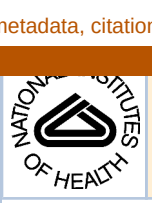

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Choline availability modulates human neuroblastoma cell proliferation and alters the methylation of the promoter region of the cyclin-dependent kinase inhibitor 3 gene

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Abstract

Choline is an important methyl donor and a component of membrane phospholipids. In this study, we tested the hypothesis that choline availability can modulate cell proliferation and the methylation of genes that regulate cell cycling. In several other model systems, hypomethylation of cytosine bases that are followed by a guanosine (CpG) sites in the promoter region of a gene is associated with increased gene expression. We found that in choline-deficient IMR-32 neuroblastoma cells, the promoter of the cyclin-dependent kinase inhibitor 3 gene (CDKN3) was hypomethylated. This change was associated with increased expression of CDKN3 and increased levels of its gene product, kinase-associated phosphatase (KAP), which inhibits the G_1/S transition of the cell cycle by dephosphorylating cyclin-dependent kinases. Choline deficiency also reduced global DNA methylation. The percentage of cells that accumulated bromodeoxyuridine (proportional to cell proliferation) was 1.8 times lower in the choline-deficient cells than in the control cells. Phosphorylated retinoblastoma (p110) levels were 3 times lower in the choline-deficient cells than in control cells. These findings suggest that the mechanism whereby choline deficiency inhibits cell proliferation involves hypomethylation of key genes regulating cell cycling. This may be a mechanism for our previously reported observation that stem cell proliferation in hippocampus neuroepithelium is decreased in choline-deficient rat and mouse fetuses.

Keywords

choline deficiency; DNA methylation; IMR-32; neuronal development

Abbreviations used

AdoMet, S-adenosylmethionine; 5-Aza, 5-Azacytidine-treated cells; BrdU, bromodeoxyuridine; CD, choline deficiency or choline deficient; Cdk, cyclin-dependent kinase; Cdk2, cyclin-dependent kinase 2; CDKN3, cyclin-dependent kinase inhibitor gene; CT, choline control; E2F1, E2F1 transcription factors; KAP, cyclin-dependent kinase inhibitor (protein product of CDKN3 gene); PBS, phosphate-buffered saline; ppRb, phosphorylated retinoblastoma protein; pRb, retinoblastoma protein

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Choline or its derivatives are important for the structural integrity of cell membranes, methyl metabolism, cholinergic neurotransmission and transmembrane signaling, as well as for lipid-cholesterol transport and metabolism (Zeisel and Blusztajn 1994). It is required for normal brain development; dietary choline supplementation of rat and mouse dams increases stem cell proliferation, while choline deficiency decreases stem cell proliferation in fetal hippocampus and septum (Albright *et al*. 1999a,b). These changes have important functional effects, as pups from choline-supplemented dams have enhanced memory (Meck *et al*. 1988; Meck and Williams 1997a,b) and long-term potentiation (an electrophysiological property of the hippocampus) (Jones *et al*. 1999). Pups born of choline-deficient mothers have decreased memory (Meck and Williams 1999) and long-term potentiation (Montoya *et al*. 2000). The mechanism whereby choline modulates cell proliferation and neurogenesis has not been previously determined.

Choline is a major dietary source for labile methyl groups (Zeisel and Blusztajn 1994), and via its metabolite betaine, acts as methyl donor to homocysteine to form methionine (Finkelstein 2000). Methionine is the precursor of *S*-adenosylmethionine (AdoMet), which is the physiological substrate for protein and DNA methylation (Jeltsch 2002). Dietary choline and methyl group deficiency not only deplete choline and choline metabolites in rats, but also decrease AdoMet concentration (Shivapurkar and Poirier 1983; Zeisel *et al*. 1989), with resulting hypomethylation of DNA (Locker *et al*. 1986; Bhave *et al*. 1988; Dizik *et al*. 1991; Wainfan and Poirier 1992; Tsujiuchi *et al*. 1999). DNA methylation occurs at cytosine bases that are followed by a guanosine (CpG sites) (Holliday and Grigg 1993) and influences many cellular events, including gene transcription, genomic imprinting and genomic stability (Jaenisch 1997; Jones and Gonzalgo 1997; Robertson and Wolffe 2000). In mammals, about 60–90% of 5′-CpG-3′ islands are methylated (Jeltsch 2002). When this modification occurs in promoter regions, gene expression is altered (Bird *et al*. 1985; Bird 1986); increased methylation is associated with gene silencing or reduced gene expression (Jeltsch 2002).

The effects of choline deficiency on cell proliferation and apoptosis have been noted in multiple neuronal model systems (Albright *et al*. 1999b; Yen *et al*. 1999, 2001; Holmes-McNary *et al*. 2001), with cell cycle arrest usually occurring in the G_1/S and G_2/M transitions (Holmes-McNary *et al*. 2001). Cyclin-dependent kinase inhibitor 3 gene (CDKN3), via its protein product kinase-associated phosphatase (KAP), is an important regulator of progression through the cell cycle (Hannon *et al*. 1994; Barford 2001; Johnson *et al*. 2002). It regulates the retinoblastoma protein (pRb) activation via a phosphorylation mechanism that is responsible for G_1 to S transition (Dyson 1998). We conducted these studies to determine whether overexpression of CDKN3 resulted from hypomethylation of its promoter region during choline deficiency.

Experimental procedures

All reagents were purchased from Sigma, St. Louis, MO, USA if not otherwise specified.

Cell culture

IMR-32 human neuroblastoma cells (ATCC, Manassas, VA, USA) at passage 57 were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA) and 1% Antibiotic-Antimycotic (Gibco) containing penicillin 10 000 units/mL, streptomycin 10 000 μg/mL and amphotericin B. After cells reached 30% confluence, the medium was changed to a defined medium containing DMEM/F12 without choline chloride (Atlanta Biologicals, Norcross, GA, USA), with added 5 μM choline chloride (choline deficient, CD), 70 μM choline chloride (control, CT) or 70 μM choline chloride + 5 μM 5-Azacytidine (5-Aza) and the following ingredients (Yen *et al*. 1999): bovine serum albumin 0.08% (Invitrogen), linolenic acid 0.04%,

sodium oleate 24 mg/4 L, phosphate-buffered saline (PBS) 80 mL/4 L, L-methionine 17.24 mg/L, sodium bicarbonate 2.2 g/L, HEPES 1.192 g/L, progesterone 0.62 mg/L, putresceine 16.6 mg/L, selenium 0.039 mg/L, L-thyroxine 0.4 mg/L, triiodothyronine 0.378 mg/L, transferrin 100 mg/L, insulin 10 mg/L and 1% Antibiotic-Antimycotic. The cells were incubated for 48 h either in CD, CT or 5-Aza defined medium, then harvested, washed three times with PBS and pelleted for immediate use or stored at −80°C. For *in situ* immunostaining, cells were grown in the same conditions but in plastic chamber slides (Nalge Nunc International, Naperville, IL, USA).

Bromodeoxyuridine (BrdU) incorporation

The cells were incubated with 10 μM BrdU for 30 min prior to harvesting and fixed in 4% paraformaldehyde in PBS. BrdU-positive cells were identified by immunohistochemistry using a BrdU Staining Kit (Oncogene, San Diego, CA, USA) according to the manufacturer's protocol, where the positive cells were stained with diaminobenzoic acid (DAB) and all cells were counterstained with methyl-green. Four plates of cells were used for each condition (CD and CT, at 48 h of treatment). The slides were coded so that the investigator was blind to the sample's treatment, and were analyzed with an Olympus BX50 microscope at 40× magnification. The BrdU-positive cells were reported as mean percent of the total number of cells counted for each sample ± standard error of the mean.

Oligonucleotide microarray analysis

Total RNA (40 μg) was extracted from six plates for each condition (CD and CT, IMR-32 cells at 48 h incubation) using TRIzol Reagent (Invitrogen), and the RNA concentration was verified by spectrophotometry. The total RNA was reverse transcribed into cDNA with Cy3 (70 μM choline as reference) or Cy5 (5 μM choline as experiment) monoreactive fluorophors using the CyScribe First-Strand cDNA Labeling Kit (Amersham Biosciences, Inc., Piscataway, NJ, USA). Finally, both cDNA samples were hybridized to a Compugen 20 000 spot V2 Human Microarray (UNC Genomics Core, UNC, Chapel Hill, NC, USA). A total of three independent experiments (three arrays) were performed. Purification of probes, hybridization (65°C, overnight) and washing were carried out according to the protocol provided by the UNC Genomics Core & Microarray Facility (http://genomicscore.unc.edu). Fluorescent images of the microarrays were collected using a GenePix 4000 fluorescent scanner (Axon Instruments, Inc., Union City, CA, USA). Gridding and primary data acquisition were performed using GenePix Pro Microarray Acquisition software. Data normalization, statistics and other data transformation were performed using GeneSpring 5.1 software (Silicon Genetics, Redwood City, CA, USA). The normalization used the positive control spots for glyceraldehyde-phosphate dehydrogenase (GAPD). Lowess normalization was used to compare with the GAPD-normalization. Gene expression levels were considered significantly different between CD- and CT-treated cells if we observed an average $\log_{(2)}$ ratio of more than 1.45 or less than −1.45 (CD/CT ratio above 2 or below 0.5).

Combined bisulfite restriction analysis (COBRA)

Genomic DNA was extracted from cells using a FlexGene DNA Kit (Qiagen, Valencia, CA, USA). In the final step, the extracted DNA was resuspended in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) and stored at − 20°C until used. COBRA was performed as previously described (Xiong and Laird 1997). Briefly, 1 μg genomic DNA was treated with sodium bisulfite followed by PCR amplification of the region of interest and restriction digestion. Genomic DNA was modified using a CpGenome DNA Modification Kit (Intergen Co., Purchase, NY, USA). All primer pairs for PCR analysis of CDKN3 CpG island regions were designed with reference to the *H. sapiens* chromosome 14 working draft sequence segment (GenBank accession number NT025892) and purchased from Invitrogen. The primers designed

for the amplification of wild DNA were: CDKN3/WL-1, 5′-CCAGTGCCGCCTCCCTCTGC-3′ (anti-sense primer) and CDKN3/WU-1, 5′-GGAGGGTGAGGCGGAGACT-3′ (sense primer). The primers for bisulfite-modified DNA were: CDKN3/ML-1, 5′-CCAATACCACCTCCCTCTAC-3′ (anti-sense) and CDKN3/MU-1, 5′-GGAGGGTGAGGTGGAGATT-3′ (sense), and CDKN3/ML-2, 5′-CCAATACCGCCTCCCTCTAC-3′ (anti-sense) and CDKN3/MU-2, 5′-GGAGGGTGAGGCGGAGATT-3′ (sense). Wild and bisulfite-modified DNAs were amplified by an initial incubation at 94°C for 2 min followed by 30 cycles (for wild DNA) or 40 cycles (for modified DNA) of 94°C for 30 s, 65°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min using Platinum PCR SuperMix (Invitrogen). Aliquots (30 μL) of the PCR-amplified products were digested with restriction enzymes, 40 units of *BstU I* at 60° C for 4 h (5′…cg^cg…3′; New England Biolabs, Beverly, MA, USA). Then, the products were subjected to electrophoresis on a 3% agarose gel and visualized by SYBR Green I (BMA).

Bisulfite sequence analysis

To confirm the changes in CDKN3 promoter methylation between CD and CT cells observed using COBRA, bisulfite sequencing was performed on the CpG island spanning the position −408 to −125 (numbered from the transcriptional start site). Primers were purchased from Qiagen: CDKN3-UP (sense wild type), 5′-GCACTCCAGCCTGGGCAACAAGAG-3′; CDKN3-UM (sense modified), 5′-GTATTTTAGTTTGGGTAATAAGAG-3′; CDKN3-LOW (anti-sense wild type), 5′-TCTTCCAGCAACCAATCAGATCC-3′; CDKN3-LM (anti-sense modified), 5′-TTTTTTAGTAATTAATTAGATTT-3′. Wild and bisulfite-modified DNAs were amplified by an initial incubation at 94°C for 2 min followed by 40 cycles of 94°C for 30 s, 57°C for 60 s, 72°C for 1 min, and a final extension at 72°C for 10 min using Platinum PCR SuperMix (Invitrogen). Four independent PCR samples from each treatment (CD, CT, 5-Aza), using bisulfite-modified or wild-type DNA template, were sequenced using an ABI 3100 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) at the UNC-CH Automated DNA Sequencing Facility. The products were sequenced in both directions using both primers for each PCR product. Retrieved sequences were analyzed using the Chromas 2.23 software (Technelysium Ptd Ltd, Tewantin, Qld, Australia). Complete conversion of cytosine to thymidine was confirmed as all cytosine from non-CG sites was converted to thymidine. We identified unmethylated, methylated and incompletely methylated sites. The latter sites were identified using the chromatograms where, while the sequence for the wild-type DNA was unequivocally determined, the bisulfite-modified DNA PCR products presented mixed signals for C and T nucleotides (in various proportions), indicating that in a given sample, the site was methylated on some strands but not on others.

Protein preparation and western blot analysis

Four samples were used for each condition (CD and CT at 48 h). The cells were harvested, washed three times in PBS and resuspended in lysis buffer [1% sodium dodecyl sulfate (SDS), 1.0 mM sodium ortho-vanadate, 10 mM Tris pH 7.4]. Protein concentration was determined by the Lowry method (Lowry *et al.* 1951). A 10 μg aliquot of protein from each sample was treated with 2× electrophoresis buffer (250 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue and 2% β-mercaptoethanol) and boiled for 5 min. The samples were loaded on a polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and subjected to electrophoresis for 50 min. The transfer was carried out on a nitrocellulose membrane (Amersham) at 8 V for 75 min (Spector *et al*. 1997). The membrane was incubated in blocking buffer (Sigma, B-6429) for 2 h at room temperature (RT) and left overnight at 4°C in blocking buffer containing one of the following antibodies: rabbit KAP (C-18), diluted 1 : 2000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse β-actin diluted 1 : 1000 (Abcam, Cambridge, MA, USA, ab6276); mouse HRP-conjugated pRb (IF8) diluted 1 : 500 (Santa Cruz Biotechnology, sc-102). The membranes were washed three times in PBS with 0.1% Tween 20. The samples

for KAP and actin measurement were incubated for 2 h at room temperature with either a goat anti-rabbit or a goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) diluted 1 : 2000 in blocking buffer. After washing three times in PBS with 0.1% Tween 20 and washing once in distilled water, the membranes were briefly exposed to a chemiluminescent agent, Luminol (Santa Cruz Biotechnology). The final images were acquired by exposing the membrane to a Kodak BioMax Film for 30–60 s. The film was scanned and the integrated optical densities of the bands were measured using the ScionImage software (Scion Corporation, Frederick, MD, USA).

Immunohistochemistry

IMR-32 cells grown in plastic chamber slides were fixed in 4% paraformaldehyde for 15 min and subsequently washed in PBS. Two different sets of slides were blocked for 2 h at RT with Blocking Buffer (Sigma, B-6429), then incubated overnight with retinoblastoma protein (pRb) antibody (Santa Cruz Biotechnology, sc-102) and phosphorylated retinoblastoma protein (ppRb) antibody, (Thr-160 specific Sc-1661; Santa Cruz Biotechnology), respectively, each diluted 1 : 200 in blocking buffer. After the incubation the slides were rinsed in PBS with 0.1% Tween 20 three times for 20 min each and incubated for 2 h at RT with fluorescent secondary antibodies: Alexa-Fluor anti-mouse (Molecular Probes, A-21201) and Alexa-Fluor anti-goat (Molecular Probes, A-21468). After 3 washes in PBS with 0.1% Tween 20, the slides were incubated for 20 min in a PBS solution containing 4′, 6-diamidino-2-phenylindole (100 ng/100 mL) for nuclei counterstaining. Images were acquired with an Olympus BX50 microscope at 40× magnification, with optical filters for UV light and red channel (above 600 nm). The protein levels were measured by assessing the Integrated Optical Density (IOD) of each image, divided by the number of cells, using ScionImage software (Scion Corporation). Finally, the average value of IOD/cell for each image from the same sample was computed. Four different slides were used for each condition (CD and CT).

Global DNA methylation

Analysis was performed using a protocol described elsewhere (Hernandez-Blasquez *et al.* 2000). Briefly, after denaturing the DNA and non-specific blocking, a goat anti-5-methylcytosine antibody (Megabase Research, Lincoln, NE, USA) was used to incubate overnight the cells grown in chamber slides. A secondary fluorescent (mouse anti-goat, Molecular Probes) was used, and images collected and data analyzed as previously described (see immunohistochemistry section). Four samples from each treatment (CD, CT and 5-Aza) were used.

Statistics

Students' *t*-test was used (JMP 3.2.6, SAS Institute INC, Cary, NC, USA) to determine statistical significance between the treatment group (CD) and control (CT) at 48 h for BrdU incorporation, Western blot assay and immunohistochemistry, and between CD or 5-Aza and CT, respectively, for global DNA methylation.

Results

DNA in CD cells was globally hypomethylated compared with CT cells (as determined by immunofluorescence) after 48 h of exposure (1091 ± 42 in CD vs. 1979 ± 224 in CT; mean integrated optical density per cell ± SEM; $n = 4$/treatment; $p < 0.01$ different) and the trend persisted after 96 h (data not shown). 5-Aza-treated cells had the lowest global DNA methylation among all three conditions after 48 h of exposure (866 ± 101; $p < 0.01$ different from CT).

CDKN3 has a CpG island in its promoter and first exon, and the primer sets used in this experiment amplified the expected 522 bp product, which covered 44 CpG sites containing several sites for cutting by *BstU I* restriction enzyme, including a triple *BstU I* (5′-CGCGCG-3′) site (Fig. 1). After *BstU I* cleavage, the PCR product amplified with wild DNA as a template was completely digested to fragments at 246, 113 and lower combined signals at 61, 56 and 46 bp. The *BstU I*-digested fragments from bisulfite-modified DNA corresponded with those of wild DNA in 70 μM choline (Fig. 1). However, the fragments from modified DNA in the 5 μM choline group contained a 359 bp band in addition to the bands of wild DNA; this band corresponded to a DNA segment length of joined 246 and 113 bp bands. This 359 bp band was only detected in cells grown in 5 μM (CD) and not in 70 μM (CT) choline, even if incubation was extended to 72 h (data not shown).

To confirm these changes, bisulfite sequencing was used for a shorter sequence, due to the limitations encountered in finding suitable priming sites for the bisulfite-modified DNA. The CpG island spanning the position −408 to −125 contained 16 CpG sites, including the triple CGCGCG site previously described (sites 12–14, Fig. 2). Three other CpG sites at the end of the PCR product were excluded due to the frequent ambiguities in the sequencing process. In all CD samples, most of the CpG sites were incompletely methylated (in a given sample, the site was methylated on some strands but not on others; sites 4, 5, 7, 8, 9, 12, 13, 14, 15), with the rest being inconstantly methylated or unmethylated. CT samples were extensively methylated, with the majority of CpG sites being fully methylated. This confirms and extends the changes observed using COBRA, where the choline-deficient DNA was partially digested. To confirm these changes, the minus strand of the PCR products was also sequenced using the anti-sense primers, as previously described. Samples from cells treated with 5-Aza were markedly hypomethylated.

These changes in promoter methylation were associated with a change in CDKN3 gene expression (Fig. 3) and in production of the KAP protein product (Fig. 4). The CD/CT ratio of signal means for CDKN3 expression on DNA arrays was 2.649, the highest ratio among all CDKNs and the only one above the arbitrary threshold of 2. The protein level of KAP by western blotting was 2.4 times higher in CD- than in CT-treated cells ($p < 0.01$, Fig. 4 and Table 1).

Decreased ppRb was observed in CD cells compared with CT cells (Fig. 4, 5 and Table 1). The average IOD/cell ratio (CD/CT) for ppRb (phosphorylated pRb is the inactivated form) was 0.32 ($p < 0.01$ using *t*-test, $n = 4$ samples per condition; Fig. 4b, Table 1). The IOD/cell ratio (CD/CT) for total pRb (CD/CT) was 0.688 (no statistical significance using the *t*-test, $n = 4$ samples per condition. The ratio (CD/CT) for expression of the Rb gene was 0.515 (DNA array data).

Increased CDKN3 expression, production of KAP and decreased ppRb associated with choline deficiency should result in decreased cell proliferation. The percentage of CT cells labeled with BrdU was 1.8 times greater than in the CD cells after 48 h of incubation in their respective defined media ($p < 0.01$; Table 1).

Discussion

We observed that choline deficiency in neuroblastoma cells was associated with inhibition of cell proliferation. The progression through the cell division cycle depends on a process in which cyclins sequentially bind and activate distinct cyclin-dependent kinases (Cdks). Cdks are regulated by both transcriptional and post-translational mechanisms that promote the correct timing and order of events for cell growth and cell division (Sherr 1994; Morgan 1997; Borriello *et al*. 2002). In this study, oligonucleotide microarray analysis identified that CDKN3, a

member of the cyclin-dependent kinase inhibitor group, was one of the genes that was overexpressed in response to low choline in growth medium. CDKN3 encodes a Cdk-interacting protein (KAP) mapped at 14q22 (Demetrick *et al*. 1995) where chromosome abnormalities linked to several neoplasms have been located (Turc-Carel *et al*. 1988; Gilladoga *et al.* 1992). KAP is a G1- and S-phase dual-specificity phosphatase that associates with Cdk2 (Hannon *et al*. 1994), and dephosphorylates a threonine residue (Thr160) in a cyclin A-dependent manner (Poon and Hunter 1995; Brown *et al*. 1999). The selective inhibition of Cdk2 reduces its ability to phosphorylate the pRb, which, in its non-phosphorylated form, complexes with E2F1 transcription factor preventing it from binding to specific gene promoter sites (Davies *et al*. 2002; Johnson *et al*. 2002). Cdk2 also drives the G_1/S transition by associating with the cyclins E and A (Johnson *et al*. 2002). Our results show that, indeed, less choline-deficient cells undergo S-phase.

Choline and methyl-group metabolism are closely interrelated (Zeisel and Blusztajn 1994). Therefore, it is not unexpected that choline availability alters global DNA methylation and the methylation status of CpG islands in genes. Choline- and methyl group-deficient rodents had diminished intracellular concentrations of AdoMet, the methyl donor used by DNA methyltransferases (Zeisel *et al*. 1989). Others have reported that choline and methyl group deficiency is associated with decreased global methylation of DNA (Pogribny *et al*. 1997; Piyathilake *et al*. 2000) and decreased methylation of specific genes (Wu and Santi 1987; Pogribny *et al*. 1997; Zhu and Williams 1998) in liver, lung and breast tissue.

The *BstU I* site located at 180–175 bp (-cgcgcg-) upstream from exon 1 starting site was not fully methylated when cells were grown in 5 μM choline, but this methylation status was not identical in all cells, as 246 and 113 bp fragments were still detected. These findings were confirmed by the bisulfite-sequencing analysis, where a total of 16 CpG sites show hypomethylation in the CD- and 5-Aza-treated cells compared with the control. The heterogeneity in methylation between cells could be due to differences in cell–cell contact. Neuroblastoma cells proliferate to form clusters of cells and then form neurites. It is possible that the position of cells in the cluster, or the cell density in culture, may influence gene methylation. In human fibroblasts and bladder cancer cells, confluence-induced growth constraint is associated with transient alterations in CpG islands of specific genes (Pieper *et al*. 1999; Velicescu *et al*. 2002).

We previously reported that changes in choline availability during pregnancy altered the rate of stem cell proliferation in fetal brain (Albright *et al*. 1999a,b), and that this was, in part, the result of changes in the localization of p15Ink4b and p27Kip1 in the fetal rat hippocampus, two cyclin-dependent kinase inhibitors that modulate cell cycle progression (Albright *et al*. 2001). We now report that choline deficiency also modulates the expression of another cell cycle inhibitor, KAP. Maternal dietary choline intake not only alters stem cell proliferation in the fetal hippocampus, but also changes the size of the neurons in fetal septum (Loy *et al*. 1991), increases nerve growth factor concentrations in the fetal hippocampus (Sandstrom *et al*. 2002), alters the electrophysiological properties of fetal hippocampus (Pyapali *et al*. 1998; Jones *et al*. 1999), and alters memory function in offspring throughout life (Meck *et al*. 1988). Thus, formation of brain structures critical to memory formation, as well as interactions between these structures in developing rat brain, are changed when cell cycle regulation is perturbed by changes in maternal intake of a single nutrient, choline. Our study suggests a mechanism whereby choline mediates these changes, via altered methylation of promoter regions in the genes that regulate cell cycling.

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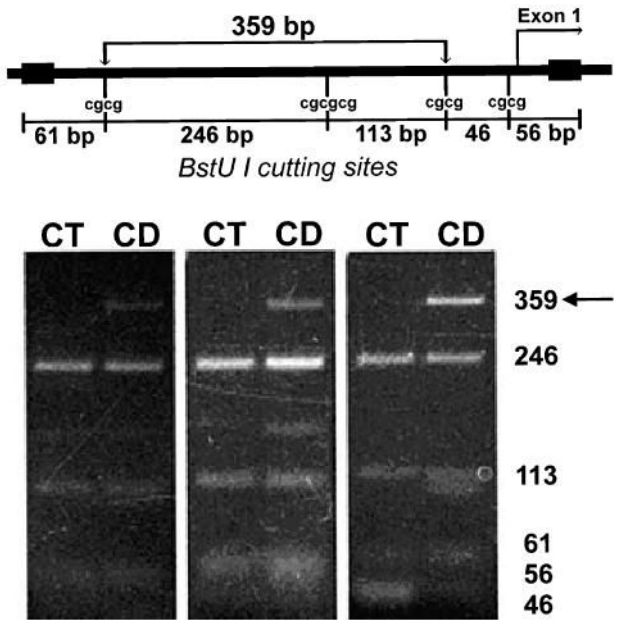

Fig. 1.

The promoter region of the CDKN3 gene is hypomethylated in choline-deficient IMR-32 cells. After 48 h of incubation in CD (5 μM choline) or CT (70 μM choline) medium, cells were lysed and DNA extracted. Genomic DNA (1 μg) was treated with sodium bisulfite followed by PCR amplification of the region of interest and restriction digestion (COBRA analysis; described in Experimental procedures). After this procedure, unmethylated CG sites transform into TG, while methylated CG sites remain as CG. The inset diagram shows the cutting points for *BstU I* restriction enzyme, which does not cut at TG sites but does cut at CG sites. Three representative gels are presented from 5 replicates. Note that a band is present at 359 bp in CD, and is not present in CT samples, indicating that the 5′-CGCGCG-3′ site upstream of exon 1 (indicated in the inset) is hypomethylated.

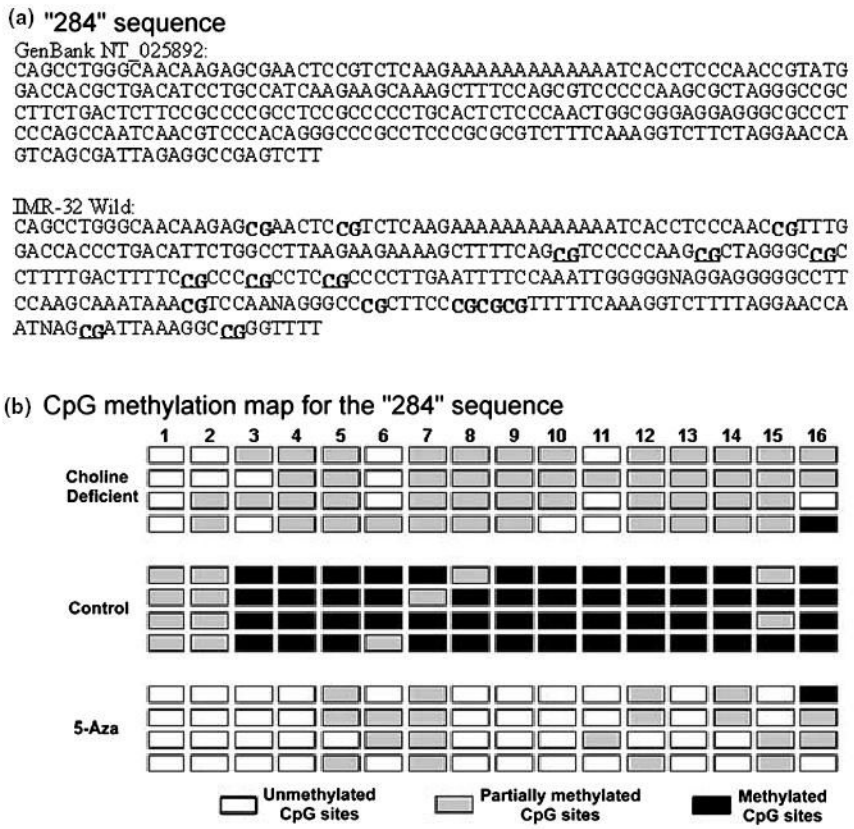

Fig. 2.

CpG methylation status of CDKN3 promoter determined by bisulfite DNA sequencing. (a) DNA sequence of the '284' PCR product (wild-type DNA) compared with the sequence from GenBank NT_025892. 'N' denotes ambiguous nucleotides. (b) Cells were incubated for 48 h in CD medium (5 μM choline), CT medium (70 μM choline), or 70 μM choline + 5 μM 5-azacytidine medium (5-Aza). Methylation maps obtained from sequencing four independent samples for each condition. Sixteen CpG sites were mapped.

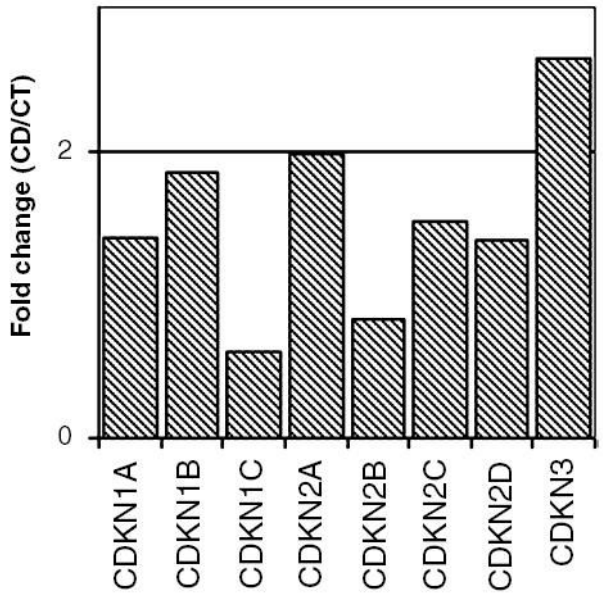

Fig. 3.

Expression of cyclin-dependent kinase inhibitor (CDKN) genes determined by oligoarrays. Cells were incubated for 48 h in CD medium (5 μM choline) or CT medium (70 μM choline). Total RNA was extracted from six plates for each condition, reverse transcribed into cDNA with Cy3 (70 μM choline as reference) or Cy5 (5 μM choline as experiment) monoreactive and then hybridized to a Compugen 20 000 spot V2 Human Microarray. A total of three independent experiments (three arrays) was performed.

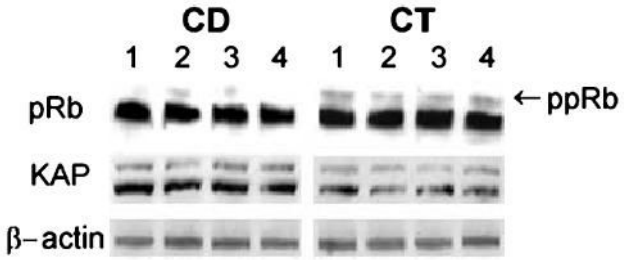

Fig. 4.

pRb and KAP protein expression in choline-deficient IMR-32 cells. After 48 h of incubation in CD (5 μM choline) or CT (70 μM choline) medium, cells were lysed; the lysates were subjected to electrophoresis and immunolabeled with a KAP or pRb antibody. β-actin was used to control for protein loading. Images of membranes were scanned and the integrated optical densities of the bands were measured and normalized to β-actin. The upper bands on the first blot (pRb) denote the ppRb form, which was decreased in the CD cells.

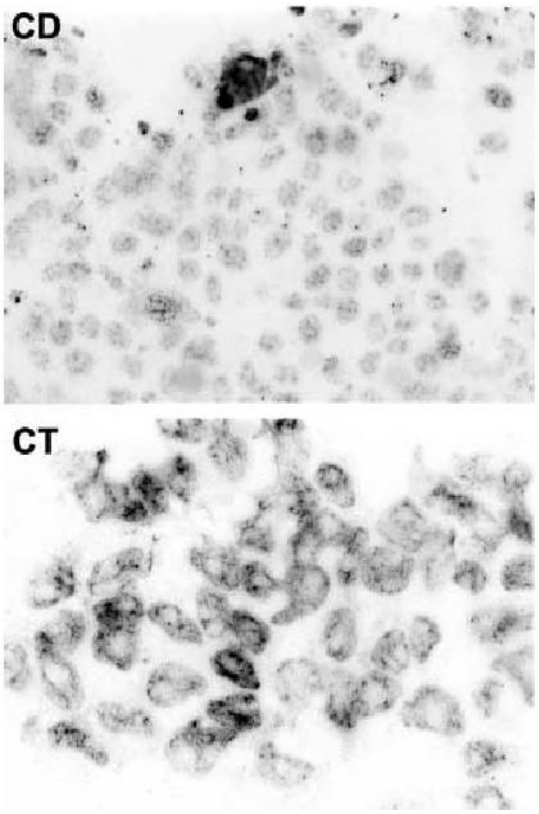

Fig. 5.

ppRb immunoreacivity in choline-deficient IMR-32 cells. After 48 h of incubation in CD (5 μM choline) or CT (70 μM choline) medium, cells were incubated with an antibody against ppRb (p110) (Ser 249/Thr 252 specific). Phosphorylated pRb is localized mainly in the nucleus.

Table 1

Changes in KAP protein, ppRb protein and BrdU accumulation in choline-deficient IMR-32 cells

	Units	CD	CT
KAP	Average IOD/lane	2650 ± 114*	1102 ± 123
ppRb (p110)	Average IOD/cell	25376 ± 2999*	78146 ± 2809
BrdU incorporation	Average % positive cells	14.7 ± 1.1*	26.7 ± 2.0

Cells were incubated for 48 h in CD (5 μM choline) or CT (70 μM choline) medium. BrdU accumulation was assessed using immunohistochemical methods described in Experimental procedures. KAP and phosphorylated Rb protein (ppRb) were determined using western blotting as described in the legend to Fig. 4. The values are expressed as average ± standard error ($n = 4$ for each treatment).

* $p < 0.01$ compared with CT.