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Ellagic acid inhibits adipocyte differentiation through coactivator-associated arginine methyltransferase 1-mediated chromatin modification☆

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Abstract

Chromatin remodeling is a key mechanism in adipocyte differentiation. However, it is unknown whether dietary polyphenols are epigenetic effectors for adiposity control. Ellagic acid (EA) is a naturally occurring polyphenol in numerous fruits and vegetables. Recently, EA-containing foods have been reported to reduce adiposity. In the present study, we sought to determine whether EA inhibits adipogenesis by modifying chromatin remodeling in human adipogenic stem cells (*h*ASCs). qPCR microarray of chromatin modification enzymes revealed that 10 μmol/L of EA significantly inhibits histone deacetylase (HDAC)9 down-regulation. In addition, EA was associated with up-regulation of HDAC activity and a marked reduction of histone acetylation levels. However, chemical inhibition of HDAC activity or depletion of HDAC9 by *si*RNA were not sufficient to reverse the antiadipogenic effects of EA. Intriguingly, EA treatment was also associated with reduced histone 3 arginine 17 methylation levels (H3R17me2), implying the inhibitory role of EA in coactivator-associated arginine methyltransferase 1 (CARM)1 activity during adipogenesis. Boosting CARM1 activity by delivering cell-penetrating peptides of CARM1 not only recovered H3R17me2 but also restored adipogenesis evidenced by H3 acetylation at lysine 9, HDAC9 down-regulation, PPARγ expression and triglyceride accumulation. Taken together, our data suggest that reduced CARM1 activity by EA results in a decrease of H3R17me2 levels, which may interrupt consecutive histone remodeling steps for adipocyte differentiation including histone acetylation and HDAC9 dissociation from chromatin. Our work provides the mechanistic insights into how EA, a polyphenol ubiquitously found in fruits and vegetables, attenuates human adipocyte differentiation by altering chromatin remodeling.
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Keywords: Ellagic acid; Adipogenesis; Chromatin histone modification; CARM1; HDAC9

1. Introduction

Epigenetic modification refers to the inheritable changes of gene expression in the absence of a change in the DNA sequence itself. Epigenetic modification comprises DNA methylation in CpG islands, covalent modification of histone tails and noncoding microRNA-mediated gene silencing [1–6]. In particular, histone modification is a key mechanism in the switching on and off of genes for differentiation; *N*-terminal tails of H3 and H4 interact with the negatively charged DNA backbone in unmodified states. Histone-modifying enzymes target specific amino acids of histones, producing changes in acetylation, methylation, phosphorylation or ubiquitination status. Modifications of these histone codes alter chromatin conformation, which subsequently induce dissociation of transcriptional (co) repressors as well as recruitment of transcriptional (co)activators [7–10]. In general, histone acetylation on lysine residues decreases the chromatin compactness, increases accessibility to genes and thereby induces transcriptional activation. Several transcriptional coactivators possess histone acetyltransferase (HAT) activity to transfer acetyl groups to lysine residues in histones, promoting conformational change in euchromatin structure [11–14]. In contrast, transcriptional co-repressors often possess Histone deacetylase (HDAC) activity to remove acetyl moieties from histone tails, leading to less accessible heterochromatin conformation [15,16]. Regulation of transcription by histone methylation is more complex than by histone acetylation. Histone methylation can be correlated with either gene activation or repression depending on histone residues (lysine or arginine), specific genetic loci or distinctive methylation pattern (*e.g.*, asymmetric or symmetric) [17–21].

A growing body of literature has revealed that epigenetic regulation is a key mechanism for adipocyte differentiation. Although considered controversial, an increase in global histone acetylation is preceded by adipocyte differentiation as the consequence of decreased HDAC activity [22]. More specifically, H3 acetylation at lysine 9 (H3K9Ac) and H3 methylation at lysine 4 (H3K4me2) have been implicated for positive regulation of adipocyte differentiation [23]. The obligatory suppression of Wnt signaling is also regulated by chromatin modification via H3 lysine 27 (H3K27Ac vs. H3K27me3) [24]. Several histone modification enzymes, that is, protein arginine methyltransferase 4 [also known as *coactivator-associated arginine*

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methyltransferase 1 (CARM1)] [25], PRMT5 [26], histone methyltransferase G9a [27] and HDAC9 [28], have been identified as either positive or negative regulators for adipocyte differentiation. Moreover, recent advances in chromatin immunoprecipitation methodology have revealed that activation of transcriptional cascade networks during early adipogenesis coincides with the regulation of histone modification of key transcription factors such as peroxisome proliferator-activated receptor gamma (PPARγ) [25]. These studies have clearly demonstrated that chromatin remodeling dictates adipocyte differentiation.

However, less information is available whether environmental effectors are able to reprogram epigenetic codes for adipocyte differentiation. Interestingly, accumulating evidence suggests that our daily diet is an important epigenetic determinant regulating obesity. Exposure to an High fat (HF)-diet early in life can alter chromatin structure, leading to an increased risk of obesity in adulthood [29–31]. Conversely, consumption of fruits and vegetables (FV) is inversely associated with obesity [32]. FV-mediated weight loss is due to decreased energy-density/increased satiety by high-fiber intake, and enhanced energy metabolism probably by a variety of phytochemicals exists in FV [33]. However, it is largely unknown whether epigenetic regulation could be a viable mechanism to explain the reduced adiposity by FV consumption. It is plausible to hypothesize that FV contain principle phytochemicals that can modulate the activity of chromatin-modifying enzymes, thereby reducing adiposity. Recently, our group has reported that supplementation of muscadine grape polyphenols (MGP) decreased visceral obesity and obesity-mediated metabolic complications compared to muscadine wine polyphenols (MWP) [34]. In that study, we noticed that ellagic acid (EA) was the major compositional difference between MGP and MWP (18.2 vs. 1.1 mg/g extract, respectively) because EA was removed during the typical wine-making procedures by filtration. It led us to hypothesize that EA regulates adiposity probably by altering epigenetic marks for adipogenesis. Here, we present evidence that EA, a polyphenol commonly found in many FV, attenuates adipocyte differentiation by modulating histone arginine methylation and subsequent histone acetylation levels.

2. Materials and methods

2.1. Materials

All cell culture dishes were purchased from Fisher Scientific. Fetal bovine serum was purchased from Cellgro. Rosiglitazone (BRL49653) was purchased from Cayman Chemical. All other chemicals and reagents were purchased from Sigma Chemical Co., unless otherwise stated.

2.2. Preparation of human adipogenic stem cells (hASCs) and adipogenic differentiation

Abdominal adipose tissue was obtained from females with a body mass index of ~30 during liposuction or abdominal plastic surgeries. Isolation of *h*ASCs and differentiation of adipocytes were conducted as described by Skurk *et al.* [35]. All protocols and procedures were approved by the Institutional Review Board (#693-2011) at the University of Florida. After removing initial monocytic cells (selective adherence to plastic), the released stromal vascular (SV) fractions were passaged down no more than three times. These adipogenic stem cell rich SV fractions are regarded as *h*ASCs without further purification procedures [36]. A pool of *h*ASCs from three or four different human subjects was used for each experiment to avoid individual variation. Conditions for *h*ASCs proliferation and differentiation were described previously [37]. EA (E2250, Sigma) stock was prepared in dimethyl sulfoxide (DMSO); aliquots of stock (10 mmol/L) were kept at −20°C and freshly diluted at the time of addition to *h*ASCs. For the treatment of EA, confluent cultures of *h*ASCs (d0 preadipocyte) were induced to differentiate in the presence of 10 μmol/L of EA (final in 0.1% DMSO) in human adipocyte medium AM-1 (ZenBio) plus differentiation cocktail for 3 days (d1-d3 adipocyte). Upon 4 days of differentiation (d4), medium was changed with AM-1 plus fresh EA (10 μmol/L) without differentiation cocktail. Since then, medium was replenished with fresh addition of EA. The cultures of human adipocytes were harvested on d4 or d7 based upon experimental design. All EA treatments were paralleled with vehicle controls (0.1% DMSO). The presence of intracellular lipid accumulation was visualized by oil red-O (ORO) staining.

2.3. qPCR and microarray analysis

Gene-specific primers for real-time PCR (qPCR) were obtained from Integrated DNA Technologies (Chicago, IL, USA). Total mRNA of *h*ASCs was isolated using Trizol reagent (Invitrogen). To remove genomic DNA contamination, mRNA was treated with DNase (Mediatech); 2 μg of mRNA was converted into cDNA in a total volume of 20 μL (iScript cDNA synthesis kit, Bio-Rad). Gene expression was determined by qPCR (CFX96, Bio-Rad), and relative gene expression was normalized by 36B4. The complete gene lists can be found in Supplemental Table 1. For PCR microarray analysis, RT^2 profiler PCR array for human epigenetic chromatin modification enzymes (QIAGEN, PAHS-085Z) was used according to the manufacture's protocol. For each group, pools of equal amounts of total mRNA provided from four different human subjects were used. The results were analyzed using software provided by QIAGEN (http://www.sabiosciences.com/pcrarray data analysis.php#Excel).

2.4. Western blot analysis

To prepare total cell lysates, monolayers of differentiated cultures of human adipocytes were harvested with ice-cold Radioimmunoprecipitation assay buffer (RIPA) buffer (Thermo Scientific) with protease and phosphatase inhibitors (Sigma). For nuclear extract preparation, NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific) was used according to the manufacturer's protocol. Proteins were fractionated onto 4–15% precasted Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDA-PAGE) (Biorad), transferred to PVDF membranes with a semidry transfer unit (Hoefer TE77X) and incubated with the relevant antibodies. Chemiluminescence from Enhanced chemiluminescence (ECL) solution (Western Lightning) was detected with FluorChem E (Cell Biosciences) imaging system. Polyclonal or monoclonal antibodies targeting to β-actin (4967), H3K9Ac (AcH3, 9649), HDAC1 (5356), HDAC2 (5113), HDAC3 (3949), HDAC4 (7628), HDAC5 (2082), HDAC6 (7558), AcH4 (2594), H3K27Ac (4353), H4 (2935), lamin A/C (4777) and CARM1 (3379) were purchased from Cell Signaling Technology. Antibodies to HDAC9 (ab 59718) and histone H3 (ab1791) were purchased from Abcam. PPARγ (sc-7273) and FABP (aP2, sc-271529) were purchased from Santa Cruz Biotechnology. The polyclonal antibody for detecting H3R17me2 (NB21-1132) was purchased from Novus Biotechnology.

2.5. HDAC enzyme activity assays

Total cellular histone deacetylase enzymatic activity was measured using a commercial HDAC assay kit (Upstate Biotechnology) according to the manufacturer's protocol. Briefly, 30 μg of nuclear lysate were incubated with fluorescent substrate in HDAC assay buffer for 45 min at 30°C. An activator solution was added to release the fluorophore from the deacetylated substrates, and fluorescence was measured in a multichannel fluorometer (Synergy H1, Biotech).

2.6. Depletion of HDAC9 using siRNA

For silencing HDAC9, *h*ASCs were seeded at confluent density and allowed to attach for 24 h in a proliferation medium. Culture of *h*ASCs were transfected with either 200 nmol/L of human HDAC9 ON-TARGET plus SMART pool *si*RNA (Thermo Scientific) or 200-nmol/L nontargeting control *si*RNA (Thermo Scientific) using DharmaFECT1 transfection reagent according to the manufacturer's protocol. The transfection efficiency was determined by Cy3-tagged *si*GLO (Thermo Scientific). After 48 h of transfection, *h*ASCs were stimulated for differentiation for 3 days in the presence and absence of EA (Fig. 3A).

2.7. Immunocytochemistry of H3R17me2 and HDAC9

*h*ASCs were cultured onto coverslips and immunostained as described previously [38]. Briefly, cells were fixed with 3.7% paraformaldehyde for 20 min. After quenching paraformaldehyde with glycine, coverslips were permeabilized with ice-cold Triton X-100 (0.1%) and blocked with 1.25-mg/ml normal goat serum for 1 h. The coverslips were incubated overnight with 1:100 dilution of the antibodies of H3R17me2 (ab8284) and HDAC9 (ab18970) antibody (Abcam) at 4°C, followed by incubation for 1 h with a 1:300 dilution of rhodamine red-conjugated goat antirabbit IgG (Jackson Immunoresearch). Fluorescent images were captured using a digital inverted fluorescence EVOS microscope (AMG Inc.). 4′,6-diamidino-2-phenylindole (DAPI) staining was used for counter staining of the nucleus.

2.8. Cell permeable peptide-CARM1 (CPP-CARM1)

Purified CPP-CARM1 was a generous gift from Dr. Dong Ryul Lee at the CHA University in South Korea [39]. CPP-CARM1 (2 μg/ml) was delivered to *h*ASCs 24 h prior to adipogenic stimulation with or without EA. This allows sufficient time for CPP-CARM1 to translocate into the nucleus before *h*ASCs are exposed to EA. Every 2 days, fresh CPP-CARM1 was added during routine media changes.

2.9. Statistics

All data are presented as the mean±S.E.M. The data were statistically analyzed using Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests. All analyses were performed with GraphPad Prism 5 (Version 5.04).

3. Results

3.1. EA alters HDAC9 expression and HDAC activity during adipocyte differentiation

Recently, we and others have reported that supplementation with EA-containing foods is associated with reduced adiposity [34,40,41]. To gain an insight into whether EA regulates epigenetic factors of adipogenesis, we performed qPCR microarrays for chromatin modification enzymes using *h*ASCs. Among the 84 genes that regulate chromatin accessibility to genomic DNA or histones (by altering the status of acetylation, methylation, phosphorylation or ubiquitination), 10 genes were up-regulated (>twofold) by EA treatment without any specific genes being down-regulated significantly (<twofold) (Fig. 1A, also see Supplemental Table 2). In particular, HDAC9 gene expression levels were ~twentyfold higher than that of vehicle control. To validate the array results, HDAC gene expressions were measured using individual gene-specific primers. As we expected, no difference was found in Class I (HDAC 1, 2, 3, 4 and 8) or Class III HDAC genes between EA-treated and control human adipocyte samples. In parallel to results from the qPCR array, HDAC9 gene expression was specifically higher in EA-treated adipocytes compared to vehicle controls among the Class II HDAC genes (HDAC 5, 7, 9 and 10). Interestingly, for HDAC11, a Class IV HDAC enzyme, mRNA levels were also significantly higher than control (Fig. 1B). HDAC9 protein expression was higher in EA-treated nuclear fraction, while other HDAC protein levels were similar between the two groups (Fig. 1C).

Next, we examined whether EA also alters HDAC activity and histone acetylation levels. There was a <50% reduction of global HDAC activity during the early differentiation period (4 days after exposure to differentiation stimuli), which was almost completely dampened in cultures grown with EA (Fig. 2A). In the presence of 100 nmol/L trichostatin A (TSA), a pan-HDAC inhibitor, there was an additional decrease of HDAC activity in the nuclear extract of differentiated cultures; only ~10% of HDAC activity remained in comparison to undifferentiated *h*ASCs (Fig. 2B, the second bar). In contrast, EA-treated nuclear extracts still possessed 50% of HDAC activity in the presence of TSA (Fig. 2B, the last bar). Consistent with the literature [22,24], differentiation of *h*ASCs significantly increased acetylation levels of H3K9Ac, H3K27Ac as well as AcH4 (Fig. 2C, left panel). Intriguingly, differentiation of *h*ASCs with EA remarkably decreased histone acetylation levels (Fig. 2C, right panel). To answer the question of whether the inhibition of HDAC activity could reverse the inhibitory effects of EA on adipogenesis, TSA was added to the *h*ASCs along with EA. Consistent with results from Chatterjee *et al.* [28], the addition of TSA during the adipocyte differentiation procedures did not inhibit adipogenesis (Fig. 2D, upper panel). Similarly, addition of TSA to EA-treated cultures during adipocyte differentiation failed to restore both adipocyte morphology (Fig. 2D) and PPARγ expression (Fig. 2E). Notably, HDAC9 gene expression was even higher with TSA treatment, suggesting that HDAC9 expression is not regulated by TSA-sensitive HDAC activity (Fig. 2E). These results collectively demonstrated that (a) EA inhibited down-regulation of HDAC activity, presumably the TSA-insensitive portion; and (b) chemical inhibition of HDAC activity by TSA was unable to reverse EA-mediated HDAC9 expression as well as inhibition of adipogenesis.

3.2. Silencing of HDAC9 is not sufficient to reverse the reduction of adipocyte differentiation by EA

It has been shown that HDAC9 is a transcriptional co-repressor of adipogenesis by preventing the activation of C/EBPα [28]. Our next question was whether the knockdown of HDAC9 could reverse the anti-adipogenic effects of EA. To address this question, we used *si*RNA to deplete HDAC9. Transfection efficiency of *h*ASCs was >90% estimated by Cy3-tagged nontargeting *si*GLO (data not shown). To knockdown HDAC9, 200 nmol/L of *si*Cont (nontargeting) or *si*HDAC9 were transfected with *h*ASCs for 48 h followed by adipogenic differentiation for 72 h (Fig. 3A). Transfection of *si*HDAC9 attenuated HDAC9 gene expression approximately ~70% compared to *si*Cont (Fig. 3B), which remained constant throughout the experiment (data not shown). Reduction of HDAC9 protein levels in the nucleus by *si*HDAC9 was comparable to *si*Cont transfected cells without EA (Fig. 3C). Surprisingly, a substantial decrease of HDAC9 by *si*HDAC9 had minimal impact on EA-mediated suppression of adipogenic gene expression, C/EBPα and PPARγ or on H3K9 acetylation (Fig. 3B, C). These data showed that the reduction of HDAC9 was unable to reverse the inhibition of adipogenesis by EA. In addition, this implicates that additional regulatory factor(s) might be involved in the suppression of adipocyte differentiation by EA other than HDAC9 regulation *per se*.

3.3. Inhibition of CARM1 by EA plays a key role in suppressing adipogenesis

Recently, EA has been identified as a novel inhibitor for CARM1 [42], whose activity is required for asymmetric transfer of two methyl groups to the H3R17me2 (Fig. 4A). Consistently, immunostaining of differentiated human adipocyte cultures (heterogeneous culture containing ~50% adipocytes) with an H3R17me2 antibody showed that CARM1 activity is restricted to lipid-laden adipocytes but not in undifferentiated *h*ASCs (Fig. 4B). To further determine whether inhibition of CARM1 activity by EA would be a key mechanism to block *h*ASCs differentiation, we examined the H3R17me2 levels

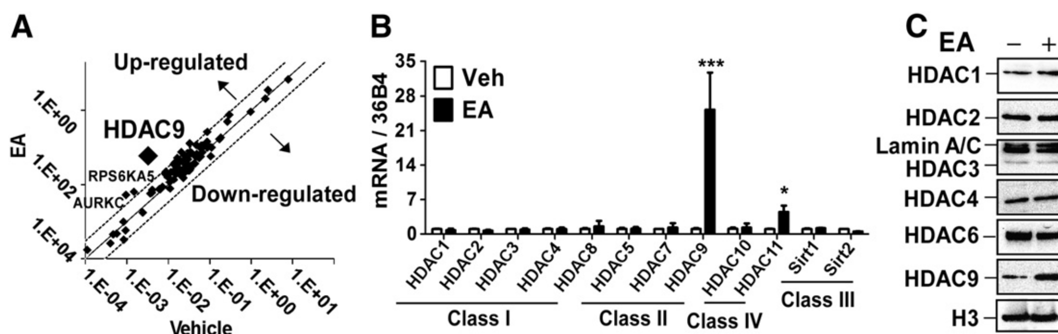

Fig. 1. EA alters HDAC9 expression during adipocyte differentiation. Cultures of *h*ASCs were induced to differentiation in the presence of 10-μmol/L EA or DMSO (vehicle) for 7 days. (A) Microarray analysis of human chromatin modification genes (84 genes) by qPCR from the *h*ASCs treated with either 10-μmol/L EA or vehicle for 7 days during differentiation. Pooled mRNA from four different human subjects was used for analysis. Broken lines indicate twofold expression of differences between treatments. (B) Gene expression levels of Class I, II and IV of HDAC and sirt1 and 2 (Class III) by qPCR. (C) Protein expression levels of HDAC 1, 2, 3, 4, 6 and 9 in nuclear extract. H3 and lamin A/C were used for loading control. *P<0.05, *** P<0.001 by Student's *t* test.

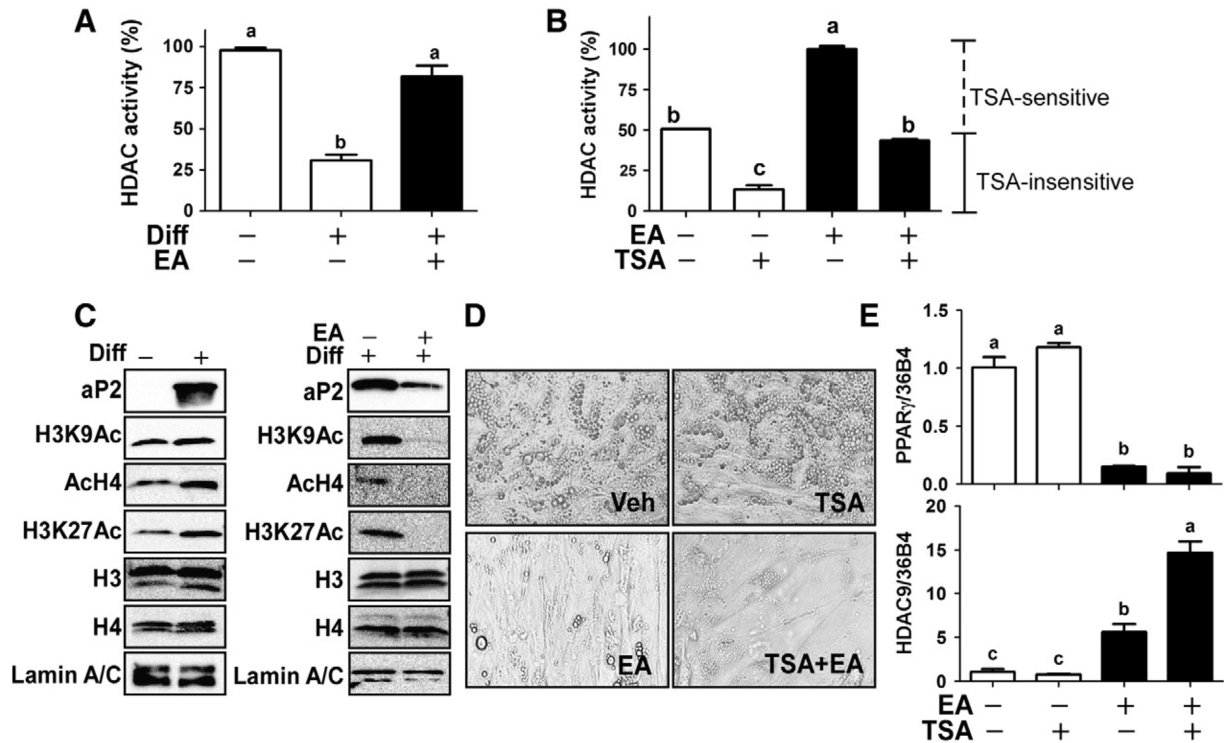

Fig. 2. EA alters HDAC activity during adipocyte differentiation. (A) Nuclear HDAC enzyme activity in undifferentiated (Diff −) and differentiated (Diff +) adipocytes in the presence or absence of EA for 4 days. (B) HDAC activity with or without pan-HDAC inhibitor TSA (100 nmol/L). (C) Western blot analysis for detecting H3K9Ac and H3K27Ac and AcH4. H3, H4 and lamin A/C were used for loading control and aP2 used as an adipocyte marker. (D) Phase contrast images of *h*ASCs differentiated with either EA or TSA only, or cotreatment of TSA+EA for 7 days. (E) PPARγ and HDAC9 gene expression grown in the presence or absence of TSA and EA. All values are presented as the mean±S.E.M. Means without a common letter differ (a>b>c, $P<0.05$) by one-way ANOVA.

during differentiation with or without EA incubation. Supporting the important role of CARM1 activity in adipocyte differentiation, EA treatment significantly reduced H3R17me2 levels compared to vehicle control from the nuclear extract fraction used in Fig. 1C (Fig. 4C). However, there was no significant difference in mRNA or protein levels of CARM1 (Fig. 4C, D), suggesting that EA inhibits enzyme activity of CARM1 rather than by transcriptional or translational modification of CARM1.

If the inhibition of CARM1 activity by EA is the major mechanism to block the adipogenesis, the replenishment of CARM1 activity can rescue adipocyte differentiation. To test this concept, 2 μg/ml of recombinant cell-penetrating peptide-CARM1 (CPP-CARM1) [39] were added to *h*ASCs throughout the adipogenic differentiation with 10-μmol/L EA. Although adipocyte morphology was not completely restored, addition of CPP-CARM1 substantially increased triglyceride (TG) accumulation compared to EA-only treatment assessed by ORO staining (Fig. 5A). Accordingly, costimulation of CPP-CARM1 with EA significantly increased PPARγ gene and protein expression compared to EA treatment alone (Fig. 5B, C). The restoration of CARM1 activity by adding CPP-CARM1 also increased H3R17me2 and H3K9 acetylation

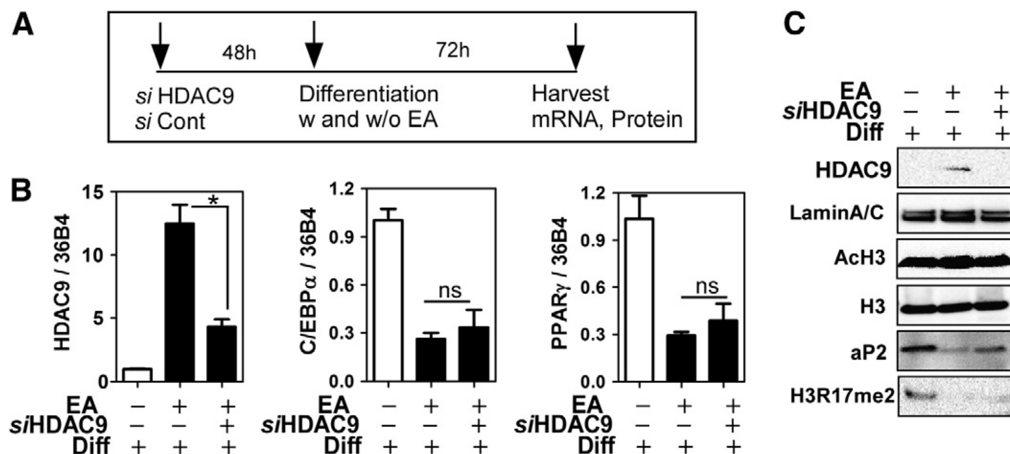

Fig. 3. Depletion of HDAC9 in *h*ASCs has minimal impact on EA-mediated inhibition of adipogenesis. (A) Experimental scheme for depletion of HDAC9 before adipogenic differentiation with or without EA. *h*ASCs were transfected with *si*Cont or *si*HDAC9 at 48 h before differentiation. Differentiated cultures were kept for 3 days before harvest of mRNA and protein after differentiation. (B) Relative gene expression of HDAC9, C/EBPα and PPARγ by qPCR analysis. (C) Protein levels of HDAC9, AcH3, H3R17me2, aP2, lamin A/C and total H3 in *si*Cont or *si*HDAC9 transfected cells. All values are presented as the mean±S.E.M. $^{*}P<0.05$ by one-way ANOVA.

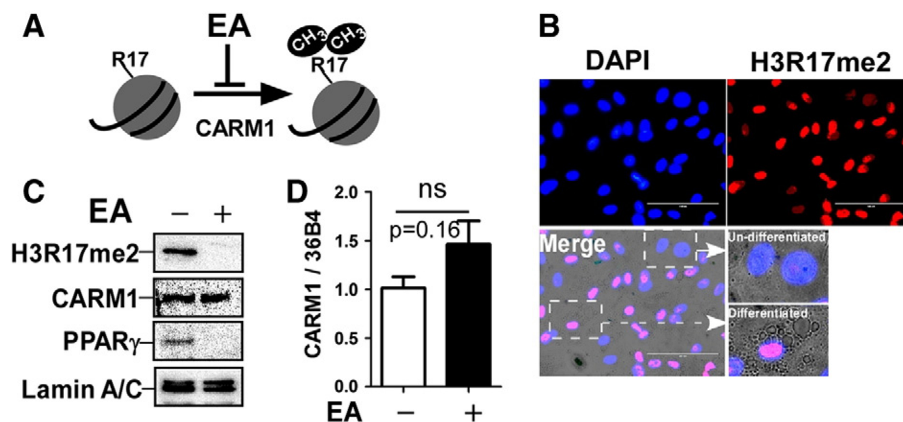

Fig. 4. EA inhibits H3R17me2 without affecting CARM1 expression levels. (A) EA inhibits CARM1-mediated methylation of H3 arginine 17 in adipocytes. (B) Immunolocalization of H3R17me2 (red) and DAPI (blue). Differentiated *h*ASCs were immunostained with H3R17me2 antibody, and the nuclei were counterstained by DAPI. Phase contrast image (40X) was overlapped to distinguish lipid loaded adipocytes versus undifferentiated cells. (C) Inhibition of H3R17me2 by EA without changes of CARM1 protein levels. Lamin A/C was used for loading control and PPARγ used as an adipocyte marker. (D) CARM1 gene expression measured by qPCR. Data are presented as the mean±S.E.M. ns=not significant by Student's *t* test.

levels (Fig. 5C). Moreover, immunostaining of HDAC9 revealed that the addition of CPP-CARM1 reduced EA-mediated retention of HDAC9 in nucleus (Fig. 5D). Taken together, these data strongly suggest that inhibition of H3R17me2 by EA is the key step to repress the subsequent H3K9 acetylation, HDAC9 dissociation from chromatin and PPARγ activation.

4. Discussion

White adipose tissue is not only a storage organ for surplus energy but is also an active endocrine tissue critical in energy and glucose homeostasis [43–45]. The metabolic and endocrine function of adipocytes correlates to the dynamics of adipocytes, that is, adipocyte size and numbers [46]. Plasticity of the adipocytes seems to be dictated by chromatin remodeling and transcriptional networks in response to environmental effectors such as diet [47,48]. Currently, little is known about the regulatory role of dietary polyphenols on epigenetic remodeling in adipocytes. The goal of this study was to identify potential links between dietary EA and epigenetic regulation of adipogenesis. We demonstrated that EA, a ubiquitous polyphenol in FV, inhibits adipocyte differentiation through CARM1-mediated epigenetic modification. Based upon our results, we propose the following working model (Fig. 6): uncommitted *h*ASCs are associated with high levels of HDAC9 that repress transcriptional activation of adipogenic genes [28]. Upon adipogenic stimuli, CARM1 enzyme facilitates the transfer of two methyl moieties to H3R17, which is accompanied by a subsequent H3K9 acetylation and HDAC9 dissociation. In the presence of EA, the inhibited CARM1 activity by EA results in the suppression of H3R17 methylation, which in turn abolishes H3K9 acetylation and HDAC9 dissociation, and ultimately represses adipogenesis.

Extensive research from several groups has identified that histone-modifying enzymes play pivotal roles in adipocyte development: (a) deletion of histone methyl-transferase enhancer of zeste homolog (Ezh2) abolished trimethylation on H3K27 of Wnt promoter region, resulting in constitutive activation of Wnt signaling and transcriptional inhibition of adipogenesis [24]; (b) silencing of PRMT5 repressed adipogenic gene expression, which was reversed by PRMT5 overexpression [26]; (c) histone methyltransferase G9a seemed to play dual roles for turning on or off adipogenic signaling based on its methylation sites by serving as a coactivator or co-repressor [49]; (d) Class II HDACs have been reported to control PPARγ signaling [50]. Among the Class II HDACs, HDAC9 has been identified as a unique transcriptional co-repressor on C/EBPα promoter [28]; and (e) mice born with the deletion of CARM1 lacked in fat pad development [51,52] identifying the adipose-specific role of CARM1 as a coactivator for PPARγ [25].

Despite accumulated evidence demonstrating the critical roles of histone-modifying enzymes in adipogenesis, few studies have identified the specific effectors that directly alter histone reprogramming by modulating histone modifying enzymes. In this study, we have identified that EA alters at least three distinctive epigenetic factors during adipogenesis of *h*ASCs.

The first modification that we immediately noticed was the abnormally high expression of HDAC9 via qPCR microarray of histone-modifying enzymes (Fig. 1). However, an increase of HDAC9 levels did not seem to be a major cause for EA-mediated inhibitory effects on adipogenesis due to the following reasons: (a) depletion of HDAC9 up to ~70% had minimal effects on adipogenesis (Fig. 3) suggesting the existence of anti-adipogenic regulatory factor(s) that could occur ahead of HDAC9 regulation; and (b) if HDAC9-mediated HDAC activity is the key mechanism to inhibit adipogenesis, inhibition of HDAC9 activity by TSA (it has been shown that HDAC9 activity is inhibited by TSA treatment [22]) should restore adipogenic potential, which was not the case in our experiment (Fig. 2D, E). These results are consistent with the conclusion from Chatterjee *et al.* [28] demonstrating that HDAC9 represses the adipogenic transcription factor in a deacetylase-independent mechanism. Based on our observations, EA seems to cause an earlier modification before the HDAC9 dissociation step (HDAC9 down-regulation is necessary but not sufficient to initiate adipogenesis).

The second modification that we noticed was decreased histone acetylation levels and increased HDAC activity by EA (Fig. 2A, C). The role of histone acetylation on adipogenesis seems to be inconsistent; inhibition of HDAC activity by TSA inhibited adipogenesis in 3 T3-L1 cells [22], while it failed to inhibit adipogenesis in primary adipogenic precursor cells in mice and humans [28]. One thing we confirmed was that TSA-sensitive HDAC activity is not required for adipogenesis at least in *h*ASCs ([28], our data Fig. 2D, E). Thus, the down-regulation of the TSA-insensitive portion of HDAC activity might be critical to initiate adipogenic differentiation. This is based upon our data showing that EA treatment during adipogenesis almost completely blocked the down-regulation of HDAC activity (Fig. 2A); and that cotreatment of EA and TSA decreased the only TSA-sensitive HDAC activity without promoting adipogenesis (Fig. 2B). Conversely, it indicates that the down-regulation of TSA-insensitive HDAC activity

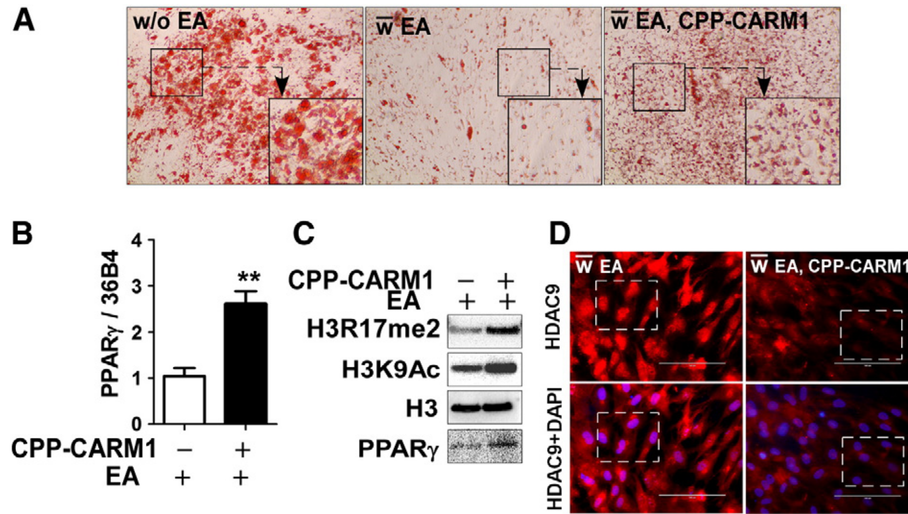

Fig. 5. Rescue of CARM1 activity by delivering CPP-CARM1 reverses EA-mediated adipogenesis of *h*ASCs in part.CPP-CARM1 was delivered to *h*ASCs at 24 h prior to adipogenic differentiation then differentiation was induced in the presence or absence of EA for 7 days. (A) TG accumulation was visualized by ORO staining. Black boxes show magnified images. (B) PPARγ gene expression by qPCR. (C) Protein levels of H3R17me2, H3K9Ac, H3 and PPARγ by Western blot analysis. (D) Immunostaining of HDAC9 was merged with DAPI staining to show decreased HDAC9 levels in CPP-CARM1-added cultures. Data are presented as the mean±S.E.M., $^{**}P$<0.001 by Student's *t* test.

may be required for adipogenesis. Intriguingly, we did not find any evidence that EA directly alters HDAC or HAT activity (data not shown). This is also consistent with the report from Selvi *et al.* [42]. Although the mechanistic link between EA treatment and "HDAC/HAT activity and histone acetylation status" is uncertain, our results suggest that EA may inhibit earlier signals that could lead to global histone acetylation for facilitation of adipogenesis.

The third and the most fundamental epigenetic modification that we have identified was the attenuation of H3R17me2 levels by EA, due to reduced CARM1 activity (Fig. 4). Yadav *et al.* have established the role of CARM1 as a PPARγ coactivator in adipose tissue [25], and Selvi *et al.* have reported the general effects of EA on CARM1 enzyme [42]. However, our work is the first to report that EA inhibits asymmetric dimethylation of H3R17 during adipogenic differentiation in *h*ASCs by linking CARM1 activity to the anti-adipogenic effects of EA. It was unexpected to find that HDAC9 depletion could not restore the H3R17me2 in the presence of EA (Fig. 3). This implies that the modification of CARM1 activity may precede the dissociation of transcriptional repressor HDAC9. It is important to note that regaining CARM1 activity by adding CPP-CARM1 recovered HDAC9 dissociation from the nucleus, histone acetylation, adipogenic gene expression and TG accumulation (Fig. 4). These data clearly demonstrated that the modulation of CARM1 by EA is a key mechanism to inhibit successive epigenetic modification for adipocyte differentiation, that is, releasing transcriptional (co)repressors. The exact mechanistic regulations collaboratively control methylation on H3R17, releasing HDAC9 from chromatin (probably from PPAR response elements peroxisome proliferator-activated receptor response element (PPRE)), and acetylation of histone are currently unknown. A recent work by Wu *et al.* demonstrated that arginine methylation on H3R17 and H3R26 by CARM1 is associated with discharging the transcriptional co-repressor NuRD, a nucleosome remodeling and the deacetylase complex, by facilitating histone acetylation in mouse embryonic fibroblast (MEF) cells [53]. This study supports our proposed model (Fig. 6) in terms of connecting CARM1-mediated histone arginine methylation to the dissociation of HDAC activity-possessing transcriptional repressors and augmentation of histone acetylation.

Although our proposed model has built upon the data obtained from human adipogenic progenitor cells (*h*ASCs), there are some limitations.

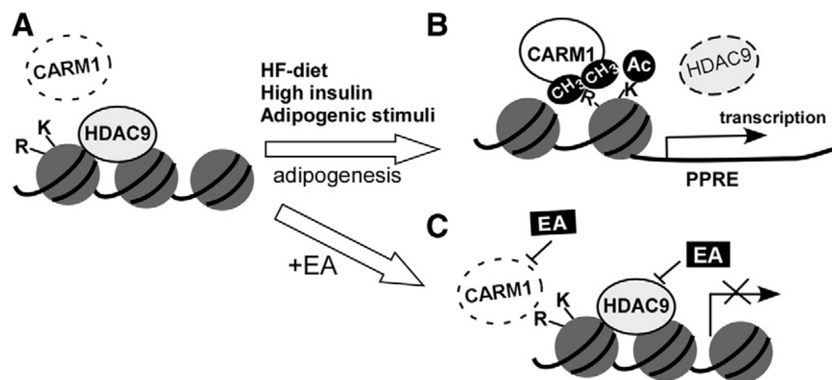

Fig. 6. Epigenetic modification of adipogenesis by EA through the mechanism involved in CARM1 inhibition. A working model illustrating the mechanism by which EA inhibits adipogenesis in *h*ASCs. (A) Uncommitted *h*ASCs are associated with high levels of HDAC9, a transcriptional co-repressor of adipogenic genes. (B) Upon adipogenic stimuli (including HF diet and high insulin), CARM1 enzyme facilitates the transfer of two methyl moieties to H3 arginine 17 sites (H3R17me2), which subsequently increases histone acetylation and HDAC9 dissociation from chromatin. (C) In the presence of EA, EA inhibits CARM1 activity, which blocks subsequent epigenetic modification, resulting in transcription inactivation of adipogenic genes.

The primary reason that we used 10 μmol/L of EA (3 μg in 1 ml of medium) was to mimic our previous *in vivo* experiment [34]. As we fed the 0.4% of MGP (18.2-mg EA/g dry extracts) to mice, which provided ~288 μg of EA per day based upon 4 g/day food intake. We also assumed that 1% of EA may be absorbed in intestinal lumen, and the total blood volume of mouse would be approximately ~1 ml, which would be roughly 2.8 μg/ml EA in blood (equivalent to ~10 μmol/L). Secondly, choosing the lowest end of the EA concentration based on 10–50 μmol/L range of EA has been routinely used for cellular studies without cytotoxicity in numerous literature. Unfortunately, EA seems to reach maximally ~1 μmol/L in serum due to low aqueous solubility and rapid metabolism by gut microbes [54]. Despite the obvious discrepancy between *in vivo* and *in vitro* study regarding effective EA concentrations, we believe our study with *h*ASCs would provide mechanistic insights into EA-mediated epigenetic modification of adiposity in humans, which is difficult to achieve through *in vivo* experiment setup. More research about the optimal EA concentration to exhibit physiological effectiveness *in vivo* is warranted. Our current study does not include information about the metabolites of EA. There is emerging evidence that urolithin A, EA-derived gut microbial metabolite, exerts various health benefits [55,56]. We are under investigation whether urolithin A is also proficient in modulating epigenetic factors that are proposed in this study. To investigate nutritional significance of EA *in vivo*, we are currently conducting animal studies by feeding HF diet with or without EA supplementation. In addition, to establish the adipose tissue-specific role of CARM1 on metabolic syndrome, we are under preparation to generate adipocyte-specific knockout mice of CARM1.

In conclusion, our present study provides mechanism-based evidence that EA attenuates adipogenesis and offers novel insights into targeting epigenetic modification for adipogenesis control using a dietary EA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2014.04.008.

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