Progesterone receptor membrane associated component 1 enhances obesity progression in mice by facilitating lipid accumulation in adipocytes

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Progesterone receptor membrane associated component 1 (PGRMC1) exhibits haem-dependent dimerization on cell membrane and binds to EGF receptor and cytochromes P450 to regulate cancer proliferation and chemoresistance. However, its physiological functions remain unknown. Herein, we demonstrate that PGRMC1 is required for adipogenesis, and its expression is significantly enhanced by insulin or thiazolidine, an agonist for PPARγ. The haem-dimerized PGRMC1 interacts with low-density lipoprotein receptors (VLDL-R and LDL-R) or GLUT4 to regulate their translocation to the plasma membrane, facilitating lipid uptake and accumulation, and de-novo fatty acid synthesis in adipocytes. These events are cancelled by CO through interfering with PGRMC1 dimerization. PGRMC1 expression in mouse adipose tissues is enhanced during obesity induced by a high fat diet. Furthermore, adipose tissue-specific PGRMC1 knockout in mice dramatically suppressed high-fat-diet induced adipocyte hypertrophy. Our results indicate a pivotal role of PGRMC1 in developing obesity through its metabolic regulation of lipids and carbohydrates in adipocytes.

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Globally, metabolic syndrome is one of the most serious clinical manifestations, which is characterized by several lines of metabolic dysfunction, including obesity, hyperglycemia, hyperlipidemia, and hypertension. Excess intake of diets containing carbohydrates and fats leads to adipocyte hypertrophy and fat expansion, resulting in obesity. White adipose tissue (WAT) stores excess energy-generating substrates in the form of triglycerides (TG) in cellular lipid droplets. Adipocytes store lipids inside the cells and hypertrophy of adipocytes is induced by excess metabolic energy. Adipogenesis is induced in response to adipogenic inducers such as insulin, cyclic adenosine monophosphate (cAMP), and dexamethasone through mechanisms involving adipogenic transcription factors such as CCAAT/enhancer-binding protein (CEBP) gene family and peroxisome proliferator-activated receptor gamma (PPARγ). PPARγ induces lipid accumulation in adipocytes by transactivating the genes involved in the transport of fatty acids such as fatty acid-binding protein 4 (FABP4) or CD36, or in insulin signaling and glucose uptake regulators, such as insulin receptor substrates 2 or glucose transporter 4 (GLUT4). Hyperglycemia induces insulin signaling that promotes translocation of GLUT4 from intracellular vesicles to the plasma membrane via activation of its downstream pathway, phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt), resulting in the facilitation of glucose uptake downstream pathway, phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt), resulting in the facilitating of glucose uptake.

Results

PGRMC1 is required for lipid accumulation during 3T3L1 cell differentiation. We analyzed the effects of PGRMC1 down-regulation on adipose differentiation in a mouse embryonic fibroblast 3T3L1 cell line by using two types (KD1, KD#2) of stable PGRMC1-knockdown (KD) cells (Fig. 1a). The lipid accumulation detected by Oil Red O staining in differentiated 3T3L1 cells was significantly inhibited by PGRMC1 KD compared to that of control shRNA treated wild-type cells (control) (Fig. 1b). These results suggest that PGRMC1 is required for lipid accumulation during 3T3L1 cell differentiation. We next analyzed the expression levels of PGRMC1 during 3T3L1 cell differentiation by quantitative PCR (qPCR) (Fig. 1c) and western blotting (Fig. 1d). The PPARγ expression was increased at day 2 after induction, and the expression of PGRMC1, similar to FABP4 that is known to be induced by PPARγ, was increased at day 4 after induction, suggesting that PGRMC1 expression, similar to FABP4, is induced after PPARγ activation. When using the PGRMC1-KD cells, the expression of PPARγ and FABP4 was significantly reduced by PGRMC1 KD (Fig. 1e). In contrast with these results, the expression of CCAAT/enhancer-binding protein beta (C/EBP-β), which is known to express at an early stage during adipocyte differentiation, was upregulated at day 2 after induction, and did not change by PGRMC1 KD (Fig. 1e). These results suggested that PGRMC1 expression is upregulated in correlation with activation of PPARγ during adipogenesis. To verify whether PGRMC1 influences adipose differentiation or lipid accumulation, we further analyzed the effect of PGRMC1 KD in 3T3L1 cells stimulated by treatment with activator, thiazolidinedione (TZD). The lipid accumulation in differentiated 3T3L1 cells induced by TZD was significantly inhibited by PGRMC1 KD (Supplementary Fig. 1a). Under these conditions, the induced expressions of PPARγ and FABP4 by TZD did not change by PGRMC1-KD (Supplementary Fig. 1b, c). These indicated that PGRMC1 is required for lipid accumulation during 3T3L1 cell differentiation.

PGRMC1 expression is enhanced during 3T3L1 cell differentiation. PPARγ acts as a key regulatory transcription factor for the induction of lipid accumulation in adipocytes. We next examined the PGRMC1 expression by treatment with TZD in the 3T3L1 cells. As shown in Fig. 2a, b, the expression of PGRMC1 as well as PPARγ and FABP4 was significantly enhanced by treatment with TZD. These results indicated that the PGRMC1 expression is regulated by PPARγ during adipocyte differentiation. To decipher regulatory mechanisms of PGRMC1 gene expression, we predicted the transcription factor-binding site in mouse PGRMC1 promoter sequences using TRANSFAC software. As a result, PGRMC1 promoter contained the predicted PPARγ response element (PPRE), activating transcription factors (ATF)/cAMP-response element modulator (CREB)-binding element (CRE), glucocorticoid receptor-binding element (GRE), and three SP1-binding elements (Supplementary Fig. 2). Therefore, we performed the reporter gene assay using luciferase constructs containing mouse PGRMC1 promoter sequences (Fig. 2c). The promoter activities with the PPRE-containing constructs (−1695/+1-PGRMC1-Luc or −345/+1-PGRMC1-Luc) were significantly elevated by treatment with TZD. By contrast, no stimulation was observed after using the construct lacking PPRE site (−327/+1-PGRMC1-Luc). We further confirmed the activity of the predicted PPRE site in the PGRMC1 promoter sequence.
promoter by using reporter constructs encoding three repeats of the PPRE (PPRE x3) or the mutated PPRE (PPRE-mt x3) upstream of a control SV40 promoter (Fig. 2d). The reporter activity of the PPRE x3-containing constructs was significantly enhanced by TZD, but no inducible activity was observed when using the control or the PPRE-mutation constructs. We also confirmed that the reporter activities of the PGRMC1 promoter (−1695/+1-PGRMC1-Luc) or the PPRE x3-containing construct were significantly elevated by the addition of TZD in 293T cells co-transfected with the PPARγ expression vector (Supplementary Fig. 3). Furthermore, the PGRMC1 promoter activity (−1695/+1-PGRMC1-Luc) was enhanced by the addition of insulin (Supplementary Fig. 4a). Insulin signaling is known to induce transactivation of ATF/CREB33. The induction of PGRMC1 promoter activity by insulin was observed when using the CRE-containing construct (−481/+1-PGRMC1-Luc), but not when using the CRE lacking construct (−465/+1-PGRMC1-Luc). Furthermore, we were unable to detect any induction of its promoter activity by dexamethasone (DEX), an inducer of GR (Supplementary Fig. 4b). These results suggested that the PGRMC1 gene expression was enhanced through mediation by PPARγ and ATF/CREB, which are induced by insulin signaling during adipogenesis. To further examine regulatory mechanisms of PGRMC1 expression in vivo, TZD was intraperitoneally injected in mice for 3 days, and gene expressions in the WAT were detected by qPCR and western blotting (Fig. 2e, f). The results indicated that the expression level of PGRMC1 in WAT was significantly induced by treatment of TZD.

PGRMC1 contributed to LDL and VLDL uptake by regulating the translocation of LDL-R and VLDL-R. Recently, it has been reported that PGRMC1 contributes to LDL uptake through interaction with LDL-R in HeLa cells31; however, its regulatory mechanism in adipocyte has remained unclear. Since PGRMC1 was required for lipid accumulation during 3T3L1 cell differentiation (Fig. 1), regulatory roles of PGRMC1 for LDL uptake were further examined in 3T3L1 cells. Control or PGRMC1-KD 3T3L1 cells were treated with Alexa Fluor 488-labeled LDL to analyze the LDL uptake using immunofluorescence microscopy and flow cytometry. Analyses with immunofluorescence microscopy revealed that the Alexa Fluor 488-labeled LDL uptake in PGRMC1-KD cells was significantly suppressed as compared with that in control cells (Fig. 3a and Supplementary Fig. 5a). Furthermore, analyses with flow cytometry showed that the Fluor 488-labeled LDL uptake was significantly decreased in PGRMC1-KD cells than that in control cells (Fig. 3b). We also showed that the LDL uptake in the differentiated 3T3L1 cells induced by TZD was suppressed by PGRMC1 KD (Supplementary Fig. 1d). We further examined the effects of PGRMC1 restoration that was performed by knockdown-rescue procedures using PGRMC1-expressing vectors, in which silent mutations were introduced into the nucleotide sequence targeted by shRNA (Supplementary Fig. 6). As a result, the suppression of LDL uptake by PGRMC1 KD was rescued by expressing shRNA-resistant PGRMC1 WT (Fig. 3a, b). We previously showed that PGRMC1 forms a dimer through heme–heme stacking by 5-coordinate haem with tyrosine 113 residue (Y113) of PGRMC123. To evaluate the effect of the dimerization of PGRMC1 on LDL uptake, we prepared the shRNA-resistant PGRMC1 mutant, Y113F, which was unable to dimerize due to disruption of the haem-binding activity. In contrast to the result using the construct of PGRMC1 WT, the suppression of LDL uptake by PGRMC1 KD was not rescued after using the Y113F mutant, suggesting that haem-dimerized

Fig. 1 Progesterone receptor membrane-associated component 1 (PGRMC1) is required for lipid accumulation during 3T3L1 cells differentiation.

a Analyses of protein expressions in differentiated 3T3L1 cells (control, KD#1, or KD#2) by western blotting using antibodies against PGRMC1 or GAPDH. b Oil Red O staining of differentiated 3T3L1. Control or two types of stable PGRMC1-KD 3T3L1 cells (PGRMC1 KD#1 and KD#2) were differentiated and stained with Oil Red O. The microscope images are shown in the upper panels of (b). Graphs in the lower panel of (b) depict the absorbance at 490 nm by Oil Red O (n = 8). c Analyses of mRNA expression of PGRMC1, CEBPβ, PPARγ, or FABP4 in 3T3L1 cells at the indicated time periods during differentiation (control, PGRMC1 KD) by quantitative PCR (qPCR) (n = 10). Analyses of protein expressions in undifferentiated or differentiated 3T3L1 cells (control, KD) by western blotting using antibodies against PGRMC1, CEBPβ, PPARγ, FABP4, or GAPDH. d Analyses of protein expressions in 3T3L1 control cells during differentiation by western blotting using antibodies against PGRMC1, PPARγ, FABP4, or GAPDH. e Analyses of protein expressions in undifferentiated or differentiated 3T3L1 cells (Control, KD) by western blotting using antibodies against PGRMC1, CEBPβ, PPARγ, FABP4, or GAPDH. Data represent mean ± S.E. Statistical analyses were performed using ANOVA with Tukey’s T test. *P < 0.05. †P < 0.05 (vs control day 0 (c)). ‡P < 0.05 (PGRMC1 KD vs control on the same day (e)).
PGRMC1 is required for LDL uptake in 3T3L1 cells. We further analyzed the uptake of the fluorescent Dil-labeled VLDL in control or PGRMC1-KD 3T3L1 cells with immuno-fluorescence microscopy (Fig. 3c and Supplementary Fig. 5b) and flow cytometry (Fig. 3d). The VLDL uptake was inhibited by PGRMC1 KD, and the suppression was rescued by expressing shRNA-resistant PGRMC1 WT, but not the Y113 mutant. These results collectively suggested that the haem-dimerized PGRMC1 is necessary for facilitating the uptake of LDL and VLDL. LDL or VLDL is incorporated into the cells via endocytosis mediated by LDL-R or VLDL-R, respectively34. To elucidate the mechanism of the regulation of LDL uptake by PGRMC1, we analyzed the cellular distribution of LDL-R or VLDL-R by fractioning plasma membranes of 3T3L1 cells (Fig. 3e). The expression of LDL-R or VLDL-R in the whole-cell lysates did not differ between control and PGRMC1 KD cells (Fig. 3e, left panel). By contrast, the localization of LDL-R or VLDL-R in the plasma membrane was decreased by PGRMC1 KD, whereas it was not changed its localization of plasma membrane protein Na-K ATPase 1, or transferrin receptor (Tf-R), which are known to be regulated by endocytosis35. Collectively, these results suggested that the translocation of LDL-R or VLDL-R from the plasma membrane mediated by the haem-dimerized PGRMC1 appears to be responsible for the accumulation of LDL or VLDL in 3T3L1 cells. This finding led us to examine whether dimerized PGRMC1 interacts with LDL-R or VLDL-R in the cells. The FLAG-tagged PGRMC1 expressed in 3T3L1 cells was immunoprecipitated with anti-FLAG antibody-agarose, and the co-immunoprecipitated endogenous LDL-R or VLDL-R was detected. It was observed that the FLAG-tagged PGRMC1 WT interacted with endogenous LDL-R (Fig. 3f) or VLDL-R (Fig. 3g), but not with the Y113 mutant, indicating that the haem-dimerized PGRMC1 interacts with LDL-R and VLDL-R. We also confirmed that LDL-R was co-immunoprecipitated with endogenous PGRMC1 (Fig. 3h). These results suggest that the haem-dimerized PGRMC1 plays a role in the plasma membrane distribution of LDL-R and VLDL-R via direct interaction.

PGRMC1 contributes to glucose uptake by regulating the translocation of GLUT4 to the plasma membrane. Glucose uptake in adipocytes plays a crucial role in accumulation of lipids...
We also showed that the 2-DG uptake in the differentiated 3T3L1 cells induced by TZD was suppressed by PGRMC1 KD (Supplementary Fig. 1e). To evaluate the fatty acids synthesized via de novo fatty acid synthesis, recently, PGRMC1 has been reported to interact with the insulin receptor and to regulate its translocation to the plasma membrane, which in turn modulates glucose uptake in lung cancer cells. Therefore, we examined the effects of PGRMC1 on glucose uptake by using 2-deoxy-d-glucose (2-DG), an inhibitor of hexokinase, in 3T3L1 cells. The uptake of 2-DG induced by treatment of insulin in 3T3L1 cells was significantly suppressed by PGRMC1 KD, whereas no significant effect by PGRMC1 KD was observed without treatment of insulin (Fig. 4a). We also showed that the 2-DG uptake in the differentiated 3T3L1 cells induced by TZD was suppressed by PGRMC1 KD (Supplementary Fig. 1e). To evaluate the fatty acids synthesis via the incorporation of glucose, 3T3L1 cells were incubated with [13C6]-glucose, [13C]-palmitic, [13C]-stearic, and [13C]-oleic acid in 3T3L1 cells were significantly decreased by PGRMC1 KD. These results suggested that PGRMC1 contributes to the acceleration of fatty acid synthesis by upregulating glucose uptake in 3T3L1 cells. This observation led us to examine whether PGRMC1 regulates GLUT4 translocation in the plasma membrane. The insulin-induced GLUT4 translocation onto the plasma membrane was inhibited by PGRMC1 KD (Fig. 4c), whereas the GLUT1 translocation was not suppressed by PGRMC1 KD. Furthermore, the insulin-induced Akt phosphorylation was not changed by PGRMC1 KD, suggesting that suppression of the GLUT4 translocation by PGRMC1 KD is independent of the Akt signaling induced by insulin. We thus examined whether PGRMC1 binds to GLUT4 using a co-immunoprecipitation assay. The FLAG-tagged PGRMC1 expressed protein in 3T3L1 cells was immunoprecipitated, and the co-immunoprecipitated endogenous GLUT4 was detected (Fig. 4d). These results suggested that the dimerized PGRMC1 was directly bound to GLUT4, thereby regulating its translocation to the plasma membrane, which in turn modulates glucose uptake.
Fig. 4 Progesterone receptor membrane-associated component 1 (PGRMC1) contributes to glucose uptake by regulating the translocation to the plasma membrane of GLUT4. a Analysis of the effect of insulin-stimulated 2-deoxyglucose (DG) uptake by PGRMC1. After treatment with or without 0.5 μmol l−1 insulin for 18 min, 3T3L1 cells (control or PGRMC1 KD) were incubated with 1 μmol l−1 2-DG for 20 min, and the 2-DG uptake was measured. The graph shows relative fold change by normalizing with 2-DG uptake of 3T3L1 control cells without treatment of insulin (n = 4). b Analysis of the effect of fatty acid synthesis by PGRMC1 using [13C6]-glucose. After differentiated 3T3L1 cells (control and KD) were incubated with 4.5 g l−1 [13C6]-glucose for 24 h, the fatty acids were extracted. [13C6]-glucose, [13C4-12]palmitic acid, [13C4-16]stearic acid, and [13C4-16]oleic acid in cells were measured by LC/MS. (n = 3). See Supplementary Fig. 7 for more detail. c Analyses of regulation of the GLUT4 translocation by PGRMC1. Plasma membrane fractions were extracted from 3T3L1 cells (control and PGRMC1 KD) treated with or without insulin. The expressed proteins in the plasma membrane or whole-cell lysate were detected by western blotting using antibodies against GLUT4, GLUT1, PGRMC1, phosphorylated Akt (pAkt), Akt, Na-K ATPase α1, or GAPDH. d Co-immunoprecipitation assay for interaction between PGRMC1 and GLUT4. FLAG-PGRMC1 WT or Y113F was overexpressed in 3T3L1 cells and immunoprecipitated with anti-FLAG antibody-conjugated beads. Co-immunoprecipitated proteins were detected with western blotting using anti-PGRMC1 or anti-GLUT4 antibody. e Co-immunoprecipitation assay for interaction between endogenous PGRMC1 and GLUT4. 3T3L1 cell lysate was incubated with anti-PGRMC1 antibody or normal rabbit IgG, and then incubated with 10 μl of protein A-sepharose beads. Co-immunoprecipitated proteins were detected with western blotting using anti-PGRMC1 or anti-GLUT4 antibody. All data are represented as mean ± S.E. Statistical analysis was performed using Student’s t test. *P < 0.05.

turn facilitated glucose uptake, resultant de novo fatty acid synthesis in 3T3L1 cells.

CO interferes with the PGRMC1-mediated lipid and glucose uptake in adipocyte. Haem-dimerized PGRMC1 interacted with LDL-R and GLUT4 and contributed to cellular uptake of LDL/VLDL and glucose (Figs. 3 and 4). We have previously shown that physiological actions of carbon monoxide (CO) derived from haem oxygenase (HO)36–39, and PGRMC1 accounts for a receptor of CO which interferes with the haem-stacking dimerization of PGRMC123, raising a possibility that CO interferes with the PGRMC1-mediated lipid and glucose uptake in the adipocyte. To examine whether CO interferes with LDL uptake, we examined the effect of LDL uptake in 3T3L1 cells by treating the cells with a CO-releasing molecule (CO-RM)37,40. Analyses with fluorescence microscopy or flow cytometry revealed that the fluorescent LDL uptake was decreased by the addition of the CO-RM (Fig. 5a, b). Furthermore, the plasma membrane distribution of LDL-R and VLDL-R was inhibited by treatment with CO-RM (Fig. 5c). We also showed that treatment with CO-RM inhibited the 2-DG uptake (Fig. 5d). The PGRMC1-mediated GLUT4 translocation in the plasma membrane was inhibited by treatment of CO-RM, but not in the whole-cell lysates (Fig. 5e). To evaluate the role of CO gas, regulation of glucose uptake in 3T3L1 cells was analyzed by ectopically expressing haem oxygenase 1 (HO-1), and its H25A mutant lacking the catalytic activity39 (Fig. 5f). As shown in Fig. 5g, the uptake of 2-DG in 3T3L1 control cells was significantly suppressed by expression of HO-1 WT, but not by that of the H25A mutant. The inhibitory effect of 2-DG uptake by HO-1 expression was at the same level as that by PGRMC1 KD. By contrast, no inhibitory effect by HO-1 expression was observed in the PGRMC1 KD 3T3L1 cells. In addition, the fluorescent LDL uptake or the 2-DG uptake in 3T3L1 cells was significantly elevated by the addition of haemin and the elevation of the LDL or 2-DG uptake by haemin was not observed in the PGRMC1 KD cells (Supplementary Fig. 8a, b). These results suggested that CO interferes with the uptake of lipid and glucose uptake in adipocyte, by interfering with the heme-mediated PGRMC1 dimerization. A recent report showed that PGRMC1 in mitochondria contributes to regulating haem biosynthesis via interaction with ferrochelatase, which catalyzes
Further- more, it has been reported that PGRMC1 in mitochondria-associated membranes transfers haem to PGRMC2, which is highly homologous to PGRMC1 and is required for thermogenesis in brown adipocyte. Therefore, we analyzed the cellular distribution of PGRMC1 in 3T3L1 cells (Supplementary Fig. 9a, b). PGRMC1 mainly localized in the perinuclear region in 3T3L1 cells, and its distribution was observed in both of ER (Concanavalin A staining) and mitochondria (MitoTracker). No significant change of PGRMC1 distribution was observed by the addition of insulin, haemin, or CO-RM. This suggests that PGRMC1 located in the ER contributes to nutrients uptake in adipocyte by regulating protein transport of low-density lipoprotein receptors (LDL-R) and GLUT4 to the plasma membrane, independently of its function of haem biosynthesis in the mitochondria.

PGRMC1 contributes to the progression of adipocyte hypertrophy in mice. To determine roles of PGRMC1 in adipose tissue in vivo, we generated PGRMC1 flox/flox mice in which PGRMC1 exon 2 was flanked with two flox sites in C57BL/6J (WT) mice, and crossed them with Adiponectin-Cre mice to create adipose tissue-specific PGRMC1-knockout (AKO) mice (Supplementary Fig. 10a). We confirmed that the PGRMC1 expression was specifically disrupted in WAT of the PGRMC1-AKO mice by western blotting (Supplementary Fig. 10b). Next, we examined the adipose tissue progression of WT or PGRMC1-AKO mice fed with normal diet (ND) or high-fat diet (HFD) for 12 weeks. Under these conditions, the amounts of food intake did not change between WT and PGRMC1-AKO mice fed HFD (Supplementary Fig. 11a). As shown in Fig. 6a, while the weights of WAT (perirenal and subcutaneous) and brown adipose tissue (BAT) in WT mice fed HFD significantly increased compared to...
those fed ND, such increases were suppressed in PGRMC1-AKO mice. In addition, the liver weights did not change between WT and AKO mice fed either ND or HFD. The body weights of PGRMC1-AKO mice were significantly decreased compared to those of WT mice irrespective of being fed with either ND or HFD (Supplementary Fig. 11b), suggesting that PGRMC1-KO mice exhibit a reduction of body weight gain, at least in part due to reduced adipose tissue development. The plasma membrane translocation of LDL-R and GLUT4 by disruption of PGRMC1 in adipocytes resulted in an increase in blood lipids. We also conducted glucose tolerance test (GTT); however, there was no significant difference between WT mice and PGRMC1-AKO mice (Supplementary Fig. 13). Serum insulin levels were elevated in mice fed HFD compared to mice fed ND, but did not change between WT and AKO mice fed either ND or HFD (Table 1). Analyses using a metabolic cage showed that no significant change of V\textsubscript{O2}, respiratory quotient (RQ), heat production, and voluntary activity was observed between WT and PGRMC1-AKO mice during light or dark cycles (Supplementary Fig. 14).

We subsequently analyzed adipose tissue histologically. As observed in Fig. 6b, adipocyte hypertrophy was observed in mesenteric and subcutaneous WAT of WT mice fed HFD; however, the hypertrophy was significantly suppressed in PGRMC1-AKO mice fed HFD. In BAT tissues, the formation of large lipid droplets was observed in WT mice fed HFD, but it was suppressed in PGRMC1-AKO mice (Fig. 6c). In the liver tissue, significant lipid accumulation was observed in both the WT and PGRMC1-AKO mice fed HFD (Supplementary Fig. 15a). Supporting this, intrahepatic contents of TG in PGRMC1-AKO mice fed HFD were unchanged as compared with those in the WT mice (Supplementary Fig. 15b). Further analyses of the adipose tissues were carried out to compare alterations in the expression of genes related to lipid metabolism between WT and PGRMC1-KO mice. In WAT, expression of PGRMC1 was significantly enhanced in WT mice by HFD, which was similar as compared to that in WT mice treated with TZD (Fig. 6d, e). In
addition, expression of PPARγ or FABP4 was also enhanced significantly in WT mice by HFD, but not in PGRMC1-AKO mice (Fig. 6d, e). This suggested that inhibition of PGRMC1 downregulated the PPARγ or FABP4 expression through the reduction of lipid accumulation in WAT, while PPARγ was an upstream factor of PGRMC1. In BAT, we analyzed not only the expression of PGRMC1 and PPARγ but also the expression of genes responsible for thermogenesis (PPARα, PGC1-α, UCP1, and CPT1b) (Supplementary Fig. 16a). In contrast to the results in WAT, the PGRMC1 expression in BAT was unchanged in WT mice fed HFD, and the expressions of PPARγ, PPARα, PGC1-α, and Cpt1b were not altered in PGRMC1-AKO. The gene expression levels of PGRMC1, PPARγ, and LDL-R in the liver did not change between WT and PGRMC1 AKO (Supplementary Fig. 16b). Thus, these data suggest that HFD enhances the PGRMC1 expression in WAT and contributes toward the progression of WATs to cause obesity.

**Discussion**

PGRMC1 is highly expressed in a variety of cancer cells and is involved in their proliferation and chemoresistance; however, its physiologic roles or regulation of PGRMC1 expression have remained largely unknown. This study revealed that the PGRMC1 is induced in adipocytes to regulate adipogenesis through mechanisms involving transcription factors such as PPARγ and CREB/ATF via PPRE or CRE of the regulatory regions of the PGRMC1 gene. In mice, the expression of PGRMC1 was enhanced by HFD or by treating with TZD. However, its expression in the liver and BAT is not altered by HFD, suggesting that the action of PGRMC1 in the regulation of adipogenesis occurs specifically in WAT. The PPARγ expression was also enhanced in WAT by HFD but not in the liver and BAT, suggesting that induction of the PPARγ expression enhances the PGRMC1 expression in vivo. On the other hand, the PPARγ expression was reduced by PGRMC1 KD in 3T3L1 cells or by PGRMC1 KO in adipose tissues. It has been reported that the PPARγ expression in 3T3-F442A cells was downregulated by knockdown of CD36, which is required for lipid accumulation by upregulating fatty acid uptake44. This suggests that inhibition of PGRMC1 downregulates the PPARγ expression by reducing lipid accumulation in adipocytes. PPARγ is known to regulate the lipid accumulation by transactivating the expression of several genes, such as for LDL-R45, lipoprotein lipase (LPL), and CD3646 for facilitating the uptake of lipids, or GLUT447,48 for stimulating glucose uptake. However, the intervening mechanisms by which PPARγ simultaneously stimulates both LDL-R-mediated lipid uptake and the GLUT4-dependent fatty acid synthesis remain to be unveiled. This study revealed that the expression of PGRMC1 is induced by insulin and/or PPARγ to stimulate lipid accumulation in adipocytes. In addition, the C/EBP-β expression, early-stage marker during adipocyte differentiation, was not affected by suppression of PGRMC1. Therefore, our results indicate that PGRMC1 is a novel target gene regulated by insulin and PPARγ for lipid accumulation in the adipocyte.

To date, PGRMC1 is known to interact with several proteins, including EGFR21,23, cytochrome P45024,25, plasmaminogen activator inhibitor RNA-binding protein 1 (PAIR-BP)48, or tubulin49. We previously reported that the haem-mediated dimer of PGRMC1 is necessary for the interaction with these molecules, such as EGFR and cytochrome P450s.23 This study showed that haem-dimerized PGRMC1 interacts with LDL-R or VLDL-R and regulates their localization on the plasma membrane. It is known that LDL-R or VLDL-R translocates from the ER or endosome to the plasma membrane, and regulates LDL or VLDL uptake via endocytosis11-13. It has been shown that PGRMC1, which is located in the ER and endosomes, is thought to play an important role in regulating the intracellular protein translocation, as PGRMC1 contains several YXXφ motifs that are implicated in vesicle transport and endocytosis50-52. Therefore, our results suggested that PGRMC1 would contribute to the plasma membrane translocation of interacting proteins such as LDL-R or VLDL-R via the regulation of vesicle transport. Recently, Bartuzi et al.53 showed that the COMM/CCDC22/CCDC93 (CCC) and the Wiskott–Aldrich syndrome protein and SCAR homolog (WASH) complexes regulate endosomal sorting and lysosomal degradation of LDL-R. Although further study is needed, PGRMC1 might involve in such regulation of endosomal trafficking of LDL-R or VLDL-R.

We showed that haem-dimerized PGRMC1 also interacts with GLUT4, and regulated its translocation into the plasma membrane to facilitate glucose uptake in adipocytes. Recently, it has been reported that PGRMC1 directly interacts with insulin receptor (IR) in lung cancer cells, but PGRMC1 knockdown did not change the phosphorylation of Akt, which is downstream of IR30. Supporting this report, our analysis also revealed that PGRMC1 did not affect the Akt phosphorylation induced by insulin in 3T3L1 cells. These results suggested that the direct interaction of PGRMC1 with GLUT4 contributed to the regulation of GLUT4 translocation, leading to stimulation of glucose uptake in adipocytes. In addition, PGRMC1 knockdown did not affect plasma membrane localization of GLUT1, which is constitutive glucose transporter. These suggest that PGRMC1 could regulate insulin-induced glucose uptake by promoting GLUT4 translocation in the adipocyte.

Another important result in this study is that either HO-1 induction or CO interferes with the uptake of LDL and glucose in

**Table 1 Analyses of blood biochemistry of WT and PGRMC1-AKO mice fed ND or HFD.**

|          | WT ND        | AKO ND  | WT HFD      | AKO HFD     |
|----------|--------------|---------|-------------|-------------|
| TG       | 54.4 ± 5.6   | 47.1 ± 2.9 | 50.2 ± 4.1 | 81.3 ± 7.1† |
| Total cholesterol | 109.7 ± 11 | 110.0 ± 1.6 | 134.2 ± 1.3† | 132.1 ± 3.7† |
| HDL cholesterol | 99.5 ± 1.8 | 96.7 ± 1.3 | 109.1 ± 1.2 | 99.3 ± 1.1   |
| LDL/VLDL cholesterol | 10.2 ± 2.0 | 13.3 ± 2.3 | 25.1 ± 0.9† | 32.8 ± 2.5† |
| Glucose (12 weeks) | 160.8 ± 4.3 | 163.6 ± 10.3 | 189.8 ± 20.3 | 194.3 ± 10.6 |
| Glucose (16 weeks) | 216.8 ± 9.5 | 221.0 ± 11.2 | 262.8 ± 7.2† | 277.4 ± 14.2# |
| Insulin  | 1.1 ± 0.3    | 1.9 ± 0.8 | 8.6 ± 2.0†  | 8.4 ± 2.5#  |
| ALT      | 18.9 ± 1.5   | 25.6 ± 0.8 | 40.1 ± 5.6† | 38.7 ± 3.9   |
| AST      | 11.4 ± 0.8   | 15.2 ± 0.6 | 24.1 ± 6.5  | 22.8 ± 5.5   |

LDL, low-density lipoproteins; VLDL, very-low-density lipoproteins; HDL, high-density lipoproteins; PGRMC1, progesterone receptor membrane-associated component 1; ND, normal diet; HFD, high-fat diet. TG, triglycerides.

Plasma biochemical parameters were analyzed using WT and PGRMC1-AKO mice after 16 weeks fed ND or HFD. Data are represented as mean ± S.E. Statistical analysis was performed using ANOVA with Tukey’s test. *P < 0.05 (mice fed HFD vs mice fed ND). †P < 0.05 (AKO mice fed HFD vs WT mice fed HFD).
3T3L1 cells. There have been considerable numbers of experimental data showing that obesity is suppressed by the induction of HO or by the administration of CO-RM34-37. However, reception mechanisms of CO and mechanisms by which CO suppresses obesity remained largely unknown. While further studies are necessary to inquire which endogenous HO product, CO or biliverdin/bilirubin, is responsible for attenuation of obesity in vivo38, the current observation showing crucial roles of heme-mediated PGRMC1 dimerization led us to suggest that CO serves as a modulator of adipogenesis to inhibit plasma membrane translocation of LDL/VLDL-R and GLUT4 by dissociating the heme-mediated dimer. Therefore, investigation to unveil the HO-1/CO/PGRMC1 axis deserves further translational studies to control obesity and adipocyte hypertrophy.

In a mice study, we demonstrated that PGRMC1-AKO mice reduced the increases of fat mass and adipocyte hypertrophy induced by HFD, suggesting that PGRMC1 deficiency decreases the lipid storage in adipose tissue. PGRMC1-AKO did not affect the TG and LDL/VLDL cholesterol in the serum of the mice fed ND, but these levels were increased in the mice fed HFD. Whereas PGRMC1 contributed to GLUT4-regulated glucose uptake in 3T3L1 cells, no significant difference was observed between WT mice and PGRMC1-AKO mice by GTT analysis. Given that the skeletal muscle is known to be the major tissue for insulin-regulated glucose uptake (80–95%)39,60, the regulation of glucose uptake in adipocytes by PGRMC1 would not result in significant changes of blood glucose. Collectively, these results suggest that PGRMC1 is enhanced by HFD, leading to promoting the HFD-induced lipid accumulation in adipose tissue. PGRMC2 has been recently reported to contribute to brown adipocyte progression induced by HFD, suggesting that PGRMC1 deficiency decreases the lipid storage in adipose tissue. The model for the regulatory mechanisms mediated PGRMC1 dimer formation in regulating lipid accumulation in the WAT through a PGRMC2-independent pathway from PGRMC1 established in our study, as PGRMC2 is a type of lipid uptake Fatty acid synthesis via LDL-R or VLDL-R, or de novo fatty acid synthesis. The adipocyte-specific PGRMC2 knockout mice showed decreased BAT mass, thermogenesis, and expression of thermogenic genes, such as UCP1 or PGC1α. However, such HFD-fed mice showed no significant change in body weight or VAT mass42. We observed during our analyses that PGRMC1 AKO significantly decreased the body weight and VAT mass without any significant suppression of thermogenesis and UCP1 or PGC1α expressions in the BAT. Although further PGRMC1 functional analyses would be required in the BAT, these results indicate that PGRMC1 contributes to fat accumulation in the WAT through a PGRMC2-independent mechanism.

In conclusion, this study presents the pivotal roles of heme-mediated PGRMC1 dimer formation in regulating lipid accumulation in adipocytes. The model for the regulatory mechanisms by PGRMC1 is illustrated in Fig. 7. When insulin signaling induces adipogenesis, the PGRMC1 gene expression is transactivated by ATF/CREB and PPARY. The heme-dimerized PGRMC1 interacts with LDL-R, VLDL-R, or GLUT4 and facilitates their translocation to the plasma membrane. Consequently, PGRMC1 contributes to lipid accumulation in adipocytes by regulating the lipid uptake via LDL-R or VLDL-R, or de novo fatty acid synthesis.

Fig. 7 Schematic model for the regulation of lipid accumulation in adipocytes by progesterone receptor membrane-associated component 1 (PGRMC1). When insulin signaling induces adipogenesis, the PGRMC1 gene expression is transactivated by ATF/CREB and PPARy. The heme-dimerized PGRMC1 interacts with LDL-R, VLDL-R, or GLUT4 and facilitates their translocation to the plasma membrane. Consequently, PGRMC1 contributes to lipid accumulation in adipocytes by regulating the lipid uptake via LDL-R or VLDL-R, or de novo fatty acid synthesis.

A lexia fluor 488-acetylated LDL were purchased from Life Technologies, Carlsbad, CA. Dil–VLDL was purchased from Kalen Biomedical, Germantown, MD. Bilirubin and fetal bovine serum (FBS) were purchased from BioWest, Nuaillé, France. Isobutylmethylxanthine (IBMX), CO-releasing molecule (CORM), FLAG peptide, anti-FLAG antibody-conjugated agarose and [13C6]-glucose were purchased from Sigma-Aldrich, St. Louis, 2-deoxyglucose (2-DG) was purchased from the Tokyo chemical industry, Tokyo, Japan.

### Antibodies
The antibodies were purchased from the following manufacturers: anti-GAPDH antibody (Santa Cruz Biotechnology; sc-25778), anti-PGRMC1 antibody for western blotting (Cell Signaling: 13858S), anti-PGRMC1 antibody for immunofluorescent staining (Abcam: ab48012), anti-PPARY antibody (Abcam: ab99256), anti-FABP4 antibody (Abcam: ab66682), anti-LDL-R antibody (R&D: AF2255), anti-VLDL-R antibody (R&D: AF2258), anti-TF-R antibody (Abcam: ab60039), anti-Na-K ATPase α1 antibody (Abcam: ab6771), anti-GLUT4 antibody (Abcam: ab115730), anti-GLUT4 antibody (Abcam: ab33780), anti-Akt antibody (Cell Signaling: #9272S), anti-p-Akt antibody (Cell Signaling: #4060S), and anti-HO-1 antibody (Santa Cruz Biotechnology; sc-136960).

### Constructions of plasmids
Mouse PGRMC1 cDNA was cloned from cDNA library of 3T3L1 cells using the primers (forward: 5′-TTTGAATTCATGGGCGAGGAGTGTGTTTT-3′, reverse: 5′-TTTGGACTCCTGCTCTCAGGCAGCAGTGTC-3′), digested with Eco RI and BamHI, and then ligated into the C-terminus FLAG-epitope-tagged expression vector, pxFLAG-CMV-14 (Sigma). The Y113F mutant of mouse PGRMC1 was prepared using the primers (sense: 5′-CTTGGACTCCTGCTCTCAGGCAGCAGTGTC-3′, antisense: 5′-CCACAATGGGCCTGAGG-3′). The PGRMC1 fragment containing resistant sequences for shRNA (KD#1) was prepared using the primers (sense: 5′-CTTGGACTCCTGCTCTCAGGCAGCAGTGTC-3′, antisense: 5′-CTTGGACTCCTGCTCTCAGGCAGCAGTGTC-3′).

Mouse PGRMC1 promoter gene (−1645~+1) was cloned from the mouse genomic library (Clontech) using primers (forward: 5′-TTTGTCTGTAAGCATGTGGTTGCCTAGTGGTT-3′, reverse: 5′-TTTAAAGCTGTCCTCTCAGCAGAAGAGGTTG-3′), digested with Eco RI and BamHI, and then ligated into the luciferase reporter vector pGL3-Basic (PROMEGA). The truncated sequences of mouse PGRMC1 promoter were prepared using primers (sense: 5′-TTTGCATAGCGGTCCCTGGGCTTTG-3′ for +481 to −119, 5′-TTTGCATAGCGGTCCCTGGGCTTTG-3′ for −119 to −56, 5′-TTTGCATAGCGGTCCCTGGGCTTTG-3′ for −56 to −345, 5′-TTTGCATAGCGGTCCCTGGGCTTTG-3′ for −119 to −345, 5′-TTTGCATAGCGGTCCCTGGGCTTTG-3′ for −56 to −345, and 5′-TTTGCATAGCGGTCCCTGGGCTTTG-3′ for −119 to −345). For the construction of the PPRE-containing reporter plasmids, three tandem-repeated PPRE fragments (5′-ACCGGGCGAGCCAGCGAGAGAG-3′ × 3) or mutated fragments (5′-ACCGGGCGAGCCAGCGAGAGAG-3′ × 3) were introduced into the Nhe I/Xho I site upstream of the SV40 promoter region of pGL3-control vector (PROMEGA).

### Methods

#### Materials
Dulbecco’s modified Eagle’s medium (DMEM), insulin, dexamethasone, troglitazone, protase inhibitor cocktail III, and diamidophenylindole (DAPI) were purchased from Wako, Osaka, Japan. Bovine serum (BS) and 2-deoxyglucose (2-DG) were purchased from Sigma-Aldrich, St. Louis. 2-deoxyglucose (2-DG) was purchased from the Tokyo chemical industry, Tokyo, Japan.
Human HO-1 (wild-type and H25A mutant) cDNA was amplified with the primers (Hango Fwd-HindIII: 5′-CCAAGATCTGCTCTTCGAG-3′, Hango Rev-BamHI: 5′-CGGATCCGCCATGGCATAAAGCCC-3′). PCR fragments were digested with HindIII/BamHI, and then ligated into p3xFLAG-CMV10 vector (SIGMA).

**Cell culture and treatments.** 3T3L1 cells were maintained in DMEM containing 10% bovine serum (BS) at 37 °C under 5% CO₂. To prepare two types of stable PGRMC1-knockdown (PGRMC1 KD) cell lines, 3T3L1 cells were transfected with lentiviral encoding a non-sense DNA sequence targeting PGRMC1, according to the manufacturer’s instructions (Life Technologies, Carlsbad, CA). Stable cell lines were selected by maintaining the cells in a medium supplemented with 10 mg·l⁻¹ blasticidin for 1 week.

Differentiation of 3T3L1 cells was induced by changing the culture medium to DMEM containing 15% FBS, 0.25 mmol·l⁻¹ dexamethasone, 0.5 mmol·l⁻¹ IBMX, and 0.2 mmol·l⁻¹ insulin (day 0). After 2 days, the culture medium was changed to DMEM containing 10% FBS and 0.2 mmol·l⁻¹ insulin (day 2). Two days later (day 4), the medium was changed to DMEM containing 10% FBS. Thereafter, the medium was changed to DMEM containing 10% FBS every day. The differentiated 3T3L1 cells were used on day 4 after inducing differentiation.

To determine the effect of thiazolidinedione (TZD), troglitazone dissolved in ethanol was added to the culture medium for the T2D group at a final concentration of 15 μmol·l⁻¹, and the cells were incubated for 48 h. For the analysis of the effect by TZD on 3T3L1 differentiation, 3T3L1 cells were treated with 15 μmol·l⁻¹ TZD every 2 days in addition to differentiation inducers as described above.

For the analysis of LDL uptake, cells were incubated in serum-free DMEM supplemented with Alexa Fluor 488-acetylated LDL to a final concentration of 3 mg·l⁻¹ at 37 °C for 60 min. For the analysis of VLDL uptake, cells were incubated in serum-free DMEM supplemented with Dil-VLDL to a final concentration of 10 mg·l⁻¹ at 37 °C for 60 min.

For the analysis of expression in plasma expression, plasma membrane proteins were prepared using a plasma membrane isolation kit (Invent Biotechnologies, Inc., Plymouth, MN, USA). Briefly, 3T3L1 cells were collected and lysed in buffer A. The lysate was centrifuged at 16,000 × g for 2 h, and then incubated with 10 μl protein A-sepharose IgG (Abcam: ab37415) for 2 h, and then incubated with 10 μl protein A-sepharose IgG (Abcam: ab37415) for 2 h.

Analyses of fatty acid synthesis using [13C6]-glucose in 3T3L1 cells were performed using a flow cytometer (Gallouis, Beckman Coulter Life Science, Brea, CA). Analyses of fatty acid synthesis were performed according to the method by Nagai et al. 62. Differentiated 3T3L1 WT or PGRMC1-KD cells were incubated with a glucose-free DMEM containing 10% FBS and 4.5 g·l⁻¹ of [13C4]-glucose for 24 h. The collected cells were hydrolyzed with 0.5 ml of 20% KOH at 80 °C for 60 min, and later 0.6 ml of 5 N HCl was added. Fatty acids were extracted twice with 0.4 ml diethyl ether, and the extracted solution was dried at 40 °C under N₂.

The flow cytometry analyses. Cells treated with Alexa Fluor 488-acetylated LDL or Dil–VLDL were diluted in PBS containing 1% paraformaldehyde. Thaema fluorescent intensity per 10,000 cells was analyzed using a flow cytometer (Gallouis, Beckman Coulter Life Science, Brea, CA).

Analyses of fatty acid synthesis using [13C6]-glucose in 3T3L1 cells. Analyses of fatty acid synthesis were performed according to the method by Nagai et al. 62. Differentiated 3T3L1 WT or PGRMC1-KD cells were incubated with a glucose-free DMEM containing 10% FBS and 4.5 g·l⁻¹ of [13C6]-glucose for 24 h. The collected cells were hydrolyzed with 0.5 ml of 20% KOH at 80 °C for 60 min, and later 0.6 ml of 5 N HCl was added. Fatty acids were extracted twice with 0.4 ml diethyl ether, and the extracted solution was dried at 40 °C under N₂.

The flow cytometry analyses. Cells treated with Alexa Fluor 488-acetylated LDL or Dil–VLDL were diluted in PBS containing 1% paraformaldehyde.

Animal studies. All the protocols for animal experiments in this study were approved by the Experimental Animal Committee of Keio University School of Medicine [approved number, 08024(10)]. PGRMC1 exon 2 was flanked with loxP sites in C57BL/6 (WT) mice (PGRMC1 fox/flox mice). PGRMC1 fox/flox mice were then crossed with adiponectin-Cre mice, thereby generating PGRMC1 adipose tissue-specific knockout (AKO) mice. All animal studies were performed at 24 °C.

WT and PGRMC1-AKO male mice at 8–11 weeks of age were fed a normal diet (ND: 4.6% kcal fat; source, soybean; CLEA Japan, Inc., Tokyo, Japan) or a HFD (40% kcal fat; Research Diet, Inc., New Jersey, USA). Body weights and amounts of food ingested by each mouse were measured weekly for 12 weeks. All mice were sacrificed 1 week after the mice were completely fed to collect visceral and subcutaneous adipose tissues. Sheath gas at 60, and Aux gas at 20. [13C4]-glucose, [13C4]-paclitaxel, [13C5]-stearic acid, and [13C6]-oleic acid in cells were measured by LC/MS.
LDL/VLDL cholesterol in serum were measured using an HDL and LDL/VLDL cholesterol assay kit (Cell Bioslac, Inc., San Diego, CA). Serum insulin was measured using an Ultra-Sensitive Mouse Insulin ELISA Kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan). WAT, BAT, and livers were immersed in paraffin and stained with hematoxylin & eosin. The stained samples were observed using a microscope (ECLIPSE E600, Nikon, Tokyo, Japan). Each adipocyte size was obtained by calculating the average size of 100 adipocytes with Image J software (National Institutes of Health, Maryland, USA).

To analyze the effect of T2D, troglitazone diluted with PBS was administered to mice by an intraperitoneal injection at a dose of 5 mg kg\(^{-1}\) body weight on 3 consecutive days, and 1 day later, the mice were sacrificed to collect WAT for analyses of PGRMC1 expression with qPCR or western blotting.

**Glucose tolerance test.** Glucose tolerance was examined in WT and PGRMC1-AKO mice 12 weeks after fed either ND or HFD. After fasting for 6 h, glucose was administered intraperitoneally (1.5 mg kg\(^{-1}\) weight). Blood glucose levels were measured by a glucometer (ACCU-CHEK Aviva-Nano, Roche Basel, Switzerland).

**Indirect calorimetry.** WT and PGRMC1-AKO mice 12–13 weeks after fed either ND or HFD were acclimatized in metabolic cages for 24 h to the experimental environment before measurements were taken. Mice were kept on 12 h light–light 12 h dark cycle at 24 °C. Next 24 h, oxygen consumption, respiratory quotient (RQ), heat production, and locomotor activity were measured using the Oxymax System 4.93 (C.L.A.M.S.; Columbus Instruments, Ohio, USA). In addition, spontaneous locomotor activity was measured for 24 h with an OPTO-M3 Activity Application Device (Columbus Instruments, Ohio, USA). We plotted oxygen consumption per mouse in relation to body weight\(^{32}\) and evaluated the average of oxygen consumption, RQ, heat production, and locomotor activity.

**Statistical and reproducibility.** Experimental results are represented as means ± standard error of means. Student’s T test was used for two-group comparisons, and one-way ANOVA with Tukey’s T test was used for multiple comparisons. P values < 0.05 were considered as significant. No statistical methods were used to predetermine the sample size. The sample size was based on experimental feasibility, sample availability, and N necessary to obtain definitive, significant results. The experiments using cell lines were replicated twice. Animals were randomly assigned among the various groups.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** All data supporting the findings of this study are available within the paper and its Supplementary Files or available from the corresponding author upon reasonable request. Source data underlying plots shown in figures are available in Supplementary Data 1. The full, uncropped images of western blots are shown in Supplementary Fig. 17.

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