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γ-Glutamyl carboxylase in osteoblasts regulates glucose metabolism in mice

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ABSTRACT

Vitamin K-dependent γ-glutamyl carboxylase (GGCX) is an enzyme that catalyzes the conversion of glutamic acid to gamma-carboxyglutamic acid in substrate proteins. Among GGCX target proteins, recent evidence indicates that osteocalcin regulates insulin sensitivity and secretion. However, the precise contribution of GGCX to glucose metabolism remains to be clarified. To address this question, we generated osteoblast-specific *Ggcx*-deficient (i.e., conditional knockout [cKO]) mice using collagen type 1 α1 (*Col1*)-*Cre* mice. *Ggcx* cKO mice exhibited altered metabolism compared with their controls; serum glucose levels could be maintained with low amounts of insulin, and the weight of white adipose tissue (WAT) significantly decreased in *Ggcx* cKO mice. Our findings suggest that GGCX expressed in osteoblasts is critical for the maintenance of blood glucose and WAT.

1. Introduction

Vitamin K (VK)-dependent γ-glutamyl carboxylase (GGCX) is an enzyme that catalyzes a posttranslational modification using VK as a co-factor, which changes glutamic acid residues (Glu) to γ-carboxyglutamic acid residues (Gla) in its target proteins. The carboxylation reaction is closely coupled to an epoxidation reaction that oxidizes VK hydroquinone to VK 2,3-epoxide in the VK cycle [1]. To date, at least 20 proteins are known to be γ-carboxylated, including blood coagulation factors, osteocalcin (OC), matrix Gla proteins (MGP), and GGCX itself. The Gla modification is critical for the physiological activities of these VK-dependent γ-carboxylated proteins. Previous studies have shown that the molecular weight of GGCX is 94 kDa and GGCX contains 3 Gla residues in its protein structure [2]. It has also been shown that GGCX protein possesses 5 transmembrane domains and localizes to the endoplasmic reticulum [1,3]. *Ggcx* mRNA is expressed in various tissues, including the liver, brain, heart, kidney, lung, pancreas, and skeletal muscle. [4]. *GGCX* gene mutations with amino acid substitution of arginine to leucine (Leu394Arg) and serine to tryptophan (Trp501Ser) reportedly exhibit abnormal GGCX activity and deficient activities of VK-dependent coagulation factors, resulting in bleeding disorders [5,6]. In Japanese elderly women, it has been reported that a single nucleotide polymorphism (Arg325Gln) of the *GGCX* gene is associated with enhanced GGCX activity and high bone mineral density [7].

Besides the regulation of coagulation and bone homeostasis by GGCX, recent studies have revealed that GGCX can be involved in the regulation of metabolic pathways through a GGCX target protein, OC [8]. OC-deficient mice initially are reported to have a phenotype with high bone mass [9], and they exhibit altered metabolic conditions, including high levels of blood glucose, low levels of serum insulin, increase in fat pad, and insulin resistance [10,11]. Interestingly, undercarboxylated OC (ucOC), rather than carboxylated osteocalcin (cOC), is assumed to play a critical role in the maintenance of energy metabolism. Therapeutically, ucOC could prevent diet-induced obesity and diabetes mellitus in mice [12]. In the context of osteoblast-expressed proteins, it has also been reported that osteoblast-specific insulin receptor-deficient mice exhibit impaired bone formation and metabolic abnormalities including glucose intolerance and higher visceral fat mass [11,13].

To address the precise contribution of GGCX in various physiological pathways, we generated *Ggcx*-floxed mice that can be used for generation of *Ggcx*-deficient mice in an organ-specific manner.

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This animal model would conquer the limitation of full *Ggcx* knockout mice, which die between embryonic days 9.5–18 owing to hemorrhage [14]. Recently, we showed the essential function of GGCX in blood coagulation by using liver-specific *Ggcx*-deficient ($Ggcx^{\Delta liver/\Delta liver}$) mice [15]. $Ggcx^{\Delta liver/\Delta liver}$ mice showed bleeding diathesis and shorter life spans. In the present study, we generated osteoblast-specific *Ggcx*-deficient ($Ggcx^{flox/flox}$; collagen type 1 α 1 [*Col1*]-*Cre*) mice by crossing *Ggcx*-floxed mice with *Col1-Cre* mice [16]. The Cre recombinase of the *Col1-Cre* mice was shown to be expressed in osteoblastic lineage cells after the pre-osteoblast stage during osteoblast differentiation [16]. Herein, we show that *Ggcx* cKO mice exhibited altered metabolism compared with their control *Col1-Cre* mice.

2. Materials and methods

2.1. Generation of osteoblast-specific Ggcx-deficient ($Ggcx^{flox/flox}$; Col1-Cre, cKO) mice and genotyping

All the animal experiments were approved by the institutional Animal Care and Use Committee of the Saitama Medical University. Mice were maintained in a temperature-controlled room (23 °C) with a 12-h light/dark schedule and fed a standard diet (CE2, CLEA Japan, Japan), with free access to water.

The GGCX-floxed mice were generated, as previously described, and they were backcrossed to C57BL/6 N mice for more than 8 generations [15]. $Ggcx^{flox/+}$ mice were intercrossed to generate $Ggcx^{flox/flox}$ mice containing homozygous recombinant alleles. Mice containing transgenic constructs of mouse *Col1* enhancer/promoter and Cre recombinase modified to include a nuclear localization sequence (*Col1*-Cre) were kindly provided by Dr. G. Karsenty [16]; they were backcrossed to C57BL/6N for more than 8 generations. For genotyping, prepared genomic DNA derived from the tail was used as the template for PCR analysis. The floxed and wild-type (WT) GGCX alleles and the Cre recombinase gene were amplified with gene specific primers as described previously [15]. The body weight of the mice was measured once a week. At 38 weeks of age, male mice were sacrificed; blood was obtained, and the liver, gonadal white adipose tissue (WAT), pancreas, and quadriceps muscle were dissected. Serum was separated via centrifugation (800*g* for 10 min at room temperature). Tissue samples were immediately stored at −80 °C until analysis.

2.2. Production of anti-GGCX antibody and immunohistochemistry (IHC) for bones

For production of polyclonal anti-GGCX antibody, the PCR product corresponding to the amino acids 533–733 of human GGCX was inserted in-frame in the expression vector pGEX-4T-1 (GE Healthcare, UK) to produce a glutathione-S-transferase (GST) fusion protein. The resulting pGEX-GGCX was transformed into *Escherichia coli* (BL21). The GST-GGCX fusion protein was purified using glutathione-Sepharose beads (GE Healthcare). The GGCX antibody was raised in a rabbit against the purified recombinant GST-GGCX fusion protein. The antibody was affinity-purified and cross-reactivity to mouse GGCX was verified by western blot analysis.

To sample the tibiae, 16-week-old male *Col1-Cre* mice ($n = 6$) and cKO mice ($n = 6$) were anesthetized with diethyl ether followed by somnopentyl and perfused with 4% paraformaldehyde in cacodylate buffer (pH 7.4) through the left cardiac ventricle. The tibiae were dissected free of soft tissue and immersed in the same fixative for an additional 12 h at 4 °C. After decalcification with 5% EDTA-2Na solution for 4 weeks at 4 °C, some specimens were dehydrated through a graded series of ethanol prior to paraffin embedding [17]. For IHC of GGCX, paraffin sections were rehydrated and probed with anti-human GGCX antiserum, according to a previously described protocol [18]. Briefly, the sections were reacted with anti-human GGCX rabbit antibody (10 μg/mL) for 3 h at room temperature and subsequently incubated with horseradish peroxidase (HRP)-labeled goat antirabbit IgG at a 1:200 dilution for 1 h. The sites of HRP were visualized after

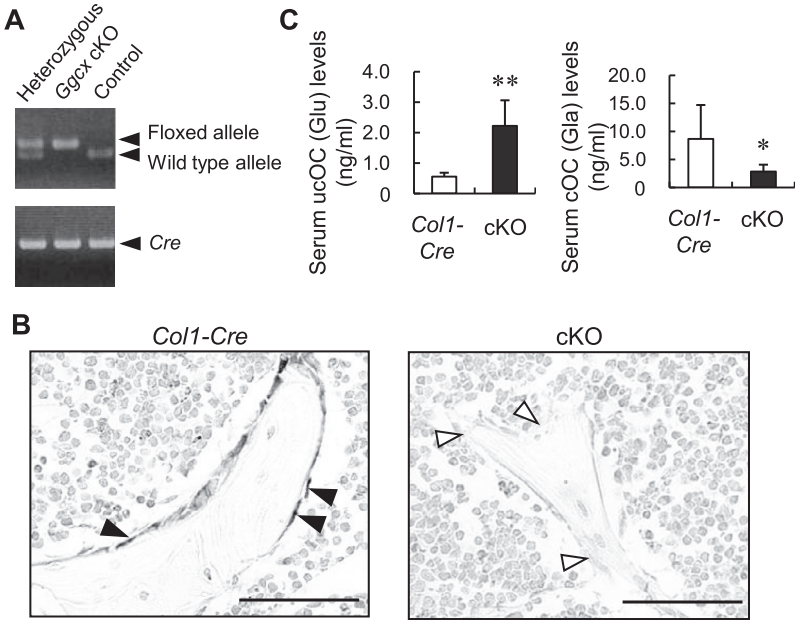

Fig. 1. Osteoblast-specific ablation of the *Ggcx* gene. (A) Generation of osteoblast-specific GGCX deficient mice. $Ggcx^{flox/flox}$ mice were mated with *Col1-Cre* mice. For genotyping, PCR was performed using genomic tail DNA (Section 2). (B) Immunohistochemical expression of GGCX in a paraffin section of the tibia from male control (*Col1-Cre*) mice and conditional knockout (cKO) mice. Images are at 400× magnification; the samples are from 16-week-old mice. Bar represents 20 μm. Solid and open arrowheads indicate osteoblasts positive and negative for GGCX, respectively. (C) Serum undercarboxylated osteocalcin (ucOC) and carboxylated osteocalcin (cOC) levels in 38-week-old male *Col1-Cre* mice and cKO mice. Serum cOC and ucOC levels were measured by using ELISA kits. Data are presented as mean ± S.D. ($n = 6$). Differences between the mean values were analyzed using the unpaired Student *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

incubation with a solution containing diaminobenzidine, H_2O_2, $CoCl_2$-$6H_2O$, and $NiSO_4$-$6H_2O$. As a control, sections were reacted with normal rabbit IgG instead of the GGCX antibody, at the same concentration. Images are at 400× magnification, and the scale bar represents 20 μm.

2.3. Measurement of serum carboxylated osteocalcin (cOC) and under-carboxylated osteocalcin (uc-OC) levels

The ucOC and cOC levels in serum were measured using the Mouse Glu-Osteocalcin High Sensitive EIA Kit (TAKARA BIO, Japan) and the Mouse Gla-Osteocalcin High Sensitive EIA Kit (TAKARA BIO), respectively.

2.4. Oral glucose tolerance test (OGTT)

At 12 weeks of age, male mice ($n = 6$) were made to fast for 16 h before the OGTT. Mice were challenged with orally administered D-(+)-glucose (2.0 mg/g body weight). Blood samples were collected from the saphenous vein at 0, 15, 30, 60, and 120 min after glucose administration. Blood glucose levels were measured using a glucose analyzer (SANWA KAGAKU KENKYUSHO, Japan). Serum insulin levels were measured using the Mouse Insulin ELISA KIT (S-type) (Shibayagi, Japan).

2.5. Microarray analysis

Gene expression in the gonadal WAT of 38-week-old *Col1-Cre* and cKO male mice were examined with Affymetrix GeneChip (Mouse Gene 1.0 ST Array), according to the manufacturer's protocol. A global analysis of gene expression and differentially expressed genes in pathways and clusters of functionally related genes were performed with the DAVID functional Annotation Clustering Tool (http://david.abcc.ncifcrf.gov/summary.jsp).

2.6. Statistical analysis

We determined the significance of differences between the two groups by using the unpaired Student *t*-test. We considered $P < 0.05$ to be statistically significant. All data are presented in the text and figures as mean ± S.D.

3. Results

3.1. Generation of osteoblast-specific Ggcx-deficient mice

We previously reported about *Ggcx*-floxed mice in which exon 6 of the *Ggcx* gene was flanked by *loxP* sequences [15]. To specifically delete the *Ggcx* gene in the osteoblasts, we used *Col1-Cre* transgenic mice that express *Cre* recombinase under the control of the col1 (α1(I)) promoter [16]. $Ggcx^{flox/flox}$; *Col1-Cre* (cKO) mice were obtained with the expected Mendelian frequency and without sexual bias. Both male and female cKO mice were fertile, and their appearance was not overtly different from that of control (*Col1-Cre*) littermates. The wild-type *Ggcx* allele was ablated in *Ggcx* cKO mice as determined by performing genotyping PCR (Fig. 1A). To confirm the ablation of GGCX protein in *Ggcx* cKO mice, we examined the immunohistochemical expression of GGCX in the tibia from male *Ggcx* cKO and control mice (Fig. 1B). As shown in the figure, expression of GGCX was not detected in *Ggcx* cKO osteoblasts. We also examined the effect of GGCX ablation on the carboxylation status of OC in the serum. Serum ucOC and cOC levels increased and decreased, respectively, in *Ggcx* cKO mice compared with control mice (Fig. 1C).

3.2. Decreased gonadal WAT weights in Ggcx cKO mice

To examine the effect of GGCX depletion in mice, the body weights of *Ggcx* cKO and control mice were monitored. The body weights of *Ggcx* cKO mice were not basically different from those of control mice (Fig. 2A). There was no significant difference in the non-fasting blood glucose levels between *Ggcx* cKO and control mice (Fig. 2B). No substantial differences in the weights of the pancreas, liver, and quadriceps muscle were observed between *Ggcx* cKO and control mice (Fig. 2C–E). It is notable, however, that the gonadal WAT weights significantly decreased in *Ggcx* cKO mice compared with control mice (Fig. 2F).

3.3. Ggcx cKO mice exhibited altered glucose metabolism

Since the amounts of visceral gonadal fat decreased in *Ggcx* cKO mice, we performed OGTTs using male *Ggcx* cKO and control mice at 12 and 32 weeks of age (Fig. 3A and B). Between the 2 lines, there was no significant difference in blood glucose levels. Calculation of the area under the curve (AUC) following the OGTT also indicated no substantial difference between the two genotypes. Despite almost the same glucose levels in both *Ggcx* cKO and control mice, serum insulin levels at 0, 15, and 60 min after glucose administration significantly decreased in *Ggcx* cKO mice at 12 weeks of age when compared with those of control mice (Fig. 3C, left panel). At 32 weeks of age, serum insulin levels during

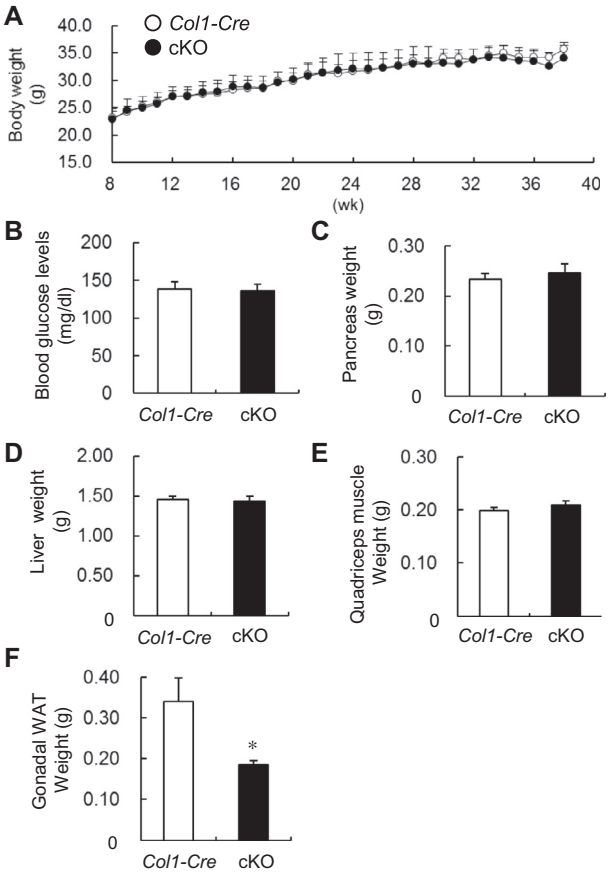

Fig. 2. White adipose tissue decreased in 38-week-old male conditional knockout (cKO) mice. (A) The body weights of *Col1-Cre* and *Ggcx* cKO mice were measured once a week from 8 to 38 weeks of age ($n = 6$). Blood glucose levels (B), pancreas weight (C), liver weight (D), gonadal white adipose tissue weight (E), and quadriceps muscle weight (F) were measured at 38 weeks of age. Data are presented as mean ± S.D. ($n = 6$). Differences between the mean values were analyzed by using the unpaired Student *t*-test. *$P < 0.05$.

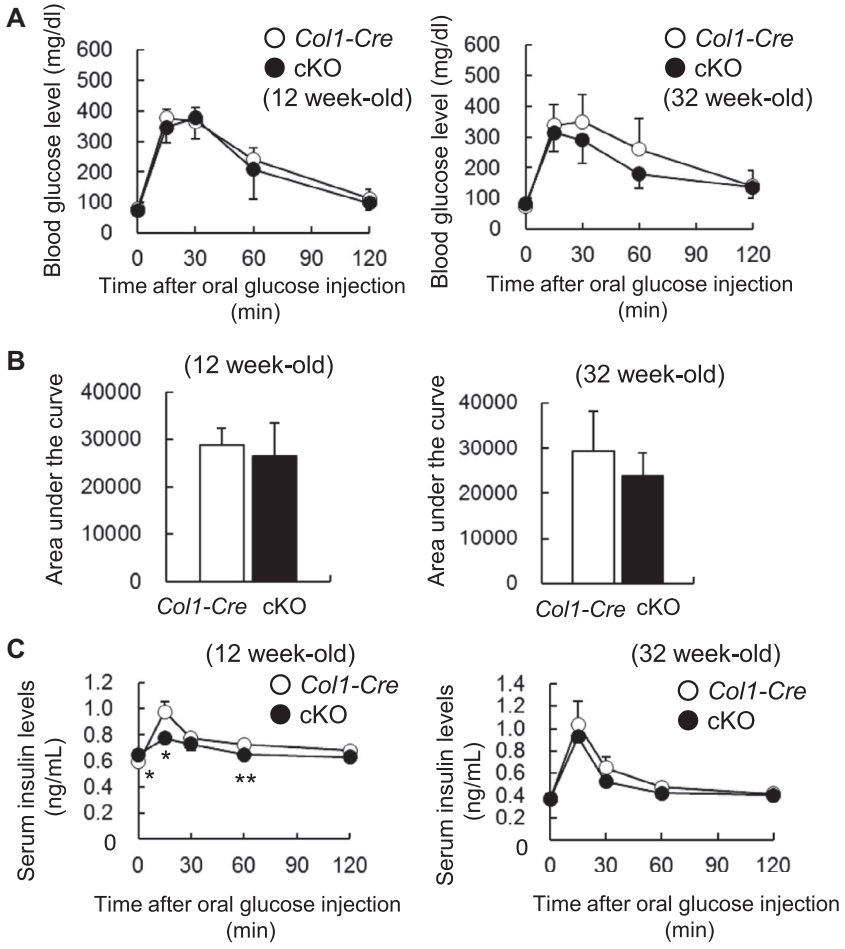

Fig. 3. Increased insulin sensitivity in *Ggcx* conditional knockout (cKO) mice. (A) Oral glucose tolerance tests (OGTT) at 12 (left panel) and 32 weeks of age (right panel). *Ggcx* cKO and *Col1-Cre* mice were made to fast for 16 h and then orally administered glucose (2.0 g glucose per kg body weight). Blood samples were collected from the saphenous vein at 0, 15, 30, 60, and 120 min after glucose administration. Blood glucose levels were measured using a glucose analyzer. (B) Area under the curve (AUC) in OGTT. (C) Serum insulin levels in OGTT. Serum insulin levels were measured by using ELISA kits. Data are presented as mean ± S.D. ($n = 6$). Differences between the mean values were analyzed using the unpaired Student *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

OGTT were not substantially different between *Ggcx* cKO and control mice (Fig. 3C, right panel). The data indicate that *Ggcx* cKO mice have a better ability to maintain circulating glucose levels with a smaller amount of insulin secretion upon glucose load.

3.4. Upregulation of fatty acid oxidation-related genes and downregulation of lipid biosynthesis-related genes in WAT of Ggcx cKO mice

Because adipose tissue is considered to be associated with insulin sensitivity, we further investigated the gene expression profiles of gonadal WAT in *Ggcx* cKO and control mice. Differentially expressed genes were determined by performing microarray analysis (Table 1). Among them, we were interested in 3 genes (*Akr1b7, Pdk4, and Pon1*), as the expression levels of these 3 genes increased in the WAT of cKO mice. *Akr1b7* encodes an aldo–keto reductase that is associated with adipocyte differentiation [19,20]. *Pdk4* encodes a mitochondrial protein of the pyruvate dehydrogenase kinase family, that is associated with glucose metabolism [21]. *Pon1* encodes a high-density lipoprotein-associated antioxidant enzyme – paraoxonase – that is associated with lipid homeostasis through the adipose tissue (Table 1) [22]. Pathway analysis and clustering of functionally related genes were performed with the DAVID functional Annotation Clustering Tool (Supplementary

Table 1
Alteration of gene expression in the white adipose tissue (WAT) of *Ggcx* conditional knockout (cKO) mice.

Upregulated genes		Downregulated genes	
Gene symbol	Fold change[a]	Gene symbol	Fold change[a]
2010106E10Rik	3.153	*Lipf*	−2.585
Hal	2.619	*Slc5a7*	−1.810
Ighg3	2.514	*Gys2*	−1.656
Spint4	2.277	*Mosc1*	−1.637
Ptgs2	2.183	*Fgf13*	−1.624
Pcp4	1.756	*Sfrp5*	−1.612
Acsm3	1.730	*Mogat2*	−1.498
Akr1b7	1.718	*Ear11*	−1.454
Pon1	1.615	*Odf3l1*	−1.418
Defb23	1.587	*Reg1*	−1.338
Pdk4	1.468	*Ptger3*	−1.335
Slc28a3	1.467	*Bhlhe41*	−1.253
Ptprz1	1.461	*Fam20c*	−1.230
P2rx1	1.458	*Zg16*	−1.202
Grem2	1.421	*Fabp2*	−1.201
Rbp7	1.387	*Gm129*	−1.187
Rarres1	1.379	*Orm1*	−1.170
Zyg11a	1.351	*Mgam*	−1.167
Upk1b	1.330	*Gm1110*	−1.140
Mfsd2a	1.319	*1110059M19Rik*	−1.122

[a] RNAs were prepared from the gonadal WAT of 38-week-old male *Ggcx* cKO ($n = 3$) and *Col1-Cre* ($n = 3$) mice. Microarray analysis was performed with Affimetrix GeneChip (Mouse Gene 1.0 ST Array). The fold change of the microarray signals (*Ggcx* cKO and *Col1-Cre* mice) is shown as log 2.

Tables S1 and S2). Interestingly, the *Irs1* gene, related with insulin sensitivity process, was shown to be upregulated [23].

4. Discussion

In the present study, we generated osteoblast-specific *Ggcx* cKO mice to assess the role of osteoblastic GGCX in glucose metabolism. We previously reported tissue-specific *Ggcx* targeting by crossing *Ggcx*-floxed mice with liver-specific Cre recombinase transgenic mice [15]. The IHC study validated that GGCX protein expression is diminished in the osteoblasts of *Ggcx* cKO mice. Because, along with its cofactor VK, GGCX is an essential enzyme that exhibits Gla modification of its target proteins including those in the bones [8], the serum ucOC and cOC levels substantially increased and decreased in *Ggcx* cKO mice, respectively, compared with those in control *Col1-Cre* mice.

In *Ggcx* cKO mice, weight loss of male gonadal WAT was obvious, while there was no loss of body and tissue weights of the pancreas, liver, and quadriceps muscle compared with control mice. The weight of gonadal WAT is often used as a biomarker for visceral obesity; therefore, it is considered that *Ggcx* cKO mice exhibit a phenotype without visceral obesity. Because visceral adiposity is considered a contributor to insulin resistance, the decrease in gonadal WAT weight could result in the activation of insulin sensitivity. Intriguingly, genes related to lipid metabolism and glucose oxidation such as aldo–keto reductase (*Ark1b7*), pyruvate dehydrogenase kinase (*Pdk4*), and paraoxonase (*Pon1*) were upregulated by >2-fold in *Ggcx* cKO mice as determined using expression microarray analysis. *Ark1b7* is expressed in adipose tissue and related to lipid metabolism [19], and a lack of the produced enzyme leads to insulin resistance [20]. *Pdk4* can be upregulated by the diabetic therapeutic agent rosiglitazone in WAT of Zucker fa/fa rats, a model of insulin resistance and dyslipidemia [21]. The increase of the *Pdk4* levels by thiazolidinediones is beneficial for the suppression of hyperlipidemia, a factor closely associated with insulin resistance. *Pon1* could be another modulator for glucose and lipid homeostasis, as polymorphisms of the gene have been significantly associated with clinical data including fasting blood glucose, triglycerides, apolipoprotein B, and fatty acid composition in the adipose tissue [22]. Pathway analysis based on the microarray data revealed that the insulin signaling pathway including insulin receptor substrate (*Irs1*) was activated in *Ggcx* cKO mice. *Irs1* is a key target of insulin receptor tyrosine kinase, and the dysregulated signaling of IRS1 together with IRS2 will result in insulin resistance [23].

It would be interesting to compare our findings with the recent literature in terms of the hormonal role of osteoblast-derived OC. In the context of bone metabolism, ucOC is a biomarker for VK insufficiency and considered a risk factor for bone fractures [24]. The *in vivo* mice studies in some labs showed that OC, in its decarboxylated form in particular, functions as an active hormone that promotes glucose metabolism [10–13]. These studies revealed that OC could facilitate insulin secretion and increase insulin sensitivity in peripheral tissues as well as increase adiponectin levels in adipocytes. Considering that the loss of osteoblastic GGCX results in the increase of serum ucOC levels in mice, osteoblastic GGCX could have negatively modulated glucose metabolism in the present study. In human studies, however, the physiological relevance of ucOC remains controversial. Some clinical studies showed that ucOC is associated with blood sugar levels and insulin secretion [25–27], although the question whether ucOC is a determinant of glucose metabolism in humans is under debate [28,29]. Considering that the serum VK level is a critical factor for regulating the carboxylation status of OC, further analysis will be required to assess the clinical relevance of OC in glucose homeostasis by monitoring the VK status.

Because VK is a cofactor of GGCX and promotes γ-carboxylation of OC, it remains to be determined whether VK intake is harmful for diabetic control based on the hypothesis of ucOC-regulated glucose homeostasis. Some clinical studies have shown that VK intake can improve insulin response and reduce the risk of type 2 diabetes mellitus [30,31]. GGCX is also expressed in the pancreas [32], and VK is abundantly distributed in several tissues including the pancreas [33]; thus, it remains to be elucidated whether GGCX in the pancreas or other tissues could have a beneficial effect on glucose and lipid metabolism by promoting γ-carboxylation of its target proteins rather than OC.

In summary, GGCX in osteoblasts could be a modulatory factor for glucose metabolism and adiposity in mice. OC can be considered as one of the mediators that are involved in the GGCX-dependent homeostasis; further studies will clarify the precise roles of GGCX and its cofactor VK in the complexity of endocrine metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.091.

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