ROS Links Glucose Metabolism to Breast Cancer Stem Cell and EMT Phenotype

Michael S. Schieber[1] and Navdeep S. Chandel[1,2,*]
[1]Division of Pulmonary and Critical Care Medicine, Department of Medicine
[2]Department of Cell and Molecular Biology
Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA
*Correspondence: nav@northwestern.edu
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Cancer stem cells display an epithelial-mesenchymal transition phenotype and are resistant to current therapies. In this issue of *Cancer Cell*, Dong and colleagues demonstrate that these phenotypes in basal-like breast cancer are promoted by a metabolic switch to glucose metabolism, resulting in decreased reactive oxygen species levels.

Cancer initiating cells, also referred to as cancer stem cells (CSCs), exhibit stem cell-like properties and have been implicated in the tumorigenesis of basal-like breast cancer (BLBC), a particularly aggressive, metastatic and chemotherapy-resistant type of breast cancer. In this issue of *Cancer Cell*, Dong et al. (2013) report a metabolic switch to glycolysis following epigenetic silencing of the gluconeogenic enzyme fructose-1,6-biphosphatase (FPB1) by the epithelial-mesenchymal transition (EMT) associated factor Snail is required for the development of BLBC.

CSCs display EMT characteristics such as loss of the adhesion protein E-cadherin (Mani et al., 2008). A number of transcription factors, including Snail, induce EMT (Kalluri and Weinberg, 2009). Previously, the same group reported that Snail interacts with G9a, a methyltransferase responsible for H3K9me2, and recruits DNA methyltransferases (DNMTs) to the E-cadherin promoter. This results in epigenetic silencing of E-cadherin expression and promotes primary tumor growth and metastasis of BLBC cells (Dong et al., 2012).

To investigate other genes regulated by the Snail-G9a-Dnmt1 complex in BLBC cells, Dong et al. (2013) performed a gene expression mircroarray analysis following G9a knockdown in a BLBC cell line and observed a substantial increase in FBP1 mRNA. Snail-G9a-Dnmt1 was then shown to directly bind and methylate the FBP1 promoter, resulting in the epigenetic silencing of FBP1. Moreover, a number of other BLBC cell lines had negligible FBP1 expression in contrast to a set of luminal breast cancer cell lines, which displayed high FBP1 levels. To test if FBP1 silencing was required for the EMT characteristics of BLBC cells, Dong et al. (2013) ectopically expressed Snail alone or together with FBP1 in ER^+-luminal breast cancer cells. Ectopic expression of Snail was sufficient to convert these cells to a BLBC phenotype, as expected. Importantly, however, Snail expression-induced EMT and basal-like phenotype was blocked when FBP1 was coexpressed under the control of a promoter that was not repressed by Snail. Moreover, ectopic expression of FBP1 in BLBC cells diminished tumor growth in mice. By contrast, loss of FBP1 in luminal breast cancer cells increased tumor growth in mice. This biological observation appears to be clinically relevant, because a retrospective analysis of patients with BLBC revealed that low FBP1 expression correlated with poor survival. The loss of FBP1 has also been previously associated with liver and gastrointestinal cancers (Chen et al., 2011). Collectively, these results suggest that epigenetic silencing of FBP1 is a critical event in tumorigenesis of BLBC.

The provocative implication of the current study is that metabolism is not simply a consequence but rather plays a causal role in dictating different phenotypic states exhibited by cancer cells. But how does an enzyme involved in glucose metabolism regulate EMT-like characteristics of BLBC? Glucose levels are maintained by two reciprocal metabolic pathways: glycolysis and gluconeogenesis. Glycolysis is an ATP- and NADH-generating reaction that results in the catabolism of glucose into two molecules of pyruvate. Under aerobic conditions, pyruvate and NADH produced by glycolysis are then imported into the mitochondria and flux through the tricarboxylic acid cycle to generate ATP through oxidative phosphorylation. Under low oxygen conditions, pyruvate can be converted to lactate, regenerating NAD^+ required for glycolysis. Conversely, gluconeogenesis produces glucose from pyruvate. Most of the enzymes in glycolysis are reversible and thus can be utilized for gluconeogenesis. However, there are three irreversible glycolytic enzymes including phosphofructose kinase 1 (PFK-1), which converts fructose 6-phosphate to fructose 1,6 bisphosphate (Figure 1). To circumvent PFK-1 during gluconeogenesis, FBP1 catalyzes the energy-consuming reaction of converting fructose 1,6-bisphosphate to fructose 6-phosphate.

Many human cancer cells display high flux through glycolysis and other metabolic pathways originating from glycolytic intermediates. These subsidiary metabolic pathways are involved in the synthesis of essential amino acids, nucleotides (via pentose phosphate shunt), and lipids. Therefore, an increase in glycolytic flux may be highly adaptive for proliferative cells with high demand for cellular macromolecules (Lunt and Vander Heiden, 2011). Indeed, Dong et al. (2013) observed that ectopic expression of FBP1 in BLBC cell lines decreased glucose uptake and diminished flux through both glycolysis and the biosynthetic subsidiary metabolic pathways. This resulted in an increase in oxygen

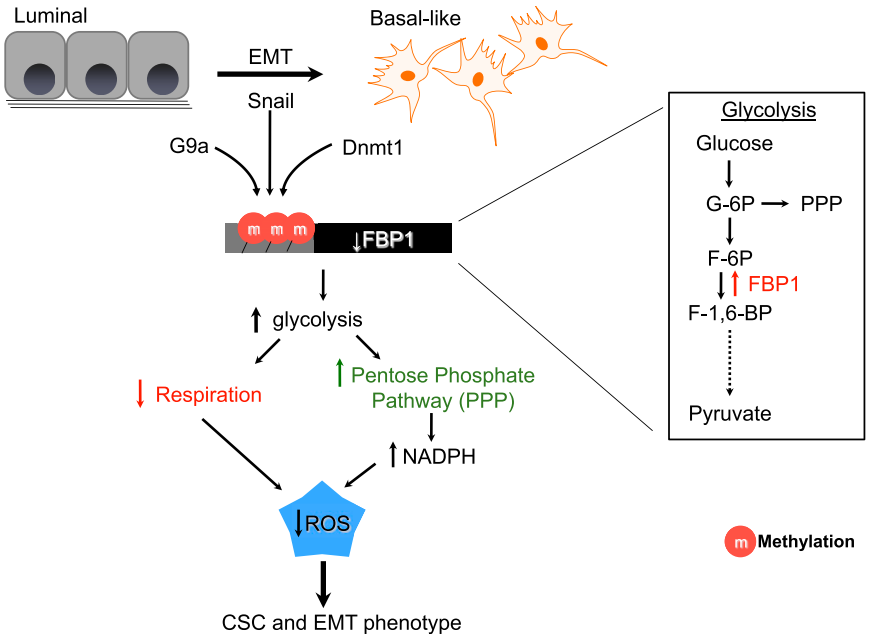

Figure 1. Epigenetic Silencing of FBP1 Decreases ROS to Promote CSC and EMT Phenotype in BLBC

FBP1 coverts F-1,6,-BP to F-6P in the rate-limiting step of gluconeogenesis (right). Epigenetic silencing of FBP1, through promoter methylation by the Snail-G9a-Dnmt1 complex, promotes glycolytic flux. An increased reliance on glucose metabolism following FBP1 silencing lowers ROS levels by two mechanisms: decreased mitochondrial respiration and increased NADPH synthesis through pentose phosphate metabolism. Lower ROS levels promote EMT and CSC phenotype in BLBC.

consumption by mitochondria. Thus, BLBC utilizes FBP1 silencing as a mechanism to maintain glucose flux through glycolysis and other associated biosynthetic metabolic pathways.

One emerging idea on how changes in glucose metabolism induce EMT-like phenotype is that mitochondrial derived reactive oxygen species (ROS) could serve as signaling molecules (Sena and Chandel, 2012). The mitochondrial electron transport chain leaks electrons from complex I, II, and III to molecular oxygen (O_2) to generate superoxide (O_2^-), which is rapidly converted to hydrogen peroxide (H_2O_2) to activate cellular signaling. However, higher levels of ROS can induce cell damage and death. Indeed, higher FBP1 expression in BLBC cell lines resulted in increased mitochondrial ROS. This increase in ROS levels due to FBP1 expression was critical for tumor suppression, which could be prevented by administration of the antioxidant N-acetylcysteine. The increased generation of H_2O_2 is likely due to enhanced mitochondrial oxygen consumption as well as the diminished flux through the pentose phosphate pathway, which generates the NADPH required for enzymes that detoxify H_2O_2 (Figure 1). Thus, the low ROS levels in BLBC due to epigenetic silencing of FBP1 allows for tumor growth of BLBC cells.

An important consequence of having low ROS levels is the maintenance of a subpopulation of CSCs within breast tumors. These CSCs are relatively insensitive to radiation and chemotherapy compared to differentiated and proliferative cancer cell populations (Diehn et al., 2009). Dong et al. (2013) show that when FPB1 is overexpressed in BLBC cell lines, the corresponding increase in ROS levels is accompanied by a significantly reduced number of $CD44^{high}/CD24^{low}/EpCAM^+$ CSC and, as a functional assay, decreased tumorsphere formation in soft agar. Because CD44 is a direct target of the β-catenin dependent transcriptional machinery, the investigators hypothesized that increased ROS, produced through FBP1 overexpression, decreased CSC by inhibiting β-catenin activation. β-catenin acts as coactivator of the transcription factor TCF4 to induce genes involved in maintaining stem-like characteristics. Indeed, FBP1 overexpression decreased β-catenin signaling by promoting its dissociation from TCF4. This interaction was restored with the antioxidant N-acetylcysteine. Taken together, these data support a mechanism where epigenetic downregulation of FBP1 increases glycolysis and decreases ROS, resulting in activation of β-catenin signaling to maintain CSCs (Figure 1).

Although Dong et al. (2013) provide a reasonable explanation for how low levels of ROS due to FBP1 silencing maintain CSCs in BLBCs, they do not mechanistically explore how modulating FBP1 protein levels can regulate E-cadherin protein expression. Presumably, the Snail-G9a-DNMT complex has access to the E-cadherin promoter in the presence or absence of FBP1. Thus, it was surprising that ectopic expression of FBP1 was sufficient to block Snail-dependent epigenetic silencing of E-cadherin. Since methylation is a balance between methyltransferase and demethylase activity, perhaps FBP1 expression triggers high levels of ROS, which affects the activity of these enzymes at the E-cadherin promoter.

A major implication of these findings is that antioxidants might not be beneficial in some cancers. A recent study indicated that non-stem cancer cells can also give rise to CSCs, indicating the bidirectionality between these two populations (Chaffer et al., 2011). If low levels of ROS maintain characteristics of CSC, which are notoriously resistant to radiation and chemotherapy, then antioxidants would possibly promote CSC. Might pharmacologically increasing ROS in BLBC promote the loss of CSCs and improve patient survival? As new therapies are developed for BLBC, it will be of interest how they affect ROS levels and glucose metabolism. Furthermore, therapies directed in diminishing glucose metabolism might be effective against BLBC.

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Selective Blockade of Transport via SERCA Inhibition: The Answer for Oncogenic Forms of Notch?

Ma. Xenia G. Ilagan[1,*] and Raphael Kopan[1,*]
[1]Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110, USA
*Correspondence: ilaganmg@wustl.edu (M.X.G.I.), kopan@wustl.edu (R.K.)
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NOTCH1, **which is frequently mutated in T cell acute lymphoblastic leukemia, has been an elusive therapeutic target. In this issue of *Cancer Cell*, Roti and colleagues demonstrate that inhibiting SERCA calcium pumps preferentially impairs the maturation of the most common class of oncogenic Notch1 mutants, thus uncovering a potential therapeutic avenue.**

The four mammalian Notch receptors are large type I membrane proteins, sporting an extracellular domain with 29–36 epidermal growth factor (EGF) repeats followed by the conserved Lin12-Notch repeats (LNR) and a heterodimerization domain (HD; Figure 1A). The LNR and HD domains constitute the negative regulatory region (NRR), which maintains the "off" state of the receptor in the absence of ligand. Upon binding of Notch to ligand presented by a neighboring cell, the NRR undergoes a conformational change to expose the S2 site to ADAM metalloprotease cleavage (Figure 1B). This is followed by γ-secretase-mediated cleavage at the S3 site within the transmembrane domain (TMD), which releases the Notch intracellular domain (NICD). NICD translocates to the nucleus, associates with the DNA-binding protein RBPjκ and the transcriptional coactivator Mastermind (MAM/MAML) to activate transcription. Activation is linked to phosphorylation of the PEST domain, its recognition and ubiquitination by the E3 ubiquitin ligase FBW7, and NICD degradation (reviewed in Kopan and Ilagan, 2009).

Because the Notch signaling pathway regulates many fundamental processes during embryonic development and in self-renewing adult tissues, both gain- and loss-of-function mutations in pathway components lead to developmental disorders, cancer, and other adult onset diseases. Best known is the contribution of ligand-independent, activated forms of Notch1 to T-ALL, more than half of which gain activating mutations in the NRR, PEST, or both (Figure 1A; Weng et al., 2004). The mutations in the NRR lead to ligand hypersensitivity and ligand-independent activation, whereas the PEST domain mutations increase the stability of NICD and lead to sustained signaling activity.

The preponderance of *NOTCH1* mutations in T-ALL has fueled the search for effective anti-Notch1 therapeutics (Figure 1B; reviewed in Tzoneva and Ferrando, 2012). Because Notch activation relies on proteolysis, γ-secretase inhibitors (GSIs), which had been originally developed for Alzheimer's disease therapy, have entered clinical trials for treatment of relapsing T-ALL. However, sustained GSI inhibition is not tolerated, because pan-Notch blockade causes severe gastrointestinal toxicity and promotes progression of squamous cell carcinomas (Extance, 2010). The same problems could affect the efficacy of stapled dominant negative MAML-like peptides (SAHM) that directly target the transcription complex. More recently, receptor-specific anti-NRR1 antibodies have been developed. Despite their ability to circumvent gut toxicity, sustained treatment with these reagents will likely cause vascular neoplasms, raising additional safety concerns (Yan et al., 2010).

To identify modulators of Notch1 signaling and potential therapeutic targets for T-ALL, Roti et al. (2013; in this issue of *Cancer Cell*) conducted complementary high throughput small molecule and cDNA overexpression screens using cell-based assays reporting Notch transcriptional activity. For the compound screen, the transcriptional signature of Notch in T-ALL was assembled from previous genome-wide expression profiling studies of multiple human T-ALL cell lines treated with vehicle or GSI. They validated a group of 28 target and 4 nontarget genes to generate a robust,