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Epoxyeicosanoids (EETs) Promote The Cancer Stem Cell State by Preventing Breast Cancer Cells Differentiation

by

Zineb El Kadiri

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCE

CALGARY, ALBERTA

MAY, 2014

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Abstract

Our goal is to examine the role of epoxyeicosatrienoic acids (EETs) – omega-6 poly-unsaturated fatty acid metabolites - in modulating breast cancer cell plasticity manifest in the discrete attractor state transitions between cancerous and differentiated states. We used an *in vitro* breast cancer cell differentiation system, MDA-MB231 that exhibited two quasi-discrete subpopulations representing a stem-like and a differentiated state. We found that elevated EETs suppressed the vitaminD-induced shift of the MDA-MB231 cells towards the differentiated state. Moreover, subpopulation gene expression profiles of cells exposed to EETs robustly identified stem cell-like and differentiated subpopulations as two discrete clusters, regardless of EETs. Interestingly, EETs promoted "stemness" and metastasis genes, while suppressed differentiation related genes, suggesting that EETs not only blocked the switch between the two attractors but also affected cell-intrinsic attractor properties. Our findings suggest that anti-EETs agents may have therapeutic or preventive utility against breast cancer that could be further explored.

Acknowledgements

I wish to thank my thesis supervisor, Dr. Sui Huang for the opportunity to participate in this research project. I am grateful for his mentoring and patience as we explored new ideas together and defined the direction of my research. I would like to thank all those who have contributed to my work and supported me during my study including, Wei Wu, Mitra Mojtahedi, Ivan Gomez and Rebecca Leong. A special thank to Dr. Joseph Zhou for his help in analyzing the microarray data.

The work in this thesis would not have been possible without the generous help and support I have received from my husband, my little ones Ilyas and Sulayman, my mom and sisters. Thank you all for your unwavering love and encouragement.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
7-AAD	7-Aminoactinomycin D
AA	Arachidonic Acid
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2
ACPS$	3-oxoacyl-ACP synthase, mitochondrial
AGR3	Anterior Gradient 3 homolog
AH$	ALDH High
AKT	Protein Kinase B (PKB)
AL	ALDH Low
ALDH	Aldehyde Dehydrogenase
ALX1	ALX homeobox 1
AMPK	Adenosine Monophosphate-Activated Protein Kinase
ARSH$	Arylsulfatase family, member H
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma 2
BCRP	ATP-binding cassette, sub-family G (WHITE), member2
BCSC	Breast Cancer Stem Cells
BICC1	Bicaudal C homolog 1 (Drosophila)
Bkca	Calcium Activated Potassium Channel
BNC2	Basonuclin 2
BT474	Breast cancer cell line

c-Myc	c-Myc binding protein
CD133	prominin 1
CD177	CD177 molecule
CD44	Cell Surface Glycoprotein CD44
CD24	CD24 Antigen
CIS	Carcinoma In Situ
cPLA2	cytosolic Phospholipase A2
CSC	Cancer Stem Cells
CXCR4	Chemokine (C-X-C motif) Receptor 4
CYP2C8	Cytochrome 2C8
CYP2C9	Cytochrome 2C
CYP2J2	Cytochrome 2J2
CYP3A	Cytochrome 3A
CYP450	Cytochrome P450
DCST1	DC-STAMP domain containing 1
DDIT4L	DNA-Damage-Inducible Transcript 4-Like
DEFB114	Defensin, beta 114
DHET	Dihydroxyeicosatrienoic acids
DLX6	Distal-Less Homeobox 6
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DSCAM	Down Syndrome Cell Adhesion Molecule
DU-145	Prostate cancer cell line

EET	EpoxyEicosaTrienoic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EIF4E2	Eukaryotic Translation Initiation Factor 4E family member 2
EMT	Epithelial to Mesenchymal Transition
EPHX-2	Epoxide Hydrolase-2
ER	Estrogen Receptor
ERBB2	Tyrosine Kinase-Type Cell Surface Receptor HER2
ERMN	ermin, ERM-like protein
ESA	Epidermal Surface Antigen
FACS	Fluorescent Activated Cell Sorting
FAM124B	Family with sequence similarity 124B
FBS	Fetal Bovine Serum
FBXO2	F-Box Protein 2
FOLH1B	Folate Hydrolase 1B
FOXL1	Forkhead Box L1
GEDI	Gene Expression Dynamics Inspector
Gli2	GLI family zinc finger 2
Gli3	GLI family zinc finger 3
GPR112	G Protein-Coupled Receptor 112
GPR182	G Protein-Coupled Receptor 182
GRN	Gene Regulatory Network
GSEA	Gene Set Enrichment Analysis

GSK/3beta	Glycogen Synthase Kinase 3 beta
HCC	Hepato Cellular Carcinoma
HB-EGF	Heparin-binding EGF-like growth factor
HBB	Hemoglobin, beta
HBZ	Hemoglobin, zeta
HER2	Human Epidermal Growth Factor Receptor 2
HETE	Hydroxyeicosatetraenoic Acid
Hh	Hedghog
IGF1	Insulin Growth Factor 1
IL-17C	Interleukin 17C
IL2RA	Interleukin 2 Receptor, Alpha
JAK	Janus Kinase
JAKMIP3	Janus Kinase and Microtubule Interacting Protein 3
JNK	c-Jun N-terminal kinases
LGI4	Leucine-rich repeat LGI family, member 4
LNCaP	Prostate cancer cell line
MAPK	Mitogen-Activated Protein Kinase
MCF2L	MCF.2 cell line derived transforming sequence-like
MCF7	Breast cancer cell line
MCHR1	Melanin-Concentrating Hormone Receptor 1
MDF7	Breast cancer cell line
MDR	ATP-binding cassette, sub-family B (MDR/TAP), member11
MMP1	Matrix Metallopeptidase 1

MPEG1	Macrophage Expressed 1
MPP7	Membrane Protein, Palmitoylated 7
MPZ	Myelin Protein Zero
mTOR	Mammalian Target Of Rapamycin
NEK8	NIMA-Related Kinase 8
NF-KB	Nuclear Factor Kappa-light-chain-enhancer of activated B
NSC	Normal Stem Cells
OR5D18	Olfactory Receptor, family 5, subfamily D, member 18
p21	Protein Complex subunit p21
PADI1	Peptidyl Arginine Deiminase, type I
PATL2	Protein Associated with Topoisomerase II homolog 2
PBS	Phosphate-Buffered Saline
PC3	Prostate cancer cell line
PCGEM1	Prostate-specific transcript (non-protein coding)
PDGFA	Platelet-Derived Growth Factor alpha polypeptide
PI3K	Phosphatidylinositide 3-kinases
PIGX	Phosphatidylinositol glycan anchor biosynthesis, class X
PIK3R5	Phosphoinositide-3-kinase, regulatory subunit 5
PR	Progesterone Receptor
PROCR	Protein C Receptor, endothelial
PRSS45	Protease, serine, 45
PRUNE2	Prune Homolog 2
Ptch1	Patched 1

PTEN	Phosphatase and tensin homolog
Ras	RAS oncogene homolog
SAA1	Serum Amyloid A1
SEC22C	SEC22 vesicle trafficking protein homolog C
sEH	Soluble Epoxide Hydrolase
sEHI	Soluble Epoxide Hydrolase Inhibitor
SEPT1	Septin 1
sFRP1	secreted Frizzled-Related Protein 1
SH3BGR	SH3 domain binding glutamic acid-rich protein
SK3BR	Breast cancer cell line
sHh	Sonic Hedghog
SOHLH1	Spermatogenesis and Oogenesis Specific Basic
SOM	Self Organizing Map}
SOX1	SRY (sexdeterminingregion Y) box1
SPOCK2	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2
STAT3	Signal transducer and activator of transcription3
SYT2	Synaptotagmin II
T47D	Breast cancer cell line
TDLU	Terminal Ductal Lobular Unit
TEF	Thyrotrophic embryonic factor
TGF-beta	Transforming Growth Factor beta
TGFBR1	Transforming Growth Factor Receptor 1
TIMP	TIMP metallopeptidase inhibitor 1

TMEM30C	Transmembrane protein 30C
TMSB15A	Thymosin Beta 15a
TNBC	Triple Negative Breast Cancer
TNN	Tenascin N
TPD52L3	Tumor proteiD52like3
TWIST1	Twist basic helix-loop-helix transcription factor
UXT	Ubiquitously-expressed, prefoldin-like chaperone
VDR	Vitamin D Receptor
VEGF	Vascular Endothelial GrowthFactor
VTD	Vitamin D
Waf1	cyclin-dependent kinase inhibitor 1A p21, Cip1
WDR33	WD repeat domain 33
Wnt	Wingless-type MMTV integration site family
ZCCHC24	Zinc finger, CCHC domain containing 24
ZNF750	Zinc finger protein 750

Chapter One: **Introduction**

1.1 Systems view of the gene regulatory network: cancer cells as cancer attractors

1.1.1 System biology approach

Historically, molecular biologists have investigated biological processes from the bottom up, focusing on molecules to explain the phenotype. Experimental technologies to analyze intracellular molecules led to a narrow view in which molecular pathways represent a linear chain of causation, they tend to ignore any "cross talk" between pathways (S. Huang & Ingber, 2007). Subsequently, the realization that there are more crosstalks than simple pathways that can offer a convenient molecular causation for any phenotype pushed scientists toward a new view of biology. This systemic approach investigates interactions of all components in a particular biological system simultaneously and asks how they give rise to collective behavior. It has been recognized that significant interactions across pathways exist which form a network in the cell rather than linear parallel causative-pathways associated with distinct phenotypes (S. Huang & Ingber, 2007). In fact, if a highly connected transcription factor is activated, hundreds if not thousands of target genes are affected. Also, when a specific cytokine triggers one behavior such as proliferation, it must repress other responses like apoptosis and differentiation suggesting that these behavior specific pathways must be interconnected to mediate such mutual activation of cell fates [for more details, see (S. Huang & Ingber, 2007)].

The "systems biology" approach involves the development of mathematical approaches, such as the reconstruction of integrated dynamics of networks (=the collective behavior of the interconnected molecules) from the quantitative properties of their elementary building blocks or the modeling of their behaviors based on such networks in tight conjunction with the experimental observation (Gardner, Di Bernardo, Lorenz, & Collins, 2003). The past years of

systems biology of gene regulatory network has led to deep insights in how these gene regulatory networks govern cell fate commitment in stem cells and cell differentiation. Such "emergent" (collective) behavior of the individual genes cannot be studied as consequence of the activity of individual pathways. From this viewpoint, it also becomes easier to see how disruption of the interactions that form the network is at the core of tumorigenesis (S. Huang & Ingber, 2007).

1.1.2 Theory of gene regulatory network and attractors

The central idea that cell types are "attractors" of said network which is now enjoying increasing popularity dates back to the concept that cell fates correspond to *attractor states*, was first proposed on theoretical grounds almost 40 year ago by Stuart Kauffman (Dirks, 2010). In modern interpretation what he proposed was essentially that attractor states of the network correspond to the stable gene expression profiles associated with cell type (S. Huang, Ernberg, & Kauffman, 2009). An attractor state as it is known in the theory of a complex non-linear dynamical system is a self-stabilizing discrete state, surrounded by unstable states of a gene regulatory network (GRN) (Kaplan & Glass, 1995) which, simply said, can be envisaged as a "*potential well*" in a "*potential landscape*" (S. Huang, 2009a). The latter is a mathematical representation of the integrated dynamics of the genome-wide gene regulatory interactions which impose constrains in the collective behavior of genes across the genome, which in turn force the genome-wide gene expression towards particular stable states. The concept of a landscape with attractors whose topography is determined by the structure of the GRN is captured by Waddington's metaphoric *epigenetic landscape* (Waddington, 1956), a metaphor introduced in the 1950s to explain the discrete nature of cell types as a system level stable state that arise from genetic interactions. (Note that this meaning is different from the term "epigenetic" used by biologist to describe covalent chromatin and DNA modifications (S. Huang & Ingber, 2007).) If

cells are attractors, then the cell fate-specific gene expression patterns, such as that of mature cells, can self-organize because they correspond to the "low-energy valleys " (states) towards which neighboring states (unstable) will "drain"(S. Huang & Ingber, 2007).

In experimental biology, cell type identity had long been thought to be cast in stone until studies in stem cell biology revived the discussion about the cell type plasticity and reversibility (Joshi & Enver, 2002), as demonstrated by the reprogramming of somatic cells into embryonic stem cell-like cells (S. Huang, 2009a), which came as a surprise to many. This behavior had long been predicted by the theory of cell type attractors (Kauffman, 1969). In the framework of the epigenetic landscape, re-programmability means jumping back to the "higher valleys". Importantly, the landscape concept also predicts the existence of cancerous states as a consequence of how the gene regulatory network is wired to produce the normal cell types: The latter would, according to Kauffman and Huang (S. Huang et al., 2009; S. Huang & Ingber, 2007), reflect the re-activation of a pre-existing, unused attractor state which was unoccupied during evolution and development and could under pathological conditions become accessible to the cell again.

A key notion here is that the attractors explain how cells can switch between discrete phenotypes. Such plasticity in the cell fate decisions is represented by the transition of a cell state from one attractor to another overcoming the "hills" in between the "valleys" (S. Huang & Ingber, 2007). The advent of genome-wide gene expression profiling has allowed us to obtain experimental evidence suggesting that mammalian cell types are indeed "high-dimensional attractor states" (S. Huang, Eichler, Bar-Yam, & Ingber, 2005). Moreover, the formal concept of "cancer attractors", derived from an integrative complex systems approach to gene regulatory network may provide a natural explanation for a particular tumor behavior and reconciles many

paradoxical properties of cancer cells (Zhou & Huang, 2011). Also, one practical theoretical conclusion from the attractor dynamics is that the *combined* perturbation of a distinct *set* of genes will drastically increase the probability of a reprogramming event (Zhou & Huang, 2011).

1.1.3 Abnormal cell types as cancer attractors

If the distinct cell types are "attractors" that encode specific "genetic programs" of the cell "pre-programmed" in the GRN, and cancer cells warrant being viewed as abnormal cell types, then, as noted above, cancer cells are trapped in abnormal "attractors" with a stable *cell type-specific* gene expression pattern that implement a malignant cell phenotype, and are unable to exit and move to an attractor that corresponds to a differentiated state (S. Huang & Ingber, 2007).

The concept of cancer attractors, which initially failed to catch the attention of cancer biologists due to the mathematical abstraction, provides a simple framework for integrating non-genetic and genetic contributions to tumorigenesis and opens a new perspective not available in the traditional paradigm of linear causative pathways (S. Huang & Ingber, 2007). ***Non-genetic*** factors can direct cells into the basins of cancer attractors without mutations by triggering a jump of the network state from one valley in the landscape to another due to a transient change in the expression of genes (S. Huang et al., 2009). In contrast, ***genetic*** alterations induce reshaping of the landscape topography via distorting basin of attractions affecting its size, the height of hills or depths of valleys (S. Huang et al., 2009; S. Huang & Ingber, 2007). These tumorgenic mutations could even create new side-paths in the landscape (e.g., lowering a separating hill) which will accidentally allow cells to enter an unused attractor state associated with a malignant gene expressing profile (S. Huang et al., 2009; S. Huang & Ingber, 2007).

Most importantly, from the attractor landscape model, it follows that "cancer stem cells" (see 1.1.4.) which still stimulates lively debate as to their very definition would be nothing but abnormal immature cells, trapped in a pathological "embryonic attractor" and separated from the differentiation attractor by epigenetic barriers too high to surmount. Thus, cancer (stem) cells are in a metastable state, only "kinetically" prevented from differentiating efficiently. But they harbor the *potential* to transition into a more mature, and hence, less migratory and less proliferative state (S. Huang et al., 2009).

The reachability of hidden, not spontaneously accessed cellular programs that the attractor model predicts is lucidly demonstrated by the recent reprogramming of somatic cells into an embryonic stem cell-like state, as mentioned above (S. Huang, 2009a). The efficiency of desired state transitions however is often very low, which as our group and an increasing number of investigators agree upon (S. Huang, 2009a), can be explained by the non-genetic heterogeneity of a cell population, such that in a given (clonal) cell population only a fraction is poised (within the attractor basin) to respond to the perturbation with the intended state transition (differentiation) (A. C. Huang, Hu, Kauffman, Zhang, & Shmulevich, 2009).

This will become important in the experiments in this thesis. The theoretical foundation and connection to non-genetic heterogeneity will be articulated in later sections (S. Huang, 2009a). In my research, the non-genetic heterogeneity in responding to drug perturbation will be at the center of our studies.

1.1.4 The concept of cancer stem cell: refining of cancer cell hypotheses vs. clonal evolution

Cancer is both a genetic disease and developmental disease (S. Huang et al., 2009). The current emphasis on the former, epitomized by the study of mutations in oncogenic pathways has led to neglect of the latter. However, the developmental versatility or plasticity of the very same (unmutated) genome to generate the diversity of cell types in metazoan is also exploited by cancer cells (S. Huang et al., 2009). Evidence demonstrates the existence of stem cells in many different types of cancers including testicular cancer, acute myeloid leukemia, breast cancer, certain brain cancers, colon cancer and other types of carcinomas (D'Angelo & Wicha, 2010). Flow cytometry sorting techniques and *in vivo* transplantation of potential cancer stem cell populations into immune-compromised mice have been used in each of these cancers to identify putative cancer stem cell markers (D'Angelo & Wicha, 2010). Only a minority of cancer cells had the ability to proliferate extensively and transfer the cancer (Al-Hajj & Clarke, 2004). This subpopulation contained cells that have been termed as "cancer stem cells" because of their similarity to the normal stem cells in terms of self-renewal (Al-Hajj & Clarke, 2004; D'Angelo & Wicha, 2010). (The concept is still somewhat controversial, because the term 'stem cell' evokes a different imagery in different people, and some investigators prefer the term 'tumor initiating cell'. Here, we use the term "cancer stem cell" – with this caveat in mind.)

From theories that explain how the very same genome generates the diversity of cell types and how disruption of this process can lead to cancer, in the past years it was proposed that cancer cells are trapped in abnormal immature "*attractor states*" of the gene regulatory network that governs cell differentiation due to epigenetic or mutational rewiring of this network (S. Huang et al., 2009). The gene expression program associated with these self-perpetuating immature attractor states command embryonic phenotypes (increased proliferation, migration,

angiogenesis, etc) that contribute to malignancy (S. Huang et al., 2009). This defect of fate regulation naturally explains the "stem cell character" of the cancer propagating cells, which therefore also harbor a hidden potential for adopting multiple cell fate options, some of them corresponding to less malignant phenotype (S. Huang et al., 2009).

The cancer stem cell idea supports the attractor hypothesis: The recent (re)-emergence of the idea of cancer "stem" cells (Tan, Park, Ailles, & Weissman, 2006) or of stem cell-like cancer propagating cells is a strong validation of the conceptual foundation of the *cancer attractors* hypothesis which, as mentioned above in section 1.1.3, is rigorously founded in formal treatment of gene regulatory networks. Moreover, the recent spate of work on breast cancer stem cells (Al-Hajj & Clarke, 2004; Cobaleda, Cruz, González-Sarmiento, Sánchez-García, & Pérez-Losada, 2008), demonstrating quasi-discrete (switch-like) behaviors between phenotypes of distinct maturity confirms both the concept of non-genetic, dynamical heterogeneity of cell populations and the attractor hypothesis (S. Huang & Ingber, 2007).

1.2 Comparing the normal stem cells (NSC) with the cancer stem cells (CSC): the hallmark properties, the cell of origin and the surrounding microenvironment

"*Embryonal rest*" a 19th century concept that was first to propose the thought that cancer might originate from stem cells (Sell, 2004) is being harkened back over a century later as we recognize the similarity between the embryonal state and the stem-like character of cancer cells which has led to the view that some forms of cancer originate in adult stem cells (Sell, 2004). In 1994, John Dick and colleagues were able to identify cancer stem cells in certain types of human leukemia by inducing acute myelogenous leukemia (AML) after transplanting leukemia-initiating stem cells present in the peripheral blood of acute AML patients into immuno-deficient (SCID) mice (Hope, Jin, & Dick, 2004). This breakthrough opened the door for investigating the

role of stem cells in perpetuating cancer in various organs. In 2003, Michael Clarke's lab convincingly identified the presence of cancer stem cells in breast cancer (Al-Hajj & Clarke, 2004). The following year, Peter Dirks' lab proved stem cell involvement in brain cancer (Singh, Clarke, Hide, & Dirks, 2004).

1.2.1 Normal stem cells: self-renewal and differentiation potential

Stem cells sit at the top of the developmental hierarchy, having the ability to self-renew and give rise to all the cell lineages in corresponding tissues. There are three kinds of stem cells: embryonic, germinal and somatic (Kakarala & Wicha, 2007). Embryonic stem cells (ESC) are generated from the inner cell mass of the blastocyst. ESCs have the capacity of an extensive proliferation and the potential to differentiate into all of the cells of the adult organ (every type of daughter cell in the mature organism) (Kakarala & Wicha, 2007). Molecular profiling has found Polycomb genes, Nanog, Oct4, and Sox2 to be important genes in regulating ESCs (Assou et al., 2007; Thomson et al., 1998). The germinal layer of the embryo gives rise to the germinal stem cells with the ability to differentiate into organ-specific cells and are responsible for reproduction (Z. Wang, Li, Banerjee, & Sarkar, 2009). The somatic stem cells or progenitor cells have limited multi-potential, they produce cells that differentiate into mature specialized cells responsible for normal tissue renewal (Kakarala & Wicha, 2007).

The different types of stem cells not only vary in their potentials, but they also proliferate differently. During the process of self-renewal, stem cells can divide symmetrically, producing two daughter cells with parental cells properties, or asymmetrically, whereby one cell remains in the stem cell state allowing for the continuous formation of stem cells which is important to increase the stem cell pool of an organ during some developmental stages or after wound healing, the other daughter cell becomes a progenitor cell that ultimately undergoes

differentiation to become a more specialized mature cell that makes up the various tissues of the body (D'Angelo & Wicha, 2010).

1.2.2 The cancer stem cell of origin

Stem cells are relatively long-lived compared to other cells with the highest potential for proliferation and therefore have a greater opportunity to accumulate mutations necessary for transformation (Mcdermott & Wicha, 2011). It is thought that perhaps only one or two mutations, such as those conferring self-sufficiency in growth or insensitivity to antigrowth signals, are needed for stem cells to initiate tumorigenesis (D'Angelo & Wicha, 2010). While cancer stem cells exhibit similar characteristics to normal stem cells, including self-renewal and unlimited replication, and are similar with respect to many cell behaviors, the cancer stem cell model per se does not imply that cancer stem cells must arise from normal stem cells (D'Angelo & Wicha, 2010). The cancer stem cell model describes the observation that cancer cells are heterogeneous and exist within a hierarchy of proliferative potentials. But it does not address the origin of these cells as to whether they arise from the transformation of normal stem cells, whether they result from the *dedifferentiation* of downstream-restricted progenitor cells that have acquired stem cell properties (D'Angelo & Wicha, 2010; McDermott & Wicha, 2010). The latter may be explained by oncovirus-induced cancer, which infects terminally differentiated cells and completely reprograms their cell cycle machinery to reactivate self-renewal (Ferrari, Berk, & Kurdistani, 2009).

1.3 Breast cancer is a stem cell disease that could be influenced by environmental factors

1.3.1 Environmental factors account to a certain degree for breast cancer disparities

Breast cancer is the most common malignancy identified among American women, except for skin cancers and one of the deadliest cancers in women in Western countries,

exceeded only by lung cancer (DeSantis, Siegel, Bandi, & Jemal, 2011). According to the 2013 statistics, the American Cancer Society for breast cancer estimated nearly 232,340 new cases of invasive breast cancer would be diagnosed in women. Approximately 64,640 new cases of the earliest form of breast cancer carcinoma in situ (CIS) will be diagnosed, and about 39,620 women will die from breast cancer. Breast cancer death rates have been declining since early 1990s, a trend that has been attributed to progress in early detection utilizing mammography screening and clinical breast examination, as well as improved treatment. Despite the significant advances in diagnosing and treating breast cancer, many clinical and scientific complications remain unresolved. Breast cancer disparities can disaggregate incidence rates by socioeconomic status, race/ethnicity and migrant status (DeSantis et al., 2011; Gomez et al., 2010). In 2008, screening rates were lower in poor women (51.4%) compared with non-poor women (72.8%) (DeSantis et al., 2011). Incidence rates were reported to be lowest within Asians; however, specific ethnicity can further disaggregate this group to have variant occurrence rates of breast cancer, according to the substantial heterogeneity in health care practices and lifestyles (Gomez et al., 2010). Immigration and longer residence of Asian women in the United States have been associated with elevated breast cancer risks in compare to women living in Asian countries (DeSantis et al., 2011; Gomez et al., 2010). Studies have shown that immigrant status (US born as compared to foreign born women) is relatively correlated with breast cancer risk as a result of variations in some lifestyle factors such as diet, and reproductive factors (earlier onset of menstruation, higher age at first live birth, and lower breastfeeding rates). These non-genetic factors can be a strong evidence for the major role of environmental factors in breast cancer causation.

1.3.2 Mammary stem cells in normal development

The mammary gland in humans and other mammals undergoes periodic biphasic regenerative cycles of replication and differentiation throughout the female reproductive life from embryonic development to puberty, pregnancy, lactation, involution and menopause (Dontu, Al‐Hajj, Abdallah, Clarke, & Wicha, 2003). The breast consists of a complicated network of ducts ending in smaller ductal structures called terminal ductal lobular units (TDLU) (Dontu et al., 2003; Sell, 2004). In the TDLU, the putative breast stem cells can differentiate into two types of cells, a luminal epithelial cell or myoepithelial cell (Dontu et al., 2003). The luminal lineage can further be divided into ductal and alveolar cells that constitute the alveolar units that expand during pregnancy (Dontu et al., 2003; Harmes & DiRenzo, 2009). Myoepithelial cells are basal and surround the ducts and have contractile properties in the process of lactation (Dontu et al., 2003).

Development of the mammary epithelium appears to require the existence of a stem cell compartment, the expansion of the cellular proliferation at the onset of puberty results in ductal elongation and side-branching under hormonal stimulation (Dontu et al., 2003; Harmes & DiRenzo, 2009). During successive reproductive cycles and lactation, further maturation characterized by regeneration and functional differentiation takes place. Cessation of lactation requires a massive apoptosis and tissue remodeling, ultimately resembling a structure of before pregnancy (Harmes & DiRenzo, 2009; Sell, 2004). In addition, the mammary epithelium in non-pregnant adults regenerates and wanes with every ovulatory cycle (Harmes & DiRenzo, 2009). Therefore, a compartment of cells with high proliferative capacity and differentiation potential is needed for such tremendous requirement of new epithelial tissue, which suggest the derivative of

these cells from self-renewing adult mammary stem cell (Dontu et al., 2003; Harmes & DiRenzo, 2009; Sell, 2004).

1.3.3 Mammary cancer stem cells in solid tumors

Following the identification of cancer stem cells in leukemia (see above), researchers investigated if similar cells could be found in solid tumors. The existence of self-renewing, multi-potent mammary stem cells was first described by DeOme et al. (1959) in an *in vivo* mouse mammary fat pad transplant system (D'Angelo & Wicha, 2010). This technique requires the removal of the epithelial component of a young mouse fat pad and then injection of breast cells into the stroma left behind (D'Angelo & Wicha, 2010). These transplanted cells form ductal outgrowth similar to the endogenous one (D'Angelo & Wicha, 2010; Mcdermott & Wicha, 2011). In 1971 the work of Daniel et al, which was later confirmed by Kim et al in 2000 demonstrated that a single stem cell was capable of generating the entire mammary gland from serially transplanted random fragments of epithelium (D'Angelo & Wicha, 2010; McDermott & Wicha, 2010). Normal mammary epithelial stem cells showed senescence on serial transplantations after the 4^{th} generation; however, in about 25% of cases, seven and eight serial transplantations were achieved (D'Angelo & Wicha, 2010; McDermott & Wicha, 2010). A retroviral tagging assay was used by Kordon and Smith (1998), they were able to show that progeny of a single infected multipotent stem cell were responsible for the generation of a new gland in a recipient animal (Dontu et al., 2003; McDermott & Wicha, 2010). Kuperwasser's group improved the transplant assay for human breast epithelial cells by "humanizing" the fat pad with injecting a mixture of irradiated and non-irradiated human fibroblasts, which generated a stromal environment that more closely simulates human breast tissue (Dontu et al., 2003; McDermott & Wicha, 2010). Further support for the existence of pluripotent epithelial stem cells

in the mammary gland was described by Eirew et al (2008) with an *in vivo* stem cell breast assay for quantifying human breast stem cell activity based on implantation of cells embedded in collagen gels into the renal capsule of immunodeficient mice supplemented with estrogen and progesterone (D'Angelo & Wicha, 2010). The development of these *in vivo* transplant assays has facilitated the prospective isolation of mouse and human breast stem and progenitor cells based on various cell-surface markers (Dontu et al., 2003).

1.3.4 Breast cancer is a disease with functional inter-tumor and intra-tumor heterogeneity

Of the many types of breast cancers, approximately 80% are invasive ductal carcinomas, and 10-15% are invasive lobular carcinoma. The remaining 5-10% constitutes rare types of breast cancers. Initially based on histopathology but recently confirmed by microarray-based gene expression profiling (Sotiriou & Pusztai, 2009), invasive ductal carcinomas display inter-tumor heterogeneity and can be further subdivided into five subtypes: luminal A, luminal B, ERBB2-positive (human epidermal growth factor receptor 2, HER2), basal and normal-like (Hwang-Verslues et al., 2009). Each of these subtypes has various risk of disease progression, incidence, response to treatment, and preferential organ sites of metastases (Fadare & Tavassoli, 2008; Hwang-Verslues et al., 2009). There is increasing evidence for a cell hierarchy in many cancers, including breast cancer (Cariati & Purushotham, 2008). This is the direct consequence of the presence of cancer cells with stem cell characteristics – or cancer stem cells. At the top of this hierarchy a small population of cells display stem-cell properties (Cariati & Purushotham, 2008). The interpretation of tumors as hierarchical cellular structures has changed scientists' vision of breast cancer.

The cancer stem cell theory explains the clonality and the intra-tumor heterogeneity of tumors by postulating that a subset of cells within certain tumors—the cancer stem cells—self-

renew, give rise to various differentiated tumor cells, and ultimately drive tumor growth and metastasis (lindeman & visvader, 2010). It is argued that cancer stem cells undergo non-genetic changes analogous to the differentiation of normal cells, producing phenotypically different non-tumorigenic cancer cells that compose the bulk of cells in a tumor and thus contribute to the tumor's cellular heterogeneity (Cariati & Purushotham, 2008; Ricardo et al., 2011). This is predicted by the plasticity explained by attractor transitions.

Various markers have been identified to distinguish the tumorigenic cancer cells from non-tumorigenic cells, which indeed indicate their intrinsic differences.

Al Hajj et al was first to report in 2003 that a subpopulation of cells could give rise to breast tumors (Cariati & Purushotham, 2008). Using flow cytometry, breast cancer stem cells were isolated and identified based on the expression of the adhesion surface markers CD44 and CD24 (Cariati & Purushotham, 2008). Distinguishing tumorigenic from non-tumorigenic cells based on marker expression is a cornerstone of the cancer stem cell hypothesis. Without this evidence, all cancer cells would be considered to have the same stochastic probability of proliferating and forming a tumor.

***Limitations to the use of the identified markers in cancer*:** Despite the strong data supporting the stem cell model in some cancers, it is important to acknowledge that some markers appear to be robust for some types of cancer but not others. In initial studies of brain tumors, CD133 was identified as marker for brain tumor stem cells (Shackleton, Quintana, Fearon, & Morrison, 2009), however, later studies showed no difference in the expression of this marker between tumorigenic and non-tumorigenic cells in other types of brain tumors (Shackleton et al., 2009). These finding suggest that some brain tumors follow the cancer stem cell model whereas others may not (Shackleton et al., 2009). Therefore, we should not deny the possibility that in a variety

of cancers, some markers used to separate the tumorigenic cells from non-tumorigenic cells may work in some circumstances or environments but not in others (Shackleton et al., 2009; Tan et al., 2006). Functional assays may be required to confirm the robustness of certain markers to solidify the differences between tumorigenic and non-tumorigenic cells in therapy sensitivity and other biological properties.

1.3.4.1 Breast cancer cell population heterogeneity is reflected by their stem cell marker expression:

Breast cancer is a heterogeneous disease. This can mean two things: (*i*) an apparent type of cancer actually consists of different subtypes (inter-patient heterogeneity). (*ii*) In the second meaning of interest in this thesis, heterogeneity (as used in the previous sections) refers to the fact that the cells within one tumor (intra-tumor heterogeneity), even without genetic differences, can belong to different (sub)-types (e.g. stem cell and non-stem cells).

With help of gene expression profiles the classification of breast tumors into different subtypes has been consolidated (Hsiao, Chou, Fowler, Mason, & Man, 2010; Hwang-Verslues et al., 2009). Differing characteristics of cancer stem cells in each type may explain why they differ in degree of invasion, clinical outcome and treatment response. Luminal tumors are positive for estrogen receptors (ER) and progesterone receptors (PR), generally well differentiated and show less aggressive primary tumor characteristics. The majority responds well to hormonal interventions and is associated with better clinical outcome (Hsiao et al., 2010; Hwang-Verslues et al., 2009). By contrast, $HER2^{+}$ tumors have amplification and/or overexpression of the *ERBB2* oncogene and are candidates for specific systemic "target-selective" therapy with anti-HER2 antibodies (Herceptin). ER-/PR-and HER-negative phenotype is used to identify the basal-like tumors; thus, these tumors are also called *triple-negative* breast cancer (TNBC) (Hsiao et al.,

2010; Hwang-Verslues et al., 2009). The basal sub-type of breast cancer is associated with poor clinical outcome, and shows a specific pattern of distant metastasis (Fadare & Tavassoli, 2008; Hwang-Verslues et al., 2009). Approximately only 20% of these tumors respond well to standard chemotherapy (Hsiao et al., 2010; Hwang-Verslues et al., 2009). Currently there is no molecular-targeted therapy for TNBC (Hsiao et al., 2010). Thus, developing improved treatments for TNBC is one of the highest priorities of current breast cancer research (Fadare & Tavassoli, 2008). A number of agents capable of targeting breast cancer stem cells in preclinical models are currently entering clinical trials including several different poly (ADP-ribose) polymerase inhibitors, JAK kinase, and EGFR inhibitors as well as "revived" classical chemotherapeutic agents such as platinum salts (Hsiao et al., 2010).

1.3.4.2 Functional heterogeneity in CD44 high human breast cancer stem cell like compartment:

Breast cancer stem cells have been isolated from human breast tumors or cancer-derived pleural effusions using cell surface markers in combination with flow cytometry. Al-Hajj et al found a subpopulation of cells with a specific pattern of markers that were defined as $CD44^{+}$/ $CD24^{-/low}$ /ESA^{+} after lineage depletion (Lin-) (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003). These cells also expressed epithelial-mesenchymal transition (EMT) markers. Using NOD/SCID mice, they showed that this subpopulation had a higher tumorigenic potential than bulk tumor cells when transplanted into these mice and were able to generate new tumors from as few as 100 cells (Al-Hajj et al., 2003). *In vitro* culture system studies have also shown that single cell suspensions of $CD44^{+}$/ $CD24^{-/low}$ /Lin^{-} cells derived from human breast cancers were able to proliferate extensively and form clonal mammospheres and are more tumorigenic than the established breast cancer-derived cell lines including MCF-7 and B3R (Hwang-Verslues et al., 2009). These findings have encouraged more studies in the field to adapt these systems to

isolate breast cancer stem cells to further characterize signal transduction pathways, gene expression profiles and other factors contributing to self-renewal and drug resistance, the hallmark of breast cancer stem cell.

1.3.4.3 $CD44^+/CD24^-$ pattern correlates with the basal breast cancer subtype rather than tumorigenisis:

Additional useful markers have been identified to characterize breast cancer stem cells (BCSC). Based on AL Hajj el al. finding of the $CD44^+/CD24^{-/low}/Lin^-$ pattern identifying BCSC, Shipitsin et al have used the gene expression profiling of $CD44^+$ cells from breast carcinoma-derived pleural effusions, and were able to identify PROCR (Shipitsin et al., 2007). PROCR is also a marker of hematopoietic, neural, and embryonic stem cells, which encodes a cell surface receptor and its expression is specific to $CD44^+$ epithelial cells (Shipitsin et al., 2007) . With the advent of flow cytometry, another marker, CD133, was identified as a marker of breast cancer stem cells isolated from cell lines generated from Brca1-exon11/$P53^{+/-}$ mouse mammary tumors. CD133 is already a known marker for blood, liver, brain and prostate cancer stem cells (Hwang-Verslues et al., 2009). The group found that the expression of CD133 did not overlap with the phenotype $CD44^+/CD24^{-/low}$ and that both subsets had a similar potential of self-renewal. This discovery implies that markers other than CD44 maybe associated with different types of breast cancer stem cell subpopulations (Hwang-Verslues et al., 2009). To overcome the limitation of using $CD44^+/CD24^{-/low}$ as the only pattern for BCSCs, more recent studies have used *in vitro* systems to show that aldehyde dehydrogenase activity (ALDH) was increased in both normal and cancerous human mammary epithelial cells that show stem/progenitor cell properties. In breast cancer, the high ALDH activity population is tumorigenic, with the ability of self-renewal and forming heterogeneous tumors resembling the parental ones (Ginestier et al., 2007). All of

the $ALDH^+$ cells were able to generate tumors in NOD/SCID mice. However, only a small proportion of these cells overlapped with $CD44^+/CD24^{-/low}$ population of cells (Hwang-Verslues et al., 2009). *In vivo*, the $ALDH^+/CD44^+/CD24^{-/low}/Lin^-$ subpopulation was able to generate larger tumors at a faster speed, while the $ALDH^-/ CD44^+/CD24^{-/low}/Lin^-$ failed to form tumors (Hwang-Verslues et al., 2009). In this work, we will use the ALDH activity as a marker for our *in vitro* studies.

Fillmore and Kuperwasser have examined the expression of CD44 and CD24, and they found that the $CD44^+/CD24^-$ subpopulation consistently correlates with the basal subtype rather than tumorigenesis (Fillmore & Kuperwasser, 2008). By including a third parameter epithelial specific antigen (ESA^+), they showed that the subpopulation $CD44^+/CD24^-/ESA^+$ has the capacity of self-renewal, reconstituting the parental cell line heterogeneity, forms tumors from as few cells as low as 100 cells, has slower cell cycle, selectively survives chemotherapy and has a 40% recurrence rate of invasive breast cancer (Fillmore & Kuperwasser, 2008). All these properties are hallmarks of cancer stem cells. It is interesting to note that the percentage of $CD44^+/CD24^{-/low}/ESA^+$ cells within a line is influenced by various factors such as cell density, presence or absence of growth factors, and the frequency of passaging, suggesting the role of the surrounding niche in regulating the cell lines' dynamics (Fillmore & Kuperwasser, 2008).
The implication that there might be various subtypes in each type of breast cancer requires more combinatorial studies of these markers to determine the overall marker profile of each type of these tumors and their possible heterogeneous origins (Fillmore & Kuperwasser, 2008; Hwang-Verslues et al., 2009). Studies on a well-established hematopoietic cancer stem cell system revealed that heterogeneity of cancer stem/progenitor cells could give rise to different types of cancers (Hwang-Verslues et al., 2009; Shipitsin et al., 2007). To address the same concept in

solid tumors, Wendy et al. examined the expression profile of cancer stem cell markers in eight known breast cancer cell lines (Hwang-Verslues et al., 2009). The $CD44^{+}/CD24^{-}$ subpopulation has been shown to have stem cell properties in only few cell lines, such as MCF-7 and SK-BR3 (Fillmore & Kuperwasser, 2008). In other cell lines, this criterion does not directly correlates with tumorigenicity (Fillmore & Kuperwasser, 2008). Using flow cytometry, *in vitro* soft agar colony formation techniques along with the tumor formation in NOD/SCID mice, they have found great heterogeneity in the expression of stem cell markers among breast cancer cell lines. They were able to isolate a subset of cells from the MDA-MB-231 cell line (the prevalence of $CD44^{+}/CD24^{-/low}$ in the bulk cell population was more than 90%) expressing the markers PROC and ESA. This subpopulation was highly enriched for the cancer stem cell/progenitor properties and exhibited high tumorigenesis in both *in vitro* and *in vivo* assays (Hwang-Verslues et al., 2009). The finding of multiple markers will allow further enrichment and purification of highly tumorigenic cancer stem cells from the $CD44^{+}/CD24^{-/low}$ breast cancer cells, thus will give a great potential to clearly define the molecular signature for targeting different cancer stem cells in breast cancers.

1.4 Molecular biology of breast cancer

1.4.1 Cell signalling pathways regulating self-renewal and differentiation of Breast normal and cancer stem cells

Based on the studies of the regulation mechanism of the cancer stem cell, cancer stem cells relied greatly on the signal pathways stability to be able to maintain the defining features of CSC, self-renewal, and differentiation. Disorder or deficiency in these cascades has been reported to be linked to tumorigenicity (D'Angelo & Wicha, 2010; Tyagi, 2005). In order to effectively target CSC, a detailed understanding of the mechanisms that underlie the CSC

behavior is of greatest importance for development of anti-cancer drugs targeting CSCs. Given the similarities between normal stem cells and CSCs, various molecular signalling pathways initially discovered in studies on normal stem cells have later been shown to be involved in carcinogenesis, including breast cancer (D'Angelo & Wicha, 2010; Dontu et al., 2003). Some of the pivotal pathways that have been shown to regulate normal and malignant stem cells as well as recurrence include: Wnt, Notch, Hedghog, and PTEN PI3K/Akt signalling pathways (D'Angelo & Wicha, 2010; Dontu et al., 2003; Nakshatri, Srour, & Badve, 2009; Tyagi, 2005).

1.4.1.1 The canonical Wnt signalling pathway:

Supporting its importance in many developmental processes, in some self-renewing tissues in mammals and its necessity throughout life, the constitutive activation of Wnt signalling has been associated with many human cancers (D'Angelo & Wicha, 2010; Dontu et al., 2003; McDermott & Wicha, 2010; Nakshatri et al., 2009; Tyagi, 2005). In human breast cancer, the role of Wnt/ Beta-catenin signalling has been subject of recent investigations. Overexpression of several Wnt family members has been identified in different cell lines and tissues (Luo, Yin, Ma, & Lu, 2010). Down-regulation of sFRP1, a ligand binding competitive inhibitor for Frizzled receptor, which results in increased Wnt activity, was detected in 40% to 80% of breast cancers (Luo et al., 2010). Other studies reported the accumulation of cytoplasmic/nuclear β-catenin (a read out of Wnt pathway activation) in more then 60% of human breast cancers (Kühl & Kühl, 2012; Luo et al., 2010). In invasive cancers, localization of β-catenin was associated with $CD44^{+}/CD24^{-}$ profile and poor outcome suggesting a valuable therapeutic target for this aggressive breast cancer subtype (Khramtsov et al., 2010). The mechanism by which the Wnt/β-catenin cascade is trigged in these tumors remains unclear and further studies are required to

understand the details and the mechanism of this signalling pathway (Khramtsov et al., 2010; Luo et al., 2010).

1.4.1.2 The Notch signalling pathway:

Activation of the Notch pathway causes changes of cell fate, such as proliferation of undifferentiated cells, blockage of differentiation, or lineage specific differentiation (Dontu et al., 2003). Some of the Notch target genes are c-Myc, cyclin D1, NF-kB, and p21/Waf1 (Dontu et al., 2003). Notch signalling pathway deregulation has been reported in many human cancers including breast cancer (D'Angelo & Wicha, 2010; McDermott & Wicha, 2010). The Notch-4 gene is known to be involved in normal mammary development. *In vitro*, overexpression of the active form of Notch-4 inhibits differentiation of normal breast epithelial cells (Dontu et al., 2003; McDermott & Wicha, 2010). *In vivo*, overexpressing Notch-4 in the mammary gland of transgenic mice failed to develop secretory lobules during gestation, and led to tumor formation (Dontu et al., 2003).

The Notch signalling pathway has been reported to cross-talk with various oncogenic cascades that are also cancer stem cell regulators including NF-kB, Akt, Sonic hedgehog (SHh), mammalian target of rapamycin (mTOR), Ras, Wnt, EGFR and platelet-derived growth factor (PDGF) signalling (D'Angelo & Wicha, 2010; Tyagi, 2005). Notch signalling pathway has been shown to be involved with not only the development of the mammary gland but is aberrantly activated in the invasive breast cancers. Furthermore, several recent studies have linked the increased levels of Notch receptors and cells enriched for breast stem cells, proposing a novel therapeutic target to prevent recurrence of invasive breast cancers (D'Angelo & Wicha, 2010; Dontu et al., 2003; Nakshatri et al., 2009).

1.4.1.3 The Hedgehog signalling pathway:

The Hedgehog (Hh) pathway regulates fundamental processes in ESCs such as proliferation, cell fate, stem cell maintenance, self-renewal and differentiation (Dontu et al., 2003; Luo et al., 2010). In normal breast tissue, Ptch1 and Gli2 are important in regulating postnatal mammary ductal development, and Gli3 plays a critical role in inducing embryonic mammary glands (Luo et al., 2010). In human breast cancers, the Hh pathway has been reported to be activated in a majority of human breast cancers (D'Angelo & Wicha, 2010; Luo et al., 2010). Liu et al. demonstrated that the Hh cascade is important for the self-renewal and multi-lineage differentiation of human breast CSCs characterized by $CD44^{+}/CD24^{-/low}$ compare to the bulk cancer cells, highlighting the importance of Hh signalling in normal and malignant breast stem cells (Khramtsov et al., 2010; Luo et al., 2010). The same group showed that Ptch1, Gli1, and Gli2 expression is enriched in mammary stem cells cultured as mammospheres whereas their down-regulation is observed once the cells are induced to differentiate (Khramtsov et al., 2010; Luo et al., 2010). Hence, suggesting a role in breast carcinogenesis (Luo et al., 2010).

1.4.1.4 The PTEN PI3K/Akt signalling pathway:

PTEN is lipid phosphatase that suppresses the Akt survival pathway which plays a role in modulating cellular apoptotic pathways (D'Angelo & Wicha, 2010; McDermott & Wicha, 2010). Several studies on PTEN mouse models reported the role of PTEN PI3K/Akt pathway in controlling stem cells homeostasis and malignancies in numerous tissues (McDermott & Wicha, 2010). PTEN is a tumor suppressor acting upstream of Akt (protein kinase B), a central regulator in the Wnt and PI3K signalling pathways that is critical in energy homeostasis (McDermott & Wicha, 2010). A role of PTEN in breast cancer and stem cells was identified in mammary epithelial cells when PTEN deletion led to mammary gland development and breast cancer in

animal models (Dontu et al., 2003; McDermott & Wicha, 2010). PTEN knockdown in breast cancer cells results in an increase in mammosphere formation *in vitro* and tumor-initiation in NOD/SCID mice (McDermott & Wicha, 2010). The Wicha group also demonstrated that PTEN down-regulation activated the Akt pathway that led to an increase of normal and malignant human mammary stem/progenitor cells both *in vitro* and *in vivo* (D'Angelo & Wicha, 2010). Akt activation, in turn led to activation of downstream cascade of Wnt /beta signaling pathway through phosphorylation and inhibition of GSK3beta (McDermott & Wicha, 2010). Therefore, PI3K or Akt selective inhibitors may be useful drugs in targeting breast cancer stem cells. Taken together, these results suggest a cross talk of the two cascades PTEN/Akt and Wnt in regulating normal and malignant breast stem cells (McDermott & Wicha, 2010)

1.4.2 Cancer stem cell resist therapeutic challenges and drive the relapse of the initial heterogeneous tumor

The stem cell model of mammary tumorigenesis has significant implications for breast cancer therapy, particularly with regards to their relative resistance to radiation and cytotoxic chemotherapy (Cariati & Purushotham, 2008; Sell, 2004). These cancer stem cells can efficiently initiate and restore the disease upon therapeutic intervention and are believed to be responsible for tumor recurrence (Cobaleda et al., 2008; Harmes & DiRenzo, 2009). Stem cells are known to express drug-resistance proteins and high levels of proteins involved in apoptosis, which makes them resistant to cell death more than the differentiated cells that comprise the bulk of the tumor (Harmes & DiRenzo, 2009). The cancer stem cells would lose their battle to resisting drugs if they were pushed to differentiate. Therapeutic resistance likely involves higher expression of genes of the Bcl-2 family and membrane transporter proteins such as ABCG, BCRP and MDR transporter that are able to pump out chemotherapy drugs (Dontu et al., 2003), in addition to the

enhanced DNA damage response and the usage of cellular quiescence arresting cells in G0 phase as well as other protective strategies (Harmes & DiRenzo, 2009). Thus, making it very difficult to kill CSCs and completely eliminate the tumor. The possible reason behind cancer recurrence is the massive heterogeneity of the target cell population so that within the tolerated dose of the drug, a subpopulation of cells will inevitably survive initial treatment, thus altering the entire transcriptome and giving rise to outlier cells with malignant phenotype that could drive tumor progression (Brock, Chang, & Huang, 2009).

1.4.3 Basal subclass of breast cancer is associated with high recurrence rate and metastatic spread

In recent years, stem cells have been of a remarkable focus of biomedical research because of their evident potential for regenerative medicine. The discovery of cancer stem cells (CSCs) has stimulated great insight into the general scheme of cancer progression, particularly with respect to metastasis (Al-Hajj et al., 2003; Hsiao et al., 2010; F. Li, Tiede, Massagué, & Kang, 2007). Many studies have suggested the potential of CSCs to act as the seeds for the distal metastasis. For adult patients with carcinoma, metastasis accounts for over 90% of lethality (F. Li, Tiede, Massagué, & Kang, 2006). Clinically, advanced-stage breast cancer is characterized by metastasis, a process that involves cell invasion, proliferation, and survival in distant sites (Dhasarathy, Phadke, Mav, Shah, & Wade, 2011).

The metastatic process resembles at least superficially the processes that occur during tissue regeneration and wound healing, enabling adult stem cells to leave tissue reservoirs, such as the bone marrow, enter the circulatory system, seeding into secondary tissue sites, where the microenvironment stimulates their proliferation, differentiation and tissue reconstruction (Mani et al., 2008).

Cancer is not solely a disease of uncontrolled proliferation of a transformed clone; rather it represents the breakdown of the fundamental rules regulating the orchestration of cells, tissues, and organs within the whole living organism (S. Huang & Ingber, 2007). Thus, we have to rethink cancer progression in this hierarchical context and give the structure of the tissue and its destruction the same importance as molecular pathways within the cell (Brock et al., 2009; S. Huang & Ingber, 2007).

At this higher level of cell type dynamics, an embryonic developmental program, the "epithelial to mesenchymal transition" (EMT) must be considered. This process is generally assumed to end at birth and is often activated in adult tissues that continually undergo turnover. This paradigm has long been applied to carcinoma in order to explain the epithelial cell's ability for invasion and metastasis (Mani et al., 2008). EMT involves an alteration from an organized, epithelial cell structure to a mesenchymal invasive and migratory phenotype (Mani et al., 2008). The discovery that the EMT generates cells with many of the properties of stem cells holds the promise of resolving a major problem in cancer biology.

Gene expression profiling of breast cancer cell lines revealed the existence of the basal B subgroup which displays mesenchymal tendencies and invasive properties that resemble breast cancer stem cells (BCSC) in that they are being $CD44^{+}/CD24^{low}$ (Mani et al., 2008). The EMT gene expression signature is enriched within the basal B subgroup, manifest in their over-expression of various EMT transcriptional drivers (Mani et al., 2008).

The MDA-MB-231 cells used in this project have been characterized as triple-negative/basal-B mammary carcinoma cells (Al-Hajj et al., 2003). Among the $CD44^{+}/CD24^{-}$-positive cell lines, MDA-MB-231 has the unique property of expressing a broad range of genes

that favor metastasis (Mani et al., 2008), which offers a good system to probe differentiation and to examine the plasticity of the malignant phenotype characterizing these cancer cells.

Blick and his group tried to summarize the findings regarding the emerging relationship between EMT and BCSC. Some of the markers have been identified as overexpressed in the basal subclass, such as AXL, EMP3 and FOSL1 (Blick et al., 2010; Mani et al., 2008). The overexpression of these markers has been associated with enhanced invasiveness and poorer prognosis in breast cancer (Mani et al., 2008).

Several proteins that are involved in EMT during the embryonic stage have come under close investigation in cancer biology. Gene set enrichment analysis (GSEA) of MCF-7 cells infected with Snail and Slug- transcriptional mediators of EMT-adenovirus drives the transcriptome of these cells from the luminal A subclass that is known to be non-invasive, highly differentiated and positive for estrogen and progesterone receptors towards a more complex pattern that closely resembles the basal B breast cancer signature (Mani et al., 2008). Also, treating MDA-MB-231 cells with TGFBR1 and TGFBR2 inhibitors decreased their migratory ability (Mani et al., 2008). The cytokine transforming growth factor beta (TGF-beta) is a potent stimulator of EMT and its role in cancer is highly complex (Deckers et al., 2006). TGF-beta expression is positively correlated with breast and other types of cancer, as well as invasive lymph node metastases in breast cancer (Dhasarathy et al., 2011).

1.5 Induced differentiation of human breast cancer cells reflects the dynamics of non-genetic heterogeneity

1.5.1 Disrupting the immature state: differentiation of breast cancer cells

Instead of the standard attempt to eradicate the malignant cancer "stem" cells, differentiation therapy has recently exploited these developmental fate options by

reprogramming cancer cells to gain access to a benign attractor state that encodes a more mature differentiated phenotype (S. Huang et al., 2009; S. Huang, 2011). The differentiation potential of cancer cells has been widely observed and exploited for therapy in a few decades (Sell, 2004; JoEllen Welsh, 2004). The property that cancer (stem) cells can be differentiated has been intensely studied even before the stem cell hypothesis become popular (Campbell & Harder, 1999). The concept of "cancer stem cells" is now well established in the case of breast cancer (Al-Hajj & Clarke, 2004), the first solid tumor in which a stem cell-like, tumor propagating cells have been isolated although fundamental questions, such as the ones pertaining to the source of the cancer stem cell, remain to be answered (Campbell & Harder, 1999). If the malignant phenotype in tumors descends from cancer stem cells, then it should be possible to treat cancers by inducing differentiation of the stem cells (Sell, 2004). If tumor cells can be forced to differentiate and to cease proliferation, then their malignancy potential will be controlled.

The attractor model is consistent with both of the discussed possibilities, namely, that cancer stem cell either arise from normal immature cells ('differentiation block') or from mutations in fully differentiated cells that re-establish a stem cell like program as typically seen in virus induced carcinoma (Ferrari et al., 2009). The more frequent case of disruption of maturation – equivalent to inability to exit from immature attractors predicts that the developmental path to the differentiated state may have become impassable but is latently present (S. Huang & Ingber, 2007). In fact, an array of compounds has long been known that can promote terminal differentiation in breast cancer cell lines *in vitro* and to suppress tumor growth in animal model (Sell, 2004; JoELLEN Welsh, 2008). The role of the active metabolite of vitamin D in cell differentiation is well established. A vast amount of evidence has been collected, implicating the essential role of vitamin D and related compounds in several cellular

processes, such as repressing the progression and promoting differentiation of breast cancer and other carcinomas *in vivo* (Lazzaro et al., 2000).

1.5.2 Vitamin D metabolism and mechanism of action

Vitamin D is critical for bone health as well as on other body systems. In 1990, Garland et al. first reported an inverse correlation between the total annual sunlight intake and age-adjusted breast cancer development, recurrence and mortality in women in the U.S (Shao, Klein, & Grossbard, 2012). Vitamin D is predominantly obtained from exposure to sunlight (UVB radiation) and to lesser extent from dietary sources such as oily fish (salmon), eggs and fortified dairy products. In the absence of adequate sun exposure, vitamin D deficiency may appear rapidly and it's not easily corrected by dietary intake alone in the absence of supplementation (Shao et al., 2012; Joellen Welsh, 2008).

In the liver, vitamin D is converted first to 25(OH)D by 25-hydroxylases, then undergoes a process of hydroxylation in the kidney into 1,25dihydroxyvitaminD (1,25$(OH)_2$D) also known as calcitriol by CYP27B (Shao et al., 2012). The active form of Vitamin D binds to VDR, an intracellular vitamin D receptor for activating the downstream cascade (Shao et al., 2012). VDR acts as ligand-activating transcription factor. It belongs to the superfamily of nuclear receptors for steroid hormones that were first identified in breast cancer cell lines in 1979 (Peng, Jhaveri, Hussain-Hakimjee, & Mehta, 2007; Shao et al., 2012). In addition to its main role in regulating extracellular calcium levels, the activation of VDR regulates up to 200 genes that mediate cellular growth, differentiation and apoptosis (Peng et al., 2007; Shao et al., 2012).

1.5.3 The role of vitamin D in repressing the progression and promoting differentiation of basal subtype of breast cancer cells

Many studies have investigated the effects of vitamin D on mammary carcinogenesis *in vitro* and *in vivo* (Shao et al., 2012). VDR knockout mice as well as vitamin D deficient mice show an enhanced cancer development (Shao et al., 2012). The mechanisms underlying the inhibitory effects of vitamin D on breast cancer development have been intensely examined. These studies have shown the role of the active metabolite of vitamin D in inducing growth arrest and apoptosis by increasing the expression of cyclin-dependent kinase inhibitors such as p21 and p27 in MCF-7 breast cancer cells (Colston, 2002). 1,25(OH)D_2 also have been demonstrated to regulate the expression of oncogenes, such as c-myc and c-fos and the actions of several growth factors, including epidermal growth factor (EGF), transforming growth factor (TGF), and insulin-like growth factor (IGF-1) (Colston, 2002; Shao et al., 2012). Furthermore, the active metabolites of vitamin D can promote apoptosis through regulation of the Bcl-2 family of genes leading to down-regulation of anti-apoptotic proteins such as Bcl-2 and Bcl-CL versus up-regulation of pro-apoptotic proteins such as Bax and Bak (Colston, 2002; Shao et al., 2012).

In some breast cancer cell lines, 1,25(OH)D_2 increases the expression of the differentiation marker E-cadherin and decreases the activity of matrix metalloproteinases (MMPs), urokinase-type plasminogen activator, and tissue-type plasminogen activator while increases the expression of plasminogen activator inhibitor and MMP inhibitor1 to prevent tumor progression and metastasis (Shao et al., 2012). MDA-MB-231 cells were found to secrete MMP-9 activity, which contributes to invasion and was to be down-regulated by treatment with vitamin D or its derivative (CB1093, a synthetic deltanoid) (Shao et al., 2012). The same study identified a concurrent increase in the secretion of TIMP-1 (a tissue inhibitor of metalloproteinases), and,

to a slightly lower extent, TIMP-2 in vitamin D treated MDA-MB-231 cells, which indicates that vitamin D can change the MMP/TIMP balance in breast cancer cells, possibly contributing to a less invasive phenotype (Shao et al., 2012). In comparison to BT474 cells (ER-positive), both MDA-MB-231 (ER- negative) and S30 (ER-transfected MDA-MB-231) cells expressed low basal level of VDR at mRNA and protein levels. BT474 expressed highest VDR protein among the three cell lines. 1-alpha-(OH)D5 (an active vitamin D analog) treatment for 48hrs caused significant VDR protein accumulation in all three cell lines (Jensen, Madsen, Lukas, Binderup, & Bartek, 2001).

Though the link between vitamin D and breast cancer is not fully understood, there is growing evidence supporting the view that vitamin D deficiency is a risk factor for breast cancer. Well-designed clinical trials are needed to confirm the relevance of vitamin D in breast cancer development, progression, and survival (Lazzaro et al., 2000; Shao et al., 2012).

1.6 The cytochrome P450-derived eicosanoids (EETs) pathway in conjunction with cancer

Epidemiological studies highlight differences in the omega-6 (ω-6) to omega-3 (ω-3) polyunsaturated fatty acid (PUFA) ratio between regions of high and low breast and prostate cancer risks (Simopoulos, 2002). Western diets are deficient in omega-3 fatty acids, and have excessive amounts of omega-6 fatty acids compared with the optimal ratio (Massiera et al., 2010). The very high ω-6: ω-3 ratio (the United States ratio, 40:1, optimal ratio, 2.3:1), as found in today's Western diets, promote the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases, whereas increased levels of omega-3 PUFA (a low ω-6: ω-3 ratio) exert suppressive effects (Massiera et al., 2010). The much higher risk of breast, colon, and prostate cancers in Asians-Americans, has raised the question whether compounds in the western diet act as cancer-promoting agents. Most research on the role of

omega-6 fatty acid derived autacoids has focused on prostaglandins and leukotrienes neglecting the role of cytochrome P450-derived eicosanoids in cancer. Despite the pleiotropic effects on cells, little is known about the role of this epoxyeicosanoid (EETs) in cancer. Our laboratory has recently shown that EETs trigger extensive spontaneous metastasis in several tumors, an effect that was not caused by excessive primary tumor growth but rather depended on endothelial EETs at the site of metastasis (Panigrahy et al., 2012; Panigrahy, Kaipainen, Greene, & Huang, 2010).

Before moving forward to discuss experimentally the effect of EETs in maintaining or promoting the stem-like cell state, let's briefly touch basis on the background of these epoxyeicosatrienoic acids, their formation, mechanisms and their overlooked role in in cancer signalling.

1.6.1 Epoxyeicosatrienoic acids: formation, metabolism, and mechanism

Arachidonic acid (AA) is an essential omega-6 fatty acid that is metabolized to three classes of eicosanoid biomediators; the cycloxygenase, lipoxygenase and cytochrome P450 (CYP) products (Capdevila, Falck, & Harris, 2000). The two enzymatic pathways cycloxygenases and lipoxygenases generate a well-studied group of autacoids, prostaglandins and leukotrienes respectively. The CYP pathway produces two types of eicosanoid products, hydroxyeicosatetraenoic acids (HETEs) that are hydroxylated by CYP ω-oxidases and the epoxyeicosatrienoic acids (EETs), formed by CYP epoxygenases (Capdevila et al., 2000) (Figure 1.1). This third eicosanoid pathway was originally studied in conjunction with inflammatory and cardiovascular disease (Capdevila et al., 2000). The epoxyeicosatrienoic acids (EETs) are known to have diverse biological functions. EETs possess potent anti-inflammatory effects on blood vessels and in the kidney (Capdevila et al., 2000). In addition, they produce vascular relaxation by activating large conductance Ca^{2+} activated K^{+} channels ($BKCa^{2+}$) in smooth muscle cells

(Campbell & Harder, 1999), protect endothelial cells from inflammatory injury and apoptosis, promote angiogenesis and protect ischemic myocardium and brain (Y. Wang et al., 2005).

1.6.1.1 Production of EETs:

EETs are synthesized by cells that express cytochrome P450 (CYP) epoxygenase (Campbell & Harder, 1999). The epoxygenases are primarily members of the CYP2C and 2J isoforms in humans located in the endoplasmic reticulum (Capdevila et al., 2000). When cytosolic phospholipase A2 (cPLA2) is activated, the arachidonic acid (AA) hydrolyzed from intracellular phospholipids is converted to EETs (Campbell & Harder, 1999) (Figure 1.1). Each CYP epoxygenase produces four regioisomers by adding an epoxide group across one of the four double bonds of arachidonic acid (Capdevila et al., 2000), with one form predominating (Campbell & Harder, 1999; Capdevila et al., 2000). The epoxide group can attach to either side of the double bond in arachidonic acid (AA), resulting in two different configurations, producing R/S and S/R enantiomers for each EETs regioisomer (Capdevila et al., 2000). The functional effectiveness of the two enantiomers also can differ. CYP2J2 expressed in human kidney forms equal amounts of both 11,12-EET enantiomers, but only 11(R),12(S)-EET produces relaxation of small renal arteries (Physiol et al., 2013). Likewise, 11(R),12(S)-EET but not S/R enantiomer, increases the activity of the large conductance Ca^{2+} activated K^{+} ($BKCa^{2+}$) channels in cell-attached patches of renal vascular smooth muscle cells (Physiol et al., 2013). Thus, stereo-selectivity is a very complex issue that complicates the investigations of some aspects of EETs function.

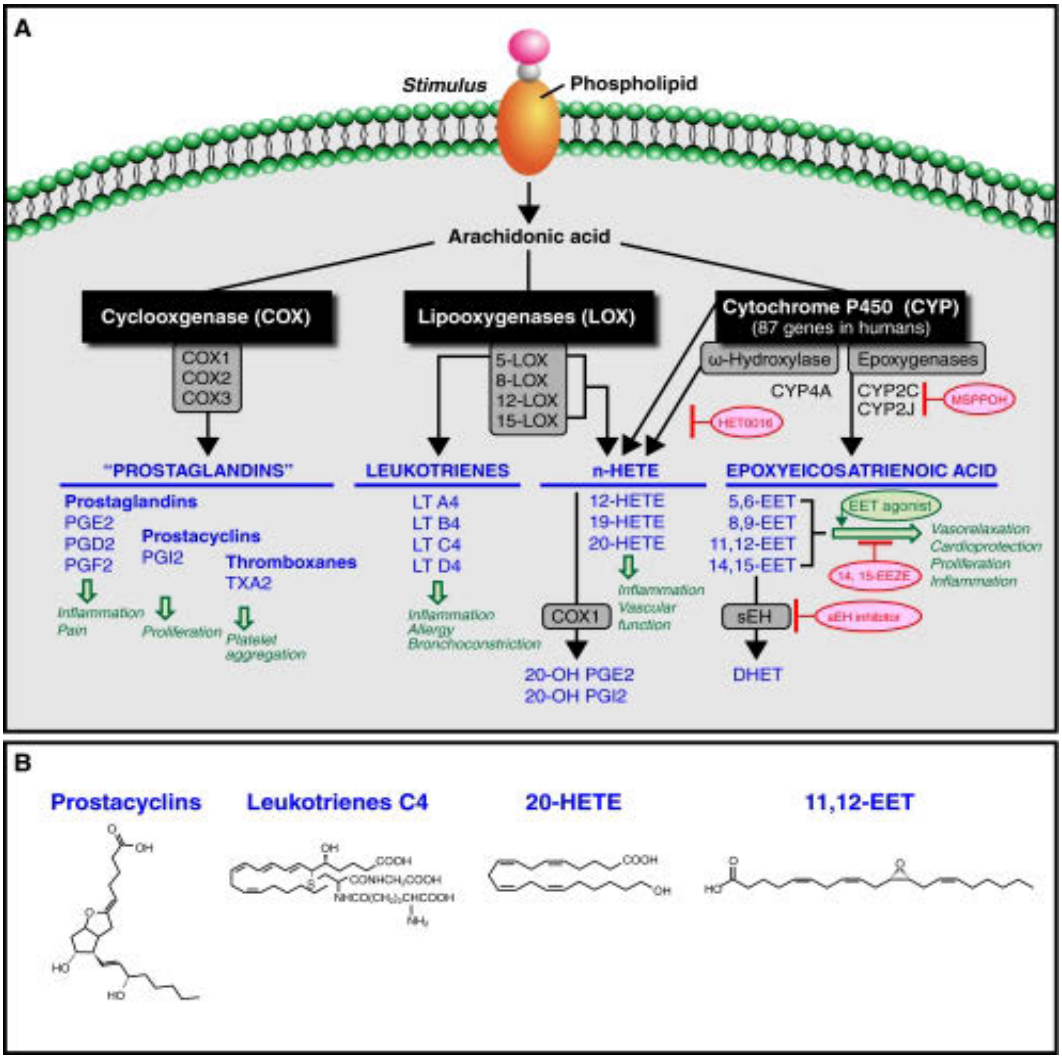


Figure 1-1: Bioactive eicosanoids derived from the arachidonic acid cascade. **A,B.** Arachidonic acid is metabolized by three pathways—the cyclooxygenase (*COX*), lipoxygenase (*LOX*), and cytochrome P450 (*CYP*) pathways. Schematic overview of major mediators and their metabolites (*blue*); enzymes (*black*, *boxed*) and biological role (*green*). Inhibitors (*red ovals*) and agonists (*green ovals*). *HETEs* Hydroxyeicosatetraenoic acids, *EETs* epoxyeicosatrienoic acids, *CYP* cytochrome P450 enzymes. MS-PPOH is a selective inhibitor of a subset of epoxygenases. HET0016 is a selective inhibitor of the ω-hydroxlase CYP4A. The sEH inhibitor (soluble epoxide hydrolase inhibitor) increases EET levels by acting as an agonist of the EET pathway. 14,15-EEZE is a putative EET receptor antagonist. *PGE2* prostaglandin E2, *PGI2* prostacyclin, *LTA4* leukotriene A4, *DHET* dihydroxyeicosatrienoic acid, *20-OH PGE2* 20-hydroxy-prostaglandin E2.

From: Cancer Metastasis Rev. 2010 December; 29(4): 723–735.

1.6.1.2 Mechanism of action of EETs:

EETs elicit multifunctional responses that are distributed across a wide variety of tissues depending upon the EETs regioisomer and the specific cell type (Spector, Fang, Snyder, & Weintraub, 2004). Little is known about the biochemical mechanisms through which EETs produce their response. The EETs act in both an autocrine and paracrine manner (A Karara, Dishman, Falck, & Capdevila, 1991; Armando Karara et al., 1992). The autocrine effect may occur through direct interaction of EETs with intracellular effector systems or through receptor-mediated mechanisms, while paracrine action can be mediated when EETs are released to the extracellular fluid affecting other cells in the local environment through a putative membrane receptor, which then activates an intracellular cascade affecting ion channels or DNA expression (A Karara et al., 1991).

Several studies support the idea that EETs bind to a G protein coupled receptor. Functional stereo-specificity is being observed (Wong, Lai, & Shen, 1997). EETs binding to a membrane receptor initiate a functional response that consist of the activation of different signal transduction pathways in different tissues. The evidence for a mechanism involving a G-protein coupled receptor stems from the demonstration that 11,12-EETs induced activation of large conductance Ca^{2+}-activated k^{+} ($BKCa^{2+}$) channels through a Gαs protein component of GTP binding protein (Fukao, Mason, Kenyon, Horowitz, & Keef, 2001; P.-L. Li & Campbell, 1997). Other signal transduction mechanisms have been found to be active in EETs biochemical responses by activating gene expression. Activation of tyrosine kinase cascade, Src kinase, mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI-3K)/Akt pathways mediate actions of EETs in endothelial cells, arterial smooth muscle cells, glomerular mesangial cells, renal epithelial cells, and myocardium (Fleming, 2001; Hoebel & Graier, 1998;

Seubert et al., 2004). Whereas, the anti-inflammatory effect produced by 11,12-EETs in the endothelium is due to the inhibition of cytokine-induced nuclear factor-kB (NF-kB) activation (Node et al., 1999).

Although several studies support the idea of an EET membrane receptor coupled to specific signalling cascades, possibly a G-protein coupled receptor, no putative EETs receptor has been conclusively identified and the possibility that EETs have direct interactions with intracellular effectors (fatty acid binding proteins, ion channels, or transcription factors) cannot be excluded (Spector et al., 2004).

1.6.1.3 Metabolism of EETs:

EETs are rapidly metabolized through several different pathways including β-oxidation, chain elongation, and hydration by soluble epoxide hydrolase (sEH) (Xiang Fang, Kaduce, VanRollins, Weintraub, & Spector, 2000). The main EET catabolic pathway is conversion to the corresponding dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolases, primarily sEH (Figure 1.1). Many potentially beneficial actions of EETs are attenuated upon conversion to DHETs. However, DHETs produce functional effects in several other systems are not merely EET inactivation products in all cases (Spector et al., 2004). sEH is considered a potential therapeutic target for increasing levels of EETs thereby enhancing its functions.

1.6.2 EETs signalling in cancer

1.6.2.1 High levels of EET induce the activation of pathways involved in proliferation, angiogenesis and anti-apoptosis:

Many CYP isozymes have the ability to epoxidize arachidonic acid to various regiomeric epoxides (Bukhari et al., 2011). In humans, the CYP2C subfamily and CYP2J2 seem to be the main contributors to EETs formation (Jiang et al., 2005). Jiang et al. have shown in their study

that CYP2J2 was up-regulated in 77% of human carcinoma tissues and eight different human carcinoma cell lines (Jiang et al., 2005). Several CYP epoxygenases have been detected in tumor cells *in vitro* and *in vivo*, supporting the potential role of EETs in cancer. In fact, CYP2C8, CYP2C9, and CYP2J2 were shown to be expressed in three prostate cancer cell lines (PC3, DU-145, and LNCaP)(Panigrahy et al., 2010). Another epoxygenase, CYP2C11, was identified to be upregulated in cerebral tumors of rats (Panigrahy et al., 2010).

CYP epoxygenase-derived EETs were found to significantly enhance phosphorylation of EGFR and activate downstream signaling cascades, including MAPK and PI3K/Akt pathways (Y. Wang et al., 2005). Such activation triggered up-regulation of metastasis-related genes, such as metalloproteinases (MMPs) and CD44 expression, and down regulation of anti-metastatic genes, such as CD82 and nm-23 expression (Jiang et al., 2007). Metalloproteinases are activated by EETs in cancer cells and lead to EET-induced activation of EGFR, which subsequently releases the pro-angiogenic factor HB-EGF in cancer cell lines (Cheng et al., 2010). Carcinoma cells over expressing CYP2J2 or treated with exogenous EETs showed an activation of MMP. This can enhance tumor progression or metastases, stimulate angiogenesis and also modulate cell adhesion. Interestingly, MMP inhibitors significantly attenuated 11,12-EET-induced cancer cell migration and invasiveness (Jiang et al., 2007).

The expression of CYP2J2 in the hematopoietic system in normal and pathological conditions was tested by Chen laboratory. CYP2J2 was found to be abundant in leukemia cell lines and cells from the bone marrow and peripheral blood of patients with leukemia and lymphoma while was absent in peripheral white blood cells and bone marrow smears from healthy volunteers (Chen et al., 2011). EET levels in urine and blood in patients with leukemia and lymphoma also were much higher than that in urine and blood from healthy volunteers.

CYP2J2 overexpression and EETs significantly promote proliferation and inhibit apoptosis in leukemia cells *in vitro*. A specific CYP2J2 inhibitor significantly inhibited leukemia cell proliferation *in vitro* and tumor growth *in vivo* (Chen et al., 2011). These data suggest that CYP2J2 may play an important role in human leukemia and lymphoma. CYP2J2-derived EETs may participate in hematological malignant diseases through one of the potential mechanisms of CYP2J2 involved in leukemogenesis, including activation of the EGFR, PI3K/Akt, AMPK, and JNK pathways. These signaling pathways are known to be activated by CYP2J2 and EETs, and are associated with leukemia cell proliferation as well as inhibition of leukemia cell apoptosis through elevating the Bcl-2/Bax ratio (Chen et al., 2011; Cheng et al., 2010; Y. Wang et al., 2005).

In a different study by Jiang and colleagues, they reported that CYP overexpression or treatment with exogenous EETs markedly promoted invasion and pro-metastatic gene expression profiles in different cancer lines *in vitro* independent of effects in tumor growth (Jiang et al., 2005, 2007). In addition, CYP epoxygenase overexpression also enhanced metastasis *in vivo* in that rAAV-CYP2J2-infected MDA-MB-231 human breast cancer cells showed 60% more lung metastases in athymic BALB/c mice and enhanced angiogenesis compared to control cells (Jiang et al., 2007). CYP3A4 expression was found to be up-regulated in breast cancer and correlates with decreased overall survival (Mitra et al., 2011). Other CYP enzymes that may participate in breast cancer progression are CYP2J2 and CYP2C8, which exhibit the capacity to synthesize EETs. However a study showed that CYP2C8 and CYP2J2 were not associated with the overall breast cancer survival (Mitra et al., 2011). CYP3A or CYP2C8 gene expression was identified to be specifically required for growth of the breast cancer cell lines MDF7, T47D, and MDA-MB-231, promoting STAT3-mediated breast caner cell growth partially through 14,15-EET. This

finding highlighted the essential role for STAT3 as a mediator of epoxygenase activity in breast cancer (Mitra et al., 2011).

Whether the levels of CYP epoxygenases depend on isoenzyme or tumor type remains up for debate. In fact, in a comparative study of three cytochrome P450 expoxygenases expression in human malignant neoplasms, CYP2J2 staining was not detected in pancreatic or prostate adenocarcinoma and it was identified in less than 15% of lung adenocarcinoma samples. In contrast, CYP2C9 was abundantly expressed. The inconsistent expression of these epoxygenases makes it difficult to generalize their biological significance in cancer (Panigrahy et al., 2010). Although the CYP450 pathway for arachidonic acid is less established than the cycloxygenase and the lipoxygenase pathways, the metabolites generated, especially EETs, are extremely interesting and have unique biological functions that are discovered daily and should be evaluated as potential targets in cancer therapy, directed both against tumor cells and their surrounding microenvironment.

1.6.2.2 Synthetic EETs trigger tumor initiation and metastasis in animal models:

Panigraphy et al. were the first group to provide *in vivo* demonstration of the effects of EETs in tumor growth (Panigrahy et al., 2012). They have used genetic and pharmacological manipulation of EET levels to demonstrate that EETs are critical for primary tumor growth and enhancing metastatic spread in several tumor models known to metastasize poorly. Remarkably, the group has shown that EETs promote exit from tumor dormancy, which is believed to be maintained by the suppression of angiogenesis (Panigrahy et al., 2012). Liposarcoma cells derived from a clone known to enter a stable period of dormancy were injected in mice with 10-fold fewer than normally required. It was observed that systemic administration of exogenous EETs to the genetically modified mice led to macroscopic tumor growth while the control

animals did not exhibit any visible tumor growth. These findings suggest that EETs accelerate the escape from tumor dormancy, which is critically dependent on the induction of angiogenesis. The factors regulating this phenotype are poorly understood. Furthermore, resection of the primary tumor in transgenic mice with high endothelial EET levels promoted spontaneous metastatic growth compared with the wild type mice; an effect that was only observed when EETs production was high at the metastatic site as opposed to only at the primary tumor [for more details, see (Panigrahy et al., 2012)]. Vascular endothelial growth factor (VEGF) is a key regulator of physiological and pathological angiogenesis. *In vitro*, VEGF induces endothelial cell proliferation and migration and acts as a survival factor for endothelial cells (Webler et al., 2008). The same group was able to show that EETs promote metastasis by triggering endothelial secretion of VEGF, which was shown to be critical for EET's role in cancer stimulating activity (Panigrahy et al., 2012).

By manipulating EET levels *in vivo*, as opposed to CYP activity, this study opened a new path in metastasis research, which has suffered from the lack of spontaneous metastasis models.

Chapter Two: **Goals and Approaches of The Project**

2.1 Overall goal and biological rationale

A novel dynamical systems perspective of "cancer stem cells": From a system dynamics point of view, cancer cells are trapped in abnormal immature "attractors states" of the gene regulatory network that governs cell differentiation due to mutational rewiring of this network (Brock et al., 2009; S. Huang & Ingber, 2007). This naturally explains, for the first time, in terms of mathematical principles of gene regulatory dynamics, the pathologists' notion that cancer results from a "differentiation block". The gene expression program associated with these self-perpetuating immature attractor states command embryonic phenotypes (increased proliferation, migration, angiogenesis etc.) that contribute to malignancy (S. Huang & Ingber, 2007). This defect of fate regulation naturally explains the "stem cell character" of the cancer propagating cells.

An alternative therapy to the current killing of cancer cells, is to trigger their "non-lethal" fates, such as maturation and differentiation, the hidden fate repertoire of cancer propagating cells, because of their stemness properties, an old idea that has led to the current concept of "cancer stem cells" (CSC). The differentiation potential of cancer cells has been broadly observed and exploited for therapy in some cases (Sell, 2004) – in line with the stemness potential. The concept of cancer stem cells is now well established in the case of breast cancer (Cobaleda et al., 2008). The attractor model predicts that the developmental path from the malign CSC-state to the benign differentiated state may have become impassable for physiological development but is latently present (S. Huang & Ingber, 2007) and hence could be exploited for therapy. In fact, some compounds have long been known to promote differentiation in breast cancer cell lines *in vitro* and to suppress tumor growth in animal models (JoEllen Welsh, 2004).

These transitions between attractor states raises the question as to whether some factors could interfere with the developmental fate options of the cancer stem cells preventing their access to the developmental path leading to the benign attractor state that encodes a more mature differentiated phenotype.

The problem: Limited efficacy of traditional differentiation therapy: Despite the spate of data showing that pharmacological (i.e., non-genetic) perturbations can robustly "reprogram" the cell phenotype between different stages of maturation, including vitamin D, retinoic acid, valproic acid, etc. (Travaglini, Vian, Billi, Grignani, & Nervi, 2009; JoEllen Welsh, 2004), unfortunately the rate of "differentiation" achieved usually is far below 100% of the cells in a population, hence limiting the utility of differentiation therapy. No systematic evaluation of compounds for breast cancer differentiation potential has been performed. Either more active compounds are yet to be discovered, or the incompleteness of differentiation is profoundly inevitable due to the inherent non-genetic heterogeneity of the cancer cell population.

Genetic and non-genetic factors may contribute to the "trapping" of a cell in an abnormal attractor state preventing them from completing their predestined journey down to the physiological cell type attractor (differentiation state) even in the presence of a differentiation signal (S. Huang et al., 2009; S. Huang & Ingber, 2007). This project examines this phenomenon at the molecular level of a case that is of therapeutic relevance: what factor could contribute to the difficulty to differentiate the immature malignant cancer stem cell to a benign differentiated state?

Working hypothesis: In the past years, arachidonic acid (omega-6 poly-unsaturated fatty acid) and its metabolites have stimulated great interest in cancer biology. Despite the pleiotropic effects on cells, little is known about the role of this epoxyeicosanoid (EETs) in cancer. Our

laboratory has recently shown that EETs trigger extensive spontaneous metastasis in several tumors (Panigrahy et al., 2010). Because of the increasingly recognized importance of the cancer stem cell state in metastasis, and the plasticity of these cancer cell states, and given the massive stimulation of tumor growth and metastasis in mice that have increased levels of EET**, I propose the following hypothesis:**

EETs may promote the cancer stem cell compartment in breast cancer cells.

Specifically, EETs may help maintain the cancer stem cell state, therefore, prevent the inherent tendency of all stem cells, including cancer stem cells to differentiate. EETs are often produced by tumor cells, and a polymorphism in enzyme CYP450 that is involved in its synthesis has been shown to increase breast cancer risk (Jiang et al., 2007; Mitra et al., 2011). Thus, it is worthwhile to test if EETs promote breast tumorgenesis. Our lab has identified small molecules that induce breast cancer stem cell differentiation. Several cell differentiation systems have been established, one of which is Vitamin D-mediated differentiation in MDA-MB-231 breast cancer cells. In the present study, I will use this breast cancer cell differentiation system (Aim 1)_ to show that high levels of endogenous EET prevent the differentiation of breast cancer cells (Aim 2), suggesting that EETs "trap" cancer stem like cells in their cancer attractor state, preventing them from exiting it and reaching a differentiated state. I will also analyze the gene expression profile of cells exposed to EETs (Aim 3) to begin to understand the molecular mechanisms through which EETs may suppress Vitamin D induced differentiation. This will provide a basis, a starting point of molecular-level candidates for further mechanistic studies that are beyond the scope of this thesis.

Complication: Cell heterogeneity. As mentioned in Chapter 1, previous work done by Huang et al. have shown that cell heterogeneity for a given trait as measured by flow cytometry also reflects differences with respect to functional properties that are reflected in the quite distinct transcriptome of the subpopulations (S. Huang, 2009a).

Phenotypic heterogeneity is systematically observed in cultures of various lines of genetically identical cells even in controlled environments when one examines any trait at single cell resolution (Altschuler & Wu, 2010). These different phenotypes can vary strikingly from cell-to-cell and may be the result of multiple distinct meta-stable states within one population. This also leads to differential response to signals, as seen in the VTD induced differentiation of clonal population of MDA-MB-231 cell line (Figure 4.1.B).

Because of such heterogeneity, gene expression profiles of entire populations can be misleading. Therefore in this project I will also take advantage of such population behavior and analyzing gene expression profile, and perform sub-population specific analysis which will more sensitively measure the genes whose expression changes (in a subpopulation only) in response to the various treatments.

The overall goal of this project is to test my hypothesis pursuing the following three specific aims:

Aim 1: To establish cell system and protocols to measure the differentiation of MDA-MB-231 *cells in vitro* that can serve as an assay to identify factors that affects the differentiation rate. An *in vitro* differentiation system will allow us to experiment the effect of EETs in preventing the cancer stem cell differentiation.

Aim 2: To determine if increasing levels of epoxy-eicosatrienoic acids (EETs) prevents maturation/differentiation of breast cancer propagating (stem) cells into a benign phenotype. This experiment will test my hypothesis that high levels of EETs promote the stem cell compartment. The use of different methods to modulate EETs will help ensure that what we see is specifically due to EETs as intended.

Aim 3: To analyze gene expression profiles of the distinct sub-states of differentiated breast cancer stem cells in the presence and absence of EETs to gain a first glimpse of the genetic pathways that control cancer stem cell fates. The sub-population specific analysis will overcome the limitation of gene expression profiles of the entire population in sidelining the non-genetic heterogeneity observed in clonal cells.

2.2 Experimental system

2.2.1 MDA-MB-231 cell line as a surrogate for primary breast tumor cells

Clinical or translational scientists often criticize the use of breast cancer cell lines instead of primary tumor cells. It is therefore important to reiterate why in this particular scenario we need to use cell lines: (1) Only the use of cell lines can offer certainty of clonality which is paramount for studying non-genetic heterogeneity and its dynamics. It would be trivial if heterogeneity stems from having a mixture of clones. (2) The panel of available cell lines including MDA-MB-231 has been shown to recapitulate well many *in vivo* characteristics including drug responsiveness and stem cell properties. Extensive transcriptomics data exist that point to the faithful mapping between *in vivo* and cell line behavior. But more importantly, in order to study the fundamental properties of the phenomenon of non-genetic heterogeneity, it is paramount to have well-characterized clonal cells, which of course entails the use of cell lines. This is because cancer cells purified from primary tumors, however carefully, still comprise

undefined mixtures of highly diverse cells covering a vast area in the high-dimensional gene expression state space, which precludes the study of state transition kinetics (Kao et al., 2009). Nevertheless, some differences exist but they are known and not of relevant for the systems biology aspects of this project.

Our experimental platform was based on high-vimentin MDA-MB cell lines, in particular a sub-line of MDA-MB-231 which are currently closely characterized. MDA-MB-231 cell line represents a malignant breast cancer state that has been shown to faithfully many of the *in vivo* properties of basal type cancer, including the cancer stem cell characteristics (Kao et al., 2009) and offers a good target to probe differentiation and to examine the plasticity of these cancer cell states in the presence of EETs.

2.2.2 Vitamin D-induced differentiation highlights the micro-heterogeneity in the clonal population of MDA-MB-231 cell line

In this thesis, I investigated the dynamics of non-genetic heterogeneity in clonal MDA-MB-231 breast cancer cells during differentiation induction following vitamin D (VTD) administration in the absence and presence of EETs. The cancer stem cell marker aldehyde dehydronase 1 (ALDH1) activity was measured using flow cytometry as a marker to detect the diffrentiated population that expressed low ALDH activity and the high ALDH activity enriched in the stem cell-like population, which revealed the non-genetic heterogeneity and bimodality after treatment indicating discrete state transition in responding to VTD as well as to VTD in combination with EETs of only a fraction of the clonal cell population. We found that the non-responding cells surprisingly display a genome- wide change of gene expression with a profile enriched in stem cell genes in comparison to the responding differentiated fraction of cells.

Our group has experimented the reversibility of the cancer stem cell state. The dynamic transitions between substates in that CSC state is continously regenerated by reversal of the differentiated cells could be a key explanantion of therapy failure. Indeed, our group has observed such reversion to the CSC state in vitamin D treated MDA-MB-231 breast cancer cells which "dedifferentiate" to the CSC state withinin days after drug removal**.** This reversible cell state switch is too fast to be expained by regrowth of contaminating stem cells and may acount for the development of resistance by regenerating cancer stem cells. Thus, it warrants further investigations to establish the clinical significance of reversal to the stem cell state which is typically more drug resistant (Waddington, 1956).

2.2.3 Decreased aldehyde dehydrogenase functional activity as a marker for breast cancer cells differentiation

Cancer stem cells exhibit similar characteristics to normal stem cells with respect to many aspects of phenotype and function (Dontu et al., 2003). One approach for finding shared stem cell markers is to focus on conserved stem and progenitor cell functions. Such markers may be inherited by the malignant stem cell compartment, across multiple histological subtypes of cancer from the same tissue of origin. A candidate marker is aldehyde dehydrogenase 1 (ALDH1), a polymorphic detoxifying enzyme responsible for oxidizing extracellular aldehydes to carboxylic acids, which leave the liver and are metabolized by the body's muscle and heart. ALDH plays an important role in multiple biological activities, including drug resistance, cell differentiation and oxidative metabolism. These biological processes are crucial for CSC longevity and maintenance (Ginestier et al., 2007).

Ginestier and colleagues showed that high ALDH activity selects for both normal and tumorigenic human mammary epithelial cells with stem/progenitor properties, and that breast

CSCs with elevated ALDH activity are highly tumorigenic in a NOD/SCID xenograft model (Croker et al., 2009). Clinical data suggest that high expression of ALDH1 in breast tumors is correlated with poor clinical outcome (Croker et al., 2009). Collectively, these studies as well as others support the idea of ALDH as a marker of normal and malignant human breast stem cells (Croker et al., 2009; Ginestier et al., 2007; Ricardo et al., 2011). The measurement of this enzyme activity has been widely used as a marker to identify, evaluate, and isolate stem and progenitor cells that express high levels of ALDH; however, the molecular roles of ALDH in the stemness remain unclear.

In breast cancer cells, cells with high ALDH activity have been reported to contain the tumorigenic cell fraction, which is able to self-renew and to recapitulate the heterogeneity of the parental tumor (Ginestier et al., 2007). In this thesis, we assesed the commonly used human breast cancer cell line (clonal MDA-MB-231) for functional activity of ALDH as a differentiation marker to investigate the dynamics of non-genetic during differentiation induction following VTD addministration in the absence and presence of EETs. The cancer stem cell marker aldehyde dehydronase 1 (ALDH1) activity was measured using flow cytometry to detect the diffrentiated population that expressed low ALDH activity and the high ALDH activity enriched in the stem cell-like population. Flow cytometry is used for qualitative measurement (presence and size of particular subpopulations) in a static situation. Using this technique, we will be able to see the population distribution of an entire population. Hence, observe changes in individual cells instead of just the average of an entire population with respect to ALDH activity.

At the end of drug treatment, the cells are incubated with fluorescence conjugated substrate in the standard ALDH assay buffer, and the retained fluorescence reflecting ALDH

activity. The fluorescent ALDEFLUOR reagent freely diffuses into cells and is a non-toxic substrate for ALDH. The amount of fluorescent ALDH reaction product that accumulates in cells directly correlates to the ALDH activity in these cells. The negative charge of this reaction product prohibits diffusion from the cells; however, it can be actively pumped via the ATP-binding cassette (ABC) transporter system. This active efflux is inhibited by the special formulation of the ALDEFLUOR Assay Buffer. Therefore, the ALDEFLUOR reaction product will be retained only by cells with intact membranes and fixed, permeabilized or dead cells will appear ALDH negative (Ginestier et al., 2007). Thus, differentiated cells will have no or weak fluorescence.

2.2.4 Direct and indirect pharmacological manipulation of EET levels

(i). Inhibiting soluble epoxide hydrolase (sEH): sEH enzymes are responsible for metabolizing EETs to DHETs (Figures 1.1, 2.1), attenuating many of the functional effects of EETs. EETs are turned over so rapidly, it is difficult to demonstrate their effect *in vivo* (Spector et al., 2004)]. This enzyme effectively utilizes 8,9-, 11,12-, 14,15-EET, whereas 5,6-EET is a poor substrate (Spector et al., 2004).

Epoxide hydrolases are a group of enzymes that convert the epoxide group of chemical compounds to corresponding diols by the addition of water (Spector et al., 2004). sEH is widely distributed in mammalian tissues being highly active in liver, kidney, intestine, and vascular tissue (VanRollins, Kaduce, Knapp, & Spector, 1993). sEH is found at lower levels in testes, lung, brain and spleen (VanRollins et al., 1993). Loss of sEH has been reported in hepatocellular carcinoma and hepatoma cells (Panigrahy et al., 2010), in principle increasing the levels of EET in tumor tissue resulting an increase of cancer-promoting effect of carcinogenesis. Contrariwise, sEH expression was up-regulated in seminoma, cholangiocarcinoma, and advanced ovarian

cancer when compared with normal tissue. In human, sEH is encoded by the EPHX-2 gene (Beetham, Tian, & Hammock, 1993; Physiol et al., 2013), which has been cloned and characterized (Beetham et al., 1993). EPHX-2 has been identified as a candidate metastasis suppressor gene in breast cancer (Panigrahy et al., 2010). Based on the rationale that high levels of EET in tissues promote tumor growth and metastasis, sEH would be expected to be down-regulated in tumors; however there is no consistent finding in the expression of sEH in all tumors.

sEH is considered a potential therapeutic target ; administration of sEH inhibitors decreases the hydrolysis of EETs and, therefore, maintains a higher endogenous EET level. sEH inhibition is being evaluated as a mechanism for increasing and prolonging the beneficial actions of EETs that are likely to be useful in treating diseases that affect the cardiovascular and respiratory systems (X Fang et al., 2001). Systematic administration of the sEH inhibitors (tAUCB or TUPS) has previously been shown to stimulate lung, liver and axillary lymph node metastasis in mice murine tumor models (Panigrahy et al., 2012). sEH inhibitors can be used for prolonging EETs short half-life by inhibiting their metabolism to DHET, thereby enhancing their biological activity. This research tool allowed us to examine the effects of elevated endogenous EETs on sustaining the ALDH high subpopulation by preventing the stem cell-like cells switch to the differentiation state.

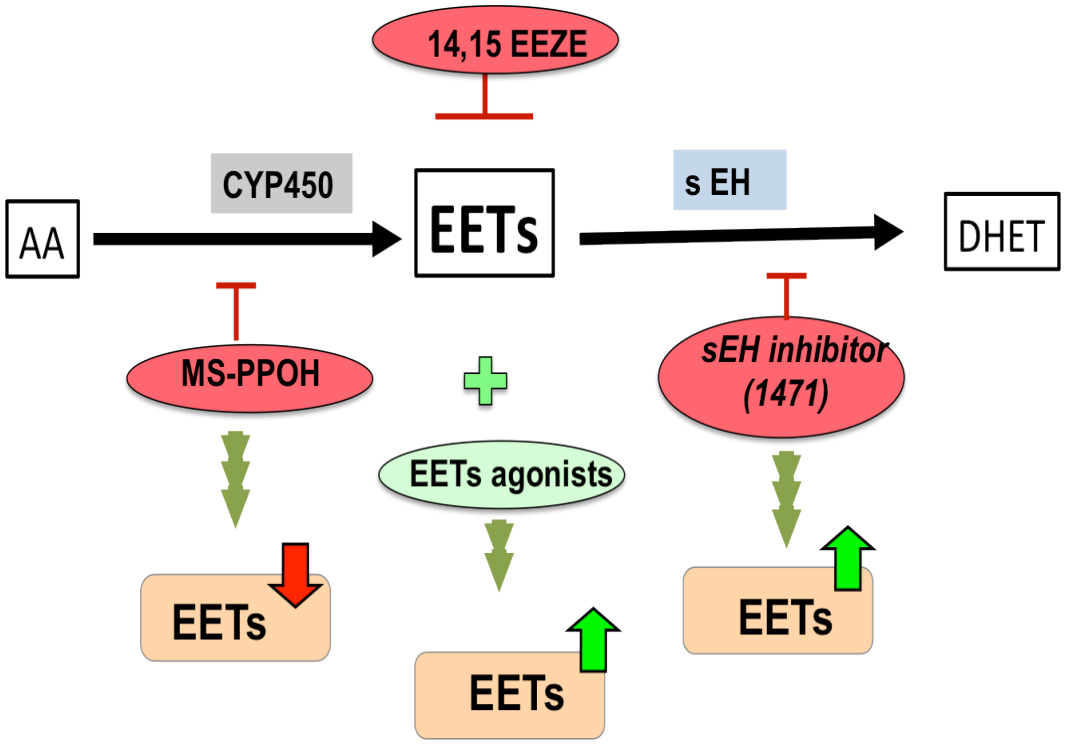


Figure 2-1: Pharmacological manipulation to the cytochrome P450-derived eicosanoids (EETs) pathway. Arachidonic acid (AA) is metabolized by cytochrome P450 (*CYP*) pathway. Schematic overview of AA-EETs and metabolites (Inhibitors (*red ovals*) and agonists (*green ovals*). MS-PPOH is a selective inhibitor of a subset of epoxygenases, decreases EET levels by acting as an antagonist to the EETs pathway. The sEH inhibitor (soluble epoxide hydrolase inhibitor) increases EET levels by acting as an agonist of the EET pathway. 14,15-EEZE is a putative EET receptor antagonist.

(ii). Blocking CYP-epoxygenase activity: CYP epoxygenase-derived EETs were found to posses potent and diverse biological effects within the vasculature involved in tumor growth (Jiang et al., 2005). Although the role of EETs in cancer is poorly understood, recent studies have shown evidence supporting CYP epoxygenases as potential tumor-promoting enzymes (Jiang et al., 2005).

In humans, the CYP2C subfamily and CYP2J2 seem to be the main contributors to EETs formation [(Jiang et al., 2005). In animal models, cerebral tumors of rats expressed significantly higher levels of CYP2C11 in comparison to normal tissue samples (Zagorac, Jakovcevic, Gebremedhin, & Harder, 2008), which is equivalent to the observation of up-regulated CYP2J2 in tumors (Jiang et al., 2005). Jiang et al. have shown in their study that CYP2J2 was up-regulated in 77% of human carcinoma tissues and eight different human carcinoma cell lines (Jiang et al., 2005). Furthermore, over-expression of CYP2J2, thus elevated endogenous EETs prevented tumor cell apoptosis, increased carcinoma cell proliferation and enhanced tumor growth (Jiang et al., 2005). Similar studies done by Wang laboratory showed that addition of exogenous EETs or overexpressing CYP2J2 induced cancer cell migration and invasion. In contrast, antisense oligonucleotides to CYP2J2 or non-specific CYP inhibitors significantly attenuated these neoplastic and malignant phenotypes (Jiang et al., 2005, 2007).

Recently, there has been growing interest in cytochrome P450 enzymes that may have a critical role in cancer progression through the potential eicosanoids products, which have been reported to promote cancer cell proliferation and metastasis in human carcinoma tissues and tumor cell lines (Iliff & Alkayed, 2009). CYP2C8, CYP2J2 and CYP3A have been shown to be expressed in breast cancer tissues correlating with a decreased overall survival (Iliff & Alkayed, 2009). These findings suggest that inhibition of CYP450-mediated EET biosynthesis may represent a novel approach for the treatment of human cancers (Jiang et al., 2007). MS-PPOH, a potent and selective inhibitor of CYP epoxygenase has been used by several studies to reduce the endogenous levels of EET, provided us with another aspect in approaching our experimental goal to modulate endogenous EET levels (Figure 2.1).

(iii). Direct administration of synthetic EETs: Four different epoxide regioisomers including 5,6-, 8,9-, 11,12-, and 14,15-EET are generated by epoxygenase activity. EETs regioisomers and enantiomers composition varies in different tissue. 14,15-EET is the most abundant regioisomer in the plasma, liver and kidney with moderate amount of 11,12-EET and a small amount of 8,9/5,6-EET (A Karara et al., 1991). EETs are rapidly taken up into cells when applied exogenously. All tested mammalian cells rapidly take up radiolabelled EETs as free fatty acids when added to the cultured medium and incorporate them into phospholipids (Spector et al., 2004). The amount taken up by most cells reaches a maximum within 20-60 min (Spector et al., 2004). Whether EETs require a membrane transporter or passively diffuse through the cell membrane is still unknown (Spector et al., 2004).

Administration of synthetic EETs, has been shown to increase systemic EET levels and mimic the effects of endothelium-derived EETs in animal models (Panigrahy et al., 2012). Several studies have used this approach as a pharmacological control of EET levels to characterize the effect of EET-modifying drugs in primary tumors and metastasis models. In our study, we have used the direct administration of the two regioisomers 11,12-, and 14,15-EET to assess the effect of EETs in preventing the induced differentiation of the MDA-MB-231 breast cancer cell line (Figure 2.1).

Chapter Three: **Materials and Methods**

3.1 Cell culture

MDA-MB-231 cell culture: The human epithelial breast cancer cells (MDA-MB-231) were obtained from ATCC and cultured in DMEM medium (Sigma, ON, Canada) supplemented with 10% FBS (fetal bovine serum), 1% L-glutamine, 1% penicillin/streptomycin in 5% CO_2 at 37°C. MDA-MB-231 clonal cells were derived from mammosphere formations. Single colony cells were obtained from serial dilution of the MDA-MB-231 cells into 96 wells plate, assuming single cells in each well. Clonal cells were cultured gradually into 12 wells plate then to 6 wells plate and so on for individual clonal expansions. We have chosen one clonal set for all the performed experiments.

3.2 Preparing vitamin D and soluble epoxide hydrolase inhibitor stock solutions

Vitamin D (VTD) powder (Sigma, ON, Canada) was dissolved in dimethyl sulfoxide (DMSO) solution to make 20 mM VTD stock solution from which dilutions were made for experiments. Soluble epoxide hydrolase inhibitor (sEHI) powder (obtained from Dr. Panigraphy, Harvard Medical School) was dissolved in DMSO solution to make 10 mM of sEHI stock solution from which dilutions were made for experiments. To test any effect of DMSO on the growth of MDA-MB-231 cells, DMSO solution was added to the cells at the optimized concentration used for MDA-MB-231 treatment for both vitamin D (10 μM) and sEHI (10 μM).

3.3 Vitamin D induced differentiation system

MDA-MB-231 clonal cells were seeded at a density of $4x10^5$ cells/plate in a 10 cm culture dish or $1.25\ x10^6$ cells in 15 cm culture dish. For experimental purposes, cultures were serum starved for 24 hrs before treatment. 48 hrs post seeding, the cells were treated in 1 % FBS DMEM with VTD (10 μM) with and without sEHI (10 μM), 14,15/11,12-EETs (10 μM), MS-

PPOH (10 μM), or 14,15 EE-(Z)E (10 μM). Dimethyl sulfoxide (DMSO) was used as vehicle control. Treated cells were incubated for 96 hrs followed by aldehyde dehydrogenase (ALDH) activity measurement using flow cytometry.

3.4 MTT proliferation assay

MTT Cell Proliferation Assay provides a simple method for determination of cell number using standard micro-plate absorbance readers. Determination of cell growth rates is widely used in the testing of drug action, cytotoxic agents. The MTT assay involves the conversion of the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan2-4. The formazan is then solubilized, and the concentration determined by optical density at 570 nm. To test any toxicity effect of sEHI on MDA-MB-231 cells, the breast cancer cells were plated in 96 wells plate in triplicates at $2x10^3$ cells/well, cultured in complete medium and allowed to attach for 24 hrs followed by addition of 5, 10, 20, 50 and 100 μM of sEHI, and incubation for 24, 48, 72, 96 hrs. Control cells were incubated in the medium with DMSO at same time points. MTT assay (Sigma, ON, Canada) was carried out according to the manufacturer protocol. MTT solution (20 μl/well) was added, incubated for 3 hrs for each time point, medium was removed; MTT solvent (200 μl) was added to dissolve the MTT formazan crystals with gently pipetting up and down. OD (optical density) values were read using a micro-plate reader at 570 nm, and background absorbance at 690 nm.
(http://www.invitrogen.com/site/us/en/home/References/protocols/cell-culture/mtt-assay-protocol/vybrant-mtt-cell-proliferation-assay-kit.html)

3.5 Western blot analysis

The breast cancer cells were plated and cultured in complete medium and allowed to attach for 24 hrs followed by the addition of 10 μM VTD with and without 10 μM sEHI, and 10 μM 14,15/11,12-EET for 96 hrs incubation. Control cells were incubated in the medium with DMSO using same time points. The ALDH high and ALDH low subpopulations of VTD-treated cells were sorted by fluorescent activated cell sorting (FACS) based on the fluorescent intensity of ALDH. Protein lysate was collected from treated cells as well as non-treated (as control). Cells were lysed with lysis buffer (50 mM Tris-CL, 1% SDS). Protein concentration was then measured with a spectrometer. Cell extracts were boiled for 10 min and chilled on ice, subjected to 8% SDS – PAGE for fragmentation, followed by an electrophoretically transfer to a nitrocellulose membrane. The blots were blocked with 5 % non-fat milk for 1 hr, then incubated for 2 hrs with primary antibodies [anti-sEH (human polyclonal, Cayman, MI, USA, 1: 2000), anti-ALDH1 (human polyclonal, BD, 1:500), washed with PBST and incubated with secondary antibody for 1 hr [goat anti-rabbit secondary antibody (1:4000), (1:2000) respectively]. The membrane was then exposed to ECL for signal detection. The membrane was stripped to re-probe with beta-actin and GAPDH as loading controls.

3.6 Flow cytometry analysis

3.6.1 Aldehyde dehydrogenase activity distribution using flow cytometry

Cells treated with VTD (10 μM) in the presence and absence of sEHI (10 μM), 14, 15-EET, 11,12-EET, MS-PPOH, 14,15 EE-(Z)-E or DMSO were subjected to aldehyde dehydrogenase (ALDH) activity measurement according to ALDEFLUOR (Stem Cell Technologies, North Calorina, USA) protocol. After 96 hrs of treatment incubation, cells were

washed with phosphate-buffered saline (PBS), harvested by trypsinization, counted for cell viability using Vi-Cell. After washing cells with 1x PBS, samples were again centrifuged for 5 min at 1200 rpm, supernatant was removed and cells were suspended in 1 ml of aldefluor assay buffer. Samples were adjusted to a concentration of 10^6 cells/ml, 5 µl/sample of active substrate (BODIPY-aminoacetaldehyde, BAAA) was added, then suspended cells were incubated in a 37°C water bath for 45 min to allow conversion of the substrate to the fluorescent product (BODIPY-aminoacetate, BAA). After incubation time, samples were washed again with 1x PBS, and re-suspended in 500 µl of aldefluor assay buffer. The amount of intracellular fluorescent product is then measured using a flow cytometer (FL1).

3.6.2 7-amino actinomycin D staining for detecting apoptotic cells

Cells treated with VTD (10 µM) in the presence and absence of sEHI (10 µM), 14, 15-EET, 11,12-EET or DMSO were subjected to 7-amino actinomycin D (7AAD, Becton Dickinson, NJ, USA) analysis. 5 µl of 7AAD solution was added to 10^6 cells suspended in aldefluor assay buffer and mixed well. Cells were incubated for 15 min at room temperature (RT) while protected from light. Unstained cells were used as negative control. Samples were analysed by flow cytometry (Becton Dickinson, CA, USA) right after fixation. Data on 10^4 cells were acquired and processed using CellQuest software (Becton Dickinson, CA, USA) as well as FlowJo software.

3.7 Statistical analysis:

Student t-test was used for statistical analysis. Each experiment was done at least in triplicate. Results are expressed as mean ± STD. A p value of < 0.05 was taken as the level of significance for all tests.

3.8 Microarray analysis

3.8.1 FACS sorting and RNA isolation:

MDA-MB-231 clonal cells were plated and cultured in complete medium. The cells were allowed to attach for 24 hrs followed by induction of differentiation by vitamin D (10 μM) and EETs treatment (10 μM). After 72 hrs after, samples were stained with ALDEFLOUR assay for ALDH activity measurement. Using FACS, the two phenotypically distinct sub-populations were sorted based on the fluorescence intensity of ALDH. The sorting gates were established using as negative controls the cells stained with PI only. The pellets generated from each sample were snap-frozen for a later RNA isolation. RNA was prepared using mirVana microRNA isolation kit from Ambion by phenol/chloroform organic extraction.

3.8.2 Microarray analysis

Gene expression profiling was performed by the Laboratory for Advanced Genome Analysis at the Vancouver Prostate Centre, Vancouver, Canada, by using Agilent SurePrint G3 Human GE 8x60K Microarray. The HumanGE- Expression BeadChip targets 27,958 Entrez Gene RNAs and 7,419 lincRNAs. Prior to labeling, the integrity of the total RNA was checked with the Agilent 2100 Bioanalyzer (RNA), quantified with the NanoDrop ND-1000 UV-VIS spectrophotometer to measure A260/280 and A260/230 ratios or RiboGreen assay. Agilent 2100 Bioanalyzer uses a RNA Integrity Number (RIN) to measure RNA sample quality. Samples with a RIN value of greater than or equal to 8.0 were deemed to be acceptable for microarray analysis. The ratio between the readings at 260 nm and 280 nm provides an estimate of the purity of the nucleic acid, which has to be between the range of 1.8 and 2.0 for a good preparation. Values below this indicate contamination of the sample with either phenol, protein, or carbohydrates.

Samples were prepared following Agilent's One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling v6.0. An input of 100ng of total RNA was used to generate Cyanine-3 labeled cRNA. Samples were hybridized on Agilent SurePrint G3 Human GE 8x60K Microarray (Design ID 028004). Total RNA was initially converted into cDNA using a T7 polymerase primed reverse transcriptase reaction to synthesize first and second strand of cDNA. This cDNA then serves as a template for cRNA synthesis by T7 RNA polymerase in the presence of cyanine 3-labeled CTP (Cy3). There is routinely at least a 100-fold RNA amplification with use of this kit. A spike-in control is introduced at the beginning of the linear amplification and labeling step that demonstrates linearity over the signal range. cRNA yield and specific activity (pmol dye/ μg cRNA) was measured with the NanoDrop 1000. The yield must be ample enough for the assay (typically 1.65 μg) and specific activity must be ≥ 8.0 pmol Cy3. Typically, an amount of 1.65 μg of Cy3 labeled cRNA was hybridized to the microarray at 65°C for 17 hours. Following hybridization, slides were washed. Preliminary fluorescence data was extracted from each microarray by the Agilent DNA Microarray Scanner at a 3 μm scan resolution (8 arrays per slide). Green processed signal was quantile normalized with Agilent GeneSpring 12.0. A QC report was generated for each microarray with a series of control panels evaluates the quality of the hybridization. (http://pctriadd.com/genomic-analysis/services-amp-expertise)

3.9 Methodology: Gene Expression Dynamics Inspector (GEDI)

3.9.1 Introduction of GEDI

GEDI is a program for the analysis of high-dimensional data, such as genome-wide profiling of gene expression. It allows the visualization, inspection and navigation through large sets of such data. It was originally developed for displaying dynamic data, specifically, multiple

parallel gene expression profile time courses, in order to map the "gene expression state space", e.g., following treatment of the same system with various drugs. However, it can also effectively help analyze large amounts of static profiles (e.g. patient samples) and help find patterns of expression without a priori knowledge of any underlying structure (Eichler, Huang, & Ingber, 2003).

3.9.2 Philosophy of GEDI

The basic idea behind GEDI is fundamentally different from conventional clustering programs, such as hierarchical clustering. The goal is not to find "clusters" of co-regulated genes. Data analysis in GEDI is sample-oriented rather than gene-oriented, i.e., the object of analysis is an array or sample (e.g., condition, patient, time point). Although in conventional hierarchical clustering, a "two-way" clustering is performed, producing both gene clusters and sample clusters, a given sample as an entity is lost, since it will end up as a branch in a dendrogram that can become very dense when many samples are involved. In GEDI, the notion of a sample as an entity is preserved. In contrast, the very idea of grouping ("clustering") of samples based on their gene behaviors or of genes based on their behavior in the various sample is a secondary, "emerging" process. At the core of GEDI, each sample (= array) is mapped into a "mosaic" ("GEDI map"), which facilitates the recognition of genome-wide patterns, but at the same time allows the user to zoom-in onto genes of interest given its participation in the emerging global patterns. Thus, GEDI covers multiple scales of information, adhering to the spirit of "systems approaches" to present "the whole" as an integrated entity - yet allowing to directly link system features to "the parts", the individual genes.

3.9.3 Data presentation of GEDI

In brief, the output of GEDI is characteristic mosaic or "GEDI Maps", a two-dimensional grid picture for each sample. Each tile in the mosaic represents a "mini-cluster" of genes (e.g., 10 genes) whose behavior across the entire set of analyzed samples is highly similar. Similarly behaving mini-clusters in turn are placed in the same neighborhood on the grid, hence creating a higher-order mosaic pattern. The color of the tile in a particular mosaic (i.e,SOM grid) represents the expression level of the genes in that mini-cluster in that particular sample. The tiles in each mosaic of the same analysis represent the same genes, thus enabling the direct comparison of samples (microarrays). In summary, GEDI maps each array into a mosaic pattern as a well-recognizable, memorable and characteristic object that gives each sample an engrammic identity.

3.9.4 Self-organizing map and GEDI

The underlying algorithm for creating the mosaics is an SOM (Self-Organizing Map) algorithm. However, unlike the conventional applications of SOMs to classify genes or samples into a predetermined number of discrete groups ("clusters"), GEDI creates a SOMs-based mosaic for every sample. The learning process is not used to classify genes, but to generate the mosaic patterns based on mini-clusters that allows human eye to find similarity based on "Gestalt" perception. In other words, GEDI can be thought of as taking the dots on a microarray that represent expression levels of each gene, grouping together small groups of genes that behave very similarly over the set of arrays to mini-clusters (dimension reduction) and rearranging them so that similar mini-clusters are placed next to each other.

GEDI runs SOMs in two phases. 1st phase is the rough training phase. 2nd phase is the fine training phase. For different phases, different parameters can be set in the settings. The number of SOM mini-clusters translates into the 'resolution' of the mosaic, and we use SOMs with

hundreds of nodes, or 'mini-clusters', typically containing 0-20 genes, to create high-resolution mosaic pictures. The color of each tile of the mosaic is determined by the centroid (approximate to the average of all the genes in that tile) value of that respective mini-cluster. Because SOMs place similar genes into the same neighborhood, coherent and robust pictures emerge that are characteristic of every sample. Every sample (or array) is associated with one mosaic picture. Due to the concatenation of the samples and time courses in the input data matrix (see data file preparation documents), every tile of the mosaic in each of the mosaics corresponds to the same group of genes across the sample

3.9.5 Gene Sets Enrichment Analysis (GSEA)

Gene Set Enrichment Analysis (GSEA) is different from typical enrichment testing in that it takes into account the magnitude of expression differences between conditions for each gene. As such, it addresses the question of whether the expression of the gene set of interest shows significant differences between these conditions. It relies on ~1300 pre-defined gene sets collected from other databases (such as Gene Ontology (GO) or pathway databases) and computational studies that are stored on MSigDB, the database the GSEA calls on (Subramanian et al., 2005). Running GSEA allows the user to restrict the search to specific groups of genes that have attributes that are of interest to the user. These are separated into sets C1-C5, defined as: C1 positional; including genes on the same chromosome or cytogenic band. C2 curated; taken from pathway databases, publications, and expert knowledge. C3 motif; conserved cis-regulatory motifs based on comparative studies. C4 computational; derived from past cancer studies. C5 GO, as above.

Chapter Four: **Results**

4.1 Clonal population of MDA-MB-231 breast cancer cell line exhibits heterogeneous response when differentiation is induced by Vitamin D

In breast cancer cells, cells with high aldehyde dehydrogenase (hereafter, ALDH) activity contain the tumorigenic cell fraction, which is able to self-renew and to recapitulate the heterogeneity of the parental tumor (Ginestier et al., 2007). ALDH, aldehyde dehydrogenase is used as a marker of normal and malignant human breast stem cells (Ginestier et al., 2007). It has been widely used as a marker to identify, evaluate, and isolate stem and progenitor cells that express high levels of ALDH.

Flow cytometry analysis of the MDA-MB-231 cells following Vitamin D (hereafter, VTD) treatment revealed the appearance of an ALDH-low subpopulation, giving rise to the typical -bi-modal cell population when ALDH was measured (Figure 4.1.A.). This indicates that VTD induces a fraction of the population of MDA-MB-231 to leave the cancer stem cell state and differentiate into a more mature state.

In our lab, ALDH activity is used as a marker in an assay to monitor cancer stem cell-like cells differentiation. We next established the time course for VTD induced differentiation. VTD induced a change in the population structure, in that a second, ALDH-low subpopulation appeared within 96 hrs. By contrast, the control (DMSO treated) cells exhibited a single population (monomodal distribution). We designate the two subsets of cells as ALDH high (AH), and ALDH low (AL) subpopulations, for the cells in the stem cell-like and the differentiated states, respectively. The co-existence of these two different states was validated by differences between cell size (FSC) and granularity (SSC) using FACS.

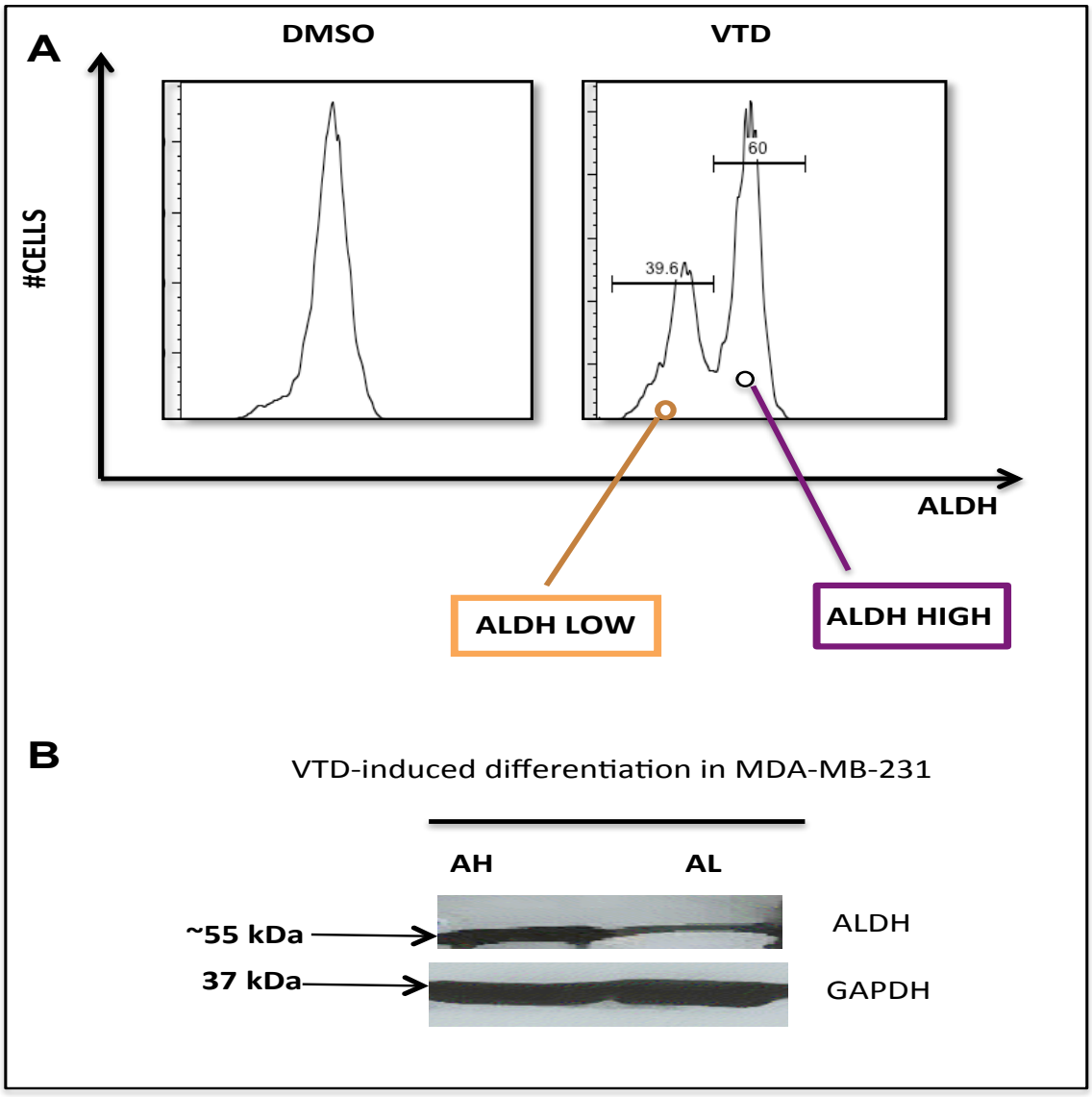


Figure 4-1: The static population reflects heterogeneity in terms of the response of the VTD-induced differentiation signal in MDA-MB-231 cells. A. A schematic representation of non-genetic heterogeneity of tumor cells reflecting the resistance of a subpopulation fraction to VTD drug perturbation. B. Flow cytometry reveals heterogeneity of phenotype in MDA-MB-231 clonal cell population that is assessed by detecting the ALDH activity versus cell count after 96 hrs of VTD (10 μM) treatment. The initial population (control) splits into two peaks, ALDH low and ALDH high sub-populations. C. Western blot analysis of ALDH expression in sorted populations of ALDH high (AH) and ALDH low (AL) of VTD induced differentiation of MDA-MB-231 cells (cells treated with 10 μM VTD, 72 hrs later ALDH activity is detected by FACS, then the ALDH high/ALDH low subgroups were separated by FACS based on ALDH fluorescent signal, and subsequently lysates were obtained). Experiment was performed in duplicate (n=2).

To confirm that the ALDH activity measured by flow cytometry reflects the expression level of the ALDH protein in the two subpopulations of VTD-treated MDA-MB-231 cells, we sorted the ALDH high/ ALDH low subgroups using fluorescent activated cell sorting (FACS) based on the fluorescent intensity of ALDH and analyzed by Western blot for ALDH expression. This confirmed that ALDH was indeed down-regulated in lysate of the AL fraction that appeared 72 hrs of treatment with 10 μM VTD (Figure 4.1.B.).

During the course of characterizing the cell state dynamics of cells in culture, we observed that the percentage of the AL or AH subpopulation after a given dose of VTD and at a given time varied within a cell culture. This seemed to depend on various conditions. For example, cell density and even the frequency of passaging influenced the percentage of differentiating cells detected in a cell culture. These observations suggest that the dynamic nature of cell lines is also regulated by factors in their immediate microenvironment not accounted for. In fact, cell density (perhaps via cell-cell contact and - quorum-sensing-like mechanisms) is well known to affect proliferation and differentiation. However the qualitative shift between the populations, i.e. the appearance and increase of the AL subpopulation following VTD treatment was consistent.

4.2 Inhibition of EETS degradation maintain breast cancer cell viability

Since we wished to utilize the inhibition of sEH, which would lead to accumulation of EETs (see Figure 2.1 in Chapter 2), as a tool to study the effect of increased EETs, we first performed an MTT assay to determine the toxicity of sEH inhibitors on MDA-MB-231 breast cancer cells. Cultured cells were treated with the sEH inhibitor (sEHI 1471, 1709) at different concentrations 5, 10, 20, 50 and 100 μM for 1-5 days and optical density readings of the MTT

assay were taken every day. The effect of sEHI on the cell growth is depicted in Figure 4.2.A. High concentrations of sEHI (50-100 μM) inhibited cell growth, which may reflect toxicity.

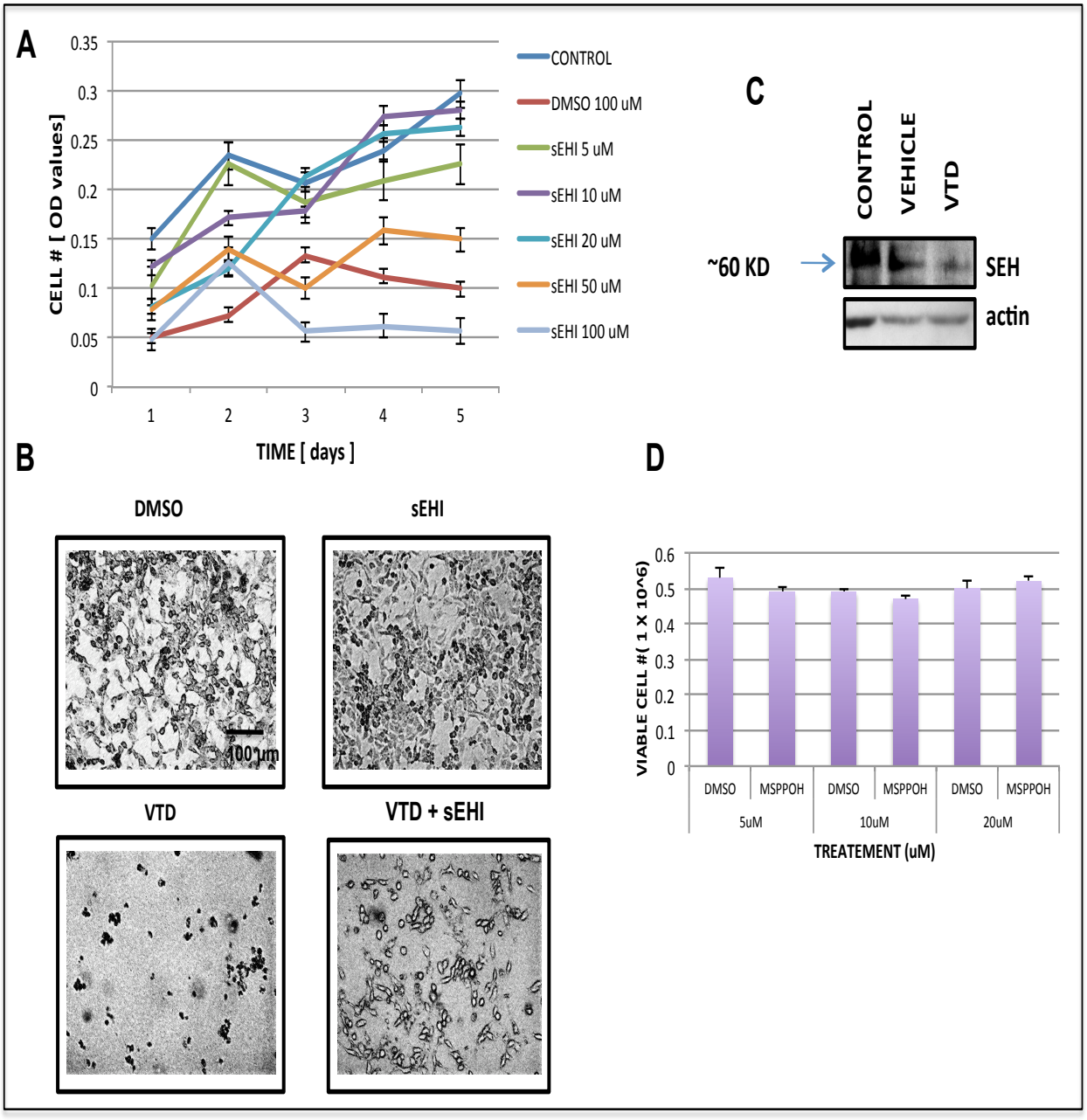


Figure 4-2: Soluble epoxide hydrolase is expressed in MDA-MB-231 breast cancer cells, its inhibition maintain the breast cancer cells viability. A. sEHI effect on breast cancer MDA-MB-231 cells measured by MTT proliferation assay. Cells were seeded in 96 wells plate in triplicate, 24 hrs later various sEHI concentrations were added, incubated for the indicated time period. Cells proliferation was measured based on average of OD values detected by MTT assay (data represent n=3). **B.** Morphology of MDA-MB-231 cells treated with drugs. MDA-MB-231 cells were seeded and treated with either VTD (10 µM), sEHI (10 µM), co-treatment of VTD (10 µM) + sEHI (10 µM), as well as DMSO (control). Cells were imaged at 20X objective magnification. Bar = 100 µm. **C.** Western blot analysis to detect sEH expression (Control: MDA-

MB-231; vehicle: MDA-MB-231 treated with DMSO; VTD: MDA-MB-231 treated with 10 μM vitamin D for 72 hrs) (n=2). **D**. MSPPOH effect on breast cancer MDA-MB-231 cells growth. Cells were seeded in 10 cm plate, treated with MSPPOH (at 5 μM, 10 μM, and 20 μM). DMSO used as control for each correspondent concentration. Cells were incubated for 72 hrs. Cells viability (cell #) counted by Vi-cell (n=3).

At a concentration of 20 μM, the drug delays onset of proliferation at an early time (before day 3). In the DMSO control, the amount of this vehicle was equivalent to that used in the 100 μM of sEHI condition – which was exceptionally high and could be the reason for the inhibitory effect on cell growth. We chose 10 μM as the optimized concentration of the sEHI for further experiments**.**

With this tool we can examine, using the model system of VTD induced differentiation in MDA-MB-231 breast cancer cells, whether elevating EET levels could prevent or suppress VTD mediated differentiation in this basal breast cancer cell line. But first we examined the cells treated with respect to morphology to make sure that co-treatment does not have any overt unexpected effect. Therefore, we treated VTD-induced cells with sEHI (10 μM) (Figure 4.2.B.). We found that while VTD treated cells displayed a convex shape with the presence of condensed vesicles, some of the (VTD + sEHI) co-treated cells maintained their original basal epithelial cell morphology. No overt toxicity was observed. Cells treated with sEHI alone showed no significant difference compared to the vehicle control. No difference of cell proliferation between the MDA-MB-231 cells treated with and without DMSO was observed, indicating that DMSO did not have an effect at the concentration used in this experiment.

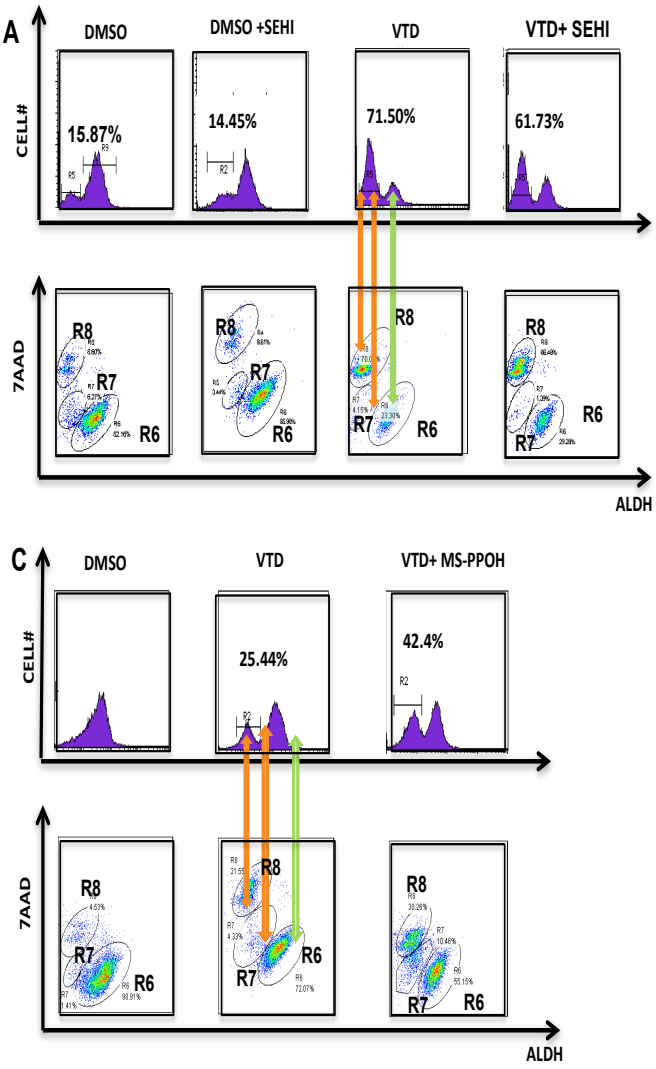
4.3 Increase of EET levels suppresses VTD-induced differentiation of MDA-MB-231 cells

4.3.1 Stabilizing endogenous EETs by soluble epoxide hydrolase inhibitor

To determine if elevated EETs affects VTD induced differentiation in MDA-MB-231 breast cancer cells, we used sEH inhibitors (1471, 1709). In order to determine whether there is such an EET metabolism that would offer the opportunity to experimentally increase EETs by inhibiting its degradation, we first examined whether MDA-MB-231 cells expressed soluble epoxide hydrolase. This enzyme is widely expressed in many tissues and its pharmacological inhibition has previously been shown to be useful in treating diseases that affect the cardiovascular and respiratory systems (X Fang et al., 2001). Total cell lysates were collected from MDA-MB-231 cells treated for 96hrs with VTD (10 μM) or with DMSO as vehicle control. Lysates from non-treated cultured cells were also obtained as a control. Expression levels of sEH were detected in the untreated as well as VTD-treated cells without significant change in the protein expression (Figure 4.2.C). This suggested that treatment with sEH would increase cellular EET levels. Unfortunately it is technically not possible (except in specialized laboratories) to measure the production of EETs in cells.

We next measured ALDH activity to examine the influence of sEH inhibition in VTD treated cells. Flow cytometry measurement of ALDH activity is shown in Figure 4.3.A. sEHI administration to the VTD-treated cells caused a visible change in the relative proportion of AL and AH subpopulations compared to control (VTD only) treatments. The ALDH activity in the VTD-induced differentiated population was reduced in the presence of sEHI by approximately half (0.56-fold ± 0.21 ($p < 0.05$)). By contrast, treating the control (DMSO) cells (which do not differentiate) with sEHI did not affect the population distribution (Figure 4.3.A). These results suggest that prolonging EET's inherently short half-life by inhibiting their metabolism sustained

the ALDH high subpopulation by preventing the stem like cells switch to the differentiation state.



	DMSO	DMSO+ sEHI	VTD	VTD+sEHI
R6	82.16%	81.2%	23.30%	29.29%
R7	6.27%	5.34%	4.15%	1.09%
R8	8.60%	7.8%	70.05%	66.49%

	DMSO	VTD	VTD+MSPPOH
R6	90.01%	72.07%	55.15%
R7	1.41%	4.33%	10.46%
R8	4.53%	21.55%	30.26%

Figure 4-3: Manipulating EET levels affect the VTD-induced differentiation dynamics of MDA-MB-231 clonal cell population. MDA-MB- 231 cultured cells treated with VTD (10 µM), as well as Co-treated with: A. VTD (10 µM) + sEHI (10 µM), C. VTD (10 µM) + MS-PPOH (10 µM), DMSO used as control. After 96 hrs of treatment, cells were stained with ALDH substrate. ALDH activity versus cell count of 10,000 cells detected with flow cytometry. Fluorescent detected by FL1 channel. % in histograms presents the ALDH low subpopulation numbers of cells. Cells were also stained with 7AAD- a nucleic dye that stains apoptotic cells (FL3 channel) to quantify apoptosis in ALDH low activity. Three subpopulations shown, R6 (ALDH high, 7 AAD -), R7 (ALDH low, 7AAD -), and R8 (ALDH low, 7AAD +). B &D. Tables showing the gate % (mean ± SD) compare to the total number of cells of the three subpopulations generated by 7-AAD analysis (n=3, $p < 0.05$).

4.3.2 Increasing EET levels by administering synthetic EETs

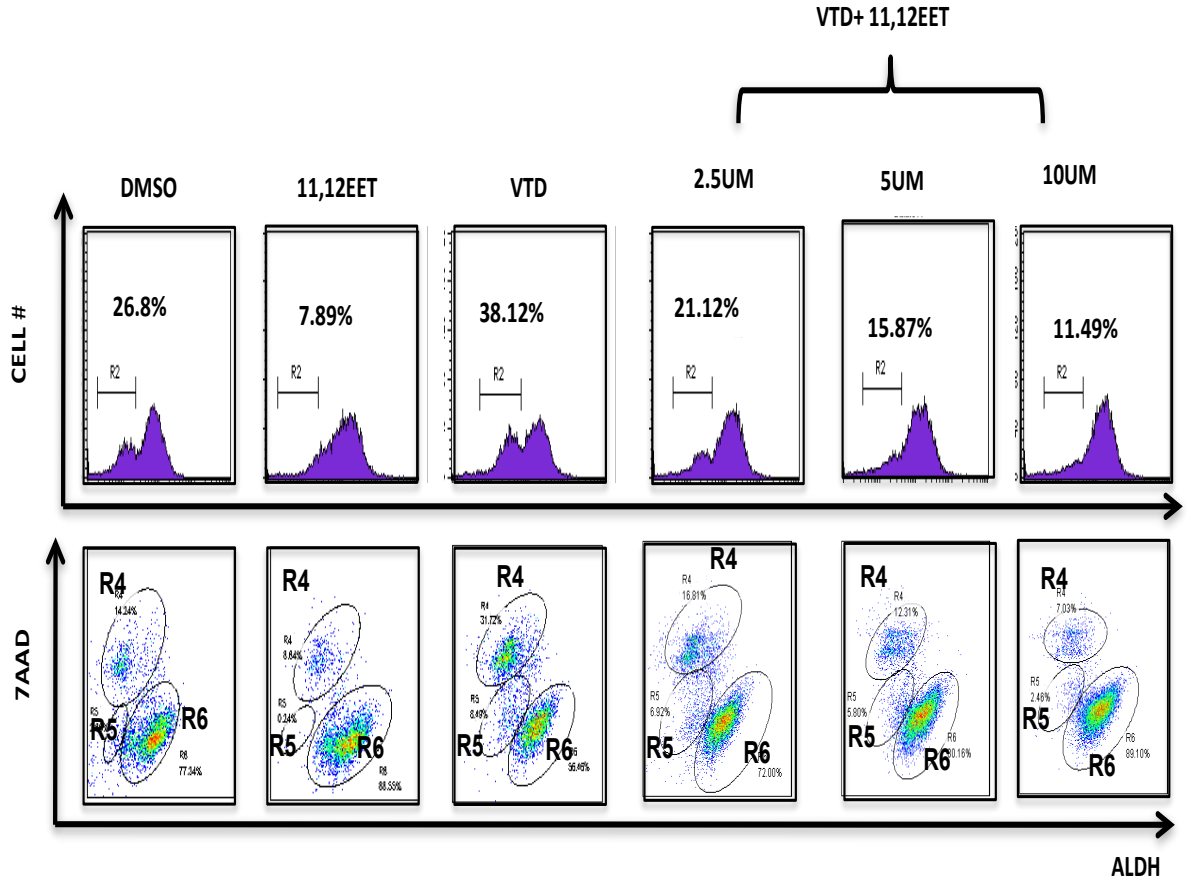
To confirm the observed effects of the increased EET levels in the VTD induced differentiation of MDA-MB-231 cells, we repeated the previous experiments but instead of using sEHI, we administered directly synthetic 11,12-EET, and 14,15-EET. These bioactive lipids are unstable and have exceedingly short half-lives. We reasoned that they may have the "hit and run" effect, since the systemic administration of exogenous EETs was observed to initiate tumor growth in animal models (Panigrahy et al., 2012).

Administration of 11,12-EET significantly inhibited VTD-induced differentiation of breast cancer cells as evidenced the generation of a much reduced AL subpopulation. The effect exhibited a dose-dependence, with a maximal relative inhibition of 0.43-fold at 10 μM concentration ($p < 0.05$) (Figure 4.4.A., Figure 4.5.A.). Similar response was obtained with the regioisomer, 14,15-EET, although its effects were slightly lower, with a maximal relative inhibition of 0.53-fold at 10 μM concentration ($p < 0.05$) (Figure 4.5.A).

We investigated the expression of ALDH1 protein in MDA-MB-231 cells by western blot analysis in the various experimental conditions, including control (DMSO), VTD-treatment, VTD +14,15-EET, and VTD+11,12-EET. The results of a typical experiment are shown in Figure 4.5.B., which demonstrates that no significant change in the expression of ALDH was detected in the presence of VTD and EETs. We reasoned that this may be due to a lack of sensitivity for changes inherent for western blot analysis of entire dishes: It measures the average ALDH expression of the whole cell population, ignoring heterogeneity of cell populations, such as the presence of multiple distinct subpopulations. Thus, we used this opportunity for a pedagogical demonstration that flow cytometry measurement of the single-cell resolution

population distribution of ALDH activity as we have routinely used in this project in fact offers the advantage not only to monitor cell-to-cell variation with respect to a single marker, but also may be just more sensitive to capture a smaller shift in the cell population distribution that one normally would have missed with “whole-population averaging” techniques such as western blots**.**

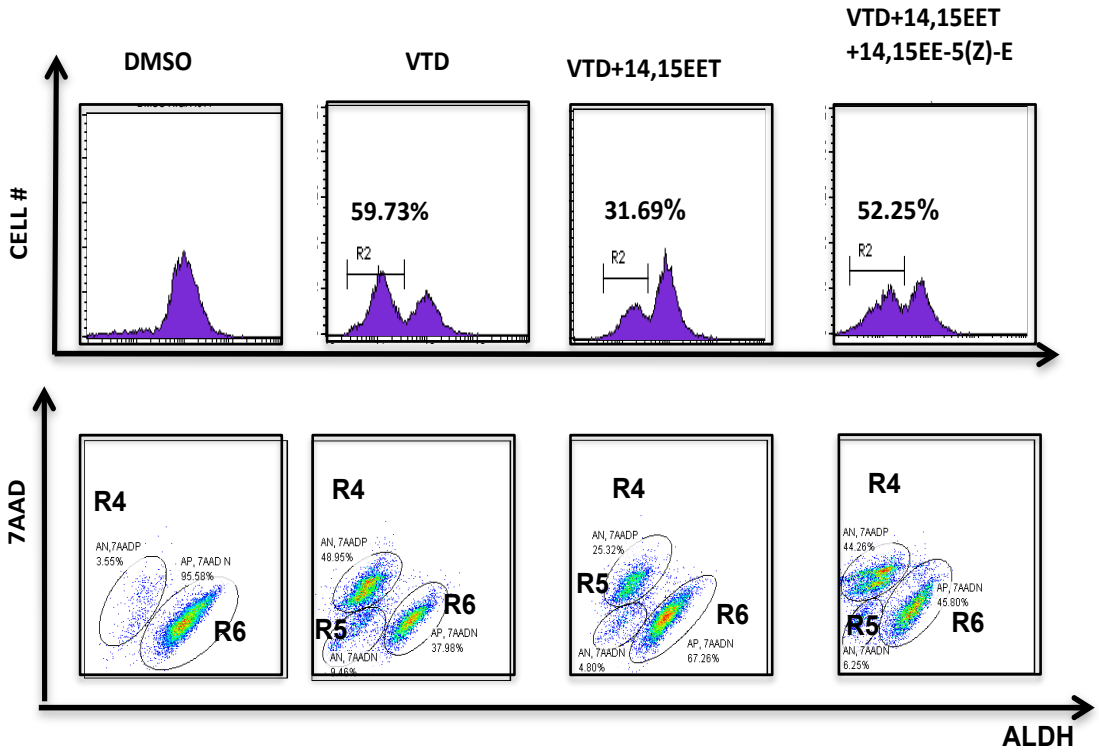
A



B

				VTD+11,12EET		
	DMSO	11,12 EET	VTD	2.5 uM	5 uM	10 uM
R6	77.34%	88.55%	56.46%	72.00%	80.16%	89.10%
R5	2.88%	0.24%	8.49%	6.92%	5.80%	2.46%
R4	14.24%	8.84%	31.72%	16.81%	12.31%	7.03%

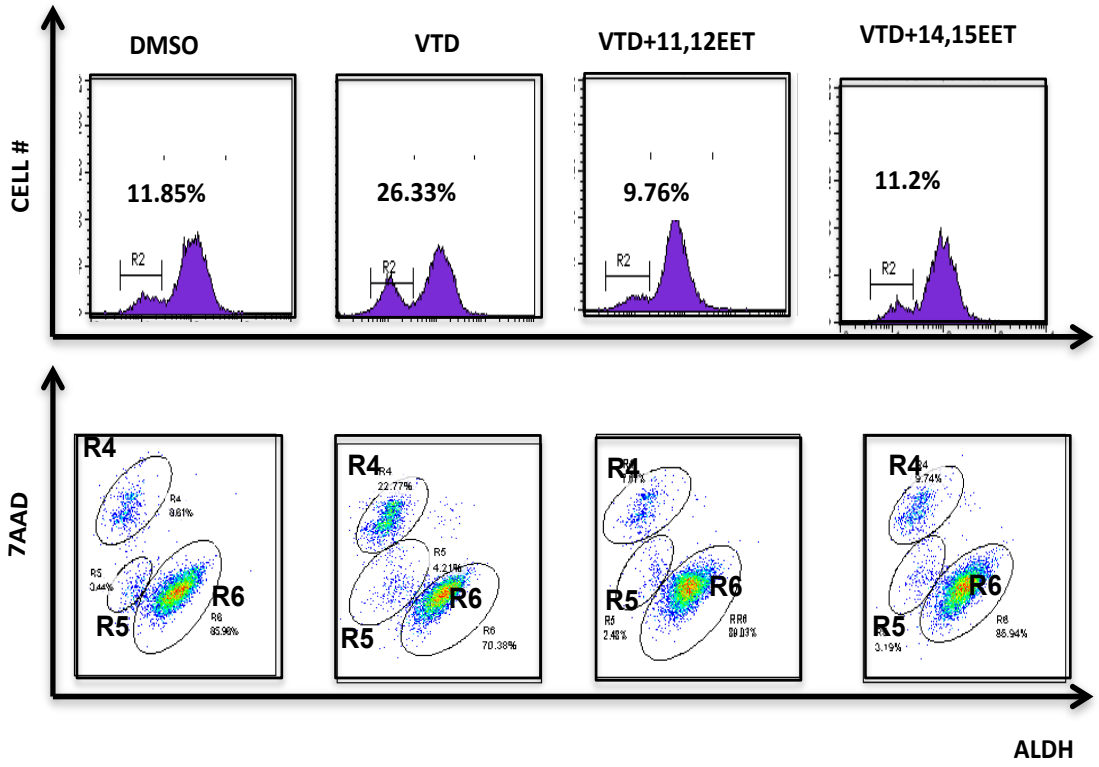
E



F

	DMSO	VTD	VTD+14,15EET	VTD+14,15EET +14,15EE-5(Z)-E
R6	95.58%	37.98%	67.26%	45.80%
R5	3.55%	9.46%	4.80%	6.25%
R4	-	48.95%	25.32%	44.26%

C

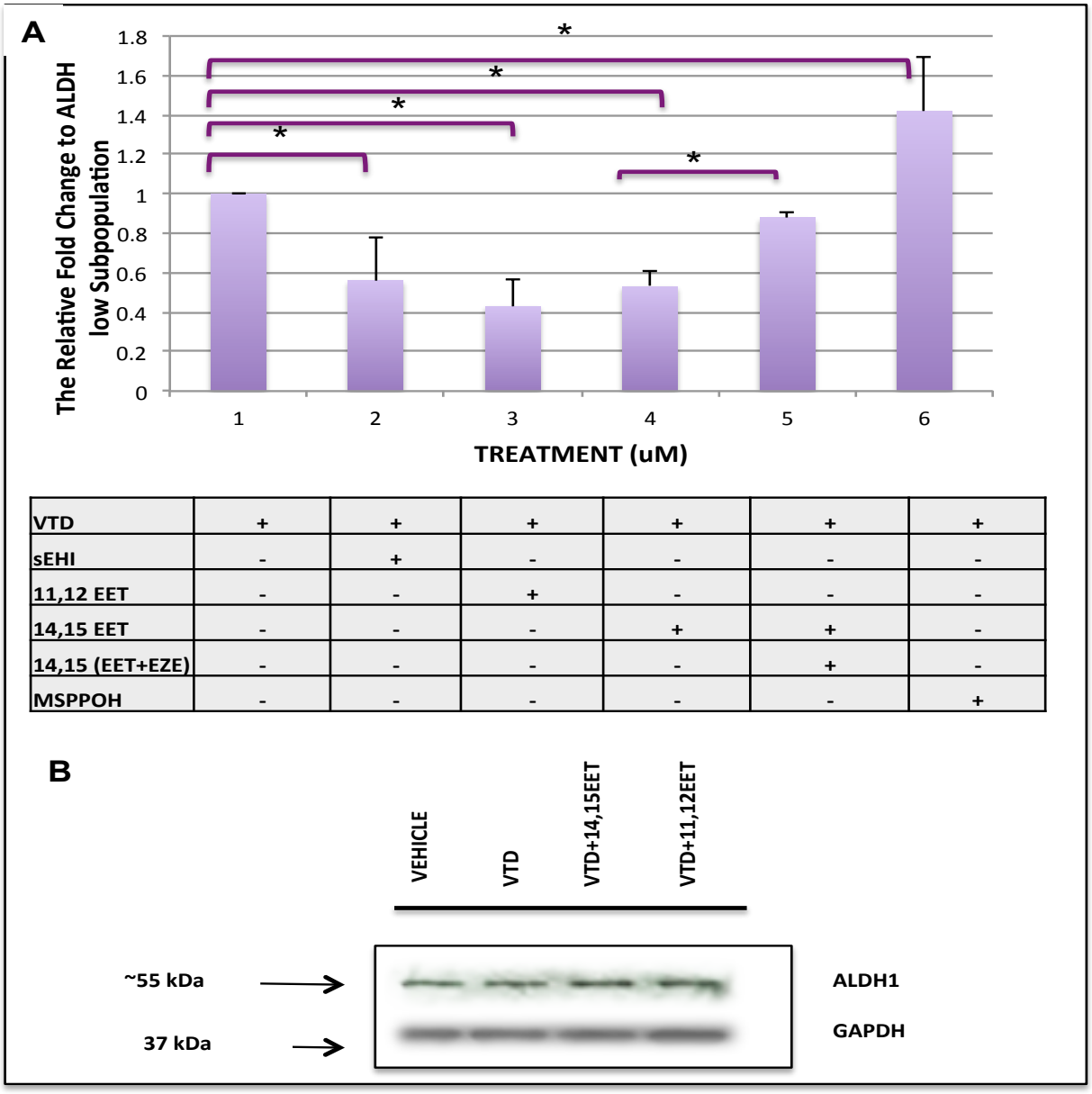


D

	DMSO	VTD	VTD+11,12EET	VTD+14,15EET
R6	85.96%	70.38%	89.03%	85.94%
R5	0.44%	4.21%	2.48%	3.19%
R4	8.61%	22.77%	7.67%	9.74%

Figure 4-4: Different isomers of EETs increase the stem cell-like subpopulation and reduce the non-viable subpopulation on VTD-induced differentiation on MDA-MB-231 cell line, this effect is blocked by EETs antagonist. MDA-MB-231 Cells plated in 10cm plates for 24 hrs, starved in serum-free DMEM media for 24 hrs, then induced with VTD (10 µM) for 96 hours, in the absence and presence of 11,12-EET, 14,15-EET and the combination of 14,15-EET+14,15-EE-5(Z)-E. DMSO, and EETs treatment were used as control. After 96 hrs of treatment, cells were stained with ALDH substrate. ALDH activity versus cell count detected with flow cytometry (FITC). % In histograms reflect the number of cells in the ALDH low sub-population per the whole population of 10,000 cells collected. Cells were also stained with 7AAD (APC) to quantify apoptosis. Three subpopulations shown: R4 (ALDH low, 7AAD

positive), R5 (ALDH low, 7AAD negative) and R6 (ALDH high, 7AAD negative). **A.** MDA-MB- 231 cells treated with different concentration of 11,12-EET (2.5 μM, 5 μM, 10 μM) along with VTD. **C**. Comparing 11,12-EET and 14,15-EET effects on VTD induced differentiation of MDA-MB-231 breast cancer cell line. **E**. MDA-MB-231 cells treated with VTD+14,15-EET, as well as with EET antagonist 14,15-EE-5(Z)-E. **B, D & F**. Tables showing the gate % (as mean ± SD) compare to the total number of cells of the three sub-populations generated by 7-AAD analysis (APC) ($n=3$, $p < 0.05$).



VTD	+	+	+	+	+	+
sEHI	-	+	-	-	-	-
11,12 EET	-	-	+	-	-	-
14,15 EET	-	-	-	+	+	-
14,15 (EET+EZE)	-	-	-	-	+	-
MSPPOH	-	-	-	-	-	+

Figure 4-5: High EET levels reduce the differentiated subpopulation in the VTD-induced differentiation of MDA-MB-231 cells, while low EET levels promote it. A. Shown is the relative fold change (inhibition, induction) of ALDH low subpopulation of VTD induced differentiation in the presence of drugs manipulating EET levels in MDA-MB-231 cells in compare to ALDH low subgroup of VTD-induced differentiation alone. Results are represented as means ± SD (n=3, *$p < 0.05$). B. Western blot analysis of ALDH expression in bulk population of VTD-induced differentiation of MDA-MB-231 cells in the absence and presence of EETs (14,15-EET, 11,12-EET). Cultured cells treated with VTD (10 µM) as well as EETs (10 µM), DMSO is used as control. 96 hrs later ALDH activity is detected by FACS, subsequently lysates were obtained (n=2).

To further examine the extent to which EETs blocks cancer stem cell-like cells differentiation, we determined the synergic effect of the two drugs, sEH inhibitor and EETs itself, or other interference - hoping to see either exaggerated or diminished effects when used concurrently. Adding the two drugs to the VTD-induced MDA-MB-231 cells had a slightly higher but insignificant effect in decreasing the VTD differentiated AL sub-population when comparing to treatment of the exogenous 14.15-EET alone.

4.4 14,15-EE-5-(Z)-E attenuated the effects of EETs in the VTD-induced differentiation of MDA-MB-231 cells

To corroborate the observed effects of 14,15-EET in VTD-mediated differentiation of MDA-MB-231 cells, cultured cells were treated with the putative EET receptor antagonist 14,15-EE-5(Z)-E. While the receptor(s) for EETs have not yet been identified, such EET antagonists are the most specific reagents available for pharmacologic suppression of EET activity (Nithipatikom et al., 2010). When VTD-mediated differentiation of MDA-MB-231 cells was induced in the presence of both EETs and its antagonist 14,15-EE-5-(Z)-E, the inhibitory effect of EETs in suppressing the (differentiated) AL subpopulation was blocked by 65% ± 0.03 ($p < 0.05$) (Figure 4.4.E.). Therefore, we can say that the regioisomer-specific EET antagonist 14,15-EE-5-(Z)-E attenuated the effect of EETs, supporting the hypothesis that high EET levels suppress the induced differentiation of MDA-MB-231 cells.

4.5 Lowering EETs production promotes VTD induced differentiation in MDA-MB-231 breast cancer cells

To assess the involvement of endogenous CYP-derived EETs in influencing the induced differentiation of MDA-MB-231 cells, we next used MS-PPOH, a potent and selective inhibitor of CYP epoxygenase. Inhibiting the CYP enzyme will reduce the endogenous levels of EET by blocking the path that converts arachidonic acid (AA) to EETs. CYP3A has been shown to be

expressed in breast cancer tissues and correlated with a decreased overall survival (Mitra et al., 2011). We performed the differentiation assay for the MDA-MB-231 in the presence of MS-PPOH. Cells were co-administered VTD (10 μM) and MS-PPOH (10 μM). As seen in Figure 4.3.B., in the presence of MS-PPOH (and expected reduced endogenous EET levels) the VTD-induced differentiation in MDA-MB-231 cells, led to increased AL subpopulation by 1.42 fold ± 0.27 fold compared to the AL group of the control ($p < 0.05$).

Non-induced MDA-MB-231 cells were treated with different concentrations of MS-PPOH, and 10 μM did not have any toxicity in the overall survival and proliferation of the cells (Figure 4.2.D). These findings suggest that inhibiting the arachidonic acid metabolism to EETs, thus lowering the levels of endogenous EET promotes the differentiated state, thereby allowing more cells from the stem-like cell state to respond to the VTD-induced differentiation.

4.6 7-AAD analysis highlights the heterogeneous meta-stable states of the VTD-mediated differentiation of MDA-MB-231 cells

The consistent observation that in the presence of high EETs, the AL subpopulation in the VTD treated bimodal population is diminished compared to the same population in the control (VTD only treated cells) could be explained not by the reduced influx from the AH subpopulation (suppression of differentiation) but alternatively, by a reduced viability of the differentiated cells. In other words, EETs may achieved the shift in the AL; AH ratio by promoting cell death in the differentiated (AL) cells – this is a reasonable assumption since differentiated cells are more fragile in general, and cell regulation in stem cells often expand the stem cell population by increasing survival.

To determine whether the differentiated cells undergo increased programmed cell death or are maintained in the differentiated (AL) state we performed a 7-amino-actinomycin D (7-

AAD) analysis to detect and quantify apoptosis and cell death. Using flow cytometry, 7-AAD staining was measured in MDA-MB-231 cells co-stained for ALDH activity (Figures 4.3, 4.4). Double staining of ALDH and 7-AAD revealed the existence of three subpopulations, the stem cell-like population (region R6 in the flow cytometry dot plot in Figure 4.3.A.: AH, 7-AAD-negative), the differentiated viable population (R7: AL, 7-ADD negative), and the dead cell population (R8: AL, 7-AAD positive). Of note is the subdivision of the ALDH-low subpopulation into 7-AAD positive (dead cells) and 7-ADD negative (viable cells). Under the VTD-treatment condition, typically a smaller % of differentiated cells remained in the viable state, while the majority eventually died, as expected. Interestingly, increased levels of EET (either by inhibiting the sEH enzyme or by direct administration of synthetic EETs) not only significantly increased the stem cell-like population but also decreased apoptosis in the AL cells, the VTD-induced differentiation of MDA-MB-231 cells (see Tables in Fig. 5. A, B). Thus, elevated EET is not toxic to the differentiated in contrary it increases viability of this apoptosis-prone cell state. This indicates that the diminution of the AL subpopulation representing differentiated cells is not due to acceleration of their death but that most likely EET act to reduce the differentiation rate, i.e. the actual transition from the AH to the AL state.

We confirmed the results again, using synthetic EETs, where the 11,12-EET treatment of the VTD-treated cells compared to VTD alone significantly increased the number of cells in the stem cell-like AH state in a dose dependent manner with a maximum at 10 μM (R6, 89.10% relative to 56.46%), significantly decreased the number of the dead cells (R4, 7.03% in compare to 31.72%) as well as significantly decreased the viable differentiated sub-population (R5, 2.46% relative to 8.49%) ($p < 0.05$) (Figure 4.4.B.). Remarkably, administration of 11,12-EET directly on cultured cells without VTD inducing MDA-MB-231 cells to differentiate eliminated the

spontaneously differentiated cells often seen in the DMSO control, thus, again increasing the stem cell like population (88.55% vs. 77.34%). It also decreased the apoptotic sub-population (8.84% vs. 14.24%) and almost completely eliminated the spontaneously differentiated subpopulation (0.24% vs. 2.88%) (as shown in Figure 4.4.B).

In a direct comparison of both EET regioisomers, 11,12-EET exhibited a slightly greater effect in inhibiting the VTD-induced differentiation (Figure 4.4.C.). The latter effect was also reflected in the 7-AAD analysis where the effect of 14,15-EET was slightly less pronounced than 11,12-EET (see Figure 4.4.D.).

The specificity of the effect of 14,15-EET with respect to suppression of apoptosis was again confirmed using the analog 14,15-EE-5-(Z)-E. The comparison of both conditions, treatment of VTD-treated cells with 14,15 EET in the absence and presence of 14,15-EE-5-(Z)-E revealed that in the latter the apoptotic population was significantly increased (44.26% vs. 25.32%), whereas the stem-cell-like AH population was decreased (45.80% vs. 67.26%). Accordingly as expected, the differentiated viable AL population increased (6.25% vs. 4.80%) (Figure 4.4.E, F.).

Conversely, low EET levels achieved by pharmacological inhibition of CYP epoxygenase using MSPPOH not only led to a decreased stem-cell-like AH subpopulation (R6: 55.15%) in VTD treated cells when compared to cells treated with VTD alone (72.07%). The number of both differentiated viable and the dead subpopulations were increased (R7: 10.46%, R8: 30.26%) in comparison to the otherwise untreated VTD-induced cells (R7: 4.33%, R8: 21.55%) (Figure 4.3.C.).

These complementary experiments in which EET levels are suppressed or reduced confirm that its activity in promoting viability in the differentiated cells is specific and present at

basal level under natural condition because competing or decreasing endogenous EETs led to increased apoptosis.

Taking together, these results indicate that increasing EET levels reduces the population fraction of basal breast cancer cell line MDA-MB-231 cells in the differentiated (AL) state by suppressing the VTD-induced differentiation (state transition from the AH to the AL state) although it appears to have anti-apoptotic effects from which the differentiated cells, naturally are more prone to undergo apoptosis.

4.7 Microarray analysis reveals gene expression pattern differences between responding and non-responding cells to vitamin D and EET treatments

Now that we have a robust system that exhibits a phenotypic change when modulating the differentiation dynamics in response to EETs treatment, we can investigate the molecular profiles of cells in order to obtain a global picture of cellular functions associated with the EETs response. Since not all cells respond, we need to consider the population heterogeneity with respect to biological response (as explained in the introduction, Section 1.1.3). Therefore, the gene expression profile differences must be measured in the sorted populations to separate responding from non-responding cells. This approach differs from most current procedures which assume population homogeneity and simply compare entire cell populations ("dishes") that are either treated or non-treated.

To examine the distinct states of differentiation in the absence and presence of EETs, we subjected the parental clonal treated MDA-MB-231 population to FACS sorting - separating responding (differentiated cells with low ALDH activity) from non-responding cells (stem-like cells with high ALDH activity). We sorted the two different fractions seen in the original

distribution with respect to ALDH for both conditions, VTD-treated cells in either the presence or absence of EETs – giving rise to four samples (plus a DMSO-treated mock sorted control):

Table 4-1: FACS sorting samples collected with respect to the ALDH activity of VTD and EETs treated MDA-MB-231 cells

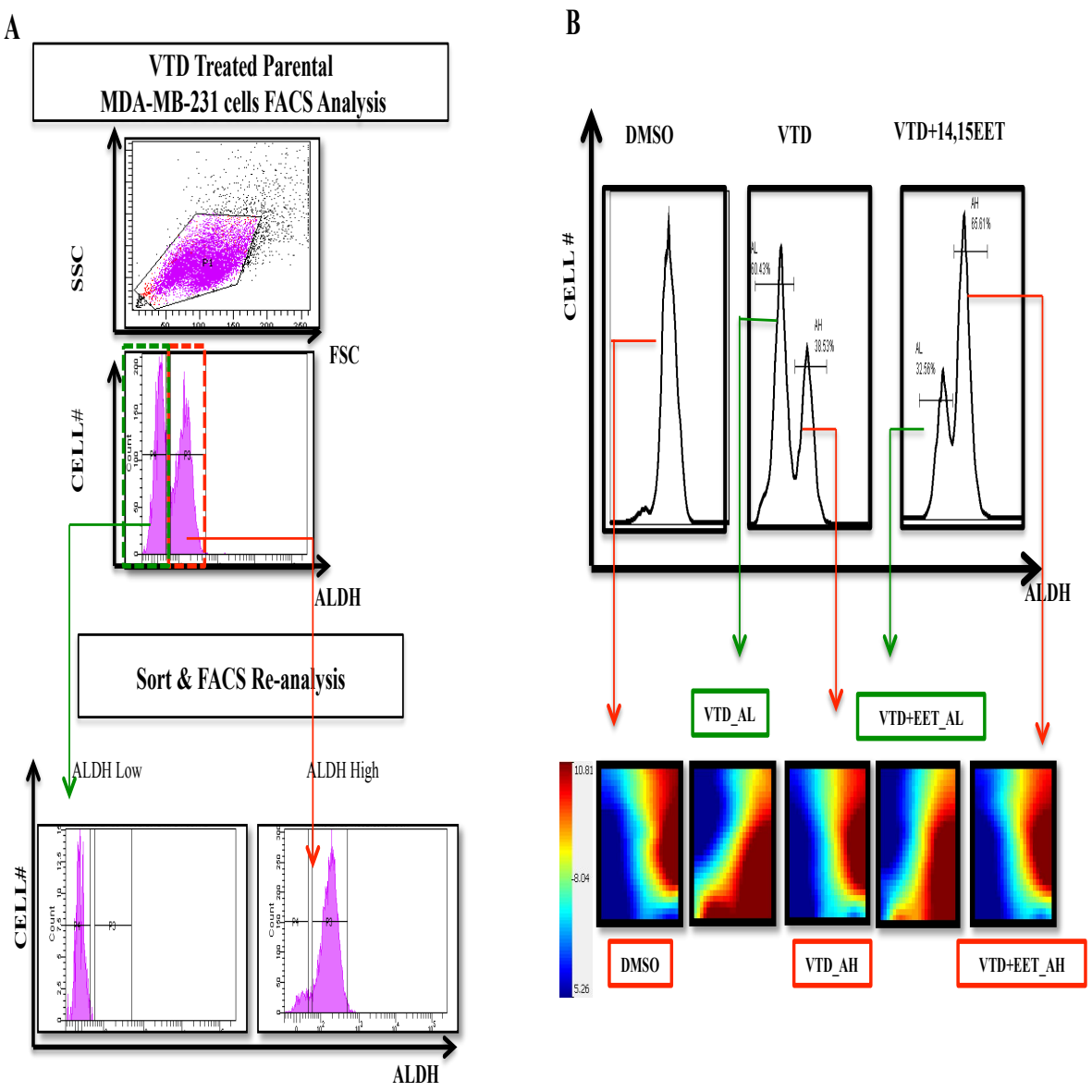
SAMPLE LABEL	DESCRIPTION
DMSO control	DMSO-treated MDA-MB-231 cells
VTD_AH	ALDH high subpopulation of the vitamin D treated MDA-MB-231 cells
VTD_AL	ALDH low subpopulation of the vitamin D treated MDA-MB-231 cells
EET +VTD_AH	ALDH high subpopulation of the vitamin D + EETs treated MDA-MB-231 cells
EET+VTD_AL	ALDH low subpopulation of the vitamin D +EETs treated MDA-MB-231 cells

Flow cytometry analysis post sorting with respect to ALDH activity was performed to check for the purity of the sorted samples (Figure 4.6.A). Total RNAs from the sorted cells as well as from the conventional bulk-population was obtained for microarray analysis using the Agilent platform (see Material and Methods, Chapter 3).

4.7.1 GEDI and hierarchal clustering analysis reflects the non-genetic heterogeneity of the differentiated subpopulation and the stem cell like subpopulation of the same parental cells

Gene expression values (normalized in the standard method using the microarray manufacturer ‘s analysis program – see Material and Methods, Chapter 3) were first subjected to

GEDI analysis for a global visual survey of the data. GEDI uses self-organizing maps to display each sample's expression profile as a map that contains a visually recognizable color pattern, referred to as "GEDI map". Specifically, in GEDI, each tile within a mosaic represents a mini-cluster of genes that behave similarly, i.e. have highly similar expression pattern across all the analyzed samples. The same genes are forced to the same mosaic position for all GEDI maps, hence allowing direct comparison of transcriptomes based on the overall mosaic pattern. Thus, each mosaic represents the gene expression profile of a sample (Figure 4.6.B.), capturing visually the sample-specific features of the expression profile.


A
VTD Treated Parental
MDA-MB-231 cells FACS Analysis
SSC
FSC
CELL#
ALDH
Sort & FACS Re-analysis
ALDH Low
ALDH High
CELL#
ALDH
B
DMSO
VTD
VTD+14,15EET
CELL #
ALDH
VTD_AL
VTD+EET_AL
DMSO
VTD_AH
VTD+EET_AH

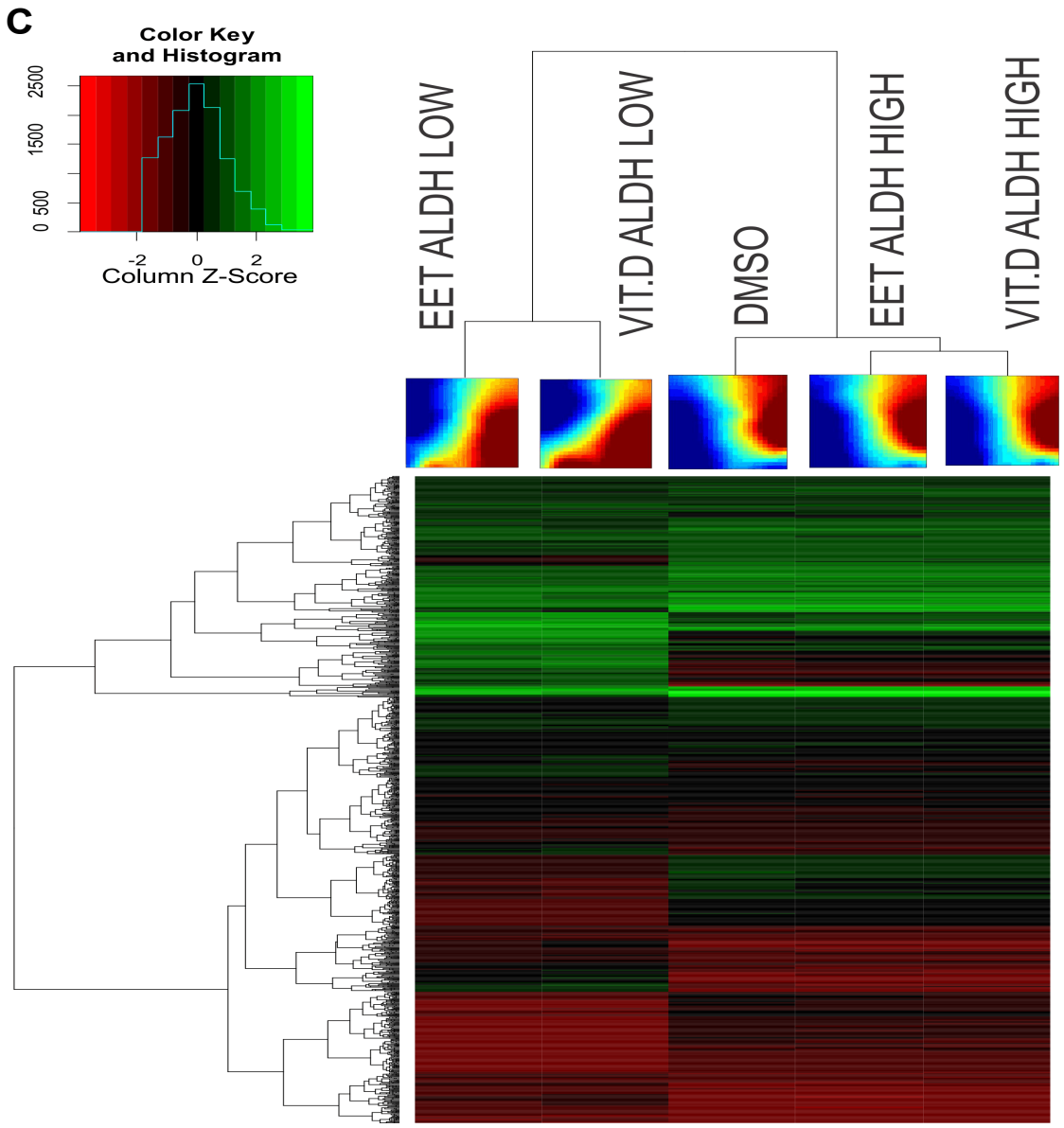


Figure 4-6: Clustering of expression profiles defines distinct patterns for the stem cell-like subpopulation and the differentiated subpopulation in the MDA-MB-231 VTD-induced differentiation. A. MDA-MB-231 cells treated with VTD (10 μM), VTD+EET (10 μM) as well as the control were subjected to FACS sorting based on the measured ALDH activity. Flow cytometry analysis was performed post sorting to confirm the purity of each sorted subpopulation, ALDH high and ALDH low (n=5). **B.** GEDI maps reflect the expression profiles of each of the sorted samples (VTD_AH, VTD_AL, EET+VTD_AH, EET+VTD_AL, as well as the mock (DMSO)). Distinct patterns are recognized upon visual inspection of the GEDI map. DMSO and ALDH high subpopulations (VTD and EET+VTD) have more similar pattern to each other then to the ALDH low subpopulation (VTD and EET+VTD) pattern. **C**. Comparison of GEDI maps and hierarchical clustering. Top: dendrogram from hierarchical clustering, computed

from 9836 genes showing 2 main clusters of samples DMSO, ALDH high subpopulations in one cluster and the ALDH low subpopulation in a different cluster. Samples with similar gene expression profiles cluster together. Heat map representation of the entire cluster of 9836 genes based on similarities in gene expression. Green: up-regulated gene expressions, Red: down-regulated gene expressions. Black: neutral expression of genes.

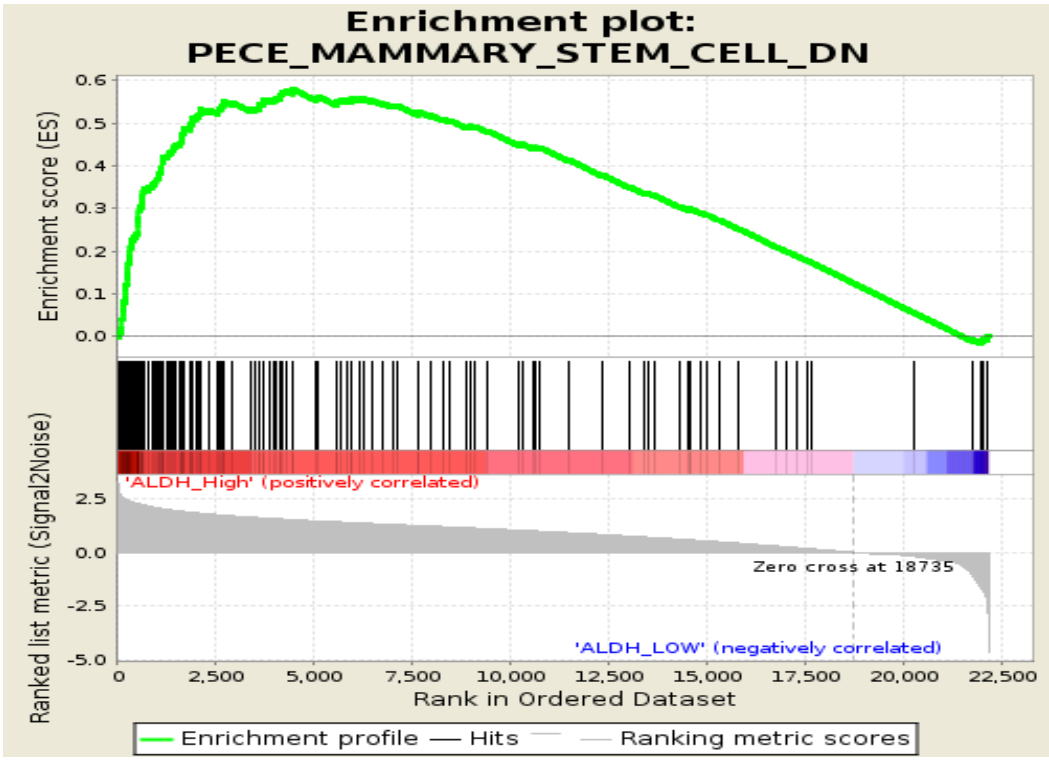
The bimodal distribution of MDA-MB-231 cell populations after 3 days of vitamin D-induced differentiation is evident when we measure the activity of ALDH with flow cytometry, as described in section 4.1. Nonetheless, phenotypical changes during differentiation are governed by the dynamics of a gene regulatory network (GRN), which encompasses thousands of genes beyond this marker. Indeed, discrete differences in patterns of gene expression between the responding (ALDH low subpopulation of the vitamin D treated cells in the presence and absence of EETs) and non-responding (ALDH high fraction of the vitamin D treated cells in the presence and absence of EETs) sub-populations to drug perturbation are immediately detected upon visual assessment of the GEDI maps (Figure 4.6.B.). The inspection of GEDI maps allows a straightforward classification of the five samples (DMSO control, VTD_ALDH High, VTD_ALDH low, VTD+EET_ALDH high, VTD+EET_ALDH low) into two groups without the aid of a clustering algorithm, simply based on the visual differences in the GEDI maps.

The patterns of the GEDI maps of the two groups are also reflected in the hierarchical clustering analysis (Figure 4.6.C.). Hierarchical cluster analysis reliably arranged the samples into distinct clusters that we identified by using GEDI (Figure 4.6.C.). The hierarchical clustering algorithm classifies the experimental samples on the basis of overall similarity in their gene expression patterns. The arrangement and length of the branches in the dendrogram reflects the relatedness of the samples. As expected, DMSO, VTD_AH and VTD+EET_AH groups, representing the stem-cell population, are more similar to each other, and are clustered together

in one category, than to the ALDH low subpopulations of the same parental tumor cells, thus, pushing the VTD_AL and VTD+EET_AL groups into a different cluster. (Figure 4.6.C.).

4.7.2 EETs promote gene expression pattern indicating cancer stem cell properties

Functional classification of the genes differentially expressed in the various gene set enrichment analysis (GSEA) libraries revealed that ALDH high samples (with and without EETs), as expected, are enriched for genes involved in mammary stem cells, DNA damage response and cancer progression (Figure 4.7). By contrast, the ALDH low samples are enriched for genes involved in cytokine and membrane activity (data not shown). The enrichment sets of the ALDH high group of cells fall within the properties of cancer stem cells; therefore, supporting the established notion that the ALDH high subpopulation as "stem cell-like cells". Of interest here, are the changes that are specific to EETs treatment to determine whether EETs affect state transition rates via a shift of the attractor landscape.



Enriched Gene Sets	SIZE	ES	NES	NOM p-value	FDR q-value
TAKAO_RESPONSE_TO_UVB_RADIATION_UP	86	0.61	1.98	0.045	0.358
PECE_MAMMARY_STEM_CELL_DN	141	0.58	1.84	0.086	0.32
IIZUKA_LIVER_CANCER_PROGRESSION_L0_L1_DN	21	0.6	1.79	<0.01	0.225

Figure 4-7: Gene set enrichment analysis (GESA) with respect to non-differentiated subpopulation (ALDH high activity samples) in compare to the differentiated subpopulation (ALDH low activity samples) of the VTD-induced differentiation of MDA-MB-231 cells. Top: enrichment plot showing mammary stem cell enrichment in the ALDH high subpopulation. Bar code: each line signal represents one gene. Density represents the enrichment of genes showing most significant enrichment on the sides. Red: up-regulated genes. Blue: down-regulated genes. Table is representing the enriched gene set. Size: number of genes enriched. ES: enrichment score. NES: normalized enrichment score. ($p < 0.05$).

From the genes whose expression was measured in the five samples, we selected a subset that consisted of genes with the greatest variation in expression (measured as fold change). The two comparisons of interested that could inform us about the effect of EETs are:

(i) VTD_AH – vs – VTD+EET_AH

(ii) VTD_AL –vs– VTD+EET_AL

From (i), we expect to see changes in the stem cell-like state induced by EETs that suppress VTD induced differentiation. (ii), is of interest as a control comparison but will reveal effects of EETs on the cells that have differentiated despite the presence of EETs.

To determine the molecular functions and the biological processes associated with each gene, we manually searched PubMed. It was beyond the scope of this thesis to perform more than one replicate of gene expression profiling in this pilot experiment or to perform independent confirmations of differential expression using q-PCR. However, this hand-curated list of either the most differentially expressed genes or of the biologically most relevant genes expressed differentially with borderline significance will be a starting point for future analyses.

Using these criteria, we identified 36 genes that differentiate the ALDH high population of the MDA-MB-231 cell treated with both VTD and EET from the cells treated with VTD alone (comparison (i) Table 4.2). Among these 36 genes, 22 genes were highly expressed and 13 genes were down-regulated in the VTD+EET_AH cells compared to the VTD_AH cells. As depicted in Table 4.2, EETs promoted the expression of genes involved in embryogenesis, cell cycle, proliferation, anti-apoptosis, DNA damage and stress response, immune response, metastasis, drug metabolism, inhibition of cell-adhesion, proteolysis, and transport. Concurrent with the up-regulation of genes implicated in these biological activities, EETs treatment

Table 4-2: Selected genes differentially expressed in the ALDH high subpopulations between VTD-treated and EET + VTD - treated MDA-MB-231 cells

		EET_VTD_AH VS VTD_AH	
		EET_VTD_AH	
CATEGORIES	GENE NAME	FOLD CHANGE	PATHWAYS
EMBRYOGENESIS	SOHLH1	6.640903671	
	DLX6	2.133720121	
	TMSB15A	4.294967552	
CELL CYCLE, SURVIVAL, PROLIFERATION	PRUNE2	3.838169635	
	SEPT1"	3.124351008	
	NEK8	2.854546597	
	FOXL1	2.239908271	WNT, Hedghog, EMT
	MPEG1	0.431236652	MAPS, WNT, STAT, JNK
	TPD52L3	0.188931824	
APOPTOSIS	TPD52L3	0.188931824	
ANTI-APOPTOSIS	PRUN2	3.838169635	
	PCGEM1	2.619702656	
AGGRESSIVENESS/METASTAIS	FOLH1B	5.63491916	
	EIF4E2	4.583892816	mTOR, Insulin signaling, WNT, JNK
	FAM124B	4.400255055	JUN, NF-kB, WNT, JNK
	PRUNE2	3.838169635	
	ALX1	3.256334004	EMT
	SOX1	0.302358265	
TUMOR SUPPRESSION	MPZ	0.258865924	
DRUG METABOLISM	HBB	4.146095174	
VITAMIN METABOLIC PROCESS	ACPS	0.430094735	
DIFFERNTIATION MARKERS	SOX1	0.302358265	
	PADI1	0.244398641	
DNA DAMAGE/STRESS RESPONSE	DDIT4L	7.983063056	mTOR, AKT
IMMUNE RESPONSE	IL-17C	3.635664937	NF-kB, MAPK
	SAA1	0.172604925	
	DEFB114	0.046630557	
TRANSCRIPTION REGULATION	ZCCHC24	0.258865924	
CYTOSKELETAL STRUCTURE/CELL ADHESION	FAM124B	4.400255055	JUN, NF-kB, Wnt, JNK
	TMSB15A	4.294967552	
	SAA1	0.172604925	
PROTEOLYSIS	FLOH1B	5.63491916	
	PRSS45	4.692462175	
TRANSPORT	HBZ	4.186646108	
CELL POLARITY/SPECIALIZATION	TMEM30C	0.162137888	

down- regulated genes associated with apoptosis, tumor suppression, anti-proliferation, anti-metastasis, cell adhesion, cell polarity, vitamin metabolic process, and differentiation markers. The majority of the differential transcripts encode known genes, but significant fractions are uncharacterized. The majority of the genes in the former group are involved in biological processes that are hallmarks of cancer stem cells, therefore, supporting our hypothesis of elevated EET levels promote the stem cell compartment of breast cancer cells and thereby suppress their differentiation.

In the differentiated (ALDH low) subpopulations, we found, using the same criteria as above, that 51 genes differentiate the ALDH low cells treated with EETs from those not treated with EETs. In the differentiated fraction of the VTD-treated breast cancer cells, EETs down-regulate some differentiation genes that were highly expressed following treatment with vitamin D. EETs also down-regulated genes involved in cell-adhesion, negative regulation of cell growth, negative regulation of embryogenesis, negative regulation of DNA damage response, apoptosis, and translation regulation. By contrast, in these differentiated fractions, EETs also up-regulated a subclass of genes associated with embryogenesis, cell growth, anti-apoptosis, angiogenesis, metastasis and cell migration, DNA repair, immune response, cell adhesion, protein assembly, transport, and cell polarity, compared to the VTD_AL cells that did not see EETs (Table 4.3). Interestingly, EETs promoted the expression of G-protein couple receptors GPCR 112 and GPCR 182 in the differentiated subpopulation.

We see differential expression of genes AFTER we have sorted for those ALDH-high cells that even after stimulation with VTD failed to differentiate - irrespective of presence of EETs. This is surprising because if EETs simply prevented the transition into the differentiated state, the FACS sorting would prevent this effect of EETs from being manifested in the gene

Table 4-3: Selected genes differentially expressed in the ALDH low subpopulation between VTD-treated and EET + VTD - treated MDA-MB-231 cells

		EET+VTD_AL VS. VTD_AL	
		EET+VTD_AL	
CATEGORIES	GENE NAME	FOLD CHANGE	PATHWAY
EMBRYOGESIS	TWIST1	5.608279658	EMT
	BICC1	2.55787882	WNT (negative regulation)
	PDGFA	2.003420435	EGFR, NOTCH
	TBX18	0.141802895	OCT3/4
CELL CYCLE, SURVIVAL, PROLIFERATION	PIK3R5	2.932210243	
	PDGFA	2.003420435	EGFR, NOTCH
	TNN	2.854643572	
	cyclinD1	0.412478748	WNT, NOTCH
	UXT	0.3598395	
	MPEG1	0.234466987	NF-kB
APOPTOSIS	FBXO2	3.262035023	
	TEF	2.48834821	
	UXT	0.3598395	NF-kB
ANGIOGENESIS	PDGFA	2.003420435	EGFR, NOTCH
AGGRESSIVENESS/METASTAIS	TWIST1	5.608279658	EMT
	ARSH	4.816690018	
	PIK3R5	2.932210243	
	TNN	2.854643572	
	MMP1	2.316064643	
DIFFERENTATION	UXT	0.3598395	
	PADI1	0.340084004	
	MCF2L	0.243655555	
	SLC17A2	0.183227739	
	ZNF750	0.172046436	
	SPOCK2	0.163903466	MAPK, PTEN
	TBX18	0.141802895	
	LGI4	0.11964505	
	AGR3	0.0143378	
DNA DAMAGE/ STRESS RESPONSE	OR5D18	8.904783332	
	BNC2	7.192699105	
	WDR33	2.649579303	WNT
	cyclinD1	0.412478748	WNT, NOTCH
	MCHR1	0.154153999	
	AGR3	0.0143378	
IMMUNE RESPONSE/INFLAMMATION	DCST1	6.602000698	
	IL2RA	4.051327593	TGF-BETA, AKT, APOPTOSIS,MAPK, JAK/STAT,JNK
TRANSLATION REGULATION	PATL2	0.315158252	
CYTOSKELETAL STRUCTURE/CELL ADHESION	ERMN	7.217178248	
	MPP7	6.867494928	
	CD177	0.223661072	
	SPOCK2	0.163903466	MAPK, PTEN
	DSCAM	0.142282149	
	JAKMIP3	0.124435744	JAK/PI3K
PROTEIN ASSEMBLY/METABOLISM	SH3BGR	5.323643109	
	FBXO2	3.262035023	
	PIGX	2.089391387	
TRANSPORT	SYT2	5.653311758	
	SEC22C	0.499433016	
CELL POLARITY	MPP7	6.867494928	
G-PROTEIN COUPLED RECEPTOR	GPR182	2.392432049	
	GPR112	5.136310919	

expression profile change. By contrast, simply comparing EETs treated vs. not treated cultures without sorting, as one would do in the standard approach, would have detected a differential expression that however would have simply reflected the distinct proportions of subpopulations in the stem-cell state. But with our combination of FACS sorting and transcriptome profiling of subpopulations, we can conclude that the EETs effect is likely to be more than blocking the switch from an ALDH-high state to an ALDH-low differentiated state, but that it directly induced a phenotype in the already ALDH-high stem-like cells that is even more immature and malignant. This is compatible with the observed increased survival of the AL cells and may suggest that EETs even could stimulate cells to go back to the CSC state but whether these changes suffice will have to be determined in functional experiments in the future.

Chapter Five: **Discussion and Concluding Remarks**

5.1 Discussion

The high ratio ω-6 vs. ω-3 poly-unsaturated fatty acids, as is characteristic in today's Western diets, promotes many diseases, including cancer. In the past years, the ω-6 poly-unsaturated fatty acid, arachidonic acid (AA) and its metabolites have stimulated great interest in cancer biology because of putative links between ω-6 PUFA and cancer, notably, breast cancer. Our laboratory has recently shown in animal models that epoxyeicosatrienoic acids (EETs) promote tumor growth and trigger extensive metastasis in several tumors (Panigrahy et al., 2012). The goal of this project was to examine the role of EETs in modulating breast cancer cell plasticity which in the formal framework of gene regulatory dynamics and attractor states would be the manifestation of discrete attractor state transitions between a cancerous state and a differentiated state.

In doing so, we have to take into consideration the phenomenon of non-genetic heterogeneity of (clonal) cell populations (S. Huang, 2009b). One manifestation of this phenomenon is that in a clonal population of cancer cells, there are subpopulations of phenotypically (and hence functionally) distinct types of cells. The recent notion of "cancer stem cells", including in breast cancer, is in line with non-genetic heterogeneity: even within a clonal cell population of a cancer cell line, there is a dynamic, functional hierarchy of cell phenotypes of varying maturity. Such studies of subpopulations critically depend on the cellular abundance of a specific marker in an entire population at the single cell resolution (Brock et al., 2009; A. C. Huang et al., 2009).

5.1.1 Summary of main findings

Approach and objective: Here, we used an *in vitro* breast cancer cell differentiation system, MDA-MB-231 cell line that exhibited two distinct, quasi-discrete subpopulations (bimodal distribution with respect to the stemness marker ALDH1 in flow cytometry) representing a stem-like and differentiated states in response to vitamin D treatment (Figure 4.1).

This bi-modality in the VTD-induced differentiation of breast cancer cells, with the recent wave of work on breast cancer stem cells (Al-Hajj & Clarke, 2004; Cobaleda et al., 2008), revealing the switch-like behaviors between phenotypes of distinct maturity, support both the concept of non-genetic, dynamical heterogeneity of cell populations and the attractor hypothesis (S. Huang & Ingber, 2007).

The AL subpopulation could be increased in the presence of vitamin D – a known inducer of cellular differentiation in some tissues. Using this well-established VTD-induced differentiation system of MDA-MB-231 cells, we investigated the role of epoxyeicosanoid acids (EETs) in interfering with the differential responsiveness of these breast cancer cells. Our findings support the hypothesis that high levels of EET suppress the vitamin D induced shift of the MDA-MB231 cells towards the differentiated state.

Interpretation of results: How can we interpret the observed shift of the two sub-populations caused by EETs? (as seen in Figure 4.4) In one extreme interpretation, EETs could kill cells in the AL subgroup (since differentiated cells are in general more fragile and susceptible to chemical challenge). Alternatively, the other extreme is that EETs may actively promote survival and proliferation of the AH cells. Another possibility could be that EETs affect the transition and/or the relative net growth between the two fractions of cells. In the perspective

of the attractor landscape, one could envision the picture that EETs promotes malignancy by "trapping" the stem-like cancer cells in the cancer attractor state, thereby preventing them from exiting it to reach the differentiated state.

Using pharmacological manipulation we achieved the increase in the endogenous EET levels by two ways: first, by stabilizing the levels of EET through inhibiting the function of the soluble expoxide hydrolase that converts EETs to DHETs. Second, by administrating two different regioisomers of synthetic EETs, 11,12-EET and 14,15-EET. The elevated EET levels led to a decrease in the ALDH low subpopulation in the VTD-induced differentiated MDA-MB-231 cells (Figure 4.3, 4.4). Our data also demonstrated that the two regioisomers of EETs inhibited the differentiation process in a dose dependent manner (Figure 4.4).

In order to begin to dissect the various possibilities underlying the apparent shift in the relative proportions of the two subpopulations, we measured cell death in the two subpopulations following when EETs was increased. Thus, flow cytometry analysis of 7-AAD staining was conducted to detect and quantify apoptotic cells. Four days after VTD differentiation was induced, three different subpopulations appeared with respect to ALDH activity and 7-AAD staining. The majority of cells were either in the stem cell like or apoptotic subpopulations, while a small number of cells were in the differentiated but viable subpopulation.

While EETs shifted the ratio of AL/AH towards AH, that is, reduced the relative proportion of the differentiated cells in favor of the stem cell-like subpopulation, importantly, the number of dead cells decreased. Thus, EETs did not simply cause more cell death in the (more fragile) differentiated cells. This result points to an active influence of EETs on the stem cell-like population. But does it act on the switch AH→AL (the differentiation of the stem cell-like cells) or simply by boosting proliferation of the latter?

To determine whether EETs indeed exert an effect on the intrinsic state of the AH cells, we next performed gene expression profiling. The "null model" would be that if EETs affect only the signalling machinery that governs the state transition AH→ AL, then the effect of EETs on the cell population would be manifested only in the numerical differences of AH and AL cells but would not change the gene expression profiles of the two subpopulations which characterize the two phenotypes. Note that such reasoning is novel because population heterogeneity is explicitly taken into account as opposed to the traditional view, which would compare the entire population treated with a drug control. We will come back to this profound distinction later.

The significantly distinct gene transcription profiles between the two subpopulations AL and AH at day 3 of VTD-induced differentiation of the breast cancer cell line could indicate that, first, in the high-dimensional space these two subpopulations occupy distinct positions (=states) with characteristic distances between them and second, that the phenotypical multi-stability is the result of a GRN that governs the gene expression profile state change.

To determine whether the apparent differentiation arrest caused by EETs is indeed only because cells are kinetically prevented from their transition into the more mature, differentiated state and whether this required a change of the intrinsic state of the stem cell state we next analyzed gene expression profiles of cells exposed to EETs to determine whether EETs affected state transition rates via a shift of the attractor landscape. Since the differential (bimodal) response of MDA-MB-231 to vitamin D reflects population heterogeneity, we performed a sub-population specific analysis to circumvent the problem of averaging over the entire bimodal populations.

GEDI maps and hierarchical clustering analysis of transcriptomes robustly identified the stem cell-like subpopulation and the differentiated sub-population as two discrete clusters,

regardless of the presence of EETs (Figure 4.6.B, C). The global pattern show remarkable variance between the differentiated and non-differentiated fractions of the clonal populations of MDA-MB-231 cells. The distinct gene expression profiles of these two subpopulations is in line with the bimodality with respect to ALDH activity and indicate a quasi-discontinuous relationship in the high-dimensional gene expression space, consistent with the two attractor states represented by the ALDH marker values 'high' and 'low'.

From the known biological function (Gene Ontology and Gene Set Enrichment Analysis) of the genes differentially expressed, we made several important conclusions. First, we found that the stem cell-like subpopulation (ALDH high) is highly enriched with cancer stem cell properties compared with the differentiated subpopulation of the same population. Second, in the presence of EETs, the distinct gene expression profiles of the cells reflect the differential expression of a subset of genes and activation of distinct signalling pathways, and some of these are specific for breast cancer malignant cells.

Third, and of particular note is that the cells that remained in the stem cell-like subpopulation as well as the differentiated cells (in response to vitamin D) exhibited marked change of gene expression when they were treated with EETs. In the non-differentiated ALDH-high cells, EETs induced genes that promote embryogenesis, cell cycle, anti-apoptosis, DNA damage and stress response, aggressiveness, drug metabolism, inhibition of cell-adhesion, transport, proteolysis and metastasis, while it suppressed apoptosis and differentiation related genes. Although global transcription changes were more pronounced in the AH cells, when we focused on the set of the most affected genes, we found that in ALDH low cells, EETs triggered similar biological programs induced in the ALDH high cells, however, targeting different set of genes. In the differentiated cells, similar to the stem cell-like cells EETs promote the expression

of genes involved in embryogenesis, cell cycle, anti-apoptosis, DNA damage and stress response, aggressiveness, inhibition of cell-adhesion, transport, proteolysis and metastasis in addition to angiogenesis while it suppressed apoptosis related genes and to a higher extent than the ALDH high cells differentiation genes.

In conclusion, this substantial shift in gene expression space towards a more immature and malignant cellular program answers our question (see Chapter 2) on the type of mechanisms through which EETs reduce the relative fraction of the vitamin D induced subpopulation of differentiated cells: EETs not only blocked the switch between the two attractors but also affected cell-intrinsic attractor properties. The two may well be linked: by shifting the attractor position, which reflects a change of the topography of the epigenetic landscape, the rate of the state transition into the attractor of the differentiated state is reduced. The alteration of cellular programs are well pronounced in the cancer stem cell-like subpopulation as well as in the ALDH low cells that underwent a valuable shift in transcriptome.

Thus, more concretely with respect to our question, this finding suggest that EETs suppress the differentiation of breast cancer cells by (i) directly inducing the genes that contribute to the immature and malignant phenotype in the already ALDH-high stem-like cells, thus stabilizing the cancer stem cell compartment and (ii) by stimulating genes in the differentiated cells that may contribute to their viability and propensity to switch back to the CSC state.

A small fraction of the differentiated subpopulatin was observed to return to the ALDH1 high state within 48 hours upon vitmain D removal (previous work done in our lab)**.** Whether the observed changes caused by high levels of EETs suffice to accelerate the reversible cell state switch of the differentiated subpopulation to the CSC state will have to be determined in

functional experiments in the future. These will also have to establish the clinical significance of reversal to the stem cell state which is typically more drug resistant. Such reversal has been reported for melanoma (Sharma et al., 2010).

5.1.2 Relevance of our findings in regard to current knowledge

What is the mechanism of action of EETs at the molecular level? No EET receptor has been conclusively identified for EETs, however, there is evidence that 11, 12-EET activates a mechanism involving a G-protein coupled receptor -(Fukao et al., 2001; P.-L. Li & Campbell, 1997). If one takes the stimulation of expression of receptors by their own ligands – an occasionally observed positive-feedback- as an indication then it is interesting to note that our microarray analysis revealed the increased expression of two G-protein coupled receptors, GPCR112 and GPCR182 in the EETs-treated differentiated cells (Table 4.3). This result may be a starting point for future investigations on the unknown EETs mechanisms.

Some of the pivotal pathways that have been shown to regulate normal stem cells have been reported to be involved in tumorgenesis (D'Angelo & Wicha, 2010). Our molecular analysis reveals that epoxyeicosanoid acids may be involved in stabilizing the stemness of the breast cancer cells, thus inhibiting their differentiation through several cascades. The presence of EETs seems to activate WNT, Hedghog, Notch, mTOR, EGFR and some other signaling pathways in both sub-populations, ALDH high and ALDH low.

Our findings are consistent with many studies done in the field. Overexpression of several Wnt family members was identified in different cell lines and cancers (Luo et al., 2010). Our data show that high levels of EETs differentially increased the transcripts of genes associated with this pathway. In our stem cell-like subpopulation, EETs seem to activate the Wnt signaling pathway. Interestingly, SOX1 is down-regulated only in the presence of EETs. In fact,

SOX1, functions as a tumor suppressor by interfering with Wnt/β-catenin signalling in the development of hepatocellular carcinoma (HCC) (Shih et al., 2013). Combined epigenetic silencing of SOX1 and SFRPs- a ligand binding competitive inhibitor for the Frizzled receptor in the Wnt pathway- through promoter hyper-methylation has been observed in these cells contributing to abnormal activation of canonical Wnt signal pathway (Shih et al., 2013). In the differentiated population, EETs may have a part in both suppression and activation of the Wnt pathway. For example, our data show that the BICC1 gene was up-regulated following EETs treatment despite stimulation by vitamin D. BICC1 has been reported to block Wnt signalling. However, the cellular function of BICC1 gene in mammalian systems is unknown. In one study, the lack of BICC1 led to E-cadherin suppression and disruption of normal cell-cell junctions with abnormalities in cell proliferation, and apoptosis (Fu et al., 2010). This contradictory aspect of Wnt cascade has been reported but within other tumors. Activated Wnt/ß-catenin signalling in melanoma in both primary tumors and metastases correlate with decreased proliferation and improved survival in patient tumors and *in vivo* melanoma model in contrast to colorectal cancer (Chien et al., 2009). Thus, the paradoxical effect of EETs in targeting the Wnt signalling pathway needs further investigation.

Another pathway we also found to be activated in the presence of EETs in breast cancer cells is that of EGFR. Indeed, CYP epoxygenase-derived EETs have been reported to significantly enhance phosphorylation of EGFR and activate downstream signalling cascades, including MAPK and PI3K/Akt pathways (Y. Wang et al., 2005), which subsequently releases the pro-angiogenic factor HB-EGF in cancer cell lines leading to increased cellular invasion (Cheng et al., 2010). This suggests that within our *in vitro* model, EETs may act in a similar way and induce invasion via the EGRF signalling pathway. Furthermore, our results revealed the

overexpression of PDGFA that is specific to the ALDH low subpopulation. PDGFA is a growth factor involved in positively regulating the mesenchymal cell proliferation and also plays an important role in wound healing and angiogenesis. Apart from being a marker of aggressive high bulk breast cancer, PDGF has been reported to be involved in the acceleration of growth of some metastatic breast tumors (Ariad, Seymour, & Bezwoda, 1991).

Tumor cells treated with exogenous EETs showed an activation of MMP. Metalloproteinases (MMP) are associated with enhanced tumor progression angiogenesis and metastasis (Cheng et al., 2010). As mentioned in the introduction, metalloproteinases lead to EET-induced activation of EGFR, which subsequently releases the pro-angiogenic factor HB-EGF in cancer cell lines (Cheng et al., 2010). Notably, MMP-1 is up-regulated by EETs treatment in the differentiated population. It has been shown that elevated expression of MMP-1 can promote the local growth and the formation of brain metastases by breast cancer cells. In addition, other genes associated with metastasis and tumor progression have been dramatically up-regulated in both sub-populations stem cell-like and differentiated support the idea that high levels of EET promote metastasis and tumorgenesis in breast cancer cells.

The TGF-beta signalling pathway was also found to be activated (in the ALDH low cells) in the presence of high EET levels. This pathway is known to play a role in human embryonic stem cells as well as tumorigenesis (Shipitsin et al., 2007). The cytokine transforming growth factor beta (TGF-beta) is a potent stimulator of EMT and its role in cancer is highly complex. TGF-beta expression is positively correlated with breast and other types of cancer, as well as invasive lymph node metastases in breast cancer (Dhasarathy et al., 2011). The EMT gene signature has shown to be specifically enriched within the Basal B subgroup, consistent with their over-expression of various EMT transcriptional drivers (Mani et al., 2008). Our findings

could add to the emerging notion of a link between EMT and BCSC. Some of the genes that have been identified as being overexpressed in Basal B subclass are indeed up-regulated in the presence of EETs, segregating the ALDH high and ALDH low subpopulations with respect to their molecular profile.

Of interest, EETs dramatically induced the expression of Twist1 in the differentiated cells. This is one example of a gene that allows us to entertain the idea that EETs actually facilitate the reversed conversion of the differentiated cells but to the more immature, stem-cell like state. Twist plays a direct role in the EMT mechanism by down-regulating E-cadherin expression and promoting invasive and metastatic phenotype (Vesuna, Van Diest, Chen, & Raman, 2008).

The stem cell-like population is highly enriched for the expression of a different gene associated with EMT transition, ALX1, in the presence of EETs. ALX1 up-regulates expression of the key EMT regulator Snail (SNAI1) that mediates EMT activation and cell invasion. In addition, enforced expression of ALX1 in ovarian cancer cells or non-tumorigenic epithelial cells has been shown to induce EMT (Yuan et al., 2013). Breast cancer stem cells that have passed through an EMT are likely to play a key role during the process of tumor metastasis (Mani et al., 2008). Thus, the finding that EETs promote the activation of certain genes associated with this embryonic process is in line with the notion that EETs promote malignancy and the idea that of a tight relationship of EMT and breast cancer stem cells.

The sensitivity of selected tumor cells to rapamycin has gained great interest over its potential as an anti-cancer therapeutic. Recent findings identified the insensitive function of rapamycin in the mTOR cascade in regulating a cell-survival pathway that is overactive in many cancers (Corradetti, Inoki, & Guan, 2005). DDIT4l – a stress-induced protein- is highly enriched

in the stem cell-like population when endogenous EET is elevated (Table 4.2). This gene was found to potently inhibit signalling through mTOR. DDIT4l works downstream of AKT and upstream of TSC2 to inhibit mTOR functions (Corradetti et al., 2005). Another gene that is overexpressed in the presence of EETs in the ALDH high population and is involved in the mTOR pathway is eIF4E. The eIF4E complex induces oncogenic transformation, however, its exact mechanism is strongly debated; it is believed to be mediated in part by the induction of genes involved in critical cellular processes implicated in oncogenesis, such as angiogenesis and metastasis, cell proliferation and prevention of apoptosis (Hsieh & Ruggero, 2010). eIF4E overexpression is common in multiple cancer types including malignancies of the breast. Increased expression of eIF4E is associated with increasing grade of disease. Furthermore, elevated total eIF4E levels correlates with decreased progression-free survival as well as overall survival in breast cancer (Guertin & Sabatini, 2005; Hsieh & Ruggero, 2010).

In summary, the effect of EETs on the differentiated vs. stem-like states balance (ALDH-low vs. high) seems to go beyond just modulating the state transitions and to involve the alteration of the expression of a distinct sets of genes in the stem cell-like and differentiated states.

Limitations: Overall, we have explicitly discussed only a tiny fraction of the genes whose expression pattern varied among these samples. Attention to the thousands of individual genes that define the molecular portraits of each condition, and learning to interpret their patterns of variation, will undoubtedly lead to a deeper and more complete understanding of breast cancers. However, the validity of expression changes must first be independently validated before further studies of functional relevance are undertaken. We would like to note that we performed the microarray experiments chiefly to address the main question regarding the broad class of mode

of EETs. The microarray analysis thus is part of a novel scheme of analysis that compares transcriptomes as a whole and between sorted sub-populations. This analysis was designed for distinguishing between (i). global changes of gene expression profile (as one would have measured without sorting into subpopulations) caused by shifts in the cellular composition of the population (here, the ratio between ALDH high and low) versus (ii). the cell intrinsic change in gene expression program. We found that the latter plays a substantial role when we considered the nature of the genes. Thus the identification of specific genes, as discussed above, was the secondary goal of this experiment and no replicates were included in the transcriptome measurements due to cost limitations. Thus, we limited our discussion of the biology of the genes whose expression was affected by EETs to those that exhibited a change in expression above two fold.

The biologically consistent results, however, give credence to the validity of the observed gene expression changes and encourage further investigations, including real-time PCR for confirmation of the transcript changes.

5.2 Concluding remarks:

Our findings indicate a role for EETs in breast cancer tumorigenesis, offering a step forward in understanding the molecular mechanisms through which EETs and its ω-6 PUFA precursors in the diet, may suppress vitamin D induced differentiation. Importantly, gene signatures associated with elevated EET levels may have clinical relevance, and signalling pathways specifically activated in breast cancer cells could be used for their therapeutic targeting. Our results also demonstrate how a formal framework that takes into account non-genetic dynamics of state transitions and non-genetic population heterogeneity can fruitfully motivate particular experimental design.

Remarkably, EETs are present in our diet. Preventing breast cancer induced differentiation *in vitro* by EETs underlines the possible role of omega-6 high diet in affecting breast cancer progression and survival. Indeed, some studies in breast cancer reported that overexpression of CYP isozymes was associated with decreased breast cancer survival (Mitra et al., 2011). As epidemiological evidence suggests, the augmented use of modern vegetable oils has increased western omega-6 PUFA consumption at the expense of omega-3 PUFAs, which may have resulted in an increase of the risk in developing a clinically aggressive cancers, including breast cancer (Brown et al., 2010; Massiera et al., 2010). Our findings could stimulate future epidemiological studies more specifically addressing the role of omage-6 PUFAs in breast cancer, and more intriguingly, to investigate whether omega-3 PUFA, already suspected to have beneficial effects in lowering cancer risk (Brown et al., 2010) might counteract the role of EETs in the breast cancer.

Anti-EETs agents may have therapeutic or preventive utility against breast cancer. However, much remains to be studied about the efficacy, concentration, and mechanism of EET-inhibitors as an anti-cancer agent *in vivo*. Further studies with different breast cancer cell lines, and more importantly clinical trials, are necessary to validate our findings and to determine if targeting EETs in tumor cells will have an impact on the development of approaches to better differentiation therapy and clinical management of breast cancer patients.

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APPENDIX A: APPENDIX

Beth Israel Deaconess Medical Center

A teaching hospital of Harvard Medical School

April 23, 2014

Re: Zineb El kadiri

To Whom It May Concern:

I hereby give permission to Zineb El kadiri for use of our figure from our publication Panigrahy et al. "Cytochrome P450-derived eicosanoids: the neglected pathway in cancer." *Cancer metastasis reviews* (2010) for her thesis. Please contact me if you have any further questions.

Sincerely,

Dipak Panigrahy, MD