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MALLORY BODY FORMATION IS ASSOCIATED WITH EPIGENETIC PHENOTYPIC CHANGE IN HEPATOCYTES *IN VIVO*

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Abstract

Microarrays were done on the livers of mice fed DDC for 10 weeks, withdrawn 1 month (DDC primed livers) and refed 6 days, and compared with mice fed the control diet. The expression of a large number of genes changed when DDC was fed or refed. A Venn diagram analysis identified 649 genes where gene expression was changed in the same direction. The epigenetic memory of the DDC primed liver involved an increase in the expression of ubiquitin D, alpha fetoprotein, connective tissue growth factor, integrin beta 2, DNA methyl transferase 3a and DNA damage – inducible 45 gamma. DNA methyl transferase 3b was down regulated as was Cbp/p300. When DDC was refed, DNA methyltransferase and histone deacetylase were up regulated as shown by microarray analysis. Histone3 lysine9 acetylation was increased by DDC and DDC refeeding and DNA methyltransferases were not changed as shown by Western blot analysis. The data suggests the concept that the epigenetic memory that explains why DDC primed hepatocytes form MBs in 7 days of DDC refeeding is primarily the result of epigenetic modifications of gene expression through changes in histone acetylation and methylation, as well as DNA methylation.

Keywords

epigenetics; Mallory bodies; phenotypic change; genetic memory

Introduction

Mice fed diethyl-1,4-dihydro-2,4,6-trimethyl-3,5-pyridine decarboxylate (DDC) added to a normal mouse diet for 10 weeks form Mallory bodies (MBs) in clusters of hepatocytes. The MBs mostly disappear by one month of DDC withdrawal (DDC primed hepatocytes). Following 7 days of DDC refeeding MBs are again formed by groups of hepatocytes (Yuan et al., 1996). The gene expression associated with the induction of MBs in this model of MB formation includes the up regulation of integrins, laminin, I-Cam 1, Src, MEKK1, ERK and p38 (Wu et al., 2005; Nan et al., 2006a). When hepatocytes from DDC withdrawn mice, where MBs had disappeared, were cultured *in vitro*, MBs reformed *in vitro* spontaneously in 6 days. The gene expression of ERK, NFkB105, Src, MEKK1, JNK1/2 and p38 was up regulated during MB formation *in vitro* (Nan et al., 2005; Nan et al., 2006b). These results suggested that the DDC primed hepatocytes had a genetic “memory” which predisposed

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them to form MBs both *in vitro* and *in vivo*. It is possible that the mechanism which could account for this memory is epigenetic.

Epigenetic memory involves heritable changes in gene expression that do not involve changes in DNA sequences (Lu et al., 2006). However, histone methylation may be reversible, dynamic (di or mono) or at least more stable (trimethyl histone). The molecular basis of these changes typically involves DNA methylation and histone modifications such as methylation and acetylation. Both mechanisms act in concert to provide stability of gene expression as the result of gene silencing or up regulation.

To test this hypothesis mice were fed DDC for 10 weeks, withdrawn for 4 months, then refed DDC for 7 days. Controls were fed the diet without DDC. These 4 groups of mice were sacrificed and their livers were quick frozen in isopentane in liquid nitrogen. Microarray analysis was done on one mouse liver per group to determine the global changes in gene expression. A large number of changes in gene expression occurred when DDC was fed, and after refeeding. Liver homogenates and nuclear extracts were used for Western blot to determine changes in DNA and histone methylation and acetylation. Histone acetyltransferase, and DNA methyltransferase were also measured. The results were correlated with microarray data. Quantitative RT-PCR was done to validate the microarray results on two selective genes.

MATERIALS and METHODS

Animals

One month old C3H male mice (Harlan Sprague-Dawley, San Diego, CA) were divided into 4 groups. Group 1: 3 control mice fed a protein rich semisynthetic, complete standard control diet (Teklad, Madison, WI). Group 2: 3 mice were fed the control diet with 0.1% 1,4-dihydro-2, 4, 6-trimethyl-3,5-pyridinedicarboxylate (DDC, Aldrich, St Louis, MO) for 10 weeks to induce MB formation *in vivo*. Group 3; 3 mice were fed DDC for 10 weeks then withdrawn from DDC for 5 weeks at which time the MBs had mostly disappeared. Group 4: 3 mice were fed DDC for 10 weeks, withdrawn 4 weeks, then refed the DDC diet plus chlormethiazole (80 mg/kg) for 7 days to enhance MB formation by inhibiting CYP2E1 (Bargag-Gorce et al, 2002). The mice used here were the same mice that have been used in previous studies (Wu et al. 2005). All mice were treated in a humane manner as approved by the Animal Care Committee at Harbor-UCLA LABiomed Research Institute according to the Guidelines of the National Academy of Science.

Microarray analysis

Liver tissue from one mouse of each of the 4 treatment groups was subjected to microarray analysis. Total liver RNAs are extracted with Ultraspec™ RNA Isolation Systemic (Biotech Laboratories, Houston, TX) and are cleaned up with Rneasy columns (Qiagen, Valencia, CA). Five micrograms of total RNA were used for preparing biotin-labeled cRNA. Labeled and fragmented cRNA is subsequently hybridized to Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA). Labeling, hybridization, image scanning and initial data analysis are performed by the Microarray Core at Los Angeles Biomedical Research Institute.

Sample Preparation and loading

Equal amounts of RNA (5 mg) from each sample were used for Affymetrix GeneChip analysis. RNA was converted to cDNA using GeneChip® One-Cycle cDNA Synthesis Kit (Affymetrix) and then to biotinylated cRNA using GeneChip® IVT Labeling Kit (Affymetrix). The quality of labeled RNA was confirmed with the Affymetrix Test 3 Array.

The biotinylated cRNA from all samples was hybridized to Affymetrix Mouse 430 2.0 GeneChip arrays.

Hybridization and staining

Hybridization cocktail was prepared, which includes controls at the fragmented cRNA. The samples were hybridized in the array at 45°C for 17 h using GeneChip Hybridization Oven 640. Immediately following hybridization, the array underwent an automated washing and staining protocol (R-Phycoerythrin Streptavidin conjugated, Molecular Probes) on the GeneChip Fluidics Station 400. The arrays were then scanned with a GeneChip Scanner 3000 (Affymetrix).

Microarray Data Analysis

Data preparation, analysis, and integration were performed using Affymetrix's GeneChip Operating Software (GCOS). The software was used for perform image processing, evaluation of data quality, normalization, transformation, and filtering, so that data was ready for further analysis. Wilcoxon's signed rank test was used in comparison analysis to derive biologically significant results from the raw probe cell intensities on expression arrays. For comparison analysis, each probe set on the experiment array was compared with its counterpart on the control array to calculate the change in P value that was used to generate the difference call of increase (I; $P < 0.04$), marginal increase (MI; $P < 0.04$ to $P < 0.06$), decrease (D; $P > 0.997$), marginal decrease (MD; $P > 0.992$ to $P > 0.997$), or no change (NC; $P > 0.06$ to $P < 0.997$). Comparison analysis was used to generate a signal log ratio for each probe prior to the experimental array to the corresponding probe pair on the control array. This strategy cancels out differences resulting from different probe finding coefficients. Signal log ratio was computed by using a one-step Tukey's biweight method by taking a mean of the log ratio of probe pair intensities across the two arrays.

Once the absolute and comparison data files were created in GCOS, genes were identified with signal intensity differences using BULLFROG v12.3 TG (Lockhart and Lockhart) and GeneSpring (Silicon Genetics). In the BULLFROG analysis, the filtering criteria used to find genes unique to DDC 7 days was the following: a change call of increase/marginal increase or decrease/marginal decrease, fold change > 1.7 , and a present call in at least one of the arrays. In GeneSpring the probes were first normalized using "Per Gene: Normalize to median". Next, transcripts were determined to be differentially expressed based on the following criteria: a Change Call of Increase, Marginal Increase, Decrease, or Marginal Decrease with a Change P -value < 0.006 or > 0.994 , a Signal Log Ratio < -0.8 or > 0.8 , a Present Call for the probe set in either or both experimental conditions, and a minimum signal intensity of 50 of a probe in either or both of the experimental files.

After generating a list of differentially expressed genes, down stream analysis was performed. The filtered transcripts were clustered in GeneSpring using SOM, K-means and GeneTree to find similar patterns of gene expression. The lists of transcripts were also uploaded into GenMapp (Gene MicroArray Pathway Profiler, Gladstone Institutes University of California at San Francisco). GenMapp clusters the transcripts based on biological function.

The data illustrated was obtained by using the KEGG web site (<http://www.genome.jp/kegg/pathway.html>) and blasting the list of total changed genes issued from our experiment for analysis. The web site calculates the number of up regulated and down regulated genes for each pathway shown in the KEGG graph. To determine the percent gene change in each pathway, the number of changed genes present in each pathway was divided by the total number of genes in the same pathway.

The same calculation was performed in the ABI panther (<http://www.pantherdb.org/genes/>) web site to illustrate the pie chart. To determine the percent gene change in each pathway, the number of genes present in each pathway was divided by the total number of changed genes.

Subcellular fractionation

Rat liver homogenates were prepared by homogenizing 100mg of frozen liver tissues in 2 mL of 20mM Tris-HCl pH 7.5; glycerol 10%; EGTA 1mM; DTT 1mM; NaFluoride 50 mM; proteases and phosphatases inhibitor cocktail (Sigma). The tissues were homogenized using the Ultra-Turrax T25 homogenizer. The Nuclear extracts were prepared using the NE-PER Nuclear Extraction Reagents (Pierce Biotechnology, Rockford, IL). Protein concentrations were evaluated using the Bradford method (1976).

Western Blot Analysis

Proteins (50 µg) from liquid nitrogen frozen stored livers were separated by SDS-PAGE gels and transferred to a PVDF membrane (Bio-Rad, Hercules, CA) for 1 h in 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 20% methanol. The membranes were stained using the primary antibodies listed in Table 1. Appropriate species anti polyclonal and monoclonal HRP-conjugated antibodies were used as the secondary antibodies. The membranes were subjected to chemiluminescence detection using luminal, according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ).

Quantitative real-time RT-PCR assay

Total liver RNAs were extracted with Trizol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA). Synthesis of cDNAs was performed with 5 µg total RNA, 50ng random hexamer primers using SuperSriptIII RNase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA). PCR primers were designed with the assistance of the Primer Express software (Applied Biosystems, Foster City, CA) [the primers for mouse liver Gadd45g were: Sens: 5' TGAATGTGGACCCTGACAATGT 3' and the anti-sens: AATGGATCTGCAGCGCTATGT. The primers for CTGF were: Sens: CTGCCTACCGACTGGAAGACA3' and the anti-sens is 5' GATGCCCCATTCCACAGGTCTTA3'. Quantitative PCR was achieved using the SYBR Green JumpStart™ Tag ReadyMix (Sigma, St. Louis, MO) on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). The thermal cycling consists of an initial step at 50°C for 2 min, followed by a denaturation step at 95°C for 10 min, then 40 cycles at 95°C for 15 s and 60°C for 1 min. Single PCR product was confirmed with the heat dissociation protocol at the end of the PCR cycles. Quantitative values were obtained from the threshold PCR cycle number (Ct) at which point the increase in signal associated with an exponential growth for PCR product starts to be detected. The target mRNA abundance in each sample was normalized to its 18S level as $\Delta Ct = Ct_{\text{target gene}} - Ct_{18S}$. For each target gene, the highest ΔCt was assigned as ΔCt_{max} . The relative mRNA levels were calculated as $2^{-\Delta Ct}$, $\Delta \Delta Ct = \Delta Ct_{\text{max}} - \Delta Ct$.

Statistics

Statistical analysis was performed using ANOVA t test and Bonferoni for multiple group comparison (Sigma Stat software). Significance was defined as $P < 0.05$.

RESULTS

The heat map from 1 mouse liver per treatment group (group1 = 43; group 2 = 11; group 3 = 90; group 4 = 105) is shown in figure 1. The gene expression changed in a large number of

genes when DDC was fed for 10 weeks and again when DDC was refed after a 4 week interval where DDC was withheld from the diet. Gene expression returned to control levels after one month withdrawal of the drug DDC. DDC refed for 7 days after withdrawal caused a return of gene expression seen after the initial 10 weeks of DDC feeding. When mouse 90 (DDC primed mouse) was compared to mouse 43 (control mouse), the expression of 2300 genes was changed using a + or – 1.6 cut off. When mouse 11 (DDC fed 10 weeks) was compared with mouse 43, the expression of over 5300 genes was changed. When mouse 105 (DDC refed) was compared with mouse 90, the expression of over 4600 genes was changed. To further analyze the data, the number of genes analyzed was reduced by changing the cut off fold change.

When the changes were analyzed by Venn to determine the shared gene expressions when the three mice treated with DDC were compared with the control or the mouse withdrawn from DDC for 5 weeks, 649 out of 9319 changes in gene expression were identified (Fig 2). The 649 changed genes identified, constituted the gene expression memory present in the DDC withdrawn mice livers.

These changes in gene expression may explain how hepatocytes are able to spontaneously form MBs 7 days after refeeding DDC compared to the mice that were initially fed DDC for 10 weeks. The 649 genes common to all three comparisons in the Venn diagram (Fig 2) were grouped according to functional pathways (Fig 3).

The genes most changed were those involved in the regulation of the actin cytoskeleton. MAPK signaling and focal adhesion function genes were the next most commonly affected. In all cases, more genes were up regulated than down regulated. The % total of gene expression changes is illustrated in Fig 4.

When all the changes in gene expression were analyzed, a few changes stood out as potentially being involved in the memory of MB formation because the changes are in the same direction. For instance the gene for ubiquitin D was up regulated (Table II).

Ubiquitin D is involved in protein degradation by the 26S proteasome. Alpha fetoprotein (AFP) was up regulated, especially in the DDC withdrawn mouse (the mouse which has the memory of how to form MBs when DDC was refed). Connective tissue growth factor (CTGF), and growth arrest and DNA-damage—inducible 45 gamma (Gadd45g) were especially up regulated in the memory mouse (Fig 5). Integrin beta 2 (Itgb2) was up regulated (Table II). Integrin is involved in MB formation *in vitro* (Wu et al, 2005). Glutathione S-transferase (GSTm4) expression was up regulated when DDC was fed but was down regulated by DDC withdrawal. GST isoforms are involved in drug detoxification and are induced by DDC feeding (Roomi et al, 2006). Platelet derived growth factor, alpha (Pdgfa) was marginally up regulated (Table II). Adrenomedullin (Adm) was up regulated (Table II). Adm is up regulated by HIF1 α (Li et al, 2004). However, HIF1 α was down regulated in the nuclear extract when DDC was fed or refed (Fig 6).

When the epigenetic memory of the mouse withdrawn from DDC for a month was compared with the control mouse, Dnmt3b was markedly down regulated suggesting that gene methylation was reduced (Table III). The levels of methyl cytosine and dimethylated histone 3 lysine 9 were not changed by the DDC treatment (Fig 9).

The reduction in methylation by Dnmt3b (Table III) could be a mechanism for the up regulation of gene expression observed. The expression of the acetyl transferase p300 was decreased by DDC withdrawal (see Table III). When the DDC refed mouse was compared with the DDC withdrawn mouse, down regulation of the deacetylases Sirt1 and 3 was observed (Table IV).

When measured by Western blot, HDAC levels were also reduced by DDC feeding but not in the DDC refed mice (Fig. 7), which does not support the significance of HDAC action in MB induction. Deacetylation of histones, therefore, may have been reduced during DDC feeding which could further enhance epigenetic up regulation of gene expression without involvement of signal transduction. To support this possibility, acetylation of H3 lysine 9 was measured by Western blot. Acetylation of histone 3 lysine 9 was increased when DDC was fed (Fig. 8). When the HDAC level is reduced, the level of H3K9 acetylation would logically increase. However when DDC was refed, the level of HDAC did not change, as shown by Western blot, suggesting mechanisms other than HDAC activity are regulating the level of H3K9 acetylation.

There were no significant changes in the level of Dnmt3b or dimethyl H3 lysine 9 (H3K9) measured by western blot (Fig. 9). This data has to be confirmed by measuring the nuclear fraction as well.

The protein levels of Gadd45g were decreased by DDC feeding and refeeding, supporting a negative role for this important protein in the formation of MBs (Fig. 10). The protein levels of DDC W vs. control did not correlated with the gene expression levels (Fig. 5). The increase in Gadd45g and CTGF expression seen by microarray analysis was confirmed using RT PCR (Fig. 11).

DISCUSSION

The most likely explanation as to how DDC induces MB formation is that DDC induces an epigenetic phenomenon by reducing DNA methylation and increasing histone acetylation. However the role of epigenetic memory must be regarded as speculative until direct molecular evidence is available. Other non epigenetic mechanisms were not investigated. As shown in the heat map of gene expression changes after feeding and refeeding DDC, there was a profound change in gene expression (i.e. 9319 genes). There was a reversible switch in gene expression when the drug was fed and withdrawn. The memory of the changes in gene expression induced by DDC feeding was partially retained (i.e. 625 genes) which shortened the time interval necessary for MB formation (10 wks vs. 6 days). Epigenetic is defined as “heritable and reversible changes in gene expression which do not involve coding sequence modifications” (Lu et al, 2006). However, histone methylation may be reversible, dynamic, irreversible, or at least more stable (trimethyl histone). The epigenetic memory hypothesis here is speculative until direct molecular evidence is available.

Of particular interest is the memory of gene expression which shortens the time interval for MB formation when the drug was refed. This phenomenon helps narrow down the critical memory of gene expression changes involved in MB formation. Since the acetyltransferase p300 was not changed by DDC feeding, and DNA methyl transferase (Dnmt3b) was down regulated, it is likely that the gene silencing by DNA methylation was reversed and this may explain the epigenetic memory of DDC withdrawn hepatocytes which show an accelerated formation of MBs when DDC is refed.

Confounding the problem, however, is the fact that, the phenotypically changed hepatocytes in the drug withdrawn mouse coexist with hepatocytes which are not phenotypically changed (Wu et al, 2005; Roomi et al., 2006; Nan et al, 2006a–b; Tazawa et al, 1983; Nagao et al, 1999). Two phenotypic markers overexpressed in the MB forming preneoplastic phenotype, alpha fetoprotein and glutathione S-transferase (Roomi et al, 2006; Nan et al, 2006b) were up regulated in the DDC treated hepatocytes. However, only alpha fetoprotein was up regulated in the DDC withdrawn memory hepatocytes which had stopped MB formation. The mouse fed DDC for 10 wks had a 69 fold increase in AFP gene expression,

the DDC withdrawn mouse had a 24 fold increase and the refed mouse had a 13 fold increase in AFP. Liver tumors that form 9 months after DDC withdrawal show a 5 fold increase in AFP expression (Nan et al, 2006b) indicating that the memory of AFP up regulation persisted for 9 months in the preneoplastic phenotype. The increase in AFP expression by DDC *in vitro* and *in vivo* has been demonstrated by Western blot, PCR and immunohistochemistry (Nan et al, 2006b). The stimulation of the DDC withdrawn livers to form MBs after 10 wks DDC feeding, followed by 4 wk withdrawal, is not specific to DDC but is also induced by a variety of liver toxins including alcohol and heat shock (French et al, 2001; Riley et al, 2003; Zhang-Gouillon et al, 1998).

Many growth factors were expressed in the livers of the mice fed DDC or withdrawn from DDC (livers with an epigenetic memory induced by DDC). These included connective tissue growth factor, hepatoma-derived growth factor related protein 3, transforming growth factor beta and platelet derived growth factor alpha. The up regulation of these genes may explain the tendency for the memory hepatocytes withdrawn from DDC, to form tumors 9–14 months later (Nan et al, 2006b).

The up regulation of integrin beta 2 observed in the DDC treated and withdrawn hepatocytes (memory cells) may explain why these cells form MBs in primary tissue culture of DDC primed hepatocytes since laminin-integrin signaling triggers MB formation *in vitro* (Wu et al, 2005).

A most interesting finding, as shown by microarray analysis, is the up regulation of Gadd45g in the DDC primed hepatocytes compared to the control (90 vs. 43). The withdrawn liver cells remember how to form MBs in response to DDC refeeding. Gadd45g is induced by stress shock and inhibits cell growth and induces apoptosis. Heat shock protein can induce Gadd45g up regulation. However, this response is abolished if its promoter is hypermethylated (Ying et al., 2005). Hypermethylation of the Gadd45g promoter region might explain its reduced expression when DDC was fed (Fig10). However the protein level was not changed when DDC W was compared to the control. Gadd45g is antiapoptotic through the p38-NFκB mediated survival pathway (Gupta et al, 2006; Zerbini and Libermann, 2005). Gadd45g has been identified as a target gene for hypermethylation epigenetic gene silencing in tumor cells (Ying et al, 2005).

This may be relevant to the observation that inhibition of either p38 or NFκB activation *in vitro* completely prevents MB formation by DDC withdrawn mice hepatocytes in primary cultures (Nan et al, 2005; Nan et al, 2006b).

The analogy of MB formation in DDC fed mice to MBs seen in chronic alcoholic liver disease is evident because the mouse fed DDC and the rat fed alcohol, both show an increase in acetylation of histone 3 lysine 9 (Shukla *et al.*, 2006; Bardag-Gorce *et al.*, 2007). The inhibition of p38 in the drug primed mouse hepatocyte primary culture, inhibited MB formation (Nan et al., 2006a). Likewise, in the experimental rat model of alcoholic liver disease, phospho-p38 levels were decreased in nuclear extracts of rat liver fed alcohol (Gorce-Bardag et al, 2007). In the case of the rat model, no MBs were formed. MBs in the liver studied here were documented to be formed in a previous study by Wu et al., (2005).

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Fig. 1.

Heat map of 3147 genes clustered from the comparison between 00-43 (Control), 00-11 (DDC fed mouse), 00-90 (DDC fed and withdrawn mouse), and 00-105 (DDC fed mouse, withdrawn and re-fed DDC mouse). Mouse MB Model In vivo (n=1).

Fig. 2.

Venn diagram of gene expression, when the control was compared with the DDC treated mice or the mouse fed DDC 10 wk and then DDC withdrawn for 5 weeks. The change in gene expression in the same direction shared by the DDC treated mice numbers 625 with 25 odd. 90 vs. 43= 2300 genes changed. 11 vs. 43= 5300 genes changed. 105 vs. 90= 6600 genes changed. 649 genes were changed in common among the three different comparisons, which includes 9319 genes in total (n=1).

Fig. 3. Functional pathways where the expression of 3343 genes changed when DDC treated mice were compared with the control or the DDC withdrawn mouse (n=1)..

Fig. 4. The % of the gene expression changes by functional pathways is shown when the DDC treated mice were compared to the control or the DDC withdrawn mice (625 genes) (n=1).

Fig. 5. Gene expression of growth factors changed when in DDC withdrawn mice (Memory cells that form MB's) compared to the control mouse liver. Note the increased expression of Gadd45g and Ctgf (n=1).

Fig. 6.
HIF1 alpha expression in nuclear extracts from the livers of mice fed DDC (Mean $\pm$ SEM, $n=3$).

Fig. 7.

The protein level of histone deacetylase (HDAC) is decreased in the liver homogenates of DDC fed mice (Mean $\pm$ SEM, $n=3$).

Fig. 8.

The protein levels of acetylated histone 3 lysine 9 are increased in the liver homogenates of DDC fed and refed mice (Mean $\pm$ SEM, $n=3$).

Fig. 9.
The protein levels of Dnmt3b and dimethyl histone 3 lysine 9 were unchanged in the liver homogenates by DDC feeding and refeeding (Mean $\pm$ SEM, n=3).

Fig. 10.

The protein levels of Gadd45g in the liver homogenates were decreased by DDC feeding and DDC refeeding (Mean $\pm$ SEM, $n=3$).

Fig. 11.

MB formation in vivo was associated with an increased expression of Gadd45g (SEM $\pm$ SE, $n=3$; C vs DDC $p=0.004$; C vs DDC W $p=0.016$). MB formation was also associated with an increased expression of CTGF (C vs DDC W $p=0.03$; C vs DDC R $p=0.014$). The level of Gadd45g and CTGF expression in DDC W was significantly increased suggesting that the expression of these two genes may be involved in the epigenetic memory of DDC primed hepatocytes.

Table II. Important gene expression changed in the same direction in the epigenetic memory

Signal (Exp)			Ratios			Gene Symbol	Gene Title
11	90	105	11 v 43	90 v 43	105 v 90		
11771	688.9	11108	119.4	5.7	13.9	Ubd	ubiquitin D
1499.7	246.7	4205.2	68.6	24.3	13.0	Alp	alpha fetoprotein ←
1434.1	392.1	773.1	24.3	5.7	2.1	Rragd	Ras-related GTP binding D
201.4	479.2	1945.1	10.6	26.0	1.6	Ctgf	connective tissue growth factor ←
2916.3	627.3	3108.2	8.6	2.3	3.7	Itgb2	integrin beta 2 ←
576.5	164	621.8	8.6	2.5	4.0	Adcy7	adenylate cyclase 7
2429.4	1047.9	3595	4.9	2.3	2.8	Vim	vimentin
1067	526	1144.3	4.6	2.1	2.5	Gpx3	glutathione peroxidase 3
402.3	166.9	526.3	3.7	1.9	3.3	Hdgfr3 /// Tm6sf1	hepatoma-derived growth factor, related protein 3 /// transmembrane 6 superfamily member 1
605.2	239.5	809.7	3.5	1.6	2.8	Dnmt3a	DNA methyltransferase 3A
1753.7	850.4	1831.3	3.3	1.7	2.0	Tgfb1	transforming growth factor, beta induced
168.8	125.2	316.5	3.3	3.0	3.7	Adm	adrenomedullin
86.5	114.5	185.2	2.0	2.8	1.7	Tlr4	toll-like receptor 4
336.3	308.9	711.9	2.0	1.9	1.7	Pdgfra	platelet derived growth factor, alpha ←

TABLE 1

Primary Antibodies Used for Western blot

Antigen	Company and Location	Host	Dilution
HDAC	BIOMOL, Plymouth Meeting, PA	Mouse	1:1000
HIF alpha	Santa Cruz Biotech, Santa Cruz, CA	Rabbit	1:1000
AcH3K9	Cell Signaling Technology, Danvers, MA	Rabbit	1:1000
Histone 3	Cell Signaling Technology, Danvers, MA	Rabbit	1:1000
Dnmt3a	Abcam Inc. Cambridge, MA	Mouse	1 :1000
Dnmt3b	Abcam Inc. Cambridge, MA	Rabbit	1 :1000
Dimethyl H3K9	Abcam Inc. Cambridge, MA	Rabbit	1 :500
5Methyl Cytosine	Diagenode, Liège (Sart Tilman) Belgium	Mouse	1 :200
Gadd45g	Proteintech Group, Inc Chicago, IL	Mouse	1:1000

Table III

DDC Withdrawal Vs Control (90 vs. 43)

Gene Title	Gene Symbol	Ratio
DNA Methylation		
DNA methyltransferase 3A	Dnmt3a	1.6
DNA methyltransferase 3B	Dnmt3b	-7.5←
Histones		
histone 1, H2bc	Hist1h2bc	-1.5
histone 1, H2b (c, e, l, m, p)	Hist1h2b (c, e, l, m, p)	-1.5
histone 2, H3c2	Hist2h3c2	-1.9
histone 1, H1c	Hist1h1c	-2.5
CBP/p300		
transcription factor activity	Cbp/p300	-1.7
Foxo		
forkhead box 01	Foxo1	2.3
forkhead box 03a	Foxo3a	1.6

Table IV

Gene expression changes when DDC was refed

Fold Change	Gene Symbol	Gene Title
DNA Methyltransferase		
→6.5	Dnmt1	DNA methyltransferase (cytosine-5) 1
2.8	Dnmt3a	DNA methyltransferase 3A
Histone Deacetylase (HDAC)		
4.6	Uap1 1	UDP-M-acteylglucosamine pyrophosphorylase 1-like 1
4.6	Hist1h2ad	histone 1, H2ad
CBP/p300		
-2.3	Pcaf	p300/CBP-associated factor
SIRT		
-1.9	Sirt1	sirtuin 1 (homolog) 1 (S. cerevisiae)
→-2.3	Sirt3	sirtuin 3 (homolog) 3 (S. cerevisiae)
Foxo		
-1.9	Foxo1	forkhead box O1
-1.9	Foxa1	forkhead box A1
-4.9	Foxq1	Forkhead box Q1
-6.5	Foxp2	forkhead box P2
3.7	Foxm1 ///Pbp///4933413	forkhead box M1 /