EFFECT OF PARTIAL POLY(ADP-RIBOSE) GLYCOHYDROLASE GENE DELETION ON CELLULAR RESPONSES TO GENOTOXIC STRESS

by

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>2ME</td>
<td>2-mercaptoethanol or β-Mercaptoethanol</td>
</tr>
<tr>
<td>3-AB</td>
<td>3-aminobenzamide</td>
</tr>
<tr>
<td>Ado</td>
<td>Adenosine</td>
</tr>
<tr>
<td>ADP-HPD</td>
<td>Adenosine 5’-diphosphate (hydroxymethyl)pyrrolidinediol</td>
</tr>
<tr>
<td>ADP-ribose</td>
<td>Adenosine 5’-diphosphate ribose</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>BAP</td>
<td>Bacterial alkaline phosphatase</td>
</tr>
<tr>
<td>Bz</td>
<td>Benzamide</td>
</tr>
<tr>
<td>BES</td>
<td>N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid</td>
</tr>
<tr>
<td>BER</td>
<td>Base-excision repair</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer susceptibility gene</td>
</tr>
<tr>
<td>BRCT</td>
<td>BRCA1 C-terminus</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>Cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-Diamidino-2-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>DCF-DA</td>
<td>2′, 7′-dichlorofluorescein diacetate</td>
</tr>
<tr>
<td>DHB-B</td>
<td>Dihydroxyboronyl Bio-Rex 70 resin</td>
</tr>
<tr>
<td>DHB-Sepharose</td>
<td>Dihydroxyboronyl Sepharose 4B resin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA protein kinase</td>
</tr>
<tr>
<td>DSBR</td>
<td>Double strand break repair</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol bis(2-aminoethyl ether-N, N', N' tetraacid acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human leukemic cells</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase pair</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M</td>
<td>Molarity</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryo fibroblast</td>
</tr>
<tr>
<td>MNNG</td>
<td>N-Methyl-N’-nitro-N-nitrosoguanidine</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propane sulfonic acid</td>
</tr>
<tr>
<td>mPARG</td>
<td>Murine polyadenosine 5’-diphosphoribose glycohydrolase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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LIST OF ABBREVIATIONS – Continued

MTT 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MVP Major vault protein
MW Molecular weight
NAD Nicotinamide adenine dinucleotide
Nam Nicotinamide
NES Nuclear export signal
NLS Nuclear localization signal
PAR Polymers of ADP-ribose
PARG Polyadenosine diphosphoribose glycohydrolase
PARP Polyadenosine diphosphoribose polymerase
PARP-1−/− PARP-1-null genotype (“knockout”)
PARP-1+/+ PARP-1 wild type genotype
PBS Phosphate-buffered saline
PI Propidium iodide
PVDF Polyvinylidene fluoride
γ-H2AX Phosphorylation of H2AX at serine 139
RAdo Ribosyladenosine
RNA Ribonucleic acid
RNase Ribonuclease
ROS Reactive oxygen species
rpm Revolutions per minute
SDS Sodium dodecyl(lauryl) sulfate
SSBR Single strand break repair
STR Staurosporine
SVPD Snake venom phosphodiesterase
Tankyrase TRF-1 interacting ankyrin-related poly(ADP-ribose)
Taq Thermus aquaticus (DNA polymerase)
TCA Trichloroacetic acid
TE Tris/EDTA buffer
TEMED N,N,N′,N′-Tetramethylethylenediamine
Tris, Trizma Tris (hydroxymethyl)aminomethane
Triton X-100 Octyl phenoxy polyethoxyethanol
UV Ultraviolet
V-PARP Vault poly(ADP-ribose) polymerase
XRCC1 X-ray repair cross complementing-1 protein
ABSTRACT

Polymers of ADP-ribose (PAR) are rapidly synthesized by poly(ADP-ribose) polymerases (PARPs) and rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG) following genotoxic stress. Since PAR metabolism plays an important role in cell fate determination following genotoxic stress, enzymes involved in PAR metabolism potentially represent promising therapeutic targets for modulating diseases of inappropriate cell proliferation or death. PARP-1 has been well validated and several PARP-1 inhibitors are currently being evaluated in clinical trials for cancer and ischemia treatment. In contrast, the biological function of PARG is still poorly understood. Due to low abundance of protein levels in mammalian cells and its unique substrate, PARG potentially represents another attractive target for pathological conditions mentioned above. PARG-\(\Delta 2,3\) cells derived from homozygous PARG-\(\Delta 2,3\) mice with targeted disruption of exons 2 and 3 of the PARG gene are used in this dissertation. The nuclear isoform PARG60 in PARG-\(\Delta 2,3\) cells lacks the putative regulatory domain A compared to the nuclear isoform PARG110 in wild type cells.

We report in this dissertation that PARG-\(\Delta 2,3\) cells accumulate less PAR in spite of more rapid depletion of NAD following treatment with N-methyl-N'-Nitro-N-Nitrosoguanidine (MNNG). The estimation of PARP and PARG activity in intact cells shows increased activity of both enzymes in PARG-\(\Delta 2,3\) cells following MNNG treatment, indicating the important role of domain A in the regulation of PARG and PARP activity under these conditions. Following
MNNG treatment, PARG-Δ2,3 cells show reduced formation of XRCC1 foci, decreased H2AX phosphorylation, decreased DNA break intermediates during repair, and increased cell death. The altered PAR metabolism and defective cellular responses related to DNA repair in PARG-Δ2,3 cells may contribute to increased sensitivity of these cells to MNNG. Studies presented in this dissertation clearly demonstrate the important role of PARG110 in PAR metabolism and cellular responses to genotoxic stress, and thus provide supportive data for the validation of PARG as a promising potential therapeutic target.
CHAPTER 1 : INTRODUCTION

1.1 Overview of polymers of ADP-ribose (PAR) metabolism

Polymers of ADP-ribose (PAR) are rapidly synthesized by poly(ADP-ribose) polymerases (PARPs) and rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG) following genotoxic stress. Although seven genes encoding PARPs have been identified, only PARP1 and PARP2 are immediately activated by DNA strand breaks and implicated in cellular responses to genotoxic stress (Ame et al., 2004). Unlike the growing numbers of genes encoding PARPs, mammalian cells appear to contain only a single gene that encodes multiple PARG isoforms targeting to different cell compartments (Lin et al., 1997) (Meyer et al., 2003) (Meyer-Ficca et al., 2004). Specifically related to this dissertation research, PAR metabolism plays an important role in cell fate determination by facilitating cell recovery following mild to moderate genotoxic stress and mediating cell death following severe genotoxic stress (Jagtap and Szabo, 2005) (Koh et al., 2005) (Figure 1).

1.1.1 Overview of enzymes involved in PAR metabolism

PAR was first detected in an acid-insoluble fraction of a nuclear extract by Chambon et al. (Chambon et al., 1963). This report led to the discovery of poly(ADP-ribose) polymerase-1 (PARP-1), which catalyzes the synthesis of PAR using NAD$^+$ as the sole substrate. PARP-1 cleaves the glycosidic bond of NAD$^+$ and catalyzes initiation, elongation and branching of PAR synthesis (Figure 2) (Burkle, 2001). NAD$^+$ provides ADP-ribose and releases nicotinamide during the reaction (Figure 2). PARP-1 is an abundant nuclear
Figure 1. Role of PAR metabolism in cellular responses to genotoxic stress.
DNA strand breaks activate PARP-1 and PARP-2 which start to synthesis PAR. PARG is also activated to degrade PAR. PAR metabolism facilitates DNA repair following mild genotoxic stress and promotes cell death following severe genotoxic stress.
Figure 2. PARPs synthesize PAR using NAD$^+$ as the substrate. Nicotinamide is released during the reaction.
protein. The report that PARP-1 knockout cells still synthesize PAR following genotoxic stress facilitated the discovery of PARP-2 (Shieh et al., 1998). PARP-2 is also located in the nucleus and accounts for the residual activity of PAR synthesis in PARP-1 knockout cells following genotoxic stress (Ame et al., 1999b). Tankyrase-1, located in telomeres, also has PARP activity (Smith et al., 1998). Since then, four more genes encoding PARPs have been identified and up to 11 more homologous sequences of PARP gene have been found in the human genome (Ame et al., 2004). These 11 homologous sequences theoretically encode distinct proteins from previously identified PARPs in that they contain a variety of adaptor domains besides the PARP catalytic domain (Ame et al., 2004). Among the multiple PARPs, PARP-1 and PARP-2 are immediately activated by DNA strand breaks. PARP-1, the first identified and best characterized member in PARP family, accounts for the majority of PAR synthesis following genotoxic stress.

PAR synthesized in response to DNA breaks is rapidly degraded by poly (ADP-ribose) glycohydrolase (PARG) (Figure 3) (Juarez-Salinas et al., 1979). While multiple genes encode PARPs, only a single gene that encodes PARG has been identified in mammals (Meyer et al., 2003). Multiple PARG isoforms derived from this single gene target to different cell compartments (Meyer-Ficca et al., 2004). Another protein with PARG activity in vitro has been recently reported (Oka S, 2006), but its role, if any, in PAR metabolism is unknown. PARG is proposed to cleave only ribose-ribose bonds of PAR (Miwa et al., 1974), but the possibility of removing the proximal unit of ADP-ribose by PARG was also suggested (Desnoyers et al., 1995) (Figure 3).
Figure 3. Degradation of PAR by PARG.
Arrows indicate the cleavage sites. Question mark represents the controversial cleavage site.
Another enzyme, ADP-ribose protein lyase has been proposed to catalyze cleavage of the bond between the acceptor protein and the proximal ADP-ribose residue (Figure 4) (Oka et al., 1984), but this enzyme was also suggested to remove glycation rather than the proximal ADP-ribose from the acceptor protein (Cervantes-Laurean et al., 1993). PAR metabolism and enzymes involved in PAR metabolism are summarized in Figure 4. The biological functions of PARPs, especially PARP-1, have been extensively studied. The role of PARG in cellular responses to genotoxic stress is still poorly understood.

1.1.2 Poly(ADP-ribosyl)ation of nuclear proteins

Most proteins known to be modified by PAR are nuclear proteins. PAR is covalently attached on acceptor proteins mainly via the γ-carboxyl groups of glutamic acid residues and also via aspartic acid and lysine residues (D'Amours et al., 1999) (Burkle, 2005). In the absence of genotoxic stress, the catalytic activity of PARP-1 is low, and the majority of PAR attached on acceptor proteins appears to be shorter than 11 ADP-ribose units (D'Amours et al., 1999). Following occurrence of DNA strand breaks, PARP-1 activity increases many fold, and PAR contains branched long chains composed of hundreds of ADP-ribose units (D'Amours et al., 1999). PARP-1 itself is the main acceptor protein for PAR following DNA damage (Ogata et al., 1981). Many other nuclear proteins have been identified to serve as the substrates for poly(ADP-ribosyl)ation as well, such as histones, DNA topoisomerases, and p53 (D'Amours et al., 1999) (Scovassi et al., 1993) (Wesierska-Gadek et al., 1996b) (Wesierska-Gadek et al., 1996a).
Figure 4. PAR metabolism cycle and the enzymes involved in this cycle.
PARPs synthesize PAR using NAD⁺ as the sole substrate. PARG degrades PAR into ADPR. PARG may remove the proximal ADPR residue as well. An ADPR protein lyase has been proposed to remove the proximal ADPR residue from the acceptor protein. Nicotinamide is abbreviated as NAM.
Besides covalent modification, PAR has been reported to noncovalently interact with a wide variety of nuclear proteins (Burkle, 2005). A 20 amino acid sequence has been proposed to be a PAR-binding sequence motif (Pleschke et al., 2000). Several proteins involved in DNA damage checkpoints and many other proteins contain this putative PAR-binding sequence (Pleschke et al., 2000).

Although the biological consequences of poly(ADP-ribosyl)ation on the specific acceptor proteins are not clear in many cases, the addition of the negative charges by poly(ADP-ribosyl)ation appears to significantly alter the functions or physico-chemical properties of acceptor proteins. Accumulation of negative charges alleviates or even abolishes the interactions between acceptor proteins with other anionic molecules such as the interactions between histones and DNA (Ferro and Olivera, 1982). Poly(ADP-ribosyl)ation may also alter the enzymatic activity of acceptor proteins. A well-understood example is that automodification of PARP-1 abolishes its catalytic activity (Kawaichi et al., 1981).

1.1.3 Methods for PAR quantification in vivo

There were some difficulties to develop sensitive and specific methods for PAR quantification in vivo due to the following reasons. PAR turnover is rapid, and the steady-level of PAR is low in intact cells, which requires the sensitive methods of PAR quantification. In addition, the PAR level cannot be measured directly due to the different size and shape of PAR. PAR usually is digested into the homogenous compound ribosyladenosine. But other more abundant molecules in the nucleus such as DNA and RNA contain adenine-
ring as well, which requires the specific methods of PAR quantification. Despite these difficulties, several methods including a fluorescence method, a radioimmunoassay method and a \(^3\)H-labeling method have been developed, validated and widely applied for PAR quantification in vivo (Jacobson et al., 1984) (Wielckens et al., 1984) (Aboul-Ela et al., 1988).

Although these three methods determine the adenine-ring containing compound derived from PAR digestion by different ways, they have similar key steps. First, boronate resins are used to efficiently isolate PAR from crude cell extracts. Borate readily forms complexes with the vicinal hydroxyl group that every ADP-ribose unit contains in PAR (Alvarez-Gonzalez et al., 1983). Second, PAR is digested by enzymes to yield ribosyladenosine, a structurally unique nucleoside. Ribosyladenosine cannot be generated from other adenine-ring containing compounds such as DNA or RNA.

A fluorescence method quantifies a fluorescent derivative of ADP-ribose after PAR is released from acceptor proteins, isolated using boronate column, and digested by enzymes (Jacobson et al., 1984). A radioimmunoassay method determines the level of 5’-AMP converted from ADP-ribose after PAR is released, isolated and digested (Wielckens et al., 1984). Compared with the fluorescence and the radioimmunoassay method, a \(^3\)H-labeling method has some advantages. The \(^3\)H-labeling method is very sensitive and specific, and quantification of PAR and NAD\(^+\) could be conducted simultaneously from the same cultured cells using this method (Aboul-Ela et al., 1988). After trichloroacetic acid (TCA) extraction, PAR is quantified using the TCA pellet, and NAD is quantified using the TCA supernatant. The ribosyladenosine,
generated from the enzymatic digestion of PAR, is quantified using scintillation counting (Aboul-Ela et al., 1988).

1.2 The Poly(ADP-ribose) polymerase family

1.2.1 PARP-1

PARP-1 (EC2.4.2.30), the first identified and best characterized member in PARP family, is a ubiquitous nuclear protein found in all eukaryotic models except yeast. PARP-1 is also referred as poly(ADP-ribose) synthetase (PARS) or poly(ADP-ribose) transferase (pADPRT) in some earlier publications (Meyer-Ficca et al., 2005). PARP-1, purified from fresh calf thymus, was first reported in 1979 (Ito et al., 1979). Since then, PARP-1 has been purified from other eukaryotic sources such as human placenta (Burtscher et al., 1986). PARP-1 is an abundant nuclear protein with an approximate estimation of 2 million and 200,000 molecules in lymphoblastic cells and Hela cells, respectively (Lautier et al., 1993).

The approximate molecular weight of PARP-1 is 113 kDa. The domain structure of PARP-1 is shown in Figure 5 (Shall and de Murcia, 2000, adapted from this paper). The N-terminal DNA binding domain contains two zinc fingers (FI and FII) and a nuclear localization signal (NLS). One PARP-1 molecule binds two zinc ions, which is essential for the binding ability of PARP-1 to DNA strand breaks (Mazen et al., 1989). The two zinc fingers recognize DNA strand breaks rather than a specific DNA sequence. FII is essential for PARP-1 binding to DNA single strand breaks, while FI is required for PARP-1 binding to DNA double strand breaks.
Figure 5. Domain structure of PARP-1.
NLS, nuclear localization signals; BRCT, BRCA1 C-terminus. Adapted from Shall and de Murcia, 2000.
(de Murcia and Menissier de Murcia, 1994). A caspase-3 cleavage site is also located in this domain (Kaufmann et al., 1993). The C-terminal catalytic domain bears the NAD$^+$ binding site that is highly conserved in different species. A 50 amino acid sequence in the catalytic domain is identical in many vertebrates and is called “the PARP signature sequence”. The central domain is the automodification domain (de Murcia and Menissier de Murcia, 1994). This domain contains more than 20 glutamic acid residues that can be covalently modified by PAR (Althaus et al., 1995). The kinetic analysis of PARP-1 suggests that it functions as a catalytic dimer. Two PARP-1 molecules in the dimer serve as a catalyst and an acceptor substrate simultaneously. Thus the automodification reaction occurs intramolecularly (Mendoza-Alvarez and Alvarez-Gonzalez, 1993). This domain also contains a BRCT motif that serves as an interface for protein-protein interactions (Shall and de Murcia, 2000).

Modulation of PARP-1 activity has greatly facilitated studies of the biological functions of PARP-1. Several ways of modulation of PARP-1 activity have been reported. First, potent and cell-permeable PARP-1 inhibitors have been developed. The first generation of PARP-1 inhibitors are analogues of nicotinamide such as benzamide and 3-aminobenzamide. These compounds have micromolar range of IC$_{50}$ values and they are inexpensive and cell-permeable. These compounds are still widely utilized to inhibit PARP-1 activity in cell models. Much more potent PARP-1 inhibitors have been reported such as AG14361. This compound is more than 1000 times as potent as 3-aminobenzamide (Calabrese et al., 2004). Almost all PARP-1
inhibitors competitively inhibit NAD$^+$ binding (Bryant and Helleday, 2004). Since NAD$^+$ is the substrate for many other PARPs, PARP-1 inhibitors are usually not highly specific for different PARPs. Effects of PARP inhibitors on other classes of NAD consuming enzymes including protein-mono ADP-ribose transferases, sirtuins and cADP-ribose synthases must also be considered. Secondly, PARP-1 knockout mouse models have been established by three independent research groups (Shall and de Murcia, 2000). Thirdly, PARP-1 activity has been inhibited by molecular biological tools. Alexander Burkle’s group inhibited PARP-1 catalytic activity by overexpressing the DNA binding domain of PARP-1 (Kupper et al., 1990). PARP-1 inhibition has also been accomplished by overexpression of antisense PARP-1 mRNA (Simbulan-Rosenthal et al., 1996).

1.2.2 PARP-2

The discovery of PARP-2 was triggered by the fact that PARP-1 knockout animals were able to synthesize PAR following DNA damage (Shieh et al., 1998). A PARP protein found in the plant *Arabidopsis thaliana* led to an effort of searching for a mammalian counterpart which resulted in cDNA cloning of human and mouse PARP-2 (Babiychuk et al., 1998) (Ame et al., 1999b). The molecular weight of PARP-2 is 62 kDa. The catalytic domains of PARP-2 and PARP-1 have 69% similarity (Ame et al., 2004). The DNA binding domain of PARP-2 consists of only 64 amino acids and does not show similarity with that of PARP-1 (Burkle, 2005) (Figure 6). The crystal structure of murine PARP-2 has been resolved in 2004 (Oliver et al., 2004). Comparison of the crystal structure of murine PARP-2 with the known structure of catalytic fragment of
**Figure 6. Domain structures of six members of PARP family.**
NLS, nuclear localization signals; BRCT, BRCA1 C-terminus; VIT, vault protein inter-alpha-trypsin domain; MVP-BD, major vault protein binding domain; HPS, His-Pro-Ser-rich domain; SAM, sterile α-module. Adapted from Burkle A, 2005.
chicken PARP-1 provides a basis for the development of specific inhibitors for different PARP isoforms (Oliver et al., 2004).

Besides PARP-1, PARP-2 is the only other member in PARP family to be activated by DNA strand breaks, suggesting that PARP-2 may also play a role in cellular responses to genotoxic stress. PARP-1 and PARP-2 form homo- and heterodimers (Schreiber et al., 2002). PARP-2 interacts with several proteins involved in the base excision repair pathway such as XRCC1 (X-ray cross complementing factor 1), DNA polymerase beta, and DNA ligase III (Schreiber et al., 2002). These proteins are also known to interact with PARP-1 (Ame et al., 2004). PARP-2 deficient cells display a significant delay in the religation of DNA strand breaks following treatment with the alkylating agent (Schreiber et al., 2002). These data demonstrate that PARP-2 plays an important role in the efficient base excision repair although its capacity of PAR synthesis is lower than PARP-1.

PARP-2 also plays a role in the maintenance of genomic stability and telomere integrity (Diefenbach and Burkle, 2005). PARP-2 knockout embryonic fibroblasts derived from the homozygous knockout mouse show abnormal chromosome segregation following treatment with the alkylating agent (Menissier de Murcia et al., 2003). PARP-2 has been shown to physically bind to TRF2 (telomeric-repeat binding factor 2), a TTAGGG repeat binding protein involved in the telomere protection in mammals (Dantzer et al., 2004). PARP-2 knockout primary cells exhibit increased frequency of chromosome breaks and chromosome ends without detectable TTAGGG repeats (Dantzer et al., 2004). The biological function of PARP-2 appears to
overlap with that of PARP-1. But PARP-2 may also have some functions which are not redundant with PARP-1, since PARP-2 knockout animals have some phenotypes mentioned above not observed in PARP-1 knockout animals (Menissier de Murcia et al., 2003).

1.2.3 PARP-3

Human PARP-3 has an approximate molecular weight of 67 kDa (Augustin et al., 2003). The domain structure of PARP-3 is similar to PARP-2 (Figure 6), featuring a small DNA binding domain composed of only 54 amino acids (Diefenbach and Burkle, 2005). PARP-3 is located in the centrosome as a core component during the entire cell cycle, and preferentially resides in the daughter centriole (Augustin et al., 2003). The DNA binding domain of PARP-3 contains a targeting sequence that is responsible for centrosome localization of PARP-3. Overexpression of PARP-3 in Hela cells interferes the G1/S cell cycle progression although it does not affect centrosomal duplication (Augustin et al., 2003). PARP-1 is also located in the centrosome (Kanai et al., 2003). PARP-3 could form a stable complex with PARP-1 (Diefenbach and Burkle, 2005), suggesting that both PARP-1 and PARP-3 are involved in the regulation of centrosome function.

1.2.4 PARP-4 or Vault PARP (VPARP)

Vaults are barrel-shaped ribonucleoprotein complexes predominantly located in the cytoplasmic compartment. Mammalian vaults consist of multiple copies of three proteins with the molecular weight of 100, 193 and 240 kDa and several untranslated RNA molecules (van Zon et al., 2003). The 100 kDa protein is termed the major vault protein (MVP) since it constitutes majority of
the molecular mass of vaults (Stephen et al., 2001). The 240 kDa protein, identical to the mammalian telomerase-associated protein 1 (TEP1), maintains the overall stability of vaults (van Zon et al., 2003). The 193 kDa protein was identified as a novel PARP and named as vault PARP (VPARP) or PARP-4 since it exhibited PARP activity and poly(ADP-ribosyl)ated MVP and itself within the vaults (Kickhoefer et al., 1999). Although the function of vaults is largely unknown, it has been suggested that they may be involved in intracellular transport, especially nucleo-cytoplasmic transport (Abbondanza et al., 1998). Vaults may also play a role in cellular detoxification processes since the expression levels of vault proteins are often upregulated in many multi-drug-resistant cell lines (Scheffer et al., 2000).

The domain structure of PARP-4 has been proposed (Figure 6). Domain I is a BRCT domain that may be important for protein-protein interactions. Domain II consists of 350 amino acids, and shows 29% similarity with the catalytic domain of PARP-1. Domain III shares similarity with the inter-α-trypsin inhibitor heavy chain–related protein. Domain IV interacts with MVP (Kickhoefer et al., 1999). PARP-4 does not contain a DNA binding domain, and it does not appear to require binding with DNA strand breaks for activation. PARP-4 is not exclusively located in the cytoplasm. It is also located to the mitotic spindle (Kickhoefer et al., 1999). The role of PARP-4 in the nucleus needs further investigation.

1.2.5 PARP-5a or Tankyrase-1

Telomeres are the special DNA structures at the ends of chromosomes. Telomeres typically consist of tandem repetitive sequence rich in GT with a
single-strand overhang stretch that is species specific. The size of the
overhang in telomeres is 50-100 nucleotides in human and mouse (Greider,
1999). The overhang structure is stabilized by the telomere-specific binding
proteins, TRF1 and TRF2 (Chong et al., 1995) (Bilaud et al., 1997). The ends
of telomeres form terminal loops rather than linear molecules (Griffith et al.,
1999). The primary function of telomeres is to protect chromosome ends from
being recognized as DNA strand breaks (Greider, 1999). Telomerase is the
enzyme which synthesizes telomere ends and maintains telomere length
(Meyerson, 1998). Telomere length and telomerase activity have been linked
to multiple human diseases such as cancer and aging (Blasco, 2005).

One protein located in human telomeres shares homology to ankyrins and
to the catalytic domain of PARP-1 (Smith et al., 1998). This protein was
identified using TRF1 as the bait in a yeast two-hybrid system (Bianchi et al.,
1997). It was named tankyrase for TRF1-interacting, ankyrin-related ADP-
ribose polymerase (Smith et al., 1998). The tankyrase reported in 1998 is
known as tankyrase-1 since another tankyrase has been discovered later.
The molecular weight of tankyrase-1 is 142 kDa. The structural features of
tankyrase are shown in Figure 6. The C-terminal domain of tankyrase displays
homology to the catalytic domain of PARP-1, but other domain structures are
different from PARP-1. The N-terminus of tankyrase-1 contains a region
called HPS that consists of homopolymeric units of histidine, proline and
serine. The largest domain of tankyrase is ankyrin-related domain (ANK) that
contains 24 ankyrin repeats, suggesting that tankyrase is also a member of
the ankyrin family. The members in the ankyrin family serve as the adaptors
between membrane proteins and the cytoskeleton (Bennett, 1992). Tankyrase also contains a SAM domain that is a homology to the sterile alpha motif. The SAM domain may promote protein-protein interactions (Smith et al., 1998). Lacking a DNA binding domain, Tankyrase-1 does not require binding to DNA strand breaks for activation. The activity of tankyrase-1 may be regulated by phosphorylation (Chi and Lodish, 2000).

Tankyrase-1 appears to regulate telomere length by poly(ADP-ribosyl)ation of TRF1 (Smith and de Lange, 2000). TRF1 is a negative regulator of telomere length. Long-term overexpression of TRF1 in a telomerase-containing cancer cell line led to a gradual and progressive telomere shortening. This shortening was reversed by the expression of a TRF1 mutant that inhibited endogenous TRF1 binding to telomeres (van Steensel and de Lange, 1997). About ten percent of tankyrase-1 binds to TRF1 through its ANK domain (Smith and de Lange, 1999). Tankyrase-1 reversed the effect of TRF1 on telomere length by poly(ADP-ribosyl)ation of TRF1 that prohibited TRF1 binding to telomeric DNA (Smith and de Lange, 2000). Furthermore, overexpression of tankyrase-1 in the nucleus resulted in the telomere lengthening (Smith and de Lange, 2000). TIN2 (TRF1-interacting nuclear protein 2), another protein involved in the regulation of TRF1 function, was reported recently (Ye and de Lange, 2004). TIN2, TRF1 and tankyrase-1 formed a ternary complex. TIN2 inhibited poly(ADP-ribosyl)ation of TRF1 by tankyrase-1 but did not affect the automodification of tankyrase-1 (Ye and de Lange, 2004).
Although tankyrase-1 plays an important role in the regulation of telomere length, most tankyrase-1 proteins are located in the cytoplasm instead of nucleus (Diefenbach and Burkle, 2005). It has been reported that tankyrase-1 interacts with multiple proteins such as nuclear mitotic apparatus protein (NuMa) (Sbodio and Chi, 2002), IRAP (insulin-responsive amino peptidase) located in Golgi-associated GLUT vesicles (Chi and Lodish, 2000), and TAB 182 (tankyrase-binding protein with 182 kDa molecular weight) (Seimiya and Smith, 2002). Tankyrase-1 is proposed to be an important insulin-signaling target. Tankyrase-1 is phosphorylated by mitogen-activated protein kinase (MAPK) upon the insulin stimulation. This phosphorylation enhances poly(ADP-ribosyl)ation of IRAP by tankyrase-1 and automodification of tankyrase-1 (Chi and Lodish, 2000) (Sbodio et al., 2002).

1.2.6 PARP-5b or Tankyrase-2

Tankyrase-2 was originally detected as a tumor antigen in the sera of meningioma and breast cancer patients (Kuimov et al., 2001) (Monz et al., 2001). The domain structure of tankyrase-2 is very similar to that of tankyrase-1 except for the absence of the N-terminal HPS domain (Figure 6) (Kaminker et al., 2001) (Lyons et al., 2001). Tankyrase-2 and tankyrase-1 display a partially redundant function. Overexpression of either protein in the nucleus prohibited TRF1 binding to telomeric DNA (Cook et al., 2002). Like tankyrase-1, tankyrase-2 has been shown to interact and poly(ADP-ribosyl)ate protein partners such as IRAP, TRF1 (Sbodio et al., 2002), and TAB182 (Seimiya and Smith, 2002). Both tankyrase-1 and tankyrase-2 can form high-molecular-mass complexes by self-polymerization via the SAM
domain, suggesting a function as scaffold proteins (De Rycker et al., 2003). Despite the extensive functional overlap with tankyrase-1, tankyrase-2 displayed some different functions from tankyrase-1. Unlike tankyrase-1, tankyrase-2 preferentially poly(ADP-ribosyl)ated itself (Cook et al., 2002). Overexpression of tankyrase-2, but not tankyrase-1, resulted in the rapid poly(ADP-ribosylation)ation mediated cell death (Kaminker et al., 2001).

1.3 Poly(ADP-ribose) glycohydrolase

Prior to the report of PARG activity, PAR was known to be degraded in vitro by phosphodiesterase that catalyzed hydrolysis of the pyrophosphate linkages (Reeder et al., 1967). PARG was first discovered in a calf thymus nucleus extract in 1971 as a novel enzyme capable of splitting ribose-ribose bonds of PAR (Miwa and Sugimura, 1971). PARG purification was difficult due to its low abundance in eukaryotic cells. A 59 kDa PARG was first purified to apparent homogeneity from bovine thymus by Hayaishi and co-workers (Hatakeyama et al., 1986). PARG was purified from the same source again in 1990 using an affinity matrix that greatly improved the quantity and quality of the enzyme (Thomassin et al., 1990). PARG has been purified to apparent homogeneity from many different sources such as guinea pig liver (Tanuma et al., 1986), pig testis (Abe and Tanuma, 1996) and human placenta (Uchida et al., 1993). These purified PARGs displayed heterogeneity in molecular weight, ranging from 58 kDa to 71 kDa. The construction of a putative full-length cDNA of bovine PARG indicated that PARG has an approximate molecular weight 110 kDa (Lin et al., 1997). A smaller fragment of protein was detected
in the later step of PARG purification, suggesting that PARG was degraded by proteases (Affar et al., 2001). Another group also indicated that PARG was extremely sensitive to proteases, and precautions must be taken to maintain the PARG integrity during the purification process (Bonicalzi et al., 2003). Thus many purified PARGs from the earlier studies may be truncated PARGs due to the proteolysis (Bonicalzi et al., 2005).

1.3.1 One PARG gene and multiple PARG protein isoforms

The putative full length cDNA of bovine PARG was constructed using partial cDNA clones and the approximate size of this full length PARG cDNA was 4.1 kilobases, encoding a predicted 110 kDa protein (Lin et al., 1997). Northern analysis detected a single transcript of the PARG gene with approximate 4.3 kilobases in bovine kidney mRNA, suggesting that full length PARG encodes a 110 kDa instead of 58 kDa protein (Lin et al., 1997). A PARG cDNA with approximate 4.0 kilobases, encoding a 109 kDa protein, was also identified in rat testis (Shimokawa et al., 1999). A 60 kDa PARG purified from the same tissue corresponded to the C-terminal amino acid sequence of the 109 kDa deduced protein. Both 109 kDa and 60 kDa proteins possess PARG activity when they were tested in an in vitro system, suggesting that the catalytic domain of PARG is located in the C-terminus (Shimokawa et al., 1999).

Southern hybridization analysis of bovine genomic DNA has shown that a single copy PARG gene encodes the PARG protein (Lin et al., 1997). No other PARG homologues have been reported in mammalian genomes. Amino acid sequence of PARG does not show significant similarity with any other
known protein in protein sequence databases. The location of the PARG gene in genome has been identified by the in situ hybridization. Human and mouse PARG genes are located in 10q11.23 and 14B, respectively (Ame et al., 1999a). Human PARG cDNA was constructed by the reverse transcription of human fibroblast mRNA. The human PARG gene, consisting of 18 exons and 17 introns, was also mapped in the same location (10q11.23) by Genome BLAST program (Meyer et al., 2003). Exon 1-3 and exon 4-14 encode the putative regulatory domain and the center of the catalytic domain of the PARG protein, respectively (Meyer et al., 2003).

Expression of human PARG cDNA by an in vitro coupled transcription and translation system yielded several specific protein bands with molecular weight ranging from 85-111 kDa (Meyer et al., 2003). Analysis of human PARG gene sequences indicated other available translation start codons in exon 2 and exon 3, suggesting that several PARG protein isoforms in human may result from the alternative translation (Meyer et al., 2003). Two splicing variants of human PARG mRNA were identified besides the full length PARG mRNA which encodes 111 kDa protein (Meyer-Ficca et al., 2004). These two variants led to expression of two PARG isoforms PARG102 and PARG99 that lack exon 1 and exon 2 compared to full length human PARG111, respectively (Figure 7) (Meyer-Ficca et al., 2004). Several PARG transcripts were found in human and mouse expressed sequence tag (EST) databases using BLAST tools, suggesting that several PARG protein isoforms may also result from the alternative splicing (Meyer et al., 2003) (Bonicalzi et al., 2003). Analysis of PARG sequences revealed multiple splicing donor and acceptor
Figure 7. One single PARG gene encodes multiple PARG proteins and the domain structure of PARG.
NLS, nuclear localization signals; PCS, protease cleavage site; MTS, mitochondria targeting sequence. Adapted from Meyer et al., 2003 and Meyer-Ficca et al., 2004.
sites, which correspond to the size of PARG 102 and PARG 99 (Meyer-Ficca et al., 2004). This report elucidated the mechanism of the alternative splicing. Several PARG isoforms were also observed in other systems such as transfected bovine PARG cDNA into COS-7 cells, endogenous PARG in HL-60 and Jurkat cells (Winstall et al., 1999b) (Winstall et al., 1999a). An additional small PARG isoform with approximate molecular weight of 65 kDa was also frequently observed (Meyer-Ficca et al., 2005).

Another 39 kDa protein exhibiting PARG activity in vitro has been identified and characterized recently (Oka S, 2006). This protein, named ARH3, resembles ADP-ribose-(arginine) protein hydrolase (ARH1). ARH1 participates in a mono-ADP-ribosylation cycle by possessing the opposite activity of mono-ADP-ribosyltransferases. ARH1 cleaves ADP-ribose-arginine bond and releases free ADP-ribose (Moss et al., 1997) (Takada et al., 1993). ARH1 and ARH3 have different structures from PARG (Glowacki et al., 2002). The location of critical amino acids for ARH3 activity is different from that required for PARG activity as well, suggesting that ARH3 exhibits PARG activity but is structurally unrelated to PARG (Oka S, 2006). ARH3 showed less capability to release ADP-ribose than PARG since the amount of ARH3 was close to 1000 times as high as that of PARG to release the similar amount of ADP-ribose (Oka S, 2006). Thus further investigation is needed to identify the role of ARH3 in PAR metabolism, if ARH3 plays any role in this process.
1.3.2 Subcellular localizations of PARG isoforms

Since the abundance of endogenous PARG is low in mammalian cells, subcellular localizations of PARG isoforms were investigated by the overexpression of exogenous PARG. The fusion protein GFP-PARG was transiently expressed in mammalian cells and subcellular distribution of PARG was visualized by the fluorescence microscopy. Human PARG111 (hPARG111) was predominantly located in the nucleus while hPARP102 and hPARG99 were located in the cytoplasm (Meyer-Ficca et al., 2004). Since hPARG102 does not contain the first 82 amino acids encoded by exon 1 compared to hPARG111, a nuclear localization signal (NLS) was predicted to be present in exon 1. Two candidate NLS sequences were revealed by computational analysis using the PSORTII program (http://psort.nibb.ac.jp). Putative NLS #1 and #2 were located in \textsuperscript{10}CTKRPRW\textsuperscript{16} and \textsuperscript{32}PSRQR\textsuperscript{38}, respectively. Site-directed mutagenesis in NLS #1, but not in NLS #2, abolished nuclear targeting of fusion protein hPARG111, indicating that NLS #1 is the real nuclear targeting sequence (Meyer-Ficca et al., 2004). The previously reported putative bipartite NLS located at amino acid positions 415-439 was also examined. This putative bipartite NLS did not target the fusion protein to the nucleus (Meyer-Ficca et al., 2004).

Another classical putative NLS at amino acid positions 834-840 of rat PARG protein was reported (Shimokawa et al., 1999). Also a putative nuclear export signal (NES) at amino acid positions 124-132 of rat PARG protein was also reported in the same paper (Shimokawa et al., 1999). This NES shares homology with the human immunodeficiency virus (HIV) Rev type NES and is
very conserved in several other mammalian PARGs (Wen et al., 1995) (Fischer et al., 1995). Another putative NES was located at the amino acid positions 877-884 near the C-terminus of the rat PARG protein. This NES shows similarity with the leucine-rich NES of p53 (Stommel et al., 1999).

The overexpressed bovine PARG103 (lacking the amino acids encoded by exon 1) in COS-7 cells was preferentially located in the cytoplasm (Winstall et al., 1999b), suggesting that NLS in exon 1 may be the primary nuclear targeting signal. The finding of both putative NLS and NES in PARG protein suggests that PARG may be able to translocate between the nucleus and the cytoplasm. The cytoplasmic PARG was proposed to shuttle to the nucleus in response to genotoxic stress (Affar et al., 2001) (Bonicalzi et al., 2003). The cytoplasmic PARG possesses the majority of PARG quantity and activity in both untreated and alkylating agent-treated conditions (Meyer-Ficca et al., 2004), but PAR metabolism initiated by DNA strand breaks mainly occurs in the nucleus. Although this nucleo-cytoplasmic shuttling could provide an explanation for this intriguing phenomenon, this shuttling model is still controversial due to the contradictory observations. Some evidence supports this model. The subcellular distribution of GFP-hPARG changed dramatically during the cell cycle. GFP-hPARG was almost exclusively located in the nucleus during interphase while GFP-hPARG was mainly located in the cytoplasm during mitosis (Ohashi et al., 2003). This change of subcellular location suggests a possible shuttling between the nucleus and the cytoplasm. But other reports do not support this shuttling model. This shuttling was not observed in HEK 293 cells since PARG activity in the isolated nucleus of
treated cells with the alkylating agent decreased compared to untreated cells (Meyer-Ficca et al., 2004). Therefore, further investigation is needed to confirm the nucleo-cytoplasmic shuttling model.

PARG may be located in other cell compartments as well. PARG was identified in an enriched cellular fraction that contains Golgi and endoplasmic reticulum (Bonicalzi et al., 2003). FTCD, a protein marker of Golgi, was found to colocalize with PARG in this cellular fraction, suggesting that PARG may also reside in the Golgi compartment (Bonicalzi et al., 2003). Although the subcellular location of PARG60 is still under investigation, the current data suggest that it may be located in the mitochondria. Only PARG 60 is present in a partial PARG gene deletion mouse model that eliminates the normal PARG nuclear isoform containing an N-terminal putative regulatory domain by targeting exon 2 and 3 of the PARG gene (Cortes et al., 2004). Computational analysis revealed the mitochondria targeting sequence located in exon 4, suggesting that PARG 60 may be the mitochondrial PARG isoform (unpublished data in Dr. Jacobson’s lab).

1.3.3 Structural, enzymatic and genetic features of PARG

Although PARG was discovered more than 30 years ago (Miwa and Sugimura, 1971), the domain structure of the PARG protein has been proposed only recently. Domain A is the putative regulatory domain. The function of domain A is still poorly understood. Domains B, C, and D comprise a catalytically active fragment (Patel et al., 2005a) (Figure 7). Although the crystal structure of the PARG catalytic domain has not been resolved, the amino acids essential for substrate binding and amino acids required for
catalytic activity have been identified using adenosine diphosphate (hydroxymethyl)pyrrolidine diol (ADP-HPD) and the bovine recombinant PARG catalytic fragment (rPARG-CF) (Koh et al., 2003b) (Patel et al., 2005a). ADP-HPD, an analog of ADP-ribose, was identified as a potent and specific PARG inhibitor (Slama et al., 1995). Thus ADP-HPD was utilized as a lead inhibitor and derivatives of this compound were synthesized to identify a putative substrate-binding site of PARG. Due to low abundance in mammalian cells and extreme sensitivity to proteases, natural isolation of PARG protein usually is time-consuming and the yield may not be enough for the structural study. Comparison of isolated PARG protein and bacterial expression of rPARG-CF indicated that heterologously expressed rPARG-CF is suitable to determine structural features of PARG (Koh et al., 2003a).

A photoreactive analogue of ADP-HPD, [α-32P]-8-azidoadenosine diphosphate (hydroxymethyl)pyrrolidinediol (8-N3-ADP-HPD), has been shown to specifically and covalently photolabel PARG (Ramsinghani et al., 1998). Tyrosine 796 (Y796), a conserved amino acid in PARG across a wide range of species, was identified as the site of photodervatization, suggesting that this residue is involved in the putative substrate-binding site of PARG (Koh et al., 2003b). Mutagenesis of 9 acidic amino acids in rPARG-CF identified three essential residues involved in the catalysis, namely aspartic acid 738 (Asp 738), glutamic acid 756 (Glu 756) and Glu 757 (Patel et al., 2005a). These findings plus sequence alignments suggest a conserved catalytic domain of approximate 185 amino acids located in domain C (Figure 8) (Patel et al., 2005a).
Figure 8. Comparison of DNA damage responsive PARPs and PARG in wild type and PARG-Δ2,3 cell nuclei.

The top diagram shows proteins involved in PAR metabolism following DNA damage present in the nucleus of wild type cells and the lower diagram shows the proteins present in PARG-Δ2,3 cells. Nuclear localization signal is abbreviated as NLS.
PARG catalyzes hydrolysis of ribosyl-ribose glycosidic linkages between ADP-ribose units. PARG contains both exoglycosidase and endoglycosidase activities (Braun et al., 1994) (Lautier et al., 1993), and the endoglycosidase activity generates protein free PAR. The $K_M$ of PARG for small PAR is sub-micromolar, but this parameter decreased up to 100-fold for large PAR (Hatakeyama et al., 1986) (Desnoyers et al., 1995). The degradation of large PAR (greater than 20 ADP-ribose units) by PARG was biphasic, with an initial rapid phase followed by a slow and non-processive phase (Hatakeyama et al., 1986). Large PAR was quickly degraded by PARG via endoglycosidic and exoglycosidic activity, releasing small PAR and free ADP-ribose (Braun et al., 1994) (Brochu et al., 1994). Then PARG switched to the distributive exoglycosidic mode of action. The concentration of PAR in the nucleus of mammalian cells was approximately in the range of 1 or 2 times of $K_M$ of PARG, suggesting that PARG may be constantly active (Alvarez-Gonzalez and Althaus, 1989).

PARG cDNA has been reported from a wide range of species such as bovine (Lin et al., 1997), rat (Shimokawa et al., 1999), murine, human, Drosophila and Caenorhabditis elegans. The amino acid sequences deduced from these PARG cDNA share profound similarity with each other. Rat, mouse, human and bovine PARG sequences share more than 80% identity while Drosophila and Caenorhabditis elegans share about 40% identity with these mammalian PARGs (Davidovic et al., 2001).

1.3.4 PARG research tools
Development of potent, specific and cell-permeable PARG inhibitors will greatly facilitate identification of the role of PARG in cellular response to genotoxic stress. Several groups have been working on the development of PARG inhibitors. Two PARG inhibitors gallotannin and N-bis-(3-phenyl-propyl)9-oxo-fluorene-2,7-diamide (GPI-16552) have been reported to be protective in murine astrocytes treated by \( \text{H}_2\text{O}_2 \) and a rat model of focal cerebral ischemia, respectively (Ying and Swanson, 2000) (Ying et al., 2001) (Lu et al., 2003). However, gallotannin enhanced cell death and GPI 16552 had no protective effect in astrocytes treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Falsig et al., 2004), thus no clear picture has yet emerged from the use of these inhibitors. Gallotannin also has been reported to inhibit nicotinamide mononucleotide adenylyl-transferase (NMNAT), an important enzyme for NAD biosynthesis (Berger et al., 2005), and raising concerns about inhibitor specificity.

One drosophila PARG mutant has been reported recently. This mutant drosophila devoid of the PARG catalytic domain was lethal in the early stages at the normal development temperature, suggesting that PARG activity is essential for embryonic development (Hanai et al., 2004). Two different PARG gene disruption mouse models have also been reported recently. Targeted disruption of the PARG gene at exon 4 results in total loss of PARG activity and early embryonic lethality of homozygous mutant mice (Koh et al., 2004), which confirms the importance of PARG activity for embryonic development. In contrast, PARG gene disruption targeting exons 2 and 3 (PARG-\( \Delta 2,3 \)) results in the loss of the major cellular PARG isoforms including the nuclear
isoform, but homozygous PARG-Δ2,3 mice are still viable due to the presence of a truncated PARG protein with an approximate molecular weight of 60 kDa (Cortes et al., 2004). Although nuclear PARG110 is absent in PARG-Δ2,3 cells, isolated nuclei of PARG-Δ2,3 cells contain approximate 28% of PARG activity of wild type cells determined by an in vitro assay (Cortes et al., 2004). The presence of nuclear PARG activity in PARG-Δ2,3 cells is presumably due to the presence of a weak NLS in domain D (Masutani et al., 2003). The enzymes involved in PAR metabolism in the nucleus of wild type and PARG-Δ2,3 cells are shown in Figure 8. PARG-Δ2,3 cells contain normal DNA damage responsive PARP-1 and PARP-2 and a PARG60 lacking the domain A (Figure 8).

These PARG-Δ2,3 mice showed increased sensitivity to alkylating agents, ionizing radiation, streptozotocin-induced diabetes, and LPS-induced septic shock (Cortes et al., 2004). PARG-Δ2,3 mice showed enhanced postischemic brain damage (Cozzi et al., 2005), but showed increased resistance to renal and intestinal ischemia/reperfusion injury (Patel et al., 2005b) (Cuzzocrea et al., 2005). Mouse embryonic fibroblasts derived from the PARG-Δ2,3 mouse have been used in the research described in this dissertation.

1.4 Biological functions of PARP-1 and PARG

1.4.1 Biological functions of PARP-1 have been extensively studied.

Many studies have shown that PARP-1 involves in many different cellular processes such as cell death, DNA damage recognition and repair,
modification of chromatin structure and transcriptional regulation. The role of PARP-1 in these processes is described with details here.

PARP-1 facilitates DNA repair in response to mild genotoxic stress, but it also mediates cell death following extensive DNA damage (Pieper et al., 1999) (Bouchard et al., 2003) (Nguewa et al., 2003). The protective effect of PARP-1 inhibitors and PARP-1 genetic deletion in animals under cardiac and neural ischemia-reperfusion injury confirms this role of PARP-1 in the presence of severe DNA damage (Szabo, 1998) (Shall and de Murcia, 2000). This type of cell death is generally considered as necrosis, which induces an inflammatory response by releasing intracellular components into the neighboring tissues (Edinger and Thompson, 2004). The mechanism of PARP-1 mediated necrosis is not fully understood, but energy failure resulting from NAD and consequent ATP depletion following PARP-1 activation by DNA breaks has been proposed as a causative factor (Ha and Snyder, 1999) (Berger, 1985) (Decker and Muller, 2002).

The role of PARP-1 in apoptosis has also been elucidated. PARP-1 is cleaved by caspase-3 and -7 into two fragments of approximate 25 kDa and 85 kDa. The 25 kDa fragment contains the N-terminal DNA binding domain of PARP-1 and inhibits the activation of uncleaved PARP-1 by competing for DNA binding. The 25 kDa fragment also impairs DNA breaks detection and repair (D'Amours et al., 2001). The 85 kDa fragment remains basal PARP-1 activity, but its activity can not be increased by DNA damage (Kaufmann et al., 1993). The 85 kDa fragment translocates from the nucleus to the cytoplasm when advanced nuclear fragmentation occurs in the late stage of apoptotic
cells (Ivana Scovassi and Diederich, 2004). The cleavage of PARP-1 prevents extensive NAD consumption and thus blocks necrotic cell death following severe DNA injury. In addition, PARP-1 cleavage may also promote apoptotic cell death by impairing DNA repair following mild DNA injury (Soldani and Scovassi, 2002). PARG is also a substrate for caspase-3 during apoptosis (Affar et al., 2001). Although the consequences of the cleavages of both PARP-1 and PARG during apoptosis are not fully understood, proper PAR metabolism seems to play an important role in regulating apoptosis (Kim et al., 2005).

The role of PARP-1 in caspase-independent apoptotic cell death has also been reported. Apoptosis-inducing factor (AIF), a pro-apoptotic protein located in the mitochondrial intermembrane space, is one of the most powerful triggers of apoptosis (Chiarugi and Moskowitz, 2002). AIF can translocate into the nucleus and induce caspase-independent apoptosis with the features that include peripheral chromatin condensation and DNA fragmentation (Susin et al., 1999). PARP-1 activation is required for AIF release in mouse fibroblasts after the treatment with a potent alkylating agent (Yu et al., 2002). The mechanism of PARP-1 mediated AIF release is not clear, but free PAR or PAR-bound proteins may serve as a potential signal between the nucleus and mitochondria cross-talk (Hong et al., 2004).

Many factors may affect the PARP-1 activation mediated necrotic or apoptotic cell death such as the cell type and the features of the stress (Kim et al., 2005). One essential difference between necrosis and apoptosis is that apoptosis needs ATP to orderly degrade cellular structures and maintain
membrane integrity (Edinger and Thompson, 2004). Thus, in cells where PARP-1 activation depletes the ATP pool, cell death occurs by necrosis. Cellular metabolic status has been recently proposed as a key factor in determining to what extent NAD and consequent ATP level depletion occurs by PARP-1 activation (Zong et al., 2004) (Ying et al., 2005). Active proliferating cells mainly produce ATP by glucose catabolism through aerobic glycolysis which occurs in the cytoplasm, while nonproliferating cells maintain ATP level through oxidative phosphorylation which occurs in the mitochondria. PARP-1 activation preferentially depletes the NAD pool in the nucleus and cytoplasm, but not in the mitochondria, thereby reducing aerobic glycolysis, but not oxidative phosphorylation (Zong et al., 2004). Therefore, proliferating cells are usually more sensitive to PARP activation and die by necrosis in response to genotoxic stress.

Although the role of PARP-1 in DNA repair was controversial for a long time, many recent studies have provided convincing data to support the involvement of PARP-1 in DNA repair. PARP-1 knockout mice show hypersensitivity to multiple DNA damaging treatments such as alkylating agents and γ-irradiation, suggesting the important role of PARP-1 in DNA repair (Wang et al., 1997) (de Murcia et al., 1997) (Masutani et al., 1999a) (Masutani et al., 1999b). A DNA repair assay quantitatively demonstrated a defect of PARP-1 knockout fibroblasts in DNA repair following MNNG treatment (Wang et al., 1995). An SV40-CAT plasmid was treated with MNNG and then transfected into the wild type and PARP-1 knockout fibroblasts. The CAT enzymatic activity in the repaired plasmid was measured and used as an
indicator of the repair capacity of the fibroblasts. Compared to the untreated plasmid, about 80% CAT activity was observed in wild type fibroblasts, but only 35% CAT activity was observed in PARP-1 knockout fibroblasts (Wang et al., 1995).

PARP-1 binds to DNA single or double strand breaks through its DNA-binding domain (D'Amours et al., 1999). Thus PARP-1 can function as a DNA injury sensor. PARP-1 interacts physically and functionally with a variety of proteins involved in multiple DNA repair pathways such as single strand break (SSB) and double strand break (DSB) repair pathways. PARP-1 may recruit DNA repair proteins to the sites of DNA injury as well (Masutani et al., 2003) (Kim et al., 2005). Since PARP-1 is activated and poly(ADP-ribosyl)ates many other proteins such as cell cycle checkpoint protein p53 besides DNA repair proteins following DNA damage, PARP-1 may alter the normal cell cycle progression and prevent the accumulation of mutations (Malanga and Althaus, 2005) (Malanga et al., 1998).

XRCC1 (X-ray repair cross-complementing protein 1) plays an important role in SSB repair pathway via the interaction with various enzymatic components of the repair machinery (Caldecott, 2003). A two-hybrid system revealed the physical association between PARP-1 and XRCC1 (Masson et al., 1998). XRCC1 has been shown to rapidly assemble into discrete nuclear foci at the sites of PAR synthesis, but XRCC1 foci can not be detected in PARP-1 knockout cells under the same condition (El-Khamisy et al., 2003). PARP-1 is involved in the base excision repair (BER) pathway in the association with XRCC1 (Schreiber et al., 2002). PARP-1 also interacts with
DNA ligase III, another important player in BER (Leppard et al., 2003). DNA dependent protein kinase (DNA-PK), an important protein in DSB repair pathway, interacts with PARP-1 and its activity is stimulated by this interaction (Ruscetti et al., 1998). Recent data have demonstrated a novel PAPP-1-dependent DNA DSB repair pathway via the break-sensing ability of PARP-1 and the recruitment of XRCC1 and DNA ligase III to seal the breaks. This pathway may act as an alternative to the DNA-PK/XRCC4/DNA ligase IV-dependent pathway (Audebert et al., 2004). PARP-1 regulates p53-mediated G1 arrest in response to radiation, thus preventing DNA replication before the damage is repaired (Wieler et al., 2003).

Many studies have demonstrated that PARP-1 plays a role in the regulation of chromatin structure in the presence or absence of DNA injury (Kim et al., 2005) (Faraone-Mennella, 2005). Histone H1 is the main target for poly(ADP-ribosyl)ation by PARP-1 among all core histones (Poirier et al., 1982), (D'Amours et al., 1999). Poly(ADP-ribosyl)ation of histone H1 by PARP-1 has been shown to decondense chromatin structure and destabilize nucleosomes. PARP-1 also modifies other non-histone chromosomal proteins such as high mobility group proteins (Tanuma and Johnson, 1983). Poly(ADP-ribosyl)ation of chromatin proteins by PARP-1 may decrease the interactions between DNA and proteins, thus disturbing the normal assembly of nucleosomes (Mathis and Althaus, 1987). The polyanionic PAR, free or attached to acceptor proteins, may dispel or even strip basic proteins such as histones from destabilized nucleosomes (Realini and Althaus, 1992) (Panzeter et al., 1992; Wesierska-Gadek and Sauermann, 1988).
The important role of PARP-1 in the regulation of chromatin structure in vivo has been elucidated using Drosophila as a model. Unlike mammals, Drosophila has only two PARP genes, a PARP-1-like gene expressing three isoforms and a tankyrase-like gene (Hanai et al., 1998) (Miwa et al., 1999). PARP-1 inhibition blocked chromatin loosening (“puffing”) and inhibited the transcription of highly inducible genes response to external stimulus (Tulin and Spradling, 2003). Interestingly, the same group also found that PARP-1 may promote the formation of more compact chromatin structures (Tulin et al., 2002). Genetic disruption of PARP-1 activity dramatically increased the micrococcal nuclease sensitivity of the heterochromatic, but not euchromatic regions of chromatin (Tulin et al., 2002). Therefore, PARP-1 may promote decondensation or condensation of chromatin structure depending on the type of chromatin (Kim et al., 2005).

A recent study has shown that PARP-1 reversibly modulates chromatin structure in an NAD$^+$-dependent manner (Kim et al., 2004). According to this study, PARP-1 possesses specific nucleosomes-binding properties, thus incorporating into chromatin and promoting the formation of the condensed chromatin structure. If NAD$^+$ is available, PARP-1 automodifies itself and dissociates from chromatin, thus promoting the formation of the decondensed chromatin (Kim et al., 2004). This process occurs without the involvement of histone modification, which is in contrast to previous studies that emphasize the importance of histone modification in regulating chromatin structure (Kim et al., 2005).
A variety of studies have also suggested that PARP-1 modulates chromatin structure by determining the methylation pattern of genomic DNA (Zardo et al., 2003). Inhibition of PARP-1 activity by 3-aminobenzamide (3-AB) increased the extent and altered the pattern of DNA methylation in the CpG island located in the promoter region of Htf9 gene in mouse fibroblasts (Zardo and Caiafa, 1998). PARP-1 may decrease the protein expression level or inhibit enzymatic activity of DNA methyltransferase T1, an important enzyme catalyzing DNA methylation (Zardo et al., 2002) (Reale et al., 2005). Since 3-AB is not a specific inhibitor for PARP-1, other PARPs may also be involved in the regulation of DNA methylation.

Earlier studies have demonstrated that PARP-1 is frequently associated with transcriptionally active regions of chromatin (de Murcia et al., 1986) (Lindahl et al., 1995), suggesting that PARP-1 may play a role in transcriptional regulation. Genetic disruption of PARP-1 activity leads to defective expression of many genes in multiple cell types (Virag and Szabo, 2002), which provides convincing evidence for the role of PARP-1 in transcriptional regulation. Following lipopolysaccharide (LPS) and interferon-γ (IFN-γ) treatment, inducible nitric oxide synthase (iNOS) expression both at the protein and mRNA levels were defective in PARP-1 knockout fibroblasts compared to wild type cells (Szabo and Dawson, 1998). After reperfusion injury, the expression of P-selectin and intracellular adhesion molecule-1 decreased in the hearts of PARP-1 knockout mice compared to their wild-type counterparts (Zingarelli et al., 1998). Given the fact that PARP-1 regulates transcription of inflammation-inducible genes, the interaction between PARP-1
and NF-κB, a key transcription factor involved in the regulation of multiple inflammation related genes, has been proposed and confirmed. NF-κB cannot be activated in macrophages derived from PARP-1 deficient mice following LPS treatment. PARP-1 deficient mice were resistant to LPS-induced endotoxic shock compared to wild type mice (Oliver et al., 1999). The effect of PARP-1 on global gene transcription was investigated using oligonucleotide microarray analysis in primary wild type and PARP-1 deficient fibroblasts. Eleven thousand genes were examined and 91 genes were identified as up- or down-regulated in PARP-1 deficient fibroblasts compared to wild type fibroblasts (Simbulan-Rosenthal et al., 2000).

Two mechanisms have been proposed for the regulatory role of PARP-1 in gene transcription. First, it is proposed that PARP-1 modulates chromatin structure as described above and thus regulates gene transcription. A second proposed mechanism is that PARP-1 acts as one component of a promoter-binding protein complex (Kraus and Lis, 2003). PARP-1 stimulates or inhibits gene transcription depending on the promoter context, the cell type and the nature of treatment (Hassa and Hottiger, 2002) (Virag and Szabo, 2002). NF-κB mediated gene expression was suppressed in immuno-stimulated macrophages following pretreatment with the PARP-1 inhibitor (Jagtap et al., 2002), but NF-κB activation was not altered by the PARP-1 inhibitor in endothelial cells stressed in the presence of high concentration of glucose (Virag and Szabo, 2002). PARP-1 has also been shown to repress gene transcription. PARP-1 bound directly to the DNA binding domain of retinoid X receptors (RXR). Overexpression of PARP-1 selectively inhibited
transcriptional activities mediated by heterodimers of RXR and thyroid hormone receptor (TR) (Miyamoto et al., 1999). In some cases, enzymatic activity of PARP-1 is not essential for the regulation of gene transcription. For example, PARP-1 mutants lacking enzymatic and DNA binding activity have been shown to interact with NF-κB comparably with the wild type PARP-1 (Hassa et al., 2001).

Two models have been proposed recently to elucidate the molecular mechanisms of PARP-1 acting as a modulator of gene transcription in a promoter specific manner (Kim et al., 2005). PARP-1 is required for the retinoic acid (RA)-induced transcription of RARβ2 (retinoic acid receptor) gene. In PARP-1 deficient mouse fibroblasts, one component of inactive mediator fails to dissociate from the promoter region of RARβ2 gene following RA treatment (Pavri et al., 2005). Based on this observation, the first model was proposed as follows: PARP-1 promotes the conversion of a mediator (coactivator) from an inactive form to an active form (Pavri et al., 2005). The second model was proposed as follows: PARP-1 poly(ADP-ribosyl)ates a corepressor such as TLE (transducin-like enhancer of Split) and promotes the dissociation of the corepressor from the promoter region (Ju et al., 2004). Both of the models demonstrate the role of PARP-1 in gene transcription in the absence of DNA damage.

1.4.2 Biological functions of PARG are still poorly understood.

In contrast to the extensive study about biological functions of PARP-1, the role of PARG in multiple cellular processes under both normal physiological conditions and stresses is largely unknown. The low abundance
of PARG protein in mammals and extremely sensitive to protease digestion during the purification process have obstructed the study of biological functions of PARG for a long time. Although specific and cell permeable PARG inhibitors are still under development, some other research tools, such as overexpression of GFP-PARG fusion protein, down regulation of PARG activity by siRNA, PARG-Δ2,3 mouse models (described with details in 1.3.4), have been recently developed (Meyer-Ficca et al., 2004) (Blenn et al., 2006) (Cortes et al., 2004). The application of these research tools has improved our understanding about the biological functions of PARG.

Many studies have demonstrated the role of PARG in cell death induced by a wide range of stresses. PARG-Δ2,3 mice showed increased sensitivity or resistance to the different stresses, depending on the nature and the location of the stresses (described with details in 1.3.4) (Cortes et al., 2004) (Cozzi et al., 2005; Patel et al., 2005b) (Cuzzocrea et al., 2005). PARG is a substrate for caspase 3 during apoptosis (Affar et al., 2001). Down regulation of PARG activity by siRNA increased cell survival following high concentrations of H2O2 treatment, but no protection was observed by transfection of PARG siRNA after the treatment with a DNA alkylating agent MNNG (Blenn et al., 2006).

Recent studies have also suggested a direct role of PARG in DNA repair following radiation-induced DNA damage and modulation of chromatin structure. A cytoplasmic isoform PARG (MW ~103 kDa) was observed to translocate into the nucleus while a fusion protein GFP-hPARG110 (nuclear isoform) was observed to translocate into the cytoplasm in response to DNA damage induced by γ-irradiation (Haince et al., 2006). This dynamic relocation
of PARG isoforms suggests the direct involvement of PARG in DNA repair. The PARG protein level has been modulated using genetic tools in *Drosophila*. Decreased PARG levels weakened copia transcriptional repression (Tulin et al., 2006). The same report also suggested that SIR2 played a role in PARG mediated chromatin silencing (Tulin et al., 2006).

The enzymatic activity of PARP-1 is required to perform most of its biological functions. PARG is the only known enzyme to degrade PAR. Thus we would expect that PARG may play a role in many cellular processes just like PARP-1, although the study about the biological functions of PARG is still in the early stage. PAR metabolism, determined by the enzymatic activity of both PARP and PARG, is investigated in PARG-Δ2,3 mouse fibroblasts and the effects of altered PAR metabolism on cellular responses to genotoxic stress are also examined in this dissertation.

1.5 Three compounds inducing genotoxic stress

Three compounds have been chosen to induce genotoxic stress. First compound was N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG) (Figure 9). MNNG, a potent monofunctional alkylating agent (Wesierska-Gadek et al., 2003), is a well-known compound to activate PARP-1 and stimulate PAR synthesis in many different cell lines (Gagne et al., 2001) (Affar el et al., 2002) (Pogrebniak et al., 2003). Previous studies have shown that MNNG immediately alters nuclear NAD and PAR metabolism (Jacobson et al., 1985) (Rankin et al., 1980). MNNG has also been shown to play an important role in
Figure 9. Structures of MNNG and STR.
PAR metabolism mediated cell death (Yu et al., 2002). Thus MNNG was chosen to induce genotoxic stress.

The second compound was staurosporine (STR) (Figure 9). STR is a potent but nonspecific protein kinase C (PKC) inhibitor (Ruegg and Burgess, 1989) (Gescher, 2000). Since it induces apoptosis in a wide range of cell types (Charlot et al., 2006) (Pregi et al., 2006) (Zhang et al., 2005) (Giuliano et al., 2004), it is a widely used model compound to study apoptosis in many laboratories. STR does not lead to DNA strand breaks directly. STR has been reported to induce reactive oxygen species (ROS) in retinal neurons and melanoma cells (Gil et al., 2003) (Zhang et al., 2004), which can induce DNA strand breaks indirectly. UCN-01 (7-hydroxystaurosporine), an analog of STR, has been reported to inhibit DNA repair in lymphocytes (Yamauchi et al., 2002). Therefore, STR may damage DNA indirectly by generating ROS and/or inhibiting DNA repair. Although one study has reported that STR-induced apoptosis was delayed in PARP-1 knockout mouse fibroblasts (Nargi-Aizenman et al., 2002), the role of PAR metabolism in STR-induced apoptosis has not been well investigated. Thus STR was selected to investigate the possible role of PAR metabolism in STR induced cell death.

The third compound was hydrogen peroxide (H$_2$O$_2$), a model compound to generate reactive oxidative species (ROS). ROS has been shown to effectively activate PARP-1 and consequently deplete the NAD and ATP pool (Virag, 2005) (Erdelyi et al., 2005). PARP-1 knockout mice were resistant to cerebral ischemia where ROS plays an important role in the pathogenesis (Eliasson et al., 1997). But the role of PARG remains unclear in cellular
responses to oxidative stress. Thus H₂O₂ was chosen to investigate the role of PARG in cellular responses to H₂O₂ induced oxidative stress.

1.6 Statement of purpose

The importance of PAR metabolism in cell fate determination following genotoxic stress makes enzymes involved in PAR metabolism potential promising therapeutic targets for modulating diseases of inappropriate cell proliferation or death such as cancer or ischemia. PARP-1 as an attractive therapeutic target has been well validated and several PARP-1 inhibitors are currently being evaluated in clinical trials for cancer and ischemia treatment. But high abundance of PARP-1 protein and increasing members of PARP family raises concerns about the efficiency and specificity of inhibiting PARP-1 by current PARP-1 inhibitors. Although biological functions of PARG are poorly understood, its low abundance in mammalian cells and unique substrate still make PARG a potential interesting therapeutic target. The recent elucidation of structural features of PARG catalytic fragment provides the foundation for development of potent and cell permeable PARG inhibitors. But the discovery of multiple PARG isoforms targeting to different cellular compartments complicates target validation of PARG. From limited research tools of PARG study, a PARG-Δ2,3 cell line derived from homozygous PARG-Δ2,3 mice was chosen as a main research model in this dissertation. This model provides the opportunity to study nuclear PAR metabolism and related cellular responses to genotoxic stress in the presence of the normal DNA
damage responsive PARPs but with replacement of the normal nuclear PARG isoform with a catalytically active protein lacking the domain A (Figure 8).

Since PARG-Δ2,3 mice showed increased sensitivity to alkylating agents, ionizing radiation, streptozotocin-induced diabetes and LPS-induced septic shock (Cortes et al., 2004), we expect that PARG60 cannot totally compensate biological functions of PARG110. Although PAR metabolism was qualitatively similar between wild type and PARG-Δ2,3 cells assessed by indirectly immuno-fluorescence staining (Cortes et al., 2004), we expect that replacement of PARG110 with PARG60 would alter PAR metabolism in PARG-Δ2,3 cells quantitatively following genotoxic stress based on the current knowledge that PARG is the most important catabolic enzyme in PAR metabolism. Therefore, the hypothesis tested in this dissertation is that the normal nuclear isoform PARG110 is required for proper PAR metabolism and cellular responses to genotoxic stress.

The purpose of this dissertation is to investigate the role of PARG110 in PAR metabolism and cellular responses to genotoxic stress and thus explore PARG as a therapeutic target. The previous characterization of nuclear PARG isoform of wild type and PARG-Δ2,3 cells has already shown that nuclear PARG60 of PARG-Δ2,3 cells lacks domain A compared to nuclear PARG110 of wild type cells. Thus, the studies presented in this dissertation also provide possible clues about the regulatory function of domain A.

To achieve this purpose, four separate studies were conducted. First, growth properties, content and redox states of pyridine nucleotide pools were determined and compared between wild type and PARG-Δ2,3 cells in the
absence of genotoxic stress to examine the possible impact of partial PARG gene deletion \emph{per se} on these characteristics of PARG-\textit{\Delta}2,3 cells. Secondly, kinetics of NAD and PAR metabolism were determined and compared between wild type and PARG-\textit{\Delta}2,3 cells following high concentrations of MNNG treatment. Thirdly, several cellular responses related to DNA repair were investigated and compared between wild type and PARG-\textit{\Delta}2,3 cells following low concentrations of MNNG treatment to elucidate the role of PARG110 in DNA repair. The possible mechanisms of increased sensitivity of PARG-\textit{\Delta}2,3 cells to MNNG were also discussed. Fourthly, cell viabilities were determined and compared between wild type and PARP-\textit{\Delta}2,3 cells following STR and H\textsubscript{2}O\textsubscript{2} treatment to investigate the possible role of PAR metabolism in cell death induced by these two compounds. The successful completion of this dissertation will hopefully address the important role of PARG110 in PAR metabolism and cellular responses to genotoxic stress, and thus improve our understanding of biological functions of full length PARG110, and facilitate the validation of PARG as a therapeutic target.
CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

All materials, chemicals, biochemicals and kits utilized in the experimental studies of this dissertation are listed below (supplier in parentheses).

2.1.1 General materials

A. Cell scraper, disposable (Sarstedt)
B. Cell counting flasks, 20 ml Dilu-Vial (Fisher)
C. Chromatography columns, poly-prep (Bio-Rad)
D. Comet slide (Trevigen)
E. Coverslip (Fisher)
F. Cryostorage microtube racks, six 1 ml tube capacity (USA)
G. Cryostorage microtubes, 1 ml (Sarstedt)
H. Disposable syring filters (UNIFLO-Plus, schleicher & Schuell)
I. Filter, VacuCap 90 (PALL life sciences)
J. Filter paper, 3 mm (Whatman)
K. Hyperfilm-MP (Amersham)
L. Membrane filters (MFS)
M. Microfuge tubes, 1.5 ml and 2 ml polypropylene, screw-cap (VWR)
N. Microslides (Corning)
O. Pasteur pipettes, 6.5” glass (VWR)
P. Pipets, plastic, 1, 2, 5, 10, and 25 ml Falcon Serological (Sarstedt)
Q. Pipet tips, Tip One 0.5 -10, 1 - 20, 1 - 200, 10 -1000, and 501 – 5000 µl (USA)
R. Polypropylene tubes, conical, 15 ml and 50 ml screw-cap conical 
   (Stockwell Scientific)
S. Polystyrene round bottom tube, 5 ml (Becton Dickinson)
T. Pointed curved forceps (Fisher)
U. Polyvinylidene fluoride (PVDF) transfer membranes (Millipore)
V. Scintillation vials, 7 ml polyethylene (RPI)
W. Syringe filter tip, Luer-lok 0.45 µm (Gelman)
X. Tissue culture dishes, 35 × 10 mm, 60 × 15 mm, and 100 × 20 mm 
   (Sarstedt)
Y. Tissue culture flasks, T-75 (Sarstedt)
Z. Tissue culture plates, 6-well, 96-well, flat bottom (Sarstedt)

2.1.2 Chemicals (Source)

2',7'-dichlorofluorescein diacetate Isocitrate (Sigma)
(Sigma)
2-Mercaptoethanol (Sigma) Low melting point agarose (Sigma)
3-Aminophthalhydrazide, “Luminol” Magnesium chloride (Fisher)
(Sigma)
5,5′,6,6′-tetrachloro-1,1′,3,3′- Methanol (EMD)
tetraethyl-imidacarbocyanine iodide (JC-1) (Sigma)
Acrylamide/bisacrylamide 30/0.8% MNNG (Sigma)
(Bio-Rad)
Adenosine (Sigma) MOPS (Sigma)
Agarose, low melting point (NuSieve GTG)  MTT (Sigma)
Ammonium acetate (Mallinckrodt)  β-NAD (Sigma)
Ammonium bicarbonate (Fisher)  NADP (Sigma)
Ammonium formate (Fisher)  Perchloric acid (Sigma)
Ammonium persulphate (EM)  Phenazine ethosulfate (Sigma)
Ampicillin (Sigma)  Ponceau S (EBI)
Benzamide (Sigma)  Phenazine ethosulfate (Sigma)
BES (Sigma)  Potassium chloride (Mallinckrodt)
Bicine (Sigma)  Potassium hydroxide (Fisher)
Bromophenol Blue (J.T Baker chemical)  Potassium phosphate dibasic (Fisher)
Calcium chloride (Sigma)  Potassium phosphate monobasic (Fisher)
p-Coumaric acid (Sigma)  Propidium iodide (Sigma)
DAPI (Roche)  Semicarbazide-HCl (Fisher)
Deoxy-adenosine (Sigma)  SDS (Sigma)
DMSO (Sigma)  Sodium acetate (Sigma)
dNTP (Fermentas)  Sodium chloride (EMD)
Dry milk, instant nonfat (Nestle USA)  Sodium deoxycholate (Sigma)
Ecolume (ICN)  Sodium hydroxide (Fisher)
EDTA (Sigma)  Sodium phosphate dibasic (Sigma)
EGTA (Sigma)  Sodium phosphate monobasic
Ethanol (AAPER alcohol and chemical Co.)
Sodium pyrophosphate tetrabasic decahydrate (Sigma)
Ethidium bromide (Sigma)
Sodium thiosulfate (Sigma)
Ethyl ether anhydrous (EMD)
Staurosporine (Sigma)
Formaldehyde (Sigma)
Sucrose (Sigma)
Formic acid (Fisher)
TCA (Calbiochem)
Glycerol (Fisher)
TEMED (Sigma)
Glycine (Sigma)
Tris base (Sigma)
Guanidinium chloride (Fluka)
Triton X-100 (Sigma)
Hydrogen peroxide (Sigma)
Tween 20 (Sigma)
Igepal (Sigma)
z-VAD-fmk (EMD)

2.1.3 Biochemicals, enzyme inhibitors and reagent kits

A. Alcohol dehydrogenase (Sigma)
B. An actin antibody (AC-15, Sigma)
C. A PAR antibody (SA-216, Biomol)
D. A PARP-1 antibody (NB110-111, Novus)
E. A γ-H2AX antibody (JBW301, Upstate)
F. A XRCC1 antibody (AHP832, Serotec)
G. Bacterial alkaline phosphatase (Sigma)
H. BCA kit (Pierce)
I. Bovine serum albumin “BSA” (Sigma)
J. FITC-coupled 2nd antibody (goat anti-rabbit, Jackson Laboratories)
K. FITC-coupled 2nd antibody (donkey anti-mouse, Jackson Laboratories)
L. HRP-conjugated IgG (goat anti-rabbit, Jackson Laboratories)
M. HRP-conjugated IgG (donkey anti-mouse, Jackson Laboratories)
N. Isocitrate dehydrogenase (Sigma)
O. Phosphodiesterase, snake venom (Worthington)
P. Protease inhibitor cocktail, complete mini tablet (Roche)
Q. RNase A (Sigma)

2.1.4 Medium, cell culture reagents and serum
Coulter clenz (Coulter) Hanks buffer (Sigma)
Dulbecco’s Modified Eagle Medium, Isoton II (Coulter)
“DMEM” (Hyclone)
Fetal bovine serum, “FBS” (Hyclone) Trypsin-EDTA (Sigma)

2.1.5 LC resins and HPLC columns
A. DHB-Bio-Rex (Jacobson laboratory)
B. DHB-Sepharose (Jacobson laboratory)
C. Guard column (Waters)
D. C18 (Beckman coulter)

2.1.6 Radiochemicals
A. 2,8-[³H] Adenine, 1 mCi/ml (Moravek Biochemicals)

2.1.7 Cell lines
A. Wild type and PARG-Δ2,3 mouse embryonic fibroblasts (Z-Q. Wang laboratory)

2.2 Data analysis software
A. CASP software
B. ImageQuant software

2.3 Equipment

A. Autoclave SG-120 (Amsco Scientific)
B. Balances, Models AE100 and PB1501 (Mettler)
C. Biological safety cabinet, class II type A/B3 (Nuair)
D. Carousel microwave oven (Sharp)
E. Cell counter, particle count and size analyzer model Z2 (Coulter)
F. Centriguge (Jouan)
G. Chart recorder for HPLC detection, model BD11 (Kipp)
H. Compact rocker (Labnet)
I. Electrophoresis apparatus (Bio-Rad)
J. Electrophoresis apparatus, DNA gel (Bio-Rad)
K. Fraction collector, HBK model FRAC-100 (Pharmacia)
L. Freezer, -80°C (Harris)
M. Gastight syringes for HPLC injection (Hamilton)
N. Gel imaging system (Kodak)
O. HPLC solvent delivery system, prostar (Varian)
P. Horizontal gel electrophoresis apparatus, Gibco BRL (Life technologies)
Q. Incubator (Precision)
R. Incubator, CO2 controlled for cell culture (Jouan)
S. Incubator shaker, Innova 4000 (New Brunswick)
T. Lyophilizer, freezemobile 25EL (VirTis)
U. Microfuge, 5417R (Eppendorf)
V. Microscope (Olympus IX70) connected to a digital camera (Nikon DXM 1200F)
W. Microplate reader, VERSA_{\text{Max}} (Molecular devices)
X. Mixer (USA)
Y. PH meter, pH/ion meter 150 (Corning)
Z. Pipetter, Accu-Jet (Brinkmann)
AA. Pipetter, positive displacement, Gilson pipetman P10, P20, P200, P1000 and P5000 (Rainin)
BB. Power supply, model EC105 (EC apparatus corporation)
CC. Power supply, computer controlled electrophoresis model 3000 (Bio-Rad)
DD. Scintillation counter, multi-purpose, LS6500 (Beckman coulter)
EE. Semi-dry transfer cell (Bio-Rad)
FF. Sonic dismembrator model 100 (Fisher)
GG. Spectrophotometer, Ultrospec III, UV/visible (Pharmacia)
HH. Thermomixer (Eppendorf)
II. UV detector, Uvicord SII (Pharmacia)
JJ. UV lamp, type 2 (Pharmacia)
KK. Vortex genie mixer (Fisher)
LL. Water purification system, milli-UF plus (Millipore)
MM. Waterbath, 180 series (Precision)

2.4 Methods
The experimental methods applied in this dissertation are described below with details. The related references are cited, and modifications are noted.

2.4.1 Cell culture methods

A. Thawing of cells.

Previous frozen PARG wild type and PARG-Δ2,3 cells with the same passage number in 1 ml cryostorage vials were thawed quickly in a 37°C water bath with gentle shaking. The cryostorage vials were washed with 70% ethanol thoroughly. The cells were collected by rinsing the vials with the cell suspension several times. Pre-warmed culture medium (DMEM + 10% FBS + 100 μM 2-mercaptoethanol, 2ME) (14 ml) was added in a T-75 flask and the cells were transferred to the flask slowly with the flask shaken gently. The flasks were placed in the cell culture incubator (37°C, 5% CO₂). On the following day, the DMSO-containing medium was replaced by the fresh culture medium after the cells had attached to the flasks.

B. Propagation of cells.

The medium was replaced by the fresh culture medium every 48 hrs. Usually cells were split every 3 days and seeded into a new flask at a density of $2.25 \times 10^5$ ($3 \times 10^3$/cm²). Briefly, cells were washed with 5 ml phosphate-buffered saline (PBS, pH 7.2) and then trypsinized by 2.5 ml 1× trypsin-EDTA. After most of the cells were detached, 5 ml of culture medium was added into the flask. The cell number was determined, and cells were seeded at the density mentioned above.

C. Cryostorage of cells.
The freezing medium, which is composed of 60% culture medium, 30% FBS, and 10% DMSO was prepared freshly. It was kept on ice for at least 15 min before use. The cultured cells were washed, harvested and centrifuged. The cell pellet was then resuspended in the cold freezing medium at a density of $1.5 \times 10^6$ /ml. The cell suspension (1 ml) was transferred to a 1 ml screw-cap cryostorage vial. The cryostorage vials were then placed in a sealed isothermal container. The container was kept in -80°C at least 24 hrs, then the vials were finally stored in the liquid nitrogen tank.

D. Quantification of cell numbers.

The cell suspension (1 ml) was transferred to a 20 ml Dilu-vial counting flask, diluted with 9 ml Isoton III isotonic saline solution, and mixed by pipetting up and down. The cell number was then determined using a Coulter Counter Z2 (Beckman coulter corporation). For PARG wild type and PARG-Δ2,3 cells, the counting parameter was set as above 12 μm.

E. Calculation of the doubling time.

Both PARG wild type and PARG-Δ2,3 cells were seeded at a density of 3000/cm² in 35-mm dishes. Cell numbers were counted at 24 hrs intervals by the Coulter counter Z2 (Beckman coulter corporation). The doubling time was calculated based on the linear part of the growth curve. PARG-Δ2,3 cells were also seeded at the density much higher than 3000/cm² in 35-mm dishes to observe the plateau phase in a shorter period of time.

F. Three compounds to induce genotoxic stress.

Proliferating and non-proliferating cells were determined by the growth curve. Proliferating wild type and PARG-Δ2,3 cells were seeded at different
densities by which the cell numbers were very close at the time of the treatment. Non-proliferating wild type and PARG-Δ2,3 cells were seeded at a similar density until both of them reached plateau phase.

_Treatment of cells with MNNG._ Once PARG wild type and PARG-Δ2,3 cells reached the desired level of confluence, the culture medium was replaced by the medium without 2ME (DMEM + 10% FBS) or Hanks buffer depending upon the downstream experiments. MNNG stock (usually 400 × ) was freshly made in DMSO. After the cells were treated by MNNG for the desired time (usually less than 1 hour), cells were harvested for the further analysis or the fresh medium without 2ME was added back, and the cells were placed in the incubator until the further analysis.

_Treatment of cells with staurosporine._ Before the treatment, the culture medium was replaced by the fresh medium without 2ME. After the cells were treated by staurosporine for different time points as indicated, cells were harvested for the further analysis.

_Treatment of cells with hydrogen peroxide._ Before the treatment, the culture medium was replaced by Hanks buffer. The concentration of hydrogen peroxide was calibrated with ultraviolet (UV) spectrophotometer every time before the treatment. After the cells were treated by hydrogen peroxide for the desired time (usually less than 1 hour), cells were harvested for the further analysis or the fresh medium without 2ME was added back and the cells were placed in the incubator until the further analysis.

### 2.4.2 Flow cytometry analysis.
For all experiments using the flow cytometry, proliferating cells were seeded as follows: PARG wild type and PARG-\(\Delta 2,3\) cells were seeded at the density of \(7 \times 10^4\) and \(9 \times 10^4\) in 60 mm dishes, respectively. After 48 hrs, the total cell number of both PARG wild type and PARG-\(\Delta 2,3\) cells reached about \(3 \times 10^5\) per dish and cells were ready for different treatments.

A. Analysis of cell cycle distribution.

Untreated cells were seeded at lower density than treated cells to avoid over confluence at the time of the analysis. Cells were treated with MNNG by the method mentioned above at the indicated concentrations for 30 minutes. Then MNNG-containing medium was removed, and the culture medium without 2ME was added back. After 72 hrs, the culture medium was removed, cells were washed with PBS and then detached by trypsin treatment. Cell suspensions were collected and centrifuged at 180 g for 5 min at room temperature. After the centrifugation, cell pellets were resuspended in 200 \(\mu\)l cold phosphate-buffered saline (PBS, pH 7.2) and kept on ice. Ice-cold 70% ethanol in PBS was added, and all samples were kept on ice for 30 min. Then cells were centrifuged at 180 g for 5 min at room temperature. The cell pellets were resuspended in 800 \(\mu\)l PBS, 100 \(\mu\)l 1 mg/ml ribonuclease A (RNase, 1 mg/ml in PBS), and 100 \(\mu\)l 400 \(\mu\)g/ml propidium iodide (400 \(\mu\)g/ml in PBS). Cells were covered by aluminum foil and incubated at 37°C for 30 min. Cells were analyzed by the flow cytometry laboratory at Arizona Cancer Center.

B. Determination of cell death

Following MNNG treatment. An Annexin V-FITC apoptosis detection kit (Sigma) was used to determine cell death. Cells were treated with MNNG by
the method mentioned above at the indicated concentrations for 30 minutes. Then MNNG was removed, and the culture medium without 2ME was added back. After 24 hrs, the culture medium was removed, cells were washed with phosphate-buffered saline (PBS, pH 7.2) and then trypsinized. The culture medium, all wash solutions and cell suspensions were collected and centrifuged at 180 g for 5 min at room temperature. After the centrifugation, cell pellets were resuspended at a density of $10^6$ cells/ml in a binding buffer supplied by the kit. Annexin V coupled with FITC and propidium iodide were added in the recommended amounts. Cells were covered by aluminum foil and incubated at room temperature for 10 min. Then cells were analyzed by the flow cytometry laboratory at Arizona Cancer Center.

Following staurosporine and hydrogen peroxide treatment. Staurosporine was added to culture medium without 2ME until the analysis (24 hrs or 48 hrs). Hydrogen peroxide was added in Hanks buffer for 30 min, then Hanks buffer was replaced by the culture medium without 2ME until analysis. All other procedures were same as with those following MNNG treatment.

C. Measurement of mitochondria membrane potential

Cells were treated with hydrogen peroxide as described above. After treatment, Hanks buffer was replaced by culture medium without 2ME and remained in the cell culture dishes until the analysis. Then the culture medium was removed, cells were washed with phosphate-buffered saline (PBS, pH 7.2) and then trypsinized. Cell suspensions were collected and centrifuged at 180 g for 5 min at room temperature. All the following procedures were conducted without direct exposure to light. After the centrifugation, cell pellets
were resuspended in 300 µl of 5 µg/ml JC-1. A JC-1 stock of 1 mg/ml was made in DMSO and stored in -20°C freezer. JC-1 of 5 µg/ml was freshly prepared in PBS. Cells were incubated at 37°C for 15 min. During the incubation, cells were shaken gently once to achieve even staining. After the staining, cells were washed with PBS, centrifuged and resuspended in 300 µl PBS. Then cells were covered by aluminum foil and analyzed by the flow cytometry laboratory at Arizona Cancer Center.

**2.4.3 Determination of total NAD/NADP content and redox states using the enzymatic cycling assays**

NAD and NADP were extracted by 0.1 M NaOH (Jacobson et al., 1979) and determined by the enzymatic cycling assays as described previously (Jacobson and Jacobson, 1976). NaOH of 0.1 M was added to the dishes of cells on ice. Cells were scraped with cell scrapers, and the samples were divided into 3 aliquots quickly. One aliquot of the sample was neutralized to pH 7.2 by 0.5 M H₃PO₄ in less than 2 minutes. This aliquot was used to measure total NAD and NADP. The second aliquot of the sample was heated at 60°C for 10 minutes to destroy the oxidized form of NAD and NADP, and then neutralized to pH 7.2 quickly, and used to measure NADH and NADPH. Neutralized samples (50-100µl) were transferred to 96-well plates and then diluted to 200 µl with water. Reaction mixtures of 100 µl for NAD or NADP assay were added. The NAD and NADP cycling assays were initiated by alcohol dehydrogenase and isocitrate dehydrogenase in the reaction mixtures, respectively. The NAD and NADP contents were determined by the absorbance at 570 nm. All samples were freshly assayed in the same day of
the extraction. The third aliquot of the sample was used to determine the total cellular protein by BCA assay using the BCA assay kit (Pierce).

2.4.4 Determination of PARP-1 automodification and PAR accumulation using Western blot analysis

For Western blot analysis of PARP-1 and PAR, proliferating cells were seeded as follows: PARG wild type and PARG-∆2,3 cells were seeded at the density of $9 \times 10^4$ and $1.5 \times 10^5$ in 60 mm dishes, respectively. After 48 hrs, the total cell number of both PARG wild type and PARG-∆2,3 cells reached about $5.5 \times 10^5$ per dish and cells were ready for different treatments.

**Lysis of cells.** Total cell extracts were prepared according to a procedure described previously (Ying et al., 2001). Cell lysis buffer (200 µl) was added to the dishes on ice and cells were removed by gentle scraping. The lysis buffer was composed of 150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 2 × COMPLETE protease inhibitor mixture (Roche Molecular Biochemicals), and 50 mM Tris·HCl, pH 7.5. Cell extracts were subject to sonication and then mixed with 50 µl 5 × loading buffer composed of 25% 2ME, 50% glycerol, 10% SDS, 0.05% bromophenol blue and 312.5 mM Tris·HCl. Cell samples were heated at 65°C for 15 min.

**Electrophoresis of SDS-PAGE gel and transfer of the total cellular protein.** The cell extracts containing equal cell number equivalents of PARG wild type and PARG-∆2,3 samples were loaded to the standard 8% SDS-PAGE gel. The gel was run at 150 V until the bromophenol blue migrated out of the gel
(usually about 1 hour). The cellular proteins were then electro-transferred to PVDF membranes (Millipore) using a semi-dry gel transfer apparatus at 17 V for 1 hour. The PVDF membranes were stained by Ponceau S staining to confirm equal loading of samples.

*Determinination of PARP-1 and PAR by specific antibodies.* The PVDF membranes were blocked in 5% dry milk (5% dry milk in PBS and 0.1% Tween 20 solution) overnight at 4°C. For determination of PARP-1, PVDF membrane was incubated with the first antibody NB110-111 (Novus) diluted 1:200 for 2 hours and then incubated with a secondary goat anti mouse antibody coupled with horseradish peroxidase (Jackson Laboratories) diluted 1:10,000 for 1 hour. For determination of PAR, PVDF membrane was incubated with the first antibody SA-216 (Biomol) diluted 1:500 for 2 hours and then incubated with a secondary goat anti mouse antibody coupled with horseradish peroxidase (Jackson Laboratories) diluted 1:10,000 for 1 hour. PAR and PARP-1 were visualized with an enhanced chemoluminescence (ECL) reaction.

### 2.4.5 Quantification of PAR and NAD from the same cell samples using a 3H-labeling method

A 3H-labeling method previously described by Aboul-ela *et al.* (Aboul-Ela et al., 1988) was used to quantify PAR. Cells (1.5 × 10^6) in 60-mm dishes were radiolabeled by 40 μCi 3H-adenine for 16 hrs. The labeled medium was replaced by fresh medium 2 hrs before MNNG treatment. Following MNNG treatment, cells were harvested by 1 ml ice-cold 20% trichloroacetic acid (TCA). The TCA supernatant was used to determine NAD content using the
enzymatic cycling assay. The radioactivity of NAD was determined by scintillation counting after NAD was isolated from the TCA supernatant by Dihydroxyboryl Sepharose (DHB-Sepharose) column chromatography. The TCA pellet was used to determine PAR content. PAR was isolated by DHB-Bio-Rex column chromatography, digested by snake venom phosphodiesterase and bacterial alkaline phosphatase (SVPD/BAP) to ribosyladenosine which was separated and collected by HPLC. The radioactivity of ribosyladenosine, which represents PAR content, was determined by scintillation counting (Figure 10) (Aboul-Ela et al., 1988, see Figure 10).

DHB-Sepharose and DHB-Bio-Rex were synthesized as described previously (Jacobson et al., 1984) (Wielckens et al., 1984). The procedure of DHB-Sepharose synthesis is as follows: Packed Sepharose 4B was washed with 0.1 M NaCl and then by deionized water. After washing, the Sepharose was resuspended in cold deionized water. Finely divided cyanogen bromide suspended in cold deionized water was added to the Sepharose suspension with continuous stirring. The pH was adjusted and maintained at 11.0 using NaOH. The Sepharose was washed with ice-cold 0.1 M NaHCO₃ (pH 9.0) and then resuspended in the same NaHCO₃ solution containing 10% 6-aminohexanoic acid with constant stirring at 4°C. The Sepharose was filtered and resuspended in deionized water containing 0.25% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. The pH was adjusted to 5.0 and 8% m-aminophenylboronic acid hemisulfate (pH 5.0) was added into the Sepharose with the volume ratio 1:40 to the Sepharose. The Sepharose was then filtered and washed with 0.5 M NaCl followed by deionized water.
Figure 10. Analytical scheme of quantification of NAD and PAR from the same cell samples.
Adapted from Aboul-Ela et al., 1988

Cells

\[ \text{3H-Adenine labeling} \]

\[ \text{MNNG treatment} \]

\[ 20\% \text{ TCA} \]

\[ \text{Supernatant} \rightarrow \text{DHB-Sepharose} \]

\[ \text{Pellet} \rightarrow \text{KOH, then DHB-Bio-Rex} \]

\[ \text{NAD (pmol)} \]

\[ \text{NAD (cpm)} \]

\[ \text{PAR (cpm)} \]

\[ \text{PAR (pmol)} = \frac{\text{NAD pmol}}{\text{cpm}} \times \text{PAR cpm} \]
The Sepharose was resuspended in deionized water and stored at 4°C until used (Jacobson et al., 1984).

The procedure of DHB-Bio-Rex synthesis is as follows: Bio-Rex 70 (100-200 mesh) was suspended in deionized water and mixed with 10% N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide. The pH was adjusted to 5.0. Aminophenylboronic acid hydrochloride (10% of Bio-Rex 70) was added into the mixture with constant stirring. The gel was washed with deionized water and AAGE 9 buffer (1 M guanidinium chloride, 250 mM ammonium acetate, 10 mM EDTA, pH 9.0). Finally, the boronate gel was stored in AAGE 9 buffer at 4°C until used (Wielckens et al., 1984).

**Determination of NAD content using the TCA supernatants.** The TCA supernatant fraction was divided into two aliquots. One aliquot was neutralized to pH 7.2 by addition of 2 M KOH/0.66 M KH₂PO₄. Then NAD content was determined by the enzymatic cycling assay as mentioned above. Another aliquot of the TCA supernatant fraction was diluted into 10 ml 250 mM ammonium acetate (pH 9.0). Prior to sample loading, the DHB-Sepharose column was washed with 10 ml 250 mM ammonium acetate (pH 4.5) and then by 10 ml 250 mM ammonium acetate (pH 9.0). Then sample was applied to DHB-Sepharose column. The column was washed with 10 ml 250 mM ammonium acetate (pH 9.0). Finally NAD was eluted by 4 ml 250 mM ammonium acetate (pH 4.5). HPLC was utilized to confirm that the radioactivity of the eluate co-eluted with NAD. The eluate was applied to a 5 μm Beckman HPLC C18 ODS-reversed phase column using 0.1 M K₂HPO₄
(pH 6.0) containing 3% methanol as the mobile phase. The radioactivity of NAD was determined by scintillation counting.

Determination of PAR content using the TCA pellet. The TCA pellet was dissolved in 0.1 ml ice-cold 88% formic acid. BSA (2 mg) was added and TCA (100% w/v) was added to a final concentration of 20% and kept on ice for 20 min. After centrifugation, the pellet was washed with 0.5 ml water-saturated diethyl ether to extract residual TCA. The pellet was then dissolved in 1 ml AAGE-6 buffer (6 M guanidinium chloride, 250 mM ammonium acetate, 10 mM EDTA, pH 6.0). To release PAR from the acceptor proteins, 1 ml 1 M KOH/100 mM EDTA was added and sample was incubated at 37°C for 2 hrs. The sample was diluted in 10 ml AAGE-9 buffer and applied to a DHB-Bio-Rex column (1 ml) which was prewashed with 5 ml 10 mM HCl, 5 ml water and then 10 ml AAGE-9 buffer. After the sample was applied to the column, the column was washed with 10 ml AAGE-9 and then 10 ml 1 M ammonium bicarbonate/10 mM EDTA (pH 9.0). PAR was eluted by 2 ml 10 mM HCl and 3 ml water. The eluate was lyophilized and resuspended in 1.2 ml 50 mM MOPS/5 mM MgCl₂ (pH 7.5). Dialyzed SVPD/BAP (1 unit) was added to the eluate and the sample was incubated at 37°C for 3 hrs. After digestion, the sample was filtered and applied to a 5 µm Beckman HPLC C18 ODS-reversed phase column using 7 mM ammonium formate containing 10% methanol as the mobile phase. Adenosine (10 pmol) and 2'-deoxyadenosine (10 pmol) were co-injected with the sample as chromatographic markers. The radioactivity of PAR was indicated by the radioactivity present in the ribosyladenosine peak which was determined by scintillation counting.
Quantification of PAR. Since NAD is the substrate to synthesize PAR and cells do not prefer labeled or unlabeled adenine, the specific activity of NAD (pmol/10^6 relative to radioactivity cpm) should be equal to the specific activity of PAR (pmol/10^6 relative to radioactivity cpm). Therefore, the pmol/10^6 of PAR was calculated using the following equation: pmol/10^6 / cpm of NAD * cpm of PAR = pmol/10^6 of PAR

2.4.6 Determination of DNA strand breaks using the comet assay

For all experiments using single cell gel electrophoresis assay (the comet assay), proliferating cells were seeded as follows: PARG wild type and PARG-Δ2,3 cells were seeded at the density of 6 × 10^4 and 7 × 10^4 in 35 mm dishes, respectively. After 24 hrs, cells were ready for the treatment.

After treatment, cells were removed from dishes by treatment with trypsin, neutralized with the culture medium and centrifuged at 180 g for 10 min. The cell pellet was resuspended in PBS at the density of 10,000 cells in 50 µl PBS. Fifty microliters 2.4% low melting point agarose (NuSieve GTG) was added to 50 µl cell suspension and mixed well. The resulting mixture was added quickly to a comet assay slide (5,000 cells/slide). A coverslip was placed on the top of the agarose drop and the slide was placed in a refrigerator at 4°C for 5 min. Then the coverslip was carefully removed and the slide was kept in the refrigerator for another 10 min. After solidification of the agarose, the slide was placed in the pre-chilled lysis buffer (2.5 M NaCl, 100 mM EDTA-Na$_2$, 10 mM Tris base, 200 mM NaOH, 1% Triton X-100, pH 10.0) at 4°C for 1 hour in the dark. After lysis, the slide was placed in the electrophoresis buffer (300 mM NaOH, 1 mM EDTA) at room temperature for 40 min. Next, the slide was
placed in the gel electrophoresis apparatus and the pre-chilled electrophoresis buffer was slowly added into the apparatus. Electrophoresis was performed at constant amperage (300 mA) for 24 min. After electrophoresis, the slide was neutralized in the neutralization buffer (400 mM Tris-HCl, pH 7.5) for 15 min. Finally the slide was washed with water and stained by addition of 60 µl 0.1 mg/ml ethidium bromide. Comets were visualized and photographed with a fluorescence microscope (Olympus IX70) connected to a digital camera (Nikon DXM 1200F). The pictures of 60-110 comets per cell were taken and analyzed by the comet assay software project (CASP).

2.4.7 Detection of XRCC-1 and γ-H2AX foci using immuno-fluorescence staining.

XRCC-1 and γ-H2AX foci were detected by indirect immuno-fluorescence staining. For all experiments using indirect immuno-fluorescence staining, sterile coverslips were placed on the bottom of a 6-well plate. Proliferating cells were seeded as follows: PARG wild type and PARG-Δ2,3 cells were seeded at the density of 2.5 × 10^4 and 5 × 10^4 in each well, respectively. After 48 hrs, cells were ready for the treatment.

After the treatment, cells were washed with PBS and fixed by 5% formaldehyde prepared in PBS for at least 30 min at room temperature in the dark. After fixation, cells were washed with PBS, then incubated in 100 mM glycine in PBS for 1 min to inactivate formaldehyde, permeabilized by addition of 0.4% Triton X-100 in PBS for 3 min and blocked using 3% BSA prepared in PBS for at least 30 min at room temperature. For the detection of XRCC-1
and γ-H2AX foci, cells were incubated with a XRCC-1 (Serotec) and γ-H2AX first antibody (Upstate) diluted 1:200 in blocking solution for 1 hour at 37°C, respectively. After washed with PBS three times, cells were incubated with the secondary antibody coupled with FITC (Jackson laboratories) for 30 min at 37°C. Finally, the slides were embedded in Vectashield mounting medium (Vector laboratories) containing 1 μg/ml DAPI. The XRCC-1 and γ-H2AX foci were visualized by a fluorescence microscope (Olympus IX70) connected to a digital camera (Nikon DXM 1200F). Photographs were taken at 60× magnifications.
CHAPTER 3: RESULTS

3.1 Basic characterizations of PARG-Δ2,3 cells

3.1.1 PARG-Δ2,3 cells proliferate more slowly than wild type cells

As shown in a representative growth curve (Figure 11), PARG-Δ2,3 cells proliferated in the culture medium containing 2ME more slowly than wild type cells. Both wild type and PARG-Δ2,3 cells were seeded at 3000/cm². After 72 hrs, the densities of wild type and PARG-Δ2,3 cells were 41,000/cm² and 14,000/cm², respectively. The density of wild type cells was 2.9 times higher than that of PARG-Δ2,3 cells after 72 hrs growth. The doubling time was calculated based on the linear portion of the growth curve. The average doubling times of wild type and PARG-Δ2,3 cells under these culture conditions were 15.6 and 25.1 hrs, respectively (Table 1).

3.1.2 Effect of withdrawal of 2ME on the doubling time

Since PARG-Δ2,3 mice are hypersensitive to DNA-damaging agents and LPS-induced septic shock (Cortes et al., 2004), it was reasoned that PARG-Δ2,3 cells may be sensitive to oxidative stress present under normal conditions of culture, thus an anti-oxidative agent 2ME was added to culture medium when the PARG-Δ2,3 and wild type cell lines were established. The effect of 2ME withdrawal on cell proliferation was examined. The proliferation rate of wild type cells was unaffected by the presence of 2ME but its withdrawal increased the doubling time of PARG-Δ2,3 cells by approximately 20% (Table 1). Cells were maintained in 2ME-containing medium and 2ME was removed only during the small period of time when the experiments were
Figure 11. Growth curves of wild type and PARG-Δ2,3 cells cultured in medium containing 100µM 2ME.
Both wild type and PARG-Δ2,3 cells were seeded at a density of 3000/cm² in 35-mm dishes. Cell numbers were counted at 24 hrs intervals by the Coulter counter Z2 (Beckman coulter corporation).
Table 1
Effect of 2-mercaptoethanol (2ME) on the doubling time

<table>
<thead>
<tr>
<th></th>
<th>With 2ME (hrs)</th>
<th>Without 2ME (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>15.6 ± 0.3</td>
<td>15.6 ± 0.6</td>
</tr>
<tr>
<td>PARG-∆2,3</td>
<td>25.1 ± 0.3</td>
<td>29.6 ± 0.3</td>
</tr>
</tbody>
</table>

Both wild type and PARG-∆2,3 cells were seeded at a density of 3000/cm². The anti-oxidative agent 2-mercaptoethanol (2ME) at 100µM concentration was added in or removed from the culture medium as indicated. Cell numbers were counted at 24 hrs intervals by the Coulter counter (Beckman Coulter Corporation). The doubling time was calculated based on the linear part of the growth curve.
conducted. Considering this experimental condition, the proliferation of PARG-Δ2,3 cells might be affected more significantly if cells were maintained in medium without 2ME. This result suggests that PARG-Δ2,3 cells may be more sensitive to oxidative stress under usual conditions of culture.

### 3.1.3 PARG-Δ2,3 cells have normal growth properties

The cell cycle distribution was determined using flow cytometry analysis following propidium iodide (PI) staining. Analysis of cell cycle distribution did not reveal any significant differences between PARG-Δ2,3 and wild type cells (Figure 12). The percentage of G1 phase in both wild type and PARG-Δ2,3 cells was about 55% while the percentage of G2/M phase in both cell lines was lower than 10% (Figure 13). The slight differences of the percentage of G1, S, and G2/M phases between wild type and PARG-Δ2,3 cells were statistically analyzed by a Student’s t test. P values of all three phases were much higher than 0.05 (Figure 13), indicating that no significant differences in cell cycle distribution between wild type and PARG-Δ2,3 cells were found.

More PARG-Δ2,3 cells had low values of FSC and SSC revealed by flow cytometry analysis under normal cell culture conditions (Figure 14). These cells, excluded from further flow cytometry analysis, represent cell debris. This observation suggests the possibility of a slightly higher rate of spontaneous apoptosis in PARG-Δ2,3 cells in the absence of genotoxic stress. Despite the difference in proliferation rate of wild type and PARG-Δ2,3 cells, saturation densities of wild type and PARG-Δ2,3 cells were $1.09 \times 10^5$/cm$^2$ and $1.02 \times 10^5$/cm$^2$, respectively (Table 2). Thus overall growth control as reflected by
Figure 12. Cell cycle distribution of wild type and PARG-\(\Delta2,3\) cells under normal culture conditions.

Cell cycle distribution was analyzed by flow cytometry analysis after propidium iodide (PI) staining. Results shown are from a representative experiment.
Figure 13. Cell cycle distribution of wild type and PARG-Δ2,3 cells under normal culture conditions.

Cell cycle distribution was analyzed by flow cytometry analysis after propidium iodide (PI) staining. Results shown are expressed as the mean ± standard deviation (SD) of three independent experiments. Variance was analyzed by a Student’s t test. Open bars represent wild type cells. Black bars represent PARG-Δ2,3 cells.
Figure 14. Flow cytometry analysis of cellular morphology in both wild type and PARG-Δ2,3 cells under normal cell culture conditions. Arrows indicate cells with both low FSC and SSC values which represent cell debris.
Table 2
The saturation density (10^5/cm^2) of wild type and PARG-Δ2,3 cells

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>PARG-Δ2,3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.09 ± 0.11</td>
<td>1.02 ± 0.17</td>
</tr>
</tbody>
</table>

The saturation density was calculated when cell numbers stopped increasing (the plateau phase of the growth curve). Results shown are expressed as the mean ± standard deviation (SD) of three independent experiments.
saturation density was similar between these two cell lines.

### 3.1.4 PARG-Δ2,3 cells have similar pyridine nucleotide pools

NAD and NADP are coenzymes involved in many metabolic reactions and the redox states of the NAD and NADP pools are tightly regulated (Jacobson and Jacobson, 1976). To investigate the effect of the partial PARG gene deletion on NAD and NADP metabolism under normal culture conditions, the NAD and NADP contents and redox states of both pools were determined in wild type and PARG-Δ2,3 cells. Since a previous study has shown that normalized NAD content by total cellular protein was dependent on the cell density (Jacobson and Jacobson, 1976), pyridine nucleotide pools in both proliferating and non-proliferating cells were examined.

Both proliferating and non-proliferating PARG-Δ2,3 cells had similar total NAD and NADP contents normalized by cell numbers and redox states relative to wild type cells (Tables 3 and 4), suggesting that the partial PARG gene deletion per se did not greatly affect NAD and NADP metabolism. Both proliferating and non-proliferating PARG-Δ2,3 cells had slightly higher total protein content (Tables 3 and 4). Flow cytometry analysis showed that PARG-Δ2,3 cells had slightly higher value of FSC (Figure 14), indicating that PARG-Δ2,3 cells have slightly larger size compared to wild type cells. Higher total cellular protein in PARG-Δ2,3 cells is consistent with slightly larger cell size. Total NAD content is several times higher than total NADP content in both wild type and PARG-Δ2,3 cells (Tables 3 and 4). The NAD pool was mainly oxidized and the NADP pool was mainly reduced in both wild type and
Total NAD and NADH contents were determined by the enzymatic cycling assay. The cellular protein was determined by the BCA assay. Results shown are expressed as the mean ± standard deviation (SD) of three independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>NAD (pmol/10^6)</th>
<th>Protein (mg/10^6)</th>
<th>NAD/protein (pmol/mg)</th>
<th>%NADH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proliferating cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1490 ± 250</td>
<td>0.57 ± 0.05</td>
<td>2540 ± 250</td>
<td>18 ± 0.6</td>
</tr>
<tr>
<td>PARG-∆2,3</td>
<td>1430 ± 140</td>
<td>0.70 ± 0.04</td>
<td>2020 ± 150</td>
<td>17 ± 0.3</td>
</tr>
<tr>
<td><strong>Non-proliferating cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1680 ± 50</td>
<td>0.45 ± 0.03</td>
<td>3790 ± 340</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>PARG-∆2,3</td>
<td>1700 ± 80</td>
<td>0.59 ± 0.02</td>
<td>2920 ± 220</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>
Total NADP and NADPH contents were determined by the enzymatic cycling assay. The cellular protein was determined by the BCA assay. Results shown are expressed as the mean ± standard deviation (SD) of three independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>NADP (pmol/10⁶)</th>
<th>Protein (mg/10⁶)</th>
<th>NADP/protein (pmol/mg)</th>
<th>%NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proliferating cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>180 ± 20</td>
<td>0.57 ± 0.05</td>
<td>320 ± 2</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>PARG-Δ2,3</td>
<td>170 ± 20</td>
<td>0.70 ± 0.04</td>
<td>240 ± 30</td>
<td>91 ± 5</td>
</tr>
<tr>
<td><strong>Non-proliferating cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>190 ± 20</td>
<td>0.45 ± 0.03</td>
<td>420 ± 80</td>
<td>90 ± 3</td>
</tr>
<tr>
<td>PARG-Δ2,3</td>
<td>160 ± 10</td>
<td>0.59 ± 0.02</td>
<td>280 ± 20</td>
<td>110 ± 14</td>
</tr>
</tbody>
</table>
PARG-Δ2,3 cells (Tables 3 and 4). These results are consistent with previous observations (Jacobson and Jacobson, 1976).

3.2 Altered NAD and PAR metabolism in PARG-Δ2,3 cells following MNNG treatment

3.2.1 PARG-Δ2,3 cells deplete NAD more rapidly

Since NAD depletion has been linked to PARP-1 mediated cell death (Pieper et al., 1999) (Berger, 1985), we examined the effect of the partial PARG gene deletion on NAD and NADP metabolism following MNNG treatment. Time- and dose-dependent responses of total NAD and NADP content were determined using the enzymatic cycling assays following MNNG treatment (Figures 15 and 16). Proliferating PARG-Δ2,3 cells showed more rapid depletion of total NAD following MNNG treatment (Figure 15). Since the majority of the NAD pool is present as NAD$^+$ and NADH content did not show an appreciable change compared with the change of NAD$^+$, NAD depletion can be attributed primarily to the loss of NAD$^+$. In contrast to the results seen with NAD content, total NADP content remained relatively constant over the initial 45 min where total NAD content was rapidly depleted and wild type and PARG-Δ2,3 cells did not show significant differences in NADP content at later time points where NADP content began to decrease (Figure 16, left panel) and no significant differences were observed as a function of dose of MNNG at 30 min (Figure 16, right panel), although some scatter was noted in the data.
Figure 15. NAD content of proliferating wild type and PARG-Δ2,3 cells following MNNG treatment. 

Left panel, cells were treated by 50 µM MNNG at different time points as indicated. Right panel, cells were treated by the various concentrations of MNNG as indicated for 30 min. Variances in NAD content at different time points were analyzed by a Student’s t test. P<0.05 was considered to be significant. ** : P<0.01, * : P<0.05. Circles represent wild type cells and triangles represent PARG-Δ2,3 cells. Some error bars are too small to be visible.
Figure 16. NADP content of proliferating wild type and PARG-Δ2,3 cells following MNNG treatment. 
**Left panel,** cells were treated by 50 μM MNNG at different time points as indicated. **Right panel,** cells were treated by the various concentrations of MNNG as indicated for 30 min. Circles represent wild type cells and triangles represent PARG-Δ2,3 cells. Some error bars are too small to be visible.
Since the redox states of NAD and NADP pool contribute to the maintenance of proper intercellular redox state (Starkov and Fiskum, 2003), they were determined using the enzymatic cycling assays following MNNG treatment. The percentage of NADH in the NAD pool increased in both wild type and PARG-Δ2,3 cells following MNNG treatment but increased more rapidly in PARG-Δ2,3 cells following different concentrations of MNNG treatment (Figure 17, right panel), which can be attributed to a more rapid NAD⁺ depletion in PARG-Δ2,3 cells with NADH content remaining constant. The percentage of NADH in PARG-Δ2,3 cells also increased more rapidly than that in wild type cells at 30, 45 and 60 min following 50 μM MNNG treatment (Figure 17, left panel). The NADP pool remained mainly reduced in both wild type and PARG-Δ2,3 cells over the initial 2 hrs following 50 μM MNNG treatment and following treatment with different doses of MNNG for 30 min where the percentage of NADH in the NAD pool increased in both cell lines (Figures 17 and 18).

Total NAD and NADP content and redox states of both pools were also determined in non-proliferating wild type and PARG-Δ2,3 cells following MNNG treatment. Non-proliferating cells showed the same pattern as observed in proliferating cells (Figures 19-22), indicating that following MNNG treatment, NAD, but not NADP, depleted more rapidly in PARG-Δ2,3 cells independent of proliferation status.

3.2.2 PARG-Δ2,3 cells accumulate less PAR
Figure 17. Redox state of the NAD pool of proliferating wild type and PARG-Δ2,3 cells following MNNG treatment. 

Left panel, cells were treated by 50 μM MNNG at different time points as indicated. Right panel, cells were treated by the various concentrations of MNNG as indicated for 30 min. Variances in percentage of NADH at different time points were analyzed by a Student’s t test. P<0.05 was considered to be significant. ** : P<0.01, * : P<0.05. Circles represent wild type cells and triangles represent PARG-Δ2,3 cells. Some error bars are too small to be visible.
Figure 18. Redox state of the NADP pool of proliferating wild type and PARG-Δ2,3 cells following MNNG treatment. Left panel, cells were treated by 50 μM MNNG at different time points as indicated. Right panel, cells were treated by the various concentrations of MNNG as indicated for 30 min. Circles represent wild type cells and triangles represent PARG-Δ2,3 cells. Some error bars are too small to be visible.
Figure 19. NAD content of non-proliferating wild type and PARG-Δ2,3 cells following MNNG treatment. 
Left panel, cells were treated by 50 µM MNNG at different time points as indicated. Right panel, cells were treated by the various concentrations of MNNG as indicated for 30 min. Variances in NAD content at different time points were analyzed by a Student's t test. P<0.05 was considered to be significant. ** : P<0.01, * : P<0.05. Circles represent wild type cells and triangles represent PARG-Δ2,3 cells. Some error bars are too small to be visible.
Figure 20. NADP content of non-proliferating wild type and PARG-Δ2,3 cells following MNNG treatment. 

Left panel, cells were treated by 50 µM MNNG at different time points as indicated. Right panel, cells were treated by the various concentrations of MNNG as indicated for 30 min. Circles represent wild type cells and triangles represent PARG-Δ2,3 cells.
Figure 21. Redox state of the NAD pool of non-proliferating wild type and PARG-Δ2,3 cells following MNNG treatment. **Left panel**, cells were treated by 50 µM MNNG at different time points as indicated. **Right panel**, cells were treated by the various concentrations of MNNG as indicated for 30 min. Variances in percentage of NADH at different time points were analyzed by a Student’s t test. P<0.05 was considered to be significant. ****: P<0.01, *: P<0.05. Circles represent wild type cells and triangles represent PARG-Δ2,3 cells. Some error bars are too small to be visible.
Figure 22. Redox state of the NADP pool of non-proliferating wild type and PARG-Δ2,3 cells following MNNG treatment.

**Left panel**, cells were treated by 50 μM MNNG at different time points as indicated. **Right panel**, cells were treated by the various concentrations of MNNG as indicated for 30 min. Circles represent wild type cells and triangles represent PARG-Δ2,3 cells.
In order to study possible mechanisms of more rapid NAD depletion in PARG-Δ2,3 cells, total PAR content was quantified using a \(^3\)H-labeling method (Aboul-Ela et al., 1988) that involves treatment of cells with TCA to rapidly quench all enzymatic activity. One advantage of this method is that NAD and PAR can be quantified using the same cell samples (see section 1.1.3). PAR was quantified using the TCA pellet and NAD was quantified using the TCA supernatant (Figure 10). PAR was isolated by applying the dissolved TCA pellet into DHB-Bio-Rex column (Figure 10). Then PAR was subjected to enzymatic digestion by snake venom phosphodiesterase and bacterial alkaline phosphatase (SVPD/BAP) to generate the unique nucleoside ribosyladenosine (RAdo). After the enzymatic digestion, the samples were injected into HPLC and the fractions of each minute were collected from the HPLC eluate. The radioactivity of each fraction was determined by scintillation counting. As shown in Figure 23, most radioactivity was present in the RAdo peak. The last unit of ADP-ribose in PAR generates the adenosine after the enzymatic digestion by SVPD/BAP. The adenosine could also come from RNA. An adenosine peak eluted several minutes earlier than the RAdo peak (Figure 23). To examine the possibility that the TCA supernatant contained a significant amount of PAR, we also examined PAR in the TCA supernatants. No significant amounts of PAR were detected in any of the samples (Figure 24), ruling out the possibility that acid soluble PAR was present in the TCA supernatant.

PARG-Δ2,3 cells unexpectedly showed less PAR accumulation despite the more rapid NAD\(^+\) consumption following MNNG treatment. PARG-Δ2,3
Figure 23. Determination of RAdo in MNNG-treated cells by HPLC. The radioactivity of each fraction of HPLC eluate collected every minute was determined by scintillation counting. The ribosyladenosine (RAdo) peak is indicated by the arrow. The small peak eluted earlier than RAdo is an adenosine peak.
Figure 24. Lack of detection of PAR in the TCA supernatant. 
**Left panel**, wild type cells treated by 250 μM MNNG for 20 min. The arrow indicates the location of RAdo. **Right panel**, PARG-Δ2,3 cells treated by 250 μM MNNG for 20 min. The arrow indicates the location of RAdo. Slightly different chromatographic conditions compared to Figure 23. Adenosine is abbreviated as Ado.
cells accumulated 18.5% of total PAR compared to wild type cells following 250 µM MNNG treatment for 20 min (Figure 25). Figure 26 shows a representative experiment of both NAD and PAR content following 250 µM MNNG treatment at different time points as indicated. The left panel of Figure 26 shows NAD content determined in the same samples where PAR was determined. The peak levels of PAR in PARG-∆2,3 and wild type cells following MNNG treatment were approximately 5.5 pmol/10^6 and 14.6 pmol/10^6 cells, respectively. These values represent total content of ADP-ribose residues present in PAR.

In order to examine PAR accumulation by a different method, time- and dose-dependent PAR accumulation was examined by a semi-quantitative Western blot assay. As shown in Figure 27 A, more PAR was detected in wild type cells than PARG-∆2,3 cells following treatment with 250 µM MNNG. Figure 27B also shows more PAR accumulation in wild type cells than PARG-∆2,3 cells following treatment with various concentrations of MNNG for 20 min. Figure 27 B also indicates that higher concentrations of MNNG resulted in less PAR accumulation at 20 min, which is consistent with more extensive NAD depletion and PAR turnover. The density of PAR signals assessed by Western blot analysis was quantitatively determined by an ImageQuant software. The quantitative analysis of Western bolts shows that PAR level in PARG-∆2,3 cells was 12% of that in wild type cells following 250 µM MNNG treatment for 20 min (Figure 28).
Figure 25. Determination of PAR by a $^3$H-labeling method.
Wild type and PARG-Δ2,3 cells were treated by 250 μM MNNG for 20 min. PAR level was determined by a $^3$H-labeling method. Data from three independent experiments are expressed as mean ± standard error of the mean (SEM). Variance was analyzed by a Student's t test. P<0.05 was considered to be significant. Open bars represent wild type cells. Black bars represent PARG-Δ2,3 cells. The average PAR level in PARG-Δ2,3 cells was 18.5% of that in wild type cells.
Figure 26. NAD$^+$ and PAR content using the same cell samples following 250 μM MNNG treatment. 

**Left panel,** NAD content was determined by an enzymatic cycling assay. 
**Right panel,** PAR content was determined by the $^3$H-labeling method. Circles represent wild type cells and triangles represent PARG-Δ2,3 cells. Data shown are from a representative experiment.
Figure 27. PAR accumulation assessed by Western blot analysis.

A. **Time course of PAR accumulation.** Wild type and PARG-Δ2,3 cells were treated by 250 µM MNNG for different time points as indicated.

B. **Dose response of PAR accumulation.** Wild type and PARG-Δ2,3 cells were treated by different concentrations of MNNG as indicated for 20 min. A PAR antibody SA-216 from Biomol was used for detection. Equal loading was confirmed by Ponceau S staining (not shown).
Figure 28. Quantitative analysis of Western blots of PAR accumulation by an ImageQuant software.
Wild type and PARG-Δ2,3 cells were treated by 250 μM MNNG for 20 min. The density of PAR signals assessed by Western blot analysis was quantitatively determined by an ImageQuant software. Data from three independent experiments are expressed as mean ± standard error of the mean (SEM). Variance was analyzed by a Student’s t test. P<0.05 was considered to be significant. Open bars represent wild type cells. Black bars represent PARG-Δ2,3 cells. The average PAR level in PARG-Δ2,3 cells was 12% of that in wild type cells.
3.3 Estimation of PARP and PARG activity in intact cells following MNNG treatment

3.3.1 Both PARG and PARP activities are increased in PARG-Δ2,3 cells

Data from the kinetics of NAD depletion and PAR accumulation from the same cell samples allowed an estimation of PARP and PARG activity in intact cells following MNNG treatment. If all of the NAD depletion following MNNG treatment can be attributed to PARP activity, the rate of NAD depletion at any point in time would represent the metabolic flux through PARP in intact cells. Previous studies have established that addition of the PARP inhibitor benzamide to the culture medium at 1 mM will selectively inhibit cellular PARPs without appreciable effects on other classes of NAD consuming enzymes (Rankin et al., 1989). Addition of 1 mM benzamide to the culture medium prior to treatment with MNNG as shown in Figure 29 completely blocked NAD depletion in both PARG-Δ2,3 and wild type cells and strongly inhibited PAR accumulation (Figure 30), demonstrating that the rate of NAD depletion reflected primarily cellular PARP activity following MNNG treatment. Figure 29 and figure 30 also show that DMSO and benzamide alone did not result in NAD consumption or PAR accumulation. Thus, the rate of NAD depletion represents PARP activity following MNNG treatment in both wild type and PARG-Δ2,3 cells.

The level of PAR accumulation at a specific time following MNNG treatment reflects the difference between the rate of PAR synthesis and rate of PAR degradation. Thus, PARG activity can be estimated by subtraction of
Figure 29. Effect of benzamide (Bz) on NAD depletion following MNNG treatment.
Wild type and PARG-Δ2,3 cells were pretreated by 1 mM benzamide for 1 hour then treated by 250 µM MNNG for 20 min. NAD was quantified by an enzymatic cycling assay. Vehicle represents DMSO in this experiment. Data shown are from a representative experiment.
Figure 30. Effect of benzamide (Bz) on PAR accumulation following MNNG treatment.
Wild type and PARG-Δ2,3 cells were pretreated by 1 mM benzamide for 1 hour then treated by 250 µM MNNG for 20 min. PAR was quantified by a \(^3\)H-labeling method. Vehicle represents DMSO in this experiment. Data shown are from a representative experiment.
PAR accumulation at a specific time from the rate of NAD depletion. Table 5 shows the estimation from a representative experiment of PARP and PARG activity in intact cells following MNNG treatment. At 5 min following MNNG treatment, PARG activity in PARG-Δ2,3 and wild type cells was very similar but at later times PARG activity was estimated to be 2.6 to 3.1 times higher in PARG-Δ2,3 cells. PARG activity was decreased at later time points (10 and 20 minutes) than that at 5 min following MNNG treatment in wild type cells, but this decrease was not observed in PARG-Δ2,3 cells (Table 5). Likewise, estimated PARP activity was similar at 5 min but at later time points was between 1.9 and 2.6 times higher in PARG-Δ2,3 cells. And PARP activity was decreased at later time points (10 and 20 minutes) than that at 5 min following MNNG treatment in wild type cells, but this decrease was not observed in PARG-Δ2,3 cells (Table 5).

3.3.2 PARG-Δ2,3 cells have a lower degree of PARP-1 automodification

Since PARP-1 is the source of most PAR synthesis following genotoxic stress, the PARP activity estimated in Table 5 represents mainly PARP-1 activity. PARP-1 also is the main acceptor protein for PAR following genotoxic stress and PARP-1 activity is dependent upon its degree of automodification where increased automodification results in decreased activity (Kawaichi et al., 1981). It is therefore predicted that the increased PARG activity in PARG-Δ2,3 cells would lead to a decrease in PARP-1 automodification and a concomitant increase in PARP-1 activity.

To test the prediction concerning PARP-1 automodification, the degree of automodification of PARP-1 was determined by Western blot analysis using a
Table 5
Estimation of PARP and PARG activity in intact cells following MNNG treatment

<table>
<thead>
<tr>
<th>Minutes following MNNG treatment</th>
<th>PARP activity (pmol/min/10⁶)</th>
<th>PARG activity (pmol/min/10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT PARG-Δ2,3 PARG-Δ2,3/WT</td>
<td>WT PARG-Δ2,3 PARG-Δ2,3/WT</td>
</tr>
<tr>
<td>5</td>
<td>67.3 60.2 0.89</td>
<td>66.2 58.3 0.88</td>
</tr>
<tr>
<td>10</td>
<td>21.2 55.8 2.64</td>
<td>16.3 50.7 3.11</td>
</tr>
<tr>
<td>20</td>
<td>34.0 65.1 1.91</td>
<td>22.5 59.6 2.65</td>
</tr>
</tbody>
</table>

The data from the experiment shown in Figure 26 were used to estimate the activity of PARP and PARG in intact cells following MNNG treatment. PARP activity was estimated by the rate of NAD depletion and PARG activity was estimated by the difference between the rate of NAD depletion and amount of PAR accumulated at the time points shown. Data are from a representative experiment.
PARP-1 antibody against the DNA binding domain of the protein that binds both unmodified and modified PARP-1. PARG-Δ2,3 cells showed a lower degree of PARP-1 automodification than wild type cells, particularly at 20 and 30 min post MNNG treatment (Figure 31, top panel). PAR determined using the same cell samples also showed less intense signals at all time points (Figure 31, bottom panel), which is consistent with the anti-PARP-1 blot. The density of unmodified PARP-1 assessed by Western blot analysis was quantitatively analyzed by an ImageQuant software. PARG-Δ2,3 cells showed more unmodified PARP-1 at 20 and 30 min after 500 µM (Figure 32, left panel) and 250 µM MNNG treatment (Figure 32, right panel). Taken together, these data demonstrate that PARG-Δ2,3 cells show a lower degree of automodification of PARP-1 that is consistent with higher PARP-1 activity.

In summary, both PARG and PARP activity in PARG-Δ2,3 cells are unexpectedly higher than those in wild type cells, and PAR level is lower in PARG-Δ2,3 cells. These observations show that the partial PARG gene deletion alters PAR metabolism.

3.4 Several cellular responses related to DNA repair are defective in PARG-Δ2,3 cells following sublethal MNNG treatment.

3.4.1 MNNG at 50 µM is a sublethal dose for both the cell lines

To investigate DNA repair process in the absence of inducible apoptosis in both wild type and PARG-Δ2,3 cells, a sublethal dose of MNNG was determined using flow cytometry analysis following Annexin V-PI staining.
Figure 31. Automodification of PARP-1 and PAR accumulation as determined by Western blot analysis following MNNG treatment. Wild type and PARG-Δ2,3 cells were treated by 500 µM MNNG for different time points as indicated. MNNG was added into culture medium for 5 min. Then the medium containing MNNG was replaced by the normal culture medium. The time points shown are from the addition of MNNG. A PARP-1 antibody NB 110-111 against the DNA binding domain was used in the top panel. PAR accumulation was determined by a PAR antibody SA-216 shown in the bottom panel using the same cell samples from the top panel. Equal loading was confirmed by Ponceau S staining (not shown). Data shown are from a representative experiment.
Figure 32. Quantitative analysis of Western blots of unmodified PARP-1 by an ImageQuant software. 

**Left panel,** the density of unmodified PARP-1 shown in the top panel of Figure 31 was quantitatively determined by an ImageQuant software. 

**Right panel,** the density of Western blots of unmodified PARP-1 was quantitatively determined by an ImageQuant software. Wild type and PARG-Δ2,3 cells were treated by 250 µM MNNG for 10 min. Then medium containing MNNG was replaced by the normal culture medium. The time points shown are from the addition of MNNG.
As shown in Figure 33, the percentages of both wild type and PARG-Δ2,3 cells in Annexin V and PI double negative quadrant (lower left) following 50 µM MNNG treatment were similar to those of untreated and DMSO treated samples, indicating that 50 µM MNNG did not induce apoptosis in either PARG-Δ2,3 or wild type cells under these conditions at 24 hrs. When the MNNG concentration was raised to 100 µM, the percentage of viable wild type cells was 94%, but the percentage of viable PARG-Δ2,3 cells was 86%. This result indicates that apoptosis was induced in a small percentage of PARG-Δ2,3 cells but not in wild type cells following 100 µM MNNG treatment (Figure 33). Treatment with 1 µM STR served as a positive control in this flow cytometry analysis. The induction of apoptosis in both cell lines following 1 µM STR rules out the possibility of insensitivity of this analysis. Interestingly, PARG-Δ2,3 cells were more resistant to STR relative to wild type cells, which is in contrast to the response to MNNG treatment described above.

3.4.2 Less PARG-Δ2,3 cells are arrested in G2 phase

The percentages of PARG-Δ2,3 cells in G1, S, and G2/M phases were similar to those in wild type cells in the absence of genotoxic stress (Figures 12 and 13). The cell cycle distribution of both cell lines was also analyzed following sublethal MNNG treatment. Since many studies have demonstrated that MNNG induces G2/M arrest in a variety of cell lines (Park et al., 2004) (Adamson et al., 2005) (Fang et al., 2001) (Jaiswal et al., 2004), the percentage of PARG-Δ2,3 cells in G2/M phase was compared with wild type cells. The percentage of wild type cells distributed in G2/M phase was 18.9%
Figure 33. A sublethal dose of MNNG for both wild type and PARG-Δ2,3 cells was determined by flow cytometry analysis. Cells were treated by different concentrations of MNNG as indicated for 30 min. Then medium containing MNNG was replaced by normal culture medium. Flow cytometry analysis was conducted at 24 hrs after treatment. Staurosporine serves as a positive control in this analysis.
following 5 μM MNNG treatment while the percentage of the untreated wild
type cells distributed in G2/M phase was 3.1% (Figure 34). The percentage of
PARG-Δ2,3 cells distributed in G2/M phase was 5.4% following 5 μM MNNG
treatment while the percentage of the untreated PARG-Δ2,3 cells distributed
in G2/M phase was 6.5% (Figure 34). This result indicates that wild type cells
may be more sensitive to G2/M arrest following sublethal MNNG treatment.

Since the majority of cells in G2/M peak actually represent the cells in G2
phase, the percentage of cells in G2/M peak can be approximately considered
as the percentage of cells in G2 phase. The G2 checkpoint provides time for
cells to repair DNA damage and thus prevents the accumulation of mutations
(Sancar et al., 2004) (Latif et al., 2004) (Dasika et al., 1999). The percentage
of cells in G2 phase was determined following 0, 10, 20, 30, 40 μM MNNG
treatment. As shown in Figure 35, both wild type and PARG-Δ2,3 cells were
arrested in G2 phase in a dose-dependent manner following sublethal MNNG
treatment. However, less PARG-Δ2,3 cells were arrested in G2 phase relative
to wild type cells at every dose of sublethal MNNG treatment. The less
efficient G2 checkpoint suggests that less time is available for DNA repair in
PARG-Δ2,3 cells relative to wild type cells following sublethal MNNG
treatment.

3.4.3 PARG-Δ2,3 cells form less XRCC1 foci

XRCC1 (X-ray repair cross-complementing protein 1), a critical
component of the DNA single strand break repair (SSBR) complex, has been
shown to rapidly assemble into discrete nuclear foci at the sites of DNA
Figure 34. Cell cycle distribution of wild type and PARG-Δ2,3 cells following 5 μM MNNG treatment was determined by flow cytometry analysis.

Cells were treated by 5 μM MNNG for 30 min. Then medium containing MNNG was replaced by normal culture medium. Flow cytometry analysis was conducted at 72 hrs after treatment.
Figure 35. G2 arrest in wild type and PARG-Δ2,3 cells following various concentrations of MNNG treatment. Cells were treated by different concentrations of MNNG as indicated for 30 min. Then medium containing MNNG was replaced by normal culture medium. Flow cytometry analysis was conducted at 72 hrs after treatment.
damage following genotoxic stress (El-Khamisy et al., 2003) (Taylor et al., 2000). XRCC1 interacts with other enzymatic proteins involved in SSBR and stabilizes the repair complex (Caldecott, 2003; Caldecott and Chalmers, 2005). A recent study has shown that the formation of XRCC1 foci was deficient in PARP-1 knockout cells (El-Khamisy et al., 2003). To examine the effect of the partial PARG gene deletion on the formation of XRCC1 foci following MNNG treatment, XRCC1 foci were determined by indirect immunostaining analysis. XRCC1 distributed in the whole nucleus and no significant amount of foci was detected in either wild type or PARG-Δ2,3 cells under normal cell culture conditions. Many XRCC1 foci were detected in wild type cells but not in PARG-Δ2,3 cells following 50 μM MNNG treatment (Figure 36).

To examine the possibility that the deficient formation of XRCC1 foci in PARG-Δ2,3 cells resulted from less XRCC1 protein level, total XRCC1 protein levels of both wild type and PARG-Δ2,3 cells were determined by Western blot analysis. Total XRCC1 protein levels in PARG-Δ2,3 cells were similar to those in wild type cells before MNNG treatment, after 50 μM MNNG treatment (R0), and 4, 8, 16 hrs after the MNNG containing medium was replaced by the normal culture medium (R4, R8, and R16) (Figure 37). The alteration of gene expression induced by MNNG has been studied by microarray and real time RT-PCR, and no alteration of XRCC1 expression has been reported (Suzuki et al., 2003); (Bae et al., 2003). As shown in Figure 37, no significant differences of XRCC1 protein levels were observed at different time points of
Figure 36. XRCC1 foci in wild type and PARG-Δ2,3 cells following 50µM MNNG treatment as determined by indirect immuno-fluorescence staining.

Wild type and PARG-Δ2,3 cells were treated by 50 µM MNNG for 20 min and then incubated in the drug-free medium for 10 min. Untreated and treated cells were then fixed with 5% formaldehyde and immunostained with a XRCC1 antibody (AHP832, Serotec) for the analysis of indirect immuno-fluorescence. Representative images were photographed at 60× magnifications.
**Figure 37.** XRCC1 protein levels at different time points following 50 µM MNNG treatment as determined by Western blot analysis.
Wild type and PARG-Δ2,3 cells were treated with 50 µM MNNG for 20 min (R0, no recovery) and then the drug-free culture medium was added back for up to 16 hrs. Untreated cells, cells without recovery (R0) and cells at 4, 8 and 16 hrs of recovery (R4, R8 and R16) were extracted and XRCC1 protein levels were determined by Western blot analysis using the same first antibody as described in Figure 36 for indirect immuno-fluorescence staining (AHP832, Serotec). Equal loading was confirmed by Ponceau S staining.
recovery relative to the control sample in both wild type and PARG-Δ2,3 cells, indicating that MNNG has no effect on the gene expression of XRCC1.

3.4.4 PARG-Δ2,3 cells form less γ-H2AX foci

γ-H2AX, the DNA damage-induced phosphorylated histone H2A at serine 139, has been shown to facilitate DNA repair by its involvement in modulation of chromatin structure (Lowndes and Toh, 2005) (Morrison et al., 2004) (Thiriet and Hayes, 2005). Although γ-H2AX is frequently associated with DNA double strand break repair (Chowdhury et al., 2005) (Mahrhofer et al., 2006), the formation of γ-H2AX foci has been recently observed in MNNG-treated 293T Lα cells (Stojic et al., 2004). In the same paper, MNNG-induced γ-H2AX foci were proposed to represent regions of altered chromatin structure where the base excision repair (BER) is proceeding (Stojic et al., 2004).

To examine the formation of γ-H2AX foci in mouse fibroblasts treated by sublethal MNNG and further investigate the effect of the partial PARG gene deletion on the formation of these foci, indirect immunostaining analysis was conducted. No significant amount of γ-H2AX foci was detected in control samples of either wild type or PARG-Δ2,3 cells (Figure 38). Many γ-H2AX foci were detected in wild type cells but not in PARG-Δ2,3 cells following 50 μM MNNG treatment (Figure 38). Western blot analysis also indicated that PARG-Δ2,3 cells were deficient in γ-H2AX formation relative to wild type cells following 50 μM MNNG treatment (Figure 39). Two bands were detected in wild type cells at 8 and 16 hrs of recovery using a specific γ-H2AX antibody,
Figure 38. γ-H2AX foci in wild type and PARG-Δ2,3 cells following 50μM MNNG treatment as determined by indirect immunofluorescence staining. Wild type and PARG-Δ2,3 cells were treated by 50 μM MNNG for 20 min and then incubated in the drug-free medium for 10 min. Untreated and treated cells were then fixed with 5% formaldehyde and immunostained with a γ-H2AX antibody (JBW301, Upstate) for the analysis of indirect immuno-fluorescence. Representative images were photographed at 60× magnifications.
Figure 39. Phosphorylation of H2AX at different time points following 50 μM MNNG treatment as determined by Western blot analysis.

Wild type and PARG-Δ2,3 cells were treated with 50 μM MNNG for 20 min (R0, no recovery) and then the drug-free culture medium was added back for up to 16 hrs. Untreated cells, cells without recovery (R0) and cells at 4, 8 and 16 hrs of recovery (R4, R8 and R16) were extracted and γ-H2AX level was determined by western blot analysis using the same first antibody as described in Figure 38 for indirect immunofluorescence staining (JBW301, Upstate). Equal loading was confirmed by Ponceau S staining.
suggesting that histone H2A had other modifications besides phosphorylation at serine 139 (Figure 39).

3.5 Determination of DNA strand breaks following sublethal MNNG treatment

3.5.1 Less DNA strand breaks are detected in PARG-Δ2,3 cells

The comet assay (single-cell gel electrophoresis assay) is a widely applied method to measure DNA strand breaks in mammalian cells (Brendler-Schwaab et al., 2005; Kumaravel and Jha, 2006). Among many parameters of the comet assay, tail moment (the product of the percent of DNA in the tail and the tail length) shows a good correlation with DNA strand breaks and is widely utilized as a sensitive indicator of DNA strand breaks (Collins, 2004). Thus, DNA strand breaks were determined by the alkaline comet assay and quantitatively reflected by calculated tail moments in both wild type and PARG-Δ2,3 cells. The typical images of the comet assay using a control sample and a MNNG treated sample were shown in Figure 40. Surprisingly, less DNA strand breaks were detected in PARG-Δ2,3 cells at every time points of recovery examined following 50 µM MNNG treatment (Figure 41). Interestingly, another peak of DNA strand breaks was observed in both wild type and PARG-Δ2,3 cells at 4 hrs of recovery after the initial introduction of DNA strand breaks by treatment with 50 µM MNNG for 20 min (R0). Although DNA strand breaks at 8 and 16 hrs of recovery were less than those at 4 hrs of recovery, they were still more than those at R0 in both wild type and PARG-Δ2,3 cells (Figure 41).
Figure 40. Typical images of the comet assay of an untreated and MNNG-treated cell.
Figure 41. Comparison of DNA strand breaks in wild type and PARG-Δ2,3 cells following 50μM MNNG treatment as determined by the comet assay.

Wild type (black) and PARG-Δ2,3 cells (gray) were treated with 50 μM MNNG for 20 min and then incubated in the drug-free medium for up to 16 hrs. DNA breaks in untreated cells, cells without recovery (R0) and cells at 4, 8 and 16 hrs of recovery (R4, R8 and R16) were determined by the comet assay. 110 comets were analyzed by the comet assay software project (CASP) per slide. P value of control between wild type and PARG-Δ2,3 cells was 0.7512. P values of R0, R4, R8 and R16 between wild type and PARG-Δ2,3 cells were less than 0.0001.
DNA strand breaks were detected following 100 µM MNNG treatment as well. MNNG at 100 µM concentration started inducing apoptosis in PARG-Δ2,3 cells but not in wild type cells (Figure 33). Since DNA strand breaks can be generated in the late stage of apoptosis, the breaks were determined in only short period of recovery following 100 µM MNNG treatment. Similar to 50 µM MNNG treatment, PARG-Δ2,3 cells formed less DNA strand breaks at 0 and 1 hr of recovery relative to wild type cells following 100 µM MNNG treatment (Figure 42).

3.5.2 MNNG induces unligatable DNA strand breaks

DNA strand breaks were persistent even 48 hrs after MNNG treatment in mismatch (MMR) proficient cells, suggesting that MNNG induces unligatable DNA strand breaks (Stojic et al., 2004). To examine whether unligatable DNA strand breaks exist in wild type mouse fibroblasts, a 10 µM dose of MNNG was chosen to induce mild DNA damage since DNA strand breaks were persistent at 16 hrs after 50 µM MNNG treatment (Figure 41). After 10 µM MNNG treatment for 20 min, a relatively small number of DNA strand breaks were generated and the number remained similar within 24 hrs after the treatment, but DNA strand breaks increased at 48 hrs compared with that at 24 hrs (Figure 43). The increased DNA strand breaks at later time points of recovery suggest that some DNA break intermediates during repair can not be religated in mouse fibroblasts following MNNG treatment.

3.5.3 The alkaline comet assay mainly measures DNA single-strand breaks
Figure 42. Comparison of DNA strand breaks in wild type and PARG-Δ2,3 cells following 100μM MNNG treatment as determined by the comet assay.

Wild type (black) and PARG-Δ2,3 cells (gray) were treated with 100 μM MNNG for 20 min and then incubated in the drug-free medium for 1 hour. DNA breaks in untreated cells, cells without recovery (R0) and cells at 1 hour of recovery (R1) were determined by the comet assay. 50 comets were analyzed by the comet assay software project (CASP) per slide. P value of control between wild type and PARG-Δ2,3 cells was 0.41. P value of R0 between wild type and PARG-Δ2,3 cells was 0.004. P values of R1 between wild type and PARG-Δ2,3 cells was less than 0.0001.
Figure 43. DNA strand breaks in wild type cells following 10μM MNNG treatment as determined by the comet assay.

Wild type cells were treated with 10 μM MNNG for 20 min and then incubated in the drug-free medium for up to 48 hrs. DNA breaks in untreated cells, cells without recovery (R0) and cells at 2, 24 and 48 hrs of recovery (R2, R24 and R48) were determined by the comet assay. 60 comets were analyzed by the comet assay software project (CASP) per slide. P value of R0 compared with control was less than 0.0001. P value of R48 compared with R24 was less than 0.0001.
The alkaline comet assay can measure DNA single strand breaks, DNA double-strand breaks and alkaline labile sites. The neutral pH comet assay only measures DNA-double strand breaks. The neutral pH comet assay can not detect DNA strand breaks following low dose MNNG treatment, indicating that low dose MNNG does not induce significant amount of DNA double-strand breaks (Stojic et al., 2004). Alkaline-labile sites induced by other DNA damaging agents have been reported previously (Ochi et al., 1986) (Kasahara et al., 1993). But whether MNNG induces alkaline-labile sites in cells has not been well investigated. The tail moment at 2 hrs of recovery following 10 \( \mu \text{M} \) MNNG treatment for 10 min was similar to that in the untreated wild type cells (Figure 44), indicating that low dose MNNG treatment does not induce significant amount of alkaline-labile sites in the cell model used in this dissertation. Thus, the alkaline comet assay mainly measures DNA single-strand breaks following sublethal MNNG treatment in mouse fibroblasts.

3.6 Effect of the partial PARG gene deletion on cell viability following MNNG treatment

3.6.1 PARG-\( \Delta 2,3 \) cells are more sensitive to MNNG treatment

A general pattern observed with PARP-1 knockout animals or cells derived from them is that highly proliferating cells or tissues are hypersensitive to alkylating agents while non-proliferating cells or tissues are more resistant than wild type (Eliasson et al., 1997) (de Murcia et al., 1997) (Le Rhun et al., 1998). Thus, the responses of both proliferating and non-proliferating PARG-\( \Delta 2,3 \) cells were investigated following the treatment with
Figure 44. DNA strand breaks in wild type cells following minimal level of DNA damage for up to 48 hrs recovery as determined by the comet assay.

Wild type cells were treated with 10 µM MNNG for 10 min and then incubated in the drug-free medium for up to 48 hrs. DNA breaks in untreated cells, cells at 2 and 48 hrs of recovery (R2, R48) were determined by the comet assay. 60 comets were analyzed by the comet assay software project (CASP) per slide. P value of R2 compared with control was 0.87. P value of R48 compared with R2 was less than 0.0001.
various concentrations of MNNG. For proliferating cells, the percentage of viable wild type cells was 91% while the percentage of viable PARG-Δ2,3 cells was 69% following 200 μM MNNG treatment (Figure 45). Following 500 μM MNNG treatment, the percentage of viable wild type cells was 43% while the percentage of viable PARG-Δ2,3 cells was only 13% (Figure 45). For non-proliferating cells, the percentage of viable wild type cells was 80% while the percentage of viable PARG-Δ2,3 cells was 60% following 1 mM MNNG treatment (Figure 46). Following 1.5 mM MNNG treatment, the percentage of viable wild type cells was 72% while the percentage of viable PARG-Δ2,3 cells was only 39% (Figure 46). These data indicate that both proliferating and non-proliferating PARG-Δ2,3 cells were more sensitive to the cytotoxic effects of MNNG relative to wild type cells, demonstrating that unlike PARP-1 knockout cells, the effect of the partial PARG gene deletion on cell responses to genotoxic stress is independent on proliferation status at least for MNNG. These data also demonstrate that non-proliferating cells are more resistant to the cytotoxic effects of MNNG independent of PARG status as higher MNNG concentrations were required to achieve a comparable amount of cell death in non-proliferating cells.

3.6.2 A pan-caspase inhibitor did not block cell death in either cell line following MNNG treatment

The majority of cells killed by MNNG were distributed in Annexin V⁺PI⁻ or Annexin V⁺PI⁺ quadrant of flow cytometry diagrams, indicating that these cells underwent apoptosis instead of necrosis. Caspases are common executers of apoptotic cell death. But many recent studies have demonstrated that
Figure 45. Viability of proliferating wild type and PARG-Δ2,3 cells following MNNG treatment as determined by flow cytometry analysis.

A. Cells were treated by different concentrations of MNNG as indicated for 30 min. Then medium containing MNNG was replaced by normal culture medium. Flow cytometry analysis was conducted at 24 hrs after treatment. Data shown are from a representative experiment.

B. Bar graph of the cell viability experiment shown in Figure 45A. Open bars represent wild type cells. Black bars represent PARG-Δ2,3 cells.
Figure 46. Viability of non-proliferating wild type and PARG-Δ2,3 cells following MNNG treatment as determined by flow cytometry analysis.

A. Cells were treated by different concentrations of MNNG as indicated for 30 min. Then medium containing MNNG was replaced by normal culture medium. Flow cytometry analysis was conducted at 24 hrs after treatment. Data shown are from a representative experiment.

B. Bar graph of the cell viability experiment shown in Figure 46A. Open bars represent wild type cells. Black bars represent PARG-Δ2,3 cells.
apoptosis can proceed without the involvement of caspases (Kolenko et al., 2000) (Cregan et al., 2004). In order to determine whether caspases are crucial in MNNG-induced apoptotic cell death, cells were pretreated by a pan-caspase inhibitor z-VAD-fmk. As show in Figure 47, z-VAD-fmk did not prevent cells from undergoing apoptosis in either proliferating wild type or PARG-Δ2,3 cells. Similar results were observed in both non-proliferating wild type and PARG-Δ2,3 cells (Figure 48). These data suggest that caspases may not be crucial in MNNG-induced apoptosis that is also independent of PARG status.

3.7 Effect of the partial PARG gene deletion on cellular responses to STR and H₂O₂ treatment

3.7.1 PARG-Δ2,3 cells may be more sensitive to low concentrations of STR treatment

As mentioned earlier in this dissertation (see section 1.5), PAR metabolism may play a role in STR-induced apoptosis. Thus, the viability of wild type and PARG-Δ2,3 cells was determined following a wide range of different concentrations of STR treatment using flow cytometry analysis after Annexin V-PI staining. Figure 49 shows the viability of wild type and PARG-Δ2,3 cells following 20 nM to 100 nM STR treatment at 24 hrs. Following 20 nM STR treatment, the percentage of viable wild type cells was 87% while the percentage of viable PARG-Δ2,3 cells was only 68%. Following 50 nM STR treatment, the percentage of viable wild type cells was 68% while the percentage of viable PARG-Δ2,3 cells was 51% (Figure 49).
Figure 47. Effect of z-VAD-fmk on MNNG-induced cell death in both proliferating wild type and PARG-Δ2,3 cells. Cells were pretreated by 50 μM z-VAD-fmk for 1 hour. Then wild type cells were treated by 600 μM MNNG while PARG-Δ2,3 cells were treated by 300 μM MNNG for 30 min. Medium containing z-VAD-fmk and MNNG was replaced by normal culture medium. Flow cytometry analysis was conducted at 24 hrs after MNNG treatment. MNNG alone (open bars), MNNG + z-VAD-fmk (black bars).
Figure 48. Effect of z-VAD-fmk on MNNG-induced cell death in both non-proliferating wild type and PARG-∆2,3 cells.

Cells were pretreated by 100 µM z-VAD-fmk for 1 hour. Then wild type cells were treated by 2 mM MNNG while PARG-∆2,3 cells were treated by 1.2 mM MNNG for 30 min. Medium containing z-VAD-fmk and MNNG was replaced by normal culture medium. Flow cytometry analysis was conducted at 24 hrs after MNNG treatment. MNNG alone (open bars), MNNG + z-VAD-fmk (black bars).
Figure 49. Viability of wild type and PARG-Δ2,3 cells following low concentrations of STR treatment as determined by flow cytometry analysis.

STR was dissolved in DMSO, added into culture medium directly and remained in the medium for 24 hrs. Flow cytometry analysis was conducted at 24 hrs after addition of STR.
Although the differences of cellular viability between these two cell lines following low concentrations of STR treatment were small, these differences were observed consistently. This result suggests that PARG-∆2,3 cells may be more sensitive to low concentrations of STR treatment.

3.7.2 PARG-∆2,3 cells are more resistant to high concentrations of STR treatment

Figure 50 shows the viability of wild type and PARG-∆2,3 cells following 1 µM to 2 µM STR treatment. Following 1.5 µM STR treatment for 24 hrs, the percentage of viable wild type cells was only 26% while the percentage of viable PARG-∆2,3 cells was 56%. Following 2 µM STR treatment for 24 hrs, the percentage of viable wild type cells was only 14% while the percentage of viable PARG-∆2,3 cells was 56% (Figure 50). The percentage of PARG-∆2,3 cells resistant to apoptosis was about 50% following 1 µM to 2 µM STR treatment for 24 hrs while wild type cells underwent apoptosis in a dose-dependent manner following the same concentrations of STR treatment. To investigate whether these 50% PARG-∆2,3 cells undergo apoptosis at a later time point, the viability of wild type and PARG-∆2,3 cells following 1 µM to 2 µM STR treatment for 48 hrs was also determined using flow cytometry analysis. As shown in Figure 51, the percentage of viable wild type cells was 14% while the percentage of viable PARG-∆2,3 cells was still 30% following 1.5 µM STR treatment for 48 hrs. Following 2 µM STR treatment for 48 hrs, the percentage of viable wild type cells was 2% while the percentage of viable PARG-∆2,3 cells was still 16%. These results indicate that PARG-∆2,3 cells
Figure 50. Viability of wild type and PARG-Δ2,3 cells following high concentrations of STR treatment for 24 hrs as determined by flow cytometry analysis.
STR was dissolved in DMSO, added into culture medium directly and remained in the medium for 24 hrs. Flow cytometry analysis was conducted at 24 hrs after addition of STR.
Figure 51. Viability of wild type and PARG-Δ2,3 cells following high concentrations of STR treatment for 48 hrs as determined by flow cytometry analysis. STR was dissolved in DMSO, added into culture medium directly and remained in the medium for 48 hrs. Flow cytometry analysis was conducted at 48 hrs after addition of STR.
are more resistant to high concentrations of STR treatment compared to wild type cells. The viability of wild type cells following 1 \( \mu \)M to 2 \( \mu \)M STR treatment for 24 hrs was comparable to that of PARG-\( \Delta 2,3 \) cells following 1 \( \mu \)M to 2 \( \mu \)M STR treatment for 48 hrs (Figures 50 and 51), suggesting that induction of apoptosis in PARG-\( \Delta 2,3 \) cells were delayed under these conditions.

The viability of wild type and PARG-\( \Delta 2,3 \) cells following 200 nM to 1 \( \mu \)M STR was also determined. As shown in Figure 52, the cellular viability was about 50% in both wild type and PARG-\( \Delta 2,3 \) cells following 200 nM, 500 nM and 1 \( \mu \)M STR treatment for 24 hrs, indicating that the similar percentages of wild type and PARG-\( \Delta 2,3 \) cells underwent apoptosis under these conditions.

However, the percentages of wild type and PARG-\( \Delta 2,3 \) cells in early apoptosis (AV\(^+\) PI\(^-\)) were different even though the overall viabilities were similar following 100 nM and 200 nM STR treatment (Figures 49 and 52). As shown in Figure 49, the percentage of wild type cells in AV\(^+\) PI\(^-\) quadrant was 15% while the percentage of PARG-\( \Delta 2,3 \) cells in this quadrant was 33% following 100 nM STR treatment. As shown in Figure 52, the percentage of wild type cells in AV\(^+\) PI\(^-\) quadrant was 23% while the percentage of PARG-\( \Delta 2,3 \) cells in this quadrant was 38% following 200 nM STR treatment. These results suggest that PARG-\( \Delta 2,3 \) cells may be easier to undergo early apoptosis compared to wild type cells. In addition, wild type cells may be easier to undergo late apoptosis (AV\(^+\) PI\(^+\)) compared to PARG-\( \Delta 2,3 \) cells following high concentrations of STR treatment as shown in Figure 50.
Figure 52. Viability of wild type and PARG-Δ2,3 cells following 200nM to 1μM STR treatment for 24 hrs as determined by flow cytometry analysis.

STR was dissolved in DMSO, added into culture medium directly and remained in the medium for 24 hrs. Flow cytometry analysis was conducted at 24 hrs after addition of STR.
Following 1.5 µM STR treatment for 24 hrs, the percentage of wild type cells in AV⁺ PI⁺ quadrant was 44% while the percentage of PARG-Δ2,3 cells was only 9% in this quadrant. Following 2 µM STR treatment for 24 hrs, the percentage of wild type cells in AV⁺ PI⁺ quadrant was 57% while the percentage of PARG-Δ2,3 cells was only 9% in this quadrant.

In summary, PARG-Δ2,3 cells showed different responses to STR treatment compared to wild type cells in overall viability, percentage of cells in early apoptosis and percentage of cells in late apoptosis, suggesting that PAR metabolism may play multiple roles in STR-induced apoptosis. The mechanisms of these different cellular responses require further investigation.

3.7.3 NAD content decreases moderately in both the cell lines following 1.5 µM STR treatment

Our data show that PARG-Δ2,3 cells are more resistant to high concentrations of STR treatment (Figures 50 and 51), suggesting that PAR metabolism plays a role in STR-induced apoptosis under these conditions. Since NAD depletion has been linked to PARP-1 mediated cell death (Pieper et al., 1999) (Berger, 1985), NAD content was determined in both wild type and PARG-Δ2,3 cells following 1.5 µM STR treatment using the enzymatic cycling assay. As shown in Figure 53, NAD content decreased to about 80% of control at 2 hrs, and further decreased to about 70% of control at 8 hrs, and then remained almost unchanged until 16 hrs in both wild type and PARG-Δ2,3 cells following 1.5 µM STR treatment. NAD content compared to control was similar at multiple time points in wild type and PARG-Δ2,3 cells although
Figure 53. NAD content of wild type and PARG-Δ2,3 cells following 1.5 µM STR treatment.
NAD content was determined by an enzymatic cycling assay.
there was a small scatter at 4 hrs (Figure 53). Interestingly, NAD content decreased moderately in both wild type and PARG-Δ2,3 cells following STR treatment, which is different from the extensive depletion of NAD following MNNG treatment.

3.7.4 Both the cell lines are resistant to H$_2$O$_2$ treatment

PARP-1 has been implicated in several acute life-threatening pathological conditions such as vascular cerebral ischemia (Eliasson et al., 1997), myocardial ischemia reperfusion (Wang and Zweier, 1996), neurotoxicity (Zhang et al., 1994) and various forms of inflammation (Erdelyi et al., 2005). In all these cases, reactive oxidative species (ROS) were generated. ROS led to DNA strand breaks that activated PARP-1. To investigate whether PARG-Δ2,3 cells have different cellular responses to ROS, the viability of wild type and PARG-Δ2,3 cells was determined following H$_2$O$_2$ treatment using flow cytometry analysis. Surprisingly, both wild type and PARG-Δ2,3 cells were resistant to H$_2$O$_2$. As shown in Figure 54, the viability of both wild type and PARG-Δ2,3 cells was similar to that of untreated control following 100 µM to 4 mM H$_2$O$_2$ treatment, indicating that both wild type and PARG-Δ2,3 cells did not undergo apoptosis following up to 4 mM H$_2$O$_2$ treatment for 24 hrs.

Disruption of normal mitochondria membrane potential is one indicator of apoptosis. To further determine the occurrence of apoptosis, mitochondria membrane potential was determined in both wild type and PARG-Δ2,3 cells following 4 mM H$_2$O$_2$ treatment using flow cytometry analysis after 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1) staining.
Figure 54. Viability of wild type and PARG-∆2,3 cells following H₂O₂ treatment for 24 hrs as determined by flow cytometry analysis.
Culture medium was replaced by Hanks buffer right before the treatment. H₂O₂ was added into Hanks buffer directly and remained in cell culture dishes for 30 min. Then Hanks buffer containing H₂O₂ was replaced by culture medium. Flow cytometry analysis was conducted at 24 hrs after addition of H₂O₂.
In normal cells, JC-1 accumulates in the mitochondria matrix and forms red fluorescence. When mitochondrial membrane potential is dissipated, the JC-1 dye is dispersed throughout the entire cells and forms green fluorescence (JC-1 monomer). As shown in Figure 55, the percentages of both wild type and PARG-Δ2,3 cells with green fluorescence were similar to those of control sample at 2, 12, 16 and 24 hrs following 4 mM H₂O₂ treatment. The percentage of wild type cells with green fluorescence was similar to that of PARG-Δ2,3 cells at every time point examined. This result supports that most wild type and PARG-Δ2,3 cells did not undergo apoptosis following 4 mM H₂O₂ treatment.
Figure 55. Depolarization of mitochondria membrane potential following 4mM H$_2$O$_2$ treatment as determined by flow cytometry analysis after JC-1 staining.

Culture medium was replaced by Hanks buffer right before the treatment. H$_2$O$_2$ was added into Hanks buffer directly and remained in cell culture dishes for 30 min. Then Hanks buffer containing H$_2$O$_2$ was replaced by culture medium. Flow cytometry analysis was conducted at different time points as indicated after addition of H$_2$O$_2$. 
CHAPTER 4 : DISCUSSION

4.1 The PARG-Δ2,3 cell line represents a useful research tool

The extensive study of biological functions of PARP-1 has demonstrated that PARP-1 is a valuable target for treatment of cancer and ischemia-reperfusion injury (Beneke et al., 2004) (Tentori et al., 2002). Currently, a PARP-1 inhibitor combined with temozolomide is under evaluation in a clinical phase II trial for the treatment of metastatic melanoma (Plummer et al., 2005). But the high abundance of PARP-1 protein in mammalian cells and increasing members of PARP family raises concerns about the efficiency and specificity of inhibiting PARP-1 by current PARP-1 inhibitors. In contrast to PARP-1, PARG is a low abundance protein in mammalian cells and only a single PARG gene has been identified in the human genome (Bonicalzi et al., 2005). PAR, unlike NAD⁺ for PARP-1, is a unique substrate for PARG. Several recent studies have reported that modulation of PARG activity may have promising therapeutic potential for treatment of cancer and ischemia-reperfusion injury (Tentori et al., 2005) (Patel et al., 2005b) (Cuzzocrea et al., 2005). Thus, PARG may potentially represent another attractive therapeutic target for these pathological conditions.

To evaluate PARG as a potential therapeutic target, the biological function of PARG needs to be further studied. Development of useful research tools such as specific cell-permeable PARP-1 inhibitors, PARP-1 gene knockout mouse models and dominant-negative PARP-1 mutants (Shall and de Murcia, 2000) (Hans et al., 1999) (Schreiber et al., 1995) has greatly facilitated the study of biological function of PARP-1. These experimental tools are less well
developed for the study of PARG. Since the efficiency and specificity of current PARG inhibitors in cell models are still under controversy, and complete knockout of PARG activity causes early embryonic lethality (see section 1.3.4), the homozygous mice with targeted disruption of exons 2 and 3 of PARG gene may provide a good research tool for PARG study. Genetic characterizations have shown that PARG activity in PARG-Δ2,3 mice comes from a truncated protein PARG60 which results from alternative splicing of the partially deleted PARG gene (Cortes et al., 2004). Full length PARG110 is absent in PARG-Δ2,3 mice, thus these PARG-Δ2,3 mice carry a hypomorphic mutation of the PARG gene.

Since cell models are suitable for mechanistic investigation, 3T3 embryonic fibroblasts were established from wild type and homozygous PARG-Δ2,3 mice (Cortes et al., 2004). The PARG-Δ2,3 cell line has been compared to a cell line derived from wild type littermates to evaluate its potential as a model for the study of PARG function in this dissertation. Although PARG-Δ2,3 cells proliferate more slowly than wild type cells, they do not differ significantly in cell cycle distribution during the proliferative phase of growth and show no differences in growth control under normal conditions of culture (see section 3.1). Additionally, both proliferating and non-proliferating PARG-Δ2,3 cells have similar content and redox states of total NAD and NADP pools compared to wild type cells (Tables 3 and 4). In total, our characterizations of PARG-Δ2,3 and wild type cells under conditions of normal culture do not reveal gross alterations in growth, proliferation, or pyridine nucleotide metabolism.
Based on these basic characterizations, the PARG-Δ2,3 cell model provides the opportunity to study nuclear PAR metabolism and related cellular responses to genotoxic stress in the presence of the normal DNA damage responsive PARPs but with replacement of the normal nuclear PARG isoform PARG110 with a truncated PARG60 (Figure 8). PARG60 is catalytically active but lacking the putative regulatory domain A. Thus, the PARG-Δ2,3 cell line represents a useful model to determine the requirement of PARG110 in PAR metabolism and cellular responses to genotoxic stress.

4.2 PARG Domain A may be a negatively regulatory domain

The estimation of PARG activity in intact cells indicates that at 5 min following MNNG treatment, PARG activity in PARG-Δ2,3 and wild type cells was very similar. But at later times PARG activity was estimated to be 2.6 to 3.1 times higher in PARG-Δ2,3 cells (Table 5). PAR metabolism stimulated by the treatment of DNA damaging agent MNNG mainly occurs in the nucleus. Thus, PARG activity in wild type and PARG-Δ2,3 cells following MNNG treatment mainly reflects the different activity of the nuclear PARG isoform. The enzymatic activity of PARG60 is approximately 2 to 3 times higher than that of PARG110. The relative PARG activity in intact cells presented here is different from the relative activity of PARG determined by an in vitro PARG assay where PARG activity in isolated nuclear fractions of PARG-Δ2,3 cells is approximately 28% compared to wild type cells (Cortes et al., 2004). This difference of PARG activity in intact cells and in vitro is not surprising since the substrates for PARG are very different between these two experimental
conditions. The majority of substrate for PARG in intact cells is protein-bound PAR and the amount of the substrate is limited by the NAD pool in cells while the substrate for PARG in the in vitro PARG assay is protein-free PAR and the amount of the substrate is excessive. Additionally, since the relative $k_{\text{cat}}$ values for PARG110 and PARG60 are not known, the PARG activity determined in the in vitro PARG assay under optimal conditions may reflect the relative nuclear content of PARG protein in PARG-Δ2,3 and wild type cells.

The difference of PARG activity in intact cells and in vitro may also result from yet-to-be-identified regulation which does not exist in the in vitro system. PARG activity was decreased at later time points (10 and 20 minutes) following MNNG treatment in wild type cells, but this decrease was not observed in PARG-Δ2,3 cells (Table 5). This decreased PARG activity at later time points suggests that PARG may be down regulated in wild type cell but not in PARG-Δ2,3 cells following genotoxic stress.

Nuclear PARG60 in PARG-Δ2,3 cells lacks the N-terminal domain A compared with nuclear PARG110 in wild type cells (Figure 8). Domain A is a putative regulatory domain while domains B, C and D comprise an enzymatic active fragment of PARG (Patel et al., 2005a). The function of domain A is still poorly understood. The higher enzymatic activity of PARG60 compared to PARG110 following DNA damage suggests that domain A may be a negative regulatory domain for PARG110. Previous studies suggest that PARG activity in vivo may be in excess because of its high specific activity in vitro, and PARG may be constantly active even in the absence of genotoxic stress.
(Davidovic et al., 2001). These previous observations support the down regulation of PARG110 activity in vivo following genotoxic stress.

Although the exact regulatory mechanism of domain A requires further study, we propose the following model for the down regulation of PARG110 activity in intact cells following DNA damage. Some unknown proteins or molecules may inhibit PARG activity by binding with domain A following genotoxic stress. This binding alters the three dimensional structure of the catalytic domain of PARG and then down regulates PARG activity. PARP-1, the main anabolic enzyme of PAR, is physically in the proximity of PARG when PARG is degrading PARP-1-bound PAR following genotoxic stress. Thus, PARP-1 may be a likely candidate to down regulate PARG activity by binding with domain A. Other proteins involved in DNA repair represent other likely candidates as the binding partners of domain A.

Down regulation of PARG activity by domain A may also represent a protective cellular response to genotoxic stress. The regulation of PARP-1 activity following genotoxic stress is not clear yet. The data presented here indicate that PARG may regulate PARP-1 activity by removing PAR from PARP-1. PARP-1 is the main acceptor protein for PAR following DNA damage via an automodification reaction and automodification reduces its activity (Burkle et al., 2005). Thus, it is predicted that decreased PARG activity would increase the degree of PARP-1 automodification and decrease the enzymatic activity of PARP-1. Western blot analysis (Figure 31) shows an increased automodification of PARP-1 following MNNG treatment in wild type cells compared to PARG-Δ2,3 cells. The estimation of PARP-1 activity in intact
cells shows that wild type cells have lower PARP-1 activity relative to PARG-Δ2,3 cells following genotoxic stress (Table 5), confirming the previous prediction. The decreased PARG activity down regulates PARP-1 activity by increasing its degree of automodification and consequently slows down the rate of NAD and energy depletion in wild type cells following DNA damage. In contrast, PARG60 does not contain domain A in PARG-Δ2,3 cells. This proposed down regulatory mechanism could not inhibit PARG60 activity in PARG-Δ2,3 cells following genotoxic stress. Thus, PARG60 activity remains high and rapidly removes PAR from PARP-1 in PARG-Δ2,3 cells. PARP-1 activity also remains high and rapidly depletes the NAD pool in PARG-Δ2,3 cells.

### 4.3 Proposed mechanisms for defective formation of XRCC1 foci and γ-H2AX foci

Several cellular responses related to DNA repair have been investigated in this dissertation. These investigations indicate that PARG-Δ2,3 cells are defective in recruitment of DNA repair protein XRCC1 to DNA damage sites and formation of γ-H2AX foci following treatment with sublethal doses of MNNG (Figures 36-39). While more studies will be needed to further define the mechanism(s) of these deficient cellular responses to genotoxic stress, several non-mutually exclusive possibilities are suggested based on the well-defined altered PAR metabolism in PARG-Δ2,3 cells (Figures 56 and 57).
MNNG alkylates many discrete sites on DNA

DNA glycosylase
AP endonuclease

Introduction of DNA breaks at sites where damage can be detected

PARP-1 and PARP-2 activation

PAR

PARP-1 detachment from DNA breaks

γ-H2AX foci

XRCC1 foci

Chromatin relaxation

Detection of additional sites of DNA damage

Repaired DNA

DNA glycosylase
AP endonuclease

Introduction of additional DNA strand breaks

Figure 56. A proposed DNA repair model following MNNG treatment.

Two classes of DNA breaks are proposed in this model. The first class represents damage that can be detected without chromatin relaxation. The second class can be detected only after chromatin relaxation dependent upon PAR metabolism.
MNNG alkylates many discrete sites on DNA

DNA glycosylase

AP endonuclease

Introduction of DNA breaks at sites where damage can be detected

PARP-1 and PARP-2 activation

PAR

PARP-1 detachment from DNA breaks

XRCC1 foci

γ-H2AX foci

Chromatin relaxation

Detection of additional sites of DNA damage

DNA glycosylase

AP endonuclease

Introduction of additional DNA strand breaks

Repaired DNA

Figure 57. A proposed model to explain DNA repair defects in PARG-Δ2,3 cells following MNNG treatment

Altered PAR metabolism in PARG-Δ2,3 cells was proposed to cause defective DNA repair.
Three possible mechanisms are proposed for the defective formation of XRCC1 foci at the DNA damage sites. First, decreased PARP-1 automodification (Figure 31) may not allow its efficient removal from sites of DNA damage in PARG-Δ2,3 cells. PARP-1 rapidly binds to damaged DNA and then synthesizes PAR which mainly modifies PARP-1 itself following genotoxic stress. Automodified PARP-1 more easily dissociates from DNA than unmodified PARP-1 since PAR contains negative charges. Thus the less automodified PARP-1 may occupy the DNA damage sites and interfere with the formation of DNA repair complex containing XRCC1 in PARG-Δ2,3 cells (Figures 56 and 57). Secondly, PAR may play an important role per se in assembly of XRCC1 foci (Figures 56 and 57) and PAR accumulation is reduced in PARG-Δ2,3 cells (Figures 25-28). This possibility is supported by the observation that XRCC1 interacts effectively with automodified PARPs (Schreiber et al., 2002) (Masson et al., 1998). Other studies also have concluded that PAR per se mediates nuclear interactions important to cell recovery from DNA damage (Pleschke et al., 2000) (Malanga and Althaus, 2005). Thirdly, the nuclear PARG110 isoform may be required for assembly of complexes containing XRCC1 and the PARG60 isoform cannot satisfy that requirement.

Although phosphorylation of H2AX is frequently associated with DNA double strand break repair (Chowdhury et al., 2005) (Mahrhofer et al., 2006), it has also been proposed to represent regions of altered chromatin structure where the base excision repair (BER) is proceeding (Stojic et al., 2004). Thus, defective formation of γ - H2AX foci suggests that the modulation of chromatin
structure required for BER is less efficient in PARG-Δ2,3 cells. More studies are needed to further understand the mechanism(s) involved in defective phosphorylation of H2AX, but the altered PAR metabolism in PARG-Δ2,3 cells may represent a likely causative factor. The altered PAR metabolism may impair DNA damage signaling needed for H2AX phosphorylation. One possibility is that insufficiently automodified PARP-1 does not detach from the DNA breaks as effectively as sufficiently automodified PARP-1 in wild type cells and thus causes a delay in the binding of kinases that lead to H2AX phosphorylation in PARG-Δ2,3 cells (Figures 56 and 57).

Compelling data indicate that PARP-1 facilitates DNA repair by modulation of chromatin structure and recruitment of other DNA repair proteins (Malanga and Althaus, 2005) (Petermann et al., 2005). PARP-1 inhibitors have been shown to improve the efficacy of other chemotherapeutic agents such as temozolomide by impeding the efficient DNA repair (Tentori and Graziani, 2005) (Calabrese et al., 2004) (Virag and Szabo, 2002). However, the mechanism of modulating chromatin structure and recruiting other DNA repair proteins by PARP-1 has not been clearly understood. Since PARP activity is higher in PARG-Δ2,3 cells due to lower degree of automodification, our data do not support a direct role of PARP enzymatic activity in recruitment of other proteins and modulation of chromatin structure. PARP-1 protein level is similar between wild type and PARG-Δ2,3 cells (Figure 31). Thus, the direct role of PARP-1 as a scaffold protein in the recruitment can not be ruled out. The studies reported in this dissertation suggest that PAR metabolism may be directly involved in modulation of
chromatin structure and recruitment of other DNA repair proteins, which should facilitate the elucidation of molecular mechanism of PARP-1 in DNA repair.

4.4 Altered PAR metabolism leads to less DNA strand breaks.

In spite of the defects in formation of XRCC1 foci and $\gamma$-H2AX foci, less DNA strand breaks were detected at every time point of recovery examined by the comet assay following treatment with sublethal doses of MNNG in PARG-$\Delta2,3$ cells (Figure 41). A recent study has shown that mismatch repair (MMR)-deficient cells form less DNA strand breaks compared with wild type cells assessed by the comet assay following the treatment with a low dose MNNG (Stojic et al., 2004), which is consistent with our results. Although the comet assay can measure DNA double strand breaks, DNA single strand breaks and alkaline labile sites, it mainly measures DNA single strand breaks in wild type and PARG-$\Delta2,3$ cells following treatment with sublethal doses of MNNG (see section 3.5.3).

It is unexpected that PARG-$\Delta2,3$ cells show defective cellular responses related to DNA repair (less XRCC1 foci and less $\gamma$-H2AX foci) and also form less DNA strand breaks following genotoxic stress. Although the mechanisms need further study, we propose a possible undetected role for PAR metabolism in detection of damaged DNA to explain these unexpected results. As shown in Figures 56 and 57, MNNG alkylates many discrete sites throughout the genome. Some alkylative sites are rapidly recognized and DNA single strand breaks are rapidly introduced by the combined action of
DNA glycosylases and AP endonucleases following MNNG treatment, which activates PARP-1 and PARP-2. PARP-1 and PARP-2 synthesize PAR rapidly and then PARG degrades PAR rapidly. However, many other alkylated sites may require additional signals to be detected. PAR metabolism may serve as the additional signal since it plays an essential role in chromatin modification which allows detection of these sites of damage. The fact that the defective phosphorylation of H2AX represents less efficient chromatin modification in PARG-Δ2,3 cells supports this assumption. Detection of these alkylated sites consequently allows DNA strand incision by DNA glycosylases and AP endonucleases. The lower level of PAR (Figures 25-28) may lead to less efficient detection of these alkylated sites and consequently result in less DNA strand breaks in PARG-Δ2,3 cells.

The traditional dogma asserts that DNA strand breaks activate PARP-1 following DNA damage. We show that PARG-Δ2,3 cells form less DNA strand breaks and have higher PARP-1 activity following genotoxic stress compared with wild type cells, which seems contradictory to the traditional dogma about PARP-1 activation. In fact, DNA strand breaks referred by the traditional dogma are the breaks initially introduced following MNNG treatment which can be detected without chromatin relaxation (Figures 56 and 57). The amount of these breaks should be similar between wild type and PARG-Δ2,3 cells following the same treatment with MNNG. The PARP activity at 5 min following MNNG treatment was similar between wild type and PARG-Δ2,3 cells (Table 5), which is consistent with the traditional dogma. Then PARP-1 activity decreased in wild type cells at later time points compared to the
activity at 5 min following MNNG treatment. But PARP-1 activity remained similar in PARG-Δ2,3 cells at later time points compared to the activity at 5 min following MNNG treatment (Table 5). Thus, PARG-Δ2,3 cells have higher PARP-1 activity at later time points following MNNG treatment. The higher PARP-1 activity in PARG-Δ2,3 cells results from the lack of down regulation of PARP activity due to the absence of domain A of nuclear PARG60. The less DNA strand breaks shown in this dissertation result from the altered PAR metabolism in PARG-Δ2,3 cells which may facilitate chromatin relaxation and introduction of additional DNA strand breaks (Figures 56 and 57). These breaks are different from the initial breaks referred in the traditional dogma which were introduced without chromatin relaxation following DNA damage (Figures 56 and 57).

4.5 Less DNA strand breaks may also reflect defective DNA repair

Although less DNA strand breaks could represent more efficient DNA repair, defective formation of XRCC1 foci and γ-H2AX foci does not support this possibility in PARG-Δ2,3 cells. The proposed model (Figure 57) suggests that the altered PAR metabolism in PARG-Δ2,3 cells (Figures 25-28) leads to less efficient detection of damaged DNA and consequently causes less DNA strand incision. Thus, less DNA strand breaks in PARG-Δ2,3 cells may result from less efficient detection of DNA damage which is the first step of DNA repair. These DNA breaks are mediated by the initiation of DNA repair since alkylation can not lead to DNA strand breaks itself. Thus, less DNA strand
breaks may also represent an indicator of defective DNA repair (less DNA repair initiation) in PARG-Δ2,3 cells.

Our data also show that many repair mediated breaks are unligatable following MNNG treatment. After the initial introduction of DNA strand breaks by treatment with 50 µM MNNG for 20 min, more DNA strand breaks were observed at 4, 8, and 16 hrs of recovery compared with the initial breaks at R0 in both wild type and PARG-Δ2,3 cells (Figure 41). Since MNNG has been removed during the recovery, more DNA breaks during up to 16 hrs of recovery compared to those at R0 suggest that significant amount of DNA breaks results from incomplete DNA repair in both wild type and PARG-Δ2,3 cells. These DNA repair mediated breaks induced by MNNG have been proposed to be unligatable in 293T cells (human embryonic kidney cells) (Stojic et al., 2004). As shown in Figure 43, the unligatable DNA strand breaks induced by MNNG are present in mouse fibroblasts as well. The feature of these unligatable DNA breaks requires further study.

The unligatable DNA strand breaks may only result from the treatment with MNNG but not with other alkylating agents since these breaks were not observed in cells following methylmethanesulfonate (MMS) treatment (Trucco et al., 1998). The DNA strand breaks decreased in a time-dependent manner during the recovery phase in both wild type and PARP-1 knockout cells following MMS treatment (Trucco et al., 1998), suggesting that the repair machinery religates the repair mediated breaks under this condition.
4.6 Several proposed mechanisms for the increased sensitivity of PARG-Δ2,3 cells to MNNG treatment

PARG-Δ2,3 cells are more sensitive to cell killing effect of MNNG compared to wild type cells (Figures 45 and 46). The primary effect of the partial PARG gene deletion is to alter NAD and PAR metabolism. The quantification of NAD has shown that PARG-Δ2,3 cells deplete NAD⁺ more rapidly than wild type cells following MNNG treatment (Figures 15 and 19). Despite more rapid NAD⁺ consumption, PARG-Δ2,3 cells accumulate less PAR following MNNG treatment (Figures 25-28). The altered PAR metabolism leads to several defective cellular responses related to DNA repair. Altered NAD and PAR metabolism may contribute to increased sensitivity of PARG-Δ2,3 cells to MNNG by several different mechanisms.

NAD depletion and consequent ATP depletion has been proposed as a causative factor for PAR metabolism mediated cell death (Ha and Snyder, 1999) (Berger, 1985). For example, restoration of NAD to near-normal levels in astrocytes following genotoxic stress blocked mitochondrial permeability transition (MPT), translocation of apoptosis-inducing factor (AIF), and cell death (Alano et al., 2004) (Ying et al., 2003) (Ying et al., 2005). PARG-Δ2,3 cells deplete NAD at a more rapid rate initially compared to wild type cells following MNNG treatment (Figures 15 and 19), which may consequently cause energy depletion and then contribute to increased sensitivity to cytotoxic effect of MNNG. Although initial more rapid NAD depletion may represent one mechanism of increased sensitivity of PARG-Δ2,3 cells to MNNG, extensive depletion of NAD occurred in both cell types at later time
points or following higher concentrations of MNNG treatment (Figures 15 and 19), suggesting that the different sensitivity of wild type and PARG-Δ2,3 cells to MNNG involves mechanisms other than NAD depletion.

Another mechanism of increased cell death following MNNG treatment may relate to an increased level of free PAR in PARG-Δ2,3 cells. PAR was quantified by a ³H-labeling method and Western blot analysis in this dissertation. PAR level in wild type cells was 5.4 times higher than PARG-Δ2,3 cells determined by a ³H-labeling method (Figure 25) while PAR level in wild type cells was 8.3 times higher than PARG-Δ2,3 cells determined by a quantitative analysis of Western blots (Figure 28). The difference of PAR signals between wild type and PARG-Δ2,3 cells detected by immunoblotting (Figure 28) appears to be greater than the difference of total PAR accumulation determined by the radiolabeling method (Figure 25). The labeling method measures total PAR, which is composed of protein-free PAR and protein-bound PAR (The majority PAR binds to PARP-1 following genotoxic stress). Western blot analysis only shows protein-bound PAR since protein-free PAR migrates out of SDS-PAGE gel due to lower molecular weight. One possible explanation for the differences of the PAR levels determined by these two methods is that PARG generates more protein-free PAR in PARG-Δ2,3 cells than wild type cells following MNNG treatment which can not be detected by Western blot analysis.

PARG contains both exoglycosidase and endoglycosidase activities (Braun et al., 1994) (Lautier et al., 1993). The endoglycosidase activity generates protein-free PAR and exoglycosidase activity generates ADP-
ribose. Since the differences of the PAR levels determined by the radiolabeling method and Western blot analysis suggest that the protein-free PAR level is higher although the total PAR level is lower in PARG-Δ2,3 cells, it is possible that PARG60 lacking the domain A has a higher ratio of endo- to exo- activity than PARG110. If so, this would result in the greater levels of protein-free PAR. Protein-free PAR may serve as a signal between nuclear and mitochondria talk, then stimulate apoptosis inducing factor (AIF) release, and ultimately induce cell death (Hong et al., 2004). Therefore, more protein-free PAR in PARG-Δ2,3 cells may contribute to increased sensitivity to genotoxic stress besides NAD depletion.

Altered PAR metabolism leads to defective DNA repair (Figure 57) which also likely contributes to the increased sensitivity of PARG-Δ2,3 cells to cytotoxicity of MNNG. Other possibilities should be considered as well. Although PARG isoforms in cytoplasmic and mitochondrial compartments are beyond the scope of this dissertation, the altered PARG activity in these two compartments of PARG-Δ2,3 cells may also contribute to the increased sensitivity to MNNG. PARG-Δ2,3 cells have only 3% of PARG activity compared to wild type cells in the cytoplasm while PARG-Δ2,3 cells have more than 3 times higher PARG activity compared to wild type cells in the mitochondria determined by an in vitro PARG assay (Cortes et al., 2004). Since many proteins located in the cytoplasm or mitochondria are involved in cell death pathways and may serve as the acceptor proteins for PAR following MNNG treatment, the significant differences of PARG activity in these two
compartments between the two cell lines may represent another mechanism for the increased sensitivity of PARG-Δ2,3 cells to cytotoxicity of MNNG.

4.7 PARG as a potential therapeutic target

The important role of PAR metabolism in cell fate determination and pathogenesis of several diseases has made enzymes involved in PAR metabolism potential therapeutic targets for multiple diseases. PARP-1 as a therapeutic target has been well validated. PARP inhibitors have been shown to potentiate chemotherapy and radiotherapy for cancer treatment in animal models and clinical trials (Beneke et al., 2004). More interestingly, PARP inhibitors may act as stand alone agents for the treatment of BRCA deficient tumors since BRCA deficient tumor cells showed extreme sensitivity to PARP inhibitors compared to heterozygous mutant and wild type cells (Farmer et al., 2005). PARP inhibition leads to persistent DNA single strand breaks which may degenerate into DNA double strand breaks when the single strand breaks are encountered by a replication fork. The vulnerability of BRCA deficient cells to PARP inhibitors may result from dysfunction of homologous recombination which is essential to repair DNA double strand breaks caused by PARP inhibition and DNA replication in these cells (Farmer et al., 2005). Recent studies have shown that PARP inhibition may represent a less toxic and more specific therapeutic strategy compared to cytotoxic chemotherapy for a wider range of tumors bearing other deficiencies in the homologous recombination pathway such as ATM and CHK2 deficiencies (Bryant and Helleday, 2006; McCabe et al., 2006). Mutations in the ATM gene have been
found in patients with leukemia, lymphoma and breast cancer (Bryant and Helleday, 2006; McCabe et al., 2006). Heterozygous mutations in the CHK2 gene have been observed in Li-Fraumeni syndrome and some osteosarcomas (McCabe et al., 2006). Thus, PARP inhibitors may represent a better therapeutic strategy than traditional chemotherapy for these tumors as well. PARP inhibitors have also shown markedly protective effects on ischemia/reperfusion injuries of multiple organs in animal models (Graziani and Szabo, 2005).

PARG, the most important catabolic enzyme in PAR metabolism, has been proposed as an alternative therapeutic target in PAR metabolism (Lu et al., 2003). Several studies have shown significant protective effects of PARG inhibition in different cell models following genotoxic stress (Ying et al., 2001; Ying and Swanson, 2000). More convincingly, PARG inhibitors have shown beneficial effects in animal models of cancer and ischemia/reperfusion injury. Some PARG inhibitors are not suitable for in vivo animal testing due to the lack of specificity, sensitivity and membrane permeability. One small molecule, GPI 16552 (IC_{50} = 5.5 \mu M, MW 503, N-bis-(3-phenyl-propyl)9-oxo-fluorene-2,7-diamide), has been recently reported as specific, potent and cell permeable PARG inhibitors (Genovese et al., 2004). GPI 16552 in combination with temozolomide (TMZ) significantly inhibited melanoma growth, reduced the ability of melanoma cells to form metastases, and increased lift-span of mice bearing tumor (Tentori et al., 2005). Post-ischemia treatment with GPI 16552 significantly decreased brain infarct volumes by 40–53% in a rat model of focal cerebral ischemia (Lu et al., 2003). The effects of
the PARG inhibitor on melanoma sensitization and cerebral ischemia/reperfusion injury protection are comparable to the effects of PARP inhibitors in similar models. These beneficial effects of PARG inhibitors in animal models strongly support the concept that PARG may be a potential therapeutic target.

However, the mechanisms of the beneficial effects of PARG inhibition have not been well studied. For instance, PAR metabolism in PARG-Δ2,3 cells following genotoxic stress has been shown to be qualitatively similar to wild type cells as assessed by indirect immuno-fluorescence staining (Cortes et al., 2004). Since PARG-Δ2,3 mice showed increased sensitivity to genotoxic stress (Cortes et al., 2004), and the primary effect of the partial PARG gene deletion (devoid of nuclear isoform PARG110) was speculated to alter PAR metabolism, the similar PAR metabolism between wild type and PARG-Δ2,3 cells was unexpected. The quantitative studies reported in this dissertation show distinct alterations of PAR metabolism in PARG-Δ2,3 cells compared with wild type cells (Figures 25-28, Table 5), supporting the previous speculation that genetic deletion of PARG110 alters PAR metabolism. PARG-Δ2,3 cells also show multiple defects in cellular responses related to DNA repair and increased sensitivity to cytotoxicity of MNNG (Figures 35, 36, 38, 41, 45, 46). Thus, our data indicate that a precise coordinate regulation of PARP and PARG activity is required for proper PAR metabolism and cellular responses to genotoxic stress.

Many studies have demonstrated that disruption of PAR metabolism by inhibition of PARP is a validated therapeutic strategy. PARG inhibitors have
shown beneficial effects in animal models which are comparable to PARP inhibitors. The data presented in this dissertation indicate that disruption of PAR metabolism targeted at PARG impairs cellular responses to genotoxic stress, indicating that properly regulated PAR is essential for cellular responses to genotoxic stress. Other studies also have concluded that PAR per se mediates nuclear interactions important to cell recovery from DNA damage (Malanga and Althaus, 2005; Pleschke et al., 2000). Our data suggest that PARG inhibition, like PARP inhibition, may achieve beneficial effects for multiple pathological conditions by disrupting PAR metabolism which is maintained by the proper balance between PARP and PARG activity (Figure 58). Altogether, our data suggest that maintenance of proper PAR metabolism is essential for cellular responses to genotoxic stress, and thus provide a possible mechanism for the therapeutic potential of PARG inhibitors.

4.8 Future studies

The estimation of PARG activity in intact cells clearly shows that domain A may be a negative regulatory domain (Table 5). We propose that some unknown proteins or molecules may bind with domain A and this binding down regulates PARG activity following genotoxic stress (see section 4.2). Identification of these unknown proteins or molecules will improve our understanding about the regulation of PARG activity responsive to DNA damage. These binding partners of PARG could be identified by mass spectrometry after PARG and its binding partners are immunoprecipitated by a PARG antibody. Since molecular weight of PARG is high and abundance of
Figure 58. A precise coordinate regulation of PARP and PARG activity is required for proper PAR metabolism.
PARG is very low in mammalian cells, a PARG antibody with high specificity and affinity is still under development. When a high quality antibody against endogenous PARG is available, identification of the binding partners of PARG will be a feasible task. After the binding partners of PARG are identified, the binding partners of domain A could be further identified by molecular biology tools. Alternatively, the binding partners of domain A may be directly identified by protein microarray, a newly developed technique which is similar to cDNA microarray in principle. Kits identificating kinase substrates are commercially available using protein microarray (www.invitrogen.com). But the proposed binding partners bind to domain A of PARG protein in responsive to genotoxic stress. The binding may not occur in the absence of genotoxic stress. Thus the imitation of binding conditions on protein microarray chip may be a daunting task.

The relative protein level of nuclear PARG110 in wild type cells and nuclear PARG60 in PARG-Δ2,3 cells is unknown. Since PARG60 contains a putative weak NLS on domain D (Masutani et al., 2003), it is likely that the relative protein level of PARG60 in PARG-Δ2,3 cells is lower or similar to that of PARG110 in wild type cells. Determination of the relative protein levels of these two nuclear PARG isoforms by Western blot analysis will confirm this prediction. But high quality antibodies of PARG, which could detect endogenous PARG and also demonstrate the difference of nuclear PARG protein levels between wild type and PARG-Δ2,3 cells if the difference exists, are still under development. In addition, PARG110, extremely sensitive to protease, is easily degraded into PARG60 during nuclear isolation even in the
presence of strong protease inhibitors. Thus, the effective inhibition of proteases to prevent degradation of PARG110 during nuclear isolation still requires further study.

The complex differences between wild type and PARG-Δ2,3 cells to different concentrations of STR treatment (see section 3.7.1 and 3.7.2) suggest that PAR metabolism plays multiple roles in STR-induced apoptosis which is dose-dependent. Since NAD content decreased moderately in both wild type and PARG-Δ2,3 cells and no differences were observed following high concentrations of STR treatment (Figure 53), energy depletion may not be involved in STR-induced apoptosis. The functional relationship between PAR metabolism and protein kinase C has not been well understood. Thus, no feasible working hypothesis has been proposed here. cDNA microarray analysis may be a good start for mechanistic elucidation. Comparison of gene transcription profiling between wild type and PARG-Δ2,3 cells following different concentrations of STR treatment may generate some good working hypotheses to facilitate the elucidation of mechanisms of different cellular response to STR treatment between these two cell lines.

PAR metabolism has been implicated in oxidative stress induced cell death in several different cell types (Eliasson et al., 1997) (Wang and Zweier, 1996) (Zhang et al., 1994). Surprisingly, flow cytometry analyses after Annexin V - PI and JC-1 staining indicate that both wild type and PARG-Δ2,3 cells were resistant to apoptosis for up to 24 hrs following 4 mM H2O2 treatment (Figures 54 and 55). The concentration of H2O2 was calibrated using ultraviolet (UV) spectrometry on the same day of the treatment and
H$_2$O$_2$ was added into Hanks buffer instead of culture medium. The resistance may result from the relative inefficiency of cell permeability of H$_2$O$_2$ in the cell type (mouse 3T3 fibroblasts) used here. The exact mechanism of this resistance still needs further investigation.
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