[CANCER RESEARCH 59, 5882–5886, December 1, 1999]

Advances in Brief

Spontaneous and Mutagen-induced Transformation of Primary Cultures of Msh2−/− p53−/− Colonocytes[1]

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Abstract

Loss of function of mismatch repair (MMR) genes underlies hereditary nonpolyposis colorectal cancer (HNPCC). However, the inability to maintain primary colon epithelial cells in culture has limited the analysis of the contribution of MMR gene defects to colorectal tumorigenesis. We have now established primary cultures of epithelial cells from the colon crypts of Msh2−/− p53−/− double-knockout mice. These cells undergo spontaneous transformation (soft agar colonies and s.c. tumor formation), with a progressively shorter latency as a function of increasing passages in culture.

Treatment of early passage cells with the mutagen methylmethane thiosulfonate (MMS) further decreases the transformation latency of Msh2−/− p53−/− cells. Spontaneous transformation of p53−/− colonocytes is only observed using late passage cells, and methylmethane thiosulfonate-treated early passage p53−/− colonocytes do not form tumors when injected into immunodeficient mice. Together, these findings support the pathogenic role of *MMR* gene inactivation in colorectal tumorigenesis and provide an experimental model for the serial assessment of the molecular phenotype associated with Msh2 deficiency.

Introduction

Several independent mutations in oncogenes and tumor suppressor genes are required for the malignant transformation of normal colonic mucosa (1, 2). These genetic alterations usually develop over several decades, but their occurrence might be accelerated by an impairment in the processes involved in the safeguard of genomic stability, leading to an increased rate of mutagenesis and/or chromosomal rearrangements (3, 4). In support of this hypothesis, a number of hereditary cancer predisposition syndromes are associated with defects in the process of DNA repair. Two of these, HNPCC[3] and Muir-Torre syndrome, are characterized by the development of tumors of the proximal colon at an early age (5, 6). In addition, Muir-Torre patients are prone to cancers of the sebaceous glands and keratoacanthomas of the skin (6). HNPCC patients exhibit defects in their ability to repair mutations because of a germ-line alteration in one of four human DNA MMR genes (*MSH2, MLH1, MSH6,* and *PMS2*; Refs. 7–12). A germ-line mutation in one allele combined with an acquired somatic mutation in the other allele are necessary for the manifestation of this trait (13, 14). Recent data suggest that MMR genes are also mutated in some cases of sporadic colon cancer (15–17). Abnormal DNA MMR is reflected in somatic changes in the length of DNA microsatellite repeat sequences, which are distributed throughout the genome (18), but mutations in genes involved in cell proliferation and survival are those postulated to play an important role in tumor development. For example, the *TGFβIIR, IGFIIR,* and *BAX* genes carry short repeated sequences in their coding region that are frequently mutated in patients with HNPCC (19–21).

The importance of an intact MMR system in preventing tumor development has been formally demonstrated by the enhanced susceptibility of Msh2 knockout mice to neoplastic transformation (22, 23). T-cell lymphoma was the most common tumor type in these knockout mice (22, 23); tumors of the intestinal tract were, however, detected in the cohort of mice that did not develop lymphoma or in those succumbing to lymphoma at old age (24). We recently developed an *in vitro* model of colorectal tumorigenesis in which primary cultures of p53-deficient mouse colonocytes were transformed by ectopic expression of oncogenic Ki-*ras* (25). We reasoned that primary colonocytes from p53−/− Msh2−/− knockout mice might undergo spontaneous transformation and that treatment of Msh2-deficient epithelial cells with mutagens might accelerate the process.

Consistent with the postulated role of MMR genes in human colorectal tumorigenesis, we found that the Msh2-deficient background promotes spontaneous and mutagen-induced tumorigenic conversion of primary p53-deficient mouse colonocytes.

Materials and Methods

Generation of Msh2−/− p53−/− Mice. Mice heterozygous for the *Msh2* and *p53* genes on a mixed strain background (SWR-J, C57BL/6J, and 129/ola) were intercrossed to produce F_2 progeny null for both genes (Msh2−/− p53−/−; Ref. 26). Mice were maintained in a triple barrier facility and were genotyped using Msh2- and p53-specific PCR-based assays.

Isolation of Genomic DNA and PCR-based Typing of Mice. DNA was extracted from ear-notched tissue and from cell lines using a QIAamp Tissue Kit (Qiagen) according to the manufacturer's instructions. A three-primer assay specific for Msh2 was carried out as described (23). A three-primer assay specific for p53 was carried out using 50 ng of template DNA in a 50-μl reaction containing 100 μM each of primer 10588 (5′-GTGGGAGGGA-CAAAAGTTC-GAGGCC-3′), 10930 (5′-TTTACGGAGCCCTGGCGC-TC-GATGT-3′), and 10480 (5′-ATGG-GAGGCTGCCAGTCCTAACCC-3′) and 1 unit of Taq polymerase (Life Technologies, Inc.). The reaction was carried out at: 94°C, 3 min; 40 cycles at 94°C, 45 s; 55°C, 60 s; 72°C, 90 s; and 72°C, 7 min. The wild-type primers 10480 and 10588 amplified a product of 600 bp, whereas the targeted allele primers 10588 and 10930 amplified a product of 150 bp. All PCR reactions were carried out in a Perkin-Elmer Gene Amp 9600 thermal cycler. Ficoll/Orange-G loading buffer was used to prevent obscuring of bands <200 bp. Amplification products were resolved by electrophoresis on a 2% agarose gel alongside a 100-bp ladder and visualized by ethidium bromide staining.

Isolation and Culture of Colon Crypts. Crypts were isolated from the colonic intestinal mucosa by a nonenzymatic technique as described (25). Briefly, the colon was removed from 27-day-old mice and flushed with 0.1 M calcium-/magnesium-free PBS. The colon was opened by longitudinal incision, soaked in 0.04% sodium hypochlorite for 30 min to kill bacteria and fungi, washed three times in PBS, cut into small pieces ~1.5 mm in length, and

Received 9/10/99; accepted 10/15/99.

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[1] This work was supported in part by NIH Grants RO1 CA-39481, RO1 CA-46782, and RO1 CA56542 (to R. V. I., B. C., and R. F., respectively).

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[3] The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; MMR, mismatch repair; MMS, methylmethane thiosulfonate; SCID, severe combined immunodeficient.

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placed in 5–10 ml of PBS containing 1 mM EDTA, 1 mM EGTA, and 0.5 mM DDT. After incubation at room temperature for 60–90 min with occasional shaking, the solution was carefully removed and replaced with PBS. The solution containing the colon pieces was vigorously shaken until the majority of the crypts were detached from the underlying stroma. All collected crypts were pooled and suspended in DMEM/nutrient mixture F-12 (HAM) supplemented with 10% FCS (Life Technologies, Inc.), 10% conditioned medium from p53−/− colon fibroblast and epithelial cell cultures, 2 mM glutamine, 50 μg/ml penicillin/streptomycin, 1 mM sodium pyruvate, 15 mM HEPES (pH 7.5), 0.1 mM nonessential amino acids, 20 μg/ml epidermal growth factor, 6 μg/ml insulin, 6 μg/ml transferrin, 1 μg/ml hydrocortisone, and 5 μg/ml cholera toxin. The number of viable crypts was determined by trypan blue exclusion. Viable crypts were plated at a density of ~250/cm^2 into mouse collagen type IV-coated 35-mm tissue culture dishes (Becton Dickinson) containing a feeder layer of ~1–2 × 10^5 mitomycin C-treated NIH 3T3 cells. Crypts were left to settle overnight in a minimal volume of medium at 37°C in 5% CO_2, and 24 h later, the culture medium was increased to 2 ml. New feeder layers were added each week, and when the cells formed a clone of ~10 mm diameter, the culture was trypsinized and transferred into two 35-mm mouse collagen type IV-coated tissue culture dishes containing the feeder layer described above. Contaminating colon fibroblasts were selectively removed from the culture using either 0.25% trypsin or physical scraping and aspiration. These procedures were repeated until only epithelial cells remained in the culture and no contaminating colon fibroblasts were seen by microscopy. Medium was changed every other day, and cells were passaged when confluent. Early passage cultures were split at 1:3 and grown without feeder layer and without conditioned medium. After isolation of crypts, colonocytes and fibroblasts were also obtained from the same colon by primary explant culture technique. Briefly, small pieces (~2–4 mm) of colon were plated into 35-mm tissue culture dishes and covered with complete medium (see above) supplemented with 50% serum. Cultures were tested for the presence of *Mycoplasma* using an ELISA detection kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Electron Microscopy. Ultrastructural studies of p53−/− and p53−/− Msh2−/− cell cultures were carried out as described (25, 27).

Treatment of Cell Lines with MMS. Cells (2 × 10^6) at early passage (P6) were plated into 175-cm^2 flasks (Collaborative Biomedical Products, Bedford, MA) and cultured overnight at 37°C with the appropriate growth medium. The following day, medium was replaced with medium containing the alkylating agent MMS (4 or 5 μg/ml; Sigma), and cells were cultured overnight. After treatment, medium was removed, and cells washed, split, and cultured with complete growth medium. The treatment was repeated four times.

Soft Agar Assays. Soft agar assays of untreated or MMS-treated p53−/− or Msh2−/− p53−/− colonocytes were performed in a double-layer agar system as described (25). Approximately 2 × 10^3 cells/ml (five replicates) in 1 ml of upper layer medium (~0.2% agarose) were overlaid onto the preformed basal layer. Colonies were sized with a calibrated eye-piece grid using an Olympus Ck2 inverted microscope at ×100. Colonies with a diameter ≥125 μm were counted after 1 and 2 weeks.

Xenograft Studies. Early, intermediate, or late passage (P10, P26, P32, P37, P44, and P49) Msh2−/− p53−/− or p53−/− colonocytes (3–5 × 10^5 cells in 0.2 ml of PBS) were injected s.c. in at least five Fox Chase ICR SCID outbred male mice [Tac:Icz:Ha (ICR)-SCID DF], 5–6 weeks of age, for each experimental group. As a control, medium was injected into the other flank. Mice were palpated daily for the development of tumors. Injections with late-passage cells were also repeated using immunocompetent syngeneic mice.

In a second set of xenograft studies, early passage Msh2−/− p53−/− or p53−/− colonocytes were treated with MMS (4 or 5 μg/ml), and after four to five doublings, inoculated (3–5 × 10^5 cells in 0.2 ml of PBS) s.c. into the flank of five Fox Chase ICR SCID outbred male mice. Tumor specimens from each mouse were fixed in 10% formalin and subjected to histopathological analysis.

Results

Establishment of Epithelial Cell Cultures from Crypts of Msh2−/− p53−/− Knockout Mice. Long-term primary cultures of Msh2−/− p5−/− colonocytes were obtained from ~30% of the mice used. Many epithelial cells derived from colonic crypts attached to collagen type IV-coated plates during the first 48 h of culture, but after 2–3 weeks, only a few colonies of epithelial cells survived and generated long-term cultures. The morphology of the cells was epithelial like and clearly distinguishable from the surrounding feeder layer of mitomycin C-treated fibroblasts (not shown).

Colonocytes derived from the explants began to spread out, usually after 10 days. At this time, explants were transferred to other Petri dishes to avoid massive contamination by colon fibroblasts. Multinucleate cells were usually present in the monolayer at early passages of the culture. Most of the cells had oval, clear nuclei with distinct and evident nucleoli. After several passages, Msh2−/− p53−/− cells, like the p53−/− colonocytes, could be maintained in regular medium.

Ultrastructural analysis of Msh2−/− p53−/− cells grown on a collagen type I matrix revealed the presence of numerous lumina lined by columnar epithelium with prominent basement membrane and obvious cell polarization (Fig. 1*A*). In addition, these cells showed a very prominent microvillar surface (Fig. 1*B*) and well-developed tight junctions (Fig. 1*B*). All of these features are indicative of well-differentiated epithelial cells and are consistent with their gastroenteric origin.

Spontaneous Transformation of Msh2−/− p53−/− Colonocytes: Growth in Soft Agar and *in Vivo* Tumor Growth. Growth in soft agar is a property of transformed cells. In quintuplicate experiments, early and late passage Msh2−/− p53−/− colonocytes were assessed for colony formation. Early-passage double-knockout cells formed ~10 colonies/plate, whereas late-passage double-knockout cells formed numerous (~90/plate) large colonies. Early-passage p53−/− colonocytes did not form colonies, and only rare, isolated colonies (~4/plate) were detected upon plating late-passage p53−/− colonocytes. In control experiments, numerous colonies (~90/plate) formed after plating early-passage p53−/− colonocytes infected with a Ki-*ras*-expressing retrovirus.

The tumorigenic potential of the Msh2−/− p53−/− colonocytes was also assessed by injecting 3–5 × 10^5 cells s.c. in immunocom-

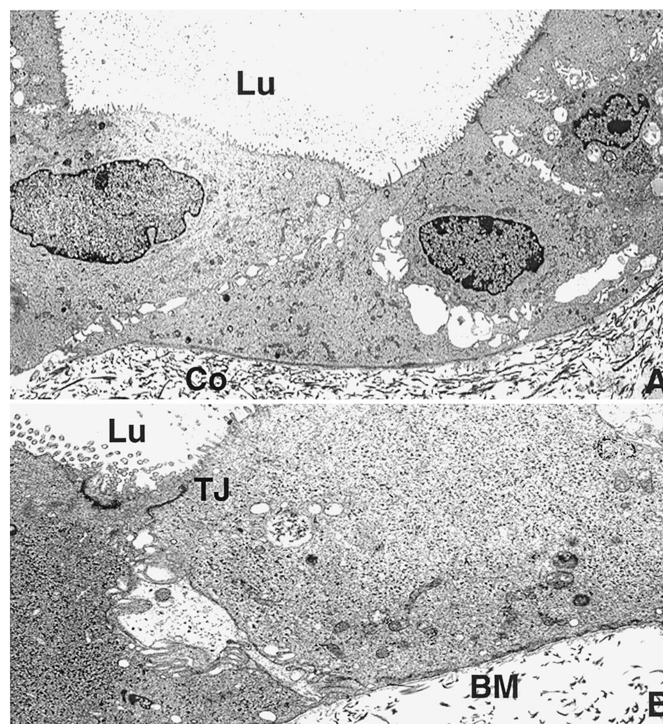

Fig. 1. Transmission electron micrographs of Msh2−/− p53−/− colonocytes grown on collagenous matrix (*Co*). Note the well-developed lumina (*Lu*) and the polarization of the cells. Tight junctions (*TJ*) are also well developed, and a prominent basement membrane (*BM*) is present. *A,* ×3200; *B,* ×8600.

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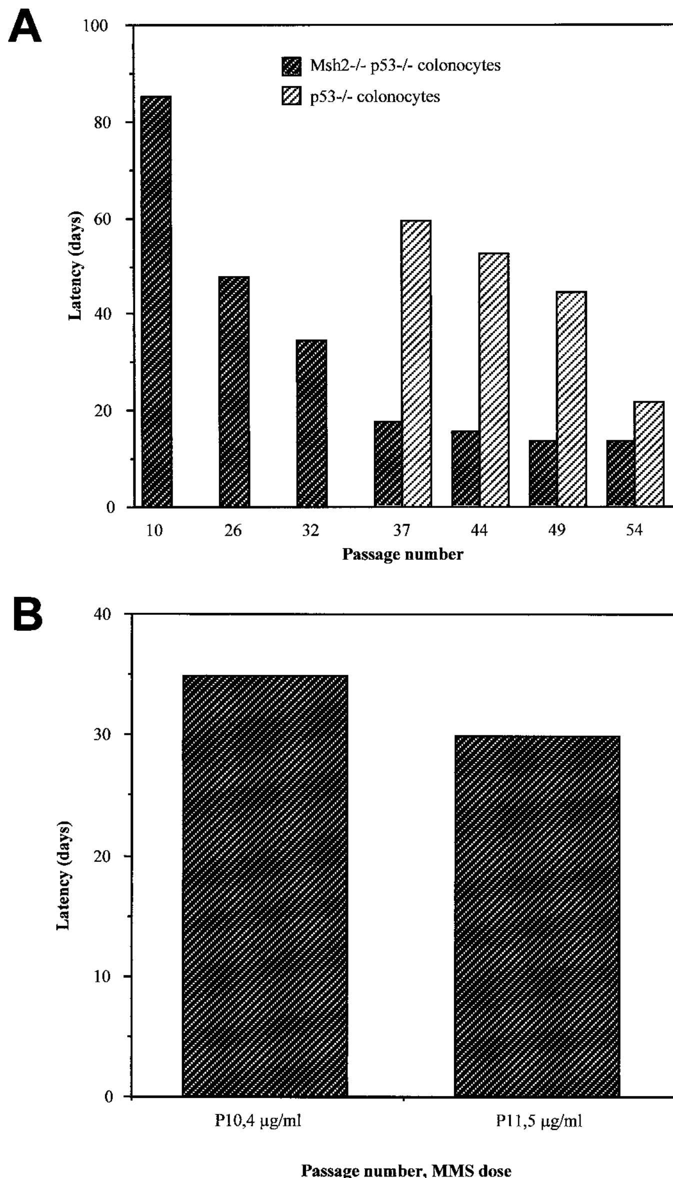

Fig. 2. Spontaneous and MMS-induced s.c. tumor formation by p53−/− and Msh2−/− p53−/− colonocytes. *A,* latency of tumor formation by p53−/− and Msh2−/− p53−/− colonocytes as a function of passage in culture of the injected cells. Each *column* represents a group of at least five mice, and the latency time is the mean (in days) before s.c. tumors were palpable in each group. For Msh2−/− p53−/− colonocytes, the incidence of tumor formation was 60% (three of five mice) at P10 and 80–100% at intermediate-late passages (P26–P54). p53−/− colonocytes were nontumorigenic when grown in culture for <35 passages. The incidence of tumor formation was 60% for P37 and 80–100% for P44–P54. *B,* latency time for tumor formation of p53−/− Msh2−/− colonocytes (P10 or P11) treated with MMS (4 or 5 μg/ml, respectively). Each *column* represents a group of 10 mice, and the latency time is the mean (in days) before detection of palpable tumors.

promised SCID mice (Fig. 2*A*) and in immunocompetent syngeneic hosts. Early-passage (P10) Msh2−/− p53−/− colonocytes produced s.c. tumors in SCID mice after a long latency (mean, 87 days) compared with the latency (mean, 18 days) of late-passage (P37) Msh2−/− p53−/− colonocytes (Fig. 2*A*). The latency of all intermediate passages was inversely correlated with the number of passages prior to s.c. injection. The incidence of tumor formation was 60% at early passages (P10) and 80–100% at intermediate-late passages (P26–P54). Msh2−/− p53−/− colonocytes did not form tumors in a syngeneic model (not shown).

The p53−/− colonocytes produced tumors in SCID mice after ~35 passages with a mean latency period of 60 days (Fig. 2*A*), and the time of latency decreased with increasing passages in culture. The incidence of tumor formation was 60% after 35 passages, 80% after 45 passages, and 100% after 50 passages. Like the Msh2−/− p53−/− cells, the p53−/− colonocytes did not form tumors when injected into syngeneic mice.

Histopathological examination of tumor xenografts arising from the double-knockout cells revealed poorly differentiated adenocarcinomas (Fig. 3*A*). Cells grew in solid cords and formed rare gland-like structures. In addition, there were numerous areas of focal necrosis and areas with a more spindle-like pattern. The p53−/− tumors were relatively more differentiated and showed the formation of neoplastic lumina and several areas of keratinization (Fig. 3*B*). These features were consistent with adenosquamous carcinomas.

MMS-induced Transformation in Msh2−/− p53−/− Colonocytes: *in Vivo* Evaluation of Tumor Growth. To assess whether mutagen treatment of p53−/− Msh2−/− colonocytes accelerates the process of transformation, early passage (P6) cells were treated with the alkylating agent MMS and injected s.c. in immunodeficient mice. Injected cells exhibited the same morphological features as untreated cells. As a control, early passage p53−/− colonocytes were also treated with MMS and injected s.c. in recipient mice. Compared with untreated cells of the same passage (see Fig. 2*A*), MMS-treated Msh2−/− p53−/− cells formed s.c. tumors more than twice as rapidly (Fig. 2*B*). Interestingly, the MMS-treated Msh2−/− p53−/− tumors showed a histology similar to that of the tumor from nonmutagenized cells (Fig. 3*C*).

In contrast, the MMS-treated p53−/− tumor cells did not generate obvious tumors in SCID mice. In a few animals, the palpable mass was, in fact, a cystic cavity lined by a simple columnar epithelium, probably derived from a sebaceous gland and formed as a consequence of the injection process (Fig. 3*D*). Thus, treatment with MMS is more potent in inducing transformation of double-knockout Msh2−/− p53−/− than of single-knockout p53−/− cells.

Discussion

We reported recently the establishment of primary epithelial cell cultures from the colon crypts of p53-deficient mice and showed that these cells are readily transformed by ectopic expression of oncogenic Ki-*ras* (25).

In the present study, we found that primary epithelial cells from the colon crypts of Msh2−/− p53−/− double-knockout mice undergo spontaneous transformation much earlier than do p53−/− cells; moreover, MMS treatment of early passage Msh2−/− p53−/− colonocytes markedly diminishes the latency time required for formation of detectable s.c. tumors. Our repeated efforts to establish long-term cultures of Msh2−/− colonocytes for use in monitoring the process of spontaneous or mutagen-induced transformation did not succeed because Msh2−/− epithelial cells underwent only a limited number of cell divisions before dying. By contrast, the growth advantage provided by the p53 null background allowed the expansion of primary colon epithelial cultures that also carry an ablated *Msh2* gene. Because p53−/− colonocytes are nontumorigenic, even if maintained in culture for several generations (25), our p53−/− Msh2−/− colonocytes might provide a useful *in vitro* model to assess molecular mechanisms whereby loss of function of the *Msh2* gene leads to neoplastic transformation.

p53- and Msh2/p53-deficient colonocytes were both highly aneuploid, as indicated by the presence of an abnormal number of chromosomes in 75–85% of metaphases (data not shown). Thus, although it cannot be excluded, a role of Msh2 deficiency in chromosomal instability, the acceleration of both spontaneous and mutagen-induced tumorigenesis most likely reflects the progressive accumulation of

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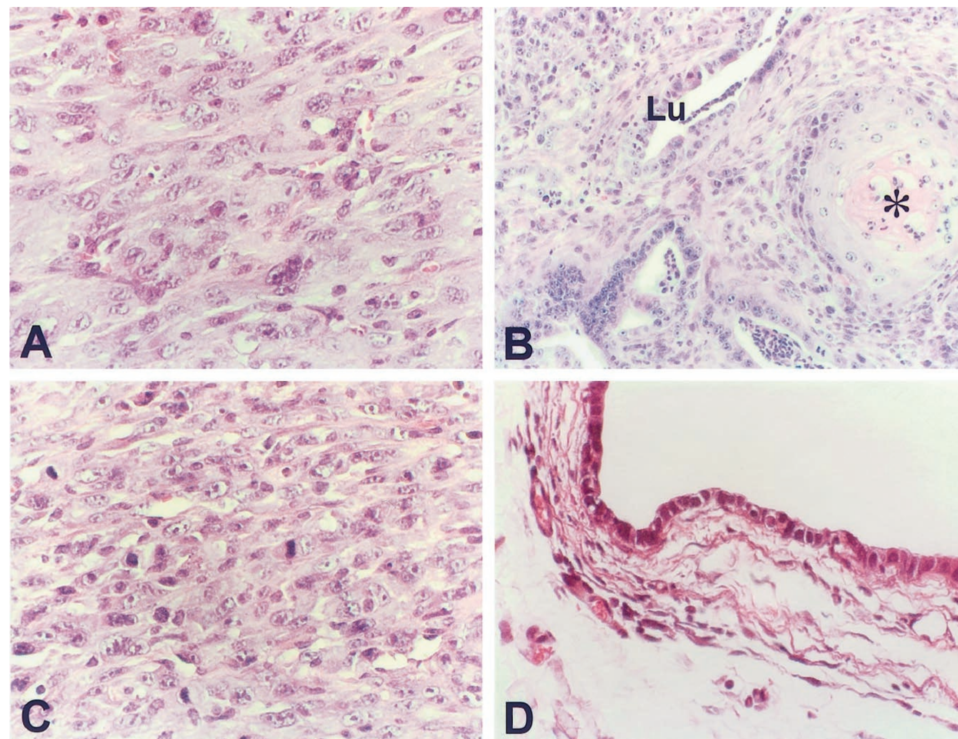

Fig. 3. Digitized light micrographs of xenografts from Msh2−/− p53−/− and p53−/− colonocytes. *A,* poorly differentiated adenocarcinoma in Msh2−/− p53−/−-induced xenografts at passage 26. *B,* adenosquamous carcinoma in p53−/−-induced xenograft at passage 49. Notice the presence of well-developed lumina (*Lu*) and of a keratin pearl (*). *C,* MMS-treated Msh2-/- p53-/- tumor xenograft at passage 12. *D,* xenograft from MMS-treated p53−/− colonocytes at passage 16. Notice the presence of a cyst lined by columnar epithelial cells and the absence of any malignancy. Images were digitized with a Pixera digital camera at resolution of ≈10^6 pixels/inch.

mutations caused by the failure of Msh2−/− cells to repair DNA errors. Mouse models of Msh2 deficiency have demonstrated previously the early onset and high incidence of spontaneous tumors predominantly localized to lymphoid tissues (22, 23). Highly aggressive lymphomas were also the predominant type of tumors in p53−/− Msh2−/− double-knockout mice, and their formation, as expected, was more rapid than in either p53−/− or Msh2−/− mice (26). The tissue preference in tumor formation of Msh2−/− mice probably reflects the rapid expansion and high turnover of lymphoid organs during development and the propensity of mice to develop sarcomas rather than carcinomas. However, the spectrum of tumors developing after longer latency or in mice that do not succumb to lymphoma includes those typically associated with MMR deficiency in humans (24). In our *in vitro* model, we showed that Msh2-deficient colonocytes did undergo neoplastic transformation, depending on the number of passages or the mutagen treatment of the injected cells. This is consistent with the importance of cumulative mutations for tumor formation or with damage to a rate-limiting pathway for tumorigenesis.

Interestingly, tumors arising from Msh2−/− p53−/− colonocytes were less differentiated of those derived from p53−/− colonocytes; this raises the possibility that genes regulating differentiation might be mutated in Msh2−/− colonocytes.

Mutations of genes involved in cell proliferation or survival were previously detected in HNPCC and in sporadic tumors with microsatellite instability (19–21, 28, 29). By reverse transcription-PCR analysis, we were unable to detect mutations in the *BAX, TGFβIIR,* or the *IGFIIR* genes (not shown), suggesting that these targets were not involved in either the spontaneous or MMS-induced transformation of Msh2−/− p53−/− colonocytes in this mouse model system. It should be also noted that the intragenic microsatellite sequences in these genes that are altered in human tumors are homologous, but not identical, in the mouse.

Msh2-deficient embryonic stem cells exhibit increased survival and reduced apoptosis when treated with the alkylating agent *N*-methyl-*N*-nitro-*N*-nitrosoguanidine or low levels of ionizing radiation (22, 30, 31). Accordingly, tumorigenesis by the Msh2−/− p53−/− colonocytes might rest on the enhanced proliferative potential and survival associated with disruption of p53 and Msh2 functions and in as yet unknown molecular alterations in genes shaping the behavior of tumor cells. The Ki-*ras* gene was not mutated in either the spontaneous or MMS-induced p53−/− Msh2−/− colon tumors (data not shown). This was not entirely unexpected in light of the relatively infrequent occurrence of Ki-*ras* mutations in HNPCC patients (32–34). Moreover, the concomitant inactivation of the *Msh2* and the *p53* genes may favor genetic alterations that, unlike RAS activation, may be causally linked with late stages of tumorigenesis. The frequency of p53 mutations is also low in patients with HNPCC (33, 35, 36), but it cannot be excluded that a pathway mimicking the one controlled by p53 is altered with high frequency in HNPCC patients.

In summary, the development of a model of tumorigenesis using Msh2−/− p53−/− primary colonocytes might be an important tool for identifying a potentially broad spectrum of mutated genes, the loss of function or aberrant activity of which may underlie the process of colon epithelial cell transformation.

Acknowledgments

We thank Bodil Tuma for help with electron microscopy and K. G. Danielson for help with cell culture.

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