

Down-regulation of PARP by antisense and its effect on oxidative stress induced cell death in *D. discoideum*

7.1. INTRODUCTION

Poly(ADP-ribose) polymerase-1 (PARP-1) protects the genome by functioning in the DNA damage repair network. Gene disruption studies involving PARP have identified the various roles of PARP in cellular responses to DNA damage. *Parp*^{-/-} mice are resistant to DNA damage induced by reactive oxygen species which involve the contribution of PARP in cell death through NAD⁺ depletion (Masutani *et al.*, 2000). PARP is a DNA damage sensor which upon binding to damage sites triggers elongation and transfer of long, linear or branched chains of poly(ADP-ribose) (PAR) onto various nuclear acceptor proteins, including itself at the expense of NAD⁺ (de Murcia and de Murcia, 1994). PARP is also a mediator of cell death after ischemia, reperfusion injury and exposure to various DNA damaging agents (Wang *et al.*, 2002). PARP has been shown to promote caspase independent cell death via release of Apoptosis inducing factor (Yu *et al.*, 2002; Ame *et al.*, 2004). Recently role of PARP has also been identified in cytochrome c release during NMDA induced excitotoxicity (Duan *et al.*, 2007). Chemical inhibition of PARP during oxidative stress provides protection against cell death as described in previous chapters. The oxidant- and free-radical mediated necrosis of pancreatic β -cells, neurons, thymocytes and other cell types can be prevented by PARP inhibitors (Ha and Snyder, 1999).

D. discoideum possesses more than one type of PARP (Otto *et al.*, 2005) however as mentioned earlier, their catalytic domain is highly conserved (Fig. 7.1). Hence, in this study, the catalytic domain has been used as a target for the antisense RNA.

7.1.1. Strategy for targeted down-regulation of *adprt1A* encoding PARP

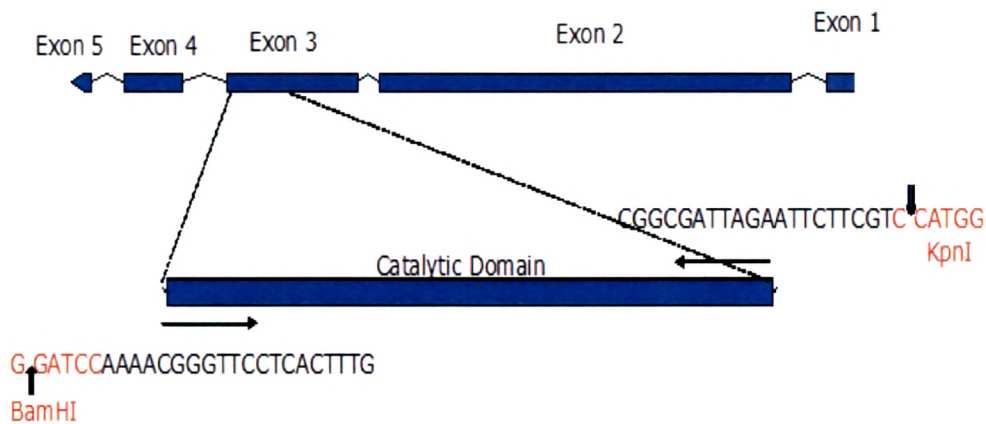
Our previous results suggest that under oxidative stress PARP gets activated within 5-10 minutes and induces downstream signalling for cell death which can be partially rescued by PARP inhibition. To support these results and to rule out any non specific effect of PARP inhibitor, benzamide, we made an attempt in this study to specifically down-regulate PARP by antisense and to check its effect on oxidative stress induced cell death and development.

ATGGCAACAAAAATACATCTCCTTATGAGATTGAATATGCAAAGAGTGATAGATCAACATGTTCAACC
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 CCTCACAAAGATGGTAATCATCTCTTTCTGATTCTGATGGTTTAGTAGTACCATTAGGTAAAATTTCAA
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 TACATCTTAAGAGTTTACAATGGAAAATAA

Figure 7.1A: *adprt1A* gene sequence of *D. discoideum*. Blue highlighted region depicts a part of the conserved catalytic domain.

As predicted from the domain information (<http://dictybase.org>; <http://www.expasy.ch/tools>), the conserved catalytic domain of *adprt1A* gene spans over exon 3 and exon 4. However, only a particular region that resides between the residues 725 to 937 bp of the gene has been used as template to target and design the antisense. This targeted catalytic domain can be mapped on exon 3 of the *adprt1A* gene (Fig. 7.1A).

Primers were designed so as to amplify the exon 3 region of *adprt1A* comprising of sequence corresponding to the catalytic domain. It was done with the help of online software, Primer 3 as well as manually. Two restriction sites (*viz.* *KpnI* and *BamHI*) were incorporated within the primers to facilitate directional cloning of the amplicon so as to obtain antisense copy of the sequence for the targeted catalytic domain. These restriction sites were selected based on the convenience to clone directionally in the vectors of choice (Figure 7.1C). The predicted amplicon sequence (500 bp) was pre-checked for the absence of internal recognition sites for these two restriction endonucleases with the help of an online tool Restriction mapper (<http://www.restrictionmapper.org>). Figure 7.1B below clearly depicts the region on the gene targeted for the construction of antisense.



LEFT PRIMER (L1) AAAACGGGTCCTCACTTTG

RIGHT PRIMER (R1) CGGCGATTAGAATTCTTCGT

Expected amplicon size: 500 base pair

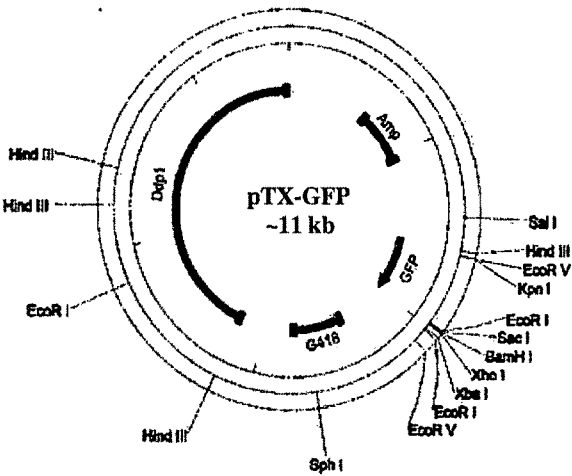
Figure 7.1B: Schematic representation of the strategy for antisense construction

(i)

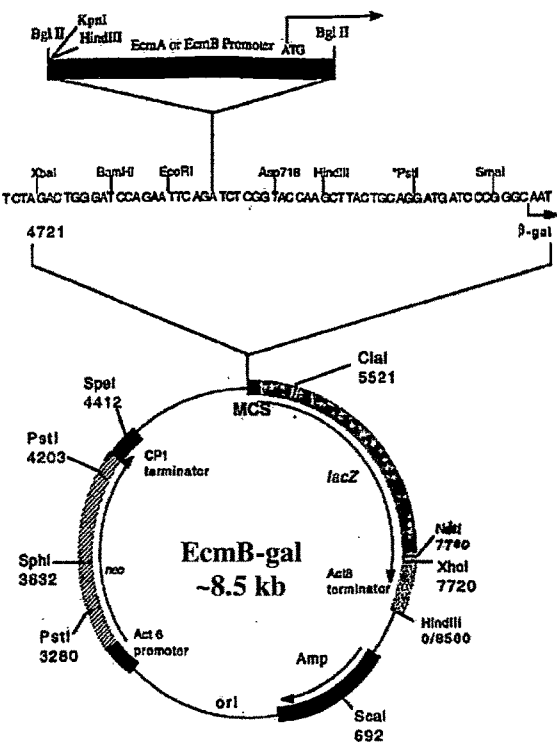
pTX-GFP

[Procured from dictybase]
(<http://dictybase.org>)

- GFP expression under control of the actin15 promoter
- Size ~11.0 kb
- Constitutive expression
- MES flanking GFP
- Genitacin & ampicillin resistance as selectable markers.



(ii)



EcmB-gal

- *gal* expression under control of the EcmB promoter (stalk cell specific)
- Size ~8.5 kb
- Stage specific expression
- Genitacin & ampicillin resistance as selectable markers.

Figure 7.1C: Restriction maps of the plasmid vectors used. (i) pTX-GFP (ii) EcmB-gal

7.2. RESULTS

7.2.1. Standardization of plasmid DNA transformation in *D. discoideum*

Transformation of *D. discoideum* cells was standardized by electroporation method using pTX-GFP which was confirmed by monitoring green fluorescence from cells (Fig. 7.2). Fluorescence was even observed from the spores during developmental phases of life cycle (Fig. 7.3).

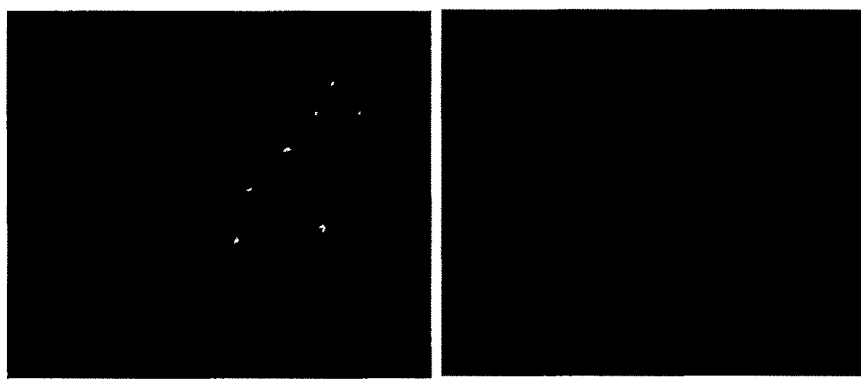


Figure 7.2: *D. discoideum* cells transformed with pTX-GFP. Cells in vegetative phase observed under phase contrast and fluorescence microscope showing green fluorescence of GFP.

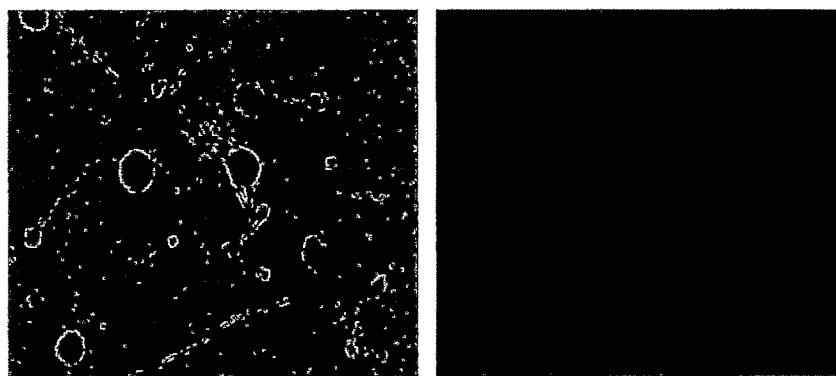


Figure 7.3: Development of *D. discoideum* transformed with pTX-GFP. Cells in sporulating phase observed under phase contrast and fluorescence microscope with spores showing green fluorescence due to GFP.

7.2.2. Cloning of PARP antisense

DNA from a log phase culture of *D. discoideum* was extracted as mentioned earlier. It was then further used as a template for the PCR reaction in order to amplify the catalytic domain of PARP using primer L1R1 (PCR conditions as mentioned in Materials & methods) and the resultant 500 bp amplicon is shown in Fig. 7.4. The confirmed amplicon was subjected to gel elution and subsequently purified DNA fragment was ligated to purified *EcoRV* digested pBluescript (pBKS) plasmid. The ligation mixture was transformed in *E. coli DH5α* and the transformant colonies were subjected to blue white selection on Luria agar containing ampicillin antibiotic. Randomly selected white colonies were screened for the presence of recombinant plasmid which was confirmed by restriction digestion pattern (confirmatory gel pictures not included). This recombinant plasmid was used as an intermediate plasmid for further cloning in the target vectors.

For cloning in pTX-GFP, the intermediate recombinant plasmid was digested with *KpnI* and *BamHI* consequently and the released 500 bp amplicon was purified. On the other hand, pTX-GFP plasmid when digested with *KpnI* and *BamHI* released about 750 bp fragment containing GFP gene (Fig. 7.5) while the remaining backbone of the plasmid was gel eluted and purified. Subsequently the purified 500 bp amplicon obtained from the intermediate plasmid and the purified pTX-GFP backbone, were ligated under appropriate conditions. The ligation mixture was used to transform *E. coli DH5α* and of the ampicillin resistant colonies obtained, randomly selected ones were screened for the presence of PARP antisense inserts by colony PCR (Fig. 7.6). The desired amplicon was obtained in 5 colonies of which one was randomly selected and further confirmed by relevant restriction enzyme digestion patterns (Fig. 7.7). The confirmed clone containing PARP antisense, pTx-PARP, was used for transformation of *D. discoideum* cells.

Similar strategy was also followed for cloning PARP antisense in stage specific vector EcmB-gal using *SmaI* and *XhoI* enzymes. Clones obtained were screened by relevant restriction enzyme digestion (Fig. 7.8) and confirmed by PCR and was named as EcmB-PARP. The confirmed clones i.e., pTx-PARP and EcmB-PARP were independently used to generate *D. discoideum* transformants with constitutive and inducible down-regulation of PARP, respectively.

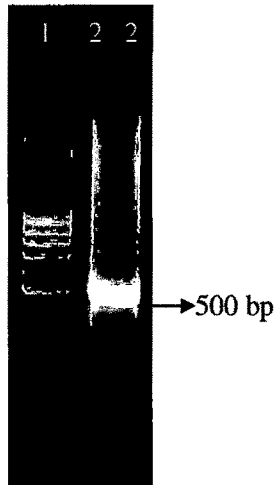


Figure 7.4: PCR amplification of PARP catalytic domain region. An amplicon of 500 bp was obtained after PCR with primers corresponding to exon 3 region of catalytic domain of PARP. Lane 1: PARP amplicon; Lane 2: 100bp ladder

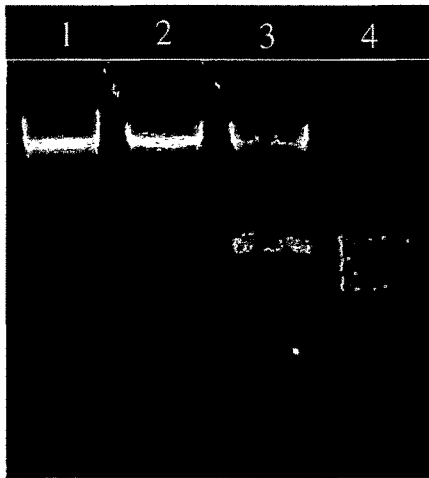


Figure 7.5: pTx-GFP digestion with *KpnI* and *BamHI*. Lane 1: *KpnI* cut pTx-GFP; Lane 2: *BamHI* cut pTx-GFP; Lane 3: *KpnI*/ *BamHI* cut pTx-GFP; Lane 4: 100bp ladder

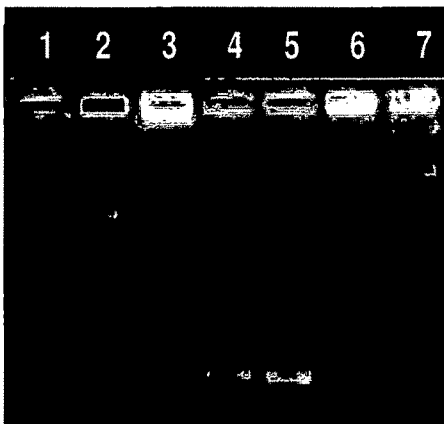


Figure 7.6: Colony PCR of *E.coli* transformants. Lanes 3-5 showed PCR amplification (500bp) indicating the presence of plasmid containing desired insert. Lane 1: PARP amplicon; Lane 3-5: colony PCR (positive); Lane 6-7: colony PCR (negative).

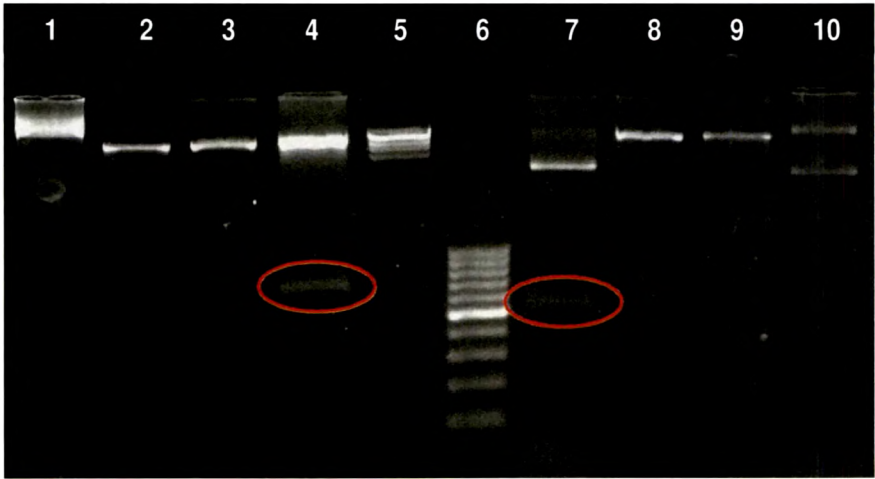


Figure 7.7: Restriction digestion pattern for pTx-GFP and pTx-PARP. pTx-GFP plasmid showed release of 750 bp corresponding to GFP. pTx-PARP showed the release of ~550 bp confirming the antisense of PARP. Lane1: Undigested pTX-GFP; Lane 2: *KpnI* cut pTX-GFP; Lane 3: *BamHI* cut pTX-GFP; Lane 4: *KpnI/BamHI* cut pTX-GFP; Lane 5: λ DNA cut with HindIII-marker; Lane 6: 100 bp ladder; Lane 7: *KpnI/BamHI* cut pTx-PARP; Lane 8: *BamHI* cut pTX-PARP; Lane 9: *KpnI* cut pTX-PARP; Lane 10: Undigested pTx-PARP

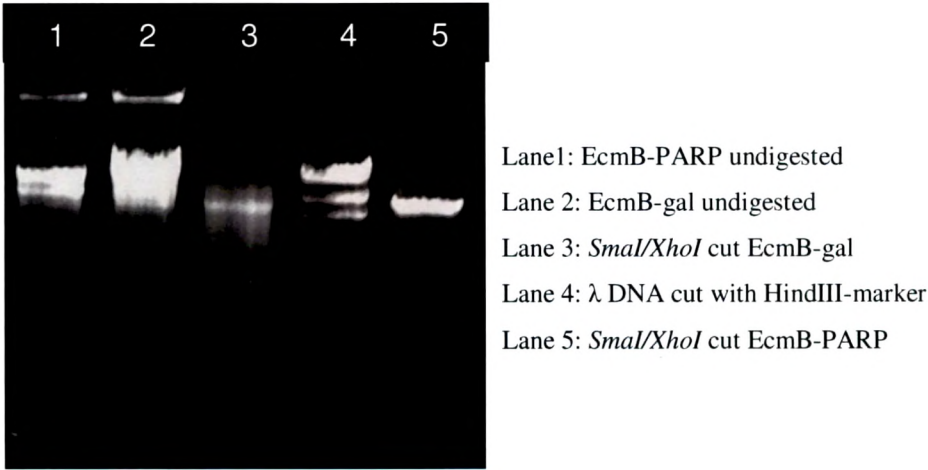


Figure 7.8: Construction of ecmB-PARP containing PARP antisense in stage specific vector ecmB. EcmB-gal plasmid showed release of ~2kb corresponding to gal. EcmB-PARP showed the release of ~500 bp confirming the antisense of PARP.

7.2.3. Functional characterization of PARP antisense

Total RNA was isolated from PARP down-regulated *D. discoideum* cells (Fig. 7.9 A) and PARP down-regulation was confirmed by monitoring gene specific expression of PARP by RT-PCR. It was found that PARP mRNA transcript was reduced by 60% (Fig. 7.9 B & C). Following this the PARP activity was checked in these PARP down-regulated cells, which was also found to be lower than basal activity (Fig. 7.10). These results correlated well with the observed reduction in the PARP transcripts.

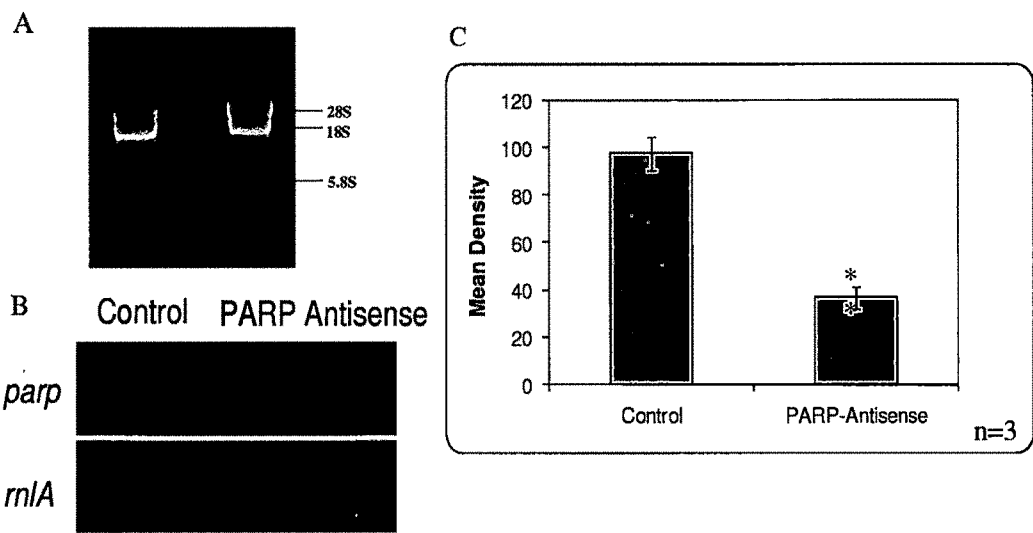


Figure 7.9: (A) RNA isolated from *D. discoideum* cells (B) RT PCR of PARP down-regulated *D. discoideum*. Gene specific expression of *parp* exhibits ~60% reduction while no change was observed with internal control *mlA*. (C) Densitometric analysis of the expression.

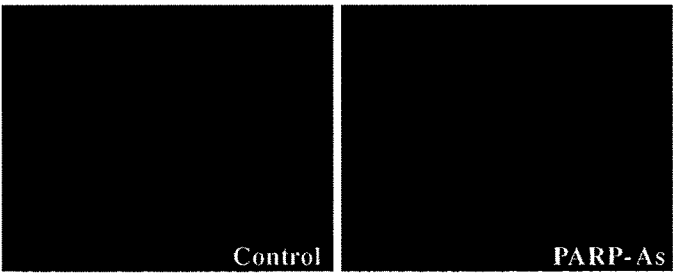


Figure 7.10: PARP activation monitored in PARP down-regulated *D. discoideum* cells. PARP activity in down regulated cells was found to be reduced compared to the control.

7.2.4. Effect of PARP down-regulation on growth and development of *D. discoideum*

PARP down-regulation did not show any effect on growth of the unicellular amoeba (Fig. 7.11) but interestingly when these cells were subjected to starvation, development was blocked at aggregation stage as observed after 12 hours (Fig. 7.12). PARP down-regulated cells did not enter further development till one week. Moreover, stage specific PARP down-regulation in *D. discoideum* cells, arrested the development at slug stage (observed after 48 hours) signifying the involvement of PARP during development at different stages of differentiation (Fig. 7.13). Our results highlight the role of PARP in multicellularity as no effect was observed on unicellular amoebae.

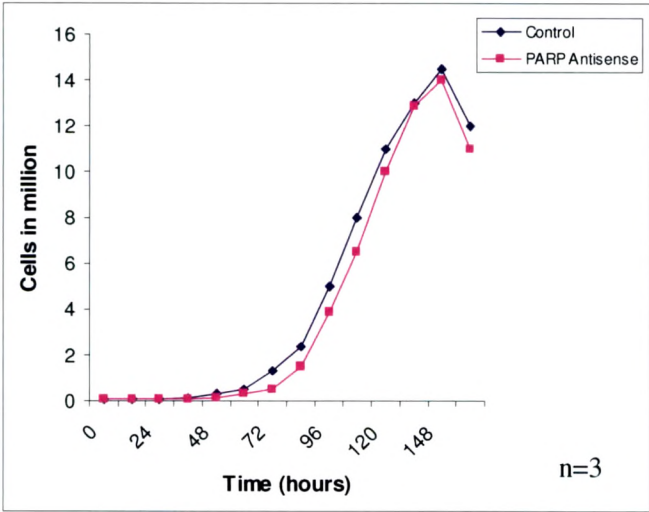


Figure 7.11: Growth curve of PARP down-regulated *D. discoideum* cells. Growth in PARP down-regulated cells was comparable to control. Results are mean of three independent experiments \pm SE.

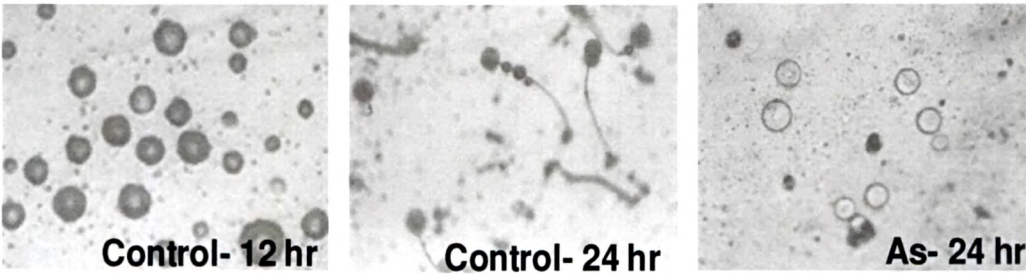


Figure 7.12: Developmental arrest in *D. discoideum* cells with constitutive down-regulation of PARP. The development was monitored after 24 hours. The photographs have been taken under 60X objective.

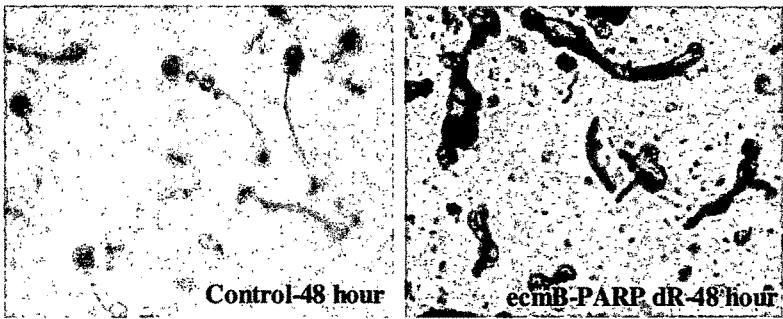
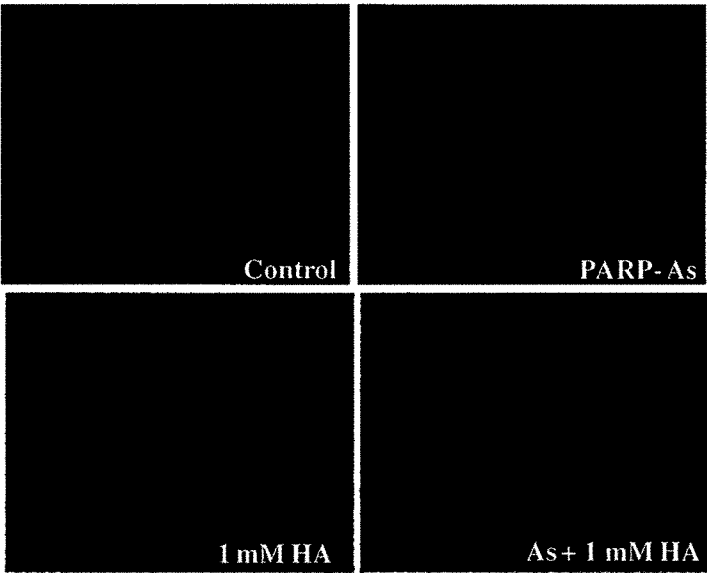


Figure 7.13: Developmental arrest in *D. discoideum* cells with stage specific down-regulation of PARP. The development was monitored after 48 hours. The photographs have been taken under 60X objective

7.2.5. Effect of constitutive PARP down-regulation on oxidative stress induced cell death

The effect of antisense mediated PARP down-regulation was evaluated on oxidative stress induced cell death. PARP activity *in situ* was evaluated by assaying PAR levels after inducing PARP activation with oxidative stress. PARP activity was substantially less in PARP down-regulated cells. PAR immunoreactivity peaked at 10 minutes with 1 mM HA stress indicating increased PARP activity. However, PARP antisense partially prevented the increase in PARP activity in the presence of 1 mM HA (Fig. 7.14).

(A)



PARP down-regulation and development

(B)

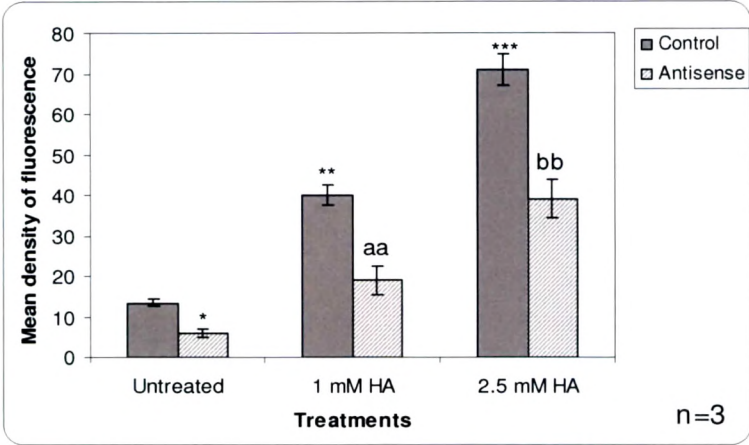
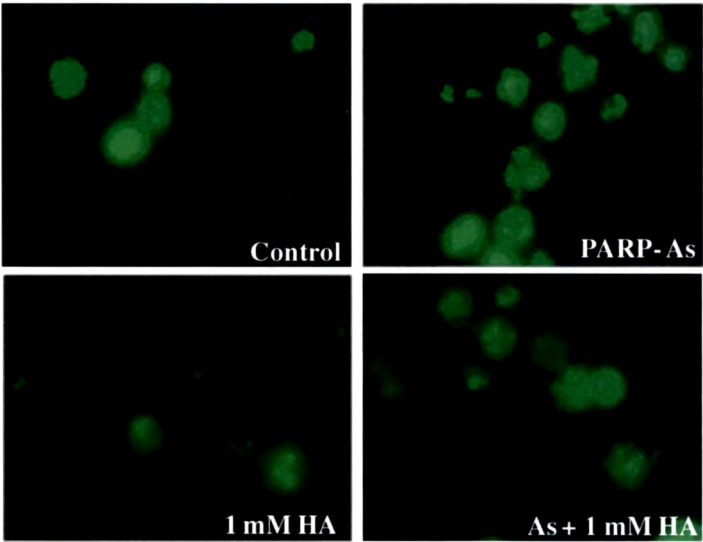


Figure 7.14: PARP activation in oxidative stress induced and PARP down-regulated *D. discoideum* cells. Results are the mean of three independent experiments \pm SE. *** p value <0.001 , ** p value <0.01 compared to control; aa, bb p value <0.01 compared to respective treatments.

Moreover, MMP changes induced by oxidative stress were also found to be partially intercepted by PARP down-regulation (Fig. 7.15). The magnitude of this effect mediated by PARP antisense was comparable with PARP inhibitor, benzamide.

(A)



PARP down-regulation and development

(B)

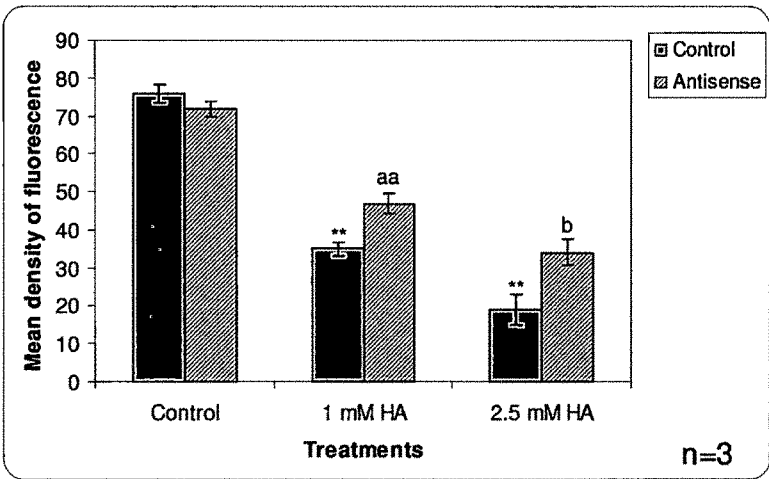


Figure 7.15: MMP changes in oxidative stress induced and PARP down-regulated *D. discoideum* cells. Results are the mean of three independent experiments \pm SE. ** p value <0.01 compared to control; aa p value <0.01 , b p value <0.05 compared to respective treatments.

Consistent with the reduced PARP activity, PARP down-regulated *D. discoideum* cells also showed reduced cell death with 1 mM HA. 1 mM HA exhibited PS exposure at 5 hours and PI staining at 12 hours while PARP down-regulation delayed the PS exposure up to 12 hours and prevented loss of membrane integrity (Fig. 7.16). Thus, reduced PARP activity contributes to reduced or delayed cell death.



Figure 7.16: Monitoring cell death by PS-PI dual staining. At 12 hours only PS exposure was observed with PARP down-regulation during paraptosis. Data are representative of at least three independent experiments. Photographs were taken with 60X objective.

7.3. DISCUSSION

Interesting observation that cells do exhibit alternate pathways to undergo cell death which are caspase independent has evoked interest on PARP and its role in these alternate pathways of cell death. Much interest has been emerging to understand the precise mechanism by which PARP mediates genome stabilization and protection against damage, as well as its involvement in different types of cell death. To study the function of PARP, PARP inhibitors are extensively used but these inhibitors have been reported to have nonspecific effects on other metabolites. This led to the use of molecular genetic approaches for modulation of poly ADP-ribosylation in living cells. In the current study PARP expression is down-regulated using antisense approach and we have established for the first time *D. discoideum* as a model for the constitutive as well as stage specific inducible antisense of PARP.

The presence of a DNA strand break/damage triggers localized PARP activation at the damaged site, which in turn leads to the ADP ribosylation of histones in addition of auto modification of PARP (D'Amours *et al.*, 1999). These effects facilitate DNA repair by promoting localized relaxation of chromatin (de Murcia *et al.*, 1997). Modification of a target by PARP is a rapid response capable of altering protein activity and/or stability (Jagtap and Szabo, 2005; Kim *et al.*, 2005). Accordingly, PARP plays an integral role in the cellular response to a variety of stress conditions, most notably DNA damage (Ame *et al.*, 2004). PARP-1 double knockout mice resulted in embryonic lethality (Henrie *et al.*, 2003). Parp(-/-) mice are more sensitive to the lethal effects of DNA damaging agents (Masutani *et al.*, 2000) as PARP is involved in DNA repair. Mice lacking PARP exhibit diverse phenotypes such as growth retardation, aberrant apoptosis and increased sensitivity to DNA damaging agents (de Murcia *et al.*, 1997). Cells that are depleted of PARP using antisense constructs exhibit changes in DNA topology (Ding and Smulson, 1994). This may facilitate nuclear changes during differentiation, or transient changes in PARP expression may facilitate altered organization of the chromatin. The extent of PARP activation is thought to play a decisive role in the regulation of cell death also. Persistent PARP activation may signal the presence of irreparable DNA damage. Among other effects, PARP has been shown to promote caspase independent cell death by inducing the release of the proapoptotic protein, apoptosis inducing factor (AIF) from mitochondria (Yu *et al.*, 2002; Ame *et al.*, 2004).

PARP in responses to DNA damage and cellular stress

PARP-1 knockout can prevent energy depletion and hence increased viability of the cells exposed to oxidative stress (Watson *et al.*, 1995). In higher organisms PARP-1 and PARP-2 double knockouts being lethal due to loss of DNA repair function (Henrie *et al.*, 2003). In view to these reports we have made an attempt to down-regulate PARP by antisense with respect to dissecting the role of PARP. For the first time our lab has reported that oxidative stress induced paraptotic cell death in *D. discoideum* is mediated by PARP (Rajawat *et al.*, 2007). Down-regulation of PARP was confirmed by gene expression analysis using RT-PCR and our results showed ~60% reduction in PARP mRNA levels (Fig. 7.9). PARP activity by indirect immunofluorescence also suggests a 2 fold decrease in PARP activity compared to basal level (Fig. 7.10). PARP downregulated *D. discoideum* cells were exposed to H₂O₂/HA and the effect on cell death was monitored. PARP downregulated cells showed reduced peak PARP activity when subjected to paraptotic and necrotic doses of H₂O₂/HA oxidants compared to control cells (Fig. 7.14). MMP changes were also partially prevented by PARP inhibition (Fig. 7.15). PS exposure monitored at 5 hours with paraptotic dose of H₂O₂/HA was delayed by 12 hours and PI was not seen till 12 hours suggesting a delay in paraptotic cell death (Fig. 7.16). Our results on PARP down-regulation were compared to our benzamide results. PARP inhibition prevented cell death by 50% while 60% rescue was observed with PARP down-regulation.

D. discoideum, a protist that emerged in evolution after plants and from an ancestor common to fungi and animals (Baldauf *et al.*, 2000) shows developmental cell death. PARP inhibition by antisense confers survival advantage to oxidative stress exposed endothelial cells by maintaining cellular energy levels and activating prosurvival pathway (Matthews and Burk, 2008). Our PARP inhibition by benzamide suggested that oxidative stress induced paraptotic and necrotic cell death is PARP mediated. Our antisense results also suggest that PARP down-regulation has delayed the oxidative stress induced paraptotic cell death. These results also suggest that *D. discoideum* necrotic cell death that occurs in orchestrated manner *via* PARP overactivation is also shifted to paraptotic type in PARP down-regulated cells. Thus we have demonstrated that *D. discoideum* is an excellent model to dissect out and study the molecular mechanisms of PARP mediated paraptotic and necrotic cell death.

Role of PARP in *D. discoideum* development

The role of PARP is majorly established as NAD⁺ dependent modifying enzyme that mediates important steps in DNA repair, transcription, and apoptosis, but its role during development is poorly understood. There are only few reports which portray its role in development. PARP deletion mutants in *Drosophila* fail to develop beyond larval stages due to defects in chromatin remodeling and regulation of gene expression (Tulin and Spradling, 2003). In mouse PARP-1 and PARP-2 double knockouts exhibit embryonic lethality (Henrie *et al.*, 2003). Another interesting fact about PARP is its occurrence in all multicellular organism but not the unicellular forms like prokaryotes and yeast. This makes *D. discoideum* an excellent model system to study the role of PARP in development, being at the point of transition from unicellular to true multicellular forms.

D. discoideum multiplies as a unicellular microorganism when food is abundant, but undergoes development on starvation; cells aggregate and differentiate, morphogenesis leads first to a migrating slug, then to a fruiting body with a mass of spores at the tip of a stalk composed of dead cells (Whittingham and Raper, 1960). *D. discoideum* developmental cell death can occur in the absence of any member of the caspase family, making a constitutive link throughout evolution between this caspase family and programmed cell death unlikely. So far there are no reports which throw light on the protein/s involved in mediating *D. discoideum* paraptotic cell death.

As the paraptotic cell death in unicellular stage of *D. discoideum* is found to be PARP mediated, we were interested to explore the involvement of PARP during its development also. PARP down-regulated cells when subjected to starvation interestingly failed to develop beyond loose aggregation stage (Fig. 7.12). PARP may be regulating certain key proteins of development. There could be two possibilities for the arrested development i.e., (1) PARP activity per se is required for transition from one stage to another or (2) PARP may play a role in the regulation of developmental gene expression perhaps by interacting with the promoters of these genes or by poly ADP-ribosylation of certain transcription factors. Our results on prestalk stage specific down-regulation of PARP showed arrested development at the slug stage (Fig. 7.13). This accentuates that PARP induces stalk cell death in *D. discoideum* and thus opens up the possibility to

further elucidate the role of PARP in its development. Because sequence homology is shared between *D. discoideum* and other metazoans (Otto *et al.*, 2005) it seems that at least nonapoptotic (paraptotic) function of PARP is conserved in both *D. discoideum* and metazoans.

Our results also suggest that presence of PARP in multicellular organisms may be linked to multicellularity. PARPs have been identified throughout the animal and plant kingdoms, with the catalytic domain exhibiting the greatest degree of sequence similarity. PARP is present in all types of eukaryotic cells with the notable exception of yeast, in which the expression of human PARP-1 was shown to lead to retarded cell growth (Kaiser *et al.*, 1992). It has also been found that a *Neurospora* PARP orthologue is indispensable for cell survival and PARPs may regulate aging by affecting NAD⁺ availability, thereby influencing Sirtuin activity, or they may function alternatively in NAD⁺ independent aging pathways (Kothe *et al.*, 2010). A single PARP homolog (*prpA*) has also been reported in *Aspergillus nidulans* which is conserved in all filamentous fungi and is closely related to PARP-1. *Aspergillus nidulans* PARP ortholog (PrpA) revealed that the protein is essential and involved in DNA repair, reminiscent of findings using mammalian systems. Strain heterologous for *prpA* gene replacement exhibits phenotypic defects in spore formation and possessed a pronounced fluffy phenotype caused by the inability to show asexual development. *prpA* over expression in *A. nidulans* suggests that proper regulation of PARP activity is required for conidiation (Semighini *et al.*, 2006).

In our studies PARP down-regulated *D. discoideum* cells get arrested at loose aggregation stage when subjected to development however, no effect was observed on the growth of unicellular *D. discoideum* (Fig. 7.11). These results support the idea that complex development and differentiation in filamentous fungi and *D. discoideum* may require additional programmed cell death pathways or components not found in yeast. Presence of PARP in *D. discoideum* and *A. nidulans* (filamentous fungi) signifies its role in multicellularity. However, further studies are needed to confirm the link between PARP and multicellularity.

In the light of our results we propose that PARP plays an important role in multicellularity plausibly by regulating the developmental processes. *D. discoideum*

being at the transition stage of multicellularity possess PARP whereas unicellular fungi *S. cerevisiae* and *S. pombe* do not possess PARP. This new finding will undoubtedly influence our perception in developmental cell death in higher complex organisms including humans.

7.4. REFERENCES

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