Biochimie 119 (2015) 16–26

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

MicroRNA-16 modulates macrophage polarization leading to improved insulin sensitivity in myoblasts

Malathi Talari [a], Bandish Kapadia [a], Vasundhara Kain [a, 1], Sriram Seshadri [b], Bhumika Prajapati [b], Parth Rajput [b], Parimal Misra [a], Kishore V.L. Parsa [a, *]

[a] Department of Biology, Dr. Reddy's Institute of Life Sciences, University of Hyderabad Campus, Hyderabad, Telangana, India
[b] Institute of Science, Nirma University, Sarkhej Gandhinagar Highway, Ahmedabad, Gujarat, India

ARTICLE INFO

Article history:
Received 21 April 2015
Accepted 5 October 2015
Available online 8 October 2015

Keywords:
Macrophage polarization
microRNA
Inflammation
High sucrose diet
GLUT4
Insulin resistance

ABSTRACT

Uncontrolled inflammation leads to several diseases such as insulin resistance, T2D and several types of cancers. The functional role of microRNAs in inflammation induced insulin resistance is poorly studied. MicroRNAs are post-transcriptional regulatory molecules which mediate diverse biological processes. We here show that miR-16 expression levels are down-regulated in different inflammatory conditions such as LPS/IFNγ or palmitate treated macrophages, palmitate exposed myoblasts and insulin responsive tissues of high sucrose diet induced insulin resistant rats. Importantly, forced expression of miR-16 in macrophages impaired the production of TNF-α, IL-6 and IFN-β leading to enhanced insulin stimulated glucose uptake in co-cultured skeletal myoblasts. Further, ectopic expression of miR-16 enhanced insulin stimulated glucose uptake in skeletal myoblasts via the up-regulation of GLUT4 and MEF2A, two key players involved in insulin stimulated glucose uptake. Collectively, our data highlight the important role of miR-16 in ameliorating inflammation induced insulin resistance.

1. Introduction

Macrophages alter their physiology and phenotype in response to the surrounding cytokine environment and stimulus to suit their functional requirements. This plastic behavior of macrophages is termed as macrophage polarization. Originally, they were categorized into classically activated M1 macrophages and alternative M2 macrophages. M2 macrophages are well known for their microbicidal functions, whereas M2 macrophages take part in the resolution of inflammation and repair of the damaged tissue caused by the hyperactivity of M1 macrophages [1,2]. Very recently to avoid the confusion across the authors in naming the activated macrophages and in order to attain the consensus in the nomenclature terminology, a new convention for naming activated macrophages was framed [3]. Thus, hereafter stimulated macrophages are named according to the new nomenclature. For example in our studies LPS and IFNγ stimulated macrophages are referred to M (LPS + IFNγ) and IL-4 exposed macrophages are represented as M (IL-4). Signifying the importance of macrophage polarization, dysregulation of this physiological process is linked to the development of several diseases including Type 2 Diabetes (T2D) [4,5]. Insulin resistance plays a dominant role in the progression of T2D and other metabolic disorders [6]. Increase in sedentary life style and enhanced intake of refined energy rich diet (high fat, high sucrose and fructose) are directly associated with the progression of insulin resistance [7–10]. A plethora of studies using various model systems have demonstrated that insulin resistance is associated with chronic low grade inflammation [11,12]. Pro-inflammatory cytokines such as TNF-α and IL-6 along with other inflammatory mediators are key players in altering the insulin signaling pathway in peripheral insulin responsive tissues [13].

Abbreviations: ARG-1, Arginase 1; GLUT4, glucose transporter 4; HSD, high sucrose diet; IP-10, interferon-γ-inducible protein 10; IRAK2, interleukin-1 receptor associated kinase-like 2; LITAF, lipopolysaccharide-induced tumor necrosis factor-alpha factor; miRNA/miR, microRNA; MRC, Mannose receptor C; 2-NBDG, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose; MEF2A, myocyte enhancer factor 2A; PHLPP, pleckstrin homology leucine rich repeat protein phosphatase; ROS, reactive oxygen species; Tab3, TGF-beta activated kinase1; TRAF3, TNF receptor-associated factor 3; T2D, Type 2 Diabetes; YM-1, Chitinase-like 3(Chil3).

* Corresponding author.
E-mail address: kishorep@drils.org (K.V.L. Parsa).
[1] Current address: Division of Cardiovascular disease, School of Medicine, The University of Alabama Birmingham, Alabama, USA.

http://dx.doi.org/10.1016/j.biochi.2015.10.004
0300-9084/

Since inflammatory M (LPS/IFNγ/TNF-α) macrophages are mainly responsible for the secretion of these inflammatory cytokines, they stand out to be the major culprits in inducing insulin resistance [14]. Thus, understanding the molecular machinery governing the generation of pro-inflammatory macrophages is critical for determining and defining the molecular targets for the treatment of the metabolic disorders.

MicroRNAs (miRNAs) have evolved as important regulators of diverse physiological processes such as cell differentiation, proliferation and apoptosis. miRNA belong to a family of short (~22 nt) non-coding RNA that regulate gene expression at the post transcriptional level [15,16]. In the recent years, microRNA have emerged as key players in driving immune functions [17–20]. The diverse roles of microRNAs are studied in independent processes [21,22] but the involvement of miRNAs in inflammation induced insulin resistance is poorly revealed.

In this study, we have used synthetic microRNA mimics as gain of function experiments to investigate different roles of miR-16 in inflammation induced insulin resistance. Here, we show that the expression of miR-16 is impaired in M (LPS + IFNγ) macrophages, palmitate treated myoblasts and major insulin responsive tissues of high sucrose diet (HSD) induced insulin resistant rats. We have also observed that ectopic expression of miR-16 augmented overall skeletal muscle insulin sensitivity uptake via the inhibition of M (LPS + IFNγ) mediated pro-inflammatory responses and up-regulation of insulin responsive skeletal muscle glucose transporter, GLUT4.

2. Materials and methods

2.1. Cells, antibodies and reagents

RAW 264.7, and L6 cells were cultured in DMEM (Gibco, Life Technologies, Grand Island, NY, USA) and maintained in 10% heat-inactivated Fetal Bovine Serum (Gibco, Life Technologies, Grand Island, NY, USA). C2C12 were grown in DMEM containing 20% FBS. Recombinant mouse IFNγand IL-4 were procured from Peprotech (Rocky Hill, NJ, USA) and *Escherichia coli* LPS (0127:B8), Palmitate (P5585), fatty acid free BSA (A8806) and Insulin (I0516) were obtained from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO, USA). Antibodies specific for iNOS (BD Biosciences, San Jose, CA, USA) and β-actin were (Calbiochem, San Diego, CA, USA) used. Anti-mouse HRP and anti-rabbit HRP antibodies were purchased from Genscript (Genscript, Piscataway, NJ, USA). *mir*Vana miR-16 mimic and negative control mimics were obtained from Ambion (Ambion, Austin, TX, USA). The lyophilized pellets were solubilized in nuclease free water as per manufacturer's instructions. DCFDA dye was purchased from Life technologies (Life Technologies, Grand Island, NY, USA) and was resuspended in DMSO as per manufacturer's guidelines.

2.2. MicroRNA profiling

RAW 264.7 cells were stimulated with 1 μg/mL of LPS and 100 ng/mL of IFNγ or left un-stimulated (Resting, R) for 24 h. Treated samples were lysed in Trizol (Invitrogen, Carlsbad, CA, USA), total RNA was extracted by RNeasy mini kit (Qiagen, Valencia, CA, USA) and samples were analyzed by Affymetrix miRNA 2.0 array at iLIFE DISCOVERIES (IMT Manesar, Gurgaon, Haryana, India).

2.3. Nucleofection and transfection

RAW 264.7 cells were maintained in medium containing DMEM with 10% fetal bovine serum at 37 °C with 5% CO_2 environment. All transfections were carried out with the Solution V (Lonza, Allendale, NJ, USA) using nucleofection apparatus (Lonza, Allendale, NJ, USA) according to manufacturer's instructions with few modifications. Briefly, 12×10^6 cells were resuspended in 100 μl of nucleofector solution V and were nucleofected with 300 nM of control or miR-16 mimic. Immediately post nucleofection, 500 μl of pre-warmed DMEM was added to the transfection mix before transferring the cells to the plate containing 10 ml DMEM with FBS. Plates were incubated for 16 h at 37 °C. Transfected cells were counted, seeded at equal density and treated as mentioned in figure legends. Transient transfections in L6 myoblasts were performed using Lipofectamine™ 2000 (Invitrogen, CA, USA) as per manufacturer's instructions. The final concentration of miRNA mimic transfected was 100 nM and cells were utilized for experiments post 24 h of transfection.

2.4. Palmitate preparation and treatment

Stock solution of 50 mM palmitate (C16:0) was prepared as described earlier [23]. Briefly, palmitic acid was dissolved in pre-heated (70 °C) 0.1 N NaOH and diluted 5 fold in pre-warmed 25% BSA solution giving a final concentration of 10 mM. Cells were treated with palmitate at final concentration of 500 μM for 24 h. Cells were serum starved for at least 3 h prior to stimulation.

2.5. Cell stimulation, lysis and western blotting

RAW 264.7 cells were stimulated with LPS (1 μg/ml) and IFNγ (100 ng/ml) for different time points. Un-stimulated and stimulated cells were lysed in TN1 lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA, 10 mM $Na_2P_2O_7H_2$, 10 mM NaF, 1% Triton-X 100, 125 mM NaCl, 10 mM Na_3VO_4, 10 μg/ml each aprotinin, leupeptin and 1 mM PMSF). Protein-matched lysates were resolved by 10% SDS/PAGE, transferred to PVDF membrane, probed with the antibody of interest and developed by Enhanced chemiluminescence (GE Life Sciences, Piscataway, NJ, USA).

2.6. ELISA for cytokine analysis

RAW 264.7 cells were stimulated with LPS and IFNγ for 10 h, culture supernatants were harvested, and the amount of TNF-α (R&D systems, Minneapolis, MN, USA), IL-6 and IP-10 (Peprotech, Rocky Hill, NJ, USA) in the cell free supernatants were measured using ELISA kits according to manufacturer's recommendations. IFN-β in the supernatants was measured as previously described [24].

2.7. Phagocytosis measurement

Phagocytic efficiency was measured in RAW 264.7 cells using fluorescent *E. coli* bacteria K-12 strain (Molecular Probes- Invitrogen, CA, USA). These lyophilized bacteria were resuspended in PBS as per manufacturer's instructions and RAW 264.7 cells transfected with control or miR-16 mimic were infected with or left un-infected at a multiplicity of infection (MOI) of 10. The infected cells were centrifuged at 650 g for 2 min and incubated at 37 °C for 3 h. The suspension was removed and external fluorescence was quenched by using 500 μg/mL of trypan blue (Sigma Aldrich, St. Louis, MO, USA) with pH 4.4 (set with citrate buffer). Cells were carefully washed with ice-cold 1X PBS, lysed in 0.1% Triton X 100 and phagocytosis of fluorescent bacteria by RAW 264.7 cells was determined in the cell lysates by fluorometry using multiplate reader (Perkin–Elmer, Waltham, Massachusetts, USA) with excitation at 494 nm and emission at 518 nm.

2.8. Reactive oxygen species measurement

Post transfection RAW 264.7 cells were stimulated with LPS and IFNγ for 24 h or left un-stimulated (Resting, R) and cells were incubated with 5 μM DCFDA dye (Life Technologies, Grand Island, NY, USA) for 30 min in dark. Subsequently, cells were carefully washed using ice-cold 1X PBS and were lysed in 0.1% Triton X 100. Amount of ROS in the cell lysates were measured by fluorometry using multiplate reader (Perkin–Elmer, Waltham, Massachusetts, USA) with excitation at 485 nm and emission at 535 nm.

2.9. Animal treatment

Healthy adult Wistar albino rats, weighing 150 ± 10 g were maintained in polypropylene cages with a standard photoperiod (12 h light:12 h dark cycle) in temperature (27 ± 1 °C) controlled room with the provision of laboratory food (Gold Mohur feeds Ltd, New Delhi, India) and water *ad libitum*. Rats were fed with either chow diet (control group) or high sucrose diet (65%, treated group) for 60 days [25]. Animals were sacrificed in accordance with the approved guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). Methodological procedure involving animals was reviewed and approved by the Animal Ethical Committee of Institute of Science, Nirma University, Ahmedabad, India (Protocol No. IS/BT/FAC-13-1009).

2.10. Glucose uptake assay

RAW 264.7 macrophage cells were nucleofected with control or miR-16 mimic. Approximately 16 h post-transfection, macrophage cells were stimulated with LPS (1 μg/mL) and IFNγ (100 ng/mL) (LPS + IFNγ) for 10 h. Supernatants from stimulated macrophages were harvested and pre-cleared cultured media was added to L6 rat myoblasts for 18 h. As a control, L6 cells were incubated with DMEM medium instead of supernatants from inflamed macrophages (denoted as control in the Fig. 4) to measure the glucose uptake in the absence of inflammatory insult. Further, cells were serum starved followed by glucose free medium for 30 min. Cells were primed with insulin for 5 and 10 min and were incubated with 50 μM of 2-NBDG (Molecular Probes- Invitrogen, CA, USA) for 15 min. Reaction was terminated by washing cells with ice cold 1X PBS and were lysed in 0.1% Triton X-100. Fluorescence was measured on a Wallac 1420 Victor Multimode Plate Reader (Perkin–Elmer, Waltham, Massachusetts, USA) with excitation at 485 nm and emission at 535 nm.

2.11. Real time PCR analysis

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse transcription was performed using NCode VILO miRNA first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). DNase I (Ambion, Austin, TX, USA) treated RNA was used for RT-PCR. Reverse transcription was performed according to manufacturer's instructions using 2 μg of total RNA. The sequence of miR-16 forward primer is CGGTAGCAGCACGTAAATATTGGCGA: and reverse primer used was provided along with the NCode VILO miRNA kit. qPCR was performed on Step One Plus instrument (Applied Biosystems, Foster City, CA, USA) using SYBR green master mix (Applied Biosystems, Foster City, CA, USA). Data were normalized to respective control genes (18S, actin for mRNA and U6 for microRNA).

2.12. Statistical analysis

Numerical data were expressed as mean ± SEM of 3–7 independent experiments unless indicated otherwise. Statistical significance was calculated using unpaired Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ are considered as significant and NS indicates non-significant.

3. Results

3.1. miR-16 expressions is down-regulated in M (LPS + IFNγ) polarized RAW 264.7 macrophages

Since the role of microRNA in regulation of macrophage polarization is not well studied [26,27], we examined the expression pattern of microRNA in M (LPS + IFNγ) macrophages by microRNA profiling assay (Fig. 1A–E) and observed that 63 microRNAs were differentially regulated due to LPS + IFNγ treatment (Fig. 1C). Consistent with the literature [28], we noted that miR-155 expression was robustly up-regulated in M (LPS + IFNγ) macrophages while miR-16 expression levels were moderately reduced (Fig. 1D–E). The microarray results were validated by qPCR analysis (Fig. 1F–G). Palmitate treatment is known to polarize macrophages towards a pro-inflammatory state [29]. Thus, we next determined miR-16 expression levels in palmitate exposed RAW 264.7 cells. Consistent with the data of LPS and IFNγ stimulated M (LPS + IFNγ) macrophages, miR-16 levels were observed to be diminished in palmitate treated RAW 264.7 cells (Fig. 1H). Based on these observations, we hypothesized that miR-16 has a functional role in regulating M (LPS + IFNγ) macrophage mediated inflammatory responses.

3.2. Ectopically expressed miRNA-16 dampens inflammatory cytokine production in M (LPS + IFNγ) macrophages

To study the functional impact of miR-16 on M (LPS + IFNγ) macrophage responses, we over-expressed miR-16 mimics in RAW 264.7 cells. Post 16 h of transfection, cells were stimulated with both LPS (1 μg/ml) and IFNγ (100 ng/ml) for 10 h and observed for M (LPS + IFNγ) markers. Consistent with the literature, we found that the enhanced expression of miR-16 significantly decreased secretion of pro-inflammatory cytokines (50) such as TNF-α (Fig. 2A), IL-6 (Fig. 2B), and IFN-β (Fig. 2C). However, ectopic expression of miR-16 failed to effect IP-10 cytokine secretion (Fig. 2D). To further analyze the role of miR-16 in pro-inflammatory responses, we examined the levels of iNOS and reactive oxygen species (ROS), which are well known to be enhanced in M (LPS + IFNγ) polarized macrophages [1,2]. Fig. 2E and F depict that over-expression of miR-16 had no impact on iNOS protein level and ROS generation. Moreover, over-expression of miR-16 also failed to inhibit the phagocytosis of fluorescent *E. coli* by RAW 264.7 cells (Fig. 2G). This suggests that miR-16 regulates specific pro-inflammatory responses during M (LPS + IFNγ) polarization. In parallel, we validated the over-expression of mature miR-16 by qPCR (Fig. 2H). Electrophoresis of qPCR amplicons showed a single band corresponding to the mature miR-16 (data not shown).

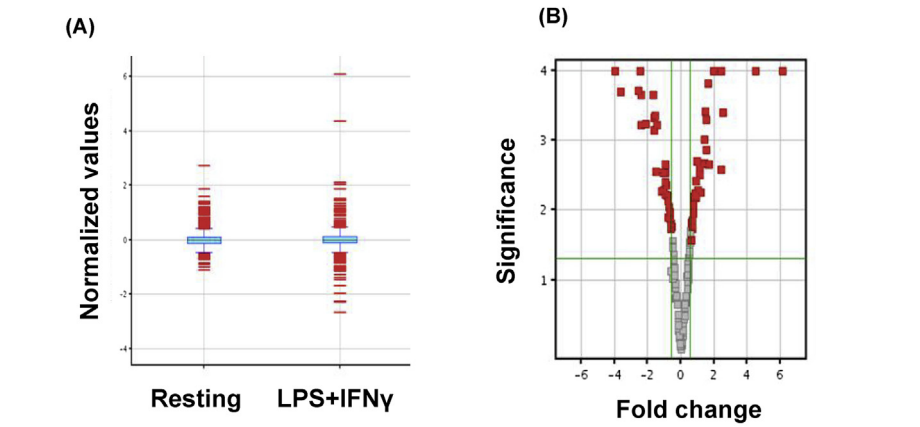
3.3. miR-16 selectively regulates IL-4 induced macrophage gene expression

Having observed the effect of miR-16 on M (LPS + IFNγ) macrophages, we then probed the effect of miR-16 on M (IL-4) macrophage responses. For this, we first treated RAW 264.7 cells with IL-4 for 8 h and 24 h and monitored the expression levels of miR-16. We observed that miR-16 levels were unchanged in IL-4 exposed RAW 264.7 cells at both the time points tested (Fig. 3A), however consistent with literature M (IL-4) markers such as Arginase-1 and Mannose receptor C-1 (MRC1) were induced

suggesting that the stimulation was appropriate [3] (Fig. 3B). Nevertheless, we sought to determine the effect of miR-16 on IL-4 induced macrophage responses for which we over-expressed miR-16 and stimulated RAW 264.7 cells with IL-4 for 24 h. We observed that miR-16 was able to increase IL-4-induced Arginase-1 expression by ~2 fold (Fig. 3C) whereas it showed no impact on other markers such as MRC-1, IL-10 and YM-1 (Fig. 3C). This suggests that miR-16 regulates specific gene expression in both M (LPS + IFNγ) and M (IL-4) macrophages.

3.4. miRNA-16 suppresses inflammation induced insulin resistance in L6 myoblasts

It is well documented that pro-inflammatory macrophages accumulate in adipose and skeletal muscle tissues of obese subjects and induces insulin resistance via the secretion of pro-inflammatory mediators [6,11,13]. However, the role of miRNA in inflammation induced insulin resistance is still unclear [20,30]. Thus, to study the functional consequences of miR-16 on M (LPS + IFNγ) macrophage driven inflammatory response on insulin responsive organs, we applied conditioned cell supernatants from M (LPS + IFNγ) stimulated control or miR-16 mimic transfected RAW 264.7 cells on L6 myoblasts for 18 h and measured insulin stimulated glucose uptake. Culturing L6 myoblasts in conditioned cell supernatants from control mimic transfected M (LPS + IFNγ) cells caused a significant reduction in both basal and insulin stimulated glucose uptake compared to control L6 cells. However, forced expression of miR-16 in macrophages partially restored insulin mediated glucose uptake by L6 myoblasts in comparison to control mimic transfected M (LPS + IFNγ) cells (Fig. 4). This is consistent with our observations that miR-16 inhibited the secretion of TNF-α, IL-6 and IFN-β (Fig. 2A–C) which are well documented to induce insulin resistance [31,32].



(C)

Treatment	Total no. of miRNA up-regulated	Total no. of miRNA down-regulated	Total no.of miRNA differentially regulated
M (LPS+IFNγ)	31	32	63

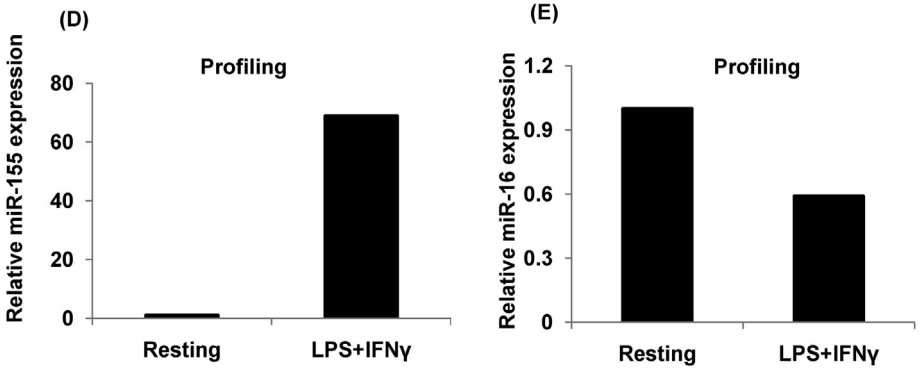


Fig. 1. miRNA-16 expression is down-regulated in M (LPS + IFNγ) polarized macrophages. RAW 264.7 macrophages were stimulated with LPS and IFNγ stimuli for 24 h and total RNA was harvested and samples were analyzed by Affymetrix miRNA 2.0 array (A) Box plot representing total microRNA profile in Resting and LPS + IFNγ stimulated RAW 264.7 cells. (B) Volcano plot representing total microRNA profile in Resting and LPS + IFNγ stimulated RAW 264.7 cells. (C) Table showing total number of microRNAs differentially expressed upon LPS + IFNγ stimulation. (D–E) miR-155 and miR-16 expression levels were obtained from profiling analysis. (F–G) miR-155 (F) and miR-16 (G) expression was analyzed using qPCR analysis in LPS + IFNγ or 500 μM palmitate (H) stimulated RAW 264.7 cells. miRNA expression was normalized using U6 and expressed relative to untreated cells which was set as 1. $^{*}p < 0.05$ and $^{**}p < 0.01$ calculated using Student's *t*-test.

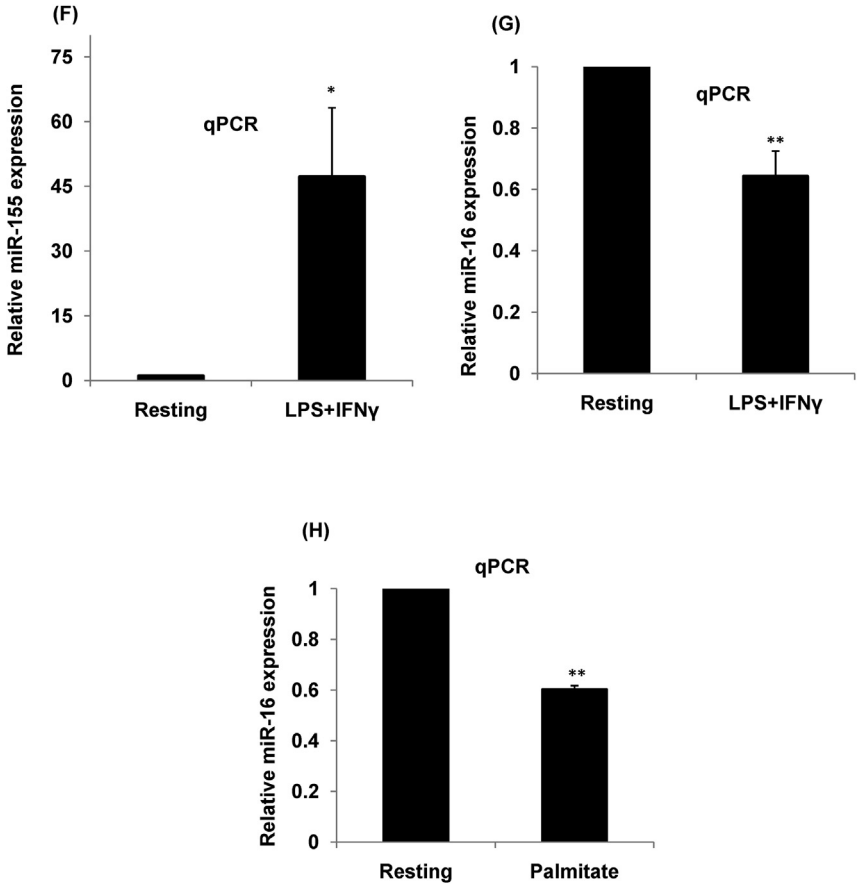


Fig. 1. (*continued*).

3.5. miR-16 expressions is down-regulated in high sucrose diet fed rat tissues

miRNA expression profiles show that miR-16 is ubiquitously expressed at modest levels in all animal tissues [33]. Since, the expression of miR-16 was consistently down-regulated in inflammation; we aimed to study the expression profile of miR-16 in inflammation induced insulin resistance conditions. For this, we used high sucrose diet (HSD) fed rats as the model system [34,35] and assessed miR-16 expression in insulin responsive organs. We observed that miR-16 is significantly down-regulated in metabolically active rat tissues such as soleus muscle (Fig. 5A), adipose (Fig. 5B), and liver (Fig. 5C). Further, a significant increase in the expression of PHLPP was noted (Fig. 5D), a key protein player involved in regulation of both inflammation [36] and insulin resistance [37]. We have followed up these observations in C2C12 myoblasts and noticed that miR-16 expression was robustly reduced in palmitate cultured cells (Fig. 5E) indicating a potential regulatory role for miR-16 in controlling inflammation mediated insulin resistance in the peripheral tissues.

3.6. Ectopic expression of miR-16 enhances insulin sensitivity in L6 myoblasts

To further probe the role of miR-16 in insulin sensitivity, we determined its functional effects on insulin stimulated glucose uptake. For this, we over-expressed miR-16 in L6 myoblasts and performed 2-NBDG glucose uptake assay. In comparison to control cells, we observed that forced expression of miR-16 improved both basal as well as insulin stimulated glucose uptake suggesting that miR-16 exerts insulin sensitizing effects on myoblasts (Fig. 6A). To examine the underlying mechanisms, we studied the expression levels of GLUT4 and MEF2A, key players in insulin mediated glucose uptake [38–41]. The degree of inflammation induced insulin resistance is generally assessed by the rate of glucose disposal from circulation/medium by GLUT4. It is well documented in literature that levels of GLUT4 were reduced in TNF-α exposed adipocytes [42]. Further, we observed that MEF2A levels were also reduced upon TNF-α stimulation (data not shown). Importantly, the expression of GLUT4 is in direct control of MEF2 family of transcription factors with a major contribution of MEF2A [40,41,43]. In addition, in our current data, we observed that basal glucose uptake levels (in the absence of insulin stimulation) were also enhanced upon miR-16 over-expression. Considering all this, we analyzed the expression of GLUT4 and MEF2 isoforms upon miR-16 over-expression in L6 myoblasts. We noted that GLUT4 and MEF2A expression levels were significantly up-regulated (Fig. 6B, C) in miR-16 over-expressing myoblasts; however no significant change was noticed in the expression of another isoform of MEF2, MEF2C, (Fig. 6D) demonstrating the specificity of the miR-16. Further, no change was also observed in the expression pattern of PHLPP1 (Fig. 6E), a crucial player in regulating Akt driven insulin signaling cascade in peripheral tissues [44]. Parallel experiments using qPCR confirmed miR-16 over-expression (Fig. 6F). Collectively our data establishes the role of miR-16 in regulating M (LPS + IFNγ) macrophages driven inflammatory responses and positions it as an important player in enhancing insulin sensitivity in tissues such as skeletal muscle.

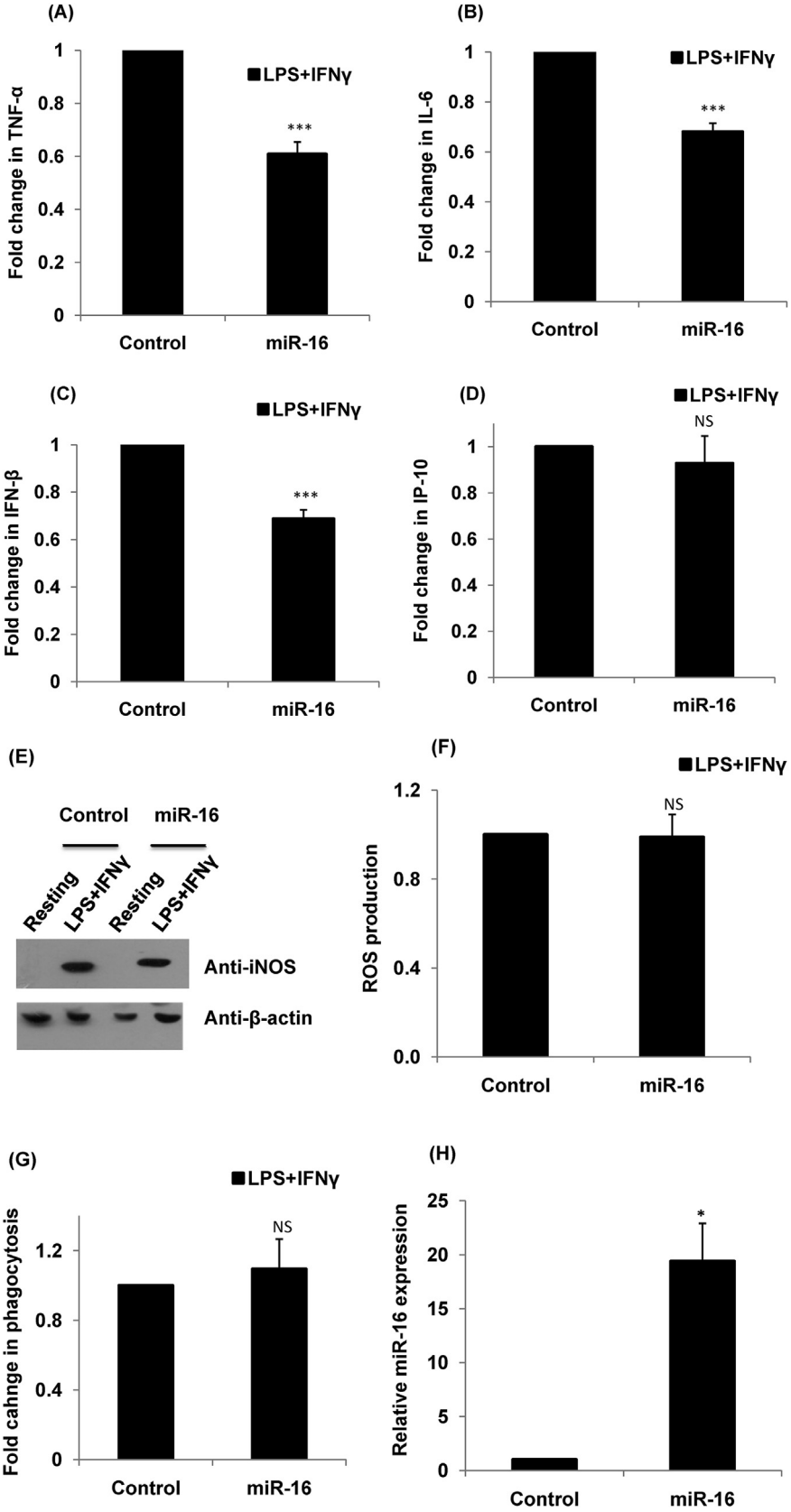


Fig. 2. Forced expression of miRNA-16 suppresses pro-inflammatory cytokine production. RAW 264.7 cells were transfected with control or miR-16 mimic for 16 h and then treated with LPS + IFNγ for 10 h and were analyzed for cytokine secretion in the culture supernatants by ELISA. (A) TNF-α, (B) IL-6, (C) IFN-β and (D) IP-10. $^{*}p < 0.05$ and $^{**}p < 0.01$ calculated using Student's *t*-test. NS represents non-significant. (E) iNOS protein levels in the cell lysates were analyzed by western blotting. Beta actin was used as the loading control. (F) Reactive oxygen species generation at 24 h of M (LPS + IFNγ) stimulation was measured using DCFDA dye by fluorometry. NS represents non-significant (G) Phagocytosis efficiency was measured after three hours of infection using *E. coli* strain K-12 by fluorometry. Values are normalized with the corresponding protein content. NS represents non-significant (H) Over-expression of miR-16 in RAW 264.7 cells post transfection was validated by qPCR. Values were normalized with the corresponding reference gene U6 and expressed relative to control transfected cell which was set as 1. $^{***}p < 0.001$ calculated using Student's *t*-test.

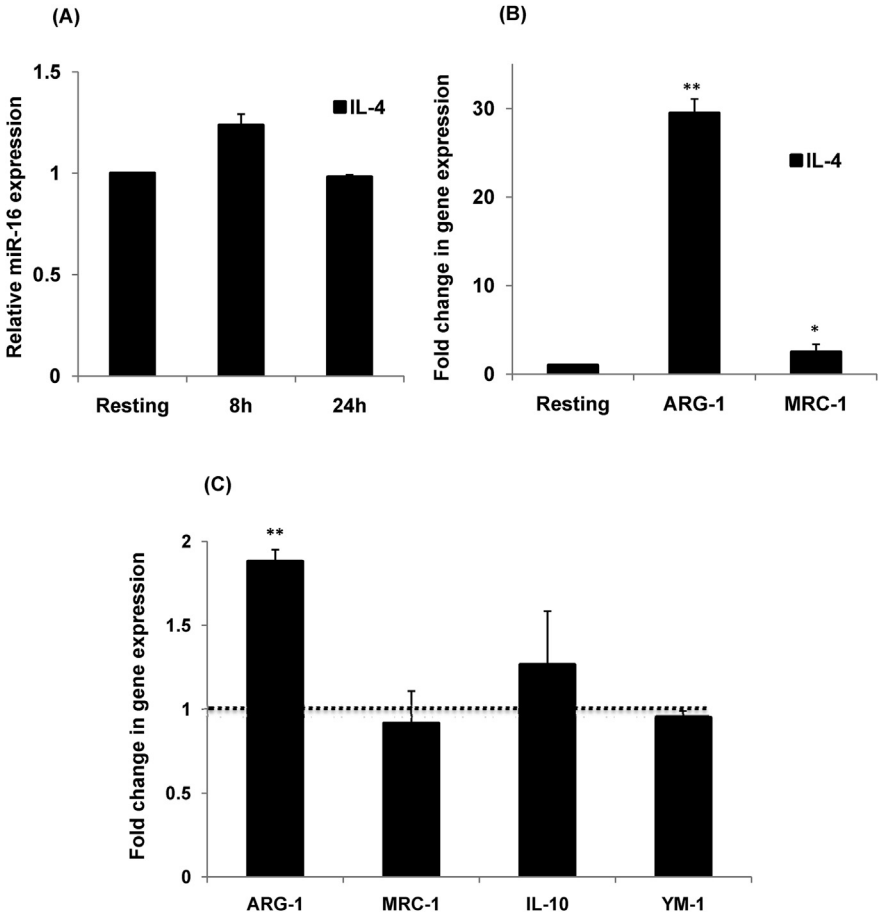


Fig. 3. Forced expression of miRNA-16 increases IL-4-induced Arginase-1 mRNA expression levels. RAW 264.7 cells were stimulated with 50 ng/mL of IL-4, miRNA and gene expression was analyzed by qPCR. (A) miR-16 expression levels were measured at 8 h and 24 h (B) mRNA expression was analyzed at 24 h of IL-4 treatment. (C) RAW 264.7 cells transfected with control or miR-16 mimic for 16 h and then treated with IL-4 for 24 h and were analyzed for ARG-1, MRC-1, IL-10 and YM-1 expression and presented as fold change relative to control miRNA mimic. $^{*}p < 0.05$ and $^{**}p < 0.01$ calculated using Student's *t*-test.

4. Discussion

In this study, we demonstrate that miR-16 plays an important role in the control of inflammation induced insulin resistance. We provide evidence that the expression of miR-16 is impaired in M (LPS + IFNγ) polarized macrophages, palmitate exposed myoblasts and insulin responsive tissues of HSD induced insulin rats. We further show that ectopic expression of miR-16 in macrophages or myoblasts exerted insulin sensitizing effects.

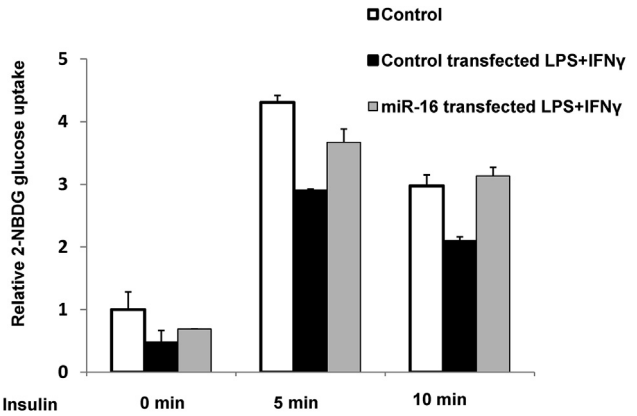


Fig. 4. miRNA-16 relieves inflammation mediated inhibition of glucose uptake by cultured L6 myoblasts. Mean basal and insulin stimulated glucose uptake was measured using 2-NBDG by fluorometry in L6 myoblasts after 18 h of incubation with supernatants obtained from treated RAW 264.7 cells as indicated in the methods. Values are normalized with the corresponding protein content and expressed relative to basal control cells which was set as 1. Data (mean ± SD) shown are a representative of three independent experiments.

In our profiling results, we noted that miR-16 expression was moderately reduced in M (LPS + IFNγ) macrophages. Such changes though small in magnitude are likely to have significant effects on cellular physiology. For instance, microRNA profiling data of polarized human monocyte derived macrophages (MDMs) published recently, also showed similar small magnitude changes despite robust responses to MDM polarizing conditions [45]. Specifically, treatment of MDMs or differentiated THP1 with LPS and IFNγ increased the levels of miR-29b by less than 2 fold, however over-expression of miR-29b significantly increased the expression of CXCL9, TNF-α and IL-6 in a dose dependent manner. Further, we have recently shown that a ser/thr phosphatase PHLPP is reduced in LPS treated immune cells (RAW 264.7 macrophages, THP1, PBMs, BMDMs) by about 50% [36]. Reducing cellular levels of PHLPP to about 50% by siRNA robustly augmented iNOS expression reiterating the fact that small changes need not necessarily be inconsequential. Although the changes in miRs in our current study and in Graff et al. [45] are small, they may exert significant effects because miRs may act in concert with other miRs belonging to same family or other family (which are also stimulus responsive) and the final outcome is a balanced effect of all the players involved. Supporting

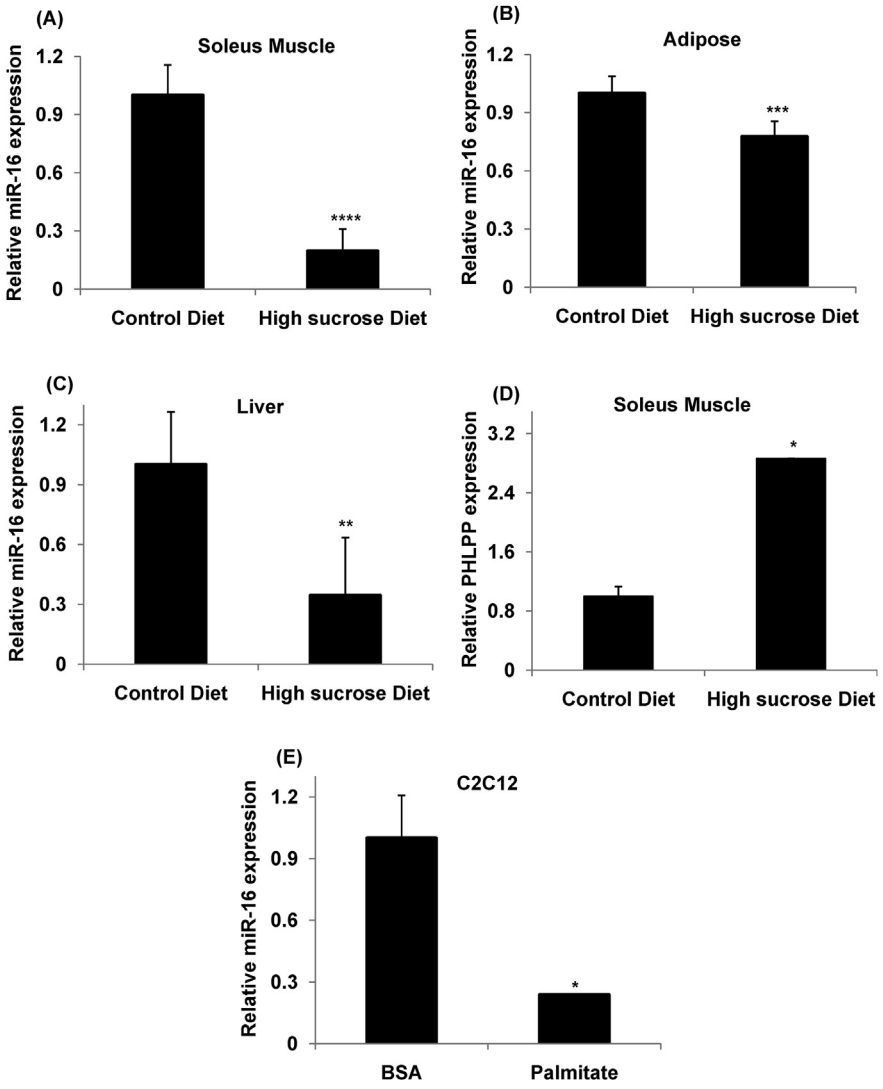


Fig. 5. miRNA-16 expression is down-regulated in HSD fed rat tissues. Tissues from control diet and high sucrose diet (n = 3) were lysed in trizol and miR-16 expression was analyzed in (A) soleus muscle, (B) adipose tissue and (C) liver using qPCR. Values were normalized with U6 and expressed relative to control diet which was set as 1. $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ calculated using Student's *t*-test. (D) PHLPP mRNA expression in soleus muscle and values are normalized using 18S. $^{*}p < 0.05$ calculated using Student's *t*-test. (E) C2C12 myoblasts were treated with palmitate (500 μM) for 24 h and miR-16 expression was examined by qPCR. Values were normalized with U6 and were expressed relative to BSA treated C2C12 myoblasts which was set as 1. $^{*}p < 0.05$ calculated using Student's *t*-test.

this possibility, an independent study showed that co-transfection of miR-16 and miR-15a mimics impaired phagocytic activity of BMDMs, while in our study, no significant effect on phagocytic uptake was noted upon miR-16 over-expression alone because a concerted effect of miR-15a and miR-16 may be probably required for influencing certain processes such as phagocytosis [46]. It is possible that the expression change of miR-16 is small (~2–3 fold) across different cells/tissues under inflammatory conditions in our study probably because miR-16 is a ubiquitous miRNA and is likely to have a key role in several physiological process such as cell proliferation/death and thus any huge alterations in its expression may be detrimental to the cell.

The role of miR-16 in macrophage maturation along with cancer progression has been studied at several levels [47–49], but its function in M (LPS + IFNγ) polarized macrophages has remained unclear. In a randomized study of coronary artery disease patients, Satoh et al. noted that the levels of TLR-4 responsive miR-16 were diminished [50]. Consistent with Satoh et al., co-stimulation with TLR4 ligand, LPS and IFNγ (M (LPS + IFNγ) stimuli) significantly reduced miR-16 level in our studies. However, current knowledge about the molecular mechanism mediating the depletion of miR-16 in macrophages and more precisely in TLR-4 activated macrophages is unclear. It may be possible that under M (LPS + IFNγ) stimulation, NF-κb activated STAT3 may act as repressor of miR-16 transcription [51,52].

Our findings are in striking contrast with observations from certain studies. Zhou et al. treated monocytes with LPS for different time points and found time dependent increase in miR-16 expression [53]. LPS increased the expression of miR-16 in BMDMs while it inhibited miR-16 in spleen and had no effect on mouse lung [54]. Thus, the effect of LPS on miR-16 expression seems to be complex and context dependent.

miR-16 contains a UAAAUAUU sequence which is complementary to the 3′UTR AREs (AU-rich elements) of mRNAs for inflammatory cytokines such as IL-6, and TNF-α [55,56]. Binding of miR-16 to AREs of mRNAs results in destabilization of the targeted mRNAs. Indeed in this study, we found that miR-16 impairs TNF-α, IL-6 and IFN-β secretion in M (LPS + IFNγ) activated macrophages. However,

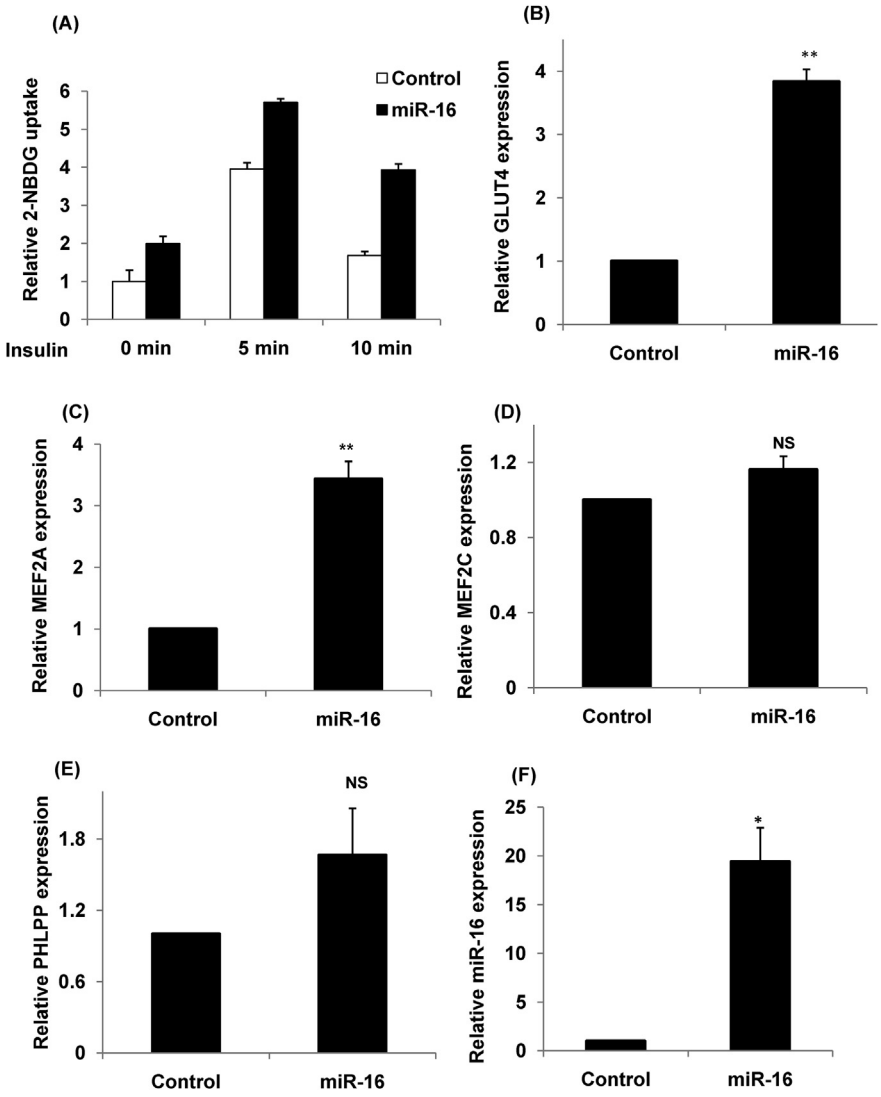


Fig. 6. Ectopic expression of miRNA-16 enhances insulin stimulated glucose uptake by L6 myoblasts. (A) Mean basal and insulin stimulated glucose uptake was measured using 2-NBDG by fluorometry in L6 myoblasts transfected with control or miR-16 mimic. Values are normalized with the corresponding protein content and expressed relative to basal control cells which was set as 1. Data (mean ± SD) shown are a representative of three independent experiments. (B) GLUT4, (C) MEF2A, (D) MEF2C, (E) PHLPP expression in miR-16 mimic over-expressed L6 myoblasts was analyzed by qPCR. Values are normalized with the reference gene 18S. **, $p < 0.001$ and NS represents non-significant calculated using Student's *t*-test. (F) miR-16 expression levels were analyzed using qPCR. Values are normalized with the reference gene U6. *$p < 0.05$ calculated using Student's *t*-test.

miR-16 appears to have no effect on IP-10 production, iNOS expression, ROS generation or a phagocytic activity of macrophages, indicating that miR-16 regulates specific responses of M (LPS + IFNγ) macrophages. In addition, miR-16 promoted IL-4-stimulated arginase-1 expression but showed no effect on other M (IL-4) markers such as MRC-1, IL-10 and YM-1. These observations reiterate the fact that different miRNA regulate varied signaling cascades in M (LPS + IFNγ) or M (IL-4) macrophages. Supporting this, infection of BMDMs from mice deficient in miR-16 and miR-15a (family member of miR-16) with *E. coli* enhanced the expression of IL-1β, IL-6 and IL-21 however exerted no effect on TNF-α synthesis [46]. Moreover it was observed in the same study that co-transfection of miR-16 and miR-15a impaired phagocytic activity of BMDMs, while in our study, no significant effect on phagocytic uptake was noted upon miR-16 over-expression alone suggesting that a concerted effect of miR-15a and miR-16 may be probably required for influencing certain processes such as phagocytosis [54].

The past decade has seen an unprecedented rise in the prevalence of obesity and overweight which leads to numerous complications like type 2 diabetes (T2D), cardiovascular diseases and even certain types of cancers [57,58]. More importantly, it is widely accepted that the progression of these diseases is mainly due to low grade chronic inflammation characterized by an increase in pro-inflammatory macrophage population [59–61]. Growing evidence suggests that miRNA are largely dysregulated in obesity and obesity associated inflammation [62–64]. Interestingly, in the current study we observed the expression of miR-16 was depleted in macrophages and myoblasts exposed to palmitate, a major driver of inflammation induced insulin resistance [65]. Further the expression of miR-16 was also diminished in insulin responsive peripheral tissues such as soleus muscle, adipose and liver of HSD induced insulin resistant rats. Our results agree in part with findings of the recent study of Olivo-Marston et al. where they observed that miR-16 expression is decreased in colon mucosa upon DIO (diet induced obesity) while upon calorie restriction the expression of miR-16 is enhanced, which is likely to be associated with inflammation induced colon cancer [66]. We have also

observed in this study that incubation of L6 myoblasts with the conditioned supernatants of miR-16 transfected M (LPS + IFNγ) skewed macrophages partially rescued the insulin stimulated glucose uptake. Interestingly, miR-16 over-expression in L6 myoblasts improved insulin sensitivity of the cells via up-regulation of GLUT4 and MEF2A expression. Our observations are in agreement with the findings of Vaag et al. where they noted that miR-16 expression was reduced in L6 cells cultured under high glucose conditions and in skeletal muscles of T2D patients [67]. Further, the findings of this study are in line with the observations of Ye et al. They reported that miR-16 was depleted under high glucose conditions in human retinal endothelial cells, while over-expression of miR-16 significantly improved the insulin receptor phosphorylation with a concomitant decrease in inflammatory cytokine TNF-α [68].

How miR-16 regulates pro-inflammatory responses and enhances insulin sensitivity at molecular level? In addition to the mRNA destabilizing effects of miR-16 [69], miR-15a/16 were recently shown to target TLR4 expression by regulating PU.1 transcription factor upon LPS or bacterial stimulation leading to enhanced phagocytosis & ROS production and increased expression of IL-1β, IL-6 and IL-21 cytokines [46]. In addition, computational analysis using TargetScan identified several putative targets of miR-16 that likely explain the effects of miR-16 on M (LPS + IFNγ) macrophages. These putative targets include TRAF3, IRAK2, TAB3, LITAF and MAPK pathway players (MEK1, Raf1 and ERK1) which have a very important role in TLR4/NFκB signaling [70–73]. Further, although speculative, other possible scenario could be that miR-16 may be down-regulating CCDN1 and Bcl2 expression [74,75]. CCDN1 regulates Cdk4 & Cyclin D/E activity. Recent studies show that Cdk4/cyclin D/E complex is involved in the regulation of glucose metabolism [76] and inflammatory responses [77]. Bcl2, an anti-apoptotic protein, is also reported to associate directly with insulin receptor substrates (IRS1/2), which has the potential to alter the insulin signaling [78]. Further Kwon et al. proposed that miR-16 may have a role in insulin/PI3k-Akt signaling cascade [79]. Taken together, our findings demonstrate that miR-16 has an important role in controlling macrophage induced inflammation and insulin sensitivity.

5. Conclusion

In summary, we show that miR-16 improves overall insulin sensitivity via the attenuation of macrophage mediated pro-inflammatory responses and augmentation of skeletal muscle insulin sensitivity. Our work demonstrates that restoration of miR-16 expression in M (LPS + IFNγ) macrophages or in skeletal muscle may ameliorate insulin sensitivity which is beneficial during the treatment of T2D.

Author contributions

KVLP conceptualized and designed the entire study. MT, BK, VK, BP and PR performed the experiments with MT performing majority of them. SS supervised HSD rat experiments. PM provided intellectual inputs and critical reagents. MT, BK and KVLP wrote the paper.

Funding

This work is financially supported by grants from Science & Engineering Research Board (SERB; SR/FT/LS-131/2009) to KVLP and Department of Biotechnology (DBT; BT/PR13527/BRB/10/765/2010) to PM and KVLP. MT and BK are thankful to CSIR for JRF/SRF research fellowship. VK acknowledges financial support from DBT (BT/PR13527/BRB/10/765/2010). BP acknowledges financial support from Jawaharlal Memorial Fund, DST, for research fellowship (SU-1/123/2014-15). SS and PR are thankful to NERF for financial support. The funding agencies have no role in the study design, collection, analysis and interpretation of the data.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

KVLP and PM are grateful for the constant encouragement and support from Drs. Venkateswarlu and Uday Saxena.

References

[1] D.M. Mosser, J.P. Edwards, Exploring the full spectrum of macrophage activation, Nat. Rev. Immunol. 8 (12) (2008) 958–969.
[2] S. Gordon, The macrophage: past, present and future, Eur. J. Immunol. 37 (Suppl. 1) (2007) S9–S17.
[3] P.J. Murray, et al., Macrophage activation and polarization: nomenclature and experimental guidelines, Immunity 41 (1) (2014) 14–20.
[4] M.C. Calle, M.L. Fernandez, Inflammation and type 2 diabetes, Diabetes Metab. 38 (3) (2012) 183–191.
[5] M.Y. Donath, S.E. Shoelson, Type 2 diabetes as an inflammatory disease, Nat. Rev. Immunol. 11 (2) (2011) 98–107.
[6] G.M. Reaven, Role of insulin resistance in human disease, Diabetes 37 (12) (1988) 1595–1607.
[7] S.M. Alwahsh, et al., Combination of alcohol and fructose exacerbates metabolic imbalance in terms of hepatic damage, dyslipidemia, and insulin resistance in rats, PLoS One 9 (8) (2014) e104220.
[8] H. Basciano, L. Federico, K. Adeli, Fructose, insulin resistance, and metabolic dyslipidemia, Nutr. Metab. (Lond) 2 (1) (2005) 5.
[9] E.J. Schaefer, J.A. Gleason, M.L. Dansinger, Dietary fructose and glucose differentially affect lipid and glucose homeostasis, J. Nutr. 139 (6) (2009) 1257S–1262S.
[10] B.W. Parks, et al., Genetic control of obesity and gut microbiota composition in response to high-fat, high-sucrose diet in mice, Cell Metab. 17 (1) (2013) 141–152.
[11] C.N. Lumeng, J.L. Bodzin, A.R. Saltiel, Obesity induces a phenotypic switch in adipose tissue macrophage polarization, J. Clin. Invest 117 (1) (2007) 175–184.
[12] M. Zeyda, T.M. Stulnig, Adipose tissue macrophages, Immunol. Lett. 112 (2) (2007) 61–67.
[13] S.E. Borst, The role of TNF-alpha in insulin resistance, Endocrine 23 (2–3) (2004) 177–182.
[14] J.M. Olefsky, C.K. Glass, Macrophages, inflammation, and insulin resistance, Annu. Rev. Physiol. 72 (2010) 219–246.
[15] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, cell 116 (2) (2004) 281–297.
[16] X. Chen, MicroRNA biogenesis and function in plants, FEBS Lett. 579 (26) (2005) 5923–5931.
[17] G. Liu, et al., miR-147, a microRNA that is induced upon Toll-like receptor stimulation, regulates murine macrophage inflammatory responses, Proc. Natl. Acad. Sci. 106 (37) (2009) 15819–15824.
[18] J. Qi, et al., microRNA-210 negatively regulates LPS-induced production of proinflammatory cytokines by targeting NF-κB1 in murine macrophages, FEBS Lett. 586 (8) (2012) 1201–1207.
[19] R.M. O'Connell, D.S. Rao, D. Baltimore, microRNA regulation of inflammatory responses, Annu. Rev. Immunol. 30 (2012) 295–312.
[20] M. Balasubramanyam, et al., Impaired miR-146a expression links subclinical inflammation and insulin resistance in Type 2 diabetes, Mol. Cell. Biochem. 351 (1–2) (2011) 197–205.
[21] R. Dai, S.A. Ahmed, MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases, Transl. Res. 157 (4) (2011) 163–179.
[22] M.D. Williams, G.M. Mitchell, MicroRNAs in insulin resistance and obesity, Exp. Diabetes Res. 2012 (2012) 484696.
[23] S. Sinha, et al., Fatty acid-induced insulin resistance in L6 myotubes is prevented by inhibition of activation and nuclear localization of nuclear factor kappa B, J. Biol. Chem. 279 (40) (2004) 41294–41301.
[24] S.L. Weinstein, et al., Phosphatidylinositol 3-kinase and mTOR mediate lipopolysaccharide-stimulated nitric oxide production in macrophages via interferon-beta, J. Leukoc. Biol. 67 (3) (2000) 405–414.
[25] P.K. Jena, S. Singh, S. Seshadri, Sugar rich diet induced insulin resistance and alteration in gut microflora, Diabetes Mellit. Hum. Health Care A Holist. Approach Diagnosis Treat. (2014) 393.
[26] G. Liu, E. Abraham, MicroRNAs in immune response and macrophage polarization, Arterioscler., thromb., Vasc. Biol. 33 (2) (2013) 170–177.

[27] S. Banerjee, et al., MicroRNA let-7c regulates macrophage polarization, J. Immunol. 190 (12) (2013) 6542–6549.
[28] R.M. O'Connell, et al., MicroRNA-155 is induced during the macrophage inflammatory response, Proc. Natl. Acad. Sci. U. S. A. 104 (5) (2007) 1604–1609.
[29] M.T. Nguyen, et al., A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways, J. Biol. Chem. 282 (48) (2007) 35279–35292.
[30] M. Trajkovski, et al., MicroRNAs 103 and 107 regulate insulin sensitivity, Nature 474 (7353) (2011) 649–653.
[31] G.S. Hotamisligil, N.S. Shargill, B.M. Spiegelman, Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance, Science 259 (5091) (1993) 87–91.
[32] J.P. Bastard, et al., Adipose tissue IL-6 content correlates with resistance to insulin activation of glucose uptake both in vivo and in vitro, J. Clin. Endocrinol. Metab. 87 (5) (2002) 2084–2089.
[33] S. Díaz-Prado, et al., Characterization of microRNA expression profiles in normal and osteoarthritic human chondrocytes, BMC Musculoskelet. Disord. 13 (1) (2012) 144.
[34] I.-S. Hwang, et al., Fructose-induced insulin resistance and hypertension in rats, Hypertension 10 (5) (1987) 512–516.
[35] C. Lavigne, et al., Prevention of skeletal muscle insulin resistance by dietary cod protein in high fat-fed rats, Am. J. Physiol. Endocrinol. Metab. 281 (1) (2001) E62–E71.
[36] N.P. Alamuru, et al., A novel immunomodulatory function of PHLPP1: inhibition of iNOS via attenuation of STAT1 ser727 phosphorylation in mouse macrophages, J. Leukoc. Biol. 95 (5) (2014) 775–783.
[37] F. Andreozzi, et al., Increased levels of the Akt-specific phosphatase PH domain leucine-rich repeat protein phosphatase (PHLPP)-1 in obese participants are associated with insulin resistance, Diabetologia 54 (7) (2011) 1879–1887.
[38] S. Fernandez-Veledo, et al., Ceramide mediates TNF-alpha-induced insulin resistance on GLUT4 gene expression in brown adipocytes, Arch. Physiol. Biochem. 112 (1) (2006) 13–22.
[39] G. Mingrone, et al., Skeletal muscle triglycerides lowering is associated with net improvement of insulin sensitivity, TNF-alpha reduction and GLUT4 expression enhancement, Int. J. Obes. Relat. Metab. Disord. 26 (9) (2002) 1165–1172.
[40] S. Mora, J.E. Pessin, The MEF2A isoform is required for striated muscle-specific expression of the insulin-responsive GLUT4 glucose transporter, J. Biol. Chem. 275 (21) (2000) 16323–16328.
[41] D.P. Sparling, et al., GLUT4 enhancer factor (GEF) interacts with MEF2A and HDAC5 to regulate the GLUT4 promoter in adipocytes, J. Biol. Chem. 283 (12) (2008) 7429–7437.
[42] H. Hauner, et al., Effects of tumour necrosis factor alpha (TNFα) on glucose transport and lipid metabolism of newly-differentiated human fat cells in cell culture, Diabetologia 38 (7) (1995) 764–771.
[43] J.B. Knight, et al., Regulation of the human GLUT4 gene promoter: interaction between a transcriptional activator and myocyte enhancer factor 2A, Proc. Natl. Acad. Sci. 100 (25) (2003) 14725–14730.
[44] N.A. Warfel, A.C. Newton, Pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP): a new player in cell signaling, J. Biol. Chem. 287 (6) (2012) 3610–3616.
[45] J.W. Graff, et al., Identifying functional microRNAs in macrophages with polarized phenotypes, J. Biol. Chem. 287 (26) (2012) 21816–21825.
[46] H.-G. Moon, et al., miR-15a/16 regulates macrophage phagocytosis after bacterial infection, J. Immunol. 193 (9) (2014) 4558–4567.
[47] R. Aqeilan, G. Calin, C. Croce, miR-15a and miR-16-1 in cancer: discovery, function and future perspectives, Cell Death Differ. 17 (2) (2010) 215–220.
[48] A. Bottoni, et al., miR-15a and miR-16-1 down-regulation in pituitary adenomas, J. Cell. Physiol. 204 (1) (2005) 280–285.
[49] T. Li, et al., MicroRNAs modulate the noncanonical NF-κB pathway by regulating IKKα expression during macrophage differentiation, Nat. Immunol. 11 (9) (2010) 799.
[50] M. Satoh, et al., Circulating Toll-like receptor 4-responsive microRNA panel in patients with coronary artery disease: results from prospective and randomized study of treatment with renin-angiotensin system blockade, Clin. Sci. (Lond) 128 (8) (2015) 483–491.
[51] S.I. Grivennikov, M. Karin, Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer, Cytokine Growth Factor Rev. 21 (1) (2010) 11–19.
[52] M.A. Rivas, et al., Downregulation of the tumor-suppressor miR-16 via progestin-mediated oncogenic signaling contributes to breast cancer development, Breast Cancer Res. 14 (3) (2012) R77.
[53] R. Zhou, et al., miR-16 targets transcriptional corepressor SMRT and modulates NF-kappaB-regulated transactivation of interleukin-8 gene, PLoS One 7 (1) (2012) e30772.
[54] H.G. Moon, et al., miR-15a/16 regulates macrophage phagocytosis after bacterial infection, J. Immunol. 193 (9) (2014) 4558–4567.
[55] Q. Jing, et al., Involvement of microRNA in AU-rich element-mediated mRNA instability, Cell 120 (5) (2005) 623–634.
[56] P. Anderson, Post-transcriptional control of cytokine production, Nat. Immunol. 9 (4) (2008) 353–359.
[57] C. Zhang, et al., Abdominal obesity and the risk of all-cause, cardiovascular, and cancer mortality sixteen years of follow-up in US women, Circulation 117 (13) (2008) 1658–1667.
[58] K.M. Flegal, et al., Cause-specific excess deaths associated with underweight, overweight, and obesity, Jama 298 (17) (2007) 2028–2037.
[59] A.H. Berg, P.E. Scherer, Adipose tissue, inflammation, and cardiovascular disease, Circ. Res. 96 (9) (2005) 939–949.
[60] J.S. Yudkin, et al., Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? Atherosclerosis 148 (2) (2000) 209–214.
[61] P. Dandona, A. Aljada, A. Bandyopadhyay, Inflammation: the link between insulin resistance, obesity and diabetes, Trends Immunol. 25 (1) (2004) 4–7.
[62] M. Hulsmans, D. De Keyzer, P. Holvoet, MicroRNAs regulating oxidative stress and inflammation in relation to obesity and atherosclerosis, FASEB J. 25 (8) (2011) 2515–2527.
[63] H. Heneghan, N. Miller, M. Kerin, Role of microRNAs in obesity and the metabolic syndrome, Obes. Rev. 11 (5) (2010) 354–361.
[64] R. McGregor, M. Choi, microRNAs in the regulation of adipogenesis and obesity, Curr. Mol. Med. 11 (4) (2011) 304.
[65] A.B. Thrush, et al., Palmitate acutely induces insulin resistance in isolated muscle from obese but not lean humans, Am. J. Physiol. Regul. Integr. Comp. Physiol. 294 (4) (2008) p. R1205-12.
[66] S.E. Olivo-Marston, et al., Effects of calorie restriction and diet-induced obesity on murine colon carcinogenesis, growth and inflammatory factors, and microRNA expression, PloS One 9 (4) (2014) e94765.
[67] J. Bork-Jensen, et al., Glucose tolerance is associated with differential expression of microRNAs in skeletal muscle: results from studies of twins with and without type 2 diabetes, Diabetologia 58 (2) (2015) 363–373.
[68] E.-A. Ye, J.J. Steinle, miR-15b/16 protects primary human retinal microvascular endothelial cells against hyperglycemia-induced increases in tumor necrosis factor alpha and suppressor of cytokine signaling 3, J. Neuroinflamm. 12 (1) (2015) 44.
[69] Q. Jing, et al., Involvement of microRNA in AU-rich element-mediated mRNA instability, Cell 120 (5) (2005) 623–634.
[70] Y.-C. Lu, W.-C. Yeh, P.S. Ohashi, LPS/TLR4 signal transduction pathway, Cytokine 42 (2) (2008) 145–151.
[71] K. Takeda, S. Akira, TLR signaling pathways, Semin. Immunol. 16 (1) (2004) 3–9.
[72] T. Ishitani, et al., Role of the TAB2-related protein TAB3 in IL-1 and TNF signaling, EMBO J. 22 (23) (2003) 6277–6288.
[73] X. Tang, et al., LPS-induced TNF-α factor (LITAF)-deficient mice express reduced LPS-induced cytokine: evidence for LITAF-dependent LPS signaling pathways, Proc. Natl. Acad. Sci. 103 (37) (2006) 13777–13782.
[74] C.-K. Cai, et al., miR-15a and miR-16-1 downregulate CCND1 and induce apoptosis and cell cycle arrest in osteosarcoma, Oncol. Rep. 28 (5) (2012) 1764–1770.
[75] A. Cimmino, et al., miR-15 and miR-16 induce apoptosis by targeting BCL2, Proc. Natl. Acad. Sci. U. S. A. 102 (39) (2005) 13944–13949.
[76] Y. Lee, et al., Cyclin D1-Cdk4 controls glucose metabolism independently of cell cycle progression, Nature 510 (7506) (2014) 547–551.
[77] A. Leitch, C. Haslett, A. Rossi, Cyclin-dependent kinase inhibitor drugs as potential novel anti-inflammatory and pro-resolution agents, Br. J. Pharmacol. 158 (4) (2009) 1004–1016.
[78] H. Ueno, et al., Association of insulin receptor substrate proteins with Bcl-2 and their effects on its phosphorylation and antiapoptotic function, Mol. Biol. Cell 11 (2) (2000) 735–746.
[79] D.-N. Kwon, B.-S. Chang, J.-H. Kim, MicroRNA dysregulation in liver and pancreas of CMP-Neu5Ac hydroxylase null mice disrupts insulin/PI3K-AKT signaling, BioMed Res. Int. 2014 (2014).