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RESEARCH ARTICLE

SPECIAL ISSUE: CELL BIOLOGY OF LIPIDS

ADAR1 inhibits adipogenesis and obesity by interacting with Dicer to promote the maturation of miR-155-5P

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ABSTRACT

Adipogenesis is closely related to various metabolic diseases, such as obesity, type 2 diabetes, cardiovascular diseases and cancer. This cellular process is highly dependent on the expression and sequential activation of a diverse group of transcription factors. Here, we report that ADAR1 (also known as ADAR) could inhibit adipogenesis through binding with Dicer (also known as DICER1), resulting in enhanced production of miR-155-5p, which downregulates the adipogenic early transcription factor C/EBPβ. Consequently, the expression levels of late-stage adipogenic transcription factors (C/EBPα and PPARγ) are reduced and adipogenesis is inhibited. More importantly, *in vivo* studies reveal that overexpression of ADAR1 suppresses white adipose tissue expansion in high fat diet-induced obese mice, leading to improved metabolic phenotypes, such as insulin sensitivity and glucose tolerance.

KEY WORDS: ADAR1, Adipogenic differentiation, Obesity, miR-155-5P

INTRODUCTION

Adipose tissue plays a vital part in regulating energy balance (Fernández-Verdejo et al., 2019; Xu et al., 2018; Yoneshiro et al., 2019). Accumulating evidence has suggested that adipocyte dysfunction is associated with the development of whole-body insulin resistance and pathogenesis of many metabolic disorders, such as obesity and type 2 diabetes (Friedman, 2000; Kahn et al., 2019; Kusminski et al., 2016; Ian et al., 2018). Hence, it is of great importance to gain a comprehensive understanding of the maturation and function of adipocytes to find effective ways to treat of obesity.

Precursor adipocytes become mature adipocytes under hormonal and metabolite stimulation, a process termed adipogenesis (Gregoire et al., 1998). It is regulated by many transcription factors (TFs), including CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ (PPARγ) (Rosen and MacDougald, 2006; Spiegelman, 1998; Mota et al., 2017). During the early stage of adipogenesis, C/EBPβ and C/EBPδ are activated, which increases the expression of late stage TFs such as C/EBPα and PPARγ, thereby upregulating the expression of downstream adipocyte-specific genes and promoting the final differentiation of precursor adipocytes into mature adipocytes (Farmer, 2006; FitzGerald et al., 2011; Sarjeant and Stephens, 2012; Siersbaek et al., 2012). Therefore, any factor that can affect the expression of early TFs can enhance or inhibit adipogenesis by indirectly affecting the expression of late TFs.

In vertebrates, the adenosine deaminases acting on RNA (ADAR) family contains ADAR1–ADAR3 (also known as ADAR, ADARB1 and ADARB2, respectively). They all have common double-stranded RNA-binding domains and deaminase domains (Desterro et al., 2003). ADAR1 was the first adenosine deaminase found to act on double-stranded RNAs that can convert adenosine into inosine (Hood and Emeson, 2012; Ramaswami et al., 2012). Such adenosine to inosine editing in the protein-coding region will lead to the production of new proteins that do not exist in the original genome (Nishikura, 2006). However, Ota et al. have recently shown that ADAR1 can interact with Dicer (also known as DICER1) to promote the maturation of miRNA (Ota et al., 2013). This novel finding that ADAR1 can function to interfere with RNA processing, independent of the deaminase function of ADAR1, provides the new implication that this protein could regulate numerous biological processes. Abnormal expression of ADAR1 has been noted in many diseases, such as cancer, autoimmune diseases, virus infection and nervous system abnormalities (Gacem et al., 2020; Ishizuka et al., 2019; Rice et al., 2012; Vogel et al., 2020; Behm et al., 2017). However, the role of ADAR1 in adipocyte differentiation is still unclear.

MicroRNAs are non-coding RNAs with 18–25 nucleotides. They can achieve post-transcriptional inhibition by inhibiting the translation of target mRNAs or promoting their degradation (Mello and Conte, 2004; Meister and Tuschl, 2004; Pfeifer and Lehmann, 2010). Accumulating evidence has shown that microRNAs play critical roles in the process of obesity and adipocyte differentiation (Jordan et al., 2011; Li et al., 2015; Shi et al., 2016; Trajkovski et al., 2011; Wang et al., 2020). Current research on miRNA 155 (miR-155) has focused on its roles in hematopoiesis, immune response, metabolism, inflammation and tumor formation (Alivernini et al., 2018; Faraoni et al., 2009). Nonetheless, Liu et al. have found that miR-155-5p can suppress adipogenesis by binding to C/EBPβ (Liu et al., 2011). In this study, we found that ADAR1 can bind with Dicer to accelerate the processing of miR-155-5p, which downregulates the adipogenic early transcription factor C/EBPβ, thereby inhibiting adipocyte differentiation. More importantly, upregulation of ADAR1 *in vivo* by adenovirus alleviates obesity caused by high-fat diet (HFD) by suppressing adipocyte expansion, so as to alleviate metabolic abnormalities.

RESULTS

ADAR1 expression is reduced in adipose tissue of obese mice

Given that ADAR1 expression levels are changed in various diseases (Gacem et al., 2020; Ishizuka et al., 2019;

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Rice et al., 2012; Vogel et al., 2020; Behm et al., 2017), we decided to measure the *Adar1* gene expression profile in mice adipose tissue.

Analysis with quantitative real-time RT-PCR (qRT-PCR) revealed that *Adar1* has the highest expression in the brain, and relatively high expression in subcutaneous adipose tissue, spleen and kidney (Fig. S1E). Importantly, both mRNA and protein levels of ADAR1 were downregulated significantly in inguinal white adipose tissue (iWAT) from obese mice (HFD feeding for 12 weeks), when compared to lean mice fed with a chow diet (CD) (Fig. 1A,B). Such reduction in ADAR1 gene and protein expression was also observed in brown adipose tissue (BAT) but not in epididymal white adipose tissue (eWAT) of HFD mice (Fig. 1C,D; Fig. S1A,B). Using the genetically obese mouse model (DB/DB), we also noticed gene and protein downregulation of ADAR1 in iWAT and BAT, but not in eWAT, when comparing to normal wild-type (WT) mice (Fig. 1E–H; Fig. S1C,D). Altogether, our data show that ADAR1 is of great importance for adipose tissue.

The expression of ADAR1 declines during the time course of adipogenic differentiation

To determine how *Adar1* gene expression changes during adipocyte differentiation, mouse embryonic fibroblasts (MEFs) were cultured with an induced differentiation medium, which is supplemented with insulin, rosiglitazone, dexamethasone and 3-isobutyl-1-methylxanthine (IBMX). As shown in Fig. 2A, the results of Oil Red O staining at different time points [day (D)0, D4 and D8] indicate that the differentiation process is successful. As expected, the mRNA expression of adipocyte marker genes (*Adipoq*, *Cebpa*, *Fabp4* and *Pparg*) increased gradually during the differentiation process (Fig. 2B–E). However, ADAR1 mRNA and protein levels were decreased as MEFs differentiate into mature adipocytes (Fig. 2F,G). Interestingly, this reduction of ADAR1 gene/protein levels was also accompanied by increased expression of proteins that promotes adipogenesis (i.e. C/EBPα, PPARγ and Fabp4). Together, the above experimental data indicate that ADAR1 might play an important role in adipogenesis by controlling the expression of key TFs.

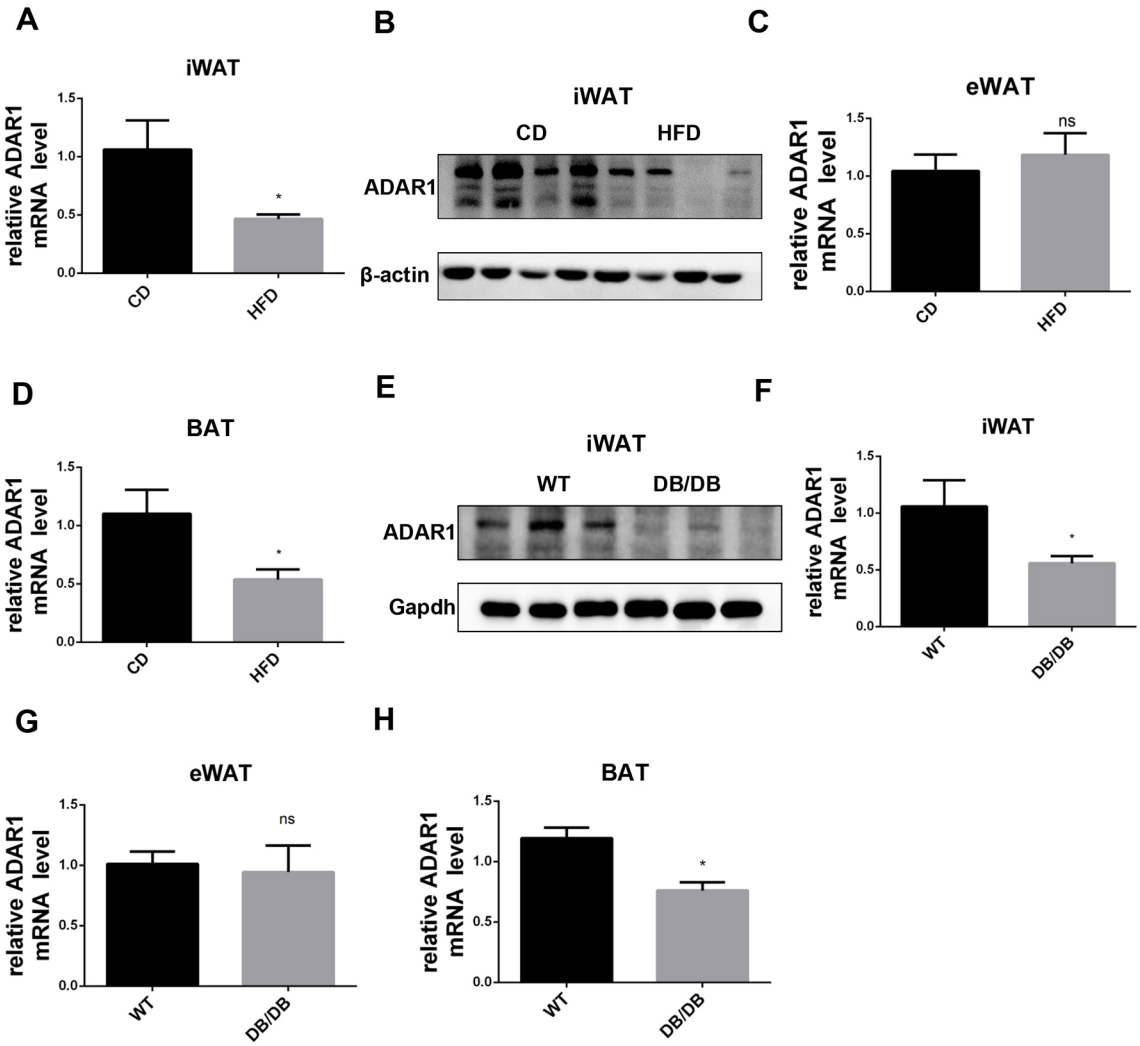

Fig. 1. ADAR1 expression decreased in the adipose tissue of obese mice. (A,B) qRT-PCR and western blot analyses of ADAR1 in iWAT of mice on chow diet and HFD (n=5). (C,D) qRT-PCR analyses of ADAR1 in eWAT and BAT of mice on chow diet and HFD (n=5). (E,F) The protein and mRNA levels of ADAR1 in iWAT of mice on WT and DB/DB mice (n=4). (G,H) The qPCR analyses of ADAR1 in the epididymal and brown adipose tissue of mice on wild-type and DB/DB mice (n=4). Data are presented as mean±s.e.m. *P<0.05; ns, not significant (unpaired two-tailed Student's t-test).

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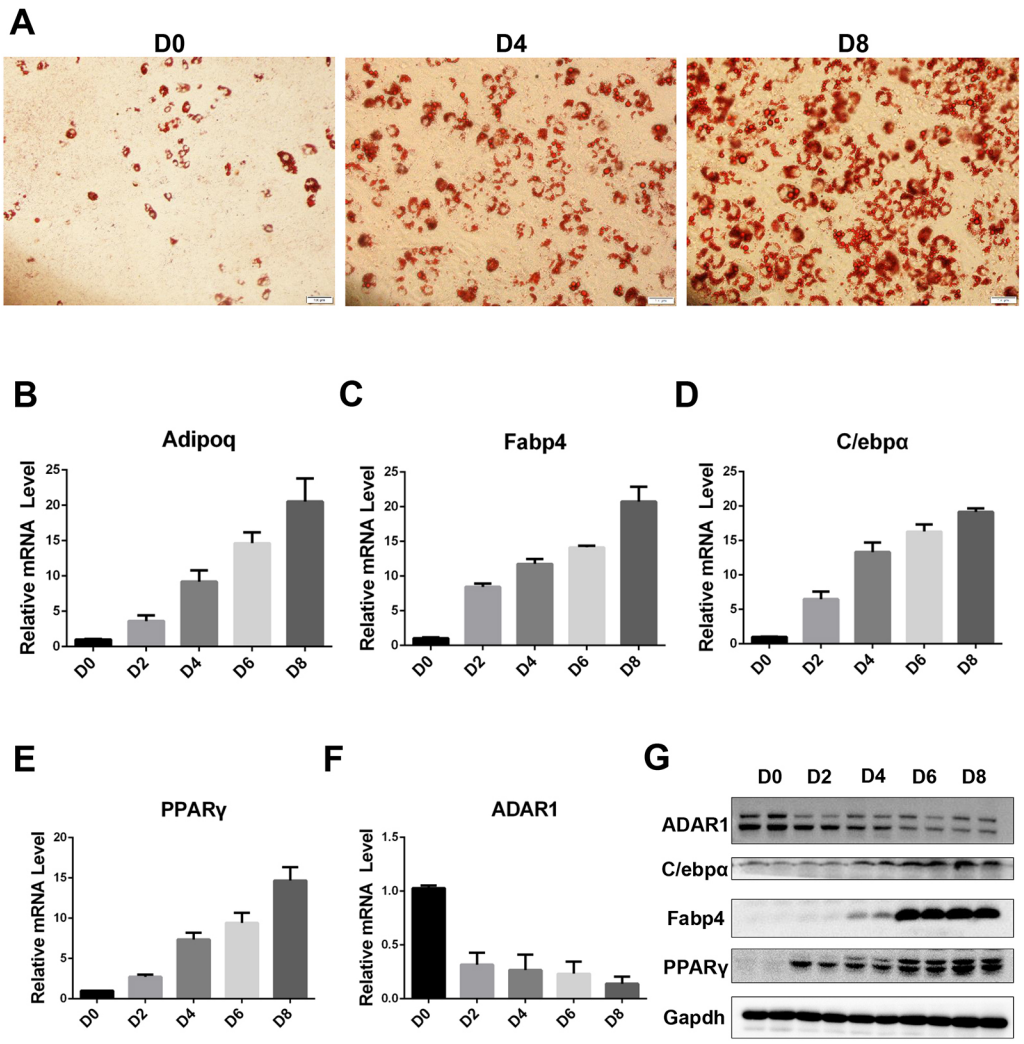

Fig. 2. The expression level of ADAR1 gradually decreases in the time course of MEF differentiation. (A) Oil Red O staining during different days (D0, D4 and D8) of MEF differentiation. Scale bars: 100 µm. (B–F) qRT-PCR analyses of adipogenic marker genes (*Adipoq*, *Fabp4*, *Cebpa* and *Pparg*, encoding Adipoq, Fabp4, C/EBPα and PPARγ) and ADAR1 during the differentiation time course (D0–D8). (G) Western blot analysis of differentiation marker proteins (PPARγ, Fabp4 and C/EBPα) and ADAR1 every 2 days during differentiation of MEFs. All data represents mean±s.e.m. Each experiment was repeated at least three times.

Overexpression of ADAR1 inhibits adipogenic differentiation

For further exploring the influence of ADAR1 on adipogenic differentiation, we overexpressed ADAR1 in MEFs through adenovirus (ad-ADAR1) transduction 2 days before induction of differentiation. Results from qRT-PCR and western blotting confirmed the upregulation of ADAR1 (Fig. 3A,B). At the end of differentiation, results from Oil Red O staining revealed a substantial reduction in adipogenesis after ADAR1 overexpression in MEFs, when comparing to ad-GFP control (Fig. 3C). Such a phenomenon might be ascribed to the downregulation of adipogenic genes during the differentiation process. As shown in Fig. 3D–G, the mRNA level of adipocyte marker genes (*Adipoq*, *Cebpa*, *Fabp4* and *Pparg*) were significantly decreased at indicated time points (D4, D6 and D8) during differentiation. Importantly, such inhibitory effects of ADAR1 on adipogenic genes could be observed as early as day 2 (*Pparg*) and persisted throughout the differentiation process (Fig. 3H,I). As shown in Fig. 3J, overexpression of ADAR1 can also suppress the protein levels of C/EBPα, PPARγ and Fabp4. Together, these data indicate that ADAR1 could suppress adipocyte differentiation by interfering with the expression of adipogenic genes.

Knockdown of ADAR1 promotes adipocyte differentiation

Since the expression of ADAR1 is decreased in iWAT and BAT of obese mice, and its gene/protein expression reduces during adipocyte differentiation (Figs 1 and 2), we next sought to exam the effects of knocking down ADAR1 in MEFs, which was achieved by transduction with knockdown adenovirus (sh-ADAR1). As shown in Fig. 4A,B, gene and protein levels of ADAR1 were virtually eliminated upon knockdown. The knockdown of ADAR1 led to an intense accumulation of lipid droplets in MEFs, indicating enhanced adipocyte differentiation in comparison to the negative control (sh-NC) groups (Fig. 4C). Further analysis showed that downregulation of ADAR1 in MEFs promoted expression of *Adipoq*, *Cebpa*, *Fabp4* and *Pparg* at indicated time points (D4, D6, and D8) during differentiation (Fig. 4D–G). Moreover, as the gene/protein expression of these adipogenic genes increases over time during differentiation in the sh-NC group, and knocking down ADAR1 further enhances their expression starting at day 2 of differentiation (Fig. 4H–J). Overall, these findings indicate that the downregulation of ADAR1 greatly promotes the differentiation process of adipocytes.

Overexpression of ADAR1 inhibits HFD-induced obesity

To verify whether overexpression of ADAR1 can inhibit obesity induced by HFD, we injected control (ad-GFP) and overexpression ADAR1 (ad-ADAR1) adenovirus into the subcutaneous adipose tissue of WT mice, followed by HFD feeding for 8 weeks. During these 8 weeks, mice received adenovirus injection every 2 weeks. At week 4, mice receiving the ad-ADAR1 injection began to show

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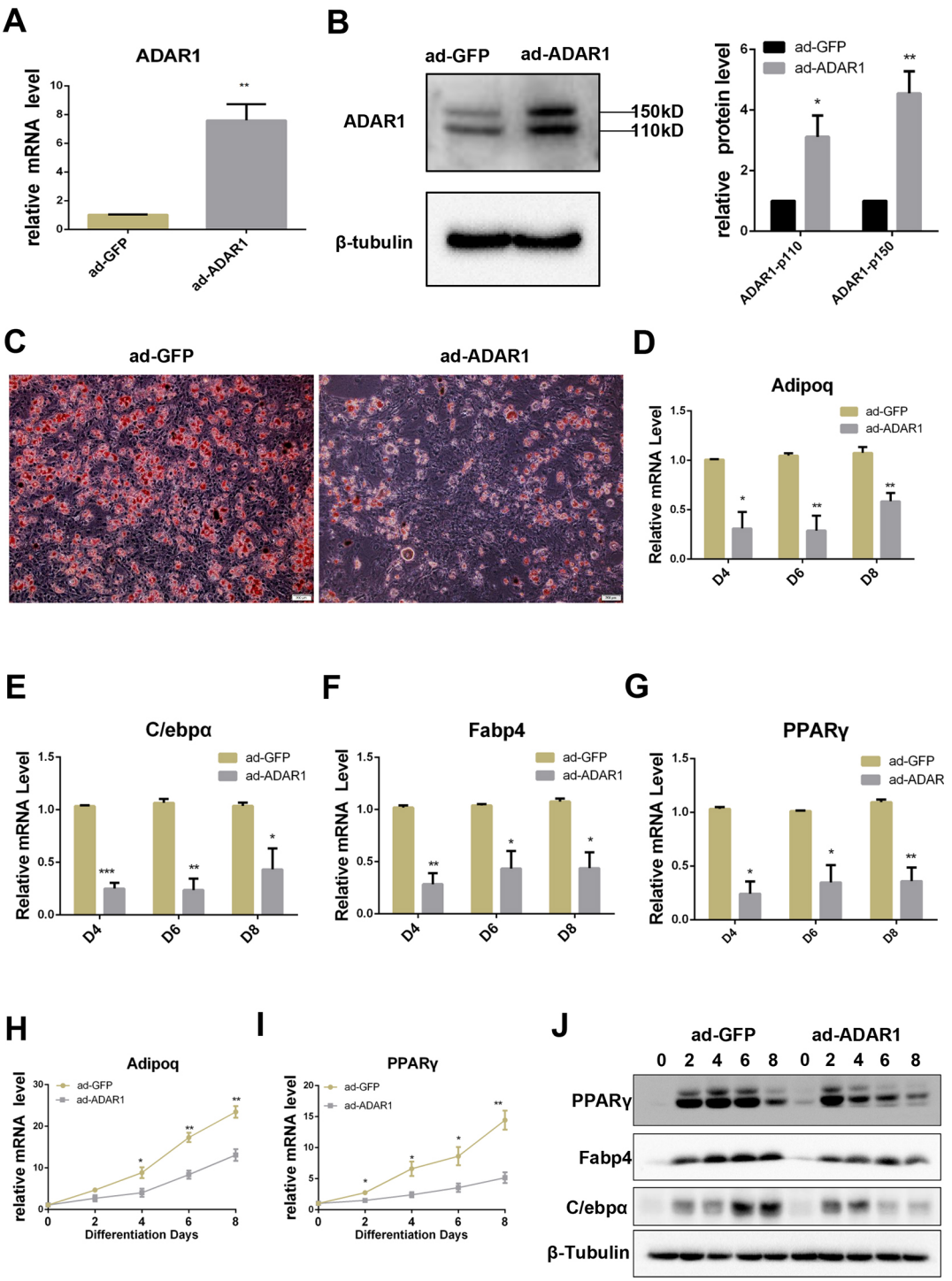

Fig. 3. Overexpression of ADAR1 can inhibit adipogenic differentiation of MEFs. (A) qRT-PCR detection of *Adar1* overexpression (ad-ADAR1) efficiency. (B) The protein expression level of ADAR1 in MEFs after adenovirus overexpression. (C) Oil Red O staining on the D8 of MEFs differentiation in the GFP group and the ADAR1 overexpression group. Scale bars: 200 µm. (D–G) qRT-PCR analyses of four adipogenic marker genes at indicated time points (D4, D6 and D8) of differentiation in the GFP and ad-ADAR1 group. (H,I) qRT-PCR analyses of adipogenic marker genes *Adipoq* and *Pparg* in the time course of differentiation with or without overexpressed ADAR1. (J) Western blot analyses of adipogenic marker genes (C/EBPα, Fabp4 and PPARγ) every 2 days during differentiation. All data represents mean±s.e.m. Each experiment was repeated at least three times. **P*<0.05, ***P*<0.01, ****P*<0.001 (unpaired two-tailed Student's *t*-test).

smaller body weight gain, when comparing to the control (ad-GFP) mice (Fig. 5A). This change was not due to the difference in food intake, because there was no obvious difference in the food intake between the experimental and control groups as can be seen in Fig. 5B. Instead, mice with ad-ADAR1 treatment had a significantly smaller fat pad in inguinal subcutaneous adipose tissue (Fig. 5C,D), and this is accompanied by smaller cell size on ad-ADAR1 mice, as shown by hematoxylin and eosin (H&E) staining (Fig. 5E). In addition, mice with ad-ADAR1 injection showed lower glucose levels during a glucose tolerance test (GTT) and insulin sensitivity

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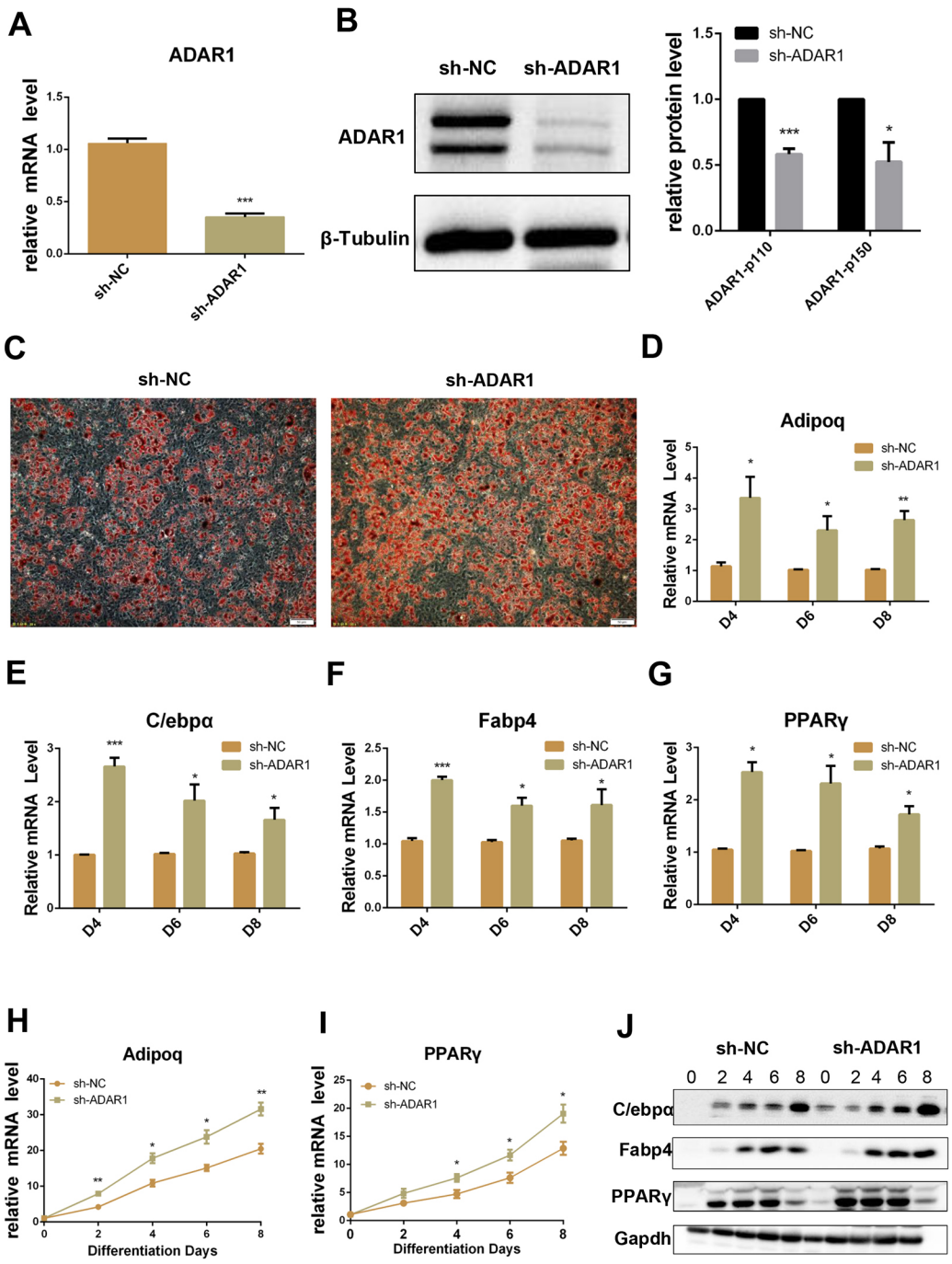

Fig. 4. Knockdown of ADAR1 can promote adipogenic differentiation of MEFs. (A) qRT-PCR detection of *Adar1* in the control and sh-ADAR1 group. (B) Western blot analyses of ADAR1 in the control and sh-ADAR1 groups. (C) Oil Red O staining on day 8 of MEFs differentiation in the control and ADAR1 knockdown groups. Scale bars: 50 µm. (D–G) qRT-PCR analysis of four differentiation marker genes on the middle and late stage (D4, D6 and D8) of differentiation in the control and ADAR1 knockdown group. (H,I) qRT-PCR detection of key adipogenic genes (*Adipoq* and *Pparg*) during the differentiation time course. (J) Western blot analyses of key adipogenic proteins (C/EBPα, Fabp4 and PPARγ) every 2 days during the differentiation of MEFs. All data represents mean±s.e.m. Each experiment was repeated at least three times.**P*<0.05, ***P*<0.01, ****P*<0.001 (unpaired two-tailed Student's *t*-test). sh-NC stands for negative control.

test (ITT), indicating overall better insulin sensitivity and metabolic response to glucose (Fig. 5F,G). Moreover, serum triglyceride (TG) and cholesterol (CHO) levels were also decreased in the ADAR1 overexpressing mice (Fig. 5H,I). Consistent with this, the mRNA expression levels of *Adipoq*, *Cebpa*, *Fabp4* and *Pparg* were significantly decreased in iWAT of ad-ADAR1 mice when compared to ad-GFP mice (Fig. 5J–M). Altogether, these results suggest that overexpression of ADAR1 *in vivo* inhibits adipogenic gene expression, thereby suppressing adipocyte maturation and further inhibiting obesity induced by HFD.

Knockdown of ADAR1 exacerbates HFD-induced obesity

To investigate the necessity of ADAR1 on HFD-induced obesity, we knocked down ADAR1 in inguinal adipose tissue with adenovirus encoding shRNA against ADAR1 (sh-ADAR1). As shown in Fig. 6A, the mice with ADAR1 knockdown (KD) gained

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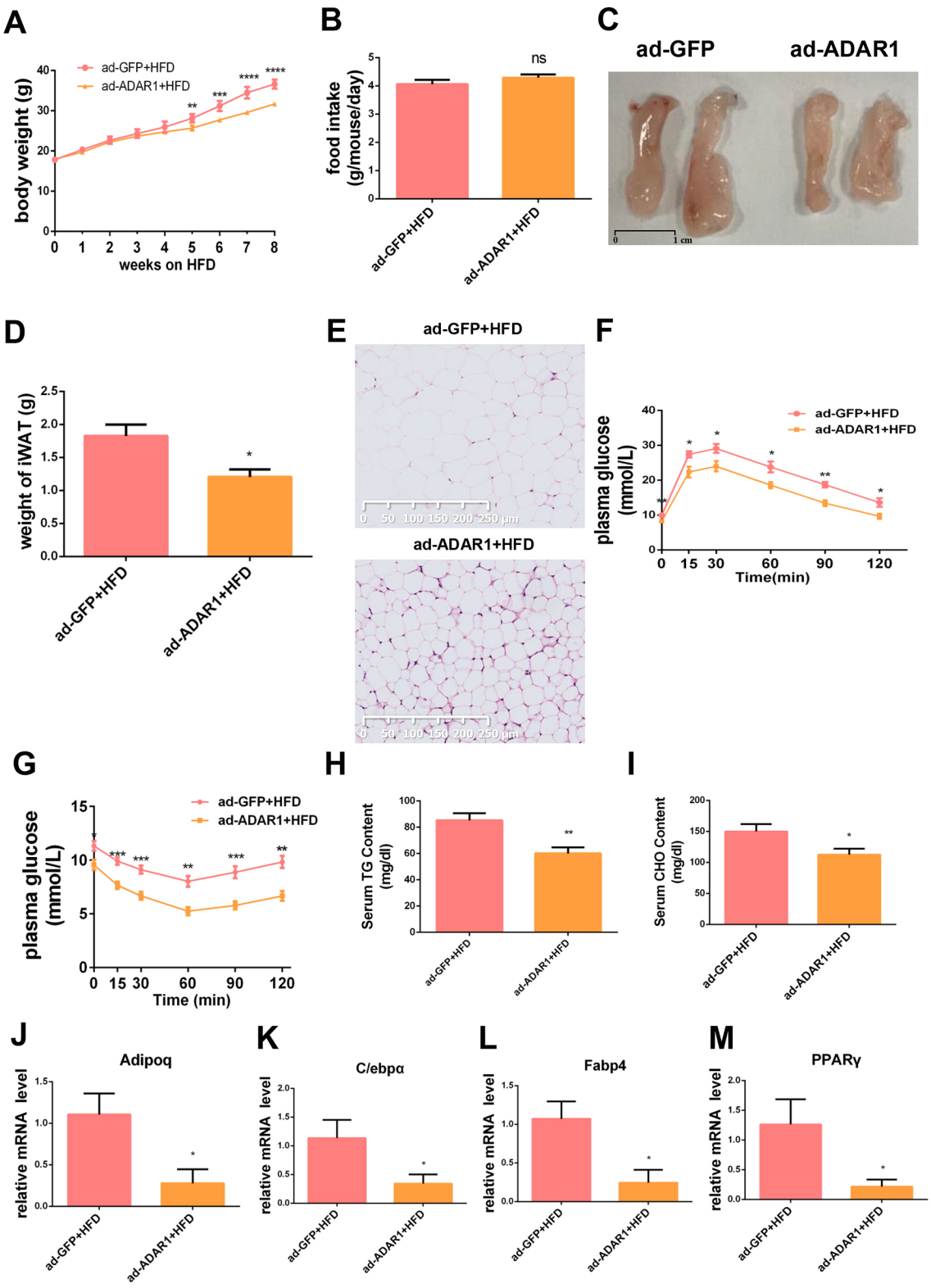

Fig. 5. Overexpression of ADAR1 can inhibit adipogenesis in subcutaneous adipose tissue and alleviate HFD-induced obesity. (A) Weight gain of mice in the control and the ADAR1 overexpression group during HFD feeding. (B) Comparison of the food intake in the two groups of mice. (C–E) Morphology (C), weight (D) and H&E staining (E) of subcutaneous adipose tissue in the control group and ADAR1 overexpression group. (F) GTT in the sixth week after HFD in the two groups. (G) ITT in the seventh week after HFD in the two groups. (H,I) Serum triglyceride and cholesterol levels in the control and the ADAR1 overexpression group after 8 weeks of HFD. (J–M) qRT-PCR detection of adipocyte marker genes (*Adipoq*, *Cebpa*, *Fabp4* and *Pparg*, encoding Adipoq, Fabp4, C/EBPα and PPARγ) in inguinal adipose tissue in the two groups after 8 weeks of HFD. n=6 per group. All data represents mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns, not significant (unpaired two-tailed Student's t-test).

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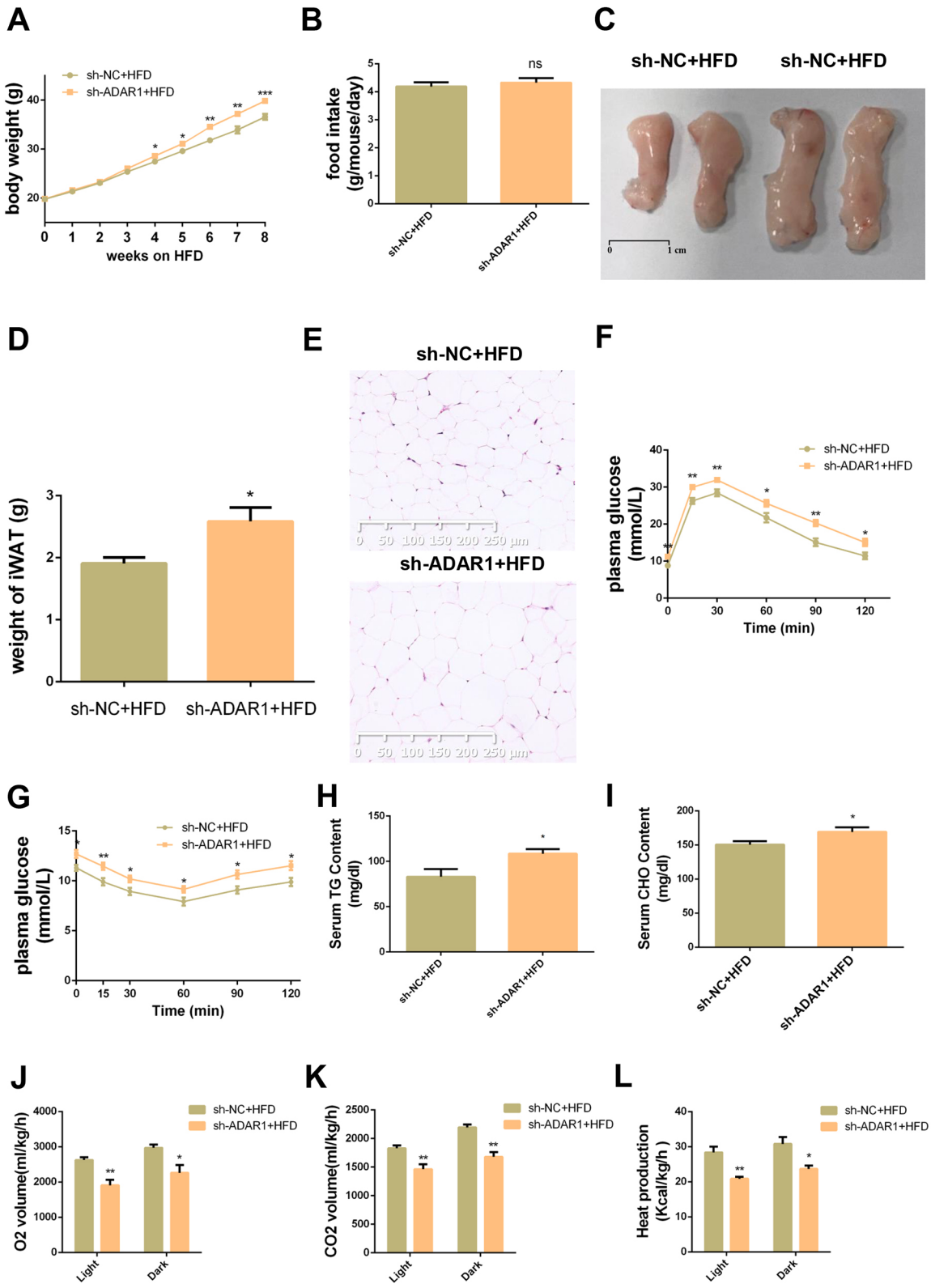

Fig. 6. Knockdown of ADAR1 aggravates HFD-induced obesity. (A,B) The body weight (A) and food intake (B) of mice in the control group and ADAR1 knockdown group during HFD feeding. (C–E) Morphology (C), weight (D) and H&E staining (E) of subcutaneous adipose tissue in the control group and ADAR1 knockdown group. (F,G) GTT (F) and ITT (G) in the sixth and seventh week after HFD. (H,I) Serum triglyceride (H) and cholesterol (I) levels in control and ADAR1 knockdown groups after 8 weeks of HFD. (J–L) Columbus Oxymax metabolic chambers were used to measure the whole-body oxygen consumption rate (V_{O_2}) (J), carbon dioxide production rate (V_{CO_2}) (K) and heat production (L) of the two groups mice. n=8 per group. *P<0.05; **P<0.01; ***P<0.001; ns, not significant (unpaired two-tailed Student's t-test). sh-NC stands for negative control.

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significantly more weight after 8 weeks of HFD feeding. However, such increase in body weight was not due to increase in food intake (Fig. 6B). Instead, we observed substantially larger inguinal adipose deposits, accompanied by an increase in the volume of individual adipocyte (Fig. 6C–E). These results indicate that knocking down ADAR1 can increase lipid deposition in adipocytes. Furthermore, mice with a downregulated ADAR1 level showed impaired glucose tolerance and insulin sensitivity after a HFD (Fig. 6F,G). Further analysis revealed the cell-autonomous effects of ADAR1 on insulin sensitivity. As shown in Fig. S3, gene silencing of *Adar1* significantly reduced Akt1 phosphorylation after insulin stimulation on day 8 of MEFs differentiation, whereas upregulation of ADAR1 could promote Akt1 phosphorylation. In addition, serum triglyceride and cholesterol levels were both increased in the ADAR1 KD group, when compared to the control mice (Fig. 6H,I). To assess the effects of ADAR1 on the energy balance, we used metabolic cage to evaluate the energy expenditure of ADAR1 KD mice. As shown in Fig. 6J–L, knocking down ADAR1 reduced oxygen consumption rate (V_{O_2}), carbon dioxide production rate (V_{CO_2}), and heat production, but had no influence on physical activity (data not shown). Overall, these results indicate that knocking down ADAR1 leads to metabolic imbalance, resulting in an aggravated obese phenotype.

ADAR1 controls the expression of late TFs by regulating C/EBPβ

During adipogenesis, early TFs (such as C/EBPβ) can promote the expression of genes encoding late TFs (like C/EBPα and PPARγ) (Farmer, 2006; Siersbaek et al., 2012). Thus, we hypothesized that the effects of ADAR1 on adipogenic differentiation in MEFs might be associated with C/EBPβ. As can be seen from Fig. 7A,B, both the gene and protein expression of C/EBPβ were increased in MEFs after knocking down ADAR1. Furthermore, as shown in Fig. 7C, the influence of ADAR1 knockdown on the expression of C/EBPα and PPARγ were alleviated by knocking down C/EBPβ (the expression of ADAR1 and C/EBPβ proves knockdown). Consistent with the gene expression results, the results of Oil Red O staining (Fig. 7D) on the 8th day of differentiation also showed that the effect of knocking down ADAR1 on the increased lipid droplet can be greatly reduced by knocking down C/EBPβ. The above results indicate that the influence of ADAR1 on adipogenesis of MEFs is at least partly dependent on C/EBPβ.

ADAR1 interacts with Dicer to promote the maturation of miR-155-5p

Next, we aimed to further investigate how ADAR1 regulates C/EBPβ expression. Previous studies have reported that ADAR1 interacts with

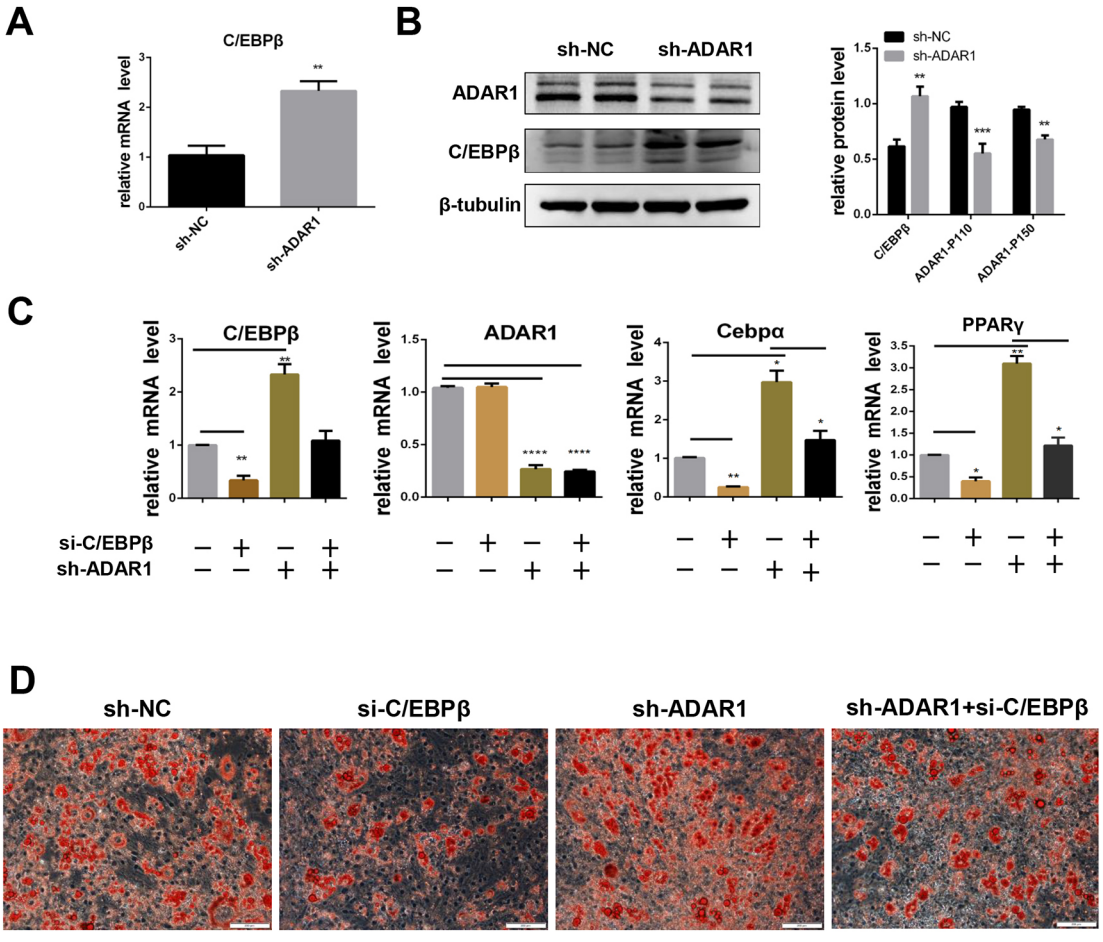

Fig. 7. ADAR1 controls the adipogenic differentiation of MEFs by regulating C/EBPβ. (A) qRT-PCR detection of early differentiation marker *Cebpb*, encoding C/EBPβ, after knocking down ADAR1. (B) Western blot analyses of C/EBPβ and ADAR1 in the control and knockdown ADAR1 group. (C) qRT-PCR analysis results showing that the influence of ADAR1 knockdown on increased expression of C/EBPα and PPARγ was alleviated by C/EBPβ knockdown in MEFs. (D) Photomicrographs of Oil Red O staining (on the 8th day of differentiation) showed that the effect of ADAR1 knockdown on increased lipid droplets was alleviated by C/EBPβ knockdown in MEFs. Scale bars: 200 µm. All data represents mean±s.e.m. Each experiment was repeated at least three times. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 (unpaired two-tailed Student's *t*-test). sh-NC stands for negative control.

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Dicer to enhance the production of mature miRNA (Ota et al., 2013). Consistent with this, results from co-immunoprecipitation (co-IP) experiments showed that ADAR1 only binds with Dicer, but not other molecules that are involved in miRNA processing such as TRBP (also known as TARBP2), Drosha, and argonaute 1 and 2 (Fig. 8A). Using TargetScan database, we found five miRNAs (miR-191-5P, miR-369-3P, miR-155-5P, miR-374b-5p and miR-376b-3p) that contain highly conserved binding sites for C/EBPβ. Importantly, only miR-155-5p shows a 3-fold increase in MEFs with ad-ADAR1 treatment, whereas the expression of other miRNAs shows no change

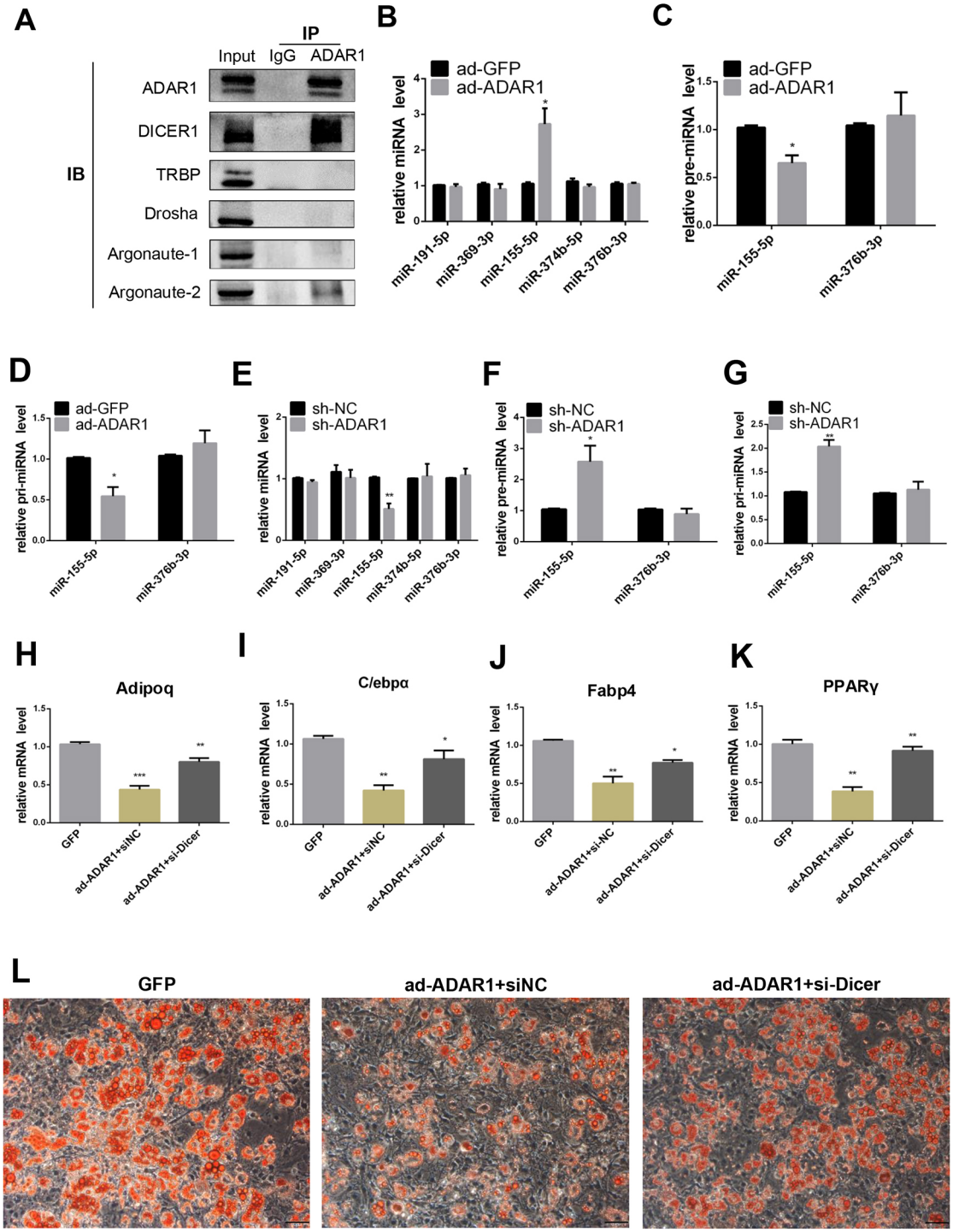

Fig. 8. ADAR1 inhibits the differentiation of adipocytes by binding to Dicer to promote the processing of miR-155-5P. (A) Co-IP results showing that ADAR1 can interact with Dicer. Cell supernatants were incubated with IgG or anti-ADAR1 antibodies followed by immunoblotting (IB). The input shows 10% of the IP amount. (B–D) The relative expression level of miRNAs and their precursors in the control and ADAR1 overexpression groups. (E–G) The relative expression level of mature miRNAs and their precursors in the control and knockdown ADAR1 groups. (H–K) The expression level of four differentiation key genes (*Adipoq*, *Fabp4*, *Cebpa* and *Pparg*, encoding Adipoq, Fabp4, C/EBPα and PPARγ) in the control and ADAR1 overexpression group with or without knocking down Dicer. (L) Oil Red O staining results in the control and overexpressing ADAR1 group with or without knocking down Dicer. Scale bars: 100 µm. Data are indicated as the mean±s.e.m. Each experiment was repeated at least three times. **P*<0.05, ***P*<0.01, ****P*<0.001 (unpaired two-tailed Student's *t*-test). sh-NC stands for negative control.

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(Fig. 8B). Accordingly, the expression level of miRNA-155-5p precursors in the ad-ADAR1 group was lower than that in the ad-GFP group, while the expression levels of miR-376b-3p precursors did not change (Fig. 8C,D). By contrast, knocking down ADAR1 inhibits the expression of miR-155-5p, but cannot affect the expression of other miRNAs (Fig. 8E). As expected, the expression levels of miR-155-5p precursors were upregulated while the precursor of miR-376b-3p was not changed after knocking down ADAR1 (Fig. 8F,G). These results indicate that ADAR1 interacts with Dicer to promote the maturation of miR-155-5P.

To explore whether ADAR1 interacts with Dicer to affect the differentiation of adipocytes, we knocked down Dicer while overexpressing ADAR1 in MEFs. As shown in Fig. 8H–K, the absence of Dicer abrogated the inhibitory effects of ADAR1 on adipogenic gene expression when comparing with cells treated with ad-ADAR1 alone. Furthermore, although ad-ADAR1 cells showed fewer lipid droplets compared to the ad-GFP control, knocking down Dicer simultaneously led to an increase in the content of intracellular lipid droplets (Fig. 8L). In addition, in order to prove that the influence of ADAR1 on adipogenic differentiation is achieved by promoting the processing of miR-155-5P, MEFs were treated with miR-155-5p inhibitor before induction of differentiation. As shown in Fig. S2, the miR-155-5P inhibitor can partially restore the inhibitory effect of ADAR1 on adipogenic genes.

Liu et al. have shown that miR-155-5p can bind to the 3′UTR of C/EBPβ and inhibit its expression in adipocytes (Liu et al., 2011), our current findings not only further confirm the importance of the miR-155-5p-C/EBPβ axis on adipocyte differentiation, but also provide more insights on the upstream miR-155-5p-C/EBPβ axis, showing that ADAR1 can interact with Dicer to promote the production of miR-155-5p.

DISCUSSION

Adipose tissue is of great importance for metabolic diseases (Rosen and Spiegelman, 2006; Kahn et al., 2006; Després and Lemieux, 2006). The expansion of adipose tissue is accompanied by increasing adipocyte volume and number of adipocytes through adipogenesis (Kopelman, 2000; NCD-RisC, 2016; Sun et al., 2011; Zhang et al., 2016). Therefore, in order to develop safe and efficacious therapies to ameliorate obesity and its accompanying detrimental complications, it is particularly important to identify key components and dissect their underlying mechanisms in regulating adipocyte differentiation. In this study, using both *in vitro* and *in vivo* gain- and loss-of-function approaches, we discovered for the first time that overexpression of ADAR1 can inhibit adipogenesis and improve the metabolic phenotype in HFD-induced obese mice.

ADAR1 is a member of the adenosine deaminase family. It has been reported that ADAR1 plays a key role in embryonic heart and liver development, and maintenance of hematopoietic stem cell function (Hartner et al., 2004, 2009; Moore et al., 2020). However, the influence of ADAR1 in adipogenesis was still unknown. Our *in vitro* studies showed that overexpression of ADAR1 in MEFs could inhibit the adipogenic differentiation, with a decrease in intracellular lipid droplets. By contrast, knockdown of ADAR1 via shRNA could promote adipogenesis with increased lipid accumulation in adipocytes. Moreover, when mice received injections of adenovirus at subcutaneous adipose tissue to locally upregulate ADAR1 in adipocytes, the fat mass of the white adipose tissue was reduced substantially, and the serum levels of triglyceride and cholesterol were also decreased when comparing to mice with control GFP adenovirus injections. This leads to improved glucose metabolism and insulin sensitivity. In comparison, knocking down ADAR1 in adipose tissue elicited a substantial impact on adipose tissue expansion, as evidenced by significantly larger adipose deposits, which leads to exacerbated glucose intolerance and insulin resistance. Such phenomena could not be ascribed to change food intake, since downregulating ADAR1 locally by direct injection of adenovirus to adipose tissue did not cause any effect on food intake. Importantly, our data showed that ADAR1 has cell-autonomous effects on insulin sensitivity, and ADAR1 played a role in regulating energy expenditure; local administration of adenovirus to knockdown ADAR1 in adipose tissue significantly decreased the oxygen consumption rate and CO_2 production rate, which might further aggravate the adverse effects on adipocyte expansion and overall obese phenotype. Interestingly, a previous publication reported that global knockdown of ADAR1 decreased food intake and ameliorated HFD-induced obesity (Cui et al., 2021). Given the high expression of ADAR1 in the brain, future studies are warranted to dissect the roles and the underlying mechanisms of ADAR1 in regulating food intake.

Adipocyte differentiation is a cellular program that is initiated by two phases of consecutive TF activation waves. The early TFs in the first wave mainly include C/EBPβ and C/EBPδ. Upon activation, they will enhance the expression of the late TFs in the second wave, such as C/EBPα and PPARγ (Farmer, 2006; Siersbaek et al., 2012). The late TFs will increase the expression of the downstream adipocyte-specific genes. In this study, ADAR1 promoted miR-155-5P maturation by binding with Dicer. A previous study has shown that miR-155-5P could inhibit 3T3-L1 differentiation by targeting the 3′UTR of C/EBPβ, thus causing the degradation of C/EBPβ mRNA (Liu et al., 2011). Meanwhile, it has also been reported that miR-155-5P controls the differentiation of brown adipocytes by forming a bistable feedback loop with C/EBPβ (Chen et al., 2013). Our results consistently showed that administration of miR-155-5p inhibitor could partially restore the expression of adipogenic genes that are suppressed by ADAR1. In conclusion, our data show that ADAR1 suppresses adipogenic differentiation by enhancing the production of miR-155-5p after interacting with Dicer.

In conclusion, our study reveals the novel function of ADAR1 in suppressing adipogenesis through promoting the maturity of miR-155-5p and reducing iWAT expansion induced by a HFD. This suggests that ADAR1 might be a potential therapeutic candidate to alleviate obesity and its related metabolic complications.

MATERIALS AND METHODS

Mice and treatment

Male C57BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. and housed in a specific pathogen-free (SPF) class animal room. Male DB/DB mice with a background of C57BLKS were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd and housed in an SPF class animal room. All the animal experiments were approved by the Ethical Committee of Huazhong University of Science and Technology and performed in accordance to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals could eat and drink freely in a 23°C environment. All experimental mice were assigned into two weight-matched groups randomly. All experimental mice were fed with high-fat research diets MD12032 (purchased from Mediscience Ltd.) starting at age of 6 weeks after the virus injection. Body weight and food intake were measured at the indicated time, and adenovirus for overexpression or knockdown of ADAR1, or control GFP or sh-NC (1×10^{12} titer) were injected into the inguinal adipose tissue every 2 weeks. GTT and ITT experiments were performed in the sixth and seventh weeks of HFD feeding, respectively.

Adenovirus construction

In this article, the AdMax adenovirus packaging system was used for adenovirus overexpression and knockdown. For the overexpression of

ADAR1, the cDNA of ADAR1 was cloned into the shuttle plasmid pDC312-PCDH, and then shuttle plasmid pDC312-PCDH-ADAR1 and helper plasmid pBHGloxdelE13cre (plasmids sourced from MiaoLing Plasmid Platform) were co-transfected into 293a cells (American Type Culture Collection). The 293a cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotics. For the construction of knockdown adenovirus, we generated adenovirus expressing shRNA against ADAR1 (sh-ADAR1) using the sequence: 5′-GTACCGGGTGAATCCAGATAGTTGTATCCT-CGAGGATACAACTATCTGGATTCACTTTITTTG-3′. The control virus of overexpression (GFP) and knockdown (sh-NC) were also constructed through the Admax system.

Western blot analysis

MEFs (see below for detail on culture) were lysed using pre-cooled RIPA lysis buffer (Servicebio, G2002) with protease inhibitors (Roche, 04693116001), sonicated on ice to fully lyse the cells, and then centrifuged at 12,000 ***g*** for 15 min. The supernatants containing proteins were collected. 30 µg of protein are separated by electrophoresis and then transferred to PVDF membrane. Then the membrane was blocked with 2% bovine serum albumin at 23°C. Next, the membrane was incubated with the corresponding primary antibody at 4°C for 12–16 h. After that, the membrane was incubated with secondary horseradish peroxidase antibodies for 2 h at 23°C. Finally, the UVP BioSpectrumR600 imaging system (Upland, California, USA) was used for exposure imaging. Primary antibodies against the following proteins were used: β-actin (1:1000 dilution, AC026, Abclonal); Gapdh (1:1000 dilution, 60004-1-Ig, Proteintech); β-tubulin (1:1000 dilution, AC015, Abclonal); C/EBPα (1:1000 dilution, A0904, Abclonal); ADAR1 (1:100 dilution, sc73408, Santa Cruz Biotechnology); Fabp4 (1:100 dilution, sc-271529, Santa Cruz Biotechnology); PPARγ (1:1000 dilution, 2435, Cell Signaling Technology); Dicer1 (1:1000 dilution, 20567-1AP, Proteintech); Drsha (1:1000 dilution, A19598, Abclonal); TRBP (1:1000 dilution, A9105, Abclonal); C/EBPβ (1:1000 dilution, A0711, Abclonal); argonaute-1 (1:1000 dilution, A6022, Abclonal); argonaute-2 (1:1000 dilution, A6023, Abclonal); phosphorylated (p)-AKT(Ser473) (1:1000 dilution, 9271, Cell Signaling Technology); total (t)-Akt (1:1000 dilution, A5031, Bimake.cn).

qRT-PCR analysis

Total RNA of adipose tissue and MEFs was isolated with Trizol (Invitrogen, USA). RNA reverse transcription reaction was implemented by primescript RT Reagent Kit (Takara). For the analysis of miRNA, primers of reverse transcription and quantitative PCRs were designed and synthesized by Ribobio Biotech Company (Guangzhou, China). U6 was served as the internal control. PCR amplification was performed with SYBR-Green (Takara) using an ABIPRISM 7500 Sequence Detector (Applied Biosystem). The gene expression level was calculated as $2^{-\Delta\Delta Ct}$ after normalization to 18 s or U6 levels. The primer sequences are in Table S1.

siRNA and miRNA inhibitor transfection experiment

siRNA targeting Dicer (50 nM), C/EBPβ (50 nM), and their control siRNAs (siNC; 50 nM) were synthesized by RiboBio Biotech Company. siRNA was transfected into MEFs before induction of differentiation by using ribo*FECT*™ CP Reagent (RiboBio Biotech Company). miR-155-5P inhibitor (100 nM, RiboBio Biotech Company) was transfected into MEFs 1 day before adding adipogenic cocktail. Control inhibitor served as experimental controls. Information about siRNA and miRNA inhibitors is shown in Table S2.

Co-immunoprecipitation

Cells were lysed with pre-cooled RIPA lysis buffer (P0013D, Beyotime Biotechnology, China). Then the lysates were centrifuged for 15 min at 7500 ***g***, cell supernatants were incubated with IgG (A7028, Beyotime Biotechnology, China) or anti-ADAR1 antibodies (sc73408, Santa Cruz Biotechnology, USA) for 12 h (overnight). On the next day, Protein A/G Magnetic Beads (HY-K0202, MCE, USA) were incubated with the lysates for 2 h. Then they were washed with PBS and RIPA lysis buffer, respectively. Finally, the precipitated protein is denatured for further experiments.

Histological analysis

Fresh subcutaneous adipose tissue was fixed in 4% paraformaldehyde fixative. Then, the subsequent sectioning and hematoxylin-eosin (HE) staining experiments were completed by biossci biotechnology Co., Ltd (Wuhan, China). A Nikon microscope was used to capture the images.

Oil Red O staining

First, the culture medium from MEFs was discarded and then the cells were washed three times with PBS. Next, MEFs were fixed with fixative for 10 min and washed three times with PBS. Cells were then stained with Oil Red O dye (G1015-100ML, Servicebio, China) for 10–15 min, and rinsed with 60% isopropanol for 5–10 s to remove excess dye. Finally, an optical microscope (BX51, Olympus) was used to collect images.

GTT and ITT

For the GTT, mice were given intraperitoneal injections of glucose (1 g/kg of body weight) after 14 h of fasting, and the glucose level in the tail vein was monitored with a blood glucose meter (OneTouch Ultra) at 0, 15, 30, and 60, 90 and 120 min. For the ITT, mice were given intraperitoneal injections of insulin (0.75 units/kg of body weight) after 4 h of fasting, the blood glucose level in the tail vein was measured at the indicated time.

Measurement of serum metabolites

Commercial assay kits (cholesterol, S03042, Rayto; triglyceride, S03027, Rayto) were utilized to measure serum triglyceride and total cholesterol levels according to the manufacturer's instructions.

Primary MEFs isolation, culture, differentiation and adenovirus infection

MEFs isolation was performed as described before (Durkin et al., 2013; Qiu et al., 2016). Briefly, MEFs were obtained from embryos of pregnant C57BL/6J mice and digested with trypsin at 37°C cell incubator for 1 h. After filtration with a 100 µm cell sieve, the digestion mixture was centrifuged at 2000 ***g*** for 5 min. After washing three times with PBS, cells were seeded in culture medium (DMEM) containing 10% fetal bovine serum and 1% antibiotics. MEFs were transfected with adenovirus 2 days before the addition of the differentiation medium. The differentiation process is as follows: at 2 days after the cells reached contact inhibition, they were treated with the differentiation medium containing 10 µg/ml insulin (Sigma-Aldrich, 1342106), 500 mM IBMX (Sigma-Aldrich, I5879), 2 µM dexamethasone (Sigma-Aldrich, D1756) and 2.5 µM rosiglitazone (Sigma-Aldrich, R2408). Medium changes were performed every 2 days, and the initial medium was replaced with one containing 10 µg/ml insulin and 2.5 µM rosiglitazone.

Statistical analysis

GraphPad Prism 6.01 was adopted for statistical analysis. All data are shown as mean±s.e.m. An unpaired two-tailed Student's *t*-test was utilized to compare the statistical difference between two groups. $P<0.05$ is considered statistically significant.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Z. Yu; Methodology: Z. Yu, R.L.; Software: Z. Yu; Validation: Z. Yu, R.L.; Formal analysis: Z. Yu, X.L.; Investigation: X.L.; Data curation: Z. Yu, Z. Yang; Writing - original draft: Z. Yu; Writing - review & editing: Y.L., J.P.; Visualization: Z. Yu; Supervision: J.P., K.H.; Project administration: K.H.; Funding acquisition: K.H.

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