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Modelling 16/8 intermittent fasting and breakfast-skipping in mouse adipocytes 3T3-L1 *in vitro*: a transcriptomics study

Pei Han Er, Jin Yi Chye, Geetha Letchumanan, Yee-How Say *

Department of Biomedical Sciences, Sir Jeffrey Cheah Sunway Medical School, Faculty of Medical and Life Sciences, Sunway University, 5 Jalan Universiti, Bandar Sunway, 47500 Subang Jaya, Selangor, Malaysia

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ABSTRACT

Background: Obesity, a chronic metabolic disease, lacks effective treatments. Intermittent fasting (IF), especially time-restricted feeding (TRF), shows promise, yet the comparative molecular effects of different regimens remain unclear. This study examined their impact on 3T3-L1 adipocytes and transcriptomic changes.

Methods: 3T3-L1 cells were differentiated into adipocytes for 7 d and synchronized with 200 nM dexamethasone before 24 h treatments: (1) Control [high glucose (4.5 g/L DMEM), 15 % bovine calf serum (BCS)], (2) 16 h fasting/8h feeding IF [control medium ZT3–ZT11; low glucose (1.0 g/L) and low serum (1 % BCS) ZT12–ZT2], (3) "Distributed" IF [medium glucose (2.75 g/L), medium serum (8 % BCS) ZT0–ZT24], (4) Breakfast-skipping (BS) [low glucose/low serum ZT1–ZT5; control medium ZT6–ZT0]. Lipid accumulation was assessed by Oil Red O staining; whole transcriptome sequencing was performed.

Results: The 16/8 IF regimen produced the greatest lipid reduction (74.41 % *vs.* control; $p = 0.007$) with upregulation of lipolysis genes (*Tgm2*, *Notch2*) and downregulation of adipogenesis and glycolysis genes (*Ccnd1*, *Ldha*). Pathway enrichment included TGF-β, p53, and apelin signaling. In contrast, the BS regimen did not differ significantly in lipid accumulation compared to control ($p = 0.999$), but nevertheless showed downregulation of mitochondrial genes *mt-Rnr1* and *mt-Rnr2*.

Conclusion: This work highlights the novel comparative evaluation of IF/TRF regimens in adipocytes using transcriptomics, demonstrating that 16/8 IF most effectively reduced lipid content and modulated metabolic pathways within 24 h. These findings provide mechanistic insight that strengthens the rationale for TRF as a dietary approach for obesity management.

1. Introduction

Obesity, defined as a body mass index (BMI) $\geq$ 30 kg/m^2, is a complex chronic disease characterized by excess adipose tissue (AT) accumulation.[1] It is strongly associated with increased risks of cardiovascular disease, type 2 diabetes, and several cancers, making it a pressing public health challenge.[2] Despite being preventable, the global prevalence of obesity has risen steadily over the past three decades. According to the World Health Organization (WHO), the proportion of adults aged $\geq$18 years with obesity increased from 7 % in 1990 to 16 % in 2022, while childhood and adolescent obesity also quadrupled during the same period.[1] This rapid increase underscores the urgent need for effective and sustainable treatment strategies.

Conventional treatment approaches include lifestyle modifications, pharmacotherapies, and bariatric surgery. While lifestyle interventions can induce weight loss, maintaining long-term adherence is difficult, and weight regain is common.[3–4] Pharmacological options, such as glucagon-like peptide-1 (GLP-1) receptor agonists, have shown promising results but face barriers of high cost, limited availability, and weight relapse after discontinuation.[5] Bariatric surgery remains the most effective option for severe obesity but is invasive and still requires long-term dietary management.[5] Collectively, these limitations highlight the need to explore more practical and sustainable alternatives.

Intermittent fasting (IF), defined as an eating pattern alternating between fasting and feeding periods, has emerged as a promising dietary strategy.[6] Several IF approaches exist, including alternate-day fasting (ADF), the 5:2 diet, and time-restricted feeding (TRF).[7–8] Among these, TRF—such as the 16/8 regimen (16 h fasting, 8 h feeding)—is the most widely adopted due to its feasibility and simplicity[9]. Unlike traditional caloric restriction, IF primarily emphasizes when food is consumed

* Corresponding author.
E-mail address: yeehows@sunway.edu.my (Y.-H. Say).

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rather than what or how much.[10–11]

Growing evidence suggests that IF/TRF influences adipocyte biology. *In vivo* and *in vitro* studies have demonstrated altered expression of genes related to adipogenesis, lipid metabolism, and inflammation. For example, IF has been shown to increase lipolytic markers (e.g., *PDK4*, *PNPLA7*) while downregulating pro-adipogenic and inflammatory genes.[12–14] Other studies report activation of the p53 signalling pathway, suggesting IF may promote adipocyte apoptosis and reduced adiposity.[15] However, findings remain inconsistent, and the direct cell-autonomous effects of IF on adipocytes are still poorly understood.

Adipocytes, beyond their role in energy storage, possess intrinsic circadian clocks that regulate lipolysis, adipogenesis, adipokine secretion, and inflammatory responses.[16] Feeding patterns that disrupt circadian rhythms, such as breakfast-skipping, may therefore impair adipocyte function and contribute to metabolic dysregulation. To investigate such mechanisms, the 3T3-L1 mouse preadipocyte line is widely used as an *in vitro* model due to its robust capacity to differentiate into mature adipocytes, express adipogenic genes, secrete adipokines, and accumulate lipids.[17–18] This model also allows controlled testing of interventions such as IF/TRF, without the confounding systemic factors present *in vivo*. In circadian biology, feeding and treatment schedules are often expressed in terms of Zeitgeber Time (ZT), which represents the 24 h light–dark cycle (ZT0 = lights on, ZT12 = lights off), providing a framework for experimental synchronization.[19]

The present study aimed to evaluate the effects of different IF/TRF regimens—16/8 IF, "Distributed" IF, and breakfast-skipping (BS)—on lipid accumulation and global transcriptomic profiles in differentiated 3T3-L1 adipocytes. By combining Oil Red O lipid quantification with whole transcriptome sequencing, this study provides direct mechanistic insights into how fasting regimens differentially regulate adipocyte metabolism. While previous research has explored IF in animal models or single-regimen cell culture systems, this work is among the first to perform a comparative transcriptomic analysis of multiple IF/TRF patterns *in vitro*. These findings may advance understanding of how feeding schedules influence adipocyte biology and inform the development of more effective obesity interventions.

2. Materials and methods

2.1. 3T3-L1 cell culture and treatment Paradigm

3T3-L1 fibroblasts were differentiated into mature adipocytes over a 10-day period at 37 °C, 5 % (v/v) CO_2, according to.[18] On Day 0, cells were seeded into a 12-well plate at a density of 5×10^4 cells/well in Pre-adipocyte Expansion Medium (PEM) containing 90 % (v/v) DMEM with 4.5 g/L glucose and 10 % (v/v) Bovine Calf Serum (BCS; TICO Europe, Netherlands). Cell growth and confluency were monitored using an inverted microscope (Nikon Eclipse TS100, Japan). Day 2 started when cell confluency reached approximately 75 %. Cells were rinsed twice with 1 × Phosphate-Buffered Saline (PBS), and Differentiation Medium (DM) containing 90 % (v/v) DMEM with 4.5 g/L glucose, 10 % (v/v) BCS, 1 µg/mL insulin (Nacalai Tesque, Japan), 0.25 µM dexamethasone (Nacalai Tesque, Japan), 2 µM rosiglitazone (TCI Chemicals, Japan), and 0.5 mM isobutylmethylxanthine (Merck, Germany) was added to each well. On Day 4, DM was replaced by Adipocyte Maintenance Medium (AMM) containing 85 % (v/v) DMEM with 4.5 g/L glucose, 15 % (v/v) BCS, and 1 µg/mL Insulin after rinsing the cells with PBS. On Day 6, AMM was replaced by DM to further induce differentiation after rinsing the cells with PBS. On Day 8, DM was replaced by AMM, following PBS rinses, to further induce lipid accumulation.

On Day 8, the circadian clock of the cells was synchronized with 200 nM dexamethasone for 30 min at room temperature (RT),[20] followed by rinsing with AMM. Then, the differentiated 3T3-L1 adipocytes were subjected to four groups of treatment regimens during a 24-hour period, with the sunrise time of 0700 [Zeitgeber Time 0 (ZT0)] and sunset time of 1900 [Zeitgeber Time 12 (ZT12)]. The four groups were: 1. Control group – AMM with 4.5 g/L glucose (control medium); 2. 16 h fasting/8h feeding IF – control medium from ZT3 to ZT11, and switched to AMM with low glucose (1.0 g/L in DMEM) and low serum (1 % BCS) from ZT12 to ZT2 (next day); 3. "Distributed" IF – AMM with medium glucose (2.75 g/L in DMEM) and medium serum (8 % BCS) from ZT0 to ZT24; 4. Breakfast-skipping BS – AMM with low glucose and low serum from ZT1 to ZT5, and switched to control medium from ZT6 to ZT0 (next day).

2.2. Lipid accumulation assay

After 24 h treatment, the spent medium was discarded from the wells. Cells were then rinsed twice with PBS, and 1 mL of 4 % (w/v) paraformaldehyde (PFA; Sigma-Aldrich, MO, USA) was added to each well to fix the cells. The plate was incubated at RT for 1 h. Meanwhile, a 0.5 % (w/v) Oil Red O (ORO; Sigma-Aldrich, MO, USA) stock solution was diluted with sterile deionized water in a 3:2 ratio (ORO:water) and was incubated at RT for 20 min. The solution was filter-sterilized prior to use. After incubation, PFA was discarded, and fixed cells were stained with 1 mL of the filtered, diluted 0.5 % (w/v) ORO solution. The plate was then incubated at RT for 20 min. Subsequently, the solution was discarded, and cells were washed twice with PBS. Stained lipids in the cells were observed and imaged under an inverted microscope (Nikon Eclipse TS100, Japan) at 100× and 200× magnification. After microscopy, PBS was discarded, and 1 mL of isopropanol was added into the wells to dissolve the stained lipids. The plate was incubated at RT for 20 min. Following that, 100 µL aliquot from each well was transferred to a 96-well plate. Absorbance was measured at 540 nm with an Infinite® M Plex microplate reader (Tecan, Switzerland), whereby isopropanol was used as a blank, to quantify the intracellular lipid content. Lipid accumulation rate was calculated relative to the control group using the formula: Lipid accumulation rate (%) = [average absorbance of treatment group − average blank/average absorbance of control group − average blank] × 100 %.

2.3. RNA extraction

Total RNA was extracted from treated 3T3-L1 adipocytes of the control group, 16/8 IF group (most effective treatment), and breakfast-skipping group (least effective treatment) using the NucleoSpin® RNA extraction kit (MACHEREY-NAGEL, Germany) following the manufacturer's protocol with slight modifications. After 24 h treatment, cells were lysed by adding 350 µL of Lysis Buffer RA1 for 15 min. The lysate was then transferred to a 1.5 mL microcentrifuge tube and homogenized by passing it through a 21G needle attached to a syringe for 10 times, cleared by centrifugation through a NucleoSpin® Filter at 11,000 *g* for 1 min, and 350 µL 70 % ethanol was added to the filtrate and mixed. The mixture was then loaded onto NucleoSpin® RNA Column and centrifuged for 30 sec at 11,000 *g*, column then placed in a new collection tube, 350 µL membrane desalting buffer added, and centrifuged for 1 min at 11,000 *g*. Digestion of DNA was performed by adding 95 µL of DNase reaction mixture directly onto the silica membrane of the column and incubating at RT for 15 min, column washed with 200 µL Buffer RAW2 (centrifuged at 11,000 *g* for 30 sec), followed by two washes with 600 µL (centrifuged at 11,000 *g* for 30 sec) and 250 µL (centrifuged at 11,000 *g* for 2 min) of Buffer RA3. The column was spun dry in the final wash step, and finally, the purified RNA was eluted from the column adding 60 µL of RNase-free H_2O and centrifuging for 1 min at 11,000 *g*. Subsequently, RNA quality and quantity assessments were performed using the BioDrop µLite + Microvolume Spectrophotometer (Biochrom, UK) and non-denaturing 1 % agarose gel electrophoresis in TAE buffer (0.5 mM, 0.02 M Tris, 0.01 M glacial acetic acid). All extracted RNA was stored at –80 °C until use.

2.4. Whole transcriptome sequencing (WTS) and differential gene expression (DEG) analysis

The RNA samples were sent to AGTC Genomics Sdn. Bhd. (Kuala Lumpur, Malaysia) for WTS. A total of two biological replicates per group were used for WTS analysis. Prior to sequencing, RNA sample quality was assessed to ensure optimal data generation. RNA concentration was determined using the Qubit™ RNA High Sensitivity (HS) assay kit and the Qubit Fluorometer 4.0 (Invitrogen). RNA purity (A260/280 and A260/230 ratios) was evaluated using the Implen NanoQuant Spectrophotometer (Implen, Germany). RNA quality was further assessed using the LabChip™ RNA Assay Reagent Kit (Revvity) and the LabChip® GX Touch™ nucleic acid analyzer (Revvity), which yielded RNA Quality Scores (RQS) ranging from 6.8 to 7.3 (out of 10 = most intact), indicating "pass" scores. The samples were then sequenced on Illumina NovaSeq 6000 system (Illumina, CA, USA) system, with 20 million reads. Libraries were normalized to 1 nM and pooled before loading into the NovaSeq 6000 sequencing platform. Illumina DRAGEN encompassed an RNA-seq (splicing-aware) aligner, along with RNA-specific analysis components for gene expression quantification and gene fusion detection. For differential gene expression (DEG) analysis, the transcript quantification file was imported into R. DEG calling was performed using DESeq2.[21] Gene Ontology (GO) and KEGG pathway enrichment analysis were conducted using clusterProfiler.[22] Reference genome used in this analysis was *Mus musculus* genome assembly GRCm38: GCF_000001635.20 downloaded from NCBI.

2.5. Statistical analysis

All statistical tests were conducted in the R statistical environment, v4.0.3. All results were presented as mean ± SD based on three independent experiments, each with triplicate readings. Differences in adipocytes lipid accumulation rate (%) between treatment groups relative to the control group were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* test. All hypothesis testing was two-sided, and a p-value < 0.05 was considered statistically significant.

3. Results

3.1. Lipid accumulation assay

Fig. 1A shows ORO-stained 3T3-L1 adipocytes with different treatment regimens under 100 × and 200 × magnifications. The morphology of the adipocytes was similar between the control group and the rest of the treatment groups. However, it can be observed that the 16/8 IF treated cells had the least intensity of red color staining compared with other treatment groups, while the BS treated cells had the most intense red color staining among all treatment regimens. Fig. 1B shows that the 16/8 IF group had the most reduction in the average percentage of lipid accumulation rate relative to the control group (−25.59 %), followed by the "distributed" IF group (−15.21 %), and BS group (−1.47 %). ANOVA showed that the differences among the groups were statistically significant ($p = 0.006$), and Dunnett's *post hoc* test indicated that lipid accumulation rate was significantly reduced in the 16/8 IF group ($p = 0.007$) and the distributed IF group ($p = 0.048$), relative to the control group. However, the BS regimen did not differ significantly in lipid accumulation compared to control ($p = 0.999$).

3.2. Whole transcriptome sequencing (WTS) and differential gene expression (DEG) analysis

Fig. 2 shows the comprehensive transcriptomic analysis of 3T3-L1 adipocytes in 16/8 IF group compared to control group. Out of 13,652 DEGs, 85 were significantly upregulated and 130 were significantly downregulated in response to 16/8 IF treatment (Fig. 2A). The heatmap analysis showed similar expression patterns between treatment duplicates, signifying replicability (Fig. 2B). The Gene Ontology (GO) enrichment analysis highlighted significantly modulated biological

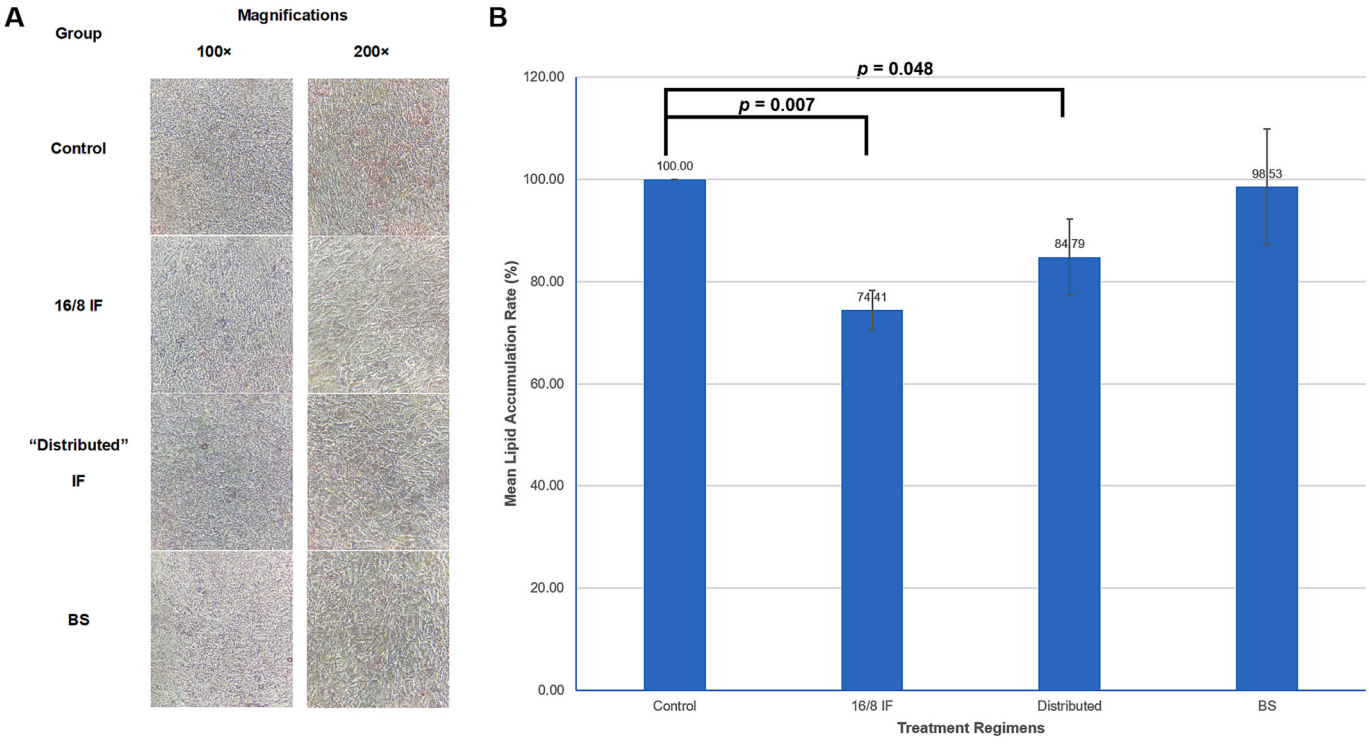

Fig. 1. Bar graph of the lipid accumulation rate (%) of different treatment groups relative to the control group. Data was expressed as mean ± SD ($n = 6$) based on six independent experiments, each with triplicate readings. A one-way ANOVA followed by Dunnett's *post hoc* test was used to compare each treatment group to the control. Significant differences compared to the control group were indicated (#$p < 0.05$, *$p < 0.01$, **$p < 0.001$).

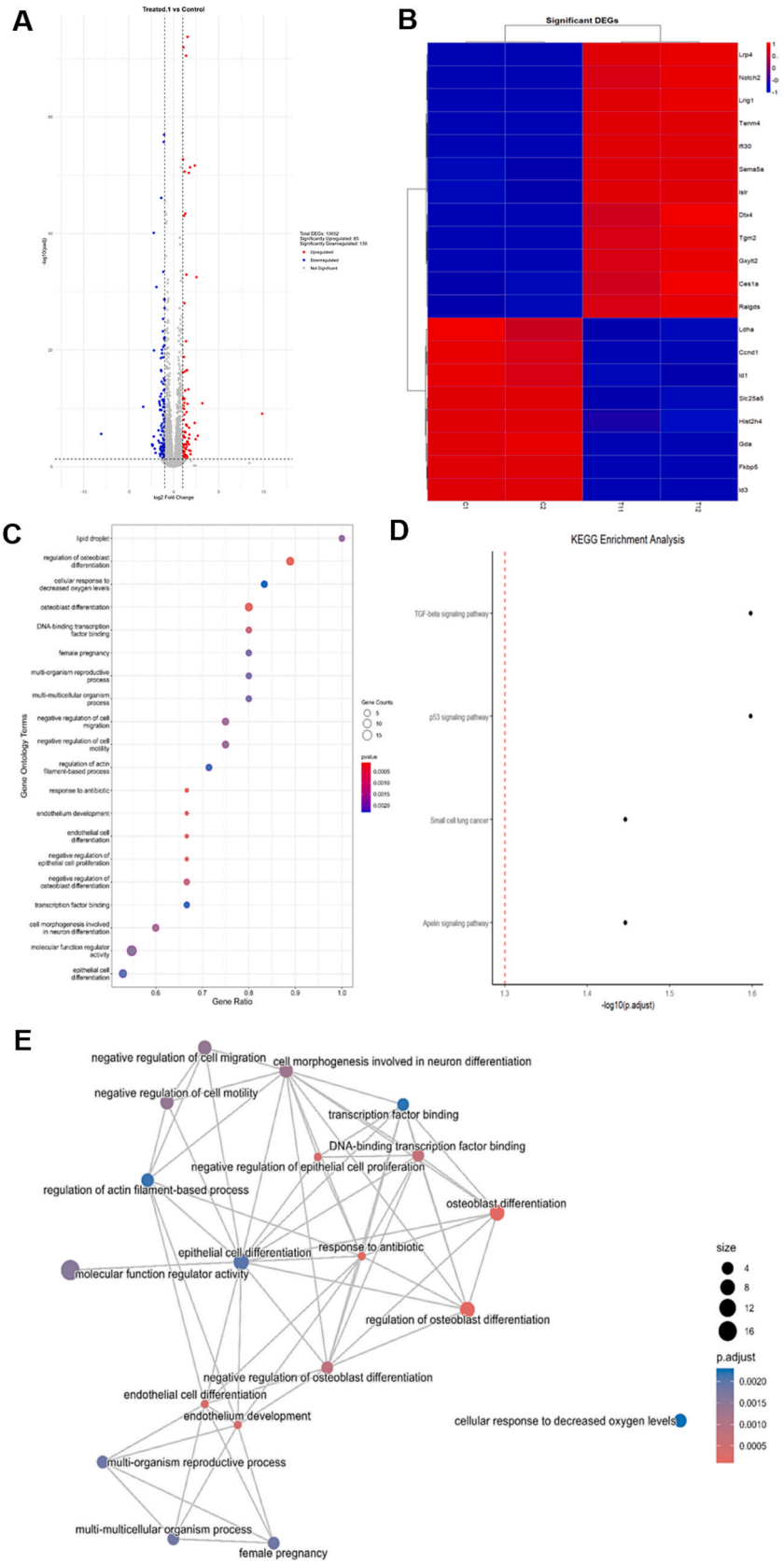

Fig. 2. Transcriptomics analysis of 16/8 IF treatment effects in 3T3-L1 adipocyte. A. Volcano plot showing DEGs between 16/8 IF group and control group. Red points indicated significantly upregulated genes, blue points indicated significantly downregulated genes (p-adjusted < 0.05, Fold change > 2 or <-2); B. Heatmap showing the expression patterns of the first 20 DEGs between 16/8 IF group and control group. Red indicated upregulated, and blue indicated downregulated; C. Dot plot of significant Gene Ontology (GO) terms identified using significant DEGs between 16/8 IF group and control group; D. Dot plot of the significant KEGG

pathways enriched in DEGs between 16/8 IF group and control group; E. Enrichment map of the GO terms enriched in DEGs between 16/8 IF group and control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

processes relevant to adipocyte function, including lipid droplet regulation (Fig. 2C). The KEGG pathway enrichment analysis identified several impacted metabolic and signaling pathways, including the TGF-β and p53 signaling pathways (Fig. 2D).

Table 1 and Table 2 show the top 10 significantly upregulated and downregulated DEGs, respectively, between 16/8 IF treatment and control. Among them, *Tgm2* was most significantly upregulated ($\log_2$ Fold Change = 1.549, adjusted $p = 1.84 \times 10^{74}$), followed by *Notch2* ($\log_2$ Fold Change = 1.085, adjusted $p = 1.15 \times 10^{72}$), and *Sema5a* ($\log_2$ Fold Change = 1.372, adjusted $p = 3.19 \times 10^{71}$), while *Ccnd1* was most significantly downregulated ($\log_2$ Fold Change = −1.064, adjusted $p = 1.17 \times 10^{57}$), followed by *Ldha* ($\log_2$ Fold Change = −1.104, adjusted $p = 1.97 \times 10^{56}$), and *Fkbp5* ($\log_2$ Fold Change = −1.378, adjusted $p = 7.97 \times 104^{7}$). The full list of significant DEGs between 16/8 IF treatment and control is shown in Supplementary Table 1.

Fig. 3 shows the comprehensive transcriptomic analysis of 3T3-L1 adipocytes in BS group compared to control. Out of 13,652 DEGs, only three were significantly downregulated and none were upregulated (Fig. 3A). The heatmap analysis showed similar expression patterns between treatment duplicates, signifying replicability (Fig. 3B). No biological process was identified through GO enrichment analysis, and the KEGG pathway enrichment analysis identified only two impacted metabolic and signaling pathways, consistent with the minimal overall gene expression changes (Fig. 3C).

Table 3 shows the three significantly downregulated ribosomal DEGs between BS treatment and control, namely *mt-Rnr2* followed by *mt-Rnr2*, and *Lars 2*.

4. Discussion

This study investigated the effect of IF/TRF *in vitro* using mouse adipocyte 3T3-L1 cell line and its effect on global gene expression (transcriptomics). The findings from lipid accumulation assay reveal that among the tested regimens, the 16/8 IF group was the most effective in reducing lipid storage, followed by "distributed" IF group, and lastly breakfast-skipping group. This suggests that 16/8 IF regimen has the highest therapeutic potential for obesity. This aligns with previous literature demonstrating the efficacy of 16/8 IF in decreasing fat mass *in vivo*, which directly reflects the reduction of overall lipid accumulation in the body's AT. Although there were no significant changes in total cholesterol, HDL-C, and LDL-C, a decrease in circulating triglycerides was observed.[23] This further supports that 16/8 IF regimen improves lipid metabolism, consistent with the findings obtained on adipocyte lipid storage. The effectiveness of the 16/8 IF regimen may be attributed to the concept of "metabolic switching". During the fasting state, glucose, which is the primary energy source, becomes limited. This forces the cells to utilize ketone bodies, which are derived from fatty acids converted from the triglycerides stored in AT, for energy. In this

Table 1
The top 10 significantly upregulated DEGs identified between 16/8 IF group and control group.

Gene Full Name	Official Symbol	Brief Gene Function	baseMean	log2FoldChange	lfcSE	stat	*p*-value	Adjusted *p*-value
Transglutaminase 2, C polypeptide	*Tgm2*	• Catalyze calcium-dependent cross-linking of proteins • Involved in extracellular matrix stabilization and apoptosis	1614.388	1.549	0.083	18.763	1.53E-78	1.84E-74
Notch 2	*Notch2*	• Enable NF-κB binding activity and enzyme binding activity	4824.175	1.085	0.059	18.504	1.90E-76	1.15E-72
Sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	*Sema5a*	• Enable axon guidance receptor activity and semaphorin receptor binding activity • Negatively regulate endothelial cell apoptotic process • Regulate signal transduction	2136.618	1.372	0.075	18.302	7.94E-75	3.19E-71
Glucoside xylosyltransferase 2	*Gxylt2*	• Predicted to enable UDP-xylosyltransferase activity • Predicted to involve in O-glycan processing	3089.608	1.042	0.066	15.864	1.13E-56	1.94E-53
Carboxylesterase 1A	*Ces1a*	• Predicted to enable carboxylesterase activity and sterol ester esterase activity • Predicted to be active in lipid droplet	500.5828	2.345	0.149	15.702	1.47E-55	2.21E-52
Deltex 4, E3 ubiquitin ligase	*Dtx4*	• Predicted to enable ubiquitin protein ligase activity • Predicted to involve in Notch signaling pathway	701.8275	1.816	0.116	15.642	3.75E-55	4.53E-52
Teneurin transmembrane protein 4	*Tenm4*	• Enable protein homodimerization activity	1673.635	1.214	0.078	15.529	2.20E-54	2.42E-51
Interferon gamma inducible protein 30	*Ifi30*	• Predicted to enable oxidoreductase activity • Involved in antigen processing and presentation of exogenous peptide antigen via MHC class I	764.194	1.683	0.109	15.490	4.06E-54	4.08E-51
Leucine-rich repeats and immunoglobulin-like domains 1	*Lrig1*	• Involved in inner action, otolith morphogenesis, and sensory perception	1158.579	1.296	0.090	14.399	5.25E-47	3.96E-44
Ral guanine nucleotide dissociation stimulator	*Ralgds*	• Predicted to enable guanyl-nucleotide exchange factor activity and Ras protein signal transduction	1709.671	1.157	0.081	14.332	1.39E-46	9.31E-44

Table 2
The top 10 significantly downregulated DEGs identified between 16/8 IF group and control group.

Gene Full Name	Official Symbol	Brief Gene Function	baseMean	log2FoldChange	lfcSE	stat	*p*-value	Adjusted *p*-value
Cyclin D1	*Ccnd1*	• Promote G1/S phase transition • Negatively regulates epithelial cell proliferation and transcription	3699.779	−1.064	0.065	−16.483	4.87E-61	1.17E-57
Lactate dehydrogenase A	*Ldha*	• Encode protein that catalyzes the interconversion of pyruvate and lactate in anaerobic glycolysis	5436.927	−1.104	0.068	−16.301	9.79E-60	1.97E-56
FK506 binding protein 5	*Fkbp5*	• Predicted to enable heat shock protein binding activity, peptidyl-prolyl cis–trans isomerase activity, and protein-macromolecule adaptor activity	1093.184	−1.378	0.093	−14.836	8.58E-50	7.97E-47
Inhibitor of DNA binding 3	*Id3*	• Enable bHLH transcription factor binding activity • Enable transcription regulator inhibitor activity • Involved in several processes, including negative regulation of myoblast differentiation and negative regulation of macromolecule biosynthetic process	360.248	−2.211	0.160	−13.845	1.37E-43	7.88E-41
Guanine deaminase	*Gda*	• Enable guanine deaminase activity • Involved in allantoin metabolic process, amide catabolic process, and nucleobase-containing small molecule metabolic process	1128.861	−1.156	0.091	−12.676	8.07E-37	3.75E-34
Inhibitor of DNA binding 1, HLH protein	*Id1*	• Enable transcription regulator inhibitor activity	368.638	−1.913	0.157	−12.174	4.25E-34	1.55E-31
Solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 5	*Slc25a5*	• Encode a transmembrane domain-containing protein of the mitochondrial inner membrane	1539.524	−1.032	0.088	−11.771	5.48E-32	1.84E-29
H4 clustered histone 14	*Hist2h4*	• Encode a replication-dependent histone of the histone H4 family	1640.862	−1.008	0.086	−11.743	7.65E-32	2.49E-29
Lipoprotein lipase	*Lpl*	• Enable lipoprotein lipase activity • Involved in several processes including cytokines production and triglyceride catabolic process	1224.481	−1.008	0.088	−11.456	2.20E-30	6.48E-28
ADAMT like 4	*Adamtsl4*	• Encode for ADAMTS-like proteins	769.6562	−1.179	0.106	−11.073	1.70E-28	4.55E-26

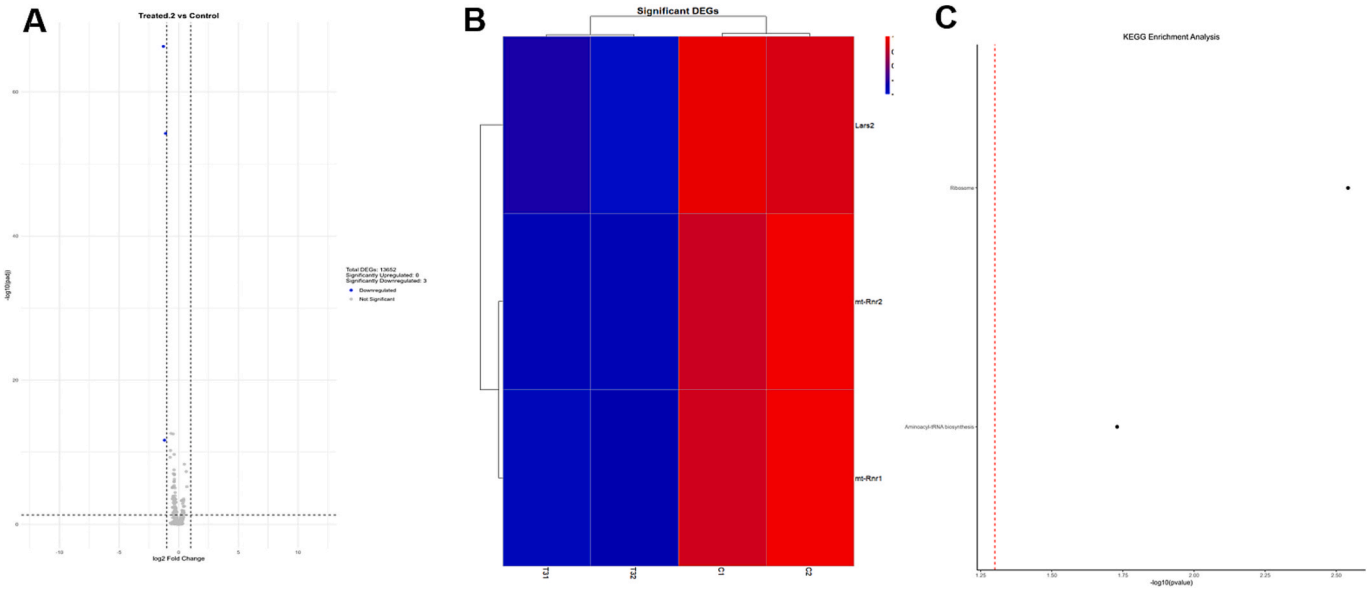

Fig. 3. Transcriptomics analysis of breakfast-skipping (BS) treatment effects in 3T3-L1 adipocyte. A. Volcano plot showing DEGs between BS group and control group. Red points indicate significantly upregulated genes, blue points indicate significantly downregulated genes (p-adjusted < 0.05, Fold change > 2 or <-2); B. Heatmap showing the expression patterns of DEGs between BS group and control group. Red indicates upregulated, and blue indicates downregulated; C. Dot plot of the significant KEGG pathways enriched in DEGs between BS group and control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

context, the 16-hour nutrient deprivation period of the regimen has successfully induced this switch in 3T3-L1 adipocyte, stimulating lipolysis, and lead to the observed significant reduction in lipid accumulation.[10]

Table 3
The significant DEGs identified between breakfast-skipping group and control group.

Gene Full Name	Official Symbol	Brief Gene Function	baseMean	log2FoldChange	lfcSE	stat	*p*-value	Adjusted *p*-value
16S rRNA, mitochondrial	*mt-Rnr2*	• Predicted to be a structural constituent of ribosome and part of mitochondrial large ribosomal subunit	4419.984	−1.284	0.072	−17.774	1.12E-70	4.91E-67
12S rRNA, mitochondrial	*mt-Rnr1*	• Predicted to be a structural constituent of ribosome and part of mitochondrial small ribosomal subunit	5974.083	−1.099	0.068	−16.098	2.65E-58	5.82E-55
Leucyl-tRNA synthetase, mitochondrial	*Lars2*	• Predicted to enable leucine-tRNA ligase activity	3212.527	−1.191	0.150	−7.918	2.41E-15	2.12E-12

The DEG analysis findings provided molecular evidence supporting this concept. Notably, *Ccnd1*, which encodes cyclin D1, was the most significantly downregulated in the 16/8 IF group. According to Wu et al. (2019), cyclin D1 inhibits lipolysis and promotes lipid accumulation by decreasing lipophagy.[24] Moreover, *Tgm2*, which encodes transglutaminase 2 (TG2), was the most significantly upregulated in the 16/8 IF group. This aligns with a study demonstrating that TG2 is a negative regulator of adipogenesis, where a low TG2 level can increase and accelerate lipid accumulation *in vivo*.[25] Additionally, 16/8 IF has significantly downregulated *Ldha*, which encodes lactate dehydrogenase-A responsible for converting pyruvate to lactate in glycolysis.[26] Besides, *Notch2*, which encodes NOTCH2 receptor, was significantly upregulated in the 16/8 IF group. Studies demonstrated that NOTCH2 signaling can decrease glycolysis in bone cells, indicating that *Notch2* is potentially increasing lipolysis indirectly by influencing glucose utilization.[27] Moreover, the KEGG pathway enrichment analysis showed that the TGF-β signaling pathway was significantly enriched. The TGF-β (transforming growth factor-beta) signaling pathway's significant enrichment is consistent with its known role in inhibiting adipogenesis and lipid accumulation.[28–30] TGF-β signaling regulates key transcription factors and signaling molecules that govern fat cell differentiation and growth. Specifically, it can inhibit the activity of PPARγ, a master regulator of adipogenesis, thereby preventing the conversion of preadipocytes into mature, lipid-storing adipocytes.[28] This mechanism contributes to the observed reduction in lipid accumulation. The enrichment of the p53 signaling pathway aligns with previous research on the effects of intermittent fasting.[15] The p53 protein is a tumor suppressor, but in the context of metabolism, its activation in adipocytes has been shown to induce apoptosis, or programmed cell death. By promoting the death of fat cells, the p53 pathway leads to a reduction in adipocyte number and, consequently, an overall decrease in adiposity.[15] This is a powerful mechanism by which IF can combat obesity. Finally, the apelin signaling pathway's enrichment is crucial for understanding the direct metabolic effects of the 16/8 IF regimen. Previous studies have demonstrated that apelin signaling has a dual role in lipid metabolism: 1. By promoting lipolysis, the breakdown of stored lipids into fatty acids and glycerol by regulating the expression of perilipin, a protein that coats lipid droplets and is essential for their breakdown by lipases[31]; 2. By regulating the expression of PPARγ, similar to the TGF-β pathway.[32] This dual action—promoting fat breakdown while preventing new fat cell formation—provides a robust explanation for the reduced lipid accumulation observed in the study. Collectively, these findings suggest that the 16/8 IF regimen affects lipid metabolism by modulating multiple key pathways: promoting lipolysis, inhibiting adipogenesis, and inducing adipocyte apoptosis.

However, the DEG analysis also revealed more complex gene regulations. For example, *Dtx4* was significantly upregulated in the 16/8 IF group, but previous studies demonstrated that DTX4 can promote adipogenesis in 3T3-L1 cells.[33] This contradiction, where a gene typically associated with promoting adipogenesis is upregulated under a condition with reduced lipid accumulation, suggests a more complex regulatory role for the DEGs in response to IF/TRF. Besides, the KEGG pathway enrichment analysis showed that the p53 signaling pathway was significantly enriched. This aligns with a study demonstrating an activation of the p53 signaling pathway under IF treatment, which suggests that the reduced lipid accumulation is attributed to the ability of IF in increasing adipocyte apoptosis in addition to promoting lipolysis.[15] Moreover, the effectiveness of the 16/8 IF regimen may also be attributed to its alignment with the adipocyte's natural circadian rhythm, an innate timing mechanism that anticipates energy consumption during daylight hours.[34] The circadian clock regulates the expression of genes involved in lipid metabolism, such as *Pnpla3 and Cpt1*.[35–36] Hence, the 16/8 IF regimen, which aligns with the feeding and fasting cycles of the circadian rhythm, can optimize the efficiency of the lipid-regulating pathways and further contribute to the observed reduction in adipocyte lipid accumulation. While the DEG analysis did not show any differentially expressed core circadian clock genes like *CLOCK* and *BMAL1*, it is important to note that many downstream genes, such as *Ccnd1* and *Ldha*, are known to be regulated by these clock genes.[37–38]

In contrast, the lipid accumulation assay findings suggest that the BS regimen, which demonstrated little to no reduction in lipid storage, has the lowest therapeutic potential for obesity. This aligns with previous literature demonstrating that breakfast-skipping regimen increased body weight gain *in vivo*.[39] Moreover, Kim et al. (2021) demonstrated that the BS regimen significantly increased epididymal AT weight and hepatic lipids in rats.[40] Chen et al. (2021) also reported an increased lipid profile (higher triglycerides, higher total cholesterol, higher LDL-C, and lower HDL-C) in breakfast skippers compared to non-breakfast skippers *in vivo*.[41] This evidence further suggests that the BS regimen may lead to increased lipid storage rather than reducing it. These negative effects of the BS regimen may be attributed to its misalignment with the adipocyte's natural circadian rhythm. Skipping breakfast, a misaligned meal pattern, can disturb the peripheral clock and change the expression of clock-controlled genes involved in lipid metabolism.[35,41–42] This shifts the balance towards increased lipogenesis and reduced fatty acid oxidation, thereby favoring lipid accumulation.

While the DEG analysis did not show any differentially expressed core circadian clock genes, it is important to note that *mt-Rnr2* and *mt-Rnr1* genes, which are among the few significantly downregulated genes in the breakfast-skipping group, are known to be clock-controlled.[43–44] *mt-Rnr2* encodes mitochondrial 16S rRNA, Humanin (HN) peptide, and small humanin-like peptides (SHLPs).[45] Intracellularly, HN binds to insulin-like growth factor binding protein 3 and inhibits insulin-stimulated glucose uptake.[46] Hence, the downregulation of *mt-Rnr2* and its downstream effects on insulin sensitivity would then lead to an increased glucose uptake by the 3T3-L1 adipocytes. Elevated glucose uptake and glycolysis favors lipid accumulation, which is consistent with the observed lipid accumulation rate in this group. Moreover, *mt-Rnr1* encodes the mitochondrial 12S rRNA and mitochondrial open reading frame of the 12S rRNA type-c (MOTS-c) peptide[45]. MOTS-c can regulate lipid metabolism by increasing β-oxidation of fatty acids.[47] Therefore, the downregulation of *mt-Rnr1* would lead to lower MOTS-c levels, impairing fatty acid oxidation pathway and further favoring lipid accumulation in cells. Collectively, these transcriptional changes align

with the observed minimal reduction in lipid accumulation rate in this group.

These findings can be situated within the broader context of circadian biology and metabolic regulation. The adipocyte circadian clock is a critical timekeeping mechanism that regulates metabolic processes like adipogenesis and lipolysis on a 24-hour cycle.[16] The timing of nutrient intake acts as a powerful external cue, or Zeitgeber, that synchronizes this internal clock.[19] The 16/8 IF regimen, by restricting nutrient availability to a specific 8-hour window, effectively acts as a Zeitgeber, realigning the cellular clock with the feeding schedule.[48] The enrichment of the TGF-β, p53, and apelin pathways suggests that the observed transcriptomic changes are a direct result of this circadian regulation. This study provides a cellular-level explanation for how time-restricted feeding regimens can modulate key metabolic pathways, highlighting the crucial link between the timing of feeding and the regulation of gene expression in adipocytes. These findings contribute to a growing body of literature demonstrating that misalignment of the circadian clock and feeding schedules is a significant contributor to metabolic dysfunction and obesity.[48]

This study is subject to several limitations that warrant careful consideration. First, the treatment duration in this study was relatively short (24 h) compared to the long-term nature of human dietary interventions, which may occur over months or years.[49] Although acute cellular responses were observed in this study, the findings may not fully capture the potential long-term effects of IF/TRF on lipid metabolism and gene expression *in vivo*. Future studies with extended treatment durations could provide a more comprehensive understanding of the adaptive responses of adipocytes to IF/TRF. Second, the use of 3T3-L1 murine adipocytes in this study provides a controlled system for mechanistic investigation of the effects of IF/TRF. However, there are key differences in their physiology and function that limit their direct relevance to human adipocytes or complex human adipocytes within a complete organism, which is influenced by systemic factors such as hormonal signaling.[50] According to Kersten (2023), the human body undergoes significant hormonal shifts during fasting, influencing AT metabolism *in vivo*.[51] Also, Defour et al. (2020) reported that the effect of fasting on gene expression was more remarkable in mice compared to humans despite the longer fasting duration in human volunteers,[13] highlighting the complexities of translating findings directly from murine models to human physiology. Future studies should employ more physiologically relevant *in vitro* models, such as human Simpson-Golabi-Behmel syndrome (SGBS) cell line, or *in vivo* models, such as non-primate models, to validate these findings and assess their translational relevance.

5. Conclusions

This study reveals the effects of different IF/TRF regimens: 16/8 IF, "distributed" IF, and BS, on lipid accumulation and global gene expression in mouse 3T3-L1 adipocytes. The findings from the lipid accumulation assay indicate that the 16/8 IF treatment was the most effective in reducing lipid storage, highlighting its significant therapeutic potential for managing obesity. This was strongly supported by the DEG analysis, which revealed that genes promoting lipolysis as well as inhibiting adipogenesis and glycolysis were significantly modulated, consistent with the lipid accumulation rate observed. While some complex gene regulations suggest additional mechanisms contributing to lipid reduction, the overall molecular effects align with the observed lipid accumulation. Conversely, BS treatment was the least effective in reducing lipid storage, suggesting its potential negative impact on metabolic health. The DEG analysis suggests that this treatment can increase glucose uptake and decrease fatty acid oxidation, favoring lipid accumulation, consistent with the lipid accumulation rate observed. Future research should focus on extended treatment durations and employ more physiologically relevant *in-vivo* models to validate these findings and comprehensively understand the long-term adaptive responses and systemic implications of various IF/TRF regimens, especially the 16/8 IF regimen. Understanding these differential cellular effects can guide the development of more targeted and effective interventions for obesity.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Statement – Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Google Gemini 2.5 Flash in order to improve the readability and language of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

CRediT authorship contribution statement

Pei Han Er: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Jin Yi Chye:** Writing – original draft, Investigation, Formal analysis, Data curation. **Geetha Letchumanan:** Validation, Supervision, Project administration, Methodology. **Yee-How Say:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Ethics approval

Not applicable.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Yee-How Say reports financial support was provided by Malaysian Ministry of Higher Education Fundamental Grant Research Scheme. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

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