CHAPTER-6

Chapter 6

Role of Swertiamarin and Poly (ADP-Ribose) Polymerase-1 in Adipocyte differentiation

6.1 Introduction

Adipose tissue, the storehouse of body fat, plays a key role in controlling glucose and fat homeostasis in the entire body. White adipose tissue (WAT) mainly governs the storage of energy and its mobilization. The production of adipokines by WAT also helps to regulate food intake, energy substrate metabolism and metabolic rate, which are one of the three main important aspects of energy balance (Ahima and Lazar 2008).

Hyperplasia and hypertrophy of adipocytes is stimulated by a metabolic state which is created by an excessive caloric intake causing positive energy imbalance leading to obesity (Shepherd et al. 1993). Although, all the overweight and obese individuals are characterized with adipocyte hypertrophy but adipocyte hyperplasia is more strongly correlated and is most marked in the case of severe obesity (Lonn et al. 2010). Here, at the cellular level the new fat cells are continuously generated from the pre-existing mesenchymal stem cells within the adipose tissue, which first commits itself to pre-adipocytes and later differentiates into mature adipocytes (Spalding et al. 2008).

Monitoring the progression of preadipocyte differentiation *in-vivo* in an experimental model is cumbersome. Hence, *in-vitro* differentiation of 3T3-L1 preadipocyte is a better, more feasible route to acquire morphological and biochemical characteristics of adipocytes when treated with IBMX, dexamethasone and insulin as discussed in earlier chapter (Holm 2003).

C/EBP-β and -δ are the early transcription factors that associate with PPAR-γ chromatin within the first few hours after the induction of differentiation (Salma et al. 2004). C/EBP-β seems to mark a subset of early transcription factor hotspots before the initiation of differentiation and chromatin remodelling of these hotspots is required before the binding of other transcription factors (Siersbaek and Mandrup 2011). Remodelling of the PPAR-γ2 promoter takes place before transcriptional activation of this gene (Xiao et al. 2011). C/EBP-β remodel the chromatin structure at putative enhancers to assist subsequent binding of PPAR-γ in late adipogenesis (Siersbaek et al. 2012). These corepressors and coactivators determine transcriptional activity by altering chromatin structure via enzyme such as histone deacetylases and histone acetyltransferases (CREB-binding protein/p300). Other mechanisms

include DNA methylation, ATP-dependent remodelling, protein phosphorylation, sumoylation, ubiquitinylation, and poly (ADP-ribosyl)ation (Bai et al. 2007).

Adipogeneic process requires the activation of certain specific inducible genes. PARP protein modulate chromatin structure and grant access to a mechanism of controlling gene expression.Poly(ADP-ribose)polymerase-1(PARP-1), is an abundant and ubiquitous chromatin associated nuclear protein amongst 17 PARP family members. PARP-1 is responsible of more than 80% of their catalytic activity. It catalyzes the nicotinamide adenine dinucleotide(NAD+)-dependent addition of polymer of ADP-ribose (Rayalam et al. 2009) onto a variety of target protein that functions as posttranslational protein modification. Hence, the present study is mainly focused on PARP-1 (Ji and Tulin 2010). PARP-1 activity is necessary for adipocyte differentiation, and increased PARylation can be observed in differentiating 3T3-L1 adipocytes (Erener et al. 2012). PARP-1 is recruited to PPAR-γ target genes in a PAR-dependent manner, allowing sustained expression of PPAR-γ and its target genes (Erener et al. 2012b). PARP-1 regulates adipogenesis by controlling histone marks and topoisomerase-2 activity. PAR formation by PARP-1 could affect adipogenesis in multiple ways: 1) by excluding or retaining transcription factors from a special chromatin site (Rouleau et al. 2010); 2) by dissociating corepressors that occupy PPAR-γ2 and its target promoters during adipocyte differentiation, allowing the recruitment of transcription coactivators; and finally 3) regulate histone modifying enzymes by subsequently altering histone modifications.

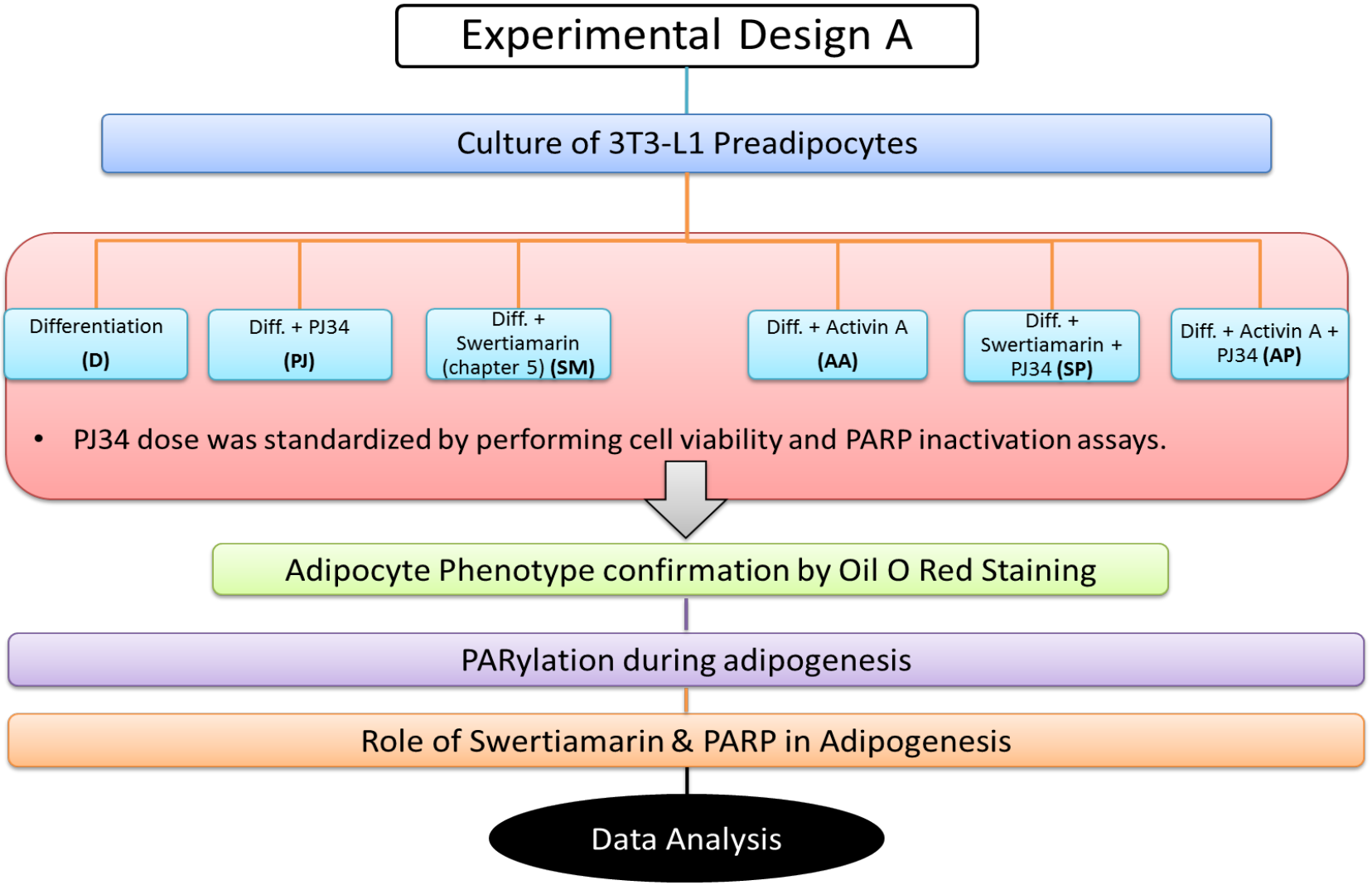
PARP-2 interacts with PPARγ/RXR heterodimer in DNA-depended manner which modulates the activity of PPARγ/RXR nuclear receptor complex which positively modulates adipogenesis (Bai et al. 2007; Bai and Canto 2012).

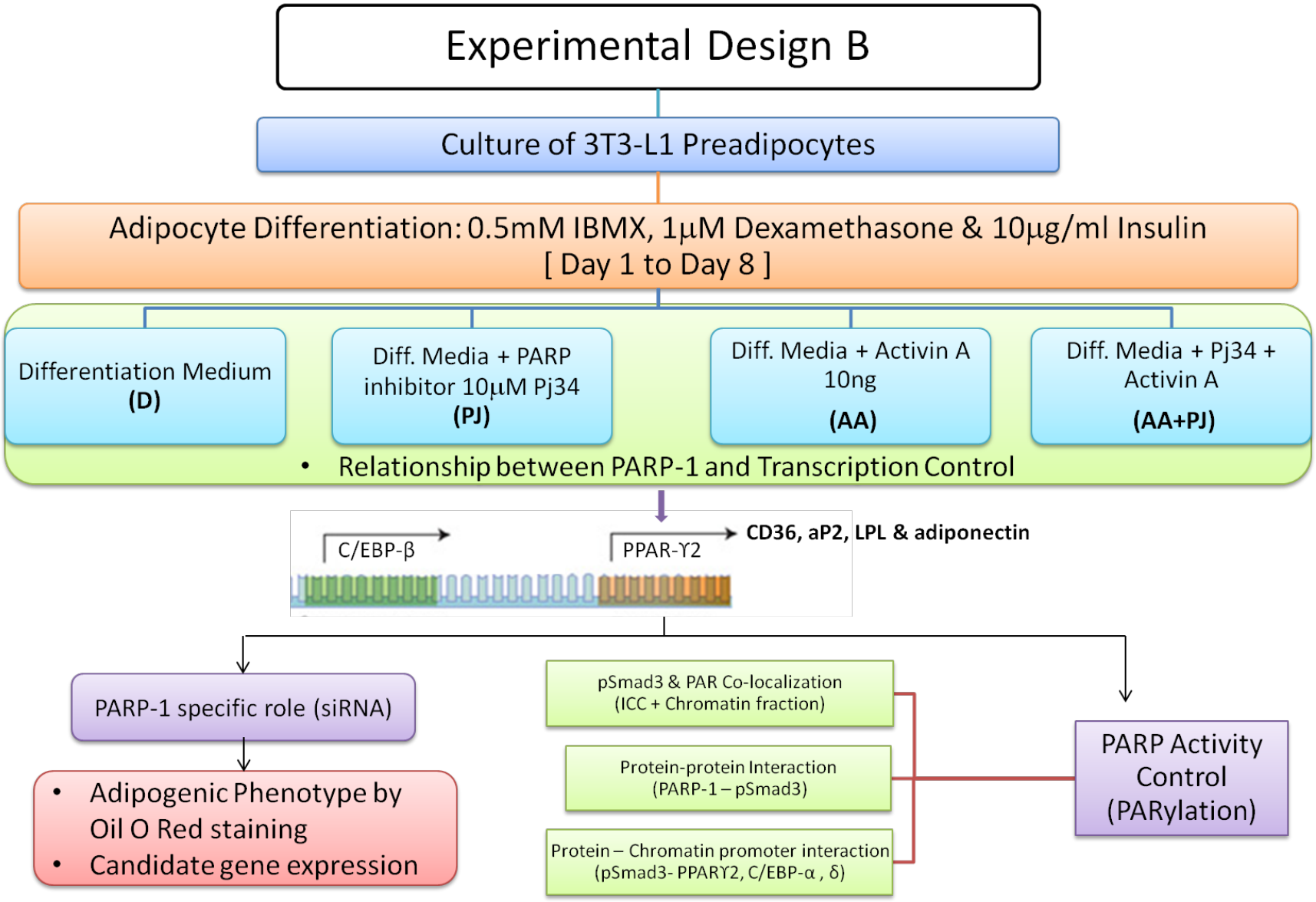
Members of Transforming growth factor (TGF)-β super family, BMPs (2/3/4/7) induces adipogenesis by increasing PPAR-γ expression (Hata et al. 2003). GDF38/myostatin promotes adipogenesis and inhibits myogenesis in C3H10T1/2 cells (Artaza et al. 2005). Activin A(AA) promotes 3T3-L1 cell proliferation & inhibits adipogenesis, Activin B inhibits lipolysis and both inhibits Smads2 mediated differentiation (Hirai et al. 2005; Zaragosi et al. 2010). The essence of the mechanism of TGF-β mediated inhibition of adipogenesis was to physically interact with Smads3/4, which would decrease expression of PPAR-γ and C/EBP-α by functionally repressing their upstream factors, C/EBP-β and -δ (Choy and Derynck 2003). Poly(ADP-ribose) polymerase-1 (PARP-1) also interacts with

Smads. PARP-1 dissociates Smad complexes from DNA by ADP-ribosylating Smad3, which controls the strength and duration of Smad-mediated transcription (Lonn et al. 2010).

It has been previously reported that proteins in the PARP family and TGF-β pathway proteins are involved in adipogenesis but the exact connecting mechanism is still elusive. In an earlier chapter swertiamarin have been shown to modulate PPAR-γ targeted regulation of adipogenesis, which here is further assessed for PARP mediated regulation. In order to understand the role of PARP in adipogenesis we targeted PARP-1 protein and its activity (PARylation) during adipogenesis. Here, we performed a time dependent protein profiling of key transcription factors involved in adipogenesis by inhibiting PARP activity. Further to prove possible PARP-Smad2/3 mediated mechanism of adipogenesis, interaction studies of PARP with Smad proteins were performed.

6.2 Experimental Design




Experimental Design B
Culture of 3T3-L1 Preadipocytes
Adipocyte Differentiation: 0.5mM IBMX, 1µM Dexamethasone & 10µg/ml Insulin
[Day 1 to Day 8]
Differentiation Medium
(D)
Diff. Media + PARP inhibitor 10µM Pj34
(PJ)
Diff. Media + Activin A 10ng
(AA)
Diff. Media + Pj34 + Activin A
(AA+PJ)
• Relationship between PARP-1 and Transcription Control
C/EBP-β
PPAR-Υ2
CD36, aP2, LPL & adiponectin
PARP-1 specific role (siRNA)
• Adipogenic Phenotype by Oil O Red staining
• Candidate gene expression
pSmad3 & PAR Co-localization (ICC + Chromatin fraction)
Protein-protein Interaction (PARP-1 – pSmad3)
Protein – Chromatin promoter interaction (pSmad3- PPARY2, C/EBP-α , δ)
PARP Activity Control (PARylation)

6.3 Material and Methods:

6.3.1 Reagents:

3T3-L1 (ATCC) early passage cells gifted from Katherine Cianfone, Quebec, CA. All antibodies were purchased from CST. All cell culture reagents were bought from Sigma Aldrich. PJ-34 was procured from Enzo life sciences. Activin A was procured from R & D Biosystem. siRNA (27 mer) and primers were synthesised by IDT. All molecular biology reagents and kits were from Life technologies. Immunoprcipratation magnetic beads and reagent were from Life technologies and enzymatic ChIP kit from Thermo Pierce.

6.3.2 Cell culture and adipocyte differentiation:

3T3-L1 cell culture maintaince and adipocyte differentiation was carried out as per Hata et al., 2008 and detailed protocol is mentioned in Chapter 4C (Section 4.3.1/2) (Hata et al. 2008). 10mM of PJ-34 stock solution were used and added into the medium for final concentration 0, 5, 10, 15 & 20 μM respectively. Activin A was used 10ng/ml (Harai S., 2005) for inhibiting adipocyte differentiation. Anti adiopogenic activity was checked using (EC_{50}=12.5μg/ml) from dose dependent studies with swertiamarin (Chapter 5).

6.3.3 Oil O Red staining and triglycerides accumulations assay:

Oil O Red staining was performed as mentioned in Chapter 4A.3.4, for visualizing lipid accumulation in adipocytes.

6.3.4 Co-Immunoprecipitation & Immunoblotting:

Cell pellet was lysed with RIPA buffer containing 1X protease inhibitor cocktail and kept for constant agitation for 30 min. at 4^{o}C. After centrifugation at 16000 g for 15 min. at 4°C, the supernatant was collected. Total protein content was quantified using Bradford assay (Biorad Bradford Solution, USA). Immunoprecipitation with pSmad3(1:100) and pPAR(10H) was performed using Dynabeads G-protein IP kit (Invitrogen) (Table 9). Protein was loaded on a 10% SDS-Polyacrylamide gel and then electrophoretically transferred onto a Nitrocellulose membrane (GE Healthcare). The membrane was then incubated for 1h at room temperature in blocking buffer (TBS-T containing 5% skimmed milk) and further incubated overnight with the primary antibodies for pSmad3 and PARP-1(1:1000) slowly rocking at 4°C. Membrane was then washed four times with TBS-T, and incubated with HRP-conjugated secondary antibodies (1:2500) for 1h. Finally, membrane was developed and visualized with Enhanced

Chemiluminescence western blotting detection system (Millipore Inc. USA) (Shah et al. 2011)(Table 7).

6.3.5 Isolation of total RNA and Semi-quantitative reserve transcriptase PCR:

Cell pellet was collected for each day of adipogenesis. RNA was isolated using the TRIzol reagent (Sigma Aldrich) and cDNA was created using reverse transcriptase PCR as discussed in Chapter 4A.3.7 (Table 4).

6.3.6 Transfection for siRNA:

3T3-L1 cells were transfected with siRNA against PARP1 at the catalytic domain. Cells were transfected with 27 mer siRNA containing sense and antisense cocktail (Kandan-Kulangara et al. 2010) using lipofectamine 2000 (Invitrogen) for transfecting 250 pmol siRNA in 3.5 cm^2 dish, according to manufacturer's protocol. Following the transfection, These cells were confirmed by immunoblotting using anti-PARP1 antibody and the same set of dishes were maintained for adipocyte differentiation (Table 6).

6.3.7 Immunocytochemistry:

In order to understand the mechanism of PARP action in adipocyte differentiation, PARP-1 and pSmad3 immunocytochemistry staining was done to characterize the interaction of PARP-1 and pSmad3 during differentiation of 3T3-L1 cells. Cells were seeded for adherence on glass coverslip with complete medium after successful completion of adipocytes differentiation protocol with treatment. They were fixed with 4 % Paraformaldehyde solution for 30 min at room temperature and permeablised with 0.1% Triton X-100 in PBS for 5 min, the cells were incubated in blocking buffer (1% bovine serum albumin and 4% FBS in PBS) for 1 hour at room temperature. Primary antibodies were added in blocking solution and coverslips were incubated overnight at 4^0C. A list of the antibodies used and their dilutions are given in Table 8. Cells were then washed thrice with washing buffer (1:10 dilution of blocking buffer in PBS) and incubated with secondary antibodies conjugated to FITC and TRITC fluorophores (Sigma Aldrich, USA) in dark for 30 min at room temperature. For negative control cells were incubated with normal IgG. Cells were mounted with Vectashield mounting medium containing 4'6'-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories, Inc.). Confocal imaging was carried for co-localization by using a LSM 710 microscope. All images were analyzed with help of LSM software supplied by Zeiss (Shah et al. 2011).

6.3.8 Cell fractionation protocol to isolate chromatin-bound protein fraction:

The cellular fractionation protocol to derive the chromatin bound protein (Ch)-fraction was derived from an earlier protocol to isolate the nuclear fraction. In brief, cells were harvested, washed in PBS, and suspended in cell lysis buffer [10 mM Hepes (pH 7.8), 0.34 M sucrose, 10% glycerol, 10 mM KCl, 1.5 mM MgCl2, 1mM PMSF, protease and phosphatase inhibitors, and 0.1% Triton-X100]. Cells were lysed by repeated pipetting and kept on ice for 7 min. to generate the whole cell extract (WCE fraction). WCE was centrifuged at 1,000 × g for 5 min. at 4 °C to separate the nuclear pellet (Np fraction) from the cytoplasm (C fraction). To extract the Ch-fraction from the nuclear fraction, we developed a protocol based on a previously described procedure (Stevnsner et al. 1994). The nuclear pellet (Np fraction) was washed once with cell lysis buffer and suspended in nuclear lysis buffer (50 mMTris-HCl, pH 7.8, 420 mM NaCl, 0.5% IGEPAL, 0.34 M sucrose, and protease and phosphatase inhibitors). The nuclear fractions were kept on ice for 30 min and then spun at 16,000 × g for 30 min at 4°C to separate the nucleoplasm (Np fraction) suspension from the pellet. This chromatin pellet was suspended in 20 mM Tris-HCl (pH 7.5) containing 100mM KCl, 2mM MgCl2, 1mM $CaCl_2$, 0.3 M sucrose, 0.1% Triton-X-100, 1mM PMSF, and protease and phosphatase inhibitors. To obtain a uniform suspension of chromatin, the suspension was briefly sonicated at a low setting (level 11) with a microtip using Ultrasonic Dismembrator and repeatedly pipetted through a small-bore (P200) tips (dounce homogenization in place of sonication also gave similar results). The chromatin proteins were extracted by incubation with micrococcal nuclease (25 U/mL) at ambient temperature for 40 min. The reaction was terminated by addition of 5 mM each of EDTA and EGTA and spun at 16,000 × g for 10 min to separate the insoluble chromatin pellet from supernatant designated as the Ch-fraction (Robu et al. 2014).

6.3.9 Chromatin Immunoprecipitation:

Culture adherent mammalian cells were treated as desired. At end of the experiment the DNA and proteins were crosslinked using a final concentration of 1% formaldehyde for 10 minutes. Glycine Solution was added at a final concentration of 125 mM for 10 min. Aspirate formaldehyde-glycine and wash the cells with ice-cold PBS containing phosphatase and protease inhibitors halt cocktail . Add the 1X solution to the cells and detach cells by scraping and transfer the cell suspension to a 1.5mL microcentrifuge tube. Lysis using MNase digestion was done with 0.2 gel unit per 6X 10^6 *3T3-L1* cell sample. The cells were sonicated on ice with three pulses of 11% amplitude for 10 sec to lyses open the cells and disrupt the nuclear membrane. Incubate for 20 sec. on ice between pulses. Incubate IP reactions

containing Dynabeads, Antibodies and 100ug DNA containing cell lysate for 2 hours to overnight at 4°C on a rotator. Wash and Elution DNA using DNA column and 50µl DNA recovery buffer. Estimate the DNA concentration for each sample. Proceed to 2µl sample for qPCR amplification and detection using gene specific primer against promoter region (Table 5, 9).

6.4 Results:

6.4.1 PARP and its activity are important for adipogenesis phenotype.

PARP-1 is mandatory for cell survival and maintenance of genomic integrity. Also, PARP inhibition by PJ-34 may become lethal or toxic to the cells. Hence, it became mandatory to standardise an appropriate dose of PJ-34, which would completely inhibit PARylation without effecting 3T3-L1 cell viability significantly. Here, different concentrations of PJ-34 were used 5-20µM. PARylation in combination with cell viability was measured respectively for each PJ-34 concentration. Here, a 50% reduction in cell growth rate at PJ-34 concentration of 15 µM was observed. Hence, it was concluded that PJ-34 conc. of not more than 10 µM for this model system would be used (Figure 6.1a).

Cell viability of 3T3-L1 cells was determined to different concentrations of PJ-34. 3T3-L1, preadipocyte treated with DNA Damaging agent (Like 100 µM H2O2.etc) showed increase in PARP activity and hence polymer formation. It was observed that treatment of 10µM of PJ-34 but not 5µM PJ-34 completely abolished PARylation. This dose of PJ-34 inhibited PARP-activation. Hence, a PJ-34 concentration of 10µM was set as it completely inhibited PARP activity without losing significant cell viability (Figure 6.1b).

FIGURE 6.1a:

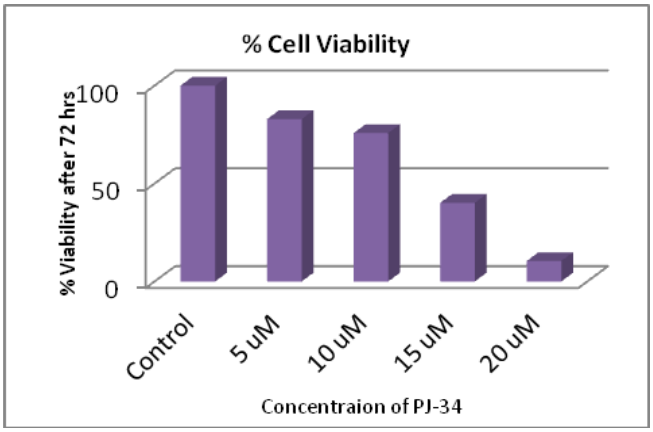


FIGURE 6.1a: Effect of PARP Inhibitor(PJ34) on cell viability of preadipocytes, 3T3-L1 cells. Cells were treated with different concentration of PJ-34 (5uM to 20 uM) and checked for its effect on cell viability after 72 hours.

FIGURE 6.1b:

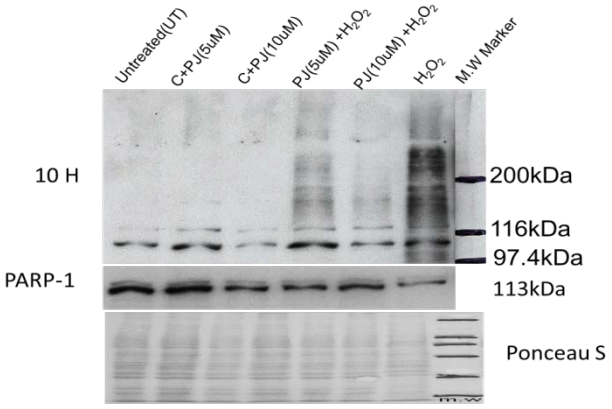


FIGURE 6.1b: 3T3-L1, preadipocyte treated with DNA Damaging agent (H_2O_2) showed an increase in PARP activity and hence polymer formation. PARP-activation can be abolished by treatment of 10uM of PJ-34 without affecting cell viability. Blot were probed with pADPr (10 H)(1: 500) and PARP-1 antibody(1:5000).

3T3-L1 cells were treated with differentiation cocktail for eight days. PARylation starts increasing from day 3, peaks at day 5 and persists till day 7 of adipogenesis, which was detected by Immunoblotting. PARylation was completely abolished during adipogenesis on using PARP inhibitor, PJ-34. The level of PARylation observed during adipogenesis, a physiological phenomenon was moderate when compared to the PARylation during cell death, positive control (Figure 6.1c).

Further to confirm the role of PARP activity on adipogenesis, mature adipocyte phenotype using Oil O Red staining was monitored in presence of PARP inhibitor, PJ-34. Here, 3T3-L1, preadipocytes when treated with ADM without PJ-34, differentiated in Mature adipocyte in 8 day after induction characterized by oil droplet formation, whereas, on using PJ-34 this was greatly hindered. This was confirmed by quantification of oil droplet formation by Oil O Red staining colorimetrically after extraction by isopropanol, where it was observed that the adipocyte differentiation was inhibited by 70% after treatment with PJ-34(10 μM). PJ-34 exhibited inhibitory effect on adipogenesis, suggesting that PARP protein and its activity positively modulates adipogenesis (Figure 6.1d).

The process of adipogenesis takes eight days after induction and is regulation by a series of events. Hormonal regulation of this process has two major waves of transcriptional factors expression. To understand the significance of PARylation with respect to these major adiopogenic stages, PJ-34 treatment was administered. PJ-34 was in mainly administered in three groups one with only first four days(ADM), second for last four days(AMM) and third for eight days of adipocyte differentiation. It was observed that PJ-34 inhibited Adipogenesis and polymer formation throughout the adipogenesis process. When used only at the initial time point i.e. at the early phase ~65% inhibition of adipogenesis was observed, which failed when treated only during late phase of Adipogenesis (Figure 6.1e). The endpoint assay was performed using Oil O Red staining.

6.4.2 Role of SM and PARP has anti-adiopogenic phenotype.

Swertiamarin is actively involved in inhibition of adipogenesis and fat metabolism (Chapter5). PJ-34, a PARP inhibitor also inhibits adipogenesis. Swertiamarin and PJ-34 were used in combination to explore whether they share a common pathway in adipogenesis inhibition or not. Adiopogenic cocktail treated 3T3-L1 cells were divided into two groups, one containing only swertiamarin(12.5μg/ml) and other group was combination of Swertiamarin and PJ-34(10μM), both groups showed inhibition of adiopogenic phenotype.

PARP activity is important for adipogenesis phenotype

Figure 6.1c:

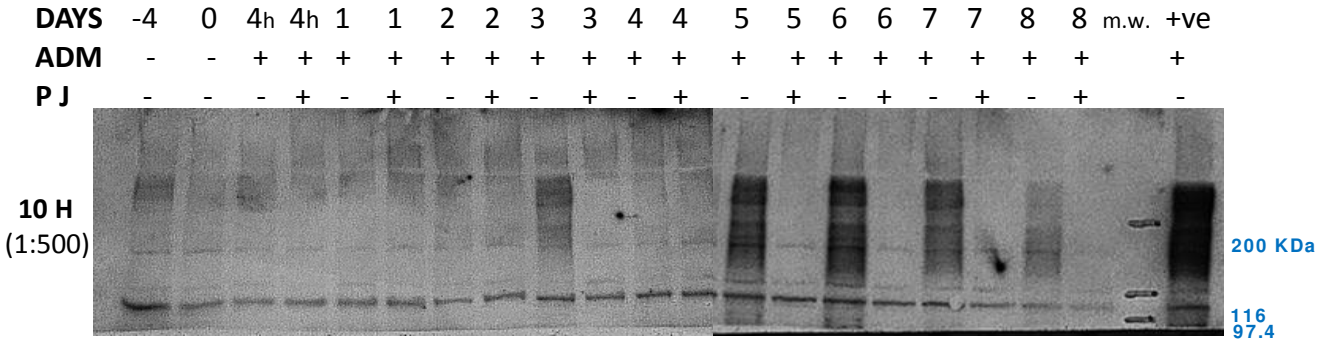


Figure 6.1c: PARP activity (PARylation) was observed during 8 days of adipogenesis in presence and absence of PARP inhibitor PJ-34(10 uM). 20 µg of total protein was loaded in each well and blot was probed with 10H(1:500) antibody.

Figure 6.1d:

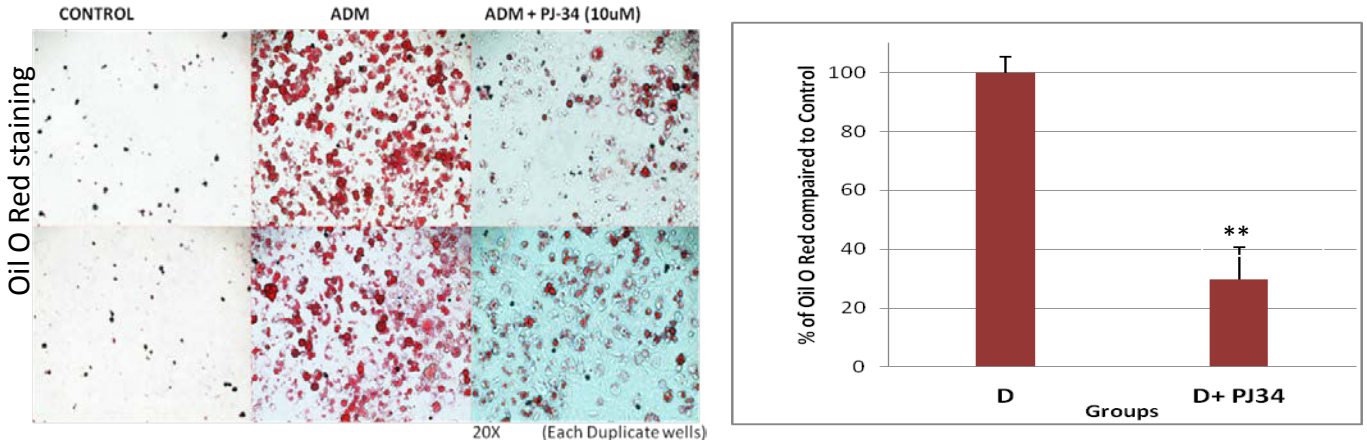


Figure 6.1d: PARP inhibitor, PJ 34 blocked adipogenic phenotype as confirmed by Oil O Red staining. 3T3-L1 preadipocytes were subjected to adipocyte differentiation using adipogenic cocktail for 8 days and were checked by the Oil O Red staining. (i) Oil O Red staining under 20X magnification under Phase contrast microscope (ii) Quantification of Oil O Red stain after extraction procedure is represented in terms of % of Oil O Red stain compared to control (p-value ** ≤ 0.005, n=3).

Figure 6.1e

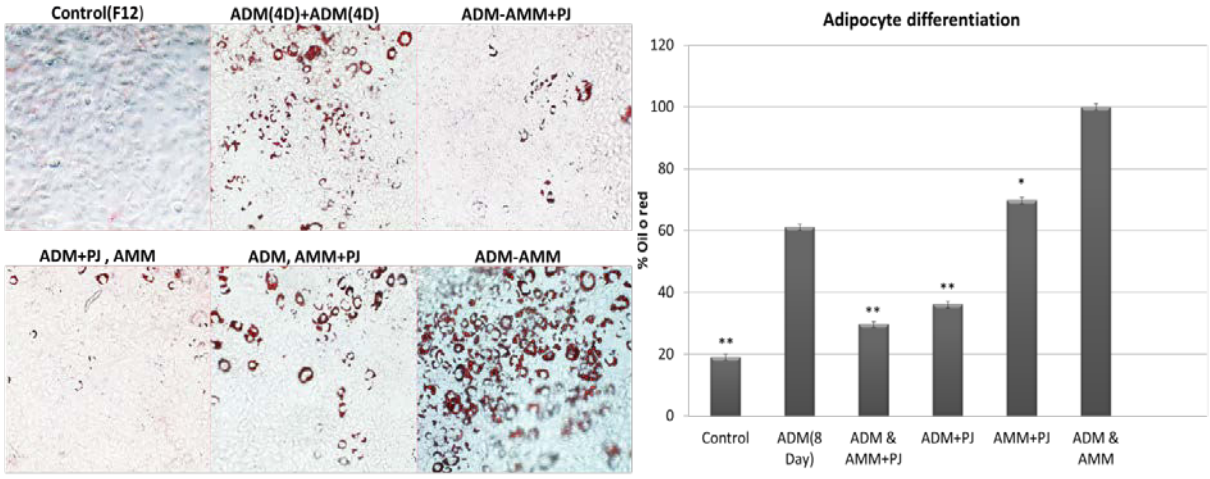


Figure 6.1e : PJ-34 was treated in early and late phase of adipogenesis, confirmation by (i) Oil O Red staining under 20X magnification under Phase contrast microscope (ii) Quantification of Oil O Red stain after extraction procedure is represented in terms of % of Oil O Red stain compared to control. (p-value ** ≤ 0.005, * ≤0.05) compared to ADM & AMM (VI)

Activin A, a TGF-β receptor ligand is known to inhibit adipogenesis. Hence, it is used as positive anti-adiopogenic factor with and without PJ-34, Activin A (10ng/ml) alone gave 48% inhibition, whereas in combination with PJ-34 the inhibition was 80%. The above results suggested that there was synergistic inhibitory effect imparted when used together (Figure 6.2).

Figure 6.2

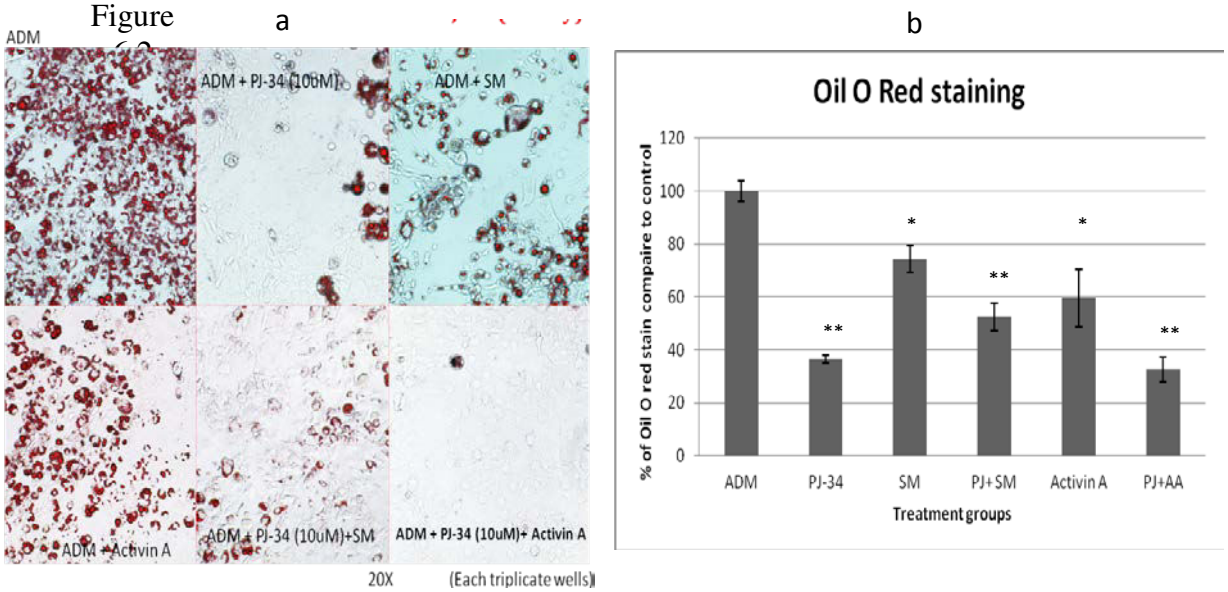


Figure 6.2: Anti-adipogenic effect of swertiamarin(SM) combined with TGF-β ligand (Activin A) with and without PJ-34. 3T3-L1 preadipocytes were subjected to adipocyte differentiation using adipogenic cocktail for 8 days and were checked by the Oil O Red staining. ;(a) Oil O Red staining under 20X magnification under Phase contrast microscope (b) Quantification of Oil O Red stain after extraction procedure is represented in terms of % of Oil O Red stain compared to control. (p-value ** ≤ 0.005)

6.4.3 PARylation is mandatory during adipogenesis:

Polymer formation was observed as one of salient feature during adipogenesis. Inhibition of polymer formation was able to reduce rate of adipogenesis. Swertiamarin was unable to show reduction in polymer formation, when tested for time dependent manner as compared to PARP inhibitor. Swertiamarin was able to inhibit adipogenesis without PARylation mediated regulation (Figure 6.3a). This shows that swertiamarin and PARP follow distinct pathways for inhibition of adipogenesis.

PJ-34 treatment inhibits PARylation which was observed on 3rd day to 7th day of adipogenesis. In addition to that Activin A was not able to inhibit polymer formation. Combined group showed same results as PJ-34 alone (Figure 6.3b). In addition to that PARylation of each day of adipogenesis for all three groups along with its combine group were observed (Figure 6.3c).

Figure 6.3a

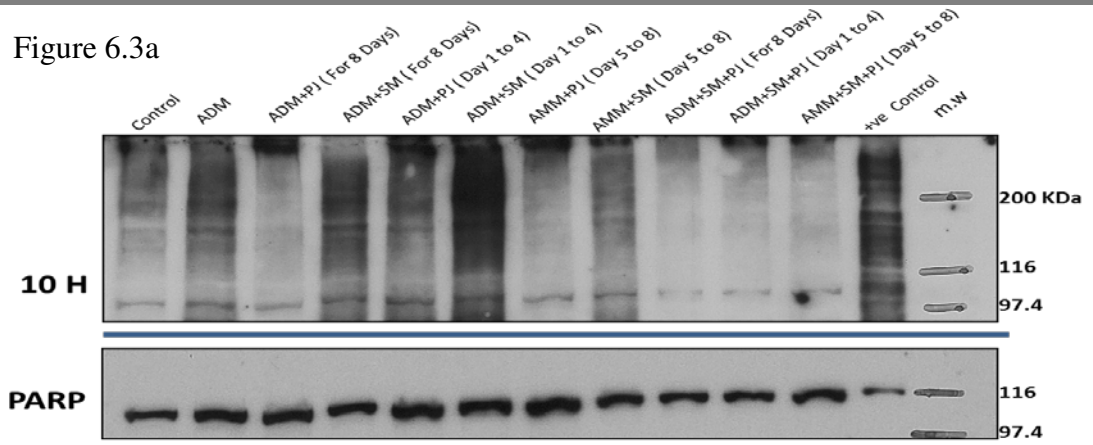


Figure 6.3a: PARP activity and it's expression when treated with SM in presence & absence of PJ-34 in Early and Late phase of Adipogenesis. Blot were probed with pADPr (10 H) (1: 500) and PARP-1 antibody(1:5000).

Figure 6.3b:

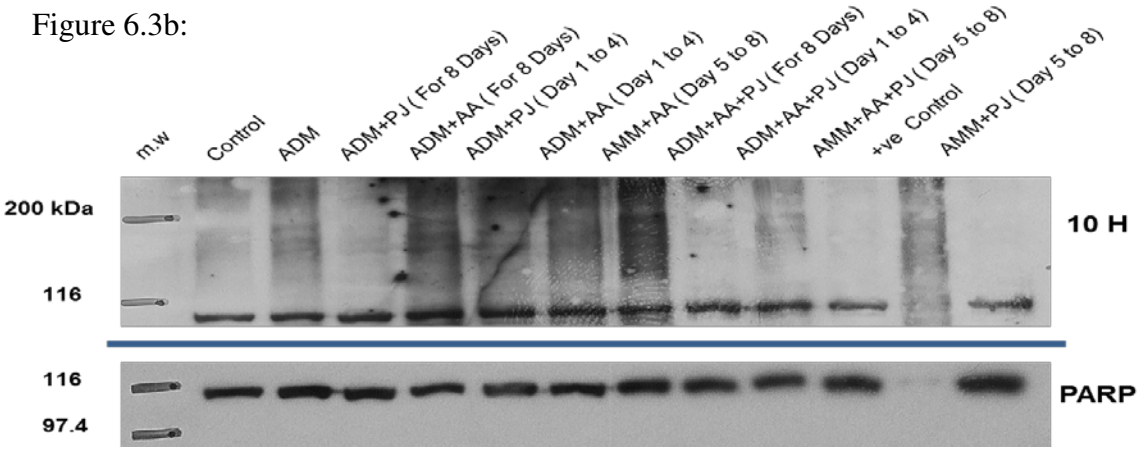


Figure 6.3b: PARP activity and it's expression when treated with Activin A in presence & absence of PJ-34 in Early and Late phase of Adipogenesis. Blot were probed with pADPr (10 H) (1: 500) and PARP-1 antibody(1:5000).

Figure 6.3c:

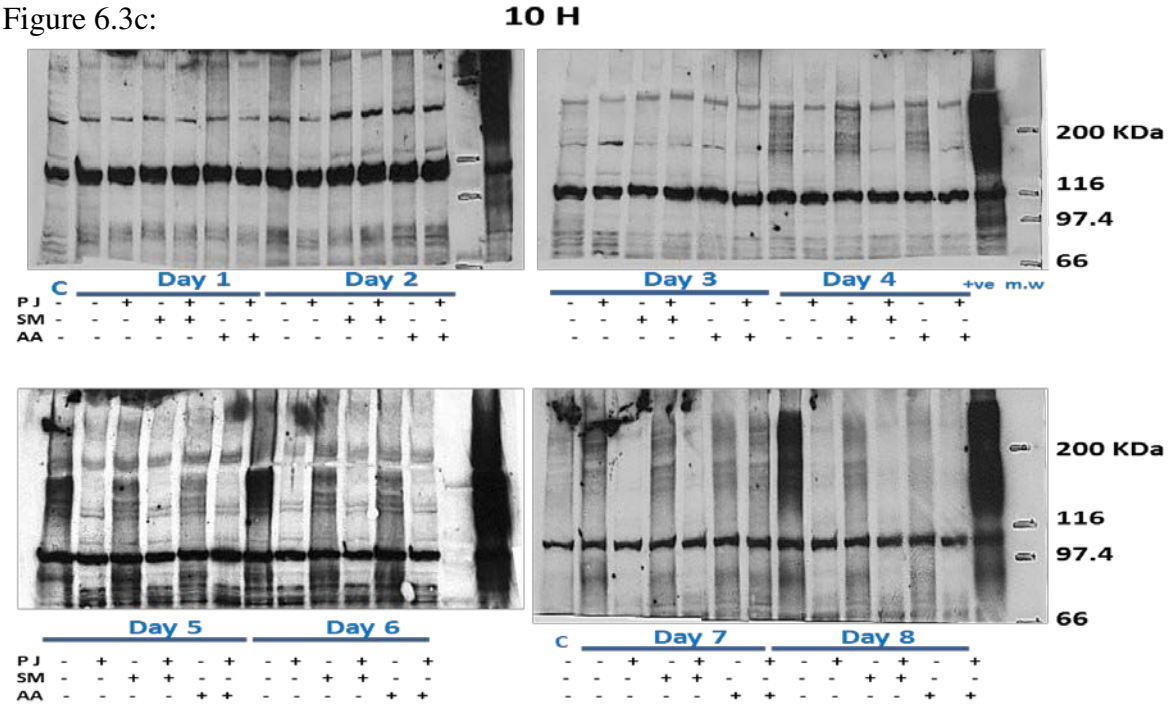


Figure 6.3c: After treatment with PJ-34, Swertiamarin, Activin-A and their combinations, PARylation was observed by probing the blot with 10H antibody.

6.4.4 Transcriptional profile during adipogenesis:

The two major classes of transcriptional factors that are actively involved in adipocyte differentiation are C/EBPs and PPAR-γ. C/EBP-β and δ are the first wave of transcriptional factors during the initiation of adipogenesis. C/EBP-δ and PPAR-γ are the second wave of transcriptional factors, which are expressed at the later stages. To monitor the adiopogenic process the gene and protein expression of C/EBP-β at the early phase and PPAR-γ at the late phase was monitored. C/EBP-β protein expression did not show any difference between adipogenesis and with PJ-34. Here it was observed that PARP activity regulated adipogenesis by C/EBP activity or transcriptional factors downstream of C/EBP like PPAR-γ(Figure 6.4b).

Figure 6.4a

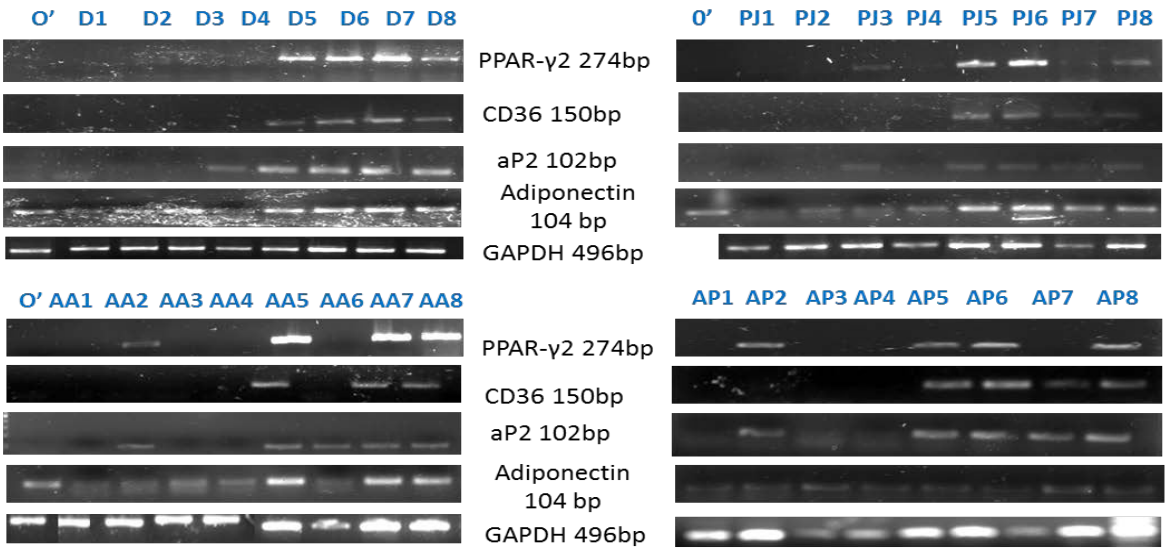


Figure 6.4b

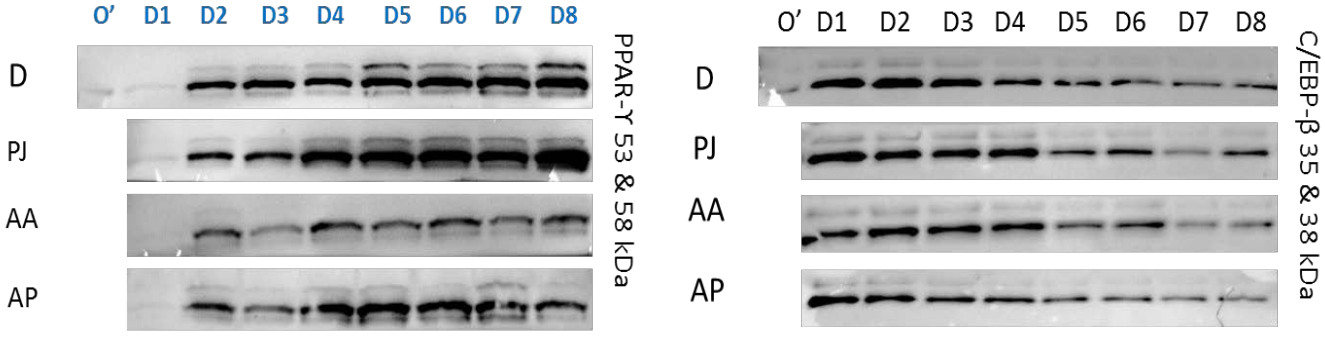


Figure 6.4 After treatment with Activin-A, PJ-34 and their combinations during adipogenesis (a) Gene expressions of PPARγ-2, CD36, aP2 and adiponectin were checked using semi-quantitative RT-PCR. (b) PPAR-γ and C/EBP-β protein expressions were checked by western blotting.

As adipocytes mature, they start expressing adipocyte functional genes like PPARγ, CD36 and $aP_{2.}$ These factors confirm the maturation and function of the differentiated adipocytes. PJ-34 group showed down regulation of PPAR-γ2, CD36 and $aP_{2.}$ Also, Activin A group showed reduction of PPAR-γ and CD36 but not significantly in aP_2 gene. When these two were combined, significant reduction of the above TFs compared to differentiation group was observed (Figure 6.4a).

6.4.5 Specific role of PARP-1 in adipocyte differentiation.

PARP family includes 17 PARP proteins with a conserved catalytic activity. PJ-34 inhibits PARylation and was used in all previous experiment. The advantage of using inhibitor is to make a clear distinction between the catalytic and other protein functions which modulate the physiological processes. To specifically prove the role of PARP-1, siRNA(27mer) against catalytic domain of hPARP-1 was used at 912 amino acid position C terminal domain. Immunoblotting of PARP-1 was performed to confirm knockdown and further PARP-1 down regulated cells were shown to inhibit Adiopogenic phenotype (Figure 6.5a).

PARP-1 knowdown cells showed significant reduction in adipocytes functional genes mainly PPARγ2 and adiponectin, leptin on 4^{th} day and 8^{th} day of adipogenesis by semi-quantitative RT-PCR (Figure 6.5b).

6.4.6 PARP-1 and activity interaction with pSmad3 during adipogenesis.

TGF-β pathway has been reported to be an important signalling pathway that modulates adipogenesis. Upon induction of adipocyte differentiation we found Smad2/3 are getting phosphorylated and make complex with Smad4. This complex shuttles from cytosolic region to nucleus and regulates transcriptional process of various physiological processes. On 2^{nd} day of adipogenesis pSmad3 was observed to be accumulated in Nucleus at higher intensity then day 5, which was required for the commitment of adipocyte differentiation and persistent expression of transcriptional factors. Transcription regulation is dependent on interaction of this complex with chromatin, which is greatly governed by PARP-1 and polymer formation on activated Smad complex. Confocal microscopy data suggested the interaction by co-localization of PARP-1 and pSmad3 on 2^{nd} and 5^{th} day of adipocyte differentiation (Figure 6.6a).

Specific role of PARP-1 in adipocyte

FIGURE 6.5a :

Effect of PARP-1 down regulation using siRNA(27mer) on Adipogenesis.

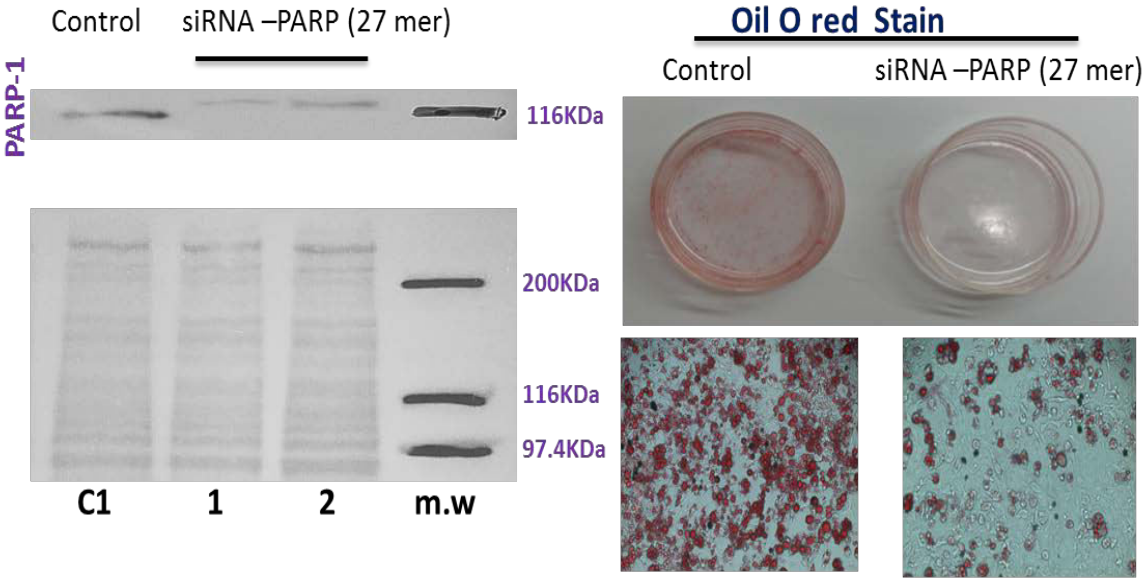


Figure 6.5a :siRNA(27mer) mediated down regulation of PARP-1. Knockdown was confirmed by western blotting of PARP-1. Transfected cells were shown to inhibit adipogenic phenotype as confirmed by Oil O Red staining.

FIGURE 6.5b:Gene expression of functional genes for mature Adipocyte by RT- PCR.

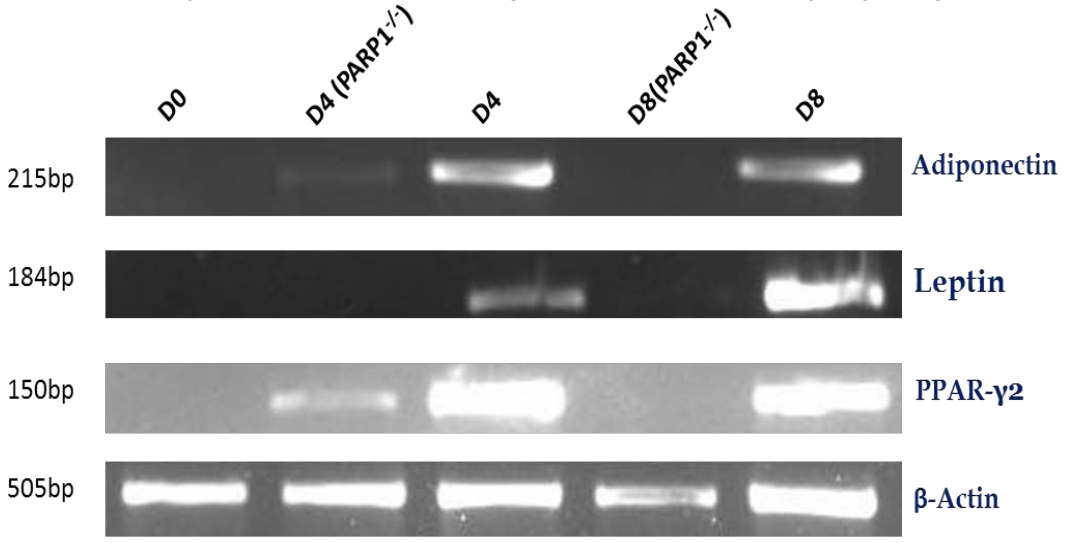


Figure 6.5b : PARP-1 is required for gene expression of adipogenic transcription factors :PPARγ2, Adiponectin and Leptin. The expression levels were checked using semi-quantitative RT-PCR.

Interaction of PARP-1 and PAR(pADPr) with pSmad3 during adipogenesis

Figure 6.6a

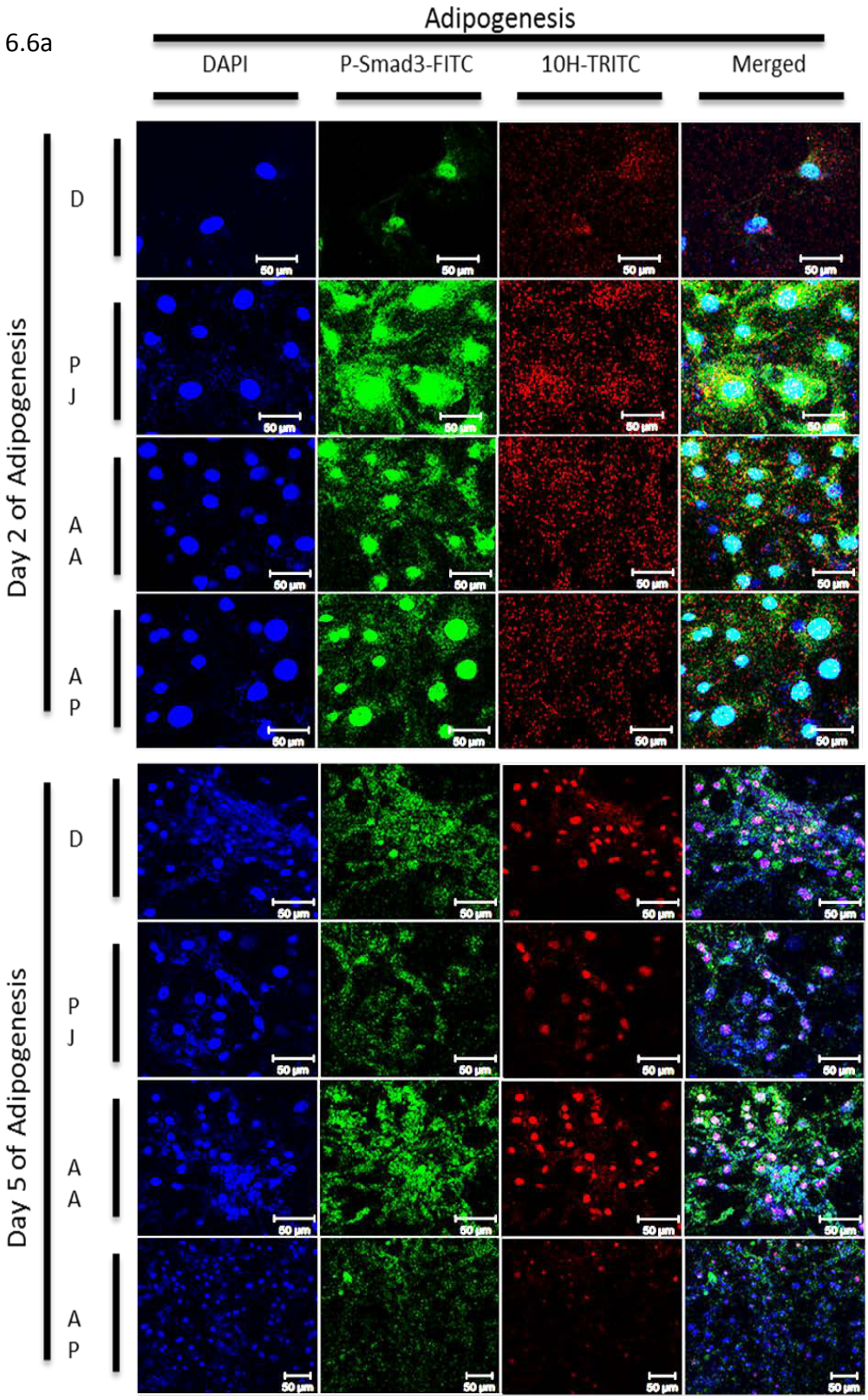


Figure 6.6 a :Expression of pSmad3 (tagged with FITC) with PAR (10H tagged with TRITC). ICC were performed on 2nd and 5th day of adipogenesis..

Further to confirm the localization of Smad complex during adipogenesis, chromatin fractions were again analyzed for PARP-1 and pSmad3 by Immunoblotting. Both protein were present in chromatin fraction after induction of adiopogenic cocktail (Figure 6.6b).

PARP-1 protein and its activity are involved in Smads complex mediated transcriptional activity. Interaction of PARP-1 and pSmad3 on second and fifth day of differentiation was found using Immunoprecipitation. On inhibition of polymer formation by PJ-34 interaction between PARP-1 and Smad complex was ablated.

Figure 6.6.b

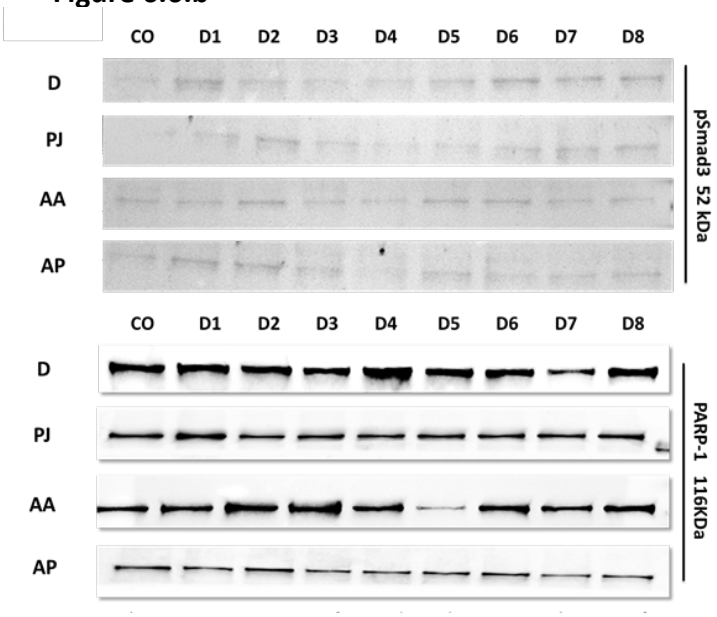


Figure 6.6b: Protein expression of pSmad3 and PARP-1 in chromatin fraction during adipogenesis.

Figure 6.6.c

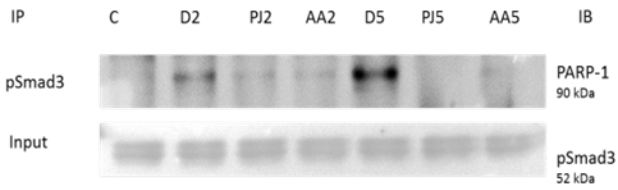


Figure 6.6c: Immunoprecipitation study showing the interaction of pSmad3 with PARP-1 (100µg protein).

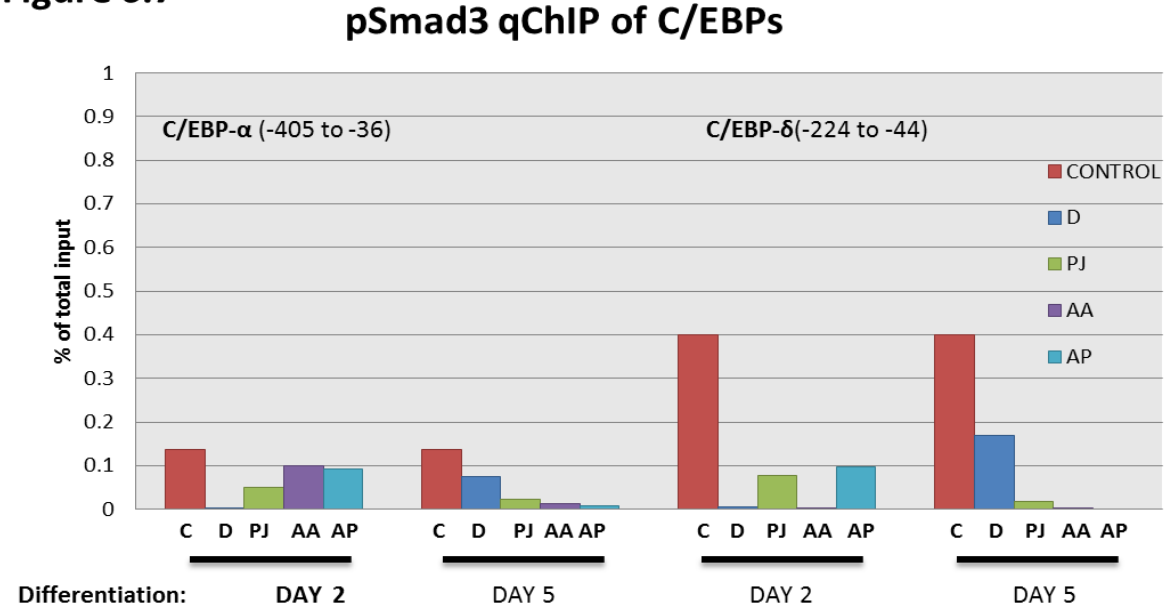
Activin A although phenotypically demonstrated anti adipogenesis but interaction of PARP-1 and PARylation of smads almost found same on 5th day when compared with differentiation suggests an alternative pathway implicating its anti-adipogenic action. This is first report and as not much supporting evidence are available in the literature in this direction (Figure 6.6c).

6.4.7 PARP-1 mediated PARylation regulates pSmad3 interaction with PPAR-γ promoter for adipogenesis:

PARylated Smads complex in chromatin, interact with promoter region of key transcriptional factors during adipogenesis. pSmad2/3 and PARP-1 complex in the nucleus lead to PARylation of Smads that was bound to the target DNA sequence in the promoter region of C/EBPs(δ & α) and PPARγ2 during differentiation. Chromatin immunoprecipitation of pSmad3 and qPCR analysis of C/EBP-α/δ and PPAR-γ2 promoters confirmed the pSmad3 interaction at PPAR-γ2 (-277 to -112) promoter (Figure 6.7). Which was absolutely inhibitor

in PJ-34 and AA on 2nd and 5th day of adipogenesis. AA demonstrated 20 fold increased chromatin binding on 5th day of adipogenesis.

Figure 6.7



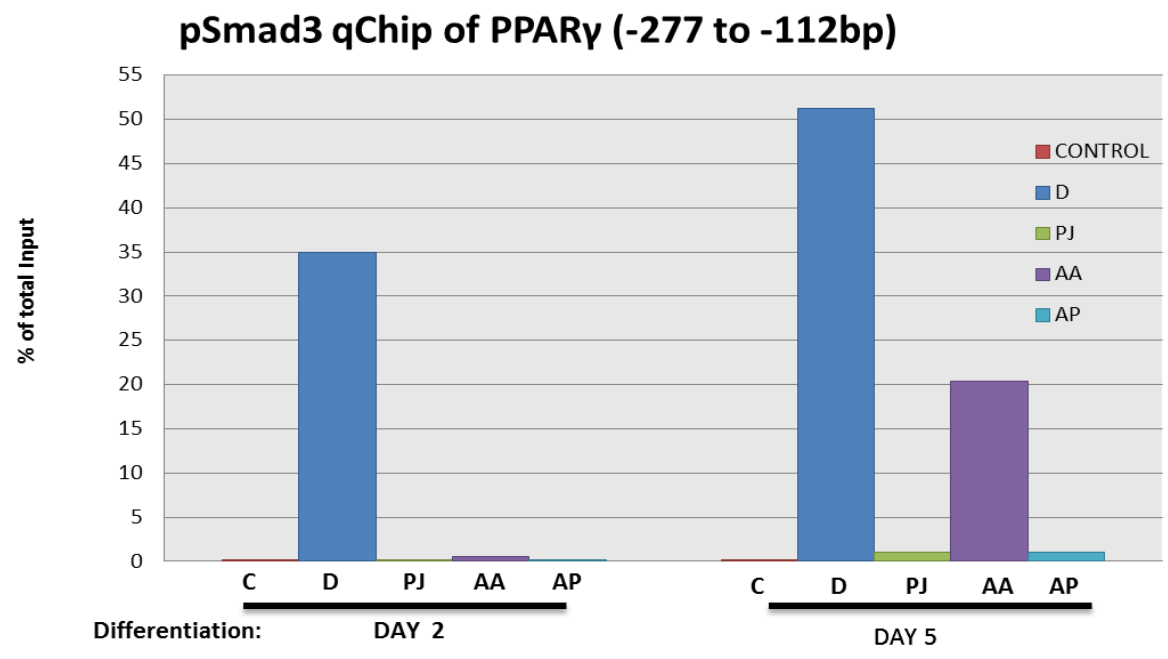


Figure 6.7: Chromatin-immunoprecipitation study showing the interaction of pSmad3 with C/EBPs and PPAR-γ promoters. (250µg of chromatin)

6.5 Discussion:

PARP protein is generally recognized for its role in cell death and DNA repair for a long period of time but currently there are several reports that suggest that it plays a role in modulating normal physiological processes like cellular proliferation, differentiation and metabolism (Bai and Canto 2012). PARP by interacting and modifying the chromatin helps the transcription machinery to gain access to the inducible genes needed for the respective cellular programs (Ji and Tulin 2010).

In recent studies, PARP-1 and PARP-2 showed to have regulatory role in fat deposition and more importantly adipogenesis. Bai et al, 2011, in their study reported that $PARP^{-/-}$ C57BL/6 mice are protected against high fat diet induced body weight gain by displaying reduced fat mass deposition. Also, Erener et al, 2012 mentioned that PARylation was essential for white adipocyte differentiation, which could be observed in differentiating 3T3-L1 adipocyte postulating a positive correlation between PARylation and adipogenesis. They further observed hypotrophy in white adipose tissue (WAT) from $PARP^{-/-}$ mice. In addition, they also observed lower expression of PPAR-γ target genes upon differentiation from adipose mesenchymal stem cells (MSCs).

In previous chapter, we for the first time demonstrated anti adiopogenic potentials of swertiamarin by regulating transcriptional factors C/EBP-β and PPAR-γ. In the present study an attempt has been made to understand intricate mechanism of PARP activity, modulating the transcription factor regulated by swertiamarin, thus causing anti adipogenesis. We observed initiation of polymer formation from the 3rd day which persisted till the 7th day of differentiation, in contrast to that reported by Erener et al, 2012 who observed that the activity did not initiate at the 3rd day but was found at the 7th day. PARP inhibitor, PJ-34 inhibits PARylation and thus, adipogenesis more effectively during early phase of differentiation. However, Swertiamarin and PJ-34 did not demonstrate any additive effect and change in PARylation thus, suggesting different mechanism of transcription control via PARylation mediated control of adipogenesis. After induction of adipocyte differentiation large number of major chromatin landscape starts activation of early transcriptional factor like C/EBP-β and δ which further leads to activation of late transcriptional factors like C/EBP-α and PPAR-γ, PJ-34 by inhibiting polymer formation as well impedes adipocyte differentiation phenotype and its mature gene expression mainly PPAR-γ, CD36, aP2 and adiponectin. Also, swertiamarin did not show any change in PARylation, suggesting different

mechanism of transcriptional control which is not PARP mediated. In another study, with 3T3-L1, preadipocytes (Simbulan-Rosenthal et al. 1996) suggested an increase in PARP protein and activity during adipogenesis along with association of PARP with multiprotein replication complex, regulating the replication apparatus by: 1) Poly(ADP-ribosyl)ation; 2) directly associating with the factors in the replication complex; 3) acting as a molecular nick sensor controlling the progression of replication fork or 4) modulating component replicative enzymes. In the present study, we further focused on PARP-1 as it has 80-90% activity amongst all other PARP. To understand specific role of PARP-1 as anti-adiopogenic, PARP-1 knockdown targeted at catalytic domain was designed. Our results confirmed its role in adipocyte differentiation and functions as was reported earliers (Erener et al. 2012). They also reported that PARylation of PARP-1 is required for its recruitment to the PPAR-γ and its target genes. Further, Lönn et al., 2010 identified interaction between PARP-1 and Smads of the TGF-β family. They reported that PARP-1, PARylates Smad3 and Smad4, dissociates Smad complex from DNA, which then attenuates the downstream signalling and Smad specific gene transcription responses. PARP-1 is indispensable for TGF-β1 induced Smad3 activation which has been shown in rat vascular smooth muscle cells hence targeting it seems to be a promising therapeutic approach against vascular diseases induced by dysregulation of TGF-β/Smad3 pathway (Huang et al. 2011). TβRII promoter also contain a PARP-1 DNA binding site which positively regulates the transcription in estrogen receptor-positive breast cancer cell lines (Sterling et al. 2006). Furthermore, blocking all ADP-ribosylation activities in bronchial epithelial cells, by using the PARylation inhibitor 3- aminobenzamide (3AB), reduced TGF-β mediated fibronectin expression (Beckmann et al. 1992). However, interpretations of such experiment in relation to PARP-1 and Smads have to be done with caution since it most likely involves effects attributed to the loss of function of all PARylation (Hottiger et al. 2010).

The TGF-β family protein superfamily regulates many aspects of adipogenesis (Zamani and Brown 2010). Activin A, a TGF-β ligand has been reported to negatively modulate adipogenesis. Hirai et al., 2005 reported that the terminal differentiation markers such as GPDH activity, lipid accumulation and aP2 expression got abated on using Activin A. Also, when Activin A was used at an earlier phase PPAR-γ expression along with C/EBP-α decreased but did not affect C/EBP-β. Activin A did not affect clonal expansion. These findings by Hirai et al., 2005 confirmed that Activin A inhibited adipogenesis by disturbing the transcriptional factors upstream of PPAR-γ expression. We found our results in line with

this study where, inhibition of adipogenesis on treatment of PJ-34 did not alter the expressions of C/EBP-β protein but reduced PPAR-γ_2 protein expression.

Receptor regulated Smads (R-Smads), Smad2 and Smad3 are directly phosphorylated and make complex with Smad4 (Co-Smad) which continuously shuttles between the cytosolic to nucleus and directly involved in transcriptional regulation (Inman and Hill 2002). Co-localization of PARP-1 and pSmad3 mainly responsible for initial phase (2^{nd} day) of adipocyte differentiation with transcriptional activation of promoter PPAR-γ_2 and late phase (5^{th} Day) of transcription. In present study, we reported PARP-1 and R-Smad complex to be present in chromatin fraction after induction of adiopogenic cocktail and physical interaction of PARP-1 and pSmad3 was found using Immunoprecipitation.

In another report(Choy and Derynck 2003), proved the physical interaction of Smadd3 and Smad4 with C/EBP-β and C/EBP-δ, which repressed the transcriptional activity of C/EBPs by inhibiting their binding to their respective cognate DNA sequence and in turn inhibiting transcription from the PPAR-γ2 and leptin promoters. In the present study, PARP-1 interacted with pSmad2/3 in PARylation dependent manner and increases during adipogenesis. pSmad2/3 and PARP-1 complex in nucleus leads to PARylation of Smads those binding to the target DNA sequence in promoter of C/EBPs and PPAR-γ2 during differentiation. Further, Chromatin immunoprecipitation of pSmad3 and qPCR analysis of C/EBP-α/δ and PPAR-γ promoters states that pSmad3 interacted at PPAR-γ2 promoter.

Adipogenesis is inhibited due to reduced polymer formation and binding of Smads. Thus, possible mechanism of action of PARP-1 mediated PARylation regulates Smad2/3 and plays a major role as transcriptional control in adipogenesis. PARP inhibition holds promising possibilities for the treatment of metabolic damage, but for achieving healthy life through PARP based therapies, one will require further understanding of PAR biology, fine-tuning of the dynamics and specificity of PARP inhibitors. Present study imparted significant role of swertiamarin as adiopogenic inhibitor, which is not mediating its effect like PARP inhibitor, thus opening up new avenues to explore novel anti adiopogenic molecules, free from limitations associated with PARP inhibitors.

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