Supporting Information

Berryhill et al. 10.1073/pnas.1210527109

SI Materials and Methods

Animals, Diets, and Experimental Design. Experiments were conducted using Balb/cJ (Jackson Laboratories), 129SVE, C57BL/6, FVB (all Taconic Farms), or FVB-PyMT (1) transgenic mice (gift from Alexander Borowsky, University of California, Davis). Mice had ad libitum access to feed and water and were housed under a 14 light:10 dark cycle. Experiments were performed in accordance with guidelines set forth by the Institutional Animal Care and Use Committee at the University of California, Davis.

Diets were isocaloric and based on a modified AIN93G diet that contained 15% fat by weight primarily supplied by soybean oil (Harlan Laboratories). The *trans*-10, *cis*-12 isomer of conjugated linoleic acid (10,12 CLA; Lipid Nutrition) replaced 1% fat by weight. The 10,12 CLA content in the experimental diet was 6.81% of total fatty acids, whereas it was undetectable in the control diet (Table S3).

Mice were weaned and bilaterally ovariectomized at 21 d of age under ketamine/xylazine (60/10 mg/kg) anesthesia. The left #4 inguinal mammary gland (MG) was cleared of endogenous epithelium at ovariectomy (2) in a subset of mice while the contralateral gland remained intact. Additional mice were bilaterally adrenalectomized concurrent with ovariectomy and maintained on 0.9% saline in drinking water. Mice were fed the control diet for 1 d following ovariectomy and then randomly assigned to either the control diet or 10,12 CLA. Daily injections started concurrently with diet assignments as follows: ICI 182, 780 (SC, 3 mg/kg/d, 100 μL; Ascent Scientific), estrogen (E) (0.1 μg/d E, 25 μL; Sigma-Aldrich; given only to control-fed mice), ICI 182, 780+E (given only to control-fed mice), letrozole (SC, 10 μg/d, 100 μL; a gift from Trish Berger, University of California, Davis), rosiglitazone (Rosi) [intraperitoneal (IP), 10 mg/kg, 12–20 μL injection volume depending on body weight; Cayman Chemical], picropodophyllotoxin (IP, 30 mg/kg, 12–20 μL; Tocris Bioscience). Vehicle was sesame oil or DMSO. Other mice received daily E (SC, 1 μg/d, 100 μL; Sigma-Aldrich) injections for 14 d commencing 7 d post ovariectomy. Mice were euthanized for blood and tissue collection via CO_2 inhalation 2 h after injection with 5-ethynyl-2′-deoxyuridine (EdU; IP, 12.5 mg/kg, 100 μL, Invitrogen).

Mammary Gland Whole Mounts, Histology, and Histochemistry. Whole mounts were prepared from #4 inguinal MG (3). Ductal elongation was determined by measuring distance from the nipple to the farthest-reaching duct termini. Ductal area was measured as the polygon area occupied by the ductal network while epithelial area was measured by tracing the epithelial structures and determining the area using National Institutes of Health ImageJ (4). Incorporated EdU was detected in paraffin sections (4 μm) of thoracic MG using Alexa 488 fluorochrome (Click-iT Detection Assays, Invitrogen) with DAPI mounting media (VectaShield, Vector Laboratories).

Serum Insulin, IGF-I, and Corticosterone Assays. Blood was collected via cardiac puncture and serum was stored at −80 °C. ELISA was used to detect serum insulin (Mercodia Inc.), IGF-I (Immunodiagnostic Systems Inc.), and corticosterone (Assaypro).

Western Blot. Whole-cell lysates were prepared from inguinal MG (5) in the presence of supplemental inhibitors (50 mM NaF, 2 mM Na_2VO_4, and Protease inhibitor mixture [Thermo Scientific]). Proteins (45 μg) were resolved using 8% (wt/vol) SDS/PAGE and transferred to PVDF (Immobilon-P, Millipore) that was incubated overnight at 4 °C with primary antibodies (rabbit monoclonal anti-IGF-IRβ (111A9) 1:500, Cell Signaling Technology; rabbit polyclonal anti-IRα (H-78) 1:500, Santa Cruz Biotechnology; rabbit monoclonal anti-IRβ (4B8) 1:1,000, Cell Signaling Technology; rabbit polyclonal anti-GAPDH 1:30,000, Sigma-Aldrich; mouse anti-β actin (ACTBD11B7) 1:2,000, Santa Cruz Biotechnology) followed by 1 h at room temperature with the secondary antibody (goat polyclonal horseradish peroxidase-conjugated anti-rabbit or anti-mouse 1:2,000, Dako). Immunoreactive bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Quantitative PCR. Primer sequences and annealing temperatures are presented in Table S4. Total RNA was prepared, DNase-treated and reverse transcribed as described (6). For all primer sets except IGF-I, cDNA (0.5 μL) was amplified using Fast SYBR Green Master Mix (Applied Biosystems) and 0.2 μmol/L primers on a 7500 Fast Real-Time PCR System (Applied Biosystems). Melting curve analysis showed a single amplification product. IGF-I was amplified from cDNA (0.5 μL) using Taqman Gene Expression Master Mix (Appied Biosystems), 0.9 μmol/L primers and 0.2 μmol/L probe. Each run included a no-template control and a reverse transcription negative control performed in the absence of reverse transcriptase. The mRNA expression levels were normalized to *18S rRNA* using relative standard curves constructed from fivefold serial dilutions of cDNA (6).

Fatty Acid Analyses. The fatty acid profile of the feed samples was analyzed using a modified version of the direct transesterification method (7). An aliquot of the solution, containing the fatty acid methyl esters (FAME), was taken for GLC analysis. Total lipids were extracted from mammary tissue according to the method of Bligh and Dyer (8) using a mixture of methanol:chloroform:water (2:2:1.8, by vol). The lipids were transesterified with methanolic sodium methoxide (0.5 M $NaOCH_3$/methanol, 15 min at room temperature) to produce FAME extracts that were analyzed on a GC-2010 gas chromatograph (Shimadzu). Fatty acid results were expressed as percentages (wt/wt) of fatty acids detected with a chain length between 10 and 24 carbon atoms. The lower limit of detection was <0.001 g/100 g fatty acids.

Statistics. Data were analyzed by *t* test, or one- or two-way ANOVA using the ProcGLM procedure in SAS (SAS Institute Inc.). Data were transformed where appropriate. Least square means comparisons were considered significant when $P \leq 0.05$. Regression analysis was performed using the ProcReg procedure in SAS.

1. Guy CT, Cardiff RD, Muller WJ (1992) Induction of mammary tumors by expression of polyomavirus middle T oncogene: A transgenic mouse model for metastatic disease. *Mol Cell Biol* 12:954–961.
2. Deome KB, Faulkin LJ, Jr., Bern HA, Blair PB (1959) Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. *Cancer Res* 19:515–520.
3. Atwood CS, et al. (2000) Progesterone induces side-branching of the ductal epithelium in the mammary glands of peripubertal mice. *J Endocrinol* 167:39–52.
4. Rasband WS (1997–2011) ImageJ (National Institutes of Health, Bethesda, MD). Available at http://imagej.nih.gov/ij/.
5. Trott JF, Schennink A, Hovey RC (2011) Cloning and expression of a unique short form of the porcine prolactin receptor. *J Mol Endocrinol* 46:51–62.

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7. Sukhija PS, Palmquist DL (1988) Rapid method for determination of total fatty-acid content and composition of feedstuffs and feces. *J Agric Food Chem* 36:1202–1206.
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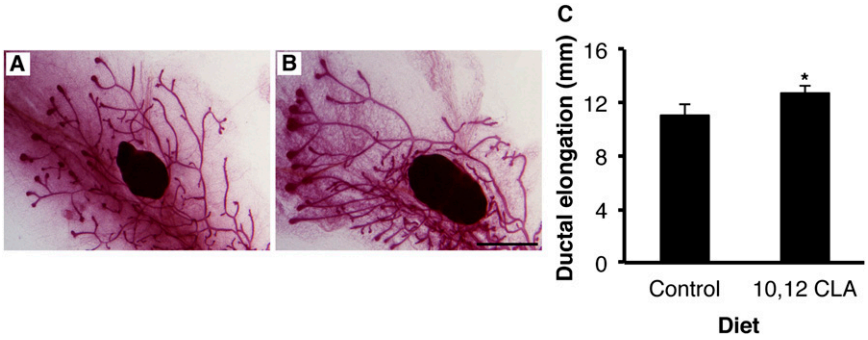

Fig. S1. Effect of dietary 10,12 CLA on mammary gland growth in OVX peripubertal mice administered estrogen. (*A* and *B*) Representative mammary gland whole mounts of OVX Balb/cJ mice fed either the (A) control or (B) 10,12 CLA diet from 22 to 42 d. Commencing 7 d after OVX, mice were administered daily injections of E (1 μg/d) for 14 d. (Scale bar, 2 mm.) (*C*) Ductal elongation measured per Fig. 1. Data are means ± SEM (n = 7–8/group). *P = 0.09.

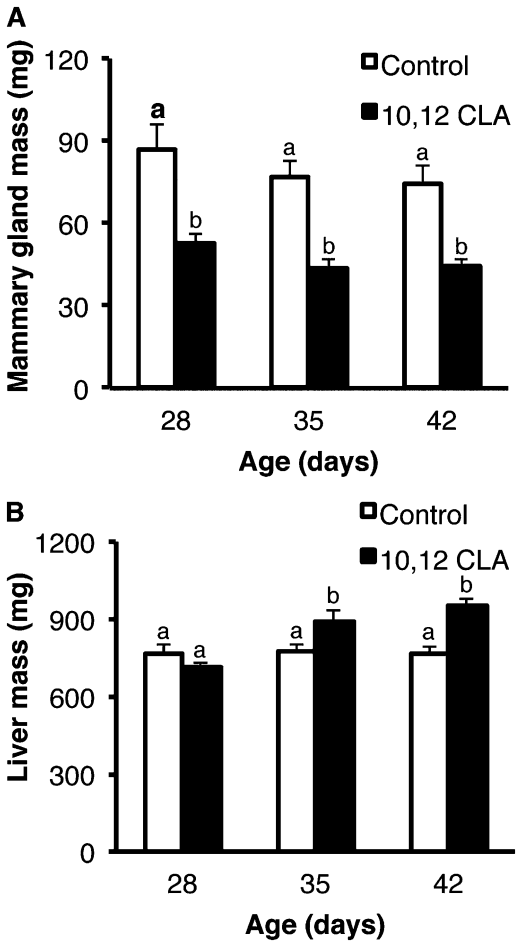

Fig. S2. Dietary 10,12 CLA decreases mammary gland mass and increases liver mass. (*A*) Lymph node-free wet mammary gland mass and (*B*) liver mass at necropsy in OVX Balb/cJ mice fed either the control or 10,12 CLA diet from 22 to 28, 35, or 42 d. Data are means ± SEM (n = 6–7/group). [a,b]Means with different superscripts are different ($P < 0.05$).

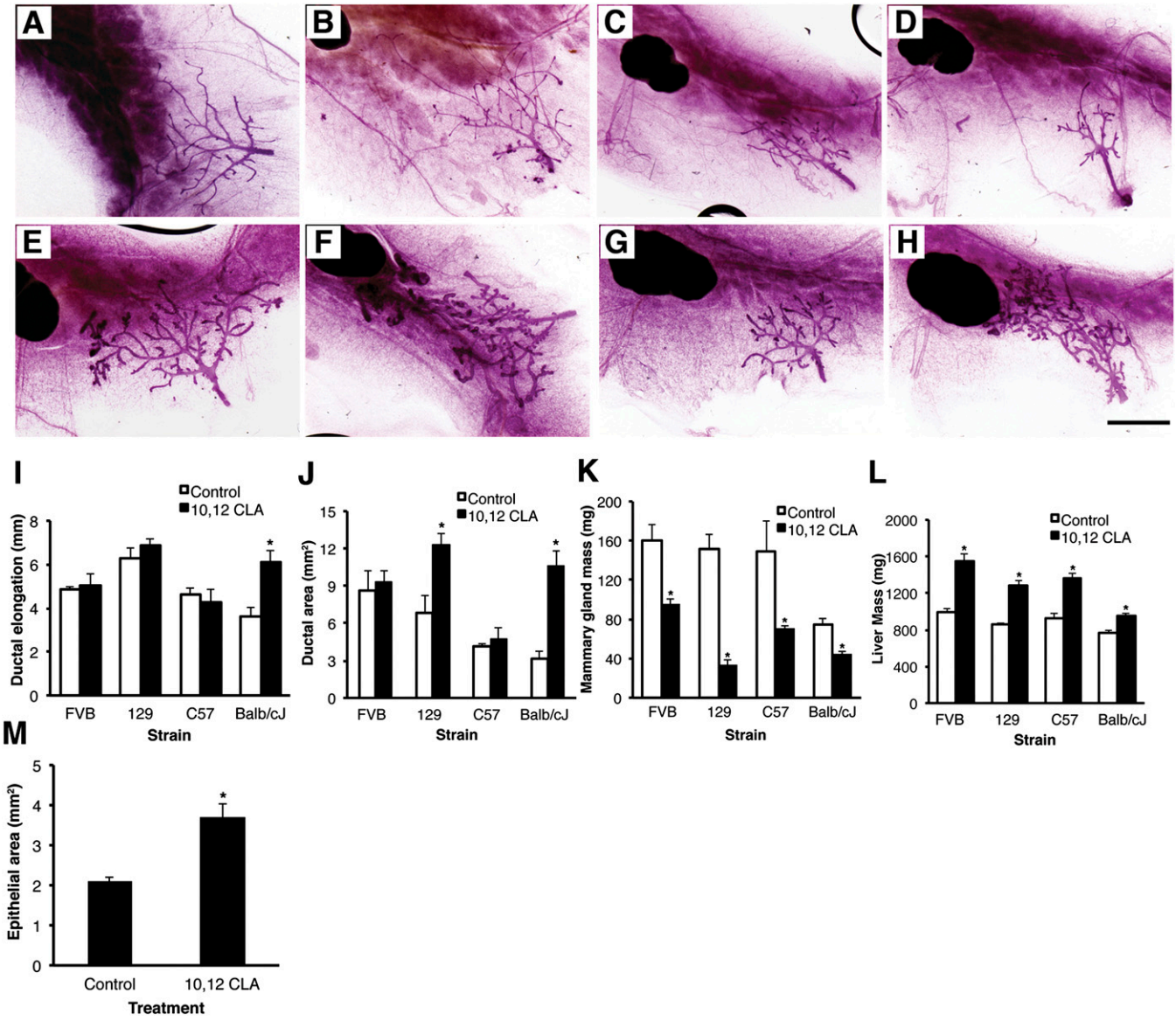

Fig. S3. Dietary 10,12 CLA stimulates ovary-independent mammary gland growth in different strains of mice. Representative whole mounts of mammary glands from OVX mice fed either the (*A*–*D*) control or (*E*–*H*) 10,12 CLA diet from 22 to 42 d. Strains of mice were FVB (*A* and *E*), 129SVE (*B* and *F*), C57BL/6 (*C* and *G*), and Balb/cJ (*D* and *H*). (*I*) Ductal elongation measured per Fig. 1. (Scale bar, 2 mm.) (*J*) Ductal area determined per Fig. 2. (*K*) Lymph node-free wet mammary gland mass and (*L*) liver mass at necropsy. (*M*) Epithelial area measured in whole mounts prepared from FVB mice as the area of all epithelial structures. Data are means ± SEM (n = 4–8/group). *Different from control diet within a strain ($P < 0.05$).

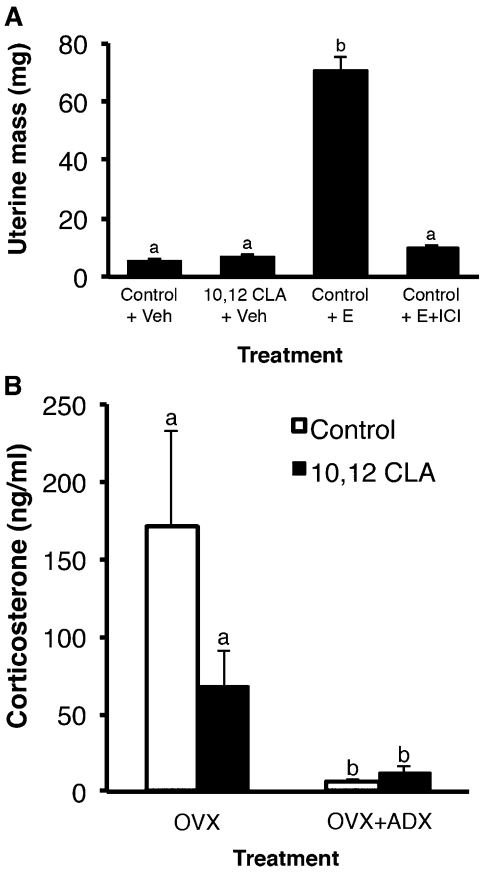

Fig. S4. Coadministered ICI 182,780 inhibits estrogen (E)-induced uterotrophy, and adrenalectomy reduces serum corticosterone. (*A*) Balb/cJ mice were ovariectomized at 21 d and fed either the control or 10,12 CLA diet from 22 to 42 d. Mice were coadministered daily injections of sesame oil vehicle (Veh), E (0.1 μg/d), or E+ICI. Wet uterine mass was determined at necropsy. (*B*) Serum corticosterone levels in Balb/cJ mice that were ovariectomized and adrenalectomized (ADX) at weaning (21 d) and then fed either the control or 10,12 CLA diet 42 d. Data are means ± SEM (n = 3–5/group). [a,b]Means with different superscripts are different ($P < 0.05$).

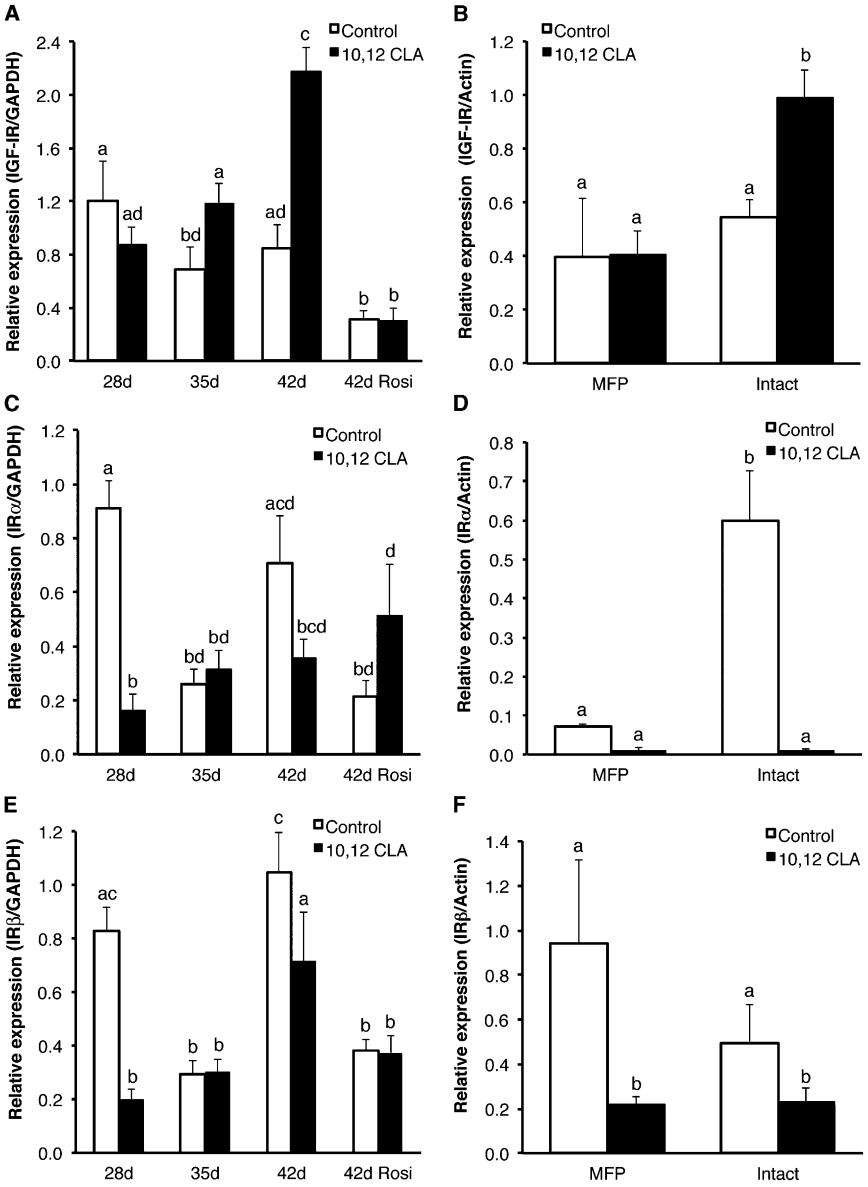

Fig. S5. Total insulin-like growth factor-I receptor is increased in the mammary glands of OVX peripubertal mice supplemented with 10,12 CLA. (*A*, *C*, and *E*) Densitometry of Western blots in Fig. 8. (*B*, *D*, and *F*) Balb/cJ mice were ovariectomized at 21 d, and one #4 inguinal mammary fat pad (MFP) was cleared of endogenous epithelium, and the contralateral gland remained intact (Intact). Mice were then fed the control or 10,12 CLA diet from 22 to 42 d. Western blots were quantified by densitometry. Data are means ± SEM (n = 3–4/group). ^{a,b,c,d}Means with different superscripts are different ($P < 0.05$).

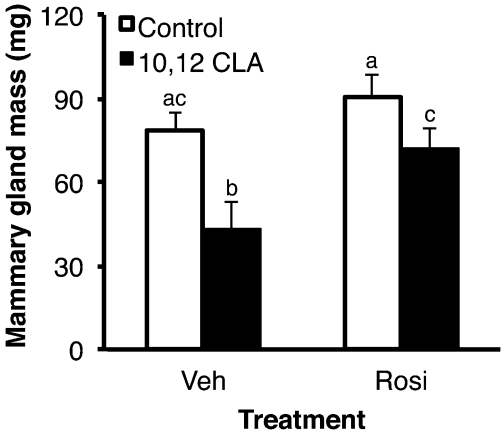

Fig. S6. Mammary gland mass is increased in OVX mice fed 10,12 CLA and coadministered rosiglitazone. Lymph node-free wet mammary gland mass was determined for OVX Balb/cJ mice fed either the control or 10,12 CLA diet from 22 to 42 d. Mice were administered daily injections of either DMSO (Veh) or rosiglitazone (Rosi). Data are means ± SEM (n = 6–7/group). [a,b,c]Means with different superscripts are different ($P < 0.05$).

Table S1. Fatty acid distribution of select fatty acids (% of total fatty acids) in the mammary glands of 35-d-old ovary-intact mice fed either the control diet or 1% 10,12 CLA

		Diet		
Fatty acid	Baseline*	Control	10,12 CLA	P value
Σ[†]SFA	38.70 ± 0.41	26.04 ± 0.49	24.99 ± 0.49	0.21
16:0	22.72 ± 0.43	18.23 ± 0.41	18.12 ± 0.27	0.83
18:0	1.44 ± 0.07	2.73 ± 0.25	2.90 ± 0.07	0.45
Σ MUFA	25.52 ± 0.29	30.01 ± 0.47	31.51 ± 0.25	0.02
18:1 *cis*-9	19.11 ± 0.19	24.55 ± 0.13	27.16 ± 0.26	<0.01
Σ PUFA	35.09 ± 0.55	43.31 ± 0.09	40.71 ± 0.68	0.03
18:2 n-6	29.52 ± 0.49	38.84 ± 0.07	37.70 ± 0.72	0.28
18:3 n-3	2.00 ± 0.05	2.56 ± 0.01	1.37 ± 0.05	<0.01
20:4 n-6	0.73 ± 0.03	0.36 ± 0.01	0.36 ± 0.02	0.82
20:5 n-3	0.10 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	<0.01
22:6 n-3	0.28 ± 0.02	0.18 ± 0.01	0.11 ± 0.01	<0.01
Σ CLA	0.07 ± 0.00	0.08 ± 0.01	1.87 ± 0.10	<0.01
cis-9, *trans*-11 CLA	0.02 ± 0.00	0.02 ± 0.00	0.36 ± 0.02	<0.01
trans-10, *cis*-12 CLA	ND[‡]	ND	1.29 ± 0.07	<0.01

Data are means ± SEM (n = 3–5/group).
*Samples from 21-d-old mice.
[†]Sum of fatty acid class.
[‡]Not detectable (<0.001 g/100 g).

Table S2. Select organ masses of 35-d-old ovary-intact mice fed either the control diet or 1% 10,12 CLA diet

		Diet		
Organ mass	Baseline*	Control	10,12 CLA	P value
Mammary gland (mg)	105.88 ± 10.13	106.70 ± 9.46	36.36 ± 2.76	<0.01
Liver (mg)	569.20 ± 19.58	930.40 ± 41.80	973.46 ± 36.72	0.46

Data are means ± SEM (n = 4–5/group).
*Samples from 21-d-old mice.

Table S3. Fatty acid composition of experimental diets (% total fatty acids)

	Diet	
Fatty acid	Control	10,12 CLA
16:0	10.98	10.49
18:0	3.81	3.79
18:1 *cis*-9	20.62	19.25
18:2 n-6	54.15	48.36
18:3 n-3	6.60	5.92
cis-9, *trans*-11 CLA	0.00	1.19
trans-10, *cis*-12 CLA	0.00	6.81
Σ SFA	15.87	15.31
Σ MUFA	22.11	20.59
Σ PUFA	60.77	54.31
Σ CLA	0.00	8.47
Σ TFA	0.42	0.35

Table S4. RT-quantitative PCR forward (F) and reverse (R) primers, probes (P), and melting temperature (T_m) for the resultant PCR product generated for each gene using F and R primers

Gene		T_m (°C)
IGF-I F	5′ GCTCTTCAGTTCGTGTGTGGAC 3′	58
IGF-I R	5′ CATCTCCAGTCTCCTCAGATC 3′	
IGF-I P	5′ 6-FAM TTCAACAAGCCCACAGGCTAT BHQ-1 3′	
IGF-IR F	5′ GGCACAACTACTGCTCCAAAGAC 3′	58
IGF-IR R	5′ CTTTATCACCACCACACACTTCTG 3′	
IR-A F	5′ TCCTGAAGGAGCTGGAGGAGT 3′	58
IR-A R	5′ CTTTCGGGATGGCCTGG 3′	
IR-B F	5′ TCCTGAAGGAGCTGGAGGAGT 3′	58
IR-B R	5′ TTCGGGATGGCCTACTGTC 3′	
IGF-II F	5′ AAGTCGATGTTGGTGCTTCTCATCT 3′	60
IGF-II R	5′ CCCCTCCGCACAGAGTCTCT 3′	
18S F	5′ ACGGCTACCACATCCAAGGA 3′	60
18S R	5′ CCAATTACAGGGCCTCGAAA 3′	