Forkhead box A3 mediates glucocorticoid receptor function in adipose tissue

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Glucocorticoids (GCs) are widely prescribed anti-inflammatory agents, but their chronic use leads to undesirable side effects such as excessive expansion of adipose tissue. We have recently shown that the forkhead box protein A3 (Foxa3) is a calorie-hoarding factor that regulates the selective enlargement of epididymal fat depots and suppresses energy expenditure in a nutritional- and age-dependent manner. It has been demonstrated that Foxa3 levels are elevated in adipose depots in response to high-fat diet regimens and during the aging process; however no studies to date have elucidated the mechanisms that control Foxa3's expression in fat. Given the established effects of GCs in increasing visceral adiposity and in reducing thermogenesis, we assessed the existence of a possible link between GCs and Foxa3. Computational prediction analysis combined with molecular studies revealed that Foxa3 is regulated by the glucocorticoid receptor (GR) in preadipocytes, adipocytes, and adipose tissues and is required to facilitate the binding of the GR to its target gene promoters in fat depots. Analysis of the long-term effects of dexamethasone treatment in mice revealed that Foxa3 ablation protects mice specifically against fat accretion but not against other pathological side effects elicited by this synthetic GC in tissues such as liver, muscle, and spleen. In conclusion our studies provide the first demonstration, to our knowledge, that Foxa3 is a direct target of GC action in adipose tissues and point to a role of Foxa3 as a mediator of the side effects induced in fat tissues by chronic treatment with synthetic steroids.

Foxa3 | GR signaling | dexamethasone | fat expansion | adipose tissue

Glucocorticoids (GCs) are a class of steroid hormones that bind to the GC receptor (GR) and exert broad physiological functions (1). Unliganded GR has been shown to reside in the cytoplasm in association with chaperone complexes and to translocate into the nucleus upon binding of agonists to activate or transrepress gene expression at select promoters (2). The GR is widely expressed in a variety of cells and is involved in the regulation of immune responses, metabolism, and development (3). A number of synthetic GR agonists have been widely used in the clinic for the suppression of inflammation and for the treatment of chronic diseases, given the properties of corticosteroids to elicit the transrepression effects of GR on the NF-κB and AP-1 promoters (4, 5).

Despite the overwhelming beneficial effects of targeting GR for anti-inflammatory purposes, long-term GC treatment has been associated with numerous adverse metabolic outcomes, including, among others, weight gain, central obesity, increased fat mass, muscle wasting, hepatic steatosis, hyperlipidemia, hyperglycemia, and insulin resistance caused by the broad effects of GR on metabolic organs (6). For example, GC treatment has been shown to increase hepatic gluconeogenesis and lipogenesis and to cause muscle atrophy (1, 6). Specifically, in adipose tissues GCs have been shown to increase adiposity in human and rodents by promoting fat differentiation (7, 8) and inhibiting energy expenditure by decreasing brown adipose tissue (BAT) thermogenesis (9, 10) and by suppressing the browning of subcutaneous fat (11). Furthermore, depending on the length of treatment and the intracellular concentrations, GCs have been shown to increase lipogenesis and lipolysis (12). Extensive gene-expression analyses combined with the computational information provided by genome-wide ChIP sequencing have identified a number of positive gene targets of GR signaling in a variety of tissues and cell types (13), including liver (14), adipocytes (15), and myotubes (16). Although some of these GC target genes are regulated ubiquitously, others appear to be cell-type selective, suggesting cooperation between GR and tissue-specific factors or cofactors (17). Indeed, it has been shown that transcriptional regulators such as liver X-receptor β (LXRβ) can contribute specifically to hyperglycemia, hyperinsulinemia, and hepatic steatosis but not to the immunological system responses elicited by GC (18) and that Kruppel-like factor 15 (KLF15) selectively prevents GC-induced muscle atrophy (19). Although it has been shown that GR regulates adipocyte metabolism (12) and that GCs affect adiposity and energy expenditure both centrally (20–22) and peripherally, the detailed mechanisms underlying these metabolic changes are incompletely understood.

Forkhead-box (Fox) proteins are a large family of transcription factors that bind DNA through a conserved winged-helix binding motif shown to be critically involved in the regulation of aging, metabolism, and development (23). Among them, members of the Foxa subfamily play important roles in early organ development and metabolism (24, 25). We recently have shown that Forkhead box protein A3 (Foxa3), previously known to play a role in liver during long-term starvation and in pancreas development (26, 27), is involved in the selective expansion of epididymal fat depots during a high-fat diet and in the suppression of energy expenditure during aging (28, 29) and that its variants are associated with human metabolic traits (30). Our studies also have revealed that Foxa3 levels are inducible in visceral fat in

Significance

This paper shows a previously unidentifed relationship between the transcription factor forkhead box a3 (Foxa3) and the glucocorticoid receptor (GR) by demonstrating, for the first time to our knowledge, that Foxa3 is a target of GR and that it is required for GR transcriptional function in fat depots. Via animal studies we show that the absence of the Foxa3 gene protects the fat depot from the expansion associated with chronic exposure to glucocorticoids, drugs widely used in medical practice to treat inflammatory disease and autoimmune disorders. Overall our findings suggest that Foxa3 can be a potential target for the prevention of glucocorticoid-induced pathological side effects in adipose tissues.

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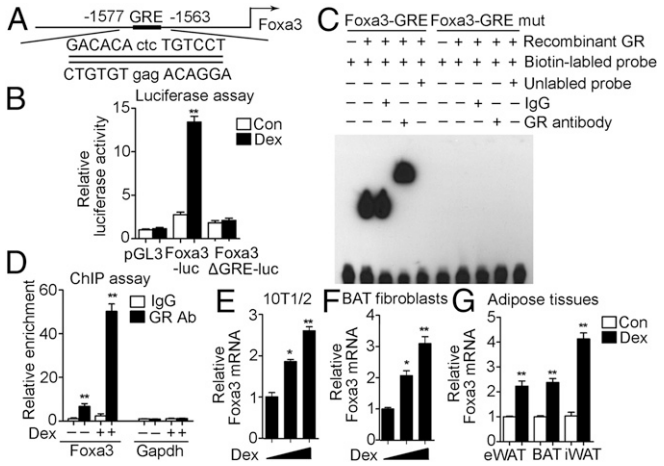

Fig. 1. GCs increase Foxa3 transcriptional levels. (*A*) Putative GRE on the Foxa3 promoter. (*B*) Luciferase activity of a WT-Foxa3 promoter reporter (Foxa3-luc) and of a mutant-Foxa3 reporter containing a deletion of the putative GRE (Foxa3ΔGRE-luc) in cells treated with DMSO (control, Con) or with 1 μM Dex for 24 h. (*C*) EMSA analysis assessing GR binding at the Foxa3 promoter. (*D*) ChIP analysis of GR binding on the putative GRE identified in the Foxa3 promoter or on the Gapdh promoter in cells treated with DMSO or 1 μM Dex for 24 h. (*E* and *F*) Dex treatment increased Foxa3 mRNA levels in differentiated 10T/2 cells (*E*) and BAT primary cells (*F*) in a dose-dependent manner. (*G*) Dex treatment increased Foxa3 mRNA levels in adipose tissues. Data are presented as mean ± SEM; $*P < 0.05$; $**P < 0.01$ compared with the control (Con) group.

response to a high-fat diet and in s.c. adipose tissue (iWAT) and BAT during aging (28, 29); however, the identity of the critical upstream regulators that control Foxa3 expression is currently unknown. Given the longstanding evidence that GC levels and signaling increase during nutritional overload and in the aging process and that long-term GC treatment leads to increased visceral adiposity and reduced thermogenesis, we explored the possible existence of a connection between GC and Foxa3. In the present study we identify Foxa3 as a direct target of GR and as a facilitator of GR binding to its target gene promoters in adipose tissues, and we delineate Foxa3's involvement in the effects of GCs on adipose tissue expansion. Our genetic analysis in vivo revealed that mice with Foxa3 ablation are protected specifically from fat tissue expansion but not from other side effects elicited in response to GC treatment, suggesting that Foxa3 contributes specifically to the pathological consequences on fat tissues elicited by long-term treatment with GC.

Results

GCs Control Foxa3 at the Transcriptional Levels. Through in silico analysis of transcription factor-binding motifs present upstream of the Foxa3 gene start site, we identified a putative GC response element (GRE) at nucleotides −1577 to −1563 from Foxa3's ATG (Fig. 1*A*), which contains a consensus half-site of a typical GRE, TGTCCT, and appears to be highly homologous to the 15-bp GRE motif A/GGnACAnnnTGTnCT/C (31, 32). Given that the synthetic GC dexamethasone (Dex) has been shown to have a higher selectivity for GR than other compounds also known to bind to the mineralocorticoid receptor (33), we used Dex for our analysis. Treatment with Dex augmented the luciferase activity of a reporter gene regulated through 2 kb of the Foxa3 promoter, and deletion of the putative GRE site blunted the response (Fig. 1*B*). Given that sequence similarities exist between steroid hormone response elements (34), we assessed whether treating cells with 3, 3′, 5-Triiodo-l-thyronine (T3), 17β-estradiol (E2), or *all-trans* retinoic acid (ARTA) would affect Foxa3 transcriptional activity. As shown in Fig. S1*A*, none of the treatments tested led to changes in luciferase activity, suggesting the specificity of GR for the responsive element present at the Foxa3 promoter. Furthermore analysis via EMSA showed that GR is able to bind at the GRE motif identified upstream of the Foxa3 promoter (Fig. 1*C*), and ChIP analysis confirmed occupancy of the ligand-bound GR at the Foxa3 promoter (Fig. 1*D*). These results suggest the presence of a functional GRE in the Foxa3 promoter.

Given the observation that agonist-activated GR occupies the GRE present at the Foxa3 promoter, we next tested whether activation of GR would increase Foxa3 levels. Dex treatment of white and brown adipocytes in vitro resulted in a dose-dependent increase of Foxa3 mRNA and protein levels (Fig. 1 *E* and *F* and Fig. S1 *B* and *C*). We next investigated whether GR activation in vivo would lead to similar modulatory effects on Foxa3. As shown in Fig. 1*G* and Fig. S1*D*, analysis of tissues obtained from mice acutely treated with Dex revealed increased Foxa3 mRNA and protein levels, compared with control mice, in depots such as epididymal adipose tissue (eWAT), BAT, and iWAT. These results demonstrate that GCs can regulate Foxa3 at the transcriptional level.

Foxa3 Is Required for GR Signaling in Adipose Tissue. It has been demonstrated recently that GR and the Foxa family members Foxa1 and Foxa2 cooperate in transcription in organs such as prostate, mammary gland, and liver (35–37). Given the previously recognized role of Foxa3 in adipose tissues, we tested the hypothesis that Foxa3 could be involved in GR signaling and might facilitate GR function in fat. To exclude the possibility that changes in gene expression would be the results of secondary effects, we acutely treated WT and Foxa3-null mice with saline or Dex, and in the adipose tissue we measured the levels of an array of genes known to be involved in GR signaling. This analysis revealed that in response to Dex the induction of 53.7% of genes was blunted in the iWAT of Foxa3-null mice (Fig. 2*A* and Dataset S1). Genes significantly altered in the absence of Foxa3 included *Lipin1*, *Gpat4* (glycerol-3-phosphate acyltransferase 4), *Angptl4* (angiopoietin-like 4), and *Lipe* (hormone-sensitive lipase) (Fig. 2*B*), whereas the expression of genes such as glycerol-3-phosphate acyltransferase 4 (*Asph*), glucose-6-phosphate dehydrogenase (*G6pd1*), ferredoxin-dependent glutamate synthase 1 (*Glu1*), and glutamic-oxaloacetic transaminase 1 (*Got1*) was not perturbed in the absence of Foxa3 (Fig. 2*C*). These results suggest that GR depends on Foxa3 for the activation of only a subset of its downstream-activated genes.

Given the established role of GCs in promoting the differentiation of preadipocytes into adipocytes (7, 8), we then investigated the role of Foxa3 in facilitating GR signaling during the adipogenic process. We treated primary iWAT stromal vascular fraction (SVF) cells obtained from WT and Foxa3 KO mice or undifferentiated 10T1/2 cells expressing control or Foxa3 knockdown with DMSO or Dex and analyzed the expressions of 73 genes known to be involved in adipogenesis. As shown in Fig. 3 *A* and *B*, Fig. S2*A*, and Dataset S2, in cells lacking Foxa3 about 70% of the subset of the critical regulators of the early phases of adipocyte differentiation, including *Cebpb*, *Cebpd*, *Pparγ* (peroxisome

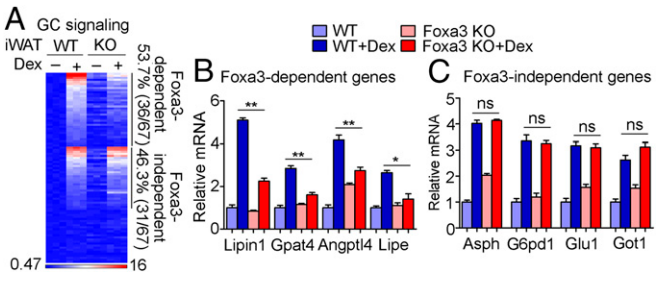

Fig. 2. Foxa3 is required for GR signaling in adipose tissue. (*A*) Heatmap of GC signaling genes of iWAT from WT and Foxa3-null mice treated with Dex every other day in the course of 1 wk. ($n = 3$). (*B* and *C*) Representative gene-expression levels of Foxa3-dependent (*B*) and –independent (*C*) genes. Data are presented as mean ± SEM; $*P < 0.05$; $**P < 0.01$ compared with the control group.

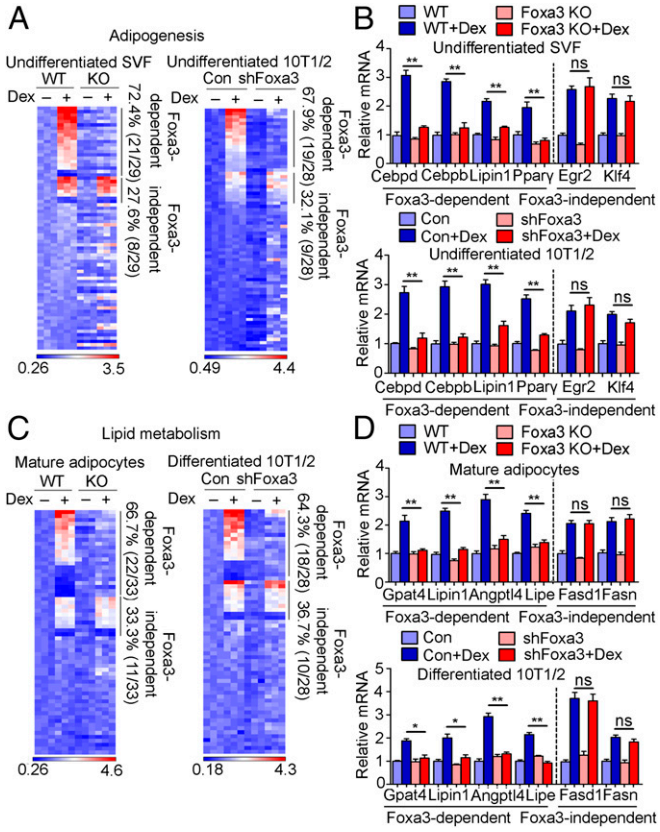

Fig. 3. Foxa3 is required for GC-mediated adipogenic gene programs in preadipocytes and for lipid metabolism gene programs in mature adipocytes. (*A*) Heatmap of adipogenic genes in undifferentiated primary SVF or 10T1/2 cells treated with differentiation mixture with or without Dex. (*B*) Representative gene-expression levels of Foxa3-dependent and -independent genes in SVF and 10T1/2 cells ($n = 3$). (*C*) Heatmap of lipid metabolism genes in primary mature adipocytes and in fully differentiated 10T1/2 cells treated with DMSO or Dex. (*D*) Representative gene-expression levels of Foxa3-dependent and -independent genes in mature adipocytes and differentiated 10T1/2 cells ($n = 3$). Data are presented as mean ± SEM; $*P < 0.05$; $**P < 0.01$ compared with the control group.

proliferator-activated receptor-γ), and *Lipin1*, were reduced significantly in response to Dex. Interestingly, early adipogenic factors such as early growth response 2 (Egr2) and Kruppel-like factor 4 (Klf4) did not appear to be affected by Foxa3 levels, because their induction was not abolished in the absence of Foxa3 (Fig. 3*B*).

It also has been shown previously that GC and GR play critical roles in lipid metabolism in adipose tissues (12), and genome-wide analysis of GR-binding regions in adipocytes has revealed that genes involved in triglyceride homeostasis are direct targets of GR in mature adipocytes (15). In light of these data, and to distinguish the extent of Foxa3 involvement in controlling early events in adipogenesis and in mature adipocytes in cooperation with GR, we analyzed the effects of Dex treatment in isolated mature adipocytes from the iWAT of WT and Foxa3 KO mice and in fully differentiated 10T1/2 cells with or without reduced levels of Foxa3. Analysis of 62 genes known to be involved in lipid metabolism showed that more than 60% of the Dex-responsive genes were dependent on Foxa3 (Fig. 3*C*, Fig. S2*B*, and Dataset S2). Our analysis showed that genes such as *Gpat4*, *Lipin1*, *Angptl4*, and *Lipe* were reduced in Foxa3-deficient cells, whereas fatty acid desaturase 1 (*Fads1*) and fatty acid synthase (*Fasn*) genes were Foxa3-independent (Fig. 3*D*). Furthermore, Foxa3 overexpression in preadipocytes or adipocytes further enhanced the levels of a set of GR-responsive genes (Fig. S2 *C* and *D*), whereas Foxa3 loss of function impaired GR-induced lipolysis but did not affect genes involved in cAMP- or PPARα-agonist–induced lipolysis (Fig. S2*E*). These results suggest that Foxa3 is required for both GR-dependent induction of adipogenesis in preadipocytes and for lipid metabolism in adipocytes.

Foxa3 Is Required for GR Binding to Target Gene Promoters. Foxa proteins previously have been shown to serve as pioneering factors by opening compact chromatin and facilitating transcriptional gene activation through their interaction with specific binding sites in promoters or enhancers (25, 38). Based on the evidence that Foxa3 is required for GR-mediated gene induction in both preadipocytes and adipocytes, we analyzed the requirement of Foxa3 for the recruitment of GR on its target gene promoters upon Dex treatment. Recent ChIP sequencing (ChIP-seq) data revealing the occupancy of GR, C/EBPβ (CAAT/enhancer binding protein beta), and acetylated histone H3 (AcH3) on the enhancers of PPARγ (39), the master regulator of adipogenesis and adipocyte differentiation, combined with the evidence that emerged from our previous study indicating that Foxa3 binds to a forkhead element adjacent to a C/EBPβ-binding site on the PPARγ2 promoter (28) and showing that the induction of PPARγ by GR is Foxa3-dependent (Fig. 3 *A* and *B*), led us to examine the binding of Foxa3, GR, and AcH3 at the PPARγ2 enhancer. This analysis revealed that in undifferentiated 10T1/2 cells the occupancy of these proteins on a site 10 kb upstream of PPARγ2 gene (chr6: 115412360–115412835) is enriched upon Dex treatment. Conversely, Foxa3 knockdown prevented the binding of GR and AcH3 to this enhancer site, suggesting that Foxa3 is critical for the binding of GR to induce PPARγ expression (Fig. 4*A* and Fig. S3*A*). Given that lipogenic and lipolytic genes such *Lipin1* and *Angptl4* previously have been reported to be direct targets of GR in adipocytes (40, 41), and based on our data showing that they are Foxa3-dependent (Fig. 3 *C* and *D*), we performed a ChIP assay to assess the requirement of Foxa3 for GR occupancy at the promoters of these target genes. This analysis revealed that GR was present at these promoters in response to Dex treatment in control adipocytes but not in cells with reduced levels of Foxa3. The binding of AcH4 to the sites near the GRE also was reduced in cells with Foxa3 deficiency (Fig. 4*B* and Fig. S3*B*), suggesting that Foxa3 may regulate chromatin remodeling at those sites and could facilitate GR-induced gene activation. To test whether Foxa3 and GR interact, we overexpressed both proteins in 10T1/2 cells and performed coimmunoprecipitation in the presence or absence of Dex. We did not detect any interaction between Foxa3 and GR (Fig. S3*C*). These results show that Foxa3 is required for GR binding to its target genes in both preadipocytes and adipocytes, possibly through chromatin remodeling.

Ablation of Foxa3 in Mice Prevents Fat Depot Expansion and Hyperlipidemia in Response to Chronic GC Treatment. Increased adiposity represents one of the major adverse effects of long-term GC treatment (1–3, 12). Based on our observation that Foxa3 contributes to the gene-expression changes induced by

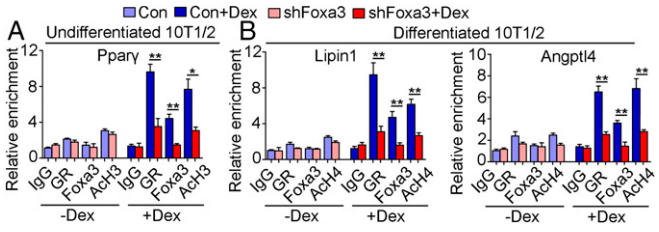

Fig. 4. Foxa3 is required for GR binding to target gene promoters in preadipocytes and adipocytes. (*A*) Occupancy of GR, Foxa3, and AcH3 at GRE sites present on the Pparγ2 enhancer in undifferentiated 10T1/2 cells. (*B*) Occupancy of GR, Foxa3, and AcH4 at the GRE site of the Lipin1 and Angptl4 promoters in differentiated 10T1/2 cells. Data are presented as means ± SEM; $*P < 0.05$; $**P < 0.01$ compared with the control group.

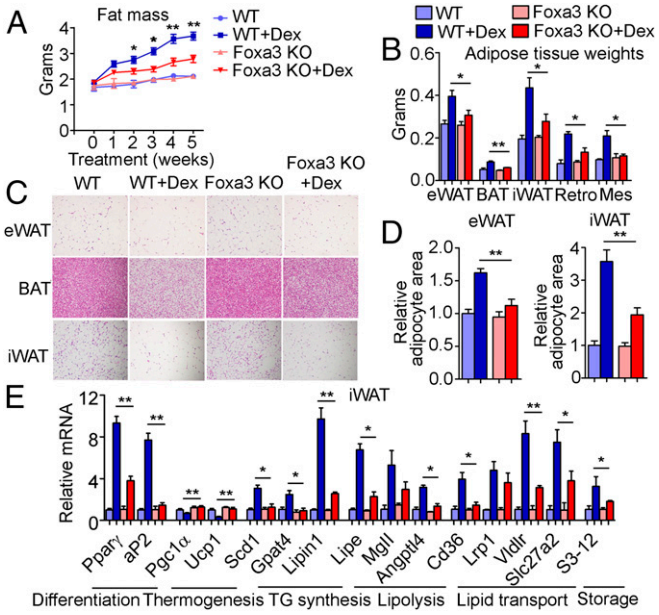

Fig. 5. Foxa3-null mice are protected from increased adiposity in response to chronic GC treatment. Shown are analyses of WT and Foxa3-null mice after 6 wk of control or Dex treatment (n = 4). (*A*) Fat mass. (*B*) Adipose tissue weight. (*C*) Representative H&E staining of eWAT, BAT, and iWAT. (*D*) Quantification of adipocyte size in eWAT and iWAT. (*E*) Gene expression in iWAT. Data are presented as means ± SEM; $*P < 0.05$; $**P < 0.01$ compared with control group.

GR activation in preadipocytes, adipocytes, and adipose tissues, we evaluated the contribution of Foxa3 to the metabolic effects by chronically treating WT and Foxa3-null mice with Dex for 6 wk. Given that GCs previously have been reported to affect food intake in rodents (21, 22), we pair-fed the mice to exclude any confounding effects caused by the potential influence of GC on caloric intake. As previously described, 2-mo-old WT and Foxa3-null mice on a normal chow diet before treatment had comparable body weight, fat mass, and lean mass (29). Analysis of these metabolic parameters in mice after Dex treatment revealed that ablation of Foxa3 gave protection from the Dex-induced increase in fat mass and adipose tissue weight (Fig. 5 *A* and *B* and Fig. S4*A*). Furthermore histological analysis revealed increased adipocyte size in eWAT, BAT, and iWAT in response to long-term treatment with GC in WT mice as compared with Foxa3-null mice (Fig. 5 *C* and *D*), and serum parameter analysis showed that Foxa3-null mice were protected from the increase in cholesterol and triglyceride levels elicited by Dex treatment (Fig. S4*B*). Importantly, reduced fat pad expansion was associated with reduced expression of GR target genes in adipose tissue from Foxa3-null mice upon long-term exposure to Dex (Fig. 5*E* and Fig. S4 *C* and *D*). These data suggest that Foxa3 is required for the Dex-induced lipid metabolic dysfunction in adipose tissues.

The Pathological Side Effects Induced by Long-Term GC Treatment in Liver, Muscle, and Spleen Are Independent of Foxa3. Having demonstrated that ablation of Foxa3 in adipose tissues prevented the GR-induced expansion of fat tissue, we next performed a tissue survey to examine whether GC treatment affected Foxa3 levels in other tissues. Our analysis revealed that chronic treatment with Dex induced Foxa3 mRNAs specifically in adipose tissues but not in liver, muscle, pancreas, kidney, or spleen, suggesting that GR cooperation with tissue-specific transcription factors and cofactors is required to achieve adipose tissue-selective transcriptional regulation of Foxa3 (Fig. S5*A*). Furthermore, WT and Foxa3-null mice treated with Dex had no differences in liver, kidney, and pancreas weights (Fig. S5*B*). To examine further the contribution of Foxa3 to GR signaling in other tissues, we performed a series of genetic and histological analyses on organs such as liver, muscle, and spleen, which previously have been shown to be largely affected by GC treatment (1–3). Specifically, GC has been shown to elevate hepatic gluconeogenesis through increased GR binding to the promoter of the phosphoenolpyruvate carboxykinase (*Pepck*) and glucose-6-phosphatase (*G6Pase*) genes and induction of their expression (14). Molecular analysis revealed that the expression of these two gluconeogenic genes, as well as the expression of insulin-like growth factor binding protein 1 (Igfbp1) and glucose transporter 2 (Glut2), proteins involved in insulin metabolism and glucose transport, were comparable in the livers of WT and Foxa3-null mice after Dex treatment (Fig. 6*A*). Furthermore, WT and Foxa3-null mice showed comparable insulin resistance after chronic Dex exposure, as demonstrated by glucose and insulin tolerance tests (Fig. S5 *C* and *D*).

In addition to their known function in glucose metabolism, GCs have been shown to affect hepatic lipid metabolism by increasing fatty acid synthesis and decreasing fatty acid β oxidation (42). Molecular analysis revealed that these gene programs were affected similarly by Dex treatment in mice of both genotypes (Fig. 6*A*), and H&E staining and assessment of liver triglyceride levels demonstrated the development of hepatic steatosis both in WT and Foxa3-null mice in response to Dex (Fig. 6 *B* and *C*). Interestingly, the analysis of the expression of genes involved in GR signaling and in lipid metabolism of liver from WT and Foxa3-null mice with or without acute Dex treatment revealed that only 24.4% and 14.2% of liver-specific GR-responsive genes were Foxa3-dependent as shown in Fig. S5*E* and Dataset S3. These results are in sharp contrast with the data obtained in adipose tissues showing that more than 50% of GR-induced genes are dependent on Foxa3 (Figs. 2 and 3).

Given that prolonged GC exposure also can be associated with muscle wasting via alteration of catabolic and anabolic pathways (1), we next assessed the gene-expression levels of some of the markers known to be regulated in these processes in muscle (16, 43). Our analysis revealed that these markers are similarly

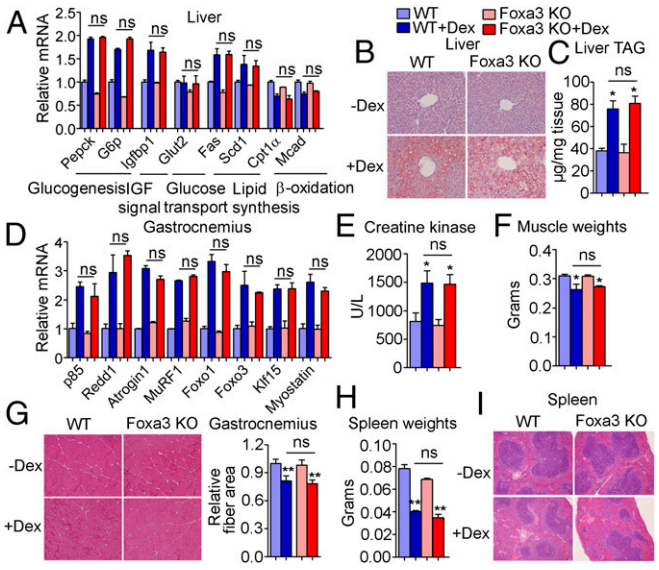

Fig. 6. Effects of GC treatment in liver, muscle, and spleen are independent of Foxa3. Shown are analyses of WT and Foxa3-null mice treated with Dex for 6 wk and untreated controls (n = 4). (*A*) Glucose and lipid metabolism gene expression in liver. (*B*) Representative Oil red O staining of liver. (*C*) Liver triglyceride levels. (*D*) Expression of genes related to muscle atrophy in gastrocnemius muscle. (*E*) Serum creatine kinase levels. (*F*) Muscle weights. (*G*) Representative H&E staining and relative fiber cross-sectional area of gastrocnemius muscle. (*H*) Spleen weights. (*I*) Representative H&E staining of spleen. Data are presented as means ± SEM; $*P < 0.05$; $**P < 0.01$; ns, not significant compared with the control group.

up-regulated by Dex treatment in the gastrocnemius of WT and Foxa3-null mice (Fig. 6*D*). Consistent with these gene-expression results, the levels of a marker of muscle damage, serum creatine kinase, were comparable in Dex-treated WT and Foxa3-null mice (Fig. 6*E*). Furthermore, muscle weights were reduced in both genotypes (Fig. 6*F*), and H&E staining of gastrocnemius muscle showed comparable reduction in fiber size irrespective of the genotype (Fig. 6*G*).

Because GC treatment has been shown to affect immune organs physiologically and functionally (44), we analyzed the effects of Dex treatment on the spleen of WT and Foxa3-null mice. We found similar reductions in weight (Fig. 6*H*) and in hematopoietic elements in the red pulp and in the germinal centers (Fig. 6*I*). These results suggest that Foxa3 does not mediate any of the side effects of GR activation in tissues other than fat.

Discussion

Here we demonstrate, for the first time to our knowledge, that the GR regulates the levels of the transcription factor Foxa3 in fat. Furthermore we show that Foxa3-null mice have reduced fat expansion in response to long-term GC treatment, suggesting that Foxa3 has a critical role in mediating GR-dependent metabolic functions in adipose tissue (Fig. S6). It has been suggested previously that cortisol levels or cortisol responses are increased during the aging process (45). Our data demonstrating that Foxa3 is induced by GCs combined with the evidence provided in our previous report indicating that Foxa3 levels increase in adipose tissues during aging (29) suggest a possible link between the effects of cortisol on Foxa3 levels in fat tissues and the induction of age-associated obesity.

Our data demonstrating that GR binding to its target genes is impaired in Foxa3-null mice strongly suggest that Foxa3 may act as a pioneering factor required to modify chromatin so that GR is accessible to a number of target genes in fat tissues. Furthermore, through the analysis of Foxa3 requirements in undifferentiated and differentiated cells, our analysis led to the identification of a number of genes dependent on Foxa3 in response to GR activation and involved in adipogenesis of preadipocytes and in lipid metabolism of mature adipocytes. These data suggest that Foxa3 has a dual role in chromatin remodeling, facilitating both hyperplastic and hypertrophic processes required for the expansion of fat in response to GR activation. Future ChIP-seq studies performed on fat tissues obtained from WT and Foxa3-null mice combined with molecular and genetic analyses will provide a more systematic map of the GR target genes that are strictly dependent on Foxa3 on a genome-wide scale.

Our data provide evidence that GC regulates Foxa3 mRNAs in a tissue-selective manner, given our observation that Foxa3 gene-expression levels are induced in fat tissues of mice treated with Dex but not in organs such as liver, pancreas, kidney, or spleen. This tissue-specific induction of Foxa3 may provide a mechanistic explanation of why Foxa3 ablation protects selectively against the expansion of adipose tissue associated with chronic GC treatment but does not prevent any of the morphological and functional alterations induced by GC treatment in liver, muscle, and spleen. It remains to be established experimentally whether Dex may lead to selective induction of Foxa3 in fat tissue because of the unique accessibility of GR to binding elements present at the Foxa3 promoter though specific cofactors available in fat (13, 17, 46). It is conceivable that GR may rely on tissue-specific factors to activate Foxa3, given that GR previously has been shown to form distinct complexes in a tissue- and agonist-selective manner, leading to differential gene-expression programs (1). Further ChIP analyses combined with biochemical studies will elucidate the specific components of the tissue-selective complexes bound at the GRE present at the Foxa3 promoter.

Our data demonstrate that Foxa3 is required for GC action in fat. In addition to these direct tissue-selective effects of GC on Foxa3 mRNA levels, it is conceivable that GR activation also may lead to tissue-specific posttranslational modifications that ultimately affect Foxa3's ability to activate its downstream gene targets. This notion is plausible, because it has been shown previously that ligand-bound GR can regulate the mRNA levels of kinases such as serum- and GC-inducible kinase 1 (SGK1) and that those changes are associated with alterations of the phospho-status of forkhead family members (47). Further studies using mass spectrometry analysis of Foxa3 combined with mutational analysis at putative phospho-sites will reveal whether Foxa3 is posttranslationally modified in response to GC.

It has been reported that patients receiving long-term treatment with GC could develop iatrogenic Cushing syndrome and have altered fat mass in selective depots, suggesting a correlation between Foxa3 levels and fat expansion. Further studies assessing whether the levels of Foxa3 are altered in the fat tissues of patients with Cushing syndrome may provide a rationale for the potential use of Foxa3 antagonists to reduce some of the metabolic side effects elicited by GC.

Overall our work demonstrates, for the first time to our knowledge, that Foxa3 levels may be under the control of GCs and provide a possible explanation of how these compounds contribute selectively to fat expansion, independently of their central effects. By showing that Foxa3 is at least partly necessary for the effects on fat tissue expansion induced by GR, we propose that Foxa3 may be an amplifier of GR signaling in fat tissue. Such a role is consistent with the previously proposed role of Foxa3 as a calorie-hoarding factor (48).

Materials and Methods

Plasmids. The Foxa3 promoter amplified from mouse genomic DNA (2 kb) was cloned into the pGL3-basic luciferase reporter vector (Promega) at the KpnI and XhoI restriction sites. A mutant Foxa3-luciferase reporter with a deletion at the putative GRE was generated using the QuikChange II Site-Directed Mutagenesis Kit (Agilent). Primers are shown in Table S1.

Cell Culture. 10T1/2 cells (ATCC), BAT fibroblasts (a kind gift of Bruce Spiegelman, Harvard Medical School, Boston), and primary SVF and fat cells from inguinal fat were used in the study. Dex (D4902), T3 (T2877), E2 (E8875), and ATRA (R2565) were purchased from Sigma. Cell culture, treatment differentiation, transfection, and luciferase assays are described in *SI Materials and Methods*.

Real-Time PCR and PCR Arrays. Total RNA was extracted from cells or tissues with TRIzol (Invitrogen) and was reverse transcribed into cDNA using the First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. Quantitative real-time PCR was performed on the ABI PRISM 7900HT sequence detection system using SYBR Green (Roche). Gene-expression levels were calculated using the ΔΔCt method after their normalization to the expression levels of the housekeeping gene *36B4*. The sequences of the primers used for real-time PCR are listed in Table S1. PCR arrays for GC signaling (PAMM-154Z; Qiagen), adipogenesis (PAMM-049Z; Qiagen), and lipid metabolism (lipid metabolism tier1 384M; Bio-Rad) were performed following the manufacturers' protocols.

EMSA and ChIP Assays. For EMSA assays, recombinant GR protein (RP-500; Thermo Scientific) was incubated with a biotin-labeled probe with the putative GRE identified at the Foxa3 promoter or with a mutant probe. Competition and supershift assays also were performed, followed by DNA–protein complex separation, transfer, UV cross-link, and signal detection according to the manufacturer's instructions (LightShift Chemiluminescent EMSA Kit, 20148; Thermo Scientific). The ChIP assay was performed using a commercial kit (Millipore). The antibodies used in the assay were IgG (12-370; Millipore), anti-GR antibody (MA1-510 and PA1-511A; Thermo Scientific), anti-Foxa3 (sc-5361; Santa Cruz), anti-AcH3 (ab4441; Abcam), or anti-AcH4 antibody (06-866; Millipore). Details are provided in *SI Materials and Methods*, and primers are listed in Table S1.

Animal Studies. WT and Foxa3-null mice (29) were kept in a humidity- and temperature-controlled environment on a 12-h light/dark cycle, with free access to chow diet and water. Mouse experiments were performed according to guidelines of the National Institute of Diabetes and Digestive and Kidney Diseases Animal Care and Use Committee and approved by the Institutional Animal Care and Use Committee of the National Institute of Diabetes and Digestive and Kidney Diseases. For acute Dex treatment, 2-mo-old male C57BL/6J mice were given a 50-μL i.p. injection of saline or 5 mg/kg

Dex (D0720000; Sigma) once or every other day for 1 wk, and tissues were dissected for gene-expression analysis or PCR array analysis. For chronic Dex treatment, 2-mo-old male WT and Foxa3-null mice were given a 50-μL i.p. injection of saline or 5 mg/kg Dex every other day for 6 wk. To exclude the potential influence of Dex on food intake, pair-feeding experiments were performed during chronic treatment, as described in *SI Materials and Methods*. Body weights and body composition (Echo Medical Systems) were recorded every week. Mice were euthanized, and tissues were dissected for further analysis.

Analysis of Serum Parameters, Insulin Sensitivity, and Liver Triglyceride Levels. Analysis of serum chemistry including cholesterol, triglyceride, and creatine kinase levels were performed by the Department of Laboratory Medicine at the NIH Clinical Center. Glucose tolerance and insulin tolerance tests and liver triglyceride levels were determined as previously described (49, 50).

Histological Analysis. Dissected tissues were fixed in 10% (vol/vol) neutral buffered formalin and embedded in paraffin, and frozen muscle sections were prepared according to standard procedures. Tissue sections of 5-μm thickness were stained with H&E or Oil red O (Histoserv). Stained slides were analyzed using a microscope, and images were captured by a digital camera (Olympus). The size of adipocytes in eWAT and iWAT and the size of fibers in gastrocnemius muscle were quantified using Image J.

Statistical Analysis. Student's *t* test was used for comparison between two groups and was performed with GraphPad Prism software. $P < 0.05$ was considered as statistically significant. Results are shown as mean ± SEM.

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