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Expression of *DNMT3A* Transcripts and Nucleolar Localization of DNMT3A Protein in Human Testicular and Fibroblast Cells Suggest a Role for De Novo DNA Methylation in Nucleolar Inactivation

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Abstract Transcriptional silencing during differentiation of human male germ cells and serum starvation of human fibroblasts is controlled by epigenetic mechanisms that involve de novo DNA methylation. It is associated with high expression of different trancripts of the DNA methyltransferase 3A (*DNMT3A*) gene that encode two isoforms with de novo methyltransferase activity and one without catalytic activity. Western blots revealed that DNMT3A protein (with catalytic domain) is present at low levels in several tissues and at increased levels in testicular cells and growth-arrested fibroblasts. Immunofluorescence experiments localized DNMT3A to discrete nucleolar foci in B spermatogonia and resting fibroblasts. The data here suggest a role for de novo DNA methylation in nucleolar inactivation. J. Cell. Biochem. 98: 885–894, 2006. © 2006 Wiley-Liss, Inc.

Key words: DNMT3A; metabolic inactivation; nucleolus; rRNA gene silencing; spermatogonia

Methylation of 5′-cytosine residues in CpG dinucleotides is critical for regulating the temporally and spatially highly coordinated expression of genes. DNA methylation leads to posttranslational histone modifications and an inactive chromatin structure [Wolffe and Matzke, 1999; Jaenisch and Bird, 2003]. The cell type-specific genomic methylation patterns and levels in somatic cells are generally stable and heritable throughout many cell divisions. Because 5-methylcytosines (mC) cannot be incorporated directly into DNA, the appropriate DNA methylation patterns must be established and/or maintained by specific enzymatic activities. Four different DNA cytosine-5-methyltransferases with motifs essential for transmethylation activity, namely *DNMT1*/*Dnmt1*, *DNMT2*/*Dnmt2*, *DNMT3A*/*Dnmt3a*, and *DNMT3B*/*Dnmt3b*, have been identified in the finished human and mouse genomic sequences [Okano et al., 1998; Bestor, 2000]. Inactivation of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* in knockout mice caused hypomethylation of the genome and embryonic lethality, indicating that they are essential for mammalian development [Li et al., 1992; Okano et al., 1999]. Dnmt2 proteins may have a more specialized role in mammalian cells. So far, methyltransferase activity of mammalian Dnmt2 proteins has only been detected in vitro [Hermann et al., 2003] and in vivo in transgenic flies [Mund et al., 2004].

The maintenance methyltransferase Dnmt1 has a high affinity for hemimethylated sites that are generated transiently during DNA replication. It detects methylated CpGs in the parental DNA strand and adds methyl groups to the corresponding sites in the newly synthesized strand. Because Dnmt3a and Dnmt3b do not show this preference for hemimethylated CpG sites, they may have a function in de novo methylation [Hsieh, 1999; Okano et al., 1999;

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Aoki et al., 2001]. *DNMT3L* is related to the DNMT3 family, but lacks the catalytic motif of DNMT [Aapola et al., 2000]. It may be involved in a premeiotic genome scanning process in male germ cells that is important for de novo methylation of dispersed repeat sequences [Bourc'his and Bestor, 2004].

Both *Dnmt3a* and *Dnmt3b* were expressed at low levels in somatic cells and at high levels in embryonal stem (ES) cells and germ cells in which active de novo methylation occurred. However, the two enzymes seem to have different DNA targets and in vivo functions. Mutations in human *DNMT3B* cause ICF (immunodeficiency, centromeric heterochromatin instability, and facial anomalies) syndrome which involves extensive loss of methylation from pericentromeric satellite DNA sequences [Hansen et al., 1999; Xu et al., 1999]. The target sequences for the DNMT3A enzyme remain to be elucidated. In transfection assays, tagged Dnmt3b colocalized with pericentromeric heterochromatin of mouse ES cells, but produced a diffuse nuclear staining in embryonal fibroblasts. Dnmt3a was always enriched in the heterochromatin compartment [Bachman et al., 2001]. Interestingly, both Dnmt3b [Okano et al., 1998] and Dnmt3a [Chen et al., 2002] encode multiple isoforms by alternative splicing and alternative promoter usage, respectively. The full-length Dnmt3a1 protein and the smaller Dnmt3a2 isoform, which is initiated from an intronic promoter, exhibited similar methyltransferase activities in vitro, but different expression patterns and nuclear localizations in vivo. *Dnmt3a1* was expressed at low levels ubiquitously and the tagged protein was concentrated on constitutive heterochromatin. *Dnmt3a2* was highly expressed in ES and embryonal carcinoma cells and to a lesser extent in testis, ovary, thymus, and spleen. Overexpressed Dnmt3a2 was more or less uniformly distributed throughout the nucleus excluding heterochromatin, but also produced a weak cytoplasmic staining [Chen et al., 2002]. In this study we show that endogenous DNMT3A colocalizes with the nucleolus in cells that are less translationally active.

Nucleoli are the most prominent solitary or multiple structures in the interphase cell nucleus. They contain specialized chromosome regions called nucleolus organizer regions (NORs) in which the ribosomal rRNA genes are located [Schwarzacher and Wachtler, 1991; Moss and Stefanovsky, 2002]. Ribosomal RNA is synthesized at the NORs, deposited into the nucleoli, and later exported to the cytoplasm to become incorporated into ribosomes. Because many cell types need large amounts of rRNA, the rRNA genes have evolved into tandemly reiterated arrays to ensure functional redundancy. Only a subset of the several hundred *rRNA* gene copies in mammalian genomes needs to be active in a given cell type at a given time. This makes the rRNA genes an attractive model for studying the dynamic balance between gene silencing and activation, and the possible role of DNA methylation in this process [Grummt, 2003; Grummt and Pikaard, 2003]. In the course of metabolic inactivation of a cell, the number of rDNA transcription units decreases considerably, implying that formerly active rRNA genes are silenced for transcription [Haaf et al., 1991]. Similarly during spermatogenesis, transcriptionally active rRNA genes decrease in number until rRNA synthesis is completely blocked.

MATERIALS AND METHODS

Tissue and Cell Substrates

Human testicular samples were obtained from patients of proven fertility by open incision biopsy in association with an orchiectomy for therapeutic reasons. "Excess" materials from autopsy specimens of different human tissues were obtained 24 hr postmortem. Tissue samples were either used immediately for RNA, protein, and/or cell preparations or shock-frozen in liquid nitrogen and stored at -80°C. Primary human skin fibroblasts were cultured in D-MEM medium supplemented with 15% fetal bovine serum and antibiotics. To induce metabolic inactivation and arrest cells in G_0 phase, cultures were starved for several days in medium without serum.

Antibody Probes

Human autoimmune serum against the nucleolar fibrillar component was used as a cytological marker for the nucleolus [Reimer et al., 1987]. Mouse monoclonal antibody (IMG-268) against the C-terminus (amino acids 705–908) of mouse Dnmt3a was purchased from Imgenex. Rabbit polyclonal antiserum (sc-10753) against the C-terminus (amino acids 281–580) of human methyl-CpG binding domain protein 4 (MBD4) was obtained from Santa Cruz Biotechnology.

A well-characterized mouse monoclonal anti-mC antibody [Mayer et al., 2000; Santos et al., 2002] was obtained from Dr. Alain Niveleau (University of Grenoble). Horseradish-, FITC-, and Texas Red-conjugated secondary (anti-mouse, anti-rabbit, and anti-human IgG) antibodies were purchased from Santa Cruz Biotechnology.

Western Blots

Grinded tissue samples and fibroblast pellets were suspended in 300 μl NP40 lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% NP40) containing protease inhibitor complex cocktail (Roche). Cells were ruptured by forcefully pressing the suspension with a syringe through a thin needle and incubated for 30 min on ice. After centrifugation at 13,000 rpm for 30 min at 4°C the upper phase was stored at −80°C. Total protein extracts (120 μg each) were analyzed by electrophoresis on 12% SDS–PAGE gels. Proteins were then transferred onto nitrocellulose filters, which were blocked for 1 hr with 5% dried milk powder in TBST (Tris-buffered saline, pH 7.5, 0.1% Tween 20). Anti-Dnmt3a antibody, diluted 1:250 in TBST, was applied for 4 hr. Bound antibodies were detected after a second incubation with horseradish peroxidase-conjugated anti-mouse IgG, diluted 1:10,000 with TBST. The BM chemiluminescence Western blotting kit (Roche) was used to visualize and quantify the enzyme reaction. Coomassie blue staining was used as a loading control to compare the expression levels of DNMT3A protein in different cell substrates.

Immunofluorescence Staining

Spermatogenic cells were prepared from fresh testicular biopsies as described previously [Haaf et al., 1990, 1995]. Briefly, the seminiferous tubules were minced as finely as possible with small scissors in phosphate-buffered saline (PBS, pH 7.3). The resulting cell suspension was transferred to a centrifuge tube and allowed to settle for 10–15 min at room temperature. The supernatant was removed with a pipette and used as a source of testicular cells. This cell suspension was diluted further (1:10–1:100) with PBS. Aliquots containing 10^5–10^6 spermatogenic cells (not counting sperm) or fibroblast cells in 200 μl of PBS were centrifuged onto clean glass slides, using a Shandon Cytospin at 1,000 rpm for 4 min. After cytocentrifugation the slides were fixed in ice-cold methanol for 30 min, permeabilized for a few seconds in ice-cold acetone, air-dried, and then immediately used for immunofluorescence staining.

After blocking the preparations for 30 min in PBS containing 1% BSA, they were incubated with the primary anti-Dnmt3a, anti-MBD4, and/or anti-nucleolar antibodies, diluted 1:200 in PBS, 1% BSA in a moist chamber at 37°C for 1 hr. The slides were then washed 3× for 5 min in PBS and incubated with the appropriate fluorescent (FITC or Texas Red) labeled secondary (anti-mouse, anti-rabbit, and/or anti-human IgG) antibodies. After three further washes with PBS, the preparations were mounted in antifade solution (Vectashield, Vector Labs) containing 0.5 μg/ml DAPI (Sigma) for DNA counterstaining. For mC staining, cell preparations were denatured in 70% formamide, 2× SSC for 1 min at 80°C to make the mC-epitopes in the phosphodiester backbone of the DNA double helix accessible to antibody molecules and then dehydrated in an ice-cold ethanol series. After brief air-drying and blocking, the slides were first incubated with mouse anti-mC antibody (hybridoma supernatant, diluted 1:50 in PBS) for 30 min and then with FITC-conjugated anti-mouse IgG, appropriately diluted with PBS.

Images were taken with a Leica epifluorescence microscope equipped with a CCD camera, which was controlled by a personal computer. Gray scale source images were captured separately with filter sets for FITC, Texas Red, and DAPI. Gray scale images were pseudocolored and merged using Leica cw 4000 FISH and ADOBE Photoshop software. It is worth emphasizing that, although a digital imaging system was used, all signals were clearly visible by eye through the microscope.

Generation of Gene Fragments and Arrays for Expression Analysis

Human cDNA clones for *DNMT1* (IMAGE 95602327), *MBD4* (IMAGE 1648532), and *MBD2* (IMAGE 486689) were purchased from the Resource Center of the German Human Genome Project (RZPD). Several transcripts resulting from human *DNMT3A* have been reported (Fig. 1). Transcripts 1 and 2 encode the full-length 101 kDa protein (isoform a, DNMT3A1), transcript 3 the smaller 77 kDa isoform b (DNMT3A2), which also possesses transmethylation activity [Chen et al., 2002] and transcript 4 a 16 kDa isoform c (DNMT3A3)

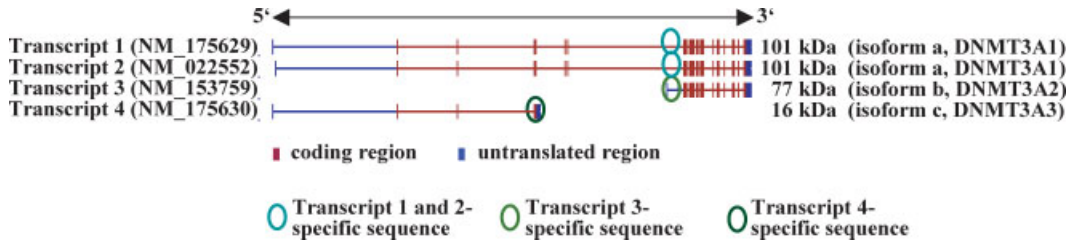

Fig. 1. Four transcripts encoding three different isoforms of DNMT3A. Green ellipses indicate transcript-specific sequences for reverse Northern blot analyses.

without catalytic activity. Primer pairs DNMT3A-TR1/2 (forward, 5′-AGG GCT CCC GGG GCC GGC-3′; reverse 5′-TCC CTT TTC CAG CGT GCC-3′), DNMT3A-TR3 (forward, 5′-CCC ATA AGG CCA GGT GCA GC-3′; reverse, 5′-CCC CCA GCC CCA TCG CC-3′), and DNMT3A-TR4 (forward, 5′-TGA GTC CTC AGC ACC AGG GG-3′; reverse, 5′-TCC AGC CAG TAG CCT CC-3′) were used to amplify isoform-specific gene fragments from genomic DNA of the *DNMT3A*-containing BAC clone RP11-458N5 (RZPD). PCR products were cloned into pCR2.1 vector using the TOPO-TA cloning kit (Invitrogen). Gene- or isoform-specific inserts were amplified with M13 forward and reverse primers using standard conditions and sequence verified.

Gene arrays for reverse Northern blot experiments were fabricated by spotting 2 μl of aliquots (200–400 ng) of purified PCR product of genes of interest in quadruplicates onto nylon filters. The low spot density allowed one to discriminate background hybridization intensity (indicated by squares in Fig. 2A) versus spot intensity (indicated by circles in Fig. 2A) for each spot. PCR products of two abundantly expressed housekeeping genes (*ACTIN* and *GAPDH*) and 1:100 dilutions of the unlabeled cDNA sample (corresponding to the hybridization probe) were spotted as controls.

Reverse Northern Blots

For reverse transcription, the reaction mixture containing 30 μg of total RNA, 4 μl of random primer (Roche), and 4 μl of 5× reverse transcriptase buffer was incubated for 10 min at 70°C and then for 15 min at room temperature. After adding 2 μl of dNTPs (5 mM each), 2 μl 0.1 M DTT, 0.5 μl of RNAse inhibitor, and 1 μl of reverse transcriptase (Invitrogen), the mixture was incubated for 2 hr at 37°C. Then, 1 μl of RNAse H was added and incubated for 20 min at 37°C. The reaction was performed in a 30 μl volume. The resulting 9–12 μg cDNA were purified with a QIAquick PCR purification kit (Qiagen). Approximately one half (15 μl) of the cDNA sample was labeled with digoxigenin using the DIG DNA labeling kit (Roche) and the other 15 μl were used for filter generation. The unlabeled cDNA was diluted 1:100 before spotting 2 μl aliqouts (6–8 ng) onto filters.

Filters (gene arrays) were hybridized overnight at 42°C with digoxigenated cDNA probe

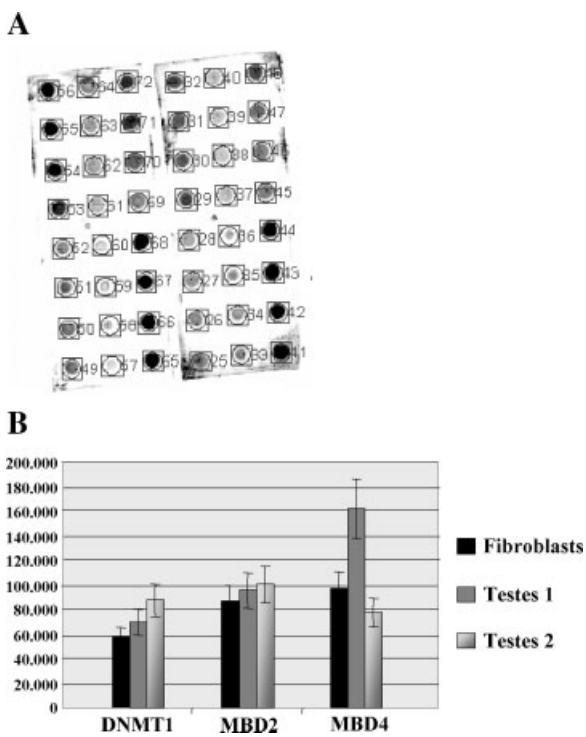

Fig. 2. Reverse Northern blot analysis of *DNMT1*, *MBD2*, and *MBD4* in testicular cells and subconfluent fibroblasts: (**A**) Two representative examples of a reverse Northern blot. Each gene of interest and each control is spotted in quadruplicates. For example, *ACTIN* at positions 37–40 and 61–64, *GAPDH* at 45–48 and 69–72, diluted cDNA at 41–44 and 65–68. **B**: Relative mRNA expression levels of *DNMT1*, *MBD2*, and *MBD4* in fibroblasts and two different testicular biopsies. Y-axis represents the average pixel intensity of spots of a given gene after normalization and background substraction.

dissolved in 5 ml of hybridization mixture (0.5 M NaH_2PO_4, 1 mM EDTA, pH 7.2, 7% SDS, and 1% BSA). Then they were washed 2× for 15 min at 50°C in 2× SSC, 1% SDS and 2× for 15 min at 50°C in 0.5× SSC, 1% SDS. The DIG luminescent detection kit (Roche) was used for signal detection. Auto-exposure of all filters was done with an AIDA chemiluminescence imager. The resulting TIFF files were imported into the AIDA image analysis program 3.40 (Raytest). The background intensity surrounding each spot was subtracted from the signal (spot) intensity. Negative intensity values were called "no signal."

Arrayed *ACTIN*, *GAPDH*, and cDNA spots served as controls to determine hybridization efficiency and to normalize hybridization results between different filters (Fig. 2A). Consistent with other studies [Dheda et al., 2004], we found *ACTIN* and *GAPDH* expression levels to considerably vary between cell substrates and, therefore, normalized the hybridization intensities of all spots on a particular filter to the amount of cDNA. The cDNA spot intensity after hybridization of the same cDNA sample correlates well with the amount of cDNA used in this experiment. The intensities of the four spots for each gene or cDNA sample on the blot were averaged and the standard deviation was calculated. To compare the hybridization intensities between two different filters, the average cDNA spot intensity on one filter was divided by that on the other filter. The resulting ratio was used as normalization factor for all genes on the same pair of filters.

RESULTS

Expression of Methyltransferases and mC-Binding Proteins in Human Tissues

Reverse Northern blots of total RNAs from a subconfluent fibroblast culture and two different testicular biopsies demonstrated that the maintenance methyltransferase *DNMT1* and the mC-binding domain proteins MBD2 and MBD4, which transmit the epigenetic information in DNA methylation patterns to downstream regulatory proteins [Ballestar and Wolffe, 2001; Jaenisch and Bird, 2003], are expressed at comparable levels in human fibroblasts and testicular cells (Fig. 2B). The reproducible differences in *MBD4* expression between the two testes samples studied may be explained by interindividual differences, that is, in the percentages of premeiotic, meiotic, and postmeiotic cell types.

Database analysis (http://www.ncbi.nlm.nih.gov/; Gene ID1788) revealed the existence of four transcripts of de novo methyltransferase *DNMT3A* (Fig. 1). Transcripts 1 and 2, which cannot be distinguished by reverse Northern blots, encode the full-length DNMT3A1 protein. Transcript 3 encodes the smaller DNMT3A2 isoform, which has similar methlytransferase activity as DNMT3A1 [Chen et al., 2002]. Reverse Northern blot analysis showed that the smaller and the larger isoforms with methyltransferase activity are expressed at equivalently high levels in human testes. Transcript 4 which lacks the catalytic motif of DNMTs is expressed at considerably lower levels (Fig. 3A).

Interestingly, Western blots of total protein extracts from four different human testes with

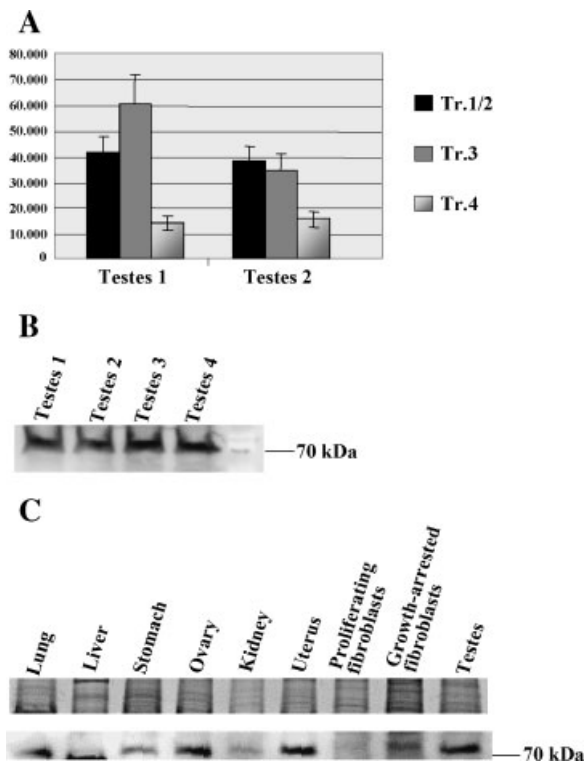

Fig. 3. Expression of *DNMT3A* transcripts and of DNMT3A protein in testicular cells: (**A**) Relative expression of the different *DNMT3A* transcripts in two testicular biopsies. Y-axis represents the average pixel intensity of spots of a given transcript on normalized reverse Northern blots. **B**: Western blot analysis of total protein extracts from four different testicular biopsies reveals a strong band at 77 kDa that corresponds to DNMT3A2. **C**: Coomassie blue staining (**upper panel**) and Western blot analysis (**bottom panel**) of protein extracts from different human cell substrates.

an extensively characterized anti-Dnmt3a antibody [Chen et al., 2002, 2003, 2004] revealed a very prominent DNMT3A2 band at approximately 77 kDa (Fig. 3B), whereas the full-length DNMT3A1 protein at 101 kDa was hardly detectable (data not shown). Because this antibody was raised against the catalytic domain of mouse Dnmt3a which is shared by the Dnmt3a1 and Dnmt3a2 isoforms and highly conserved between mouse and humans, it should recognize both mouse and human Dnmt3a1/DNMT3A1 and Dnmt3a2/DNMT3A2, and in addition several Dnmt3b/DNMT3B isoforms. A multi-tissue Western blot (Fig. 3C) of total protein extracts from human lung, liver, kidney, stomach, uterus, ovary, testes, as well as fibroblasts under different culture conditions demonstrated that DNMT3A protein is expressed at variable levels ubiquitously. The highest DNMT3A levels were detected in ovary, testes, and growth-arrested fibroblasts. Again the full-length DNMT3A1 protein produced only a faint band at 101 kDa. At least on human Western blots, the antibody to mouse Dnmt3a seems to have a higher affinity for the smaller isoform than for the full-length protein. The 16 kDa DNMT3A3 isoform resulting from transcript 4 lacks the antibody binding site and, therefore, is not detectable at all.

Localization of DNMT3A in Human Spermatogonia

The anti-Dnmt3a antibody was also used to directly visualize the catalytic domain of DNMT3A in human cells, for example during differentiation of male germ cells. Because we were mainly interested in the subcellular localization of DNMT3A, immunofluorescence staining was performed on individual testicular cells, which were centrifuged in suspension onto glass slides. The different premeiotic, meiotic, and postmeiotic cell types were easily identified after DAPI counterstaining by their size, nuclear morphology, heterochromatic chromocenters, and/or nucleolar structure [Brinkley et al., 1986; Haaf et al., 1990]. In contrast to an earlier study [Chen et al., 2002], which localized GFP-tagged Dnmt3a1 and Dnmt3a2 proteins to heterochromatin and euchromatin of transfected NIH3T3 cells, respectively, most human testicular cell types in our preparations did not show detectable DNMT3A staining. We conclude that endogenous DNMT3A expression in these cells was too low for immunocytochemical visualization. However, about 30% of the cells with large nuclei and prominent nucleolus in the nuclear center displayed 10–15 brightly fluorescing foci within the solitary nucleolus (Fig. 4A). Two percent also exhibited a weak overall nucleolar staining, which was clearly above background staining (Fig. 4B). In DAPI-stained controls, in which the first (anti-Dnmt3a) antibody was ommitted, the nucleolus always displayed a "negative" fluorescence staining, because it mainly consists of nucleolar RNA and proteins (Fig. 4C). On the basis of cytological characteristics, the DNMT3A foci-positive cells were identified as spermatogonia of type B. In A spermatogonia which represent the germinal stem cells, the nucleolus is always

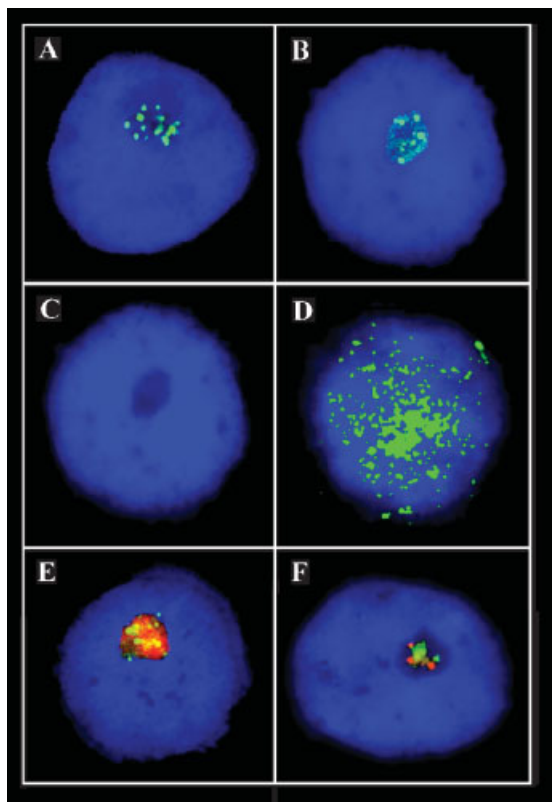

Fig. 4. Localization of DNMT3A, MBD4, and methylated CpGs in testicular cells: (**A**, **B**) Immunolocalization of DNMT3A protein (green FITC fluorescence) in the nucleolus of human B spermatogonia. Nuclear DNA (blue) is counterstained with DAPI. **C**: DAPI staining alone produces a ''negative'' nucleolar staining. **D**: Methylcytosine (mC) staining (green) shows an enrichment of the nucleolar domain with methylated CpGs. **E**: Double immunofluorescence staining of the nucleolus (Texas red fluorescence) and MBD4 protein (green). **F**: Simultaneous immunolocalization of MBD4 (green) und DNMT3A (red) in the nucleolus.

located at the nuclear periphery. B spermatogonia, which stop rDNA transcription and enter meiosis, are endowed with one central nucleolus [Hartung et al., 1990]. Because the affinity of anti-Dnmt3a antibody to proteins within fixed cells may differ from that to denatured proteins on Western blots, it not clear whether the nucleolar protein detected by immunofluorescence staining represents mainly the DNMT3A2 isoform. Nucleolar foci were not stained with an antiserum against DNMT3B (data not shown). We therefore conclude that a DNMT3A isoform(s) with transmethylation activity, DNMT3A1 and/or DNMT3A2, accumulates in the nucleolus of B spermatogonia prior to meiotic differentiation.

Following anti-mC antibody staining of denatured preparations, methylated DNA sequences appear as strongly fluorescing foci throughout the nucleus [Mayer et al., 2000]. The nucleolar domain in B spermatogonia was highly methylated compared with the rest of the nucleus (Fig. 4D). mC-binding proteins recruit histone deacetylation and chromatin remodeling complexes to methylated regions in the human genome to repress transcription [Ballestar and Wolffe, 2001; Jaenisch and Bird, 2003]. MBD4, one of the essential components involved in epigenetic silencing [Kondo et al., 2005], immunolocalized to distinct sites in the nucleolus of B spermatogonia (Fig. 4E, green signals), which was simultaneously stained with human anti-nucleolar autoantibodies (Fig. 4E, red signals). Double immunofluorescence staining localized MBD4 (Fig. 4F, green signals) and DNMT3A (Fig. 4F, red signals) to different sites within the highly substructured nucleolus of B spermatogonia [Hartung et al., 1990]. Collectively, our results suggest that de novo methyltransferase DNMT3A is involved in methylation of nucleolar rDNA in B spermatogonia. Binding of MBD4 to methylated rDNA may be necessary for chromatin modification and nucleolar inactivation before the onset of meiosis.

Behavior of DNMT3A During Metabolic Inactivation of Fibroblasts

Nucleolar morphology and transcriptional activity largely depend on the metabolic state of the cell. This has been demonstrated by classical silver staining of nucleolar proteins [Derenzini et al., 1989; Schwarzacher and Wachtler, 1991] and immunofluorescence staining of RNA polymerase I transcription units [Haaf et al., 1991]. Serum starvation of exponentially growing fibroblast cells for several days goes parallel with growth arrest (in G_0 phase of the cell cycle), metabolic inactivation of the whole cell, and low trancriptional activity of rRNA genes.

Reverse Northern blots of total RNAs from parallel human fibroblast cultures under exponential growth and serum starvation conditions showed an approximately twofold increase of *DNMT3A* transcripts 1/2 and 3 during metabolic inactivation (Fig. 5A). Interestingly, transcript 4 which lacks the motif for transmethylation activity was expressed at very low levels, if at all in metabolically active cells and drastically upregulated in growth-arrested cells. Consistent with these results, Western blots showed an approximately twofold increase in DNMT3A protein in growth-arrested fibroblasts (Fig. 5B).

Double-immunofluorescence staining with anti-Dnmt3a (Fig. 6, green signals) and anti-nucleolar (Fig. 6, red signals) antibodies demonstrated accumulation of DNMT3A in

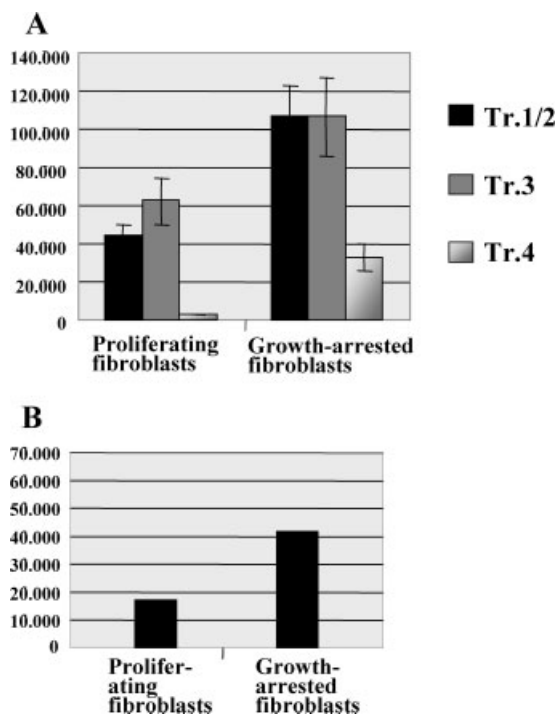

Fig. 5. Increased *DNMT3A* RNA and DNMT3A protein levels in growth-arrested fibroblasts: (**A**) Relative expression of the different *DNMT3A* transcripts in proliferating and growth-arrested fibroblasts, as measured by reverse Northern blots. Y-axis represents the average pixel intensity of spots of a given transcript after normalization and background subtraction. **B**: Relative amount of DNMT3A protein in proliferating and growth-arrested fibroblasts, as measured by Western blots.

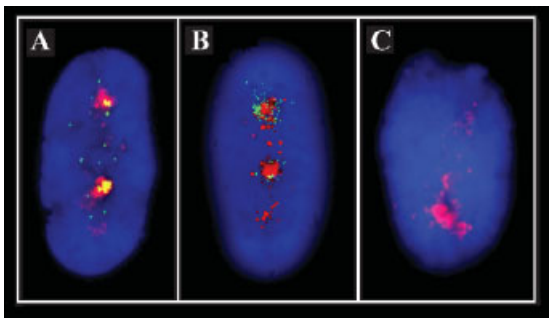

Fig. 6. Double immunofluorescence staining of DNMT3A (green FITC fluorescence) and nucleolus (Texas red fluorescence) in growth-arrested (**A**) and proliferating (**B**, **C**) fibroblasts. Nuclei are counterstained with DAPI.

the nucleolus of transcriptionally inactive cells. In three independent experiments, 70%–80% of the serum-starved cells displayed 12–15 brightly fluorescing DNMT3A foci in the nucleolar domain and often also a few (approximately five) foci in the nucleoplasm (Fig. 6A). In contrast, <10% of exponentially growing fibroblasts contained immunocytochemically detectable amounts of DNMT3A protein (Fig. 6B). The vast majority of cycling cells showed only anti-nucleolar staining, but no DNMT3A foci (Fig. 6C). The signal intensity of nucleolar foci in fibroblast cells was comparable to that in spermatogonia.

DISCUSSION

Reverse Northern blots provide an inexpensive, fast, and accurate method for the quantification of RNA expression. When genes of interest were spotted in quadruplicates, the standard deviation of the measured spot intensites for each gene was relatively low (5%–10%). In our experience, 25% differences in mRNA levels between cell substrates can be reliably detected. We showed that transcripts for three different (16, 77, and 101 kDa) isoforms of de novo methlytransferase DNMT3A are expressed in testicular cells, which represent a mixture of premeiotic, meiotic, and postmeiotic cell types. Using an anti-Dnmt3a antibody, which on Western blots preferentially reacted with the 77 kDa isoform of human DNMT3A, we immunolocalized endogenous DNMT3A to the nucleolus of B spermatogonia, which also contained high concentrations of methylated CpGs and mC-binding proteins. Because antibody-binding properties in fixed cells and on Western blots may not be identical, we cannot say for sure which isoform(s), DNMT3A1 and/or DNMT3A2, is concentrated in nucleolar foci. However, it must be an isoform(s) with methyltransferase activity, strongly suggesting a role for de novo methylation in suppression of rRNA genes during male germ cell differentiation. An association between DNMT3A expression and nucleolar activity was also observed in cultured fibroblasts. Prolonged serum starvation induced upregulation of all *DNMT3A* transcripts and localization of DNMT3A protein to nucleolar foci. It is interesting to note that transcript 4 which encodes a DNMT3A isoform without catalytic activity is expressed in testicular cells and resting fibroblasts, but not at a significant level in cycling cells. Although this isoform cannot directly methylate DNA sequences, it may have a regulatory role in de novo methylation.

Transcription of rRNA genes is essential for the production of cytoplasmic ribosomes containing rRNA, which are required for protein synthesis. Consequently, the highest transcriptional nucleolar activity is seen in proliferating cells and the lowest in resting cells [Schwarzacher and Wachtler, 1991; Moss and Stefanovsky, 2002]. Nucleolar activity is regulated at two levels, firstly by controlling the number (dosage) of active versus inactive rRNA genes and secondly by modulating the RNA polymerase I initiation frequency among the active rRNA genes [Grummt, 2003; Grummt and Pikaard, 2003]. So far the mechanisms responsible for switching rRNA genes on or off are poorly understood. Several lines of evidence suggest suppression of rRNA genes by methylation. Stable (heritable) inactivation of amplified rRNA genes in increased human NORs was accompanied by an immunocytochemically detectable rDNA hypermethylation [Miller et al., 1976]. Transcriptionally active NORs in human cells were generally less methylated than inactive ones [Ferraro and Prantera, 1988]. In *Xenopus*, activation of a major part of the rRNA genes during early development was accompanied by loss of rDNA methylation [Bird et al., 1981]. In *Arabidopsis*, experimental DNA demethylation and histone deacetylation increased the number of active rRNA genes [Lawrence et al., 2004]. In plant hybrids in which only the NORs of one parental species are active (nucleolar dominance), concerted changes in DNA methylation/chromatin structure

may act as an epigenetic switch that can turn the parental sets of rRNA genes on and off [Lawrence and Pikaard, 2004]. Immunoprecipitation experiments showed that the SNF2h- and TIP5-containing nucleolar remodeling complex (NoRC) recruits histone deacetylase (HDAC1) and DNA methyltransferase (DNMT1 and DNMT3B) activities to the rDNA promoter and, thus, leads to epigenetic silencing of the rRNA genes [Santoro et al., 2002]. NoRC-mediated histone modification seems to precede de novo methylation of specific CpG dinucleotides [Santoro and Grummt, 2005]. So far it is not know whether the NoRC also interacts with DNMT3A. The results of this study suggest that the de novo methyltransferase DNMT3A is a component of the regulatory network that controls the dosage of active rRNA genes.

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