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Expulsion of micronuclei containing amplified genes contributes to a decrease in double minute chromosomes from malignant tumor cells

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Double minute chromosomes (DMs) are a hallmark of gene amplification. The relationship between the formation of DMs and the amplification of DM-carried genes remains to be clarified. The human colorectal cancer cell line NCI-H716 and human malignant primitive neuroectodermal tumor cell line SK-PN-DW are known to contain many DMs. To examine the amplification of DM-carried genes in tumor cells, we performed Affymetrix SNP Array 6.0 analyses and verified the regions of amplification in NCI-H716 and SK-PN-DW tumor cells. We identified the amplification regions and the DM-carried genes that were amplified and overexpressed in tumor cells. Using RNA interference, we downregulated seven DM-carried genes, (*NDUFB9*, *MTSS1*, *NSMCE2*, *TRIB1*, *FAM84B*, *MYC* and *FGFR2*) individually and then investigated the formation of DMs, the amplification of the DM-carried genes, DNA damage and the physiological function of these genes. We found that suppressing the expression of DM-carried genes led to a decrease in the number of DMs and reduced the amplification of the DM-carried genes through the micronuclei expulsion of DMs from the tumor cells. We further detected an increase in the number of γH2AX foci in the knockdown cells, which provides a strong link between DNA damage and the loss of DMs. In addition, the loss of DMs and the reduced amplification and expression of the DM-carried genes resulted in a decrease in cell proliferation and invasion ability.

The amplification of oncogenes plays an important role in the malignant transformation of human cells and is associated with tumor development and drug resistance.[1,2] The mechanism of amplification leading to the overexpression of oncogenes varies between different types of cancers.[3,4] Double minute chromosomes (DMs) are DNA segments containing amplified oncogenes in tumor cells. DMs are small, paired, acentric and autonomously replicating extrachromosomal structures composed of circular DNA a few Mb in size.[1,5–9] DMs carrying amplified oncogenes are mostly present in solid tumors and neoplastic hematologic disorders.[10] The frequency of DMs carrying oncogenes appears higher than the frequency of homogeneously staining regions (HSRs) in tumors.[11]

Previous reports showed that the elimination of DMs from tumor cells was greatly accelerated by treating cells with a low concentration of hydroxyurea (HU); this also led to the reduced amplification of the oncogene *C-MYC* and the reversion of the malignant phenotype of colorectal carcinoma COLO320DM cells and promyelocytic leukemia HL-60 cells.[12–14] Similar to this finding, the elimination of amplified multidrug resistance

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What's new?

Double-minute chromosomes (DMs) are a hallmark of gene amplification and a major cytogenetic characteristic of malignant tumor cells. The function of DMs and DM-carried genes, however, remains to be clarified. Here, the authors identified amplification regions containing DM-carried genes that were themselves amplified and overexpressed in human malignant tumor cells. Knocking down the DM-carried amplified genes individually, they found that suppression of such oncogenes reduced the number of DMs, the amplification of these DM-carried genes, and cellular function. DNA damage and expulsion of micronuclei containing these DM-carried amplified genes may contribute to the decrease of DMs in tumor cells.

genes on DMs from human tumor cells using radiation therapy can reverse drug-resistance phenotypes.[15,16]

The elimination of DMs with reagents is mediated by the selective entrapment of DMs into cytoplasmic micronuclei (MN). In our study, these MN are designated as "DM-type MN." "Chromosome-type MN" are another type of MN and are formed either from acentric chromosomal fragments or from individual chromosomes.[17–19] The elimination of MN containing DMs and their amplified genes from tumor cells leads to a reversion of the tumor phenotype and cellular differentiation.[13,20,21] These findings reinforce the importance of DM-mediated gene amplification in tumorigenesis. Further studies of DMs, as well as the amplified genes that they carry, are important to cancer research.

Many human cancer cell lines are known to contain DMs.[15,21,22] However, the function of DMs and DM-carried genes in cancer cells remains to be clarified. In our molecular and cytogenetic study of cancer cells, we identified the amplification regions and the genes amplified and overexpressed via DMs. To clarify their function, we knocked down these genes using RNAi and then investigated the formation of DMs, the amplification of the DM-carried genes, and the physiological functions of these genes in cancer cells. We confirmed that suppression of the DM-carried amplified genes led to a decrease in the number of DMs and reduced the amplification levels of the DM-carried genes by MN expulsion of the DMs. We further found that γH2AX foci, a molecular marker for DNA damage, increased in the gene knockdown cells, which provides a strong link between DNA damage and the loss of DMs. In addition, the loss of DMs and the reduced amplification and expression of the DM-carried genes resulted in a reduction in cell proliferation and invasion ability.

Material and Methods

Cell line and cell culture

The human colorectal cancer cell line NCI-H716 and the human malignant primitive neuroectodermal tumor cell line SK-PN-DW were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were authenticated by the Beijing Microread Genetics (Beijing, China) using short tandem repeat (STR) analysis. NCI-H716 cells were routinely cultured in RPMI-1640 medium (Invitrogen, Auckland, New Zealand), and SK-PN-DW cells were cultured in DMEM medium (Invitrogen); both media were supplemented with 10% fetal bovine serum and grown at 37°C in 5% CO_2.

Antibodies and reagents

Antibodies against c-MYC and NDUFB9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-TRIB1 antibody and the anti-NSMCE2 antibody were purchased from Abnova (Taibei, Taiwan). The anti-MTSS1 antibody was obtained from Abcam (Cambridge, MA). The anti-phospho-H2AX (Ser139) antibody was obtained from Millipore (Billerica, MA). The anti-mouse and anti-rabbit secondary antibodies, conjugated to fluorophores, were purchased from Rockland Immunochemicals (Gilbertsville, PA). The CF488 goat anti-mouse IgG (H+L) secondary antibodies were obtained from Biotium (Hayward, CA). Polyethylenimine transfection reagent was obtained from Polysciences (Warrington, PA). The bacterial artificial chromosome (BAC) clones RP11–691H24, PR11–300I14, RP11–150N13, PR11–89K10, PR11–440N18, and PR11–62L18 were obtained from BACPAC Resources Center (Children's Hospital Oakland, Oakland, CA).

Affymetrix SNP Array 6.0 analysis

DNA were isolated from tumor cells and normal human peripheral blood using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) and then applied to the Affymetrix GeneChip Human Mapping SNP6.0 array (Shanghai Biotechnology, Shanghai, China) to analyze the DNA copy number changes in the tumor cells.

Quantitative reverse transcriptase-polymerase chain reaction and quantitative polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Invitrogen), according to the manufacturer's protocol, and reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Alameda, CA). DNA was routinely isolated from tumor cells and normal human peripheral blood. Polymerase chain reaction (PCR) analysis was performed on the cDNA and DNA using specific primers (Supporting Information Tables 1 and 2) under the following conditions: 94°C for 5 min; 45 cycles of 95°C for 5 sec, 60°C for 30 sec and 72°C for 30 sec; and 72°C for 7 min. The relative mRNA transcription and DNA amplification levels of each gene were normalized against *ACTB* mRNA and DNA levels, respectively.

Immunoblotting analysis

Cells were lysed for the immunoblotting analysis as described previously.[23] The lysates were separated using 12%

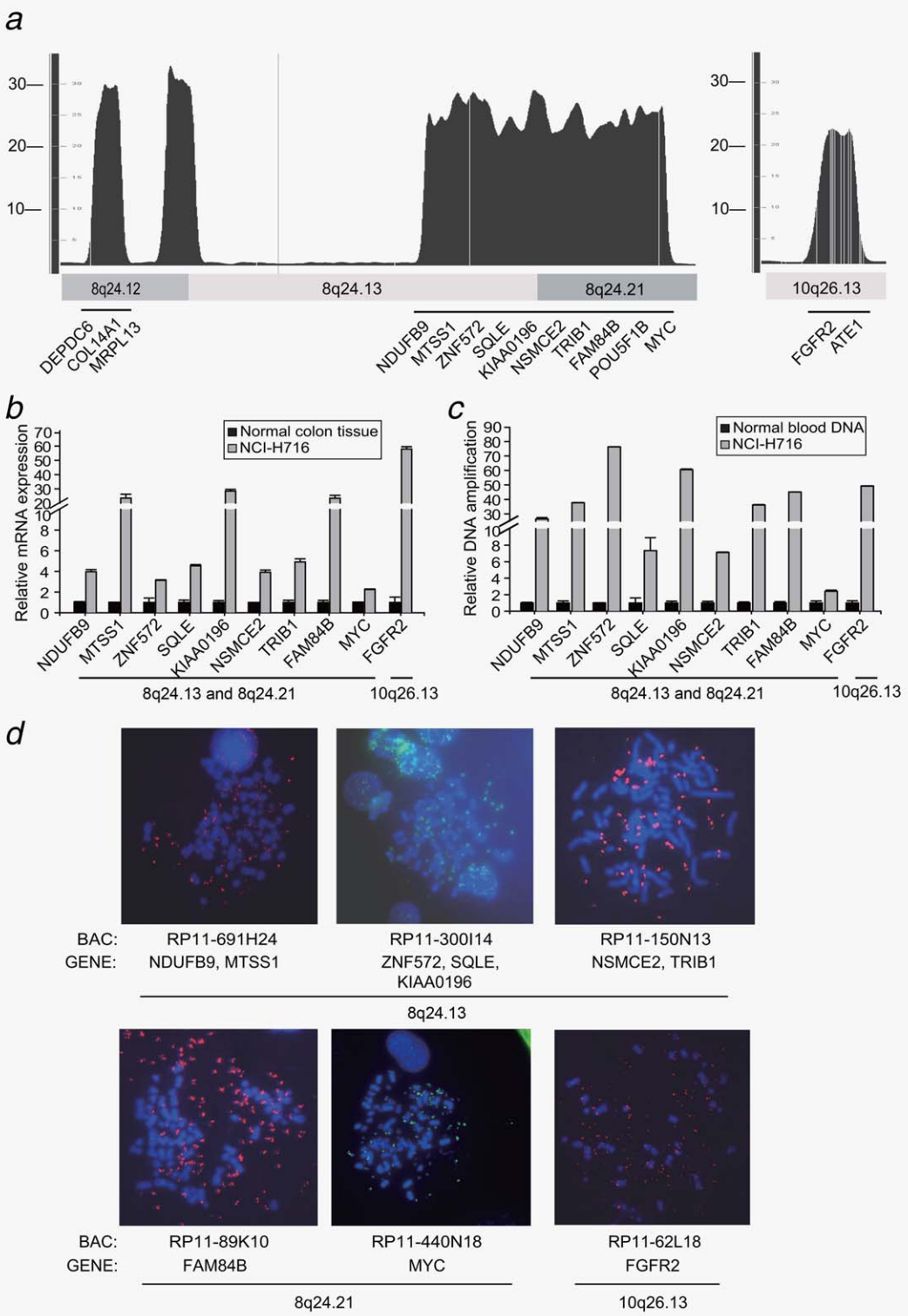

Figure 1. Ten genes are amplified via DMs and overexpressed in NCI-H716 cells. (*a*) Four amplified regions (>3-fold) were determined by Affymetrix SNP Array 6.0 analysis in NCI-H716 cells. (*b*) Ten genes (*NDUFB9*, *MTSS1*, *ZNF572*, *SQLE*, *KIAA0196*, *NSMCE2*, *TRIB1*, *FAM84B*, *MYC* and *FGFR2*) located on 8q24.13, 8q24.21 or 10q26.13 were at least 2-fold overexpressed compared to normal colon tissue using qRT-PCR analysis. (*c*) Ten genes (*NDUFB9*, *MTSS1*, *ZNF572*, *SQLE*, *KIAA0196*, *NSMCE2*, *TRIB1*, *FAM84B*, *MYC* and *FGFR2*) were amplified by at least 2-fold compared to the normal human peripheral blood DNA using qPCR analysis. (*d*) The amplified regions were located on DMs in NCI-H716 cells. Representative images of FISH analyses are shown. The signals indicated the six BAC clones (RP11–691H24, PR11–300I14, RP11–150N13, PR11–89K10, PR11–440N18 and PR11–62L18) that specifically cover the ten amplified genes (*NDUFB9*, *MTSS1*, *ZNF572*, *SQLE*, *KIAA0196*, *NSMCE2*, *TRIB1*, *FAM84B*, *MYC* and *FGFR2*). These probes mainly hybridized to the DMs in NCI-H716 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

SDS-PAGE and then transferred onto polyvinylidene difluoride membranes, followed by incubation with the primary antibodies then the fluorescent-conjugated secondary antibodies. The fluorescent signals were visualized using the Odyssey imaging system (Li-COR, Lincoln, NE).

Immunofluorescence analysis

Cells were seeded onto coverslips in six-well plates for 24 hr for the immunofluorescence analysis, as described previously,[24] and fixed with 4% paraformaldehyde. The coverslips were incubated with the anti-phospho-H2AX antibody, followed by the CF488 goat anti-mouse IgG secondary antibodies. DNA was visualized by counterstaining the cells with 4,6-diamidino-2-phenylindole (DAPI). Images were obtained using a Leica DM5000B microscope (Leica Microsystems, Solms, Germany). To analyze the extent of the DNA damage, the cells were grouped into four categories according to their γH2AX signals, including no signal, 0–30% signals, 30–60% signals and 60–100% signals using ImageJ (National Institutes of Health, Bethesda, MD) software. The Mann–Whitney *U*-test was performed using the Statistical Package for the Social Sciences (SPSS v19.0, International Business Machines Corporation, New York, NY) program to assess the significant difference between the different groups.

Preparation of metaphase spreads

The cells were harvested for metaphase spread preparation according to a previous study[10,25] and were then stained with Giemsa. Approximately 100 karyotypes of each group were observed under the microscope, and the numbers of DMs were counted. A *t*-test was used to analyze the statistical difference in the number of DMs for each group, and the statistical significance was accepted when $p < 0.05$.

Fluorescence *in situ* hybridization analysis

The BAC clones were used as DNA probes and were labeled with Spectrum Cy3-dUTP or Green-dUTP. The probes were then hybridized to interphase and metaphase spreads of tumor cells as described previously.[26] Chromosomes were counterstained with DAPI. High-quality interphase and metaphase images were captured using a Leica DM5000 B fluorescence microscope and analyzed using the MetaMorph Imaging System (Universal Imaging, West Chester, PA).

Cell proliferation, cell cycle distribution and cell invasion assays

Cells were seeded in 96-well plates at 3×10^3 cells per well. CellTiter 96®AQ_{UEOUS} One Solution Cell Proliferation Assay (Promega, WI) was used to measure the cell viability during a 4- to 6-day time course according to the manufacturer's protocol. Three independent experiments were performed.

For the cell cycle distribution assay, the BD Cycletest™ Plus-DNA Reagent Kit (BD Biosciences, Bedford, MA) was used according to the manufacturer's protocol and as described previously.[27] The cells were harvested by

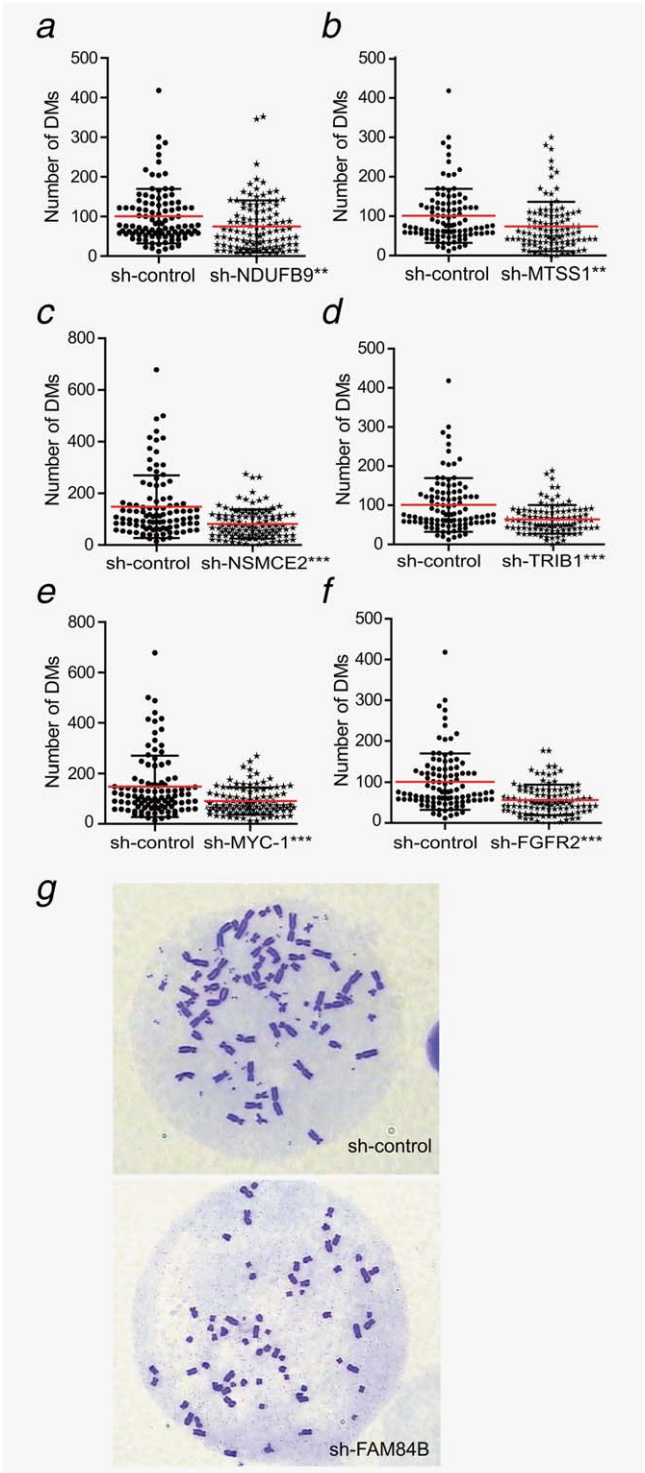

Figure 2. The formation of DMs was abolished in NCI-H716 shRNA cells. (*a–f*) The number of DMs decreased in NCI-H716 cells stably transfected with shRNA vectors (sh-NDUFB9, sh-MTSS1, sh-NSMCE2, sh-TRIB1, sh-MYC or sh-FGFR2) compared to the control cells. The number of DMs was plotted as the mean ± SD. $^{**}p < 0.01$ and $^{***}p < 0.001$, according to the *t*-test statistical analysis. SD, standard deviation. (*g*) Representative images of metaphase karyotype analysis in the FAM84B knockdown and control NCI-H716 cells are shown. Large numbers of small DMs were found in the FAM84B knockdown karyotype. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

trypsinization, washed in ice-cold PBS and then fixed using ice-cold 75% ethanol at 4°C for 24 hr. The cellular DNA was stained according to the manufacturer's protocol. Cell cycle distribution was analyzed using flow cytometry analysis (Bio-Rad, Richmond, CA).

For the cell invasion assay, BD BioCoat™ Matrigel™ Invasion Chambers (BD Bioscience) were used according to the manufacturer's protocol and as described previously.[28] Briefly, the cells were incubated for 24 hr at 37°C and allowed to pass through the membrane. Afterward, the noninvading cells were removed from the upper surface of the membrane. The cells on the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin, and the number of cells was counted. The chi-square test was performed to assess the statistical difference in the invasion rates, and statistical significance was accepted when $p < 0.05$.

Results

Identification of genes amplified via DMs

DMs represent the tumorigenesis manifestation of proto-oncogene amplification.[22] To acquire the molecular characteristics of genes amplified in tumor cells, we performed an Affymetrix SNP Array 6.0 analyses to verify the amplification regions in human colorectal cancer NCI-H716 cells. Four amplified regions (>3-fold) were identified in NCI-H716 cells: 8q24.12 (121 034 440∼121 537 936), 8q24.13 (121 999 452∼122 552 404), 8q24.21 (125 541 866∼128 978 180) and 10q26.13 (123 172 840∼123 617 816) (Fig. 1*a*). There are 15 genes (*DEPDC6*, *COL14A1*, *MRPL13*, *NDUFB9*, *MTSS1*, *ZNF572*, *SQLE*, *KIAA0196*, *NSMCE2*, *TRIB1*, *FAM84B*, *POU5F1B*, *MYC*, *FGFR2* and *ATE1*) located in these four amplified regions (Fig. 1*a*). As overexpression of the genes is effective for their function in tumor cells, we performed quantitative reverse transcriptase-PCR (qRT-PCR) analysis to determine whether these genes were overexpressed when compared with normal colon tissue (Fig. 1*b*). The results showed that ten genes, *NDUFB9*, *MTSS1*, *ZNF572*, *SOLE*, *KIAA0196*, *NSMCE2*, *TRIB1*, *FAM84B* and *MYC*, located on 8q24.13 and 8q24.21 and *FGFR2* located on 10q26.13 were at least 2-fold overexpressed in the cancer cells compared to the normal colon tissue (Fig. 1*b*). Subsequently, we performed quantitative PCR (qPCR) analysis to determine whether these genes were amplified in NCI-H716 cells. The results showed that all of the overexpressed genes, *NDUFB9*, *MTSS1*, *ZNF572*, *SOLE*, *KIAA0196*, *NSMCE2*, *TRIB1*, *FAM84B*, *MYC* and *FGFR2*, were amplified by at least 2-fold in the cancer cells when compared to the normal human peripheral blood DNA (Fig. 1*c*). Therefore, we found ten genes that are amplified and overexpressed in NCI-H716 cells.

Amplified genes might exist on DMs and/or at HSRs in cancer cells.[29] To ascertain whether the amplified genes were located on DMs, fluorescence *in situ* hybridization (FISH) analysis was performed. Six BAC clones specifically covering the ten amplified genes (*NDUFB9*, *MTSS1*, *ZNF572*, *SOLE*, *KIAA0196*, *NSMCE2*, *TRIB1*, *FAM84B*, *MYC* and *FGFR2*)

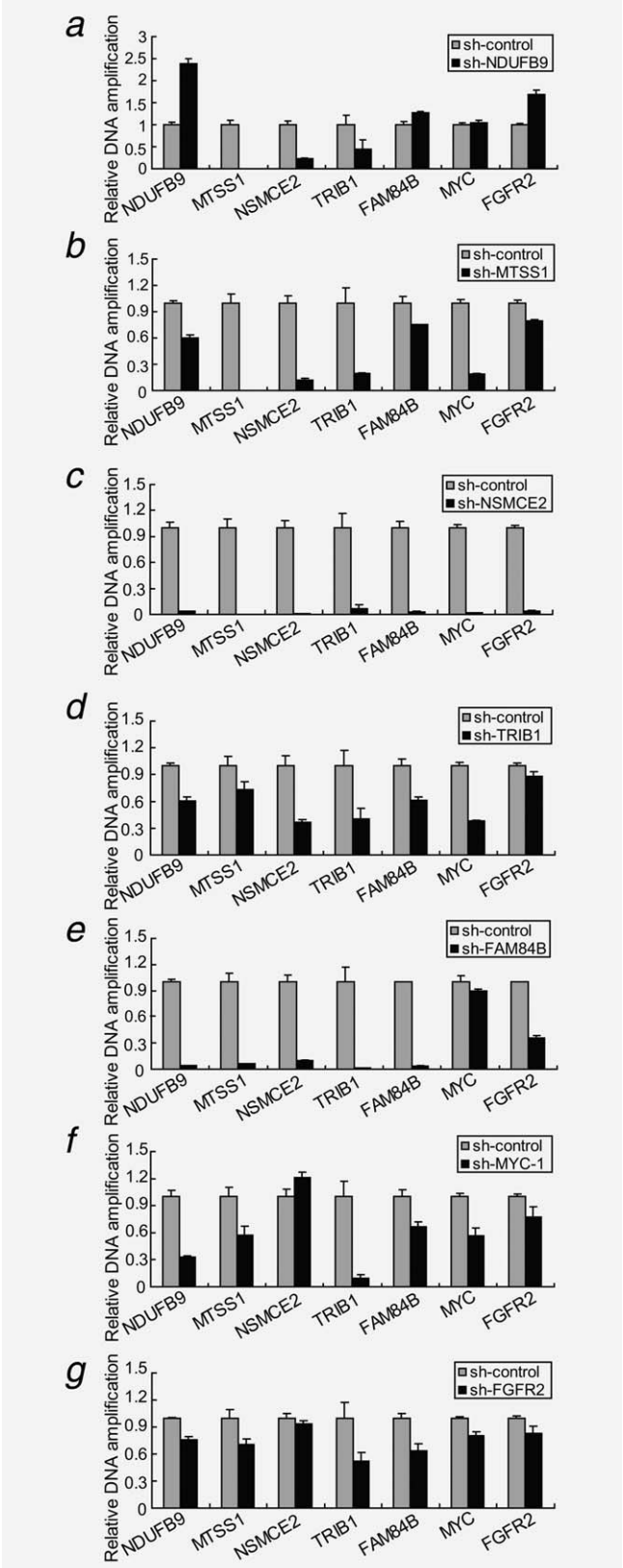

Figure 3. The amplification levels of the DM-carried genes are reduced in NCI-H716 shRNA cells. (*a–g*) The amplification levels of the DM-carried genes *NDUFB9*, *MTSS1*, *NSMCE2*, *TRIB1*, *FAM84B*, *MYC* and *FGFR2* were examined in each shRNA NCI-H716 cell line and control cell line.

Table 1. Micronuclei counts from NCI-H716 knockdown cells

NCI-H716 cells	No. of cells	No. of cells with MN	MN frequency ($\times 10^{-2}$)	No. of MN+	MN+/MN (%)	MN+ frequency ($\times 10^{-2}$)	Fold change over control	No. of MN−	MN−/MN (%)	MN− frequency ($\times 10^{-2}$)	Fold change over control
sh-control	135	9	6.67	5	55.56	3.70	1.00	4	44.44	2.96	1.00
sh-NDUFB9	291	65	22.34	55	84.62	18.90	5.11	10	15.38	3.44	1.16
sh-MTSS1	203	34	16.75	26	76.47	12.81	3.46	8	23.53	3.94	1.33
sh-control	228	20	8.77	18	90.00	7.89	1.00	2	10.00	0.88	1.00
sh-NSMCE2	256	56	21.88	43	76.79	16.80	2.13	13	23.21	5.08	5.77
sh-control	135	9	6.67	5	55.56	3.70	1.00	4	44.44	2.96	1.00
sh-TRIB1	273	27	9.89	13	48.15	4.76	1.29	14	51.85	5.13	1.73
sh-control	180	19	10.56	11	57.89	6.11	1.00	8	42.11	4.44	1.00
sh-FAM84B	107	11	10.28	5	45.45	4.67	0.76	6	54.55	5.61	1.26
sh-control	287	42	14.63	37	88.10	12.89	1.00	5	11.90	1.74	1.00
sh-MYC-1	143	28	19.58	23	82.14	16.08	1.25	5	17.86	3.50	2.01
sh-control	370	11	2.97	10	90.91	2.70	1.00	1	9.09	0.27	1.00
sh-FGFR2	361	30	8.31	20	66.67	5.54	2.05	10	33.33	2.77	10.26

MN+ indicates cells with BAC hybridization signals in the MN; MN− indicates cells without BAC hybridization signals in the MN.

were selected as FISH probes and hybridized to metaphase spreads of NCI-H716 cells. The results showed that these six BACs all hybridized to DMs in the NCI-H716 cells (Fig. 1*d*). This result suggests that DMs carry these amplified genes in NCI-H716 cells.

Similarly, we also examined the high amplification areas and the amplified genes in the DM-containing SK-PN-DW tumor cells using Affymetrix SNP Array 6.0 analysis and qPCR analysis (data not shown). We identified that *MYC* was also amplified via DMs in SK-PN-DW tumor cells (Supporting Information Fig. 1A).

The production of DMs is abolished in DM-carried-gene knockdown cells, resulting in the reduced amplification of DM-carried genes

Amplified genes play critical roles in tumor cells.[29] To further address the role of the amplified genes contained in the DMs of tumor cells, we selected gene-silencing siRNAs to target seven of the above DM-carried genes (Supporting Information Table 3); we also generated pSuper-short hairpin RNA (shRNA) expression vectors to knock down the expression of these genes. Immunoblotting and qRT-PCR analyses showed that the expression of *NDUFB9*, *MTSS1*, *NSMCE2*, *TRIB1*, *FAM84B*, *MYC* and *FGFR2* decreased in stable NCI-H716 shRNA cells (Supporting Information Figs. 2A–2G), and the expression of *MYC* also decreased in stable SK-PN-DW shRNA cells (Supporting Information Fig. 1B).

We next examined whether knockdown of these amplified genes affected the production of DMs. We performed a DM counting assay on the karyotypes of each NCI-H716 shRNA cell. Interestingly, we found that the number of DMs was significantly decreased in the knockdown cells when compared to the vector control cells (Figs. 2*a*–2*f*). We also confirmed this result in the SK-PN-DW knockdown cells (Supporting Information Fig. 1C). Furthermore, in the sh-FAM84B NCI-H716 cells, we found no DMs in 16.5% of the karyotypes, but we observed a large number of very small, sediment-like DMs in ~65.2% of the karyotypes (Fig. 2*g*). These results indicate that knockdown of the amplified genes induced genomic instability and abolished DMs formation in the tumor cells.

Next, we performed qPCR analysis on the DM-abolished cells to determine whether the amplification levels of the DM-carried genes were also affected. In each shRNA NCI-H716 cell line, we analyzed the amplification levels of *NDUFB9*, *MTSS1*, *NSMCE2*, *TRIB1*, *FAM84B*, *MYC* and *FGFR2* (Figs. 3*a*–3*g*). The results showed that almost all of the DM-carried genes had reduced amplification in these NCI-H716 shRNA cells compared to the control cells, with the exception of the increased amplification of the *NDUFB9* gene in sh-NDUFB9 cells (Figs. 3*a*–3*g*). These results suggest that along with the loss of DMs, the amplification levels of the DM-carried genes decreased; the exception to this might be a result of an adaptation of a particular gene under suppression conditions.

MN carrying DMs fragments are induced following knocking down of DM-carried genes

Because the number of DMs and the amplification levels of DM-carried genes decreased, we examined the fate of these DMs prior to being lost from the cells. We performed FISH analysis to detect the amplification and location of DMs in the interphase of each knockdown cell line using Cy3- or green-labeled BAC clones containing the DM-carried genes. We found obvious induction of MN in the NCI-H716 shRNA cells. The MN could be divided into two FISH results: positive

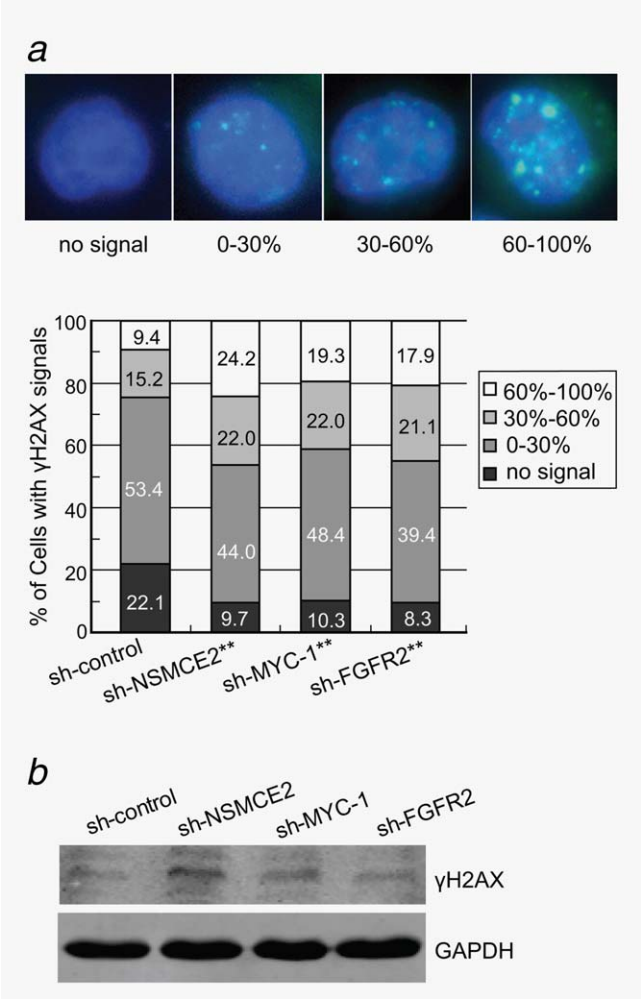

Figure 4. Induction of γH2AX foci in NCI-H716 shRNA cells. (*a*) Cells were grouped into four categories, including no signal, 0–30% signal, 30–60% signal and 60–100% signal, according to their γH2AX signals. The percentages of cells in each category based on the amount of γH2AX foci were shown. **Corrected $p < 0.003$ according to the Mann–Whitney *U* statistical analysis. (*b*) Immunoblotting analysis showed that γH2AX levels increased in the *NSMCE2*, *MYC*, and *FGFR2* knockdown cells compared to the control cells. GAPDH was used as a loading control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

MN (MN+) stained with both DAPI and Cy3, and negative MN (MN−) stained with DAPI only (Supporting Information Fig. 3). We counted the cells that produced MN, and the results are summarized in Table 1. We found the number of stably knockdown cells carrying MN with and without BAC signals increased; moreover, the percentage of cells that had MN+ greatly increased (Table 1). We further confirmed this result in SK-PN-DW knockdown cells (Supporting Information Table 4). These results imply that DMs participating in MN+ are lost from cancer cells. The induction of MN+ is consistent with the above results concerning the decrease in the number of DMs and the amplification level of the carried genes. We speculate that the formation of MN+ may be associated with the loss of DMs.

Suppression of the DM-carried genes caused DNA damage in tumor cells

Low concentrations of HU have been found to cause DNA damage, detectable as γH2AX foci, in cells.[12] We wanted to determine whether the suppression of the DM-carried genes could cause DNA damage in addition to the observed DM loss. We performed immunofluorescence analysis to detect the amount of γH2AX foci in three knockdown NCI-H716 cell lines. We grouped cells into four categories based on the amount of signals in each cell (Fig. 4*a*). The Mann–Whitney *U*-test was used to assess the significant differences in the γH2AX signals in the knockdown and control cells. A corrected *p* value less than 0.003 was considered significant. From the analysis, we found more cells with γH2AX foci in the NCI-H716 shRNA knockdown cells than the control cells. This was consistent with the immunoblot analysis of γH2AX expression (Fig. 4*b*). We further confirmed this result in SK-PN-DW knockdown cells, and using the Mann–Whitney *U*-test, a corrected *p* value less than 0.005 was considered statistically significant (Supporting Information Fig. 1D). The results indicate that suppression of the DM-carried genes is effective in inducing γH2AX foci formation in these cells. This suggests that the knocking down of the DM-carried genes can result in genomic instability, as well as DNA damage, and can lead to the loss of DMs from tumor cells.

Suppression of the DM-carried genes reduced malignant behavior

The amplification of DM-carried genes plays important roles in tumor cells; therefore, we evaluated the effect of the suppression of the DM-carried genes, as well as the loss of DMs, on the physiological functions of the cells. We examined the cell growth rate of three knockdown NCI-H716 cell lines. We found that cell proliferation was impaired in each cell line when compared to the control cell line (Fig. 5*a*). Knocking down the three amplified genes induced a notable increase in the percentage of cells in G1 phase (Fig. 5*b*). These results demonstrate that the expression of the DM-carried amplified genes is essential for the physiological function of tumor cells.

The ability of the cells to invade and migrate to the periphery of normal tissue is an important characteristic of tumor cells. To further examine the invasion of cells stably transfected with shRNA, we performed a basement membrane invasion assay after 24 hr of subculture. All three knockdown cell lines showed a significant decrease in cell invasion compared to the control cell line (Fig. 5*c*, Supporting Information Table 5). The results demonstrate that the DM-carried genes in tumor cells contribute to the cell's invasive characteristics.

Discussion

DMs are cytogenetic hallmarks of extra-chromosome genomic amplification, and many DMs are found in the

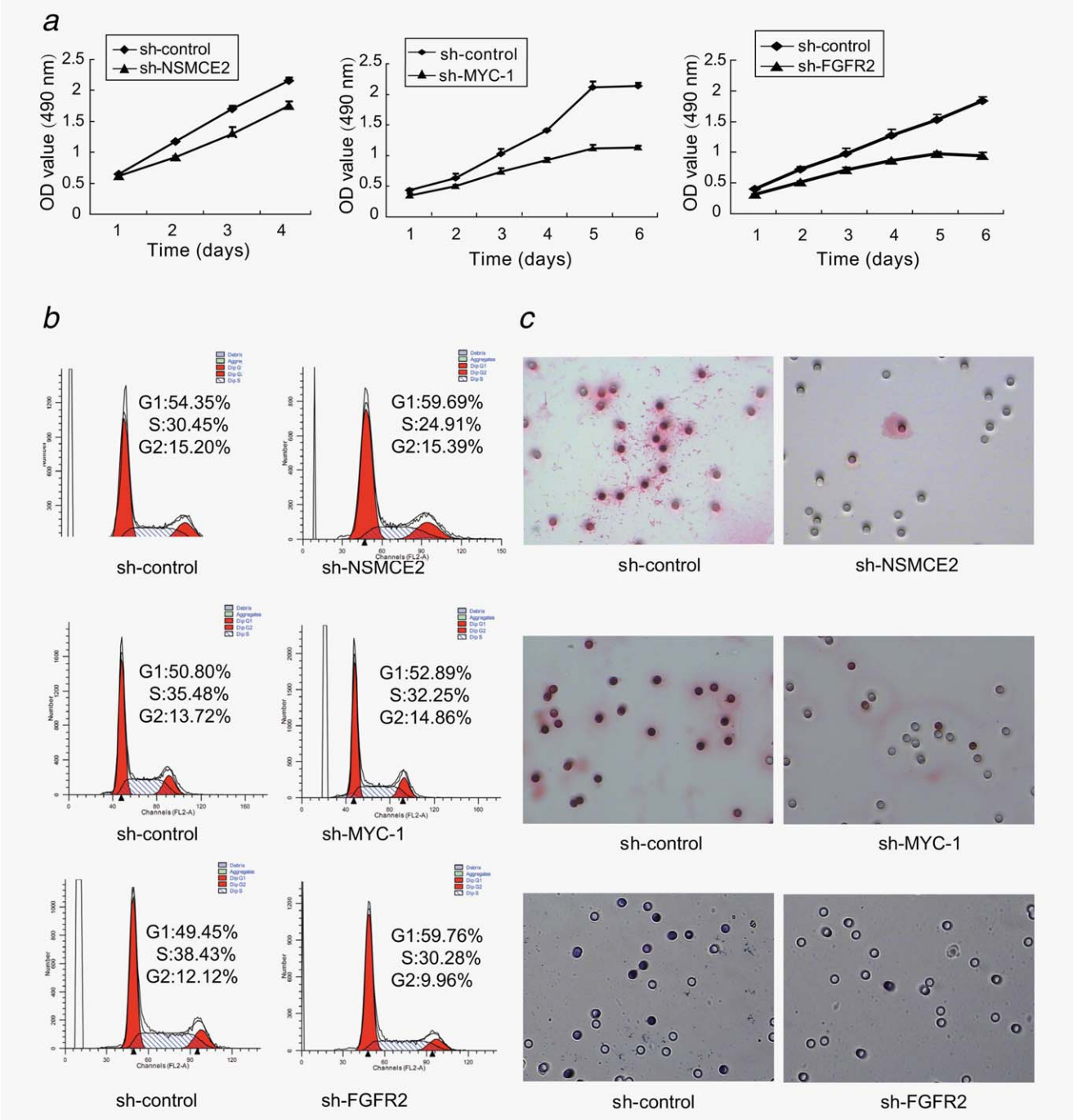

Figure 5. Suppression of the DM-carried genes reduces cell proliferation, causes a G1 arrest and reduces cell invasion. (*a*) The growth of shRNA NCI-H716 cells decreased. The OD value of the cells was measured every day for 4–6 days and plotted. (*b*) The cell cycle distribution of sh-NSMCE2, sh-MYC, sh-FGFR2 and sh-control NCI-H716 cells was determined using FACS analysis. (*c*) One representative trial of a cell invasion assay is shown for the shRNA NCI-H716 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

malignant tumor cells.[22] Our study focused on the function of DMs and gene amplification in malignant tumor cells. Using the Affymetrix SNP Array 6.0 analyses, we identified the regions of high amplification and the amplified genes located in these regions. We then tested the mRNA transcription and verified the DNA amplification of these genes. DMs are a major cytogenetic characteristic of malignant tumor cells; thus, we investigated the location of these amplified genes and identified the genes that were amplified via DMs.

To test the role of these DM-mediated amplified and overexpressed genes in malignant tumor cells, we used an

RNAi system to knock down the expression of these genes. We generated sh-NDUFB9, sh-MTSS1, sh-NSMCE2, sh-TRIB1, sh-FAM84B, sh-MYC, and sh-FGFR2 stable knockdown NCI-H716 and SK-PN-DW cell lines using the pSuper-shRNA system. Amazingly, when the DM-carried genes were suppressed, the number of DMs declined in each knockdown cell line compared to the control cell line. In the sh-FAM84B knockdown cells, in particular, we did not detect DMs in 16.5% of the cells, but there were many very small DMs in 65.2% cells. These results indicate that the suppression of DM-carried genes led to genomic instability. Gene amplification is a significant event in these cell lines, so we investigated the amplification levels of the DM-carried genes in these knockdown cells. As the number of DMs decreased, the amplification levels of most DM-carried genes also decreased in these gene knockdown cells compared to the control cells.

As described previously, elimination of the amplified genes was accelerated by treating cells with a low dose of HU, and the elimination was accompanied by the specific inclusion of DMs into MN.[12,30] The elimination of DMs into MN can also be induced by a broad range of DNA replication inhibitors used at low doses, as well as low-dose radiation therapy.[15,30] We were very interested in whether DMs would gather into MN when the expression of the DM-carried genes was suppressed. To trace the DMs and the DM-carried genes in the knockdown cells, we performed FISH analysis using BAC clones containing the DM-carried genes as probes to detect MN formation. We designated the positive signals as MN+ (DM-type MN), which may mediate the elimination of the DMs. We designated the negative signals as MN− (chromosome-type MN); this type arises from a centric whole chromosome that was bound by microtubules from both spindle poles. This type of MN has no DMs and thus would have no signal due to the lack of hybridization of the DM-specific probes.

DMs incorporate into MN, which leads to the elimination of the DMs from the cell through extrusion. Both MN with or without signals were increased in the shRNA stably transfected cells compared to the control cells. The percentage of MN+ increased in the shRNA cells, which suggests that the DMs are in the MN expelled from the cells. We observed the aggregation of DM-carried genes in interphase, illustrating the subsequent extrusion from the nucleus and the formation of MN that will be expelled from the cell. As previously reported, a plasmid was first multimerized to form a large circular molecule that resembles a DM and was then integrated into the chromosome, forming HSR.[31,32] However, in our study, we did not detect HSR in the metaphase nucleus. These results indicate that knocking down the DM-carried genes leads to the incorporation of DMs into MN, resulting in DM elimination from the cells and reduced amplification of the DM-carried genes. In addition, reducing the amplification of the DM-carried genes consequently causes a decline in DM formation.

A previous study showed that low-dose HU could induce γH2AX foci throughout the nucleus during S-phase.[12] It is reasonable to assume that HU induced γH2AX foci not only on chromosome arms but also in DMs.[12] Unexpectedly, DNA damage in DMs and in chromosomes has different consequences. The cell cycle will be arrested to restore damaged DNA in chromosomes, but DMs heavily stained with γH2AX signals always aggregated at metaphase, and they remained behind when the chromatids separated during anaphase, thus generating MN. We have performed immunofluorescence to determine the amount of γH2AX foci in the knockdown cells. We grouped cells into four categories based on the amount of γH2AX foci present in the knockdown cells using immunofluorescence, and we found that knockdown cells showed an increased amount of γH2AX foci compared to control, which is consistent with the immunoblot results.

We speculated that suppression of the DM-carried genes is effective at inducing γH2AX foci formation, which is a sensitive DNA damage marker. The underlying cause might be that deficiency of the genes that are implicated in tumorigenesis would lead to genomic instability and DNA recombination.[33] DNA damage is highly cytotoxic and is associated with genomic instability. These results indicate that γH2AX foci increased in the gene knockdown cells, which provides a strong link between DNA damage and the loss of DM. Knocking down DM-carried genes can cause DNA damage and can lead to DM loss from tumor cells through the extrusion of MN.

Elimination of DM-amplified genes from tumor cells via MN leads to a reversion in tumor phenotype and cellular differentiation.[20,21] We examined the physiological functions of the shRNA cells. We found that knocking down of all the DM-carried genes led to a marked decrease in cell proliferation ability and cell invasion ability. The decrease in the number of DMs and in the amplification levels of the DM-carried genes in malignant tumor cells might result in cell cycle arrest and reduction of the cell invasion ability. Using the DM-containing human malignant tumor cell lines as models, we conclude that the formation of DMs is associated with the amplification and expression of the DM-carried genes.

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Conflict of interest

The authors declare no conflict of interest.

References

1. Albertson DG, Collins C, McCormick F, et al. Chromosome aberrations in solid tumors. *Nat Genet* 2003;34:369–76.
2. Fletcher JA. Cytogenetics of solid tumors. In: Gersen SL, Keagle MB, eds. The principles of clinical cytogenetics, 2nd edn. Totowa, NJ: Humana Press, 2005. Section IV, 421–51.
3. Myllykangas S, Knuutila S. Manifestation, mechanisms and mysteries of gene amplifications. *Cancer Lett* 2006;232:79–89.
4. Schwab M. Amplification of oncogenes in human cancer cells. *Bioessays* 1998;20:473–9.
5. Schimke RT. Gene amplification and methotrexate resistance in cultured animal cells. *Harvey Lect* 1980;76:1–25.
6. Stark GR, Wahl GM. Gene amplification. *Annu Rev Biochem* 1984;53:447–91.
7. Windle B, Draper BW, Yin YX, et al. A central role for chromosome breakage in gene amplification, deletion formation, and amplicon integration. *Genes Dev* 1991;5:160–74.
8. Masuda A, Takahashi T. Chromosome instability in human lung cancers: possible underlying mechanisms and potential consequences in the pathogenesis. *Oncogene* 2002;21:6884–97.
9. Kuttler F, Mai S. Formation of non-random extrachromosomal elements during development, differentiation and oncogenesis. *Semin Cancer Biol* 2007;17:56–64.
10. Deng X, Zhang L, Zhang Y, et al. Double minute chromosomes in mouse methotrexate-resistant cells studied by atomic force microscopy. *Biochem Biophys Res Commun* 2006;346:1228–33.
11. Benner SE, Wahl GM, Von Hoff DD. Double minute chromosomes and homogeneously staining regions in tumors taken directly from patients versus in human tumor cell lines. *Anticancer Drugs* 1991;2:11–25.
12. Shimizu N, Misaka N, Utani K. Nonselective DNA damage induced by a replication inhibitor results in the selective elimination of extrachromosomal double minutes from human cancer cells. *Genes Chromosomes Cancer* 2007;46:865–74.
13. Eckhardt SG, Dai A, Davidson KK, et al. Induction of differentiation in HL60 cells by the reduction of extrachromosomally amplified c-myc. *Proc Natl Acad Sci USA* 1994;91:6674–8.
14. Von Hoff DD, McGill JR, Forseth BJ, et al. Elimination of extrachromosomally amplified MYC genes from human tumor cells reduces their tumorigenicity. *Proc Natl Acad Sci USA* 1992;89:8165–9.
15. Schoenlein PV, Barrett JT, Kulharya A, et al. Radiation therapy depletes extrachromosomally amplified drug resistance genes and oncogenes from tumor cells via micronuclear capture of episomes and double minute chromosomes. *Int J Radiat Oncol Biol Phys* 2003;55:1051–65.
16. Sanchez AM, Barrett JT, Schoenlein PV. Fractionated ionizing radiation accelerates loss of amplified MDR1 genes harbored by extrachromosomal DNA in tumor cells. *Cancer Res* 1998;58:3845–54.
17. Norppa H, Falck GC. What do human micronuclei contain? *Mutagenesis* 2003;18:221–33.
18. Shimizu N, Itoh N, Utiyama H, et al. Selective entrapment of extrachromosomally amplified DNA by nuclear budding and micronucleation during S phase. *J Cell Biol* 1998;140:1307–20.
19. Heddle JA, Cimino MC, Hayashi M, et al. Micronuclei as an index of cytogenetic damage: past, present, and future. *Environ Mol Mutagen* 1991;18:277–91.
20. Utani K, Kawamoto JK, Shimizu N. Micronuclei bearing acentric extrachromosomal chromatin are transcriptionally competent and may perturb the cancer cell phenotype. *Mol Cancer Res* 2007;5:695–704.
21. Shimizu N, Nakamura H, Kadota T, et al. Loss of amplified c-myc genes in the spontaneously differentiated HL-60 cells. *Cancer Res* 1994;54:3561–7.
22. Camps J, Nguyen QT, Padilla-Nash HM, et al. Integrative genomics reveals mechanisms of copy number alterations responsible for transcriptional deregulation in colorectal cancer. *Genes Chromosomes Cancer* 2009;48:1002–17.
23. Sun W, Li H, Yu Y, et al. MEKK3 is required for lysophosphatidic acid-induced NF-kappaB activation. *Cell Signal* 2009;21:1488–94.
24. Kanda T, Kamiya M, Maruo S, et al. Symmetrical localization of extrachromosomally replicating viral genomes on sister chromatids. *J Cell Sci* 2007;120:1529–39.
25. Yihui F, Xiaohong Z, Jing B, et al. Ultrastructural organization of premature condensed chromosomes at S-phase as observed by atomic force microscopy. *Appl Surf Sci* 2007;253:6.
26. Guan XY, Trent JM, Meltzer PS. Generation of band-specific painting probes from a single microdissected chromosome. *Hum Mol Genet* 1993;2:1117–21.
27. Zhang W, Zhu J, Bai J, et al. Comparison of the inhibitory effects of three transcriptional variants of CDKN2A in human lung cancer cell line A549. *J Exp Clin Cancer Res* 2010;29:74.
28. Meng XN, Jin Y, Yu Y, et al. Characterisation of fibronectin-mediated FAK signalling pathways in lung cancer cell migration and invasion. *Br J Cancer* 2009;101:327–34.
29. Albertson DG. Gene amplification in cancer. *Trends Genet* 2006;22:447–55.
30. Shimizu N. Molecular mechanisms of the origin of micronuclei from extrachromosomal elements. *Mutagenesis* 2011;26:119–23.
31. Shimizu N, Hashizume T, Shingaki K, et al. Amplification of plasmids containing a mammalian replication initiation region is mediated by controllable conflict between replication and transcription. *Cancer Res* 2003;63:5281–90.
32. Wahl GM. The importance of circular DNA in mammalian gene amplification. *Cancer Res* 1989;49:1333–40.
33. Oh DH, Rigas D, Cho A, et al. Deficiency in the nuclear-related factor erythroid 2 transcription factor (Nrf1) leads to genetic instability. *FEBS J* 2012;279:4121–30.