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Metastatic Conversion of Chemically Transformed Human Cells

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A linear model for human cell metastasis has been developed in vitro from chemically transformed normal human cells. The chemically transformed cells are nontumorigenic in nude mice, but can be converted to a tumorigenic phenotype by transfection with a nondirectional cDNA library or antisense cDNA to the *ML-1* gene. The primary transfected cell line (TR^1T) forms localized, progressively growing tumors in nude mice that do not invade into the surrounding tissue. This tumorigenic TR^1T cell line could be advanced into a metastatic stage following an additional transfection (TR^2M cell line) with the cDNA expression library or antisense cDNA to the *ML-1* gene. Metastatic cells, selected from tumors that were attached to internal organs, exhibited an increase in invasiveness as measured in vitro using an invasion chamber. The metastatic cells also exhibited an increased expression of matrix metalloproteinase-1 (MMP-1), although MMP-1 was not part of the cDNA that was transfected into either the TR^1T cells or the doubly transfected metastatic TR^2M cells. These data suggest that the increase in MMP-1 expression was a secondary downstream event responding to an upstream genetic change that initiated the conversion of cells from a tumorigenic to a metastatic stage. In summary, human cell lines representing premalignant, malignant, and metastatic phenotypes have been established in culture that can be used to identify gene changes that occur as normal human cells progress to a metastatic stage during tumor development. One gene, *ML-1*, that is found in the expression library appears to be involved in malignant progression, because *ML-1* antisense cDNA will convert chemically transformed cells to both tumorigenic and metastatic stages, and cells from both local and metastatic tumors have a reduced or complete loss of expression of the *ML-1* gene.

Metastasis cDNA transfection Matrix metalloproteinase

SINCE the initial report by Milo and DiPaolo in 1978 (26) of the chemical transformation of human cells, several investigators have examined this process with a variety of chemical and physical carcinogens. Chemicals such as benzo[*a*]pyrene, propane sultone, aflatoxin B_1, and *N*-methyl-N_1-nitro-*N*-nitrosoguanidine or UV- and X-irradiation will transform normal human fibroblast or epithelial cells into a stage in which the cells will grow in soft agar and produce localized, self-limiting nodules when injected into nude mice (5,18,27,29,31,39,44,53). The sequential process of human cell carcinogenesis from chemically transformed cells to fully malignant cells has routinely been abrogated in the early stages of malignant progression, usually at the level of expression of anchorage independent growth (AIG).

The conversion of nontumorigenic human cells to fully tumorigenic cells was initially described by

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Rhim et al. (36), Milo et al. (32), and Shuler et al. (38), who showed that nontumorigenic cells derived from human tumors and treated with chemical carcinogens would induce local tumors in nude mice. In other reports (21,28,37), the presence or absence of mutations in the more commonly described oncogenes or tumor suppressor genes did not appear to be causally related to either the nontumorigenic AIG or the progressively growing tumorigenic stage in nude mice. Further, nontransplantable human cell lines, which were derived from various human tumor sites with mutations in codons 126 of *p53* and 12 of H-*ras*, did not possess additional mutations in these genes following conversion to a tumorigenic stage (21) by treatment with methyl methanesulfonate (MMS) nor were there changes in the level of H-*ras* or c-*myc* mRNA (21,28). Because these data were inconsistent with the causal role of these known oncogenes/tumor suppressor genes in the malignant conversion of rodent cells, it was suspected that other molecular changes must be involved in the conversion of human cells to malignancy. Based on this assumption, a cDNA expression library was developed from the MMS-converted tumor cells and used to transfect nontransplantable human cells derived from human squamous cell carcinomas (SCC) (22). It was assumed that the cDNA library would contain copies of mRNA that were unique to the tumorigenic cells and responsible for expression of tumorigenicity. The cDNA library transfected cells, when injected into nude mice, formed progressively growing, localized tumors (22) that did not metastasize to distant sites.

In 1996, Milo and co-workers reported the first successful malignant conversion of cultured human cells, using the same cDNA library, by transfection of normal human cells that had been chemically transformed with aflatoxin B_1 or propane sultone (30). As with the human tumor-derived cells, the transfected, chemically transformed human cells were converted in vitro to a phenotype that induced progressively growing tumors in nude mice, but without evidence of metastasis. More recently, Hahn et al. (15) have repeated the malignant transformation of human fibroblast and epithelial cells using the ectopic expression of the telomerase catalytic subunit, hTERT, in combination with an H-*ras* oncogene, and the large T-antigen protein of SV40.

In this article, we report the in vitro establishment of defined, progressive stages in the tumorigenic process, beginning with normal human cells and proceeding stepwise to development of cells that produce progressively growing, metastatic tumors in nude mice. With the development of this system, we can now correlate, in a linear in vitro model, the molecular events that are associated with each stage in carcinogenesis from normal cells to a fully malignant, metastatic phenotype and begin to identify those genes that are directly involved in the process. One such gene that we have identified, *ML-1*, appears be involved in metastatic conversion, because antisense *ML-1* cDNA will convert chemically transformed human cells to tumorigenic and metastatic stages. The combination system of transfection-mediated expression cloning and tumorigenic selection in nude mice appears to be a useful way of isolating tumor-associated genes without prior knowledge of the gene family.

MATERIALS AND METHODS

Cell Culture

Mixed cultures of human neonatal foreskin (HNF) were established from specimens collected from local hospitals. The tissues were minced into 1-mm^2 sections, placed in centrifuge tubes, rinsed 3× with minimal essential medium (MEM), and digested with a 0.25% collagenase solution (25,30,31). After pelleting the cells at $700 \times g$, they were seeded into 75-cm^2 flasks. The nonattached cells were removed 24 h later and the attached cells passaged at 72 h by trypsinization and reseeding into flasks at 10,000 cells per cm^2 in MEM with 20% fetal bovine serum (FBS) (25,30).

Carcinogen Treatment

The cells were arrested in the G_1 phase of the cell cycle by deletion of arginine and glutamine from IBR Modified Dulbecco's medium (6) supplemented with 10% dialyzed FBS (25,30). The cell cycle block was released after 24 h by feeding the cultures with complete MEM containing 10% FBS and 0.5 units of insulin. Ten to 12 h after release and 2–3 h into early S phase, propane sultone at its ED_{50} concentration (20 μg/ml) was added to the cultures and incubated overnight. At the conclusion of the treatment period, the experimental medium was removed, the cells washed 3× with 5 ml of MEM minus FBS, and the cultures then split at a 1:2 dilution in complete MEM supplemented with 4× amino acids, 2× vitamins, and 20% FBS. The cells were incubated in an air atmosphere enriched with 5% CO_2 at 37°C and, at each near confluency, the cells were split 1:10 through 20 population doublings (PDL).

cDNA Library Construction

In a previous publication, the construction of a nondirectional cDNA library was described (22). The expression cDNA library was derived from SCC cells that had been converted to tumorigenicity for nude

mice by MMS (28,32). Initially, 5 μg of poly(A)$^+$ RNA was used to synthesize cDNA using oligo(dT) primers and Superscript reverse transcriptase (GIBCO/BRL, Gaithersburg, MD). The cDNA library was constructed by using the Librarian cDNA library construction kit (Invitrogen Corp.; Carlsbad, CA). Briefly, *Bst*XI cloning linkers (5′-GAATTCCACCACA/5′-GTGGAATTC) were added to both ends of oligo(dT)-primed cDNA. The cDNA with linkers was then purified by cDNA spin column (Pharmacia, Bridgewater, NJ) and ligated to the *Bst*XI site of the eukaryotic expression vector pRC/RSV (Invitrogen). The ligation mixture was used to transform *Escherichia coli* strain DH10B. The organisms containing the cDNA library were plated out on Luria-Bertani plates containing ampicillin at 50 μg/ml, after which 1.1×10^6 colonies from primary plates were pooled in 200 ml of LB medium containing 7% (v/v) dimethyl sulfoxide (DMSO) and stored at −70°C as library stock.

Preparation of ML-1 *Expression Construct*

The *ML-1* cDNA was produced in the sense and antisense orientation as previously described (43). The construction of vectors was accomplished by using an eukaryotic expression vector and ligating to the *Bst*X1 site of the pRC/RSV promoter. Individual clones transfected with either the sense or antisense construct were verified for the orientation of the insert by restriction enzyme digestion patterns. The insert for this construct was amplified from 1 μg of total RNA using the following primer sets. Forward: 5′-GACTCTCTGTGTGGCTTATGC and reverse: 5′-GAGACCTCACAGAGCACAGTTG by RT-PCR. This amplified fragment was digested by *Bsp*120I and *Not*I and cloned into *Bsp*120I-digested pcDNA3 (Invitrogen). Orientation was checked by restriction digestion and the insertional region was sequenced for correct in-frame expression.

Transfection

Plasmid DNA was prepared from the cDNA expression library and the *ML-1* expression construct as described elsewhere (22,43). Chemically transformed cells were seeded at approximately 60% confluency in 10-cm dishes. After cell attachment, the dishes were washed twice with 20 ml of phosphate-buffered saline (PBS). Forty microliters of Lipofect Ace (GIBCO/BRL) and 30 μg of *Sal*I-linearized plasmid in 5 ml of serum-free medium were mixed and preincubated according to the manufacturer's recommendations and added to each plate. After overnight incubation, 5 ml of fresh medium containing 20% FBS was added. The plates were incubated for another 24 h and the medium then replaced with fresh MEM containing 10% FBS. G418 (GIBCO/BRL) was added at 250 μg/ml for the selection of pRC/RSV-transfected cells. After 2 days, the cell cultures were split 1:4 in MEM with 10% FBS containing G418 at 250 μg/ml and after 2 weeks the G418-resistant colonies were pooled and the cells grown to generate approximately $2–4 \times 10^7$ cells. The same procedure was followed for double-transfection of the once-transfected, chemically transformed cells except that strict G418 selection was not used, because the primary transfected cells were already neo resistant. Instead, selected colonies of doubly transfected cells were grown in medium containing a minimal amount of G418 to maintain their neo resistance.

Tumorigenicity Assays

Agar-cloned, chemically transformed cells (25,31), transfected chemically transformed human cells (TR^1T), and doubly transfected cells (TR^2M) were suspended in MEM at a density of 1.0×10^7 cells per ml and 0.5 ml injected subcutaneously (SC) into the subscapular or flank area of 3–40-week-old, splenectomized, male nude mice (28). Animals with subcutaneous tumors of approximately 1.0 cm in length were sacrificed and all tumors were harvested for histopathological evaluation.

Invasion Chamber Analysis

The invasion chamber assay (1) was performed using Biocoat Matrigel (Becton Dickinson Labware, Bedford, MA) according to manufacturer's suggestions except that 2×10^4 cells were used per well rather than 1×10^5.

Southern Analysis for Integration of Vector Into Genomic DNA

Genomic DNA isolation was performed by digesting 30 μg of genomic DNA and resolving the isolated products on an 8% agarose gel. Southern blot hybridization was performed as described (22). Vector probes were prepared using the USB random primer labeling kit (United State Biochemical Corp.; Cleveland, OH).

Northern Analysis for Vector Expression

Electrophoresis and Northern transfer were implemented using previously described methods (22). Briefly, 20 μg of total RNA was loaded per lane. A 1×10^6 cpm/ml probe was used in the hybridization protocol as described elsewhere (16). The probes were prepared by PCR labeling using primers specific for the neo region of the cloning vector.

Semiquantitative RT-PCR for Expression of Matrix Metalloproteinase-1 (MMP-1), Tissue Inhibitor of Metalloproteinase-1 (TIMP-1), and the ML-1 *Gene*

RT-PCR amplification was carried out on a standard thermal cycler according to the methods described by the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). The cloning linker-specific primer was 5′-GCCAGTGTGGTGGAATTC. The amplification cycle used in this procedure was: 95°C for 2 min, 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min for a total of 35 cycles. The exponential range of amplification for each target sequence was determined by performing various numbers of cycles using a fixed amount of first-strand cDNA reaction mixture and a fixed number of cycles using a series of dilutions of the cDNA (33). Semiquantitative RT-PCR products were separately amplified (10,23,47) with specific primers for MMP-1 (611 bp), TIMP-1 (386bp), and *ML-1* (43), separated on agarose gel, and stained with ethidium bromide. HPRT was amplified with the same RT-PCR reaction and served as an internal control. The results were analyzed by NIH Image software and the relative optical densities of MMP-1, TIMP-1, and *ML-1* were normalized to that of the HPRT gene.

RESULTS

Establishment of a Tumorigenic Phenotype From Chemically Transformed Cells

In an effort to identify at the tumorigenic stage human genes that are a prerequisite for further metastatic progression, we first established a tumorigenic human cell line from chemically transformed primary normal foreskin cells. The primary cells were first treated with propane sultone to create a transformed, nontumorigenic, AIG stage (30). The chemically transformed cells were then transfected with a nondirectional cDNA expression library prepared from a squamous carcinoma cell line (22) that had been converted to malignancy by treatment with MMS (28). In the nondirectional library, the cDNA population was cloned in the vector pRC/RSV (Invitrogen) in order for the transcripts to be expressed in either the sense or antisense direction for added tumorigenic potential either by overexpression of a tumor-promoting cDNA or by antisense suppression of the endogenous transcript from a tumor suppressor gene.

The chemically transformed primary human cells formed colonies in soft agar, but did not produce progressively growing tumors in nude mice (28). Following transfection and G418 selection, the stably transfected cells from about 10^4 colonies were pooled and injected into nude mice for production of tumorigenic cells as described (22,30). Cell lines established from these tumors, designated TR^1T, were highly tumorigenic and produced progressively growing, but not metastatic, tumors in nude mice within 3–4 weeks after injection (Fig. 1, top).

Conversion of Tumorigenic Cells to a Metastatic Phenotype

In order to investigate progression to metastasis, it was considered necessary to completely block the expression of any tumor suppressor genes that were only partially inactivated in the TR^1T cell line by the first transfection procedure. Although metastasis is a complex process involving several groups of diversified genes, it is not a disorganized random event (51), because it has been shown that metastasis can be initiated or blocked by one or more key genes such as *Tiam1* (49) and *KAII* (11). Therefore, we hypothesized that a limited number of additional critical genetic changes may produce a metastatic phenotype if additional cDNA library material were transfected (double transfection) into the TR^1T cells.

After SC inoculation into nude mice, the doubly transfected cells produced several metastatic tumors on stomach, intestine, and liver. A metastatic cell line (designated TR^2M) was established from one of the peritoneal metastatic foci produced by the doubly transfected TR^1T cells. In these cells, any additional library cDNAs introduced by double transfection could be identified by comparing library integration and expression patterns with that of the parental TR^1T cells. Phenotypically, the newly established metastatic TR^2M cells were different from the parental TR^1T cells in that TR^2M cells produced a smaller and

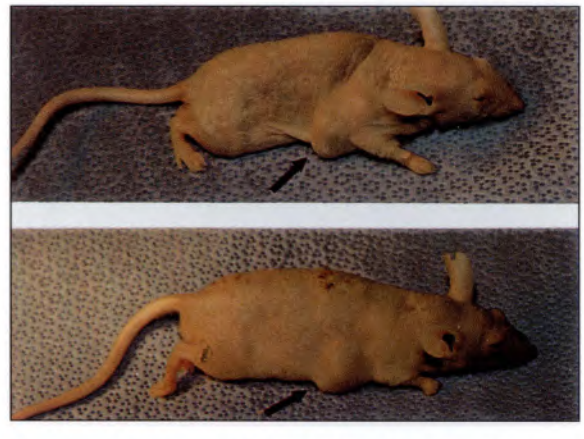

FIG. 1. Both cell lines TR^1T (top) and TR^2M (bottom) produced subcutaneous tumors in nude mice. Photographs were taken 4 weeks after injecting 0.5×10^6 cells subcutaneously into nude mice.

delayed primary tumor in five out of eight nude mice at the site of injection (Fig. 1, bottom) but metastasized to the peritoneum and several internal organs (Fig. 2). This cell line also established metastatic colonies in the lung after tail vein injection (data not shown). Histologically, the metastatic tumors were more poorly differentiated than the primary tumor from which they were derived (Fig. 3).

Conversion of Cells to a Metastatic Phenotype by ML-1

From cells that were converted to a malignant phenotype by the eukaryotic expression cDNA library, either total RNA or polyadenylated mRNA was extracted.. A 0.6-kb RT-PCR product, designated *ML-1*, was isolated and subcloned into the *Bst*XI-digested eukaryotic expression vector pRC/RSV. Several clones were sequenced and the RT-PCR gene product was found to be approximately 2 kb in size and located on the q arm of chromosome 13 at site 12–14 (43).

In order to determine if *ML-1* was one of the genes in the cDNA library responsible for tumorigenic and metastatic conversion of human cells, nontumorigenic and TR^1T cells were transfected with plasmids containing inserts in either the sense or antisense orientation using the same procedures that were used for the cDNA library transfections. Three- to 4-week-old male mice were injected SC into their flanks as described (28,38). For each batch of transfected cells, two SC sites on each mouse were injected. When their SC tumors became 1–2 cm in diameter, the mice were sacrificed and all tumors were harvested for histological analysis and for cell culture.

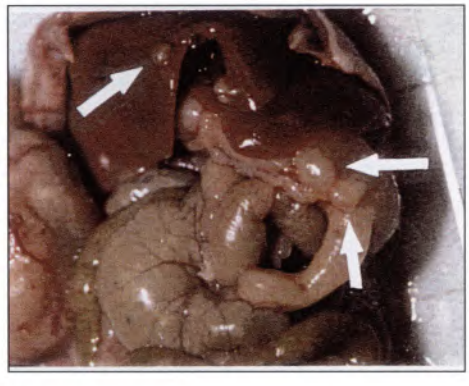

FIG. 2. Most tumors produced by cells that were doubly transfected with the cDNA expression library (TR^2M) were metastatic (i.e., 8 out of 10 mice had metastatic tumors). Tumors were identified on various internal organs throughout the peritoneal cavity on liver, intestine, stomach, and kidney (arrows) following the subcutaneous injection of 1×10^7 cells. Photograph was taken 2 months after cell injection.

Three mice that were injected SC with cells doubly transfected with the *ML-1* antisense cDNA had internal tumors, whereas cells transfected with the sense cDNA had none. As shown in Fig. 4A, the local tumors established by the *ML-1* transfected cells were identical in morphology to the SC tumors induced by cells that were transfected with the cDNA library (Fig. 3A). The metastatic tumors induced by cells doubly transfected with *ML-1* antisense cDNA (Fig. 4B) were poorly differentiated, similar to the metastatic tumors induced by the cDNA library doubly transfected cells (Fig. 3B).

Evidence that ML-1 *Is Directly Involved in the Metastatic Conversion*

Tumors that were induced locally by cells transfected with the cDNA expression library or with *ML-1* antisense cDNA and their metastatic counterparts were evaluated for expression of *ML-1*, because we had demonstrated earlier that several different normal human tissues express mRNA for the *ML-1* gene (43). In all cases, the nontumorigenic cells (from normal human foreskin or human tumors) expressed high levels of *ML-1* mRNA (Fig 5, lanes 1 and 2). Examination of subcutaneous or metastatic tumors induced by cells transfected with the cDNA expression library or *ML-1* antisense cDNA indicated that the *ML-1* transcripts were severely depressed (Fig. 5, lane 4) or not detectable (Fig. 5, lanes 3 and 5). In confirmation, demonstrable levels of *ML-1* gene expression were not found in 10 other human cell tumors produced in nude mice with transfected cells (data not shown).

Characterization of Cells From Metastatic Tumors

To investigate the invasive potential of TR^2M cells, cell lines established from the metastatic tumors were compared in vitro with their parental TR^1T cells to see if the TR^2M cells possessed an increased ability to degrade matrix proteins and move through a pored barrier by invasion chamber analysis (1). The results of this evaluation showed that TR^2M cells had an increased invasion index similar to that of a highly metastatic positive control invasive cell line HT1080, whereas the parental TR^1T had an invasion index similar to that of noninvasive 3T3 negative control cells (Fig. 6).

In order to eliminate the possibility that tumors formed by TR^1T or TR^2M cells are spontaneous mouse tumors and to show the integration of transfected cDNAs into the genome of the tumor cells, both the metastatic and its parental tumorigenic cells were characterized for their human origin, cDNA integration, and characteristic neo gene expression. The re-

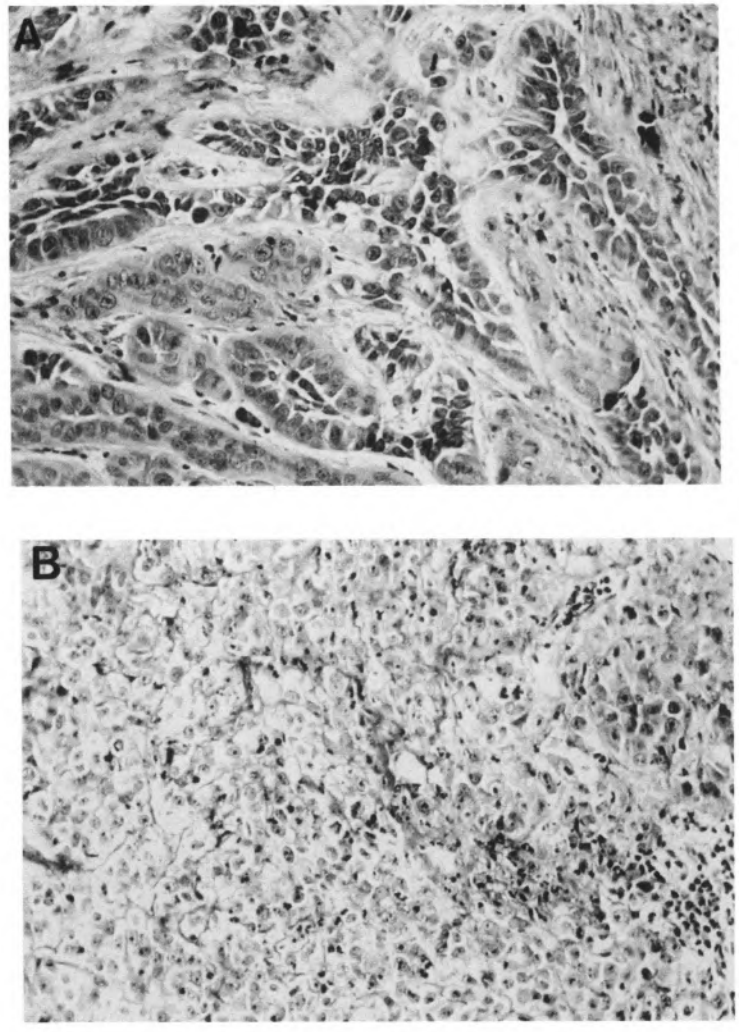

FIG. 3. Cytopathology of progressively growing, metastatic tumors compared to nonmetastatic tumors formed by the injection of 5.0×10^6 cells transfected with a cDNA expression library. (A) Tumor formed by the injection of chemically transformed cells transfected once with a cDNA expression library. The local subcutaneous primary tumor shows an epithelial origin with well-differentiated, gland-like structures. (B) Metastatic tumor formed following the injection of doubly transfected cells into the flanks of nude mice. The metastatic tumor appeared as multiple tumor nests with poorly differentiated small, epithelial cells. Both the metastatic tumor and the primary subcutaneous tumor were produced by cells originating from the same cell line. Magnification 400×.

sults of these studies confirmed that: both TR^1T and TR^2M cells were of human origin by species-specific repetitive sequence hybridization (Fig. 7), and both cell lines contained integrated cDNA vector and expressed the characteristic neo gene (Fig. 8).

These results are consistent with the notion that both the tumorigenic TR^1T and metastatic TR^2M cells were produced from the original, chemically transformed, human cells by integration of the transfected cDNA expression library or antisense cDNA to the *ML-1* gene. The expression of the neo-resistant gene also indicated that the integrated cDNAs were expressed in these cells. Thus, the two cell lines, TR^1T and TR^2M, represent a human cell model with two consecutive progression stages: tumorigenic and metastatic. In this model, the initial genetic changes (gene overexpression, gene suppression, or mutations) introduced by the integration of the cDNAs could be identified by using the vector sequence as a tag for further investigation.

Expression of MMP-1 and TIMP-1

The involvement of matrix-degrading enzymes was strongly suggested by the invasion chamber assay. To determine if the metastatic potential in TR^2M cells was associated with an increase in matrix-degrading MMP-1, the expression levels of both MMP-1 (23,47) and its negative regulator TIMP-1 (10) were measured in both TR^1T and TR^2M cells

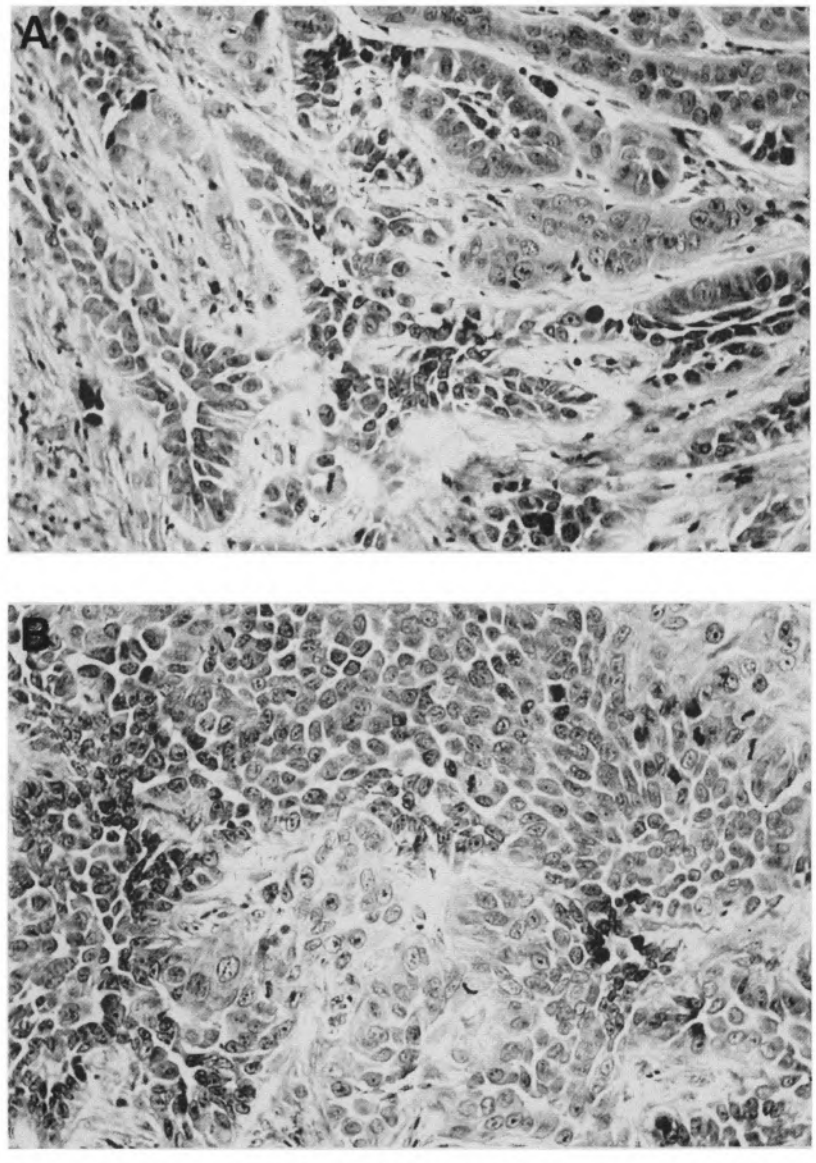

FIG. 4. Cytopathology of tumors induced by cells transfected with *ML-1*. (A) Subcutaneous tumor formed by cells transfected with *ML-1* antisense cDNA. (B) Metastatic tumor induced by cells doubly transfected with *ML-1* antisense cDNA. The morphology of the subcutaneous tumor and the metastatic tumor is similar to that induced by the cDNA expression library as shown in Fig. 3.

by semiquantitative RT-PCR. The results showed a dramatic increase in the expression of MMP-1 in TR^2M cells compared to that of human primary cells and parental TR^1T cells, while the expression level of its inhibitor TIMP-1 remained similar in all cells (Fig. 9). This further confirmed that our metastatic cell line has the characteristic imbalance of MMP-1 and TIMP-1 that has been observed in other metastatic cells (23).

Analysis of the cDNA Library for MMP-1

In an effort to amplify any MMP-1 in the library cDNA insert in TR^2M cells, both RT-PCR and genomic DNA PCR were performed using library cDNA linker as an amplification tag. The amplification products were resolved on agarose gel and analyzed by Southern blot using MMP-1 cDNA as a probe. No hybridized band was visible even after prolonged exposures (data not shown). These data suggested that MMP-1 cDNA was not part of the integrated library cDNA in TR^2M cells and that the increase in MMP-1 expression is most likely a secondary effect from another upstream target gene changed by the transfection. Indeed, two previously unidentified cDNAs were shown to be the predominant "tagged" gene-specific cDNAs in the tumorigenic/metastatic cell lines (22,43). One of the "tagged" fragments in

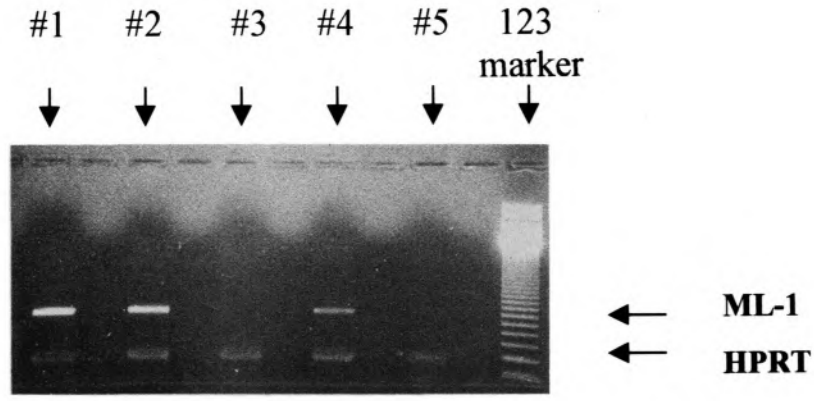

FIG. 5. Expression of *ML-1* gene transcripts in: normal human foreskin cells (lane #1); premalignant cells derived from a human squamous cell carcinoma (lane #2); metastatic tumor induced by cells doubly transfected with a nondirectional cDNA expression library (lane #3); subcutaneous tumor induced by cells transfected with a nondirectional cDNA expression library (lane #4); and metastatic tumor induced by cells doubly transfected with *ML-1* antisense cDNA (lane #5); 123 = DNA marker. HPRT was amplified with the same RT-PCR reaction and served as an external control.

the cDNA expression library, *ML-1* (43), has demonstrated a strong potential for inducing progression of tumorigenic cells to the metastatic stage.

DISCUSSION

The in vitro conversion of tumorigenic human cells to a fully malignant, metastatic stage remains a rare event, although it is a fairly common event in animal models. For example, with mouse and rat tumorigenic cells, conversion to full malignancy (metastasis) has been accomplished by: treatment with chemical chemotherapeutic agents (8) or transforming growth factor-β1 (14); transfection with genomic DNA or v-*src* from metastatic cultures (46,52), with c-H-*ras*EJ (34,42) or with E6 and E7 genes from HPV-16 (7); hypoxia (40,41); use of transgenic animal models (50); irradiation with UV (13); v-*raf* transformation (4); or introduction of the kinase oncogenes *mos, src, fes,* and *fms* (12).

Conversely, the conversion of human tumor cells to a metastatic phenotype is a more infrequent event.

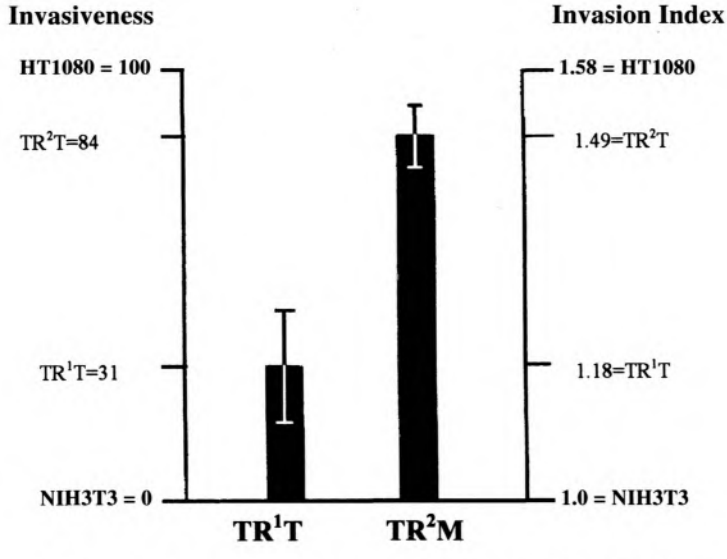

FIG. 6. Invasion chamber assays showing an increase in invasiveness of the metastatic cell line TR^2M compared to its parental TR^1T cell line. A comparison of the invasion index of TR^2M (with noninvasive NIH 3T3 cells and invasive HT1080 cells as controls) showed more than a twofold increase in the invasion index of TR^2M over parental TR^1T cells. The invasion chamber assay was performed according to the manufacturer's suggestion, except that 2×10^4 cells were used per well instead of 1×10^5. The results presented here were the average of three experiments. The invasion index was calculated using NIH 3T3 (data normalized to 1) and invasiveness calculated against the metastatic cell line HT1080 (data normalized to 100).

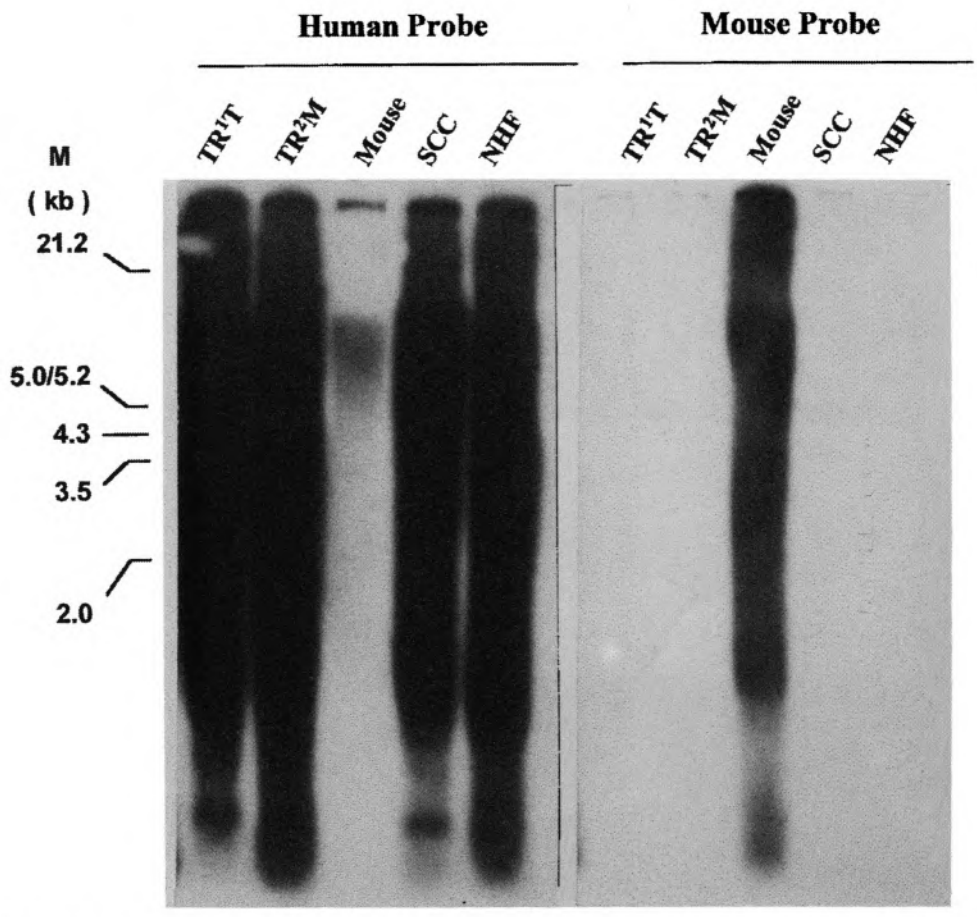

FIG. 7. Southern analysis reveals that both the TR^1T and TR^2M cells are of human, not mouse, origin. Left: human probe; right: mouse probe. M: DNA size marker. NHF: normal human foreskin cells. SCC: nontumorigenic cells from a human squamous cell carcinoma.

However, Azuma et al. (3) have reported an in vitro system in which nonmetastasizing adenocarcinoma cells could be converted to metastasis by treatment with *N*-methyl-*N*-nitrosourea (MNU). The metastatic cells secreted high levels of tissue-type plasminogen activator and metalloproteinases, while secreting reduced levels of TIMP-1. In addition, Tsujii et al. (48) reported an increased metastatic potential in human colon cancer cells following transfection with a cyclooxygenase (COX-2) expression vector. These cells demonstrated an increased activation of metalloproteinase-2 and a membrane-type metalloproteinase, increased invasiveness, and an elevation in prostaglandin production. The invasive property of these cells could be modulated by treatment with sulindac sulfide, a known inhibitor of COX enzyme activity. Finally, Takahashi et al. (45) reported the conversion of cells from a human adenocarcinoma of the stomach to an invasive and metastatic phenotype by subcutaneous and intraperitoneal serial passage in nude mice.

Despite the above demonstrations of metastatic conversion of human tumor cells, the in vitro conversion of normal human cells in culture to a metastatic phenotype has remained a rare phenomenon. In one such study, Hakim (16) reported that incubation of normal colon epithelial cells with both an extracellular matrix and conditioned medium from a colon carcinoma resulted in the progressive conversion of normal cells into an anchorage-independent growth stage, a tumorigenic stage, and finally into a stage that metastasized to the liver of athymic mice following IV injection. The RNA of the converted cells was shown to hybridize with cDNA from the colon carcinoma and the converted cells exhibited significant changes in cell membrane oligosaccharides. In this report, we have described the establishment of three cell phenotypes from normal human cells representing a nontumorigenic, a tumorigenic, and a metastatic stage following chemical carcinogen transformation and transfection with a cDNA expression library or antisense cDNA to the *ML-1* gene. Although the tumorigenic conversion of chemically transformed primary human cells has been reported (30) and cell

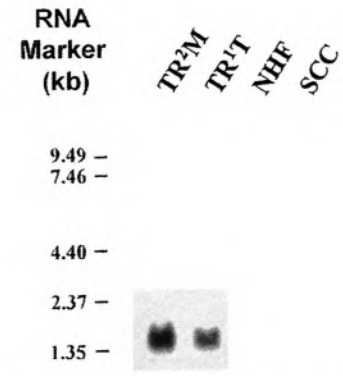

FIG 8. Expression of specific neo gene by Northern analysis in TR^1T and TR^2M cells. Total RNA (20 μg) was used for each lane. M: molecular size marker. NHF: normal human foreskin. SCC: nontumorigenic cells from a human squamous cell carcinoma.

lines have been established from malignant human tumors with invasive properties (35), the lack of cell lines representing normal, nontumorigenic, tumorigenic, and metastatic stages with a linear heritage has hindered direct comparative studies of human tumor progression at the molecular level. As reported herein, for the first time, a model has been developed in which a metastatic human cell line was formed from a tumorigenic cell line that was derived from nontumorigenic, chemically transformed, primary human cells. This system greatly facilitates the comparison between normal, nontumorigenic, tumorigenic, and metastatic cells and has the added convenience of a vector tag that allows easy identification of the initial genetic changes leading to the tumorigenic and metastatic stages of tumor development.

The exact mechanism of tumorigenic and metastatic conversion by transfection requires further study. However, observed morphological and molecular changes in cells converted to the metastatic stage are possible targets. In our studies, including the present study, discrete colonies of cells in culture, which are transfected with the cDNA expression library, show a morphological change from a fibroblast-like to a more epithelial-like appearance (30). These results are similar to those reported by van Leeuwen et al. (49) in a 3T3 system in which an increased malignancy was observed after transfection of the invasion-inducing, tumor-related *Tiam1* gene construct. Molecular changes in human cells that are associated with metastasis have been described by Kustikova et al. (20), who have shown that invasion and metastasis of cells from an epithelial adenocarcinoma are associated with an elevated activity of AP-1 with high expression of Fra-1 and Fra-2 proteins. The expression of *fra-1* is related to a morphological change in the cells from an epithelial to a more fibroblastoid appearance. Additionally, Zucker et al. (54) have found that incubation of cells with VEGF/VPF results in an increased production of matrix metalloproteinase (MMP) and suggests that an early event in tumor angiogenesis involves generation of thrombin, increased

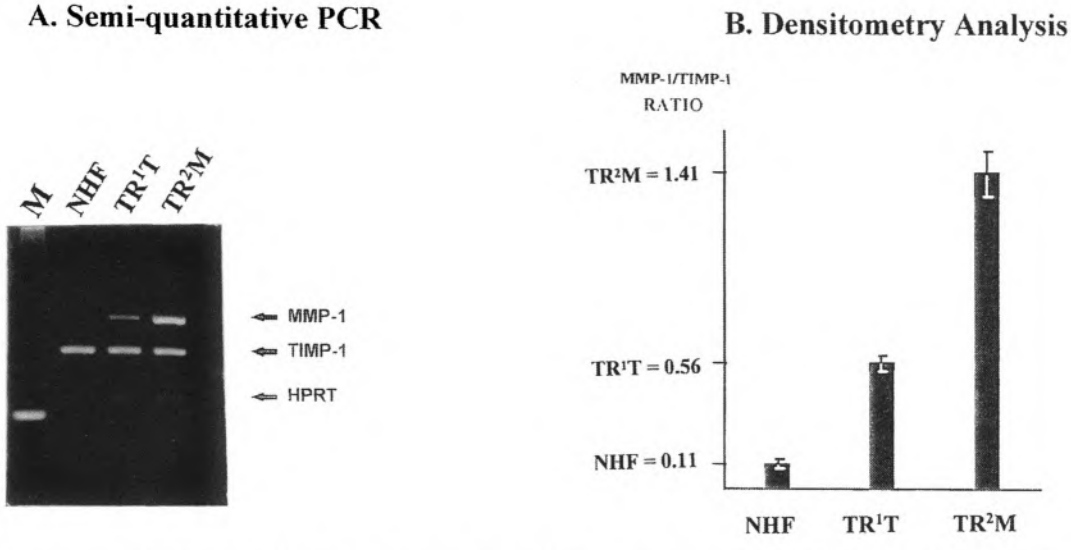

FIG. 9. Semiquantitative RT-PCR products were separately amplified with specific primers for MMP-1 (611 bp) and TIMP-1 (386 bp), separated on agarose gel, and stained with ethidium bromide. HPRT was amplified with the same RT-PCR reaction and served as an internal control. All amplifications were performed in the PCR exponential range by varying the cycle numbers and appropriate dilutions of the starting RT reaction. The results were analyzed by NIH Image software. The relative optical densities of MMP-1 and TIMP-1 were normalized to that of the HPRT gene. Semiquantitative PCR showed a marked increase in MMP-1 expression in TR^2M cells (A). Digital analysis indicating a 2.5-fold increase in the MMP-1/TIMP-1 ratio in TR^2M cells over TR^1T cells (B).

production of MMP, and activation of endothelial progelatinase A leading to degradation of the basement membrane. In addition to these examples, Joshi et al. (17) believe that nitric oxide may play an important role in metastasis. This concept is based on the fact that nitric oxide synthase activity is greatly elevated in malignant melanoma cell lines as opposed to melanocytes and thus may be an important factor in regulation of angiogenesis and vasodilation. In concert with these rather specific factors, Dellas et al. (9) have applied comparative genomic hybridization to assess whether specific genomic aberrations can be associated with poor prognosis and progression of cervical cancer. In their study, deletions in chromosome 9p were found to be closely associated with lymph node metastasis, whereas losses in chromosomes 11p and 18q were associated with poor prognosis in the absence of lymph node metastasis.

Of all the factors associated with the metastatic conversion of cells, the ability of tumor cells to degrade the cellular matrix appears to be one of the most important attributes leading to metastasis. In human colon adenocarcinomas, matrilysin expression is highly correlated with stage of progression (24), and the expression of matrilysin, in combination with expression of stromelysin-1 and stromelysin-3, plays an important role in tumor progression. Further, the metastatic conversion of human salivary gland adenocarcinoma cells by MNU (3) demonstrates that an increased metastatic capacity of the cells is directly associated with an increased secretion of metalloproteinases and a decreased or altered production of TIMP-1. The important function of the metalloproteinases in human cell metastasis is further exemplified by the work of An et al. (2), who describe the reversion of a metastatic colon cancer cell line to a less aggressive phenotype by an orally active synthetic MMP inhibitor. Animals that were gavaged with the inhibitor (CT1746) after implantation of a metastatic colon cancer cell line, in contrast to control animals, showed an increased median survival time, a decrease in mean tumor area, and inhibition of metastasis and spread of tumor to individual organs.

In our work, increased MMP-1 expression and an increased in vitro invasive potential of TR^2M cells (versus parental TR^1T cells) was consistent with the ability of the TR^2M cells to metastasize in nude mice. As discussed above, it has been reported in several other metastatic cells (with an increased ability to degrade matrix proteins) that this characteristic is associated with an increased metastatic potential when compared to their nonmetastatic counterparts (19,23). The more fundamental question is whether the increased expression of MMP-1 in the metastatic cells is the primary event in metastatic progression or one of the many secondary changes in gene expression in response to a change in a yet unidentified primary gene target. To answer this question, the advantage of the tagged metastasis model is apparent. Unlike any other metastasis model, the initial genetic changes in the metastatic TR^2M cells, which are directly induced by the transfections, are tagged and therefore identifiable. Our results indicate that none of the transfected cDNAs is related to MMP-1 as measured by Southern hybridization with MMP-1 cDNA as a probe. Therefore, change in MMP-1 expression is not likely to be due to a direct, initial, genetic change in the transfection-mediated metastatic conversion of TR^1T cells. One possibility is that MMP-1 is upregulated, directly or indirectly, by an upstream event associated with a tagged gene in the metastatic cells. Our studies on the tagged gene-specific cDNA in the tumorigenic and metastatic cell lines has revealed one such gene in the cDNA library, designated *ML-1* (43). When nontumorigenic or tumorigenic cells were converted to the next stage by antisense cDNA to the *ML-1* gene, subcutaneous injection of these cells produced tumors at the site of injection and internal metastatic tumors with the same histopathology as the metastatic tumors induced by cells doubly transfected with the cDNA expression library.

In summary, human cell lines representing premalignant, malignant, and metastatic phenotypes have been established in culture that can be used to identify gene changes that occur as normal human cells progress to a metastatic stage during tumor development.

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