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Trans-regulated Silencing and Reactivation of *TP53* Tumor Suppressor Gene in Malignant Transformation and Its Reversion

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Despite growing interest in the methylation-mediated silencing of tumor suppressor genes in the neoplastic process, its signaling mechanism remains largely unknown. Here we show in a cultured murine cell line system that the silencing and reactivation of tumor suppressor gene *TP53* were reversibly controlled by a *trans*-acting regulatory mechanism. The gene product p53, which was constitutively expressed and activated upon X-ray irradiation in non-malignant parental cell line, was undetectable in its X-ray-induced malignant transformants, while they retained a wild-type *TP53*. The silencing was cancelled by transferring a human chromosome 11 and the expression of p53 was restored. The non-malignant revertants thus obtained were again susceptible to transformation by X-irradiation, giving rise to re-transformants, in which p53 was again repressed while the human chromosome 11 retained the ability to turn on *TP53* when it was transferred into other malignant clone. The silent *TP53* could be reactivated by treatment with the demethylating agent 5-azadeoxycytidine. These observations indicate the presence of a *trans*-acting signaling mechanism in the methylation-mediated regulation of *TP53* expression which is associated with the acquisition of malignancy.

Key words: Malignant transformation — p53 — Transcriptional silencing — Methylation — Transaction

Tumor suppressor gene *TP53* (formerly known as *p53*) is a key regulator in the neoplastic process and is directly involved in approximately half of all human cancers,[1] where mutational inactivation plays a critical role in the neoplastic process.[2–4] However, the presence of wild-type *TP53* in a subset of tumors has suggested the existence of alternative mechanisms to shut down the p53 pathway.[5] They include disruption of the p53/MDM2 autoregulatory loop by enhanced degradation due to overexpression of MDM2 [6, 7] or due to binding to viral oncoproteins encoded by transforming viruses.[8, 9] Currently, there is also a growing interest in the transcriptional silencing of tumor suppressor genes.[10–12] Indeed, silencing by *de novo* methylation has been found in *RB1*, $p16^{INK4a}$, $p14^{ARF}$, *hMLH1*, *VHL*, *LKB1*, *NF1* and *BRCA1* genes.[13–22] In contrast, although the functional silencing of human and rat *TP53* by promoter methylation have been suggested from *in vitro* assay data,[23, 24] no direct evidence has been obtained for *de novo TP53* silencing in human cancers, due at least in part to the intrinsically low-level expression of the wild-type *TP53* in normal adult tissues. Furthermore, the role of transcriptional silencing in carcinogenesis, whether it is cause or consequence, as well as its regulatory mechanism, is largely unknown. To address the involvement of epigenetic mechanisms in the etiology of malignancy, we examined the alteration of the expression of *TP53* during malignant transformation and its reversion to non-malignancy by chromosome transfer in the m5S cell line system. In this respect, the m5S cell line, an immortalized but non-malignant near-diploid cell line of mouse embryonic origin, [25] provides a unique *in vitro* system for the dissection of genomic changes associated with malignancy because it retains wild-type *TP53* but is amenable to transformation to malignancy upon exposure to radiation and other mutagens in dose-dependent manner. We show here that the methylation-mediated silencing of the *TP53* gene is associated with the acquisition of malignancy and its transcriptional ON/OFF is reversibly controlled by a *trans*-acting regulatory mechanism.

MATERIALS AND METHODS

Cell lines The m5S is an immortalized but non-malignant murine cell line established from mouse embryonic skin, characterized by a near-diploid karyotype, anchorage-dependent monolayer growth, remarkable sensitivity to confluence-dependent inhibition of growth, and lack of tumorigenicity in nude mice.[25, 26] The cells were maintained in α-modified MEM medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. For experiments, 10^6 cells were plated into 100-mm plastic dish, and allowed to grow to reach confluence with medium changes every other day. Morphological transformants were those previously isolated from X-irradiated m5S cells and characterized for their karyotypes and clonability in soft agar and tumorigenicity in nude mice.[26] The transformed derivatives were

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cultured under the same conditions, but the serum concentration was reduced to 5% when they reached 80% confluence. The cells were used for western blot analysis after culturing for the last 4 days without changing the medium.

Human chromosome transfer A single human chromosome was transferred by a microcell-mediated chromosome transfer as described.[27, 28] Briefly, microcells were prepared from A9 cells containing a *neo*-tagged single human chromosome by centrifugal enucleation in the presence of 10 μg/ml cytochalasin B and sequential filtration through polycarbonate filters with pore sizes of 8, 5 and 3 μm. The microcells were then fused to the cells using polyethylene glycol, and monochromosomal hybrids were selected and maintained in culture medium containing G418 at 800 μg/ml for selection and 400 μg/ml for maintenance.

Re-transformation of revertants by X-irradiation The transformants that had reverted to flat morphology after transfer of human chromosome 11 were irradiated at confluence with graded doses of X-rays (50 kVp, 2 mm Al filter). The experimental procedures and scoring criteria for transformation assay were essentially the same as described previously,[26] except for the presence of 400 μg/ml G418 during the incubation period of 6 weeks.

Assay for anchorage independence The effects of introduced human chromosomes on the growth in soft agar were studied as described.[26] A 0.33% agar medium containing 100 cells was overlayed on 2% agar medium, and the cells were allowed to grow for 3 weeks. The colonies were counted by a BioTran III automatic count totalizer (New Brunswick Scientific Co., Inc., Edison, NJ).

Western blot analysis After removal of culture medium, the confluent cultures were exposed to X-rays. The removed medium was set aside and returned to the original dish immediately after irradiation, and the cells were harvested at varying times after irradiation. Unirradiated cultures served as controls. Whole cell extract was prepared as described previously.[29] The extract (50 μg/lane) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after denaturation at 95°C for 5 min, transferred to polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) using a Semi-Dry Transfer Cell (Bio-Rad Lab., Hercules, CA), and probed with 330 ng/ml anti-p53 antibody p53(Ab-3) (Calbiochem, Cambridge, MA) or 660 ng/ml anti-p21 antibody p21(C-19) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The immune complexes were visualized by using enhanced chemiluminescence reagent (Amersham Corp., Arlington Heights, IL) according to the protocols recommended by the manufacturer. For reference, the membrane was then probed with 150 ng/ml anti-β-tubulin antibody (Roche Molecular Biochemicals, Basel, Switzerland) under the same conditions.

Treatment with 5-azadeoxycytidine On day 1, 10^6 cells were plated per 100-mm plastic dish. After 24 h, the cultures were treated with various concentration of 5-aza-2′-deoxycytidine (Sigma Chemical Co., St. Louis, MO), then the medium was replaced with that containing the same concentration of the drug on day 4, and cells were harvested on day 6 for extraction of protein.

RESULTS

Constitutive and X-ray-induced expression of p53 Fig. 1A shows the basal expression levels of p53 in the parental m5S cells and 18 X-ray-induced morphological transformants. The parental m5S cells constitutively expressed p53 at a high level. However, the expression of p53 was variable among the transformants. In particular, p53 was undetectable or greatly reduced in three highly malignant transformants, TR6110, TR2520 and TR2531, which were previously shown to differ from other transformants in that they were of spindle-shaped morphology, clonable in soft agar and highly tumorigenic in nude mice.[26] The *TP53* cDNA was recovered from m5S and TR6110 cells by reverse transcriptase-mediated polymerase chain reaction (RT-PCR). Sequence analysis of the entire coding region of cDNA revealed the presence of authentic mouse *TP53* (data not shown). The recovery of cDNA from TR6110 indicates that *TP53* is expressed at a trace level in this clone. The constitutive expression of p53 was also seen in m3 strain (Fig. 1B), a progenitor of m5S cell line prior to immortalization, indicating that a high level of expression was characteristic of cells of embryonic origin.

When the m5S cells were X-irradiated, there was a rapid and transient decrease in the p53 level, which was followed by an increase to a level comparable to that of the unirradiated control. Such response kinetics were essentially the same for 2 cGy- and 3 Gy-irradiation, while the transient depression of p53 appeared earlier at higher dose. In order to examine the functional competence of the p53, we examined the kinetics of p21 protein, the product of the $p21^{WAF1}$ gene, whose transcription is upregulated by activated p53. As shown in Fig. 1C, p21 was not detected in the non-irradiated cells but increased after irradiation, indicating that the constitutively expressed p53 was in a latent inactive form that was immediately activated upon irradiation, and hence was subject to degradation followed by transcriptional activation and stabilization, being consistent with the p53 turnover pathway in response to genotoxic damage.[2, 4, 30, 31] In the malignant transformants, TR6110, TR2520 and TR2531, which showed a loss of p53, the induction of p53 after X-irradiation was also absent in TR2520 and TR2531, while a slight increase in the p53 level was observed in TR6110 after 3 Gy exposure (Fig. 2A) but not after 2 cGy exposure (data not shown).

Modulation of p53 expression by chromosome transfer In order to identify a possible radiation-induced loss-

of-function mutation which was responsible for the silencing of *TP53*, we transferred *neo*-tagged human chromosomes into the transformants by microcell-mediated chromosome transfer. In this experiment, human chromosomes 1, 2, 7, 11, 12, 17 and 20 were transferred into TR6110. We found that TR6110 cells reverted to flat morphology, which was comparable to that of the parental m5S cells, only when they were transfected with human chromosome 11. This clone, named TR6110(neo-11) exhibited confluence-dependent inhibition of growth, loss of ability to form colonies in soft agar, and constitutive and X-ray-induced expression of p53 (Figs. 2A and 3). The effect of human chromosome 11 was similar for TR2520 and TR2531 cells; the monochromosomal hybrids, TR2520(neo-11) and TR2531(neo-11), showing a flat morphology (data not shown), regaining basal and X-ray-induced expression of p53 (Fig. 2A). Unexpectedly, however, introduction of human chromosome 17, which harbored human *TP53*, was unable to revert the cells to flat morphology, and moreover, neither mouse nor human p53 was expressed in the hybrid TR6110(neo-17) (Fig. 2B).

Re-transformation by X-irradiation Then, we asked if any mutation on transferred human chromosome 11 con-

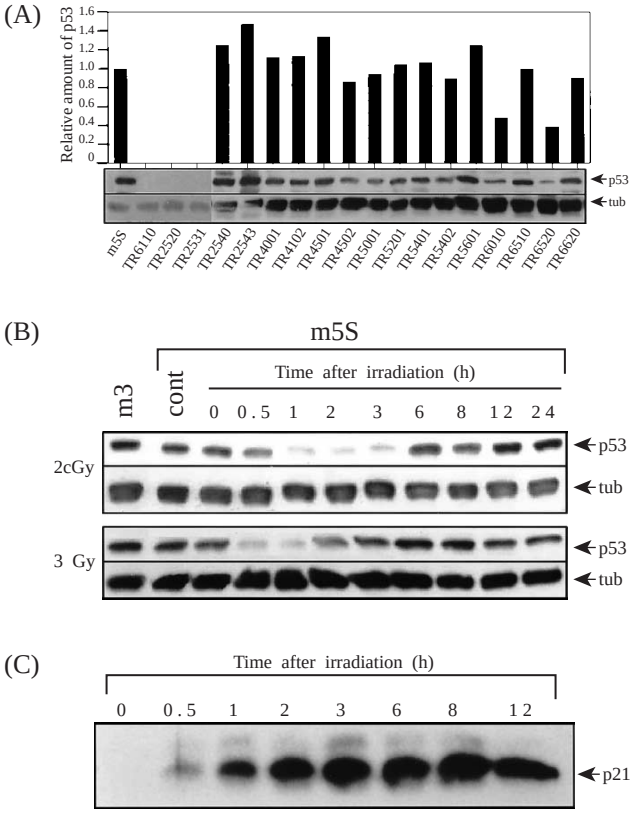

Fig. 1. Western blot analysis for the expression of p53 and p21. (A) The expression of p53 in m5S cells and their X-ray-induced transformants. The expression of p53 was normalized to β-tubulin, and is presented as signal density relative to that of m5S cells. For the transformants, the first digit represents experimental number, the second digit shows the X-ray dose in Gy, and the last 2 digits show clone number. (B) Changes in the level of p53 after 2 cGy or 3 Gy X-irradiation in m5S cells. Unirradiated cells were used as controls. The left lane, m3, is an unirradiated primary culture of mouse embryonic cells, a progenitor of m5S prior to immortalization. (C) The expression of p21 after 3 Gy X-irradiation in m5S cells.

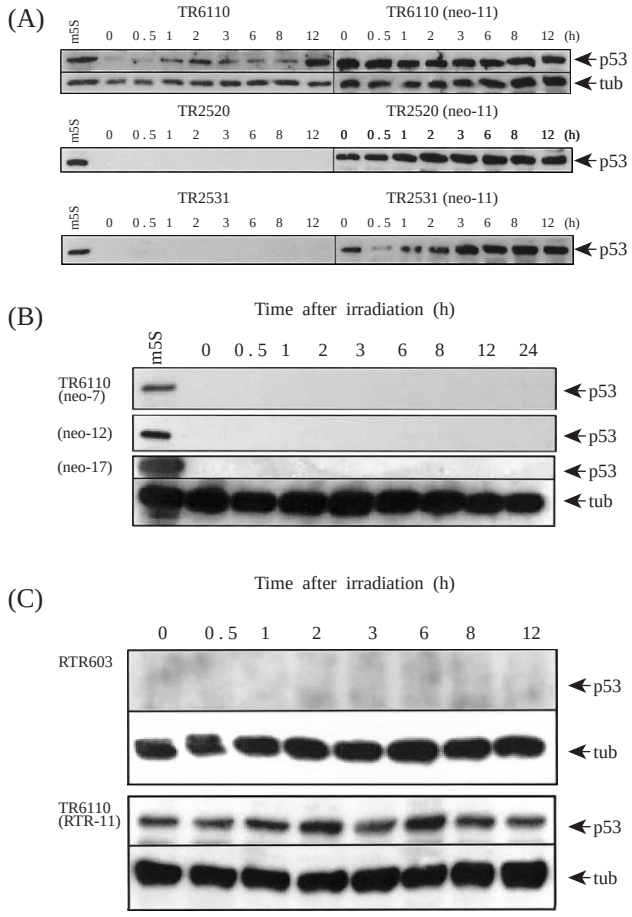

Fig. 2. The effects of chromosome transfer on the expression of p53. (A) The expression of p53 at various times after 3 Gy X-irradiation in malignant transformants, TR6110, TR2520 and TR2531, before and after (neo-11) transfer of human chromosome 11. (B) The expression of p53 by 3 Gy X-irradiation in TR6110 cells containing human chromosome 7, 12 or 17. In each experiment, the expression level in unirradiated m5S cells is shown as a control. (C) The expression of p53 after 3 Gy X-irradiation in re-transformant RTR603 and morphological revertant TR6110(RTR-11) obtained by transfecting TR6110 with human chromosome 11 isolated from RTR603.

fers upon the cells a malignant phenotype. When we studied the re-transformation of the revertant TR6110(neo-11) to malignancy by X-irradiation, a dose-dependent induction of malignant foci was observed, where the rate of focus induction was comparable to that in the X-irradiated m5S cells (data not shown). A transformed clone thus

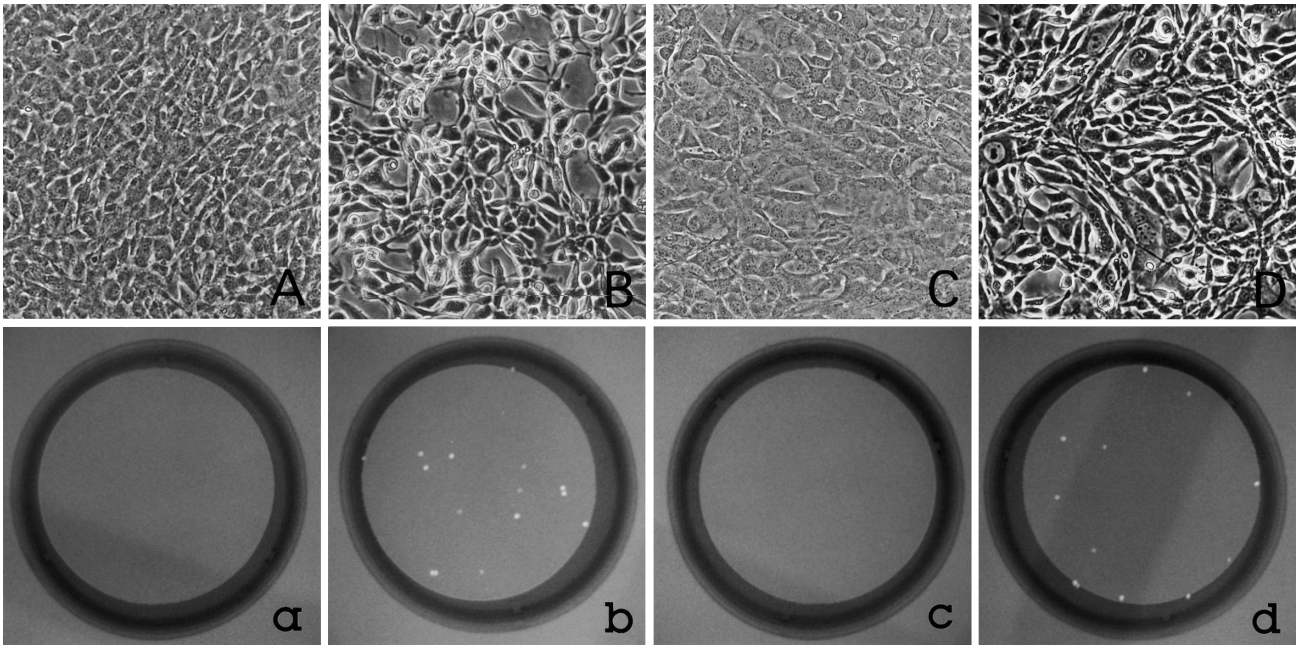

Fig. 3. Morphological change (top row) and colony formation in soft agar (bottom row) associated with malignant transformation and reversion. (A, a): m5S cells, (B, b): X-ray-induced transformant TR6110, (C, c): flat revertant TR6110(neo-11) obtained by introducing a human chromosome 11 into TR6110, (D, d): re-transformant RTR603 obtained by 6 Gy irradiation of TR6110(neo-11). Malignant transformants, TR6110 and RTR603, are characterized by a massive piling-up and multi-layered crisscrossed array of cells and clonability in soft agar.

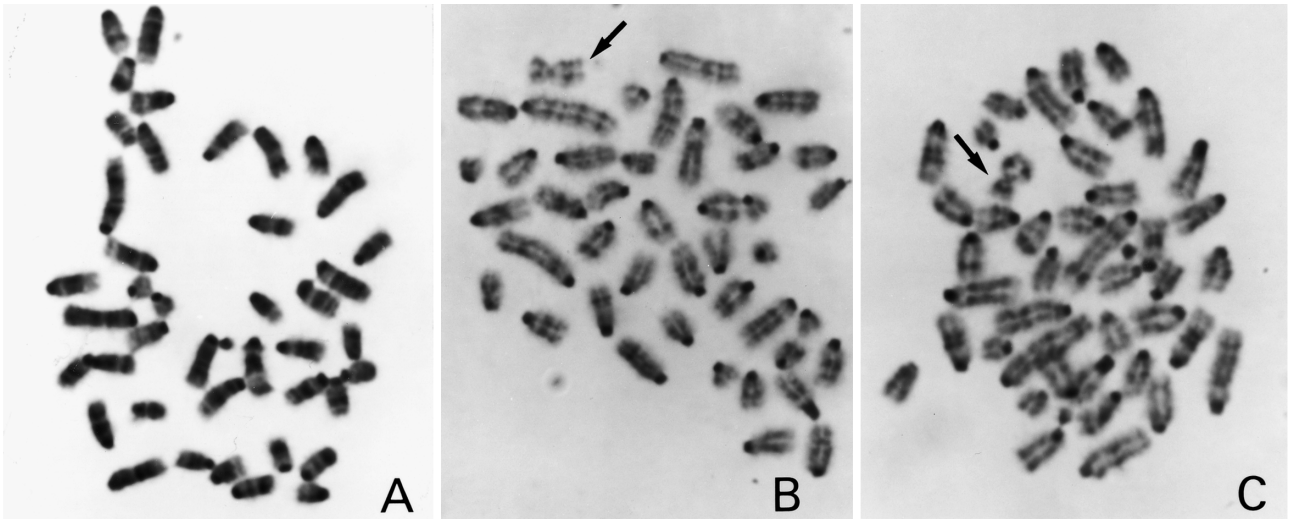

Fig. 4. Chromosome constitutions of the cells. (A) Malignant transformant TR6110, (B) revertant TR6110(neo-11), (C) re-transformant RTR603. Arrow indicates human chromosome 11.

obtained, here named re-transformant RTR603, had lost the expression of p53 both before and after X-irradiation (Fig. 2C), showed a similar morphology to that of TR6110 and had regained the ability to grow in soft agar (Fig. 3), but still contained a morphologically intact human chromosome 11 (Fig. 4). Then, the human chromosome 11 was isolated from the RTR603 cells and transferred to TR6110 cells. The resultant monochromosomal hybrid, named TR6110(RTR-11), again became flat, lost clonability in soft agar and expressed p53 (Fig. 2C), indicating that the malignant reversion was not a consequence of mutation on the introduced human chromosome 11, but a consequence of epigenetic inactivation by X-irradiation, although, unlike the issue in the primary transformation, mutation(s) on mouse chromosomes is not excluded at this stage.

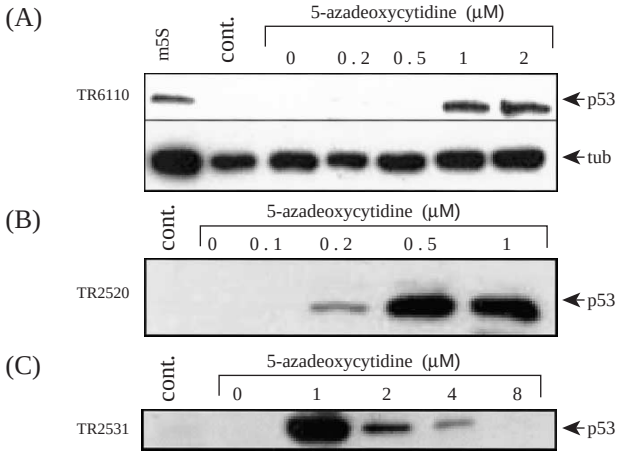

Fig. 5. Restoration of the expression of p53 in the malignant transformants after treatment with the demethylating agent 5-azadeoxycytidine.

Restoration of p53 expression by demethylating agent

These observations strongly point to the involvement of epigenetic mechanisms in the regulation of *TP53* expression. Then, we treated the malignant transformants with a demethylating agent, 5-aza-2′-deoxycytidine. The treated cells became flat and recovered the expression of p53 (Figs. 5 and 6), suggesting that the loss of expression of p53 in the transformants was a consequence of transcriptional silencing of *TP53* by methylation modification.

DISCUSSION

The mouse m5S is a unique immortalized cell line in that it retains near-diploid karyotype, contact inhibition at confluence and anchorage-dependent growth. Unlike many other murine cell lines, the m5S cells retains wild-type *TP53* and, similarly to the primary mouse embryonic cells, the latent form of the gene product p53 is expressed at high level, which is activated upon X-ray irradiation at a dose as low as 2 cGy. However, in the malignant derivatives, the expression of p53 is totally lost or severely diminished at the transcriptional level, but can be restored by treatment with a demethylating agent, 5-azadeoxycytidine. A significant finding is that the silencing of *TP53* is cancelled by introducing human chromosome 11, while it is refractory to other human chromosomes, including chro-

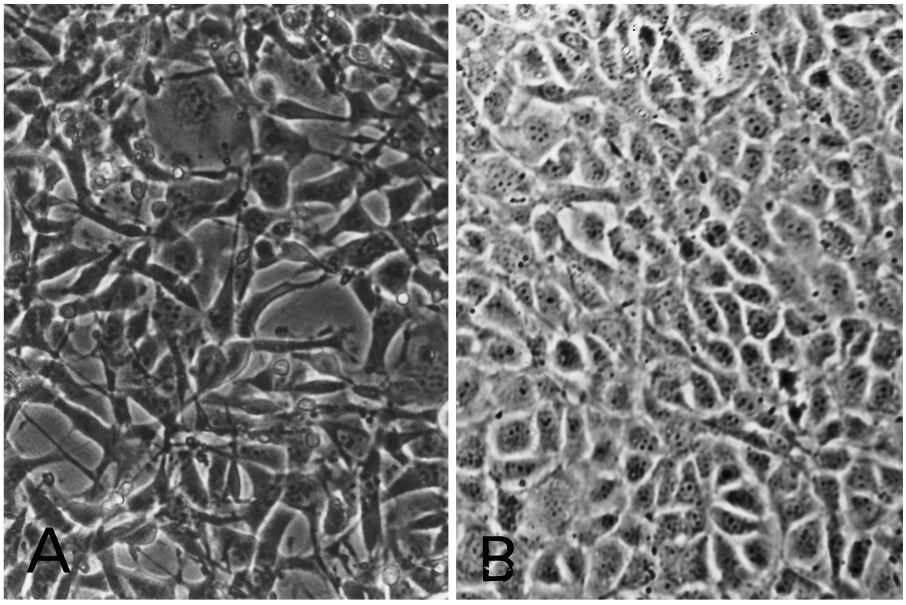

Fig. 6. Morphological characteristics of TR6110 before (A) and after (B) treatment with 5-azadeoxycytidine.

mosome 17, which harbors human *TP53*. The p53-expressing revertants thus obtained are also susceptible to transformation by X-irradiation into malignant cells with no expression of p53, while they retain morphologically and functionally intact human chromosome 11, which is still able to turn on the expression of *TP53* upon introduction into other malignant cells. The change in the p53 expression status is coupled with the change the cell morphology and anchorage dependency, indicating a strong link between p53 function and cellular and malignant phenotype.

All these lines of experimental evidence clearly indicate the involvement in the malignant transformation of methylation-mediated silencing of *TP53*, which is reversible and controlled by a *trans*-acting regulatory mechanism. The reversible transcriptional ON/OFF switching suggests that the *TP53* is not a prime target of X-ray mutagenesis responsible for the malignant transformation, but is the subject of epigenetic methylation-mediated silencing. As to the maintenance of transcriptional silencing of *TP53*, it should be noted that neither mouse nor human *TP53* was expressed by introducing human chromosome 17, which harbors human *TP53*. Since p53 is expressed in the donor A9 cells containing 17 (data not shown), the observation indicates that both host and donor alleles are affected in harmony by the silencing signal. Recently, one of the DNA methyltransferase genes, *DNMT3B*, has been mapped to human chromosome 20 ICF immunodeficiency syndrome locus.[32] However, the direct involvement of *DNMT3B* is unlikely because the malignant clone TR6110 was refractory to the transfer of human chromosome 20.

The molecular mechanism of silence signaling that otherwise leads to methylation modification of *TP53* remains to be deciphered. However, it is tempting to speculate that mechanistic links exist between genomic imprinting and gene silencing. Human chromosome 11p15.5 and its mouse homologue distal chromosome 7 harbor an imprinting control region, where genes are differentially silenced or activated by methylation modification in a parent-of-origin specific manner.[33] The reversible transcriptional silencing of *TP53* may be explained by a hypothetical *trans*-acting methylation control module on human chromosome 11 with its function regulated by a substrate-specific feedback mechanism. In the presence of p53, the control module, whether it is endogenous or exogenous, turns off the silencing signal, whereas in its absence the silencing signal is turned on to keep *TP53* methylated. Obviously, such a scenario is highly speculative. However, the transcription of several transcription factors has been shown to be regulated by ubiquitine/proteasome-mediated proteolytic motifs, forming a substrate-specific *trans*-regulatory loop of transcription.[34] For instance, gene-specific demethylation by a *trans*-acting mechanism mediated by protein-DNA recognition has been demonstrated for *Igκ* gene.[35, 36]

The relationship between radiation-induced malignant transformation and transcriptional silencing of *TP53* remains unknown. The loss of p53 with the retention of wild-type *TP53* during radiation-induced malignant transformation has been reported in some *in vitro* systems.[37, 38] Also, high frequencies of promoter hypermethylation of $p15^{INK4b}$ gene have been reported in mouse radiation-induced thymic lymphomas.[39, 40] The involvement of the same mechanism for silencing is a testable proposition. The malignant clones dealt with in the present study, TR6110, TR2520 and TR2531, are unique in that they show high clonability in soft agar and tumorigenicity in nude mice. Among them, TR6110 and TR2531 share a del(13)(C2-ter) deletion mutation, which was common to 17 of 24 (70%) morphological transformants.[26] It is therefore likely that the methylation-mediated silencing of *TP53* is a later event associated with selection bias. Though the possibility has been discussed repeatedly,[41] there is no direct evidence of methylation-mediated silencing of *TP53* in human primary cancer. However, there are several indirect lines of evidence for the involvement of methylation in the abrogation of p53 function.[23, 24] In human cancer, approximately 30% of *TP53* mutations occur at CpG sites, suggesting the preferential targeting of methylated CpG dinucleotides by hydrolytic deamination of 5-methylcytosine or errors made during the methylation process.[10, 42, 43] This leads to the intriguing proposition that transcriptional inactivation by methylation modification may predispose *TP53* to irreversible mutational inactivation during tumorigenesis. The delineation of the signaling mechanism of methylation-mediated gene silencing in relation to its role in carcinogenesis may shed light on the feasibility of p53-targeted clinical management of malignancies.

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