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RUNX1 Control of Mammary Epithelial and Breast Cancer Cell Phenotypes

Deli Hong
University of Massachusetts Medical School

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**RUNX1 CONTROL OF MAMMARY EPITHELIAL AND BREAST CANCER
CELL PHENOTYPES**

A Dissertation Presented

By

Deli Hong

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 12, 2017

Program of Cell Biology

RUNX1 CONTROL OF MAMMARY EPITHELIAL AND BREAST CANCER CELL
PHENOTYPES

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This work was undertaken in the Graduate School of Biomedical Sciences
Program of Cell Biology
The signature of the Thesis Advisor signifies validation of Dissertation content

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Gary S. Stein, Ph. D., Thesis Advisor

The signatures of the Dissertation Defense Committee signify completion and
approval as to style and content of the Dissertation

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Leslie Shaw, Ph. D., Member of Committee

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Kendall Knight, Ph. D., Member of Committee

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Jeffery Nickerson, Ph. D., Member of Committee

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Robert Weinberg, Ph. D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation
meets the requirements of the Dissertation Committee

_____

Hong Zhang, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences
signifies that the student has met all graduation requirements of the School.

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Anthony Carruthers, Ph.D., Dean of the Graduate School of
Biomedical Sciences

December 12, 2017

Acknowledgements

Foremost, I would like to express my deepest gratitude to my thesis advisor Prof. Gary Stein, Dr. Jane Lian and Dr. Janet Stein for their continuous guidance and generous support throughout my doctoral study, without which this experience would not have been the positive experience it was. They always support me to pursue my own scientific interests in the lab. This great experience working in their lab helped me to mature into a well-rounded scientist.

I would like to give thanks to Jason Dobson for his guidance during the initial stage of my doctoral study doctoral study. Specific thanks go to Dr. Andrew Fritz, who is my primary collaborator in the lab. Additional thanks to Dr. Coralee Tye and Natali Page for their help in RNA-seq analysis, Joseph Boyd for his assistance with bioinformatics analysis, and Kristiaan Finstad and Morgan Czaja for their help in animal studies. Thanks to Terri Messier for her kind help in the lab. Additional thanks to all current and previous members of the Stein-Lian laboratory, Nick, Gillian, Prachi, Kaleem, Jonathan, Kirsten, Mingu, Areg, Josh, Helena, Gileade, Phil, Hai, Xuhui, Rodrigo, Jennifer, Mark, Cesear, Ryan, Alex for their help with my projects and scientific discussions.

I would like to thank my thesis research advisory committee members Dr. Leslie Shaw, Dr. Kendall Knight, Dr. Jeffrey Nickerson, Dr. Stephen Jones and my external committee Dr. Robert Weinberg and my committee chair Dr. Hong Zhang for their time, valuable input and support for my graduate studies and career development.

I would like to extend my gratitude to my beloved parents Dr. Xiuping Hong and Dr. Yiping Sun for their unconditional love and support throughout my life. I also want to thank all of my friends for their support and company.

Abstract

Breast cancer remains the most common malignant disease in women worldwide. Despite the advantages of early detection and improved treatments, studies into the mechanisms that initiate and drive breast cancer progression are still required. Recent studies have identified RUNX1, which is an essential transcription factor for hematopoiesis, is one of the most frequently mutated genes in breast cancer patients. However, the role of RUNX1 in the mammary gland is understudied.

In this dissertation, we examined the role of RUNX1 in both normal mammary epithelial and breast cancer cells. Our *in vitro* studies demonstrated that RUNX1 inhibits epithelial to mesenchymal transition (EMT), migration, and invasion, reflecting its tumor suppressor activity, which was confirmed *in vivo*. Moreover, RUNX1 also contributes significantly to inhibition of the phenotypes of breast cancer stem cells (CSC), which is responsible for metastasis and tumor relapse. We showed that Runx1 overexpression reduces the tumorsphere formation and cancer stem cell population. Overall, our studies provide mechanistic evidence for RUNX1 repression of EMT in mammary cells, anti-tumor activity *in vivo* and regulation of CSC-like properties in breast cancer.

Our results highlight crucial roles for RUNX1 in preventing epithelial to mesenchymal transition and tumor progression in breast cancer. This RUNX1 mediated mechanism points to novel intervention strategies for early stage breast cancer.

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List of Symbols and Abbreviations

Acute myeloid leukemia (AML)

Acute lymphoblastic leukemia (ALL)

Aldehyde dehydrogenase (ALDH)

Anaphase-promoting complex (APC) complex

Breast cancer stem cell (BCSC)

Caenorhabditis elegans (C.elegans)

Cancer stem cell (CSC)

Circulating tumor cell (CTC)

Cleidocaranial dysplasia (CCD)

Core-binding factor β (CBFβ)

Cyclin-dependent kinase 1 (CDK1)

Drosophila melanogaster (Dm)

Ductal carcinoma in situ (DCIS)

Endothelial to hematopoietic transition (EHT)

Epidermal growth factor (EGF)

Epithelial-mesenchymal transition (EMT)

Estrogen receptor (ER)

Fibroblast-specific protein 1 (Fsp1)

Hematopoietic stem cells (HSCs)

Hepatocyte growth factor (HGF)

Human epidermal growth factor receptor 2 (HER2)

Integrin-like kinase (ILK)

Invasive ductal carcinoma (IDC)

Long noncoding RNAs (lncRNAs)

Mammary stem cells (MSC)

Mesenchymal to epithelial transition (MET)

Mitotic checkpoint complex (MCC)

Myelodysplastic syndrome (MDS)

Nervy homology regions (NHR)

Polyoma enhancer-binding protein-2α (PEBP2α)

Progesterone receptor (PR)

Propidium iodide (PI)

Standard error of the mean (SEM)

Strongylocentrotus purpuratus (Sp)

Sub-nuclear matrix-targeting signal (NMTS)

The Cancer Genome Atlas (TCGA)

Transcripts per million (TPM)

Transforming growth factor beta (TGF-β)

Triple-negative breast cancer (TNBC)

Preface

Portions of this thesis have appeared in the following published works:

Chapter II:

Hong D, Messier TL, Tye CE, Dobson JR, Fritz AJ, Sikora, KR, Browne G, Stein JL, Lian JB, Stein GS

Runx1 stabilizes the mammary epithelial cell phenotype and prevents epithelial to mesenchymal transition. *Oncotarget.* 2017; 8:17610-17627

Other published work during graduate study that are not presented in this thesis:

1. Zaidi SK, Frietze SE, Gordon JA, Heath JL, Messier TL, **Hong D**, Boyd JR, Kang M, Imbalzano AN, Lian JB, Stein JL, Stein GS

Bivalent Epigenetic ControlD of Oncofetal Gene Expression in Cancer**.** *Molecular and Cellular Biology* 2017 In press PMID: 28923849

2. Fritz AJ, Ghule PN, Boyd JR, Tye CE, Page NA, **Hong D**, Weinheimer AS, Barutcu AR, Gerrard DL, Frietze SE, Zaidi SK, Imbalzano AN, Lian JB, Stein JL, Stein GS

Intranuclear and higher‐order chromatin organization of the major histone gene cluster in breast cancer Journal of cellular physiology 2017 In press PMID: 28504305

3. Barutcu AR, **Hong D**, Lajoie BR, McCord RP, van Wijnen AJ, Lian JB, Stein JL, Dekker J, Imbalzano AN, Stein GS

RUNX1 associates with TAD boundaries and organizes higher order chromatin

structure in breast cancer cells. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 2016; 1859(11): 1389

4. Dobson JR, **Hong D**, Barutcu AR, Hai W, Anthony N Imbalzano, Lian JB, Stein JL, van Wijnen AJ, Nickerson JA, Stein GS

Isolation and characterization of nuclear matrix-associated DNA in breast cancer cell lines. *J Cell Physiol.* 2017;232(6):1295.

5. Browne G, Dragon JA, **Hong D**, Messier TL, Gordon JA, Farina NH, Boyd JR, VanOudenhove JJ, Perez AW, Zaidi SK, Stein JL, Stein GS, Lian JB

MicroRNA-378-mediated suppression of Runx1 alleviates the aggressive phenotype of triple-negative MDA-MB-231 human breast cancer cells. Tumour Biol. 2016; 37(7): 8825

6. Barutcu AR, Lajoie BR, McCord RP, Tye CT, **Hong D**, Messier TL, Browne G, van Wijnen AJ, Lian JB, Stein JL, Dekker J, Imbalzano AN, and Stein GS

Chromatin interaction analysis reveals changes in small chromosome and telomere clustering between epithelial and breast cancer cells. Genome Biol. 2015; 16(1): 214.

7. Zhang X, Wu H, Dobson JR, Browne G, **Hong D**, Akech J, Languino LR, Stein JL, Stein GS and Lian JB

Expression of the IL-11 Gene in Metastatic Cells Is Supported by Runx2-Smad and Runx2-cJun Complexes Induced by TGFβ1 *J Cell Biochem.* 2015; 16(9): 2098

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Hsa-mir-30c promotes the invasive phenotype of metastatic breast cancer cells by targeting NOV/CCN3. *Cancer Cell International* 2014; 14(1): 73

Chapter I Introduction

1.1 Mammary Gland Biology and Breast Cancer

1.1.1   Breast cancer overview

Breast cancer is the most commonly diagnosed cancer in women worldwide (~30% of new cancer diagnoses). Approximately 1 in 8 women in the USA will develop invasive breast cancer during their lifespan. In 2017, about 252,000 new invasive breast cancer cases are expected to be diagnosed in the U.S (Siegel, Miller et al. 2016).   In the past few decades, significant advances in early detection and treatment of breast cancer have greatly improved the overall 5-year survival rate for breast cancer patients with an increase from 35% in 1960's to 89% in 2016 (Miller, Siegel et al. 2016). Despite this progress, breast cancer remains the second leading cause of cancer-related death in American women. About 40,000 women in the USA are expected to die due to breast cancer in 2017 alone (Siegel, Miller et al. 2016).   Worldwide, half-a-million women die from breast cancer each year. Therefore, further studies into the mechanisms that initiate and drive breast cancer progression are still needed. A greater understanding of these mechanisms will provide new potential targets for improved therapies.

1.1.2 Breast cancer molecular subtypes

Breast cancer is either ductal or lobular, with the ductal type compositing the majority of cases (40%-75%) (Bombonati and Sgroi 2011). Ductal breast carcinoma progression can be further divided into 4 progressive stages based on

histology: flat epithelial atypia (benign lesion), atypical hyperplasia (precancerous lesion), ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC, which is highly aggressive and metastatic) (Bombonati and Sgroi 2011). However, patients exhibit considerable heterogeneity in clinical responses even amongst the same stage, indicating the need for a new classification method (Polyak 2007, Rivenbark, O'Connor et al. 2013). In the past two decades, using the gene expression portrait including the expression of estrogen receptor (ER), the progesterone receptor (PR) and the human epidermal growth factor receptor-related protein (HER2), breast cancer is characterized into 6 distinct molecular subtypes, summarized in Table 1.1, including four major subtypes: Luminal A, Luminal B, Basal like, Her2 enriched, and two unusual subtypes: Claudin-low and normal-breast (Sørlie, Perou et al. 2001, Sørlie, Tibshirani et al. 2003, Prat, Parker et al. 2010, Eroles, Bosch et al. 2012) .

Molecular Subtype	Luminal A	Luminal B	Basal Like	Her2 Positive	Claudin Low	Normal Breast Like
Frequency	50-60%	10-20%	10-20%	15-20%	12-14%	5-10%
ER/PR/Her2	ER+PR+ HER2-	ER+/-PR+/- HER2-	ER-PR- HER2-	ER-PR- HER2+	ER-PR- HER2-	ER-/+ HER2-
Proliferation	Low	High	High	High	High	Low
Histologic grade	Low	Intermediate	High	High	High	Low
Prognoisis	Good	Intermediate	Poor	Poor	Poor	Intermediate
P53 mutation	Low	Intermediate	High	High	High	Low
Top Metastatic site	Bone	Bone, Liver	Lung	Lung	Unclear	Unclear

Table 1.1 Features of molecular subtypes of breast cancer.

Luminal A

The luminal A subtype comprises 50–60% of all diagnosed breast tumors and is therefore the most common subtype (Eroles, Bosch et al. 2012). It is characterized by high expression of ER-activated genes that are typically expressed in the luminal epithelial lining in the mammary ducts (Sørlie, Perou et al. 2001). Luminal A tumors usually have a low histological grade, and lower expression of proliferation related genes. In particular, the immunohistochemistry profile of the luminal A subtype is characterized by expression of ER, PR, Bcl-2, GATA3 and cytokeratin CK8/18, and an absence of HER2 and Ki67 expression (Eroles, Bosch

et al. 2012). This subtype of breast cancer has a higher incidence of metastasis to bone (18.7% of total patients) compared to other sites such as nervous system, liver and lungs, which together represent less than 10% of metastatic sites (Eroles, Bosch et al. 2012). Luminal A patients have a generally good prognosis with a metastatic rate of 27.8% which is significantly lower than that of other subtypes (Kennecke, Yerushalmi et al. 2010). The treatment of this subtype is mainly based on hormonal treatment in postmenopausal patients, and selective estrogen receptor modulators like Tamoxifen, a competitive inhibitor of the estrogen receptor binding to its ligands (Guarneri and Conte 2009).

Luminal B

The luminal B group makes up 10–20% of all breast cancers and has a higher histological grade, greater proliferative rate, and an aggressive phenotype with a worse prognosis compared with the Luminal A subtype (Colleoni, Rotmensz et al. 2012). Similar to the Luminal A subtype, the Luminal B subtype also expresses ER, but with a higher expression of proliferation genes, such as Ki67 and cyclin-B1, and growth factor receptors EGFR and HER2. Bone is also the most common site of metastasis (30%), together with a high metastasis rate in other organs such as the liver (13.8%) (Eroles, Bosch et al. 2012). Luminal B tumors are treated with Tamoxifen and aromatase inhibitors, which inhibits the generation of estrogen. However, the worse prognosis compared to luminal A tumors underlines the need of new therapeutic options for this subgroup (Bosch, Eroles et al. 2010).

Basal-like

The basal-like subtype accounts for 10% to 20% of breast cancer cases (Bosch, Eroles et al. 2010). Basal-like tumors typically express genes characteristic of mammary myoepithelial cells, including Cytokeratins CK5 and CK17, P-cadherin, Caveolin 1/2, Nestin, CD44, Vimentin and EGFR (Nielsen, Hsu et al. 2004). Meanwhile genes characteristic of the luminal epithelium, such as CK8/18 and Kit, are lower in these tumors (Eroles, Bosch et al. 2012). Clinically, basal-like tumors are characterized by their larger size, higher grade, presence of necrosis, pushing borders of invasion, and frequent invasion of the lymph node (Livasy, Karaca et al. 2005, Bosch, Eroles et al. 2010). One of the most relevant features of this subtype is the lack of expression of the three key receptors in breast cancer: estrogen receptor, progesterone receptor and HER2. For this reason the basal-like group overlaps with triple-negative breast cancer (TNBC) (Eroles, Bosch et al. 2012). Compared with luminal subtypes, basal-like tumors frequently have a worse prognosis and a higher relapse rate in the first 3 years (Dent, Trudeau et al. 2007). Molecularly, basal-like tumors have a high rate of p53 mutation and often carry a germ-line mutation in BRCA1 (Sørlie, Tibshirani et al. 2003). Metastatic relapse of the basal-like subtype commonly occurs in visceral organs, such as lung, central nervous system and lymph nodes (Kennecke, Yerushalmi et al. 2010).

HER2 positive

HER2 positive tumors represent 15-20% of breast cancers. They are characterized by a high expression of the HER2 gene and other genes associated with the HER2 pathway (Eroles, Bosch et al. 2012). These tumors are highly proliferative, with a high histological grade and frequent p53 mutations (Montemurro, Di Cosimo et al. 2013). Clinically, the HER2-positive subtype is a poor prognosis subtype, while the introduction of anti-HER2 treatment has significantly improved survival in both primary and metastatic disease (Slamon, Leyland-Jones et al. 2001).

Claudin-low

The Claudin-low subtype is the newest defined subtype, which was identified in 2007 (Herschkowitz, Simin et al. 2007). It is characterized by having low expression of tight junction and intercellular adhesion genes, including Claudin-3, -4, -7, Occludin and E-cadherin (Eroles, Bosch et al. 2012). The gene expression profile of this subtype is similar to that of the basal subtype, as both have low Her2 and luminal gene expression (Parker, Mullins et al. 2009, Prat, Parker et al. 2010). In contrast to the basal subtype, however, the Claudin-low subtype expresses a set of 40 immune response-related genes, indicating high infiltration of immune system cells (Hennessy, Gonzalez-Angulo et al. 2009, Prat, Parker et al. 2010, Sabatier, Finetti et al. 2014). Additionally, this subtype is also enriched in genes associated with epithelial-mesenchymal transition and cancer stem cell phenotypes (Eroles, Bosch et al. 2012). These tumors show poor long-term

prognosis and are not sensitive to neoadjuvant chemotherapy (Prat, Parker et al. 2010, Prat and Perou 2011).

Normal Breast Subtype

The normal-breast subtype accounts for about 5-10% of breast carcinomas (Eroles, Bosch et al. 2012). This subtype expresses genes associated with adipose tissues and has an intermediate prognosis between luminal and basal-like subtypes. Tumors from this subtype are occasionally inappropriately classified as triple-negative as they do not express ER, PR and HER. However, this subtype differs from the basal-like subtype, as they are negative for CK5 and EGFR expression (Eroles, Bosch et al. 2012). There are some contradictory views of this subtype, as some researchers question its existence as they consider it a technical artifact due to contamination from normal breast tissue (Weigelt, Mackay et al. 2010). The knowledge regarding the molecular mechanism and treatment is inadequate for this subtype due to its rarity and the technical artifact hypothesis.

1.1.3 The origin of breast cancer and breast cancer subtypes

As discussed above, breast cancer is not a single disease, but is composed of distinct subtypes associated with different clinical outcomes. Understanding this heterogeneity is key for developing targeted therapy and preventive intervention. The roots of breast cancer heterogeneity lie in the developmental hierarchy of the normal mammary gland, which contains both luminal and basal cell lineages (Skibinski and Kuperwasser 2015). It has been speculated for a long time that

accumulation of specific mutations in a particular cell type of the normal mammary epithelium generates transformed multi-potent cells, which then give rise to a specific breast cancer subtype (Smalley and Ashworth 2003). The molecular features of each subtype mirror the characteristics of the normal cell type of their origin. For example, mammary stem cells are thought to be the cell-of-origin for basal-like breast cancer based on their shared features such as expression of basal cytokeratin and low expression of hormone receptors (Polyak 2007). While Luminal A tumors are thought to be derived from relatively well-differentiated cells of the ER+ lineage, Luminal B tumors are believed to develop from less differentiated luminal progenitors (Polyak 2007). However, this hypothesis has been recently challenged using *in vivo* lineage tracing. In this method, particular cell types from the mammary gland, such as mammary stem cells, luminal progenitors and basal progenitor cells, have been identified using different cell surface markers (Summarized in Fig. 1.1) (Visvader and Stingl 2014, Skibinski and Kuperwasser 2015). Comparison of the gene expression signature of these lineages with breast cancer subtypes has suggested that one lineage may give rise to the multiple subtypes (Lim, Vaillant et al. 2009). Luminal progenitors likely serve as the origin of both luminal and basal-like breast cancers (Lim, Vaillant et al. 2009, Prat and Perou 2011); whereas the basal progenitor signature is most closely aligned with the expression profile of the Claudin-low subtype (Lim, Vaillant et al. 2009). This observation was confirmed by studies of the origin of BRCA-1 associated breast cancer. Different strategies have all demonstrated that BRCA-

8

1 associated basal-like breast cancer is derived from luminal progenitor cells (Lim, Vaillant et al. 2009, Molyneux, Geyer et al. 2010, Proia, Keller et al. 2011, Bai, Smith et al. 2013).

Still, it remains an open question as to why luminal progenitor cells can give rise to both luminal and basal subtypes. One explanation is that the luminal progenitor population itself is heterogeneous (e.g., with respect to estrogen receptor expression) (Booth and Smith 2006, Shehata, Teschendorff et al. 2012). Another hypothesis is based on the striking finding that 80% of basal-like breast tumors carry p53 mutations. An early loss of p53 may cause genome instability thereby allowing the acquisition of secondary mutations. The cells with p53 and secondary mutations may gain a competitive advantage over neighboring clones with regard to proliferation, migration and invasion, which are also features of basal-like breast cancer (Skibinski and Kuperwasser 2015). Currently the origin of Her2-positive breast cancer remains unclear. Better understanding of the etiology and biology of each subtype will enhance the precision of diagnosis and treatment of women with different forms of breast cancer.
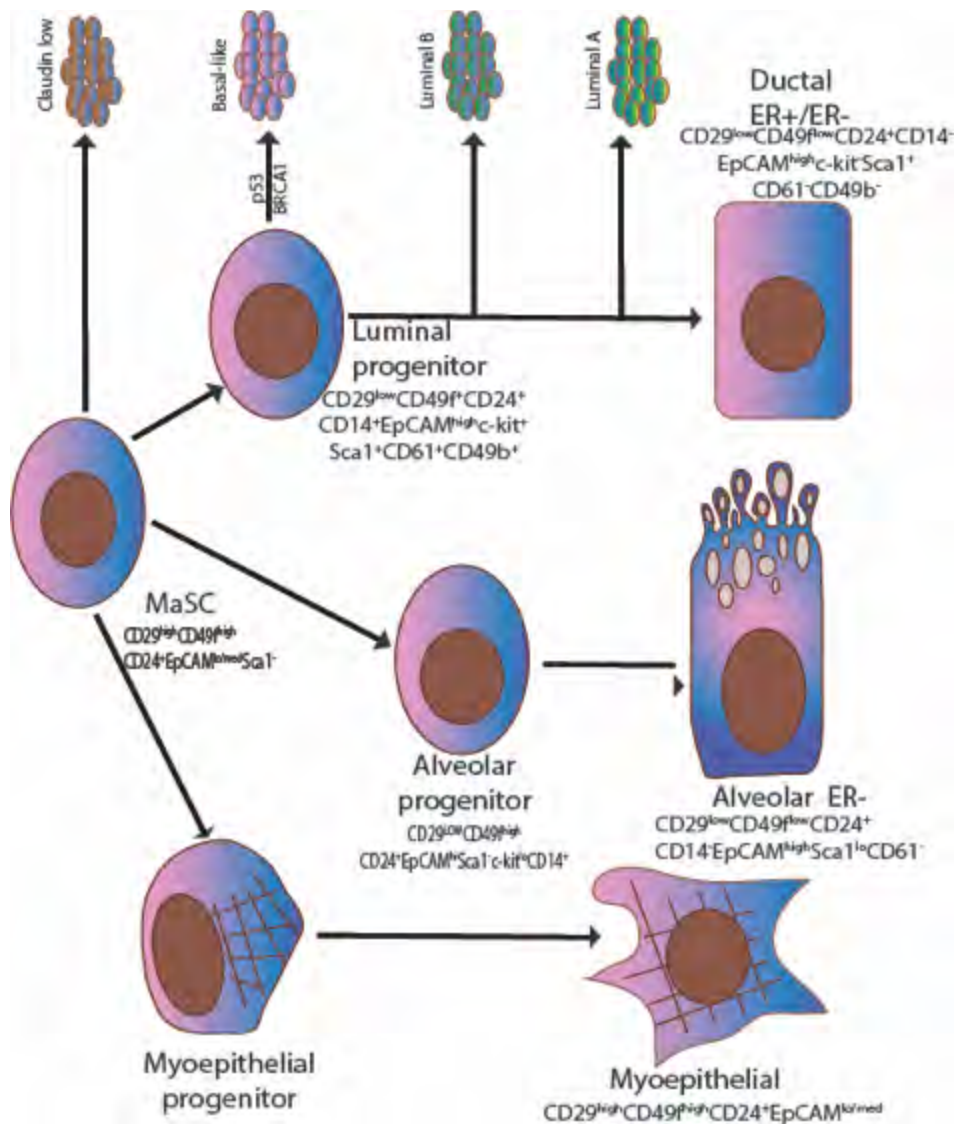
Figure 1.1 Schematic model of mammary epithelial hierarchy and potential relationship with breast tumor subtypes. Cell surface markers used for the isolation of epithelial cell populations from the mouse mammary gland are indicated. The four major tumor types are shown linked to their closest normal epithelial cell type. Basal-like subtype could originate through mutation of p53 and BRCA1 in the luminal progenitor cells.

1.1.4 Cell line models used in breast cancer studies

The first breast cancer cell line, BT-20, was established in 1958 (Lasfargues and Ozzello 1958). Later more breast cancer cell lines were generated, such as the MDA series generated by MD Anderson Cancer Center. One of such cell line, MDA-MB-231, the highly metastatic breast cell line generated in 1973 (Cailleau, Young et al. 1974), is widely used to identify genes and pathways that regulate metastasis to different sites (Kang, Siegel et al. 2003, Minn, Gupta et al. 2005, Bos, Zhang et al. 2009). The most commonly used breast cancer cell line in the world is MCF-7, which was also established in 1973 at the Michigan Cancer Foundation (Soule, Vazquez et al. 1973). MCF-7 cells have high expression of estrogen receptor (ER), which makes them very sensitive to hormone and thus an ideal model to study hormone response (Levenson and Jordan 1997). Currently there are more 100 breast cancer cell lines available from ATCC. Based on their gene expression profiles, they have been grouped into different subtypes of breast cancer (Neve, Chin et al. 2006, Prat, Karginova et al. 2013). Breast cell line models are widely used to identify molecular mechanisms, test treatment response both *in vitro* and in xenograft models (Holliday and Speirs 2011).

Another breast cell line model used in this dissertation is the MCF10 cell line series. MCF10A cells are considered to be a normal-like mammary epithelial cell, which was obtained from a patient with benign fibrocystic disease (Soule, Maloney et al. 1990). It is a spontaneously immortalized, non-malignant breast cell line. The MCF10A cell line is the founder of a progressively more aggressive family of breast

cancer lines named MCF10 series. These cell lines include MCF10AT1, which is a premalignant cell line derived from MCF10A by overexpressing the H-Ras oncogene (Dawson, Wolman et al. 1996), a set of highly aggressive and metastatic MCF10CA cell lines (including MCF10CA1a), which gained the capability of metastasis after *in vivo* passage of MCF10AT (Santner, Dawson et al. 2001). While MCF10A cells cannot form tumors *in vivo*, MCF10AT can form tumors with an incidence of about 25% and MCF10CA1a always forms tumors after subcutaneous injection into nude mice (Dawson, Wolman et al. 1996, Santner, Dawson et al. 2001). Therefore, the MCF10 cell line series provides a useful model to assess the progression of breast cancer.

1.2   The Runx Family

1.2.1 Runx family overview

Runx proteins, which function as lineage-specific transcription factors, regulate cell differentiation, proliferation and growth (Reviewed in (Coffman 2003)).   Runx proteins are also known as acute myeloid leukemia (AML), core-binding factor (CBF) or Polyoma enhancer-binding protein-2α (PEBP2α) family (Ito 2004). The Runx proteins share a highly conserved Runt domain with 128 amino acids in the N-terminus (Ogawa, Maruyama et al. 1993). This Runt homology domain is responsible for DNA binding and hetero-dimerization with Core Binding Factor (CBF-β), which stabilizes the protein complex. The Runx-CBF-β complex binds to a consensus sequence within the DNA (PyGPyGGTPy;Py- cytosine or thymine)

(Melnikova, Crute et al. 1993) (Ogawa, Maruyama et al. 1993). A nuclear targeting sequence, located on the C-terminal end of the Runt domain, is essential for proper nuclear localization (Kanno, Kanno et al. 1998). Although Runx proteins are primarily located in nucleus, in some cell types, Runx proteins are found partly in cytoplasm sequestered by STAT5, which is usually elevated in cancer cells (Ogawa, Satake et al. 2008).

All Runx family members also have a conserved C-terminal region, which is a sub-nuclear matrix-targeting signal (NMTS) (Zeng, McNeil et al. 1998, Zaidi, Javed et al. 2001). The NMTS in Runx proteins is a 30-35 amino acid sequence, responsible for sub-nuclear localization to distinct nuclear sites for specific gene regulation (Zeng, van Wijnen et al. 1997, Zeng, McNeil et al. 1998, Zaidi, Javed et al. 2001, Stein, Lian et al. 2007). The NMTS organizes the multiple complexes of Runx proteins with different classes of co-regulatory factors, such as SMAD family members. Runx proteins also have PY and VWRPY motifs for protein-protein interaction with other transcription factors (Aronson, Fisher et al. 1997, Javed, Guo et al. 2000, Lian, Javed et al. 2004, Zaidi, Young et al. 2005, Westendorf 2006, Chuang, Ito et al. 2013).

In almost all species, Runx function has been shown to be dependent on its binding to CBF-β, which increases specificity and affinity of Runx protein binding to their target genes (Golling, Li et al. 1996, Adya, Castilla et al. 2000, Kagoshima, Nimmo et al. 2007). Sedimentation equilibrium measurement was performed to confirm that Runx, CBF-β and DNA form a complex in a 1:1:1 stoichiometry (Tang,

Crute et al. 2000). The affinity of Runx for DNA or Runx-CBF-β for DNA has also been measured using electromobility shift assay (EMSA) and an isothermal titration calorimetric assay (Crute, Lewis et al. 1996, Huang, Crute et al. 1998, Tang, Crute et al. 2000). Both measurements have shown a significant enhancement (6 to 10-fold) of Runx-DNA binding affinity in the presence of CBF-β (Crute, Lewis et al. 1996, Huang, Crute et al. 1998, Tang, Crute et al. 2000). In addition to CBF-β, Runx factors also bind with co-activators (e.g., p300) or co-repressors (e.g., Groucho) depending on the cellular context (Aronson, Fisher et al. 1997, Javed, Guo et al. 2000, Coffman 2003, Durst and Hiebert 2004, Chuang, Ito et al. 2013, Ito, Bae et al. 2015). This complex and dynamic ability allows Runx factors to engage in diverse functions and regulatory mechanisms (Coffman 2003).

1.2.2 Structure of Runx

The structure of the Runt domain has been determined, using X-ray crystallography and NMR, to be a member of the s-type Ig fold DNA binding domains (Berardi, Sun et al. 1999, Nagata, Gupta et al. 1999). Other members include NF-kB, NFAT, STAT1 and p53 (Berardi, Sun et al. 1999, Nagata, Gupta et al. 1999). The structure of Runx-CBF-β-DNA complex was later solved using X-ray crystallography (Warren, Bravo et al. 2000, Bravo, Li et al. 2001, Tahirov, Inoue-Bungo et al. 2001). As shown in Figure 1.2, the structure reveals that the Runt domain contacts with both DNA major and minor grooves, and the C-terminal region of the Runt domain establishes sequence-specific DNA-contacts. On the

other hand, CBF-β does not make any contacts with DNA but induces a conformational change in the Runt domain to allosterically facilitate binding between Runx factors and DNA (Tahirov and Bushweller 2017). Mutagenesis studies also identified that residues at the C-terminus of Runt domain (T169, D171, R174 and R177 in human RUNX1) are the key amino acids, essential for forming the complex between Runx and CBF-β and DNA (Li, Yan et al. 2003). In breast cancer patients, several RUNX1 mutations have been identified in Runt domain. These mutations, such as D171 and R174, are located at the interface of the Runt domain and DNA (Fig. 1.2 Red residues), suggesting that loss of Runx binding on target genes will cause disease. In addition to breast cancer, mutations in the interface of DNA/Runx or CBF-β/Runx binding have been documented in patients with either RUNX1 related leukemia or RUNX2 related Cleidocranial Dysplasias (CCD), respectively (Otto, Kanegane et al. 2002, Mangan and Speck 2011).

The Runt domain is evolutionary conserved in metazoans suggesting that Runx proteins have conserved functions through different species. Because Runx genes are highly context dependent and partially redundant within vertebrates, the use of invertebrate animal models with simple genetic background such as *Drosophila melanogaster* or *Caenorhabditis elegans (C.elegans)* can help us find an ancestral function of Runx.
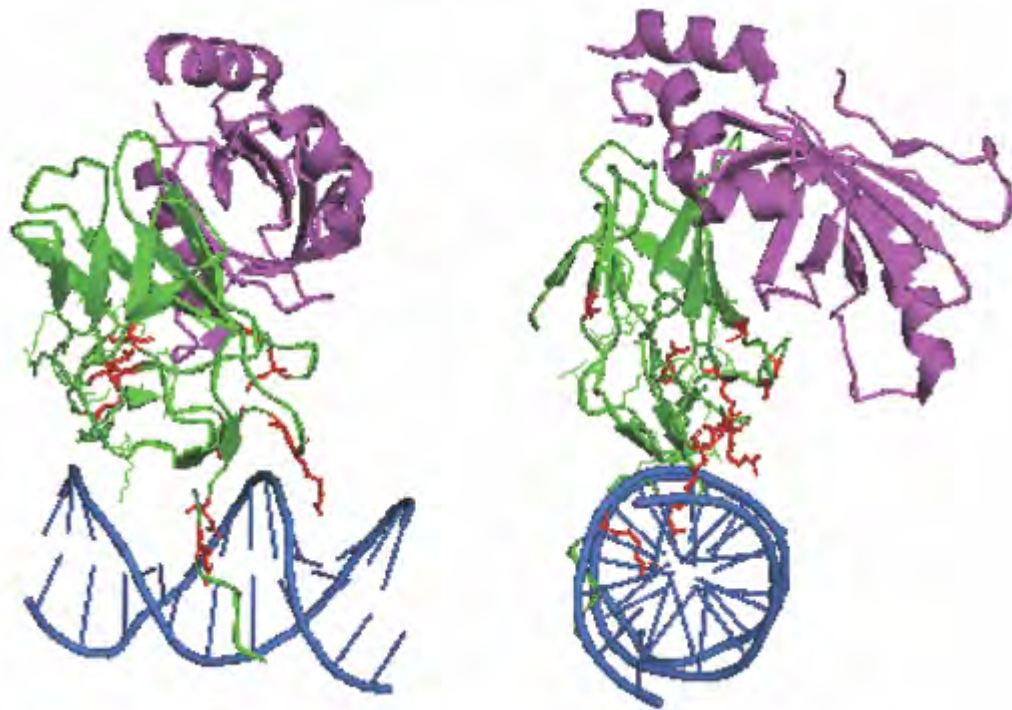
Figure 1.2 Structure of the CBF-β: Runt domain: DNA complex.

Ribbon representation shows CBF-β in purple, the Runt domain in green, and the DNA in blue. RUNX1 mutations identified in breast cancer patients are shown in red. For clarity, the structure is shown in two different orientations, rotated by 90 degrees relative to one another. The image was rendered from PDB code 1H9D. The mutations are clearly seen in the DNA binding domain suggesting a loss of RUNX1 function in breast cancer. Association of loss function of RUNX1 and breast cancer progression is studied in Chapter II,III and IV.

1.2.3 Evolutionary role of Runx

Evolutionarily, Runx genes have been identified in all metazoans and unexpectedly in the unicellular amoeboid halozoan *Capsaspora owczarzaki*, suggesting the Runx family is involved in fundamental biological processes (Sebé-Pedrós, de Mendoza et al. 2011). The role of Runx genes have been intensively studied in the invertebrate animal models *Drosophila melanogaster (Dm), Strongylocentrotus purpuratus (Sp)* and *Caenorhabditis elegans.* The mechanisms obtained from these models can give us a hint of Runx function in mammals, especially in human.

In the fruit fly, *Drosophila melanogaster,* there are four Runx genes (Rennert, Coffman et al. 2003, Bao and Friedrich 2008)*.* The most well studied Runx family member is *runt,* which was identified for its function in development. *DmRunt* is one of the five pair-rule genes, which regulate the spatial expression of other pair rule genes and segment polarity genes (Nusslein-Volhard and Wieschaus 1980, Gergen and Wieschaus 1985). Deletion of *DmRunt* results in the loss of larval segments and consequently, smaller than wild-type flies (Gergen and Wieschaus 1985). In addition, *DmRunt* also plays a role in sex determination and neurogenesis (Gergen and Butler 1988, Kania, Bonner et al. 1990, Duffy and Gergen 1991, Duffy, Kania et al. 1991, Canon and Banerjee 2000). Another Runx family member studied in *Drosophila* is *lozenge* (*lz*), which is required for eye development and hematopoiesis (Canon and Banerjee 2000). The function of two other Runx genes, *CG34145* (*RunxA*) and *CG42267* (*RunxB*) remains unclear.

However, it has been shown that *RunxB* is involved in the control of cell survival (Boutros, Kiger et al. 2004).

In *Caenorhabditis elegans*, the single Runx homolog, *rnt-1*, also plays an essential role during development (Hughes and Woollard 2017). It regulates the balance between proliferation/self-renewal and differentiation in the lateral neuroectodermal seam cells (Kagoshima, Sawa et al. 2005, Nimmo, Antebi et al. 2005, Xia, Zhang et al. 2007). The seam cells are multi-potent stem cell-like cells formed during *C.elegans* embryogenesis (Sulston and Horvitz 1977). *Rnt-1* is expressed in seam cells during embryogenesis and throughout larval development and functions to regulate their division (Braun and Woollard 2009). Consequently, in *rnt-1* mutant worms, the number of seam cells is reduced from 16 to an average of 13 per worm (Kagoshima, Sawa et al. 2005, Nimmo, Antebi et al. 2005). Importantly, overexpression of *rnt-1* leads to hyper-proliferation and expansion of seam cells (Kagoshima, Sawa et al. 2005, Kagoshima, Nimmo et al. 2007). As a result, *rnt-1* overexpression mutant worms develop massive hyperplasia leading to a tumor-like appearance of the seam cell tissue, which normally forms a straight line of cells at each side of the worm (Kagoshima, Nimmo et al. 2007).

There are two Runx genes in sea urchin *S. purpuratus*, but only one of them, *SpRunt-1*, is expressed (Braun and Woollard 2009). *SpRunt-1* is expressed in various tissues during embryogenesis and transiently in adult coelomocytes upon challenging their immune system (Coffman, Kirchhamer et al. 1996, Pancer, Rast et al. 1999, Robertson, Dickey et al. 2002, Fernandez-Guerra, Aze et al. 2006).

During embryogenesis, *spRunt-1* regulates the expression of transcription factors and other markers of terminal differentiation in all major tissues (Robertson, Coluccio et al. 2008). *SpRunt-1* activates the WNT signaling pathway thereby positively regulating cell proliferation (Minokawa, Wikramanayake et al. 2005, Robertson, Coluccio et al. 2008).

The role of Runx genes as the master regulator specifying lineage was further studied in the more complex vertebrate animal models. Runx1 is expressed in hematopoietic progenitors in Zebrafish and Xenopus where it controls stem cell differentiation (Tracey, Pepling et al. 1998, Kalev-Zylinska, Horsfield et al. 2002, Burns, Traver et al. 2005). Runx1 is also required for neuronal development in Xenopus (Park, Hong et al. 2012). In both fish and frogs, Runx2 is required for chondrogenesis and is detected in developing bones (Flores, Tsang et al. 2004, Flores, Lam et al. 2006, Kerney, Gross et al. 2007).

In summary, evidence gathered utilizing different animal models from invertebrate to vertebrate, separated by millions of years of evolution, helps build a picture of Runx genes as key transcription factors. This work further highlights their function in lineage determination and fine-tuning the balance between cell proliferation and differentiation. These Runx functions identified in lower animal models are also found in mammalian cells. In the next section, the role of each Runx factor during normal development in mammalian systems is reviewed.

1.3 The Runx Family and Development in Mammals

1.3.1 Overview

In mammals, the Runx family is composed of three genes, Runx1, Runx2, and Runx3. Each of these genes is transcribed from two promoters, a distal P1 promoter and a proximal P2 promoter (Ghozi, Bernstein et al. 1996, Fujiwara, Tagashira et al. 1999, Drissi, Luc et al. 2000, Bangsow, Rubins et al. 2001). All Runx proteins play essential roles in both normal developmental processes and diseases. Runx1 is essential for hematopoiesis (Okuda, van Deursen et al. 1996), Runx2 is required for osteoblast maturation and osteogenesis (Otto, Thornell et al. 1997), and Runx3 is involved in gastrointestinal, neurogenesis of the dorsal root ganglia and T-cell differentiation (Inoue, Ozaki et al. 2002, Levanon, Bettoun et al. 2002, Li, Ito et al. 2002). Deletion of any of the Runx genes is lethal in mice. For example, Runx1 loss causes embryonic lethality due to major defects in the formation of the fetal liver and hemorrhaging in the central nervous system by embryonic day 12.5 (E12.5) (Okuda, van Deursen et al. 1996, Wang, Stacy et al. 1996). Furthermore, mice bearing a homozygous mutation in Runx2 die just after birth due to an inability to breathe, presumably caused by complete lack of ossification (Otto, Thornell et al. 1997). The concept of fundamental core mechanism(s) for Runx protein function in development has been posited, however no single common machinery that governs the development of different tissues has been identified. Instead, Runx proteins utilize multiple spatiotemporal mechanisms to regulate development of different tissues depending on tissue type

or age. In this section, I will discuss the role of each Runx protein in tissue development.

1.3.2 Runx1

Runx1 is widely considered as the master regulator of developmental hematopoiesis (Okuda, van Deursen et al. 1996, Yzaguirre, de Bruijn et al. 2017). The process of hematopoiesis begins with primitive hematopoiesis, where a limited number of blood lineages (erythrocyte progenitors, erythrocyte/ megakaryocyte progenitors and primitive macrophages) that sustain early embryonic development are produced primarily from the yolk sac (Palis, Robertson et al. 1999, Xu, Matsuoka et al. 2001, Ferkowicz and Yoder 2005, Tober, Koniski et al. 2007). Runx1 is expressed in the mesodermal masses in this yolk sac, and in the primitive hematopoietic cells with the exception of primitive erythrocyte progenitor cells (North, Gu et al. 1999, Georges Lacaud, Lia Gore et al. 2002). Although, Runx1 is not considered to be required for primitive hematopoiesis, in its absence, all three primitive hematopoietic lineages are affected. Without Runx1, primitive macrophages are absent (Georges Lacaud, Lia Gore et al. 2002, Li, Chen et al. 2005), the number of megakaryocytes is reduced (Potts, Sargeant et al. 2014), and primitive erythrocytes are abnormal in function with decreased expression of the erythroid marker Ter118 and the transcription factors EKLF and Gata1 (Castilla, Wijmenga et al. 1996, Yokomizo, Hasegawa et al. 2008). Therefore, Runx1 plays an essential role in primitive hematopoiesis.

After primitive hematopoiesis, endothelial cells undergo a process known as definitive hematopoiesis, which constitutes the second and third waves of blood development (Yzaguirre, de Bruijn et al. 2017). During this stage of development, hematopoietic stem cells (HSCs) are formed (Chen, Mao et al. 2014). HSCs have long-term repopulation capacity and the ability to produce any of the hematopoietic lineages (Bryder, Rossi et al. 2006). Definitive hematopoietic cells are derived from a subset of epithelial cells called hemogenic endothelium (HE) cells, which are part of the interior lining of blood vessels in the embryo (Swiers, Rode et al. 2013). HE cells are a transitional population that undergoes an endothelial to hematopoietic transition (EHT) to transform into hematopoietic progenitors and stem cells (Kissa and Herbomel 2010). Runx1 is indispensable for definitive hematopoiesis and a critical transcription factor regulating such processes by suppressing the endothelial transcriptional program and initiating the hematopoietic program (North, Gu et al. 1999, Yokomizo, Ogawa et al. 2001, Chen, Yokomizo et al. 2009, Lancrin, Mazan et al. 2012, de Bruijn and Dzierzak 2017). In the absence of Runx1, no definitive HSCs are formed (Okuda, van Deursen et al. 1996, Wang, Stacy et al. 1996). On the other hand, in Runx1 heterozygous mutant embryos, definitive hematopoiesis is suppressed and the spatial and temporal developments of HSCs are changed (Wang, Stacy et al. 1996, Cai, de Bruijn et al. 2000, Mukouyama, Chiba et al. 2000). Depletion of Runx1 within specific tissues or developmental stages in mice demonstrated that Runx1 expression is required specifically in endothelial cells for de novo generation of HSCs, but is not essential for the

renewal and survival of HSCs thereafter (Chen, Yokomizo et al. 2009, Yzaguirre, de Bruijn et al. 2017). Even so, Runx1 is still required for lineage-specific differentiation and homeostasis. For instance, Runx1 is necessary for megakaryocytic maturation and differentiation of B-cells and T-cells in mouse bone marrow (Ichikawa, Asai et al. 2004, Seo, Ikawa et al. 2012, Niebuhr, Kriebitzsch et al. 2013).

Runx1 may function in embryogenesis at an even earlier stage than hematopoiesis. In human embryonic stem cells, RUNX1 is transiently expressed during early mesendodermal differentiation, which starts at E 5.5 day (Wang and Chen 2016), by promoting an epithelial to mesenchymal transition in a Transforming growth factor beta (TGF-β) dependent manner (VanOudenhove, Medina et al. 2016). In addition to its role in defining hematopoietic lineages, Runx1 is also involved in the development of other tissues including hair follicles, bone, nervous system, mammary gland and muscle (Yamashiro, Åberg et al. 2002, Lian, Balint et al. 2003, Osorio, Lee et al. 2008, Hoi, Lee et al. 2010, Kanaykina, Abelson et al. 2010, van Bragt, Hu et al. 2014, Sokol, Sanduja et al. 2015, Umansky, Gruenbaum-Cohen et al. 2015). It has been well documented that Runx1 modulates the developmental activation and proliferation of hair follicle cells (Osorio, Lee et al. 2008). The formation of hair follicle stem cells requires constant interaction between epithelial and mesenchymal cells, which both require RUNX1 expression (Raveh, Cohen et al. 2006, Osorio, Lee et al. 2008, Sennett and Rendl 2012). In epithelial cells, depletion of Runx1 delays the formation of hair follicles

due to the lack of hair follicle cell emergence (Osorio, Lee et al. 2008, Osorio, Lilja et al. 2011). However, the function of Runx1 in this cell type appears dispensable, as the defects are overcome with time (Osorio, Lilja et al. 2011). Loss of Runx1 in mesenchymal cells during embryogenesis affects the integrity of hair follicle formation. It has been shown that mesenchymal cells still mature into hair follicles after knockout of Runx1 in mice, but with enormous sebaceous cysts that do not contain the bulb and bulge region at the first hair cycle (Osorio, Lilja et al. 2011). Besides embryogenesis, Runx1 is also crucial for regulating the hair cycle at the transition into adult skin homeostasis. Runx1 directly promotes the proliferation of hair follicle stem cells and loss of RUNX1 delays the activation of stem cells into the cell cycle (Osorio, Lee et al. 2008, Hoi, Lee et al. 2010, Scheitz, Lee et al. 2012). Recently it has been discovered that RUNX1 is also essential for mammary gland development as will be discussed later (see Section 1.5.1).

1.3.3 Runx2

Bone development occurs through two independent processes termed intramembranous and endochondral ossification (Berendsen and Olsen 2015). For intramembranous bone development, flat bones are directly formed by osteoblasts, which are differentiated from mesenchymal cells (Berendsen and Olsen 2015). Runx2 is the first transcription factor required for osteoblast differentiation (Komori 2010, Komori 2017). Osteoblasts are completely absent in Runx2$^{-/-}$ mice, which indicates that Runx2 is required for the differentiation of mesenchymal stem cells

into osteoblasts (Komori, Yagi et al. 1997, Otto, Thornell et al. 1997). Runx2 also activates the bone commitment transcription factor SP7 and bone matrix proteins including Spp1, Col1a1, IBSP and Bglap2 (Ducy, Zhang et al. 1997, Sato, Morii et al. 1998, Harada, Tagashira et al. 1999, Lee, Kim et al. 2000, Stein, Lian et al. 2004). After mesenchymal stem cell differentiation into osteoblasts, Runx2 expression is decreased, and abnormally maintaining Runx2 expression inhibits osteoblast maturation (Liu, Toyosawa et al. 2001, Geoffroy, Kneissel et al. 2002, Kanatani, Fujita et al. 2006). For the formation of long bones, endochondral ossification requires maturation of chondrocytes at the center of the bone, known as the diaphysis. Terminally differentiated chondrocytes undergo apoptosis and are then replaced by mesenchymal cells. These mesenchymal cells later differentiate into osteoblasts (Berendsen and Olsen 2015). In Runx2$^{-/-}$ mice, chondrocyte maturation is severely inhibited and mechanistically Runx2 up-regulates chondrocyte maturation through the activation of osteoblast differentiation (Komori, Yagi et al. 1997, Inada, Yasui et al. 1999, Komori 2017).

Recently, evidence has demonstrated that Runx2 is also involved in hematopoiesis. Runx2 expression is at an even higher level than Runx1 in hematopoietic stem cells; however the level of Runx2 sharply decreases during myeloid differentiation (Kuo, Zaidi et al. 2009). This loss of Runx2 expression is necessary for myeloid progenitor differentiation, as ectopic expression of Runx2 blocked differentiation in *in vitro* assays (Kuo, Zaidi et al. 2009). Besides myeloid differentiation, Runx2 is also involved in regulating lymphoid lineage (Stewart,

Terry et al. 1997). Runx2 is expressed at the earliest stage of thymocyte development and forced expression of Runx2 slows down T cell development, resulting in an expansion of double-negative and CD8 immature single-positive cells (Satake, Nomura et al. 1995, Vaillant, Blyth et al. 2002, Blyth, Vaillant et al. 2010). Moreover, Ehrhardt et al. showed that Runx2 expression is enriched in a subpopulation of memory B cells and therefore might be involved in B-cell differentiation (Ehrhardt, Hijikata et al. 2008). In addition to bone development and hematopoiesis, Runx2 is also expressed in prostate, testis, vascular endothelium and ovary where its function in these tissues remains unclear (Sun, Vitolo et al. 2001, Jeong, Jin et al. 2008, Blyth, Vaillant et al. 2010). The reason why bone-specific factor Runx2 is found in hematopoietic stem cells and other tissue lineages is still unclear. It could potentially be related to mitotic bookmarking functions of Runx factors (Young, Hassan et al. 2007, Young, Hassan et al. 2007).

1.3.4 Runx3

Like Runx1 and Runx2, Runx3 has also been shown to be involved in development (Inoue, Shiga et al. 2008). Runx3$^{-/-}$ mice exhibit ataxia due to improper function of several important organs, including dorsal root ganglia, natural killer cells, and CD8+ T cells (Inoue, Ozaki et al. 2002, Levanon, Bettoun et al. 2002, Taniuchi, Osato et al. 2002, Durst and Hiebert 2004, Chen, de Nooij et al. 2006). In addition to neuronal defects, Runx3-null mice develop gastric hyperplasia and die shortly after birth due to starvation (Li, Ito et al. 2002). These data indicate a possible role

of Runx3 in regulating development of the gastric epithelium (Li, Ito et al. 2002). Conversely, this phenotype was not observed in another Runx3 knockout mouse (Levanon, Brenner et al. 2003, Levanon, Bernstein et al. 2011). The reason for this discrepancy is still unclear, but could be a result of different genetic backgrounds and/or antibodies used in these studies (Ito 2012, Levanon, Negreanu et al. 2012).

In summary, all three Runx proteins are essential for normal development in multiple tissues and have diverse roles in proliferation, differentiation and cell lineage commitment. In the original studies, all Runx-null mice are lethal. The advancement of new tissue-specific CRISPR/Cas9 technology may find novel developmental roles for this conserved Runx family in the future.

1.4 The Runx Family in Cancer

1.4.1 Overview

As discussed, all three Runx proteins are involved in the development of multiple tissues. Therefore, the precise regulation and integrity of these factors is necessary for normal function. Deregulation of Runx functions causes many diseases and cancers. One such example, mutation of RUNX2, causes a hypomorphic allele and results in a congenital disorder in skeletal development named Cleidocranial Dysplasia (CCD) (Otto, Kanegane et al. 2002, Matheny, Speck et al. 2007) . In this section, I will give examples of how dysfunction of Runx proteins causes diseases and cancer.

1.4.2 Runx1

RUNX1 was first cloned in 1991 at the breakpoints on chromosome 21 in leukemia (Miyoshi, Shimizu et al. 1991). Later it was discovered that a RUNX1 fusion protein, RUNX1-ETO (AML1-ETO), is generated by a translocation between chromosomes 8 and 21 (t8:21) (Miyoshi, Shimizu et al. 1991, Erickson, Gao et al. 1992, Miyoshi, Kozu et al. 1993). RUNX1-ETO leads to leukemia and is the most common genetic alteration in acute myeloid leukemia (AML), especially within the M2 subtype of AML (Lin, Mulloy et al. 2017, Sood, Kamikubo et al. 2017). This subtype is associated with younger age and relatively good prognosis (Lin, Mulloy et al. 2017). The RUNX1-ETO fusion protein contains the N-terminal 177 amino acids of RUNX1, including the entire Runt DNA-binding domain, fused in frame with almost the entire ETO protein. ETO contains four evolutionarily conversed domains termed nervy homology regions (NHR), which mediates homodimerization of RUNX1-ETO (Davis, McGhee et al. 2003, Liu, Cheney et al. 2006, Kwok, Zeisig et al. 2009, Yan, Ahn et al. 2009). Like RUNX1, RUNX1-ETO regulates gene expression by forming complexes with diverse proteins and gains the ability to form complexes with aberrant partners compared with the wild-type RUNX1. For instance, RUNX1-ETO forms a co-repressor complex with nuclear receptor co-repressor (NCOR1), histone deacetylase (HDAC1), and SIN3A/HDAC at the ETO NHR domain (Gelmetti, Zhang et al. 1998, Lutterbach, Westendorf et al. 1998, Wang, Hoshino et al. 1998, Amann, Nip et al. 2001, Davis, McGhee et al. 2003, Lin, Mulloy et al. 2017). RUNX1-ETO also interacts with E proteins through the

NHR domain to inhibit E-protein-induced transcriptional activation (Zhang, Kalkum et al. 2004). It has also been reported that in physiological conditions, p300 and PRMT bind weakly to RUNX1-ETO forming a transcription co-activation complex to dynamically regulate target gene expression (Sun, Wang et al. 2013). Dominant-negative inhibition of native RUNX1 function may therefore be the central mechanism for RUNX1-ETO induced leukemogenesis (Goyama and Mulloy 2011). However, surprisingly, RUNX1-ETO also requires some activities of the native RUNX1 to promote leukemogenesis, as RUNX1 is a member of the RUNX1-ETO transcription complex (Li, Wang et al. 2016) .

In addition to the t(8:21) translocation, more than 50 other chromosomal translocations affect RUNX1. Most of them are related to leukemia, but only about half of the partner genes have been identified among these translocations (Etienne De Braekeleer 2011). Other common translocations include t(12;21) in pediatric acute lymphoblastic leukemia (ALL), known as TEL-RUNX1 (Jamil, Theil et al. 2000); and t(3:21) in therapy related AML and myelodysplastic syndrome (MDS), known as RUNX1-MECOM (Yang, Cho et al. 2012).

RUNX1 somatic mutations are also detected in approximately 3% of pediatric and 15% of adult AML patients. Adult AML is associated with older age and worse prognosis. These leukemic cells generally have a growth advantage over the hematopoietic progenitor cells with defects in differentiation due to mutated RUNX1 (Tang, Hou et al. 2009, Greif, Konstandin et al. 2012, Mendler, Maharry et al. 2012, Schuback, Arceci et al. 2013, Skokowa, Steinemann et al. 2014). RUNX1

is also one of the most frequently mutated genes in MDS and ALL, about 10% and 25% respectively (Speck and Gilliland 2002, Bejar, Stevenson et al. 2011, Grossmann, Kern et al. 2011, Mullighan 2012, Papaemmanuil, Gerstung et al. 2013, Haferlach, Nagata et al. 2014).

In summary, RUNX1 is a major player in hematologic malignancies. It is a key regulator of hematopoiesis, and maintains a proper balance between proliferation and differentiation. Therefore, the high frequency of loss-of-function somatic point mutations or translocations in multiple subtypes of leukemia result in the repression of RUNX1 normal function and initiation of leukemogenesis. Several companies including Invitae and NEO genomics provide screening of RUNX1 mutations in leukemia patients to evaluate prognosis and select therapeutic strategy.

Besides its impact on leukemia, Runx1 is either over- or under-expressed in many solid tumors, implying that Runx1 either promotes or represses epithelial cancers depending on the cellular context (Scheitz and Tumbar 2013). For example, Runx1 is identified as a tumor promoter in ovarian and skin cancers and tumor suppresses tumor growth in prostate cancer (Scheitz, Lee et al. 2012, Keita, Bachvarova et al. 2013, Takayama, Suzuki et al. 2015). The involvement of Runx1 in skin cancer was first discovered in a chemically induced mouse model. Loss of Runx1 significantly decreases the number of skin tumors formed (Hoi, Lee et al. 2010). Using lineage tracing, it has been shown that the Runx1-expressing hair follicle stem cells are the origin of these chemically induced skin tumors (Scheitz,

Lee et al. 2012). Mechanistically, in skin cancer Runx1 maintains an active/phosphorylated form of the oncogene STAT3, and thus increases cell survival, proliferation and invasion (Scheitz, Lee et al. 2012).

1.4.3 Runx2 and Runx3

In contrast with RUNX1, which has opposing functions in different cancer types, RUNX2 has been well documented to be an oncogene (Chuang, Ito et al. 2017). For example, Runx2 functions as an oncogene in lymphoma, where it is a frequent target for viral insertion in T-cell lymphomas (Stewart, Terry et al. 1997, Blyth, Vaillant et al. 2006). In osteosarcoma, increased RUNX2 expression is also associated with tumorigenicity, metastasis, lower survival and poor prognosis by directly activating PI3K/AKT pathways (Martin, Zielenska et al. 2011, Cohen-Solal, Boregowda et al. 2015). Up-regulation of RUNX2 has been linked to bone metastasis in multiple epithelial cancer types including colon, breast, prostate and thyroid cancer (Pratap, Javed et al. 2005, Akech, Wixted et al. 2010, Chimge, Baniwal et al. 2011, Niu, Kondo et al. 2012, Cohen-Solal, Boregowda et al. 2015). RUNX2 contributes to metastatic events through regulation of bone metastatic-related genes, such as osteopontin, bone sialoprotein, matrix metalloproteinases, and activation of signaling pathways including WNT and TGF-β (Pratap, Lian et al. 2006). Meanwhile RUNX3 is also involved in multiple solid tumors and functions as a tumor suppressor in the majority of the cases (reviewed in (Chuang and Ito 2010, Chen, Wang et al. 2014, Chen, Liu et al. 2016)) .

1.5 RUNX1 in Mammary Gland development and Breast Cancer

1.5.1 Mammary gland development and hierarchy

The mammalian mammary gland is a highly dynamic organ that undergoes profound changes in structure and function during the reproductive cycle and pregnancy (Richert, Schwertfeger et al. 2000, Hennighausen and Robinson 2005, Watson and Khaled 2008). The development of mouse mammary gland starts at puberty when the embryonic epithelial placode transforms into a branched network of collecting ducts and tubes, which consist of two distinct types of cell lineages: the inner layer of luminal lineage (including ductal and alveolar luminal cells) and the outer layer of basal lineage (the myoepithelial cells) (Muschler and Streuli 2010). During pregnancy, increased progesterone and prolactin levels result in greater branching and formation of mature lobuloalveolar units that contain terminally differentiated cells for milk production (Hennighausen, Robinson et al. 1997). The milk is released by contraction of ductal and lobular myoepithelial cells (Haaksma, Schwartz et al. 2011). Following lactation, the mammary gland returns to a virgin-like state through involution, with the death of epithelial cells and extensive tissue remodeling (Richert, Schwertfeger et al. 2000, Watson and Khaled 2008, Inman, Robertson et al. 2015).

1.5.2 RUNX1 and mammary gland development

Runx1 has a spatial/temporal expression pattern in the mammary gland, as it is differentially expressed during physiological stages of mammary gland development. The highest levels are observed in virgin and early-pregnant glands and decrease in late pregnancy and during lactation (McDonald, Ferrari et al. 2014, van Bragt, Hu et al. 2014, Rooney, Riggio et al. 2017). Compared with cells of the luminal lineage, Runx1 is expressed at higher levels in basal progenitor cells (McDonald, Ferrari et al. 2014, van Bragt, Hu et al. 2014). As Runx1 expression is lost from differentiated alveolar luminal cells, it has been speculated that a reduction in RUNX1 expression is necessary for milk production and secretion (van Bragt, Hu et al. 2014). Besides the expression pattern, the role of Runx1 in regulation of mammary development and its role in normal mammary gland are still understudied. In normal-like basal MCF10A cells, RUNX1 is essential for 3D growth in Matrigel (Wang, Brugge et al. 2011). Furthermore, Runx1 is required for mammary stem cells to exit the bipotent state and differentiate into mature lobules and ducts (Sokol, Sanduja et al. 2015). *In vivo*, deletion of Runx1 specifically in the mouse mammary gland reduces the proportion of luminal cells. In particular, loss of Runx1 results in a deficit in mature estrogen receptor (ER) positive luminal cells (van Bragt, Hu et al. 2014). The mechanism(s) of Runx1 regulation of mammary gland development is still unclear. It has been suggested that Runx1 decides the fate of the ER-positive luminal subpopulation and directs ductal differentiation by repressing the alveolar transcription factor Elf5 (van Bragt, Hu et al. 2014). There

are relatively few studies devoted to determining the role of Runx1 in the basal lineage of myoepithelial cells, even though Runx1 is expressed at a higher level in this subpopulation compared with luminal cells (van Bragt, Hu et al. 2014). Interestingly, Runx1 conditional knockout mice have defects in myoepithelial cell contraction and milk ejection, and most of the pups die within 24 hours after birth with no observed milk spots (van Bragt, Hu et al. 2014).   It is interesting to note that smooth muscle contraction is among the top down-regulated pathways in embryonic stem cells with RUNX1 depletion (VanOudenhove, Medina et al. 2016). These data reveal a potential role for RUNX1 in maintaining the normal phenotype of basal myoepithelial cells.

1.5.3 RUNX1 and breast cancer

In recent years, growing evidence has indicated that RUNX1 suppresses tumor growth in breast cancer. RUNX1 was initially identified as a potential transcription factor to suppress tumor growth in breast cancer, as it was down regulated among a 17-gene signature associated with metastasis in adenocarcinoma including breast cancer (Ramaswamy, Ross et al. 2003). The expression of RUNX1 was later shown to decrease when comparing normal mammary tissue to breast cancer, with a greater decrease in higher-grade tumors (Kadota, Yang et al. 2010). Sequencing of breast cancer patient samples then identified that 6% of all breast invasive cancers and 10% of invasive lobular breast cancers have an alteration in the RUNX1 gene (Ciriello, Gatza et al. 2015, Rooney, Riggio et al. 2017). Both

whole genome and whole exome sequencing have identified point mutations and deletions of RUNX1 in luminal and basal breast cancers (Banerji, Cibulskis et al. 2012, Ellis, Ding et al. 2012, Network 2012). In these studies, RUNX1 is a frequently mutated gene along with other well-known tumor suppressor and oncogene genes including PTEN, CDH1, TP53, PIK3CA, which have been intensively investigated in breast cancer (Bertheau, Lehmann-Che et al. , Kechagioglou, Papi et al. 2014, Mukohara 2015, Maeirah Afzal and Ezharul Hoque 2016). These RUNX1 mutations, including point mutations, frame-shift mutations, and deletions, were assumed to be loss-of-function mutations. The majority of these mutations are located at the interface between the Runt domain and DNA, suggesting that the RUNX1 mutants cannot bind properly to target genes (Fig.1.2). Notably, mutations were also identified in the RUNX1 binding partner CBF-β (Network 2012). Thus, it is possible that loss of RUNX1 function by disrupting RUNX1-DNA binding or the interaction between RUNX1 and CBF-β may promote tumorigenesis in mammary gland.   Recently, there are two studies that independently identified RUNX1 loss-of-function mutation as the driver for the existence of other mutations in breast cancer, thus strongly suggesting that RUNX1 loss promotes breast cancer progression (Pereira, Chin et al. 2016, Kas, de Ruiter et al. 2017).

In summary, by sequencing the tumors from breast cancer patients, RUNX1 mutations that associate with initiation and progression of the disease have been identified in multiple subtypes of breast cancer. In a tissue microarray study,

RUNX1 intensity was decreased in breast cancer tumors compared with normal mammary tissues (Browne, Taipaleenmäki et al. 2015). However, the molecular mechanisms underlying RUNX1 suppressed tumor growth remain unclear and require further investigation in cell lines, mouse models, and human patient samples.

Multiple studies using cell lines and mouse models have been carried out to identify RUNX1 function in breast cancer. In normal mammary epithelial cells, loss of RUNX1 in a 3D Matrigel assay resulted in hyper-proliferation and abnormal morphogenesis, which requires normal FOXO1 function (Wang, Brugge et al. 2011). In another study, conditional knockout of Runx1 in mammary epithelial cells reduced the proportion of ER+ luminal cells, but did not result in mammary tumors (van Bragt, Hu et al. 2014). However, loss of TP53 or Rb1 rescued this phenotype and resulted a hyper-proliferation of Runx1-deficient ER+ luminal cells. Cells harboring a double mutation may eventually develop into breast cancer (van Bragt, Hu et al. 2014). Further exploration using double-knockout mice (Runx1/TP53 or Runx1/RB1) will be required to determine whether these mice develop abnormal mammary hyperplasias or tumors. Recent work from the Frenkel lab has demonstrated that loss of RUNX1 in Luminal A breast cancer cells facilitates estrogen-induced WNT signaling by suppressing the scaffold protein AXIN1 (Chimge, Little et al. 2016). Therefore, along with genetic data, growing evidence in cell lines and mouse models establishes the concept that RUNX1 reduces aggressive phenotype in breast cancer, especially in the luminal subtype.

In contrast, a few studies indicate that RUNX1 may function as an oncogene in breast cancer. In particular, triple-negative breast cancer was correlated with high RUNX1 expression and poor prognostic outcome (Ferrari, Mohammed et al. 2014). RUNX1 inhibition in the triple-negative MDA-MB-231 late stage breast cancer cell line, showed a less aggressive phenotype with decreased proliferation, migration and invasion in vitro (Recouvreux, Grasso et al. 2016). Furthermore, in the MMTV-PyMT mouse model, RUNX1 expression is positively correlated with advanced disease (Browne, Taipaleenmäki et al. 2015). The discrepancy could be due to heterogeneity in breast cancer, as breast cancer encompasses a diverse group of subtypes. These subtypes have different cellular origins (luminal versus basal) and molecular alterations (e.g., hormonal status including ER, PR, and HER2) (Eroles, Bosch et al. 2012). In the luminal subtype of breast cancer, it has been well accepted that RUNX1 reduces tumor aggressive phenotypes. On the other hand, in the basal-like subtype, RUNX1 may have a dual function depending on the stage of breast cancer. In normal mammary myoepithelial cells, loss of RUNX1 disrupts the normal function of that cell layer's ability to contract and eject milk (van Bragt, Hu et al. 2014). However, in late-stage triple-negative breast cancer, RUNX1 is linked to fast proliferation and a more aggressive phenotype (Recouvreux, Grasso et al. 2016). The molecular signatures of normal basal cells/early stage basal cancer and late stage basal cells are significantly different. Due to the distinct cellular context and gene expression patterns, RUNX1 may form complexes with different co-activator or co-repressor proteins. This differential binding of co-

regulatory factors may convert its activity from being a gene against tumor growth to an oncogene by differentially regulating the same subset of genes. Alternatively, these RUNX1 complexes may be targeted to entirely new subsets of genes.

In conclusion, knowledge regarding the function of RUNX1 in breast cancer is still far from complete, and the potential dual role as promoting or suppressing tumor growth highlights its extreme context dependency. It is still a challenge to integrate the genomic data obtained from patients with molecular data from cell lines and animal models. A better understanding of RUNX1 function in different stages of breast cancer will potentially translate into targeted therapies that could greatly benefit prevention and screening.

1.6 Epithelial Mesenchymal Transition in Breast Cancer

1.6.1 Overview of EMT

The concept of epithelial-mesenchymal transition (EMT) was first described almost 50 years ago in 1968 by Elizabeth Hay (Hay 1968). EMT is an evolutionally conserved morphogenetic program during which epithelial or epithelial-like cells undergo a series of biochemical changes allowing them to acquire a mesenchymal phenotype (Thiery 2002). During the EMT process, polarized epithelial cells with tight junctions acquire mesenchymal properties, such as enhanced migration, invasiveness, and elevated resistance to apoptosis. EMT is precisely regulated by the interplay of signaling pathways, transcription factors and miRNAs. Several transcription factors, for example, the Zeb, Snail and Twist families, are activated

by a variety of signaling pathways, including TGF-β, NOTCH and WNT (Nieto 2002, Yang, Mani et al. 2004, Liu, El-Naggar et al. 2008, Lamouille, Xu et al. 2014). In turn, these transcription factors initiate the EMT program by silencing E-cadherin expression at the cell surface. The loss of E-cadherin is a fundamental hallmark of EMT (Kalluri and Weinberg 2009). Furthermore, mesenchymal-like cells commonly express Vimentin, which is a cytoskeletal protein necessary for migration (Kalluri and Weinberg 2009). Recent findings suggest that EMT is not an all-or-none process from purely epithelial to purely mesenchymal phenotypes, but rather is a multi-stage process, with one or multiple intermediate stages. These intermediate phenotypes have been referred to as partial EMT (Shibue and Weinberg 2017). The details on partial EMT and its role in metastasis and cancer stem cells will be discussed in detail in section 1.7.2.

There are three different types of EMT, which carry out very different functions. 1. EMT that is required for the formation of mesodermal and neural tube tissue during embryogenesis; 2. EMT associated with tissue regeneration and organ fibrosis; 3. EMT that contributes to the pathogenesis of cancer metastasis (Kalluri and Weinberg 2009, Thiery, Acloque et al. 2009, Kovacic, Mercader et al. 2012). I will discuss the role of EMT in development and cancer in the next two sections.

1.6.2 Epithelial mesenchymal transition in development

EMT drives essential aspects of embryonic development. During gastrulation, complete EMT occurs to generate fully committed mesenchymal cell types forming

the early mesoderm or endoderm (Viebahn, Lane et al. 1995, Thiery, Acloque et al. 2009). In contrast, partial and reversible EMT occurs during morphogenesis of certain epithelial tissues such as the mammary gland (Nakaya and Sheng 2013).

During puberty, mammary epithelial stem/progenitor cells that reside in the terminal end buds of the breast start to elongate and migrate, thereby driving branching morphogenesis (Micalizzi, Farabaugh et al. 2010). These epithelial cells transiently acquire mesenchymal features, including loss of apical-basal polarity (Ewald, Brenot et al. 2008, Ewald, Huebner et al. 2012), and elevated expression of the EMT transcription factors Snai1 and Twist (Kouros-Mehr and Werb 2006, Foubert, De Craene et al. 2010). The cells in the terminal end buds are regulated by a number of extracellular factors known to induce EMT, including epidermal growth factor (EGF) and hepatocyte growth factor (HGF). For instance, in the mouse mammary gland, overexpression of HGF causes hyperplastic branching morphogenesis, while inhibition of HGF signaling blocks budding of side branches (Rosário and Birchmeier 2003). Branching morphogenesis is a highly plastic process with an incomplete EMT program, as both the epithelial and mesenchymal lineages are essential for normal mammary gland function. Recently two transcription factors, Elf5 and Ovol2, have been shown to be the gatekeepers of mammary epithelial differentiation by inhibiting EMT at terminal end buds (Chakrabarti, Hwang et al. 2012, Watanabe, Villarreal-Ponce et al. 2014). Elf5 is the master regulator for transforming luminal progenitor cells into alveolar cells during pregnancy and lactation (Oakes, Naylor et al. 2008, Choi, Chakrabarti et al.

2009). Therefore, a partial EMT state, gaining partial mesenchymal features with maintenance of some epithelial characteristics, is critical during mammary gland development.

1.6.3 Epithelial mesenchymal transition in cancer

Almost 80% of human cancer deaths derive from epithelial tissues including tumors of the breast, lung, pancreas, prostate, colon, ovary, kidney and liver (Ye and Weinberg 2015). Hyperplasia or early stage tumors arising in these tissues continue to express the epithelial marker E-cadherin, whereas cells from highly aggressive primary tumors exhibit mesenchymal features including motility and invasiveness (Choi, Lee et al. 2013, Cheng, Chang et al. 2014). Cancer cells have the capability to utilize the EMT process to initiate invasion and metastasis (Chaffer, San Juan et al. 2016).

In breast cancer, an EMT signature is enriched in basal-like and Claudin low subtypes compared with Luminal A/B subtypes (Prat, Parker et al. 2010). Since tumor progression is positively associated with acquisition of mesenchymal features, this may be an explanation for why basal and Claudin low breast cancers are more aggressive. Depletion of activators of EMT, such as Twist, Snail and Zeb in human and mouse breast cancer cell lines, greatly inhibit metastasis after mammary fat pad or tail vein injection (Yang, Mani et al. 2004, Guo, Keckesova et al. 2012, Zhang, Corsa et al. 2013, Roy, Gonugunta et al. 2014, Tran, Luitel et al. 2014). For instance, depleting Snail in MMTV-PyMT mice completely abolished 95%

of lung metastasis (Tran, Luitel et al. 2014). Consistently, activating EMT in human breast cancer cells can enhance metastatic dissemination (Tran, Luitel et al. 2014). Therefore, EMT has been defined as a critical component of the metastatic process.

Although EMT processes are well documented in many in vitro cancer cell models and even *in vivo* animal experiments, the existence of EMT during tumor progression and its relevance in metastasis have remained matters of controversy. One of the key concerns is the lack of convincing histological evidence of EMT in clinical samples (Thiery, Acloque et al. 2009). Two recent reports raise the question of whether EMT is dispensable for invasion and metastasis in mouse models of breast and pancreatic cancer (Fischer, Durrans et al. 2015, Zheng, Carstens et al. 2015). Fisher et al. used a spontaneous breast to lung metastasis mouse model and labeled fibroblast-specific protein 1 (Fsp1) as a marker for EMT. They observed that many Fsp1 negative cells metastasize to lung, indicating that EMT is not necessary for metastasis (Fischer, Durrans et al. 2015). In another study, Zheng et al. knocked out either Snail or Twist in a spontaneous pancreatic ductal adenocarcinoma (KPC model) and observed no difference in metastasis by tracing α-smooth muscle actin as a mesenchymal marker (Zheng, Carstens et al. 2015). However, there is some debate regarding whether Fsp1 or α-smooth muscle actin are proper EMT markers, as they are rarely induced upon activation of EMT (Aiello, Brabletz et al. 2017, Ye, Brabletz et al. 2017). Furthermore, using the same KPC mouse model, depleting Zeb1 suppresses stemness, invasion and

metastasis, indicating that EMT is necessary for metastasis in vivo (Krebs, Mitschke et al. 2017).

Although these two studies suggest that EMT is dispensable for metastasis, both uncovered that EMT is key to chemoresistance (Fischer, Durrans et al. 2015, Zheng, Carstens et al. 2015). Several other studies also demonstrated that induction of EMT confers resistance to chemotherapy and radiotherapy (Creighton, Li et al. 2009, Oliveras-Ferraros, Corominas-Faja et al. 2012, Chen, Gibbons et al. 2014). The underlying molecular mechanisms of EMT-induced chemoresistance remain unsolved. One hypothesis is that the EMT activator Twist can bind to the promoter and activate the expression of the ABC transporter, which is responsible for efflux of drugs out of the cell (Saxena, Stephens et al. 2011). In the past decade, studies have highlighted a link between EMT and cancer stem cells, which be discussed in detail in section 1.7.

1.6.4 Runx and EMT

The Runx proteins are important players in the determination of cell fate during development, which often overlaps with the occurrence of EMT. During embryogenesis, transient RUNX1 expression in early mesendodermal differentiation of human embryonic stem cells promotes EMT through TGF-β signaling (VanOudenhove, Medina et al. 2016). During mammary branching morphogenesis, the level of Runx2 is increased and accompanied by the up-regulation of EMT activators such as Snail2 (Kouros-Mehr and Werb 2006,

McDonald, Ferrari et al. 2014). Overexpressing RUNX2 in mammary epithelial cells activated differentiation and induced EMT (Chimge, Baniwal et al. 2011, Owens, Rogers et al. 2014).   On the other hand, depleting Runx2 in mouse mammary glands disrupted ductal outgrowth at puberty and progenitor cell differentiation during pregnancy (Owens, Rogers et al. 2014, Ferrari, Riggio et al. 2015).   All these data suggest that Runx2 is a positive regulator of EMT in mammary gland development.

Increasing evidence has suggested that deregulation of Runx expression and function is linked to the aberrant induction of EMT in cancer. Parallel to its involvement in EMT during development, RUNX2 has been implicated in the aberrant activation of EMT and metastasis in breast and prostate cancer. In breast cancer cells, RUNX2 is necessary for the induction of Snail expression (Chimge, Baniwal et al. 2011), while in prostate cancer, RUNX2 also positively regulates EMT drivers such as Snail2, SMAD3, and Sox9 (Baniwal, Khalid et al. 2010, Little, Noushmehr et al. 2012, Little, Baniwal et al. 2014) .

Until now, there has been no direct evidence showing whether RUNX1 regulates EMT in the mammary gland or breast cancer. However, it was shown that RUNX1 binds to the promoter of E-cadherin and positively regulates its promoter activity (Liu, Lee et al. 2005). Runx1 also represses ELF5 expression, which is a key driver of alveolar luminal cell differentiation (van Bragt, Hu et al. 2014). Therefore, RUNX1 may be important in maintaining homeostasis and preventing EMT in the mammary gland.

1.7 Breast Cancer Stem cells

1.7.1 Intra-tumor heterogeneity

Breast cancer is a heterogeneous disease, which often displays intra-tumor and inter-tumor heterogeneity as the result of genetic and non-genetic alterations (Polyak 2007) . Inter-tumor heterogeneity has been proposed to reflect the different cells-of-origin that become transformed into the tumor cells (Burrell, McGranahan et al. 2013). In breast cancer, inter-tumor heterogeneity often leads to the classification of different tumor subtypes as discussed in Section 1.1.3.

It also has been noticed for a long time that tumors contain sub-clones that differ in karyotype and chemoresistance (Shapiro, Yung et al. 1981, Yung, Shapiro et al. 1982) . Using deep-sequencing expression profiling of various regions in the same tumor, it has been found that within a single tumor, there are multiple clones with distinct genetic and epigenetic profiles, as well as somatic mutations (Anderson, Lutz et al. 2010, Gerlinger, Rowan et al. 2012). This phenomenon has been described as intra-tumor heterogeneity (Marjanovic, Weinberg et al. 2013, Prasetyanti and Medema 2017). Intra-tumor heterogeneity is not limited to the differences in malignant cancer cells. More importantly, a tumor is a complex structure containing different clones of tumor cells as well as other cell types, such as infiltrating immune cells, stromal cells and endothelial cells (Lu, Weaver et al. 2012, Junttila and de Sauvage 2013).

Both intrinsic and extrinsic factors influence the intra-tumor heterogeneity. The intrinsic factors exist at both genetic and epigenetic levels (Prasetyanti and

Medema 2017). Cancer cells usually inherit or acquire aberrations in their genome, such as point mutation, translocation, deletion and amplification (Vogelstein, Papadopoulos et al. 2013). Those mutations reflect a degree of genome instability, which is a hallmark of cancer (Hanahan and Weinberg 2011). Among those mutations, some defined as driver mutations, induce activation of oncogenic pathways and suppression of tumor suppressors (Stratton, Campbell et al. 2009). Intensive efforts have been carried out to find these driver mutations in cancer patients. Recently, a list of 40 mutation driver genes has been identified in breast cancer patients (Pereira, Chin et al. 2016). Interestingly, RUNX1 is in that list, suggesting its role for maintaining genome stability (Pereira, Chin et al. 2016). Epigenetic heterogeneity is also often observed in tumors (Dawson and Kouzarides 2012). Drugs that target epigenetic enzymes, which rearrange chromatin structure and function, are being developed rapidly and undergoing clinical trials (Simó-Riudalbas and Esteller 2015, de Lera and Ganesan 2016).

The different environments surrounding tumors also influence the intra-tumor heterogeneity (McGranahan and Swanton 2017). Tumor cells that survive within the hypoxic region due to poor vascularization commonly maintain a mesenchymal phenotype, and have high hypoxia-inducible factor (HIF) expression, which inhibits cell differentiation (Terry, Buart et al. 2017). Besides the local environment of the tumor cells, tumors are constantly under selection pressure, which is a result of the dynamic tumor microenvironment, applied therapy, and attacks from the immune system (Colak and Medema 2014) (McGranahan and Swanton 2017).

These pressures act as the extrinsic factors for intra-tumor heterogeneity. For instance, therapy acts as a selection mechanism that shapes the evolution of tumor cells (McGranahan and Swanton 2017)(Kreso and Dick 2014). In breast cancer, treating luminal breast cancer with aromatase inhibitor induces the remodeling of the clonal population by the acquisition of new mutations or the enrichment of existing mutations (Miller, Gindin et al. 2016).

Therefore, the combination of genetic/epigenetic alterations and microenvironment components can generate intra-tumor heterogeneity and support tumor progression by conferring a competitive advantage on subsets of cancer cells (Prasetyanti and Medema 2017). The origin of intra-tumor heterogeneity could be explained by the cancer stem cell (CSC) theory, which will be discussed in section 1.7.2.

1.7.2 Cancer stem cells

Cancer stem cells (CSCs) are defined by their ability to form new tumors, self-renew, and differentiate into non-stem like cancer cells (Shibue and Weinberg 2017). Also, when injected into immunocompromised mice, CSCs have the ability to generate tumors with high efficiency (Alison, Lim et al. 2011). Thus, CSCs have been implicated both in initiating and sustaining primary tumor growth and in driving the seeding and establishment of metastases at distal sites (Al-Hajj, Wicha et al. 2003, Abraham, Fritz et al. 2005, Sheridan, Kishimoto et al. 2006, Ginestier, Hur et al. 2007, Liu, Wang et al. 2007). Cancer stem cells were first isolated from

AML leukemia based on the expression of cell-surface markers (Lapidot, Sirard et al. 1994), and later in solid tumors such as breast (Al-Hajj, Wicha et al. 2003), brain (Singh, Hawkins et al. 2004), colon (O'Brien, Pollett et al. 2006, Ricci-Vitiani, Lombardi et al. 2006) and pancreatic cancer (Hermann, Huber et al. 2007).

The Wicha group first isolated breast cancer stem cells (BCSCs) in 2003 using cell surface markers for $CD24^{low}/CD44^{high}$ Lineage ^{negative} (Al-Hajj, Wicha et al. 2003). They showed that within this population, as few as 200 cells were able to initiate tumor formation in immunocompromised mice (Al-Hajj, Wicha et al. 2003). Now it is clear that BCSCs exist in two distinct development states and can reversibly transition between them due to their property of cell plasticity (Liu, Cong et al. 2014). The first state is the mesenchymal-like state in which BCSCs express the $CD24^{-}CD44^{+}$ cell surface marker profile. They are mainly quiescent with low proliferation. The location of this population is commonly at the tumor-invasive edge adjacent to the tumor stroma. The second population is the epithelial-like state, and they express the de-toxifying enzyme, aldehyde dehydrogenase (ALDH). These BCSCs are highly proliferative, and localized at the center of the tumor. BCSCs containing both of the CSC markers ($CD24^{-}$ $CD44^{+}$ and $ALDH^{+}$) show the greatest tumor-initiating capacity (Liu, Cong et al. 2014).

Breast cancer stem cells have been associated with metastasis. Gene expression profiles of BCSCs featured an invasive gene signature with increased metastastic potential (Liu, Wang et al. 2007). It was also shown that disseminated bone marrow cancer cells from breast cancer patients have the $CD44^{+}/CD24^{-/low}$

cancer stem cell phenotype (Balic, Lin et al. 2006). In a mouse xenograft model, human breast cancer cells metastasized to the lung express high levels of the stem cell marker CD44, strongly suggesting the metastatic role of BCSCs (Liu, Patel et al. 2010). It has been proposed that BCSCs may enter the circulation and become the circulating tumor cells (CTCs) to metastasize to distal organs and serve as the seeds of metastatic lesions (Batlle and Clevers 2017). Some CTCs have high expression levels of BCSC markers (Baccelli, Schneeweiss et al. 2013). Moreover, from liquid biopsy samples of luminal breast cancer patients, the CTCs with BCSC signature are enriched upon disease progression, while the CTCs with bulk tumor signature are not changing (Baccelli, Schneeweiss et al. 2013).

1.7.3 EMT and plasticity and cancer stem cells

It has been postulated for decades that EMT is related to the generation of CSCs. In 2008, Mani et al. from the Weinberg group first demonstrated that a CD44^{high}/CD24^{low} population was generated from the bulk population upon EMT induced by either TGF-β or transcription factors (Mani, Guo et al. 2008). This sub population exhibits a gene expression profile similar to mammary stem cells and is able to initiate tumors quite efficiently in mouse (Mani, Guo et al. 2008). Later, multiple studies confirmed the link between EMT and breast (Thiery, Acloque et al. 2009, Scheel, Eaton et al. 2011, Chaffer, Marjanovic et al. 2013). Mechanistically, a number of pathways and transcription factors that are known to induce EMT,

including Notch, hedgehog, WNT, TGF-β and NF$\kappa\beta$, are also capable of regulating cancer stem cells (Scheel and Weinberg 2012).

Little is known regarding RUNX1 regulation of CSC in breast cancer or in other solid tumors. Based on the evidence that RUNX1 regulates mammary stem cell differentiation and its role during mammary morphogenesis, it seems worth investigating whether RUNX1 inhibits/activates the cancer stem cell phenotype in breast cancer.

1.8 Rationale for the dissertation

Given the crucial role of RUNX1 in tissue development, especially in the mammary gland, and the fact that RUNX1 is often mutated in breast tumors, we hypothesized that RUNX1 functions to reduce tumor aggressive phenotype in breast cancer.

In Chapter II, I initiated the project by comparing the RUNX1 levels in a panel of normal mammary epithelial cells (MCF10A) and human breast cancer (MCF7) cells and found that the level of RUNX1 is decreased in breast cancer cell lines. By using a breast cancer progression model (MCF10 series), I also observed that RUNX1 expression is lost during breast cancer progression. From this observation, further experiments were performed to establish the concept that RUNX1 reduces tumor aggressive phenotypes in both normal and breast cancer cells and loss of RUNX1 is accompanied by disease progression. Since the mechanism(s) underlying the function of RUNX1 in breast cancer was unclear, in this dissertation

I explored the functional role of RUNX1 in mammary epithelial and breast cancer cells.

When I joined the Stein-Lian lab, RUNX1 molecular mechanisms were focused in hematopoiesis and leukemia. The first report of RUNX1 mutations in breast cancer patients generated my and the lab's enthusiasm for understanding the role of RUNX1 in mammary epithelial and breast cancer cells. The aim of the first part of this dissertation was to investigate the consequences of loss of RUNX1 in both normal mammary epithelial and breast cancer cells at cellular levels. There are several lines of evidence that suggest RUNX1 may be involved in EMT (Liu, Lee et al. 2005, van Bragt, Hu et al. 2014). Therefore, in Chapter II, I focused on testing whether RUNX1 depletion is associated with the activation of EMT in breast cancer; and identify the mechanisms on how RUNX1 represses EMT.

In Chapter II, our studies found RUNX1 is a repressor of EMT and thus preserves the epithelial phenotype in normal mammary epithelial cells. The next goal was to gain a better understanding of how RUNX1 regulates EMT, and to identify novel genes and pathways that are regulated by RUNX1. To achieve this goal, we performed gene expression profiling and genome-wide RUNX1 binding analysis (RNA-seq, ChIP-seq) in MCF10A cells (with or without RUNX1 depletion). These studies discovered novel genes and pathways indicating RUNX1 is a master transcription factor in mammary lineage.

Many recent studies have linked EMT phenotypes to cancer stem cells (Shibue and Weinberg 2017). Breast cancer cells that undergo EMT or partial

EMT exhibit cancer stem cell properties with more aggressive metastatic potential (Grigore, Jolly et al. 2016). It was intriguing that results achieved in Chapter II suggested RUNX1 could repress the breast cancer stem cell phenotype. Therefore, the involvement of RUNX1 in breast cancer stem cells was investigated in Chapter IV through a combination of *in vitro* (tumorsphere assay, matrigel invasion and migration assays) and *in vivo* (mammary fat pad injection, tibia injection) studies.

Overall, the goals of this dissertation are to determine the role of RUNX1 in normal mammary epithelial cells and to understand how the loss of RUNX1 contributes to breast cancer progression. The novel findings obtained in this dissertation provide a better understanding of Runx biology, as well as mechanisms of tumor initiation and progression, and open many future directions for developing therapeutic interventions.

Chapter II RUNX1 stabilizes the mammary epithelial cell phenotype and

prevents epithelial to mesenchymal transition

A large portion of this chapter comes from the published work:

Deli Hong, Terri L. Messier, Coralee E. Tye, Jason R. Dobson, Andrew J. Fritz,

Kenneth R. Sikora, Gillian Browne, Janet L. Stein, Jane B. Lian, Gary S. Stein

Runx1 stabilizes the mammary epithelial cell phenotype and prevents epithelial

to mesenchymal transition. *Oncotarget.* 2017; 8:17610-17627

Contribution: Deli Hong, Jane B. Lian, Janet L. Stein and Gary. S. Stein.

conceived and designed the experiments, and analyzed data. Deli Hong

performed the majority of the experiments. Terri L. Messier helped with the

experiment in Fig. 2.4. Jason R. Dobson, Gillian Browne and Deli Hong

performed tissue microarray. Coralee E. Tye and Kenneth R. Sikora build the

RNA-seq library and normalized the RNA-seq count. Andrew J. Fritz performed

ChIP-seq qPCR. Deli Hong created all the figures. Deli Hong, Jane B. Lian, Janet

L. Stein and Gary S. Stein wrote the manuscript.

2.1 ABSTRACT

RUNX1 is a well characterized transcription factor essential for hematopoietic differentiation and RUNX1 mutations are the cause of leukemias. RUNX1 is highly expressed in normal epithelium of most glands and recently has been associated with solid tumors. Notably, the function of RUNX1 in mammary gland and how it is involved in initiation and progression of breast cancer is still unclear. Here we demonstrate the consequences of RUNX1 loss in normal mammary epithelial and breast cancer cells. We first observed that RUNX1 is decreased in tumorigenic and metastatic breast cancer cells. We also observed loss of RUNX1 expression upon induction of epithelial-mesenchymal transition (EMT) in MCF10A (normal-like) cells. Furthermore, depletion of RUNX1 in MCF10A cells resulted in striking changes in cell shape, leading to mesenchymal cell morphology. The epithelial phenotype could be restored in breast cancer cells by re-expressing RUNX1. Analyses of breast tumors and patient data revealed that low RUNX1 expression is associated with poor prognosis and decreased survival. We addressed mechanisms for the function of RUNX1 in maintaining the epithelial phenotype and find RUNX1 directly regulates E-cadherin; and serves as a downstream transcription factor mediating TGF-β signaling. We also observed through global gene expression profiling of growth factor depleted cells that induction of EMT and loss of RUNX1 is associated with activation of TGF-β and WNT pathways. Thus, these findings have identified a novel function for RUNX1 in sustaining normal

epithelial morphology and preventing EMT and suggest RUNX1 levels could be a prognostic indicator of tumor progression.

2.2 INTRODUCTION

Evidence is rapidly accruing for the oncogenic and tumor suppressor functions of the Runx family of transcription factors, RUNX1, RUNX2 and RUNX3, which are essential for normal lineage-specific development (Ito 2004, Blyth, Cameron et al. 2005). In late stage cancer, including breast, prostate and thyroid, abnormal expression of RUNX2 drives metastasis to bone (Pratap, Lian et al. 2006, Pratap, Wixted et al. 2008, Pratap, Imbalzano et al. 2009). Inhibition of RUNX2 in metastatic breast and prostate cancer cells drastically reduces tumor growth and metastasis *in vivo* (Pratap, Imbalzano et al. 2009, Akech, Wixted et al. 2010), revealing Runx2 function as an oncogene. It has been well documented that translocations of RUNX1, the essential hematopoiesis factor, with ETO, TEL (ETV6) (Bhojwani, Pei et al. 2012) or other genes cause a wide range of leukemias (Zhang and Rowley 2006). However, little is known of RUNX1 oncogenic or tumor suppressor activities in solid tumors. An early microarray profiling study comparing adenocarcinoma metastasis with primary adenocarcinoma tumors identified RUNX1 as one of 17 genes signature that associate with metastasis (Ramaswamy, Ross et al. 2003). Recent genetic studies have identified loss-of-function somatic mutations or deletion of RUNX1 in breast cancer patients (Banerji, Cibulskis et al. 2012, Network 2012). These data are consistent with evidence that RUNX1 is reduced in metastasis-prone solid tumors (Ramaswamy, Ross et al. 2003). There

is a requirement for understanding RUNX1-mediated regulatory mechanism(s) in breast cancer.

Breast cancer remains the leading cause of cancer related death in women worldwide (Jemal, Bray et al. 2011). Among the different subtypes of breast cancer, both the basal-like and Her2-enriched subtypes are the most clinically challenging; they have the worst survival rates and are often associated with metastasis (Martin-Castillo, Oliveras-Ferraros et al. 2013). It has been speculated that this aggressive phenotype of basal like breast cancer is linked with the Epithelial to Mesenchymal Transition (EMT), which is a key biological process in cancer progression and is involved in the conversion of early stage tumors into invasive malignancies (Bill and Christofori 2015). Oncogenic EMT occurs when primary tumor cells undergo a switch from an epithelial phenotype, which lacks motility and exhibits extensive cell-to-cell contact, to a mesenchymal phenotype having high cellular motility, lower cellular interactions, and a non-polarized cell organization (Zavadil and Böttinger 2005). Several studies, using breast cancer cell lines and clinical samples, have demonstrated that increased expression of mesenchymal markers including Vimentin, Fibronectin and N-cadherin, as well as reduced expression of epithelial markers including E-cadherin are observed in basal subtype breast cancer (Ramaswamy, Ross et al. 2003, Zhang and Rowley 2006, Banerji, Cibulskis et al. 2012, Network 2012). The specific mechanisms that preserve the structural and functional properties of the epithelial cells of the glandular tissues and protect normal epithelial cells from transitioning to

malignancy in basal-like breast cancer are compelling and unresolved questions. We therefore have focused our studies on the functional activities of RUNX1 in basal subtype breast cancer cells.

In this chapter, I hypothesize that RUNX1 maintains the normal epithelial phenotype and that loss of RUNX1 promotes EMT. Our results demonstrate that depletion of RUNX1 in mammary epithelial cells disrupts/alters cellular morphology and suppresses E-cadherin expression. We find that RUNX1 level decreases during both TGF-β-induced and growth factor starvation-induced EMT, supporting a crucial role for RUNX1 in preventing EMT. Furthermore, our analysis of breast tumors and survival data supports the above finding that loss of RUNX1 promotes tumor progression. Thus, these studies demonstrate that RUNX1 functions to preserve epithelial phenotype in mammary epithelial cells and reveal that RUNX1 has potential to reduce tumor growth in breast cancer.

2.3 MATERIALS AND METHODS

2.3.1 Cell lines and cultures

Human breast cancer cell lines MCF10A, MCF7, MDA-MB-231 and T47D cells were purchased from ATCC. MCF10AT1 and MCF10CA1a cells are a gift from Jeff Nickerson's lab.

MCF10A cells were grown in DMEM: F12 (Hyclone: SH30271, Thermo Fisher Scientific, Waltham, MA, USA) with 5% (v/v) horse serum (Gibco: 16050, Thermo Fisher Scientific, Waltham, MA, USA) + 10 µg/ml human insulin (Sigma Aldrich, St. Louis, MO: I-1882) + 20 ng/ml recombinant hEGF (Peprotech, Rocky Hill, NJ, USA: AF-100-15) + 100 ng/ml cholera toxin (Sigma Aldrich: C-8052) + 0.5 µg/ml hydrocortisone (Sigma Aldrich: H-0888) 50 IU/ml penicillin/50 µg/ml streptomycin and 2 mM glutamine (Life Technologies, Carlsbad, CA, USA: 15140-122 and 25030-081, respectively). TGF-β induced EMT in MCF10A cells was initiated by addition of 10 ng/ml TGFβ1 (R&D Systems, Minneapolis, MN, USA) to the medium. Growth factors starvation induced EMT in MCF10A cells was performed as previously described (Santner, Dawson et al. 2001). Briefly, MCF10A cells were plated in completed medial and at day 2, the medium was switched to DMEM: F12, with 5% (v/v) horse serum and 50 IU/ml penicillin/50 µg/ml streptomycin without added growth factors. The cells were maintained in this medium for up to 14 days until the morphological change was observed.

MCF10AT1 cells were grown in the same medium as MCF10A cells. MCF10CA1a cells were grown in DMEM: F12 with 5% (v/v) horse serum with 50

IU/ml penicillin/50 µg/ml streptomycin and 2 mM glutamine. MCF7 cells were maintained in Dulbecco modified Eagle medium (DMEM) high glucose (Fisher Scientific: Thermo Fisher Scientific, Waltham, MA, USA: MT-10-017-CM) supplemented with 10% (v/v) FBS (Atlanta Biologicals, Flowery Branch, GA, USA: S11550), 50 IU/ml penicillin/50 µg/ml streptomycin. T47D cells were maintained in RPMI 1640 with phenol red (Fisher Scientific: MT-10-040-CM) supplemented with 10% (v/v) FBS and 50 IU/ml penicillin/50 µg/ml streptomycin. MDA-MB-231 cells were cultured in alpha minimal essential medium (α-MEM) (Life Technologies: A10490-01) containing 10% (v/v) FBS and 50 IU/ml penicillin/50 µg/ml streptomycin. MCF10CA1a cells were transfected using FuGENE-6 (Roche, Indianapolis, IN, USA) according to the instructions of the manufacturer.

2.3.2 Lentiviral plasmid preparation and viral vector production

Lentivirus-based RNAi transfer plasmids with pGIPZ shRunx1 (clone V2LHS_150257 and V3LHS_367631, GE Dharmacon) and pGIPZ non-silencing (Cat No. RHS4346, GE Dharmacon) were purchased from Thermo Scientific. To generate lentivirus vectors, 293T cells in 10 cm culture dishes were co-transfected with 10 µg of pGIPZ shRunx1 or pGIPZ non-silencing, with 5 µg of psPAX2, and 5 µg of pMD2.G using lipofectamine 2000 reagent (Life Technologies). Viruses were harvested every 48 h post-transfection. After filtration through a 0.45 µm-pore-size filter, viruses were concentrated by using LentiX concentrator (Clontech, Mountain View, CA, USA).

2.3.3 Gene delivery by transfection and infection

For shRNA-mediated knockdown of RUNX1 expression, MCF10A or MCF7 cells were plated in six-well plates (1×10^5 cells per well) and infected 24 h later with lentivirus expressing shRunx1 or nonspecific shRNA. Briefly, cells were treated with 0.5 ml of lentivirus and 1.5 ml complete fresh DMEM-F12 per well with a final concentration of 4 µg/ml polybrene. Plates were centrifuged upon addition of the virus at $1460 \times g$ at 37°C for 30 min. Infection efficiency was monitored by GFP co-expression at 2 days post infection. Cells were selected with 2 µg/ml puromycin (Sigma Aldrich P7255-100MG) for at least two additional days. After removal of the floating cells, the remaining attached cells were passed and analyzed.

2.3.4 Western blotting

Cells were lysed in RIPA buffer and 2X SDS sample buffer supplemented with cOmplete, EDTA-free protease inhibitors (Roche Diagnostics) and MG132 (EMD Millipore San Diego, CA, USA). Lysates were fractionated in an 8.5% acrylamide gel and subjected to immunoblotting. The gels are transferred to PVDF membranes (EMD Millipore) using a wet transfer apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked using 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad Laboratories) and incubated overnight at 4°C with the following primary antibodies: a rabbit polyclonal RUNX1 (Cell Signaling Technology, Danvers, MA, USA: #4334, 1:1000); a mouse monoclonal to E-

cadherin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA: sc21791, 1:1000); a mouse monoclonal Vimentin (Santa-Cruz Biotechnology sc-6260, 1:1000); a mouse monoclonal to β-Actin (Cell Signaling Technology #3700, 1:1000); a rabbit polyclonal LaminB1 (Abcam, Cambridge, UK: 16048, 1:2000); a rabbit polyclonal N-cadherin (Santa Cruz Biotechnology sc-7939, 1:2000). Secondary antibodies conjugated to HRP (Santa Cruz Biotechnology) were used for immunodetection, along with the Clarity Western ECL Substrate (Bio-Rad Laboratories) on a Chemidoc XRS+ imaging system (Bio-Rad Laboratories).

2.3.5 Immunofluorescence staining microscopy

Cells grown on coverslips were fixed with using 3.7% formaldehyde in phosphate buffered saline (PBS) for 10 min. Cells were then permeabilized in 0.1% Triton X-100 in PBS, and washed in 0.5% Bovine Serum Albumin in PBS. Detection was performed using a rabbit polyclonal RUNX1 antibody (Cell Signaling Technology #4336), a mouse monoclonal Vimentin (Santa Cruz Biotechnology sc-6260), a rabbit polyclonal N-cadherin (Santa Cruz Biotechnology sc-7939) and a mouse monoclonal to E-cadherin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Staining was performed using fluorescent secondary antibodies; for rabbit polyclonal antibodies a goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor® 488 conjugate (Life Technologies A-11008), was used and for mouse monoclonal a F(ab')2-goat anti-mouse IgG (H+L) secondary antibody, Alexa Fluor® 488 conjugate was used (Life Technologies A-11001).

2.3.6 Quantitative PCR

RNA was isolated with Trizol (Life Technologies) and cleaned by DNase digestion (Zymo Research, Irvine, CA, USA). RNA was reversed transcribed using SuperScript II and random hexamers (Life Technologies). cDNA was then subjected to quantitative PCR using SYBR Green technology (Applied Biosystems, Foster City, CA, USA). Sequences of primers used in the paper. RUNX1 Forward: AACCCTCAGCCTCAGAGTCA, RUNX1 Reverse: CAATGGATCCCAGGTATTGG; E-cadherin Forward: GGAAGTCAGTTCAGAGCATC, E-cadherin Reverse: AGGCCTTTTGACTGTAATCACACC; N-cadherin Forward: TGTTTGACTATGAAGGCAGTGG, N-cadherin Reverse: TCAGTCATCACCTCCACCAT; Vimentin Forward: AGGAAATGGCTCGTCACCTTCGTGAATA, Vimentin Reverse: GGAGTGTCGGTTGTTAAGAACTAGAGCT; GAPDH Forward: TGTGGTCATGAGTCCTTCCA, GAPDH Reverse: ATGTTCGTCATGGGTGTGAA; HPRT Forward: TGCTGACCTGCTGGATTACA, HPRT Reverse: TCCCCTGTTGACTGGTCATT; β-Actin Forward: AGCACAGAGCCTCGCCTTT, β-Actin Reverse: CGGCGATATCATCATCCAT.

2.3.7 Tissue microarray

Formalin-fixed paraffin-embedded (FFPE) human breast cancer samples were obtained from the UMMS tissue bank and FFPE human breast cancer tissue microarrays (TMA) from US BioMax (Rockville, MD, USA). TMAs (BR1503a & BR10010) were obtained from US BioMax. Sample information pertaining to Type, Grade, Stage, TNM, were provided by US BioMax. BR1503a is a primary breast tissue array of 150 samples of 75 patient cases: three cases of adjacent normal breast tissue, three cases of breast fibroadenoma, two cases of breast cystosarcoma phyllodes, seven cases of breast intraductal carcinoma, and 60 cases of breast invasive ductal carcinoma. Duplicate cores per case. BR10010 is a breast carcinoma and matched metastatic carcinoma array of 100 samples of 50 patient cases: 46 cases of invasive ductal carcinoma, one case of micropapillary carcinoma, two cases of invasive lobular carcinoma, and one case of neuroendocrine carcinoma. Duplicate cores per case. RUNX1 staining was done as previously described (Liu, Lengner et al. 2011) using RUNX1 Rabbit Polyclonal 4334 from Cell Signaling Technology. Each tissue section was imaged and independent researchers blindly scored the sections based on the metric in Fig. 2.12 A.

2.3.8 Analysis of RUNX1 expression in various cancers using public data sets

RUNX1 expression was analyzed in various breast cancer subtype types using the TCGA database (www.cbioportal.org) (Network 2012). The PROGgene

database (www.compbio.iupui.edu/proggene) was used to identify the data sets for survival analysis and re-analyzed the public GEO data sets (www.ncbi.nlm.nih.gov/gds) (GSE3494-U133A).

2.3.9 RNA-Seq, ontology, and pathway analysis

RNA was isolated using DirectZol RNA mini prep kit (Zymo Research), quantified by Qubit HS RNA assay (Thermo Fisher Scientific) and assayed for RNA integrity by Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA was depleted of ribosomal RNA, reverse transcribed and strand-specific adapters added following manufacturer's protocol (TruSeq Stranded Total RNA Library Prep kit with Ribo-Zero Gold, Illumina, San Diego, CA, USA) with the exception that the final cDNA libraries were amplified using the Real-time Library Amplification Kit (Kapa Biosystems, Wilmington, MA, USA) to prevent over-amplification of libraries. Generated cDNA libraries were assayed for quality then sequenced as single-end 100 bp reads (IlluminaHiSeq1000, UVM Advanced Genome Technologies Core). Sequence files (fastq) were mapped to the most recent assemblies of the human genome (hg38) using TopHat2 (Kim, Pertea et al. 2013). Expression counts were determined by HTSeq (Anders, Pyl et al. 2015) with recent gene annotations (Gencode v22) (Harrow, Frankish et al. 2012). Differential expression was analyzed by DESeq2 (Love, Huber et al. 2014). Correlation between replicates and differential gene expression between time points was assessed by principal

component analysis (PCA). RNA-Seq data have been deposited in the GEO under accession codes GSE85857. In addition, mRNA expression data was uploaded to IPA (www.ingenuity.com) and analyzed using default parameters. The expression heat map was generated using GENE-E (Broad Institute, MA, USA www.broadinstitute.org/cancer/software/GENE-E/). Fifty-eight EMT genes were selected by using the list from (Taube, Herschkowitz et al. 2010, Minafra, BravatÀ et al. 2014).

2.3.10 ChIP-qPCR

RUNX1 ChIP-qPCR was performed essentially as described(O'Geen, Frietze et al. 2010) . Briefly, 200,000 MCF10A cells were cross-linked, lysed and sonicated to obtain DNA fragments mostly in the 200-1000-bp range. Immunoprecipitation was performed at 4°C overnight with anti-RUNX1 antibody (4334, Cell Signaling Technology) at a 1:15 antibody to chromatin ratio. Primers used in ChIP-qPCR are listed below: CDH1 Forward: CCCAACCTGACCACAGGAAT, CDH1 Reverse: GCTGCATGCGTAACAACACA; TGFB2 Forward: AGTCCTCCTCCCCCTAATGT, TGFB2 Reverse: CAGGGTATAGGCCACGACTG; TGFBR3 Forward: TCTTTGTAGCCTGCTGGGTT, TGFBR3 Reverse: CCCCCATCCTTACAAGTGGTT; ZNF333 (negative control 1) Forward: TGAAGACACATCTGCGAACC, ZNF333 Reverse: TCGCGCACTCATACAGTTTC; ZNF180 (negative control 2) Forward: TGATGCACAATAAGTCGAGCA, ZNF180 Reverse: TGCAGTCAATGTGGGAAGTC.

2.3.11 Statistical analysis

The results were reported as Mean ± S.E.M. unless otherwise indicated, and Student's t-Tests were used to calculate statistical significance.

The following datasets were generated:

- RNA-sequences:

  http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE85857, publicly available at NCBI Gene Expression Omnibus (accession no. GSE 85857)

2.4 RESULTS

2.4.1 RUNX1 expression is decreased in breast cancer

RUNX1 involvement in breast cancer was first tested using a panel of normal and breast cancer cell lines representing different breast cancer subtypes (Fig. 2.1). The selected cell lines included non-metastatic luminal MCF7 and T47D breast cancer cells and basal-like breast cancer MDA-MB-231 cells. Compared to the high level of RUNX1 in normal-like basal MCF10A control cells, RUNX1 mRNA (Fig. 2.1A) and protein (Fig. 2.1B) were significantly decreased in all breast cancer cell lines tested, but less so in the triple-negative MDA-MB-231 cells.

We next evaluated RUNX1 mRNA and protein expression in the MCF10 progression series of MCF10A normal-like mammary epithelial cells, tumorigenic MCF10AT1 and MCF10CA1a cells (Santner, Dawson et al. 2001). RUNX1 mRNA

(Fig. 2.1C) and protein (Fig. 2.1D) expression were strikingly decreased in both MCF10AT1 and MCF10CA1a cells compared with MCF10A cells. In both non-metastatic cancer cell types, loss of RUNX1 expression paralleled decreases of the epithelial marker E-cadherin, while the mesenchymal marker Vimentin was highly expressed only in the MCF10CA1a cells. These changes in EMT markers are consistent with the mesenchymal phenotype of the two cancer cell lines. Thus, decreased RUNX1 with tumor progression correlates with EMT. Together our findings indicate an important role for RUNX1 in normal breast epithelial cells and provide evidence for the emerging concept that RUNX1 may function to suppress tumor growth in breast cancer (Chuang, Ito et al. 2013).
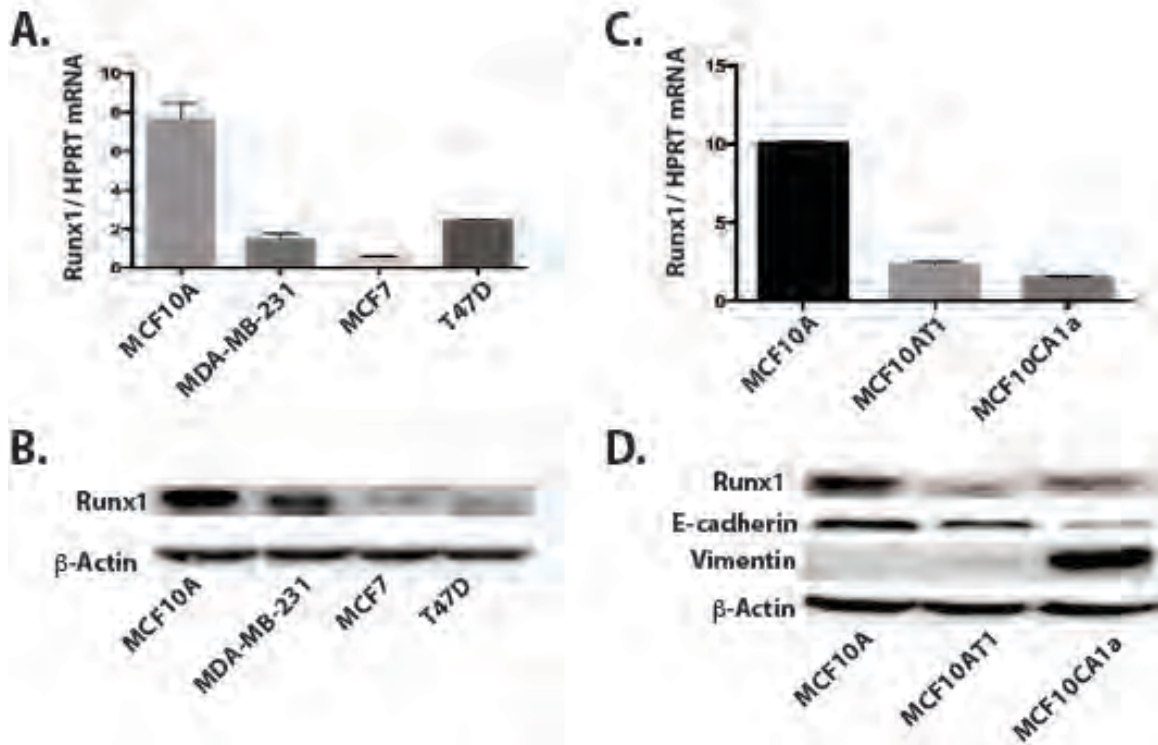
Figure 2.1. Decreased RUNX1 expression is related to breast cancer progression in cell models. (**A**) RUNX1 RNA expression by RT-qPCR for a panel of breast cancer cell lines compared to MCF10A cells show that RUNX1 protein is decreased in breast cancer cells. (**B**) Western blot of cell lysate for the same panel of cell lines shown in **A**. (**C**) RUNX1 RNA expression by RT-qPCR of normal mammary-like MCF10A cells, MCF10A-derived tumorigenic cell line MCF10AT1, and metastatic MCF10CA1a cells shows RUNX1 is decreased in the cancer cells. (**D**) Western blot comparison in the MCF10 series. All the experiments are performed 3 times (N=3).

2.4.2 TGF-β induced EMT decreases RUNX1 expression in MCF10A cells

The above results show that RUNX1 levels are decreased in breast cancer cells and that decreased RUNX1 is accompanied with EMT in the MCF10 series. To mechanistically address if decreased RUNX1 and EMT are coupled in breast cancer, we used a well-known method to induce EMT in mammary cells, by adding TGF-β to MCF10A cells (Xu, Lamouille et al. 2009). TGFB1-Smad signaling is the most frequently described inducer of EMT, and RUNX1 is known to be a downstream target of TGF-β signaling. Furthermore, it is well documented that RUNX1 forms an interaction complex with SMADs (Ito and Miyazono 2003), thereby regulating genes responsive to TGF-β. Taken together, we hypothesized that RUNX1 expression would be repressed upon treating with TGF-β.

MCF10A cells were incubated with 10 ng/ml TGFβ1 for 6 days, and we observed that the original cobblestone-like epithelial morphology with tight cell-cell contact was lost, and cells gained an elongated fibroblast-like morphology (Fig. 2.2A). When the levels of epithelial and mesenchymal markers were examined by western blotting and immunofluorescence microscopy, the TGFβ1-treated cells exhibited a 50% down-regulation of the epithelial marker E-cadherin, while expression of the mesenchymal markers Vimentin and N-cadherin was induced (Fig. 2.2B, C). Significantly, in this TGF-β-induced EMT model, we observed the down-regulation of RUNX1 at both the protein and mRNA levels (Fig. 2.2B). Although the immunofluorescence results showed that not all cells acquired the mesenchymal phenotype (Fig. 2.2C), indicating that only a subset of the cells

underwent EMT, we still find that RUNX1 is decreased during EMT. As further evidence that loss of RUNX1 occurs concomitantly with EMT, co-immunofluorescence reveals that the subset of cells undergoing EMT (Vimentin-positive cells), had lower or no RUNX1 expression (Fig. 2.2D). These results support the idea that RUNX1 may function as a suppressor of the EMT.
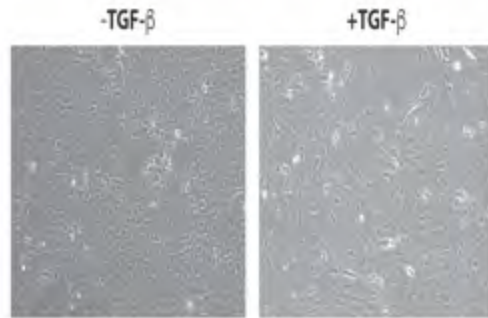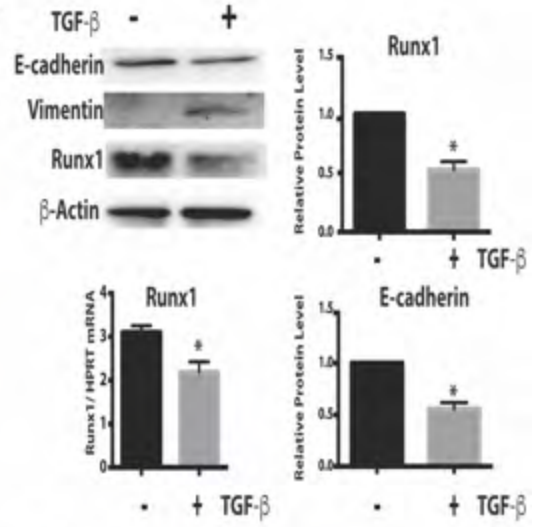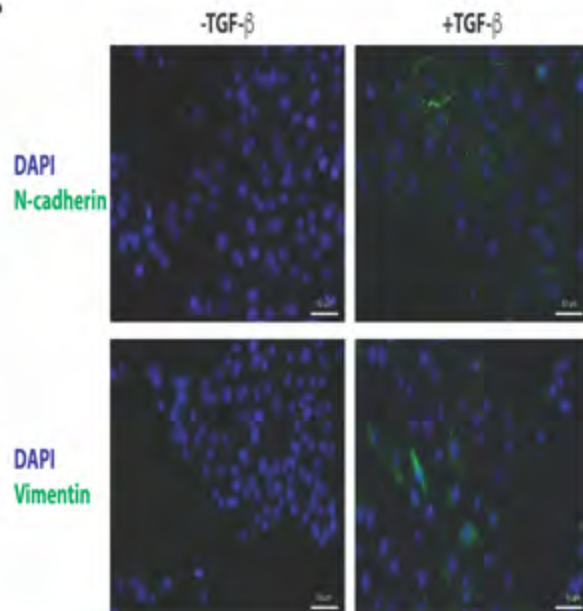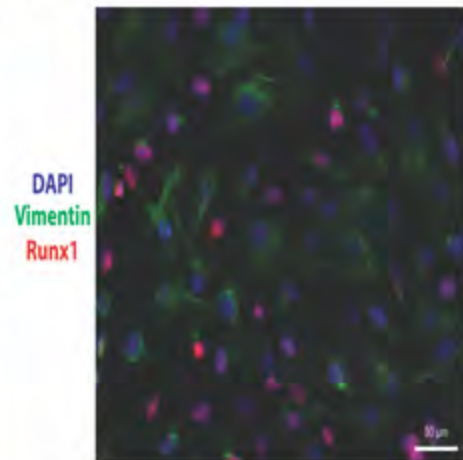
Figure 2.2. RUNX1 decreases during TGFβ-induced EMT. MCF10A cells treated with 10 ng/ml TGFβ for 6 days. (**A**) MCF10A cells treated with TGFβ show morphological changes toward an EMT-like state. (**B**) Western blot analyses show changes in EMT markers and RUNX1 expression during EMT. Left lower panel: RT-qPCR of RNA from MCF10A cells shows decreased RUNX1 expression in TGFβ treated cells. Student's *t* test * *p* value <0.05 for TGFβ-treated cells compared to control cells. Where error bars are shown these represent the standard error of the mean (SEM) from three independent experiments. (**C**) Immunostaining shows increased Vimentin and N-cadherin expression in the cytoskeleton during TGFβ-induced EMT. (D) Immunostaining shows the cells with Vimentin (Green) expression have less or no RUNX1 (Red) expression. All the experiments are performed 3 times (N=3).

2.4.3 RUNX1 reverses the TGF-β-induced EMT phenotype

To further prove a functional role for RUNX1 in preventing EMT and maintaining the epithelial phenotype, we examined whether overexpressing RUNX1 could reverse the EMT phenotype after TGF-β induction.

A plasmid containing HA-tagged RUNX1 was transfected into TGF-β-treated MCF10A cells. We observed that the cells with RUNX1 overexpression changed

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their morphology from mesenchymal-like back to epithelial-like (Fig. 2.3A). Overexpressing RUNX1 in these cells also increased E-cadherin and repressed Vimentin expression, suggesting that cells re-acquired an epithelial phenotype and that the TGF-β-induced EMT was blocked (Fig. 2.3B). This result demonstrated that the repression of RUNX1 is a necessary step during TGF-β induced EMT.
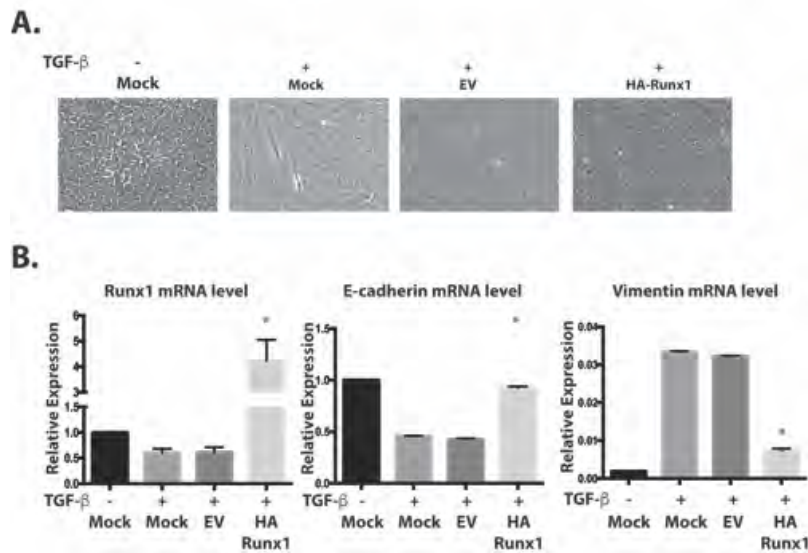
Figure 2.3. RUNX1 reverses TGFβ induced EMT. (**A**) Images of MCF10A cells treated with TGF-β show morphological changes toward an mesenchymal state. Overexpressing RUNX1 in TGF-β-treated cells returned cell morphology to an epithelial-like state. (**B**) RT-qPCR of RNA from MCF10A cells show changes in gene expression by overexpressing RUNX1 in TGFβ-treated cells, which activates E-cadherin and represses Vimentin expression. Student's *t* test * *p* value <0.05 for HA-RUNX1 overexpression in MFC10A cells compared to EV control cells. Error bars represent the standard error of the mean (SEM) from three independent experiments. All the experiments are performed 3 times (N=3).

2.4.4 Decreased expression of RUNX1 during TGF-β independent EMT in MCF10A cells

We considered the possibility that RUNX1 may function in an exogenous TGF-β-independent manner to repress EMT. We used a cell model of EMT induction

that is independent of treatment with exogenous TGF-β. It has been previously shown that withdrawal from MCF10A medium of specific factors required for optimal cell growth (insulin, EGF, Hydrocortisone and Cholera Toxin), changed cell morphology from cobblestone to spindle like (Yusuf and Frenkel 2010). Here we demonstrate that this morphological change (Fig. 2.4A) resembles an EMT process. Western blotting and qRT-PCR results show that the epithelial marker E-cadherin was down regulated, while mesenchymal markers N-cadherin and Vimentin were upregulated (Fig. 2.4B and C). Importantly RUNX1 protein is not detected in growth factor-depleted cells by western blot and immunofluorescence microscopy (Fig. 2.4B and D, top panel). Compared with TGF-β-induced EMT (Fig. 2.2C), in this exogenous TGFB independent model, all cells acquired the mesenchymal phenotype and lost epithelial markers and RUNX1 expression (Fig. 2.4D). These results reveal that modifying growth medium is a more powerful method for inducing EMT in MCF10A cells. Based on the loss of RUNX1 during both exogenous TGF-β-dependent and -independent EMT, we conclude that RUNX1 is a key factor in repressing the EMT and maintaining epithelial morphology in normal-like mammary epithelial cells.

A. +Growth Factor   -Growth Factor

B.

Growth Factor	+	−
Runx1		
E-Cadherin		
Vimentin		
N-Cadherin		
β-Actin		

C.

D.

+Growth Factor   − Growth Factor

DAPI Runx1

DAPI Vimentin

DAPI N-cadherin

DAPI E-cadherin
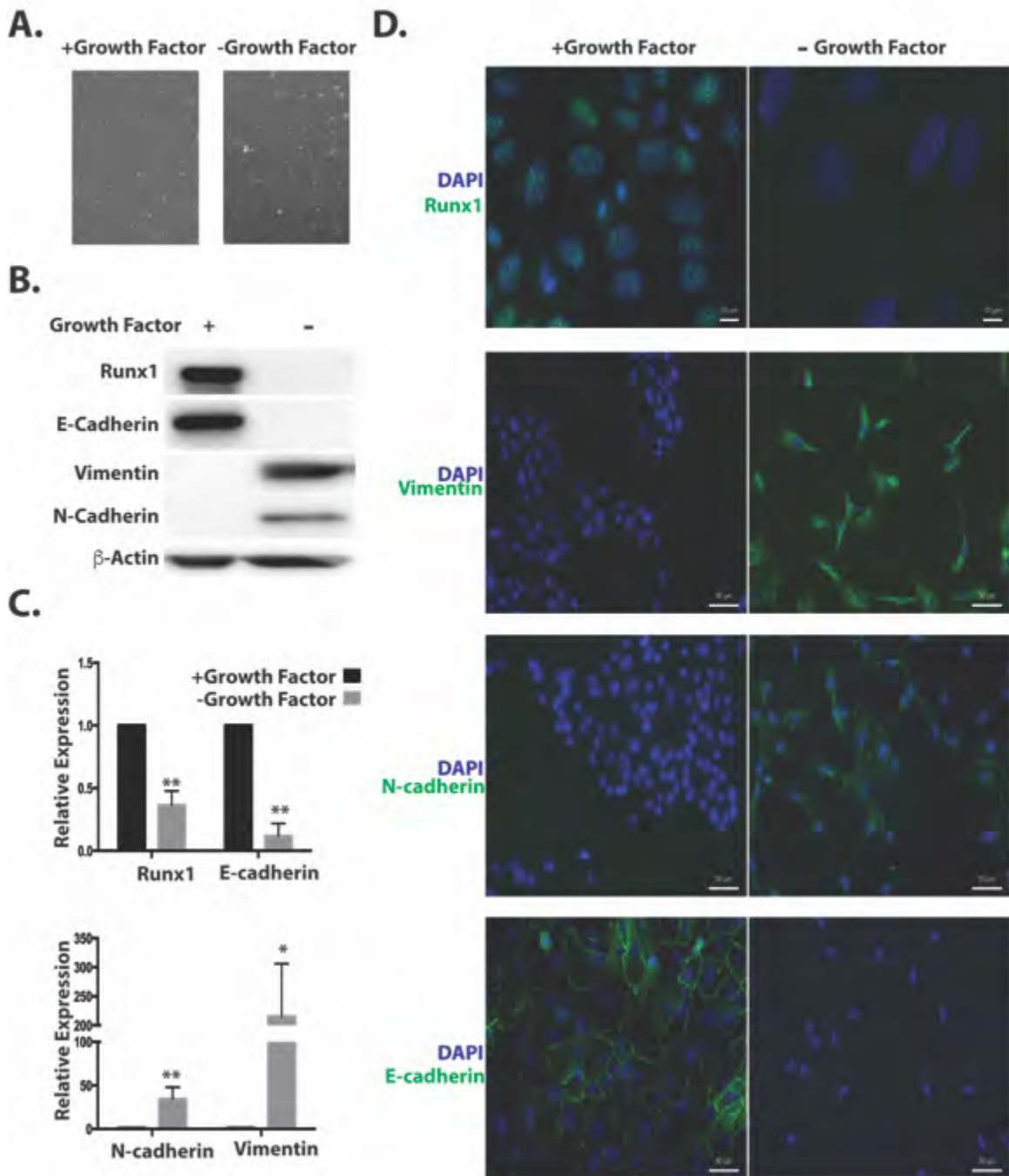
Figure 2.4. Decreased RUNX1 during TGF-β-independent EMT. (**A**) Images of MCF10A cells grown in medium without growth factors (Insulin, EGF, Hydrocortisone and Cholera toxin) for 7 days show morphological changes from cobblestone to spindle-like. (**B**) Western blot analyses of cell lysates from MCF10A cells treated with or without growth factors show changes in EMT markers and RUNX1 expression during EMT. (**C**) RNA expression of the EMT markers E-cadherin, N-cadherin and Fibronectin was quantified using RT-qPCR in MCF10A cells in the presence or absence of growth factors. Student's *t* test * *p* value <0.05, ** p value <0.01 for growth factors depleted MCF10A cells compared to cells with growth factors. Error bars represent the standard error of the mean (SEM) from three independent experiments. (**D**) Immunostaining of E-cadherin, Vimentin, N-Cadherin and RUNX1 reveals changes in organization of cell–cell adhesion, cytoskeleton and decreased RUNX1. All the experiments are performed 3 times (N=3).

2.4.5 Gene expression profiling of growth factor-depleted MCF10A cells reveals the spectrum of EMT markers

To further understand the mechanisms of growth factor depletion-induced EMT, we carried out unbiased genome-wide expression profiling by RNA-Seq, comparing cells grown in normal and growth factor depleted conditions. Among

the 1880 differentially expressed mRNAs that have a 2-fold cut off, 457 genes were up- and 1423 were down-regulated. Gene ontology analysis identified functional categories and associated pathways (Fig. 2.5). Among the top 5 canonical pathways that were affected, regulation of the EMT pathway was the most significant with 20 genes altered in the network (Fig. 2.5A and C). This observation further confirmed that this novel method of removing growth factors in MCF10A induces EMT. Other relevant pathways include cancer metastasis signaling and integrin-like kinase (ILK) signaling (Fig. 2.5A). Together these most significant signaling pathways are indicative of the MCF10A cells acquiring a more cancer related phenotype.

In addition to pathway analysis, we selected 58 epithelial and mesenchymal genes by using two database sources (described in Materials and Methods) and examined the expression patterns based on relative reads from our RNA-Seq profiling. The heat map constructed from these data (Fig. 2.5B) compares expression of EMT genes under two different growth conditions—normal and growth factor-depleted. Well-established epithelial genes such as DSP, Claudins and KRT family (Tomaskovic-Crook, Thompson et al. 2009) were down-regulated. We observed consistent up-regulation of common mesenchymal genes (CDH2, FN1 and VIM) as well as genes related to signaling pathways such as BMP/TGFB and WNT when growth factors were removed. We also noted that both TGFβ2 and Runx2 are among the up-regulated genes (Fig. 2.5B). Moreover, we found that expression of 43 genes in the Runx2 interaction network were altered (Fig. 2.5C),

consistent with up-regulation of Runx2 protein level upon growth factor depletion (Fig. 2.6) and its role in promoting invasion and metastasis to bone (Pratap, Lian et al. 2006).

To study how loss of RUNX1 is involved in this EMT process, we also examined the RUNX1 interaction network and found that 20 genes (Fig. 2.5C) were altered upon growth factor depletion. Further pathway analysis with the 1880 differentially expressed genes revealed that decreased RUNX1 and the altered RUNX1 interaction network are associated with activation of TGFβ and WNT pathways (Fig. 2.5D), which are known to relate to RUNX1 function (Chimge, Little et al. 2016). The stimulated TGFβ and WNT pathways further activate the downstream well-studied EMT-inducing transcription factors Snail and Twist (Fig. 2.5D) (Tomaskovic-Crook, Thompson et al. 2009). These studies provide evidence that depletion of RUNX1 contributes to initiation of EMT in the normal-like MCF10A mammary epithelial cells. These results also indicate that Runx2 plays an important role during growth factor starvation-induced EMT and elucidate mechanisms by which RUNX1 and Runx2 are involved in EMT. Together, these RNA-Seq data confirm that the growth factor starvation method is a unique cell treatment to induce EMT in MCF10A cells without exogenous addition of TGFβ.

A. Top Canonical Pathways

Name	p-value	Overlap
Regulation of the Epithelial-Mesenchymal Transition Pathway	1.66E-06	20/143
Colorectal Cancer Metastasis Signaling	5.47E-06	22/181
Hepatic Fibrosis/ Hepatic Stellate Cell Activation	5.69E-06	18/129
ILK Signaling	7.25E-06	19/144
Caveolae-medicated Endocytosis Signaling	1.78E-05	11/57

B. EMT Gene Expression
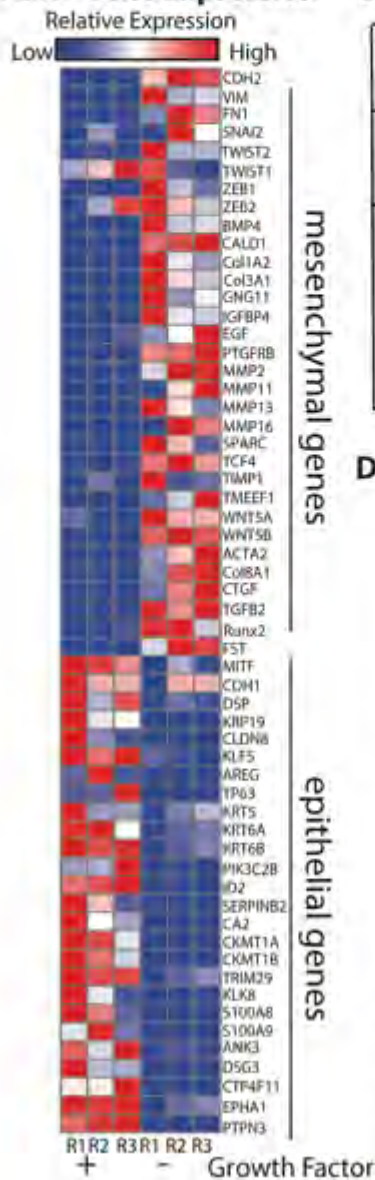
C. Differentially Expressed Genes in Networks

Regulation of EMT p val=1.66E-06 20/143	CDH2, EGF, EGFR, FGF12, FGFR4, FZD5, ID2, JAG2, LEF1, MMP2, MRAS, NOTCH1, PDGFRB, PIK3C2B, SNAI1, TCF4, TGFB2, TWIST2, WNT5A, WNT5B
Runx1 network p val=2.56E-02 20/419	AR, CCND1, EGF, EGFR, ESR1, FOS, FYN, HAPLN1, HLA-B, HLA-DMA, LEF1, MYH9, NOTCH1, PRKACB, RORC, RUNX1T1, S100A9, SMRACD2, SOX5, TGFB2
Runx2 network p val=3.73E-09 43/536	AR, COL10A1, COL18A1, COL1A2, COL3A1, EGF, EGFR, EMP1, ESR1, EGF12, GFGR4, RN1, FOS, FOXO1, GLI2 GLIS2, HAPLN1, IL1RN, IL20RB, ITGA1, ITGB1, JAG2, KLF2, LEF1, LUM, MMP14, NOTCH1, POSTN, PPIP5K1, PPKACB, PTGER4, PTGS2, SHOX2, SMURF2, SNAI1, SOX5, SVIL, TCF4, TGFB2, TGM2, TWIST2, WNT5A, WNT5B

D. Runx1 in EMT
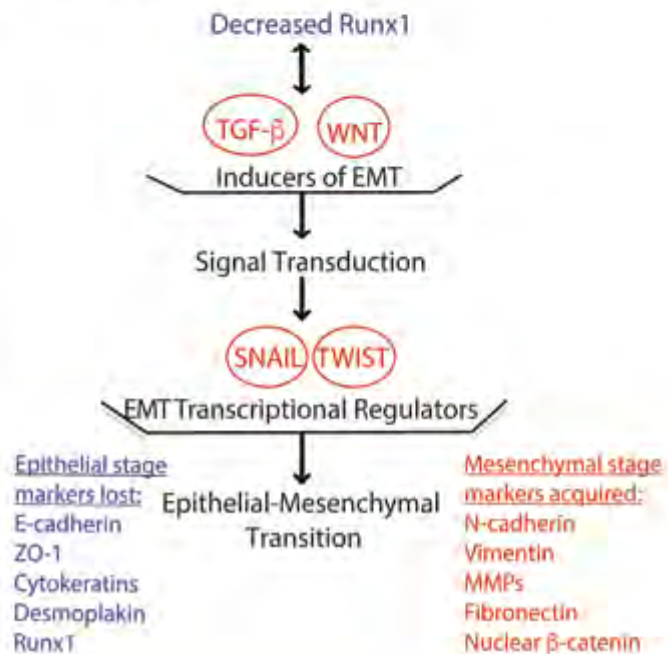
Figure 2.5. RNA-Seq reveals MCF10A cells undergo EMT upon growth factor removal. (**A**) Top canonical pathways with the most significant p values identified by using Ingenuity Pathway Analysis (QIAGEN, Hilden, Germany). (**B**) Relative expression heat map of 58 EMT related genes confirming MCF10A cells undergo EMT. (**C**) Differentially expressed genes (2-fold cut off) in the EMT regulation pathway (p val 1.66E-06), RUNX1 interaction network (p val 2.56E-02) and Runx2 interaction network (p val 3.73E-09). (**D**) Model of RUNX1 function in growth factor depletion induced EMT. Illustration shows the consequences of up and down regulated genes when RUNX1 is decreased upon growth factor depletion. The listed genes and pathways are promoting EMT by loss of RUNX1 function. Blue indicates down regulated genes. Red indicates up regulated genes or pathways. Ingenuity Pathway Analysis (QIAGEN) was used in panel **A**, **C** and **D**; GENE-E (Broad Institute, Cambridge, MA, USA) was used in panel **B**.

Growth Factor	+	-
Runx2		
Lamin B1		
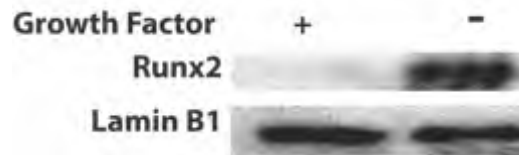
Figure 2.6. Increased Runx2 during growth factor depleted induced EMT. Western blot analyses of cell lysates from MCF10A cells treated with or without growth factors showing changes in Runx2 activation during EMT. The experiments is performed 3 times (N=3).

2.4.6 Directly Depleting RUNX1 in MCF10A cells results in loss of epithelial morphology and activation of EMT

We have shown by multiple lines of evidence that down-regulation of RUNX1 is a key step during breast cancer EMT. However, we still could not distinguish whether decreased RUNX1 expression drives the activation of EMT or is an outcome of EMT. To address that question and understand whether RUNX1 can function directly to maintain normal epithelial morphology, we inhibited endogenous RUNX1 expression in MCF10A cells using lentivirus that contained short-hairpin RNA targeting RUNX1 (shRunx1) (Fig. 2.7). We generated two different MCF10A shRunx1 cell lines using two different shRNA sequences (shR1-1, shR1-2). Compared to the parental and control (non-silencing) cells, we observed that RUNX1-depleted MCF10A cells showed an obvious shift in morphology from cobblestone-like cells to more spindle-shaped cells (Fig. 2.7A).

Western blot and Q-PCR analysis demonstrated endogenous RUNX1 was down regulated at both the protein and mRNA levels (Fig. 2.7B and C). Because the shRunx1 cells exhibited a morphological change consistent with loss of the epithelial phenotype, E-cadherin expression was examined. RUNX1 knockdown cells showed a significant decrease of E-cadherin, as well as up-regulation of the mesenchymal genes Vimentin and N-cadherin (Fig. 2.7C).

A.

MCF-10A NS | MCF-10A shR1-1 | MCF-10A shR1-2

B.

Runx1 mRNA level

E-cadherin mRNA level

N-cadherin mRNA level

Vimentin mRNA level

C.

Anti-Runx1

Anti-E-cadherin

Anti-Lamin B1

MCF-10A | NS | shR1-1 | shR1-2

D. Runx1 ChIP-qPCR in MCF10A cells

CDH1 TGFB2 TGFBR3 NC1 NC2
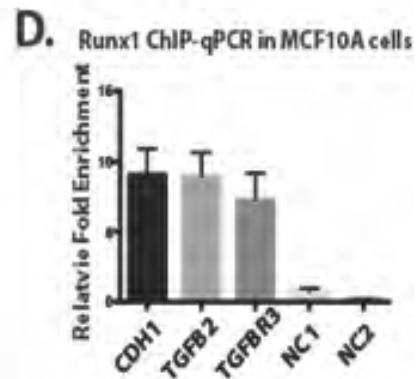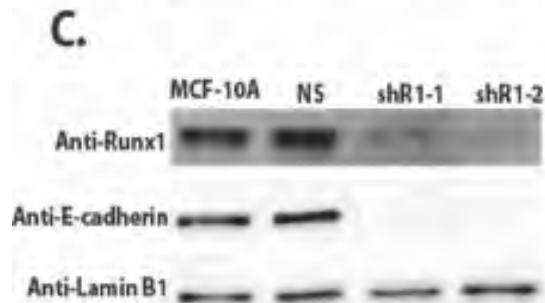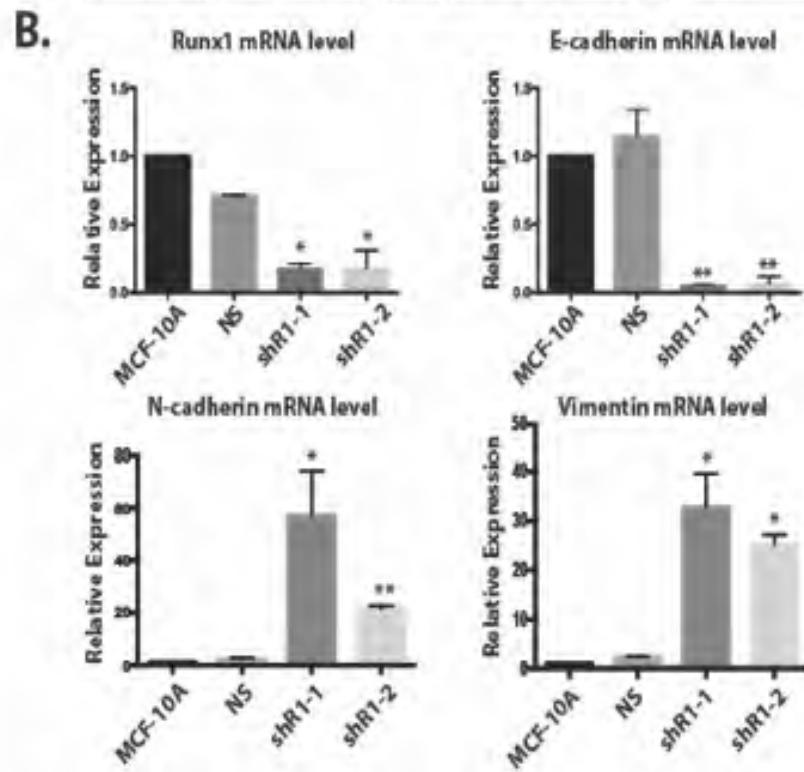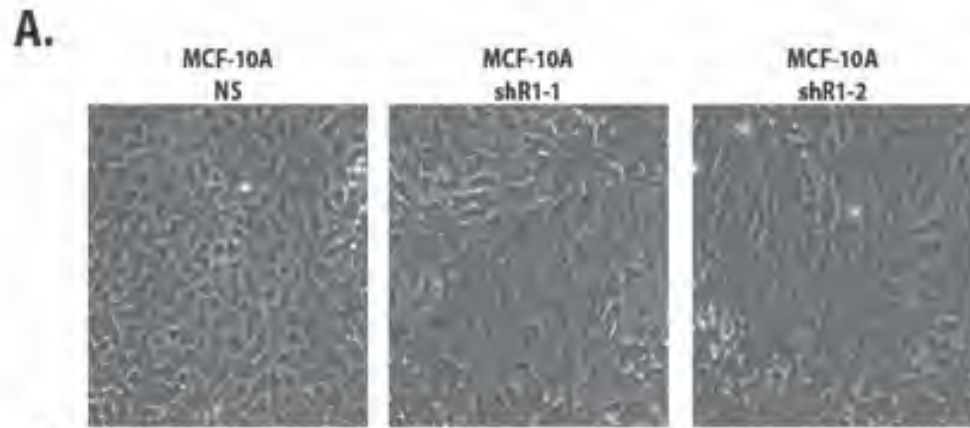
Figure 2.7. Depleting RUNX1 in MCF10A cells promotes a mesenchymal-like phenotype. (**A**) MCF10A cells treated with shRunx1 show morphological changes toward an EMT- like state. (**B**) Western blot analyses of lysates from MCF10A cells treated with shRunx1 show decreased protein expression of RUNX1 and E-cadherin. (**C**) RT-qPCR analyses of RNA from MCF10A cells treated with shRunx1 show decreased gene expression of E-cadherin and activation of mesenchymal marks of N-cadherin and Vimentin. Student's *t* test * *p* value <0.05, ** p value <0.01 for MCF10A shRunx1 cells compared to the MCF10A ns cells. Error bars represent the standard error of the mean (SEM) from three independent experiments. (**D**) ChIP-qPCR confirmation of RUNX1 occupancy at CDH1, TGFB2 and TGFBR1. ZNF188 (NC1) and ZNF333 (NC2) were used as the negative control as RUNX1 are predicted not to bind these genes. Data obtained with antibodies against RUNX1 are normalized to input control. All the experiments are performed 3 times (N=3).

Taken together, these results indicate that depletion of RUNX1 directly initiates EMT in MCF10A cells, and establishes for the first time that RUNX1 is required to maintain the normal mammary epithelial phenotype. The mechanism for these biological activities involves RUNX1 binding to EMT-related target genes.

Previously it has been shown that both E-cadherin (Liu, Lee et al. 2005) and genes in TGFB family (Hanai, Chen et al. 1999) have RUNX1 binding sites. Thus, to further support a direct role for RUNX1 regulation of E-cadherin and TGF-β signaling in MCF10A cells, a RUNX1 ChIP-qPCR was performed (Fig. 2.7D). Significant enrichment of RUNX1 binding on E-cadherin (CDH1), TGFB2 and TGFBR3 genes were observed. The positions of the amplicons on tested genes are shown in Figure 2.8. These results indicate that RUNX1 may directly bind to the E-cadherin gene and regulate its expression. Our findings also provide an additional line of evidence for a key function of RUNX1 in blocking TGF-β signaling and maintaining epithelial morphology. Further the binding of RUNX1 to the E-cadherin gene is also associated with the H3K4ac activating histone mark (Messier, Gordon et al. 2016). We searched for putative RUNX1 binding sites and found 5 consensus motif sequences that are coincident with H3K4ac peaks present in MCF10A cells but not in metastatic MDA-MB-231 cells Figure 2.9.
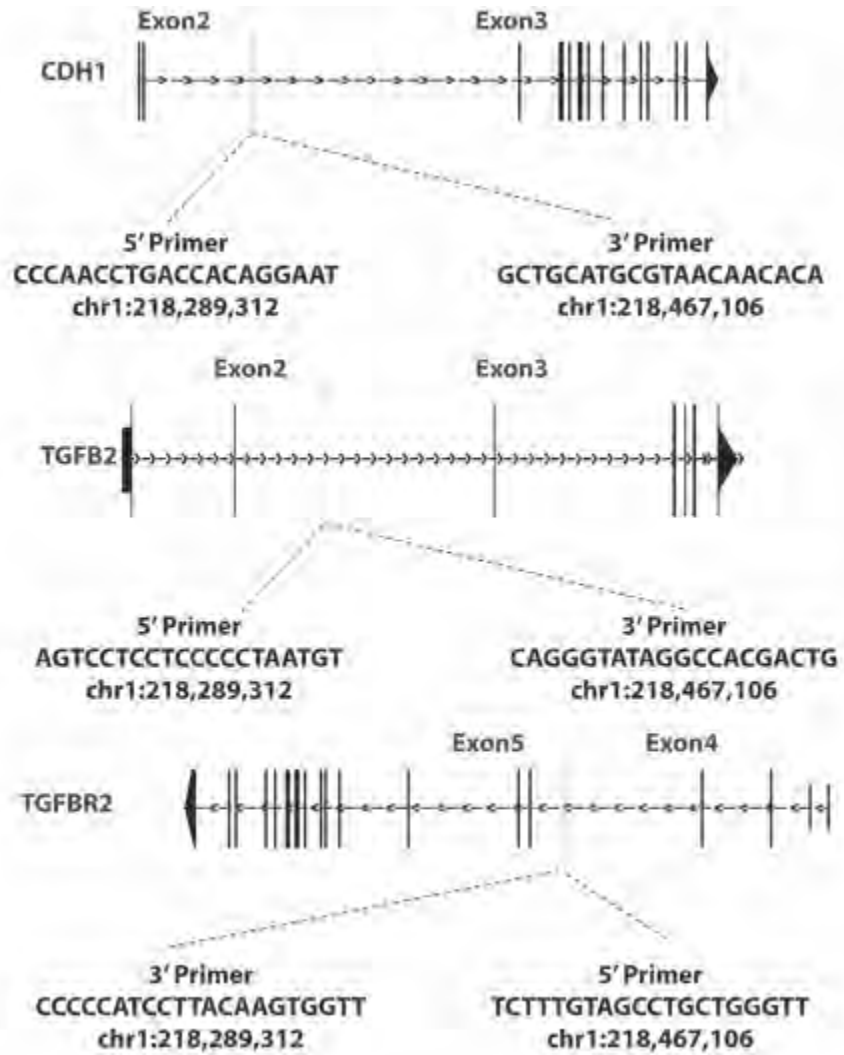
Figure 2.8. Schematic diagram of ChIP qPCR primers and amplicons over the tested gene for ChIP-qPCR.
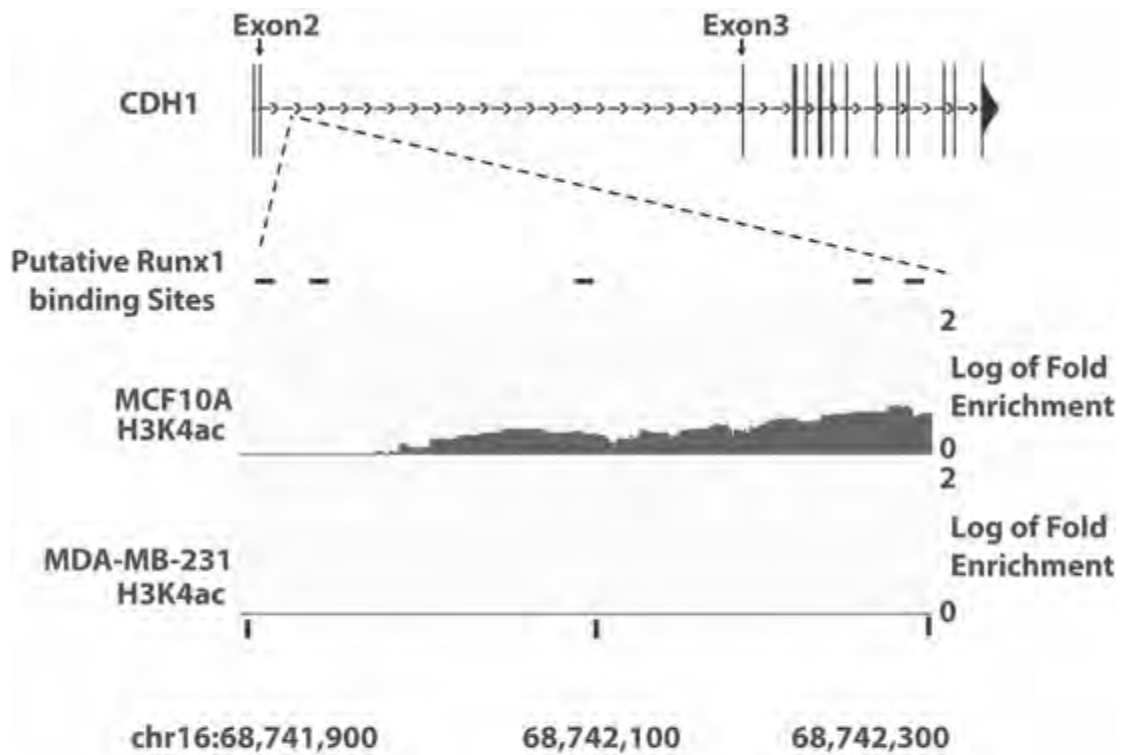
Figure 2.9. RUNX1 consensus sequences in CDH1 are coincident with H3K4Ac peaks in MCF10A cells. ChIP analysis showing significant binding of H3K4Ac (GSE69377) to a region in CDH1 genes with multiple RUNX1 binding motifs in MCF10A cells but not in MDA-MB-231 cells.

2.4.7 Depleting RUNX1 in MCF7 breast cancer cells promotes EMT

The loss of epithelial morphology in normal-like mammary cells by knockdown of RUNX1 (Fig. 2.7) raises a compelling question regarding the role of RUNX1 in

breast cancer cells. Therefore, we tested whether this regulation also occurs in epithelial-like MCF7 breast cancer cells. Two shRunx1 (shR1-1, shR1-2) stable knockdown in the MCF7 cell line were generated. Endogenous RUNX1 was down-regulated at both the protein and mRNA levels for both short-hairpin RNAs (Fig. 2.10A and B). In these RUNX1-depleted MCF7 cells, western blot and qRT-PCR analyses revealed a significant decrease of E-cadherin expression at both the protein and mRNA levels and an up-regulation of the mesenchymal genes Vimentin and N-cadherin at the mRNA level (Fig. 2.10C). Based on these results, we conclude that RUNX1 is preventing EMT in both normal mammary cells (MCF10A) and early breast cancer cells (MCF7), consistent with its function in maintaining an epithelial phenotype.

2.4.8 Overexpressing RUNX1 in mesenchymal like breast cancer cells drives mesenchymal to epithelial transition (MET)

To further establish a definitive role for RUNX1 function in preserving the epithelial phenotype, we carried out a "rescue" study to examine the consequences of restoring RUNX1 expression in mesenchymal like breast cancer cells (Fig. 2.10D and E). RUNX1 was ectopically expressed in tumorigenic MCF10AT1 cells, which resulted in increased E-cadherin expression and decreased Vimentin expression (Fig. 2.10D and E). Notably, the E-cadherin level is only increased at the mRNA level but not the protein level under transient transfection conditions (data not shown). This key finding shows that overexpression of RUNX1 in

mesenchymal cancer cells drives the cells back to the epithelial stage. These observations provide direct evidence that RUNX1 prevents EMT.
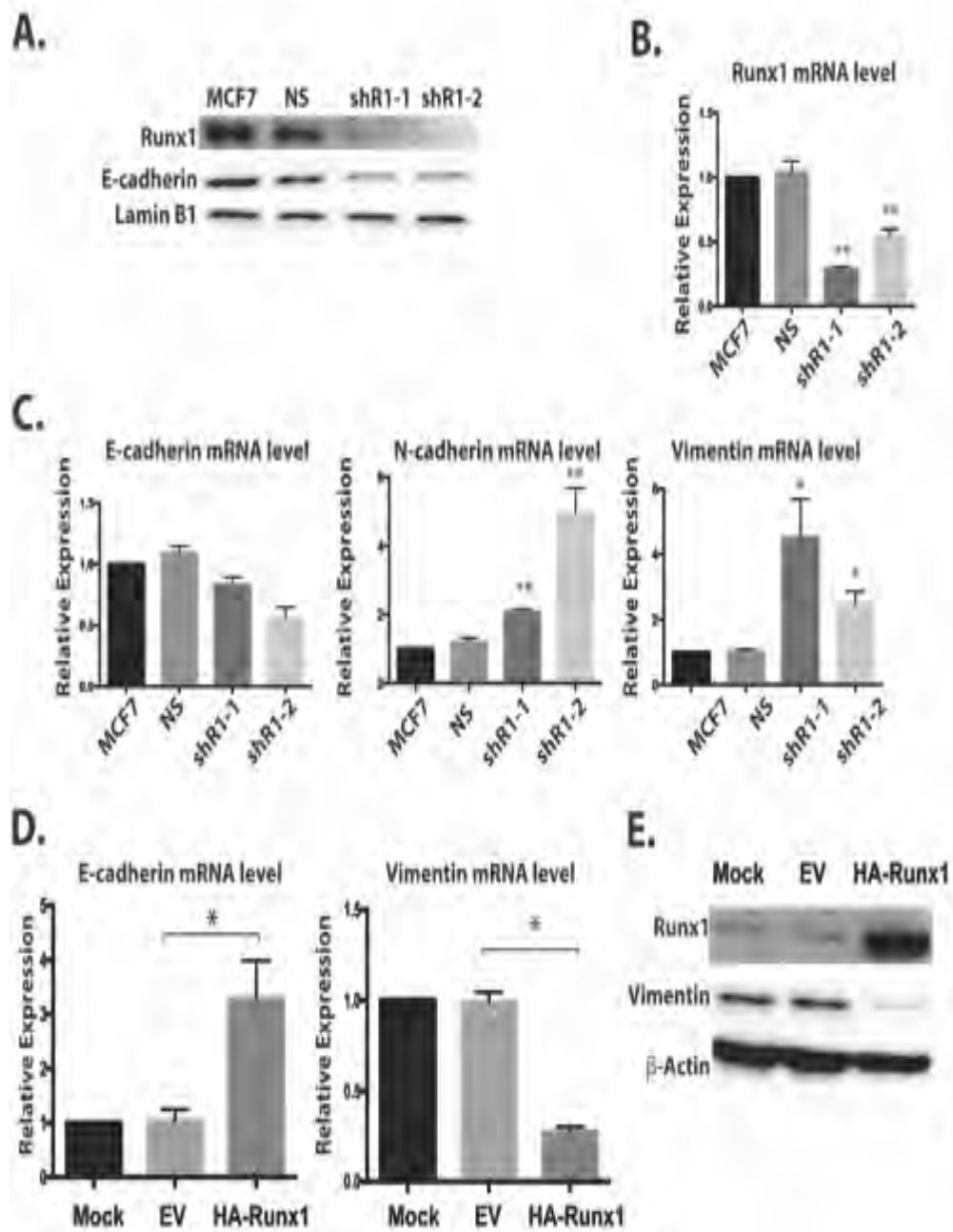
Figure 2.10. RUNX1 controls EMT-MET in non-metastatic breast cancer cells. Two breast cancer cell lines MCF7 (epithelial-like) (**A**-**C**) and MCF10AT1 (mesenchymal-like) (**D**, **E**) were examined for RUNX1 knockdown or ectopic expression, respectively. (**A**) Western blot analyses of lysates from MCF7 cells with RUNX1 depletion show decreased protein expression of RUNX1 and E-cadherin. (**B**) RT-qPCR of RNA from MCF7 cells treated with shRunx1 shows decreased gene expression of RUNX1. (**C**) RT-qPCR shows decreased gene expression of E-cadherin and increased gene expression of N-cadherin and Vimentin in RUNX1 depleted MCF7 cells. Student's *t* test * *p* value <0.05, ** p value <0.01 for MCF7 shRunx1 cells compared to the MCF7ns cells. Error bars represent the standard error of the mean (SEM) from three independent experiments. (**D**) RT-qPCR of RNA from MCF10AT1 cells overexpressing RUNX1 show increased gene expression of E-cadherin and decreased gene expression of Vimentin. Student's *t* test * *p* value <0.05 for MCF10AT1 RUNX1 overexpression cells compared to the MCF10AT1 EV cells. Error bars represent the standard error of the mean (SEM) from three independent experiments. (**E**) Western blot analyses of lysates from MCF10AT1 cells treated with RUNX1 overexpression show increased protein expression of RUNX1 and decreased expression of Vimentin. All the experiments are performed 3 times (N=3).

2.4.9 RUNX1 expression in breast tumors correlates with metastasis, tumor subtype and survival

We next evaluated RUNX1 expression in breast cancer patient tissues. With a highly specific RUNX1 antibody, we applied immunohistochemistry to determine the expression pattern of RUNX1 in different types of breast cancer using a Tissue Microarray (TMA) of 185 tumors and 6 control normal adjacent tissue sections. The results identified that RUNX1 expression is associated with breast cancer stages and subtypes. We observed RUNX1 expression at high levels in all normal and benign mammary epithelial tissues (Fig. 2.11A). RUNX1 is also expressed in breast cancer samples including ductal carcinoma in situ and invasive ductal carcinoma (Fig. 2.11A). However, breast cancer cells metastatic to the lymph node showed significantly less RUNX1 expression compared with the primary tumor site (Fig. 2.11A and B). Quantification of RUNX1 levels at primary sites and lymph metastatic sites in 50 patients showed that RUNX1 is significantly lower (p=0.005 using two tailed t test) in lymph samples (Fig. 2.11C). We also observed slightly higher RUNX1 levels in grade 1 compared with grade 2 tumors (Fig. 2.12)

We further investigated the relationship of RUNX1 expression to clinical outcomes through mining of The Cancer Genome Atlas (TCGA) database. RUNX1 was found to be under-expressed in several breast cancer subtypes, including Luminal B, Her2-enriched and basal-like breast cancers, which all have a poor prognosis (Fig. 2.11D). Luminal A subtype, which is generally associated with a good prognosis, showed RUNX1 levels equivalent to normal-like breast tissue.

However, 5% of samples in this subtype have RUNX1 somatic mutations (Network 2012), with the majority located in the RUNX1 DNA-binding domain, which can compromise RUNX1 transcriptional activity. We conclude from these data that RUNX1 expression is subtype-dependent and correlates with prognosis.
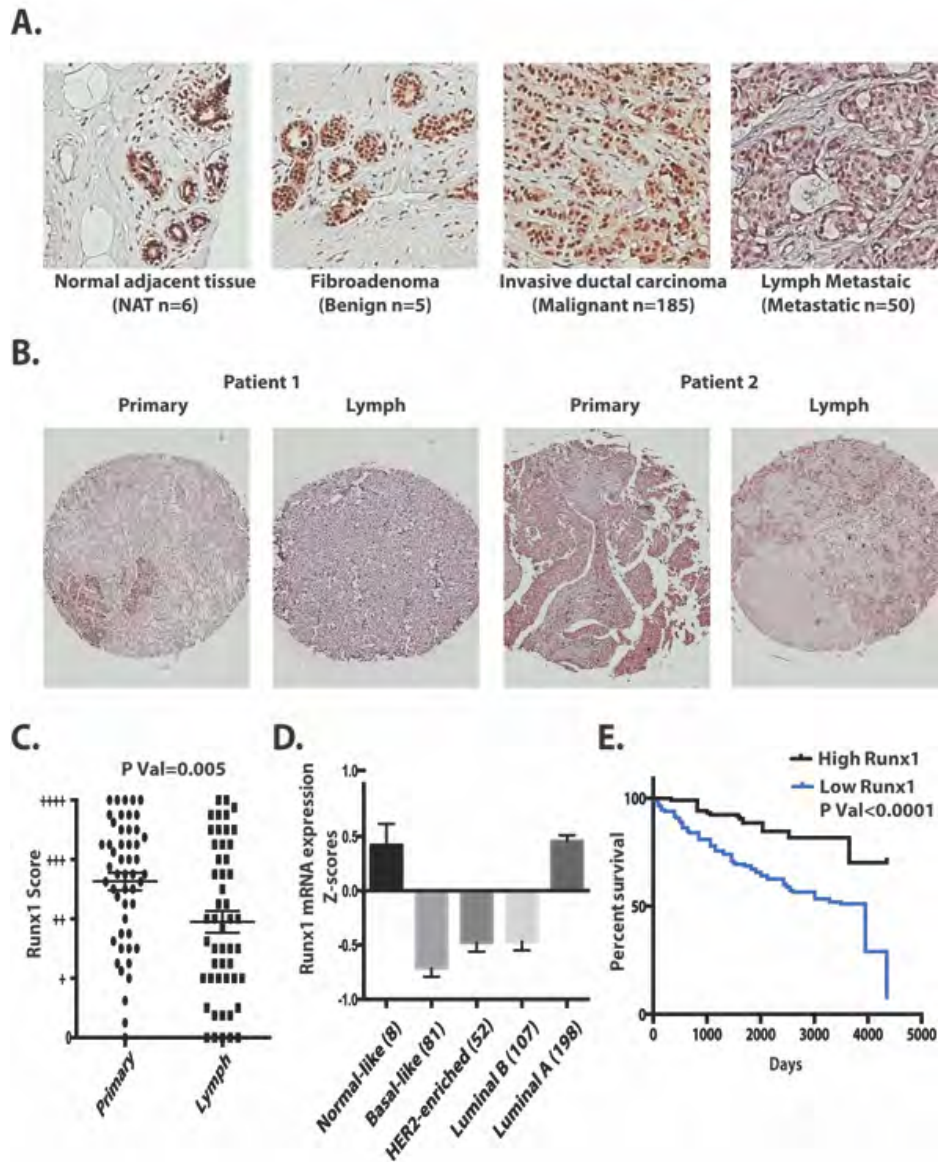
Figure 2.11. RUNX1 expression in breast tumors correlates with metastasis, tumor subtype and survival. (**A**) Representative tissue microarray images of RUNX1 in normal adjacent tissue (NAT), fibroadenoma, invasive ductal carcinoma, and tumor metastasis to lymph. (**B**) Representative of TMAs (n=50) showing two patients' primary tumor and their lymph metastasis with RUNX1 positive cells (brown stain). Two tailed t test ** $p<0.005$ between primary tumor and lymph metastatic sites. (**C**) Distribution of RUNX1 staining scores for 50 patients with primary breast tumor and lymph metastasis. Using a semi-quantitative scoring system, three researchers blindly scored TMAs. (**D**) RUNX1 mRNA is decreased in breast cancer subtypes. (**E**) Kaplan-Meier analysis showed higher overall survival in patients with higher RUNX1 mRNA expression (GSE3494-U133A). Gehan-Breslow-Wilcoxon test with p value<0.0001 compared with high RUNX1 expression patients and low RUNX1 expression patients.

RUNX1 expression levels were also compared with patient survival rates using a data set (GSE3494-U133A) in the Gene Expression Omnibus database (Fig. 2.11E). Our analyses show that patients with low RUNX1 levels in their tumors exhibit poor survival relative to patients with high RUNX1 expression.

Taken together our data demonstrate that RUNX1 sustains the epithelial phenotype and preserving the epithelial integrity in normal epithelial cells. Loss of

RUNX1 is not only accompanied with EMT (Fig. 2.2-2.5) but can also initiate the EMT transformation (Figs. 2.7 and 2.10). Therefore, loss of RUNX1 normal activities in tumor tissues may serve as an indicator of poor prognosis for breast cancer patients as revealed in several clinical studies (Fig. 2.11). We conclude from these clinical data that as tumors advance from early stage to a more aggressive phenotype, loss of RUNX1 may promote tumor progression.
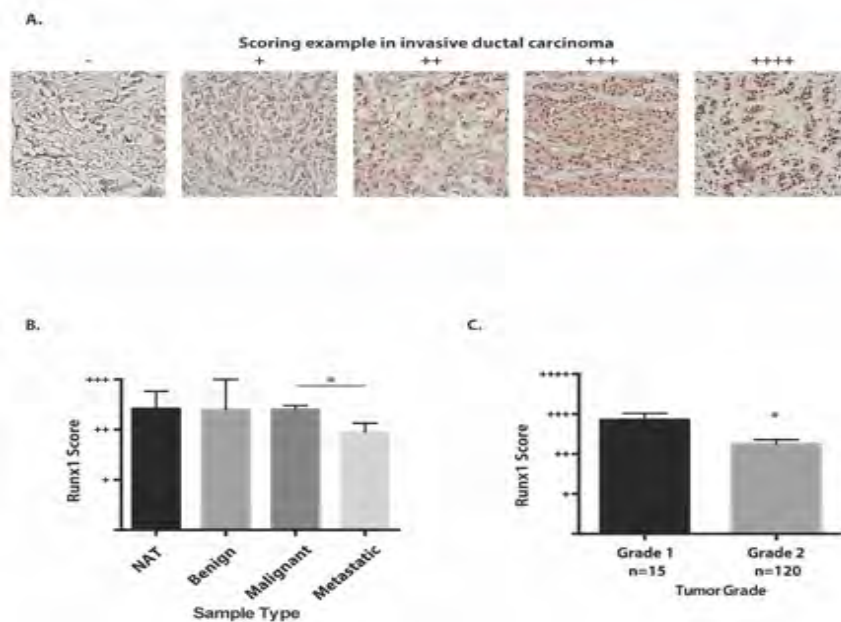
Figure 2.12. RUNX1 tissue microarray show that RUNX1 is associated with early stage tumor. (**A**) Representative tissue microarray images of RUNX1 in invasive ductal carcinoma represent each scoring. (**B**) RUNX1 in scoring in each category including normal adjacent tissue (NAT), fibroadenoma, invasive ductal carcinoma, and tumor metastasis to lymph. (**C**) RUNX1 scoring in grade 1 and grade 2 tumors

2.5 DISCUSSION for Chapter II

Our study has established a crucial role for RUNX1 in maintaining the normal epithelial phenotype. This finding is supported by our demonstration that RUNX1 is decreased during EMT and that loss of endogenous RUNX1 initiates and promotes EMT which is also accompanied by changes in the morphology of mammary epithelial cells. Using two independent methods to induce EMT, either by adding TGF-β or removing required growth factors which increases/activates TGF-β expression, we observed significantly decreased RUNX1 expression. Further, RUNX1 re-expression rescues the epithelial phenotype following TGF-β treatment, which assures maintenance of normal epithelial cell morphology and prevents EMT. By inhibition of RUNX1 in MCF10A (normal) and MCF7 (epithelial-like breast cancer) cells, together with re-expression in MCF10AT1 (malignant cells with low RUNX1 levels), we provide direct evidence that loss of RUNX1 directly contributes to the initiation of EMT in breast cancer, while the presence of RUNX1 restores the epithelial phenotype. Together these findings have revealed, for the first time, that the expression of RUNX1 has a critical function in preserving epithelial morphology in mammary epithelial cells and preventing EMT; thus, RUNX1 can be considered as a transcription factor preventing tumor initiation in normal epithelial cells.

Here we focused our study on normal mammary epithelial and epithelial-like breast cancer cells, and discovered a key function for RUNX1 in preventing EMT. We examined the mechanisms by which RUNX1 regulates EMT in cancer

progression. First, we show RUNX1 is a positive regulator of the epithelial marker E-cadherin. Upon loss of RUNX1, the expression level of E-cadherin is strikingly decreased. We also showed that RUNX1 directly binds to a consensus motif in the E-cadherin gene using ChIP-qPCR. Second, we demonstrate RUNX1 operates downstream of the TGF-β pathway and functions as a suppressor of TGF-β regulation. RUNX1 is well established to mediate TGF-β-BMP signaling by forming co-regulatory complexes with SMADs (Zaidi, Sullivan et al. 2002, Ito and Miyazono 2003). Our RNA-Seq analysis of growth factor-depleted cells suggests that loss of RUNX1 is coupled with activation of the TGF-β pathway. This was confirmed experimentally by showing that RUNX1 is decreased upon TGF-β treatment and RUNX1 reverses TGF-β induced EMT. Supporting these molecular mechanisms, RUNX1 has known properties that establish cell phenotypes, including the hematopoietic lineage (Tober, Yzaguirre et al. 2013), and regulating quiescent hair follicle bulge stem cells to differentiate to early progenitor hair germ cells (Lee, Sada et al. 2014). Very recently RUNX1 was shown to be transiently upregulated early in hESC differentiation to mesendodermal lineages via RUNX1-TGFB2 signaling and that loss of RUNX1 impaired epithelial differentiation (VanOudenhove, Medina et al. 2016). Thus, our studies, which have now identified a cellular function for RUNX1 in normal mammary cells, is consistent with these other normal tissues to support their cell type specific phenotype. We have further studied the consequence of disturbing normal RUNX1 function in breast cancer

cells and provided evidence that RUNX1 loss of function has a significant effect on cancer-related mechanisms.

Repression, overexpression, and/or deregulated functioning of RUNX1 have been shown to cause cancers (Ito, Bae et al. 2015). TGF-β is a well-known EMT inducer and has a dual role in breast cancer progression (David, Huang et al. 2016). In normal epithelial cells and early stage breast cancer, TGF-β acts as a tumor suppressor, yet at later stages of tumor progression can promote cancer cell migration, invasion and metastasis (Padua and Massague 2009). Our results have provided evidence that TGF-β is an upstream regulator of RUNX1. Because RUNX1 is downstream of TGF-β, RUNX1 may also have different functions depending on the specific cellular context (Browne, Taipaleenmäki et al. 2015). For example, while RUNX1 has been shown to function as a tumor suppressor in prostate cancer (Takayama, Suzuki et al. 2015), it acts as an oncogene in ovarian cancer (Keita, Bachvarova et al. 2013) and in a mouse model of breast cancer (Browne, Taipaleenmäki et al. 2015). Our identification of TGF-β as a RUNX1 upstream regulator provides insight into the compromised mechanisms of RUNX1 function that are associated with breast cancer.

RUNX1 is also subject to the hormonal status of cells. Treating ER+ breast cancer cells with 17β-estradiol promotes EMT (Huang, Fernandez et al. 2007) and also decreases RUNX1 expression (Vivacqua, De Marco et al. 2015). In turn, depletion of RUNX1 represses the expression of estrogen receptor α (van Bragt, Hu et al. 2014), suggesting a negative feedback loop in progression of ER+ breast

cancer. Our data show MCF7 ER+ breast cancer cells can be induced into EMT by RUNX1 depletion. One study using computational analysis revealed that RUNX1 is highly correlated with mammary stem cell differentiation (Sokol, Sanduja et al. 2015). Other studies showed that RUNX1 is important for mammary gland maturation, and its interaction with ERα is necessary for luminal development and may prevent breast cancer progression (van Bragt, Hu et al. 2014, Sokol, Sanduja et al. 2015). It also has been shown that RUNX1 represses WNT pathways, which allows ER to be expressed in luminal breast cancer cells (Chimge, Little et al. 2016). All these pieces of evidence raise the hypothesis that RUNX1 could reduce aggressiveness in ER-positive breast cancer; here we clearly demonstrate RUNX1 has a direct role to prevent EMT in MCF7 ER+ breast cancer cells.

In addition to RUNX1-mediated mechanisms downstream of TGF-β (feedback loop) and upstream hormonal regulation of RUNX1, miRNAs are also a likely mechanism contributing to the down regulation of RUNX1 during EMT. MicroRNAs are known to promote/inhibit EMT (e.g., miR-200 family, miR-27 and miR-30) (Zaravinos 2015). Our analysis using TargetScan7.0 indicates that most of these miRNAs also target the RUNX1 3'UTR. It has been shown that miR27a (Tang, Yu et al. 2014), miR144 (Vivacqua, De Marco et al. 2015) and miR387 (Browne, Dragon et al. 2016), which are upregulated during breast cancer progression, are directly down-regulating RUNX1. The convergence of these multiple pathways that inhibit RUNX1 expression leads us to conclude that loss of RUNX1 is an important mechanistic step in breast cancer initiation and/or progression.

Examination of TCGA and other public datasets identified loss of RUNX1 correlates with poor prognosis (Fig. 2.12C) and poor survival (Fig. 2.12D). It has been shown in breast tumors that the majority of EMT markers are expressed in basal layer cells (Sarrio, Rodriguez-Pinilla et al. 2008). Also reported is that basal subtypes of breast cancer are more aggressive and metastatic compared to the luminal subtypes (Kennecke, Yerushalmi et al. 2010). TCGA data show that RUNX1 is expressed at the lowest level in patients with basal-like breast cancer. These findings are consistent with our identification of a RUNX1 function in preserving the epithelial phenotype in normal-like basal cells (MCF10A). Loss of RUNX1 expression may cause the basal cells to lose their epithelial morphology, phenotype integrity and become more susceptible to initiation of EMT. This explains why our functional studies focused on the role of RUNX1 in basal-like mammary epithelial cells (MCF10A).

Intact RUNX1 function is also important for Luminal A breast cancer. Genetic studies show RUNX1 is mutated in 5% of Luminal A subtype breast cancer patients (Banerji, Cibulskis et al. 2012, Network 2012). A recent study suggested that in MCF7 cells, disruption of RUNX1 function might contribute to development of ER$^+$ luminal breast cancer in the context of either *TP53* or *RB1* loss (van Bragt, Hu et al. 2014). Significantly, we demonstrated that loss of RUNX1 in luminal like breast cancer cells (MCF7) can promote EMT (Fig. 2.10). Taken together, these biochemical and clinical data support the emerging concept that RUNX1 reduces

tumor aggressiveness and that loss of RUNX1 is associated with the progression of breast cancer.

Our studies demonstrate a clear reduction of endogenous RUNX1 in two cell models (MCF7 and MCF10AT1) of breast cancer. This finding is consistent with human TMA data that showed the strongest RUNX1 staining (66% strong or moderate levels) in normal cases, compared with 29% and 35% in DCIS and IDC samples, respectively (Sarrio, Rodriguez-Pinilla et al. 2008, Kennecke, Yerushalmi et al. 2010). However, this human data is in contrast to findings in the MMTV-PyMT mouse model of breast cancer (Browne, Taipaleenmäki et al. 2015), where Browne et al. reported that RUNX1 steadily increased during tumor growth. Thus, the decreased RUNX1 in human samples with increased disease progression indicates RUNX1 has distinct functional activities that differ between mouse and human breast tumors.

In conclusion, we identified RUNX1 as a key transcription factor in basal epithelial breast cells through its ability to maintain normal epithelial morphology. Our studies offer RUNX1 as a novel bio-therapeutic molecule for breast cancer intervention.

Chapter III RUNX1 Genome-wide Regulation of Normal Mammary Epithelial Cells: Novel Functions for Mitosis and Genome Stability

A large portion of this chapter comes from the manuscript:

Deli Hong, Andrew J. Fritz, Coralee E. Tye, Natalie A. Page, Joseph R. Boyd Janet L. Stein, Jane B. Lian, Gary S. Stein

RUNX1 Global Binding and Gene Regulation in Mammary Epithelial Cells Revealed Novel Runx1 Mediated Cellular Activities

Contribution: Deli Hong, Jane B. Lian, Janet L. Stein and Gary. S. Stein. conceived and designed the experiments, and analyzed data. Deli Hong performed the majority of the experiments. Andrew J. Fritz built the ChIP-seq library. Coralee E. Tye and Natalie A. Page built the RNA-seq library. Andrew J. Fritz, Coralee E. Tye and Joseph R. Boyd analyzed the RNA-seq and ChIP-seq results. Deli Hong created all the figures. Deli Hong, Jane B. Lian, Janet L. Stein and Gary S. Stein wrote the manuscript.

3.1 Introduction:

RUNX1 belongs to the Runx family of transcription factor that have been known for their function in balancing proliferation and differentiation during development (Ito, Bae et al. 2015). In particular, RUNX1 is essential for hematopoiesis, as Runx1-null mice die between embryonic day (E) 12.5 and E13.5 due to the lack of definitive hematopoiesis (Okuda, van Deursen et al. 1996, Wang, Stacy et al. 1996). The role of RUNX1 in definitive hematopoiesis is to differentiate the hemogenic endothelium cells into hematopoietic stem cells through the endothelial to hematopoietic transition (Yzaguirre, de Bruijn et al. 2017). Disrupting normal RUNX1 function in hematopoietic cells promotes leukemogenesis (Sood, Kamikubo et al. 2017). For example, RUNX1 mutations, including translocations and point mutations, are frequently found in a variety of human hematological malignancies. These mutations function as oncogenes to promote leukemogenesis (Sood, Kamikubo et al. 2017).

In recent years, it has been revealed that the role of RUNX1 is not confined to the hematopoietic lineage. Multiple lines of evidence have emerged demonstrating that RUNX1 plays a key role in epithelial glands and in solid tumors, especially in breast cancer (Scheitz and Tumbar 2013, Riggio and Blyth 2017). Next generation sequencing studies on breast cancer tumor samples have consistently identified RUNX1 point mutations and deletions in human breast cancers, especially in luminal subtypes (Banerji, Cibulskis et al. 2012, Ellis, Ding et al. 2012, Network 2012, Ciriello, Gatza et al. 2015). Moreover, in several studies, RUNX1 mutations

are characterized as cancer driver mutations, which directly contribute to tumor progression (Pereira, Chin et al. 2016, Kas, de Ruiter et al. 2017). In one such study, insertional mutagenesis screening identified that RUNX1 truncation is involved in invasive lobular cancer development (Kas, de Ruiter et al. 2017).

Since RUNX1 mutations have been identified as driver mutations (Pereira, Chin et al. 2016), several studies have examined the function of RUNX1 in breast cancer cells (van Bragt, Hu et al. 2014, Barutcu, Hong et al. 2016, Chimge, Little et al. 2016). These studies have generally found that RUNX1 has a role to reduce aggressive phenotype in luminal subtypes of breast cancer. In ER-positive MCF7 breast cancer cells, RUNX1 contributes to local chromatin interactions, and loss of RUNX1 leads to the deregulation of genes associated with chromatin structure and the activation of an epithelial to mesenchymal transition (Barutcu, Hong et al. 2016, Chimge, Little et al. 2016). Mechanistically, loss of RUNX1 activates WNT signaling by preventing the inhibition of AXIN1 (van Bragt, Hu et al. 2014, Chimge, Little et al. 2016) . Conversely, in MDA-MB-231 triple-negative breast cancer (TNBC) cells, RUNX1 has been shown to have tumor-promoting activity by supporting migration and invasion (Recouvreux, Grasso et al. 2016).

Compared with breast cancer, our understanding of RUNX1 function in normal mammary gland remains inadequate. RUNX1 levels fluctuate during physiological stages of mammary gland development, and in mice the highest level of RUNX1 is observed in virgin and early-pregnant glands (van Bragt, Hu et al. 2014). In the mammary gland, RUNX1 is expressed primarily in the basal layer compared with

the luminal layer (van Bragt, Hu et al. 2014, Rooney, Riggio et al. 2017). Furthermore, depleting RUNX1 in mammary stem cells (MSC) leads to a reduction in luminal MSC and an increase in the basal MSC population (van Bragt, Hu et al. 2014). This spatial/temporal expression pattern suggests that RUNX1 is precisely regulated and that its normal function is necessary for mammary gland development and morphogenesis. Previously, our group has demonstrated that RUNX1 stabilizes mammary epithelial cells by repressing the epithelial to mesenchymal transition (EMT) (Hong, Messier et al. 2017). Loss of RUNX1 induces the initiation of EMT and changes the morphology of the cells. While limited evidence suggests that RUNX1 regulates proliferation and differentiation in mammary epithelial cells (Wang, Brugge et al. 2011, Sokol, Sanduja et al. 2015, Hong, Messier et al. 2017), the precise function(s) of RUNX1 in these cells is (are) unclear.

To better elucidate the function of RUNX1 and the consequences of its loss of expression in mammary epithelial cells, in this chapter, I characterized the gene expression profile of the MCF10A cells with and without RUNX1 expression by RNA-seq analysis. In addition, to gain insight into RUNX1-mediated gene regulation, I determined RUNX1 genomic occupancy by performing RUNX1 ChIP-seq analysis in MCF10A cells. I observed that loss of RUNX1 significantly alters the gene expression pattern and many aspects of cellular activities. ChIP-seq analysis reveals that RUNX1 binding is enriched at promoter regions and miRNA genes. RUNX1 binds to a broad spectrum of up- and down-regulated genes,

suggesting that RUNX1 utilizes different mechanisms to regulate gene expression in normal mammary epithelial cells. I provided evidence that RUNX1 knockdown deregulates mitosis and induces genome instability in mammary epithelial cells. As a result, in this chapter, I provide additional insight into the underlying RUNX1 regulatory mechanisms and the consequences of RUNX1 perturbation in mammary epithelial cells.

3.2. Materials and Methods:

3.2.1 Generation of MCF10A stable cell lines and cell culture

Human breast cancer cell lines MCF10A cells were purchased from ATCC. MCF10A cells were grown in DMEM: F12 (Hyclone: SH30271, Thermo Fisher Scientific, Waltham, MA, USA) with 5% (v/v) horse serum (Gibco: 16050, Thermo Fisher Scientific, Waltham, MA, USA) + 10 µg/ml human insulin (Sigma Aldrich, St. Louis, MO: I-1882) + 20 ng/ml recombinant hEGF (Peprotech, Rocky Hill, NJ, USA: AF-100-15) + 100 ng/ml cholera toxin (Sigma Aldrich: C-8052) + 0.5 µg/ml hydrocortisone (Sigma Aldrich: H-0888) 50 IU/ml penicillin/50 µg/ml streptomycin and 2 mM glutamine (Life Technologies, Carlsbad, CA, USA: 15140-122 and 25030-081, respectively).

Lentivirus generation and infection have been previous described in (Hong, Messier et al. 2017). Lentivirus-based RNAi transfer plasmids with pGIPZ shRunx1 (clone V2LHS_150257 and V3LHS_367631, GE Dharmacon), pGIPZ EV control (Cat No. RHS4351, GE Dharmacon) and pGIPZ non-silencing (Cat No. RHS4346,

GE Dharmacon) were purchased from Thermo Scientific. To generate lentivirus vectors, 293T cells in 10 cm culture dishes were co-transfected with 10 µg of pGIPZ shRunx1 or pGIPZ non-silencing, with 5 µg of psPAX2, and 5 µg of pMD2.G using lipofectamine 2000 reagent (Life Technologies). Viruses were harvested every 48 hr post-transfection. After filtration through a 0.45 µm-pore-size filter, viruses were concentrated by using LentiX concentrator (Clontech, Mountain View, CA, USA). For shRNA-mediated knockdown of RUNX1 expression, MCF10A cells were plated in six-well plates (1×10^5 cells per well) and infected 24 hr later with lentivirus expressing shRunx1 or nonspecific shRNA. Briefly, cells were treated with 0.5 ml of lentivirus and 1.5 ml complete fresh DMEM-F12 per well with a final concentration of 4 µg/ml polybrene. Plates were centrifuged upon addition of the virus at 1460 × g at 37°C for 30 min. Infection efficiency was monitored by GFP co-expression at 2 days post infection. Cells were selected with 2µg/ml puromycin (Sigma Aldrich P7255-100MG) for at least two additional days. After removal of non-viable cells, the remaining attached cells were passed and analyzed.

3.2.2 RNA-seq and analysis

RNA was isolated using DirectZol RNA mini prep kit (Zymo Research), quantified by Qubit HS RNA assay (Thermo Fisher Scientific) and assayed for RNA integrity by Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA was depleted of ribosomal RNA, reverse transcribed and strand-specific adapters added following manufacturer's protocol (TruSeq Stranded Total RNA Library Prep kit with Ribo-Zero Gold, Illumina, San Diego, CA, USA) with the exception that the

final cDNA libraries were amplified using the Real-time Library Amplification Kit (Kapa Biosystems, Wilmington, MA, USA) to prevent over-amplification of libraries. Generated cDNA libraries were assayed for quality then sequenced as single-end 100 bp reads (IlluminaHiSeq1000, UVM Advanced Genome Technologies Core). Sequence files (fastq) were mapped to the most recent human genome (hg38) assembly using TopHat2. Expression counts were determined by HTSeq with recent gene annotations (Gencode v22). Differential expression was analyzed by DESeq2. Correlation between replicates and differential gene expression between time points was assessed by principal component analysis (PCA). In addition, mRNA expression data was uploaded to IPA (www.ingenuity.com) and analyzed using default parameters.

3.2.3 ChIP-seq and analysis

ChIP-seq was performed as previously described (O'Geen, Frietze et al. 2010). We performed independent replicates for MCF10A using 10ul of antibody against RUNX1 (Cell Signaling Technologies, 4334BF, 1ug/ul) and 150ug of chromatin for each sample. Adapters were cut (cutadapt v1.11) and low-quality reads trimmed (Galaxy FASTQ Quality Trimmer 1.0.0; window 10, step 1, minimum quality 20). Reads were mapped to the human genome (hg38 canonical) using STAR version 2.4 (Dobin, Davis et al. 2013) with splicing disabled (–alignIntronMax 1) (Dobin, Davis et al. 2013). Enriched regions (narrowPeak calls) for each replicate were generated using MACS2 (Feng, Liu et al. 2012) and replicates were then evaluated using deepTools (Ramírez, Ryan et al. 2016) to correlate alignments

and IDR (Li, Brown et al. 2011) to evaluate peak call reproducibility. After pooling

replicates, MACS2 (Zhang, Liu et al. 2008) was used to call narrowPeak at high

stringency (P-value <10e-5), these peaks were further filtered according to IDR

cutoffs. FE wiggle tracks were generated using MACS2's bdgcmp and UCSC's

bedGraphToBigwig utility. HOMER motif analysis was used to determine motifs

within 200bp of the peak summits. ChIPBETA (Binding and Expression Target

Analysis) was used to predict targets that are activated or repressed by RUNX1

(Wang, Sun et al. 2013). Gene expression heatmap was generated by web-based

tool Morpheus (https://software.broadinstitute.org/morpheus/). Venn diagrams

were generated by BioVenn (Hulsen, de Vlieg et al. 2008).

3.2.4 Western blotting

Cells were lysed in RIPA buffer and 5X SDS sample buffer supplemented with

cOmplete, EDTA-free protease inhibitors (Roche Diagnostics) and MG132 (EMD

Millipore San Diego, CA, USA). Lysates were fractionated in an 8.5% acrylamide

gel and subjected to immunoblotting. The gels are transferred to PVDF

membranes (EMD Millipore) using a wet transfer apparatus (Bio-Rad Laboratories,

Hercules, CA, USA). Membranes were blocked using 5% Blotting Grade Blocker

Non-Fat Dry Milk (Bio-Rad Laboratories) and incubated overnight at 4°C with the

following primary antibodies: a rabbit polyclonal RUNX1 (Cell Signaling

Technology, Danvers, MA, USA:#4334, 1:1000); a mouse monoclonal to E-

cadherin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA: sc21791, 1:1000);

a mouse monoclonal CDK1 (Santa-Cruz Biotechnology sc-54, 1:1000); a mouse

monoclonal to β-Actin (Cell Signaling Technology #3700, 1:1000), a rabbit polyclonal Tyr15-p-CDK1(Abcam: 47594, 1:1000); a rabbit polyclonal Cyclin B1(Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA: sc752, 1:1000); a rabbit polyclonal Cyclin A (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA: H432, 1:1000); a mouse monoclonal Cyclin E (BD Bioscience, 554183 1:1000), a rabbit polyclonal Bub1 (Cell Signaling Technology, Danvers, MA, USA:#4116s, 1:1000);), a rabbit polyclonal Wee1 (Cell Signaling Technology, Danvers, MA, USA:#4936S, 1:1000); a rabbit polyclonal Cdc25B (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA: SC326, 1:1000); a rabbit polyclonal Cdc25C (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA: SC327, 1:1000);. Secondary antibodies conjugated to HRP (Santa Cruz Biotechnology) were used for immunodetection, along with the Clarity Western ECL Substrate (Bio-Rad Laboratories) on a Chemidoc XRS+ imaging system (Bio-Rad Laboratories).

3.2.5 Immunofluorescence staining microscopy

Cells were fixed with using 3.7% formaldehyde in phosphate buffered saline (PBS) for 10 min. Cells were then permeabilized in 0.1% Triton X-100 in PBS, and washed in 0.5% Bovine Serum Albumin in PBS. Detection was performed using a mouse monoclonal γH2AX antibody (Millipore JBW301). Staining was performed using fluorescent secondary antibodies; for rabbit polyclonal antibodies a goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor® 594 conjugate (Life Technologies A-11062), was used for 1:5000 dilution and 1 hour at 37 °C. Cell were also stained with DAPI (Sigma-Aldrich: D9542-10MG) for DNA content.

3.2.6 Flow cytometry analysis

Cells analyzed by flow cytometry were fixed for 10 minutes in ice cold 70% ethanol for 30 mins before being stained for 30 minutes with an antibody against H3S28P (Alexa fluor 647-conjugated, BD Biosciences, 558609). Cells were then suspended in 2% FBS in PBS and stained with Propidium iodide (PI) (BD Pharminge 550825) for 15 minutes to determine DNA content. Flow cytometric analysis was performed using the LSRII instrument (BD Biosciences). FlowJo (Ashland, OR, http://www.flowjo.com/) version 10 was used to display DNA histograms and to determine the percent of cells positive for H3S28P, a marker of mitosis, within the cycling cell populations.

3.3 Results:

3.3.1. RUNX1 knockdown in normal-like mammary epithelial cells results in aberrant gene regulation

To investigate the role of RUNX1 in normal mammary epithelial cells, we used previously described normal-like mammary epithelial MCF10A cells stably expressing either control (non-silencing shRNA control (NS), empty vector control (EV)), or two different shRNAs against RUNX1 (shRunx1-1 and shRunx1-2) (Hong, Messier et al. 2017). I confirmed the down-regulation of RUNX1 at the protein level by Western blot analysis (Fig 3.1A), and then performed RNA-seq analysis using above cell lines and validated the quality of the RNA-seq results by principal component analysis (Figure 3.1B). Two of the control cell lines (NS and EV) form

a cluster, which is more similar to the parental MCF10A cells than the cluster formed by two RUNX1 knockdown cell lines (shRunx1-1 and shRunx1-2). Heatmap of gene expression shows the results of three replicates within each condition. (Figure 3.1C) The reproducibility suggests the quality of these RNA-seq libraries will enable identification of genes differentially expressed upon RUNX1 depletion.
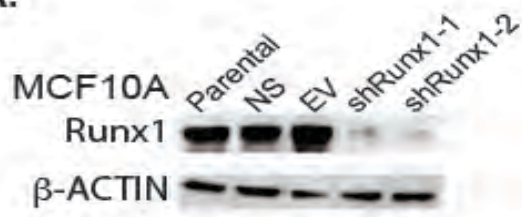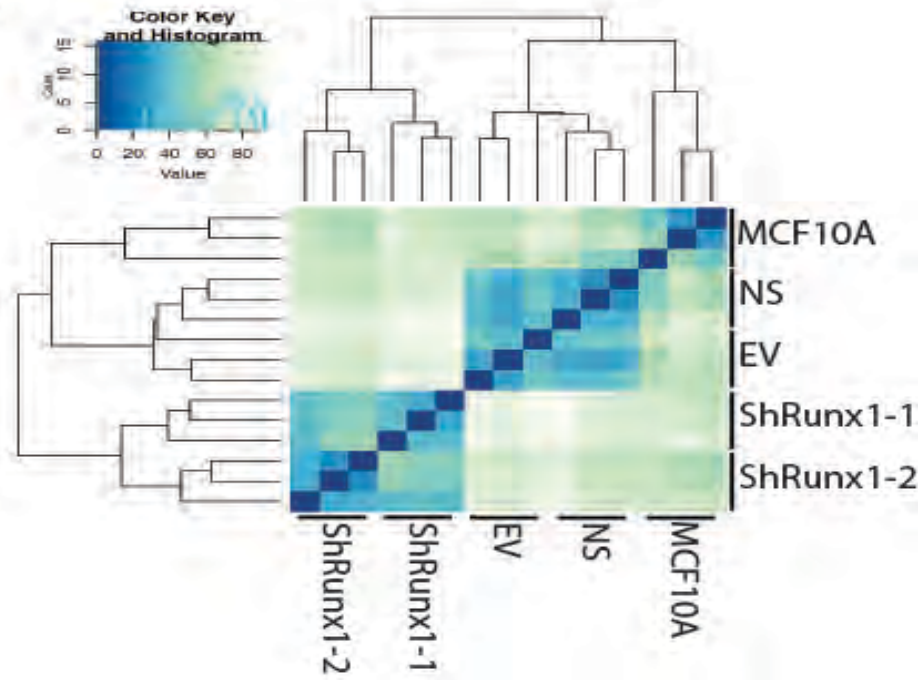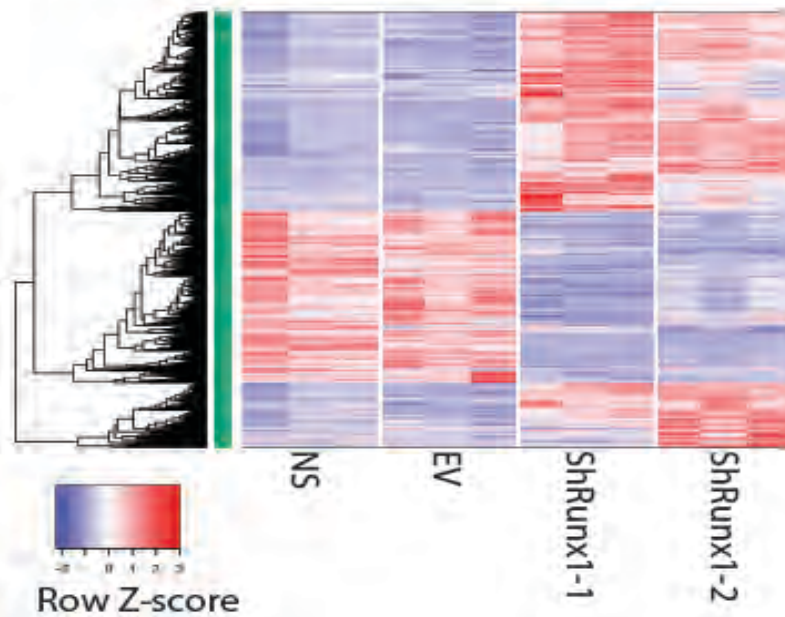
Figure 3.1 RNA-seq in RUNX1 depleted MCF10A cells. (**A**) Western

blot analyses of lysates from MCF10A cells treated with shRunx1 show

decreased protein expression of RUNX1. The experiment is performed 3

times (N=3). (**B**) Sample-to-sample distances. Heatmap showing the

Euclidean distances between the samples as calculated from the

regularized log transformation. (**C**) Heatmap showing the transcripts per

million (TPM) expression values of the differentially expressed gene

replicates. Samples were calculated from the regularized log

transformation.

 Notably, from the initial assessment of RNA-seq data, we found several

mesenchymal markers including N-cadherin (CDH2), Fibronectin 1 (FN1) and

Matrix metallopeptidase 13 (MMP13) significantly up- regulated upon depletion of

RUNX1 (Figure 3.2). These findings are consistent with our previous reports that

loss of RUNX1 initiates EMT in MCF10A cells (Chapter II). We next delineated the

differentially expressed genes between the two control cell lines (NS, EV) and two

shRunx1 (shRunx1-1, shRunx1-2) in MCF10A cells. Differentially expressed

genes were defined as those with at least a 2-fold change within all 4 groups (EV

vs shRunx1-1; EV vs shRunx1-2; NS vs shRunx1-1; NS vs shRunx1-2) (Fig. 3.3A,

B). Overall, we identified 1209 up- and 660 down- regulated genes upon RUNX1

depletion in MCF10A cells (Fig. 3.3A, B).
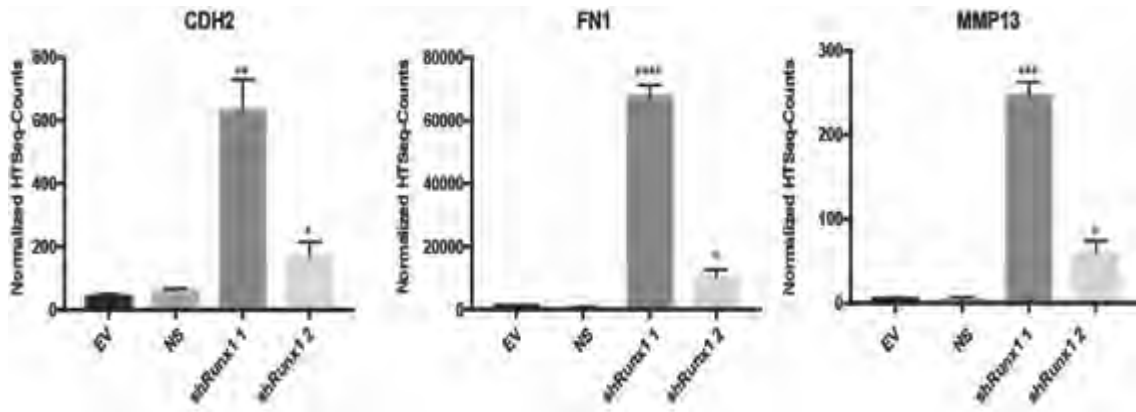
Figure 3.2 The expression of mesenchymal genes is increased in RUNX1 depleted MCF10A cells. RNA-seq analysis of MCF10A cells treated with shRunx1 shows increased gene expression of CDH2, FN1 and MMP13. Student's t test * p value <0.05, ** p value <0.01, *** p value <0.001, **** p value <0.0001 for MCF10A shRunx1 cells compared to the MCF10A NS cells. Error bars represent the standard error of the mean (SEM) for the three biological samples.

A. Defining Up-regulated Genes

2078 D.E. Genes — 1892 D.E.Genes
EV / shRunx1-1 — EV / shRunx1-2

1464

2261 D.E. Genes — 2047 D.E. Genes
NS / shRunx1-1 — NS / shRunx1-2

1637

1464 D.E. Genes — 1637 D.E. Genes
within EV — within NS

1209

B. Defining Down-regulated Genes

1423 D.E. Genes — 1091 D.E.Genes
EV / shRunx1-1 — EV / shRunx1-2

836

1513 D.E. Genes — 1067 D.E. Genes
NS / shRunx1-1 — NS / shRunx1-2

849

836 D.E. Genes — 849 D.E. Genes
within EV — within NS

660

Figure 3.3 **Defining differentially expressed genes in RUNX1 knockdown in MCF10A cells showing in Venn diagram**. (**A**) Left: Genes that are upregulated (> 2-fold; $p<0.05$) in shRunx1-1 and shRunx1-2 cells compared to EV control. Middle: Venn diagram showing genes that are upregulated (> 2-fold; $p<0.05$) in shRunx1-1 and shRunx1-2 cells compared to NS control. Right: upregulated genes identified between EV control and NS control. (**B**) Left: Genes that are downregulated (> 2-fold; $p<0.05$) in shRunx1-1 and shRunx1-2 cells compared to EV control. Middle: Venn diagram showing genes that are downregulated (> 2-fold; $p<0.05$) in shRunx1-1 and shRunx1-2 cells compared to NS control. Right: downregulated genes identified between EV control and NS control.

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To elucidate the cellular consequence of RUNX1 depletion in MCF10A cells, we performed Ingenuity Pathway Analysis (IPA) to identify pathways altered upon RUNX1 loss (Fig. 3.4). Several pathways involved in growth factor signaling-such as FGF signaling, HGF signaling and PDGF signaling-are activated upon the loss of RUNX1, suggesting RUNX1 is necessary for normal cell growth in MCF10A cells (Fig. 3.4A). Activation of other pathways-such as NF-κB signaling, Lymphotoxin β Receptor signaling and FcγRIIB signaling in B-lymphocytes, implies that RUNX1 is involved in cellular inflammation and immune response. It has been demonstrated that downregulation of RUNX1 activates the NF-kB pathway in both myeloid tumor and gastric cancer cells (Nakagawa, Shimabe et al. 2011, Wu, Zhang et al. 2017). The top up-regulated pathways in this analysis suggest the involvement of RUNX1 of inflammation in mammary tissue. We also found that multiple pathways linked to cell cycle regulation, including cyclins and Cell Cycle Regulation, Cell Cycle Regulation by BTG (B-cell translocation gene 2) and Mitotic Roles of Polo-like Kinase, are decreased in RUNX1-depleted cells (Fig. 3.4B). Moreover, many pathways related to breast cancer progression, for instance Hereditary Breast Cancer Signaling, Her-2 Signaling in Breast Cancer, and Breast Cancer Regulation by Stathmin1, are drastically altered upon loss of RUNX1, providing evidence that RUNX1 is involved in breast cancer biology (Fig 3.4C).

Taken together, these results suggest that RUNX1 acts as a master transcriptional regulator in mammary epithelial cells, controlling the expression of nearly 1,900 genes. Loss of RUNX1 disturbs many aspects of cellular activities,

including cell cycle and cell growth, response to inflammation and immune stress and breast cancer progression.
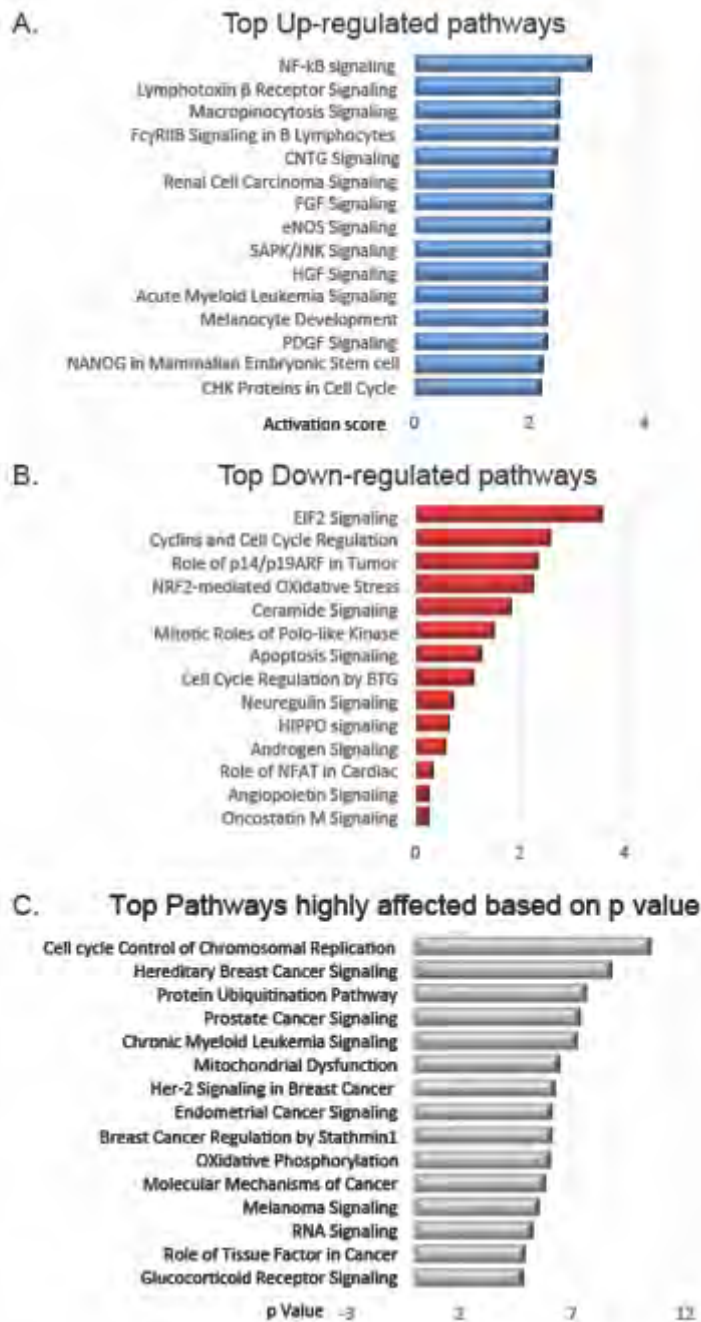
Figure 3.4 IPA canonical pathway analyses from each tier of core analysis. (**A**) Pathways upregulated in response toRUNX1 knockdown in MCF10A cells. (**B**) Down-regulated pathways in RUNX1 knockdown in MCF10A cells. (**C**) Top pathways based on p values, which are highly altered upon RUNX1 depletion in MCF10A cells. The X axis represents negative log p values based on the probability that molecules in the uploaded dataset were included in the predefined IPA canonical pathways by true association as opposed to inclusion of molecules based on chance alone. Only the top 15 pathways in each category with the largest negative log p values are shown.

3.3.2. RUNX1 ChIP-seq analysis identifies enriched binding at promoters

To determine whether the differences in gene expression in RUNX1 depleted cells are directly related to RUNX1 binding, we performed RUNX1 ChIP-Seq in the parental MCF10A cells and identified 11969 reproducible peaks of RUNX1 binding. Next, we investigated the distribution of RUNX1 binding sites across eight different categories of genomic elements including promoter, exon, intron, intergenic, 5'UTR, 3'UTR, TSS and pseudo gene regions by mapping RUNX1 sites to the annotated genes. The annotation of these RUNX1 binding sites revealed that majority of the RUNX1 bindings are within intergenic regions (46%) and introns

(42%), and only 8% of the peaks are located within promoter regions (Fig 3.5 A). However, after normalizing the peaks based on the frequency of those elements in the genome, we observed that RUNX1 peaks are specifically enriched in promoter and 5'UTR regions of protein coding genes, as well as miRNA genes (Fig 3.5 B, C). The binding of RUNX1 within promoters and 5'UTRs is consistent with the role of RUNX1 as a transcription factor, which validate further the quality of our ChIP-seq analysis. Notably, we also observed significant binding of RUNX1 to miRNA genes suggesting that RUNX1 is involved in miRNA biogenesis in mammary epithelial cells.

We next performed de novo motif analysis on these RUNX1 ChIP-seq peaks (Fig. 3.5D). The most significantly enriched motif was the RUNX1 motif itself (Fig. 3.5D), validating the quality of the RUNX1-ChIP-seq data. Moreover, we identified additional binding motifs close to the RUNX1 binding site including AP1, TEAD4 and STAT5 which are known to form transcription complexes with RUNX1 (Fig. 3.5D) (Ogawa, Satake et al. 2008, Pencovich, Jaschek et al. 2011, Li, Wang et al. 2016, Obier, Cauchy et al. 2016). Additional several functional motifs that were not previously associated with RUNX1, such as ZFP410, BCL6B, NFIA and TFAP2B, are also present in the analysis suggesting that they might be part of RUNX1-mediated gene regulation (Fig. 3.5D). Overall, our motif analysis indicates a complex regulatory network for RUNX1 that includes interactions with other transcription factors.
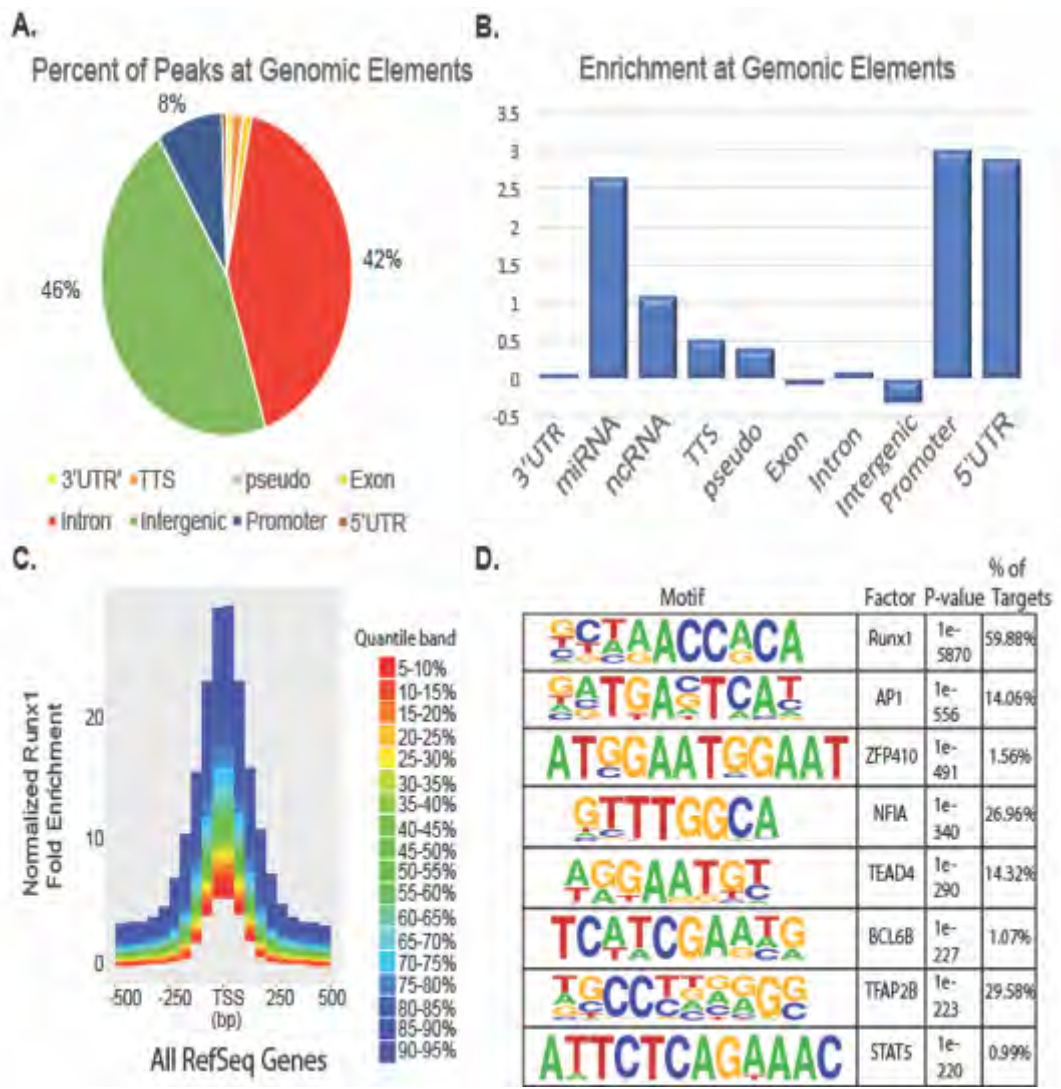
Figure 3.5 RUNX1 ChIP-seq in parental MCF10A cells. (**A**) Pie chart showing the distribution of RUNX1 ChIP-seq peak annotation. (**B**). The enrichment of RUNX1 ChIP-seq peak annotation. (**C**) Normalized RUNX1 ChIP-seq signal intensity plot for all human UCSC genes ± 2 kb. (**D**) HOMER de novo motif analysis of the RUNX1 peaks. The motifs are ordered by significance from top to bottom.

3.3.3. RUNX1 binds to up- or down-regulated genes

Next, we asked whether RUNX1 binding was associated with differentially expressed genes. To address this question, we analyzed the RUNX1 peak frequency at the differentially expressed genes; although we determined that approximately 90% of the differentially expressed genes harbor RUNX1 binding within 100 kb of their TSS, only 20% of these genes have RUNX1 binding at their promoters (0-1kb to TSS) (Fig. 3.6A). These data indicate RUNX1 employs multiple mechanisms to regulate gene expression, either directly binding to the promoter region or binding to the distal regulatory loci. We further analyzed RUNX1 regulatory mechanism by using ChIP-Binding and Expression Target Analysis (ChIP-BETA analysis), which predicts whether RUNX1 has activating or repressive function. ChIP-BETA analysis showed that down-regulated genes are directly associated with RUNX1 depletion (Fig. 3.6B). These data suggest that the primary function of RUNX1 is to activate gene expression and RUNX1 represses gene expression mainly in an indirect manner.

A. Down Regulated Genes (93.2% within 0-100kb); Up Regulated Genes (88.86% within 0-100kb); Unregulated Genes (88.05% within 0-100kb). Percentage of differentially expressed genes vs Distance to TSS (kb)

B. Static (Background); Down-regulated Genes (1.84e-12); Up-regulated Genes (1). Cumulative Fraction of Genes% vs Rank of genes based on Regulatory Potential Score (from high to low)
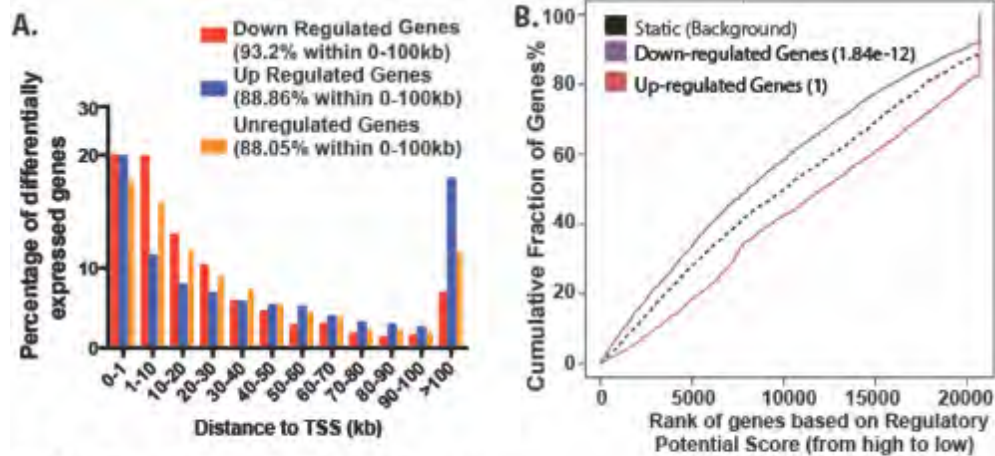
C. Motif on Down Regulated Genes

Motif	Factor	P-value	% of Targets
	Runx	1e-86	32.57%
	ELK1	1e-42	22.22%
	NFY	1e-25	23.18%
	KLF4	1e-22	14.18%
	PB0076.1	1e-19	53.64%
	E2F	1e-18	54.41%

D. Motif on Up Regulated Genes

Motif	Factor	P-value	% of Targets
	GAPBR	1e-42	31.25%
	SP2	1e-36	33.78%
	POL004	1e-26	15.18%
	GMEB2	1e-23	36.46%
	GFY	1e-22	5.80%
	Runx	1e-20	30.95%

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Figure 3.6 RUNX1 regulates up- and down- regulated genes in a different pattern. (**A**) Bar graph showing RUNX1 peak binding within ± 100 kb of transcriptional start site (TSS), or > 100 kb of the gene bodies of up- and down-regulated genes or non-differentially expressed genes. (**B**) ChIP-BETA activating/repressive function prediction of the RNA-seq and RUNX1 ChIP-seq data set identified from the RUNX1 knockdown compared with NS control. The red and the purple lines represent upregulated and downregulated genes, respectively. The dashed line indicates the non-differentially expressed genes as background. Genes are cumulated by rank on the basis of their regulatory potential score from high to low. P-values represent significance comparing up- or down-regulated group distributions with the non-differentially expressed group by the Kolmogorov-Smirnov test. (**C**) HOMER de novo motif analysis of the RUNX1 peaks in down-regulated genes. The peaks are ordered by significance from top to bottom. (**D**) HOMER de novo motif analysis of the RUNX1 peaks in up-regulated genes. The peaks are ordered by significance from top to bottom.

Motif analysis on differentially expressed genes also illustrates distinctive motif patterns among up- and down-regulated genes, even though RUNX1 binding is detected in a similar percent of targets (Fig. 3.6C, D). For down-regulated genes,

the top motif is Runx itself, suggesting direct binding (Fig. 3.6C). However, for up-regulated genes, the Runx motif is not the most significant motif; these results suggest RUNX1 represses genes in an indirect manner (Fig. 3.6D). Moreover, besides the Runx motif, no other motif is shared between up- and down-regulated genes, indicating that RUNX1 may utilize distinct mechanisms to activate or repress gene expression (Fig. 3.6C, D). We also performed the motif analysis at the promoter regions of the genes, which expression are not changed upon loss of RUNX1. The results showed that Runx motif is still the most significant motif at the promoter (Fig. 3.7). This specific binding suggests RUNX1 has the potential to regulate those genes in other cellular contexts.

Overall, the RUNX1 binding pattern and motif analysis are consistent with the engagement of RUNX1 in both transcriptional activation and repression. Furthermore, it is the first time showing that RUNX1 may utilize different mechanisms to control target gene activation and repression.

Motif	Factor	P-value	% of Targets
	Runx2	1e-735	50.01%
	ELK3	1e-149	22.63%
	POL003.1	1e-98	31.18%
	NFY	1e-79	17.05%
	GFY	1e-56	4.59%
	KFL4	1e-44	9.74%
	CREB	1e-41	14.10%
	RFX1	1e-39	8.30%

Figure 3.7 HOMER de novo motif analysis of the RUNX1 peaks in un-differentially expresses genes. The peaks are ordered by significance from top to bottom.

3.3.4. Loss of RUNX1 affects cell cycle-related genes

From pathway analysis, we discovered that many pathways related to cell cycle regulation were altered upon loss of RUNX1 (Fig.3.4). Therefore, we hypothesized that loss of RUNX1 dysregulates the expression of cell cycle genes and thus influences the overall cell cycle. To test this hypothesis, we first generated an expression heatmap for the cell cycle related genes using normalized counts from the RNA-seq data (Fig 3.8A). From the heatmap, we observed that there are no consistent patterns associated with G1 phase-related genes or G2 phase-related genes (Fig 3.8A). However, the expression of genes linked to S phase and DNA replication is severely down-regulated upon loss of RUNX1 (Fig 3.8A). Decreased expression of S phase genes is consistent with previous reports that RUNX1 is necessary for acceleration of the G1/S transition and that RUNX1 promotes proliferation in mesenchymal stem cells (Bernardin-Fried, Kummalue et al. 2004, Kim, Barron et al. 2014). We also observed that genes related to mitosis, such as Cyclin B1 and Cyclin-dependent kinase 1 (CDK1), are down-regulated in RUNX1 knockdown cells (Fig 3.8A). The key event that initiates mitotic entry is the activation of the Cyclin B1-CDK1 complex by increasing Cyclin B1 expression and of inactivate p-CDK1(Thr14/Tyr15) by dephosphorylation (Malumbres and Barbacid 2009). To validate the RNA-seq data, we performed western blot analysis on proliferating cells to determine the protein levels of these cell cycle genes and the phosphorylation state of CDK1 in RUNX1 knockdown MCF10A cells (Fig 3.8B). We observed that the level of Cyclin A is increased while Cyclin E

remains unchanged upon loss of RUNX1. Consistent with RNA-seq data, the level

of Cyclin B1 is decreased with RUNX1 knockdown. Although total CDK1 protein

level does not decrease as dramatically as was observed in the RNA-seq data, the

level of phospho-CDK1 (Tyr 15), which is the inactive form of CDK1, accumulates

in RUNX1-depleted cells (Fig 3.8B).   With the lower level of Cyclin B and the

increased level of pTyr15-CDK1, we hypothesized that RUNX1 is necessary for

G2/M transition and mitotic entry in MCF10A cells. However, cell cycle profiling

showed that RUNX1 knockdown has no significant impact on overall cell cycle (Fig.

3.8C upper and middle). We observed only very mild and not significant increase

in the G2 population in two shRunx1 cell lines compared with NS control cells (Fig.

3.8C bottom).
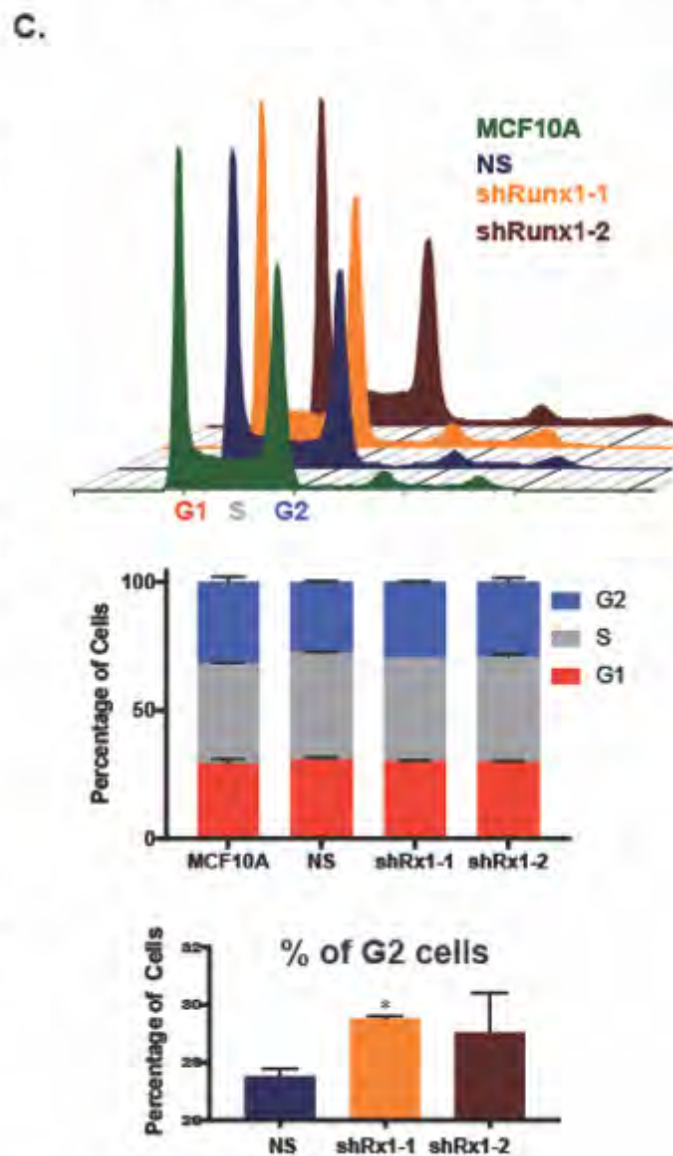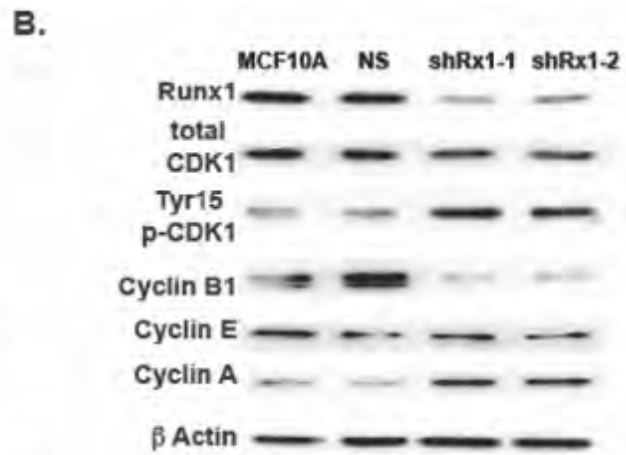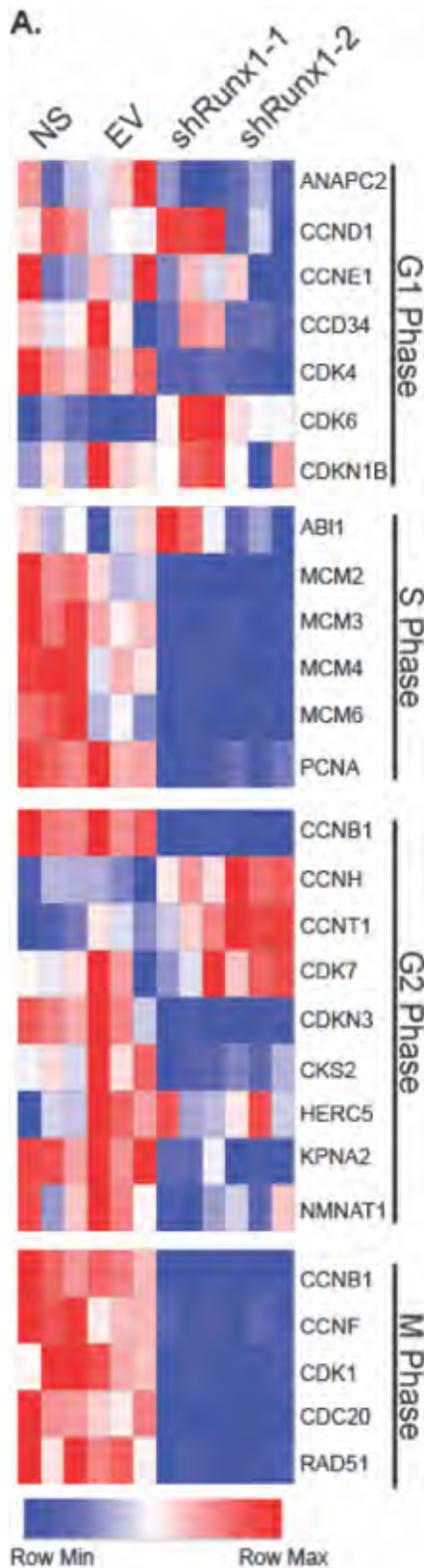
Figure 3.8 RUNX1 alters the expression of cell cycle genes. (**A**) Heat map of relative expression from RNA-seq data of cell cycle-related genes in MCF10A control (EV, NS) and shRunx1 (shRunx1-1, shRunx1-2) cells. (**B**) Western blot analyses of whole cell lysates from MCF10A cells with RUNX1 depletion show decreased protein expression of RUNX1 and alteration of protein expression of cell cycle related genes. Tyr15 pCDK1: Phospho-CDk1 (Tyr15). The experiment is performed 3 times (N=3). (**C**) Top: Histogram plots of cell cycle profiles of control and RUNX1-depleted MCF10A cells were obtained by FACS analysis of propidium iodide (PI)-stained cells. Middle: The cell cycle distribution plotted as a bar chart. Columns, mean; Error bars, SEM, from three independent experiments. Bottom: Percentage of cells in G2 phase were plotted. Student's t test * p value <0.05.

3.3.5. Loss of RUNX1 decreases the proportion of mitotic cells.

Although we did not observe a significant change in overall cell cycle in RUNX1-depleted MCF10A cells, we explored the explanation for the decreased level of Cyclin B1 and the accumulation of Tyr15-p-CDK1. Therefore, we tested whether loss of RUNX1 specifically affects mitosis and performed flow cytometry analysis on the MCF10A cells labeled with the mitotic-specific marker H3S28P. We observed an over 40% decrease in the mitotic population, suggesting RUNX1 is required for mitosis (Fig.3.9A).

Interestingly, from RNA-seq data, the RNA levels of several components of the mitotic checkpoint complex (MCC), including Bub1, Bub1b and MAD2L1, are significantly decreased upon loss of RUNX1 (Fig 3.9B, C, Fig 3.10A). Moreover, ChIP-seq data also reveal that RUNX1 binds to their promoters, indicating a direct regulation by RUNX1(Fig 3.9D, Fig 3.10B). Previously it has been reported that in leukemia cells, a RUNX1 mutant abrogates mitotic checkpoints by targeting the MCC component MAD2L1 (Krapf, Kaindl et al. 2010). Here, we show that the native form of RUNX1 is a direct activator of several MCC components, including BuB1, BuB1b and MAD2L1, highlighting the importance of RUNX1 during mitosis. Further exploration will be required to elucidate the precise function of RUNX1 during mitosis.
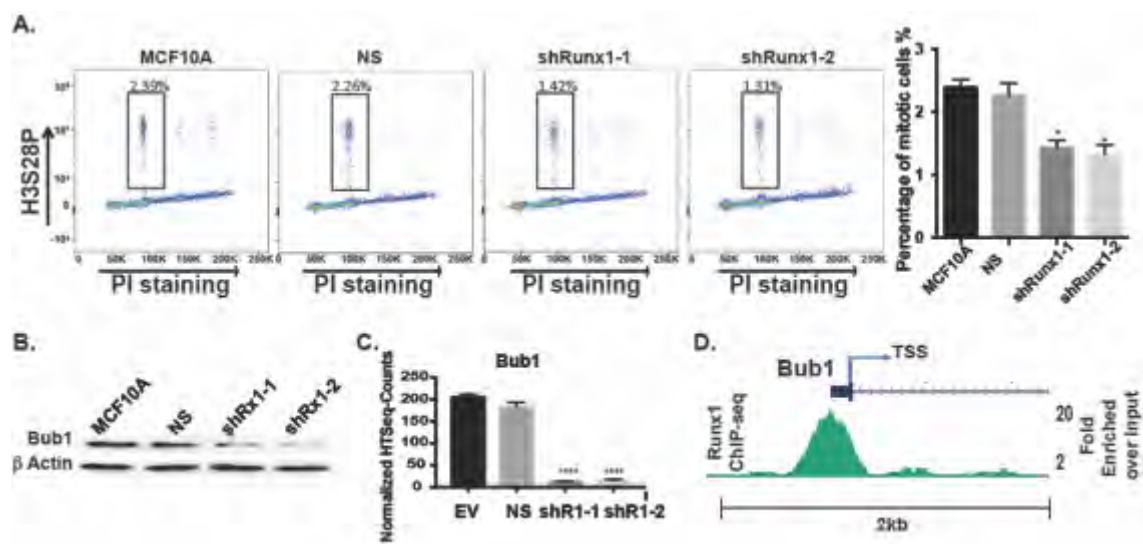
Figure 3.9 Loss of RUNX1 reduces the mitotic population. (**A**) Representative flow cytometric analysis of control and RUNX1 depleted MCF10A cells with H3S28P versus DNA content (PI staining). The percentage of mitotic cells is indicated above the rectangles. The cells below the rectangles are the non-mitotic cells. Right: Bar graph of mitotic population in each condition. Error bars represent the standard error of the mean (SEM) from three biological samples. Student's t test * p value <0.05. (**B**) Western blot analyses of whole cell lysates from MCF10A cells with RUNX1 depletion show decreased protein expression of Bub1. The experiment is performed 3 times (N=3). (**C**) RNA-seq analyses from MCF10A cells treated with shRunx1 show increased gene expression of Bub1. Error bars represent the standard error of the mean (SEM) from three biological samples. Student's t test **** p value <0.0001. (**D**) ChIP-seq genome browser view of RUNX1 binding near the transcription start site (TSS) of Bub1 gene.
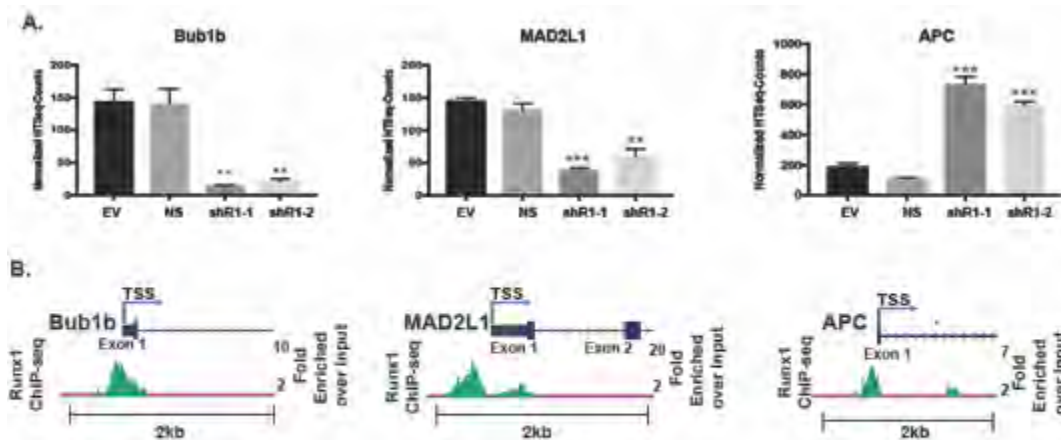
Figure 3.10 RUNX1 is a direct regulator of Bub1b, MAD2L1 and APC.

(**A**) RNA-seq analyses from MCF10A cells treated with shRunx1 show decreased gene expression of Bub1b and MAD2L1, and increased gene expression of APC. Student's t test ** p value <0.01, *** p value <0.001, for MCF10A shRunx1 cells compared to the MCF10A NS cells. Error bars represent the standard error of the mean (SEM) from three biological samples. (**B**) ChIP-seq genome browser views of RUNX1 binding at the transcription start site (TSS) of Bub1b, MAD2L1 and APC gene.

3.3.6. Loss of RUNX1 decreases genomic stability

It has been demonstrated that loss of Bub1 and the mitotic checkpoint complex is associated with genome instability (Baker, Jin et al. 2009). Upon loss of RUNX1, we observed activation of genes that sense DNA damage, such as ATM and Rad50, and the decreased expression of DNA repair-related genes, such as PARP1 and members of Fanconi anemia proteins (Fig. 3.11A). Therefore, we hypothesized that loss of RUNX1 induces genome instability in MCF10A cells. To test this hypothesis, we stained the cells with the DNA damage marker γH2AX, and observed no differences between RUNX1-depleted cells and control cells (Fig. 3.11B left). However, when comparing the DNA damage response after treating cells for 4 hrs with 5μg/ml bleomycin, which induces double-strand breaks, RUNX1 knockdown cells displayed a pronounced delay of DNA repair after 24 hrs of induced DNA damage (Fig. 3.11B middle and right).

Therefore, the alteration of the genes associated with DNA damage (Fig. 3.11A) and repair and the delay of the DNA repair process (Fig.3.11B) demonstrate that RUNX1 knockdown cells exhibit the feature of genomic instability. We propose that the enhanced propensity of RUNX1 depleted cells to acquire chromosomal abnormalities may increase the potential of developing a cancer phenotype. These findings indicate that loss of RUNX1 is accompanied with genome instability, which is consistent its role to preserve the normal phenotype in mammary epithelial cells.
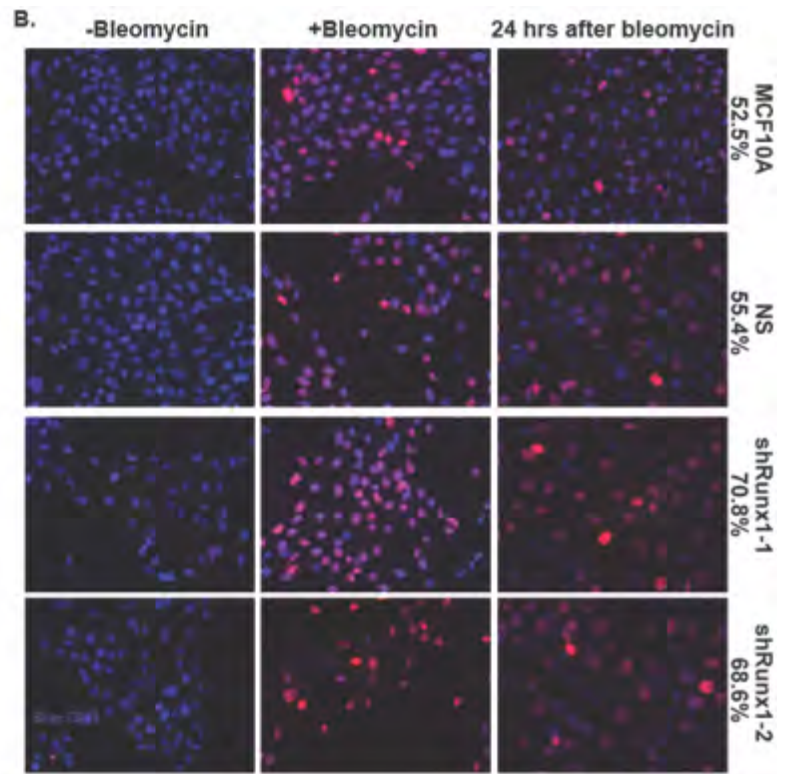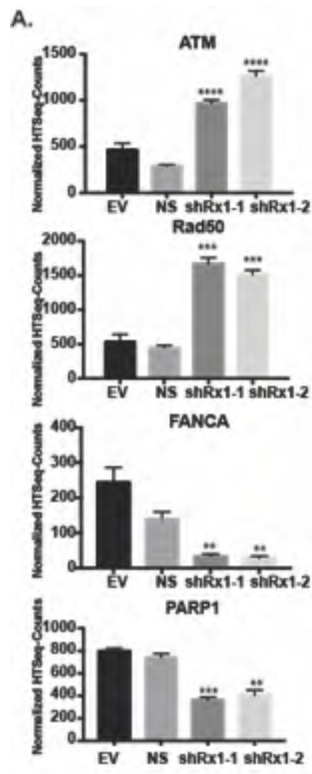
Figure 3.11 Loss of RUNX1 slows DNA repair. (**A**) RNA-seq analyses of RNA from MCF10A cells with shRunx1 show increased gene expression of DNA damage sensing genes such as ATM and Rad50, and decreased gene expression of DNA repair genes such as FANCA and PARP1. Student's t test ** p value <0.01, *** p value <0.001, **** p value <0.0001 for MCF10A shRunx1 cells compared to the MCF10A NS cells. Error bars represent the standard error of the mean (SEM) from three biological samples. (**B**) Representative images of γH2AX foci in untreated cells, the cells treated for 4hr with 5μg/ml bleomycin, and the cells stained 24h after bleomycin treatment. Blue: DAPI staining; Red: γH2AX. All the experiments are performed 2 times (N=2).

3.4. Discussion:

The transcription factor RUNX1 is well known for its function in hematopoiesis and its involvement in leukemogenesis (de Bruijn and Dzierzak 2017, Sood, Kamikubo et al. 2017). In the past few years, using deep-sequencing technology, RUNX1 has been identified as one of the frequently mutated genes in breast cancer patients along with other well-studied tumor suppressors such as P53, PTEN and RB1 (Banerji, Cibulskis et al. 2012, Ellis, Ding et al. 2012, Network 2012, Ciriello, Gatza et al. 2015). Although multiple lines of evidence support the concept that impaired RUNX1 function in normal mammary epithelial cells promotes breast cancer initiation and progression, the mechanism(s) of RUNX1-mediated gene expression

in this cell lineage remain(s) unknown. In this chapter, we delineated the molecular consequences of RUNX1 loss in MCF10A cells and examined RUNX1 cellular functions. We also examined how loss of RUNX1 contributes to the onset and progression of breast cancer.

We investigated RUNX1-mediated genome-wide transcriptional regulation in normal-like mammary epithelial MCF10A cells. Loss of RUNX1 expression in MCF10A cells alters the expression of approximately 2,000 genes and the pathway analysis on these differentially expressed genes revealed that RUNX1 is involved in multiple aspects of cellular activities. For instance, RUNX1 is involved in cell proliferation by activating cell cycle-related pathways. RUNX1 is also involved in cellular stress response by repressing several pathways related to immune or inflammation response. Combining RUNX1 ChIP-seq data in MCF10A cells and RNA-seq data in RUNX1 depleted cells, we observed that RUNX1 employs multiple mechanisms to regulation its target genes. We further demonstrated that loss of RUNX1 alters mitosis in mammary epithelial cells. Depleting RUNX1 resulted in a reduced mitotic cell population and decreased expression of several components of the mitotic checkpoint complex. Moreover, loss of RUNX1 increased genome instability as DNA repair is slowed in RUNX1-depleted cells. Overall, our results highlight the importance of RUNX1 in mammary epithelial cells. Loss of RUNX1 alters the expression of many genes and various aspects of cellular function and thus affect normal cell growth and may lead to genome instability.

Previously, it has been well documented that RUNX1 regulates its target gene expression by binding to a well-defined Runx consensus sequence located within promoter or enhancer elements (Meyers, Downing et al. 1993, Otto, Lübbert et al. 2003). Now, additional lines of evidence suggest that RUNX1 regulates gene expression in a more complex manner, which encompasses multiple regulatory layers involving interaction with other co-factors or transcription factors, distal regulatory elements and epigenetic factors (Elagib, Racke et al. 2003, Reed-Inderbitzin, Moreno-Miralles et al. 2006, Huang, Yu et al. 2009, Bowers, Calero-Nieto et al. 2010, Phillips, Taberlay et al. 2017). For instance, in leukemia cells, RUNX1 regulates the expression of two integrins in different manners (Phillips, Taberlay et al. 2017). It regulates ITGA6 gene by directly binding to the consensus motif in its promoter (Phillips, Taberlay et al. 2017). In contrast, RUNX1 regulates ITGB4 gene expression in a more complex manner, as it activates the ITGB4 promoter without binding to the RUNX1 consensus motif (Phillips, Taberlay et al. 2017). Therefore, RUNX1 can utilize different mechanisms to regulate gene expression. Consistently, from our RUNX1 binding site analysis using ChIP-seq, we observed that RUNX1 might employ different mechanisms for up or down-regulated genes. ChIP-BETA analysis revealed that the primary function of RUNX1 is to directly activate gene expression. The exact mechanism(s) explaining RUNX1-mediated fine-tuning of transcription control remains to be determined. We propose that RUNX1, based on cellular content, either directly binds to target gene promoters to support competency for transcription regulation or RUNX1 scaffolds

with the other co-activator(s)/repressor(s) at distal loci. Further studies will be critical to elucidate these roles and specify the altered protein-protein interactions that affect RUNX1 function in different cellular contexts.

From RUNX1-ChIP-seq results, we observed that RUNX1 binding is enriched at miRNA and other non-coding RNA genes in MCF10A cells (Fig. 3.5 B). RUNX1 is well known as a hub of miRNA biogenesis in both normal hematopoiesis and in leukemic cells (Rossetti and Sacchi 2013). RUNX1 expression is not only controlled by hematopoietic transcription factors such as GATA2, ETS and RUNX1 itself (Nottingham, Jarratt et al. 2007, Pimanda, Donaldson et al. 2007), but also by an increasing number of miRNAs (Rossetti and Sacchi 2013). Using bioinformatics tools such as TargetScan, more than 60 conserved miRNAs with potential binding to the RUNX1 3'UTR have been predicted (Rossetti and Sacchi 2013). Many of them, such as miR-17, miR-20a and miR-27, have been validated experimentally (Fontana, Pelosi et al. 2007, Ben-Ami, Pencovich et al. 2009). RUNX1 also controls miRNA gene expression by binding to the Runx consensus sequences in miRNA regulatory regions. From RUNX1-ChIP-seq data in hematopoietic cells, RUNX1 physically binds over 200 miRNA genes including the above-mentioned miR-17 and miR-27 (Ptasinska, Assi et al. 2012, Wu, Seay et al. 2012). In fact, the feed-back regulatory loops between RUNX1 and miRNAs are essential for hematopoietic differentiation and proliferation (Mi, Li et al. 2010). The enrichment of RUNX1 on miRNA genes in MCF10A cells suggests that RUNX1 may also regulate the expression of miRNAs in mammary epithelial and breast

cancer cells. Currently, in those mammary lineages, studies have only focused on identifying the miRNAs targeting RUNX1 stability, such as miR-378 and miR-144 (Vivacqua, De Marco et al. 2015, Browne, Dragon et al. 2016). Therefore, further exploration of the overlap between miRNA expression arrays in RUNX1-depleted cells and RUNX1 ChIP-seq data from this chapter will be useful in identifying miRNAs regulated by RUNX1.

For a long time, RUNX1 was postulated to control cell cycle because of its function in regulating cell proliferation. Studies have demonstrated that RUNX1 contains three serine residues (S48, S303, and S424) that match the cyclin-dependent kinase (CDK) consensus on target proteins (Biggs, Peterson et al. 2006). Multiple CDKs such as CDK1, CDK4 and CDK6 phosphorylate RUNX1 both *in vitro* and *in vivo (*Biggs, Peterson et al. 2006*).* This phosphorylation is necessary for RUNX1 degradation during mitosis by the Anaphase-promoting complex (APC) (Biggs, Peterson et al. 2006). Later on, it was shown that RUNX1 accelerates the G1/S transition in hematopoietic cells and knockdown of RUNX1 reduces S phase cells (Bernardin-Fried, Kummalue et al. 2004, Kim, Barron et al. 2014). Therefore, it is not surprising that S-phase and DNA replication-related genes are down-regulated upon RUNX1 depletion in MCF10A cells (Fig.3.8A). However, the involvement of RUNX1 in mitosis is not well known. Very recently, Nyam-Osor Chimge et al. showed that in MCF7 breast cancer cells, loss of RUNX1 represses Cyclin B1 expression and accumulates cells in G2 phase, indicating a G2/M arrest (Chimge, Little et al. 2016). In this chapter, by labeling the cells with the mitotic

specific marker, H3S28P, we detected that the mitotic cell population is reduced by RUNX1 knockdown in MCF10A cells. This raises the compelling question of the mechanism(s) of mitotic reduction in RUNX1 depleted cells. Additionally, what triggers the degradation of Cyclin B1 and the accumulation of p-CDK1(Thr14, Tyr15) in RUNX1 depleted MCF10A cells (Fig.3.8B)? During the cell cycle, CDK1 is phosphorylated and inactivated by Wee1 and MYT1 at Thr-14 and Tyr-15, and the phosphoryl group in phosphorylated-CDK1 is removed by the Cdc25 phosphatases (Pines 1999, Malumbres and Barbacid 2009). From RNA-seq data, we did observe that two members of CDC25 family, CDC25B and CDC25C, are significantly down-regulated upon RUNX1 depletion, while Wee1 and MYT1 expression are not changed (Fig. 3.12). Moreover, RUNX1 directly binds to the transcription start site of Wee1, CDC25B and CDC25C. These data suggest that RUNX1 is a direct positive regulator of CDC25B and CDC25C, and without RUNX1, the inactive form of p-CDK1 may not be efficiently activated by CDC25B and CDC25C, and thus block the cell from entering mitosis. However, our western blot analyses on MCF10A cells are not consistent with our RNA-seq data, which shows a decreased level of CDC25B but increased expression of CDC25C. It is unclear whether the decreased CDC25B is sufficient to keep CDK1 in its inactive form.
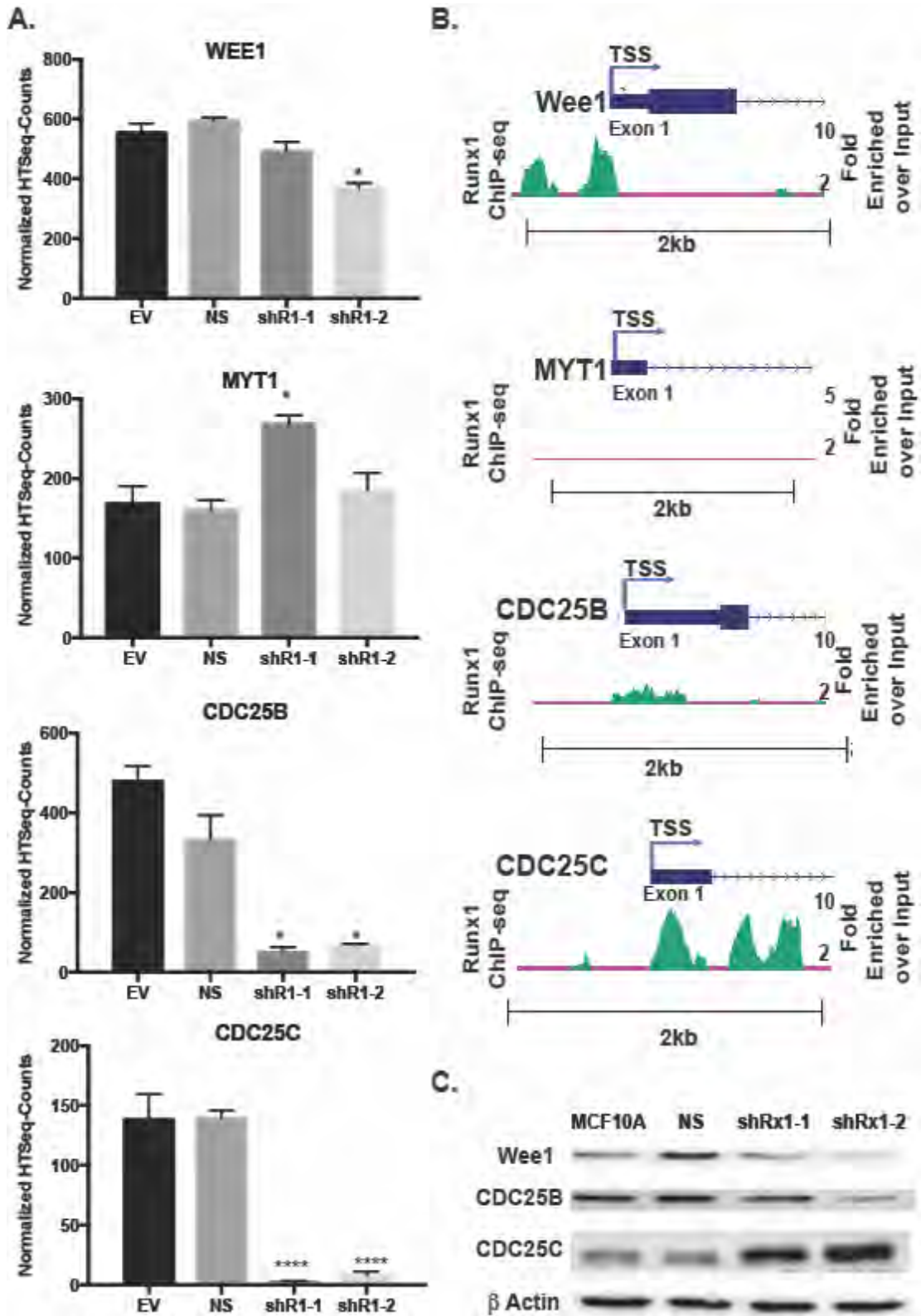
Figure 3.12 RUNX1 is a direct regulator of Bub1b, MAD2L1 and APC.

(**A**) RNA-seq analyses of RNA from MCF10A cells treated with shRunx1 show only slightly altered gene expression of Wee1 and MYT1, and decreased gene expression of CDC25B and CDC25C. Student's t test ** p value <0.01, *** p value <0.001, for MCF10A shRunx1 cells compared to the MCF10A NS cells. Error bars represent the standard error of the mean (SEM) from three biological samples. (**B**) ChIP-seq genome browser views of RUNX1 binding at the transcription start site (TSS) of Wee1, CDC25B and CDC25C but not MYT1. (**C**) Western blot analyses of whole cell lysates from MCF10A cells with RUNX1 depletion show protein expression of Wee1, CDC25B and CDC25C. The experiments are performed 3 times (N=3).

Another possibility for RUNX1-mediated progression through mitosis is by improper regulation of mitosis related genes. In Figure 3.9 and Figure 3.10, we showed that RUNX1 is a positive regulator of Bub1, Bub1b and MAD2L1, which are components of the mitotic checkpoint complex (Lara-Gonzalez, Westhorpe et al. 2012). When RUNX1 is depleted, expression levels of members of the mitotic checkpoint complex are severely inhibited (Fig. 3.9-3.10). It also has been shown that the mitotic checkpoint complex is an inhibitor of the anaphase-promoting

complex (APC) (Lischetti and Nilsson 2015). APC is a multi-subunit E3 ubiquitin ligase, which is inactive prior to entry into mitosis (Lischetti and Nilsson 2015). During mitosis, APC is activated through interaction with Cdh1(FZR1), and facilitates mitotic exit by ubiquitinating and degrading cell-cycle regulators such as cyclin B1 and Securin (Lischetti and Nilsson 2015, Zhou, He et al. 2016). Interestingly, RUNX1 is also a target of APC and is degraded during mitosis (Biggs, Peterson et al. 2006). The activity of APC is subject to multiple layers of regulation throughout the cell cycle (Lischetti and Nilsson 2015). Our data show that RUNX1 is a direct negative regulator of APC, as RUNX1 binds to the APC promoter region and loss of RUNX1 activates the expression of APC (Fig. 3.10A, B). Therefore, it is possible that RUNX1 is an essential repressor of APC, and a feedback regulatory mechanism between RUNX1 and APC is necessary for keeping APC activity specifically in mitosis. During normal cell cycle, RUNX1 negatively regulates APC expression before entering mitosis. In mitosis, RUNX1 is degraded by APC, which further activates APC expression to promote Cyclin B1 degradation and mitotic exit (Fig. 3.13). When RUNX1 expression is disrupted, APC is aberrantly activated and leads to constitutive degradation of Cyclin B1 in the cell cycle and thus blocks cells from entering mitosis (Fig. 3.13).

Alternatively, the decreased mitotic population in RUNX1 depleted cells may be due to premature mitotic exit. Depleted or mutated components in the mitotic checkpoint complex, such as Bub1, have been shown to lead to inappropriate chromosome segregation and premature mitotic exit which leads to aneuploidy

and genome instability (Goto, Mishra et al. 2011). It is possible that loss of RUNX1 will increase the incidence of spindle checkpoint defects and premature mitotic exit, resulting in a reduced population of mitotic cells.

Despite the inconclusive mechanism(s) on how RUNX1 is involved in cell cycle, especially in mitosis, in this chapter we demonstrated that RUNX1 is a major transcription factor which regulates expression of key genes and is involved in various aspects of cellular activity. Further experiments based on our RUNX1 ChIP-seq and RNA-seq data from RUNX1 depleted cells will aide in elucidating the function of RUNX1 in mammary epithelial cells. These future investigations will provide an improved understanding of how dysregulated RUNX1 leads to breast cancer initiation and progression.
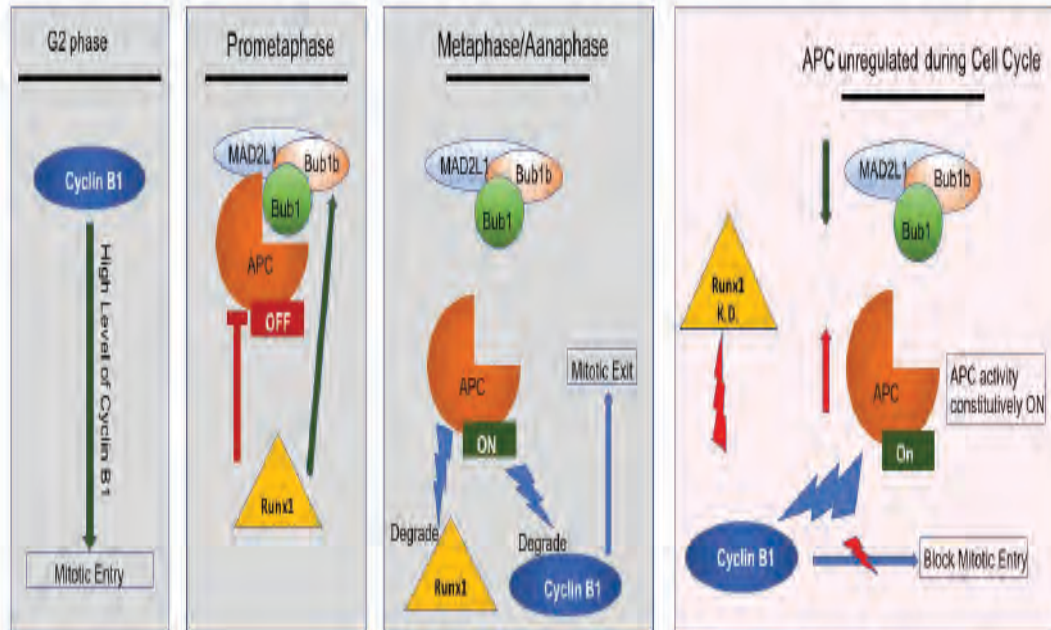
Figure 3.13 Possible mechanisms of RUNX1-controlled mitotic entry.

Chapter IV RUNX1 suppresses breast cancer stemness and tumor growth

A large portion of this chapter comes from the manuscript:

Runx1 exhibits anti-tumor activity and inhibits stemness in breast cancer cells

Deli Hong, Andrew J. Fritz, Kristiaan Finstad, Mark P. Fitzgerald, Adam L. Viens, John Ramsey, Janet L. Stein, Jane B. Lian, Gary S. Stein

Contribution: Deli Hong, Jane B. Lian, Janet L. Stein and Gary. S. Stein. conceived and designed the experiments, and analyzed data. Deli Hong, Andrew J. Fritz., Mark P. Fitzgerald and Adam L. Vienes performed the experiments. Deli Hong and Kristiaan H. Finstad performed animal experiments. John Ramsey constructed and packaged RUNX1 overexpression lentivirus. Deli Hong created all the figures. Deli Hong, Jane B. Lian, Janet L. Stein and Gary S. Stein wrote the manuscript.

4.1 Abstract:

Breast cancer remains the most common malignant disease in women worldwide. Despite advances in detection and therapies, studies are still needed for further understanding mechanisms underlying this cancer. Cancer stem cells (CSC) play an important role in tumor formation, growth, drug-resistance and recurrence. Here, we demonstrate for the first time that the transcription factor RUNX1, well known as essential for hematopoietic differentiation, represses the breast cancer stem cell (BCSC) phenotype and suppresses tumor growth *in vivo*. The present studies show that BCSCs sorted from pre-malignant breast cancer cells exhibit decreased RUNX1 levels, while overexpression of RUNX1 suppresses tumorsphere formation and reduces the BCSC population. RUNX1 ectopic expression in breast cancer cell lines reduces migration, invasion and *in vivo* tumor growth (57%) in mouse mammary fat pad. Mechanistically, RUNX1 functions to suppress breast cancer tumor growth through repression of cancer stem cell activity and direct inhibition of Zeb1 expression. Consistent with these cellular and biochemical results are the clinical findings that the highest RUNX1 levels occur in normal mammary epithelial cells in patient specimens and that low RUNX1 expression in tumor is associated with poor patient survival. Our key finding that RUNX1 represses stemness in several breast cancer cell lines points to the importance of RUNX1 in other solid tumors and suggests RUNX1 may regulate cancer stem cells.

4.2 Introduction:

Breast tumors are heterogeneous, as they are comprised of several types of cells, including transformed cancer cells, supportive cells, tumor-infiltrating cells and cancer stem cells (CSC). The CSC is acknowledged to be a significant component of growing tumors (Ming, Michael et al. 2015, Chaffer, San Juan et al. 2016). As the name implies, CSC can self-renew and reconstitute the cellular hierarchy within tumors (Visvader and Lindeman 2008, Meacham and Morrison 2013). Moreover, these stem-like cells are highly chemo-resistant and metastatic (Abdullah and Chow 2013, Zhao 2016). Significantly, signaling pathways (TGF-β, WNT, Hedgehog and Notch) and transcription factors (Snail, Twist and Zeb) regulating stemness properties in CSC are involved in controlling an essential cellular process designated epithelial-mesenchymal transition (EMT) (Scheel and Weinberg 2012, Hadjimichael, Chanoumidou et al. 2015). The EMT process is linked to chemo-resistance and cancer metastasis (Singh and Settleman 2010, Pattabiraman and Weinberg 2014, Shibue and Weinberg 2017). One such example is Zeb1, a well-known EMT-activator that is also a key factor for cell plasticity and promotes stemness properties in breast and pancreatic cancers (Lehmann, Mossmann et al. 2016, Krebs, Mitschke et al. 2017). However there remains a compelling requirement to understand regulatory mechanisms that contribute to and sustain the stemness of the CSC population. Identifying regulator(s) that maintain or repress the cancer stem cell phenotype can provide

insights for novel therapeutic approaches. Recently, a list of 40 mutation-driver genes for which deregulation contributes directly to breast tumor progression has been identified (Pereira, Chin et al. 2016); among these is the transcription factor RUNX1, which has been shown to repress EMT. Here we address for the first time, the function of RUNX1 in regulating breast cancer stem cells.

The Runx family, including RUNX1, Runx2 and Runx3, are evolutionarily conserved transcription factors and function as critical lineage determinants of various tissues (Ito, Bae et al. 2015). During normal development, it is well documented that RUNX1 plays a fundamental role in controlling the stem cell population in hematopoietic (Yokomizo, Ogawa et al. 2001, Jacob, Osato et al. 2010, Wang, Krishnan et al. 2014), hair follicle (Hoi, Lee et al. 2010, Osorio, Lilja et al. 2011), gastric (Matsuo, Kimura et al. 2017) and oral epithelial stem cells (Scheitz, Lee et al. 2012). As a master transcriptional regulator, RUNX1 is a central player in fine-tuning the balance among cell differentiation, proliferation, and cell cycle control in stem cells during normal development (Wang, Jacob et al. 2010). In the mammary gland, it has recently been shown that RUNX1 is involved in luminal development (Sokol, Sanduja et al. 2015). These studies also showed that loss of RUNX1 in mammary epithelial cells blocked differentiation into ductal and lobular tissues. These findings suggest that RUNX1 is an essential regulator of normal mammary stem cells (Sokol, Sanduja et al. 2015). In addition to its essential function during normal development, disrupting

RUNX1 function(s) can cause cancer (Ito 2004, Ito, Bae et al. 2015). RUNX1 is a frequent target of translocations and other mutations in hematopoietic malignancies. For example, RUNX1 related chromosomal translocations including RUNX1-ETO (Hatlen, Wang et al. 2012), TEL-RUNX1 (Fischer, Schwieger et al. 2005) and RUNX1-EVI (Mitani, Ogawa et al. 1994) are associated with distinct leukemia subtypes.

In breast cancer, RUNX1 has been shown to regulate the WNT pathway and key transcription factors including ERα and ELF5 (Ito, Bae et al. 2015)(van Bragt, Hu et al. 2014)(Chimge, Little et al. 2016)(Barutcu, Hong et al. 2016). Recent studies from our group have demonstrated that RUNX1 maintains the epithelial phenotype and represses EMT (Hong, Messier et al. 2017). RUNX1 expression is decreased during breast cell EMT, and loss of RUNX1 expression in normal-like epithelial cells (MCF10A) and epithelial-like breast cancer cells (MCF7) initiates the EMT process. Complementary studies demonstrated that ectopic expression of RUNX1 reverts cells to the epithelial state. However, mechanisms underlying RUNX1 regulation of cancer stem cell properties and the consequences for tumor growth *in vivo* remain to be resolved.

Based on evidence that RUNX1 regulates stem cell properties during normal development and that loss of RUNX1 activates partial EMT in breast cancer, we hypothesized that RUNX1 represses the cancer stem cell population and/or stemness properties in breast cancer. We investigated whether altering RUNX1

levels by overexpression and knockdown in breast cancer cells changes the stemness phenotype, aggressive properties and tumor progression *in vivo*. Our findings have identified for the first time a significant function for RUNX1 in repressing the cancer stem cell population as well as tumorsphere formation, and demonstrated that RUNX1 represses breast cancer tumor growth *in vivo*.

4.3 Materials and Methods

4.3.1 Cell culture:

MCF10AT1 and MCF10A cells were grown in DMEM: F12 (Hyclone: SH30271, Thermo Fisher Scientific, Waltham, MA) with 5% (v/v) horse serum (Gibco: 16050, Thermo Fisher Scientific, Waltham, MA, USA) + 10 µg/ml human insulin (Sigma Aldrich, St. Louis, MO: I-1882) + 20 ng/ml recombinant hEGF (Peprotech, Rocky Hill, NJ, USA: AF-100-15) + 100 ng/ml cholera toxin (Sigma Aldrich: C-8052) + 0.5 µg/ml hydrocortisone (Sigma Aldrich: H-0888) 50 IU/ml penicillin/50 µg/ml streptomycin and 2 mM glutamine (Life Technologies, Carlsbad, CA, USA: 15140-122 and 25030-081, respectively). MCF10CA1a cells were grown in DMEM: F with 12, 5% (v/v) horse serum with 50 IU/ml penicillin/50 µg/ml streptomycin and 2 mM glutamine. MCF7 cells were maintained in Dulbecco modified Eagle medium (DMEM) high glucose (Fisher Scientific: Thermo Fisher Scientific, Waltham, MA, USA: MT-10-017-CM) supplemented with 10% (v/v) FBS (Atlanta Biologicals, Flowery Branch, GA, USA: S11550), 50 IU/ml penicillin/50 µg/ml streptomycin.

4.3.2 Lentiviral plasmid preparation and viral vector production

RUNX1 cDNA was cloned into Lentivirus-based overexpression plasmids pLenti-CMV-Blast-DEST (Addgene). To generate lentivirus vectors, 293T cells in 10 cm culture dishes were co-transfected with 10 µg of pGIPZ shRunx1 or pGIPZ non-silencing, with 5 µg of psPAX2, and 5 µg of pMD2.G using lipofectamine 2000 reagent (Life Technologies). Viruses were harvested every 48 h post-transfection. After filtration through a 0.45 µm-pore-size filter, viruses were concentrated by using LentiX concentrator (Clontech, Mountain View, CA, USA).

4.3.3 Gene delivery by transfection and infection

For overexpression RUNX1, MCF10AT1 or MCF10CA1a cells were plated in six-well plates (1x10^5 cells per well) and infected 24 h later with lentivirus expressing RUNX1 overexpression or Empty Vector. Briefly, cells were treated with 0.5 ml of lentivirus and 1.5 ml complete fresh DMEM-F12 per well with a final concentration of 4 µg/ml polybrene. Plates were centrifuged upon addition of the virus at 1460 × g at 37°C for 30 min. Infection efficiency was monitored by GFP co-expression at 2 days post infection. Cells were selected with 2 µg/ml puromycin (Sigma Aldrich P7255-100MG) for at least two additional days. After removal of the floating cells, the remaining attached cells were passed and analyzed. ShRunx1 virus were generated and delivered as has been described previously (Hong, Messier et al. 2017).

4.3.4 Western blotting

Cells were lysed in RIPA buffer and 2X SDS sample buffer supplemented with cOmplete, EDTA-free protease inhibitors (Roche Diagnostics) and MG132 (EMD Millipore San Diego, CA, USA). Lysates were fractionated in an 8.5% acrylamide gel and subjected to immunoblotting. The gels are transferred to PVDF membranes (EMD Millipore) using a wet transfer apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked using 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad Laboratories) and incubated overnight at 4°C with the following primary antibodies: a rabbit polyclonal RUNX1 (Cell Signaling Technology, Danvers, MA, USA: #4334, 1:1000); a mouse monoclonal to E-cadherin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA: sc21791, 1:1000); a mouse monoclonal Vimentin (Santa-Cruz Biotechnology sc-6260, 1:1000); a mouse monoclonal to β-Actin (Cell Signaling Technology #3700, 1:1000); a rabbit polyclonal Twist1 (Santa Cruz Biotechnology sc-15393, 1:2000); a rabbit polyclonal Zeb1 (Sigma-Aldrich HPA027524-100UL, 1:1000). Secondary antibodies conjugated to HRP (Santa Cruz Biotechnology) were used for immunodetection, along with the Clarity Western ECL Substrate (Bio-Rad Laboratories) on a Chemidoc XRS+ imaging system (Bio-Rad Laboratories).

4.3.5 Tumorsphere formation assay:

Monolayer cells were enzymatically dissociated into single cells with 0.05% trypsin-EDTA. Cells were plated at 10,000 cells per well in a 24-well low-attachment plate (Corning). Cells were grown for 7 days in DMEM/F12

supplemented with B27 (Invitrogen) in the presence of 10 ng/ml EGF and 10 ng/ml bFGF. Where indicated, the CDK4 inhibitor palbocilib (Sigma) was added at a final concentration of 100 nM. Tumorsphere-forming efficiency was calculated as the number of spheres divided by the number of singles cells seeded, expressed as a percentage.

4.3.6 CD24/CD44 flow cytometry

Flow cytometry for CD24 (PE-cy7, Biolegend 311120) and CD44 (APC, BD Pharmigen 559942) was performed using the best conditions for marker detection as previously described (Fillmore and Kuperwasser 2008)(Quan 2013). Cells were grown to sub-confluency and dissociated with Accutase. The Accutase was quickly neutralized with serum and $1x10^6$ cells were washed with 1xPBS. These cells were then re-suspended in 475ul of 1%FBS/ 1xPBS, to which 25ul of CD44-APC and 4ul of CD24-PE-cy7 were added and incubated at room temperature for 30 minutes. Cells were then washed with PBS and strained (Falcon 352235) to obtain single cell suspensions. Isotype controls were used to gate for negative signal in each replicate of the experiment.

4.3.7 Migration assays

For the scratch assays, cells were seeded in triplicate and when they reached 95–100% confluence, were serum starved with 0.1% FBS-containing media for 12 h. Subsequently, a scratch was made across the cell layer using a P-200 pipette tip, and cell migration was monitored by recording images at indicated time points

post-scratch. The area of the scratch was quantified using the MiToBo plug-in for ImageJ software and plotted as a percentage of total area.

For the transwell migration assay, cells were trypsinized and re-seeded in triplicate in migration chambers (BD Bioscience, Bedford, MA) in serum-free medium for 24 hours (MCF10AT1 cells) or 48 hours (MCF10CA1a cells) after cell seeding. Cells were allowed to migrate through 8 µm pores toward medium containing 5% Horse Serum. The experiment was performed and results quantified as previously described (Browne, Taipaleenmäki et al. 2015).

4.3.8 Invasion Assay

For the invasion assay, cells were trypsinized and reseeded in triplicate in growth factor-reduced Matrigel invasion chambers (BD Bioscience, Bedford, MA) in serum-free medium for 24 hours (MCF10AT1 cells) or 48 hours (MCF10CA1a cells) after cell seeding. Cells were allowed to migrate through 8 µm pores toward medium containing 5% Horse Serum. The experiment was performed and results quantified as previously described (Browne, Taipaleenmäki et al. 2015).

4.3.9 Immunofluorescence staining microscopy

Cells grown on coverslips were fixed with using 3.7% formaldehyde in Phosphate Buffered Saline (PBS) for 10 min. Cells were then permeabilized in 0.1% Triton X-100 in PBS, and washed in 0.5% Bovine Serum Albumin in PBS. Detection was performed using a rabbit polyclonal RUNX1 antibody (Cell Signaling #4336), a mouse monoclonal CD24 (Santa-Cruz sc-11406). Staining was performed using fluorescent secondary antibodies; for rabbit polyclonal antibodies a goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor® 568 conjugate (Life Technologies A-11011), was used and for mouse monoclonal

a F(ab')2-goat anti-mouse IgG (H+L) secondary antibody, Alexa Fluor® 488 conjugate was used (Life Technologies A-11001).

4.3.10 Animal studies

Female SCID mice 7 weeks of age were used for mammary fat pad injection. The mice were randomly divided into two groups (seven for each group). In all, $1X10^6$ MCF10CA1a cells suspended in 0.1 ml of saline were mixed with 0.1 ml of Matrigel (BD) and were injected under mammary fat pads. Bioluminescence images were acquired by using the IVIS Imaging System (Xenogen) 5 min after injection 150 mg/kg of D-Luciferin (Gold BioTech, St. Louis, MO) in PBS. All animals were housed in a pathogen-free environment and handled according to protocol number 12-051 approved by the Institutional Animal Care and Use Committee at the University of Vermont. In conducting using animals, the investigators adhere to the laws of the United States and regulations of the Department of Agriculture.

4.3.11 Analysis of RUNX1 expression and patient survival using public data sets

The PROGgene database (www.compbio.iupui.edu/proggene) (Goswami and Nakshatri 2013) (Goswami and Nakshatri 2014) was used to identify the data sets for survival analysis and re-analyzed the public GEO data sets (www.ncbi.nlm.nih.gov/gds) (GSE37751 (Terunuma, Putluri et al. 2014), GSE7390 (Desmedt, Piette et al. 2007), TCGA (Network 2012)). RUNX1 expression in different breast cancer stages was analyzed using the TCGA database (www.cbioportal.org).

4.3.12 Quantitative PCR

RNA was isolated with Trizol (Life Technologies) and cleaned by DNase digestion (Zymo Research, Irvine, CA, USA). RNA was reversed transcribed using SuperScript II and random hexamers (Life Technologies). cDNA was then subjected to quantitative PCR using SYBR Green technology (Applied Biosystems, Foster City, CA, USA).

RUNX1 Forward: AACCCTCAGCCTCAGAGTCA,

RUNX1 Reverse: CAATGGATCCCAGGTATTGG;

FN1 Forward: CATGAAGGGGGTCAGTCCTA;

FN1 Reverse: CTTCTCAGCTATGGGCTTGC;

VEGF Forward: CCTTGCTGCTCTACCTCCAC;

VEGF Reverse: CCATGAACTTCACCACTTCG;

CXCR4 Forward: TACACCGAGGAAATGGGCTCA;

CXCR4 Reverse: TTCTTCACGGAAACAGGGTTC;

CXCL12 Forward: GTGGTCGTGCTGGTCCTC;

CXCL12 Reverse: AGATGCTTGACGTTGGCTCT;

MMP13 Forward: ATGAGCCAGAGTGTCGGTTC;

MMP13 Reverse: GTTAGTAGCGACGAGCAGGAC;

MMP9 Forward: ATAGACTACTACAGGCT;

MMP9 Reverse: TAGCACGGATAGACCA;

GAPDH Forward: TGTGGTCATGAGTCCTTCCA,

GAPDH Reverse: ATGTTCGTCATGGGTGTGAA;

HPRT Forward: TGCTGACCTGCTGGATTACA,

HPRT Reverse: TCCCCTGTTGACTGGTCATT;

β-Actin Forward: AGCACAGAGCCTCGCCTTT,

β-Actin Reverse: CGGCGATATCATCATCCAT.

4.3.13 ChIP-qPCR

ChIP-qPCR was performed essentially as described (O'Geen, Frietze et al. 2010). Briefly, 200,000 MCF10AT1 or MCF10CA1a cells were cross-linked, lysed and sonicated to obtain DNA fragments mostly in the 200-1000-bp range. Immunoprecipitation was performed at 4°C overnight with anti-RUNX1 antibody (4334, Cell Signaling Technology) at a 1:15 antibody to chromatin ratio. Primers used in ChIP-qPCR are listed below:

Zeb1 Forward: GTCGTAAAGCCGGGAGTGTC,

Zeb1 Reverse: GCCATCCGCCATGATCCTC;

ZNF333 (negative control 1) Forward: TGAAGACACATCTGCGAACC,

ZNF333 Reverse: TCGCGCACTCATACAGTTTC;

ZNF180 (negative control 2) Forward: TGATGCACAATAAGTCGAGCA,

ZNF180 Reverse: TGCAGTCAATGTGGGAAGTC.

4.3.14 Tissue microarray

Tissue microarray data of RUNX1 in breast cancer patients were obtained from Human Protein Atlas (www.proteinatlas.org) (Uhlén, Fagerberg et al. 2015).

4.3.15 Statistical analysis

Each experiment was repeated at least three times. The differences in mean values among groups were evaluated and expressed as the mean ± SEM. A P-value less than 0.05 was considered statistically significant (*$P < 0.05$, **$P < 0.01$,

***P* < 0.001). Student's *t*-test was used to compare the expressions of cell surface markers, side population analysis, cell viability, relative mRNA levels, migrated cells and invaded cells.

4.4 Results:

4.4.1. Reduced RUNX1 expression is associated with decreased survival probability in breast cancer patients.

To investigate possible association between RUNX1 expression and breast cancer progression, we first examined RUNX1 expression in normal and breast cancer patients using the Human Protein Atlas. Within normal breast tissues, RUNX1 is highly expressed in the mammary gland (Fig. 4.1A). However, in ductal carcinoma tissues, the level of RUNX1 is decreased in malignant regions (red circle) compared with normal glandular tissues (blue circle) in the same tumor specimen (Fig. 4.1B). In the majority of ductal carcinoma specimens (9 out 12 samples) from the Human Protein Atlas, 75% of breast cancer tumors show low RUNX1 staining (Fig. 4.1C). We also analyzed TCGA data and found that RUNX1 levels are progressively decreased across early stage breast cancer (Stage 1 vs Stage2; Stage 2 vs Stage 3) (Figure 4.2). These findings suggest that during breast cancer progression, the mammary gland loses its original structure and RUNX1 levels are decreased. The data are consistent with our previous report that RUNX1 is highly expressed in normal-like mammary epithelial MCF10A cells and reduced in a panel of breast cancer cell lines (Hong, Messier et al. 2017). With the reduced

RUNX1 expression, mammary epithelial cells do not maintain their epithelial phenotype (Hong, Messier et al. 2017) From these observations of low RUNX1 in breast tumors and the concomitants loss of RUNX1 in normal epithelial cells with loss of epithelial properties, we hypothesized that loss of RUNX1 is promoting a breast cancer phenotype.
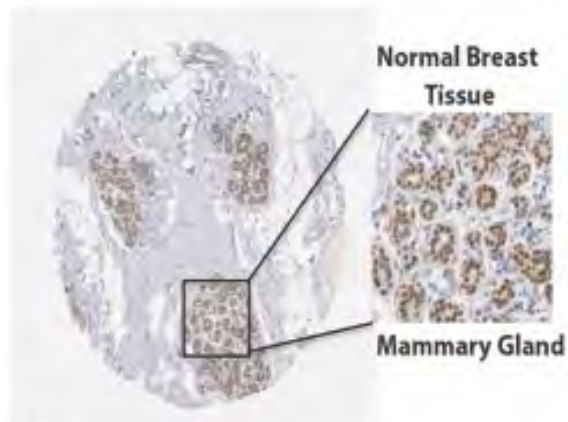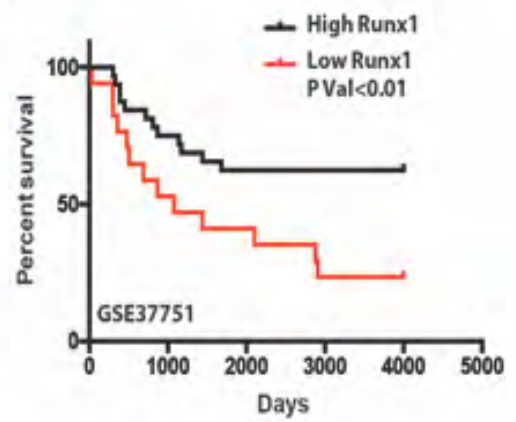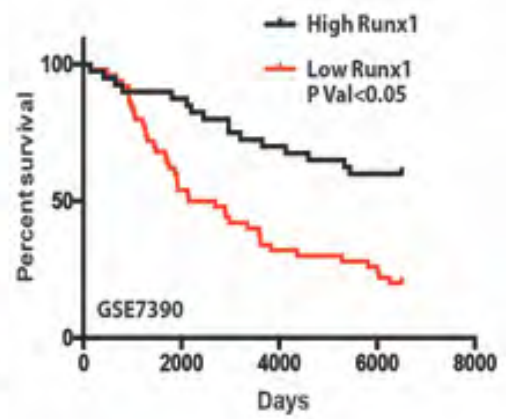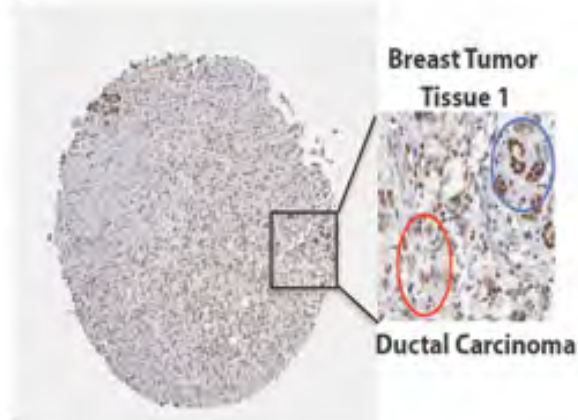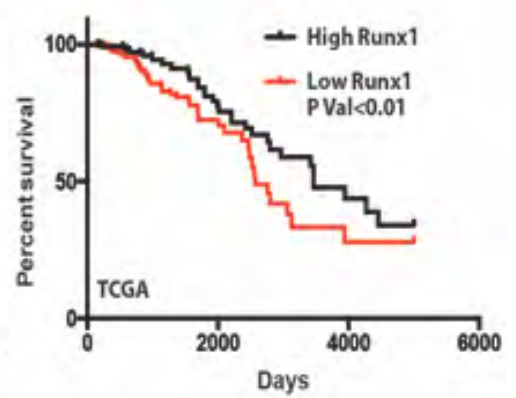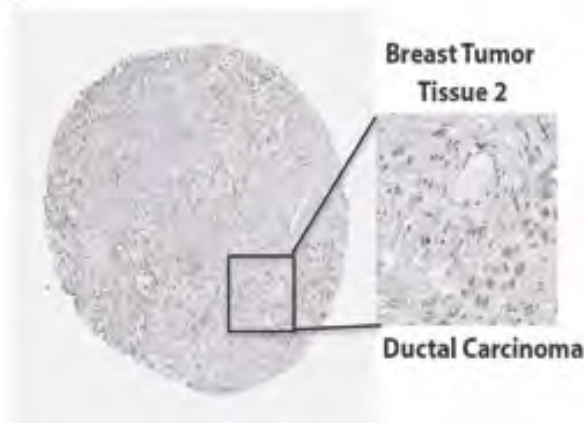
Figure 4.1. Reduced RUNX1 expression is associated with decreased survival probability in breast cancer patients. **(A)** Representative tissue microarray images of RUNX1 in normal breast tissue. **(B)** and **(C)** Representative tissue microarray images of RUNX1 in breast tumor tissues. **(D)** Kaplan-Meier analysis showed higher overall survival in patients with higher RUNX1 mRNA expression (GSE37751, GSE7390 and TCGA). Gehan-Breslow-Wilcoxon test with *p* value<0.01, *p* value<0.05, *p* value<0.01 respectively compared with high RUNX1 expression patients and low RUNX1 expression patients in three data sets.

We therefore addressed whether there was a clinical relation of RUNX1 expression in breast cancer patient tumors to survival. Using publically available mRNA expression datasets, we analyzed the correlation of mean expression levels of RUNX1 and survival rate in breast cancer patient tissue samples. Kaplan–Meier analysis of the expression of RUNX1 in three separate datasets of GSE37751-"Molecular Profiles of Human Breast Cancer and Their Association with Tumor Subtypes and Disease Prognosis" (36 high RUNX1 and 24 low RUNX1 patients), GSE7390-"Strong Time Dependence of the 76-Gene Prognostic Signature" (82 high RUNX1 and 116 low RUNX1 patients) and TCGA data of breast cancer patients mRNAs (304 high RUNX1 and 290 low RUNX1 patients) indicated a statistically significant correlation ($p < 0.01$, $p < 0.05$, and $p<0.01$ respectively)

between high RUNX1 expression levels and longer patient survival time (Fig. 4.1D).

These results suggested that reduction in RUNX1 expression is associated with

low survival probability of breast cancer patients. Thus several *in vitro* studies

combined with these clinical observations support a role for RUNX1 in repressing

tumor growth.
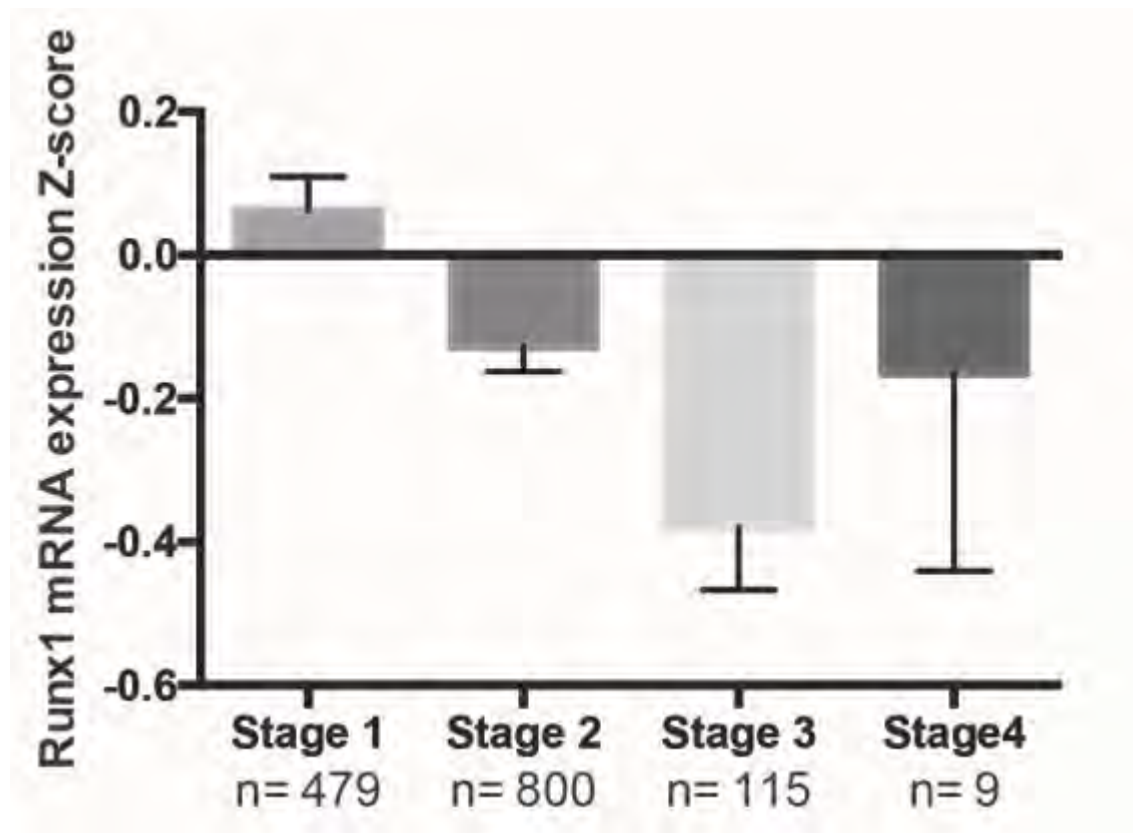
Figure 4.2. RUNX1 mRNA is decreased during breast cancer progression. TCGA data shows that RUNX1 mRNA is decreased in Stage 2 and Stage 3 tumors.

4.4.2. RUNX1 is decreased in tumors formed in mouse mammary fat pad

To further establish if RUNX1 decreases during breast tumor growth *in vivo*, we utilized a mouse xenograft model to examine RUNX1 levels before and after tumor formation. MCF10CA1a cells, which are aggressive breast cancer cells, were injected into mammary fat pad of SCID mice and tumor growth was monitored weekly. Tumors formed within two weeks (Fig. 4.3A), and one month post-injection, mice were sacrificed and tumors were removed to analyze for RUNX1 and other factors at both protein and mRNA levels. The parental MCF10CA1a cells had a 3.3-fold higher RUNX1 protein level than the removed tumor (Fig. 4.3B, C). qRT-PCR using human-specific primer sets confirmed that RUNX1 mRNA is also decreased specifically within the tumor (Fig. 4.3C). The epithelial marker E-cadherin was decreased in tumor samples, while the mesenchymal marker Vimentin was increased (Fig. 4.3B). In addition to Vimentin, the mRNA levels of several human cancer-related genes such as VEGF, FN1, MMP13, MMP9, CXCR4, CXCL12 are also up regulated (Fig. 4.3B, D). These findings indicate that the human breast cancer cells that formed a tumor in mouse mammary fat pads acquired a more aggressive phenotype and that RUNX1 expression is decreased during the period of tumor growth. Therefore, we have directly demonstrated that in this MCF10CA1a mouse xenograft model, RUNX1 expression is decreased during *in vivo* model of tumor progression.
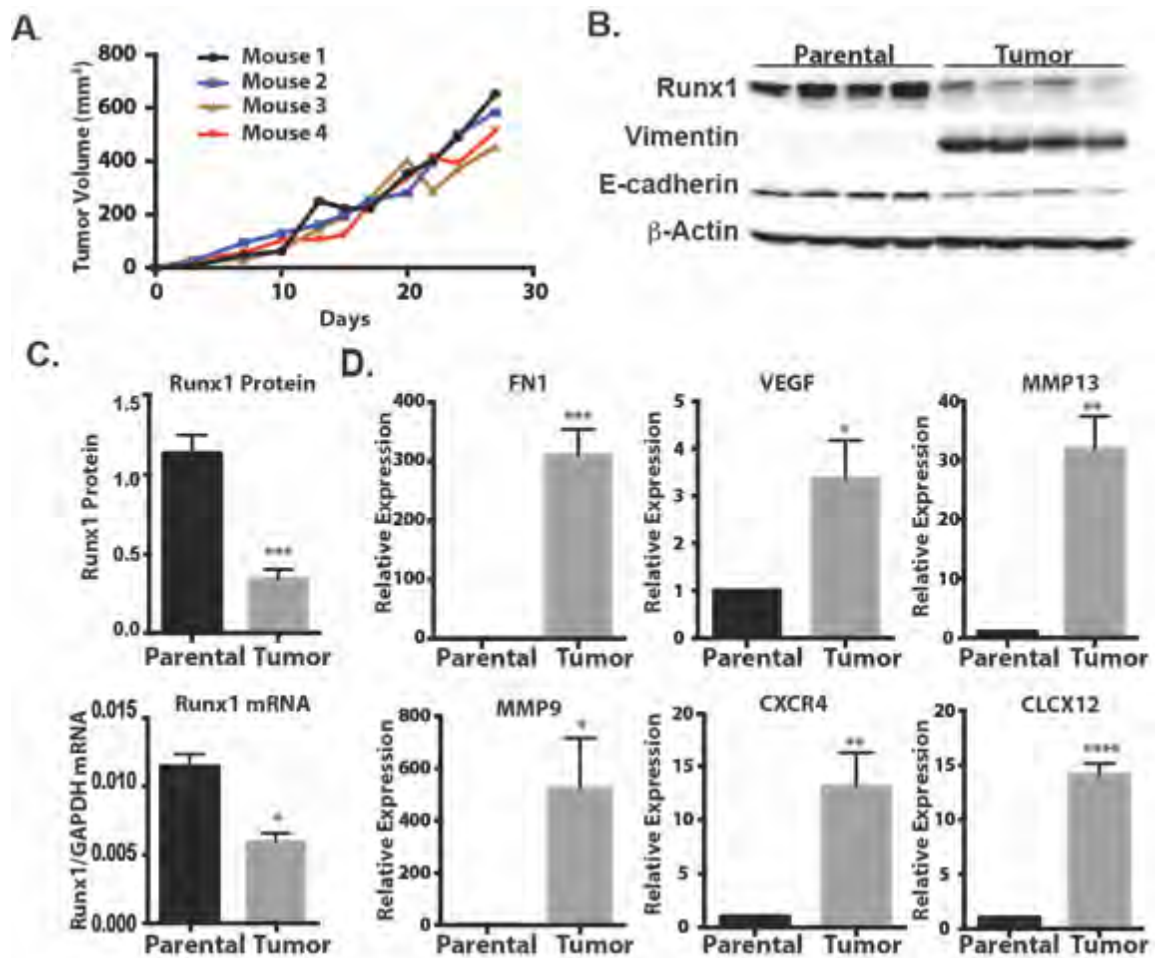
Figure 4.3. RUNX1 is decreased in tumors formed in mouse mammary fat pad. (A) MCF10CA1a cells were injected into the mammary fat pad of SCID mice. Points represent mean tumor volume. **(B)** Western blot analyses show RUNX1 and E-cadherin levels are decreased and Vimentin level is increased in tumor samples compared to MCF10CA1a cells. **(C)** Upper panel, Protein quantification show that RUNX1 is significant decreased in tumor samples compared to MCF10CA1a cells. Lower panel, RT-qPCR analyses of RNA from tumor samples show decreased RUNX1 expression of compared with MCF10CA1a cells. Student's *t* test * *p* value <0.05, *** *p* value <0.001 and. Error bars represent the standard error of the mean (SEM) from three independent experiments. **(D)** RT-qPCR analyses of RNA from tumor samples show activation of mesenchymal marks Vimentin and FN1 and other tumor growth related genes including MMP9, MMP13, VGF, CXCR4 and CXCL12 compared with MCF10CA1a cells. Student's *t* test * *p* value <0.05, ** p value <0.01, *** *p* value <0.001 and **** *p* value <0.0001. Error bars represent the standard error of the mean (SEM) from three independent experiments.

4.4.3. RUNX1 reduces the aggressive phenotype of breast cancer cells *in vitro.*

It has been suggested that RUNX1 reduces aggressive phenotypes in breast cancer (van Bragt, Hu et al. 2014, Chimge, Little et al. 2016, Hong, Messier et al. 2017). Based on these data and the results that RUNX1 level is decreased in the xenograft model (Fig. 4.3B), we further addressed whether ectopic expression of RUNX1 in malignant breast cancer cells reduces the aggressive phenotype. RUNX1 was overexpressed using a lentivirus delivery system (pLenti-CMV) in pre-malignant MCF10AT1 and highly aggressive malignant MCF10Ca1a cells (Fig. 4.4A). Upon overexpressing RUNX1, Vimentin expression is decreased in both cell lines (Fig. 4.4A). However, E-cadherin expression was not affected by RUNX1 overexpression, suggesting that the cells have not fully transitioned back to normal-like stage.

A.

MCF10CA1a
EV   OE Runx1
Runx1
E-cadherin
Vimentin
β-Actin

MCF10AT1
EV   OE Runx1
Runx1
E-cadherin
Vimentin
β-Actin

B.

MCF10AT1   MCF10CA1a
EV   OE Runx1   EV   OE Runx1

0 hr   0 hr

48 hr   16 hr

MCF10AT1
% of wound closure

MCF10Ca1a
% of wound closure

C.

MCF10AT1   MCF10CA1a
EV   OE Runx1   EV   OE Runx1

Migration

MCF10AT1
Absorbance

MCF10CA1a
Absorbance

D.

MCF10AT1   MCF10CA1a
EV   OE Runx1   EV   OE Runx1

Invasion

MCF10AT1
Absorbance

MCF10Ca1a
Absorbance

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Figure 4.4. RUNX1 reduces the aggressive phenotype of breast cancer cells *in vitro.* **(A)** Western blot analyses confirm RUNX1 overexpression in MCF10CA1a (Upper) and MCF10AT1 (Lower) cells. Vimentin expression is repressed upon RUNX1 overexpression in both cell lines. **(B)** Representative phase contrast images (magnification 100×) of MCF10AT1 and MCF10CA1a cells with EV control or RUNX1 overexpression subjected to a scratch assay for times indicated. The area of the scratch was plotted as a percentage of total area for N = 3 independent experiments carried out in duplicate. **(C)** Light microscopy images (mag. 12×) of stained cells from a representative (1 of N = 2) trans-well migration assay experiment MCF10AT1 and MCF10CA1a cells with EV control or RUNX1 overexpression (*left*); quantitation of migrated cells assessed by measurement of the absorbance of solubilized crystal violet stain retained by migrated cells (*right*). **(D)** Light microscopy images (mag. 12×) of stained cells from a representative (1 of N = 2) trans-well matrigel invasion assay experiment with MCF10AT1 and MCF10CA1a cells with EV control or RUNX1 overexpression to evaluate invasion (*left*); quantitation of invaded cells assessed by measurement of the absorbance of solubilized crystal violet stain retained by invaded cells (*right*). For all assays, three independent experiments were carried out in duplicates. All quantitative data are depicted as mean ± S.E. per group. *$P < 0.05$, **$P < 0.01$

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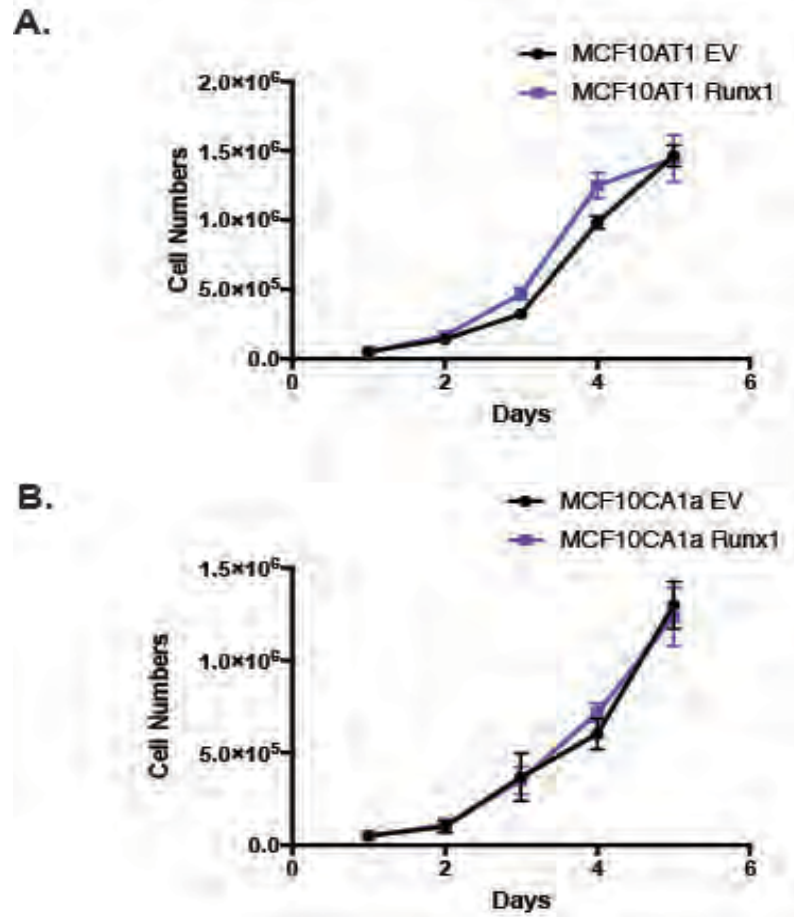
Figure 4.5. RUNX1 overexpression does not change cell proliferation. (A.) Growth curves for MCF10AT1 cells either express empty vector (black) or RUNx1 (blue). Line graph represents mean SEM from two experiments with a technical replicate each (N=4). No statistician difference was found (*, $p < 0.05$). (B.) Growth curves for MCF10Ca1a cells either express empty vector (black) or RUNx1 (blue). Line graph represents mean SEM from two experiments with two technical replicates each (N=2). No statistician difference was found (*, $p < 0.05$).

Overexpressing RUNX1 in both MCF10AT1 and MCF10CCA1a cells does not change the proliferation (Fig.4.5 A, B). To evaluate the effect of RUNX1 in regulation of migration and invasion capacities of the breast cancer cells *in vitro,* we used the scratch migration and Transwell assays*.* Figure 4.4B shows representative images of the scratch assay, both at the time of the scratch and 48 h (MCF10AT1) or 16 h (MCF10CA1a) later. RUNX1 overexpression decreases the ability of breast cancer cells to migrate. These results were confirmed using the trans-well migration assay (Fig. 4.4C). Invasion of both MCF10AT1 and MCF10CA1a cells was also significantly inhibited when RUNX1 was overexpressed (Fig. 4.4D). We conclude from these studies that loss of RUNX1 in MCF10A and cancer cells is detrimental in promoting EMT in vitro (Hong, Messier et al. 2017) and *in vivo* (Fig 4.3B), while exogenous expression of RUNX1 suppresses the migration and invasion of breast cancer cells *in vitro*.

4.4.4. RUNX1 represses tumor growth *in vivo*

Together our data above and the earlier studies demonstrate that RUNX1 has tumor suppresser activity *in vitro.* However, to date there are no studies showing that RUNX1 inhibits tumor growth *in vivo*. We tested the ability of RUNX1 to alter tumor growth *in vivo* by using the metastatic MCF10CA1a breast cancer cells. MCF10CA1a/EV (control) and MCF10CA1a/ RUNX1- overexpression cells carrying a luciferase reporter (experiment) were injected into the mammary fat pad of SCID mice. Eighteen days post-injection tumors appeared in the control mice, with an average volume of 63 mm^3 (caliper measurement), while the experimental

group had barely palpable tumors (Fig. 4.6A). At the end point of this experiment (4 weeks), we sacrificed the mice, excised the tumors, and measured tumor volume and weight (Fig. 4.6B, C). Mice injected with MCF10CA1a/OE RUNX1 cells had a significantly reduced tumor size (57%) and weight (47%) compared with tumors from control mice. Figures 4.7A and 4.7B show the excised tumors and luminescence of tumors in all seven mice from each group. MCF10CA1a cells with EV or OE RUNX1 were validated before injection into the SCID mice (Figure 4.7C). Luminescent images of representative mice (Fig. 4.6D) confirm reduced tumor growth. Collectively, these data indicate that RUNX1 suppresses breast tumor growth *in vivo*.
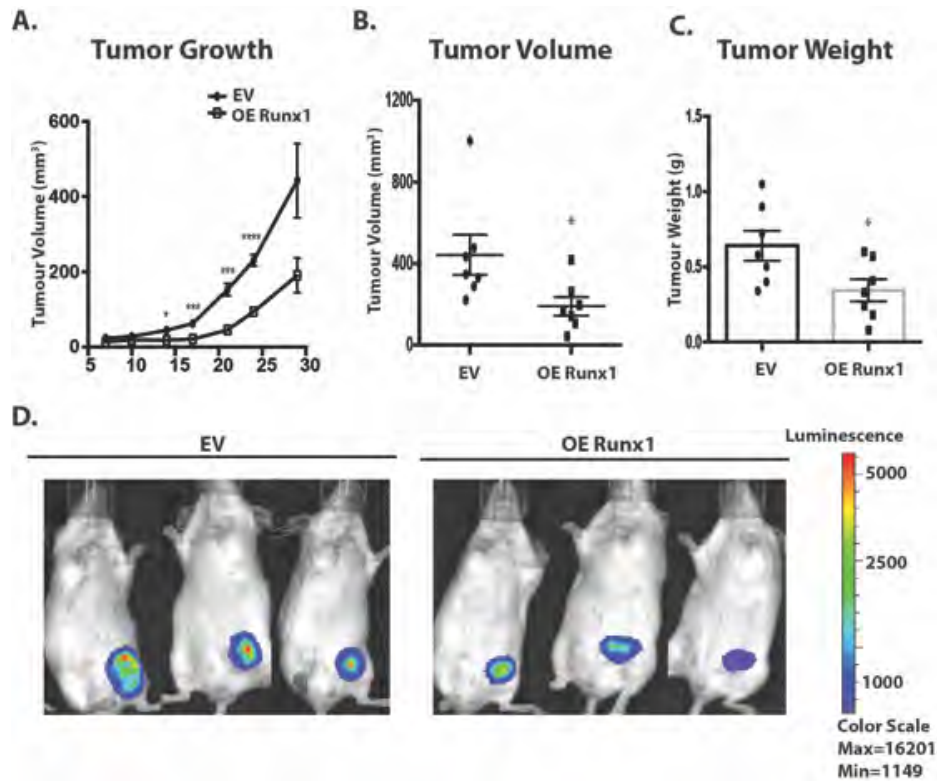
Figure 4.6. RUNX1 represses tumor growth *in vivo*. (A) A total of 1 ×

10^6 MCF10CA1a cells with EV or RUNX1 overexpression were injected

into mammary fat pad of SCID mice (*n* = 7 in each group). The points

represent average tumor volume at each time point ± SD. *P* values were

obtained by 2-tailed Student *t* test. *, *P* < 0.05; ***, P<0.001; ****,

P<0.0001. **(B)** Tumor size measured at day 28 (end point). *P* values

were obtained by 2-tailed Student *t* test. *, *P* < 0.05. **(C)** Tumor weight at

day 28 (end point). *P* values were obtained by 2-tailed Student *t* test.

*, *P* < 0.05. **(D)** Representative luminescence images at 4 weeks after

mammary fat pad injection.
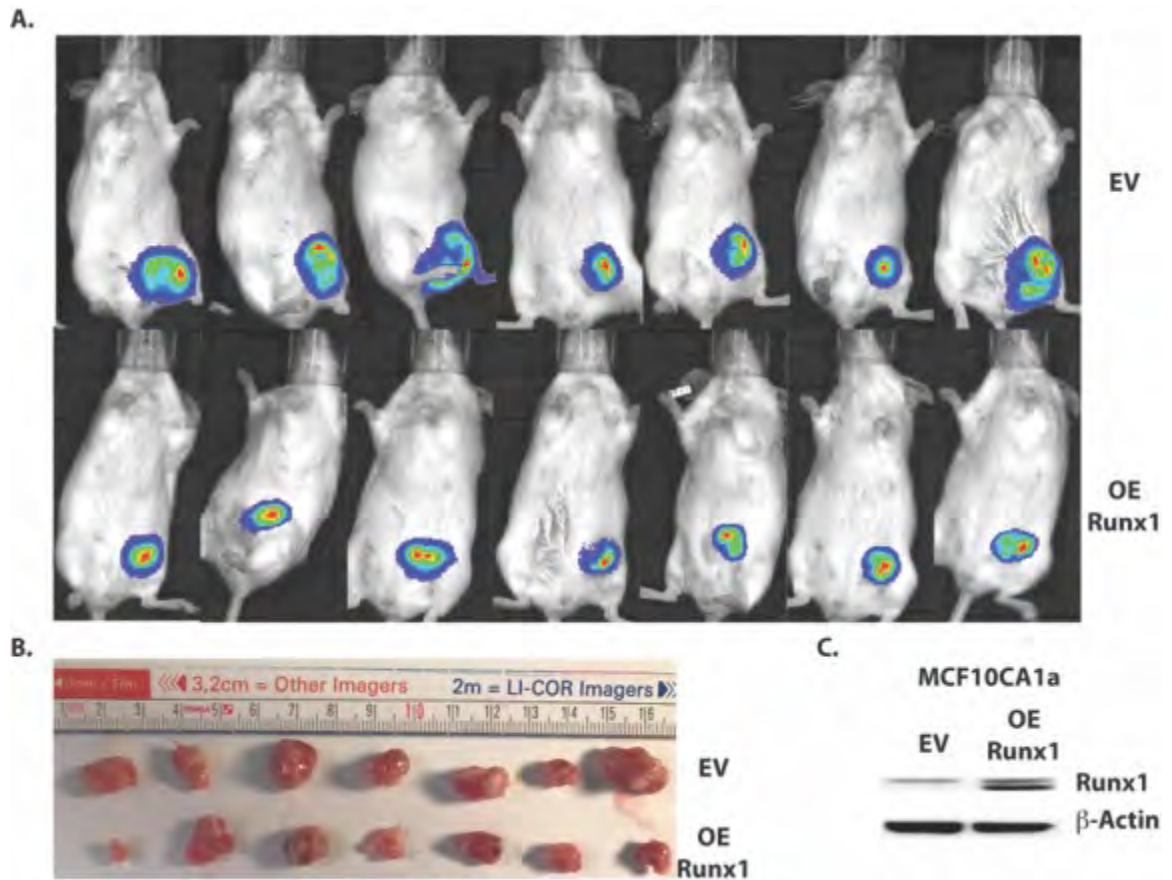
Figure 4.7. RUNX1 represses tumor growth in mammary fat pad. (A) Luminescence images at 4 weeks after mammary fat pad injection. **(B)** Picture of excised tumors show that MCF10CA1a cells with RUNX1 overexpression formed smaller tumors in mice mammary fat pad. **(C)** Western Blot for MCF10CA1a cells shows RUNX1 is overexpressed.

4.4.5. RUNX1 level is decreased in breast cancer stem cells (BCSC).

As breast cancer stem cells have been shown to be critical for tumor initiation and growth (Shibue and Weinberg 2017) and all of our data demonstrate a role for RUNX1 in decreasing tumorigenesis, we next investigated the potential role of RUNX1 in breast cancer stemness. We used fluorescence-activated cell sorting (FACS) to isolate BCSCs from pre-malignant MCF10AT1 cells based on expression of the cell-surface antigen markers CD44 and CD24. These two markers have been successfully used to identify putative CSCs in primary breast tumors or mammary cell lines (CD44^{high}/CD24^{low}). We compared the BCSC cells with bulk cells (CD44^{high}/CD24^{high}) as gated in Figure 4.8. The CD44^{high}/CD24^{low} subpopulation from MCF10AT1 cells displayed lower levels of RUNX1 protein (33%) compared to the bulk cell population and the parental MCF10AT1 cells (Fig. 4.9A). To examine whether CD24^{low} cells have low RUNX1 expression, we also performed immunofluorescence co-staining of RUNX1 and CD24 in MCF10AT1 cells. The cells with high CD24 expression also have high RUNX1 expression (Figure 4.10). Moreover, the CD44^{high}/CD24^{low} population displays many CSC-like properties; they are endowed with higher expression of cancer stem cell markers Zeb1 and Twist1 (Fig. 4.9A) and greater long-term self-renewal capacity as measured by tumorsphere formation assays (Fig. 4.9B). Collectively, these data provide evidence that cell populations with BCSC properties express lower levels of RUNX1 compared to the bulk and parental population, and suggest that RUNX1 influences BCSC properties.
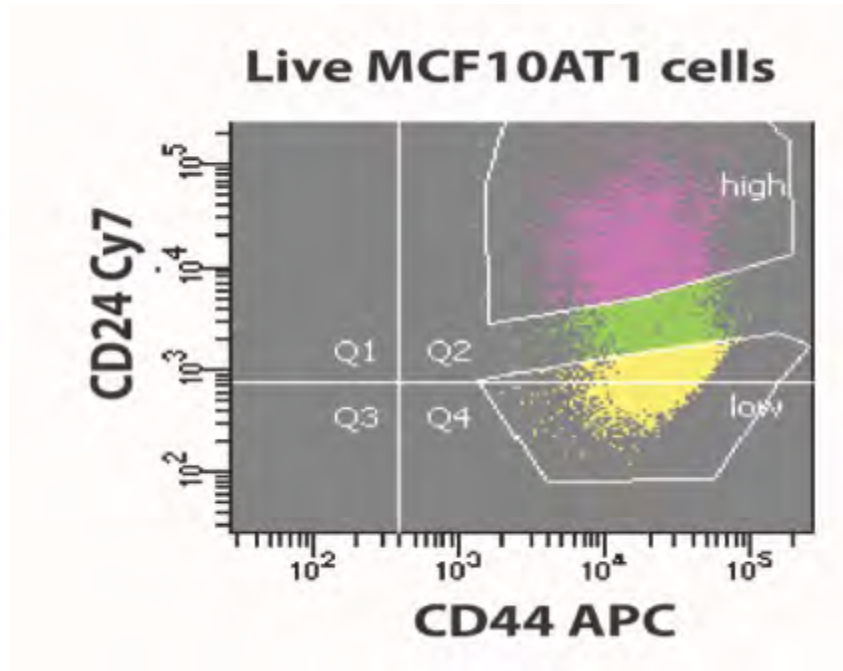
Figure 4.8. Gate for MCF10AT1 sorting and MCF10CA1a cells have high BCSC population. A. Gating for BCSC and Bulk sub-population in MCF10AT1 cells.
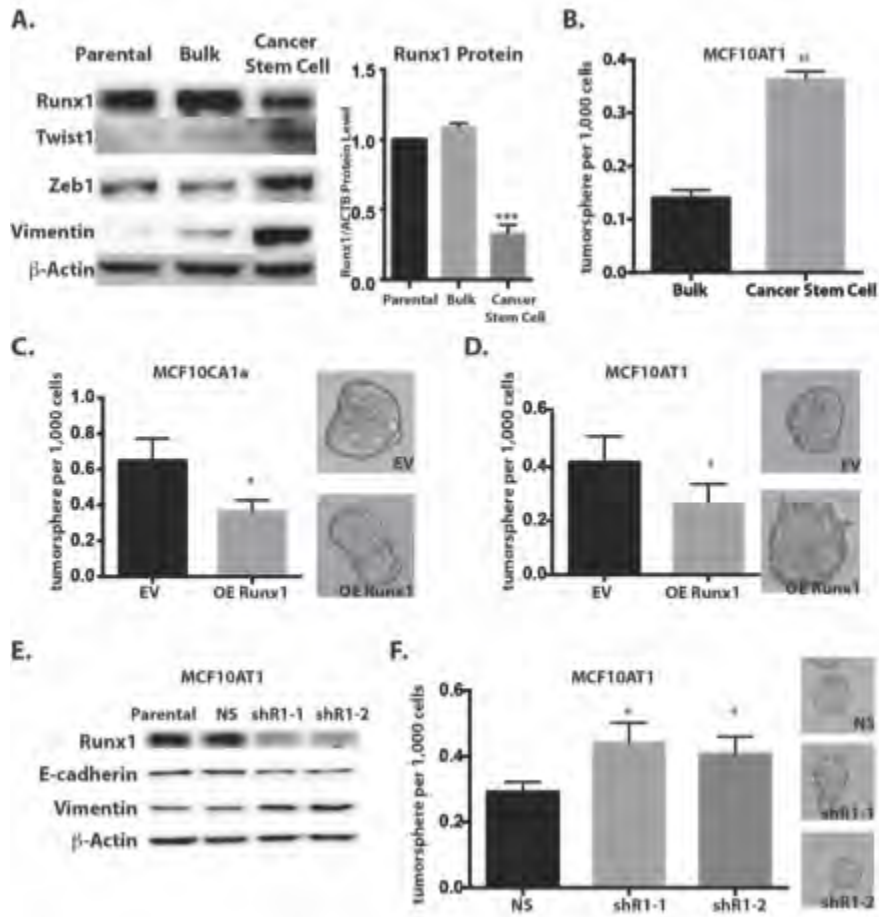
Figure 4.9. RUNX1 level is decreased in BCSC. (A) Western blot analyses show RUNX1 is decreased and Zeb1, Twist1 and Vimentin level are increased in BCSC samples compared to Parental and Bulk MCF10AT1 cells. Right, protein quantification shows that RUNX1 is significant decreased in BCSC. **(B)** Tumorsphere formation efficiency for BCSC populations is significantly higher than bulk population. **$P < 0.01$. **(C)** RUNX1 overexpression in MCF10CA1a cells reduces tumorsphere formation efficiency. *$P < 0.05$. Right, represent picture of tumorsphere. **(D)** RUNX1 overexpression in MCF10AT1 cells reduces tumorsphere formation efficiency. *$P < 0.05$ Right, represent picture of tumorsphere. **(E)** Western blot analyses of lysates from MCF10AT1 cells treated with shRunx1 show decreased protein expression of RUNX1 and E-cadherin and increased protein expression of Vimentin. **(F)** RUNX1 knockdown in MCF10AT1 cells activates tumorsphere formation efficiency. *$P < 0.05$. Right, represents picture of tumorsphere. All the experiments are performed 3 times (N=3).
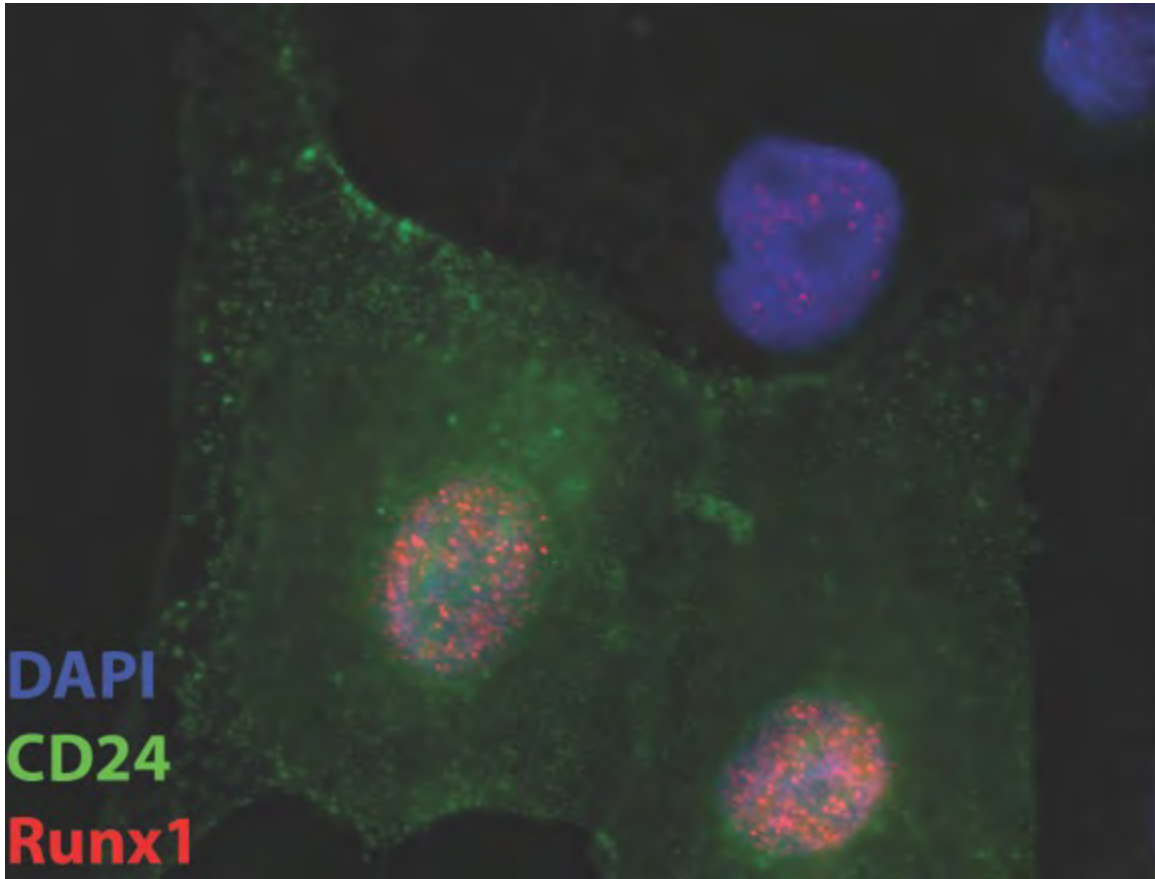
Figure 4.10. CD24^{high} Cells have high RUNX1 expression in MCF10AT1 cells. Immunostaining shows the cells with CD24 (Green) expression have high RUNX1 (Red) expression. All the experiments are performed 3 times (N=3).

4.4.6. RUNX1 inhibits stemness properties in breast cancer cells

To further investigate the role of RUNX1 in regulating BCSC properties, we addressed the capability of RUNX1 to regulate tumorsphere formation from breast cancer cells. Tumorsphere formation assays were performed using non-adherent plates with non-serum medium. The ectopic expression of RUNX1 in both MCF10CA1a and MCF10AT1 cells significantly decreased the number of tumorsphere ($p < 0.05$) (Fig. 4.9C, D). To better understand if RUNX1 represses stemness properties in breast cancer, we used two lenti-viruses to establish RUNX1 knockdown cell lines in MCF10AT1 cells (Fig. 4.9E). Depletion of RUNX1 in these cell lines activated an epithelial to mesenchymal transition with lower E-cadherin and higher Vimentin expression (Fig. 4.9E). Significantly, the knockdown of RUNX1 resulted in increased tumorsphere formation efficiency in MCF10AT1 cells (51% and 41% respectively) (Fig. 4.9F). This ability of RUNX1 to repress stemness properties was also observed in additional cell lines, including normal-like MCF10A cells and ER positive luminal-like MCF7 cells (Figure 4.11A, B), which suggests that RUNX1 suppression of stemness is a universal phenotype in breast cancer cells.
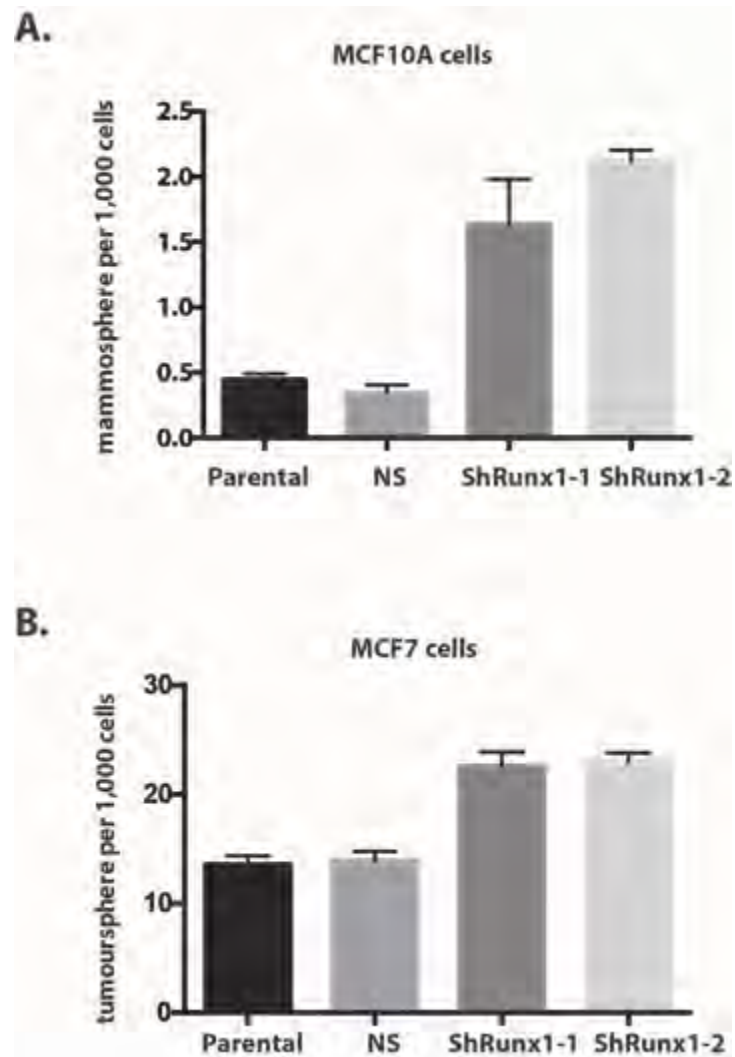
Figure 4.11. Loss of RUNX1 promotes stemness in MCF10A and MCF7 cells. (A) RUNX1 knockdown in MCF10A cells activates tumorsphere formation efficiency. **(B)** RUNX1 knockdown in MCF107 cells activates tumorsphere formation efficiency. All the experiments are performed 3 times (N=3).

Further evidence for the influence of RUNX1 on the cancer stem cell population in MCF10AT1 cells was provided by flow cytometry analysis. As shown in Figure 4.12A, ectopic expression of RUNX1 reduced the CD44^{high}/CD24^{low} subpopulation of MCF10AT1 cells from 22.3% to 15.1% (Fig. 4.12A). Consistent with the consequence of RUNX1 overexpression, knockdown of RUNX1 significantly increased the CD44^{high}/CD24^{low} subpopulation of MCF10AT1 cells by more than two-fold (21.9% ns; 45.3% shR1-1; 45.6% shR1-2) (Fig. 4.12B). However ectopic expression of RUNX1 in MCF10CA1a cells did not change the percent of the CD44^{high}/CD24^{low} cancer stem cell population (Figure 4.13). The highly metastatic MCF10CA1a cells have a large percentage of cells (80%) that are CD44^{high}/CD24^{low}, indicating that the cells may have lost their plasticity and are locked into a mesenchymal phenotype (Figure 4.13). These results indicate that RUNX1 functions both to suppress cancer stem cell properties and reduce the breast cancer stem cell population.
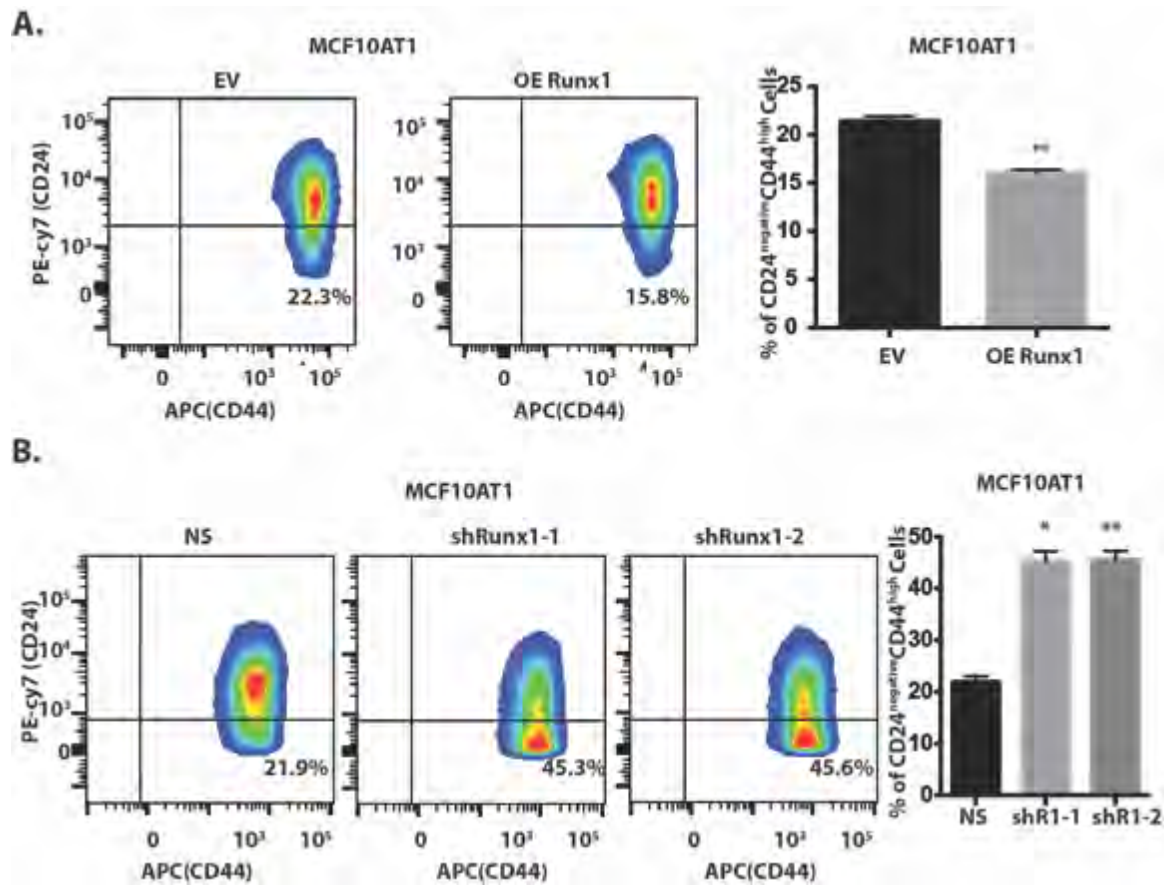
Figure 4.12. RUNX1 reduces BCSC sub-population. (A) Flow cytometric analysis of CD44 and CD24 expression in MCF10AT1 cells with EV or RUNX1 overexpression. **(B)** Flow cytometric analysis of CD44 and CD24 expression in MCF10AT1 cells stably expressing RUNX1 or non-silencing shRNAs. All the experiments are performed 3 times (N=3).
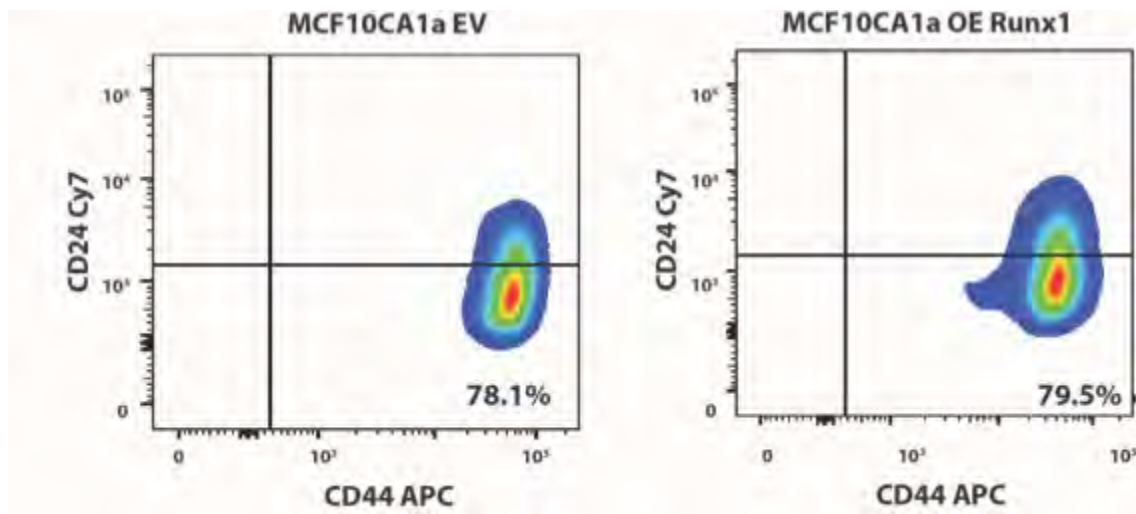
Figure 4.13. Overexpression RUNX1 in MCF10CA1a cells does not change BCSC population. Flow cytometric analysis of CD44 and CD24 expression in MCF10AT cells stably expressing non-silencing (Left) or RUNX1 (Right) shRNA. All the experiments are performed 3 times (N=3).

4.4.7. RUNX1 represses the expression of Zeb1 in breast cancer cells.

In Figure 4.9A, we observed that decreased RUNX1 expression is coincident with activation of Zeb1 in BCSC in MCF10AT1 cells. Zeb1 is well known for its function in promoting EMT, cancer stemness and metastasis in breast cancer (Zhang, Sun et al. 2015). Therefore, we tested whether RUNX1 functions by negatively regulating Zeb1 expression in breast cancer cells. Zeb1 protein is down regulated when RUNX1 is ectopically expressed in MCF10AT1 cells (Fig. 4.14A). This RUNX1-mediated Zeb1 repression was confirmed in MCF10AT1 RUNX1 knockdown cells, where Zeb1 expression is enhanced (Fig. 4.14B). We did not

observe RUNX1 repression of Zeb1 expression in MCF10CA1a cells, which is a consequence of very low Zeb1 mRNA levels in MCF10CA1a cells compared to MCF10AT1 cells (Figure 4.15). To test whether RUNX1 can directly regulate Zeb1 in MCF10CA1a cells, we performed ChIP-qPCR for RUNX1 in the Zeb1 promoter region in both MCF10AT1 and MCF10CA1a cells (Figure 4.16). As shown in Fig. 4.14C, RUNX1 directly binds to the Zeb1 promoter in the two breast cancer cell lines relative to two negative control genes ZNF333 and ZNF180. Upon RUNX1 overexpression, the binding of RUNX1 is enhanced on Zeb1 promoter, suggesting that RUNX1 has potential to directly regulate Zeb1 expression in both pre-malignant and metastatic breast cancer cell lines.

In summary, our findings suggest that RUNX1 reduces breast cancer aggressive phenotypes both *in vivo* and *in vitro*. Both EMT and cancer stem cell properties are repressed by RUNX1 in breast cancer cells. We thus conclude RUNX1-mediated repression could be through negative regulation of Zeb1 expression in breast cancer cells (Fig. 4.14D). Zeb1 is well known for activating both EMT and cancer stem cells in breast cancer. (Zhang, Sun et al. 2015) Therefore RUNX1 indirectly represses these two cellular processes. It has been shown that RUNX1 can directly repress EMT in breast cancer (Hong, Messier et al. 2017). It is possible that RUNX1 can directly repress cancer stem cell phenotype in a Zeb1-independent manner (Fig. 4.14D). This study provides new insight into functional mechanisms of the RUNX1 transcriptional regulator in contributing to the stemness and the plasticity of breast cancer stem cells.
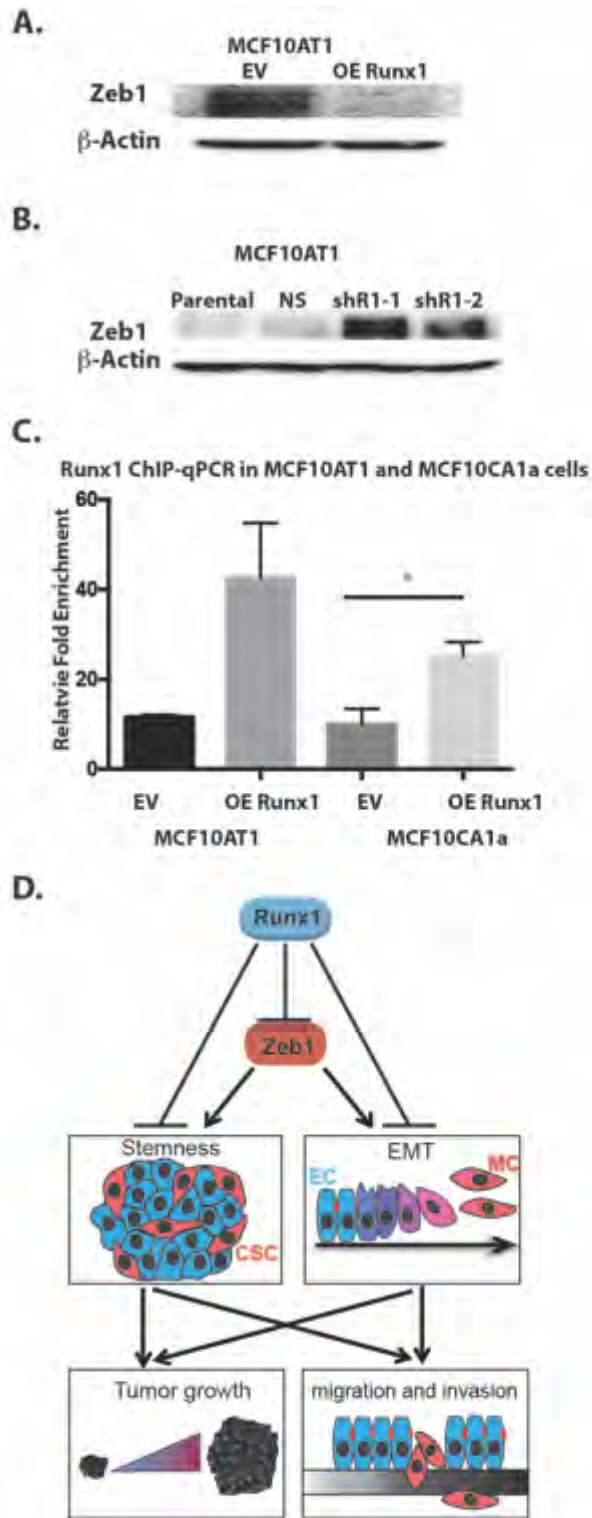
Figure 4.14. RUNX1 negatively regulates Zeb1 expression. (A) Western blot analyses show Zeb1 is decreased upon RUNX1 overexpression in MCF10AT1 cells. **(B)** Western blot analyses show Zeb1 is activated upon RUNX1 knockdown in MCF10AT1 cells. **(C)** ChIP-qPCR confirmation of RUNX1 occupancy at Zeb1. RUNX1 binding is increased in RUNX1 overexpression samples. Data obtained with antibodies against RUNX1 are normalized to input control and ZNF188 (NC1) and ZNF333 (NC2), which were used as the negative control as RUNX1 are predicted not to bind these genes. **(D)** Mechanism on how RUNX1 represses tumor growth in breast cancer. (EC- epithelial-like cells; MC-mesenchymal-like cells). All the experiments are performed 3 times (N=3).
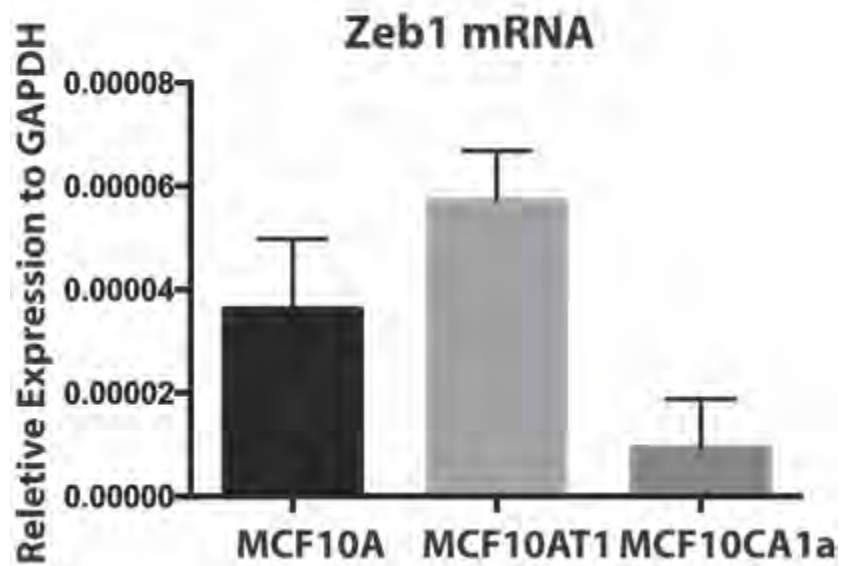
Figure 4.15. Zeb1 is expressed at low level in MCF10CA1a cells.

Zeb1 RNA expression by RT-qPCR of normal mammary-like MCF10A cells, MCF10A-derived tumorigenic cell line MCF10AT1, and metastatic MCF10CA1a cells shows Zeb1 is expressed at a low level in MCF10CA1a cells.
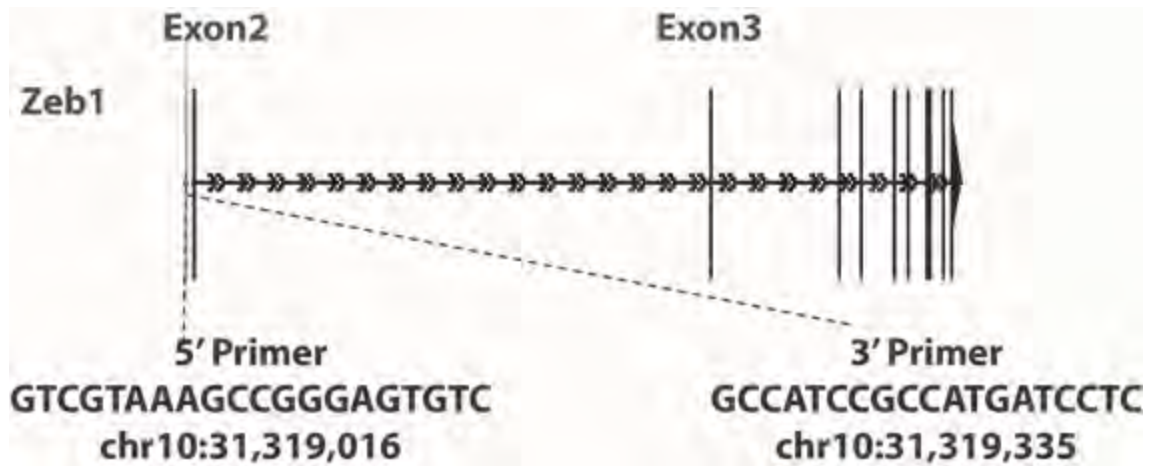
Figure 4.16. Schematic diagram of ChIP qPCR primers and amplicons over Zeb1 for ChIP-qPCR.

4.5 Discussion for Chapter IV:

We provide multiple lines of evidence that RUNX1 reduces breast cancer cells grown in mouse mammary fat pad and inhibits breast cancer stem cell phenotypes. RUNX1 levels are decreased in tumors grown in murine mammary fat pads. RUNX1 also reduces cell migration and invasion of breast cancer cells *in vitro* and tumor growth *in vivo*. Moreover, RUNX1 reduces the breast cancer stem cell population and tumorsphere formation efficiency, thus indicating that RUNX1 represses stemness properties in breast cancer. RUNX1 overexpression and knockdown studies revealed that RUNX1 mediates the mechanisms of inhibition of breast cancer stemness and tumorigenesis through repression of Zeb1

expression. Taken together, our findings provide compelling evidence that the loss of RUNX1 induces increased cancer stem cells and that RUNX1 overexpression can suppress the CSC population, which is responsible for metastasis, treatment resistance and tumor recurrence in breast cancer.

Breast cancer is ranked as the second leading cause of cancer death in women after lung cancer (Torre, Bray et al. 2015). In 2017, approximately 63,400 cases of female breast carcinoma in situ are expected to be diagnosed (Siegel, Miller et al. 2017). Despite the significant advances that have been achieved in early detection and treatment of breast cancer, understanding the mechanisms of breast cancer progression and metastasis still requires intensive study. Recently, using sophisticated next-generation sequencing technology, a 40 mutation-driver gene list was generated in human breast cancer (Pereira, Chin et al. 2016). RUNX1, which is often mutated in breast tumors, is one of those genes. Utilizing the TCGA clinical data sets, we found that reduced RUNX1 levels in tumor correlate with poor survival of breast cancer patients. Together these clinical findings suggest that RUNX1 may be a promising therapeutic biomarker for breast cancer screening and personalized medicine.

An unresolved question is whether RUNX1 functions to promote or suppress tumor growth in breast cancer. Increasing evidence indicates that loss of RUNX1 function accompanies progression of breast cancer (van Bragt, Hu et al. 2014, Chimge, Little et al. 2016, Hong, Messier et al. 2017), supporting the concept that RUNX1 suppresses tumor growth. Clinically, RUNX1 expression is decreased in

high histological grade tumors compared with low/mid-grade tumors (Kadota, Yang et al. 2010). In the past few years, RUNX1 loss-of-function somatic mutations have been identified in several subtypes of breast cancer (Network 2012)(Banerji, Cibulskis et al. 2012)(Ellis, Ding et al. 2012). Mechanistically, loss of RUNX1 in ER+ breast cancer activates the WNT signaling pathway and ELF5 expression (van Bragt, Hu et al. 2014)(Chimge, Little et al. 2016) suggesting that RUNX1 represses breast cancer progression. Our previous study showed loss of RUNX1 promotes EMT in both normal and breast cancer cells indicating that RUNX1 has the potential to inhibit tumor growth (Hong, Messier et al. 2017). In this study, we clearly demonstrated that the level of RUNX1 is decreased during tumor growth, and that ectopic RUNX1 expression suppresses tumor growth in the mouse mammary fat pad. Together these combined studies and our experiments establish that RUNX1 reduces aggressive phenotype in breast cancer. However, we cannot rule out the possibility that RUNX1 may have other functions in breast cancer, especially in late stage disease. For example, in the MMTV-PyMT mouse model, the level of RUNX1 is positively correlated with tumor progression (Browne, Taipaleenmäki et al. 2015) and regulates genes promoting tumor growth in late stage MDA-MB-231 breast cancer cells (Recouvreux, Grasso et al. 2016). However, in our study, we found that metastatic MCF10CA1a cells with RUNX1 overexpression formed smaller tumors in mouse mammary fat pad indicating that RUNX1 functions to reduce tumor growth. These contradictory results suggest

that RUNX1 has dual functions (pro- vs anti-tumor growth) in late stage breast cancer depending on cellular context.

The anti-tumor growth activity of RUNX1 in breast cancer is likely through its properties in maintaining the normal mammary epithelial phenotype. For example, loss of RUNX1 causes the cells to lose their epithelial morphology and activates mesenchymal genes in normal-like MCF10A cells (Hong, Messier et al. 2017). Furthermore, depletion of RUNX1 in ER positive luminal MCF7 breast cancer cells transforms the cells into a partial EMT state (Hong, Messier et al. 2017). It has been suggested that partial activation of the EMT promotes plasticity that allows reprogramming of the epithelial cell to acquire both migratory and stem-like features (Grigore, Jolly et al. 2016).

We investigated whether RUNX1 might function by suppressing Zeb1, due to its well-known activity in increasing breast cancer stemness and as a marker of EMT. Our results show that RUNX1 directly binds to the Zeb1 promoter in both MCF10AT1 and MCF10CA1a cells and that binding is enhanced upon RUNX1 overexpression. In MCF10AT1 cells, RUNX1 negatively regulates Zeb1 expression at the protein level. Together these findings indicate that the binding of RUNX1 on the Zeb1 promoter and the suppression of Zeb1 by RUNX1 reduce breast cancer stemness in cells that retain plasticity. Consistent with this conclusion, overexpressing RUNX1 in MCF10CA1a cells does not change the expression of EMT markers to the same extent that it does in premalignant MCF10AT1 cells (Fig. 4.4A). These data and the fact that RUNX1 represses EMT

in normal-like MCF10A cells (Hong, Messier et al. 2017), highlight its critical function in repressing tumor initiation and growth in early stage breast cancer. Also of significance is that overexpression of RUNX1 in MCF10CA1a cells decreased tumor growth *in vivo* and tumorsphere formation efficiency *in vitro,* suggesting that RUNX1 can reduce aggressive phenotype in late stage breast cancer cells.

In summary, our findings constitute strong experimental evidence that RUNX1 functions to reduce aggressive phenotype of breast cancer cells. This study provides a novel dimension to understanding how the transcriptional regulator RUNX1 contributes to the stemness and the plasticity of breast cancer stem cells. Together, these data support a central role for RUNX1 in preventing breast cancer progression. Both tight control of RUNX1 expression and RUNX1 functional integrity are required to prevent breast cancer malignancy. Consequently, clinical strategies should consider RUNX1 as a biomarker and potentially as a therapeutic candidate.

Chapter V Discussion and future direction

5.1. Results summary

The results of my dissertation studies have uncovered novel functions of RUNX1: a) in the regulation of normal mammary epithelial cells; b) identifying the loss of RUNX1 during cancer progression; and c) dysregulated mechanisms caused by depletion of RUNX1. Together these findings demonstrated RUNX1 inhibits the breast cancer development.

In chapter II of this dissertation, we investigated the consequences of the loss of RUNX1 in both mammary epithelial and breast cancer cells. In the normal mammary epithelial MCF10A cells, we observed that depletion of RUNX1 changes the morphology of cells from epithelial-like to mesenchymal-like, and loss of RUNX1 initiates EMT in both normal epithelial and breast cancer cells. We also discovered that RUNX1 expression was lost upon induction of EMT by two different methods, suggesting that reduction of RUNX1 expression is a hallmark of EMT initiation in these cells. Mechanistically, RUNX1 functions through both exogenous TGF-β–dependent and -independent mechanisms indicating that RUNX1 is involved in multiple signaling pathways. Taken together, our studies revealed for that RUNX1 has anti-tumor growth activities in mammary lineage cells. The dissertation studies established the concept that RUNX1 preserves the epithelial morphology and negatively regulates EMT in both normal mammary epithelial and

breast cancer cells.

In Chapter III of this dissertation, we explored whether RUNX1 regulates other cellular activities in normal mammary epithelial cells. To identify those putative functions of RUNX1 in MCF10A cells, we performed both global gene expression profiling and RUNX1 genome-wide binding analysis. Using high throughput sequencing, 1809 novel target genes that are differentially expressed upon loss of RUNX1 were identified. The pathway analysis for these genes indicated that RUNX1 regulates many aspects of cellular activities including the cell cycle and genome stability. We also performed RUNX1-ChIP-seq to study the mechanisms of RUNX1 regulated gene expression. Our results demonstrated that in normal-like mammary epithelial cells, RUNX1 may form the complexes with some of the known RUNX1 co-regulatory factors, such as AP1, TEAD4 and STAT5. RUNX1 may also interact with some factors, that have not previously been identified, such as NFIA. Our results also indicate that in MCF10A cells, the primary function of RUNX1 is to activate target gene expression. RUNX1 may primarily repress target gene expression in an indirect manner. Using Flow Cytometry analysis, we demonstrated that RUNX1 loss results in a significant reduction of mitotic cells, with the percentage of mitotic cells reduced from 2.5% in parental and non-silencing control to 1.4% in shRunx1 cells which is greater than 40% decrease. Consistent with G2/M arrest, commonly associated with genome instability, the ablation of RUNX1 decreased the expression levels of multiple DNA-repair related genes. Moreover, after treating the cells with a DNA-damaging agent, the DNA

repair process was compromised in Runx1 depleted MCF10A cells. Overall, this chapter discovered functions of RUNX1 in mammary epithelial cells such as controlling mitosis that was not previously reported.

In Chapter IV of this dissertation, we further elucidated RUNX1 function in breast cancer cells in relation to tumor growth. An important component of tumor growth, is the contribution of cancer stem cells (CSC). Because CSCs are associated with EMT, and RUNX1 is a negative regulator of EMT (Chapter II), we examined the cancer stem cell properties upon altering RUNX1 expression. Our results demonstrated that RUNX1 suppresses tumorsphere formation efficiency and the cancer stem cell population by negatively regulating Zeb1 expression. We observed that ectopic RUNX1 expression reduces migration and invasion *in vitro* and tumor growth *in vivo*, thus establishing RUNX1 reduces aggressive phenotypes in breast cancer cells. We therefore show to our knowledge for the first time, that RUNX1 inhibits the cancer stem cell phenotype in solid tumors, highlighting the potentials of RUNX1 regulating CSC in other epithelial cancers.

5.2. Significance and clinical impact

Breast cancer is the most common cancer and the second leading cause of cancer death in American women. On average 1 in 8 American women will be diagnosed with invasive breast cancer in their lifetime (Siegel, Miller et al. 2016). With the advantages of early detection and improved treatments, the 5-year survival rate of breast cancer patients has increased to 90% (Miller, Siegel et al. 2016). However, the survival rate for patients with metastatic breast cancer remains low

(22%) (Siegel, Miller et al. 2016). Therefore, understanding the mechanisms of breast cancer initiation, progression and metastasis remains an important task.

In this dissertation, the functional activities of transcription factor RUNX1 in normal mammary epithelial and breast cancer cells were examined. The results from this dissertation demonstrate that RUNX1 has tumor suppressor potential in both mammary epithelial and breast cancer cells. Loss of RUNX1 expression initiates EMT and deregulates cell cycle. Moreover, overexpressing RUNX1 in breast cancer cells represses cancer stem cell phenotype and tumor growth *in vivo.* The data from patient samples further suggests that RUNX1 expression and its normal function are clinically relevant in breast cancer prognosis. My analyses from public data sets showed low RUNX1 expression in patient tumors is associated with poor survival. Therefore, we propose that RUNX1 could translate into a new prognostic biomarker in breast cancer and potentially be a therapeutic target.

Mutations of RUNX1 and its partner CBFβ account for 24% of adult AML cases (Look 1997) and 25% of pediatric ALL cases (Loh, Goldwasser et al. 2006). Thus, drug developments targeting the RUNX1 mutation or the interaction between RUNX1 and CBFβ currently are a priority focus for finding treatments for various types of leukemia. For instance, a small molecule AI-10-49, which selectively binds to a CBFβ mutant (CBFβ–SMMHC) and disrupts its binding to RUNX1, delays leukemogenesis in mice (Illendula, Pulikkan et al. 2015). Another compound 7.44, a small molecule disrupting RUNX1-ETO tetramerization, also

suppresses leukemogenesis both *in vitro* and *in vivo* (Schanda, Lee et al. 2017*)*.

Besides above-mentioned small molecules targeting RUNX1 or CBFβ mutation, small compounds, such as AI-4-57 and Ro5-3335, which both specifically block the Runx-CBFβ interaction, inhibit the growth of leukemia cell lines *in vitro* (Cunningham, Finckbeiner et al. 2012, Illendula, Gilmour et al. 2016). Therefore, RUNX1 is a promising target for intervention in leukemia.

To date, few efforts have been employed to specifically target RUNX1 in breast cancer cells. Therefore, developing small molecules that specifically target RUNX1 to activate its expression can be a new therapeutic direction for breast cancer prevention and intervention, as indicated by our Runx1 repletion studies in mice (Fig. 4.5). Recently a study shows that a small molecule T63 activates Runx2 expression and therefore attenuates the loss of bone mass (Zhao, Chen et al. 2017). Same strategy, identifying small molecules promote Runx1 expression, could apply to prevent loss of Runx1 induced disease.

5.3. Open questions and future directions

In this dissertation, we investigated the importance of RUNX1 in both normal epithelial and breast cancer cells. We identified several novel RUNX1 functions including repressing EMT and suppressing the cancer stem cell phenotype. However more work is needed to paint the full picture of function(s) of RUNX1 in normal mammary epithelial cells and in progression of breast cancer.

What are the up-steam regulators of RUNX1?

An interesting direction for future research is to determine the upstream regulator(s)

of RUNX1. There are many known transcription factor regulatory elements in the

two RUNX1 promoters, as well as co-regulatory factors, histone modifications and

enhancers which are found across the RUNX1 gene, all of which contribute to

regulation of RUNX1 expression. In hematopoietic cells, RUNX1 is up regulated

by a RUNX1 intronic cis-regulatory element (+23 RUNX1 enhancer) (Bee, Ashley

et al. 2009). This enhancer contains conserved motifs that bind various

hematopoiesis related regulators such as Gata2, ETS, and RUNX1 itself acting in

an auto-regulatory loop (Nottingham, Jarratt et al. 2007, Bee, Ashley et al. 2009).

It is unclear whether this auto-regulatory mechanism also operates in mammary

cells, and if so what factor(s) bind to +23 RUNX1 enhancer? In the mammary gland,

RUNX1 is precisely regulated as its level fluctuates in pregnancy and lactation (van

Bragt, Hu et al. 2014, Rooney, Riggio et al. 2017). RUNX1 is highly expressed in

the basal lineage compared with the luminal lineage, suggesting a mechanism that

either activates RUNX1 in basal cells or inactivates it in luminal cells (van Bragt,

Hu et al. 2014).   However, it is unclear what transcription factor(s) control(s)

RUNX1 expression in mammary cells, especially in basal/ myoepithelial cells. In

breast cancer, RUNX1 is often mutated and its level is decreased compared with

normal mammary epithelial cells (Chapter II and Chapter III). The mechanisms

driving the loss of RUNX1 expression in breast cancer cells are still unknown, but

may involve multiple mechanisms including protein degradation by the proteasome,

inhibited translation by miRNAs, the removal of an activator, the binding of a repressor transcription factor, DNA hypermethylation, and(or) altered histone modifications.

We performed transcription factor binding prediction analysis on the sequences within 1kb upstream of the RUNX1 P1 promoter and identified potential binding sites of 66 transcription factors (Fig. 5.1). Among those transcription factors, some such as ERα, STAT，GATA1, are well known for their physical interactions with Runx1 protein and their roles in breast cancer (Elagib and Goldfarb 2007, Stender, Kim et al. 2010, Scheitz, Lee et al. 2012, Li, Ke et al. 2015, Banerjee and Resat 2016). However, whether these 66 factors are actually functional in the mammary lineage, and whether they are positive or negative regulators of RUNX1 requires further examination.

In Chapter II, we showed that TGF-β is one of the upstream regulators of RUNX1 in mammary epithelial cells. The level of RUNX1 is decreased upon TGF-β treatment and overexpressing RUNX1 in TGF-β treated cells reversed the EMT phenotype. These data clearly demonstrate that RUNX1 is downstream of the TGF-β signaling pathway and that down-regulation of RUNX1 is necessary for the activation of TGF-β induced EMT. Estrogen is another upstream regulator of RUNX1 (Vivacqua, De Marco et al. 2015), as treating MCF7 cells with 17-β-estradiol, decreases the level of RUNX1. However which activators support RUNX1 expression in mammary lineage requires exploration; therefore, identifying the possible positive regulator(s) in normal mammary epithelial cells is necessary

for strategizing to protect RUNX1 expression in mammary gland and for breast

cancer intervention.
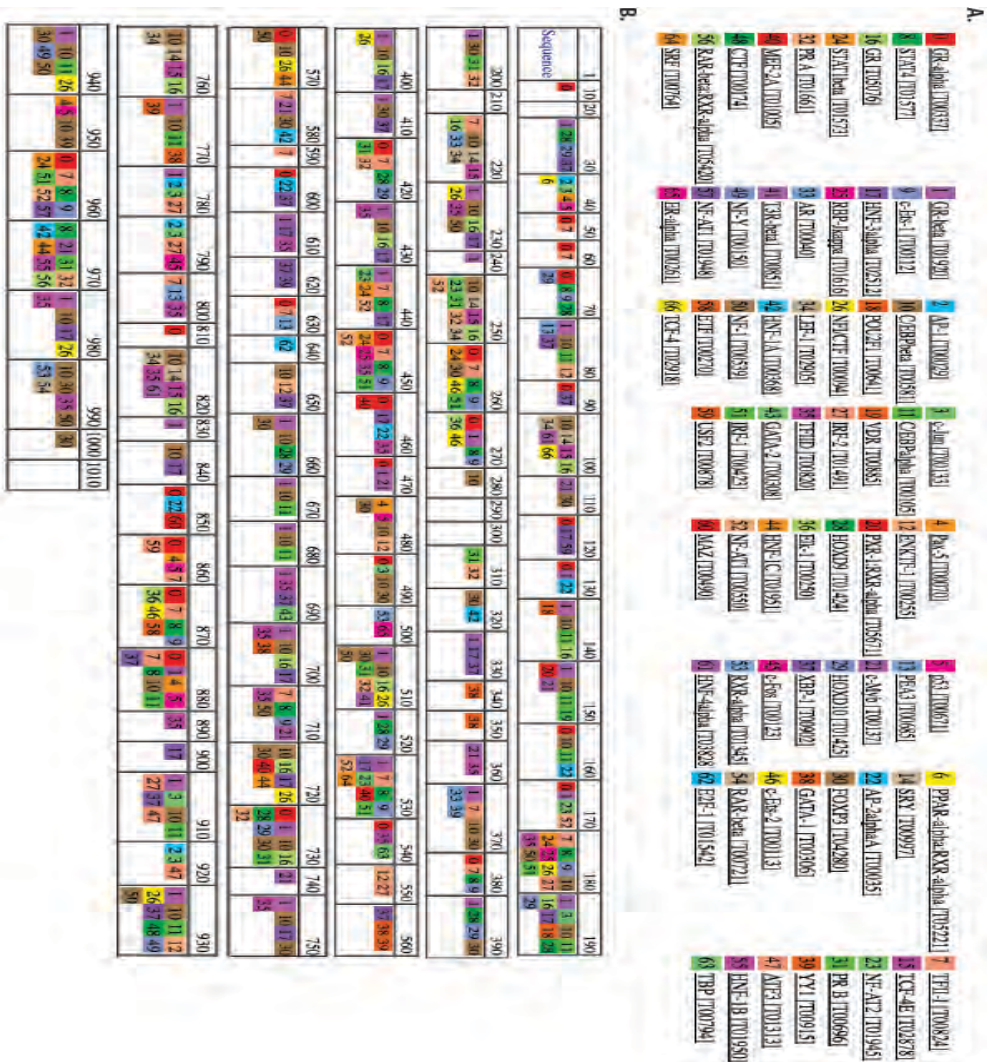
Figure 5.1 Potential RUNX1 regulators locate within 1kb upstream of RUNX1 promoter. (**A**) List of transcription factors with predicted binding sites within 1kb upstream of RUNX1 promoter. (**B**) Diagram of the location of the predicted binding sites of each transcription factor within 1kb upstream of RUNX1 promoter.

What are the co-regulatory partners of RUNX1 in different cellular contexts?

RUNX1 even with its co-regulatory partner CBF-β, is still not a strong DNA binding protein and primarily functions through interacting with diverse transcription factors, such as AP-1, GATA-1 and STAT (Pencovich, Jaschek et al. 2011, Scheitz, Lee et al. 2012, Chuang, Ito et al. 2013). Therefore, the complexity of RUNX1 regulatory mechanisms relies on the composition of its binding partners. The Runx1 binding partners are usually transcription factors, thereby giving RUNX1 the capability to temporally regulate target gene expression. Motifs of some transcription factors, such as STAT and AP-1, were identified in our motif analysis on RUNX1 peaks as co-localizing with RUNX1 motif, suggesting they have the potential to form complexes with RUNX1. Depending on cellular context in different subtypes or stages of breast cancer, RUNX1 may form transcription regulatory complexes with distinct co-activators or co-repressors. Thus, the diversity in binding of cofactors including histone modifiers, may explain the contradictory reports that RUNX1 has anti-tumor growth activity in mammary

epithelial cells and is tumor-promoting in late stage triple-negative breast cancer {Chuang, 2013 #237}. It would be informative to determine the components of RUNX1 transcription complexes by Runx1 immunoprecipitation in different breast cancer cell lines representing distinct subtypes and disease stages.

Does RUNX1 have a function in mitosis?

In Chapter III, we demonstrated that loss of RUNX1 decreases mitotic population in MCF10A cells by more than 40%. Therefore, is RUNX1 required for mitosis? If so, what function does RUNX1 play in mitotic cells? During mitosis, some regulatory complexes remain bound to the condensed chromatin for rapid reactivation of genes following mitosis which is define as mitotic bookmarking (Zaidi, Young et al. 2010).

Runx2, another lineage specific Runx factor, is well known for its association with RNA Pol I-transcribed ribosomal RNA genes and RNA Pol II-transcribed phenotype-specific genes during mitosis (Young, Hassan et al. 2007, Young, Hassan et al. 2007). Is RUNX1 also involved in mitotic bookmarking? Nancy Speck's group showed that in RUNX1 deficient hematopoietic stem and progenitor cells, ribosome biogenesis is reduced, with lower rRNA and ribosomal protein mRNA levels (Cai, Gao et al. 2015). Moreover, from our RNA-seq data, we also observed that upon RUNX1 knockdown, the transcription of majority of ribosomal proteins is inhibited (Fig.5.2). It will be worth investigating whether RUNX1 is a mitotic bookmarking factor in mammary epithelial cells and identify the genes that RUNX1 occupies during mitosis. One possible strategy is to perform Runx1-ChIP-

seq in the cells blocked in mitosis with Nocodazole. We can identify the genes be

bound by Runx1 during mitosis. We can compare the expression of levels of these

genes during mitosis in both control and RUNX1 depleted cells. It will be interesting
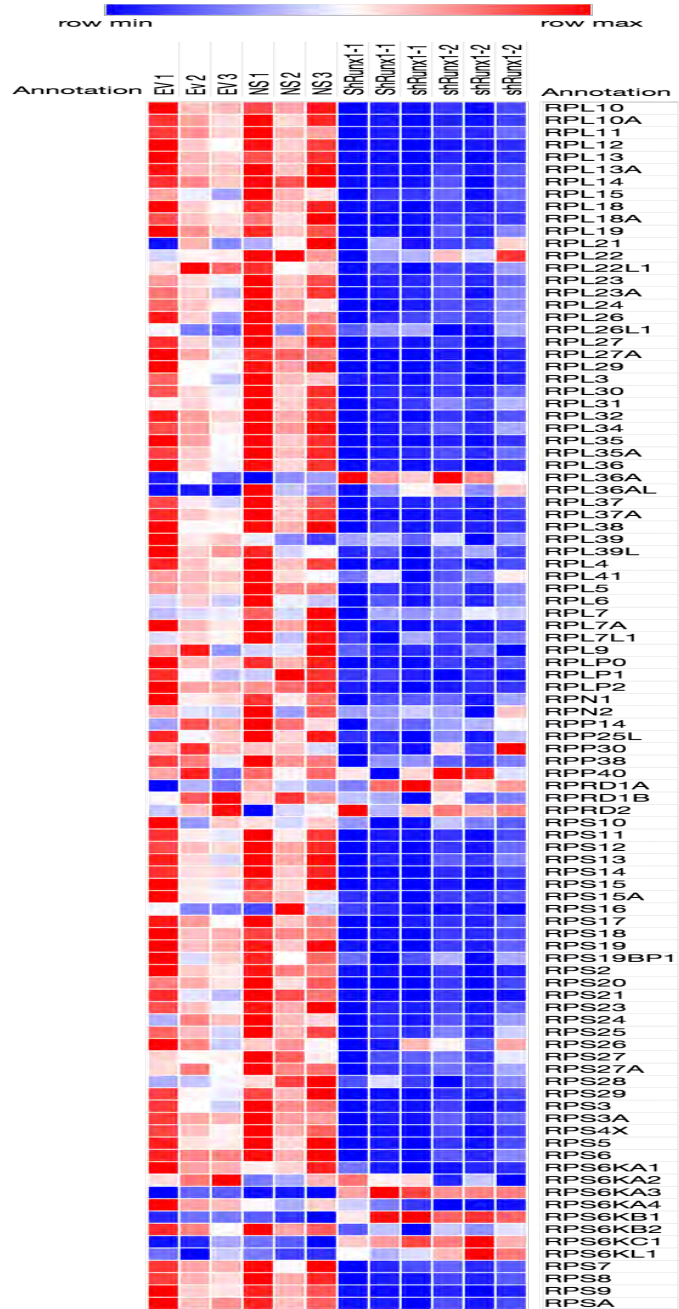
Figure 5.2 Heat map of changes in ribosome protein mRNAs. Heatmap

of changes in ribosome protein mRNAs from control (NS, EV) or shRunx1

(shRunx1-1, shRunx1-2) MCF10A cells.

to test whether these genes still transcribed /translated properly without RUNX1

binding during mitosis?

What is the mechanism(s) of RUNX1 controlled genome stability?

Decreased genome stability is a hallmark of cancer (Hanahan and Weinberg 2011).

In Chapter III, we showed that loss of RUNX1 may lead to genome instability as

DNA damage repair is slowed down in RUNX1 depleted cells. The exact

mechanism(s) of RUNX1 controlled genome stability requires further exploration.

Many mechanisms are involved to drive genome instability at both the

chromosomal and nucleotide levels (Lee, Choi et al. 2016). Genomic instability at

the nucleotide level is frequently represented in the hyper-mutation phenotype

(Roberts and Gordenin 2014). Most of the mutations are caused by the defect of

DNA repair pathways (Lee, Choi et al. 2016).

Nevertheless, sequencing data from cancer patients have identified the

existence of mutations densely clustered in short DNA segments which cannot be

explained by DNA repair defect (Nik-Zainal, Alexandrov et al. 2012)(Roberts,

Sterling et al. 2012). Later, it was identified that members of Apolipoprotein B

editing complexes (APOBECs) are cytidine deaminases (Conticello 2008) that are

responsible for generating this pattern of mutation (Roberts, Sterling et al. 2012), which is wide-spread in human cancers, including breast cancer (Burns, Lackey et al. 2013, Roberts, Lawrence et al. 2013, Kanu, Cerone et al. 2016). HIV-1 protein Vif down regulates the human APOBEC3 family by targeting them for degradation (Wiegand, Doehle et al. 2004), which requires CBFβ (Zhang, Du et al. 2011, Kim, Kwon et al. 2013). Moreover CBFβ is a positive regulator for APOBEC3 transcription, as knockdown of CBFβ decreases APOBEC3 mRNA (Anderson and Harris 2015). In human breast cancer, RUNX1 levels are decreased, which may generate free-state CBFβ. It is possible that the free-state or increased CBFβ promotes APOBEC3 expression and induces genome instability by generating mutations. Thus, RUNX1 mediated APOBEC3 repression may be a new axis for controlling genome stability in breast cancer.

Is RUNX1 involved in Immune suppression?

In the past few years, new findings have led to increased attention in the mechanisms by which cancer cells with EMT phenotype might contribute to immune suppression (reviewed in (Terry, Savagner et al. 2017)). Multiple routes have been examined on the mechanisms of EMT induced tumor immune escape (Terry, Savagner et al. 2017). For instance, the EMT program can medicate cancer cell immune resistance to natural killer cells(Terry, Buart et al. 2017). Natural killer cells are the effector lymphocytes of the innate immune system, repressing tumor growth during cancer initiation and progression(Terry, Savagner et al. 2017). The EMT program can also activate immunosuppressive cytokines or immune

checkpoint ligands to modulate efficacy of immune response and its duration. For instance in triple-negative breast cancer, 20% of tumors activate the expression of one such immune checkpoint ligand, programmed cell death ligand-1 (PD-L1) (Wimberly, Brown et al. 2015) (Mittendorf, Philips et al. 2014) , which binds with its receptor PD-1 in T-cells. The binding of PD-1 and PD-L1 inhibits T-cell cytotoxic activity, resulting in a T-cell exhaustion state (Zou, Wolchok et al. 2016). Antibody blocking PD-1/PD-L1 signal clinically restores T-cell activities and represses tumor growth (Alsaab, Sau et al. 2017) . To date, nivolumab or pembrolizumb (anti-PD-1 antibody) and atezolizumab (anti-PD-L1 antibody) have been approved by the FDA to treat various metastatic cancers (Alsaab, Sau et al. 2017). In cancer, several EMT signal pathways, such as Zeb1 and TGF-β, can drive PD-L1 expression as an immune escape mechanism (Chen, Gibbons et al. 2014, Chen and ten Dijke 2016).

In chapter II, we demonstrated that RUNX1 blocks the initiation of EMT and we hypothesize that RUNX1 represses the immune surveillance both in the immune system and in cancer cells. As the master regulator of hematopoiesis, RUNX1 is essential for T-cell maturation (reviewed in (Collins, Littman et al. 2009, Hsu, Shapiro et al. 2016, Ebihara, Seo et al. 2017)). Without RUNX1, development of T-cells is blocked resulting in the loss of functional nature killer T cells (Egawa, Eberl et al. 2005, Egawa, Tillman et al. 2007). Recently, it has been shown that Runx3 is a central regulator of CD8$^+$ T cells by promoting T cell differentiation and accumulating matured CD8$^+$ T cells in tumors (Milner, Toma et al. 2017). Given

the fact that both Runx1 and Runx3 is up-regulated and required for T-cell maturation (Yu, Zhang et al. 2017). Runx1 may be also involved in resident lymphocytes in tumors. Meanwhile, in cancer cells, our preliminary data indicate that RUNX1 functions as a negative regulator of PD-L1 and other immune checkpoint ligands. In our MCF10A shRunx1 RNA-seq data, we found that loss of RUNX1 activates both PD-l1 and B7H4, another immune checkpoint ligand. However, it is unclear whether RUNX1 directly or indirectly regulates expression of these two ligands. Taken together, these data implicate that RUNX1 is a key component to repress immune escape and its exact function requires further research.

Is RUNX1 a regulator of long noncoding RNAs (lncRNAs)?

Long noncoding RNAs are greater than 200 nucleotides in length and have no protein coding capacity. They are often observed to be deregulated in a variety of cancer types. Several lncRNAs have been well document for their function during breast cancer progression (reviewed in (Cerk, Schwarzenbacher et al. 2016, Wang, Liu et al. 2016)).

Strikingly from our RNA-seq data, we observed that RUNX1 significantly altered the expression of several lncRNAs including NEAT1, MALAT1, XIST, HOTAIR, HOTAIRM1, GAS5 and ZFAS1 (Tabel 5.1). The expression patterns of these lncRNAs upon loss of RUNX1 are consistent with their patterns upon breast cancer progression. RUNX1 genomic binding analysis shows that RUNX1 directly binds to the promoters of many of these lncRNAs, such as NEAT1 and MALAT1,

suggesting transcriptional regulation by RUNX1. This may be another unidentified aspect of RUNX1 anti-tumor growth activity in breast cancer. It will be interesting to determine the extent to which RUNX1 plays a regulatory role in controlling lncRNA expression and how it relates to breast cancer progression. To test this, I will knockdown oncogenic lncRNA by Gapmer or overexpress anti-tumor lncRNA by CrisprA in RUNX1-depleted cells and examine whether phenotypes induced by loss of RUNX1 are attenuated by specific lncRNA.

LncRNA	Fold change cells in shRunx1 MCF10A cells	Runx1 Binding at Promoter	Alteration in Breast Cancer	Functions/ characteristics	References
NEAT1	+4.5	Yes	Upregulation	Higher NEAT1 indicates poor prognosis	(Choudhry, Albukhari et al. 2015)
MALAT1	+5.6	Yes	Upregulation	Pre-mRNA splicing	(Gutschner, Hämmerle et al. 2013)
XIST	-5.68	Yes	Downregulation	Related to loss of BRCA1 function	(Chaligné, Popova et al. 2015)
HOTAIR	+2.27	No	Upregulation	Promote invasiveness	(Gupta, Shah et al. 2010)
HOTAIRM1	+2.98	No	Upregulation	Correlated with HOXA1 expression	(Su, Malouf et al. 2014)
GAS5	-2.98	Yes	Downregulation	Induce growth arrest and apoptosis	(Mourtada-Maarabouni, Pickard et al. 2008)
ZFAS1	-1.93	Yes	Downregulation	Repress tumor cells growth	(Askarian-Amiri, Crawford et al. 2011)

Table 5.1 List of LncRNAs expression of which is changed upon RUNX1 knockdown in MCF10A cells and their involvement in human breast cancer.

5.4. Concluding Remarks

This thesis describes the function of RUNX1 in both mammary epithelial cells and breast cancer cells. In mammary epithelial cells, RUNX1 maintains the epithelial phenotype and loss of RUNX1 promotes EMT. Additionally, our results demonstrate the anti-tumor growth function of RUNX1 in breast cancer cells by inhibiting the cancer stem cell population. In conclusion, my thesis work provides novel and significant insight into the mechanisms by which RUNX1 prevents transition of the mammary epithelium to breast cancer. This work impacts our understanding of Runx biology, mammary epithelial biology and breast cancer. Our findings pave the way for future investigation of the regulation of RUNX1 in other epithelial cancers.

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