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Ovarian aging and the activation of the primordial follicle reserve in the long-lived Ames dwarf and the short-lived bGH transgenic mice

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Abstract

The aim of this study was to evaluate the effect of growth hormone (GH) in the maintenance of the ovarian primordial follicle reserve. Ovaries from 16 mo old GH-deficient Ames Dwarf (df/df) and Normal (N/df) mice were used. A subgroup of df/df and N mice received GH or saline injections for six weeks starting at 14 mo of age. In addition, ovaries from 12 mo old mice overexpressing bovine GH (bGH) and controls were used. df/df mice had higher number of primordial and total follicles than N/df mice ($p<0.05$), while GH treatment decreased follicle counts in both genotypes ($p<0.05$). In addition, bGH mice had lower number of primordial and total follicles than the controls ($p<0.05$). pFoxO3a levels were higher in mice treated with GH and in bGH mice ($p<0.05$) when comparing with age match controls. These results indicate that increased circulating GH is associated with a reduced ovarian primordial follicle reserve and increased pFoxO3a content in oocytes.

Keywords

ovarian aging; primordial follicle; ovarian follicular reserve; GH; IGF; FOXO

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Competing interests

The authors disclose no competing interests.

1. Introduction

Ames Dwarf mice (df/df) have a defective Prop1 (Prophet of Pit1) gene, which impairs anterior pituitary gland development, resulting in deficient growth hormone (GH) secretion (Andersen et al., 1995; Miquet et al., 2005). As a result df/df mice have very low levels of circulating insulin-like growth factor I (IGF-I), are smaller and live around 30 to 65% longer than Normal littermates (N/df) (Brown-Borg et al., 1996; Chandrashekar and Bartke, 1993). Interestingly, early life short-term treatment of df/df mice with exogenous GH can reduce the longevity, insulin sensitivity and cellular stress resistance (Masternak et al., 2010; Panici et al., 2010). In contrast, transgenic mice overexpressing bovine GH (bGH) have elevated plasma levels of GH, resulting in increased circulating IGF-I levels and adult body size (Blackburn et al., 1997; Wolf et al., 1993). The lifespan of bGH mice is approximately 50% shorter than for normal littermates (Wolf et al., 1993). Collectively, this evidence points to an important role of the GH/IGF-I axis in the aging process, which seems to be dependent on the stage of life and maturation at which it occurs.

A progressive decline and depletion of the ovarian primordial follicle reserve is the main determinant of the age at the onset of menopause (Faddy et al., 1992; te Velde et al., 1998). Concomitant with the reduction in the number of follicles, the quality of the remaining oocytes generally decreases with age (Faddy et al., 1992; Hirshfield, 1994). The size of the primordial follicle reserve decreases about 10 fold from the ages of 0.5 to 1.5 y in female mice (Kevenaar et al., 2006) and is already depleted in 2.5 y old females (Słuczanowska-Gł0105;bowska et al., 2013). It is well known that a functional GH/IGF-I axis is important for the normal ovarian function (Bachelot et al., 2002; Chandrashekar et al., 2004; Zaczek et al., 2002). However, young female mice with a disrupted GH receptor gene (GHRKO), which also have lower serum IGF-I and increased longevity (Coschigano et al., 2003), have increased number of primordial follicles (Slot et al., 2006), and still have ovarian activity at older ages, when normal mice have exhausted the ovarian follicular reserve (Sluczanowska-Glabowska et al., 2012). GH and GHR deficient mice share several characteristics with mice subjected to calorie restriction (CR), including reduction of serum levels of IGF-I and insulin (Bonkowski et al., 2006; Bonkowski et al., 2009). Not surprisingly, it has been shown that mice subjected to CR also have an increased ovarian primordial follicle reserve (Li et al., 2011; Li et al., 2015; Xiang et al., 2012). Despite that, no studies to date have quantified the ovarian reserve in df/df and bGH mice, which would enhance the present understanding of the role of the GH/IGF-I axis in regulating the rate of primordial follicle depletion.

The activation of the transcription factor Forkhead Box O3a (FoxO3a) is an essential step for the activation of the primordial ovarian reserve irreversible growth (Castrillon et al., 2003; John et al., 2007). FoxO3a is a downstream effector of the phosphoinositide 3-kinase (Pi3k)/protein kinase B (Akt1) signaling and its pathway (John et al., 2008). Hyperphosphorylation of FoxO3a results in its nuclear exclusion, culminating in the global activation of primordial follicles and premature ovarian failure (Castrillon et al., 2003). We have previously shown that oocytes enclosed in primordial/primary follicles from df/df mice have lower levels of pFoxO3a (Schneider et al., 2014). The reduced activation of the FoxO pathway by both insulin and IGF-I seems to have a central role in the extended longevity phenotype observed

in the df/df and GHRKO mice (Bartke, 2008). Therefore, oocyte FoXO3a phosphorylation can be also reduced and represent a link between somatic and ovarian aging.

Based on this evidence, the aim of this study was to evaluate the ovarian primordial follicle reserve, as well as the activation of the FoxO3a transcription factor in aged df/df mice, treated with GH or saline in adult life, and in bGH mice.

2. Materials and Methods

2.1 Animals and treatments

For these experiments six groups of female mice were used. Four groups consisted of Ames dwarf (Prop-1^{df}, df/df, n=12) and their normal littermates (N/df, n=12) mice, between 16 and 18 mo old, receiving GH (n=6 for df/df, and n=6 for N/df mice) or saline injections (n=6 for df/df, and n=6 for N/df mice). The other two groups consisted of 10 to 12 mo old bGH (n=6) and normal mice (N, n=6). Mice were maintained under temperature (22 ± 2°C) and humidity (40–60%) controlled conditions. All experiments were approved by the Ethics Committee for Animal Experimentation from the University of Southern Illinois, IL, USA. Mice treated with GH received recombinant porcine GH subcutaneous injections (4 μg/g of body weight; Alpharma, Inc., Victoria, Australia) twice daily, beginning at 14 mo of age during 6 wk. Control mice received saline injections in the same way as GH treated mice. After 6 wk of treatment, mice were kept two more weeks in the same conditions until euthanasia. The GH treatment used in the current study was proven effective for increasing serum IGF-I concentrations and body weight gain in previous studies using the same dose and treatment length in young (1 mo old) (Louis et al., 2010; Masternak et al., 2010), middle age (5 mo old) (Gesing et al., 2014) and old mice (16 mo old) (Louis et al., 2010). Body weights were measured before the first GH injection and at the end of the 6 wk treatment to confirm effectiveness of the treatment.

2.2 Tissue collection and processing

The mice were anesthetized and euthanized after fasting for 12 h and the pair of ovaries was collected, dissected from surrounding adipose tissue and placed in 10% formalin buffered solution. After that, the ovaries were removed from the formalin solution, dehydrated in alcohol, cleared in xylene and embedded individually in Paraplast Plus (Sigma Chemical Company®, St. Louis, MO, USA). One ovary of the pair was then serially sectioned at 5μm using a semi-automated rotary microtome (RM2245, Leica Biosystems, San Diego, CA, USA). Sampling started at the beginning of the visible area of the ovarian surface until the end of the structure, and every 6^{th} section was selected and placed on a standard histological slide for staining and counting (adapted from Myers et al. (2004)). Intermediate sections were randomly selected for immunohistochemistry analysis using slides impregnated with 3% organosilane (Sigma Chemical Company®, St. Louis, MO, USA) in ethanol.

2.3 Morphological classification and follicle counting

The slides were dried at 56°C for 24 h, stained with hematoxylin-eosin, and mounted with coverslips and synthetic resin (Sigma Chemical Company®, St. Louis, MO, USA). Images of the ovarian sections were captured with a digital camera coupled to a microscope (Nikon

Eclipse E200, Nikon Corporation, Japan) using the 10 and 40× objectives, assisted by the software Moticam 5.0 (Motic, Hong Kong, China).

Only follicles containing an oocyte with clearly visible nucleus were counted in each slide. Follicle classification was based on Myers et al. (2004). Follicles were classified as primordial (oocyte surrounded by a single layer of flattened granulosa cells), in transition (oocyte surrounded by a layer of flattened granulosa cells and at least one cuboid granulosa cell), primary (oocyte surrounded by a single layer of cuboidal granulosa cells), secondary (oocyte surrounded by two or more layers of cuboid granulosa cells without a visible antrum) and tertiary (follicles with a clearly defined antral space and a with multiple layers of granulosa cells around the oocyte). To estimate actual follicle quantity the number of follicles in each category was multiplied by six to account for the section sampling and by two to account for the fact that only one ovary of the pair was used.

2.4 Immunohistochemistry analysis

For immunohistochemistry analysis, the ovarian samples were deparaffinized with xylene and rehydrated with graded alcohols. The primary polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and diluted in 1.5% BSA solution. The anti-FoxO3a (FKHRL1 antibody; N16, SC-101683-goat (IgG)) and anti-phosphorylated FoxO3a (pFoxO3a; p-FKHRL1 antibody; Ser 253, SC-101683-rabbit (IgG)) antibodies were used at a final dilution of 1:50. Blockage of the endogenous peroxidase activity was achieved with hydrogen peroxide blocking solution (Spring Bioscience, Pleasanton, CA, USA), while the antigen recovery was performed in humid heat, during 3 min after the boiling point, in citrate solution at pH 6.0. Non-specific background staining was reduced by covering the tissue sections that received protein block (Spring Bioscience, Pleasanton, CA, USA). Thereafter, slides were incubated overnight with the primary antibody in a humid chamber at 4°C. The slides with pFoxO3a and FoxO3a antibodies were instilled with secondary antibodies Reveal Polyvalente HRP® Kit (Spring Bioscience, Pleasanton, CA, USA) and Dako LSAB®2 System-HRP (Dako Corporation, Carpinteria, CA, USA), respectively. The slides were incubated at room temperature with 3,3'-diaminobenzidine (DAB-K3468, DAKO Corporation, Carpinteria, CA, USA), counterstained with Mayer's hemalum solution (Merck, Darmstadt, Germany) and mounted with coverslips and synthetic resin (Sigma Chemical Company®, St. Louis, MO, USA). The images of the follicles were captured by a digital camera coupled to a microscope (Nikon Eclipse E200, Nikon Corporation, Japan) using a 40X objective assisted by the software Moticam 5.0 (Motic®, Hong Kong, China). Only oocytes enclosed in follicles classified as primordial/in transition (n=108; n=18/group) and primary (n=108; n=18/group) were used. Protein quantification was performed by image analysis software (Image J®) and the most common value (the mode) of each area (oocyte) was registered by the 32-bit histogram application, using a scale ranging from 0 (the greatest staining intensity) to 255 (no staining) that was converted to a scale from 0 (no staining) to 4 (greatest staining) (Moreira et al., 2013; Schneider et al., 2014).

2.5 Statistical analysis

All statistical analyzes were performed using Graphpad Prism 5 (Graphpad Inc., La Jolla, CA, USA). Two-way ANOVA was used for comparing the number and size of follicles and immunostaining between df/df and N/df mice (effect of the genotype, treatment with GH and the interaction). T-test was performed for comparing the number and size of follicles and immunostaining between bGH and normal mice. A P value lower than 0.05 was considered as statistically significant, and between 0.05 and 0.10 as a trend.

3. Results

GH treatment increased body weight gain in both N and df/df mice ($P<0.05$) at the end of the 6 wk treatment (Suppl. Figure 1). However, body weight did not change ($P>0.05$) in Saline treated mice during the same period (Suppl. Figure 1), therefore confirming the effectiveness of the treatment applied in the current study.

The number of primordial, in transition and total follicles was higher in df/df than N/df mice ($P<0.01$, Figures 1A, 1B and 1F). In addition, GH treatment decreased the number of primordial and total follicles in both df/df and N/df mice ($P<0.02$, Figures 1A and 1F). The number of primary follicles was not different between genotypes or treatments ($P>0.10$, Figure 1C). There was a genotype by treatment interaction for the number of secondary follicles, since GH treatment increased ($P<0.01$, Figure 1D) the number of follicles in df/df mice, while decreased it in N/df mice ($P<0.01$, Figure 1D). The number of tertiary follicles was lower in df/df than N/df mice ($P<0.01$, Figure 1E). The number of primordial, in transition, primary and total follicles was decreased in bGH compared to normal mice ($P<0.01$, Figures 2A, 2B, 2C and 2F), while the number of secondary and tertiary follicles was higher in bGH than in normal mice ($P<0.01$, Figures 2D and 2F).

FoxO3a immunostaining in primordial follicles oocytes was higher in df/df mice ($P=0.009$, Figure 3A), however it was reduced in df/df and N/df mice treated with GH ($P=0.03$, Figure 3A). pFoxO3a immunostaining in primordial follicles oocytes was also higher in df/df mice ($P=0.01$, Figure 3C), and it was increased by GH treatment in both df/df and N/df mice ($P=0.01$, Figure 3C). FoxO3a immunostaining in primary follicles oocytes was not different between genotypes or treatments ($P>0.10$, Figure 3B). However, there was a genotype by treatment interaction for pFoxO3a immunostaining in primary follicles oocytes, which was higher for df/df treated with GH ($P<0.05$, Figure 3D). There was no difference in FoxO3a immunostaining in primordial and primary follicles oocytes of bGH compared to normal mice ($P>0.05$, Figures 4A and 4B). However, pFoxO3a immunostaining in primary follicles oocytes was higher in bGH compared to normal mice ($P=0.006$, Figure 4D). FoxO3a and pFoxO3a immunostaining images for the oocytes in the different groups are presented in Figures 5 and 6, respectively.

Data for nuclei, oocyte and follicle diameters are shown in Table 1 for df/df and N/df mice and in Table 2 for bGH and normal mice. GH treatment decreased oocyte and follicle diameters in primordial follicles of df/df and N/df mice ($P<0.05$, Table 1). In addition, oocyte diameter of secondary follicles was reduced in df/df mice ($P=0.03$, Table 1). Tertiary follicle size was reduced in df/df compared to N/df mice ($P=0.03$, Table 1) and oocyte

nucleus diameter was increased in mice treated with GH (P=0.0001, Table 1). Nuclei diameters from oocytes of primordial, in transition and primary follicles were increased in bGH compared to normal mice (P<0.05, Table 2). Secondary and tertiary follicles oocyte diameter was higher in bGH than normal mice (P<0.05, Table 2).

4. Discussion

The current study points to the GH/IGF-I axis as a central player in regulating the female reproductive lifespan. The results show that df/df mice have a greater number of primordial follicles than N/df mice, while bGH mice have a reduced number of primordial follicles in comparison to normal mice. Additionally, the treatment of both df/df and N/df mice with exogenous GH enhanced the depletion of the primordial follicle reserve. Therefore, these results demonstrate that the GH/IGF-I axis via its downstream effectors can regulate activation of the ovarian primordial follicle reserve, confirming the close association between somatic aging and reproductive lifespan.

The number of primordial and in transition follicles was higher, while the number of secondary follicles was lower, in df/df compared to N/df mice. In contrast, bGH mice had a lower number of primordial and in transition follicles, with a higher number of secondary and tertiary follicles, compared to N mice. Collectively these data indicate that follicles are trapped in the primordial stage, not being activated and therefore not progressing to the secondary stage due to GH/IGF-I deficiency. No previous record of ovarian follicular quantification in df/df and bGH mice was found in the literature, showing that the data from the present study are unique and provide novel evidence for these mice as models for the study of ovarian aging. This is important, since other mouse models for ovarian aging (like the knockouts of PTEN and FoxO3) have complete ovarian failure early after puberty (Castrillon et al., 2003; John et al., 2008; Reddy et al., 2009) and do not allow for comparative studies at adult life as the df/df and bGH mice. It was previously demonstrated that young female GHRKO mice have an increased primordial follicle reserve (Slot et al., 2006). The difference observed in the GH deficient mice in the present study was even bigger than observed for GHRKO before and this can be due the fact we used much older mice. Still, the comparison is important since previous studies have shown that not all characteristics are shared between df/df and GHRKO mice (Al-Regaiey et al., 2005). In addition, rats subjected to caloric restriction also have increased primordial follicle reserve compared to *ad libitum* fed rats, which is associated with reduced levels of circulating insulin and IGF-I (Li et al., 2011). Therefore, our findings are in agreement with these previous models, indicating that alterations in the GH/IGF-I axis that increase or decrease longevity also affect the reproductive lifespan.

The treatment of adult df/df and N/df mice with GH was able to activate primordial follicle growth, since we observed a decrease in the number of primordial follicles in both genotypes. Interestingly, the reduction in the number of primordial follicles was followed by increased number of secondary follicles in df/df mice, but not in N/df mice. This can indicate that while GH is stimulating follicle progression and continuous growth in df/df mice, it may be stimulating higher follicular atresia in N/df mice, since they do not continue to grow to further stages. Previous studies found that the number of primordial follicles was

reduced in younger GHRKO mice treated with IGF-I for two weeks (Slot et al., 2006). Together, this evidence suggest that low levels of GH/IGF-I may cause the accumulation of primordial follicles, extending the longevity of the ovarian reserve, but as its levels are increased even at older ages, the activation of primordial follies is restored. It should be noted that both df/df and bGH mice have reduced pituitary secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Chandrashekar and Bartke, 1996; Tang et al., 1993). The transition of follicles from the primordial to primary stages is gonadotropin independent (Scaramuzzi et al., 2011), however prolactin deficiency can reduce primordial follicle activation (Ormandy et al., 1997). Therefore, our results should be interpreted carefully as in rodents porcine GH can bind to both GH and prolactin receptors (Amit et al., 1992) and have lactotropic and somatotropic effects in these GH/prolactin deficient df/df mice. On the other hand, bGH has purely somatogenic effects (Amit et al., 1992; Chandrashekar and Bartke, 2003), but the sustained increase of GH in the transgenic bGH mice results in higher serum levels of prolactin (Chandrashekar and Bartke, 1996), which can also affect the rate of primordial follicle activation.

The presence of FoxO3a in its non-phosphorylated form is crucial to maintaining the primordial follicles in their quiescent state (John et al., 2008). Primordial follicles begin to grow irreversibly when oocyte FoxO3a is phosphorylated by stimulus from the surrounding granulosa cells (John et al., 2007; John et al., 2008; Zhang et al., 2014). Therefore, the higher level of non-phosphorylated FoxO3a observed in primordial follicles oocytes may be an explaination why follicles remain for longer periods in the primordial stage in df/df mice. In addition, GH treatment reduced FoxO3a protein level, while it increased its phosphorylated form in primordial follicles oocytes. Again, this evidence suggests that GH treatment is activating the primordial follicle pool by promoting increased phosphorylation of the FoxO3a transcription factor. We also observed that df/df mice had increased pFoxO3a level in primordial follicle oocytes, but this increase was proportional to the increase in FoxO3a total protein. In a previous study we observed that pFoxO3a protein levels in primordial/primary follicles were lower in 12 mo old df/df compared to N/df mice, despite no difference in total FoxO3a protein levels (Schneider et al., 2014), which can suggest an age-dependent FoxO3a regulation in the ovary. FoxO3a and pFoxO3a were not different in primordial follicles of bGH and normal mice. However, the level of pFoxO3a was higher in primary follicles oocytes of bGH than normal mice, which could be related to the higher rate of primordial follicle activation and depletion observed in bGH mice.

Bowen and Atwood (2004) postulated the Reproductive-Cell Cycle Theory of Aging, which states that the hormones that regulate reproduction act in an antagonistic pleiotrophic manner to control aging via cell cycle signaling, with many suggested pathways that can be implicated is this trade-off (Atwood and Bowen, 2011). Additionally, it has been shown before that lower GH/IGF levels correlate with increased lifespan and delayed sexual maturation in different strains of mice, which also indicates the existence of a tradeoff between growth rate, sexual maturation and longevity (Yuan et al., 2012; Yuan et al., 2015). The FoxO transcription factor can be a central player in this regulation, since its increased activation is linked to accelerated aging in several model organisms (Kenyon, 2010). The nuclear presence of the non-phosphorylated FoxO is associated with activation of pathways related to cell self-preservation, maintenance of the quiescent state, stress resistance,

maintenance of the stem cell pool and tumor suppression (Salih and Brunet, 2008). Based on this previous evidence and our current results, we can suggest that the modulation of the GH/IGF-I axis and its downstream effector FoxO3a can have a central role in the regulation of the trade-off between the somatic and reproductive aging in mice. Therefore, accelerated aging is linked to higher GH/IGF-I levels, increased primordial oocyte FoxO3a activation and faster depletion of follicular reserves. The opposite situation is observed in mice with reduced levels of circulating GH/IGF-I.

It is well established that the growth of primordial, in transition and secondary follicles is directly linked to the growth of the oocyte and its nucleus (Lintern-Moore and Moore, 1979), with an intense cross-talk between the oocyte and its surrounding granulosa cells (Sobinoff et al., 2013; Zhang and Liu, 2015). Granulosa cells secreted factors can activate the Pi3k/FoxO3a pathway in the primordial follicle oocyte and initiate follicle growth (Zhang et al., 2014; Zhang and Liu, 2015). Our results show that oocytes from primordial, in transition and primary follicles of bGH mice had increased nuclei diameter. Growth of oocyte nuclei is associated with increased gene expression activity (Moore and Lintern-Moore, 1974), thus indicating that the oocyte is awakening the machinery necessary for its growth and differentiation. In addition, primary and secondary follicles oocyte diameter was increased in bGH mice. On the other hand, df/df mice tended to have smaller secondary follicle oocyte diameter, although GH treatment decreased primordial follicles oocyte size in both df/df and N/df mice. Overall this may be an indication that higher GH levels are associated with increased oocyte transcriptional activity, which would lead to early activation of oocyte and follicular growth, as was observed for bGH mice. It is possible that FoxO3a activation is an important regulator of oocyte growth, since the FoxO3a knockout mice also have a rapid increase in primordial follicle oocyte diameter, which is followed by premature ovarian failure due to exhaustion of the follicular reserve (Castrillon et al., 2003). The same association between increased primordial follicle oocyte size and early activation of the ovarian reserve was observed for the Pten knockout mice (Reddy et al., 2008).Thus, we can hypothesize that there may be a relationship between the activation of oocyte FoxO3a signaling, leading to oocyte growth and awakening of quiescent follicles in the reserve.

5. Conclusion

GH-deficient df/df female mice had an increased ovarian primordial follicle reserve in comparison to N/df mice, and GH treatment in adulthood was able to activate primordial follicle growth and reduce the size of the reserve in both df/df and N/df mice. The opposite was observed in transgenic mice overexpressing bGH, which had accelerated ovarian aging and a decreased ovarian primordial follicle reserve. Overall, this indicates that the size of the ovarian primordial follicle reserve at older ages can be a direct reflection of the circulating levels of GH/IGF-I, and that even transient changes in GH/IGF-I levels after maturation can alter the rate of follicle depletion and ovarian aging. Our study also has shown that FoxO3a phosphorylation is increased by GH and is associated with decreased primordial follicle reserve, possibly linking changes in the GH/IGF-I axis to the rate of ovarian aging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- GH deficient df/df mice had increased, while transgenic mice overexpressing GH had decreased ovarian primordial follicle reserve;
- GH treatment at adult age can reduce the primordial follicle reserve in both df/df and N/df mice;
- Reduced ovarian reserve is associated to increased presence of pFoxO3a in oocytes enclosed in primordial follicles.

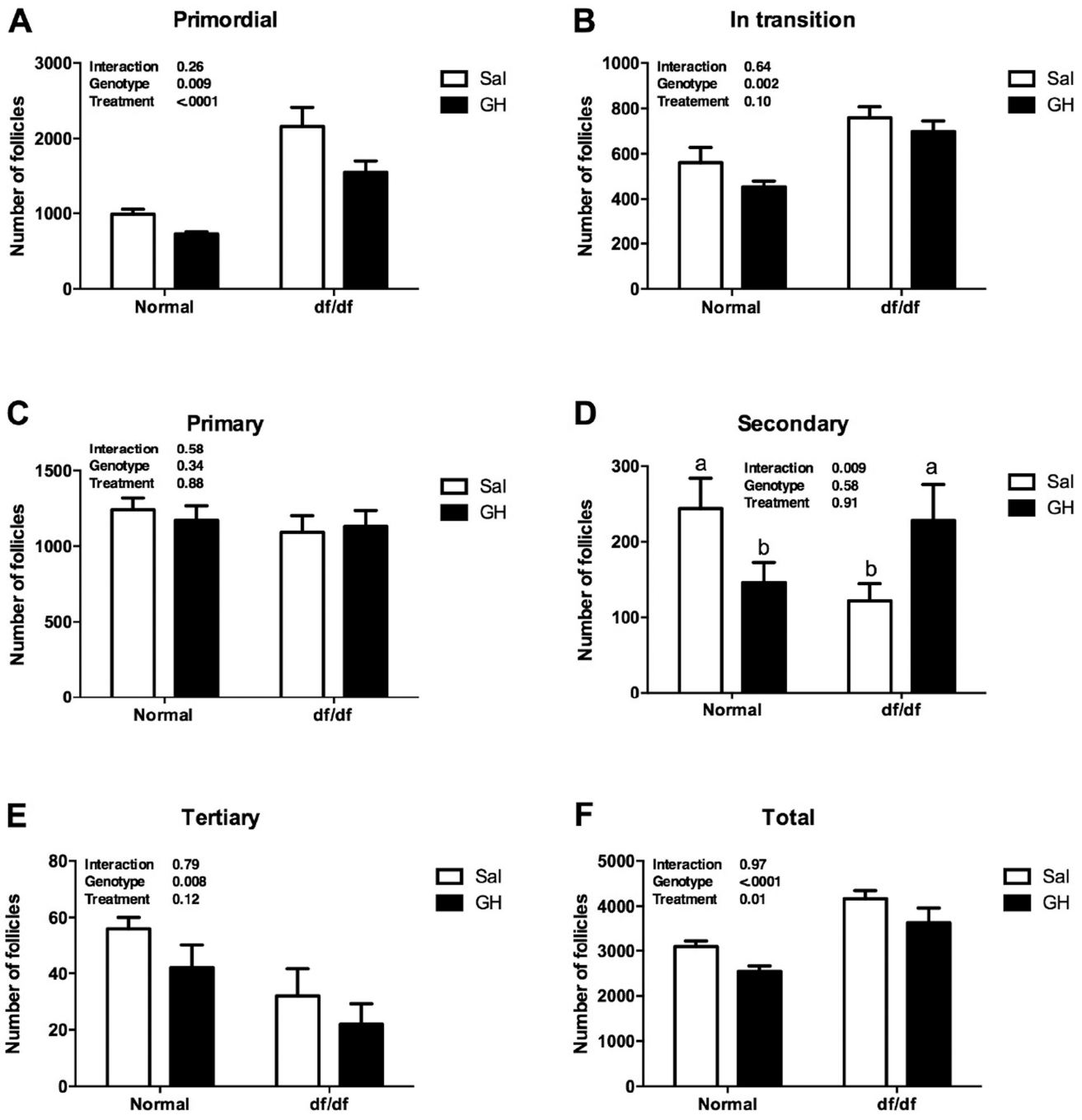

Figure 1.

Number of primordial (A), transitional (B), primary (C), secondary (D), tertiary (E) and total follicles (F) in Ames dwarf (df/df) and Normal (N/df) mice receiving GH or saline treatment. Different letters indicate significant differences when the interaction was significant ($P<0.05$).

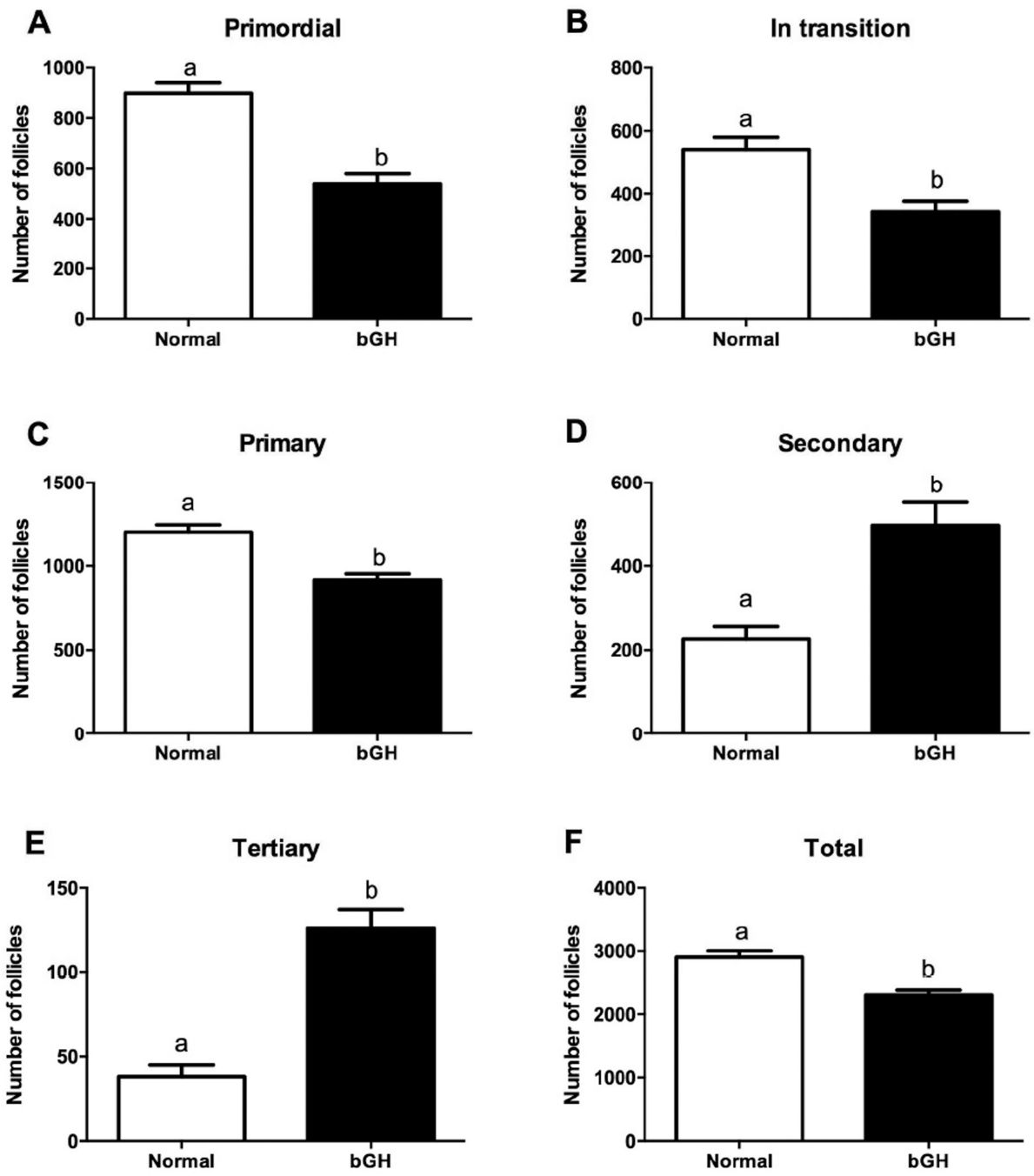

Figure 2.

Number of primordial (A), transitional (B), primary (C), secondary (D), tertiary (E) and total follicles (F) in normal and transgenic mice overexpressing GH (bGH). Different letters indicate significant differences ($P<0.01$).

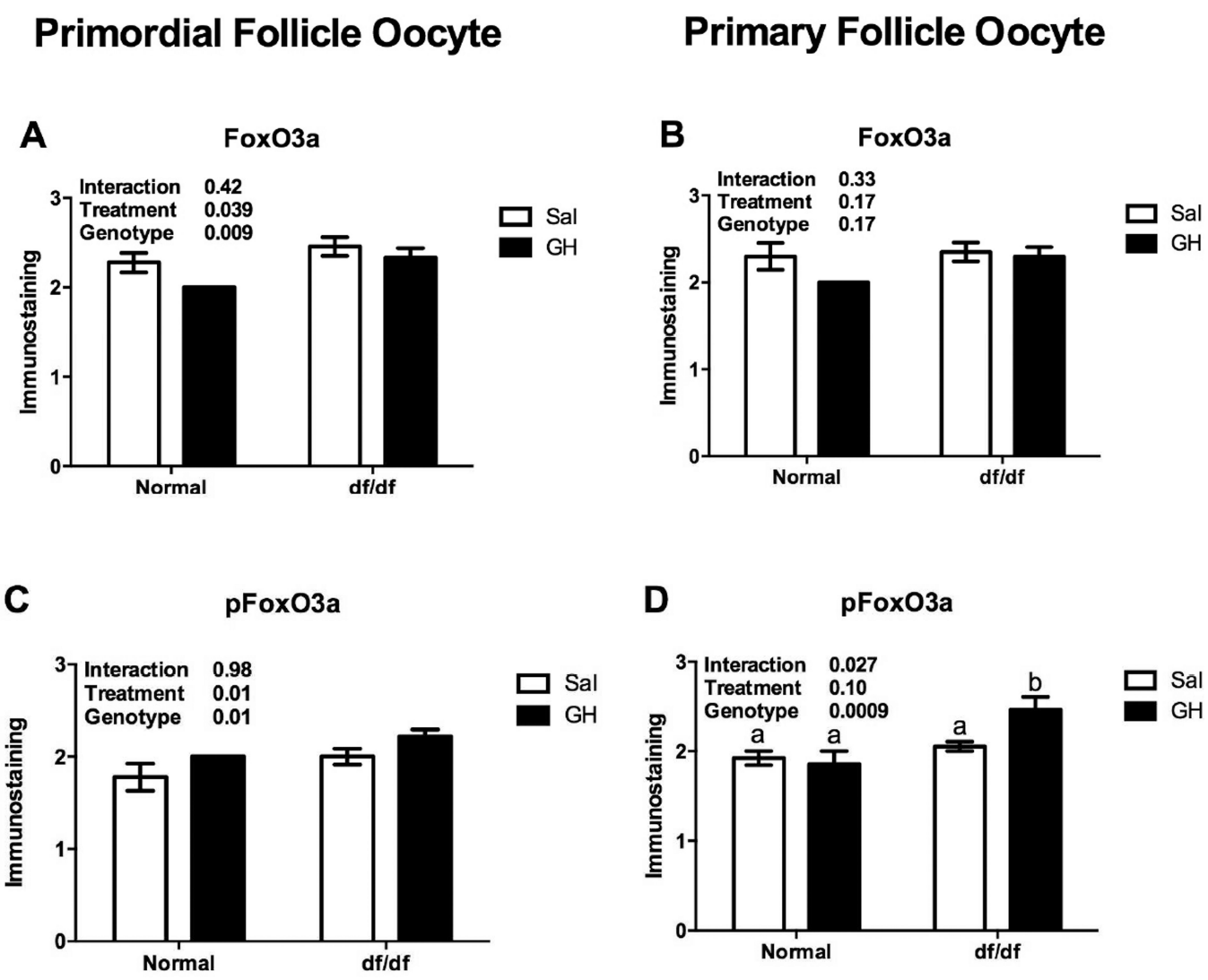

Figure 3.

Immunostaining for FoxO3a (A and B) and pFoxO3a (C and D) in oocytes from primordial/transitional and primary follicles in Ames dwarf (df/df) and Normal (N/df) mice receiving GH or saline treatment (n=18 oocytes/category/group). Different letters indicate significant differences when the interaction was significant ($P<0.05$). The initial values were converted to a scale from 0 (no staining) to 4 (intense staining).

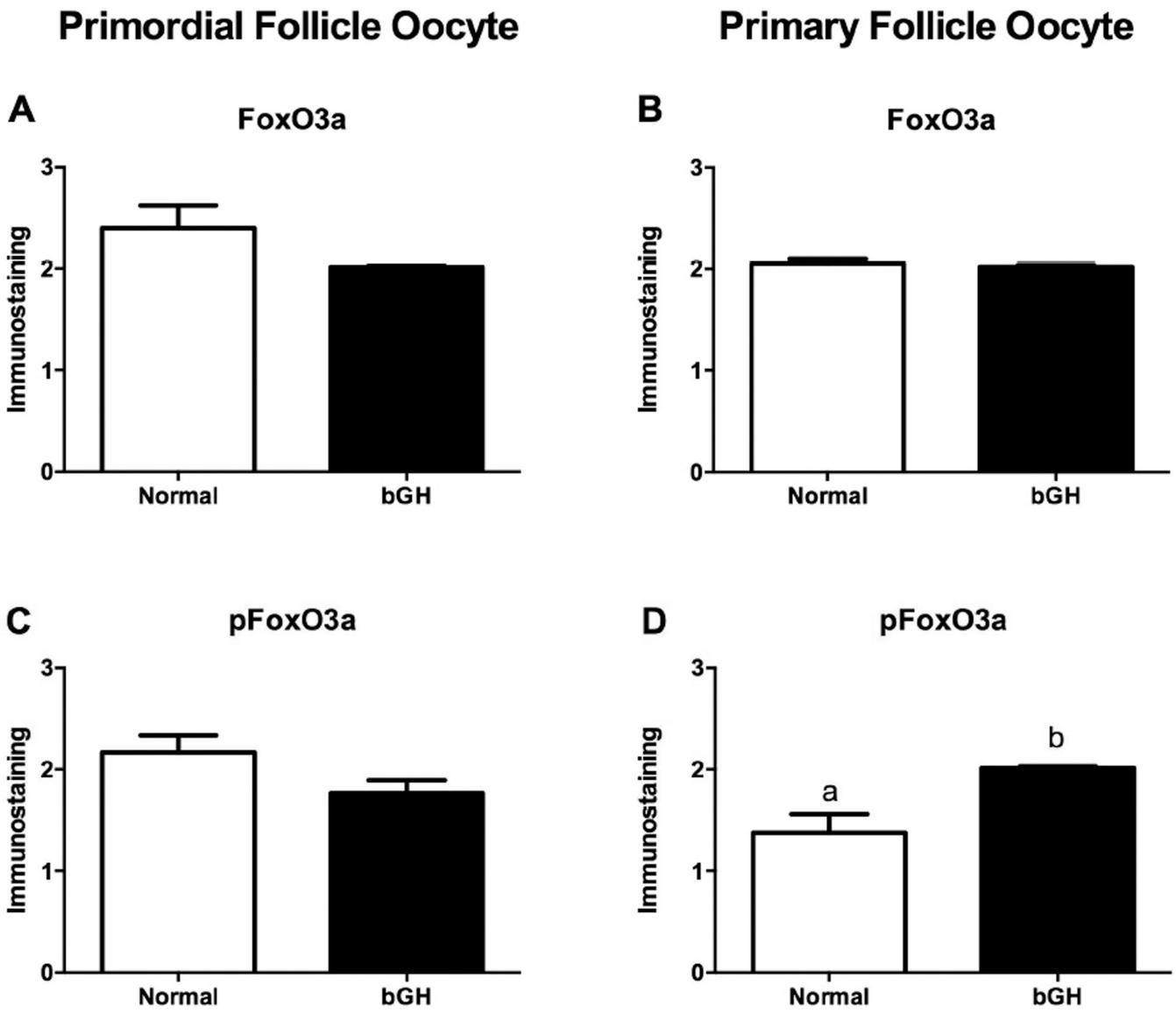

Figure 4.

Immunostaining for FoxO3a (A and B) and pFoxO3a (C and D) in oocytes from primordial/transitional and primary follicles in normal mice and transgenic mice overexpressing GH (bGH) (n=18 oocytes/category/genotype). Different letters indicate significant differences ($P<0.05$). The initial values were converted to a scale from 0 (no staining) to 4 (intense staining).

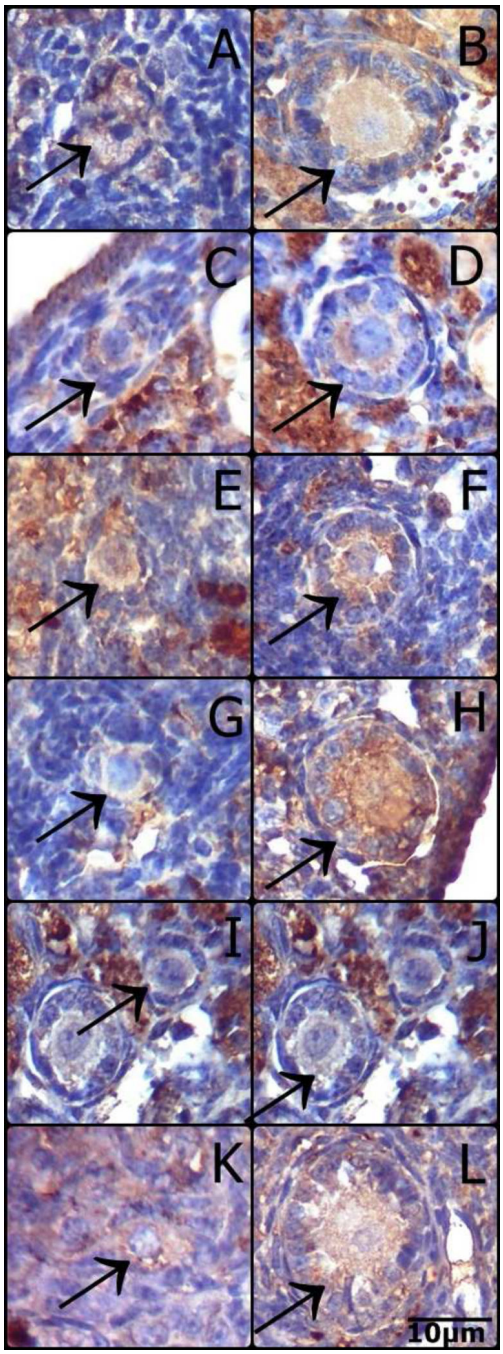

Figure 5.

Representative images of immunostaining for FoxO3a in oocytes from primordial and primary follicles of N/df mice receiving saline (A, B) or GH (C and D), df/df mice receiving saline (E and F) or GH (G and H), bGH mice (I and J) and normal mice (K and L). (Primordial follicles: A, C, E, G, I, K; Primary follicles: B, D, F, H, J, L).

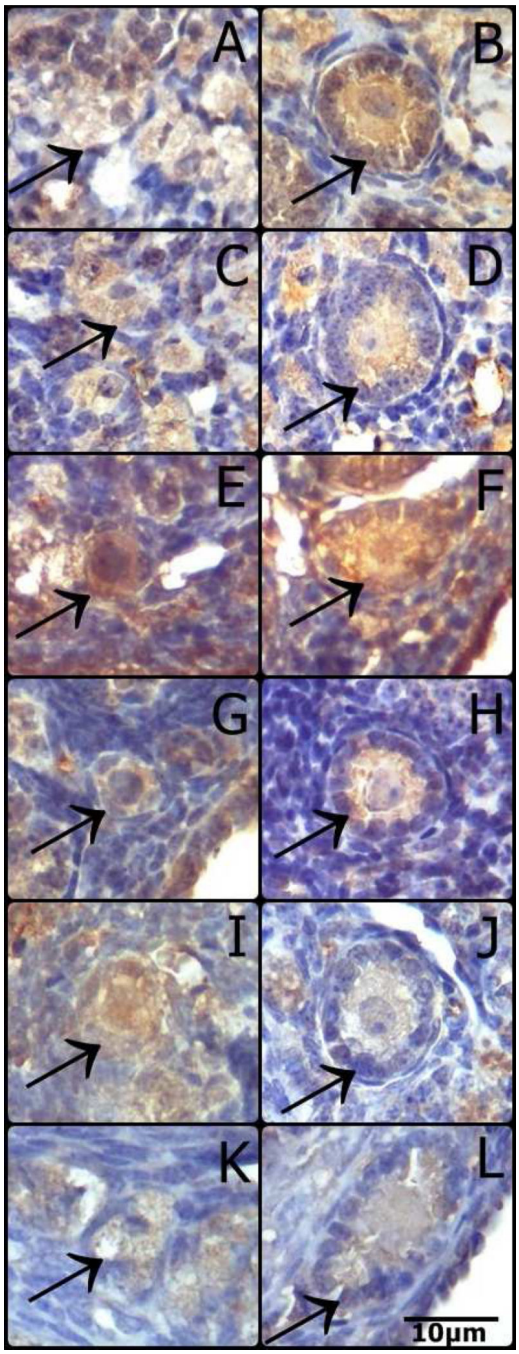

Figure 6.

Representative images of immunostaining for pFoxO3a in oocytes from primordial and primary follicles of N/df mice receiving saline (A, B) or GH (C and D), df/df mice receiving saline (E and F) or GH (G and H), bGH mice (I and J) and normal mice (K and L). (Primordial follicles: A, C, E, G, I, K; Primary follicles: B, D, F, H, J, L).

Table 1

Nuclei, oocyte and follicle diameters of primordial (n=18/group), in transition (n=18/group), primary (n=18/group), secondary (n=18/group) and tertiary (n=18/group) follicles in 16 months old Ames Dwarf and Normal mice treated with GH or saline solution for 6 weeks. Data is presented as average ± standard error of the mean.

	Normal		Ames dwarf		P value		
Diameter (μm)	**Saline**	**GH**	**Saline**	**GH**	**Genotype**	**Treatment**	**Genot*Treat**
Primordial follicle							
Nucleus	3.2 (±0.2)	2.8 (±0.2)	2.9 (±0.1)	3.0 (±0.1)	0.77	0.44	0.15
Oocyte	6.4 (±0.4)	5.2 (±0.2)	5.7 (±0.3)	5.6 (±0.3)	0.54	0.04*	0.07
Follicle	9.6 (±0.8)	7.8 (±0.3)	8.3 (±0.3)	8.0 (±0.4)	0.25	0.03*	0.14
In transition follicle							
Nucleus	3.1 (±0.1)	3.2 (±0.2)	3.1 (±0.1)	2.8 (±0.1)	0.17	0.29	0.10
Oocyte	6.5 (±0.3)	6.3 (±0.3)	6.3 (±0.3)	6.4 (±0.3)	0.71	0.83	0.59
Follicle	10.4 (±0.4)	10.0 (±0.4)	9.7 (±0.4)	9.9 (±0.4)	0.29	0.79	0.44
Primary follicle							
Nucleus	3.4 (±0.2)	3.8 (±0.3)	3.2 (±0.2)	3.6 (±0.3)	0.36	0.08	0.77
Oocyte	10.6 (±1.0)	10.8 (±0.8)	9.6 (±0.7)	10.2 (±0.9)	0.34	0.63	0.76
Follicle	17.3 (±1.5)	16.3 (±1.1)	15.7 (±0.9)	15.6 (±1.2)	0.32	0.65	0.70
Secondary follicle							
Nucleus	6.1 (±0.5)	5.9 (±0.4)	4.8 (±0.4)	6.0 (±0.3)	0.12	0.19	0.10
Oocyte	25.7 (±0.9)	24.4 (±1.0)	23.3 (±0.9)	22.9 (±0.7)	0.03*	0.33	0.62
Follicle	50.2 (±3.2)	52.8 (±3.6)	56.8 (±4.9)	55.9 (±5.7)	0.29	0.88	0.72
Tertiary follicle							
Nucleus	5.4 (±0.3)	6.3 (±0.3)	4.1 (±0.5)	6.6 (±0.5)	0.21	0.0001*	0.21
Oocyte	45.6 (±15.2)	30.2 (±0.9)	29.8 (±1.1)	31.0 (±1.5)	0.40	0.42	0.35
Follicle	150.8 (±9.0)	170.7 (±7.3)	129.1 (±6.7)	132.1 (±10.2)	0.001*	0.19	0.33

*Significant P value – P<0.05

Table 2

Nuclei, oocyte and follicle diameters of primordial (n=18/group), in transition (n=18/group), primary (n=18/group), secondary (n=18/group) and tertiary (n=18/group) follicles in 12 months old bovine growth hormone transgenic (bGH) and normal mice. Data is presented as average ± standard error of the mean.

Diameter (μm)	Normal	bGH	P value
Primordial follicle			
Nucleus	2.6 (±0.1)	3.1 (±0.1)	0.03*
Oocyte	5.7 (±0.3)	5.7 (±0.2)	0.89
Follicle	8.6 (±0.3)	11.4 (±3.3)	0.40
In Transition follicle			
Nucleus	2.9 (±0.1)	3.4 (±0.1)	0.01*
Oocyte	6.2 (±0.2)	6.3 (±0.3)	0.69
Follicle	8.9 (±0.2)	9.4 (±0.3)	0.21
Primary follicle			
Nucleus	3.3 (±0.1)	4.1 (±0.2)	0.009*
Oocyte	8.5 (±0.4)	10.6 (±0.6)	0.007*
Follicle	14.6 (±0.5)	16.4 (±0.7)	0.03*
Secondary follicle			
Nucleus	5.8 (±0.4)	5.5 (±0.3)	0.53
Oocyte	25.2 (±0.8)	22.1 (±0.9)	0.01*
Follicle	54.8 (±3.7)	53.8 (±4.1)	0.85
Tertiary follicle			
Nucleus	6.8 (±0.3)	6.2 (±0.3)	0.17
Oocyte	31.3 (±1.1)	32.6 (±1.0)	0.35
Follicle	172.6 (±4.3)	164.7 (±5.7)	0.28

*Significant P value – P<0.05