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Hypothesis

ZEB1 Mediates Drug Resistance and EMT in p300-Deficient CRC

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Abstract

We discuss the hypothesis that ZEB1-Wnt-p300 signaling integrates epithelial to mesenchymal transition (EMT) and resistance to histone deacetylase inhibitors (HDACis) in colorectal cancer (CRC) cells. The HDACi butyrate, derived from dietary fiber, has been linked to CRC prevention, and other HDACis have been proposed as therapeutic agents against CRC. We have previously discussed that resistance to butyrate likely contributes to colonic carcinogenesis, and we have demonstrated that butyrate resistance leads to cross-resistance to cancer therapeutic HDACis. Deregulated Wnt signaling is the major initiating event in most CRC cases. One mechanism whereby butyrate and other HDACis exert their anti-CRC effects is via Wnt signaling hyperactivation, which promotes CRC cell apoptosis. The histone acetylases (HATs) CBP and p300 are mediators of Wnt transcriptional activity, and play divergent roles in the downstream consequences of Wnt signaling. CBP-mediated Wnt signaling is associated with cell proliferation and stem cell maintenance; whereas, p300-mediated Wnt activity is associated with differentiation. We have found that CBP and p300 differentially affect the ability of butyrate to influence Wnt signaling, apoptosis, and proliferation. *ZEB1* is a Wnt signaling-targeted gene, whose product is a transcription factor expressed at the invasive front of carcinomas where it promotes malignant progression and EMT. ZEB1 is typically a transcriptional repressor; however, when associated with p300, ZEB1 enhances transcription. These changes in ZEB1 activity likely affect the cancer cell phenotype. ZEB1 has been shown to promote resistance to chemotherapeutic agents, and expression of ZEB1 is upregulated in butyrate-resistant CRC cells that lack p300 expression. Since the expression of ZEB1 correlates with poor outcomes in cancer, ZEB represents a relevant therapeutic target. Here we propose that targeting the signaling network established by ZEB1, Wnt signaling, and p300 signaling can reverse HDACi resistance and inhibit EMT.

Key words: ZEB1, Wnt, epithelial to mesenchymal transition, histone deactylase inhibitors, colorectal cancer, Wnt signaling, p300

Introduction

Wnt signaling and histone deacetylase inhibitors

The histone deacetylase inhibitor (HDACi) butyrate, derived from dietary fiber, exerts a strong preventive effect against colorectal cancer (CRC) [1-13], and other HDACis are possible cancer therapeutic agents [14-16]. Therefore, neoplastic cell resistance to the effects of butyrate likely contributes to CRC, as well as to cross-resistance to HDACis proposed for CRC therapy [2]. Mutations resulting in deregulated Wnt signaling are a major initiating event in most sporadic CRC cases [17-26]. One mechanism whereby butyrate and other HDACis exert their anti-CRC effects is via hyperactivation of the deregulated Wnt signaling in neoplastic colonic cells, promoting apoptosis and repressing cell proliferation [1, 2,16].

The histone acetylases (HATs) CBP and p300 associate with the Wnt signaling factor beta-catenin and mediate the transcriptional activity of the Wnt pathway. Although CBP and p300 share considerable sequence homology, they play divergent roles in the downstream consequences of Wnt activity. CBP-mediated Wnt signaling is associated with cell proliferation and stem cell maintenance; whereas, p300-mediated Wnt activity is associated with differentiation [27-37]. CBP and p300 affect the ability of butyrate to influence Wnt signaling, apoptosis, and proliferation [38-41].

To understand the phenomenon of butyrate resistance, we developed a HCT-116 CRC cell line (HCT-R) resistant to the effects of physiologically relevant concentrations of butyrate [2]. These cells are also cross-resistant to structurally distinct and clinically relevant HDACis [2, 42], and exhibit repressed expression of p300 [39]. HCT-R cells exhibit (a) increased expression of the epithelial to mesenchymal transition (EMT) factor ZEB1 and (b) modified expression of other characteristics of EMT, such as decreased expression of E-cadherin and increased expression of vimentin [3]. Coincidentally, p300 knockout cells, also derived from the HCT-116 cell line, also exhibit physiological changes consistent with EMT, such as decreased cell-cell/matrix adhesion and increased migration [43].

ZEB1, CBP/p300, and EMT

EMT, associated with cancer cell metastasis, results in altered gene expression and changes stationary and polarized epithelial cells into mesenchymal cells with enhanced motility and invasiveness. The reverse process, mesenchymal to epithelial transition (MET), occurs after the metastatic cells have settled into a new location. ZEB1 is expressed at the invasive front of carcinomas, where it affects gene expression to induce EMT [44,45] (and refs therein). ZEB1 upregulates expression of vimentin and downregulates expression of E-cadherin, which is a key event for EMT and metastasis [44]. Since ZEB1 promotes tumorigenesis and metastasis [44-52], its expression is correlated to poor outcomes in cancer, including resistance to chemotherapy [48 and refs. therein]. ZEB proteins most frequently repress transcription through both passive and active mechanisms [44]. Passively, ZEB1 displaces transcriptional activators from promoters; actively, ZEB proteins recruit transcriptional corepressors. In addition, ZEB can associate with transcriptional coactivators to upregulate expression [44]. ZEB proteins can act through a variety of transcriptional cofactors; for example, ZEB1 binds to the HATs p300 and PCAF [45,46], and this complex activates transcriptional activity. Alternatively, ZEB1 acts as a repressor when it interacts with BRG1, an ATPase that forms part of the SWI/SNF chromatin remodeling complex, or with CtBP, which forms a complex that may include HDACis [44 and refs. therein]. The expression levels of p300 and CtBP influence ZEB1 activity; in particular, high levels of CtBP result in an inverse relationship between ZEB1 and E-cadherin expression [51], consistent with the role of ZEB1/CtBP in repressing E-cadherin expression. *ZEB*1 is a Wnt signaling-targeted gene, and its product can modulate gene expression in a Wnt activity-dependent manner [46-50].

The miR-200 family of microRNAs (miRNAs) influence EMT and MET; these miRNAs are downregulated during EMT and upregulated during MET, likely reflecting their functional contribution to the cell phenotypes along the epithelial to mesenchymal continuum [46,47]. ZEB proteins and miR-200 members negatively modulate each other's expression, consistent with the pro-EMT role of ZEB factors and the anti-EMT role of miR-200 family members [46,47]. The downregulation of ZEB expression is mediated by miR-200 binding to *ZEB* RNA 3′ UTR and blocking translation [46,47]. Complexes composed of ZEB1, p300, and PCAF can bind to the miR200c/141 promoter, resulting in acetylation of ZEB1 and reversal of ZEB1 repression of miR200c/141 expression [46]. This interaction decreases ZEB1 expression and therefore inhibits EMT. The HDACi trichostatin A (TSA), which synergizes with HAT activity, shifts cells toward a more epithelial phenotype, demonstrating the effect of net acetylation [46,47]. These reports are consistent with our observation that cells resistant to the HDACi butyrate downregulate p300 and exhibit an EMT-like profile that includes increased expression of ZEB1. Treatment of human airway epithelial cells with the CBP-Wnt inhibitor ICG-001, which enhances p300-Wnt activity [27-37], suppresses EMT induced by TGFbeta1 [53]. This is also consistent with findings that p300 knockout cells have an EMT-like phenotype [43], since CBP and p300 compete for binding to beta-catenin. In summary, decreased p300 favors CBP-Wnt activity and promotes EMT-type gene expression and phenotype. However, low expression of p300 is not sufficient for acquisition of an EMT-like phenotype. For example, HCT-15 cells that are naturally p300-deficient have an epithelial phenotype [55]; however, these cells exhibit low ZEB1 expression [56]. Thus, expression of ZEB1 and associated factors [46-58] might be essential for EMT following p300 downregulation.

Intriguing connections have also been drawn between ZEB1, EMT, dedifferentiation of cancer cells

to cancer stem cells (CSCs), resistance to therapy, and HDACis [59-65]. EMT likely promotes the emergence of a drug-resistant, relatively dedifferentiated mesenchymal cancer cell phenotype [59], which contributes to HDACi-resistance during colonic tumorigenesis. Therefore, the phenomenon of butyrate resistance [66-75] and resistance to other HDACis, may be in part mediated by ZEB1, possibly through altered gene expression and cell signaling [76-81]. A summary of the impact of ZEB1 on CRC [45, 47-50, 58, 82-87] is shown in Table 1.

Table 1. Summary of Functional impact of ZEB1 in CRC

ZEB1 FUNCTION	EXPERIMENTAL MODEL	REFERENCE
Represses stemness-inhibiting microRNAs	CRC and pancreatic cancer cells	82
Inhibits senescence	SW480 and SW620 CRC cells	83
Promotes metastasis and loss of cell polarity	SW480, HCT-116	84
Not required for EMT	LS174T CRC cells	85
Regulates miR-200c in EMT	human CRC sample analyses	86
ZEB1-hTERT complex inhibits E-cadherin expression	HCT-116 CRC cells	58
ZEB1 and TCF4 reciprocally modulate each other's transcriptional activity	SW480, SW620, and HCT-116 CRC cells	50
Regulates the plasminogen proteolytic system by inducing uPA and inhibiting PAI-1	SW480, HCT116, and Colo320 CRC cells	49
Promotes vasculogenic mimicry through EMT induction	HCT-116 CRC cells	87
Promotes metastasis and loss of cell polarity	HCT-116, SW480 CRC cells	84
Represses E-cadherin expression, induces EMT	SW480 CRC cells	47
Promotes EMT	HCT-116 CRC cells	45
Promotes tumor invasiveness	HCT-116 and SW480 CRC cells	48

Butyrate resistance, EMT, and colonic tumorigenesis

Studies of butyrate resistance [66-75] provide more information on signaling crosstalk affecting CRC development and behavior. HCT15 cells, which are p300 negative, are more resistant to the effects of butyrate than other CRC cells such as SW480 [70], and this report is consistent with what we have observed when evaluating these cell lines, as well as other, p300-deficient, cells [39 and unpublished data]. HCT-15 cells, which have been further selected for resistance through chronic exposure to butyrate, exhibit an EMT phenotype (albeit one relatively more epithelial-like [55]), and show greater ability for tumor growth, angiogenesis, and metastasis in an *in vivo* nude mouse model [70]. These observations suggest that presence of butyrate in the colonic microenvironment may select for phenotypically more aggressive, therapy-resistant tumor types during neoplastic progression. However, the overall incidence of colon cancer is reduced with a high fiber dietary intake, which may indicate a major preventive effect of butyrate at the initiating stage of neoplastic development. Thus, on the one hand, butyrate likely reduces the incidence of CRC; however, on the other hand, if CRC does develop despite the presence of higher levels of butyrate in the colonic microenvironment, the resulting tumors may be more aggressive and therapy-resistant.

Possible associations between HDACi resistance and EMT is also indicated by the finding that treatment of certain CRC cell lines with HDACis increases EMT-like phenotypes, as measured by changes in gene expression and cellular physiology [75]. Thus, there may be a connection between butyrate resistance developed as a result of chronic butyrate exposure, EMT, and the relative levels of CBP-Wnt activity vs. p300-Wnt activity. Of direct relevance to the connection between ZEB1 and HDACi resistance, a breast cancer cell line resistant to the HDACi phenylbutyrate exhibited increased expression of ZEB1, and ZEB1 inhibited the expression of genes (e.g., *RAB25, ESRP1*) that enhance phenylbutyrate sensitivity in these cells [76].

Hypothesis

Our central hypothesis is that *ZEB1 cooperates with the downregulation of p300 and p300-Wnt activity in promoting both resistance to therapeutics and EMT in CRC.* This hypothesis can be divided into two parts. *First, we hypothesize that ZEB1 upregulation promotes both EMT and HDACi resistance in CRC cells.* This is based on the observation that butyrate-resistant cells exhibit changes in gene expression consistent with EMT, such as increased expression of ZEB1 and vimentin and downregulated expression of E-cadherin [3]. ZEB1 expression and EMT are also associated with cancer cell dedifferentiation, which promotes drug (e.g., HDACi) resistance. *Second, we posit that ZEB1 is a downstream effector of CBP-Wnt signaling that controls HDACi resistance and EMT, and is repressed by p300-Wnt signaling partially through miR-200 activity.* ZEB1 is upregulated in butyrate-resistant cells that exhibit repressed expression of p300. Further, the association of ZEB1 with p300 changes ZEB1 function from that of a repressor to that of an activator of transcription [45,46]. Therefore, the relative levels of CBP- vs. p300-mediated Wnt signaling [28-31,38-41, 77] may affect both ZEB1 expression and function, influencing HDACi resistance and EMT [78] through altered gene expression in neoplastic colonic cells. In addition, we

propose that there is reciprocal repression between ZEB1 and the miR-200 family of miRNAs, particularly miR-200c, and this modulation of expression influences EMT [79]. Furthermore, miR-200 family members, particularly miR-200a, downregulate Wnt signaling [80,81]; therefore, miR-200a and miR-200c may contribute to the ability of ZEB1 to integrate Wnt signaling, resistance to HDACis, and EMT. This hypothetical interaction network is summarized in Fig. 1.

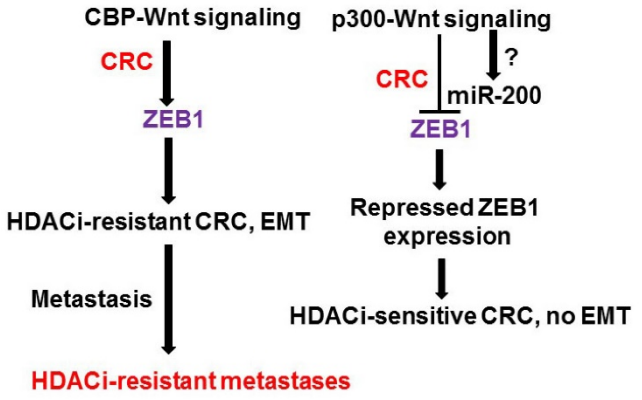

Figure 1. Proposed ZEB1 interactions in CRC. Deregulated Wnt signaling modulates gene expression, thus promoting CRC. CBP-Wnt signaling (left) is linked to proliferation, neoplasia, and cancer stem cell maintenance; whereas, p300-Wnt signaling (right) is associated with differentiation. We hypothesize that ZEB1 is upregulated in p300-deficient CRC cells through CBP-Wnt signaling. This results in enhanced EMT and altered gene expression leading to resistance to HDACis. The end result of these interactions is an HDACi-resistant metastatic CRC phenotype. Members of the miR-200 family repress ZEB expression, and are in turn repressed by ZEB factors, and some miR-200 members can repress Wnt signaling. The exact relationship between miR-200 family members and p300-mediated Wnt activity is unknown.

Testing the hypotheses

The first experimental objective would be to establish how altered expression of ZEB1 integrates HDACi-resistance and EMT in CRC cells. In addition, we would need to ascertain whether altered ZEB1 expression mediates effects of CBP-Wnt activity vs. p300-Wnt activity on HDACi-resistance and EMT, and the role of miRNA in these interactions.

The role of ZEB1 in promoting resistance to HDACis and mediating the effects of altered CBP-Wnt vs. p300-Wnt activity, can be determined by ZEB1 knockout in p300-deficient butyrate-resistant cells by CRISPR. The reverse experiment, in which ZEB1 is overexpressed in butyrate-sensitive, p300-expressing HCT-116 cells, can be achieved through stable transfections with a ZEB1 expression vector. Cells with ZEB1 knockout or overexpression would be assayed in absence or presence of butyrate and the clinically relevant HDACis vorinostat and LBH589 for: (a) Wnt activity, measured by reporter assays and levels of active (dephosphorylated) beta-catenin; (b) proliferation; (c) apoptosis; (d) clonal cell growth; (e) expression of vimentin and E-cadherin via Western blot analysis; (f) matrigel growth [43]; and (g) invasiveness.. HDACi-resistance would be evaluated by metrics a,b,c,d and EMT by metrics e,f,g,h.

To examine the role of p300 and relative levels of CBP-Wnt activity vs. p300-Wnt activity in affecting ZEB1 expression levels and activity, a number of methodologies can be used. One experimental model could be the p300 knockout CRC cells [43] that have EMT-like characteristics and that reverse their EMT phenotype by "rescue" transfection with a p300 expression vector. In addition, specific inhibitors that repress CBP-Wnt [28-31] and p300-Wnt [77] activity could be employed. CRISPR could also be utilized to create CBP knockout cells analogous to the p300 knockout lines.

In addition, in cells with modified CBP-Wnt activity vs. p300-Wnt activity, coimmunoprecipitation followed by Western blot can be utilized to measure the degree of association of ZEB1 with its corepressive (CtBP, BRG1) or coactivating (p300, PCAF) cofactors, to correlate this association with cell phenotype. ZEB1-cofactor association may mediate the effects of ZEB1 expression on cell physiology. For example, in biliary tract cancer cells, SMAD4 mediates the ability of the HDACi vorinostat to suppress EMT and reduce chemoresistance to gemcitabine; in this experimental model, vorinostat inhibited the nuclear translocation of SMAD4 and its interaction with ZEB1 and other EMT factors [78].

In this experimental approach, the levels of expression of relevant miR-200 family members would be measured in the relevant experimental cell models described above, and correlated to ZEB1 levels and cell physiology. A ZEB1 3′ UTR expression vector, which is sensitive to translational control by miRNAs, can be utilized as a high throughput luciferase reporter to identify conditions (e.g., CBP-Wnt vs. p300-Wnt activity, HDACis) that affect the ability of miR200 family miRNAs to modulate ZEB1 expression. Thus, changes in ZEB1 expression that are mediated through interactions of miR-200 members with ZEB1 mRNA would alter expression from the ZEB1 3′ UTR vector. If changes in 3′ UTR reporter activity suggestive of involvement of miR-200 family members are observed, miRNA expression vectors (e.g., miR-200a and miR-200c) can be used to downregulate ZEB1 expression. This approach is expected to reverse the butyrate/HDACi-resistance and EMT characteristics observed in HCT-R and p300 knockout CRC cells.

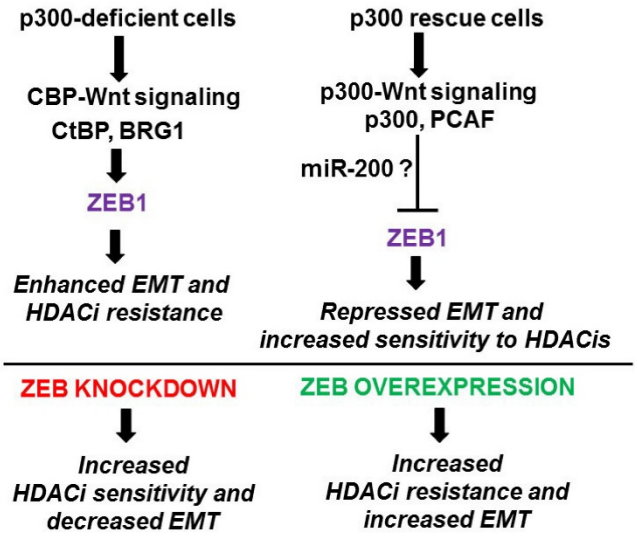

Figure 2. Expected results from proposed experiments. Cells deficient in p300 expression (e.g., HCT-R and p300 knockout HCT-116 cells) (top left) are expected to have relatively higher CBP-mediated Wnt activity, leading to higher levels of ZEB1 and association of ZEB1 with CtBP and BRG1. The resulting activity of ZEB1 as a transcription repressor would enhance EMT and resistance to HDACis. p300 rescue cells (top right) would exhibit the opposite pattern with increased p300-mediated Wnt signaling, decreased expression of ZEB1, and association of ZEB1 with p300 and PCAF. Lower levels of ZEB1, along with increased activity of ZEB1 as a transcription activator, would promote sensitivity to HDACis and repress EMT. We expect that the effects of p300-mediated Wnt signaling in repressing ZEB1 levels will be at least partially mediated by miR-200 family miRNAs. At bottom we show expected outcomes for knockdown or overexpression of ZEB1 in p300-deficient and p300 rescue cells, respectively.

Expected results

We expect (Fig. 2) ZEB1 knockout to decrease HDACi resistance and the EMT-like phenotype of butyrate-resistant cell lines; whereas, ZEB1 overexpression may induce a degree of butyrate/HDACi-resistance in butyrate-sensitive cells, along with a more pronounced EMT-like phenotype. The ZEB1 knockout cells should also be more sensitive to the pro-apoptotic and growth suppressing effects of HDACis, and should exhibit increased expression of E-cadherin and decreased expression of vimentin. The opposite will be expected in butyrate-sensitive cells with stable overexpression of ZEB1.

We expect that p300 rescue cells would exhibit increased sensitivity to HDACis, and decreased ZEB1 expression coupled to a lesser degree of EMT-like phenotype (measured by changes in vimentin/E-cadherin expression, matrigel-growth and invasion). If this is observed, we would determine whether exogenous overexpression of ZEB1 at least partially reverses these effects of restored p300 expression. This outcome would strongly suggest that ZEB1 is a major mediator of CBP-Wnt activity vs. p300-Wnt activity, and their downstream physiological outcomes.

In summary, CBP-Wnt signaling would be associated with increased resistance to HDACis, EMT, and expression of ZEB1; p300-Wnt signaling is expected to promote the opposite: HDACi sensitivity, less pronounced EMT behavior, and decreased expression of ZEB1. Enhanced CBP-Wnt signaling is expected to increase ZEB1 association with repressors such as CtBP and BRG1 and promote EMT; whereas, enhanced p300-Wnt signaling is expected to increase ZEB1 association with p300 and PCAF. We expect that the effects of p300-Wnt signaling on ZEB1 expression, and, consequently, on EMT and HDACi-resistance, would be at least partially mediated by miR-200 family miRNAs, particularly miR-200 and miR-200c.

Conclusion

This line of inquiry may lead to a comprehensive understanding of how ZEB1 modulates CBP- vs. p300-mediated Wnt signaling to influence EMT and resistance to HDACis. Expression of ZEB1 is correlated to poor outcomes in human cancer; thus ZEB represents a therapeutic target. The findings of the proposed studies can be utilized to design approaches that reverse HDACi resistance and inhibit EMT.

Abbreviations

CRC: colorectal cancer; EMT: epithelial to mesenchymal transition; HDACi: histone deactylase inhibitor; miRNA: microRNA; TSA: trichostatin A; CSCs: cancer stem cells.

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Competing Interests

The authors have declared that no competing interest exists.

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