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XIST Repression in the Absence of DNMT1 and DNMT3B

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Abstract

X chromosome inactivation (XCI) in human and mice involves *XIST/Xist* gene expression from the inactive X (Xi) and repression from the active X (Xa). Repression of the *XIST/Xist* gene on the Xa has been associated with methylation of its 5′ region. In mice, Dnmt1 has been shown to be involved in the methylation and transcriptional repression of *Xist* on Xa. We examined maintenance of *XIST* gene repression on Xa in HCT116 cell lines knockout for either *DNMT1* or *DNMT3B* and for *DNMT1* and *DNMT3B* simultaneously. Methylation of the *XIST* promoter and *XIST* transcriptional repression is sustained in *DNMT1*-, *DNMT3B*- and *DNMT1/DNMT3B* knockout cells. Despite global DNA demethylation, the double knockout cells present only partial demethylation of the *XIST* promoter, which is not sufficient for gene reactivation. In contrast, global DNA demethylation with 5-aza-2′-deoxycytidine leads to *XIST* expression. Therefore, in these human cells maintenance of *XIST* methylation is controlled differently than global genomic methylation and in the absence of both DNMT1 and DNMT3B.

Key words: X-chromosome inactivation; DNA-methyltransferase; XIST; epigenetic inheritance

1. Introduction

In mammals, dosage compensation of X-linked gene products between XY males and XX females is achieved by transcriptional inactivation of one X chromosome in females.[1] The *Xist* gene is expressed exclusively from the inactive X (Xi), and seems to trigger initiation of X chromosome inactivation (XCI) in *cis*.[2] It transcribes a 17.8 kb nuclear mRNA which is not translated.[3] In the early mouse embryo, low levels of *Xist* expression are detected from the single X and from both the Xs in the male and female embryo, respectively.[4,5] In female embryos, immediately prior to gastrulation, up-regulation of *Xist* RNA occurs on the one X chromosome to be inactivated.[4]

Repression of *Xist/XIST* on the active X (Xa) in males and females has been correlated with methylation of its 5′ end.[6,7] This region is hypermethylated on the Xa, where *Xist/XIST* is repressed, and hypomethylated on the Xi, where *Xist/XIST* is expressed. These results suggest that methylation is involved in *Xist/XIST* gene silencing in humans[7,8] and mice.[9,10] Indeed, treatment of normal human fibroblasts and somatic cell hybrids containing one human Xa with the demethylating agent 5-aza-2′-deoxycytidine (5-aza-dC) leads to *XIST* demethylation and expression.[11,12]

To date, five different mammalian DNA (cytosine-5) methyltransferases (MTases) have been identified: Dnmt1, Dnmt2, Dnmt3a, Dnmt3b and Dnmt3L.[13–16] Dnmt1 is constitutively expressed, has higher activity in hemi-methylated DNA and has been recognized as a major maintenance MTase (reviewed in Bestor[17]). Dnmt2 was isolated based on its homology to the pmt1p of fission yeast, and is expressed at low levels in all human and murine tissues.[14] Although neither *de novo* nor maintenance DNA MTase activity has been demonstrated for this protein in murine ES cells,[18] low *in vitro* activity of human DNMT2 was detected and it was specific for a loose DNA consensus sequence.[19] In contrast, Dnmt3a and Dnmt3b have been shown to be essential for *de novo* DNA MTase activity in murine ES cells and in early embryos, but not required for the maintenance of

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imprinted methylation patterns.[20] More recently, Dnmt3a was shown to be required for methylation of imprinted loci in germ cells.[21] In humans, mutations in DNMT3B cause ICF syndrome, characterized by the hypomethylation of pericentromeric repetitive DNA.[22,23] Finally, Dnmt3L was isolated based on its homology to Dnmt3a and Dnmt3b in the cysteine-rich region.[16] Like Dnmt3a, this protein is required for the establishment of genomic imprints during gametogenesis.[24] However, Dnmt3L lacks the catalytic domain common in the other MTases and it represses transcription by binding to the histone deacetylase HDAC1 protein rather than by methylating DNA.[25,26]

The role of Dnmt1 in the process of DNA methylation and XCI has been extensively studied in mice. Murine ES cells deficient for Dnmt1 show high levels of global DNA demethylation, which in turn leads to biallelic expression of imprinted genes.[27,28] In addition, upon differentiation, these cells fail to repress *Xist* expression, a phenomenon correlated to lack of proper methylation of the 5′ region of the *Xist* gene.[6] These results demonstrate that Dnmt1 activity is causally involved in global DNA methylation and in transcriptional repression of imprinted genes and of the *Xist* gene.[5,6]

The role of the human homologue DNMT1 in controlling gene expression was investigated in the human carcinoma cell line HCT116 knockout for the *DNMT1* gene by homologous recombination.[29] Surprisingly, the authors showed that despite a greatly decreased DNMT1 activity the cells presented only a 20% reduction in overall DNA methylation, restricted to specific regions of the genome. Using the same approach, Rhee et al.[30] generated HCT116 cell lines deficient for DNMT3B and for both DNMT1 and DNMT3B. While *DNMT3B* knockout cells retained >97% of genomic 5-methylcytosine (m^5C), the double knockout (DKO) cells presented ~95% reduction in the m^5C content. This in turn leads to transcriptional activation of *TIMP-3*, the imprinted *IGF2* allele and the wild-type $p16^{INK4a}$ allele. The authors thus concluded that DNMT1 and DNMT3B cooperate to maintain global DNA methylation and gene silencing in those human cancer cells.

These data indicate that human MTases may be involved in DNA methylation differently than their murine counterparts. That prompted us to investigate the methylation status of *XIST* in the absence of DNMT1 and DNMT3B activity in the HCT116 knockout cells.

2. Materials and Methods

2.1. Cell culture

Parental HCT116 cell line, two independent *DNMT1* knockout clones (1C1 and 9A), one *DNMT3B* knockout clone (3bKO) and one *DNMT1/DNMT3B* DKO clone were kindly provided by Drs B. Vogelstein and K. Schuebel.[29,30] These cells contain one normal X and one normal Y chromosomes. Cells were cultured without selection in McCoy media supplemented with 10% fetal calf serum and penicilin/streptomycin (Invitrogen) at 37°C/5% CO_2. Treatment with 5-aza-dC (Sigma) was as follows: cells at the mid-log phase in 100 mm culture dishes were supplemented with fresh media containing 10 μM of 5-aza-dC. Fresh media with 5-aza-dC was added every 24 h for 48 h. Cells were allowed to recover from treatment for 48 h in media without the drug before harvesting. Treatment was carried out in duplicate plates. DKO cells were treated for up to 72 h with 30 μM 5-aza-dC.

2.2. Analysis of XIST gene expression

RNA was isolated from treated and untreated pooled cells with the Trizol reagent according to manufacturer's instructions (Invitrogen). Analysis of *XIST* gene expression was performed by northern blotting with 20 μg of total RNA as described previously[31] using a probe from the most 5′ *XIST* cDNA clone Hbc1a.[32] Normal female fibroblasts were used as a positive control for *XIST* expression and *G3PDH* cDNA probe as an internal control. *DNMT1* and *DNMT3B* gene activity were evaluated by hybridization of northern blots with radiolabeled *DNMT1* (data not shown) and *DNMT3B* cDNAs, respectively. Lack of *DNMT1* expression was confirmed by RT–PCR.

2.3. Analysis of XIST 5′ end methylation

DNA was isolated from cells as described previously.[31] Analysis of methylation of the 5′ end of the *XIST* gene was performed by Southern blotting with the most 5′ *XIST* cDNA clone Hbc1a as the probe, as described previously.[7,12] An aliquot of 5–10 μg of genomic DNA was digested with 100 U each of *EcoR*V and one of the methylation-sensitive restriction enzymes *Hha*I and *Ava*I, and with 100 U each of *Pst*I and the methylation-sensitive restriction enzyme *Sac*II (Amersham-Pharmacia).

3. Results and Discussion

3.1. Maintenance of XIST repression in the absence of DNMT1

XIST gene activity was assayed by northern blot analysis of total RNA from the parental HCT116 cell line and from the *DNMT1* knockout clone 9A (Fig. 1). Although *XIST* RNA was detected from control female cells, no *XIST* expression was detected from either the parental cell line or the *DNMT1* knockout cells (Fig. 1A, lanes 1, 2 and 9). Lack of *DNMT1* expression in the *DNMT1* knockout cells was confirmed by RT–PCR (Fig. 1B).

Lack of *XIST* expression in the *DNMT1* knockout cells could be due to the maintenance of methylation of *XIST* 5′ region, or alternatively due to the absence of other

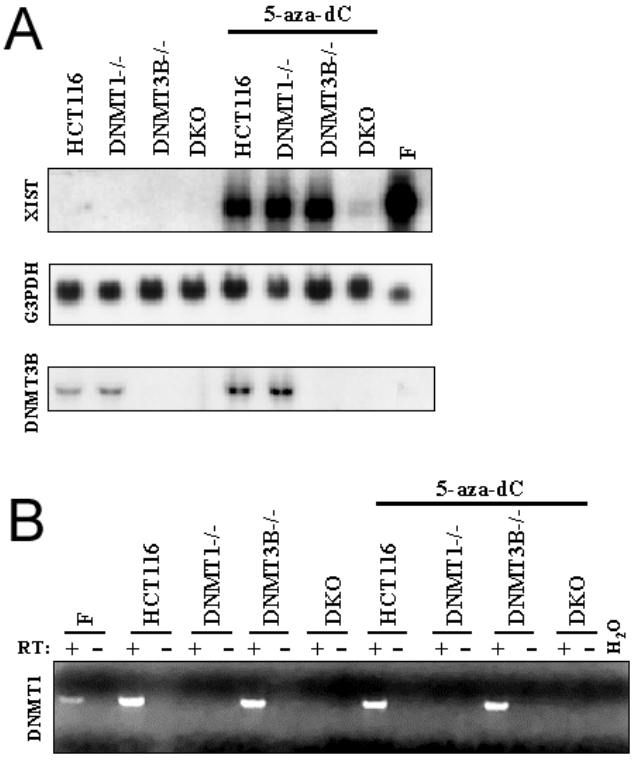

Figure 1. Analysis of *XIST* expression in different MTase knockout HCT116 cells. (**A**) Northern blot analysis of HCT116 clones. cDNA probes are indicated on the left of the corresponding panels. (**B**) RT–PCR analysis of *DNMT1* expression. Plus signs, RT added; minus signs, RT omitted; DKO, *DNMT1/DNMT3B* double knockout cells; F, human female fibroblast cell line. *DNMT1* and *DNMT3B* genotypes and 5-aza-dC treatment are indicated above the lanes.

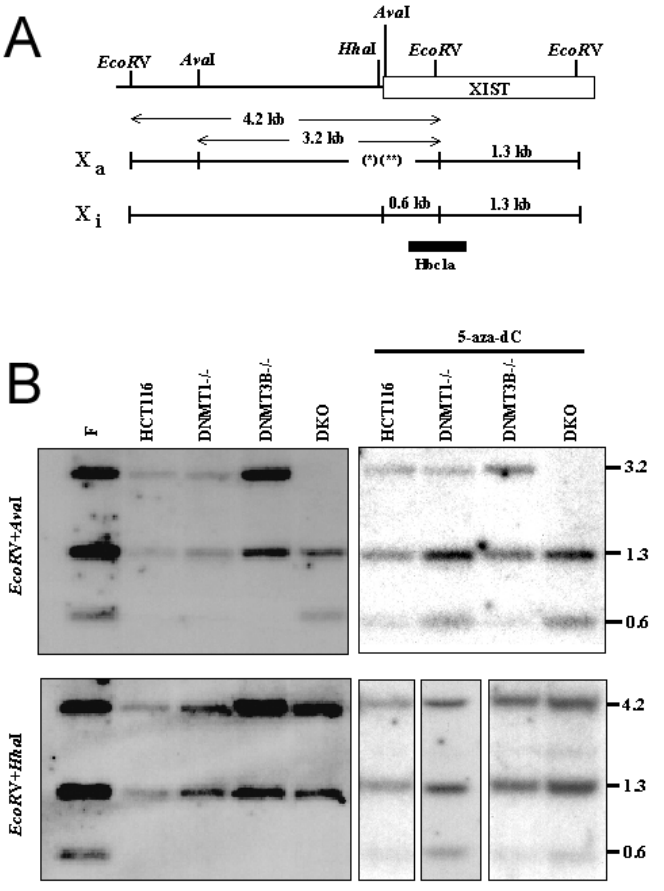

Figure 2. Methylation status of the *XIST* 5′ end. (**A**) Scheme of restriction patterns of the 5′end of *XIST* in the Xa and Xi. Single and double asterisks indicate methylated *Hha*I and *Ava*I sites, respectively. (**B**) Southern blotting analysis: genomic DNA from HCT116 clones and fibroblasts from a normal female (F) was digested with *Eco*RV and *Ava*I or with *Eco*RV and *Hha*I as indicated. *DNMT1* and *DNMT3B* genotypes and 5-aza-dC treatment are indicated above the lanes; sizes in kb, 0.6 kb bands correspond to unmethylated *XIST* alleles. The *Hha*I site remains methylated in the DKO cells and 5-aza-dC treatment leads to demethylation of both *Hha*I and *Ava*I sites on HCT116 cells (see text).

factors required for *XIST* induction after demethylation. Therefore, methylation status of the methylation-sensitive restriction enzyme *Hha*I, *Ava*I and *Sac*II sites at *XIST* 5′ end was analyzed by Southern blotting (Figs 2 and 3). Unlike PCR-based assays, this approach allows detection of partial DNA demethylation patterns. Our results show that Xa-specific methylation of the 5′ end of *XIST* is retained in the *DNMT1* knockout cells (Figs 2B and 3B). These data demonstrate maintenance of *XIST* methylation and transcriptional repression in the absence of DNMT1 activity.

3.2. Maintenance of XIST repression in the absence of both DNMT1 and DNMT3B

XIST expression was analyzed in the *DNMT3B* knockout and also in the *DNMT1/DNMT3B* DKO cells. Northern blot analysis revealed lack of *XIST* expression in *DNMT3B* knockout cells and, surprisingly, also in the DKO cells, which are known to present ∼95% reduction in the m^5C content[30] (Fig. 1). Lack of *DNMT3B* and of both *DNMT3B* and *DNMT1* expression was confirmed in the *DNMT3B* knockout and DKO cells, respectively (Fig. 1).

Methylation analysis of the *XIST* gene in different MTase knockout HCT116 cells was performed (Figs 2 and 3). *DNMT3B* knockout cells maintained the four methylated sites present in the parental HCT116 (Figs 2B and 3B). In the DKO cells, maintenance of methylation of the *Hha*I and the downstream *Sac*II [*Sac*II$^{(2)}$] sites was observed (Figs 2B and 3B). However, the *Ava*I site was completely unmethylated, as indicated by the presence of the 600 bp band and absence of the 3.2 kb band (Fig. 2B), and the upstream *Sac*II [*Sac*II$^{(1)}$] site was only partially unmethylated, as indicated by the presence of the 800 bp band and absence of the 240 and 170 bp bands (Fig. 3B). Our results show that, while causing global DNA demethylation, lack of both DNMT1 and DNMT3B activity was not sufficient to disrupt maintenance of methylation specifically on the *Hha*I and *Sac*II$^{(2)}$ sites at the 5′end of *XIST*.

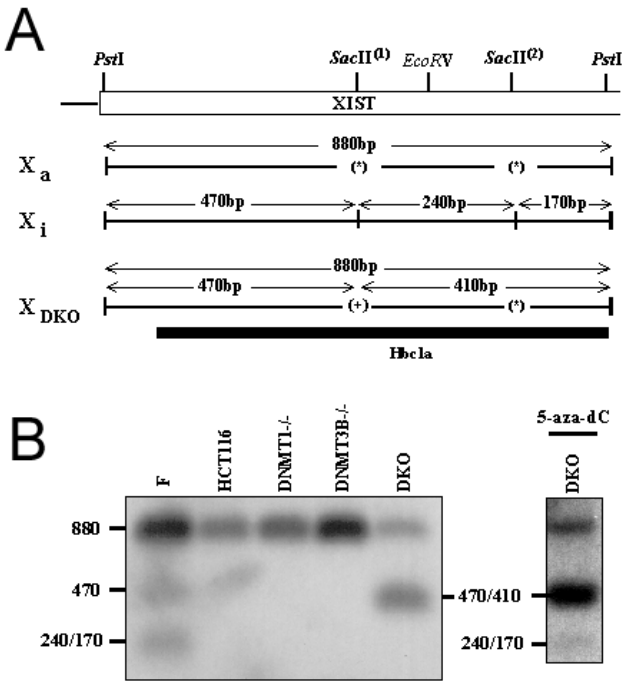

Figure 3. Methylation status of *Sac*II sites at *XIST* 5′ end. (**A**) Scheme of restriction patterns of the 5′ end of *XIST* on the Xa, Xi and on the partially methylated X chromosome of DKO cells (X_{DKO}). Asterisks indicate methylated *Sac*II sites; plus sign indicates the partially methylated $SacI^{(1)}$ site at the X_{DKO}. (**B**) Southern blotting: genomic DNA from HCT116 clones and from fibroblasts from a normal female (F) was digested with *Pst*I and *Sac*II. *DNMT1* and *DNMT3B* genotypes are indicated above the lanes; sizes in bp. Absence of the 240/170 bp bands indicates that the $SacII^{(2)}$ site remains methylated in the DKO cells (see text).

3.3. 5-aza-dC-mediated DNA demethylation induces XIST expression in HCT116 parental and knockout cells

In order to investigate the effect of global demethylation on the maintenance of *XIST* transcriptional repression in HCT116 cells, these cells were treated with 5-aza-dC. Northern blot analysis revealed partial reactivation of the *XIST* gene in treated parental and all knockout HCT116 cells (Fig. 1). 5-aza-dC treatment of DKO cells was less effective even at higher drug concentrations and longer treatment time (data not shown), probably due to the slower growth rate of these cells, as previously reported by Rhee et al.[30] Our results show that *XIST* expression from the Xa is induced by global DNA demethylation in HCT116 parental and knockout cells, as reported in human fibroblasts and in somatic cell hybrids containing the human Xa.[11,12] It is interesting to note that 5-aza-dC treatment also leads to higher expression of *DNMT3B* in HCT116 parental and *DNMT1* knockout cells (Fig. 1A).

The methylation status of the 5′ region of the *XIST* gene in the 5-aza-dC treated HCT116 cells was analyzed (Figs 2 and 3). Partial demethylation of the *Ava*I, *Hha*I and the two *Sac*II sites was observed in the treated cells expressing *XIST* (Figs 2B and 3B). Therefore, our data show that DNA methylation is involved in the control of *XIST* expression from the Xa in HCT116 cells, as already shown for a normal human cell line.[12] Moreover, these results show that the *Hha*I and $SacII^{(2)}$ methylation sites are essential for *XIST* transcriptional control.

In mice, Dnmt1 is required for the maintenance of global DNA methylation,[27,28] and for the establishment of *Xist* methylation and maintenance of *Xist* repression on the Xa.[6] Therefore, Dnmt1 has been recognized as the major mammalian MTase involved in both imprinting and XCI. Rhee et al.[29] have challenged this idea by generating *DNMT1* knockout HCT116 cells that retained most of the overall genomic methylation. Subsequently, Rhee et al.[30] showed that maintenance of global DNA methylation in HCT116 cells was lost only in the *DNMT1/DNMT3B* DKO cells, suggesting a cooperation between these two MTases. More recently, Ting et al.[33] demonstrated that inhibition of DNMT1 expression by small interfering RNA (siRNA) in HCT116 cells did not affect global DNA methylation, corroborating the results of Rhee et al.[29] In addition, using the same siRNA approach in an epithelial ovarian cancer cell line, Leu et al.[34] observed that although DNMT1 has an important role in maintaining DNA methylation, deficiency of both DNMT1 and DNMT3B leads to a 2-fold increase in global DNA demethylation than DNMT1 deficiency alone. These data suggest that if indeed DNMT1 is a major maintenance MTase in humans, there must be other compensatory pathways for lack of DNMT1 expression in those human cancer cells.

Nevertheless, our results in the *DNMT1/DNMT3B* DKO cells demonstrate that, despite global DNA demethylation, *XIST* gene methylation and repression in the Xa can be maintained in the absence of both DNMT1 and DNMT3B activity. DKO cells present ~5% of the normal levels of m^5C,[30] which we show are partially targeted to the *XIST* gene, specifically to the *Hha*I and $SacII^{(2)}$ sites, which appear to be sufficient for *XIST* repression.

Recently, lower expression of *XIST* in recurrent versus primary ovarian tumors has been reported, suggesting that directly or indirectly this gene may be important for the control of cell growth.[35] Thus, one cannot exclude that during the derivation of the *DNMT1/DNMT3B* DKO cells there could have been a selective advantage of those that maintained *XIST* methylation and transcriptional repression. Characterization of loss of hypermethylated CpG islands in the DKO cells leads to the identification of silenced tumor suppressor genes.[36] Conversely, the identification of the genomic regions that maintain DNA methylation in the *DNMT1/DNMT3B* DKO cells may point to genes whose transcriptional repression is essential for cell growth and/or viability.

In addition, since the 5-aza-dC experiments showed that *XIST* repression is dependent on DNA methylation

in the HCT116 cells, our data indicate that MTases other than DNMT1 and DNMT3B must be involved in the process of *XIST* methylation in these cells. Currently, DNMT2 and DNMT3A are the only other known candidate MTases for this process. However, as in *Arabidopsis*, where at least 10 different MTase genes have been identified (reviewed in Martienssen and Colot[37]), other unidentified DNA MTases may exist in mammals.

In conclusion, the observed discrepancy between maintenance of methylation of global DNA and of the *XIST* gene in the DKO HCT116 cells is unexpected since in mice these processes are mediated by the same enzyme, namely Dnmt1.[27,28] In the human DKO cells, although global DNA methylation is disrupted, *XIST* methylation at the *Hha*I and *Sac*II[(2)] sites is maintained. Therefore, our findings suggest that in humans different epigenetic mechanisms may control global and *XIST* gene expression and that *XIST* methylation can be mediated by other unknown factors. Additional experiments in normal human somatic cells using alternative gene inactivation approaches, such as siRNA, are required to confirm our observations and identify these factors.

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