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High expression of DNMT3A and DNMT3B regulatory factors of TGFB in non-neoplastic liver tissues of HCC

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ABSTRACT

Increased expression of TGFB regulatory factors DNMT3A and DNMT3B in non-neoplastic liver tissues of HCC patients is the goal of this study. Furthermore, we demonstrate that TGF- is capable of elevating the percentage of CD133+ cells present in liver cancer cell lines in a manner that is both consistent and long-lasting over several cell divisions. This process is linked to stable alterations in DNA methylation that occur over the whole of the genome and continue even after cell division. In addition, the silencing of de novo DNA methyl-transferases with siRNA is able to inhibit the phenotypic changes that are induced by TGF-. According to the findings of our research, there is a self-sustaining interaction between the DNA methylation machinery and the TGF- signaling pathway, which may be significant in the development of cellular phenotypes. CD133 positive and negative fractions expand within liver cancer cell lines in proportions that remain stable throughout time. In contrast to their CD133- counterparts, MACS-sorted CD133+ Huh7cells demonstrated the ability to shape themselves into spheres when grown under non-attachment circumstances. This study also found that the TGF- is responsible for the de novo induction of CD133, which is linked to an increase in the expression of DNMT3 genes and there is a correlation between the TGF—induced transition in the cell subpopulation and a distinct DNA methylome. TGF- has the potential to generate genome-wide alterations in DNA methylation, which ultimately leads to a persistent shift in the fraction of liver cancer cell subpopulations.

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Introduction

In recent years, liver cancer has become the sixth most prevalent malignancy and the fourth main cause of fatalities globally due to cancer (1, 2). It is also the second most deadly tumor, with just 18% of patients surviving the disease for five years. Hepatocellular carcinoma, often known as HCC, is responsible for between 75 and 85 percent of all primary liver malignancies. Its onset and progression may be influenced by a number of risk factors, including alcoholism, chronic hepatitis B or C virus infections, and other chronic infections. Individuals who have HCC have persistent inflammation in the liver as well as fibrosis, both of which create a chain reaction of genetic and epigenetic changes. The incidence of HCC is quickly rising despite the huge efforts that have been made over the last several decades, and the prognosis for patients who have advanced stages of HCC is quite dismal (1). A deeper comprehension of the specific molecular process that is involved in HCC is necessary for making progress in the therapy of the disease. Although though HCC is a clinically and physiologically heterogeneous form of cancer, the majority of HCCs have similar characteristics in terms of the genetic and epigenetic modifications that they exhibit. The discovery of these widespread molecular changes in HCC may provide a logical approach to the formulation of an efficient molecularly targeted medication for the treatment of HCC (2). Hepatocellular carcinoma (HCC) is the major form of primary liver cancer. Inflammation is a necessary component of the wound-healing response to the risk factors that have been identified. Yet, persistent inflammation makes it more likely for hepatocytes to accumulate mutations and epigenetic abnormalities, which in turn promotes the transformation of hepatocytes into cancerous cells (3,4). Chemokines, cytokines, and growth factors are all released by the stromal cells that make up the liver's microenvironment, and they are the ones responsible for mediating this process (4). During the process of hepatocarcinogenesis, it has been shown that the transforming growth factor beta (TGF-), which is one of those secreted factors, plays a crucial function that is depending on the cell type and varies from case to case (5). Overexpression of TGF- is linked to a poor prognosis in head and neck cancer that has already developed (6-8). Unfortunately, there has been little work done to characterize the tumor cells that TGF- aims to destroy in HCC (9-11).

Out of all of them, the surface marker CD133/Prominin1 [PROM1] has been one of the ones that has been reported the most often. One and only one of CD133's functions is to act as a transmembrane protein. only partly understood (12,13), however this might be a sign of a unique cell subpopulation with specific properties. The functional characterization of these cells will lead to an advance in our knowledge of the processes that are involved in the promotion and maintenance of the development of liver cancer.

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Recent research has pointed to a possible connection between TGF- signaling and TICs in the liver. To begin, signaling pathways that have been shown to be active in liver cancer, such as TGF-, are also active in liver TICs that have been isolated (14). Second, TGF- induces epithelial-mesenchymal transition, which results in the production of cells that are capable of self-renewal. This process is also linked to an increased risk of tumor spreading.

Stem cells, tumor initiating cells (TICs), and metastatic cells commonly share characteristics such as invasiveness and self-renewal (15,16). In conclusion, a recent research shown that TGF- is capable of inducing the expression of CD133 in liver cancer cell lines, in addition to an improved capacity for tumor initiation in mice (17). When all of these data are taken into consideration together, it seems that TGF- may play a unique role in the process of creating a TIC program in HCC. DNA methylation has the potential to bring about a long-term and irreversible change in the phenotype of a cell via the process of cellular division (18). DNA methylation is a good candidate for the mechanism that will be necessary to convert the presence of TGF- in the cellular environment into phenotypic changes that are stable over time. DNA methylation is an excellent potential mechanism because of the relative durability of DNA methylation marks. This is the reason why DNA methylation is a mechanism. However, there is a paucity of evidence that currently points to a connection between being exposed to components of the microenvironment of the tumor and the generation of persistent alterations in the DNA methylation of the cells that are the targets of the treatment. This is the case despite the fact that these cells are the ones that need to be treated.

So, the aim of the study to express the differences in the DNA methylome between liver cancer cells that express CD133 and those that do not express CD133 has been investigated.

Materials and Methods

The cultivation of cells as well as therapies

Mycoplasma contamination was checked for on a consistent basis while Huh7 and HepG2 cells were grown in DMEM media at 37 degrees Celsius and 5% carbon dioxide. For cytokine treatments, cells were plated and allowed to adhere before adding media with 10ng/ml final of IL-6 or TGF-β1. In the tests involving inhibition, the cells were either treated with SB-431542 (Sigma-Aldrich) at a concentration of 2 M alone or in conjunction with TGF-1.

In order to conduct an experiment on the development of spheres, hepatosphere media was created. After five or six days, the number of spheres was determined. RNAiMAX lipofectamine was used to transfect cells with siRNA non-targeting and pool siRNAs targeting DNMT3A and DNMT3B at a concentration of 20 nM. At a period of 12 hours after transfection, the cells were washed, and the media was changed.

Flow Cytometry Activated by Fluorescence (FACS) (FACS)

Conjugation with FITC, Cy3, or Alexa750 was performed on secondary antibodies in a randomized order. The incorporation of bromodeuxyridine (BrdU) and analysis of DNA content were done concurrently in order to research the advancement of the cell cycle. The FACS equipment was used to collect the fluorescent events, and then those events were evaluated.

Sorting of cells by magnetic activation (MACS)

Using magnetic-activated cell sorting, CD133+ cells in Huh7 and HepG2 cells were either eliminated or enhanced, with minor modifications made to the procedures outlined in the manufacturer's instructions. After incubating the cells with an FcR blocking reagent for 30 minutes at 4 degrees Celsius, the cells were then treated with Micro Beads conjugated to monoclonal anti-human CD133 antibodies for 15 minutes. After the washing step, the cell suspension was put to an LS column that had been prerinsed and then positioned inside the magnetic field of a MACS separator. Collecting the flow-through from the LS column that included unlabelled cells was done in order to deplete CD133+ cells. In order to enrich CD133+, the column had to be taken from the separator and put on a collecting tube with a capacity of 15 ml. To collect the labeled cells, a strong push was applied to the plunger while it was in the column. The eluted fraction was enhanced for a second time over a fresh LS column so that the purity of the CD133+ cells could be improved. Aliquots were maintained from each experiment in order to do a FACS analysis to determine how effective the enrichment was.

Pyrosequencing and modification of the bisulfite group

We carried out bisulfite pyrosequencing in the same manner as was previously reported in order to measure the proportion of methylated cytosine in each unique CpG site. The EZ DNA methylation Kit was used to accomplish the conversion of 600 ng of DNA from samples that were going to be processed for Infinium bead arrays. By performing a PCR test with both modified and unmodified primers for the GAPDH gene, the quality of the alteration was evaluated.

Assays of methylation using beads in arrays

Several samples' methylation profiles were analyzed with the use of HM450 Infinium methylation bead arrays. In a nutshell, the HM450 beadchip is capable of interrogating over 480,000 methylation sites. The analysis of the bead array was carried out strictly according to the prescribed protocols for amplification, labeling, hybridization, and scanning. To guarantee precision, we repeated each methylation analysis at least twice.

Whole genome expression array

Using the protocol provided by the manufacturer of the TRIzol Reagent (Invitrogen), total RNA was extracted from the sample using that reagent. The spectrophotometer and bioanalyzer model ND-8000 were used to evaluate both the amount and quality of the RNA. In order to validate these potential genes using quantitative RT-PCR, we chose 12 different genes. Internal control was performed by alternating between the use of four distinct housekeeping genes. Extractions of proteins were carried out, followed by immunoblotting.

Analysis using bioinformatics

As was previously explained, the raw data from the expression bead array was exported from Genome Studio and imported into the BRB-Array Tools software. With the use of the "lumi" R/Bioconductor software, the data were

both normalized and annotated. The class comparison was carried out by conducting a t-test. This was done in order to examine the relationships between the different groups of bead arrays. Only those probes that had a fold-change of two and an FDR that was less than 0.05 were deemed to have differential expression.

Combinations of R and Bioconductor tools were what we relied on for our methylome analysis. In order to import the raw data straight from the IDAT files into a MethyLumiSet object, the "WateRmelon" package was used. The quality of the data was analyzed by utilizing boxplots to determine the distribution of methylation and unmethylated signals, as well as multidimensional scaling plots and unsupervised clustering to determine the inter-sample association. Probes were screened to exclude those that had a low bead count, were of poor quality (with a detection P value more than 0.05), or were recently characterized as being cross-reactive. After that, we adjusted the color bias, then did inter-sample quantile normalization, followed by probe bias correction and intra-array beta-mixture quantile normalization as mentioned before. In order to make the data more suitable for parametric statistical analysis, the beta values of methylation were logarithmically converted into M values. The M values were analyzed using principal component analysis to identify the batch effects, and the results were then adjusted with the surrogate variable analysis tool. We utilized the annotations from the HM450 database, and the human reference genome that we used was hg19. This allowed us to investigate the genomic context of DMPs. Using HM450 bead arrays, we were able to gather information on other genetic characteristics from a recent paper. After converting our dataset to a list of genomic regions that were then methylated, we used the "methyAnalysis" package to locate areas that had experienced differential methylation. These areas are referred to as differentially methylated regions (DMRs).

We carried out a class comparison blocked by cell line status so that we could investigate the differential methylation that exists between CD133 negative and positive cells. An analysis of variance was carried out, and a randomized block design was used for the study's layout. The methylation data are used to construct two different linear models, one for each gene. The class variable and the block variable are both part of the comprehensive model, whereas the block variable is the only component of the simplified model. In order to examine the importance of the difference between the classes, the likelihood ratio test statistics are used. For the sake of this comparison, we decided to use a p-value threshold of less than 0.001 and a 5% difference in methylation. Only those probes whose FDR-corrected p values were lower than 0.05 were deemed to have significant results in the other comparisons.

Results

Liver cancer cells lacking CD133 and expression of DNA methylation genes in distinct ways

CD133 has been shown to be a marker of TICs in a variety of human cancers, including HCC (12). We analyzed two different liver cancer cell lines that were not linked to one another in order to test the hypothesis that this marker differentiates a cell subpopulation that has a unique DNA methylation pattern(12). This was the initial stage in our investigation. Both HepG2 and Huh7 cell lines had detectable levels of CD133 expression, with the former having a mean of 5% and the latter having 25%. The expression of CD133 at the mRNA level was shown to have a positive correlation with the expression of the surface protein. In contrast to the extraordinary levels of expression that we found for other molecules like CD90, CD44, or EpCAM, we found that only a small to a moderate fraction of cells express CD133. It is essential to note that the expression of CD133 by FACS has been examined at several time periods over the course of a number of years, under a variety of settings including cell passages and confluence. There seems to be a dynamic equilibrium between the proportions of both cell lines that express CD133 and those that do not express CD133, based on the very low fluctuation in CD133 expression that was seen across time and environments.

Studying the expression of genes that code for critical actors in the DNA methylation machinery provided us with a foundation upon which to build our investigation into the possibility of a distinct methylation program being expressed in CD133+ liver cancer cells. These includes genes involved in the maintenance of DNA methylation (DNMT1), as well as genes involved in the creation of new DNA methylation (DNMT3A and DNMT3B). It is important to note that DNMT3A was consistently and substantially overexpressed in Huh7 and HepG2 cells that had gradually increased their levels of CD133. In addition, an increased level of DNMT3B expression was seen in HepG2 cells that were enriched in CD133. As was said before, the consistent equilibrium that exists between the two cell fractions hints that there is no significant variation in the rates at which they undergo the cell cycle. Hence, substantial changes in expression are consistent with real differences in function that exist between the two subpopulations, despite the fact that these differences are minor in scale.

These findings taken together lend credence to the hypothesis that CD133 positive and negative fractions expand within liver cancer cell lines in proportions that remain stable throughout time (Table 1). In contrast to their CD133- counterparts, MACS-sorted CD133+ Huh7cells demonstrated the ability to shape themselves into spheres when grown under non-attachment circumstances (Table 2 and Fig 1). However, this was not the case with HepG2 cells, and there was no evidence of sphere formation. This might be because MACS resulted in a less significant enrichment of CD133+ cells than had been anticipated.

Liver cancer cells that lack CD133 and those that have CD133 may be distinguished by their DNA methylomes

The findings presented above lend credence to the concept that CD133+ and CD133- cell subsets exhibit different phenotypic and functional characteristics. CD133+ cells have been shown to have a greater production of de novo DNMTs, which may be reflected in a distinct arrangement of their DNA methylome. In order to investigate this hypothesis, we carried out DNA methylome research over the whole genome using Huh7 and HepG2 cells that had been FACS-sorted to isolate CD133- and CD133+ fractions. In addition to most human bona fide CpG islands, the Illumina Infinium HM450 bead array was used to analyze the DNA that was separated from these fractions (19). This array includes other genomic characteristics of interest in addition to most human CpG islands.

Table 1. CD133 liver cancer cells.

CD133	Huh7	HepG2
positive cells by FACS	26%	6%

Table 2. CD133- and CD133+ liver cancer cells.

Relative expression	Huh7	HepG2	P value
DNMT1			
-/-	1.00	1.10	1.2
-/+	1.20	0.90	0.36
+/+	1.30	0.80	0.26
DNMT3A			
-/-	1.00	1.50	098
-/+	1.20	2.50	0.41
+/+	1.40	3.50	0.11
DNMT3B			
-/-	1.00	1.00	1.2
-/+	1.10	1.70	0.55
+/+	1.30	1.40	1.9

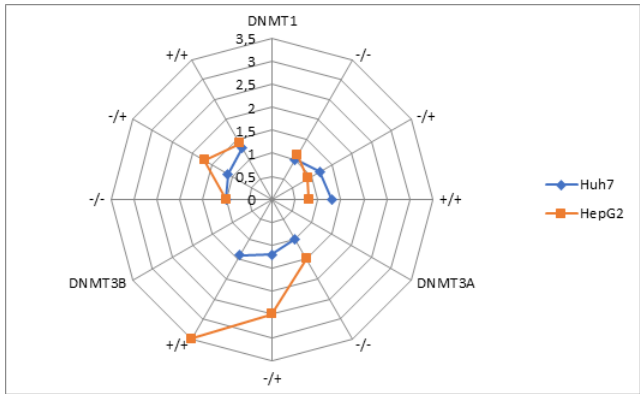

Figure 1. CD133- and CD133+ liver cancer cells.

Table 3. DNA methylome.

	Huh7	HepG2	P value
CD133-	0.30	0.90	0.63
CD133+	0.05	0.80	0.44

After doing initial unsupervised analyses, we discovered that the parental cell line was the most important factor in determining the variance in DNA methylation. As a result, the primary focus of our investigation was a comparison of CD133- to CD133+ fractions while taking into consideration the cell of origin (Table 3). As a result of the class comparison analysis, 823 differentially methylated probes were found to have a significant p-value, while having relatively high FDRs, which is likely owing to changes in sample and cell line characteristics. This was done so that we could mine the data more effectively.

After identifying differentially methylated CpGs and the genes that are connected to these sites, the next step was for us to locate the pathways that are uniquely changed in CD133+ cells. In order to do this, we carried out pathway analysis taking into account the methylome profiles of both cell lines either together or separately.

TGF-, but not IL-6, is responsible for the sustained induction of CD133 expression

While it has been observed that exposure to TGF- increases the proportion of CD133+ cells in the Huh7 cell line (17), the underlying mechanism for this effect is still completely understood. Therefore, one of our goals was to determine whether or not this finding is replicated in two different cell lines and whether or not it is consistent with an epigenetic mechanism. It is essential to take notice of the fact that both Huh7 and HepG2 cells respond to TGF- by phosphorylating the receptor-dependent SMAD3 after expressing equivalent amounts of the TGF- receptor (TGFBRII). This is the case. In addition to the research that we did with TGF-, we also did a set of parallel trials with the pro-inflammatory cytokine interleukin 6 (IL-6), which has also been related to an elevated risk of HCC. These trials were undertaken in conjunction with the studies that we did with TGF-. We were successful in accomplishing this objective by selecting dosages of commonly used cytokines that, during a treatment period of four days (in the case of TGF-), induced morphological changes in both cell lines but did not in any way affect the cell lines' ability to survive. As was to be anticipated, the proportion of CD133+ cells in Huh7 and HepG2 cells increased by a factor of two after being subjected to TGF- over four days. This was seen in both cell lines. It is noteworthy to note that treatment with IL-6 also led to a rise in CD133 positive in both cell lines, although a rather slight increase in both instances. This is something that should be taken into consideration (Table 4 and Figs 2, 3). After that, we looked at how long the impact of the CD133 expression that was triggered by both cytokines continued to be present in the cell. In order to do this, we used the identical methodology that we utilized in the earlier experiment on both of the cell lines. Following the first four days of the cell culture, the medium that had been utilized was switched out for the standard media, and the cells were cultivated for a further four days after that. After collecting the cells, an examination using FACS was performed to look for evidence of CD133 expression being present (table 4 and Fig2). It is

Table 4. TGF-β, but not IL-6, induces CD133 expression in a stable fashion.

CD133 positive cell by FACS	Days 4	Days 8	P value
	Huh7		
Control	18	6	Non Significant
IL6	24	8	0.33
TGFb	42	10	Non Significant
	HepG2		
Control	15	4	Non Significant
IL6	15	4	0.27
TGFb	45	10	Non Significant

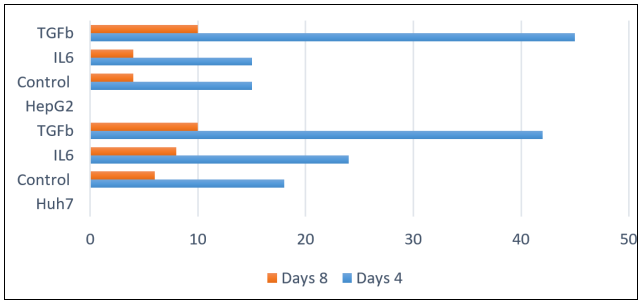

Figure 2. TGF-β, but not IL-6, induces CD133 expression in a stable fashion.

important to note that only cells that had been treated with TGF- demonstrated a constant rise in the percentage of CD133+ cells, and the amount of this increase was equivalent to the increase that was observed on day 4 (Fig 3). Importantly, the only factor that was capable of generating a significant increase in the expression of CD133 at the transcriptional level in both cell lines was exposure to TGF-. This finding is significant because it demonstrates that TGF- is the only factor capable of doing so.

expression of DNMT3 genes

Either a switch in the expression of CD133 or an accelerated rate of growth exclusively in the smaller CD133+ portion of cells may be used to explain the rise in CD133 positivity that was produced by TGF-. Both explanations are possible. In order to choose between these two hypotheses, we carried out the previous experiment once more using cells that could not express CD133. These cells were chosen by removing CD133+ cells from the sample using MACS. After 4 days of treatment, it became clear that TGF- was able to substantially produce a population of CD133+ cells in both of the cell lines that were tested. In this instance as well, we changed the medium after the first four days and then allowed the cells to continue growing without the presence of cytokines for a further four days. After these extra 4 days, the rise in the CD133 positive percentage was more than the one found at day 4, for both cell lines. This was in comparison to the one observed at day 4. In HepG2 control cells, the expression of CD133 on the cell surface remained extremely close to zero. Based on these findings, it seems that TGF- is able to enhance the production of CD133 surface protein, but it does not cause increased proliferation of CD133+ cells. This is supported further by the hypothesis that cells treated with TGF- will proliferate at a slower rate than normal. In these conditions, IL6 only demonstrated a brief impact, which was quite similar to the results of our earlier experiment (Table 5 and Fig 3,4,5).

After the demonstration that TGF- may be able to create a de novo percentage of CD133+ cells, we questioned if this impact was connected with a differential expression of DNA methylation players. This was due to the fact that we had previously established that CD133+ cells cultured in typical culture conditions result in overexpression of DNMT3 genes. DNMTs and TET2 exhibited a large upsurge in mRNA expression in at least one of the two cell lines after being rested for four days after being exposed to TGF-(Table 5 and Fig 3,4,5). On the other hand, TET1 was underexpressed throughout this period. This transpired as a result of a lack of TET1 expression. The most reliable discovery was that TGF-induced overexpression of DNMT3A occurred in both cell lines. This finding met the criteria for statistical significance. This was shown to be the case for the basal CD133-expressing cells, which are

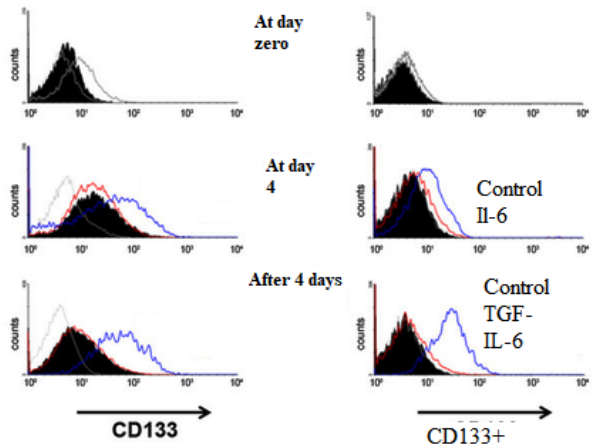

Figure 3. CD133 induction by TGF- correlation.

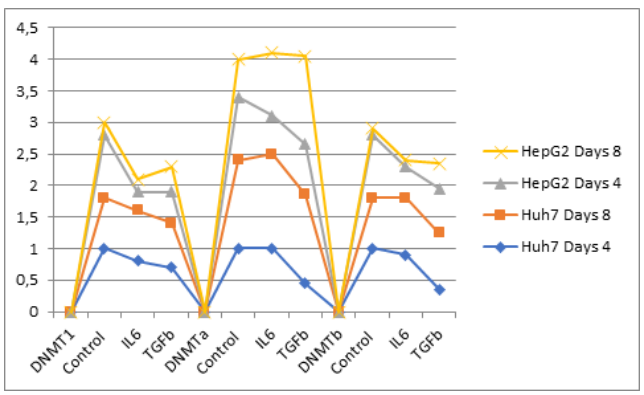

Figure 4. de novo CD133 induction by TGF-β correlates with overexpression of DNMT3 genes.

Table 5. de novo CD133 induction by TGF-β correlates with overexpression of DNMT3 genes.

	Huh7		HepG2		
	Days 4	**Days 8**	**Days 4**	**Days 8**	**P value**
			DNMT1		
Control	1.0	0.8	1.0	0.2	
IL6	0.8	0.8	0.3	0.2	0.64
TGFb	0.7	0.7	0.5	0.4	
			DNMTa		
Control	1.0	1.4	1.0	0.6	
IL6	1.0	1.5	0.6	1.0	0.22
TGFb	0.45	1.4	0.8	1.4	
DNMTb					
Control	1.0	0.8	1.0	0.1	
IL6	0.9	0.9	0.5	0.1	0.41
TGFb	0.35	0.9	0.7	0.4	

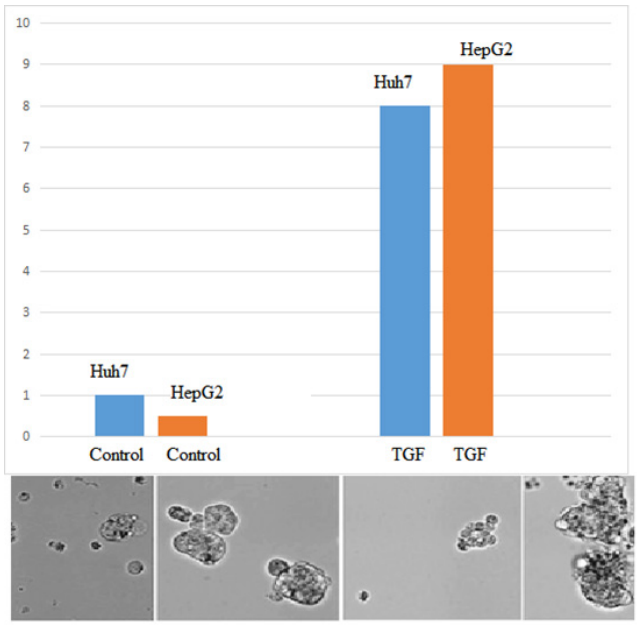

Figure 5. CD133 expression was studied by FACS after 4 consecutive days of exposure to each experimental condition for both cell lines. Representative phase contrast images are shown in the lower panels. Consecutive.

referred to as the cells that were extracted from HCC cell lines that had not been treated in any way (Table 5 and Fig 3, 4,5). It is essential to emphasize that exposure to IL-6 was not capable, under any of the conditions that were studied, of inducing statistically significant alterations at the level of mRNA expression of genes associated with DNA methylation or demethylation. This was the case regardless of the setting that was investigated. According to these findings, in contrast to IL-6, TGF is able to create a persistent de novo percentage of CD133-expressing cells in two distinct cell lines derived from liver cancer. These cell lines were cultivated apart from one another during their entire development. This induction seems to have a correlation with a functional attribute of basal CD133+ cells, which is an improved capacity for growth under the circumstances of non-attachment cell culture. In addition, the fact that TGF- continues to promote CD133 expression even after it has already been produced is evidence of the presence of an epigenetic process. In contrast, the fact that IL-6 only briefly increases CD133 expression demonstrates that the epigenetic process is taking place.

Correlation between the TGF-induced transition in the cell subpopulation and a distinct DNA methylome:

We opted to explore the DNA methylome that is produced by TGF- exposure since we had previously shown that CD133+ cells have their own distinct DNA methylome and that TGF- is able to create a de novo percentage of CD133+ cells. In order to achieve this goal, we made use of HM450 bead arrays to investigate the changes in DNA methylation that were brought about by exposing Huh7 and HepG2 cells to TGF- over four days. In addition, in order to determine the epigenetic permanence of the effects of TGF-, we included the DNA of cells that had been exposed to normal cell culture media after being treated with TGF- and released after four days. In the same way that the DNA methylation profile of CD133-expressing cells was described above, the methylomes of Huh7 and HepG2 cells are easily recognizable from one another, despite the fact that the experimental settings were different. On the other hand, in addition to alterations that were unique to a certain cell type, we were also able to detect genome-wide modifications that were produced by TGF- in a manner that was independent of the cell type. In order to develop a DNA methylation signature that was produced by TGF-, we focused on those loci that were either substantially hypomethylated or hypermethylated in both cell lines. In addition to this, we were interested in the modifications that persisted even after the cell division process was complete and were unaffected by the lack of TGF-. We chose significant loci (FDR 0.05) that were differentially methylated after both 4 days of treatment and 4 extra days following release. This was done so that we could compare the two sets of data. In the end, we chose those CpG sites that achieved a difference of at least 10% on average between the control and TGF- conditions. There are 555 differentially methylated positions (DMPs) that meet all of the requirements, with 115 of them being hypomethylated after being exposed to TGF- (21%), and the vast majority being hypermethylated (n = 440, 82%), including many sites on intergenic regions. In addition, we discovered that the total GC content of DMPs was noticeably lower when compared to the total GC content of HM450 probes or to a random selection of probes. This

was the case regardless of the probe type. In order to get a more in-depth understanding of the genomic context, we performed an analysis of the overlap between DMPs and genomic characteristics, as was previously reported (23).

These annotations take into account both the position in regard to the gene and the association with CpG islands, also known as CGIs (which may refer to islands, coasts, shelves, or open sea). The proximal promoter CGIs and shores, as well as non-CGI gene bodies, are the targets of a significant proportion of the probes contained in the HM450 bead arrays. When a randomly generated list of sites is compared to the genomic characteristics, a distribution that is analogous to the genomic features is shown. On the other hand, the promoter CGIs and coastlines are shockingly under-represented on the DMP list. Conversely, our research has shown that the vast majority of DMPs are located inside the gene bodies themselves, in non-CGI areas. This finding is consistent with our earlier finding that GC content is minimal.

The next step consisted of looking for differentially methylated regions (DMRs) that were caused by TGF- and were shared by both cell lines. We defined a DMR by the existence of at least two contiguous DMPs that were separated by no more than 100 bp and had a significant false discovery rate of less than 0.05. It's interesting to note that one of these DMRs is made up of three CpG sites that correlate to the main part of the DNMT3B gene. The vast majority of DMRs, in point of fact, were discovered outside of gene promoters. An independent quantitative approach is known as bisulfite DNA pyrosequencing was used in order to verify the DMPs present in DNMT3B and TRRAP. In each of the cell lines, hypermethylation was proven to have occurred in response to TGF- treatment for four days and then again four days after the treatment was terminated. In order to have a clearer picture of how the TGF—induced methylome transition affects the phenotypic, we analyzed the expression of the complete genome in both Huh7 and HepG2 cells. This was done in order to compare the two cell lines. We chose the 8-day time point because, according to our model, it is the one that best defines the long-term, stable changes that are induced by this cytokine. This time point is comprised of 4 days of TGF- treatment and 4 days of post-release. Both cell lines had the predicted profile of gene expression, according to the study of expression, which included known TGF- targets (Fig3,4,5). In addition, "type I transforming growth factor beta receptor binding" was the very first gene ontology category to be classified at the molecular function level. On the other hand, there was no significant overlap when the expression and methylation of important gene lists were intersected with one another. We displayed all of the expression and methylation data and separately assessed CpG island and non-CpG island locations.

This was done since it is known that the impact of a particular methylation modification on gene transcription is dependent on the chromosomal position (24). By charting concurrently all genes, regardless of their position in the genome, there is no evident association to be detected, which was to be anticipated. On the other hand, hypermethylation of the gene bodies was shown to have a positive correlation with gene expression. When we intersected the CD133 and TGF- methylation signatures, we found that there was a tiny but substantial overlap of 30 genes ($p = 0.0011$). This overlap was minor, but it was significant.

Inactivating de novo DNA methyltransferases and impact on reducing the response to TGF- at methyl-sensitive regions

We used a pool of siRNAs in an experiment to mute de novo DNA methyl-transferases in the setting of TGF- exposure in HepG2 cells. The goal of this experiment was to gain insight into the causal role that DNA methylation plays in the cancer cell population transition that is triggered by TGF-. The primary finding of our study was that TGF- was able to cause a shift in the expression of CD133 in HepG2 cells that were anywhere from a two- to three-fold increase as measured by FACS (Table 6). In light of the fact that the prior kinetics experiment showed that TRRAP hypermethylation occurred at 2 days after treatment with TGF-, we began treatment with TGF- 24 hours after transfection and collected DNA two days after that. The siRNA effectiveness was evaluated by qRT-PCR, and the results revealed that both siRNAs had an impact whether they were employed alone or in combination. Nevertheless, the siRNA against DNMT3B demonstrated superior efficacy in silencing the matching transcript. The morphology of HepG2 cells, which generally expand in the form of refractive colonies with clear boundaries, was not altered by transfection with a siRNA that did not target a specific gene. The anticipated reaction to TGF- was likewise unaffected by the non-targeting siRNA, which resulted in the absence of refractive colonies and the presence of flattened, larger cells as predicted. There was no discernible change in the morphology of the cells after transfection with either DNMT3A or DNMT3B siRNAs, or a combination of the two. Nevertheless, in contrast to the control that did not target anything, there was no reaction to the TGF- exposure, and the majority of the cells continued to exist as refractive colonies under all of the other conditions without suffering a substantial loss of cell viability. In a similar manner, the non-targeting siRNA did not have an effect on the anticipated rise in CD133+ expression measured by FACS. On its alone or in combination, siRNA directed against DNMT3s had no impact on the baseline expression of CD133. In spite of this, the reaction to TGF- was dramatically diminished regardless of the settings. Consequently, the CD133 phenotype served as a mirror to the morphological "rescue."

Lastly, we investigated whether or not inhibition of DNA methylation alterations in response to TGF- occurred when DNMT3 was silenced under the same experimental settings. In HepG2 cells, methylation of TRRAP is induced after 2 days of treatment with TGF-; this effect is unaffected by the transfection of a non-targeting siRNA. In contrast, the methylation of TRRAP is considerably reduced across the board when additional experimental conditions are applied in the absence of TGF- (Table 6).

Discussion

In this paper, we provide an in-depth analysis of the DNA methylome of liver cancer cells that express CD133 as well as those that do not express CD133. In order to conduct DNA methylome analyses, we isolated pure populations of CD133- and CD133+ cells from two HCC cell lines that were not linked to one another. CD133+ cells derived from liver cancer cell lines have been shown to be functionally different cells with an enhanced capacity to produce tumors in animal models (14). This information

Table 6. Differentially methylated regions after TGF-β exposure.

CHR	Start	End	# probes	Symbol	Distance2TSS	Promoter
chr1	3.31E + 07	3.31E + 07	4	EPHX1	0	T
chr7	1.35E + 08	1.35E + 08	2	CALD1	12547	F
chr7	1.57E + 08	1.57E + 08	2	DNAJB6	1875	F
chr9	1.33E + 08	1.33E + 08	1	NCS1	35897	F
chr19	32147452	32147685	3	ZNF254	12365	F
chr20	32148754	32148915	2	DNMT3B	15877	F
chr19	23278023	23278036	3	ZNF730	−3214	F
chr17	48275324	48275919	3	COL1A1	2598	F
chr17	48270042	48270097	4	COL1A1	7845	F
chr17	73631586	73631785	3	SMIM5	1989	F
chr17	80560479	80560634	3	FOXK2	7125	F
chr16	87437787	87437924	3	MAP1LC3B	9878	F
chr13	92002338	92002454	3	MIR17HG	3647	F
chr15	78286548	78286614	3	LOC91450	0	T
chr5	52096641	52096811	1	PELO	11447	F
chr6	33241410	33241770	3	RPS18	1458	F
chr10	1.35E + 08	1.35E + 08	5	TUBGCP2	1587	F
chr12	1.21E + 08	1.21E + 08	3	UNC119B	5287	F

has been published in the past. These results are consistent with clinical studies that show a worse prognosis for those patients of HCC that have a larger percentage of cells that express CD133. Although it was outside the scope of our research to assess the tumor-initiating or metastasis-initiating capabilities of CD133+ cells, our findings show that these cells have a distinct DNA methylation signature. The primary emphasis of future research should be on determining whether or not DNA methylation is essential in the process of developing the cellular programs that define the primary qualities of these cells.

Recently, prognostic implications of TGF-pathway activation in HCC have been linked to the ability of this signaling pathway to induce metastatic behavior in a fraction of HCC cells (25-28). This link was made possible by the discovery that TGF-pathway activation can induce metastatic behavior in HCC cells. Recent research has shown that TGF- is able to enhance the number of CD133+ cells in vitro, which provides more evidence of the connection between liver TICs and TGF- in HCC. (17) This provides an additional link between these two factors. Now, we were able to repeat those earlier findings and even go further with them. In two different HCC cell lines that are not linked to one another, we demonstrated that TGF- has the ability to raise CD133 expression both at the protein and the mRNA level. The impact that is brought about by TGF- is one that lasts, in contrast to the effect that is brought about by the pro-inflammatory cytokine IL-6, which only lasts temporarily (36). We show that this impact is reliant on particular signaling via TGF- type I receptor and is independent of enhanced cell proliferation of CD133+ cells. This is shown by our findings. We were able to indicate that TGF- is capable of inducing de novo expression of CD133 by employing cellular fractions that were depleted of CD133. In addition, this stimulation of CD133 cells is correlated with an improved capacity to proliferate under non-attachment circumstances, which is a surrogate functional assessment for TIC and stem cell capabilities (28-35)

Increased amounts of the de novo DNA methylation transcripts DNMT3A and DNMT3B were expressed in CD133+ cells that expressed CD133 at baseline levels as well as CD133+ cells that had been stimulated by TGF-. Because of this, we decided to investigate further whether or not TGF- had the potential to trigger alterations in DNA methylation throughout the whole genome. We were able to demonstrate cell line-independent alterations in DNA methylation that were persistently produced by TGF-a, which suggests that an epigenetic process is involved in the creation of a cellular program. In cells that had been treated with TGF-, the methylome did not completely overlap with the methylome of CD133+ cells that had been cultured under normal circumstances.

Interestingly, despite the fact that TGF- is known to cause DNA methylation alterations at certain loci (25-28), there has been very little evidence to suggest that the whole genome contains TGF—methyl-sensitive sites up to this point. To be more specific, a number of earlier investigations focused on chromatin alterations related to epithelial-mesenchymal transition (EMT). EMT is a developmental process that includes the remodelling of the actin cytoskeleton, the loss ofapical–basal polarity and cell-to-cell contact, and epigenetic reprogramming (29). EMT is similar to other developmental processes in that it requires the reprogramming of epigenetics. It is interesting to note that gene-specific alterations in DNA methylation have been shown to be linked with the capacity to sustain epigenetic silence of essential EMT genes (27). To put this another way, it has been hypothesized that DNA methylation plays a role in the process of regulating the flip that takes place between epithelial and mesenchymal phenotypes. At the circumstances that we used, TGF- caused a reduction in the expression of E-Cadherin in both cell lines, but it did not cause an obvious rise in the expression of N-Cadherin, which is a recognized hallmark of EMT. On the other hand, our findings are in line with a model that predicts TGF- would cause alterations in DNA methylation that are long-lasting. In point of fact, the purpose

of our experimental design was to replicate an epigenetic process. To do this, we chose to focus on the alterations in DNA methylation that were able to survive the process of cell division. In order to determine whether or if this impact of TGF- is exclusive to transformed cells, more research is required.

We present what seems to be a singular pattern of differential methylation, and this is true irrespective of concerns about the cells that were targeted, the influence of the cell cycle, or the subtypes of cells. Our TGF- signature, which was persistent after the removal of TGF- and was shared by two different liver cancer cell lines, was distinguished by a decreased amount of GC content and an enrichment in enhancer regions (23). At this time, it is unknown whether or whether the same observations occur in response to other types of cytokines.

Conclusion

According to the findings of our research, there is a self-sustaining interaction between the DNA methylation machinery and the TGF-signaling pathway, which may be significant in the development of cellular phenotypes. This is the first evidence that TGF- has the potential to generate genome-wide alterations in DNA methylation, which ultimately leads to a persistent shift in the fraction of liver cancer cell subpopulations.

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