INVESTIGATING THE ROLE OF CTBP IN COLORECTAL CANCER

by

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Abstract

Recent studies in breast cancer tissue have demonstrated that C-terminal binding protein (CtBP) over-expression induces stem cell-like features and genome instability; whereas CtBP depletion or caloric restriction reverses gene expression and increases DNA repair. C-terminal binding proteins (CtBP) are dimeric nuclear factors that function as transcriptional regulators in response to changes in the cellular level of NADH. Based on the binding affinity of CtBP for NADH, CtBP can function as a sensor of cellular metabolism and therefore has the potential to act as a metabolic marker in known obesity-related cancers. Thus, we decided to study CtBP expression and localization in colorectal cancer (CRC), a known obesity-related cancer.

To study the potential role of CtBP as a metabolic marker in obesity-related cancer, we first subjected a panel of colorectal (HT-29, HCT-15, and SW480), lung (NCI-H1299) and breast (MCF-7) cancer cell lines to a number of metabolic perturbations including hypoxia and hyperglycemia and then observed the expression of known CtBP target genes. The results from these experiments suggested that CtBP does not function as a transcriptional regulator in the colorectal cancer cell lines studied, even while under conditions which are known to elevate cellular NADH levels. We then determined that simultaneous knockdown of CtBP 1 and 2 had no effect of the mRNA expression of known CtBP targets, suggesting again that it was not functioning as a transcription repressor in the colorectal cells. Furthermore we determined the subcellular localization of CtBP1 and CtBP2 in all the cell types interrogated. From these localization experiments we determined that CtBP 1 and CtBP2 were found in primarily in the cytoplasm of the CRC cell lines studied, in contrast to the breast and lung cancer cell lines, where they were found in the nucleus. Based on these findings, we conclude that CtBP does not act as a transcriptional regulator in the colorectal cancer cell lines tested. To test the therapeutic implications of this finding, we also treated the panel of cancer cell lines with purported inhibitors of CtBP and performed MTT assays at 24, 48, and 72 hours to determine the amount of cell death. The results of these experiments showed no significant differences in response to the inhibitors among the different cell types, thus suggesting that the inhibitors used potentially act through a mechanism other than one in which CtBP is responsible.

Introduction

Colorectal cancer is the third most commonly diagnosed cancer in the United States and simultaneously worldwide.[1, 2] It also is the fourth leading cause of cancer-related death in the world and the third leading cause in the U.S.[1, 2] It is evident from these facts that even though colorectal cancer incident rates have shown a gradual decline over the past decades, specifically in the U.S., the burden of the disease still remains great nationally and internationally.[3]

The incidence of colorectal cancer has been closely associated with several risk factors including non-modifiable factors such as age and heredity, as well as a substantial amount of modifiable environmental and behavioral factors. Two of these modifiable factors are physical inactivity and obesity [defined by a body mass index (BMI) $\geq$ 30 kg/m^2], for which abundant evidence has shown that they account for nearly a third of all colorectal cancer diagnoses.[3] Studies have also shown that with increased physical activity and lower BMI, a lower risk of developing colorectal cancer can be associated, further validating the relationship between obesity and colorectal cancer.[3]

Obesity is one of the fastest growing epidemics in the United States and worldwide. In fact, obesity has more than doubled worldwide since 1980 and in the United States alone, over two-thirds of the population are considered to be overweight or obese.[4, 5] This rapidly growing epidemic can mainly be attributed to behavioral factors, especially over-nutrition and a lack of physical activity, both of which can be modified, so as to prevent obesity and obesity-related illness. Numerous health-related issues, such as cardiovascular disease, type II diabetes mellitus and a number of cancers, including colorectal cancer, are known to have some association with obesity. In fact, in the United States, it has been estimated that obesity is associated with 15%-20% of all cancer-related deaths.[6] From these statistics, it is quite evident that obesity plays a significant role in health status and more specifically, cancer. Although our current understanding of how increased caloric intake influences obesity-related cancers is progressing, the biological mechanisms responsible for the association are only beginning to be elucidated.[3] In order to define the correlation between obesity and cancer, the impact that metabolic imbalance has on the molecular level must be thoroughly investigated and ultimately understood.[7]

It is widely known that the intake of excess calories has a significant effect on cellular metabolism, and subsequently an imbalance in cellular metabolism has been linked to cancer.[8] In glycolysis, the tricarboxylic acid (TCA or Krebs) cycle, and the oxidation of free fatty-acids, glucose and other carbon intermediates transfer electrons to nicotinamide adenine dinucleotide (NAD+), a coenzyme involved in oxidation-reduction reactions, reducing it to NADH. In 1927, Otto Warburg discovered elevated levels of glucose consumption and lactic acid production in cancer cells, despite the presence of substantial oxygen needed to sustain the respiratory production of ATP through the oxidation of the reduced form of NADH via oxidative phosphorylation.[8] The net result of this occurrence was an increase in the steady state levels of cellular NADH.[8] One consequence of high cellular NADH levels due to excess caloric intake is the increased production of reactive oxygen species, also commonly referred to as ROS, due to an incomplete transfer of electrons in the mitochondrion during cellular respiration.[9] The ROS resulting from this process are known for greatly contributing to DNA damage and consequently associated with the risk of malignant transformation, therefore providing one well understood way to link metabolic perturbations caused by obesity and cancer.[7]

In addition to the knowledge that increased ROS levels play a large role in genome instability, elevated levels of NAD+/NADH are known to have great influence on a number of proteins that utilize the reduced or oxidized coenzymes as cofactors or substrates, one of which is C-terminal binding protein (CtBP) and is the primary basis of our research.[7]

CtBP was first discovered to be a phosphoprotein that interacted with the PxDLS (Pro-X-Asp-Leu-Ser) amino acid sequence located at the carboxyl-terminus of the oncogenic adenovirus E1A protein, thus deriving its name.[10, 11] Like other proteins that bind to the E1A protein, CtBP is known to interact with several transcriptional repressors, thus making CtBP a transcriptional co-repressor.[12] Vertebrates possess two CtBP gene loci, *CtBP1* and *CtBP2,* which share 78% amino acid identity and 83% similarity.[13] Both CtBP1 and CtBP2 possess a highly conserved N-terminal domain that binds transcription factors containing a consensus PxDLS peptide motif, and a central dehydrogenase homology domain.[14, 15] Exactly how C-terminal binding proteins facilitates gene repression is currently not known, though several studies have suggested that CtBP binds to histone deacetylases, such as Sir2, and other chromatin-modifying enzymes in order to form a chromatin-modifying complex.[16] This complex is thus proposed to be recruited to chromatin by the interactions formed between CtBP and DNA-binding transcription factors. In order for such a chromatin-modifying complex to be generated, CtBP must first dimerize, a process that is dependent on the conformational change that occurs when high cytosolic and nuclear NADH levels are present.[17, 18] Therefore, the transcriptional co-repressing function of CtBP is dependent upon the processes that lead to elevations in cellular NADH levels, such as hypoxia and increased glycolysis.[17] Considering that CtBP has a reported 100-fold higher binding affinity for NADH as opposed to NAD+, it is this response to cellular NADH that leads to idea that C-terminal binding proteins may establish a link between metabolism and epigenetic changes typically observed in obesity-related cancers, such as colorectal cancer.

The regulatory function of both CtBP1 and CtBP2 has been noted to be involved in wide variety of cellular processes, even those of which are characteristic of tumor initiation, transformation and development.[17, 19] In 2013, an exhaustive list of CtBP target genes were identified and classified into three main groups by Di *et al.* These three categories consisted of the targets' ability to impact genome stability, stem-cell like self-renewal and epithelial differentiation, all of which are pathways that can lead to highly aggressive types of cancer.[20] Specifically, C-terminal binding protein's inherent ability to regulate genes that are necessary for tissue development processes, such as epithelial-to-mesenchymal transitions (EMT), suggests that CtBP overexpression may play a significant role in tumorigenesis and tumor progression.[21] In fact, the overexpression of CtBP has been shown to suppress epithelial and proapoptotic gene expression, thus providing consistent evidence that CtBP has the ability to induce a wide variety of tumorigenic phenotypes such as cell survival, proliferation, migration/invasion, and EMT. Furthermore, overexpression of CtBP has also been observed and reported to be found in several types of cancers, including ovarian, prostate, colorectal, lung and breast cancers.[21]

Evidence supporting the idea that underlying molecular mechanisms of CtBP in oncogenesis could be linked to its function as a transcriptional co-repressor of tumor suppressors continues to grow, including recent studies that have shown CtBP localization to the nucleus of breast cancer cells.[14] Deng *et al* specifically showed that CtBP localized to the nucleus in primary breast cancer tumors by immunohistochemical staining and even compared these results to normal breast tissue, showing that CtBP nuclear localization was significantly greater in the tumor cells. This study further showed that since CtBP localized to the nucleus, it was able to function as a transcriptional repressor of the *Brca1* gene, a known breast cancer tumor suppressor.[22] Considering that previous work has suggested that CtBP binds chromatin-modifying enzymes forming a chromatin-modifying complex, studies showing

CtBP localized to the nucleus, especially in cancer cells supports the claim that these proteins bind DNA and function as transcriptional regulators of genes involved in tumor suppression. Although CtBP localization has been extensively studied in breast cancer and some other cancer, no CtBP localization studies have yet been done in colorectal cancer cells. Therefore, this study will investigate cellular localization of CtBP1 and CtBP2 in a number of colorectal cancer cell lines.

The transcriptional co-repressor activity of CtBP is known to target pro-apoptotic factors (Bik, Noxa), cytoskeletal/cell adhesion molecules (Keratin-8, E-cadherin) and tumor suppressors ($p16^{INK4a}$, $p15^{INK4b}$), conferring resistance to apoptosis, facilitating EMT, and promoting metastasis and oncogenesis.[23] However, specifically in this study, the purported CtBP targets gene in which we chose to investigate in colorectal cancer were E-cadherin, SIRT1, and HES1. Of these, E-cadherin is best characterized. E-cadherin (E-cad) is a protein involved in cell-cell adhesion that is known to play a significant role in epithelial differentiation and studies have indicated that E-cadherin is a potent tumor suppressor in breast cancer, a known obesity-related cancer.[22] SIRT1, a mammalian homologue of the Sir2 histone deacetylase, has known roles in gene regulation, metabolism, and longevity and recent studies in CCD primary human fibroblasts and NCI-H1299 (lung cancer) have shown that in environments in which elevated NADH levels are present, such as hypoxia, SIRT1 transcription is down-regulated due to the recruitment of the CtBP co-repressor.[24] Hairy and enhancer of split-1, otherwise known as HES1, is a transcription factor that has been shown to enhance stem cell-like features in cancer, features in which have been associated with tumor generation, progression and chemoresistance.[25] In addition to these purported CtBP targets, CDC-like kinase 1 (CLK1) was studied as a potential novel CtBP target. CLK1 was chosen because it is an enzyme that has been shown to be involved in cell proliferation, one of the main processes used to characterize cancer.[26]

Due to C-terminal binding protein's ability to allow many of the critical characteristics of cancer to flourish, it is also a prime target for the development of a therapeutic agent. Recent studies have suggested that small molecules capable of disrupting protein-protein interactions formed between CtBP and its transcriptional factors has the potential to act as a starting point for the development of future novel therapeutic agents.[21] Thus far, only a few CtBP inhibitors of this kind have been identified, including 4-methylthio-2-oxobutanoic acid (MTOB) and NSC95397.[21, 27]

The goal of this study was to investigate the role of CtBP as a transcriptional co-repressor and metabolic sensor in colorectal cancer. We hypothesized that in being a metabolic sensor involved in other cancer types, CtBP would play a role in colorectal cancer, a known obesity-related cancer. The exposure of a panel of colorectal, lung and breast cancer cell lines to various metabolic stressors, including hypoglycemia, hyperglycemia and hypoxia and subsequent determination of gene expression of CtBP and the aforementioned targets was performed in order to study and compare the role of CtBP in these cancer types. In addition to studying the role of CtBP as a transcriptional co-repressor and metabolic sensor primarily in colorectal cancer cells, the effects of MTOB and NSC95397 on cell viability among colorectal, lung and breast cancer cell types were also investigated.

Results

When cells are subjected to metabolic perturbations such as hypoxia and hyperglycemia, two characteristics commonly found in diabetes and other metabolic-related illnesses, cellular NADH levels increase. Under hypoxic conditions, this phenomenon is attributed to a lack of oxygen, which inhibits the recycling of NADH to NAD+ by oxidation. Under hyperglycemic conditions, more glucose fluxes through the glycolytic pathway, producing more pyruvate and acetyl-CoA, leading to more NADH production and a resultant imbalance of NAD+/NADH within the cell. Since the transcriptional repressor activity of C-terminal binding proteins has been shown to be dependent on the NAD+/NADH ratio within a cell, we chose to study the expression of known CtBP targets in colorectal cancer cells after subjecting them to hypoxia (1% O_2), hypoglycemia (1 g/L glucose), and hyperglycemia (4 g/L) for 24 hours.

The 4 main target genes studied were E-cadherin, HES1, CLK1, and SIRT1. The expression of these genes were specifically studied in HT-29 and HCT-15 colorectal cancer cell lines, as well as NCI-H1299 and MCF-7, lung and breast cancer cell lines respectively for comparative purposes (shown in Figure 1). Under hypoxia and hyperglycemia, the transcriptional repressor activity of CtBP would be expected to increase due to elevated levels of NADH, thus decreasing the expression of CtBP target genes. In the colorectal cancer cell lines, only E-cadherin in the HT-29 cells showed a decrease in mRNA expression when under hypoxic and hyperglycemic conditions. The other targets studied in these two colorectal cancer cell lines showed either no difference or an increase in mRNA expression between normoxic and hypoxic samples, results opposite from what is expected if CtBP were involved in repressing the expression of these genes. After subjecting NCI-H1299 and MCF-7 cells to similar hypoxic and hyperglycemic conditions, there were decreases in CLK1 and E-cadherin expression in MCF-7 cells and statistically significant decreases in HES1 expression in the MCF-7 cells and in SIRT1 expression of both NCI-H1299 and MCF-7 cells. The targets in which a decrease in mRNA expression was observed when under hypoxic and hypoglycemic conditions indicates that the transcriptional repressor activity of CtBP may be involved due to an assumed increase in cellular NADH resulting from the conditions imposed. These results suggest that CtBP does not function as a transcriptional repressor in the colorectal cancer cell lines studied in contrast to lung and breast cancer cells, where CtBP has been shown to function as a repressor.

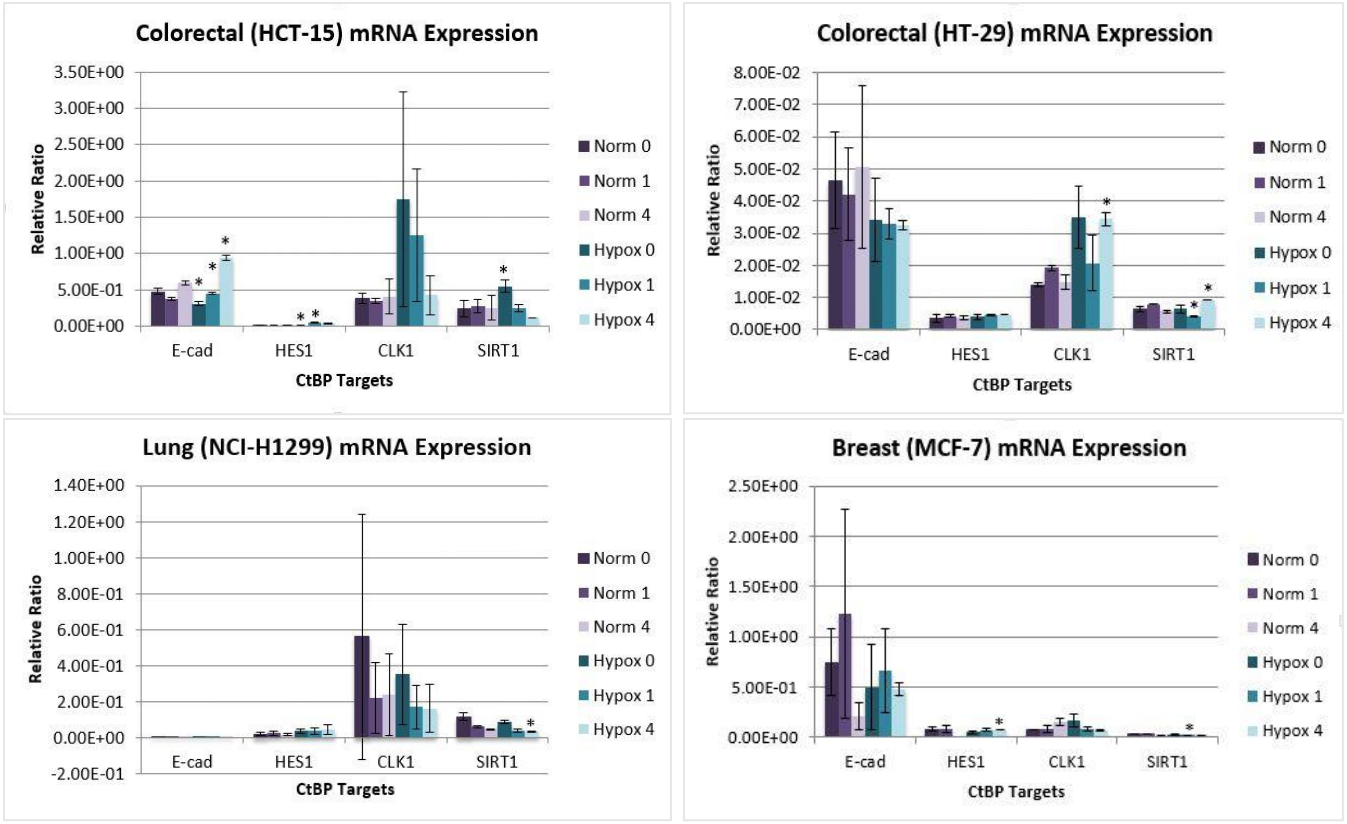


Figure 1: Effect of hypoxia and hyperglycemia on CtBP target gene expression. Graphs showing the relative ratios of mRNA expression of known CtBP targets (E-cadherin, HES1, CLK1, and SIRT1) normalized to B2M as determined by qRT-PCR. Cells were incubated under normoxic (20% O_2) and hypoxic (1% O_2) conditions in either hypoglycemia (0 g/L glucose), euglycemia (1 g/L glucose), and hyperglycemia (4 g/L glucose) for 24 hours (N=1,* P<0.05).

In order to further study the functional role of CtBP in colorectal cancer, CtBP was knocked down in HT-29 (colorectal), NCI-H1299 (lung) and MCF-7 (breast) cancer cells and the effect of CtBP knockdown on gene and protein expression was determined. Cancer cells were simultaneously transfected with CtBP1 and CtBP2 siRNAs for 72 hours to allow for sufficient knockdown the expression of these two genes and proteins within the cells. To confirm knockdown, mRNA and protein expression of CtBP1 and CtBP2 was then measured using qRT-PCR and Western blotting respectively. As shown in Figure 2A, simultaneous transfection with CtBP1 and CtBP2 siRNAs decreased mRNA levels approximately 80-90% in all cell types tested. However, while this also resulted in a significant decrease of CtBP1 and CtBP2 protein in the HT-29 and NCI-H1299, MCF-7 cells were resistant, showing no change in CtBP1 and CtBP2 protein expression (Figure 2B).

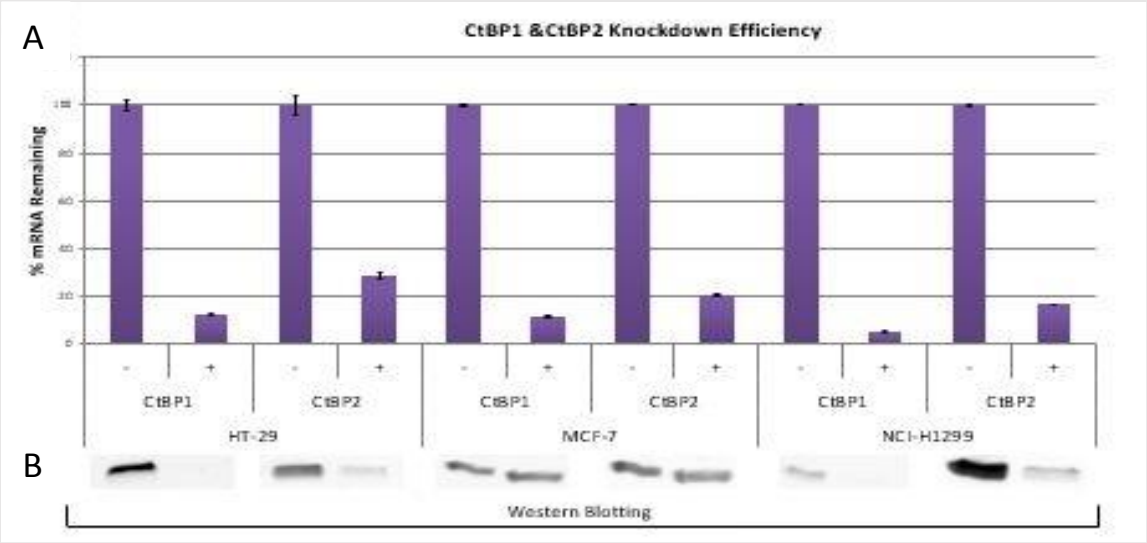
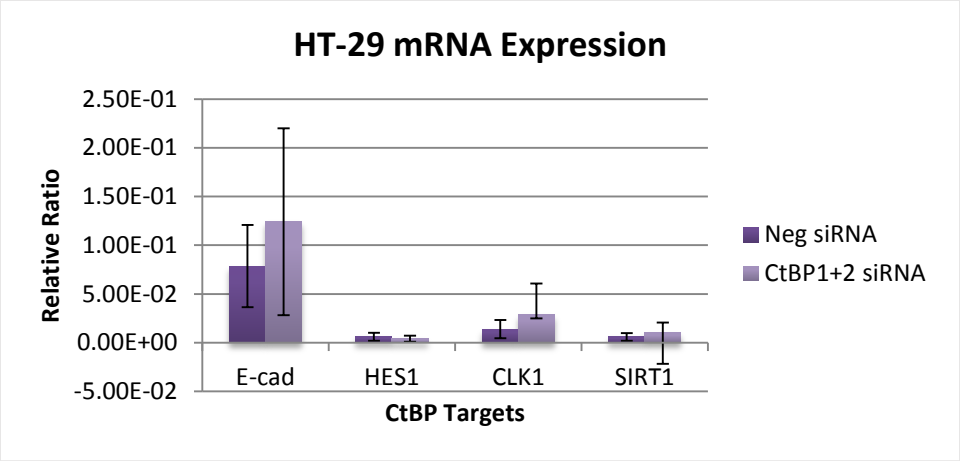
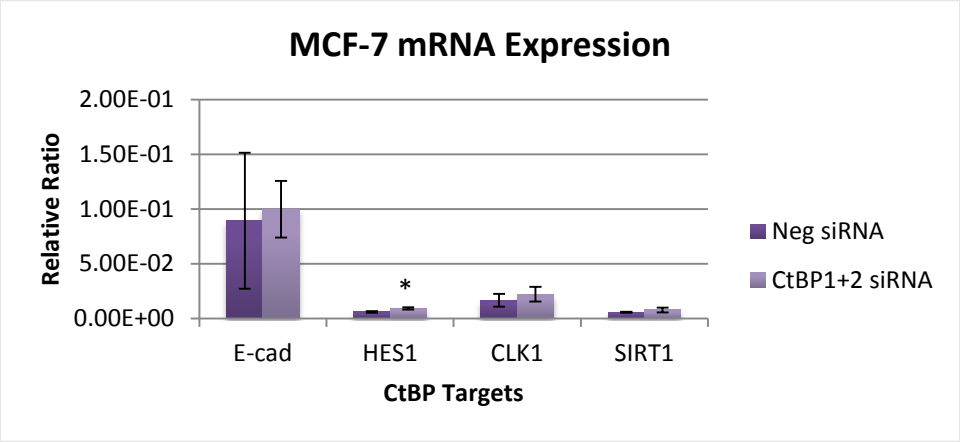


Figure 2: CtBP1 and CtBP2 gene and protein knockdown efficiency. A. Graph showing the % mRNA of CtBP1 and CtBP2 remaining after transfecting each cell type with negative, CtBP1 or CtBP2 siRNA and incubating for 72 hours in HT-29, MCF-7 and NCI-H1299 cells. **B.** Western blots showing the protein expression of their corresponding samples above using CtBP1 and CtBP2 primary antibodies.

Once successful knockdown was verified, the mRNA expression of the same set of known CtBP target genes was measured in HT-29, NCI-H1299, and MCF-7 cells (E-cadherin, SIRT1, CLK1, and HES1). If the genes that encode for C-terminal binding proteins are silenced by siRNA transfection, then it would be expected that the gene expression of CtBP targets would increase due to the depletion of CtBP from the cell and thus a lack of transcriptional repression caused by CtBP if these proteins were functioning as such. As shown in Figure 3, in the HT-29 colorectal cancer cells and the MCF-7 breast cancer cells there is no apparent difference in the gene expression between the samples transfected with negative control siRNA and the samples transfected with CtBP1+2 siRNAs among the target genes observed. The NCI-H1299 lung cancer cell line however did show an increase in target gene expression, especially in CLK1 and SIRT1. The lack of significant differences in the MCF-7 cells is likely to be attributed to unsuccessful knockdown of CtBP1 and CtBP2 protein expression (shown in Figure 2B) while the lack of differences in the HT-29 cells suggests that CtBP was not repressing these genes in the basal state. These results further support the idea that CtBP does not function as a transcriptional repressor in HT-29 colorectal cancer cells, however it may act as such in NCI-H1299 lung cancer cells. Unfortunately, the data from the MCF-7 breast cancer cell samples do not yield any conclusive results since there was unsuccessful knockdown of the CtBP1 and CtBP2 genes.





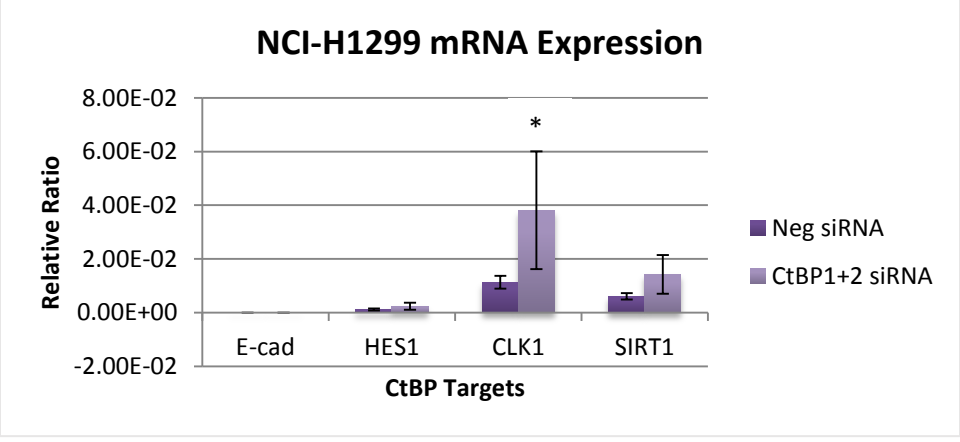


Figure 3: Effect of CtBP1 and CtBP2 knockdown on the gene expression of known CtBP targets. Graphs showing the relative ratios of mRNA expression of known CtBP targets (E-cadherin, HES1, CLK1, and SIRT1) normalized to B2M in HT-29, MCF-7 and NCI-H1299 cell lines after 72 hour simultaneous knockdown of CtBP1 and CtBP2 as determined by qRT-PCR (N=1,* $P<0.05$).

Since the previous experiments both suggested that CtBP does not function as a transcriptional regulator in colorectal cancer cells, the subcellular localization of CtBP within these cells was determined in order to further test our hypothesis. If CtBP were to function as a transcriptional repressor, it would be expected that these proteins would localize to the nucleus where they could bind DNA and exert their effect. To determine cellular localization of CtBP, a cell fractionation procedure was performed in which the cytoplasmic portion of the cell was isolated from the nuclear fraction. Surprisingly, in all three colorectal cancer cell lines tested, CtBP1 and CtBP2 localized to the cytoplasm, again suggesting that these proteins are not functioning as transcriptional regulators in these cells. In contrast, the localization of CtBP1 and CtBP2 in the lung cancer (NCI-H1299) and breast cancer (MCF-7) cell lines was primarily to the nucleus (shown in Figure 4). These results support the hypothesis that CtBP functions as a transcriptional regulator in lung and breast cancer cells, however is likely inactive in colorectal cancer cells. For the HT-29 and SW480 cell lines, Western blotting for Histone H1, a nuclear control, shows no contamination in the cytoplasmic fraction, thus indicating that the cellular fractionation was successful.

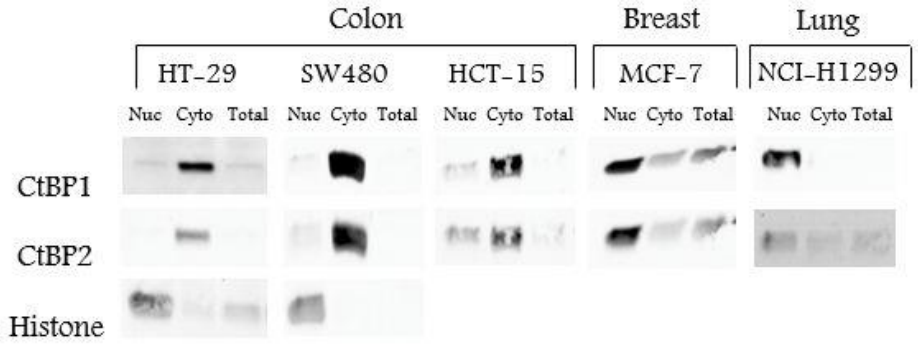


Figure 4: Subcellular localization of CtBP1 and CtBP2 within colorectal, lung and breast cancer cell lines. Western blots showing the localization of CtBP1 and CtBP2 within the following cell lines: HT-29, SW480, HCT-15, MCF-7, and NCI-H1299. A Histone H1 antibody was used as a nuclear control on the HT-29 and SW480 Western blots. (Nuc=nuclear; Cyto=cytoplasm)

After determining that CtBP localized to the cytoplasm in the colorectal cancer cell lines and was likely inactive, we next investigated whether overexpression of the *CtBP1* and *CtBP2* could drive these proteins into the nucleus and if so, if it would induce activity in the colorectal cancer cell lines. CtBP1 and 2 were overexpressed by transfecting the cells with CtBP1 and CtBP2 overexpression plasmids and the effect on gene expression was determined. As shown in Figure 5B, when CtBP1 or CtBP2 was overexpressed in HCT-15 colorectal cancer cells for 72 hours and the cells were fractionated, we observed that more CtBP localized to the nucleus than it did previously (compare Figure 4 with Figure 5B). However, the mRNA expression of the CtBP target genes studied still showed no significant difference (shown in Figure 5A). These results thus indicate that even when overexpressed in colorectal cancer cells, CtBP still does not appear to exhibit transcriptional repressor activity as shown by the lack of a significant decrease in the gene expression of the CtBP target genes studied.

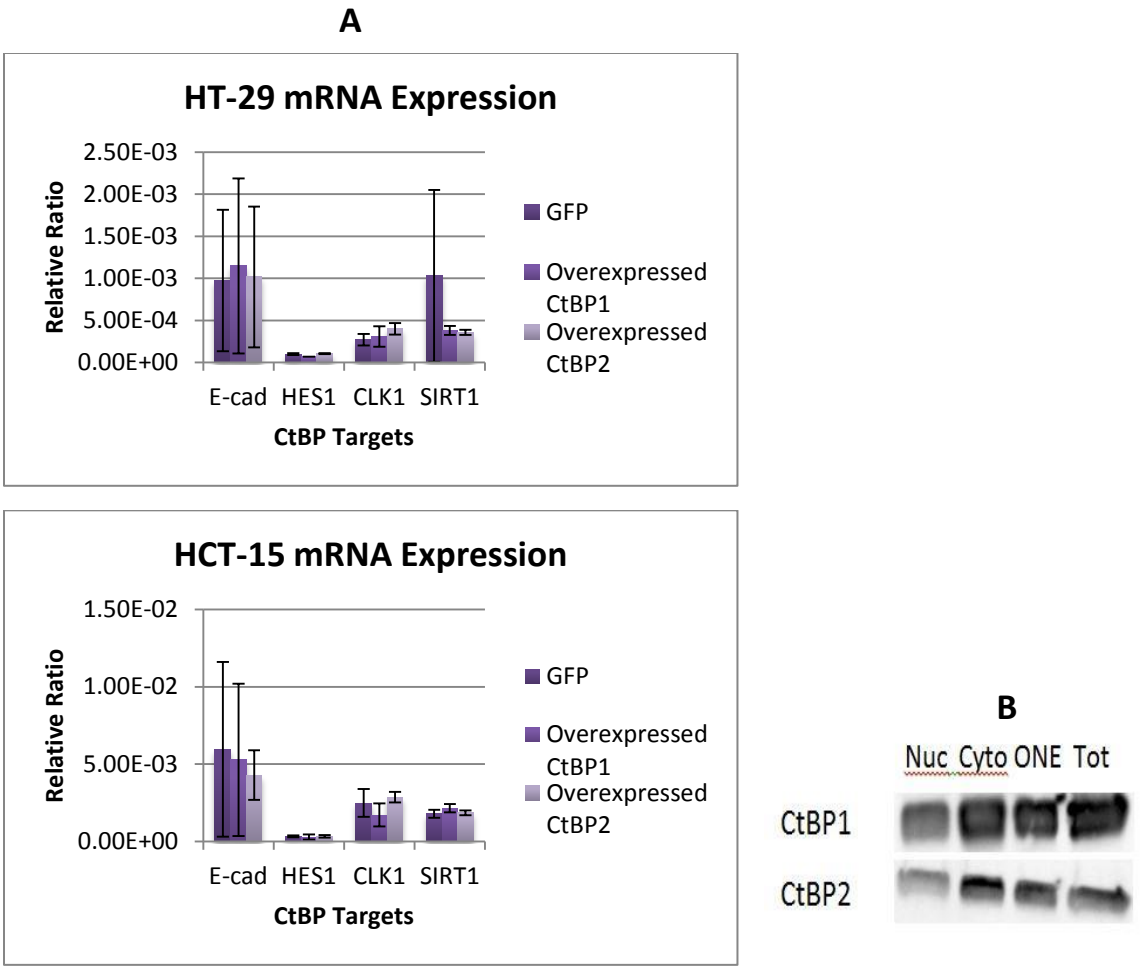
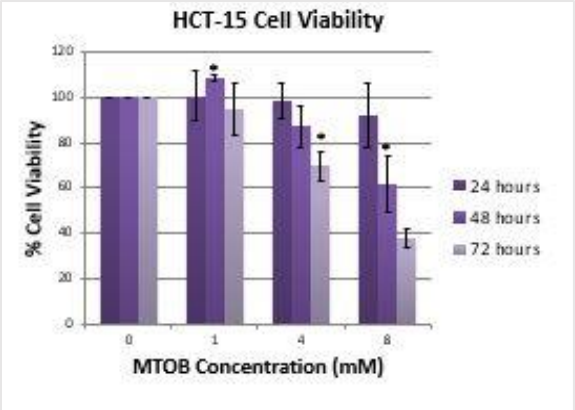
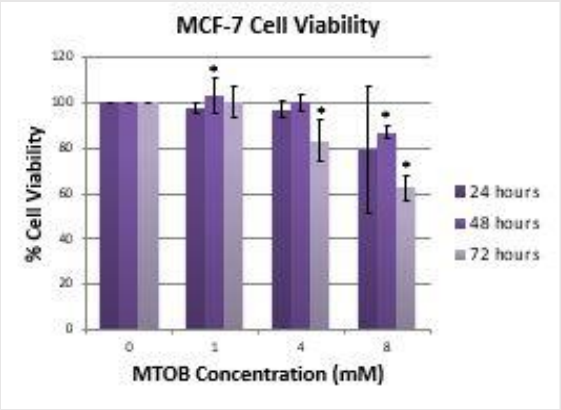


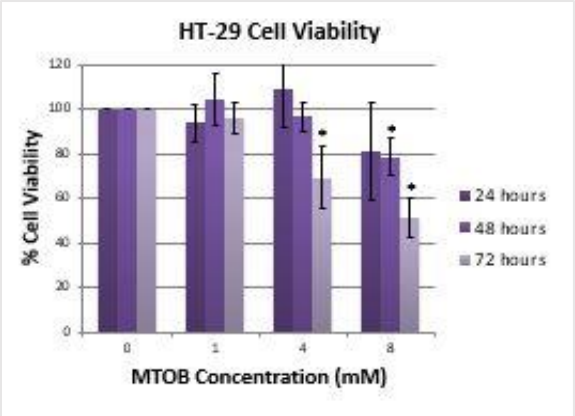
Figure 5: Effect of the overexpression of CtBP1 and CtBP2. A. Graphs showing the relative ratios of mRNA expression of known CtBP target genes (E-cadherin, HES1, CLK1, and SIRT1) in HT-29 and HCT-15 cells after transfection with CtBP1 and CtBP2 overexpression constructs and a control GFP plasmid for 72 hours. Expression was determined using qRT-PCR and were normalized to B2M and 18s (N=1, *P<0.05). **B.** Western blots from a cell fractionation procedure performed in HCT-15 cells after transfecting with CtBP1 and CtBP2 overexpression constructs for 72 hours and probing for CtB1 and CtBP2 expression. (Nuc=nuclear; Cyto=cytoplasm; ONE=outer nuclear envelope; Tot=total)

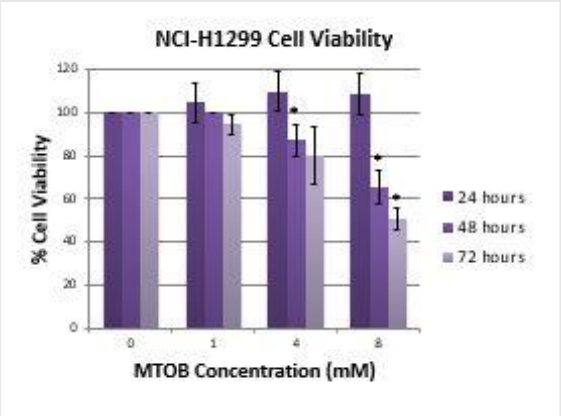
Lastly, since CtBP has been shown to be nonfunctional in the colorectal cancer cell lines, we hypothesized that these cells may be resistant to pharmacological targeting by purported CtBP inhibitors such as NSC95397 and 4-methylthio-2-oxobutyric acid (MTOB). To test this hypothesis, HT-29 and HCT-15 (colorectal), NCI-H1299 (lung) and MCF-7 (breast) cancer cell lines were treated with varying concentrations of each inhibitor (1, 4, or 8 mM for MTOB and 1, 10, or 50 µM for NSC95397) and incubated for 24, 48 or 72 hours. The amount of remaining live cells after treating with each inhibitor was measured via MTT assay and compared to control (untreated) samples. As shown in Figure 6, amongst all 4 cell types treated there was significant cell death, however there was no apparent difference in the amount of viable cells remaining after treating with either inhibitor between the varying cell types. Therefore, in applying what we have discovered about CtBP in that it was found to be nonfunctional in colorectal cancer cells but functional in lung and breast cancer cells, it can be assumed that the results from treating these cells with these two inhibitors is likely attributed to some other mechanism, causing significant cell death.

A

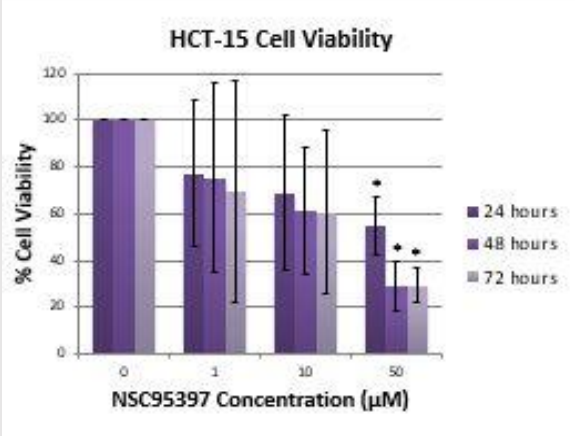

HCT-15 Cell Viability
% Cell Viability
MTOB Concentration (mM)
24 hours
48 hours
72 hours

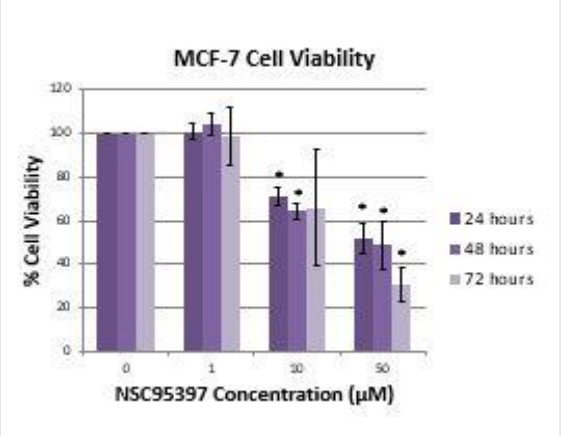

MCF-7 Cell Viability
% Cell Viability
MTOB Concentration (mM)
24 hours
48 hours
72 hours

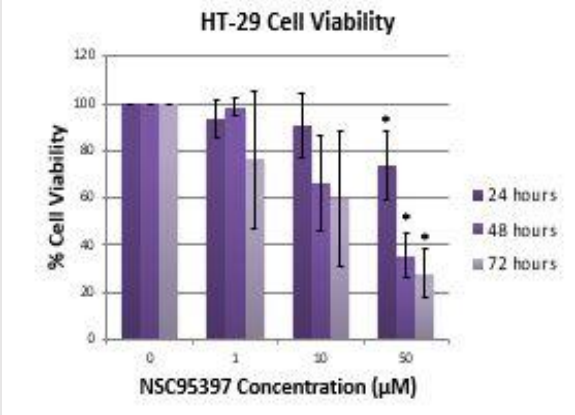

HT-29 Cell Viability
% Cell Viability
MTOB Concentration (mM)
24 hours
48 hours
72 hours


NCI-H1299 Cell Viability
% Cell Viability
MTOB Concentration (mM)
24 hours
48 hours
72 hours

B


HCT-15 Cell Viability
% Cell Viability
NSC95397 Concentration (µM)
24 hours
48 hours
72 hours


MCF-7 Cell Viability
% Cell Viability
NSC95397 Concentration (µM)
24 hours
48 hours
72 hours


HT-29 Cell Viability
% Cell Viability
NSC95397 Concentration (µM)
24 hours
48 hours
72 hours

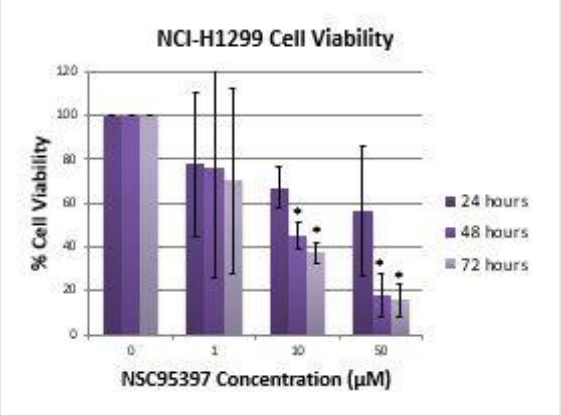

NCI-H1299 Cell Viability
% Cell Viability
NSC95397 Concentration (µM)
24 hours
48 hours
72 hours

Figure 6: Effect of treatment with CtBP inhibitors on cell viability. A. Graphs showing % cell viability measured by MTT assay after being treated with 0, 1, 4 and 8 mM MTOB and incubated for 24, 48, and 72 hours in HT-29 and HCT-15 colorectal cancer cell lines, NCI-H1299 lung and MCF-7 breast cancer cell lines as compared to the respective untreated control for each time point (N=3, *P<0.05). **B.** Graphs showing % cell viability measured by MTT assay after being treated with 0, 1, 10, and 50 μM NSC95397 and incubated for 24, 48, and 72 hours in the same cell lines treated in **A.** as compared to the respective untreated control for each time point (N=3, *P<0.05).

Discussion

Previous studies have shown that overexpression of CtBP can suppress epithelial and proapoptotic gene expression, thus providing evidence that CtBP has the ability to induce a wide variety of tumorigenic phenotypes such as cell survival, proliferation, migration/invasion, and EMT.[21] In 2010, Gujaral *et a*l showed that CtBP was overexpressed in colorectal adenocarcinomas, suggesting a possibility that CtBP could play a role in the processes that contribute to tumor initiation, transformation and development in colorectal cancer.[28] On the contrary, our results suggest that CtBP likely does not play a role in these processes, at least not by way of transcriptionally repressing genes like those observed in this study which have been associated with tumor suppression and metabolic regulation.

The transcriptional co-repressor activity of CtBP is known to be dependent on the levels of NADH present within the cell. Conditions like hypoxia and hyperglycemia, conditions commonly associated with diabetes and other obesity-related illnesses, are known to elevate intracellular NADH levels. Therefore, when subjecting a panel of cancer cell lines to conditions such as these, it would be expected that cellular NADH levels would rise and subsequently the transcriptional co-repressor activity of CtBP would be promoted. This response to a rise in cellular NADH specifically caused by hypoxia has been observed in NCI-H1299 lung cancer cells, as well as in MCF-7, a breast cancer cell line, both obesity-related types of cancer.[17, 29] Therefore we chose to subject a panel of colorectal cancer cell lines, another obesity-related type of cancer, to similar conditions and looked for changes in gene expression of some known and one novel CtBP target. While we did not directly measure NADH levels, the results of our experiments showed that in the two colorectal cancer cell lines studied (HT-29 and HCT-15), CtBP target genes with the exception of E-cadherin in HT-29 cells, exhibited greater mRNA expression while under the imposition of conditions in which NADH levels would be expected to increase. Therefore, if CtBP were involved in the transcriptional repression of these genes, the expression of these genes should have been suppressed. For the sake of ensuring the reproducibility of the results found in previous studies which studied mRNA expression in breast and lung cancer cells under hypoxic and hyperglycemic conditions, we repeated this experiment in NCI-H1299 (lung) and MCF-7 (breast) cancer cell lines. These experiments produced similar results to those of the studies done prior to our research in that when subjected to these metabolic perturbations, CtBP target gene expression was suppressed presumably due to the transcriptional repressor function of CtBP. These results contrast the results found in our colorectal cancer cells, leading us to believe that CtBP may not play a direct role in transcriptionally repressing genes known to be involved in tumor suppression specifically in colorectal cancer.

When silencing the genes that encode for CtBP1 and CtBP2 by transfecting colorectal cancer cells with siRNAs, essentially no changes were observed in CtBP target gene expression. If CtBP were to function as a transcription co-repressor in colorectal cancer like it has been shown to function in breast and lung cancer, then when these two genes are silenced, the expression of known CtBP targets would show no sign of repression and thus a rise in mRNA expression. Although we were able to show this response to CtBP knockdown in lung cancer cell lines, like other studies had previously shown, we did not observe it in the colorectal cancer cell line studied. Consistent with the results from the first set of experiments, CtBP again does not appear to function as a transcriptional regulator in colorectal cancer cells. Interestingly, from these gene silencing studies we also discovered that CLK1, CDC-like kinase 1, is most likely a target gene of CtBP in addition to the others studied which were already known targets of CtBP. In order to confirm this as a novel target of CtBP, we plan to conduct further studies using ChIP analysis to show direct binding of CtBP to the CLK1 promoter region.

The culmination of the results from our first two studies led us to investigate where within the cell CtBP is localized. If CtBP is believed to function as a transcriptional repressor, then it would be expected to be found primarily in the nucleus where it would be binding the promoters of its target genes. Subcellular fractionation revealed that in the colorectal cancer cells studied, CtBP1 and CtBP2 are both primarily found in the cytoplasm. In the lung and breast cancer cell lines studied on the other hand, both CtBP1 and CtBP2 were located in the nucleus. The results of this localization study agree with the data from the previous experiments, increasing the amount of evidence for CtBP not exhibiting transcriptional regulator activity in colorectal cancer cells.

Although CtBP was found to localize to the cytoplasm in colorectal cancer cells by the cell localization experiments, previous studies investigating the cellular localization of CtBP used immunohistochemical staining in primary tumors.[11, 17, 22] One potential explanation for this discrepancy is that tissue culture presents a caveat in that it is essentially an artificial system and is removed from the environment in which a tumor resides. Therefore it is possible that although we did not observe functional CtBP in the colorectal cancer cell lines tested, CtBP could still be playing a role in the primary tumors. Thus, future studies will include examining the cellular localization of CtBP in primary colorectal tumors and identifying any differences between the results obtained using cultured cells and those of the tumors.

Considering recent studies had proposed two chemicals, MTOB and NSC95397, to be involved in inhibiting CtBP, we chose to investigate the effect of treatment with these chemicals on cell viability in colorectal (HT-29 and HCT-15), lung (NCI-H1299) and breast (MCF-7) cancer cells. Based on the evidence gathered from our own research, it would be expected that if CtBP were not functioning as a transcriptional repressor in colorectal cancer like we have shown, then inhibition of CtBP by way of pharmacological treatment would most likely not result in any significant cell death. Also, considering that our results consistently confirms that CtBP functions as a transcriptional repressor in lung and breast cancer, the inhibition of CtBP would be expected to cause significant cancer cell death, especially when compared to colorectal cancer cells treated with the same inhibitors. Surprisingly though, the treatment of all 4 cancer cell types with MTOB and NSC95397 resulted in significant cell death, even in the colorectal cancer cells. Therefore, these results indicate that these pharmacological agents are not specific in inhibiting CtBP and that they are likely involved in other mechanisms responsible for causing the significant cell death observed. To confirm this hypothesis, future studies would include alternatively knocking down CtBP in the cells first and then treating with MTOB or NSC95397. The effect of treatment with these chemicals on the cell viability would then once again be measured via MTT assay. If significant cell death was still observed even after knocking out CtBP in each cell type, then it could be concluded that the proposed CtBP inhibitory activity of these pharmacological agents is not responsible for causing these cells to die, confirming the idea that these agents are not specific in targeting CtBP. In fact, if CtBP inhibition was the cause of the cell death from MTBO and NSC95397 treatment, we would expect that siRNA treatment would mimic the pharmacological inhibition and cause cell death on its own. Given our results, we would predict that the colorectal cancer cells would be refractory to this effect.

Considering that inherent overexpression of CtBP has been observed in a number of obesity-related cancers, including colorectal cancer cells, we imagined if we overexpressed CtBP further, then surely we would then see transcriptional repressive effect of the gene targets investigated. However, when we did this we still witnessed no significant changes in gene expression of the target genes studied in the colorectal cancer cells, even while increasing CtBP's presence in the nucleus. This is very interesting because it suggests that in colorectal cancer cells, even if CtBP localizes to the nucleus due to

overexpression, there is still something hindering CtBP's function as a transcriptional repressor. Whatever is causing this in colorectal cancer cells is still unknown, but further studies will include performing ChIP assays to determine if CtBP actively binds DNA in these colorectal cancer cells when overexpressed.

In summary, the combined results of the experiments performed in this study indicate that CtBP plays no direct role in transcriptionally repressing genes known to be involved with tumor suppression in colorectal cancer cells. While these results did rely on established cell lines far separated from the primary tumors from which they were isolated, other tumor cells such as the lung and breast cancer cells retained their CtBP activity. Obviously future studies will need to investigate CtBP activity and localization in primary colorectal tumor samples, however these results do call into question CtBP's role in colorectal cancer cell growth. Therefore, we suggest that focus should be expanded to other mechanisms or pathways involved in colorectal cancer for further study and investigation if we want a greater chance of developing or discovering therapeutics methods that are successful in treating colon cancer.

Materials and Methods

Cell lines and reagents

Colorectal (HT-29, HCT-15, and SW480) and lung (NCI-H1299) cancer cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in Roswell Park Memorial Institute (RPMI) media supplemented with 10% (v/v) fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin. MCF-7 (breast cancer) cells were obtained from ATCC and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, L-glutamine, penicillin, streptomycin and 10 µg/mL insulin. CtBP1 and CtBP2 Silencer® Select siRNAs were obtained from Life Technologies (Carlsbad, CA). They were both simultaneously introduced into cells using Lipofectamine 2000 from Life Technologies according to the manufacturer's instructions. Primary antibodies were obtained from the following sources: mouse anti-CtBP1 (BD Biosciences; cat. #612042); mouse anti-CtBP2 (BD Biosciences; cat. #612044); and mouse-anti Histone H1 (Santa Cruz; cat. #sc-8030). MTOB, NSC95397, and MTT, used for the cell viability experiments, were obtained from Sigma Aldrich (St. Louis, MO). Overexpression constructs for CtBP1 and CtBP2 were kindly provided by Kevin Gardner (National Institutes of Health, Bethesda, MD).

RNA extraction and quantitative real-time PCR

Total cellular RNA was isolated using TRIzol® Reagent acquired from Life Technologies according to the manufacturer's instructions. 1 µg of RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit obtained from Bio-Rad (Hercules, CA). The resulting cDNA was subjected to qRT-PCR using human gene-specific primers for CLK1, E-cadherin, HES1, CtBP1, CtBP2, SIRT1, B2M, and 18S obtained from Integrated DNA Technologies (Coralville, IA). PCR was performed using the Roche LightCycler96 instrument and software (Basel, Switzerland) and the reactions were performed using the LightCycler FastStart DNA Master SYBR Green I kit. For the hypoxia and CtBP knockdown experiments, the mRNA levels of each gene were normalized relative to the mean mRNA levels of B2M. For the CtBP overexpression experiment, the mRNA levels of each gene were normalized to the mean mRNA levels of B2M and 18S. The qRT-PCR data presented in all experiments are mean of technical duplicates.

Protein isolation and Western blotting

Cells were rinsed and harvested in Phosphate Buffered Saline (PBS). The harvested cells were centrifuged at 1,500 x g for 5 min at 4°C. The supernatant was discarded and the remaining cell pellet was lysed using WCEB (150 mM NaCl, 10 mM Tris pH 7.6, 0.1% SDS, 5 mM EDTA) including protease inhibitors. Lysates were sonicated for 5 s at 40% magnitude and then protein concentrations were determined using the Pierce BCA protein assay kit (Rockford, IL) and 30 µg of sonicated lysates were separated by SDS/PAGE (any kD precast acrylamide Ready Gels by Bio-Rad) using a Bio-Rad MiniProtean Tetra System and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked in TBST (0.2 M NaCl, 10 mM Tris, pH 7.4, and 0.2% Tween-20) containing 5% nonfat dry milk for 1 h and then incubated overnight at 4°C with an antibody against CtBP1, CtBP2 or Histone H1 in TBST containing 5% nonfat dry milk. The membranes were then incubated with goat anti-mouse immunoglobin in TBST containing 5% nonfat dry milk for 1 h. Bound antibodies were detected using Pierce ECL Western blotting substrate.

Cell Culture

For the normoxic/hypoxic experiments, cells were first plated at $0.4x10^6$ cells/well in 4g/L glucose media and incubated for 24 hours in a ThermoScientific Forma Steri-Cycle CO_2 incubator at 37°C and 5% CO_2. Media was removed and cells were rinsed with PBS. DMEM with 0g/L, 1g/L or 4g/L glucose was added to MCF-7 cells and RPMI with 0g/L, 1g/L, or 4g/L glucose was added to NCI-H1299, HT-29, and HCT-15 cells. Cells were then incubated for 24 hours in either normoxic (37°C, 5% CO_2) or hypoxic conditions (37°C, 1% O_2, 5% CO_2). Hypoxia treatment was performed using an Invivo$_2$ 400 Hypoxia Workstation and Ruskinn Hypoxia Gas Mixer (Bridgend, UK). After incubation, RNA and protein were harvested and used in qRT-PCR and Western blotting as described above.

Subcellular Fractionation

HT-29, HCT-15, SW480, NCI-H1299, and MCF-7 cells were rinsed and harvested in PBS. A fraction was removed from the harvested cells and placed in a tube labeled "Total" with TLB (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Np40, 0.5% sodium deoxycholate) with protease inhibitors. The remaining cells were pelleted at 1,500 x g for 5 min and the supernatant (PBS) was discarded. The pellet was resuspended in HLB (10 mM NaCl, 10 mM Tris, 1.5 mM $MgCl_2$) with protease inhibitors and set on ice for 5 min. The cells were pelleted again at 1,500 x g for 5 min and the supernatant (cytoplasmic fraction) was removed and put into a tube labeled "cyto." The remaining pellet was resuspended in HLB+ (10 mM NaCl, 10 mM Tris, 1.5 mM $MgCl_2$, 1% Np40, 0.5% sodium deoxycholate) with protease inhibitors and then pelleted again at 1,500 x g for 5 min. The supernatant was removed and put into a tube labeled "ONE" (outer nuclear envelope fraction). The remaining cell pellet was resuspended in TLB and labeled "nuc" (nuclear fraction). All of the fractionated samples were then sonicated at 30% magnitude for 5 s and Western blotting was performed in the same way as mentioned above.

siRNA Mediated Knockdown

For the CtBP knockdown experiments, MCF-7, HT-29, and NCI-H1299 cells were first plated at 0.2 x 10^6 cells/well in 4g/L glucose media (DMEM for MCF-7, RPMI for HT-29, NCI-H1299) and incubated at 37°C and 5% CO_2 for 24 hours. Cells were simultaneously transfected with CtBP1 and CtBP2 specific siRNAs using Lipofectamine2000 according to the manufacturer's instructions. After incubating for another 24 hours, the media was removed and fresh 4g/L glucose media was added and the cells were incubated for another 48 hours (72 hours total). RNA and protein were then harvested and used for qRT-PCR and Western blotting as described above.

CtBP1 and CtBP2 Overexpression

For the CtBP overexpression experiments, HCT-15 and HT-29 cells were plated at 0.2 x 10^6 cells/well and incubated at 37°C and 5% CO_2 for 24 hours. Plated cells were then transiently transfected with CtBP1, CtBP2 or GFP control plasmids using a 1:3 DNA:Lipofectamine 2000 ratio (2 µg DNA: 6 µL Lipofectamine 2000) according to manufacturer's instructions. Transfected cells were then incubated for 72 hours and RNA and protein were harvested for qRT-PCR and Western blotting as described above.

Chemical treatment and MTT viability assay

MCF-7, NCI-H1299, HT-29 and HCT-15 cells were plated at 0.2 x 10^6 cells/well and were incubated at 37°C and 5% CO_2 for 24 hours. 40 mM MTOB stock was made in both RPMI and DMEM media and then used to treat cells at 1 mM, 4 mM, and 8 mM final MTOB concentrations. 100 mM NSC95397 stock was made up in DMSO and then used to treat cells at 1 µM, 10 µM, and 50 µM final NSC95397 concentrations. After treatment, cells were incubated for 24, 48, or 72 hours. At each time point, cells were treated with 100 µL of 5 mg/mL MTT in PBS and incubated for 1 h. Media was then removed and 1 mL of solubilization solution (0.04 M HCl in isopropanol) was added to each well and allowed to rock at room temperature for 15 min before determination of A_{560}. % Cell viability was determined by comparing the absorbance data of treated cells to the untreated control cells at each time point.

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