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Amino Acid-Dependent Activation of Liver Estrogen Receptor Alpha Integrates Metabolic and Reproductive Functions via IGF-1

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SUMMARY

Throughout evolution, organisms have devised strategies to limit fertility in case of prolonged starvation. In mammals, the liver plays a central role in the orchestration of mechanisms allowing for the maintenance of energy homeostasis. We here demonstrate that dietary amino acids regulate the transcriptional activity of hepatic estrogen receptor alpha (ERα) through an mTOR-dependent mechanism. As a result of ERα activation, hepatic IGF-1 mRNA and blood IGF-1 are increased. Conversely, calorie restriction or selective ablation of ERα in the liver decrease blood IGF-1 to levels inadequate for the correct proliferation of the lumen epithelium in the uterus and the progression of the estrous cycle. We propose that the liver acts as critical mediator of energetic and reproductive functions responsible for the blockade of the estrous cycle in case of protein scarcity. Our findings may provide novel insights to understand the cause of selected forms of infertility and metabolic alterations in women after menopause.

INTRODUCTION

Food intake and fertility are under a strict reciprocal control and this mechanism ensures that reproduction occurs only in favorable conditions with respect to energy availability. The biochemical pathways coupling nutrition and reproduction were described in detail in invertebrates (Gerisch et al., 2001; Motola et al., 2006; Lee et al., 2008); in most vertebrates, it is recognized that malnutrition affects the reproductive cycle, but the molecular events involved in this phenomenon remain to be clarified.

In mammals the ovarian sex hormone 17β-estradiol (E$_2$) and its receptors ERα and ERβ have a major role in the control of reproductive functions as well as in the regulation of food intake (Dubuc, 1985; Asarian and Geary, 2006) and lipid and glucose homeostasis (Hewitt et al., 2004); thus E$_2$ signaling apparatus is a potential candidate for the coupling of reproductive functions with the energetic metabolism.

E$_2$ is synthesized via a feedback mechanism involving the hypothalamus in which the gonadotropin-releasing hormone (GnRH) is produced to stimulate the anterior hypophysis to release the gonadotropins into the bloodstream (Levine, 1997). Gonadotropins elicit the maturation of follicles in the ovary and the production of sex hormones that orchestrate the maturation of the oocytes and the implantation of the fertilized eggs in the uterus. GnRH synthesis in the hypothalamus is in turn controlled by circulating E$_2$ via complex mechanisms, including the hormonal direct action on electrical activity of GnRH neurons and indirect control exerted via neuron-neuron or glia-GnRH interactions (Herbison, 1998).

GnRH neurons are also the recipient of input from the neural cells responsive to peripheral (e.g., leptin, insulin, ghrelin) (Magni et al., 1999; Chan and Mantzoros, 2001; Malik et al., 2008) and central (NPY, orexins, kisspeptin, melanocortin) metabolic hormones (Li et al., 1999; Campbell et al., 2003; Dungan et al., 2006; Lee and Wardlaw, 2007). Thus the finding that ERs are largely present in the hypothalamic regions which are responsible for energy intake and storage (Musatov et al., 2007) and that circulating levels of steroid hormones influence neurons responsive to central metabolic hormones led to the formulation of the hypothesis of a central mechanism for the control of energetic metabolism by sex hormones (Schneider, 2004).

However, estrogen signaling is known to control some of the mechanisms regulating the energetic metabolism in the peripheral organs as well. E$_2$ controls the amount of white adipose tissue (WAT) in female mice (Heine et al., 2000) and modulates leptin production positively and negatively depending upon its binding to ERα or ERβ (Yi et al., 2008); in the skeletal muscle, both ERα and ERβ were shown to be instrumental in estrogen regulation of the glucose transporter GLUT4 (Barros et al., 2006); in the pancreas insulin content is regulated via ERα (Alonso-Magdalena et al., 2008). In the liver, where most of the estrogen receptors are of the ERα type (Alvaro et al., 2000), this receptor and estrogen were shown to control glucose

homeostasis, improving glucose tolerance and insulin sensitivity in animal models and humans (Takeda et al., 2003; Simpson et al., 2005; Gao et al., 2006), and by regulating the activity of specific genes, estrogen was described as directly stimulating lipogenesis in several animal species (Courtney et al., 1988) and the expression of lipogenic genes (Gowri et al., 2007). By using the ERE-Luc reporter mouse model (Ciana et al., 2003a), we clearly demonstrated a major impact of E_2 on liver ER activity; most interestingly, liver ERα activity was strongly regulated by food intake (Ciana et. al 2005) and during the female estrous cycle ERα transcriptional activity in the liver and in the reproductive organs were temporally associated, in contrast to what was observed in the nonreproductive organs (Ciana et al., 2003a). These observations led us to further investigate the biological relevance of food intake-dependent regulation of ER activity in liver and to verify the extent to which liver ER activity could be involved in the control of reproductive functions.

We here demonstrate the existence of a novel liver-dependent mechanism controlling growth of the uterus and therefore ensuring that regular ovulatory cycles occur only in relation to an adequate nutritional supply.

RESULTS

Amino Acids Induce ER Transcriptional Activity in Liver

Previous studies with the ERE-Luc reporter mouse model demonstrated that consumption of nonestrogenic food activated estrogen receptors (ER) in liver. To further demonstrate the association between food consumption and liver ER activity, we investigated the consequences of calorie restriction (CR). Adult female ERE-Luc mice were subjected to 40% CR for 4 weeks and, at the end of each week, liver ER activity was monitored in vivo by bioluminescence-based imaging (BLI, Figure 1A) or ex vivo by quantitative analysis of luciferase enzymatic activity (Figure 1B) and mRNA (Figure 1C). All measurements were done in the morning (between 9 and 11 a.m.) when luciferase activity was generally heightened by food consumption at night. CR was associated with a very significant decrease in luciferase mRNA and activity in the liver of ERE-Luc mice. This effect was not associated with changes in ERα mRNA, indicating that the receptor activity, but not its turnover, was affected by CR (Figure 1D).

To identify the nature of the macronutrients responsible for liver ER activity, we measured luciferase accumulation after oral administration of an estrogen-free diet or an isocaloric amount of its carbohydrate (C), protein (mainly amino acids), or lipid (L) components. Figure 1E shows that 6 hr after gavage, the complete diet induced an increase in luciferase activity of 67% versus controls; when each single macronutrient was administered, the amino acids, but not the L or C, were able to significantly increase ER activity (+46% versus control). As control, we measured the effect of the diets on another well-known sensor of cellular energy homeostasis: AMP-dependent protein kinase (AMPK) (Andersson et al., 2004). As previously reported, AMPK protein content in liver increased in animals fed with the regular diet (+83%) and with C (+100%), while its phosphorylated state (PAMPK) augmented with C (+40%) and decreased with the complete diet (−34%), amino acids (−36%), and L (−40%) (Figure S1A, available online). Thus amino
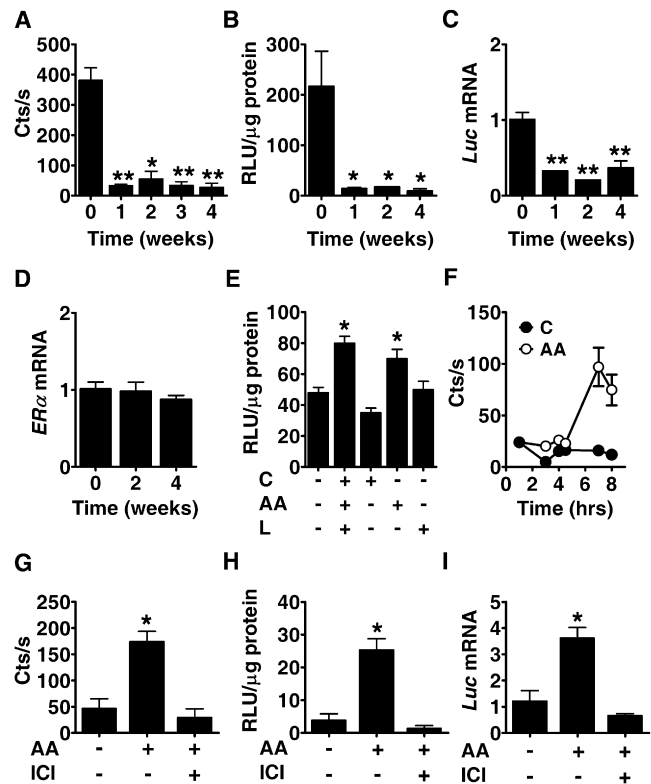

Figure 1. Food Consumption Is Associated with ER Transcriptional Activity in the Liver

(A–D) Luciferase (LUC) activity as measured in vivo by BLI (A) and ex vivo by enzymatic activity (RLU, B), *LUC* (C), and *ERα* mRNA (D) in liver prior to (0) or at the end of weeks 1, 2, 3, and 4 of CR. Data represent the mean ± SEM (n = 6). *p < 0.05; **p < 0.01 (versus time 0).

(E) LUC enzymatic activity in liver of mice 6 hr after gavage with isocaloric amounts of carbohydrates (C), amino acids (AA), or lipids (L). Data represent the mean ± SEM (n = 18).

(F) Time course of the effect of treatment with C or AA on liver LUC activity.

(G–I) BLI (G), LUC enzymatic activity (H), and *LUC* mRNA content (I) measured in liver of mice 6 hr after gavage with AA and with or without ICI 182,780 (ICI). Data represent the mean ± SEM (n = 6). *p < 0.05; **p < 0.01.

acids exerted an effect selective for the sex hormone receptor activity.

Because it is well known that sugars are rapidly catabolized, we investigated the effects of C or AA administration at shorter times after oral treatment. We measured luciferase activity in living mice by BLI at 1, 3, 4, 7, and 8 hr after C or AA administration by gavage. At no time did C increase luciferase-dependent photon emission in liver; conversely, 6–8 hr after AA administration photon emission was increased 4-fold versus time 0 (Figure 1F). We calculated that the total amount of AA we could administer by gavage was equivalent to the 20% average daily protein intake: considering that this amount of AA induced an increase in liver photon emission of the same order of magnitude as endogenous estrogen at proestrus (P, the phase of the estrous cycle in which circulating E_2 is highest) (Figure S1B), we concluded that liver ER activity induced by amino acids may be associated with a specific physiological function. Thus we further tested whether the AA effect on liver luciferase was

associated with transcriptional activation of ER by coadministration of the ER pan-antagonist ICI 182,780 (6.7 mg/kg). Figures 1G–I show that when amino acids were coadministered with the ER antagonist, amino acids failed to induce an increase in photon emission as well as in luciferase enzymatic activity and mRNA, thus indicating that, in liver, luciferase accumulation is strictly dependent on ER activity.

Amino Acids Stimulate ER Activity in Primary Cultures of Hepatocytes and Transfected HepG2 Hepatoma Cells

To demonstrate whether amino acids directly affects liver ER, we carried out a series of observations in hepatic cells in culture. Primary cultures of hepatocytes isolated from the ERE-Luc mice were shown to express luciferase at a concentration dependent on the amount of E_2 used for the stimulus; the reporter was not induced in cells isolated from ERE-Luc/ER$\alpha^{-/-}$ mice (Figures 2A and 2B). Similarly, amino acids induced a significant increase of luciferase activity only in the cells isolated from mice expressing ERα (Figure 2C). The AA effect was blocked when hepatocytes were incubated in the presence of 10 mM of 2-aminobicyclo(2.2.1)-heptane-2-carboxylic acid (BCH), an AA-uptake inhibitor thought to be specific for the L System (Christensen et al., 1969), indicating that amino acids had to penetrate the cell membrane in order to regulate ER transcriptional activity (Figure 2D). In HepG2 cells, not expressing ERs, the effect of AA was studied in cotransfection experiments. In the cells transfected with the luciferase reporter plasmid only, E_2 (1 nM) or amino acids (0.25–10 mM) did not induce luciferase synthesis; in the presence of ERα the effect of AA was dose dependent and maximal at a concentration of 10 mM (+125%) (Figure 2E). In transfected HepG2 cells, the AA-induced increase of ERα activity was blocked by rapamycin (Rapa, 50 nM) and LY294002 (50 μM), both described as inhibiting the mTOR pathway (Ghayad et al., 2008). Pharmacological blockade of the PKA signaling pathway by H-89 (50 μM) did not affect AA-induced ER activity (Figure 2F). Further, we found that amino acids failed to induce the transcriptional ability of selected phosphorylation mutants of ERα such as S167A and Y537A (Figure S2), supporting the existence of a direct effect of amino acids on ER activity that involves mTOR-mediated signaling.

Dietary Proteins Rescue Mice from CR-Induced Blockade of the Reproductive Cycle

Next, we asked what could have been the physiological role of the AA-mediated modulation of ERα in the liver. Protein malnutrition, as the most severe form of food deprivation in mammals, is known to be associated with the blockade of the reproductive cycle. The fact that proteins, but not other macronutrients, stimulated ER signaling in the liver was consistent with the idea of a functional connection between nutrient-induced activation of hepatic ER and reproduction. To test this hypothesis, we investigated whether the 40% CR, able to decrease liver ER activity, was sufficient to arrest the reproductive cycle and whether a diet enriched in amino acids by 40% was able to rescue the effect of the CR. Mice were subjected to 40% CR by using either a regular (reg) or an isocaloric diet enriched by 40% in raw proteins (hyperproteic diet, hyp); hence, in the CR experiments, mice treated with the reg diet consumed 40% less of all macronutrients, while those treated with the hyp diet consumed an
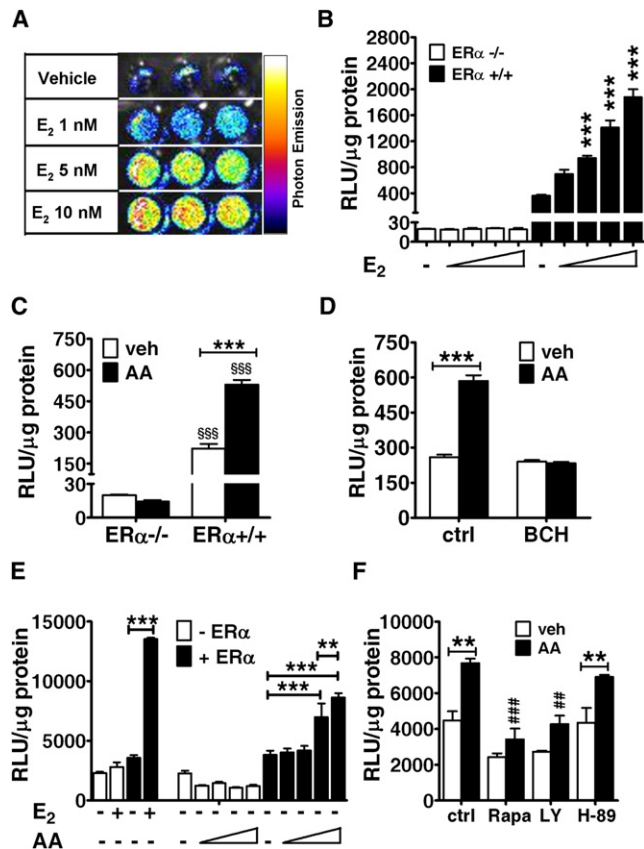

Figure 2. AA-Induced ER Transcriptional Activation in Hepatic Cells Grown in Culture

(A) Photon emission of hepatocytes isolated from ERE-Luc mice in primary culture incubated for 6 hr in the presence of the indicated E_2 concentrations.

(B and C) LUC enzymatic activity measured 6 hr after treatment with E_2 (0.5, 1, 5, 10 nM) or AA (1 mM) in hepatocytes from ERE-Luc (ER$\alpha^{+/+}$) or ERE-Luc/ERαKO (ER$\alpha^{-/-}$) mice. Data represent the mean $\pm$ SEM (n = 6). ***p < 0.001 versus vehicle (veh) ER$\alpha^{+/+}$; §§§p < 0.001 versus ER$\alpha^{-/-}$.

(D) LUC enzymatic activity in hepatocytes isolated from ERE-Luc mice after 30 min incubation with BCH (10 mM) prior to 6 hr treatment with veh or AA (1 mM). Data represent the mean $\pm$ SEM (n = 6). ***p < 0.001.

(E) LUC enzymatic activity in transfected HepG2 incubated as shown with E_2 (5 nM) or AA (0, 0.25, 0.5, 5, 10 mM) for 5 hr. Data represent the mean $\pm$ SEM (n = 6). **p < 0.01; ***p < 0.001.

(F) LUC enzymatic activity in transfected HepG2 cells treated with 10 mM AA with or without 50 nM rapamycin (Rapa), 50 μM LY294002 (LY), or 50 μM H-89. Data represent the mean $\pm$ SEM (n = 6). ###p < 0.001 versus ctrl AA.

amount of proteins identical to animals fed ad libitum (AL), but a lower amount of C and L. The effects of these dietary regimens on estrous cycle progression were assessed daily by vaginal smears. The regularity of the estrous cycle was scored by counting the number of proestrus (P) in a 4-day period, considering that in mice the length of the estrous cycle is about 4 days. Figure 3A shows that 1 week after 40% CR with regular diet a very limited percentage (14%) of the mice were cycling; indeed, 100% of mice were in anestrus on average 8.5 days after the beginning of CR. CR with hyperproteic diet was significantly less disruptive for the cycle because after 1 week of CR, 66% of the mice were still cycling and on the 12th day of CR, 25%
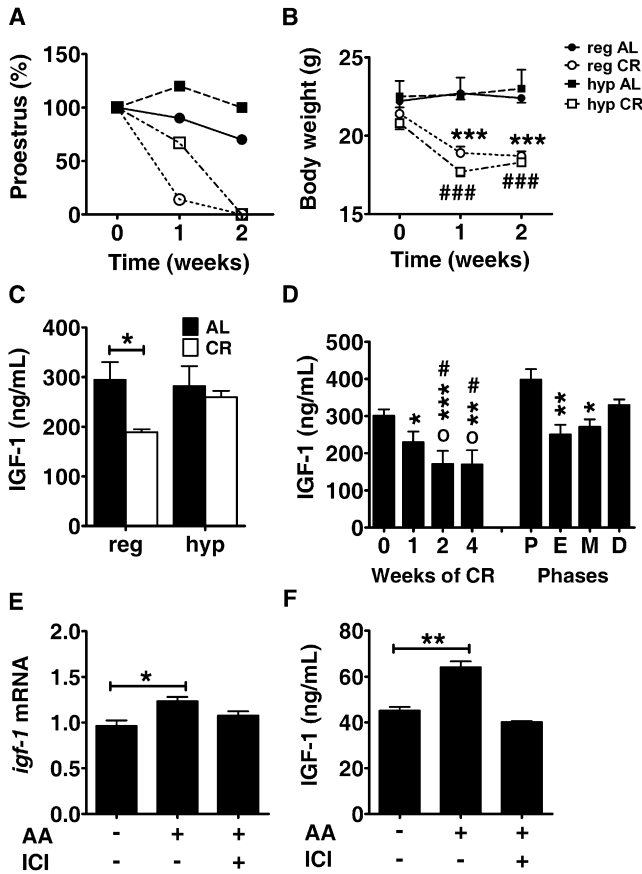
Figure 3. Dietary Proteins Delay CR-Induced Blockade of the Reproductive Cycle and Prevent ER-Dependent Synthesis of Circulating IGF-1

(A) The frequency of proestrus in the 2 weeks prior to the beginning of the experiment (time 0) was scored as 100% and then the cycle was analyzed by vaginal smears daily and scored as % versus time 0 at weeks 1 and 2 of treatment with regular (reg) or hyperproteic (hyp) diets.

(B) Animal weight at the beginning of the study and at the end of weeks 1 and 2 of CR. Data represent the mean ± SEM (n = 6). ***p < 0.001 (versus reg diet AL); ###p < 0.001 (versus hyp diet AL).

(C) Total serum IGF-1 after 2 weeks of CR with reg or hyp diets. Data represent the mean ± SEM (n = 6). *p < 0.05.

(D) Total serum IGF-1 levels at the beginning (0) or at the end of weeks 1, 2, and 4 of CR with reg diet and in females in different phases of estrous cycle Data represent the mean ± SEM of 3–21 mice. *p < 0.05; **p < 0.01; ***p < 0.001 (versus P); #p < 0.05 (versus D); °p < 0.05 (versus time 0). P, proestrus; E, estrus; M, metestrus; D, diestrus.

(E and F) *Igf-1* mRNA in liver (E) and total serum IGF-1 content (F) measured 6 hr after gavage with AA with/without ICI. Data represent the mean ± SEM (n = 6). *p < 0.05; **p < 0.01.

of the mice was still in the metestrus/diestrus (M/D). Interestingly, the hyperproteic diet had a favorable effect also in controls as indicated by the slight increase (+20%) in the number of P/mouse. The similarity of body weights (Figure 3B) in the various experimental groups confirmed that the amount of calories provided by the two diets was identical.

Altogether, these data were consistent with the theory envisioning the requirement for the AA-dependent activation of liver ER for a correct progression of the estrous cycle.

Decreased IGF-1 Signaling Impairs the Progression of the Estrous Cycle

In model organisms such as *Caenorhabditis elegans* and *Drosphila melanogaster*, energy balance controls reproductive functions through a well-conserved IGF-1/insulin-like signaling pathway (Lee et al., 2008; Motola et al., 2006; Gerisch et al., 2001). In rodents and primates, liver is the primary source of circulating IGF-1 (Yakar et al., 1999) and the hormone blood content is severely decreased by protein malnutrition (Ammann et al., 2000) and increased by dietary proteins (Filho et al., 1999). These observations led us to verify whether AA-induced liver ER activity affected IGF-1 synthesis and therefore the progression of the cycle. In line with the data in the literature, at the end of the second week of CR, we found a significant decrease in IGF-1 content in the serum of mice treated with the regular diet, but no decrease in animals in which CR was carried out with the hyperproteic diet (Figure 3C).

When we analyzed the effect of 40% CR with the regular diet, we found that, at the end of the first week of CR, serum IGF-1 content was decreased by 24% (from 300 ng/mL to 230 ng/mL) and by the second week of CR circulating levels of IGF-1 were stabilized at a concentration of 170 ng/mL (Figure 3D). In keeping with a relationship between reproduction and circulating IGF-1, the measurement of IGF-1 in the bloodstream of mice fed ad libitum showed significant changes during the estrous cycle: the highest serum concentration of IGF-1, 398 ng/mL, was at proestrus (P), the lowest, 250 ng/mL, at estrus (E) (Figure 3D). Thus the impairment of the progression of the reproductive cycle induced by CR could have been due to a reduction of circulating IGF-1 to levels below those observed at E.

Most interestingly, by treating the mice with amino acids alone or with amino acids and ICI 182,780 we showed that ERα activity was necessary for the AA-induced increase of Igf-1 mRNA in liver and IGF-1 protein in serum (Figures 3E and 3F).

The involvement of IGF-1 in the progression of the reproductive cycle was studied further in cycling mice. ERE-Luc mice were subjected daily to vaginal smear analysis and to in vivo imaging; blood was collected to measure circulating levels of E$_2$ and IGF-1. In this group of animals, E$_2$ serum level was maximal at P, decreased at E, and then slowly increased at metestrus (M) and diestrus (D); the concentration of the peptide hormone was maximal at P, decreased significantly at E, but very rapidly augmented at M and D (Figures 4A and 4B). These fluctuations were consistent with our previous observations and other reports on increased IGF-1 secretion after administration of exogenous E$_2$ (Venken et al., 2005). In line with the changes of circulating E$_2$, photon counting indicated that in the hepatic area luciferase activity peaked at each P (Figure 4E). We then tested the involvement of IGF-1 in the progression of the cycle by treating the mice with JB3, a competitive antagonist of the IGF-1 receptor (IGF-1R) (Pietrzkowski et al., 1992). Infusion of JB3 at the rate of 1 μg/day, a concentration previously shown to block IGF-1R activity (Figure S3), increased the length between each P from 4–5 to 6–7 days (Figure 4C) and blunted the profile of ER activity in time (Figure 4E).

Further evidence of the consequences of the reduced synthesis of liver IGF-1 on the reproductive cycle was obtained by crossing the ERE-Luc with mice carrying a liver-selective
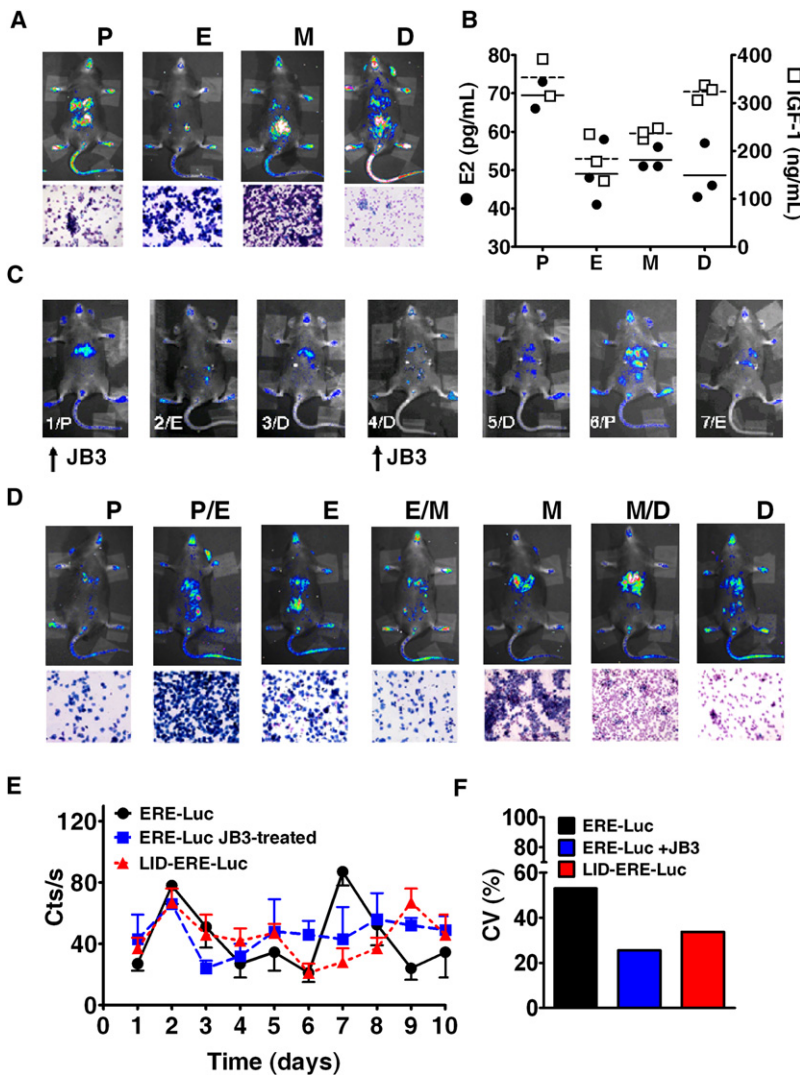
Figure 4. Decreased IGF-1 Signaling Impairs the Progression of the Reproductive Cycle

(A) BLI and corresponding vaginal smears of a representative mouse during the 4 day-long estrous cycle.

(B) IGF-1 and E_2 serum concentration at the indicated estrous cycle phases.

(C) JB3 (1 μg/day s.c.) effect on photon emission as measured daily at 2:00 p.m. by BLI in a representative ERE-Luc mouse.

(D) BLI and corresponding vaginal smears of a representative LID/ERE-Luc mouse in different phases of the estrous cycle.

(E and F) Photon emission as measured daily by BLI (E) in the hepatic area of ERE-Luc, JB3-treated ERE-Luc, and LID/ERE-Luc female mice and (F) respective coefficient of variation. Data represent the mean ± SEM of 5–10 animals.

ablation of the IGF-1 gene (LID mice, Yakar et al., 1999). LID mice have serum IGF-1 levels 60%–70% lower than ERE-Luc mice (Yakar et al., 1999 and Figure S4A), but are fertile. Consistent with the JB3 results, we found that the decreased liver output of IGF-1 was associated with an increased length of the reproductive cycle (to 6–7 days) (Figure 4D). Similarly to JB3-treated mice, in LID mice the profile of oscillation of the hepatic ER activity (Figure 4E) was modified. A quantitative assessment of the extent of the fluctuations of ER activity in liver during the estrous cycle was provided by the measurement of the coefficient of variation (CV), which was clearly higher in ERE-Luc (53%) than in JB3-treated (25.6%) and LID (33.7%) mice (Figure 4F).

A Prompt Blockade of Fertility Is Not Observed in LID Mice Subjected to CR

Previous results led us to conclude that dietary amino acids enabled the progression of the estrous cycle by maintaining liver ER above the threshold of transcriptional activity necessary for the synthesis of the amount of circulating IGF-1 sufficient for its signaling. In LID mice, the slower progression through the cycle could have been explained by the presence of circulating factors substituting for IGF-1 (e.g., insulin levels in LID are 4-fold higher than in WT mice, Yakar et al., 2001). If this were the case, LID mice should have been insensitive to food deprivation and continue to cycle even when subjected to CR. This hypothesis was verified by comparing the effect of 40% CR on the fertility cycle of ERE-Luc and LID/ERE-Luc mice. Figure 5 shows that in ERE-Luc mice CR caused a very rapid blockade of the cycle, but was not as efficacious in LID/ERE-Luc mice: vaginal smear analysis showed that by the second week of treatment only 10% of the ERE-Luc mice were cycling while 100% of the LID/ERE-Luc mice were still having a regular cycle (Figures 5A and 5B). Thus LID mice were clearly less susceptible to CR in spite of the fact that the effect of the diet was similar in ERE-Luc mice as indicated by the changes in body weight (−39% in ERE-Luc, −45% in LID/ERE-Luc, after 4 weeks in CR) (Figure S4B) and in IGF-1 synthesis (−44% in ERE-Luc, −48% in LID/ERE-Luc, after 4 weeks of CR) (Figure S4C).

Liver ER, an Integrator of Metabolic and Reproductive Functions

To finally demonstrate that liver ER was a sensor of the energetic metabolism and the switch for the blockade of reproduction in case of shortage of energetic supply, we generated a liver-selective ERα$^{-/-}$ mouse (named LERKO) by breeding ERα floxed mice (ERα$^{flox/flox}$) (Dupont et al., 2000) with mice expressing the cre-recombinase protein specifically in liver (Figure S5A–S5C). Consistent with the view that LERKO mice should have an altered equilibrium between reproductive cycle and energetic metabolism, LERKO mice started to cycle earlier than their controls (day 42 versus 47) (Figure S5D), in spite of the fact that their weight was lower than in ERα$^{flox/flox}$ mice all through postnatal development (Figure S5F). Indeed, in LERKO mice the growth rate was slower than in controls up to 28–30 days
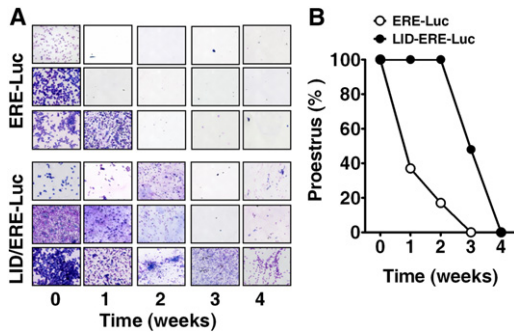
Figure 5. Calorie Restriction and Reproductive Status of LID/ERE-Luc Mice

(A) Vaginal smears of three ERE-Luc and three LID/ERE-Luc representative mice at the beginning (0) and at the end of weeks 1, 2, 3, and 4 of CR. The experiment was repeated three times on a total of nine animals/experimental group.

(B) The number of proestrus in ERE-Luc and LID/ERE-Luc was scored and expressed as percentage of proestrus/week in CR versus regularly fed mice. Quantification was repeated in a second independent experiment with overlapping results.

of age: at this time, while the growth of ERα$^{flox/flox}$ mice proceeded at the same pace up to 70 days, the growth of LERKO mice noticeably accelerated, rapidly reached the growth of control mice, and then stopped (Figure S5F). Differently from mice expressing ERα in liver, the initial cycles of LERKO mice were very regular with a periodicity superimposable to adult mice (4.3 versus 5 days, Figure S5E). The lack of a functional liver ERα impacted on IGF-1 synthesis in liver as indicated by the decreased mRNA content (−40% versus controls, Figure 6A) and by the lowered serum levels (210 ng/mL versus the average 300 ng/mL found in ERα$^{flox/flox}$ mice, Figure 6B). CR did not further decrease IGF-1 secretion in LERKO mice. In addition, in LERKO mice, AA administration failed to significantly increase circulating levels of IGF-I (Figure 6C).

We predicted that in LERKO mice the low IGF-1 synthesis was sufficient to maintain a viable cycle, but a minimal perturbation would have interrupted the cycle. Indeed, vaginal smear analysis indicated that in LERKO mice 2 days of diet were sufficient to completely block the cycle. Figure 6F shows that mice cycling properly arrested immediately in the M/D phases when subjected to CR. The controls cycled regularly when fed ad libitum (Figure 6D) and similarly to the ERE-luc mice had a gradual response to CR as indicated by the few P observed during the first week of CR. In these animals the complete shift to the M/D did not occur before at least 5–6 days of CR (Figure 6E).

In Uterus Proliferation of Lumen Epithelium Requires Circulating IGF-1

Circulating IGF-1 has been shown to have a role in the proliferation of uterus epithelium in preparation of implantation of the fertilized oocytes (Sato et al., 2002). We therefore investigated the mutual interplay of ERα and IGF-1R activity in relation to the proliferation of the uterus epithelium during the estrous cycle. First, we studied the effect of IGF-1 on Ishikawa cells, a line derived from endometrial adenocarcinoma. Flow cytometry showed that maximal cell growth occurred when cells were
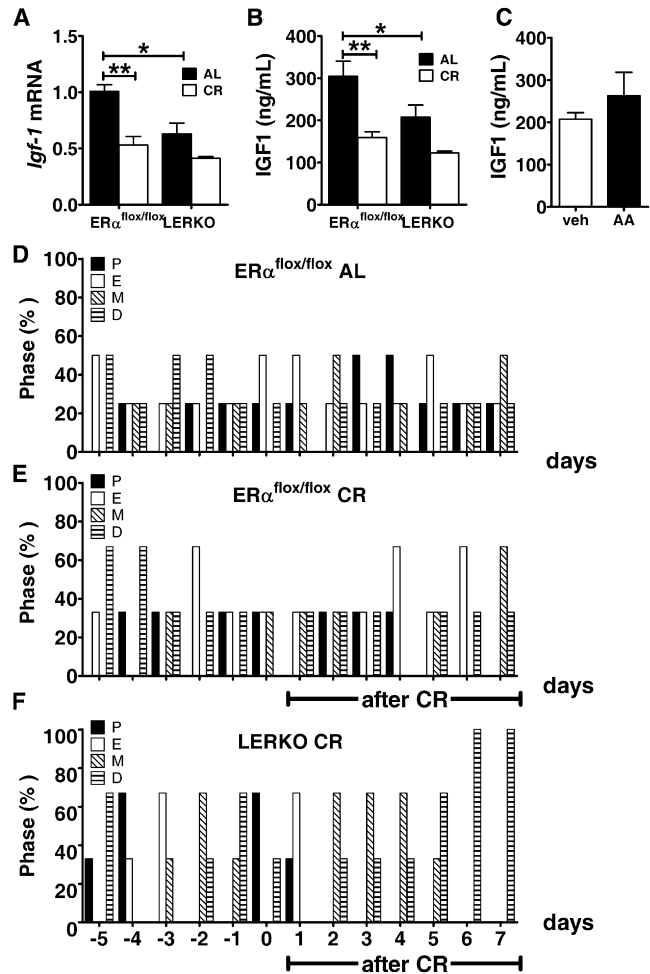

Figure 6. Rapid Induction of Reproductive Cycle Blockade in Mice with a Selective Ablation of Liver ERα

(A and B) Igf-1 mRNA (A) and total serum IGF-1 content (B) in ERα$^{flox/flox}$ and LERKO mice fed AL and in CR. Data represent the mean ± SEM (n = 6). *p < 0.05; **p < 0.01.

(C) Total serum IGF-1 content in LERKO mice 6 hr after gavage with AA. Data represent the mean ± SEM (n = 6).

(D–F) The phase of the cycle was analyzed by vaginal smears daily 5 days before and 7 days after 40% CR in ERα$^{flox/flox}$ mice fed AL (D) or in CR (E) and in CR LERKO mice (F). Data were expressed as % of animals in a given phase of the cycle. Each experimental group was composed of three mice. The experiment was repeated three times with superimposable results.

treated with E_2 24 hr prior to IGF-1; E_2 alone was not able to induce a significant increase in the number of cells in the G2/M phase of the cell cycle (Figure 7A). In a second set of experiments based on cell counting (Figure 7B), we showed that in the absence of E_2, the medium growth factors were sufficient to maintain the cells at a basal proliferation status that was not modulated by E_2, but was increased by growth factors like IGF-1 and insulin at high concentration. The requirement of a functional ER for IGF-1-dependent cell proliferation was demonstrated by the fact that ICI 182,780 blocked cell growth induced by IGF-1 alone or IGF-1 + E_2. This indicates the need for a coupling of ER and IGF-1R activities to obtain the maximal effect on uterine cell growth.
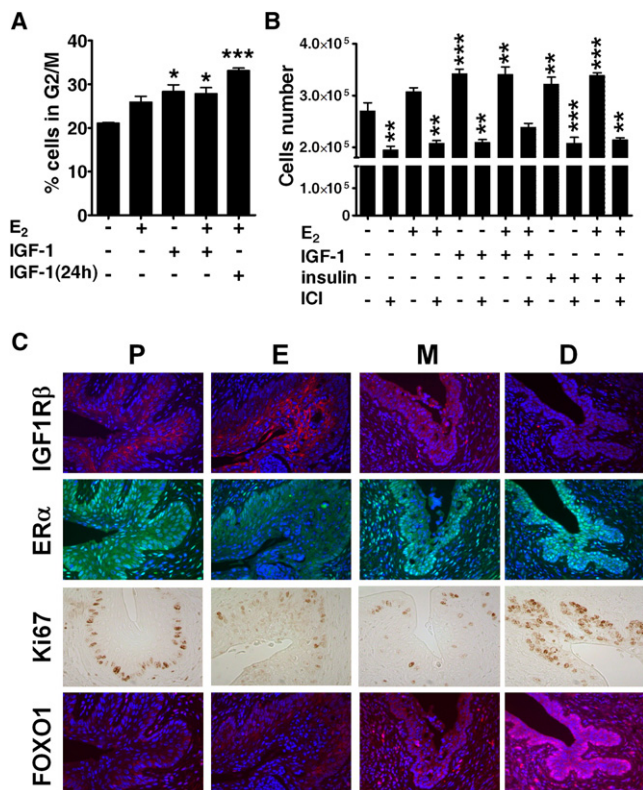
Figure 7. ERα Transcriptional Activity and IGF-1 Are Required for the Growth of Epithelial Cells in Uterus

(A) Flow cytometry analysis of Ishikawa cells grown in culture for 48 hr in the presence of the indicated compounds: 5 nM E_2, 50 nM IGF-1; and 500 nM ICI. IGF-1 (24 hr) cells were treated with E_2 for 24 hr and then IGF-1 was added in the medium and the incubation was continued for 24 hr. Data show the % of cells counted in the G2/M phase of the cycle. Data represent the mean ± SEM of five separate experiments carried out in triplicate. *p < 0.05; ***p < 0.001 (versus veh).

(B) Ishikawa cells were counted 48 hr after treatment with E_2 and/or IGF1 at the concentrations used in (A). Insulin concentration was 50 nM. Data represent the mean ± SEM of five separate experiments carried out in triplicate. *p < 0.05; **p < 0.01; ***p < 0.001 (versus veh).

(C) Representative IHC for IGF1-Rβ, ERα, Ki-67, and FOXO1 in uteri excised from ERE-Luc mice at different phases of estrous cycle.

Next, for a better insight of the dynamics of ER-IGF-1 interplay in a more physiological setting, we studied the localization and expression of IGF-1R and ERα in the mouse uterus during the different phases of the reproductive cycle (Figure 7C). Staining of IGF-1Rβ indicated that this protein is modestly present at D and accumulated through P to E. On the other hand, ERα nuclear staining was clearly distinguishable from D and was highest at P. IHC with the proliferation marker Ki-67 demonstrated that in the uterus epithelium at diestrus there is an outburst of proliferation which continues in the basal layer at P and stops at E. This is consistent with the staining of the pioneering factor FOXO1 essential for the expression of approximately 50% of ER target genes and inhibited by IGF-1 via phosphorylation; FOXO1 nuclear concentration is highest at D; by P the pioneering factor was found in the cytoplasm and remained cytoplasmic up to D, when proliferation started.

These data clearly indicated that IGF-1 and its cognate receptor are heavily involved in the progression of the reproductive cycle and their activity is strongly regulated by ER transcriptional activity.

DISCUSSION

The present study shows that in liver AA-dependent activation of ERs is necessary for a systemic production of IGF-1 sufficient for the correct progression of the estrous cycle, thus suggesting for hepatic ERα the novel role of peripheral integrator of metabolic and reproductive functions.

The involvement of the liver in the regulation of fertility is well rooted in the phylogenesis: hepatopancreas in invertebrates and liver in vertebrates control reproduction by providing the gonads with the nutrients essential for the maturation of the oocyte (e.g., vitellogenin) (Jasmani et al., 2004) and the role of E_2 and ER in the production of the major yolk proteins is well known. Consistent with the view of ERs coupling the nutritional status with fertility, in invertebrates and several oviparous species the highest expression of the ER genes is generally observed in the gonads and in the liver/hepatopancreas (Nagler et al., 2007).

In mammals, liver is the peripheral integrator of nutrient availability and energetic needs of the organism and is important for feeding, digestion, and metabolic balance (Langhans, 1996). Sex is an important determinant of liver activities as indicated by the fact that several of the enzymes involved in the catabolism of xenobiotics and transcription factors relevant for energy metabolism are differentially expressed in males and females; furthermore, the sexually dimorphic GH secretion from the anterior pituitary gland was reported to cause a sex-dependent hepatic metabolism (Norstedt and Palmiter, 1984). Finally, it is worth emphasizing that liver expresses ERα, the receptor isoform most involved in the control of female sexual differentiation and reproductive functions (Korach et al., 1996).

With regard to the mechanism of liver ERα transcriptional activation by AA, previous reports demonstrated that ER directly regulated the IGF-I promoter (Gao et al., 2008, Hewitt et al., 2010). Here, we demonstrated the requirement for a viable intracellular transport of amino acids and a functional mTOR pathway. mTOR is a sensor of cellular nutrients and amino acids shown to control ERα activity via phosphorylation of Ser167 by S6 kinase, a well-known element of the mTOR pathway (Yamnik et al., 2009). In line with these results, we show that in hepatoma cells amino acids fail to activate the transcriptional activity of ERα mutants in Ser 167. In addition, we show that tyrosine 537 is necessary for AA activation of ERα in the absence of E_2; this phosphorylation was previously shown to be necessary for p21ras-dependent activation of unliganded ER (Patrone et al., 1998). We here propose that the regular intake of dietary amino acids maintains a basal level of ER transcriptional activity necessary to poise the receptors for a proper response to the hormonal stimulus.

Our study points to IGF-1 synthesized by liver as a molecule signaling the nutritional status to the reproductive apparatus in mammals; a wealth of studies carried out in invertebrates such as *D. melanogaster* and *C. elegans* demonstrated that IGF-I/insulin-like signaling triggered by food availability is the key

regulator of reproductive behavior. In nematodes, however, the switch for the fertility status is a lipid-activated protein, DAF-12, an ortholog of the mammalian LXR receptor (Motola et al., 2006). It is conceivable that in more evolved organisms able to synthesize lipids, but not all amino acids, the lipid sensor LXR lost its ability to regulate the reproductive functions in favor of a novel transcription factor sensitive to proteins, as a better indicator of the state of malnutrition in mammals.

Gonadal hormones are well-known regulators of the somatotropic axis at the hypothalamic level; by the selective ablation of liver ERα, here we demonstrate the existence of a peripheral mechanism of control of IGF-1 synthesis by estrogen signaling so far not described. This mechanism may be a phylogenetic remnant keeping a physiological relevance in mice as underlined by the observation that in LERKO mice the pubertal spurt is uncoupled from total body mass and that, in adult females, the low synthesis of circulating IGF-1 heightens the vulnerability of the reproductive cycle to CR. Of course, with evolution, the main control of peripheral functions, including reproduction, was acquired by the brain; indeed, when exposed to quite long-term CR mice stop cycling in spite of the fact that the levels of circulating IGF-1 are quite high.

Our finding of a role of dietary amino acids in the control of reproduction in mammals is in line with previous reports showing that the deficiency of a single essential AA like methionine reproduces many of the effects of CR (Malloy et al., 2006) and that protein rather than total calorie intake regulates IGF-1 levels in humans (Fontana et al., 2008). We propose that liver ER contributes to the maintenance of the systemic levels of IGF-1 required for the waves of uterine epithelium proliferation necessary for the correct implantation of the fertilized oocyte at each estrous cycle. In LID mice the lower sensitivity to CR may be explained by the very high levels of circulating insulin known to be able to bind IGF-1 receptors and that might have a compensatory activity by stimulating IGF-1 receptors in the uterus in the absence of the cognate hormone.

The role of IGF-1 in E_2-dependent mouse uterine epithelial cell proliferation is well established (Korach et al., 1996), although the source of IGF-1 has been controversial (Zhu and Pollard, 2007). In agreement with previous reports, by using tissue grafting with uteri from IGF-1 null mice (Sato et al., 2002), we demonstrated that systemic IGF-1 is required for the E_2-induced epithelial uterine cell proliferation. The exact mechanism involved needs further study. In keeping with current belief we here show that the growth of Ishikawa cells induced by IGF-1 or insulin is blocked in the absence of a viable ER. However, we believe that the study of isolated epithelial cells is not sufficient to gain the necessary insight on the complexity of the changes occurring in the uterus during the 4 days of the cycle and the role played by the stroma component of the uterus. Indeed, IHC shows that the expression of proliferative markers ER and IGF-1Rβ in uterus change differentially in stroma and epithelium. A better understanding of the impact of E_2 and IGF-1 on the stroma and epithelium is necessary to fully elucidate the role of systemic IGF-1. On the basis of the results here presented, we postulate that circulating IGF-1 is a relevant factor for the blockage of FOXO1 activity at diestrus, allowing the completion of the proliferative phase at P and facilitating differentiation of epithelial cells of the uterus; this would explain the increase of IGF-1Rβ noticeable at P and E in the cells that are undergoing differentiation. Previous studies in our laboratory have also shown that a cross-coupling between ERα and growth factors may act as a switch to differentiation (Ciana et al., 2003b).

We believe that the present study, by showing the relevance of amino acids on ER signaling in liver for the regulation of reproductive functions has implications for the elucidation of mechanisms of impaired pubertal development, nutritional amenorrhea, and obesity-related infertility. A better understanding of these etiologies has far-reaching implications for the prevention and management of reproductive dysfunctions and associated comorbidities.

EXPERIMENTAL PROCEDURES

Animals
Animals were fed ad libitum with a certified estrogen-free, isoflavone-deprived, 4RF21 diet (Mucedola). Room temperature was maintained at 22–25°C and the light/dark cycle was 12 hr (lights on at 7:00 a.m.). Heterozygous ERE-Luc reporter mice (Ciana et al., 2003a); ERE-Luc/ERαKO mice, and LID/ERE-Luc mice, were obtained by mating ERE-Luc with ERαKO (Dupont et al., 2000) and LID mice (Yakar et al., 1999) respectively; for the LERKO mice see Supplemental Experimental Procedures. Mice were generally 2–4 months old. Vaginal smears were done at 8:00 a.m.

A 40% CR regimen was calculated from the daily food consumption measured for 14 days prior to the experiment. The hyp diet was 40% enriched in raw proteins and decreased in carbohydrates and lipids to contain the same calories as in the control diet. After overnight fasting, mice were repeatedly (0.3 ml, three times every 45 min) treated by gavage to receive in total 21% of the daily intake.

Pharmacological Treatments
JB3 (DSL) was administered 1 μg/day s.c. with an osmotic minipump (3 day-release, Alzet) first implanted at proestrus. ICI 182,780 (TOCRIS) was administered i.p. at the dose of 6.7 mg/kg/day for 4 days before in vivo imaging.

All animal experimentation was carried out in accordance with European guidelines for animal care and use of experimental animals, approved by the Italian Ministry of Education, University and Research, and controlled by the panel of experts of the Department of Pharmacological Sciences, University of Milan.

Bioluminescence-Based Imaging and Luciferase Assay
BLI and luciferase assay were carried out as previously described (Maggi and Rando, 2009).

Biochemical and Hormonal Assays
Serum total estradiol measurement was done by Dr. Parlow (NHPP, Harbor-UCLA Medical Center, Torrance, CA, USA). IGF-1 (active mouse and rat IGF-1 EIA and ELISA, DSL) was measured according to the manufacturer's protocols.

Cell Cultures and Treatments
Adult hepatocytes were isolated from liver of ERE-Luc or ERE-Luc/ERαKO mice and grown according to Valverde et al. (Valverde et al., 2003). Prior to treatment, cells were incubated in serum and phenol red-free medium for 45 min at 37°C, in the presence or absence of 10 mM BCH (30 min); then cells were exposed to E_2 or AA stimulation for 6 hr.

For human HepG2 cells, 48 hr before transfection the growth medium was replaced with white MEM with dextran-coated charcoal stripped-FBS (DCC-FBS). Transfection of the p-VERE-tk-LUC (0.8 μg) and ERα-containing plasmids (0.016 μg) was done with Lipofectamine LTX and Plus Reagent (Invitrogen-Life Technologies) for 6 hr and then medium was replaced with white MEM plus 1% of DCC-FBS. To study AA effects, we starved cells in Krebs Solution for 2 hr. HepG2 cells were treated with vehicle, 10 nM E_2, or with the AA solution at pH 7.4 in Krebs (for amino acids see Table S1). Luciferase content was measured after 5 hr of incubation. Rapamycin and

LY294002 (2-[4-Morpholinyl]-8-phenyl-1[4H]-benzopyran-4-one hydrochloride) (both Sigma-Aldrich) and H-89 (N-[2-(p-bromocinnamylamino)ethyl]-5-iso-quinolinesulphonamide) (Alexis Corporation) were added 30 min prior to treatments. After 5 hr cells were harvested for luciferase enzymatic assay.

Treatments of Ishikawa cells with 5 nM E_2 and/or 50 nM IGF-1 (Long R^3 IGF-I, Sigma) and/or 50 nM insulin (from porcine pancreas, Sigma), with or without 500 nM ICI 182,780 (TOCRIS) were done in the presence of 5% FBS-DCC. The medium and treatments were renewed every 24 hr. Cells were counted by CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

The DNA distribution profiles of the Ishikawa cells were determined 48 hr after treatments by using the FACSCalibur™ (BD). After fixation with EtOH and DNA staining, cells were analyzed by flow cytometry with a 488 nm laser line for excitation. Quantitative analysis (10,000 events) was done with the CELLQuest system program (BD).

Real-Time PCR Gene Expression Analysis
Total liver RNA was extracted with RNeasy Mini kit (QIAGEN). cDNA was prepared as described (Ciana et al., 2007). rtPCR experiments were done by TaqMan technology and using the primers: *Luc* forward and reverse primers (ACA-GAT-GCA-CAT-ATC-GAG-GTG-AA and GCC-AAC-CGA-ACG-GAC-ATT-T), *Luc* TaqMan MGB probe 5′-TAC-GCG-GAA-TAC-TTC; TaqMan Gene Expression assays for *ERα* (Mm00433149_m1), *Igf-1* (Mm00439561_m1), and as a reference gene assay 18S rRNA VIC-MGB-PDAR (Applied Biosystems). The reaction was carried out according to the manufacturer's protocol by using 7900HT fast real-time PCR system (Applied Biosystems). Data were analyzed by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Immunohistochemistry
Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Five micrometer-thick sections were treated with 3% H_2O_2 in order to block endogenous peroxidises and then permeabilized (0.2% Triton X-100). The primary antibodies used were monoclonal ERα 1D5 clone, Zymed Laboratories (1:500); monoclonal rat anti-mouse Ki-67 antigen, DakoCytomation (1:25); rabbit polyclonal IGF-I receptor β antibody, Cell Signaling Technology (1:50); and rabbit monoclonal antibody FoxO1 (C29H4), Cell Signaling Technology (1:25). Secondary biotinylated antibodies were from Vector Laboratories (1:200). Immunoreactivity amplification was performed with the avidin-biotin-peroxidase (Vectastain ABC kit, Vector Laboratories) and developed with DAB (3,3′-diaminobenzidine substrate, Sigma-Aldrich) or Alexa Fluor® 555 Streptavidin (Molecular Probes) for IGF1-Rβ and FOXO1 or Alexa Fluor® 488 Streptavidin for ERα. Hoechst 33258 (Molecular Probes) was used to stain nuclei. Slices were photographed at 4000×.

Statistical Analysis
If not otherwise stated, statistical significance was assessed by one-way or two-way ANOVA with Bonferroni's multiple comparison post hoc test by using GraphPad Prism 5 (GraphPad Software).

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