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Effect of growth hormone receptor gene disruption and PMA treatment on the expression of genes involved in primordial follicle activation in mice ovaries

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Abstract The activation of the Pi3k-Akt1-FOXO pathway seems to be involved in the extended longevity observed in growth hormone receptor/growth hormone binding protein knockout (GHRKO) mice and is related to the growth of primordial ovarian follicles. The aim of this work was to measure the expression of genes in the ovaries of GHRKO and normal (N) mice treated with phorbol 12-myristate 13-acetate (PMA), an inhibitor of GH and IRS1 signaling. For this study, a group of N (n=10) and GHRKO (n=10) mice, N mice treated (n=10) or not (n=10) with PMA, and GHRKO mice treated (n=10) or not (n=10) with PMA were used. All were 6-month-old female mice. After the last PMA injection, the ovaries were collected for gene expression analysis. Expression of *Amh*, *Gdf9*, and *Bmp15* was higher in GHRKO than N mice (P<0.05), but was not different between PMA-treated N mice (P>0.10). Expression of *Amh* and *Gdf9* was higher (P<0.05) for GHRKO PMA-treated mice. In addition, we observed a higher expression of *Socs3* (P<0.001) in GHRKO than N mice and a tendency for increased expression of *Foxo3a* (P=0.07). For GHRKO PMA-treated mice, *Foxo3a* mRNA expression was higher (P=0.02) and a tendency for higher expression of *Mtor* (P=0.06) and *Socs3* (P=0.10) in GHRKO PMA-treated mice was observed. To summarize, the present data further confirm the previous histological observations that GHRKO mice have an ovarian phenotype characteristic of younger mice indicated by higher expression of *Amh*, *Gdf9*, and *Bmp15* mRNA. In addition, we have shown a higher expression of *Socs3* in GHRKO mice and higher *Foxo3a* expression in PMA-treated GHRKO mice, suggesting a role for these mediators in the process of ovarian aging.

Keywords GHRKO · FOXO3a · AMH · Aging · Ovary

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Introduction

Mice with a disruption of the growth hormone receptor (GHR)/GH binding protein gene (GHRKO) are characterized by a reduction of 90 % in hepatic production of insulin-like growth factor (IGF-I) and a reduced adult body size (Zhou et al. 1997). This phenotype is accompanied by an increased lifespan of GHRKO mice when compared to littermate controls (List et al. 2011). GHRKO mice are also hypoinsulinemic and have increased insulin sensitivity (Bonkowski et al. 2009). Similarly to GHRKO mice, Ames dwarf (df/df) mice have a mutation that decreases pituitary GH secretion and consequently reduces serum concentrations of IGF-I (Chandrashekar and Bartke 1993). These mice are also characterized by a smaller body size and increased lifespan (Brown-Borg et al. 1996). On the other hand, mice that express a GH transgene are characterized by significant reduction of their lifespan when compared to their normal (N) controls (Bartke et al. 2002). These lines of evidence suggest that the GH/IGF-I axis plays a major role in the process of aging (Barbieri et al. 2003). Therefore, the study of the intracellular mediators of tissue-specific responses is important to determine how the process of aging is coordinated in the body, including the female reproductive tissues.

A functional GH/IGF-I axis is important for maximum reproductive efficiency (Chandrashekar et al. 2004). GHRKO mice are fertile, although they have reduced ovulation rate and smaller litter size than N females (Danilovich et al. 1999), and IGFKO mice are infertile (Baker et al. 1996), while mice with liver-specific IGF-I gene disruption have N fertility (Liu et al. 2000). This evidence suggests that GH is important for maximal reproductive efficiency; however, systemic IGF-I is essential for N follicle development, and females are not able to reproduce in its absence. Additionally, GHRKO females have an increased number of primordial follicles and a reduced number of healthy antral follicles (Zaczek et al. 2002; Slot et al. 2006), which can explain the reduced ovulation rate. However, treating GHRKO females with IGF-I decreases the number of primordial follicles and increases the number of healthy antral follicles (Slot et al. 2006), suggesting that IGF-I-induced signaling promotes primordial follicle activation and antral follicle survival. In addition, previous evidence indicates that GHRKO female mice have a prolonged reproductive lifespan, as indicated by the presence of ovarian activity at a later age (older than 20 months) when N mice have already depleted the ovarian follicular reserves (Sluczanowska-Glabowska et al. 2012).

The activation of the phosphoinositide 3-kinase (Pi3k)/protein kinase B (Akt1) pathway and the downstream transcription factor Forkhead Box O3a (FOXO3a) is essential for growth initiation of the ovarian primordial follicle reserve (Castrillon et al. 2003; Reddy et al. 2008; Brown et al. 2010). Hyperphosphorylation of FOXO3a results in its nuclear export, culminating with the global activation of primordial follicles and premature ovarian failure (Kalich-Philosoph et al. 2013). In addition, the mammalian target of rapamycin (mTOR) activation accelerates primordial follicle activation in a synergistic way with the Pi3k/Akt1 pathway (Adhikari et al. 2010; Caron et al. 2010). The activation of the Pi3k-Akt1-FOXO pathway is promoted by both insulin and IGF-I and reduced signaling via this pathway seems to be involved in the extended longevity observed in GHRKO mice (Bartke 2008). Therefore, it is possible that the extended reproductive lifespan and slower primordial follicle activation observed in GHRKO mice can be also related to altered activation of this pathway.

Phorbol 12-myristate 13-acetate (PMA) is a compound that can induce the proteolysis of cell surface GHR, therefore reducing GH signaling (Alele et al. 1998). As a consequence, its administration can reduce IGF-I mRNA production in some tissues (Li et al. 1999). In addition, PMA also functions as a protein kinase C (PKC) activator, decreasing insulin-induced tyrosine phosphorylation of IRS-1 and its ability to bind and activate Pi3k (Zheng et al. 2000). Since IRS-1 is an essential cellular mediator of IGF-I signaling, treatment of cells with PMA can significantly reduce Pi3k/Akt1 activation induced by IGF-I (Zheng et al. 2000). Therefore, we tested the short-term effects of reduced IGF-I signaling on the genes involved in the activation of primordial follicles and compared to the long-term effects of reduced IGF-I signaling in GHRKO mice.

Based on this evidence, the aim of this work was to measure the expression of genes related to the ovarian pathway for activation of primordial follicles in GHRKO and N mice and in N and GHRKO mice treated acutely with PMA.

Methods

Normal and GHRKO mice (all 6-month-old females) were bred and maintained under temperature- and light-controlled conditions (22±2 °C, 12 h light/12 h dark cycle) (Masternak et al. 2004). Normal mice (n=10, N PMA) and GHRKO mice (n=10, GHRKO PMA) were treated with three intraperitoneal injections every 48 h of PMA dissolved in DMSO (0.1 mg/kg; Sigma-Aldrich Corp., St. Louis, MO, USA) (Erroi et al. 1993). The control group consisted of N mice (n=10, N DMSO) and GHRKO mice (n=10, GHRKO DMSO) treated with DMSO. Another group of 20 mice consisted of non-treated GHRKO mice (n=10, GHRKO NT) and non-treated N mice (n=10, N NT). Twenty-four hours after the last PMA injection, all animals were anesthetized and euthanized after overnight fasting, and the ovaries were collected and stored at −80 °C.

The ovaries were removed from the −80 °C and homogenized with 400 μL of Qiazol (Qiagen, Valencia, CA, USA) using 0.5-mm zirconium oxide beads in the Bullet Blender 24 (Next Advance, Averill Park, NY, USA) for 4 min at speed 10. After that, 300 μL of Qiazol was added to the samples and incubated for 5 min at room temperature. Next, 170 μL of chloroform was added, and the samples were homogenized and incubated for 3 min at room temperature. Samples were then centrifuged at 4 °C at 12,000×g for 15 min and the clear upper phase was transferred to a new tube with 525 μL of cold ethanol. The solution was transferred to columns (miRNeasy Mini Kit, Qiagen), and RNA was isolated according to kit instructions. On-column DNase treatment (RNase-free DNase Set, Qiagen) following manufacturer's instructions was performed. The quantity of RNA was determined using a spectrophotometer (Epoch Microplate Spectrophotometer, Biotek, Winooski, VT, USA) and diluted to 200 ng/μL. Reverse transcription reactions were performed with 1 μg of RNA (5 μL) using iScript Synthesis Kit (Biorad, Hercules, CA, USA) in a 20-μL volume incubated for 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C (MJ Mini Personal Thermal Cycler, Biorad). The final cDNA solution was diluted to 10 ng/μL before use.

Real-time PCR using SYBR Green dye was used to evaluate gene expression. β_2 microglobulin expression was used as an internal control. The primer sequences are listed in Table 1. The PCR reactions were performed in duplicate in a 20-μL volume using 5 μL of Fast SYBR Green Mastermix (Applied Biosystems, Foster City, CA, USA), 0.4 μL of each primer (10 μM stock), and 2 μL of cDNA. Fluorescence was quantified with the ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems). For each assay, 45 PCR cycles were run (95 °C for 3 s and 60 °C for 30 s), and a dissociation curve was included at the end of the reaction to verify the amplification of a single PCR product. Analyses of amplification plots were performed with the 7500 Software (Applied Biosystems). Each assay plate included a negative control. The coefficient of variation was below 5 % for all the primer pairs used. Relative expression was calculated from the equation $2^{A-B}/2^{C-D}$ (where A is the cycle threshold [Ct] number for the gene of interest in the first control sample, B is the Ct number for the gene of interest in the analyzed sample, C is the Ct number for

Table 1 Primer pairs used in the experiment

Gene	Primer sequence
β2M	*For* AAGTAT ACT CAC GCC ACC CA *Rev* CAG GCGTAT GTATCA GTC TC
Ghr	*For* AGGTCTCAGGTATGGATCTTTGTCA GCCAAGAGTAGCTGGTGTAGCCT
Igf1	*For* CTGAGCTGGTGGATGCTCTT *Rev* CACTCATCCACAATGCCT
Socs2	*For* CTG CGC GAG CTC AGT CAA A *Rev* ATC CGC AGGTTA GTC GGT CC
Socs3	*For* TGT CGG AAG ACT GTC AAC GG *Rev* GAA GAA GCC AAT CTG CCC CT
Akt1	*For* CCG GTT CTT TGC CAA CAT CG *Rev* ACA CACTCC ATG CTG TCA TCT T
Pi3k	*For* TAGCTGCATTGGAGCTCCTT *Rev* TACGAACTGTGGGAGCAGAT
Mtor	*For* CGG CAA CTT GAC CAT CCT CT *Rev* TGCTGG AAG GCGTCA ATC TT
Irs1	*For* CGG GGA AGA CGA GAT GCT TT *Rev* TAC TGG AGC CTT GCG GCA C
Foxo3a	*For* TCCCAGATCTACGAGTGGATGG *Rev* CCTTCATTCTGAACGCGCAT
Bmp15	*For* GAGCGAAAATGGTGAGGCTG *Rev* GGCGAAGAACACTCCGTCC
Gdf9	*For* GCTCTATAAGACGTATGCTACC *Rev* CAGAGTGTATAGCAAGACCGAT
Amh	*For* TCCTACATCTGGCTGAAGTGATATG *Rev* CAGGTGGAGGCTCTTGGAACT

β2M β-2 microglobulin, *Ghr* growth hormone receptor, *Igf1* insulin-like growth factor I, *Socs2* suppressor of cytokine signaling 2, *Socs3* suppressor of cytokine signaling 3, *Akt1* protein kinase B, *Pi3k* phosphoinositide 3-kinase, *Mtor* mammalian target of rapamycin, *Irs1* insulin receptor substrate 1, *Foxo3a* Forkhead Box O3a, *Bmp15* bone morphogenetic factor 15, *Gdf9* growth and differentiation factor 9, *Amh* anti-Müllerian hormone

β2 microglobulin in the first control sample, and *D* is the Ct number for β2 microglobulin in the analyzed sample). The first control sample was expressed as 1.00 by this equation, and all other samples were calculated in relation to this value. Afterward, the results in the control groups (N mice non-treated, N mice treated with DMSO, and GHRKO mice treated with DMSO) were averaged, and all other outputs were divided by the mean value of the relative expression in the control group to yield the fold change of the genes of interest expression compared to the control group (Masternak et al. 2005).

The results are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). A *t* test was performed for comparison between groups. A *P* value lower than 0.05 was considered statistically significant and as a tendency between 0.05 and 0.10.

Results

Expression of *Amh*, *Gdf9*, and *Bmp15* was higher in GHRKO than N non-treated mice ($P<0.05$; Fig. 1a). Despite that, expression of *Amh*, *Gdf9*, and *Bmp15* was not different between N mice treated with PMA or DMSO for a week ($P>0.10$; Fig. 1b). Expression of *Amh* and *Gdf9* was higher ($P<0.05$) for GHRKO PMA-treated than control GHRKO mice, although *Bmp15* only tended to be higher in GHRKO PMA-treated mice ($P=0.07$; Fig. 1c).

Regarding the expression of the genes in the pathway of primordial follicle activation (summarized in Fig. 2), we observed a higher expression of *Socs3* ($P<0.001$) in GHRKO than N non-treated mice and a tendency for increased expression of *Foxo3a* ($P=0.07$) in GHRKO than in N non-treated mice. No difference in the expression of *Pi3k*, *Socs2*, *Mtor*, and *Igf1* between N and GHRKO non-treated mice was observed ($P>0.10$).

No differences in the expression of *Igf1*, *Irs1*, *Pi3k*, *Akt1*, *Foxo3a*, *Mtor*, *Socs2*, and *Socs3* were observed between N mice treated with PMA and DMSO for a week ($P>0.10$; Fig. 3). *Foxo3a* mRNA expression was higher ($P=0.02$) and a tendency for higher expression of *Mtor* ($P=0.06$) and *Socs3* ($P=0.10$) in PMA-treated vs DMSO-treated GHRKO mice was observed (Fig. 4). No difference was observed in the expression of *Igf1*, *Irs1*, *Pi3k*, *Akt1*, and *Socs2* when comparing PMA with DMSO-treated GHRKO mice ($P>0.10$; Fig. 4).

Discussion

In the present study, we observed that expression of *Amh*, *Gdf9*, and *Bmp15* was higher in GHRKO than in N mice of the same age. AMH is exclusively produced by developing preantral and small antral follicles (Jeppesen et al. 2013), and it is used to estimate the size of the ovarian reserve (Visser et al. 2006). GDF9 and BMP15 are also produced exclusively by growing oocytes in the mouse ovary (McGrath et al. 1995; Dube et al. 1998) and are involved in follicle maturation and oocyte quality (Dong et al. 1996; Yan et al. 2001). Therefore, the increased expression of *Amh*, *Gdf9*, and *Bmp15* in GHRKO mice suggests that these mice have a larger ovarian reserve than N mice of the same age. This observation confirms previous histological findings that 2-year-old GHRKO mice still have visible ovarian structures (antral follicles and corpora lutea) while N mice of the same age have already completely depleted their ovarian reserve (Sluczanowska-Glabowska et al. 2012). Despite that, the expression of *Amh*, *Gdf9*, and *Bmp15* was not altered by short-term PMA treatment in N mice, indicating that changes observed in the ovarian reserve of GHRKO female mice can be due to chronic suppression of IGF-I signaling. On the other hand, GHRKO mice treated with PMA expressed higher levels of *Amh* and *Gdf9*, indicating a possible interaction between the gene disruption and PMA treatment, which would further preserve the follicular reserve. Although the difference between PMA-treated and control GHRKO mice was more subtle (two times higher) than that observed between GHRKO and N mice (almost four times higher), this effect was not previously observed and can indicate that PMA treatment in GHRKO mice could further disrupt the progression of follicles to the primary stage, increasing the size of the ovarian reserve and possibly further reducing the ovulation rate. It is also possible that the divergent impact of PMA treatment on ovarian gene expression in GHRKO as compared to N mice was related to different regulation of IGF-1 signaling in the ovaries of these animals. In GHRKO mice, both direct and indirect actions of GH on the ovary have been eliminated by deletion of the GH receptors with concomitant reduction of IGF-1 from the systemic circulation. However, it was shown that despite elimination of GH signal and reduction of serum IGF-1, local IGF-1 expression persists in ovaries from GHRKO mice (Zaczek et al. 2002). Since PMA disrupts IGF-1 signaling, the presence of IGF-1 mRNA in

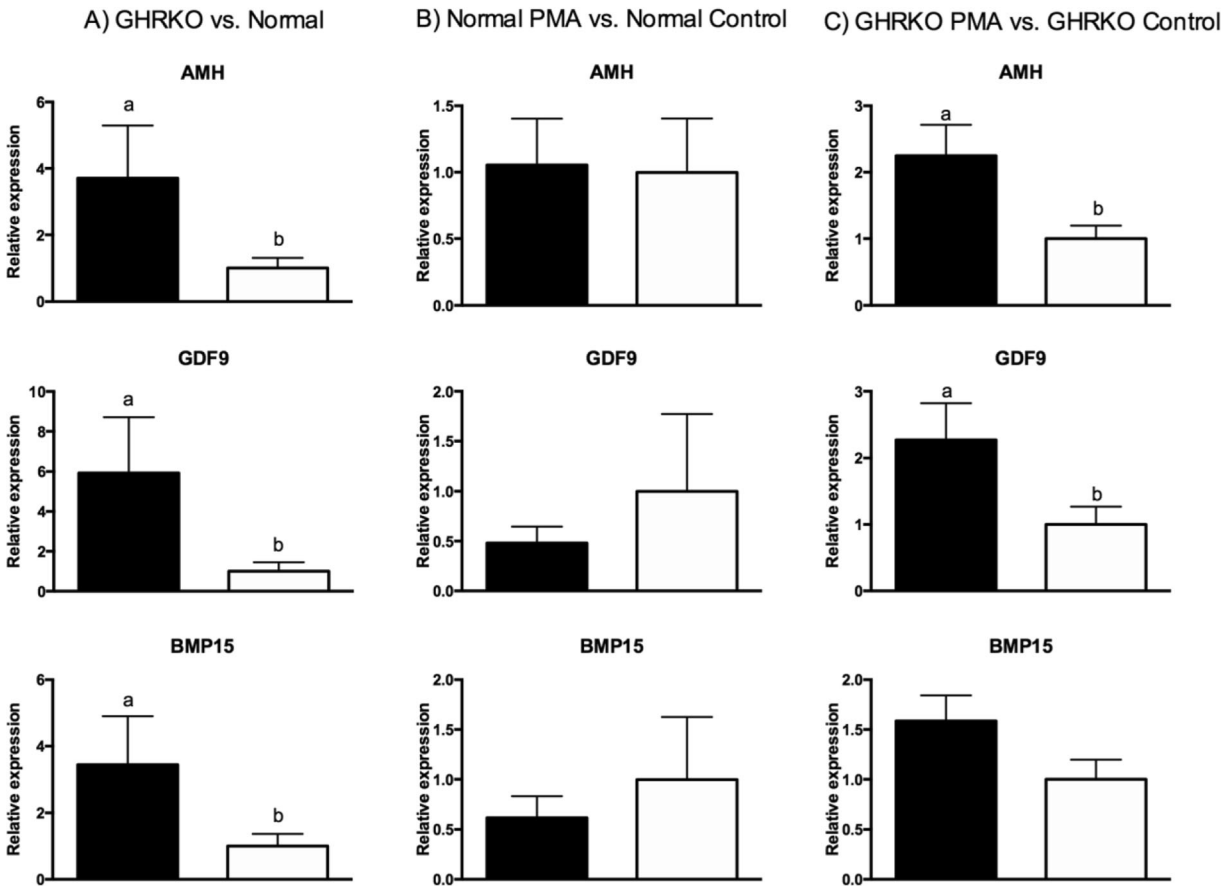

Fig. 1 Expression of *Amh*, *Bmp15*, and *Gdf9* mRNA in **a** GHRKO (*black bar*) and normal mice (*white bar*), **b** normal mice treated with PMA (*black bar*) or DMSO (*white bar*), and **c** GHRKO mice treated with PMA (*black bar*) and DMSO (*white bar*)

GHRKO's ovaries can provide possible explanation for the interaction between genotype and treatment.

The most significant difference observed between GHRKO and N mice was the increased expression of *Socs3* mRNA. It has been previously demonstrated that SOCS-1 and SOCS-3 act as negative regulators of insulin signaling and that *Socs3* overexpression reduces IRS1 phosphorylation (Ueki et al. 2004). Therefore, the higher expression of *Socs3* mRNA observed in GHRKO mice could be blocking insulin signaling and therefore preventing the activation of the Pi3k/Akt1 pathway and follicle progression from primordial to primary stage, reducing the rate of the ovarian reserve depletion in these mice. Other genes were not differently expressed between GHRKO and N mice and therefore were probably not related directly at the transcriptional level to the process of ovarian aging. What is also interesting and likely biologically significant is the tendency for increased expression of *Foxo3a* in the ovaries of GHRKO compared to N mice. Moreover, this difference reached significance in GHRKO mice treated with PMA in comparison to control GHRKO mice. FOXO3a is crucial, while not phosphorylated, for the maintenance of follicles in the primordial quiescent stage (Castrillon et al. 2003). Therefore, the higher expression of *Foxo3a* indicates that follicles could be trapped in the primordial stage. This hypothesis agrees with histological observations in long-living GH-resistant GHRKO mice, where a higher number of follicles in the primordial stage are observed concomitantly with fewer follicles in the primary, secondary, and tertiary stages (Slot et al. 2006). A recent study has demonstrated that oocyte expression of a constitutively active form of *Foxo3* in a transgenic mouse model is associated with the preservation of the ovarian reserve (Pelosi et al. 2013) and, therefore, can be related to an ovarian phenotype characteristic of younger mice as observed in GHRKO mice in the current and previous studies (Sluczanowska-

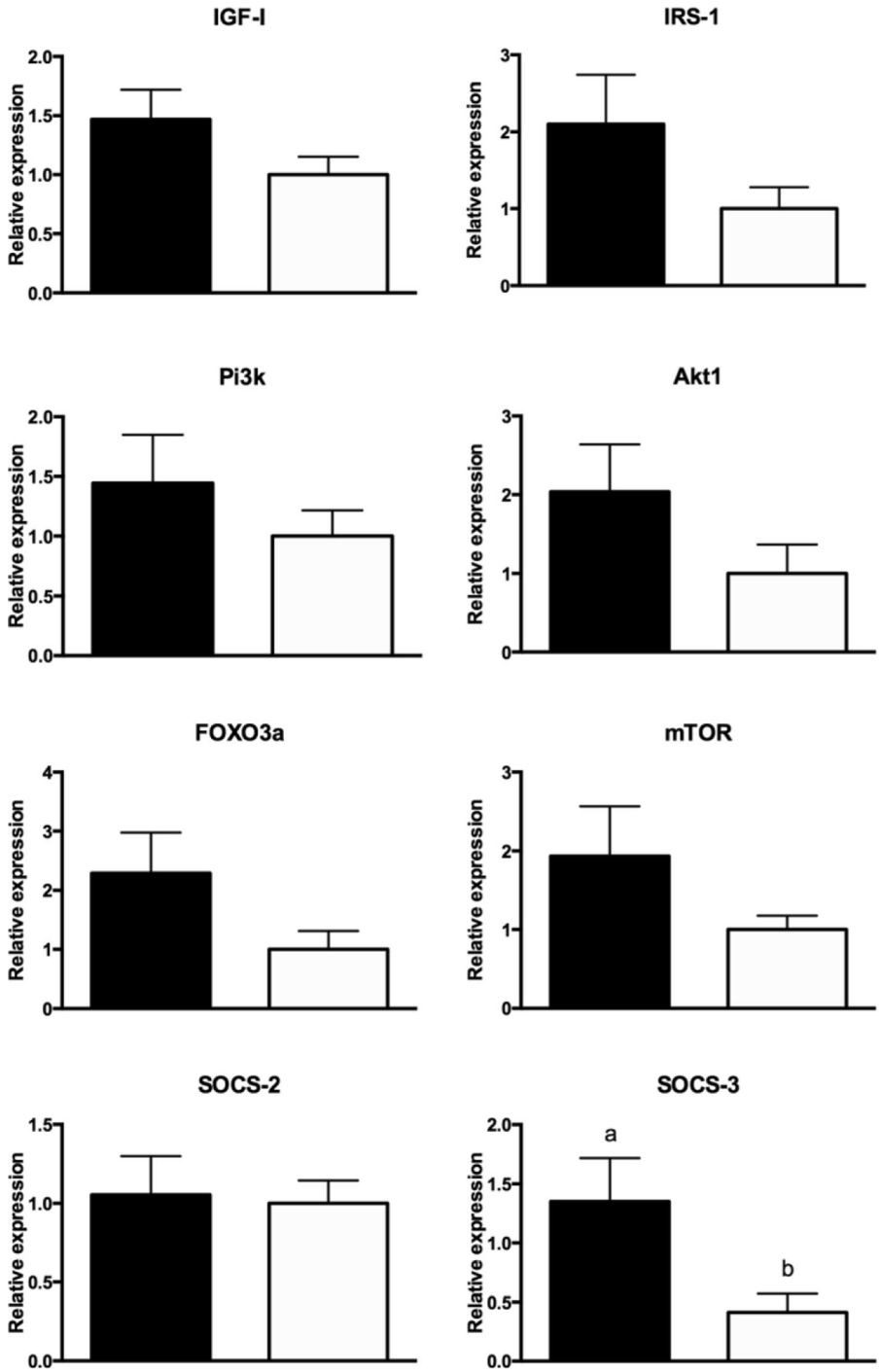

Fig. 2 Expression of *Igf1*, *Irs1*, *Pi3k*, *Akt1*, *Foxo3a*, *Mtor*, *Socs2*, and *Socs3* mRNA in GHRKO (*black bar*) and normal mice (*white bar*)

Glabowska et al. 2012). Studies in other tissues generally indicate that higher FOXO3 mRNA expression predicts also higher level of nuclear FOXO3 (Essaghir et al. 2009; Turrel-Davin et al. 2010). Despite that, it is known that cellular localization (nuclear vs. cytoplasmic) of FOXO3 rather than the level of expression truly indicates the activation of this protein. It is well established that FOXO3 is moved to the cytoplasm once

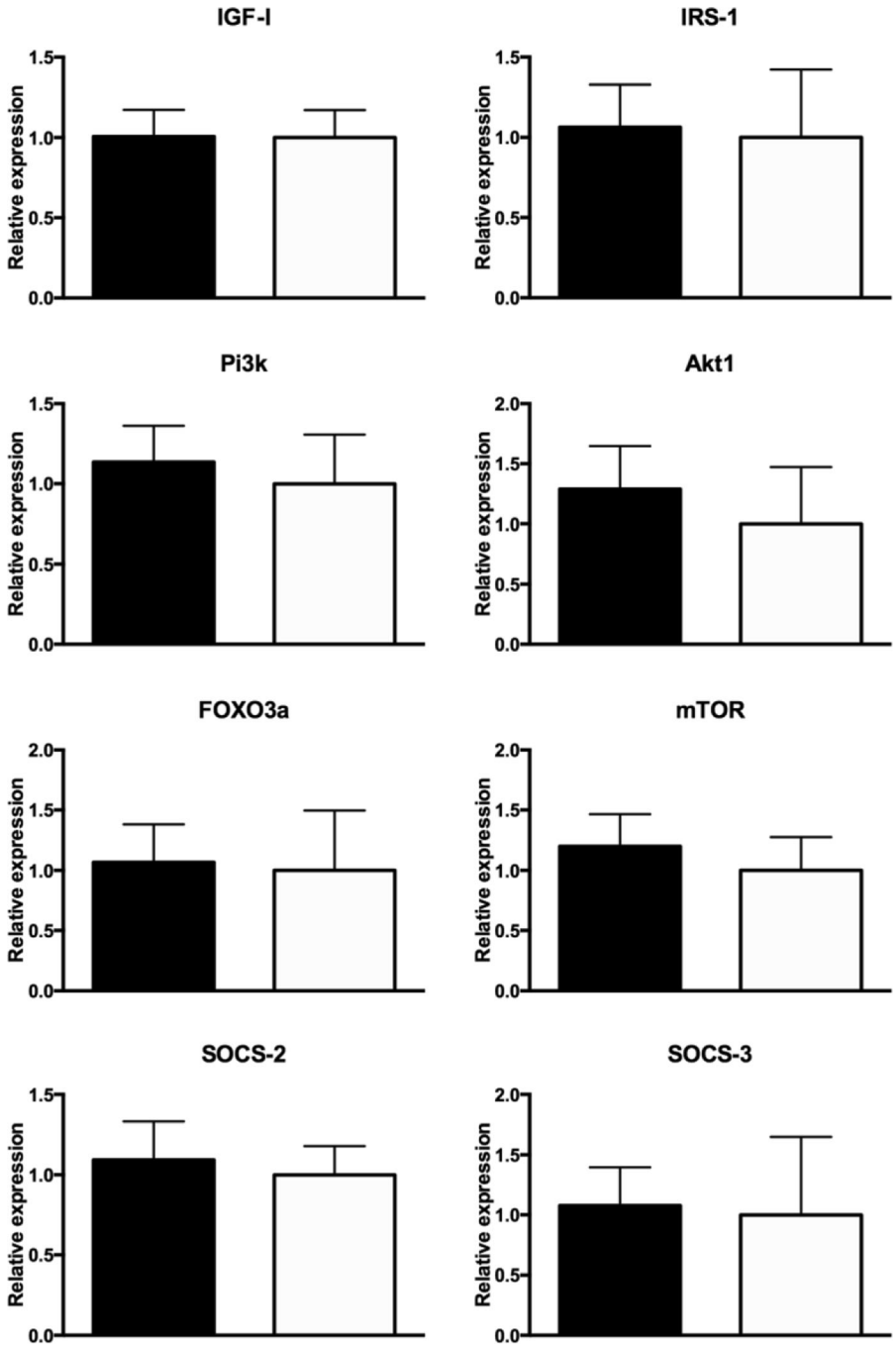

Fig. 3 Expression of *Igf1*, *Irs1*, *Pi3k*, *Akt1*, *Foxo3a*, *Mtor*, *Socs2*, and *Socs3* mRNA in normal mice treated with PMA (*black bar*) or DMSO (*white bar*)

activated by Akt; therefore, we could speculate less cytoplasmic FOXO3 in GHRKO mice due to the reduced IGF-1/insulin signaling. However, further studies will need to be completed to confirm the activity of FOXO3 in GHRKO mice and the specific role of PMA on localization of FOXO3 in N and GHRKO animals.

As stated before, the importance of the endocrine versus the paracrine/autocrine IGF-I is controversial. However, in the present study, we clearly demonstrate

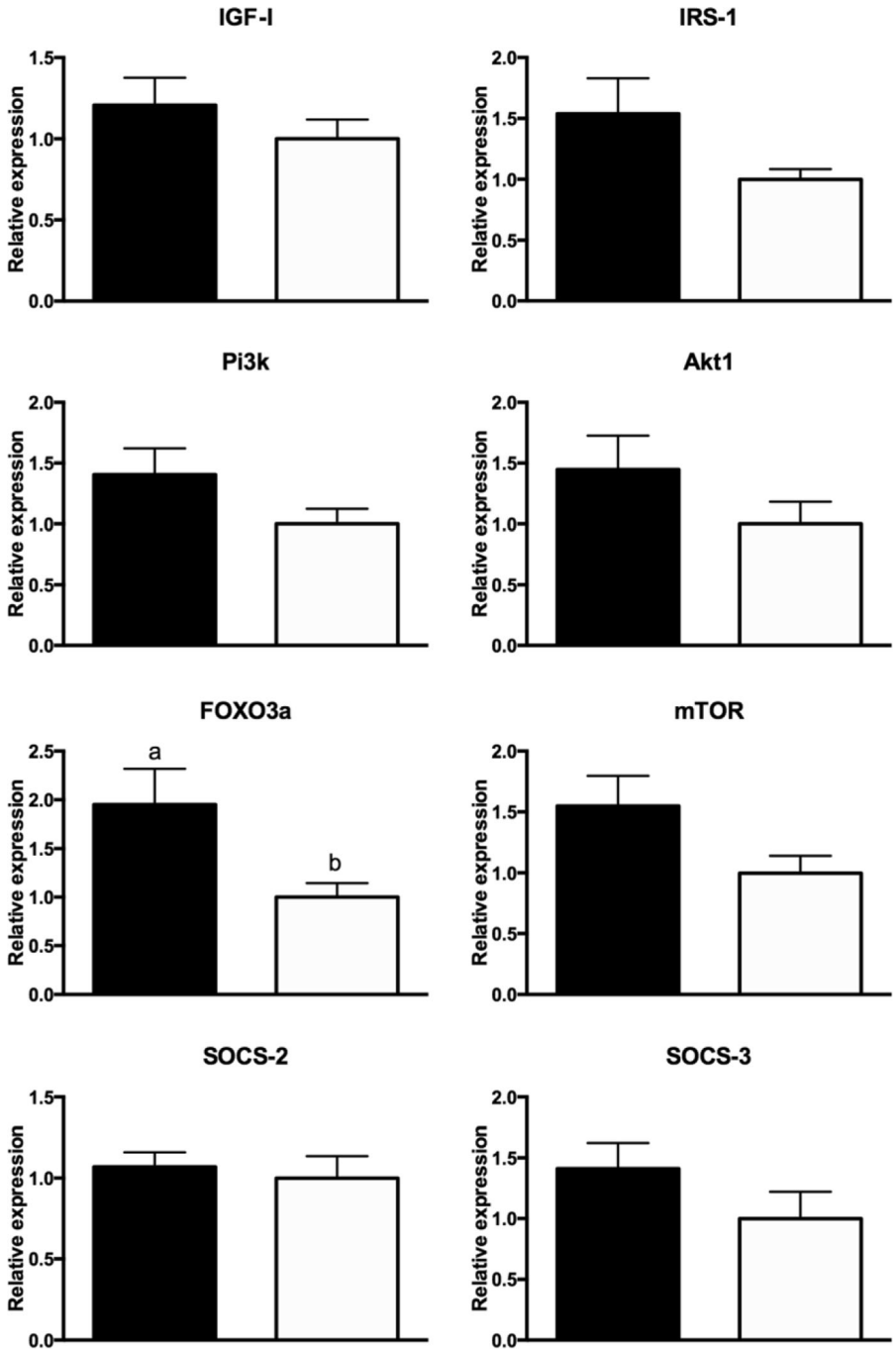

Fig. 4 Expression of *Igf1*, *Irs1*, *Pi3k*, *Akt1*, *Foxo3a*, *Mtor*, *Socs2*, and *Socs3* mRNA in GHRKO mice treated with PMA (*black bar*) or DMSO (*white bar*)

that there is ovarian expression of *Igf1* mRNA and that it is independent of the presence of a functional GH receptor since both GHRKO mice and N mice treated with PMA expressed similar levels of *Igf1* mRNA to their respective controls. Therefore, these results indicate that a local functional GHR is not needed for ovarian IGF-I production, which must be regulated mainly by other factors. Ovarian IGF-I production seems to be stimulated by estradiol and inhibited by GH in rats, although the combined effect of estradiol and GH is stimulatory

(Hernandez et al. 1989). It is also interesting to notice that neither of the PMA-treated groups had any changes in the expression of *Igf1* mRNA, which should disrupt insulin/IGF-I signaling. This indicates that the level of circulating IGF-I is not playing a role in the local production of IGF-I by the Pi3k/Akt1 pathway.

Conclusion

To summarize, the present data further confirm the previous histological observations that GHRKO mice have ovarian phenotype characteristic of younger mice indicated by higher expression of ovarian markers of the size of the ovarian reserve (*Amh*, *Gdf9*, and *Bmp15* mRNA). Also, we have shown that short-term treatment with PMA did not alter the profile of these ovarian markers in N mice, but increased them in GHRKO mice. In addition, we have shown a higher expression of *Socs3* in GHRKO mice and higher expression of *Foxo3a* in the ovaries of GHRKO mice treated with PMA, which suggests a role for these mediators in the process of ovarian aging. However, we are aware that the genes measured relate to total protein expression and do not represent any post-translational modifications (e.g., phosphorylation status), which makes it important to continue with future studies to determine the action of these intracellular signaling intermediates and the relation with ovarian physiology of aging.

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