PLOS | ONE

RESEARCH ARTICLE

Potential miRNA involvement in the anti-adipogenic effect of resveratrol and its metabolites

Itziar Eseberri[1,2], Arrate Lasa[1,2]*, Jonatan Miranda[1,2], Ana Gracia[1,2], Maria P. Portillo[1,2]

1 Nutrition and Obesity group, Department of Nutrition and Food Science, University of Basque Country (UPV/EHU) and Lucio Lascaray Research Center, Vitoria, Spain, **2** Centro de Investigación Biomédica en Fisiopatología de la Obesidad y Nutrición (CIBERobn), Instituto de Salud Carlos III, Madrid, Spain

* arrate.lasa@ehu.eus

Citation: Eseberri I, Lasa A, Miranda J, Gracia A, Portillo MP (2017) Potential miRNA involvement in the anti-adipogenic effect of resveratrol and its metabolites. PLoS ONE 12(9): e0184875. https://doi.org/10.1371/journal.pone.0184875

Editor: Cristina Óvilo, INIA, SPAIN

Received: March 7, 2017

Accepted: September 3, 2017

Published: September 27, 2017

Data Availability Statement: All relevant data are within the paper.

Funding: This study was supported by grants from the Ministerio de Economía y Competitividad (AGL2011-27406-ALI), Instituto de Salud Carlos III (CIBERObn), Government of the Basque Country (IT-572-13) and University of the Basque Country (UPV/EHU) (ELDUNANOTEK UFI11/32). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. I. Eseberri is a recipient of a doctoral fellowship from the University of the Basque Country.

Abstract

Objective

Scientific research is constantly striving to find molecules which are effective against excessive body fat and its associated complications. Taking into account the beneficial effects that resveratrol exerts on other pathologies through miRNA, the aim of the present work was to analyze the possible involvement of miRNAs in the regulation of adipogenic transcription factors peroxisome proliferator-activated receptor γ *(pparγ)*, `CCAAT` enhancer-binding proteins *α* and *β* (*cebpβ* and *cebpα)* induced by resveratrol and its metabolites.

Methods

3T3-L1 maturing pre-adipocytes were treated during differentiation with 25 μM of *trans*-resveratrol (RSV), *trans*-resveratrol-3-O-sulfate (3S), *trans*-resveratrol-3'-O-glucuronide (3G) and *trans*-resveratrol-4'-O-glucuronide (4G). After computational prediction and bibliographic search of miRNAs targeting *pparγ*, *cebpβ* and *cebpα*, the expression of microRNA-130b-3p (miR-130b-3p), microRNA-155-5p (miR-155-5p), microRNA-27b-3p (miR-27b-3p), microRNA-31-5p (miR-31-5p), microRNA-326-3p (miR-326-3p), microRNA-27a-3p (miR-27a-3p), microRNA-144-3p (miR-144-3p), microRNA-205-5p (miR-205-5p) and microRNA-224-3p (miR-224-3p) was analyzed. Moreover, other adipogenic mediators such as sterol regulatory element binding transcription factor 1 (*srebf1*), krüppel-like factor 5 (*klf5*), liver x receptor α (*lxrα*) and cAMP responding element binding protein 1 (*creb1*), were measured by Real Time RT-PCR. As a confirmatory assay, cells treated with RSV were transfected with anti-miR-155 in order to measure *cebpβ* gene and protein expressions.

Results

Of the miRNAs analyzed only miR-155 was modified after resveratrol and glucuronide metabolite treatment. In transfected cells with anti-miR-155, RSV did not reduce *cebpβ* gene and protein expression. 3S decreased gene expression of *creb1*, *klf5*, *srebf1* and *lxrα*.

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: RSV, *Trans*-resveratrol; 3S, *trans*-resveratrol-3-*O*-sulfate; 3G, *trans*-resveratrol-3-*O*-glucuronide; 4G, *trans*-resveratrol-4'-*O*-glucuronide; miR-130b-3p, microRNA-130b-3p; miR-155-5p, microRNA-155-5p; miR-27b-3p, microRNA-27b-3p; miR-31-5p, microRNA-31-5p; miR-326-3p, microRNA-326-3p; miR-27a-3p, microRNA-27a-3p; miR-144-3p, microRNA-144-3p; miR-205-5p, microRNA-205-5p; miR-224-3p, microRNA-224-3p.

Conclusions

While RSV and glucuronide metabolites exert their inhibitory effect on adipogenesis through miR-155 up-regulation, the anti-adipogenic effect of 3S is not mediated via miRNAs.

Introduction

Obesity is a genuinely serious real health problem. In 2014 about 13% of the world's adult population worldwide (11% of men and 15% of women) suffered from obesity [1]. In addition, this pathology induces a great number of co-morbilities, such as type 2 diabetes, dyslipidemia, hypertension and cancer among others. Consequently, direct and indirect costs associated with these common medical conditions have charted a steady rise in obesity costs over the years, as the epidemic has grown.

Adipose tissue growth in obesity can be mediated by hypertrophy, which is to say an increase in adipocyte size and/or hyperplasia, that is an increase in adipocyte number. When hyperplasia takes place there is a stimulation of pre-adipocyte proliferation and further differentiation. The above process, which promotes pre-adipocyte differentiation into mature adipocytes (2) plays a crucial role in the development of obesity and needs to be highly controlled. It is well established that in the long term continued energy overloading can increase this process, mainly in young subjects [2]. Although several molecular aspects of adipogenesis are still unknown, peroxisome proliferator-activated receptor γ (*ppar*γ) has been identified as the master coordinator of adipocyte differentiation[3]. The control that *ppar*γ exerts over pre-adipocytes for them to reach adipocyte functionally needs the expression of other important genes both at the early and at the latter stages of adipocyte differentiation, such as CCAAT enhancer-binding proteins α and β (*cebp*β and *cebp*α) respectively [4, 5].

These adipogenic genes are regulated by different mechanisms, microRNAs (MiRNAs) among others. MiRNAs are small non-coding RNAs about 19–23 nucleotides in length that have emerged as important regulators of gene expression [6]. They act by base paring with their target mRNA, which leads to mRNA degradation or translation repression [7, 8]. More than 2500 miRNAs have been described in humans to date [9]. Some of them are involved in numerous physiological and pathological processes, such as energy homeostasis [10], sugar and lipid metabolism [11, 12] and tumorigenesis [13]. As far as adipose tissue is concerned, several studies have concluded that some miRNAs can regulate adipogenesis by targeting genes that regulate this process [14–16].

Scientific research is constantly being undertaken with the aim of finding new molecules, either drugs or food components, which are effective in preventing excess accumulation of body fat and associated complications. This is the case of *trans*-resveratrol (3,4,5-trihydroxystilbene, RSV), a polyphenol with a stilbene structure that consists of two phenolic rings held together by a double styrene bond. This compound is naturally present in various plants, including grapes, berries and peanuts and is produced in response to stress, as a defence mechanism against fungal, viral, bacterial infections and damage from exposure to ultraviolet radiation [17]. Most RSV undergoes rapid and extensive metabolism into enterocytes, before entering blood. Furthermore, it undergoes rapid first-pass metabolism in the liver [17]. Consequently, RSV bioavailability is very low and only a small proportion reaches plasma. The concentrations of glucuronide and sulfate metabolites are relatively higher [18–20]. The proportions of glucuronide and sulfate metabolites depend on the tissue [21] and the species [22]. RSV, which shows antioxidant and antiinflammatory properties, is effective in the prevention of several diseases including cardiovascular diseases, diabetes, cancer and recently, obesity. With regard to obesity, a general consensus concerning the body-fat lowering effect of resveratrol in mice and rats exists [23, 24]. This effect is mainly mediated by a reduction in adipogenesis and lipogenesis and by an increase in energy expenditure, lipolysis and fatty acid oxidation in liver and skeletal muscle [24].

Given the above relating to RSV metabolism, an important question is whether RSV metabolites are active molecules. In a previous study we described how RSV, as well as certain metabolites (*trans*-resveratrol-3-O-sulfate -3S-, *trans*-resveratrol-3'-O-glucuronide -3G- and *trans*-resveratrol-4'-O-glucuronide -4G-) were able to modify the expression of genes related to the adipogenic process [25]. While all of them (RSV, 3G, 4G and 3S) reduced *cebpβ* mRNA levels, only the sulfate metabolite reduced *cebpα* and *pparγ* gene expression.

In this scenario and taking into account that the beneficial effects of RSV on other pathologies, such as cancer and diabetes, are mediated by miRNA [26, 27], the present study focuses on the possible involvement of different miRNAs in the changes induced by RSV and its metabolites in adipogenic transcription factors *pparγ*, *cebpβ* and *cebpα*, a process which has not been analyzed to date. For this purpose, a well-defined pre-adipocyte model, 3T3-L1 murine adipocytes, was used.

Material and methods

Experimental design and cell treatment

The experimental design for 3T3-L1 maturing pre-adipocyte was previously described (25). Briefly, cells grown in 6-well plates were incubated with either 0.1% ethanol (95%) (control group) or with RSV, 3G, 4G or 3S, all of them provided by Bertin Pharma (Montigny le Bretonneux, France), at 25 μM (diluted in 95% ethanol) during the adipogenic phase from day 0 to day 8 of differentiation. The medium was changed every two days. On day 8, supernatant was removed and cells were used for triacylglycerol determination and RNA extraction. Each experiment was performed 3 times.

MiRNAs selection

For miRNAs selection as potential regulators of *cebpβ*, *cebpα*, *pparγ*, two criteria were established: a) to be validated or predicted by five algorithms (miRanda, miRDB, miRWalk, RNA22 and Targetscan algorithms) in miRWalk 2.0. database [28] and b) to be reported in Pubmed search using "miR + adipogenesis" terms (Table 1).

miRNA transfection

3T3-L1 pre-adipocytes at a confluence of approximately 80% were transfected with the DeliverX^{TM} Plus siRNA Transfection Kit (Affimetrix, Santa Clara, CA) following the manufacturer´s protocol with mirVana miRNA inhibitor mmu-miR-155-5p or mirVana miRNA inhibitor Negative Control (Applied Biosystems, Foster City, CA, USA). The final concentration of miRNA Inhibitors was established at 30 nM and the transfection period at 48 hours. These optimal conditions were determined in previous experiments carried out at 24, 48 and 72 hours in cells at different confluence statuses, and transfection efficiency was assessed using miRNA probes and fluorescent transfection controls.

At the same time, cells were stimulated to differentiate with DMEM containing 10% FCS, 10 μg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 μM dexamethasone and treated with RSV at 25 μM or ethanol 95% (Control group) during 48 hours. Afterwards, the

Table 1. miRNAs whose target genes have been predicted or validated by means of the miRWalk 2.0 or reported in the literature.

miRNA	Validated target genes	Predicted target genes (5 algorithms)	Literature Mir+adipogenesis
mmu-miR-31-5p		*cebpα*	[29, 30]
mmu-miR-101a-3p	*cebpα*		-
mmu-miR-101b-3p	*cebpα*		-
mmu-miR-124-3p	*cebpα*	*cebpα*	-
mmu-miR-130b-3p		*pparγ*	[15, 31–33]
mmu-miR-144-3p	*cebpα*		[34]
mmu-miR-155-5p	*cebpβ*		[16, 35–37]
mmu-miR-190a-5p		*cebpα*	-
mmu-miR-190b-5p		*cebpα*	-
mmu-miR-205-5p		*cebpα*	[38]
mmu-miR-224-3p		*cebpα*	[39]
mmu-miR-27a-3p	*pparγ*	*pparγ*	[40, 41]
mmu-miR-27b-3p	*pparγ*	*pparγ*	[42–46]
mmu-miR-326-3p		*cebpα*	[47]
mmu-miR-329-3p	*cebpα*		-
mmu-miR-330-5p		*cebpα*	-
mmu-miR-362-3p	*cebpα*		-
mmu-miR-466f-5p		*cebpβ*	-
mmu-miR-466i-3p	*cebpα*		-
mmu-miR-466m-3p	*cebpα*		-
mmu-miR-466o-3p	*cebpα*		-
mmu-miR-671-5p		*cebpα*	-
mmu-miR-690	*cebpα*		-

CEBP α and β: Relative CCAAT enhancer-binding protein α and β; PPARγ: peroxisome proliferator-activated receptor γ.

https://doi.org/10.1371/journal.pone.0184875.t001

supernatant was removed and cells were used to RNA and protein extraction. Each experiment was performed 3 times.

Extraction and analysis of RNA and quantification by Real Time reverse transcription-polymerase chain reaction (Real Time RT-PCR)

Total RNA sample containing small and large-size RNA from maturing pre-adipocytes was extracted with miRNeasy™ RNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Small-size RNA was used for miRNA expression analysis and large-size RNA to quantify the mRNA expression.

1.5 μg of large-size RNA of each sample was reverse-transcribed to first-strand complementary DNA (cDNA) using iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Sterol regulatory element binding transcription factor 1 (*srebf1*), krüppel-like factor 5 (*klf5*), liver x receptor α (*lxrα*) and cAMP responding element binding protein 1 (*creb1*) mRNA levels were quantified using Real-Time PCR with an iCyclerTM–MyiQTM Real-Time PCR Detection System (BioRad, Hercules, CA, USA) in the presence of SYBRGreen master mix (Applied Biosystems, Foster City, CA, USA). All sample mRNA levels were normalized to the values of 18S (Table 2).

Reverse transcription of 10 ng of small-size RNA and PCR were performed with the TaqMan® MicroRNA Assay kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). miRNA levels for miR-130b-3p, miR-155-5p, miR-27b-3p, miR-

Table 2. Primers for PCR amplification of each studied gene.

	Sense primer	Anti-sense primer
srebf1	5´ – AAATCTTGCTGCCATTCG – 3´	5´ – TTGATCCCGGAAGCTCTGTG – 3´
klf5	5´ – CCGGAGACGATCTGAAACAC – 3´	5´ – GGAGCTGAGGGGTCAGATACTT – 3´
creb1	5´ – TTTGTCCTTGCTTTCCGAAT – 3´	5´ – CACTTTGGCTGGACATCTTG – 3´
lxrα	5´ – ATCGCCTTGCTGAAGACCTCTG – 3´	5´ – GATGGGGTTGATGAACTCCACC – 3´
18s	5´ – GTGGGCCTGCGGCTTAAT – 3´	5´ – GCCAGAGTCTCGTTCGTTATC – 3´

Sterol regulatory element binding transcription factor 1 (*srebf1*); krüppel-like factor 5 (*klf5*); cAMP responding element binding protein 1 (*creb1*), liver x receptor α (*lxrα*); 18S ribosomal RNA (*18s*).

https://doi.org/10.1371/journal.pone.0184875.t002

326-3p, miR-31-5p, miR-27a-3p, miR-144-3p, miR-205-5p and miR-224-3p were quantified using TaqMan® MicroRNA Assay (Applied Biosystems, Foster City, CA, USA) for each miRNA and normalized to the values of U6 snRNA. The miRNA assay sequences were as follows:

miR-130b-3p 5′ – CAGUGCAAUGAUGAAAGGGCAU – 3′

miR-155-5p 5′ – UUAAUGCUAAUUGUGAUAGGGGU – 3’

miR-27b-3p 5′ – UUCACAGUGGCUAAGUUCUGC – 3′

miR-326-3p 5′ – CCUCUGGGCCCUUCCUCCAGU – 3′

miR-31-5p 5′ – AGGCAAGAUGCUGGCAUAGCUG – 3′

miR-27a-3p 5′ – UUCACAGUGGCUAAGUUCCGC—3′

miR-144-3p 5′ – UACAGUAUAGAUGAUGUACU—3′

miR-205-5p 5′ – UCCUUCAUUCCACCGGAGUCUG—3′

miR-224-3p 5′ – AAAUGGUGCCCUAGUGACUACA—3′

All gene and miRNA expression results were expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method [48].

After miRNA transfection assay, total RNA sample containing small and large-size RNA was extracted with miRNeasy™ RNA isolation kit (Qiagen, Hilden, Germany). Small-size RNA was used to mir-155 expression analysis and large-size RNA to quantify the mRNA expression of *cebpβ*. The expression levels of both mir-155 and *cebp*β were analyzed as explained before.

Protein expression analysis

Total protein was isolated from maturing 3T3-L1 adipocytes using 150 μL of lysis buffer (2 nM tris-HCl, 0.1 M sodium chloride (NaCl), 1% Triton, 10% glycerol, 1 mM sodium orthovanadate (OvNa), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium fluoride (FNa) and 1% protease inhibitor) and centrifuged (12.000g, 15 minutes, 4˚C) to remove membranes and other proteic residues. Protein concentration was determined by BCA protein assay kit (Thermo Scientific, Wilmington, DE, USA). Total protein (20 μg) was subjected to 10% SDS-polyacrylamide gel, electroblotted onto PVDF membranes (Millipore, Bradford, MA, USA), and incubated with polyclonal rabbit anti-*cebpβ* (1:1000) and monoclonal mouse anti-tubulin (1:5000) (Santa-Cruz Biotech, CA, USA) overnight and afterwards with polyclonal goat anti-mouse IgG-HRP for *cebpβ* (1:5000) and polyclonal goat anti-rabbit for α-tubulin (1:5000) (Santa-Cruz Biotech, CA, USA) for 2 hours. Bound antibodies were visualized by an

ECL system (Thermo Fisher Scientific Inc., Rockford, IL, USA) and quantified by Chemi-Doc MP imaging system (BioRad, CA, USA).

Statistical analysis

Results are presented as mean ± standard error of the mean. Statistical analysis was performed using SPSS 24.0 (SPSS Inc. Chicago, IL, USA). Comparisons between each treatment and the controls were analyzed by Student's *t* test. Statistical significance was set-up at the $p < 0.05$ level.

Results and discussion

As stated in the introduction section, obesity is a real problem, and functional molecules may be a new effective tool for the management of this disease. Among them, resveratrol has been demonstrated as having beneficial effects in order to face obesity in both *in vitro* and *in vivo* models. Several published *in vitro* studies conclude that this polyphenol is able to inhibit the process of adipogenesis, leading to a lower amount of differentiated adipocytes and thus to a decrease in triglyceride accumulation [49–51]. Along the same lines, we previously demonstrated that resveratrol and its glucuronide and sulfate metabolites are able to block adipogenesis and to reduce triglyceride accumulation to the same extent in 3T3-L1 maturing pre-adipocytes [25].

Adipogenesis is a complex process governed by a tightly controlled network of transcription factors that coordinate a great number of genes [52–55]. At the centre of this network there are two principal adipogenic factors, *pparγ* and *cebpα*, whose expression is regulated by other transcription factors, such as *cebpβ* [27]. In recent years, miRNAs have been described as a potential group of adipogenic controllers. Indeed, a snapshot of miRNA profiling revealed a dramatic change of 21 miRNAs during 3T3-L1 adipocyte differentiation [56]. In this line, miR-155 and miR-27b have been shown to suppress the expression of *cebpβ* and *pparγ* in adipocytes. Therefore, these miRNAs could be considered one of the mechanisms by which the adipogenic process is inhibited [16, 35, 42].

Modulation of miRNA expression by dietary compounds is increasingly being investigated by scientists working in the field of functional ingredients and their potential capacity to prevent pathologies. Indeed, some dietary polyphenols, such as curcumin, epigallocatechin gallate or resveratrol have been demonstrated to suppress different cancer cells growth by up-regulating miRNAs [57]. Resveratrol has also been linked to modifications on miRNAs expression in heart myoblasts, which could explain its cardioprotective effect. Quercetin, coffee polyphenols and grape seed proanthocyanidins can target miR-122 in mice livers and control cholesterol and bile acid synthesis and fatty acid oxidation, and thus, prevent liver steatosis [58, 59]. With regard to regulation of adipogenesis through miRNAs, Zhu *et al.* demonstrated that epigallocatequines up-regulated the expression of miR-27a and miR-27b and down-regulated that of *pparγ* and *cebpα* [60]. The same effects were found by persimmon tannin treatment during adipogenesis [43]. By contrast, it seems that nonivamide, a capsaicin analogue, increases the expression of the miRNA mmu-let-7d-5p, which has been associated with decreased *pparγ* levels [61]. Other plant or fruit extracts have been also identified as adipogenic regulators via miRNAs [62, 63].

In view of all mentioned above, and considering that miRNAs can play a crucial role in the effect attributed to dietary polyphenols, in the present study we aimed to analyze the mechanisms by which RSV and its metabolites modified the gene expression of adipogenic regulators. For this purpose we focussed on the analysis of those potential miRNA (validated or

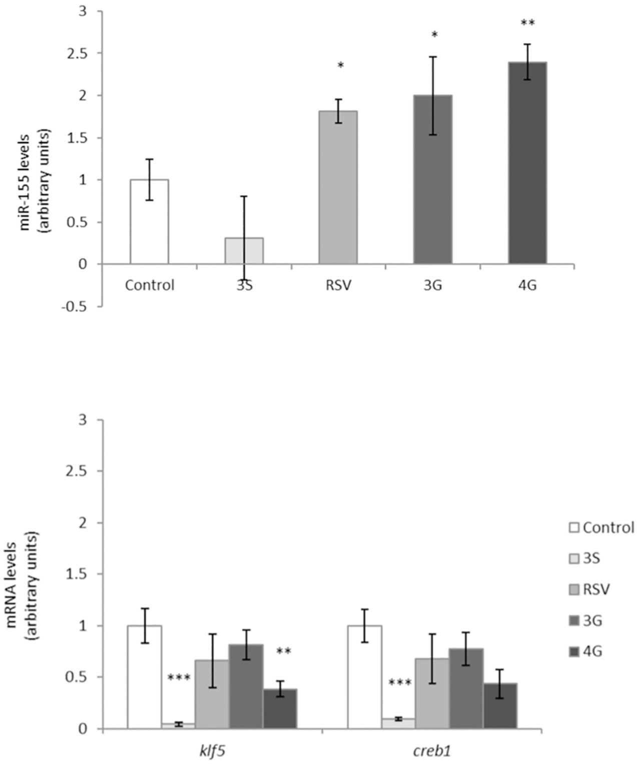


Fig 1. Effects of 25 μM of resveratrol (RSV) on the expression of mir-155 (A) and 25 μM of *trans*-resveratrol-3-O-sulfate (3S), *trans*-resveratrol-3-O-glucuronide (3G) and *trans*-resveratrol-4-O-glucuronide (4G) on *creb1* and *klf5* gene expression (B) in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8. Values are means ± SEM (Standard Error of the Mean) of three independent experiments carried out in sextuplicate. Comparisons between each treatment and the controls were analyzed by Student's *t*-test. The asterisks represent differences versus the controls ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

https://doi.org/10.1371/journal.pone.0184875.g001

predicted) targeting *pparγ*, *cebpβ* and *cebpα*, which were selected by using the miRWalk 2.0. database and a literature review.

MiR-155 and other genes that regulate the expression of *cebpβ* were measured (Table 1). RSV and the glucuronide metabolites increased miR-155 gene expression, but 3S metabolite did not (Fig 1A). These results could suggest that whereas RSV, 3G and 4G exert their effect via miR-155, 3S metabolite does not do so. In order to verify the mechanism of RSV and the glucuronide metabolites, maturing 3T3-L1 adipocytes were transfected with an anti-miR-155 compound while they were cultured in the presence or absence of RSV. After transfection, *cebpβ* gene and protein expression remained unchanged in treated cells (Fig 2), demonstrating that the polyphenol, and reportedly its glucuronide metabolites, inhibit the process of adipogenesis, at least in part, via miR-155. The modulation of miR-155 by RSV has been extensively studied in monocytes and macrophages. In these cells RSV was shown to increase miR-155 expression, to reduce the inflammatory response and to protect from atherosclerosis and hypertension [64–67]. Nevertheless, studies analyzing this regulatory pathway in adipocytes have not been carried out yet.

Taking into account that the sulfate metabolite did not exert any effect on miR-155 (Fig 1A), other regulatory routes that could lead to the reduction observed in *cebpβ* gene expression were analyzed. In the network of adipogenic transcription factors *creb1* plays a crucial role as *cebpβ* precursor [68–70]. Moreover, *klf5* is induced by *cebpβ/δ* and in turn controls *pparγ*

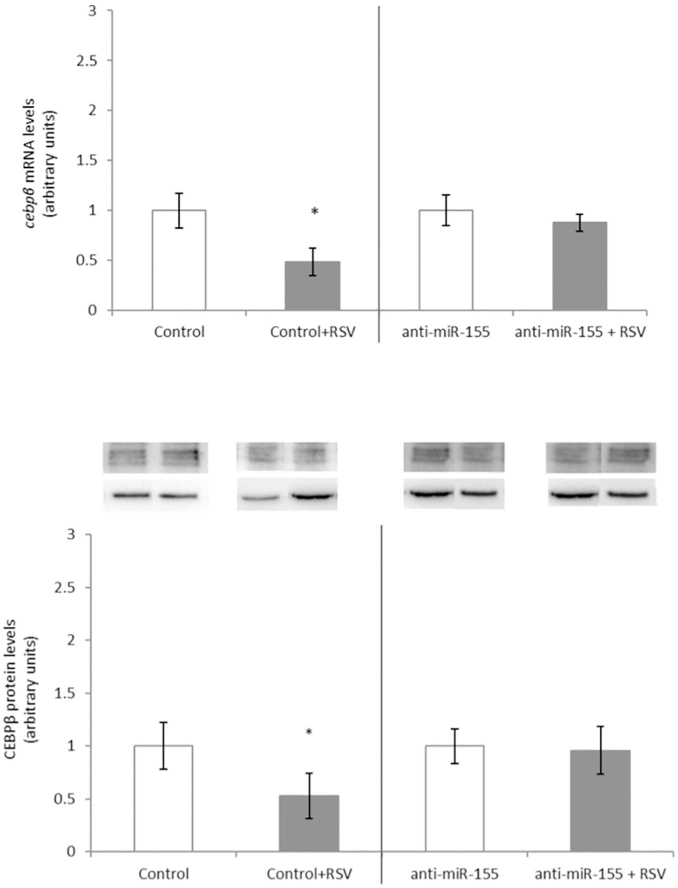


Fig 2. *cebpβ* gene (A) and protein (B) expressions after transfection with miR-155 inhibitor, with or without 25 μM RSV in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 2. Values are means ± SEM (Standard Error of the Mean) of three independent experiments carried out in triplicate. Comparisons between each treatment and the controls were analyzed by Student's *t*-test. The asterisks represent differences versus the controls ($*P < 0.05$).

https://doi.org/10.1371/journal.pone.0184875.g002

expression, thus mediating both the early and late stages of the differentiation program [71]. In the present study, 3S metabolite reduced gene expression of *creb1* and 3S and 4G that of *klf5* (Fig 1B). Therefore, it could be suggested that 3S metabolite orchestrated its effects on the initial phase of the adipogenesis in a transcriptional way, apparently without any influence of miRNA. By contrast, the 4G metabolite not only exerted its effect via miR-155, but also through *klf5*. The modulation of *cebpβ* was also observed by other polyphenols [72, 73].

MiR-27b, miR-27a and miR-130b were selected by miRWalk database (Table 1) as *pparγ* regulator. Sulfate metabolite did not change the expression of these miRNAs (Fig 3A). As in the case of *cebpβ*, other genes that are involved in the regulation of *pparγ* during adipogenesis (*srebf1* and *lxrα*) [74, 75] were analyzed. 3S metabolite reduced the expression of both genes (Fig 3B), which explains the changed induced by this metabolite in *pparγ* expression without changes in miR-27b, miR-27a and miR-130b. This fact was also observed with other anti-adipogenic pytochemicals such as apigenin [76], or black adzuki bean [77].

Finally, we set out to analyze the miRNAs related to *cebpα*. For this purpose, miR-326, miR-31, miR-144, miR-205 and miR-224 were selected as miRNAs targeting *cebpα*, according to the computational analysis and literature (Table 1). None of these miRNAs were modified by 3S treatment (Fig 4), which suggests that its mechanism of action was not via miRNAs. The

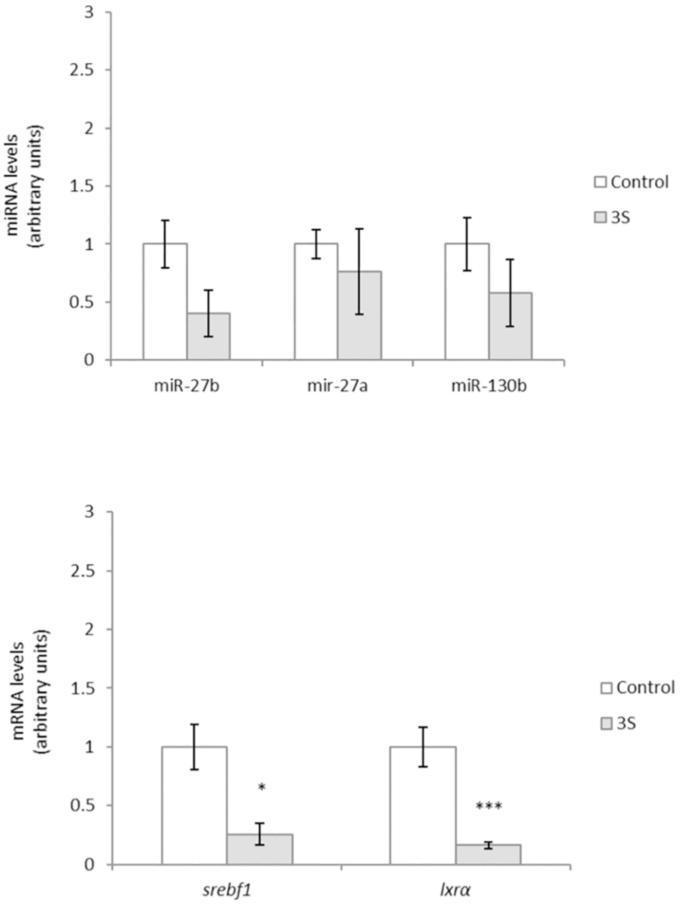


Fig 3. Effects of 25 μM of *trans*-resveratrol-3-*O*-sulfate (3S) on the expression of mir-27b, miR-27a and miR-130b (A) and on gene expression of *srebf1* and *lxrα* (B) in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8. Values are means ± SEM (Standard Error of the Mean) of three independent experiments carried out in sextuplicate. Comparisons between each treatment and the controls were analyzed by Student's *t*-test. The asterisks represent differences versus the controls (*$P < 0.05$; ***$P < 0.001$).

https://doi.org/10.1371/journal.pone.0184875.g003

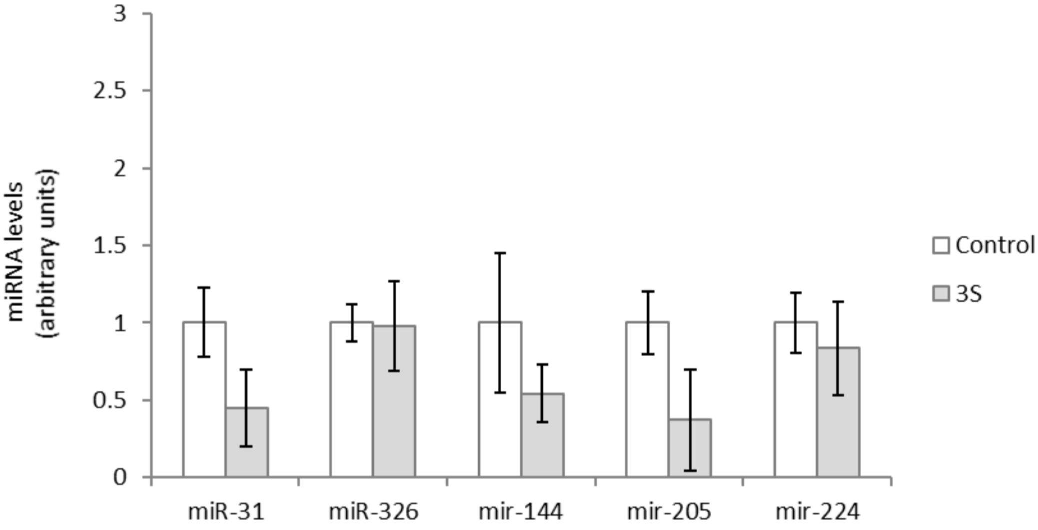
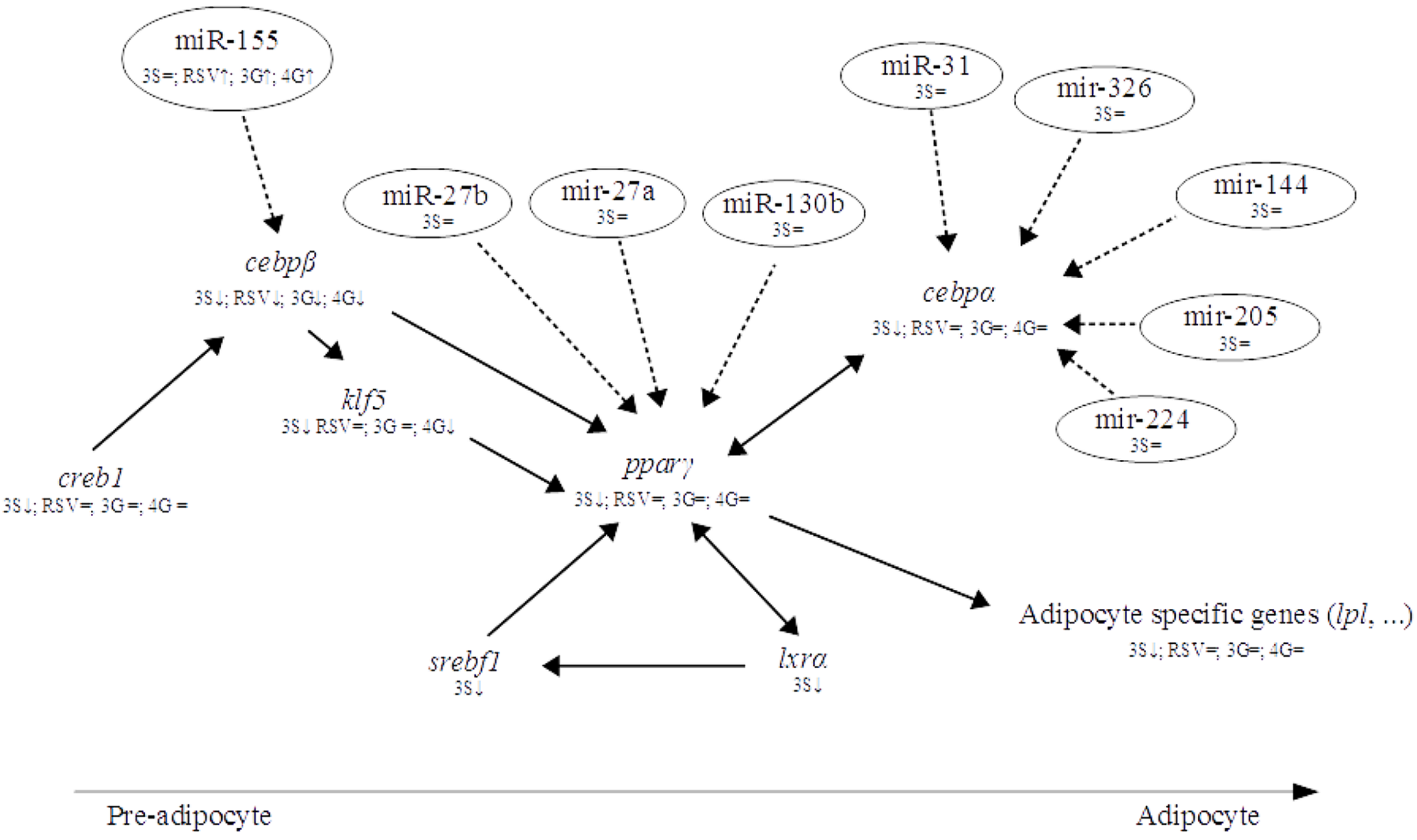


Fig 4. Effects of 25 μM of *trans*-resveratrol-3-*O*-sulfate (3S) on the expression of miR-326, miR-31, miR-144, miR-205 and miR-224 in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8. Values are means ± SEM (Standard Error of the Mean) of three independent experiments carried out in sextuplicates. Comparisons between each treatment and the controls were analyzed by Student's *t*-test.

https://doi.org/10.1371/journal.pone.0184875.g004



Modified from Farmer, SR. Cell. Metabolism 2006; 4(4): 263–273.

Fig 5. Genes and miRNAs involved in the inhibition of adipogenesis by resveratrol (RSV), *trans*-resveratrol-3-O-sulfate (3S), *trans*-resveratrol-3-O-glucuronide (3G) and *trans*-resveratrol-4-O-glucuronide (4G) in the pathways of the adipogenic process (modified from Farmer *et. al.* 2006).

https://doi.org/10.1371/journal.pone.0184875.g005

down-regulation observed by 3S on *pparγ*, can be considered itself one of the reasons for reduction in *cebpα*. These results, as a whole, suggest that 3S metabolite could exert its anti-adipogenic effect through adipogenic regulatory genes but not through miRNAs, as is the case of resveratrol and glucuronide metabolites (Fig 5).

This study presents the limitation that the experiments were performed in 3T3-L1 adipocytes. Therefore, data extrapolation to human is not completely possible. There are some differences in the metabolism and physiology of mouse and human adipogenesis such as differences in the modulation of *pparγ* [78]. However, the main species-specific differences in adipogenesis focus on when (and where) the products of the genes are made. However, the role of the master regulators is the same in both species, as far as we know. Taking into account the methodological difficulties that human adipocytes present for transfections and the heterogeneity of results in response to treatments, 3T3-L1 adipocytes were used in the present study.

Conclusions

In summary, our study clearly suggests that the inhibitory effect on adipogenesis attributed to RSV and its glucuronide metabolites (3G and 4G) in 3T3-L1 adipocytes is mediated by the up-regulation of miR-155, which in turn leads to a down-regulation of *cebpβ* gene expression. In the case of 4G, *klf5* also contributed to this regulation. By contrast, the inhibitory effect observed in cells treated with 3S metabolite was not mediated via miRNAs. In this case, changes in *creb1*, *klf5*, *srebf1* and *lxrα*, explain the effects of this metabolite on adipogenesis.

Author Contributions

Conceptualization: Itziar Eseberri, Arrate Lasa, Jonatan Miranda, Ana Gracia, Maria P. Portillo.

Formal analysis: Itziar Eseberri, Arrate Lasa, Jonatan Miranda, Ana Gracia.

Funding acquisition: Maria P. Portillo.

Investigation: Itziar Eseberri, Arrate Lasa, Jonatan Miranda, Ana Gracia.

Methodology: Itziar Eseberri, Arrate Lasa, Jonatan Miranda, Ana Gracia.

Resources: Itziar Eseberri, Arrate Lasa, Jonatan Miranda, Ana Gracia.

Supervision: Arrate Lasa, Jonatan Miranda, Maria P. Portillo.

Writing – original draft: Itziar Eseberri, Arrate Lasa, Jonatan Miranda, Maria P. Portillo.

Writing – review & editing: Itziar Eseberri, Arrate Lasa, Jonatan Miranda, Maria P. Portillo.

References

1. WHO. Obesity and overweight 2014 updated June 2016. [Available from: http://www.who.int/mediacentre/factsheets/fs311/en/] (Last acess: March 2017).
2. Zhu JG, Xia L, Ji CB, Zhang CM, Zhu GZ, Shi CM, et al. Differential DNA methylation status between human preadipocytes and mature adipocytes. Cell Biochem Biophys. 2012; 63(1):1–15. https://doi.org/10.1007/s12013-012-9336-3 PMID: 22270829
3. Fajas L, Fruchart JC, Auwerx J. Transcriptional control of adipogenesis. Curr Opin Cell Biol. 1998; 10(2):165–73. PMID: 9561840
4. Tang QQ, Jiang MS, Lane MD. Repressive effect of Sp1 on the C/EBPalpha gene promoter: role in adipocyte differentiation. Mol Cell Biol. 1999; 19(7):4855–65. PMID: 10373535
5. Cao Z, Umek RM, McKnight SL. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev. 1991; 5(9):1538–52. PMID: 1840554
6. Hilton C, Neville MJ, Karpe F. MicroRNAs in adipose tissue: their role in adipogenesis and obesity. Int J Obes (Lond). 2013; 37(3):325–32.
7. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004; 116(2):281–97. PMID: 14744438
8. Ambros V. The functions of animal microRNAs. Nature. 2004; 431(7006):350–5. https://doi.org/10.1038/nature02871 PMID: 15372042
9. Bracken CP, Scott HS, Goodall GJ. A network-biology perspective of microRNA function and dysfunction in cancer. Nat Rev Genet. 2016; 17(12):719–32. https://doi.org/10.1038/nrg.2016.134 PMID: 27795564
10. Teleman AA, Maitra S, Cohen SM. Drosophila lacking microRNA miR-278 are defective in energy homeostasis. Genes Dev. 2006; 20(4):417–22. https://doi.org/10.1101/gad.374406 PMID: 16481470
11. Krützfeldt J, Stoffel M. MicroRNAs: a new class of regulatory genes affecting metabolism. Cell Metab. 2006; 4(1):9–12. https://doi.org/10.1016/j.cmet.2006.05.009 PMID: 16814728
12. Poy MN, Spranger M, Stoffel M. microRNAs and the regulation of glucose and lipid metabolism. Diabetes Obes Metab. 2007; 9 Suppl 2:67–73.
13. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer. 2006; 6(11):857–66. https://doi.org/10.1038/nrc1997 PMID: 17060945
14. Takanabe R, Ono K, Abe Y, Takaya T, Horie T, Wada H, et al. Up-regulated expression of microRNA-143 in association with obesity in adipose tissue of mice fed high-fat diet. Biochem Biophys Res Commun. 2008; 376(4):728–32. https://doi.org/10.1016/j.bbrc.2008.09.050 PMID: 18809385

15. Lee EK, Lee MJ, Abdelmohsen K, Kim W, Kim MM, Srikantan S, et al. miR-130 suppresses adipogenesis by inhibiting peroxisome proliferator-activated receptor gamma expression. Mol Cell Biol. 2011; 31 (4):626–38. https://doi.org/10.1128/MCB.00894-10 PMID: 21135128

16. Liu S, Yang Y, Wu J. TNFα-induced up-regulation of miR-155 inhibits adipogenesis by down-regulating early adipogenic transcription factors. Biochem Biophys Res Commun. 2011; 414(3):618–24. https://doi.org/10.1016/j.bbrc.2011.09.131 PMID: 21986534

17. Del Rio D, Rodriguez-Mateos A, Spencer JP, Tognolini M, Borges G, Crozier A. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid Redox Signal. 2013; 18(14):1818–92. https://doi.org/10.1089/ars.2012.4581 PMID: 22794138

18. Asensi M, Medina I, Ortega A, Carretero J, Baño MC, Obrador E, et al. Inhibition of cancer growth by resveratrol is related to its low bioavailability. Free Radic Biol Med. 2002; 33(3):387–98. PMID: 12126761

19. Walle T, Hsieh F, DeLegge MH, Oatis JE, Walle UK. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos. 2004; 32(12):1377–82. https://doi.org/10.1124/dmd.104.000885 PMID: 15333514

20. Andrés-Lacueva C,. Urpí-Sardá M., Zamora-Ros R., Lamuela-Raventós RM. Bioavailability and metabolism of resveratrol. In: Plant Phenolics and human health: Biochemistry, Nutrition, and Pharmacology. Fraga CG, editor. New Jersey: John Wiley & Sons, Inc.; 2009.

21. Juan ME, Maijó M, Planas JM. Quantification of trans-resveratrol and its metabolites in rat plasma and tissues by HPLC. J Pharm Biomed Anal. 2010; 51(2):391–8. https://doi.org/10.1016/j.jpba.2009.03.026 PMID: 19406597

22. Azorín-Ortuño M, Yáñez-Gascón MJ, Vallejo F, Pallarés FJ, Larrosa M, Lucas R, et al. Metabolites and tissue distribution of resveratrol in the pig. Mol Nutr Food Res. 2011; 55(8):1154–68. https://doi.org/10.1002/mnfr.201100140 PMID: 21710561

23. Szkudelska K, Szkudelski T. Resveratrol, obesity and diabetes. Eur J Pharmacol. 2010; 635(1–3):1–8. https://doi.org/10.1016/j.ejphar.2010.02.054 PMID: 20303945

24. Aguirre L, Fernández-Quintela A, Arias N, Portillo MP. Resveratrol: anti-obesity mechanisms of action. Molecules. 2014; 19(11):18632–55. https://doi.org/10.3390/molecules191118632 PMID: 25405284

25. Lasa A, Churruca I, Eseberri I, Andrés-Lacueva C, Portillo MP. Delipidating effect of resveratrol metabolites in 3T3-L1 adipocytes. Mol Nutr Food Res. 2012; 56(10):1559–68. https://doi.org/10.1002/mnfr.201100772 PMID: 22945685

26. Phuah NH, Nagoor NH. Regulation of microRNAs by natural agents: new strategies in cancer therapies. Biomed Res Int. 2014; 2014:804510. https://doi.org/10.1155/2014/804510 PMID: 25254214

27. Tomé-Carneiro J, Larrosa M, Yáñez-Gascón MJ, Dávalos A, Gil-Zamorano J, Gonzálvez M, et al. One-year supplementation with a grape extract containing resveratrol modulates inflammatory-related microRNAs and cytokines expression in peripheral blood mononuclear cells of type 2 diabetes and hypertensive patients with coronary artery disease. Pharmacol Res. 2013; 72:69–82. https://doi.org/10.1016/j.phrs.2013.03.011 PMID: 23557933

28. Dweep H, Gretz, N. miRWalk2.0: a comprehensive atlas of microRNA-target interactions 2015 [Available from: http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/] (Last acces: June 2017).

29. Martin PJ, Haren N, Ghali O, Clabaut A, Chauveau C, Hardouin P, et al. Adipogenic RNAs are transferred in osteoblasts via bone marrow adipocytes-derived extracellular vesicles (EVs). BMC Cell Biol. 2015; 16:10. https://doi.org/10.1186/s12860-015-0057-5 PMID: 25887582

30. Sun F, Wang J, Pan Q, Yu Y, Zhang Y, Wan Y, et al. Characterization of function and regulation of miR-24-1 and miR-31. Biochem Biophys Res Commun. 2009; 380(3):660–5. https://doi.org/10.1016/j.bbrc.2009.01.161 PMID: 19285018

31. Chen Z, Luo J, Ma L, Wang H, Cao W, Xu H, et al. MiR130b-Regulation of PPARγ Coactivator- 1α Suppresses Fat Metabolism in Goat Mammary Epithelial Cells. PLoS One. 2015; 10(11):e0142809. https://doi.org/10.1371/journal.pone.0142809 PMID: 26579707

32. Liu L, Liu H, Chen M, Ren S, Cheng P, Zhang H. miR-301b~miR-130b-PPARγ axis underlies the adipogenic capacity of mesenchymal stem cells with different tissue origins. Sci Rep. 2017; 7(1):1160. https://doi.org/10.1038/s41598-017-01294-2 PMID: 28442776

33. Pan S, Yang X, Jia Y, Li R, Zhao R. Microvesicle-shuttled miR-130b reduces fat deposition in recipient primary cultured porcine adipocytes by inhibiting PPAR-g expression. J Cell Physiol. 2014; 229(5):631–9. https://doi.org/10.1002/jcp.24486 PMID: 24311275

34. Tao C, Huang S, Wang Y, Wei G, Zhang Y, Qi D, et al. Changes in white and brown adipose tissue microRNA expression in cold-induced mice. Biochem Biophys Res Commun. 2015; 463(3):193–9. https://doi.org/10.1016/j.bbrc.2015.05.014 PMID: 25983326

35. Chen Y, Siegel F, Kipschull S, Haas B, Fröhlich H, Meister G, et al. miR-155 regulates differentiation of brown and beige adipocytes via a bistable circuit. Nat Commun. 2013; 4:1769. https://doi.org/10.1038/ncomms2742 PMID: 23612310

36. Gaudet AD, Fonken LK, Gushchina LV, Aubrecht TG, Maurya SK, Periasamy M, et al. miR-155 Deletion in Female Mice Prevents Diet-Induced Obesity. Sci Rep. 2016; 6:22862. https://doi.org/10.1038/srep22862 PMID: 26953132

37. Pang WJ, Lin LG, Xiong Y, Wei N, Wang Y, Shen QW, et al. Knockdown of PU.1 AS lncRNA inhibits adipogenesis through enhancing PU.1 mRNA translation. J Cell Biochem. 2013; 114(11):2500–12. https://doi.org/10.1002/jcb.24595 PMID: 23749759

38. Yu J, Chen Y, Qin L, Cheng L, Ren G, Cong P, et al. Effect of miR-205 on 3T3-L1 preadipocyte differentiation through targeting to glycogen synthase kinase 3 beta. Biotechnol Lett. 2014; 36(6):1233–43. https://doi.org/10.1007/s10529-014-1491-8 PMID: 24563321

39. Peng Y, Xiang H, Chen C, Zheng R, Chai J, Peng J, et al. MiR-224 impairs adipocyte early differentiation and regulates fatty acid metabolism. Int J Biochem Cell Biol. 2013; 45(8):1585–93. https://doi.org/10.1016/j.biocel.2013.04.029 PMID: 23665235

40. Kim SY, Kim AY, Lee HW, Son YH, Lee GY, Lee JW, et al. miR-27a is a negative regulator of adipocyte differentiation via suppressing PPARgamma expression. Biochem Biophys Res Commun. 2010; 392(3):323–8. https://doi.org/10.1016/j.bbrc.2010.01.012 PMID: 20060380

41. Wang T, Li M, Guan J, Li P, Wang H, Guo Y, et al. MicroRNAs miR-27a and miR-143 regulate porcine adipocyte lipid metabolism. Int J Mol Sci. 2011; 12(11):7950–9. https://doi.org/10.3390/ijms12117950 PMID: 22174642

42. Karbiener M, Fischer C, Nowitsch S, Opriessnig P, Papak C, Ailhaud G, et al. microRNA miR-27b impairs human adipocyte differentiation and targets PPARgamma. Biochem Biophys Res Commun. 2009; 390(2):247–51. https://doi.org/10.1016/j.bbrc.2009.09.098 PMID: 19800867

43. Zou B, Ge Z, Zhu W, Xu Z, Li C. Persimmon tannin represses 3T3-L1 preadipocyte differentiation via up-regulating expression of miR-27 and down-regulating expression of peroxisome proliferator-activated receptor-γ in the early phase of adipogenesis. Eur J Nutr. 2015; 54(8):1333–43. https://doi.org/10.1007/s00394-014-0814-9 PMID: 25510894

44. Chan LS, Yue PY, Kok TW, Keung MH, Mak NK, Wong RN. Ginsenoside-Rb1 promotes adipogenesis through regulation of PPARγ and microRNA-27b. Horm Metab Res. 2012; 44(11):819–24. https://doi.org/10.1055/s-0032-1321909 PMID: 22893262

45. Gan CC, Ni TW, Yu Y, Qin N, Chen Y, Jin MN, et al. Flavonoid derivative (Fla-CN) inhibited adipocyte differentiation via activating AMPK and up-regulating microRNA-27 in 3T3-L1 cells. Eur J Pharmacol. 2017; 797:45–52. https://doi.org/10.1016/j.ejphar.2017.01.009 PMID: 28088385

46. Marques AP, Rosmaninho-Salgado J, Estrada M, Cortez V, Nobre RJ, Cavadas C. Hypoxia mimetic induces lipid accumulation through mitochondrial dysfunction and stimulates autophagy in murine preadipocyte cell line. Biochim Biophys Acta. 2017; 1861(3):673–82. https://doi.org/10.1016/j.bbagen.2016.12.005 PMID: 27939617

47. Tang YF, Zhang Y, Li XY, Li C, Tian W, Liu L. Expression of miR-31, miR-125b-5p, and miR-326 in the adipogenic differentiation process of adipose-derived stem cells. OMICS. 2009; 13(4):331–6. https://doi.org/10.1089/omi.2009.0017 PMID: 19422302

48. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25(4):402–8. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609

49. Rayalam S, Yang JY, Ambati S, Della-Fera MA, Baile CA. Resveratrol induces apoptosis and inhibits adipogenesis in 3T3-L1 adipocytes. Phytother Res. 2008; 22(10):1367–71. https://doi.org/10.1002/ptr.2503 PMID: 18688788

50. Yang JY, Della-Fera MA, Rayalam S, Ambati S, Hartzell DL, Park HJ, et al. Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. Life Sci. 2008; 82(19–20):1032–9. https://doi.org/10.1016/j.lfs.2008.03.003 PMID: 18433793

51. Chen S, Li Z, Li W, Shan Z, Zhu W. Resveratrol inhibits cell differentiation in 3T3-L1 adipocytes via activation of AMPK. Can J Physiol Pharmacol. 2011; 89(11):793–9. https://doi.org/10.1139/Y11-077 PMID: 22017765

52. Otto TC, Lane MD. Adipose development: from stem cell to adipocyte. Crit Rev Biochem Mol Biol. 2005; 40(4):229–42. https://doi.org/10.1080/10409230591008189 PMID: 16126487

53. Farmer SR. Transcriptional control of adipocyte formation. Cell Metab. 2006; 4(4):263–73. https://doi.org/10.1016/j.cmet.2006.07.001 PMID: 17011499

54. Rosen ED, MacDougald OA. Adipocyte differentiation from the inside out. Nat Rev Mol Cell Biol. 2006; 7(12):885–96. https://doi.org/10.1038/nrm2066 PMID: 17139329

55. Rankinen T, Zuberi A, Chagnon YC, Weisnagel SJ, Argyropoulos G, Walts B, et al. The human obesity gene map: the 2005 update. Obesity (Silver Spring). 2006; 14(4):529–644.

56. Kajimoto K, Naraba H, Iwai N. MicroRNA and 3T3-L1 pre-adipocyte differentiation. RNA. 2006; 12 (9):1626–32. https://doi.org/10.1261/rna.7228806 PMID: 16870994

57. Lançon A, Michaille JJ, Latruffe N. Effects of dietary phytophenols on the expression of microRNAs involved in mammalian cell homeostasis. J Sci Food Agric. 2013; 93(13):3155–64. https://doi.org/10.1002/jsfa.6228 PMID: 23674481

58. Kobori M, Masumoto S, Akimoto Y, Oike H. Chronic dietary intake of quercetin alleviates hepatic fat accumulation associated with consumption of a Western-style diet in C57/BL6J mice. Mol Nutr Food Res. 2011; 55(4):530–40. https://doi.org/10.1002/mnfr.201000392 PMID: 21462320

59. Murase T, Misawa K, Minegishi Y, Aoki M, Ominami H, Suzuki Y, et al. Coffee polyphenols suppress diet-induced body fat accumulation by downregulating SREBP-1c and related molecules in C57BL/6J mice. Am J Physiol Endocrinol Metab. 2011; 300(1):E122–33. https://doi.org/10.1152/ajpendo.00441.2010 PMID: 20943752

60. Zhu W, Zou B, Nie R, Zhang Y, Li CM. A-type ECG and EGCG dimers disturb the structure of 3T3-L1 cell membrane and strongly inhibit its differentiation by targeting peroxisome proliferator-activated receptor γ with miR-27 involved mechanism. J Nutr Biochem. 2015; 26(11):1124–35. https://doi.org/10.1016/j.jnutbio.2015.05.006 PMID: 26145192

61. Rohm B, Holik AK, Kretschy N, Somoza MM, Ley JP, Widder S, et al. Nonivamide enhances miRNA let-7d expression and decreases adipogenesis PPARγ expression in 3T3-L1 cells. J Cell Biochem. 2015; 116(6):1153–63. https://doi.org/10.1002/jcb.25052 PMID: 25704235

62. Stefanon B, Pomari E, Colitti M. Effects of Rosmarinus officinalis extract on human primary omental pre-adipocytes and adipocytes. Exp Biol Med (Maywood). 2015; 240(7):884–95.

63. Zhang J, Huang Y, Shao H, Bi Q, Chen J, Ye Z. Grape seed procyanidin B2 inhibits adipogenesis of 3T3-L1 cells by targeting peroxisome proliferator-activated receptor γ with miR-483-5p involved mechanism. Biomed Pharmacother. 2016; 86:292–6. https://doi.org/10.1016/j.biopha.2016.12.019 PMID: 28011376

64. Sonkoly E, Pivarcsi A. microRNAs in inflammation. Int Rev Immunol. 2009; 28(6):535–61. https://doi.org/10.3109/08830180903208303 PMID: 19954362

65. Kawai Y, Nishikawa T, Shiba Y, Saito S, Murota K, Shibata N, et al. Macrophage as a target of quercetin glucuronides in human atherosclerotic arteries: implication in the anti-atherosclerotic mechanism of dietary flavonoids. J Biol Chem. 2008; 283(14):9424–34. https://doi.org/10.1074/jbc.M706571200 PMID: 18199750

66. Kawai Y. Immunochemical detection of food-derived polyphenols in the aorta: macrophages as a major target underlying the anti-atherosclerotic activity of polyphenols. Biosci Biotechnol Biochem. 2011; 75 (4):609–17. https://doi.org/10.1271/bbb.100785 PMID: 21512255

67. Ma C, Wang Y, Shen A, Cai W. Resveratrol upregulates SOCS1 production by lipopolysaccharide-stimulated RAW264.7 macrophages by inhibiting miR-155. Int J Mol Med. 2017; 39(1):231–7. https://doi.org/10.3892/ijmm.2016.2802 PMID: 28004106

68. Son YH, Ka S, Kim AY, Kim JB. Regulation of Adipocyte Differentiation via MicroRNAs. Endocrinol Metab (Seoul). 2014; 29(2):122–35.

69. Siersbæk R, Nielsen R, Mandrup S. Transcriptional networks and chromatin remodeling controlling adipogenesis. Trends Endocrinol Metab. 2012; 23(2):56–64. https://doi.org/10.1016/j.tem.2011.10.001 PMID: 22079269

70. Zhang JW, Klemm DJ, Vinson C, Lane MD. Role of CREB in transcriptional regulation of CCAAT/enhancer-binding protein beta gene during adipogenesis. J Biol Chem. 2004; 279(6):4471–8. https://doi.org/10.1074/jbc.M311327200 PMID: 14593102

71. Oishi Y, Manabe I, Tobe K, Tsushima K, Shindo T, Fujiu K, et al. Krüppel-like transcription factor KLF5 is a key regulator of adipocyte differentiation. Cell Metab. 2005; 1(1):27–39. https://doi.org/10.1016/j.cmet.2004.11.005 PMID: 16054042

72. Eseberri I, Miranda J, Lasa A, Churruca I, Portillo MP. Doses of Quercetin in the Range of Serum Concentrations Exert Delipidating Effects in 3T3-L1 Preadipocytes by Acting on Different Stages of Adipogenesis, but Not in Mature Adipocytes. Oxid Med Cell Longev. 2015; 2015:480943. https://doi.org/10.1155/2015/480943 PMID: 26180590

73. Zhang T, Yamamoto N, Yamashita Y, Ashida H. The chalcones cardamonin and flavokawain B inhibit the differentiation of preadipocytes to adipocytes by activating ERK. Arch Biochem Biophys. 2014; 554:44–54. https://doi.org/10.1016/j.abb.2014.05.008 PMID: 24845100

74. Yoshikawa T, Shimano H, Amemiya-Kudo M, Yahagi N, Hasty AH, Matsuzaka T, et al. Identification of liver X receptor-retinoid X receptor as an activator of the sterol regulatory element-binding protein 1c

gene promoter. Mol Cell Biol. 2001; 21(9):2991–3000. https://doi.org/10.1128/MCB.21.9.2991-3000.2001 PMID: 11287605

75. Seo JB, Moon HM, Kim WS, Lee YS, Jeong HW, Yoo EJ, et al. Activated liver X receptors stimulate adipocyte differentiation through induction of peroxisome proliferator-activated receptor gamma expression. Mol Cell Biol. 2004; 24(8):3430–44. https://doi.org/10.1128/MCB.24.8.3430-3444.2004 PMID: 15060163

76. Kim MA, Kang K, Lee HJ, Kim M, Kim CY, Nho CW. Apigenin isolated from Daphne genkwa Siebold et Zucc. inhibits 3T3-L1 preadipocyte differentiation through a modulation of mitotic clonal expansion. Life Sci. 2014; 101(1–2):64–72. https://doi.org/10.1016/j.lfs.2014.02.012 PMID: 24582594

77. Kim M, Park JE, Song SB, Cha YS. Effects of black adzuki bean (Vigna angularis) extract on proliferation and differentiation of 3T3-L1 preadipocytes into mature adipocytes. Nutrients. 2015; 7(1):277–92. https://doi.org/10.3390/nu7010277 PMID: 25569623

78. Lindroos J, Husa J, Mitterer G, Haschemi A, Rauscher S, Haas R, et al. Human but not mouse adipogenesis is critically dependent on LMO3. Cell Metab. 2013; 18(1):62–74. https://doi.org/10.1016/j.cmet.2013.05.020 PMID: 23823477