Misregulation of gene expression in primary fibroblasts lacking poly(ADP-ribose) polymerase

Cynthia M. Simbulan-Rosenthal*, Danith H. Ly†, Dean S. Rosenthal*, Genevieve Konopka*, RuiBai Luo*, Zhao-Qi Wang‡, Peter G. Schultz†, and Mark E. Smulson*§

*Department of Biochemistry and Molecular Biology, Georgetown University School of Medicine, 3900 Reservoir Road NW, Washington, DC 20007; †Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037; and ‡Unit of Gene–Environment Interactions, International Agency for Research on Cancer, 150 Cours Albert Thomas 69008, Lyon, France

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Poly(ADP-ribose) polymerase (PARP) is implicated in the maintenance of genomic integrity, given that inhibition or depletion of this enzyme increases genomic instability in cells exposed to genotoxic agents. We previously showed that immortalized fibroblasts derived from PARP$^{-/-}$ mice exhibit an unstable tetraploid population, and partial chromosomal gains and losses in PARP$^{-/-}$ mice and immortalized fibroblasts are accompanied by changes in the expression of p53, Rb, and c-Jun, as well as other proteins. A tetraploid population has also now been detected in primary fibroblasts derived from PARP$^{-/-}$ mice. Oligonucleotide microarray analysis was applied to characterize more comprehensively the differences in gene expression between asynchronously dividing primary fibroblasts derived from PARP$^{-/-}$ mice and their wild-type littermates. Of the 11,000 genes monitored, 91 differentially expressed genes were identified. The loss of PARP results in down-regulation of the expression of several genes involved in regulation of cell cycle progression or mitosis, DNA replication, or chromosomal processing or assembly. PARP deficiency also up-regulates genes that encode extracellular matrix or cytoskeletal proteins that are implicated in cancer initiation or progression or in normal or premature aging. These results provide insight into the mechanism by which PARP deficiency impairs mitotic function, thereby resulting in the genomic alterations and chromosomal abnormalities as well as in altered expression of genes that may contribute to genomic instability, cancer, and aging.

Inhibition or depletion of poly(ADP-ribose) polymerase (PARP) by chemical inhibitors (1–3) or by expression of dominant negative mutants (4, 5) or antisense RNA (6, 7) promotes genomic instability, as revealed by increased DNA strand breakage, DNA recombination, gene amplification, micronuclei formation, and sister chromatid exchanges (SCE) in cells exposed to genotoxic agents. PARP-deficient cell lines are hypersensitive to such agents, also exhibiting increased SCE (8). These observations implicate PARP as a guardian of the genome that facilitates DNA repair and suppresses DNA recombination. Mice homozygous for a disrupted *PARP* gene (PARP$^{-/-}$ mice), which express no immunodetectable PARP protein (9, 10) and exhibit only 5–10% of the PARP activity of wild-type cells (11, 12), are extremely sensitive to γ irradiation and methylnitrosourea. Primary fibroblasts derived from these animals also show an increased frequency of SCE and micronuclei formation after exposure to genotoxic agents (9, 10, 13), further implicating PARP in the maintenance of genomic integrity. Immortalized cells derived from PARP$^{-/-}$ mice exhibit a reduced growth rate, G_2-M accumulation, and chromosomal instability on exposure to DNA-alkylating agents, presumably as a result of a defect in DNA repair (14). Although telomerase activity is unaltered in PARP$^{-/-}$ cells, PARP$^{-/-}$ mice display telomere shortening compared with wild-type mice (15), suggesting that PARP contributes to regulation of telomere length, an important determinant of genomic stability.

Another marker of genomic instability is the development of tetraploidy or aneuploidy, which is typical of many tumors and is associated with progression to malignancy or metastasis (16). Tetraploidy results when cells exit from mitosis with neither chromosome segregation nor cytokinesis; tetraploid cells are genetically unstable and become aneuploid at subsequent mitoses (17). We recently showed that immortalized fibroblasts derived from PARP$^{-/-}$ mice contain a genomically unstable tetraploid population (18). We further characterized the genetic alterations associated with PARP deficiency by comparative genomic hybridization analysis, which revealed partial gains in chromosomes 4, 5, and 14 and a partial loss of chromosome 14 in PARP$^{-/-}$ mice or immortalized PARP$^{-/-}$ fibroblasts (18). PARP deficiency has also been associated with an increased frequency of chromosome fusions and aneuploidy (15). Stable transfection of immortalized PARP$^{-/-}$ fibroblasts with PARP cDNA appeared to confer genomic stability, given that the chromosomal gains and the unstable tetraploid population characteristic of these cells were no longer detected (18). Analysis of some key genes that map to regions of chromosomal gain or loss in PARP$^{-/-}$ mice revealed that expression of the tumor suppressor gene *Rb* and the oncogene *Jun* were altered in PARP$^{-/-}$ cells (18).

To provide further insight into the mechanism by which PARP deficiency affects genomic stability, we have now compared the gene expression profiles of asynchronously dividing primary fibroblasts derived from wild-type and PARP$^{-/-}$ mice with the use of oligonucleotide microarray analysis. The results of this approach were verified for a subset of genes whose expression appeared to be altered by PARP deficiency with the use of reverse transcription–PCR (RT-PCR) and immunoblot analysis. PARP deficiency results in down-regulation of the expression of genes that contribute to regulation of cell cycle progression or mitosis, DNA replication, or chromosome processing or assembly. These genes overlap with those whose expression was recently shown to be altered in association with normal or premature (progeria) aging (19). PARP deficiency can therefore cause misregulation of the mitotic machinery of dividing cells, potentially leading to genomic alterations, such as formation of unstable tetraploid and aneuploid cells predisposed to chromosome segregation abnormalities, as well as partial chromosomal gains and losses. These mitotic errors may further lead to altered expression of genes that contribute to cancer and aging. The expression of genes that encode extracellular matrix (ECM) or cytoskeletal proteins implicated in cancer initiation or progression or in normal or premature aging was also up-regulated in PARP$^{-/-}$ cells. Expression of PARP is decreased in association with aging and progeria (19). Furthermore, PARP overexpression and amplification of human chromosome 1q41-q44, which contains the *PARP* gene, are correlated with low genetic instability in human breast carcinomas (20). Our results suggest that

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Abbreviations: PARP, poly(ADP-ribose) polymerase; ECM, extracellular matrix; EST, expressed sequence tag; APP, β-amyloid precursor protein; RT-PCR, reverse transcription–PCR.

§To whom reprint requests should be addressed. E-mail: smulson@bc.georgetown.edu.

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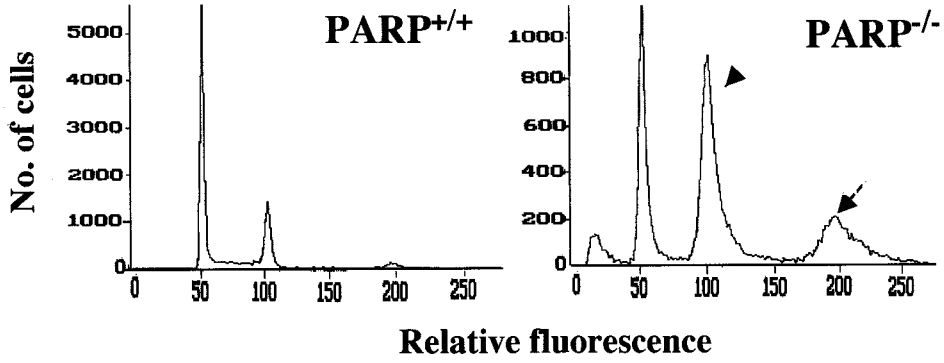

Fig. 1. Flow cytometric analysis of primary wild-type and PARP$^{-/-}$ fibroblasts. Asynchronously dividing cells were grown to ≈60% confluency for 3 days, after which nuclei were prepared and stained with propidium iodide for flow cytometric analysis. In addition to the major peak corresponding to G_0-G_1 (haploid) nuclei apparent for wild-type cells, PARP$^{-/-}$ cells exhibited a larger peak corresponding to G_2-M (diploid) nuclei (arrowhead) as well as a third peak corresponding to tetraploid nuclei (arrow).

reduced PARP expression may be an early factor that contributes to the pathogenesis of cancer and age-related diseases.

Materials and Methods

Cells. Newborn female PARP$^{-/-}$ mice, which were generated by disruption of exon 2 of the *PARP* gene by homologous recombination (9), and their wild-type (PARP$^{+/+}$) littermates (strain 129/Sv × C57BL/6) were used as a source of primary skin fibroblasts. The cells were derived by standard protocols and cultured in DMEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Flow Cytometry. Nuclei were prepared for flow cytometric analysis as described (21). Cells were exposed to trypsin, resuspended in 100 μl of a solution containing 250 mM sucrose, 40 mM sodium citrate (pH 7.6), and 5% DMSO, and subsequently lysed in a solution containing 3.4 mM sodium citrate, 0.1% Nonidet P-40, 1.5 mM spermine tetrahydrochloride, and 0.5 mM Tris·HCl (pH 7.6). Lysates were incubated for 10 min with RNase A (0.1 mg/ml), after which nuclei were stained for 15 min with propidium iodide (0.42 mg/ml), filtered through a 37-μm nylon mesh, and analyzed with a dual-laser flow cytometer (FACScan, Becton Dickinson).

Oligonucleotide Microarray Hybridization. Primary fibroblasts derived from wild-type and PARP$^{-/-}$ mice were grown under identical conditions to ≈60% confluency. Total RNA was then isolated from the cells and subjected to reverse transcription, and the resulting cDNA was subjected to *in vitro* transcription in the presence of biotinylated nucleoside triphosphates. Biotinylated cRNAs were hybridized to Mu11K oligonucleotide arrays (Affymetrix, Santa Clara, CA), which contain probes for 11,000 known mouse genes and expressed sequence tags (ESTs). According to stringent criteria, only those differences in RNA abundance between the two cell types that were reproducible in independent replicates and represented a change of 2-fold or greater were considered further.

RT-PCR. Unique oligonucleotide primer pairs for mouse cyclin B1, cyclin B2, HMG-2, p55^{cdc}, and p21 mRNA were designed and prepared. Total RNA, purified from cell pellets with Trizol Reagent (GIBCO/BRL), was subjected to RT-PCR with a Perkin–Elmer Gene Amp EZ tTh RNA PCR kit. The reaction mix (50 μl) contained 300 μM each of dGTP, dATP, dTTP, and dCTP, 0.45 μM of each primer, 1 μg of total RNA, and rTth DNA polymerase (5 units). RNA was transcribed at 65°C for 40 min, and DNA was amplified by an initial incubation at 95°C for 2 min, followed by 30 cycles of 95°C for 1 min, 60°C for 1.5 min, 65°C for 0.5 min, and a final extension at 70°C for 22 min. The PCR products were then separated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

Immunoblot Analysis. SDS/PAGE and transfer of separated proteins to nitrocelluose membranes were performed according to standard procedures. Membranes were stained with Ponceau S (0.1%) to verify equal loading and transfer of proteins. They were then incubated with antibodies to PARP (1:1,000 dilution; PharMingen), to cyclin A (1:200; Santa Cruz Biotechnology), to cyclin B1 (1:200; Santa Cruz Biotechnology), to p55^{CDC} (1:200; Santa Cruz Biotechnology), to HMG-2 (1:300; PharMingen), to p21 (1:250 dilution; PharMingen), to DNA primase (1:200; NeoMarkers, Union City, CA), to MDM2 (1:200; Santa Cruz Biotechnology), to β-amyloid (1:200; Santa Cruz Biotechnology), or to cyclin D1 (1:200; Santa Cruz Biotechnology). Immune complexes were detected by subsequent incubation with appropriate horseradish peroxidase-conjugated antibodies to mouse or rabbit IgG (1:3000) and enhanced chemiluminescence (Pierce).

Results and Discussion

Multinuclear Morphology and Unstable Tetraploid Population of Primary PARP$^{-/-}$ Fibroblasts. Asynchronously dividing early-passage primary fibroblasts derived from wild-type and PARP$^{-/-}$ mice were grown under identical conditions to ≈60% confluency. Phase-contrast microscopy revealed that, although most PARP$^{-/-}$ cells exhibited a morphology similar to that of the wild-type cells and characteristic of normal fibroblasts, a proportion (≈10%) of the PARP$^{-/-}$ cells was multilobed or contained multiple nuclei. Similar to our results with immortalized PARP$^{-/-}$ fibroblasts (18), flow cytometric analysis of the primary PARP$^{-/-}$ cells revealed the presence of a tetraploid population (Fig. 1). Whereas DNA histograms of wild-type cells (Fig. 1) exhibited a normal pattern characterized by two major peaks of nuclei at G_0-G_1 (haploid) and G_2-M (diploid) phases of the cell cycle, those of primary PARP$^{-/-}$ cells showed, in addition to these two major peaks, a third peak corresponding to tetraploid cells at G_2-M. The primary PARP$^{-/-}$ cells also exhibited a reduced growth rate (data not shown) as well as a higher G_2-M (diploid) peak (Fig. 1) compared with the wild-type cells, again consistent with results obtained with immortalized PARP$^{-/-}$ fibroblasts (9, 14).

Oligonucleotide Microarray Analysis of the Effects of PARP Deficiency on Gene Expression. The transcriptional profiles of primary PARP$^{-/-}$ and wild-type fibroblasts were examined by oligonucleotide microarray hybridization analysis to assess the effects of PARP deficiency on gene expression. Biotinylated cRNA was prepared from cDNA that was synthesized from total RNA of asynchronously dividing cells grown to ≈60% confluence. The labeled cRNA was then allowed to hybridize with high-density oligonucleotide arrays containing probes for 11,000 known mouse genes or ESTs. The gene expression pattern for PARP$^{-/-}$ cells was compared with that for wild-type cells (baseline), and only those changes that were reproducible in independent replicates were considered further. Genes whose expression was up- or down-

Table 1. Differential expression profiles of cell cycle and DNA synthesis/repair genes between wild-type and PARP$^{-/-}$ primary fibroblasts

Accession no.*	Fold Δ†	Gene name
Cell cycle control		
z26580	−2.1	Cyclin A
AA426917	−2.7	Cyclin B1
X66032	−2.1	Cyclin B2
AA000468	−2	p55^{CDC}
AA123463	−2	SET protein
W90992	2.9	Cyclin D2
W08016	2.2	Cyclin D1
z37110	3.5	Cyclin G
M37761	2	Calcyclin
X58876	3.3	MDM2
U09507	2	p21
DNA-RNA synthesis/repair		
d13544	−3.1	DNA primase small subunit
X53068	−2	Proliferating cell nuclear antigen (PCNA)
X14805	−3.2	DNA methyltransferase 1
u08110	−2	RNA1 homolog (Fug1)
X52875	5.5	Homeobox protein Prx2
M31885	3	Helix-loop-helix DNA binding protein regulator (Id)
AA107455	1.8	Elongation factor 2
W40670	4.9	Adenylate kinase isoenzyme 1
Chromosomal processing and assembly		
Z46757	−6.8	HMG-2
M37736	−2.4	Histone H2A.1
z31235	−3.2	pr22 microtubule protein
Protein Processing		
aa545125	−2.5	Ubiquitin conjugating enzyme E2

*GenBank accession no.
†Fold increase or decrease in expression in PARP$^{-/-}$ cells relative to that in wild-type cells.

Table 2. Differential expression profiles of additional genes grouped according to function between wild-type and PARP$^{-/-}$ primary fibroblasts

Accession no.*	Fold Δ†	Gene name
ECM or cytoskeleton		
X51834	−2.3	Osteopontin
M13806	−4.6	Keratin type 1
Aa120653	−2.1	SM-22 α homolog
X07233	−2.3	NCAM-140
x62622	24.8	TIMP 2
z30970	3.0	TIMP 3
X70296	5.3	Protease nexin (PN-1)
AA067929	3.9	APP
D00613	7.0	Matrix Gla protein (MGP)
Z35168	2.9	Collagen IV α 5
AA003383	4.8	Annexin III
Z29532	3.7	Follistatin
W13196	2.1	Caveolin
ET61114	2.1	Ly-6C
AA027619	3.3	OTS-8
Y09257	7.5	NOV protein
Aa152671	10.2	Tropoelastin
X15591	4.7	CTLA-α
U03184	3.3	Cortactin
X53929	2.2	Decorin (PGII)
X66976	2.8	Col8A1 (CDS)
W08049	3.1	Microfibril-associated glycoprotein precursor
AA059700	2.2	β-2 microglobulin
Stress response		
d12907	−2	HSP47
U13705	4.6	Plasma glutathione peroxidase (MUSPGPX)
Growth factors		
112447	−4.5	IGFBP-5
105439	3.2	IGFBP-2
X81582	20	IGFBP-4
X81584	2.1	IGFBP-6
Z22703	3.1	Keratinocyte growth factor (KGF-7)
Immune response		
X16151	−2.3	Early T-lymphocyte activation 1 (Eta-1)
W98255	2	CD81 antigen
m18184	2.1	Lymphocyte differentiation antigen
AA137432	17.6	Nephritis antigen
AA097051	2.7	T-cell activating protein

*GenBank accession no.
†Fold increase or decrease in expression in PARP$^{-/-}$ cells relative to that in wild-type cells.

regulated in the PARP$^{-/-}$ cells by a factor of at least 2 are listed in Tables 1 and 2. Of the 11,000 genes and ESTs monitored, 91 (≈0.8%) showed consistent changes in expression level of at least 2-fold. Differences in the expression of several of these genes were verified both at mRNA level by RT-PCR analysis (Fig. 2*A*) and at the protein level by immunoblot analysis (Figs. 2*B* and 3). In most instances, the differences in protein abundance detected by immunoblot analysis were markedly greater than values of differential expression determined by microarray analysis. The expression of 23 genes and 5 ESTs (30%) was down-regulated (by a factor of 2 to 8), and that of 50 genes and 13 ESTs (69%) was up-regulated (by a factor of 2 to 25) in PARP$^{-/-}$ cells. Of the 91 genes and ESTs that had altered expression as a result of PARP deficiency, approximately 40% can be grouped into either genes whose products are involved in the critical regulation of cell cycle progression (12%) or genes involved in maintenance and remodeling of the cytoskeleton and ECM (26%) (Tables 1 and 2).

Reduced Expression of Genes Important in Cell Cycle Regulation or in DNA Replication or Repair in PARP$^{-/-}$ Cells. A subset of genes (12%) with altered expression because of PARP deficiency encode proteins that are involved in the critical regulation of cell cycle progression and mitosis (Table 1). Genes in this category whose expression was decreased by 2- to 3-fold in PARP$^{-/-}$ cells include those for cyclins A, B1, and B2. The expression of these genes is normally up-regulated at G_2-M and regulates cell cycle progression by associating with and regulating the activities of cyclin-dependent kinases, which, in turn, phosphorylate and regulate the activities of specific target proteins involved in cell cycle progression (22–24). Consistent with results of microarray hybridization, immunoblot analysis revealed a dramatic reduction in expression of cyclins A and B1 in PARP$^{-/-}$ cells compared with that in wild-type cells (Fig. 2*B*).

Cyclin A is required for DNA synthesis during S through G_2 phases and for mitosis (22, 23), whereas cyclin B1 is a key regulator of mitosis and functions as the regulatory subunit of M phase-promoting factor (MPF) (24). Disruption of cyclin A expression results in cell cycle arrest at G_2 (25), which might explain, at least in part, the accumulation of PARP$^{-/-}$ fibroblasts at G_2-M. PARP or poly(ADP-ribosyl)ation is also thought to play a role at or near the S-G_2 transition. Thus, the abundance of PARP mRNA (26), poly(ADP-ribose) (27), and poly(ADP-ribose)-linked acceptor proteins such as histone H1 dimers (28) increases markedly at the S-G_2 transition. Furthermore, chemical inhibitors of PARP also arrest cells at G_2 (29).

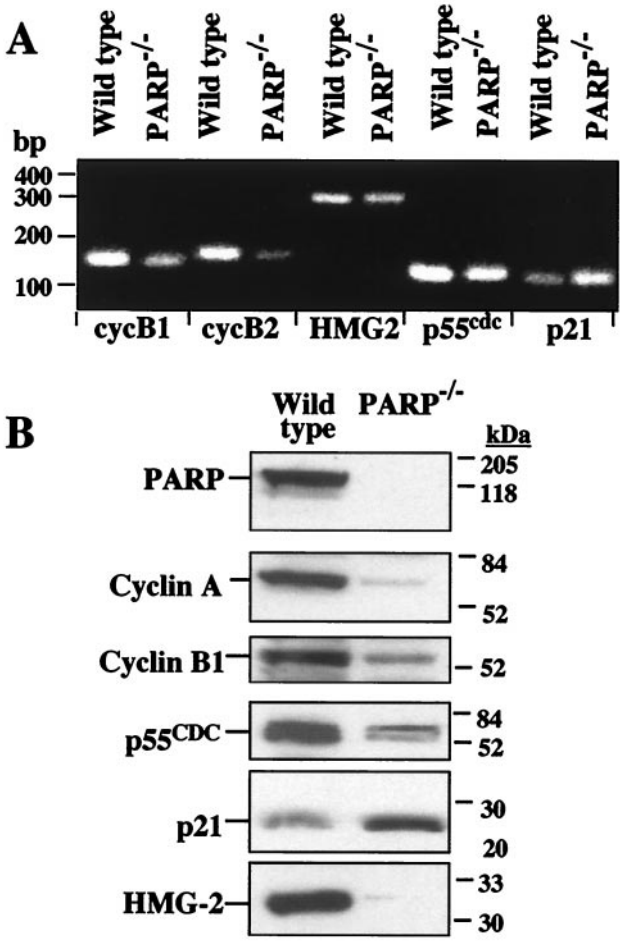

Fig. 2. Down-regulated expression of specific genes involved in regulation of cell cycle progression or chromosome processing and assembly in primary PARP$^{-/-}$ fibroblasts. RNA (1 μg) from primary wild-type and PARP$^{-/-}$ fibroblasts was subjected to RT-PCR analysis with specific primers for mouse cyclin B1, cyclin B2, HMG-2, p55^{cdc}, and p21 mRNA (*A*). Cell extracts of wild-type and PARP$^{-/-}$ primary fibroblasts (30 μg protein) were subjected to immunoblot analysis with antibodies to PARP, to cyclin A, to cyclin B1, to p55^{CDC}, to p21, or to HMG-2 (*B*). The positions of the corresponding immunoreactive proteins as well as those of molecular size standards (in kilodaltons) are indicated.

Consistent with the results of microarray hybridization, a marked decrease in transcript levels in PARP$^{-/-}$ cells, as shown by RT-PCR analysis, correlates with decreased abundance of cyclin B1 protein in these cells (Fig. 2). Inhibition of cyclin B1 transcription also prevents G_2-M transition (30), suggesting that the down-regulation of cyclin B expression apparent in PARP$^{-/-}$ cells also contributes to their accumulation at G_2-M. Furthermore, MPF, which comprises cyclin B and the cyclin-dependent kinase CDC2, regulates mitotic initiation by phosphorylating and activating enzymes implicated in chromatin condensation, nuclear membrane breakdown, and mitosis-specific reorganization of microtubules (24, 31, 32). Regulation of the intracellular abundance of cyclin B1, which is likely the primary regulator of the B-type cyclins (33), controls mitotic initiation, with a threshold level of cyclin B necessary for mitosis to proceed (34). Mitotic misregulation as a result of down-regulation of cyclin B1 expression may thus impair chromosome segregation or cytokinesis and contribute to the development of tetraploidy and aneuploidy in PARP-deficient cells (15, 18).

Expression of p55^{CDC} (CDC20), another cell cycle regulator, was also down-regulated in PARP$^{-/-}$ cells, as shown by microarray (Table 1), RT-PCR (Fig. 2*A*), and immunoblot (Fig. 2*B*) analysis. This protein binds to and activates the anaphase-promoting complex (APC; cyclosome), a multicomponent ubiquitin ligase that mediates the degradation of cyclins, including cyclins A and B, and is essential for chromosome segregation, anaphase initiation, and exit from mitosis (35, 36). The ubiquitin-dependent proteolysis of cyclins is critical for cell cycle progression and promotes its unidirectionality (37). p55^{CDC} is further implicated in the DNA damage and spindle assembly checkpoints, which delay exit from mitosis to prevent chromosome missegregation (38–40). This protein also ensures the precise duplication of centrosomes, which are important in spindle assembly, by coordinating the timely disengagement of mother and daughter centrioles (41). Centrosome amplification increases genetic instability in malignant tumors as a result of dysfunctional aberrant mitotic spindles and consequent chromosome missegregation during mitosis (42, 43). Finally, modulation of the activity of the APC-p55^{CDC} complex contributes to another checkpoint mechanism that blocks sister chromatid separation when chromosomes are misaligned; defects in this mechanism result in aneuploidy in human cells (44). These various functions of p55^{CDC} suggest that the down-regulation of p55^{CDC} apparent in PARP$^{-/-}$ cells may also contribute to the chromosome missegregation that gives rise to tetraploidy and aneuploidy in these cells.

In addition to cell cycle-regulatory genes, the expression of several genes whose products participate in spindle assembly or chromosome segregation or processing was also down-regulated 2- to 7-fold in PARP$^{-/-}$ cells (Table 1); these genes include those for histone H2A.1, HMG-2, and pr22. The down-regulation of HMG-2 expression was confirmed by RT-PCR analysis and was also apparent at the protein level by immunoblot analysis (Fig. 2). The chromatin-associated nuclear protein HMG-2 induces changes in DNA structure that enhance binding of transcription factors, and it promotes the assembly of nucleoprotein complexes that facilitate chromatin function (45). The pr22 protein regulates microtubule dynamics that are important for formation of the mitotic spindle during mitosis, for cellular motility, and for intracellular transport processes (46, 47). PARP$^{-/-}$ cells also exhibit reduced expression of the ubiquitin-conjugating enzyme E2, a component of the ubiquitin–proteasome pathway that mediates selective degradation of key regulatory proteins including cyclins (48). Misregulation of this pathway induced by down-regulation of E2 expression may thus also contribute to impaired timing and coordination of late mitotic events in PARP-deficient cells.

Genes that encode proteins important in synthesis or repair of DNA or RNA constituted ≈10% of the genes and ESTs whose expression was altered in PARP$^{-/-}$ cells (Table 1). These genes include those for the small subunit of DNA primase, proliferating cell nuclear antigen (PCNA), and DNA methyltransferase 1, the expression of each of which was down-regulated ≈2- to 3-fold in the PARP-deficient cells. The expression of the small subunit of DNA primase was also reduced at the protein level in these cells, as shown by immunoblot analysis (Fig. 3*A*). This 49-kDa subunit of DNA primase, which contains the active site, forms a complex with DNA polymerase α and initiates DNA replication by catalyzing the synthesis of small ribonucleotide primers during synthesis of the lagging DNA strand (49). The decreased expression of DNA primase in PARP$^{-/-}$ cells is consistent with our previous results showing down-regulation of both DNA polymerase α and DNA primase expression in PARP-depleted cells expressing PARP antisense RNA during reentry into S phase (50, 51). The reduced expression of these genes important for DNA replication or repair may contribute to the slower growth rate and the proliferation defects apparent in PARP-deficient cells (9, 14).

The genes whose expression is down-regulated in PARP$^{-/-}$ cells overlap with those whose expression is reduced in fibroblasts from older humans or from individuals with progeria, a rare genetic disorder characterized by premature aging (19). The common genes include those that encode proteins important for cell cycle regulation, spindle assembly, or chromosome segre-

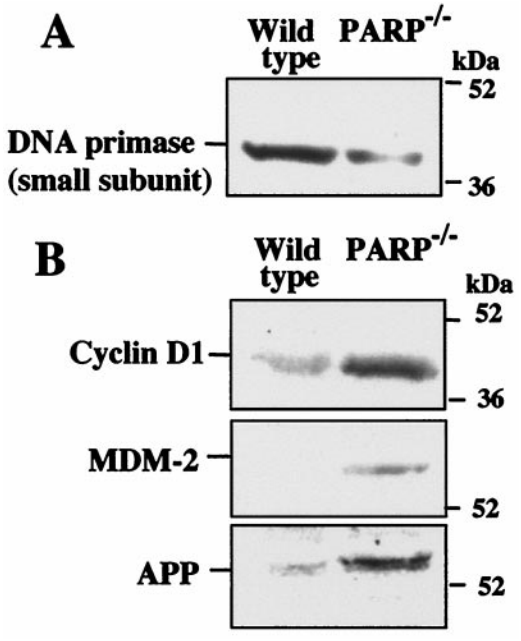

Fig. 3. Altered expression of specific DNA replication/repair genes (*A*) and genes that encode ECM/cytoskeletal proteins implicated in cancer progression and age-related diseases (*B*) in primary PARP$^{-/-}$ fibroblasts. Cell extracts of wild-type and PARP$^{-/-}$ primary fibroblasts (30 μg protein) were subjected to immunoblot analysis with antibodies to the small subunit of DNA primase (*A*) or cyclin D1, MDM2, and β-amyloid (*B*). The positions of these proteins as well as those of molecular size standards (in kilodaltons) are indicated.

gation (such as cyclins A and B, p55^{CDC}, and HMG-2) as well as those whose products contribute to DNA synthesis or repair (such as PCNA). Expression of the PARP gene is also down-regulated in association with normal or premature aging (19), suggesting that this decrease in PARP expression may be an early event that contributes to the aging process.

Increased Expression of Genes Implicated in Cancer Progression or Aging in PARP$^{-/-}$ Cells. Although PARP$^{-/-}$ mice have not been shown to be cancer prone or to exhibit shortened life spans, they are hypersensitive to γ irradiation and genotoxic agents and show increased genomic instability (9, 10, 13). The loss of PARP and the lack of p53 expression in immortalized PARP$^{-/-}$ fibroblasts (18) may increase genomic instability by allowing the survival of cells with gross genetic abnormalities because of an impaired ability both to perform DNA repair (14) and to undergo Fas-mediated apoptosis (52) in cells that have accumulated substantial DNA damage. Fifty known genes and 12 ESTs were up-regulated in primary PARP$^{-/-}$ fibroblasts, and the genes with increased expression include several whose products appear to show correlation with cancer or age-related diseases. Genes that encode ECM components or cytoskeletal proteins constitute $\approx$26% of the genes and ESTs with altered expression in PARP$^{-/-}$ cells. Genes whose expression was increased $\approx$2- to 25-fold include those that encode for annexin III, caveolin, cortactin, and tissue inhibitor of metalloproteinase-2 (TIMP-2) (Table 2).

Cortactin, implicated in intracellular signaling that mediates reorganization of the actin cytoskeleton (53), is amplified or overexpressed in several human cancers, contributing to their metastatic potential by promoting tumor cell migration and invasion (53, 54). Increased expression of caveolin, an integral membrane scaffolding protein (55), is associated with diabetes, Alzheimer's disease, muscular dystrophy, hypercholesterolemia, as well as progression of prostate and breast cancer (55–57). Increased expression of TIMP-2, an endogenous inhibitor of matrix metalloproteinases, is correlated with breast cancer progression (58). Altered expression of binding proteins for insulin-like growth factor (IGFBPs) is implicated in adrenocortical hyperplasia and cancer, with increased expression of IGFBP-2 and IGFBP-4 and reduced expression of IGFBP-5 being associated with these conditions (59); a similar pattern of changes in the expression of these IGFBP genes was detected in PARP$^{-/-}$ cells (Table 2).

Increased expression of cyclins D1 and D2, both of which regulate G_1 phase of the cell cycle, was also apparent in mRNA transcript profiles of PARP$^{-/-}$ cells (Table 1); immunoblot analysis also revealed an increased abundance of cyclin D1 protein in these cells (Fig. 3*B*). Overexpression of cyclin D1 is associated with a variety of human cancers (60–63) and is an early event in breast (61) and colorectal (62) carcinogenesis. Overexpression of this protein promotes cell transformation and tumorigenesis as well as amplification of other genes, whereas cyclin D1 antisense RNA reverts the malignant phenotype of cancer cells (64). Enhanced cyclin G expression, detected as well in the PARP$^{-/-}$ cells (Table 1), also induces arrest of cells at G_2-M (65); its altered expression in PARP$^{-/-}$ cells may thus also contribute to their accumulation at G_2-M. Cyclin G is also overexpressed in human breast and prostate cancer cells (66). The *MDM2* oncogene, frequently amplified in human leukemias (67), is also up-regulated in PARP$^{-/-}$ cells, as revealed by both microarray and immunoblot analysis (Table 1, Fig. 3*B*). Overexpression of this gene increases the tumorigenic potential of cells by binding to and inhibiting the tumor suppressor functions of p53 and Rb, resulting in their ubiquitination and degradation (68–70).

Overexpression of ECM proteins is a hallmark of both the aging phenotype (19) and PARP deficiency (Table 2). The products of several genes whose expression is induced by PARP deficiency are implicated in age-related diseases. For example, expression of the gene for β-amyloid precursor protein (APP) was increased at both the mRNA (Table 2) and protein (Fig. 3*B*) levels in PARP$^{-/-}$ cells. APP is associated with Alzheimer's disease, amyloidosis, Down's syndrome, and other neurodegenerative disorders (71) and shows increased expression during human aging (72). Other genes for ECM proteins that are induced in PARP$^{-/-}$ cells include the genes that encode β_2-microglobulin, which is similar to APP and adopts the fibrillar configuration of β-amyloid in individuals with amyloidosis or carpal tunnel syndrome (73), and protease nexin-I (Table 2). This latter protein is implicated in the pathogenesis of systemic sclerosis, which together with systemic lupus erythrematosus and rheumatoid arthritis belong to a family of age-related connective tissue diseases characterized by ECM accumulation, vascular injury, and autoimmunity (74). Overexpression of the cyclin-dependent kinase inhibitor p21 (Waf1 or Cip1) was also observed in PARP$^{-/-}$ cells at both the mRNA (Table 1) and protein (Fig. 2*B*) levels. Interestingly, p21 induction, like PARP deficiency, also alters the expression of genes for various cell cycle or chromosome segregation proteins, such as cyclin B1 and HMG-2, as well as that of genes for ECM proteins, such as APP, that are associated with age-related diseases (72).

PARP plays dual roles in transcription, depending on the concentration of its substrate NAD and the presence of DNA strand breaks, which are required for enzyme activity. In the absence of NAD, PARP promotes activator-dependent transcription by interacting with RNA polymerase II-associated factors (75); it also binds transcription enhancer factor 1 and the transcription factor AP-2 to increase the transcription of muscle-specific genes and AP-2-mediated transcription, respectively (76, 77). We have also previously shown that transient transfection of an E2F-1 gene promoter-luciferase reporter construct into wild type, PARP$^{-/-}$ and PARP$^{-/-}$ cells stably transfected with PARP cDNA (PARP$^{-/-}$ + PARP) increases both E2F-1 promoter activity and E2F-1 expression in wild-type and PARP$^{-/-}$ (+PARP) cells after reentry

into S-phase, but not in PARP$^{-/-}$ cells (51). Thus, PARP upregulates the activity of the E2F-1 gene promoter during early S phase (51). PARP also functions as a coactivator of Tax-activated transcription *in vivo* given that transient cotransfection of PARP$^{-/-}$ cells with an HTLV reporter construct and expression vectors for Tax and PARP markedly increases Tax-specific transcription (78). In contrast, in the presence of NAD, PARP-dependent silencing of transcription involves poly(ADP-ribosyl)ation of specific transcription factors, which prevents both their binding to the respective DNA consensus sequences and the formation of active transcription complexes (79). Thus, although PARP is not a transcription factor, some of its functions may be mediated by direct or indirect effects on gene expression. The parallels between the genes whose expression is altered by PARP deficiency and those that are known markers of cancer progression or age-related diseases suggest that certain features of cancer or aging phenotypes may result, at least in part, from changes in gene expression induced by PARP loss. The loss of PARP expression may thus be an early event that contributes to both cancer progression and aging. Whether PARP-deficient mice will, as they get older, become more cancer prone, or whether these animals will have shorter life spans compared with their wild-type littermates, remains to be elucidated.

The major intent of this paper has been to first establish basal patterns of gene expression resulting from PARP deficiency, aside from the catalytic activation of PARP on DNA damage, immunostimulation, or other cellular perturbations. PARP-1 is known to physically associate with certain nuclear proteins, and the absence of these interactions in PARP$^{-/-}$ cells may therefore contribute to alterations in gene expression; however, the determination of the relative contribution of physical association compared with catalytic activity, at this time, is well beyond the scope of this paper. The current study of changes in basal gene expression due to the absence of PARP and its activity has provided stimulus for further studies that have recently been initiated; however, they represent major programs in themselves involving the detailed analysis of patterns of gene expression changes that occur after either induction or inhibition of poly(ADP-ribosyl)ation under a variety of conditions. Past studies on the effects of reduced poly(ADP-ribosyl)ation in nuclear processes have relied largely on the use of chemical inhibitors of PARP, which have been useful but are limited because of a lack of specificity and their potentially unrelated effects on other biological processes (80–82). Thus, in recent years, the use of PARP$^{-/-}$ mice, as in the current study, has provided a preferred, more specific approach to gain valuable insight into its pleiotropic functions in cells.

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