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REVIEW

The converging roles of BRD4 and gene transcription in pluripotency and oncogenesis

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Pluripotent embryonic stem cells (ESCs) and cancer cells share traits and molecular mechanisms, such as the ability to self-renew and a block in cellular differentiation. Both ESCs and tumor cells have a large proliferative capacity and cellular plasticity. One common denominator linking these two cell types is the BET family member, BRD4. BRD4 plays a critical role in gene regulation, recruiting the active form of Positive Elongation Factor b (P-TEFb) to RNA polymerase II at paused promoters ultimately resulting in the production of elongated mRNAs. BRD4 is deregulated in many cancers making it an attractive therapeutic target. Here, we highlight the recent findings coupling the role of BRD4 in pluripotency and tumorigenesis.

BRD4 in gene regulation

BRD4 is a member of the BET (bromodomain and extraterminal domain) tandem bromodomain-containing family that binds acetylated histones H3 and H4 and influences gene regulation [1]. BRD4 was originally identified as a mitotic chromosome-binding protein that remains associated with acetylated chromatin throughout the entire cell cycle [2, 3]. This unique property makes BRD4 a candidate for mitotic or epigenetic bookmarking after cellular division. Prior to mitosis the nuclear envelope breaks down, the chromatin condenses, most regulators separate from the mitotic chromosomes, and transcription ceases [4]. After mitosis, the genes have a remarkable memory through chromatin factors that "bookmark" genes to be expressed or silenced resuming the same gene expression patterns indistinguishable from the mother cell prior to cellular division [5].

RNA polymerase II (RNA Pol II) elongation is a rate-limiting step in gene transcription [6]. At many developmental genes RNA Pol II stalls or pauses after the synthesis of a nascent transcript about 30-65 nucleotides in length [7]. In human ESCs, nearly 30% of genes transcription undergoes a transcriptional pausing [8]. This suggests that transcriptional pausing and elongation are additional checkpoints control during development [9]. To relieve RNA Pol II pausing and enter the productive mRNA elongation state, additional proteins such as BRD4 are necessary to reverse the stalled RNA Pol II. BRD4 directly influences gene transcription associating with positive elongation factor b (P-TEFb) [10, 11]. The BRD4-P-TEFb interaction enhances RNA pol II, releases transcriptional pausing, and mediates productive elongated mRNAs. The recruitment of BRD4 and P-TEFb mediates elongation by phophorylating serine 2 on RNA Pol II's carboxy terminal domain and releasing the

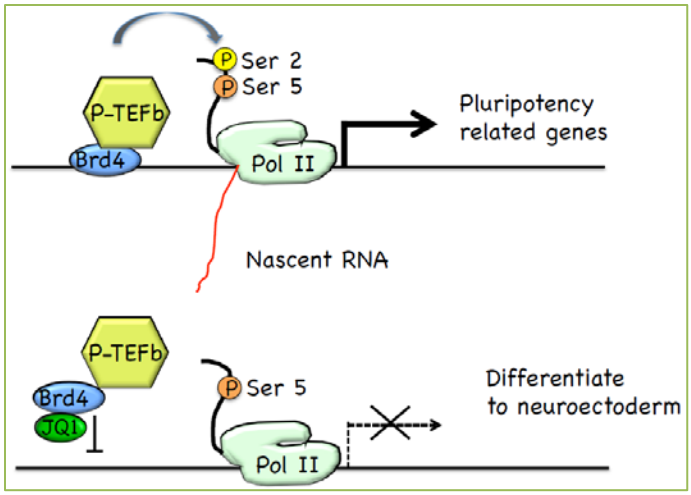

Figure 1. Model of BRD4 action in pluripotency. BRD4 interacts with P-TEFb resulting in the phosphorylation of RNA Pol II serine 2 (Ser 2), a release from pausing of the nascent RNA strand, and the productive transcriptional elongation of the pluripotency related genes. Inhibition of BRD4 with a BET inhibitor, JQ1, or depletion of BRD4 by knockdown ablates the production transcription of the pluripotency genes. RNA Pol II is unable to elongate as evident by the presence of serine 5 (Ser 5) phosphorylation and a lack of Ser 2 phosphorylation. This results in a shift away from pluripotency and differentiation to the neuroectoderm cell fate.

pausing factors NELF (Negative Elongation Factor) and DSIF (DRB-sensitivity-inducing factor) [6, 7].

BRD4 in oncogenesis

In addition to its role in gene regulation, BRD4 is important for human disease. BRD4 is a target for viral-encoded regulators in human immunodeficiency virus and human papillomavirus allowing these infectious agents to hijack the cellular machinery facilitating selective viral genome integration during mitosis [12,13]. BRD4 deregulation is linked to numerous cancers. Cancer cells often exploit the host cell's transcriptional and chromatin regulatory machinery to propagate their oncogenic gene expression profiles. NUT midline carcinoma (NMC) is a group of malignant and highly lethal cancers, arising from chromosomal translocations involving the NUT (Nuclear protein in Testis) gene on chromosome 15q14 [14]. In the majority of NMC cases, the NUT-containing chromosome translocates to the *Brd4* gene on chromosome 19 [14]. This BRD4-NUT protein fusion results in a poorly differentiated and highly aggressive carcinoma with a mean survival of six months. Besides NMC, BRD4 deregulation is found in leukemia, hepatocellular carcinoma, breast cancer, and brain cancers [15-17]. BRD4's function in cancer is associated with epithelial-to-mesenchymal transition (EMT) [18], stem cell-like conversion, and primary stress responses. BRD4 protects chromatin from DNA damage signaling, further implicating its role as gatekeeper in normal cells and oncogenesis [19].

Indeed, BRD4 is a therapeutic target for multiple cancers. Small-molecule inhibition of BRD4's bromodomains or targeting small interfering hairpin RNAs eliminates several malignant cancer cells including leukemia, melanoma, prostate, and breast cancers [15, 16, 20-27]. Pharmacological inhibition of BET bromodomain proteins with small molecules (such as JQ1 and I-BETs) interferes with BRD4's requirement for enhancer function and transcriptional elongation in different cancers [15, 28, 29]. The inhibition of BRD4 is accompanied by terminal differentiation through down regulation of c-MYC, a factor that regulates transcriptional pause release and important for tumor viability [15, 29]. Recently BRD4 was found to control *HOTAIR*, a long noncoding RNA essential for glioblastoma proliferation [30]. This suggests BRD4 may be a therapeutic target in Glioblastoma Multiforme one of the most common and aggressive malignant adult brain tumors.

Converging themes in pluripotent and cancer cells

Both pluripotent and cancer cells have common features such as rapid cell cycles, featuring a diminution of G1 phase, and high telomerase activity [31]. Together, these can result in uncontrolled cellular proliferation. ESCs and cancer cells have a loss of G1 cell cycle checkpoint after DNA damage [32]. The plasticity of their cell type is underscored as upon transplantation into immunodeficient mice where both ESCs and cancer cells form benign and malignant tumors, respectively [33]. Notably both have changes in epithelial and mesechymal states. Epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET) regulate cellular plasticity during embryonic development and in oncogenesis [34]. EMT is a process in which polarized cell lose their cell-cell adhesion, become mobile and invasive gaining mesenchymal features. EMT in cancer cells contributes to tumor development, invasion, dissemination, and resistance to therapies [34]. The Twist transcription factor is a key activator of EMT. Twist has a diacetylated histone H4-like domain that recruits BRD4. Together this Twist-BRD4 interaction directs gene expression in basal-like breast cancer [26]. Disruption of the BRD4-diacetylated Twist interactions with BET inhibitors can suppress tumorigenesis in basal-like breast cancer [26].

MET is a reversal of epithelial characteristics and in oncogenesis is associated with metastases [34]. In normal development, stem cells are at the top of the somatic lineage hierarchy and utilize both EMT and MET to generate all of the embryonic germ layers: ectoderm, endoderm, and mesoderm. Optimal epigenetic reprogramming from the somatic state to induced pluripotency requires a sequential

EMT-MET mechanism. The tumor suppressor protein p53 is frequently mutated in cancers and can modulate epithelial and mesenchymal states [34-37]. P53 is a barrier to somatic cell reprogramming, whereas mutations in p53 augment epigenetic reprogramming in induced pluripotency [38-40]. The p53 somatic reprogramming block is mediated by inhibition of MET [41]. Interestingly BRD4 interacts with p53 resulting in a phosphorylation switch, targeting both proteins to acetylated chromatin for gene-specific activation [42].

The master regulators OCT4, SOX2, c-MYC, and KLF4 control the pluripotent gene expression during embryogenesis, in ESCs, and induced pluripotent stem cells (iPSCs). The exciting discovery by Yamanaka's group demonstrated that the expression of these four transcription factors in fibroblast somatic cells converts them to iPSCs [43]. Interestingly, these core stem cell transcription factors are upregulated in several types of human cancers. OCT4 is expressed in pancreatic cancer, oral squamous cell carcinoma, seminoma, and bladder cancer [33, 44-48]. SOX2 overexpression results in cancer stem cell-like characteristics, which influence tumor formation and aggressiveness in squamous cell carcinoma, lung cancer, serous ovarian cancers, gastric cancer, pancreatic cancer, and brain cancers [49-53]. Indeed, SOX2 is associated with chemotherapy and tamoxifin resistance in breast cancer [50-53]. In addition to its role in induced pluripotency, KLF4 is believed to act as an oncogene in breast cancer [54]. The transcription factor c-MYC is a well-known mediator in oncogenesis and is activated in nearly 70% of all human cancers [55]. A c-MYC protein network accounts for the similarities between ESC and cancer stem cell transcriptional programs [56].

Cancer cells exhibit an increased utilization of glycolysis for carbon metabolism, rather than mitochondrial oxidative phosphorylation even in the presence of oxygen [57]. This phenomenon is called the "Warburg effect", a pyruvate-lactate metabolism that rapidly generates ATP, is observed in numerous tumors reflecting their highly proliferative state. Similarly, pluripotent ESCs prefer a glycolysis metabolism [58-60]. Within hours of ESC cellular differentiation, a metabolic switch occurs and oxidative phosphorylation dominates [57]. The ESC glycolytic step differs from the Warburg effect (a metabolic pyruvate-lactate step), in that a pyruvate-acetyl CoA step enhances the high rates of cytosolic acetyl-CoA synthesis proposed to maintain the levels of histone acetylation post-translational modification (PTM) necessary for pluripotency [61]. These studies further extend the connection in ESCs between metabolic intermediates and an open or permissive chromatin state.

Pluripotent and cancer cells have distinctive epigenetic profiles. One highlight is the status of X-chromosome inactivation (XCI), a crucial epigenetic process that silences one of the two female Xs to balance the gene dosage with XY males [62]. XCI is tightly linked with pluripotency, with epigenetic silencing commencing during cellular differentiation. In contrast, a global reprogramming and reactivation of the silenced X [63-64] accompany the conversion of female somatic cells to iPSCs. In undifferentiated ESCs, the long noncoding RNAs (lncRNAs) *Xite* (the enhancer for *Tsix*), *Tsix* (the anti-sense repressor of *Xist*), and *Xist* (the silencer) are all expressed on the single male X and both female X-chromosomes [62]. Following cellular differentiation, the male X extinguishes the expression of these lncRNAs to retain a single, active X (Xa). Differentiating female ESCs have a choice of active (Xa) versus inactive X (Xi). On the future Xa, *Xite* and *Tsix* expression keep the silencer lncRNA *Xist* levels low. On the future Xi, *Xist* and *Tsix* are extinguished resulting in a robust upregulation of *Xist* levels [62]. *Xist* subsequently recruits heterochromatic silencing factors. OCT4 lies at the top of the XCI hierarchy regulating the pluripotent-associated lncRNAs *Xite* and *Tsix* [65]. OCT4 partners with the chromatin insulator, CTCF, specifying the early decision of XCI counting (XCI occurs when there is more than one X), X-X homologous pairing (a chromosomal dance necessary for XCI), and choice (the mutually exclusive decision of active versus inactive X) [65]. This epigenetic memory is observed in XCI as once chosen for activation or inactivation, the same female X-chromosome retains its fate after cell division.

Pluripotent and cancer cells both display an epigenetic ground state with hypomethylation and a paucity of PTM silent marks such as histone 3 lysine 27 trimethylation (H3K27me3) that decorates the inactive X-chromosome in differentiated female cells [62]. For nearly sixty years it has been observed that the Barr body, the heterochromatic inactive X-chromosome is lacking in the nuclei of breast and ovarian cancers [66].

Oxygen status is crucial for human ESCs and tumorigenesis. The presence of two active female X-chromosomes (XaXa) exemplifies the ground state of pluripotency in mouse ESCs [63]. Human female ESCs show invariable XCI status (exhibiting partial or complete XCI) due to their advanced developmental stage during derivation as well as between subcultures of a single human ESC line [67]. The epigenetic ground state of human ESCs (XaXa) is obtained during derivation and culture upon exposure to physiological levels of oxygen, suggesting that oxygen maintains a more developmental immature state [67]. Thus, the ground state of epigenetics (XCI) in human ESCs is established by increasing oxygen. Tumor cells frequently

outgrow their normal blood supply and develop hypoxia and hypoglycemia. This results in a series of events in which cancer cells take advantage of the normal cellular stress response. Normal cells respond to the hypoxia by increased tissue oxygenation to stabilize homeostasis. In contrast, tumor cells engage a vicious cycle of growth and starvation [68]. The overall result is that hypoxia increases tumor aggression and metastasis. Overall, an ESC-like signature is present in poorly differentiated tumors [31]. The parallels that link the pluripotent and tumor cell types are of huge interest. A commonality is the BET family member BRD4.

BRD4 is crucial for pluripotent gene expression and the lncRNAs that regulate XCI.

Although BRD4 is known to play crucial roles in cancers, very little is known about its function in normal development. Mutation of the mouse *Brd4* results in a peri-implantation lethal [69]. This is a crucial time during lineage segregation with the formation of the pluripotent epiblast (the source of ESCs and the embryo proper) and female X-chromosome inactivation (XCI) establishment, a crucial epigenetic process that silences the dosage disparity between XX females and XY males [63]. Taken together, these results suggest that BRD4 may play a role in both of these early development processes. Therefore, we postulated that BRD4 interacts with pluripotent factors and functions in the coupled processes of pluripotency and XCI. Indeed, we discovered that BRD4 physically interacts with OCT4 in ESCs. We determined that male and female ESCs show similar BRD4 protein levels during cellular differentiation at differentiation day 0 (pre-XCI), day 4 (time of XCI establishment), and day 8 (post-XCI) [70]. This is in contrast to OCT4 expression, which shows a diminution of protein expression upon differentiation. The OCT4 transcription factor directly regulates the XCI lncRNAs *Xite* and *Tsix*. OCT4 triggers X-chromosome pairing and counting [65]. During the shift away from pluripotency by removal of leukemia inhibitory factor (LIF) and mouse embryonic feeder layers, the lncRNAs *Xite* and *Tsix* are extinguished. In contrast, upon differentiation the silencer *Xist* is robustly expressed reflective of XCI. These XCI lncRNAs are transcribed in both the sense and anti-sense orientation suggesting that tight control of RNA Pol II is necessary for their regulation.

OCT4 is a master regulator of the pluripotent fate targeting *Xite*, *Tsix*, *Nanog*, *Sox2*, and *Oct4* itself [65, 71-73]. As ESCs differentiate, the chromatin shifts from a transcriptionally permissive euchromatic or "open state" to a more heterochromatic or "closed state" [74-76]. These chromatin changes are exemplified by alterations in histone PTMs, chromatin configuration, the presence of the Polycomb group proteins, and gene expression [77, 78]. In ESCs both OCT4 and BRD4 occupy the regulatory regions of pluripotent genes such as *Nanog*, *Oct4*, *Sox2*, *Xite*, and *Tsix* [70]. Using an OCT4-regulated ESC line [79], we determined that OCT4 recruits BRD4 to selective regulatory regions such as the *Nanog* promoter [70].

The small molecule, JQ1, which selectively binds the bromodomains, can inhibit the BET domain proteins [15, 28]. We posited that inhibition of BRD4 by JQ1 alters the pluripotent status in ESCs. First, we performed a dose curve of JQ1 in male and female ESCs. Interestingly, JQ1 inhibits c-MYC protein as previously reported but in addition we demonstrated that OCT4 levels are decreased [70]. Next, we queried BRD4, P-TEFb, and histone H4 acetylation status at pluripotent regulatory regions in ESCs using quantitative Chromatin immunoprecipitation. (qChIP). JQ1 exposure specifically displaces BRD4 and P-TEFb occupancy without altering H4 acetylation levels [70]. The BET inhibition diminished pluripotent gene expression, shifting the cell fate of the treated ESCs to neuroectoderm identity. In contrast, the RNA Pol II inhibitors *Hexim1* and *Sens3* were upregulated suggesting that BRD4 represses these genes [70]. JQ1 exposure in differentiated female cells greatly diminishes *Xist* lncRNA expression but does not reactivate genes along the silent X-chromosome. Consistent with the JQ1 treatment, loss of *Brd4* by small interfering RNAs (siRNAs) in ESCs reduces the expression of pluripotent genes as well as *Xite* and *Tsix* [70]. Overexpression of the BRD4 in ESCs enhances the levels of pluripotent genes. These results support the findings of DiMicco *et al*. for a role of BRD4 in human ESCs [80]. Taken together, our results suggest that BRD4 activates the pluripotent and XCI genes in ESCs. BRD4 maintains ESC identity and fate.

Conclusion

Herein we have summarized similarities and differences between pluripotent and stem cells. We have highlighted the recent findings for BRD4 and stem cell identity. However many outstanding questions remain. Such as is BRD4 involved in X-X chromosome pairing? Does BRD4 provide the epigenetic memory for female XCI fate retaining the same active and inactive X-chromosomes after cellular division? Our understanding of how BRD4 functions in normal developmental processes is crucial for elucidating its deregulation in cancers.

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Conflict of Interest

T.W. and M.E.D. declare that there are not potential conflicts of interest.

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