Effects of Long-term Serial Passaging on the Characteristics and Properties of Cell Lines Derived From Uveal Melanoma Primary Tumors

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PURPOSE. Development of liver metastasis remains the most common cause of mortality in uveal melanoma (UM). A few cell lines cultured from primary UM tumors have been used widely to investigate the pathobiology of UM. However, the translation of basic knowledge to the clinic for the treatment of the metastatic disease has remained incremental at best. In this study, we examined whether the properties of UM cell lines at various passages were similar to their corresponding primary tumors.

METHODS. Gene expression profiling by microarray was performed on UM primary tumors and derived cell lines cultured at varying passages. Expression of UM protein markers was monitored by immunohistochemical analyses and Western blotting. The in vivo tumorigenic properties of UM cultures were evaluated using athymic nude mice.

RESULTS. Cell passaging severely reduced the expression of genes encoding markers typical of UM, including those of the prognostic gene signature. Marked differences between gene expression profiles of primary tumors and cell lines could be linked to the infiltrating immune and stromal cells in situ. In addition, the tumorigenic properties of UM cell lines also increased with cell passaging in culture as evaluated by their subcutaneous injection into athymic mice.

CONCLUSIONS. Together, these findings demonstrate that the short-term UM primary cultures exhibit molecular features that resemble the respective surgical material and, thus, represent the best model for in vitro-assessed cancer treatments.

Keywords: uveal melanoma, cell lines, gene profiling, cell passaging, tumorigenicity

For decades, tumor cells cultured in Petri dishes have been used to advance our understanding of molecular cancer biology and are now well recognized as a cornerstone of anticancer drug development. Cell lines acquire infinite replication when they are grown in the appropriate culture conditions. They then become an invaluable tool for the fundamental study of altered cellular pathways and epigenetic modifications, as well as for anticancer drug testing.1 Results achieved using continuous cell lines as biologic models often are extrapolated to the tumors from which they originate. However, there is a widespread debate among the scientific community on their validity owing to the possibilities of clonal selection and tissue culture artifacts.1–3 Indeed, cell lines exhibit a lack of differentiation and genomic instability after accumulating new chromosomal rearrangements and mutations during their adaptation to an artificial environment without stromal, vascular, and inflammatory cells.1–8

Uveal melanoma (UM) is the most common primary ocular malignancy in adults, accounting for 70% of all eye cancers. The overall mean age-adjusted incidence in the United States is 4 to 6 per million individuals.9 The actuarial 15-year metastatic mortality is approximately 50% despite efficient treatment of the primary ocular tumor.10 Factors associated with metastatic disease include pathologic features;11 cytogenetic abnormali-
ties, such as monosomy 3 and 8q gain\textsuperscript{12,13}; BAP1 mutations\textsuperscript{14}; and the class 2 gene signature.\textsuperscript{15} Because UMs seldom are surgically resected and biopsies dedicated to research very small, laboratory studies are limited by the lack of material. Despite that the establishment of permanent cell cultures from UM tumors is difficult, a number of cell lines have been characterized to date that can be used for research purposes.\textsuperscript{16–59} The availability of these UM cell lines allows ophthalmic researchers to advance from correlative studies to mechanistic investigations using advanced molecular methodologies. However, there often are discrepancies in treatment response between UM cell lines and UM primary tumors and metastases. For instance, based on promising in vitro studies,\textsuperscript{40,41} our group and others conducted phase II clinical trials with imatinib mesylate as a pharmacologic treatment of metastatic UM.\textsuperscript{42,43} However, the encouraging in vitro results were not reproduced in patients, as imatinib mesylate did not improve overall survival.\textsuperscript{42,43} A subsequent study to understand the molecular mechanisms behind this resistance, using UM metastatic tissues and cell lines, demonstrated that expression of the stem cell factor (SCF) in the tumor environment might promote resistance of metastatic UM to imatinib mesylate.\textsuperscript{44} Interpreting the potential clinical significance of the data obtained from cell lines also requires to determine the extent to which a given cell line and its molecular features resemble the respective surgical material. Such features have been investigated in several studies for other types of cancers using genomic and transcriptomic analyses.\textsuperscript{1,3–8,45–49}

So far, only a few detailed comparisons of the cell lines properties with those of the UM primary tumors from which they were derived have been published.\textsuperscript{19,21,31,33,34,37–38} We wished to compare the properties of UM primary tumors to those of their corresponding cell lines at various passages. Morphologic characteristics, gene expression profiling, and protein expression were examined by immunohistochemical analyses, DNA microarray, and Western blotting. Moreover, we compared the in vivo tumorigenic properties of short- and long-term UM primary cultures using athymic nude mice.

**MATERIALS AND METHODS**

This study followed the tenets of the Declaration of Helsinki and was approved by our institutional human experimentation committee. Written informed consent was obtained from the enucleated subjects. All animal experiments were conducted in voluntary compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and our institutional animal care and use committee approved all procedures.

**Primary Tumor Samples and Tissue Culture**

Six archival paraffin-embedded and frozen human UM tumor tissues (105, 122, 127, 130, 142, and 143) and their corresponding cell lines (T105, T122, T127, T130, T142, and T143) were studied. All samples were collected from enucleated eyes and confirmed by the Service de pathologie du CHU de Québec to be UMs by immunohistochemistry.\textsuperscript{35} Clinicopathologic characteristics, survival data, chromosomal anomalies and mutation status are compiled in Supplementary Table S1. Pathologic data were assessed as described previously.\textsuperscript{50} All UM cell lines established in our laboratory were derived from postenucleation biopsies performed by an oculard pathologist and were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Corp.) and 0.002% (vol/vol) gentamicin, under 5% CO\textsubscript{2} at 37°C as described previously.\textsuperscript{35} Normal melanocytes or vascular endothelial cells cannot grow in this medium, and tumor-infiltrating noncancerous fibroblasts are scarce in UM. Purity of the cultures was confirmed by microscopic inspection and MART-1 staining (prediluted; Dako Canada, Mississauga, ON, Canada). All cell lines were tested routinely for mycoplasma infection using the Mycoplasma Detection Kit (Biotool, Houston, TX, USA). To test their anchorage-independent growth, the cell lines were plated in 1.5% methylcellulose (diluted in Iscove’s modified Dulbecco’s medium [IMDM]; R&D Systems, Minneapolis, MN, USA) on untreated 35-mm tissue culture dishes (10,000 cells per triplicate). After 14 days of growth, colonies were observed under a dissecting microscope after staining with 0.1 mg/ml MTT (Sigma-Aldrich Corp.).

**Cell Count and Doubling Time**

Uveal melanoma cell number was compared between low and high passages using a cell and particle counter (Z2; Beckman Coulter, Mississauga, ON, Canada). Cell counts were done (N = 3) at three consecutive passages. The doubling time was determined using the initial number of cells, final number of cells, and duration of culture in hours (Roth V. 2006: available in the public domain at http://www.doubling-time.com/compute.php).

**Immunohistochemical Analyses**

Cells were grown until they reached 80% confluency and harvested during log phase, then fixed in 10 volumes of phosphate-buffered formalin for 10 minutes before being embedded in paraffin. Cell pellet sections were analyzed for structural features, including cell morphology, nuclear atypia, and mitosis, using hematoxylin and eosin stain. Immunohistochemical analyses were performed on cell line pellets and primary tumors sections following a technique of diamino-benzidine (DAB) biotin-streptavidin complex formation.\textsuperscript{50} Immunohistologic analyses were performed using antibodies directed against the proteins MART-1 (gene symbol, MLANA; prediluted; Dako Canada), S100 (prediluted; Dako Canada), HMB45 (prediluted; Dako Canada), VEGF (dilution 1:100; Santa Cruz Biotechnology, Dallas, TX, USA), cyclin D1 (gene symbol, CCND1; dilution 1:50; Cell Signaling Technology, Danvers, MA, USA), and p16 (gene symbol, CDKN2A; dilution 1:400; Santa Cruz Biotechnology). A negative control consisting of the omission of the primary antibody was performed for each case. The immunohistochemical staining results were evaluated using the blinded fashion technique by two experienced investigators without knowledge of clinicopathologic data on each individual case. Staining intensity was graded as negative (−), weak (+), moderate (++) or strong (+++).

**Western Blotting**

Western blots were conducted as described previously\textsuperscript{55,51} using the following primary antibodies directed against the proteins HTR2B (dilution 1:1000; Sigma-Aldrich Corp.), PARP-1 (dilution 1:2000), and PARG (dilution 1:2000; both kind gifts of Serge Desnoyers, Centre de Recherche FRQS du CHU de Québec-Université Laval, Quebec, Canada), integrin α5 subunit (10 μg/ml; Chemicon, Temecula, CA, USA), Sp1 (dilution 1:2000; Santa Cruz Biotechnology), VEGF (dilution 1:200, Santa Cruz Biotechnology), cyclin D1 (dilution 1:1000; Cell Signaling Technology), and p27 (gene symbol, CDKN1B; dilution 1:1000; Cell Signaling Technology). A β-actin antibody was used as loading control (dilution 1:40,000; Jackson Immunoresearch Laboratories, West Grove, PA, USA). The membrane then was incubated with peroxidase-conjugated goat secondary antibod-
Characteristics of Passaged UM Cells

Analysis
determined using the following formula: \( V = (L + W^2) \times 0.5 \). They then were embedded in paraffin for histologic analyses using hematoxylin and eosin (H&E) staining and MART-1 antibody as described previously. 55

Gene Expression Profiling and Unsupervised Bioinformatics Analyses

Total RNA was isolated directly from two UM primary tumors (patients 142 and 143) and the corresponding cell lines (T142 and T143) cultured to near 100% confluence at passages P4, P20, and P60 using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Gene expression profiling was performed by the Plateforme de génétique moléculaire du CUO-Recherche (Québec, QC, Canada). Quantity and quality of total RNA were assessed using the 2100 Bioanalyzer and the RNA 6000 Nano LabChip kit (Agilent Technologies, Mississauga, ON, Canada). Cyanine 3-CTP-labeled cRNA targets were prepared and hybridized on a SurePrint G3 Human GE 8 x 60K array slide (60,000 probes, Agilent Technologies) as reported previously. 51 Data were analyzed using the ArrayStar software (version 4.1; DNASTAR, Madison, WI, USA). All data generated from the array also were analyzed by RMA ("Robust Multiarray Analysis") for background correction of the raw values. They then were transformed in log2 base and quantile normalized, before a linear model was fitted to the normalized data to assess the effect of passage on gene expression.

Quantitative PCR (qPCR)

Reverse transcription was performed using random hexamer primers following the manufacturer’s protocol (Superscript II; Invitrogen, Carlsbad, CA, USA). Equal amounts of cDNA were run in quadruplicate and amplified in a 20 µl reaction containing 10 µl of 2X Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies), 250 nM of upstream and downstream primers, and 10 ng of cDNA target. The mixture was incubated at 95°C for 3 minutes, and then cycled 40 times at 95°C for 5 seconds and at 60°C for 10 seconds using the Rotor-Gene Q real-time cycler (Qiagen). Amplification efficiency of the HTR2B transcript was validated and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript and quantity of HTR2B was calculated using the standard curve method. Specific primers were designed using Primer3 software (available in the public domain at http://biotools.umassmed.edu/bioapps/primer3_www cgi): HTR2B Forward-TCTTTTCAACCGCATGCATCA and HTR2B Reverse-TGCTGTAGCCCCGTAGTTATA (107 base pairs [bp]); GAPDH Forward-AAGGTCGGAGTCAACGGGAT and GAPDH Reverse-GGAAGATGTTGATGGGATTTC (220 bp).

Statistical Analyses

Student’s t-test was used to determine statistical significance for comparison of the groups in qPCR analyses and the measurement of the cell doubling times. The values are represented as mean ± SEM and P < 0.05 was considered significant. The sample size included data from three independent experiments performed in triplicate. Xenograft tumor growth was compared using 1-way ANOVA with Tukey’s multiple comparisons test and P < 0.05 was considered significant.

RESULTS

Main Characteristics of the UM Cell Lines and the Primary Tumors From Which They Originate

Examination of UM cell lines under phase contrast microscopy indicated that they all exhibit an epithelioid morphology (with the exception of T105 and T143 that have a mixed spindle/epithelioid and a spindle phenotype, respectively; Fig. 1A). All cell lines formed colonies (Fig. 1B) compared to melanocytes (data not shown) demonstrating their anchorage-independent proliferation. Moreover, they all expressed the melanocyte differentiation antigen MART-1 (Fig. 1C; Table), a pattern very similar to that of the primary tumors from which they originated (Fig. 2; Table). Doubling times were measured for the UM cell lines T105, T122, T127, T130, T142, and T143 at three consecutive low- (ranging from P6–P11 depending on the cell line used) and high (ranging from P60–P62) passages. The average doubling time ranged from 23.65 ± 0.35 (with T150) to 34.32 ± 3.46 (with T142) at low passages, but then significantly decreased as UM cells progressed to higher passages (from 14.46 ± 1.06 with T142 to 20.71 ± 2.48 with T127; Supplementary Table S2), which is quite faster than UVM doubling time (>78h). 28 The most dramatic differences were observed for the T105 and T142 cell lines, whose respective doubling times decreased from 29.64 ± 2.04 and 34.32 ± 3.46 at low passage to 16.55 ± 0.15 (P < 0.005) and 14.46 ± 1.06 (P = 0.005) at high passage, respectively. We were able to analyze the cytogenetic abnormalities and mutation status in four of our primary tumors (Supplementary Table S1). The primary tumor 105, isolated from a patient who died of other cause, was positive for trisomy 8, and was mutant for BAP1 and GNA11 (Q209L). The primary tumors 130 and 143, derived from patients alive without metastasis, were negative for trisomy 8 and were wild type (WT) for BAP1. The primary tumor 142, established from a patient who died of metastasis, was positive for monosomy 3 and trisomy 8, and was mutant for BAP1 and GNA11 (Q209L). Short Tandem Repeat (STR) profiling could be completed for four of our UM cell lines.
(Supplementary Table S3) and revealed no relationship to any other human cell line. However, many loci differed from the patient’s blood or tumor samples. To determine the effect of long-term culture on the stability of DNA fingerprinting was beyond the scope of this study. However, experiments presently are underway to investigate the potential limitations of STR profiling for UM cell line authentication (such as genetic drifting, and presence of endogenous PCR inhibitory contami-
nants, like melanin). 54

We next determined by immunohistochemical analyses to which point expression of the UM (MART-1, S100 and HMB45), and vascular (VEGF) markers was affected by cell passaging in UM primary tumors 105, 122, 127, and 130, and their corresponding cell lines at passages P3/P4 and P60 (Fig. 2; Table). Expression of the p16 tumor suppressor and the G1/S cell cycle-specific protein cyclin D1 also was monitored as these proteins were reported to be frequently upregulated in UM. 55–57 As shown in Figure 2 (and summarized in the Table), the primary tumor 122 expressed all markers at varying levels, the most intense labeling being observed for MART-1, S100, and p16. Interestingly, expression of MART-1, S100, and p16, although detectable in more than 50% of T122 cells at a low passage (P3), became strongly reduced (for MART-1) or entirely lost (for S100 and p16) when T122 cells were grown to P60.
TABLE. Mitotic Rate and Immunostaining Data (Graded According to Intensity*) in UM Primary Tumors (PT) and Derived Cell Lines at Low (P3/P4) and High (P60) Passages

<table>
<thead>
<tr>
<th>Marker</th>
<th>T105 PT</th>
<th>T122 PT</th>
<th>T127 PT</th>
<th>T130 PT</th>
<th>T105 P3</th>
<th>T122 P3</th>
<th>T127 P3</th>
<th>T130 P3</th>
<th>T105 P60</th>
<th>T122 P60</th>
<th>T127 P60</th>
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<tr>
<td>Mitosis</td>
<td>10</td>
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<td>0</td>
<td>2</td>
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<td>MART1</td>
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<td>HMB45</td>
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<td>VEGF</td>
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<tr>
<td>Cyclin D1</td>
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<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
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<td>P16</td>
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* Staining intensity was graded as negative (−), weak (+), moderate (++) or strong (+++).

On the other hand, VEGF and cyclin D1, whose expression was low at P3, sought their expression considerably increased at P60. Very similar results also were obtained with the T105, T127, and T130 cell lines (Table). Therefore, culturing UM cell lines at high passages also altered expression of many of these proteins compared to primary tumors.

Influence of Cell Passaging on the Gene Expression Signatures of UM Cell Lines

Culturing cells directly from the primary tumor tissues also may force them to change their properties to survive in such a different environment. To evaluate such a possibility, we conducted gene expression profiling on microarray using total RNA prepared from UM cell lines T142 and T143 cultured at different passages (P4, P20, and P60), and compared their pattern of expressed genes to that of the original primary tumors. Scatter plot analysis revealed that the pattern of genes expressed by the 142 and 143 primary tumors changed dramatically as the cell lines are grown until passage P4 (Fig. 3A). Culturing T143 cells further from P4 to P60 did not lead to modifications as dramatic as those observed between the uncultured primary tumor and passage P4 (Fig. 3B). A total of 4986 (for T142) and 6771 (for T143) genes fitted into that category of differentially expressed genes; FXR1, LTA4H, ID2, ROBO1, MTSUS1, LMCDO1, STAB1, and EIF1B) or high (class 2 genes; HTR2B, CDH1, RAB3J, and ECM1) risk of evolving towards the formation of liver metastases has been described. Therefore, we examined whether the expression of these markers was affected by cell passaging in T142 and T143 cell lines when compared to their primary tumor. As shown in Figure 4A (also refer to Supplementary Table S4), expression of the FXR1, LTA4H, and ID2 genes was the least affected by cell passaging among the class 1 genes although they were most actively transcribed in the cell lines than in the primary tumors. On the other hand, expression of MTSUS1, LMCDO1, SATB1, and EIF1B genes, which were moderately to highly expressed in the primary tumors 142 and 143, was rapidly lost with cell passaging in both cell lines. Although class 2 genes were highly to moderately expressed in tumor 142, their expression dramatically decreased (with the exception of ECM1) with the passages in culture (Fig. 4B; Supplementary Table S4). The tumor 143 had high levels of CDH1 and RAB3J genes but only very low levels of HTR2B and ECM1 genes. Again, CDH1 and RAB3J gene expression was entirely lost at a very early passage (P4). The dramatic extinction of HTR2B expression at the transcriptional level in T142 cells (Figs. 4B–C; Supplementary Table S4) was further validated by qPCR (Fig. 4D; P < 0.001). However, these alterations did not necessarily translate into corresponding changes at the protein level as Western blot analysis detected less HTR2B protein, the most discriminant gene of the molecular signature, in the primary tumor than in the T142 cell line (Fig. 4E). Its expression, however, decreased in T142 cells as they are passaged up to P60. Therefore, although this 12-gene signature may prove useful as a tool to discriminate between classes 1 and 2 primary tumors, it is useless when cell lines are selected as the source of biologic material for these analyses.

Influence of Cell Passaging on the Expression of UM Class 1 and 2 Markers

A gene expression signature comprising 12 genes that can discriminate between UM primary tumors at low (class 1 genes; FXR1, LTA4H, ID2, ROBO1, MTSUS1, LMCDO1, STAB1, and EIF1B) or high (class 2 genes; HTR2B, CDH1, RAB3J, and ECM1) risk of evolving towards the formation of liver metastases has been described. Therefore, we examined whether the expression of these markers was affected by cell passaging in T142 and T143 cell lines when compared to their primary tumor. As shown in Figure 4A (also refer to Supplementary Table S4), expression of the FXR1, LTA4H, and ID2 genes was the least affected by cell passaging among the class 1 genes although they were most actively transcribed in the cell lines than in the primary tumors. On the other hand, expression of MTSUS1, LMCDO1, SATB1, and EIF1B genes, which were moderately to highly expressed in the primary tumors 142 and 143, was rapidly lost with cell passaging in both cell lines. Although class 2 genes were highly to moderately expressed in tumor 142, their expression dramatically decreased (with the exception of ECM1) with the passages in culture (Fig. 4B; Supplementary Table S4). The tumor 143 had high levels of CDH1 and RAB3J genes but only very low levels of HTR2B and ECM1 genes. Again, CDH1 and RAB3J gene expression was entirely lost at a very early passage (P4). The dramatic extinction of HTR2B expression at the transcriptional level in T142 cells (Figs. 4B–C; Supplementary Table S4) was further validated by qPCR (Fig. 4D; P < 0.001). However, these alterations did not necessarily translate into corresponding changes at the protein level as Western blot analysis detected less HTR2B protein, the most discriminant gene of the molecular signature, in the primary tumor than in the T142 cell line (Fig. 4E). Its expression, however, decreased in T142 cells as they are passaged up to P60. Therefore, although this 12-gene signature may prove useful as a tool to discriminate between classes 1 and 2 primary tumors, it is useless when cell lines are selected as the source of biologic material for these analyses.

Influence of Cell Passaging on the Expression of Immune, Stromal and Melanocyte Signature Genes, as Well as on Other Metabolic Pathways

Specific signatures related to the infiltration of normal cells in tumor tissues have been described recently. Therefore, we examined whether the expression of these immune and stromal genes signatures (141 genes in each), as well as genes associated to melanocyte differentiation (15 genes), was affected by cell passaging in T142 and T143 cell lines when
FIGURE 2. Immunohistochemical analyses of UM markers in the primary tumor 122 and its derived cell line T122 upon cell passaging. H&E staining, and expression of MART-1, HMB45, S100, VEGF cyclin D1, and p16 markers monitored by immunohistochemistry in the UM primary tumor 122 as well as its derived cell line T122 at low (P4) and high (P60) passages. Dark brown indicates positive staining. Scale bar: 20 μm.
compared to their primary tumor (Fig. 5; Supplementary Table S4). Although immune and stromal genes were highly expressed in primary tumors 142 and 143, their expression dramatically decreased with cells passaging in culture (Figs. 5A–B; Supplementary Table S4). Our unsupervised bioinformatic analyses identified "Interferon signaling" (Reactome ID 913531), "Cytokine signaling in immune system" (Reactome ID 1280215), "Class I MHC mediated antigen processing & presentation" (Reactome ID 983169), and "MHC class II antigen presentation" (Reactome ID 2132295) among the enriched pathways (Supplementary Table S5). Thus, pronounced differences between gene expression profiles of primary tumors and cell lines could be attributed partly to the absence of immune and stromal components in vitro. In addition, as shown in Figure 5C (also refer to Supplementary Table S4), melanocyte differentiation genes, such as melanin biosynthetic enzymes, are mostly repressed after cell passaging. Other enriched pathways found by unsupervised bioinformatic analyses (Supplementary Table S5) were associated with "Respiratory electron transport" (Reactome ID 163200), "Citric acid (TCA) cycle" (Reactome ID 5368287), "Gene expression" (Reactome ID 74160), "Cell Cycle-Mitotic" (Reactome ID 69278), and "Beta-catenin independent WNT signaling" (Reactome ID 3858494).

**Cell Passaging Alters the Expression of Genes Previously Studied in UM and the Tumorigenic Properties of UM Cell Lines**

We next addressed whether passaging UM cell lines also alters the expression of genes whose products are important to the properties of UM. Among them, we demonstrated that PARP-1 and PARG enzymes, as well as the integrin α5β1, may have critical functions in the tumorigenic properties of UM.55,51 As these three genes were strongly regulated by the transcription factor Sp1, we also included it into this analysis. In addition, we also monitored the expression of the melanosomal marker MART-1, as well as VEGF, cyclin D1, p16, and p27. Microarray analyses clearly indicated that expression of the gene encoding MART-1, elevated in the primary tumors 142 and 143, was lost very rapidly with cell passaging in T142 and T143 cell lines (Fig. 6A; Supplementary Table S4). Similar results also were obtained for PARP-1, PARG, cyclin D1, and p16 (only in T143 cells) genes. On the other hand, expression of the α5 integrin subunit encoding gene ITGA5, Sp1 and VEGF genes increased.
FIGURE 4. Influence of cell passaging on the expression of classes 1 and 2 genes. Heatmap representation of (A) class 1 and (B) class 2 genes expressed by the UM primary tumors 142 and 143, and their derived cell lines T142 and T143 cultured at passages P4, P20, and P60 as determined by DNA microarrays. Data also are presented for the housekeeping genes β2-microglobulin (B2M), C-terminal binding protein 1 (CTBP1), and golgin A1 (GOLGA1; Ctrl). (C) Data obtained for the HTR2B gene in T142 cells presented as linear signals normalized to B2M. (D) Quantitative PCR analysis of HTR2B gene expression normalized to GAPDH in T142 cells grown at passages P4, P20, and P60. *P < 0.001. (E) Western blots conducted on protein extracts from UM primary tumors 142 and 143, as well as derived cell lines at passages P4, P20, and P60 using antibodies against HTR2B and actin (loading control).
FIGURE 5. Influence of cell passaging on the expression of immune, stromal, and melanocyte gene signatures. Heatmap representation of (A) immune, (B) stromal, and (C) melanocyte gene signatures of the UM primary tumors 142 and 143, and their derived cell lines T142 and T143 cultured at passages P4, P20, and P60 as determined by DNA microarrays. Data also are presented for the housekeeping genes B2M, CTBP1, and GOLGA1 (Ctrl).
FIGURE 6. Influence of cell passaging on the expression of UM markers and the tumorigenic properties of UM cell lines. (A) The pattern of expression of genes typically reported as important UM markers is shown in a heatmap for the UM primary tumors 142 and 143, and their derived cell lines T142 and T143 cultured at passages P4, P20, and P60 as determined by DNA microarrays. Data for the housekeeping genes B2M, CTBP1, and GOLGA1 also are shown (Ctrl). (B) Western blots conducted on protein extracts from UM primary tumors 105, 142, and 143 and their derived cell lines using antibodies against PARP-1, PARG, integrin α5 subunit, Sp1, VEGF, cyclin D1, p27, and actin (loading control). (C) Scatter plots depicting the tumor volume (mm³) produced in athymic nude mice injected subcutaneously with the UM cell lines T122, T130, T142, and T143 cultured at low (P4/P6), intermediate (P20/P21), or high (P60/P62) passages (line, mean). The number of tumors relative to the number of injected sites is indicated below the graph. *P < 0.05.
with cell passaging compared to their corresponding primary tumor. However, these alterations at the transcriptional level did not necessarily translate into corresponding changes at the protein level as revealed by Western blot analyses. Indeed, although the protein expression of the α5 integrin subunit, Sp1, and p27 matched quite well with their transcriptional profile, that of PARP-1 and PARG, as well as cyclin D1 was inversely correlated with their mRNA expression pattern (Fig. 6B). Consistent with the data from the microarrays, VEGF expression increased dramatically when cells reached P4 in culture (Fig. 6B). However, it then decreased rapidly from P4 to P20 and remained low at P60 in T142 and T143 cells.

To evaluate to which point cell passaging may alter the tumorigenic properties of cell lines, T122, T130, T142, and T143 cells grown at passages P4/P6, P20, and P60 were injected into athymic mice and tumor formation was monitored over a 28-day period (Fig. 6C). At passage P4/P6, the T122 and T130 cell lines were by far the most tumorigenic as all injected mice developed detectable tumors (12/12 and 11/12 tumors, with a mean tumor volume of 127 and 110 mm³, respectively). Mice injected with T142 and T143 cells also yielded detectable tumors (both 4/12 tumors, with a mean tumor volume of 5 and 21 mm³, respectively). Most interestingly, the mean tumor volume for all cell lines but T130 increased considerably when P20 or P60 cells were injected. The results were statistically significant for T122 (P4 vs. P20, P < 0.05; P4 vs. P60, P < 0.005), T142 (P4 vs. P20, P < 0.05), and T143 (P6 vs P60, P < 0.01) cells.

**DISCUSSION**

Although cell lines maintain expression of some hallmarks of cancer, most exhibit reduced or altered key functions and often no longer represent reliable models of their original source material after several passages in culture. To the best of our knowledge, no studies have been performed to examine at the molecular level the similarities between UM primary tumor surgical samples and their derived cell lines at short- and long-term passage in culture. In the present study, we demonstrated that cell passaging causes, at the very first passages, dramatic changes in genes expressed in UM cells, including most of those that belong to the prognostic signature. Changes also occurred in their growth properties (evaluated through the measurement of the cell doubling time) as well as in their ability to produce tumors when injected in athymic nude mice, with increased tumorigenic properties at higher cell passages.

Through immunohistologic analyses, we found that expression of the melanosomal protein MART-1 decreased after short-term passages. This is consistent with the observed decrease in the melanin content during cell passaging in the majority of UM cell lines, as well as our previous demonstration of a loss of melanin biosynthetic enzymes in cultured UM. It might provide a growth advantage by reducing the energy and oxidative stress associated with pigment production. Vascular endothelial growth factor, cyclin D1, and p16 have been studied widely in UM by our group. In all our UM cell lines, expression of p16 decreased in cells grown at high passage compared to primary tumors, and in contrast, expression of cyclin D1 remained stable upon cell passaging compared to primary tumors. Expression of VEGF was variable according to passage or cell line. These results suggested that selection of cell lines that best mirror UM will optimize the clinical relevance of future discoveries.

One major problem of tissue culture is that cell lines evolve as they are grown continuously over a large number of passages. In fact, genotypic and phenotypic variants constantly appear in cancer cell lines, and selection of subpopulations with superior growth properties occurs frequently. This may change drastically the characteristics of a cell line, as shown in our study with the UM cell lines examined. For instance, at high passage, the Caco2 colon cancer cell line presents pathological changes affecting morphology, expression of the glucose transporters, metabolic activity, and transepithelial electrical resistance. Passage-related changes that lead to altered androgen response and prostate-specific antigen secretion also were observed in the prostate LNCap cell line. Most importantly, a previous comparison of gene expression patterns between several normal tissues and corresponding tumors and cell lines demonstrated a greater discrepancy in the number of differentially expressed genes between the cell line and tumor than between the tumor and normal tissue. In our study, such discrepancy depends on passage, cell line, or markers studied. Our research indicates that loss of the immune and stromal components during cell passaging also accounts for the transcriptional difference noted between cell lines and tumors.

Genomic instability is a characteristic feature of many tumors and it may develop early during pathogenesis. It persists after tumor development and results in appearance of multiple subclonal populations. As cell lines have shorter population doubling time (hours) than tumors (months), it is expected that cell lines acquire more genetic and epigenetic changes over the same period. In 2004, Onken et al. confirmed the presence of two molecular classes of UMs using gene expression profiling, in which the 8-year survival probability was 95% in class 1 patients versus 31% in class 2 patients. In our study, we investigated whether monitoring classes 1 and 2 genes would be as informative for UM cell lines as they are for the primary tumors. However, our results clearly showed that as cells are grown in culture, the class 2 phenotype of tumor 142 is lost in favor of more differentiated traits, a type of in vitro transition that would result in greater transcriptional distance between the class 2 cell line and its respective tumor.

Consistent with similar studies conducted with other types of cancers, our results provided evidence that short-term UM primary cultures show characteristics much closer to the primary tumors than to long-term cultures. This is most likely due to the fact that low-passage primary cultures still support a culture population made up of different UM cell subtypes that are genetically distinct from one another. Consequently, some investigators have preferred to work with short-term primary cultures to circumvent the limitations of continuous cell lines, but the former cannot be reliably shared between investigative groups, making it difficult to obtain independent validation of results using common biologic samples. However, we found that genetic instability also occurred in short-term passages in UM cell lines. Moreover, the genetic alterations seemed to be more significant at early passages than at late passages, which is reflected by the high variability of the STR profile observed for most of our UM cell lines at low cell passages. However, such a high microsatellite instability is not uncommon and has been reported for other types of cancer cell lines. Interestingly, unstable STR has been shown to be the consequence of alterations in the expression/activity of several DNA mismatch repair enzymes, including MLH1, MSH2, MSH6, and PMS2. Therefore, examination of the expression status for these proteins should prove very interesting in deciphering whether some of them contribute to the genomic instability noted in our UM cell lines.

The major limitations of cancer cell lines are their lack of stromal, vascular, and immune components. Tumors are, indeed, composed of an ecosystem of evolving clones, competing or cooperating with each other and with other
Characteristics of Passaged UM Cells

normal cells infiltrating their microenvironment.\(^7^5\) This raises the intriguing question of whether these clones have been selected through their growth into culture medium or over time through cell passaging. Thus, cell lines derived from one clone are not necessarily representative of the diversity found in the original tumor. These deficiencies have been partially overcome by xenograft transplantation of tumor fragments or tumor cell line suspensions into immunocompromised mice.\(^7^6–7^8\) However, xenografts also have certain limitations, including the presence of stromal components of xenogeneic origin that support the growth of human cancer cells.

Interestingly, despite often dramatic variations in gene expression profiles between UM primary tumors and their derived cell lines, the protein products showed moderate modifications by Western blotting. Recently, we demonstrated the critical functions played by the PARP-1 and PARG enzymes on the tumorigenic properties of UM.\(^5^1\) In the present study, the mRNA transcripts encoding PARP-1 and PARG decreased to very low levels when tumors 142 and 143 were passaged in culture. In contrast, expression of PARP-1 and PARG proteins increased, suggesting that they might negatively regulate transcription of their own respective genes through mechanisms involving negative feedback loops as suggested recently by Vidakovic et al.\(^7^9\) for the mouse PARP-1 gene.

In conclusion, our analyses provided valuable information relevant to the suitability of cell lines in modeling UM primary tumor heterogeneity. Because it is difficult to obtain sufficient biologic material from metastases, we could not derive cell lines from liver metastatic lesions of UM patients. We concede, therefore, that their characteristics may considerably differ from those of primary tumors. The goal of our study was by no means to question the data previously obtained using UM long-term cultured cell lines. It is obvious that cell lines may represent valuable tools in functional assays, as well as pharmacologic and genetic studies to better understand the numerous cellular and molecular mechanisms involved in UM development and progression. However, we must be aware of the limitations (such as their elevated genomic instability at low cell passages) of using such cell lines as biologic materials. It remains important to define to which point the in vitro model closely resembles particular features of the original tumor and whether these cellular and molecular properties are maintained in basic culture conditions for primary and metastatic cell lines.

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