RESEARCH PAPER

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DMSO is a strong inducer of DNA hydroxymethylation in pre-osteoblastic MC3T3-E1 cells

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Artificial induction of active DNA demethylation appears to be a possible and useful strategy in molecular biology research and therapy development. Dimethyl sulfoxide (DMSO) was shown to cause phenotypic changes in embryonic stem cells altering the genome-wide DNA methylation profiles. Here we report that DMSO increases global and gene-specific DNA hydroxymethylation levels in pre-osteoblastic MC3T3-E1 cells. After one day, DMSO increased the expression of genes involved in DNA hydroxymethylation (TET) and nucleotide excision repair (GADD45) and decreased the expression of genes related to DNA methylation (*Dnmt1*, *Dnmt3b*, *Hells*). Already 12 hours after seeding, before first replication, DMSO increased the expression of the pro-apoptotic gene *Fas* and of the early osteoblastic factor *Dlx5,* which proved to be *Tet1* dependent. At this time an increase of 5-methyl-cytosine hydroxylation (5-hmC) with a concomitant loss of methyl-cytosines on *Fas* and *Dlx5* promoters as well as an increase in global 5-hmC and loss in global DNA methylation was observed. Time course-staining of nuclei suggested euchromatic localization of DMSO induced 5-hmC. As consequence of induced *Fas* expression, caspase 3/7 and 8 activities were increased indicating apoptosis. After five days, the effect of DMSO on promoter- and global methylation as well as on gene expression of *Fas* and *Dlx5* and on caspases activities was reduced or reversed indicating downregulation of apoptosis. At this time, upregulation of genes important for matrix synthesis suggests that DMSO via hydroxymethylation of the *Fas* promoter initially stimulates apoptosis in a subpopulation of the heterogeneous MC3T3-E1 cell line, leaving a cell population of extra-cellular matrix producing osteoblasts.

Introduction

Methylation of cytosine-guanine dinucleotides (CpGs) on DNA is an important epigenetic mechanism involved in the selective regulation of gene expression and in the stabilization of chromatin. Therewith it is implicated in cell and tissue development and pathogenesis[1-3] such as cellular differentiation and cancer development, respectively. For example, it has been demonstrated that suppression of the pro-apoptotic gene *Fas* via promoter methylation is an essential mechanism for the generation of RAS transformed tumor cells.[4] Methylation of CpGs on DNA is achieved by DNA methyltransferases (DNMTs) whereby DNMT1 is associated with maintenance of global DNA methylation patterns and DNMT3a and DNMT3b are defined as de novo DNA methyltransferases.[5]

Except a potential passive DNA demethylation during cell replication, for a long time CpG methylation markers were thought to be very stable. However, very recently groundbreaking insights have been published regarding the long-standing enigma of active DNA demethylation. In addition to findings of direct demethylation of cytosines,[6] conversion of 5-methyl-cytosine (5-mC) to 5-hydroxymethyl-cytosines (5-hmC) by members of the TET-hydroxylase gene family was shown to promote active DNA demethylation in mammalian cells through a process that requires the base excision repair pathway involving the GADD45 gene family.[7-12] Further, it has been demonstrated that 5-hmC patterns are dynamically regulated during early embryonic differentiation and in mouse embryonic stem cells (ESC).[13] *Tet1* is specifically expressed in murine ESC where it is required for stem cell maintenance by promoting the transcription of pluripotency factors.[14] These studies suggested for the first time a naturally occurring way for dynamic regulation of DNA methylation.

Dimethyl sulfoxide (DMSO) alters the epigenetic DNA methylation profile of mouse embryoid bodies causing phenotypic changes in embryonic stem cells by stimulation of *Dnmt3a*[15] and affect differentiation in several cell-lines.[16-20] Furthermore, a direct and specific stimulatory effect of DMSO on the catalytic activity of DNMT3a has been demonstrated in vitro.[21] The effect of this epigenetic active compound on genes related to active DNA demethylation is unknown.

Osteoblasts, the bone forming cells, derive from mesenchymal stem cells.[22] Driven by transcription factors like *Dlx5*,[23-27] initially

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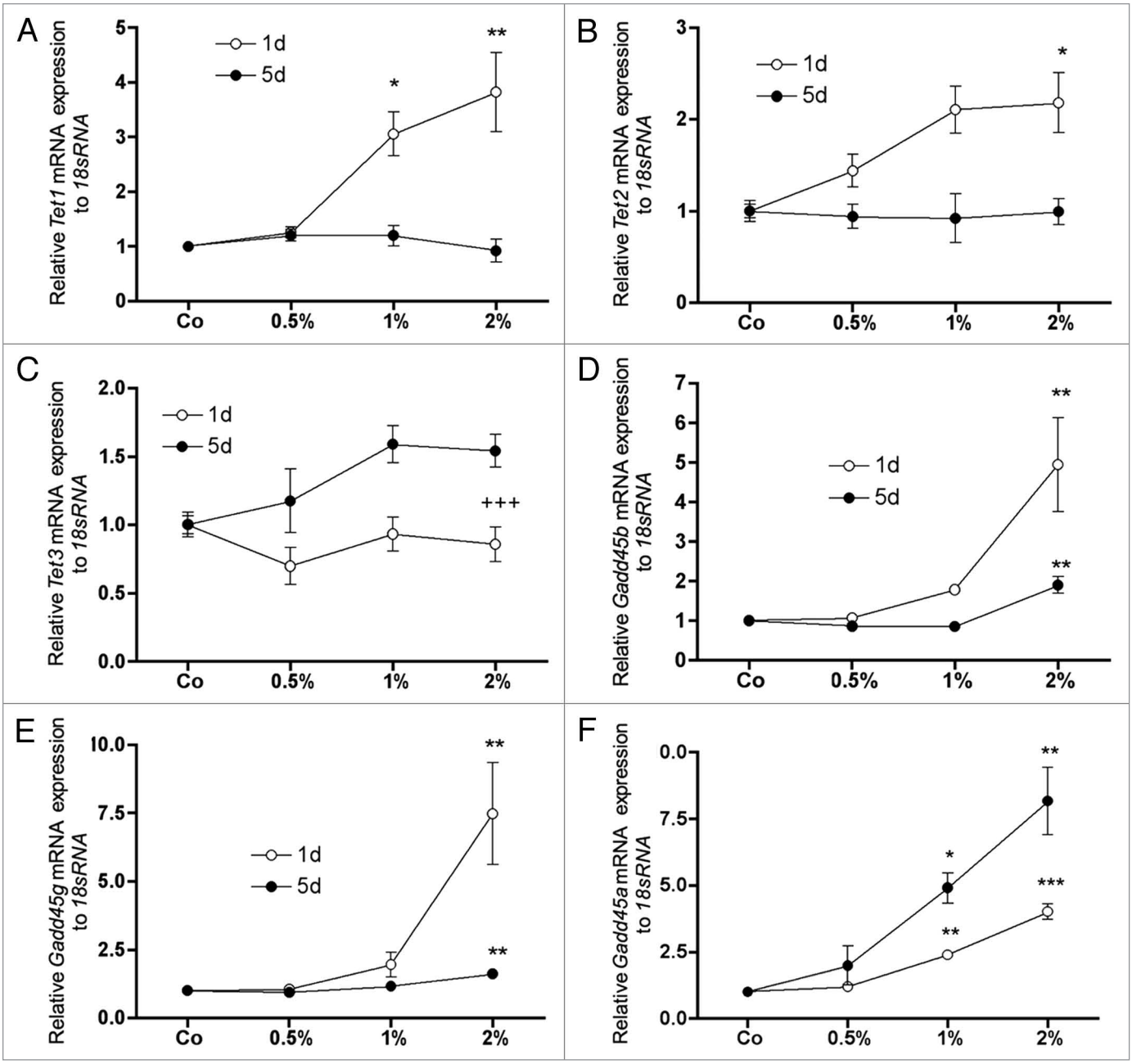

Figure 1. Effect of DMSO on the mRNA expression of genes involved in active DNA demethylation. After 1 d, DMSO strongly induced the expressions of *Tet1* (A) and *Tet2* (B) in a concentration dependent manner. Expression of *Tet3* was not affected at this time (C). Concomitantly, for *Gadd45b* (D) and *Gadd45g* (E) at 2% of DMSO and for *Gadd45a* (F) already at 1% DMSO in culture medium a significant upregulation was measured. After 5 d the effect was depleted or reduced except for *Tet3* (C) and *Gadd45a* (F). At this time expression of *Tet3* was significantly increased when compared with day 1. An even stronger effect was seen for *Gadd45a* expression after 5 d. To analyze mRNA expressions, RNA was isolated and analyzed by qRT-PCR. Gene expressions were normalized to *18S* rRNA. Treated probes are referred as fold change to untreated control (Co). Results are represented as mean ± SD *p < 0.05, **p < 0.01, *** or $^{+++}$p < 0.001, n = 3. Significances were calculated by one-way ANOVA (*) or by two-way ANOVA (+).

the pre-osteoblastic MC3T3-E1 cell line expresses a phenotype which forms several cell layers and synthesizes huge amounts of collagen type I (COL1A1, COL2A2) containing extra cellular matrix,[28,29] which mineralizes at late differentiation stages.[30-32] Recently it was demonstrated that at late differentiation stages, DMSO enhances mineralization of the extra cellular matrix by the osteoblastic MC3T3-E1 cell line.[33]

Using the MC3T3-E1 cell line we report the ability of DMSO to induce active DNA demethylation. We demonstrate that via CpG hydroxymethylation DMSO affects promoter methylation of *Fas* and *Dlx5* as well as global methylation patterns. This has an impact on cell viability and, as suggested by genome wide mRNA expression analysis, this compound may induce matrix synthesis in MC3T3-E1 cells.

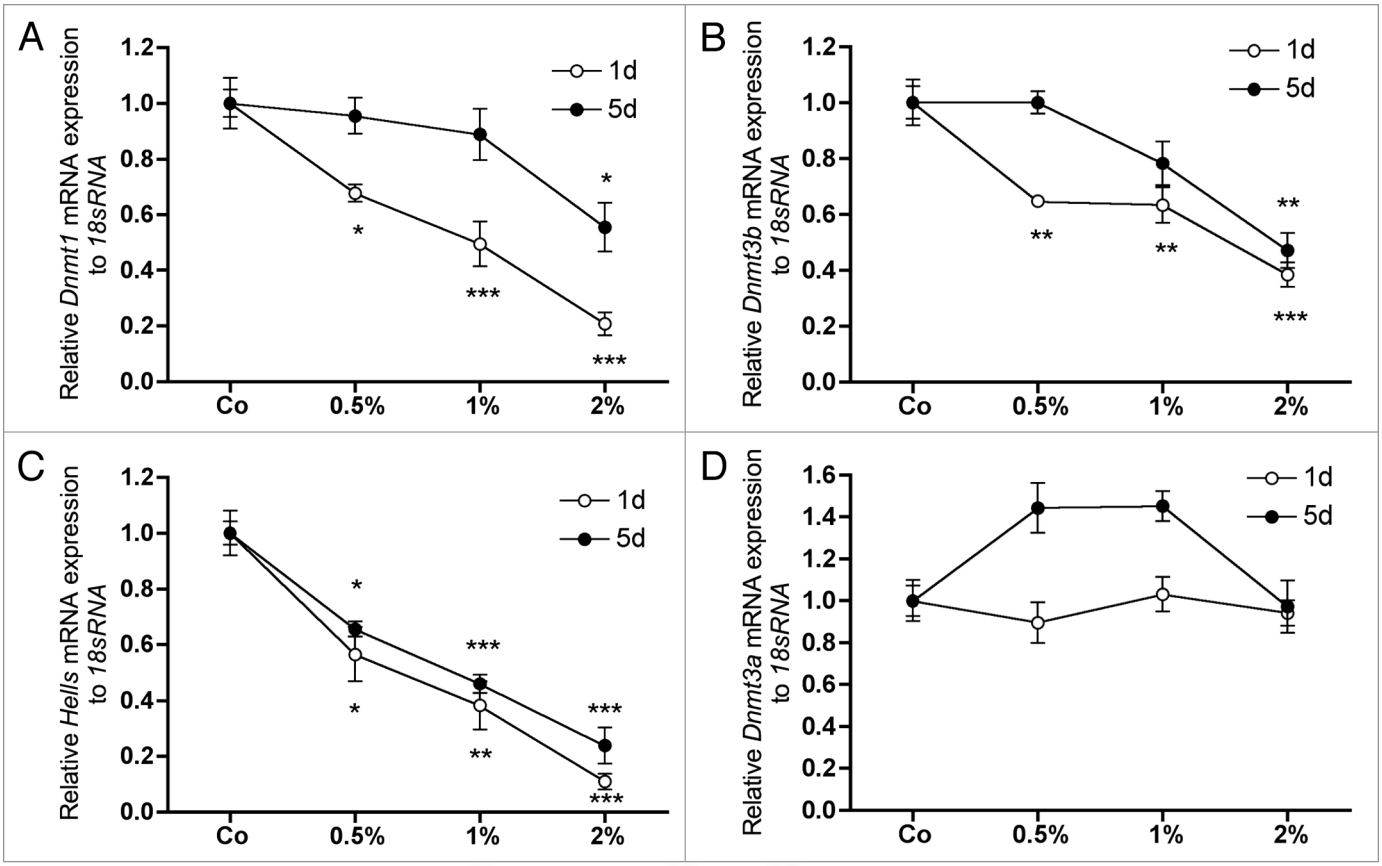

Figure 2. Effect of DMSO on the mRNA expression of genes involved in DNA methylation. mRNA expression of *Dnmt1* (A), *Dnmt3b* (B) and *Hells* (C) showed a strong, DMSO-concentration dependent downregulation after 1 d of treatment. Also if the repression of *Hells* was after 5 d similar to day 1 (C), for all three genes a general alleviation was observed at this time (A–C). No significant effects could be measured for *Dnmt3a* expression at these times (D). To analyze mRNA expressions, RNA was isolated and analyzed by qRT-PCR. Gene expressions were normalized to *18S* rRNA. Treated probes are referred as fold change to untreated control (Co). Results are represented as mean ± SD *p < 0.05, **p < 0.01, ***p < 0.001, n = 3. Significances were calculated by one-way ANOVA.

Results

DMSO simultaneously affects the mRNA expression of genes involved in active DNA demethylation and DNA methylation processes. qRT-PCR analyses have shown that DMSO, particularly after 1 day of treatment, strongly and dose dependently upregulated the mRNA expression of two members of the TET-oncogene family *Tet1* (Tet oncogene 1) and *Tet2* (Tet oncogene 2) while no regulation was found at day 5 (**Fig. 1A and B**). *Tet3*, the third member of this family, was not regulated at day 1 but was expressed significantly higher at day 5 (**Fig. 1C**).

At both time points, DMSO significantly increased expression of all members of the GADD45 (growth arrest and DNA-damage-inducible 45) family. While DMSO stimulated *Gadd45b* and *Gadd45g* expression at day 1 (**Fig. 1D and E**) *Gadd45a* was higher expressed at day 5 compared with day 1 (**Fig. 1F**).

At the same time the mRNA expression of *Dnmt1, Dnmt3b* and *Hells* was downregulated in a concentration dependent manner (**Fig. 2A–C**). At day 5, however, attenuation of the effect was observed for most of the listed genes (**Figs. 2 and 3**). Interestingly, only a marginal regulation of the mRNA expression of *Dnmt3a* could be observed at any time analyzed (**Fig. 2D**).

Effects of DMSO on the mRNA expression of *Fas* and *Dlx5*. DNA methylation dependent regulation of the pro-apoptotic gene *Fas* in MC3T3-E1 has previously been shown in reference 34. Furthermore, *Gadd45a* dependent demethylation of the early osteoblastic differentiation factor *Dlx5* has recently been demonstrated in reference 35. After 1 d of DMSO treatment, *Fas* expression was clearly dose-dependently upregulated. However, a significant inverse effect was observed after 5 d of treatment (**Fig. 3A**). Further, after 1 d of DMSO treatment, we also observed a strong and concentration dependent upregulation of *Dlx5* (**Fig. 3B**). After 5 d this effect on *Dlx5* expression by DMSO disappeared (**Fig. 3B**).

Time course of DMSO modulated gene expression. To narrow the timeframe of the expression of genes, which are suggested being involved in the active demethylation process, MC3T3-E1 cells were treated with 1% DMSO for 1, 2, 3, 3, 4, 5 and 10 d. Compared with controls, upregulation for *Tet1* was significant on day 1 while on day 2 only a trend to stimulation was observed (**Fig. 4A**). *Tet2* was significantly upregulated on day 1 and 2 (**Fig. 4B**). No regulation was found for *Tet1* and *Tet2* until day 10 (**Fig. 4A and B**). Similarly, *Gadd45b* was strongly upregulated only at day 1, with no significant regulation during the following

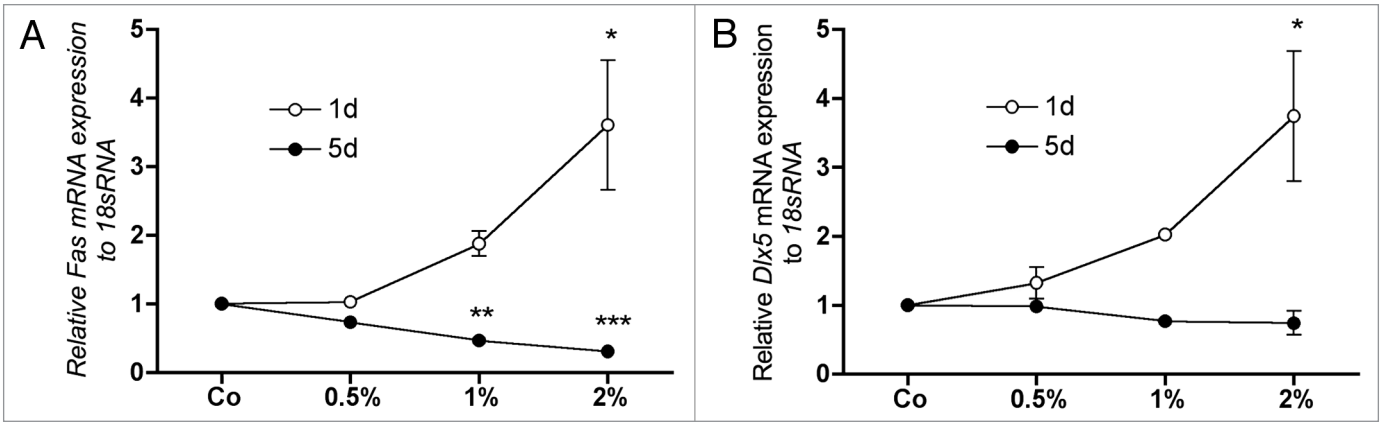

Figure 3. The impact of DMSO treatment on the mRNA expression of *Fas* and *Dlx5* in MC3T3-E1 cells. mRNA expression of the apoptosis inducer *Fas* was clearly and dose dependently upregulated after 1 d of treatment. After 5 d, the effect was reversed (A). The early osteoblastic differentiation factor *Dlx5* was concentration dependently upregulated by DMSO after 1 d whereby significance was reached at 2% DMSO in medium. At day 5, expression of *Dlx5* was generally unaffected by DMSO treatment. To analyze mRNA expressions, RNA was isolated and analyzed by qRT-PCR. Gene expressions were normalized to *18S* rRNA. Treated probes are referred as fold change to untreated control (Co). Results are represented as mean ± SD *p < 0.05, **p < 0.01, ***p < 0.001, n = 3. Significances were calculated by one-way ANOVA.

culture time (**Fig. 4C**). For *Dnmt1* a downregulation was found after 1 d of treatment to 18% while on day 2 only a trend to downregulation was observed. No regulation was seen until day 10 (**Fig. 4D**). DNMT1 protein expression was confirmed by immunoblotting (**Fig. 4E**).

The pro-apoptotic gene *Fas* (**Fig. 5A**) and the early osteoblastic differentiation factor *Dlx5* (**Fig. 5B**) were significantly upregulated during the first 3 d, however, while *Dlx5* was still significantly increased on day 3, *Fas* lost significance at that time point. Both genes showed no difference in expression until day 10 (**Fig. 5A and B**).

Decreased promoter CpG methylation of *Fas* and *Dlx5* is accompanied by increased DNA CpG hydroxymethylation after DMSO treatment. As *Tet1* and *Tet2* were upregulated and *Dnmt1* expression was suppressed by DMSO, we examined DNA hydroxymethylation and DNA methylation on *Fas* and *Dlx5* promoters after DMSO treatment. Already after 12 h of DMSO treatment, we measured a considerable increment of DNA 5-hydroxymethylation of CpGs of both *Fas* and *Dlx5* promoters (**Fig. 6A–D**, left bars). After 1 d, the effect increased slightly or remained in a comparable range as for the *Dlx5* proximal promoter region (**Fig. 6A–D**, middle bars). On day 5 no difference between treated and untreated cell were observed (**Fig. 6A–D**, right bars). Inversely, to elevated hydroxymethylation, all fragments analyzed showed significantly decreased DNA methylation levels already after 12 h of DMSO treatment (**Fig. 6E–H**, left bars). According to *Fas* and *Dlx5* mRNA expressions (as shown in **Fig. 3A and B**, respectively), after 1 d of DMSO treatment all promoter-fragments analyzed showed still significant cytosine demethylation when compared with untreated control (**Fig. 6E–H**, middle bars). A different methylation pattern was found after 5 d of DMSO-treatment: the *Fas* CpG island at -2.6 kb showed a slighter but still significant decrease in cytosine methylation by DMSO (**Fig. 6E**), however, in correlation with *Fas* mRNA repression (**Fig. 3A**) at position -30 bp cytosine methylation was found to be significantly increased when compared with controls (**Fig. 6F** and right bars). At this time no effect on cytosine methylation was measured for the *Dlx5* promoter (**Fig. 6G and H**).

DMSO increases global DNA hydroxymethylation and decreases global DNA methylation. Hydroxymethylation was quantified with a multiwell plate reader by measuring fluorescence of 5-hmC-antibody stained preparations. As shown in **Figure 7A**, already after 12 h of treatment, DMSO significantly increased global DNA hydroxymethylation in a concentration dependent manner; after 1 d of treatment this effect was even more pronounced (**Fig. 7B**), while on day 5 no significant difference was found (**Fig. 7C**). As consequence of DNA hydroxymethylation, we evaluated if active DNA demethylation occurred by DMSO treatment. For this purpose, cells were synchronized by serum depletion and subsequently treated for 12 h with DMSO. Indeed, already after 12 h of DMSO treatment and thus before first cell replication starts, a significant global demethylation of DNA could be observed and this effect became more evident after 1 d of treatment (**Fig. 7D**). Similar to *Dnmt1* expression, the demethylating effect of DMSO was clearly attenuated after 5 d of treatment and significantly reduced when compared with day 1 (**Fig. 7D**).

Nuclear localization of methyl-cytosine hydroxylation by DMSO in MC3T3-E1 cells. In **Figure 8A–D**, 5-hmC immunostaining after 12 h and 24 h of 1% DMSO treatment is shown. Compared with control (**Fig. 8A**), already after 12 h of treatment signals of 5-hmC were observable (**Fig. 8B**) whereby the effect clearly increased after 1 d (**Fig. 8C**). Co-staining with DAPI showed the localization of the nuclei and demonstrated the signals of 5-hmCs in the nuclei (**Fig. 8D**). Nuclear staining with DAPI, a dye which preferentially labels heterochromatic regions on the chromosomes,[36,37] revealed that most of the hydroxymethylated spots were found outside of these regions, suggesting that DMSO induced hydroxymethylation occurs primarily in the

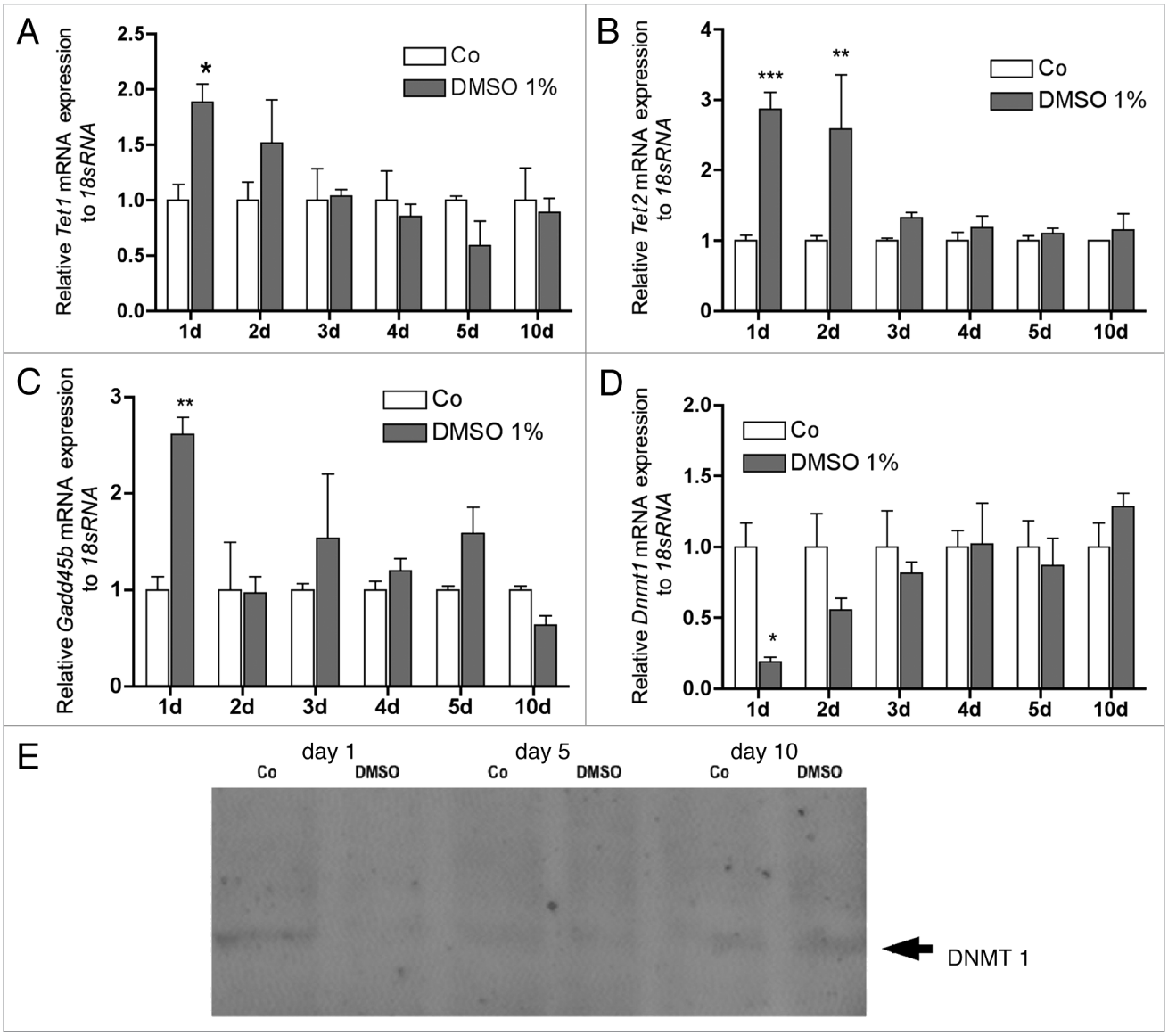

Figure 4. Time course for DMSO regulated expression of genes involved in active DNA demethylation. *Tet1* was significantly upregulated on day 1 while on day 2 the upregulation showed only a tendency. No regulation was found until day 10 (A). *Tet2* was significantly upregulated on the first both days but no regulation was found until day 10 (B). On the first day, *Gadd45b* was significantly upregulated (C), while *Dnmt1* was significantly downregulated (D). No regulation was found during the further culture time. Downregulation of DNMT1 was also found at the protein level (E). To analyze mRNA expressions, RNA was isolated and analyzed by qRT-PCR. Gene expressions were normalized to *18S* rRNA. Treated probes are referred as fold change to untreated control (Co). Results are represented as mean ± SD *p < 0.05, **p < 0.01, ***p < 0.001, n = 3. Significances were calculated by two-way ANOVA and Bonferroni post hoc test.

gene-rich euchromatic chromatin. Co-labeling of DMSO treated cells with antibodies against 5-hmC and 5-mC demonstrated areas where only 5-hmCs (green) or 5-mCs (red) were localized (**Fig. 8E**). However, also double-labeled regions were found (yellow) suggesting the co-existence of both, modified and unmodified methyl-cytosines.

***Tet1* and *Tet2* are key players in DMSO dependent demethylation and upregulation of *Fas* and *Dlx5*.** To assure the role of active DNA demethylation in DMSO induced *Fas* and *Dlx5* upregulation, *Tet1* and *Tet2* mRNA expression was knocked-down by siRNA. In line with promoter methylation analysis (see **Fig. 6**), already after 12 h of 1% DMSO treatment a significant upregulation of *Fas* mRNA expression could be detected. A similar increment was measured after 1 d of treatment. However, knockdown of *Tet1* or *Tet2* mRNA expression by siRNA abrogated the stimulatory effect at both times measured (**Fig. 9A**). Similar effects were observed when *Dlx5* mRNA expression was analyzed, although, stimulation of *Dlx5* expression was significantly increased not until 1 d of treatment (**Fig. 9B**). In **Figure 9C and D**, the efficacy of *Tet1* and *Tet2* knockdown by siRNA transfection after 1 d of 1% DMSO treatment is demonstrated. Finally, after 1 d of

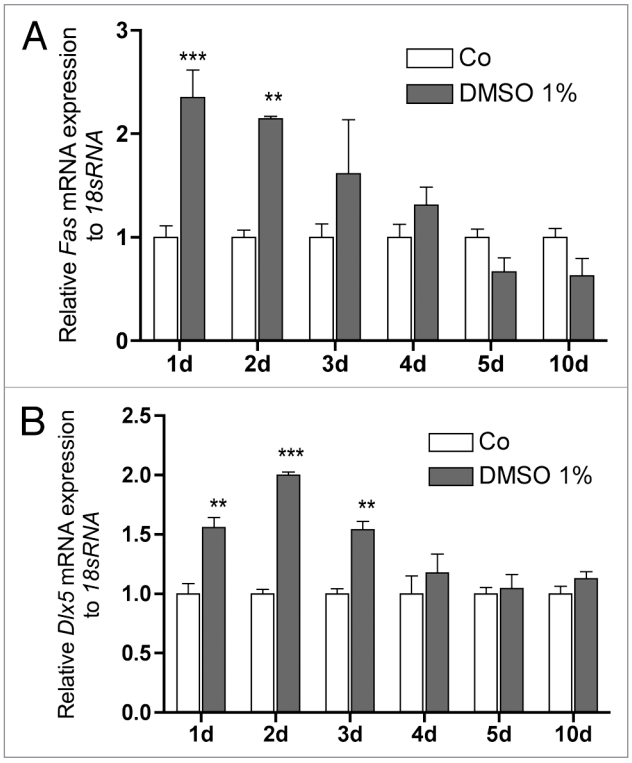

Figure 5. Time course for DMSO regulated expression of the proapoptotic *Fas* and the early osteoblastic cells transcription factor *Dlx5*. Both genes were significantly upregulated on the first both days. While on day 3 *Dlx5* (B) was still significantly upregulated *Fas* (A) showed only a tendency. No regulation was found during the further culture time. To analyze mRNA expressions, RNA was isolated and analyzed by qRT-PCR. Gene expressions were normalized to *18S* rRNA. Treated probes are referred as fold change to untreated control (Co). Results are represented as mean ± SD **p < 0.01, ***p < 0.001, n = 3. Significances were calculated by two-way ANOVA and Bonferroni post hoc test.

1% DMSO exposure, the effect of *Tet1* and *Tet2* knockdown was also visible on global 5-hmC status. Repression of *Tet1* and *Tet2* provoked a great attenuation of global 5-hmC levels when compared with cells treated only with DMSO (**Fig. 9E**). A diminution of 5-hmC labeled cells was also seen by immunestaining when 1% DMSO treated cells (**Fig. 10A**) were compared with 1% DMSO/Tet1-siRNA treated MC3T3-E1 cells (**Fig. 10B**). When compared with untreated control cells (see **Fig. 8A**), the number of 5-hmC labeled cells was still higher in 1% DMSO/Tet1-siRNA treated cells.

DMSO affects cell multiplication and the activity of caspase 8 and caspase 3/7 in MC3T3-E1 pre-osteoblasts. As *Fas* expression was altered by DMSO, cell proliferation and caspase activities were measured. DMSO, dose (**Fig. 11A**) and time (**Fig. 11B**) dependently regulated cell multiplication of MC3T3-E1 cells. At the second day of treatment, at 0.5% DMSO in culture medium, cell multiplication was increased significantly whereby at 2% a significant attenuation was observed (**Fig. 11A**). Cell multiplication was more pronounced after 5 d of treatment and significantly decreased by both 1% and 2% of DMSO (**Fig. 11B**).

FAS directly activates caspase 8 and indirectly caspase 3 and 7.[38,39] To verify the role of apoptosis in DMSO dependent attenuation of cell multiplication, caspase 8 and caspase 3/7 activities were measured after 1 d and after 5 d of DMSO treatment. In line with *Fas* expression (**Fig. 3A**), after 1 d both DMSO concentrations significantly increased caspase 3/7 (**Fig. 11C**) and caspase 8 (**Fig. 11D**) activities when compared with untreated controls. Furthermore, after 5 d of treatment the DMSO effect was clearly attenuated (**Fig. 11E and F**). Only at 2% DMSO in the culture medium a slight but significant increase of caspase 3/7 activity was still measured (**Fig. 11E**).

Figure 12 shows the time course of cell multiplication during 10 d of culture (**Fig. 12A**) and compares the ratio between living and dead cells (**Fig. 12B**). At the beginning of the culture, the difference in cell number increases indicating increased apoptosis in DMSO treated cells; the higher ratio of dead to living cells (**Fig. 12B**) on culture start supports this assumption. With culture time, the difference between untreated and DMSO treated cells increased until day 5. Then the differenced disappeared and the ratio of dead cells to living cells went below 1 in DMSO treated cells, which indicates a higher survival/dead rate after 10 d in the treated cells when compared with control cells. This results support the findings of *Fas* expression and caspase activities (**Figs. 3A and 11C–F**).

DMSO stimulates the expression of genes involved in ECM synthesis. *Dlx5* is expressed at early stages during osteoblastic differentiation where ECM synthesis is induced. As shown in **Table 1** gene array data analysis shows that DMSO upregulated the mRNA expression of both genes collagen type I, *Col1a1* and *Col1a2*, after five days of treatment. Furthermore, *Col5a1* and *Col5a2* were considerably expressed and strongly upregulated by DMSO at both days measured. Of the enzymes involved in post-translational collagen modifications, two of three known lysyl hydroxylases were expressed and upregulated by DMSO, especially *Plod2* (procollagen lysine, 2-oxoglutarate-5-dioxygenase 2), which had been demonstrated to be essential in bone collagen fibril formation.[40] The gene coding for *Lox*, a protein, which cross-links extracellularly the collagen I chains, is highly expressed and also upregulated by DMSO (**Table 1**). Furthermore, *Mmp13*, the

Figure 6 (See opposite page). In *Fas* as well as in *Dlx5* promoters CpG hydroxymethylation and methylation were affected by DMSO treatment. Specific CpG hydroxymethylation (A–D) and CpG methylation (E–H) were measured in selected promoter regions of *Fas* and *Dlx5* after treatment with 1% DMSO for the given periods. Already after 12 h of treatment, a strong increase in 5-hmC status and a concomitant decrease in CpG methylation was seen in both genes in all promoter fragments analyzed (A–H). After 1 d, the increase of 5-hmC status became significant for all fragments analyzed (A–D). Compared with 12 h, CpG demethylation decreased further in the CpG island of the *Fas* promoter at -2.6 kb from transcriptional start (E), remained unchanged in the *Dlx5* distal promoter fragment (G) and slightly diminished in the *Fas* CpG island at -30 bp from the transcriptional start (F) and in the *Dlx5* proximal promoter (H). After 5 d, the *Fas* promoter region at -2.6 kb still showed a significantly decreased CpG demethylation after DMSO treatment (E). However, the *Fas* promoter region at -30 bp even showed a highly significant increase in CpG methylation. No changes in CpG methylation status were observed for both *Dlx5* promoter fragments after 5 d of treatment. Results are represented as mean ± SD *p < 0.05, **p < 0.01, ***p < 0.001, for all n = 3, all significances were calculated by Student's t-test.

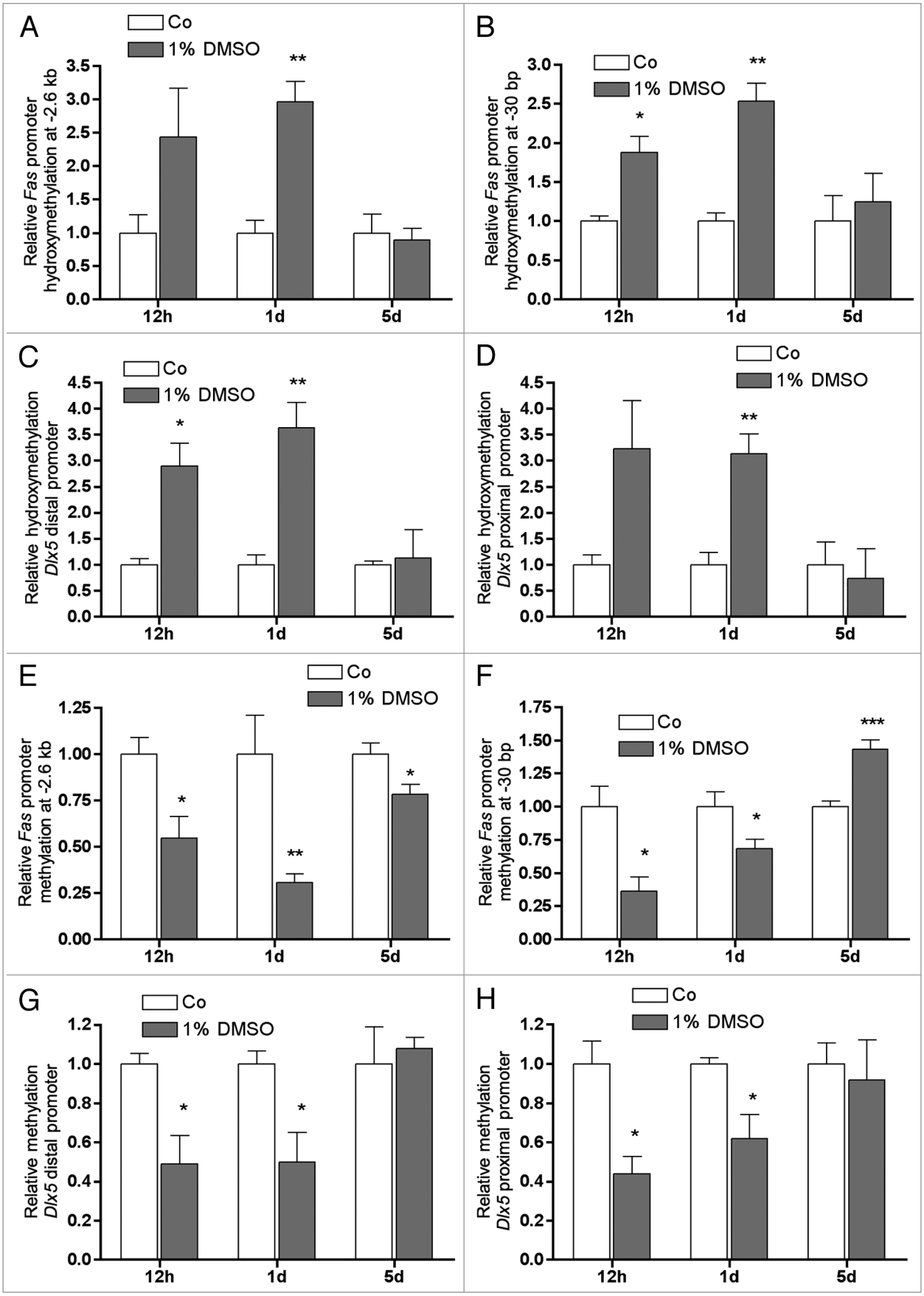

Figure 6. For figure legend, see page 640.

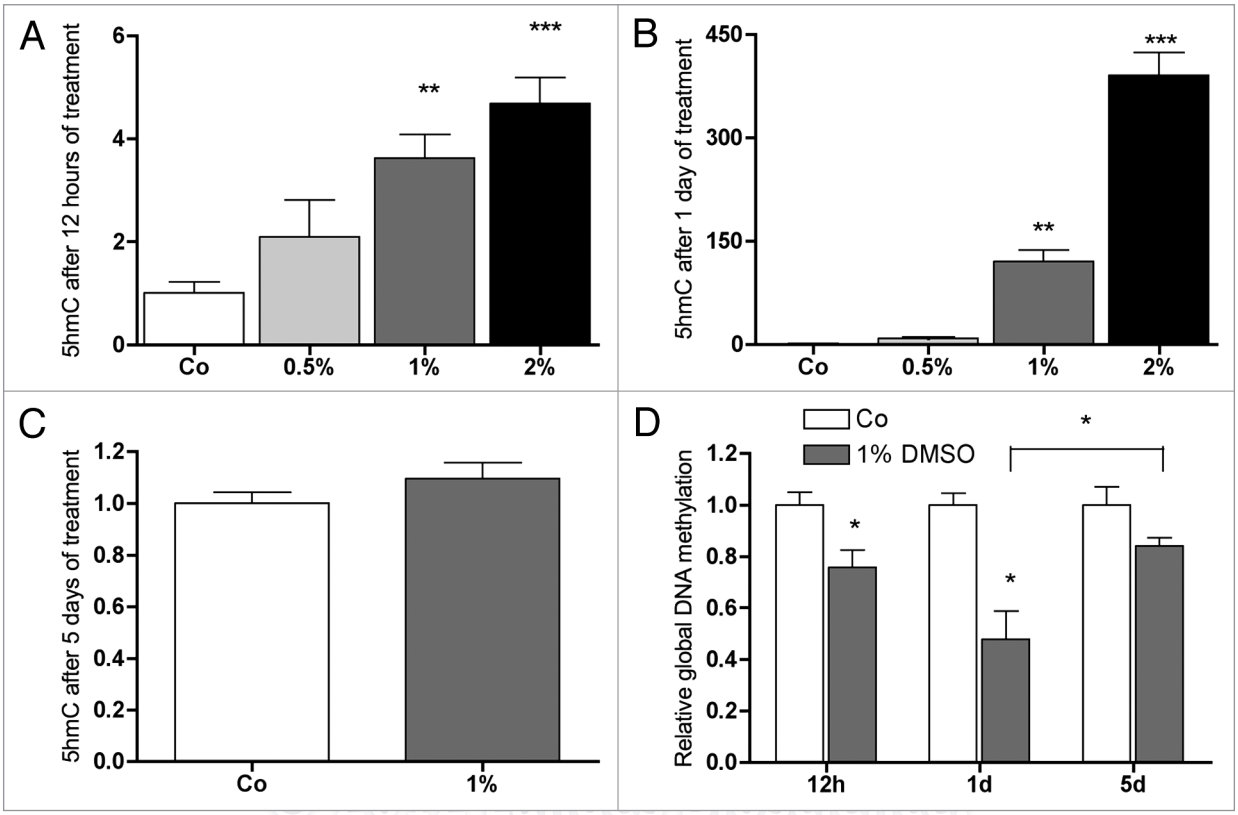

Figure 7. DMSO stimulates global 5-mC hydroxylation and decreases global DNA methylation in MC3T3-E1 cells. Already after 12 h, a concentration dependent increase in 5-hmC was seen reaching significance at 1% and 2% DMSO in culture medium (A). At the same time, at 1% DMSO, global DNA methylation decreased significantly (D). After 1 d, the effect on global hydroxylation of 5-mC as well as on global DNA methylation was stronger (B and D). After 5 d, when compared with control, at 1% DMSO in medium no significant effect on global DNA demethylation was measured. The recovery in global DNA methylation in the DMSO treated cells between day 1 and day 5 was significant (D). Results are represented as mean ± SD *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. For (A and B) n = 6, for (C) n = 3. For (A and B) significances were calculated by one-way ANOVA, for (C) significances were assessed by Student's t-test.

most important osteoblastic metalloproteinase was upregulated at day 1 and strongly downregulated at day 5.

Discussion

DMSO, a polar aprotic solvent widely used in biological and medical applications, was found to regulate epigenetic mechanisms altering CpG methylation patterns in diverse cells and tissues[15,41,42] thus having an impact on their development and differentiation.[17] Here, we show that in pre-osteoblastic MC3T3-E1 cells DMSO significantly affects the expression of genes related to DNA hydroxymethylation and DNA methylation. This has consequences on the expression of *Fas* and *Dlx5*.

Expression of TET and GADD45 gene family members were significantly upregulated and genes involved in DNA methylation were significantly downregulated after 1 d of treatment with 1% DMSO. Furthermore, at this time the expression of the pro-apoptotic gene *Fas* and of the early osteoblastic differentiation factor *Dlx5* was also increased by DMSO treatment. It has been demonstrated that epigenetic regulation of *Fas* expression via DNA methylation plays an important role in tumor development[43-46] as well as in avoiding anoikis of osteoblasts.[34] In regard to regulation of *Dlx5* expression, *Gadd45a* dependent active demethylation has recently been demonstrated.[35]

Recently, an active DNA-demethylation process has been shown by oxidative modification of 5-mCs to 5-hmCs by members of the TET gene family with further involvement of the GADD45 gene family, which is responsible for nucleotide and base excision repair (reviewed in refs. 1, 10 and 47–49). This mechanism was suggested for active DNA demethylation in *Xenopus laevis* oocytes[7] and in embryos of zebrafish.[8] In adult neuronal tissue (cortex, hippocampus) activation and demethylation of promoters of specific neuronal genes with concomitant upregulation of *Gadd45b* and *Gadd45c* has been demonstrated.[50]

Promoter DNA methylation is achieved by DNMTs, a highly conserved gene family. *Dnmt1* is related to maintenance of CpG methylation patterns during mitosis while *Dnmt3a* and *Dnmt3b* play a role in de novo DNA-methylation.[51] HELLS was shown to act as DNA methylation supporter by recruiting DNMT1 and DNMT3B to promoters.[52] Moreover, *Hells* has been shown to play an essential role in bone development.[53] Downregulation of these proteins results in passive DNA-demethylation leading to activation of gene transcription. DMSO strongly downregulated the expression of *Dnmt1*, *Dnmt3b* and *Hells* after

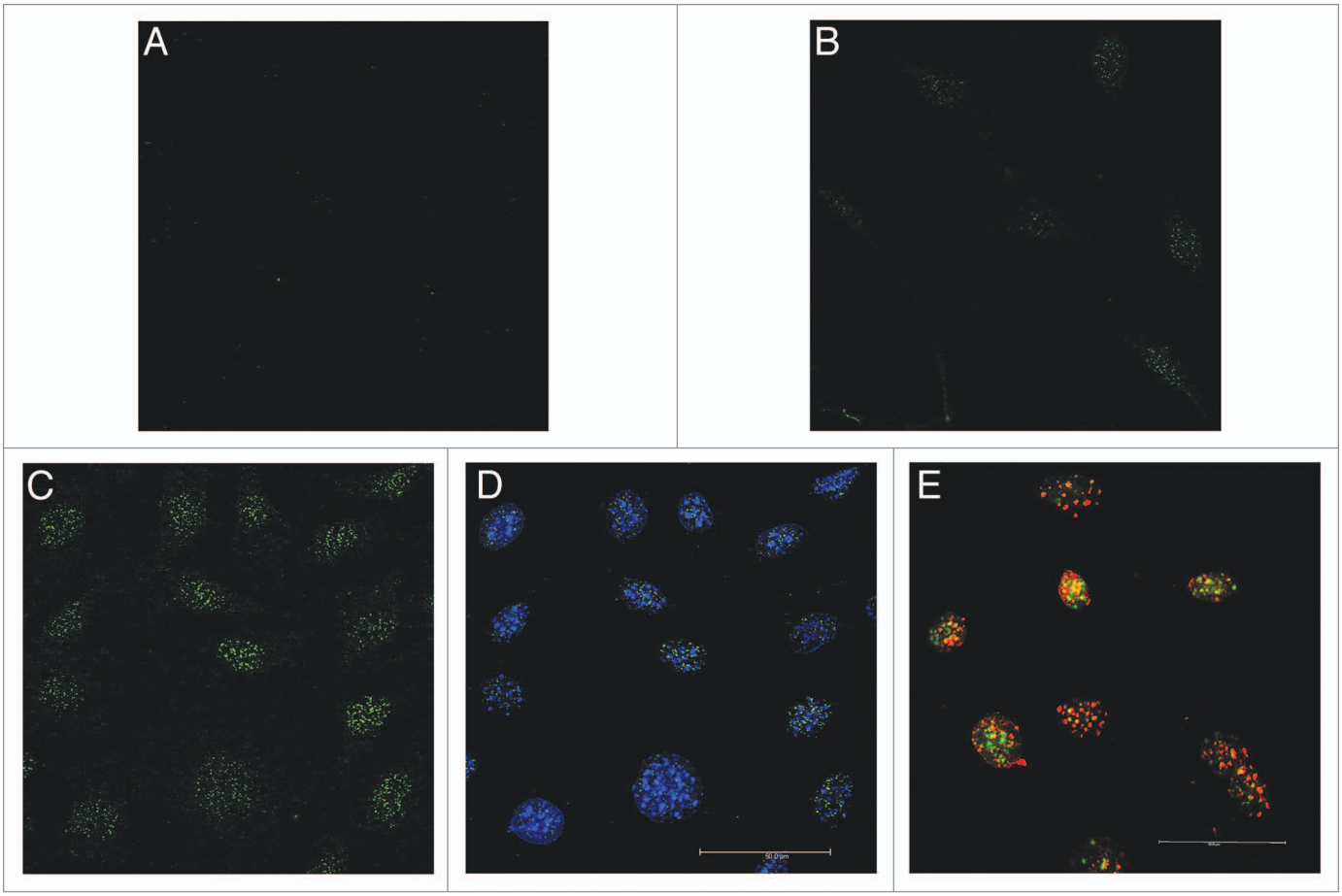

Figure 8. Nuclear localization of 5-hmC (green punctuation) after 1% DMSO treatment in MC3T3-E1 cells. The increase in 5-hmC was demonstrated and localized by immune-staining techniques. When compared with control (A), an increase in 5-hmC was already visible after 12 h of 1% DMSO treatment (B) and after 1 d (C) the effect was clearly more pronounced. Combined staining with DAPI demonstrated the signals of 5-hmCs in the nuclei and revealed that most of the hydroxymethylated spots were found outside of the heterochromatic regions (D). Co-labeling of DMSO treated cells with 5-mC (red spots) and 5-hmC (green spots) antibodies revealed areas where only 5-hmCs or 5-mCs are localized. However, also double-labeled regions were found (yellow spots, E). Scale bar for all images is 50 μm.

one day of treatment. Therefore, passive DNA demethylation of *Fas* and *Dlx5* promoters is feasible. However, after seeding, MC3T3-E1 cells need at least 24–30 h for doubling and *Fas* as well as *Dlx5* expression were already increased 12 h after seeding with concomitant DMSO treatment. Thus the data suggested an alternative mechanism for *Fas* and *Dlx5* upregulation. Indeed, already after 12 h of treatment increased 5-hmC and decreased in 5-mC levels were measured on both, *Fas* and *Dlx5* promoters and global 5-hmC levels were also significantly increased at this time suggesting active DNA demethylation. Furthermore, cell synchronization by serum depletion confirmed loss of DNA methylation by DMSO treatment before first cell replication cycle. A further argument for active DNA demethylation is that by DMSO induced global hydroxymethylation and *Fas* and *Dlx5* upregulation could be prevented by *Tet1*-siRNA or *Tet2*-siRNA knockdown proving *Tet1* and *Tet2* involvement in this process. In our experiments, downregulation of either *Tet1* or *Tet2* was sufficient to attenuate upregulated *Dlx5* and *Fas* and to prevent global hydroxymethylation. This suggests that in this case both gene products are a prerequisite for the demethylation process. This is in contrast to recent reports, which have been performed in embryonic stem cells that have demonstrated rather an additive effect of both genes on 5-hmC formations.[54] However, in myeloid malignancies it has been demonstrated that despite an expression of wild type TET1 and TET3, an inactivating mutation of TET2 is sufficient to block active demethylation and thus to induce these diseases.[55-57] This suggests that TET1 or TET3 cannot replace the function of TET2.

In embryonic stem cells TET1 protein and 5-hmCs are enriched at regions with high density of CpGs around the TSSs[54,58,59] and requirement of *Tet1* for active DNA demethylation in the adult mouse brain in vivo has been reported recently. There, after deamination of 5-hmC by the *Aicda* (AID, activation-induced deaminase)/*Apobec* (apolipoprotein B mRNA-editing enzyme complex) gene family, 5 hmUs are removed by base excision repair by *Smug1* (single-strand-selective monofunctional uracil-DNA glycosylase 1) and *Tdg* (thymine-DNA glycosylase).[60] However, as found by genome wide expression analysis, in MC3T3-E1 cells these genes were either only weakly expressed or were downregulated by DMSO (**Table S1**), in contrast to

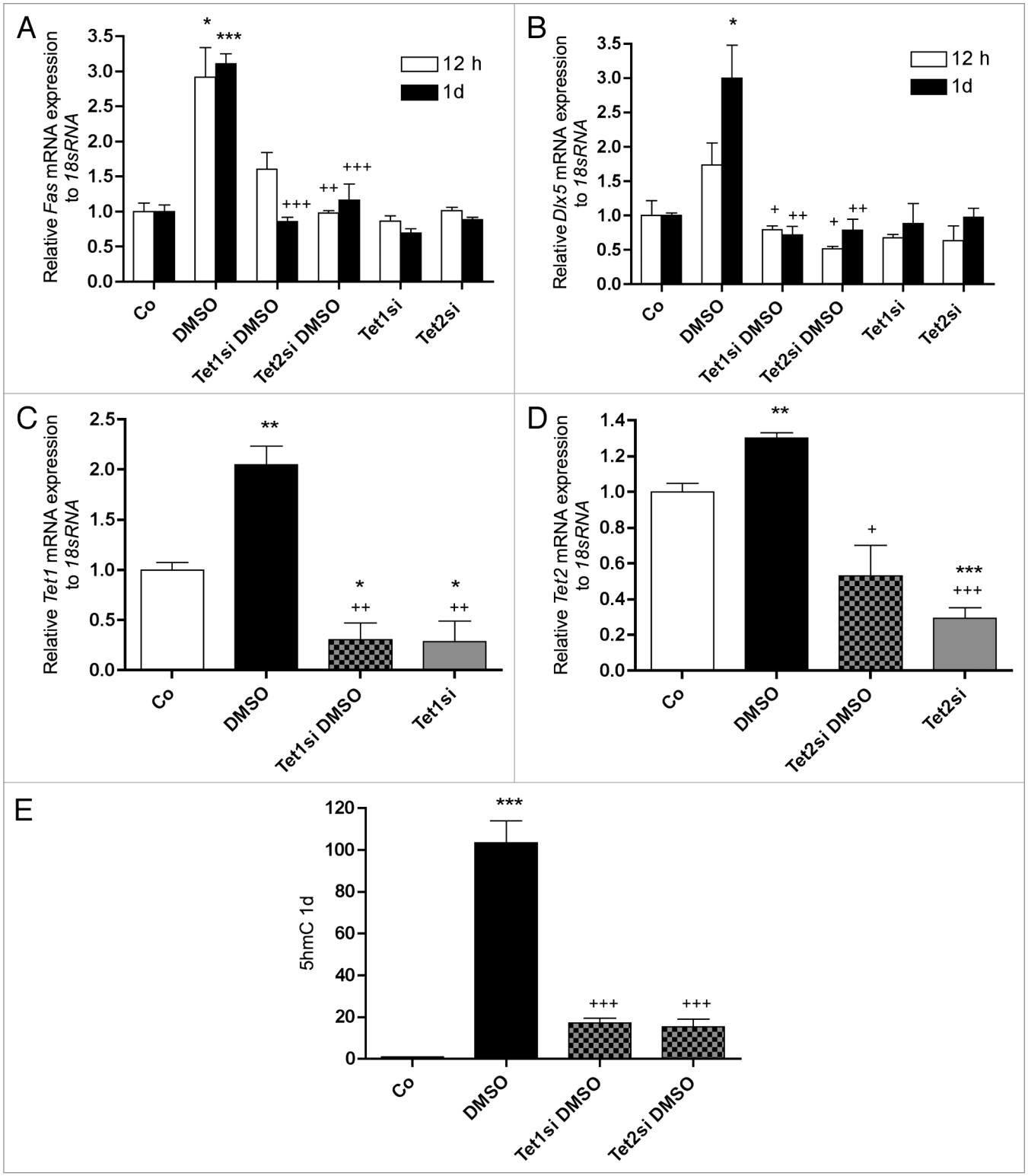

Figure 9. *Fas* and *Dlx5* expression and global 5-hmC status after *Tet1* and *Tet2* knock down by siRNA. mRNA expression of *Fas* (A) was already significantly increased after 12 h of 1% DMSO treatment whereby the *Dlx5* expression (B) showed only a trend. After 1 d, 1% DMSO significantly increased the expressions of both genes. Upregulation of *Fas* (A) as well as of *Dlx5* (B) mRNA expression by 1% DMSO was depleted at both times measured by siRNA knock down of *Tet1* or *Tet2* expression. Sole treatment of the cells with *Tet1* or *Tet2* siRNA had no effect on the expression of both genes. Efficiency of *Tet1* mRNA depletion by *Tet1* and *Tet2* siRNA is shown in (C and D), respectively. Effect of *Tet1* or of *Tet2* knock down was measurable on global 5-hmC level (E). When compared with 1% DMSO treated cells suppression of *Tet1* or *Tet2* expression clearly decreased 5-hmC levels (E). To analyze mRNA expressions, RNA was isolated and analyzed by qRT-PCR. Gene expressions were normalized to *18S* rRNA. Treated probes are referred as fold change to untreated control (Co). Results are represented as mean ± SD * or $^{+}p < 0.05$; ** or $^{++}p < 0.01$; *** or $^{+++}p < 0.001$; n = 3. + refers to DMSO treated samples. Significances were calculated by Student's t-test.

genes of the GADD45-family. Additionally, most members of the ERCC-family (excision repair cross-complementing rodent repair deficiency, complementation group), especially *Ercc3, Ercc4 and Ercc5* (*Xpg*), were expressed and upregulated by DMSO (**Table S1**). These data suggest that instead of base excision repair, nucleotide excision repair is possibly responsible for continuing demethylation after hydroxymethylation in DMSO treated MC3T3-E1 cells.

In a very recent study, it has been shown that during osteoblastic differentiation of adipose-derived mesenchymal stem cells, GADD45A was upregulated and responsible for active demethylation of osteogenic genes with support of members of the *ERCC* gene family via nucleotide excision repair.[35] Here we show that expression of *Gadd45a* was continuously upregulated during culture time and stimulated by DMSO suggesting that a comparable demethylation process could occur driving further differentiation. Because this demethylation process proceeds via excision of methylated nucleotides (ERCC-driven), no hydroxymethylation will be found. Both genes are discussed for their role in nucleotide excision repair, the former by an ERCC-family mediated process, the latter by a TET-family mediated oxidative demethylation.[35,47]

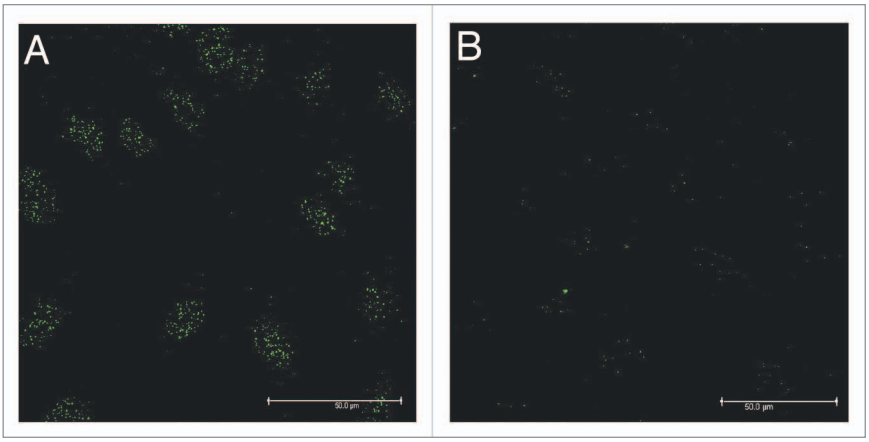

Figure 10. A diminution of 5-hmC labeled cells was also seen by immune-staining when 1% DMSO treated cells (A) were compared with 1% DMSO/Tet1-siRNA treated MC3T3-E1 cells (B). MC3T3-E1 cells were treated for 1 d either with 1% DMSO without or with *Tet1* si-RNA. Thereafter cells were fixed and incubate with anti-5-hmC antibody, followed by incubation with a fluorescence labeled secondary antibody. Scale bar for all images is 50 μm.

However, DMSO also upregulated *Gadd45b* already at day one, but downregulated on day 5. This can be explained by the fact that respective cells have already undergone apoptosis as a consequence of promoter demethylation in the first 2 d of incubation.

Taken together, these findings suggest that DMSO, after *Tet1* and/or *Tet2* induced hydroxymethylation, finalizes active demethylation of *Fas* and *Dlx5* promoters by nucleotide excision repair involving GADD45B. A second process of active demethylation for osteoblastic cells differentiation runs in parallel via ERCC-family and GADD45A.

In line with a very recent report in reference 13, we show that 5-hmCs were found solely in euchromatic regions of the chromatin suggesting that hydroxymethylation occurs mainly in euchromatin for the purpose of gene activation as shown for *Dlx5* and *Fas*. Moreover, these findings indicate that either hydroxymethylation is specific for euchromatin or at least that DMSO regulates demethylation solely in transcribed chromatic areas. It has to be taken into account that 5-hmC levels were assessed by immune-staining techniques and the considerable difference measured between DMSO treated and control cells may be overestimated by the almost total absence of 5-hmCs labels in untreated cells.

The effect of DMSO on the expression of *Fas* provoked attenuation of cell multiplication and induction of apoptosis as shown by the initially increased caspases 3/7 and caspase 8 activities. In parallel DMSO upregulated the early osteoblastic transcription factor *Dlx5* suggesting stimulation of the early osteoblastic differentiation stage.[23-27] In vivo, osteoblasts differentiate either to bone lining cells or to osteocytes; about two-thirds undergo apoptosis.[61] After 5 d of DMSO treatment *Fas* was downregulated and the effect on *Dlx5* expression disappeared. However, at this time genes involved in synthesis of extra cellular matrix like *Col1a1, Lox* or *Plod2* were upregulated. The heterogeneity of the pre-osteoblastic MC3T3-E1 cell line was previously shown in references 32 and 62. Considering these data, we interpret that DMSO initially induces a sub-population of MC3T3-E1 to apoptose. The surviving cells, possibly triggered by *Dlx5*, express genes related ECM synthesis that would generate greater amounts of collagenous ECM, which by a feedback mechanism supports osteoblastic survival[34] and constitutes the basis for an appropriate ECM mineralization. This suggestion fits with the recently published work by Stephens et al. where it was demonstrated that after long time exposure to DMSO (15 d), mineralization substantially increases in MC3T3-E1 cells. Interestingly, for this increased mineralization process, DMSO treatments during the first three days were crucial and sufficient, then the cultures lose their DMSO sensitivity (between day 3 and 6).[33] This unambiguously indicates that during the first culture period, future development of the cells is determined. Here we demonstrate that for this early culture period, the DMSO induced demethylation is limited in time. Timely coincidence strongly suggests that this DMSO induced demethylation is the trigger for the latter found increased mineralization of the culture, likely by increased collagen synthesis. Increased collagen synthesis could also be responsible for the convergence of the cell multiplication at day 10 and downregulation of *Fas*. ECM increases Cyclin A2 (*Ccna2*) expression and downregulates *Fas* by epigenetic promoter methylation[34] and takes over the regulation for the further differentiation of osteoblasts.[63] Reduced apoptosis and increased cell proliferation is also suggested by the decrease in the dead/live ratio at day 10.

As already mentioned above, on day 5 an increased methylation of *Fas* was found. This was found in parallel to a DMSO induced, although insignificant upregulation of *Dnmt3a*. This

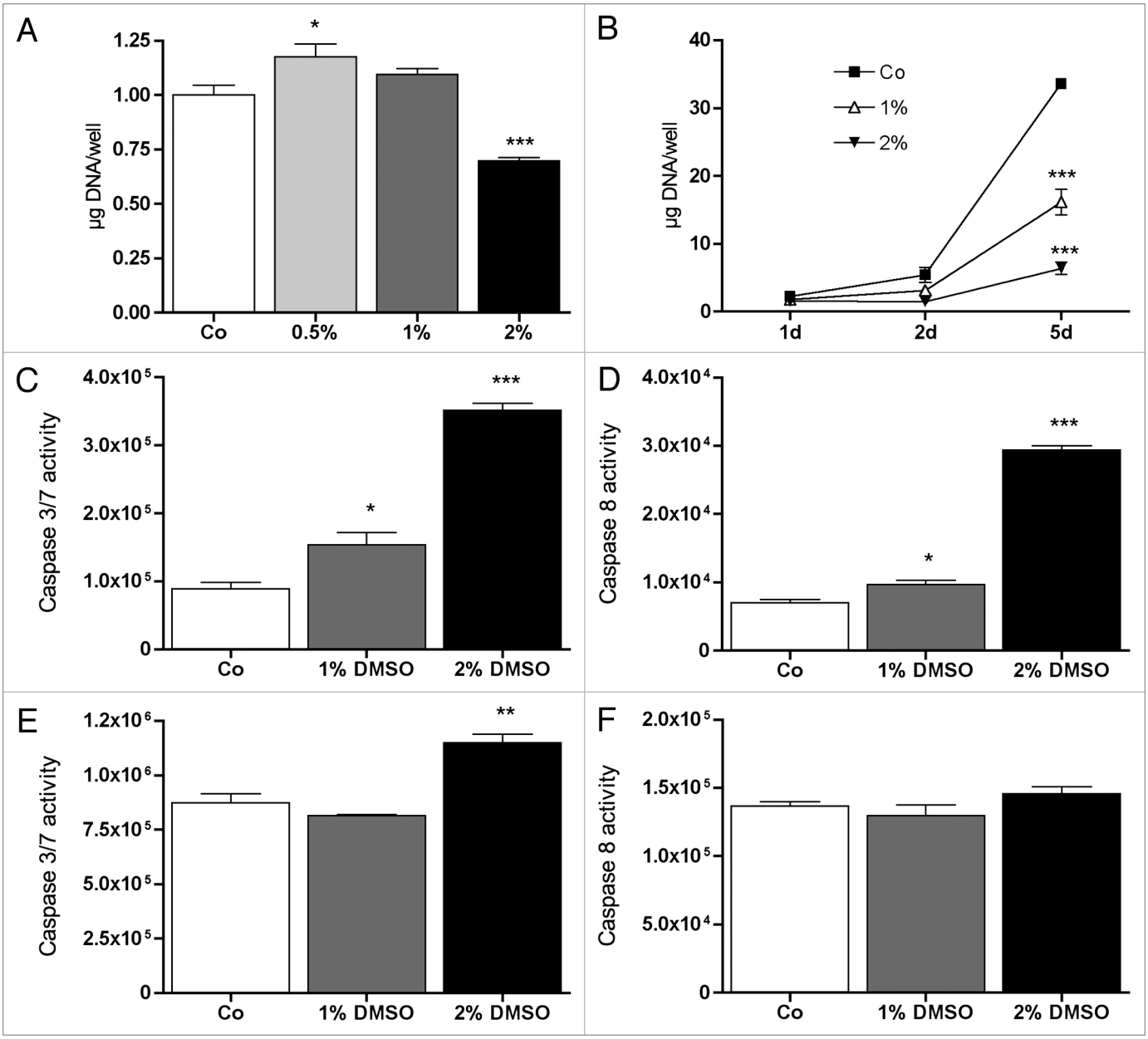

Figure 11. Effect of DMSO on cell multiplication and caspases 3/7 and caspase 8 activities after DMSO treatment. After 2 d of treatment with 0.5% DMSO in medium, cell multiplication was slightly but significantly increased while at 2% DMSO a significant decrease in cell multiplication was measured (A). Cell multiplication was significantly attenuated after 5 d of treatment for both 1% and 2% of DMSO in medium (B). After 1 d, caspase 3/7 (C) as well as caspase 8 (D) activities were significantly upregulated at 1% as well as at 2% DMSO in culture medium when compared with untreated control (Co). After 5 d treatment the activity of caspase 8 did not change significantly (F). Caspases 3/7 were still increased at 2% DMSO while at 1% no significant difference was found (E) when compared with untreated control (Co). Relative cell multiplication is expressed as µg DNA/well. Caspases activities were measured by substrate cleavage and the generated luminescent signal was captured with a luminometer. Bars represent mean ± SD *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. For (A and B) n = 8, for all other graphs n = 5. Significances were calculated by one-way ANOVA.

gene is obligatory for proper methylation during hematopoiesis[64] und has been found upregulated by DMSO in mouse embryoid body.[15] Furthermore, an enzymatic stimulation of *Dnmt3a* at higher concentrations was found in an in vitro assay.[21] One might speculate that an upregulation of *Dnmt3a* or a stimulation of the enzymatic activity could be responsible for *Fas* de novo methylation.

Furthermore, as *Tet1* was shown to be required for ESC cell maintenance,[14] here it may be responsible for the presumed retention of an early ECM producing osteoblastic phenotype via *Dlx5* by DMSO. However, a novel feature of this study is provided from data indicating that apoptosis can be regulated by active DNA demethylation, thus revealing a new approach for the understanding of cell death regulation. The role of DNA

hydroxymethylation in osteoblastic differentiation has still to be elucidated.

In summary, the results presented assign new aspects to DMSO as epigenetic active drug. We show for the first time a chemical compound capable to induce methyl-cytosine hydroxylation, which results in active demethylation of *Fas* and *Dlx5* promoters. This offers a role for DMSO as source for the development of compounds able to induce specific active demethylation of promoters, as in the past for the development of defined histone deacetylase inhibitors.[17] This could gain clinical importance for the therapy of diseases or/and for tumor treatment and help to understand physiological and pathophysiological mechanisms.

Materials and Methods

Cell culture. MC3T3-E1 cells, a clonal pre-osteoblastic cell line derived from newborn mouse calvaria (kindly donated by Dr. Kumegawa, Meikai University, Department of Oral Anatomy), were cultured in humidified air under 5% CO_2 at 37°C in α-minimum essential medium (α-MEM; Biochrom) supplemented with 5% fetal calf serum (Biochrom), 50 μg/mL ascorbic acid (Sigma), and 10 μg/mL gentamycin (Sigma). For propagation, cells were subcultured twice a week using 0.001% pronase E (Roche) and 0.02% EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) before achieving confluence. To prevent a potential phenotypic drift during repeated sub-cultures the cells were not used more than 4 weeks after thawing.

For experiments, the cells were seeded in culture dishes at a density of 5,000/cm^2 and cultured for 12 h, 1, 2 and 5 d untreated as controls or in the presence of 0.5%, 1% or 2% of DMSO in culture medium. Cell synchronization was performed by serum starvation for 24 h. All cell experiments were performed in time-independent triplicates, data are shown as mean of the three experiments.

Isolation of nucleic acids and expression analysis by qRT-PCR. DNA and RNA were extracted using a DNA/RNA Isolation Kit (Qiagen) following manufacturer's instructions. cDNA was synthesized from 0.5 μg RNA using the 1st Strand cDNA Synthesis Kit (Roche) as described by the supplier. The obtained cDNA was subjected to PCR amplification with a real-time cycler using FastStart SYBR-Green Master Mix (Roche) for the genes *Dlx5, Fas, Tet1, Tet2, Tet3, Gadd45a, Gadd45b, Gadd45g, Dnmt1, Dnmt3a, Dnmt3b and Hells* (primers are shown in **Table 2A**). The qRT-PCR was performed with 45 cycles composed of 30 sec denaturation at 95°C, 30 sec annealing at the indicated temperature (**Table 2A**) and 30 sec extension at 72°C after 10 min of initial denaturation at 95°C. For normalization of the expression we used the *18S* RNA TaqMan probe (4319413E, Applied Biosystems). Expression was quantified using the comparative quantization method.[65]

Affymetrix GeneChip analysis. Total RNA was isolated using a RNA Isolation Kit (Qiagen). Quality control of the RNA's as well as labeling, hybridization and scanning of the hybridized arrays was performed by the "Kompetenzzentrum fuer Fluoreszente Bioanalytik (KFB)" using the mouse 430 2.0

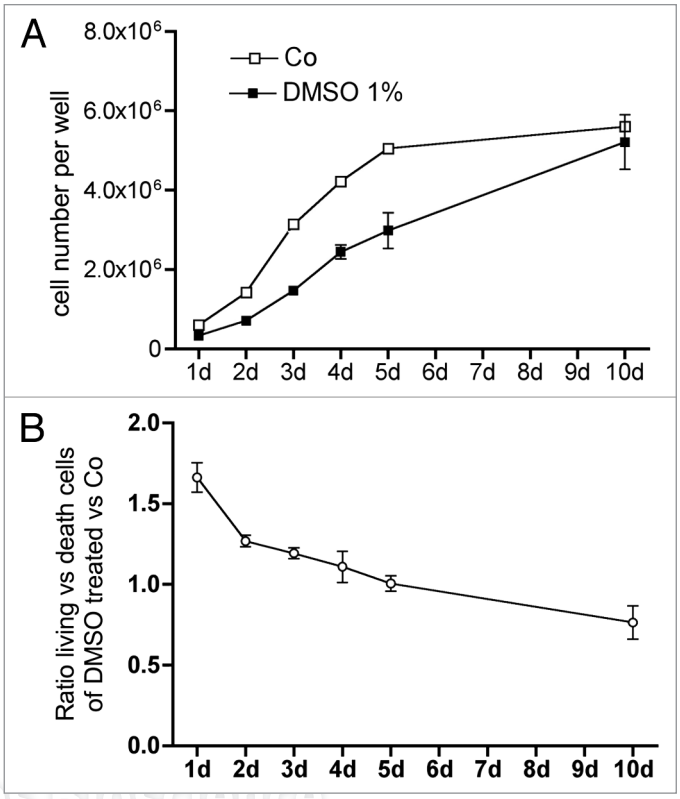

Figure 12. DMSO significantly attenuated cell multiplication of MC3T3-E1 cells and changed the ratio of living vs. dead cells. Cells were treated for 1 to 10 d with 1% DMSO. At the respective time point cells were liberated by pronase treatment and an aliquot was counted (A). The ratio between living and dead cells changed with culture time (B). Except on day 1 and 10 the differences between untreated and DMSO treated cells were significantly different.

Table 1. Expression of genes involved in osteoblastic extra-cellular matrix synthesis by genome wide expression analysis

Gene	Co	1 d 1% DMSO	5 d 1% DMSO
Col1a1	1.00	1.09	1.21
Col1a2	1.00	1.10	1.18
Col5a1	1.00	1.16	1.77
Col5a2	1.00	1.16	1.24
Plod1	1.00	1.21	1.06
Plod2	1.00	1.29	2.00
Plod3	1.00	1.05	1.20
Lox	1.00	1.04	1.39
Mmp13	1.00	1.82	0.12

Fold increase of mRNA expression of the genes compared with untreated control cells. Genes up or downregulated beyond 1.1-fold or 1.5-fold are marked in bright red or dark red, respectively. Genes downregulated beyond 1.1-fold (<0.91) are marked in green.

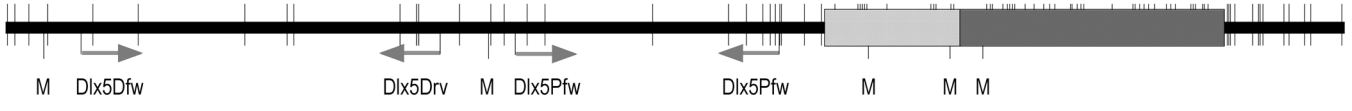

Figure 13. *Dlx5* genomic structure with 5′ flanking region (1,100 bp, black line), the non translated region (182 bp, light gray box), whose left border indicates the transcription start site, the first exon (355 bp, dark gray box) and a part of the first intron. The vertical bars above the horizontal line of the gene indicate the CpGs and the vertical lines labeled with M indicate the MboII restriction sites. Two fragments indicated by light gray arrows and labeled as distal and proximal fragments are used for the "MethylMiner" assay.

Table 2. Primers for qRT-PCR Sybr Green assays (A) and primers for the assessment of the Fas and Dlx5 promoter CpG hydroxymethylation and methylation (B)

A	Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Tm (°C)
	Dnmt1	ACC GCT TCT ACT TCC TCG AGG CCT A	GTT GCA GTC CTC TGT GAA CAC TGT GG	62
	Dnmt3a	CAC ACA GAA GCA TAT CCA GGA GTG	AGT GGA CTG GGA AAC CAA ATA CCC	62
	Dnmt3b	AAC CGT GAA GCA CGA GGG GAA TAT	ACT GGA TTA CAC TCC AGG AAC CGT	62
	Hells	TGA GGA TGA AAG CTC TTC CAC T	ACA TTT CCG AAC TGG GTC AAA A	62
	Fas	TAT CAA GGA GGC CCA TTT TGC	TGT TTC CAC TTC TAA ACC ATG CT	64
	Dlx5	TCT CTA GGA CTG ACG CAA ACA	GTT ACA CGC CAT AGG GTC GC	62
	Tet1	ATT TCC GCA TCT GGG AAC CTG	GGA AGT TGA TCT TTG GGG CAA T	60
	Tet2	CCC GTT AGC AGA GAG ACC TCA	CTG ACT GTG CGT TTT ATC CCT	62
	Tet3	CGC TGC TCG TCT GGA AGA TG	GGC CCC GTA AGA TGA CAC A	60
	Gadd45a	CCG AAA GGA TGG ACA CGG TG	TTA TCG GGG TCT ACG TTG AGC	60
	Gadd45b	CAA CGC GGT TCA GAA GAT GC	GGT CCA CAT TCA TCA GTT TGG C	62
	Gadd45g	GGG AAA GCA CTG CAC GAA CT	AGC ACG CAA AAG GTC ACA TTG	64
B	**Gene, site**	**Forward primer (5'–3')**	**Reverse primer (5'–3')**	**Tm (°C)**
	Fas -2.6 kb	GAA GTA GAA ACA GAA GCT GAG	TTG CTA CAT CCC AAC TGT AAC	62
	Fas -30 bp	CAT ACC CAC AGG CAG TCT AGA	CAG CCC AGA GTA ACT CAC TTC	62
	Dlx5 distal promoter	CAC ACA CCC AAA TCA ATT CAC	CTC TTT TCT TGC TCC ACA CC	63
	-*Dlx5* proximal promoter	CCT GGG ACT GCT TGG TTT GTC GAA GC	GGT TAA ATC CTT AAT TGC GCG CTT AC	63

chip (Affymetrix). Genes that were up or downregulated beyond 1.2-fold are listed in the **Supplemental Data**.

Specific promoter methylation and hydroxymethylation. To analyze the *Fas* and *Dlx5* promoters DNA for 5-mC or 5-hmC, appropriate fragments of the targeted promoter regions were generated by digestion of 1 μg or of 7 μg of genomic DNA respectively with 70 U of the CpG methylation insensitive restriction enzyme MboII (Promega) for 1 h at 37°C from cells cultured for 12 h for 1 d or for 5 d with diverse concentrations of DMSO in the medium. Subsequently, the enzyme was heat inactivated at 65°C for 20 min. Two regions of the *Fas* promoter were selected. One CpG-rich region is localized at 2.6 kb and the second one at 30 bp upstream from the *Fas* transcriptional start site (TSS).[1,4,34] As shown in **Figure 13**, of the *Dlx5* promoter region, a fragment was selected with an overall CpG content of 51%. It starts at 1,049 bp upstream from the TSS and extends to 60 bp of the first exon. MboII digestion subdivided the selected promoter into two fragments: the distal region showed a CpG content of 47%, the proximal region of 55% (**Fig. 13** and NM_198854.1). After MboII digestion, DNA was purified using a commercially available PCR clean-up Kit following the supplier's instructions. In the next step methylated or hydroxymethylated DNA fragments were captured using the "MethylMiner Methylated DNA Enrichment Kit" (Invitrogen). In brief, methylated or hydroxymethylated DNA was captured by methyl-CpG binding domain protein 2 (MBD2) or by a specific hydroxymethyl-cytosine antibody (Abgent) respectively, coupled to magnetic beads to separate the unmodified from the non-modified DNA fractions. DNA was eluted from the magnetic beads with 200 μl of 2 M NaCl solution as single fraction independent from the CpG methylation density and concentrated by ethanol precipitation. Finally, the mean methylation or hydroxymethylation status of the fragments was determined by amplifying the fragments by quantitative real-time PCR. Amplification ratios of the bound (methylated/hydroxymethylated) DNA fraction to unbound (unmethylated/non-hydroxymethylated) DNA fraction were calculated (for primer design see **Table 2B**).

For assessment of the specificity of the 5-hmC antibody, either 40 ng of non methylated, fully methylated or fully hydroxymethylated DNA standards (Zymo Research) were captured with the "MethylMiner Methylated DNA Enrichment Kit" in combination with the methyl-CpG binding domain protein 2 (MBD2) or with the hydroxymethyl-cytosine antibody (Abgent) as described above and eluted DNA fractions were

amplified by real-time PCR using the supplied PCR Primer (**Fig. S1**).

Global DNA methylation. MC3T3-E1 cells were cultured either as described above or were synchronized by serum depletion for 1 d, to exclude DNA replication as cause for DNA demethylation. Subsequently the cells were treated with DMSO for 12 h. Thereafter cells were washed twice with PBS, detached with 0.02% Pronase in PBS and collected by centrifugation (1,400 rpm). Subsequently, DNA and RNA were isolated from about 5×10^5 cells with a DNA/RNA isolation Kit (Qiagen). Global methylation was addressed by digestion of DNA with the 5-methyl-cytosine sensitive restriction enzyme HpaII and compared with a restriction with the insensitive enzyme MspI.[66] 100 ng DNA were digested in multi core buffer (Promega) with 5 U MspI or HpaII or incubated without enzyme (blank) for 3 h at 37°C in a total volume of 30 µl. Sticky ends of the DNA were filled up by adding 20 µl of a mixture containing 0.1 µM Biotin-11-dCTP and 0.1 µM Biotin-11-dGTP (Perkin Elmer) and 0.5 U Klenow-fragment of DNA-polymerase III (Promega) in 500 mM TRIS-HCl, pH 7.2, 100 mM $MgSO_4$ and 1 mM dithiothreitol and incubated at room temperature for half an hour. Thereafter, the reaction was transferred into multi-well plates (Optiplate, Beckman) and 100 µl of Reacti-Bind™ DNA Coating Solution (Pierce) was added to each well and incubated over night at room temperature. On the next day, the wells were washed once with TBS (10 mM Tris-HCL pH 8.0, 150 mM NaCl). After a blocking step with 1% blocking solution (Roche) in TBS for one hour, a solution of 50 ng/µl streptavidin-horse radish peroxidase conjugate (Promega) in TBS was added and incubated for one further hour at room temperature. Thereafter, the wells were washed three times with TBS containing 0.5% Tween-20 and two times with water. One hundred microliters of the chemo-luminescence substrate (Promega) was added to each well and the light emission was measured in an illuminometer (Promega).

Assessment of global cytosine 5-hydroxymethylation. After incubation with increasing DMSO concentrations for 12 h, 1 d or 5 d, MC3T3-E1 cells were fixed by methanol-acetone (7:3) for 10 min at -20°C. Thereafter cell layers were blocked for 20 min with 10% blocking reagent (Roche) and incubated for one further hour with 0.5 µg/ml anti-5-hmC antibody (Abgent). Afterwards, the cells were washed thrice with PBS and incubated for one further hour with an Alexa488 labeled secondary antibody (Invitrogen) diluted 1:300 in blocking buffer. Finally nuclei were stained with Hoechst 33258 dye (Polysciences). The fluorescence of both signals was measured with a multiwell plate reader (Tecan). The amount of DNA (nuclei signal) was estimated using a standard curve prepared from calf thymus DNA (Roche). The signal of the fluorescence of the 5-hmC staining was normalized to the amount of DNA. No signals were found when only the Alexa488 labeled secondary antibody (Invitrogen) was used.

***Tet1* and *Tet2* siRNA transfections.** For *Tet1* and *Tet2* depletion by siRNA, MC3T3-E1 cells were seeded at 20,000 cells/cm^2 in six multi-well plates. Six hours after seeding, cells were transfected with 40 pmol of *Tet1* or *Tet2* siRNA (Sigma) using X-tremeGENE siRNA Transfection Reagent (Roche) as described by the supplier. One day after transfection, medium change was performed and one part of the cells was treated with medium containing 1% of DMSO while the other part was left unchanged. After an incubation time of 12 h and 1 d, nucleic acids were isolated as described above and subjected to qRT-PCR.

Immunostaining of 5-hydroxymethyl-cytosine and 5-methyl-cytosine. After incubation with increasing DMSO concentrations for 12 h or 1 d, MC3T3-E1 cells were fixed by methanol-acetone (7:3) for 10 min at -20°C. For 5-hmC and 5-mC co-staining, cells were additionally incubated with 4 N HCl for 5 min at room temperature. After blocking, the cells for 20 min with 10% blocking reagent (Roche), cells were incubated for 1 h at room temperature with anti-5-hmC antibody (Abgent) or contemporaneously with anti-5-mC antibody (Eurogentec) both diluted 1:500 in blocking buffer. Afterwards, the cells were washed thrice with PBS and incubated for one further hour with Alexa488 or Alexa633 labeled secondary antibodies (Invitrogen) diluted 1:300 in blocking buffer. Finally nuclei were stained with Hoechst 33258 dye (Polysciences). The cell layers were washed several times with PBS and finally with water. Slides were mounted with Vectashield mounting media and immunofluorescence was visualized on a Leica laser-scanning microscope.

No signal was found by using only the Alexa488 or the Alexa633 labeled secondary antibody (Invitrogen).

Cell multiplication. MC3T3-E1 cells were seeded in culture dishes at a density of 5,000/cm^2 and were either left untreated (controls) or treated with 0.5%, 1% and 2% of DMSO in medium for 1, 2 and 5 d. For determination of the cell number (using DNA concentration as surrogate), cell layers were washed with PBS and frozen with 1 mM TRIS-HCl buffer (pH 8.0) containing 0.1 mM EDTA. During thawing, Hoechst 33258 dye (Polysciences) was added to a concentration of 1 µg/mL and, after an incubation time of 15 min at room temperature, the fluorescence was measured (excitation 360, emission 465 nm).[67] The amount of DNA was estimated using a standard curve prepared from calf thymus DNA (Roche).

Time course of cell multiplication was also measured by cell counting. For this purpose cells were seeded at a density of 5,000/cm^2 in 24-well culture plates and were either left untreated (controls) or treated with 1% of DMSO in medium for 1, 2, 3, 4, 5 and 10 d. After the respective culture time cells were detached by means of 1 ml 0.001% pronase E (Roche) and 0.02% EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) and aliquots were counted with a cell counter (Casy).

Protein isolation and immunoblotting. For protein extraction, cell layers were washed two times with PBS and scraped in SDS sample buffer (2% SDS, 100 mM β-mercaptoethanol, 125 mM TRIS-HCl, pH 6.8) and heated at 95°C for 5 min. Thirty µg of protein extracts were fractionated on 8% SDS-polyacrylamide gels. Following SDS-gel electrophoresis, the proteins were transferred to nitrocellulose filters (Millipore) and blocked overnight with 10% blocking reagent (Roche Applied Science) in TN Buffer (50 mM Tris, 125 mM NaCl, pH 8). Subsequently, the filters were incubated for 1 h at room

temperature with antibodies against DNMT1 (K-18, Santa Cruz Biotechnology) diluted 1:200 in blocking buffer. Afterward, all filters were washed three times with immune-blot wash buffer (TN buffer containing 0.01% Tween) and incubated for 1 h with an anti-goat IgG HRP-labeled secondary antibody (Sigma) diluted 1:160,000 in blocking buffer. Finally, the blots were washed again three times with immune-blot washing buffer before detection of light emission with the BM chemiluminescence Immune-blotting kit (Roche Applied Science) as described by the supplier. Chemiluminescence was measured with an image acquisition system (Vilber Lourmat).

Measurement of caspase activity. Caspase 3/7 and caspase 8 activities were measured by using the Caspase-Glo 3/7 and Caspase-Glo 8 assay Kit (Promega) following manufacturers instructions. Briefly, after treatment with 1% or 2% DMSO for 1 and 5 d, cells were lysed and substrate cleavage by caspases was measured by the generated luminescent signal with a 96 multi-well luminometer (Glomax, Promega).

Statistical analysis. Statistical analyses were performed using either ANOVA or Student's t-test using Prism 4.03 (GraphPad Software) while $p \leq 0.05$ was considered as significant. For each experiment, the triplicate results of the qRT-PCR were averaged and this mean value was treated as a single statistical unit. The data of the triplicate experiments are presented as means ± standard deviation (SD).

Disclosure of Potential Conflicts of Interest

No author contributing to this study has to declare a conflict of interest regarding sponsoring of this work by any companies.

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Supplemental Material

Supplemental materials may be downloaded here:
www.landesbioscience.com/journals/epigenetics/article/20163

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