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The 1,25D$_3$-MARRS Receptor/PDIA3/ERp57 and Lifespan

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ABSTRACT

Using MRI on mice bearing a targeted knockout (KO) of the 1,25D$_3$-MARRS receptor/PDIA3/ERp57 we found that they had decreased body fat relative to their littermate (LM) controls, a condition associated with increased lifespan. Others have found that lower body fat is correlated with decreased lipid droplets in intestinal cells that may be mediated by a factor secreted by germ cells (possibly estradiol). In a reducing environment estradiol competed for binding to the 1,25D$_3$-MARRS receptor/PDIA3/ERp57. A consequence of this was that estradiol stimulated calcium uptake in enterocytes isolated from LM mice. In time course studies, lipid droplets increased in response to 1 nM estradiol from 1–5 D of culture, relative to corresponding controls, while at 6 and 7 D this steroid decreased lipid droplets. Enterocytes from LM or KOs incubated with estradiol for 1–4 D showed the hormone increased lipid droplets. Using the 4 D culture period, 1 and 10 nM estradiol significantly increased the number of lipid droplets in cells from LM mice by 40–60%, compared to equivalent conditions in KO mice. In assessing signal transduction pathways, the hormone increased phospho-Akt levels, but no differences were observed in phospho-mTORC1, or phospho-S6K (although cells from chicks did exhibit a hormone-mediated difference). Finally, the remaining mice (which had stopped reproducing) were allowed to die naturally and lifespan recorded. LM mice lived 687 ± 77 D (without an outlying value) while KO mice lived 740 D ± 80 D. These data suggest the 25D$_3$-MARRS receptor/PDIA3/ERp57 may contribute to the length of lifespan in mammals. J. Cell. Biochem. 116: 380–385, 2015.

KEY WORDS: VITAMIN D; NON GENOMIC EFFECTS; ESTRADIOL; LONGEVITY; MEMBRANE RECEPTORS

Reduced body fat increases the life span of *C. elegans* and mammals [Wang et al., 2008] through largely unknown mechanisms. In this study we explored the role of signaling through the 1,25D$_3$ membrane-associated, rapid response steroid-binding (MARRS) receptor in body fat homeostasis and longevity in the mouse. The 1,25D$_3$-MARRS receptor was originally isolated as a cell surface receptor for the vitamin D metabolite 1,25-dihydroxy-vitamin D$_3$ (a seco-steroid hormone in which the B ring is broken) and found to be identical to protein disulfide isomerase A3 (PDIA3). 1,25D$_3$-MARRS receptor/PDIA3/ERp57, but not the classical vitamin D receptor (VDR), is essential in 1,25D$_3$-stimulated calcium and phosphate uptake in chicks [Nemere et al., 2004; Khanal et al., 2008], and mice [Nemere et al., 2010, 2012a,b]. The 1,25D$_3$-MARRS receptor/PDIA3/ERp57 preferentially binds 1,25D$_3$ analogs that have a *cis* configuration [Nemere et al., 1994] and resemble steroid hormones such as estradiol with an intact B ring.

Wang et al. (2008) found that decreased body fat in *C. elegans* was related to a decreased number of lipid droplets in intestinal epithelial cells, and that a factor secreted by germ cells, possibly estradiol, was responsible for regulating the number of lipid droplets. Mice lacking the 1,25D$_3$-MARRS receptor/PDIA3/ERp57 are leaner than littermates.

In this study, we tested the hypothesis that the increased longevity reported by Wang et al. (2008) is related to the function of the 1,25D$_3$-MARRS receptor/PDIA3/ERp57 in lipid droplet accumulation. Specifically we tested whether loss of the 1,25D$_3$-MARRS receptor/PDIA3/ERp57 was related to lifespan.

MATERIALS AND METHODS

ANIMALS

All protocols were approved by the Institutional Animal Care and Use Committee at Utah State University. ERp57$^{flx/flx}$ mice [Garbi et al., 2006] were bred to commercially available mice having the cre-recombinase gene driven by the villin promoter (Jackson labs, Bar Harbor, ME). Pups were weaned at 3 weeks of age and had no developmental differences.

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They were genotyped using the following primers and classical PCR: The forward primers for ERp57 were: CGC CAG CCT CTC CAT TTA G, and the reverse primers were CAG AGA TCC TCC TGC CTC TG. The product for the WT is 100 bp, for the floxed allele the product is 387 bp. The primers for Cre were: GCT GGT TAG CAC CGC AGG TGT AGA G (forward), CGC CAT CTT CCA GCA GGC GCA CC (reverse), to give a 500 bp product. Reaction products were separated out on 2% (w/v) agarose gels containing ethidium bromide. In the experiments described here, adult mice (8 wks) were used. Signal transduction pathway studies were conducted with NZB (Jackson) mice after the genetically altered mice stopped reproducting.

MRI

After logging on to the EchoMRI system the program was launched, and an output file opened. After selecting the species the proper calibration sample was inserted, and the system test run. The mouse was then inserted in the tube and the 'run scan' button pressed. After finishing the measurements, data were extracted and transferred to a thumb drive.

CELL ISOLATION AND TISSUE CULTURE

Mice were used at 8 weeks of age. They were killed by cervical dislocation and the entire small intestine removed to ice cold saline. After 15 min, the intestines were slit longitudinally, rinsed in ice-cold saline and transferred to citrate chelation medium (96 mM NaCl, 27 mM NaCitrate.$2H_2O$, 1.5 mM KCl, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, pH 5.0). The acidic pH allows retention of viability and morphology in chick intestinal cells [Nemere, 2005; Sterling and Nemere, 2005]. The intestines were stirred for 15 min at room temperature to dissociate epithelial cells, and then transferred to fresh chelation medium. The released cells were poured into 50 ml conical centrifuge tubes and held on ice. Two additional 15 min periods of cell isolation were conducted, the cells pooled and collected by centrifugation at 1000 × g, 10 min (4C). The cell pellets were resuspended in either 7 ml for calcium uptake studies or 10 ml of Gey's balanced salt solution (GBSS; containing 119 mM NaCl, 4.96 mM KCl, 0.22 mM KH_2PO_4, 0.84 mM $NaHPO_4$, 1.03 mM $MgCl_2$• $6H_2O$, 0.28 mM $MgSO_4$• $7H_2O$, 0.9 mM $CaCl_2$, pH 7.3) for tissue culture. Isolated intestinal epithelial cells from male and female mice were kept separate and not pooled, in the event sex specific differences occurred. For uptake studies, freshly isolated cells 2.2 ml aliquots were pipetted into polypropylene tubes containing 2.2 μCi $^{45}CaCl_2$. Additions were made at $t = 0$. Samples were then taken at 1, 3, 5, 7, and 10 min during the treated phase. Samples were centrifuged, the supernatant decanted, and the inside of the tubes swabbed with a Kimwipe while still in the inverted position. Cell pellets were resuspended in reagent grade water and disrupted by repeated up-and-down pipetting. Aliquots were taken for radio-isotope determination by liquid scintillation spectrophotometry and protein analysis by the Bradford assay. Data were calculated as cpm/mg protein, and the values related to average basal values.

For tissue culture intestinal cells were plated onto 35 mm plastic petri (Falcon, Fisher Scientific; Franklin Lakes, NJ) dishes by adding 1 ml of cell suspension to 2 ml of RPMI 1640 medium (Thermofisher, Logan, UT)), and antibiotics containing 100 units/ml of penicillin and 100 μg/ml of streptomycin (Sigma Chemical Co; St. Louis, MO). The cells were incubated overnight at 37°C, 5% CO_2/95% air without serum to promote cell adherence. At this time they were also either treated with 1 nM estradiol (Sigma) or the vehicle ethanol. The next day fetal bovine serum was added to 10% v/v (Thermofisher), and each dish again treated with hormone or vehicle. On each of the subsequent days of incubation the cell cultures were again treated with hormone or vehicle.

LIPID STAINING

On the day of analysis, media were aspirated and the cells fixed with 10% v/v formalin in PBS. For time course studies, the plates were held in formalin until the last plates were fixed, and then all cells for each time point were stained on the same day. After removing the formalin, plates were washed with 60% v/v isopropanol, then dried completely. For staining, A stock solution of oil red O (0.5 g in 100 ml isopropanol) was prepared. Cells were stained with Oil Red O staining working solution (0.5% Oil Red O in isopropanol stock solution, diluted 3:2 in water, and left 1 h at room temperature before filtering with a 0.45-μm filter). After staining for 10 min, cells were washed four times with water, the water removed and the plates dried. The lipids were eluted with 1 ml of 100% isopropanol (10 min incubation) and then by pipetting up and down, and read at 500 nm against 100% isopropanol blank.

WESTERN ANALYSES

Western blotting was performed as previously described [Nemere and Ray, 2000] using anti phhosphorylated S6 kinase (abcam, Cambridge, MA), anti phosphorlylated mTORC (abcam), or anti-phosphorylated Akt (Fisher Scientifc).

PROTEIN DETERMINATION

Aliquots (20 μl) of the elution solution were analyzed for protein using the Bradford reagent (BioRad, Hercules, CA) against bovine gamma globulin as standard (Sigma–Aldrich, St Louis, MO).

MORTALITY

Ten mice were allowed to die naturally, and dates of birth and death recorded.

STATISTICAL ANALYSES

Statistical comparisons were made using Student's *t*-test. Significant differences were judged to be at $P < 0.05$.

RESULTS

After noting that KO mice tended to be smaller than LM, we performed MRI studies on mice to determine if any differences existed between KO and LM mice. While there were no significant differences between LM and KO mice of either sex for water or lean body mass, female KO mice had significantly less fat mass than LM females (Fig. 1).

Primm and Gilbert (2001) had previously reported that PDI (of which the $1,25D_3$-MARRS receptor/PDIA3 is a subtype) binds estradiol and thyroid hormone. We therefore undertook experiments to determine if estradiol could compete with [^{3}H]$1,25D_3$ for binding to the $1,25D_3$-MARRS receptor/PDIA3/ERp57 using male mice. Since this protein has two thioredoxin domains we further tested

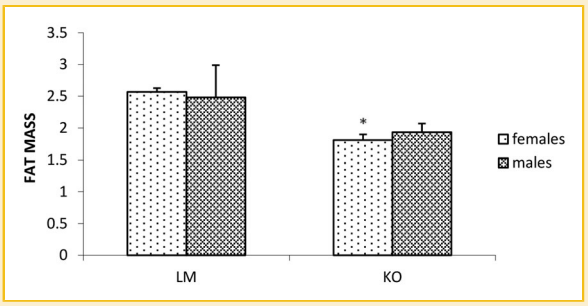

Fig. 1. MRI results indicate a difference in fat body mass between LM and KO female mice. Results are presented as mean ± SEM for three female KO and three female LM mice, and for four male KO mice and three LM mice. *$P < 0.05$ relative to LM females.

binding in the absence or presence of β-mercaptoethanol. Figure 2 illustrates the results of these experiments. In the presence of a reducing environment, estradiol effectively competes for binding to the 1,25D_3-MARRS receptor/PDIA3/ERp57 whereas in the absence of a reducing environment, estradiol increases the binding of [^{3}H] 1,25D_3 to the receptor/PDIA3/ERp57. Assuming that the cell surface is in a non-reducing environment, this may explain why we observed a much larger stimulation of protein kinase A activity in intestinal cells from female littermate mice treated with 1,25D_3 compared to cells from male littermate mice [Nemere et al., 2010]. It further suggests that estradiol may use the 1,25D_3-MARRS receptor/PDIA3/ERp57 to increase intestinal cell calcium uptake. Using intestinal cells from male LM mice, this was indeed found to be the case (Fig. 3A). Estradiol had no effect on calcium uptake in intestinal

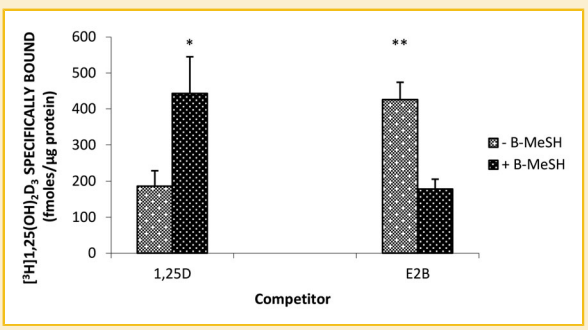

Fig. 2. Effect of estradiol (E2B) on the specific binding of [^{3}H]1,25D_3 in the absence or presence of β-mercaptoethanol (β-MeSH). Intestinal cell lysates from male LM mice were incubated with radiolabeled ligand in the absence of unlabeled ligand (total binding) or a 200-fold excess of unlabeled 1,25D_3 (nonspecific binding) or estradiol for 5 h on ice. Bound and free hormone were separated by the perchloric acid precipitation method which does not detect the classical VDR [Larsson and Nemere, 2003]. Data are presented as the mean ± SEM for 4 independent experiments in the absence of β-MESH; in 2 of these experiments, cell lysates were also incubated with β-MESH. *$P < 0.05$ relative to estradiol in the presence of β-MESH; **$P < 0.05$ relative to 1,25D_3 as competitor in the absence of β-MESH.

cells isolated from male KO mice (Fig. 3B). We have previously reported that calcium absorption in vivo was lower in KO than LM mice [Nemere et al., 2012b].

We then cultured intestinal epithelial cells from male LM mice and tested whether estradiol increased lipid droplets as judged by Red Oil O staining for lipid droplets over a period of 1–7 days. As shown in Figure 4A the time course indicated a period of 1–5 D during which estradiol increased Red Oil O staining in intestinal cells from LM mice, relative to vehicle controls. After 5 D staining for lipid droplets decreased. We ran a similar timecourse to determine whether the culture conditions affected the expression of the 1,25D_3-MARRS receptor/PDIA3/ERp57. Figure 4B illustrates the results of Western analysis and Figure 4C graphically depicts densitometric analysis of two Westerns. Estradiol treament did not lead to an increase in the 1,25D_3-MARRS receptor/PDIA3/ERp57 in days 1–5 in duplicate experiments, and there was no significant decrease in expression on subsequent days of culture. The apparent decline in 1,25D3-MARRS/PDIA3/ERp57 levels in cells treated for 6–7 D was due to a break in these two bands in one of the Western analyses.

Using a 4 D time point, we then cultured intestinal cells isolated from a male KO and LM mouse to test whether the absence of the 1,25D_3-MARRS receptor/PDIA3/ERp57 would prohibit the estradiol-mediated increase in Red Oil O staining. As shown in Figure 5, KO mice did not respond to estradiol with an increase in Red Oil O staining. While estradiol effectively doubled the amount of staining in cell isolated from LM mice, this steroid hormone did not increase lipids in KO mice, and actually caused a small decrease (Fig. 5)

We then performed a dose-response analysis on intestinal cells from male LM mice to determine whether the increase in Red Oil O staining occurred at a physiological concentration of estradiol. Figure 6 illustrates the results of this experiment, and indicates that a maximum is reached at the physiological concentration of 1 nM estradiol.

Signaling pathways for life span regulators have been reported to work through mTOR and S6 kinase [Selman et al., 2009]. Westerns were run on mouse intestinal cells treated either with vehicle, 300 nM 1,25D_3, or 1 nM estradiol for 1–7 min. Figures 7A and 7B indicate that no consistent changes were observed in S6K phosphorylation. As shown in Figures 7C and D the only change seen in p-MTOR was a slight decrease in intensity in some of the bands, but this was not reproducible. either. We did however see an increase in phospho-Akt as shown in Figures 8A and B in cells from male mice. Changes in phospho-Akt were evident in samples from either male or female mice (data not shown). Although there were changes in control samples taken for determination of p-Akt, they were not consistent and most likely are a consequence of sampling. Estradiol treatment consistently increased p-Akt, while 1,25D_3 increased it to a smaller extent (Figs. 8A and B). A comparison of the blot and densitometric analysis in Figure 8 also indicates that the software was not infallible, having not detected a faint band in C7, but detecting one in D10.

Finally as shown in Figure 9, mice bearing the targeted knockout of the 1,25D_3-MARRS receptor, lived on average 30% longer than their littermates.

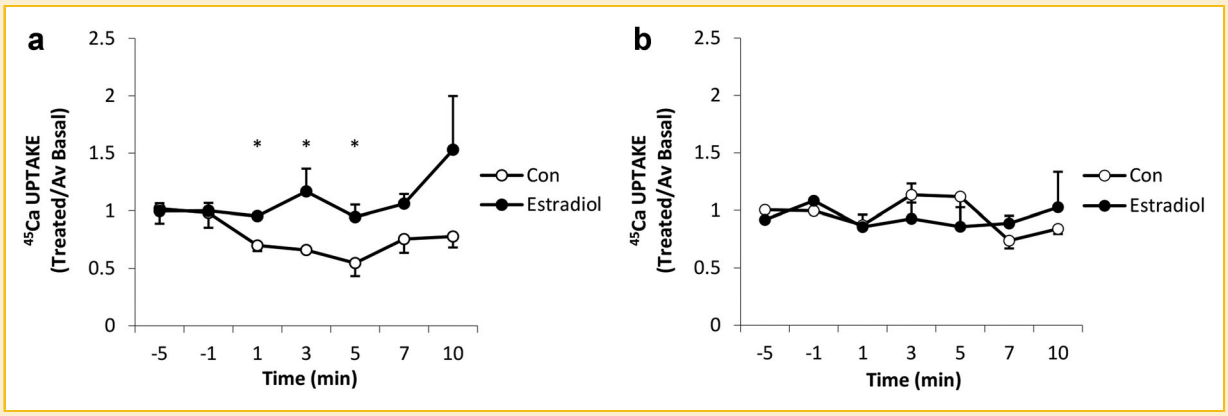

Fig. 3. Estradiol stimulates calcium uptake in intestinal cells isolated from LM male mice. Data are presented as the average of two independent experiments ± range. *$P < 0.05$ relative to corresponding controls. a, intestinal cells from LM mice; b, intestinal cells from KO mice.

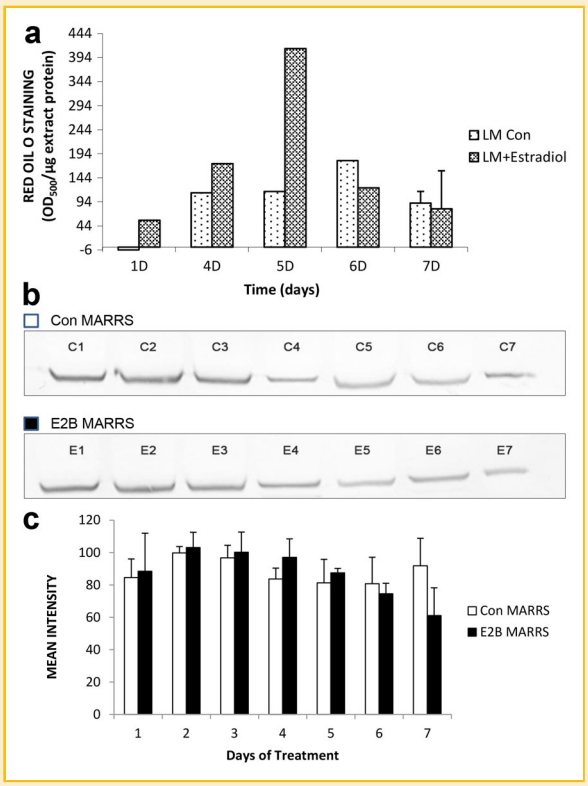

Fig. 4. Estradiol increases Red Oil O staining in intestinal epithelial cells isolated from one male LM mouse. Intestinal cells isolated by citrate chelation were cultured for 1–7 days. Estradiol (1 nM final concentration) or the vehicle ethanol (0.3% final concentration) were added on a daily basis. On the indicated day, media were removed, 10% formalin added, and the Petri dishes sealed with parafilm and kept at 23 °C until the time course was complete. At that time, the cells were stained with Red Oil O, extracted and the extract measured for stain spectrophotometrically. OD at 500 nm was then related to protein in the same cell extract. For the 7 day time point, cells were plated in triplicate cultures. (a) lipid droplet analysis; (b) Western analysis of the 1,25D3-MARRS-receptor/PDIA3/ERp57 during a similar timecourse in New Zealand Black mice; (c) densitometric analyses of two Western blots.

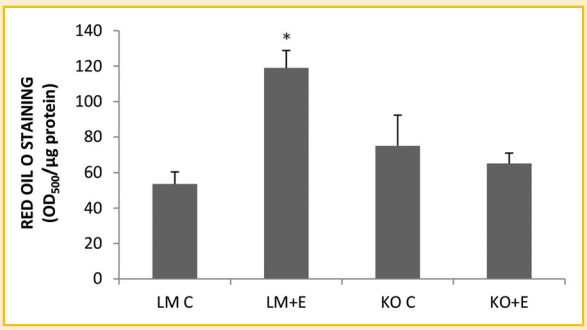

Fig. 5. Estradiol increases Red Oil O staining in intestinal cells from LM but not from KO male mice. Data are as mean ± SEM for three independent experiments for littermate controls or estradiol treated (LM C, LM + E, respectively) and knockout controls or estradiol treated, respectively (KO C, LK + E, respectively). *$P < 0.05$, relative to corresponding littermate controls.

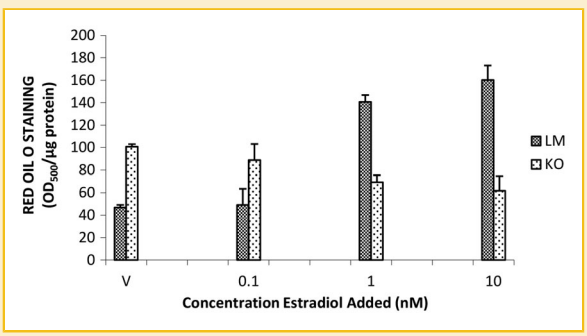

Fig. 6. Dose-response analysis for estradiol-induced increase in Red Oil O staining. Intestinal cells were isolated from an LM and KO mouse (both male) and cultured in triplicate with vehicle or increasing concentrations of estradiol. Data are presented as mean ± SEM.

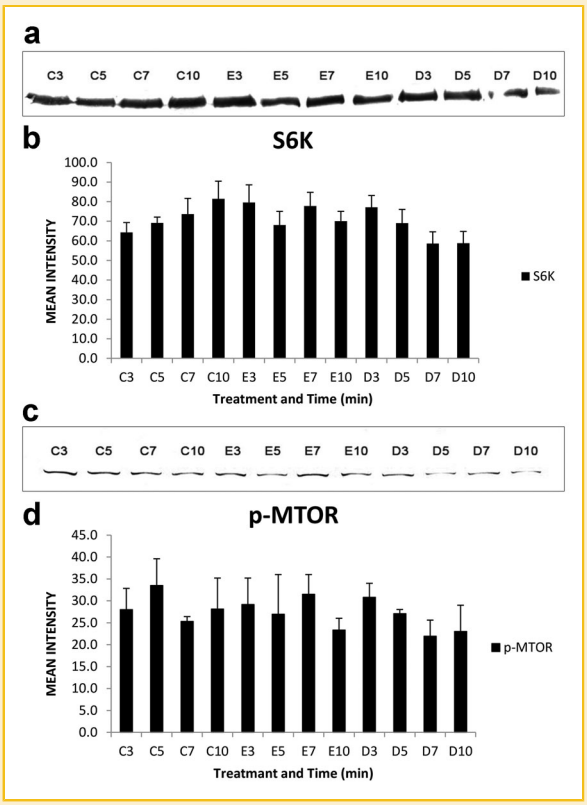

Fig. 7. Neither estradiol nor 1,25D3 increase levels of p-S6K or p-MTOR, relative to controls as judged by Western analyses. (a) Western of pS6K in controls (C), estradiol-treated cells (E) or $1,25D_3$-treated cells (D). Each of the incubations was sampled at 3, 5, 7, and 10 min after addition of vehicle or hormone. (b) densitometric analyses of two representative Westerns. (c) Western of p-mTOR in controls (C), estradiol-treated cells (E) or $1,25D_3$-treated cells (D). Each of the incubations was sampled at 3, 5, 7, and 10 min after addition of vehicle or hormone. (d) densitometric analyses of two representative Westerns. Values are average ± range.

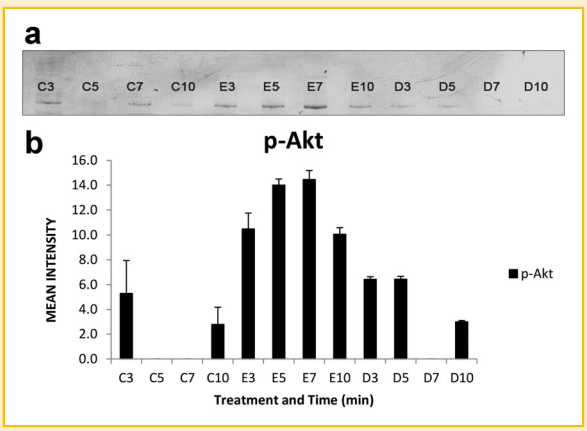

Fig. 8. Western analyses indicate hormones increase phospho-Akt in intestinal cells from littermate mice. (a) Western blot; (b) densitometric analyses of two blots. Values are average ± range.

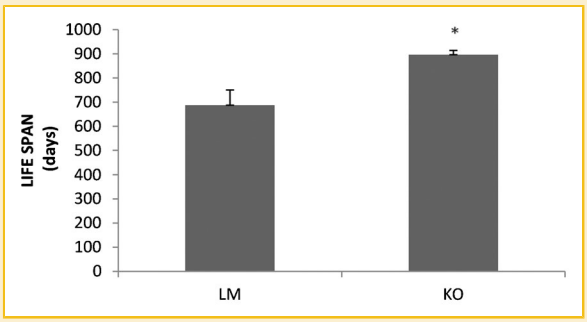

Fig. 9. KO mice live longer than LM mice. Data are from four LM female mice and six KO male mice. Data represent mean ± SEM. *$P < 0.05$.

DISCUSSION

There is a well known connection between leaness and longevity, and it was noted that KO mice tended to be smaller than LM mice. This led us to conduct MRI studies on a small number of mice. After finding that there was a decreased fat mass in mice bearing the targeted knockout of the $1,25D_3$-MARRS receptor/PDIA3/ERp57, and that this might be mediated by estradiol, we found that this steroid did compete with $1,25D_3$ for binding to the $1,25D_3$-MARRS receptor/PDIA3/ERp57. We had previously reported [Nemere et al., 2010] that while the $1,25D_3$-MARRS receptor from male and female LM mice had a similar affinity for $1,25D_3$, the number of binding sites for female mice was substantially lower. Within the context of the current studies, the earlier findings [Nemere et al., 2010] were due to estradiol binding to the $1,25D_3$-MARRS receptor/PDIA3/ERp57. A functional consequence of this was established in the current report by having observed estradiol-enhanced uptake of calcium in isolated intestinal cells, relative to controls in male LM mice. The ability of estradiol to bind to and activate the $1,25D_3$-MARRS receptor/PDIA3/ERp57 may explain why the number of binding sites in female mice is one third of those found in males [Nemere et al., 2010], and may also be responsible for the greater phosphate absorption found in females relative to males [Nemere et al., 2012a,b].

We performed a timecourse to study the effect of estradiol on lipid droplets as measured by red oil O staining. Estradiol increased lipid droplets in littermates between 1–5D, this was followed by decreased responsiveness at later time points, that was not due to a decrease in the $1,25D_3$-MARRS receptor/PDIA3/ERp57, as demonstrated by Western analyses of samples from similar timecourses. Using the 4D time point, we compared estradiol action in LMs and KOs and found there was no effect on lipid droplet content in the knockouts A dose response analysis indicated a maximal effect at a physiological concentration of estradiol. Western analyses indicated an increase in phospho-Akt in response to hormone, while downstream pathways were not identified. Finally, with a small number of animals allowed to die naturally, we found that KO mice lived 30% longer than littermate mice. An anomaly in this finding is that while the KO females showed decreased fat mass relative to litter mates, the KO males outlived the littermate females. Due to the small sample size it is not possible to draw conclusions from this lack of

congruity. Unfortunately these mice stopped reproducing so further studies in this model system are not possible.

Our previous works have demonstrated the positive aspects of the $1,25D_3$-MARRS receptor/PDIA3/ERp57 on calcium and phosphate uptake from the intestine. Combined with the current results, indicating negative aspects of the same protein, highlights the dual nature of this entity. It also implies profound genetic effects that have yet to be elucidated. The combined data indicate that the $1,25D_3$-MARRS receptor/PDIA3/ERp57 may be a potential therapeutic target [Farach-Carson and Nemere, 2003].

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