MATERIAL AND METHODS

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Reagents

Human Resistin peptide was procured from ABCAM, Cambridge, U.K. Human recombinant TNFα was purchased from M. P. Biomedicals India Pvt. Ltd. Swertiamarin was isolated and well characterized in our lab (Patel et al, 2013). Pure metformin was generous gift from Sun pharmaceuticals. Ltd. DMEM F/12 (#12500062), Dulbecco's Modified Eagle media (DMEM) High Glucose (#12100046), DMEM Knock out (KO) (#10829018), Glutamax (#35050061) and Fibroblast growth factor (# PHG0024) were procured from GIBCO, Thermo Scientific, Massachusetts, USA. Rest of the chemicals, if mentioned otherwise, were acquired from Sigma Aldrich. Cell culture grade plastic wares were from NunClon, Thermo Fisher. All solvents were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd– India.

TRIZOL (#15596026) and High-Capacity (complementary DNA) cDNA Reverse Transcription Kit (#4368814) were purchased from Invitrogen, Thermo Fisher, USA. SYBR® Premix Ex Taq™ II (#RR82LR) was used from TAKARA, Clontech (Japan). Primers for Real Time PCR were synthesized from Europhins, India. Details of all the primers used in the study are mentioned in table No. 3.

The Sodium dodecyl sulfate (SDS)- poly acrylamide gel electrophoresis (PAGE) and Western blotting reagents were purchased from BIO-RAD, California, USA.

3.2 Isolation and purification of hADSC

Procedure for isolation of hADSC from adipose tissue/ lipoaspirates was adapted from (Zhu et al, 2013) with some modifications. Fine chopped adipose tissue fractions collected after centrifugations were digested with 1ug/ml Type I collagenase under mechanical shaking conditions at 37 (degree celcius) °C. The reaction was stopped by adding sterile culture media containing fetal bovine serum (FBS) followed by centrifugation at 2000 revolutions per minute (RPM) for 10 minutes at room temperature (RT). The pellet obtained was washed thrice with sterile phosphate buffered saline (PBS). The final pellet from tissue or stromal vascular fraction (SVF) from lipoaspirates was collected and plated onto tissue culture treated T25 flask with sterile DMEM F12 media with 10% FBS and 1% antibiotics (penicillin and streptomycin) in CO_2 incubator at 37°C with 5% CO_2. The media was changed after every 2-3 D. Cells were trypsinized at 70% confluency and at passage #2, the cells at

the density of 10^3 cells/cm^2 were shifted into DMEM Knock out (KO) complete media supplemented with 1X Glutamax and 1ng/ml FGF for better growth and maintenance of stemness of the cells.

3.3 Estimations of stored adipokine levels

Protein was extracted from adipose tissue/lipoaspirates as described previously (Lira et al, 2010) (Lira et al., 2010) (Lira et al., 2010) (Lira et al., 2010) (Lira et al., 2010) (Lira et al., 2010) (Lira et al., 2010) (Lira et al., 2010) (Lira, Rosa et al. 2010). Briefly 200mg of adipose tissue was homogenized in radio-immunoprecipitation assay (RIPA) buffer (0.625% Nonidet P-40, 0.625% sodium deoxycholate, 6.25mM sodium phosphate and 1mM Ethylenediaminetetraacetic acid (EDTA) containing protease inhibitor cocktail), pH 7.4 followed by centrifugation to collect the supernatant. Stored levels of adiponectin, leptin and resistin were measured by Enzyme linked Immunosorbent Assay (ELISA) as per the manual's instruction (Human Acrp30, # ELH-Adiponectin, Human Leptin ELISA, # ELH-Leptin and Human Resistin ELISA kit, # ELH-Resistin, Ray- Biotech, U.S.A) and the concentrations of all the adipokines were calculated per µg(microgram) protein of sample as estimated by Bradford method.

3.4 Immunophenotyping of the isolated cells by flow cytometry

At passage 3 (P#3), $1x10^5$ cells were trypsinized and the cell pellets were incubated with primary antibodies of $CD44^+$ Phycoerythrin (PE) labelled, $CD105^+$- Fluorescein isothiocyanate (FITC) labelled, $CD34^-$FITC labelled (BD Bioscience, USA) and $CD31^-$FITC labelled (Biolegend, USA) at 4ºC for 45 mins in dark. Cells were washed by centrifugation at 1000rpm at 4°C and re-suspended in staining buffer. The fluorescent intensities were measured on Fluorescence-activated cell sorting (FACS) ARIA III, Becton and Dickinson (BD), USA using 488nm laser with PE and FITC filters and 10000 events were recorded. The data was analysed by Flo Jo Software.

3.5 Growth Curve experiment

Freshly grown hADSC from chADSC and ohADSC were seeded at the density of 200 cells/cm^2. Cell count was taken at an interval of 48 hrs till 192 hrs. Population doubling time (PDT) was calculated as per the formula PDT=ln2*T/ln(NT/N0) (Legzdina et al, 2016). A graph of growth curve was plotted against time (H)/ No of cells $\pm$ standard error of mean (S.E.M).

3.6 Cytokines and insulin sensitizers treatment on hADSC

hADSC were treated with cytokines (resistin and TNFα) and insulin sensitizers (SM and Met). Sterile human resistin peptide was used at a concentration of 50ng/ml (Isakson et al, 2009) while TNFα was used at a concentration of 10ng/ml (Lee et al, 2010). Insulin sensitizers like SM and Met were used at a concentration of 25µg/ml (Patel et al, 2016) 64.58µg/ml (500uM) (Zhou et al, 2015) respectively to treat hADSC. Treatment regime for cytokines and insulin sensitizers are mentioned in their respective experiments.

3.7 Induction of insulin resistance in hADSC

hADSC at P#3 from healthy control (F) of BMI 23 were treated with TNFα or resistin for 48H to induce insulin resistance. The insulin resistance was confirmed by reduced expressions of insulin signaling proteins by western blot analysis (3.2.16) and reduced glucose uptake by glucose uptake assay (3.2.10).

3.8 MTT Assay

hADSC at P#3 were seeded in 96 well plate at a cell density of 2000 cells/well and were treated with TNFα or resistin for 48H, 96H and 168H. Post treatment 100ul of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added at a final concentration of 0.5mg/ml and incubated for 3H in dark after. Further, 100ul of dimethyl sulfoxide (DMSO) was added, mixed properly and the absorbance was measured at 595nm and 525nm on ELISA plate reader (Thermo Scientific). DMSO was taken as blank and results were depicted as % proliferative cells $\pm$ S.E.M, where control cells were considered to be 100% proliferative.

3.9 Cell Cycle Analysis

hADSC were treated with respective cytokines and insulin sensitizers for 48H and 96H. Cells from all the treated groups were harvested and fixed with 70% ethanol. The cell pellets were washed and incubated with 50µg/ml RNAse for 30 minutes at 37°C. The cells were then stained with propidium iodide (PI) (50ug/ml) for 45 minutes at RT in dark followed by acquisition on FACS ARIA III using PI filter for cell cycle measurement. Cells were selected on the basis of forward scatter (FSC)/ side scatter (SSC) plot. Singlets were selected from Doublet Discrimination Plot. Control cells were set in G0/G1 phase and relative analysis was done for resistin treated cells.

Results were depicted from pooled 3 biological replicates of each group and denoted as % population of cells in a particular cell cycle phase.

3.10 Glucose uptake Assay

hADSC were subjected to glucose uptake assay as per (Blodgett et al, 2011). $1x10^3$ hADSC / well of 96 well plate were seeded. TNFα and resistin treatment were given for 48H followed by treatment of SM and Met for 24H in dose dependent manner. Post treatment, media was aspirated and cells were washed with sterile PBS. Cells were starved by addition of 100ul of glucose free, phenol red free and serum free media in each well. 100 nM insulin was added to the cells for 20 minutes followed by addition of 10μM of 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) NBDG-FITC for 1H. Cells were lysed with Na^+ buffer (140mM sodium chloride (NaCl), 5mM potassium chloride (KCl), 2.5mM calcium chloride ($CaCl_2$), 1mM magnesium sulfate (MgSO4), 1mM Potassium dihydrogen phosphate (KH2PO4) and 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4)) and chilled lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 40mM KCl, 20 mM Tris, pH 7.4). Fluorescence of FITC was measured at 535nm emission in multimode plate reader. 1ug/ml Hoescht dye was added to measure DNA content and intensity was measured at 405nm. Cells without 2-NBDG were taken as blank and background was nullified. The fluorescent glucose intensity was divided by that of DNA content to obtain glucose uptake values which were expressed as % glucose uptake by cells. Glucose uptake in control cells was considered to be 100% and graph was plotted as % glucose uptake $\pm$S.E.M.

3.11 RNA extraction and Gene expression studies

Isolation of total RNA from all the groups was carried out using Trizol Reagent. First strand cDNA was synthesized from 2ug of total RNA by High Capacity cDNA Reverse Transcription Kit. Real Time polymerase chain reactions (PCR) of lipogenic genes were carried only from AT whereas gene expressions of inflammatory markers were examined both from AT and hADSC. Gene expressions of ATF, pluripotent markers, inflammatory markers, MYF-5, lipogenic genes and OTF were carried out from hADSC. Details of all the gene amplifications are mentioned in (Table No. 3)

No table of figures entries found. Genes were amplified in Real Time PCR 7500, Applied Biosystems using SYBR premix Ex Taq II TAKARA, #RR820A, TAKARA BIO INC, USA. ACTB (β actin) gene was taken as internal control to obtain ΔCt. Fold

change was calculated from ΔΔCt of the gene and graphs were plotted as fold change for target gene expression.

3.12 Adipocyte differentiation

hADSC were seeded in the tissue culture treated culture dishes till they became 100% confluent in DMEM high glucose (Gibco) media having 10% FBS and 1% antibiotic. After the cells became over confluent, adipocyte differentiation was induced by adding adipogenic cocktail (10µg/ml insulin, 1µM dexamethasone, 500µM 3-isobutyl-1-methylxanthine (IBMX) and 500µM indomethacin) for 22nd D. Lipid droplets were stained with Oil Red O (ORO) stain and observed under 20X objective, inverted phase contrast microscope and quantified.

3.13 ORO staining

After 22 day of adipocyte differentiation, media was aspirated and PBS wash was given. Cells were fixed with 4% PFA and incubated at 4°C for 3-5 minutes. Cells were treated with 70% ethanol for 5 minutes, which was then aspirated and the monolayers of adipocytes were air dried. 1ml of ORO stain was added and incubated for 15 minutes. Cells were washed with distilled water (d/w) and excess stain was removed. Lipid droplets were stained red in colour as observed in inverted phase contrast microscope using 20X objective lens. d/w was aspirated and air dried. Then 100% isopropanol was added and ORO stain was extracted and quantified at 450nm. The graph of relative optical density (O.D) of ORO stain $\pm$ S.E.M was plotted where adipogenic control was considered as 1.

3.14 Osteogenic differentiation

hADSC at P#3 were seeded till they attained over confluency. Osteogenic differentiation was induced by adding osteogenic cocktail (1µM dexamethasone, ascorbic acid (50µg/ml) and 10mM β glycerophosphate) for 14th D. (Legzdina et al, 2016). Calcium deposition was observed by Alizarin Red (Sigma-Aldrich) staining and quantified.

3.15 Alizarin Red Staining

On 14th D of osteogenic differentiation, media was aspirated and cells were washed with PBS. Cells were fixed by adding 4% PFA and incubated at 4°C. Wash was given with d/w. 1ml Alizarin Red stain was added and incubated for 30 minutes at RT. Extra stain was removed by giving washes with d/w. Calcium deposition was stained in red

as observed under 20X objective of Inverted Phase Contrast microscope. 4M Guanidium hydrocholic acid was added and alizarin red stain was extracted and O.D. was taken at 450nm. Results were depicted as O.D ± SEM, where control cells were considered to be 1.

3.16 Protein expression studies

Proteins extracted from adipose tissue for adipokine estimation were used for western blot analysis of insulin signaling proteins. hADSC after respective experiments were harvested and washed with PBS. The cells were lysed by adding Lamelli lysis buffer, followed by sonication giving 45% amplitude and 2sec pulse with on off cycle. 3 cycles were given for lysis of nucleic acid. Brief spin was given and protein estimation was done by Bradford method (Bio-Rad, USA). 20ug of protein was separated and transferred on nitrocellulose membrane (Thermo Inc). Blots were incubated with blocking buffer (5% fat free skimmed milk powder with PBS containing 0.1% Tween-20) for 1H at RT. The blots were probed with primary antibodies of insulin receptor (IR), pIR, pAKT, Total AKT, non-phosphorylated β catenin, pSMAD 1/5/8 and β Actin (CST, Thermo and Sigma) for overnight at 4°C on mild rocking condition

β Actin was taken as internal control, incubated. Blots were washed four times with PBST. Respective secondary antibodies either anti-rabbit or anti-mouse horseradish peroxidise (HRP) conjugated IgG were used (Table No. 4). Protein bands were visualised using enhanced chemiluminiscence (ECL) reagent (Bio-Rad). The image was captured by Alliance 4.7 UVI Tec Chemidoc. Densitometric analysis was carried out by Image J software.

3.17 Statistical Analysis

The results are represented as mean ± S.E.M, significance was calculated using student's T-test between the two groups and denoted as $p< 0.05$ -*, $p<0.01$- **, $p<0.0001$-***. The statistical analysis was done using Microsoft excel 2016 software and graph pad prism. Number of observations of the experiment is mentioned in respective figure legends.

TABLE 3. 1

Table 3. Details of the primers used in Real Time PCR

Sr. No.	Gene	Source	Forward Primer	Reverse Primer	Accession Number	Amplicon size (bp)
1	*CEBPB (ATF)*	hADSC, hAT	CAAAACTTTGGCACTGGGGC	TAAATAACACCACGGGCGGG	NM_005194.3	137
2	*CEBPD (ATF)*	hADSC	GGAGAGACTCAGCAACGACC	TTGCGCTCCTATGTCCCAAG	NM_005195.3	120
3	*PPARG (ATF)*	hADSC, hAT	GTGGCCGCAGATTTGAAAGA	TGATCCCAAAGTTGGTGGGC	NM_138712	138
4	*CEBPA (ATF)*	hADSC	TATAGGCTGGGCTTCCCCTT	AGCTTTCTGGTGTGACTCGG	NM_004364.4	94
5	*SREBP-1F (SREBP-1c) (ATF)*	hADSC	TGCATTTTCTGACACGCTTC	CCAAGCTGTACAGGCTCTCC	NM_001005291	171
6	*SIRTUIN-1 (SIRT-1) (ATF)*	hADSC	AGGCCACGGATAGGTCCATA	GTGGAGGTATTGTTTCCGGC	NM_012238.4	103
7	*DLK-1 (PREF1) (ATF)*	hADSC	CGGCTTCATCGACAAGACCT	GTGAGACCTGTGAACTCGGG	NM_003836.6	135
8	*RUNX2 (OTF)*	hADSC	CCGGAATGCCTCTGCTGTTA	TGTCTGTGCCTTCTGGGTTC	NM_001278478.1	192
9	*MYF-5*	hADSC	ATGCCATCCGCTACATCGAG	ATTCGGGCATGCCATCAGAG	NM_005593.2	132
10	*CCL2 (MCP-1) (IM)*	hADSC, hAT	CCCAAAGAAGCTGTGATCTTCA	TCTGGGGAAAGCTAGGGGAA	NM_002982.3	183
11	*IKBKB (IKKβ) (IM)*	hADSC	ATGAATGCCTCTCGACTTAG	CCAGTTCTCACTCTTCTTG	XR_949402.3	125
12	*NLRP3 (IM)*	hADSC, hAT	AGAAGCTCTGGTTGGTCAGC	CAAGGCATTCTCCCCCACAT	NM_001079821.2	113
13	*HIF1 (IM)*	hADSC, hAT	AACTGAGAGGTTGAGGGACG	ACGTTCAGAACTTATCCTACCAT	NM_001243084.1	179
14	*SOX2*	hADSC	ATCAGCATGTATCTCCCCGG	TACCGGGTTTTCTCCATGCT	NM_003106.3	241
15	*POU5F1 (OCT3/4)*	hADSC	CAAAGCAGAAACCCTCGTGC	CTCGGACCACATCCTTCTCG	NM_001173531.2	164
16	*NANOG*	hADSC	CAGCTGTGTGTACTCAATGATAGATTT	CAACTGGCCGAAGAATAGCAATGGTGT	NM_001355281.1	178
17	*FAS (lipogenic gene)*	hAT	CACAGGGACAACCTGGAGTT	ACTCCACAGGTGGGAACAAG	NM_004104	97
18	*ACC-1 (lipogenic gene)*	hAT	TTTAAGGGGTGAAGAGGGTGC	CCAGAAAGACCTAGCCCTCAAG	NM_198834	171
19	*LEPTIN (lipogenic gene)*	hAT	ACACCAAAACCCTCATCAAGA	GAAGGCAAGCTGGTGAGGA	NM_013076.2	184
20	*ADIPOQ (ADIPONECTIN) (lipogenic gene)*	hAT	TGACCAGGAAACCACGACTC	CCCTTAGGACCAATAAGACCTGG	NM_001177800.1	184
21	*RESISTIN (lipogenic gene)*	hAT	AGCCATCAATGAGAGGATCCA	TCCAGGCCAATGCTGCTT	NM_020415	70
22	*Osterix (SP7) (OTF)*	hADSC	TCCCTGCTTGAGGAGGAAG	GAGTTGTTGAGTCCCGCAGA	NM_001173467.2	106
22	*ACTB (β ACTIN)*	hADSC, hAT	ACTCTTCCAGCCTTCCTTCC	CGTACAGGTCTTTGCGGATG	NM_001101	101

Note: For simplicity genes are depicted in regular nomenclature in all the chapters

TABLE 3. 2

Details of antibodies for Western Blot analysis						
Sr. No.	**Name of the antibody**	**Antibody type**	**Company and Catalog (#)**	**Isotype**	**Mol. Wt (Kda)**	**Dilution**
1	Insulin receptor β (Anti-CD220)	Primary polyclonal	BD Transduction Laboratories, #-611277)	Rabbit	95	1:1000
2	Phospho-insulin receptor (Y-1361)	Primary polyclonal	Thermo Fisher #-PA5–35787	Rabbit	95	1:1000
3	PI3 kinase p85	Primary Monoclonal	Cell Signaling Technology, # 13666	Mouse	85	1:1000
4	Phospho-Akt (Ser473)	Primary Monoclonal	Cell Signaling Technology, # 4060	Rabbit	60	1:1000
5	Glut4 (C-terminal)	NA	Sigma Aldrich, # 4048	Rabbit	58	1:1000
6	pSMAD1/5/8 (Ser463/465)	Primary Polyclonal	Millipore, Merck, #AB3848	Rabbit	52-58	1:1000
7	β catenin (Non-Phosphorylated)	Primary Polyclonal	Sigma Aldrich, # SAB4500545	Rabbit	85	1:1000
6	β actin	Primary Monoclonal	Thermo Scientific -# MA1–91399	Mouse	42	1:10000
7	Anti-Rabbit IgG	Goat-Anti Mouse Poly-HRP	Thermo Scientific # 32260	Goat		1:2500
8	Anti-mouse IgG	Goat-Anti Mouse Poly-HRP	Thermo Scientific # 32230	Goat		1:5000