EXPLORING THE TEMPORAL EXPRESSION OF SIGNALING PATHWAYS IN RESISTIN MEDIATED INSULIN RESISTANT HADSC FATE

CHAPTER 6

6. Exploring the temporal expression of signaling pathways in resistin mediated insulin resistant hADSC fate

6.1 INTRODUCTION

Resistin is an adipose tissue secreted protein required for lipid synthesis and maturation of adipocytes. However, excess FA elevate its levels and is known to diminish adipocyte maturation which causes adipose tissue dysregulation and insulin resistance (A. Park et al., 2014) Till date, there are no reports on effects of resistin on hADSC to best of our knowledge. Therefore, ramifications of resistin on stemness, metabolic profile and adipogenic and osteogenic differentiation potentials were examined in chapter 5. It was observed that it hampered proliferation, mitigated stemness, metabolic profile and insulin signaling of hADSC. Resistin enhanced adipogenesis and osteogenesis.

Several signaling pathways like BMP, WNT, NOTCH etc. regulate differentiation of hADSC. Among these BMP and WNT signaling play pivotal role in regulation of differentiation process of hADSC mainly adipogenesis and osteogenesis (James, 2013). BMP signaling belongs to superfamily TGF-β proteins wherein, if the BMP ligands bind to BMPR-1A receptors, adipogenesis is augmented and if they bind to BMPR-1B receptors osteogenesis is induced in MSC. The ligand receptor interaction phosphorylates SMAD1, SMAD5, SMAD8 and form pSMAD1/5/8 complex which binds to the Co-SMAD4 and translocate into the nucleus to regulate expressions of the key ATF or OTF like PPARγ and RUNX2 respectively depending on the stimuli (R. N. Wang et al., 2014).

WNT signaling is governed by canonical and non-canonical pathway in which β catenin dependent canonical pathway is generally involved in maintenance of stemness, differentiation of stem cells and developmental phenomena. WNT ligands bind to frizzled receptors and co-receptors LRP5 which activate the pathway by dephosphorylating the bound β catenin from the proteasome mediated degrading complex. These accumulated activated β catenin are translocated to nucleus and regulate the transcriptional factors governing adipocyte and osteocyte differentiation (Kamiya et al., 2008).

Several studies have demonstrated that BMP and WNT signaling inversely regulate adipogenesis but both the pathways stimulate osteogenesis. However, over-activated

BMP signaling also inhibits adipogenesis (James, 2013). Studies revealed that resistin did not alter WNT signaling in mature human adipocytes obtained from pre-adipocytes (Isakson et al., 2009).

Reports state that most signaling pathways converge at the PPARγ and RUNX2 activity in adipogenesis and osteogenesis respectively (James, 2013). It is known that WNT signaling is activated and inhibits cell cycle and clonogenic expansion of cells which inhibit adipogenesis (Cawthorn & Sethi, 2008). Saleh et al, explored that WNT signaling pathway can efficiently be expressed in 3 dimensional culture only and not in 2 dimensional (Saleh et al., 2016). However, much is not known about time dependent expressions of signaling pathways during the process of adipogenesis or osteogenesis. But various BMP ligands and WNT ligands have been found to express and activate signal cascade during differentiation. WNT10b is expressed after 24H of adipogenic stimuli, however β catenin levels fall after 2^{nd}D (Cawthorn & Sethi, 2008; Moseti et al., 2016). Studies have revealed that TNFα inhibited adipogenesis by increasing WNT signaling but resistin did not affect it in 3T3L1 during adipogenesis, but no information is mentioned regarding the time point (Hammarstedt et al., 2007; Isakson et al., 2009). As adipogenic and osteogenic differentiation are highly dynamic and orchestered, it becomes necessary to understand the interplay of signaling pathways and their dependent genes during differentiation.

Therefore, in the present study an attempt was made to dissect and understand the modulatory effects of resistin on temporal expressions of pSMAD1/5/8 and β catenin and the targeted ATF or OTF during adipogenic and osteogenic differentiation of hADSC. Further, as observed in chapter 5, that SM and Met ameliorated TNFα and resistin mediated inflammation, altered pluripotency and insulin resistance in hADSC, we further explored their effects on signaling pathways, ATF and OTF in mature adipocytes (22D) and osteocytes (14D) obtained after resistin treatment.

6.2 PLAN OF WORK

hADSC were subjected to adipogenesis for 22D and osteogenesis for 14D in continuous stimuli of 50ng/ml resistin. Cells were phenotypically observed and then harvested at initial phase i.e. 4^{th}D, preadipocyte stage- 12^{th}D and mature adipocyte - 22^{nd}D of adipogenesis. Temporal expressions of pSMAD1/5/8 and β catenin were analysed at all the three time points. Also ATF C/EBPβ, C/EBPδ, PPARγ2, C/EBPα

and adiponectin as functional gene were examined at the said time points. Similarly, hADSC were also subjected to osteogenesis for 14D and cells were harvested at osteoblast stage at 6thD and mature osteocyte stage at 14thD. Temporal expressions of pSMAD1/5/8 and β catenin were monitored at both the time points along with osteogenic genes RUNX2, Osterix and SIRT1. Further, both SM and Met were explored for their effects on SMAD1/5/8, β catenin, signaling pathway. Key insulin signaling protein and lipogenic genes in adipocytes obtained at 22ndD of adipogenesis and signaling proteins and osteogenic genes were monitored in osteocytes obtained after 14D of osteogenesis. The outline of the plan of work is depicted in figure 6.1. Groups under the study are mentioned below figure legends.

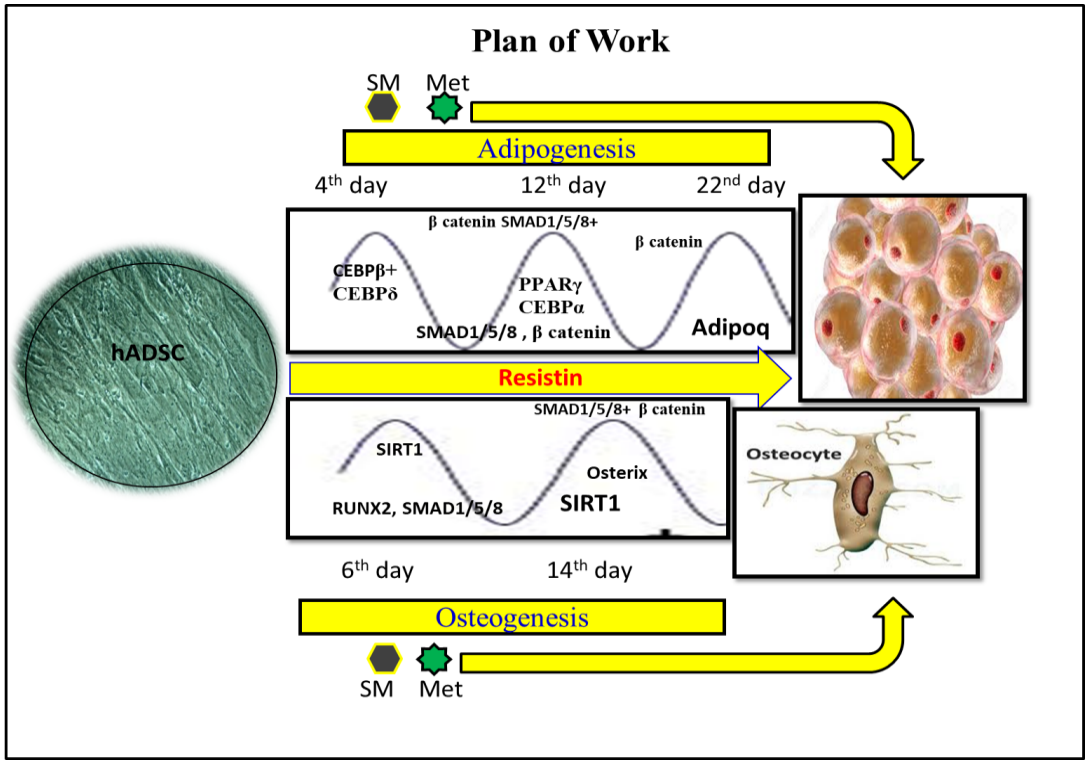

Figure 6. 1: **Plan of Work**

6.3 RESULTS

6.3.1 Resistin modulates differentiation potentials of hADSC

Modulatory effects of resistin on fate of hADSC were observed as mentioned in chapter 5. which revealed that it provoked hADSC to differentiate more towards adipocyte and osteocyte. Further, to understand the role of resistin on differentiation potentials of hADSC, the cells were subjected to adipogenesis and osteogenesis for 22D and 14D respectively in presence of resistin.

Resistin treated hADSC showed early induction of adipogenesis with formation of large lipid laden adipocytes till 22ndD (Figure. 6.2. A-B.). Lipid molecules in adipocytes were stained by ORO stain on 22ndD of adipogenesis followed by quantification which revealed that resistin treated hADSC (AC+R) produced hypertrophic adipocyte compared to adipogenic control (AC) (Figure. 6.2.C). Similarly, osteogenic differentiation in presence of resistin revealed that differentiation was induced early with elevated granularity in cells as observed throughout 14D of differentiation (Figure. 6.2.D.). Alizarin Red staining at 14thD revealed elevated calcium formation as observed in OC+R compared to OC (Figure. 6.2. E and F.).

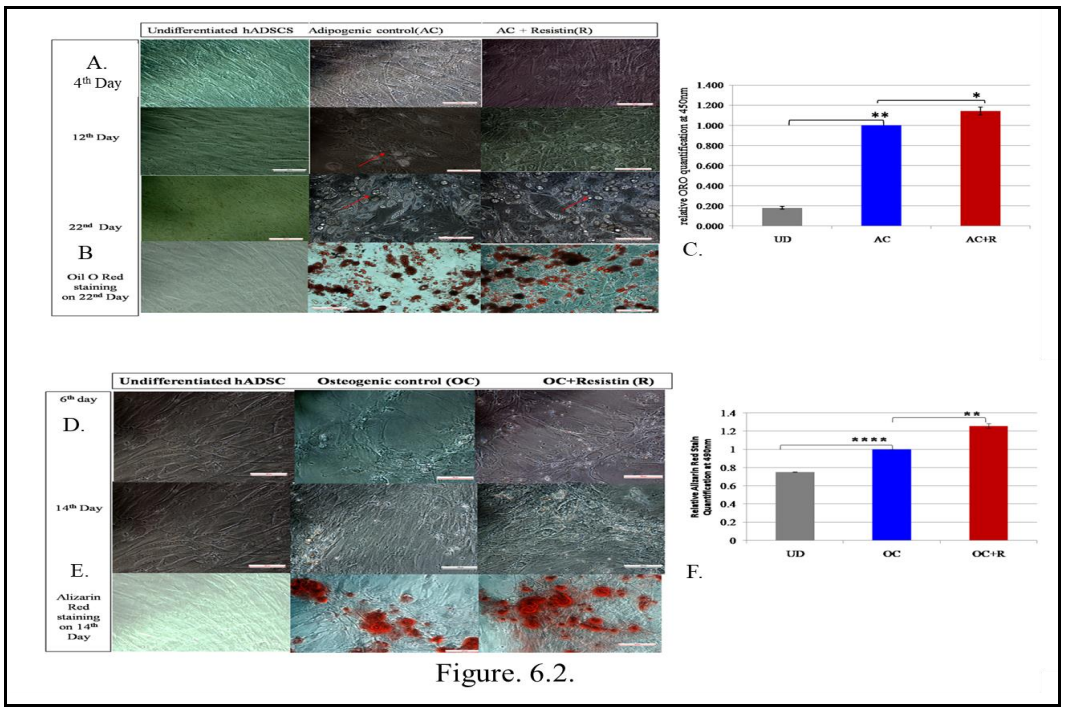

Figure. 6.2.

Figure 6. 2: Temporal differentiation potentials of hADSC. A. Bright field images on 4thD, 12thD and 22nd D of adipocyte differentiation. B. ORO staining: ORO stained adipocytes at 22ndD C. Quantification of ORO stain: ORO stain quantified at 450nm. The graph is plotted as relative values of O.D. $\pm$ S.E.M. where control is 1. Temporal osteogenic differentiation of hADSC. D. Bright field images on 6th D and 14thD of osteocyte differentiation. E. ORO staining: Representative alizarin red stained osteocytes at 14thD. F. Quantification of Alizarin Red stain: Alizarin Red stain was quantified at 450nm. The graph is plotted as relative values of O.D. $\pm$ S.E.M where control is considered as 1. n=3, p Value *<0.05, **<0.01, ***<0.001, ****0.0001.

6.3.2 Resistin induces adipogenesis through temporal expression of signaling proteins and ATFs.

Adipogenesis is a complex interplay of ATF that is tightly regulated by several signaling pathways. As observed in chapter 5. resistin upregulated adipogenesis on

22^{nd}D of differentiation. This chapter describes the temporal expressions of key proteins of BMP and WNT signaling pathways that govern adipogenesis and its target genes. Bright field images of temporal adipogenic differentiation depicted that resistin provoked formation of hypertrophic adipocytes (Figure .6.3. A-C).

BMP and WNT signaling pathways predominantly regulate adipogenesis. As resistin alters the differentiation potentials of ADSC, an attempt was made to understand the temporal expressions of effector signaling molecules that regulate adipogenesis. Protein expressions of pSMAD1/5/8 and non-phosphorylated β catenin (active) proteins were evaluated on 4D, 12D and 22D of adipogenic differentiation. Expressions of pSMAD1/5/8 increased subsequently from UD, A4D to A12D, which indicated augmentation of adipogenesis in undifferentiated hADSC to preadipocyte stage. However, resistin treated hADSC did not confer any change on 4^{th}D (AR4D) and 12^{th}D (AR12D). Initially resistin diminished adipogenesis till pre-adipocyte stage as evident by reduced expression of this complex protein in AR4D and AR12D but demonstrated significant elevation on 22^{nd}D compared to their respective adipogenic controls (Figure 6.3. A. and B). These temporal variations in the expression of pSMAD1/5/8 complex denotes that resistin targets adipocyte maturation which provoke lipogenesis and hypertrophy in mature adipocytes. Further, temporal expressions of the regulatory protein of WNT signaling, activated non-phosphorylated β catenin levels which, was significantly elevated in UD exemplifying it as a stem cell marker and in maintenance of stemness. With progress in adipogenesis, β catenin levels aroused from A4D to A12D which, subsequently reduced on A22D. In resistin treated group expression of β catenin was altered in temporal manner and was significantly high on 22^{nd}D compared to its corresponding adipogenic control, as in the initial stage (4^{th}D) resistin embarked its marked reduction compared to adipogenic control A4D. These suggest that resistin has profound effects on preadipocyte to adipocyte stage and there is a plausible crosstalk between resistin and WNT signaling during adipogenesis (Figure 6.3. A. and C).

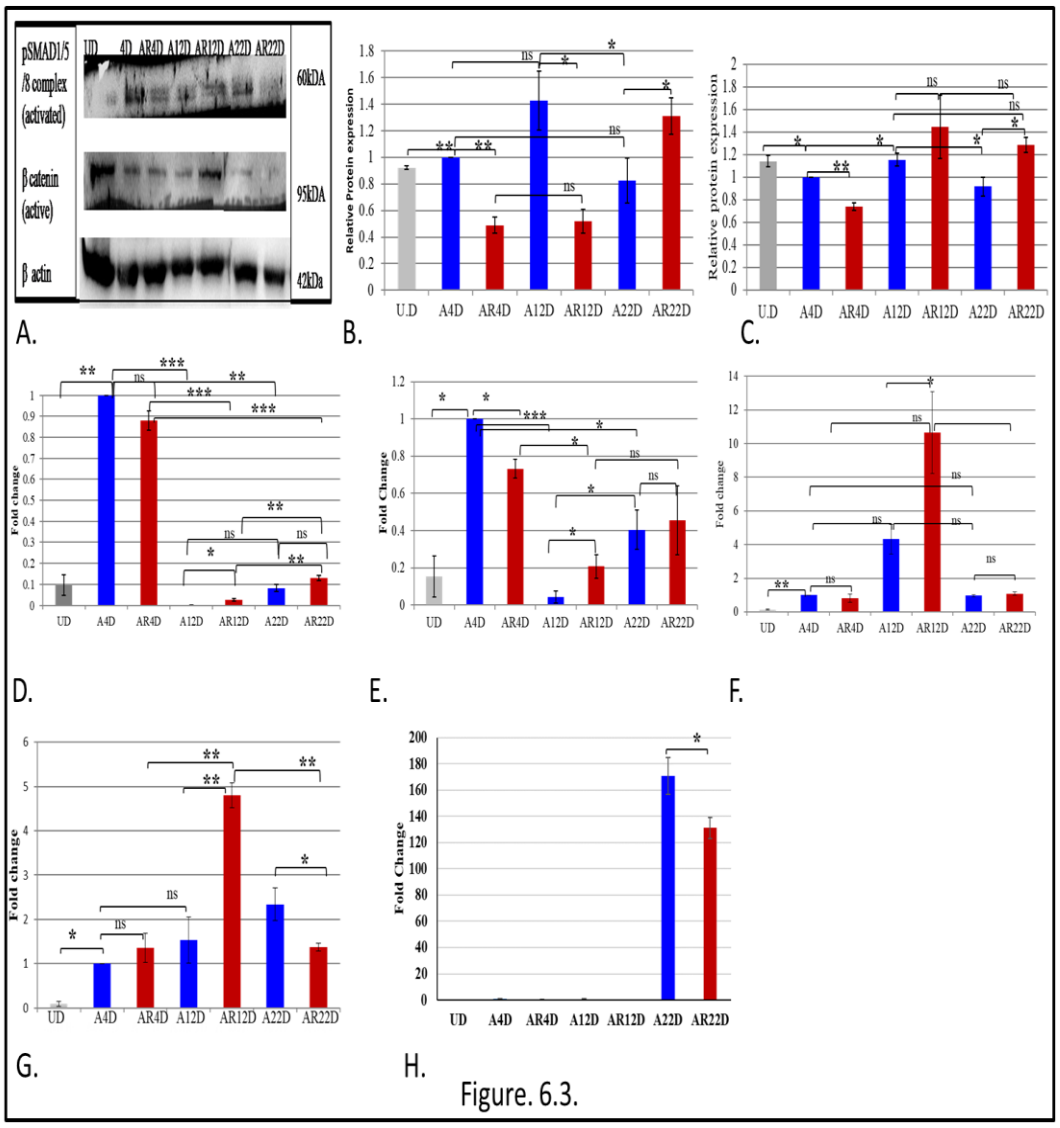

Figure. 6.3.

Figure 6. 3: Resistin induces adipogenesis through temporal expression of signaling proteins and ATFs. A. Western Blot analysis of signaling proteins. Protein bands of activated pSMAD1/5/8 complex, activated non-phosphorylated β Catenin and β Actin were observed by western blot. Densitometric analysis are depicted as relative protein expressions with β actin as control. B. Expressions of pSMAD1/5/8 protein complex C. Expressions of non-phosphorylated β Catenin n=3, *<0.05, **<0.01, ***<0.0001, ns-non significant. Temporal expressions of ATF. Expressions of adipogenic genes on 4^{th} D, 12^{th} D and 22^{nd} D as fold change± S.E.M. taking β actin as internal control. D. Gene Expressions of C/EBPβ E. Gene Expressions of C/EBPδ F. Gene expressions of PPARγ G. Gene Expressions of C/EBPα H. Gene Expressions of Adiponectin. n=3, p value, *<0.05, **<0.01, ***<0.001, ns-non significant. The groups under the study were UD- undifferentiated, A4D- adipogenic control at 4^{th}D, AR4D- resistin group at 4^{th}D during adipogenesis, A12D- adipogenic control on 12^{th}D, AR12D-resistin group on 12^{th}D, A22D- adipogenic control on 22^{nd}D, AR22D- resistin group on 22^{nd}D.

Once the activated RSMAD complex and β catenin proteins translocate into nucleus, they activate or repress ATF which govern adipogenesis. Therefore, gene expressions

of key ATFs were monitored during all the 3 time points of adipogenesis. C/EBPβ, one of the early transcription factor was significantly upregulated in both adipogenic control (A4D) and resistin treated group (AR4D) which then eventually decreased till 22D in both the groups (Figure. 6.3.D.). Similarly, C/EBPδ was intensely expressed on 4^{th}Din adipogenic control while downregulated in resistin treated group C/EBPδ (AR4D). With progression in adipogenesis, expressions of this transcription factor were reduced in adipogenic control on 12^{th}D. On the contrary, resistin upregulated C/EBPδ in pre-adipocytes on 12^{th}D and again spiked on 22^{nd}D(A22D), which evidently denotes its involvement in maintenance of adipocyte function (Figure.6.3. E.). Further, this upregulation of C/EBPδ induces later transcription factors, PPARγ and C/EBPα in resistin treated cells.

PPARγ, the master regulator of adipogenesis was examined during all three time points of adipogenesis. The expression of PPARγ was found to be at peak in A12D and AR12D compared to A4D and AR4D respectively which further declined in both the groups on 22^{nd} D (Figure. 6.3. F.). Similarly, the latter TF C/EBPα had also followed the same trend as that of PPARγ (Figure. 6.3.G.). These results suggest that resistin promotes adipogenesis and insulin resistance in adipocytes. Further, PPARγ dependent functional gene, adiponectin was examined. Adiponectin was expressed on 22^{nd}D of differentiation as observed in A22D, however in resistin group on AR22D, significantly reduced expression of adiponectin was observed (Figure. 6.3.H.) which denotes loss of insulin sensitivity in mature adipocytes. Thus, lipid laden hypertrophic adipocytes were formed in resistin treated group as confirmed by ORO staining.

6.3.3 Resistin induces osteogenesis through temporal expression of signaling proteins and OTFs.

ADSC effectively differentiate into osteocyte which is also regulated under several cellular cues. As observed in chapter 5. resistin upregulated osteogenesis on 14^{th}D of differentiation, therefore temporal effects of resistin on pSMAD1/5/8 complex and activated non-phosphorylated β catenin levels were monitored during osteocyte differentiation, which is also a relay phenomenon that converts ADSC to osteoblast and finally to osteocytes. Protein expressions were monitored by western blot (Figure 6.4.A.). Expression of pSMAD1/5/8 complex in osteogenic control (O6D) was steeply elevated in mature osteocytes at 14D (O14D) which states that pSMAD1/5/8 drives

osteogenesis in hADSC, whereas resistin treatment reduced expression of pSMAD1/5/8 on 6th D (OR6D) and 14thD (OR14D) (Figure 6.4. A. and B.).

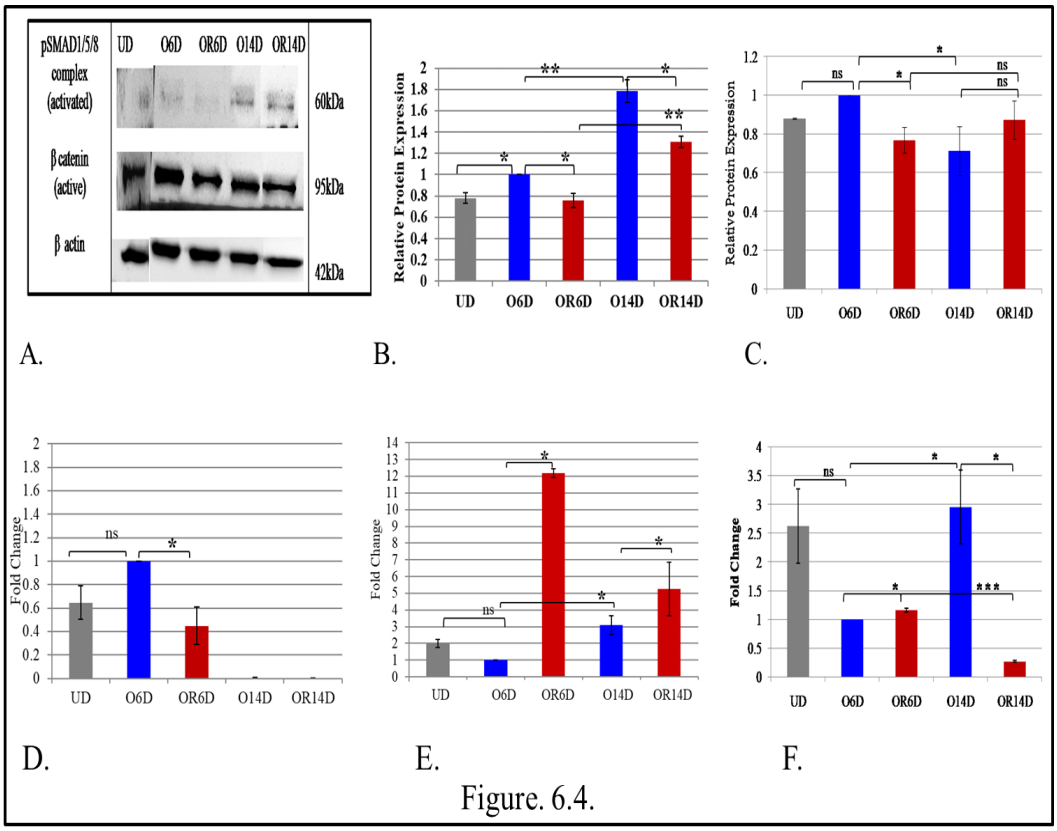

Figure. 6.4.

Figure 6. 4 Resistin induces osteogenesis through temporal expression of signaling proteins and OTFs. A. Western Blot analysis. Protein bands of activated pSMAD1/5/8 complex, activated non-phosphorylated β Catenin and β Actin were observed after 6thD and 14thD of osteogenesis. Densitometric analysis was performed and protein expressions were represented as relative protein expression ± S.E.M. with β actin as internal control. **B.** Expressions of pSMAD1/5/8 protein complex **C.** Expressions of non-phosphorylated β Catenin. **Temporal expressions of OTF**. Expressions of OTF on 6th D and 14th D as fold change ± S.E.M. taking β actin as internal control. **D.** Gene expressions of RUNX2. **E.** Gene expressions of Osterix. **F.** Gene expressions of SIRT-1. n=3, p value, *<0.05, **<0.01, ***<0.001, ns-non significant. The groups under the study were UD- undifferentiated hADSC, O6D- osteogenic control on 6thD, OR6D- resistin group on 6thD, O14D-osteogenic control on 14thD, OR14D- resistin group on 14thD.

These reduced expressions of SMAD complex indicates that osteocyte maturation and functions are hampered by resistin.

Activated β catenin of WNT signaling also plays an instrumental role in osteogenesis and hence was examined during osteogenesis in presence of resistin. There was no significant difference in UD and osteogenic control at 6D (O6D), which was later on significantly reduced in osteogenic control at 14D (O14D). In contrast, resistin

treatment showed reduced expression from initial time (OR6D) but with no significant difference in mature osteocytes (OR14D) (Figure 6.4.A and C).

RUNX2 is the well-established OTF that promptly induces osteogenesis and plays inevitable role during the initiation process of differentiation. Gene expression studies revealed that RUNX2 was significantly high in osteogenic control at 6D (O6D) compared to UD, whereas resistin treated cells expressed low RUNX2 (OR6D) which was further diminished on 14^{th} D both in osteogenic control and resistin treated cells (Figure. 6.4.D.). RUNX2 stimulates osterix, the later transcription factor which is responsible for maturation of osteocytes. Osterix was found to be significantly upregulated on 14^{th}D in osteogenic control (O14D) compared to (O6D) whereas resistin demonstrated stimulated expression on both 6^{th}D (OR6D) and 14^{th}D (OR14D) (Figure. 6.4.E.). SIRT-1 is another important gene that drives osteogenic differentiation. It was highly expressed in O14D compared to O6D but resistin treatment significantly downregulated its expression which mitigates insulin sensitivity and renders insulin resistance in osteocytes (Figure 6.4.F.).

The overall study suggests that resistin increases adipogenesis and osteogenesis of hADSC but not conventionally as it dysregulated signaling proteins throughout differentiation process. It also decreased functions of mature adipocyte and osteocyte as adiponectin and SIRT-1 were found to be decreased on resistin treatment.

6.3.4 SM modulates signaling pathway proteins and ATF

Resistin hampers maturation of adipocyte during obesity as evident in 3T3-L1 (Ikeda et al, 2013). Also, SM and Met ameliorated insulin resistance in hADSC, thus their effects were observed on signaling proteins and gene expressions of ATF in mature adipocytes obtained from resistin treated hADSC.

On 22^{nd}D of adipogenic differentiation, adipocytes formed were subjected for protein expressions of key signaling proteins governing differentiation, gene expressions of ATF and culture supernatants were analysed for levels of adiponectin and leptin through ELISA.

Western blot analysis revealed that activated pSMAD1/5/8 protein complex was elevated in resistin treatment compared to AC which showed that BMP signaling drives adipogenesis in hADSC. However, SM and Met significantly reduced

expression of this protein complex which supports the result that SM and Met inhibits adipogenesis (Figure 6.5. A. and B.). Activated β catenin levels were monitored and resistin group had high expressions but, SM and Met did not show any significant difference on its expression. Thus, SM and Met might not be interacting with WNT signaling during resistin mediated adipogenesis (Figure 6.5. A. and C.). Also, insulin signaling plays a role in differentiation. Activated insulin signaling promotes differentiation thus, IR and pAKT levels were observed. Resistin increased expressions of both IR and pAKT in 22^{nd}D adipocyte, which were concomitantly reduced by SM. pAKT was reduced by SM. IR expressions were not observed in Met group but pAKT was decreased. Here, reduced expressions of IR and pAKT demonstrated that insulin signaling is also downregulated as SM and Met inhibits adipogenesis (Figure. 6.5. A., D. and E.).

To understand the effects of resistin and signaling pathways on expressions of ATF, gene expression studies revealed that C/EBPβ, C/EBPδ, PPARγ were significantly elevated with concomitant reduction in C/EBPα in resistin treated cells compared to adipogenic control. SM increased expression of C/EBPδ, PPARγ and C/EBPα but no difference in C/EBPβ compared to resistin treated cells. However, Met reduced expression of C/EBPβ, increased PPARγ but did not confer any changes in C/EBPδ and C/EBPα (Figure 6.5. F.-I.).

To monitor functionality of adipocytes gene expressions and the secreted levels of adiponectin and leptin by adipocytes were measured. Resistin treated cells significantly downregulated adiponectin with concomitant elevation in expression of leptin gene. SM significantly upregulated adiponectin gene with concomitant reduced expression of leptin gene (Figure. 6.5.J. and K.). Met also embarked increase in expression of adiponectin but no significant difference in leptin expressions was observed. ELISA of adiponectin and leptin also demonstrated similar results, thus SM ameliorated dysregulated adipocyte metabolism (Figure 6.5. L and M.).

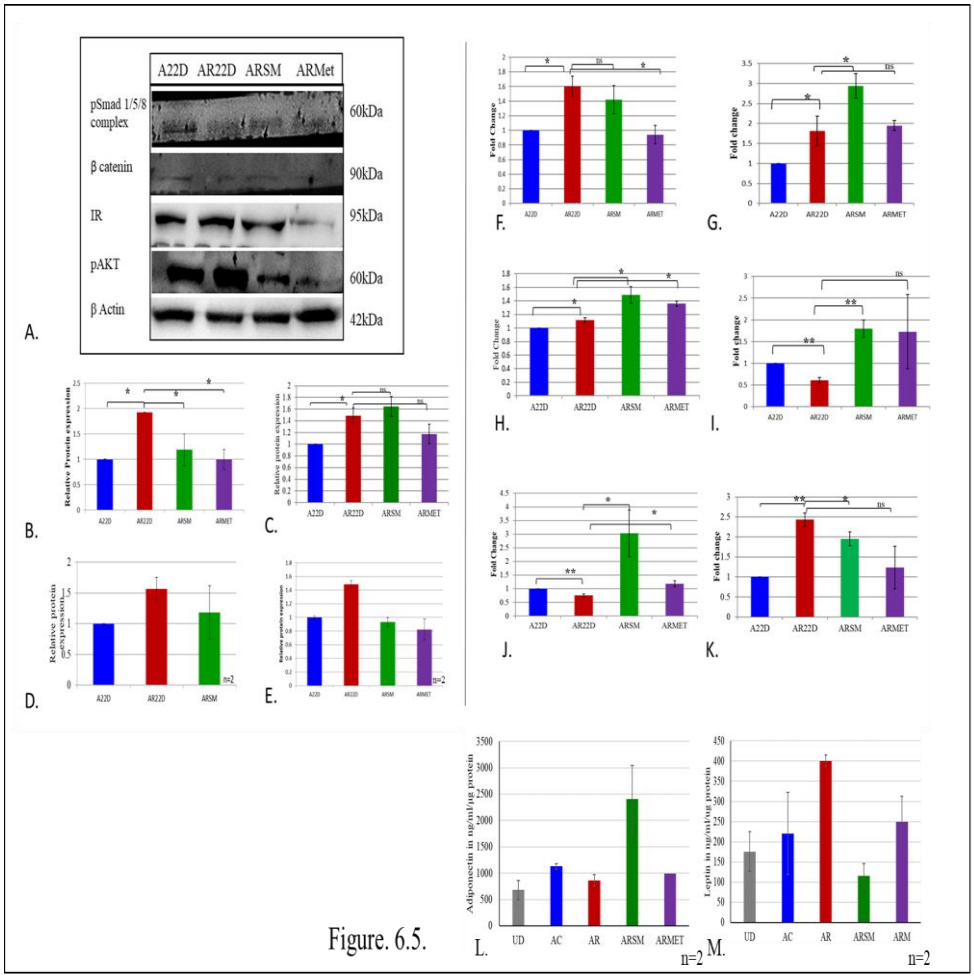

Figure. 6.5.

Figure 6. 5: SM modulates signaling pathway proteins and ATF. A. Western Blot analysis of signaling proteins. Protein bands of activated pSMAD1/5/8 complex, activated non-phosphorylated β Catenin, IR, pAKT and β Actin were observed in 22nd D of adipogenesis through western blot. Results are depicted as relative protein expressions ± S.E.M. with β actin as internal control. **B.** Expressions of pSMAD1/5/8 protein complex, **C.** Expressions of non-phosphorylated β Catenin. n=3, **D.** Expressions of IR, n=2, **E.** Expressions of pAKT, n=2. Temporal expressions of ATF. Expressions of adipogenic genes on 22nd D of adipogenesis depicted as fold change± S.E.M. taking β actin as internal control**. F.** Gene Expressions of C/EBPβ **G.** Gene Expressions of C/EBPδ **H.** Gene expressions of PPARγ **I.** Gene Expressions of C/EBPα **J.** Gene Expressions of Adiponectin. **K.** Gene Expressions of Leptin. n=3, p value, *<0.05, **<0.01, ***<0.001, ns-non significant. *<0.05, **<0.01, ns-non significant. Levels of adipokines in culture supernatants after 22nd D of adipogenesis. **L**. Levels of adiponectin represented as ng/ml/ug protein ± S.E.M., **M**. Levels of leptin represented as ng/ml/ug protein ± S.E.M. n=2.

The groups under the study were A22D- adipogenic control at 22ndD, AR22D-resistin group on 22ndD, ARSM- SM and Resistin treatment group observed on 22ndD, ARMet- Met and Res treatment group observed at 22ndD. AC-adipogenic control, UD-undifferentiated hADSC.

6.3.5. SM regulates signaling pathway and OTF

hADSC also differentiates efficiently into osteocytes. Resistin is also associated with pathogenesis of degenerative diseases like rheumatoid and osteo arthritis. Thus, BMP, WNT and insulin signaling which play role during osteogenesis were also monitored with expressions of key OTF.

On 14^{th}D of osteogenic differentiation, cells were harvested to observe the expressions of the signaling proteins as in adipogenesis. Restistin treatment reduced expression of pSMAD1/5/8 complex protein compared to OC. SM significantly increased the expression of this complex but Met rendered no significant change compared to resistin group respectively (Figure 6.6.A & B.). Resistin did not alter gene expression of β catenin but SM and Met significantly downregulated expressions of this protein compared to resistin group (Figure 6.6. A & C.). These results comply with the observations that SM and Met inhibits osteogenesis. Resistin downregulated expressions of IR and pAKT compared to osteogenic control. SM decreased the expressions of IR whereas Met increased IR compared to resistin. However, SM and Met both increased the levels of pAKT compared to their respective resistin group (Figure 6.6. A & D. and A & E.). Thus, the results indicate that SM exerts different mechanistic action in adipocyte and osteocytes in presence of resistin.

Gene expression studies revealed that expressions of RUNX2 was not observed on the 14^{th}D and resistin increased expressions of Osterix but reduced expressions of SIRT-1 compared to osteogenic control. SM and Met significantly reduced Osterix compared to resistin group. Further, SM and Met significantly induced expression of SIRT-1compared to resistin. These results are concurrent with the results that SM and Met at this particular concentration reduced ectopic osteogenic production of hADSC under the influence of resistin. However, both SM and Met increased the expression of SIRT-1 compared to resistin treated group, thus ameliorating resistin mediated insulin resistance in osteocytes (Figure 6.6. F. and G.).

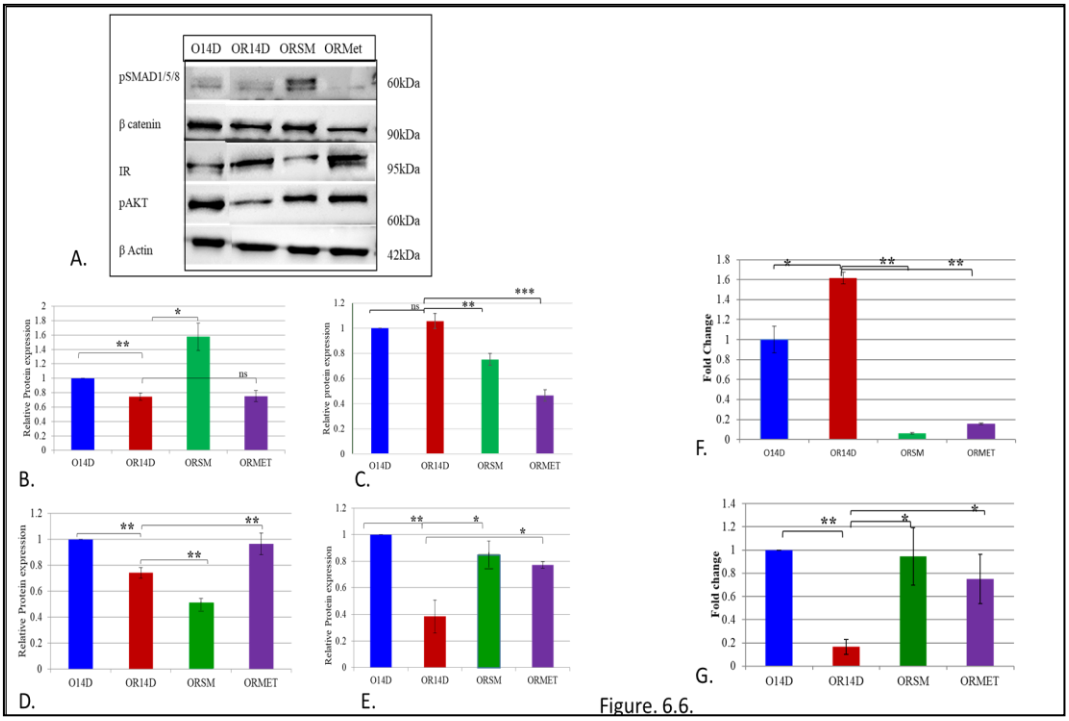

Figure. 6.6.

Figure 6. 6 SM regulates signaling pathway and OTF. A. Protein bands of activated pSMAD1/5/8 complex, activated non-phosphorylated β Catenin, IR, pAKT and β Actin were observed after 14D of osteogenesis through western blot. Densitometric analysis was performed and protein expressions were represented as relative protein expression ± S.E.M. with β actin as internal control. **B.** Expressions of pSMAD1/5/8 protein complex **C.** Expressions of β catenin, **D.** Expressions of IR **E.** Expressions of pAKT n=3, *<0.05, **<0.01, n=3, *<0.05, **<0.01; Temporal expressions of OTF. Expressions of OTF on 14thD as fold change ± S.E.M. taking β actin as internal control. **F.** Gene Expressions of Osterix. **G.** Gene Expressions of SIRT-1. n=3, p value, *<0.05, **<0.01.

The groups were O14D-osteogenic control on 14thD, OR-Resistin group on 14thD, ORSM- SM and Resistin group on 14thD, ORMet- Met and resistin group on 14thD.

6.4. DISCUSSION

Resistin assaults maturation, functions and metabolism of adipocytes (Ikeda et al., 2013). It also affects chondrocytes and osteocytes by activation of inflammatory pathways which culminate into degenerative diseases like rheumatoid and osteoarthritis (Thommesen et al., 2006). Being a major biologically active adipokine, its role on stemness and functionality in hADSC is still illusive. Attempts were made to explore its enigmatic effects on temporal expressions of key proteins of BMP and WNT signaling pathways along-with ATF and OTF were scrutinized during adipogenesis and osteogenesis in presence of resistin.

In the present study the modulatory effects of resistin on signaling pathways BMP and WNT were monitored in hADSC differentiation under the influence of resistin.

Temporal expressions of pSMAD1/5/8 determined that resistin embarked upregulation of this complex at the stage of adipocyte maturation only, as noted on 22^{nd}D which provides evidence of it being positively stimulating adipogenesis in hADSC. Till date, there are no reports on interaction between resistin and BMP signaling pathway, specifically during adipogenesis. Thus, the current results designate that resistin treatment enhances adipogenesis through activated pSMAD1/5/8 complex.

WNT signaling plays a significant role during adipogenesis. Resistin treatment depicted less expression of activated non-phoshorylated form of β catenin on 4^{th}D (AR4D) compared to its adipogenic control (A4D), which eventually increased in mature adipocyte state (AR22D) compared to adipogenic control (A22D). These abnormal expressions of BMP and WNT signaling during adipogenesis might be the cause of hypertrophic adipocytes with dysregulated functions.

Aligned with expressions of signaling proteins, ramification of resistin on temporal expressions of ATF were monitored by gene expression study. The early transcriptional factors C/EBPβ and C/EBPδ were highly expressed on 4^{th}D of adipogenic differentiation whereas, resistin demonstrated insignificant alterations of C/EBPβ on 4^{th} D with elevated expression on 12^{th}D whereas, downregulated C/EBPδ in initial time was then upregulated on 12^{th}D. These results indicate that resistin demonstrate differential expressions during early and pre-adipocyte formation stage of adipogenesis.

To the surprise elevated levels of PPARγ and C/EBPα on 12^{th}D in resistin treated cells instilled a major influence of resistin on pre-adipocyte stage. In view of this observation, it was earlier reported that resistin inhibition in 3T3-L1 and primary rat pre-adipocytes did not alter expressions of PPARγ and C/EBPα but alters lipid formation and adipocyte maturation by downregulating carbohydrate response element binding protein (ChREBP) (Ikeda et al., 2013) Moreover, resistin did not affect human pre-adipocyte differentiation (Isakson et al., 2009). Resistin treated hADSC exemplified reduced expression of C/EBPα at 22^{nd}D in mature adipocytes which indicates insulin resistance in adipocytes. This result is supported by the observation that C/EBPα increases insulin sensitivity, thus its downregulation inculcates insulin resistance in adipocytes (Matulewicz et al., 2017). Further, effects of resistin were observed on adiponectin gene levels, which was found to be significantly

downregulated in resistin treated cells which indicate loss of insulin sensitivity and functions of adipocytes. Thus, resistin induces insulin resistance and hampers maturation of adipocytes derived from hADSC. These results abide by the fact that diminished adiponectin levels cause insulin resistance and energy imbalance (Ghoshal & Bhattacharyya, 2015).

Apart from adipocyte metabolism, resistin also abrogates osteocyte metabolism, however effects of resistin on osteogenesis are contradictory (James, 2013; Kamiya et al., 2008). pSMAD1/5/8 complex expressions were elevated in both osteogenic control (O14D) and resistin treated cells (OR14D) compared to earlier time point. These results can be understood by the evidence that BMP signaling pathway regulates both osteoblastogenesis and osteoclastogenesis in an intricate manner (Kamiya et al., 2008). Moreover, BMP signaling illustrates diverse effects which are context dependent or depends on the cellular environment. There are contradicting reports regarding positive or negative association of BMP signaling in osteogenesis (James, 2013).

On the other end, expressions of β catenin were variable in resistin treated cells wherein, it was reduced significantly on 6^{th}D and increased non-significantly on 14^{th}D compared to their osteogenic control hADSC. Moreover, it has been explored that osteogenesis is augmented with pre-existing osteogenic machinery and does not require complex chromatin remodeling (Rauch et al, 2019). It was also explored that resistin increased osteocytes by formation of abnormal collagen Type I which results into subchondral obese phenotype leading to bone deformities (Philp et al., 2017).

Further, targeted OTFs were studied for their gene expressions. In the present study, RUNX2, Osterix and SIRT-1 were sequentially upregulated in osteogenic controls throughout osteogenesis. These results comply with the fact that RUNX2 is the initial transcription factor and osterix is the later transcription factor that drive maturation of osteocytes as observed in classical phenomena (Langenbach & Handschel, 2013). However, resistin treatment demonstrated variable results where RUNX2 was downregulated on 6^{th}D compared to its osteogenic control, whereas Osterix was profoundly expressed on 6^{th}D and 14^{th}D compared to osteogenic controls respectively. Thus, resistin might be stimulating the expression of osterix which may lead to development of subcondral obese phenotype responsible for degenerative diseases.

SIRT-1 also plays an important role during osteogenesis by stimulating RUNX2 (Zainabadi et al., 2017). It was significantly upregulated on 14^{th}D compared to 6^{th}D in osteogenic control, however resistin treatment diminished its expressions on 14^{th}D which demonstrate that resistin induced insulin resistance in osteocyte as SIRT-1 downregulates in insulin resistance condition (C. Sun et al., 2007; S. Zhou et al., 2018). Thus, the study indicates that resistin might also induce osteoclastogenesis in hADSC which could culminate in degenerative bone diseases.

The results of the signaling pathways and transcription factors which govern adipogenesis and osteogenesis revealed that resistin drives hADSC towards hypertrophic adipocyte formation and osteocyte differentiation but diminished their maturation and functionality which culminate into obesity and degenerative diseases.

We, further observed the effects of resistin, SM and Met on pSMAD1/5/8 complex of BMP and β catenin of WNT signaling pathways that govern adipogenesis and osteogenesis of hADSC. These proteins were monitored only on 22^{nd}D of adipogenesis and 14^{th}D of osteogenesis. pSMAD1/5/8 complex was elevated in resistin treated group on 22^{nd}D while SM and Met reduced this complex significantly. These results indicate that resistin activates BMP signaling which stimulate adipogenesis in hADSC. Supported by evidence that BMP signaling activate adipogenesis (James, 2013). Thus, it may be speculated that SM and Met reduced adipogenesis by downregulating pSMAD1/5/8 complex expressions.

Resistin also increased expressions of β catenin but SM and Met did not demonstrate any significant difference. Study suggested that resistin did not affect WNT signaling during 3T3-L1 differentiation into adipocytes (Hammarstedt et al., 2007). Resistin increased the expression of IR, pAKT on 22^{nd}D of adipogenesis. However, SM reduced expressions of both these proteins. These results could be explained by the observation that activated insulin signaling is required for adipogenesis (Klemm et al., 2001). Downstream ATF and OTF elucidated that resistin increased the expression of C/EBPβ, C/EBPδ and PPARγ while expressions of C/EBPα were significantly downregulated. Increase in these ATF reveal increased adipogenesis but downregulation of C/EBPα is a hallmark of insulin resistance (Matulewicz et al., 2017). Moreover, elevated expressions of C/EBPδ, PPARγ and C/EBPα by SM

evidently proves that this seco-iridoid enhanced insulin sensitivity in hADSC. Resistin downregulated adiponectin while it upregulated leptin and thus, mitigates maturation and functionality of adipocytes. Resistin in earlier reports showed increased circulating leptin and reduced adiponectin levels alongwith reduced insulin sensitivity in adipocytes and peripheral tissues (K.-H. Kim et al., 2004; H. K. Park et al., 2017). SM and Met very efficiently increased adiponectin levels and decreased leptin in adipocytes compared to those derived from resistin treatment. These results comply with our earlier results and other reports (Y.-W. Kim et al., 2006; Patel et al., 2013).

Further, when osteogenesis was observed under the influence of insulin sensitizers and resistin, it was deduced that resistin reduced expressions of pSMAD1/5/8 while, there was no significant difference in the expression of β catenin expression. SM increased the expression of pSMAD1/5/8 while it reduced expressions of β catenin in osteocyte at 14^{th}D of osteogenesis. These results suggest that SM inhibited osteogenesis as also evident by Alizarin red quantification. Insulin signaling proteins were downregulated in resistin group, and SM decreased the expressions of IR but increased the levels of pAKT on 14^{th}D of osteogenesis. These alterations in insulin signaling might have also contributed in SM mediated decreased osteogenesis. SM and Met both significantly reduced the expression of Osterix, thus inhibiting osteogenesis. These results are supported by the evidence that osterix drives osteogenesis from osteocytes (Sinha & Zhou, 2013). SIRT-1 when observed was significantly reduced in resistin treated osteocytes. SM and Met significantly increased the expression of SIRT-1 which denotes that these insulin sensitizers ameliorated resistin mediated insulin resistance in osteocytes. Zhou et al, have meticulously explained that SIRT-1 effectively induces insulin signaling by inhibiting PTPN-1 and serine threonine kinases (S. Zhou et al., 2018).

6.5 CONCLUSION

The study concluded that resistin elevated adipogenesis and osteogenesis by altering key proteins of BMP and WNT signaling at different time points of differentiation. It upregulated ATF but mitigated functions of adipocytes and induced insulin resistance. Similarly, it activated OTF but induced insulin resistance and thus hampered osteocyte formation. SM very efficiently ameliorated resistin mediated dysregulation in adipocytes by targeting SMAD complex and ATF, adiponectin and leptin. Thus, providing its anti-adipogenic activity and insulin sensitizing effect. SM also restored

lost functions of osteocytes by targeting SIRT-1. Several cues for further understanding the crosstalks between resistin, signaling pathways and SM which regulate adipogenesis and osteogenesis are generated which can be further explored.

SUMMARY OF CHAPTER 6: