

# Differentiation-associated Downregulation of Poly(ADP-Ribose) Polymerase-1 (PARP-1) Expression in Skeletal Muscle Increases Its Resistance to Oxidative Stress

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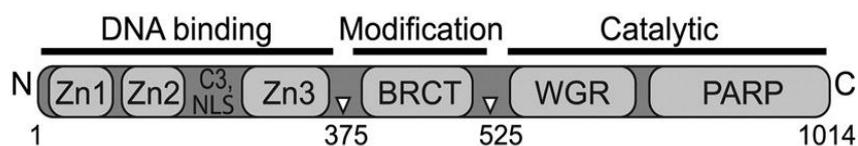
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# 1. Introduction

## 1.1. PARP-1, Poly(ADP-ribose) polymerase 1

PARP-1, Poly(ADP-ribose) polymerase 1, is the major isoform of the poly (ADP-ribose) polymerase family, consisting of 17 proteins in humans and 16 in mice. It is a constitutive nuclear and mitochondrial protein with well-recognized roles in various essential cellular functions, such as deoxynucleic acid (DNA) repair, signal transduction, different cell death pathways, and a variety of pathophysiological conditions including burn, sepsis, diabetes and cancer. Most of the functions mediated by PARP are related to oxidative stress. Activation of PARP-1 in response to oxidative stress catalyzes the covalent attachment of the poly (ADP-ribose) (PAR) groups on itself and other acceptor proteins, utilizing nicotinamide adenine dinucleotide (NAD<sup>+</sup>) as a substrate. Overactivation of PARP-1 depletes intracellular NAD<sup>+</sup>, influencing mitochondrial electron transport, cellular adenosine triphosphate (ATP) generation and, if persistent, can result in necrotic cell death.

PARP-1 has three functionally distinct domains: the N-terminal DNA-binding domain (DBD) responsible for PARP-1's interaction with DNA breaks, which are also found in DNA ligase III. The automodification domain (AD) contains the BRCT motif, which is common in DNA repair and cell-cycle proteins, constituting the major protein interface with various nuclear partners, and the C-terminal with the catalytic domain (CAT) showing common structural features with the active site of bacterial (ADP-ribosylating) toxins (Diphtheria toxin or Pertussis toxin), notably the NAD-fold (PARP signature) (Langelier et al., 2008) (**Figure 1**). The primary structure of the enzyme is highly conserved in eukaryotes (human and mouse enzyme have 92% homology at the level of amino acid sequence) with the catalytic domain showing the highest degree of homology between different species; the catalytic domain contains or so-called PARP signature is a 50-amino acid-containing block showing 100% homology between vertebrates.



**Figure 1.** Domain organization of the human PARP-1 (Langelier et al., 2008). PARP-1 composed of 3 main domains, DNA-binding (DBD, residues 1-374), Modification (AD, residues 375-525) and Catalytic (CAT, residues 526-1014) domains. DBD contains three Zn-fingers (Zn1, Zn2 and Zn3), a nuclear localization signal (NLS) and a caspase cleavage site (C3). Modification domain has the BRCT fold (BRCA1 C terminus) with auto-modification property. Catalytic domain has the conserved PARP signature responsible for NAD<sup>+</sup> binding..

## 1. 2. Other PARPs

Based on the comparative analysis of their catalytic domain homology, 17 mammalian proteins were named PARPs (Ame et al, 2004), although the nomenclature has been changing in recent years, and new classifications arise based on structural and enzymatic characterization (Hottiger et al, 2010).

Three-dimensional structures of the catalytic domain of chicken PARP-1 and mouse PARP-2 showed structural homology with the active site of the bacterial ADP ribosylating toxin from *Corynebacterium diphtheria* (Collier, 2001). Recently, the  $\beta$ - $\alpha$ -loop- $\beta$ - $\alpha$  NAD-fold (which is the most conserved region in PARP-1 orthologous, and is considered the PARP signature) was used to search the non-redundant protein database from the National Center for Biotechnology Information (NCBI) for human PARP homologues. Novel putative PARPs were identified, and this increased the number of PARP-family members to 17.

PARP-2 was discovered as a residual PARP activity in embryonic fibroblast derived from PARP-1 deficient mice (Shieh et al., 1998; Ame et al., 1999). It has a molecular weight of 65kDa and it contains 570 amino acids. Crystal structure is very similar to the PARP-1, except for differences in the vicinity of the acceptor site that reflect differences in terms of substrates. It is a nuclear, DNA-dependent member of the PARP

family and interacts with PARP-1 and shares common partners involved in Single Strand Brake Repair (SSBR) and Base Excision Repair (BER). PARP-2 deficient mice show a delay in DNA-strand breaks resealing similar to that observed in PARP-1 deficient cells. (de Murcia et al. 2003; Schreiber, 2002). Knockout animals for both genes die in the early stage of embryogenesis, which shows the importance of these two genes.

PARP-3 is a mono-ADP ribosylase, a core component of the centrosome located at the daughter centriole throughout the cell cycle (Augustin et al., 2003). Its E and F domains share a 61% homology with PARP-1. It has two splice variants, 533 and 540 amino acid long, respectively, with a molecular weight of 67kDa. It was reported that PARP-3 is not activated upon binding to DNA (Loseva et al., 2010). PARP-3 interacts with PARP-1 suggesting a link between the DNA-damage surveillance network and the mitotic fidelity checkpoint.

VPARP (PARP-4), the largest of the PARP family with a molecular weight of ~200 kDa, possesses a mono-ADP-ribosylation feature. It is a part of the Vault particles, which are ribonucleoprotein (RNP) complexes containing small RNAs and proteins. One of the Vaults proteins (identified by the molecular tool of yeast two-hybrid system) showed homology with the PARP family members. It also localizes in the nuclei, is associated with the mitotic spindle, and is able to carry out an ADP-ribosylation reaction (Kickhoefer et al., 1999). VPARP4<sup>-/-</sup> mice show higher sensitivity to carcinogen-induced tumorigenesis, suggesting a possible role in drug-induced abnormal tissue grow (Raval-Fernandes et al., 2005).

PARP5a or Tankyrase-1 is a 142 kDa molecular weight protein containing 1327 amino acids. It was named by Smith and colleagues as TRF-interacting, ankyrin-related ADP-ribose polymerase (Smith et al., 1998). It has a nuclear origin, but also can be found in the cytosol and its activity is modified by mitogen-activated protein kinases (MAPK). Tankyrase-1 binds to TRF-1, a telomere-specific DNA binding protein, and was found to have PARP activity, which raises the question whether or not telomers are under the control of ADP-ribosylation. It was shown that is required for the polymerization of mitotic spindle-associated PAR localizing to mitotic spindle poles. Tankyrase-1 ribonucleic acid interference (RNAi) in HeLa cells showed spindle defect in a PAR concentration dependent manner (Chang et al., 2005).

PARP-5a or Tankyrase-2 is a 127 kDa protein structurally similar to tankyrase-1, except that it misses the N-terminal HPS domain (His, Pro, Ser residues). It possesses PARP activity, binds to TRF-1 and IRAP, as TNK-1 shares overlapping functions (given that TNK-1 and TNK-2 proteins associate and share most of their protein partners) (Sbodio et al. 2002). Knockdown experiments for TNK-2 showed no changes in telomerase capping and length, but the smaller size of the animals indicate a metabolic disturbance, proving the enzyme role in metabolism (Hsiao et al., 2006).

TiPARP or PARP-7 was found when analyzing the expression patterns of messenger RNAs (mRNAs) after 2,3,7,8-tetrachlorodibenzo-p-dioxin treatment. It contains 657 amino acids and has a ~75kDa molecular weight, showing similarity to PARP-1 catalytic domain with catalytic activity on histones. It is a mono-ADP-ribosylase and co-localized in the nucleus with AHR (aryl hydrocarbon receptor), a transcription factor activated by synthetic hydrocarbons and some endogenous ligands. MacPherson and colleagues found that TiPARP is a transcriptional repressor of AHR (MacPherson et al., 2013). It was also found to have a role in regulating gluconeogenesis through AHR, ADP-ribosylating PEPCK (phosphoenolpyruvate carboxykinase) with NAD<sup>+</sup> consumption, and decreased SIRT1 activity of PGC1 $\alpha$  (Diani-Moore et al., 2013). Treatment with dioxin caused lethality in TiPARP<sup>-/-</sup> mice by day 5, with increased level of steatosis and toxicity in liver, while TiPARP<sup>+/+</sup> mice survived the 30 day treatment period (Ahmed et al., 2015).

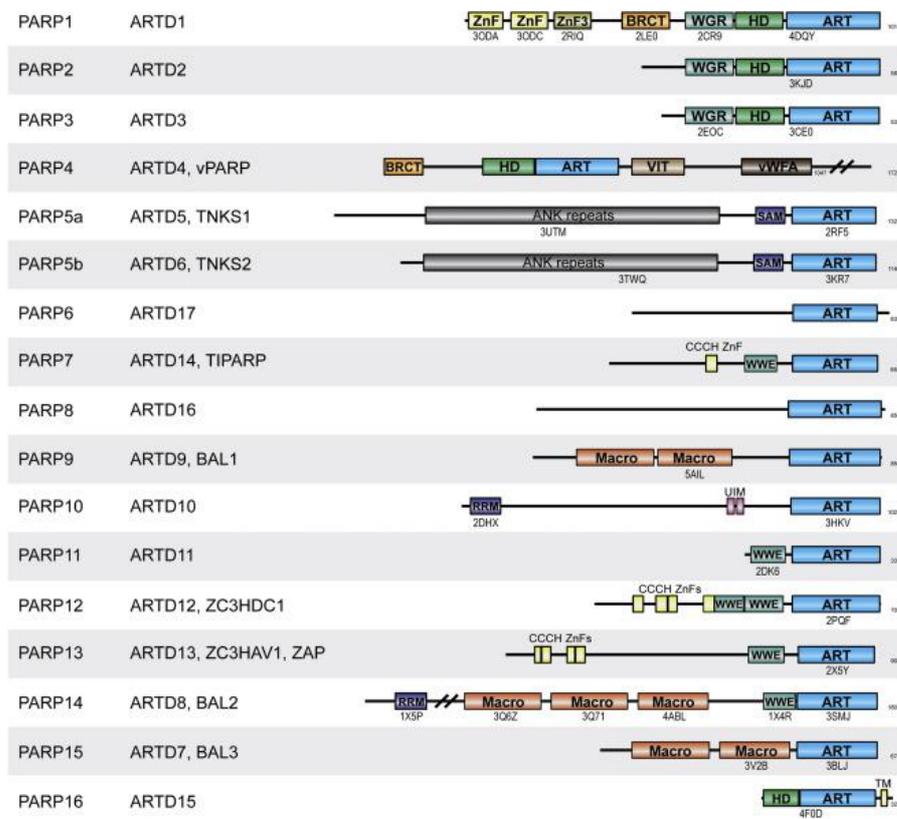
Other members of the PARP family not yet mentioned above can be categorized as CCCH-type PARPs, Macro PARPs and other PARPs. The group of CCCH-type PARPs contains PARP-12 and -13, and the already reviewed TiPARP above. They share a common CCCH-like zing finger, WWE (Tryptophan: W; Glutamate: E amino acids) and the catalytic domain. One of the members, PARP-13, has two isoforms. The shorter one in terms of size, which lacks the PARP domain, was identified as a binding protein to viral ribonucleic acid (RNA) and called ZAP. Screening mammalian cDNA libraries revealed CCCH-like zing finger protein inhibiting viral replication (Gao et al., 2002). Later, in another study reported by the same group that analyzed the role of zinc fingers of PARP-13 in viral activity, the 2nd and 4th zing finger motif were shown to have major decreasing activity of viral infection in the cytoplasm, while the 1st and 3rd have minor (Guo et al., 2004).

Macro domain PARPs or BAL PARPs, PARP-9,-14,-15 (BAL 1, 2, 3), belong to this group, having a so-called macrodomain attached to their PARP domain, 2, 3, and 1 respectively. Macrodomains are highly conserved domains throughout the animal kingdoms, as they can be found at the C-terminus of macro-H2A histone proteins and in several non-structure proteins of ssRNA viruses. They are also found in the family of proteins in bacteria, archaeobacteria, and eukaryotes, suggesting a ubiquitous cellular role. They have the capability of binding PAR. PARP-9 was described using a differential display method from diffuse large B-cell lymphoma (DLB-CL) patients. It is a ~88kDa protein that is highly expressed in fatal high-risk DLB-CLs, compared to the cured, low-risk group. The high-risk group cells also show higher rates of migration capability, indicating a PARP-9 role in the disease's malignancy (Aguiar et al., 2000). Apoptosis inhibition is an important step in B-lymphoid oncogenesis, and elevated macro PARP corresponds to hostility. PARP-14 is a binding partner of Stat6, the IL-4-induced (interleukin-4) transcription factor, and mediates protection against apoptosis in IL-4 treated cells (Cho et al 2009.). The signaling pathway goes through AMPK (AMP-activated protein kinase), a cellular energy sensor, as enhancement restored the glycolytic activity and the survival signaling in PARP 14<sup>-/-</sup> B cells (Cho et al., 2011). It possesses a cell motility and focal adhesion function, namely that PARP-14 has a role in focal adhesion turnover, and is localized at the end of actin stress fibers (Vyas et al., 2013).

Other PARPs include PARP-6, PARP-8, PARP-10, PARP-11, PARP-16. Their function is still relatively unknown. PARP-6 is a mono-ADP-ribosyl-transferase found in colorectal cancer specimens. It is thought to be a tumor suppressor (Tuncel et al., 2012). PARP-10 inhibits c-myc, a transcriptional regulator deregulated in cancers, and has a role in cell proliferation (Yu et al. 2005). Its activity depends on phosphorylation by CDK-2-cyclin E in vitro (Chou et al., 2006). It was shown to be a mono-ADP-ribosylase and able to regulate IL-1 $\beta$ - and TNF- $\alpha$ -dependent NF $\kappa$ B signaling (Verheugd et al., 2013). It interacts with PCNA, and is responsible for DNA damage sensitivity (Nicolae et al. 2013). PARP-16's crystal structure was revealed by Karlberg (Karlberg et al., 2012) and showed auto-mono-ADP-ribosylating activity. The enzyme is required during endoplasmatic reticulum stress activating stress sensors, PERK and IRE1 $\alpha$  by ADP-ribosylating, these sensors, and itself (Jwa M et al., 2012).

The domain structure of PARPs is summarized in **Figure 2**, representing the new ARTD (ADP-ribosyltransferase diphtheria toxin-like) nomenclature proposed by Hottiger and colleagues (Hottiger et al., 2010) according to the International Union of Biochemistry and Molecular Biology (IUBMB).

Based on the domain structure of the PARP family, different interactions are possible in different cell compartments. Moreover, PARP-1 interacts with PARP-2 or PARP-3 and Tankyrase 1 and 2 (Cook et al., 2002), widening the possibilities of their biological functions.



**Figure 2.** Domain Architecture of Human PARPs (Barkauskaite et al., 2015). PARP1–16 with alternative names listed on the side domain structures. ARTD, ADP-ribosyl transferase diphtheria type; vPARP, vault PARP; TNKS, Tankyrase; TIPARP, TCDD inducible PARP; BAL, B cell aggressive lymphoma; ZC3HDC1, zinc finger CCCH-type domain containing 1; ZC3HAV1, zinc finger CCCH-type antiviral 1; ZAP, zinc finger antiviral protein. Structural information availability of specific domains is indicated by PDB code under the specific domains. The number of amino acids

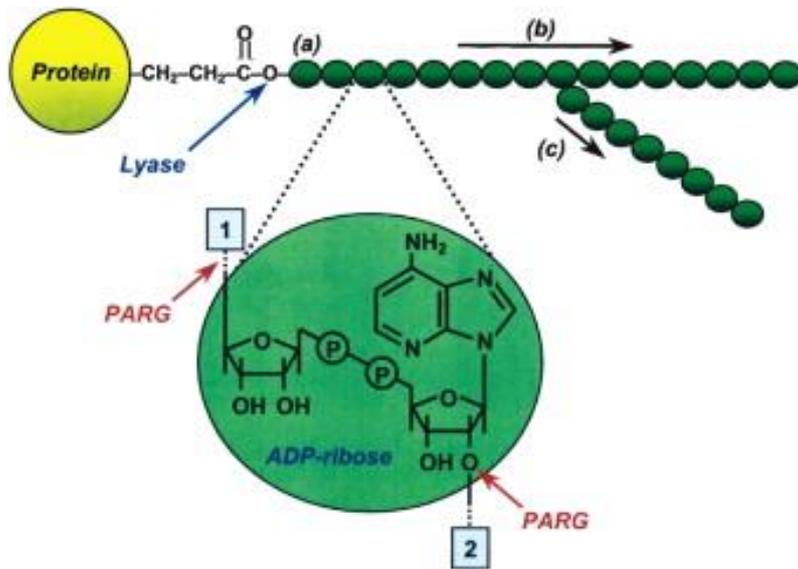
composing the protein is indicated on the right. The following domain names were used: ZnF, zinc finger; BRCT, BRCA1 C-terminal; WGR, conserved Trp-Gly-Arg motif domain; HD, helical domain; ART, ADP-ribosyl transferase; VIT, vault protein inter-alpha-trypsin; vWFA, von Willebrand type A; ANK, ankyrin; SAM, sterile alpha motif; CCCH ZnF, CCCH type zinc finger; WWE, three conserved residues W-W-E motif domain; Macro, macrodomain; RRM, RNA recognition motif; UIM, ubiquitin interaction motif; TM, transmembrane motif.

### **1. 3. Metabolism of poly(ADP-ribose)**

#### **1. 3. 1. Poly(ADP-ribose), pADPr Synthesis**

Poly(ADP-ribosylation) is a covalent, reversible modification by PARPs attaching poly-ADP-ribose on a variety of target proteins, using  $\text{NAD}^+$  as the substrate (**Figure 3**). It is a conserved process among metazoa.

In 1963, historically speaking, Chambon published that there is an enzymatic activity responsible for the synthesis of polymers (PAR) that requires  $\text{NAD}^+$ . Namely, it was shown that  $^{14}\text{C}$ -adenine-labeled ATP incorporated into an acid-insoluble fraction in a nuclear extract (Chambon et al., 1963). That was the pioneer work that sparked interest from different laboratories and outgrew a competitive field of research.



**Figure 3.** Structure of PAR (Kraus and Lis, 2003).

The synthesis of PAR polymers consists of the following steps: (a) initiation, (b) elongation, and (c) branching. PAR is attached to the glutamate residues of proteins on the position labeled 1. The covalent bonds between ADP-ribose units are made via glycosidic ribose-ribose 1'' → 2' bonds at the positions labeled 1 and 2. The red and blue arrows represent the cleavage sites for PARG and ADP-ribosyl protein lyase, respectively.

pADPr is a homopolymer of ADP-ribose, and is linked by glycosidic 1''-2'' bonds reaching up to 200 units. The first step is the initiation -- attaching mono-ADP-ribose to acceptor proteins, including PARP-1. In the elongation step, the mono-ADP-ribose units are added to the chain, reaching up to 20-50 units. Further, ADP-ribosylation may occur by branching of the polymer showing similar structure to ribonucleic acid (RNA) or DNA which was proven by the fact that antibody against pADPr recognized RNA and DNA.

In resting cells, the acceptor proteins are mono- or oligo--ADP-ribosylated, while in stress conditions, such as under nuclear genotoxic stimuli, proteins are poly-ADP-ribosylated. The degradation also depends on the stimuli, as stressors can trigger the degradation in a significantly quicker manner than in resting cells.

There are numbers of proteins as targets for PARylation, such as RNA/DNA polymerases, Topoisomerase II., p53, PCNA just to name a few. Adding ADP ribose

units to a protein changes the protein net charge, which results in modified function. Usually this prevents the interaction of a DNA-binding capability, or if it occurs near the catalytic site of an enzyme, may result activity-modifying property (D'Amours et al. 1999).

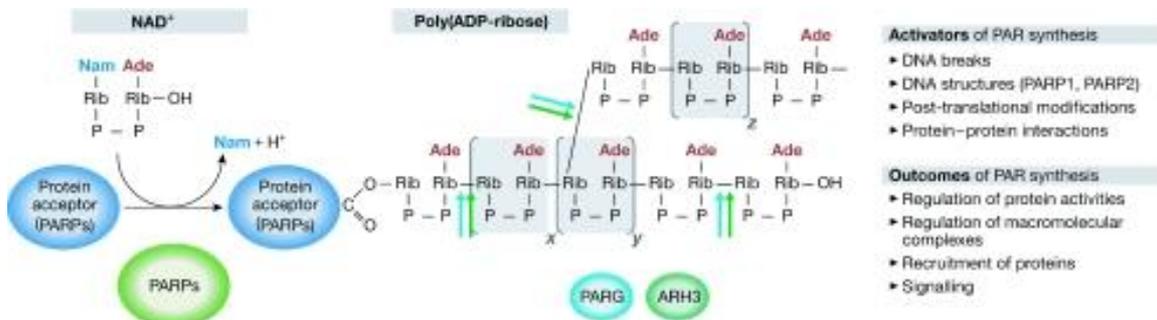
Looking at the PARP structure, there are elements responsible for the PARylation. The N-terminal DNA binding domain (DBD) contains two zinc fingers (F1, F2) that underlie the structural basis for DNA strand break detection. Upon a single strand break, both zinc fingers participate in the PARP activation, although PARP activation by double-stranded breaks depends upon the F1 domain closer to the N-terminus (Ikejima et al., 1990, Gradwohl et al., 1990, Langelier and Pascal 2013). There is a third zinc finger that can be found within the DBD, and is necessary for the coordination of interaction with the catalytic domain upon DNA binding, which forms a head-to-tail structure of the homodimer (Langelier et al., 2008).

A discovery was made regarding PAR-binding proteins that revealed 20 amino acid motifs with basic and hydrophobic/basic residues. The proteins identified share a common function, as they participate in maintaining genomic stability. The proteins are as follows: p53, p21CIP1/WAF1, xeroderma pigmentosum group A complementing protein, MSH6, DNA ligase III, XRCC1, DNA polymerase  $\epsilon$ , DNA-PK<sub>CS</sub>, Ku70, NF- $\kappa$ B, inducible nitric oxide synthase, caspase-activated DNase, and telomerase (Pleschke et al., 2000). Another approach, MALDI-TOF MS technology (matrix-assisted laser desorption-ionization time-of-flight mass spectrometry) was utilized for identifying pADPr binding proteins, resulting in 30 nuclear proteins as well-- mostly hnRNPs (heterogeneous nuclear ribonucleoproteins), proteins that bind RNA in the nucleus and have a role in RNA maturation and translocation (Gagne et al., 2003).

### **1. 3. 2. Poly(ADP-Ribose) Catabolism: Poly(ADP-Ribose) glycohydrolase and others degrades PAR**

Mono-and poly-ADP-ribose units can be removed by PAR-removing enzymes (**Figure 4**). A prediction was made that the lifetime of the PAR polymer is less than 1 minute, which indicates a strictly controlled regulation of the synthesis and degradation (Virag and Szabo, 2002). The principal member of the enzymes is Poly(ADP-Ribose)

glycohydrolase (PARG), which was discovered in the 70s (Miwa and Sugimura, 1971; Miwa et al., 1974). PARG is a product of a single gene on chromosome 10. There are the full-length nuclear PARG-111, PARG-102 and the cytosolic PARG-99. There is a PARG-60 with nuclear and mitochondrial localization. PARG-55 is an alternative splice variant of PARG-60 localized in mitochondria (Meyer-Ficca et al., 2004 and Meyer et al., 2007).



**Figure 4.** Metabolism of poly(ADP-ribose) (Hakmé et al., 2008). PARPs hydrolyse  $\text{NAD}^+$  and catalyse adding ADP-ribose units to glutamate residues of either acceptor proteins (heteromodification) or themselves (automodification).  $x$ ,  $y$  and  $z$  labels represent values from 0 to  $>200$ . PARG and ARH3 can both hydrolyse PAR at shown positions; activators and outcomes of PAR synthesis are also noted on the right. Ade, adenine; ARH3, ADP-ribosyl hydrolase-3; Nam, nicotinamide; PAR, poly(ADP-ribose); PARG, poly(ADP-ribose) glycohydrolase; PARP, poly(ADP-ribose) polymerase; Rib, ribose.

Primarily, the degradation of poly(ADP-ribose) is carried out by PARG, and has a central role of the catabolism. Inactivating the gene causes lethality in early embryonic age mice (Koh et al., 2004). Using the lentiviral system elegantly presented by Erdelyi et al., it has been shown that PARG serves as an apoptosis to necrosis switch during severe oxidative stress. (Erdelyi et al., 2009).

A study reported by Oka showed an alternative enzymatic activity for the degradation of poly(ADP-ribose), with a similar reaction to PARG by a 39kDa enzyme called poly(ADP-ribose) hydrolase or ARH3, which shares amino acid identity with the catalytic domain of PARG (Oka et al., 2006).

## **1. 4. Biological function of PARP-1**

### **1. 4. 1. Role of PARP-1 in the differentiation and gene expression**

Differentiation is the process by which a cell changes from one cell type to another. Usually it occurs when a less specialized type becomes more specialized to fulfill specific tasks, so they gain new functions and lose others. This strictly controlled process requires concerted gene activation and repression, and results in differentiation into specialized cells. For example, myosatellite cells or satellite cells are precursors of skeletal muscle cells and upon activation re-enter the cell cycle, start proliferation and differentiate to myotubes. Furthermore, many fully differentiated cell types such as lymphocytes, fibroblasts, and hepatocytes retain the ability to proliferate, such as in the course of immune response, wound healing, or liver regeneration, respectively. Experimental data show the differentiation-modifying effect of PARP, but it is important to note that the effect is cell-specific. Inhibition of PARP has been shown to interfere with the differentiation of human granulocyte-macrophage progenitor cells to the macrophage cells (Francis et al., 1983). Overexpression of PARP arrested MB4 cells and blocked all trans-retinoic acid-induced neutrophilic differentiation (Bhatia et al., 1995). Benzamide PARP inhibitors induced melanogenesis and differentiation of melanoma cells (Durkacz et al., 1992). Moreover, PARP-1 has a role in angiogenesis (Caldini et al., 2011; Pyriochou et al., 2008) and adipocyte differentiation (Erener et al., 2012). Also, after DNA damage, it is of primary importance to stop replications at certain checkpoints to allow for the repair of DNA damage.

Co-purification techniques revealed the interaction of PARP-1 with replication factors such as DNA polymerase, and DNA primase, DNA helicase, DNA ligase, topoisomerases I and II and key components of multiprotein replication complex (Simbulan-Rosenthal et al., 1996; Dantzer et al., 1998; Bauer et al., 2001).

PARP-1 has been shown to function in various aspects of the transcription process through a variety of mechanisms, including roles as a modulator of chromatin, a coregulator for DNA-binding transcription factors, and a regulator of DNA methylation. Chromatin is a protein–DNA complex, the structural base of information coded, that comprises genomic DNA, core histones (i.e., H2A, H2B, H3, and H4), linker histones

(e.g., H1), and other chromatin-associated proteins. The basic repeating units of chromatin are the nucleosome (i.e., 146 base pairs of DNA wrapped around a core histone octamer with two copies of each core histone) and the chromatosome (i.e., nucleosome, plus 20–40 base pairs of linker DNA and linker DNA-binding proteins, such as H1) (Widom, 1998; Wolffe and Guschin, 2000). One possible way PARP-1 regulates chromatin structure and transcription is the PARylation of proteins bind to chromatin and the auto-PARylation of PARP-1, which affects the structure of chromatin. Earlier studies showed that PARP-1 promotes decondensation, and the PARylation of H1 was proposed as the underlying mechanism (Poirier et al., 1982). In 2004, Kim and colleagues showed (Kim et al., 2004) that PARP-1 and H1 compete for binding to nucleosomes. PARP-1 depletion by RNAi increases the binding of H1 at many sites of the genome (Krishnakumar et al., 2008; Krishnakumar and Kraus, 2010), suggesting contribution to the dynamic regulation of gene expression. A considerable body of evidence suggests that PARP1 directly interacts with core histones (D'Amours et al., 1999; Pinnola et al., 2007). Histones H3 and H4 are preferential targets for PARP1 binding in vitro, and they control the enzymatic activity of PARP1 (Pinnola et al., 2007; Kotova et al., 2011). There are a number of studies showing that histone variants provide the physical link between PARP-1 and chromatin. H2Ax has been shown to be phosphorylated at the site of DNA damage, which pADPribosylated by genotoxic treatments. Chemical inhibition of PARP-1 triggers the accumulation of phosphorylated H2Ax (Bryant et al., 2005). Also, PARP-1 and H2Ax interaction, outside of DNA repair, was shown during rat spermatogenesis with high levels of pADPr (Meyer-Ficca et al., 2005). H2Az, another histone variant highly homologous to H2Ax, is considered as an interacting partner of PARP-1.

Histone variant H2Av is a functional homologue of H2Ax in *Drosophila*, and combines the function of H2Ax and H2Az proteins (Leach et al., 2000 and Madigan et al., 2002). Effective repair of double strand breaks by homologous recombination requires phosphorylation of *Drosophila* H2Av (acetylated by Tip60), which is followed by chromatin remodeling involving un-phosphorylated and phosphorylated H2Av exchange (Kusch et al., 2004). In this model, (1) a complex of H2Av and PARP1 is activated by DNA break formation; (2) phosphorylation of H2Av triggers PARP1 enzymatic activity; (3) activated PARP1 modifies H1 histone with pADPr, which

removes it from damaged loci; (4) the Tip60 complex remodels local chromatin, and facilitates entry of DNA repair complexes; and, finally, (5) Tip60 removes Ser137-phospho-H2Av from repaired chromatin (Thomas and Tulin, 2013).

It is known that transcription induced during steroid response or the response of stress-activated genes, such as hsp70, is accompanied by a local loosening of the chromatin structure that manifests in polytene chromosomes as “puffs” at the site of transcription. After hormone treatment, infection, and heat shock stress, pADPr polymers accumulate at the target loci. As a result of PARP-1 action, chromatin loosens, and gene transcription takes place. But how is gene expression induced? What is the role of PARP-1 in the transcription machinery?

The mode of action by PARP-1 is the modification of protein by: a) the covalent attachment of pADPr polymers; or b) non-covalent interaction. Gagne represents a proteome-wide identification of pADPr binding proteins, which are classified into 6 groups: DNA repair, DNA replication, cell cycle, chromosome organization/biogenesis, protein synthesis, and mRNA metabolism (Gagne et al., 2008). Evidence suggests that certain proteins possess pADPr-binding domains, specifically a 190 amino acid module known as macrodomain (Karras et al., 2005) and a novel zinc finger motif (PBZ), which present in many DNA damage and checkpoint proteins (Ahel et al., 2008).

Protein shuttling by PARP-1 and pADPr is also part of the process of loosening the chromatin structure. The process of shuttling, or pulling proteins from nucleic acid, is a paramount mechanical process in the dynamic, complex environment of the nucleus. The role PARP1 plays in numerous nuclear activities stems from this fundamental event by which PARP1 covalently or noncovalently modifies proteins to dissociate from nucleic acid. It is believed that PARP’s broad role in shuttling proteins from nucleic acid is preceded by two non-mutually exclusive events: electrorepulsion between the highly anionic poly-ADP-ribose and DNA/RNA, and the stealing/masking of DNA/RNA binding domains (D’Amours et al., 1999). Electrorepulsive shuttling occurs based on the repulsive force of similar charges, namely that increasing auto(ADP)ribosylation increases the repulsion between PARP and DNA. Removing PAR units by PARG increases the affinity of PARP to DNA (Ferro and Olivera, 1982; Zahradka and Ebisuzaki, 1982).

## **1. 4. 2. Role of PARP-1 in DNA repair**

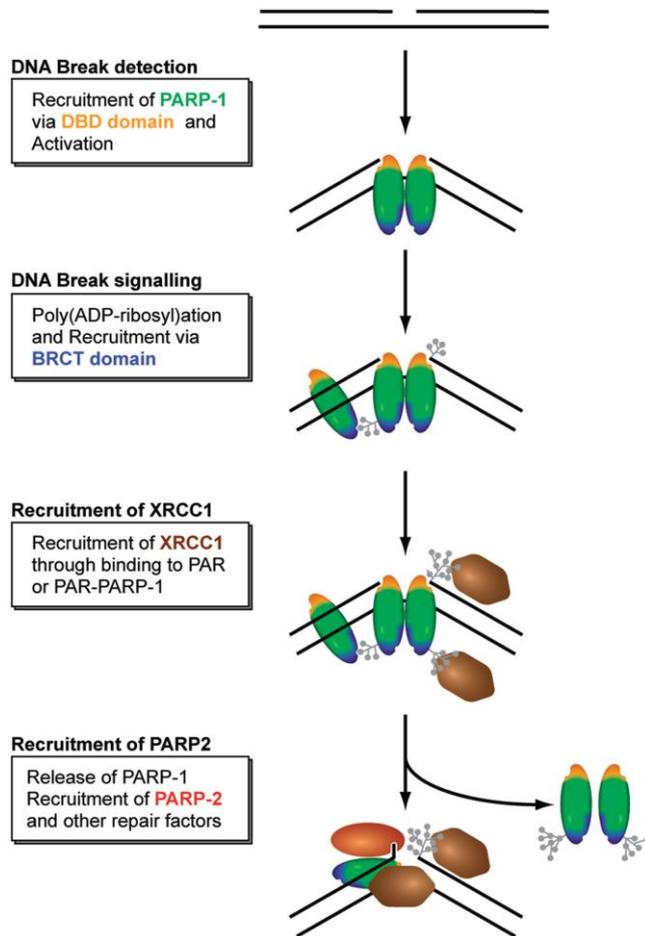
PARP-1 is a key mechanism in maintaining the stability and integrity of the cell DNA. There are constant intracellular (normal metabolism byproducts) and extracellular damaging sources (radiation, chemical agents, heat shock etc.), which might modulate the chemical status of a cell effecting its DNA. These could subsequently cause mutations, chromosomal aberrations, or cell death. Based on its DNA binding ability, it is presumed that PARP-1 is involved in DNA repair. Inhibitors led to some conclusions that PARP-1 is involved in the repair processes. PARP-1 inhibition sensitizes cells to genotoxic stimuli (Küpper et al., 1995; Ding and Smulson, 1994), although PARP inhibitors have limitations due to specificity. PARP knockout mice gave a better insight generated by the de Murcia's laboratory. PARP<sup>-/-</sup> mice are viable and fertile with normal phenotype, but are highly sensitive to ionizing radiation and alkylating agents (de Murcia et al., 1997).

### **1. 4. 2. 1. The base excision repair/single-strand break repair process (BER/SSBR)**

DNA single-strand break repair is a primary cellular pathway for repairing damaged bases or single-strand breaks. In this mechanism DNA glycosylases, AP (apuric/apyrimidic) endonucleases, DNA polymerases, DNA ligases (I, III.), XRCC1 are recruited to the damaged sites, which are repaired by either short patch (one nucleotide) or long-patch repair mechanisms.

Investigating wild type and PARP-1 deficient cell extracts gave insight into the involvement of PARP-1 in repair. PARP-1 interacted with DNA polymerase  $\beta$  giving a striking consequence. Cell extract from PARP-1 deficient cells failed in the Long Patch Repair and showed moderate effect in Short Patch Repair (Dantzer et al., 2000). Another study showed the involvement of PARP-1 in connection with aprataxin-1 in single strand break, a repair protein that was found to be defective in a neuro degenerative disorder (ataxia oculomotor aprataxin-1). The two proteins showed a concerted mechanism in the recruitment of the repair process in SSB (Harris et al., 2009). Interaction of condensin, another performer of the chromatin structure with

PARP-1 along XRCC1 was identified as an SSB-specific response during DNA damage (Heale et al., 2006) (**Figure 5**).



**Figure 5.** Simplified model for the recruitment of repair factors to SSB (Mortusewitz et al., 2007). DNA binding domain of PARP-1 detects single-strand breaks and poly(ADP-ribosylation) occurs leading to chromatin relaxation. Further poly(ADP-ribosylation) at DNA lesions releases PARP-1 enabling other factors to be recruited such as XRCC1.

#### 1. 4. 2. 2. Double-strand breaks (DSBs) repair by HR and NHEJ

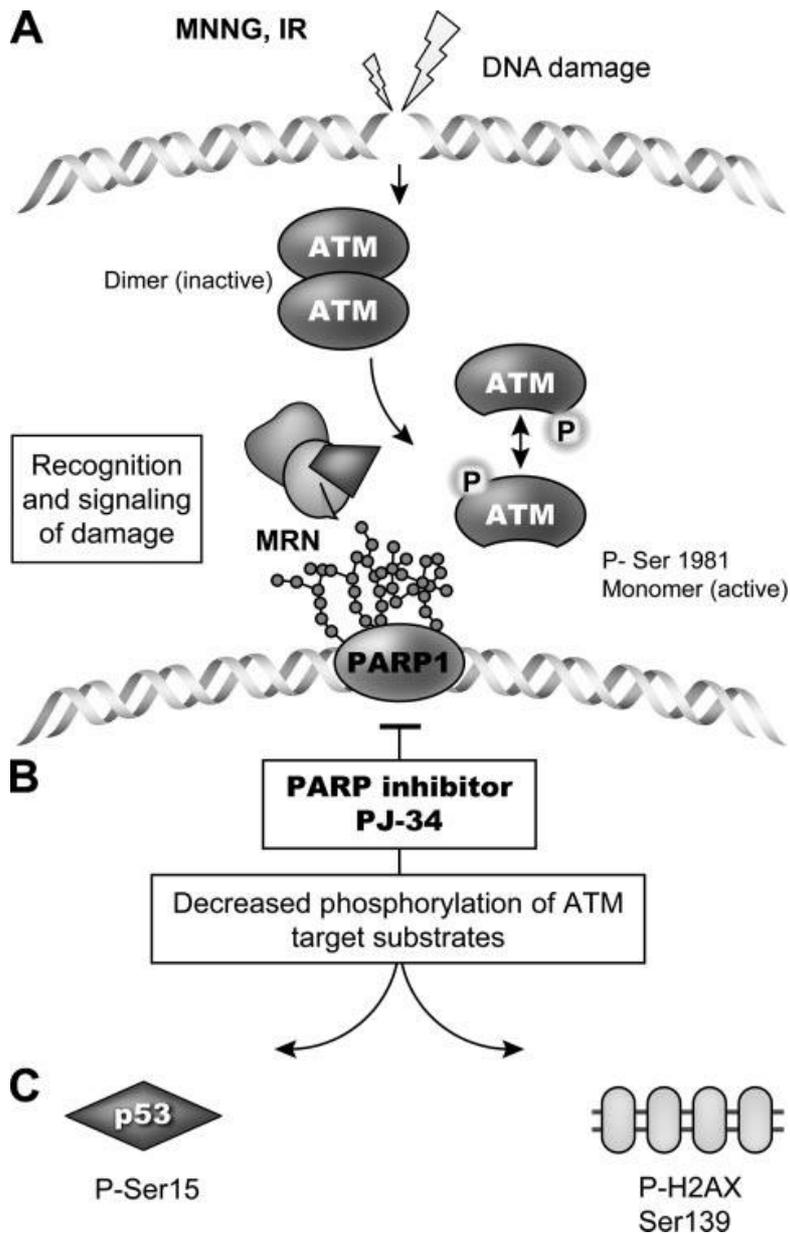
Double-strand breaks (DSB) have the most serious consequences. DSB can be provoked by ionizing radiation, anti-cancer drugs or even by reactive oxygen/nitrogen species. Nature evolved two mechanisms that step in when this type of damage develops. The

two major mechanisms involved are homologous recombination (HR) and non-homologous end joining (NHEJ). HR is utilized during S and G2 phase; it uses the sister chromatid as a template. It requires MRN complex (Mre 11-Rad50-Nbs1), Exo1, RPA, Rad 51, 54. NHEJ (which are referred to as "toxic" or mutagenic), can be divided into classical/canonical-NHEJ and alternative-NHEJ depending on the involvement of KU70-KU80 complex.

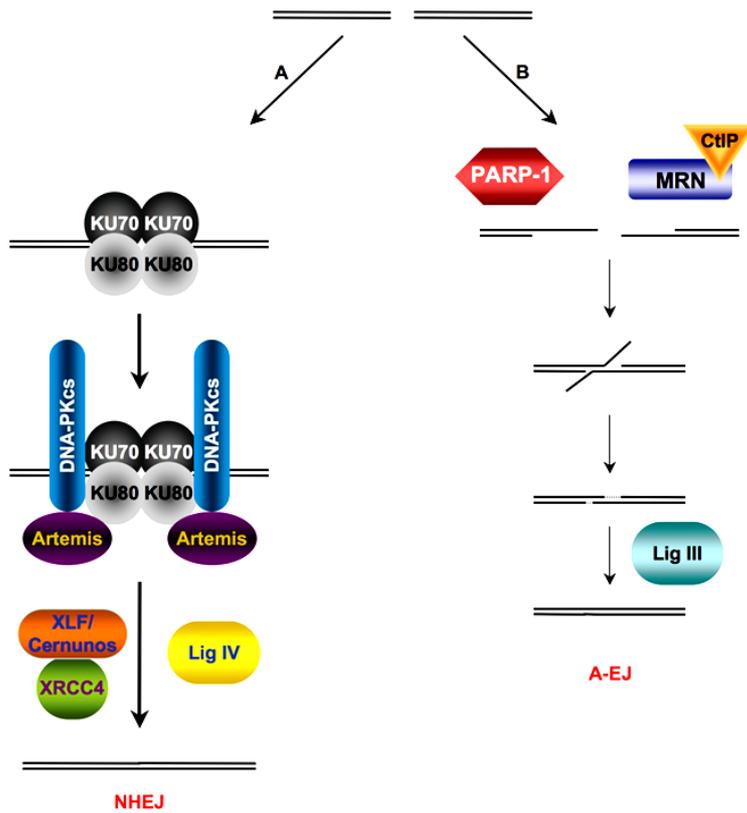
HR takes place when the NHEJ pathways are suppressed; HR requires PARP-1 proved by a study where chicken DT40 cells lacking PARP-1 have reduced level of HR capability (Hochegger et al., 2006). Ataxia telangiectasia mutated (ATM) is a DNA damage-responding kinase induced by the DNA damage. ATM phosphorylates the proteins required for DNA damage response and repair, including proteins of MRN (Mre11/Rad50/NBS1) complex, p53, SMC1 and histone variant H2Ax upon homologous recombination. ATM was shown to physically interact with PARP-1 (Haince JF et al., 2007) (**Figure 6**).

NHEJ can be divided into classical-NHEJ and alternative-NHEJ, depending on the active participation of KU70-KU80. Mostly, C-NHEJ occurs in cells, but A-NHEJ takes place to a lesser degree when C-NHEJ fails or factors are not available. In the C-NHEJ, KU70-KU80 complex is formed after DSB, which recruits DNA-PK phosphorylating subsequent proteins participating in the repair. It was shown that PARylation has effect on the kinase activity *in vitro* (Ruscetti et al., 1998). XRCC4-Lig IV catalyzes the ligation of DNA ends.

When C-NHEJ fails, A-NHEJ (A-EJ) takes over the repair. There is a competition between PARP-1 and KU proteins for the DNA. If KU is not available, PARP-1 is recruited and plays an active role in the A-NHEJ. KU80<sup>-/-</sup> cells highly depend on PARP-activity for the repair process, proving PARP-1 has a critical role in this alternative mechanism. Ligation needs the presence of XRCC1-LigIII (**Figure 7**).



**Figure 6.** ATM- and PAR-dependent DNA break recognition (Haince et al., 2007). Activation of ATM and PARP-1 by the DNA strand breaks (A), then PAR accumulation contributes to the recruitment of ATM providing phosphorylation of ATM downstream targets (B). PARP inhibition changes the phosphorylation of ATM-dependent substrates (C).



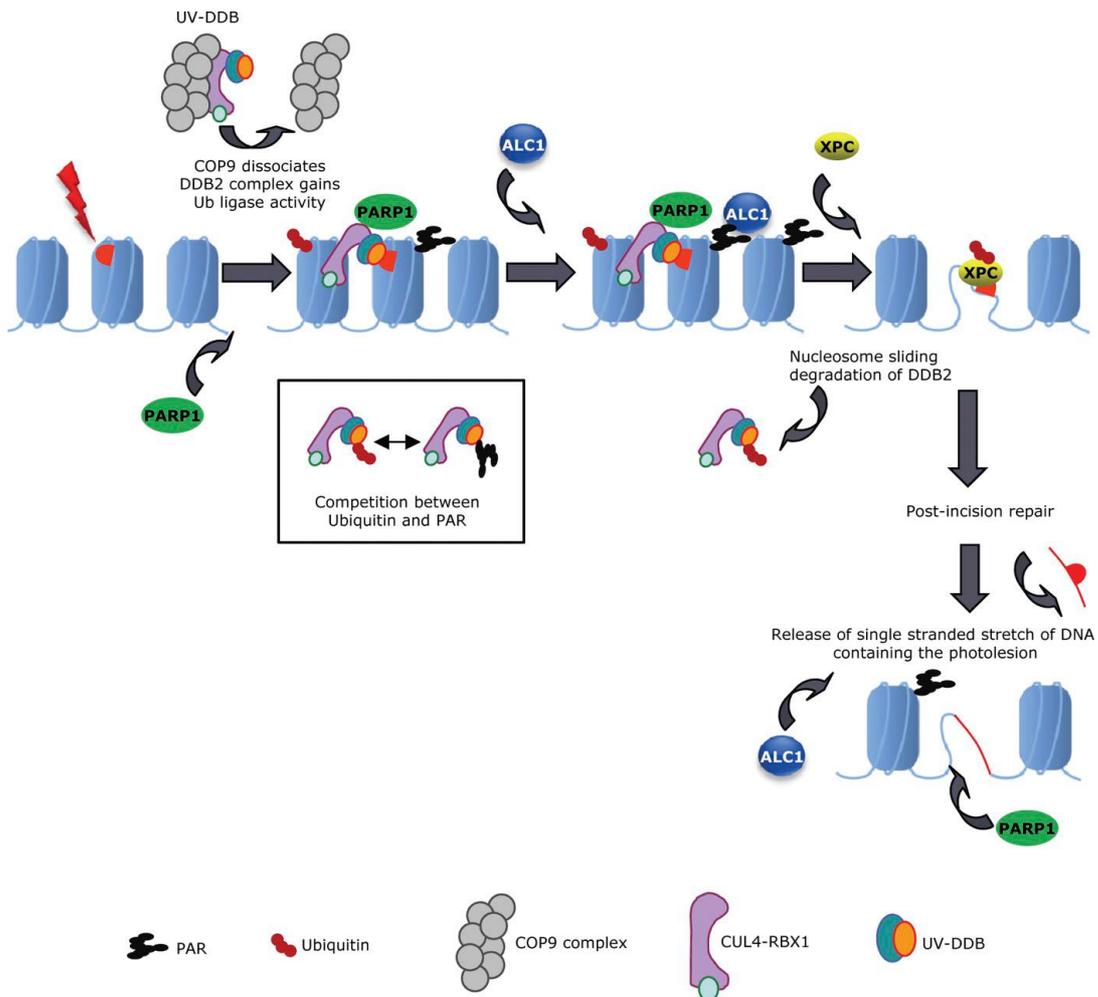
**Figure 7.** Schematic diagram of NHEJ and A-NHEJ after DSBs (Grabarz et al., 2012). A. The heterodimer Ku70/Ku80 contacts with the damaged DNA and recruits DNA-PKcs and Artemis. The latter interacts with the DNA ends and prepares it for enzymatic ligation by the Cernunnos-XLF/XRCC4/Ligase IV complex. B. A-EJ (for Alternative end-joining) is always mutagenic. Damaged DNA, which is not processed by Ku70/Ku80, is degraded. A-EJ is independent on Xrcc4, Ligase IV, and is dependent on PARP-1, Ligase III.

### 1. 4. 2. 3. PARP-1 in nucleotide excision repair (NER)

The main repair mechanism in prokaryotes and eukaryotes against UV-induced DNA lesions and damages is the nucleotide excision repair (NER) that includes the production of thymine dimers (T-T) and other cyclobutane pyrimidine dimers (CPD), as well as 6–4 photoproducts (6-4PP). Mutations in the repair machinery members are associated with xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. There are two classes of NER, the global genomic (GG-NER) and transcription coupled

(TC-NER). The role of PARP-1 in NER is not fully understood.

Robu and colleagues showed that PARP-1 works collaboratively with DDB2 (DNA-binding protein 2) and XPC (xeroderma pigmentosum group C) at UV-damaged lesions in GG-NER (Robu et al., 2013; Luijsterburg et al., 2012; Pines et al., 2012) (**Figure 8**).



**Figure 8.** A model of DDB2- and PARP1-dependent regulation of NER (Pines et al., 2012). UV-DDB is recruited to the UV damage site as a part of CUL4A-RBX1 complex. This binds to the DNA and DDB1 and DDB2 that have a role in binding PARP-1. CUL4A-RBX and PARP-1 regulate DDB2 by ubiquitylation and PARylation. The PARylation of chromatin recruits ALC1, which has a chromatin structure modifying effect mobilizing XPC.

### **1. 4. 3. PARP-1 in mitochondria**

Although the scientific field is divided regarding the presence of mitochondrial PARP-1 (mtPARP-1) there are bodies of evidence that support this idea and the role of PARP-1 in mitochondrial DNA repair, bioenergetics, and mitochondrial cell death signaling (Masmoudi et al., 1988; Du et al., 2003; Rossi et al., 2009; Brunyanszki et al., 2016). Depletion of PARP-1 by siRNA in A549 lung epithelial cells or using tissue from PARP-1 KO mice showed enhanced mitochondrial biogenesis and mitochondrial repair (Szczeny et al., 2014). Mitochondrial PARP-1 activation was showed during H<sub>2</sub>O<sub>2</sub> treatment in U939 by our laboratory. Early mitochondrial DNA damage occurred in response to H<sub>2</sub>O<sub>2</sub> challenge, which was also associated with increased PARylation reactions within the mitochondria (Brunyanszki et al., 2014). These processes were blocked by  $\beta$ -adrenoreceptor blockade with propranolol, the drug that used among burn patients after severe burn injury.

### **1. 4. 4. PARP-1 in Cell Death**

PARP-1 has been implicated in different modes of cell death, apoptosis and necrosis, parthanatos and autophagy. The first correlation between cell death and PARP-1 was made by Berger (Berger et al., 1983). DNA strand breaks activate PARP-1 which plays in the repair process. Inhibition of PARP-1 caused the repair process to be failed while the persistent activation of the enzyme producing excessive poly(ADP-ribose) account for the rapid cell death due to decreased NAD<sup>+</sup> pool and ATP synthesis. In the latter, PARP-1 inhibition proved to be beneficial maintaining the cellular NAD<sup>+</sup> and ATP pools.

Cells that are no longer needed or are a threat to the organism are destroyed by a well-regulated cell suicide process known as programmed cell death or apoptosis. Regulated necrosis or necroptosis as recently used (Van den Berghe et al., 2014) was accepted as new phenomena after showing that tumor necrosis factor alpha (TNF $\alpha$ ) can trigger necrosis mediated by Receptor-Interacting Proteins (RIPs) (Galluzzi et al., 2012). An other form of a caspase-independent cell death is parthanatos (Wang Y et al., 2009) which is accompanied with PARP-1 activation, poly(ADP-ribosyl)ation and AIF

(apoptosis-inducing-factor) translocation including a nuclear-mitochondrial-nuclear communication axis.

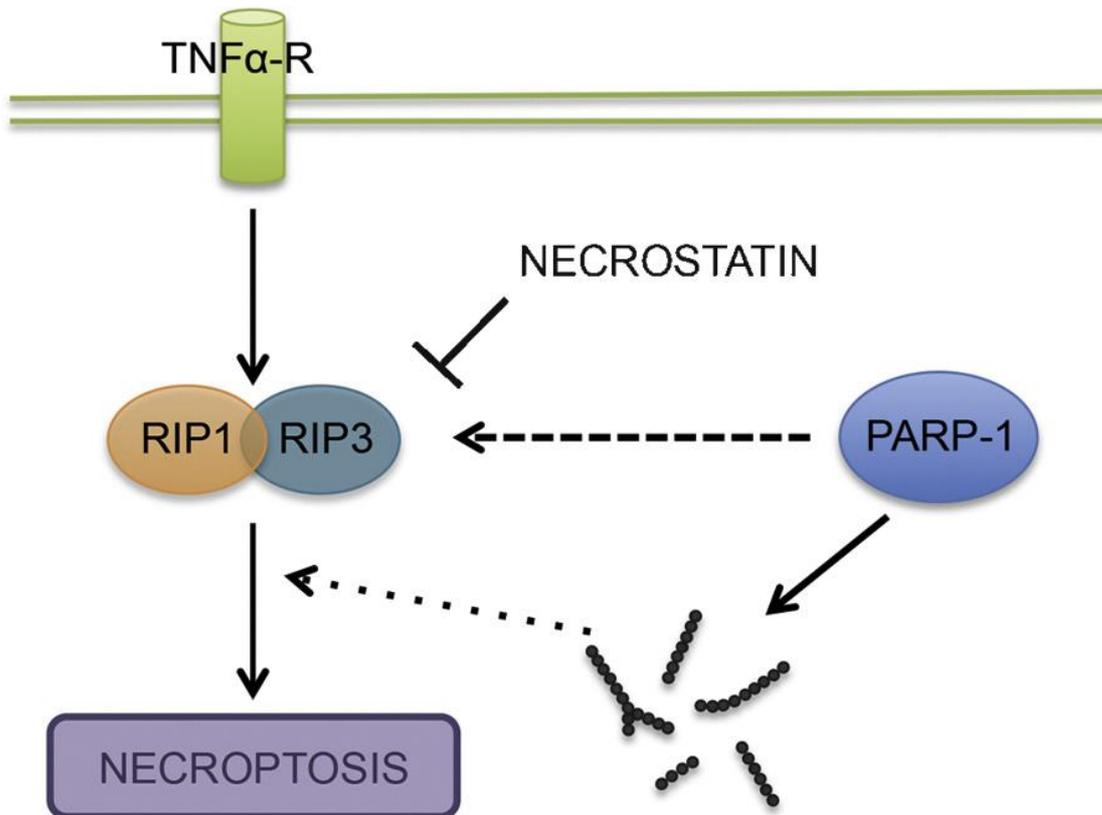
Apoptosis is a tightly regulated process is accompanied by cell shrinkage, chromatin condensation and DNA fragmentation, blebbing of cell membrane, cellular organelles degradation. The apoptotic process can be divided by two subgroups: a, extrinsic initiated by extracellular ligands and b, intrinsic activated by intracellular stressors, DNA damage, oncogenic factors converging on mitochondria. One of the hallmark of apoptosis is the generation of the cleaved PARP-1 by caspase-3 and caspase-7. These two caspases cleave PARP-1 for a 24 kDa N-terminal DNA-binding domain (DBD), which still maintain a strong DNA binding capability, and a 89 kDa C-terminal catalytic fragment which loses its catalytic activity upon cleavage (Tewari et al., 1995). This results the inactivation of PARP-1 promoting apoptosis by ensuing DNA fragmentation and preserving cellular energy for ATP-sensitive steps opposing the energy depletion-induced necrosis (Kim et al., 2000; D' Amours et al., 2001). Herceg and Wang proved the above observation using caspase-uncleavable version of PARP in TNF- $\alpha$ -treated PARP-1 fibroblasts leading these cells to NAD depletion and necrosis (Herceg and Wang 1999).

An intensive line of research focused on whether or not poly(ADP-ribosylation) by PARP-1 makes any differences in the apoptotic process. If there is PARylation involved, that must be taken place at the early phase of apoptosis since PARP-1 is cleaved at that stage and not active catalytically. Inhibition of PARP gave contradict results in terms of the effect on apoptosis. Inhibition of PARP also inhibited (Shiokawa et al., 1997), stimulated (Ray et al., 1992; Payne et al., 1998) or did not change the outcome of apoptosis (Watson et al., 1995) depending on cell type, stimuli, conditions applied.

Studies using PARP-1 knockout mice derived cells showed that PARP-1 mostly can be dispensable for apoptosis. Hepatocytes, thymocytes and neurons from knock out animals compared to their wild-type counterpart did not show differences in the apoptotic process after Fas, TNF $\alpha$ ,  $\gamma$ -irradiation, or dexamethasone treatment; however PARP-1 cleavage is still a key step in the apoptotic machinery.

Necrosis or necroptosis as a new nomenclature was implemented based on the fact that it is a well-regulated process which was considered before as a passive, unregulated

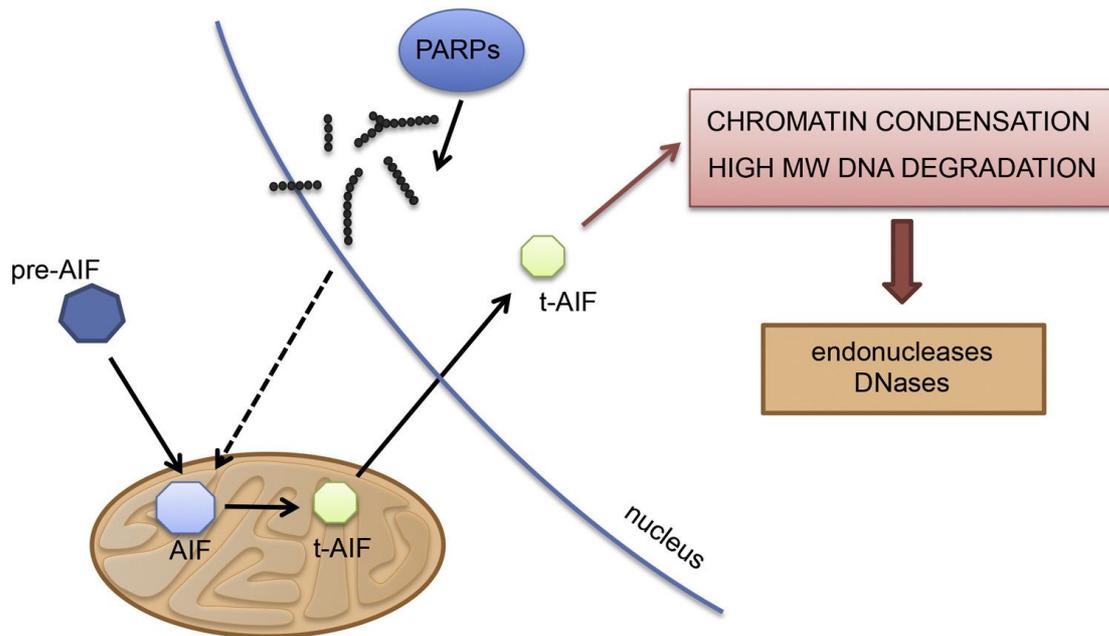
way of dying. It is activated by different extracellular stimuli and the most obvious feature of the necrosis is the disintegration of the plasma membrane and cell content release to the extracellular space. It has been implicated in the pathology of muscular dystrophy, myocardial ischemia-reperfusion injury, diabetes, Alzheimer's, Huntington's and Parkinson's disease. The most crucial members of necroptosis are Receptor-Interacting-Proteins1 and 3 (RIPK1 and RIPK3) which are death domain-containing Ser/Thr kinases and activate nuclear factor kappa B (NF- $\kappa$ B) pathway. That can be inhibited by necrostatin-1 causing no effect on the apoptotic pathway (Van den Berghe et al., 2014). PARP-1 overactivation-induced necrosis was implicated in several pathophysiological conditions. Mouse embryonic fibroblast treated with DNA-alkylating agent N-methyl-N-nitro-N-nitrosoguanidine (MNNG) showed mitochondrial dysfunction, apoptosis inducing factor (AIF) -translocation and cell death in which c-Jun N-terminal kinase (JNK) is required for the PARP-1-induced consequences. Also, these effect were attenuated in RIP1 and TRAF2 (Tumor necrosis factor receptor-associated factor 2) knock out cells which are upstream of JNK which concludes the PARP-1 and necrosis close relation (Xu et al., 2006). High concentration of reactive oxygen and nitrogen species (ROS/RNS) caused overactivation of PARP-1 leading to necrotic cell death in thymocytes. Cells from PARP-1 deficient mice were protected against oxidant-induced damage. Same was true for the pharmacological intervention by 3-aminobenzamide or 5-iodo-6-amino-1,2-benzopyrone against the oxidative stimuli proving the important role of PARP-1 in the necrotic death (Virag et al., 1998 a, b). In a research paper published by Adam's Laboratory the question was raised whether or not necrosis takes place as a single pathway or different signaling systems contributes to the overall outcome. DNA-alkylating agents, MNNG and methyl methanesulfonate (MMS), rapidly activates PARP-1, while in TNF-induced necrosis this is only a late step of the process. Also, PARP-1 inhibitor, 3 amino-benzamide (3-AB), attenuated the effect of MNNG, but failed to do so in TNF-treated cells. Finally, interfering with RIP1 and Rip3 or blocking ceramide (imipramine) generation was unable to protect necrosis through the PARP pathway (Sosna et al., 2013). This work proves the complexity of the necrosis which can not be concluded as a simplified and uncontrolled way of death (**Figure 9**).



**Figure 9.** Schematic diagram of necroptosis (Aredia and Scovassi 2014). TNF induces the association of RIP1 and RIP3 (necrostatin is inhibiting it) in which process, PARP-1 directly can regulate necroptosis or indirectly, producing Poly(ADP-ribose). RIP: Receptor-Interacting Protein.

Parthanatos, which refers to PAR and the Greek word of death, is a recognized cell death by the Nomenclature Committee of Cell Death and it classifies as a form of regulated necrosis (Galluzzi et al., 2012). It is a PARP-1 activation driven upon nuclear damage through AIF translocation from mitochondria to nucleus showing phosphatidylserine externalization, loss of mitochondrial membrane potential, chromatin condensation, shrinkage of the cell typically seen also in apoptosis, but the distinction of loss of cell membrane integrity, the lack of dependence on caspase activation and on energy with the appearance of large 50kb DNA fragments (**Figure 10**). AIF is a 62kDa transmembrane flavoprotein found in the inner mitochondrial membrane anchored which is processed to a 57kDa soluble form before its release (Otera et al., 2005). After different stimuli with staurosporin, c-Myc, etoposide, or

ceramide, in Rat-1 cells induced AIF release and it was caspase independence showed by using caspase inhibitor Z-VAD.fmk which failed to prevent the translocation of AIF and the condensation of chromatin (Daugas et al., 2000). Immortalized mouse fibroblast showed AIF release after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, a DNA-alkylating agent that potently activates PARP-1, exposure which was prevented by PARP-1 inhibitors, 1,5-dihydroxyisoquinoline (DHIQ) (300  $\mu$ M) and 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ) (30  $\mu$ M). The translocation and the cytotoxic effect was also abolished in PARP-1-KO fibroblasts. The broad range of caspase inhibitors failed to prevent the MNNG-induced cytotoxicity proving the caspase-independent mechanism (Yu et al., 2002). The above mentioned observation was proven in a cell free system where HeLa cells nuclei activated by MNNG were incubated with isolated brain mitochondria showed while nuclei non treated with MNNG did not show AIF release. The MNNG activated nuclei treated with PARG or phosphodiesterase markedly reduce the AIF from the mitochondria (Yu et al., 2006). The release of AIF from the mitochondria is promoted by Bax (Bcl-2-associated X protein). It contains a nuclear localization signal (NLS) which helps the translocation from the cytoplasm to nucleus where it forms a complex with cyclophyllin-A and H2Ax resulting chromatin condensation and DNA degradation (Moubarak et al., 2007; Delavalle et al., 2011).

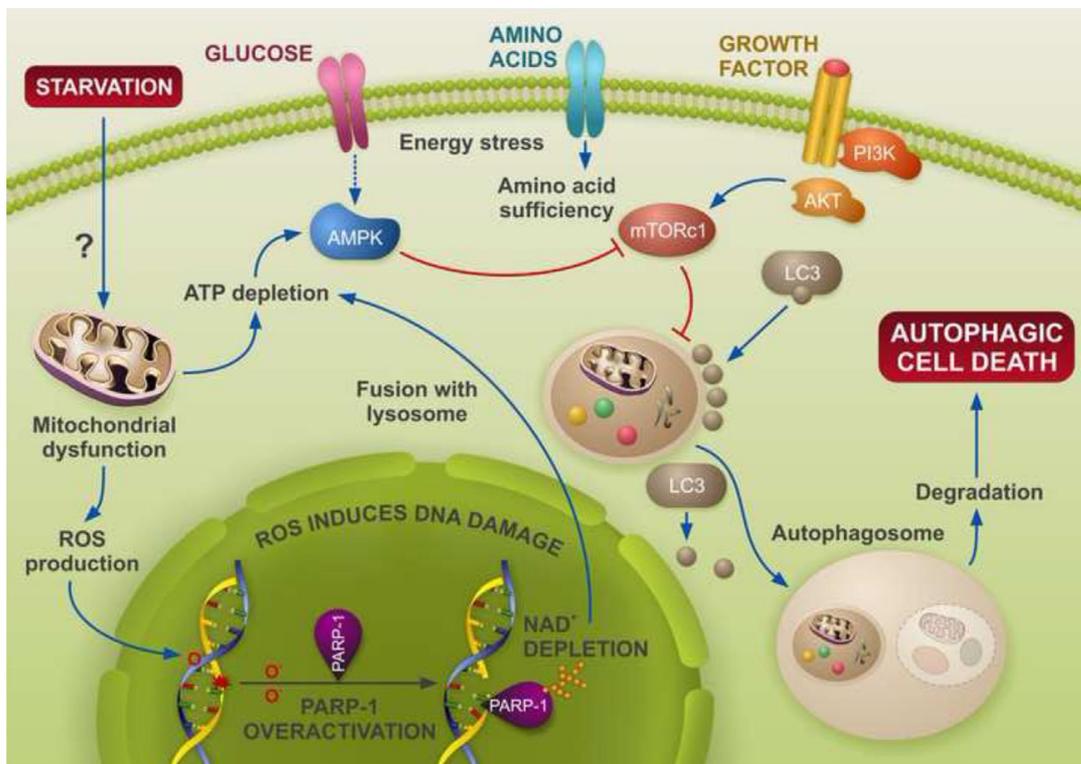


**Figure 10.** *Hallmark of parthanatos: AIF translocation (Aredia and Scovassi, 2014).* AIF, synthesized in the cytosol as a precursor (pre-AIF), in its mature form is imported into the mitochondria. During parthanatos, AIF is converted into its soluble form tAIF and translocates to the nucleus where it promotes chromatin condensation and high molecular weight DNA degradation through the recruitment of endonucleases and DNases. AIF relocalization is promoted by the accumulation of polymers of ADP-ribose synthesized by PARPs. AIF: Apoptosis Inducing Factor; tAIF: truncated AIF.

Autophagy is thought to be a self-defense mechanism helping cells with the maintenance of energy homeostasis degrading damaged compounds. There are different inducers such as oxidative stress, starvation, DNA-damage. It is mainly regulated by Ser/Thr kinases, mTOR (mammalian Target Of Rapamycin) and AMPK (AMP-activated Protein Kinase). They sense metabolic changes and work on the contrary. Akt is also plays a crucial role since it directly phosphorylates mTOR. mTOR is the negative regulator and active when energy sources are high or abundant keeping autophagy in an “off” status. AMPK is active when nutrient is scarce, during starvation (Aredia and Scovassi, 2014). A constant level of autophagy can be seen in neurons and malfunction of autophagy may associate with Alzheimer's disease-, Parkinson's disease and Huntington disease. During cancer cachexia (muscle mass wasting) where myostatin

expression increased, mTOR/Akt pathway is inhibited (Gallot et al., 2014). Duchenne muscular dystrophy (DMD) is a X-linked degenerative muscle disorder affects male infants. In the cellular level, the condition goes hand in hand with elevated ROS (reactive oxygen species) production, altered  $\text{Ca}^{2+}$  homeostasis and impaired autophagy. *Mdx*-Knock-out mice, a model of DMD lacking dystrophin, an important muscle protein, have elevated Nox2 activity and Src kinase expression which might be responsible for the elevated oxidant production and decreased autophagy through PI3K/Akt/mTOR pathway (Pal et al., 2014). On the other hand, by sport-physiological mean, during ultra-endurance running as an acute exercise, when energy demands are high, autophagy activity is increased via dephosphorylation of forkhead box O3 (FoxO3) (Jamart et al., 2012). Chronic aerobic exercise results different outcome of the autophagy process depending on the type of exercise, dietary restriction, oxidant production or type of muscles involved (Tam BT and Siu PM, 2014). PARP-1, as discussed above, is activated during oxidative stress and DNA damage which depending on the intensity of the stimuli could also activate the autophagy machinery. Embryonic fibroblasts from *PARP-1*<sup>+/+</sup> and *PARP-1*<sup>-/-</sup> mouse (MEF) showed different delays in autophagic pattern after starvation, namely the number of autophagosomes were higher in *PARP-1*<sup>+/+</sup> compared to *PARP-1*<sup>-/-</sup>. The same pattern was seen after induction of autophagy by rapamycin. Similar results were obtained by pharmacological inhibition and siRNA-based depletion of PARP-1 suggesting the active role of PARP-1 in autophagy upon starvation. ROS production following starvation was also higher in MEF from *parp-1*<sup>+/+</sup>. The diffusion of ROS into nucleus caused DNA damage and it was more pronounced in the *PARP-1*<sup>+/+</sup> cells. Cells lacking PARP-1 showed reduced level of ATP depletion which was corresponded with the significantly less activation of AMPK. In *PARP-1*<sup>+/+</sup> cells mTOR was completely inhibited suggesting the commitment of these cells to autophagy and also that PARP-1 might control mTOR activity during starvation (Rodriguez-Vargas et al., 2012). Chemotherapy often induces autophagy in cancer cells, moreover PARP-1 inhibition potentiating chemotherapy-induced cell death. MEF from *PARP-1*<sup>+/+</sup> and *PARP-1*<sup>-/-</sup> mouse were treated with doxorubicin, a DNA damaging agent, inducing a rapid, direct effect on PARP activation.  $\text{NAD}^+$  and ATP depletion was observed in *PARP-1*<sup>+/+</sup> but in *PARP-1*<sup>-/-</sup> without going toward necrotic or apoptotic pathways. Cells from *PARP-1*<sup>+/+</sup> underwent doxorubicin-induced

autophagy as seen by electron microscopy, but this was almost absent in *PARP-1*<sup>-/-</sup> cells. Pharmacological inhibition of PARP-1 also protected against the autophagic vacuole production after doxorubicin treatment. These results suggest whether or not cells undergo autophagy depends on the PARP-1 activation (Munoz-Gamez et al., 2009). In a subcutaneous tumor model, PARP-1 activation is required in the TNFSF10-induced ADP-ribosylation of high mobility group protein B1 (HMGB1) which causes cancer cell death. Pharmacological inhibition or knockdown of PARP-1 decreased autophagy for the good of apoptosis increasing the anticancer activity (Yang et al., 2015). Starvation and hypoxia can trigger metabolic stress, ROS production, DNA damage and PARP-1 activation leading to autophagy as a defensive mechanism to counterbalance the energy needs for these cells and to avoid cell death. Inhibiting PARP-1 might open new ways in therapeutic applications, probably with combination with other drugs, to inhibit autophagy, which in this case is an escape from death, and reactivate apoptosis to eliminate those cells (**Figure 11**).



**Figure 11.** *PARP-1* modulates starvation-induced autophagy (Virag et al., 2013). After deprivation of nutrients, cells undergo autophagy through the activation of

AMPK/inhibition of mTOR, allowing the formation of the autophagosome core. Events upstream of AMPK involve energy depletion, ROS production and DNA damage. In this scenario, PARP-1 over-activation leads to ATP depletion, acting as a feedback loop to reactivate autophagy. This stress signal when maintained leads eventually to cell death through autophagy. PARP inactivation delays autophagy and favors apoptosis. In the absence of PARP-1 or after PARP inactivation, ROS levels decrease and ATP drop is reduced. As a consequence, the feedback loop reactivated by PARP-1 is cut-down, and apoptosis is triggered.

	Apoptosis	Necrosis	Autophagy	Parthanatos
Variations/Subsets in the literature	Caspase-dependent intrinsic <sup>a</sup> Caspase-independent intrinsic <sup>b</sup> Extrinsic apoptosis by death receptors <sup>c</sup> Extrinsic apoptosis by dependence receptors <sup>d</sup>	Random or unregulated Programmed or regulated, e.g. necroptosis (some think parthanatos could also be considered a case of regulated necrosis)	Macroautophagy Microautophagy Chaperone-mediated autophagy Mitophagy (but here we consider autophagy as macroautophagy)	
(Biochemical) Signatures				
Mitochondrial	Caspase activation (except in <sup>b</sup> ) Mitochondrial depolarization MOMP <sup>a,c</sup> Irreversible $\Delta\psi_m$ dissipation <sup>a</sup> CYT c release Release of IMS proteins <sup>b</sup> Respiratory chain inhibition <sup>b</sup> BID cleavage <sup>c</sup> PP2A activation <sup>d</sup> DAPK1 activation <sup>d</sup>	Loss of ultrastructure Swelling	Degradation	Depolarization Irreversible $\Delta\psi_m$ dissipation ATP and NADH depletion AIF release CYT c release Caspase activation (late stage, non-obligatory)
Cytoplasmic	Shrinkage	Swelling (including of organelles) Vacuolation Organelle disintegration	Massive vacuolization Lysosomal degradation MAP1LC3 lipidation	PAR polymer accumulation PAR-AIF interactions (binding) Condensation AIF translocation to the nucleus
Nuclear	PARP cleavage Chromatin condensation DNA fragmentation (small-scale, DNA ladder)	Chromatin digestion DNA hydrolysis (smear)	SQSTM1 degradation	Rapid PARP-1 activation (not cleavage) PARP-1-mediated PAR synthesis Chromatin condensation PAAN activation (putative) DNA fragmentation (large-scale, ~50 kb)
Structural (plasma membrane) changes	Membrane integrity preserved Formation of apoptotic bodies Membrane blebbing Phosphatidylserine externalization	Loss of integrity Blebbing Cell lysis	Double membrane-bound autophagosomes formed	Loss of integrity Phosphatidylserine externalization
Examples of trigger factors and/or conditions	Death receptor signalling <sup>c</sup> Dependence receptor signalling <sup>d</sup> DNA damage Trophic factor withdrawal Viral infections	Excitotoxicity Ischaemia Stroke Reactive oxygen/nitrogen species	Amino acid starvation Serum starvation Protein aggregates	Excitotoxicity Ischaemia DNA damage Stroke Reactive oxygen/nitrogen species
Energy (ATP) requirement	+	-	+	-
(Obligatory) Caspase-dependence	+ <sup>a,c,d</sup> - <sup>b</sup>	-	-	-
Inflammatory component	-	+	-	-
Major mediator(s)	Caspases (except in <sup>b</sup> )	Calpains, CYPD, RIP-1, RIP-3 (and PARP-1 and AIF, if parthanatos is considered regulated necrosis), etc.	ATG5, ATG6 (Beclin-1), ATG7, ATG12, VPS34, AMBRA-1	PARP-1 PAR AIF
Pharmacological inhibition	Caspase inhibitors, e.g. Z-VAD-fmk (except in <sup>b</sup> )	RIP-1 inhibitors, e.g. necrostatin-1, calpain inhibitors, etc.	VPS34 inhibitors, e.g. 3-methyladenine and wortmannin	PARP-1 inhibitors, e.g. DPQ
Genetic inhibition (knockout/mutation, RNAi targeting) or inhibition by protein overexpression	BCL2 overexpression <sup>a, b</sup> Inhibition of caspases (3, 8 and 9) <sup>c,d</sup> Inhibition of PP2A <sup>d</sup> Crma expression <sup>c</sup>	Inhibition of RIP-1 or RIP-3	Inhibition of AMBRA1, ATG5, ATG7, ATG12 or BECN1	PARP-1 knockout, AIF down-regulation (e.g. in Harlequin mouse)

**Figure 12.** Similarities and differences in apoptosis, necrosis, autophagy and parthanatos (Fatokun et al., 2014). While parthanatos is generally considered to be

separate and distinct from necrosis, some investigators consider it to be a specific case of regulated necrosis, just as is necroptosis.  $\Delta\psi_m$ : mitochondrial transmembrane potential. AIF, apoptosis-inducing factor; AMBRA1, activating molecule in Beclin-1-regulated autophagy protein 1; ATG, autophagy; BCL2, B-cell lymphoma 2; BECN1, Beclin-1; CrmA, cytokine response modifier A; CYPD, cyclophilin D; CYT, cytochrome; DAPK1, death-associated protein kinase 1; DPQ, 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone; IMS, intermembrane space; MAP1LC3, microtubule-associated protein 1 light chain 3; MOMP, mitochondrial outer membrane permeabilization; PAAN, parthanatos AIF-associated nuclease; PAR, poly (ADP-ribose); PP2A, protein phosphatase 2A; RIP, receptor-interacting protein; SQSTM1, sequestosome 1; VPS, vacuolar protein sorting; Z-VAD-fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

The role of PARP-1 in different forms of cell death such as apoptosis, necrosis, parthanatos or autophagy was described above. It is mainly associated with DNA damage in some degree but it is thought to have DNA-independent function as well which needs more and deeper investigation.

These 4 types of death can be distinguished based on morphological, biochemical phenotype or the changes to pharmacological inhibition, as outlined in **Figure 12**.

#### **1. 4. 5. Role of PARP-1 in pathophysiology**

An unbalanced cell cycle regulation, cell proliferation or DNA-repair are hallmarks of tumorigenesis. Deletion of one PARP-1, -2, -3 enzymes or pharmacological inhibition under non-stress condition does not lead to major physiological issues or subsequently cancer since the abundance of other repair enzymes step in for this role in the cell. But when they are challenged instability occurs. PARP-1<sup>-/-</sup> mice challenged with whole body  $\gamma$ -irradiation or N-methyl-N-nitroso urea (MNU) showed genomic instability. Cells derived from PARP-1<sup>-/-</sup> mice also displayed high sensitivity to MNU. (de Murcia et al., 1997, Rouleau et al., 2007). Other repair proteins and PARP simultaneous deletion led to embryonic lethality or tumor formation (De Vos et al., 2012).

Reactive oxygen/nitrogen species (ROS/RNS) damage the cells and the components of

it such as proteins, lipids, nucleic acids. Generally speaking, under oxidative/nitrosative stimuli PARP-1 is activated and contributes to the DNA repair function. Depending on the severity of the oxidative stress signal, it might induce apoptosis, necrosis or autophagy. This is associated with a wide spectrum of diseases including cardiovascular, neurological, immunological and diabetic complications (for review, see Virag and Szabo 2002). In the last 20 years peroxynitrite received a lot of attention, which also activates PARP-1 and causes PARP-1 related cell injury potentiating NF $\kappa$ B activation turning on inflammatory pathways. The other side of the coin is that PARP-1 and PARP-2 takes part of the normal development of immune cells playing role in T and B-cells differentiation and function in the adaptive immunity. Inhibitors of PARP-1 improve immune-mediated diseases such as arthritis, colitis or allergy (Rosado et al., 2013).

PARP-1 also has a role in energy metabolism serving as a metabolic regulator. Muscles from PARP-1<sup>-/-</sup> mice showed higher mitochondrial activity, in connection with SIRT-1 (another NAD<sup>+</sup> consuming enzyme), improved glucose removal and insulin sensitivity (Bai and Canto 2012). Long term pharmacological inhibition of PARP improves fitness in mice by increasing the number of mitochondrial complexes and enhancing mitochondrial respiratory capacity, which in terms of a pharmacological approach opens new possibilities for treatment for muscle dysfunction linked to mitochondrial function (Pirinen et al., 2012). In young exercised mice SIRT-1 deacetylates PARP-1 decreasing its activity. This effect is suppressed in aged mice, but was reversible with PARP-1 inhibitor suggesting that inhibition of PARP-1 may serve a pharmacological tool in muscle-related pathophysiological condition such as sarcopenia or disuse-induced atrophy by aging (Mohamed et al., 2014).

The importance of PARP-1 activity has been described in several other pathological condition such as ischemic-reperfusion where PARP-1 is an active participant of the neutrophil-mediated myocardial damage upon ischemia and reperfusion and genetic depletion or pharmacological intervention using inhibitors of the enzyme improved the outcome of the injury (Zingarelli et al., 1998). In a hemorrhagic shock model, PARP-1 was activated parallel with metabolic acidosis, lactate production which was reduced by PARP-1 inhibitor 3-AB in the ileum and liver (Watts et al., 2001). ROS and RNS are the two major contributor to endothelial dysfunction in diabetes in which PARP-1 is

responsible for the metabolic changes, specially since ROS/RNS can cause DNA damage activating the enzyme. Inhibition of the process by PARP-1 inhibitor could reverse the endothelial dysfunction (Soriano et al., 2001). PARP-1 activity also mediates inflammation in mouse model of contact hypersensitivity promoting leukocyte migration and expression inflammatory mediators, cytokines, chemokines and MMP (Matrix metalloproteinase) (Bai et al., 2009).

Targeting repair deficient tumor cells to promote their genomic instability, improving muscle function in aged individuals, reducing inflammatory cytokines expression and leukocyte migration have a common interest: PARP inhibition. Some of the inhibitors already have advanced to clinical trials and give hopes to fight against these pathological complications.

## **1. 5. Oxidative stress, as a trigger of PARP activation**

As it was discussed earlier, the best characterized activator of PARP-1 is DNA-damage induced by free radicals. Prolonged ROS/RNS (Reactive oxygen/nitrogen species) production results in an excessive PARP-1 activation reducing the cell NAD<sup>+</sup> and ATP stores, blocking apoptosis and resulting necrosis. The better understand the role of PARP-1, below an overview is given about the origin of reactive oxygen/nitrogen species.

Reactive oxygen and nitrogen species are known to contribute to wide variety of diseases such as cancer, atherosclerosis, diabetes, neurodegenerative disorders, or aging. As a nomenclature point of view, reactive species, either ROS or RNS, are a collective terms and can be divided into two subgroups, free radicals and non-radicals (**Table 1**).

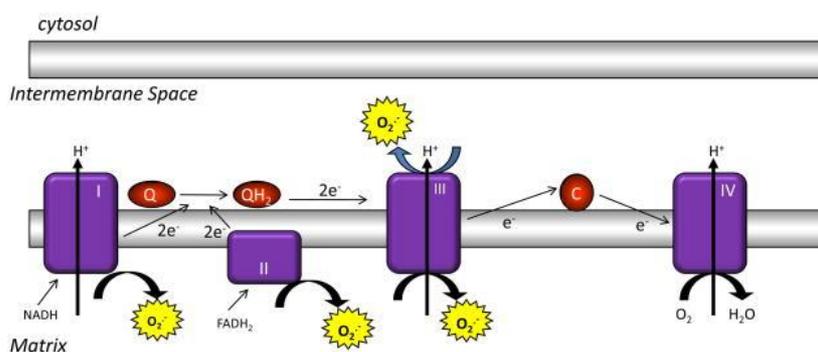
**Table 1.** Reactive oxygen and nitrogen species (Halliwell and Whiteman 2004).

<u>Free radicals</u>	<u>Oxidants</u>
<b>Reactive oxygen species (ROS)</b>	
Superoxide, O <sub>2</sub> <sup>•-</sup>	Hydrogen peroxide, H <sub>2</sub> O <sub>2</sub>
Hydroxyl, OH <sup>•</sup>	Hypobromous acid, HOBr
Hydroperoxyl, HO <sub>2</sub> <sup>•</sup>	Hypochlorous acid, HOCl
	Ozone O <sub>3</sub>
Peroxyl, RO <sub>2</sub> <sup>•</sup>	Singlet oxygen (O <sub>2</sub> <sup>1</sup> Δg)
Alkoxy, RO <sup>•</sup>	Organic peroxides, ROOH
Carbonate, CO <sub>3</sub> <sup>•-</sup>	Peroxynitrite, ONOO <sup>-</sup>
Carbon dioxide, CO <sub>2</sub> <sup>•-</sup>	Peroxynitrous acid, ONOOH
<b>Reactive nitrogen species (RNS)</b>	
Nitric oxide, NO <sup>•</sup>	Nitrous acid, HNO <sub>2</sub>
Nitrogen dioxide, NO <sub>2</sub> <sup>•</sup>	Nitrosyl cation, NO <sup>+</sup>
	Nitroxyl anion, NO <sup>-</sup>
	Dinitrogen tetroxide, N <sub>2</sub> O <sub>4</sub>
	Dinitrogen trioxide, N <sub>2</sub> O <sub>3</sub>
	Peroxynitrite, ONOO <sup>-</sup>
	Peroxynitrous acid, ONOOH
	Nitronium (nitryl) cation, NO <sub>2</sub> <sup>+</sup>
	Alkyl peroxynitrites, ROONO
	Nitryl (nitronium) chloride, NO <sub>2</sub> Cl

ROS is a collective term that includes both oxygen radicals and certain non-radicals that are oxidizing agents and/or are easily converted into radicals (HOCl, HOBr, O<sub>3</sub>, ONOO<sup>-</sup>, <sup>1</sup>O<sub>2</sub>, H<sub>2</sub>O<sub>2</sub>). In other words, *all oxygen radicals are ROS, but not all ROS are oxygen radicals*. Peroxynitrite and H<sub>2</sub>O<sub>2</sub> are frequently erroneously described in the literature as free radicals, for example. RNS is also a collective term including nitric oxide and nitrogen dioxide radicals, as well as non-radicals such as HNO<sub>2</sub> and N<sub>2</sub>O<sub>4</sub>.

'Reactive' is not always an appropriate term:  $\text{H}_2\text{O}_2$ ,  $\text{NO}^\bullet$  and  $\text{O}_2^{\bullet-}$  react quickly with only a few molecules, whereas  $\text{OH}^\bullet$  reacts quickly with almost everything.  $\text{RO}_2^\bullet$ ,  $\text{RO}^\bullet$ ,  $\text{HOCl}$ ,  $\text{NO}_2^\bullet$ ,  $\text{ONOO}^-$ ,  $\text{NO}_2^+$  and  $\text{O}_3$  have intermediate reactivities. We can distinguish endogenous and exogenous source of reactive species. The endogenous sources are mitochondria, peroxisomes, phagolysosomes and NO producing enzymes, NO synthases.

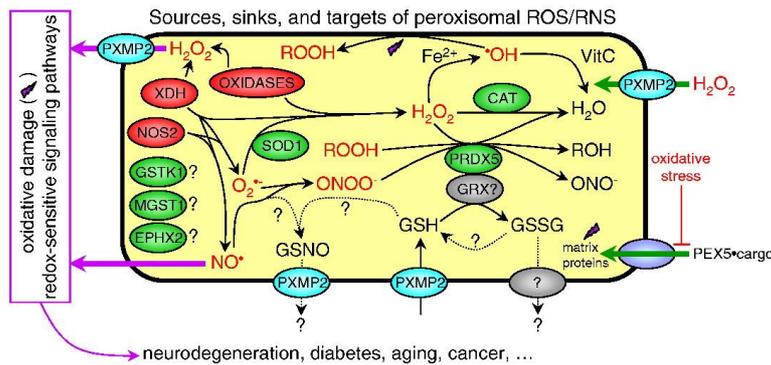
The main producers of the reactive species are mitochondria which are continuously working energy producing organelles in cells and are responsible for the terminal oxidation and oxidative phosphorylation. In its electron ( $e^-$ ) transport chain (ETC),  $e^-$  is transferred in the inner mitochondrial membrane and the proton ( $\text{H}^+$ ) gradient generated used for ATP synthesis. In this system, electrons can prematurely reduce oxygen in Complex I, II, and Complex III producing superoxide (**Figure 13**).



**Figure 13.** *The mitochondrial electron transport chain produces ROS (Hamanaka and Chandel, 2010).* Mitochondrial complexes I and II use electrons donated from NADH and  $\text{FADH}_2$  to reduce coenzyme Q, which shuttles these electrons to complex III, where they are transferred to cytochrome *c*. Complex IV uses electrons from cytochrome *c* to reduce molecular oxygen to water. The action of complexes I, III, and IV produce a proton electrochemical potential gradient, the free energy of which is used by the ATP synthase. Complexes I, II, and III produce superoxide through the incomplete reduction of oxygen to superoxide. Complexes I and II produce superoxide only into the mitochondrial matrix. Complex III produces superoxide into both the matrix and the intermembrane space.

Peroxisomes are multifunctional organelles in eukaryotic cells oxidizing fatty acids by the  $\alpha$ - and  $\beta$ -oxidation, participating ether-phospholipid biosynthesis, amino acid

catabolism, polyamine oxidation. There are different peroxisomal oxidases as part of the metabolic pathways that produce ROS/RNS. NOS2, inducible nitric oxide synthase was also described in peroxisomes capable of producing ROS and RNS. Release of ROS and RNS can damage the peroxisome at the site of production or escaping from the organelle and subsequently can lead to other signaling pathways and damaging effect in the cells (**Figure 14**).



**Figure 14.** ROS and RNS production in the peroxisome (Fransen et al., 2012).

Peroxisomes contain various enzymes that produce hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide (O<sub>2</sub><sup>•-</sup>), or nitric oxide (NO<sup>•</sup>) as part of their normal catalytic cycle. These molecules can readily react to form other ROS and RNS such as peroxynitrite (ONOO<sup>-</sup>), hydroxyl radical (•OH), and alkyl peroxides (ROOH). Peroxisomes are also well equipped with enzymatic and non enzymatic antioxidant defense systems, including catalase (CAT), superoxide dismutase 1 (SOD1), peroxiredoxin 5 (PRDX5), glutathione S- transferase kappa (GSTK1), ‘microsomal’ glutathione S- transferase (MGST1), epoxide hydrolase 2 (EPHX2), reduced glutathione (GSH) and vitamin C (VitC). GSH and VitC most likely freely penetrate the peroxisomal membrane through PXMP2, a non- selective pore- forming protein with an upper molecular size limit of 300–600 Da. Excess peroxisomal ROS/RNS can directly inactivate peroxisomal matrix proteins or promote the production of potential signaling molecules such as S- nitrosoglutathione (GSNO). Alternatively, some of these small reactive molecules may induce membrane damage through lipid peroxidation or diffuse out of the organelle. The latter event may perturb the cellular redox status, a condition generally considered as a risk factor for the development of age- related diseases. Finally, under conditions of increased oxidative stress, peroxisomes may also function as a sink for cellular ROS. XDH, xanthine

oxidase; NOS2, inducible nitric oxide synthase; GRX, glutaredoxin; ROH, alcohol;  $\text{ONO}^-$ , nitrite.

Phagolysosomes are organelles which actually use reactive species to destroy invaders. It is a single-membrane phagosome fused with a lysosome. They could be found in certain white blood cells such as granulo-, mono- and lymphocytes (Forman and Torres 2001).

### **1. 5. 1. The effect of the oxidative/nitrosative stress in the context of PARP**

In general, ROS and RNS are capable of damaging polyunsaturated fatty acids, proteins, nucleic acids altering their function leading to dysfunction. Peroxidation of lipids can alter the membrane assembly causing fluidity and permeability changes. The byproduct is 4-hydroxy-2-nonenal (HNE) and acrolein (Niki, 2009). Proteins also undergo oxidation upon RNS/ROS stimuli. Tyrosine residues are altered by nitration resulting nitrotyrosine which goes hand in hand with tertiary structure changes and loss of function in enzymatic activity. The products of the oxidation are aldehydes, keto- and carbonyl compounds of the proteins. The amount of such molecules during lifespan is increased specially the last third of life (Levine and Stadtman, 2001; Grune T., 2014).

The other very important molecule, which suffers reactive species-caused damage, is the DNA. In the human body tens of thousands of DNA damage take place a day, which corrected by specific DNA repair mechanisms such as base excision repair or nucleotide excision repair. The modifications are single- and double strand DNA breaks, modification of nucleotide bases, loss of purines and the crosslink of DNA and proteins. The most common form of modification is taken place at guanine site by  $^*\text{OH}$  which yields 8-hydroxy-2-deoxyguanosine (8-OHdG) (Halliwell, 2007). Whether the repair is successful or not and depending on the rate of the oxidative damage, cells might survive or undergo apoptosis or necrosis in connection with PARP-1 activation (Virag and Szabo, 2002).

We have to keep in mind that the sensitivity to oxidative stress is different between nuclear and mitochondrial DNA, for instance while a given concentration of oxidant

(glucose-oxidase treatment) give a rise of DNA damage in a concentration dependent manner in mitochondria, the same amount of oxidant does not generate damage in the nuclear genetic material (Szczesny et al, 2013).

### **1. 5. 2. Endogenous antioxidant systems**

In order to eliminate or neutralize the ROS/RNS products, the living cells are endowed with antioxidant systems and molecules. Also it is important to note the other source of antioxidant, which come from nutritional sources. As shown in **Figure 14**, there are enzymes such as catalase (CAT), glutathione-peroxidase (GPX) and superoxide dismutase (SOD) which facilitate the detoxification of the ROS/RNS species. The SOD converts superoxide to hydrogen peroxide while the CAT and GPX transform hydrogen peroxide to water. Two type of SOD can be distinguished, Cu- and Zn-SOD (SOD1) which are from cytoplasmic origin, and manganese-SOD (SOD2) which is found in mitochondria (McCord and Fridovich, 1968, Marklund, 1990). CAT and GPX are found in mitochondria and cytosol.

The non-enzymatic system includes vitamins such as Vitamin C and Vitamin E, or belongs to the carotenoids which originated from plants.

Although ROS are associated with harmful effects, but it was proven also that it has a role in signal transduction activating transcription factors which works as indicators of the redox status of the cell. If the oxidant/antioxidant system are unbalanced by the constant oxidative damage or the impairment of repair proteins, then the damage is inevitable.

In skeletal muscle upon exercise the oxygen consumption rate goes relatively high compared to other tissue which has one particular consequence: elevated production of ROS during physical activity. Therefore, it is essential that muscle develop some mechanism, which reduce the harmful effect of ROS. Exercised animals (long-term swimming animals) has an increased 8-OHdG repair capacity hereby smaller amount of 8-OHdG compared to their sedentary control. Also the carbonylated protein level was smaller in the exercised group (Radak et al., 1999). This work was further extended by including aged animals. The chymotrypsin-like activity, 8-OHdG content is also changed with age. Although those protein level, which undergo oxidative modification

do not change by the aging, the activity of proteasome complex responsible for elimination such proteins are elevated in trained/aged animals. The repair capacity of DNA caused by the presence of 8-OHdG was increased in muscle of exercised/aged animals compared to sedentary aged animals which suggests the beneficial effect of regular exercise against the increased level of 8-OHdG and elevated level of oxidatively modified proteins by the increased repair function and the resistance in protein against the oxidative stimuli (Radak et al, 2002). The age-related changes in connection with the exercise also showed an adaptive mechanism in human muscle (vastus lateralis), namely that the acetylation of 8-oxoguanine-DNA glycosylase (OGG1), which responsible for the base excision repair, is independent of age but it is dependent on the physical status. Regular exercise moreover generates an adaptive response with a more profitable antioxidant apparatus in skeletal muscle of human (Radak et al., 2011).

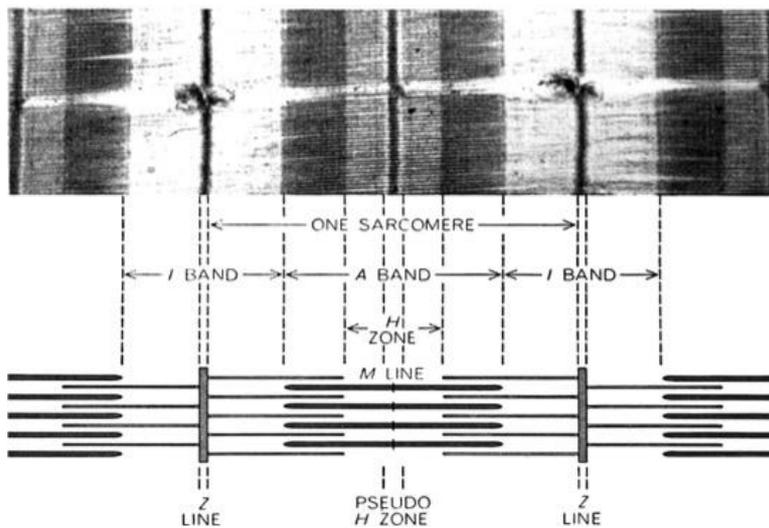
## **1. 6. The biology of skeletal muscle**

The most fundamental action in life is movement. Moving the whole body, or just part of it, digestion in the intestinal system as it transits nutrient, the movement of the eye, just to name a few are essential for life. To accomplish these complex function, nature evolved muscle which are a very complicated system, but share a common function: contraction based on the molecular mechanism of action of actin and myosin.

The animal kingdom possesses different type of muscles but they largely can be divided as striated and smooth. Within the striated group there are two types: skeletal and cardiac. The prior is classified by two regular phenotypes: fast twitch and slow twitch. Both have striated appearance due to the contractile proteins. There are also significant structural differences, which distinguish, cardiac from skeletal muscle. The latter one formed by multinucleated myocytes called fibers and they are not contracted by the neighbor cell electrical stimuli as it is seen in cardiac tissue. Conversely, cardiac cells work as a syncytium and they contract by the electrical stimuli which spreads spontaneously to neighboring cells. The action of skeletal muscle is graded: the extent and strength of its contraction can be altered and the stimuli that depolarizes the fiber initiates a contraction or no-contraction, in other words a yes or no response.

### 1. 6. 1. Molecular machinery of the contractile force

The vertebrate muscle has a striated appearance and consists of multinucleated cells. Within the cells there are parallel structures called myofibrils. The longitudinal section of the myofibril shows a well-characterized unit called sarcomere and has a repeats in every 2.3  $\mu\text{m}$  along the fibril axis. The underlying structure was revealed by early pioneering work in the 50's and 60's and opened the model for the sliding filament hypothesis of muscle. Two filamentous formations was distinguished: the thick which mainly consists of myosin and the thin filaments which is composed of actin, tropomyosin and troponin complex (**Figure 15**). In the 1940's Albert Szent-Györgyi showed that at the molecular level, the contraction is based on the interplay of myosin, actin and ATP and it requires  $\text{Mg}^{2+}$  (1942).



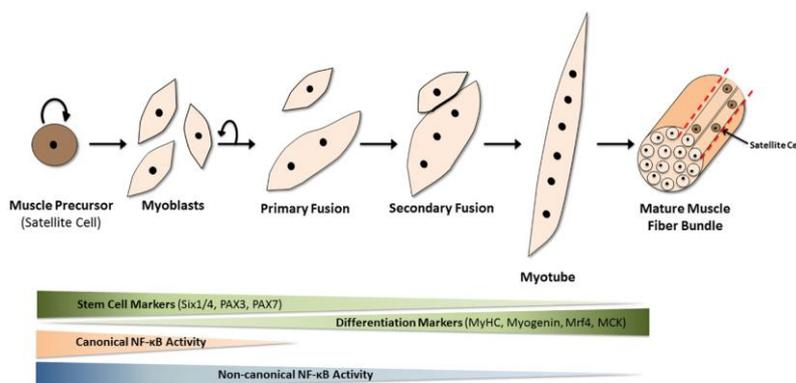
**Figure 15.** Longitudinal section of frog sartorius muscle showing the overlapping thick and thin filaments (Huxley HE, 2004). There is a dark A band and the light I band which are arranged regularly. There is a central region of the A band called the H zone is a less dense part of that band. The I band is divided by a dense Z line.

## 1. 6. 2. Origin of skeletal muscle

Skeletal muscle originates from the mesoderm which gives rise to somite, a complex transient embryonic structure. A cross-talk has to be taken place between the somite and adjacent structure to form muscle. Transplantation and isolation experiments were done to prove of the communication between cells. For instance, if chick or frog embryo mesoderm were removed and cultured with the respective dorsal neuronal tube then striated muscle were formed. In this interaction it is essential that growth factors (wnt proteins, Sonic hedge hog) and transcription factors (MyoD, Myf5, Pax3) cooperate with each other (Hill and Olson, 2012).

## 1. 6. 3. The cellular basis of myogenesis in adults

In the early 1960's Alexander Mauro made discovery for existence of additional proliferative cells in the skeletal muscle of the tibialis anticus of the frog (Mauro, 1961). These cells were also found in the limbs of mouse embryos after day 15 postcoitum (E15) and postnatal growth. They were called satellite cells by A. Mauro which responsible for maintaining and repair of damaged myofibers (Hawke and Garry, 2001). They usually reside between the sarcolemma and basal lamina in the muscle fiber. Upon stimulation (exercise, damage), activating different signaling pathways (TWEAK, NF- $\kappa$ B), they proliferate/differentiate to myoblasts, the progenitor of satellite cells, fusing into multinucleated myofibers (**Figure 16**).



**Figure 16.** Mammalian skeletal myogenesis (Enwere et al., 2014).

Upon muscle injury, a resident population of quiescent myogenic precursor cells, known as satellite cells (because they encircle muscle fibers), proliferate and differentiate into myoblasts. They also fuse together (primary fusion step) to create multinucleated myocytes or myotubes over the course of several days. Additional myoblasts fuse to the existing myotubes in the secondary fusion step to produce even larger myotubes which eventually align to form muscle fibers. This differentiation process is regulated by many internal and external factors. Over the course of myogenesis, expression of stem cell markers such as Pax7 is gradually lost, while the appearance of differentiation markers, such as myosin heavy chain (MyHC), muscle-regulatory factor 4 (Mrf4), and muscle creatine kinase (MCK) gradually increases. The NF- $\kappa$ B pathways are now known to play significant roles in this differentiation process. Upon the loss of canonical NF- $\kappa$ B signaling and the activation of non-canonical (or alternative) NF- $\kappa$ B signaling, myoblasts stop dividing and start fusing to form multinucleated myotubes, a key event in myogenesis.

Satellite cells are heterogeneous and they can be divided into committed cells (upon activation they progress to myogenic lineage) and stem cells (responsible for maintaining the satellite pool). In resting conditions satellite cells remain in a reversible and quiescent G<sub>0</sub> state which is regulated by cyclin-dependent kinase inhibitors (such as *p27<sup>Kip1</sup>*, *p57<sup>Kip2</sup>*), the negative regulator of fibroblast growth factor (*Spry1*) or also maintained by muscle specific miRNAs (microRNAs). Upon activation they re-enter the cell cycle a, directly or through b, an intermediate state called G<sub>alert</sub> in which they show a greater regenerative potential. After the activation they exit and become quiescent again where *Spry1* is upregulated and Notch signaling has important role. The proliferating myoblasts also exit the cell cycle under differentiation into myocytes to later form myotubes which occurs after a muscle injury (Dumont et al., 2015).

As it was described earlier one of the theory for aging is the elevated ROS causing impaired muscle function, regeneration and the loss of muscle mass and decline in strength, hallmark of sarcopenia. Satellite cells density remains relatively stable during the aging process, but the myofibers show a decline suggesting that the potential to regenerate and/or to build muscle by the satellite cell population, as well as the self-renewal to reconstitute the muscle stem cell pool are impaired.

During the regeneration major metabolic changes take place and need necessity for mitochondrial biogenesis which results increased ROS production. Using *Pitx2* and *Pitx3* mutant which are key elements in preventing excess ROS production, showed impaired regeneration and early onset of differentiation supporting the idea that controlled elevation of ROS is essential for the differentiation process, but overproduction impairs the normal myoblasts to myotube switch (L'Honoré et al., 2014). Comparing the young and aged satellite cells antioxidant capacity there are differences that makes the ROS accumulation higher in the latter one which goes hand in hand with reduced myogenic potential and capacity in aged cells (Fulle et al., 2005).

#### **1. 6. 4. Skeletal muscle cells as a model system**

The commonly used model for myogenesis, satellite cell function and morphology is the use of C2C12, primary line of murine myoblasts, isolated in the late 70's, which easily differentiate to myotubes under certain condition (Yaffe and Saxel, 1977). Their behavior is very similar of satellite cells and can be used as a model system for such. Technically speaking, their differentiation is taken place after the C2C12 myoblasts became confluent and are put in medium supplemented with low percent of horse serum (usually 2%) slowing down their proliferation and switching them to a differentiation stage. During the differentiation, when fusion occurs along in changes phenotype/morphology, there are markers which ensure that the differentiation has started, namely *PCNA*, proliferating cell nuclear antigen, which needed for cell proliferation and its expression level decreased toward myotubes. *Pax7*, a member of a paired-box family of transcription factors, is also expressed in C2C12 myoblasts but it is downregulated to differentiation conditions (Seale P et al., 2000). Myoblasts that committed to the differentiation pathway express *myogenin*, an early marker of the differentiation (Andrés and Walsh, 1996).

## 2. Aims

Poly(ADP-ribose) polymerase 1 (PARP-1) is the major isoform of PARP family, a constitutive nuclear and mitochondrial protein with well-studied roles functioning as a two-edged sword: a, having various essential function in DNA repair, signal transduction, cell death and b, pathophysiological role, mostly due to overactivation, seen in cancer, diabetes or sepsis.

In differentiating muscle, from myoblasts to myotubes, we showed a marked reduction of PARP-1 expression toward the differentiated myotubes.

Since PARP-1 is activated by oxidative stimuli, such as by ROS/RNS which showed to increased in pathophysiological condition or even by exercise raises some questions whether or not:

- the decreased PARP-1 in myotubes means a possible advantage for the differentiated cells compared to the undifferentiated myoblasts against oxidative stress
- the mitochondrial function altered and has beneficial effect comparing undifferentiated and differentiated cells
- pharmacological inhibition of PARP-1 mimic the advantage of the down-regulated features proving our concept
- the inhibition of PARP could be beneficial in pathological condition such as burn in human (propranolol suppressed PARP-1 activation in leukocytes (Brunyanszki et al., 2014; Olah et al., 2011))

## **3. Materials and Methods**

### **3. 1. Reagents**

Unless otherwise indicated, all reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), horse serum, and 0.25% trypsin-EDTA were purchased from Life Technologies (Carlsbad, CA, USA).

### **3. 2. Cell culture**

The murine C2C12 (Catalog# ATCC® CRL-1772™) and rat L6 (Catalog#ATCC® CRL1458™) skeletal muscle cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Undifferentiated, proliferating C2C12 and L6 myoblasts were cultured in DMEM (ATCC, Cat#30-2002) containing 15% and 10% FBS, respectively. Differentiation for both cell lines was induced by changing the culture medium to DMEM containing 2% horse serum (Szczesny et al., 2013). All cells were maintained at 37 °C, 5% CO<sub>2</sub>. In supporting experiments we also used the human monocyte histiocytic lymphoma cell line, U937 (ATCC® CRL-1593.2™). Differentiation of U937 cells was induced by incubating cells with 150 nM phorbol 12-myristate 13-acetate (PMA) for 4 days (Stoppelli et al., 1985).

### **3. 3. Preparation of whole-cell extracts and Western blots**

Whole-cell extracts were prepared using NP-40 lysis buffer (20 mM Tris–HCl, pH 8.8, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 12 mM Na-deoxycholate). Cell homogenates were incubated for 30 min on ice followed by a clean-up centrifugation at 20,000 × g for 10 min at 4°C. Protein concentration was determined with Pierce BCA Protein Assay Reagent (Thermo Scientific) using bovine serum albumin as a standard. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose (Bio-Rad) membrane. The membrane was blocked with StartingBlock™ Blocking Buffer (Thermo Scientific) for 1 h followed by incubation with primary antibody: PARP-1 (1:1,000;

Cell Signaling, Cat#9532), PAX7 (1:1,000; Abcam, Cat#ab34360), myogenin (1:1,000; Abcam, ab124800), PCNA (1:1,000; Cell Signaling, Cat#2586), Histone H3 (1:1,000; Cell Signaling, Cat#12648P), ATP synthase (subunit alpha) (1:1000, Life Technologies, Cat#459240/G0531), actin-HRP (1:5,000; Santa Cruz Biotechnology, Cat#sc-1616 HRP); followed by incubation with anti-mouse or anti-rabbit secondary antibodies (Cell Signaling). The membrane was developed with SuperSignal™ West Pico Chemiluminescent Substrate (Pierce) and visualized in a GeneBox Detection System (Syngene).

### **3. 4. MTT viability assay**

The MTT assay was performed as described earlier (Gerö et al., 2013). Briefly, cells were incubated with MTT reagent at a final concentration of 0.5 mg/ml, for 1 h at 37 °C. The cells were washed with PBS and the residual formazan was dissolved in DMSO; subsequently, absorbance was measured at 570 nm with a background correction at 690 nm using a Molecular Devices M2 microplate reader.

### **3. 5. LDH cytotoxicity assay**

Lactate dehydrogenase (LDH) release was measured as described previously (Gerö et al., 2014). Briefly, 30 µl of supernatant from cultured cells was mixed with 100 µl of freshly prepared LDH assay reagent containing 85 mM lactic acid, 1 mM nicotinamide adenine dinucleotide (NAD<sup>+</sup>), 0.27 mM N-methylphenazonium methyl sulfate (PMS), 0.528 mM INT, and 200 mM Tris (pH 8.2). Changes in absorbance were measured kinetically at 492 nm for 15 min, 37 °C, using a monochromator-based reader (Powerwave HT, Biotek).

### **3. 6. Measurement of NAD<sup>+</sup> levels**

Total cellular NAD<sup>+</sup> was determined using NAD<sup>+</sup>/NADH Cell-Based Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to manufacture's recommendations and as previously described (Modis et al., 2012). The amount of

formazan produced, which is proportional to the amount of NAD<sup>+</sup> in the cell lysate, was measured at 450nm with SpectraMax M2 microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA).

### **3. 7. Annexin V-phycoerythrin (Annexin V-PE) -7-aminoactinomycin D (7-AAD) staining for apoptosis/necrosis detection by flow cytometry**

Detection of cell death was performed using PE Annexin V Apoptosis Detection Kit I (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's recommendations. Briefly, control and treated cells were trypsinized, washed in ice-cold PBS and re-suspended in 1 ml Binding Buffer.  $1 \times 10^5$  cells in 500  $\mu$ l were incubated with PE Annexin V and 7-AAD for 10 min at 25°C in the dark, and analyzed immediately using a Guava EasyCyte Plus Flow Cytometer (Millipore, Billerica, MA). CytoSoft 5.3 software was used to estimate the subpopulations of early and late apoptotic, as well as necrotic cells, as a percentage of the total cell count (Brunyanszki et al., 2014).

### **3. 8. Bioenergetic analysis in isolated mitochondria**

The XF24 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA) was used to measure mitochondrial bioenergetic function. Mitochondria were isolated and extracellular flux analysis was performed as previously described (Frezza et al., 2007; Rogers et al., 2011; Modis et al., 2013). Respiration by the mitochondria (7.5  $\mu$ g/well) was sequentially measured in a coupled state with substrate present (5.5mM succinate; basal respiration, State 2), followed by State 3 (phosphorylating respiration, in the presence of ADP and substrate), State 4 (non-phosphorylating or resting respiration) following conversion of ADP to ATP, and State 4o, induced with the addition of oligomycin. Next, maximal uncoupler-stimulated respiration (State 3u) was detected by the administration of the uncoupling agent FCCP. At the end of the experiment the Complex III inhibitor, antimycin A, was applied to completely inhibit mitochondrial respiration. Inclusion of rotenone with succinate in the initial condition (State 2) triggers the respiration to be driven only by Complex II–IV. This ‘coupling

assay' examines the degree of coupling between the electron transport chain (ETC) and the oxidative phosphorylation (OXPHOS), and can distinguish between ETC and OXPHOS with respect to mitochondrial function/dysfunction.

### **3. 9. Mitochondrial membrane potential assay**

Changes in mitochondrial membrane potential were monitored with TMRE Membrane Potential Kit from Life Technologies (Carlsbad, California, USA) according to manufacturer's instructions and as previously described (Modis et al., 2013). Briefly, myoblasts were seeded at a concentration of  $1 \times 10^4$  cells per well in 96-well plates. For differentiated myoblasts, after the initial 24 h incubation, the culture medium was replaced with differentiation media and incubated for an additional 5 days. Next, cells were exposed to various concentrations of H<sub>2</sub>O<sub>2</sub> for 24h. 50 nM TMRE was added to the media and cells were incubated for an additional 20 min at 37°C, 5% CO<sub>2</sub>; 10 and 30 μM FCCP were used as positive controls. Changes in fluorescence (ex549/em575) were monitored by monochromator-based reader (Powerwave HT, Biotek).

### **3. 10. Fluorescence microscopy**

Myoblasts and myotubes were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min, washed with PBS, and permeabilized with 0.5% Triton X-100 in PBS for 15 min at 21°C. After washing with PBS, coverslips were incubated first in 0.5% Triton X-100 containing 2.5% horse serum in PBS for 30 min, and then with primary antibodies overnight at 4°C. For the detection of PARP-1, an anti-PARP-1 antibody (Genetex, Cat#GTX61031) was used followed by Alexa Fluor® 546 anti-rabbit antibody (Life Technologies, Cat#A11035). For myogenin detection, anti-myogenin antibody (Abcam, ab124800) was used followed by Alexa Fluor® 488 anti-rabbit antibody (Life Technologies, Cat#A11034). Cells were washed three times with PBS and fluorescence was visualized using a Nikon Eclipse 80i inverted microscope with a Photometric CoolSNAP HQ2 camera and the NIS-Elements BR 3.10 software (Nikon Instruments, Melville, NY, USA).

### **3. 11. PARP-1 silencing by small-interfering RNA and bioenergetic analysis in PARP-1 silenced cells**

Cells ( $1 \times 10^5$  /well) were seeded into 6-well tissue culture treated plates and cultured to reach approximately 50% confluence in 24h. Next, cells were transfected with 40 nM PARP-1 specific siRNA (cat#4390771 s62054, Applied Biosystems/Ambion, Austin, TX, USA) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacture's recommendations. Scrambled siRNA (Ambion, Silencer Negative Control#1) was used as a control. After 24h, cells were harvested and seeded onto 24-well XF24 cell culture plates. On the following day, XF24 Extracellular Flux Analyzer was used to measure cellular bioenergetics as described (Modis et al., 2012).

### **3. 12. Transient transfection of myoblasts with PARP1**

Myoblasts were transfected on 96-well plates with full-length mouse PARP-1 cDNA inserted into pCMV6-Entry vector (Myc-DDK-tagged) purchased from Origene Technologies (Cat#MR211449) (Rockville, USA, MD). Insert free plasmid, pCMV-Entry was used as control (Origene, Cat#PS100001). Transfection of myoblasts was performed using Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions. Briefly, DNA (0.2 µg/well) and Lipofectamine 2000 (1 µl/well) were separately diluted in 25 µl of Opti-MEM (Gibco). Next, DNA was added to the Lipofectamine 2000 reagent and the lipid/DNA mixtures were allowed to form complex for 5 min at room temperature. Cells were washed once with 100 µl of PBS and 100 µl of DMEM containing 15% FBS/well was added to each well. Next, lipid/DNA mixture was added and cells were incubated at 37 °C, 5% CO<sub>2</sub>. After 24 h, transfection medium was removed and replaced with DMEM containing 2% horse serum to start differentiation. To validate the expression of PARP-1, anti-DDK mouse monoclonal antibody (1:1,000, Origene, Cat#TA50011-100) was used. After 5 days of differentiation, differentiation was confirmed visually (methods as described above) and oxidant sensitivity of the cells was tested by exposing the cells to hydrogen peroxide (0.8 mM) followed by the measurement of LDH release into the culture medium (methods as described above).

### **3. 13. Proximity Ligation Assay (PLA)**

In situ protein/protein proximity/interaction studies were performed with Duolink *in situ* (Olink Bioscience, Uppsala, Sweden). Cells were fixed in slide chambers (Lab-Tek) and incubated with the same antibodies as for Western analysis. Images were visualized using a Nikon Eclipse 80i fluorescent microscope with CoolSNAP HQ camera and analyzed with NIS-Elements BR3.10 software.

### **3. 14. Collection of muscle samples from children with severe burn injury**

Children aged 0 to 17 years with more than 40% total body surface area burns that would require skin grafting, who arrived at our hospital within 96 hours of injury, were eligible. All subjects received standard burn care as previously described (Herndon et al., 1998). Each patient underwent wound excision and grafting with skin autografts and allografts within 72 hours of admission. Sequential grafting procedures were performed over time until the wounds were 95% healed. Enteral nutrition was started at admission and continued until the wounds were 95% healed. Patients were fed a commercial enteral formula (Vivonex T.E.N.; Sandoz Nutritional, Minneapolis, MN) through a nasoduodenal tube. The daily caloric intake was calculated to deliver 1500 kcal per square meter of body-surface area burned plus 1500 kcal per square meter of total body surface area. Patients remained in bed for 5 days after each excision and grafting procedure and then were allowed daily walks. All patients were administered antianxiety medication after the first week post-burn. Biopsy of the vastus lateralis muscle was taken at various times post-burn and pooled into three groups: Early (samples taken at 2–6 days post-burn; n=4), Middle (13–18 days post-burn; n=4) and Late (69–369 days; n=8). Biopsies from cleft-lip and cleft palate patients between 3 and 18 years of age admitted to our hospital for reconstructive surgery were used as non-burned normal controls (n=3). Collection and analysis of the samples occurred with the approval of the institutional IRB committee. Patients were randomized to receive propranolol or no propranolol treatment, as part of a prospective randomized clinical trial; samples (homogenates of skeletal muscle biopsies suitable for Western blotting

analysis, or frozen samples suitable for immunohistochemical analysis) that were collected over a period of 2 years were obtained from a tissue bank for the current study. All samples that contained suitable volume were analyzed; no samples were excluded from the analysis. Patient demographic data are shown in Table 1. Biopsies were snap frozen at  $-80^{\circ}\text{C}$  for subsequent Western blot analysis or for immunohistochemical analysis.

**Table 2.** Demographic Characteristics of the Study Groups

	Control	Early	Middle	Late	Middle +Propranolol	Late +Propranolol
Subject number	3	4	4	8	4	5
male	2	2	1	7	4	4
female	1	2	3	1	0	1
Age	11.6±1.2	8.8±3.2	9.5±3.3	5.5±1.6	11.2±1.8	9.4±2.3
Burn (TBSA), %	n/a	62±5	75±6	66±5	70±8	68±7
Height (kg)	48±3	47±20	56±20	33±8	52±12	44±12
Weight (cm)	159±12	135±15	132±17	123±11	148±13	134±16
Samples obtained post-burn (days)	n/a	3±1	15±2	203±40	13±3	233±30

Values are mean ± SEM except where otherwise noted. Height and weight measured at baseline. All 4 groups of burned children were similar in demographics. n/a= non applicable.

### 3. 15. Propranolol treatment

The drug was given in a regimen as previously described (Jeschke et al., 2007), at 4 mg/kg/day by mouth from the time of admission for a period of 10±1 months. Patients were closely monitored for heart rate and blood pressure. Patients did not receive any other anabolic or anticatabolic agent. During the in-hospital portion of the treatment, patients received insulin if necessary (blood glucose >210 mg/dl) to decrease blood glucose below 210 mg/dl, with target blood glucose of 140 to 160 mg/dl. Pharmacokinetic studies demonstrated that in the current patient population the effective plasma drug concentrations were achieved in 30 minutes, and the half-life is approximately 4 hours (Williams et al., 2011). Skeletal muscle biopsies from the propranolol-treated patients, obtained in the 'Middle' and 'Late' time points (6–19 days post-burn; n=4 and 139–289 days post-burn, n=5, respectively) were compared with the responses seen in the respective comparable groups of control patients (not treated with propranolol).

### **3. 16. Western blotting for poly(ADP-ribose) (PAR)**

Muscle samples were homogenized in homogenizing buffer (50mM Tris pH 7.4, 150mM NaCl, 1% Triton-X-100, 10mM EDTA, Protease Inhibitor Cocktail (Complete Mini by Roche). PAR Western blotting was performed as previously described (Tóth-Zsámboki et al., 2006). Proteins were loaded onto 4–12% polyacrylamide gels and separated by electrophoresis then transferred to nitrocellulose membranes. For immunoblotting, membranes were blocked with 10% nonfat dried milk in Tris-buffered saline (TBS) for 90 min. The primary antibody (anti-PAR (poly-ADP-ribose) polyclonal antibody, EMD Biosciences) against PAR were applied at 1 µg/ml concentrations overnight at 4°C. After washing 3 times in TBS containing 0.05% Tween-20 (TBST), the secondary antibody (peroxidase-conjugated goat anti-rabbit) were applied at 1:2000 dilution for 1 h. Blots were washed 3 times in TBST, once in TBS, and incubated in enhanced chemiluminescence reagents (Supersignal WestPico Chemiluminescent substrate; Pierce Biotechnology, Rockford, IL, USA). The levels of PARylated protein at 120 kDa (representing auto-PARylation of PARP-1) were normalized to actin.

### **3. 17. Immunohistochemical analysis**

Frozen sections of 5 micron thickness were prepared from all biopsy specimens collected. Sections were fixed in cold 95% ethanol for 10 minutes, then immersed in 3% hydrogen peroxide solution for 10 minutes, and rinsed with de-ionized water. Slides were processed at room temperature in a Dako horizontal auto-stainer, using the biotin-streptavidin method. Both avidin and biotin were obtained from Vector Laboratories, as part of the AB blocking kit, and diluted 1:5 using Dako antibody diluent. Tris buffered saline was used to rinse slides between each of the consecutive processing steps. The primary antibodies were diluted in the biotin solution, each to the concentration specified as follows, and applied for 1 hour. Primary antibodies used in our studies were: 1) PAR (Tulip mouse monoclonal, anti-human 1:10); 2) CD-31 (DAKO mouse monoclonal, anti-human, 1:200); 3) Von Willebrand factor (DAKO mouse monoclonal, anti-human, 1:200); S-100 (DAKO rabbit polyclonal, anti-human, 1:4000).

### **3. 18. PAR immunostaining procedure**

Sections were incubated with diluted avidin for 7 minutes, rinsed, and incubated with the primary antibody (PAR) biotin solution for 1 hour. Afterwards, slides were incubated in universal secondary antibody LSAB2 (Dako) for 15 minutes, followed by LSAB2 labeling agent (Dako) for 15 minutes, and then diaminobenzidine (DAB, Dako) for 5 minutes. Slides were rinsed in distilled water, counterstained with Harris Hematoxylin (Fisher Scientific) for 1 minute, rinsed in distilled water first, 0.25% ammonia water, and distilled water as final step. Slides were then dehydrated through graded series of alcohols, four changes of xylene, and finally coverslipped with synthetic glass and permount mounting media.

### **3. 19. Double immunostaining procedure**

Double immunohistochemical staining was performed according to the following combinations: 1) PAR (FR) and CD-31 (DAB), 2) PAR (FR) and factor VIII-related antigen (DAB), 3) PAR (FR) and S-100 (DAB). Sections were incubated with diluted avidin for 7 minutes, rinsed, and incubated with the primary antibody (CD-31, fVIII-related antigen, or S-100) biotin solution for 1 hour. Afterwards, slides were incubated with PAR antibody solution (10 microg/mL) for 1 hour, and then in universal secondary antibody LSAB2 (Dako) for 15 minutes, followed by LSAB2 labeling agent (Dako) for 15 minutes, and then diaminobenzidine (DAB, Dako) for 5 minutes. At this point, slides were incubated with the tertiary antibody solution alkaline phosphatase streptavidin (1:200) for 15 minutes, and then the fast-red chromagen (Biopath Labs) was applied for 5 minutes. Slides were finally rinsed, counterstained, dehydrated and coverslipped as described above.

### **3. 20. Statistical analysis**

Data obtained from C2C12, L6 and U937 cells are shown as means  $\pm$  SEM and SD. One-way ANOVA was applied for statistical analysis, and Tukey's post-hoc test was used for the determination of significance between individual groups. The value of  $p < 0.05$  was considered statistically significant. All statistical calculations were performed using Graphpad Prism 5 analysis software. All experiments were performed at least 3 times on different days.

For the analysis of human samples, nonparametric ANOVA test was applied for statistical analysis and for the determination of significance, the Kruskal-Wallis post-hoc test was used.  $P < 0.05$  considered as significant.

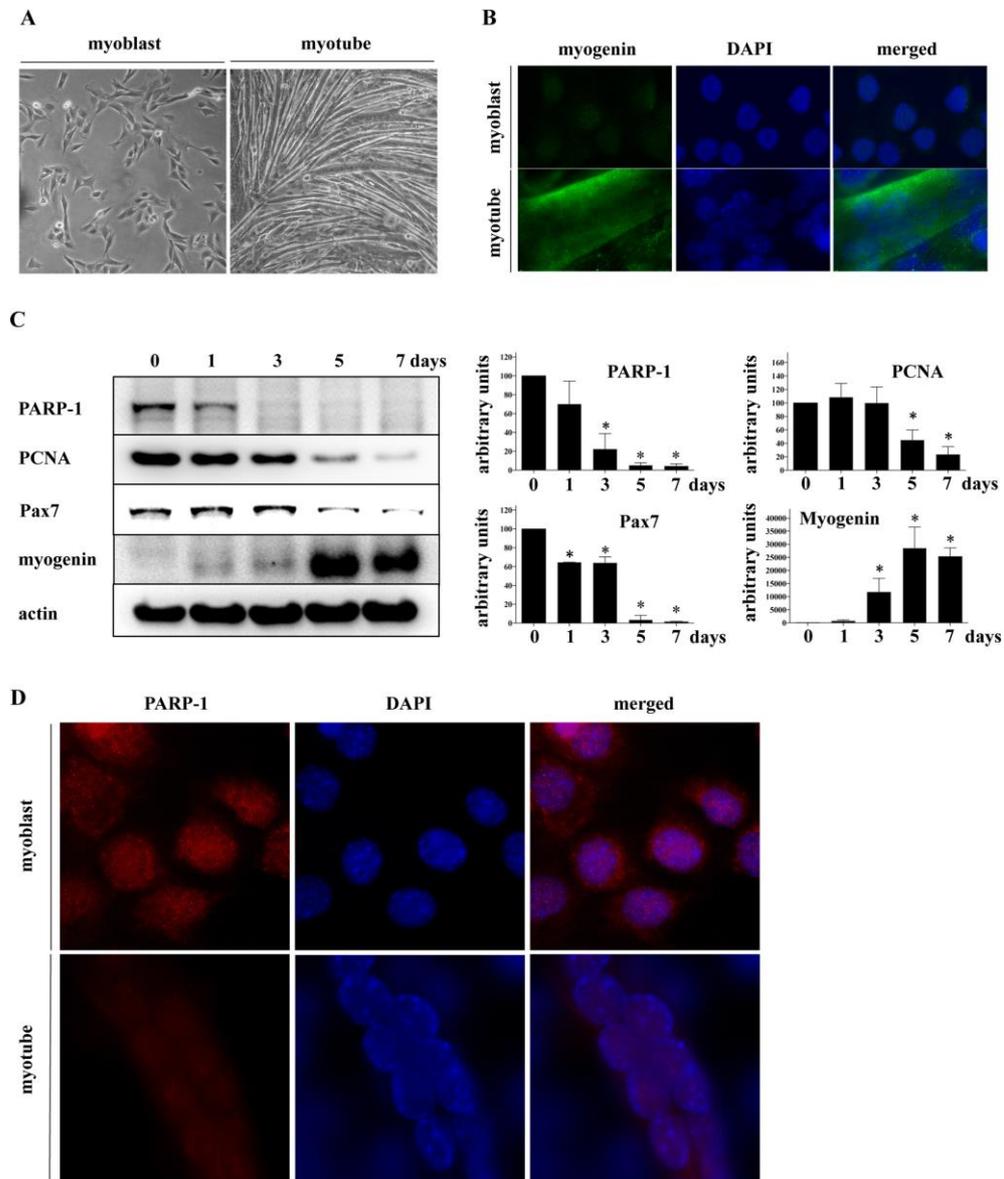
## 4. Results

### 4. 1. Myoblast differentiation is associated with downregulation of PARP-1 expression

The C2C12 cell line is a well-defined model for skeletal muscle differentiation that recapitulates the *in vivo* process through irreversible withdrawal from the cell cycle, repression of proliferation-associated genes, and expression of terminally differentiated muscle-specific genes (Yaffe and Saxel 1977; Shen et al., 2003, Ferri et al., 2009). Proliferating myoblasts differ from terminally differentiated, non-proliferating myotubes in morphology and protein expression profiles (**Figure 17A, B**). To confirm proper differentiation, we monitored the expression of transcription factor paired-box 7 (Pax7), proliferating cell nuclear antigen (PCNA), which is known to be inhibited during myoblast differentiation or repression of cellular proliferation, (Frezza et al., 2007; Rogers et al., 2011) and myogenin, which is known to be expressed in differentiated myotubes (Wang and Rudnicki 2012). Time-course Western blot analysis of myoblast differentiation, from day 0 through 7, is shown in **Figure 17 C**. These results confirm that the process of myoblast differentiation is accurately recapitulated, as shown by decreased expression of Pax7 and PCNA, and increased expression of myogenin. Moreover, we observed a marked decrease in PARP-1 expression in myotubes (**Figure 17C**). PARP-1 expression was ten-fold greater in myoblasts than in myotubes (**Figure 17C**). Immunocytochemical analysis showed that in myoblasts, PARP-1 is localized mostly in the nucleus with little cytoplasmic staining, whereas terminally differentiated myotubes showed a global reduction of signal intensity (**Figure 17D**). To confirm our observation that skeletal muscle cell differentiation is accompanied by reduction in PARP-1 expression, we performed similar Western blot analyses using another well-defined model of skeletal muscle differentiation, namely, rat-derived L6 cells (Hudson et al., 2014). The obtained data clearly indicate that myotubes of L6 cells have reduced expression of PARP-1 (**Figure 18A**). Moreover, differentiation of U937 cells, induced by PMA, also showed a reduction in PARP1 expression (**Figure 18B**).

In order to investigate whether PARP-1 downregulation is an effect of contact

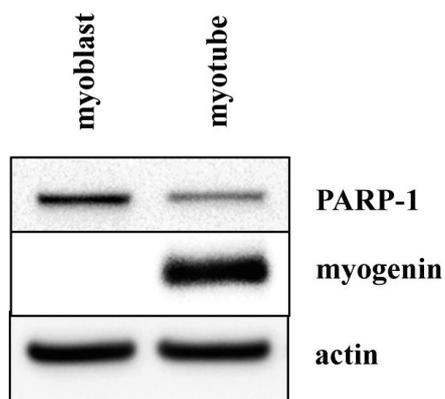
inhibition, confluent myoblast culture was maintained for an additional one or two days while the level of PARP-1 was monitored by Western blot. As shown in **Figure 19**, the level of PARP-1 was not changed under these conditions suggesting that reduction of the PARP-1 level is an effect of differentiation, not contact inhibition.



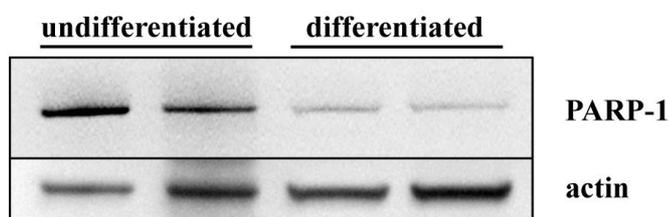
**Figure 17.** *PARP1* level is reduced in myotubes. (A) Representative phase-contrast microscopy images of myoblasts and myotubes showing typical morphological differences. (B) Immunocytochemistry of the differentiation marker, myogenin, in myoblasts and myotubes. DAPI was used for nuclear counterstaining. Increased

myogenin signal was detectable in the fully differentiated myotubes. (C) The effect of differentiation on protein expression of PARP-1 and the differentiation markers, PCNA, Pax7, and myogenin were monitored on Days 0–7 using whole cell extracts. Actin was used as a loading control. The relative quantity of proteins was calculated by densitometry and normalized to actin based on the analysis of three independent Western blots. \* indicates  $p < 0.05$  relative to myoblasts at Day 0 (100%). (D) PARP-1 distribution in undifferentiated myoblasts and differentiated myotubes. DAPI was used for nuclear counterstaining. PARP-1 signal decreased in the fully differentiated myotubes.

**A**

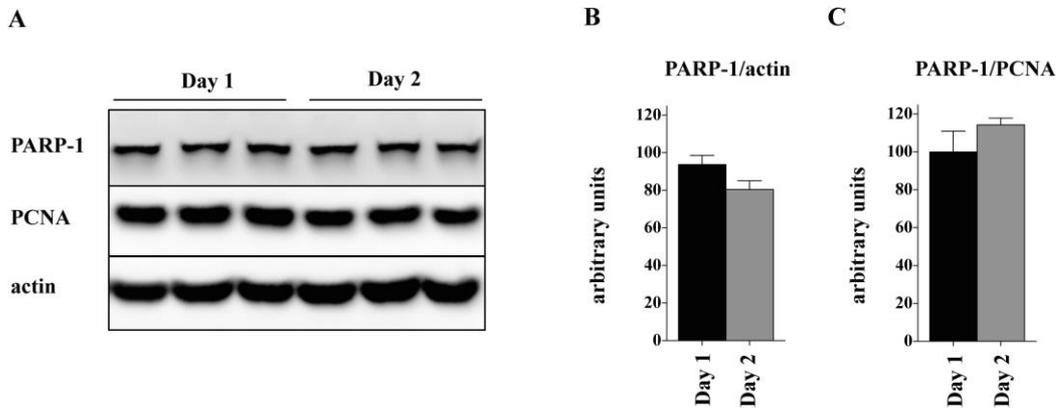


**B**



**Figure 18.** Expression of PARP-1 is reduced in differentiated L6 and U937 cells. (A) Western blot analysis of PARP-1, myogenin as a differentiation marker, and actin show the relative quantity of PARP-1 and the differentiation of L6 myoblasts and myotubes.

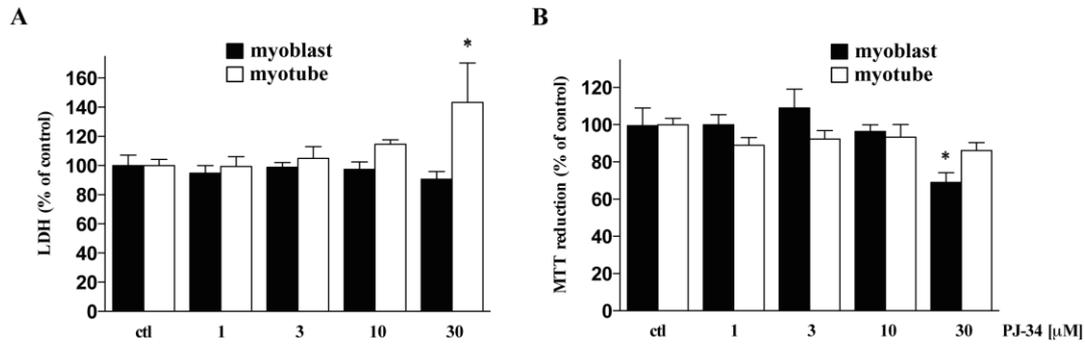
(B) Western blot analysis of PARP-1 and actin as loading control in undifferentiated and differentiated U937.



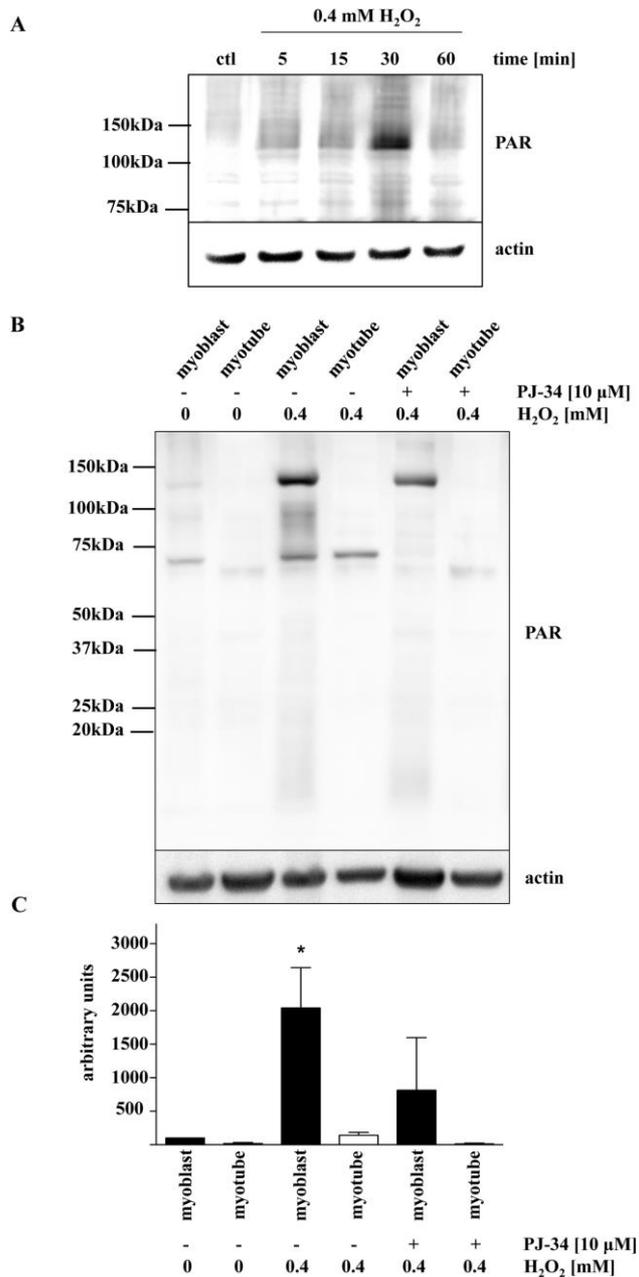
**Figure 19.** Lack of the contact inhibitory effect on PARP-1 expression in myoblasts. (A) Western blot analysis of PARP-1 in myoblasts at day 1 and 2 after reaching 100% confluence. Actin was used as a loading control. PARP-1 densitometric analysis was normalized to actin (B) or PCNA (C); values obtained in Day 1 cells were set as 100%. The results show no significant difference in PARP-1 protein between cells kept for 1 or 2 days after reaching 100% confluence.

## 4. 2. Differentiated myotubes develop resistance to oxidative stress

In order to study the effect of PARP-1 inhibition, we first determined the maximum non-toxic concentration of well-known PARP inhibitor, PJ34 (Jagtap et al., 2002). For our subsequent studies, we selected 10 $\mu$ M as the highest, non-toxic concentration of PJ34 based on preliminary studies with MTT conversion and LDH release cell-viability assays (**Figure 20**).



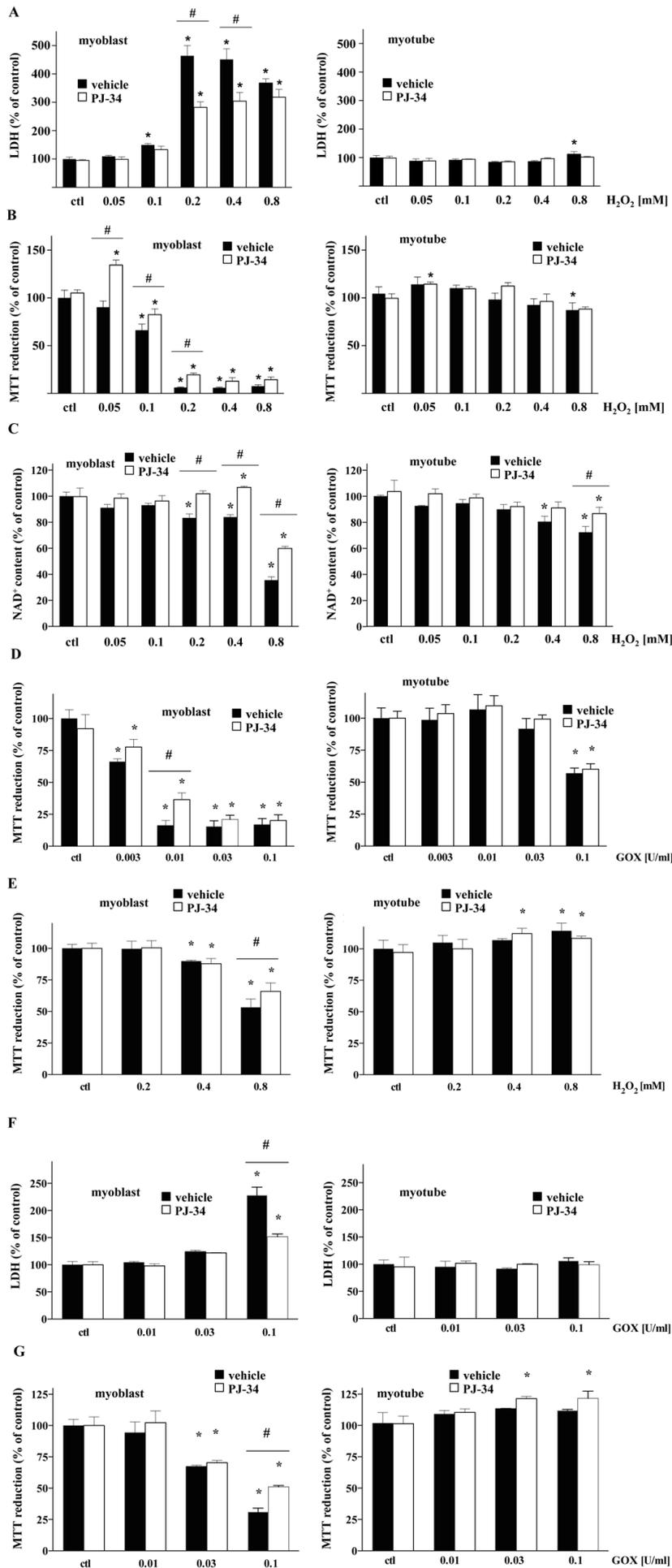
**Figure 20.** *Effect of PARP inhibitor PJ34 on cell viability.* Cytotoxic effect of increased concentration of PJ34 has been detected by LDH release (A) and MTT conversion assays (B). The highest concentration of PJ34 without cytotoxic effect was 10 $\mu$ M. Data are shown as mean  $\pm$  SD of 3 repeats. \* shows significant difference,  $p < 0.05$ , in the cell response to PJ34 relative to controls. Data shown in A, B were calculated as percentage of control cells (untreated myoblasts or myotubes, in each case set as 100%).



**Figure 21.** PARylation induced by oxidative stress is reduced in myotubes. (A) Representative Western blot shows a maximal amount of PAR signal at 30 minutes after H<sub>2</sub>O<sub>2</sub> treatment. (B) Comparison of PAR formation of myoblasts and myotubes in response to exposure to 0.4 mM H<sub>2</sub>O<sub>2</sub> in the presence or absence of PJ34 at 30 min post-treatment. (C) Densitometric analysis of PAR level in myoblasts and myotubes. One-way ANOVA was used for statistical analysis and determination of significance. \* indicates  $p < 0.05$  relative to untreated myoblasts.

Consistent with our observations that PARP-1 expression is downregulated in myotubes as compared to myoblasts, H<sub>2</sub>O<sub>2</sub> challenge induced a lesser degree of PARP-1 activation in myotubes than in myoblasts, as determined by Western blot analysis of PAR adducts in whole-cell extracts of each cell type (**Figure 21**).

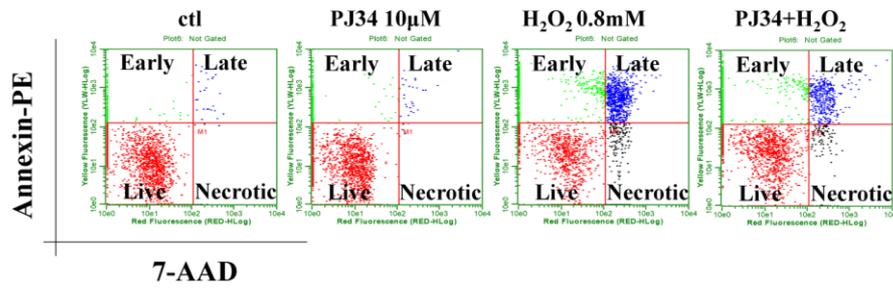
Next, we compared the changes in the viability of myoblasts and myotubes exposed to various concentrations of H<sub>2</sub>O<sub>2</sub> by monitoring the LDH release into the culture medium, measuring the capacity of the cells to convert MTT to formazan, and quantifying cellular NAD<sup>+</sup> levels. As expected, increasing concentration of H<sub>2</sub>O<sub>2</sub> caused an increase in LDH release (**Figure 22A**). 200 μM H<sub>2</sub>O<sub>2</sub> resulted in a ~5-fold increase in LDH release by myoblasts but not myotubes (**Figure 22A**). As expected, pre-treatment with the PARP inhibitor, PJ34, significantly reduced H<sub>2</sub>O<sub>2</sub>-induced LDH release in myoblasts (**Figure 22A**). Similarly, we observed significant reduction of both MTT conversion capability and NAD<sup>+</sup> levels in myoblasts exposed to increasing concentrations of H<sub>2</sub>O<sub>2</sub>, but not in myotubes (**Figure 22B and 22C**). Similarly, PJ34 pretreatment attenuated the deleterious effect of H<sub>2</sub>O<sub>2</sub> in myoblasts, with only relatively minor effects in myotubes (**Figure 22B and 22C**). The cytotoxic effects of glucose oxidase (GOx), an alternative oxidative stressor that generates constant, low levels of H<sub>2</sub>O<sub>2</sub> in culture media, were also attenuated in PJ34-treated myoblasts in a concentration-dependent manner (assessed by measurement of MTT reduction, **Figure 22D**). Myotubes were affected only by the highest concentration of GOx and experienced no beneficial effect from PJ34 pre-treatment (**Figure 22D**). To verify our observations, we performed similar sets of experiments in another type of skeletal muscle cell line, namely, rat L6 cells. Similar to C2C12, L6 myoblasts showed a distinctively higher sensitivity to oxidants— as measured by MTT conversion and LDH release assays— that could be partially attenuated by pre-treating with PJ34 (**Figure 22E, 22F and 22G**). Myotubes were more resistant to the same concentrations of H<sub>2</sub>O<sub>2</sub> (**Figure 22E**) and GOx (**Figure 22F and 22G**) regardless of PJ34 pre-treatment, which seemed to have no beneficial effect for myotubes.



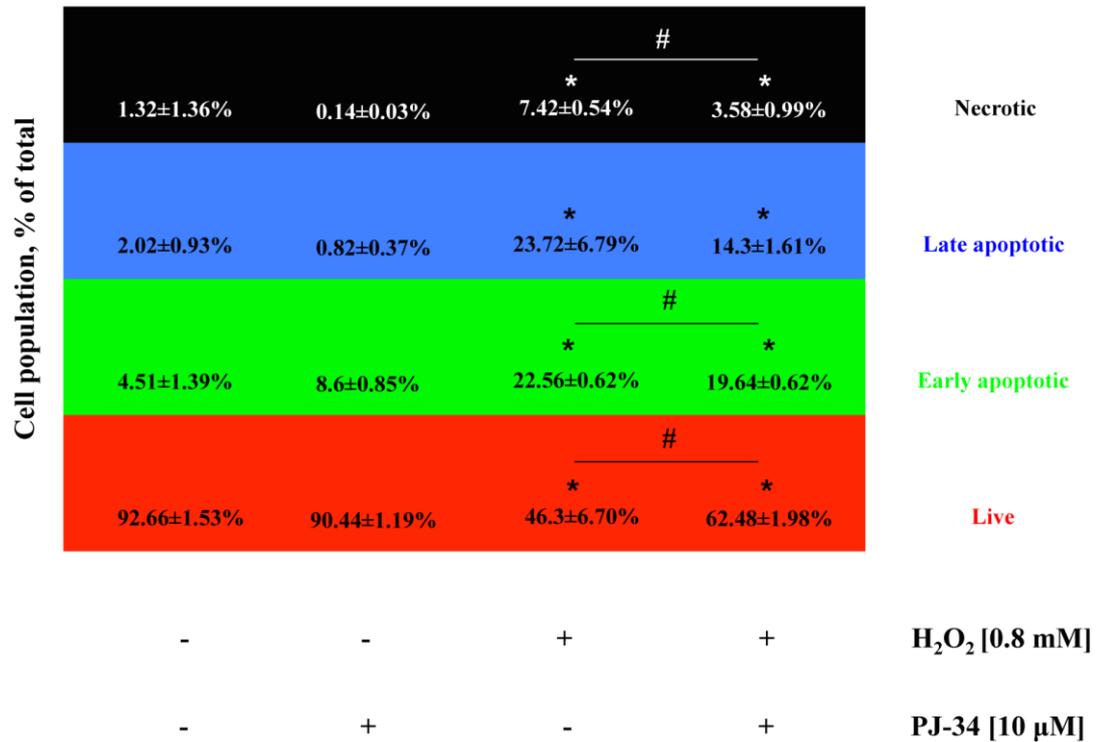
**Figure 22.** *Myotubes are resistant to oxidant-induced loss of cell viability.* Cell viability of myoblasts and myotubes in response to increasing concentration of oxidative stress was measured by LDH release (A, F), MTT conversion (B, D, E, G), and cellular NAD<sup>+</sup> content (C). 10 $\mu$ M PJ34 was used in all experiments to test the effect of PARP1 inhibition. Experiments shown in panels A-D were performed with C2C12, and those in panels E-G, with L6 cells. Data represent mean  $\pm$  SD. One-way ANOVA was used for statistical analysis and for the determination of significance between individual groups. \*shows significant difference,  $p < 0.05$ , in the cell response to H<sub>2</sub>O<sub>2</sub> or GOx relative to controls while # shows significant protective effect of PJ34 in H<sub>2</sub>O<sub>2</sub> or GOx treated cells,  $p < 0.05$ . Data shown in A, B, C, D, E, F, and G were calculated as percentage of control cells (untreated myoblasts or myotubes, in each case set as 100%).

Our observation that myotubes are resistant to oxidant induced stress was further validated by flow cytometry, which showed that oxidative stress induces cell death of mixed type (necrotic and apoptotic), and that PARP inhibition with PJ34 reduces cell death primarily by decreasing the portion of necrotic and early apoptotic cell populations (**Figure 23A and 22B**). Taken together, these data indicate that myotubes are more resistant to oxidative stress than myoblasts, and that the oxidative stress-induced cell dysfunction/cell death in myoblasts involves a significant PARP-1 dependent component.

A



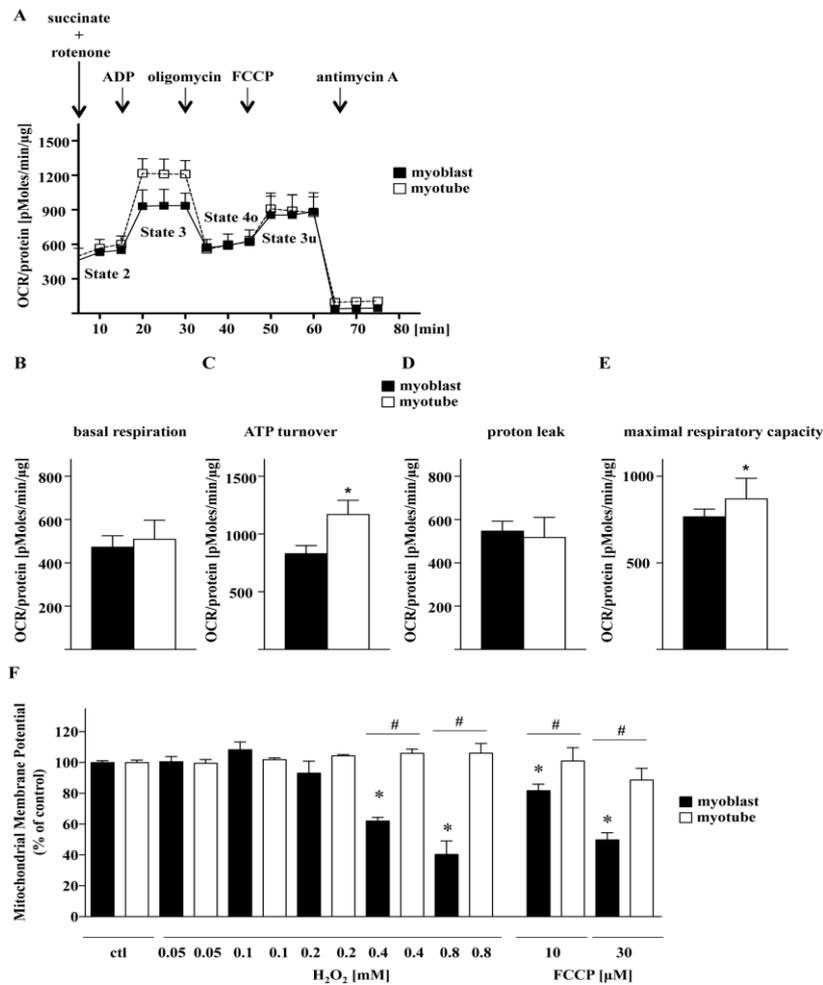
B



**Figure 23.** Inhibition of PARP-1 reduces subpopulations of apoptotic and necrotic cells induced by H<sub>2</sub>O<sub>2</sub>. C2C12 myoblasts were exposed to 0.8 mM H<sub>2</sub>O<sub>2</sub> for 24h in the presence or absence of the PARP inhibitor PJ34 (10 µM). PJ34 reduced the oxidant-induced death of the myoblasts by decreasing the proportion of the necrotic, early and late apoptotic cell populations. Representative dot plots (A) and analysis (B) are shown. One-way ANOVA was used for determining significance between groups. \* shows significant differences, p<0.05, in the cell response to H<sub>2</sub>O<sub>2</sub> relative to controls, while # shows significant protective effect of PJ34, p<0.05. Data representative of 3 different determinations conducted on different experimental days are shown. (Total cell number in each group was set to 100%).

### **4. 3. Myotubes preserve mitochondrial functions during oxidative stress**

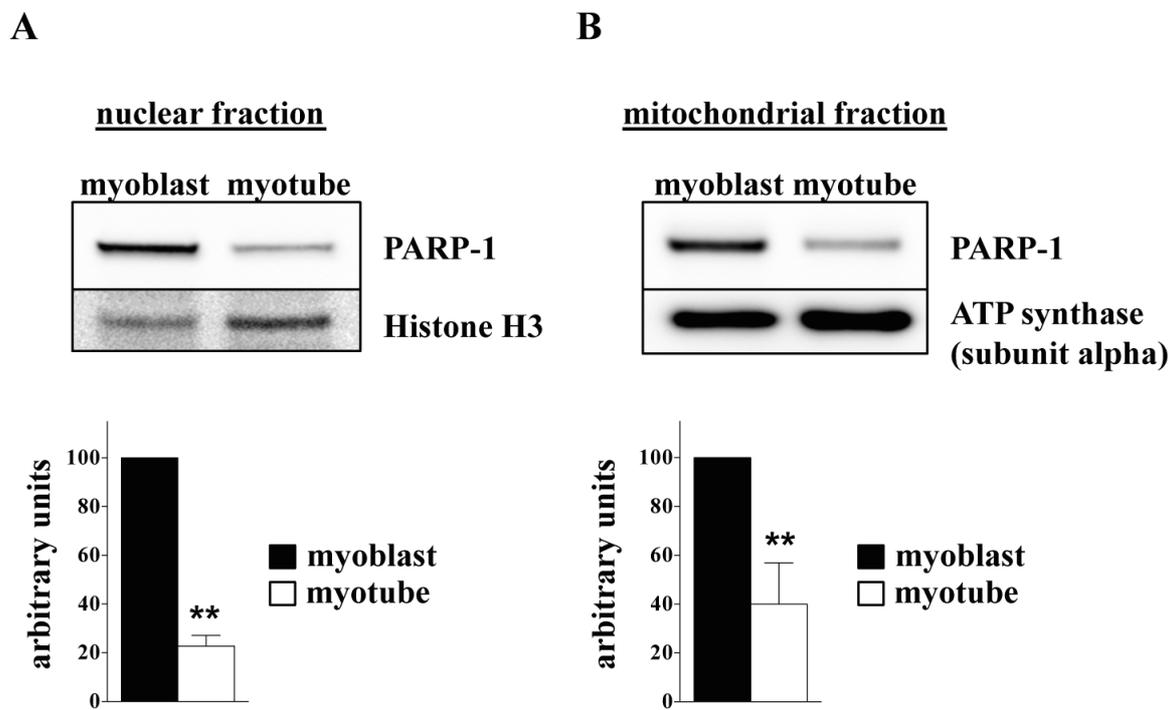
We investigated the differences in major bioenergetics parameters in mitochondria isolated from C2C12 myoblasts and myotubes with Extracellular Flux Analysis (**Figure 24A**). While basal respiration (State 2) did not show any significant differences between the two cell types, myotubes were found to have higher ATP turnover (State 3) and maximal respiratory capacity (State 3u) than myoblasts. (**Figure 24B, 24C, 24D, 24E**). To investigate the effect of H<sub>2</sub>O<sub>2</sub> on mitochondrial functions of myoblasts and myotubes, we measured mitochondrial membrane potential. We observed a gradual decrease of the mitochondrial membrane potential in response to increasing concentration of H<sub>2</sub>O<sub>2</sub> in the myoblasts, while the mitochondrial membrane potential was unaffected in the myotubes (**Figure 24F**). FCCP (a mitochondrial uncoupler) was used as a control (**Figure 24F**).



**Figure 24.** Cellular bioenergetics of mitochondria isolated from myoblasts and myotubes. Bioenergetic analysis was carried out using the Extracellular Flux Analyzer in response to the sequential administration of ADP (4 mM), oligomycin (2 μM), FCCP (4 μM), and antimycin A (4 μM) in the presence of succinate (5.5 mM) and rotenone (2 μM). (A) Representative analysis of oxygen consumption. Calculated bioenergetics parameters: (B) basal respiration, (C) ATP turnover, (D) proton leak and (E) maximal respiratory capacity. Data are shown as means ± SD of n = 9 wells collected from n = 3 experiments performed on 3 different days. \* p<0.01, in ATP turnover and maximal respiratory capacity. State 2 = Basal Respiration, State 3 = ATP Turnover, State 4o = Proton Leak, State 3u=FCCP-stimulated Maximal Respiratory Capacity. (F) Mitochondrial membrane potential changes in the absence or presence of the PARP inhibitor PJ34 (10 μM) in C2C12 myoblasts and myotubes. Statistical analyses of n = 3 independent experiments were assessed; where \* indicates significant difference,

p<0.001, between control and H<sub>2</sub>O<sub>2</sub>-treated myoblasts, while # shows significant difference, p<0.001, between myoblasts and myotubes treated under the same conditions.

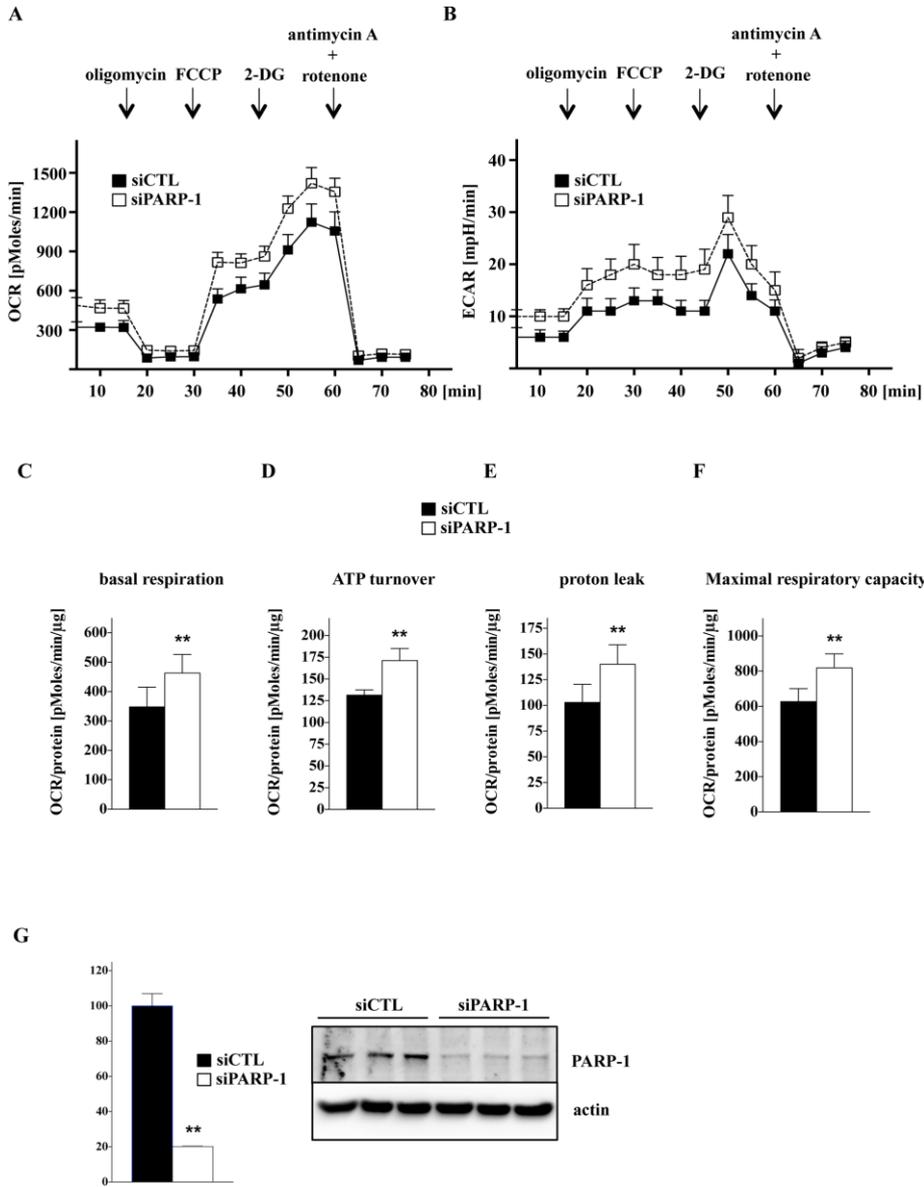
Since PARP-1 was localized in the nucleus and in the mitochondria (Rossi et al., 2009; Szczesny et al., 2014) we investigated its level in both cellular fractions. As shown in **Figure 25**, reduction of PARP-1 level was found in nuclear and mitochondrial fractions of myoblasts as compared to myotubes (**Figure 25A and 25B, respectively**).



**Figure 25.** PARP-1 protein levels in mitochondrial and nuclear fractions of myoblasts and myotubes. Western blot analysis detected elevated expression of PARP-1 in myoblasts than in myotubes for both nuclear (A) and mitochondrial (B) fractions. Densitometric analysis of PARP-1 protein level in myoblast was set as 100%. Statistical analyses of n = 3 independent experiments were assessed; where \*\* indicates p<0.001.

We have previously shown that PARP-1 silencing significantly enhances basal mitochondrial bioenergetics in cultured endothelial and epithelial cells (Modis et al., 2012). Now, we investigated the effect of transient siRNA-mediated silencing of PARP-

1 on the bioenergetic response in C2C12 myoblasts. Silencing PARP-1 expression increases both oxidative phosphorylation (**Figure 26A**) and glycolytic activity (**Figure 26B**). The latter observation is in line with recent findings showing the regulation of glycolytic function by PARP-1 in neurons (Andrabi et al., 2014).

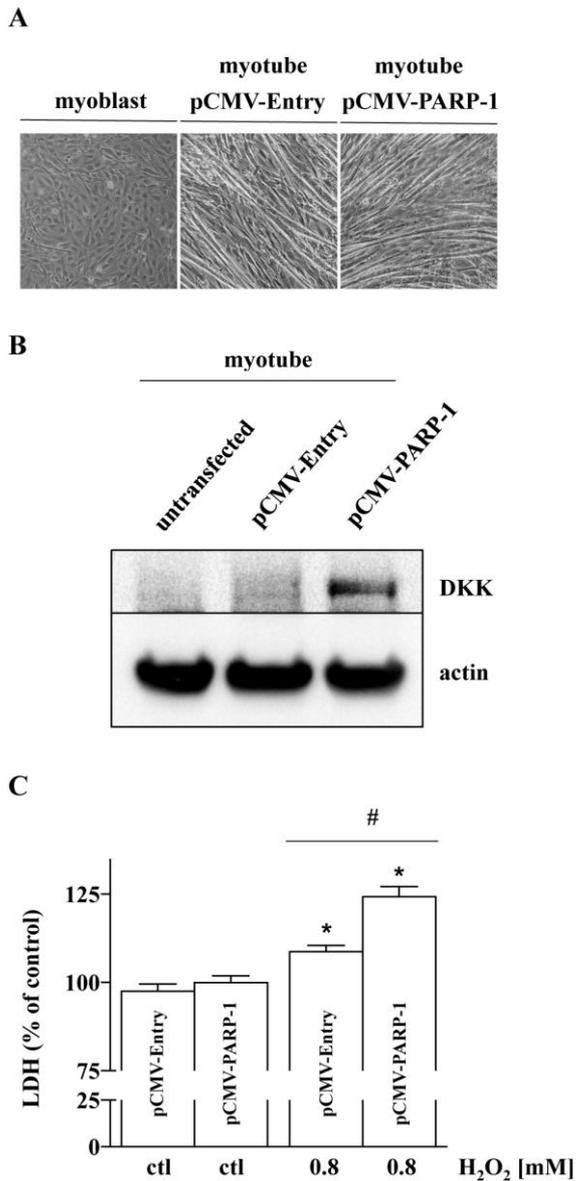


**Figure 26.** Silencing *PARP-1* increases both oxidative phosphorylation and glycolytic activity of C2C12 myoblasts. Bioenergetic analysis of the myoblasts was conducted by extracellular flux analysis. In the figure, a time-course measurement of OCR (A) and ECAR (B) for 1Å~104 cells/well under basal conditions was followed by the sequential addition of oligomycin (1 μg/ml), FCCP (0.3 μM), 2-DG (5mM) and antimycin A (2

µg/ml). Cells with PARP-1 silencing show significantly elevated OCR values during basal and uncoupled respiration (\*p<0.05). (C, D, E, F) Bioenergetic parameters are elevated in cells after siRNA silencing of PARP-1, compared to control (\*p<0.01). Data are shown as means ± SD of n = 15 wells (each group) collected from n = 3 experiments performed on 3 different days. (G) Western blot analysis shows the efficiency of PARP-1 silencing as evaluated by unpaired t-test, \*p < 0.001 relative to siCTL.

#### **4. 4. Over expression of PARP1 increases the oxidant sensitivity of myotubes**

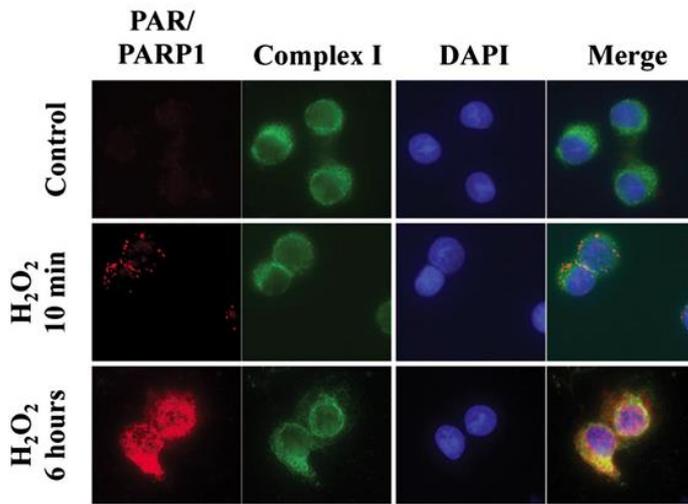
There is a possibility that, in addition to the downregulation of PARP1, a host of additional alterations in the transcriptome profiles between myoblasts and myotubes resulted in the observed phenotype of decreased oxidant sensitivity in myotubes (in contrast to myoblasts). Therefore, PARP1 supplementation (forced expression of PARP1 using a cDNA inserted into pCMV6-Entry vector) was performed in myotubes, followed by differentiation of the cells into myoblasts (**Figure 27A**). The overexpressed PARP-1 maintained in myotubes as detected by Western analysis using anti-DDK monoclonal antibody (**Figure 27B**). Myotubes with forced expression of PARP1 became more sensitive to hydrogen peroxide induced cell injury: they responded with increased LDH release, compared to the sham-transfected myotube controls (**Figure 27C**).



**Figure 27.** PARP-1 overexpression sensitizes C2C12 myotubes to oxidative stress. (A) PARP-1 overexpression does inhibit the differentiation of myoblasts to myotubes. (B) Representative western blot shows the presence of expressed, tagged PARP-1 in myotubes (after 5 days of differentiation from myoblasts). (C) Myotubes with overexpressed PARP-1 possessed increased sensitivity to oxidative stress 24 h after 0.8 mM H<sub>2</sub>O<sub>2</sub>, as estimated by the measurement of LDH release. Data represent mean  $\pm$  SD of n = 6 determinations. \* shows significant difference, p<0.05, in the cell response to H<sub>2</sub>O<sub>2</sub> relative to controls (in the absence of H<sub>2</sub>O<sub>2</sub>), while # shows a significant increase in LDH release in the myotubes with forced PARP-1 expression, when compared to control myotubes.

#### 4. 5. Oxidative stress leads to an early PARP-1 activation in U937 cells

10 minutes of oxidative stimulus ( $\text{H}_2\text{O}_2$ , 400mM) induced PARP-1 activation (auto-PARylation of PARP-1) in the extranuclear/mitochondrial compartment, but not in the nucleus showed by in situ proximity ligation assay (PLA) (**Figure 28**). At 3-24 hours, in line with other studies, nuclear PARP activation occurred. Other pathophysiologic steps were involved after the early-onset of PARP-1 activation such as mitochondrial DNA damage, cellular oxidant production and compromised cell membrane integrity.

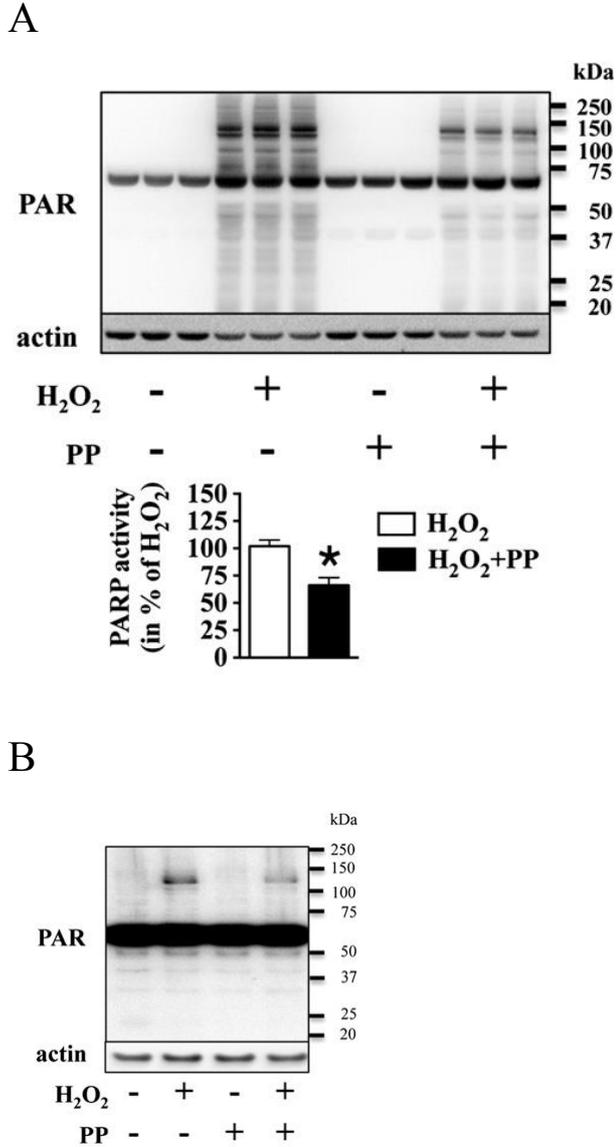


**Figure 28.** Auto-PARylation of PARP1 in the extranuclear and nuclear compartments in U937 cells during oxidative stimuli. Auto-PARylation of PARP1 was assessed by PAR/PARP1 in situ PLA analysis, was found extranuclearly at the early time point (10 minutes) after  $\text{H}_2\text{O}_2$  exposure, while it was localized both to the nuclear and extranuclear compartments by 6 hours. Representative images of at least  $n = 3$  independent determinations, conducted on different experimental days, are shown.

#### 4. 6. $\beta$ -adrenoceptor signaling is involved in PARP-1 activation during $\text{H}_2\text{O}_2$ challenge

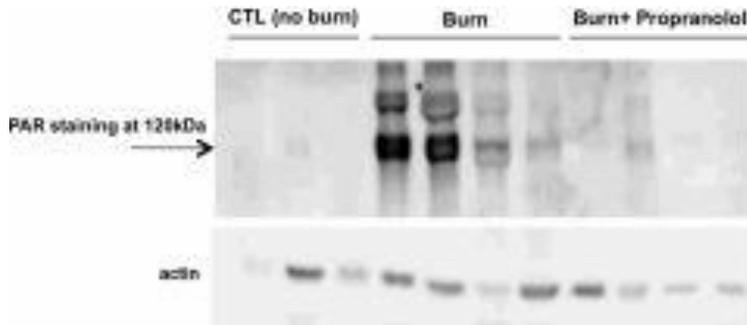
Pretreatment of U937 cells with the  $\beta$ -receptor antagonist propranolol (10  $\mu\text{M}$ ) prior to 10 minutes of  $\text{H}_2\text{O}_2$  challenge significantly reduced the increase of PARylation of

multiple proteins and the auto-PARylation of PARP-1 (**Figure 29**). The regulation of PARP-1 activation appears to be a general phenomenon since propranolol inhibited the PARylation in C2C12 myoblasts during the H<sub>2</sub>O<sub>2</sub> challenge.



**Figure 29.** Propranolol regulates PARP activation in U937 and C2C12 cells during oxidative stress. The  $\beta$ -adrenoceptor antagonist propranolol (PP) decreases cellular PARylation in (A) U937 and C2C12 (B) cells treated with H<sub>2</sub>O<sub>2</sub>. Western blot shown includes three separate technical replicates (lanes) for each experimental condition. Densitometric analysis of PARylation in H<sub>2</sub>O<sub>2</sub>-treated cells was set as 100% and effect of pharmacological modulators is presented as mean  $\pm$  S.E.M. of three independent experiments; \*p<0.05.

#### 4. 7. The positive effect of PARP inhibition in skeletal muscle biopsies from burn patients



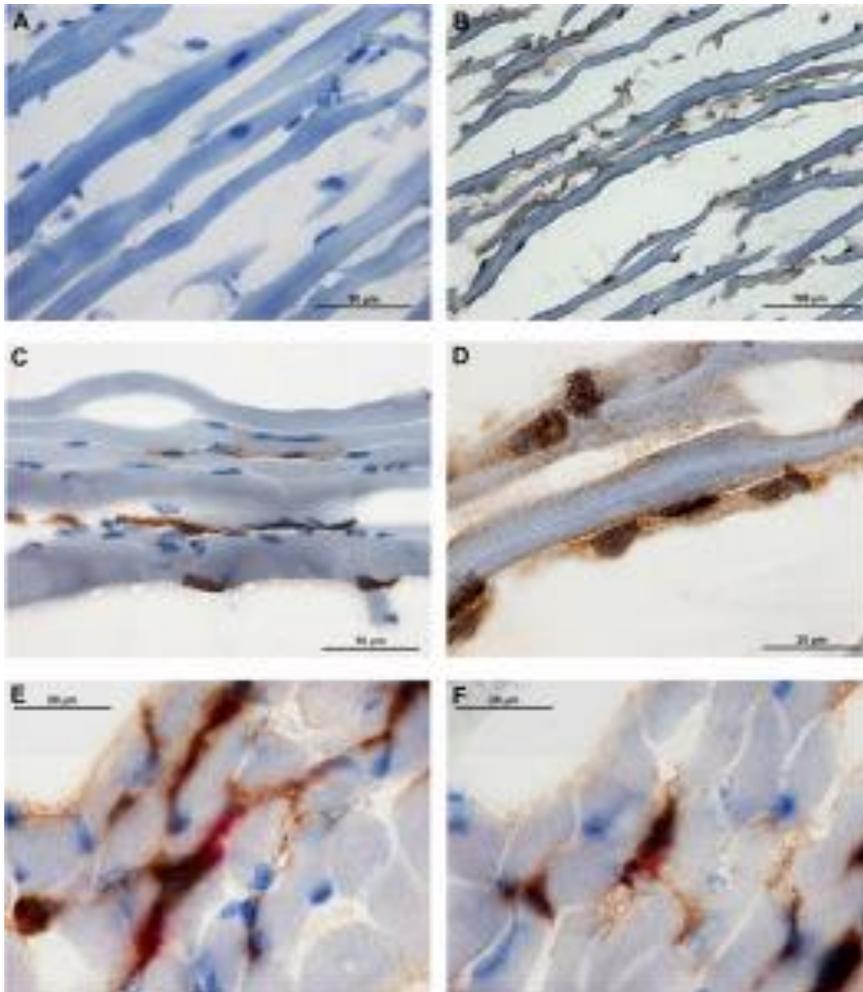
**Figure 30.** Representative PAR Western blots are shown from homogenates of skeletal muscle biopsies of control patients (no burn, n=3), of patients with burn injury at the middle stage of the disease (n=4) and of propranolol-treated patients with burn injury at the middle stage of the disease (n=4). The bottom part of the figure shows the corresponding actin loading control bands.

We compared PARP activation in normal skeletal muscles and muscles from different time point after burn injury. We classified the burn muscle samples as 'Early', 'Middle', 'Late' time points based on the time range when biopsies were taken as seen in Table 1 (Materials and Methods). 'Early' time point showed elevated PARylation while 'Middle' had the most pronounced PARP activation. The 'Late' time point showed a heterogeneous population, but still was elevated compared to healthy controls. The PARylation mainly affected PARP-1 at ~120kDa, although other proteins were also modified by the PARylation process (**Figure 30**).

Immunohistochemical evaluation of PARP activation mostly was observed in the 'Middle' group compared to control group. The signal was primarily seen in the capillary endothelium and co-localized with CD-31 and factor VIII-related antigen. Also, PAR positive staining was observed in mononuclear cells and neuroglial cells confirmed by S-100 staining (**Figure 31**).

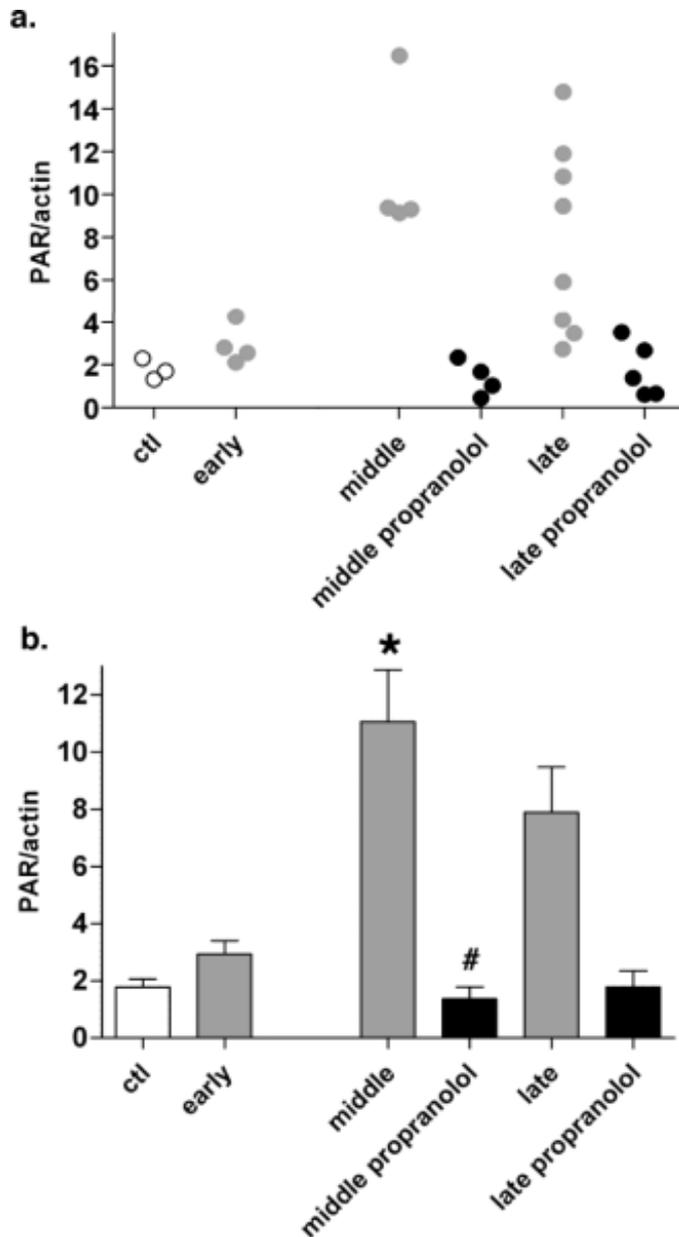
The other goal of the study was to evaluate the effect of propranolol therapy on the PARP activation. The main finding was the reduced PARylation in the 'Middle' and in

some degree in the 'Late' group compared to the patients who did not receive propranolol. (Figure 32).



**Figure 31.** PAR localized primarily to vascular endothelial cells and mononuclear cells. High-power photomicrographs of frozen tissue sections studied by immunohistochemistry to show the activation and localization of PAR in 'Middle' group of patients. A: Control tissue sections, showing no significant PAR staining. Original magnification: 40 $\times$ . B: Tissue sections collected from a burn case, showing marked activation of PAR. Original magnification: 20 $\times$ . C–D: Tissue section collected from a burn case, showed at higher magnification, highlighting the localization of PAR-positive staining within cells of the capillary endothelium (brown staining), while the nuclei of myocytes appear negative (blue counterstaining). Original magnification: (C) 40 $\times$ , (D) 100 $\times$ . E–F: Tissue sections collected from a burn case showing red

staining for PAR and brown staining for either CD-31 (E) or Factor VIII-related antigen (F), to demonstrate co-localization within vascular endothelial cells. Original magnification: (E, F) 100 $\times$ .



**Figure 32.** Time course of PARP activation in skeletal muscle biopsies of pediatric burn patients and reduction of PARP activation in skeletal muscle biopsies of pediatric burn patients treated with propranolol. Densitometric analysis of PAR staining, normalized to actin, is shown, in control subjects (no burn injury), and in the Early, Middle and Late stage of the disease in the absence of propranolol treatment, and the Middle and Late stage of the disease in the presence of propranolol treatment. Results are expressed

as means $\pm$ SEM. \*P <0.05 shows a significant increase in the PAR response, when compared to the control group in the `Middle' group of patients; #p<0.05 shows a significant inhibition of the response by propranolol in the `Middle' group of patients.

## **5. Discussion**

### **5. 1. Differentiation-associated downregulation of PARP-1 expression increases the resistance to oxidative stress**

The main conclusion of the present study is that the differentiation associated decrease in protein level of PARP-1 in murine skeletal muscle cells contributes to acquisition of marked resistance to oxidative stress and the development of a capacity to maintain cellular bioenergetics during oxidative challenge.

Cellular reprogramming occurs during skeletal muscle differentiation with multiple genes up- and down-regulating as required to orchestrate a smooth transition from proliferation to cell cycle exit, then differentiation and finally, fusion of multiple cells to form myotubes. One of the key components of the process is the expression of muscle specific transcription factors, such as myogenin, which has a primary role in down-regulating Cdk activity in order to exit the cell cycle (Zang et al., 1999). One key enzyme affecting chromatin remodeling and gene expression is PARP-1 (Jagtap and Szabo, 2005) We hypothesize that maintenance of a high level of PARP-1 expression is required at the earlier stages of the differentiation process in order to initiate the changes in the chromatin structure to facilitate reprogramming gene expression. The cost to the cell for this high level of PARP-1 expression is increased sensitivity to oxidative stress although cells do not normally experience substantial oxidative stress in this undifferentiated stage.

The situation is quite different in fully differentiated myocytes. Significant oxidative stress is generated from normal physiological functioning of differentiated myocytes, e.g. skeletal muscle contraction during movement or exercise (Hepple 2014; Aoi et al. 2014; Nikolaidis et al., 2012; Radak et al., 2002; Ji 2008; Radak 2008; Szczesny et al., 2010). Therefore, we hypothesized that the downregulation of PARP-1 serves as a protective mechanism limiting the deleterious consequences of oxidant-mediated PARP-1 overactivation such as cellular NAD<sup>+</sup> depletion, decrease in bioenergetics, or in extreme cases, cell death. This is particularly important for skeletal muscle, which is prone to oxidative damage (Sakellariou et al., 2014).

To investigate this hypothesis, we first determined the intracellular distribution of PARP-1. Our immunocytochemistry studies showed that PARP-1 is localized mainly in the nucleus of myoblasts, that its nuclear expression is reduced after differentiation has taken place in myotubes, and that this occurs parallel to an increase in extranuclear staining. Based on several lines of study in various cell types (Rossi et al., 2009; Brunyanszki et al., 2014; Szczesny et al., 2014) as well as data presented in the current report, this staining represents mitochondrial PARP-1. Relatively little is known about the functional role of mitochondrial PARP-1. Rossi and colleagues showed that the mitochondrial protein mitofilin co-localized with PARP-1 in the mitochondria of HeLa cells, thus demonstrating the mitochondrial presence of the enzyme. Using subcellular fractionation, we show in the current paper that myoblasts have higher levels of PARP-1 in their mitochondria than differentiated myotubes (**Figure 25B**). In line with previous studies, the possible role of the decreased PARP-1 expression in the mitochondria of C2C12 might be related to the higher integrity of mtDNA in the latter cells. We have recently demonstrated that there are differences in mitochondrial DNA repair capability between myoblasts and myotubes, with myotubes having higher DNA integrity (Szczesny et al., 2013), and observed that low levels of oxidative stress selectively activate mitochondrial PARP-1 in human U937 monocytes (Brunyanszki et al., 2014, **Figure 28**). These prior findings taken together with the data presented in the current report suggest that both the downregulation and the intracellular distribution of PARP-1 (from the nucleus to the mitochondria) during myoblast differentiation may contribute to a change in oxidative stress resistance. This is further supported by our recent observation that PARP-1-depletion increases the rate of the mitochondrial DNA repair (Szczesny et al., 2014).

Other series of studies showed the beneficial effects of genetically disrupting and pharmacologically inhibiting PARP-1 in models of cerebral ischemia, oxidative and nitrosative stress in islet cells, myocardial post-ischemic injury, and cerebral artery occlusion - especially with regards to its effect on motor function (Eliasson et al., 1997; Heller et al., 1995; Pieper et al., 2000; Ding et al., 2001). PARP-1 was overexpressed in myotubes to further test whether PARP-1 downregulation is an independent contributor to the resistance of myotubes to oxidative stress. This intervention increased the oxidative stress sensitivity of the myotubes which supports the view that

downregulation of PARP1 during differentiation of myoblasts to myotubes directly regulates the oxidant-sensitivity of these cells. Nevertheless, it is obvious that a host of changes occur in the transcriptome profile during cell differentiation, and, therefore, multiple factors— in addition to PARP1 downregulation— are also likely to contribute. There are a number of studies implicating PARP-1 in the regulation of cellular bioenergetics under normal conditions and during oxidative stress. The deletion of PARP-1 increases  $\text{NAD}^+$  content and SIRT1 activity in brown adipose tissue and muscle, which mimics the responses seen with SIRT1 activation (both result in increased energy expenditure and enhanced oxidative metabolism) (Bai et al., 2011). Moreover, in bEnd.3 endothelial and A549 epithelial cells, pharmacological inhibition and siRNA-mediated silencing of PARP-1 independently increased basal and uncoupled cellular respiration, suggesting that PARP-1 is a regulator of physiological mitochondrial function (Modis et al., 2012). Additionally, PARP-1<sup>-/-</sup> muscle showed a marked deacetylation of PGC-1 $\alpha$  and FOXO1, which is linked to enhanced mitochondrial biogenesis and a more oxidative profile of muscle fibers (Kauppinen et al., 2013). In line with the above-cited literature, the bioenergetic experiments conducted in the current study indicate that PARP-1 is a regulator of basal mitochondrial function and also suggest that PARP-1 regulates the glycolytic capacity of myoblasts — both of these functions were enhanced when PARP-1 was silenced (**Figure 26**). Based on these data, we conclude that the downregulation and redistribution of PARP-1 during skeletal muscle differentiation will not only create an oxidative stress resistance, but also enhance the cellular bioenergetics capacity of the differentiated muscle cell relative to the myoblast, for instance, by consuming less  $\text{NAD}^+$ . Since cellular  $\text{NAD}^+$  levels are compartmentalized and regulated by multiple enzymes (Koch-Nolte et al., 2011; Dölle et al., 2013); therefore it is conceivable that differentiation-associated changes in the expression or activity of various enzyme systems that handle  $\text{NAD}^+$ , other than PARP-1 may have also contributed to the observed decrease in cellular  $\text{NAD}^+$  levels during differentiation. Follow-up analysis of expression level of other enzymes utilizing  $\text{NAD}^+$  is warranted to explain this observation. In this context, it is interesting to mention the recent report of Frederick and colleagues demonstrating that increasing  $\text{NAD}^+$  biosynthesis in the skeletal muscle is not alone sufficient to increase metabolic function (Frederick et al., 2015).

It should also be noted that while downregulated in myotubes compared to myoblasts, PARP-1 is not completely absent in the differentiated myotubes. For instance, 800  $\mu\text{M}$   $\text{H}_2\text{O}_2$  induces 70% depletion of  $\text{NAD}^+$  content in undifferentiated cells but only 30% depletion in differentiated cells (**Figure 22C**). Accordingly, oxidative stress does have some effect on these cells - though relatively minor when compared to myoblasts - that is reduced by inhibition of PARP-1. Also, *in vivo*, there is a constant turnover of the muscle, meaning that skeletal muscle probably represents a mixed population of cells in various stages of differentiation and correspondingly, various degrees of PARP-1 expression and oxidative stress resistance. These notions are consistent with a recent report by Pirinen and colleagues (Pirinen et al., 2014) that demonstrated that treating mice with pharmacological inhibitors of PARP-1 exerted beneficial effects on skeletal muscle endurance and performance, coupled with increased cellular  $\text{NAD}^+$  content, mitochondrial function, and mitochondrial biogenesis.

## **5. 2. The localization of PARP-1 during oxidative challenge and the effect of propranolol on the PARP-1 activation in U937 and C2C12 cells**

We found that an early and specific activation of PARP1 occurs in the mitochondria in oxidatively stressed U937 cells. The functional consequences of such activation is modulated by the  $\beta$ -adrenoceptor antagonist propranolol suggesting a cAMP/PKA and PARP-1 interplay.

The findings are noted here might serve as a cellular base for the advantageous outcome in propranolol treated patients discussed below.

## **5. 3. Poly(ADP-ribosyl)ation in skeletal muscle tissue of pediatric patients with burn injury: the positive effect of propranolol**

Severe burn injury induces pathophysiological response affecting the whole body including muscle, lung, heart and liver. Studies in rodent and large animal models of burn injury have demonstrated the activation in various tissues and the beneficial effect

of its pharmacological inhibition (Bartha et al, 2011; Asmussen et al., 2011). In a murine model of severe burn injury in heart and lung tissue exhibited increased PARP-1 activity and more pronounced mitochondrial DNA damage than their non-injured counterpart, which clearly demonstrates the critical role of PARP-1 in thermal injury (Szczeny et al., 2015). As a primary study to map the role of PARP-1 in critical illness such as burn injury, we measured the PARP-1 activation in human skeletal muscle biopsies at various stages of severe pediatric burn injury. In conjunction with that, we tested the effect of propranolol, an effective treatment for burn patients, on the PARP-1 activation in skeletal muscle biopsies. We found that PARP-1 became activated in skeletal muscle tissue 13-18 days after burn and localized primarily to vascular endothelial cells and resident mononuclear cells to some degree. There was a marked suppression of PARP activation in the skeletal muscle biopsies of patients who received propranolol treatment. Our conclusion was that some of the clinical benefits of propranolol in burns might be related to its inhibitory effect on PARP activation (Olah et al., 2011). Aforementioned results from our lab demonstrated that inhibiting PARP-1 has a beneficial outcome on the systemic effect of burn in patient.

Other studies have shown the profitable effect of exercise on muscle of burn patients: for instance, improved lean body mass, peak torque, muscle strength endurance (Hardee et al., 2014; Porter et al., 2015a; Porter et al., 2015b). This could partially be explained by the elevated effectiveness of the antioxidant apparatus and/or mitochondrial respiratory capacity. If we assume that two cohorts of burn patients, exercised and non-exercised, have the same PARP level/response but different antioxidant capacities, it can be concluded that in the exercised group the more effective antioxidant capacity serves to neutralize PARP-1 overactivation thus improving physical function in severely burned patients.

## **6. Future directions**

Observations made in the current study that remain to be investigated include (a) the functional consequence of the variable regulation of various 'minor' PARP isoforms during differentiation (i.e. downregulation of PARP-2 and PARP-7 and upregulation of PARP-3), (b) the mechanism through which glycolytic activity increases after PARP-1 silencing in myoblasts, and (c) the reasons why the downregulation of PARP-1 during differentiation is cell-type dependent. It is interesting to note that inhibition of PARP-2 has recently been shown to produce an increase in mitochondrial biogenesis in skeletal muscle (Mohamed et al., 2014). (d) For burn patients it would be also beneficial to explore if incorporating antioxidants or PARP inhibitors as a supplement to exercise program currently used enhances the outcome of therapy. Along with the exercise program in severely burned patient, it would be useful to incorporate antioxidant or/and PARP-inhibition supplementation as a treatment for a more robust positive outcome and investigate muscle function such as lean body mass, peak torque, muscle strength endurance parallel with the identification the molecular triggers of PARP activation in burn patients to better address the therapy.

## 7. Conclusions

Based on multiple lines of functional data, we concluded that the functional importance of the differentiation associated decrease in PARP-1 in C2C12 muscle cells is that it endows myotubes with increased oxidative stress resistance and bioenergetic function. We realize that the regulation of PARP-1 in myoblasts and myotubes may be a specialized case since PARP-1 has multiple roles in a variety of cell types ranging from promotion of survival in cancer cells to actively mediating necrosis in parenchymal cells and catalyzing pro-inflammatory gene expression in immune cells (Jagtap and Szabo 2005; Pacher and Szabo 2008; De Vos et al., 2012). Changes in PARP-1 expression may well have very different roles depending on the cell type and the (patho)physiological context.

We showed an early PARP-1 activation in a mitochondrial localization and the inhibition of PARylation by the  $\beta$ -adrenoceptor agonist propranolol.

We also provided evidence for PARP activation in muscle tissue of burn patients and were able to demonstrate the protective effect of propranolol. In order to clarify the exact mechanistic role of PARP in human burns, future, interventional studies with PARP inhibitors would be necessary.

- PARP-1 protein expression level is markedly decreased during skeletal muscle cell differentiation in murine and rat cell-based models
- The reduced level of PARP-1 in myotubes results greater oxidative stress resistance than their undifferentiated counterparts.
- PARP-1 participates in the regulation of muscle cell bioenergetics under normal conditions and during oxidative challenge. We showed increased respiratory parameters in myoblasts with siRNA-mediated silencing of PARP-1 suggesting a beneficial effect of the depletion.
- There is an early-onset of PARP-1 activation in the mitochondria in U937 cells and can be inhibited by the  $\beta$ -adrenoceptor agonist propranolol.
- Pharmacological inhibition of PARP-1 has a beneficial effect in burn patients.

## 8. Summary

Poly(ADP-ribose) polymerase 1 (PARP-1), the major isoform of the poly (ADP-ribose) polymerase family, is a nuclear and mitochondrial protein with well-recognized roles in various essential cellular functions such as DNA repair, signal transduction, apoptosis, as well as in a variety of pathophysiological conditions including burn, sepsis, diabetes and cancer. Activation of PARP-1 in response to oxidative stress catalyzes the covalent attachment of the poly (ADP-ribose) (PAR) groups on itself and other acceptor proteins, utilizing  $\text{NAD}^+$  as a substrate. Overactivation of PARP-1 depletes intracellular  $\text{NAD}^+$  influencing mitochondrial electron transport, cellular ATP generation and, if persistent, can result in necrotic cell death. Skeletal muscle cells are particularly exposed to constant oxidative stress insults during exercise. In this study, we investigated the role of PARP-1 in a well-defined model of murine skeletal muscle differentiation (C2C12) and compare the responses to oxidative stress of undifferentiated myoblasts and differentiated myotubes. We observed a marked reduction of PARP-1 expression as myoblasts differentiated into myotubes. This alteration correlated with an increased resistance to oxidative stress of the myotubes, as measured by MTT and LDH assays. Mitochondrial function, assessed by measuring mitochondrial membrane potential, was preserved under oxidative stress in myotubes compared to myoblasts. Moreover, basal respiration, ATP synthesis, and the maximal respiratory capacity of mitochondria were higher in myotubes than in myoblasts. Inhibition of the catalytic activity of PARP-1 by PJ34 (a phenanthridinone PARP inhibitor) exerted greater protective effects in undifferentiated myoblasts than in differentiated myotubes. The above observations in C2C12 cells were also confirmed in a rat-derived skeletal muscle cell line (L6). Forced overexpression of PARP1 in C2C12 myotubes sensitized the cells to oxidant-induced injury. We also showed that oxidative stress, such as  $\text{H}_2\text{O}_2$  treatment (for 10minutes), caused an early-onset of PARP-1 activation in the mitochondria assessed by PLA analysis and at later time point (at 3-24 hours) the activation occurred in the nucleus. We compared PARP activation in normal skeletal muscles and muscles from different time point after burn injury ('Early', 'Middle' and 'Late' groups) and found that the PARylation mainly affected PARP-1 at ~120kDa, although other proteins were also modified by the PARylation process. The other goal of the study was to evaluate the

effect of propranolol therapy on the PARP activation. The main finding was the reduced PARylation in the 'Middle' and in some degree in the 'Late' group compared to the patients who did not receive propranolol. In order to study the mechanism of the protective effect of propranolol, we showed that the  $\beta$ -adrenoceptor signaling is involved in the PARP-1 activation in U937 and C2C12 cells since propranolol inhibited the PARylation in both cells after H<sub>2</sub>O<sub>2</sub> challenge.

Taken together, we showed that (a) the reduction of PARP-1 during differentiation serves as a protective mechanism against oxidative stress. (b) PARP-1 activation during oxidative challenge occurs in the early time points in the mitochondria and hours later in the nucleus. (c) this PARP-1 activation can be inhibited by the  $\beta$ -adrenoceptor agonist propranolol which (d) has beneficial effect in burn patients.

## 9. Összefoglalás

PARP-1, poli(ADP-ribóz) polimeráz 1 a PARP család egyik fő izoformája, ez a család emberben 17, egérben 16 fehérjéből áll. Egy folyamatosan termelődő sejtmagi és mitokondriális fehérje jól ismert szereppel különböző sejtes funkciókban, mint például DNS (dezoxiribonukleinsav) hibajavításban, jelátviteli folyamatokban, különböző sejthalál útvonalakban és különböző kóros folyamatokban mint égés, szepszis, cukorbetegség és rák. A PARP által szabályozott legtöbb funkció oxidatív stresszhez kapcsolódik. A PARP-1 aktivációja oxidatív stimulus hatására poli(ADP-ribóz) egységek kovalens kapcsolását jelenti egyrészt saját magára az enzimre, illetve célfehérjékre  $\text{NAD}^+$  felhasználása mellett. A PARP-1 "túlaktivációja"  $\text{NAD}^+$  szint csökkenéshez vezet, amely befolyásolja a mitokondriális elektron transzportot és amennyiben továbbra is fennáll, nekrotikus sejthalálhoz vezet. Harántcsíkolt izomszövet testedzés folyamán folyamatos oxidatív stimulus alatt áll. Vizsgálataink során kimutattuk a PARP-1 szerepét izomdifferenciáció során és összehasonlítottuk az oxidatív stressz hatását differenciálatlan és differenciált C2C12 egér sejtekben. Megfigyeltük, hogy differenciáció alatt a PARP-1 fehérje szintje csökkent, amely emelkedett oxidatív stressz rezisztenciával járt együtt a differenciált sejtekben amit LDH és MTT mérésével igazoltunk. Miotubulusok mitokondriális membrán potenciálja oxidatív körülmények között megtartott volt a nem differenciált sejtekhez képest, emelkedett bazális respiráció, ATP szintézis és maximális respirációs kapacitás mellett. A PARP-1 inhibitor PJ34 védő hatást mutatott a differenciált mioblasztokban oxidáns kezelésre, míg a miotubulusokban ez a hatás nem volt megfigyelhető. Ezeket a megfigyeléseket egy másik sejtvonalban, L6 patkány izomsejtben is kimutattuk. PARP-1 overexpressziója a C2C12 miotubulusokban oxidánsal szembeni érzékenység növekedésével járt.  $\text{H}_2\text{O}_2$  kezelés hatására egy korai fázisú (10 perc) mitokondriális, illetve egy késői fázisú (3-24 óra) sejtmagi PARP-1 aktivációt regisztráltunk PMA analízissel. Egy összehasonlító elemzést is elvégeztünk normál és az égés után különböző időpontban vett izom biopszia mintákból, amelyből azt a következtetést vontuk le, hogy a PARP-1 aktiváció leginkább a középső csoportot ("middle") érintette összehasonlítva a kontrol csoporttal. A PARiláció elsősorban magát a PARP-1 enzimet érintette 120 kDa-nál, de más fehérjék is érintettek voltak. A tanulmány fő célja az volt,

hogy megállapítsuk vajon rendelkezik-e védő hatással a propranolol. Kezelés hatására a PARiláció csökkent volt a középső ("middle") és bizonyos fokig a késői ("late") csoportban összehasonlítva a kezeletlen csoporttal. Annak érdekében, hogy megállapítsuk a propranolol hatásának mehanizmusát illetve a folyamat kapcsolatát a  $\beta$ -adrenoceptor jelátvitellel, U937 és C2C12 sejteket kezeltünk  $H_2O_2$ -dal és megállapítottuk, hogy a propranolol gátolta a PARilációs folyamatot.

Eredményeinket összefoglalva megállapíthatjuk, hogy (a) a PARP-1 fehérje szintjének a csökkenése egy védő hatást jelent a differenciált miotubulusoknak oxidatív stresszel szemben. (b) PARP-1 aktiváció oxidatív stimulus hatására egy korai, percekben mérhető, mitokondriális és egy késői, órákkal később lezajló, nukleáris, fázisokra bonthatóak. (c) Ez a PARP-1 aktiváció gátolható  $\beta$ -adrenoceptor agonista propranolollal amelynek (d) kedvező hatása van égett betegekben.

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## 11. List of publication related to the dissertation

**Olah G**, Szczesny B, Brunyánszki A, López-García IA, Gerö D, Radák Z, Szabo C. (2015) Differentiation-associated downregulation of poly(ADP-ribose) polymerase-1 expression in myoblasts serves to increase their resistance to oxidative stress. *PloS One*, 10: e0134227.

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