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Developing Effective Therapeutics for Difficult-to-treat Cancers

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CONTENTS

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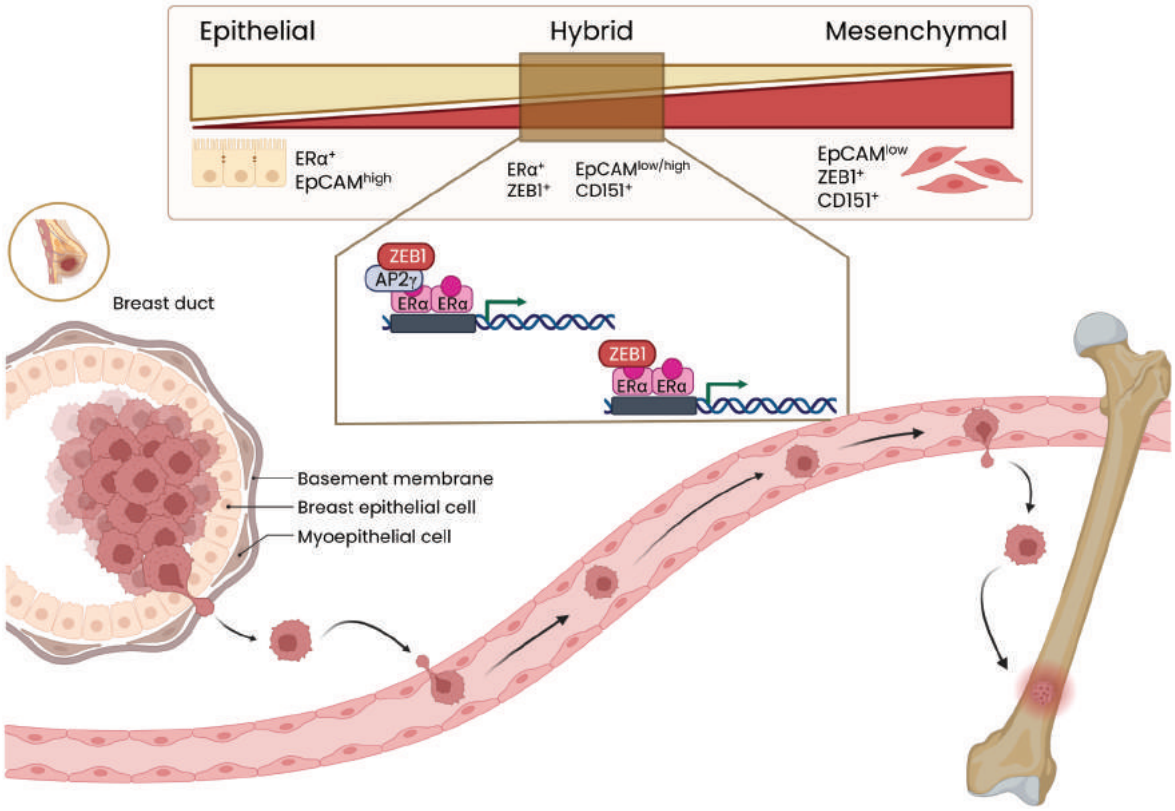
Deep Dive: Epithelial to Mesenchymal Transition in Estrogen Receptor Positive Breast Cancer



Epithelial to mesenchymal transition (EMT) is a hallmark of cancer progression and in breast cancer not only predicts poor prognosis, but also resistance to endocrine therapy. A recent paper outlines the interactions between ZEB1 and estrogen receptor alpha and identifies an EMT hybrid population that could be targeted in novel therapeutic strategies to reverse EMT and improve patient prognosis.

Epithelial to Mesenchymal Transition

EMT is characterized by loss of cellular adhesions and apical polarity associated with epithelial cells. These changes are often due to decreased expression of E-cadherin, an epithelial cell marker, and increased expression of N-cadherin, vimentin, and cellular proteases, which aid in cell motility and trans-endothelial migration. Zeb, Snail, Slug, and Twist are master EMT-inducing transcriptional factors.[1,2]

ERα is a marker of breast cancer prognosis

Estrogen receptor alpha (ERα) is a member of a superfamily of transcription factors that bind to small lipid soluble molecules like thyroid and steroid hormones. It can dimerize and bind to estrogen response elements (EREs) in DNA, or ERα can interact with additional proteins to create complexes that recruit transcription machinery to additional response elements.[3] Estradiol (E2), which activates and signals through ERα, is required for normal growth and differentiation of breast epithelial cells. ERα is a positive predictor of breast cancer prognosis because it antagonizes EMT signaling pathways and is indicative of response to hormonal therapies.[2,4]

While the presence of ERα indicates the likelihood of patients to respond to antiestrogen therapies, not all ERα+ breast cancers respond to these therapies and many that initially respond eventually develop resistance. EMT is thought to play a critical role in the development of endocrine resistance. In breast cancer patients high and low ERα-expressing cells coexist. Cells with low ERα expression resemble mesenchymal cells, while cells with high ERα expression maybe epithelial cells or represent a hybrid state. The hybrid state is associated with poor patient prognosis. Even though decreased ERα function correlates with EMT in breast cancers, ER α activation can induce EMT in other types of cancer. In this study, the authors investigated the role of EMT-inducer Zeb1 on ERα activity and ability to modulate EMT in breast cancer to determine whether the two factors can work in concert to promote the progression of more invasive forms of breast cancer.[5]

Short-term ZEB1 expression created a hybrid EMT state

Breast cancer cell lines MCF7, MCF7-V (a variant with enhanced ERα responses), and T-47D, were engineered to express ZEB1 via a doxycycline-inducible lentiviral system. In all three of the cell lines, long-term expression of ZEB1 (8-12 weeks) resulted in decreased ERα expression, as previously reported, and complete EMT. Short-term ZEB1 expression (1-2 weeks), however, maintained ERα expression levels and resulted in partial EMT. ERE luciferase reporter constructs were used to show that these maintained ERα levels correlated with ERα activity. Consistent with the luciferase reporter assays, short-term ZEB1 expression increased mRNA levels of ERα target genes, while long-term expression of ZEB1 resulting in mesenchymal-like phenotype caused reduced ERα target gene mRNA levels.

Using tumor spheroids, researchers found that in the presence of E2, ZEB1 expression increased the size of the spheroid and dissemination into the surrounding matrix. However, FI cocktail (forskolin, to stimulate adenylate cyclase and 3-isobutyl-1-methylxanthine, to block phosphodiesterase) or the antiestrogen ICI, suppressed invasion of the cells into the matrix. 3D invasion assays with T47-D cells showed similar results, indicating that in response to E2 ZEB1 enhances ERα-mediated cell invasion.

The Gene expression-based Outcome for Breast cancer Online (GOBO) tool showed improved overall survival in ERα+ patients with high ZEB1 levels. In ERα- patients, ZEB1 levels did not impact overall survival, but high ZEB1 levels did negatively affect distant metastasis-free survival. Further examination using the GOBO tool suggested that patients with ERα activation due to high levels of ZEB1 were more sensitive to the active tamoxifen metabolite 4-hydroxytamoxifen (4-OHT) during early EMT. This observation was supported by cell cycle assays where 4-OHT treated cells with high levels of ZEB1 were more frequently found arrested in G0/G1 phase than control cells (75% compared to 60%).

ZEB1 directly interacts with ERα

The researchers then wanted to understand how ZEB1 modulates ERα responses. CHIP-seq experiments were performed in MCF7-V-ZEB1 cells after one week of ZEB1 expression. These experiments identified 37,922 ZEB1 binding sites that had significant overlap with ERα binding sites (ERBSs) induced by E2 and the FI cocktail. The Genomic Regions Enrichment of Annotations Tool (GREAT) uncovered functions related to EMT, migration, and WNT signaling for ZEB1-induced ERBSs.

Co-immunoprecipitation experiments confirmed that ZEB1 and ERα are present in the same protein complexes and that binding sites of the AP2 family are highly enriched at the intersections of ZEB1 and ERα binding. Comparing published CHIP-seq data from AP2γ, which regulates transcription at ERBSs, with the ZEB1 and ERα CHIP-seq data identified 6,019 shared

sites. To further support AP2γ, ZEB1, and ERα being a part of the same transcription factor complexes, co-IPs found that AP2γ formed a complex with ZEB1 and knockdowns of AP2γ disrupted the ability of ZEB1 and ERα to co-precipitate and reduced ERα chromosomal binding.

ANXA2, KRT8, HSPB1, and *TIMP1* were identified as markers for the hybrid EMT state

To further investigate the EMT transitional state, time course experiments of ZEB1 expression were performed in MCF-V cells. Using EpCAM as a marker for EMT, distinctions were made between epithelial and mesenchymal phenotypes across the time course. After ten weeks of ZEB1 expression, a mesenchymal phenotype was marked by $EpCAM^{low}$ expression and loss of ERα. However, at only five weeks of ZEB1 expression both $EpCAM^{high}$ and EpCAMlow cell populations were identified, and this population was used to further characterize the EMT hybrid state. Single cell RNA-seq confirmed the presence of additional epithelial cell markers in the $EpCAM^{high}$ population and genes known to facilitate invasion were present in $EpCAM^{low}$ cells. ANXA2, KRT8, HSPB1, and TIMP1 were found in both $EpCAM^{high}$ and $EpCAM^{low}$ populations and thus might be important in establishing the hybrid EMT state. Depletion of ANXA2, HSPB1, or TIMP1 in the presence of ZEB1 and ERα activation reduced migration in wound-healing assays and cell invasion towards bone, but had no effect on cell invasion toward lung tissue, indicating a role for these genes in tissue tropism of ERα+ breast cancer cells.

CD151 is a novel hybrid EMT marker with therapeutic potential

The single cell RNA-seq data of the $EpCAM^{high/low}$ cells was unable to detect any previously identified cell surface markers correlating with different EMT stages in primary mammary tumors (CD61–, CD51–, and CD106–). CD151, an integrin involved in metastasis to bone, was significantly enriched in the $EpCAM^{low}$/CD61-/CD51-/CD106- cell population and expression correlated with ZEB1 levels. Reduction of CD151 in the presence of ZEB1 and ERα activation decreased cell proliferation, migration, and invasion towards bone. This suggests that CD151 might be a therapeutic target to reduce cell division and migration in ZEB1+/ERα+ breast cancer cells.

While it has been previously shown that EMT results in resistance to antiestrogen therapies, the impact of early or hybrid EMT states on ERα signaling had not been investigated. This study uncovered the ability of ZEB1 to interact with ERα and alter EMT state, cell proliferation, and breast cancer metastasis and provided new insights into possible therapeutic targets to help reduce cancer severity.[5]

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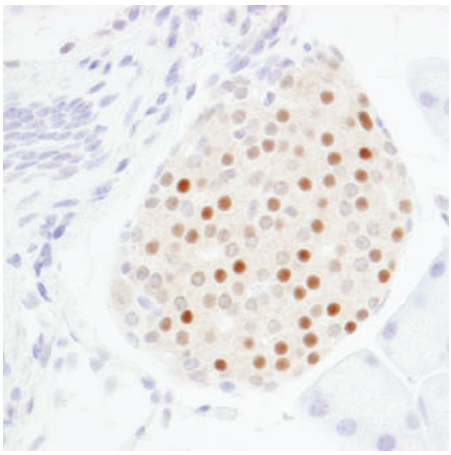
Highlight: The Promise of Signal Transduction Pathways for the Treatment of Pancreatic Cancer

Successfully treating pancreatic cancer represents one of the biggest challenges in oncology to date. Pancreatic cancer has the lowest five-year survival rate of all cancers1; equivalent incidence and mortality rates[2]; and an underwhelming response to all widely available treatments, including surgical resection[3,4]. The poor prognosis of pancreatic cancer highlights the need to identify additional therapeutic targets in order to produce more efficacious therapies. To this end, signal transduction cascades associated with pancreatic cancer cells are an area of intense research that has much promise[5,6].

Signal transduction pathways include both the membrane-bound receptor that receives the signal, and the complex molecular pathway which then transduces the signal into intracellular activity. Alterations to epidermal growth factor receptors (EGFR) and several molecules within two EGFR-initiated signal transduction pathways are heavily implicated in pancreatic cancer[5,7,8]. EGFR is overexpressed in the majority of pancreatic cancers[9,10], and this excessive expression is associated with an even worse prognosis for the individual[11,12]. Interestingly, EGFR inhibitors have shown only modest clinical benefits[7,13,14,] and this may be due to the concomitant mutations and alterations to EGFR signal transduction molecules. For example, one key EGFR-initiated pathway includes K-Ras/RAF/MEK/ERK/MAP2K. Single point mutations in the K-Ras gene have been identified in pancreatic cancers, causing constitutive activation and initiation of various cellular processes that contribute to tumorogenesis[5,15–17]. The PI3K/AKT/mTOR pathway is another EGFR signal transduction cascade implicated in pancreatic cancer[5,6,18]. This pathway is involved with the enhancement of cell growth and survival, and also appears to be excessively activated in pancreatic cancers[19,20]. Numerous other signal transduction molecules and pathways are implicated in pancreatic cancer cells and the stroma, including: 1) JAK/STAT, 2) cyclooxygenase-2 (COX-2), 3) sonic hedgehog (SHH), 4) Notch, and 5) WNT. For example, many pancreatic cancers are associated with persistently active STAT3[21,22,] and excessive COX-2 and WNT expression[23,24.]



Detection of MafA in FFPE mouse pancreatic islet.
Antibody: Rabbit anti-MafA (IHC-00352).
Secondary: HRP-conjugated goat anti-rabbit IgG (A120-501P).
Substrate: DAB.

The abundance of signaling pathways implicated in pancreatic cancer etiology and progression provides a wealth of possible therapeutic targets that may apply to all, or to subpopulations, of patients. This characteristic of pancreatic cancer may position it as an ideal model for the refinement and application of personalized medicine[25,26].

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Highlight: Prevention and Treatment of Advanced Cervical Cancer

At one time, cervical cancer was the leading cause of cancer death for women in the US. The incidence of cervical cancer is not restricted to the US or developed nations, however. Cervical cancer is the fourth most common cancer in women worldwide and represents approximately 8% of all female cancers.[1]

Most cases of cervical cancer are associated with human papilloma virus (HPV) infection, a discovery that has fueled development of screening and prevention strategies.[2] These strategies include HPV screening as well as regular Pap tests, which detect precancerous cells before they become malignant. As a result of these screening strategies, the number of cases of cervical cancer and the number of deaths from the disease have decreased significantly in the US.[3] The importance of these screening tests are also illustrated in the varying 5-year survival rate depending on stage of disease at diagnosis; 5-year survival rate is over 90% in stage 0 disease, but drops to less than 20% in advanced disease stages.[4]

New cases of cervical cancer in the US have dropped significantly since 2006, when the first HPV vaccine was approved by the Food and Drug Administration (FDA). Since then, two additional HPV vaccines have been approved. However, despite advances in vaccination against the causative agent, patients continue to be diagnosed with advanced stage cervical cancer.

For these patients, surgery, radiation therapy, and chemotherapy have been the mainstay of treatment.[5] Unfortunately, though, these treatments have failed to significantly improve overall survival.[6] As such, there is ongoing research into the development of immunotherapies for the treatment of cervical cancer. These immunotherapies seek to stimulate the patient's own immune system to recognize and subsequently destroy cancer cells more efficiently. Currently, in addition to the three HPV vaccines, one targeted antibody and one immune checkpoint inhibitor have been FDA-approved for the treatment of cervical cancer. The targeted antibody, bevacizumab, is a monoclonal antibody that inhibits tumor blood vessel growth by targeting the vascular endothelial growth factor (VEGF)/VEGF receptor pathway.[7] The immune checkpoint inhibitor, pembrolizumab, targets programmed cell death protein 1 (PD-1), a protein on T cells that normally prevents these immune cells from attacking "self."8 By blocking PD-1, pembrolizumab augments the body's immune response to cancer cells, thereby shrinking the tumor and/or slowing its growth.

Numerous other immunotherapies for the treatment of cervical cancer are currently in clinical trials. These include vaccines, targeted antibodies, adoptive cell therapy, and immunomodulators.

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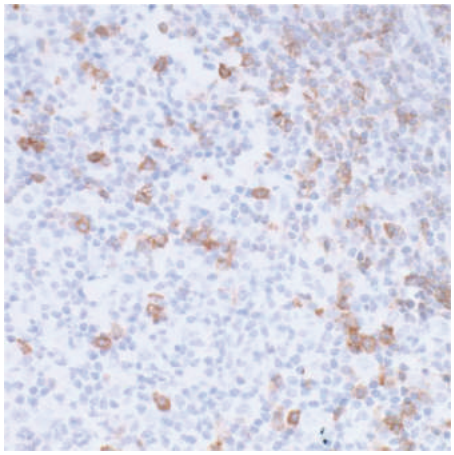
Highlight: CAR-T cells for the Treatment of Blood and Solid Tumors



Detection of human PD-1 in FFPE Hodgkin's lymphoma by IHC.
Antibody: Rabbit anti-PD-1 recombinant monoclonal [BLR076G] (A700-076).
Secondary: HRP-conjugated goat anti-rabbit IgG (A120-501P).
Substrate: DAB.

T cells with chimeric antigen receptors, more commonly known as CAR-T cells, are used in the immunotherapy of cancer. In CAR-T cell therapy, a patient's own T cells are taken from their blood and engineered in a laboratory to express an antigen receptor that targets a specific molecule expressed by a patient's cancer cells. CAR-T cells contain a target-specific extracellular domain fused to the internal domain of CD3-zeta, which may or may not be fused to one or more costimulatory domains. Finally, these cells are transfused back into the patient. These cancer-specific T cells are a powerful tool that track down and kill cancer cells.

The development of CAR-T cells began in the late 1980s-1990s and the technologies that ultimately led to their creation were first developed by immunologists Michel Sadelain and Zelig Eshhar.[1,2] At the time the first T cell engineering was taking place, most scientists did not believe that T cells would have much efficacy in the fight against cancer. Little did they know that two decades later, CAR-T cells would be an FDA-approved treatment for adult and childhood cancer.

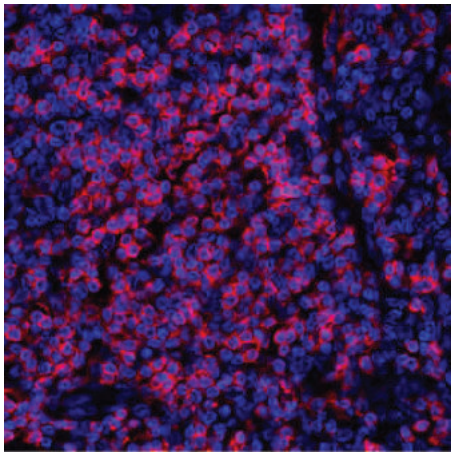
Indeed, the FDA has approved CAR-T cells for the treatment of acute lymphoblastic leukemia, or ALL, in both adults and children, and for the treatment of lymphoma.[3] The CAR-T cells used to treat ALL target a molecule called CD19.[4] CD19 is found normally on the surface of B cells, where it is involved in B cell development and activation. CD19 is expressed at normal or elevated levels on the malignant B cells of at least 80% of ALL patients, as well as at least 88% of B cell lymphomas and all B cell leukemias, making it a useful therapeutic target in blood cancer. CD19 CAR-T cells are in clinical trials for the treatment of lymphoma and leukemia.

Two types of CD19-targeting CAR-T cells have been developed.[5] The first generation CD19 CAR-T cells had just a CD19 recognition domain. However, these cells had a low persistence in vivo and were not very effective as anti-cancer therapy. Second-generation CD19 CAR-T cells added the intracellular domain of a costimulatory molecule: either CD28 or 4-1BB, while third-generation CD19 CAR-T cells include the intracellular domains of both CD28 and 4-1BB, fused to the CD3-zeta intracellular domain. In a clinical trial, a direct comparison of a second- versus third-generation CD19 CAR-T cell for the treatment of lymphoma showed that the third-generation CAR-T cells proliferated and persisted in vivo better than the second-generation CAR-T cells.[6] Other studies showed that third-generation CD19 CAR-T cells are also effective against leukemia.[7]

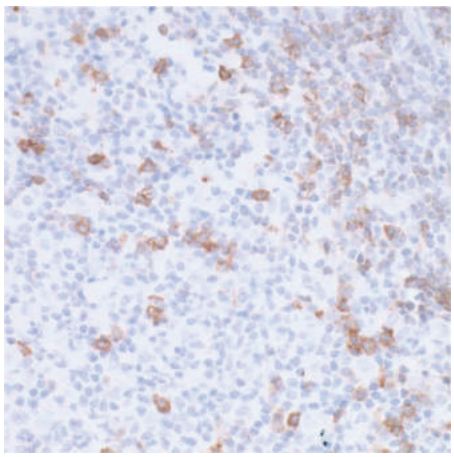
While CAR-T cell therapy has been extensively evaluated in hematologic malignancies, more recently, CAR-T cells have been developed for some solid tumors as well. For the treatment of

neuroblastoma, a childhood cancer that is notoriously difficult to treat, CAR-T cells targeting either GD2 or CD171 have reached clinical trials[8]. In both cases, some patients had partial or complete responses. Clinical trials for this disease are ongoing. GD2-targeting CAR-T cells have also shown success for the treatment of glioma[9] and are in clinical trials for sarcoma. CAR-T cells targeting HER2, a receptor tyrosine-kinase that is overexpressed in many tumors, have also been explored as a treatment for sarcoma and glioma. In both cancers, some patients showed a clinical response to treatment, which included either stable disease or partial or complete tumor regression.[10]

Multiple other targets of CAR-T cells are also being evaluated for solid tumors, and combination therapies are being explored as well. However, local immune suppression in the microenvironment of solid tumors makes CAR-T therapy more challenging for solid tumors compared to blood tumors. Current clinical trials are exploring the combination of CAR-T cells with checkpoint blockade immunotherapy, which activates the anti-tumor immune response. Early reports from a CAR-T plus anti-PD-1 checkpoint blockade in mesothelioma showed 72% response rate including both partial and complete responses.[11]



Detection of human CD247/CD3Z (red) in FFPE tonsil by IHC-IF. Antibody: Rabbit anti-CD247/CD3Z recombinant monoclonal [BL-336-1B2] (A700-017). Secondary: HRP-conjugated goat anti-rabbit IgG (A120-501P). Substrate: Opal™. Counterstain: DAPI (blue).



Detection of human PD-1 in FFPE Hodgkin's lymphoma by IHC. Antibody: Rabbit anti-PD-1 recombinant monoclonal BLR076G] (A700-076). Secondary: HRP-conjugated goat anti-rabbit IgG (A120-501P). Substrate: DAB.

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Highlight: BRD4 is an Epigenetic Regulator and Emerging Target in Hematopoietic and Solid Tumors

Human BRD4

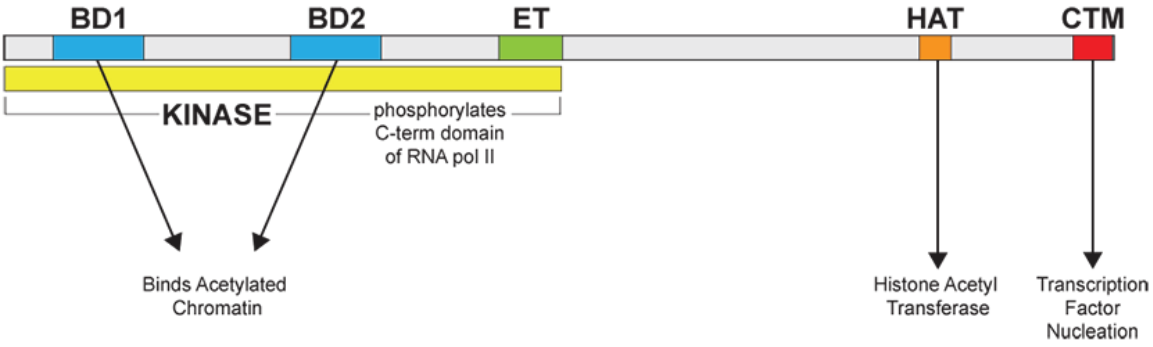

Diagram of full length human BRD4 showing its four primary functions: (1) binding to acetylated chromatin via two N terminal bromodomains (BD1, BD2); (2) histone acetyl transferase (HAT) activity for acetylating lysine residues in histones, (3) serine kinase activity which acts on RNA pol II; and (4) a C-terminal motif (CTM) which serves as a nucleation site for transcription factors. The extraterminal (ET) domain is characteristic of BET family members and serves as an additional protein-protein interaction domain.

Bromodomain protein 4 (BRD4) is a transcriptional regulator that plays a key role in cancer, autoimmunity, and inflammatory diseases[1,2]. BRD4 was discovered as a protein bound to acetylated chromatin during cell cycle progression. In this way BRD4 maintains consistent gene expression during subsequent rounds of division, a phenomenon known as epigenetic memory or "bookmarking" for gene transcription[2,7]. BRD4 also plays a critical role in regulating differentiation and development[2,4]. In the absence of BRD4, bone marrow stem cells are unable to generate B and T cells[2]. BRD4 is required for the re-expression of stem cell genes during reprogramming of MEFs or B cells to induced pluripotent stem cells[2], and also plays a role in osteoblast differentiation[4]. The role of BRD4 in cell cycle control and differentiation has made it an emerging therapeutic target for cancer and immune system pathologies.

The BET family of bromodomain proteins are characterized by the presence of two bromodomains and an ET domain[2,6]. Of these, BRD4 is the most well-studied. BRD4 has four primary molecular functions. It (i) maintains chromatin structure via binding acetylated histones, an activity mediated by sites in the N-terminal domain; (ii) Histone Acetyl Transferase (HAT) activity via its HAT catalytic domain[2,6]; (iii) serine kinase activity via a kinase domain that spans the N-terminal region and phosphorylates residues in the C-terminal domain of RNA Pol II[2,6]>; and (iv) the C-terminal domain serves as a binding and nucleation site for transcription factors and complexes. BRD4 is found in several transcription complexes, including the general cofactor Mediator and the P-TEFb elongation factor[6]. The C terminal domain also mediates the interactions of BRD4 with many well-known transcriptional regulators, most notably P-TEFb, MYC, NFκb, and p53[2,4,6-8].

BRD4 has recently come to light as a possible target for cancer therapy because of its role as a transcriptional and epigenetic regulator of the cell cycle1-5. In particular, many hematopoietic cancers depend on constant BRD4 activity for expression of Myc[2,3,5,7]. Solid tumors are also associated with BRD4 activity2. Deregulation of BRD4 is clinically linked to breast, colon, and

prostate cancers[7]. Interestingly, a BRD4 fusion protein, BRD4-NUT, is found in aggressive midline carcinomas[2,7]. Whether BRD4 plays a further role in metastasis is unclear[2].

To date, most available BET inhibitors affect all BET family members producing unpredictable and sometimes dangerous results.[1] BET inhibitors work by blocking the association of BRD4 with chromatin by mimicking acetyl-lysine residues on histones[2,3].This effect prevents transcription of Myc and other critical regulatory genes leading to cell cycle arrest[2,3,5]. Specific BET inhibitors are currently under development which will allow more selective inhibition of BRD4 and other BET family members[1,3,5,7]. BET inhibitors may be especially effective in combination with a variety of other chemotherapeutic agents by enabling the use of lower doses of toxic drugs and by helping to overcome resistance[5,7].

References

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