD/SCID mouse	cAMP/PKA activation $CD44^{Hi}/CD24^{Lo}$ population↓ Increased MET	[341]
Citral	Natural product	In vivo, 4T1 xenograft mouse model	Aldefluore assay showing decrease in ALDH-positive cell population in primary breast cancer xenograft	[342]
Curcumin	Natural product	In vitro, MCF-7, MDA-MD-231	Gene expression: OCT4↓ NANOG↓ SOX2↓ Decreased mammosphere formation Differentiation induction of CSC: $CD44^{+}/CD24^{-}$↓	[343]
		In vitro, SUM149, MCF10A, MCF-7	Gene expression: ALDH1A3↓ PROM1↓TP63↓ITGA6↓ Decreased mammosphere formation	[344]

CWP/ICG001	Small molecule	In vitro, MCF-7, MDA-MB-231	Induction of Sam68-CBP complex, leading to disruption of CBP/β-catenin Differentiation induction of CSC Increased CSC apoptosis	[345]
Dasatinib	Synthetic chemical	In vitro, paclitaxel-res SUM159	mRNA level: N-cadherin (CDH2), Fibronectin (FN1), Snail (SNAI1), ZEB1, TP63, SMA (ACTA2) ↓ /E-cadherin expression↑ protein level: p-Src↓ 3D culture of SUM159. Dasatinib treated group showed formation of round, acinar-like structure Decreased mammosphere formation Differentiation induction of CSC Increased MET	[346]
Diallyl Trisulfide	Natural product	In vitro, MCF-7, SUM159	Gene expression level: CD44, ALDH1A1, NANOG, OCT4↓, Wnt/β-catenin signal↓ Reduced CSC viability	[347]
Digitoxin	Natural product	Patient sample, patient derived xenograft model	Increased Intracellular Ca dissociates cell tight junction, leading to altered DNA methylation profile of gene expression shown below: Gene expression level: OCT4, SOX2, NANOG, SIN3A↓	[348]
Disulfiram	Synthetic chemical	In vitro, MDA-MB-231	Protein level: STAT3, cyclinD, Survivin, ALDHA1↓, Caspase-3↑ Cell surface marker: CD44+/CD24-↓ Increased CSC apoptosis Decreased mammosphere formation	[349]
Doxorubicin	Synthetic chemical	In vitro, Hs578T	Decreased proliferation, aggregation and mammosphere formation of stem-like cells Affects the balance between self-renewal and differentiation	[350]
EC-70124	Synthetic chemical	In vitro, HS578T, BT549, MDA-MB-231 and HCC3153	Gene expression: PI3K/mTOR, JAK/STAT ↓ Cell marker: CD44, ALDH1, CD49f, CD133↓ Differentiation induction of CSC Decreased EMT	[351]
Efatutazone	Synthetic chemical	In vitro, MCF10A, MCFDCIS In vivo, MCFDCIS subQ injection	PPARγ agonist mRNA level: hFABP4↑, CK8↑, CK6a, CK6b, CK17↓ *in vivo*: increased lipid droplets, reduced CD44, p63 staining, increased CK8 staining. FABP4, PLIN2 mRNA↑ Differentiation induction of CSC: upregulation of PPARγ responsive genes in epithelial and stromal components	[352]
Entinostat	Synthetic chemical	In vitro, MDA-MB-231,	HDAC inhibition	[353]

		In vivo, MDA-MB-231 Xenograft	Transcriptomic array in MDA-MB-231 cell treated with entinostat: RARB, ELF3, DHRS3	
Flubendazole	Synthetic chemical	In vitro, MDA-MB-231, BT-549, MCF-7 and SK-BR-3 MDA-MB-231 Xenograft nude mice	Gene expression: MYC, OCT4, SOX2, NANOG cyclinD1↓ Oil red O staining enriched MDA-MB-231 cells. Cell surface marker: CD44+/CD24-↓ Decreased mammosphere formation Differentiation induction of CSC: β -catenin, N-cadherin, vimentin↓, keratin 18↑ Reduced CSC self-renewal	[354]
Forskolin	Natural product	In vitro, HMLE, NAMEC8, MCF10A Xenograft NOD/SCID mouse	cAMP/PKA activation Differentiation induction of CSC: vimentin, CD44+/CD24-, Snail, Twist1 and Zeb1↓; CDH1, E-cadherin ↑	[341]
Graphene Oxide	Synthetic chemical	In vitro, MCF7	CSC signaling pathway: Wnt, NOTCH, STAT1/3, Nrf2 signal ↓ Cell surface marker: CD44+/CD22-↓, due to increase in CD22+ population Differentiation induction of CSC Decreased mammosphere formation	[355]
Helichrysetin	Natural product	in vitro, MCF10A, DCIS.com, MCF10CA	Differentiation induction of CSC: ID2 inhibition ID2 inhibition Decreased mammosphere formation Decreased CSC self-renewal	[356]
IM-412	Synthetic chemical	In vitro, MDA-MB-453, MDA-MB-231	FGFR1/3 inhibition Protein level: Smad2/3, p38/MAPK, Akt, JNK↓ Reduced EMT Differentiation induction of CSC: Inhibition of TGF- β pathway	[357]
Ivermectin	Synthetic chemical	In vitro, MDA-MB-231	Gene expression: SOX2, NANOG, OCT4 ↓ Cell surface marker: CD44+/CD22-↓ Decreased CSC viability Decreased CSC self-renewal	[358]
K252	Small molecule	In vitro, MDA-MB-468	Differentiation induction: ERN1 inhibition Confocal microscopy of K5 and K8 expression showing ERN1 and ALPK1 Knockdown induces luminal differentiation (K5-/K8+) in MDA-MB-468 Reduction of colony forming unit of anchorage independent growth of TNBC cell lines	[359]

			Protein level: β-casein↑	
Laminin	Endogenous	In vitro, LM05-E	Gene expression: SOX2, NANOG, OCT4 ↓ Protein level: p-ERK↑ Reduced CSC viability Differentiation induction of CSC Decreased mammosphere formation	[360]
Lovastatin	Synthetic chemical	In vitro, MDA-MB-468, MDA-B-231	Gene expression: Hippo, Notch, Wnt↓ Protein level: Yap/Taz protein↓, vimentin↓/E-cadherin↑ Differentiation induction of CSC Reduced EMT	[335]
Metformin	Synthetic chemical	In vitro, MDA-MD-231, MCF-7	RNA expression/ Differentiation induction: miRNA-708 ↑, CD47↓ Protein level: CD47↓	[361]
Nobiletin	Natural product	In vitro, MCF7A	CD36 inhibition Gene expression: SOX2, OCT4, NANOG↓ Decreased mammosphere formation	[362]
Ouabain	Natural product	Patient sample, patient derived xenograft model	Increased Intracellular Ca dissociates cell tight junction, leading to alter DNA methylation profile of gene expression shown below: Gene expression level: OCT4, SOX2, NANOG, SIN3A↓	[348]
Osteoprotegerin (OPG)	Endogenous	In vivo, MMTV-PyMT	RANKL decoy receptor Depletion of Sca1-/lo CSC Induce tumor cell differentiation and reduce recurrence and metastasis	[363]
Palbociclib	Small molecule	In vitro, MCF7, MCFDCIS	CDK4/6 inhibition Long term suppression of P63 Immunohistochemistry in DCIS Mammosphere formation↓ Differentiation induction of CSC: NELL2↑	[364]
PD98059	Synthetic chemical	In vitro, MDA-MB-231	MAPK inhibition Differentiation marker: N-cadherin↓, E-cadherin↑ Induction of CSC apoptosis and cell cycle arrest Increased MET	[340]
Prolactin	Endogenous	*In vivo*, Human BC cells HER2+, Luminal B Xenograft HER2 mouse model (NOD/SCID)	mRNA level: ALDHA1, ALDHA3, CD44↓ Cell surface marker: ALDH↓ Decreased mammosphere formation	[365]
P123	Peptide (1.9kDa)	*In vitro,* CSC cells	BMP signal agonist Cell surface marker: CD44+ population↓, E-cadherin+ population↑in BCSC	[366]

Quercetin	Natural product	In vitro, MCF7A	PI3K/Akt/mTOR signal inhibition Decreased mammosphere formation Cell surface marker: CD44+/CD24-↓	[367]
Quisinostat	Synthetic chemical	*In vitro*, MDA-MB-231, MDA-MD-468, HCC38, MCF-7	HDAC Class I and II inhibition Cell surface marker: CD44+/CD24-↓ Decreased CSC viability (in combination with doxorubicin)	[368]
Resveratrol	Natural product	In vitro, MDA-MB-231	SIRT1 induction Cell surface marker: CD44+/CD22-↓ Differentiation induction	[369]
Rosiglitazone	Synthetic chemical	In vivo, MMTV-PyMT transgenic mouse	Increase in FABP4, adiponectin expression (Co treatment with PD98059) Development of unilocular lipid droplets in tumor cells after treatment (Co treatment with PD98059) Differentiation induction of CSC: E-cadherin↑	[370]
Salinomycin	Synthetic chemical	In vitro, MCF7	Decreased mammosphere formation	[371]
SCH772984	Synthetic chemical	In vitro, MCF-10A, MDA-MB-231, MDA-MB-436	ERK inhibition Cell surface marker: ALDH↓ Protein level: p21↑	[372]
Seocalcitol	Synthetic chemical	In vitro, SUM-1315, BT-549, BT-20, SUM-159PT, MDA-MB-468, MFM-223, CAL-148	Vitamin D receptor signal activation Cell surface marker: ALDH↓ Decreased mammosphere formation Differentiation induction of CSC	[373]
Silibinin	Natural product	In vitro, MDA-MB-468	Gene expression: CD133, ALDH, C-MYC, NANOG, KLF4 SOX2↓, GATA3, BRCA1↑ Cell surface marker: ALDH+/CD133, ALDH+/CD44, CD133/CD44↓ phenotype of MDA-MB-468 in 2D and 3D culture: increase in size and spindle shape in treatment group. BRCA1 upregulation in 3D culture group with treatment Differentiation induction of CSC Decreased mammosphere forming size	[374]
Simvastatin	Synthetic chemical	In vitro, MDA-MB-468, MDA-B-231	Gene expression: Hippo, Notch, Wnt↓ Protein level: Yap/Taz protein↓, vimentin↓/E-cadherin↑ Differentiation induction of CSC Reduced EMT	[335]

Trametinib	Small molecule	In vitro, SUM159, HCC1143	MEK inhibition K19, K14, VIM expression in HCC1143 and SUM149 cell line → K19hi/K14hi/VIMlo Drug Tolerant Progenitor (DTP) cells enrichment RNA seq: cytokeratin upregulation	[338]
T315	Small molecule	In vitro, MDA-MB-231, SUM-159 Xenograft NOD/SCID mouse	ILK inhibition Decreased NOTCH1 signaling Decreased mammosphere formation *In vivo:* decreased ALDH+ population, decreased mammosphere formation and decreased tumor initiating ability	[375]
Vitamin D	Natural product	In vitro, SUM159	mRNA level: OCT4, CD44, LAMA5, NOTCH↓ Differentiation marker: cytokeratin14, SMA↑, cytokeratin18, cytokeratin5↓ Decreased mammosphere formation Differentiation induction of CSC	[339]
4a1		In vitro, MDA-MB-231	HEXIM induction Protein level: HEXIM1, p27↑, NANOG↓ Nile red staining increased in 4a1 treatment	[376]
5-aza-2'-deoxycytidine	Synthetic Chemical	$CD44^{hi}/CD24^{low}$ expressing CSC isolation from primary malignant breast tumor (patient)	mRNA level: p15, p16, BRCA1, BRCA2, p53↑ cell surface marker: CD44+/CD24-↓ protein: ABCG2↓	[377]

Chapter 2: Investigating the effects of vitamin D compounds in targeting breast cancer stem cells in triple negative breast cancer[5,6,7]

2.1 Introduction

Triple negative breast cancer (TNBC) accounts for 15-20% of all breast cancer cases [378]. It is characterized by a lack of expression of estrogen receptor (ER), progesterone receptor (PR), and erb-b2 receptor tyrosine kinase 2 (HER2, ERBB2) [379]. It is more common in non-Hispanic black women and Hispanic women under 40 years of age [380]. Relative to non-TNBC cases, patients with TNBC present at younger age with higher tumor grade, distant recurrence, and greater risk of metastasis and death [381, 382]. Specifically, the chance of distant recurrence and death is high within five years of diagnosis [383]; the overall five-year survival rate for patients with TNBC is 62%, in contrast with 75% for non-TNBC patients [384].

Within the category of TBNC, all intrinsic breast cancer subtypes can be identified, among which basal-like is the phenotype most commonly observed (at 50-70%) [385]. TNBC and basal-like breast cancer show significant overlap in tumors having mutation in the gene BRCA1 DNA repair associated (*BRCA1*) [386]. In TNBC studies, the most frequent histologic subtypes are high nuclear grade (poorly differentiated) and invasive ductal carcinoma [382]. Immunohistochemically, in addition to the lack of hormonal receptors, TNBC is characterized by increased expression of cytokeratins 5/6, cytokeratin

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[6]**Keywords**: cancer stem cells; mammosphere; pluripotency; differentiation; OCT4; BXL0124; Notch signaling;
[7]**Abbreviations**: TNBC, triple negative breast cancer; CSCs, cancer stem cells; BCSCs, breast cancer stem cells

14, vimentin, nestin, p16, p63, phosphohistone H3 (PPH3), epidermal growth factor receptor (EGFR), P-cadherin, moesin, IMP U3 small nucleolar ribonucleoprotein 3 (IMP3), and CD146 [387-389].

Molecular heterogeneity is the hallmark of TNBC, and remains a major impediment for targeted therapy [390]. Gene expression studies have classified TNBC into six clusters: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (MES), mesenchymal-stem-like (MSL), and luminal androgen receptor (LAR). Except for MSL and LAR, these subtypes correlate strongly with the basal-like intrinsic subtype [391]. However, individual subtypes also have unique gene signatures that highlight "predictive driver signaling pathways" which can be selected for targeted therapies, and display distinct therapeutic responses and pathological complete response after neoadjuvant chemotherapy [392, 393]. The BL1 and BL2 subtypes are enriched in cell cycle and DNA damage response gene expression, and are responsive to antimitotic agents such as taxanes and DNA-damaging agents [394]. The IM subtype is enriched in genes involved in immune transduction and cytokine signaling pathways [395]. Both the MES and MSL subtypes are enhanced in genes related to epithelial-mesenchymal transition (EMT), cellular differentiation, and growth pathways; MSL specifically expresses proliferation genes at low levels, accompanied by enriched expression of genes associated with stem cells [392]. The LAR subtype is enriched in hormone-regulated pathways [392]. In addition to these six clusters, a new intrinsic subgroup, the claudin-low subtype, has also been identified as TNBC; it comprises 70% of all TNBC tumors. The claudin-low subtype has a high frequency of metaplastic and medullary differentiation, along with characteristic stem cell

and epithelial-mesenchymal transition (EMT) features similar to the aforementioned MSL subtype [392, 396].

The mutational profiles developed from previous studies provide insights and potential targets in the form of genomic abnormalities that drive the clinical plasticity and intra-tumor heterogeneity of TNBC [390]. Basal-like tumors feature high-frequency loss of function mutations in tumor protein p53 (*TP53*), RB transcriptional corepressor 1 (*RB1*) and *BRCA1* [397-399]. Simultaneous inactivation of these three tumor suppressor pathways in mammary epithelium increases tumor latency and potentiates metastasis [400]. In addition, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) is found to be commonly mutated in basal-like tumors, which is in line with the PI3K/AKT pathway being frequently activated in TNBC. Dysregulation of the PI3K/AKT pathway may also be attributable to loss of phosphatase and tensin homolog (*PTEN*) or inositol polyphosphate-4-phosphatase type II B (*INPP4B*). [401]. Basal-like tumors also show characteristic expression of cytokeratins 5, 6, and 17 and increased expression of MYC proto-oncogene, bHLH transcription factor (*MYC*), forkhead box M1 (*FOXM1*), and genes associated with proliferation [402, 403].

The heterogeneity observed in the mutational profile of TNBCs is also paralleled by a wide spectrum of genomic evolution. When using targeted deep sequencing to evaluate allelic abundance and clonal frequency, TNBCs showed higher clonal frequencies in *TP53*, *PIK3CA*, and *PTEN*, indicating those mutations to have been acquired early in tumorigenesis; in contrast, mutations in cytoskeletal and motility genes had lower clonal frequencies and so occurred later in tumorigenesis [404]. This indicates that TNBCs are composed of mosaics of multiple clones that can give rise to an array of genotypic and

phenotypic features. Notably, TNBC also demonstrated widespread genomic instability characterized by subtype-specific distinct copy number alterations (CNAs); the BL1 subtype showed the highest number of CNAs, with amplification of genes including *MYC*, *PIK3CA*, cyclin dependent kinase 6 (*CDK6*), AKT serine/threonine kinase 2 (*AKT2*), KRAS proto-oncogene, GTPase (*KRAS*), fibroblast growth factor receptor 1 (*FGFR1*), insulin like growth factor 1 receptor (*IGF1R*), cyclin E1 (*CCNE1*), and cyclin dependent kinase inhibitors (*CDKN2A/B*), and hemi-/homozygous deletions of genes involved in DNA repair such as *BRCA2*, *PTEN*, MDM2 proto-oncogene (*MDM2*), RB transcriptional corepressor 1 (*RB1*), and *TP53* [390]. Understanding the biology of TNBC along with the specifics of its heterogeneity will pave the way to further develop targeted therapy and better predict responses to chemotherapeutic agents.

Compelling evidence indicates that within a cancer, there is a subpopulation of cells known as tumor-initiating cells or cancer stem-like cells (CSCs) that are responsible for the development, progression and metastasis of the tumor [46, 405]. CSCs can self-renew through division or give rise to the bulk of tumor cells in the mass through differentiation [406, 407]. Previous studies have shown putative breast CSCs are resistant to chemotherapy and are more aggressive [408]. TNBC cells have consistency demonstrated CSC-signatures and the functional role of CSCs in breast tumorigenesis and tumor biology, targeting CSCs may be a useful therapeutic approach to prevent breast cancer cell proliferation and relapse in this aggressive breast cancer subtype [409]. The goal of this chapter is to examine the effects of vitamin D compounds in targeting BCSCs in TNBC. We assessed the mammosphere forming ability of SUM159 cells, a TNBC cell line, and

investigated the changes in the markers of pluripotency, stem cell maintenance, and lineage-specific differentiation in the mammospheres treated with vitamin D compounds.

2.2 Materials and Methods

2.2.1 Cell culture and reagents

1α,25$(OH)_2D_3$ and a Gemini vitamin D analog (BXL0124; 1α25-dihydroxy-20R-21(3-hydroxy-3-deuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-cholecalciferol, >95% purity) were provided by BioXell, Inc. (Nutley, NJ) [410]. SUM159 breast cancer cells (RRID: CVCL_5423) were obtained from Asterand (Detroit, MI). SUM159 cells were grown in Ham's F-12 culture medium supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, 1 μg/ml hydrocortisone and 5 μg/ml insulin at 37°C and 5% CO_2.

2.2.2 Mammosphere forming assay

SUM159 cells were grown to 50-60% confluence and cells were detached with StemPro Accutase (Life Technologies, CA). Cells were then plated at 2,000 cells/mL in 6-well ultra-low attachment plates and maintained in Mammocult serum-free medium supplemented with hydrocortisone and heparin (Stem Cell Technologies, Vancouver, Canada). Cells were treated with 1α,25$(OH)_2D_3$ or BXL0124 for five days for each passage. For secondary and tertiary mammosphere culture, primary mammospheres were collected and enzymatically dissociated using StemPro Accutase (Life Technologies, CA). Then, cells were re-plated at a density of 2,000 cells/mL for subsequent passages. Images of mammospheres were taken, and the number of mammospheres was counted to

determine the mammosphere forming efficiency (MFE). The MFE was calculated by dividing the number of mammospheres (≥100 μm) formed by the number of single cells seeded. Experiments were repeated three times.

2.2.3 Western blot analysis

Whole cell lysates (15 μg/lane) were resolved in 4% to 20% SDS-PAGE from Bio-Rad (Hercules, CA). Blots were then probed with the indicated antibodies. Primary antibodies against c-NOTCH1 (1:1000, Cat# 4147, RRID: AB_2153348), Cytokeratin 18 (1:1000, Cat# 4548, RRID: AB_2296725) and OCT4 (1:1000, Cat# 2750, RRID: AB_823583) were from Cell Signaling Technology (Beverly, MA); β-actin (1:2000, Cat# A1978, RRID: AB_476692) was from Sigma-Aldrich (St. Louis, MO). Secondary antibodies were from Cell Signaling Technology. Western blot images are quantified by using GeneGnome XRQ chemiluminescence imaging system and analyzed by GeneTools analysis software from Syngene (MD, USA).

2.2.4 Quantitative polymerase chain reaction analysis

The Taqman® probe- based gene expression system from Applied Biosystems (Foster City, CA) was used to detect the genes of interest. The procedures were followed as described previously [411]. Primers used for analysis are GAPDH (Hs02758991), CD44 (Hs01075861), LAMA5 (Hs00966585), CD24 (Hs00175569), NOTCH1 (Hs01062014), JAG1 (Hs00164982), JAG2 (Hs00171432), NFKB1 (Hs00765730), OCT4 (POU5F1) (Hs00999632), NOTCH2 (Hs01050702), NOTCH3 (Hs01128537), HES1 (Hs00172878), KRTN14 (Hs00265033), KRTN18 (Hs02827483), ACTA2 (Hs00426835) and KRTN5

(Hs00361185). Experiments were repeated three times in duplicates.

2.2.5 Statistical analysis

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

2.3 Results

2.3.1 Inhibition of mammosphere forming efficiency by 1α,25(OH)$_2$D$_3$ and BXL0124 in SUM159 breast cancer cells.

Varying doses of 1α,25(OH)$_2$D$_3$ and BXL0124 were tested for their effectives on mammosphere forming efficiency (MFE) in SUM159 cells. MFE was significantly reduced with 1α,25(OH)$_2$D$_3$ at 10 nM (44.7% inhibition, p<0.05) and 100 nM (46.3% inhibition, p<0.05). BXL0124 reduced the MFE at 1 nM (50.4% inhibition, p< 0.05), 10 nM (52.8% inhibition, p<0.05) and 100 nM (76.4% inhibition, p<0.05) **(Figure 2.1)**. BXL0124 was more potent than 1α,25(OH)$_2$D$_3$ at the concentrations tested.

2.3.2 Inhibition of mammosphere self-renewal by 1α,25(OH)2D3 and BXL0124 in SUM159

To assess the mammosphere self-renewal capacity as an indicator of stemness, SUM159 spheres were grown in mammosphere cell culture media for three successive passages. MFE was increased from primary to secondary mammospheres (1.03% to 1.45%) and from secondary to tertiary mammospheres (1.45% to 3.11%) in SUM159 cells **(Figure 2.2)**. Treatment with vitamin D compounds significantly decreased the number of

mammospheres in each passage of mammosphere culture. The MFE of primary mammospheres was reduced upon treatment with 100 nM 1α,25(OH)$_2$D$_3$ (60.6% inhibition, p<0.01) or 10 nM BXL0124 (64.7% inhibition, p<0.01). Similarly, the MFE in secondary and tertiary mammospheres was decreased with 1α,25(OH)$_2$D$_3$ (60.7% inhibition, p<0.01 and 69.7% inhibition, p<0.01) and BXL0124 (62.4% inhibition, p<0.01 and 71.6% inhibition, p<0.01) **(Figure 2.2A)**. Mammospheres treated with vitamin D compounds exhibited more round and smooth edges compared to those of control group **(Figure 2.2B)**.

2.3.3 Repression of pluripotency markers and cancer stem cell genes by 1α,25(OH)$_2$D$_3$ and BXL0124 in SUM159

To further investigate the effect of vitamin D compounds on cancer stem-like cells in TNBC, we analyzed SUM159 mammospheres treated with vitamin D compounds for expression of pluripotency and stem cell genes which have been shown to be important in breast cancer progression [412]. The pluripotency marker, OCT4, was greatly reduced by treatment with 100 nM 1α,25(OH)$_2$D$_3$ (29%, p<0.01) and 10 nM BXL0124 (39%, p<0.05) **(Figure 2.3A)**. Levels of CD44 and LAMA5, markers associated with stem cell maintenance, were decreased with vitamin D compounds. Levels of CD44 mRNA were decreased by 35% with 1α,25(OH)$_2$D$_3$ (p<0.001) and 48% with BXL0124 (p<0.01). LAMA5 level was decreased by 43% with 1α,25(OH)$_2$D$_3$ (p<0.01) and 59% with BXL0124 (p<0.05). NFκB1, a key molecule involved in stem cell signaling, was significantly reduced by both compounds: 39% by 1α,25(OH)$_2$D$_3$ (p<0.05) and 52% by BXL0124 (p<0.01). Western blot analysis showed that OCT4 was increased from primary

to secondary mammospheres in the control group, whereas treatment with vitamin D compounds reduced the protein levels in both primary and secondary mammospheres **(Figure 2.3B)**. The decrease in protein levels of OCT4 mirrored the inhibition of mRNA expression by vitamin D compounds.

2.3.4 Repression of Notch signaling molecules responsible for stem cell maintenance by 1α,25(OH)$_2$D$_3$ and BXL0124 in SUM159

Notch signaling has been shown to play a fundamental role in embryonic development, cell differentiation, tissue homeostasis, and stem cell maintenance [413]. In normal breast stem cells, activation of Notch signaling promotes stem cell self-renewal and differentiation of progenitor cells [414]. Clarke *et al.,* showed that Musashi1 and Notch1 signaling regulated human breast cancer cells [415]. High Notch activity in breast cancer cells increased mammosphere formation and expression of breast cancer stem cell markers [416]. In this study, therefore, we investigated whether vitamin D compounds regulate the expression of key molecules in this family involved in stem cell maintenance. The NOTCH1 mRNA level was decreased by 6% and 48% with 1α,25(OH)$_2$D$_3$ and BXL0124 (p<0.01), respectively (Figure 2.4A). NOTCH2 mRNA level was decreased by 16% and 43% with 1α,25(OH)$_2$D$_3$ (p<0.01) and BXL0124 (p<0.05). NOTCH3 mRNA level was decreased by 52% with 11α,25(OH)$_2$D$_3$ (p<0.01) and 62% with BXL0124 (p<0.05). Ligand JAG1 expression was decreased by 48% and 66% with 1α,25(OH)$_2$D$_3$ (p<0.01) and BXL0124 (p<0.001), respectively. Ligand JAG2 expression was also decreased by 30% and 50% with 1α,25(OH)$_2$D$_3$ (p<0.05) and BXL0124 (p<0.05), respectively. HES1 expression was reduced by 19% and 40% with 1α,25(OH)$_2$D$_3$ and BXL0124 (p<0.05). The

levels of the activated form of NOTCH1 (cleaved-NOTCH1, c-NOTCH1) were increased from primary to secondary mammospheres in the control group, whereas the vitamin D compounds decreased c-NOTCH1 protein levels in both primary and secondary mammospheres **(Figure 2.4B)**.

2.3.5 Modulation of mammary epithelial lineage-specific differentiation markers by vitamin D compounds

Mammospheres in culture generally fail to express markers associated with breast lineage commitment and differentiation but can be induced to do so with differentiating stimuli, showing the plasticity and stem-like nature of the mammospheres [417]. In this study, therefore, we assessed expression of markers associated with myoepithelial/basal phenotype [cytokeratin 14 (CK14) and smooth muscle actin (SMA)] of vitamin D treatment. CK14 (KRTN14) mRNA level was increased by 70-fold with $1\alpha,25(OH)_2D_3$ ($p<0.01$) and 82-fold with BXL0124 ($p<0.05$) **(Figure 2.5A)**. Levels of SMA (ACTA2) mRNA were increased by 1.3-fold with $1\alpha,25(OH)_2D_3$ and 1.1-fold with BXL0124, but these increases were not statistically significant. CK18 (KRTN18), a marker associated with luminal/ductal cells, was down-regulated by $1\alpha,25(OH)_2D_3$ (24% inhibition, $p<0.05$) and BXL0124 (56% inhibition, $p<0.01$). Cytokeratin 5 (CK5, KRTN5), a biomarker for basal-like breast cancers and epithelial-mesenchymal transition, was significantly decreased upon treatment with vitamin D compounds. The mRNA levels of KRTN5 were decreased with 100 nM $1\alpha,25(OH)_2D_3$ (92% inhibition, $p<0.001$) and 10 nM BXL0124 (97% inhibition, $p<0.001$). Western blot analysis of primary and secondary mammospheres demonstrated that CK18 levels were similarly decreased by the treatment with

1α,25(OH)$_2$D$_3$ and BXL0124 **(Figure 2.5B)**.

2.4 Discussion

The nutritional importance of vitamin D compounds has long been known but it has also been appreciated for some time that vitamin D and its analogs have anti-proliferative and chemopreventive effects in solid tumors [418]. In our previous studies, we have reported that Gemini vitamin D analog BXL0124 mammary tumor growth and invasion [297, 419]. BXL0124 targets BCSC subpopulation and putative stem cell markers in MCF10DCIS cells [300]. It inhibits the transition of DCIS to IDC by maintaining myoepithelial cell layer and membrane while decreasing cell proliferation and tumor volume [296]. Furthermore, oral administration of BXL0124, and a synthetic triterpenoid, CDDO-IM has shown to delay MMTV-ErbB2/neu-induced mammary tumor formation by decreasing activation of ErbB2, which is overexpressed in 20% of human breast cancer, and downstream targets including activated-Erk1/2, activated-Akt, Bcl2, CycD1, c-Myc, p21, and PCNA [299].

Preclinical studies showing the potentiating effect of vitamin D compounds in tumor inhibition have led to combination studies in clinical trials [420]. Given the potent efficacy of BXL0124 in both *in vivo* and *in vitro* studies with several models of breast cancer, BXL0124 could be a potentially promising agent to be tested in clinical trials as a single preventive agent or in combination with others. The tumor inhibitory effects of vitamin D compounds, including 1α,25(OH)$_2$D$_3$ and a Gemini vitamin D analog BXL0124, are mediated through signaling pathways involved in cancer stem cell signaling, cell cycle suppression and differentiation pathways [421]. TNBC cell lines, such as SUM159, exhibit

predominantly patterns of basal cell surface markers with some minor subpopulations of stem-like or luminal type [422]. The sorted stem-like subpopulation, $CD44^{hi}CD24^{neg}EpCAM^{lo}$, of SUM159 cells can readily form tumors when injected into NOD/SCID mice with as few as 100 cells, and this subpopulation of cells expressed high colony forming unit capacity, and elevated spheroid formation, resistance to chemotherapy and ability to reconstitute the parental cell line, which are features of self-renewal characteristics of cancer stem cells and tumorigenicity [309]. In this study, we found that vitamin D compounds decreased SUM159 mammosphere formation in association with down-regulation of expression of key markers of cancer stem cell phenotype and maintenance. These findings point to possible mechanistic links between cancer stem cell signaling and VDR pathways regulating the tumor growth, suggesting that vitamin D compounds may be used as chemopreventive agents targeting the cancer stem cell population to prevent tumor development in triple negative breast cancer. More potent dose dependent decrease in MFE in mammospheres treated with BXL0124 compared to $1\alpha,25(OH)_2D_3$, suggesting that BXL0124 is more effective agent to selectively target cancer stem cells in triple negative breast cancer.

OCT4 is a critical transcription factor in adult stem cell reprogramming to give rise to induced pluripotent stem cells [136]. Interestingly, somatic cell reprogramming and tumorigenesis share common mechanisms [423]. Aberrant expression of *Oct4* and other key pluripotency markers are associated with abnormal cell growth and tumor formation [424, 425]. Kumar *et al.* demonstrated that over-expression of *OCT4* gene contributed to de-differentiation of melanoma cells to CSC-like cells, while RNAi knockdown of *OCT4* in de-differentiated melanoma cells led to diminished CSC phenotypes [426]. In our study,

we found that both mRNA and protein levels of OCT4 decreased with vitamin D compound treatment in mammospheres. This indicates an important role for vitamin D compounds in regulating a key transcription factor of cancer stem cells.

Myoepithelial cells are localized between luminal cells and stroma, maintaining tissue integrity and polarity in normal breast tissue [427]. Once breast cells are transformed into tumor cells, normal tissue architecture and polarity are lost. This is followed by a decrease in differentiated myoepithelial cells surrounding the tumor [428, 429]. Thus, myoepithelial cells appear to play a natural suppressive role limiting tumor growth and invasion [430]. Upregulation of myoepithelial markers in SUM159 mammospheres by vitamin D compounds suggest that the tumor inhibitory effects of these compounds are mediated by inducing the cancer stem cells into more mature differentiated cell types **(Figure 2.6)**.

CD44 is a transmembrane glycoprotein that is involved in malignant progression and metastasis of breast cancer [431]. Knockdown of CD44 induces differentiation and drives the breast cancer stem cell-like population toward a non-stem cell-like phenotype [41]. We show here that vitamin D compounds reduced the CD44 mRNA transcript levels in mammospheres, suggesting that vitamin D compounds may induce differentiation of breast cancer cells. In addition, LAMA5 is a signature extracellular matrix component in human pluripotent stem cells [432]. shRNA knockdown of LAMA5 reduced self-renewal capacity of human pluripotent stem cells [432]. Vitamin D compounds decreased LAMA5 levels in mammospheres, indicating that vitamin D compounds may regulate self-renewal of breast cancer stem cells.

2.5 Conclusions

Triple-negative breast cancer has a higher rate of relapse and poorer prognosis than other major breast cancer types [433]. The basal-like subtype of TNBC is found to exhibit constitutively high level of NF-κB signaling that, in turn, up-regulates JAG1 expression and activates NOTCH signaling, leading to expansion of cancer stem cells [434]. Notch signaling is important for normal mammary stem cell maintenance during development. It is required for self-renewal of mammary stem cells and activation of the pathway increased secondary mammosphere formation by 10-fold [435]. Interestingly, vitamin D compounds repressed the components of Notch-signaling axis including NF-κB, NOTCH1, NOTCH2, NOTCH3, JAG1, JAG2 and HES1. These findings highlight the therapeutic potential of vitamin D compounds that target Notch signaling in cancer stem cells. Overall, therefore, our study suggests that vitamin D compounds may be useful agents to prevent or impede progression in triple negative breast cancer by targeting cancer stem cell populations.

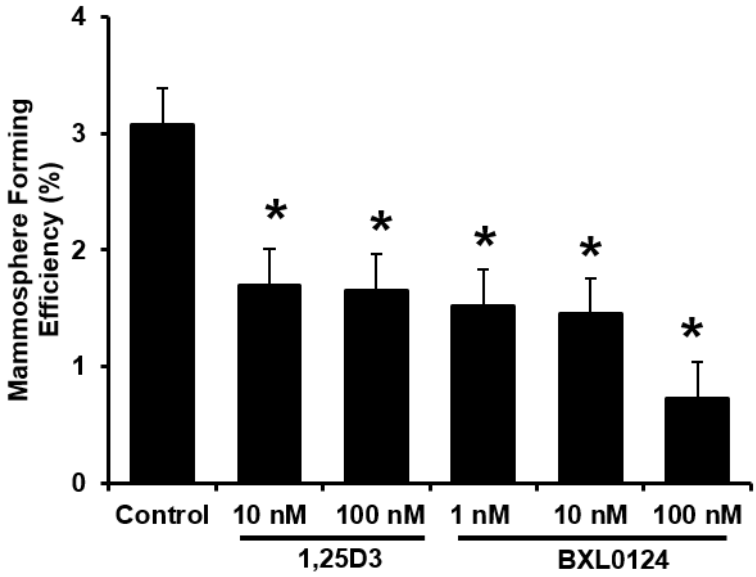


Figure 2.1 Inhibition of mammosphere forming efficiency (MFE) by 1α,25(OH)$_2$D$_3$ and BXL0124 in SUM159 breast cancer cells

SUM159 cells were plated at a density of 2,000 cells/ml in ultra-low attachment 6-well plates and grown for 5 days in the presence of 1α,25(OH)$_2$D$_3$ (1,25D3, 10 nM and 100 nM) and BXL0124 (1 nM, 10 nM and 100 nM). Mammosphere forming efficiency is calculated by dividing the number of mammospheres (≥100 μm) formed by the number of cells seeded, presenting this as a percentage. Three independent experiments were performed. The data are presented as the mean ± S.E.M. * p<0.05.

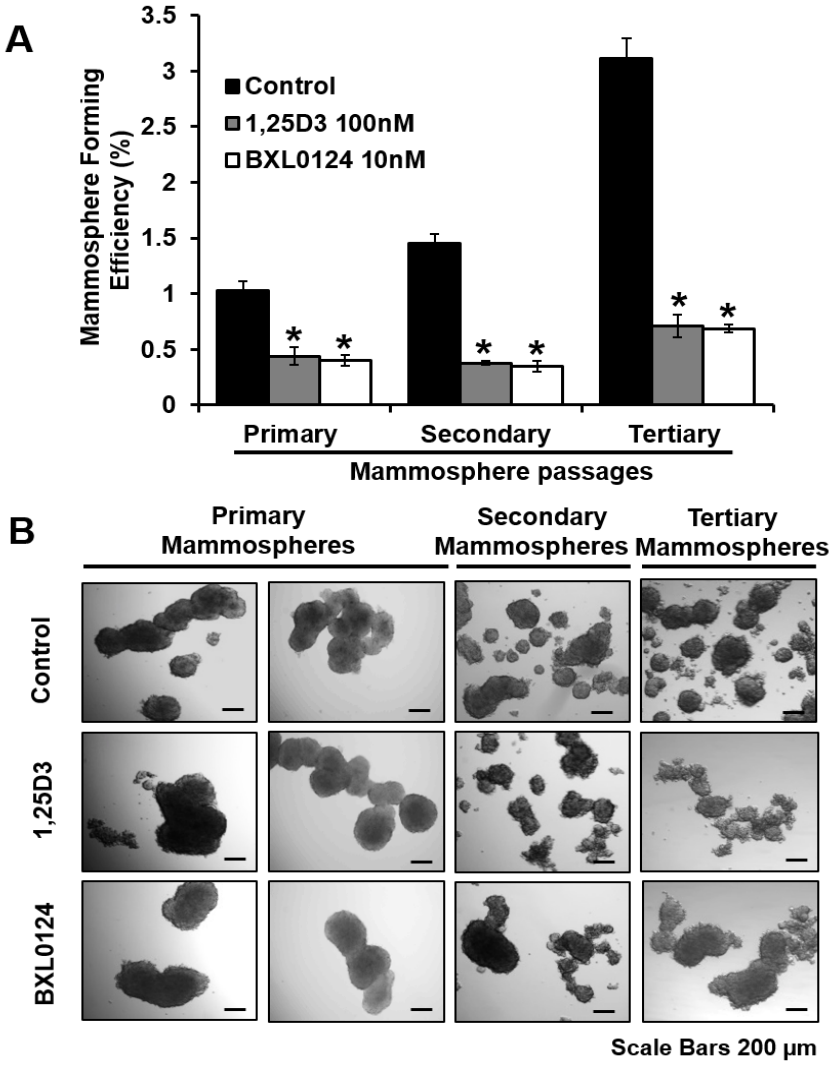


Figure 2.2 Inhibition of mammosphere self-renewal by 1α,25(OH)$_2$D$_3$ and BXL0124 in SUM159 mammospheres

(A) MFE of primary, secondary and tertiary passages of SUM159 mammospheres are shown. Mammospheres were treated with 1α,25(OH)$_2$D$_3$ (1,25D3, 100 nM) and BXL0124 (10 nM) for 5 days. Three independent experiments were performed. The data are presented as the mean ± S.E.M. *p<0.05. **(B)** Representative images of SUM159 mammospheres from primary, secondary and tertiary passages are shown for morphological comparison (scale bar 200 µm)

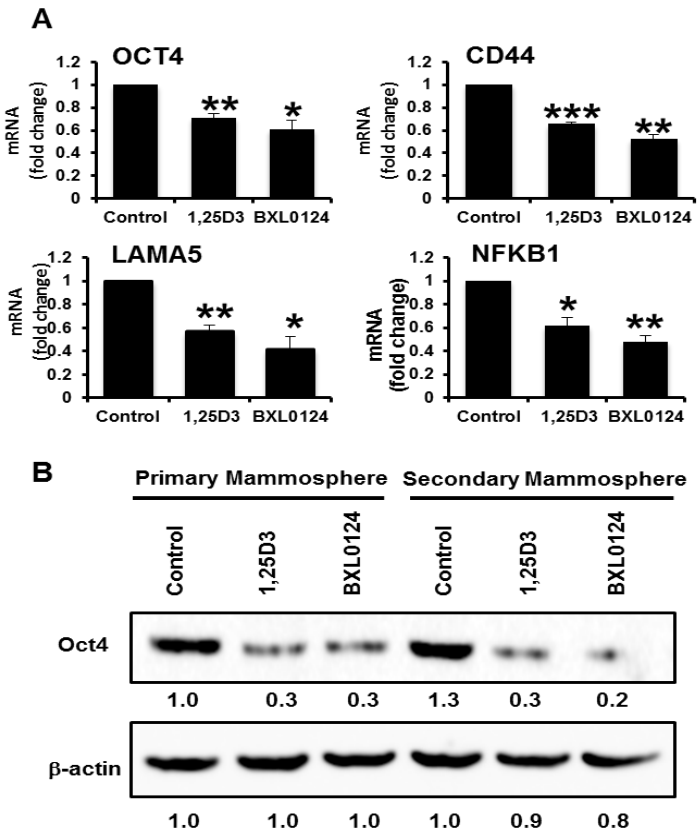


Figure 2.3 Repression of pluripotency and stem cell markers by 1α,25(OH)$_2$D$_3$ and BXL0124 in SUM159 mammospheres

Mammospheres were treated with 1α,25(OH)$_2$D$_3$ (1,25D3, 100 nM) and BXL0124 (10 nM) for 5 days. **(A)** qPCR analysis was performed on primary mammospheres harvested after five days of growth to assess the gene expression of OCT4, CD44, LAMA5 and NF-κB. Average Ct values are shown in parenthesis for OCT4 (22), CD44 (19), LAMA5 (22), and NF-κB (23). The experiments were repeated three times. *p<0.05, **p< 0.01, ***p<0.001. **(B)** Western blot analysis of primary and secondary mammospheres treated with 100 nM 1,25D3 and 10 nM BXL0124 detected by anti-OCT4 antibody is shown. β-actin was used as a loading control. Protein levels are quantified by GeneTools analysis software.

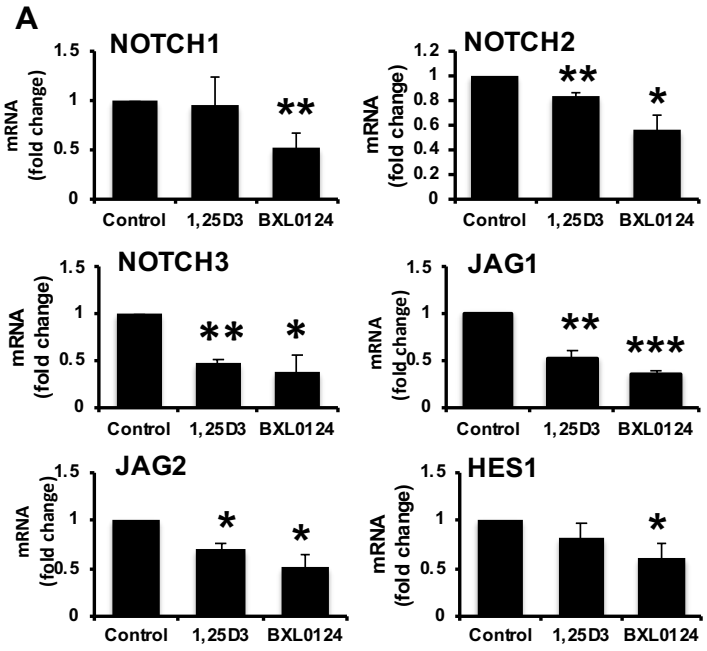


Figure 2.4 Repression of Notch signaling molecules by 1α,25(OH)$_2$D$_3$ and BXL0124 in SUM159 mammospheres

Mammospheres were treated with 1α,25(OH)$_2$D$_3$ (1,25D3, 100 nM) and BXL0124 (10 nM) for 5 days. **(A)** qPCR analysis was performed on primary mammospheres harvested after 5 days of growth to assess the gene expression of markers associated with the Notch signaling pathway – NOTCH1, NOTCH2, NOTCH3, JAG1, JAG2 and HES1. Average Ct values are shown in parenthesis for NOTCH1 (25), NOTCH2 (23), NOTCH3 (23), JAG1 (24), JAG2 (25) and HES1 (23). The data are presented as the mean ± S.E.M**.** *$p<0.05$, **$p<0.01$, ***$p<0.001$. **(B)** Western blot analysis of primary and secondary mammospheres treated with 100 nM 1,25D3 and 10 nM BXL0124 detected by c-NOTCH1 antibody. β-actin was used as a loading control. Protein levels are quantified by GeneTools analysis software.

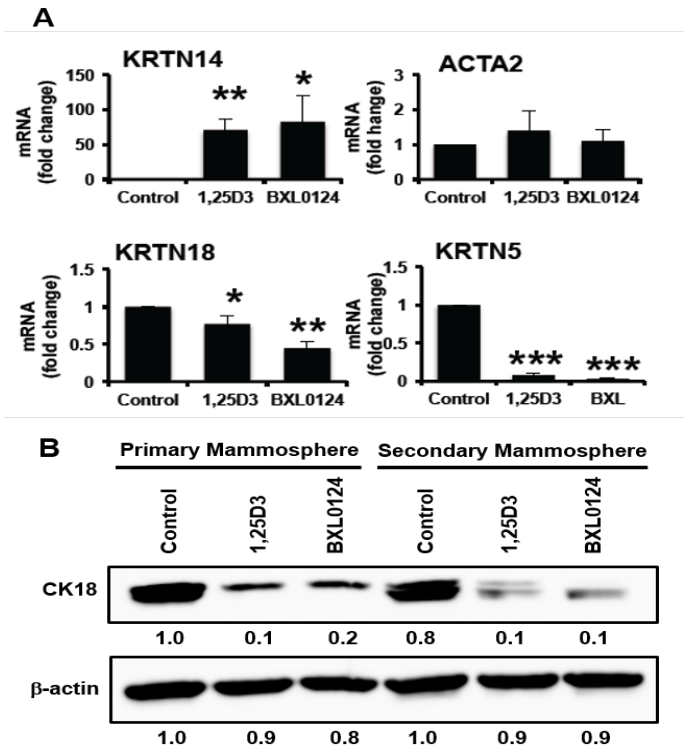


Figure 2.5 Induction of myoepithelial differentiation by vitamin D compounds

Mammospheres were treated with 1α,25(OH)$_2$D$_3$ (1,25D3, 100 nM) and BXL0124 (10 nM) for 5 days. **(A)** qPCR analysis of markers associated with myoepithelial cells (CK14, CK5, and ACTA2) and luminal/ductal cells (CK18) in SUM159 primary mammospheres harvested after 5 days of growth. Average Ct values for CK14 (control #35, 1,25D3 #29, BXL0124 #28); ACTA2 (control #25, 1,25D3 #25, BXL0124 #25); CK18 (control #19, 1,25D3 #20, BXL0124 #20) and CK5 (control #25, 1,25D3 #29, BXL0124 #30). Three independent experiments were performed. The data are presented as the mean ± S.E.M. * p<0.05, ** p<0.01, *** p<0.0001 **(B)** Western blot analysis of primary and secondary mammospheres treated with 100 nM 1,25D3 and 10 nM BXL0124 detected by CK18 antibody. β-actin was used as a loading control. Protein levels are quantified by GeneTools analysis software.

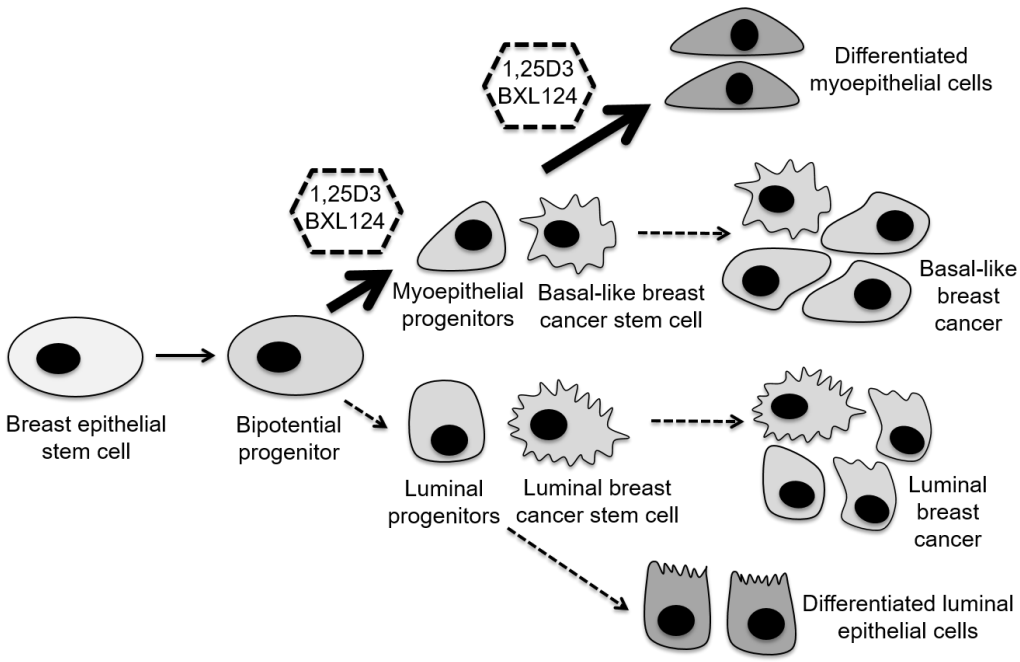


Figure 2.6 A proposed scheme of action of vitamin D compounds in cancer stem cells and differentiation pathway

The lineage diagram of cancer stem cells is modified from a previous publication [436] and the possible action sites of vitamin D compounds are shown.

Chapter 3: Elucidating the mechanism of transcription factor-mediated breast cancer stemness and reprogramming [8,9]

3.1. Introduction

Cancer stem cells (CSCs) are a small subpopulation of cells within tumors that drive uncontrolled tumor growth. They are capable of self-renewal, differentiating into multiple malignant cell types, and forming tumors when transplanted into a xenograft host. The CSC phenotype is defined by three core pluripotency transcription factors: octamer-binding transcription factor 4 (OCT4), SRY-box transcription factor 2 (SOX2), and homeobox protein Nanog (NANOG). These factors are expressed in both embryonic stem cells and CSCs, and play critical roles in maintaining an undifferentiated state [32].

Of these three basic transcription factors, OCT4 is the principal factor in the machinery governing pluripotency. It is a key regulator responsible for self-renewal and pluripotency in embryonic stem cells [437], and its expression is repressed during development and tissue differentiation. However, mounting evidence shows a reactivation of *OCT4* during physiological states such as wound healing and tumorigenesis [438]. Recent studies in clinical cases of invasive breast cancer demonstrated *OCT4* expression to be associated with aggressive tumor features, ALDH1 expression, tamoxifen resistance, and poor clinical outcome in hormone receptor-positive breast cancer [140] .

OCT4 transcript variants have detectable expression in human cancer cell lines, and their translation into proteins contributes to cell migration, invasion, and transformation

[8]**Keywords:** OCT4, transcription factor, breast cancer stem cells, reprogramming, stable overexpression

[9]**Abbreviations:** TNBC, triple negative breast cancer; CSCs, cancer stem cells; BCSCs, breast cancer stem cells

activities, providing evidence of the role of OCT4 in tumorigenicity and cancer stem cell phenotype [439]. Distinctive expression patterns of *OCT4* variants have been identified in different types of breast cancer: *OCT4A* and *OCT4B* are highly expressed in low-grade ductal tumors, whereas *OCT4B* is overexpressed in lobular type breast cancer. Expression of *OCT4* variants is also associated with the expression of estrogen receptor (*ER*), progesterone receptor (*PR*), erb-b2 receptor tyrosine kinase 2 (*HER2, ERBB2*), and tumor protein p53 (*TP53*) [440].

Phenotypically, resistance to chemo- or radiotherapy is among the hallmarks of CSCs. The function of OCT4 in the stemness-mediated resistance of BCSCs to chemotherapy and irradiation is of particular interest in breast cancer. Doxorubicin resistant-TNBCs showed increased CSC phenotype along with high expression of signal transducer and activator of transcription 3 (STAT3), OCT4, and c-Myc. Treatment with the STAT3 inhibitor WP1066 decreased phosphorylation of STAT3 and the expression of OCT4 and c-MYC, leading to a reduction in $CD44^+$ BCSC population and restoration of doxorubicin sensitivity [441]. OCT4 also confers resistance to irradiation by increasing clonogenic survival following irradiation and upregulating interleukin 24 (IL-24) production through STAT3 and nuclear factor kappa B (NF-κB) signaling [442].

Ultimately, a deeper understanding of the role of OCT4 in mediating stemness and resistance, and its interaction and interconnection with other markers and effectors of CSC function, is essential. Ectopic expression of *Oct4* in 4T1 mouse breast cancer cells increased tumorsphere formation and expression of stem cell markers such as CD133, CD34, Sca-1, and ALDH1 *in vitro* and tumorigenic potential *in vivo* [443]. Meanwhile, knockdown of *OCT4* in MCF-7 cells, which have a high basal level of OCT4, induces EMT

by upregulating α-smooth muscle actin and suppressing E-cadherin [144]. The goal of this chapter is to examine the significance of CSCs in breast tumorigenesis by elucidating the mechanism of OCT4 transcription factor-mediated breast cancer stemness and reprogramming. We utilized genetic manipulation of *OCT4* to assess its influence on putative markers of stemness *in vitro* and tumorigenicity *in vivo*.

3.2 Materials and Methods

3.2.1 Cell culture and reagents

SUM159 breast cancer cells (RRID: CVCL_5423) were obtained from Asterand (Detroit, MI). SUM159 cells were grown in Ham's F-12 culture medium supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, 1 μg/ml hydrocortisone and 5 μg/ml insulin at 37°C and 5% CO_2.

3.2.2 Mammosphere formation assay

SUM159 cells were grown to 50-60% confluence and cells were detached with StemPro Accutase (Life Technologies, CA). Cells were then plated at 2,000 cells/mL in 6-well ultra-low attachment plates and maintained in Mammocult serum-free medium supplemented with hydrocortisone and heparin (Stem Cell Technologies, Vancouver, Canada).

3.2.3 Western blot analysis

Whole cell lysates (15 μg/lane) were resolved in 4% to 20% SDS-PAGE from Bio-Rad (Hercules, CA). Blots were then probed with the indicated antibodies. Primary

antibodies against OCT4 (1:2000) was from Abcam (Cat# ab181557, RRID: AB_2687916); CD44 (1:500) was from Santa Cruz Biotechnology (Cat# sc-7297, RRID: AB_627065); p-NF-κB p65 (Ser536) (1:1000, Cat# 3033), NF-κB p65 (D14E12) (1:1000, Cat# 8242), and SOX2 (D6D9) (1:1000, Cat# 3579, RUID: AB_2195767) were from Cell Signaling Technology (Beverly, MA); and β-actin (1:2000) was from Sigma-Aldrich (Cat# A1978, RRID:AB_476692). Secondary antibodies were from Cell Signaling Technology. Western blot images are quantified by using GeneGnome XRQ chemiluminescence imaging system and analyzed by GeneTools analysis software from Syngene (MD, USA).

3.2.4 Transient transfection

SUM159 cells were transfected with pLKO.1 (Cat# 8453, RRID: Addgene_8543) and pSin-EF2-Oct4-Pur (Cat# 16579, RRID: Addgene_16579) plasmids from Addgene (Watertown, MA) for 48 hours using FuGENE HD transfection reagent (Catalog number: E2311) from Promega (Madison, WI) following the manufacturer's instructions.

3.2.5 Immunofluorescence analysis

SUM159 cells grown in 35 mm confocal dishes were fixed with 4% paraformaldehyde and blocked in 10% goat serum. The slides were then incubated overnight at 4ºC with the combination of primary antibodies to OCT4 (1:200) from Cell Signaling Technology (Cat# 2750, RRID: AB_823583), CD44 (1:200) from Santa Cruz Biotechnology (Cat# sc-7297, RRID: AB_627065) and TO-PRO-3 iodide nuclear antibody (Invitrogen, 1μM). The slides were incubated with fluorophore-conjugated secondary antibodies (Alexa Fluor 488 or 546, 1:100, Invitrogen, Carlsbad, CA) at room temperature for 30 minutes. Images were taken using confocal microscopy at laser excitation

wavelengths 488 nm, 546 nm and 633 nm (TO-PRO-3). Immunofluorescence was analyzed using Nikon Eclipse C1 plus confocal microscope system.

3.2.6 Generation of stable cell lines

SUM159 cells were transfected with pLenti-CMV-GFP-2A-Puro-Blank Vector (Cat# LV590) and pLenti-GIII-CMV-GFP-2A-Puro POU5F1 Vector (Cat# LV268791) from Applied Biological Materials Inc. (British Columbia, Canada). Puromycin (0.6 μg/ml) was used as the selection antibiotic. Individual clones were isolated using cloning cylinders.

3.2.7 Flow cytometry

For monolayer culture, SUM159 cells were grown for 48 hours in 100 mm tissue culture dishes. For mammosphere cultures, cells were plated at a density of 4000 cells/well and grown for 5 days. Cells were detached with StemPro Accutase (Life Technologies, Carlsbad, CA) and prepared following the procedure previously described [297]. Cells were stained with antibodies against CD44-APC (Cat. 559942), CD49f-FITC (Cat. 561893) and ESA-APC (Cat. 347200) from BD Biosciences (San Jose, CA) and CD24-PE (Cat. 12024742) from Life Technologies (Carlsbad, CA) for 45 minutes. The stained cells were analyzed with FC500 Analyzer (Beckman Coulter) to determine the percentage of different CD44/CD24, CD49f/CD24 and ESA/CD24 subpopulations. The acquisition of ≥10,000 cells per sample was analyzed.

3.2.8 Aldefluor assay

The ALDEFLUOR Kit (Cat. 01700) from StemCell Technologies (Cambridge, MA) was used to measure ALDH activity. Cells were harvested and stained according to the manufacturer's protocol. Cells ($2x10^5$ cells) were incubated in ALDEFLUOR Assay buffer containing ALDH substrate (300 μM) for 45 minutes. For each experiment, additional sample of cells were stained under identical conditions with 1.5 mM of DEAB (diethylaminobenzaldehyde) reagent, a specific ALDH inhibitor, as a negative control. The samples were then analyzed by flow cytometry by measuring ALDEFLUOR fluorescence vs. side scatter (SSC) histogram.

3.2.9 Xenograft study

All xenograft animal studies were performed with the approval of the Institutional Review Board for the Animal Care and Facilities Committee of Rutgers University. Female NU/NU nude mice (5-6 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and allowed to acclimate for the first two weeks before the injection with cells (7-8 weeks). Human SUM159 and MCF10DCIS cells were injected into the mammary fat pad on both sides or subcutaneously on both dorsal flanks at 10^6 cells per site. Animals were checked twice each week for body weight and tumor formation. Upon detection, tumor sizes were measured using a Vernier caliper and tumor volume (V: cubed centimeters) was calculated using the equation $V= D*d^2/2$ where D (centimeters) is the largest perpendicular diameter and d (centimeters) is the smallest perpendicular diameter. The experiment was terminated at the end of 4 weeks. Tumors from sacrificed animals were excised, weighed and snap frozen in liquid nitrogen for future analysis. A section of

tumor was also fixed in formalin for 24 hours and then embedded in paraffin for pathological analysis.

3.2.10 Immunohistochemical (IHC) Analysis

Paraffin embedded tumor tissues were sent to Research Pathology Services at Rutgers Translational Science for IHC analysis. Sections were stained with antibody against OCT4 (ab181557, 1:2000) from Abcam (Cambridge, MA). The sections were counterstained with Harris hematoxylin and eosin (H&E).

3.2.11 Statistical analysis

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

3.3 Results

3.3.1 Transient overexpression of OCT4 in SUM159 cells

To assess the consequences due to transient overexpression of OCT4 in SUM159 cells, cells seeded in monolayer culture are transfected with empty vector (pLKO.1-puro) or OCT4 (pSin-EF2-Oct4-Pur) plasmids for 48 hours. SUM159 parental cells were included as a control. The protein level of OCT4 was assessed by western blot. It confirms that OCT4 was significantly increased in cells transfected with OCT4-vector compared to non-transfected and empty vector transfected cells **(Figure 3.1)**.

3.3.2 Transient overexpression of OCT4 in SUM159 cells up-regulates protein levels of CD44 and p-NF-κB p65 (Ser536)

To characterize the regulatory role of OCT4 in modulating stemness related signaling, several markers pertinent to cancer stem cell identification (CD44), maintenance of pluripotency and differentiation (NF-κB, p-NF-κB p65 Ser536) and cancer stemness (Sox2) were analyzed in SUM159 cells with transiently introduced exogenous OCT4 [42, 444, 445]. Since OCT4 and SOX2 are known to regulate reciprocally in embryonic cells, we seek to determine SOX2 levels in these OCT4 overexpressed cells [446]. SOX2 level was not detected in OCT4-overexpressed SUM159 cells. In contrast, CD44 expression was increased upon OCT4 overexpression. Similarly, phospho-NF-κB p65 (Ser536) level was also increased when OCT4 was overexpressed while total NF-κB level remained unchanged. These findings suggest that OCT4 modulates markers related to stemness and pluripotency **(Figure 3.2)**.

3.3.3 Enhanced OCT4 expression in SUM159 cells upregulates the protein level of CD44

In order to determine if CD44 marker expression is regulated in SUM159 cells by elevated OCT4, we assessed the immunofluorescence staining of CD44 in OCT4 overexpressing cells. OCT4 staining was found to be significantly higher in both the cytosol and nuclei of OCT4 overexpressing cells in contrast to control cells and localized to the cell membrane. These observations substantiated our previous finding that OCT4 regulates the protein level of CD44 in SUM159 cells **(Figure 3.3)**.

3.3.4 Establishing stable OCT4-overexpressing SUM159 cell line

To study the role of OCT4 in regulating cancer stemness and tumorigenesis *in vitro* and *in vivo*, OCT4-stable SUM159 cell line was established. Puromycin (0.6 μg/ml) was used as a selection antibiotic. SUM159 cells were transfected with pLenti-CMV-GFP-2A-Puro-Blank empty vector or POU5F1 pLenti-GIII-CMV-GFP-2A-Puro OCT4 vector. Stable colonies were selected and expanded. Individual clones were harvested to assess mRNA expression **(Figure 3.4A)** and protein level **(Figure 3.4B)** of OCT4. In addition, these stable clones were propagated to verify the persistent expression of OCT4 at multiple time points with or without puromycin **(Figure 3.5)**. The experiment generated eight OCT4-overexpressed clones (C7, C9, C5, C3, C14, C11, C13 and C12) and three control vector clones (EV1, EV1-2 and EV4). Since C7 and C12 clones exhibited persistent overexpression of OCT4, we selected these two clones for further experiments.

3.3.5 Assessment of putative breast cancer stem cell markers in SUM159 cells

To assess the basal levels of putative breast cancer stem cell markers in SUM159 cells, cells were grown in monolayer culture and analyzed for ALDH, CD24, CD49f and ESA levels [309]. SUM159 cells stained with ALDEFLUOR™ assay measuring ALDH enzymatic activity showed very low basal ALDH activity with or without DEAB, an ALDH enzyme inhibitor, compared to SK-BR-3 breast cancer cells **(Figure 3.6A)**. Since ALDH activity is observed very minimal in SUM159 cells, we proceeded to investigate additional stem cell markers. SUM159, EV1 and C7 cells were also stained for antibodies against CD24 and CD49f **(Figure 3.6B)** and CD24 and ESA **(Figure 3.6C)**. As shown in **Figure 3.6B**, CD24/CD49f staining was higher and more distributed in SUM159 cells

compared to EV1 and C7. $CD24^{+}/CD49f^{+}$ subpopulation was found to be absent in C7 clone compared to SUM159 and EV1 cells. Additionally, we observed changes in the level of CD24 but not ESA in C7 compared to SUM159 and EV1 cells. This indicates that OCT4-overexpression decreases CD24 expression in SUM159 cells.

3.3.6 Characterization of stable OCT4-overexpressing SUM159 cells

The most common approach to identify CSCs in tumors is through examining the expression of characteristic cell surface markers. The $CD44^{+}/CD24^{-/low}$ subpopulation is identified as tumorigenic BCSCs isolated from clinical patients [46]. The progenitor cells of this phenotype are more abundant in triple negative breast cancer and are associated with poor prognosis [447, 448]. To investigate the effect of OCT4 on the expression of BCSC surface markers, we performed flow cytometry analysis of C7 clone for putative BCSC marker CD44/CD24 staining. Control vector transfected (EV1) clone and SUM159 parental cells were included as controls. The $CD44^{+}/CD24^{-/low}$ subpopulation was decreased in C7 clone (12%) compared to EV1 (44%) and SUM159 parental cells (61%). Concomitantly, the $CD44^{+}/CD24^{high}$ subpopulation was increased in C7 (88%) compared to EV1 clone (56%) and SUM159 parental cells (39%) **(Figure 3.7A)**. These data indicate overexpression of OCT4 in SUM159 cells significantly changes into more stem-like, characteristic BCSC phenotype. In addition, the profile of $CD44^{+}/CD24^{-/low}$ in OCT4-overexpressed C12 clone was compared with those of SUM159 monolayer cells and mammospheres **(Figure 3.7B)**. The profiling of C12 clone showed similarity to that of mammosphere. These findings suggest the pluripotency transcription factor OCT4 mediated changes in BCSC surface markers in SUM159 cells.

3.3.7 Stable OCT4 overexpression did not change EMT markers in SUM159 cells

EMT is implicated in tumor metastasis and EMT-derived tumor cells are enriched with stemness characteristics including therapeutic resistance [449]. Increasing evidence has demonstrated that cancer stem cells play a significant role in tumor recurrence and metastasis. *OCT4*, a master gene for pluripotency, is known to regulate EMT, invasion and metastasis in cancers including colorectal cancer and breast cancer [450]. OCT4 and NANOG together have been shown to promote EMT in breast cancer [153]. To assess the effect of OCT4 on EMT markers in SUM159 cells, we investigated the levels of epithelial marker (E-cadherin) and mesenchymal markers (Vimentin and Snail) in stable OCT4-overexpressing cells by western blot. We found that stable overexpression of OCT4 did not change the levels of EMT markers in SUM159 cells **(Figure 3.8)**.

3.3.8 Stable OCT4-overexpression in SUM159 cells did not increase tumorigenicity *in vivo*

Since OCT4 overexpression changes the profile of SUM159 breast cancer cells into more stem-like profile, we further investigated the role of OCT4 in tumor formation *in vivo*. To assess the tumorigenicity of OCT4-overexpressed cells, we injected $1x10^6$ cells of C12 clone into nu/nu mice either subcutaneously into flanks or mammary fat pads on both right and left sides. SUM159 and MCF10DCIS cells were included as control groups. No significant difference in the number and size of the tumors was observed between SUM159 parental cells and C12 clone at 4 weeks after cell injection **(Figure 3.9A)**. In subcutaneous injection groups, both SUM159 and C12 cells formed tumors from three out of four

injection sites. Tumors were formed in two out of four injection sites when cells were injected into the mammary fat pad in both groups.

To investigate the histopathological features in xenograft tumors, excised tumor tissue samples were sent to Research Pathology Services at Rutgers Translational Science for pathology evaluation. Hematoxylin and eosin (H&E) staining showed excised tumor specimens from SUM159 parental cells and C12 clone histologically resembling mesenchymal tumors whereas MCF10DCIS cells produced epithelial tumors **(Figure 3.9B)**. SUM159 and C12 tumors contain whorls of neoplastic cells in fibrous stroma. In contrast, MCF10DCIS tumors encompass centers of distended ducts surrounded by neoplastic epithelial cells **(Figure 3.10)**. These findings are in accord with the previous studies showing that SUM159 cells form mesenchymal tumors and MCF10DCIS cells form epithelial tumors [296, 451]. In addition, immunohistochemical analysis of tumors showed some positive but scant staining of OCT4 in C12 tumors compared to absence of OCT4 staining in SUM159 parental cells **(Figure 3.11)**.

3.4 Discussion

Induced pluripotency and oncogenic transformation share common mechanisms [452]. The expression of master regulators of pluripotency such as OCT4 and SOX2 in different cancers is known to drive aggressive phenotypes and correlates with poor survival and therapeutic resistance [32, 146, 151]. Studies have shown that there is an increased expression of OCT4 in $CD44^{+}/CD24^{-}$ BCSCs and mammospheres [417, 453]. Previously, we observed OCT4 and other CSC markers were downregulated by vitamin D compounds in SUM159 cells. This observation led us to further investigate the OCT4-driven

pluripotency reprogramming and breast tumorigenesis by manipulating OCT4 in SUM159 cells. Since the basal level of OCT4 was undetectable these cells, we investigated the role of OCT4 in stemness and oncological characteristics by overexpressing OCT4. Transient overexpression of OCT4 in SUM159 cells increased the level of CD44, a key BCSC marker. However, CD44 has a dichotomous role in breast cancer progression and metastasis inhibition, suggestive of its complex nature in breast tumorigenesis. Therefore, OCT4-driven modulation of CD44 in breast cancer remains to be elucidated [454].

NF-κB signaling plays a role in maintaining undifferentiated state of human induced pluripotent stem (iPS) cells. Upon differentiation, NF-κB activity is reduced along with OCT4 and NANOG expression. Knock-down of NF-κB abolished the expression of OCT4 and NANOG without affecting colony growth and shape of human iPS cells [455]. Inhibiting NF-κB represses CD44 and decreased proliferation and invasiveness of TNBC cells [456]. We observed up-regulation of phospho-NF-κB p65, the activated form of NF-κB upon OCT4 overexpression. This finding suggests the possible existence of feedback regulation between NF-κB and OCT4 which subsequently activates CD44 expression.

High $CD44^{+}/CD24^{-}$ ratio is correlated with increased cell proliferation, mammosphere formation and tumorigenesis. It is conserved throughout metastasis in breast cancer [45]. In our study, stable clones of overexpression of OCT4 significantly increased $CD44^{+}/CD24^{-}$ ratio in SUM159 cells. Interestingly, it did not further increase EMT markers. We assessed the tumorigenicity of stable OCT4-overexpressed cells *in vivo*. OCT4-overexpressed cells did not change tumor formation frequency or tumor type compared to parental cells. Histological analysis showed that they are stromal tumors/fibroma. Immunohistochemical analysis demonstrated scantly OCT4-positive cells

in C12 clone-derived tumors compared to negative OCT4 staining in parental SUM159 tumors. More number of animals will be needed to confirm this finding.

3.5 Conclusions

A significant body of evidence indicates a key role of pluripotency-related transcription factors in breast tumorigenesis. A greater understanding of how OCT4 drives breast cancer stemness and progression will be beneficial for the development of BCSC-targeted therapies. We assessed the role of OCT4 in TNBC by first inducing its transient overexpression in SUM159 cells, after which we observed upregulation of CD44 (a BCSC marker) and p-NF-kB (NF-kB signaling). We further established stable OCT4-overexpressing cell line to assess its role in promoting tumorigenesis *in vivo*. The stable overexpression of OCT4 significantly increased the $CD44^{+}/CD24^{-}$ BCSC subpopulation relative to control cells; however, epithelial and mesenchymal markers were not affected. In a xenograft assay, mice injected with the cells stably overexpressing OCT4 did not demonstrate any increase in tumorigenicity when compared to the control group. This may be possibly due to the loss of OCT4 overexpressing cells in tumors. Notably, our preliminary study has only limited sample size. Further studies incorporating a larger number of xenografts are warranted to draw conclusions regarding OCT4-driven tumorigenesis.

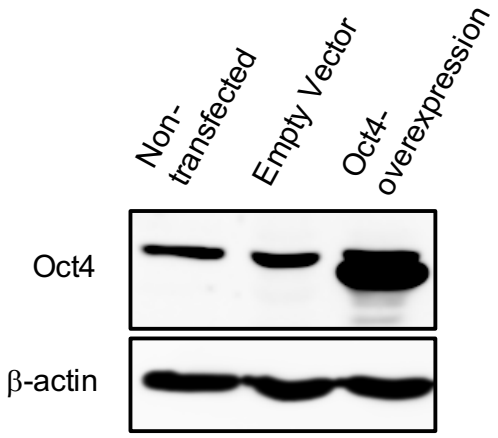


Figure 3.1 Transient overexpression of OCT4 in SUM159 cells

SUM159 cells were seeded in monolayer culture. Cells were transfected with empty vector (pLKO.1-puro) and OCT4 (pSin-EF2-Oct4-Pur) plasmids for 48 hours using FuGENE HD transfection reagent. Western blot detected by anti-OCT4 antibody is shown. β-actin was used as a loading control.

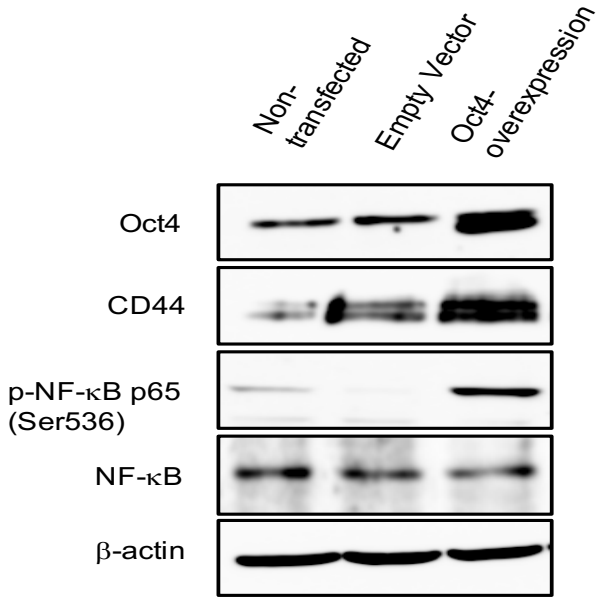


Figure 3.2 Transient overexpression of OCT4 in SUM159 cells up-regulates protein levels CD44 and p-NF-κB p65 (Ser536)

SUM159 cells were seeded in monolayer culture. Cells were transfected with empty vector (pLKO.1-puro) and OCT4 (pSin-EF2-Oct4-Pur) plasmids for 48 hours using Fusion HD transfection reagent. Cells were harvested for western blot analysis. Membranes were probed with antibodies against OCT4, CD44, p-NF-κB p65 (Ser536) and NF-κB. β-actin was used as a loading control.

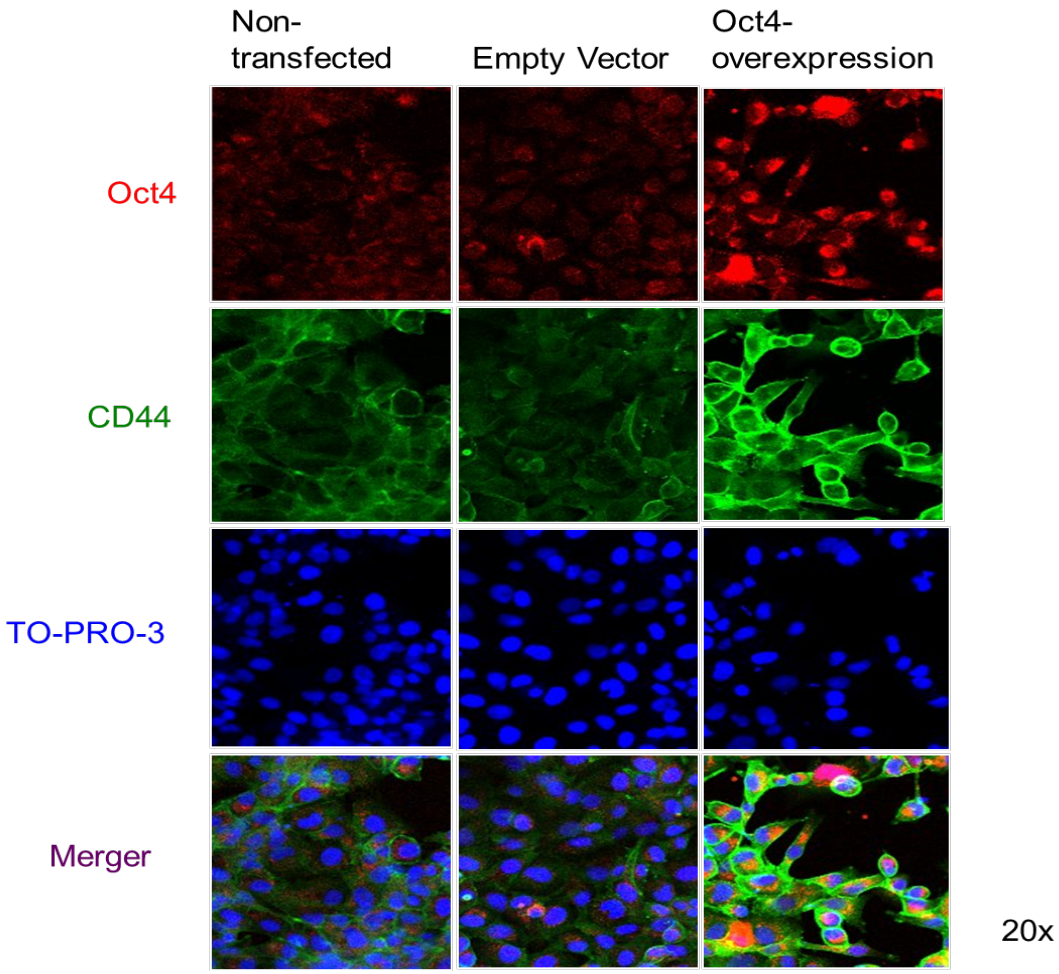


Figure 3.3 OCT4 increases the protein level of CD44 in SUM159 cells

SUM159 cells were transiently transfected with empty vector (pLKO.1-puro) and OCT4 (pSin-EF2-Oct4-Pur) plasmids for 48 hours in confocal dishes. Confocal immunofluorescent analysis was performed using OCT4 Rabbit mAb (Red) and CD44 mouse mAb (Green) antibodies. Nuclei were counterstained with blue-fluorescent TO-PRO-3 dye. Merger panels combine all three images. Confocal microscopy images were taken at 20X magnification.

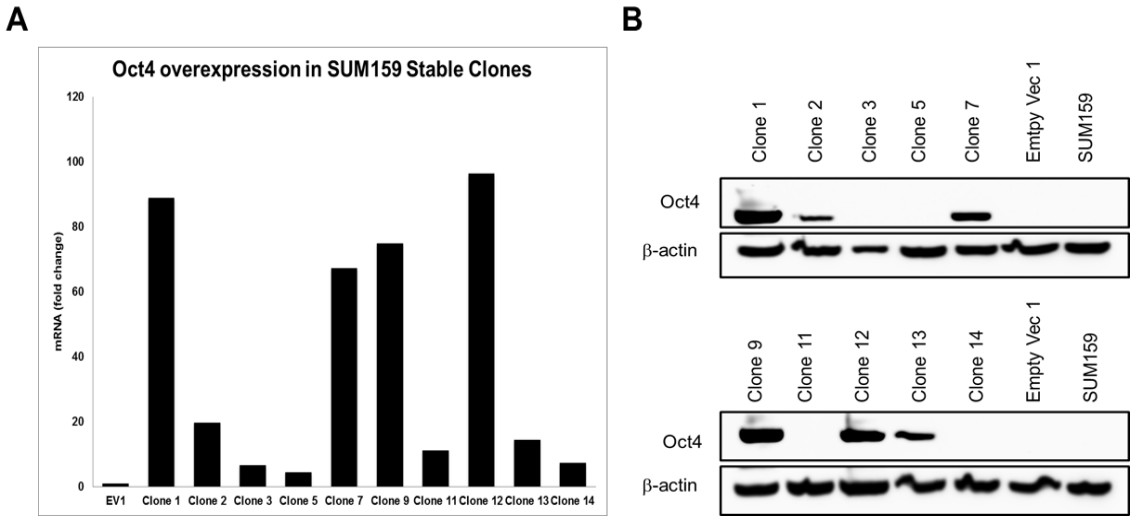


Figure 3.4 Stable overexpression of OCT4 in SUM159

SUM159 were transfected with lentiviral-based plasmids, pLenti-CMV-GFP-2A-Puro-Blank empty vector and POU5F1 pLenti-GIII-CMV-GFP-2A-Puro OCT4 vector. Puromycin (0.6 μg/ml) was used as the selection antibiotic. Individual clones were isolated using cloning cylinders. (A) qPCR analysis of the OCT4-overexpressing stable clones including empty vector clone 1 (EV1). (B) Western blot analyses of the OCT4-overexpressing stable clones including empty vector clone (Empty Vec 1) and SUM159 parental cell for comparison using anti-OCT4 antibody. β-actin was used as a loading control.

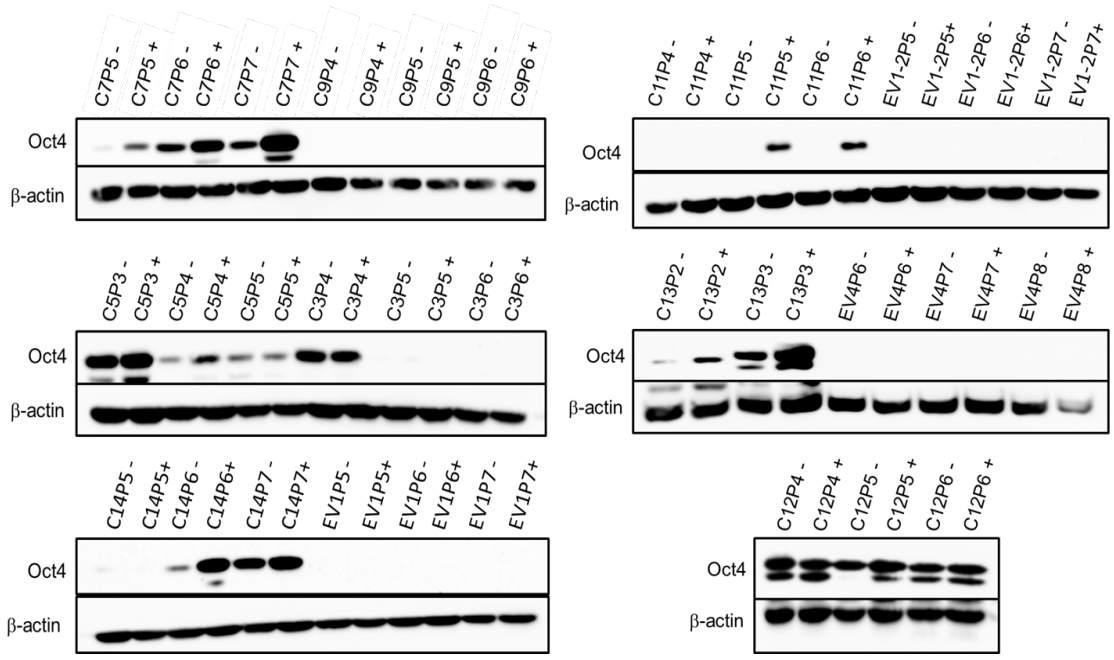
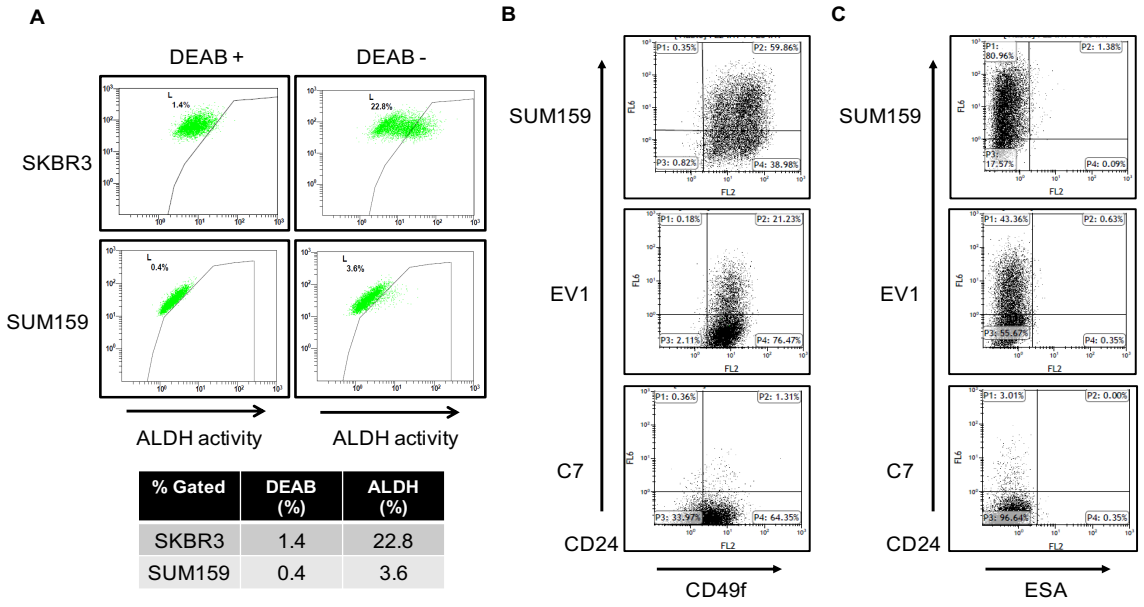


Figure 3.5 Persistent overexpression of OCT4 in SUM159 cells

SUM159 transfected with lentiviral-based plasmids, pLenti-CMV-GFP-2A-Puro-Blank empty vector and POU5F1 pLenti-GIII-CMV-GFP-2A-Puro OCT4 vector, were passed several passages with or without puromycin (0.6 μg/ml). Western blot analyses was performed to identify persistent OCT4-overexpressed clones (C) and empty vector clones (EV) upon successive passages (P) with or without (+/-) puromycin using anti-OCT4 antibody. β-actin was used as a loading control.



% Gated	DEAB (%)	ALDH (%)
SKBR3	1.4	22.8
SUM159	0.4	3.6

Figure 3.6 Levels of putative breast cancer stem cell markers in SUM159 cells

(A) Low activity of ALDH is detected in SUM159 cells**.** Representative ALDEFLUOR™ assay with FACS analysis to measure ALDH activity of SUM159 and SKBR3 human breast cancer cells. 300,000 cells were seeded in monolayer cell culture for 48 hr. Cells incubated with ALDEFLUOR™ reagent in the presence of DEAB, the inhibitor of ALDH activity, for 30 minutes were used to establish a baseline fluorescence. The ALDH-positive population was identified as the cells showing a right-shift in fluorescence upon incubation with ALDEFLUOR™ reagent in the absence of the DEAB. SKBR3 cells were stained as a positive control. Representative scatter plots from independent flow cytometry analyses are shown for SUM159, EV1 and C7 cells stained with combinations of antibodies against **(B)** CD24 and CD49f and **(C)** CD24 and ESA for 45 minutes.

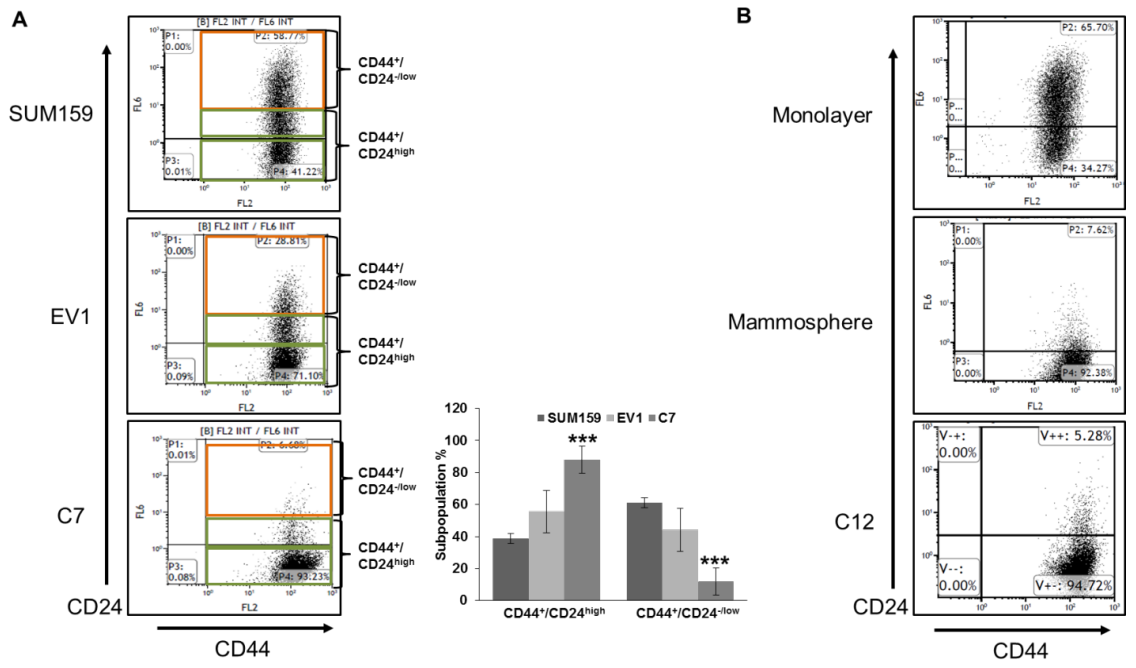


Figure 3.7 Stable overexpression of OCT4 in SUM159 cells increases the breast cancer stem cell subpopulations and mimics $CD44^{+}/CD24^{-/low}$ profile of mammospheres

(A) SUM159 parental cells, empty vector clone (EV1) and OCT4 overexpressed clone (C7) were stained with CD44 and CD24 antibodies for 45 minutes and flow cytometry was performed. Representative scatter plots from the flow cytometry are shown. Different subpopulations of cells based on varying levels of CD24 are highlighted with colored rectangles. The average percentage of $CD44^{+}/CD24^{high}$ and $CD44^{+}/CD24^{-/low}$ subpopulations from three independent experiments are represented as a bar graph to show the difference between SUM159, EV1 and C7 groups. The data are presented as the mean ± S.D., *** $p<0.001$. **(B)** SUM159 monolayer, mammospheres and OCT4-overexpressed clone (C12) were analyzed for levels of putative breast cancer stem cell markers – CD44 and CD24. Cells were stained with combination of antibodies against CD44 and CD24 for 45 minutes and flow cytometry analysis was performed. Representative flow cytometry

scatter plots of cells harvested from SUM159 monolayer, mammosphere and C12 are shown for profile comparison.

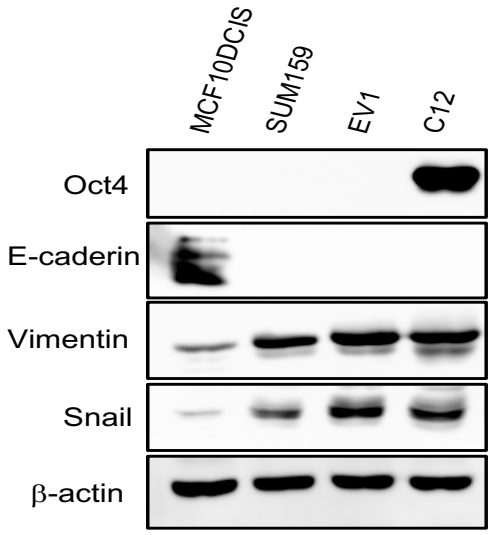


Figure 3.8 Stable OCT4-overexpression does not change EMT markers in SUM159 cells

SUM159 parental cells, empty vector clone (EV1), OCT4-overexpressed clone (C12) and MCF10DCIS cells grown in monolayer culture were harvested for western blot analysis of E-cadherin, vimentin and Snail. Anti-OCT4 antibody was used to assess OCT4 level in all cell lines tested. β-actin was used as a loading control.

A

# cells injected per site	SUM159 (# tumors formed/ # sites injected)	C12 (# tumors formed/ # sites injected)	MCF10DCIS (# tumors formed/ # sites injected)
10^6 SC	3/4	3/4	6/6
10^6 MFD	2/4	2/4	6/6

B

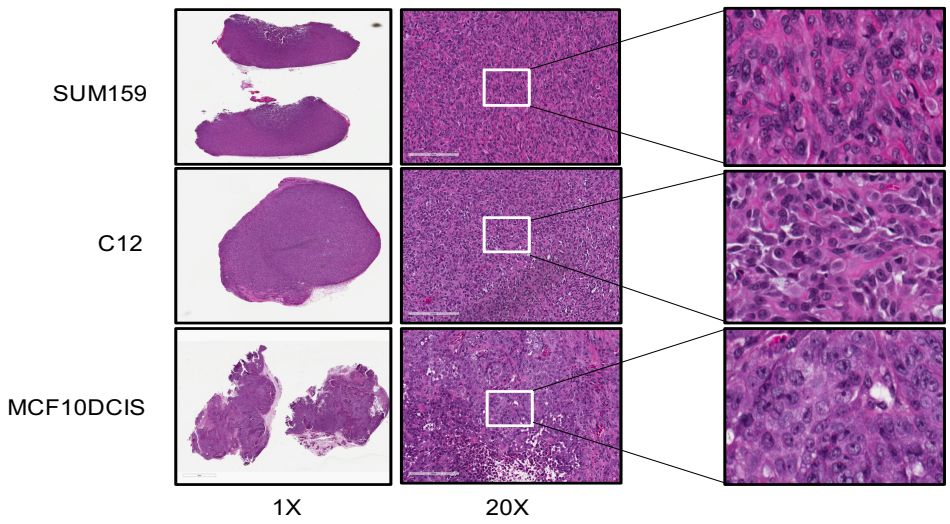


Figure 3.9 SUM159 xenograft tumorigenicity assay in nu/nu mice

(A) SUM159, C12, and MCF10DCIS cells were injected subcutaneously (SC) (10^6 cells) into both flanks or into mammary fat pad (MFD) (10^6 cells) of both left and right sides in nu/nu mice. Tumors were harvested at the end of 4 weeks. **(B)** Representative hematoxylin and eosin (H&E) staining showing the histology of xenograft tumors injected with SUM159, C12 and MCF10DCIS cells at 1X and 20X magnifications. Images were taken with ScanScope XT.

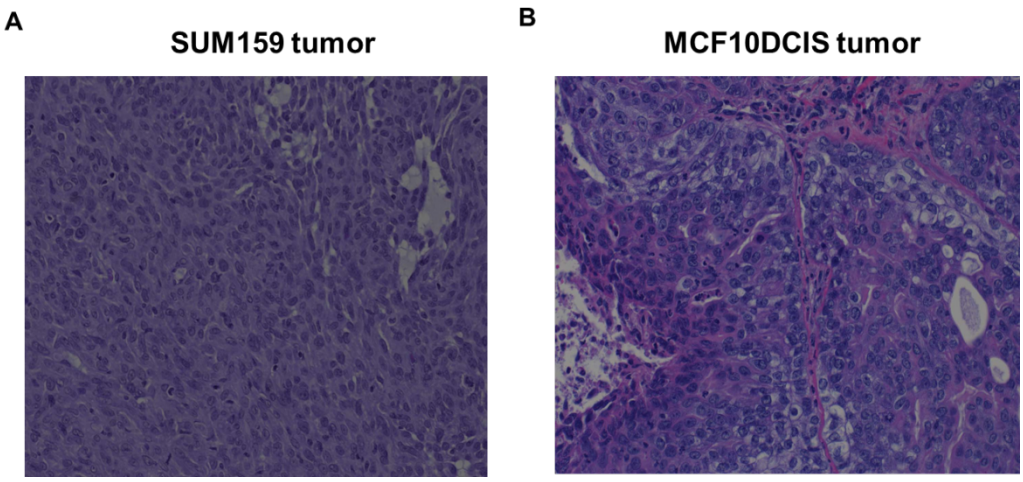


Figure 3.10 Pathological analyses of xenograft tumors

Representative hematoxylin and eosin (H&E) staining showing tumor tissues from SUM159 **(A)** and MCF10DCIS **(B)** xenograft tumors. The pathologist's report was provided by Michael Goedken from Research Pathology Services at Rutgers Translational Science. **(A) SUM159 tumor:** Samples contained variably sized, ovoid, densely cellular, expansile masses single cell type with a thin fibrous capsule when present (some tumors had little to no adjacent host tissue). Tumor patterns included whorls and streams of neoplastic cells in fibrous stroma. Polymorphic, variably sized cells with indistinct cell margins and eosinophilic fibrillar cytoplasm and variably sized, bland, ovoid to angular nuclei with basophilic chromatin and nucleoli. Less differentiate regions had anisokaryosis while more differentiated regions were interrupted by clearings. There were 1 to 3 mitotic figures per HPF. Some sample had necrotic foci and small areas of hemorrhage and/or inflammatory infiltration by neutrophils and fewer mononuclear cells. Morphologic diagnosis: stromal tumor, fibroma. **(B) MCF10DCIS tumor:** Limited to variable sized distended ducts (fibrous capsules) were neoplastic cells of a single cell population.

Epithelial cells formed variably cribriform structures in fine fibrovascular stroma. Ovoid cells with prominent margins contained abundant amphophilic granular cytoplasm. Nuclei were of uniform size and have a regular chromatin pattern with inconspicuous nucleoli, and rare mitotic figures. Centers of distended ducts contained variable amounts of necrotic cell debris rimmed by degenerate and apoptotic neoplastic epithelial cells. There were few mitotic figures. Extracellular matrix between ducts contained increased inflammatory cells. Morphologic diagnosis: Mammary gland ductal carcinoma in situ. Discussion: SUM159 cells produced mesenchymal tumors while MCF10DCIS cells resulted in epithelial tumors.

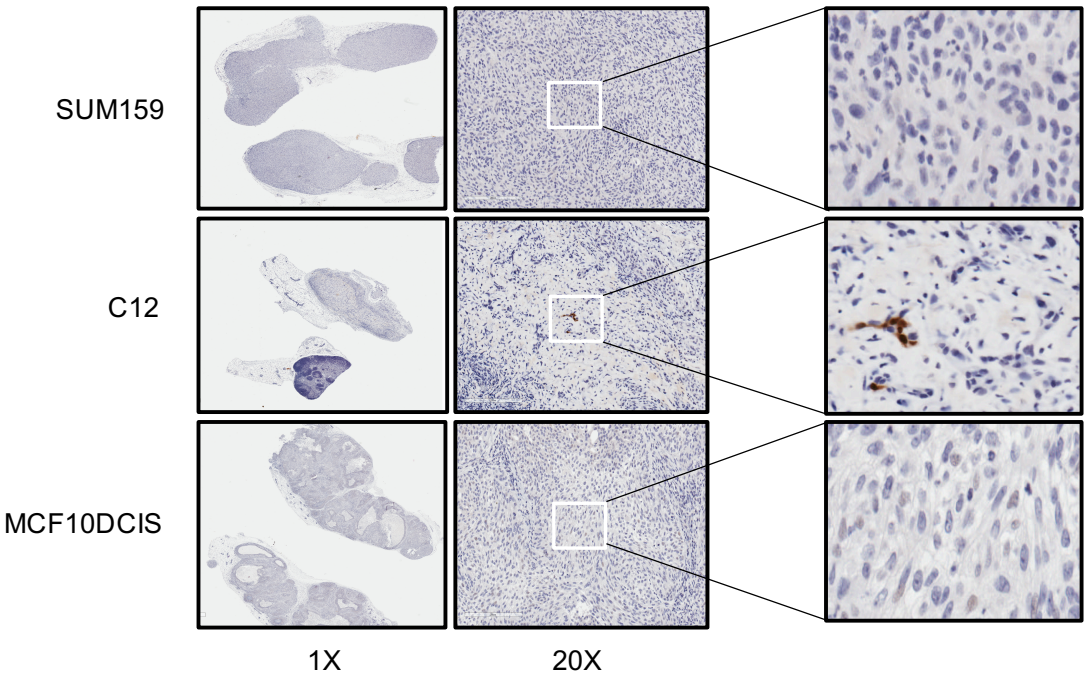


Figure 3.11 Tumors from stable OCT4-overexpressing cells express scant positive staining of OCT4

A representative immunohistochemical analysis detecting OCT4 in xenograft tumors injected with SUM159, C12 and MCF10DCIS cells at 1X and 20X magnifications. Images were taken with ScanScope XT. Immunohistochemical staining was performed at Research Pathology Services at Rutgers Translational Science.

Chapter 4: Analysis of the transcriptome: Regulation of Cancer Stemness in Breast Ductal Carcinoma In Situ by Vitamin D Compounds[10,11,12]

4.1 Introduction

Breast cancer is the most common cancer and the second leading cause of cancer related deaths in women worldwide [457]. Based on the presence or absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2), breast cancers are divided into subtypes: luminal A (ER+ and/or PR+; HER2–), luminal B (ER+ and/or PR+; HER2+), basal-like (ER–, PR–, and HER2–), and HER2-enriched (ER–, PR–, and HER2+) [458]. Breast cancer development is a multi-step process that involves epigenetic and genetic changes contributing to aberrant cell growth [459]. Histologically, breast cancer can be staged into invasive ductal carcinoma, ductal carcinoma in situ (DCIS) and invasive lobular carcinoma. About 20% of breast cancers newly diagnosed in 2019 among US women will be classified as DCIS, amounting to over 48,000 cases [460]. DCIS is an early stage, non-invasive type characterized by proliferation of malignant epithelial cells in the ducts [461]. It arises from atypical ductal hyperplasia and may progress to invasive ductal carcinoma (IDC) and metastatic cancer [461]. It is

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[10]Part of this chapter has been published in Cancer Prev Res (Phila). 2020;13 (8):673-686.

[11]**Keywords**: cancer stem cells; mammosphere; DNA-seq; RNA-seq; differentiation; BXL0124; TP63

[12]**Abbreviations**: DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; DMR, differentially methylated region; IPA, Ingenuity Pathway Analysis

predicted that up to 50% of the DCIS cases will progress to IDC within 10 years of initial diagnosis [462]. Gene expression and microRNA analyses have been performed to elucidate the molecular characteristics of DCIS progression to IDC [463, 464]. However, the natural history of progression of DCIS to IDC is yet to be fully determined.

Cancer stem cells (CSCs) were first identified in breast cancer using the cell surface markers $CD44^{+}/CD24^{-}$ [46]. This population is characterized by a stem-cell gene expression signatures, drug-resistant phenotype and self-renewal capacity *in vitro* and *in vivo* [465]. Human DCIS lesions form spheroids and duct-like structures in *ex vivo* organoid culture and tumors in immunodeficient mice, suggestive of the presence of CSC-like cells in these early tumors [466]. MCF10DCIS.COM was derived from the non-tumorigenic MCF10A human breast cell line and exhibits basal-like subtype properties [467]. It is similar to human DCIS with bi-potentiality that can give rise to both myoepithelial and luminal cells and spontaneous progression to invasive breast cancer *in vivo* [468], and it is widely used as a model for IDC development from precursor lesions. MCF10DCIS.COM cultures and tumors contain high $ALDH1^{+}$ and $CD44^{+}/CD49f^{+}/CD24^{-}$ subpopulations with increased self-renewal and tumor development capabilities, similar to CSCs of fully invasive tumors [469].

Vitamin D signaling is known to be a potential target for breast cancer chemoprevention [470]. Our laboratory has shown that vitamin D compounds inhibit triple negative breast cancer tumorigenesis by reducing expression of cancer stem-cell associated genes, including OCT4 and CD44, and by inducing differentiation and up-regulating myoepithelial markers [339]. These compounds reduce *in vitro* mammosphere formation and *in vivo* tumorigenesis, although the molecular mechanisms of these effects are not

known. BXL0124 is an analog of calcitriol ($1\alpha25(OH)_2D_3$) modified with an additional side chain at C21-methyl group, endowing it with more biological activity at lower concentrations without causing hypercalcemia, a limiting side effect of calcitriol [287]. BXL0124 also inhibits MCF10DCIS xenograft tumorigenesis more potently than $1\alpha25(OH)_2D_3$, apparently through the similar mechanism of suppressing cancer stem cells [297]. The goal of this chapter is to identify global profiles of changes in gene expression and CpG methylation in MCF10DCIS cells induced by vitamin D compounds, to gain an understanding of the pathways involved in their overall effects and the molecular basis of their activities, and to identify potential targets that could be exploited in the chemoprevention of breast cancer progression from DCIS to IDC. We analyzed the transcriptome of breast cancer stemness regulated by vitamin D in DCIS in MCF10DCIS mammospheres treated with vitamin D compounds.

4.2 Materials and methods

4.2.1 Reagents and cell culture

$1\alpha25(OH)_2D_3$ and a Gemini vitamin D analog (BXL0124; 1α,25-dihydroxy-20R-21(3-hydroxy-3-deuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-cholecalciferol, >95% purity) were provided by BioXell, Inc. (Nutley, NJ) [287]. Vitamin D compounds were dissolved in DMSO. MCF10DCIS.com human breast cancer cells (MCF10DCIS, RRID: CVCL_5552) were provided by Dr. Fred Miller at the Barbara Ann Karmanos Cancer Institute (Detroit, MI). Cells were maintained in DMEM/F12 medium supplemented with 5% horse serum, 1% penicillin/streptomycin, and 1% HEPES solution at 37 °C, 5% CO_2. The cells were passaged every 3-4 days between passage number p30

and p50. Mycoplasma testing was done every three months using Mycoplasma PCR detection kit (Cat# MP0035, Sigma-Aldrich). Cell line authentication was done using Short Tandem Repeat profiling (Cat# 135-XV) at American Type Culture Collection (ATCC).

4.2.2 Mammosphere formation assay

MCF10DCIS cells were grown to 70-80% confluence and cells were detached with StemPro Accutase™ (Life Technologies, NY) cell detachment solution. Cells were then grown in 6-well ultra-low attachment plates at a density of 10,000 cells/mL and maintained in MammoCult™ Human Medium added with MammoCult™ proliferation supplement, hydrocortisone solution and heparin solution (STEMCELL Technologies, MA). Spheres were treated with DMSO (0.01%), 1α25$(OH)_2D_3$ (100 nM), and BXL0124 (10 nM) for 5 days. After 5 days in culture, the culture plates were gently swirled to cluster the spheres in the middle of each well and photographed. Mammosphere forming efficiency (MFE) is calculated by dividing the number of mammospheres (≥100 μm) formed by the number of single cells seeded in individual wells. Three independent experiments were repeated.

4.2.3 Nucleic acid isolation and next-generation sequencing

MCF10DCIS mammospheres were treated with DMSO (0.01%), 1α25$(OH)_2D_3$ (100 nM), and BXL0124 (10 nM) for 5 days followed by extraction of RNA and DNA using AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA). The concentrations and quality of the RNA and DNA were determined using NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer separately. We prepared three independent sets of cultures with MCF10DCIS mammospheres treated with DMSO, 1α25$(OH)_2D_3$ and BXL0124, and

extracted each separately to obtain 3 RNA samples and 3 DNA samples. We pooled those samples for the RNA-seq and Methyl-seq analyses, which was carried out at RUCDR Infinite Biologics, a Rutgers University affiliated institution which provides Next Generation Sequencing (NGS) services. RNAseq libraries were prepared using Illumina RNA Library Prep Kit v2 according to manufacturer's user guide with 400 ng of RNA as input. The libraries were then quantified using KAPA Library Quantification kit according to manufacturer's user guide and pooled with barcodes. The pooled libraries were sequenced on Illumina NextSeq 550 system, using NextSeq 500/550 Mid Output v2 kit. The sequencing parameters used were 150 bp, paired-end with around 20 million reads generated per sample. The DNA samples were further processed using an Agilent SureSelect Human Methyl-seq Target Enrichment System (Agilent Technologies, Santa Clara, CA) and sequenced on an Illumina NextSeq 500 instrument with 76-bp single-end reads, generating 34–47 million reads per sample.

4.2.4 Sequencing data analysis

The RNA-seq reads were mapped to human reference genome with Hisat2 software. Cufflinks was used to assemble the transcript products and calculate the fragment abundance. Cuffdiff was used to quantify transcripts of genes differentially expressed among the three treatment conditions (DMSO, 1,25D3 and BXL0124). Methyl-seq was aligned using Bismark software (version 0.15.0), CpG sites were counted and clustered into Differentially Methylated Regions (DMRs) using DMRfinder (version 0.1)[471]. Genomic annotation was performed with ChIPseeker in R [472]. R was also used for downstream NGS data analysis and visualization as we have reported previously [473,

474]. The RNA-Seq and Methyl-Seq datasets described in this study have been deposited in the NCBI Gene Expression Omnibus with accession number GSE148548.

4.2.5 Ingenuity pathway analysis

Analysis of pathways and gene networks of the expression data was performed with IPA software from Qiagen (Version 49309495).

4.2.6 Quantitative polymerase chain reaction (qPCR) Analysis

To validate RNA-sequencing data, we performed three independent sets of experiments with MCF10DCIS mammospheres treatment with DMSO, $1\alpha25(OH)_2D_3$ and BXL0124. After 5 days of treatment, RNA samples are collected, and qPCR analysis was performed. RNA was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA), followed by amplification of the cDNA by using primers of genes of interest and TaqMan 2X Universal PCR Master Mix (Applied Biosystems, CA) and using the ViiA™ 7 Real-Time PCR System (Applied Biosystems, CA). Primers used are GAPDH (Hs02758991), GDF15 (Hs00171132), LCN2 (Hs01008571), S100A4 (Hs00243202), NGFR (Hs00609976), PPP1R1B (Hs00259967), AGR2 (Hs00356521), KRT6A (Hs04194231), EMP1 (Hs00608055), IGFBP5 (Hs00181213), CAPN6 (Hs00560073), CYP24A1 (Hs00167999), and KRT5 (Hs00361185). GAPDH was used as an internal control. Relative changes of gene expression were calculated using ΔΔCT Method.

4.2.7 Bisulfite pyrosequencing

The bisulfite-treated DNA was amplified by PCR using Platinum PCR Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Primers (5'-3'): forward (GTGGTTTTGTTTTGTTGTTAGAGAG), reverse (biotin-AAAATTCCCTAAAATTAAAAACTTCT) and sequencing (TGTTTTGTTGTTAGAGAGA) are designed with PyroMark Assay Design SW 2.0 software and obtained from Integrated DNA Technologies (Coralville, Iowa). Specifically, the reverse primer was biotinylated at the 5' end. The biotinylated PCR product was captured using streptavidin-coated beads (GE Healthcare, Piscataway, NJ, USA). After annealing with the sequencing primer, the single-stranded PCR product was pyrosequenced on a PyroMark Q24 advanced instrument (Qiagen). Average Methylation Index (MI) is calculated by combining the percentage of each CpG peak and dividing with the total number of CpG peaks in the pyrogram as previously shown [475].

4.2.8 Western blot analysis

Whole cell lysates (20 μg/lane) were resolved in 10% SDS-PAGE from Bio-Rad (Hercules, CA). Blots were then probed with the indicated antibodies. Primary antibodies against TP63 Clone 10H7L17 (1:1000) was from Thermo Fisher Scientific (Cat# 703809 RRID: AB_2809251); α-SMA (1:1000) was from Abcam (Cat# ab5694, RRID: AB_2223021) and β-actin, Clone AC-15 (1:2000) was from Sigma-Aldrich (Cat# A1978, RRID:AB_476692). Secondary antibodies were from Cell Signaling Technology. Western blot images are quantified by using GeneGnome XRQ chemiluminescence imaging system and analyzed by GeneTools analysis software from Syngene (MD, USA).

4.2.9 Flow cytometry

The detailed procedure was reported previously [476]. MCF10DCIS cells isolated from mammospheres were stained with antibodies against CD44-APC, Clone G44-26 from BD Biosciences (Cat# 559942, RRID: AB_398683) and CD10-PE from Thermo Fisher Scientific (Cat# 12-0106-41, RRID: AB_10714985). The stained MCF10DCIS cells were analyzed by flow cytometry using an FC500 Analyzer (Beckman Coulter) to determine the percentage of 4 different $CD44^{-}/CD10^{+}$, $CD44^{+}/CD10^{+}$, $CD44^{-}/CD10^{-}$ and $CD44^{+}/CD10^{-}$subpopulations.

4.2.10 Statistical analysis

The MFE data are presented as means ± SD. Simple comparisons between two groups were analyzed using Student's t-test, and comparisons of multiple groups were analyzed using one-way analysis of variance (ANOVA). *P* value <0.05 is considered statistically significant. Statistical analysis was carried out using R statistical software.

4.3 Results

4.3.1 Vitamin D compounds inhibit MCF10DCIS mammosphere forming efficiency

To examine the effect of vitamin D compounds on cancer stem-like cells, the mammosphere forming assay was performed. MCF10DCIS cells were grown in mammosphere culture with proliferation supplements as described above for 5 days, as previously reported [300]. Based on our previous studies, we selected an equivalent effective dose for each compound, $1\alpha25(OH)_2D_3$ (100 nM) and BXL0124 (10 nM).

Treatment with $1\alpha25(OH)_2D_3$ and BXL0124 decreased the MFE from 1.18% in controls to 0.82% ($p < 0.05$) and 0.87% ($p < 0.05$) respectively (**Figures 4.1A and 4.1B**). While the vitamin D compounds reduced the number of spheres, the proportion of larger spheres 100-200 μm and >200 μm was increased in the treated cultures (**Figure 4.1C**) and the spheres in the vitamin D treated cultures were rounder in shape (**Figure 4.1A**).

4.3.2 Global gene expression profiling in cells treated with vitamin D compounds

The distribution of differential expression genes (DEGs) in $1\alpha25(OH)_2D_3$ vs. control and BXL0124 vs. control treatment groups are shown respectively in volcano MA plots (**Figure 4.2A**). Of the genes upregulated, 52.8% (371 genes) were common to the two vitamin D compounds, while 49.3% (546 genes) were common in the downregulated group (**Figure 4.2B**). Comparing the gene expression changes of 15,331 genes being sequenced from $1\alpha25(OH)_2D_3$ to control group, a list of 12,351 genes was obtained with *q* value less than 0.01. Of those, 439 genes had a more than four-fold positive ($log_2>2$) change and 703 genes had greater than four-fold negative ($log_2<-2$) change in normalized RNA expression in $1\alpha25(OH)_2D_3$ versus control group. Similarly, when comparing the gene expression changes of BXL0124 treatment group relative to control group, a list of 12,738 genes was obtained at *q* value of less than 0.01. Of those, 634 genes were upregulated by more than positive four-fold ($log_2>2$) change and 948 genes were down-regulated by more than negative four-fold ($log_2<-2$) change in expression. The fragments per million (FPM) of differentially expressed genes in response to $1\alpha25(OH)_2D_3$ and BXL0124 treatments were normalized by log_2 and shown in a heatmap compared with

control (**Figure 4.3 A and B)**. The 25 most up-regulated and down-regulated genes for 1α25(OH)$_2$D$_3$ and BXL0124 cultures compared with controls are shown in **Table 4.1.**

To validate the findings from RNA-seq data using qPCR analysis, we selected 16 genes of potential interest, based on established relevance to cancer stemness, breast cancer progression and chemoresistance (**Figure 4.4**). We first evaluated CYP24A1, a gene that is well established to be involved in vitamin D metabolism and highly induced by vitamin D compounds [420, 477]. As expected, the level of CYP24A1 RNA increased with 1α,25(OH)$_2$D$_3$ treatment (82,000-fold, $p < 0.001$) and BXL0124 treatment (177,564-fold, $p < 0.01$), respectively. Genes that are known to be associated with breast cancer basal-like phenotype (KRT6A and KRT5) and a negative regulator of breast tumorigenesis (EMP1) were also upregulated, in accord with the RNA-seq results. Cytokeratin 6A (KRT6A) gene expression was upregulated 6.2-fold with 1α,25(OH)$_2$D$_3$ ($p < 0.05$) and 9.2-fold with BXL0124 ($p < 0.05$) treatment. Cytokeratin 5 (KRT5) expression level was increased 3.9-fold and 5.9-fold with 1α,25(OH)$_2$D$_3$ ($p < 0.01$) and BXL0124 ($p < 0.001$), respectively. EMP1 gene is increased 2.9-fold with 1α,25(OH)$_2$D$_3$ ($p < 0.05$) and 6.1-fold with BXL0124 ($p < 0.01$), respectively. We validated genes that are involved in epithelial mesenchymal transition, invasion and metastasis (S100A4 and LCN2), chemo-resistance (NGFR, PPP1R1B, and AGR2), and basal breast cancer (NGFR). S100A4 gene expression was reduced by 29% with 1α,25(OH)$_2$D$_3$ ($p < 0.05$) and by 35% with BXL0124 ($p < 0.05$). 1α,25(OH)$_2$D$_3$ and BXL0124 decreased LCN2 gene expression by 97% ($p < 0.001$) and 97% ($p < 0.01$), respectively. NGFR gene expression was down regulated by 97% with 1α,25(OH)$_2$D$_3$ ($p < 0.01$) and by 99% with BXL0124 ($p < 0.001$). In our analysis, 1α,25(OH)$_2$D$_3$ and BXL0124 decreased PPP1R1B gene expression by 81% ($p < 0.01$) and

97% ($p < 0.001$) respectively. AGR2 gene expression was also reduced by 1α,25(OH)$_2$D$_3$ (by 91%, $p < 0.05$) and BXL0124 (by 85%, $p < 0.01$). GDF15 gene, implicated in the maintenance of breast cancer stem-like cells, was significantly inhibited with 1α,25(OH)$_2$D$_3$ by 94% ($p < 0.01$) and with BXL0124 by 95% ($p < 0.01$), respectively. Genes that are involved in cell migration and cytokinesis (CAPN6) and mammary gland involution and differentiation (IGFBP-5) were also analyzed. CAPN6 gene expression was decreased by 1α,25(OH)$_2$D$_3$ (89%, $p < 0.01$) and with BXL0124 (97%, $p < 0.001$) in MCF10DCIS mammospheres. IGFBP5 gene expression was increased two folds with 1α,25(OH)$_2$D$_3$ ($p < 0.05$) while reduced by 89% with BXL0124 ($p < 0.05$). Our qPCR analysis confirmed and validated the expression changes as observed in RNA-seq data (**Figure 4.4**).

4.3.3 Correlation and validation between DNA methylation and RNA expression changes in vitamin D treated mammospheres

To determine whether the gene expression changes were due to changes in gene methylation status and to investigate the global DNA methylation changes induced by vitamin D compounds, we performed single base-pair resolution DNA methylation sequencing with the mammospheres treated with DMSO, 1α25(OH)$_2$D$_3$ and BXL0124 using Agilent SureSelect Human Methyl-seq library and Illumina NextSeq 500 platform. DNA methylation profiles were characterized using DMRfinder based on a total of 176,900 DMRs for 1α25(OH)$_2$D$_3$ and 162,581 DMRs for BXL0124 treatment groups which were annotated using ChIPseeker. Distribution of DMRs annotated by gene feature were shown in **Figure 4.5A**. With a cutoff criterion of $p < 0.05$ and methylation difference >10%, we

obtained 46 genes with their DMRs located in the promoter regions as shown in the heatmap (**Figure 4.5B**). In addition, we analyzed methyl-seq data of all loci assayed. We found 83 genes with $p < 0.05$ with a cutoff of methylation difference > 10%, and 145 genes with $p < 0.05$ without cutoff (**Figure 4.6**). Plotting methylation differences for all genes against each other for both $1\alpha25(OH)_2D_3$ vs. DMSO and BXL0124 vs. DMSO showed a linear relationship between the two groups (**Figure 4.7**). Notably, MCF10DCIS mammospheres treated with $1\alpha25(OH)_2D_3$ and BXL0124 shared similar pattern in heatmap compared with control implying the methylation modification in these DMRs may be a regulatory mechanism for breast cancer chemoprevention.

We further performed correlation between DNA methylation profiles and RNA expression profiles in $1\alpha25(OH)_2D_3$ vs. control and BXL0124 vs. control. We identified a list of 105 DMRs with corresponding RNA expression data in the former group and a list of 165 DMRs with corresponding gene expression data in the latter group ($\log_2$ two-fold for RNA expression difference and 10% for methylation difference were used as cutoffs). Starburst plots integrating DNA methylation and gene expression are shown in **Figure 4.8**. Each dot represents a DMR and the corresponding DMR locations are featured by different colors. From the analysis, DMRs can be classified into two major groups: one that has direct association between DNA methylation and RNA expression, and the other group that has inverse association between DNA methylation and RNA expression. The top 10 genes that have inverse relationship between transcript level and promoter DNA methylation are shown in **Table 4.2**. Since DNA hypermethylation at CpG island in promoter region is known to silence gene expression, we selected one of the most recognized vitamin D responsive target gene, CYP24A1, from the list of genes that showed

higher mRNA expression with lower CpG methylation, and then further validated its methylation status with pyrosequencing. Pyrosequencing analysis of the selected 6 CpG sites in the promoter region of CYP24A1 gene between 52789045 and 52789434 bps showed that CpG hypermethylation is decreased upon treatment with vitamin D compounds compared to control (average MIs DMSO = 75%, $1\alpha25(OH)_2D_3$ = 62%, and BXL0124 = 55%) **(Figure 4.9**). This pyrosequencing finding in CYP24A1 promoter methylation correlates with significant up-regulation of RNA expression in mammospheres treated with vitamin D compounds validating the DNA methyl-seq and RNA-seq results.

4.3.4 Analysis of upstream regulators of genes differentially regulated by vitamin D compounds and downstream targets of the vitamin D receptor

We performed Ingenuity Pathway Analysis (IPA) to identify upstream transcriptional regulators of the differentially regulated genes by vitamin D compounds (**Figure 4.10**). Using a cutoff absolute value for the z-score of >2.5, we found 30 genes regulated by $1\alpha25(OH)_2D_3$ and 27 genes regulated by BXL0124, among which TP63, VDR, CD24, CST5, and IFNB1 were regulated by both vitamin D compounds. We further identified direct upstream or downstream regulators of vitamin D receptor in human mammary gland and breast cancer cell lines in published databases that were also noted in our RNA-seq data (cutoff for *p* value overlap <0.01 and activation z-score > 2). **Figure 4.11** shows top candidate genes predicted to be regulated by VDR in the presence of $1\alpha25(OH)_2D_3$ or BXL0124. Genes identified in this analysis that are regulated by both vitamin D compounds include TP63, CYP24A1, CD14, TRPV6, STAT4, and FABP6.

Analysis of Ingenuity Canonical Pathways regulated by $1\alpha25(OH)_2D_3$ vs. control and BXL0124 vs. control is shown in **Tables 4.3** and **4.4**, respectively. Using criteria of $p < 0.01$ and absolute value z score > 2, 45 canonical pathways were regulated by $1\alpha25(OH)_2D_3$ and 8 canonical pathways were regulated by BXL0124. Among them, IL-6, NANOG, GP6 and LXR/RXR pathways were strongly affected.

4.3.5 Identification of TP63 as key pathway targeted by vitamin D compounds

One target gene of interest that emerged from our analyses is TP63, a member of tumor suppressor p53 family of transcription factors. TP63 is essential for development and maintenance of epithelial stem cells [478]. Loss of TP63 expression promotes malignant cells migration, invasion and distant metastasis in cancers [479]. Tp63 is associated with differentiated myoepithelium-specific genes in normal breast tissue and its perturbation is observed in MCF10DCIS cells which show significant decrease in $Tp63^+$ population and impaired differentiation [480]. To further examine the effects of vitamin D compounds on TP63, we analyzed protein levels in mammospheres (**Figure 4.12**) and observed the upregulation of TP63 protein upon treatment with vitamin D compounds. Additionally, we analyzed the protein level of α-smooth muscle actin (SMA), a myoepithelial cell differentiation marker in DCIS progression in mammospheres [481]. α-SMA level was upregulated in mammosphere samples treated with $1\alpha25(OH)_2D_3$ and BXL0124 (**Figure 4.12**). The disruption of the continuity of myoepithelial basement membrane is a pre-requisite to stromal invasion of tumor cells in DCIS progression to IDC [482]. Loss of CD10, a myoepithelial surface peptidase, is associated with triple negative breast cancer and increased risk of DCIS [483, 484]. We, therefore, performed flow

cytometric analysis of CD10 in MCF10DCIS mammospheres treated with vitamin D compounds. We observed significant upregulation of CD10+ in the treated cells (**Figure 4.13**). Overall, our data suggest that vitamin D compounds prevent DCIS progression to IDC by reducing cancer stem-like cells (or their "stemness") in the population, and instead inducing myoepithelial differentiation (**Figure 4.14**).

4.4 Discussion

We initiated this study to gain insights into global changes in gene expression regulated by vitamin D compounds in MCF10DCIS. This was intended to provide clues as to the molecular mechanisms and pathways by which these compounds reduce DCIS progression to IDC. We observed that vitamin D compounds significantly decreased mammosphere formation, an indicator that they reduced the stem cell-like population of the cell's stemness. Among the genes that are commonly and differentially regulated by vitamin D compounds, BXL0124 regulated a higher number of genes compared to $1\alpha25(OH)_2D_3$, consistent with the fact that it is a more potent agonist of VDR signaling. From the RNA-seq analysis, we selected a list of 12 candidate genes with which to validate expression changes and for further study. GDF15, growth differentiation factor 15, is a member of the TGF-β superfamily [485]. GDF15 increases tumorsphere formation and increases stemness markers OCT4, SOX2, NANOG, phosphorylation of Smad2 and activation of ERK1/2 by autocrine/paracrine circuit leading to the maintenance of the breast cancer stem-like cell state [486]. Both vitamin D compounds decreased GDF15 expression in mammospheres, suggesting inhibitory effects of these compounds on stemness and epithelial-mesenchymal transition (EMT). LCN2 (lipocalin) is a secreted

glycoprotein that transport lipophilic ligands [487]. Overexpression of LCN2 promotes cancer cell invasion and motility, up-regulates slug, vimentin, fibronectin and down-regulates E-cadherin indicative of inducing EMT [488]. Both $1\alpha25(OH)_2D_3$ and BXL0124 reduced LCN2 at mRNA levels.

Chemoresistance is one of the hallmarks of cancer stemness [24]. Nerve growth factor receptor (NGFR), a cancer stem cell marker in melanoma, along with its downstream target FGF13 (a mediator of chemoresistance), is increased in chemotherapy sensitive melanoma cells but not in chemo-resistant cells [489]. Triple negative breast cancer cells secrete nerve growth factor (NGF) that upregulates NGFR expression and inhibits apoptosis induction by chemotherapeutic agents [490]. In our study, NGFR expression was decreased in mammospheres treated with vitamin D compounds, suggesting the possible role of vitamin D compounds in reducing chemoresistance by targeting cancer stem cells. KRT5 and KRT6 are members of the keratin gene family coexpressed during differentiation of epithelial tissues [491]. In breast cancer, KRT5 and KRT6a are commonly used as immunohistochemical markers of the basal-like subtype [492]. Immunostaining of tissue microarrays using clinical DCIS samples revealed that cytokeratin 5 and 6 expression was inversely associated with invasion into surrounding normal breast tissue [493]. Therefore, increased expression of these markers upon treatment with vitamin D compounds suggests that vitamin D induces myoepithelial differentiation in DCIS, preventing its progression to IDC. CAPN6 (calpain-6), a family member of calcium dependent cysteine proteases, is down-regulated in simple ductal hyperplasia and atypical ductal hyperplasia but up-regulated in ductal carcinoma in situ breast cancer, suggestive of its function in breast cancer progression [494]. The expression

level of CAPN6 is significantly decreased with vitamin D treatment, indicating its inhibitory effect in breast cancer progression. To further validate the RNA sequencing data from MCF10DCIS mammosphere culture, we performed bioinformatic analysis of our data in relation to publicly available database with MCF10DCIS cells and confirmed a strong positive linear relationship (**Figure 4.15**). However, we observed the expression profiles of certain genes (including the ones that we have validated such as NGFR, GDF15, PPP1R1B, KRT5, KRT6A and LAMA3) in our MCF10DCIS mammospheres are different from those of MCF10DCIS monolayer cells. Treating the mammospheres with vitamin D compounds reversed the expression of these genes. Similarly, several genes highly regulated by vitamin D compounds listed in Table 1 (LACRT, CREB3L1, FCGBP, HMGCS2, BPIFA1, MUC5AC, CLDN10, BPIFB1, and SPINK8) showed different expression profiles in mammospheres compared to monolayer cells, suggesting that these genes might play a role in stem-like driven mammosphere growth.

Using single base-pair resolution Methyl-sequencing, we examined the DNA methylation status of the genes to determine whether their changes in expression was due to changes in promoter CpG island methylation induced by the vitamin D compounds. We found that treatment with $1\alpha25(OH)_2D_3$ and BXL0124 have shown similar patterns of changes in CpG methylation profiles. We have also identified that some of the DMRs showed direct relationship between DNA methylation and RNA expression, whereas some showed inverse relationship– while the methylation is increased, gene expression was down-regulated and vice versa. Since a large body of evidence shows that CpG island promoter methylation results in gene silencing, we selected a well-established vitamin D metabolizing gene, CYP24A1, that is strongly induced by the compounds to test this

hypothesis. We found that when DMRs are hypomethylated by vitamin D compounds, gene expression of CYP24A1 is up-regulated. Overall, these findings suggest epigenetic regulation as a potential mechanism of vitamin D compounds for breast cancer chemoprevention.

IPA analysis of transcriptomes revealed potential signaling pathways and downstream targets regulated by vitamin D compounds in DCIS. Among these, we selected the TP63 pathway for further analysis. Tp63, a member of tumor suppressor p53 family of transcription factors, is essential for development and maintenance of epithelial stem cells [479]. Six different isoforms of Tp63 have been identified, based on the presence of transactivating (TAp63) or dominant-negative (ΔNp63) domains [479]. Tp63 knockout mice showed impaired mammary, epithelial and craniofacial developments, indicating its role in regulating proliferation and differentiation of these cell types [495]. Immunohistochemistry studies in normal and diseased breast tissues show that Tp63 is immunoreactive in myoepithelial cells of normal and benign lesions but not in invasive breast carcinoma [496]. Recent analysis based on METABRIC cohort studies showed that the best overall survival is associated with myoepithelial mammary cell phenotype with Tp63 expression, similar to normal breast-like class [497]. In addition, Tp63 is found to be highly expressed in metaplastic carcinomas of the breast with spindle cell or squamous differentiation [498]. Loss of TP63 expression promotes malignant cells migration, invasion and distant metastasis in cancers [468, 499]. ΔNp63α is found to mediate CXCL12-CXCR4 pathway that activates diverse oncogenic downstream signaling pathways including PI3K/AKT, MAPK, JAK/STAT and NF-κB. By activating CXCR4 as its transcriptional target, ΔNp63α promotes pro-stem cell activity and chemotaxis of breast

cancer cells to metastasis sites [500]. In irradiated human epithelial cells, a high expression level of key stem cell factor OCT4, a downstream target of miR34a, activates p63 by cooperating with p63 isoform (TAp63α) to promote oncogenic transformation [501]. Our data showed that in DCIS mammospheres treated with $1\alpha25(OH)_2D_3$ and BXL0124, protein levels of TP63 increased, consistent with our hypothesis that the mechanism by which the vitamin D compounds inhibit progression of DCIS to IDC involves a reduction in the cancer stem cell-like population or the stemness of the cells. To explore the possibility that these effects are manifest in a more highly differentiated state of these treated populations, we examined markers of myoepithelial differentiation. We found that the vitamin D compounds increased levels of α-SMA and CD10, myoepithelial and basal progenitor cell surface markers, respectively.

4.5 Conclusions

In summary, we have identified epigenetic and gene expression changes regulated by vitamin D compounds in MCF10DCIS mammospheres using RNA-seq and Methyl-seq techniques. RNA-seq data provided a global view of genes differentially regulated by $1\alpha25(OH)_2D_3$ and BXL0124. A list of potential genes affected included those involved in breast cancer stemness and progression. Methyl-seq data revealed epigenetic modification of gene expression in MCF10DCIS mammospheres treated with vitamin D compounds. This observation is further supported by validation of CYP24A1 gene using pyrosequencing technique. IPA analysis identified top upstream regulators and downstream targets of the vitamin D receptor including, notably, TP63. These findings identify potential key pathways that could play a significant role in DCIS progression to

IDC and cancer stemness and offer targets that might be exploited for inhibition of this progression.

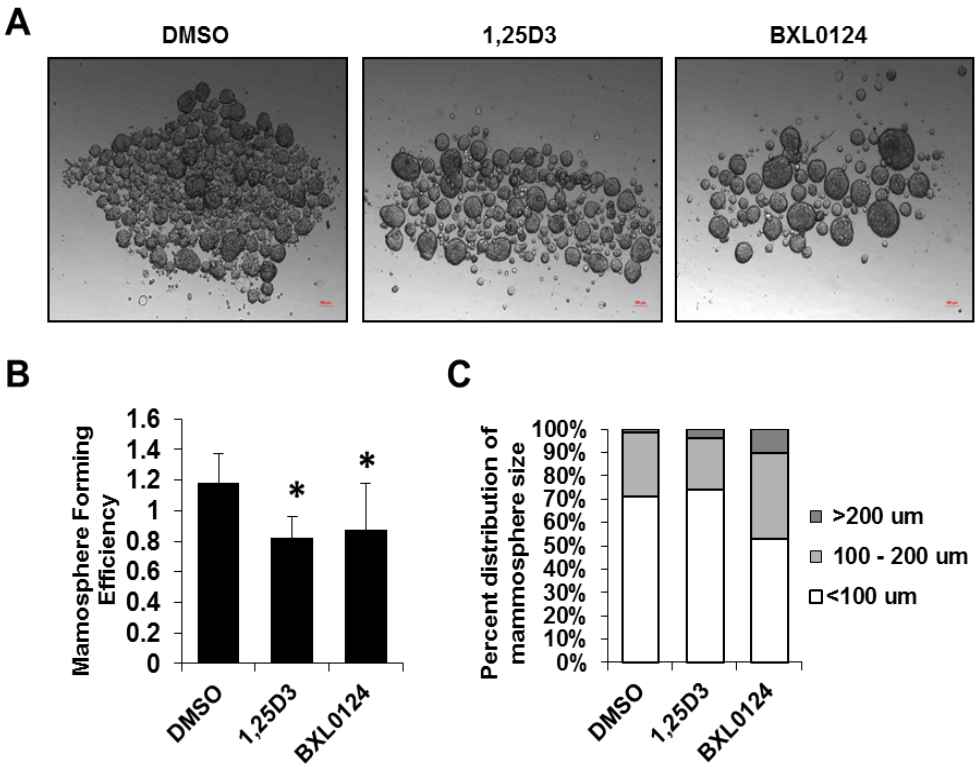


Figure 4.1 Inhibition of MCF10DCIS mammosphere forming efficiency by vitamin D compounds

(A) Representative pictures of MCF10DCIS mammospheres. MCF10DCIS cells were seeded 20,000 cells per well in 6-well ultra-low attachment plates and grown in Mammocult mammosphere media and treated with DMSO, $1\alpha25(OH)_2D_3$ (abbreviated as 1,25D3, 100 nM) or BXL0124 (10 nM) for 5 days. (B) Mammosphere forming efficiency (MFE) of MCF10DCIS mammospheres is shown. MFE was calculated by dividing the number of mammospheres (>100 μm) formed by the number of cells seeded presenting this as a percentage. The data are represented as mean ± SD. $n = 3$ indicates three independent experiments (p value *<0.05). (C) The size of tumorspheres was divided into three ranges (<100, 100–200 and >200 μm). Average number of tumorspheres in each size range is shown in the graph.

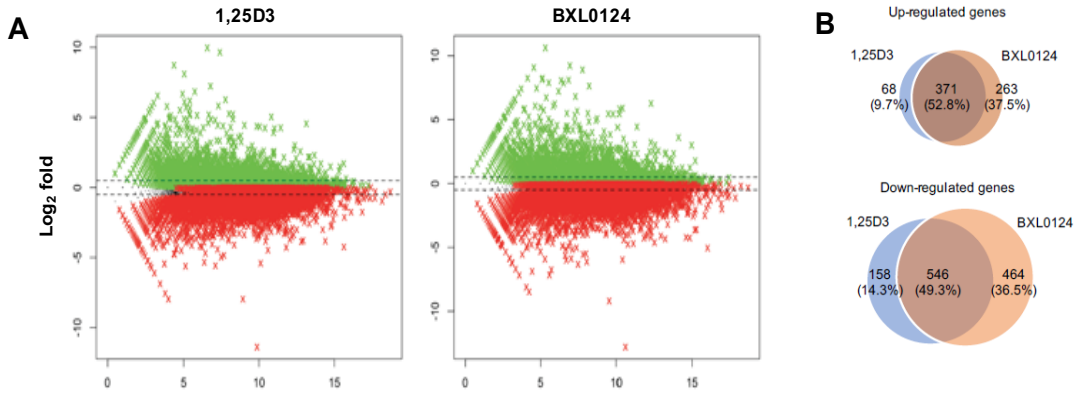


Figure 4.2 Differential expression analyses of transcripts in MCF10DCIS mammospheres treated with 1,25D3 and BXL0124

A. Volcano plots were generated using the DEGSeq package. Log_2 2-fold was used as a cutoff point to analyze the differential expression. **B.** Venn diagram depicting overlapping genes between 1,25D3 and BXL0124 for up-regulated and down-regulated genes (cutoff Log_2 2-fold).

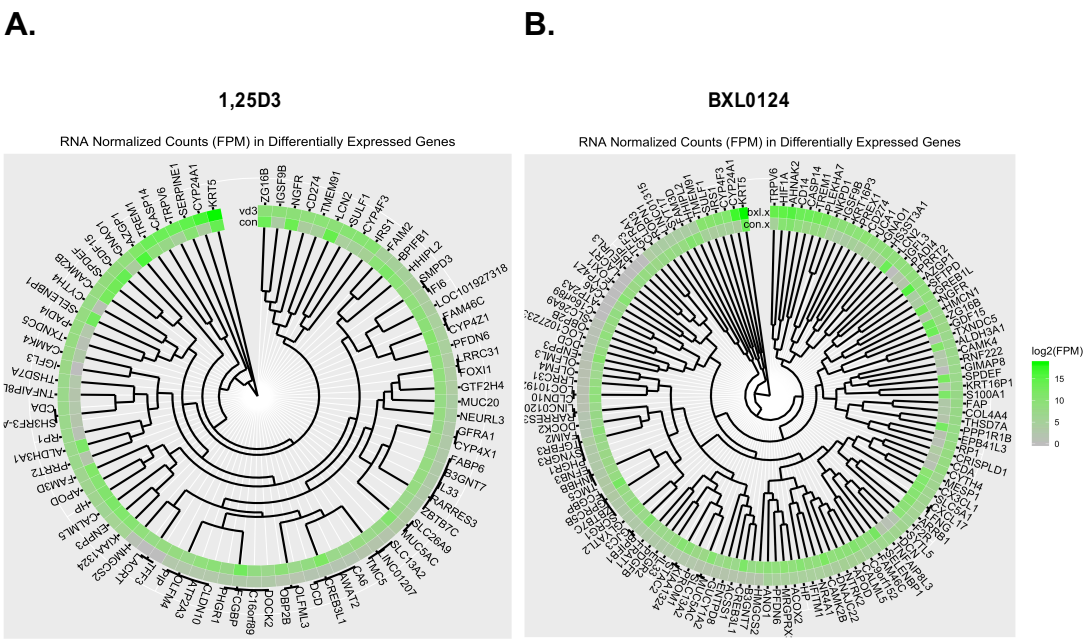


Figure 4.3 Circular heatmap representation of differential gene expression in MCF10DCIS mammospheres treated with 1,25D3 (A) and BXL0124 (B)

The circular heatmaps were produced using pheatmap package with R software. Shades of green and grey representing increase and decrease in gene expression relative to control.

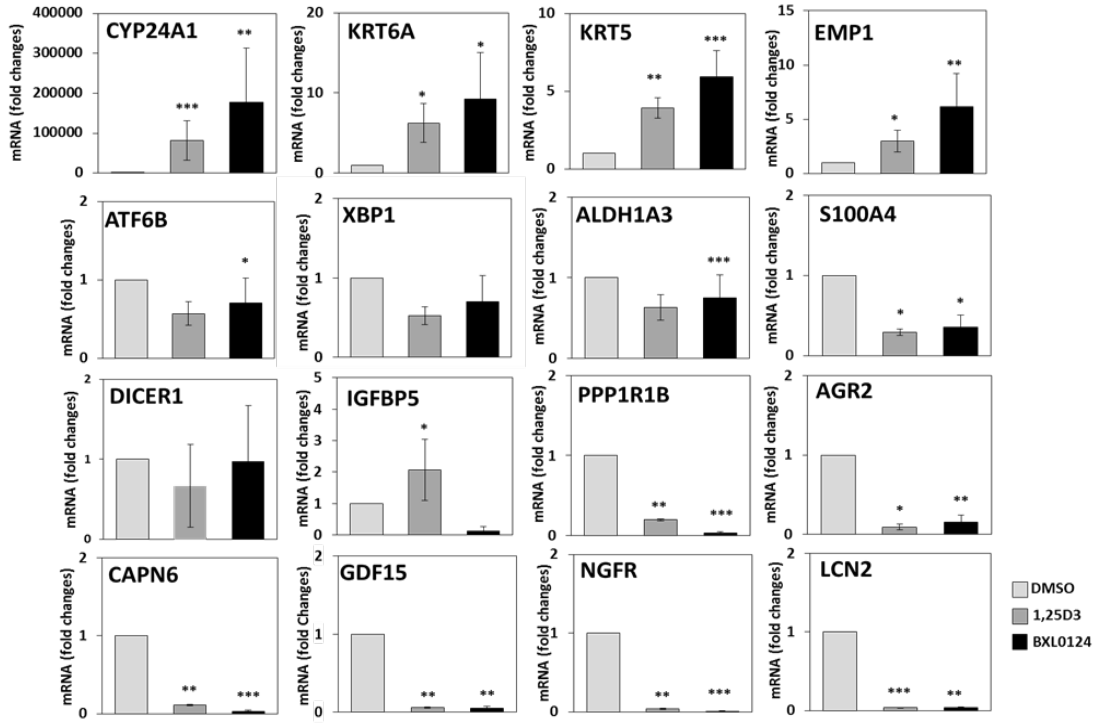


Figure 4.4 qPCR validation of selected genes differentially regulated in RNA sequencing data

qPCR analyses of selected genes differentially regulated in RNA sequencing data were performed. Three independent experiments were performed. Cycle numbers for genes related to CYP24A1, KRTA6A, KRTA5, EMP1, ATF6B, XBP1, ALDH1A3, S100A4, DICER1, IGFBP5, PPP1R1B, AGR2, CAPN6, GDF15, NGFR and LCN2 are 35, 30, 17, 22, 23, 22, 21, 23, 24, 34, 24, 23, 21, 23, 21 and 21 respectively. *p* values *<0.05, **<0.01, ***<0.001.

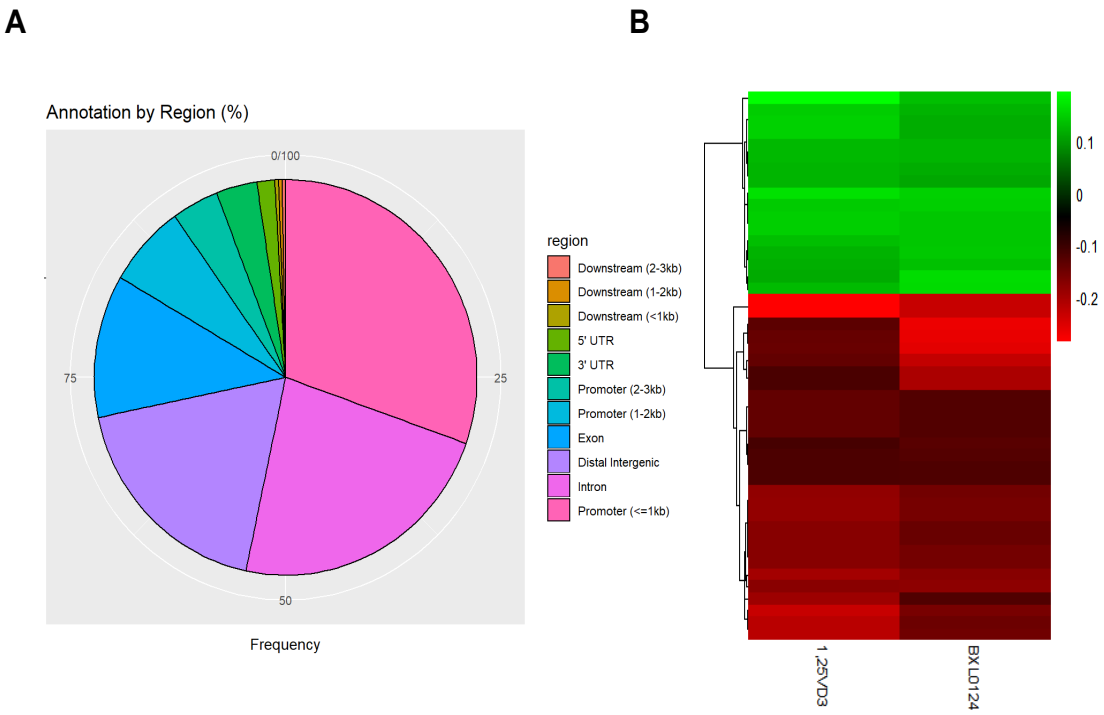


Figure 4.5 Correlation of gene expression and DNA methylation regulated by vitamin D compounds

A. Distribution of annotated differentially methylated regions (DMRs) by gene feature **B.** The clustered heatmap was produced by analyzing the top 46 genes differentially methylated in promoter regions using pheatmap package with R software ($p < 0.05$ with cutoff methylation difference >10%).

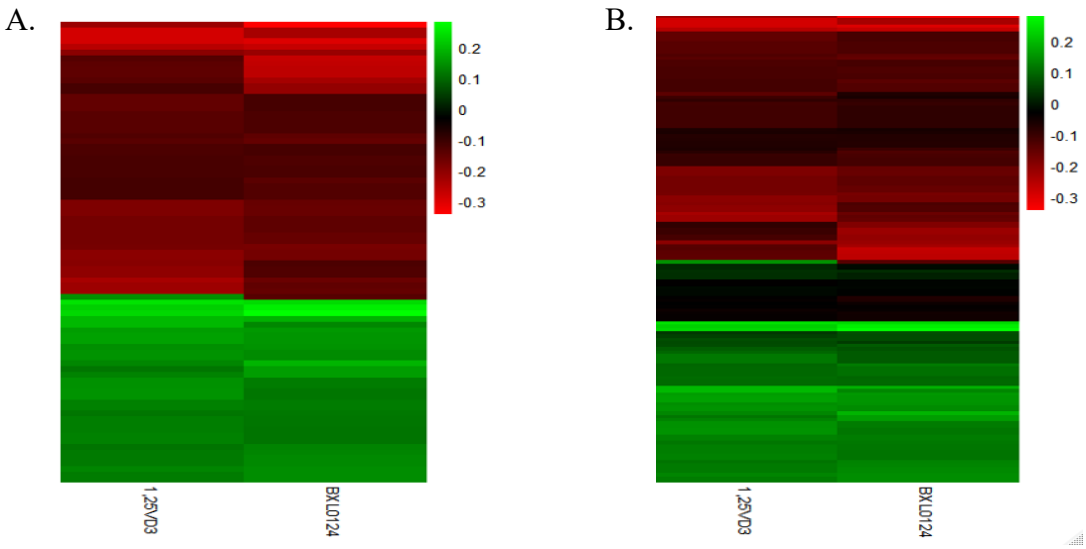
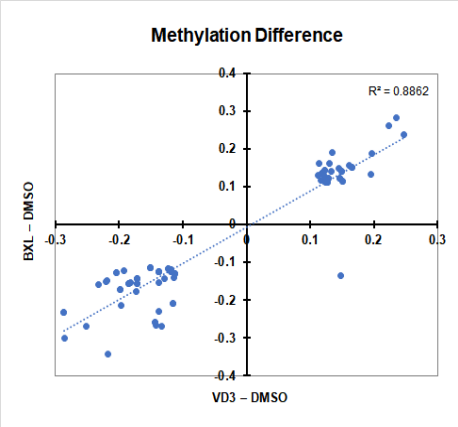


Figure 4.6. Differential DNA methylation regulated by vitamin D compounds

Methyl-seq data were analyzed with all loci (all the regions in genes) included using pheatmap package with R software. **A.** The clustered heatmap was produced by analysing 83 genes (p<0.05 with cutoff methylation difference > 10%). **B.** The clustered heatmap was produced by analysing 145 genes (p<0.05).

A.



B.

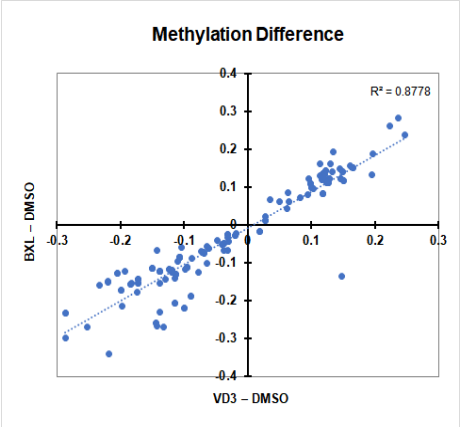


Figure 4.7 Correlation of methylation differences for all genes against each other for 1,25D3 (VD3) and BXL0124 (BXL) treatments in MCF10DCIS mammospheres

Scatterplots with methylation difference value of 1,25D3-DMSO shown at x-axis versus BXL0124-DMSO shown at y-axis. Methylation difference of 0.1 and a *p*-value of 0.05 was applied to the first plot **(A)** and a *p*-value of 0.05 without cutoff for methylation difference was applied to the second plot **(B)**.

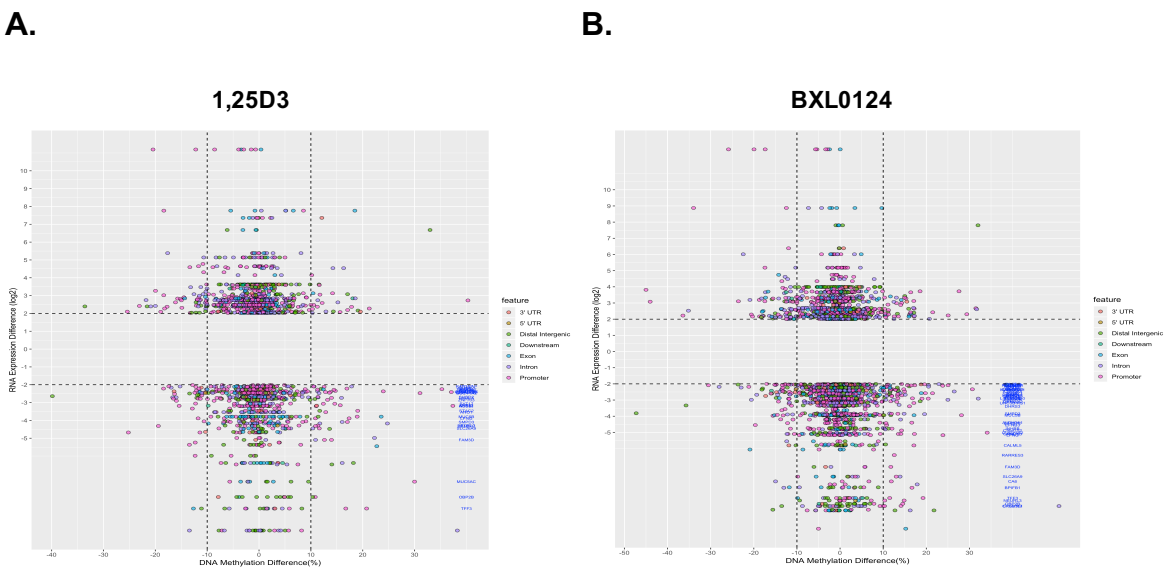


Figure 4.8 Starburst plot for the comparison of gene expression and DNA methylation regulated by 1,25D3 (A) and BXL0124 (B)

Starburst plot integrating alterations in DNA methylation and gene expression. The x-axis is the difference in DNA methylation levels (ΔM); the y-axis is the difference in gene expression (log_2 fold change).

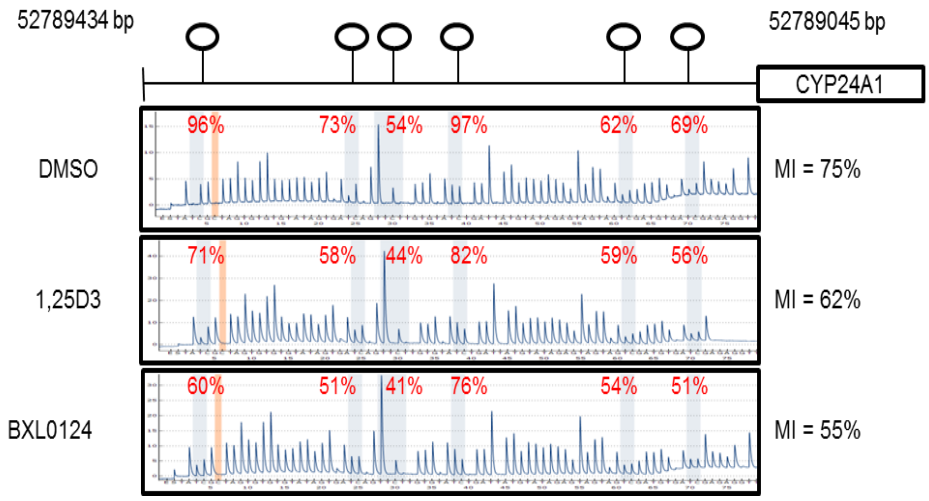


Figure 4.9 Validation of CYP24A1 gene CpG methylation regulated by vitamin D compounds

Pyrosequencing analysis of CpG methylation sites in the promoter region of CYP24A1 gene in mammospheres regulated by vitamin D compounds. Promoter methylation status of CYP24A1 between 52789045 and 52789434 bp position is shown. Percent methylation of individual CpG site and average methylation indexes (MI) are indicated.

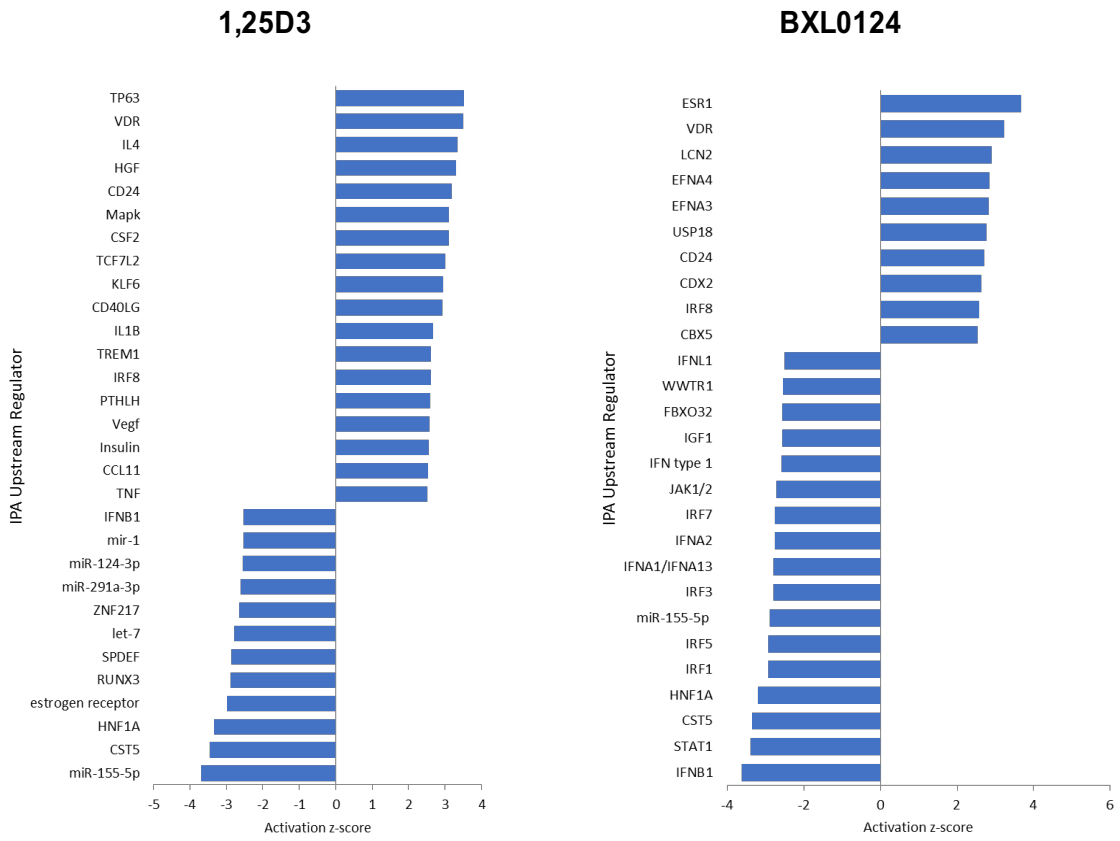


Figure 4.10 Analysis of upstream transcriptional regulators of differentially regulated genes by IPA

Upstream regulators of the differentially regulated genes in MCF10DCIS mammospheres treated with 1,25D3 and BXL0124 were identified using IPA. Cutoff absolute value for z-score >2.5.

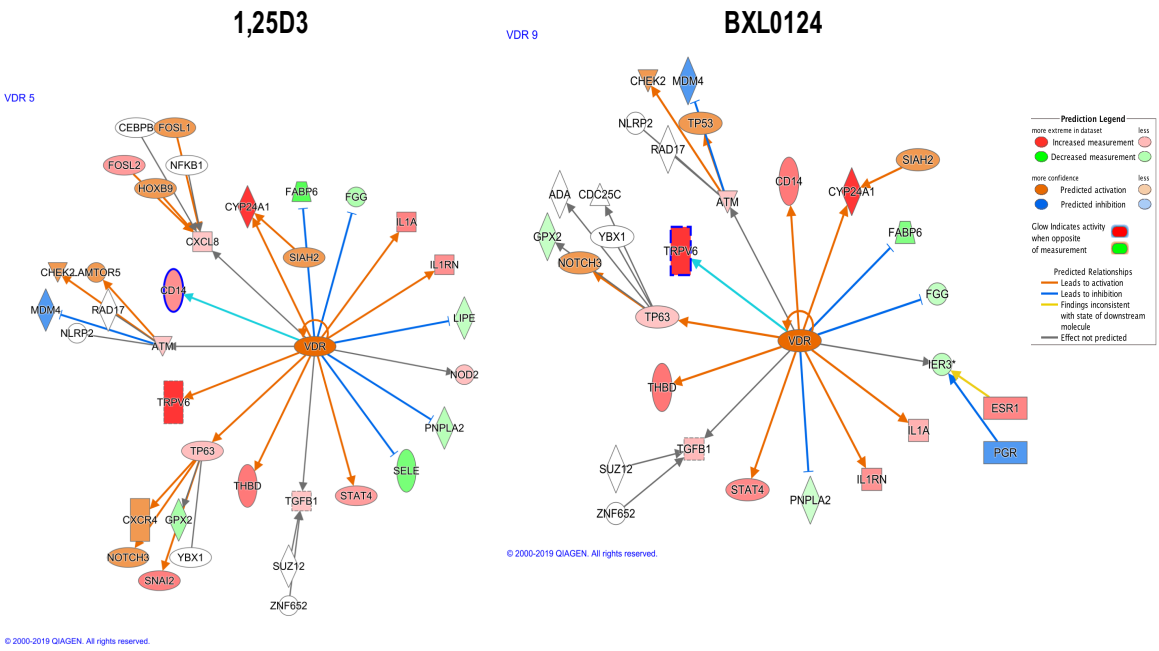


Figure 4.11 Identification of direct upstream or downstream regulators of vitamin D receptor by IPA

Top candidate genes predicted to be regulated by vitamin D receptor (VDR) in human mammary gland and breast cancer cell lines in published databases were identified in RNA-seq data of MCF10DCIS mammospheres treated with 1,25D3 and BXL0124 by using IPA at a cutoff for *p* value overlap <0.01 and activation z-score >2.

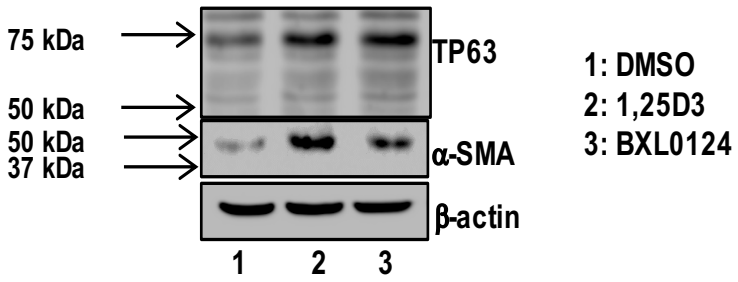


Figure 4.12 Assessment of myoepithelial differentiation markers regulated by vitamin D compounds

Western blot analysis of TP63 and α-SMA in mammospheres treated with DMSO, 1,25D3 (100 nM) and BXL0124 (10 nM) for 5 days. β-actin was used as a loading control.

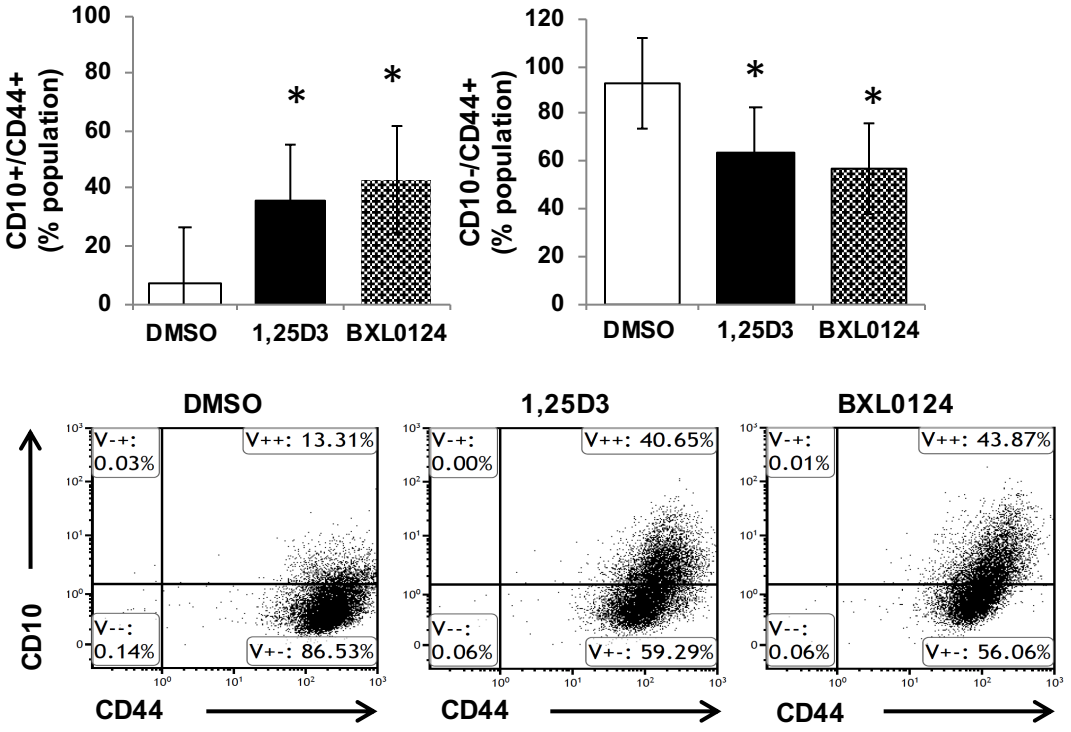


Figure 4.13 Identification of mesenchymal cell population distinguished by the expressions of CD44 and CD10

Flow cytometry analysis of progenitor marker CD44 and myoepithelial marker CD10 in MCF10DCIS mammospheres treated with DMSO, 1,25D3 (100 nM) and BXL0124 (10 nM) for 5 days. A representative flow cytometry analysis is shown. $CD10^{+}/CD44^{+}$ and $CD10^{-}/CD44^{+}$ populations were depicted after three independent data are combined to calculate $CD10^{+}/CD44^{+}$ and $CD10^{-}/CD44^{+}$ populations. The data are represented as mean ± SD. n = 3 indicates three independent experiments (p value *<0.05).

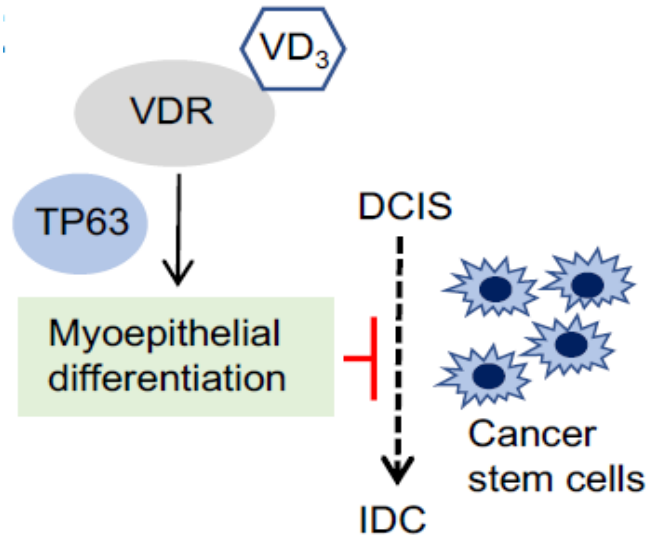


Figure 4.14 Schematic diagram of vitamin D compounds regulating the TP63 pathway in myoepithelial differentiation of DCIS

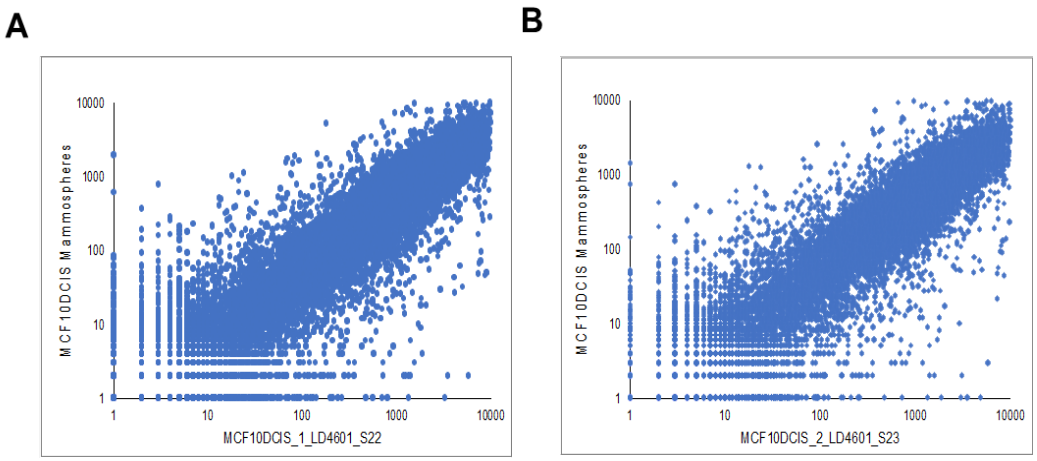


Figure 4.15 Bioinformatic analyses of RNA-Seq data of MCF10DCIS mammospheres in relation to external databases (MCF10DCIS cells)

RNA-Seq analyses of MCF10DCIS mammospheres (control) were plotted against MCF10DCIS cells from publicly available databases, MCF10DCIS_1_LD4601_S22 **(A)** and MCF10DCIS_1_LD4601_S23 **(B)**, respectively (Ding, L., Su, Y., Fassl, A. *et al.* Perturbed myoepithelial cell differentiation in *BRCA* mutation carriers and in ductal carcinoma in situ. *Nat Commun* **10,** 4182 (2019). https://doi.org/10.1038/s41467-019-12125-5) using Microsoft excel. (A) correlation coefficient 0.71 (B) correlation coefficient 0.72 to MCF10DCIS mammospheres control.

Table 4.1. Top 25 most up-regulated and down-regulated genes in comparison of 1,25D3 vs control and BXL0124 vs control

Upregulated Gene	Gene Name	1,25D3		BXL0124	
		Log2 fold change	Z score	Log2 fold change	Z score
CYP24A1	Cytochrome P450 family 24 subfamily A member 1	10.3	205.8	11.3	268.3
IGFL3	IGF Like Family Member 3	8.5	13.8	10.4	23.7
TRPV6	Transient Receptor Potential Cation Channel Subfamily V Member 6	7.5	108.9	8.5	148.1
CYTH4	Cytohesin 4	7.0	17.9	7.8	22.8
GIMAP8	GTPase, IMAP Family Member 8	6.9	8.6	8.5	13.9
OPCML	Opioid Binding Protein/Cell Adhesion Molecule Like	6.3	7.2	6.9	8.5
RP1	Retinitis Pigmentosa 1	6.3	14.0	7.4	20.3
ABCD2	ATP Binding Cassette Subfamily D Member 2	6.1	6.5	6.5	7.6
GNRHR	Gonadotropin Releasing Hormone Receptor	6.1	6.5	6.2	6.8
CNGB1	Cyclic Nucleotide Gated Channel Beta 1	6.0	6.4	7.2	9.3
CSNK1E	Casein Kinase 1 Epsilon	5.9	8.6	4.5	5.2
ZEB2	Zinc Finger E-Box Binding Homeobox 2	5.7	8.2	5.2	6.7
ZNF699	Zinc Finger Protein 699	5.7	5.8	6.3	7.1
IGFL1	IGF Like Family Member 1	5.7	5.7	6.1	6.6
DCN	Decorin	5.6	11.0	6.1	13.1
GTF2IP4	General Transcription Factor IIi Pseudogene 4	5.6	5.5	5.1	4.7
MT4	Metallothionein 4	5.5	7.4	3.8	3.9

NPTN-IT1	NPTN Intronic Transcript 1	5.3	4.9	6.5	7.5
KRT24	Keratin 24	5.2	9.7	5.2	9.6
SHE	Src Homology 2 Domain Containing E	5.2	6.8	7.0	12.6
SLC7A8	Solute Carrier Family 7 Member 8	5.1	9.4	5.5	10.9
PGLYRP3	Peptidoglycan Recognition Protein 3	5.1	6.6	4.5	5.3
CYP2C18	Cytochrome P450 Family 2 Subfamily C Member 18	5.1	4.6	6.4	7.3
LINC00504	Long Intergenic Non-Protein Coding RNA 504	5.1	4.6	6.2	6.8
CT62	Cancer/Testis Antigen 62	5.1	6.5	4.5	5.2

Down-regulated Gene	Gene Name	1,25D3		BXL0124	
		Log2 fold change	Z score	Log2 fold change	Z score
LACRT	Lacritin	-9.9	-17.8	-9.9	-17.8
CREB3L1	CAMP Responsive Element Binding Protein 3 Like 1	-9.8	-69.6	-9.5	-71.4
FCGBP	Fc Fragment of IgG Binding Protein	-9.6	-99.3	-9.2	-102.4
SLC44A4	Solute Carrier Family 44 Member 4	-9.3	-15.0	-6.3	-17.2
HMGCS2	3-Hydroxy-3-Methylglutaryl-CoA Synthase 2	-9.0	-27.8	-8.0	-29.5
BPIFA1	BPI Fold Containing Family A Member 1	-8.7	-12.6	-6.7	-13.8
DCD	Dermcidin	-8.6	-24.6	-8.6	-24.7
LEFTY1	Left-Right Determination Factor 1	-8.6	-12.2	-5.6	-13.4
IQGAP2	IQ Motif Containing GTPase Activating Protein 2	-8.3	-11.4	-8.3	-11.4
OBP2B	Odorant Binding Protein 2B	-8.2	-34.7	-9.5	-32.1
PIGR	Polymeric Immunoglobulin Receptor	-8.1	-10.8	-8.2	-10.8
CYP4Z2P	Cytochrome P450 Family 4 Subfamily Z Member 2, Pseudogene	-8.0	-10.3	-8.0	-10.3
GJB1	Gap Junction Protein Beta 1	-8.0	-10.2	-6.0	-10.9
THRSP	Thyroid Hormone Responsive	-7.9	-10.2	-5.0	-10.7
CRYM	Crystallin Mu	-7.9	-14.1	-4.6	-14.5
MUC5AC	Mucin 5AC, Oligomeric Mucus/Gel-Forming	-7.6	-53.6	-8.1	-52.5
CHRM1	Cholinergic Receptor Muscarinic 1	-7.5	-8.8	-7.5	-8.8

CLDN10	Claudin 10	-7.3	-35.1	-8.5	-33.4
MS4A7	Membrane Spanning 4-Domains A7	-7.2	-8.1	-7.2	-8.2
UGT2B11	UDP Glucuronosyltransferase Family 2 Member B11	-7.1	-7.8	-7.1	-7.8
RPRML	Reprimo Like	-6.9	-7.4	-6.9	-7.5
ATP2A3	ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+ Transporting 3	-6.6	-28.9	-10.8	-22.9
BPIFB1	BPI Fold Containing Family B Member 1	-6.6	-64.7	-8.2	-61.4
SPINK8	Serine Peptidase Inhibitor, Kazal Type 8	-6.6	-6.7	-6.6	-6.7
LOC101927822	RNA Gene	-6.6	-6.6	-6.6	-6.6

Genes are arranged in the order of log2 change. Gene names are taken from www.genecards.org.
ND: Not Detected

Table 4.2. Selected DMRs with inverse DNA methylation change and RNA expression change

Gene	Gene Name	Location	Feature	DNA Methylation change (Treatment vs. Control)	RNA Expression log_2 fold change (Treatment vs. Control)
1,25D3 (RNA expression increase, methylation decrease)					
CYP24A1	Cytochrome P450 family 24 subfamily A member 1	chr20: 52789045-52789434	Promoter	-12.2	11.2
TRPV6	Transient Receptor Potential Cation Channel Subfamily V Member 6	chr7: 142584106-142584444	Promoter	-18.3	7.8
IRS1	Insulin Receptor Substrate 1	chr2: 227660036-227660323	Promoter	-11.7	4.6
PRRT2	Proline Rich Transmembrane Protein 2	chr16: 29823628-29824005	Promoter	-13.3	4.6
CAMK4	Calcium/Calmodulin Dependent Protein Kinase IV	chr5: 110559299-110559797	Promoter	-10.7	4.3
SYNC	Syncoilin, Intermediate Filament Protein	chr1: 33169068-33169345	Promoter	-20	3.3
RGS17	Regulator of G Protein Signaling 17	chr6: 153450993-153451251	Promoter	-18.4	3
BAIAP2L2	BAI1 Associated Protein 2 Like 2	chr22: 38508088-38508248	Promoter	-15.7	2.8
EPAS1	Endothelial PAS Domain Protein 1	chr2: 46523790-46524036	Promoter	-16.1	2.6
DNAH11	Dynein Axonemal Heavy Chain 11	chr7: 21582584-21582913	Promoter	-17.7	2.4
1,25D3 (RNA expression decrease, methylation increase)					
OBP2B	Odorant Binding Protein 2B	chr9: 136084744-136085168	Promoter	10.8	-8.3
MUC5AC	Mucin 5AC, Oligomeric Mucus/Gel-Forming	chr11: 1151319-1151453	Promoter	30	-7.4
FAM3D	Family with Sequence Similarity 3 Member D	chr3: 58653571-58653767	Promoter	14	-5

SLC26A9	Solute Carrier Family 26 Member 9	chr1: 205909935-205910046	Promoter	11.6	-4.5
NEURL3	Neuralized E3 Ubiquitin Protein Ligase 3	chr2: 97174131-97174505	Promoter	11.4	-4.3
ZBTB7C	Zinc Finger and BTB Domain Containing 7C	chr18: 45664078-45664282	Promoter	10.9	-4.3
SMPD3	Sphingomyelin Phosphodiesterase 3	chr16: 68480865-68480956	Promoter	17	-4.1
LFNG	LFNG O-Fucosylpeptide 3-Beta-N-Acetylglucosaminyltransferase	chr7: 2551215-2551299	Promoter	17.4	-3.9
ANO1	Anoctamin 1	chr11: 69925157-69925401	Promoter	12.9	-3.5
SYT12	Synaptotagmin 12	chr11: 66790610-66790701	Promoter	37.4	-3.2
BXL0124 (RNA expression increase, methylation decrease)					
CYP24A1	Cytochrome P450 family 24 subfamily A member 1	chr20: 52788666-52788927	Promoter	-20	12.5
TRPV6	Transient Receptor Potential Cation Channel Subfamily V Member 6	chr7: 142583185-142583379	Promoter	-12.5	8.9
DCN	Decorin	chr12: 91572142-91572303	Promoter	-11.9	6.4
IRS1	Insulin Receptor Substrate 1	chr2: 227664786-227665021	Promoter	-17.4	5.2
CRISPLD1	Cysteine Rich Secretory Protein LCCL Domain Containing 1	chr8: 75896529-75896590	Promoter	-13.5	4.3
KLK6	Kallikrein Related Peptidase 6	chr19: 51471818-51472142	Promoter	-13.3	3.9
SYNC	Syncoilin, Intermediate Filament Protein	chr1: 33169068-33169345	Promoter	-12.7	3.9
BGLAP	Bone Gamma-Carboxyglutamate Protein	chr1: 156211057-156211193	Promoter	-50	3.8

GJA1	Gap Junction Protein Alpha 1	chr6: 121758702-121759148	Promoter	-12.6	3.3
PLEC	Plectin	chr8: 145012511-145012576	Promoter	-15.6	3.3
1,25D3 (RNA expression decrease, methylation increase)					
CREB3L1	CAMP Responsive Element Binding Protein 3 Like 1	chr11: 46259950-46260255	Promoter	10.3	-9.6
OBP2B	Odorant Binding Protein 2B	chr9: 136084744-136085168	Promoter	14.5	-9.4
BPIFB1	BPI Fold Containing Family B Member 1	chr20: 31871155-31871382	Promoter	16.7	-8.4
SLC26A9	Solute Carrier Family 26 Member 9	chr1: 205912819-205912892	Promoter	13	-7.7
FAM3D	Family with Sequence Similarity 3 Member D	chr3: 58652298-58652634	Promoter	11.8	-7.1
RARRES3	Retinoic Acid Receptor Responder 3	chr11: 63304457-63304769	Promoter	12.6	-6.4
CALML5	Calmodulin Like 5	chr10: 5538707-5538846	Promoter	17.1	-5.8
LFNG	LFNG O-Fucosylpeptide 3-Beta-N-Acetylglucosaminyltransferase	chr7: 2551215-2551299	Promoter	23.8	-5.2
ANO1	Anoctamin 1	chr11: 69923479-69923824	Promoter	13.5	-5.1
C9orf152	Chromosome 9 Open Reading Frame 152	chr9: 112970404-112970590	Promoter	14.3	-4.9

Genes are arranged in the order of log2 change. Gene names are taken from www.genecards.org

Table 4.3. Top regulated pathways with p < 0.01 and absolute value z score >2 in comparison of $1\alpha25(OH)_2D_3$ vs Control

Ingenuity Canonical Pathways	-log(p-value)	z-score	Molecules
IL-6 Signaling	5.4	3.1	*AKT3,ATM,CD14,CSNK2B,CXCL8,FGFR1,FOS,GAB1,IL1A,IL1B,IL1RAP,IL1RN,IL33,IL6ST,IRS1,JAK2,KRAS,MAP2K6,MAP4K4,MCL1,NFKBIE,NGFR,PIK3C2A,PIK3CA,PIK3R1,SOS1,TNFAIP6*
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	5.3	3.1	*AKT3,APC,ATM,BMP2,BMP3,BMP4,BMP6,BMP7,BMP8B,FGFR1,GAB1,IL6ST,IRS1,JAK2,KRAS,LIFR,PIK3C2A,PIK3CA,PIK3R1,RIF1,SMAD9,SOS1,WNT4,WNT6,WNT7A,WNT9A*
GP6 Signaling Pathway	4.9	2.6	*AKT3,ATM,CALML5,CAMK4,COL12A1,COL16A1,COL17A1,COL4A5,COL4A6,COL6A2,COL9A3,FCER1G,FGB,FGFR1,FGG,GAB1,GRAP2,IRS1,ITPR1,KLF12,LAMA3,LAMB3,LAMC2,PIK3C2A,PIK3CA,PIK3R1*
IGF-1 Signaling	4.3	2.3	*AKT3,ATM,CCN1,CCN2,CCN3,CSNK2B,FGFR1,FOS,FOXO1,GAB1,IGFBP4,IGFBP5,IRS1,JAK2,KRAS,NEDD4,PIK3C2A,PIK3CA,PIK3R1,PRKAR2A,SOCS2,SOS1*
EGF Signaling	3.7	2.8	*AKT3,ATM,CSNK2B,EGFR,FGFR1,FOS,GAB1,IRS1,ITPR1,ITPR2,MAP3K1,PIK3C2A,PIK3CA,PIK3R1,SOS1*
Estrogen-Dependent Breast Cancer Signaling	3.5	2.3	*AKT3,ATM,CREB3L4,EGFR,EP300,ESR1,FGFR1,FOS,GAB1,HSD17B1,HSD17B2,IRS1,KRAS,PIK3C2A,PIK3CA,PIK3R1,STAT5A*
Renal Cell Carcinoma Signaling	3.4	2.9	*AKT3,ATM,EP300,ETS1,FGFR1,FOS,GAB1,HIF1A,IRS1,KRAS,PDGFB,PIK3C2A,PIK3CA,PIK3R1,SOS1,TGFB1,UBB*
NF-κB Activation by Viruses	3.1	2.7	*AKT3,ATM,EIF2AK2,ELP1,FGFR1,GAB1,IRS1,ITGA2,ITGA5,ITGB1,ITGB2,KRAS,MAP3K1,NFKBIE,PIK3C2A,PIK3CA,PIK3R1*
Adrenomedullin signaling pathway	3.1	3.2	*ADCY7,ADM,AKT3,ATM,BCL2,BRAF,CALML5,CAMK4,FGFR1,FOS,GAB1,HIF1A,IL1A,IL1B,IL1RN,IL33,IRS1,ITPR1,ITPR2,KRAS,MAP2K6,MAPK6,NPR3,PIK3C2A,PIK3CA,PIK3R1,PRKAR2A,SHF,SOS1*
Ovarian Cancer Signaling	2.9	2.7	*AKT3,APC,ARRB1,ATM,BCL2,BRAF,BRCA2,EGFR,FGFR1,GAB1,GJA1,IRS1,KRAS,PIK3C2A,PIK3CA,PIK3R1,PMS2,PRKAR2A,PTGS1,WNT4,WNT6,WNT7A,WNT9A*
Acute Myeloid Leukemia Signaling	2.8	2.2	*AKT3,ATM,BRAF,CEBPA,CSF1R,CSF3R,FGFR1,GAB1,IRS1,KITLG,KRAS,MAP2K6,PIK3C2A,PIK3CA,PIK3R1,SOS1,STAT5A*
HGF Signaling	2.7	3.2	*AKT3,ATM,ELK3,ETS1,FGFR1,FOS,GAB1,IRS1,ITGA2,ITGA5,ITGB1,KRAS,MAP3K1,MAP3K12,MAP3K15,PIK3C2A,PIK3CA,PIK3R1,SOS1*
Paxillin Signaling	2.7	2.5	*ACTG2,ATM,FGFR1,GAB1,IRS1,ITGA2,ITGA5,ITGA7,ITGB1,ITGB2,ITGB8,KRAS,PIK3C2A,PIK3CA,PIK3R1,PTPN12,SOS1,TLN2,VCL*
ERK/MAPK Signaling	2.6	2.6	*ATM,BRAF,CREB3L4,DUSP1,DUSP2,DUSP4,ELK3,EP300,ESR1,ETS1,FGFR1,FOS,GAB1,IRS1,ITGA2,ITGA5,ITGB1,KRAS,KSR1,PIK3C2A,PIK3CA,PIK3R1,PPM1L,PRKAR2A,RAPGEF3,SOS1,TLN2,VRK2*
CNTF Signaling	2.6	3.1	*ATM,FGFR1,GAB1,IL6ST,IRS1,JAK2,KRAS,LIFR,PIK3C2A,PIK3CA,PIK3R1,RPS6KA2,SOS1*
IL-2 Signaling	2.6	2.3	*AKT3,ATM,CSNK2B,FGFR1,FOS,GAB1,IRS1,KRAS,PIK3C2A,PIK3CA,PIK3R1,SOS1,STAT5A*

Macropinocytosis Signaling	2.5	2.5	*ATM,CD14,CSF1R,FGFR1,GAB1,IRS1,ITGA5,ITGB1,ITGB2,ITGB8,KRAS,PDGFB,PIK3C2A,PIK3CA,PIK3R1*
CD28 Signaling in T Helper Cells	2.5	2.2	*AKT3,ATM,CALML5,CAMK4,FCER1G,FGFR1,FOS,GAB1,GRAP2,HLA-DMA,IRS1,ITPR1,ITPR2,MALT1,MAP3K1,NFATC4,NFKBIE,PIK3C2A,PIK3CA,PIK3R1*
JAK/Stat Signaling	2.4	2.3	*AKT3,ATM,FGFR1,FOS,GAB1,IRS1,JAK2,KRAS,PIK3C2A,PIK3CA,PIK3R1,SOCS2,SOS1,STAT4,STAT5A*
IL-7 Signaling Pathway	2.4	2.3	*AKT3,ATM,BCL2,BCL6,FGFR1,FOXO1,FOXO6,GAB1,IRS1,MCL1,PIK3C2A,PIK3CA,PIK3R1,SOS1,STAT5A*
Non-Small Cell Lung Cancer Signaling	2.3	3.1	*AKT3,ATM,CDK6,EGFR,FGFR1,GAB1,IRS1,ITPR1,ITPR2,KRAS,PIK3C2A,PIK3CA,PIK3R1,SOS1*
FLT3 Signaling in Hematopoietic Progenitor Cells	2.3	2.3	*AKT3,ATM,CREB3L4,EP300,FGFR1,GAB1,IRS1,KRAS,PIK3C2A,PIK3CA,PIK3R1,RPS6KA2,SOS1,STAT4,STAT5A*
Mouse Embryonic Stem Cell Pluripotency	2.2	2.7	*AKT3,APC,ATM,BMP4,FGFR1,GAB1,ID2,IL6ST,IRS1,JAK2,KRAS,LIFR,PIK3C2A,PIK3CA,PIK3R1,SMAD9,SOS1*
Melanocyte Development and Pigmentation Signaling	2.2	3.0	*ADCY7,ATM,BCL2,CREB3L4,EP300,FGFR1,GAB1,IRS1,KITLG,KRAS,PIK3C2A,PIK3CA,PIK3R1,PRKAR2A,RPS6KA2,SOS1*
eNOS Signaling	2.1	2.2	*ADCY7,AKT3,AQP3,AQP5,ATM,BDKRB1,CALML5,CAMK4,CAV1,CHRNB4,ESR1,FGFR1,GAB1,IRS1,ITPR1,ITPR2,LPAR5,PIK3C2A,PIK3CA,PIK3R1,PRKAA2,PRKAB2,PRKAR2A*
Thrombopoietin Signaling	2.1	2.3	*ATM,FGFR1,FOS,GAB1,IRS1,JAK2,KRAS,PIK3C2A,PIK3CA,PIK3R1,SOS1,STAT5A*
IL-3 Signaling	2.1	2.1	*AKT3,ATM,FGFR1,FOS,FOXO1,GAB1,IRS1,JAK2,KRAS,PIK3C2A,PIK3CA,PIK3R1,SOS1,STAT5A*
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	2.0	2.7	*ATM,CLEC7A,CXCL8,EIF2AK2,FGFR1,GAB1,IL17C,IL1A,IL1B,IRS1,NOD2,OAS1,OAS2,PIK3C2A,PIK3CA,PIK3R1,TGFB1,TGFB3,TLR4*
FGF Signaling	2.0	2.1	*AKT3,ATM,CREB3L4,EP300,FGFR1,GAB1,IRS1,ITPR1,MAP2K6,MAP3K1,PIK3C2A,PIK3CA,PIK3R1,SOS1*
TREM1 Signaling	1.9	2.3	*AKT3,CXCL8,IL1B,ITGA5,ITGB1,JAK2,LAT2,NOD2,SIGIRR,STAT5A,TLR4,TREM1*
UVA-Induced MAPK Signaling	1.9	2.3	*ATM,EGFR,FGFR1,FOS,GAB1,IRS1,KRAS,PARP10,PARP4,PARP8,PIK3C2A,PIK3CA,PIK3R1,RPS6KA2,SMPD1,SMPD3*
PI3K/AKT Signaling	1.9	2.1	*AKT3,BCL2,FOXO1,GAB1,GDF15,INPP5J,ITGA2,ITGA5,ITGB1,JAK2,KRAS,MAPK8IP1,MCL1,NFKBIE,PIK3CA,PIK3R1,PPM1L,SOS1*
Aryl Hydrocarbon Receptor Signaling	1.9	2.3	*AIP,ALDH3A1,ATM,ATR,CDK6,EP300,ESR1,FAS,FOS,GSTA2,GSTM4,IL1A,IL1B,NCOA2,NQO1,NRIP1,RBL1,TGFB1,TGFB3*

IL-17A Signaling in Airway Cells	1.8	2.5	*AKT3,ATM,FGFR1,GAB1,IRS1,JAK2,MUC5AC,MUC5B,NFKBIE,PIK3C2A,PIK3CA,PIK3R1*
Melanoma Signaling	1.7	2.5	*AKT3,ATM,BRAF,FGFR1,GAB1,IRS1,KRAS,PIK3C2A,PIK3CA,PIK3R1*
PAK Signaling	1.7	2.7	*ARHGAP10,ATM,EPHA3,FGFR1,GAB1,IRS1,ITGA2,ITGA5,ITGB1,KRAS,PDGFB,PIK3C2A,PIK3CA,PIK3R1,SOS1*
Renin-Angiotensin Signaling	1.7	3.0	*ADCY7,ATM,FGFR1,FOS,GAB1,IRS1,ITPR1,ITPR2,JAK2,KRAS,MAP3K1,PIK3C2A,PIK3CA,PIK3R1,PRKAR2A,SHF,SOS1*
ERK5 Signaling	1.6	2.5	*CREB3L4,EGFR,EP300,FOS,GAB1,GNA13,IL6ST,KRAS,RPS6KA2,SH2D2A,WNK1*
Pancreatic Adenocarcinoma Signaling	1.6	2.9	*AKT3,ATM,BCL2,BRCA2,E2F7,EGFR,FGFR1,GAB1,IRS1,JAK2,KRAS,PIK3C2A,PIK3CA,PIK3R1,TGFB1,TGFB3*
LXR/RXR Activation	1.6	-2.3	*APOC1,APOD,APOE,CD14,CLU,IL1A,IL1B,IL1RAP,IL1RN,IL33,LYZ,MYLIP,NGFR,SERPINF1,SERPINF2,TLR4*
Nitric Oxide Signaling in the Cardiovascular System	1.5	2.3	*AKT3,ATM,ATP2A3,CALML5,CAMK4,CAV1,FGFR1,GAB1,IRS1,ITPR1,ITPR2,PIK3C2A,PIK3CA,PIK3R1,PRKAR2A*
Lymphotoxin β Receptor Signaling	1.5	2.3	*AKT3,ATM,ELP1,EP300,FGFR1,GAB1,IRS1,PIK3C2A,PIK3CA,PIK3R1*
Endometrial Cancer Signaling	1.4	2.5	*AKT3,ATM,FGFR1,GAB1,IRS1,KRAS,PIK3C2A,PIK3CA,PIK3R1,SOS1*
Neuroinflammation Signaling Pathway	1.4	2.2	*AKT3,ATM,BCL2,BIRC6,CREB3L4,CSF1R,CX3CL1,CXCL8,EP300,FAS,FGFR1,FOS,GAB1,GLUL,IL1B,IRS1,JAK2,MAPK6,NCF2,NFATC4,PIK3C2A,PIK3CA,PIK3R1,PYCARD,S100B,SLC6A11,SNCA,TGFB1,TGFB3,TGFBR3,TLR4,TREM2, HLA-DMA*
P2Y Purigenic Receptor Signaling Pathway	1.3	2.7	*ADCY7,AKT3,ATM,CREB3L4,EP300,FGFR1,FOS,GAB1,IRS1,KRAS,P2RY1,P2RY2,P2RY6,PIK3C2A,PIK3CA,PIK3R1,PRKAR2A*

Table 4.4. Top regulated pathways with p < 0.01 and absolute value z score >2 in comparison of BXL0124 vs Control

Ingenuity Canonical Pathways	-log(p-value)	z-score	Molecules
GP6 Signaling Pathway	4.99	2.2	*AKT3,ATM,CALML5,CAMK4,COL12A1,COL16A1,COL17A1,COL4A4,COL4A5,COL4A6,COL7A1,COL8A1,COL9A3,FCER1G,FGB,FGFR1,FGG,GAB1,GRAP2,IRS1,ITPR1,LAMA3,LAMB3,LAMC2,PIK3CA,PIK3R1*
LXR/RXR Activation	2.28	-2.183	*ABCG1,AHSG,APOC1,APOD,CD14,CLU,IL1A,IL1B,IL1RAP,IL1RL1,IL1RN,IL33,LYZ,MYLIP,NGFR,SERPINF1,SERPINF2,TLR4*
Interferon Signaling	2.28	-2.828	*BCL2,IFI6,IFITM1,IFITM2,IFITM3,IRF1,ISG15,OAS1*
EGF Signaling	2.27	2.309	*AKT3,ATM,CSNK2B,EGFR,FGFR1,GAB1,IRS1,ITPR1,ITPR2,MTOR,PIK3CA,PIK3R1*
Ceramide Signaling	2.06	-2.496	*AKT3,ATM,BCL2,FGFR1,GAB1,IRS1,KSR1,MRAS,NGFR,PIK3CA,PIK3R1,S1PR2,SMPD1,SMPD3,SPHK1*
NRF2-mediated Oxidative Stress Response	1.97	-2.714	*ABCC4,ACTG2,ATM,DNAJC13,DNAJC4,DNAJC6,ENC1,EP300,EPHX1,FGFR1,FTL,GAB1,GPX2,GSTA2,GSTM4,IRS1,JUND,MAFF,MAP2K6,MRAS,NQO1,PIK3CA,PIK3R1,SQSTM1,UBB*
AMPK Signaling	1.8	2.236	*ADRA1B,ADRA2B,ADRB2,AK8,AKT3,ATM,CAB39,CHRNB4,CREB3L4,EP300,FGFR1,FOXO1,FOXO6,GAB1,IRS1,MRAS,MTOR,PFKP,PIK3CA,PIK3R1,PRKAA2,PRKAB2,PRKAR2A,PRKAR2B,RAB9B,SMARCD3*
IL-17A Signaling in Airway Cells	1.44	2.121	*AKT3,ATM,CXCL3,FGFR1,GAB1,IRS1,MUC5AC,MUC5B,NFKBIE,PIK3CA,PIK3R1*

Conclusions

Breast cancer is a heterogenous disease, characterized by genetic alterations, distinct histopathological and molecular profiles, and intra- and inter- tumoral diversity. It was previously known that accumulation of genetic mutations in normal somatic cells over time gives rise to selective advantage for increased cell proliferation, survival and inhibition of differentiation. The dedifferentiated transformed cells can efficiently form new tumors. The evolving concept of cancer stem cell theory has overridden the precedent model of sequentially acquired mutations and instead has led to a new hypothesis that tumor cells are organized in a cellular hierarchy maintained by a small subset of cells called CSCs. These cells can be identified by cell surface markers and are featured by their ability to self-renew, resistant to chemotherapy and radiation and initiate new tumors upon serial transplantation in xenograft studies.

Our work presented here sheds light on breast cancer stem cells are critical in the pathogenesis of breast cancer and the potential role of vitamin D compounds in inhibiting this subset of cells, specifically targeting the transcription factors and signal transduction pathways implicated in the maintenance and regulation of breast cancer stemness using triple negative breast cancer cells as a model. We identified OCT4, a major pluripotency transcription factor, as a key factor regulating cancer stem cells. As a proof of concept, overexpression of OCT4 in SUM159 cells showed a upregulated CD44 and increased CD44+/CD24- subpopulations compared to control cells. In contrast, studies in head and neck squamous carcinoma and gastric cancer demonstrated that OCT4 is one of the downstream regulators of CD44 maintaining the properties of CSCs. It can be hypothesized that there is a feedback regulatory mechanism between OCT4 and CD44.

In addition, we employed next-generation RNA and DNA sequencing techniques to perform global transcriptomic analysis of differentially expressed genes in MCF10DCIS mammospheres treated with vitamin D compounds. This study has provided us with a genetic signature and TP63-VDR mediated signaling pathway as possible targets for chemoprevention of DCIS progression to IDC. Overall these results leverage our understanding of vitamin D mediated targeting of transcription factor as a potential therapy against breast cancer stem cells and progression.

Future directions

Our studies show that vitamin D inhibitory effects on breast cancer stemness could be mediated through OCT4 transcription factor, a master regulator of pluripotency. Our genetic study in SUM159 cells overexpressing OCT4 has demonstrated OCT4-mediated upregulation of BCSC markers and the $CD44^{+}/CD24^{-}$ subpopulation. From the results, we can hypothesize targeting OCT4 can inhibit breast cancer stem cells and tumorigenesis *in vitro* and *in vivo.* To study the mechanistic interaction of OCT4 targeting CD44, we will perform ChIP-binding assay to assess OCT4 binding of promoter region of CD44 in OCT4-overexpressed cells. Since our preliminary in vivo study has limited number in sample size, we would utilize larger number of animals (10 mice in each group), injecting stable OCT4-overexpressing cells and control cells (one million cells/injection) subcutaneously into the flanks of nu/nu mice to assess OCT4-induced tumorigenesis for 3-4 weeks. We will also perform serial tumor transplantation assay to assess self-renewal ability of OCT4-overexpressed cells, using 100, 1000, 10000, 100000, 1000000 cell numbers injected subcutaneously into the flanks of nu/nu mice for 11-12 weeks. At the end of the experiment, we will harvest the tumors and perform histopathological analysis, immunohistochemistry, and RNA and protein studies to check the expression of OCT4, CD44 and CSC- and proliferation markers. This study will provide us with a clue of mechanistic aspect of OCT4 as a key transcription factor regulating stemness and tumorigenecity in breast cancer.

In our transcriptomic analysis of MCF10DCIS mammospheres treated with vitamin D compounds, we identified TP63-VDR signaling axis as a potential target of vitamin D-mediated inhibition of CSC population and DCIS progression to IDC. TP63 family is expressed in multiple isoforms which are linked to distinct clinical outcomes in cancer.

Two different isoforms of TP63, TAp63 and ΔNp63 undergoing alternative splicing can generate six variants, each showing different biological activities. The role of TP63 and its isoforms remains controversial in cancer. ΔNp63 promotes stem cell activity in basal-like breast cancer via Wnt and Shh signaling pathways whereas TAp63 induces differentiation. ΔNp63 directly regulates PI3K/CD44v6 pathway and increased level of ΔNp63 is associated with BCSC resistance to chemotherapy [502]. Since our study exhibited TP63 upregulation upon treatment with vitamin D, we will assess differential expression of TP63 isoforms by (1) designing primers for all the variants (α, β, and γ) of both TAp63 and ΔNp63 isoforms and assess the mRNA expressions by qPCR analysis and (2) western blot using antibodies that can detect different isoforms of TP63 to identify target of vitamin D compounds. Based on the isoforms we would identify, we will measure the expression of downstream target genes known to be directly regulated by each isoform (for example Bax, MDM2 and p21 for TAp63 isoforms, and STAT6, ZNRF2 and NOTCH2NL for ΔNp63 isoforms) [503, 504]. To further strengthen the significance of the specific isoform we would identify as the target of vitamin D, we will conduct inducible knock-down experiment to genetically perturb the expression of the TP63 isoform followed by treatment with vitamin D compounds. Based on this finding, we will perform xenograft studies to evaluate the effect on tumorigenicity and evaluate TP63 isoforms, differentiation and CSC markers in tumor samples. These studies will elucidate the mechanistic insights by which vitamin D compounds inhibit breast cancer progression and provide potential targets for chemoprevention.

Appendix 1: Epigenetic signature targeted by vitamin D in MCF10DCIS

A.1.1 Introduction

Epigenetic modulations are involved in every single stage of cancer progression, and emerging evidence regarding their role in hierarchically-organized cancer cells underscores the significance of epigenetic mechanisms in regulating cancer stem cell (CSC) formation, plasticity, and maintenance [226]. Accumulating research findings have demonstrated that miRNAs are implicated in the transition from ductal carcinoma in situ to invasive ductal carcinoma (**Figure A.1**) and dysregulation of microRNAs are identified in early breast cancer [505]. Targeting the differentially expressed miRNAs that control CSC populations in DCIS lesions may serve as potential therapeutic targets for the breast cancer prevention [506].

In particular, miR-200 family members are of interest for their critical roles in maintaining cancer stem cell (CSC) character, self-renewal signaling pathways, epithelial-mesenchymal transition (EMT), pluripotency, and reprogramming [507]. The expression of miR-200c and miR-200b is downregulated in BCSCs. Upregulation of miR-200c inhibits the formation of colonies *in vitro* and tumorigenesis *in vivo* by targeting BMI1 proto-oncogene, polycomb ring finger (*BMI1*), a regulator of stem cell self-renewal [508]. Meanwhile, expression of the miR200c/141 cluster is significantly increased in epithelial-like $ALDH^+$ BCSCs, but downregulated in mesenchymal cells. Depletion of miR200c/141 decreased cell proliferation and invasion *in vitro* and increased tumor lung metastasis and induced EMT *in vivo* [509]. Notably, members of the miR-200 family inhibit Notch signaling by targeting components such as jagged canonical Notch ligand 1 (*JAG1*) and mastermind-like transcriptional coactivators (*MAML2* and *MAML3*), and through

potentiating Notch activation by zinc finger E-box binding homeobox 1 (ZEB1). Notch and ZEB1 regulate one another's expression in a counter-feedback loop. In addition, increased expression of JAG1 and ZEB1 and decrease in miR-200 are observed in basal type breast cancer [510].

Other miRNAs are also involved in maintaining breast cancer stemness, including let-7, miR-146, miR-221/222, and miR-27. Let-7 targets HRas proto-oncogene, GTPase (*HRAS*) and high mobility group AT-hook 2 (*HMGA2*) to regulate self-renewal and tumorigenicity, and let-7 family miRNAs have reduced expression in mammospheres and BCSCs harvested from patients. Transfecting breast tumor-initiating cells with let-7 inhibited proliferation and mammosphere formation *in vitro* and tumorigenesis and metastasis in xenografts [511]. Long non-coding RNA H19 targets let-7 to increase expression of lin-28 homolog A (*LIN28*), a key pluripotency factor and downstream gene of the Wnt/β-catenin pathway; this promotes BCSC properties such as mammosphere forming ability, clonogenicity, and migration. LIN28 in turn blocks let-7 production and depresses let-7 target genes (*RAS*, *MYC*, and *HMGA2*). Together, these three factors form a negative feedback circuit to maintain breast cancer stemness [512]. Similarly, Bodal et al. found that miR-146 functions as a tumor suppressor and is associated with breast cancer susceptibility. It upregulates let-7 by inhibiting LIN28-mediated degradation, leading to the induction of asymmetric division by stem cells and inhibition of BCSC self-renewal [513, 514]. Meanwhile, miR-146a-5p represses tumor progression and mesenchymal markers and upregulates epithelial markers in TNBC cells. The effects are reversed upon overexpression of its downstream target SRY-box transcription factor 5 (*SOX5*), a transcription factor that induces EMT by transactivating *TWIST1* expression [515].

MicroRNAs are also known to play an oncogenic role in breast cancer by targeting tumor suppressor genes such as phosphatase and tensin homolog (*PTEN*) [516]. The miR-221/222 cluster is one such oncogene. In breast cancer, it inhibits *PTEN*, leading to phosphorylation of AKT and promotion of cancer growth and progression. Overexpression of miR-221/222 increased BCSC population and mammosphere formation through targeting the PTEN/Akt pathway, while blocking this cluster restored *PTEN* levels and reversed BCSC phenotypes [517]. In addition, miRNAs are involved in regulating the chemoresistance of breast cancer cells. miR-27b targets ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*), a substrate of the 26S proteasome that is downregulated in CSCs; this has the effect of upregulating the transporter ATP binding cassette subfamily G member 2 (*ABCG2*), the ABC transporter family being known to contribute to multidrug resistance. Downregulation of *ENPP1* is associated with breast cancer chemoresistance to docetaxel and the generation of BCSCs [518]. Since BCSCs exist widely within breast cancer, therapeutic targeting of those microRNAs that regulate BCSCs will effectively deter self-renewal, tumor progression, metastasis, and drug resistance. The purpose of our study here is to examine the regulation by vitamin D compounds of microRNA candidates identified in early pre-invasive breast cancer (using MCF10DCIS cells).

A.1.2 Materials and Methods

A.1.2.1 Cell culture and reagents

1α25$(OH)_2D_3$ and a Gemini vitamin D analog (BXL0124; 1α,25-dihydroxy-20R-21(3-hydroxy-3-deuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-cholecalciferol, >95% purity) were provided by BioXell, Inc. (Nutley, NJ) [287]. Vitamin D compounds were dissolved

in DMSO. MCF10DCIS.com human breast cancer cells (MCF10DCIS, RRID: CVCL_5552) were provided by Dr. Fred Miller at the Barbara Ann Karmanos Cancer Institute (Detroit, MI). Cells were maintained in DMEM/F12 medium supplemented with 5% horse serum, 1% penicillin/streptomycin, and 1% HEPES solution at 37 °C, 5% CO_2. The cells were passaged every 3-4 days between passage number p30 and p50. The cells were passaged every 3-4 days between passage number p30 and p50.

A.1.2.2 miRNA assays and quantitation by quantitative polymerase chain reaction analysis

RNA from MCF10DCIS monolayer cells treated with DMSO, 1α25$(OH)_2D_3$ and BXL0124 were collected after 48 hours using miRNasy mini kit (Qiagen, CA). Real time PCR analysis was performed using the ViiA™ 7 Real-Time PCR System (Applied Biosystems, CA) using 3µl of RT products in a reaction containing TaqMan miRNA assay and TaqMan Universal PCR Master Mix, following the manufacturer's instructions (Applied biosystems, CA). TaqMan assays used are hsa-miR-200a-3p (478490_mir), hsa-miR-200b-3p (477963_mir), hsa-miR-200c-3p (478351_mir), hsa-let-7a-5p (478575_mir) and hsa-miR-146a-5p (478399_mir). Relative quantities of each miRNA were calculated using ΔΔCT Method using U6 (001973) as endogenous reference. Three independent experiments were repeated.

A.1.2.3 Statistical analysis

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

A.1.3 Results

A.1.3.1 Selected miRNAs were not regulated by vitamin D compounds in MCF10DCIS cells

To investigate the effect of vitamin D compounds on microRNAs implicated in BCSC self-renewal, maintenance and breast cancer progression, we analyzed a few selected miRNA candidates in MCF10DCIS cells in monolayer culture treated with vitamin D compounds for 48 hours **(Figure A2)**. miR-200a was increased by 1.4-fold with $1\alpha,25(OH)_2D_3$ ($p>0.05$) and 1.8-fold with BXL0124 ($p>0.05$). Levels of miR-200b was increased by 1.3-fold with $1\alpha,25(OH)_2D_3$ ($p>0.05$) and decreased by 90% with BXL0124 ($p>0.05$). miR-200c was decreased by 80% with $1\alpha,25(OH)_2D_3$ ($p>0.05$) and increased by 1.1-fold with BXL0124 ($p>0.05$). The levels of let-7 miRNA was decreased by 98% with $1\alpha,25(OH)_2D_3$ ($p>0.05$) and 91% by BXL0124 ($p>0.05$). miR-146 expression was decreased by 78% with $1\alpha,25(OH)_2D_3$ ($p>0.05$) and increased by 1.3-fold with BXL0124 ($p>0.05$).

A.1.4 Discussion

MicroRNAs contribute to the post-transcriptional regulation of genes involved in many biological functions. In general, microRNAs suppress gene expression by inducing mRNA decay or translational repression [519]. Aberrant expression of miRNAs has been implicated in diverse cellular pathways integral to breast cancer development, progression, metastasis, relapse, chemoresistance, and BCSC characteristics. In our experiment, we selected a few microRNA candidates that have been reported as involved in BCSC self-renewal and breast cancer progression from early stage to invasive breast cancer **(Figure**

A1). Treatment of MCF10DCIS cells in monolayer culture with vitamin D compounds for 48 hours resulted in some alterations in the expression of the miRNA candidates we studied; however, these changes were neither dramatic nor statistically significant. It is possible that stemness-related miRNAs were not effectively targeted in monolayer culture by vitamin D. It is plausible to in the future perform the experiment in mammosphere culture, which enriches stemness in breast cancer cells.

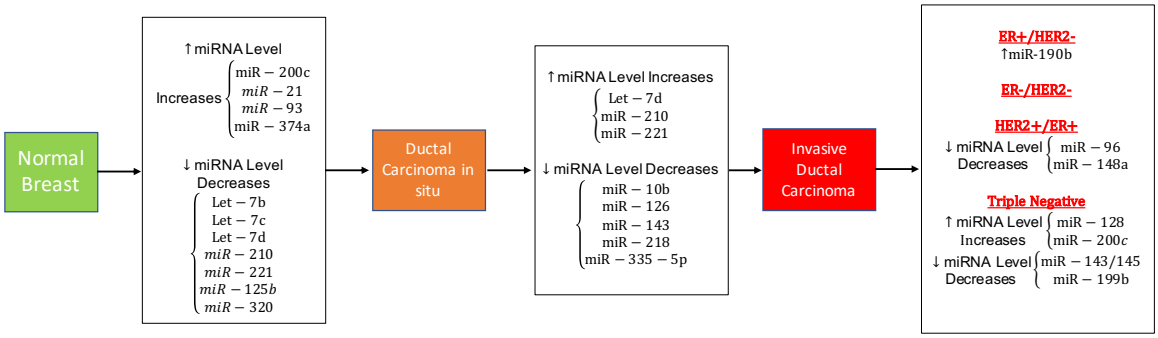


Figure A.1. MicroRNA signature in breast cancer progression

Profile of potential microRNAs deregulated in the progression of DCIS to IDC is shown. Targeting key miRNA candidates in the invasive transition will effectively prevent breast cancer progression. Diagram is modified from Stefano Volinia et al. PNAS 2012; 109(8):3024-3029

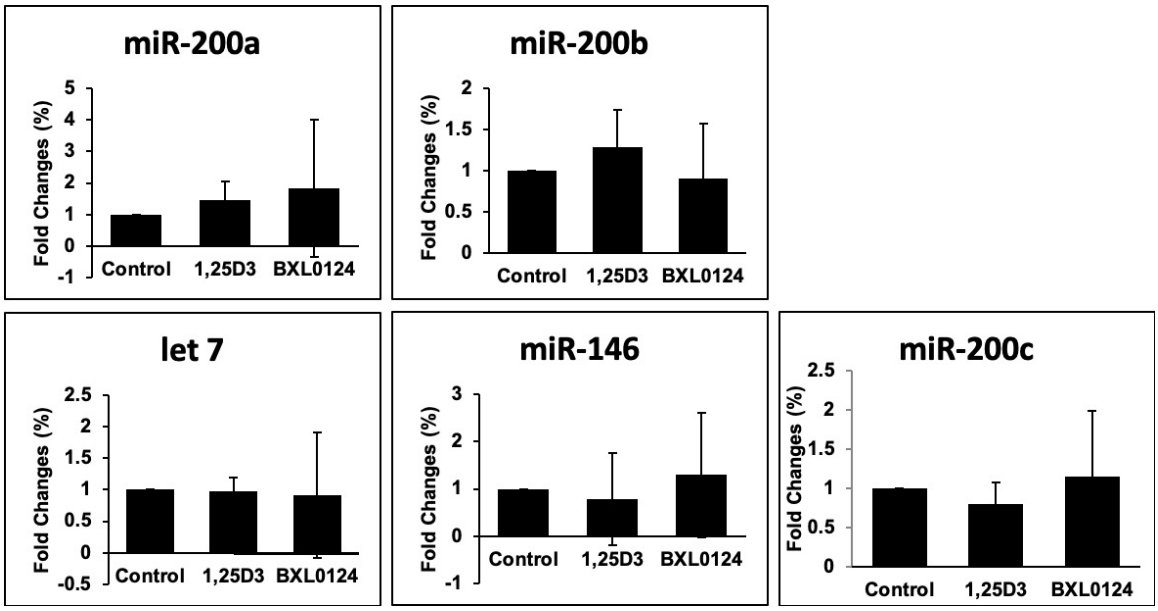


Figure A.2. Effects of vitamin D compounds on microRNAs in MCF10DCIS

MCF10DCIS cells in monolayer were treated with 1α,25(OH)2D3 (1,25D3, 100 nM) and BXL0124 (10 nM) for 4 days. Cells were harvested for qPCR analysis. Average Ct values and *p* values are shown in parenthesis for miR-200a (26, $p>0.05$), miR-200b (25, $p>0.05$), miR-200c (23, $p>0.05$), let 7 (23, $p>0.05$) and miR-146 (25, $p>0.05$). Experiments were repeated three times.

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