Alteration of the Poly(ADP-Ribosyl)ation Enzymes in Uveal Melanoma and Regulation of PARG Gene Expression by the Transcription Factor ERM

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**PURPOSE.** Poly(ADP-ribosylation) is a reversible post-translation al modification that requires the contribution of the enzymes poly(ADP-ribose) polymerase-1 (PARP-1) and poly(ADP-ribose) glycohydrolase (PARG). Our study explores expression and activity of PARP-1 and PARG in uveal melanoma cell lines with varying tumorigenic properties.

**METHODS.** Gene profiling on microarrays was conducted using RNA prepared from the uveal melanoma cell lines T97, T98, T108, and T115. The activity of PARP-1 and PARG was monitored by enzymatic assays, whereas their expression was measured by Western blot and PCR. The PARG promoter was analyzed using promoter deletions and site-specific mutagenesis in transfection analyses. The transcription factors binding the PARG promoter were studied by electrophoretic mobility shift assay (EMSA) analyses. Suppression of PARP-1 and PARG expression was performed in T97 and T115 cells by RNAi, and their tumorigenic properties monitored by injections into athymic mice.

**RESULTS.** Expression of PARP-1 was found to vary considerably between uveal melanoma cell lines with distinctive tumorigenic properties in vivo. Sp1 and the ETS protein ERM were shown to bind to the PARG gene promoter to ensure basal transcription in uveal melanoma. Importantly, suppression of PARG gene expression in T97 and T115 cells increased their capacity to form tumors in athymic mice, whereas suppression of PARP-1 significantly reduced or almost entirely abolished tumor formation.

**CONCLUSIONS.** Our results suggest that while overexpression of PARP-1 may confer a proliferative advantage to aggressive uveal melanoma tumors, PARG may, on the other hand, support a tumor suppressor function in vivo. (Invest Ophthalmol Vis Sci. 2012;53:6219–6231) DOI:10.1167/iovs.11-8853

Uveal melanoma is the most common type of primary intraocular tumor in the adult population, its incidence reaching 4.3 cases per million individuals in the United States. Unlike skin melanoma, which metastasizes primarily to the lymph nodes and lungs, uveal melanoma will propagate to the liver as the first metastatic site in 68% of cases. Once this organ is invaded, survival becomes a matter of months for the patient. Uveal melanoma demonstrates a poor radiosensitization toward ionizing radiation and elevated doses (60–90 Gy) are used routinely to control the progression of the primary intraocular tumor, which is why we refer to this characteristic as “radioresistance” throughout the text. Radioresistant tumor cells often have been reported to be much more efficient in repairing the DNA damage caused by ionizing radiation, allowing them to re-enter cell-cycle progression.

Poly(ADP-ribose) polymerase-1 (PARP-1) is the primary enzyme that catalyzes the addition of poly(ADP-ribose) (PAR) units from NAD⁺ after stimulation by DNA strand breaks. PAR is attached covalently to acceptor proteins and alters their function via transitory post-translational modification. PAR level normally is low in any given cell, but increases after DNA damage. Modified proteins rapidly regain their native state with the action of poly(ADP-ribose) glycohydrolase (PARG), which progressively removes the PAR units from the PARP-1-modified acceptor proteins. PARP-1 is involved in various important cellular functions (see the study of Virág and Szabo for a review) but is known primarily for its very important role in DNA repair. Despite that 22 different PARP proteins have been reported to date, each encoded by a different gene, only a single gene, located on chromosome 10 (10q11.23), has been shown to encode for PARG. Its promoter is bidirectional, as a 470-base pair (bp) region is shared between the PARG and TIMM23 genes. It has been shown recently that binding of PU.1 (Spi-1), a transcription factor (TF) that belongs to the ETS family, to the −28 to −102 bp region located upstream of the PARG gene transcription start site might direct basal promoter activity. Transcription factors possessing a duplicated ETS sequence GGAA are thought to be crucial for basal promoter activity of
other genes, such as ATR, TERC, RB1, XPB, and more importantly, PARP-1. A study conducted in our laboratory has shown that, besides ETS proteins, PARP-1 gene transcription is ensured partly by the binding of the TFs Sp1 and NFI to the basal PARP-1 promoter. Consequently, it is plausible that the same TFs could be involved in PARG gene transcription as PARP and PARP-1 belong to the same housekeeping class of genes. Inhibition of PAR degradation may have consequences similar to the stimulation of poly(ADP)ribosylation, as the ratio of the PARP-1/PARG enzymatic activities in any given cell likely dictates whether that particular cell will survive or die. Indeed, increased synthesis of PAR resulting from PARP-1 over-activation as a consequence of oxidative, genotoxic, or radiation treatments can cause a dose-dependent cell death due to ATP depletion consequent to NAD\(^+\) consumption. Alteration of the PARP-1/PARG activity ratio through pharmacologic inhibition of PARG by natural plant tannins, like green tea polyphenols, or by the non-tannin small molecule GPI 16552, has been reported to cause accumulation of PAR, and induce growth inhibition and apoptosis in cancer cell lines. The early embryonic lethality resulting from the complete lack of PARG expression illustrates further how regulation of PAR levels is critical for cell survival, as apparently no other mechanism can take over for the catabolism of PAR. Although PARP-1, and to some extent PARG, expression and activity have been documented in an increasing number of pathologies, including various types of cancers, such as Ewing’s sarcoma, breast cancer, and pancreatic cancer, to our knowledge no such investigation has yet been conducted in uveal melanoma.

In our study, we investigated the expression and enzymatic activities of PARP-1 and PARG enzymes in uveal melanoma cell lines that demonstrate varying tumorigenic properties in vivo. Also, as very little is known about the mechanisms that regulate transcription of the PAR gene, we examined the promoter and 5′-regulatory sequences that are required to ensure proper expression of the PAR gene in uveal melanoma. We provided evidence that, besides a minor role played by the positive TF Sp1, most of the basal PARG promoter activity observed in uveal melanoma appears to be ensured by the binding of the ETS protein ERMT1-5 to the PARG proximal promoter. Importantly, while suppressing PARP-1 gene expression through lentivirus-encoded shRNAs inhibited tumor formation almost completely in uveal melanoma cell lines with very distinctive tumorigenic properties, suppressing PARG gene transcription increased the mean tumor volume significantly in the same cell lines, suggesting that PARG may exert a tumor suppressor function in vivo.

**METHODS**

All experiments were conducted in voluntary compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the Laval University Animal Care and Use Committee approved all procedures. Our study also was conducted in accordance with our institution’s guidelines and the Declaration of Helsinki. The protocols also were approved by the institution’s Committee for the Protection of Human Subjects.

**Cell Culture of Uveal Melanoma Cell Lines**

The uveal melanoma cell lines T97, T98, T108, and T115 were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 0.002% (vol/vol) gentamicin (Invitrogen, Carlsbad, CA) under 5% CO\(_2\) at 37°C as described previously. Isolation and culture of normal uveal melanocytes (UVM) was performed according to the procedure described by Hu et al.

**Plasmids and Oligonucleotides**

Functional analysis of the human PARG promoter was done by transfection of recombinant plasmids containing various portions of the human PARG promoter cloned upstream of the chloramphenicol acetyl transferase (CAT) reporter gene. The recombinant plasmid huPARP(–1473), bearing the entire PARG gene promoter from position –1473 down to position +238 (relative to the position of the previously reported hPARG mRNA start site) inserted upstream of the CAT gene into the Sail (5′end) and XbaI (3′end) sites of the cloning vector pCAT-Basic, was constructed by Bio Basic Inc. (Markham, ON, Canada). Details on the construction of the pCAT-Basic-HuPARG derivatives that bear 5′-deletions of the PARG promoter, or derivatives from the plasmid huPARP(–160) that bear mutations in either or both the –76 or the –53 ETS target sites, are provided in the Supplementary Methods (available online at http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8853/-/DCSupplemental). The various oligonucleotides bearing the DNA binding sites for the Sp1, NFI, and AP-1 TFs, as well as those that cover various sections of the human PARG promoter are described in Supplementary Table 2 (available online at http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8853/-/DCSupplemental).

**Lentivirus Production and Cell Transduction**

The details regarding the production of lentiviruses that over-express shRNAs against the human PARG and PARP-1 transcripts are provided in Supplementary Methods (available online at http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8853/-/DCSupplemental). Melanoma cells were plated in 24-well plates at a density of 15,000 cells per well and incubated overnight at 37°C. The medium was removed and 100 μL of the virus suspension was added to the cells along with 100 μL of fresh medium. The next day, 300 μL of fresh medium was added and cells were allowed to grow for 2 to 3 more days. The cells were transferred into a 6-well plate, allowed to proliferate for few days, and then sorted by flow cytometry. GFP-positive cells were used to generate the PARG-deficient population of melanoma cells.

**Transfections and CAT Assays**

The PARG promoter recombinant plasmids were transfected into uveal melanoma cell lines grown to 80% confluence either by lipofection with Lipofectamine 2000 (Invitrogen) or by electroporation with the NEON electroporation device (Invitrogen) following the manufacturer’s instructions. CAT activities were determined and normalized to secreted hGH as described. Each CAT value corresponds to the mean of at least three separate transfections done in triplicate.

**PARP and PARG Assays**

PARP and PARG activities were measured in all uveal melanoma cell lines and UVM using a commercial colorimetric kit following the supplier’s recommendations (Trevigen Inc., Helgerman, CT).

**Western Blots**

PARP-1 and PARG protein content was evaluated by Western blots as described recently. The membrane was blotted with a mouse anti-PARP (D8B10; dilution 1:500; Millipore, Billerica, MA), a mouse anti-PARP (C2-10; dilution 1:1,000; a kind gift from Guy Poirier, CHUL Research Center, Quebec, Canada), or a mouse anti-ACTN (dilution 1:40,000; Cedarlane, Burlington, ON, Canada). Antibody (F-20)X (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used for detection of the ERMT TF in the same extracts as for PARP-1 and PARG. The membrane
then was washed with PBS plus 1% Tween-20 plus 5% skim milk (PBSTMT), incubated for 45 minutes with a peroxidase-coupled anti-mouse IgG diluted 1:1,000 in fresh PBSTMT and the bound antibodies detected using a chemiluminescence kit (SuperSignal West Dura; Thermo Fisher Scientific, Rockford, IL).

**Nuclear Extracts and Electrophoretic Motility Shift Assay (EMSA)**

Crude nuclear extracts were prepared from all cell types cultured to confluence and dialyzed against Dounce 1 buffer (50 mM KCl, 4 mM MgCl₂, 20 mM K₂HPO₄ [pH 7.4], 1 mM 2-mercaptoethanol and 20% [vol/vol] glycerol), as described.³⁰ The double-stranded oligonucleotide hPARG-47/-106 (see Supplementary Table 2, available online at http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8853/-/DCSupplemental), bearing the human PARG promoter section that ensures basal activity, was ⁵,⁶³⁰-end-labeled and used as a probe in EMSAs. Approximately 5 × 10⁴ counts per minute (cpm) of labeled DNA was incubated with crude nuclear proteins (5 μg) from each cell type in the presence of 500 ng of poly(dI-dC)-(dI-dC) (Amersham Biosciences, Piscataway, NJ) in buffer D (5 mM Hepes [pH 7.9], 10% [vol/vol] glycerol, 25 mM KCl, 0.05 mM EDTA, 0.5 mM dithiothreitol [DTT] and 0.125 mM PMSE). For competitions in EMSA, unlabeled double-stranded oligonucleotides bearing various DNA target sequences for known TFs (Sp1, NFI, AP-1, ETS) were used, available online at http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8853/-/DCSupplemental). For EMSA supershift analyses, 4 μL of antibody directed against the TFs ETS-1 (C-20), PU.1 (T211X), ERM (F-20X), TEL (H-214X) (all antibodies from Santa Cruz Biotechnology Inc.) or 2 μL of antibody against Sp1, NFI, and AP-1 were added to the reaction mix before the separation of the DNA-protein complex on 5% to 8% native polyacrylamide gels ran against Tris/glycine buffer.³⁰ Gels were dried and autoradiographed at −80°C overnight to reveal the position of the shifted DNA-protein complexes generated. 

**Real Time Polymerase Chain Reaction (RT-PCR)**

Total RNA was isolated from the T97, T98, T108, and T115 cell lines using the RNeasy Mini Kit (Qiagen, Santa Clarita, CA). Reverse transcription was performed using random hexamer primers following the manufacturer’s protocol for synthesis of the first strand cDNA (Superscript II; Invitrogen Canada Inc., Hamilton, Ontario, Canada). RT-PCR was performed as described³¹ using the primer sets shown in Supplementary Table 3 (available online at http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8853/-/DCSupplemental). Amplification efficiencies were validated and normalized to the actin mRNA transcript.

**Tumorigenicity Assays and Immunohistochemical Analyses**

Approximately 1 × 10⁴ cells (group 1, T97/shLuc and T97/shPARP; group 2, T115/shLuc and T115/shPARP; group 3, T97/shLuc and T97/ shPARP-1; group 4, T115/shLuc and T115/shPARP-1) in DMEM without FBS were injected subcutaneously into the flanks of Crl:CD1-nuBR athymic nude mice (Charles River, St-Constant, QC, Canada). Six 7-week-old female mice were used for each cell line. Mice from the same group all were sacrificed at the same time. Tumors were excised and embedded in paraffin for immunohistochemical analysis on 5 μm thick sections. They were first stained with hematoxylin/eosin for histologic analysis and then immunohistochemically analyzed using a diaminobenzidine (DAB) biotin-streptavidin complex with a mouse monoclonal primary antibody against the MART1 protein (A105; Invitrogen Canada Inc.) as described recently.²⁷

**Microarray Analyses**

Cyanine 3-CTP labeled cRNA targets were prepared from 25 ng of total RNA using the Agilent One-Color Microarray-Based Gene Expression Analysis kit (Agilent Technologies Canada Inc., Mississauga, ON, Canada). Then, 600 ng cRNA were incubated on a G4851A SurePrint G3 Human GE 8 × 60 K array slide (60,000 probes; Agilent Technologies). Slides then were washed, stained, and scanned on an Agilent SureScan Scanner according to the manufacturer’s instructions. Data were analyzed using the ArrayStar V4.1 (DNASTAR, Madison, WI) software for scatter plots and generation of the heat maps of selected genes of interest as described recently.²⁷

**Statistical Analyses**

Student’s t-test was performed for comparison of the groups in the transfection analyses. Differences were considered to be statistically significant at P < 0.05. All data are expressed as mean ± SD.

**RESULTS**

**Gene Expression Profiles of Uveal Melanoma Cell Lines with Different Tumorigenic Properties**

The T97, T98, T108, and T115 cell lines were cultured from the primary tumor of four different patients diagnosed with uveal melanoma.²² In contrast with uveal melanocytes, which have a spindle-like morphology, these cell lines exhibit a mixed spindle-epithelioid phenotype. Moreover, they express protein markers, such as S100, HMB45, and MART-1, that are typical of uveal melanoma. Their doubling time has been estimated to be between 17, 17, 14, and 33 hours, respectively, for T97, T98, T108, and T115, which is quite faster than what was measured for UVM (78 hours).³² Importantly, these cell lines have distinctive capacities to form tumors when injected subcutaneously into athymic mice,³² which make them ideal biologic tools to study cancer formation in vivo.

To assess further the variations at the transcriptional level between T97, T98, T108, and T115 cell lines, gene expression profiling on microarrays was conducted on total RNA from each cell line. Scatter plot analysis of the 60,000 different targets that cover the entire human transcriptome and contained on the arrays indicated that the T97 and T98 cells share more similarities than with any of the other cell lines, their gene expression profiles being very distinctive from those yielded by T108 and T115 cells (Fig. 1A). Similarly, the T108 and T115 cells are much more alike to one another than with either T97 or T98 cells.

In an attempt to visualize this distance between the uveal melanoma cell lines, a heat map was generated for all the genes showing a 2-fold or more expression variation unique to each cell line paired against the UVM expression profile (Fig. 1B). Once again, T97 and T98 cells have very similar, but not identical gene expression profiles when considered in pair. T115 cells have a pattern of gene expression closer to UVM than with any of the other uveal melanoma cell lines, whereas T108 appears to be in between T97/98 and T115. Interestingly, a major proportion of genes expressed at high levels in T108, T115, and UVM (red and yellow) become highly repressed in T97 and T98 cells (blue).

**Expression of PARP-1 and PARG Varies in Uveal Melanoma Cell Lines**

As alterations in the expression and activity of PARP-1 and PARG have been reported to be related intimately to the growth properties of many types of cancer cells, we next
established whether changes in the pattern of expression for these proteins could correlate with the ability of these cell lines to yield tumors in nude mice. Data extracted from the gene profiling databank conducted on the uveal melanoma cell lines indicated that transcription of the \textit{PARG} gene was very low in all cell lines and not that much different from the level observed in UVM (Fig. 2A). Interestingly, expression of TIMM23 was very similar in all uveal melanoma cell lines and also not that different from the level seen in UVM (Fig. 2A). However, its basal level of expression clearly was higher than what was observed for PARG in all cell lines examined, which is consistent with the data reported by Meyer et al.\textsuperscript{15} On the other hand, \textit{PARP-1} gene expression was more elevated in T108 and most notably T115 cell lines relative to UVM, whereas its expression was lower in T97 and T98 cells (Fig. 2A). Consistent with these results, RT-PCR analyses indicated that expression of the \textit{PARP-1} gene was very low in T97 and T98 cells (Fig. 2A). In contrast, \textit{PARG} expression was significantly diminished in T97 and T98 cells (Fig. 2C), a result also consistent with the g-mean linear signals from the microarrays corresponding to the \textit{PARG} transcript. Moreover, the molecular mass of the \textit{PARG} protein increased considerably in T108 and T115 cells.

As changes in the level of any given enzyme do not necessarily translate into corresponding changes in its enzymatic activity, we then conducted in vitro enzymatic assays for \textit{PARP-1} and \textit{PARG}. \textit{PARG} enzymatic activity was evaluated by the loss of biotinylated PAR from histones, whereas \textit{PARP} activity was assessed by the incorporation of biotinylated PAR onto histone proteins. \textit{PARP-1} enzymatic activity in T97 and T98 cells was very similar to that of UVM despite that \textit{PARG} protein concentration was found to be lower in T97 and T98 cells, suggesting that although reduced at the protein level,
PARP-1 processivity was clearly higher in T97 and T98 cells than in UVM. In addition, PARP-1 activity was 4.5 to 8-fold higher in T108 and T115 cells relative to the level observed in UVM (Fig. 2D). In addition, little to no change (2-fold increase in T98) in PARG activity was observed between T97/T98 cells and UVM, whereas 3- and 5-fold increases were observed in T108 and T115 cells, respectively. Most importantly, the ratio of the PARP-1 activity over that of PARG revealed that T97 and T98 have a much lower ratio (0.67 and 0.52, respectively) that favors the enzymatic removal of PAR by PARG, whereas T108 and T115 cells (ratio of 1.48 and 1.49, respectively) rather favor the catalytic addition of PAR by the PARP-1 enzyme onto acceptor proteins (Fig. 2D).

Basal PARG Gene Transcription is Ensured by the −46/−84 Region of the PARG Promoter

Only a few studies have characterized the human PARG gene promoter and the transcription factors it binds, and to our knowledge none has ever been conducted in uveal melanoma so far. Functional analysis of the human PARG promoter was conducted first by transfecting the uveal melanoma cell lines with plasmids containing various portions of the PARG gene promoter cloned upstream of the CAT reporter gene. Five recombinant plasmids (huPARG[−1], huPARG[−84], huPARG[−160], huPARG[−562], and huPARG[−717]) bearing the human PARG sequence ending at 5’ position −1, −84, −160, −562, and −717 bp relative to the PARG gene mRNA start site (Figs. 3A, 3B) but all sharing the same 3’-end (at position +128) were transfected into T97, T98, T108, and T115 cells. Analysis of the transfection data indicated clearly that basal PARG promoter activity is ensured by a segment encompassing the −84 to −1 area of the PARG gene as high promoter activity was obtained with plasmid huPARG[−84], whereas further deletion down to position −1 (in huPARG[−1]) almost entirely abolished PARG promoter activity in all cell lines (Fig. 3C). Further extension of the PARG promoter up to position −717 had only minimal influences on PARG promoter activity in all but T115 cells in which a 4-fold repression occurred upon extending the PARG promoter up to position −562, suggesting that PARG promoter sequences from −84 and −562 bear negative regulatory elements that repress basal PARG promoter activity in T115 cells (Fig. 3C).

As basal PARG promoter activity is determined by the −84/−1 segment, we next subjected this DNA sequence to further analysis by the TFSEARCH program to search for the presence of any putative TF binding sites within this area. Two target sites for ETS proteins were identified at positions −76 and −55 relative to the PARG gene mRNA start site, as well as two degenerate sites for Sp1 (positions −19 and −59), one site for G/ERD (position −67) as well as a target site for CREB (position −33, Fig. 4A). To demonstrate the functional relevance of these sites, the basal PARG promoter was shortened further to position −46 and the resulting construct
(huPARG[−46]) transfected into uveal melanoma cell lines. Cells also were transfected with the constructs huPARG(−1) and huPARG(−84) as negative and positive controls, respectively. While transfection of huPARG(−84) again resulted in high CAT activities in all transfected cells, deletion of the −84/−47 PARG sequence in huPARG(−46), which also eliminated the most 5′ Sp1 site, the site for C/EBP and Ets target sites entirely abolished promoter activity in all cell lines (Fig. 4B), suggesting that the shortest DNA sequence needed to ensure basal PARG promoter activity is contained between positions −46 and −84. It also suggests that the downstream putative CREB and Sp1 sites near positions −33 and −19, respectively, are insufficient to ensure basal PARG promoter activity in these cells.

Transcription Factors Sp1 and ERM Bind to the Basal PARG Promoter In Vitro and In Vivo

EMSA next was used to demonstrate binding of TFs to the PARG basal promoter. For this purpose, a double-stranded oligonucleotide spanning the −47 to −106 PARG sequence was used as the labeled probe in these assays. Only one major DNA-protein complex with very low electrophoretic mobility was observed on gel upon incubation of the probe with nuclear extracts from T97 or T115 cells (Fig. 4C, lanes 2 and 7; identical results were obtained with extracts from T98 and T108 cells, data not shown). Unlabeled oligonucleotides bearing target sites for TFs NFI, Sp1, and AP-1 proved totally inefficient at preventing formation of the DNA-protein complex seen on gel except for the Sp1 oligonucleotide that competed weakly when the extract from T115 cells was used (lane 9). On the other hand, the unlabeled PARG−47/−106 oligonucleotide could compete efficiently for the formation of this complex (lanes 6 and 11).

The identity of the protein that caused a decrease in the formation of the DNA-protein complex when the Sp1 oligonucleotide was used as the unlabeled competitor was investigated further by supershift analyses using antibodies against TFs Sp1, Sp3, NFI, and the AP-1 subunit c-Jun. The supershift analysis was conducted using the extract from T115 cells to support the previous findings. Antibodies against Sp3 (lane 4), NFI (lane 5), and JunB (lane 6) were unable to alter in any way the formation of the DNA-protein complex yielded by nuclear proteins from T115 cells (Fig. 4D). On the other hand,
the Sp1 antibody significantly reduced formation of this complex (lane 3), indicating that a significant proportion of this complex contained Sp1.

As it has been reported previously that the ETS protein PU.1 binds the basal PARG promoter to modulate its transcriptional activity, we examined the expression pattern for all the proteins from the ETS family in uveal melanoma cell lines as well as UVM by microarrays. The T97, T98, and T108 cell lines share very similar ETS member expression patterns (Fig. 5A). T115 cells showed a pattern of ETS protein expression that was closer to UVM. Expression of ELF2/NERF, ELF4/MEF, ELK4/SPA1, ETV2/ERV2, and ETV6/TEL was shared by all cell lines, including UVM, whereas ELF3, ETV4/EIA-F, and SPI1/PU.1 were expressed by T97, T98, and T108 cells only. Significant expression of ETV3/PE-1 was restricted to T97 and T98 cells, whereas ETS2 and FLI1 were expressed strictly in T115 cells.

Figure 4. Transfection and EMSA analyses of the −1−84 PARG promoter region. (A) The DNA sequence of the human PARG basal promoter (−1 to −84) was examined using the TESS program. Target sites for TFs ETS, C/EBP, CREB, and Sp1 are indicated. (B) A recombinant plasmid bearing a shorter version of the PARG promoter extending up to position −46 (HuPARG(−46)) was transfected into T97, T98, T108, and T115 cells along with the PARG promoter-containing plasmids huPARG(−1) and huPARG(−84). *CAT activities statistically different from those measured with HuPARG(−84) (P < 0.05; paired samples, t-test). (C) Nuclear proteins (5 μg) from T97 and T115 cells were incubated with a labeled probe bearing the basal promoter of the PARG gene (hPARG−47−106) either alone (C), or with a 200-fold molar excess of unlabeled competitors bearing the target sites for known TFs (NFI, Sp1, and AP-1) or the hPARG basal promoter (contained on the hPARG−47−106 oligonucleotide [PARG]). (D) The hPARG−47−106 labeled probe used in panel (A) was incubated with nuclear proteins from T115 cells either alone (C) or with antibodies against Sp1, Sp3, and NFI, or against the AP-1 subunit JunB before separation of the complexes by EMSA. P, labeled probe alone; U, unbound fraction of the labeled probe.
that an unlabeled, double-stranded derivative from the hPARG-47/–106 oligonucleotide bearing mutations into both ETS sites (ETS1/2m) was unable to compete efficiently for the formation of the C1 shifted complex, unlike the hPARG-47/–106 wild-type (wt) oligonucleotide. Formation of the C1 complex then was monitored by EMSA. (C) The hPARG-47/–106 labeled probe used in Figure 4 was incubated with nuclear proteins from T97, T98, T108, or T115 cells either alone (C), or with antibodies against Ets-1, PU.1, ERM, and TEL before separation of the complexes by EMSA. C, labeled probe alone; SSC, supershifted complex. (D) Western blot conducted on 50 μg nuclear proteins from T97, T98, T108, and T115 cells using antibodies against ERM (top panel) and actin (bottom panel).
the microarrays. The expression of ERM in all four uveal melanoma cell lines was demonstrated further by Western blot (Fig. 5D) and RT-PCR (Fig. 5E).

The −76 Element from the PARG Promoter is the Most Effective of the Two ETS Binding Sites

To evaluate the contribution of each of the two ETS sites from the human PARG promoter, both were mutated in the parental plasmid huPARG(−160) either individually (mutated plasmids Hu-PARG[160/mETS−76] and Hu-PARG[160/mETS−53]) or in combination (Hu-PARG[160/mETS−76–53]).

Mutating the −53 ETS target site alone yielded a weak, but not statistically significant, increase in basal hPARG promoter activity (Fig. 6A). However, mutation of the upstream ETS site alone in −160/mETS−76 resulted in a dramatic 13-fold reduction in PARG promoter activity in T97 and T98 cells, and also reduced promoter activity by 3- and 4-fold in T108 and T115 cells, respectively (Fig. 6A). Interestingly, mutating −76 and −53 ETS sites simultaneously in plasmid Hu-PARG(−160/mETS−76–53) entirely abolished basal PARG promoter activity in all cell lines. As a putative target site for C/EBP also has been located at position −67 in between the two ETS sites (Fig. 4A), an additional recombinant construct (Hu-PARG[160/AETS−76–53]) that lacks the −76–53 sequence that normally contains the ETS and C/EBP sites was constructed and used for transfection. Deletion of ETS and C/EBP sites yielded CAT activities not statistically different from those resulting from the transfection of Hu-PARG(−160/mETS−76–53) (mutated in ETS sites but still bearing an intact C/EBP sequence, Fig. 6A), suggesting that under the conditions used, C/EBP has no regulatory influence on the transcription driven by the PARG promoter in uveal melanoma cells.

ERM belongs to the PEA3 subfamily of ETS proteins that, in addition to ERM (ETV5) and PEA3 (ETV4), also includes ER81 (ETV1). Therefore, we investigated further which of these three proteins had the most influence on the activity of the PARG promoter by conducting co-transfections of huPARG(−160) along with expression plasmids encoding each of these three proteins. As shown in Figure 6B, overexpression of either PEA3 or ER81 had no influence on basal PARG promoter activity in T97 and T115 cells. On the other hand, overexpression of ERM resulted in 10- and 3-fold increases in T97 and T115 cells, respectively. Mutating both ETS sites on the PARG promoter entirely abolished responsiveness toward ERM in T97 and T115 cells.

Suppressing the Expression of PARG or PARP-1 in T97 and T115 Cells Alters Their Tumorigenic Properties In Vivo

We next investigated whether suppression of either PARG or PARP-1 gene expression would alter in any way the tumorigenic properties that are typical of uveal melanoma cell lines. To this end, we transduced T97 and T115 cells with a lentivirus encoding an shRNA directed against either the human PARG or PARP-1 genes. Expression of the PARP and PARP-1 proteins then was monitored in T97 and T115 cells stably expressing the PARG and PARP-1 shRNAs to confirm the extent of PARG and PARP-1 gene extinction. As shown in Figure 7A, endogenous PARG and PARP-1 gene expression was very efficiently suppressed in T97 and T115 cells when transduced with lentiviruses encoding shPARG (T97/shPARG and T115/ shPARG) and shPARP-1 (T97/shPARP-1 and T115/shPARP-1) but not by a control lentivirus expressing an shRNA directed against luciferase (T97/shLuc and T115/shLuc).

We then injected T97/shPARP and T115/shPARP, along with their corresponding negative controls T97/shLuc and T115/shLuc, into both flanks of immunodeficient CD1-mice and monitored tumor formation over time. Mice injected with the control cell lines T97/shLuc and T115/shLuc yielded tumors in all but one mouse (for T115/shLuc cells) with median volumes of 57 mm3 and 86 mm3, respectively (Fig. 7B). However, suppressing PARG gene expression significantly increased the median tumor size to 208 and 327 mm3 in T97 and T115 cells, respectively, therefore suggesting that PARG gene expression is required to suppress or restrict the tumorigenic properties of these cells. Immunohistochemical analysis of the tumor tissues produced by T97/shPARG and T115/shPARG cells indicated that nearly all cells stained positive for the melanoma marker MART-1, therefore establishing their melanocytic origin (Fig. 7C). Typical of aggressive uveal melanoma, vasculogenic mimicry patterns could be observed upon hematoxylin/eosin staining throughout the entire tumors yielded by T97/shPARG (data not shown) and T115/shPARG cells (Fig. 7C).

As a complement to the experiment cited above, we next injected athymic mice with the T97/shPARG and T115/shPARG-1 cell lines, along with their corresponding negative controls T97/shLuc and T115/shLuc. Again, both control cell lines yielded tumors in mice (median tumor volumes of 243 and 411 mm3, respectively, Fig. 7B). However, injection of T97/shPARP-1 and T115/shPARP-1 either dramatically reduced (with T97/shPARP-1 cells; median tumor volumes of 88 mm3) or almost entirely abolished (with T115/shPARP-1 cells; only one very small [14 mm3] tumor detected) the ability of these cells to yield tumors in mice (Fig. 7B), therefore suggesting that PARP-1 expression and activity are required for the tumorigenicity of uveal melanoma.

DISCUSSION

In our study, we demonstrated a significant variation in the expression of the poly(ADP-ribosyl)ation enzymes PARP-1 and PARG between uveal melanoma cell lines (T97, T98, T108, and T115) with varying tumorigenic properties in vivo. Whether at the gene transcript, protein quantity, or enzymatic activity level, PARP-1 expression always was higher in high- rather than low-tumorigenicity cell lines, suggesting that dysregulation of the expression of the PARP-1 and PARG genes might be involved in the specific properties of the uveal melanoma tumor cells.

Although initially reported to have low tumorigenicity at low cell passage (P5–P7),27 the T115 cells used in our study turned out to be highly tumorigenic and produced tumors in athymic mice much faster than T97 cells. Preliminary experiments we just recently conducted suggested this phenotypic change to be caused by the increased number of cell passages (P18–P22) required to produce the derivatives from the T115 and T97 cells that stably integrated the luciferase (our negative control), PARG, and PARP-1 shRNA-encoding lentiviruses.

Interestingly, a clear difference in the molecular mass of the PARG enzyme has been observed between T108 and T115 (estimated to be of 102 and 111 kilodaltons [kDa]), and T97 and T98 cell lines (estimated to be of a higher molecular mass). Although the exact cause of this shift presently is unknown, one might speculate that it likely results from a yet unknown post-translational modification of the PARG protein that occurs only in T108 and T115 cells. PARG is known to undergo endogenous phosphorylation at Ser16,35 Other phosphorylation sites are known, mostly located in the N-terminal putative regulatory domain.36 To our knowledge, no additional post-
translational modification besides phosphorylation has been reported to date for PARG. Since alternative splicing variants of PARG have been reported previously, we cannot exclude the possibility that a different splice variant could explain the variation in the molecular weight observed for this enzyme in T108 and T115 cells.

Transfections of the PARG promoter constructs performed in our study clearly identified the basal PARG promoter to be located between positions −84 and −46 relative to the PARG transcription start site, which supports the results of Uchiumi et al. Search for putative TF target sites that might be contained within this minimal promoter region identified possible binding sites for Sp1 and ETS, which were validated by our EMSA results. Indeed, the competition and supershift analyses in EMSA indicated that Sp1 does bind, although weakly, to the −84/−46 basal PARG promoter region. Interestingly, Sp1 also was reported to bind the PARP-1 promoter and to regulate positively its transcription. Both genes (PARP-1 and PARG) belong to the same family of housekeeping genes. Importantly, experiments in EMSA clearly indicated that ERM (ETV-5), and not PU.1 (Spi-1) as reported previously, binds with high affinity to the most upstream (position −76) of the two ETS sites identified in the basal PARG promoter region. This discrepancy might be due to the fact that different types of cells may express different patterns of ETS proteins. Indeed, the data from the microarrays indicated clearly that T97, T98, and T108 cells show very similar patterns of ETS protein expression, some of which (such as ELF2, ETV2, and ETv6) are expressed at a high level, whereas the pattern observed for T115 is much closer to that seen for UVM. Again, the fact than an ETS protein, namely ERM, was found to bind the PARG promoter is interesting as such proteins also have been found to bind the PARP-1 promoter and to participate to its transcription.

ERM belongs to the PEA3 subfamily that, in addition to ERM and PEA3 (also known as ETV4), also includes ER81 (also known as ETV1). As revealed by the microarray and Western blot analyses, expression of ERM varies from very low levels in T97 and T115 cells, to moderate levels in T98 and T108 cells (Fig. 5A). High levels of ETV4/PEA3 expression have been observed in T97, T98, and T108 cells whereas ETV1/ER81 was expressed at low levels in all uveal melanoma cell lines. The proteins from the PEA3 subfamily are known to be involved in the regulation of cancer cells, including breast, lung, ovarian, colorectal, oral, and gastric cancers, and their overexpression generally is correlated with a poor prognosis and the presence of metastases. Furthermore, ERM has been reported to function as a transcriptional activator. It also is known to have regulatory functions in many types of cancers, such as
paraffin wax. Tumor sections stained with hematoxylin/eosin (H&E) also are provided (magnification 100× and 600×).

**Figure 7.** Influence of suppressing PARG gene expression on the tumorigenic properties of the T97 cell line. (A) PARG and PARP-1 expression was monitored by Western blot in protein extracts from T97, T97/shLuc, T97/shPARG, T97/shPARP-1, T115, T115/shLuc, T115/shPARG, and T115/shPARP-1 cells. Actin expression also is provided as a normalization control. (B) Box-Whisker plots depicting the tumor volume (mm^3) for each of the nude mice injected subcutaneously with the T97/shLuc, T97/shPARG, T97/shPARP-1, T115/shLuc, T115/shPARG, and T115/shPARP-1 cells. In this plot, the *whiskers* extend to the largest and smallest data points, the box extends from the upper quartile to the lower quartile and is crossed by a *line* at the median of the data. (C) Expression of MART-1 by immunohistochemistry on one of the in vivo tumors yielded by the T115/shPARG cells. Tumor sections stained with hematoxylin/eosin (H&E) also are provided (magnification 100× and 600×).

Prostate[41] and ovarian cancers.[42] ERM also enhances intestinal epithelial tumorigenesis.[43]

The fact that the ERM antibody entirely supershifted the C1 DNA-protein complex observed despite that a proportion of this complex also contained Sp1 suggests that the labeled probe used in the EMSA was bound simultaneously by Sp1 and ERM. Cooperation between ETS and Sp1 proteins in gene regulation is not unique and has been reported previously.[44] Similarly, duplicated GGAA motifs that typically are bound by ETS proteins are not unique to the PARG promoter and have been identified recently in the promoters of a few DNA helicase genes (such as XPB, RecQL5, and RTEL) as well as in the promoter region of genes encoding DNA replication/repair synthesis factors, including ATR, TERC, and Rb1.[17] In all instances, mutations that disrupt these duplicated ETS sites dramatically reduced basal promoter activity.

The increase in PARP-1 activity in the T108/T115 cell lines, that proved to be highly tumorigenic in our study compared to the less aggressive T97/T98 cells and the normal melanocytes, is expected to lead to the binding of an abnormally elevated quantity of PAR to acceptor proteins, a process that is toxic in itself for the cell, as it triggers mitochondrial apoptosis inducing factor (AIF) release.[45] Also, a higher PARP-1 cellular activity leads to a depletion in the pool of cellular NAD+ as its formation depends on ATP, therefore causing a subsequent energy failure.[46] Then, why is it that suppressing PARP gene expression in highly (T115) and poorly (T97) tumorigenic cell lines caused a significant increase in tumor volume when they were injected into athymic mice, whereas suppressing PARP-1 strongly abrogated (in T97) or almost entirely suppressed (in T115) the ability of these cells to produce tumors in vivo? Based on the above assumptions, one would expect that suppression of PARG would favor dramatically the addition of PAR to acceptor proteins and further increase the depletion in the pool of cellular NAD+, thus causing the cell to undergo apoptosis. This obviously is not happening here as T97 and T115 cells do proliferate very well in culture without any signs of apoptosis. Auto-modification of PARP-1 has been reported to lead to PARP-1 inactivation.[46,47] Therefore, it is conceivable that suppression of PARG in T97 and T115 cells also results in excessive PARP-1 auto-modification, which then becomes unable to deplete further the intracellular pool of NAD+, therefore contributing to reduce the proportion of these cells progressing to apoptosis. Part of the explanation also may rely on the recent demonstration that mice deficient in the expression of PARG110 exhibit high genomic instability characterized by a high frequency of sister chromatid exchanges and chromosomal aberrations.[50] Interestingly, PARG110−/− mice have greatly increased numbers of hepatocellular carcinomas following treatment with diethylnitrosamine (DEN). Because it already was reported that spontaneous sister chromatid exchange (SCE) in UM cell lineages are very low,[49] it would be unlikely that it is a cause of increased tumorigenicity in T97 and T115 cells deficient in the expression of PARG110. More analyses are needed to sort out this possibility. On the other hand, abnormally elevated PARP-1 gene and protein expression has been reported in many different types of cancers, including Ewing’s sarcoma, malignant lymphoma, colorectal cancer, intestinal adenoma, hepatocellular carcinoma, endometrial hyperplasia, breast, uterine, lung, and ovarian cancers (recently reviewed by Velamos et al.[50]). Interestingly, PARP-1 dysfunction also was noted in malignant glioblastoma[52] and skin melanoma.[53] The results presented in our study raised the interesting possibility that one of the many components of the apoptosis signal transduction pathway is deficient in such a manner that the
apoptotic member of the Bcl-2 family, is reduced dramatically. Analysis of the gene profiling data for the uveal melanoma cell lines indicated that expression of BCL-2-like 13 (BCL-2L13, apoptosis facilitator, Bcl-rambo), a pro-apoptotic member of the Bcl-2 family, is reduced dramatically by 7- to 82-fold in T97, T98, T108, and T115 cells relative to normal melanocytes (data not shown). Additional experiments will be required to define precisely whether a strong reduction in the expression of BCL-2L13 is a hallmark of aggressive uveal melanoma. Another explanation would be the implication of BAP1, a tumor suppression gene in uveal melanomas. Our data showed that expression of BAP1 is increased when PARP1 expression is decreased in T97 and T115, suggesting a compensation mechanism in these cell lines. Therefore, a decrease in tumorigenicity would have been expected, unless an inactivating mutation is present in the BAP1 gene. Additional molecular sequencing of the BAP1 gene in these UM cell lines is needed for a better understanding of the BAP1 gene in these UM cell lines.

Together, our data described an interesting variation in the expression of the poly(ADP-ribose)lation enzymes PARP-1 and PARG between uveal melanoma cell lines with very distinctive tumorigenic properties and also suggested a tumor suppressor function for PARG in uveal melanoma. Suppression of PARG gene expression in MCF-7 human breast cancer cells revealed that approximately half of the 217 genes found to be regulated by PARG were in fact down-regulated by this protein, of which some, such as LGALS3BP, are dysregulated in cancer, therefore lending further support to a suppressor function for PARG. It is speculated that variations in PARP-1/PARG expression and enzymatic activities might contribute to the neoplastic transformation of uveal melanoma and to its intrinsic resistance toward radiation therapy. PARP-1 expression level may soon prove to be a promising new biologic marker of aggressive uveal melanoma with a prognostic value.

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References


