5-Aza-2’-deoxycytidine Leads to Reduced Embryo Implantation and Reduced Expression of DNA Methyltransferases and Essential Endometrial Genes

Yu-Bin Ding, Chun-Lan Long, Xue-Qing Liu, Xue-Mei Chen, Liang-Rui Guo, Yin-Yin Xia, Jun-Lin He*, Ying-Xiong Wang*

Department of Reproductive Biology, Chongqing Medical University, Chongqing, People’s Republic of China

Abstract

Background: The DNA demethylating agent 5-aza-2’-deoxycytidine (5-aza-CdR) incorporates into DNA and decreases DNA methylation, sparking interest in its use as a potential therapeutic agent. We aimed to determine the effects of maternal 5-aza-CdR treatment on embryo implantation in the mouse and to evaluate whether these effects are associated with decreased levels of DNA methyltransferases (Dnmts) and three genes (estrogen receptor α [Esr1], progesterone receptor [Pgr], and homeobox A10 [Hoxa10]) that are vital for control of endometrial changes during implantation.

Methods and Principal Findings: Mice treated with 5-aza-CdR had a dose-dependent decrease in number of implantation sites, with altered endometrial decidualization and stromal cell proliferation. Western blot analysis on pseudo-pregnant day 3 (PD3) showed that 0.1 mg/kg 5-aza-CdR significantly repressed Dnmt3a protein level, and 0.5 mg/kg 5-aza-CdR significantly repressed Dnmt1, Dnmt3a, and Dnmt3b protein levels in the endometrium. On PD5, mice showed significantly decreased Dnmt3a protein level with 0.1 mg/kg 5-aza-CdR, and significantly decreased Dnmt1 and Dnmt3a with 0.5 mg/kg 5-aza-CdR. Immunohistochemical staining showed that 5-aza-CdR repressed DNMT expression in a cell type–specific fashion within the uterus, including decreased expression of Dnmt1 in luminal and/or glandular epithelium and of Dnmt3a and Dnmt3b in stroma. Furthermore, the 5′ flanking regions of the Esr1, Pgr, and Hoxa10 were hypomethylated on PD5. Interestingly, the higher (0.5 mg/kg) dose of 5-aza-CdR decreased protein expression of Esr1, Pgr, and Hoxa10 in the endometrium on PD5 in both methylation-dependent and methylation-independent manners.

Conclusions: The effects of 5-aza-CdR on embryo implantation in mice were associated with altered expression of endometrial Dnmts and genes controlling endometrial changes, suggesting that altered gene methylation, and not cytotoxicity alone, contributes to implantation defects induced by 5-aza-CdR.

Introduction

The nucleoside analog 5-aza-2’-deoxycytidine (5-aza-CdR) is a potent DNA demethylating agent that has been widely used to demonstrate the correlation between demethylation and reactivation of specific genes [1]. 5-Aza-CdR induces cell cycle arrest, cell differentiation, and cell death mainly by inhibiting post-replication methylation of DNA. In mice, exposure to 5-aza-CdR during development alters gene expression, causes malformations, and suppresses growth; administration of 5-aza-CdR to pregnant mice or rats at mid- or late-gestational periods elicits multiple characteristic defects [2–4].

Among the DNA cytosine-5-methyltransferase (Dnmt) family of DNA methylases, three members (Dnmt1, 3a, and 3b) have been shown to mediate the cytotoxic effects of 5-aza-CdR in mammals [5,6]. 5-Aza-CdR inhibits DNMT and demethylates DNA by incorporation into DNA [1], degradation of DNMT [7], downregulation of DNM1 mRNA and protein levels [8–11], or repression of DNM1 enzymatic activity [11,12], leading to changes in gene reactivation. 5-Aza-CdR also downregulates gene expression independently of DNA methylation [9,13–17].

Embryo implantation is a critical step in embryo development and pregnancy outcome. To enable implantation, the uterus goes through changes that prepare it to receive the embryo. Recent studies have suggested that DNA methylation may be involved in endometrial change during periimplantation stages. For instance, mRNA levels of Dnmt1, Dnmt3a, and Dnmt3b are altered according to the phase of the menstrual cycle [18,19]. Several genes are regulated by DNA methylation in relevant cell types, including estrogen receptor 2 (ESR2) [20], paired box gene 2 (PAX2) [21], E-cadherin [22], and S100A4 [23] in endometrial cells, and killer cell immunoglobulin-like receptor 3DL3 (KIR3DL3) in natural killer cells [24]. In addition, human endometrial stromal...
cells treated with 5-aza-CdR showed significantly enhanced expression of 76 genes by microarray analysis, including two established decidualization marker genes. These expression patterns are similar to those of cells treated with medroxyprogesterone acetate [25].

Currently, the effect of 5-aza-CdR on mouse endometrial gene expression, DNA methylation, and embryo implantation from the beginning of fertilization is unknown. Here, we investigated whether the effect of 5-aza-CdR on embryo implantation in mice was associated with endometrial DNMT expression. We also investigated the effect of 5-aza-CdR on methylation of the flanking regions of estrogen receptor α (Esr1), progesterone receptor (Pgr), and homeobox A10 (Hoxa10), which have key roles in implantation, and examined expression of their encoded proteins in mouse endometrium [26,27] following 2 or 4 days of 5-aza-CdR administration.

Results

5-Aza-CdR Reduced the Number of Implantation Sites and Impaired Decidualization, and Stromal Cell Proliferation

On D5 (after 4 days of treatment), the number of embryo implantation sites did not differ significantly between control mice and those treated with 0.1 mg/kg 5-aza-CdR (p = 0.249), but was greatly reduced in mice treated with 0.5 mg/kg 5-aza-CdR (p < 0.001; Figure 1A–D). On D5, there were no differences in embryonic appearance or weight between the three treatment groups (data not shown). Hematoxylin and eosin staining of longitudinal uterine sections on D7 after 4 days treatment with 5-aza-CdR, indicates defected development of uterus during post-implantation period in mice treated by 0.5 mg/kg 5-aza-CdR. (E–G) Hematoxylin and eosin staining of longitudinal uterine sections on D7 after 4 days treatment with 5-aza-CdR, indicates defected development of uterus during post-implantation period in mice treated by 0.5 mg/kg 5-aza-CdR. (H–J) Endometrial decidualization as indicated by alkaline phosphatase activity (blue) on PD6 in oil-induced decidualized uteruses (red indicates negative staining). (K–M) Endometrial stromal cell proliferation on PD6 in oil-induced decidualized uteruses, indicated by immunostaining with rabbit anti-Ki67 (brown). (N) Graph showing the number of Ki67-positive stromal cells in each group (n = 6 for each group). Bars indicate the mean ± S.D. **p < 0.001.

doi:10.1371/journal.pone.0045364.g001

Figure 1. Effects of 5-aza-CdR on embryo implantation, cell proliferation, and decidualization. (A–C) Embryo implantation sites (blue dots with arrows) in mice treated with or without 5-aza-CdR, stained by intravenous Chicago Blue B injection on day 5 of pregnancy (D5). (D) Graph showing the number of implantation sites in each group (n = 6 for each group). Bars indicate the mean ± S.D. **p < 0.001. (E–G) Hematoxylin and eosin staining of longitudinal uterine sections on D7 after 4 days treatment with 5-aza-CdR, indicates defected development of uterus during post-implantation period in mice treated by 0.5 mg/kg 5-aza-CdR. de, decidua; em, embryo; S, stroma. (H–J) Endometrial decidualization as indicated by alkaline phosphatase activity (blue) on PD6 in oil-induced decidualized uteruses (red indicates negative staining). (K–M) Endometrial stromal cell proliferation on PD6 in oil-induced decidualized uteruses, indicated by immunostaining with rabbit anti-Ki67 (brown). (N) Graph showing the number of Ki67-positive stromal cells in each group (n = 6 for each group). Bars indicate the mean ± S.D. **p < 0.001.

Effects of 5-aza-CdR on Embryo Implantation
to decidualization [28], which is a vital step during implantation [29]. The oil-induced decidualized endometria on PD6 of mice treated with 0.1 mg/kg 5-aza-CdR for 4 days showed strong alkaline phosphatase activity, whereas the endometria of mice treated at 0.5 mg/kg had no detectable alkaline phosphatase activity (Figure 1H–J). In addition, an increase in the presence of Ki67-positive cells on PD6 indicated a decrease in proliferation of endometrial stromal cells in mice treated at 0.5 mg/kg for 4 days; there was no significant difference in the number of Ki67-positive cells in endometrial stroma of control mice versus mice treated at 0.1 mg/kg (Figure 1K–N).

5-Aza-CdR Repressed Dnmt Protein Expression

Because 5-aza-CdR is an inhibitor of DNA methyltransferases, we analyzed Dnmt1, Dnmt3a, and Dnmt3b protein expression in mouse endometrium after 2 days (PD3) and 4 days (PD5) of treatment with 5-aza-CdR. On PD3, western blot analysis showed that 0.1 mg/kg 5-aza-CdR significantly repressed Dnmt3a expression ($p < 0.001$) compared to the control, but did not significantly alter Dnmt1 ($p = 0.129$) or Dnmt3b ($p = 0.167$) expression. In contrast, 0.5 mg/kg 5-aza-CdR significantly repressed all three Dnmts ($p < 0.01$ each). On PD5, the 0.1 mg/kg dose led to a decrease only in Dnmt3a protein ($p < 0.01$), and the 0.5 mg/kg dose repressed Dnmt1 ($p < 0.01$) and Dnmt3a ($p < 0.001$), but Dnmt3b was not significantly repressed at either dose ($p = 0.154$ at 0.1 mg/kg; $p = 0.105$ at 0.5 mg/kg; Figure 2).

We next examined tissue sections to determine whether 5-aza-CdR affected Dnmt expression in a cell type–specific fashion within the uterus. On PD3, mice treated at 0.1 and 0.5 mg/kg showed a repressed Dnmt1 protein level in stroma compared to control mice, with no changes in the luminal or glandular epithelium (Figure 3A–C). On PD5, mice showed a decreased Dnmt1 protein level in stroma at 0.1 and 0.5 mg/kg 5-aza-CdR, and repressed expression in luminal epithelium at 0.5 mg/kg (Figure 3D–F). On PD3, mice showed no change in Dnmt3a in stroma, but not in luminal or glandular epithelium at 0.1 and 0.5 mg/kg 5-aza-CdR (Figure 3G–L). On PD3, mice showed no change in Dnmt3b protein level in stroma at 0.1 or 0.5 mg/kg 5-aza-CdR, but showed decreased expression in luminal and glandular epithelia at 0.5 mg/kg (Figure 3M–O). On PD5, Dnmt3b protein levels were unchanged in stroma, luminal epithelia, and glandular epithelia at both doses (Figure 3P–R). These results are summarized in Table 1.
Effects of 5-aza-CdR on Embryo Implantation

Control 0.1 mg/kg 5-aza-CdR 0.5 mg/kg 5-aza-CdR

Dnmt1

PD3 A B C

PD5 D E F

Dnmt3a

PD3 G H I

PD5 J K L

Dnmt3b

PD3 M N O

PD5 P Q R

Negative

PD5 S T U

ge

s

le

100 μm
5-Aza-CdR Reduced Methylation of Hoxa10, a Gene that Controls Endometrial Change

To investigate whether a 5-aza-CdR affects methylation in flanking regions of genes that are vital for endometrial changes in mouse embryo implantation, we used BSP to quantitatively assess the methylation status of each CpG site in the flanking regions of three genes: estrogen receptor α (Esr1), progesterone receptor (Pgr), and homeobox A10 (Hoxa10). For each group (control and 0.5 mg/kg 5-aza-CdR), we randomly selected five clones of each gene region from each of three mice, resulting in 15 BSP analyses for each gene (Figure 4A–C). For Esr1 (Figure 4A), there were 19 CpG sites spanning –73 to +327 nt of the promoter and 5′-UTR (exon 1). For Pgr (Figure 4B), there were 16 CpG sites spanning +119 to +396 nt of the 5′-UTR (exon 1). For Hoxa10, there were 21 CpG sites spanning –386 to +8 nt of the promoter and 5′-UTR (exon 1).

The percentages of total methylated CpG sites in Esr1, Pgr, and Hoxa10 in control mice were 2.807%, 3.750%, and 10.159%, respectively, indicating the baseline hypomethylation status of these regions. Compared to these controls, 0.5 mg/kg 5-aza-CdR significantly reduced methylation of the Hoxa10 region (2.540%, p < 0.001), but had no significant effect on Esr1 (1.053%, p = 0.128) or Pgr (2.500%, p = 0.431).

5-Aza-CdR Decreased Expression of Proteins that Control Endometrial Change

We examined the expression of Esr1, Pgr, and Hoxa10 protein in the endometrium on PD5 using western blot analysis. This revealed that Hoxa10 was significantly repressed at both 0.1 and 0.5 mg/kg 5-aza-CdR, and that Esr1 and Pgr were repressed only at 0.5 mg/kg 5-aza-CdR (Figure 5). Immunohistochemistry revealed that Esr1 was reduced in stroma and glandular epithelium (Figure 6A, B), and Pgr (Figure 6C, D) and Hoxa10 (Figure 6E, F) were reduced in stroma at 0.5 mg/kg 5-aza-CdR. These results are summarized in Table 2.

Discussion

It has been shown that intrauterine insult during mid- to late-gestation using the anti-cancer agent and cytidine analog 5-aza-CdR causes temporally related defects in the developing mouse [3,30]. Our results showed a significantly decreased number of embryo implantation sites on PD5 in mice treated daily with 0.5 mg/kg 5-aza-CdR on PD1–4. Further results showed that the proliferation of endometrial stromal cells was significantly reduced and decidualization was impaired in these mice. Endometrial stromal cell proliferation and differentiation are key processes for successful implantation [31]. Hence, our study indicates that the cytotoxic effects of 5-aza-CdR on embryo implantation might be associated with reduced stromal cell proliferation and differentiation (decidualization). This cytotoxicity of 5-aza-CdR results from its capacity to cause DNA damage, as 5-aza-CdR is a nucleoside analog that can be incorporated into the DNA backbone, which may in turn induce formation of a covalent adduct between the 5-aza-CdR molecule and DNMTs [1].

In addition to its cytotoxic function, 5-aza-CdR demethylates DNA and thereby alters gene expression through DNMT activity, and has been widely used to demonstrate how DNA methylation

Table 1. Localization of DNA methyltransferases (Dnmts) in mouse endometrium following treatment with 5-aza-2′-deoxycytidine.

| Protein | 5-aza-CdR (mg/kg) | Day of pseudo-pregnancy | Stroma | Luminal epithelium | Glandular epithelium |
|---------|------------------|-------------------------|--------|-------------------|---------------------|
| Dnmt1   | 0                | PD3                     | ++     | +                 | +                   |
|         | 0.1              | PD5                     | +      | +                 | +                   |
|         | 0.5              | PD3                     | –      | +                 | +                   |
| Dnmt3a  | 0                | PD3                     | +++    | +++               | +++                 |
|         | 0.1              | PD5                     | +      | +++               | +++                 |
|         | 0.5              | PD3                     | +      | +++               | +++                 |
| Dnmt3b  | 0                | PD3                     | ++     | ++                | ++                  |
|         | 0.1              | PD5                     | –      | +                 | +                   |
|         | 0.5              | PD3                     | ++     | +                 | +                   |

--, no staining; +, weak staining; ++, moderate staining; and +++, strong staining.

Effects of 5-aza-CdR on Embryo Implantation

Figure 3. Effects of 5-aza-CdR on Dnmt1, Dnmt3a, and Dnmt3b expression in mouse endometrium by immunohistochemistry. Mice were treated for 2 or 4 days with 0 (control), 0.1, or 0.5 mg/kg 5-aza-CdR, and sections were prepared on PD3 or PD5. Immunoreactivity (brown) of Dnmt1 (A–F), Dnmt3a (G–L), and Dnmt3b (M–R) is shown. Sections that were not exposed to the primary antibody were used as negative controls (5–U) and stained with Hematoxylin and eosin. le, luminal epithelium; ge, glandular epithelium; S, stroma.

doi:10.1371/journal.pone.0045364.g003
methylation blocks gene expression, whereas demethylation 

mice exposed to diethylstilbestrol [49]. In general, DNA

ovarian cancer [47] or endometriosis [48], and in the uteri of 

of women wearing intrauterine devices [46], in patients with  

promoter is also susceptible to methylation in the endometria  
PGR [41–45] expression in human tumors. The  
methylation plays a major role in repressing  

which may explain the implantation failure. Promoter hyper-

defective uterine decidualization in 5-aza-CdR-treated mice,  

repressed expression of Esr1, Pgr, and Hoxa10 and subsequent

results in implantation defects in mammals [33–37]. We found  
repressed expression of Esr1, Pgr, and Hoxa10 and subsequent  
defective uterine decidualization in 5-aza-CdR-treated mice,  
which may explain the implantation failure. Promoter hyper-

methylation plays a major role in repressing ESR1 [38–40] and  
PGR [41–45] expression in human tumors. The HOXA10  

promoter is also susceptible to methylation in the endometria  
of women wearing intrauterine devices [46], in patients with  
ovarian cancer [47] or endometriosis [48], and in the uteri of  
mice exposed to diethylstilbestrol [49]. In general, DNA  
methylation blocks gene expression, whereas demethylation  

(e.g., with 5-aza-CdR) activates gene expression. Treatment  
with 5-aza-CdR reactivated the expression of ESR1, PGR, and  
HOXA10 from their repressed state in the above studies [38–49]. However, we found that Esr1, Pgr, and Hoxa10 had reduced expression with hypomethylated promoters after treatment  
with 5-aza-CdR. This finding suggests that 5-aza-CdR  
plays diverse roles in cells, including demethylating functions as  
well as methylation-independent functions [50]. Our results  
agree well with previous findings that 5-aza-CdR may inhibit  
gene expression in a methylation-independent manner. Cx31,  
Cx43, Cx45, Cdh1, and Ctnnb1 are all repressed by 5-aza-CdR in  
preimplantation embryos [9]. 5-Aza-CdR decreases expression  
of MDR1 mRNA in K562/ADM cells [13]. In addition, microchip assays revealed a large number of genes downregulated in human endometrial stromal cells [25] and different cancer cells following 5-aza-CdR treatment [14–17].

The mechanism by which 5-aza-CdR decreases gene expression  
is still unknown, and few studies have addressed this question.  
Stability of estrogen receptor mRNA is decreased through  
modulation of HuR (Hu Antigen R) in ER-positive MCF7 cells  
following 5-aza-CdR treatment [51], which might explain why  
Esr1 was suppressed by 5-aza-CdR. It has been also found that 5-

aza-CdR incorporates into DNA; this may inhibit template  
function and chain elongation [52], inhibit DNA polymerase α  
in opposition to the normal substrate deoxyctydine 5’-triphos-

phate [53], and induce DNA damage in a dose-dependent manner  
[50]. In addition, the 5-aza-CdR analog 5-aza-cytidine (5-aza-C)  
induces formation of DNMT-DNA adducts at the DNA replica-
 

ation fork in vivo [54,55]. We suggest that 5-aza-CdR-induced  
DNMT-DNA adducts may affect DNA replication and expression of  
Esr1, Pgr, and Hoxa10 as well.

In conclusion, we showed that the effects of 5-aza-CdR on  
embryo implantation may be due to both cytotoxicity in stromal  
cell proliferation and differentiation. 5-aza-CdR may interfere  
with endometrial expression of Dnmts and genes vital for  
implantation, thereby leading to significant inhibition of embryo  
implantation.
Figure 6. Immunohistochemistry of Esr1 (A, B), Pgr (C, D), and Hoxa10 (E, F) in mouse endometrium on PD5. Mice were treated daily with or without 0.5 mg/kg 5-aza-CdR. The inset boxes in the upper right corners are enlarged region of each figure. le, luminal epithelium; ge, glandular epithelium; s, stroma.
doi:10.1371/journal.pone.0045364.g006
Table 2. Localization of Esr1, Pgr, and Hoxa10 in mouse endometrium following 4 days of 5-aza-2'-deoxycytidine treatment.

| Protein | 5-aza-CdR (mg/kg) | Stroma | Luminal epithelium | Glandular epithelium |
|---------|------------------|--------|-------------------|----------------------|
| Esr1    | 0                | +      | –                 | +++                  |
|         | 0.5              | –      | –                 | ++                   |
| Pgr     | 0                | +++    | +++               | +++                  |
|         | 0.5              | ++     | +++               | +++                  |
| Hoxa10  | 0                | +++    | –                 | –                    |
|         | 0.5              | ++     | –                 | –                    |

+, no staining; +, weak staining; ++, moderate staining; and +++, strong staining. doi:10.1371/journal.pone.0045364.t002

Table 3. Primers for bisulfite-sequencing PCR.

| Gene     | Primer sets          | Accession number* |
|----------|----------------------|-------------------|
| Esr1     | Forward 5’ GGGAGGGGTGTTAAGGTT 3’ | NM_007956.4       |
|          | Reverse 5’ CCCAAAACCTCTCCCTCATAA 3’ |                 |
| Pgr      | Forward 5’ GAGAATTTAGGGAGTTATAGAGATTG 3’ | NM_008829.2       |
|          | Reverse 5’ CGAATAAACCTCTCCTCTCTCT 3’ |                 |
| Hoxa10   | Forward 5’ TATGGGAGTGTTAAGGTGGTTG 3’ | NM_008263.3       |
|          | Reverse 5’ CTTTCTTCCTAAGACATATCTATC 3’ |                 |

*Genebank accession number. doi:10.1371/journal.pone.0045364.t003
Histologic and Immunohistochemical Staining

Uterine tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 12 hr, air-dried, and embedded in paraffin. Sections (4 μm thick) were cut, mounted on slides, deparaffinized with a graded series of xylene, and rehydrated in a descending graded alcohol series. Sections were immersed in 0.1 M citrate buffer and boiled for 15 min in a microwave for antibody retrieval. Once the slides had cooled at room temperature, they were washed for 10 min in TBST (Tris-buffered saline, pH 7.6, with 1% (w/v) Tween 20) and rinsed in distilled H2O. Sections were blocked in 3% H2O2/methanol for 10 min at room temperature, incubated with blocking solution containing 10% normal goat or rabbit serum in TBST for 30 min at room temperature, then incubated with primary antibodies for Dnmt1, Dnmt3a, Dnmt3b, Hoxa10 (Santa Cruz Biotechnology; 1:100, 1:200, 1:100, and 1:50, respectively), Pgr and Esr1 (Abcam; 1:200 and 1:300, respectively) or Ki67 (Millipore, Temecula, CA, USA; 1:300) overnight at 4°C. Sections were washed three times with TBST, then incubated with secondary antibody (biotinylated goat anti-rabbit or rabbit anti-goat IgG, Zhongshan Goldenbridge, 1:300, and 1:300, respectively) or Ki67 (Millipore, Temecula, CA, USA; 1:300, 1:200) for 1 h at room temperature. After one wash, sections were analyzed using the StrepABC horseradish peroxidase kit (Beyotime). Negative controls were created in parallel by replacing the primary antibody with PBS. All sections were lightly counterstained with hematoxylin and cosin (Beyotime). Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA) was used to quantify images. The intensity of Dnmt staining was classified as no staining (−), weak staining (+), moderate staining (++), or strong staining (+++).

Bisulfite Sequencing PCR (BSP)

Genomic DNA was extracted from frozen endometrial tissue of control and 5-aza-CdR-treated mice (n = 3 each) using a standard DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Genomic DNA (1 mg) was modified by bisulfite to convert unmethylated cytosines to uracil using the Methylamp DNA Modification Kit (Epigentek, Brooklyn, NY, USA). Bisulfite-modified DNA was dissolved in 20 μl water and stored at −80°C. The flanking regions (Figure S1) of Esr1, Pgr, and Hoxa10, were sequenced by BSP using primers listed in Table 3. PCR was carried out at 98°C for 4 min, followed by 20 cycles of 94°C for 45 s, 66°C (~0.5°C each cycle) for 45 s, and 72°C for 1 min, with a final incubation at 72°C for 7 min. The resulting first-stage PCR product was amplified using 20 additional cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min, with a final incubation at 72°C for 8 min. PCR products were purified using the QiAquick PCR purification kit (Qiagen) and sequenced by Sangon Biotechnology.

Alkaline Phosphatase Staining

Isolated fresh uterine tissues on PD6 were fixed in 2% paraformaldehyde in PBS for 20 min and then cryoprotected in 30% sucrose/PBS for 18 hr at 4°C. Sections (4 μm thick) were cut and embedded in OCT compound (Sakura Finetek, Torrance, CA, USA), and stained with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride (both from Beyotime), and counterstained with Nuclear Fast Red (Beyotime).

Statistical Analysis

Comparisons between two or more groups were made using Fisher’s exact test, t test, and analysis of variance. The CpG methylation rates of genes were analyzed by the Fisher’s exact test. Pearson’s or Spearman’s rank correlation coefficient was used for evaluating correlation between two variables. To see whether 5-aza-CdR treatment or other possible factors were responsible for the change in implantation before and after the treatment, a multiple linear regression model was used. A p value <0.05 was considered statistically significant.

Supporting Information

Figure S1 Schematic diagram of flanking regions of bisulfite-sequenced genes. Upper panels: UCSC genome browser view of gene structures for Esr1 (A), Pgr (B), and Hoxa10 (C). Lower panels: Sequences of promoter regions containing CpG sites (bold). All sequences are 5′ normal (A), 3′ orientation, with brackets indicating transcription start sites (bold). Arrows indicate transcription start sites.

(TIF)

Author Contributions

Conceived and designed the experiments: YBD YXW. Performed the experiments: CLL YBD. Analyzed the data: YBD CLL JLH YYX XMC. Contributed reagents/materials/analysis tools: YBD CLL JLH LRG XMC. Wrote the paper: YBD YXW.

References

1. Juttermann R, Li E, Jaenisch R (1994) Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. Proc Natl Acad Sci U S A 91: 11797–11801.
2. Schmahl W, Torka P, Kriegel H (1984) Embryotoxicity of 5-aza-cytidine in mice. Phase- and dose-specificity studies. Arch Toxicol 55: 143–147.
3. Rosen MB, Chernoff N (2002) 5-Aza-2′-deoxycytidine reactivates expression of RUNX1 by deletion of DNA methyltransferases leading to caspase independent apoptosis in colorectal cancer Lovo cells. Biomed Pharmacother 63: 492–500.
4. Yu JN, Xue CY, Wang XG, Lin F, Liu CY, et al. (2009) 5-AZA-2′-deoxycytidine (5-AZA-CdR) leads to down-regulation of Dnmt1 and gene expression in preimplantation mouse embryos. Zygote 17: 137–145.
5. Ding L, Qi L, Zhang J, Guo B (2009) Camptothecin-induced cell proliferation inhibition and apoptosis enhanced by DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine. Biol Pharm Bull 32: 1165–1169.
6. Deng T, Zhang Y (2009) 5-Aza-2′-deoxycytidine reactivates expression of RUNX3 by deletion of DNA methyltransferases leading to caspase independent apoptosis in colorectal cancer Lovo cells. Biomed Pharmacother 63: 492–500.
7. Benbrahim-Tallaa L, Waterland RA, Dill AL, Webber MM, Waalkes MP (2007) Tumor suppressor gene inactivation during cadmium-induced malignant transformation of human prostate cells correlates with overexpression of de novo DNA methyltransferase. Environ Health Perspect 115: 1454–1459.
8. Ando T, Nishimura M, Oka Y (2000) Dectinicate (5-Aza-2′-deoxycytidine)- decreased DNA methyltransferase activity and expression of MDR-1 gene in K562/ADM cells. Leukemia 14: 1915–1920.
9. Gius D, Cui H, Bradbury CM, Cook J, Smart DK, et al. (2004) Distinct effects of 5-aza-CdR on embryo implantation of mouse blastocysts. Mol Cell Biol 25: 4727–4741.
15. Schmelz K, Sattler N, Wagner M, Lubbert M, Doeksen B, et al. (2005) Induction of gene expression by 5-Aza-2′-deoxycytidine in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) but not epithelial cells by DNA-methylation-dependent and -independent mechanisms. Leukemia 19: 103–111.

16. Yap OW, Bhut G, Liu L, Tollefsbol TO (2009) Epigenetic modifications of the Estrogen receptor beta gene in epithelial ovarian cancer cells. Anticancer Res 29: 139–144.

17. Arai M, Yokosuka O, Hirasawa Y, Fukai K, Chiba T, et al. (2006) Sequential

16. Yap OW, Bhat G, Liu L, Tollefsbol TO (2009) Epigenetic modifications of the

25. Logan PC, Ponnampalam AP, Rahnama F, Lobie PE, Mitchell MD (2010) The

22. Rahnama F, Thompson B, Steiner M, Shafiei F, Lobie PE, et al. (2009)

21. Wu H, Chen Y, Liang J, Shi B, Wu G, et al. (2005) Hypomethylation-linked

9

29. Finn CA, Martin L (1972) Endocrine control of the timing of endometrial

27. Ramathal CY, Bagchi IC, Taylor RN, Bagchi MK (2010) Endometrial

decidualization: of mice and men. Semin Reprod Med 28: 17–26.

28. Taylor HS, Arici A, Olive D, Igarashi P (1998) HOXA10 is expressed in

26. Tan J, Paria BC, Dey SK, Das SK (1999) Differential uterine expression of

24. Xie R, Loose DS, Shipley GL, Xie S, Bassett RL Jr, et al. (2007) Hypomethylation-induced expression of S100A1 in endometrial carcinoma. Mod Pathol 20: 1045–1054.

23. Trumdee AE, Hilby SE, Chang C, Sharkey AM, Santouridis S, et al. (2006) Molecular characterization of KRIM3L3. Immunogenetics 57: 904–916.

25. Logan PC, Ponnampalam AP, Rahnama F, Lobie PE, Mitchell MD (2010) The
differential DNA methylation inhibitor 5-Aza-2′-deoxycytidine on human endometrial stromal cells. Hum Reprod 25: 2859–2869.

26. Tan J, Paria BC, Dey SK, Das SK (1999) Differential uterine expression of estrogen and progesterone receptors correlates with uterine preparation for implantation and decidualization in the mouse. Endocrinology 140: 5310–5321.

27. Ramathaly CJ, Bagchi IC, Taylor RN, Bagchi MK (2010) Endometrial
decidualization: of mice and men. Semin Reprod Med 28: 17–26.

28. Taylor HS, Arici A, Olive D, Igarashi P (1998) HOXA10 is expressed in

29. Finn CA, Martin L (1972) Endocrine control of the timing of endometrial

30. Lesley BA, Palomino WA, Apparao KB, Young SL, Lanning RA (2006) Estrogen receptor-alpha (ER-alpha) and defects in uterine receptivity in women. Reprod Biol Endocrinol 4 Suppl 1: S9.

31. Mulac-Jericevic B, Mullinax RA, DeMayo FJ, Lydon JP, Cosnely OM (2000) Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. Science 289: 1751–1754.

32. Ivase H, Omoso Y, Iwata H, Toyama T, Hara Y, et al. (1999) DNA methylation analysis at distal and proximal promoter regions of the oestrogen receptor gene in breast cancers. Br J Cancer 80: 1982–1986.

33. Wiley A, Katsaros D, Chen H, Riguetti de la Longrais IA, Beeghly A, et al. (2006) Aberrant promoter methylation of multiple genes in malignant ovarian tumors and in ovarian tumors with low malignant potential. Cancer 107: 299–308.

34. Lim H, Ma L, Ma WG, Maas RL, Dey SK (1999) Hoxa-10 regulates uterine
endometrium during implantation. Endocrinology 139: 1005–1017.

35. Zhu LJ, Cullinan-Bove K, Polihronis M, Bagchi MK, Bagchi IC (1998) Calcitonin is a progesterone-regulated marker that forecasts the receptive state of endometrium during implantation. Endocrinology 139: 3923–3934.