H3K27 Trimethylation Is an Early Epigenetic Event of p16INK4a Silencing for Regaining Tumorigenesis in Fusion Reprogrammed Hepatoma Cells*

Stable epigenetic silencing of p16INK4a is a common event in hepatocellular carcinoma (HCC) cells, which is associated with abnormal cell proliferation and liberation from cell cycle arrest. Understanding the early epigenetic events in silencing p16INK4a expression may illuminate a prognostic strategy to block HCC development. Toward this end, we created a reprogramed cell model by the fusion mouse HCC cells with mouse embryonic stem cells, in which the ES-Hepa hybrids forfeited HCC cell characteristics along with reactivation of the silenced p16INK4a. HCC characteristics, in terms of gene expression pattern and tumorigenic potential, was restored upon induced differentiation of these reprogrammed ES-Hepa hybrids. The histone methylation pattern relative to p16INK4a silencing during differentiation of the ES-Hepa hybrids was analyzed. H3K27 trimethylation at the p16INK4a promoter region, occurring in the early onset of p16INK4a silencing, was followed by H3K9 dimethylation at later stages. During the induced differentiation of the ES-Hepa hybrids, H3K4 di- and trimethylations were maintained at high levels during the silencing of p16INK4a, strongly suggesting that H3K4 methylation events did not cause the silencing of p16INK4a. Our results suggested that the enrichment of H3K27 trimethylation, independent of H3K9 dimethylation, trimethylation, and DNA methylation, was an early event in the silencing of p16INK4a during the tumor development. This unique chromatin pattern may be a heritable marker of epigenetic regulation for p16INK4a silencing during the developmental process of hepatocellular carcinogenesis.

Hepatocellular carcinoma (HCC) is a worldwide malignancy with a survival rate of <5% and an average survival of <1 year after diagnosis (1). Elucidating the molecular mechanisms in HCC development is essential for improving the prognosis efficiency. From Western blotting and immunohistochemical analyses in the HCC cell line and primary HCC samples, poorly differentiated tumors showed loss of p16INK4a to a great extent (2, 3). Ablatant silencing of p16INK4a, an inhibitor of D-type cyclin-CDK4/CDK6 complexes, could allow mammary epithelial cells to escape senescence during G1 to S phase, resulting in the triggering of neoplastic process, pre-malignant lesions, and progress in a variety of cancers (4–9). Unlike the silencing of p16INK4a in pancreatic adenocarcinoma, glioblastoma, certain leukemias, non-small cell lung cancer, and bladder carcinoma (10, 11), which is caused by mutations and homozygous deletions, the silencing of p16INK4a in HCC is caused mainly by epigenetic modulations, including DNA hypermethylation, in association with repressive histone modifications, H3K27 trimethylation, and H3K9 di- and trimethylation (2, 3, 12–14). However, the present results reflected the stable epigenetic silencing state in full-fledged cancers or cancer cell lines, the early epigenetic status prior to the transcriptional repression of p16INK4a in HCC initiation remains largely unknown. This greater understanding might allow for the detection and prediagnosis at an early stage of HCC development.

Nuclear reprogramming can reset the aberrant epigenetic modulations of cancer cells. In the previous studies of nuclear cloning, mouse melanoma, embryonic carcinoma (15, 16), and medulloblastoma (17) can be reprogrammed to support normal development, but the malignant characteristics regained after being transplanted in vivo. This regaining of tumorigenesis suggested the erased epigenetic memory and diminished tumorigenic potential in the reprogrammed cancer cells were restored in the context of a particular developmental state. However, the use of nuclear cloning can only be applied to certain types of cancer cells with stem cell properties, whereas nuclei of leukemia, lymphoma, and breast cancer cells failed to be reprogrammed (16). Moreover, it is impractical to employ this pro-

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additional 3 ml of medium was added. Cells were centrifuged, medium was added over a period of 1 min. Subsequently, an glycol solution for 1 min with occasional stirring. Then, 1 ml of was gently added. Cells were incubated in the 50% polyethylene (Roche Diagnostics, Basel, Switzerland) prewarmed to 37 °C gentle tapping, and 1 ml of 50% w/v polyethylene glycol 1500 After the supernatant was aspirated, the pellet was broken by

The results indicate that the enrichment of H3K27 trimethylation is an early event of stable silencing of these results indicate that the enrichment of H3K27 trimethylation is an early event of stable silencing of p16INK4a in the mouse HCC cells can be reactivated by fusion with mouse ES cells. After differentiated in vitro, the ES-Hepa hybrids captured the tumorigenic potential at all differentiation points, in which p16INK4a was silenced gradually by accumulation of H3K27 trimethylation first and then H3K9 dimethylation, whereas a high level of H3K4 methylations kept all through. These results indicate that the enrichment of H3K27 trimethylation is an early event of stable silencing of p16INK4a in the mouse HCC development course.

EXPERIMENTAL PROCEDURES

Cell Lines—E14 ESCs were cultured in Glasgow minimum essential medium (Invitrogen) containing 10% knock-out serum replacement (Invitrogen), 1% fetal bovine serum (HyClone, Logan, UT), 1% penicillin/streptomycin/glutamine, 1% non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, and 1000 units/ml leukemia inhibitory factor (ESGRO, Chemicon, Temecula, CA). The mouse hepatoma cell line Hepa1–6 was cultured in high glucose Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin/glutamine. Thymocytes collected from 6- to 8-week-old green fluorescent protein transgenic mice were passed through an 18-gauge needle several times to create single-cell suspensions.

Generation of Transgenic Cell Lines—To generate the transgenic mES cell lines, cells were transfected with a replication-incompetent lentiviral vector carrying a hygromycin resistance gene and an RFP gene. Forty-eight hours after viral transduction, hygromycin B (Invitrogen) was included in the medium at a concentration of 25 μg/ml for 2 weeks. Following drug selection, individual colonies were picked and expanded into cell lines. To generate drug-resistant cancer cells, Hepa1–6 cells were transfected with a neomycin resistance gene and a green fluorescent protein gene with the Effectene Transfection Reagent kit (Qiagen). Forty-eight hours after transduction, neomycin (Invitrogen) was included in the culture medium at a concentration of 100 μg/ml, and individual colonies were picked at day 14 and expanded into cell lines.

Cell Fusion—For polyethylene glycol fusions, cells of each type (generally 5 × 10^6) were combined in serum-free Glasgow minimum essential medium in a conical tube and pelleted. After the supernatant was aspirated, the pellet was broken by gentle tapping, and 1 ml of 50% w/v polyethylene glycol 1500 (Roche Diagnostics, Basel, Switzerland) prewarmed to 37 °C was gently added. Cells were incubated in the 50% polyethylene glycol solution for 1 min with occasional stirring. Then, 1 ml of medium was added over a period of 1 min. Subsequently, an additional 3 ml of medium was added. Cells were centrifuged, and the supernatant was discarded. The pellet was resuspended in complete ES cell medium and plated. Selection was applied after 48 h using hygromycin (200 mg/ml) and neomycin (100 mg/ml). Fourteen days following drug selection, single colonies were picked and expanded under standard conditions.

Karyotype Analysis—A 25-cm flask at 60% cell confluence was treated with 0.04 – 0.1 μg/ml colchicine for 3 h. Cells were recovered by trypsinization and treated with a hypotonic (0.56% w/v) KCl solution for 15 min. The cells were centrifuged at 500 rpm, fixed by washing three times in fresh fixative (3:1 methanol/acetacid), and dropped onto cold, clean glass slides. The slides were air-dried, stained with 4',6-diamidino-2-phenylindole, and observed under a microscope.

Fluorescence-activated Cell Sorting—For analysis of DNA content, cells in a 10-cm dish were trypsinized, washed in phosphate-buffered saline (PBS), and fixed with 70% ethanol at 4 °C for 30 min. RNase A was added to 500 μl of PBS at a final concentration of 20 μg/ml. Cells were incubated in this solution at 37 °C for 30 min and then centrifuged at 1500 rpm for 5 min. The supernatant was discarded, and the cells were incubated with propidium iodide in 50 μl of PBS at a final concentration of 50 μg/ml in the dark at room temperature for 30 min and then centrifuged at 1500 rpm for 5 min. The supernatant was discarded again, and the cells were resuspended in 0.5 ml of PBS. The stained cells were analyzed with the FACSCaliber system (BD Biosciences).

Immunohistochemistry—For immunostaining, cells were fixed with 4% paraformaldehyde and stained with anti-Oct4 (Rabbit Anti-Oct-4, Chemicon, AB3209) for 1 h in PBS containing 1% fetal bovine serum and 0.1% Triton-X. Cells were then washed three times with PBS and incubated with an appropriate fluorescein isothiocyanate-labeled secondary antibody (Jackson ImmunoResearch). Specimens were analyzed on an Olympus fluorescence microscope.

For teratomas from ES cells, ES-Hepa hybrid cells and Hepa1–6 tumors, ~1 × 10^6 cells of each clone, were subcutaneously injected into the inguinal region of immunodeficient nude mice. The mice were monitored for tumor growth for 6 weeks. Tumor volume (V) was calculated in all experiments according to V = ab^2/2, where a and b designate the long and short diameters of the tumor, respectively. At 4 weeks, teratomas or tumors were fixed with 4% paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin.

In Vitro Differentiation—For embryonic body formation, ES and ES-Hepa hybrid cells were harvested by treatment with 0.25% trypsin. The cells were cultured in bacterial-grade Petri dishes in Dulbecco’s modified Eagle’s medium/F-12 containing 20% knock-out serum replacement (Invitrogen), 2 mM L-glutamine, 1 × 10^-4 nonessential amino acids, 1 × 10^-4 M2-mercaptoethanol (Invitrogen), and 1% penicillin/streptomycin. The medium was changed every other day. Embryonic bodies were harvested at days 3, 5, 7, and 9 for gene expression analysis.

For induction of ES and ES-Hepa hybrid cells to differentiate by monolayer culture, ES and ES-Hepa hybrid cells were dispersed into a single cell suspension with 0.25% trypsin, and plated the cells on the 0.2% gelatin-coated cell culture dish. The cells were differentiated in high glucose Dulbecco’s

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In situ fluorescence in situ hybridization, a 562-bp RNA-fluorescence hybridization probe for nascent p16INK4a transcripts was designed from Primer Premier 5 and Oligo 6. Then another PCR was performed by adding the T7 promoter primer to the sense primer and the Sp6 primer to the antisense primer from the previous reaction. The PCR product was purified with the Mini-DNA fragment Rapid Purification Kit (BioDev), and 1 μg of the product was labeled by in vitro transcription with a Bio- tin-dUTP RNA labeling kit (Roche Applied Science). The RNA product from the Sp6 promoter was used as the probe for p16INK4a, and that from T7 was used as a control. The product was purified with Quick Spin RNA Columns (Qiagen). For each slide, 50 ng of DNA probe, 5 μg of salmon sperm DNA, and 20 μg of tRNA were used. Two volumes of 100% EtOH were added. After being dried, the samples were resuspended in 10 μl of hybridization mix and 1 μl of RNA guard and left to dissolve at 37 °C and then denature at 80 °C for 10 min. Following overnight hybridization at 37 °C, slides were washed three times with 50% formamide/2× standard saline citrate at 45 °C for 5 min each, followed by three washes with 1× standard saline citrate (prewarmed to 60 °C) at 45 °C for 5 min each. Detection was then performed using a TSA kit (Molecular Probes, cat. no. T20931) and analyzed with Image Pro Plus 5.1 (Media Cybernetics).

**Bisulfite Sequence Analysis**—Bisulfite treatment was performed with the EpiTect Bisulfite Kit (Qiagen, 59104) according to the manufacturer’s recommendations. For bisulfite sequencing of the mouse p16INK4a promoter, primers were designed as follows: p16INK4a f (mouse p16INK4a promoter at nucleotides −502 to −481), 5′-AATTTCGGTGTTAánchez-TGGG-3′, and p16INK4a r (mouse p16INK4a promoter at −17 to +7), 5′-ATCCATACTACTCCAAATAACTCC-3′. Amplified products were cloned into pCR2.1-TOPO (Invitrogen). Ten randomly selected clones were sequenced with M13 forward and reverse primers.

**Chromosome Immunoprecipitation**—Approximately 5×10^6 to 10^7 cells of each line were harvested and cross-linked with 1% paraformaldehyde for 10 min at room temperature, and formaldehyde was quenched by the addition of 1/20 volume of 2.5 M glycine to plates. The chromatin was then sonicated to create DNA fragments with a length of 200 to 1000 bp. Fragmented chromatin was subjected to immunoprecipitation with 3 μl of H3K4me2 (Abcam, ab7766), 3 μl of H3K4me3 (Abcam, ab8580), 4 μl of H3K9me2 (Upstate, 17-648), 4 μl of H3K9me3 (Upstate, 17-625), 4 μl of H3K27me3 (Upstate, 17-622), and 3 μl of rabbit anti-mouse IgG (Upstate, 17-622). After elution of immune complexes with 25 μl of Dynal magnetic beads (protein G) for each reaction (Invitrogen, 100.03D), DNA was resuspended with 500 μl of TE solution (10 mm Tris-Hcl, 1 mm EDTA). The samples were reverse cross-linked by heating at 65 °C overnight and then treated with RNase (50 μg/ml) for 1 h at 37 °C and proteinase K for 30 min at 55 °C. DNA extracted by the phenol/chloroform method was ethanol-precipitated and resuspended in 50 μl of double-distilled water. Quantification of precipitated DNA was performed using real-time quantitative PCR amplification, comparing the values attained with those from a 1:100 dilution of the input DNA. Primer sequences used in this research included the following: p16INK4a forward,
Expressed SNP Analysis—To detect the single nucleotide polymorphisms (SNPs) in transcribed regions, cDNA was synthesized from ES and ES-Hepa hybrid total RNA using the SuperScript III reverse transcriptase kit (Invitrogen, cat. no. 18080-051) according to the manufacturer’s instructions. DNA was extracted with the phenol/chloroform method, ethanol-precipitated, and resuspended in 500 μl of double-distilled water. RT-PCR was performed with High Fidelity polymerase (Invitrogen, cat. no. 11304-011) and primers for p16INK4a: forward, CAGGTGATGATGGCAG; reverse, GGATAGCTTCAGCTCAAGCAC. PCR products were cloned into the T vector (Takara, D103A). Sequencing of the cloned products was performed with the M13 forward and reverse primers.

RESULTS

Reactivation of Silenced p16INK4a in Hepa1–6 Cells after Fusion with ES Cells—ES cells that constitutively express RFP and are resistant to hygromycin were fused with green fluorescent protein-expressing, neomycin-resistant adult cancer Hepa1–6 cells, and the hybrids were selected in hygromycin and neomycin. Drug-resistant colonies expressing red fluorescent protein and green fluorescent protein appeared on day 2, as illustrated in Fig. 1A. After 2 weeks of selection, the double-drug-resistant, dual-fluorescent colonies of ES-Hepa hybrids established a flattened and less compact appearance (Fig. 1B).

Analysis of chromosome spreads and DNA content from 20 colonies indicated that the hybrid contained a near-tetraploid chromosome complement of 80 and 4n DNA content, demonstrating that the stable cell hybrid contained both the ES and the cancer cell chromosomes in a single cell (Fig. 1, C and D). The pluripotent genes Oct4, Nanog, Sox2, and Rex-1, which were silenced in Hepa1–6 cells, were increased to a level similar to those of ES cells after fusion (Fig. 1E), and the tissue-specific genes Ttr and Alb in the ES-Hepa hybrids were obviously abolished compared with those in Hepa1–6 cells (Fig. 1E). Also, the expression level of tumor-related genes, such as Bcl2, Bad, Bax, Cdkn1a, Rassf1, c-fos, Bmi1, Ezh2, and Eed in the ES-Hepa hybrids were similar to that of ES cells, except for the c-Jun oncogene (Fig. 1F). These results suggested that the ES-Hepa hybrids had ES-like potency, and the Hepa1–6 hybrid counterparts lost their gene expression pattern.

We adopted RT-PCR and real-time PCR to examine the expression level of p16INK4a in ES, Hepa1–6, and ES-Hepa hybrid cells. The p16INK4a gene was expressed in the ES and ES-Hepa hybrid cells, whereas it was silenced in the Hepa1–6 cells (Fig. 2A). To confirm the reactivation of silenced p16INK4a...
in Hepa1–6 cells, we measured the allelic expression of p16INK4a by using RNA fluorescence in situ hybridization. In the ES-Hepa hybrids, four dot signals were obvious per nuclei; these cells were in sharp contrast with the lack of signal in the Hepa1–6 cells, demonstrating the reactivation of silenced p16INK4a in Hepa1–6 cells by cell fusion (Fig. 2B). Further, we examined the SNP in the transcribed region of p16INK4a in ES, Hepa1–6, and ES-Hepa hybrid cells. In this research, an adenine residue in the ES cell genome was different from a guanine residue in the Hepa1–6 genome. Sequencing results of exon2 of p16INK4a in the ES-Hepa hybrids showed mixed transcription sequences originating from both ES and Hepa1–6 cells, demonstrating that both adenine and guanine can be sequenced.

**FIGURE 2. Reactivation of p16INK4a derived from Hepa1–6 in ES-Hepa hybrid cells.** A, real-time PCR (left) and RT-PCR (right) analysis of p16INK4a. B, RNA-fluorescence in situ hybridization staining for p16INK4a in ES, Hepa1–6, and ES-Hepa hybrid cells. Yellow arrowheads indicate p16INK4a RNA nascent transcripts. The percentage of cells showing the expected allelic expression is indicated. Representative images show sites of p16INK4a transcription (green) merged with 4',6-diamidino-2-phenylindole in ES, Hepa1–6, and ES-Hepa hybrid cells. The RNA product from the SP6 promoter was used as the probe for p16INK4a and that from T7 was used as a negative control. C, expressed SNP analysis of p16INK4a exon 2 within the ES-Hepa hybrids and their fusion partners. An adenine residue in the ES cell cDNA was different from a guanine residue in the Hepa1–6 DNA. In cDNA of the ES-Hepa hybrid cells, p16INK4a expression was from both the Hepa1–6 and the ES cells, showing that both adenine and guanine can be sequenced.
hybrid cells (Fig. 3A). We suggest that, in Hepa1–6 cells, DNA methylation was not the main cause of p16\(^{\text{INK4a}}\) silencing. We next focused on histone modifications that contribute to the creation of chromatin structure, leading to stable expression of the genome (30). In Hepa1–6 cells, p16\(^{\text{INK4a}}\) was transcriptionally silenced (Fig. 2A), a state consisting of trimethylation of H3K27 (Fig. 3C), dimethylation of H3K9 (Fig. 3C), and demethylation of H3K4 (Fig. 3B). We did not observe modulation of the trimethylation of H3K9 in the promoter region of p16\(^{\text{INK4a}}\) (Fig. 3C). However, as a positive control, H3K9me3 had a role in silencing of the pluripotent gene Nanog in the Hepa1–6 cells, reflecting the credibility of the method and the antibody (data not shown). In the ES-Hepa hybrids, the histone methylations on p16\(^{\text{INK4a}}\) were similar to those in ES cells that both di- and trimethylation of H3K4 were presented in the promoter region of p16\(^{\text{INK4a}}\), accompanied by a slight enrichment of H3K27me3 (Fig. 3, B and C), suggesting an availability for transcriptional increase or decrease during differentiation (31). The former repressive marker H3K9me2 found in Hepa1–6 cells was depleted in the ES-Hepa hybrids (Fig. 3, B and C). These findings demonstrated the activation of the promoter region of p16\(^{\text{INK4a}}\) in the ES-Hepa hybrids.

Inactivation of p16\(^{\text{INK4a}}\) in the Differentiated ES-Hepa Hybrids in Vitro—Differentiation in ES cells is controlled by epigenetic modulations in response to autocrine and paracrine delivery of signaling molecules. Similarly, abnormalities in genetic and epigenetic controls may lead a cell to an abnormal program by responding to differentiation-related environment. In our research, we found that ES and ES-Hepa cells exhibited entirely different fates after differentiation in vitro in terms of gene expression pattern, epigenetic modulation, and tumorigenic potential. We first induced ES and ES-Hepa cells into embryonic bodies in vitro for 3, 5, 7, and 9 days (Fig. 4A). p16\(^{\text{INK4a}}\) expression in the ES-Hepa hybrids diminished gradually upon differentiation in vitro, whereas p16\(^{\text{INK4a}}\) expression in ES cells showed a higher level of expression throughout the differentiation course (Fig. 4B). Further investigation of the gene activation of c-fos, c-jun, Bcl2 in ES-Hepa cells proved that the hybrid began to exhibit a tumorigenic gene expression mode after differentiation in vitro (Fig. 4B). We next induced ES-Hepa hybrid cells to differentiate by monolayer culture for 0, 7, 14, and 21 days in vitro to examine the cancer gene expression mode by real-time PCR. Oncogenes, including c-fos, Bcl2, c-jun, c-myc, and Rassf1, increased in varying degrees over the differentiation course in the ES-Hepa hybrids (Fig. 4C), which suggested that the differentiated ES-Hepa hybrids gradually lost cell cycle control, re-exhibiting tumorigenic potential. In the differentiation course, p16\(^{\text{INK4a}}\) expression decreased greatly in the ES-Hepa hybrids and became transcriptionally repressed, whereas p16\(^{\text{INK4a}}\) expression in ES cells increased significantly over the differentiation course (Fig. 5A). The alteration of gene expression by cross-talk with the surrounding differentiation microenvironment suggested epigenetic modulation on the promoter region during the gene silencing or activation course. These results showed that the ES-Hepa hybrids recaptured the tumor gene expression pattern upon induced differentiation in vitro.

Histone Methylation Pattern in the p16\(^{\text{INK4a}}\) Silencing Course in the Differentiated ES-Hepa Hybrids—We next analyzed the histone modifications in the p16\(^{\text{INK4a}}\) silencing course upon differentiation. In the differentiated ES cells, p16\(^{\text{INK4a}}\) expression kept increasing and was accompanied by a basal level of H3K27me3 and hyper-di- and -trimethylation of H3K4 (Fig. 5, A–C), whereas, in the differentiated ES-Hepa
hybrids, H3K27me3 began to increase at day 7 and kept increasing as differentiation continued. At day 21, the modulation of H3K27me3 on p16<sub>INK4a</sub> in the differentiated ES-Hepa hybrids was even stronger than that of Hepa1–6 cells (Fig. 5B). As for repressive marker H3K9me3, it did not contribute to the silencing of p16<sub>INK4a</sub> in Hepa1–6 or in the differentiated ES-Hepa hybrids (Figs. 3C and 5B). However, considerable levels of di- and trimethylation of H3K4 were also detected on silenced p16<sub>INK4a</sub> in differentiated ES-Hepa cells (Fig. 5C). Although the modulation level of H3K4me2 and H3K4me3 was weaker compared with the undifferentiated ES-Hepa hybrids, the existence of H3K4 methylation on silenced p16<sub>INK4a</sub> suggests that, in the ES-Hepa hybrids, H3K4 methylations are not necessarily directly related to gene silencing. These data showed that the enrichment of H3K27 trimethylation was an early epigenetic event in silencing of p16<sub>INK4a</sub>.

Tumorigenic Potential of the Differentiated ES-Hepa Hybrids in Vivo—After differentiated in vitro, we obtained several types of cells with different p16<sub>INK4a</sub> expression levels, which were modulated by epigenetic mechanisms in a time course (Table 1). We next injected ES cells and the ES-Hepa hybrids at different p16<sub>INK4a</sub> expression levels into immunodeficient nude mice subcutaneously and examined tumor formation every week for over 6 weeks. Undifferentiated (D0) ES and ES-Hepa hybrid cells established the similar tumorigenic potential (Fig. 6, A and B). After induced differentiation in vitro, ES cells (D7 and D14) at elevated p16<sub>INK4a</sub> expression levels could hardly give rise to teratomas (Fig. 6, A and B). In the ES-Hepa hybrid differentiation group, cells harboring early epigenetic alterations prior to the stable repression of p16<sub>INK4a</sub> (D7 and D14) possessed the tumorigenic properties (Fig. 6, A and B). These results associated the early epigenetic events of
Modulation of p16 in Fusion-reprogrammed Hepatoma Cells

p16INK4a silencing to the tumorigenesis in the differentiated ES-Hepa hybrids.

The hematoxylin & eosin staining of the tumors derived from the ES-Hepa hybrids with different p16INK4a expression levels further proved the malignancy of differentiated ES-Hepa hybrid cells. Like ES cells, undifferentiated ES-Hepa hybrids had the potential to give rise to three germ layers, including cuticular epithelium, glandular epithelium, and cartilage (Fig. 6C). However, the rate of tumor formation (2 weeks) for undifferentiated ES-Hepa hybrids was much faster than the rate for ES cells (4 weeks), ES-lymphocyte hybrids (4 weeks), and Hepa1–6 cells (4–5 weeks) (Table 2), suggesting a high proliferative capacity of the ES-Hepa hybrids upon differentiation. In the tumors from the ES-Hepa hybrids, 80% of the total tumor contained undifferentiated cells, establishing the similar tissue types like tumors derived from the Hepa1–6 cells, which was in sharp contrast with 20% undifferentiated areas in the undifferentiated ES cells group (Fig. 6C). After the ES-Hepa hybrids were differentiated in vitro for 7 and 14 days, the undifferentiated areas in the tumor increased, with a less divergent but homogenous tissue type compared with tumors derived from undifferentiated ES-Hepa hybrids (Table 2). These data further demonstrated that the tumorigenic potential was restored in the reprogrammed ES-Hepa hybrids upon differentiation.

DISCUSSION

Silencing of p16INK4a is a common event in both human and mouse cancers through losing control of cell cycle arrest and abnormal cell proliferation (32). Early epigenetic events that occur in the silencing course of p16INK4a may be considered to predict cancer development, thus providing means for pre-diagnosis. In the current study, epigenetic silencing of p16INK4a in mouse HCC cells can be reactivated by fusion with mouse embryonic stem cells. Upon differentiation, the hybrid regained the original histone methylation pattern of the p16INK4a silencing program, associated with a strong tumorigenic potential after transplanted subcutaneously into the immunodeficient nude mice. Because the early epigenetic events in silencing of p16INK4a remain largely unknown, our study provides novel insights in this course.

We still do not know the specific reasons why the differentiated reprogrammed cancer cells re-established malignant programs like the former cancer cells. In our speculation, there are two reasons that could explain this fact. First, some genes refuse to be reprogrammed. In this research, c-jun escaped from the reprogramming mode and exhibited an expression level similar to that in Hepa1–6 cells. The transcription factor c-jun was reported as an essential oncogene in HCC development and progression by cooperation with Ras and repression of p53 in cell proliferation and anti-apoptosis (33). The anti-reprogramming effect of c-jun revealed it might be one of the genes that contribute to the tumorigenic course of the ES-Hepa hybrids upon differentiation. Second, genetic mutations in cancer cells cannot be changed by means of epigenetic reprogramming effects. These inappropriate intrinsic genetic alterations inside a cell may predispose the cell to malignant epigenetic modulations after response to the extrinsic signals.

The bidirectional cellular communication with the microenvironment of the cancer cell is related to the ability of progression and development. Previous studies have proven that embryonic microenvironments would change aggressive cancer malignancy by reducing tumorigenesis and metastasis (34–36). Tumors may develop in a particular developmental pattern, in which epigenetic modulation may contribute to the tumorigenic course of the ES-Hepa hybrids upon differentiation. Second, genetic mutations in cancer cells cannot be changed by means of epigenetic reprogramming effects. These inappropriate intrinsic genetic alterations inside a cell may predispose the cell to malignant epigenetic modulations after response to the extrinsic signals.
removing the existing epigenetic modifications and reinitiating the pluripotent transcriptional network in reprogrammed cells (20, 24, 37, 38), and this network may reset the epigenetic program of Hepa1–6 cells, activating silenced p16INK4a. After the withdrawal of the embryonic microenvironment, the reprogrammed ES-Hepa hybrids regained malignant characteristics in terms of epigenetic inactivation of p16INK4a, activation of oncogenes, and tumorigenic potential in vivo. These results suggested the possibility of the role of microenvironment alteration such as gastric acid reflux, inflammation, and other factors in tumorigenesis from stem cells with intrinsic mutation. This might also be the reason why medicines such as 5-Aza-2′-deoxoytidine and procanamide do not work after withdrawal (39, 40).

Although we cannot determine the cause of the malignant “program” in differentiated ES-Hepa hybrids, it results in the repression of tumor suppressors and activation oncogenes by triggering DNA methyltransferases, histone-modification enzymes such as lysine acetyltransferases, lysine deacetylases, and lysine methyltransferases in the differentiated environment. The ES-Hepa hybrids in this study may represent a “transition” state that p16INK4a was affected by both H3K4 and H3K27 methylation, which may predict either active or repressive states in the differentiation course. When the differentiation-related malignant program started, trimethylation of H3K27 was the first to modulate the promoter region of p16INK4a, which was much before the dimethylation of H3K9. However, although the di- and trimethylation of H3K4 were considered as markers of gene-activation chromatin structure, our ChIP results showed that, in the p16INK4a inactivation course, both methylation of H3K4 markers existed on the promoter region of p16INK4a, suggesting that the methylation of H3K4 was not necessarily directly related to the gene expression activity. The combination of H3K27 trimethylation and H3K9 dimethylation was enough for silencing the p16INK4a and indicated the tumorigenic potential of the ES-Hepa hybrids.

In summary, our studies have shown an early epigenetic pattern of p16INK4a silencing in the tumorigenic course of fusion reprogrammed mouse HCC cell. We have

**FIGURE 6. In vivo analyses of tumorigenic potential in the ES-Hepa hybrids.** A, tumor volume measurements (mean ± S.E.) of ES, Hepa1–6, ES-Hepa hybrids, and differentiated ES and ES-Hepa hybrid cells after injected subcutaneously into nude mice. Representative data from multiple experiments are shown. B, representative bright-field images of the tumors. C, representative hematoxylin & eosin-stained sections of a teratoma from ES-Hepa hybrid cells. The ES-Hepa hybrids differentiated into cuticular epithelium (arrow in b), glandular epithelium (triangle in b), and cartilage (⁎ in c). Undifferentiated tissue can be seen in 80% of the teratoma (d). Scale bar, 1000 μm.

**TABLE 2**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Latency period (weeks)</th>
<th>Degree of differentiation (%)</th>
<th>Types of tissues seen</th>
</tr>
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<tbody>
<tr>
<td>ES</td>
<td>3–4</td>
<td>&gt;60</td>
<td>Epithelial and mesodermal tissues</td>
</tr>
<tr>
<td>Hepa1–6</td>
<td>4–5</td>
<td>0</td>
<td>Homogenous undifferentiated</td>
</tr>
<tr>
<td>ES-lymphocyte hybrids</td>
<td>3–4</td>
<td>&gt;60</td>
<td>Broad range of epithelial and mesodermal tissues</td>
</tr>
<tr>
<td>ES-Hepa (D0)</td>
<td>2–3</td>
<td>&lt;20</td>
<td>Immature epithelial tissues, glandular epithelium, adipose, and cartilage</td>
</tr>
<tr>
<td>ES-Hepa (D7)</td>
<td>2–3</td>
<td>&lt;10</td>
<td>Undifferentiated epithelial tissues</td>
</tr>
<tr>
<td>ES-Hepa (D14)</td>
<td>2–3</td>
<td>&lt;10</td>
<td>Undifferentiated epithelial tissues</td>
</tr>
</tbody>
</table>
shown here that the trimethylation of H3K27 is the first marker established on the promoter region, independent of promoter DNA methylation and H3K9 methylation. This epigenetic silencing pattern of p16INK4a may predispose tumor initiation and progression, because it promotes the cell cycle regulator silencing course from the permissive activation state. Although this work focused on murine HCC and, therefore, might not be representative of other tumor systems, the activation and then repression of p16INK4a by means of nuclear reprogramming may provide a hint of epigenetic modulation in the other related species. It is important now to define whether this specific epigenetic modulation pattern in p16INK4a silencing can be applied to human HCC to establish prognosis strategies for clinical use.

REFERENCES