FAT/CD36 regulates PEPCK expression in adipose tissue

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Wan Z, Matravadia S, Holloway GP, Wright DC. FAT/CD36 regulates PEPCK expression in adipose tissue. Am J Physiol Cell Physiol 304: C478–C484, 2013. First published January 9, 2012; doi:10.1152/ajpcell.00372.2012.—Fatty acid translocase (FAT)/CD36 has been extensively studied for its role in facilitating fatty acid uptake. Recent findings have also demonstrated that this protein regulates adipocyte lipolysis and may modulate fatty acid reestertification. As FAT/CD36 has been shown to control the expression of genes involved in fatty acid oxidation in adipocytes, we reasoned that this protein might also control the expression of enzymes involved in fatty acid reestertification. In adipose tissue from FAT/CD36 knockout (KO) mice, we found that glycerol and fatty acid release were reduced and this was associated with reductions in adipose triglyceride lipase. Decreases in lipolysis were paralleled by increases in the free fatty acid-to-glycerol ratio and reductions in primary and fractional rates of fatty acid reestertification in cultured adipose tissue from FAT/CD36 KO mice. Reductions in reestertification were associated with decreases in the mRNA expression and protein content of phosphoenolpyruvate carboxykinase (PEPCK). To determine if reductions in lipolysis could lead to decreases in PEPCK mRNA expression, we treated cultured mouse adipose tissue with the lipase inhibitor CAY10499 (2 μM) and found that this resulted in an ~50% reduction in PEPCK mRNA expression. Treatment with hexarelin (10 μM, 12 h), a CD36 agonist, increased PEPCK mRNA expression independent of lipolysis. Collectively, our results provide novel evidence that FAT/CD36 regulates PEPCK in adipose tissue and that this could be secondary to reductions in lipolysis.

DURING EXERCISE OR FASTING circulating catecholamine levels rise leading to increases in adipose tissue lipolysis and the release of fatty acids into the circulation to be used as a fuel source in peripheral tissues such as skeletal muscle and liver (18). While the majority of liberated fatty acids from triacylglycerol (TG) molecules in adipose are released into the blood stream, a significant portion are reesterified back to TG (31). The reestertification of fatty acids within adipocytes requires the generation of glycerol-3-phosphate (G3P), and this occurs primarily through the de novo synthesis of G3P from precursors such as lactate and pyruvate in a process called glyceroneogenesis (14, 22, 24). Phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate dehydrogenase kinase 4 (PDK4) have been identified as key enzymes in the biochemical regulation of this process (8, 9). PEPCK catalyzes the conversion of oxaloacetate to phosphoenolpyruvate (PEP), a precursor of G3P, whereas PDK4 inhibits pyruvate dehydrogenase complexes resulting in an increased flux of pyruvate towards the formation of oxaloacetate. Given the importance of fatty acids in whole body fuel metabolism and the central role of fatty acid reestertification in the control of adipose tissue fatty acid release, there has been a renewed interest in examining the processes regulating the expression of PEPCK and PDK4. In this regard, PEPCK and PDK4 have been shown to be targets of the insulin-sensitizing peroxisome proliferator-activated receptor-γ (PPARγ) agonists thiazolidinediones (TZDs; Refs. 8, 9). In recent work from our laboratory, we have shown that PDK4 and PEPCK are rapidly induced in adipose tissue during exercise (29, 30) and that this effect may be mediated, at least in part, by catecholamines (30). The absolute rates of fatty acid reestertification change in parallel with lipolysis (7) and thus the induction of glyceroneogenic enzymes, such as PDK4, by lipolytic agents are not entirely unexpected. While the specific mechanisms mediating the effects of β-adrenergic agonists on glyceroneogenic enzymes have not been fully elucidated, it has been suggested that the induction of these genes in adipocytes occurs secondary to lipolysis and the subsequent provision of fatty acids as ligands to PPARs (16, 20, 30).

Fatty acid translocase/CD36 (FAT/CD36) is a transmembrane protein thought to play an important role in mediating fatty acid transport into adipose tissue (11) and skeletal muscle (6, 11). In addition, recent evidence now suggests that FAT/CD36 is also involved in the control of adipocyte lipolysis. Zhou et al. (32) reported that the knockdown of FAT/CD36 in 3T3-L1 adipocytes led to reductions in glycerol and fatty acid release. Interestingly, the ratio of fatty acid to glycerol release was increased, which was suggested to be a result of reductions in the reuptake of fatty acids (32). However, increases in the fatty acid-to-glycerol ratio are also indicative of reductions in fatty acid reestertification. Thus an alternative interpretation of this data would suggest that FAT/CD36 could control the expression of PEPCK and/or PDK4. In this regard, work from Tremblay and colleagues (25) has shown that treatment with hexarelin, a FAT/CD36 agonist, induces the expression of PPARγ, a transcriptional regulator of PEPCK (8) and PDK4 (9, 30). Moreover, attenuated lipolysis has been linked to reductions in the expression of PEPCK (21) and PDK4 (20), and thus the ablation of FAT/CD36 may control the expression of PEPCK and/or PDK4 secondary to reductions in lipolysis. In this framework, the purpose of the present investigation was to examine the role of FAT/CD36 in regulating the expression of PEPCK and PDK4. We hypothesized that indexes of fatty acid reestertification would be reduced in adipose tissue from FAT/CD36 knockout (KO) mice and that this would be associated with reductions in PEPCK and PDK4 expression/content. We further hypothesized that decreases in PEPCK and/or PDK4 would be, at least in part, secondary to decreases in lipolysis.

METHODS AND MATERIALS

Materials. Reagents, molecular weight markers, and nitrocellulose membranes for SDS-PAGE were obtained from Bio-Rad (Mississauga, ON). Western Lighting Plus ECL was a product of Perkin Elmer (Waltham, MA). Anti-PEPCK antibodies (cat. no. 10004943) were obtained from Cayman Chemicals (Ann Arbor, MI). Anti-phosphorylated PDH-E1α Ser300 (cat. no. AP1064) and Ser293 (cat.
no. AP1062) antibodies were products of EMD Biosciences (San Diego, CA). An antibody against the E1α-subunit of PDH was from Mitosciences (Eugene, OR). Antibodies against β-actin (cat. no. ab8227), PKD4 (cat. no. ab38242), and perilipin A (cat. no. ab3526) were from Abcam (Cambridge, MA). Anti-adipose triglyceride lipase (ATGL; cat. no. 2138), PPARγ (cat. no. 2443), and hormone-sensitive lipase (HSL; cat. no. 4107) antibodies were from Cell Signaling (Danvers, MA). Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). CAY10499 (cat. no. 10007875) and GW9662 (cat. no. 70785) were purchased from Cayman Chemicals. Fatty acid-free bovine serum albumin (FA-free BSA; cat. no. 152401) was from MP Biomedical (Solon, OH). Free glycerol reagent (cat. no. F6428) was obtained from Sigma (Oakville, ON). Nonesterified fatty acids assay kits (NEFA-h kit) were purchased from Wako Chemicals (Richmond, VA). SuperScript II reverse transcriptase, oligo(dT) and dNTP were products of Invitrogen (Burlington, ON). Taqman Gene Expression Assays for mouse β-actin (4352933E), eukaryotic 18S RNA (4352930E), PEPCK (Mm01247058_m1), PKD4 (Mm01166879_m1), PPARγ co-activator-1α (PGC-1α; cat. no. Mm01208835_m1), carnitine palmitoyltransferase 1 (CPT-1; cat. no. Mm01308166_m1), medium-chain acyl-CoA dehydrogenase (MCAD; cat. no. Mm00431611_m1), and fatty acid transporter 4 (FATP4; cat. no. Mm01327405_m1) were from Applied Biosystems (Foster City, CA). All other chemicals were purchased from Sigma.

Treatment of animals. All protocols followed Canadian Council on Animal Care guidelines and were approved by the University of Guelph Animal Care Committee. Age matched wild-type (WT, C57BL/6) and whole body CD36-KO mice were provided by Dr. M. Febbrino (Cleveland Clinic, Cleveland, OH) and were bred onsite. Mice were housed three per cage, with a 12:12-h light/dark cycle, and were provided with water and standard rodent chow ad libitum. In the initial characterization studies of adipose tissue metabolism in these animals, both male and female mice were utilized. As the effect of genotype was similar in both sexes, subsequent experiments used male C56BL/6J mice purchased from Jackson Laboratories.

Adipose tissue organ culture. Approximately 10-wk-old FAT/CD36 KO and age-matched WT mice were anesthetized with pentobarbital sodium (5 mg/100-g body wt), and gonadal white adipose tissue was removed and cultured as we have described in detail previously (26, 30). Briefly, 250 mg of gonadal adipose tissue were minced into ~5 to 10-mg pieces and placed into culture dishes containing 7.5 ml of M199 supplemented with 1% antibiotic/antimycotic, 50 μg/ml insulin, and 2.5 mM dexamethasone. The cultures were placed in a cell incubator at 37°C to equilibrate for 24 h. On the morning of the experiment, media were replaced with fresh M199 supplemented with 2.5% FA-free BSA and 4 mM pyruvate in the absence of insulin and dexamethasone. After 2 h, the media were collected and adipose tissue minces were rinsed in ice-cold sterile PBS and strained and adipose tissue fragments were snap frozen and stored at −80°C for further analysis.

To explore the effects of the inhibition of lipolysis on the expression of PEPCK, epididymal adipose tissue from C57BL/6J mice (20wk of age) was cultured as described above. On the morning of the experiment, media were removed and replaced with fresh media supplemented with 2.5% FA-free BSA, 4 mM pyruvate, and either the lipase inhibitor CAY10499 (2 μM) or vehicle control for 4 h. As before, insulin and dexamethasone were not present during this treatment period. To assess the effects of CD36 activation on lipolysis and PEPCK expression, cultured adipose tissue was treated with the CD36 agonist (25) 2.5% FA-free BSA, 4 mM pyruvate, and either the lipase inhibitor CAY10499 (2 μM) or vehicle control for 4 h. As before, insulin and dexamethasone were not present during this treatment period. To assess the effects of CD36 activation on lipolysis and PEPCK expression, cultured adipose tissue was treated with the CD36 agonist (25) and stored at −80°C for further analysis.

Lipolysis. Media were saved and analyzed for NEFA and glycerol concentrations using colorometric assays according to the manufacturer’s instructions. NEFA and glycerol concentrations were corrected for tissue weight and reported as micromoles released per gram tissue per 2 h (WT vs. FAT/CD36 KO experiment) or 4 h (vehicle vs. CAY10499 experiment). Primary and fractional fatty acid reesterification was calculated as described by Van Harmelen et al. (27). Briefly, primary fatty acid reesterification was calculated as the difference between the theoretical fatty acid release (3 times glycerol release into the media) and the measured fatty acid release. This calculation assumes a negligible amount of fatty acid oxidation and is a measure of absolute rates of fatty acid reesterification. Fractional fatty acid reesterification is the proportion of fatty acids formed during lipolysis that is reesterified back to TG and is a measure of relative reesterification. This was calculated as the ratio of primary fatty acid reesterification to total fatty acid produced by lipolysis. Fractional reesterification can range from zero, no fatty acid reesterification, to 1, a condition where all the fatty acids are recycled to TG.

Western blot analysis. Protein was extracted from adipose tissue [the total protein concentration of adipose tissue from FAT/CD36 KO (5.28 ± 0.28 μg/μl) and WT (5.17 ± 0.49 μg/μl) mice was similar], and the protein content of PEPCK, PKD4, PDH, ATGL, HSL, PPARγ, and perilipin was determined by Western blotting as described in detail by our laboratory previously (26, 30). Signals were detected using enhanced chemiluminescence and were subsequently quantified by densitometry by Gene Tool according to the manufacturer’s instructions (SynGene, ChemiGenius2; PerkinElmer).

Real-time PCR. Changes in the mRNA expression of PKD4 and PEPCK were determined using RT-PCR as we have described in detail previously (28–30) using β-actin or 18S as an internal control. Relative differences in gene expression between groups were determined using the 2−ΔΔCT method (19). The amplification efficiencies of the gene of interest and the housekeeping gene were equivalent.

Statistical analysis. Differences between two groups were made using an unpaired Student’s t-tests. Significance was set at P < 0.05.

RESULTS

Lipolysis and markers of fatty acid reesterification are reduced in adipose tissue from FAT/CD36 KO mice. Glycerol (Fig. 1A) and fatty acid (Fig. 1B) release were reduced in cultured gonadal adipose tissue from FAT/CD36 KO mice compared with WT controls. To assess fatty acid reesterification we measured the ratio of fatty acid to glycerol release; primary reesterification, an index of absolute rates of fatty acid reesterification; and fractional reesterification, a measure of relative rates of fatty acid recycling. The ratio of free fatty acid to glycerol release was increased (Fig. 1C), whereas primary (Fig. 1D) and fractional (Fig. 1E) fatty acid reesterifications were reduced in adipose tissue from FAT/CD36 KO mice.

PEPCK mRNA expression and protein content are reduced in adipose tissue from FAT/CD36 KO mice. As shown in Fig. 2, both the mRNA expression (Fig. 2A) and protein content (Fig. 2B) of PEPCK were decreased, while there were no differences in the mRNA expression (Fig. 2A) or protein content (Fig. 2B) of PKD4, or the phosphorylation of PDH (Fig. 2B). Reductions in lipolysis were associated with decreases in the protein content of ATGL, whereas HSL and perilipin contents were intact (Fig. 2C). There were no differences in the phosphorylation of HSL between

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genotypes (data not shown). The protein content of PPAR gamma was not different in adipose tissue from WT and FAT/CD36 KO mice ($P_{/H11005}<0.11$). The mRNA expression of PGC-1 ($/H9251$) was reduced, whereas CPT-1 expression was increased in gonadal adipose tissue from FAT/CD36 KO mice. There were no differences in the mRNA expression of MCAD or FATP4 between genotypes (Table 1).

**Inhibition of lipolysis reduces PEPCK mRNA expression.** Treatment of cultured adipose tissue with the HSL inhibitor CAY10499 inhibited glycerol (Fig. 3A) and fatty acid (Fig. 3B) release. The inhibition of lipolysis was associated with reductions in PEPCK mRNA expression (Fig. 3C).

**Hexarelin increases PEPCK mRNA expression independent of lipolysis.** Treatment with hexarelin, a FAT/CD36 agonist (12 h, 20 μM), did not increase glycerol (Fig. 4A) or fatty acid release (Fig. 4B) in cultured adipose tissue. A 12-h treatment with hexarelin induced PEPCK mRNA expression (Fig. 4C). Hexarelin did not induce PEPCK mRNA expression in adipose tissue from FAT/CD36 KO mice (vehicle 1.11 ± 0.23 and hexarelin 1.36 ± 0.18; $n=5$/group; $P=0.41$). The PPARγ antagonist GW9662 did not attenuate the hexarelin-induced increase in PEPCK (hexarelin: 1.68 ± 0.37 and hexarelin + GW: 1.95 ± 0.39, fold increase compared with respective control; $n=5–7$/group).

**DISCUSSION**

FAT/CD36 is a membrane-associated protein that has been extensively studied for its role in regulating fatty acid uptake in a variety of tissues including skeletal muscle and adipose (as reviewed in Ref. 10). Interestingly, a recent investigation has...
provided evidence suggesting that FAT/CD36 also regulates lipolysis in fat cells (32). These investigators demonstrated that lipolysis was reduced in cultured 3T3 adipocytes when FAT/CD36 was knocked down via small interfering RNA (32). In parallel with reductions in lipolysis, the knockdown of FAT/CD36 resulted in increases in the ratio of fatty acid to glycerol release, providing evidence that FAT/CD36 may also control the reesterification of fatty acids in adipocytes. Given the important role of fatty acid reesterification in modulating fatty acid efflux from adipose tissue (22), understanding the mechanisms through which FAT/CD36 controls this process has significant implications for understanding the regulation of whole body lipid metabolism.

In this context we sought to examine potential mechanisms of how FAT/CD36 could modulate fatty acid handling in adipose tissue. We found that lipolysis was reduced in adipose tissue from FAT/CD36 KO mice and this was associated with reductions in the protein content of ATGL, a lipase that has been implicated in regulating resting, or basal, rates of lipolysis (4). In parallel with reductions in lipolysis we found that markers of fatty acid reesterification were also reduced as demonstrated by increases in the free fatty acid-to-glycerol ratio and decreases in primary and fractional fatty acid reesterification in adipose tissue from FAT/CD36 KO mice. The association among reductions in ATGL, lipolysis, and fatty acid reesterification is consistent with recent findings from Ahmadian et al. (1), who reported that the adipose tissue-specific overexpression of ATGL led to increases in lipolysis and fatty acid cycling.

Previous work has provided evidence that PDK4 (9) and PEPCK (8) are crucial enzymes involved in the reesterification
of fatty acids in adipose tissue, and thus we reasoned that the content of these enzymes would be reduced in adipose tissue from FAT/CD36 KO mice. While we did not detect any differences in the mRNA expression or protein content of PDK4 or changes in the phosphorylation of PDH, a protein phosphorylated by PDK4 (17), the expression and content of PEPCK were reduced 50% in adipose tissue from FAT/CD36 KO mice, thus suggesting that this could account for a portion of the reduction in fatty acid reesterification in adipose tissue from FAT/CD36 KO mice. These data are somewhat surprising as the expression of PDK4 (9) and PEPCK (8) in adipose has been thought to be regulated through similar transcriptional mechanisms. The current findings showing reductions in PEPCK in the absence of decreases in PDK4 in adipose tissue from FAT/CD36 KO mice would suggest that the expression and content of these enzymes are regulated, at least in part, through distinct pathways.

Prior research has reported a close association between lipolysis and the expression of enzymes involved in fatty acid reesterification. For example, Niang et al. (21) demonstrated that long-term leptin treatment reduced adipose tissue lipolysis and this occurred in parallel with decreases in glyceroneogenesis and PEPCK protein content. These findings would suggest that reductions in PEPCK expression in adipose tissue from FAT/CD36 KO mice might be secondary to decreases in lipolysis. In an effort to address this hypothesis we attenuated lipolysis in cultured mouse adipose tissue with the lipase inhibitor CAY10499 and found that PEPCK mRNA expression was reduced ~50%. These findings are consistent with recent work from Granneman and colleagues (20) demonstrating a role for lipolysis-derived fatty acids in the induction of enzymes involved in fatty acid oxidation in brown adipocytes and earlier work reporting a rapid and robust increase in PEPCK mRNA levels in cultured adipocytes by unsaturated fatty acids (2). Absolute rates of fatty acid reesterification parallel rates of lipolysis (7), and thus it is not entirely unexpected that by-products of lipolysis, i.e., fatty acids, could regulate the expression of enzymes that are involved in the reesterification of fatty acids. Taken in concert these findings would suggest that reductions in adipose tissue lipolysis might account for at least a portion of the decrease in PEPCK content in adipose tissue from FAT/CD36 KO mice.

Although the pharmacological inhibition of lipolysis lead to reductions in PEPCK mRNA expression, a finding that mirrors

### Table 1. mRNA expression of CPT-1, MCAD, FATP4, and PGC-1α in gonadal adipose tissue from WT and FAT/CD36 KO mice

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>WT</th>
<th>FAT/CD36 KO</th>
<th>P Value</th>
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<tbody>
<tr>
<td>CPT-1</td>
<td>1.17 ± 0.29</td>
<td>4.41 ± 0.98</td>
<td>0.01</td>
</tr>
<tr>
<td>MCAD</td>
<td>1.02 ± 0.08</td>
<td>0.81 ± 0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>FATP4</td>
<td>1.01 ± 0.06</td>
<td>0.94 ± 0.11</td>
<td>0.56</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>1.16 ± 0.23</td>
<td>0.33 ± 0.05</td>
<td>0.01</td>
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</tbody>
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Data are means ± SE for n = 6/group. CPT-1, carnitine palmitoyltransferase 1; MCAD, medium-chain acyl-CoA dehydrogenase; FATP4, fatty acid transporter 4; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; WT, wild type; FAT/CD36 KO, fatty acid translocase/CD36 knockout.

Fig. 3. Inhibition of lipolysis with CAY10499 leads to reductions in PEPCK mRNA expression in cultured adipose tissue. The lipase inhibitor CAY10499 (2 μM, 4 h) inhibited glycerol (A) and NEFA (B) release and PEPCK (C) mRNA expression in cultured adipose tissue. Data are presented as means ± SE for 7–12 mice per group. In C the mRNA data were normalized to β-actin and are expressed as fold differences compared with vehicle control. *P < 0.05 vs. vehicle control.

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the effect of FAT/CD36 ablation, we cannot discount the possibility that FAT/CD36 may regulate PEPCK through a mechanism that is independent of alterations in lipolysis. Previous work from Rodrigue-Way et al. (25) demonstrated that the growth hormone-releasing peptide hexarelin induced mitochondrial biogenesis in cultured adipocytes and in mouse adipose tissue in vivo. Interestingly, the in vivo effects of hexarelin were absent in CD36 null mice (25) a finding consistent with previous work demonstrating that hexarelin is a CD36 agonist (12). Similar to these findings we found that hexarelin treatment induced the expression of PEPCK in cultured mouse adipose tissue. Importantly, this occurred in the absence of increases in glycerol and fatty acid release suggesting a lipolysis-independent effect of FAT/CD36 on the regulation of PEPCK. Moreover, as the ablation of FAT/CD36 reduces lipolysis while hexarelin did not stimulate this process, these findings would suggest that FAT/CD36 is required, but its activation is not sufficient, for the stimulation of lipolysis.

TZDs have been shown to increase the expression of PGC-1α, FATP4, CPT-1, and MCAD in cultured adipocytes (5, 25), providing evidence that these genes, similar to PEPCK, are regulated, at least in part, by PPARγ. In the present investigation we did not find a consistent pattern of expression in these genes from FAT/CD36 KO mice. While PGC-1α mRNA expression was reduced, CPT-1 was increased and MCAD and FATP4 remained unchanged in adipose tissue from FAT/CD36 KO compared with WT mice. These findings are consistent with previous work demonstrating a differential regulation of gene expression by hexarelin and TZDs in cultured adipocytes (25). Our findings in adipose tissue from FAT/CD36 KO mice, in conjunction with the fact that GW9662 did not attenuate hexarelin-induced increases in PEPCK, provide evidence that PPARγ is not involved in the pathway(s) through which FAT/CD36 regulates PEPCK. In this regard hexarelin, at least in macrophages, has been reported to activate numerous transcription factors (3, 13). The identification of the specific mechanisms linking FAT/CD36 to regulation of gene expression in adipose tissue is an area that requires further investigation.

The findings of the current investigation, and previous work from others (32), suggest a previously unappreciated role for FAT/CD36 in the regulation of whole body lipid metabolism that goes beyond its well described role in mediating skeletal muscle fatty acid uptake (15, 23). In this context, it would now appear that FAT/CD36 is also involved in the control of adipose tissue lipolysis and the expression of enzymes, such as PEPCK, involved in fatty acid reesterification. The control of PEPCK expression by FAT/CD36 would appear to be mediated, at least in part, through a mechanism that is secondary to reductions in lipolysis.

The utilization of fatty acids as a fuel source by skeletal muscle is dependent on both the supply and delivery of fatty acids to skeletal muscle, as well as the movement of fatty acids into muscle cells. Given the impaired uptake and oxidation of fatty acids in skeletal muscle from FAT/CD36 KO mice (6), it is tempting to speculate that muscles from these mice may secrete a factor that signals to adipose tissue and downregulates components of the lipolytic machinery such as ATGL. This could be a
mechanism that is in place to match the uptake and delivery of fatty acids. If true, this would provide unique insight into a coordinated regulation of fatty acid uptake and delivery, perhaps being mediated by FAT/CD36.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: Z.W., S.M., G.P.H., and D.C.W. conception and design of research; Z.W. and S.M. performed experiments; Z.W. and D.C.W. analyzed data; Z.W., S.M., G.P.H., and D.C.W. interpreted results of experiments; Z.W. prepared figures; Z.W. and D.C.W. drafted manuscript; Z.W., S.M., G.P.H., and D.C.W. approved final version of manuscript.

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