



Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: <https://www.sciopen.com/journal/2097-0765>

Interference with the expression, location, and structure of gap junction protein in lipid rafts: mechanical insights into the inhibitory mechanism of A-ECG dimer on adipogenesis of 3T3-L1 preadipocytes

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ABSTRACT: Our previous study demonstrated that epicatechin-3-gallate-(4 β $\rightarrow$ 8, 2 β $\rightarrow$ O $\rightarrow$ 7)-epicatechin-3-gallate (A-ECG dimer), a unique procyanidin dimer derived from persimmon fruit, strongly inhibits adipogenesis in 3T3-L1 preadipocytes by disrupting lipid raft structure. However, the underlying mechanism and manner of action remains unclear. In this study, transcriptomic analysis identified gap junction protein alpha 1 (*Gjal*), a key lipid raft-associated gene, as being involved in the early stages of adipogenesis. The *Gjal* encodes a gap junction protein that facilitates intercellular communication and channel function upon localization to lipid rafts. Treatment with A-ECG dimer downregulated both *Gjal* expression and its encoded protein, leading to suppression of downstream lipid synthesis signaling. This inhibition was associated with altered localization and structural changes of gap junction proteins within lipid rafts. Specifically, A-ECG dimer impaired raft clustering and reduced gap junction protein recruitment into raft domains during adipogenesis. Further structural analysis revealed that A-ECG dimer interacts with gap junction proteins via polar, van der Waals, hydrogen bonding, and electrostatic forces, resulting in reduced radius of gyration (45.78%), as well as decreased hydrophilic and hydrophobic solvent accessible surface areas (28.45% and 32.74%, respectively). These findings provide new insights into the underlying mechanism by which polyphenols inhibit adipogenesis.

Keywords: Polyphenols, lipid rafts, gap junction protein alpha 1, transcriptomics, adipogenesis

1. Introduction

Dimeric procyanidins have received widespread attention due to their superior bioactivities compared to monomers and polymers.^{[1],[2],[3]} For instance, they exhibit enhanced antiviral and antibacterial effects,^[4] stronger radical-scavenging capacity,^[5] and improved protective activity against collagen degradation.^[6] Notably, certain dimers demonstrate metabolic stability and can be directly absorbed into the rat bloodstream following oral administration.^{[7],[8]} In our previous work, we isolated two unique dimeric procyanidins from persimmon fruits, namely epicatechin-3-gallate-(4 β $\rightarrow$ 8, 2 β $\rightarrow$ O $\rightarrow$ 7)-epicatechin-3-gallate (A-ECG dimer)

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Received 3 June 2025
Received in revised form 8 October 2025
Accepted 20 January 2026

and epigallocatechin-3-gallate-(4 β $\rightarrow$ 8, 2 β $\rightarrow$ O $\rightarrow$ 7)-epigallocatechin-3-gallate (A-EGCG dimer). The structure of A-ECG dimer is shown in Fig. 1. And we found that A-ECG dimer exhibited the most potent inhibition of lipid accumulation in 3T3-L1 preadipocytes among various polyphenols tested,^{[9],[10]} with an IC₅₀ of 20.36 μ M.^[11] Nevertheless, the underlying mechanism by which A-ECG dimer suppresses adipogenesis remains unclear.

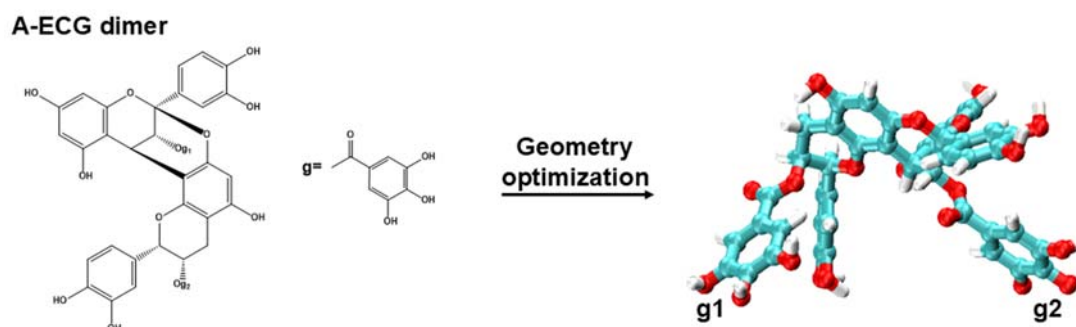


Fig. 1. Schematic diagram for the structure of A-ECG dimer in this study.

Lipid rafts, enrich in cholesterol, sphingolipids, and signaling proteins, is a specific ordered domain of cell membrane crucial for signaling transduction during cell growth and differentiation.^{[12],[13],[14]} The structural integrity of lipid rafts has been confirmed to be essential for the activation of downstream lipid synthesis signaling pathway in 3T3-L1 cells.^{[15],[16]} Our previous investigation suggested that the binding preference of the A-ECG dimer for lipid rafts and its detrimental impact on the lipid rafts structure contributed to its inhibition of 3T3-L1 preadipocyte differentiation.^[11] Many signaling protein located in the lipid rafts were reported to be targets of polyphenols.^[2] For example, insulin receptor (IR) and 67 kDa laminin receptor (67LR) was both proved to be the target of bioactive polyphenols, like epicatechin-3-gallate (ECG) and epigallocatechin-3-gallate (EGCG), responsible for its regulation of lipid metabolism and anticancer biological activity. Moreover, we also found that A-EGCG dimer targeted 67LR within lipid rafts, leading to a potent inhibitory impact on the differentiation of 3T3-L1 cells. However, the precise target within lipid rafts for A-ECG dimer, which exhibited a stronger differentiation inhibitory ability than A-EGCG dimer, has yet to be identified.

Given the complexity of biological systems, conventional trial-and-error approaches face significant challenges in identifying molecular targets for A-ECG dimer. The introduction of omics technologies has some advantages in facilitating a systems-level understanding by integrating diverse datasets, unraveling the molecular mechanisms underlying the effects of bioactive compounds, and offering a holistic and data-driven approach to target identification.^{[17],[18]} However, their specific application in elucidating the anti-adipogenic mechanism of A-ECG dimer remains unexplored.

In this study, we employed an integrated approach combining transcriptomics with experimental validation to address this research gap. We first identified key genes and raft-associated proteins during the early stages of adipogenesis using transcriptomics, followed by verification via real time fluorescence quantitative polymerase chain reaction (RT-qPCR) and western blot (WB). The spatial relationship between

the target protein and lipid rafts under A-ECG dimer treatment was explored using fluorescence staining and density gradient centrifugation. Furthermore, we investigated the structural impact of A-ECG dimer on the key protein using molecular modeling technology. Therefore, this work provides a systematic strategy by combining the advantages of multiple technologies to uncover the mechanism behind the potent inhibitory effect of A-ECG dimer on adipogenesis in 3T3-L1 preadipocytes.

2. Materials and methods

2.1 Materials.

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS, pH = 7.4), and penicillin-streptomycin (PS) were purchased from Gibco (Grand Island, NY, USA). Methyl- β -cyclodextrin (M β CD) were from Shanghaiyuanye Bio-Technology Co., Ltd (Shanghai, China), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPPE) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). 18- α -Glycyrrhetic acid (AGA), dimethyl sulfoxide (DMSO), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (Dex), insulin, bovine serum albumin (BSA), and OptiPrepTM density gradient medium was from Sigma-Aldrich Chemical Co. (St. Louis, MO). Mouse monoclonal antibody peroxisome proliferator activated receptor gamma (PPAR γ), Rabbit polyclonal antibodies CCAAT/enhancer-binding protein alpha (C/EBP α), 4',6-diamidino-2-phenylindole (DAPI) and trizol reagent were purchased from Servicebio (Wuhan, China). Rabbit polyclonal antibodies acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal antibodies against (β -actin), gap junction protein alpha 1 (Gja1), flotillin 1, HRP Goat Anti-Rabbit IgG, and Alexa Fluor 594-conjugated Goat Anti-Rabbit IgG were purchased from ABclonal Technology Co.,Ltd. (Wuhan, China). PageRulerTM Prestained Protein Ladder (10 to 180 kDa) was from Thermo Fisher Scientific Inc. Epicatechin-3-gallate-(4 β →8, 2 β →O→7)-epicatechin-3-gallate (A-ECG dimer, 95.6%) was isolated from persimmon as the method our group previously reported.^[19] The details about A-ECG dimer including the high performance liquid chromatography traces and purity can be found in Fig. S1 and Table S1 (Supporting information).

2.2 Cell culture and differentiation.

The cell culture and differentiation of 3T3-L1 preadipocytes were performed based on the classical protocol.^[20] Firstly, 3T3-L1 preadipocytes were obtained from ATCC (Manassas, VA, USA). The cells were cultured in DMEM supplemented with 10 % FBS and 1 % (v/v) PS at 37 °C and 5 % CO₂. To induce the 3T3-L1 preadipocyte differentiation, the cells were seeded and incubated until confluence. After 2 days confluence, the medium was replaced to DMEM containing 10 % FBS and cocktail (MDI-1, 500 μ M IBMX, 1 μ M Dex, and 10 μ g/ mL insulin). The cells were induced differentiation for 2 days under MDI-1 treatment and then the medium was substituted with MDI-2 inducer, which was DMEM with 10% FBS and 10 μ g/mL insulin. Then the cells were further induced to differentiate for 2 days under MDI-2 treatment. Thereafter, the culture medium was replaced every 2 days with 10% FBS-DMEM. A large amount of mature lipid droplets

would be formed and accumulated after 8 days of treatment. In this study, different concentrations (5 μ M, 10 μ M, 20 μ M, and 50 μ M) of A-ECG dimer were prepared and added to the culture medium to treat differentiated cells. A concentration of 50 μ M was used for transcriptomics analysis because higher concentrations can capture broader gene expression changes. This concentration had been previously evaluated and confirmed to be non-cytotoxic to the cells.^[9] Subsequently, a lower concentration range (5 - 20 μ M) considering physiological condition was selected to both validate the transcriptomics findings and investigate its effects on intracellular lipid synthesis signaling pathways and lipid raft structure. In addition, 100 μ M of AGA was introduced in the first 2 days of differentiation, which was regarded as an inhibitor of Gjal.

2.3 RNA extraction, quantification, cDNA library preparation for sequencing and bioinformatics analysis.

To explore the effects of A-ECG dimer on 3T3-L1 preadipocyte differentiation at gene level, the cells (1×10^5 cells/well) were first seeded in triplicate in 6-well plates and cultured until confluence. Then A-ECG dimer under the concentration of 50 μ M was added to differentiation medium for 24 h, which was used to monitor the key genes in the early stages of differentiation. M β CD (3 mM) was used as a positive control for lipid raft disruption. Then the cells were harvested with trizol reagent for extracting total RNA and conducting transcriptome sequencing. In this study, A-ECG dimer was dissolved with DMSO, which is less than 0.1% (v/v) and has non-cytotoxic effect. Measurement of the concentration and purity of RNA was performed by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and then the quality was evaluated using the RNA Nano 6000 assay kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Subsequently, cDNA library was constructed by using NEBNext UltraTM RNA library preparation kit (NEB, USA). In brief, mRNA was firstly isolated and purified from the total RNA using poly-T oligo-attached magnetic beads. Short RNA fragments were then obtained under fragmentation buffer. Then the first-strand cDNA was synthesized by random hexamers and M-MuLV reverse transcriptase. After that, DNA polymerase I and RNase H were added to synthesize the second-strand cDNA, and then it was linked with sequencing adapters after adenylation of 3' ends of DNA fragments. Meanwhile, AMPure XP system (Beckman coulter, USA) was used to select fragment size, and the cDNA library was enriched by polymerase chain reaction (PCR). Finally, the library quality was tested on an Agilent 2100 Bioanalyzer and then sequenced on an Illumina HiSeq platform (Illumina, NovaSeq6000, USA) at Biomarker Technologies Corporation (Beijing, China).

Subsequently, raw reads were processed through in-house perl scripts and saved as fastq file format.

After that, clean reads were obtained by filtering out reads containing adapters and ploy-N, and low-quality reads from raw reads. Meanwhile, Q20, Q30, GC-content and sequence duplication level of the clean reads were also calculated and used to control the quality. Then these clean reads were mapped to the reference genome sequence using Hisat2 tools. Quantification of gene expression levels were calculated by FPKM (fragments per kilobase of transcript per million mapped fragments) method.^[21]

Finally, clean reads with high quality were used to perform some different analysis using a bioinformatic pipeline tool, BMKCloud (www.biocloud.net) online platform, including comparative analysis,^[22] gene functional annotation,^[23] and especially differential expressed genes (DEGs) analysis.^[24] In this study, DESeq2 software was used to identify the DEGs using a statistical model based on the negative binomial distribution and then genes with an adjusted *P*-value < 0.05 and a fold change ≥ 1.5 were assigned as DEGs.^[25]

2.4 Validation of DEGs at gene and protein levels.

To verify the accuracy of DEGs obtained from transcriptomic analysis, gene and protein expression level was further determined using real time fluorescence quantitative polymerase chain reaction (RT-qPCR) and western blot (WB) method, respectively. In this study, 3T3-L1 cell samples processed at different times (4 h, 8 h, 12 h, 24 h) were used for further analysis.

For RT-qPCR, the cells after different treatments were first harvested with trizol reagent for collecting total RNA, followed by chloroform phase separation and isopropanol precipitation. The RNA pellet was washed with 75% ethanol, air-dried, and resuspended in diethylpyrocarbonate water. Subsequently, the concentration and purity of dissolved RNA were measured on NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE). cDNA was synthesized from 1 μ g of total RNA using the MonScript™ RTIII All-in-One Mix with dsDNase. Quantitative PCR was performed using the MonAmp™ Fast SYBR® Green qPCR Mix on a QuantStudio 5 system (Thermofisher ABI, QuantStudio 5, USA) with the following cycling conditions: 95°C for 5 min; 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. The corresponding primer sequences applied for RT-qPCR are listed in Table S2 (Supporting Information). Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Mouse β -actin expression used as an endogenous reference and at least three independent biological replicates were performed in this study.

For WB analysis, the cells after different treatment were first harvested to collect cell total proteins by lysis buffer, which were composed of enhanced RIPA buffer containing 1% protease inhibitor cocktail, 0.1% phosphatase inhibitors, and 0.1 mM PMSF. All samplings were lysed on ice for 30 min and then the total protein was measured using a BCA-protein test kit (Servicebio, China). Afterward, protein samples (20 μ g) were resolved in 10 % SDS-PAGE and then were transferred onto the PVDF membrane (Bio-Rad, USA). Meanwhile, the membranes were blocked with 5% (w/v)

BSA for 1 hour at room temperature. Subsequently, primary antibodies (1:1000) were added and incubated overnight on ice. After that, HRP-conjugated secondary antibody (1:2000) was added and incubated for 30 min at room temperature. Finally, the protein signals were visualized using the hypersensitive ECL chemoluminescence agents (Servicebio, China) and captured with an Odyssey Fc system (LI-COR Biosciences, USA). Quantification of optical density of bands was calculated using the ImageJ software (National Institute of Health, USA).

2.5 Immunofluorescence staining.

To investigate the localization of Gja1 protein relative to lipid rafts and the impact of A-ECG dimer treatment, immunofluorescence experiments were performed. 3T3-L1 preadipocytes were seeded at a density

of 5×10^4 cells per well in 15 mm culture dish and cultured until confluence. Similar assay was independently performed three times. Cells were then treated with 50 μM A-ECG dimer or 3 mM M β CD (as a lipid raft disruption control) in differentiation medium for 8 h. After that, the cells were washed with PBS three times and then fixed in 4% paraformaldehyde for 30 min. Subsequently, 5% BSA was added to block non-specific staining for 1 h at 37 °C and then rabbit anti-Gjal antibody (1:100, ABclonal, China) was introduced and incubated overnight at 4 °C. The primary antibody was removed from the samples, followed by washing with PBS buffer, then an Alexa Fluor 594-labeled goat anti-rabbit IgG (1:500, ABclonal, China) was added and incubated for 1 h at room temperature. Lipid rafts were labeled with NBD-DPPE dye, and nuclei were stained with DAPI (Servicebio, China). Imaging was carried out using an ultra-high sensitivity confocal microscope (Olympus FV300, Olympus, Tokyo, Japan). Fluorescence intensity quantification was based on at least three independent cell cultures, and the representative images for visualization were selected from one of these replicates.

2.6 Lipid rafts isolation.

Lipid rafts of 3T3-L1 preadipocytes were isolated using the OptiPrep™ density gradient medium based on previous report.^[26] In brief, 3T3-L1 preadipocytes were seeded in triplicate in 150 mm culture dish (3×10^6 cells/dish) and cultured at 37 °C and 5 % CO₂ until confluence. Then A-ECG dimer under the concentration of 10 μM was added to differentiation medium (MDI-1) for 8 h, with M β CD (3 mM) as a positive control for disrupting lipid rafts. Then the 3T3-L1 cells were washed with cold PBS buffer and then scraped and collected with cold OptiPrep base buffer (OBB, 20 mM Tris-HCl, 250 mM sucrose, 6 mM EDTA, 1 mM CaCl₂, phosphorylase and protease inhibitor, pH = 7.8). After that, the collected cells were passed 20 times using a 1 mL syringe with $\lambda/22\text{G}$ needle and centrifuged at $1000 \times g$ for 10 min at 4 °C. The supernatant was collected and then the pellet was resuspended in 250 μL of OBB buffer for lysis again. Then the cell lysate was further passed 20 times through syringe and centrifuged as described above. The supernatant was then collected and merged with the last obtained one. After that, 2 mL cold Optiprep medium (60% w/v) was mixed with supernatant to have a final concentration of 45% (w/v), which was placed in the bottom of 4 mL ultracentrifuge tube. Subsequently, two pre-prepared Optiprep medium with the concentration of 35 % (w/v) and 5 % (w/v) were added in sequence. The volume was 750 μL respectively. Then the mixture was centrifuged at 53000 rpm ($140,000 \times g$) for 2.5 h at 4 °C through Optima XPN-100 Ultracentrifuge with an SW60Ti rotor (Beckman, USA). Gradation was carried out from the top to the bottom of the tube, with each fraction for 500 μL . Finally, the protein content of each tube was measured using a BCA-protein test kit (Servicebio, China) and the expression level of targeted protein was analyzed by WB method mentioned-above.

2.7 Molecular docking (MD) and molecular dynamic simulations (MDS).

The binding sites and modes of A-ECG dimer with Gjal were investigated using MD and MDS method. The complete crystal structure of gap junction proteins has not been thoroughly analyzed, while their terminal structure containing active sites has been analyzed and included in the RCSB PDB crystal database, which ID

number is 1R5S. Therefore, the 1R5S structure was used to dock with A-ECG dimer to explore their modes of action and sites of action. Prior to docking, the geometric structure of the A-ECG dimer was initially subjected to rough optimization using the Chem3D module integrated in ChemBioOffice 12.0 software (CambridgeSoft, MA, USA). Subsequently, a more precise structural optimization was performed employing Hartree-Fock (HF) theory with the 6-31G(d,p) basis set in the ORCA 5.0 software package.^[27] MD was then carried out using the LibDock module in Discovery Studio 3.5 software.^[28] In brief, the 1R5S active site ($x = 5.0 \text{ \AA}$, $y = -4.3 \text{ \AA}$, $z = 2.0 \text{ \AA}$) was defined as the docking site with a $26.3 \times 26.3 \times 26.3 \text{ \AA}^3$ docking box, and the docking preference was set to high quality. Other parameter settings remained unchanged, and the binding sites of the two were analyzed based on the docking scores. After that, MDS was performed to explore interaction of A-ECG dimer and 1R5S from dynamic binding insights at molecular level by GROMACS 5.1.4 software package.^[29] In this study, 1R5S used the CHARMM36 lipid force field^[30] and the force field of A-ECG dimer was constructed by the CHARMM General Force Field (CGenFF) program (<https://cgenff.umaryland.edu/>).^[31]

Before performing production MDS, the system was firstly energy minimized by steepest descent algorithm.^[32] Then two different balance ensembles were used in this system, which included the constant number of atoms, volume, and temperature (NVT) ensemble and NP (pressure) T ensemble.^[33] The temperature was kept at 310 K with time constant of 0.1 ps through applying V-rescale thermostat. The equilibrated time of temperature was 1 ns with time step of 2 fs under NVT ensemble. Then the pressure of system was maintained at 1 bar with time constant of 2 ps using Parrinello–Rahamn method along with isotropic coupling scheme. All directions were set by periodic boundary condition and particle mesh Ewald method was used in long-range electrostatic interactions.^[34] After equilibrating system completely, the production MDS was independently carried out at least three times for 100 ns and trajectory were saved every 2 ps. All other conditions are kept consistent in the production MDS. The root-mean-square deviation (RMSD) values for both the system and the protein have remained within 5 Å across three independent simulation systems over the last 50 ns (Fig. S2, Supporting Information), meaning the equilibrium of trajectories. Thus, the interaction analysis between A-ECG dimer and 1R5S were performed by selecting the last trajectory of 50 ns and the binding snapshots were shown using VMD 1.9.2 software.^[35]

2.8 Statistics.

Data were presented as means $\pm$ standard deviation. Statistical comparisons were analyzed using one-way ANOVA of SPSS 20.0 (SPSS Inc., Chicago, IL, USA), followed by post hoc tests (LSD and Duncan's test) for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Downregulation of gap junction protein alpha 1 (*Gjal*) is closely related to the inhibition of A-ECG dimer on 3T3-L1 preadipocytes differentiation

In this study, the genes involved in the early stage (24 h) of differentiation of 3T3-L1 preadipocytes were explored. Consequently, a total of 12 cDNA libraries from 12 samples with four different treatments, as shown in Fig. 2A, were constructed and sequenced after evaluating its quality. A total of 261.74 M reads, and 78.20 Gb clean data were obtained through removing low-quality reads. Meanwhile, the quality score of bases was used to evaluate the accuracy of a base, with the quality value of 20 (commonly known as Q20) to be considered as the accuracy of 99% and 30 (Q30) as 99.9%. Then the Q20 and Q30 values of each sample were calculated, and the results were more than 96.13 and 90.28%, respectively. The GC content among 12 samples in this study ranged from 49.76 to 50.55% and then the gene expression levels were quantified by fragments per kilobase of transcript per million mapped fragments (FPKM) method. As shown in Fig. 2B, the FPKM density distribution of each sample was mapped and the results indicated that the FPKM value extended six orders of magnitude from 10^{-2} to 10^4 , meaning that the transcriptome data had high quality and could be used for further analysis.^[21] Based on FPKM values, correlation heatmap analysis and cluster analysis of three biological replicates for 12 samples from transcriptome data were performed and the results showed strong correlation ($R^2 > 0.98$) between three independent biological replicates (Fig. S3, Supporting Information). Besides, only one sample from MCD group showed lower correlation ($R^2 \approx 0.81$) between biological replicates and deviated from their main classification from cluster analysis of gene expression profiles, meaning that this group needed special attention in subsequent analysis. This result indicated the samples in the same group had high consistency, while those in the different group showed great difference, also suggesting that the samples could be used to further analyze differential expressed genes (DEGs). The number of DEGs between different groups was displayed shown in Fig. 2C.

Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis were the most used strategies for DEGs. They are valuable resources for comparing gene functions and interpreting large-scale transcriptomic data, facilitating the understanding of gene in various pathways and metabolic processes.^{[36],[37]} Based on these analysis, we found that most of the DEGs in this study were related to the signaling pathways of intracellular lipid synthesis and degradation, but not the lipid rafts involved (data not shown).

Because our main focus was to explore the changes in genes related to membrane rafts during the early stage of differentiation, the number of upregulated and downregulated genes between the comparison group was then analyzed and shown by Venn diagram (Fig. 2D) and Volcano plots (Fig. S4, Supporting Information), respectively. Among these, 1625 genes were significantly upregulated in the Model group, as shown in Fig. 2D, which became our primary focus. These genes were subsequently subjected to vocabulary and functional analysis and matched with vocabulary related to cell membrane or lipid rafts, ultimately pinpointing gap junction protein alpha 1 (*Gjal*). The *Gjal* was classified into the gap junctions group, which is involved in cell-to-cell communication and intercellular channel functions.^[38] We observed that the FPKM value of *Gjal* was relatively high (~ 2.8 -fold) in the Model group compared to the Control group, indicating that *Gjal* was upregulated after treatment with MDI reagents. Similar findings have been reported by other research groups,^{[39],[40]} where the use of small interfering RNA targeting gap junction proteins suppressed

adipogenesis, suggesting that *Gja1* plays an important role at an early stage of adipogenesis. Based on this, we hypothesized that *Gja1* expression should be lower in the differentiation inhibition group than in the Model group. This hypothesis was subsequently confirmed, as we found that the FPKM value of *Gja1* decreased with MβCD treatment (by ~ 1.7-fold) compared to the Model group (Fig. 2D), implying that disruption of lipid rafts affects the transcription and expression of *Gja1*. Possibly due to the A-ECG dimer being a more potent lipid raft disruptor than MβCD, no expression of *Gja1* was detected in this treatment group. Therefore, these results showed that *Gja1* was considered as the key gene related to the lipid rafts of 3T3-L1 preadipocytes.

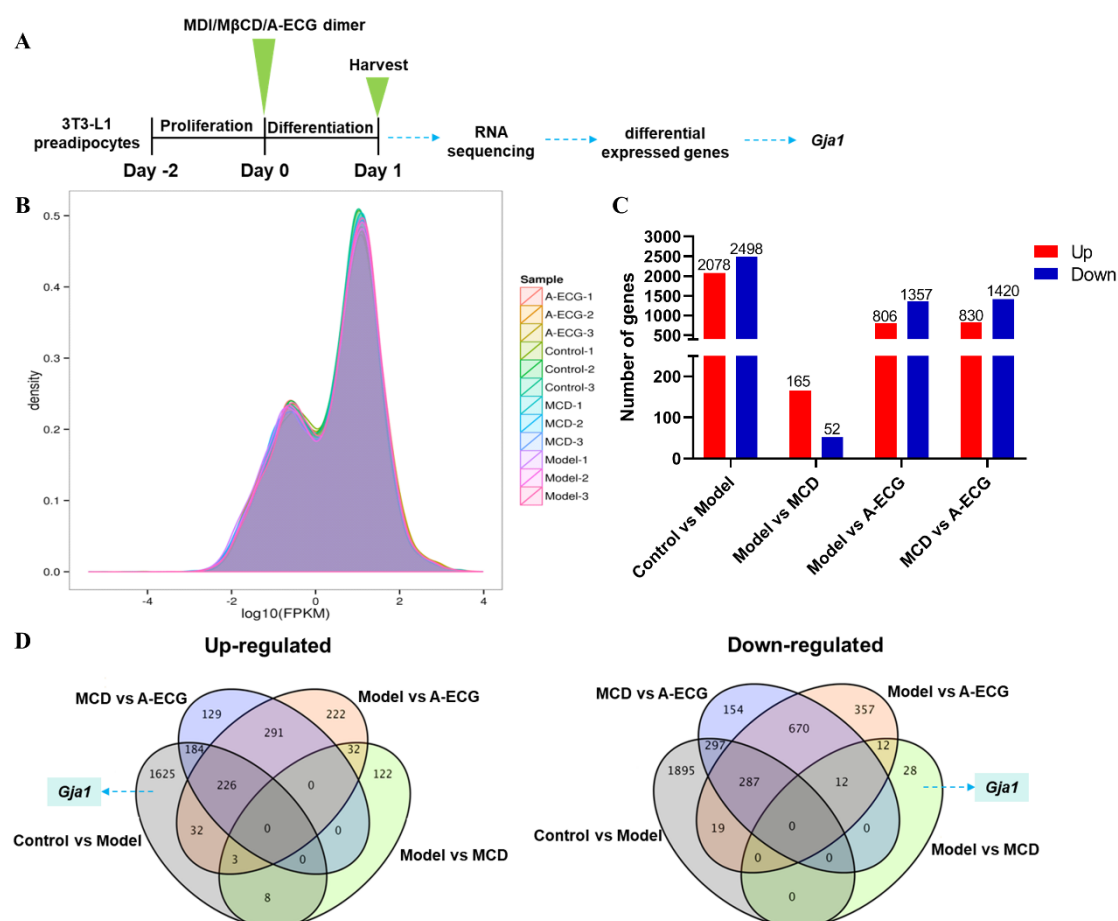


Fig. 2. Gene expression comparison in differentiating 3T3-L1 cells after treatment with different compounds for 24 h. (A) The schematic diagram of treatment with MDI (Model group), MβCD (MCD group) and A-ECG dimer polyphenol (A-ECG group) using a 3T3-L1 preadipocyte differentiation system. The cells without treatment with MDI or other compounds were used as a control group. (B) Comparison chart of fragments per kilobase of transcript per million fragments mapped (FPKM) density distribution of each sample. (C) Number of differentially expressed genes (up- and downregulated) between different comparison groups. (D) Venn diagrams of the overlapping of upregulated and downregulated genes for comparison of different groups.

To test the aforementioned results from transcriptomics, 3T3-L1 cells at an early stage of adipogenesis (4 h, 8 h, 12 h, 24 h) with different treatments were collected and measured by RT-qPCR and WB, respectively. As shown in Fig. 3, the gene and protein levels of *Gja1* were relatively low in the 3T3-L1 preadipocytes cells. After MDI stimulation, there was no significant difference in mRNA and protein expression levels of *Gja1* within the first hour, as shown in Fig. S5 (Supporting Information). However, their mRNA and protein levels gradually increased from 2 h and reached their highest level around 8 h, and then begun to decrease, suggesting that the levels of *Gja1* might be an index of differentiation of 3T3-L1 preadipocytes in the early

stage. Due to the stimulation of MDI reagents, the confluent growth-arrested 3T3-L1 cells would once again enter the growth cycle for mitotic clonal expansion, which required gap-junctional communication and intercellular channels. Subsequently, an inhibitor of Gja1, namely 18- α -Glycyrrhetic acid (AGA), was introduced in the first 2 days of differentiation to explore its effects on the lipid accumulation of 3T3-L1 cells and the mature lipid droplets were labeled with Oil Red O dye after completing 8 days of differentiation. The results revealed that a significant reduction (52.0%, $P < 0.05$) in intracellular lipid content when 3T3-L1 cells were treated with AGA, as shown in Fig. S6 (Supporting Information), which was consistent with previous report.^[39] Thus, the highly expressed Gja1 plays an essential role in the early stages of 3T3-L1 preadipocytes differentiation.

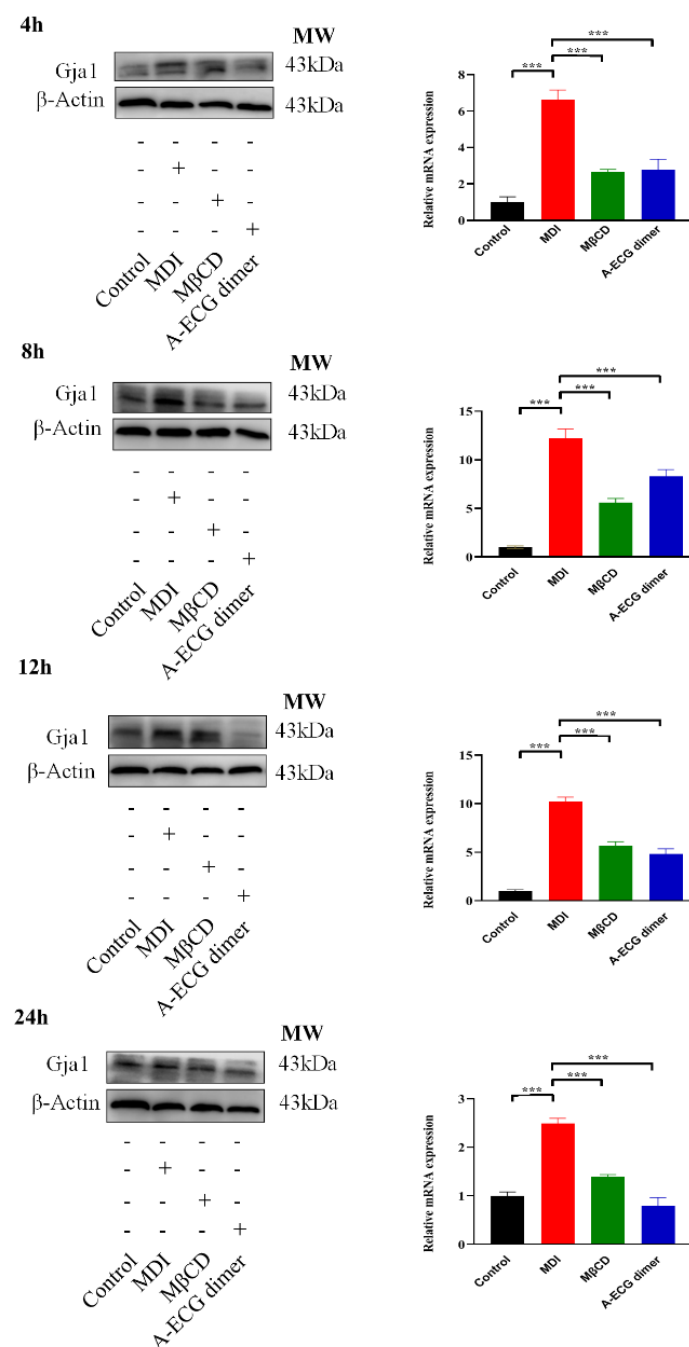


Fig. 3. The protein and gene expression levels of Gja1 after treatment with different compounds at different time points. Results were expressed as mean $\pm$ standard deviation ($n = 3$ independent replicates). Asterisk among groups indicate statistical difference (***) $P < 0.001$.

Subsequently, we found that both gene and protein expression of Gja1 were inhibited in the 3T3-L1 preadipocytes cells along with the treatment with M β CD, as shown in Fig. 3, meaning that the disruption of lipid rafts would downregulate the expression of Gja1. In the time test conducted using M β CD to disrupt the lipid rafts, the results revealed a 44% reduction in the rafts structure within a 30-minute period.^[11] However, the high expression of Gja1 began after 2 hours, so we speculated that M β CD could inhibit the synthesis of intracellular Gja1 protein by disrupting the structure of lipid rafts. Such effect contributed to the inhibition of lipid accumulation induced by M β CD did not change in the presence or absence of AGA, further supporting that the effect of M β CD might be independent of downregulating Gja1 directly. By contrast, pretreatment with A-ECG dimer could downregulate the mRNA expression and protein expression of Gja1 under different time treatments (Fig. 3), and its intracellular lipid accumulation inhibition effect could also be significantly ($P < 0.05$) enhanced in the presence of AGA (Fig. S6, Supporting Information), suggesting that A-ECG dimer might be not only disrupt the structure of lipid rafts, but also decreasing the expression of Gja1 directly. In addition, A-ECG dimer showed significant inhibition on intracellular lipid accumulation even at 5 μ M and 10 μ M, with a significant reduction of 45.0% and 54.2% (Fig. S6, Supporting Information), respectively. Our data indicated that the downregulating effect of A-ECG dimer at concentration as low as physiological concentration was stronger than both of 3 mM M β CD and 100 μ M AGA.

Overall, these results showed that the gap-junctional communication played by Gja1 was crucial in the early stages of adipogenesis and the treatment with A-ECG dimer could inhibit the adipogenesis through downregulating the expression of Gja1. In addition, these results from RT-qPCR and WB measurement were in line with transcriptome data, suggesting that the data obtained by transcriptomics was reliable and useable in this study. On the other hand, these results mentioned above indicated that Gja1 was closely associated with lipid rafts and involved in the inhibition of A-ECG dimer on 3T3-L1 preadipocytes differentiation.

3.2 A-ECG dimer inactivated intracellular lipid synthesis signaling pathway by inhibiting lipid rafts clusters and the expression of Gja1 protein

Considering that the gap junction function of Gja1 depends on the membrane structure, we inferred that the protein encoded by *Gja1* would be transferred to the lipid rafts after intracellular synthesis. Thus, immunofluorescence staining assay was first conducted to roughly reveal the position of the lipid rafts and protein encoded by *Gja1* (Fig. 4A), respectively. The structure of lipid rafts remained intact in the 3T3-L1 preadipocytes before and after MDI induction, as evidenced from the strong mean fluorescence intensity (FI) of 49.05 and 49.71 (Fig. 4B). In contrast, the mean FI of protein encoded by *Gja1* (27.8) was weak in the control group due to its low expression levels (Fig. 4C). However, the expression of protein encoded by *Gja1* was increased by 41.6% after the stimulation of MDI reagents, as can be seen in the increase in mean FI in Fig. 4C. Subsequently, we found that the position of labeled protein was highly consistent with the position of the lipid rafts, as demonstrated by fluorescence co-localization. Thus, this result also showed that the protein encoded by *Gja1* after intracellular synthesis would be transferred to the lipid rafts. Furthermore, both lipid rafts FI and protein FI were reduced after the addition of M β CD (44.6% and 14.0%) and A-ECG dimer (53.5%

and 23.7%), suggesting that the structure of lipid rafts was disrupted and the protein content in the rafts was also decreased.

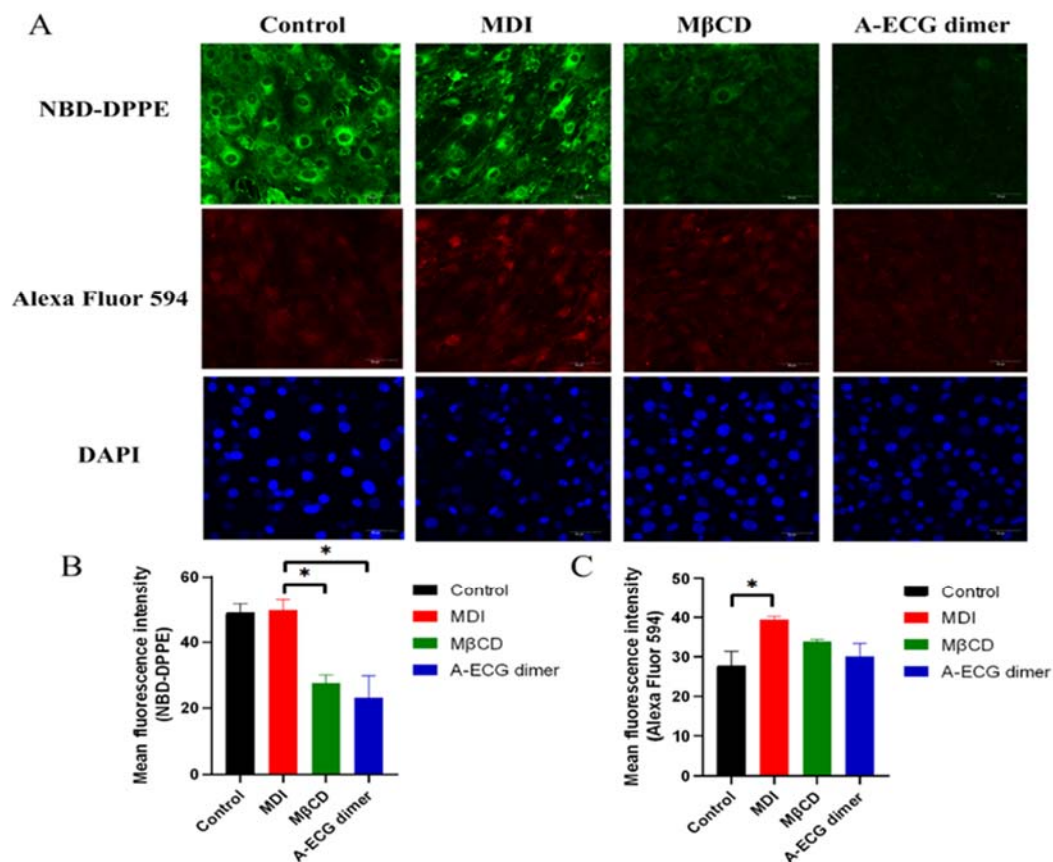


Fig. 4. The protein expression level and position of Gja1 protein after treatment with different compounds. (A) Representative lipid rafts domain fluorescence photomicrograph of the 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPPE) probe (upper panel) and the protein expression level of Gja1 (middle panel) output by a laser scanning confocal microscope (scale bars represent 50 μ m). The lower panel shows cell nuclear location using 4',6-diamidino-2-phenylindole (DAPI). Mean fluorescence intensity of different compounds on 3T3-L1 preadipocytes lipid rafts (B) and protein Gja1 (C). Results were expressed as mean $\pm$ standard deviation of three independent replication. * $P < 0.05$ versus MDI group.

The results from the above immunofluorescence experiment preliminarily indicated that the position of protein Gja1 after intracellular synthesis was closely associated with the lipid rafts. Subsequently, the lipid rafts were further isolated by density gradient centrifugation and then were used to measure the content of protein Gja1 in this study. Schematic illustration for isolating lipid rafts was shown in Fig. 5A. The lipid rafts were identified by using flotillin 1 markers, which was mainly concentrated in fraction 2 and fraction 3 of Control group. Due to the low expression level, the content of protein Gja1 in lipid rafts of 3T3-L1 preadipocytes was relatively low, agreeing with the results from immunofluorescence assay. Meanwhile, we found that lipid rafts would further cluster to form tighter aggregates after MDI stimulation, revealed by unique band fraction (Fig. 5B). At this point, much more Gja1 protein contents were measured, which might be required for the function of intercellular connections and communication. Subsequently, we found the cluster assembly of this lipid rafts continued to be maintained during M β CD treatment, but the content of protein Gja1 in lipid rafts would be reduced. An opposite result was seen in A-ECG dimer group as the cluster of lipid rafts caused by MDI stimulation would be inhibited. Meanwhile, the content of protein Gja1 in the

lipid rafts was also decreased, suggesting that A-ECG dimer interacted with 3T3-L1 cells in a distinctive manner by blocking the clustering of lipid rafts caused by MDI stimulation and reducing Gja1 expression. Furthermore, due to the rapid lipid rafts disruption effect of A-ECG dimer, we speculated that A-ECG dimer could inhibit the synthesis of intracellular Gja1 by disrupting the structure of lipid rafts and then decrease its content in the raft's domain. Overall, these results were in line with those from immunofluorescence assay.

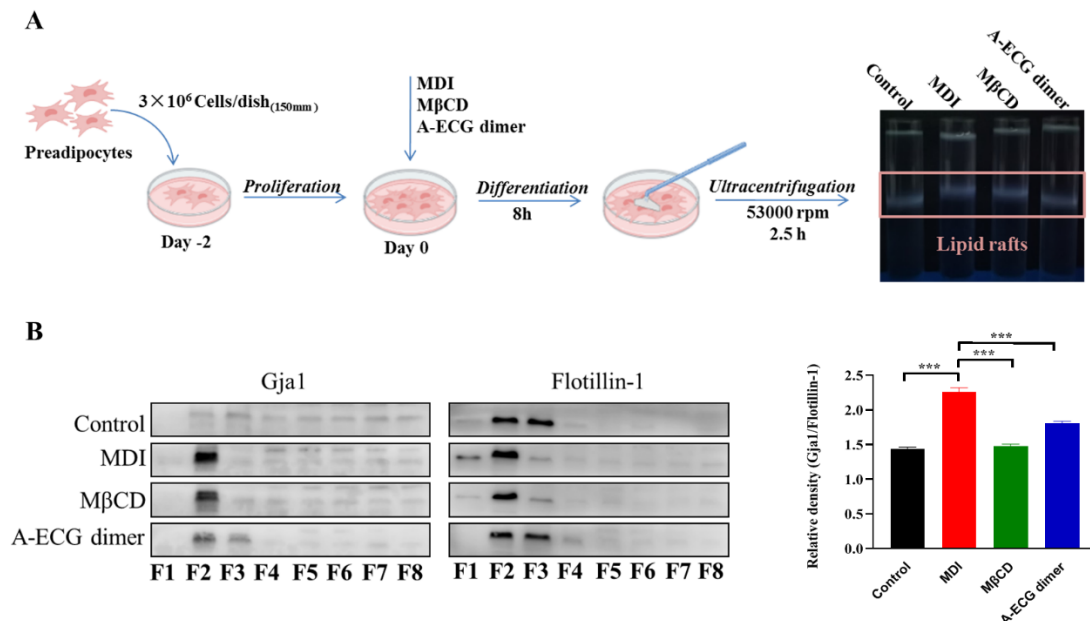


Fig. 5. The lipid rafts obtained by density gradient ultracentrifugation method and the effect of different treatments on Gja1 in lipid rafts. (A) Schematic illustration for isolating lipid rafts from 3T3-L1 preadipocytes. (B) the expressions of lipid raft marker Flotillin-1 and Gja1 were detected by western blot after different treatments. Results were expressed as mean ± standard deviation (n = 3 independent replicates). Asterisk among groups indicate statistical difference (***) $P < 0.001$.

Given the results above, the effect of A-ECG dimer on Gja1-mediated intracellular lipid synthesis signaling pathway has also been tested in this study. Considering the available concentration in circulating blood, we further tested A-ECG dimer at the range of 5 - 20 μM . Because a previous study suggested about 17.18 μM total polyphenols in plasma of rats after administered with the dose of 250 mg/kg body weight of grape seed polyphenols, which was effective to regulate lipid metabolism.^[41] Thus, we explored the effect of A-ECG dimer at physiological concentrations on Gja1-mediated intracellular lipid synthesis signaling pathway. As shown in Fig. 6a, the expression level of protein encoded by *Gja1* was increased when the cells were cultured in medium with MDI reagents. However, the protein expression could be inhibited after treatment with MβCD and A-ECG dimer at the concentration of 5 μM , 10 μM , and 20 μM , respectively. This result indicated that A-ECG dimer at physiological concentrations could also inhibit the expression of Gja1 protein (Fig. 6b). Subsequently, some intracellular transcription factors controlling the lipid synthesis and the maturation process of lipid droplet were measured by WB, which included peroxisome proliferator-activated receptor γ (PPAR γ), CCAAT/enhancer-binding protein alpha (C/EBP α), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS). Of these four factors, PPAR γ and C/EBP α are master transcriptional regulators that control 3T3-L1 preadipocytes to differentiate into mature fat cells. They work synergistically to activate the expression of genes involved in lipid synthesis and droplet formation. Meanwhile, ACC and FAS are key

enzymes directly responsible for fatty acid biosynthesis, converting acetyl-CoA into long-chain fatty acids. Ultimately, the coordinated action of these regulators and enzymes drives adipogenesis and lipid accumulation. The results showed that the expression of PPAR γ and C/EBP α significantly increased after MDI stimulation, which further activated the downstream lipid synthesis signaling pathway, manifested by high expression of ACC and FAS (Figs. 6B, 6C). In this study, M β CD was used as a positive control for lipid rafts disruption and its effects on Gja1-mediated lipid synthesis signaling pathway were also explored. As shown in Fig. 6, the protein expression of involving lipid synthesis was downregulated after treatment with M β CD, showing that the integrity of lipid rafts structure was essential for the activation of downstream signaling pathways. As for A-ECG dimer, its inhibitory ability on the expression of these proteins was stronger than M β CD, even under 5 μ M physiological concentration treatment, suggesting that A-ECG dimer can be regarded as an excellent candidate compound for intervention in adipogenesis. Furthermore, our results also showed that the protein expression inhibition ability induced by A-ECG dimer was also concentration-dependent.

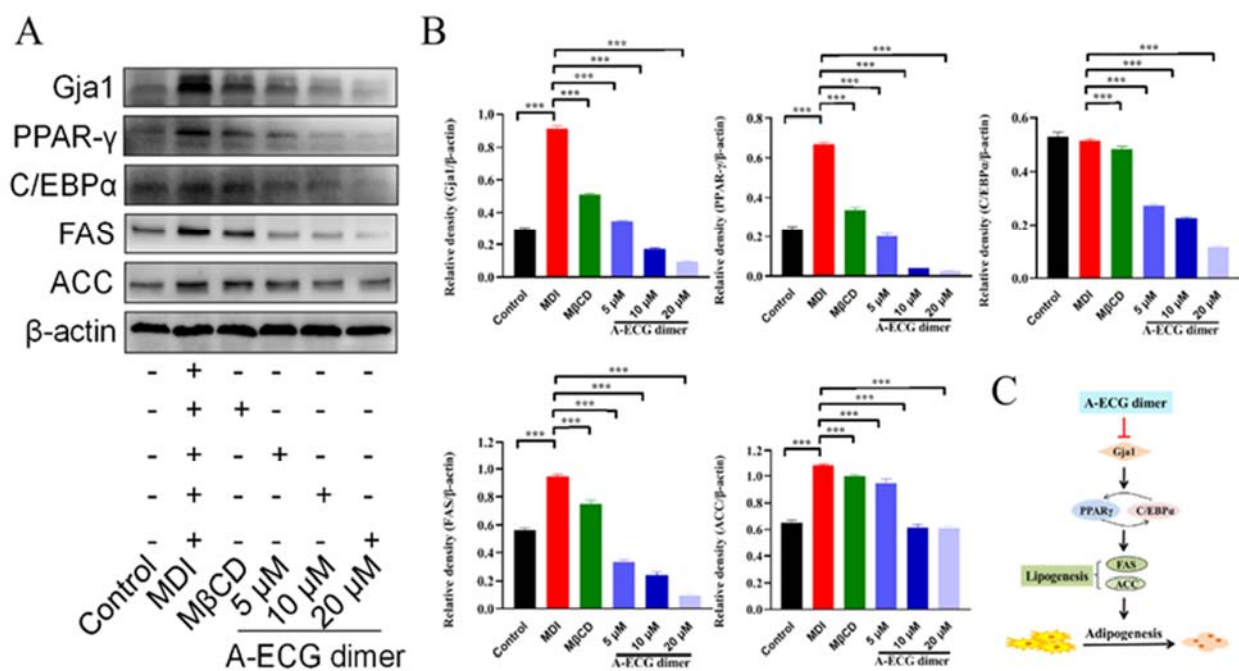


Fig. 6. Effects of A-ECG dimer at different concentrations on intracellular lipid synthesis signaling pathway regulating by Gja1. The post-confluent 3T3-L1 preadipocytes were incubated by MDI, M β CD, and A-ECG dimer (5 μ M, 10 μ M, 20 μ M).

The proteins were detected by western blot (A). The quantitative analysis of each protein was performed by dividing the density of the target protein band by β -actin (B). Schematic illustration for the intracellular lipid synthesis signaling pathway of A-ECG dimer action (C). Results were expressed as standard deviation ($n = 3$ independent replicates). Asterisk among groups indicate statistical difference (***) $P < 0.001$.

3.3 A-ECG dimer inhibited the ordered structure formation of Gja1 protein by binding to its active sites and disturbing its secondary structures

Previous studies have shown that there are many molecules locating at lipid rafts can bind to active sites of Gja1, including caveolin-1, β -catenin, tubulin, and src homology (SH), thus performing various function, including 1) transportation of gap junction sites between cells,^[42] 2) stabilization of junction sites,^[43] and 3) transmission of different signals.^[44] Further study also indicated that the formation of ordered structure of

protein Gja1 mediated by interaction between ligand molecule and receptor protein was essential for the function of gap-junctional communication and intercellular channels,^[45] which was also necessary for the adipogenesis of 3T3-L1 preadipocytes. Considering the strong binding preference between A-ECG dimer and lipid rafts,^[46] we thus suspected that A-ECG dimer would also bind to Gja1 and interfere with its ordered structure. The complete crystal structure of gap junction proteins has not been thoroughly analyzed to date due to barriers in transmembrane protein structure analysis technology. Fortunately, the terminal structure of Gja1 containing active sites has been resolved, which PDB ID number is 1R5S. Thus, the structure of 1R5S was used to dock with A-ECG dimer to explore their binding sites and manners by using molecular modeling method.

As shown in Fig. 7A, A-ECG dimer bound to the helical structure of 1R5S through polar interactions, van der Waals interactions, and hydrogen bonding interactions. Specifically, A-ECG dimer could interact with amino acids of Ser 56, Glu 57, Gln 58, Trp 60, Ser 64, and Gln 67 through polar interaction, and with amino acids of Met 70 and Asn 93 through van der Waals interaction. Besides, A-ECG dimer could not only form hydrogen bonds with the main chain of amino acids Ala 61, Ala 65, and Glu 66, but also with the side chain of Asn 62. This indicated that these amino acids might be binding sites of A-ECG dimer in protein Gja1. Compared with the previously reported active sites of 1R5S, we found that the action sites of A-ECG dimer highly overlapped with those of SH acting on 1R5S.^[45] Furthermore, the helical structure of Gja1 including active sites was also reported to be responsible for the formation of highly ordered structures, serving as intercellular channels for communication between different Gja1 binding partners. Therefore, we inferred that the binding of A-ECG dimer to these sites might interfere with the ordered structure of protein Gja1. Subsequently, we explored the binding of A-ECG dimer with protein Gja1 from a dynamic binding insight. As shown in Fig. 7B, A-ECG dimer is stably bound to those amino acids obtained from MD through polar interaction, van der Waals interactions, and hydrogen bonding interaction. What's more, A-ECG dimer could also form additional hydrogen bonds with amino acids of ASP 86, ASP 89, and Pro 88 in protein Gja1. Further analysis of the number of hydrogen bonds showed that the formation of 3 to 6 hydrogen bonds accounted for over 75% of the total hydrogen bonds (Fig. 7C), indicating that multiple hydrogen bonds dominated the interaction between A-ECG dimer and 1R5S. In addition, other forces such as electrostatic interaction, van der Waals interaction, hydrophobic interaction, etc. were also involved in the binding process. Hence, we computed the binding energies associated with the various forces outlined earlier to denote their respective contributions. The binding energies of A-ECG dimer to 1R5S through electrostatic and van der Waals interactions were -234.02 ± 16.08 KJ/mol and -182.75 ± 36.53 KJ/mol, respectively, suggesting that electrostatic interaction was another important manner of action between the A-ECG dimer and protein Gja1.

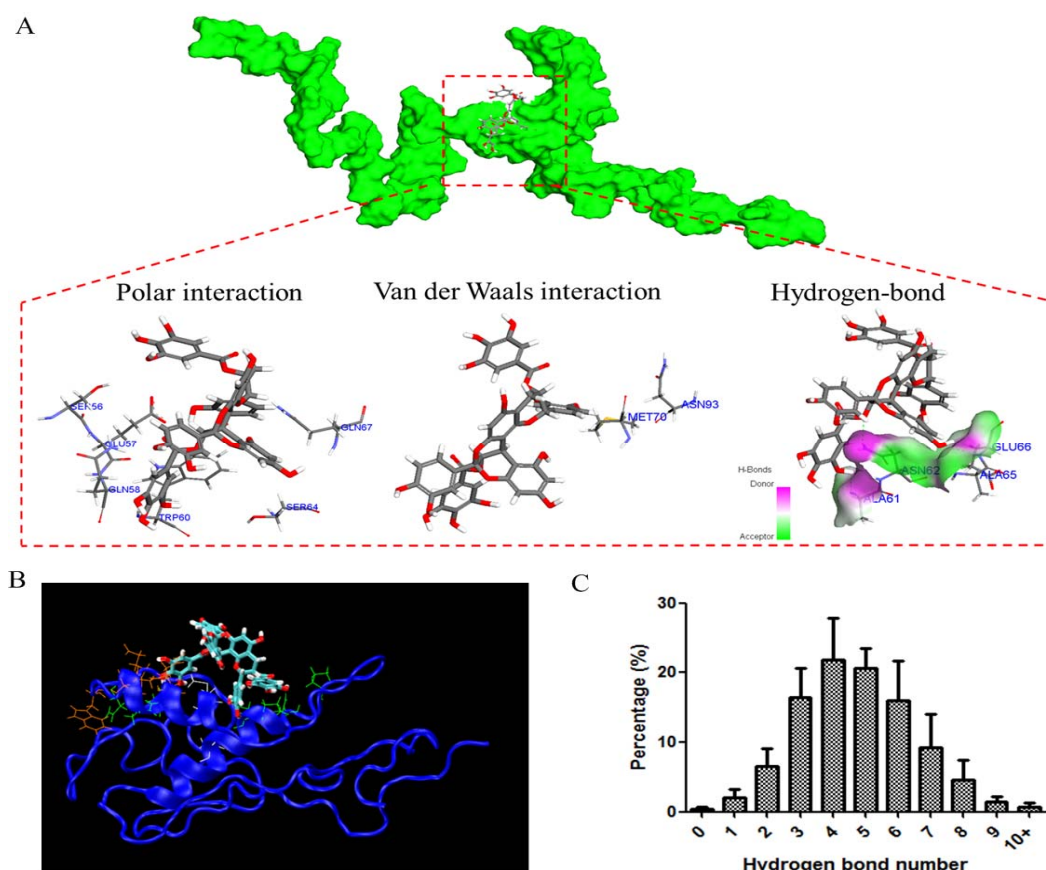


Fig. 7. The binding conformation and sites between A-ECG dimer and 1R5S revealed by molecular docking and molecular dynamic simulation. (A) The protein was displayed in green VDW model and A-ECG dimer was displayed in bonding model. Binding amino acids between 1R5S and A-ECG dimer were labeled and shown. (B) Binding sites of A-ECG dimer and 1R5S after molecular dynamic simulation. The 1R5S is shown by the blue secondary structure coupled with the NewCartoon model, and the A-ECG dimer is shown by the bonding model. The amino acids that interact with the A-ECG dimer through polar, van der Waals and hydrogen bond interaction are shown by the orange, white, and green line model. (C) Proportion of hydrogen bonds formed by A-ECG dimer and 1R5S receptor protein. Results were expressed as mean $\pm$ standard deviation of three independent replication.

Subsequently, the effect of A-ECG dimer on the ordered structure of protein Gjal was also explored and the results were shown in Fig. 8. Firstly, the radius of gyration value of 1R5S receptor before and after A-ECG dimer treatment in three independent replicates was compared (Fig. 8A), which was often used to evaluate the changes in the relative initial position of the receptor protein during the dynamic binding process. The result showed that the radius of gyration value of 1R5S decreased by 45.78% after the action of A-ECG dimer, suggesting significant structural changes. Analysis of solvent accessible surface area (SASA) further elucidated this structural change, which is defined as the surface area of molecule that can be contacted by solvents and can be further divided into hydrophilic SASA and hydrophobic SASA.^[47] As shown in Fig. 8B, the hydrophobic and hydrophilic SASA of protein 1R5S decreased by 32.74% and 28.45% after the action of A-ECG dimer, indicating that A-ECG dimer could change the protein structure after binding to its active sites. This result was further supported by the evidence from the overlapped snapshot of the protein 1R5S structure before and after A-ECG dimer treatment (Fig. 8C). Then, we further analyzed the changes in the secondary structure of protein 1R5S after the action of A-ECG dimer in three independent replicates and this result was shown in Fig. 8D and Fig. 8E. In the initial structure of 1R5S, its secondary structure was mainly composed of

Turn (71.21%), Alpha Helix (12.88%), and Coil (15.91%). After the action of A-ECG dimer, structural changes could be seen in 1R5S, with a significant decrease in Turn of 33.07% and an increase in Coil of 24.75%. A slight increase (3.54%) in Alpha Helix could be seen in the secondary structure of 1R5S. Furthermore, some other secondary structures, with 1.77% Isolated Bridge and 2.02% Extended Configuration, could be observed after the action of A-ECG dimer. Previous studies have shown that the two helical structures in Gja1 were responsible for the formation of ordered structure, while the remaining structures maintained the stability of this structure, thereby serving as channels for communication between different Gja1 binding partners.^[45] Our results suggested that the changes in the secondary structure of protein Gja1 caused by A-ECG dimer might disturb Gja1-mediated ordered structure formation, thus inhibiting the intercellular communication. However, the results obtained from molecular modeling were not validated by assay due to incomplete structural information, requiring confirmation through structural changes and *in vivo* experiments in the future.

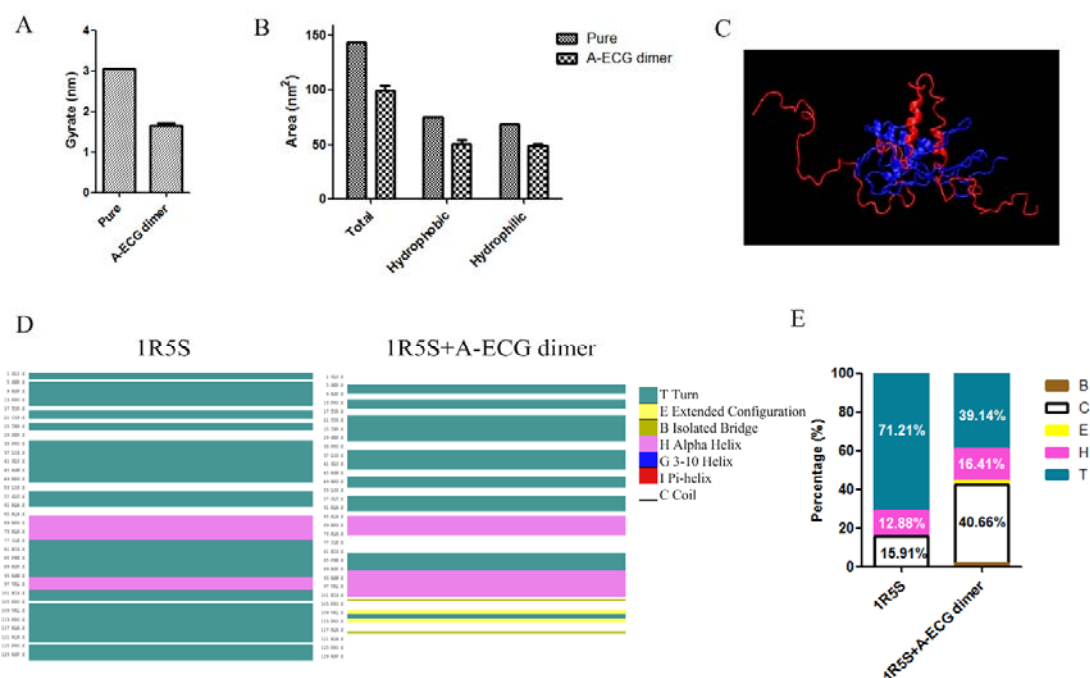


Fig. 8. Changes in the structure of 1R5S receptor protein after A-ECG dimer treatment. (A) Radius of gyration of 1R5S receptor protein before and after A-ECG dimer treatment. (B) The solvent-accessible surface area of total, hydrophilic and hydrophobic groups of 1R5S receptor protein before and after A-ECG dimer treatment. (C) The superimposed snapshot of the 1R5S receptor protein structure before and after A-ECG dimer treatment. The red is the initial structure of 1R5S, and the blue is the structure after A-ECG dimer treatment. The protein structure is shown as secondary structure coupled with NewCartoon model. (D) Changes in the secondary structure of 1R5S receptor protein before and after A-ECG dimer treatment. (E) Quantitative analysis of changes in the secondary structure of the 1R5S receptor protein. Results were expressed as mean $\pm$ standard deviation of three independent replication.

4. Discussion

The present study elucidates the molecular mechanisms by which the A-ECG dimer inhibits 3T3-L1 preadipocyte differentiation, with a particular focus on the role of lipid rafts and the gap junction protein Gja1. Firstly, the identification of Gja1 as a crucial player in adipocyte differentiation aligns with previous reports emphasizing the importance of gap-junctional communication in mitotic clonal expansion, a prerequisite for adipogenesis^[14, 39]. The observed upregulation of Gja1 following MDI induction and its subsequent

suppression by A-ECG dimer suggested that Gja1-mediated intercellular signaling was essential for initiating adipocyte differentiation. The fact that pharmacological inhibition of Gja1 (using AGA) significantly reduced lipid accumulation further supports its functional relevance^[40]. Notably, the temporal expression pattern of Gja1, peaking at 8 h post-induction, suggests that its role is most critical during the early stages of differentiation. This finding raises intriguing questions about whether Gja1 acts primarily as a structural component facilitating cell-cell communication or as a signaling hub that integrates extracellular cues to promote adipogenic process^[38]. Future studies employing conditional knockout models or real-time imaging of Gja1 dynamics could provide deeper insights into its precise mechanistic contributions.

Moreover, the study underscores the importance of lipid rafts in facilitating Gja1 localization and function. The co-localization of Gja1 with lipid rafts, along with the observation that raft disruption (via M β CD or A-ECG dimer) reduces Gja1 protein levels, suggests that intact raft microdomains are necessary for Gja1 stability. Interestingly, A-ECG dimer exhibited a dual mechanism: (1) disrupting raft clustering and (2) suppressing Gja1 expression. This dual action may explain its superior inhibitory efficacy compared to M β CD and other polyphenols^[11, 48]. The ability of A-ECG dimer to prevent raft clustering, a phenomenon not observed with M β CD, suggests a unique mode of interaction with membrane microdomains. This result is also closely related to previous binding studies, where A-ECG dimer disrupts the lipid rafts structure by preferentially binding to cholesterol components^[46, 49], while the M β CD removes cholesterol from the lipid rafts through its hydrophobic pore structure^[50]. Beyond raft disruption, A-ECG dimer was shown to directly bind Gja1, interfering with its secondary structure and potentially disrupting its oligomerization into functional gap junctions. Molecular modeling revealed that A-ECG dimer interacts with key residues in Gja1's helical domain, overlapping with known binding sites for regulatory proteins (e.g., caveolin-1, β -catenin). This suggests that A-ECG dimer may competitively inhibit endogenous Gja1-binding partners, further impairing intercellular communication. However, in interpreting the molecular interaction between A-ECG dimer and Gja1, it is important to acknowledge a methodological limitation in our structural simulations. Our molecular docking and dynamics analyses were performed using the terminal structure of Gja1 (PDB: 1R5S) rather than the full-length transmembrane protein. While this approach provided valuable insights into potential binding mechanisms, the absence of complete transmembrane domains and extracellular loops means we cannot fully capture the native conformational dynamics of Gja1 within the lipid rafts.

The multifaceted inhibitory effects of A-ECG dimer, spanning raft disruption, Gja1 suppression, and downstream lipogenic blockade, highlight its potential as a novel anti-adipogenic agent. However, several limitations should be acknowledged. First, the research was conducted solely in the 3T3-L1 murine preadipocyte model. Although widely used, this system may not fully cover the complexity of human adipogenesis or the physiological relevance of human lipid metabolism. Further studies using human adipocyte cultures or humanized models would help validate the translational relevance of the Gja1-lipid raft pathway identified here. Second, it remains unclear whether the observed effects are specific to A-ECG dimer

or shared by other structurally related procyanidin dimers. Comparative studies using dimers with different linkage types or substitution patterns, such as A-type and B-type dimers, are needed to elucidate structure–activity relationships in the future. Third, what are the *in vivo* implications of Gja1 inhibition exerted by A-ECG dimer? Animal models should be used to evaluate whether A-ECG dimer reduces fat accumulation and improves metabolic parameters through the Gja1-mediated intracellular lipid synthesis signaling pathway. Finally, does Gja1 interact with other adipogenic regulators (e.g., insulin signaling components) within lipid rafts? The co-immunoprecipitation assays should be employed to identify novel Gja1-associated protein networks in the future. Addressing these questions will not only refine our understanding of procyanidin bioactivity but also help assess the potential of A-ECG dimer as a lead compound or nutritional intervention agent for metabolic health applications.

5. Conclusion

A key gene involved the membrane rafts after A-ECG dimer interacting with 3T3-L1 preadipocytes, namely *Gja1*, was identified and validated with transcriptome analysis, RT-qPCR and WB techniques. Both the gene and encoded protein expression of Gja1 were suppressed upon treatment with A-ECG dimer at physiological concentrations, leading to the inhibition of downstream lipid synthesis signaling pathways. Additionally, the assembly of lipid rafts clusters triggered by MDI stimulation and the protein Gja1 content within these lipid rafts were hindered and reduced due to the effects of the A-ECG dimer. This interference might signify a disruption in gap-junctional communication and intercellular connections between cells, which was further supported by changes observed in the structure of 1R5S following the action of the A-ECG dimer. Specifically, the A-ECG dimer interacted with 1R5S through forces of action such as polar interactions, van der Waals interactions, electrostatic interactions, and hydrogen bonding. This binding at the active site also had detrimental impact on the structure of 1R5S, evident from the reduced radius of gyration value (45.78%), hydrophilic SASA (28.45%), and hydrophobic SASA (32.74%). Additionally, the secondary structure of the Gja1 protein, crucial for forming ordered structures, was notably altered due to the A-ECG dimer's action. Consequently, these findings offer comprehensive insight into the mechanism through which A-ECG dimer inhibits adipogenesis in 3T3-L1 preadipocytes and expands the current understanding about the manner of action of the strong inhibitory effect exerted by polyphenols on adipogenesis in 3T3-L1 preadipocytes.

Supporting Information

Additional figures illustrate the high-performance liquid chromatography traces of A-ECG dimer, the root mean square deviation of system and protein, correlation heatmap analysis and cluster analysis of three biological replicates for all samples from transcriptome data, volcano plots of the differential expression genes, protein and gene expression levels of Gja1 after treatment with MDI at different time points, effects of different compounds on MDI-induced 3T3-L1 preadipocyte differentiation, overview of purity and source of A-ECG dimer, and the primers of β -actin and Gja1.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (32172201).

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