Deletion of Macrophage Mineralocorticoid Receptor Protects Hepatic Steatosis and Insulin Resistance Through ERα/HGF/Met Pathway

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Although the importance of macrophages in nonalcoholic fatty liver disease (NAFLD) and type 2 diabetes mellitus (T2DM) has been recognized, how macrophages affect hepatocytes remains elusive. Mineralocorticoid receptor (MR) has been implicated to play important roles in NAFLD and T2DM. However, cellular and molecular mechanisms are largely unknown. We report that myeloid MR knockout (MRKO) improves glucose intolerance, insulin resistance, and hepatic steatosis in obese mice. Estrogen signaling is sufficient and necessary for such improvements. Hepatic gene and protein expression suggests that MRKO reduces hepatic lipogenesis and lipid storage. In the presence of estrogen, MRKO in macrophages decreases lipid accumulation and increases insulin sensitivity of hepatocytes through hepatocyte growth factor (HGF)/Met signaling. MR directly regulates estrogen receptor 1 (Esr1 [encoding ERα]) in macrophages. Knockdown of hepatic Met eliminates the beneficial effects of MRKO in female obese mice. These findings identify a novel MR/ERα/HGF/Met pathway that conveys metabolic signaling from macrophages to hepatocytes in hepatic steatosis and insulin resistance and provide potential new therapeutic strategies for NAFLD and T2DM.

Nonalcoholic fatty liver disease (NAFLD) is intimately intertwined with insulin resistance and type 2 diabetes mellitus (T2DM). The ectopic lipid accumulation in hepatocytes in the process of NAFLD directly or indirectly impairs key components of the insulin signaling pathway and markedly increases the risk of T2DM (1,2). Insulin resistance and T2DM, on the other hand, impair hepatic metabolism and exacerbate NAFLD. With the increasing prevalence of T2DM, NAFLD affects more and more individuals worldwide.

Kupffer cells (KCs) and their interactions with hepatocytes play essential roles in hepatic steatosis and insulin resistance. KCs are a group of specific macrophages that reside in the liver. Depletion of KCs improves hepatic steatosis and insulin resistance (3). Given the evolutionary homology, KCs and hepatocytes are situated in proximity

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with each other in the liver, allowing efficient communications between these cell types (4). Studies have focused on inflammation as the link from KCs to hepatocytes, although the key connections are still elusive. For instance, tumor necrosis factor-α has been implicated as such a connecting point (3). However, macrophage tumor necrosis factor-α plays only negligible roles in hepatic steatosis and insulin resistance (5). Moreover, much less is known about the impacts of macrophage-derived metabolic factors on hepatocytes. These metabolic factors may be particularly important in the early stage of simple hepatic steatosis without inflammation. Therefore, although the contribution of KCs has been recognized in hepatic steatosis and insulin resistance, the mechanisms mediating the signals from KCs to hepatocytes remain largely unknown.

Mineralocorticoid receptor (MR) may regulate the interactions between macrophages and hepatocytes and therefore play important roles in hepatic steatosis and insulin resistance. MR is a member of the nuclear receptor superfamily. Its functions in the cardiovascular system have been explored (6,7), and MR antagonists are already in clinical use to treat heart failure (8). Antagonists of MR also have been shown to improve hepatic steatosis and insulin resistance in animal models of obesity (9–12), supporting the metabolic benefits of MR blockade. However, little is known about the cellular and molecular mechanisms. Given the importance of macrophages, the role of macrophage MR and whether MR controls metabolic factors and related signaling pathways that regulate macrophage-hepatocyte interaction in the setting of hepatic steatosis and insulin resistance are of great interest.

In this study, we determined whether and how macrophage MR deletion affects hepatic steatosis and insulin resistance. We first studied the effects of myeloid MR knockout (MRKO) on insulin sensitivity, glucose homeostasis, and hepatic steatosis by using obese mouse models. We then explored the impacts of MRKO in macrophages on lipid accumulation and insulin sensitivity of hepatocytes by using a coculture system. Finally, we deciphered the molecular basis and delineated a new signaling pathway involving MR/estrogen receptor-α (ERα)/hepatic growth factor (HGF) and conveyed messages from macrophages to hepatocytes through HGF both in culture and in mice.

RESEARCH DESIGN AND METHODS

Animals

Floxed control (FC) and myeloid MRKO mice were generated as previously described (13) and crossed with Lepfl/fl mice on a C57BL/6 background (The Jackson Laboratory) to obtain the following four experimental groups: FC (MRfl/fl), MRKO (MRfl/fl::LysM-cre), FC-ob (MRfl/fl::Lepob/ob), and MRKO-ob (MRfl/fl::LysM-cre::Lepob/ob). For 17β-estradiol (E2) treatment, 4-week-old male obese mice were implanted with E2 pellets (0.18 mg/pellet; Innovative Research) or placebo subcutaneously as previously described (14). All animal protocols were approved by the Institutional Review and Ethics Board of the Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine, and the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

Glucose and Insulin Tolerance Tests

Glucose tolerance test (GTT) was performed in 10-week-old ob/ob mice with intraperitoneal injection of 1 g/kg glucose after an 8-h fasting. Insulin tolerance test (ITT) was conducted in 11-week-old ob/ob mice with intraperitoneal injection of insulin (1 unit/kg) after a 5-h fasting. For experiments with hepatic Met knockdown, GTT and ITT were performed 3 and 5 days after injection of adenoviruses, respectively.

Tissue Sample Collection

Twelve-week-old ob/ob mice were anesthetized after 5 h of fasting followed by tissue dissection as previously described (15). Briefly, after blood collection, insulin (5 units/kg) was injected into the inferior vena cava. Liver, uterine adipose tissue, and skeletal muscle were collected and snap-frozen in liquid nitrogen. 3, 4, and 5 min after insulin injection, respectively. Tissues were also collected before insulin injection. For experiments with hepatic Met knockdown, random-fed blood glucose was measured 4 days after and tissues collected 7 days after adenovirus injection. Additional blood samples were taken from 11-week-old mice fed ad libitum for insulin measurements.

Histology

Hepatic hematoxylin-eosin (H&E) staining was performed as previously described (16). Oil Red O staining was carried out as previously reported (17), and quantification was performed with Image J software.

Measurement of Triglycerides and Free Fatty Acids

Plasma concentrations of triglycerides (TGs) and free fatty acids (FFAs) were determined by using an automated biochemical analyzer at the Xuhui District Central Hospital (Shanghai, China). To measure hepatic TG and FFA content, lipid extract was prepared and dissolved as previously reported (18). The concentration of TGs was measured at the Xuhui District Central Hospital. TG content was normalized with tissue weight. Hepatic FFAs were measured by gas chromatography-mass spectrometry profiling.

Immunoblotting

Total protein was extracted from frozen tissues or cultured cells. Quantified protein was separated by SDS-PAGE, transferred onto polyvinylidene fluoride membranes, and immunoblotted with primary antibodies and secondary antibodies sequentially. Membranes were visualized by using ECL Western Blotting Substrate (Thermo Fisher Scientific). Band intensity was quantified by using Quantity One software (Bio-Rad) and normalized to total protein or α-tubulin.

The following primary antibodies were used: anti-insulin receptor (IR) (3025S), antiphospho-IR (3024S), anti-Akt (9272S), antiphospho-Akt (Ser473, 9271S), anti-Met (3127), antiphospho-Met (3126S), anti-acetyl-CoA
carboxylase (ACC) (3662), and anti-stearyl-CoA desaturase 1 (SCD-1) (2438) from Cell Signaling Technology; anti-α-tubulin (T6199) and anti-FLAG (F3165) from Sigma-Aldrich; anti-cell death–inducing DNA fragmentation factor-α-like effector a (Cidea) (ab8402) from Abcam; anti-lymphocyte antigen 6 complex, locus D (Ly6d) (17361–1-AP) from Proteintech; and anti-MR (sc-11412) and anti-fatty acid synthase (FAS) (sc-48357) from Santa Cruz Biotechnology.

Lipidomics
Lipid extraction and processing was performed as previously described (19). A derivatization method was used to improve specificity and sensitivity of phosphatidylethanolamine (PE) detection (20). The shotgun lipidomics was carried out with a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific) according to published protocols (21). Tandem mass spectrometry scan fragment and collision energy for each lipid class were optimized according to previously published methods (21).

Cell Culture
Mouse primary hepatocytes were isolated by situ collagenase perfusion as previously described, with minor modifications (22). The cell suspension was further purified with 50% Percoll (GE Healthcare) before counting and plating.

KCs were isolated from female mice as previously reported, with minor modifications (23). After 25–50% Percoll gradient centrifugation, the KC-enriched interlayer was collected and plated. Nonadherent and floating cells were washed off 15–30 min after plating. In a separate experiment, KCs were preincubated with 0.1 μmol/L ICI 182,780 (Tocris Bioscience) for 24 h and then with 10 nmol/L E2 for another 24 h in the presence or absence of ICI 182,780.

For hepatocyte-KC coculture, hepatocytes and KCs were plated successively at a ratio of 10:1, and incubated with 0.1 μmol/L E2 for 5 h after overnight recovery. The cells were then stimulated with FFAs (a combination of 200 μmol/L oleic acid and 100 μmol/L palmitic acid) in the presence of E2. For insulin-stimulated effects, insulin (100 nmol/L) was added to the media after coculture for 20 min.

ELISA
Plasma insulin concentration was determined by Rat Ultrasensitive Insulin ELISA kit (ALPCO). HGF levels were determined by using Mouse/Rat HGF Quantikine ELISA Kit (R&D Systems) according to the manufacturer’s instructions. Plasma E2, dihydrotestosterone, and testosterone levels were measured by using kits from DRG International (EIA-4399 and EIA-5761) and Enzo Life Sciences (ADI-900-065) according to the manufacturer’s protocols.

Met Knockdown in Primary Hepatocytes
Primary hepatocytes were transfected with Met small interfering RNA (siRNA) (GenePharma) by using X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics) according to the manufacturer’s instructions. The sequences of the Met siRNA were: GGAGGUGUUUGAAAGAUATT, UAUCUUUCC AAACACCUCCTCG.

Establishment of a Stable MR Overexpression RAW264.7 Cell Line
A stable MR overexpression (MROV) RAW264.7 cell line was established as previously described (16). The full-length mouse Mr3c2 coding region was amplified and subcloned into pHAGE-feF1a-ires-ZsGreen vector for subsequent transfection.

Construction of Truncated Mouse Esr1 Promoter Luciferase Reporter Plasmids
A 2-kilobase pair (kb) mouse estrogen receptor 1 (Esr1) promoter was amplified and cloned into pGL3-basic vector (Promega) to produce the 2-kb Esr1-luciferase plasmid. Specific primers were used to yield various truncated Esr1-luciferase plasmids. Sequences of these primers are listed in Supplementary Table 1.

Chromatin Immunoprecipitation
Chromatin immunoprecipitation (ChIP) experiments were performed in MROV RAW264.7 cells by using an EZ-ChIP kit (EMD Millipore) according to the manufacturer’s protocols. Cells were cultured with DMEM containing 10% charcoal/dextran-treated FBS. Primer sequences for PCR analysis of ChIP products are listed in Supplementary Table 2.

Generation and Administration of Recombinant Adenoviruses
Recombinant adenoviruses were generated by using the BLOCK-iT Adenoviral RNAi Expression System (Thermo Fisher Scientific) according to the manufacturer’s instructions. The adenoviruses were administrated to 10-week-old female obese mice at a dose of 1 × 10⁹ plaque-forming units/mouse through the tail vein (24). For cell culture experiments, purified adenoviruses were used at a dose of 1 × 10⁷ plaque-forming units/well in 12-well plates. The sequence of scrambled short hairpin RNA (shRNA) was 5′-CCTAAGGTCTAAGTCCCTGGCTC-3′, the sequence of Met-specific shRNA was 5′-CTGTGTGGAAACCCGATATTGT TTA-3′, and the sequence of ERE-specific shRNA was 5′-TA CTACCTGGAGAACGGCCCA-3′.

Statistical Analysis
All data are presented as mean ± SEM. Comparisons between groups were done by Student t test, and comparisons between curves were performed with two-way ANOVA and Bonferroni posttests with GraphPad Prism software. P ≤ 0.05 was considered statistically significant.

RESULTS
Myeloid MR Deficiency Improves Glucose Homeostasis and Insulin Sensitivity in Female ob/ob Mice
We first used the ob/ob mouse model to study the impacts of myeloid MR deficiency on obesity and T2DM. Randomized blood glucose of female MRKO-ob mice was drastically lower than that of FC-ob mice (Fig. 1A). Improved GTT and ITT were also observed in female MRKO-ob mice (Fig. 1B–E). Furthermore, female MRKO-ob mice had markedly lower fasting plasma insulin (Fig. 1F) and significantly lower
plasma TGs (Fig. 1G) than FC-ob mice. Female MRKO-ob and FC-ob mice had comparable body weights, body composition, food consumption, islet areas, and plasma FFAs (Supplementary Fig. 1A–F). Therefore, female MRKO-ob mice displayed improved insulin sensitivity and glucose homeostasis.

We then determined insulin sensitivity in major insulin target tissues of female mice. In liver, phosphorylation of IR (p-IR) and phosphorylation of Akt (p-Akt) were induced by insulin in both FC-ob and MRKO-ob mice, but a much larger increase was observed in MRKO-ob mice (Fig. 1H and I). In uterine adipose tissue and skeletal muscle, insulin-stimulated p-IR and p-Akt were similar between the two genotypes (Supplementary Fig. 1G–J). These results imply that the improved systemic insulin sensitivity of MRKO-ob mice is mostly attributable to enhanced hepatic insulin responsiveness.

**Myeloid MR Deficiency Improves Glucose Homeostasis and Insulin Sensitivity in Male ob/ob Mice Treated With Estrogen**

In contrast to females, male MRKO-ob mice and FC-ob mice showed comparable metabolic parameters (Supplementary Fig. 2). In a model of high-fat diet (HFD)–induced obesity and insulin resistance, we did not observe a significant

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**Figure 1**—Improved glucose homeostasis and insulin sensitivity in female MRKO-ob mice. A–E: Weekly random-fed blood glucose levels, GTT, area under the curve (AUC) of GTT, ITT, and AUC of ITT of female mice. F: Plasma insulin levels of fed or fasted female mice. G: Plasma TGs of female mice. H: Immunoblotting analysis of p-IR, total IR (T-IR), p-Akt (Ser473), and total Akt (T-Akt) in livers of female mice. I: Quantification of immunoblotting results as p-IR/T-IR and p-Akt/T-Akt. n = 6–8. *P < 0.05, **P < 0.01, ***P < 0.001. —, before insulin injection; +, after insulin injection; n.s., not significant.
difference between male FC and MRKO mice (Supplementary Fig. 3). In female mice, HFD failed to induce insulin resistance in either genotype, although body weight gain was observed (data not shown) consistent with previous results (25).

We next tested whether estrogen supplementation would influence the metabolic phenotype of male MRKO-ob mice. Eight weeks of E2 treatment significantly improved random-fed blood glucose, GTT, ITT, fasting plasma insulin, and TGs in male FC-ob and MRKO-ob mice (Fig. 2A–G). Of note, many more improvements were detected in MRKO-ob mice and as a result, E2-implanted MRKO-ob mice had significantly more improved metabolic parameters than E2-implanted FC-ob mice (Fig. 2A–G). No difference in body weight loss or hypophagia was observed between male FC-ob and MRKO-ob mice after E2 treatment (data not shown). Hepatic insulin signaling was also markedly enhanced in E2-implanted MRKO-ob mice compared with that of E2-implanted FC-ob mice (Fig. 2H and I). E2 implantation increased plasma E2 and decreased plasma dihydrotestosterone and testosterone in male FC-ob and MRKO-ob mice to similar levels (Supplementary Fig. 4). Therefore,

**Figure 2**—Improved glucose homeostasis and insulin sensitivity in male MRKO-ob mice treated with estrogen. A–E: Weekly random-fed blood glucose levels, GTT, area under the curve (AUC) of GTT, ITT, and AUC of ITT of male mice. F: Plasma insulin levels of fed or fasted male mice. G: Plasma TGs of male mice. H: Immunoblotting analysis of p-IR, total IR (T-IR), p-Akt (Ser473), and total Akt (T-Akt) in livers of male mice. I: Quantification of immunoblotting results as p-IR/T-IR and p-Akt/T-Akt. n = 5–7. *P < 0.05, **P < 0.01, ***P < 0.001. −, before insulin injection; +, after insulin injection; n.s., not significant; Pl, placebo.
estrogen supplementation was sufficient to elicit metabolic protection of MRKO in male obese mice.

To test the necessity of estrogen for metabolic improvements, female obese mice underwent ovariectomy (OVX), which eradicated E2 in the plasma of both female FC-ob and MRKO-ob mice (Supplementary Fig. 5A). OVX abolished the beneficial effects of MRKO in female obese mice (Supplementary Fig. 5B–E).

Collectively, myeloid MRKO improved insulin sensitivity, glucose homeostasis, and hepatic insulin signaling of obese mice. Estrogen was sufficient and necessary for myeloid MRKO-mediated metabolic protection.

**Myeloid MR Deletion Attenuates Hepatic Steatosis in ob/ob Mice**

Female MRKO-ob mice had a significantly lower liver weight-to-body weight ratio (LW/BW) than FC-ob mice (Fig. 3A). Histology of livers showed much smaller and fewer lipid droplets in female MRKO-ob mice (Fig. 3B). Furthermore, hepatic TG content was markedly reduced in female MRKO-ob mice compared with FC-ob mice (Fig. 3C).

In males, no obvious difference was observed in LW/BW or hepatic TG content between MRKO-ob and FC-ob mice (Supplementary Fig. 6A and B). Similarly, LW/BW of HFD-fed myeloid MRKO mice and that of HFD-fed FC mice were comparable (Supplementary Fig. 6C). After E2 treatment, male MRKO-ob mice manifested decreased LW/BW, lipid droplets, and hepatic TG content compared with male FC-ob mice (Fig. 3D–F). In addition, LW/BW, lipid accumulation and hepatic TG content were comparable between female MRKO-ob and FC-ob mice after OVX (Supplementary Fig. 6D–F).

To investigate the contribution of lipid metabolites, we performed shotgun lipidomics to analyze subclasses of TGs and phospholipids in livers of female mice. The majority of the TG species were significantly downregulated in livers of female MRKO-ob mice compared with FC-ob mice, including the most abundant species C52:2, C52:3, and C54:3 (Fig. 3G). Total content of phosphatidylcholine (PC) were significantly reduced in livers of female MRKO-ob mice (Supplementary Fig. 7A). Consistently, the majority of the individual PC species were decreased in livers of MRKO-ob mice compared with FC-ob mice (Fig. 3H). No significant difference was found in other phospholipid classes between MRKO-ob and FC-ob mice (Supplementary Fig. 7A–C). In addition, hepatic fatty acids were reduced in MRKO-ob mice compared with FC-ob mice (Supplementary Fig. 7D–F). These data suggest that myeloid MRKO alleviates hepatic steatosis in female ob/ob mice, particularly by decreasing the content of TGs and fatty acids as well as altering the composition of phospholipid species.

**Myeloid MR Deletion Downregulates Molecules Important in Hepatic Lipogenesis and Lipid Storage in Female ob/ob Mice**

SCD-1 is a rate-limiting enzyme in hepatic de novo lipogenesis (26). Results of quantitative RT-PCR (qRT-PCR) demonstrated that hepatic expression of SCD-1 was significantly increased in FC-ob mice and attenuated in MRKO-ob mice (Fig. 4A). MRKO-ob mice also had markedly lower hepatic gene expression of Ly6d (Fig. 4A), which was reported to be positively associated with hepatic fat content (27,28). Gene

**Figure 3**—Attenuated hepatic steatosis in female and E2-implanted male MRKO-ob mice. A and B: LW/BW and hepatic H&E staining of female mice (n = 6–8). Scale bar = 200 μm. C: Hepatic TGs of female mice (n = 6–8). D and E: LW/BW and hepatic H&E staining of male obese mice implanted with placebo (Pl) or E2 (n = 5–7). Scale bar = 500 μm. F: Hepatic TGs of male obese mice implanted with Pl or E2 (n = 5–7). G: Quantification of TG species based on lipidomic analysis of hepatic lipids extracted from female mice (n = 4). Data are ratios of the MRKO-ob value of each species relative to the mean value of FC-ob group after normalization with hepatic protein concentration. Red represents higher and green lower concentration in MRKO-ob mice compared with FC-ob mice. H: Quantification of PC species based on lipidomic analysis of hepatic lipids from female mice (n = 4). Data are presented the same as G. *P < 0.05, **P < 0.01, ***P < 0.001. n.s., not significant.
expression of Cidea sharply increased in FC-ob mice compared with FC mice and drastically decreased in MRKO-ob mice compared with FC-ob mice (Fig. 4A). Cidea is a key player in hepatic lipid storage, which is both sufficient and necessary for hepatic steatosis in ob/ob mice (29,30). Expression of other lipogenesis-related genes, such as FAS and ACC, was not affected by MRKO (Fig. 4A). Consistent with the results of gene expression, immunoblotting results demonstrated that protein levels of SCD-1, Ly6d, and Cidea were markedly lower in livers of MRKO-ob mice than in FC-ob mice, whereas levels of FAS and ACC were not significantly different between these two genotypes (Fig. 4B and C). No obvious difference was detected in expression of genes related to β-oxidation or inflammation between FC-ob mice and MRKO-ob mice (Supplementary Fig. 8). These data suggest that myeloid MRKO reduces hepatic lipogenesis and lipid storage in female ob/ob mice, contributing to the improvement of hepatic steatosis.

**MRKO KCs Reduce Lipid Accumulation and Increase Insulin Sensitivity of Hepatocytes Through an ERα/HGF/Met Axis**

Depletion of monocytes/macrophages by using clodronate liposomes abolished the metabolic protection of MRKO and resulted in comparable insulin sensitivity and hepatic lipid content between female FC-ob and female MRKO-ob mice (Supplementary Fig. 9), suggesting that MRKO in macrophages, but not in other cells of the myeloid lineage, is essential to metabolic protection. We next used KCs, the resident macrophages in the liver, to study their interactions with hepatocytes.

Primary KCs from female FC mice (FCKCs) and KCs from female MRKO mice (KOKCs) were characterized (Supplementary Fig. 10A–F) and then cocultured with wild-type primary hepatocytes. Physiological concentration E2 was added to mimic the microenvironment in females. KOKCs significantly reduced lipid accumulation in hepatocytes compared with FCKCs (Fig. 5A and B). Staining with BODIPY 493/503 also demonstrated much fewer lipid droplets in the hepatocyte-KOKC coculture system (Supplementary Fig. 10G). Furthermore, when cocultured with KOKCs, insulin-induced p-IR and p-Akt were significantly increased in hepatocytes, indicating improved insulin sensitivity (Fig. 5C and D). Together, these findings demonstrate the protective roles of KOKCs in lipid accumulation and insulin sensitivity of hepatocytes.

HGF, which is secreted by nonparenchymal cells, including macrophages (31), and its receptor Met play essential roles in the maintenance of hepatic insulin sensitivity and glucose homeostasis (32). Of note, when activated by E2, ER binds to the promoter of Hgf to facilitate its expression (33,34). Therefore, we asked whether HGF is associated with the beneficial effects of KOKCs on hepatocytes. Immunofluorescence staining demonstrated that HGF is expressed in KCs (Supplementary Fig. 11A). Depletion of
MR deficiency in KCs reduces lipid accumulation and increases insulin sensitivity in hepatocytes through the ERa/HGF/Met pathway. A: Oil Red O staining of hepatocytes cocultured with FCKC or KOKC in the presence of FFAs and E2. Scale bar = 200 μm. B: Quantification of Oil Red O staining. C: Immunoblotting analysis and quantification of p-IR in hepatocytes from the above-described coculture system. D: Immunoblotting analysis and quantification of p-Akt (Ser473) in hepatocytes from the above-described coculture system. E: ELISA analysis of HGF in supernatants of FCKCs and KOKCs in the presence of FFAs and E2. F: Immunoblotting analysis and quantification of phosphorylated Met (p-Met) in hepatocytes cocultured with FCKCs or KOKCs in the presence of FFAs and E2. G: Immunoblotting analysis of p-Met and total Met (T-Met) in hepatocytes after transfection with scrambled siRNA or Met siRNA. Cells were stimulated with 40 ng/mL HGF for 15 min before collection. H: Quantification of Oil Red O staining of hepatocytes transfected with scrambled siRNA (−) or Met siRNA (+) followed by coculture with FCKCs or KOKCs in the presence of E2 and FFAs. I: Immunoblotting analysis and quantification of p-IR in hepatocytes from the coculture system in H. J: Immunoblotting analysis and quantification of p-Akt (Ser473) in hepatocytes from the coculture system in H. K: qRT-PCR analysis of Cidea gene expression in hepatocytes from the coculture system in H. Data are from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. Hep, hepatocytes; −, before insulin injection; +, after insulin injection; n.s., not significant; Sc, scrambled.
monocytes/macrophages by using clodronate liposomes significantly decreased HGF secretion in nonparenchymal cells from livers and erased the difference between female FC-ob and MRKO-ob mice, suggesting that KCs are a major source of HGF in the liver (Supplementary Fig. 11B). In the presence of E2 and FFAs, KOKCs secreted more HGF than FCKCs, and the upregulation was blocked by ICI 182,780, an antagonist of ERα, suggesting that ERα mediates these effects (Fig. 5E). Knockdown of ERα also blocked the increase of HGF secretion in KOKCs (Supplementary Fig. 11C and D). Conversely, overexpression of ERα increased HGF secretion in KCs (Supplementary Fig. 11E and F).

Phosphorylation of Met (p-Met) (HGF receptor) was enhanced in hepatocytes cocultured with KOKCs compared with those cocultured with FCKCs in the presence of E2 (Fig. 5F). Knockdown of Met in hepatocytes by using siRNA (Fig. 5G) blocked the impacts of KOKCs on the reduction of lipid accumulation in hepatocytes (Fig. 5H). Furthermore, enhanced insulin signaling in hepatocytes cocultured with KOKCs was abolished by Met knockdown (Fig. 5I and J). In parallel, the downregulation of Cidea gene expression in hepatocytes cocultured with KOKCs was also blocked by Met knockdown (Fig. 5K). Taken together, these data indicate that an ERα/HGF/Met axis plays an indispensable role in mediating the beneficial effects of KOKCs on the metabolic improvements of hepatocytes.

**MR Directly Regulates Esr1 in Macrophages**

Results of qRT-PCR showed that expression of Esr1 (encoding ERα) significantly increased in KOKCs (Fig. 6A). Conversely, RAW264.7 cells with stable MROV (Supplementary Fig. 12A and B) had much lower expression of Esr1 (Fig. 6B). These data indicate repressive regulation of Esr1 by MR in macrophages.

Five putative MR response elements (MREs) were identified in the promoter region of mouse Esr1 (hereafter referred to as region 1–5, or R1–5) (Fig. 6C). Truncated Esr1 promoters containing a gradually decreasing number of the predicted MREs (omission starting from R1) were constructed to perform luciferase reporter assay in HEK293FT cells (Fig. 6D). HEK293FT cells transfected with MR-flag plasmid were cultured with charcoal/dextran-treated serum, and detectable MR-flag was localized in the nuclei, suggesting ligand-independent nuclear accumulation (Supplementary Fig. 12C). Cotransfection experiments showed that expression of MR-flag suppressed the transcription of all Esr1 promoters with different lengths (Fig. 6E). In particular, these results indicated that both R5 alone and R4–5 were sufficient for MR to suppress Esr1 transcription and that R1–3 were not necessary (Fig. 6E). Another group of truncated Esr1 promoters containing a gradually decreasing number of the predicted MREs (omission starting from R5) were constructed (Fig. 6F). MR-flag still decreased the transcription of Esr1 promoter when R5 was absent, suggesting that R5 was not necessary for the suppression (Fig. 6G). However, the suppression disappeared when both R4 and R5 were absent, suggesting that R4 was required and that R1–3 was not sufficient (Fig. 6G). Together, these results suggest that functional MREs are located in R4 and/or R5. Subsequently, a ChIP assay using the MROV RAW264.7 cells demonstrated direct binding of MR to Esr1 promoter in R4 and R5, but not R1–3, further supporting the functionality of the MREs in R4 and R5 (Fig. 6H and I).

**Hepatic Met Mediates the Beneficial Effects of Myeloid MR Deficiency in Female ob/ob Mice**

Knockdown of Met in livers was carried out by using adenoviruses expressing Met-specific shRNA (AdshMet) (Supplementary Fig. 13). Injection of AdshMet abolished the protective effects of MRKO on random-fed blood glucose, fasting plasma insulin level, GTT, and ITT in female ob/ob mice (Fig. 7A–D). Similarly, adenovirus expressing scrambled shRNA (AdshScr) eliminated the beneficial impacts of MRKO on LW/BW, hepatic TG content, hepatic lipid accumulation, and hepatic p-IR and p-Akt in female ob/ob mice (Fig. 7E–I). Together, these results strongly suggest that MR deficiency improves glucose homeostasis, insulin sensitivity, and hepatic lipid metabolism and insulin signaling, depending on hepatic Met in vivo. We propose a working model to illustrate the MR/ERα/HGF/Met axis that mediates the interactions between macrophages and hepatocytes in the liver and that may explain the impacts of MRKO on insulin resistance and hepatic steatosis (Fig. 7J).

**DISCUSSION**

Although the function of MR in electrolyte balance and the cardiovascular system has been investigated in more detail, much less has been studied in glucose and lipid metabolism. Through the current study, we uncovered the beneficial effects of myeloid-specific MR blockade on hepatic lipid metabolism as well as systemic insulin sensitivity and glucose homeostasis in ob/ob mice. We also identified a novel mechanism through which MR and ERα cross talk and macrophage and hepatocyte communicate to regulate lipid accumulation and insulin sensitivity.

**Estrogen Is Both Sufficient and Necessary for the Metabolic Improvements of Myeloid MR Deficiency in Obese Mice**

We demonstrate that myeloid MRKO protected female obese mice against glucose intolerance, insulin resistance, and hepatic steatosis. Although female ob/ob mice are infertile (35), a fair amount of E2 is detected in these mice according to the current results and other reports (36). In addition, ovaries of ob/ob mice are capable of producing viable eggs when transplanted into lean female recipients (37), suggesting functional ovarian hormones, including estrogen, in these mice. The results further show that OVX abolished the protective effects of myeloid MRKO in female obese mice and that estrogen supplementation to male obese mice was sufficient to bring out the metabolic protection of myeloid MRKO, illustrating the sufficiency and requirement of estrogen. The metabolic protection of estrogen signaling has been well documented in both human subjects and animal models (38). In particular, estrogen...
signaling in myeloid cells has been reported to exert a critical function in metabolic regulation (39). Previous reports have demonstrated protective impacts of enhanced estrogen signaling on glucose and lipid metabolism in ob/ob mice (40). The current study illustrates that the combination of estrogen and myeloid MR deficiency is effective at improving insulin resistance and hepatic steatosis.

**The MR/ERα/HGF/Met Signaling Axis Facilitates Macrophage-Hepatocyte Interaction**

The data suggest that MR deficiency in macrophages is mainly responsible for the metabolic improvements of MRKO in obese mice, although the relative contribution of resident KCs and infiltrated macrophages remain to be investigated. Previous studies have revealed that hepatocyte-derived interleukin-4 and interleukin-13 are important determinants of macrophage phenotype (15,41). We conversely identified an MR/ERα/HGF/Met pathway that controlled metabolic signal transduction from macrophages to hepatocytes and ultimately contributed to metabolic regulation in the liver. HGF/Met is an important pair in the regulation of hepatic insulin sensitivity and glucose homeostasis (32). Specific deletion of Met in hepatocytes leads to the development of severe non alcoholic steatohepatitis in mice (42). We demonstrated that in macrophages, MR directly regulated the expression of ERα, which in turn controlled...
the release of HGF that binds to Met on hepatocytes. Through such communications, MRKO in macrophages decreases lipid accumulation and improves insulin sensitivity in hepatocytes. We also observed that macrophage MRKO downregulated critical lipid-regulating molecules, such as Cidea, in hepatocytes depending of Met, implying that these molecules were downstream of the MR/ERα/HGF/Met pathway. However, the exact mechanism for how this signaling pathway controls these molecules remains to be interrogated.

We mainly used ob/ob mice as the obese mouse model to explore the roles of myeloid MR in hepatic steatosis and insulin resistance. Although widely used in metabolic studies, ob/ob mice possess several limitations. In particular, leptin mutation and deficiency are not a common cause of NAFLD or T2DM in humans, wherein the etiology is much more complex. Our study characterizes the MR/ERα/HGF/Met axis as a potentially important metabolic pathway in connecting macrophages to hepatocytes. Future work to test the metabolic consequence of MR blockade in human subjects or cells may provide more translatable insights into human NAFLD and T2DM.

Figure 7—Hepatic Met mediates the protective effects of myeloid MR deficiency in vivo. A and B: Random-fed blood glucose and fasting plasma insulin levels of female FC-ob and MRKO-ob mice injected with AdshScr or AdshMet. C-G: GTT, ITT, LW/BW, hepatic TGs, and hepatic H&E staining of female FC-ob and MRKO-ob mice injected with AdshScr or AdshMet. Scale bar = 400 μm. H: Immunoblotting analysis of p-IR, total IR (T-IR), p-Akt (Ser473), and total Akt (T-Akt) in livers of female FC-ob and MRKO-ob mice injected with AdshScr or AdshMet before and after insulin injection. I: Quantifications of immunoblotting results as p-IR/T-IR and p-Akt/T-Akt. J: Working model of the MR/ERα/HGF/Met axis that conveys metabolic signaling from macrophages to hepatocytes. In the liver where macrophages and hepatocytes are situated in proximity, MR deficiency leads to elevated ERα expression followed by enhanced HGF secretion from macrophages in the presence of estrogen. HGF subsequently phosphorylates and activates hepatocyte Met, which mediates the decrease of lipid accumulation and the increase of insulin signaling in hepatocytes and improves hepatic steatosis and insulin resistance in female and E2-implanted male ob/ob mice. n = 6–10. *P < 0.05, **P < 0.01, ***P < 0.001. --, before insulin injection; +, after insulin injection; n.s., not significant.
Nuclear Receptor–Nuclear Receptor Cross Talk Controls Hepatic Metabolism

Nuclear receptors are important hubs of transcriptional control, and their target genes include those critical in metabolic regulation (43,44). Their functions in maintaining metabolic homeostasis are further amplified or fine-tuned by nuclear receptor–nuclear receptor cross talk. The mutual inhibition between peroxisome proliferator–activated receptor-α and liver X receptor is a good example (45,46). Through reciprocal regulations, these two nuclear receptors put hepatic lipid metabolism under balanced control (45,46). A previous study demonstrated that MR and ERα physically interact with each other in HEK293 cells (47). Furthermore, ERα activation has been shown to inhibit MR-mediated gene expression (47). The current results identify ERα as a target gene of MR. In macrophages, MR regulates ERα, which itself plays key roles in metabolic regulation and, in this case, mediates the secretion of HGF to deliver messages from macrophages to hepatocytes. All these data support the importance of cross talk between nuclear receptors in metabolic regulation.

Conclusions

In summary, we identified an MR/ERα/HGF/Met signaling pathway that mediates MR-ERα cross talk and macrophage-hepatocyte interaction and that ultimately conveys the protective effects of myeloid MR deficiency on glucose homeostasis, insulin sensitivity, and hepatic lipid metabolism in obese mice. These findings may have opened a new window to explore the functions of MR in metabolic regulation. Perturbation of macrophage MR, with or without supplementation of estrogen, may be a potential novel strategy to fight against NAFLD and T2DM.

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