

Regulation of Phenotypic Plasticity in Triple-Negative Breast Cancer

by

Nicholas Christopher D'Amato

Department of Pharmacology and Cancer Biology
Duke University

Date: _____

Approved:

Victoria Seewaldt, Supervisor

Mark Dewhirst

Mariano Garcia-Blanco

Jeffrey Rathmell

Xiao-Fan Wang

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
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ABSTRACT

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Abstract

Breast cancers with a basal-like gene signature are primarily triple-negative, are frequently metastatic, and carry the worst prognosis. Basal-like breast cancers are also frequently enriched for markers of breast cancer stem cells as well as markers of epithelial-mesenchymal transition (EMT). While EMT is generally thought to be important in the process of metastasis, direct *in vivo* evidence of EMT in human disease remains rare. Here we report a novel model of human triple-negative breast cancer, the DKAT cell line, which was isolated from an aggressive, treatment-resistant triple-negative breast cancer that demonstrated morphological and biochemical evidence of epithelial-mesenchymal plasticity in the patient.

In culture, the DKAT cell line exhibits a basal epithelial phenotype under normal culture conditions in serum-free MEGM, and can undergo a reversible EMT in response to serum-containing media, a unique property among the breast cancer cell lines we tested. This EMT is marked by increased expression of the transcription factor Zeb1, and Zeb1 is required for the enhanced migratory ability of DKAT cells in the mesenchymal state. Additionally, we find that expression of the cytokine IL-6 is dramatically increased in mesenchymal DKAT cells, and blocking IL-6 signaling reduces expression of Zeb1 in DKAT and MDA-MB-231 cells. We further show that

DKAT cells express progenitor-cell markers, and single cells are able to generate tumorspheres containing both epithelial and mesenchymal cell types. *In vivo*, as few as ten DKAT cells are capable of forming xenograft tumors which display a range of epithelial and mesenchymal phenotypes. Finally, we also present evidence that epithelial-mesenchymal plasticity may be an early feature of some breast cancers, as we find vimentin-positive mammary epithelial cell clusters in asymptomatic women at high risk for breast cancer. Taken together, our results provide evidence that the aggressive behavior of a subset of triple-negative breast cancers is driven by inherent epithelial-mesenchymal plasticity, and that this plasticity may be a characteristic of some breast cancers from their earliest stages.

The DKAT cell line represents an important new model for further study of the molecular mechanisms that regulate epithelial-mesenchymal plasticity. The novel finding that IL-6 regulates Zeb1 expression adds further support for the development of anti-IL-6 therapeutics, which will have the potential to target pathways at the intersection of metastasis and tumor recurrence. Increased understanding of the pathways that are critical for this plasticity will lead to improved diagnosis and treatment options for patients with highly aggressive and deadly breast cancers.

Contents

Abstract	iv
List of Tables	xi
List of Figures.....	xii
List of Abbreviations.....	xiv
Acknowledgements.....	xxi
1 Introduction	1
1.1 Breast Cancer and Metastasis	1
1.2 Breast Cancer Subtypes	3
1.2.1 Basal-Like Breast Cancer	6
1.2.1.1 Characteristics of Basal-Like Breast Cancers.....	7
1.3 Epithelial-Mesenchymal Plasticity.....	9
1.3.1 EMT/MET in Development and Tissue Homeostasis	11
1.3.2 EMT/MET in Cancer	13
1.3.2.1 Evidence of EMT in Mouse Models of Carcinoma.....	14
1.3.2.2 Evidence of EMT in Human Cancers	16
1.3.3 Transcription Factors Regulate EMT.....	18
1.3.4 Zeb1 in EMT	19
1.3.4.1 Zeb1 Structure and Function	19
1.3.4.2 Regulation of Zeb1 Activity.....	23
1.3.4.3 Regulation of Zeb1 Expression	24

1.3.4.4	Zeb1 in Development	27
1.3.4.5	Zeb1 in Cancer.....	28
1.4	Mammary Stem Cells and Breast Cancer Stem Cells.....	30
1.4.1	Stem Cells in the Normal Mammary Gland.....	31
1.4.2	Breast Cancer Stem Cells.....	33
1.5	Linking EMT and Breast Cancer Stem Cells.....	34
1.5.1	EMT Generates Cells with Properties of Cancer Stem Cells.....	35
1.5.2	EMT and Breast Cancer Stem Cell Phenotypes are Related to the Basal-Like Subtype	38
1.6	Summary and Hypothesis	41
2	Materials and Methods.....	44
2.1	Cell Culture	44
2.1.1	Establishment of the DKAT cell line.....	44
2.1.2	<i>In Vitro</i> Plasticity.....	44
2.1.3	Generation of Clonal DKAT Cell Lines.....	45
2.1.4	Zeb1-Expressing DKAT Cells.....	45
2.1.5	Zeb1 shRNA.....	46
2.1.6	Other Cell Lines.....	46
2.2	Cytogenetic Analysis	47
2.3	Immunohistochemistry	47
2.4	TP53 and PIK3CA Sequencing	47

2.5 Immunofluorescence.....	48
2.6 Immunoblots.....	49
2.7 Flow Cytometry.....	50
2.8 Differential Gene Expression Studies.....	50
2.9 Invasion Assays.....	51
2.10 Migration Assays.....	52
2.11 Xenograft Experiments.....	52
2.12 Tumorsphere Formation.....	53
2.13 Conditioned Media Experiments.....	54
2.14 Neutralizing Antibody Experiments.....	54
2.15 RPFNA Cytology.....	55
3 Characterization of a Novel Model of Triple-Negative Breast Cancer.....	57
3.1 Introduction.....	57
3.2 Results.....	60
3.2.1 Phenotypic Plasticity in a Human Triple-Negative Breast Cancer.....	60
3.2.2 Establishment of DKAT Cell Line <i>In Vitro</i>	64
3.2.3 DKAT Cell Line Maintains a Basal-like Phenotype <i>In Vitro</i>	66
3.2.4 DKAT Cells are Highly Invasive.....	69
3.2.5 DKAT Cells Cluster with Other Basal-Like Breast Cancer Cell Lines.....	70
3.2.6 The DKAT Cell Line Contains a Population of Tumor Initiating Cells.....	71

3.2.7 A Single DKAT Cell is Capable of Generating Tumorspheres Containing Both Luminal and Basal Epithelial Cells.....	72
3.2.8 Both CD44 ⁺ /CD24 ^{-/low} and CD44 ⁺ /CD24 ^{hi} DKAT Cells Can Give Rise to Heterogenous Progeny	74
3.2.9 Both CD44 ⁺ /CD24 ^{-/low} and CD44 ⁺ /CD24 ⁺ DKAT Cells Form Tumors at a Dose of Ten Cells.....	76
3.3 Discussion.....	78
4 Phenotypic Plasticity in Triple-Negative Breast Cancer is Regulated by Zeb1.....	83
4.1 Introduction	83
4.2 Results.....	86
4.2.1 DKAT Cells Demonstrate <i>In Vitro</i> Plasticity.....	86
4.2.2 A Single DKAT Cell is Capable of Generating Tumorspheres Containing both Epithelial and Mesenchymal Cells.....	92
4.2.3 SCGM-Induced EMT is Due to Plasticity, Not Selection.....	93
4.2.4 DKAT Cell Line Plasticity is Unique Among Breast Cancer Cell Lines.....	96
4.2.5 EMT Decreases DKAT CD44 ⁺ /CD24 ^{-/low} Population.....	97
4.2.6 DKAT Cells are Highly Tumorigenic and Exhibit <i>In Vivo</i> Plasticity	99
4.2.7 Zeb1 Regulates DKAT Cell Plasticity	102
4.2.8 Autocrine IL-6 Signaling Maintains Zeb1 Expression	104
4.2.9 Atypia From Asymptomatic High Risk Women Contains Vimentin ⁺ Epithelial Cells.....	108
4.3 Discussion.....	110
5 Conclusions and Perspectives	116

5.1 Inherent Plasticity of Triple-Negative Breast Cancer Cells.....	116
5.2 EMT Regulation of Breast Cancer Stem Cells	119
5.3 Zeb1 in Breast Cancer	122
5.4 IL-6 Regulation of EMT in Breast Cancer Cells.....	124
5.5 EMT as an Early Event in Breast Cancer Development.....	128
5.6 Summary and Concluding Remarks	132
References	135
Biography	154

List of Tables

Table 3.1: Characterization of human primary tumor, chest wall recurrence, and bone marrow metastasis.	63
Table 4.1: Differential gene expression of DKAT cells following SCGM-induced EMT.	91

List of Figures

Figure 1.1: Epithelial-mesenchymal plasticity.....	11
Figure 1.2: Zeb1 Protein Structure.....	20
Figure 1.3: CTCs may represent cells with an intermediate EMT or stem cell phenotype.	37
Figure 3.1: Human breast cancer specimen displays morphological and biochemical evidence of EMT/MET.	62
Figure 3.2: DKAT cell line karyotype.	65
Figure 3.3: DKAT cells express markers of basal epithelial cells.....	67
Figure 3.4: DKAT cells are highly invasive and migratory.	69
Figure 3.5: The DKAT cell line clusters with other basal-like breast cancer cell lines.	71
Figure 3.6: CD44 and CD24 staining of breast cancer cell lines.....	72
Figure 3.7 : DKAT cells form mammospheres containing both luminal and basal epithelial cells.....	73
Figure 3.8: De-differentiation of CD44 ⁺ /CD24 ⁺ DKAT cells.	75
Figure 3.9: As few as ten CD44 ⁺ /CD24 ^{-low} or CD44 ⁺ /CD24 ⁺ DKAT cells form tumors.	77
Figure 4.1: DKAT cells undergo morphologic and phenotypic changes consistent with <i>in vitro</i> EMT.	88
Figure 4.2: A single DKAT cell can generate tumorspheres with both epithelial and mesenchymal cells.	93
Figure 4.3: Plasticity in culture is unique to DKAT cells.	95
Figure 4.4: EMT decreases the population of CD44 ⁺ /CD24 ^{-low} cells.	98

Figure 4.5: Comparison of H&E and IHC patterns from DKAT xenografts.....	101
Figure 4.6: Zeb1 regulates DKAT EMT/MET.	103
Figure 4.7: IL-6 is highly secreted by DKAT-SCGM cells.....	105
Figure 4.8: Zeb1 expression is regulated by IL-6 signaling.	107
Figure 4.9: Atypia from high-risk women contains vimentin+ cells.	109
Figure 5.1: IL-6 regulation of Zeb1 and EMT.	125

List of Abbreviations

Akt – v-akt murine thymoma viral oncogene homolog

ALDH – aldehyde dehydrogenase

ALDH1A3 – ALDH-1a3

AP-2 – activating protein 2

ATP – adenosine triphosphate

ATP1a1 – ATPase alpha 1

Bad – Bcl-2-associated death promoter

Bcl-2 – B-cell lymphoma 2

BCSC – breast cancer stem cell

bFGF – basic fibroblast growth factor

bHLH – basic helix-loop-helix

Bim – Bcl-2 interacting mediator of cell death

BMP – bone morphogenetic protein

BRCA1 – breast cancer 1, early onset

BRG1 – brahma-related gene 1

CBP – CREB-binding protein

CD – cluster of differentiation

CDH2 – cadherin 2, type 1, N-cadherin

cDNA – complementary DNA

CID – CtBP interaction domain

CK14 – cytokeratin 14

CK14 – cytokeratin 14

CK17 – cytokeratin 17

CK18 – cytokeratin 18

CK5 – cytokeratin 5

CK6 – cytokeratin 6

CK8 – cytokeratin 8

CLDN1 – claudin 1

CNS – central nervous system

CSC – cancer stem cell

CtBP – C-terminal binding protein

CTC – circulating tumor cell

CZF – C-terminal zinc finger domain

DAPI - 4',6-diamidino-2-phenylindole

DCIS – ductal carcinoma in situ

DNA – deoxyribonucleic acid

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

EMT – epithelial-mesenchymal transition

ER – estrogen receptor alpha

ERBB2 – v-erb-b2 erythroblastic leukemia viral oncogene homolog 2

ESA – epithelial specific antigen

EuHMT - Euchromatic histone-lysine N-methyltransferase

FACS - Fluorescence-activated cell sorting

Fas – tumor necrosis factor superfamily, member 6

FBS – fetal bovine serum

FGF – fibroblast growth factor

FGF2 – fibroblast growth factor 2

FGFR2 – fibroblast growth factor receptor 2

FKHRL1 - forkhead transcription factor like 1

FOXC2 – Forkhead box protein C2

GATA3 – GATA binding protein 3

GBM – Glioblastoma

GJB2 – gap junction protein, beta 2

GSC – Glioma stem cell

GSK3 β - Glycogen synthase kinase 3 beta

H&E – hematoxylin and eosin

Her2 – ERBB2

HGF – hepatocyte growth factor

HIF1 α – hypoxia inducible factor 1 alpha

HMEC – human mammary epithelial cell

IGF-IR – insulin-like growth factor-I receptor

IHC – immunohistochemistry

IL-6 – interleukin 6

IL-8 – interleukin 8

KRT14 – keratin 14

KRT17 – keratin 17

KRT5 – keratin 5

KRT6C – keratin 6C

LacZ – lactose operon gene Z

LCM – Laser Capture Microscopy

Lin⁻ – lineage negative

LOH – loss of heterozygosity

LOX – lysyl oxidase

MAPK – Mitogen-activated protein kinase

MCK – muscle creatine kinase

MDM2 – murine double minute 2

MEGM – mammary epithelial growth medium

MET – mesenchymal-epithelial transition

MH2 – Mad homology 2

miRNA – microRNA

mRNA – messenger RNA

MaSC – mammary stem cell

MSC – mesenchymal stem cell

mTOR – mammalian target of rapamycin

mTORCII – mTOR complex II

NC2 – negative cofactor 2

NF- κ B – nuclear factor kappa-light-chain-enhancer of activated B cells

NZF – N-terminal zinc finger domain

p300 – protein 300

p53 – tumor protein p53

PDGF – platelet-derived growth factor

PDK1 – phosphatidylinositol-dependent kinase 1

PH – pleckstrin homology

PI3K – Phosphatidylinositol 3-kinase

PIK3CA – catalytic subunit of PI3K

PIP2 – phosphatidylinositol 4,5 phosphate

PIP3 – phosphatidylinositol 3,4,5 phosphate

PR – progesterone receptor

PTEN – phosphatase and tensin homolog

RNA – ribonucleic acid

RPFNA – random periareolar fine needle aspiration

S6K – S6 kinase

SBD – Smad binding domain

SCA1 – stem cell antigen 1

SCGM – stromal cell growth medium

SKY – spectral karyotyping

SMA – smooth muscle actin

Sp1 – specificity protein 1

SUMO – Small Ubiquitin-like Modifier

TGFA – TGF-alpha

TGFBI – TGF-beta-induced

TGF β – transforming growth factor beta

TIP60 – Tat interactive protein 60

TP63 – tumor protein p63

VDR – vitamin D receptor

XBP1 – X-box binding protein 1

XIAP – X-linked inhibitor of apoptosis protein

Zeb1 – zinc finger E-box-binding homeobox 1

Zeb2 – zinc finger E-box-binding homeobox 2

Zfh1 – zinc finger homeodomain 1

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1 Introduction

1.1 Breast Cancer and Metastasis

Breast cancer is the most common cancer among women worldwide and accounts for more than 1 in 4 cancers in women in the United States [American Cancer Society]. Mortality rates from breast cancer have declined by approximately 2 percent per year since 1990, in part due to better screening and increased use of targeted therapies directed against dysregulated hormone and growth factor signaling pathways. Despite this progress, breast cancer remains a tremendous public health burden and there are still great strides that must be made in understanding and treating this disease. Nearly 40,000 women were expected to die of breast cancer in 2010 in the United States, and another 207,000 women were diagnosed with the disease [American Cancer Society].

It has long been recognized that the vast majority of deaths caused by breast cancer do not occur from the primary tumor, but from complications arising from metastases [DeVita et al 2005]. Metastasis is a complex multi-step process whereby tumor cells invade into the surrounding tissue and eventually enter the blood stream where they must survive until reaching a distant site where a combination of physical and biochemical interactions will allow the cell to exit the blood vessel and

establish a new colony [Fidler 2003, Valastyan and Weinberg 2011]. The final step, establishment of a macrometastasis, is thought to be the rate limiting step [Hanahan and Weinberg 2011]. However, there remains a great deal of controversy over the origin of the metastatic cells within the primary tumor.

Early studies of breast cancer metastases proposed the “seed and soil” hypothesis, which suggests that the establishment of a metastatic lesion requires both a cell capable of establishing a distant lesion (the “seed”), and a permissive tissue in which that cell could survive and multiply (the “soil”) [Paget 1889].

Traditional models of invasion and metastasis hypothesize that metastases result from the outgrowth of a subpopulation of cells with defined morphology and gene expression patterns, and that these cells are unique in their ability to migrate away from the primary tumor and establish a distant metastasis [Fidler and Kripke 1977, Poste and Fidler 1980]. An alternative hypothesis states that the majority of cells in a primary tumor are capable of forming a distant metastasis, and that this process is mainly determined by random survival of cells during the metastatic cascade [Milas et al 1983]. In breast cancer, it has also been hypothesized that the aggressive metastatic behavior of certain types of breast cancers may reflect the cell of origin from which the cancer arose. The implication of this hypothesis is that some tumors

contain cells which are more capable of forming distant metastases than other tumors [Dontu et al 2004].

For a breast cancer patient with distant metastasis at the time of diagnosis, the five year survival rate is only approximately 23 percent [National Cancer Institute 2011]. Answering the fundamental questions about the nature of the metastatic process is critical in order to understand the most effective ways to prevent and target metastatic disease. In recent years it has become clear that important insights into the biology of the metastatic process can be gained by studying the different types of breast cancer which exhibit different propensities to form distant metastases.

1.2 Breast Cancer Subtypes

Breast cancer is a heterogeneous disease that exhibits a wide range of clinical behaviors, prognoses, and histologies. Many breast cancers grow very slowly and are also slow to metastasize, while others grow very rapidly and give rise to many metastases throughout the body in a short period of time. An understanding of the biology which underlies these very different clinical behaviors is essential for the development of new and better screening techniques and therapies.

The observation that some breast cancers are driven by hormone signaling was originally made through the work of Beatson et al. in 1896 which found that removing the ovaries of premenopausal women with breast cancer often resulted in tumor regression [Beatson 1896]. Further investigation has firmly established estrogen signaling as a driver of a subset of breast cancers, and this has led to the development of breast cancer therapies which function by blocking estrogen signaling. This can be accomplished by inhibiting estrogen production in postmenopausal women with aromatase inhibitors, or by inhibiting estrogen receptor (ER) activity in the breast with selective estrogen receptor modulators such as tamoxifen or raloxifene [Obiorah and Jordan 2011]. However, not all breast cancers are dependent on estrogen for stimulating their growth, and breast cancers which are not driven by hormone signaling are typically not suitable for treatment with these therapies.

With the advent of microarray technology, it became possible to compare mRNA expression from large numbers of breast cancer samples in an effort to understand the underlying biology of different tumors. Gene expression profiling initially identified five breast cancer subtypes, each with a distinct molecular signature and prognosis: luminal A, luminal B, Her2-amplified, normal breast-like, and basal-like [Perou et al 2000, Sorlie et al 2001, Sorlie et al 2003].

The Luminal A and B subtypes are characterized by high expression of ER and ER transcriptional targets including GATA3, XBP1, and LIV1 [Sorlie et al 2001, Sorlie et al 2003]. Tumors identified as Luminal A have the best prognosis, while Luminal B tumors have an intermediate prognosis. Because of their high expression of ER α , Luminal A and B breast cancers are commonly treated with anti-estrogen therapies [Sorlie et al 2001].

The Her2-amplified group is characterized by overexpression of the Her2/Neu/ErbB2 growth factor receptor [Sorlie et al 2001]. Overexpression of receptor tyrosine kinase Her2 leads to aberrant constitutive activation of downstream signaling pathways including MAPK and PI3K, which can act in concert to promote increased cell proliferation and to inhibit cell death [Ross et al 2009]. Numerous therapies targeting the Her2 receptor have been developed in the past decade, most notably Herceptin and Lapatinib, leading to improved survival rates for Her2-overexpressing breast cancers. However, acquired resistance to these therapeutics and relapse remains a serious problem among Her2-positive breast cancer patients [Ross et al 2009].

The normal breast-like subtype is characterized by high expression of genes associated with adipose tissue and mammary basal epithelial cells such as Cytokeratins 5 and 6 (CK5/CK6) and low expression of luminal epithelial-specific

genes [Sorlie et al 2001]. The fifth subtype, the basal-like group, is also characterized by high expression of genes associated with basal epithelial cells and low expression of luminal epithelial-specific genes. However, of the five subtypes, basal-like breast cancers have the worst prognosis and represent a significant clinical challenge [Sorlie et al 2001, Sorlie et al 2003].

1.2.1 Basal-Like Breast Cancer

Basal-type breast cancers account for approximately 15 percent of newly diagnosed breast cancers in the United States, and are most common among young African-American women and *BRCA1* (breast cancer 1, early onset) mutation carriers [Millikan et al 2008, Sorlie et al 2001, Sotiriou et al 2003]. In an effort to better identify basal-like tumors without the expensive process of microarray analysis, many sets of immunohistochemical (IHC) markers have been proposed. The most commonly-used IHC marker combinations are ER/PR/Her2 (triple-negative) as well as ER/PR/Her2/EGFR/CK5/6 [Cheang et al 2008]. This five-marker panel has been found to have better predictive power of a poor prognosis and lower disease-free survival compared with ER/PR/Her2 staining alone [Cheang et al 2008]. While considerable disagreement over the precise definition of basal-like, triple-negative, and ER-negative breast cancers continues in the literature [Badve et al 2011,

Gusterson 2009, Rakha et al 2008], these terms are often found to be used interchangeably. For the sake of consistency in this work, the term “basal-like breast cancers” will refer to cancers with a basal-like signature as determined by gene expression profiling based on the initial subtype classifications [Sorlie et al 2001, Sorlie et al 2003], whereas the term “triple-negative” will refer to tumors which stain negatively for ER/PR/Her2 by IHC, and “ER-negative” will refer to tumors lacking expression of ER α as determined by IHC.

1.2.1.1 Characteristics of Basal-Like Breast Cancers

Tumors with a basal-like gene signature are primarily triple-negative, lacking ER and PR expression and HER overexpression [ER-/PR-/Her2-]. However, it is important to note that not all triple-negative breast cancers are basal-like by gene expression profiling, nor are all basal-like breast cancers triple-negative. Although estimates vary, approximately 10 – 20 percent of triple-negative breast cancers are not basal-like by gene expression profiling, and approximately 15 – 45 percent of basal-type breast cancers are reported to express at least one of the three triple-negative markers [Nielsen et al 2004, Rakha et al 2008]. Nevertheless, in the absence of more specific marker sets, the basal-like subtype is typically identified immunohistochemically by lack of ER/PR/Her2 staining, and positive staining for

CK5/6, CK14, and CK17, which are characteristic of the basal epithelial layer of the ducts of the mammary gland, as well as by high expression of the epidermal growth factor receptor (EGFR) [Rakha et al 2008, Sorlie et al 2001].

Clinically, triple-negative breast cancers tend to be highly aggressive and have the worst prognosis. While some triple-negative breast cancers are responsive to chemotherapy, they have a high percentage of recurrence and a high frequency of metastasis to visceral organs [Carey et al 2007, Rouzier et al 2005]. Currently there are no biomarkers to determine whether a woman has a chemotherapy-resistant or chemotherapy-sensitive basal-like breast cancer. Additionally, because they do not express either estrogen or progesterone receptors or overexpress the HER2 receptor, these tumors are not suitable for treatment with targeted therapies against these molecules such as Tamoxifen or Herceptin. Due to the combination of aggressive biology and lack of effective therapies, the five year survival rate for African-American women with basal-like breast cancer is only 15 percent. Thus there is a desperate need to improve our understanding of the underlying biology of basal-like breast cancers in order to identify biomarkers of resistance, and to develop therapies to treat those which do not respond to current treatments.

In an effort to better identify the clinically aggressive triple-negative breast cancers, several additional triple-negative sub-groups have recently been proposed.

These include the claudin-low and metaplastic subtypes of human breast tumors [Hennessy et al 2009, Herschkowitz et al 2007], and the basal B or mesenchymal subgroup identified in breast cancer cell lines [Charafe-Jauffret et al 2006, Neve et al 2006]. The genetic signatures of the claudin-low and metaplastic subtypes suggest clues to the biology that underlies these aggressive tumors, as both groups are enriched for markers of mammary stem or progenitor-like cells as well as markers of epithelial-mesenchymal transition (EMT), a developmental process thought to be co-opted by cancer cells as part of the metastatic cascade [Prat et al 2010, Taube et al 2010].

1.3 Epithelial-Mesenchymal Plasticity

The cells of metazoan organisms can be broadly categorized into two types that differ in both their function and morphology, both of which are necessary for proper body patterning and tissue function. Epithelial cells display apical-basal polarity and comprise cohesive layers via specialized membrane structures including tight junctions, adherens junctions, gap junctions, and desmosomes.

These epithelial layers are separated from surrounding tissue by a layer of extracellular matrix called the basal lamina [Thiery and Sleeman 2006].

Mesenchymal cells, on the other hand, do not have apical-basal polarity, do not form

organized layers, and express different cytoskeletal proteins than epithelial cells [Thiery and Sleeman 2006].

The process by which epithelial cells lose epithelial characteristics and gain mesenchymal characteristics is called Epithelial-Mesenchymal Transition (EMT). EMT involves large-scale phenotypic and molecular changes characterized by loss of intercellular adhesion complexes and apico-basal polarity, and increased migratory capacity (Figure 1.1) [Thiery and Sleeman 2006]. This process can be induced by many different extracellular cues or intracellular signaling mechanisms, and not every EMT is the same. Importantly, EMT can be both partial and transient in some contexts, and both EMT and the reverse process, mesenchymal-epithelial transition (MET), are well described critical processes for embryonic development [Thiery et al 2009]. Because clinical and *in vitro* observations of invasive and metastatic cancer cells are reminiscent of developmental EMT processes, there has been great speculation that aberrant activation of EMT pathways may be important in the metastatic cascade [Micalizzi et al 2010].

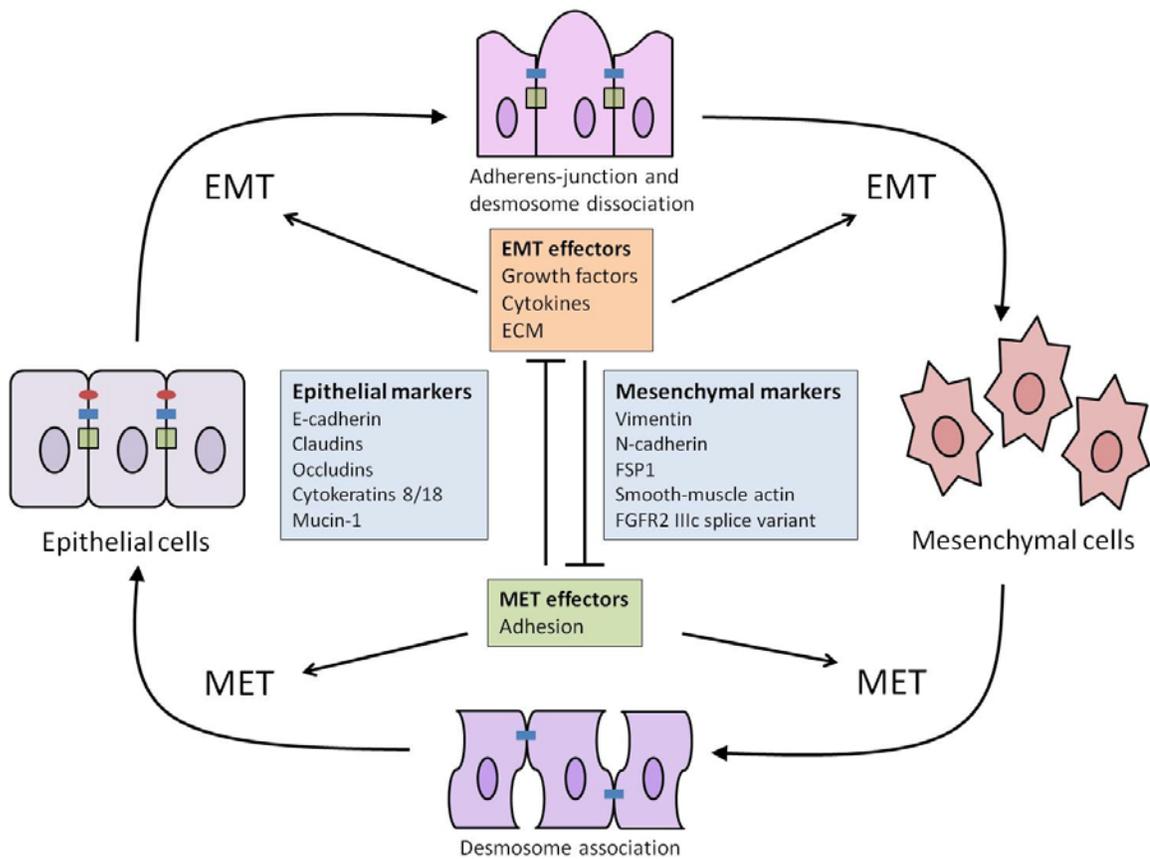


Figure 1.1: Epithelial-mesenchymal plasticity.

Illustration of the changes associated with epithelial-mesenchymal transition, including loss of cell-cell junctions and apical-basal polarity (top), and the reverse process, mesenchymal-epithelial transition (bottom). These changes may be partial and reversible.

1.3.1 EMT/MET in Development and Tissue Homeostasis

EMT was first described in a developmental context in a model of chick primitive streak formation [Hay 1995]. EMT occurs in several stages during embryonic development, and these events are broadly categorized by function into

primary, secondary, and tertiary EMTs. Examples of a primary EMT include gastrulation in metazoans and neural crest formation in vertebrates [Thiery et al 2009, Yang and Weinberg 2008]. Gastrulation is the first instance of EMT during embryonic development, where growth factor gradients induce a small subset of cells at the primitive streak of the single epithelial layer of the embryo to change their cellular architecture, lose their tight junctions and gain focal adhesions, and migrate through the original basement membrane to the interior of the epiblast [Micalizzi et al 2010]. Induction of the transcription factor Snail through Fibroblast Growth Factor (FGF) signaling is thought to be important in the downregulation of E-cadherin in gastrulation [Ciruna and Rossant 2001]. The remaining epithelial cells give rise to the ectoderm, while the ingressing cells eventually give rise to the mesoderm or undergo a subsequent MET in order to give rise to the endoderm [Acloque et al 2009, Micalizzi et al 2010].

Neural crest formation is considered to be the second EMT event during vertebrate embryogenesis. During neurulation, cells of the junction of the ectoderm and neuroectoderm undergo a partial EMT which leads to delamination of these cells which then collectively migrate as a sheet of cells into the embryo. These cells give rise to neurons, pigment cells, facial structures, and cervical structures [Kalluri and Weinberg 2009]. Expression of Snail is again observed in cells undergoing EMT

during neural crest formation, and this is thought to be driven by extracellular growth factor stimulation of pathways including Fibroblast Growth Factor (FGF), Wnt, and Bone Morphogenetic Proteins (BMPs) pathways. Further EMTs throughout development give rise to the heart valves, skeletal muscle, and the palate, among other structures [Kalluri and Weinberg 2009].

In development, the process of MET is best described as it relates to formation of the functional kidney, where specialized group of mesenchymal cells revert to an epithelial phenotype to give rise to the kidney tubules [Davies 1996]. In the adult organism, EMTs are also implicated in processes which involve the production of fibroblast-like cells or which require induction of cell motility. This includes tissue fibrosis in the kidney, liver, lung and intestine, as well as wound healing in organs such as skin [Kalluri and Weinberg 2009].

1.3.2 EMT/MET in Cancer

The majority of human solid tumors are carcinomas, which by definition means that they are thought to arise from an epithelial cell. Early disease progression involves uncontrolled cellular proliferation, but invasion through the basement membrane and spread to distant sites is the main cause of cancer-related mortality [DeVita et al 2005]. Many studies of the metastatic process have found that

activation of various components of the EMT program can give cancer cells increased migratory and metastatic capabilities; however, there has been great debate over the relevance of these *in vitro* findings to human cancer. The main sources of disagreement stem from 1) disagreements over the terminology and precise definition of EMT, and 2) the lack of proof of mesenchymal-like carcinoma cells in human cancers [Tarin et al 2005]. Indeed, an early argument against the importance of EMT centered on the observation that many metastatic lesions appear to have an epithelial morphology and expression pattern [Bukholm et al 2000, Wells et al 2008]. However, a number of recent studies in both mouse and human have provided strong evidence for the importance of epithelial-mesenchymal plasticity in invasion and metastasis.

1.3.2.1 Evidence of EMT in Mouse Models of Carcinoma

Even some of the earliest studies of mouse mammary carcinomas described the appearance of “carcinosarcomas” that included areas of spindle cell morphology, though their existence was largely thought of as an experimental artifact and not studied in great detail [Cardiff 2010]. However, with the more recent advent of a large number of genetically engineered mouse models of breast cancer and a deeper understanding of developmental epithelial plasticity, spindle cell tumors were

observed in numerous models of breast cancer, often associated with loss of p53 function, and were considered as possible evidence of EMT [Cardiff 2010]. Since many of these mouse models utilize epithelial-cell specific expression or knockout, the emergence of tumor regions with spindle cell morphology expressing mesenchymal markers such as snail, vimentin, Smooth Muscle Actin (SMA), and loss of E-cadherin, supports the occurrence of epithelial-mesenchymal transition *in vivo* [Cardiff 2010].

More direct proof was offered recently by a study in which mammary epithelial cells were permanently labeled by the LacZ reporter gene in a myc-induced model of tumorigenesis [Trimboli et al 2008]. In this study, tumor-adjacent stromal fibroblasts expressing mesenchymal markers were found to express LacZ, demonstrating full morphological transition to a mesenchymal state. By using a mesenchymal-specific reporter, this group was also able to identify epithelial cells in the early stages of EMT which co-expressed epithelial and mesenchymal markers [Trimboli et al 2008].

Further evidence of epithelial-mesenchymal plasticity in mouse models of carcinoma came from the work of Oltean et al., who utilized several reporter systems based on mesenchymal- or epithelial-specific expression patterns of FGFR2 splice-variants. These studies convincingly showed that Dunning AT3 rat prostate

cancer cells with a mesenchymal expression pattern, when injected subcutaneously into rats, could give rise to metastatic lesions in the lung in which a subset of cells had adopted an epithelial expression pattern, based on expression of the epithelial FGFR2 splice variant as well as E-cadherin [Oltean et al 2006, Oltean et al 2008].

1.3.2.2 Evidence of EMT in Human Cancers

In human cancers, there is strong evidence correlating expression of EMT markers with metastasis and poor prognosis. E-cadherin is an important component of the adherens junctions of epithelial cells which contribute to their characteristic intercellular interactions and polarity [Liu et al 2005]. In recent years, loss of E-cadherin has come to be seen as a central event in EMT by which tumor cells can lose their intercellular contacts in the process of becoming metastatic. In many cancer types, E-cadherin has been described as a tumor suppressor and metastasis inhibitor [Berx et al 1995, Frixen et al 1991]. In many solid tumor types, including breast cancers, decreased expression of E-cadherin is associated with poor prognosis and decreased survival [Siitonen et al 1996, Yang and Weinberg 2008]. In normal development, E-cadherin is regulated by a large number of extracellular cues such as Transforming Growth Factor β (TGF β), Hepatocyte Growth Factor (HGF), Epidermal Growth Factor (EGF), and Platelet Derived Growth Factor (PDGF). E-

cadherin transcription is directly regulated by a variety of transcription factors including Snail, Slug, Twist, Zeb1, Zeb2, and FOXC2 [Liu et al 2005]. Activation of these pathways or overexpression of these transcription factors *in vitro* is capable of inducing EMT, and many of these same pathways and transcription factors are also associated with invasion and metastasis in human cancers [Blanco et al 2002, Peinado et al 2007, Yang and Weinberg 2008].

By immunohistochemistry, expression of mesenchymal markers is often found at the tumor-stroma interface, suggesting that signaling cues from the tumor microenvironment may be important in inducing EMT [Brabletz et al 2001, Franci et al 2006]. Furthermore, a recent study found that in the majority of men with castration resistant metastatic prostate cancer and women with metastatic breast cancer, circulating tumor cells stained positively for both cytokeratin and vimentin, indicative of a partial or intermediate EMT state [Armstrong et al 2011].

While disagreement continues in the literature over whether the spectrum of changes observed during cancer progression and metastasis constitutes a true epithelial-mesenchymal transition, a significant body of evidence demonstrates that epithelial-mesenchymal plasticity is a feature of many solid tumors, including breast cancers.

1.3.3 Transcription Factors Regulate EMT

In normal epithelial tissue, E-cadherin expression is governed by several activating transcription factors including Sp1 and AP-2 [Batsche et al 1998, Hennig et al 1996, Liu et al 2005]. As previously mentioned, loss of E-cadherin in cancer cells is commonly viewed as a marker of EMT, and is often associated with concomitant upregulation of EMT-associated transcription factors. These transcription factors include zinc finger transcriptional repressors such as Snail, Slug, Zeb1, and Zeb2, and the bHLH family member Twist [Kalluri and Weinberg 2009, Peinado et al 2007]. Each of these transcription factors has been shown to be able to induce features of EMT *in vitro*, in part through binding to the promoter of E-cadherin, as well as other genes important for epithelial cell-cell junctions, and repressing their transcription [Peinado et al 2007].

While members of the Snail family have been extensively characterized in the developmental context and more recently in cancer, Zeb1 has received less attention until recently. However, emerging evidence points to a critical role for Zeb1 in the induction and maintenance of the mesenchymal phenotype in breast cancer cells [Aigner et al 2007, Brabletz and Brabletz 2010].

1.3.4 Zeb1 in EMT

Zinc finger E-box binding protein (Zeb1) is an evolutionarily-conserved zinc-finger transcription factor best characterized as a repressor of the epithelial cell protein E-cadherin. Both *Drosophila* and *C. elegans* contain a single functional ortholog (Zfh1 and Zag-1, respectively), while in vertebrates, the Zeb family contains two members: Zeb1 (Tcf8, deltaEF1, BZP, zfhx1a, zfhep, AREB6) and Zeb2 (SIP1, ZFHX1B) [Clark and Chiu 2003, Fortini et al 1991]. Zeb1 was first discovered and cloned in 1993 and early studies identified it as an E-box binding protein with roles in repression of the delta-crystallin gene during lens cell formation in chicken embryogenesis [Funahashi et al 1993], repression of the mouse Muscle Creatine Kinase gene (MCK) during myogenesis [Sekido et al 1994], and repression of the Immunoglobulin heavy-chain enhancer in non-B cells in humans [Genetta et al 1994]. In recent years, Zeb1 has been implicated in regulation of EMT in numerous cancer types through transcriptional regulation of multiple targets [Peinado et al 2007].

1.3.4.1 Zeb1 Structure and Function

The structure of Zeb1 is characterized by two widely-separated C2H2-type zinc finger domains. The N-terminal zinc finger domain (NZF) contains four zinc

fingers, while the C-terminal zinc finger domain (CZF) contains three. Other domains include a centrally-located homeobox domain, a CtBP interaction domain (CID), a Smad binding domain (SBD), and a glutamic acid-rich region at the C-terminus [Funahashi et al 1993]. There is a high degree of homology between Zeb1 and Zeb2 in their NZF (88%) and CZF (93%) suggesting that they display similar DNA binding preferences [Postigo and Dean 2000]. The homology between these two family members outside the zinc finger clusters is considerably lower. While the most widely recognized role for Zeb1 is transcriptional repression, the mechanism of Zeb1 action on promoters is complicated and can include both repression and activation based on context-dependent binding partners [Postigo 2003].

Similar to other transcription factors implicated in EMT such as Snail1 and Snail2, Zeb1 functions by zinc finger domain binding directly to 5'-CACCT(G)

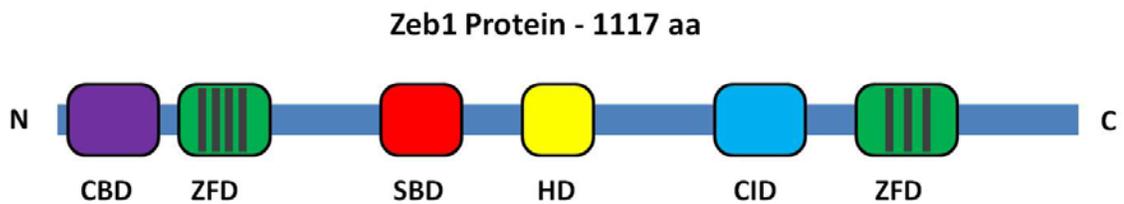


Figure 1.2: Zeb1 Protein Structure.

Representation of Zeb1 protein functional domains. Zeb1 interacts with DNA through the ZFDs, while other domains are important for coactivator and corepressor interactions. CBD: coactivator binding domain; ZFD: zinc finger domain; SBD: Smad binding domain; HD: homeodomain; CID: CtBP interaction domain.

sequences, commonly referred to as E-boxes [Genetta et al 1994, Sekido et al 1994]. Once bound to the DNA, Zeb1 can recruit a large number of cofactors including corepressors such as Carboxyl-terminal binding protein (CtBP-1) which can bind to three PLDLS and PLSDLS-like CtBP binding sequences within Zeb1 [Postigo and Dean 1999]. All three of these CtBP binding sites are reported to be necessary for efficient repression of E-cadherin expression, though another group found that the CtBP-1 interaction was dispensible for Zeb1 repression of E-cadherin in a controversial model of Zeb1 overexpression [Postigo and Dean 1999, van Grunsven et al 2003]. Once bound to Zeb1, CtBP can further recruit other proteins including histone deacetylases, histone methyltransferases, and chromatin remodeling complexes that combine the necessary factors for altering the chromatin environment to support transcriptional repression [Shi et al 2003].

Knockdown of CtBP-1, or the CtBP-interacting histone methyltransferase proteins G9a or EuHMT, resulted in increased activity of an E-cadherin promoter construct in the E-cadherin-low U2OS osteosarcoma cell line, suggesting the importance of this complex in repression of E-cadherin transcription. Other corepressors reported to interact with Zeb1 include the Tat-interacting protein TIP60 and the ubiquitous negative cofactor NC2 [Hlubek et al 2001, Ikeda et al 1998].

In other contexts, Zeb1 has been reported to act, at least in part, by displacement of transcriptional activators of the basic helix-loop-helix (bHLH) family or by competition for co-activators such as CBP/p300 [Genetta et al 1994, Jethanandani and Kramer 2005]. Interestingly, it was observed in a panel of cell lines that mutation of the Zeb1 CID resulted in only a partial relief of its E-cadherin repressor activity, suggesting CtBP-independent mechanisms of repression [Sanchez-Tillo et al 2010]. This study found that N-terminal region of Zeb1 interacted with BRG1, an ATPase subunit of the SWI/SNF chromatin remodeling complex, and that knockdown of BRG1 in the presence of the CID-mutant Zeb1 completely abolished its repressor activity. These findings suggest that BRG1 and CtBP are the two main interacting proteins required for Zeb1-mediated repression of E-cadherin [Sanchez-Tillo et al 2010].

While most studies of Zeb1 focus on its role as a transcriptional repressor, it has also been reported to act as a transcriptional activator. Early reports implicated Zeb1 in transcription of the chicken ovalbumin gene in response to estrogen treatment [Chamberlain and Sanders 1999], while another group found that a Zeb1 variant, AREB6, was able to activate transcription of rat Na, K-ATPase $\alpha 1$ subunit gene (Atplal) [Watanabe et al 1993]. Zeb1 has also been shown to bind to two E-boxes within the Vitamin D3 Receptor (VDR) promoter region and upregulate

activity of a VDR promoter in certain cell types *in vitro* [Lazarova et al 2001]. In this instance, promoter activation was unchanged by the presence of exogenous CtBP, though a separate study found that expression levels of CtBP and CBP/p300 in human tumor tissues determined whether Zeb1 acted as an activator or repressor of transcription [Pena et al 2006].

1.3.4.2 Regulation of Zeb1 Activity

Like many other transcription factors, the activity of Zeb1 is thought to be regulated, at least in part, by post-translational modifications including phosphorylation and sumoylation. These modifications may also play a role in the determination of Zeb1's role as a transcriptional activator or repressor. Though little is known about the functional implications, Zeb1 has been shown to exist in both hypophosphorylated and hyperphosphorylated forms in multiple cell lines, though it does not appear to affect nuclear localization. Zeb1 and Zeb2 were also found to be sumoylated, with the polycomb protein Pc2 acting as a SUMO E3 ligase for Zeb2 [Long et al 2005]. Interestingly, mutation of the proposed sumoylation sites in Zeb2 did not affect nuclear localization, protein stability, or activity of a VDR promoter construct, but the mutation did result in enhanced repression of an E-cadherin promoter in 293T cells. This finding suggests that sumoylation may represent a cell

type-specific and gene-specific method of regulating Zeb1 and Zeb2 repressor activity, possibly by disrupting binding to the co-repressor CtBP [Long et al 2005].

1.3.4.3 Regulation of Zeb1 Expression

As a critical regulator of multiple processes during embryogenesis, expression of Zeb1 is tightly controlled in a cell-specific fashion. Early studies of Zeb1 regulation found that its transcript was upregulated in response to estrogen signaling in the chick oviduct, and this was determined to be mediated directly by estrogen receptor binding to the Zeb1 promoter [Chamberlain and Sanders 1999]. In humans, several studies have shown that Zeb1 can be induced by progesterone and estrogen, and that this induction requires the associated hormone receptors [Dillner and Sanders 2002, Spoelstra et al 2006].

Zeb1 has also been implicated in modulation of TGF β signaling through its interactions with Smad proteins via the Smad binding domain, and Zeb1/Smad interactions have been shown to lead to upregulation of a number of TGF β target genes [Postigo 2003]. Like TGF β , the NF- κ B signaling pathway is multifaceted. The NF- κ B pathway was first characterized as an important activator of immune and inflammatory responses in immune cells, but many studies have also shown its effects to be important in epithelial cells where NF- κ B signaling can induce cell

proliferation and EMT [Orlowski and Baldwin 2002]. In the MCF10A mammary epithelial cell line, exogenous expression of the constitutively-active NF- κ B p65 subunit led to repression of E-cadherin and other changes typical of EMT, including upregulation of Zeb1 and increased activity of a Zeb1 promoter construct [Chua et al 2007]. Further, knockdown of Zeb1 in this model of active NF- κ B signaling resulted in a decrease in the number of viable cells, suggesting Zeb1 may be important in cell proliferation or survival downstream of active NF κ B signaling in mammary epithelial cells [Chua et al 2007].

Hypoxia has also been proposed to increase Zeb1 by signaling through the Hypoxia Inducible Factor 1 (HIF1 α) transcription factor in a renal carcinoma cell line [Evans et al 2007, Krishnamachary et al 2006]. While hypoxia and HIF1 α have been demonstrated to be capable of inducing EMT and repressing E-cadherin in a number of models, a direct interaction between HIF1 α and Zeb1 expression remains controversial [Yang et al 2008].

Recent advances in the study of microRNAs (miRNAs) have revealed a critical role for a Zeb1/miRNA feedback loop in repression of the epithelial phenotype in multiple cell types. Mature miRNAs are non-coding single stranded transcripts of approximately 22 nucleotides which regulate expression of a vast number of genes. The two currently recognized methods of action involve the

binding of miRNA to an mRNA transcript which can 1) create double-stranded RNA which is then recognized and degraded by the Dicer complex, and 2) inhibit translation of mRNA into protein [Inui et al 2010]. The Zeb1 mRNA contains binding sites for several members of the miR-200 family including miR-200a, miR-200b, and miR-200c, as well as miR-205 [Gregory et al 2008]. All four of these miRNAs are able to repress expression of Zeb1 and Zeb2 to varying degrees. This has been demonstrated to occur via direct binding of the mature miRNAs to the Zeb1 or Zeb2 transcript, as mutation of the putative miRNA binding sites abolishes the inhibitory effect of the miRNA [Gregory et al 2008, Park et al 2008].

Conversely, Zeb1 has also been shown to regulate these same miRNAs as part of a feedback loop regulating epithelial gene expression. MiR-200b, miR-200c, and miR-141 were all highly upregulated in response to Zeb1 knockdown by shRNA in MDA-MB-231 breast cancer cells. Zeb1 was found to bind directly to Z-boxes and E-boxes within the regulatory region of the miR-200c and miR-141 sequence. Interestingly, while miR-200c inhibits Zeb1 expression as previously mentioned, miR-141 is capable of repressing TGF β 2 expression, providing a functional link whereby these miRNAs target two different factors in the same EMT-induction pathway [Burk et al 2008]. This same study also found that the level of these epithelial-specific miRNAs was decreased in basal-type breast cancers compared to

more differentiated cancers [Burk et al 2008]. Together, this data suggests that these factors form a feed-forward loop whereby aberrant expression of Zeb1 can repress miRNAs which normally repress Zeb1, leading to robust induction and maintenance of the mesenchymal phenotype.

1.3.4.4 Zeb1 in Development

In any metazoan, proper development is thought to occur through precise spatio-temporal regulation of protein expression achieved in part through a delicate balance of positive and negative regulators of transcription. As a master regulator of a broad number of genes, Zeb1 has been implicated in a number of developmental pathways including hematopoiesis, chondrogenesis, osteogenesis, and myogenesis. In the developing *Drosophila* embryo, the Zeb1 ortholog *zfh-1* is expressed early on in the mesoderm, but is normally downregulated prior to the beginning of myogenesis. It is also expressed in the later embryo in the dorsal vessel, support cells of the gonads, muscle precursor cells, and motor neurons of the developing central nervous system [Lai et al 1991]. Loss of *zfh-1* function, while not lethal, results in CNS defects and adversely affects muscle organization and positioning. Conversely, forced expression of *zfh-1* results in CNS defects and blocks *Drosophila* myogenesis,

possibly through repression of the muscle differentiation factor *mef2* [Lai et al 1991, Postigo et al 1999].

Zeb1 expression in the mouse embryo is similar to that of the *Drosophila* embryo, occurring in the mesodermal tissues, derivatives of the neural crest, parts of the central nervous system, and thymocytes [Higashi et al 1997]. Homozygous deletion of Zeb1 results in perinatal lethality with skeletal and limb defects, consistent with a role of Zeb1 in chondrogenesis and limb formation, as well as T-cell deficiency in the thymus [Takagi et al 1998]. Among mice expressing a truncated form of Zeb1 lacking the C-terminal zinc finger cluster, approximately 20 percent of mice survived past postnatal day two, and all mice had a similar deficiency of T-cells in the thymus [Higashi et al 1997, Takagi et al 1998]. The difference between the Zeb1-null mice and Zeb1-mutants suggests an important role for the N-terminal zinc-finger cluster during development [Miyoshi et al 2006].

1.3.4.5 Zeb1 in Cancer

As previously discussed, E-cadherin is an important component of epithelial cell-cell junctions, and its loss is frequently used as a marker of EMT *in vitro* and *in vivo*. Loss of E-cadherin also has implications beyond its direct physical role, as this

loss also releases the transcription factor β -catenin to be translocated to the nucleus and induce transcription of numerous pro-survival and pro-migratory genes.

Along with Snail, Slug, and Twist, Zeb1 has been demonstrated to be capable of repressing E-cadherin in numerous contexts including during cancer progression. In breast cancer cell lines, a strong inverse correlation has been demonstrated between expression of Zeb1 and E-cadherin, and endogenous Zeb1 has been shown to bind directly to the E-cadherin promoter in the mesenchymal MDA-MB-231 cell line [Eger et al 2005]. Additionally, knockdown of Zeb1 in MDA-MB-231 cells is sufficient to restore expression of E-cadherin protein at the membrane and reduce migration through a transwell [Aigner et al 2007]. Conversely, these studies found that expression of Snail-1 did not correlate with E-cadherin levels in a panel of breast tumors, and knockdown of Snail was less effective at blocking migration than Zeb1, suggesting that Zeb1 may be a more efficient repressor of E-cadherin than Snail in some breast tumors [Aigner et al 2007].

This same study further looked at the expression of Zeb1 in a panel of human colorectal and breast tumors. In colorectal adenocarcinomas, Zeb1+/Cytokeratin+ epithelial cells were found invading into the surrounding stromal tissue, suggesting an intermediate EMT phenotype at the leading edge of the tumor and a role for Zeb1 in metastasis [Aigner et al 2007]. Indeed, knockdown of Zeb1 in the HCT116

colorectal cancer cell line also greatly diminished metastasis formation following intrasplenic injection or tail vein injection assays [Spaderna et al 2008]. Similarly, Zeb1 overexpression is found in the tumor-associated stroma of low-grade endometrioid adenocarcinomas, and in the epithelial and stromal compartments of high-grade endometrioid adenocarcinomas and uterine papillary serous carcinomas [Singh et al 2008, Spoelstra et al 2006]. In stained breast tumor samples, Zeb1 was found in the tumor-associated stroma, as well as in epithelial cells in de-differentiated areas of the tumor which had lost cytokeratin expression [Aigner et al 2007].

1.4 Mammary Stem Cells and Breast Cancer Stem Cells

Many groups have postulated that within a heterogenous tumor, there exists a subgroup of cancer cells commonly referred to as tumor initiating or cancer stem cells (CSCs) [Reya et al 2001]. These rare cells are thought to fulfill a function similar to stem cells of normal tissues, and by virtue of increased resistance to apoptosis are thought to be able to repopulate a tumor following therapy [Wicha et al 2006]. In addition to expressing markers of EMT, basal-like breast cancers have also been shown to contain a higher percentage of these CSCs compared to other subtypes [Honeth et al 2008]. Thus there has been intense interest in understanding the

processes and pathways which regulate breast cancer stem cell behavior with the goal of finding therapies which can target these cells.

1.4.1 Stem Cells in the Normal Mammary Gland

The mammary gland consists of branching ducts and terminal ductal lobulo-alveolar units, which function to produce the milk proteins during breast feeding. The ducts and lobules are comprised of two layers of epithelial cells: the inner luminal epithelial cell layer and the surrounding basal layer of myoepithelial cells. The majority of breast tumors are considered to derive from this ductal lobulo-alveolar network, and the majority of breast tumors contain cells expressing markers of the luminal epithelium [Taylor-Papadimitriou et al 1989, Wellings et al 1975]. The large-scale expansion of luminal and myoepithelial cells that occurs at puberty and during pregnancy supports the existence of stem-like cells capable of tissue generation and regeneration throughout the reproductive period. Evidence from a number of groups suggests that similar to other organs, a hierarchy of cells can be defined within the mammary gland with a stem cell at the apex.

Stem cells are characterized by the ability to self-renew (possibly through asymmetric cell division) while also giving rise to more committed progenitor cells which in turn proliferate and differentiate into all the cell types of that specific tissue

[Reya et al 2001]. Mammary fat pad transplantation assays, in which a tissue fragment or cell suspension is implanted into the cleared mammary fat pad of a recipient mouse, have provided a wealth of experimental evidence to support this hypothesis [Daniel et al 2009, Deome et al 1959, Visvader 2009]. Using combinations of cell surface markers to identify cells enriched for stem- or progenitor-like activity, Shackleton et al. showed that a single Lin⁻CD29^{hi}CD24⁺ cell is able to reconstitute a complete functional mammary gland [Shackleton et al 2006]. It is important to note that this Lin⁻CD29^{hi}CD24⁺ population does not consist entirely of mammary stem cells (MaSCs), but is enriched for mammary gland repopulating activity. Furthermore, many studies disagree on the cell surface markers to best enrich for such activity, as well as the markers which identify more committed steps of differentiation, such as the putative luminal or myoepithelial progenitor cells.

In the human mammary gland, evidence for such a MaSC is less direct, but a number of observations suggest the existence of MaSCs similar to those in the mouse. First, identical chromosomal alterations can be found in contiguous regions of mammary epithelial tissue, suggesting they came from a common progenitor cell [Deng et al 1996, Lakhani et al 1996]. Attempts to use humanized murine mammary fat pads to recapitulate the mouse transplantation experiments have thus far failed

to yield similar results with normal human MaSCs, but xenograft studies in mice have been vital in the development of the breast cancer stem cell (BCSC) theory.

1.4.2 Breast Cancer Stem Cells

In 2003, Al-Hajj et al. demonstrated that a distinct population of human breast cancer cells, described as $CD44^+/CD24^{low}/ESA^+$, was dramatically and reproducibly enriched for tumor forming capability in an orthotopic xenograft model versus the bulk unsorted population [Al-Hajj et al 2003]. Many subsequent studies have focused on the $CD44^+/CD24^{low}$ population of breast cancer cells both from patient samples and established breast cancer cell lines, showing that this population is enriched for numerous characteristics of stem-like cells *in vitro* including self renewal and generation of heterogenous progeny [Fillmore and Kuperwasser 2008, Ponti et al 2005, Zucchi et al 2007].

Other groups have found that in a number of different normal tissues and cancer types, the stem cell population may be identified by increased aldehyde dehydrogenase (ALDH) activity [Ginestier et al 2007]. The ALDH family of enzymes is responsible for oxidation of intracellular aldehydes and is involved in other cellular processes such as retinoic acid production [Jackson et al 2011, Yoshida et al 1992]. In breast cancers, ALDH1-positive cells are enriched in mammosphere

forming capacity and tumor forming capacity compared to ALDH1-negative cells, and expression of ALDH1 in human tumors correlated with poor prognosis [Charafe-Jauffret et al 2010, Ginestier et al 2007]. Further studies have found that ALDH1-1 positive cells display enhanced migratory and metastatic behavior *in vitro* and in mouse xenografts, and expression of ALDH1 in human tumors correlates with tumor grade, presence of metastasis, and cancer stage [Crocker et al 2009, Marcato et al 2011].

1.5 Linking EMT and Breast Cancer Stem Cells

Numerous *in vitro* studies have found that a population of CD44⁺/CD24^{-/low} cells exists in mammary epithelial cells isolated and grown in tissue culture and in many of the commonly-used breast cancer cell lines, and that this population is enriched in self-renewal as assessed by mammosphere formation assays [Fillmore and Kuperwasser 2008]. These studies have allowed further characterization of the putative breast cancer stem cells (BCSCs), and have shown that CD44⁺/CD24^{-/low} cells are 1) more resistant than non-BCSCs to apoptosis induced by radiation [Phillips et al 2006, Zhan et al 2011] and chemotherapy [Fillmore and Kuperwasser 2008], and 2) more invasive than non-BCSCs in transwell invasion assays [Sheridan et al 2006]. Collectively, these reports have shown that in addition to their increased capacity for

self-renewal, BCSCs also possess biological properties similar to cells which have undergone EMT, suggesting a biological link between stem cell activity and EMT.

1.5.1 EMT Generates Cells with Properties of Cancer Stem Cells

One of the most intriguing findings in the field of EMT in cancer progression and metastasis is the observation that induction of EMT by a variety of stimuli *in vitro* also endows cells with properties of cancer stem cells. Breast cancer stem cells were strongly linked to EMT by two studies in 2008 which demonstrated that induction of EMT by a variety of stimuli *in vitro* resulted in an increased fraction of cells with properties of cancer stem cells [Mani et al 2008, Morel et al 2008].

Treatment with TGF β or overexpression of the EMT-inducing transcription factors Snail or Twist in a model of nontumorigenic immortalized human mammary epithelial cells increased the percentage of cells in the CD44⁺/CD24⁻ fraction, increased the tumorigenicity of the cells, and greatly increased the ability of the cells to form mammospheres *in vitro*. These mammospheres contained both basal/myoepithelial-like cells (CK14⁺) and luminal epithelial-like cells (CK8/18⁺) [Mani et al 2008].

Additionally, this study extended the findings with *in vivo* experiments which demonstrated that mouse mammary stem cells (CD49^{high}/CD24^{med}) [Stingl et al 2006] and human CD44⁺/CD24^{low} cells from normal reduction mammoplasty tissue

also express markers of EMT, including high expression of vimentin and low expression of E-cadherin [Mani et al 2008]. Several functional mechanisms for this link have been proposed, including direct repression of CD24 transcription by Twist even before the onset of EMT markers [Vesuna et al 2009]. However, the functional implications of CD24 repression have yet to be elucidated.

These studies suggest that the very process which allows tumor cells to leave the primary tumor site and enter the circulatory system also endows cells with enhanced survival and self-renewal capabilities, both of which would be beneficial to the process of establishing a metastatic lesion (Figure 1.2) [Chaffer and Weinberg 2011]. The idea of targeting the tumor initiating cells had previously been proposed, as had the idea of preventing or targeting cells which have undergone EMT. Interestingly, it has recently emerged that not only are CD44⁺/CD24⁻ cells more resistant to some chemotherapies, chemotherapy itself may actually increase the CD44⁺/CD24⁻ population of cells in some tumors.

One study found that among patients with locally advanced breast cancers, 12 weeks of neoadjuvant chemotherapy (including docetaxel, or doxorubicin and cyclophosphamide) resulted in a significant increase in the percentage of CD44⁺/CD24⁻ cells and increased mammosphere forming efficiency [Li et al 2008]. A later study found that following treatment with either endocrine therapy (using the

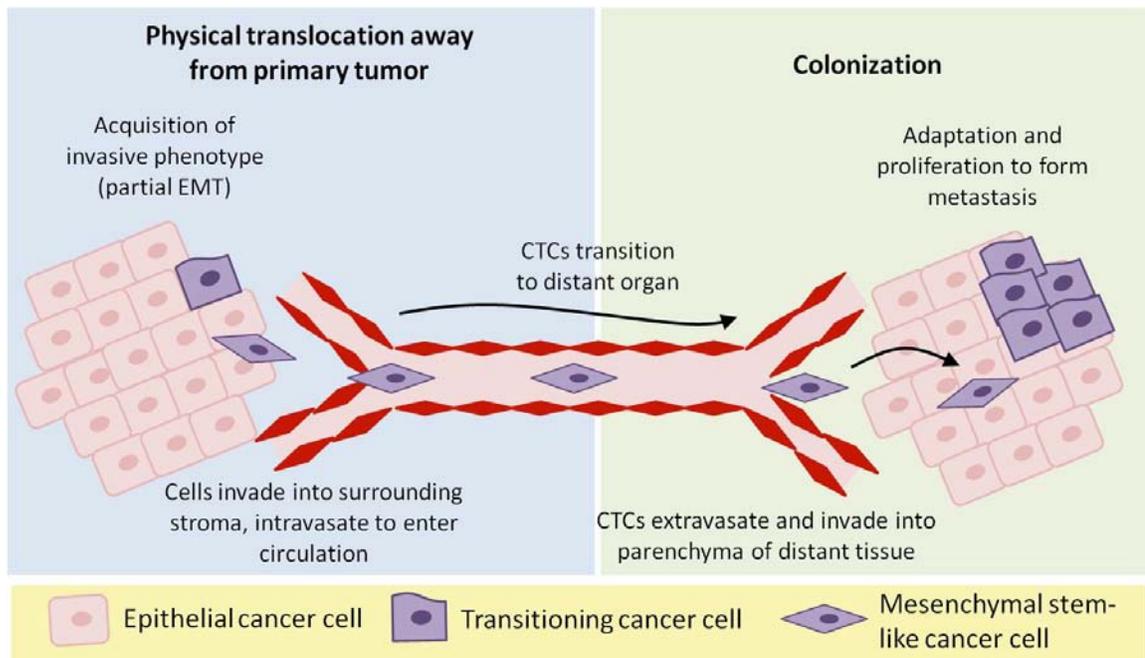


Figure 1.3: CTCs may represent cells with an intermediate EMT or stem cell phenotype.

The process of EMT endows cells with increased invasive and migratory abilities as well as features of the putative cancer stem cell population, including increased resistance to apoptosis and anoikis. The formation of macrometastases may be determined in part by the ability of migrating cells to return to a more epithelial-like state, and by signals regulating tumor cell dormancy and proliferation.

aromatase inhibitor letrozole) or chemotherapy (docetaxel), gene expression analysis revealed a higher degree of overlap with the CD44⁺/CD24⁻ stem-like cell profile than before treatment, as well as an increase in mesenchymal markers such as vimentin [Creighton et al 2009]. Similar findings of increased expression of EMT markers following endocrine or chemotherapy have also been reported in colorectal cancer cell lines *in vitro* [Yang et al 2006] and in ovarian cancer cell lines in mouse

xenografts [Kajiyama et al 2007], suggesting this may be a common characteristic among solid tumors. It remains to be determined whether these observations are due to enhanced survival of the CD44⁺/CD24⁻ population compared to the bulk of the tumor, or whether the stresses of chemotherapy can induce an EMT-like change in some fraction of the cancer cells, which also promotes a change toward a stem cell-like phenotype.

1.5.2 EMT and Breast Cancer Stem Cell Phenotypes are Related to the Basal-Like Subtype

The basal-type breast cancer subtype was originally named such for the expression pattern characteristic of the basal epithelial cells of the mammary ductal-alveolar structure. This basal epithelial layer has been proposed to contain the mammary gland stem cells, as well as a population of more committed basal/myoepithelial progenitor cells [Sleeman et al 2006]. Combined with the stem cell-like phenotype of many basal-like tumors, it has been hypothesized that basal-like breast cancers were likely derived from a basal progenitor or stem cell.

Interestingly, one recent study has suggested that this notion is not necessarily true. Using a transgenic mouse model with targeted inactivation of BRCA1 and p53 with a cell-type specific Cre-recombinase gene, Molyneux et al. demonstrated that inactivation of these proteins in CD24^{+/high}/Sca-1⁻/ER⁻ luminal

progenitor cells results in a basal-like tumor, while inactivation of these proteins in CK14+ basal progenitor cells results in the formation of tumors of the clinically-rare metaplastic subtype [Molyneux et al 2010]. The results of this study are at odds with a similar prior study in which BRCA1 and p53 inactivation in CK14+ positive cells resulted in basal-like tumors [Liu et al 2007].

These opposing observations may be due in part to the different genetic backgrounds of the mice, different K14-Cre transgenes, or deletion of different exons from *BRCA1*. Another possible interpretation of this discrepancy is that the markers used to identify luminal progenitor cells in the study of Molyneux et al. were not sufficiently specific and also identified bipotent progenitor cells. However, the implication that luminal progenitor cells can give rise to cells with basal characteristics suggests an inherent ability for aberrant de-differentiation during tumorigenesis, possibly as a result of loss of BRCA1 or p53 function.

Breast cancers occurring in *BRCA1* germline mutation carriers are frequently of the basal-like subtype [Sorlie et al 2003, Turner and Reis-Filho 2006]. The abundance of basal-like breast cancers among *BRCA1* mutation carriers may be due in part to the effects of loss of *BRCA1* function on the PI3K-Akt pathway, which is frequently over-active in triple-negative breast cancers [Lopez-Knowles et al 2010, Umemura et al 2007]. Basal-like tumors frequently display loss of *PTEN* copy

number, and a high-frequency of *PTEN* mutations including intragenic chromosome breaks, inversions, deletions and micro copy number aberrations were found specifically in *BRCA1*-mutant cell lines and xenografts [Saal et al 2008]. Additionally, mutation of *PTEN* or loss of heterozygosity (LOH) leads almost exclusively to basal-like tumors in the breast [Podsypanina et al 1999, Saal et al 2008].

Downstream of *PTEN*, Akt is capable of affecting EMT through direct phosphorylation and inhibition of the kinase GSK3 β . Active GSK3 β is capable of repressing Snail activity both by direct phosphorylation (which results in Snail translocation out of the nucleus and proteolytic degradation) and by inhibition of Snail transcription, possibly through NF κ B [Bachelder et al 2005, Zhou et al 2004]. Thus Akt activity may play a context-dependent role in EMT. Together, these studies suggest a possible functional link between loss of *BRCA1* activity and over-activation of the PI3K-Akt pathway, which are frequently observed in basal-like tumors, and EMT.

In addition to mutant *BRCA1*, basal-like breast cancers also frequently harbor mutant *p53*. Interestingly, a separate study regarding the cell of origin for basal-type breast cancer utilized a transgenic mouse model based on inactivation of the Rb tumor suppressor protein. Targeted inactivation of Rb in mammary epithelial cells lead to an expansion of the Lin⁻/CD24⁺/CD49f⁺ presumptive stem cell population and

gave rise to tumors of a number of phenotypes. However, when p53 was mutated during tumorigenesis or through Cre-mediated recombination, the Rb-null mice exclusively developed tumors with a basal-like gene expression pattern and markers of EMT [Jiang et al 2010]. Similarly, in a transgenic mouse model of Wnt1-dependent breast tumor escape, loss of heterozygosity (LOH) of the p53 allele resulted in recurrence of tumors with hallmarks of EMT [Debies et al 2008], further supporting a role for loss of p53 function in epithelial-mesenchymal plasticity and basal-like breast cancer.

1.6 Summary and Hypothesis

Breast cancers with a basal-like gene signature are primarily triple-negative, frequently occur in young African-American women and *BRCA1* mutation carriers, and carry the worst prognosis [Sorlie et al 2001]. Recent studies have shown that triple-negative breast cancers are enriched for markers of EMT, a process thought to be important in the metastatic cascade [Thiery 2002, Thompson et al 2005]. Other recent work has demonstrated a link between EMT and the induction of stem cell-like characteristics in mammary epithelial cells, and that an EMT and stem cell-like gene expression signature is found in residual breast cancer cells following chemotherapy [Creighton et al 2009, Mani et al 2008].

Collectively, these studies suggest that epithelial-mesenchymal plasticity may be important for the highly aggressive behavior and poor prognosis of the lethal subset of triple-negative breast cancers, but direct *in vivo* evidence of EMT/MET remains rare. Additionally, development of novel therapeutic approaches to target triple-negative breast cancers is currently limited in part by a lack of appropriate cell lines and mouse xenograft models.

We have developed a novel model of human triple-negative breast cancer, the DKAT cell line, isolated from an aggressive, treatment-resistant triple-negative breast cancer. While the DKAT model was derived from a breast cancer that exhibited a diverse range of phenotypes, it is unclear whether this phenotypic diversity was the result of 1) outgrowth or evolution of specialized subpopulations of cells within the primary tumor or 2) phenotypic plasticity inherent within the DKAT cells. Therefore we tested DKAT cells for evidence of phenotypic plasticity by altering culture conditions and testing for *in vitro* EMT/MET, and utilized this model and other breast cancer cell lines to study the pathways regulating epithelial-mesenchymal plasticity in breast cancer. Finally, we also tested whether this plasticity can be found as an early event in mammary carcinogenesis by testing for expression of mesenchymal markers in mammary epithelial cells from asymptomatic women at high risk for developing breast cancer. Increased

understanding of the pathways which regulate epithelial-mesenchymal plasticity will lead to improved diagnosis and treatment options for patients with highly aggressive and deadly triple-negative breast cancers.

2 Materials and Methods

2.1 Cell Culture

2.1.1 Establishment of the DKAT cell line

Pleural fluid from a patient with biopsy-confirmed primary breast cancer was obtained with the approval of The Ohio State University Institutional Review Board and with the written informed consent of the patient. Pleural fluid was centrifuged to pellet the cells. The pellet was then resuspended in Mammary Epithelial Cell Growth Medium (MEGM) supplemented with bovine pituitary extract, insulin, human recombinant epidermal growth factor, and hydrocortisone (Lonza, Basel, Switzerland).

2.1.2 *In Vitro* Plasticity

For EMT: DKAT cells were switched from MEGM to SCGM (Lonza); DKAT control cells remained in MEGM. Time in SCGM ranged up to 10 passages, and control DKAT cells were maintained in MEGM and passaged in parallel. For MET: After 10 passages in SCGM, a portion of DKAT cells were switched back to MEGM. Control cells included 1) the original DKAT cells grown continuously in MEGM and 2)

DKAT cells maintained in SCGM. All cells and controls were passaged in parallel and imaged and harvested at similar confluency.

2.1.3 Generation of Clonal DKAT Cell Lines

DKAT cells in MEGM were digested into a single-cell suspension with trypsin and plated at one cell per well in a 96 well plate, and wells were visually checked for individual cells and monitored for the formation of colonies. After 22 days, cells were removed from wells containing colonies by cell dissociation buffer (Invitrogen) and sequentially grown out in a 24-well plate, 6-well plate, T25 flask, and T75 flask in MEGM. *In vitro* EMT was induced as previously described.

2.1.4 Zeb1-Expressing DKAT Cells

The full-length Zeb1 transcript was amplified from MDA-MB-231 cells by PCR using the following primers: Forward 5'-CAA GCG AGA GGA TCA TGG CG-3' and Reverse 5'-TTC CTT CTA GAA AAA CGA TTA GGC-3'. The resulting product was cloned into the XhoI and BamHI sites of pLXSN. Retroviral particles were generated by transfecting the resulting construct or the pLXSN empty vector control into GP293 cells. DKAT-MEGM cells were then transduced and expressing cells were selected with .5mg/ml G418 (Invitrogen).

2.1.5 Zeb1 shRNA

Mission shRNA constructs targeting Zeb1 and a non-targeting control construct were purchased from Sigma (St. Louis, MO), and lentivirus was produced according to manufacturer's instructions. DKAT cells in MEGM were transduced and selected with .5 μ g/ml Puromycin, and the resulting cells were grown in either MEGM (control) or SCGM to induce EMT. Scratch assays were performed at least 14 days after the transition to SCGM. Zeb1 mRNA was assessed by rtPCR using commercially-available PCR primers (sc-38644-PR, Santa Cruz).

2.1.6 Other Cell Lines

Primary human mammary epithelial cells (HMECs) (Lonza) were immortalized with hTERT (HMEC-hTERT), and maintained in supplemented MEGM. MDA-MB-231 and MCF-7 cells (American Type Culture Collection [ATCC], Manassas, VA) were maintained in α MEM (Invitrogen, Carlsbad, CA) supplemented as previously described [Seewaldt et al 1995]. SUM-190 cells (ATCC) were maintained in RPMI supplemented with 10% FBS.

2.2 Cytogenetic Analysis

Spectral karyotyping analysis (SKY) was performed as previously described [Mrozek et al 2002]. Karyotypic abnormalities were classified according to the International System for Human Cytogenetic Nomenclature.

2.3 Immunohistochemistry

The human formalin-fixed, paraffin-embedded primary breast biopsy, chest wall recurrence, and bone metastasis samples were sectioned at 4 μm thickness and stained for alpha-smooth muscle actin (1A4, Sigma), vimentin (3B4 Boehringer Mannheim Roche, Hvidovre, Denmark), CK5 (OVTM, DAKO, Glostrup, Denmark) and wide range keratins (MNF116, DAKO). The antibodies were visualized by streptavidin-biotin (DAKO 5004).

2.4 TP53 and PIK3CA Sequencing

Sequencing of exons 5-9 of the *TP53* gene and exons 9 and 20 of *PIK3CA* using previously published primers and programs [IARC TP53 Mutation Database, <http://www-p53.iarc.fr/p53sequencing.html> and [Wang et al 2000]] was performed on genomic DNA isolated from passage 20 DKAT cells. Mutations were tested in

both the sense and antisense sequences and were confirmed by repeat sequencing of a second, independent genomic DNA preparation.

2.5 Immunofluorescence

Cells were fixed in either methanol for 20 minutes or 4% paraformaldehyde for 30 minutes on ice. Cells were blocked for 30 minutes, incubated with primary antibody overnight at 4°C and secondary antibody for 1 hour at RT, and counter stained with DAPI. Images were acquired on a Zeiss Axio Observer A1 fluorescence microscope (Carl Zeiss, Göttingen, Germany) with a 40x or 63x objective. Primary antibodies were from 1) Santa Cruz Biotechnology Inc. (Santa Cruz, CA): CK5 (sc-66856), CK14 (sc-53253), CK18 (sc-28264), CK17 (sc-101931), E-cadherin (sc-7870), EGFR (sc-120), ERalpha (sc-8005), p63 (sc-56188), and vimentin (sc-5565) 2) BD biosciences (San Jose, CA): vimentin (550513), E-cadherin (610181) 3) Thermo Fisher Scientific Inc. (Fremont, CA): PR (MS-298) 4) Abcam (Cambridge, MA): smooth muscle actin (ab15734), and 5) Invitrogen (Carlsbad, CA): claudin-1 (37-4900), claudin-4 (32-9400), ZO-1 (40-2300), occludin (40-4700). Fluorescent secondary antibodies included goat anti-mouse Alexa-fluor 488 (A21222) and goat anti-rabbit Alexa-fluor 597 (A11070) from Invitrogen.

2.6 Immunoblots

Cells grown on 10cm dishes or 6-well plates were washed with ice-cold PBS and lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail (Roche). Cells were then scraped and pipette into a microcentrifuge tube, incubated on ice for 10 minutes, and vortexed for 15 seconds. Lysate was then spun down at 14,000 rpm for 10 minutes at 4 degrees, and supernatant was collected and stored at -80 degrees celcius. Proteins were separated by SDS-PAGE using NuPAGE Bis-Tris gels (Invitrogen) and transferred to PVDF membranes (Roche).

Primary antibodies used include: p53 and Zeb1 (DO-1 and E-20, respectively, Santa Cruz Biotechnology Inc., Santa Cruz, CA); PTEN, Akt-pSer473, Akt-1, Akt-2, Akt-3, and Twist (9552, 4051 and 4058, 2967, 2964, 3788, and 4119, respectively, Cell Signaling, Danvers, MA); claudin-1, claudin-4, occludin, ZO-1 (37-4900, 32-9400, 40-4700, and 40-2300, respectively; Invitrogen, Carlsbad, CA), snail-1 (gift from Antonio Garcia-deHerreros). Loading control was provided by reprobing the membrane with an antibody to beta-actin (I19, Santa Cruz Biotechnology, Inc.).

Immunoblots were imaged utilizing SuperSignal West Dura Substrate (Thermo) and a Kodak Image Station 2000MM. Immunoblot quantitation was

accomplished using Kodak 1D Image Analysis Software (Eastman Kodak, Rochester, MN).

2.7 Flow Cytometry

Cells were harvested with cell dissociation buffer (Invitrogen), washed with PBS with 1% BSA, and incubated with APC-conjugated CD44 and PE-conjugated CD24 antibodies (BD Biosciences, San Jose, CA) for 30 minutes at room temperature. At least fifty thousand events were collected using a FACSCaliber flow cytometer and analyzed for CD44/CD24 expression with CellQuest software (BD Immunocytometry Systems).

2.8 Differential Gene Expression Studies

mRNA was collected using the Qiagen RNeasy mini kit with the optional DNase step (Quiagen, Valencia, CA) and RNA integrity was confirmed by electrophoresis. cRNA synthesis and probe generation for cRNA array hybridization were performed according to the standardized protocols provided by Affymetrix™ (Affymetrix, Santa Clara, CA). Transcript analysis was performed using Affymetrix HG-U133 Plus2.0 GeneChips. Samples were labeled and hybridized following strict adherence to Affymetrix's standardized protocols. GeneChip results were assessed

for quality prior to further analysis. Median expression values of technical replicates (n=3) for each of three cell lines, HMECs, DKAT, and MDA-MB-231, were compared with published breast cancer cell line expression data downloaded from the publisher's web site [Charafe-Jauffret et al 2006]. Probe Set IDs were used to merge the two data sets (16,383 features). Duplicate Gene Symbols were summarized by mean Probe Set ID values. The data were array- and gene-wise median centered and filtered to exclude genes with standard deviations of observed values less than 1.5. The remaining 473 features were clustered using centroid linkage and Pearson correlation as the similarity metric (Cluster ver. 3.0). Data is currently being submitted to the NCBI GEO in accordance with MIAME guidelines.

2.9 Invasion Assays

1x10⁵ DKAT or MDA-231 cells were plated in the inner chamber of 8 micron pore, 24 well-size filters coated with Growth Factor-Reduced Matrigel™ (BD Biosciences) or uncoated control inserts. Cells were plated in base MEGM media + 0.01% FBS. Base MEGM containing 10% FBS was used as the chemoattractant. Plates were incubated at 37°C degrees for 20 hours, non-invading cells were removed, and membranes stained with DAPI. The number of cells that had migrated through the

membrane was determined by fluorescence microscopy. Numbers shown are the average of triplicate wells, three fields counted per well.

2.10 Migration Assays

5×10^5 DKAT or MDA-MB-231 cells were plated in 6-well plates and cultured until confluent. The cell monolayer was then scratched with a pipette tip, rinsed, and photographed at regular intervals until the scratch was completely closed. The area of the scratch was measured using ImageJ software [Abramoff 2004], and results are expressed as a percentage of the original scratch area filled by the cells at the indicated timepoint.

2.11 Xenograft Experiments

DKAT cells grown in MEGM were tested for xenograft growth by limiting dilution; 10^4 , 10^3 , 10^2 , or 10 cells were resuspended in 50 μ l of either 1) 1:1/v:v PBS:Matrigel™ or 2) PBS alone, and injected into the right and left uncleared #4 mammary fat pad of NOD.CB17-PrkdcSCID/J mice. Four independent experiments (eight – ten replicates per experiment) were performed at Duke University and University of California at Davis (UCD). DKAT xenografts using matrigel were performed with NOD CB17-Prkdc SCID/J mice (Jackson Labs) and were done in

accordance with a protocol approved by the Duke University Institutional Animal Care and Use Committee (protocol number A328-08-12). Tumor growth was assessed weekly by measuring tumor volume by caliper. Mice were sacrificed when the tumor reached 1.5 cm in diameter or after 150 days. Tumors were excised, fixed in 4% paraformaldehyde at 4°C overnight, sectioned and stained, and underwent central pathology review at UCD. DKAT xenografts without matrigel were done in nude and NOD CB17-Prkdc SCID/J mice (Jackson Labs) in accordance with a protocol approved by the UCD Institutional Animal Care and Use Committee (protocol number 13217).

2.12 Tumorsphere Formation

Tumorsphere culture was as previously described [Dontu et al 2003] with minor modifications. Briefly, cells were plated at 1 cell/well in 96-well ultra-low attachment plates (Sigma) in basal MEGM medium supplemented with B27, Heparin, EGF, and bFGF. Cells were cultured for 10-14 days and the tumorsphere formation efficiency was determined by counting the number of tumorspheres and dividing by the number of wells seeded.

For tumorsphere immunofluorescence studies, DKAT cells were plated at 10^3 cells per 100mm dish. 14 days later, tumorspheres were pooled and fixed in 100% ice

cold methanol, spun onto slides, and IF staining was performed as described above. Tumorsphere images were acquired with an SP5 Leica laser scanning confocal microscope.

2.13 Conditioned Media Experiments

DKAT cells grown in MEGM or SCGM for at least 14 days were treated with fresh media, and conditioned media was collected 24 hours later, spun down, and supernatant collected. Cytokine levels were analyzed using the Bio-Plex Precision Pro Human Cytokine 8-Plex Panel magnetic bead assay kit and read with a Bio-Plex 200 instrument (Bio-Rad, Hercules, CA). IL-6 levels in conditioned media were also analyzed by Human IL-6 Quantikine ELISA kit (D6050, R&D Systems, Minneapolis, MN).

2.14 Neutralizing Antibody Experiments

DKAT cells grown in SCGM or MDA-MB-231 cells grown in DMEM (Invitrogen) were treated with 1ug/ml of either Mouse IgG control, anti-VEGF, or anti-IL-6 antibody (MAB002, MAB293, or MAB2061 respectively, R&D Systems) for 24 hours. MDA-MB-231 cells were also treated with STAT3 Inhibitor VII (573103, Merck KGaA, Darmstadt, Germany) at .5 μ M for 6 hours. Cells were rinsed with PBS

and lysate was collected as described earlier. Protein band intensity was measured using Carestream Molecular Imaging software (Carestream Health, Rochester, NY).

2.15 RPFNA Cytology

RPFNA was performed as published previously, in accordance with methods established and validated by Fabian and colleagues [Fabian et al 2000]. RPFNA samples were immediately placed and fixed in modified CytoLyt (Hologic Inc.) containing 1% formalin for 24 hours at room temperature. Samples were washed with CytoLyt until the majority of red blood cells were removed, followed by storage at 4°C. Cytology specimens were mounted on Superfrost Excell slides (Fisher) and preserved in 95% ethanol. These slides were dehydrated in 95% and 100% ethanol for 30 seconds each and air-dried for 10 minutes. Specimens were fixed in ice-cold acetone for 20 minutes and then air-dried. Rehydration was achieved in TBS for 5 minutes, followed by immersion in 3% hydrogen peroxide for 5 minutes, deionized water, and TBS.

Immunostaining was carried out on Dako Autostainer (Dako). Specimens were placed in Background Buster (Innovex Biosciences) for 30 minutes, followed by incubation with mouse monoclonal anti-human vimentin antibody (Dako; M7020) at 1:300 dilution for 45 minutes and single wash in TBS. Detection and visualization

were performed with Envision+ and DAB+ (Dako) as per manufacturer's recommendations.

3 Characterization of a Novel Model of Triple-Negative Breast Cancer

3.1 Introduction

Breast cancer is a heterogeneous disease that exhibits a wide range of clinical behaviors, prognoses, and histologies. Gene expression profiling of patient samples initially identified five breast cancer subgroups (luminal A and B, Her2 amplified, normal-like, and basal-like), each with a distinct molecular signature [Perou et al 2000, Sorlie et al 2001, Sorlie et al 2003]. Tumors with a basal-like gene signature are primarily triple-negative (ER-/PR-/Her2wt), frequently occur in young African-American women and *BRCA1* mutation carriers, and carry the worst prognosis [Nielsen et al 2004, Sorlie et al 2001, Sorlie et al 2003, Sotiriou et al 2003]. While some triple-negative breast cancers respond to treatment, a subset are highly invasive and metastatic and do not respond to chemotherapy or radiation [Carey et al 2007].

Recent studies have shown that triple-negative breast cancers are enriched for markers of EMT, a process generally thought to be important in the metastatic cascade [Thiery 2002, Thompson et al 2005]. Other recent work has demonstrated a link between EMT and the induction of stem cell-like characteristics in mammary epithelial cells, and that an EMT and stem cell-like gene expression signature is found in residual breast cancer cells following chemotherapy [Creighton et al 2009,

Mani et al 2008]. Collectively, these studies suggest that epithelial-mesenchymal plasticity may be important for the highly aggressive subset of triple-negative breast cancers.

EMT is a part of normal physiological processes, including embryogenesis and wound healing, in which cells of epithelial origin lose epithelial characteristics and polarity and acquire a mesenchymal phenotype associated with increased migratory behavior [Burdsal et al 1993, Christ and Ordahl 1995, Savagner 2001, Thiery 2003]. Activation of an EMT-like program in cancer cells *in vitro* similarly results in increased cell migration and invasion as well as increased resistance to apoptosis [Savagner 2001, Thiery 2002]. At the molecular level, EMT is characterized by 1) loss of expression of membranous E-cadherin, claudins, and occludins, 2) increased expression of mesenchymal markers including vimentin and smooth muscle actin, 3) acquisition of a spindle-like morphology, and 4) cytoskeleton reorganization [Burdsal et al 1993, Thiery 2003]. The reverse process, MET, is characterized by a loss of expression of mesenchymal markers and restoration of epithelial markers and morphology [Chaffer et al 2007, Christ and Ordahl 1995].

The similarities between the developmental EMT events and the process of tumor cell dissemination, in which cells lose contact with the primary tumor and invade into the normal host tissue and blood vessels, has led to the hypothesis that

EMT is an important part of the metastatic cascade [Yang and Weinberg 2008]. However, much of the evidence for the association of EMT/MET with cancer is derived from studies in animal models and breast cancer cell lines that are highly adapted to tissue culture conditions [Thiery et al 2009, Thompson et al 2005]. There is difficulty in identifying EMT in human breast cancer because the full sequence of events that have come to define EMT *in vitro* are not commonly observed *in vivo*, and metastases commonly have an epithelial phenotype similar to the primary tumor [Christiansen and Rajasekaran 2006]. In order to reconcile the observations of breast cancer pathologists with *in vitro* studies of breast cancer cell lines, it has been proposed that EMT in the human tumor setting may be transient and reversible, and may only occur in isolated foci of the tumors such as the invasive front, where the tumor cells interface with the surrounding stroma [Yang and Weinberg 2008].

The biological origin of breast cancer cells with the capacity to invade and metastasize is the subject of intense debate. The long-standing predominant view is that metastasis results from the selective outgrowth or evolution of specialized sub-populations of cells within the primary tumor, and that these cells are unique in their ability to invade and metastasize [Poste and Fidler 1980]. However, recent experimental and clinical data have accumulated to support the hypothesis that breast cancer may be driven by a sub-population of progenitor-like cells that have

intrinsic phenotypic plasticity and retain stem cell-like properties, and that this phenotypic plasticity may be a key determinant of metastatic potential [Chaffer et al 2007, Thiery 2002, Yang and Weinberg 2008].

The study of plasticity in human breast cancer is currently limited by a lack of appropriate models. Here we report the characterization of the DKAT breast cancer cell line, a novel model of triple-negative breast cancer that displays phenotypic plasticity *in vitro* and *in vivo*. The DKAT line was isolated from a rapidly progressing, treatment-resistant, metastatic triple-negative human breast cancer which exhibited morphologic and biochemical evidence of plasticity. The DKAT cell line provides a new model to investigate the role of plasticity in aggressive triple-negative breast cancers.

3.2 Results

3.2.1 Phenotypic Plasticity in a Human Triple-Negative Breast Cancer

The DKAT cell line was derived from the malignant pleural effusion of a 35-year-old Caucasian woman with no family history of breast or ovarian cancer. The patient initially presented with a 4 cm ER/PR(-/-), HER2/Neu-wt, CK5(+), EGFR(+), lymph node-negative breast cancer (T2N0M0). At the sixth cycle of cyclophosphamide, methotrexate, and 5-fluorouracil, seven months from the initial

diagnosis, recurrent cancer was detected within the chest wall radiation field. At ten months from initial diagnosis, metastases to the lung, pleura, liver, and bone were observed. At eleven months, the patient developed pancytopenia; a bone marrow biopsy revealed multiple foci of tumor cells within the bone marrow. At this time the tumor was not responsive to taxotere and navelbine. Twelve months from initial diagnosis the patient died from pancytopenia and rapid disease progression.

The primary breast cancer surgical specimen demonstrated a range of phenotypes. Six widely separated microfocal neoplastic lesions were identified that exhibited varying morphological and immunohistochemical features (summarized in Table 3.1). Primary tumor foci #1-5 stained strongly for the epithelial marker E-cadherin and displayed only scattered weak staining for CK8/18, CK5, and the mesenchymal marker vimentin (Figure 3.1, left column). In contrast, primary tumor focus #6 was strongly positive for vimentin and CK5 as well as CK8/18 and E-cadherin (Figure 3.1, second column). Similar to primary tumor focus #6, the chest wall recurrence contained solid sheets of malignant cells that were positive for vimentin, CK5, and CK8/18, but E-cadherin staining was notably less intense (Figure 3.1, third column). The staining pattern of the malignant cells in the bone marrow, however, was similar to the epithelial staining pattern observed in Primary Tumor Foci #1-5 with cells staining strongly for CK5, CK8/18 and membranous E-cadherin,

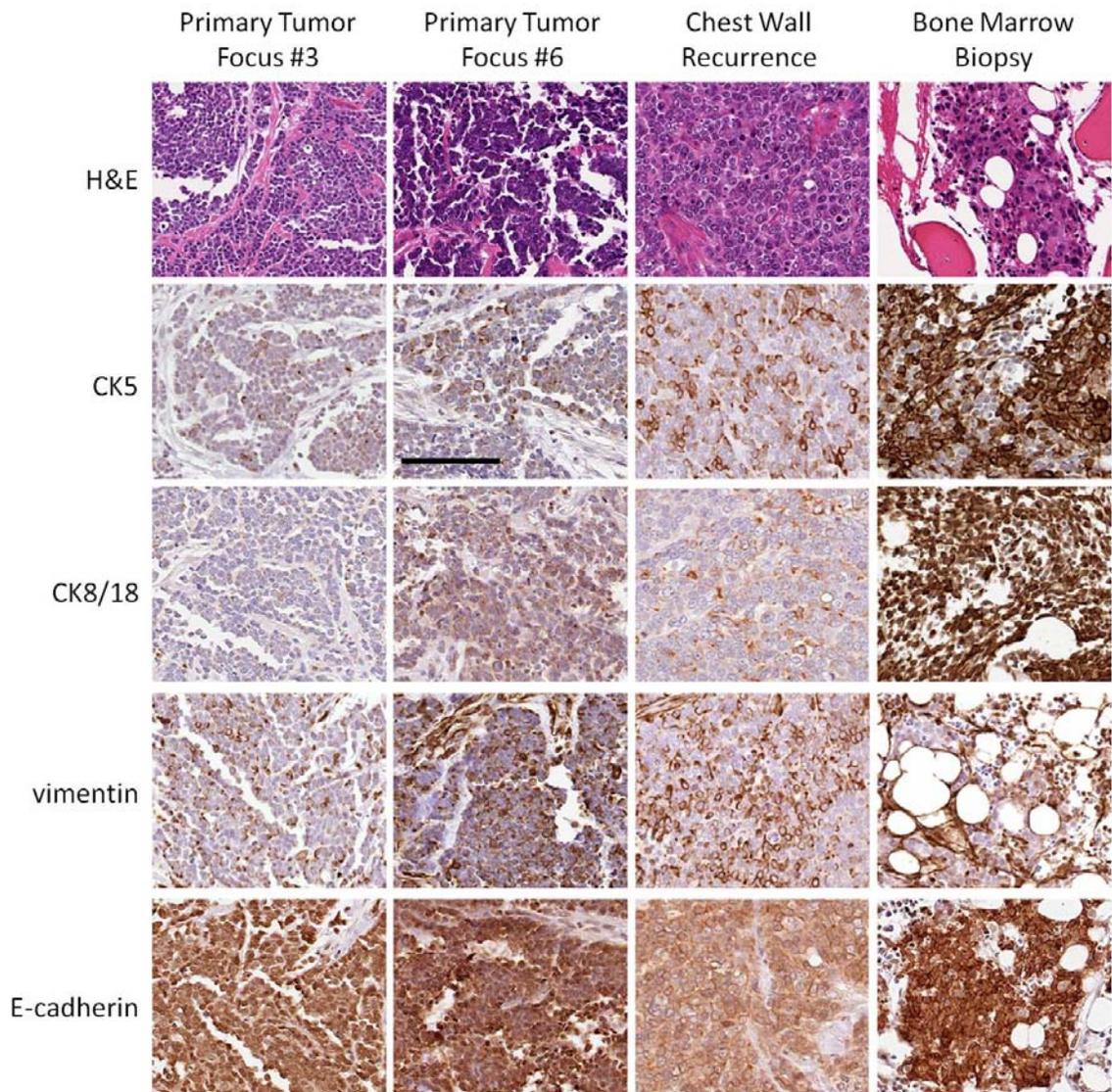


Figure 3.1: Human breast cancer specimen displays morphological and biochemical evidence of EMT/MET.

Images of the patient's primary tumor focus #3 (left column), focus #6 (second column), chest wall recurrence (third column), and bone marrow biopsy (right column) showing H&E staining and immunohistochemistry for CK5, CK8/18, vimentin, and E-cadherin.

Table 3.1: Characterization of human primary tumor, chest wall recurrence, and bone marrow metastasis.

Qualitative evaluation of expression based on *in situ* hybridization for Her2 and immunohistochemistry for all other markers. nt= not tested.

Marker	Primary Foci #1-5	Primary Foci #6	Chest Wall Recurrence	Bone Marrow Metastasis
ER	(-)	(-)	nt	nt
PR	(-)	(-)	nt	nt
HER2/neu	Not amplified	Not amplified	nt	nt
CK5	(+)	(++)	(++)	(+++)
CK14	(+)	(+)	(+)	(+)
CK8/18	(+)	(++)	(+)	(+++)
E-cadherin membrane	(++)	(+)	(+)	(+++)
cytoplasmic	(++)	(++)	(+)	(++)
vimentin	(+)	(+++)	(+++)	(+)

but not vimentin (Figure 3.1, right column). These observations show that the original primary tumor contained both epithelial and mesenchymal regions, the chest wall recurrence expressed a combination of mesenchymal and epithelial markers suggesting an intermediate phenotypic state, and the metastatic lesions in the bone expressed epithelial markers. A complete list of markers expressed in the primary tumor, chest wall recurrence, and bone marrow metastasis is provided in Table 3.1. Taken together, these observations provide morphologic and biochemical evidence of EMT/MET in a human triple-negative breast cancer.

3.2.2 Establishment of DKAT Cell Line *In Vitro*

Cells isolated from the patient's malignant pleural effusion rapidly adapted to tissue culture conditions. Forty-eight hours after isolation (passage 1) DKAT cells began proliferating in culture with a doubling time of approximately 24 hours (data not shown). The DKAT cell line has been maintained continuously in culture for >70 passages. Twenty-five metaphase DKAT cells (passage 3) were subjected to spectral karyotyping (SKY) analysis and an additional 9 cells were G-banded and karyotyped (Figure 3.2). The modal chromosome number was 56. In addition to 29 cells with the hyperdiploid chromosome number, 3 cells had hypopentaploid chromosome number (104-109 chromosomes) and 2 cells had either 117 or 127 chromosomes. Among the predominant hyperdiploid cells, two clones, a stemline and a sideline 1, were identified. The only difference between the stemline and sideline 1 is the presence of one double minute (dmin) in a sideline. Because of its small size, the origin of this double minute could not be identified with confidence. Sideline 2 is near-tetraploid and represents a doubling of the stemline, with some random chromosome losses and a few non-clonal aberrations in the cells, and sideline 3 is a composite karyotype of 2 cells with the highest chromosome numbers.

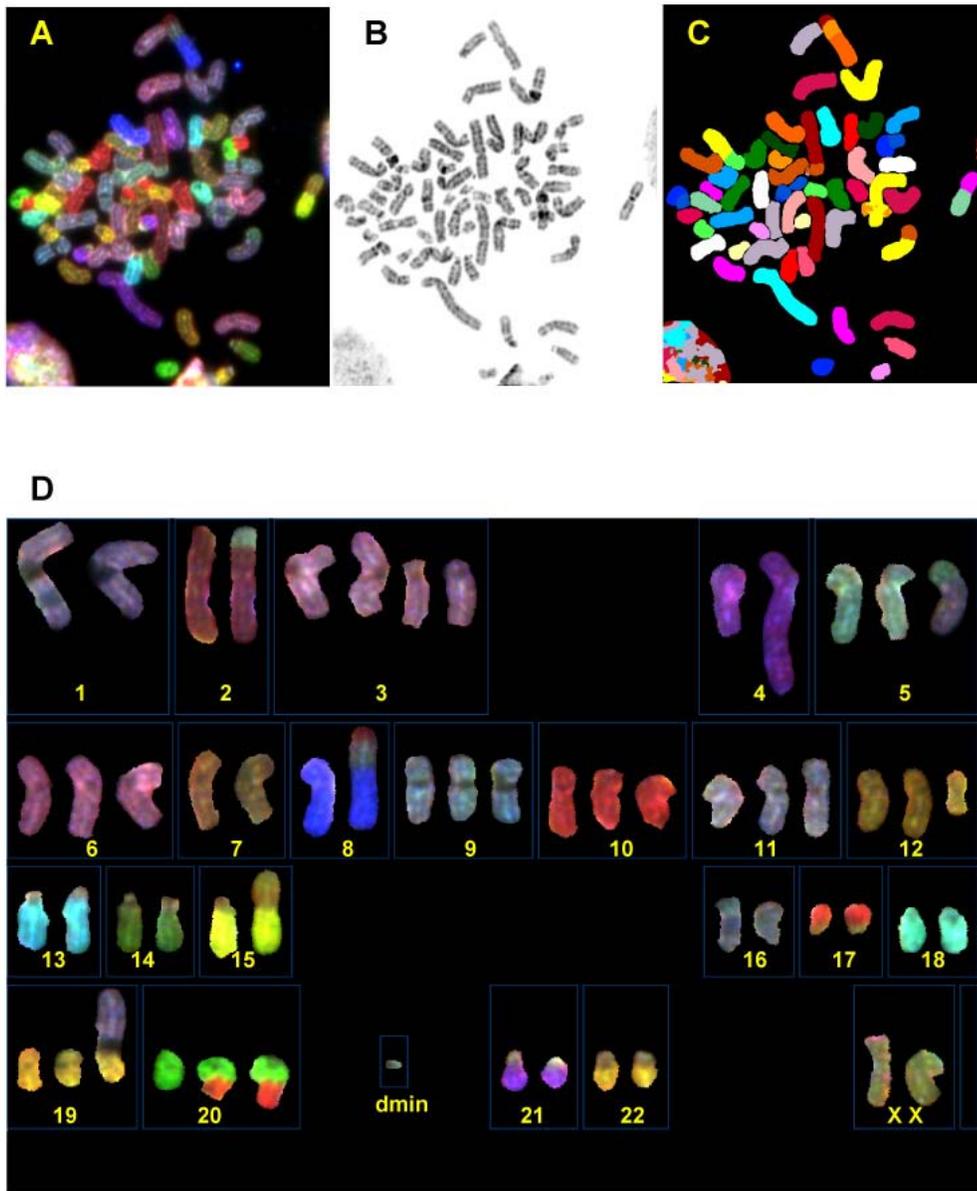


Figure 3.2: DKAT cell line karyotype.

A representative mitotic DKAT cell from sideline 1 is shown. **A.** Metaphase cell with chromosomes shown in SKY display colors. **B.** Inverted and contrast-enhanced DAPI image of the same metaphase cell. **C.** The same metaphase cell with chromosomes shown in spectra-based classification colors. **D.** Spectral karyotype of the same metaphase cell shown in SKY display colors.

3.2.3 DKAT Cell Line Maintains a Basal-like Phenotype *In Vitro*

Aggressive triple-negative breast cancers frequently contain mutations in the coding sequences of the *TP53* and *PIK3CA* genes [Hennessy et al 2009, Sorlie et al 2001]. Sequencing of exons 5-9 of the *TP53* gene in DKAT cells identified a single point mutation in exon 8 at codon 273 (CGT > CAT) resulting in replacement of arginine with histidine (R273H) within the DNA binding domain (data not shown) [Cho et al 1994]. This missense mutation has been previously reported in a number of human cancer cell lines including the MDA-MB-468 breast cancer cell line, and reported to be deleterious to p53 function [Dong et al 2007, Nigro et al 1989]. In breast tumors, *PIK3CA* mutations have been found to frequently occur in exons 9 and 20 [Bachman et al 2004]. Sequencing of *PIK3CA* mutation hotspots in exons 9 and 20 from DKAT genomic DNA revealed no mutations within the coding region of these two exons.

A variety of techniques were used to test DKAT cells for baseline expression of proteins associated with triple-negative breast cancers and an aggressive phenotype including markers of EMT, progenitor-like cell markers, and markers of PI3K-Akt pathway activation. Immunohistochemical analysis showed that DKAT cells did not stain for ER or PR, but stained positively for EGFR (data not shown). Fluorescence in situ hybridization (FISH) demonstrated diploid copy number of

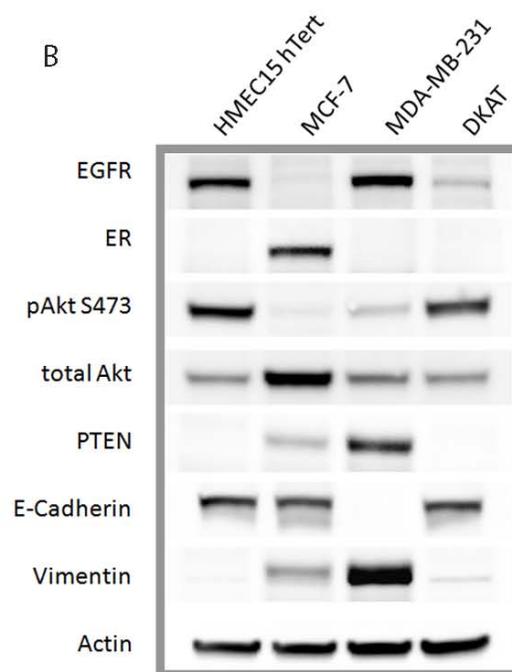
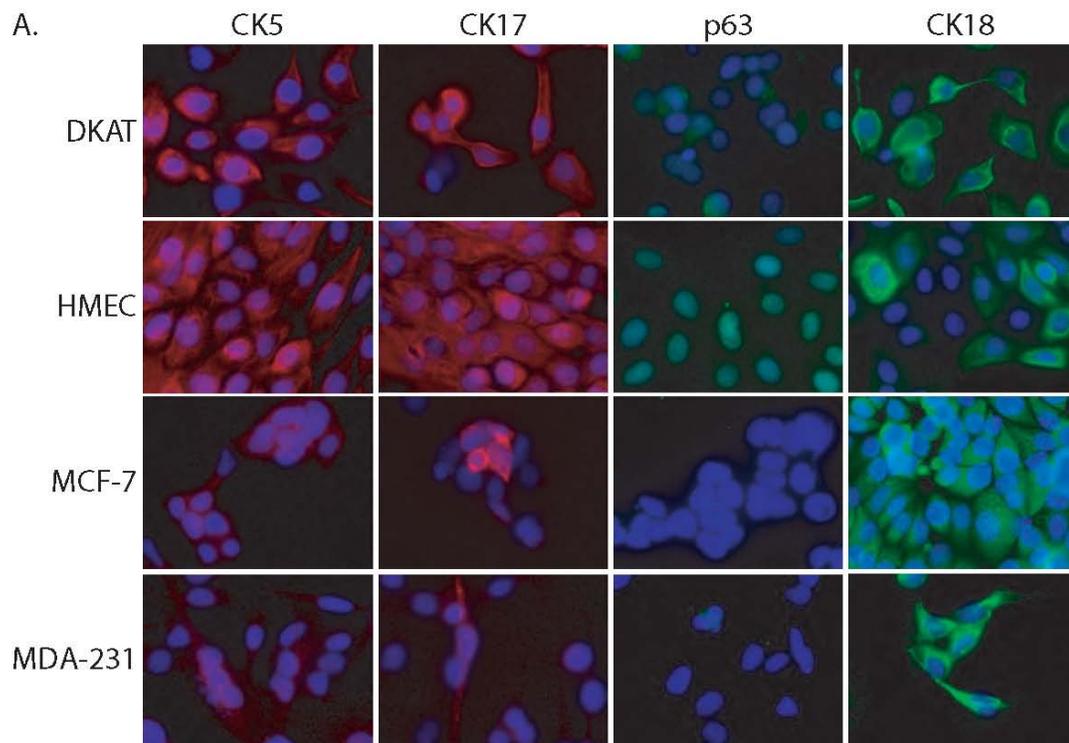


Figure 3.3: DKAT cells express markers of basal epithelial cells.

Figure 3.3 (continued): A. Immunofluorescence for CK5, CK17, p63 and CK18 in DKAT, immortalized HMEC, MCF-7, and MDA-MB-231 cells. **B.** Western blotting of total cell lysate from immortalized non-tumorigenic HMECs, luminal MCF7 cells, mesenchymal MDA-MB-231 cells, and DKAT cells.

HER2, consistent with the triple-negative primary human tumor (data not shown).

Immunofluorescence and western blotting showed that DKAT cells express markers consistent with basal mammary epithelial cells, including CK5 and CK17, and p63 (Figure 3.3A). Interestingly, DKAT cells also express the luminal epithelial cell marker CK18. However, all other cell lines tested also expressed CK18, including the mesenchymal MDA-MB-231 line (Figure 3.3A). Western blot analyses demonstrated that DKAT cells have a high level of Akt phosphorylation at Serine 473, a marker of Akt activation, and low expression of PTEN relative to other breast cancer cell lines tested (Figure 3.3B). Low expression of PTEN is common in triple-negative breast cancers, and represents an alternate method of PI3K-Akt pathway activation in the absence of *PIK3CA* activating mutation, which the DKAT cells do not contain. Taken together, these observations demonstrate that under normal tissue culture conditions, DKAT cells express markers of basal mammary epithelial cells and PI3K/AKT pathway activation.

3.2.4 DKAT Cells are Highly Invasive

Because a subset of basal-type breast cancers is highly invasive and the human tumor from which the cell line was derived invaded into the chest wall and metastasized to several organs, we tested the DKAT cell line for its invasive potential *in vitro*. Transwell migration and invasion assays were performed comparing DKAT cells to MDA-MB-231 cells, which are reported to be highly invasive *in vitro* and in xenograft experiments by many groups. As shown in Figure 3.4, the DKAT cells showed 1.7 and 2.6 fold more cells having migrated through the transwell and invaded through the matrigel, respectively, compared to MDA-MB-231 cells after 18 hours.

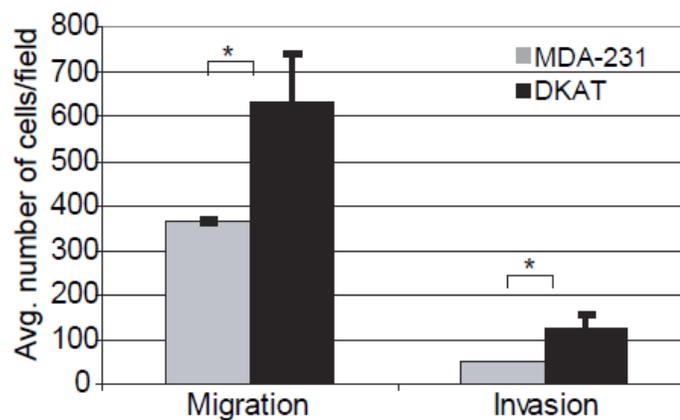


Figure 3.4: DKAT cells are highly invasive and migratory.

In vitro transwell migration and invasion assays comparing DKAT cells to MDA-MB-231 cells. Data averaged from 3 independent experiments. Error bars represent standard error, (*) $p < 0.05$.

3.2.5 DKAT Cells Cluster with Other Basal-Like Breast Cancer Cell Lines

In order to assess the relationship of DKAT cells to the previously published breast cancer subtypes, we performed unsupervised hierarchical clustering of DKAT transcript expression under normal culture conditions. Comparing our experimental data with a previously published analysis of breast cancer cell lines [Charafe-Jauffret et al 2006], we found that the DKAT gene expression profile clustered with a number of cell lines previously categorized as basal-like (Figure 3.5). DKAT cells cluster most closely with SUM-149 cells, but also with 184B5, and the immortalized non-tumorigenic MCF-10A and HMEC cells. Parallel analysis of MDA-MB-231 and HMEC transcripts prepared as controls showed a high correlation to the previously published microarray data ($r= 0.70$ and $r= 0.84$, respectively), validating the effectiveness of combining these two data sets.

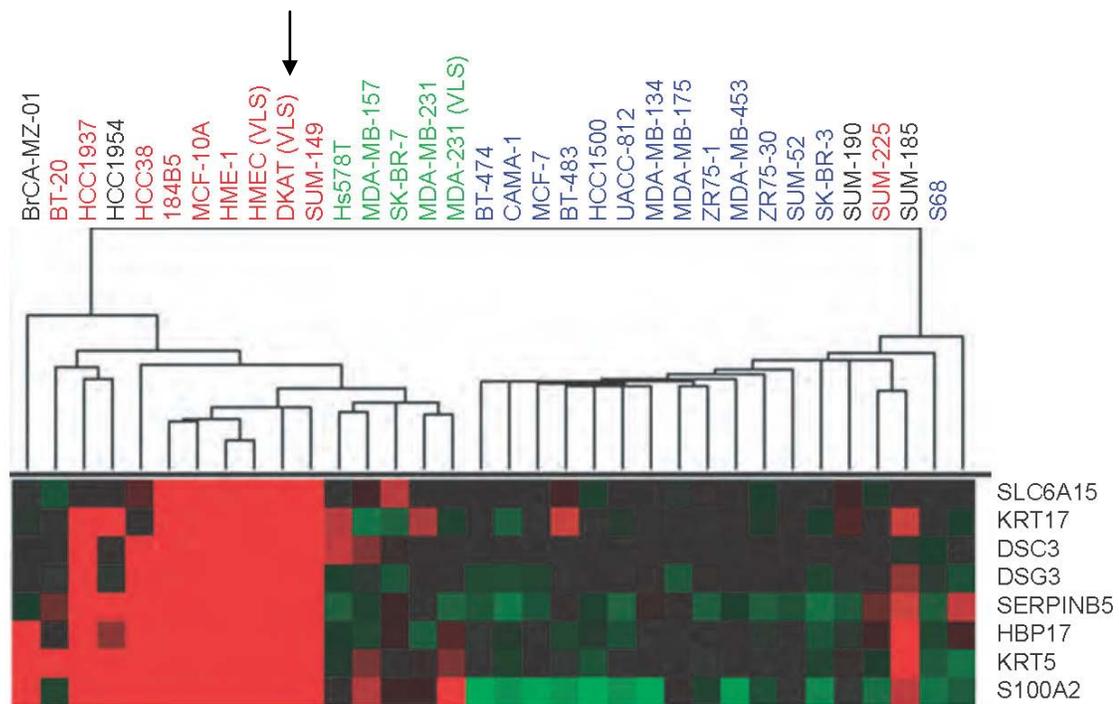


Figure 3.5: The DKAT cell line clusters with other basal-like breast cancer cell lines.

Results from DKAT, MDA-MB-231, and HMEC Affymetrix HG-U133 2+ arrays were analyzed by unsupervised hierarchical clustering with a published data set [Charafe-Jauffret et al 2006]. Cell lines are labeled by breast cancer subtype; luminal-like (blue); basal-like (red); mesenchymal-like (green); unknown subtype (black). Displayed is an expanded view of selected gene cluster that contained basal-specific gene expression.

3.2.6 The DKAT Cell Line Contains a Population of Tumor Initiating Cells

The basal-like subtype of breast cancers are reported to often contain a high proportion of cells in the CD44⁺/CD24^{-low} population identified as being enriched for tumor initiating capability in mouse xenografts [Al-Hajj et al 2003, Honeth et al 2008]. Since the DKAT cell line was isolated from a triple-negative cancer and its

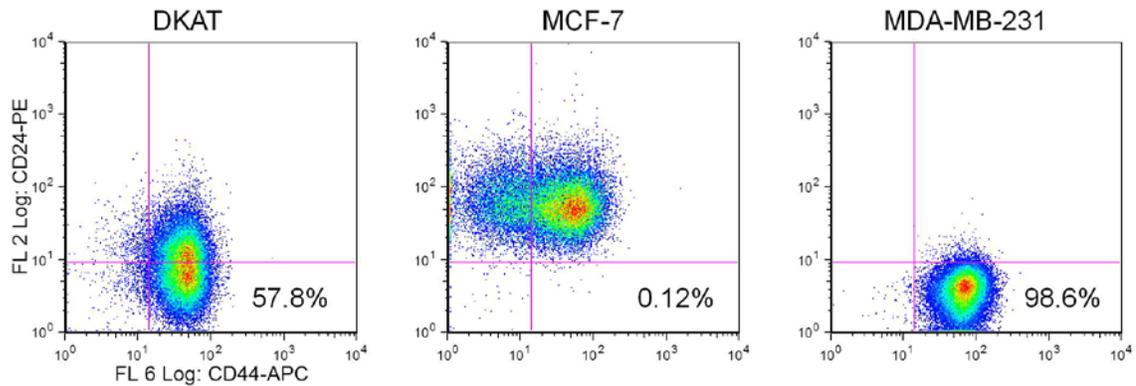


Figure 3.6: CD44 and CD24 staining of breast cancer cell lines.

Flow cytometric analysis of DKAT-MEGM, MCF-7 and MDA-MB-231 cells stained for CD44 and CD24. Numbers indicate the percentage of cells with a CD44⁺/CD24^{-low} profile.

gene expression clustered with other models of basal-like breast cancer, we tested the cells for cell surface expression of CD44 and CD24. Flow cytometric analysis revealed that 58 percent of DKAT cells are of the CD44⁺/CD24^{-low} phenotype that is reported to be enriched for tumorigenic and stem cell-like activity, and is associated with cells that have undergone EMT [Mani et al 2008] (Figure 3.6).

3.2.7 A Single DKAT Cell is Capable of Generating Tumorspheres Containing Both Luminal and Basal Epithelial Cells

Because the CD44⁺/CD24^{-low} population of cells is reported to be enriched for stem cell-like activity, we next tested whether a single DKAT cell was capable of forming multi-lineage cell clusters in non-adherent mammosphere culture

conditions. DKAT cells formed solid mammospheres (tumorspheres) with a tumorsphere formation efficiency between 3 and 10 percent (data not shown). Tumorspheres stained strongly for CK5, CK17, and CK18 (Figure 3.7), stained weakly for SMA, and were negative for CK14 by immunofluorescence (data not shown). Dual staining for CK5 and CK18 demonstrated that individual tumorspheres contained both CK18⁺/CK5^{low} luminal epithelial-like cells and CK18^{low}/CK5⁺ basal epithelial-like cells (Figure 3.7, right panel). This suggests that DKAT cells contain a population of progenitor-like cells capable of giving rise to both luminal and basal epithelial cells.

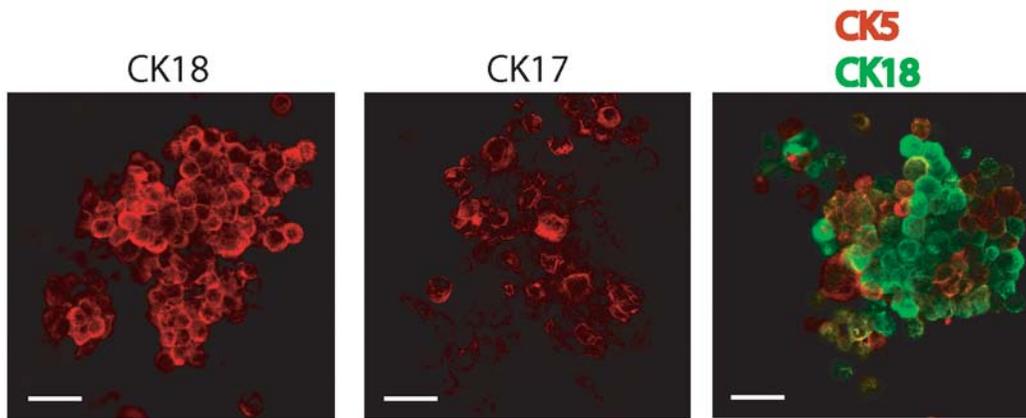


Figure 3.7 : DKAT cells form mammospheres containing both luminal and basal epithelial cells.

DKAT cells were dissociated into single-cell suspension with trypsin, and 1×10^4 cells were seeded into ultralow attachment 100mm dishes in supplemented mammosphere media. After 14 days, mammospheres were pelleted and stained for the indicated cytokeratins. Confocal images were acquired with a 63x objective. Scale bar = 40 μm .

3.2.8 Both CD44⁺/CD24^{-low} and CD44⁺/CD24^{hi} DKAT Cells Can Give Rise to Heterogenous Progeny

Stem cells represent a small population of cells which possess the unique ability to give rise to all other cell types within the body or tissue. Similarly, it is hypothesized that tumors may contain a small population of so-called cancer stem cells which can give rise to the heterogeneous cells of a tumor. In breast cancer, the CD44⁺/CD24^{-low} population of cells is reported to be enriched for stem cell-like activity. We previously showed that the DKAT cell line contains cells with a wide variety of CD24 expression levels (Figure 3.6). We therefore tested the ability of the different cell populations to give rise to heterogeneous progeny.

To test this, we stained for CD44 and CD24 expression and then sorted cells based on surface CD24 levels (Figure 3.8A). The CD44⁺/CD24^{-low} and CD44⁺/CD24^{high} cells were then cultured separately for 14 days then re-analyzed for CD44 and CD24 expression. As expected, after 14 days we found that the CD44⁺/CD24^{-low} cells had given rise to a population of cells with the same spectrum of CD24 expression as the original unsorted population (Figure 3.8C). However, we were surprised to find that after 14 days, the CD44⁺/CD24^{high} cells, which were expected to be more differentiated and therefore only able to give rise to more CD44⁺/CD24⁺ cells, also contained cells with a CD44⁺/CD24^{-low} profile (Figure 3.8B). While the percentage of CD44⁺/CD24^{-low} cells was less in this group, our results suggest that either our

sorting was not stringent enough and some CD44⁺/CD24^{-/low} cells contaminated the CD44⁺/CD24^{high} population, or that CD44⁺/CD24⁺ DKAT cells have the ability to de-differentiate into more stem-like cells in culture.

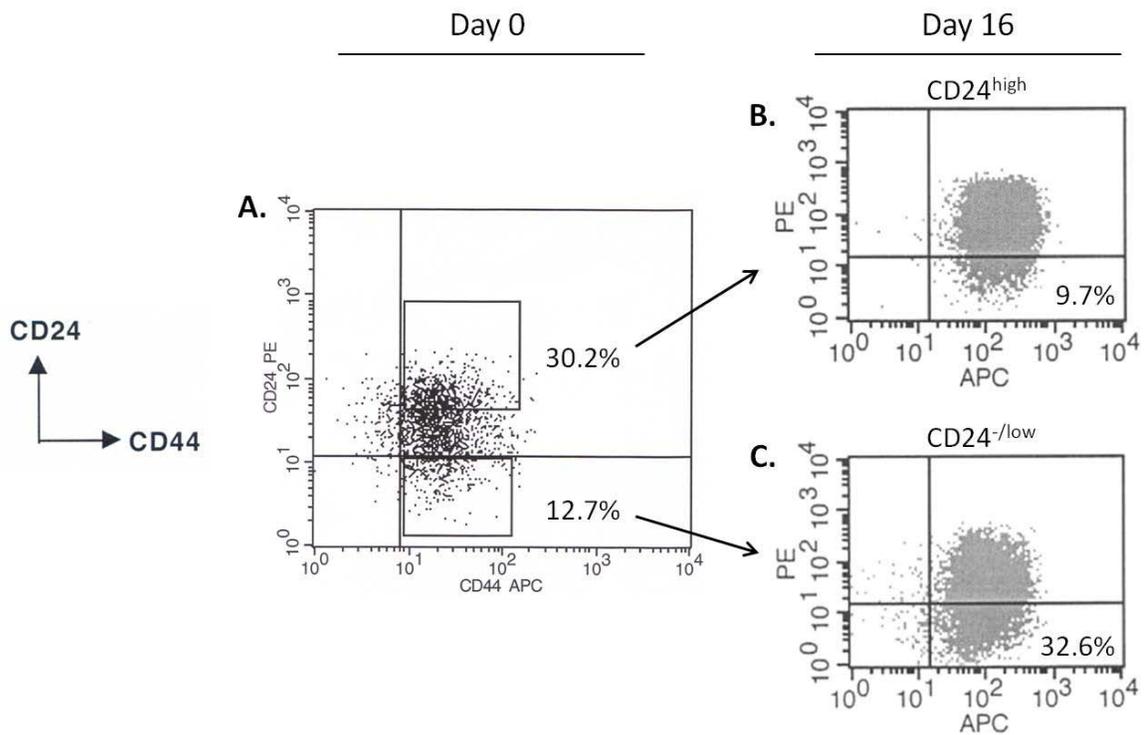


Figure 3.8: De-differentiation of CD44⁺/CD24⁺ DKAT cells.

A. DKAT cells were stained with CD44-APC and CD24-PE antibodies, then sorted into CD44⁺/CD24^{high} and CD44⁺/CD24^{-/low} populations by FACS. These cell populations were cultured separately for 16 days then re-analyzed for CD44 and CD24 cell surface expression (**B,C**). Numbers represent the percentage of cells with a CD44⁺/CD24^{-/low} profile.

3.2.9 Both CD44⁺/CD24^{-low} and CD44⁺/CD24⁺ DKAT Cells Form Tumors at a Dose of Ten Cells

In order to understand the regulation of the breast cancer stem cell population and develop therapies which specifically target these cells, additional models must be created which recapitulate the behavior of these cells in human cancers *in vivo*. The DKAT cell line represents a novel model of triple-negative breast cancer with a population of stem cell-like CD44⁺/CD24^{-low} cells. Our *in vitro* data suggests that this population does not display all the characteristics of a true stem cell-like population, so we next tested whether this population of cells was enriched for tumor initiation *in vivo* as reported for other breast cancer cell lines. In order to test this, we performed limiting-dilution injections of CD44⁺/CD24^{-low} or CD44⁺/CD24⁺ cells into the uncleared mammary fatpads of NOD/SCID mice. We observed a 100 percent rate of tumor formation for both CD44⁺/CD24^{-low} and CD44⁺/CD24⁺ cells at doses of 1×10^4 , 1×10^3 , and 1×10^2 injected cells. Surprisingly, injection of only 10 CD44⁺/CD24^{-low} or CD44⁺/CD24⁺ cells gave rise to a tumor in four of the 10 mouse mammary fat pads (Figure 3.9B). While tumors from CD44⁺/CD24^{-low} cells were larger on average than tumors derived from CD44⁺/CD24⁺ cells, this suggests that the putative stem cell-like CD44⁺/CD24^{-low} population of cells in the DKAT cell line is not enriched for tumor initiation capability compared to the

CD44⁺/CD24⁺ population. Importantly, since as few as 10 cells were able to repeatedly produce tumors, it is clear that the DKAT cell line is highly tumorigenic.

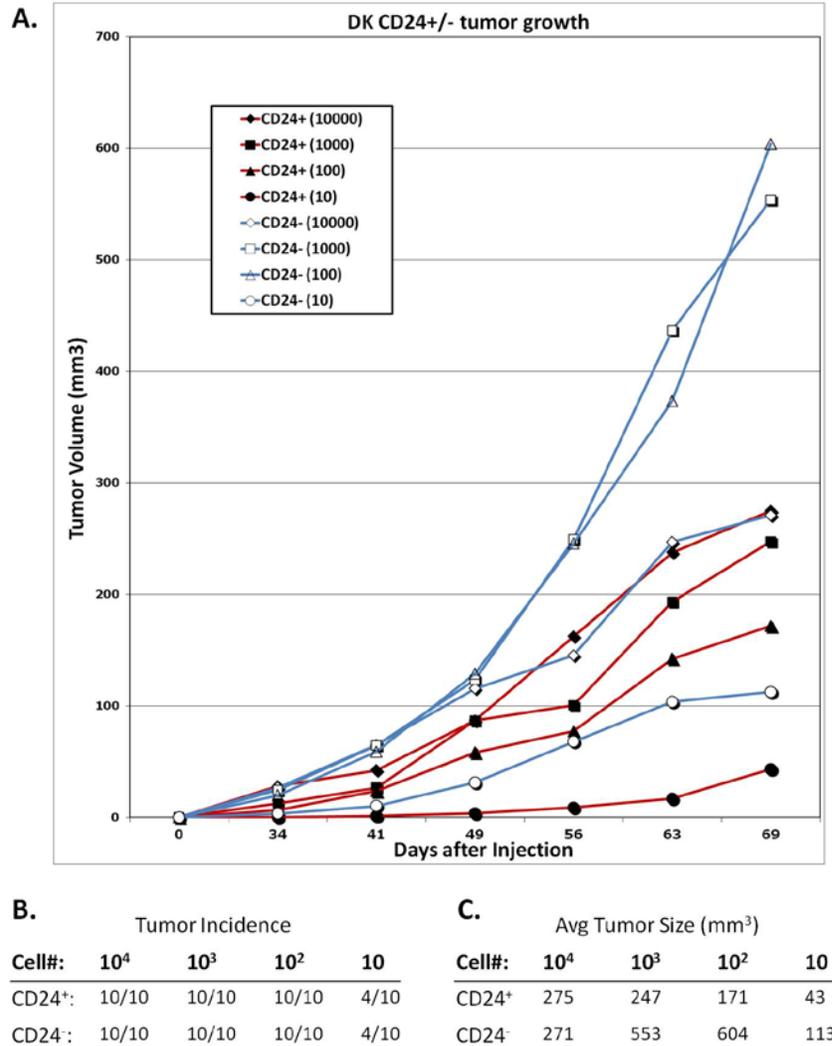


Figure 3.9: As few as ten CD44⁺/CD24^{-/low} or CD44⁺/CD24⁺ DKAT cells form tumors.

A. Growth curve of average tumor volume over time for four different doses of CD44⁺/CD24⁺ (red) or CD44⁺/CD24^{-/low} (blue) DKAT cells injected into uncleared mouse mammary fatpads. **B.** Tumor incidence for each group. **C.** Average tumor volume as measured for each group size at day 69.

3.3 Discussion

Recent advances in gene expression profiling have resulted in a deeper understanding of breast cancer as not a single disease, but rather a collection of malignancies with differential histopathological features and prognoses. The basal-like subtype has the worst prognosis of the five originally identified subtypes, and because many of these basal-like tumors do not rely on estrogen or Her2 signaling for survival and proliferation, we currently have no targeted therapeutics which are effective against these tumors. In order to understand the best ways to treat such tumors, cell lines which accurately model the biology of triple-negative tumors are extremely valuable. Here we report the isolation of the DKAT cell line from a highly aggressive triple-negative human breast cancer that displayed morphological and biochemical evidence of EMT and MET.

The human breast cancer from which the DKAT cell line was isolated was comprised of six distinct foci. All six of these foci stained strongly for CK5, consistent with the characteristics of basal-like and triple-negative breast cancers. Five of the six foci displayed an epithelial staining pattern, with strong staining for membranous E-cadherin and only weak, scattered staining for vimentin. However, one focus of the primary tumor, as well as the area of invasion into the chest wall, displayed a more mesenchymal phenotype. These areas stained strongly for

vimentin, while E-cadherin appeared more diffuse and mainly cytoplasmic (Figure 3.1). While EMT is most commonly discussed as involving downregulation of E-cadherin at the transcriptional level, there are numerous reports showing that relocalization of E-cadherin away from the membrane occurs during EMT-like changes in mouse xenograft studies of EMT [Barnes et al 2010, Brown et al 2011, Chetty et al 2008]. Interestingly, metastatic tumor cells in the bone marrow maintained CK5 expression and were found to have a staining pattern consistent with a reversion to an epithelial phenotype with both cytoplasmic and membranous E-cadherin and very low levels of vimentin (Figure 3.1).

One of the major arguments made by critics of the theory of EMT in cancer metastasis is that EMT is rarely seen in human cases. In support, they point out that metastases most often do not express mesenchymal markers, but very closely resemble the primary tumor. In response, proponents of the involvement of EMT in cancer suggest that the acquisition of mesenchymal traits may be transient or partial in nature. Our results are an important contribution to the field as we provide evidence in a human cancer of a transient acquisition of mesenchymal markers in an area of local invasion while a metastatic lesion shows an epithelial staining pattern similar to the original primary tumor cells.

Interestingly, we find that the DKAT cell line contains a population of CD44⁺/CD24^{-/low} cells corresponding to the tumor initiating or stem cell-like population described in the literature, but these cells are not enriched for tumor formation in orthotopic mouse xenografts, nor are they exclusive in their ability to give rise to heterogenous progeny. Specifically, we determined that as few as ten CD44⁺/CD24^{-/low} or CD44⁺/CD24⁺ cells are able to give rise to tumors when injected into the uncleared fat pads of nude mice. Additionally, *in vitro* we found that following FACS sorting and separate culture, both CD44⁺/CD24^{-/low} and CD44⁺/CD24⁺ cells were capable of giving rise to the putative breast cancer stem cell-like CD44⁺/CD24^{-/low} cells. While we cannot rule out contamination of the CD24⁺ population of cells with a few CD24^{low} cells, it is also possible that our observation is the result of CD44⁺/CD24⁺ cells dedifferentiating into CD44⁺/CD24^{-/low} cells. In support of the latter possibility, a study which was published during the course of our research has found that *in vitro*, single CD44⁺/CD24⁺ cells had the ability to give rise to both CD24^{low} and CD24^{high} cells. This study further reported that in mouse xenografts, CD44⁺/CD24⁺ cells gave rise to tumors containing both CD44⁺/CD24⁺ and CD44⁺/CD24⁺ cells [Meyer et al 2009]. This suggests that while the CD44⁺/CD24^{-/low} population may be enriched for tumorigenicity and stem cell-like features in some models, this population is not exclusive in this capacity, and these markers may not

be appropriate for identification of the stem cell-like cell population in basal-like or triple-negative breast cancer cells which have been passaged *ex-vivo*.

Although many human breast cancer cell lines have been developed from pleural fluid samples, the DKAT model is notable for the exceptional ease with which these cells adapted to *in vitro* growth. Given the swift acclimation to tissue culture and the presence of few, if any, contaminating non-epithelial cells in the initial passage, the DKAT line did not appear to undergo a rigorous selection process for survival *in vitro*. The DKAT cells may therefore retain many features of the original tumor cells, including the highly invasive behavior that contributed to the rapid demise of the patient from widespread breast cancer metastases. In support of that, we show that the DKAT cells are highly migratory and invasive in *in vitro* assays, even when compared to the highly metastatic MDA-MB-231 breast cancer cell line. They also maintain expression of markers of the basal mammary epithelial cell layer including basal-type cytokeratins CK5 and CK17 as well as the transcription factor p63.

The ability of breast cancer cells to reversibly adapt to diverse microenvironments, or plasticity, is hypothesized to underlie the aggressive behavior of a subset of triple-negative [ER/PR(-/-), HER2/neu-wt] breast cancers. This hypothesis, based on the highly metastatic behavior of many triple-negative

breast cancers and their poor prognosis, has been bolstered in recent years by observations that gene expression patterns of triple-negative breast cancers share a strong degree of similarity with the gene expression of cells which have undergone EMT.

One of the most interesting characteristics of the tumor from which the DKAT cell line was derived is the observation of mesenchymal markers in the area of invasion into the chest wall, as well as in one focus of the primary tumor. Because both the majority of the primary tumor and the metastatic lesions in the bone marrow were observed to express epithelial markers, we conclude that this data supports the existence of a transient epithelial-mesenchymal transition among invading cells. Based on morphology and immunostaining, it was not possible to determine whether this range of phenotypes was due to an outgrowth of cell populations with a fixed phenotype, or rather due to inherent phenotypic plasticity of the tumor cells. Thus it remains to be seen if the epithelial-mesenchymal plasticity of the DKAT cell line can be recapitulated *in vitro*, which will be explored in the following chapter.

4 Phenotypic Plasticity in Triple-Negative Breast Cancer is Regulated by Zeb1

4.1 Introduction

Epithelial-mesenchymal transition (EMT) and the reverse process, mesenchymal-epithelial transition (MET), are normal processes important for embryonic development and tissue homeostasis, and are thought to contribute to the metastatic dissemination of cancer cells [Burdsal et al 1993, Davies 1996, Hay 1995, Thiery 2003, Thompson et al 2005]. During EMT, cells of epithelial origin lose epithelial characteristics, such as tight cell-cell junctions and apical-basal polarity, and acquire a mesenchymal phenotype associated with increased migratory behavior and decreased cell-cell contact [Savagner 2001, Thiery 2002]. At the molecular level, EMT is characterized by 1) loss of expression of epithelial cell junction proteins including E-cadherin, claudins, and occludins, 2) increased expression of mesenchymal markers including vimentin and smooth muscle actin, 3) acquisition of a spindle-like morphology, and 4) cytoskeleton reorganization [Burdsal et al 1993, Thiery 2003]. The reverse process, MET, is characterized by a loss of expression of mesenchymal markers and restoration of epithelial markers and morphology [Chaffer et al 2007, Christ and Ordahl 1995]. Given the role of

EMT/MET in regulating invasion and motility during embryogenesis, it has been hypothesized that reversible or transient EMT/MET, or plasticity, might similarly be important during breast cancer invasion and metastasis [Chaffer et al 2007, Thiery 2002, Yang and Weinberg 2008].

A wide variety of signaling pathways and proteins have been implicated in EMT in cancer progression, including members of the Snail, Twist, and Zeb families of transcription factors. Snail, Twist, and Zeb1 are each capable of inducing EMT in breast cancer cells *in vitro*, in part through binding to the promoter of E-cadherin, an important component of adherens junctions in epithelial cells, and repressing its transcription [Peinado et al 2007]. While Snail and Twist have received the most study to date, recent evidence points to the zinc-finger transcription factor Zeb1 as a critical regulator of the mesenchymal phenotype, and its expression is regulated by several pathways which control epithelial morphology and function in both normal development and cancer [Aigner et al 2007, Burk et al 2008, Eger et al 2005].

While an extensive amount of research has focused on the molecular regulation of epithelial and mesenchymal markers in breast cancer, less is known about the microenvironmental cues which are relevant to induction of EMT in human disease. Interleukin-6 (IL-6) is a pleiotropic cytokine initially identified as a mediator of inflammatory and immune responses, but an increasing number of

studies have implicated IL-6 in various cancer types including breast cancer [Kishimoto 1989, Schafer and Brugge 2007]. Increased IL-6 expression is associated with increased breast tumor grade and increased metastatic burden, and higher serum IL-6 levels are associated with poor prognosis in breast cancer patients [Kozlowski et al 2003, Salgado et al 2003, Zhang and Adachi 1999]. While IL-6 has recently been demonstrated to be capable of inducing EMT in epithelial breast cancer cell lines *in vitro* [Sullivan et al 2009], the relationship between IL-6 and Zeb1 during EMT of breast cancer cells is currently unknown.

The study of plasticity in human breast cancer is currently limited by a lack of appropriate models which can reversibly transition from the epithelial to mesenchymal state. In the previous chapter we have detailed the isolation and characterization of the DKAT cell line, a novel model of triple-negative breast cancer that was isolated from a rapidly progressing, treatment-resistant, metastatic human breast cancer. In this chapter, we demonstrate that similar to the human tumor, in xenograft experiments DKAT cells form heterogeneous tumors with regions of both epithelial and mesenchymal characteristics. Further, DKAT cells are able to undergo EMT/MET in response to altered culture conditions *in vitro*, suggesting inherent epithelial-mesenchymal plasticity. Zeb1 is a key regulator of the mesenchymal phenotype, and its expression is regulated by IL-6, suggesting that IL-6 or its

receptors may represent an attractive therapeutic target. The DKAT cell line provides a new model to investigate the molecular mechanisms of phenotypic plasticity and its role in aggressive triple-negative breast cancers.

4.2 Results

4.2.1 DKAT Cells Demonstrate *In Vitro* Plasticity

DKAT cells were derived from a breast cancer that exhibited a range of epithelial and mesenchymal markers and was highly metastatic, but it is unclear whether this phenotypic diversity was the result of 1) outgrowth or evolution of specialized subpopulations of cells within the primary tumor or 2) phenotypic plasticity inherent within the DKAT cells. We tested whether the DKAT cell line retained the phenotypic plasticity observed in the primary tumor by altering culture conditions and testing for *in vitro* EMT/MET.

To test for the ability of DKAT cells to undergo *in vitro* EMT, a portion of cells were switched from serum-free MEGM, their initial culture media, to Stromal Cell Growth Media (SCGM) containing insulin, bFGF, and 5% FBS. Passage matched cells were maintained in MEGM as a control. Upon being switched to growth in SCGM, DKAT cells rapidly underwent a morphological change to a more spindle-shaped and less cobblestone appearance (Figure 4.1A, left column). After 14 days,

immunofluorescence studies showed that DKAT cells cultured in MEGM maintained positive staining for the epithelial markers CK5, CK18, and E-cadherin, which was found both at the membrane and throughout the cytoplasm. Staining for the mesenchymal marker vimentin was weak and scattered. Strikingly, after 14 days in SCGM, DKAT cells had become predominately vimentin positive and E-cadherin negative, though a small percentage of cells retained an epithelial staining pattern (Figure 4.1A).

Additionally, we observed a significant decrease in epithelial markers CK5 (Figure 4.1A, right column), consistent with a shift from expression of cytokeratin intermediate filaments to vimentin during EMT [Micalizzi et al 2010]. While CK5 was uniformly downregulated, CK18 was still expressed at low levels in some of the cells, as seen in other cell lines classified as mesenchymal, such as MDA-MB-231 cells. Western blotting confirmed these changes, showing dramatically increased expression of the mesenchymal markers vimentin and N-cadherin after 14 days in SCGM, and decreased levels of E-cadherin and additional epithelial markers CK17 and claudin-1 (Figure 4.1B).

We next tested whether DKAT cells induced to undergo *in vitro* EMT demonstrated increased migratory ability. Because DKAT cells undergo EMT in response to serum-containing media, the serum-containing chemoattractant used in

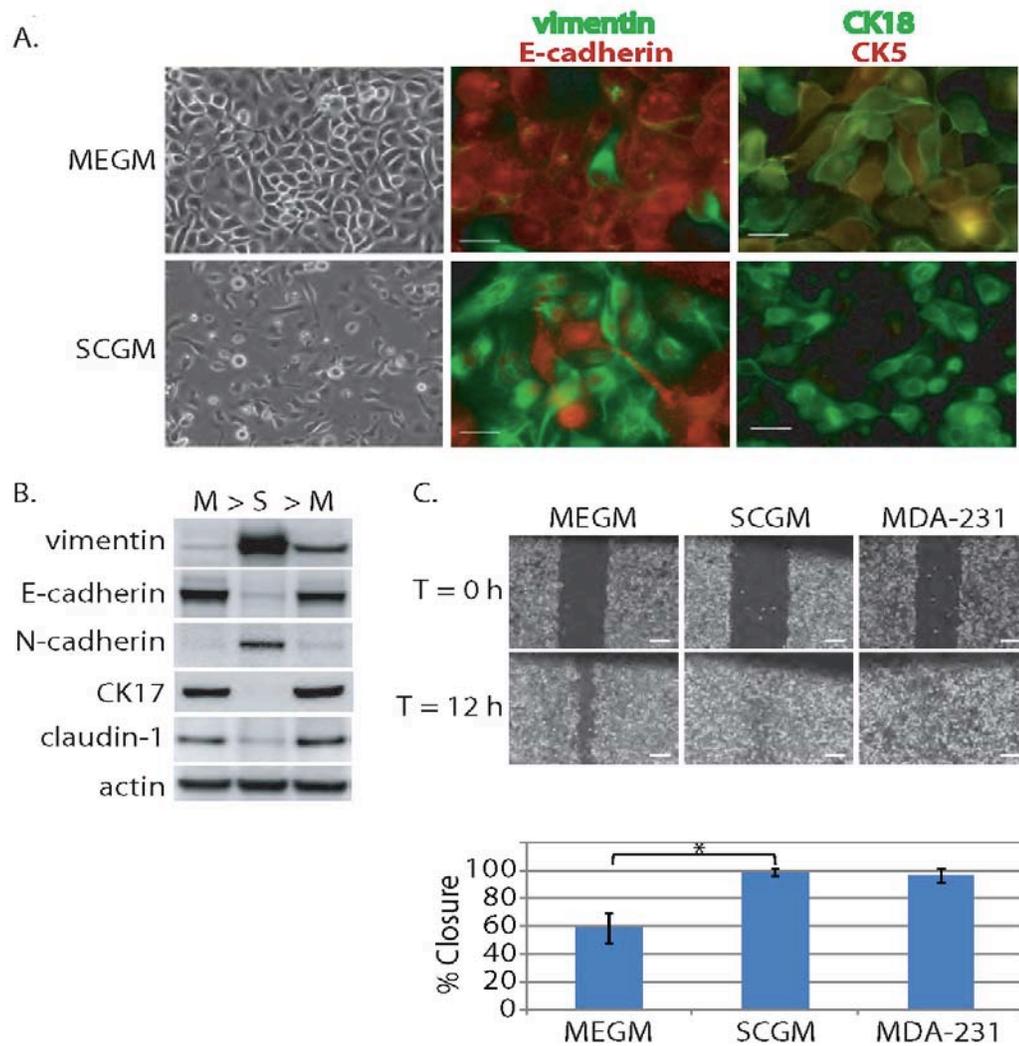


Figure 4.1: DKAT cells undergo morphologic and phenotypic changes consistent with *in vitro* EMT.

A. Phase contrast or immunofluorescence images of DKAT cells cultured in either MEGM (upper) or SCGM for 14 days (lower). Phase contrast images were acquired with a 20x objective, scale bar = 40 μ m. IF images were acquired with a 40x objective, scale bar = 20 μ m. Cells were stained simultaneously for vimentin (green) and E-cadherin (red), or CK18 (green) and CK5 (red). **B.** Cell lysates from passage-matched cultures of DKAT cells that had been induced to undergo EMT in SCGM for 10

Figure 4.1 (continued): passages and then returned to MEGM for 5 passages to induce MET were analyzed by western blot for epithelial and mesenchymal markers. M=MEGM, S=SCGM. C. Scratch wound healing assay comparing DKAT cells grown in MEGM or SCGM (14 days) and MDA-MB-231 cells. Images (upper panel) were taken at the time of the scratch (T=0) and 12 hours later with a 5x objective. Scale bar = 200 μ m. Results (lower panel) are expressed as the percentage of the scratch area closed at 12hrs as measured by ImageJ software, and are an average of 12 scratches per sample, (*) $p < .05$.

a standard transwell assays would also induce EMT, skewing the results of any attempt to compare migration of DKAT cells cultured in MEGM and SCGM.

Therefore, we utilized a scratch wound-healing assay in which MEGM cells would not be exposed to serum. Following the creation of a scratch in the confluent monolayer, DKAT cells grown in SCGM for 14 days migrated to fill the area of the scratch more quickly than DKAT cells in MEGM, with almost 100% closure by DKAT-SCGM cells after 12 hours versus 60% closure for DKAT-MEGM cells (Figure 4.1C). This increased migratory behavior further supports the mesenchymal phenotype of DKAT cells in SCGM.

To test for the ability of the cells to undergo *in vitro* MET, DKAT cells cultured in SCGM for 10 passages and exhibiting a mesenchymal phenotype were returned to MEGM media. Five passages after being returned to MEGM, western blotting showed a dramatic decrease in vimentin expression and re-expression of E-cadherin (Figure 4.1B), demonstrating a reversion to the original epithelial

phenotype. Taken together, these observations show that DKAT cells undergo a reversible EMT/MET *in vitro* in response to altered culture conditions, suggesting inherent phenotypic plasticity.

We next examined the gene expression changes of DKAT cells following the SCGM-induced EMT. DKAT mRNA expression was compared in cells grown for 14 days in either MEGM or SCGM. As expected, DKAT cells grown in SCGM media demonstrated a significant decrease in epithelial-specific gene expression, including genes that regulate adhesion and cell-cell junctions. A significant increase in genes associated with a mesenchymal phenotype and cellular transformation was also observed. A partial listing of differentially expressed genes is presented in Table 4.1. Of note, there was a decrease in expression of *KRT6C* (CK6; 131.8-fold), *KRT5* (CK5; 37.0-fold), *TP63* (p63; 26.2-fold), *KRT17* (CK17; 7.8-fold), *KRT14* (CK14; 3.0-fold), and *CLDN1* (claudin-1; 2.9-fold) and an increase in *TGFA* (TGF-alpha; 2.3-fold), *TGFBI* (TGF-beta-induced; 2.6-fold), *LOX* (lysyl oxidase; 2.9-fold), *ALDH1A3* (ALDH-1a3; 3.6-fold), *CDH2* (N-cadherin; 5.6-fold), and *FGF2* (FGF2; 6.0-fold).

To confirm the results of the gene expression studies, we performed western blotting to detect changes in expression of claudin-1, CK17, and N-cadherin from protein lysates of DKAT cells that had undergone EMT/MET as described above. As expected, claudin-1 and CK17 protein levels were strongly decreased in DKAT cells

Table 4.1: Differential gene expression of DKAT cells following SCGM-induced EMT.

List of genes related to the epithelial phenotype, cell-cell junctions, and motility with altered expression following 14 days of culture in SCGM to induce EMT. Microarray experiments were performed as described in Materials and Methods.

Fold Change (MEGM to SCGM)	Probe Set	Gene Symbol	Description
-131.8	213680_at	KRT6C	keratin 6C
-37.0	201820_at	KRT5	keratin 5
-26.2	209863_s_at	TP63	tumor protein p63
-8.8	214580_x_at	KRT6A	keratin 6A
-9.1	211194_s_at	TP63	tumor protein p63
-7.8	205157_s_at	KRT17	keratin 17
-6.7	204105_s_at	NRCAM	neuronal cell adhesion molecule
-4.4	208083_s_at	ITGB6	integrin, beta 6
-4.0	200953_s_at	CCND2	Cyclin D2
-3.0	209351_at	KRT14	keratin 14
-2.9	222549_at	CLDN1	claudin 1
-3.0	229041_s_at	ITGB2	integrin, beta 2
-3.0	204990_s_at	ITGB4	integrin, beta 4
-3.0	217312_s_at	COL7A1	collagen, type VII, alpha 1
-2.5	209270_at	LAMB3	laminin, beta 3
-3.0	211473_s_at	COL4A6	collagen, type IV, alpha 6
-2.4	215177_s_at	ITGA6	integrin, alpha 6
2.3	205016_at	TGFA	transforming growth factor, alpha
2.6	201506_at	TGFBI	transforming growth factor β -induced
2.7	205422_s_at	ITGBL1	integrin, beta-like 1
2.8	212489_at	COL5A1	collagen, type V, alpha 1
2.9	215446_s_at	LOX	lysyl oxidase
3.4	204726_at	CDH13	cadherin 13, H-cadherin
3.5	205885_s_at	ITGA4	integrin, alpha 4
3.6	203180_at	ALDH1A3	aldehyde dehydrogenase 1 family, memberA3

3.9	226622_at	MUC20	mucin 20, cell surface associated
4.2	202016_at	MEST	mesoderm specific transcript homolog
5.6	203440_at	CDH2	cadherin 2, type 1, N-cadherin
5.7	224480_s_at	MAG1	lung cancer metastasis-associated protein
6.0	204422_s_at	FGF2	fibroblast growth factor 2 (basic)

grown in SCGM, while expression of N-cadherin was markedly increased, confirming the results of the microarray data (Figure 4.1B).

4.2.2 A Single DKAT Cell is Capable of Generating Tumorspheres Containing both Epithelial and Mesenchymal Cells

Our earlier experiments show that DKAT cells exhibit *in vitro* plasticity and that individual DKAT cells are capable of giving rise to both luminal and basal epithelial cells in non-adherent tumorsphere culture. To determine whether individual cells can also give rise to both epithelial and mesenchymal cells under these conditions, we stained these tumorspheres for the epithelial markers E-cadherin and β -catenin, as well as the mesenchymal marker vimentin. DKAT tumorspheres contained populations of cells that were either E-cadherin(+)/vimentin(-) or E-cadherin(-)/vimentin(+) (Figure 4.2). The ability of DKAT cells to form tumorspheres with dual epithelial and mesenchymal markers, as well as luminal and basal markers, demonstrates that a population of DKAT cells is

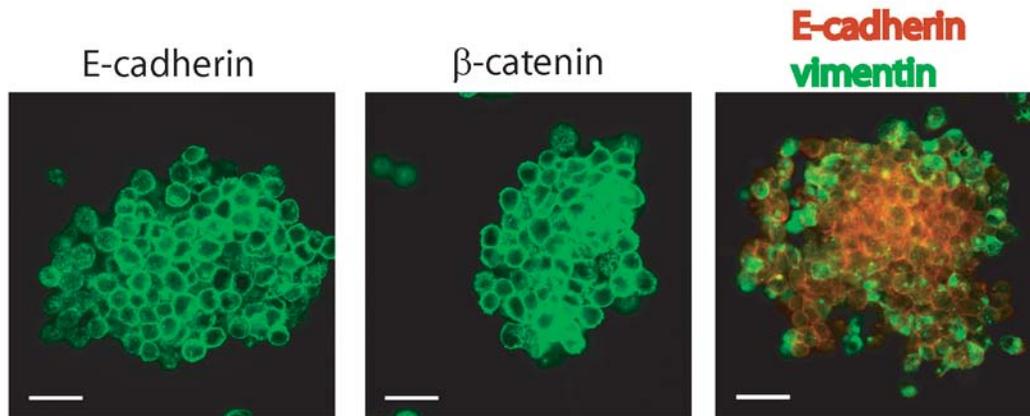


Figure 4.2: A single DKAT cell can generate tumorspheres with both epithelial and mesenchymal cells.

DKAT cells were dissociated into single-cell suspension with trypsin, and 1×10^4 cells were seeded into ultralow attachment 100mm dishes in supplemented mammosphere media. After 14 days, mammospheres were pelleted and stained for E-cadherin and β -catenin, or dual stained for E-cadherin (red) and vimentin (green). Confocal images were acquired with a 63x objective. Scale bar = 40 μ m.

able to differentiate into cells of multiple lineages and has inherent phenotypic plasticity.

4.2.3 SCGM-Induced EMT is Due to Plasticity, Not Selection

While our data suggests DKAT cells undergo both EMT and MET in response to altered culture conditions, an alternative interpretation of our data is that culturing in SCGM results in the outgrowth of a pre-existing sub-population of DKAT cells with a mesenchymal phenotype. In order to rule out the latter possibility, we looked at expression of vimentin and E-cadherin at very early

timepoints following the switch to SCGM. DKAT cells cultured in SCGM demonstrated a 100-fold increase in vimentin expression as early as 1h which corresponded with the observed morphological changes (Figure 4.3A). Interestingly, we did not observe decreased expression of E-cadherin until day 14. Because we did not observe a rapid downregulation of E-cadherin protein following SCGM treatment, we examined E-cadherin localization by immunofluorescence. Within 2 hours of SCGM treatment, E-cadherin is predominately localized away from the membrane to the cytoplasm (Figure 4.3C). Together, these observations demonstrate that culturing DKAT cells in SCGM results in a rapid switch from an epithelial to a mesenchymal phenotype more rapidly than could be explained by selection or outgrowth of a pre-existing subpopulation of mesenchymal cells.

To further rule out the possibility that long-term culture in SCGM results in the selection of cells already in the mesenchymal state, we sub-cultured clonal populations derived from single DKAT cells in MEGM. As shown in Figure 4.3B, a clonal population of DKAT cells that expressed low vimentin and high E-cadherin levels when grown in MEGM underwent the same vimentin upregulation and E-cadherin downregulation when cultured in SCGM as did the bulk population of DKAT cells. The ability of a clonal population of cells to undergo EMT further supports the inherent phenotypic plasticity of the DKAT cell line.

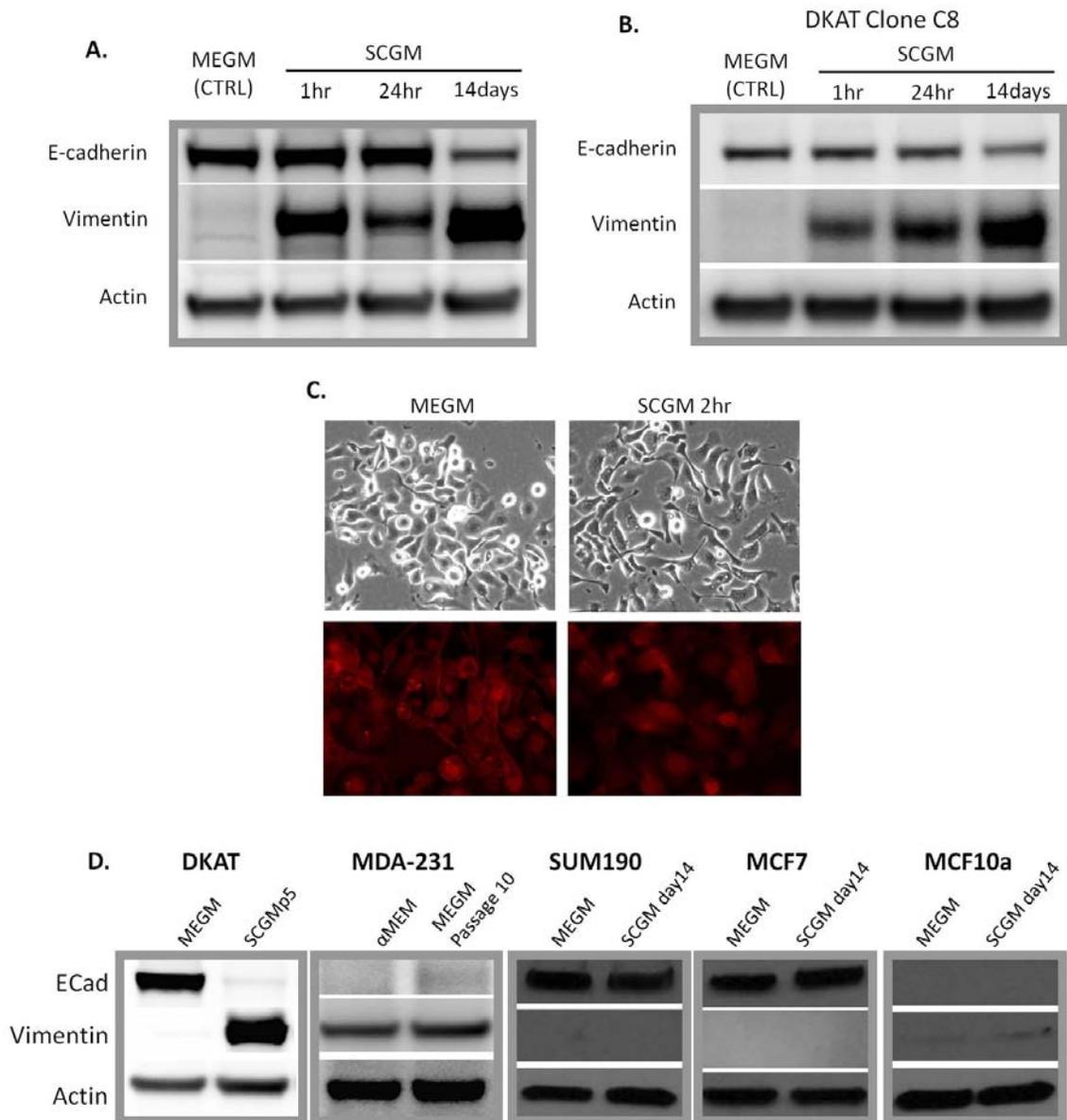


Figure 4.3: Plasticity in culture is unique to DKAT cells.

A-B. Total cell lysate from the bulk (**A**) or a clonal (**B**) population of DKATs cultured in MEGM or SCGM for the indicated time points was analyzed by western blot for vimentin, E-cadherin and actin. **C.** Brightfield and E-cadherin IF images from DKAT cells cultured in MEGM or SCGM for 2hrs. **D.** Total cell lysate from cells grown in the indicated media were analyzed by western blot for expression of E-cadherin, vimentin, and actin to test for evidence of EMT or MET in culture.

4.2.4 DKAT Cell Line Plasticity is Unique Among Breast Cancer Cell Lines

In order to determine if the properties of the DKAT cell line were unique or whether growing any breast cancer line in MEGM or SCGM would result in similar phenotypic shifts, we cultured a variety of breast cell lines in MEGM or SCGM. MDA-MB-231 breast cancer cells, which have been categorized as Basal B or mesenchymal according to gene expression profiling, are frequently used to study breast cancer metastasis [Charafe-Jauffret et al 2006, Kao et al 2009]. These cells express high levels of vimentin and undetectable levels of E-cadherin and are typically cultured in medium containing 5-10% FBS. Therefore we cultured this cell line in MEGM and looked for evidence of MET. After 14 days in MEGM, western blotting showed no change in the level of E-cadherin or vimentin protein expression, indicating MET had not occurred (Figure 4.3D).

Conversely, MCF-7 and SUM-190 breast cancer cells are normally grown in media containing 5-10% FBS and express epithelial markers. After culturing these two cell lines in SCGM for 14 days, we saw no evidence of EMT (Figure 4.3D). Furthermore, culturing these two cell lines in MEGM did not have an effect on the expression of E-cadherin or vimentin. Finally, we also tested the effect of growing hTERT-immortalized human mammary epithelial cells (HMECs) in SCGM for 14

days. While culturing these cells in SCGM did result in a loss of E-cadherin and increased vimentin expression (Figure 4.3D), the cells did not proliferate in the presence of serum and arrested during the 14 day time course (data not shown). This is consistent with prior studies which found that serum-containing medium is not suitable for long-term culture of HMECs as it can induce senescence in these cells [Brenner et al 1998, Hammond et al 1984].

4.2.5 EMT Decreases DKAT CD44⁺/CD24^{-low} Population

Numerous reports in the past several years have demonstrated that the process of EMT endows cells with properties of cancer stem cells, such as increased capacity for self renewal, mammosphere formation, and tumor initiation [Mani et al 2008]. We had previously shown that the DKAT cell line contained a large population of CD44⁺/CD24^{-low} cells, but surprisingly we found that this population of cells was not enriched for tumor initiating capability. We therefore set out to test if the process of EMT would alter the percentage of cells in the CD44⁺/CD24^{-low} population. Unexpectedly, we found that after 14 days, DKAT cells grown in SCGM and DKAT cells treated with the EMT-inducing growth factor TGF- β actually contained a smaller percentage of cells of the CD44⁺/CD24^{-low} phenotype than DKAT cells maintained in MEGM (Figure 4.4). When taken together with our data from

Chapter 3 that showed both CD44⁺/CD24^{-low} and CD44⁺/CD24^{-low} cells can give rise to CD24^{low} cells (Figure 3.8), our results suggests that CD24 expression in all DKAT cells is dynamic and therefore CD44⁺/CD24^{-low} cells may not represent the stem cell population in this model.

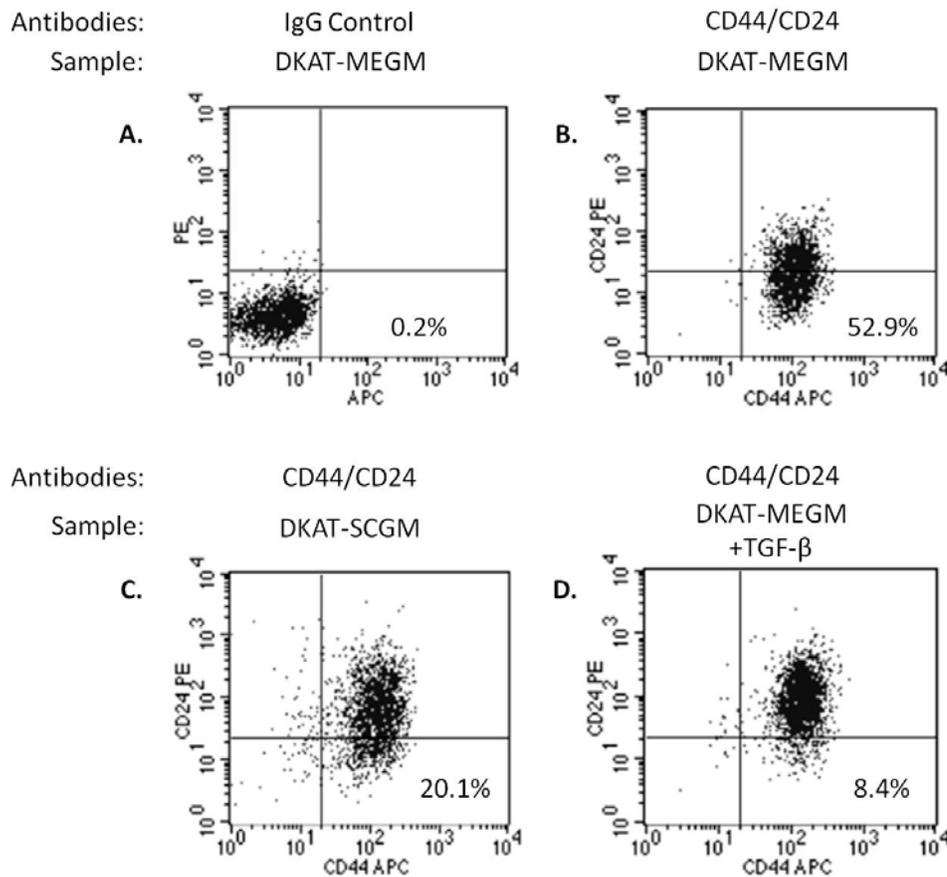


Figure 4.4: EMT decreases the population of CD44⁺/CD24^{-low} cells.

A. DKAT cells cultured in MEGM were stained with IgG-PE and IgG-APC antibodies as a negative control. **B-D.** DKAT cells cultured in MEGM (**B**), SCGM (**C**), or MEGM + 2.5ng/ml TGF- β (**D**) were stained with CD24-PE and CD44-APC antibodies and cell surface CD44/CD24 expression was analyzed by flow cytometry. Numbers indicate percentage of cells in the CD44⁺/CD24^{-low} quadrant.

4.2.6 DKAT Cells are Highly Tumorigenic and Exhibit *In Vivo* Plasticity

Having established the *in vitro* plasticity of the DKAT model is unique among breast cancer cell lines tested, we next tested whether DKAT xenograft tumors also exhibited evidence of epithelial-mesenchymal plasticity. 10^4 cells grown in MEGM were injected into the mammary fat pad of immunocompromised mice. Palpable tumors were present within 2-4 weeks and most animals were sacrificed by 13 weeks post-injection with tumors of 15 mm diameter. DKAT xenograft tumors exhibited a range of morphological patterns and immunohistochemical staining. Three recurring morphological patterns were observed in DKAT xenografts transplanted without Matrigel. Pattern I: Infiltrating growth without dense fibrous matrix (Figure 4.5Ai). DKAT cells formed cords and columns of neoplastic cells that infiltrated the surrounding fat and stained positive for CK5. Pattern II: Infiltrating growth with dense fibrous matrix (Figure 4.5Aii). DKAT cells formed nests and cords of cells surrounded by a dense connective tissue. Notably, DKAT cells were observed inside the ducts of the host mammary gland demonstrating the ability of DKAT cells to invade through the basement membrane. Pattern III: Intraductal and Pagetoid spread (Figure 4.5Aiii). One xenograft was not palpable but was visible when the host fat pad was exposed. This transplant lay flat in the fat pad spreading along the ductal tree, but not forming a three-dimensional mass. The whole mount revealed an

abnormal irregular branching pattern that is frequently found in outgrowths of mouse mammary intraepithelial neoplasia. In some areas, the tumor cells infiltrated as small nests between the myoepithelium and the luminal cells of the mouse (Pagetoid spread). In other areas, the human cancer cells were clearly invading the stroma without any recognizable association with mouse mammary cells. A dense inflammatory infiltrate surrounded all areas of the outgrowth. DKAT cells stained positively for CK5, but did not stain for vimentin.

The addition of Matrigel significantly affected the morphological and staining patterns of DKAT xenograft tumors (Figure 4.5B). DKAT xenografts transplanted with Matrigel formed nests and cords of cells that stained for both CK5 and SMA, suggesting the formation of myoepithelial cells. The peripheral cells infiltrated surrounding tissue and enclosed nests of epithelial cells that stained positive for CK8/18 and negative for SMA. The DKAT cells in the center of the tumor (Figure 4.5B, left column) exhibited larger nuclei with open chromatin and more abundant cytoplasm. IHC for SMA and CK5 showed clusters of unstained cells surrounded by dual staining cells. In contrast, the invasive cells at the edge of the tumor (Figure 4.5B, right column) from cords of cells that were intensely positive for CK5 and some SMA. Taken together, these results show that similar to the human tumor, DKAT xenografts are invasive and exhibit phenotypic plasticity.

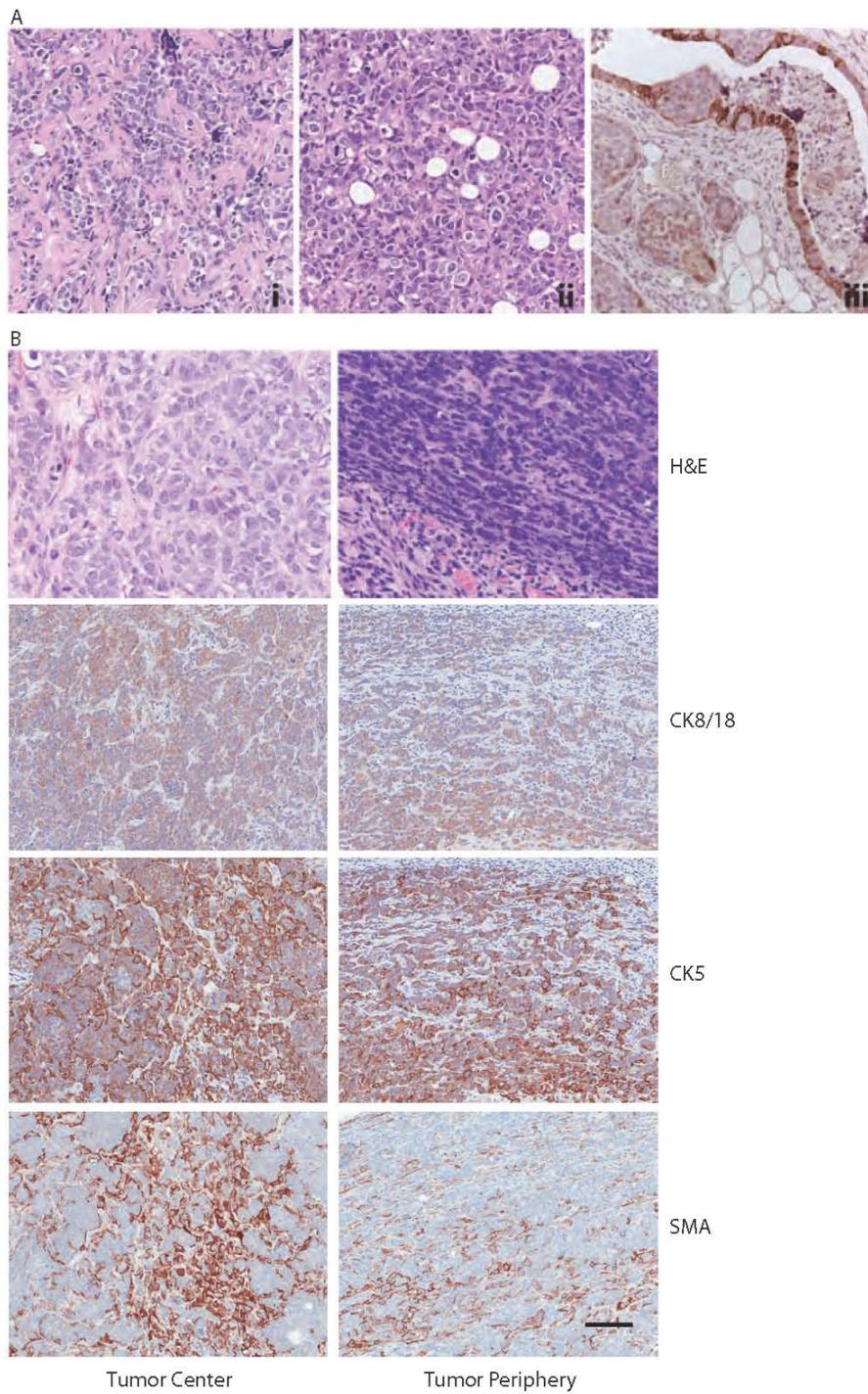


Figure 4.5: Comparison of H&E and IHC patterns from DKAT xenografts.

Figure 4.5 (continued): A. IHC showing H&E (i and ii) or CK5 staining (iii) illustrating the three recurring morphological patterns observed in DKAT xenografts transplanted without Matrigel. **B.** IHC showing H&E, CK8/18, CK5, and SMA staining of DKAT xenografts transplanted with Matrigel. The left column shows the center of a transplant and the right column shows the leading edge of the tumor.

4.2.7 Zeb1 Regulates DKAT Cell Plasticity

A large number of transcription factors are implicated in the process of EMT in both development and tumorigenesis, including members of the Snail, bHLH, and Zeb families. Proteins from each of these families have been reported to be capable of repressing E-cadherin and inducing EMT in various cell types [Peinado et al 2007]. In order to further investigate the mechanisms underlying epithelial plasticity in the DKAT cell line, we looked at the expression of three of these transcription factors in the DKAT model of EMT/MET. Interestingly, western blotting showed no noticeable difference in expression of Snail1 and Twist protein between DKAT cells in the epithelial and mesenchymal states (Figure 4.6A). However, Zeb1 was noticeably increased in DKAT-SCGM cells compared to DKAT-MEGM cells (Figure 4.6A). Zeb1 is a zinc-finger transcription factor which is known to bind directly to the E-cadherin promoter and repress its transcription [Eger et al 2005]. To determine if over-expression of Zeb1 is sufficient to induce EMT in the DKAT model, we constitutively expressed Zeb1 in epithelial DKAT-MEGM cells. The constitutive

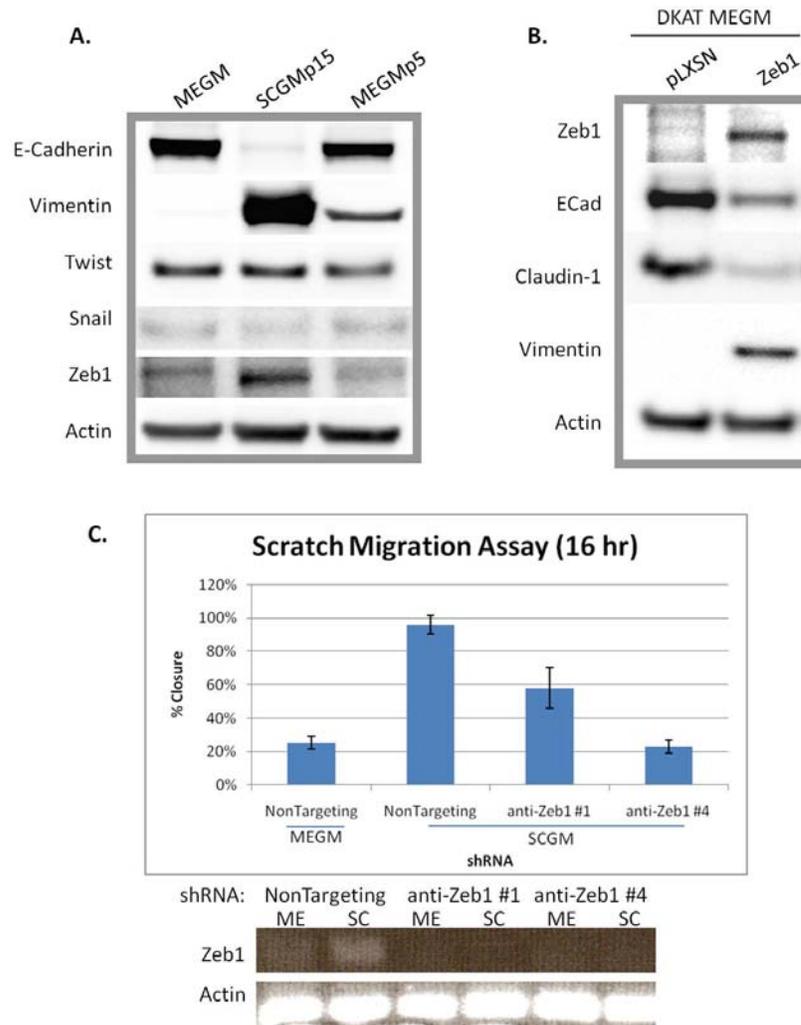


Figure 4.6: Zeb1 regulates DKAT EMT/MET.

A. Total cell lysates from DKAT-MEGM or DKAT-SCGM cells was subjected to western blot analysis for markers of EMT. **B.** Total cell lysate from DKAT-MEGM cells stably transfected with a control vector (pLXSN) or vector containing Zeb1 transcript (Zeb1) was subjected to western blot analysis for markers of EMT. **C.** Scratch wound healing assay comparing DKAT cells grown in MEGM or SCGM as indicated, stably expressing either a non-targeting shRNA sequence or one of two Zeb1-targeting sequences. Results are expressed as the percentage of the scratch area closed at 16hrs as measured by ImageJ software, and are an average of 12 scratches per sample. Lower panel shows rtPCR for Zeb1 confirming no expression in DKAT-SCGM lines expression Zeb1-targeting shRNA.

over-expression of Zeb1 resulted in a shift to a mesenchymal phenotype, as evidenced by decreased expression of E-Cadherin and Claudin-1, and increased expression of vimentin (Figure 4.6B). Conversely, stable knockdown of Zeb1 using two different lentiviral shRNA sequences prevented the increase in migration when DKAT cells were switched from MEGM to SCGM (Figure 4.6C). Together, these data indicate that Zeb1 is sufficient to induce EMT in the DKAT model and critical for the increased migratory ability of the mesenchymal DKAT cells.

4.2.8 Autocrine IL-6 Signaling Maintains Zeb1 Expression

Having established that the transcription factor Zeb1 is important in both the induction and maintenance of the mesenchymal phenotype in the DKAT model, we next sought to find the pathways which regulate Zeb1 expression in the mesenchymal state. As a number of cytokines have recently been reported to be capable of inducing EMT in breast cancer cells, we tested a panel of cytokines to determine if any were differentially produced by DKAT-MEGM and -SCGM cells. Of the eight cytokines in the panel, only IL-6 and IL-8 were significantly upregulated in conditioned media of mesenchymal DKAT-SCGM cells compared to epithelial DKAT-MEGM cells (Figure 4.7A). IL-6 overexpression has recently been reported to be sufficient to induce EMT in MCF-7 mammary epithelial cells in 3D culture

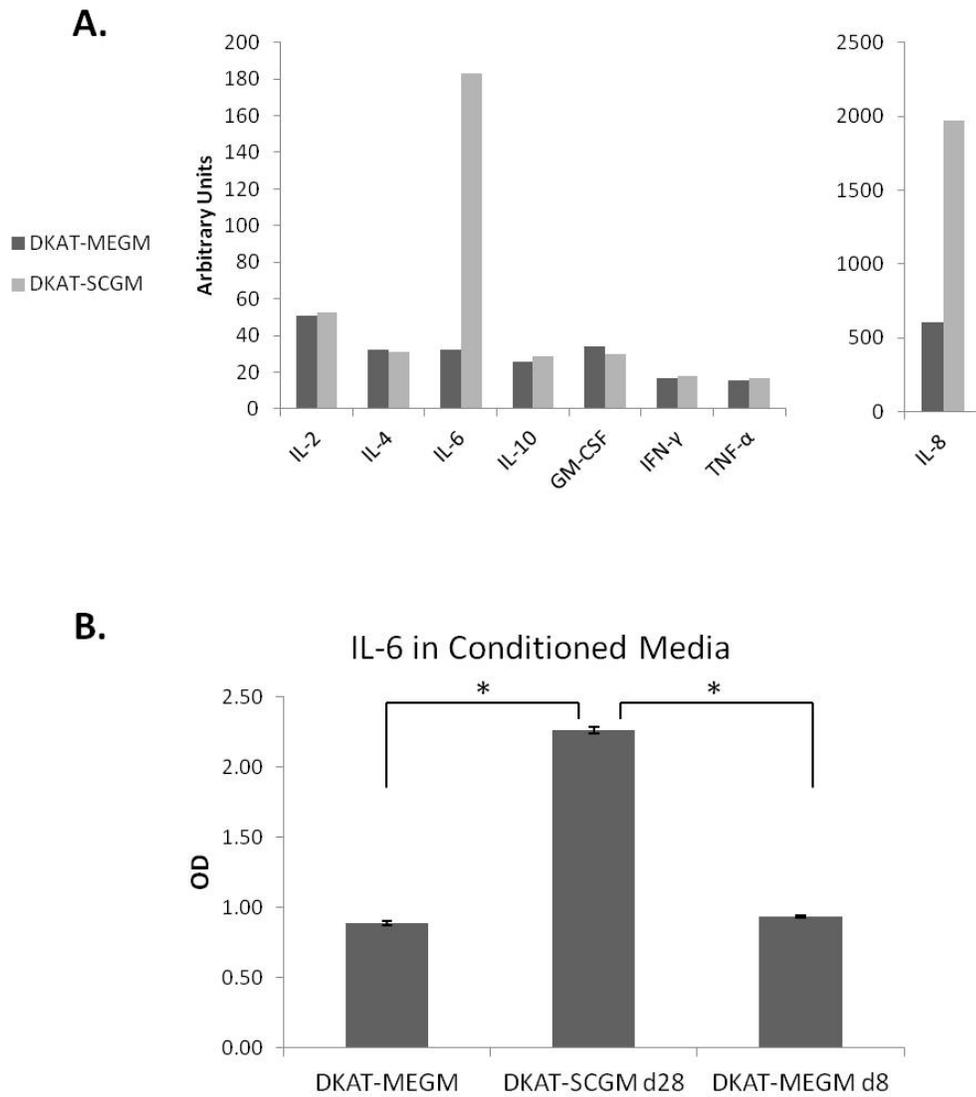


Figure 4.7: IL-6 is highly secreted by DKAT-SCGM cells.

A. Conditioned media from DKAT cells grown in MEGM or SCGM for 14 days was collected and cytokine levels were determined by multiplex assay. **B.** Conditioned media was collected from DKAT cells that were grown in MEGM for 28 days, SCGM for 28 days, or SCGM for 20 days and returned to MEGM for 8 days. The amount of IL-6 was determined by ELISA. (*) $p < .05$.

[Sullivan et al 2009]. While this paper demonstrated increases of Snail-1 and Twist in IL-6-overexpressing cells, no mention was made of an effect on Zeb1 levels.

Therefore we chose to focus our efforts on IL-6 signaling.

We first tested whether the upregulation of IL-6 levels seen in DKAT-SCGM cells was permanent following induction of EMT or if it was reduced following MET. Analysis of conditioned media from DKAT cells grown in MEGM or SCGM for 28 days, or in SCGM for 20 days followed by reversion to MEGM revealed that IL-6 levels were strongly upregulated in DKAT-SCGM cells, but returned to their original levels upon reversion to MEGM (Figure 4.7B). This suggests that IL-6 signaling is specifically important to mesenchymal DKAT cells. Because we had previously found Zeb1 upregulated in mesenchymal DKAT cells, we next tested the importance of IL-6 signaling on Zeb1. DKAT-SCGM cells were treated with either an IgG control or a neutralizing antibody against IL-6. At a concentration of 1ug/ml, treatment with the IL-6 neutralizing antibody resulted in a dramatic reduction of Zeb1 mRNA levels at 24hr (Figure 4.8A). No noticeable difference was observed in the levels of Snail-1 or VEGF mRNA in response to the anti-IL-6 neutralizing antibody, suggesting this effect may be specific to Zeb1. To test if this effect was specific to the DKAT cell line, I performed the same experiments using the mesenchymal MDA-MB-231 cell line. The neutralizing antibodies had a similar effect

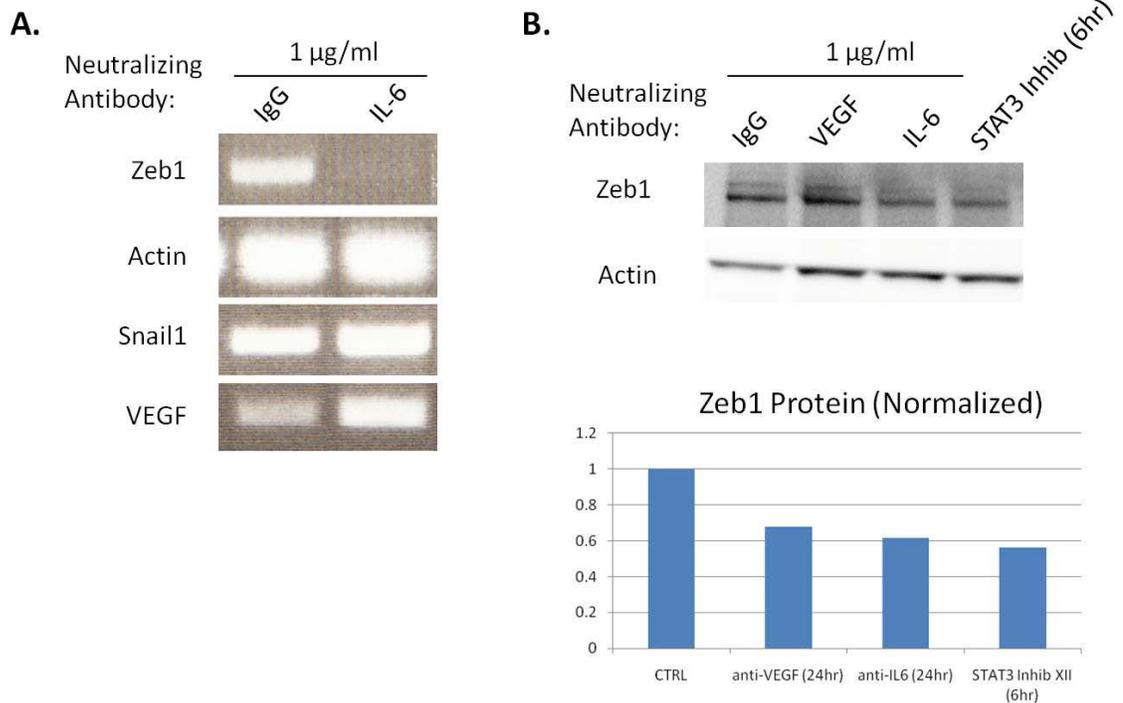


Figure 4.8: Zeb1 expression is regulated by IL-6 signaling.

A. DKAT-SCGM cells were treated with the specified antibody for 24 hrs and mRNA expression was analyzed by rtPCR. **B.** MDA-MB-231 cells were treated with the specified antibody at 1ug/ml for 24 hrs, or STAT3 Inhibitor VII at .5µM for 6 hrs. Zeb1 and Actin were then analyzed by immunoblot. Graph shows Zeb1 protein band intensity relative to Actin, and normalized to IgG control.

on Zeb1 mRNA levels and also decreased Zeb1 protein levels by approximately 40 percent at 24hr (Figure 4.8B).

The best-characterized signaling pathway downstream of IL-6 is the JAK/STAT pathway. To determine if STAT signaling was important for Zeb1 expression, MDA-MB-231 cells were also treated with a STAT3 inhibitor. After six hours, STAT3 inhibitor-treated cells displayed approximately a 40 percent decrease

in Zeb1 protein levels compared to control cells, a similar decrease as seen following 24 hour treatment with the IL-6 neutralizing antibody (Figure 4.8B). Together, these results suggest that IL-6 signaling through STAT3 promotes Zeb1 expression in mesenchymal breast cancer cells.

4.2.9 Atypia From Asymptomatic High Risk Women Contains Vimentin+ Epithelial Cells

While the prevailing theory of metastasis suggests that it is a late event in cancer, recent studies have found that pre-invasive breast cancer samples can be categorized by gene expression into the same subtypes as invasive ductal carcinomas, including the basal-like subtype [Hannemann et al 2006, Livasy et al 2007]. Because the basal-like subtype of breast cancers are enriched for markers of EMT, we tested if we could observe markers of EMT as an early event in cancer. In a cohort of women at high-risk for breast cancer due to family history, *BRCA1* mutation, or past disease, we tested for expression of vimentin in mammary epithelial cells obtained by random periareolar fine needle aspiration (RPFNA). Using IHC, we found that vimentin-positive epithelial clusters could be identified in asymptomatic pre-cancerous women (Figure 4.9). These preliminary studies further support the involvement of epithelial-mesenchymal plasticity in human breast

cancer, and suggest that such plasticity may occur even before the development of a detectable lesion.

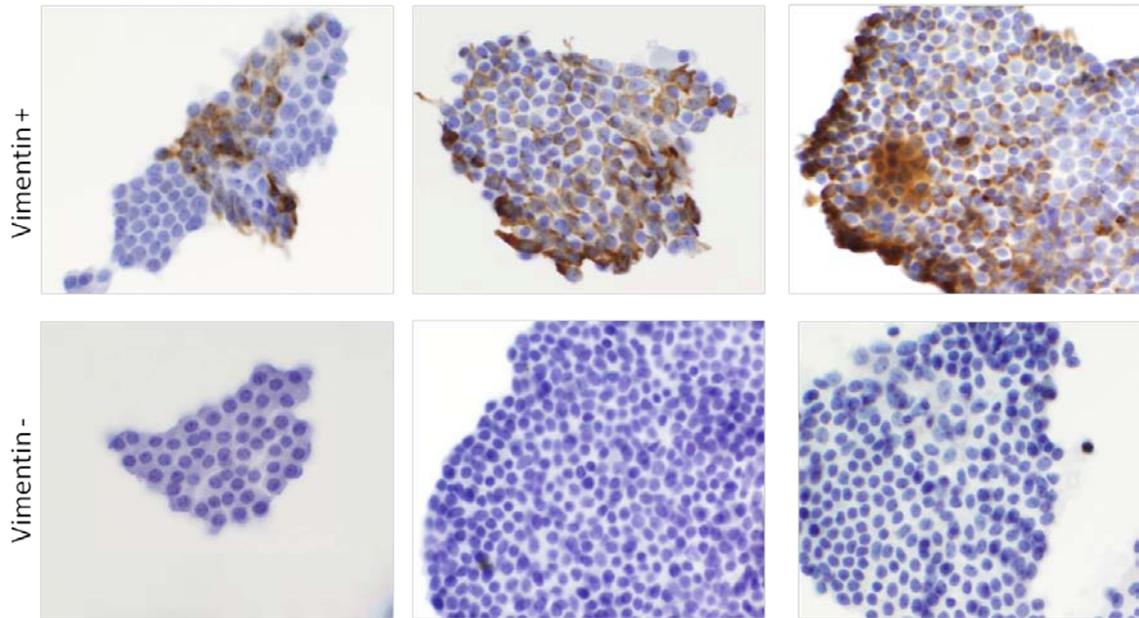


Figure 4.9: Atypia from high-risk women contains vimentin+ cells.

Epithelial cell clusters were obtained by RPFNA from asymptomatic women at high risk for breast cancer, isolated by LCM, and stained by IHC for vimentin.

4.3 Discussion

The DKAT cell line was isolated from a multi-focal basal-like breast cancer which displayed morphological and immunohistochemical evidence of epithelial-mesenchymal plasticity. While five of the six foci displayed an epithelial staining pattern, one focus of the primary tumor, as well as the area of invasion into the chest wall, displayed a more mesenchymal phenotype, exemplified by strong staining for vimentin and diffuse, cytoplasmic staining for E-cadherin. Metastatic tumor cells in the bone marrow were found to have a staining pattern consistent with a reversion to an epithelial phenotype. However, based on morphology and immunostaining it was not possible to determine whether this range of phenotypes was due to an outgrowth or selection of a cell population with a mesenchymal phenotype, or rather due to inherent phenotypic plasticity of the tumor cells adapting to different microenvironments.

To address these possibilities, we tested DKAT cells for phenotypic plasticity *in vitro* using 2D monolayer and tumorsphere culture, and *in vivo* in mouse xenografts. DKAT cells in culture exhibited phenotypic plasticity as evidenced by reversible induction of EMT in response to serum-containing media. Of the three most commonly-studied EMT-inducing transcription factors Snail1, Zeb1, and Twist, we found that only Zeb1 protein levels were increased during DKAT EMT, and this

induction was necessary for the increased migratory ability of DKAT cells grown in SCGM. In order to assess whether the observed changes in marker expression and migratory ability were a result of selection of different subpopulations of cells in the different media, we generated clonal cell lines from epithelial DKAT cells in MEGM. We found that these clonal lines generated from a single cell were able to undergo *in vitro* EMT, confirming the inherent plasticity of DKAT cells.

The observation that expression of Zeb1, but not Snail or Twist, was upregulated in the mesenchymal state is intriguing. During the course of our research, other studies have demonstrated that Zeb1 appears to be required for maintenance of the epithelial phenotype, possibly through its repression of epithelial-specific miRNAs such as miR-200c [Burk et al 2008]. Because expression of Snail and Twist is relatively high in the DKAT cell line even in the MEGM state, it is possible that this may somehow “prime” the DKAT cell line for EMT, which requires expression of Zeb1 for full induction and maintenance of the mesenchymal phenotype.

A number of recent studies have demonstrated a link between EMT and the induction of stem cell-like characteristics in mammary epithelial cells [Mani et al 2008, Morel et al 2008]. Contrasting with these studies, we unexpectedly found that induction of EMT in the DKAT cell line, either by culture in SCGM or treatment with

TGF β , resulted in a decrease in the CD44⁺/CD24^{-/low} population. It is important to note, however, that induction of a mesenchymal phenotype in the other models was an irreversible event; following withdrawal of the EMT-inducing stimuli, the cells remained in the mesenchymal state. Additionally, these studies also used immortalized normal HMECs. The DKAT cell line, which was isolated from a triple-negative cancer, contains a high percentage of CD44⁺/CD24^{-/low} breast cancer stem cells even in the epithelial state, and can undergo both EMT and MET in culture in response to altered media conditions. Tumorsphere culture of single DKAT cells resulted in the growth of multi-lineage cell clusters with distinct populations of epithelial (both luminal and basal) and mesenchymal cells, further suggesting the inherent phenotypic plasticity of DKAT cells and supporting the idea that a stem-cell phenotype may be important in the epithelial plasticity of the cell line. However, the CD44/CD24 markers may not accurately reflect the stem cell properties of individual DKAT cells.

In our *in vivo* studies, similar to the multi-focal primary tumor we found that DKAT xenograft tumors exhibit a variety of phenotypes that appear to be dependent on the microenvironment and illustrate the plasticity of the DKAT cells. When placed into a standard subcutaneous environment, they invade into the surrounding tissue as cords of cells. When injected into an intact mammary fat pad, they are able

to invade and colonize the host ducts. In Matrigel plugs, DKAT cells form nodules and nests with markers of both luminal and myoepithelial differentiation.

Though traditional models of EMT involvement in tumor metastasis suggest that EMT would occur at the tumor periphery, we observe SMA-positive DKAT cells in the center of the tumor. We propose that this is likely due to the presence of growth factors in the Matrigel used in the initial tumor cell injections. The *in vivo* plasticity of the DKAT cells suggests that the EMT/MET observed *in vitro* is not merely an artifact of the cell line adapting to culture conditions. Taken together, our data demonstrate that DKAT cells exhibit epithelial-mesenchymal plasticity, and that a single DKAT cell is capable of giving rise to cells with characteristics of multiple lineages.

Currently we are unable to reliably identify the subset of triple-negative breast cancers that are clinically aggressive and have the worst prognosis. Several new subtypes have been proposed within the triple-negative group based on gene expression patterns, including the Basal B, metaplastic, and claudin-low subtypes [Hennessy et al 2009, Herschkowitz et al 2007, Neve et al 2006]. These marker classification systems are an important step forward in understanding the biology of aggressive breast tumors; however, the phenotypic plasticity of DKAT cells makes it difficult to precisely classify these cells by fixed marker expression studies and gene

expression profiles. For example, DKAT cells induced to undergo *in vitro* EMT show reduced expression of tight junction proteins associated with the claudin-low phenotype; such classification may therefore be significantly influenced by culture conditions *in vitro* and the microenvironment *in vivo*.

While there has been intense interest in the study of EMT as it relates to breast cancer, there is still controversy over its contribution to cancer progression in patients. Here we show evidence of EMT and MET over the course of metastatic progression in a human breast cancer and demonstrate that a cell line derived from this cancer maintains epithelial plasticity in culture. The relevance of our studies to human cancer is underscored by recent data showing circulating tumor cells (CTCs) that stain positively for cytokeratin, vimentin, and N-cadherin were found in the majority of men with castration-resistant metastatic prostate cancer and women with metastatic breast cancer [Armstrong et al 2011]. This intermediate epithelial-mesenchymal staining pattern observed in CTCs suggests that epithelial plasticity, even within a small percentage of the tumor cell population, may be a driving factor in metastasis. Finally, we present evidence of vimentin staining among mammary epithelial cells from asymptomatic high-risk women, suggesting that EMT plasticity may be a feature of a subset of breast cancers from their earliest stages.

Traditional models of invasion and metastasis hypothesize that metastatic breast cancer results from the outgrowth of a subpopulation of cells with defined morphology and gene expression patterns, and that these cells are unique in their ability to migrate away from the primary tumor and establish a distant metastasis [Poste and Fidler 1980]. It has also been suggested that the aggressive metastatic behavior of certain subtypes of breast cancer (i.e. triple-negative) may reflect properties of the cell of origin from which the cancer arose [Dontu et al 2004], and more recently that the process of EMT itself may endow cancer cells with stem cell-like properties, enhancing their metastatic potential [Brabletz et al 2005, Mani et al 2008, Morel et al 2008]. The ability of the DKAT cells to undergo a reversible EMT and the presence of vimentin-positive epithelial cell clusters in asymptomatic women at high risk for breast cancer provides evidence that the aggressive behavior of a subset of triple-negative breast cancers may be driven by the inherent phenotypic plasticity of the primary tumor. As EMT has been shown to increase stem cell-like features and increase resistance to apoptosis, this plasticity represents a therapeutic challenge. Future development of strategies to target these plastic stem-like cells will likely lead to better outcomes for patients with aggressive metastatic breast cancer.

5 Conclusions and Perspectives

5.1 Inherent Plasticity of Triple-Negative Breast Cancer Cells

Breast cancer is not a single disease, but rather a collection of distinct diseases with different histopathological characteristics and clinical outcomes. Five different subtypes of breast cancer have previously been identified by differential gene expression profiling studies. These include the Luminal A and Luminal B subtypes characterized by expression of the Estrogen and Progesterone Receptors, the Her2-overexpressing subtype, the Normal Breast-like subtype, and the Basal-like or triple-negative subtype [Sorlie et al 2001]. Breast cancer mortality rates have decreased slightly over the past two decades, thanks in part to drugs that target estrogen and Her2 signaling, however there are currently no effective treatments against highly aggressive triple-negative breast cancers which do not rely on these pathways for their growth and survival [American Cancer Society].

Data from gene expression profiling of human breast tumors and mouse models of breast cancer suggests that epithelial-mesenchymal plasticity, or the ability of cells to reversibly undergo a switch from an epithelial phenotype to a mesenchymal phenotype (associated with increased migratory and invasive ability), may underlie the aggressive behavior of a subset of triple-negative breast cancers

[Sarrío et al 2008]. Importantly, recent studies in breast cancer patients suggest a common gene signature between cells which have undergone EMT and cells which resist chemotherapy, emphasizing the importance of understanding the regulation of EMT during cancer progression [Creighton et al 2009].

Despite the abundance of *in vitro* data demonstrating that activation of EMT programs endows cancer cells with many pro-metastatic properties such as increased motility and resistance to anoikis, one of the biggest obstacles toward a consensus on EMT in cancer has been the lack of direct evidence of EMT in human disease. Here we show immunohistochemical evidence of a multifocal primary tumor expressing epithelial markers, an area of local invasion that demonstrated increased expression of mesenchymal proteins, and a distant metastasis expressing epithelial markers. While this snapshot view by itself cannot prove the progression of tumor cells through epithelial and mesenchymal phenotypic states, we further demonstrate that a cell line derived from the pleural fluid of this cancer retains the basal epithelial phenotype of the primary tumor and demonstrates epithelial-mesenchymal plasticity *in vitro* and *in vivo*.

Despite the highly-invasive and migratory phenotype of the DKAT cells *in vitro*, we did not observe spontaneous metastases in our mouse xenograft models. One possible explanation is that the time before sacrificing the mice due to the large

size of the primary tumor was not long enough to observe metastases. Additionally, a number of studies have reported that in some cases, the primary tumor can act to inhibit the formation of macrometastases [Demicheli et al 2007, Retsky et al 2008]. In the future, luciferase- or RFP-labeled DKAT cells could be injected into the mammary fat pad, and when the tumor reached a predetermined size it could be resected, and the mice monitored over time for the appearance of spontaneous metastases. Experiments to compare the metastatic capability of DKAT-MEGM and DKAT-SCGM cells could include additional orthotopic mammary fat pad injections with labeled cells, or tail vein injections assays to determine their relative abilities to extravasate from the blood stream and form macrometastases.

In human breast cancer, it is presumed that EMT is induced, at least in part, in response to cues from the microenvironment such as factors induced by hypoxia or cytokines and growth factors produced by surrounding cancer associated fibroblasts or infiltrating macrophages [Polyak and Weinberg 2009]. The ability of DKAT cells to reversibly transition between the epithelial and mesenchymal state based on altered culture conditions, rather than in response to specific genetic alterations, makes them an excellent tool for experiments aimed at studying the pathways that regulate induction and maintenance of EMT. In our model, the transcription factor Zeb1 appears to be the critical regulator of the mesenchymal

phenotype. This finding is consistent with a previous study that showed in the mesenchymal MDA-MB-231 cells, knockdown of Zeb1 is sufficient to induce MET and partial re-expression of E-cadherin, while knockdown of Snail is less effective [Aigner et al 2007].

Finally, while the traditional view of cancer progression is that EMT and metastasis are late events, we present novel findings that vimentin-positive epithelial cells occur in pre-cancerous breast tissue from asymptomatic high-risk women, suggesting that EMT may occur before the development of invasive disease. This has important implications for our understanding of cancer progression and disease management, which will be discussed in a later section.

5.2 EMT Regulation of Breast Cancer Stem Cells

Several studies in recent years have shown that the process of EMT in mammary epithelial cells or breast cancer stem cells results in increased stem cell-like activity, resulting in a convergence of EMT and breast cancer stem cell research [Mani et al 2008]. The most clinically-relevant implication of these findings is that the very process which endows cells with the properties necessary to invade and migrate away from the primary tumor also induces properties beneficial to the establishment of a distant metastatic lesion including self renewal and resistance to

apoptosis. Thus there is increased emphasis on finding mechanisms of targeting the pathways which regulate EMT or those which are relied upon specifically by the stem cell population. Targeting such pathways would have the dual effect of inhibiting EMT/metastasis and decreasing stem cell-like properties of breast cancer cells that are believed to be responsible for relapse following surgery or cytotoxic treatment such as chemotherapy or radiation.

In chapter 4, we report that contrary to other published models, EMT induced in the DKAT cell line by either media change or TGF- β treatment results in a decrease in the CD44⁺/CD24^{-low} breast cancer stem cell-like fraction. While the functional implications of this post-EMT decrease on mammosphere formation and tumor initiation require further testing, we have previously found that the CD44^{hi}/CD24^{-low} fraction of DKAT cells cultured in MEGM is not enriched for tumor initiation, and that the CD44⁺/CD24⁺ fraction is capable of generating CD44^{hi}/CD24^{-low} cells. It would therefore be interesting to test whether other putative breast cancer stem cell markers, such as ALDH activity, are more suited to identifying stem cell-like activity in the DKAT model. The observation that nearly 100 percent of the MDA-MB-231 cells are of the CD44⁺/CD24^{-low} phenotype further suggests that CD24 expression may not be suitable for detection of stem cell-like activity in triple-negative or mesenchymal breast cancer cell lines *in vitro*.

In a preliminary study, we found that approximately eight percent of DKAT cells were in the ALDH1+ fraction as assessed by FACS staining, compared to the approximately 60 percent of DKAT cells in the CD44⁺/CD24^{-/low} fraction. The ALDH1+ fraction is far more consistent with our observation of 3-10 percent mammosphere formation efficiency of the DKAT cell population. The ability of ALDH activity to accurately discriminate between the cancer stem and non-stem cells could be tested by sorting cells based on ALDH1 activity using the commercially available ALDEFUOR assay, and testing the ALDEFUOR+ and ALDEFUOR- populations for their relative ability to grow as tumorspheres for multiple passages in nonadherent culture, and testing their tumor formation ability with mouse mammary fat pad injections. It would also be informative to test whether the induction of EMT in the DKAT model affects the proportion of ALDEFUOR+ cells, and if so, whether it increases or decreases this proportion of cells. In order to gain useful biological information from studies such as differential gene expression analysis of stem versus non-stem-like cells, further understanding and confirmation of the functional properties of the cell populations identified by the various putative stem cell markers is critical.

5.3 Zeb1 in Breast Cancer

The zinc finger transcription factor Zeb1 is one of several transcription factors capable of inducing EMT during embryonic development and in cancer cells in part by directly binding to the promoters of epithelial-specific genes such as E-cadherin and repressing their transcription [Aigner et al 2007]. Zeb1 has recently been described as a key component of a feedback loop involving microRNAs which regulates the epithelial phenotype in a number of cell types including breast cancer cells [Burk et al 2008, Park et al 2008]. In epithelial cells, miR-200c binds Zeb1 and decreases its expression. Decreasing miR-200c is sufficient to increase Zeb1 protein. Interestingly, once induced, Zeb1 can inhibit expression of miR-200c through binding to an upstream regulatory sequence [Burk et al 2008]. Knockdown of Zeb1 is sufficient to increase miR-200c expression in a number of cell types. Importantly, blocking the upregulation of miR-200c inhibits MET induced by Zeb1 knockdown [Park et al 2008]. This negative feedback loop suggests that Zeb1 repression of miR-200c is a critical interaction for maintaining the epithelial phenotype in cells.

We show that in the DKAT model of triple-negative breast cancer, Zeb1, but not Snail or Twist, is induced during EMT. Additionally, the work of a collaborator has shown that miR-200c is downregulated approximately two-fold in mesenchymal DKAT-SCGM cells compared to epithelial DKAT-MEGM cells (Clifford Tepper,

personal communication). With such a double-negative feedback loop in place in epithelial cells, it remains to be elucidated how miR-200c repression of Zeb1 is first relieved upon initiation of EMT. Intriguingly, Snail has also been shown to bind to two of the four E-boxes located in the miR-200c promoter and inhibit miR-200c expression [Burk et al 2008]. One hypothesis is that induction of Snail may provide an initial downregulation of miR-200c, relieving repression of Zeb1. Once expressed to a threshold level, Zeb1 could then be capable of repressing miR-200c and maintaining the mesenchymal phenotype. This hypothesis could be tested with a detailed timecourse experiment to track Snail, Zeb, and miR-200c expression following induction of EMT such as SCGM or TGF- β . If induction of EMT and the decrease of miR-200c can be blocked by a Snail shRNA or Snail knockout, this would indicate a role for a Snail–miR-200c interaction during EMT induction.

Additionally, while we found that Zeb1 knockdown had a dramatic effect on the migratory ability of DKAT-SCGM cells *in vitro*, we have not yet tested the effect of Zeb1 knockdown *in vivo*. Mouse mammary fat pad injections of DKAT-SCGM cells with either a control or Zeb1-targeting shRNA sequence would allow us to determine whether decreasing Zeb1 expression has an effect on the growth of the primary tumor or the ability of these cells to metastasize.

5.4 IL-6 Regulation of EMT in Breast Cancer Cells

While IL-6 has previously been implicated in EMT, to our knowledge, our finding that IL-6 signaling regulates expression of Zeb1 is a novel finding. In the MCF-7 model of ER+ breast cancer, exogenous expression of Twist has been shown to increase IL-6 expression and secretion, and conversely, IL-6 overexpression in the MCF-7 cell line has been shown to induce expression of Twist and Snail and decrease expression of E-cadherin [Sullivan et al 2009]. However, our work as well as a previous study using the MDA-MB-231 cell line found that Zeb1 is critical for maintenance of the epithelial phenotype [Aigner et al 2007]. This raises the intriguing possibility that treatments which block IL-6 signaling may be able to reduce expression of Zeb1 and induce MET (Figure 5.1), potentially leading to decreased cancer cell growth and increased sensitivity to cytotoxic stimuli such as chemotherapy or radiation.

In this work we have shown that in the mesenchymal DKAT-SCGM and MDA-MB-231 cells, blocking IL-6 signaling reduces Zeb1 mRNA and protein levels. We have preliminary data showing that recombinant IL-6 treatment can induce expression of Zeb1, and are currently working to determine the signaling pathways involved downstream of IL-6. To test if the classical JAK/STAT pathways are

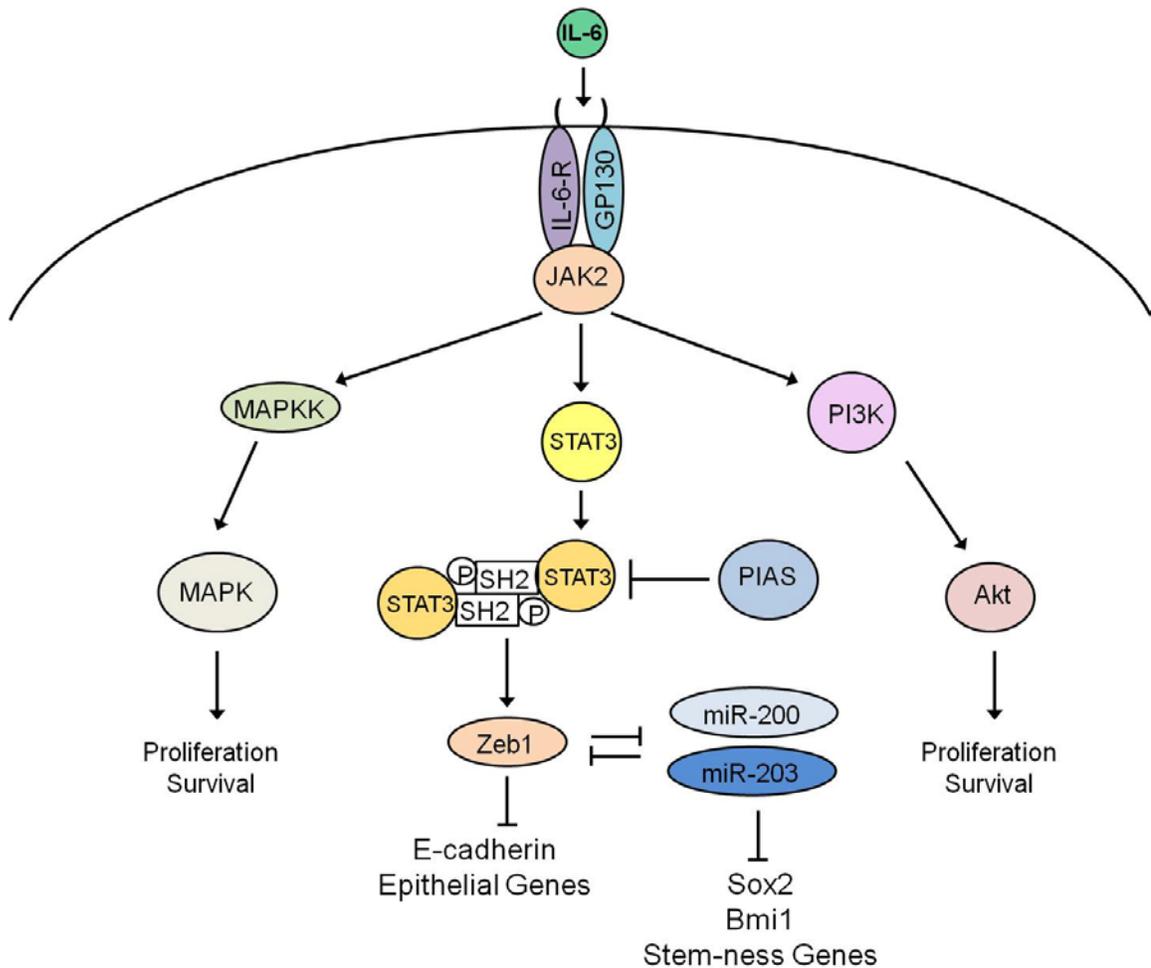


Figure 5.1: IL-6 regulation of Zeb1 and EMT.

IL-6 stimulation of the STAT3 pathway may induce expression of Zeb1, which in turn can inhibit a number of epithelial-specific genes. Zeb1 can also repress microRNAs including miR-203 and members of the miR-200 family, critical regulators of the epithelial phenotype that can also repress genes such as Bmi1 and Sox2, which are important for stem cell-like characteristics.

necessary for induction of Zeb1 expression, we will simultaneously treat with recombinant IL-6 and a JAK or STAT3 inhibitor and test for induction of EMT either by scratch assay or western blotting for epithelial and mesenchymal markers.

One recent study has shown that IL-6 expression and JAK2/STAT3 activity were found to be high specifically in basal-like breast cancer cell lines, and inhibitors of this pathway were found to effectively decrease the viability of many of these basal-like breast cancer cell lines *in vitro* [Marotta et al 2011]. Furthermore, this study found that STAT3 signaling was specifically high in CD44⁺/CD24⁻ cells from human breast tumors [Marotta et al 2011]. Similarly, in glioblastoma, Glioma Stem Cells (GSCs) have been found to express high levels of the IL-6 receptors IL-6R α and GP130 compared to non-stem cells [Wang et al 2009]. Inhibition of IL-6 signaling in the GSCs resulted in decreased GSC growth and survival, decreased neurosphere formation, and decreased GBM tumor growth. Non-stem GBM cells, however, expressed and secreted higher levels of the IL-6 ligand, suggesting that in addition to autocrine signaling, paracrine IL-6 signaling between stem and non-stem cells may play a role within the tumor [Wang et al 2009]. IL-6 paracrine signaling has also been observed to regulate the CD44⁺/CD24⁻ breast cancer stem cell population of Src-transformed MCF10a cells [Iliopoulos et al 2011]. Additionally, IL-6 secreted by breast cancer cells in a mouse xenograft model was shown to recruit Mesenchymal Stem Cells (MSCs) to the tumor site, where a feedback loop involving multiple cytokines results in increased IL-6 production by the tumor cells and an increased proportion of ALDH1⁺ cells with stem cell-like activity [Liu et al 2011]. An

important future experiment will be important to test the effect of inhibiting IL-6 signaling on DKAT tumor growth in mice. This could be accomplished with an IL-6 targeting shRNA that would inhibit autocrine signaling from the tumor cells, or with neutralizing antibodies against IL-6 or the IL-6 receptor. In addition to changes in tumor growth, it would also be useful to determine if there are changes in the CD44⁺/CD24^{-/low} or ALDH1⁺ cell populations within the treated tumors compared to untreated. Additionally, an anti-IL-6 treatment could be combined with a standard chemotherapeutic such as cisplatin to test for the possibility of synergistic effects against the tumor cells.

While IL-6 has clearly been implicated in the regulation of the cancer stem cell phenotype, the downstream targets important for this activity are not well characterized. Interestingly, Zeb1 has been shown to be capable of regulating cancer stem cells by repressing expression of several micro-RNAs, which themselves act to repress expression of numerous stem cell promoting factors [Wellner et al 2009]. Therefore, it is possible that IL-6 signaling may regulate stem cell-like characteristics in breast cancer cells in part by inducing and maintaining Zeb1 expression. To test the importance of Zeb1 in the stem cell-like phenotype of breast cancer cells downstream of IL-6, Zeb1-low epithelial cells such as SUM-159 or DKAT-MEGM cells could be transfected with a Zeb1-targeting shRNA prior to IL-6 treatment to

prevent Zeb1 induction. Alternatively, an inducible shRNA construct could be used to decrease Zeb1 expression at various times following IL-6 treatment to determine the effect on the breast cancer stem cell phenotype utilizing mammosphere formation assays and staining for CD44/CD24 or ALDH1 activity. Conversely, if Zeb1 is an important downstream target of Il-6 signaling, exogenous overexpression of Zeb1 should prevent an IL-6 or IL-6R α neutralizing antibody from decreasing the stem cell-like population of cells.

Currently there are several drugs that target IL-6 signaling in various stages of development for the treatment of inflammatory-related diseases such as rheumatoid arthritis, and several clinical trials are currently ongoing testing the effect of IL-6 antibodies in the treatment of cancer. As a secreted molecule that acts at the intersection of epithelial-mesenchymal plasticity and breast cancer stem cells, IL-6 is an attractive target for continued therapeutic development.

5.5 EMT as an Early Event in Breast Cancer Development

While the prevailing view of metastasis is that it represents the final step of tumor progression, mounting data suggests that dissemination of cancer cells throughout the body may be an early event in carcinogenesis. Rather than the clonogenic emergence of metastasis-competent cell, it may be that signals controlling

the activation of dormant or senescent metastatic cells at distant sites are the most important determinant of the timing of macrometastasis formation [Klein 2008, Podsypanina et al 2008]. Indeed, mouse models of several cancer types including breast have shown that disseminated tumor cells can be identified even before development of clinically detectable primary tumors [Eyles et al 2010, Husemann et al 2008].

Our finding that vimentin-positive atypical epithelial cell clusters can be found in asymptomatic women at high risk for breast cancer even before the development of DCIS suggests that epithelial-mesenchymal plasticity may be a feature of some breast cancers from their earliest stages. It is intriguing to speculate that expression of such factors in these early stage lesions may have prognostic value, and may possibly be predictive of progression to invasive disease or metastasis, or of progression to a specific subtype of breast cancer, such as triple-negative. Our results also suggest that individual cells or small groups of cells may acquire mesenchymal traits very early in the disease process that allow them to leave the primary tumor site to establish micrometastases. This would mean that even early surgical intervention following tumor detection may not be able to prevent the dispersal of cancer cells throughout the body. Indeed, other studies have demonstrated that tumor cell dissemination is an early event in transgenic mouse

models of breast cancer and melanoma [Eyles et al 2010, Husemann et al 2008]. Specifically, in models of BALB-NeuT mice and MMTV-polyomavirus-middle T transgenic mice, CK+ tumor cells were detectable in the bone marrow of mice as early as 4-9 weeks of age. Simultaneous analysis of the mammary glands of the corresponding mice revealed only atypical ductal hyperplasia (ADH) or DCIS [Husemann et al 2008].

Interestingly, other work in our lab has demonstrated that higher expression of vimentin in epithelial cell clusters from RPFNA samples is associated with obesity [Pilie et al 2011]. Furthermore, vimentin and phospho-STAT3^{Y705} levels correlated strongly with IL-6 concentration in the RPFNA wash fluid (Stephanie Ellison-Zelski, personal communication), and high IL-6 serum levels have previously been found to be associated with obesity [Morisset et al 2008, Roytblat et al 2000]. Taken together, these results suggest that IL-6 signaling may be one pathway involved in the early induction of mesenchymal features in mammary epithelial cells. It will be informative to utilize future RPFNA samples as part of a larger study to determine if IL-6 or vimentin levels are associated with disease progression, incidence of metastasis, or disease outcome. We are also currently working to stain RPFNA for Zeb1 to determine if its expression can also be found in pre-cancerous lesions.

A number of reports have also shown that DCIS can be classified by gene expression into the five subtypes originally defined for invasive ductal carcinomas [Bryan et al 2006, Clark et al 2011, Hannemann et al 2006, Livasy et al 2007]. In these studies, a basal-like expression pattern was associated with poor prognostic markers such as high-grade nuclei, p53 overexpression, and elevated Ki-67 index [Livasy et al 2007]. A separate study utilized a collection of triple-negative histological samples that contained areas of invasive carcinoma as well as adjacent DCIS, and in 94 percent of the samples, both the invasive carcinoma and DCIS cells stained positively for vimentin [Dabbs et al 2006]. With recent evidence demonstrating that circulating tumor cells commonly express an intermediate EMT phenotype [Aktas et al 2009, Armstrong et al 2011], it will be of great interest to follow studies which examine the association between basal-like DCIS and the presence of CTCs, as well as the prognostic value of early detection of EMT markers such as vimentin before development of invasive disease. The ability to detect tumors with an inherent plasticity or tendency to undergo EMT will be an important tool in the diagnosis and treatment of highly aggressive and metastatic breast cancers.

5.6 Summary and Concluding Remarks

In this work, we present data which contributes to the study of epithelial-mesenchymal transition in breast cancer. Importantly, we characterize a novel model of triple-negative breast cancer which was derived from a patient with metastatic therapy-resistant triple-negative breast cancer. We show that this cancer displayed evidence of EMT in the area of invasion into the chest wall, and evidence of MET in a metastatic lesion in the bone. While this is not direct proof of EMT-MET during the course of human breast cancer, we demonstrate that the cell line derived from this cancer can undergo reversible EMT-MET in culture in response to altered culture conditions, and contains cells capable of giving rise to both epithelial and mesenchymal cells in nonadherent tumorsphere culture. As few as 10 cells are able to consistently form tumors in mouse xenograft experiments, and these tumors also display evidence of epithelial-mesenchymal plasticity. As a model of triple-negative breast cancer, a cell line with epithelial properties in normal culture conditions that can be induced to undergo EMT in response to serum-containing media will be valuable to the field as investigators search for pathways which can be targeted and modulated pharmacologically to block or reverse EMT *in vivo*.

We have utilized this model to demonstrate that IL-6 regulation of Zeb1 may represent an important pathway controlling epithelial-mesenchymal plasticity in

breast cancer cells. High levels of IL-6 have previously been shown to predict poor prognosis among breast cancer patients, but its specific effects on EMT are just recently being realized. IL-6 can be secreted both by stromal cells in the tumor microenvironment and by tumor cells themselves, and both autocrine and paracrine IL-6 signaling are likely to be important for regulation of EMT and the breast cancer stem cell phenotype. This dual role in breast cancer cells makes the IL-6 pathway an attractive target for the development of therapeutics, and our work further supports the rationale for the development of anti-IL-6 drugs for the treatment of breast cancer.

Finally, we show evidence of epithelial-mesenchymal transition in mammary tissue from asymptomatic women at high risk for breast cancer. This novel finding suggests that epithelial-mesenchymal plasticity may be inherent to some cancers from their earliest stages, rather than a feature acquired late in disease progression. Future studies are needed to determine if detection of EMT markers at such an early stage is indicative of increased risk of progression or poor prognosis.

In total, this work presents findings both from human patients and *in vitro* studies which implicate inherent epithelial-mesenchymal plasticity in the aggressive behavior of a subset of triple-negative breast cancers. Further study of the pathways

regulating this plasticity will lead to improved diagnosis and therapeutic options to effectively treat these deadly breast cancers.

References

- Abramoff M, Magelhaes, PJ, Ram, SJ (2004). Image Processing with ImageJ. *Biophotonics International* **11**: 36-42.
- Acloque H, Adams MS, Fishwick K, Bronner-Fraser M, Nieto MA (2009). Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *J Clin Invest* **119**: 1438-1449.
- Aigner K, Dampier B, Descovich L, Mikula M, Sultan A, Schreiber M *et al* (2007). The transcription factor ZEB1 (deltaEF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity. *Oncogene* **26**: 6979-6988.
- Aktas B, Tewes M, Fehm T, Hauch S, Kimmig R, Kasimir-Bauer S (2009). Stem cell and epithelial-mesenchymal transition markers are frequently overexpressed in circulating tumor cells of metastatic breast cancer patients. *Breast Cancer Res* **11**: R46.
- Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003). Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* **100**: 3983-3988.
- American Cancer Society Breast Cancer Facts & Figures 2009-2010. American Cancer Society, Inc.: Atlanta. pp 1-36.
- Armstrong AJ, Marengo MS, Oltean S, Kemeny G, Bitting R, Turnbull J *et al* (2011). Circulating Tumor Cells from Patients with Advanced Prostate and Breast Cancer Display Both Epithelial and Mesenchymal Markers. *Mol Cancer Res*.
- Bachelder RE, Yoon SO, Franci C, de Herreros AG, Mercurio AM (2005). Glycogen synthase kinase-3 is an endogenous inhibitor of Snail transcription: implications for the epithelial-mesenchymal transition. *J Cell Biol* **168**: 29-33.
- Bachman KE, Argani P, Samuels Y, Silliman N, Ptak J, Szabo S *et al* (2004). The PIK3CA gene is mutated with high frequency in human breast cancers. *Cancer Biol Ther* **3**: 772-775.
- Badve S, Dabbs DJ, Schnitt SJ, Baehner FL, Decker T, Eusebi V *et al* (2011). Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. *Mod Pathol* **24**: 157-167.

Barnes EA, Kenerson HL, Jiang X, Yeung RS (2010). Tuberin regulates E-cadherin localization: implications in epithelial-mesenchymal transition. *Am J Pathol* **177**: 1765-1778.

Batsche E, Muchardt C, Behrens J, Hurst HC, Cremisi C (1998). RB and c-Myc activate expression of the E-cadherin gene in epithelial cells through interaction with transcription factor AP-2. *Mol Cell Biol* **18**: 3647-3658.

Beatson GT (1896). On the Treatment of Inoperable Cases of Carcinoma of the Mammary - Suggestions for a New Method of Treatment, with Illustrative Cases. *Lancet* **2**: 104-107.

Berx G, Cleton-Jansen AM, Nollet F, de Leeuw WJ, van de Vijver M, Cornelisse C *et al* (1995). E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. *EMBO J* **14**: 6107-6115.

Blanco MJ, Moreno-Bueno G, Sarrío D, Locascio A, Cano A, Palacios J *et al* (2002). Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. *Oncogene* **21**: 3241-3246.

Brabletz S, Brabletz T (2010). The ZEB/miR-200 feedback loop--a motor of cellular plasticity in development and cancer? *EMBO Rep* **11**: 670-677.

Brabletz T, Jung A, Reu S, Porzner M, Hlubek F, Kunz-Schughart LA *et al* (2001). Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc Natl Acad Sci U S A* **98**: 10356-10361.

Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T (2005). Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat Rev Cancer* **5**: 744-749.

Brenner AJ, Stampfer MR, Aldaz CM (1998). Increased p16 expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with p16 inactivation. *Oncogene* **17**: 199-205.

Brown RL, Reinke LM, Damerow MS, Perez D, Chodosh LA, Yang J *et al* (2011). CD44 splice isoform switching in human and mouse epithelium is essential for epithelial-mesenchymal transition and breast cancer progression. *J Clin Invest* **121**: 1064-1074.

Bryan BB, Schnitt SJ, Collins LC (2006). Ductal carcinoma in situ with basal-like phenotype: a possible precursor to invasive basal-like breast cancer. *Mod Pathol* **19**: 617-621.

Bukholm IK, Nesland JM, Borresen-Dale AL (2000). Re-expression of E-cadherin, alpha-catenin and beta-catenin, but not of gamma-catenin, in metastatic tissue from breast cancer patients [see comments]. *J Pathol* **190**: 15-19.

Burdsal CA, Damsky CH, Pedersen RA (1993). The role of E-cadherin and integrins in mesoderm differentiation and migration at the mammalian primitive streak. *Development* **118**: 829-844.

Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S *et al* (2008). A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO Rep* **9**: 582-589.

Cardiff RD (2010). The pathology of EMT in mouse mammary tumorigenesis. *J Mammary Gland Biol Neoplasia* **15**: 225-233.

Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F *et al* (2007). The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. *Clin Cancer Res* **13**: 2329-2334.

Chaffer CL, Thompson EW, Williams ED (2007). Mesenchymal to epithelial transition in development and disease. *Cells Tissues Organs* **185**: 7-19.

Chaffer CL, Weinberg RA (2011). A perspective on cancer cell metastasis. *Science* **331**: 1559-1564.

Chamberlain EM, Sanders MM (1999). Identification of the novel player deltaEF1 in estrogen transcriptional cascades. *Mol Cell Biol* **19**: 3600-3606.

Charafe-Jauffret E, Ginestier C, Monville F, Finetti P, Adelaide J, Cervera N *et al* (2006). Gene expression profiling of breast cell lines identifies potential new basal markers. *Oncogene* **25**: 2273-2284.

Charafe-Jauffret E, Ginestier C, Iovino F, Tarpin C, Diebel M, Esterni B *et al* (2010). Aldehyde dehydrogenase 1-positive cancer stem cells mediate metastasis and poor clinical outcome in inflammatory breast cancer. *Clin Cancer Res* **16**: 45-55.

- Cheang MC, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK *et al* (2008). Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. *Clin Cancer Res* **14**: 1368-1376.
- Chetty R, Serra S, Asa SL (2008). Loss of membrane localization and aberrant nuclear E-cadherin expression correlates with invasion in pancreatic endocrine tumors. *Am J Surg Pathol* **32**: 413-419.
- Cho Y, Gorina S, Jeffrey PD, Pavletich NP (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science* **265**: 346-355.
- Christ B, Ordahl CP (1995). Early stages of chick somite development. *Anat Embryol (Berl)* **191**: 381-396.
- Christiansen JJ, Rajasekaran AK (2006). Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. *Cancer Res* **66**: 8319-8326.
- Chua HL, Bhat-Nakshatri P, Clare SE, Morimiya A, Badve S, Nakshatri H (2007). NF-kappaB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. *Oncogene* **26**: 711-724.
- Ciruna B, Rossant J (2001). FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. *Dev Cell* **1**: 37-49.
- Clark SE, Warwick J, Carpenter R, Bowen RL, Duffy SW, Jones JL (2011). Molecular subtyping of DCIS: heterogeneity of breast cancer reflected in pre-invasive disease. *Br J Cancer* **104**: 120-127.
- Clark SG, Chiu C (2003). *C. elegans* ZAG-1, a Zn-finger-homeodomain protein, regulates axonal development and neuronal differentiation. *Development* **130**: 3781-3794.
- Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Sjolund A *et al* (2009). Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. *Proc Natl Acad Sci U S A* **106**: 13820-13825.
- Croker AK, Goodale D, Chu J, Postenka C, Hedley BD, Hess DA *et al* (2009). High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. *J Cell Mol Med* **13**: 2236-2252.

- Dabbs DJ, Chivukula M, Carter G, Bhargava R (2006). Basal phenotype of ductal carcinoma in situ: recognition and immunohistologic profile. *Mod Pathol* **19**: 1506-1511.
- Daniel CW, Deome KB, Young JT, Blair PB, Faulkin LJ, Jr. (2009). The in vivo life span of normal and preneoplastic mouse mammary glands: a serial transplantation study. 1968. *J Mammary Gland Biol Neoplasia* **14**: 355-362.
- Davies JA (1996). Mesenchyme to epithelium transition during development of the mammalian kidney tubule. *Acta Anat (Basel)* **156**: 187-201.
- Debies MT, Gestl SA, Mathers JL, Mikse OR, Leonard TL, Moody SE *et al* (2008). Tumor escape in a Wnt1-dependent mouse breast cancer model is enabled by p19Arf/p53 pathway lesions but not p16 Ink4a loss. *J Clin Invest* **118**: 51-63.
- Demicheli R, Retsky MW, Hrushesky WJ, Baum M (2007). Tumor dormancy and surgery-driven interruption of dormancy in breast cancer: learning from failures. *Nat Clin Pract Oncol* **4**: 699-710.
- Deng G, Lu Y, Zlotnikov G, Thor AD, Smith HS (1996). Loss of heterozygosity in normal tissue adjacent to breast carcinomas. *Science* **274**: 2057-2059.
- Deome KB, Faulkin LJ, Jr., Bern HA, Blair PB (1959). Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. *Cancer Res* **19**: 515-520.
- DeVita VT, Hellman S, Rosenberg SA (2005). *Cancer, principles & practice of oncology*, 7th edn. Lippincott Williams & Wilkins: Philadelphia, PA.
- Dillner NB, Sanders MM (2002). The zinc finger/homeodomain protein deltaEF1 mediates estrogen-specific induction of the ovalbumin gene. *Mol Cell Endocrinol* **192**: 85-91.
- Dong P, Tada M, Hamada J, Nakamura A, Moriuchi T, Sakuragi N (2007). p53 dominant-negative mutant R273H promotes invasion and migration of human endometrial cancer HHUA cells. *Clin Exp Metastasis* **24**: 471-483.
- Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ *et al* (2003). In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev* **17**: 1253-1270.

Dontu G, El-Ashry D, Wicha MS (2004). Breast cancer, stem/progenitor cells and the estrogen receptor. *Trends Endocrinol Metab* **15**: 193-197.

Eger A, Aigner K, Sonderegger S, Dampier B, Oehler S, Schreiber M *et al* (2005). DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. *Oncogene* **24**: 2375-2385.

Evans AJ, Russell RC, Roche O, Burry TN, Fish JE, Chow VW *et al* (2007). VHL promotes E2 box-dependent E-cadherin transcription by HIF-mediated regulation of SIP1 and snail. *Mol Cell Biol* **27**: 157-169.

Eyles J, Puaux AL, Wang X, Toh B, Prakash C, Hong M *et al* (2010). Tumor cells disseminate early, but immunosurveillance limits metastatic outgrowth, in a mouse model of melanoma. *J Clin Invest* **120**: 2030-2039.

Fabian CJ, Kimler BF, Zalles CM, Klemp JR, Kamel S, Zeiger S *et al* (2000). Short-term breast cancer prediction by random periareolar fine-needle aspiration cytology and the Gail risk model. *J Natl Cancer Inst* **92**: 1217-1227.

Fidler IJ, Kripke ML (1977). Metastasis results from preexisting variant cells within a malignant tumor. *Science* **197**: 893-895.

Fidler IJ (2003). The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* **3**: 453-458.

Fillmore CM, Kuperwasser C (2008). Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Res* **10**: R25.

Fortini ME, Lai ZC, Rubin GM (1991). The *Drosophila* *zfh-1* and *zfh-2* genes encode novel proteins containing both zinc-finger and homeodomain motifs. *Mech Dev* **34**: 113-122.

Franci C, Takkunen M, Dave N, Alameda F, Gomez S, Rodriguez R *et al* (2006). Expression of Snail protein in tumor-stroma interface. *Oncogene* **25**: 5134-5144.

Frixen UH, Behrens J, Sachs M, Eberle G, Voss B, Warda A *et al* (1991). E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J Cell Biol* **113**: 173-185.

- Funahashi J, Sekido R, Murai K, Kamachi Y, Kondoh H (1993). Delta-crystallin enhancer binding protein delta EF1 is a zinc finger-homeodomain protein implicated in postgastrulation embryogenesis. *Development* **119**: 433-446.
- Genetta T, Ruezinsky D, Kadesch T (1994). Displacement of an E-box-binding repressor by basic helix-loop-helix proteins: implications for B-cell specificity of the immunoglobulin heavy-chain enhancer. *Mol Cell Biol* **14**: 6153-6163.
- Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M *et al* (2007). ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* **1**: 555-567.
- Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G *et al* (2008). The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* **10**: 593-601.
- Gusterson B (2009). Do 'basal-like' breast cancers really exist? *Nat Rev Cancer* **9**: 128-134.
- Hammond SL, Ham RG, Stampfer MR (1984). Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. *Proc Natl Acad Sci U S A* **81**: 5435-5439.
- Hanahan D, Weinberg RA (2011). Hallmarks of cancer: the next generation. *Cell* **144**: 646-674.
- Hannemann J, Velds A, Halfwerk JB, Kreike B, Peterse JL, van de Vijver MJ (2006). Classification of ductal carcinoma in situ by gene expression profiling. *Breast Cancer Res* **8**: R61.
- Hay ED (1995). An overview of epithelio-mesenchymal transformation. *Acta Anat (Basel)* **154**: 8-20.
- Hennessey BT, Gonzalez-Angulo AM, Stemke-Hale K, Gilcrease MZ, Krishnamurthy S, Lee JS *et al* (2009). Characterization of a naturally occurring breast cancer subset enriched in epithelial-to-mesenchymal transition and stem cell characteristics. *Cancer Res* **69**: 4116-4124.
- Hennig G, Lowrick O, Birchmeier W, Behrens J (1996). Mechanisms identified in the transcriptional control of epithelial gene expression. *J Biol Chem* **271**: 595-602.

- Herschkowitz JL, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z *et al* (2007). Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome Biol* **8**: R76.
- Higashi Y, Moribe H, Takagi T, Sekido R, Kawakami K, Kikutani H *et al* (1997). Impairment of T cell development in deltaEF1 mutant mice. *J Exp Med* **185**: 1467-1479.
- Hlubek F, Lohberg C, Meiler J, Jung A, Kirchner T, Brabletz T (2001). Tip60 is a cell-type-specific transcriptional regulator. *J Biochem* **129**: 635-641.
- Honeth G, Bendahl PO, Ringner M, Saal LH, Gruvberger-Saal SK, Lovgren K *et al* (2008). The CD44+/CD24- phenotype is enriched in basal-like breast tumors. *Breast Cancer Res* **10**: R53.
- Husemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E *et al* (2008). Systemic spread is an early step in breast cancer. *Cancer Cell* **13**: 58-68.
- Ikeda K, Halle JP, Stelzer G, Meisterernst M, Kawakami K (1998). Involvement of negative cofactor NC2 in active repression by zinc finger-homeodomain transcription factor AREB6. *Mol Cell Biol* **18**: 10-18.
- Iliopoulos D, Hirsch HA, Wang G, Struhl K (2011). Inducible formation of breast cancer stem cells and their dynamic equilibrium with non-stem cancer cells via IL6 secretion. *Proc Natl Acad Sci U S A* **108**: 1397-1402.
- Inui M, Martello G, Piccolo S (2010). MicroRNA control of signal transduction. *Nat Rev Mol Cell Biol* **11**: 252-263.
- Jackson B, Brocker C, Thompson DC, Black W, Vasiliou K, Nebert DW *et al* (2011). Update on the aldehyde dehydrogenase gene (ALDH) superfamily. *Hum Genomics* **5**: 283-303.
- Jethanandani P, Kramer RH (2005). Alpha7 integrin expression is negatively regulated by deltaEF1 during skeletal myogenesis. *J Biol Chem* **280**: 36037-36046.
- Jiang Z, Deng T, Jones R, Li H, Herschkowitz JL, Liu JC *et al* (2010). Rb deletion in mouse mammary progenitors induces luminal-B or basal-like/EMT tumor subtypes depending on p53 status. *J Clin Invest* **120**: 3296-3309.

- Kajiyama H, Shibata K, Terauchi M, Yamashita M, Ino K, Nawa A *et al* (2007). Chemoresistance to paclitaxel induces epithelial-mesenchymal transition and enhances metastatic potential for epithelial ovarian carcinoma cells. *Int J Oncol* **31**: 277-283.
- Kalluri R, Weinberg RA (2009). The basics of epithelial-mesenchymal transition. *J Clin Invest* **119**: 1420-1428.
- Kao J, Salari K, Bocanegra M, Choi YL, Girard L, Gandhi J *et al* (2009). Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. *PLoS One* **4**: e6146.
- Kishimoto T (1989). The biology of interleukin-6. *Blood* **74**: 1-10.
- Klein CA (2008). Cancer. The metastasis cascade. *Science* **321**: 1785-1787.
- Kozlowski L, Zakrzewska I, Tokajuk P, Wojtukiewicz MZ (2003). Concentration of interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-10 (IL-10) in blood serum of breast cancer patients. *Rocz Akad Med Bialymst* **48**: 82-84.
- Krishnamachary B, Zagzag D, Nagasawa H, Rainey K, Okuyama H, Baek JH *et al* (2006). Hypoxia-inducible factor-1-dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. *Cancer Res* **66**: 2725-2731.
- Lai ZC, Fortini ME, Rubin GM (1991). The embryonic expression patterns of *zfh-1* and *zfh-2*, two *Drosophila* genes encoding novel zinc-finger homeodomain proteins. *Mech Dev* **34**: 123-134.
- Lakhani SR, Slack DN, Hamoudi RA, Collins N, Stratton MR, Sloane JP (1996). Detection of allelic imbalance indicates that a proportion of mammary hyperplasia of usual type are clonal, neoplastic proliferations. *Lab Invest* **74**: 129-135.
- Lazarova DL, Bordonaro M, Sartorelli AC (2001). Transcriptional regulation of the vitamin D(3) receptor gene by ZEB. *Cell Growth Differ* **12**: 319-326.
- Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu MF *et al* (2008). Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *J Natl Cancer Inst* **100**: 672-679.

- Liu S, Ginestier C, Ou SJ, Clouthier SG, Patel SH, Monville F *et al* (2011). Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. *Cancer Res* **71**: 614-624.
- Liu X, Holstege H, van der Gulden H, Treur-Mulder M, Zevenhoven J, Velds A *et al* (2007). Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated basal-like breast cancer. *Proc Natl Acad Sci U S A* **104**: 12111-12116.
- Liu YN, Lee WW, Wang CY, Chao TH, Chen Y, Chen JH (2005). Regulatory mechanisms controlling human E-cadherin gene expression. *Oncogene* **24**: 8277-8290.
- Livasy CA, Perou CM, Karaca G, Cowan DW, Maia D, Jackson S *et al* (2007). Identification of a basal-like subtype of breast ductal carcinoma in situ. *Hum Pathol* **38**: 197-204.
- Long J, Zuo D, Park M (2005). Pc2-mediated sumoylation of Smad-interacting protein 1 attenuates transcriptional repression of E-cadherin. *J Biol Chem* **280**: 35477-35489.
- Lopez-Knowles E, O'Toole SA, McNeil CM, Millar EK, Qiu MR, Crea P *et al* (2010). PI3K pathway activation in breast cancer is associated with the basal-like phenotype and cancer-specific mortality. *Int J Cancer* **126**: 1121-1131.
- Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY *et al* (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**: 704-715.
- Marcato P, Dean CA, Pan D, Araslanova R, Gillis M, Joshi M *et al* (2011). Aldehyde dehydrogenase activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression is predictive of metastasis. *Stem Cells* **29**: 32-45.
- Marotta LL, Almendro V, Marusyk A, Shipitsin M, Schemme J, Walker SR *et al* (2011). The JAK2/STAT3 signaling pathway is required for growth of CD44CD24 stem cell-like breast cancer cells in human tumors. *J Clin Invest* **121**: 2723-2735.
- Meyer MJ, Fleming JM, Ali MA, Pesesky MW, Ginsburg E, Vonderhaar BK (2009). Dynamic regulation of CD24 and the invasive, CD44posCD24neg phenotype in breast cancer cell lines. *Breast Cancer Res* **11**: R82.

- Micalizzi DS, Farabaugh SM, Ford HL (2010). Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. *J Mammary Gland Biol Neoplasia* **15**: 117-134.
- Milas L, Peters LJ, Ito H (1983). Spontaneous metastasis: random or selective? *Clin Exp Metastasis* **1**: 309-315.
- Millikan RC, Newman B, Tse CK, Moorman PG, Conway K, Dressler LG *et al* (2008). Epidemiology of basal-like breast cancer. *Breast Cancer Res Treat* **109**: 123-139.
- Miyoshi T, Maruhashi M, Van De Putte T, Kondoh H, Huylebroeck D, Higashi Y (2006). Complementary expression pattern of Zfhx1 genes Sip1 and deltaEF1 in the mouse embryo and their genetic interaction revealed by compound mutants. *Dev Dyn* **235**: 1941-1952.
- Molyneux G, Geyer FC, Magnay FA, McCarthy A, Kendrick H, Natrajan R *et al* (2010). BRCA1 basal-like breast cancers originate from luminal epithelial progenitors and not from basal stem cells. *Cell Stem Cell* **7**: 403-417.
- Morel AP, Lievre M, Thomas C, Hinkal G, Ansieau S, Puisieux A (2008). Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS One* **3**: e2888.
- Morisset AS, Huot C, Legare D, Tchernof A (2008). Circulating IL-6 concentrations and abdominal adipocyte isoproterenol-stimulated lipolysis in women. *Obesity (Silver Spring)* **16**: 1487-1492.
- Mrozek K, Heinonen K, Theil KS, Bloomfield CD (2002). Spectral karyotyping in patients with acute myeloid leukemia and a complex karyotype shows hidden aberrations, including recurrent overrepresentation of 21q, 11q, and 22q. *Genes Chromosomes Cancer* **34**: 137-153.
- National Cancer Institute (2011). SEER Cancer Statistics Review 1975-2008. National Cancer Institute: Rockville, MD.
- Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T *et al* (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* **10**: 515-527.

Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z *et al* (2004). Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin Cancer Res* **10**: 5367-5374.

Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K *et al* (1989). Mutations in the p53 gene occur in diverse human tumour types. *Nature* **342**: 705-708.

Obiorah I, Jordan VC (2011). Progress in endocrine approaches to the treatment and prevention of breast cancer. *Maturitas*.

Oltean S, Sorg BS, Albrecht T, Bonano VI, Brazas RM, Dewhirst MW *et al* (2006). Alternative inclusion of fibroblast growth factor receptor 2 exon IIIc in Dunning prostate tumors reveals unexpected epithelial mesenchymal plasticity. *Proc Natl Acad Sci U S A* **103**: 14116-14121.

Oltean S, Febbo PG, Garcia-Blanco MA (2008). Dunning rat prostate adenocarcinomas and alternative splicing reporters: powerful tools to study epithelial plasticity in prostate tumors in vivo. *Clin Exp Metastasis* **25**: 611-619.

Orlowski RZ, Baldwin AS, Jr. (2002). NF-kappaB as a therapeutic target in cancer. *Trends Mol Med* **8**: 385-389.

Paget S (1889). The distribution of secondary growths in cancer of the breast. *The Lancet* **133**: 571 - 573.

Park SM, Gaur AB, Lengyel E, Peter ME (2008). The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev* **22**: 894-907.

Peinado H, Olmeda D, Cano A (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer* **7**: 415-428.

Pena C, Garcia JM, Garcia V, Silva J, Dominguez G, Rodriguez R *et al* (2006). The expression levels of the transcriptional regulators p300 and CtBP modulate the correlations between SNAIL, ZEB1, E-cadherin and vitamin D receptor in human colon carcinomas. *Int J Cancer* **119**: 2098-2104.

Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA *et al* (2000). Molecular portraits of human breast tumours. *Nature* **406**: 747-752.

- Phillips TM, McBride WH, Pajonk F (2006). The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. *J Natl Cancer Inst* **98**: 1777-1785.
- Pilie PG, Ibarra-Drendall C, Troch MM, Broadwater G, Barry WT, Petricoin EF, 3rd *et al* (2011). Protein microarray analysis of mammary epithelial cells from obese and nonobese women at high risk for breast cancer: feasibility data. *Cancer Epidemiol Biomarkers Prev* **20**: 476-482.
- Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM *et al* (1999). Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. *Proc Natl Acad Sci U S A* **96**: 1563-1568.
- Podsypanina K, Du YC, Jechlinger M, Beverly LJ, Hambarzumyan D, Varmus H (2008). Seeding and propagation of untransformed mouse mammary cells in the lung. *Science* **321**: 1841-1844.
- Polyak K, Weinberg RA (2009). Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* **9**: 265-273.
- Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D *et al* (2005). Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res* **65**: 5506-5511.
- Poste G, Fidler IJ (1980). The pathogenesis of cancer metastasis. *Nature* **283**: 139-146.
- Postigo AA, Dean DC (1999). ZEB represses transcription through interaction with the corepressor CtBP. *Proc Natl Acad Sci U S A* **96**: 6683-6688.
- Postigo AA, Ward E, Skeath JB, Dean DC (1999). zfh-1, the Drosophila homologue of ZEB, is a transcriptional repressor that regulates somatic myogenesis. *Mol Cell Biol* **19**: 7255-7263.
- Postigo AA, Dean DC (2000). Differential expression and function of members of the zfh-1 family of zinc finger/homeodomain repressors. *Proc Natl Acad Sci U S A* **97**: 6391-6396.
- Postigo AA (2003). Opposing functions of ZEB proteins in the regulation of the TGFbeta/BMP signaling pathway. *EMBO J* **22**: 2443-2452.

Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI *et al* (2010). Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res* **12**: R68.

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

Retsky MW, Demicheli R, Hrushesky WJ, Baum M, Gukas ID (2008). Dormancy and surgery-driven escape from dormancy help explain some clinical features of breast cancer. *APMIS* **116**: 730-741.

Reya T, Morrison SJ, Clarke MF, Weissman IL (2001). Stem cells, cancer, and cancer stem cells. *Nature* **414**: 105-111.

Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN (2009). The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. *Oncologist* **14**: 320-368.

Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K *et al* (2005). Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin Cancer Res* **11**: 5678-5685.

Roytblat L, Rachinsky M, Fisher A, Greemberg L, Shapira Y, Douvdevani A *et al* (2000). Raised interleukin-6 levels in obese patients. *Obes Res* **8**: 673-675.

Saal LH, Gruvberger-Saal SK, Persson C, Lovgren K, Jumppanen M, Staaf J *et al* (2008). Recurrent gross mutations of the PTEN tumor suppressor gene in breast cancers with deficient DSB repair. *Nat Genet* **40**: 102-107.

Salgado R, Junius S, Benoy I, Van Dam P, Vermeulen P, Van Marck E *et al* (2003). Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. *Int J Cancer* **103**: 642-646.

Sanchez-Tillo E, Lazaro A, Torrent R, Cuatrecasas M, Vaquero EC, Castells A *et al* (2010). ZEB1 represses E-cadherin and induces an EMT by recruiting the SWI/SNF chromatin-remodeling protein BRG1. *Oncogene* **29**: 3490-3500.

Sarrío D, Rodríguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G, Palacios J (2008). Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. *Cancer Res* **68**: 989-997.

- Savagner P (2001). Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. *Bioessays* **23**: 912-923.
- Schafer ZT, Brugge JS (2007). IL-6 involvement in epithelial cancers. *J Clin Invest* **117**: 3660-3663.
- Seewaldt VL, Johnson BS, Parker MB, Collins SJ, Swisshelm K (1995). Expression of retinoic acid receptor beta mediates retinoic acid-induced growth arrest and apoptosis in breast cancer cells. *Cell Growth Differ* **6**: 1077-1088.
- Sekido R, Murai K, Funahashi J, Kamachi Y, Fujisawa-Sehara A, Nabeshima Y *et al* (1994). The delta-crystallin enhancer-binding protein delta EF1 is a repressor of E2-box-mediated gene activation. *Mol Cell Biol* **14**: 5692-5700.
- Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML *et al* (2006). Generation of a functional mammary gland from a single stem cell. *Nature* **439**: 84-88.
- Sheridan C, Kishimoto H, Fuchs RK, Mehrotra S, Bhat-Nakshatri P, Turner CH *et al* (2006). CD44+/CD24- breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. *Breast Cancer Res* **8**: R59.
- Shi Y, Sawada J, Sui G, Affar el B, Whetstine JR, Lan F *et al* (2003). Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* **422**: 735-738.
- Siitonen SM, Kononen JT, Helin HJ, Rantala IS, Holli KA, Isola JJ (1996). Reduced E-cadherin expression is associated with invasiveness and unfavorable prognosis in breast cancer. *Am J Clin Pathol* **105**: 394-402.
- Singh M, Spoelstra NS, Jean A, Howe E, Torkko KC, Clark HR *et al* (2008). ZEB1 expression in type I vs type II endometrial cancers: a marker of aggressive disease. *Mod Pathol* **21**: 912-923.
- Sleeman KE, Kendrick H, Ashworth A, Isacke CM, Smalley MJ (2006). CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells. *Breast Cancer Res* **8**: R7.
- Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H *et al* (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* **98**: 10869-10874.

- Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A *et al* (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A* **100**: 8418-8423.
- Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A *et al* (2003). Breast cancer classification and prognosis based on gene expression profiles from a population-based study. *Proc Natl Acad Sci U S A* **100**: 10393-10398.
- Spaderna S, Schmalhofer O, Wahlbuhl M, Dimmler A, Bauer K, Sultan A *et al* (2008). The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer. *Cancer Res* **68**: 537-544.
- Spoelstra NS, Manning NG, Higashi Y, Darling D, Singh M, Shroyer KR *et al* (2006). The transcription factor ZEB1 is aberrantly expressed in aggressive uterine cancers. *Cancer Res* **66**: 3893-3902.
- Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D *et al* (2006). Purification and unique properties of mammary epithelial stem cells. *Nature* **439**: 993-997.
- Sullivan NJ, Sasser AK, Axel AE, Vesuna F, Raman V, Ramirez N *et al* (2009). Interleukin-6 induces an epithelial-mesenchymal transition phenotype in human breast cancer cells. *Oncogene* **28**: 2940-2947.
- Takagi T, Moribe H, Kondoh H, Higashi Y (1998). DeltaEF1, a zinc finger and homeodomain transcription factor, is required for skeleton patterning in multiple lineages. *Development* **125**: 21-31.
- Tarin D, Thompson EW, Newgreen DF (2005). The fallacy of epithelial mesenchymal transition in neoplasia. *Cancer Res* **65**: 5996-6000; discussion 6000-5991.
- Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J *et al* (2010). Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. *Proc Natl Acad Sci U S A* **107**: 15449-15454.
- Taylor-Papadimitriou J, Stampfer M, Bartek J, Lewis A, Boshell M, Lane EB *et al* (1989). Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: relation to in vivo phenotypes and influence of medium. *J Cell Sci* **94** (Pt 3): 403-413.

- Thiery JP (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* **2**: 442-454.
- Thiery JP (2003). Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* **15**: 740-746.
- Thiery JP, Sleeman JP (2006). Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* **7**: 131-142.
- Thiery JP, Acloque H, Huang RY, Nieto MA (2009). Epithelial-mesenchymal transitions in development and disease. *Cell* **139**: 871-890.
- Thompson EW, Newgreen DF, Tarin D (2005). Carcinoma invasion and metastasis: a role for epithelial-mesenchymal transition? *Cancer Res* **65**: 5991-5995; discussion 5995.
- Trimboli AJ, Fukino K, de Bruin A, Wei G, Shen L, Tanner SM *et al* (2008). Direct evidence for epithelial-mesenchymal transitions in breast cancer. *Cancer Res* **68**: 937-945.
- Turner NC, Reis-Filho JS (2006). Basal-like breast cancer and the BRCA1 phenotype. *Oncogene* **25**: 5846-5853.
- Umemura S, Yoshida S, Ohta Y, Naito K, Osamura RY, Tokuda Y (2007). Increased phosphorylation of Akt in triple-negative breast cancers. *Cancer Sci* **98**: 1889-1892.
- Valastyan S, Weinberg RA (2011). Tumor metastasis: molecular insights and evolving paradigms. *Cell* **147**: 275-292.
- van Grunsven LA, Michiels C, Van de Putte T, Nelles L, Wuytens G, Verschueren K *et al* (2003). Interaction between Smad-interacting protein-1 and the corepressor C-terminal binding protein is dispensable for transcriptional repression of E-cadherin. *J Biol Chem* **278**: 26135-26145.
- Vesuna F, Lisok A, Kimble B, Raman V (2009). Twist modulates breast cancer stem cells by transcriptional regulation of CD24 expression. *Neoplasia* **11**: 1318-1328.
- Visvader JE (2009). Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. *Genes Dev* **23**: 2563-2577.

- Wang CS, Goulet F, Lavoie J, Drouin R, Auger F, Champetier S *et al* (2000). Establishment and characterization of a new cell line derived from a human primary breast carcinoma. *Cancer Genet Cytogenet* **120**: 58-72.
- Wang H, Lathia JD, Wu Q, Wang J, Li Z, Heddleston JM *et al* (2009). Targeting interleukin 6 signaling suppresses glioma stem cell survival and tumor growth. *Stem Cells* **27**: 2393-2404.
- Watanabe Y, Kawakami K, Hirayama Y, Nagano K (1993). Transcription factors positively and negatively regulating the Na,K-ATPase alpha 1 subunit gene. *J Biochem* **114**: 849-855.
- Wellings SR, Jensen HM, Marcum RG (1975). An atlas of subgross pathology of the human breast with special reference to possible precancerous lesions. *J Natl Cancer Inst* **55**: 231-273.
- Wellner U, Schubert J, Burk UC, Schmalhofer O, Zhu F, Sonntag A *et al* (2009). The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nat Cell Biol* **11**: 1487-1495.
- Wells A, Yates C, Shepard CR (2008). E-cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. *Clin Exp Metastasis* **25**: 621-628.
- Wicha MS, Liu S, Dontu G (2006). Cancer stem cells: an old idea--a paradigm shift. *Cancer Res* **66**: 1883-1890; discussion 1895-1886.
- Yang AD, Fan F, Camp ER, van Buren G, Liu W, Somcio R *et al* (2006). Chronic oxaliplatin resistance induces epithelial-to-mesenchymal transition in colorectal cancer cell lines. *Clin Cancer Res* **12**: 4147-4153.
- Yang J, Weinberg RA (2008). Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell* **14**: 818-829.
- Yang MH, Wu MZ, Chiou SH, Chen PM, Chang SY, Liu CJ *et al* (2008). Direct regulation of TWIST by HIF-1alpha promotes metastasis. *Nat Cell Biol* **10**: 295-305.
- Yoshida A, Hsu LC, Dave V (1992). Retinal oxidation activity and biological role of human cytosolic aldehyde dehydrogenase. *Enzyme* **46**: 239-244.

Zhan JF, Chen LH, Yuan YW, Xie GZ, Sun AM, Liu Y *et al* (2011). STAT1 promotes radioresistance of CD44(+)/CD24(-/low) cells in breast cancer. *Exp Biol Med (Maywood)* **236**: 418-422.

Zhang GJ, Adachi I (1999). Serum interleukin-6 levels correlate to tumor progression and prognosis in metastatic breast carcinoma. *Anticancer Res* **19**: 1427-1432.

Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M *et al* (2004). Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat Cell Biol* **6**: 931-940.

Zucchi I, Sanzone S, Astigiano S, Pelucchi P, Scotti M, Valsecchi V *et al* (2007). The properties of a mammary gland cancer stem cell. *Proc Natl Acad Sci U S A* **104**: 10476-10481.

Biography

Nicholas Christopher D'Amato

Born to Christopher and Susan D'Amato, September 26, 1983, in Newton, NJ

Education

Duke University, Durham, NC, Department of Pharmacology and Cancer Biology, Ph.D. in Pharmacology, December 2011

Princeton University, Princeton, NJ, B.A. in Molecular Biology, Certificate in Finance, June 2005

Publications

D'Amato, NC*, JH Ostrander*, RD Cardiff, L Young, K Simin, RE Bachelder, M Bowie, J Delrow, A Dawson, L Yee, K Mrózek, T Clay, T Osada, and VL Seewaldt. Phenotypic plasticity in an aggressive triple-negative breast cancer: Human biology is recapitulated by a novel model system. Manuscript currently in preparation.

Vasilatos, S, G Broadwater, W Barry, J Baker, S Lem, E Dietze, G Bean, A Bryson, P Pilie, V Goldenberg, D Skaar, C Paisie, A Torres-Hernandez, TL Grant, LG Wilke, C Ibarra-Drendall, JH Ostrander, **NC D'Amato**, C Zalles, R Jirtle, VM Weaver, and VL Seewaldt. CpG island tumor suppressor promoter methylation in non-BRCA-associated early mammary carcinogenesis. *Cancer Epidemiol Biomarkers Prev.* 2009 Mar;18(3):901-14.

Baker, J, JH Ostrander, S Lem, G Broadwater, G Bean, **NC D'Amato**, V Goldenberg, C Rowell, C Ibarra-Drendall, T Grant, P Pilie, S Vasilatos, M Troch, V Scott, L Wilke, C Paisie, S Rabiner, A Torres-Hernandez, C Zalles, and VL Seewaldt. ESR1 promoter hypermethylation does not predict atypia in RPFNA nor persistent atypia after 12 months tamoxifen chemoprevention. *Cancer Epidemiol Biomarkers Prev.* 2008 Aug;17(8):1884-90.

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