Chapter 6:

Gene expression analysis of *ADPRT1A* and developmental genes involved in *D. discoideum* development

Gene expression analysis of developmental genes involved in *D. discoideum* development

6.1 Introduction

Cell differentiation and survival during developmental processes involve the action of a large network of inducible genes. However, the compact eukaryotic chromatin provides a hurdle in this process, thus hampering access to the transcriptional machinery. This problem is resolved by chromatin modifiers which work in conjunction with cellular signals thereby resolving the complex packaging and in turn controlling gene expression. One such well reported chromatin modifier is Poly (ADP-ribose) polymerase 1 (PARP-1), a nuclear enzyme belonging to the PARP or ADP-ribosyl transferase (ART) family of proteins (D'Amours *et al*., 1999). It is established majorly as a DNA damage response protein (D'Amours *et al*., 1999). PARP-1 on activation can modify itself and other chromatin-associated proteins, thereby enhancing or repressing gene transcription (Beneke, 2012). However, emerging reports indicate that basal PARP-1 activity in normal cells is a crucial part of gene regulation during development (Ji and Tulin, 2010; Jubin *et al*., 2016b). Drosophila PARP has been shown to act in ectodermal specification and neural crest development in Zebrafish (Rouleau *et al*., 2011). In addition, PARP is also reported to impact the timely expression and silencing of both euchromatic and heterochromatic sequences during Drosophila development (Tulin and Spradling, 2003). Moreover, PARP-1 knockout mice also demonstrated altered gene expression of some cytoskeletal elements (Simbulan-Rosenthal *et al*., 2000). Recently, Hamazaki *et al*., (2015) have showed that there is an inhibition of DNA demethylation of the *IL17d* promoter region, on inhibiting PARP at the 2-cell stage leading to downregulation of target mRNA which are essential for early embryogenesis. Accumulating evidences strengths the importance of PARP as a transcriptional co-activator (Cervellera and Sala, 2000) and PARP may regulate the expression of a few genes involved in determining tissue polarity or cytoskeleton in Drosophila. Adding to this, PARP has also shown to be involved in stress responses like oxidative stress via proteases, UV-C stress via nitric

oxide and DNA repair in *D. discoideum* (Couto *et al*., 2011; Rajawat *et al*., 2014 a, b; Mir *et al*., 2015).

D. discoideum, a unicellular eukaryote exhibits multicellularity upon starvation (Raper *et al*., 1984). During its life cycle, *D. discoideum* alternates among unicellular and multicellular modes of existence. Under nutrient starvation, cAMP acts not only as an intracellular secondary messenger but also as a secreted extracellular signal. The secreted cAMP causes around ten thousand *Dictyostelium* cells to chemotax and form multicellular aggregates (Konijn *et al*., 1969). *ADPRT1A* overexpression indicated a certain link between PARP-1 and growth and multicellularity in *Dictyostelium* while *ADPRT1A* KO demonstrated the role of ADPRT1A in cell growth *i.e* in both cell survival and cell death as well as in cAMP signaling for the developmental morphogenesis of *D. discoideum.* Also, the mechanisms involved in the transfer of environmental signals to intracellular signalling molecules and the generation of cAMP pulses by ADPRT1A is incompletely understood. The aggregate formed then forms a migrating slug and finally culminates into a fruiting body that consists of a slender dead stalk crowned by a cluster of viable spores covered in a spore case. Cells at the anterior of the slug, the 'prestalk cells', differentiate to eventually lead to the formation of the stalk of the fruiting body (20%) and the posterior 'prespore cells' form the spores (80%) (Raper, 1984; Williams, 2006). These cell types can be identified with a set of molecular markers namely *EcmA, EcmB* (prestalk specific) and *D19* (prespore specific) (Gaudet *et al*., 2008). The secreted cAMP binds to a G-protein coupled cAMP receptor A (cAR A) and activates adenylyl cyclase A (ACA). Upon activation, ACA catalyzes the conversion of ATP to cAMP, which is either secreted from the cell or used to initiate intracellular signaling processes. cAMP levels are controlled, in part, by phosphodiesterases, with Reg A clearing excess intracellular cAMP and the extracellular phosphodiesterases, PdsA and Pde4 doing likewise for the secreted cAMP (Shaulsky *et al*.,1996; Bader *et al*., 2006; Garcia *et al*., 2009). The organized generation of cAMP pulses and their relay is important for both initiating aggregation and chemotaxis (Gomer *et al*., 2011; Jaiswal *et al*., 2012).

In view of the above, the present study focuses on understanding the role of PARP-1 in transcription and *Dictyostelium* development.

6.2 Results

PARP down-regulation (*PARP dR*), *ADPRT1A* overexpression (OE) as well as *ADPRT1A* knockout (KO) affected aggregation stage during *Dictyostelium* development (Rajawat *et al*., 2011; Jubin *et al*., 2016a). As shown in Chapter 5, *ADPRT1A* KO led to delayed aggregation due to lower cAMP levels and defective chemotaxis. Thus, gene expression analysis of developmental genes involved during aggregation was done by Real Time PCR. We examined the extracellular and intracellular phosphodiesterases *PDSA* and *REGA* respectively, adenylyl cyclase *A* (*ACA*) and cAMP receptor (*CAR1*) during early stages of development from 0 to 8 hrs after the initiation of the developmental program.

6.2.1 Expression of *PDSA* in *ADPRT1A* KO, OE, PARP inhibition and *PARP* dR

cAMP is an essential signaling messenger in the multicellular development of *D. discoideum.* The extracellular cAMP that is secreted by the cells is regulated by a cyclic nucleotide phosphodiesterase (PDE) (Sucgang *et al.,* 1997). *PDSA is* involved in active phosphodiesterase production during *D. discoideum* development (Barra *et al.,* 1980). As we observed low cAMP levels in *ADPRT1A* KO, we hypothesize that higher levels of *PDSA* might lead to degradation of cAMP and hence transcript analysis was carried out in *ADPRT1A* KO, OE, PARP inhibition and *PARP* dR cells as shown in Fig 6.1-6.4.

Interestingly, *PDSA* levels were significantly lowered in *ADPRT1A* KO as compared to control cells throughout initial hours of starvation from 0-8 hrs. Even at the time point when loose aggregate formation (LA) was initiated, the *PDSA* levels were elevated ($p<0.05$) but it did not reach the levels comparable to control cells (Fig. 6.1).

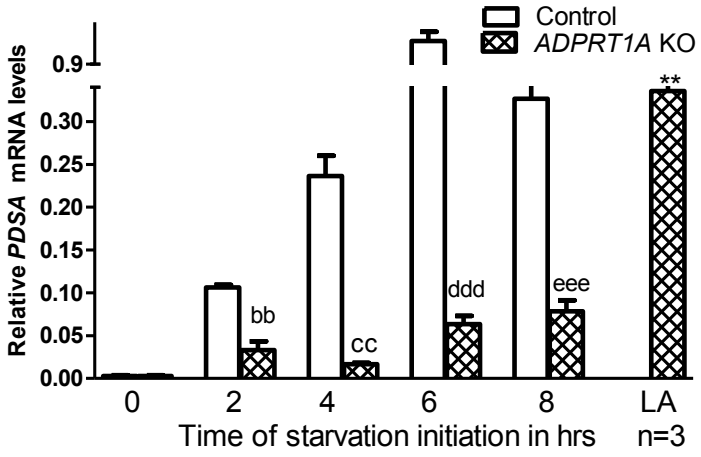


Fig. 6.1: Expression patterns of *PDSA* in *ADPRT1A* KO cells. Expression levels of *PDSA* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. $^{bb,\ cc}p$ value <0.01 as compared to control levels at 2 hrs and 4 hrs respectively. $^{ddd,\ eee}p$ value <0.001 as compared to control levels at 6 hrs and 8 hrs respectively. $^{**}p$ value < 0.01 as compared to control levels at 6 hrs. LA levels are compared with control mRNA levels at 6 hrs.

Fig. 6.2 shows decrease in *PDSA* transcript even in *ADPRT1A* OE cells. However, the mRNA levels were higher as compared to *ADPRT1A* KO cells. Also, levels of *PDSA* showed a rescue at time point of LA formation (p<0.01) in *ADPRT1A* OE which was higher as compared to *ADPRT1A* KO but not comparable to control cells. *PARP* dR cells showed similar transcript profile as *ADPRT1A* KO. However, no rescue was seen even at LA time point which explains stalled development in *PARP* dR. Conversely, PARP inhibited cells showed higher levels at 4 and 8 hrs or no change in *PDSA* levels.

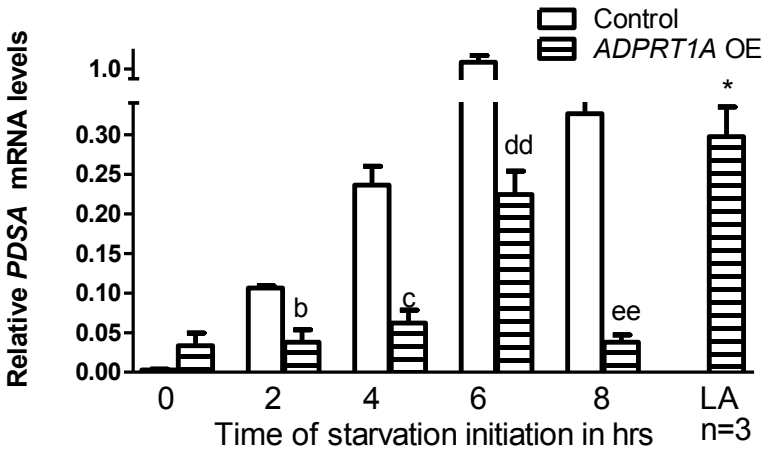


Fig. 6.2: Expression patterns of *PDSA* in *ADPRT1A* OE cells. Expression levels of *PDSA* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. $^{b,c}p$ value <0.05 as compared to control levels at 2 hrs and 4 hrs respectively. $^{dd,ee}p$ value <0.01 as compared to control levels at 6 hrs and 8 hrs respectively. $^{*}p$ value < 0.05 as compared to control levels at 6 hrs. LA levels are compared with control mRNA levels at 6 hrs.

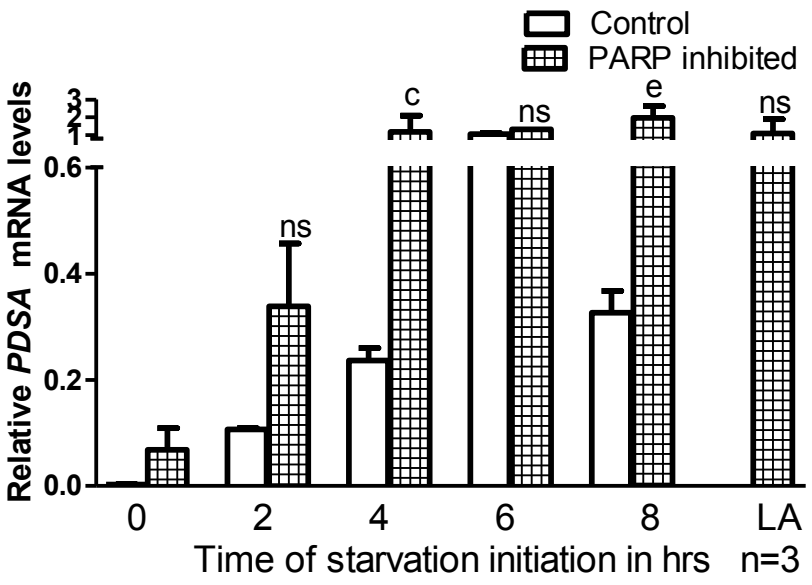


Fig. 6.3: Expression patterns of *PDSA* in PARP inhibited cells. Expression levels of *PDSA* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. $^{c,e}p$ value <0.05 as compared to control levels at 4 hrs and 8 hrs respectively. LA levels are compared with control mRNA levels at 6 hrs.

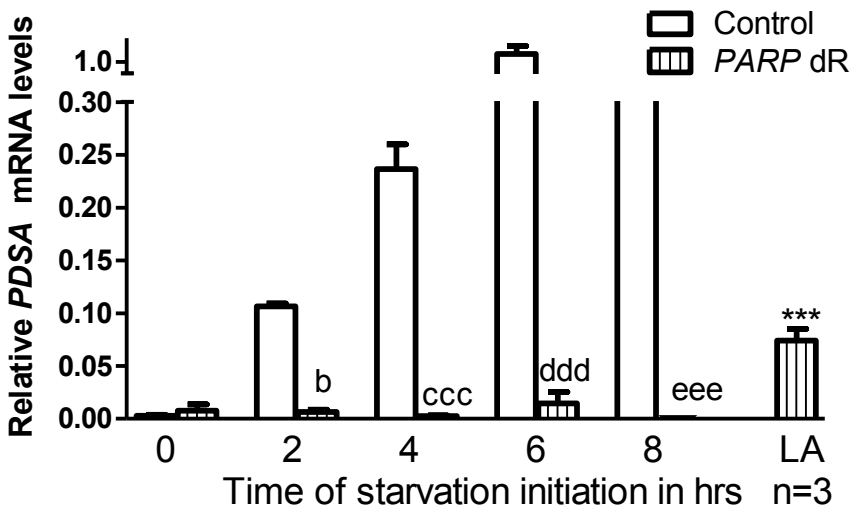


Fig. 6.4: Expression patterns of *PDSA* in *PARP* dR cells. Expression levels of *PDSA* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. ^{b}p value <0.05 as compared to control levels at 2 hrs, $^{ccc,ddd,eee}p$ value <0.001 as compared to control levels at 4 hrs, 6 hrs and 8 hrs respectively. $^{***}p$ value <0.001 as compared to control levels at 6 hrs. LA levels are compared with control mRNA levels at 6 hrs.

6.2.2 Expression of *REGA* in *ADPRT1A* KO, OE, PARP inhibition and *PARP* dR

REGA is a phosphodiesterase specific for degradation of intracellular cAMP (Thomason *et al*., 1999). Mutant *REGA Dictyostelium* strains wherein REGA is inactivated form small aggregates (Wessels *et al*., 2000). *REGA* levels in *ADPRT1A* KO and *PARP* dR were significantly decreased (Fig. 6.5 and 6.8). *ADPRT1A* KO also showed smaller aggregates as validated by reports on small aggregates in *REGA* mutants by Wessels *et al*., 2000. While *ADPRT1A* OE and PARP inhibited cells show higher *REGA* levels followed by comparable levels as compared to control at 6 hrs and 8 hrs respectively.

ADPRT1A OE and PARP inhibited cells show similar profile as overactivation led to higher auto ADP-ribosylation of ADPRT1A thus inactivating it which is similar to inhibition of PARP activity.

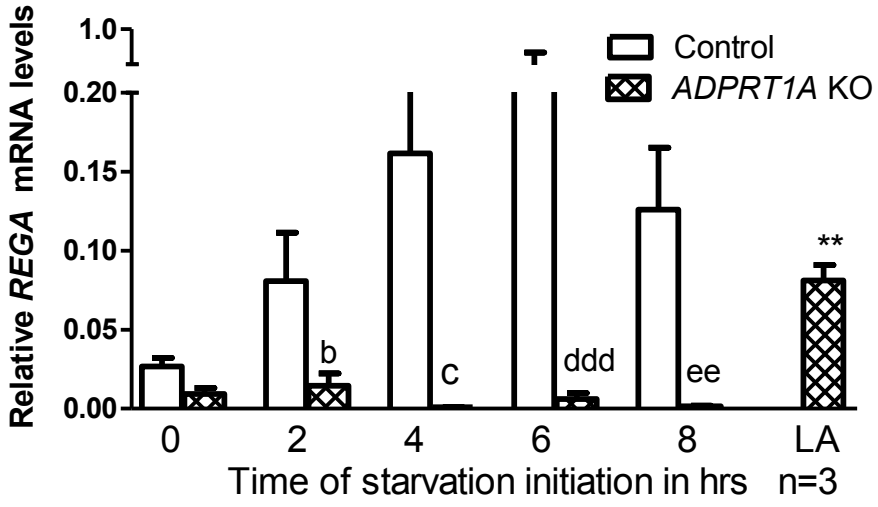


Fig. 6.5: Expression patterns of *REGA* in *ADPRT1A* KO cells. Expression levels of *REGA* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. $^{b,c}p$ value <0.05 as compared to control levels at 2 hrs and 4 hrs respectively. ^{ddd}p value <0.001 as compared to control levels at 6 hrs, ^{ee}p value <0.01 as compared to control levels at 8 hrs. $^{**}p$ value <0.01 as compared to control levels at 6 hrs. LA levels are compared with control mRNA levels at 6 hrs.

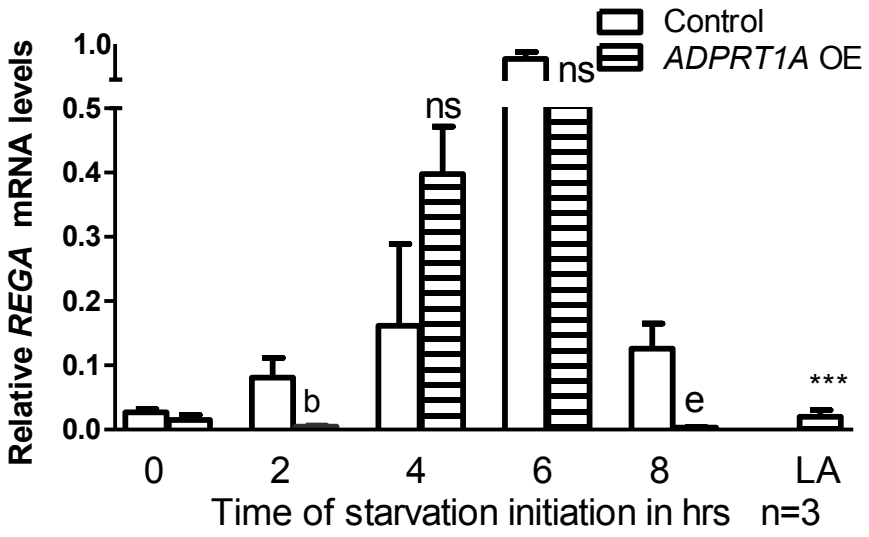


Fig. 6.6: Expression patterns of *REGA* in *ADPRT1A* OE cells. Expression levels of *REGA* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. ^{b}p value <0.05 as compared to control levels at 2 hrs, ^{e}p value <0.01 as compared to control levels at 8 hrs. $^{***}p$ value <0.001 as compared to control levels at 6 hrs. LA levels are compared with control mRNA levels at 6 hrs.

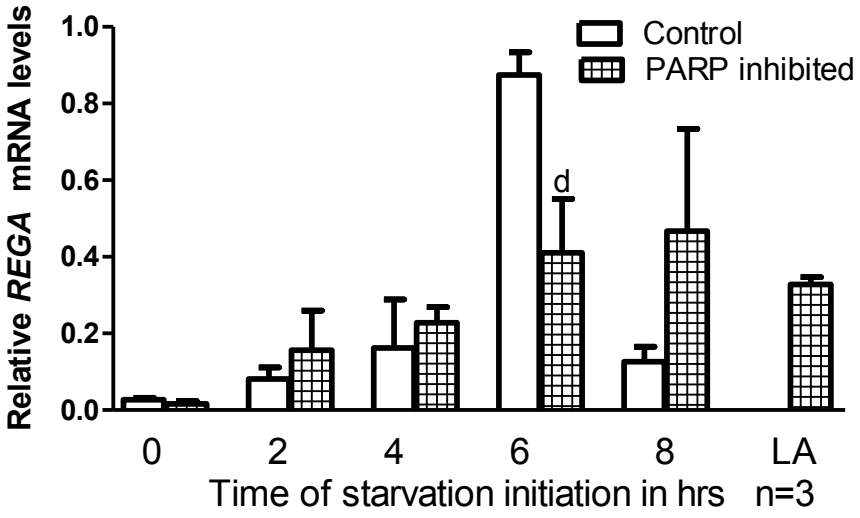


Fig. 6.7: Expression patterns of *REGA* in PARP inhibited cells. Expression levels of *REGA* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. LA levels are compared with control mRNA levels at 6 hrs.

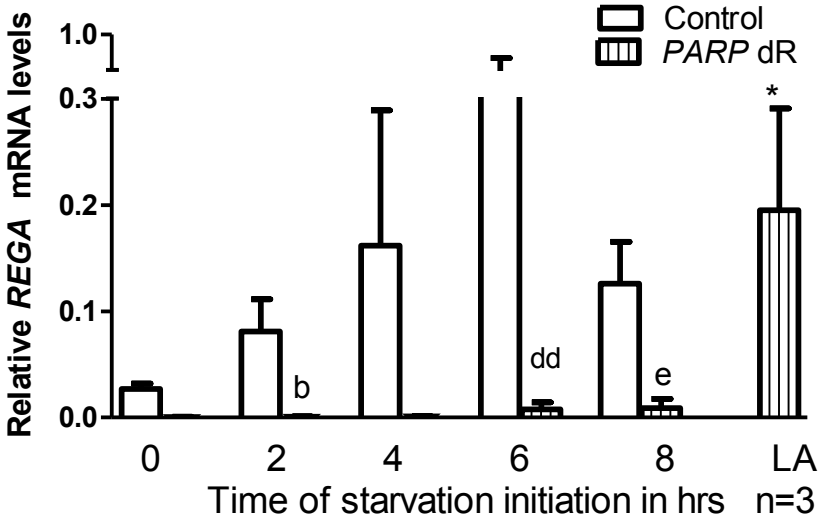


Fig. 6.8: Expression patterns of *REGA* in *PARP* dR cells. Expression levels of *REGA* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. $^{b,e}p$ value <0.05 as compared to control levels at 2 hrs and 8 hrs respectively, ^{dd}p value <0.01 as compared to control levels at 8 hrs. $^{*}p$ value <0.05 as compared to control levels at 6 hrs.LA levels are compared with control mRNA levels at 6 hrs.

6.2.3 Expression of *ACA* in *ADPRT1A* KO, OE, PARP inhibition and *PARP* dR

Out of three types of adenyl cyclases in *Dictyostelium*, the adenylyl cyclase expressed during aggregation, *ACA* is responsible for the synthesis of cAMP that is required for cell-cell signaling during early development (Pitt *et al*., 1992). *ADPRT1A* KO, PARP inhibited cells as well as *PARP* dR cells show significantly decreased *ACA* levels (Fig. 6.9, 6.11, 6.12). Also, all three mutants showed rescue in *ACA* levels at time point of LA formation. However, it was not comparable to control cells. These reduced *ACA* transcripts thus explain lower cAMP levels in *ADPRT1A* KO, PARP inhibited cells as

well as *PARP* dR cells as seen in previous chapter 5. *PARP* dR cells lesser rescue, further enlightening blocked development at loose aggregate stage during development (Fig. 6.12). On the contrary, *ADPRT1A* OE cells showed higher *ACA* (Fig. 6.10) which is taken care by *REGA* levels observed in Fig. 6.6.

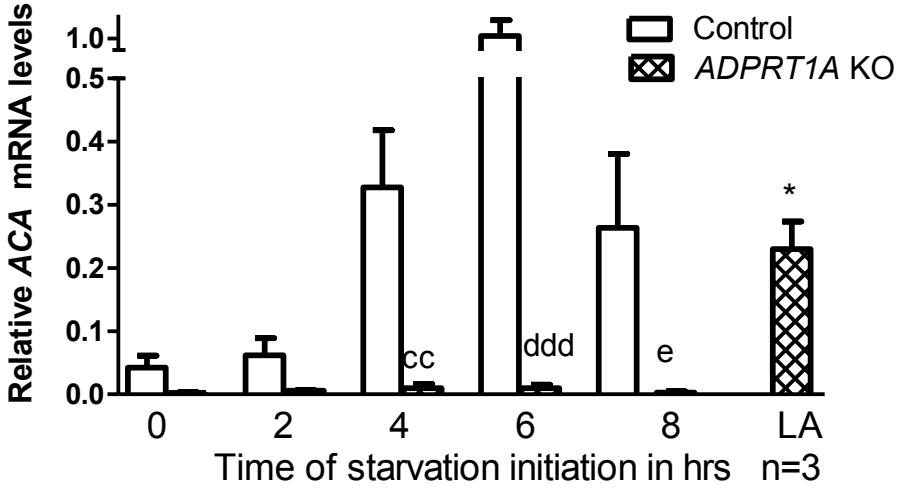


Fig. 6.9: Expression patterns of *ACA* in *ADPRT1A* KO cells. Expression levels of *ACA* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. ^{e}p value <0.05 as compared to control levels at 8 hrs, ^{e}p value <0.01 as compared to control levels at 8 hrs. $^{*}p$ value <0.05 as compared to control levels at 6 hrs.LA levels are compared with control mRNA levels at 6 hrs.

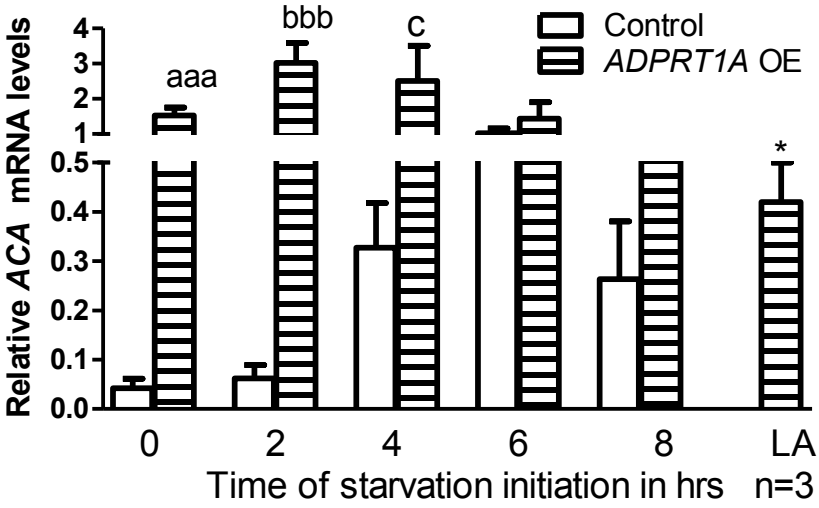


Fig. 6.10: Expression patterns of *ACA* in *ADPRT1A* OE cells. Expression levels of *ACA* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. ^{c}p value <0.05 as compared to control levels at 4 hrs, $^{aaa,bbb}p$ value <0.001 as compared to control levels at 2 hrs and 4 hrs respectively. $^{*}p$ value <0.05 as compared to control levels at 6 hrs.LA levels are compared with control mRNA levels at 6 hrs.

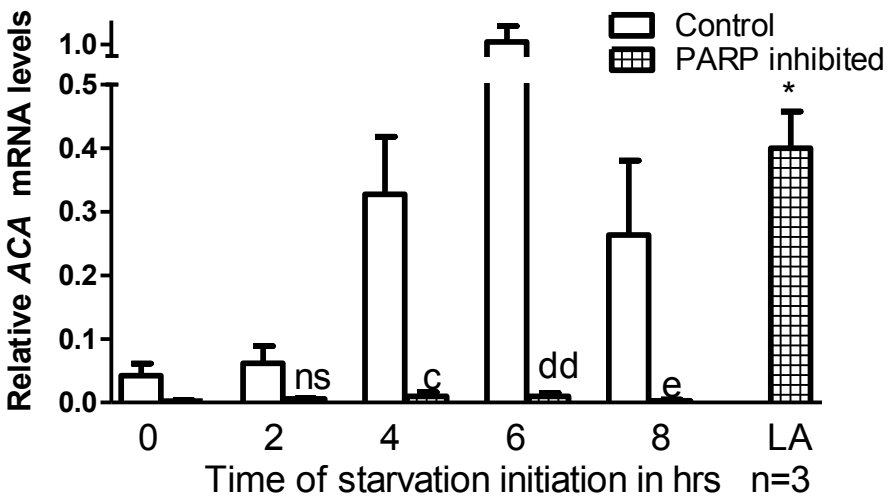


Fig. 6.11: Expression patterns of *ACA* in PARP inhibited cells. Expression levels of *ACA* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. $^{c,e}p$ value <0.05 as compared to control levels at 4 hrs and 8 hrs respectively, ^{dd}p value <0.01 as compared to control levels at 6 hrs. $^{*}p$ value <0.05 as compared to control levels at 6 hrs. LA levels are compared with control mRNA levels at 6 hrs.

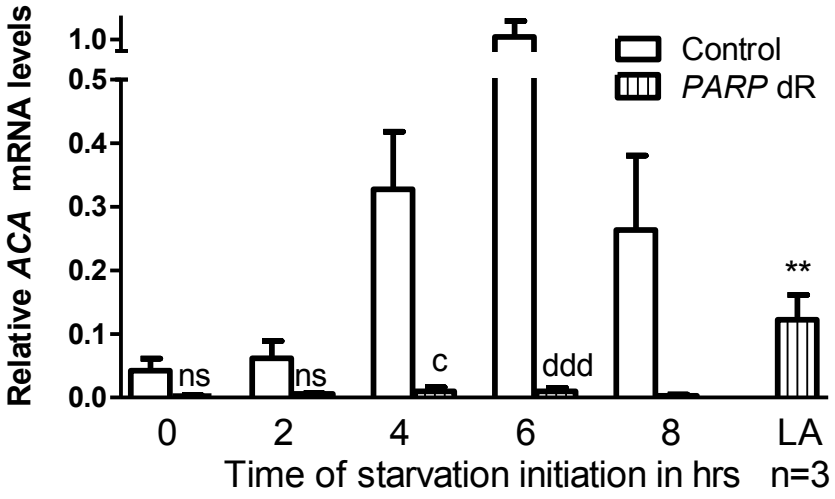


Fig. 6.12: Expression patterns of *ACA* in *PARP* dR cells. Expression levels of *ACA* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. ^{c}p value <0.05 as compared to control levels at 4 hrs, ^{ddd}p value <0.001 as compared to control levels at 6 hrs. $^{**}p$ value <0.01 as compared to control levels at 6 hrs. LA levels are compared with control mRNA levels at 6 hrs.

6.2.4 Expression of *CAR1* in *ADPRT1A* KO, OE, PARP inhibition and *PARP* dR

CAR1, a G-protein-linked cAMP receptor, plays a principal role in *Dictyostelium* development. CAR1 null mutants are unable to bind or sense cAMP and it also leads to arrest in early development (Sun and Devreotes, 1991). CAR1 mediates aggregation and the other cARs- *CAR2* and *CAR3* play roles in later developmental stages in *Dictyostelium* (Saxe *et al*., 1990). Thus, we hypothesized that *CAR1* downregulation might be a reason for the defect in chemotaxis and low cAMP levels too. Fig. 6.13 supports our hypothesis wherein *CAR1* transcripts were significantly downregulated in

ADPRT1A KO thus validating the defective chemotactic phenotype of *ADPRT1A* KO and also the delay in development was supported by the rescue in *CAR1* mRNA. Fig. 6.14-6.16 demonstrate downregulation of *CAR1* under *ADPRT1A* OE, PARP inhibited and *PARP* dR cells. *PARP* dR cells showed no rescue in *CAR1* levels further validating blocked aggregation stage (Fig. 6.16).

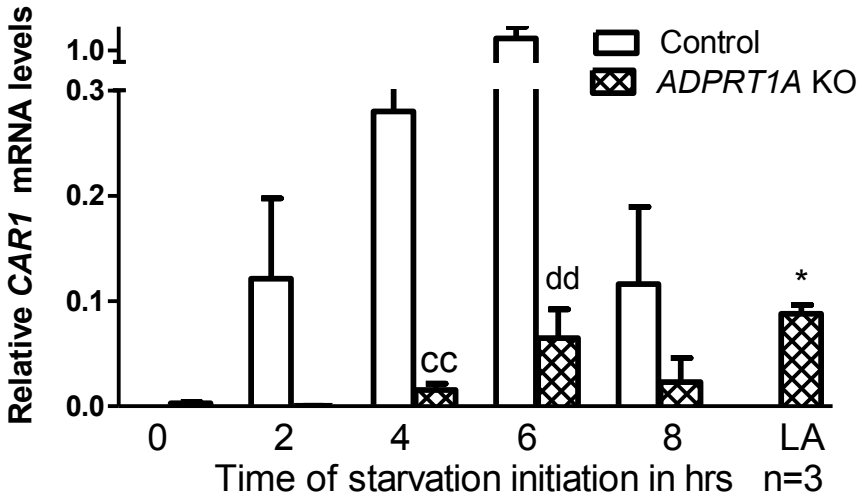


Fig. 6.13: Expression patterns of *CAR1* in *ADPRT1A* KO cells. Expression levels of *CAR1* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. $^{cc,dd}p$ value <0.01 as compared to control levels at 4 hrs and 6 hrs respectively, $^{*}p$ value <0.05 as compared to control levels at 6 hrs. LA levels are compared with control mRNA levels at 6 hrs.

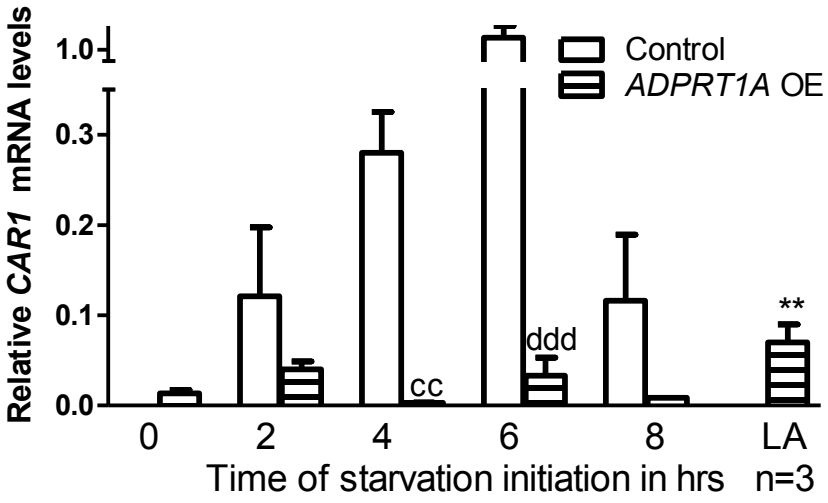


Fig. 6.14: Expression patterns of *CAR1* in *ADPRT1A* OE cells. Expression levels of *CAR1* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. ^{cc}p value <0.01 as compared to control levels at 4 hrs, ^{ddd}p value <0.001 as compared to control levels at 6 hrs. $^{**}p$ value <0.01 as compared to control levels at 6 hrs. LA levels are compared with control mRNA levels at 6 hrs.

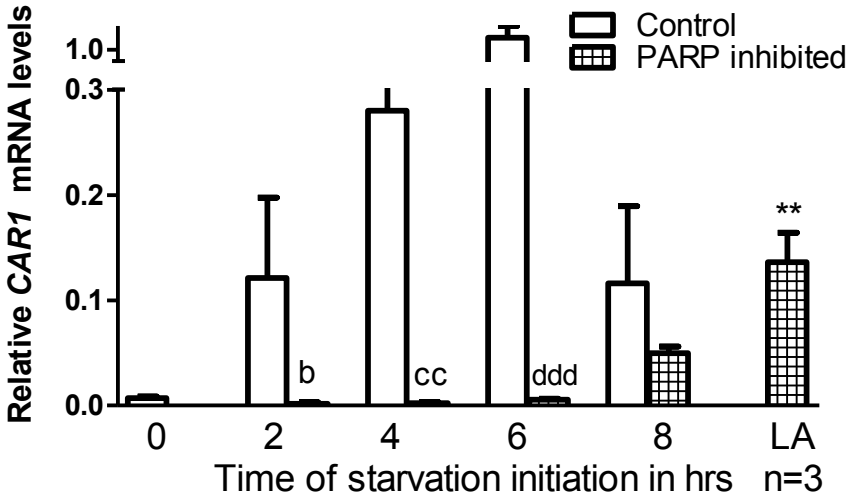


Fig. 6.15: Expression patterns of *CAR1* in PARP inhibited cells. Expression levels of *CAR1* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. ^{b}p value <0.05 as compared to control levels at 2 hrs, ^{cc}p value <0.01 as compared to control levels at 4 hrs. ^{ddd}p value <0.001 as compared to control levels at 6 hrs $^{***}p$ value <0.001 as compared to control levels at 6 hrs. LA levels are compared with control mRNA levels at 6 hrs.

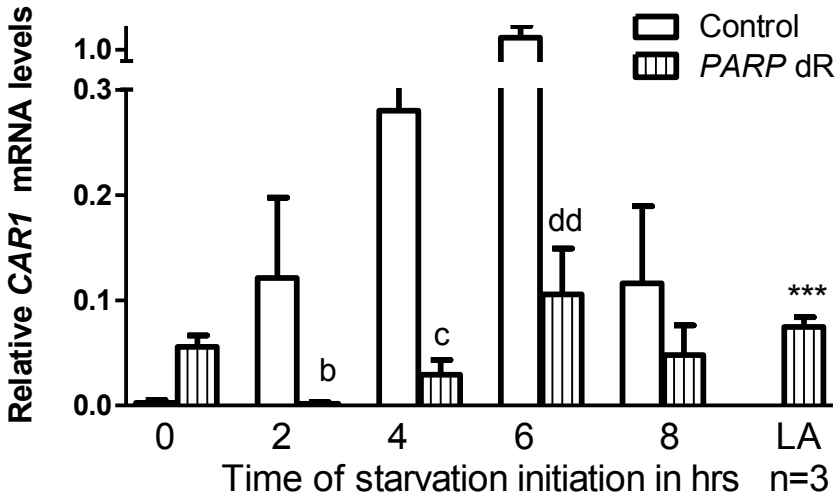


Fig. 6.16: Expression patterns of *CAR1* in *PARP* dR cells. Expression levels of *CAR1* gene were analyzed by Real Time PCR. The levels were normalized to that of the endogenous control *RNLA* (mitochondrial large rRNA). In addition, the expression value in control cells at 6 hrs time point was used as an internal standard and values were normalized accordingly. Values shown in graphs are the mean and SE of 3 independent experiments, which were performed by using independent total RNA. $^{b,c}p$ value <0.05 as compared to control levels at 2 hrs and 4 hrs respectively. ^{b}p value <0.05 as compared to control levels at 2 hrs, ^{dd}p value <0.01 as compared to control levels at 6 hrs. $^{***}p$ value <0.001 as compared to control levels at 6 hrs. LA levels are compared with control mRNA levels at 6 hrs.

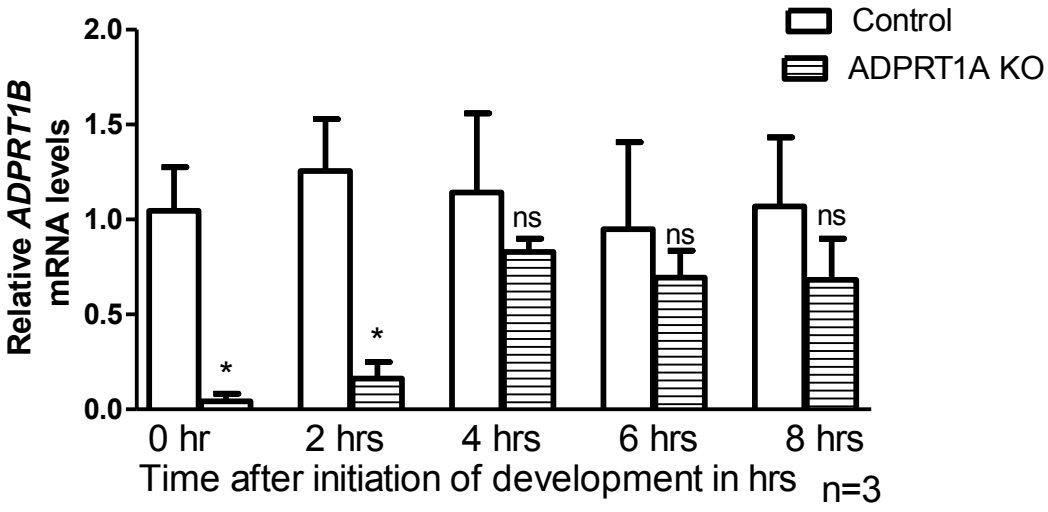
Thus, it can be concluded that low cAMP produced in *ADPRT1A* KO might be due to lower *ACA* and *CAR1* levels. Lower phosphodiesterases helps the cell to maintain the amount of cAMP produced. Higher levels of phosphodiesterases transcripts would lead to further reduction of cAMP levels. Decreased *ACA, CAR1* and *PDSA* levels also explain smaller aggregates and the delay in early development while *CAR1* explains the faulty chemotaxis in *ADPRT1A* KO. Out of the four *ADPRT* mutants, *ADPRT1A* KO profile gave us a much better understanding of the role of *ADPRT1A* KO due to its specificity

unlike PARP inhibition and *PARP* dR wherein all PARP's affected. Also, *ADPRT1A* OE showed results like PARP inhibition leading to inactivation. Hence, further studies were carried out only in *ADPRT1A* KO cells.

6.2.5 Expression of *ADPRT* isoforms in *ADPRT1A* KO

There is no stalled development but delayed development in *ADPRT1A* KO. To explain this phenomenon and to check for redundancy of ADPRT1A function, we checked for *ADPRT1A* isoforms transcript levels *i.e. ADPRT1B* and *ADPRT2* levels from 0 to 8 hrs after initiation of starvation. Fig. 6.17A shows there was no significant change in *ADPRT1B* levels while there is a significant increase at 8 hrs (Fig. 6.17B, $p<0.05$) after starvation in *ADPRT2* transcript levels which might compensate for *ADPRT1A* function. However, it was unable to completely compensate for *ADPRT1A* function which is evident from the delay yet seen even after 8 hrs and also the transcript levels of *ACA, CAR1, PDSA* and *REGA* not reaching levels comparable to control even at time point when loose aggregation was initiated.

(A)



(B)

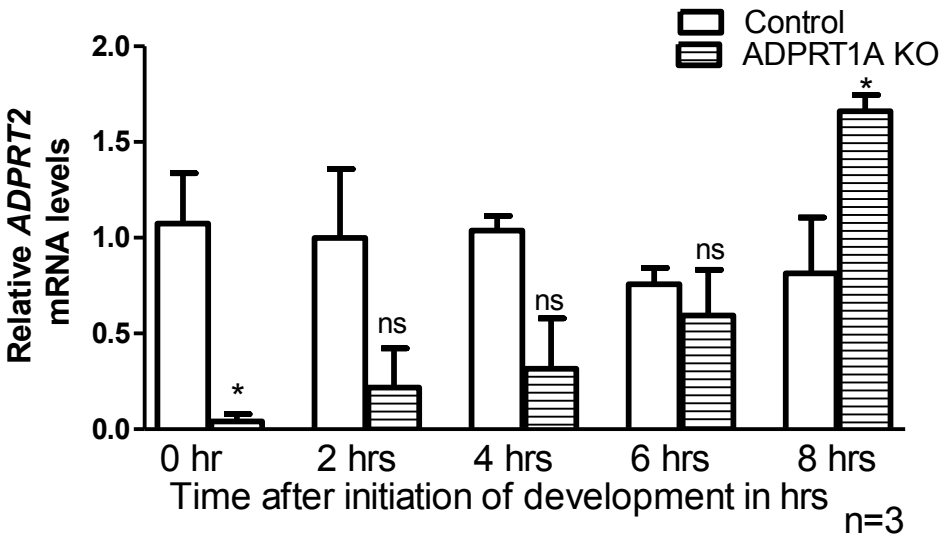


Fig. 6.17: ADPRT isoforms in *ADPRT1A* KO. (A) Real time analysis of *ADPRT1B* transcript in *ADPRT1A KO* cells. Data is a representation of SEM values of three independent experiments. (B) Real time analysis of *ADPRT2* transcript in *ADPRT1A KO* cells. Data is a representation of SEM values of three independent experiments. $^{*}p$ value <0.05 as compared to control levels at respective time point.

6.2.6 Expression of differentiation markers in *ADPRT1A* KO

PARP-1 has been implicated to play a key role in differentiation (Jubin *et al*., 2016b). Hence, to validate the role of ADPRT1A in *Dictyostelium* differentiation via transcription, prestalk and prespore markers namely *ECMA, ECMB* (prestalk) and *D19* (prespore) respectively were examined in *ADPRT1A* KO slugs. *ECMA* transcripts were significantly higher in *ADPRT1A* KO (Fig. 6.18A, p<0.05) while *ECMB* showed no significant change at the transcriptional level (Fig. 6.18B). On the other hand, *ADPRT1A* KO slugs exhibited significantly lower *D19* transcript levels (Fig. 6.18C, p<0.05).These results illustrate a prestalk tendency in absence of *ADPRT1A*.

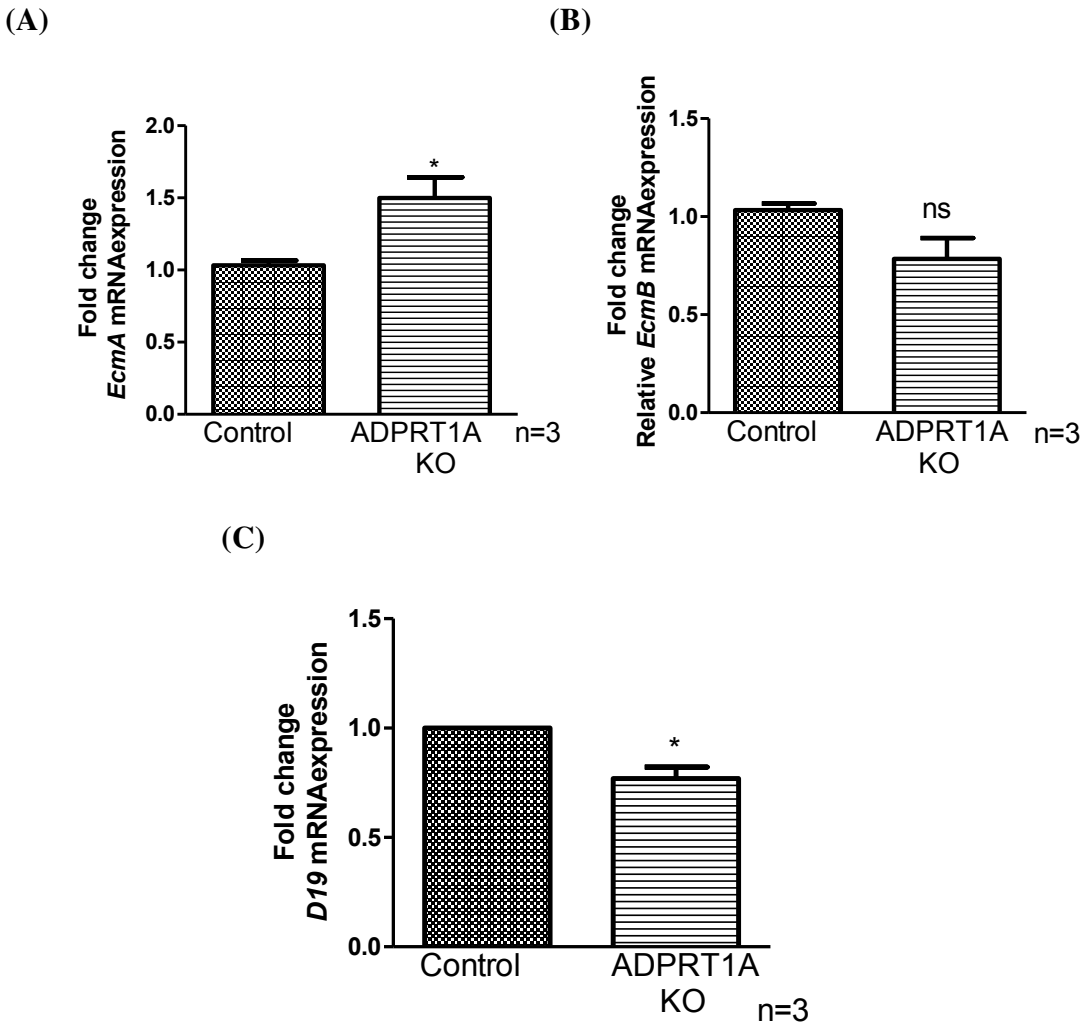


Fig. 6.18: Prespore-Prestalk markers in *ADPRT1A* KO slugs. Real time analysis of (A) *ECMA,* (B) *ECMB* and (C) *D19* transcripts in *ADPRT1A* KO slugs. Data is a representation of SEM values of three independent experiments. $^{*}p$ value <0.05 as compared to control levels.

Further, the prestalk tendency of *ADPRT1A* KO slugs were confirmed by neutral red staining of *ADPRT1A* KO slugs. Neutral red binds to dead prestalk cells giving rise to red color in regions of prestalk cells. Fig. 6.19 reconfirms prestalk tendency of *ADPRT1A* KO slugs wherein they show more neutral red stained cells as compared to control slugs.

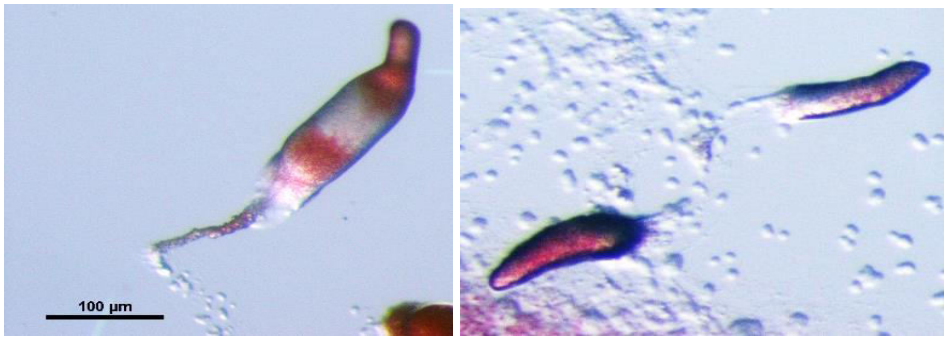


Fig. 6.19: Neutral red stained slugs of Control and *ADPRT1A* KO cells. Data is a representative of three independent experiments. Bars indicate scale: 100 µm.

6.3 Discussion

A diverse set of signaling molecules and regulatory components are reported to be involved in the initiation and early developmental stages in *Dictyostelium* development (Mir *et al*., 2007). cAMP remains to be one of the most important signaling molecule in *D. discoideum* developmental morphogenesis, particularly during aggregation and late developmental stages. As discussed earlier, cAMP acts both as a secreted extracellular signal and as an intra-cellular second messenger involved in signal transduction (Schaap, 2011). Of the three isoforms, adenyl cyclase A (ACA) produces the cAMP required for cell aggregation in early development (Pitt *et al*., 1992). Binding of cAMP to cAR1 (cAMP receptor 1) induces the synthesis of intracellular cAMP by activation of the cAMP-dependent protein kinase A (PKA) pathway (Firtel and Chung, 2000) thus leading to cAMP production (Maeda *et al*., 2004), while the cAMP levels are regulated by secreted cAMP phosphodiesterase (PDSA) and intracellular cAMP phosphodiesterase (REGA) (Faure *et al*., 1990; Shaulsky *et al*., 1996). In view of analyzing the low cAMP production in *ADPRT1A* KO and with the knowledge of PARP-1 being involved in transcriptional regulation (Kraus and Lis, 2003), the transcript levels of genes involved in cAMP signal transduction were studied by Real Time PCR. Mutants that are defective in aggregate formation have been reported to show altered expression of *ACA, CAR1,*

PDSA, REGA (Hirose *et al*., 2000; Garciandia and Suarez, 2013; Wu and Janetopoulos, 2013). *ADPRT1A* KO cells show decreased expression of the above mentioned genes involved in cAMP signaling (Fig. 6.1, 6.5, 6.9, 6.13). This goes in accordance with the study by Ogino *et al*., which demonstrated *Parp*-$1^{-/-}$ ES cells with altered gene expression profile (Ogino *et al*., 2007). *PARP* dR shows similar transcript profile for *PDSA, REGA, ACA* and *CAR1* but much a lower expression of genes was observed as compared to control as well as *ADPRT1A* KO cells (Fig. 6.4, 6.8, 6.12, 6.16). Expression of these genes was also checked in *ADPRT1A* KO and *PARP* dR cells at the time point of loose aggregate formation. *ADPRT1A* KO cells showed rescue in *PDSA, ACA* and *CAR1* levels which could be due to compensation by ADPRT isoforms thereby leading to rescue in *PDSA, ACA* and *CAR1* transcript levels. Such results were observed in case of PARP-1 deficiency, wherein PARP-2 compensated for the loss of PARP-1 thus proving to possess non redundant functions (de Murcia *et al*., 2003). Thus, it is clear from *ADPRT1A* KO gene expression data that ADPRT1A loss led to downregulation of genes involved in cAMP signaling. However, the development in *ADPRT1A* KO cells was not stalled unlike *PARP* dR cells indicating compensation of its function. However, the full function was not compensated and this was substantiated by the aberrant structures during *ADPRT1A* KO development as well as the transcript levels at the time of initiation of aggregation in control cells. Transcript level analysis of ADPRT isoforms viz., *ADPRT1B* and *ADPRT2* displayed higher *ADPRT2* (PARP-2 ortholog) transcript levels in *ADPRT1A* KO cells as compared to control after 8 hrs of starvation, thus supporting our assumption (Fig. 6.17). However, the delay was yet observed as per reports which suggest that PARP-2 cannot compensate for PARP-1 function completely. Furthermore, deficiency in DNA-dependent PARP activity cannot be functionally compensated for by any other PARP family member, at least during early development (de Murcia *et al*., 2003).

Also, thick and interrupted streams as seen in *ADPRT1A* KO cells were supported by the report wherein *pdsA*$^{-}$ cells showed unusual thick and transient streams at high cell densities (Garcia *et al*., 2009). Moreover, phosphodiesterase null mutations also seemed

to block chemotaxis in *Dictyostelium* which is also observed in the present study in case of *ADPRT1A* KO cells (Sucgang *et al*., 1997) whereas low *ACA* and *CAR1* levels explain the low intracellular cAMP levels. Moreover, disruption of any one of the genes involved in cAMP signaling such as *ACA, CAR1, PDSA* and *REGA* result in aggregation defects in *D. discoideum* (Pitt *et al*., 1992; Kim *et al*., 1998; Bader *et al*., 2006; Sawai *et al*., 2007). Change in transcript levels as seen in PARP inhibited cells is due to decreased PARP activity of all isoforms as the inhibitor used is NAD^+ analogue which is the substrate for all PARP's. *ADPRT1A* KO, PARP inhibition and *PARP* dR show similar cAMP signaling transcript profiles. AOE cells show a different profile as *CAR1* levels are low while an increase in *ACA* and *PDSA* levels was seen. However, *REGA* remained unaffected. These phenotypes could be due to overexpression of *PARP-1* and hence higher PARP activity which leads to autoPARylation and regulation in PARP activity. Also, over consumption of NAD^+ could also affect other NAD^+ utilizing enzymes. Thus, taking into account the above results in *ADPRT1A* KO, PARP inhibited and *PARP* dR cells and existing reports that suggest PARP-1 interacts with histones and chromatin modifying enzymes to regulate their activity at promoters of target genes, ultimately affecting gene expression (Frizzell *et al.,* 2009), ADPRT1A regulates transcription of genes involved in cAMP signal transduction.

PARP-1 has also been reported in regulating cellular differentiation pathways (Pavri *et al*., 2005; Rouleau *et al*., 2011; Jubin *et al*., 2016b). The *Dictyostelium* developmental cycle exhibits differentiation of cells into prespore and prestalk cells (Jeremyn *et al*., 1989). In order to explore the transcriptional regulatory role of PARP-1 in differentiation which is also controlled in *Dictyostelium* by cAMP, differentiation markers namely *ECMA, ECMB* and *D19* were checked in *ADPRT1A* KO slugs using Real Time PCR. Fig 6.18A, B and C clearly shows *ADPRT1A* KO cells show higher prestalk marker, *ECMA* (Fig. 6.18A) expression and lower prespore marker, D19 expression (Fig. 6.18C) as compared to control cells, thus indicating a prestalk tendency in *ADPRT1A* KO cells. This was further substantiated by neutral red staining of control and *ADPRT1A* KO slugs

which also showed higher proportion of prestalk cells (red) in *ADPRT1A* KO (Fig. 6.19). Thus, these results indicate role of ADPRT1A in cellular differentiation.

In conclusion, ADPRT1A has been demonstrated to regulate developmental genes involved in cAMP signaling as well as transcription of differentiation markers in *Dictyostelium.*

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