Poly(ADP-Ribose) Polymerase 1 Promotes Tumor Cell Survival by Coactivating Hypoxia-Inducible Factor-1–Dependent Gene Expression

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Abstract

Hypoxia-inducible factor 1 (HIF-1) is the key transcription factor regulating hypoxia-dependent gene expression. Lack of oxygen stabilizes HIF-1, which in turn modulates the gene expression pattern to adapt cells to the hypoxic environment. Activation of HIF-1 is also detected in most solid tumors and supports tumor growth through the expression of target genes that are involved in processes like cell proliferation, energy metabolism, and oxygen delivery. Poly(ADP-ribose) polymerase 1 (PARP1) is a chromatin-associated protein, which was shown to regulate transcription. Here we report that chronic myelogenous leukemia cells expressing small interfering RNA against PARP1, which were injected into wild-type mice expressing PARP1, showed tumor growth with increased levels of necrosis, limited vascularization, and reduced expression of GLUT-1. Of note, PARP1-deficient cells showed a reduced HIF-1 transcriptional activation that was dependent on PARP1 enzymatic activity. PARP1 neither influenced binding of HIF-1 to its hypoxic response element nor changed HIF-1α protein levels in hypoxic cells. However, PARP1 formed a complex with HIF-1α through direct protein interaction and coactivated HIF-1α–dependent gene expression. These findings provide convincing evidence that wild-type mice expressing PARP1 cannot compensate for the loss of PARP1 in tumor cells and strengthen the importance of the role of PARP1 as a transcriptional coactivator of HIF-1–dependent gene expression during tumor progression. (Mol Cancer Res 2008;6(2):282–90)

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Introduction

In solid tumors, rapid cell proliferation is associated with areas of hypoxia. Intratumoral hypoxia induces neoangiogenesis, which is an essential switch from tumorigenesis to tumor progression (1). Oxygen limitation regulates vascularization, glucose metabolism, cell survival, and tumor spread. The hypoxic response critically depends on the transcription factor hypoxia-inducible factor-1 (HIF-1; ref. 2). HIF-1α was found to be overexpressed in more than 70% of human cancers and their metastases (3). The effect of HIF-1 on tumor growth is complex and involves the activation of several adaptive pathways and results in the induction of target genes (4). In solid tumors, immunohistochemistry often shows larger fronts of HIF nuclear expression delineating areas of necrosis (5). Induction of HIF is therefore believed to be supportive, if not causative, in cancer (6-8). In tumor xenograft and orthotopic mouse models, manipulation of the levels of either HIF-1α or HIF-2α has shown a causal link between HIF expression and tumor progression (4). HIF signaling has emerged as an important hypoxia-driven response allowing tumor cells to survive, expand, and invade. As a result, tumor hypoxia or HIF expression is strongly associated with a diminished therapeutic response and malignant progression (9).

HIF induction is a multistep process, which is tightly regulated *in vivo* (10, 11). HIF-1 is composed of two polypeptides: HIF-1α and HIF-1β (12). Two additional HIF-α members, the closely related HIF-2α (13) and more distantly related HIF-3α (14), were recently identified. HIF-1 activity is regulated at the posttranscriptional level by protein degradation of HIF-1α subunits after oxygen-dependent hydroxylation of specific proline residues (15). During hypoxia, the prolyl hydroxylases are inactive and HIF-1α is not complexed with the ubiquitin E3 ligase complex containing von Hippel Lindau, thereby allowing for the formation of active HIF-1 complexes (2, 5, 12). Transactivation involves dimerization of the two HIF-1 subunits, which bind to an enhancer element, called hypoxia response element, in target genes. Among the most studied promoters with regard to the recruitment of HIF-1 are those of the *EPO*, *GLUT-1*, and *CA9* [carbonic anhydrase IX (*CAIX*)] genes (16-18). The presence of hypoxia response element sites is necessary, but not sufficient, to direct gene expression in response to hypoxia, suggesting that HIF-1 must interact with other transcription factors or cofactors bound

around these sites (19). The assembly of a higher-order HIF-1 transcription complex is an important stage in HIF-1–dependent transcription, involving multiple coactivator/cofactor-HIF-1-DNA interactions. Two key coactivators of HIF-1 are the histone acetyltransferases p300 and its homologue, the cyclic AMP-responsive element binding protein–binding protein, which can directly associate with the COOH-terminal transactivation domain of HIF-1α. HIF-1α also interacts with other coactivators such as SRC-1 and TIF1 (20).

Poly(ADP-ribose) polymerase 1 (PARP1) is a nuclear chromatin-associated protein and belongs to a large family of enzymes that can synthesize polymers of ADP-ribose units by using β-NAD^+ as substrate (21). Several studies showed that $PARP1^{-/-}$ mice were protected against myocardial infarction, streptozotocin-induced diabetes, lipopolysaccharide-induced septic shock, zymosan-induced vascular failure, a nonseptic model of multiple organ dysfunction, as well as collagen-induced arthritis (21). We recently presented evidence that PARP1 can act as a coactivator of nuclear factor κB *in vivo* (22).

In a 12-*O*-tetradecanoylphorbol-13-acetate–induced skin cancer model, PARP1 was suggested to be important for tumor induction (23). The importance and contribution of PARP1 for tumor progression in tumor cells is still not clear. Here we present evidence that tumors derived from chronic myelogenous leukemia cell line K562 lacking PARP1 but grown in wild-type mice expressing PARP1 show a significant increase in necrotic areas, reduced vascularization, and reduced expression of *GLUT-1*, a HIF-1–dependent gene. HIF-1–dependent gene expression was also reduced in K562 cells expressing siRNA against PARP1 (siPARP1) or primary $PARP1^{-/-}$ mouse lung fibroblasts (MLF). PARP1 interacted directly with HIF-1α, providing convincing evidence that PARP1 acts mechanically as a transcriptional cofactor of HIF-1α transcriptional activation. Interestingly, HIF-1–dependent gene expression was dependent on the enzymatic activity of PARP1. Taken together, our results show that PARP1 and its enzymatic activity are important for tumor progression, possibly leading to new therapeutic approaches for the treatment of human tumors.

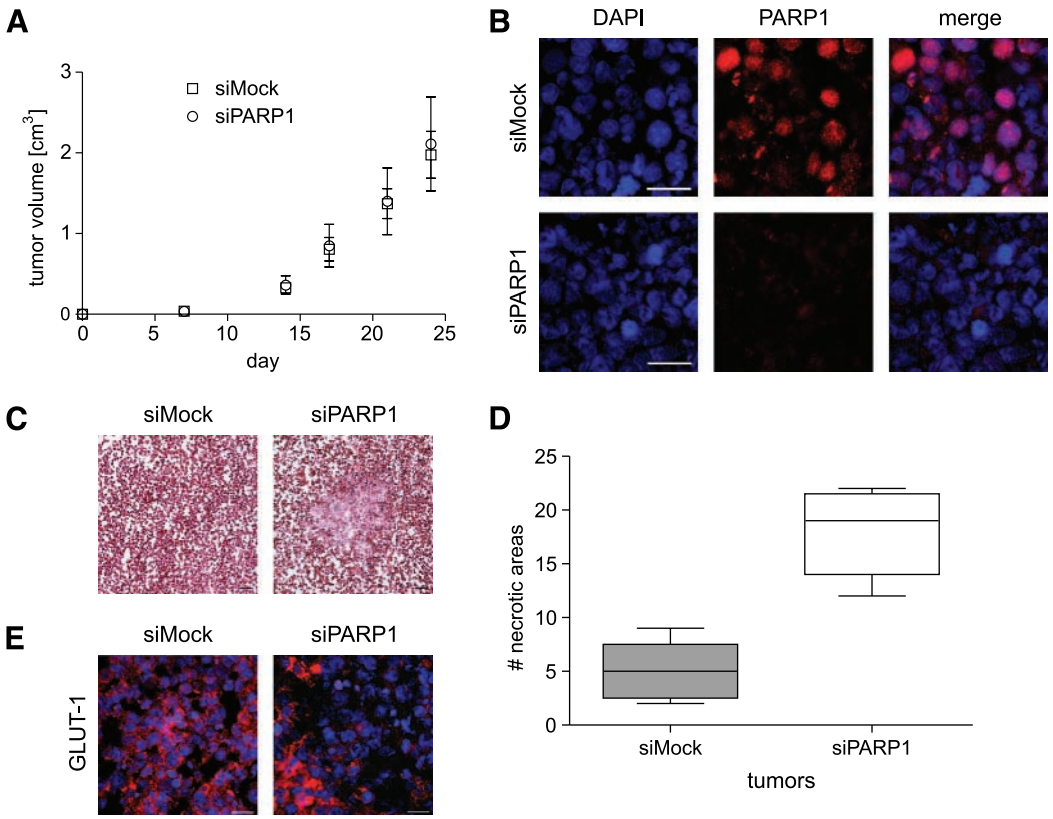

FIGURE 1. The down-regulation of PARP1 enhances tumor cell death but does not affect tumor growth. Chronic myeloid leukemia cell line K562 was subcutaneously injected in athymic nude mice. Cells with down-regulated *PARP1* gene by transduction of siPARP1 were compared with cells transfected with siMock. **A.** Tumor growth curves for siMock (□) and siPARP1 (○) injected mouse groups (four mice and eight implantation sites for each group). Bars, SE. **B.** Staining of tumor sections with anti-PARP1 antibody confirmed the sustained down-regulation of PARP1 in tumor cells after subcutaneous growth in mice. PARP1 staining (*red*), nuclear staining [4′,6-diamidino-2-phenylindole (DAPI; *blue*)], and merged image (*merge*) indicated presence of PARP1 in the nuclei of wild-type (*wt*) tumors. **C.** Representative pictures of tumors stained by H&E indicating an enhanced presence of necrotic tumor areas in siPARP1 tumors. **D.** Quantification of necrotic lesions. All tumors were sequentially sectioned and areas from three different depths of tumor were H&E stained and evaluated. Number of necrotic lesions in each tumor type is presented in a group of mice ($P < 0.001$). **E.** Staining of tumor sections with anti–glucose-transporter (GLUT-1) antibody (*red*) and 4′,6-diamidino-2-phenylindole (*blue*) reveals down-regulation of GLUT-1 expression in siPARP1 tumors.

Results

Down-Regulation of PARP1 Induces Areas of Necrosis in Tumors

To test the role of PARP1 in tumor progression, we stably transduced cell line K562 with a construct expressing siPARP1 or the corresponding mock control using a scrambled sequence (siMock). Down-regulation of PARP1 was analyzed by immunoblot of cell lysates (see Supplementary Fig. S1A). The established siMock and siPARP1 cells were subcutaneously injected in both flanks of athymic nude mice. First signs of tumor formation appeared as early as 1 week postinjection for both cell groups. The formation of tumors was obvious 2 weeks after implantation. Tumor growth was similar in both mouse groups, irrespective of PARP1 down-regulation (Fig. 1A). To confirm that PARP1 was still down-regulated in these tumors, we stained the sections with anti-PARP1 antibody (Fig. 1B). Whereas siMock tumors were positive for PARP1, no signal was visible in siPARP1 tumors. Histologic evaluation of tumors from siPARP1 cells showed larger areas of necrosis (areas with no nuclear-blue staining) compared with their controls (Fig. 1C). Quantification of necrotic areas revealed that the incidence (number of lesions) and size of lesions were increased in tumors expressing siPARP1 (12-22 lesions per tumor in siPARP1 tumors versus 2-9 lesions in siMock tumors; Fig. 1D and data not shown). The areas of cell death with histologic necrotic features were further analyzed by detection of glucose transporter GLUT-1, which is known to be up-regulated in the hypoxic areas of tumors (24). Whereas siMock tumors showed almost homogenous presence of GLUT-1, large areas without GLUT-1 staining were observed in siPARP1 tumors (Fig. 1E). Immunohistochemical analysis with the endothelial marker CD31 revealed a significantly decreased blood vessel density in the siPARP1 tumors (Fig. 2). The reduced vascularization corresponded well with the increased number of necrotic areas observed in siPARP1 tumors, indicating limited blood supply. Repeated experiments with mouse colon carcinoma cell line MC38 injected in a syngeneic mouse model (C57BL/J6) provided the same pattern of enhanced necrosis in the absence of PARP1 (data not shown). These data indicate that the constitutive down-regulation of PARP1 by siRNA did not affect tumor growth within the limits allowed by animal protocol, but caused larger areas of cell death, which in turn could affect tumor progression. Together with the reduced levels of GLUT-1 in siPARP1 tumors, those results suggested the involvement of PARP1 in HIF-dependent gene expression.

HIF-Dependent Gene Expression Is Impaired in siPARP1-Expressing K562 Cells

To test whether PARP1 influences HIF-dependent gene expression, siMock- and siPARP1-expressing K562 cells were treated with ciclopirox olamine (25), a hypoxiamimetic drug, and the expression of HIF-dependent genes was assessed by transient transfection of a reporter gene under the control of hypoxia response element (Fig. 3A). The experiments revealed that ciclopirox olamine–induced expression levels of the reporter gene were dependent on HIF and severely reduced in siPARP1-expressing K562 cells. Transfection of siPARP1-expressing K562 cells with a nondegradable PARP1 restored the observed HIF-1–dependent gene expression (Fig. 3A), confirming that PARP1 is required for HIF-1–dependent gene expression.

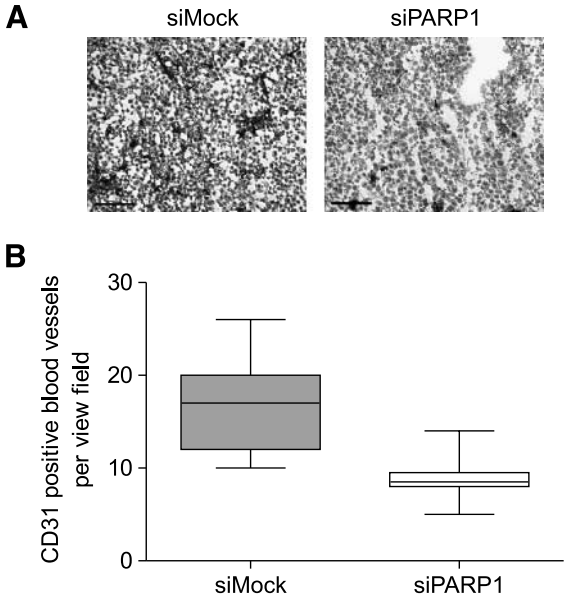

FIGURE 2. Reduced vascularization of K562 tumors is associated with the down-regulation of PARP1. **A.** Tumor vasculature was evaluated by immunohistochemical staining with CD31 antibody. Representative pictures show a higher number of blood vessels in siMock tumors compared with scarcely vascularized siPARP1 tumors. Bar, 100 μm. **B.** Vascular density based on CD31 staining is quantified for both tumors. The decreased number of blood vessels in siPARP1 tumors was found statistically significant by Student's *t* test ($P < 0.01$).

HIF-Dependent Gene Expression Is Impaired in Primary PARP1−/− MLF

To further investigate the contribution of PARP1 in HIF-dependent gene expression, primary $PARP1^{+/+}$ and $PARP1^{-/-}$ MLF were exposed to different concentrations of ciclopirox olamine or hypoxia as indicated, and the expression of HIF-dependent genes was assessed by reporter gene analysis as described above (Fig. 3B and C). Ciclopirox olamine–induced expression of the reporter gene was again severely reduced in $PARP1^{-/-}$ cells when compared with wild-type MLF (Fig. 3B). The experiments under hypoxic conditions (1% O_2) confirmed that PARP1 is important for HIF-1–dependent gene expression (Fig. 3C).

The Enzymatic Activity of PARP1 Is Required for Full HIF-Dependent Transcription

To explore whether the enzymatic activity was important for HIF-dependent gene expression, cells were treated with the PARP inhibitor DAM-TIQ-A. Efficacy of the inhibitor was tested in K562 cells treated with H_2O_2. DAM-TIQ-A was shown to efficiently suppress the H_2O_2-induced formation of poly(ADP-ribose) (Supplementary Fig. S1B). siMock- and siPARP1-expressing K562 cells were then treated with DAM-TIQ-A and subsequently incubated under hypoxic conditions (1% O_2). RNA levels of HIF-1 target gene *CAIX* were

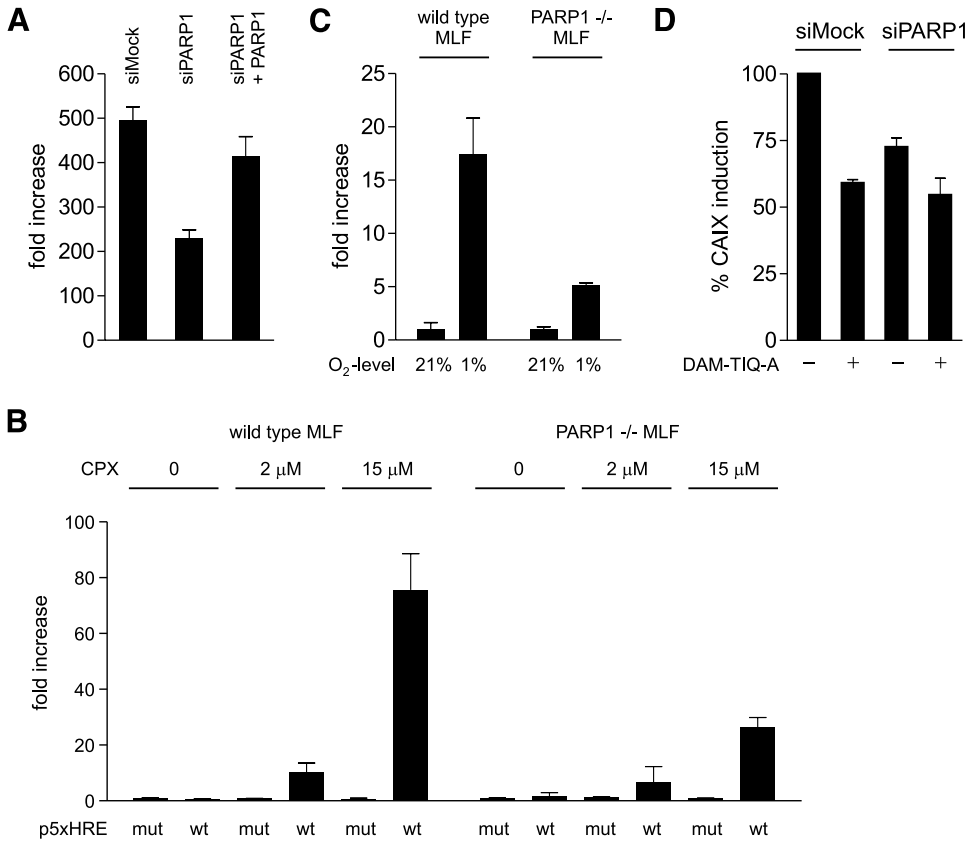

FIGURE 3. PARP1 is required for HIF-1–dependent gene expression. **A.** Transient reporter assay in cell lines K562 siMock, siPARP1, and siPARP1 complemented with wild-type PARP1. Cells were transfected with either the hypoxia reporter (*Epo*) or the mutated control (*mut*) and treated with 15 μmol/L ciclopirox olamine (*CPX*) for 10 h. Cells were harvested and hypoxia-dependent gene expression was determined. The depicted fold increase is calculated as the ratio of normalized luciferase activity of the Epo reporter over the mut reporter. Bars, range of two replicates. **B.** $PARP1^{+/+}$ and $PARP1^{-/-}$ MLF were transfected with either the hypoxia reporter or the mutated control and treated with the indicated amount of ciclopirox olamine for 12 h. Cells were harvested and hypoxia-dependent gene expression was determined. The depicted fold increase is determined by normalization of the relative luciferase activity of the reporter plasmids to that of an internal control (RSV-βGal). The ratios for untreated mutant reporter in both cell lines were arbitrarily set to 1. Bars, range of two replicates. **C.** Cells were prepared as in **B** but kept in either normoxic (21%) or hypoxic (1%) condition for 9 h after transfection. The depicted fold increase is calculated as the ratio of normalized luciferase activity of the Epo reporter over the mut reporter. Bars, SD of three replicates. **D.** Quantitative reverse transcription-PCR analysis of CAIX expression. siMock- and siPARP1-expressing K562 were treated with DAM-TIQ-A as indicated and incubated in normoxic (21% O_2) or hypoxic (1% O_2) conditions. Induction is defined as the ratio of hypoxic over normoxic CAIX levels. The induction of untreated siMock cells was arbitrarily set to 100%. Bars, SD of four replicates.

measured by quantitative real-time PCR (Fig. 3D). Inhibition of PARP1 in siMock cells also resulted in a reduction comparable to the level obtained by knockdown of PARP1. Treatment of siPARP1-expressing cells with inhibitor did not further reduce *CAIX* expression levels.

Normal HIF-1 Signaling in PARP1-Deficient Cells

To investigate the molecular mechanism of PARP1 regulation of HIF-1–dependent gene expression, we analyzed whether the activation of the HIF-1 signaling pathway is overall affected in PARP1-deficient cells on stimulation with ciclopirox olamine or hypoxia. Immunoblot analysis of nuclear extracts from siMock- and siPARP1-expressing K562 revealed that HIF-1α was induced to equal levels after stimulation with ciclopirox olamine (Fig. 4A). Additionally, these experiments showed that there is equivalent nuclear stabilization of HIF-1α in the tested cells. Repeated experiment with $PARP1^{+/+}$ and $PARP1^{-/-}$ MLF provided the same conclusions (data not shown). DNA binding activity of HIF-1 on naked templates was tested by electrophoretic mobility shift assay studies with MLF extracts and DNA oligonucleotides corresponding to the hypoxia response element sites of the erythropoietin *EPO* promoter. PARP1 deficiency did not influence HIF-1 binding to the template (Fig. 4B). Addition of an anti–HIF-1α antibody induced a supershift, confirming that HIF-1 was indeed present in the observed complex (Fig. 4C).

PARP1 Forms a Complex with HIF-1 and Binds Directly to HIF-1 In vitro

HIF-1 and p300/cyclic AMP-responsive element binding protein–binding protein were shown to form a ternary complex and to function synergistically to enhance the activity of nuclear receptors (26). Thus, PARP1 might also synergistically coactivate HIF-1–mediated transactivation. To directly test whether PARP1 physically interacts with HIF-1, we immunoprecipitated PARP1 from HeLa whole-cell extracts after

stimulation with ciclopirox olamine and tested for the presence of HIF-1α by immunoblot analysis. HIF-1α formed a complex with PARP1 (Fig. 5A), which was not mediated by DNA because the presence of ethidium bromide did not affect complex formation (data not shown). To further confirm a direct protein-protein interaction, we bound recombinant purified full-length glutathione *S*-transferase (GST)-PARP1 to glutathione beads followed by incubation with *in vitro* translated HIF-1α subunit, and bound proteins were resolved by SDS-PAGE. PARP1 bound directly to the HIF-1α subunit (Fig. 5B). Similar binding was observed for HIF-2α but not for HIF-3α, HIF-1β, or HIF-2β subunits (Supplementary Fig. S1C and data not shown).

Expression of PARP1 Increases HIF-1α–Dependent Transactivation in PARP1$^{-/-}$ Cells

To further investigate the functional relevance of the PARP1-HIF-1α interaction, primary PARP1$^{+/+}$ and PARP1$^{-/-}$ MLF were transfected with an expression plasmid for HIF-1α, and the expression of HIF-1α–dependent genes was assessed by reporter gene analysis as described above (Fig. 3). Transcriptional activation by HIF-1α expression was again severely reduced in PARP1$^{-/-}$ cells when compared with wild-type MLF (Fig. 5C). The same experiment using a plasmid with a mutated promoter confirmed that the observed gene expression is indeed HIF-1α specific. Assuming that the reduced luciferase levels in PARP1$^{-/-}$ MLF (Figs. 3 and 5C) are indeed due to the absence of PARP1, one expects that complementation of PARP1$^{-/-}$ cells with the wild-type *PARP1* gene restores HIF-1–dependent gene expression. HIF-dependent reporter plasmids (wild-type and mutant) were therefore cotransfected in PARP1$^{-/-}$ MLF with expression vectors for HIF-1α and PARP1. Overexpression of PARP1 or HIF-1α alone could not, or modestly, activate HIF-dependent gene expression; however, coexpression of HIF-1α with PARP1 synergistically activated HIF-1α–dependent transcriptional activation (Fig. 5D). In the same set of experiments, we did not observe transcriptional coactivation of HIF-2α by PARP1, suggesting that HIF-2α might be regulated differentially by PARP1. The regained ability of PARP1$^{-/-}$ MLF to activate genes in a HIF-1α–dependent manner on reintroduction of PARP1 provides convincing functional evidence that PARP1 acts as a classic transcriptional coactivator of HIF-1–dependent gene expression.

Discussion

HIF-1–regulated genes have been implicated in the promotion of tumor progression and metastasis by enhancing angiogenesis, cell proliferation, and the resistance to apoptotic cell death (27). In this study, we find that PARP1 is a regulator of HIF-1–dependent xenograft tumor progression. Mechanistically, we identified PARP1 as a transcriptional coactivator of HIF-1–induced gene expression and we determined a novel process of HIF-1 regulation under hypoxia that acts through PARP1 by regulating target gene expression of critical HIF-1 genes such as *GLUT-1, CAIX, EPO*, and *VEGF*.

The knockout strategy has revealed an important role of PARP1 in cell death after myocardial or brain ischemia-reperfusion injury (28, 29). Significant protection against oxidant-induced tissue damage can also be achieved with

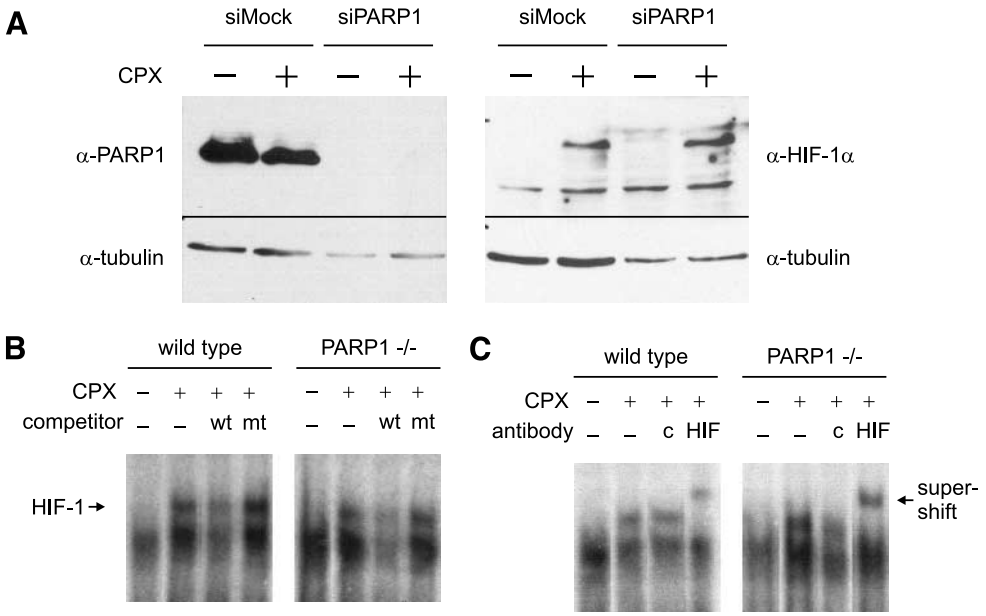

FIGURE 4. HIF-1 stabilization and DNA binding activity are not affected in PARP1$^{-/-}$ cells. **A.** K562 siMock and siPARP1 cell lines were treated with 15 μmol/L ciclopirox olamine for 14 h. Nuclear proteins were then extracted and resolved by SDS-PAGE followed by immunoblot analysis with anti-PARP1 and anti–HIF-1α antibodies. Anti-tubulin was used as a loading control. **B.** Autoradiographs of electrophoretic mobility shift assay for ^{32}P-labeled oligonucleotides containing a HIF-1 binding site. Nuclear extracts of PARP1$^{+/+}$ and PARP1$^{-/-}$ MLF treated with 10 μmol/L ciclopirox olamine for 6 h were used in the assay. Competition for binding was done with unlabeled wild-type and binding mutant oligonucleotides. **C.** Confirmation of HIF-1 binding was effected by adding anti–HIF-1α (*HIF*) or control IgG (*c*) antibodies for a supershift.

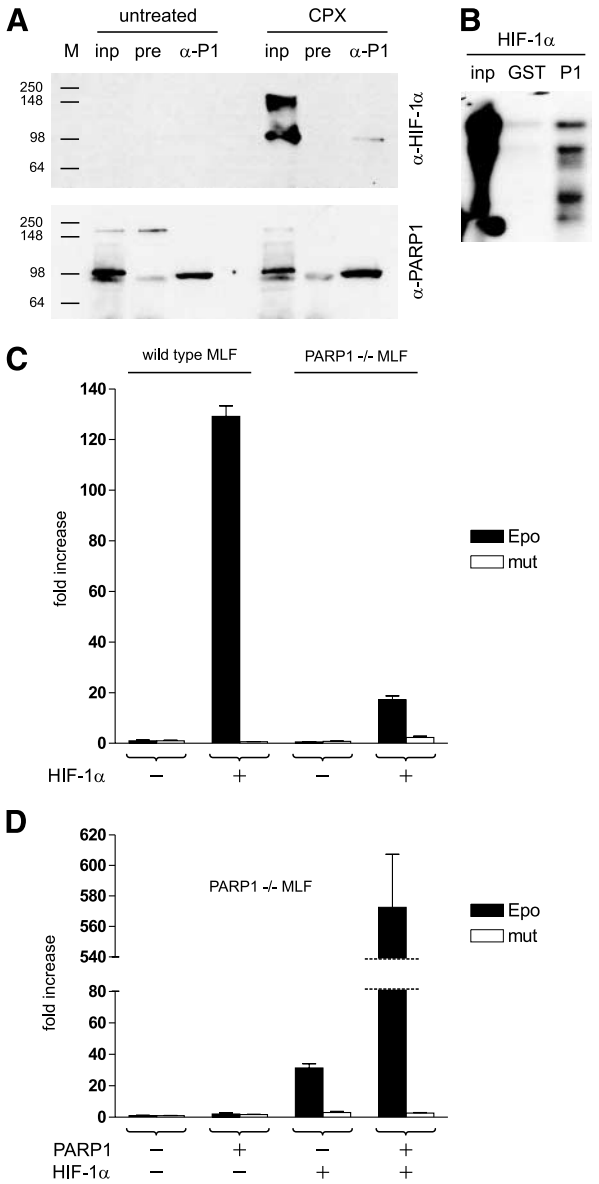

FIGURE 5. PARP1 directly interacts with HIF-1α and synergistically activates HIF-1α–dependent gene expression. **A.** Immunoprecipitation of HIF-1α with anti-PARP1 antibody serum (*α-P1*) and preimmune serum (*pre*) as a control. Total extracts of HeLa cells treated with 15 μmol/L ciclopirox olamine for 16 h were used. Input lane (*inp*) was loaded with 10% the amount of extract used for the immunoprecipitation. The sizes of protein marker bands (*M*) are indicated in kilodaltons. **B.** Autoradiography of *in vitro* translated HIF-1α pulled down with GST-PARP1 (*P1*) or GST alone (*GST*). Input lane was loaded with 1% the amount of translation product used for the pull-down. **C** and **D.** PARP1$^{-/-}$ MLF were transfected for 24 h by polyethylenimine with either the hypoxia reporter or the mutated control together with PARP1 or HIF-1α, as indicated. Cells were harvested and hypoxia-dependent gene expression was determined. The depicted fold increase is determined by normalization of the relative luciferase activity of the reporter plasmids to the protein concentration of each sample. The ratios for untreated mutant reporter in both cell lines were arbitrarily set to 1. Bars, SD of three replicates.

pharmacologic PARP1 inhibitors (30). The most obvious explanation for this observation is that on reperfusion, oxygen-derived free radicals, NO, and peroxynitrite induce DNA breaks, which in turn overactivate PARP1 (31). This excessive activation leads to intracellular NAD and ATP depletion resulting in mitochondrial free radical generation and necrosis (32, 33). Whether these observed effects are due to anoxia or reduced nutritional supply or both (e.g., ischemia) is currently not clear. In our tumor model, we found PARP1 to promote cell survival rather than cell death. The observed increase in necrotic areas in tumors grown from PARP1-deficient cells was also accompanied by a decrease in vascularization of tumors. These findings are in agreement with earlier observation that inhibition of PARP1 decreases angiogenesis in an *in vitro* model (34). We therefore propose that insufficient oxygen and nutrient supply due to impaired HIF-1 activation is the cause for the increased necrosis during tumor progression, independent of radical oxygen species formation. Experiments by Tong et al. (35) provided evidence that in a p53$^{-/-}$ background, PARP1 is important as cofactor for suppression of tumorigenesis in certain tissues. Conde et al. (36) reported that injection of ras-transformed PARP1$^{-/-}$ p53$^{-/-}$ cells failed to generate any visible tumor. We did not observe an influence of PARP1 protein on tumor latency and cell proliferation using K562 or MC38 cells (data not shown). This could be due to the fact that both cell lines are expressing functional p53. Furthermore, our results indicate that in a p53-proficient background, PARP1 acts as promoter of tumor survival as shown by the increase of necrosis in tumors formed by siPARP1-expressing K562 and MC38 cells. Obviously, the tissue expressing PARP1 around the tumors was not able to overcome the lack of PARP1 in the tumor cells.

Our gene expression studies suggest a transcriptional coactivator role for PARP1 in HIF-1–dependent gene expression because in the absence of PARP1, impaired expression of HIF-1–dependent genes was found when mouse fibroblasts and K562 cells were exposed to ciclopirox olamine or hypoxia. Interestingly, the protein levels of HIF-1 were not reduced in treated cells, indicating that the signaling pathway and turnover rate of HIF-1 per se were not affected. Moreover, the DNA binding activity of HIF-1 assayed *in vitro* on naked nonchromatinized templates was not impaired in nuclear extracts from PARP1$^{-/-}$ fibroblasts, suggesting that HIF-1 does not require PARP1 for binding to its hypoxia response element.

Complementation experiments of PARP1$^{-/-}$ cells with a PARP1 expression plasmid confirmed that PARP1 is required when HIF-dependent gene expression is activated by HIF-1α overexpression. We detected endogenous PARP1 in a complex with HIF-1. Moreover, PARP1 directly bound to the HIF-1α subunit *in vitro*. Together these observations implicate that PARP1 is playing a critical and central role downstream of the HIF signaling pathway and strengthens the involvement of PARP1 as transcriptional coactivator. It is of great interest to elucidate the relationship of PARP1 to other HIF-1–associated coactivators with regard to their relative contribution to HIF-1–dependent gene activation, in relation to different promoters and stimuli. Chromatin immunoprecipitation experiments could be used to study the presence of PARP1 at promoters of HIF-1–dependent genes. Unfortunately, such experiments are made difficult by the lack of suitable antibodies and the high affinity of PARP1 for DNA strand breaks in general.

The requirement of PARP1 enzymatic activity for HIF-1–dependent transcription is shown by experiments including PARP inhibitors. As such, expression of CAIX was reduced in the presence of the PARP inhibitor DAM-TIQ-A. Thus, for at least a subset of PARP1-dependent HIF-1 target genes, the enzymatic activity of PARP1 is necessary for full activation. Whether only PARP1 is poly(ADP-ribosylated) or also other factors, such as histones or even HIF-1α, has to be further elucidated. Two groups recently reported that lack of homologous recombination by BRCA1 and BRCA2 dysfunction sensitized tumor cells to the inhibition of PARP enzymatic activity (37, 38). Others provided evidence that the enzymatic activity of PARP1 can also be induced by D-myo-inositol-1,4,5-triphosphate in a DNA damage–independent mechanism (39). Whether the observed effect of PARP inhibitor is due to the PARP1 activation induced by damaged DNA or other stimuli has to be further investigated. PARP inhibitors were also reported to possess free radical scavenging properties (40). Because hypoxia could potentially induce local oxidative stress, it is tempting to speculate that PARP inhibitors with antioxidative potency contribute indirectly and nonspecifically to decreased HIF-1–dependent transcriptional activity by reduction of free radicals. We cannot exclude that the observed effects are due to the off-targeting activity of PARP inhibitors (41, 42). The residual activation of gene expression in the presence of PARP inhibitor might be explained by a process that is independent from PARP1. The small observed difference between siRNA knockdown and pharmacologic inhibition, on the other hand, could be due to incomplete knockdown of PARP1 or the contribution of other PARP family members.

Martin-Oliva et al. recently provided evidence that the number of 7,12-dimethylbenz(*a*)anthracene– and 12-*O*-tetradecanoylphorbol-13-acetate–induced skin tumors is reduced in mice treated with PARP inhibitor, suggesting that PARP1 is important for tumor initiation. They suggested that the transcriptional activity of HIF-1 was compromised by PARP inhibition or, in immortalized PARP1-deficient cells, on stimulation with 12-*O*-tetradecanoylphorbol-13-acetate or desferoxamine (an iron chelator and activator of HIF; ref. 23). In contrast, our studies provide evidence that PARP1 is important for the progression of tumors formed by transformed cell lines and that wild-type mice expressing PARP1 cannot compensate for the loss of PARP1 in cancer cells. One possible explanation for the observed discrepancies is the differences in examined tumor types. Furthermore, our studies with primary PARP1$^{-/-}$ MLF and siPARP1-expressing cells under hypoxic conditions or treated with a hypoxiamimetic drug revealed that HIF-1 signaling (HIF-1 protein stability and DNA binding) per se is not affected by PARP1.

By integrating our data with previous findings, we propose a model by which HIF-1 regulation under hypoxic conditions occurs additionally through PARP1 as transcriptional coactivator. The presence of PARP1 stimulates HIF-1–dependent gene expression and thus allows induction of genes involved in neoangiogenesis and cell survival. From a therapeutic point of view, inhibition of PARP1 enzymatic activity could be an effective target in conditions of tissue ischemia because tumors lacking PARP1 show increased necrosis on ischemic insult. Thus, well-tolerated PARP1 inhibitors that are known to inhibit PARP1 enzymatic activity (38) could be beneficial in the treatment of HIF-1–dependent tumor progression.

Materials and Methods

Reagents and Antibodies

Ciclopirox olamine was purchased from Sigma and dissolved in methanol before diluting to final concentration in supplemented media. Radiochemicals were obtained from Amersham Biosciences. The anti-PARP1 antibody is described in ref. 43, and the anti–HIF-1α antibody in ref. 44. Antimouse IgG was purchased from Santa Cruz Biotechnology. Anti–GLUT-1 antibody was purchased from Abcam, Inc. Anti-PAR antibody was a kind gift of Guy Poirier.

Mouse Strains and Experiments

All animal procedures were done in accordance with the regulations of the Cantonal Veterinary Authority of Zurich in accordance with the Swiss laws on animal protection.

For the tumor growth studies, athymic nude mice (HsdCpb:NMRI-Foxn1^{nu}) were obtained from Harlan Laboratories. Eight-week-old females were injected subcutaneously with 8×10^6 K562 chronic myelogenous leukemia cells, which were either stably transfected with siRNA against PARP1 (siPARP1 tumors) or transfected with mock siRNA (siMock tumors). Tumor growth was checked regularly and mice were terminated when the size of tumor was reaching approved limits.

Histology

Dissected tumors were macroscopically evaluated, cut in half, and frozen in embedding medium (Tissue-Tek O.C.T. compound, Sakura, USA, Inc.). Paraformaldehyde-fixed cryosections (8 μm) were blocked in 0.5% bovine serum albumin and incubated with either polyclonal rabbit anti-PARP1 or rabbit anti-GLUT-1 (Abcam) antibody for 1 h at room temperature. Tumors were further incubated with Cy3-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch) followed by nuclear staining with 4′,6-diamidino-2-phenylindole and mounting in Prolong medium (Invitrogen). Formalin-fixed cryosections were stained with H&E and evaluated for the presence of necrosis. The quantification of necrosis was done on the whole area of each tumor in several sections. Blood vessels were stained with CD31 antibody (Becton Dickinson). Specific binding was detected with the Vectrastain ABC Kit with the AEC substrate (Vector Laboratories) followed by hematoxylin counterstaining.

Generation of siRNA Cell Lines

Generation of viruses and transduction of cells were previously described (45). After several rounds of selection, expression level of PARP1 was screened by immunoblot analysis.

Cell Culture, Transient Transfection, and Nuclear Extracts

Mouse PARP1$^{+/+}$ or PARP1$^{-/-}$ MLF cells were isolated from 129S/EV-PARP1$^{+/+}$ and 129S/EV-PARP1$^{-/-}$ mice, both described in ref. 46. Only cell passages 2 to 10 were used for experiments. MLF and HeLa cell lines were grown in DMEM Glutamax-I (Invitrogen) containing 4.5 g/L glucose. K562 cells

were grown in RPMI (Invitrogen). All media were supplemented with 10% FCS (Invitrogen), 50 units/mL penicillin, 50 μg/mL streptomycin (Sigma), and α-naphthylacetic acid (Invitrogen). Cells were grown in 5% CO_2 at 37°C in a humidified incubator. MLF and HeLa cells were transfected with polyethylenimine or calcium phosphate, and K562 cells with DEAE-dextran, as previously described. Because of differences in transfection efficiencies, an expression plasmid of β-galactosidase (pph-RSV-nt-β-gal; ref. 47) was cotransfected as a transfection efficiency control, and luciferase activities were normalized based on β-galactosidase activity. In case of K562 and MLF, normalization was done with total protein amount measured by Bradford assay. Luciferase activity was measured as described in ref. 48. Nuclear extracts of HeLa and K562 cells were produced as described in ref. 49.

Quantitative Real-time PCR

Total RNA of cells was extracted with the RNA Isolation Mini Kit (Agilent) following the manufacturer's protocol. RNA was then reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was done on a Rotor-Gene 3000 machine (Corbett Research) with TaqMan probes (Applied Biosystems) against two endogenous controls (ribosomal proteins P0 and S14) and against CAIX. Quantitation was done using the Rotor-Gene software version 6.1 (Corbett Research) and the built-in two-standard curve method.

Plasmids

The reporter plasmid p5xHREwt was created by cloning one oligonucleotide containing a HIF-1 binding site from the VEGF promoter (5′-AGCTTGATATCGGATCCGCATACGTGGGCTCCAACAGGTCCTCTTCCCTCCCAGTCACTGACTAACCCT-3′; binding site underlined) and two oligonucleotides containing two binding sites from the EPO promoter (5′-AGCTTGGATCCGGCCCTACGTGCTGCCTCGCATGGGCCCTACGTGCTGCCTCGCATGGCCC-3′; binding sites underlined) into the multiple cloning site of pGL3-Basic (Promega). For p5xHREmut, the oligonucleotides were the same except for the binding sites that were changed to 5′-TAAAAGGG-3′ and 5′-TAAAAGCT-3′, respectively. Expression vectors CMV-HIF-1α, CMV-HIF-2α, and CMV-HIF-3α used for *in vitro* transcription were constructed by PCR amplification of the open reading frame of I.M.A.G.E. cDNA clones #3842146, #6305604, and #6250259, respectively. PCR products were then ligated into pphCMV-T7-km-3 described in ref. 50.

shRNA coding sequences (available on request) were cloned into pSUPER vector by using *Bgl*II and *Hin*dIII cloning sites. *Bam*HI/*Sal*I fragment containing HI promoter upstream of shRNA coding sequence was subcloned into pRDI292 vector (51). The envelope plasmid pMD.G and the packaging plasmid pCMV-ΔR8.91 have previously been described (45).

In vitro *Transcription/Translation and GST Pull-Down Assays*

GST and GST-PARP1 were expressed in *Escherichia coli*. *In vitro* transcription/translation reactions were carried out with the TNT T7 Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's protocol. GST pull-down assays were done with bacterial extracts, glutathione-Sepharose beads (Amersham Pharmacia Biotech), and the radiolabeled product in the presence of 80 mmol/L NaCl as described in ref. 52.

Immunoprecipitation and Immunoblot

For immunoprecipitation, 1 mg of HeLa nuclear extract was incubated with anti-PARP1 serum or preimmune serum in immunoprecipitation buffer containing 80 mmol/L NaCl and Protein A-Sepharose beads (Amersham Biosciences). Beads were then extensively washed in the presence of 100 mmol/L NaCl and 0.05% NP40, followed by SDS-PAGE and immunoblot analysis according to the manufacturer's protocol (enhanced chemiluminescence; Pierce).

Electrophoretic Mobility Shift Assay

The electrophoretic mobility shift assay was done as described in ref. 53. Briefly, the oligonucleotides 5′-GCCCTACGTGCTGCCT-3′ and 5′-GCCCTAAAAGCTGCCT-3′ were annealed to their antisense counterparts, labeled with ^{32}P by T4 PNK, and purified in Microspin G-25 columns (Amersham Biosciences). The radiolabeled oligonucleotides were then added to 10 μg of HeLa nuclear extract. After 20 min, samples were loaded and separated on a 4% native acrylamide gel. The gels were then dried for autoradiography. For competition, HeLa nuclear extracts were preincubated for 5 min with unlabeled competitor oligonucleotides for a final ratio of 25:1 of unlabeled over labeled oligonucleotide. For supershift, nuclear extracts were preincubated for 20 min with anti–HIF-1α or with an unrelated antimouse IgG antibody.

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