INDIAN NATIONAL SCIENCE ACADEMY

Bahadur Shah ZafarMarg, New Delhi 110 002

FINAL PROGRESS REPORT

for Research support for grant to an INSA Young Scientist Medal Awardee

1. Name of the Young Scientist : Dr. Suman Dasgupta
Designation : Assistant Professor
Address : Department of Molecular Biology & Biotechnology
Tezpur University, Tezpur -784028
Napaam, Dist: Sonitpur, Assam
Tel. : +91-9954332278 (M)
+91-3712-275401 (O)
Fax : +91-3712-267005/267006
Email : suman@tezu.ernet.in
suman.dsut@gmail.com

2. Date of Birth : 17/03/1980

3. Year of receipt of INSA Young Scientist Medal: 2012

4. Field of Specialisation : Type 2 diabetes mellitus

5. Title of the Research Project : ***"Role of transcription factors IRF8 and KLF4 in lipid induced adipose tissue inflammation and insulin resistance".***

6. Duration of the Project : 3 years, January 2015 - December 2017

Background: Adipose tissue dysfunction and inflammation have been identified as major players in insulin resistance and type 2 diabetes, however, the underlying mechanism of their interrelationship is not clear. It is now well understood that adipose tissue is not simply a storage depot for excess calories but are also endocrine organs, with multiple metabolic roles in regulating whole-body physiology. Two elegant studies in this direction demonstrated that dysfunctional adipose tissue of obese humans and mice is characterized by a striking accumulation of macrophages. Accumulating macrophages are highly activated, with increased expression of a large array of proinflammatorycytokine genes. These proinflammatory cytokine genes inhibit insulin signaling by serine phosphorylation of insulin receptor substrate 1 (IRS-1). Although, the transcription factors are known to involve in governing macrophage biology, we therefore interested to study the role of transcription factors, IRF8 and KLF4 in macrophage polarization status and their involvement of in adipocyte dysfunction and insulin resistance.

Objectives

(i) To study the role of IRF8 and KLF4 transcription factors in obesity induced macrophage infiltration and its polarization from anti-inflammatory M2 state to proinflammatory M1 state in adipose tissue.

(ii) To evaluate the role of transcription factors IRF8 and KLF4 in modulating the expression of various adipokines and cytokines that are linked with the insulin resistant state of adipocytes.

(iii) To study the role of IRF8 and KLF4 in FFA induced adipose tissue inflammation and adipocyte dysfunction in term of adipogenesis, lipogenesis and lipolysis.

Detailed Research Report

To study the involvement of transcription factors in M1 polarization status of macrophages, we treated RAW264.7 macrophageswith LPS in presence or absence of cycloheximide (CHX). LPS significantly induces the upregulation of TNFa and IL6 proinflammatory cytokine genes. Pre-incubation of protein synthesis inhibitor, CHX, efficiently blocked the action of LPS (**Fig. 1**).

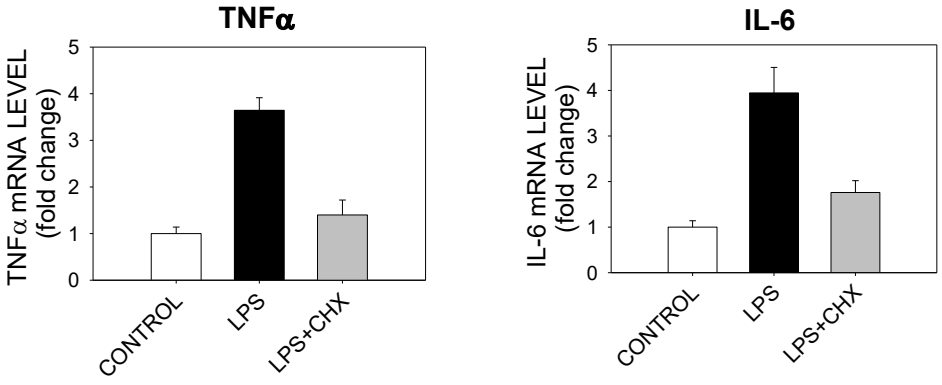

Fig. 1: Effect of CHX on LPS induced macrophage M1 polarization status. TNF-α and IL-6 gene expression. Quantification of TNF-α and IL-6 mRNA expression in RAW264.7 in response to LPS without or with CHX by RTqPCR.

To have more insight, specifically about the role of IRF8, on LPS induced macrophage M1 polarization status, we silenced IRF8 gene in RAW264.7 cells and then treated with LPS. In comparison to control siRNA, IRF8 knockdown RAW264.7 macrophage cells showed suppression of LPS action on TNF-α gene expression. Although, LPS significantly upregulates the expression of IL-6 but it is very intriguing to observe that knockdown of IRF8 could not able to prevent the stimulatory action of LPS (**Fig. 2**). These result suggest that TNF-α gene expression is under the direct transcriptional control of IRF8 but the situation is not the same in case of IL-6 gene expression.

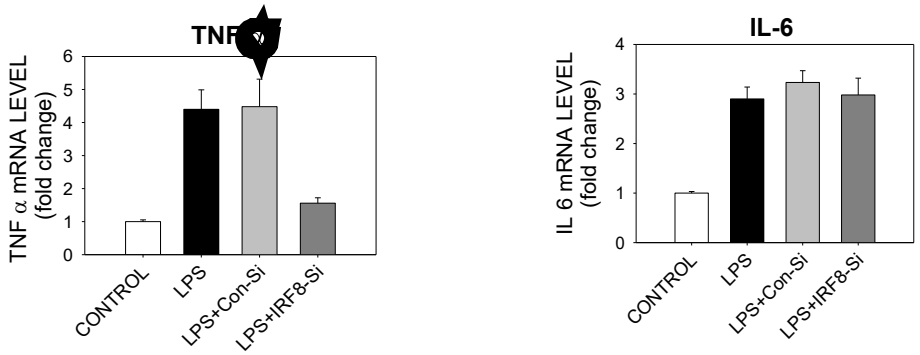

Fig. 2: Effect of IRF8 silencing in proinflammatory cytokine gene expression. Quantification of TNF-α and IL-6 mRNA expression in IRF8 knockdown RAW264.7 macrophage cells in response to LPS by RT-qPCR.

Increased level of macrophage infiltration to the adipose tissue is a characteristic feature of inflamed adipose tissue which closely related to the impairment adipocyte function. MCP1 is widely known as master regulator that drives macrophages into the adipose tissue. To investigate the involvement of IRF8 and KLF4 in obesity induced macrophage infiltration to the adipose tissue, RAW264.7 macrophages were transfected with control siRNA (siCon) or IRF8 siRNA (siIRF8) or KLF4 siRNA (siKLF4) followed by the incubation with MCP1 for 4 hours in a 5µm transwell Boyden chamber. IRF8 or KLF4 silenced macrophages did not produced any significant changes on MCP1 induced macrophages migration in the Boyden chamber (**Fig. 3**).

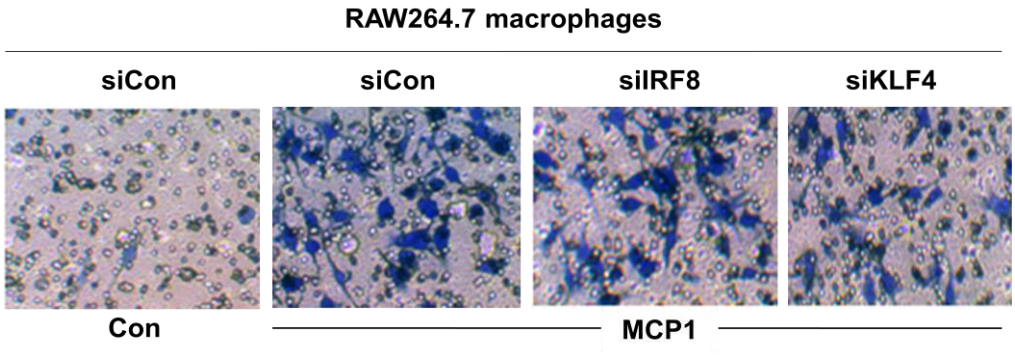

Fig. 3: RAW264.7 macrophages were transfected with control siRNA (siCon) or IRF8 siRNA (siIRF8) or KLF4 siRNA (siKLF4) followed by the incubation with MCP1 for 4 hours in a 5µm transwell Boyden chamber and allowed cells to migrate through the porous membrane.

The early and late transcription factors involved in adipogenesis are C/EBPβ and PPARγ, they are responsible for the formation of new adipocytes. It is known that adipocytes in obese individuals becomes hypertrophic and uptakes more FFA's from circulation and it has been validated from our experiment that high lipid uptake leads to significant down regulation in the expression of the early and late transcription factors i.e. C/EBPβ and PPARγ in high fat diet (HFD) fed mice as compared to standard diet (SD) fed mice (**Fig. 4**). Thus formation of new adipocyte has been greatly reduced because of consuming high amount of free fatty acid.

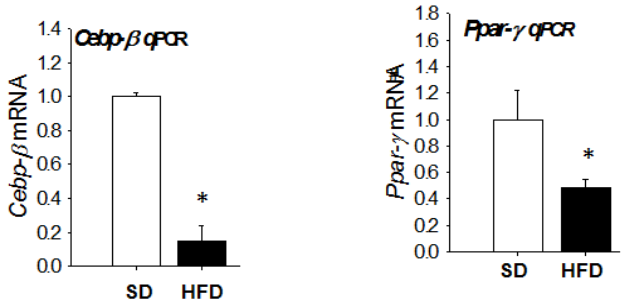

Fig 4: The expression level of early and late transcription factor involved in adipogenesis i.e. C/EBPβ and PPARγ from adipose tissue of 3-week-old HFD- or SD-fed mice. β-actin expression was taken as internal control. Data are shown as the mean ± SD of three independent experiments.

We have also observed that down regulation of these transcription factors expression also modulate the expression pattern of adipogenic markers such as adiponectin, CD36 and aP2. Adiponectin which is an important insulin sensitive adipokine released by adipocytes has decreased almost five folds in HFD as compared to SD mice. CD36 expression also corroborate this showing a significant decrease in HFD mice compared to SD mice. Expression of aP2/FABP4, which is involved in transporting the FFA's from circulation inside cell is also showing reduced expression in HFD mice as compared to SD mice (**Fig. 5**).

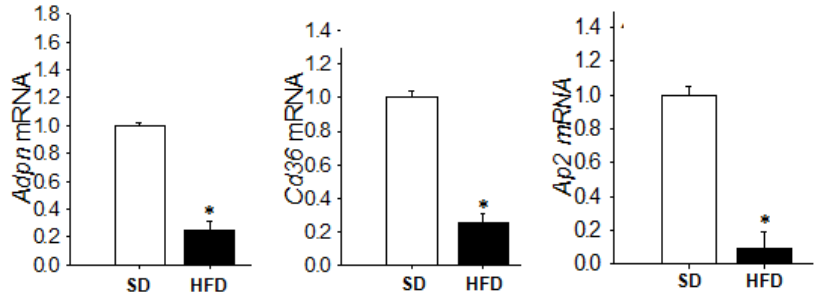

Fig. 5: The expression profile of adipogenic markers such as adiponectin, cd36, and aP2 from adipose tissue of 3-week-old HFD- or SD-fed mice. β-actin expression was taken as internal control. Data are shown as the mean ± SD of three independent experiments.

We have also checked the expression of different pro-inflammatory cytokines that are released during adipose tissue inflammation. We have seen a significant increase in the expression of TNFα and IL-6 in HFD mice as compared to SD mice (Fig. 5). Since, Fetuin-A (FetA) that acts as a mediator linking FFA and TLR4 and activates the inflammatory pathway by activating NF-κB, a transcription factor that initiates the expression of pro-inflammatory cytokines like IL-6, TNFα etc, we have analysed the gene expression of FetA and observed almost five folds enhanced level of FetAin HFD as compared to SD mice (**Fig. 6**).

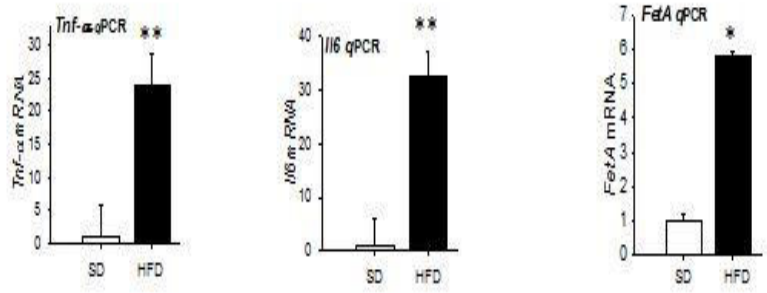

Fig. 6: The expression level of pro-inflammatory cytokines such as TNF-α, IL-6 and Fetuin-A from adipose tissue of 3-week-old HFD- or SD-fed mice. β-actin expression was taken as internal control. Data are shown as the mean ± SD of three independent experiments.

To observe involvement of IRF8 and KLF4 in obesity induced adipose tissue inflammation, 3T3L1 adipocytes were transfected with control siRNA (siCon) or IRF8 siRNA (siIRF8) or KLF4 siRNA (siKLF4) followed by the treatment with a saturated free fatty acid palmitate or lipopolysachharide (LPS) for 6 hours. On termination of incubations cells were harvested, lysed, supernatant was isolated and protein content was estimated by Lowry et. al (1951). Total 60 μg of protein was run on SDS-PAGE followed by the western blotting with phospho-NF-κBp65 or phospho-IκBα. β-actin was used as internal control. Both LPS and FFA (palmitate) significantly induces the phosphorylation of NF-κBp65 and IκBα in control siRNA transfected cells. Interestingly, increased abundance of phospho-NF-κBp65 and phospho-IκBα in response to LPS and FFA were attenuated in IRF8 silenced cells (**Fig. 7A**), however such impairment was not observed in KLF4 knockdown cells (**Fig. 7B**). To investigate further, we performed κB luciferase activity to estimate the NF-κB transactivation potential in response to LPS and FFA. LPS and FFA induced increased κB luciferase activity was prevented in IRF8 silenced cells (**Fig. 7C**), however such impairment was not observed in KLF4 siRNA transfected cells (Fig. 1D). These results corroborated with the phosphorylation status of NF-κBp65 and IκBα level. All these result strongly suggest that IRF8 play a crucial role in LPS and FFA induced adipocyte inflammation.

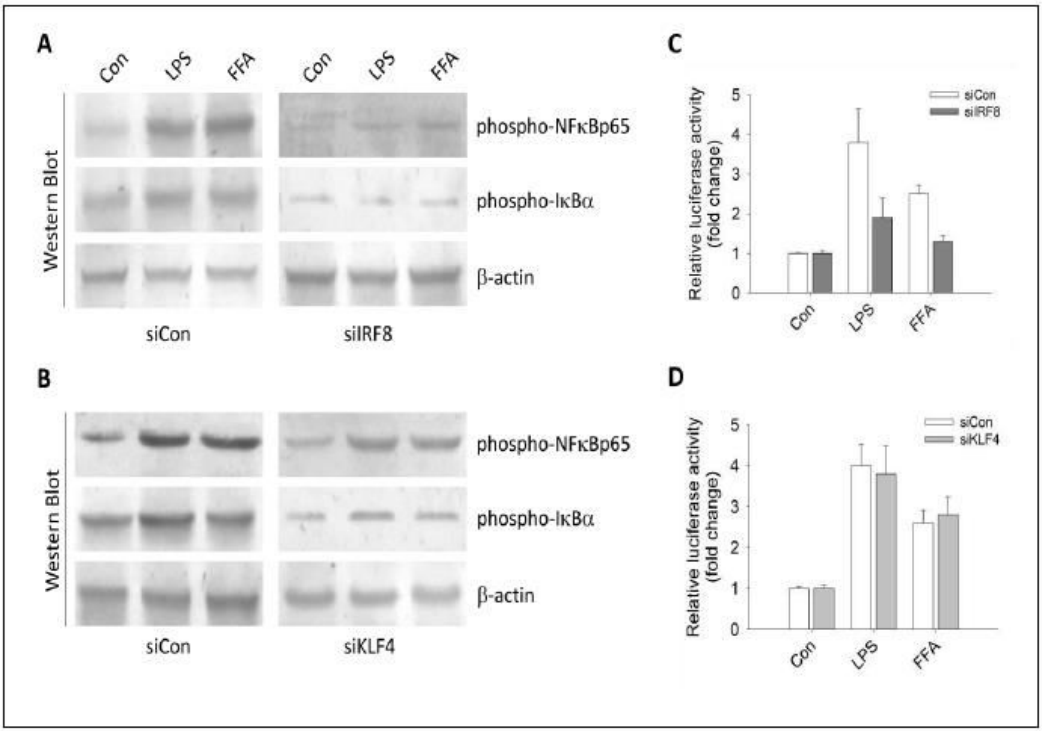

Fig. 7: Effect of IRF8 and KLF4 knockdown in adipocyte inflammation. (A,B) IRF8 and KLF4 silenced cells were treated with LPS or palmitate (FFA) for 6 hours. On termination of incubations, cells were subjected to SDS-PAGE followed by Western blotting with anti-NF-κBp65 and anti-IκBα antibodies. β-actin was used as loading control. (C,D) IRF8 or KLF4 siRNA was cotransfected with κB luciferase vector and after 48 hours, cells were incubated with LPS or palmitate (FFA) for 6 hours. Cells were lysed and luminescence was measured by multi-mode reader.

To investigate the role of IRF8 and KLF4 on the effect of FFA in adipocyte lipogenesis and lipolysis, IRF8 siRNA and KLF4 siRNA tranfected adipocytes were incubated with FFA for 6 hours. On termination of incubations, total RNA was isolated and Real time quantitative PCR was performed with lipogenesis (acetyl-CoA carboxylase, ACC; fatty acid synthase, FAS) and lipolysis (adipose tissue triglyceride lipase ATGL; perilipin) marker genes. FFA treatment did not alter the expression level of ACC and FAS in control and IRF8 silenced cells. Although FFA incubation did not change the gene expression of FAS significantly, however, significant downregulation of ACC gene expression was observed in KLF4 knockdown cells (**Fig. 8A**). A significant induction of ATGL and perilipin gene expression was observed in FFA incubated adipocytes indicating that prolong incubation of FFA may promotes adipocytes lipolysis (**Fig. 8B**). IRF8 and KLF4 silenced adipocytes did not produce any effect indicating that IRF8 and KLF4 do not have any role on FFA induced promotion of lipolysis.

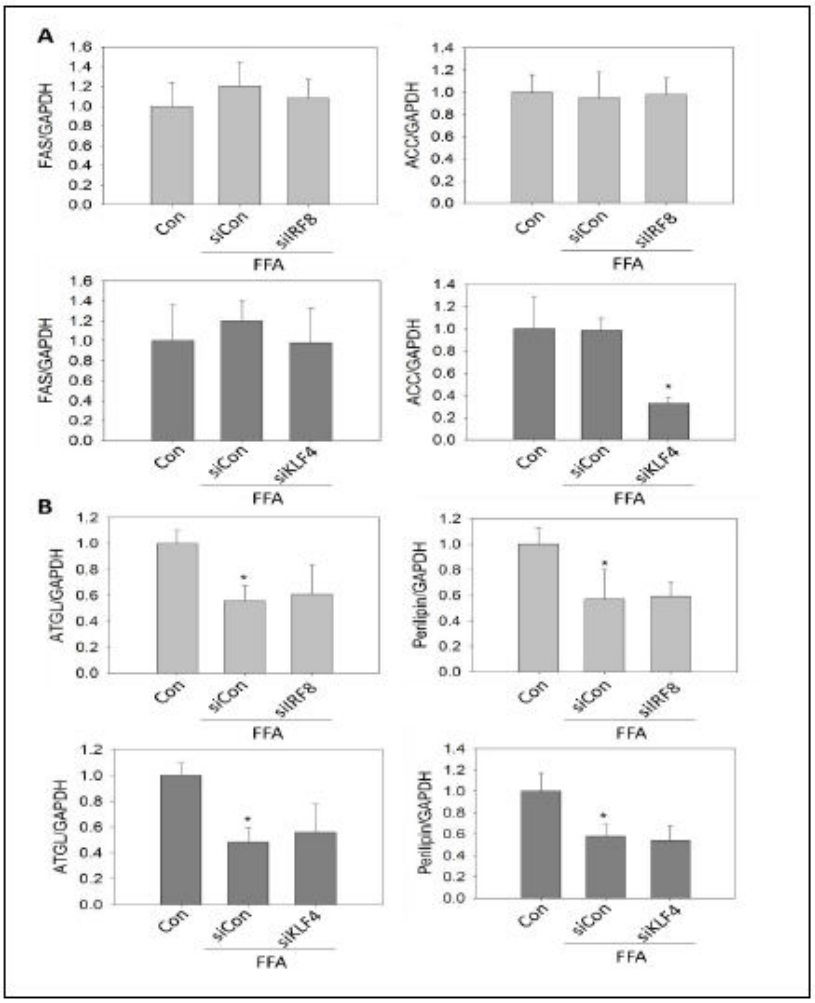

Fig. 8: Effect of IRF8 and KLF4 silencing in lipogenesis and lipolytic marker genes expression. (A,B) Quantification of FAS and ACC (A) and ATGL and perilipin (B) mRNA expression in IRF8 or KLF4 knockdown 3T3L1 adipocytes, respectively in response to FFA by RT-qPCR. Data are shown as the mean ± SD of three independent experiments.

Work over the past several years has revealed that adipose tissue as a master regulatory tissue in controlling both glucose and lipid homeostasis in humans. The formation of new adipocytes from precursor cells is a crucial aspect in controlling normal adipose tissue function. Therefore, detailed knowledge of pathway involved in adipogenesis may offer promising opportunities for therapeutic regime to counteract obesity and type 2 diabetes. We therefore investigated the effect of IRF8 and KLF4 in adipogenesis.

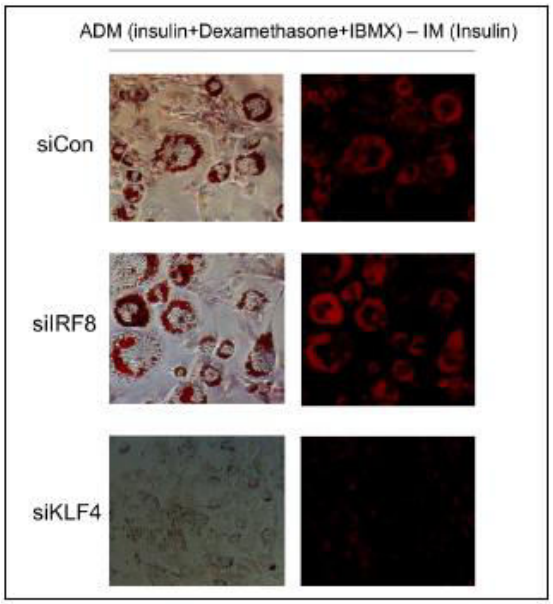

Fig. 9: Involvement of IRF8 and KLF4 in adipogenesis. IRF8 or KLF4 siRNA transfected 3T3-L1 preadipocytes were incubated with ADM for 3 days followed by 2 days post incubation with IM. Adipogenic differentiations were measured by Oil Red O staining and were observed under microscopy.

3T3-L1 preadipocytes were transfected with IRF8 or KLF4 and after 48 hours of transfection, cells were placed in adipocyte differentiation medium (ADM) containing insulin, dexamethasone and IBMX for 3 days followed by 2 days incubation in insulin medium (IM). On day 5, cells were treated with Oil red O stain for estimation of cellular lipid accumulation. In comparison to control, cells incubated with ADM and insulin medium showed massive accumulation of lipid as indicated by Oil red O staining. KLF4 silenced cells showed attenuation of lipid accumulation, however, IRF8 inhibition did not produced any effect (**Fig. 9**). These results clearly depicts that the involvement of KLF4 in lipid accumulation in mature adipocyte and thus indicates KLF4 play a key role in adipocte differentiation.

The C/EBPβ and PPARγ are known early and late transcription factors involved in preadipocyte to mature adipocyte conversion or adipogenesis. Since, we observed that KLF4 play an important role in adipocyte differentiation, we next sought to investigate its effect on

C/EBPβ and PPARγ expression level. ADM followed by IM treatment notably induces both C/EBPβ and PPARγ abundance; however, such effect was compromised in KLF silenced cells (**Fig. 10)** indicating that KLF4 regulates the expression of C/EBPβ and PPARγ which in turn promotes adipocyte differentiation.

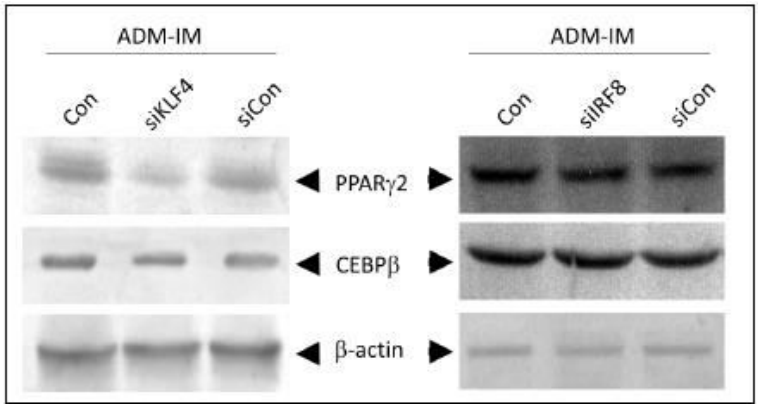

Fig. 10: The expression level of early and late transcription factor involved in adipogenesis. IRF8 and KLF4 silenced cells were placed in adipocyte differentiation medium (ADM) containing insulin, dexamethasone and IBMX for 3 days followed by 2 days incubation in insulin medium (IM). After incubations, cells were subjected to western blotting with anti-PPARγ2 and anti-C/EBPβ antibodies. β-actin expression was taken as internal control.

To explore the role of macrophage IRF8 and KLF4 on adipocyte insulin sensitivity, we silenced IRF8 or KLF4 expression in macrophages by siRNAs and co-colture in transwell chamber for 12h. Cells were then incubated without or with a saturated free fatty acid palmitate for 8 h in presence of insulin and fluorescent labelled 2-NBDG. On termination of incubations, cells were analysed for insulin stimulated glucose uptake. Palmitate induced impairment of glucose uptake was notably waived in adipoctes which were cocultured with IRF8 silenced macrophages (**Fig. 11**). This result indicates that IRF8 play crucial role in regulating adipocytes insulin sensitivity.

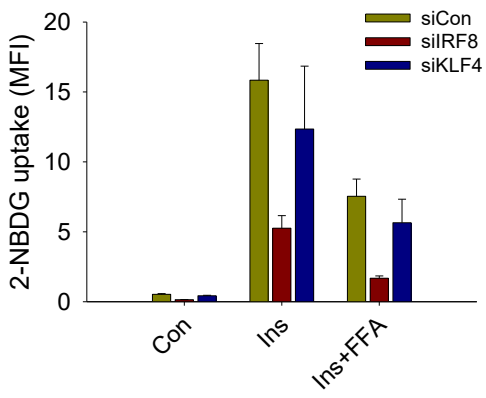

Fig. 11: Analysis of 2-NBDG uptake by 3T3-L1 adipocytes in response to Insulin (Ins) or Ins + FFA (palmitate) in trans-culture with macrophages transfected with control siRNA or IRF8 or KLF4 siRNA. Each value is the mean ± SEM of three independent experiments.

Conclusion: Our study clearly depicts the importance of macrophage IRF8 and KLF4 in regulating the cross-talk between macrophage and adipocytes and thereby governing the adipocyte function. Thus targeting these important nodes in macrophages could be helpful for the development of novel therapeutics against type 2 diabetes.

INDIAN NATIONAL SCIENCE ACADEMY
Bahadur Shah Zafar Marg, New Delhi 110 002

UTILIZATION CERTIFICATE FOR THE YEAR - 2017-2018
(To be submitted in Duplicate)

Certified that an expenditure of **Rs 4,99,384/-** ***(Rupees Four Lakhs Ninety Nine Thousand Three Hundred Eighty Four only)*** has been incurred out of grant of **Rs. 5,00,000/-** ***(Rupees Five Lakhs only)*** of which Rs. 4,99,997/- released on 02.08.2017 with a previous year balance of Rs. 3/-) under **INSA Young Scientist Project (INSA Sanction No. SP/YSP/101/2014/065 dated 14 October 2014)** entitled ***"Role of transcription factors IRF8 and KLF4 in lipid induced adipose tissue inflammation and insulin resistance"*** and utilized properly under different heads as mentioned in the Statement of Expenditure. It is further certified that the grant has been exclusively utilized for which it was sanctioned with an unspent balance of **Rs. 616/-** ***(Rupees Six Hundred Sixteen only)***.

10/05/18
(Young Scientist)
Dr. Suman Dasgupta
Assistant Professor
Dept. of Molecular Biology & Biotechnology
Tezpur University
Napaam, Sonitpur, Assam - 784028

(Head of the Institute)
Registrar
Tezpur University

6/6/18
(Finance Officer)
Joint Registrar
Tezpur University

INDIAN NATIONAL SCIENCE ACADEMY

Bahadur Shah Zafar Marg, New Delhi 110 002

STATEMENT OF EXPENDITURE FOR THE YEAR - 2017-2018
(To be submitted in Duplicate)

1. Name of the Programme : **Young Scientist Project**

2. Name of the Young Scientist and the address of the host Institution. : **Dr. Suman Dasgupta**
Dept. of Molecular Biology & Biotechnology
Tezpur University
Tezpur - 784028
Napaam, Dist: Sonitpur, Assam

3. Implementation date : **15 November 2014**

4. Title of Research Project : ***"Role of transcription factors IRF8 and KLF4 in lipid induced adipose tissue inflammation and insulin resistance"***

5. Grants: a) Self

Particulars	Contingency (including travel)	Total
Balance B/F (as on 01.04.2017)	Rs. 3.00	Rs. 3.00
Grant sanctioned for the current year **2017-2018**	Rs. 4,99,997.00	Rs. 4,99,997.00
Grant utilized (upto 11.12.2017)	Rs. 4,99,384.00	Rs. 4,99,384.00
Unspent Balance (as on 31.03.2018)	Rs. 616.00	Rs. 616.00

b) Research Personnel, if appointed, in the project : **Nil**

S. Dasgupta 10/05/18
(Young Scientist)

Dr. Suman Dasgupta
Assistant Professor
Dept. of Molecular Biology & Biotechnology
Tezpur University
Napaam, Sonitpur, Assam - 784028

(Head of the Institute)
Registrar
Tezpur University

(Finance Officer) 6/5/18
Joint Registrar
Tezpur University