DNA methylation-dependent epigenetic regulation of Dimethylarginine dimethylaminohydrolase 2 gene in trophoblast cell lineage

Junko Tomikawa, Kazumi Fukatsu*, Satoshi Tanaka and Kunio Shiota

Laboratory of Cellular Biochemistry, Animal Resource Sciences/Veterinary Medical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

*Present address: Division of Molecular Neurobiology, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan.

Running title: Epigenetic regulation of Ddah2 in trophoblast cells

Trophoblast cell lineage is established through the first cellular differentiation in mammalian embryogenesis, and its developmental potential is restricted to the extraembryonic tissues contributing solely to the placenta. Several lines of evidence suggest a relative lack of importance of DNA methylation in gene regulation in the extraembryonic tissues compared to embryonic ones. Here we analyzed the dynamics of epigenetic status in the upstream region of mouse Ddah2 gene, which was found to be specifically repressed in a stem cell population of trophoblast cell lineage. We found a tissue-dependent differentially methylated region in the regulatory region of Ddah2 gene. This region was hypermethylated in trophoblast stem cells and was hypomethylated in differentiated cells both in vivo and in vitro. This change was well correlated with Ddah2 expression. In addition, in vitro methylation confined to the differentially methylated region was sufficient to repress promoter activity in the reporter assay. Furthermore, a repressive pattern of histone modifications was formed around the differentially methylated region in undifferentiated TS cells with repressed Ddah2. Our data suggest that DNA methylation-mediated chromatin remodeling is involved in the regulation of Ddah2 gene expression, and thus is important even in trophoblast cell lineage.

In mammals, genomic DNA is methylated at cytosine residues predominantly in CpG dinucleotides (CpGs) (1). Methylation of DNA is essential for mammalian development (2) and is associated with gene silencing in conjunction with histone core modifications probably through chromatin remodeling (3-8). The level of DNA methylation of bulk genome changes during mammalian embryogenesis. In the mouse, the paternal contribution of the genome (sperm-derived DNA) undergoes rapid and very likely, active demethylation, while the maternal contribution of the genome (oocyte DNA) is gradually demethylated in a passive manner until the morula stage (9, 10). In the blastocyst, the level of DNA methylation increases by de novo methylation but is much lower in trophoderm (TE) than in the inner cell mass (ICM) (11). The hypomethylated status of the genome of extraembryonic lineages persists later in postimplantation development (12, 13), rendering the impression that DNA methylation may play a less relevant role in gene regulation in the extraembryonic lineages than in the embryonic lineages. In support of this concept, it has been reported that both maintenance of imprinted X inactivation and imprinted expression of some genes on
mouse distal chromosome 7 in the extraembryonic lineages are not perturbed by genome-wide demethylation due to genetic ablation of DNA methyltransferase 1 (Dnmt1) gene (14-16).

We have previously reported that the enhancer/promoter region of mouse Oct4 gene is hypermethylated in TE-derived mouse trophoblast stem (TS) cells and in the placenta, both having repressed Oct4 expression. Ectopic Oct4 expression in TS cells treated with 5-aza-2'-deoxycytidine (5-aza-dC), an inhibitor of DNA methyltransferases, and in the Dnmt1-deficient placenta, has suggested that at least on Oct4 locus, DNA methylation plays an important role in transcriptional regulation in cells/tissues of extraembryonic origin (17). We have also reported that both de novo methylation and demethylation of some Not I recognition sites occur in a differentiation-dependent manner in rat Rcho-1 cells, a progenitor population of trophoblast giant cells (18). Similarly, in mouse TS cells, equal numbers of Not 1 sites (15 each out of ~1500 analyzed) in the genome were revealed to be methylated or demethylated as the cells were induced to differentiate (19). These findings implied that DNA methylation might regulate dynamic changes in gene expression during the differentiation process even of trophoblast cells. However, it remained to be demonstrated if differentiation status-dependent changes in DNA methylation are indeed involved in the regulation of gene expression in trophoblast cells.

In the present study, we analyzed expression and epigenetic status of gene encoding the dimethylarginine dimethylaminohydrolase 2 (Ddah2) in mouse TS cells and in trophoblastic tissues of postimplantation mouse embryos. The Ddah2 gene has been identified in our attempts to isolate genes differentially expressed between undifferentiated and differentiated TS cells. There is no report to date showing the contribution of epigenetic regulatory mechanisms in the regulation of this gene, but it has GC-rich and TATA-less promoter region (Fig. 1A). Our results here suggest that DNA methylation, accompanied by repressive histone modifications, plays a pivotal role in trophoblast stem cell-specific silencing of the Ddah2 gene.

EXPERIMENTAL PROCEDURES

Trophoblast stem (TS) cell culture and chemical treatment

The TS cell line used in this study was established from C57BL/6 strain mouse embryo (19) and maintained as described previously (20) with slight modification. In brief, TS cells were grown in the medium supplemented with 37.5 ng/ml FGF4 (PeproTech, London, UK), 1.5 µg/ml heparin (Sigma Chemical Company) and 80 % mouse embryonic fibroblast cell-conditioned medium (CM). Differentiation of TS cells was induced by substituting medium with one lacking FGF4, heparin and CM.

For treatment with 5-aza-2'-deoxycytidine (5-aza-dC) (Sigma-Aldrich, St. Louis, MO) and trichostatin A (TSA) (Sigma-Aldrich), TS cells were cultured for 2 days with FGF4, heparin and CM, and were cultured for 2 additional days after the addition of 5-aza-dC (1 or 5 µM) and/or TSA (200 nM).

All reagents used in this study were purchased from Wako Pure Chemicals (Osaka, Japan) unless stated otherwise.

Animals and tissue preparation

Adult C57BL/6 strain mice were purchased from Charles River Japan (Yokohama, Japan). Noon of the day when a vaginal plug was found was designated as embryonic day 0.5 (E0.5). Postimplantation embryos collected at E6.5 were embedded in OCT compound (Sakura Finetechical, Tokyo, Japan), and subjected to in situ hybridization and laser-microdissection.

Frozen sections (6 µm thick) of E6.5 embryos were fixed with 4% (w/v) paraformaldehyde, then stained with Mayer’s hematoxylin and dried well. Isolation of tissues from the sections was performed using the P.A.L.M. MicroBeam System (P.A.L.M. Microlaser Technologies AG, Bernried, Germany) according to the manufacturer’s instructions. Cells of each
tissue were pooled (200-500 cells) and stored at –80°C until used for DNA/RNA extraction.

In situ hybridization analysis

In situ hybridization analysis on the frozen sections of E6.5 embryos (6 µm thick) was performed essentially as described (21). Hybridization with digoxigenin (DIG)-labeled antisense RNA (cRNA) probe for Ddah2 was carried out at 55 °C overnight, and hybridized probes were detected using an alkaline phosphatase-conjugated anti-DIG Fab fragment (Roche Diagnostics, Tokyo, Japan) and NBT/BCIP (Roche Diagnostics). The cDNA clone used to make the Ddah2 cRNA probe (IMAGE clone no. 329801) was purchased from Kurabo Industries (Osaka, Japan).

RNA analysis

Total RNA was isolated from cells and tissues with TRizol reagent (Invitrogen, Carlsbad, CA). Northern hybridization analysis was performed by a standard protocol with cRNA probes for Ddah2, Errβ (22), Mash2 (23), Tphp (24), Pl-I (25) and Gapdh (26) labeled with DIG-11-uridine triphosphate (Roche Diagnostics). The DIG-labeled probes were detected with DIG luminescence detection kit (Roche Diagnostics).

For RT-PCR analysis, first-strand cDNA was synthesized from total RNA (150 ng) using random hexamers and Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). The PCR was carried out with Ddah2-specific primers (5’-CCAGATGATGCAGCTAGTGAC-3’ and 5’-CCACAACCTACCCAGCCTCTAT-3’), Errβ-specific primers (5’-CGTCCCATACGATGACAGC-3’ and 5’-CATGCGCTGACTCAGCTCAT-3’), Tphp-specific primers (5’-CATGACTCCTACCTCAATCTCC-3’ and 5’-TTTTGCTGGCCTTCGCCCT-3’) or β-actin-specific primers (5’-GACAAACGGGTCCTCCGCGATGTGCAAG-3’ and 5’-TTACCGTTTGCCCTAACGAGCTAG-3’). Each PCR reaction was performed under the following conditions: for Ddah2, Errβ, and Tphp, 95°C 10 min; 40 cycles of 95°C, 30 s; 62°C, 30 s; 72°C, 1 min; final extension 72°C, 10 min; for β-actin, 95°C 10 min; 35 cycles of 95°C, 30 s; 55°C, 30 s; 72°C, 1 min; final extension 72°C, 10 min.

Sodium bisulfite sequencing

Genomic DNA was extracted as described previously (27). Sodium bisulfite treatment of genomic DNA and sequencing analysis were carried out essentially as described (17). The DNA fragment covering the 5’-flanking sequence of Ddah2 gene was amplified by PCR using the following set of primers: D2B1F; 5’-GTTGGTGTGCAAAAGTACAGAGGC-3’, D2B1R; 5’-GCAGTGTCCAGGGCCACCCCTGATCC-3’, D2B2F; 5’-ATCAAGGGTGGCCTGACACTGTC-3’, D2B2R; 5’-ACCACTACAAAAACAGACTTACGGC-3’, D2B3F; 5’-AGACAGAGGATCTGATGCAAGTC-3’, D2B3R; 5’-AGATTAGAGGATTGATGATCTG-3’. The PCR products were cloned into pGEM-Teasy vector (Promega), and 10 or more clones randomly picked from each of two independent PCRs were sequenced to determine the presence of methylated cytosines.

Plasmid construction

The 5’-flanking fragments of the Ddah2 gene containing sequence -924 to -36 and sequence -609 to -36 were isolated by genomic PCR using specific forward primers (5’-GGGGTACCCCATTGGTCTGGCATCTG-3’ and 5’-GGGGTACCCCAGGGCTCTAAATGAC-3’) and a common reverse primer (5’-GAAGATCTTCTAAAGACACGCCAT-3’). The forward primers include the Kpn I site at the 5’ end of the sequence and the reverse primer also includes the Bgl II site. Each PCR product was cloned into pGL3-Basic vector (Promega), a promoterless luciferase vector. The resultant plasmid constructs were designated as pGL-924 and pGL-609.
pGL-609 according to the positions of fragments. To make hybrid constructs, pGL-924 was digested with Kpn I and Bal I, releasing two DNA fragments. Both fragments were isolated and separately collected. The smaller fragment (-924 to -611) was methylated by Sss I methylase (New England BioLabs, Beverly, MA) and then re-ligated with the larger fragment, which lacked the region from -924 to -611. As a control, the unmethylated smaller fragment was also re-ligated with the larger fragment. These re-ligated constructs were separated by agarose gel electrophoresis and purified to avoid contamination of un-ligated fragments. Methylation status of these constructs was verified by bisulfite sequencing. The regionally methylated construct and unmethylated control construct were designated as pGL-me-924 and pGL-um-924, respectively.

**Transfection and Promoter luciferase assays**

The TS cells were replated on 24-well plates at 5x10^4 cells per well one day prior to transfection and cultured in differentiative condition. All constructs (0.4 µg) were transfected into these cells by using Lipofectamine Reagent (Invitrogen) with pRL-TK. Luciferase activities were determined using Dual-Luciferase Reporter Assay System (Promega) at 72 hr after transfection according to the manufacturer’s instructions. Promoter activity was normalized via Renilla luciferase activity. Assays were performed three times each in triplicate.

**Chromatin immunoprecipitation (ChIP) assay**

The ChIP assay was performed with 1x10^6 cells per assay using the ChIP Assay Kit (Upstate Biotechnology Inc., Lake Placid, NY) as recommended by the manufacturer. Briefly, fixed cells were lysed and sonicated until chromatin fragments become 200-1,000 bp in size. All antibodies used in this study were purchased from Upstate Biotechnology. Antibodies against acetylated histone H3 (Catalog no. 06-599), acetylated histone H4 (Catalog no. 06-598), dimethylated lysine 4 of histone H3 (H3K4) (Catalog no. 07-030) and dimethylated lysine 9 of histone H3 (H3K9) (Catalog no. 07-212) were used for immunoprecipitation (IP), and rabbit IgG (Catalog no. 12-370) was used as a negative control to check the specificity of IP. After IP, recovered chromatin samples were subjected to PCR with the following set of primers: for region I; 5’-CAGAGGATCTGATGCACAGTC-3’ and 5’-CATGTACCCTGTGCCTAGAGT-3’, region II; 5’-AGTTCAATTGGTCTGGCACTCCTG-3’ and 5’-GGATGGTGAAGCCTCTGTACT-3’, and region III; 5’-GATTGTAACCCCACAGCGAC-3’ and 5’-CGAGCTCACGTACGACGAT-3’. The PCR products were run on agarose gel and the intensity of each band was measured using ImageJ software (version 1.32; U.S. National Institutes of Health). Results from two independent experiments performed in triplicate were quantified and averaged.

**RESULTS**

**Expression of Ddah2 gene in trophoblast cell lineage**

The TS cell line is derived from trophoblast stem cells localized in the TE of blastocysts and extraembryonic ectoderm (ExE) of postimplantation embryos (20). These cells retain an undifferentiated state in the presence of FGF4 and heparin in medium supplemented with 80% mouse embryonic fibroblast cell-conditioned medium (CM) (stem cell conditions). Removal of FGF4, heparin or CM induces differentiation into trophoblast subtypes in vitro (20). Errβ is a marker gene of trophoblast stem cells (22). Mash2 is expressed in the ectoplacental cone (EPC), a reservoir of differentiating trophoblast cells of postimplantation embryos (23); Tphp is also detected in EPC and further differentiated diploid trophoblast cells in placenta (24). Pl-1 is a gene specifically expressed in trophoblast giant cells (25). In TS cell differentiation, Ddah2 expression was repressed under stem cell conditions while it was increased upon induction of differentiation (Fig. 1B).
Trophoblast stem cell-specific repression of Ddah2 gene was also observed in E6.5 embryo by in situ hybridization analysis. Ddah2 was widely expressed in most of the tissues including EPC, but was barely detectable in ExE close to the extraembryonic-embryonic boundary, a pool of trophoblast stem cells in E6.5 conceptus (Fig. 1C). In further developed placenta, Ddah2 continued to be expressed at high levels (Fig. 1D). Thus, the Ddah2 gene expression during trophoblast cell differentiation was regulated in a differentiation status-dependent manner both in vivo and in vitro.

Derepression of Ddah2 gene in TS cells by treatment with reagents affecting epigenetic status

To investigate if repression of Ddah2 gene is mediated by epigenetic mechanisms, we treated TS cells with 5-aza-dC, an inhibitor of DNA methylation, and/or TSA, an inhibitor of histone deacetylation. Upregulation of Ddah2 expression in undifferentiated TS cells was induced by 5-aza-dC treatment (Fig. 2). In addition, TSA also strongly induced Ddah2 expression, suggesting that both DNA methylation and histone deacetylation are involved in the repression of Ddah2 gene.

DNA methylation status of 5'-flanking region of Ddah2 gene in trophoblast cell lineage

Repression of Ddah2 expression in undifferentiated TS cells was alleviated by 5-aza-dC treatment alone. Thus, we predicted the presence of CpGs, differentially methylated between undifferentiated and differentiated TS cells, in the upstream region of the transcription start site of Ddah2 gene. The Ddah2 locus has a CpG island overlapping the first exon. Bisulfite sequencing analysis of the Ddah2 upstream region (-1,289 to +456) revealed that CpGs within the CpG island (-71 to +430) were almost completely unmethylated. However, the CpGs upstream of the CpG island appeared hypermethylated in undifferentiated TS cells, while these CpGs were relatively hypomethylated in differentiated TS cells (Fig. 3). In particular, 5 CpGs, located at -897, -710, -698, -684 and -674, were found to be almost completely demethylated in differentiated TS cells where Ddah2 is expressed.

To investigate whether TS cells indeed recapitulated the epigenetic change of Ddah2 locus in vivo, we then analyzed the DNA methylation status of CpGs in the Ddah2 upstream region in ExE and EPC cells. Laser microdissection was used to isolate pieces of each tissue from sections of E6.5 concepti (Fig. 4A), and the integrity of collected tissues was verified by RT-PCR for the expression of marker genes. Detection of Errß in ExE-derived but not EPC-derived RNA, and restricted expression of Tpbp in EPC-derived cells indicated that the tissues were properly isolated from each population (Fig. 4B). Moreover, as was shown in Fig. 1C, Ddah2 was detected in EPC and only faintly detected in ExE. Bisulfite sequencing analysis of genomic DNAs prepared from the collected tissues again revealed almost no methylation of CpGs in the CpG island, and the differentiation status-dependent change in methylation of CpGs at -897, -710, -698, -684 and -674 (Fig. 4C). In contrast to the case of TS cells, CpGs further upstream (-1249, -1201, -987, -956 and -931) showed nearly complete methylation in both ExE and EPC. These results indicated that TS cell differentiation in vitro basically recapitulated differentiation of trophoblast cell lineage in vivo in terms of the change in DNA methylation status of Ddah2 locus, and suggested that methylation of a particular set of CpGs in the 5'-flanking region of Ddah2 gene plays an important role in repression of this gene at least in trophoblast cell lineage. Therefore, we designated the region containing 5 differentially methylated CpGs as a tissue-dependent differentially methylated region (T-DMR) of Ddah2 locus.

Chromatin modification status of 5'-flanking region of Ddah2 gene in TS cells

Since a differentiation status-dependent change in histone acetylation at Ddah2 locus in TS cells was also suggested, we next examined the chromatin
modification status of the Ddah2 upstream region by ChIP assays with anti-acetylated histone H3 and anti-acetylated histone H4 antibodies. We designed three sets of primers across the Ddah2 5′-flanking region: an upstream region of the T-DMR (-1,285 to -1,021; region I), a region included within the T-DMR (-928 to -722; region II) and a region closer to the transcription start site (-180 to +44; region III) (Fig. 5A). ChIP assays revealed increased H3 and H4 acetylation at all examined regions in differentiated TS cells compared with undifferentiated ones (Fig. 5B and 5C). Methylation status of lysines in histone H3 was also examined by using anti-dimethylated H3K4 and anti-dimethylated H3K9 antibodies. Whereas dimethylation of H3K4 at the T-DMR (region II) was likewise increased with differentiation of TS cells, that of both region I and region III did not change with statistic significance. Conversely, a slight reduction of H3K9 methylation was observed at all examined region. These results suggest that histone code in a repressive state was established not only at the T-DMR but also in the broad 5′-flanking region of Ddah2 gene in undifferentiated TS cells with repressed Ddah2 expression.

**Discussion**

In this study, we found a T-DMR in the 5′-flanking region of Ddah2 gene. The Ddah2 T-DMR was hypermethylated in ExE of E6.5 mouse embryo where Ddah2 expression is repressed, while it was hypomethylated in EPC where Ddah2 is expressed. Dynamic change in the methylation status of the T-DMR was also demonstrated in vitro in TS cells. Importantly, in vitro methylation of the T-DMR was sufficient to inhibit promoter activity of the Ddah2 5′-flanking region in the reporter assay, strongly suggesting that methylation of the T-DMR is crucial for silencing Ddah2 gene.

Complete inhibition of Ddah2 promoter activity by methylation of only 5 cytosines in the Ddah2 T-DMR was rather surprising. These CpGs are located 674 to 897-bp upstream of the transcription start site, encompassing a single core histone octamer in theory, and are apparently outside of the region which showed promoter activity (-609 to -36) in the reporter assay. Another set of CpGs further upstream (-1249, -1201, -987, -956 and -931) appeared fully methylated in EPC and almost completely methylated in ExE. However, this set showed partial loss of methylation even in TS cells maintained in an undifferentiated state, although it was still hypermethylated in comparison with the set in differentiated TS cells. This may indicate that methylation of distal CpGs is less relevant for repression of Ddah2 expression. Methylation of DNA at the transcription regulatory region is generally associated with gene silencing (3, 17, 27), either by directly inhibiting binding of transcription factors to
their recognition sequences (28, 29), or by indirectly preventing transcription factors from accessing their target sites through attachment of methyl-binding proteins. These proteins recruit histone deacetylases and histone methyltransferase, thereby resulting in formation of a closed chromatin structure (5-7). A search for CpG-containing conserved sequences of transcription factor binding sites did not reveal any within the Ddah2 T-DMR, excluding the possibility that CpG methylation directly inhibited binding of known transcription factors to the T-DMR. However, Ddah2 expression in undifferentiated TS cells was upregulated by treatment with TSA, showing that histone acetylation-mediated change of chromatin structure is also involved in regulation of Ddah2 gene. In the 5'-flanking region of Ddah2 gene that includes the T-DMR, histones H3 and H4 were hyperacetylated and H3K4 was hypermethylated in differentiated TS cells compared with undifferentiated ones. In addition, the level of H3K9 methylation was reduced with differentiation, suggesting that the Ddah2 upstream region switches from an inactive to an active chromatin structure with differentiation of TS cells (8, 30, 31). In the luciferase reporter assay, methylation of only the T-DMR reduced luciferase activity to the level comparable to that of empty vector, suggesting that methylation inhibited not only enhancer activity of the T-DMR itself but also promoter activity conferred by the region proximal to the T-DMR. These results, together with the derepression of Ddah2 by 5-aza-dC treatment in undifferentiated TS cells, imply that methylation of Ddah2 T-DMR plays a causal role in changing the chromatin structure toward a repressive state at the region around the Ddah2 T-DMR.

As Ddah2 was widely expressed in virtually all regions of E6.5 embryo except ExE, and in a wide variety of adult tissues (32, 33), we assume that Ddah2 expression is activated by ubiquitously expressed transcription factor(s), and is actively suppressed in ExE through methylation of the Ddah2 T-DMR. In support of this view, involvement of transcription factors such as Sp1 and E2F in human DDAH2 expression has been suggested (34). In mouse Ddah2 locus, we found some putative Sp1 binding sites immediately upstream of the transcription start site (data not shown). Although participation of Sp1, E2F and their putative binding sites in the activation of Ddah2 gene should be directly examined, we presume that tissue- and developmental stage-dependent expression of Ddah2 gene is regulated by the presence of widely expressed transcription factors in combination with the epigenetic status around the T-DMR. In this context, specific repression of Ddah2 in ExE of E6.5 embryo is of great interest. It may be possible that methylation of the Ddah2 T-DMR is regulated under the control of signaling pathways specifically activated in ExE and undifferentiated TS cells. To date, a molecular link between extracellular signal and genomic region-specific change in DNA methylation has not been defined. The TS cells may provide a unique model system to address this issue.

Physiological role of Ddah2 in the trophoblast cell lineage is unknown. Ddah2 has been shown to metabolize mono- or asymmetrically di-methylated free arginine residues to citrulline, and has been implicated in the positive regulation of nitric oxide (NO) synthesis (35). However, production of NO both in ExE and EPC of postimplantation mouse embryos has not been detected (36). Recently, conversion of methyl-arginine residues in histones H3 and H4 to citrulline by PAD4/PADI4 was shown to play a pivotal role in epigenetic regulation of transcriptional activity (37). It may be possible that Ddah2 activity is also involved in these epigenetic modifications, although it is still unclear whether Ddah2 converts methyl-arginine residues within a polypeptide chain to citrulline.

In conclusion, our data indicate for the first time not only the presence of CpGs methylated in a differentiation status-dependent manner, but also the existence of the link between DNA methylation and transcriptional regulation of a non-imprinted unique gene in the trophoblast cell lineage.
REFERENCES

1. Gruenbaum, Y., Stein, R., Cedar, H., and Razin, A. (1981) FEBS Lett. 124, 67-71
2. Li, E., Bestor, T. H., and Jaenisch, R. (1992) Cell 69, 915-926
3. Chan, Y., Fish, J. E., D’Abreo, C., Lin, S., Robb, G. B., Teichert, A. M., Karantzoulis-Fegaras, F., Keightley, A., Steer, B. M., and Marsden, P. A. (2004) J. Biol. Chem. 279, 35087-35100
4. Fish, J. E., Matouk, C. C., Rachlis, A., Lin, S., Tai, S. C., D’Abreo, C., and Marsden, P. A. (2005) J. Biol. Chem. 280, 24824-24838
5. Fujita, N., Watanabe, S., Ichimura, T., Tsuruzoe, S., Shinkai, Y., Tachibana, M., Chiba, T., and Nakao, M. (2003) J. Biol. Chem. 278, 24132-24138
6. Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J., and Wolffe, AP. (1998) Nat. Genet. 19, 187-191
7. Martinowich, K., Hattori, D., Wu, H., Fouse, S., He, F., Hu, Y., Fan, G., and Sun, Y. E. (2003) Science 302, 890-893
8. Reik, W., Dean, W., and Walter, J. (2000) Nature 403, 501-502
9. Santos, F., Hendrich, B., Reik, W., and Dean, W. (2002) Dev. Biol. 241, 172-182
10. Reik, W., Dean, W., and Walter, J. (2001) Science 293, 1089-1093
11. Sado, T., Fenner, M. H., Tan, S. S., Tam, P., Shioda, T., and Li, E. (2000) Dev. Biol. 225, 294-303
12. Lewis, A., Mitsuya, K., Umlauf, D., Smith, P., Dean, W., Walter, J., Higgins, M., Feil, R., and Reik, W. (2004) Nat. Genet. 36, 1291-1295
13. Hattori, N., Nishino, K., Ko, Y. G., Hattori, N., Ohgane, J., Tanaka, S., and Shiota, K. (2004) J. Biol. Chem. 279, 17063-17069
14. Ohgane, J., Hattori, N., Oda, M., Tanaka, S., and Shiota, K. (2002) Biochem. Biophys. Res. Commun. 290, 701-706
15. Shiotani, K., Kogo, Y., Ohgane, J., Imamura, T., Urano, A., Nishino, K., Tanaka, S., and Hattori, N. (2002) Genes Cells 7, 961-969
16. Tanaka, S., Kunath, T., Hadjantonakis, A. K., Nagy, A., and Rossant, J. (1998) Science 282, 2072-2075
17. Hirota, S., Ito, A., Morii, E., Wanaka, A., Tohyama, M., Kitamura, Y., and Nomura, S. (1992) Brain Res. Mol. Brain Res. 15, 47-54
18. Luo, J., Sladek, R., Bader, J. A., Matthyssen, A., Rossant, J., and Giguere, V. (1997) Nature 388, 778-782
19. Guillemot, F., Nagy, A., Auerbach, A., Rossant, J., and Joyner, A. L. (1994) Nature 371, 333-336
20. Lescisin, K. R., Varmuza, S., and Rossant, J. (1988) Genes Dev. 2, 1639-1646
21. Colosi, P., Talamantes, F., and Linzer, D. I. (1987) Mol. Endocrinol. 1, 767-776
22. Fort, P., Marty, L., Piechaczyk, M., el Sabrouty, S., Dani, C., Jeanteur, P., and Blanchard, J. M. (1985) Nucleic Acids Res. 13, 1431-1442
23. Nishino, K., Hattori, N., Tanaka, S., and Shiota, K. (2004) J. Biol. Chem. 279, 22306-22313
24. Comb, M., and Goodman, H. M. (1990) Nucleic Acids Res. 18, 3975-3982
29. Prendergast, G. C., Lawe, D., and Ziff, E. B. (1991) Cell 65, 395-407
30. Santos-Rosa, H., Schneider, R., Bannister, A. J., Sherriff, J., Bernstein, B. E., Emre, N. C., Schreiber, S. L., Mellor, J., and Kouzarides, T. (2002) Nature 419, 407-411
31. Tachibana, M., Sugimoto, K., Nozaki, M., Ueda, J., Ohta, T., Ohki, M., Fukuda, M., Takeda, N., Niida, H., Kato, H., and Shinkai, Y. (2002) Genes Dev. 16, 1779-1791
32. Leiper, J. M., Santa Maria, J., Chubb, A., MacAllister, R. J., Charles, I. G., Whitley, G. S., and Vallance, P. (1999) Biochem. J. 343, 209-214
33. Tran, C. T., Fox, M. F., Vallance, P., and Leiper, J. M. (2000) Genomics 68, 101-105
34. Jones, L. C., Tran, C. T., Leiper, J. M., Hingorani, A. D., and Vallance, P. (2003) Biochem. Biophys. Res. Commun. 310, 836-843
35. Gagioti, S., Scavone, C., and Bevilacqua, E. (2000) Biol. Reprod. 62, 260-268
36. MacAllister, R. J., Parry, H., Kimoto, M., Ogawa, T., Russell, R. J., Hodson, H., Whitley, G. S., and Vallance, P. (1996) Br. J. Pharmacol. 119, 1533-1540
37. Wang, Y., Wysocka, J., Sayegh, J., Lee, Y. H., Perlin, J. R., Leonelli, L., Sonbuchner, L. S., McDonald, C. H., Cook, R. G., Dou, Y., Roeder, R. G., Clarke, S., Stallcup, M. R., Allis, C. D., and Coonrod, S.A. (2004) Science 306, 279-283

FOOTNOTES

° We thank Dr. Maddy Roberts for proofreading the manuscript and Drs. Shintaro Yagi and Naka Hattori for helpful advice and comments on the manuscript. We also thank Drs. Mayumi Oda and Naoko Hattori for technical assistance.

This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and Grant-in-aid for Scientific Research, Ministry of Education, Culture, Sports, Science and Technology, Japan (15080202 to K. S. and 16380226 to S. T.).

The abbreviations used are: Ddah2, Dimethylarginine dimethylaminohydrolase; T-DMR, tissue-dependent differentially methylated region; CpGs, CpG dinucleotides; 5-aza-dC, 5-aza-2'-deoxycitidine; TSA, trichostatin A; TS cell, trophoblast stem cell; ICM, inner cell mass; TE, trophectoderm; ExE, extraembryonic ectoderm; EPC, ectoplacental cone

FIGURE LEGENDS

Fig. 1. Expression profile of Ddah2 gene in trophoblast cell lineage in vitro and in vivo. A) Genomic structure of the Ddah2 locus with position of probe used in both northern and in situ hybridizations. Second diagram shows a detailed map of the approximately 2 kb region around the transcription start site (+1), in which vertical lines indicate positions of CpGs. The transcription start site of gene was determined based on UCSC database (accession ID no. BC003328). Thick horizontal lines indicate the region identified as CpG island. For Ddah2 probe (indicated by orange bar), a cDNA fragment overlapping exons 6 and 7 was used. B) Expression of Ddah2 gene in differentiating TS cells. Total RNA was prepared from TS cells cultured for indicated time periods after induction of differentiation. Northern hybridization was performed with 10 µg of total RNA and each specific probe indicated. Gapdh was used as loading control. C) Expression of Ddah2 gene in postimplantation embryo at E6.5. Sections of E6.5 conceptus were used for in situ hybridization analysis with Ddah2-specific cRNA probes (antisense) or sense probes (sense). On the left, schematic drawing of E6.5 conceptus is shown. Arrowhead indicates the region where a lower level of signals was observed. Background is relatively high because of prolonged time for coloring reaction to clearly visualize positive signals. EPC, ectoplacental cone; ExE, extraembryonic ectoderm; Epi, epiblast. D) Expression of Ddah2 in developing placenta. Total RNA (10µg) prepared from placenta at indicated stage.
of development was separated and hybridized to probe for Ddah2.

Fig. 2. Effects of 5-aza-dC and TSA on expression of Ddah2 gene in TS cells. (Top) Northern hybridization analysis of the expression of Ddah2 gene in 5-aza-dC and/or TSA-treated TS cells. Total RNA (10µg) extracted from cells exposed to 5-aza-dC (1 or 5 µM) and/or TSA (200 nM) was separated and hybridized with probe specific for Ddah2. Relative levels of expression of Ddah2 gene are summarized in the histogram at the bottom. Intensity of each band on the film images was measured using ImageJ program, and the value was normalized to Gapdh expression in each lane. Level of expression in untreated differentiated cells (Diff) was set as 1 for relative expression level of Ddah2 (white column).

Fig. 3. DNA methylation status of Ddah2 5'-flanking region in TS cells. (Top) A schematic diagram of the Ddah2 5'-flanking region. Vertical lines and numbers indicate positions of cytosine residues of CpGs relative to transcription start site (+1). Thick horizontal line indicates the region included in the CpG island. Asterisks (*) above numbers denote CpGs most differentially methylated between undifferentiated and differentiated TS cells. (Bottom) DNA methylation status of individual CpGs at Ddah2 upstream region in undifferentiated (Undiff) and differentiated (Diff) TS cells determined by bisulfite sequencing. Open and filled circles represent unmethylated and methylated cytosines, respectively.

Fig. 4. DNA methylation status of the Ddah2 upstream region in trophoblast lineage of postimplantation embryo. A) Images of frozen section of E6.5 conceptus before (left) and after (right) laser microdissection. Cells were isolated from morphologically defined ExE and EPC (indicated by arrows in the left panel and by arrowheads in the right panel), and subjected to DNA/RNA extraction. Scale bar represents 200 µm. md, maternal decidua. B) Characterization of laser microdissection-isolated cells. Expression of Ddah2 and marker genes for trophoblast cell lineage (as indicated) in collected cells was analyzed by RT-PCR. C) DNA methylation status in Ddah2 5'-flanking region in ExE and EPC. Bisulfite sequencing analysis was performed with DNAs extracted from cells derived from ExE and EPC. Open and filled circles represent unmethylated and methylated cytosines, respectively.

Fig. 5. Histone modification status of Ddah2 5'-flanking region in TS cells. A) A schematic diagram of the Ddah2 5'-flanking region and location of three pairs of ChIP assay primers for regions designed as region I, region II and region III. B) Histone modification status in Ddah2 upstream region in TS cells before and after differentiation. TS cells maintained in an undifferentiated state (U) or cultured for 4 days after induction of differentiation (D) were subjected to ChIP assays with antibodies against acetylated histone H3 (AcH3), acetylated histone H4 (AcH4), dimethylated H3K4 (Me²H3K4) and dimethylated H3K9 (Me²H3K9). Normal rabbit IgG (IgG) was used as a negative control for the specificity of the immunoprecipitation (IP). Equal amount of chromatin fragments to that used for each IP were also subjected to PCR without IP as a positive control (input). C) Semiquantitative analysis of the relative levels of histone modifications. Intensity of each band in gel images was measured using the ImageJ program, and normalized by input. Each column and bar represents the mean ± SE of two independent experiments performed in triplicate. Values in samples from undifferentiated TS cells on each gel image were set as 1 for each modification. *, p <0.05; **, p <0.01 (Student’s t-test).

Fig. 6. Repression of Ddah2 promoter activity by DNA methylation. A) Promoter and enhancer activities of the Ddah2 upstream sequence. Map of Ddah2 5'-flanking region is shown at the top; B indicates the location of Bal I site. Empty plasmid (pGL3-Basic) or constructs carrying different lengths of the Ddah2 5'-flanking sequences, pGL-924 and pGL-609, were transfected
into differentiated TS cells. Luciferase activities were determined relative to that of pGL3-Basic. B) Effects of in vitro methylation of T-DMR on Ddah2 promoter activity. Both T-DMR-specifically methylated construct (pGL-me-924) and unmethylated control construct (pGL-um-924) were used directly for luciferase assays without any amplification in E. coli. Open and filled boxes represent the unmethylated and methylated region of DNA, respectively. Note that the activity of pGL-um-924 is lower than equivalent construct pGL-924, likely due to inferior quality of the plasmid DNA obtained from re-construction/purification steps. Each column and bar in A and B represents the mean ± SE of triplicates of three separate experiments. *, p < 0.05; **, p < 0.01 (Student’s t-test).
Figure 1

A

Ddah2 locus

CpG island

Ex. 1

Days of differentiation

0 2 4 6

Ddah2

Errß

Mash2

Tpbp

Pl-1

Gapdh

B

C

EPC

ExE

Epi

D

Placenta

E9.5

E11.5

E13.5

E15.5

E17.5

Ddah2

Gapdh
Figure 2

![Image of a graph showing the relative expression level of Ddah2 and Gapdh under different conditions of 5-aza-dC and TSA treatments.](http://www.jbc.org/downloaded)
Figure 3

Ddah2 locus

CpG island

Undiff

Diff

* * * * *

-1,249 -1,201 -987 -897 -931 -956 -710 -698 -684 -674 -544 -531 -507 -482 -392 -333 -195 -181 -183 -119 -71
Figure 4
Figure 5

A

-1,000  T-DMR
-500  +1

Region I  Region II  Region III

B

ChIP

Input  AcH3  AcH4  Me\(^2\)H3K4  Me\(^3\)H3K9  IgG

Region I  Region II  Region III

C

Relative degree of histone modification

Region I  Region II (T-DMR)  Region III

AcH3  AcH4  Me\(^2\)H3K4  Me\(^3\)H3K9
Figure 6

A

| Construct         | Relative luciferase activity |
|-------------------|-----------------------------|
| pGL3-Basic       |                             |
| pGL-609          |                             |
| pGL-924          |                             |

B

| Construct         | Relative luciferase activity |
|-------------------|-----------------------------|
| pGL3-Basic       |                             |
| pGL-um-924       |                             |
| pGL-me-924       |                             |

- Unmethylated
- Methylated
DNA methylation-dependent epigenetic regulation of dimethylarginine dimethylaminohydrolase 2 gene in trophoblast cell lineage

Junkot Tomikawa, Kazumi Fukatsu, Satoshi Tanaka and Kunio Shiota

J. Biol. Chem. published online March 6, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M513782200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts