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Investigating the involvement of RUNX1 and HIF-1 α in hypoxia-induced epithelial-to-mesenchymal transition and generation of breast cancer stem cells

Lizzi Hahn

UVM Larner College of Medicine Summer Research Fellowship

Advisors: Andrew Fritz, Janet Stein, & Gary Stein

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Dear Larner College of Medicine Research Committee,

This summer I had the privilege of working in the Stein/Lian Lab in Department of Biochemistry under the guidance of Dr. Andrew Fritz. I would like to express my sincerest thanks to several parties: to the Research Committee and College of Medicine Alumni for facilitating this excellent opportunity and supporting my work; to Drs. Gary Stein, Janet Stein, and Jane Lian for the time and resources they graciously provided to help me develop my project in their lab; and to Dr. Fritz, who not only showed extreme patience in guiding me through my research, but also went out of his way to teach me invaluable lessons about experiment design, a career in research, and the subtle art of studying and describing molecular interactions without minimizing their complexity.

The time constraint was the greatest challenge I faced this summer. Namely, we had hoped to perform more experiments using cells incubated in the hypoxic chamber to validate the results we collected using cobalt (II) chloride treatment (a hypoxia mimetic), but technical issues with the chamber itself took almost the whole summer to resolve. Thus, in order to see this project to completion, I will assist with the collection of data using the chamber during the school year, though this unfortunately means I cannot provide that comparison with cobalt chloride here. Additionally, I did not isolate the functional vectors for *RUNX1* overexpression and knockdown in time to perform experiments with them, as we decided to switch to a new technique for their development later in my project. Nonetheless, the new constructs I helped to develop using cloning techniques and CRISPR (the latter of which I will not discuss in this report but nonetheless was very grateful to learn) are now validated and ready to use, which will hopefully be a straight forward progression from the preliminary data we collected on *RUNX1* expression in hypoxia.

I am extremely appreciative for all that I was able to learn during the past 7 weeks. From a more practical standpoint, I was able to hone my skills on some techniques (e.g. Western blotting, immunofluorescence, DNA isolation, qPCR) and learned exciting new ones (e.g. cell culture, flow cytometry, lentiviral plasmid development, CRISPR). From Dr. Fritz I also learned a great deal about how to approach research in general; for instance, I soon learned that an interaction between two molecules in one tissue or cell type often has very little bearing on whether the same interaction will be observed in another. It quickly became clear that this distinction is a central theme – rather than a footnote - when the transcription factors being studied play such tremendous roles in cell lineage determination and maintenance. I also came to appreciate that developing biological constructs is *going* to alter the system being studied, highlighting the amount of thought that must go into minimizing the methodology's impact on true physiologic phenomena. For example, the reason we developed plasmids with selectable puromycin and blasticidin markers is that the cells virally transfected with the original *RUNX1* overexpression vector (lacking selectable markers) demonstrated such a tremendous survival disadvantage that they were quickly outgrown by the cells which had not taken up the plasmid. Lastly, I learned to resist the temptation to employ tunnel vision when creating experiments; Dr. Fritz frequently emphasized that with transcriptional regulation, the question is not so much *if* two molecules influence one another as much as *to what degree* and *in what conditions*. This perspective is one I will try to assume both in my future research and in my future medical practice as I strive to see the interplay between the individual factors influencing a person's health.

Thank you again for this incredible opportunity!



Lizzi Hahn

Introduction

Breast Cancer Stem Cells. In women, breast cancer is the cause of most new cancer diagnoses and the second leading cause of cancer-related deaths.¹ This high mortality rate is largely attributed to the frequency of metastasis and tumor relapse in the more malignant subtypes, which include Her2-enriched and basal-like breast cancer.² A major driving force behind metastasis, relapse, and chemoresistance is the existence of breast cancer stem cells (BCSCs)³; this subpopulation of cancer cells adds another dimension of difficulty to our clinical approach to breast cancer as they can not only self-renew, but also differentiate into non-stem-like cancer cells that can contribute significantly to a tumor's overall heterogeneity (Fig. 1).^{4,5} Given these inherent properties, it is unsurprising that CSCs have been associated with initiating and furthering primary tumor growth, promoting intratumoral cell diversity, and facilitating metastases.³ Hence, BCSCs are a promising target for therapies aiming to overcome the failure and recurrence of traditional treatments. By identifying regulators implicated in the maintenance or repression of the BCSC phenotype, we hope to elucidate microenvironments and signaling pathways that can be modulated to decrease the unpredictability and resistance to therapy of cancer stem cell-enriched tumors.

Epithelial-to-Mesenchymal Transition. One process strongly linked to the development of CSCs is the epithelial-to-mesenchymal transition (EMT). The EMT is an evolutionarily conserved transformation of epithelioid cells into more mesenchymal-like cells – that is, they begin to demonstrate decreased expression of tight junction molecules (e.g. E-cadherin) and increased migration, invasiveness, and resistance to apoptosis) (Fig. 2).^{6,7} The EMT has been solidly characterized as a process that facilitates carcinogenesis; while epithelia-derived hyperplasias and low grade tumors usually continue to express E-cadherin and maintain their epithelial phenotype, higher grade tumor cells will commonly acquire mesenchymal traits that serve to further the tumor's ability to metastasize and become morphologically diverse.^{8,9} Further, it is thought that the EMT may specifically contribute to the aggressive nature of Her2-enriched and basal-like cancers.^{10,2} For example, studies suggest that high expression of transcriptional drivers of EMT allow basal and Her2-positive breast cancer cells to become trastuzumab-resistant, a notion supported by the restoration of chemotherapy sensitivity to resistant Basal/Her2-positive breast carcinoma cells following lentiviral knockdown of EMT transcription factors like SLUG and SNAIL-2.² Similarly, inhibition of other EMT-inducing transcription factors such as Twist and Zeb in human and breast cells interferes with the ability of injected BCSCs to metastasize.^{3,11-15}

BCSCs and the EMT. Along with showing a greater degree of EMT, basal/Her2-enriched breast cancers show greater expression of the CD44+/CD24- phenotype classically used to identify CSCs, and it is now widely accepted that there is a direct relationship between these two processes (i.e. EMT and generation of CSCs) as they occur in carcinogenesis.^{16,3} Of the signaling pathways that regulate EMT, many have also been implicated in the development of CSCs – including Notch, hedgehog, WNT, TGF- β , and NF- κ B.¹⁷ This suggests that the EMT, while serving an important role in normal embryogenesis, is commandeered by breast cancer cells that use it to gain stem-like features. The reliance of CSC development on the EMT is nuanced, with cancer cells employing the EMT to adjust phenotype according to the demands of the tumor microenvironment, rather than driving it to one morphological pole or the other.^{18,13,19-21} This is consistent with the understanding that CSCs must strike a balance between a) mesenchymal traits that support motility and a high proliferative capacity and b) epithelial traits that allow the establishment of new, cohesive tumors after metastasis. Because of the contribution of EMT, EMT-induced CSC generation, and the requisite fine tuning of the latter to breast cancer pathogenesis, the Stein lab has directed its attention to the regulators functioning upstream of these processes to better grasp which molecular interactions are pertinent to the dysregulation of cell lineage underlying breast cancer severity.

RUNX1 and breast cancer. At the forefront of this investigation is R1, one of a family of Runt-related transcription factors known to regulate a plethora of critical developmental processes involved in proliferation, cell lineage determination, and apoptosis.^{22,23} Each plays a role in distinct aspects of development, with unique DNA- and protein-binding activity that changes as a function of tissue type and biological context²² (binding domains and common binding partners shown in Fig. 3). RUNX expression is frequently altered in a variety of cancers, highlighting a vital function of these transcription factors in tumor suppression that set the stage for this study. The lab has specifically focused on RUNX1 because mutations of this factor, while long associated with leukemias (consistent with its role in regulating hematopoietic lineage), have more recently been implicated in breast cancer, a finding that corroborates proposed involvement of RUNX1 in the maintenance of the mammary epithelial phenotype.²⁴⁻²⁷ RUNX1 expression has been shown to decrease with increasing breast cancer malignance, and, conversely, patients with tumors exhibiting lower levels of RUNX1 have a poorer prognosis than those with higher RUNX1 levels (Fig. 4).¹⁰ In addition to demonstrating aberrant expression patterns, RUNX1 and one of its critical cofactors, core-binding factor beta (CBF β), are both frequently mutated in cancer – including breast cancer (Fig. 5).²⁸

Runx1, EMT, and BCSCs. Following RUNX1 knockdown in the breast cancer cell line MCF10A, our lab has observed an upregulation of genes related to the EMT accompanied by loss of epithelial features, such as decreased E-cadherin expression; notably, overexpression of RUNX1 rescued this phenotype by driving the mesenchymal-to-epithelial transition (MET).¹⁰ RUNX1 expression in MCF10A has also been shown to decrease after induction of EMT with TGF- β treatment and growth factor depletion, suggesting that there are pathways that allow RUNX1 and regulators

of the EMT to reciprocally influence one another. In addition to demonstrating that RUNX1 level is decreased by EMT, our lab has also found lower RUNX1 expression in BCSCs isolated from premalignant MCF10AT1 cells using FACS, and knockdown of RUNX1 led to increased features of stemness.³ Together, these findings identify RUNX1 as a potential suppressor of EMT and the acquisition of stem-like properties by breast cancer cells. However, the specific pathways and conditions eliciting impairment of RUNX1's protective functions in breast cancer is not yet known. This gap in knowledge served as the impetus for the project described here. In exploring potential external influences on this system, several cases emerged from the literature that pointed to tumor-associated hypoxia as a likely candidate for the suppression of RUNX1 in breast tissue:

First, several studies have described interactions between RUNX transcription factors and Hypoxia Inducible Factor-1 α (HIF-1 α). These have been noted in a variety of settings and with numerous outcomes, depending on tissue type and metabolic conditions.²⁹ HIF-1 α is the primary mediator of cellular response to hypoxic conditions, allowing cells to survive and adapt.³⁰ HIF-1 α function is beneficial in physiologic instances of hypoxia, but its upregulation in the hypoxic tumor microenvironment (TME) allows tumor cells to persist even as they overgrow local oxygen supply – and is thus associated with poor prognosis.²³ This means that molecules that prevent the degradation of HIF-1 α have oncogenic potential while those that degrade or inhibit HIF-1 α have tumor suppressing function.³¹ RUNX1 is one such factor that blocks the transcriptional activity of HIF-1 α in a dose-dependent manner.³² However, the relationship between HIF-1 α and RUNX1 is not linear; while co-immunoprecipitation and GST pull-down assays have shown that the two directly interact, and while RUNX1 overexpression interferes with HIF-1 α binding to its known DNA targets, HIF-1 α actually promotes the activity of RUNX1.^{32,23} Furthermore, HIF-1 α interacts with a number of other molecules that differentially modify RUNX1 activity, meaning that a more complete study of the connection between HIF-1 α and RUNX1 is in order to determine how their interplay directs cellular response to the hypoxic TME.

Second, the hypoxic tumor microenvironment (TME) is known to induce EMT and is associated with an enrichment of stem-like cancer cells.^{33,34} Subregions of hypoxia are present in all tumors, and the progression of cells toward the cancer stem cell phenotype and the mesenchymal end of the EMT spectrum contribute to a poorer overall prognosis (Fig. 6).³⁵ The fact that hypoxia promotes EMT and acquisition of cell stemness implicates RUNX1 in cell response to hypoxia not only because of the growing body of evidence designating RUNX1 as a suppressor of EMT and CSCs, but also because the mechanism by which hypoxia induces EMT is mediated by HIF-1 α .³³ HIF-1 α regulates EMT by modulating a panel of transcription factors (including ZEB, Snail, SLUG, TWIST, and Notch), some of which are also attributed to giving rise to the BCSC phenotype.¹⁷ BCSCs are also more directly upregulated by hypoxia via HIF-1 α -mediated expression of ALKBH5, which demethylates NANOG mRNA to increase levels of the pluripotency factor.³⁶ Exposing pre-formed BCSCs to hypoxic conditions enhances their chemoresistance, again through a HIF-dependent mechanism.³⁷ These correlations are illustrated in vivo as well: in clinical specimens taken from breast tumors, cancer cells that were located in more hypoxic regions were less differentiated and expressed higher levels of genes associated with CSCs, and furthermore, the cells that survive hypoxic TMEs (which are those that express high HIF-1 α) tend to express more mesenchymal traits.^{34, 38-40} It is thus highly probable that RUNX1 is critical in suppressing EMT and BCSC development specifically in the face of hypoxia, and inversely that a process innate to certain hypoxic TMEs is responsible for inhibiting the anti-oncogenic effect of RUNX1 on these processes.

Third, RUNX1 functions in a signaling pathway downstream of Transforming Growth Factor- β (TGF- β), a known mediator of cell response to hypoxia and inducer of EMT. TGF- β is secreted by tumor cells, reactive stromal cells, and platelets, and the signaling pathway it engages along with its canonical signal transducers, the SMAD proteins, is the most thoroughly understood promoter of the EMT.^{35,10} One of the primary ways TGF- β signaling induces EMT is by activating transcription factors like ZEB, SNAIL, and TWIST that effect the reduced expression of E-cadherin.⁴¹ RUNX1 complexes with SMADs, and by doing so regulates genes sensitive to regulatory signals from TGF- β .¹⁰ Additionally, TGF- β treatment of MCF-10A mammary cells reduces RUNX1 expression, and the Stein lab demonstrated that this RUNX1 depletion is required for induction of EMT by TGF- β . Like its association with EMT and BCSCs, the relationship of RUNX1 with TGF- β also indirectly links it to response to hypoxia via interaction of TGF- β with HIF-1 α . Specifically, the HIF-1 α stabilized by hypoxia has been shown to upregulate TGF- β 1 and SMAD3 in breast cancer, and application of a HIF-1 α inhibitor decreased the expression of both molecules.²⁹

Despite these findings that link RUNX1 to hypoxia-related processes and molecules, little is known about the relationship between hypoxia and RUNX1 itself. The goal of this project was to probe this relationship in the context of breast cancer; specifically, we hypothesized that RUNX1 plays a role in cell response to hypoxia via mediation of cell signaling networks involving HIF-1 α and TGF- β , and that reduction of RUNX1 activity in this pathway contributes to the EMT and elevated population of BCSCs observed in the hypoxic TME. Our data revealed that, as in treatment with TGF- β , hypoxia induced by cobalt (II) chloride does result in reduced expression of RUNX1. Additionally, we observed several other changes accompanying cobalt (II) chloride treatment that we did not expect: decreased Lamin B1 expression, increase in micronuclei, and downregulated CTCF. These findings suggests that there may be a degree of genomic instability resulting from failure of RUNX1 to suppress EMT in the hypoxic TME.

Methods

Cell culture. Two human breast cancer cell lines, MCF10A and MCF10AT1, were grown in media containing DMEM/F12, 5% horse serum, 10 µg/mL human insulin, 20 ng/mL recombinant hEGF, 100 ng/mL cholera toxin, 0.5 µg/mL hydrocortisone, 50 IU/mL penicillin/50 µg/mL streptomycin, and 2 mmol/L glutamine (note: all product details are provided in Table 1). MCF10A cells are immortalized, non-malignant breast cells, whereas MCF10AT1 are MCF10A cells that have been RAS-transformed to become premalignant (Fig. 7). To all media, Accutase, and wash solutions used for the hypoxia treated cells, 200 µmol/L cobalt (II) chloride was added. Cobalt (II) chloride acts as a hypoxia mimic in that it stabilizes HIF-1 α , potentially by displacing a critical Fe²⁺ from the active site of prolyl hydroxylases.⁴² These prolyl hydroxylases allow for Von Hippel-Lindau factor to bind and degrade HIF-1 α , so their inhibition by Co²⁺ allows induction of HIF-1 α -mediated cell response to hypoxia. All experiments were performed no more than 10 passages after cells were thawed.

Western blotting. Cells were harvested in RIPA buffer and 2X SDS sample buffer to which cOmplete, EDTA-free protease inhibitors and MG132 had been added. Cells were sonicated and centrifuged, and concentration of supernatant was determined with Nanodrop using the Pierce BCA Protein Assay Kit to allow loading of equivalent volumes of each sample in 1X RIPA and 5X loading buffer onto an 8.5% acrylamide gel. After immunoblotting, gels were transferred to polyvinylidene difluoride membranes using a wet transfer technique. Membranes were blocked using 5% BSA / TBST before incubating overnight at 4°C with the following primary antibodies diluted in 5% BSA / TBST with 1/500 sodium azide: rabbit polyclonal to RUNX1; mouse monoclonal to E-cadherin; mouse monoclonal to Vimentin; mouse monoclonal to β -actin; rabbit polyclonal to ZEB1; rabbit monoclonal to CTCF; rabbit monoclonal to RUNX2; mouse monoclonal to Lamin B1, and a mouse monoclonal to HIF-1 α . Secondary antibodies conjugated to horseradish peroxidase and Clarity Western ECL Substrate were used to visualize blots on a Chemidoc XRS+ imaging system.

Quantitative PCR. RNA was extracted from cells with Trizol and purified using DNase digestion. RNA was subjected to quantitative PCR using the Luna Universal One-Step qPCR kit.

Immunofluorescence. Cells were grown on coverslips and fixed using methanol or 3% formaldehyde in PBS for 10 minutes, depending on the manufacturer's specifications. Cells were rinsed in PBS and then permeabilized for 20 minutes in a blocking buffer (1X PBS / 5% serum, same species as secondary antibody / 0.3% Triton X-100) before incubating in a 1X PBS / 5% serum solution for 40 minutes. Coverslips were incubated overnight at 4°C with the following primary antibodies diluted in 1X PBS, 1% BSA, and 0.3% Triton X-100: rabbit polyclonal to RUNX1; mouse monoclonal to Lamin B1; rabbit monoclonal to Lamin B1; mouse monoclonal to E-cadherin; rabbit monoclonal to cleaved caspase-3. Specimens were rinsed with PBS and incubated in fluorochrome-conjugated secondary antibody (diluted in same solution used to dilute primary) for 2 hours in the dark; for rabbit polyclonal antibodies, a goat anti-rabbit IgG (H+L) secondary antibody was used, and for mouse polyclonal antibody, a goat anti-mouse IgG (H+L) secondary antibody was used. Coverslips were rinsed in PBS and Prolong Gold + DAPI was added before imaging with immunofluorescence microscopy.

Flow cytometry. Cells were grown to subconfluency and detached from plates with 10-15 minute treatments of Accutase followed by neutralization with an equivalent volume of serum. Cells were centrifuged and resuspended in serum to obtain a cell count that was used to isolate 1 x 10⁶ cells per sample. Cells were centrifuged again and resuspended in equivalent volumes of serum, to which the antibodies CD24 and CD44 were added and incubated for 20 minutes. Cells were centrifuged and resuspended in PBS/FBS twice to remove antibody and then passed through a 40-micron filter to isolate single cells. These were centrifuged once more before flow cytometry was performed using the pre-determined optimized conditions.^{24,43,44}

Lentiviral plasmid preparation. Two plasmids, one with a puromycin selectable marker (pCW57-MCS1-2A-MCS2) and the other with a blasticidin selectable marker (pCW57-MCS1-P2A-MCS2) were amplified in bacteria (STBL3), isolated with a Miniprep kit, and then digested with EcoR1. PCR was used to add overhangs complementary to the EcoR1 site to two vectors – one with RUNX1 and another with RUNX1 P2A GFP. The PCR products were run on an agarose gel and isolated using gel extraction before being purified. The Gibson Assembly Reaction was used to insert the purified PCR product into each of the plasmids, and the resulting constructs were transformed into bacteria in LB+Amp. Plasmids were isolated from bacteria and sequenced to ensure correct assembly.

Results

Influence of hypoxia on RUNX1 and associated factors. Western blotting showed that HIF-1 α was upregulated in both treated MCF10A and MCF10AT1 cells, indicating success of its stabilization by cobalt (II) chloride (Fig. 8). Blotting also revealed that both cell lines treated with cobalt (II) chloride at a concentration of 200 µM for 3 and 7 days showed a downregulation of RUNX1. The effect was far more prominent after treatment with 200 µM cobalt (II) chloride than with lower concentrations; MCF10A cells demonstrated a subtle decrease in RUNX1 at 3, 4, and 7 days of 100 µM treatment, while MCF10AT1 cells demonstrated a slightly greater reduction in RUNX1. Notably, both cell lines appear to have a greater reduction of RUNX1 in the 3 day treatments of 100 µM cobalt (II) chloride than in the 4 and 7 day treatments, suggesting the possibility of a delayed compensatory mechanism fronted by the cell after partial

loss of critical RUNX1 function. Blotting results were supported by immunofluorescence, which showed decreased RUNX1 signal accompanying cobalt (II) chloride treatment.

Analysis of qPCR results revealed that hypoxia also leads to changes in the expression of genes regulated by RUNX1, such as MALAT1, Lamin B1, and LIFR (Fig. 9). Both 3 and 7 day treatments of cobalt (II) chloride led to enhanced expression of MALAT1 (a large nc-RNA with oncogenic potential) and LIFR (a receptor involved in differentiation and proliferation), and a decrease in LaminB1 (a nuclear envelope protein to be discussed further below). This is consistent with recent ChIP-seq and RNA-seq analyses performed by the lab showing that knockdown of RUNX1 led to increased MALAT1 and LIFR and decreased LaminB1. However, no changes were observed for EMT markers like E-cadherin, vimentin, and fibronectin-1 at the mRNA level. In contrast, Western blotting did show that the protein levels of the mesenchymal marker vimentin increased with cobalt (II) chloride treatment, and that the effect was enhanced at longer treatments for the MCF10A1 cells. Levels of the epithelial cell marker E-cadherin were reduced in longer treatments of cobalt (II) chloride. Both of these indicate that EMT does not occur immediately in hypoxia, but rather with longer term exposures. Alternatively, this poses questions as to whether hypoxia effects changes in RNA and protein levels at different times – with RNA levels changing at a timepoint earlier than we analyzed – and whether regulation is occurring in a largely post-translational manner, as suggested by the contrasting data for vimentin. Supplementing the qPCR identification of Lamin B1 mRNA downregulation with hypoxia, Western blot also revealed that treatment led to decreases in Lamin B1 protein. The immunofluorescent staining for Lamin B1 was less prominent in the treated cells as well, confirming its reduction by Cobalt (II) chloride-induced hypoxia (Fig. 10). While we attempted to co-stain for Lamin B1 and RUNX1, cleaved caspase (a marker of apoptosis), and E-cadherin, we were unable to visualize Lamin B1 with other proteins because the Lamin B1 antibody required fixation in methanol while the rest were optimized for use with formaldehyde-fixed cells.

Western blot additionally demonstrated a downregulation of CTCF and ZEB1 with cobalt (II) chloride treatment. The alteration of CTCF levels was surprising, given that CTCF was originally selected as a control, and provided unexpected insight as to the effects of hypoxia on genomic stability – to be discussed further below. ZEB1 downregulation was similarly unexpected, due to the known role of ZEB1 in promoting EMT and stem-related processes (figure 7). Preceding data showed that RUNX1 binds the ZEB1 promoter and downregulates its expression. Further, level of ZEB1 mRNA is reduced in cells where RUNX1 has been overexpressed, and increased in cells with RUNX1 knockdown by short hairpin RNA (shRNA) (Fig. 11). This differential relationship between RUNX1 and ZEB1 could have its roots in the experimental procedure, necessitating validation with hypoxic chamber use.

A qPCR analysis of BCSC markers allowed us to observe the effect of hypoxia on the development of a population of breast cancer stem cells. Multiple isoforms of the CD44 marker exist: the CD44s is a marker of BCSCs, while the CD44v is associated with non-BCSCs. Hypoxia produced an enrichment of cells with the CD44s marker and reduced cells expressing CD44v, indicating that cells did in fact show increased stemness following hypoxia treatment (Fig. 12).

Induction of genome instability by hypoxia. Immunofluorescent staining was performed and the morphology between the treated and untreated cells was compared. We observed that the hypoxia-treated cells demonstrated an increased incidence of micronuclei (Fig. 13). Further, treatment of cells with cobalt (II) chloride for greater durations translated into an increasing percentage of cells with associated micronuclei. Treated cells also demonstrated a more polymorphic appearance, with greater variety in nuclear size and shape.

Validation of RUNX1 overexpression constructs. Flow cytometry showed that compared to untreated cells, treated cells had greater CD44⁺ expression, a marker of breast cancer cell stemness, but the other common stemness marker, CD24⁺, did not stain. Sequencing of PCR products for the lentiviral plasmid preparation showed that both the puromycin and blasticidin plasmids had successfully incorporated the RUNX1 gene, but that the RUNX1 GFP P2A gene had been inserted with mutations in the GFP domain. The Sanger sequencing of the pCW57 vector showing successful insertion of RUNX1 can be seen in Fig. 14. When these puromycin and blasticidin plasmids were used for lentiviral transfection (not described here) of MCF10A cells in the appropriate selection antibiotic, microscopy showed growth of cells, indicating the successful delivery of DNA with resistance-conferring sequences (Fig. 14).

Additionally, sequencing showed that amplification of a hygromycin plasmid for constitutive RUNX1 expression was successful, meaning effects of inducible and constitutive RUNX1 expression can be compared in the future under different conditions.

Discussion

All too often, breast cancer treatments prove to be ineffective, and as a result we see a mortality rate that is overwhelming compared to other cancers. The physical, emotional, and financial burdens of high grade breast cancers on patients underscores the need to tackle this family of diseases head on. This can be done by addressing the most therapy-resistant aspects of breast tumors, which include the propensity of the aggressive subtypes to develop stemness and undergo EMT. The present project is part of a much larger effort to uncover molecular targets implicated in these two processes and to identify conditions that promote or prevent them, with the hope that any factors elucidated can be used to eliminate the most resistant subpopulations of breast cancer cells. In this study, we found that RUNX1 is in fact

downregulated in hypoxia, and thus, its reduction is likely to account for a degree of EMT and acquisition of stemness observed in cells hypoxic TMEs. All of this was consistent with pre-existing evidence showing that RUNX1 interacts with factors known to directly effect changes following hypoxia, such as HIF-1 α and TGF- β . The results described here provide additional clarity to the involvement of RUNX1 in breast cancer, facilitating its future use in therapeutic strategies.

Further, we found that cobalt (II) chloride treatment was associated with increased frequency of micronuclei. This corroborates a previous study by Snyder and Diehl⁴⁵ which showed that the reduction of oxygen levels in a mouse enclosure from 21% (normoxic conditions) to 7.5% for up to a week resulted in mouse bone marrow cells showing a statistically significant 0.15% increase in micronuclei. Additionally, an increased frequency of chromatin bridges has been observed in fibroblasts incubated in 0.2% oxygen following genome insult with Gy irradiation – structures known to fragment to produce micronuclei (Fig. 15).^{46,47} It is well known that hypoxia destabilizes the genome through decreased expression of DNA repair genes, oncogene amplification, DNA replication stress, and downregulated DNA damage checkpoint signaling. Our finding of micronuclei induction suggests that resulting genomic reorganization and modification of nuclear structures also feed into hypoxia-induced genetic aberrancy.⁴⁶ We conjecture that the observed genomic reorganization potentially occurs as a result of TGF- β signaling now being uninterrupted by RUNX1.¹⁰ It has been established that TGF- β -induced EMT leads to mitotic defects (including suppressed expression of the nuclear envelope protein Lamin B1) in conditions where persistent proliferation is not halted, and further that these mitotic defects may be a root cause of genomic instability seen in tumor cells where EMT is underway (Fig. 16).³⁵

Cobalt (II) chloride treatment also led to a downregulation of Lamin B1 and CTCF. Loss of Lamin B1 from the nucleus is one factor known to increase presence of binucleated cells and micronuclei, thus implicated RUNX1 depletion in the formation of both structures through unchecked TGF- β activity. CTCF, like Lamin B1, is also critical for maintaining genomic stability, and its downregulation has been observed in models of breast cancer outside of hypoxia.⁴⁸ Among many other chromosome-modulating functions, including the arrangement of chromatin into localized loops and larger topological regions, the insulator protein functions in homologous recombination repair of double-strand DNA breaks.⁴⁹ Hence, downregulation of CTCF in breast cancer leads to a breakdown of both DNA integrity and organization. It is not yet known how hypoxia, RUNX1, or TGF- β factor into CTCF repair of DNA damage or its reduction in breast cancer, but these relationships warrants further investigation considering the importance of CTCF in establishing chromosomal order.

It is possible that RUNX1 elicits genome-stabilizing effects that counteract those of heightened TGF- β signaling through mechanisms other than its interaction with SMADs. Though similarities between members of the RUNX family and between RUNX factors in different tissues cannot be assumed, future studies of RUNX1 may benefit from drawing inspiration from them. For example, RUNX1 in hemopoietic cells helps to promote p53 dependent apoptosis in response to DNA damage, outlining the possibility that reduction of RUNX1 may lead to increased survival of breast cancer cells in which enhanced TGF- β signaling has caused genomic instability.^{50,51} In another example of RUNX proteins suppressing tumorigenic processes initiated by TGF- β , both RUNX1 and RUNX3 are induced by TGF- β to interact with forkhead box protein O3 (FOXO3A) to promote the expression of a pro-apoptotic mediator in specific tissues.²³ RUNX3 also modulates TGF- β activity by enhancing activity of heme oxygenase 1 (HMOX1), which responds to oxidative DNA damage such as the double-stranded breaks caused by TGF- β when dysregulated in cancer.⁵²

Two other factors require further study with respect to role of RUNX1 in hypoxia: CBF β and NOTCH. CBF β forms a complex with RUNX1 in the nucleus that suppresses the transcription of genes in the NOTCH signaling pathway.⁵³ This is significant because enhanced Notch expression underlies breast cancer qualities such as anchorage independent growth and tumorigenicity. Furthermore, Notch may play a distinct role in mediating these processes in the hypoxia TME in breast cancer; Notch genes are upregulated by HIF-1 α , and this hypoxia-induced Notch activation is critical for maintaining the undifferentiated cell state in stem and precursor cells.⁵⁴ In addition to complexing with RUNX to suppress NOTCH, CBF β also regulates level of RUNX1 by binding its mRNA to enhance its translation - evidenced by the depletion of RUNX1 that is observed when CBF β is knocked down in MCF10A cells.⁵³ Thus, CBF β is a promising candidate for paving the connection between hypoxia and maintenance of cell lineage by RUNX1.

In addition to pursuing a deeper understanding of RUNX1 role in suppressing DNA destabilization by TGF- β -induced EMT in hypoxia, and in studying RUNX1 relationship to potential CBF β -Notch interactions in hypoxia, future steps for this project will include experiments using a hypoxic chamber to validate the results collected using cobalt (II) chloride. It is possible that cobalt (II) chloride, which mimics hypoxia by stabilizing HIF-1 α , may not accurately show the full scope of hypoxia's effect on RUNX1 given that cells employ responses to hypoxia that are independent of HIF-1 α , and these too may be influencing RUNX1 or other parts of the pathway discussed above. Further, the inducible plasmids developed can now be used to determine whether an epithelioid phenotype, genomic stability, and appropriate differentiation of cells are restored to hypoxic cells when RUNX1 is overexpressed in hypoxia. These experiments will help to pinpoint the function of RUNX1 in cell response to hypoxia and inversely, the effects of its absence on cancer progression in the hypoxic TME.

Figures

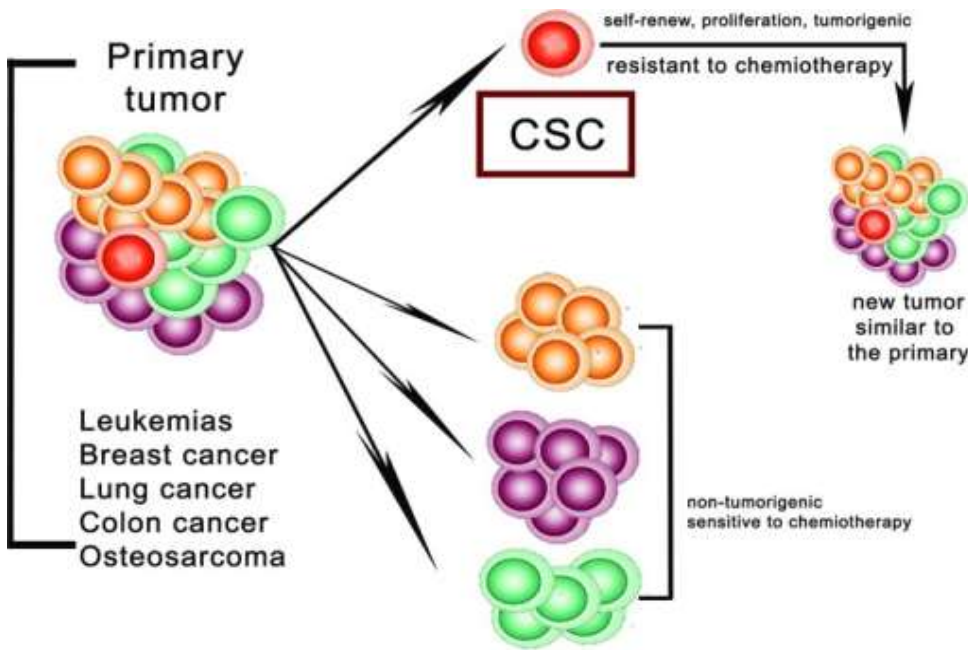


Figure 1. Advancement of tumor severity via transformation of primary tumor cells into a sub-population of cancer stem cells, which show enhanced abilities of self-renewal, proliferation, and resistance to chemotherapy. Adapted from ‘cancer stem cells in osteosarcoma,’ by H.K. Brown, M. Tellez-Gabriel, and D. Heymann, 2017, *Cancer Letters*, 386: 189-195.

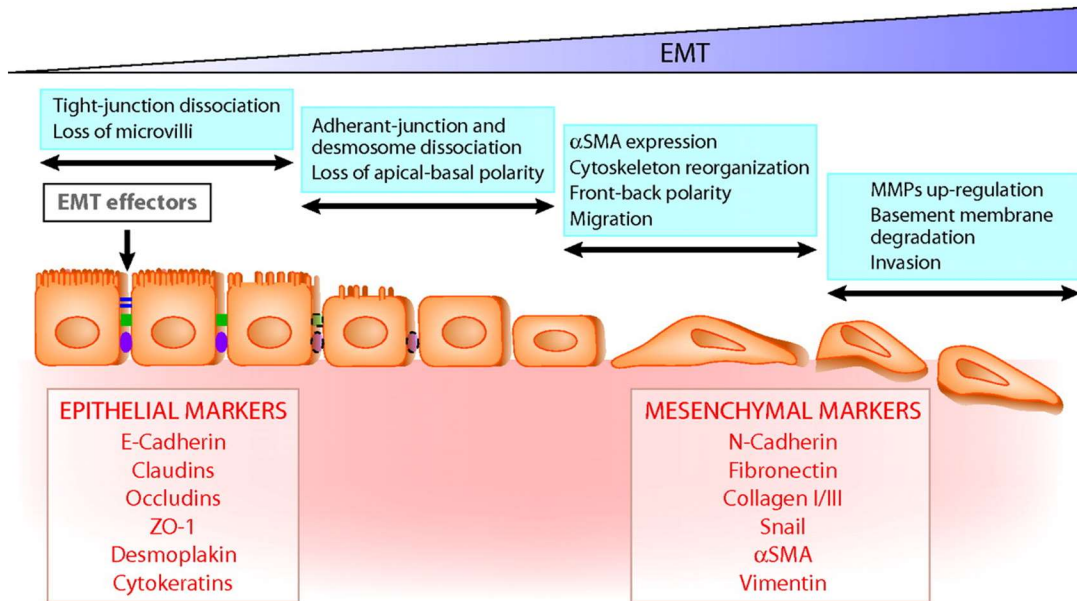


Figure 2. Cellular features and markers of the epithelial to mesenchymal transition. Adapted from ‘epithelial to mesenchymal transition and peritoneal membrane failure in peritoneal dialysis patients: pathologic significance and potential therapeutic interventions,’ by L.S. Aroeira, A. Aguilera, et al., 2007, *Journal of the American Society of Nephrology*, 18: 2008.

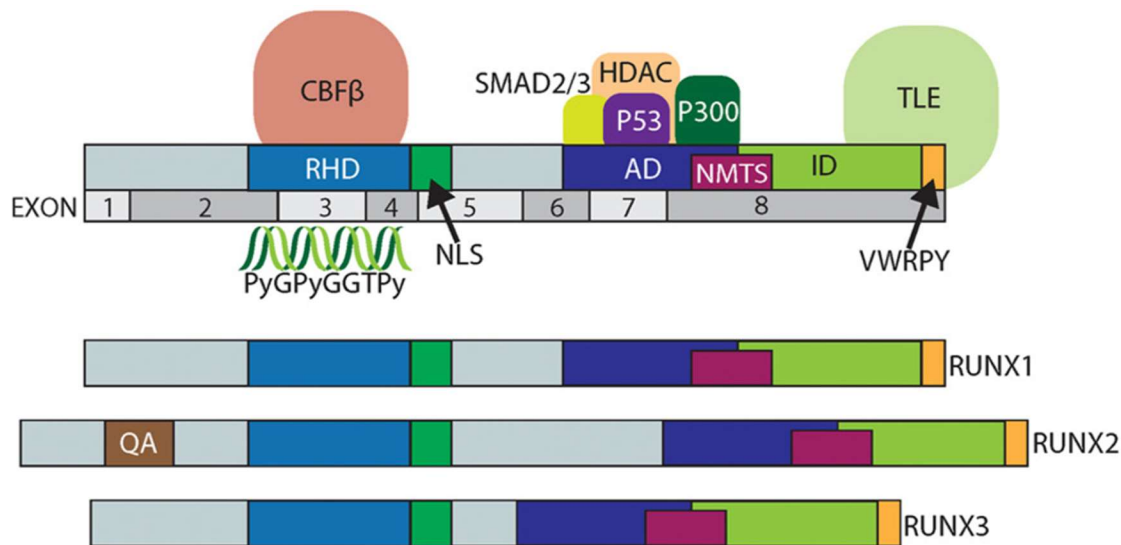


Figure 3. Binding domains in the sequences of RUNX1, RUNX2, and RUNX3. Schematics show a conserved binding site for the important RUNX cofactor CBF β , for the TGF- β messengers known as the SMADs, and an assortment of other proteins that interact with RUNX factors to influence their DNA-binding activity. Adapted from 'RUNX1-dependent mechanisms in biological control and dysregulation in cancer,' by D. Hong and A.J. Fritz, 2018, *Journal of Cell Physiology*, 234(6), p. 8599.

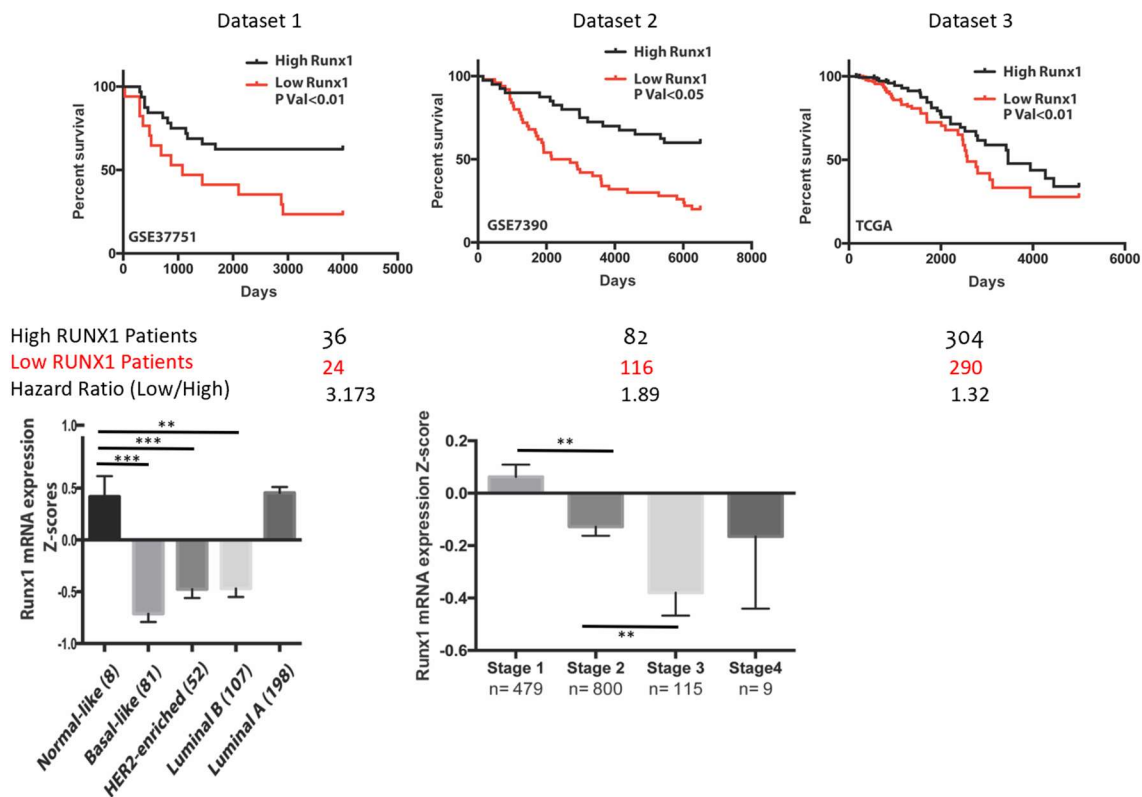


Figure 4. Reduced RUNX1 expression is associated with breast cancer subtypes with poor prognosis. The top three charts show three different datasets indicating lower survival rate in patients with lower RUNX1. The lower charts show that RUNX1 mRNA is depleted in the more aggressive subtypes of breast cancer (left) and in more advanced stages of breast cancer (right). Adapted from 'RUNX1 stabilizes the mammary epithelial cell phenotype and prevents epithelial to mesenchymal transition,' by D. Hong and T. Messier, 2017, *Oncotarget*, 8(11).

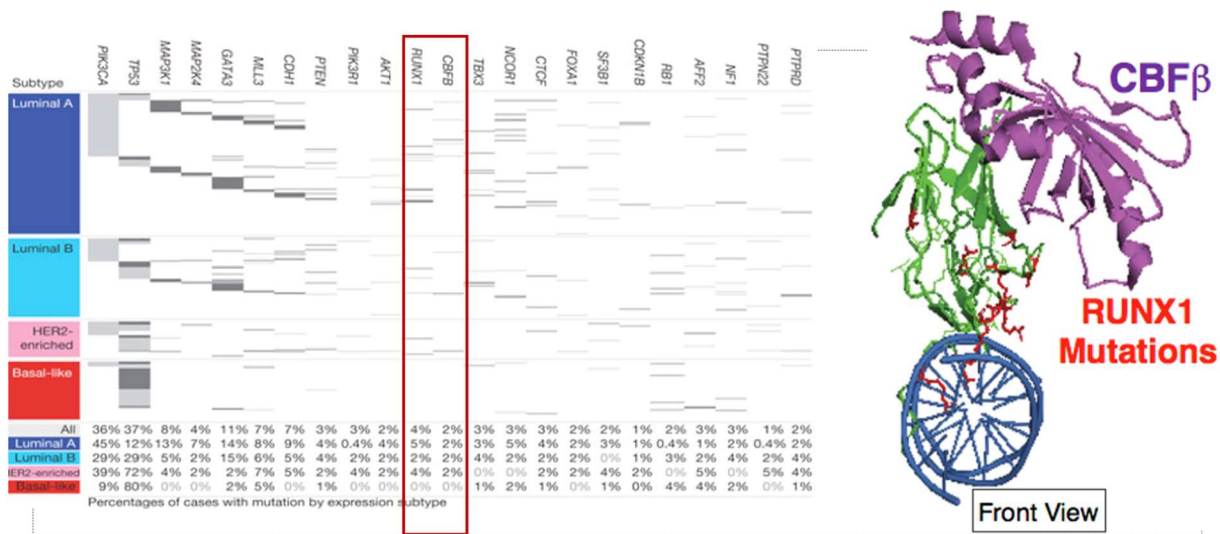


Figure 5. Mutations of RUNX1 and CBFβ (exemplified in protein structures on right) are common in breast cancer – particularly those subtypes listed in the figure above (left). Adapted from ‘RUNX1-dependent mechanisms in biological control and dysregulation in cancer,’ by D. Hong and A.J. Fritz, 2018, *Journal of Cell Physiology*, 234(6), p. 8603.

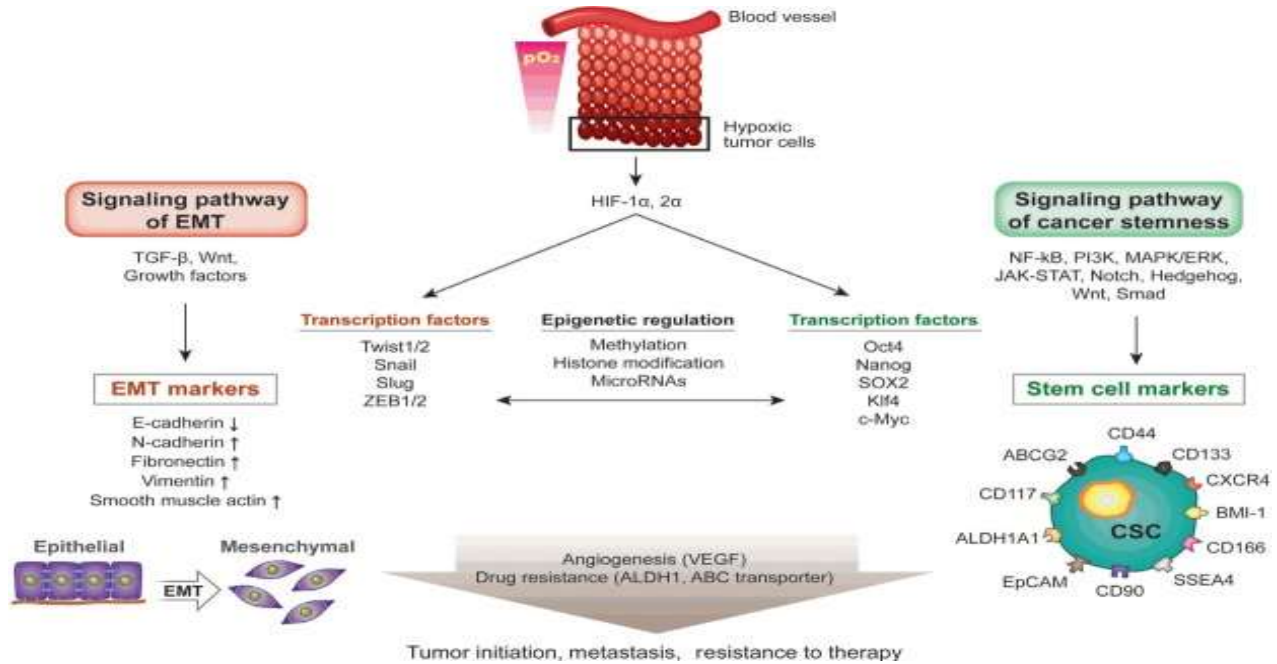


Figure 6. Hypoxia induces EMT and cancer stemness through the regulation of EMT- and CSC-mediating transcription factors by HIF-1α. Adapted from ‘genomic instability is induced by persistent proliferation of cells undergoing epithelial-to-mesenchymal transition,’ by V. Comaills, L. Kabeche et al., 2016, *Cell Reports*, 17, 2632.

Table 1. Details for products in methods section.

Product	Product Details
Cell culture	
DMEM/F12	Hyclone: SH30271, Thermo Fisher Scientific
Horse serum	Gibco: 16050, Thermo Fisher Scientific
Human insulin	Sigma-Aldrich, I-1882
Recombinant hEGF	Peptotech, AF-100-15
Cholera toxin	Sigma-Aldrich, C-8052
Hydrocortisone	Sigma-Aldrich, H-0888
Penicillin/Streptomycin	Life Technologies, 15140-122
Glutamine	Life Technologies, 25030-081
Cobalt (II) Chloride	Sigma Aldrich, C8661
Western blotting	
EDTA-free protease inhibitors	Roche Diagnostics
MG132	EMD Millipore
Polyvinylidene difluoride membranes	EMD Millipore
Membrane wet transfer kit	BioRad Laboratories
Rabbit polyclonal to RUNX1	Cell Signaling Technology: #4334, 1:1,000
Mouse primary monoclonal antibody to E-cadherin	Santa Cruz Biotechnology: sc21791, 1:1,000
Mouse primary monoclonal antibody to Vimentin	Santa Cruz Biotechnology: sc-6260, 1:1,000
Mouse primary monoclonal antibody to β -actin	Cell Signaling Technology: #3700, 1:1,000
Rabbit primary polyclonal antibody to ZEB1	Sigma-Aldrich: HPA027524-100 UL, 1:1,000
Rabbit primary monoclonal antibody to CTCF	Cell Signaling: #3418, 1:1,000
Rabbit primary monoclonal antibody to RUNX2	Cell Signaling: 12556, 1:1,000
Mouse primary monoclonal antibody to Lamin B1	Cell Signaling: #68591, 1:1,000
Mouse primary monoclonal antibody to HIF-1 α	Cell Signaling: NB-100-105, 1:1,000
Horseradish peroxidase-conjugated secondary antibodies	Santa Cruz Biotechnology
Clarity Western ECL Substrate	Bio-Rad Laboratories
Chemidoc XRS+ imaging system	Bio-Rad Laboratories
Quantitative PCR	
Trizol	Life Technologies
DNase digestion technique	Zymo Research
Immunofluorescence	
Rabbit primary polyclonal antibody to RUNX1	Cell Signaling Technology: #4334, 1:200, formaldehyde
Mouse primary monoclonal antibody to Lamin B1	Cell Signaling: #68591, 1:200, methanol
Rabbit primary monoclonal antibody to Lamin B1	Abcam: ab194106, 1:100, methanol
Mouse primary monoclonal antibody to E-cadherin	Cell Signaling: #14472, 1:50, formaldehyde
Rabbit primary monoclonal antibody to cleaved caspase-3	Cell Signaling: #9664, 1:400, formaldehyde
Goat anti-rabbit IgG (H+L) secondary antibody	Alexa Fluor 594 conjugate, Life Technologies A-11037
Goat anti-mouse IgG (H+L) secondary antibody	Alexa Fluor 488 conjugate, Life Technologies A-11029
Prolong Gold + DAPI	Life Technologies-Molecular Probes
Flow cytometry	
CD24 antibody	PE-Cy7, BioLegend 311120
CD44 antibody	APC, BD Pharmigen 559942)
Lentiviral plasmid preparation	
pCW57-MCS1-2A-MCS2	Addgene: #71782
pCW57-MCS1-P2A-MCS2	Addgene: #80921
Miniprep kit	Zyppy Miniprep, Zymo Research
Gel extraction kit	Zymoclean Gel Recovery, Zymo Research
DNA purification kit	DNA Clean and Concentrator-5, Zymo Research

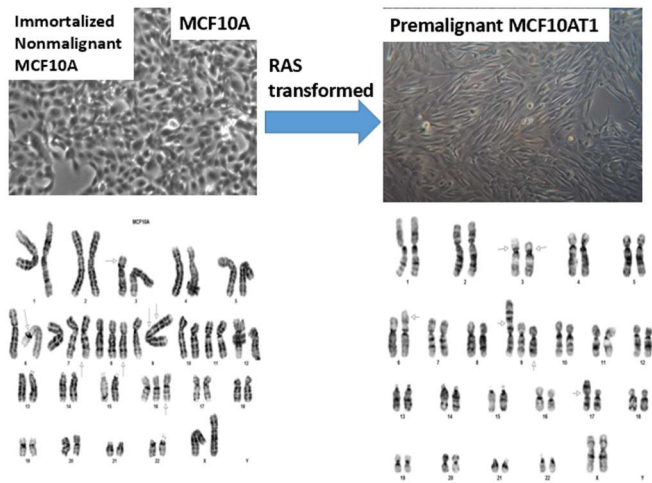


Figure 7. Cell lines used and the morphology and karyotype of each.

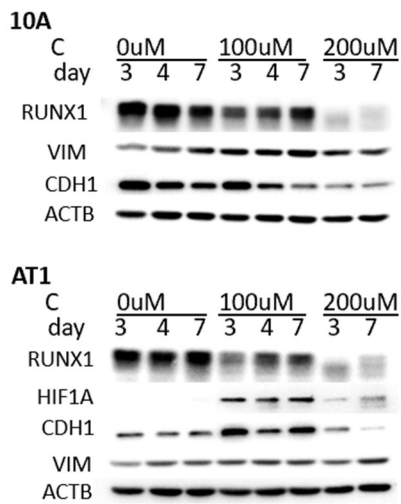


Figure 8. Western blot showing levels of RUNX1, vimentin, E-cadherin, HIF-1 α , and β -actin in MCF10A and MCF10AT1 cells after 3,4, and 7 days of cobalt (II) chloride treatment (0, 100, 200 μ M).

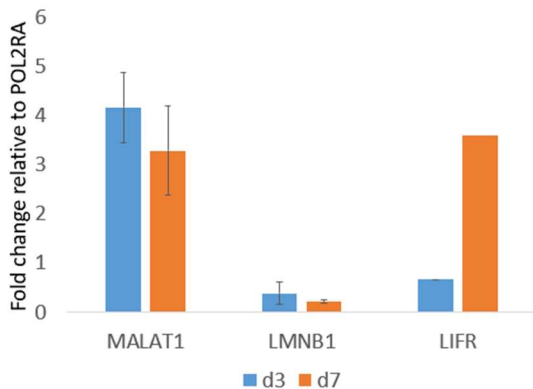


Figure 9. Fold change in transcript levels (relative to POL2RA) of RUNX1 targets MALAT1, Lamin B1, and LIFR after 3 and 7 days of cobalt (II) chloride treatment.

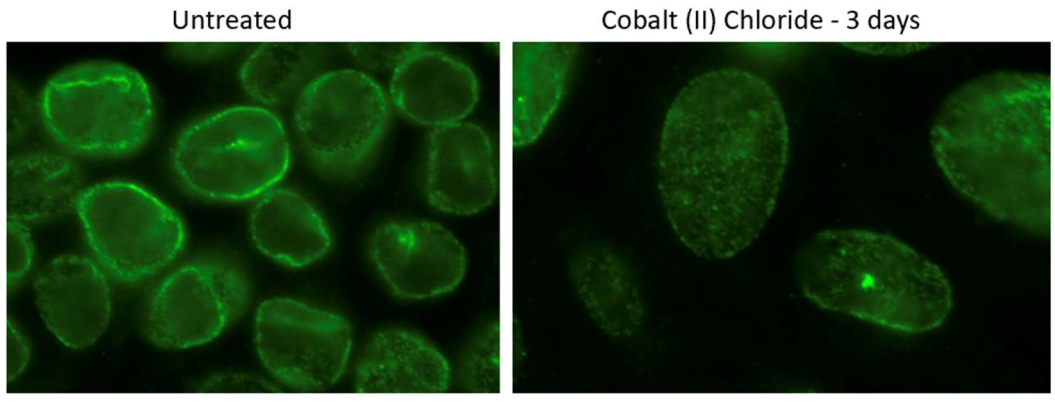


Figure 10. Lamin B1 staining of untreated cells compared to cells treated with cobalt (II) chloride for 3 days.

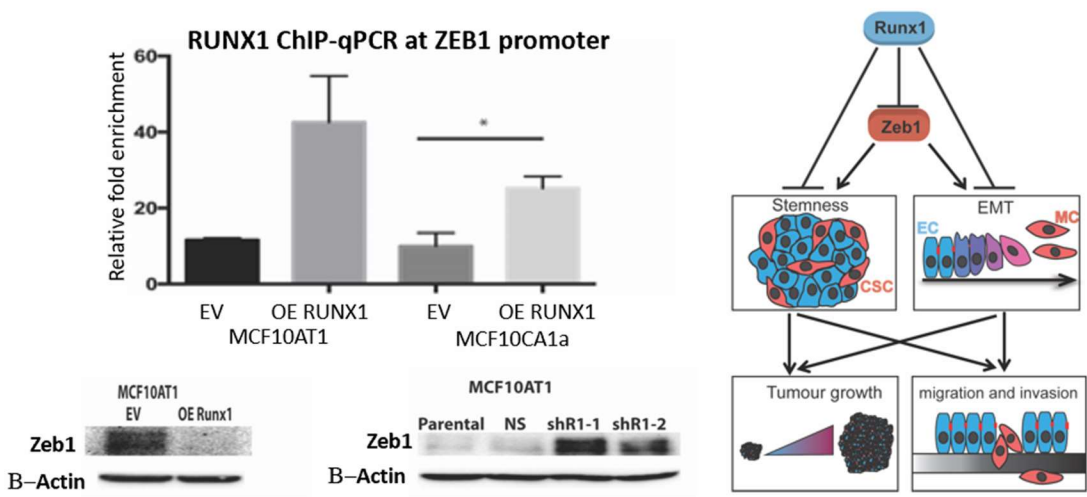


Figure 11. RUNX1 regulation of ZEB1 expression. On the left, ChIP-qPCR (above) shows that RUNX1 binds directly to the promoter of ZEB1 to influence its transcription, and blots (below) show that ZEB1 levels are lower when RUNX1 is overexpressed and higher when RUNX1 is knocked down. To the right is a diagram of the involvement of ZEB1 in promoting stemness and EMT, which contribute to tumor growth, migration, and invasion. Adapted from 'RUNX1-dependent mechanisms in biological control and dysregulation in cancer,' by D. Hong and A.J. Fritz, 2018, *Journal of Cell Physiology*, 234(6), p. 8603.

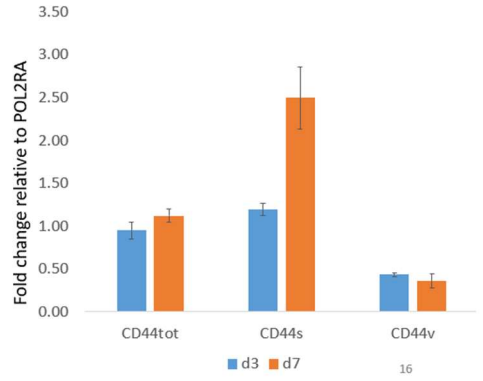


Figure 12. Fold change in expression of CD44s and CD44v (relative to POL2RA) in cells treated with cobalt (II) chloride for 3 and 7 days.

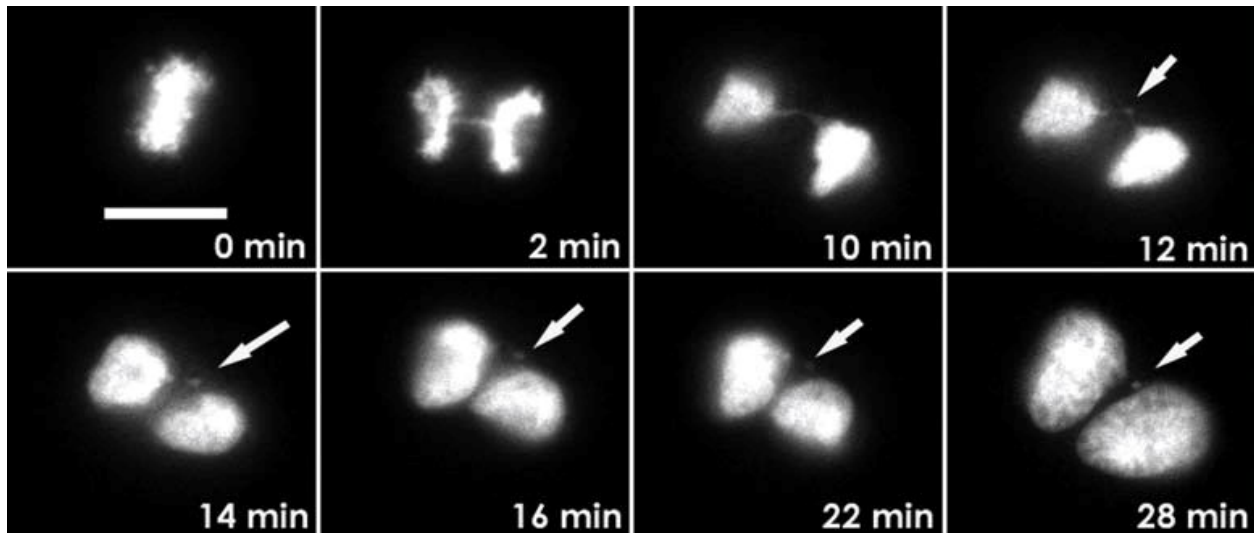


Figure 15. Chromatin bridges, the frequency of which increases under hypoxic conditions, fragment and result in the formation of micronuclei. Adapted from 'resolution of anaphase bridges in cancer cells,' by D.R. Hoffelder, L. Luo et al., 2004, *Chromosoma*, 112: 392.

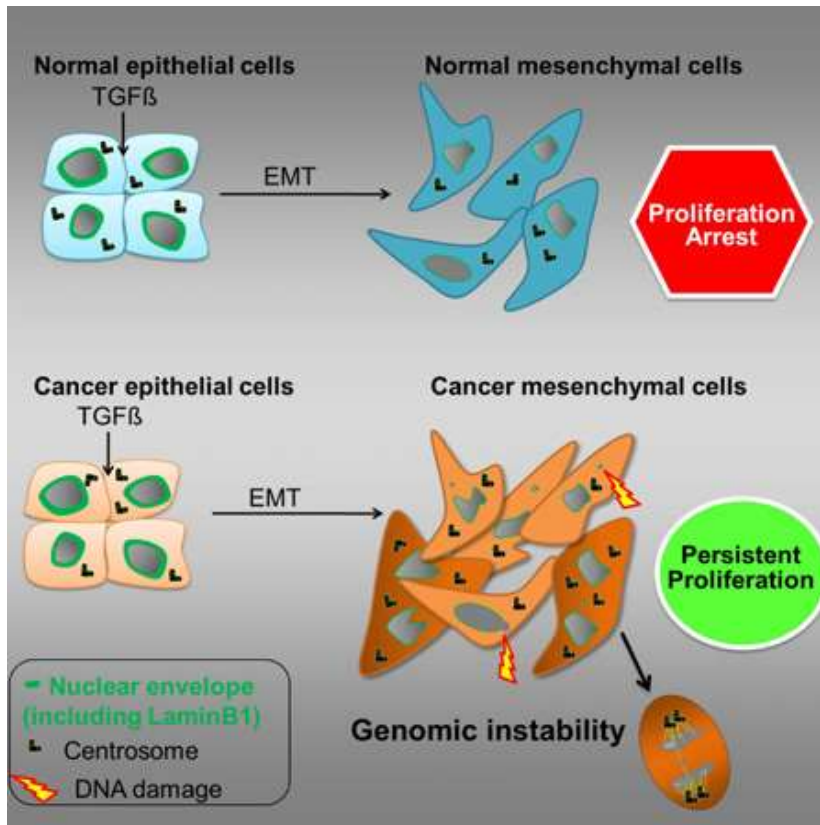


Figure 16. Cells undergoing TGF- β -induced EMT that continue to proliferate demonstrate genomic instability. Adapted from 'genomic instability is induced by persistent proliferation of cells undergoing epithelial-to-mesenchymal transition,' by V. Comaills, L. Kabeche, et al., 2016, *Cell Reports*, 17, 2632.

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