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GLP-1: a novel zinc finger protein required in somatic cells of the gonad for germ cell development

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Abstract

Mouse gonadal development is regulated by a variety of transcription factors. Here we report the identification and characterization of a novel nuclear zinc finger protein called GATA like protein-1 (GLP-1), which is expressed at high levels in the somatic cells of the developing gonads, including Leydig cells in the testes and granulosa cells in the ovaries. Biochemical analysis of GLP-1 shows that it acts as a transcriptional repressor of GATA factor function. To determine the necessity of GLP-1 in gonadal development, a null allele in mice was generated by replacing all of the coding exons with the bacterial lacZ gene. GLP-1^{lacZ} null mice are viable with no detectable defects in visceral organ development; however, both males and females are completely infertile. Loss of GLP-1 leads to defective sperm development in males with a marked reduction in mature spermatids observed as early as postnatal week 1. In females, loss of GLP-1 leads to a severe block in germ cell development as early as E17.5. Together, these data identify GLP-1 as a critical nuclear repressor in somatic cells of the gonad that is required for germ cell development, and highlight the importance of somatic-germ cell interactions in the regulation of this critical process.

Keywords

zinc finger; reproduction; gonad development; germ cell; ovary; testes

INTRODUCTION

Mammalian reproduction involves the coordinated development of several important cell types in various tissues, including somatic and germ cells in the gonads, gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus, and gonadotrophs in the anterior pituitary. In mammals, the gonad originates from the urogenital ridge identically in males and females. This stage of development is called the sexually indifferent phase, and specification of the male gonad is regulated in large part by the action of the transcription factor Sry, which is expressed in somatic cells of the early gonad. Lack of Sry expression leads to the formation of ovaries instead of testes (Berta et al., 1990). Two major cell lineages are found in the somatic cells of the gonad, the supporting cells, which surround and help mature the germ cells, and the steroidogenic cells, which produce the hormones necessary for sexual development. In the testes, Sertoli cells comprise the supporting cell lineage and Leydig cells represent the steroidogenic lineage. In contrast, these terms are less well-defined in the ovary, where theca

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cells are considered part of the steroidogenic lineage, but granulosa cells can function as both supportive and steroidogenic cells (Havelock et al., 2004).

Interestingly, several examples of critical paracrine signaling pathways between somatic and germ cells exist (reviewed in (Braw-Tal, 2002; Gilchrist et al., 2004). For instance, granulosa cells in the developing ovary express the c-Kit ligand, which is essential for oocyte development (Matzuk et al., 2002). Conversely, oocyte expressed factors including GDF-9 and BMP-15 are critical for granulosa cell growth and development. In the male, germ cells appear to regulate the rate of testicular development (Franca et al., 1998), while ligands for the tyrosine kinase receptor family tyro-3/axel/mer are expressed in Leydig cells during development and are required for proper sperm development (Lu et al., 1999). Thus, both direct and paracrine signaling between somatic and germ cells are required for proper germ cell development and fertility in mammals.

Several transcription factors are required for gonad development and of these GATA factors are some of the most critical. GATA1, GATA4 and GATA6 are all expressed in the developing ovary and testes along with the critical GATA co-factors FOG1 and FOG2 (Anttonen et al., 2003; Ito et al., 1993; Ketola et al., 2003; Laitinen et al., 2000; Yomogida et al., 1994). The regulation of GATA factor activity is crucial to proper gonad development as evidenced by the severe defects in sex determination and proper gonad differentiation in GATA4 mutants that do not interact with the important co-factor FOG2 (Tevosian et al., 2002). Thus, precise control over GATA activity is regulated by proteins co-expressed with GATA factors in the developing gonad.

Using an *in silico* approach, we have identified a novel zinc finger protein called GATA like protein-1 (GLP-1) that is critical for gonad development in the mouse. GLP-1 is a nuclear protein containing two zinc fingers, one of which is highly homologous to the conserved GATA zinc finger DNA binding motif. Forced expression of GLP-1 represses GATA activation of target promoters. GLP-1 is expressed primarily in Leydig cells in the testes and granulosa cells in the ovary. GLP-1^{lacZ} null mice are completely infertile. Females show a marked reduction in oocyte development as early as E17.5, resulting in a complete loss of postnatal oocytes. Similarly, GLP-1^{lacZ} null male mice exhibit severe sperm differentiation defects by one week after birth, resulting in decreased numbers of mature spermatids. These data identify GLP-1 as a novel zinc finger repressor of GATA transcription factors that regulates important somatic-germ cell interactions in the developing gonad.

MATERIALS AND METHODS

Cloning of mouse GLP-1 cDNAs

The NCBI database was screened using the TBLASTN protocol with the peptide sequence of the mouse GATA6 zinc finger motif (aa232-321). An expressed sequence tag (EST) clone (GenBank accession number BF163948), containing a GATA-like zinc finger motif, was obtained from a commercial source (Invitrogen, Inc.). 5’ rapid amplification of cDNA ends (5’ RACE) was used to generate the full coding sequence of mouse GLP-1 from testes cDNA (Clonetech, Inc.). The GenBank accession number of the cDNA sequence of full-length mouse GLP-1 is DQ286956. The human GLP-1 cDNA sequence is based on the EST clone BC044225. Alignment of GLP-1 to other GATA factor proteins was performed using the ClustalW program and MacVector software (Accelrys, Inc.).

Transcription reporter assays

GLP-1/GAL4 fusion constructs were generated in the pGAL4 vector using PCR to clone the indicated regions of GLP-1 into the pGAL4 plasmid as previously described (Morrisey et al.,

1997). The GAL4 luciferase reporter vector pG5.luc was obtained from Promega. NIH-3T3 cells were transfected using Fugene 6 as previously described (Weidenfeld et al., 2002) with 0.5 μg pG5.luc and 2.0 μg of the pGAL4/GLP-1 expression plasmids. The pSP-A.luc reporter plasmid and pCMVGATA6 expression plasmids have been previously described (Rath et al., 2005; Weidenfeld et al., 2002). A *Renilla* luciferase reporter was used as a transfection control (Promega). Luciferase assays were performed 48 hours after transfection using a commercially available kit (Promega). Western blots were performed on cell extracts from these assays using a GATA6 antibody (Santa Cruz Biotech, C-20) or an antibody against the myc epitope to detect GLP-1.

Generation of GLP-1^{lacZ} null mice

To generate a null mutation in GLP-1 a targeting vector designed to replace all five exons that encode the mouse GLP-1 protein with the bacterial β-galactosidase cDNA and the neomycin resistance cassette, was generated in the pPNT vector (Tybulewicz et al., 1991). This construct was electroporated into the R1 ES cell line and targeted clones were identified by Southern blot. Positive ES cells clones were injected into E3.5 mouse blastocysts to generate chimeric mice. Chimeric offspring were mated to C57BL/6J mice to generate germline transmission of the mutant allele. GLP-1^{lacZ} heterozygous null mice were intercrossed and embryos were collected from timed matings. Noon of the day that the vaginal plug was detected was considered E0.5. Aged matched wild-type littermates were utilized for controls. Routine genotyping of embryos and adult mice was performed using PCR and the following oligonucleotides: forward wild-type sequence: 5’- GGTGGTTTCTCATACCCTACTCC-3’, forward neomycin selection cassette sequence 5’-CATTCCTCCCACTCATGATCTATAG-3’, shared reverse wild-type sequence 5’-CCGCACACAGAAACACACACAAAT GG-3’. Gender of embryos was determined by PCR amplification of the Sry gene product using the following oligonucleotides: forward 5’-CTGGTGACAATTGTCTAGAGAGC-3’, reverse 5’-CAGAAGGTTGTACAGTTTTGTTGAGGC-3’. The neomycin cassette was removed by crossing the GLP-1^{lacZ} heterozygous null mice into the CMV-cre line (Schwenk et al., 1995).

Determination of fertility

GLP-1^{lacZ} mice of various genotypes were mated to each other starting at 6 weeks and monitored over a two-year period and the results are shown in Table 2. Other matings outside of these specific studies were also performed. In all instances, matings of GLP-1^{lacZ} null mice never resulted pregnancies. In additional experiments, GLP-1^{lacZ} null and heterozygous females were superovulated by injection of 5 units of pregnant mare serum gonadotropin followed by injection of human chorionic gonadotropin 48 hours later. Egg collection was performed 24 hours after human chorionic gonadotropin injection. No eggs were ever recovered from GLP-1^{lacZ} null females.

Histology and immunocytochemistry

Embryos and tissues were fixed in 4% paraformaldehyde at 4 ^{o}C with shaking for 24 to 48 hours. Samples were then dehydrated in increasing concentrations of ethanol and subsequently embedded in paraffin. *In situ* hybridization was performed using antisense riboprobes previously described for luteinizing hormone receptor (LHR), SF-1, p450scc, Sox9, and Wnt4 (Akiyama et al., 2004; Bielinska et al., 2003; Ingraham et al., 1994; Koopman et al., 1991; Tevosian et al., 2002; Vainio et al., 1999; Yao et al., 2002). *In situ* hybridization probe for follicle stimulating hormone (FSH) was generated using mRNA from mouse ovaries and the following oligonucleotides: sense: 5'-AGTTACGTGCTTGTCCCTCTAAA-3', antisense with T7: 5'-TGTAATACGACTCACTATAGGGCGACAGCAGTGTTGCACTATTTTAAT-3'. Immunohistochemistry using the mouse vasa homologue (MVH) and PF20 antibodies was

performed as previously described (Toyooka et al., 2000; Zhang et al., 2004). TUNEL assays were performed on tissue sections from 2 month old wild-type and GLP-1^{lacZ} null testes using a previously described protocol (Morrisey et al., 1998). Further details on histological procedures can be found at the University of Pennsylvania Molecular Cardiology web site http://www.uphs.upenn.edu/mcrc.

Immunocytochemistry for GLP-1 was performed using a full-length myc-tagged GLP-1 cDNA cloned into the pCMVTag3B vector (Stratagene). NIH-3T3 cells were transfected and stained for GLP-1 expression using the anti-myc epitope antibody (9E10) as previously described (Morrisey et al., 1997). Cells were counter-stained with DAPI.

Electron microscopy

Testes from 6 month old wild-type and GLP-1^{lacZ} null mice were fixed in 2% gluteraldehyde with 0.1 M sodium cacodylate pH 7.4 for 72 hours at 4^0C. Samples were further incubated with 2% osmium tetroxide and 0.1 M sodium cacodylate pH 7.4 for 1 hour at 4^0C. Ultrathin sections were stained with lead citrate and uranyl acetate and viewed on a JEM 1010 microscope. Digital images were captured on a Hamamatsu HamC4742-95-12 CCD camera using AMT Advantage software.

Semi-quantitative RT-PCR

Total RNA was extracted from wild-type and GLP-1^{lacZ} null testes at 4 months and cDNA was generated using a commercially available kit (Promega). Equal amount of cDNA was amplified for 30 cycles using the following oligonucleotides: GLP-1 forward 5'-ATTGTACCCACCTACAGCTTGCCT-3', reverse 5'-TTGTACCTGATGCCACAGGCATTG-3'; steroidogenic acute regulatory protein (StAR) forward 5'- TGG GAG AGT GGA ACC CAA ATG TCA, reverse 3'-CTTCAGGTCAATACTGAGCAGCCA; aromatase forward 5'- ATC ATG GTC CCG GAA ACT GTG ACT-3', reverse 5'- AATCGGGAGATGTAGTGACTGTGC-3'; Sox9 forward 5'-CTAATGCTATCTTCAAGGCGCTGC-3', reverse 5'-CAAGTATTGGTCAAACTCATTGAC-3'; GAPDH forward 5'-ACCACAGTCCATGCCATCAC-3', reverse 5'-TCCACCACCCTGTTGCTGTA-3'. Amplified products were visualized on a 1.5% agarose gel.

RESULTS

Identification and characterization of GLP-1

To identify and characterize novel transcriptional regulators containing GATA zinc finger motifs, the GenBank EST database was screened using the mouse GATA6 zinc finger peptide sequence. One EST was identified and used to construct a full-length cDNA by 5' RACE, which we call GATA like protein 1 (GLP-1). The protein encoded by the full length GLP-1 cDNA contains two zinc fingers with the N-terminal finger having high level homology to other GATA zinc fingers (Fig. 1A and B) **(Lowry and Atchley, 2000)**. Importantly, most of the conserved amino acids in GATA factor zinc fingers are present in this finger of GLP-1 (Fig. 1B). However, the C-terminal zinc finger of GLP-1 does not have a classic GATA finger structure (Fig. 1A).

Although similar to GATA zinc fingers, the N-terminal finger region of GLP-1 shows distinct differences to other GATA factors. Besides the zinc finger, an extended basic amino acid rich region adjacent to the C-terminal zinc finger is required for GATA factor DNA binding **(Blobel et al., 1995; Merika and Orkin, 1993)**. GLP-1 contains only a few basic amino acids adjacent to its N-terminal zinc finger (Fig. 1A). This region is then followed by the carboxy-terminal zinc finger of GLP-1, which is different than those found in the GATA family. As predicted

from these differences, gel shift analysis shows that GLP-1 does not bind to the classic 5'-WGATAR-3' GATA factor binding motif (data not shown). Furthermore, using PCR amplification of random oligonucleotides bound to GLP-1 tagged protein, we were unable to detect high affinity sequence specific DNA binding by GLP-1 (data not shown).

To test whether GLP-1 was a nuclear protein, NIH-3T3 cells were transfected with an expression plasmid encoding a myc-tagged GLP-1 protein. Immunocytochemistry demonstrates exclusive nuclear localization of GLP-1 in these cells (Fig. 1C). Nuclear localization of GLP-1 suggested that it regulates gene transcription in spite of the fact that we were unable to demonstrate sequence specific DNA binding. Next, we tested whether GLP-1 possessed transcriptional activity and could affect expression of GATA target genes. Co-transfection assays showed that GLP-1 repressed GATA6 dependent activation of the SP-A promoter, a known target of GATA6 in lung epithelium (Bruno et al., 2000), in a dose dependent manner (Fig. 1D). No significant repression was observed in the absence of GATA6. These data suggested that GLP-1 acted as a transcriptional repressor. To identify the domains within GLP-1 that conferred transcriptional repression, deletions of GLP-1 were fused to the yeast GAL4 heterologous DNA binding domain. As expected from the co-transfection assays above, full-length GLP-1 repressed gene transcription when fused to the GAL4 DNA binding domain (Fig. 1E). Deletion analysis shows that this repression function is localized to the two zinc fingers present in GLP-1 (Fig. 1E). A truncated GLP-1 lacking the zinc fingers also lacks the ability to repress GATA6 trans-activation (Fig. 1F). These data are consistent with GLP-1 acting as a transcriptional repressor that is able to represses GATA transcription factor function.

Expression of GLP-1 and generation of GLP-1^{lacZ} null mice

Northern blot analysis showed that GLP-1 was expressed exclusively in the adult testes in the mouse (Fig. 2A). *In situ* hybridization was performed to further determine the cell-type specific expression pattern of GLP-1 in the mouse gonads. Hybridization using the GLP-1 cRNA probes revealed GLP-1 mRNA expression was restricted to the Leydig cells of the testes and granulosa cells in the ovaries (Fig. 2B and C).

Given the expression pattern of GLP-1 and the known importance of GATA factor zinc finger proteins in gonadal development (LaVoie, 2003; Lei and Heckert, 2004; Tremblay and Viger, 2001), we generated a GLP-1^{lacZ} null mouse line to determine its role in gonadal development. The targeting construct was designed to replace all five coding exons of GLP-1 with the bacterial β-galactosidase cDNA and the neomycin selection cassette (Fig. 2F). Southern blot and PCR analysis demonstrated correct targeting of the mouse GLP-1 allele and loss of GLP-1 expression (Fig. 2G–I). LacZ expression was used to better define GLP-1 expression. LacZ staining of adult mouse ovaries and testes confirmed the *in situ* hybridization results, revealing expression almost exclusively in the Leydig cells of the testes and granulosa cells of the ovaries (Fig. 2D and E). Expression of GLP-1 was not observed in male or female germ cells either by RT-PCR or by lacZ expression from the GLP-1^{lacZ} allele (data not shown). Moreover, expression of GLP-1 was not observed in any other tissue during development by either *in situ* hybridization of lacZ staining of GLP-1^{lacZ} heterozygous embryos (data not shown).

Defects in male gonad development in GLP-1^{lacZ} null mice

GLP-1^{lacZ} null mice appeared grossly normal and grew at the same rate as wild-type littermates. However, both male and female GLP-1^{lacZ} null mice were infertile at all ages tested up to two years of age (Table 1). This infertility was not overcome by super-ovulation of GLP-1^{lacZ} null females. Histological examination and testes to body weight ratios show that as early as postnatal day 30, the testes are smaller in GLP-1^{lacZ} null mice when compared to wild-type or heterozygous mice (Table 2). At birth, testes from the wild type and GLP-1^{lacz} null mice look relatively similar. In contrast, at one and six months of age, very few mature sperm were found

in the testes GLP-1^{lacZ} relative to wild-type mice (Fig. 3A–F). Transmission electron microscopy (TEM) revealed a lack of mature spermatids in GLP-1^{lacZ} null testes at six months (Fig. 3G and H). The dearth of mature sperm in GLP-1^{lacZ} null mice suggested that there was an apoptotic-mediated loss of early spermatocytes. TUNEL assays show that there is a significant increase in apoptosis in GLP-1^{lacZ} null testes, primarily in the outer layers of the seminiferous tubules where early spermatocytes are differentiating (Fig. 3I and J). These data suggest that GLP-1 may regulate a maintenance signal that is generated in Leydig cells and required for spermatocyte survival and maturation.

The germ cell markers mouse vasa homologue (MVH) and PF20 and the Sertoli cell marker gene Sox9 were used to further assess male germ cell and Sertoli cell development in GLP-1^{lacZ} null mice. Expression of both MVH and PF20 was significantly down-regulated, but not absent, in GLP-1^{lacZ} null mice, reflecting a loss of male germ cells (Fig. 4). Interestingly, expression of Sox9 is slightly increased in GLP-1^{lacZ} null mice at 6 months (Fig. 4), indicating that, although the testes contain fewer germ cells, Sertoli cell numbers are not reduced.

Since GLP-1 is expressed nearly exclusively in Leydig cells in the testes, *in situ* hybridization was performed to assess the impact of loss of GLP-1 on Leydig cell development. Expression of the luteinizing hormone receptor (LHR) and side chain cleavage enzyme (p450scc) were not affected in GLP-1^{lacZ} null testes (Fig 5A–D), indicating that Leydig cell numbers were not reduced. However, SF-1 was upregulated in Leydig cells of GLP-1^{lacZ} null mice, suggesting that GLP-1 might be a repressor of SF-1 gene expression (Fig. 5E and F). RT-PCR was performed to assess the expression levels of potential GATA factor targets in the developing testes. Steroidogenic acute regulatory protein (StAR), aromatase, Sox9, and SF1 have all been im harbplicated as direct targets of GATA factors (LaVoie, 2003;Tremblay and Viger, 2001). Given that GLP-1 represses GATA factor function, expression of some of these factors may be up-regulated. Although StAR and aromatase are down-regulated, Sox9 and SF1 are up-regulated in GLP-1^{lacZ} null testes (Fig. 5I). These data suggest that GLP-1 regulates GATA target genes in a complex manner similar to Friend of GATA (FOG) factors, which act as co-activators or co-repressors in a context dependent manner (Letting et al., 2004;Pal et al., 2004).

Defects in female gonad development in GLP-1^{lacZ} null mice

Since female GLP-1^{lacZ} null mice were also infertile, we next examined ovarian development in these animals. In the ovaries of wild-type mice, multiple primordial follicles with oocytes are seen at E17.5 and at birth (Fig. 6A, C, and E), with more developed follicles throughout the ovary at one and 6 months (Fig. 6G and I). In contrast, GLP-1^{lacZ} null ovaries contain few, but not zero, primordial follicles and oocytes at E17.5 (Fig. 6B and D), and essentially none from birth through 6 months (Fig. 6F, H, and J). Accordingly, MVH staining revealed significantly fewer germ cells in the GLP-1^{lacZ} null ovaries at E17.5 relative to wild-type mice (Fig. 6K and L), with virtually no specific germ cell staining at 6 months in the null ovary (Fig. 6M and N). Together, these data indicate a severe deficiency in oocyte development and/or survival in GLP-1^{lacZ} null mice.

Notably, GLP-1^{lacZ} null ovaries were considerably smaller than those from wild-type mice at 1 month, perhaps in part due to the lack of mature follicles (Fig. 6G and H). However, by six months, somatic cells had repopulated the ovaries of GLP-1^{lacZ} null mice such that their size was similar to wild-type (Fig. 6I and J).

Since GLP-1 is expressed in granulosa cells within the ovaries, somatic cell development in GLP-1^{lacZ} null females was assessed. *In situ* hybridization for the follicle stimulating hormone receptor (FSHR), a marker for granulosa cells, revealed low and diffuse expression in the

GLP-1^{lacZ} null relative to wild-type ovaries (Fig. 7A and B). Wnt4 mRNA, which is detected primarily in granulosa cell in wild-type mice (Fig. 7G), is similarly expressed diffusely in the GLP-1^{lacZ} null ovaries, although levels are somewhat higher than those of the FSHR (Fig. 7G and H). Finally, p450scc and SF-1, which are detected primarily, but not exclusively, in the theca cells of wild-type mice, are expressed at similar relative levels in the GLP-1^{lacZ} null ovaries, though again in a much more diffuse pattern (Fig. 7C–F). Together, these data indicate that loss of GLP-1 results in a severe organizational changes in the ovary, but that the primary components for sex steroid production, including granulosa and theca cells, still appear to be present.

Hormone levels in GLP-1^{lacZ} null mice

If the GLP-1^{lacZ} null ovaries do indeed contain both theca and granulosa cells, then they should be capable of synthesizing estradiol, a steroid that requires both CYP17 and CYP19 (aromatase) activity in the theca and granulosa cells, respectively (Havelock et al., 2004). Accordingly, serum estradiol levels in wild-type and GLP-1^{lacZ} mice were identical at one and six months of age (Fig. 8A). Interestingly, serum LH and FSH levels were considerably higher in the GLP-1^{lacZ} null mice relative to wild-type littermates (Fig. 8B and C), suggesting that, although considerable sex steroid production in occurring in the null mouse ovary, the overall amounts of estradiol being produced may still be insufficient to completely suppress gonadotropin secretion by gonadotrophs.

DISCUSSION

Cross talk between somatic and germ cells is required for normal gonadal development in both males and females, with the germ cells likely orchestrating the timing of this maturation process (Franca et al., 1998; Matzuk et al., 2002). In the ovary, cross-talk is regulated in part by growth factors that are secreted from one cell type and signal through receptors expressed elsewhere. For example, oocytes secrete the TGF-β family member GDF-9, which then signals through receptors expressed in surrounding granulosa cells, while granulosa cells secrete KIT ligand, which signals through receptors expressed in germ cells (Matzuk et al., 2002). Together, these signals regulate the timing and progression of follicular growth from primordial to pre-ovulatory follicles. Although less in known about the secreted factors that regulate cross talk between germ cells and sertoli cells in the testes, germ cells appear to similarly control the timing of the spermatogenesis program (Franca et al., 1998).

In addition to soluble growth factors, folliculogenesis also appears to be regulated at the levels of transcription. For example, the oocyte-specific helix-loop-helix transcription factor FIGα is critical for primordial follicle formation (Soyal et al., 2000). Ovaries from mice lacking FIGα contain large numbers of oocytes just before birth; however, they are not organized into primordial follicles, and are completely resorbed within days after birth (Soyal et al., 2000). Wnt4, which is expressed primarily in granulosa but also found in theca cells (Vainio et al., 1999), is also critical for normal oocyte development, as Wnt4 null mice have an even more severe deficiency in oocyte development, with a 90% reduction in total oocytes by birth (Vainio et al., 1999). Finally, in granulosa cells, GATA-4 and GATA-6 may play important roles in maintaining normal interactions between somatic and germ cells, thereby regulating follicle development (Heikinheimo et al., 1997; Vaskivuo et al., 2001).

We show here that a newly identified gene, GLP-1, which contains a GATA-like zinc finger, is expressed in ovarian granulosa cells as well as testicular Leydig cells and is required for normal germ cell development in both males an females. GLP-1 represses GATA factor mediated transcription, and therefore may be acting in concert with other GATA proteins as part of a transcriptional cascade to regulate granulosa and Leydig cell development, as well as subsequent direct and paracrine signaling between somatic and germ cells. Although GLP-1

repression through GATA factors suggests a direct protein-protein interaction, we have not been able to verify such interactions. GLP-1 may interact directly with GATA factors but our assays are not sufficiently sensitive to detect such interactions or GLP-1 may interact with other factors including FOG co-factors, thus forming a terinary complex with GATA. Future studies will be necessary to determine which if any of these possibilities occur.

Both GATA4 and GATA6 are expressed in Leydig and granulosa cells during gonadal development (Anttonen et al., 2003; Ketola et al., 1999; Ketola et al., 2003; Lavoie et al., 2004). Furthermore, GATA4 has been shown to activate the SF-1 promoter (Tremblay and Viger, 2001) and GATA factors are known to physically interact and transcriptionally cooperate with SF-1 in the regulation of the Mullerian inhibiting substance hormone promoter (Tremblay and Viger, 2003). The increased SF-1 expression in Leydig cells in GLP-1^{lacZ} null mice might therefore be due to a loss of GLP-1-mediated repression of GATA4 mediated transcription.

While both male and female gonadal germ cell development is reduced in the GLP-1^{lacZ} null mice, the phenotypes of these two gonads are markedly different. In males, GLP-1 is expressed almost exclusively in the steroid-producing Leydig cell rather than the spermatogenesis-supporting Sertoli cells. While spermatogenesis is significantly reduced in these mice, small numbers of new spermatids are still being generated in adult mice, suggesting that germ cell development is reduced but not completely arrested. One possibility is that these mice have lower androgen production from the Leydig cells, resulting in reduced spermatogenesis. Both SF-1 and GATA-6 are known to regulate androgen production in steroidogenic tissues (Jimenez et al., 2003). However, the lack of significant differences in testosterone levels in GLP-1^{lacZ} null relative to wild-type mice (data not shown) argues that other mechanisms are likely involved.

In the ovary, GLP-1 is expressed almost exclusively in granulosa cells, which produce steroids as well as interact with and support germ cells. In GLP-1^{lacZ} null females, the germ cell phenotype is significantly more severe than in the males. While germ cells appear to be present in E17.5 embryos, albeit at severely reduced levels, oocytes are completely resorbed shortly after birth. This phenotype resembles a more severe form of the phenotype noted FIGα null mice (Soyal et al., 2000), and suggest that GLP-1 is acting very early to regulate primordial follicle formation. However, in contrast to FIGα, GLP-1 is not expressed in germ cells. Thus, GLP-1 must be playing a critical role in the somatic-germ cell interactions that regulate normal germ cell development.

Interestingly, while the two-week ovaries of the GLP-1 null mice are significantly smaller than those of the wild-type mice, the ovary rapidly grows such that the sizes are relatively equal by 8 weeks after birth. Although the exact nature of the remaining cells is still unclear, they appear to retain some ability to produce sex steroids, suggesting that both granulosa and theca cells must still be present, albeit in disorganized state. Notably, these follicle-free ovaries physically resemble tubular adenomas that have been described in other mouse models (Blaakaer et al., 1995; Ishimura et al., 1986). Furthermore, evidence suggests that GATA4 may be involved in protecting granulosa cells from apoptosis (Vaskivuo et al., 2001), and elevated GATA4 and GATA6 expression has been associated with granulosa cell tumors (Anttonen et al., 2005; Laitinen et al., 2000). Together these observations bring forth the intriguing possibility that loss of the GATA inhibitory factor GLP-1 leads to increased GATA4 and/or GATA6 activity, promoting changes in the granulosa cells resulting in unregulated growth.

Since GLP-1 is expressed in somatic granulosa rather than germ cells, how is it regulating oocyte development? One possibility is that GLP-1^{lacZ} null mice may have reduced sex steroid production. Two facts argue against such a simplistic mechanism. First, GLP-1^{lacZ} null female

mice have similar levels of estradiol relative to age-matched matched wild-type mice, although the higher gonadotropin levels in the null mice would argue that either sex steroid production is still insufficient over the course of the estrus cycle or that GLP-1 is having unrecognized effects in gonadotrophs. Second, the ovaries from female mice lacking estrogen or androgen receptors still contain many follicle-enclosed oocytes suggesting that intraovarian signaling is not necessary for follicle formation (Emmen and Korach, 2003; Hu et al., 2004; Shiina et al., 2006). Another possibility is that as mentioned above, GLP-1 may be an important transcriptional inhibitor that regulates normal granulosa cell growth and development. Loss of GLP-1 might therefore lead to abnormal granulosa function, which in turn would affect germ cell development. Finally, GLP-1 might mediate the production of granulosa cell factors that are involved in the signaling between somatic and germ cells that regulates germ cell development and/or survival. Future experiments to determine the down-stream targets of GLP-1 in Leydig and granulosa cells, as well as the nuclear complexes that interact with GLP-1, will help decipher critical pathways regulating germ cell development in both males and females.

In summary, we have uncovered a critical somatic cell transcriptional repressor that is essential for normal germ cell development in both the male and female gonads. Although the mechanisms by which GLP-1 is regulating germ cell development are still being investigated, the striking germ cell phenotype, especially in the ovary, underscores the importance of normal somatic cell growth and function for germ cell development.

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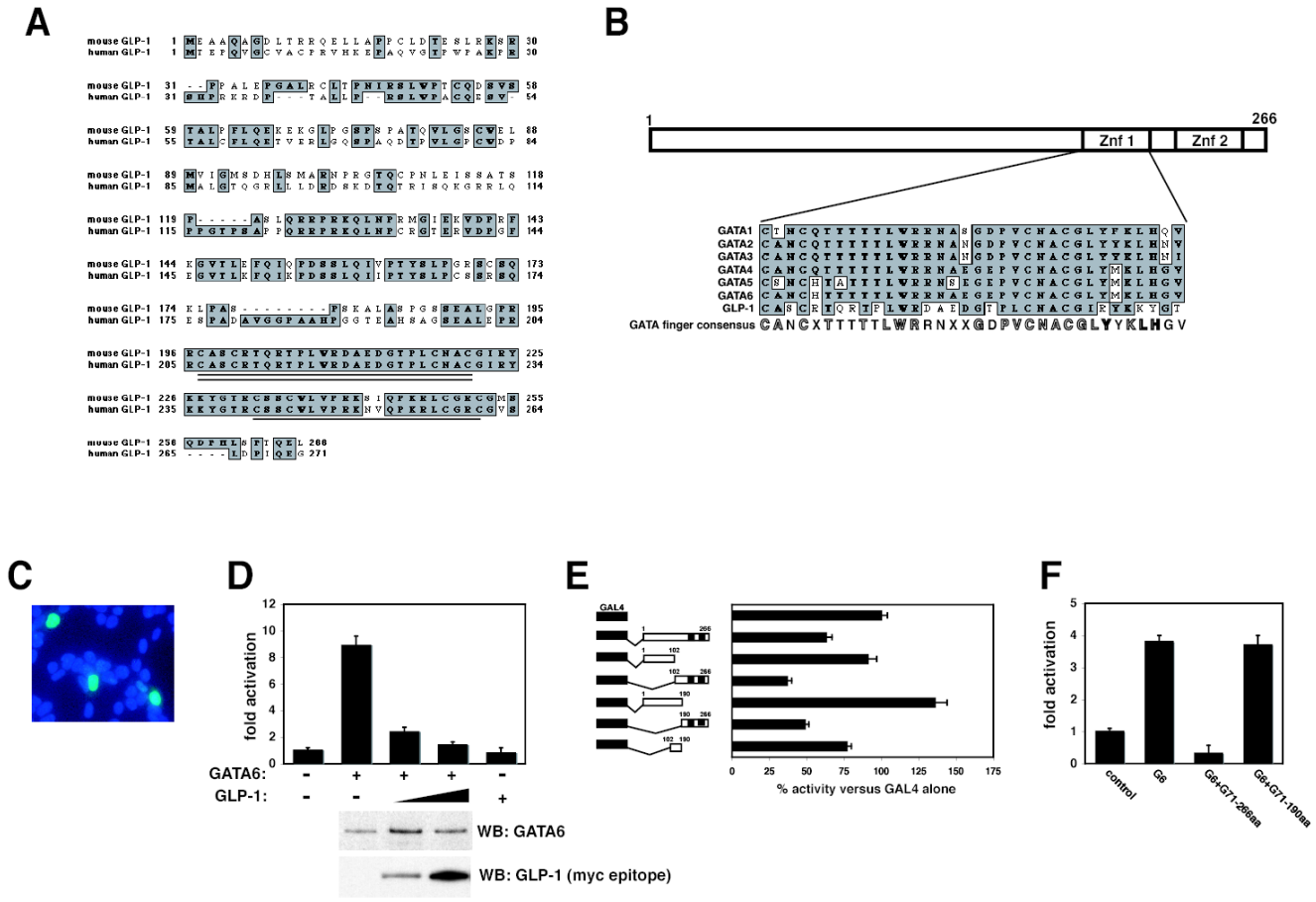

Figure 1. Identification of GLP-1 as a nuclear transcriptional repressor.

(A) Alignment of mouse and human GLP-1 protein sequences showing high-level conservation in the zinc finger region. The GATA like amino-terminal zinc finger is double underlined while the carboxy-terminal zinc finger has a single underline. (B) Alignment of GATA carboxy-terminal zinc fingers to that of zinc finger 1 (Znf 1) of GLP-1. Amino acid residues conserved in GATA factor zinc fingers are highlighted in the consensus sequence. (C) Immunocytochemistry of transfected NIH-3T3 cells showing nuclear localization of GLP-1. (D) GLP-1 represses GATA6 activation of the SP-A promoter in a dose dependent fashion. Co-transfections of NIH-3T3 cells with the pSP-A.luc vector and 1 μg of GATA6 expression plasmid and increasing amounts of the GLP-1 expression plasmid (1 and 2 μg). GLP-1 expression alone does not appreciably affect the pSP-A.luc vector. Western blots show expression of GATA6 and GLP-1/myc tagged proteins. (E) Expression of GAL4/GLP-1 fusion proteins demonstrate that GLP-1 is a transcriptional repressor and that this repression activity is mediated by the two zinc fingers (black boxes) located at the carboxy-terminal end of the protein. (F) Expression of a truncated GLP-1 (aa1-190) lacking the zinc fingers does not repress GATA6 activation of the SP-A promoter. Transfection data represent the average of three assays performed in duplicate ± S. E. M..

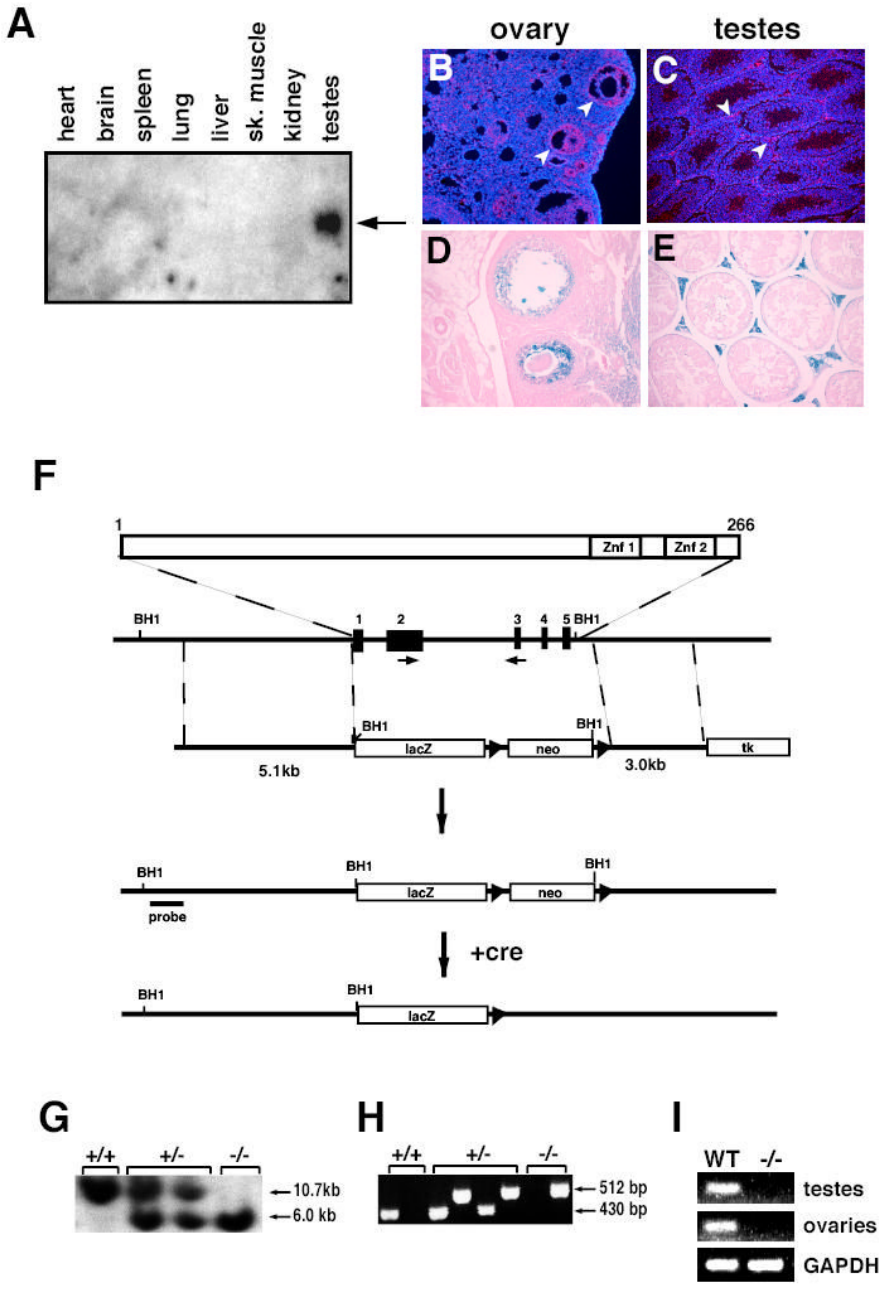

Figure 2. Expression of GLP-1 in mouse gonads and generation of GLP-1^{lacZ} null mice.

Northern blot analysis of GLP-1 expression in adult mice showing the 1.2 kb transcript in the mouse (A, arrow). *In situ* hybridization shows that GLP-1 is expressed in cells surrounding the developing ovarian follicle as well as Leydig cells in the testes at six months of age (B and C). LacZ expression from GLP-1^{lacZ} +/− knock-in mice shows that GLP-1 expression is found in the granulosa cells of the developing ovaries and confirms expression in Leydig cells of the testes at six months (D and E). Schematic of GLP-1^{lacZ} knock-in targeting vector and modified allele (F). The CMV-cre line was used to remove the neomycin cassette (Schwenk et al., 1995). Southern blot analysis of GLP-1^{lacZ} allele using probe indicated in E (G). PCR analysis of genomic DNA used to confirm fidelity of GLP-1^{lacZ} allele using oligos described in

Materials and Methods (H). RT-PCR showing lack of GLP-1 expression in GLP-1^{lacZ} null testes and ovaries using oligonucleotides denoted in F located in exons 2 and 3 (I).

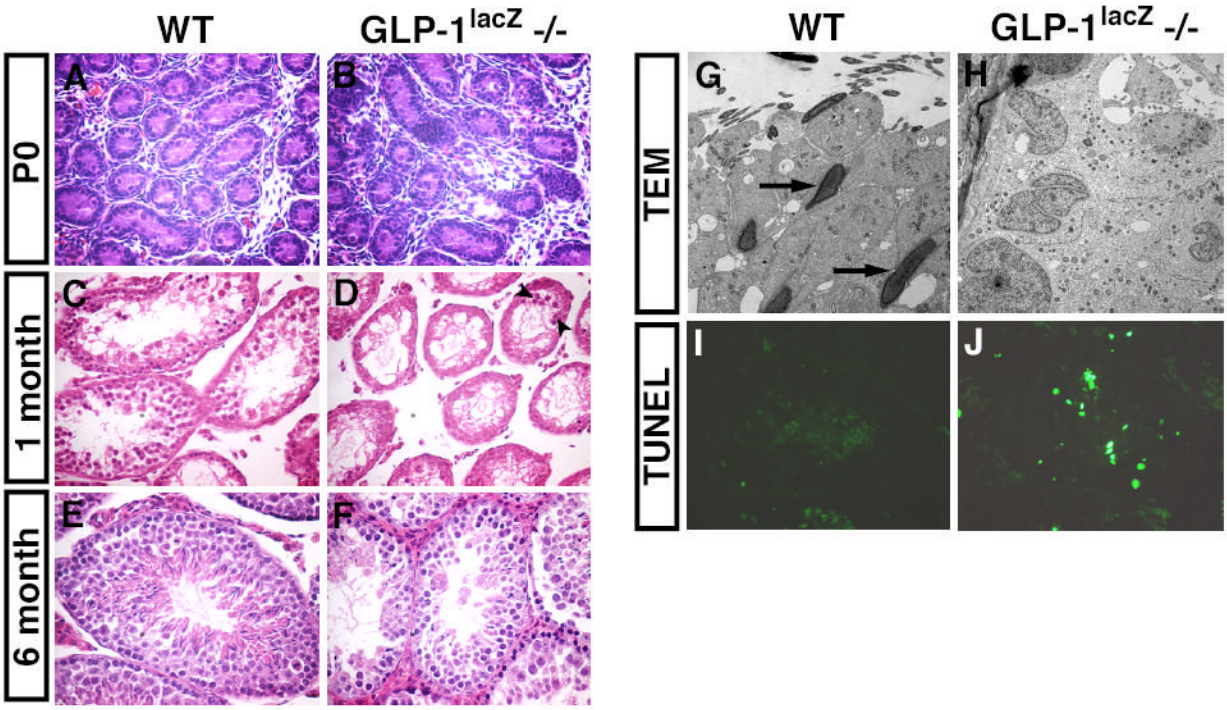

Figure 3. Histological analysis of sperm development in GLP-1^{lacZ} null mice.

H+E staining was performed on histological sections from P0 (A and B), one month (C and D), and six month (E and F) wild-type and GLP-1^{lacZ} null testes. Severe reduction in sperm development is observed as early as one month of age with only a few immature sperm observed in GLP-1^{lacZ} null testes (D, arrowheads). TEM shows that there are few if any mature spermatids in GLP-1^{lacZ} null testes at six months (G and H, arrows). TUNEL assays were performed on testes from two months of age from wild-type and GLP-1^{lacZ} null mice (I and J). Although little apoptosis was observed in wild-type testes at this age (I), significant apoptosis was observed in GLP-1^{lacZ} null testes (J).

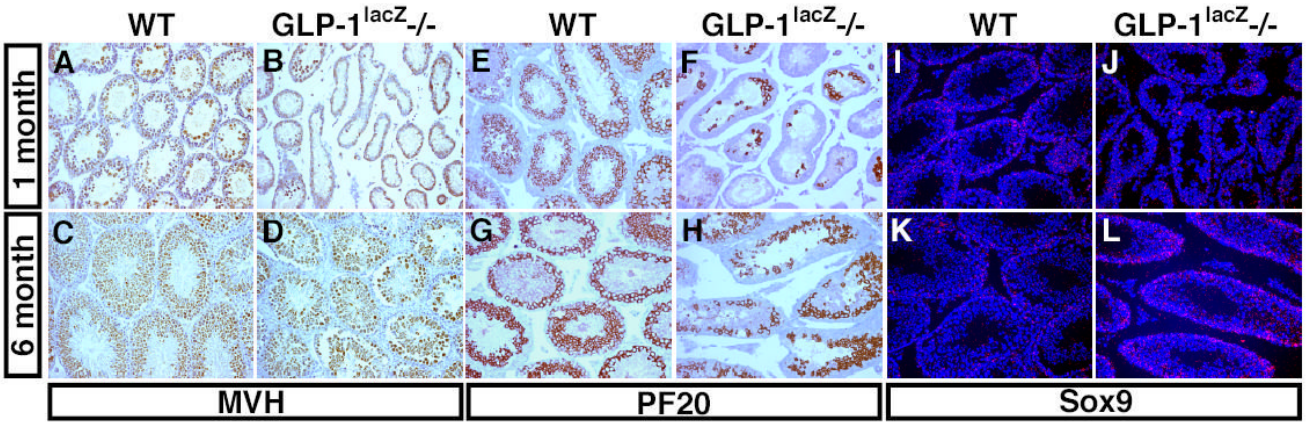

Figure 4. Expression of germ and Sertoli cell markers in GLP-1^{lacZ} null testes.

Immunohistochemistry was performed using antibodies to MVH and PF20, markers of germ cells in the developing testes. At both one and six months of age, GLP-1^{lacZ} null mice show reduced levels of germ cell development (A–H). Expression of the Sertoli cell marker gene Sox9 shows relatively unchanged levels at one month but increased levels at six months of age in GLP-1^{lacZ} null mice (I–L).

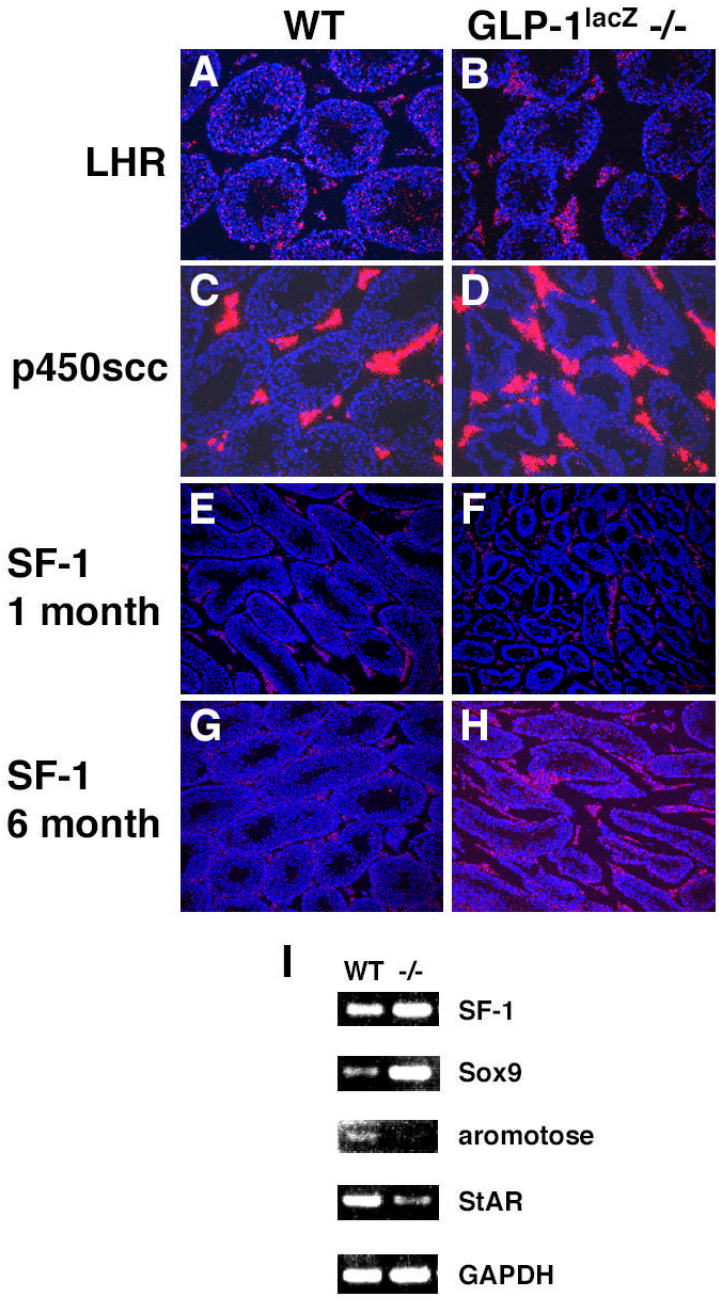

Figure 5. Somatic cell marker gene expression in GLP-1^{lacZ} null testes.

In situ hybridization was performed to assess expression of LHR, p450scc, and SF-1 in wild-type (A, C, E) and GLP-1^{lacZ} null (B, D, F) testes at 6 months. Expression of LHR and p450scc were unchanged in GLP-1^{lacZ} null testes (A–D). However, SF-1 expression was increased in GLP-1^{lacZ} null mice (E and F). RT-PCR was performed to determine relative expression of SF-1, Sox9, aromotose, StAR, and GAPDH in 4 month old GLP-1^{lacZ} null testes (I).

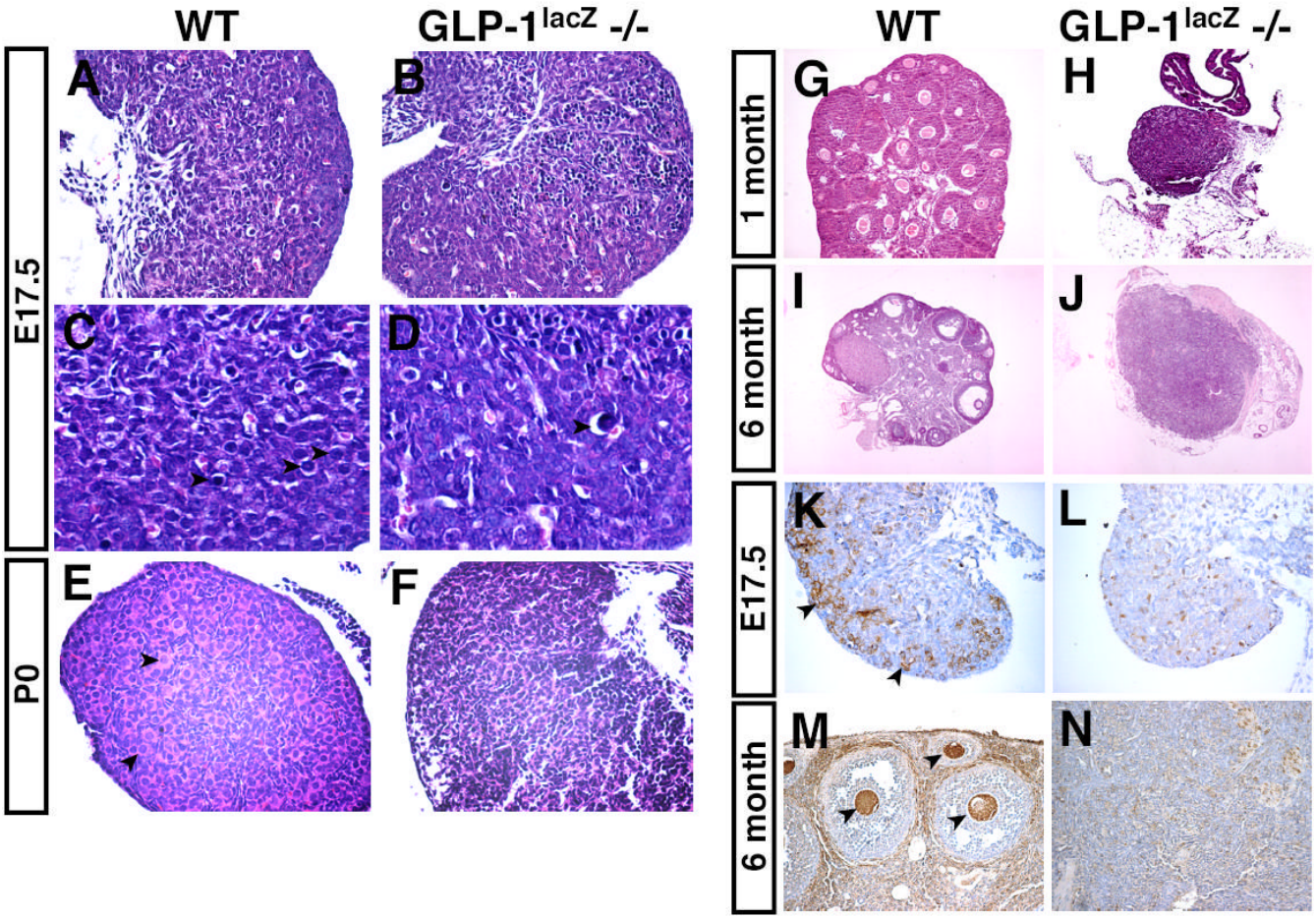

Figure 6. Histological analysis of ovarian development in GLP-1^{lacZ} null mice.

H+E staining of histological sections was performed on E17.5, P0, one month, and six month ovaries from wild-type and GLP-1^{lacZ} null mice. Although GLP-1^{lacZ} null ovaries appear grossly similar to wild-type ovaries (A and B), high magnification images show fewer germ cells in GLP-1^{lacZ} null ovaries (C and D, arrowheads). Similar differences are noticed at P0 (E and F, arrowheads). The ovaries of GLP-1^{lacZ} null postnatal mice were initially much smaller than wild-type but grew to almost normal size by six months, even though they still lacked oocytes (G–J). Immunohistochemsitry with an MVH antibody was used to assess the presence of germ cells in GLP-1^{lacZ} null ovaries (K–N). Wild-type ovaries at both E17.5 and six months contained MVH positive oocytes (K and M, arrowheads). However, a severe reduction of specific MVH staining in GLP-1^{lacZ} null ovaries at E17.5 (L) and complete lack of staining at six months (N) demonstrates that oocyte development is severely compromised.

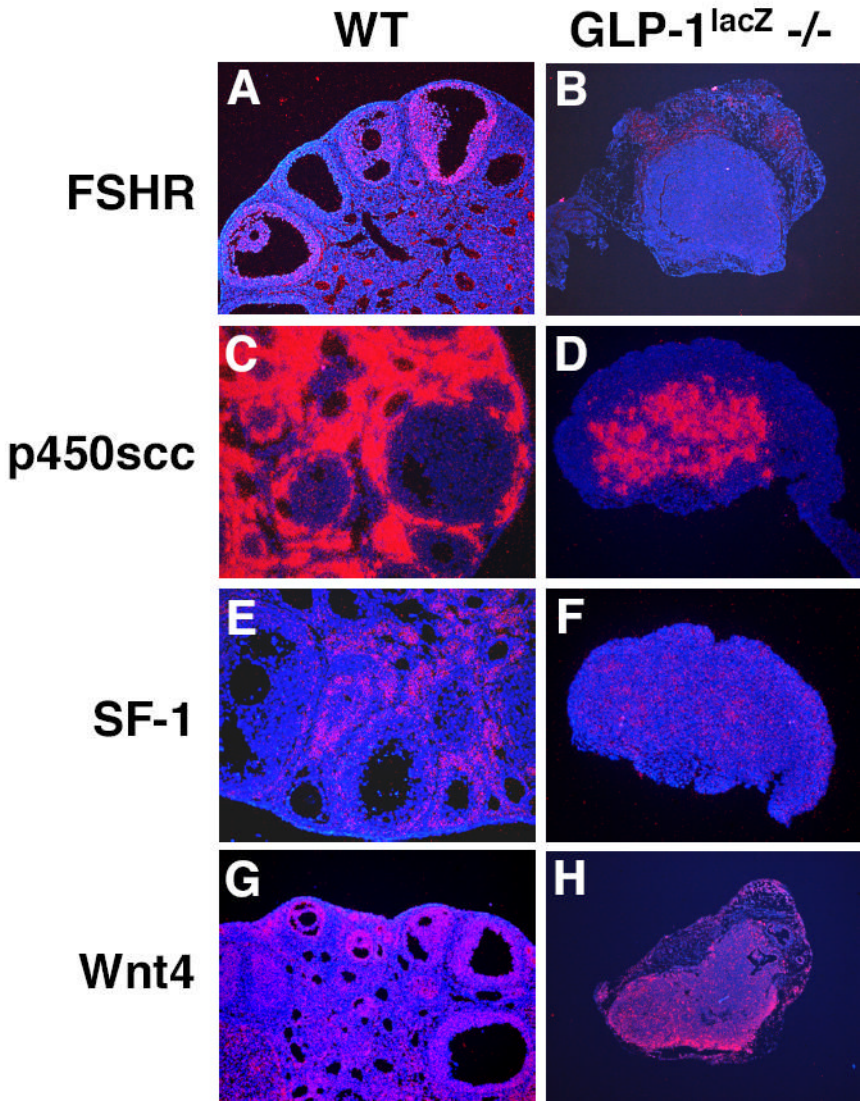

Figure 7. Somatic cell markers in GLP-1^{lacZ} null ovaries.

In situ hybridization was performed on wild-type and GLP-1^{lacZ} null ovaries at six months for FSHR, p450scc, SF-1, and Wnt4. Expression of FSHR and SF-1 was down-regulated in GLP-1^{lacZ} null ovaries (A–F). Expression of p450scc and Wnt4 was present in GLP-1^{lacZ} null ovaries but diffuse due to defective follicular development (C,D,G,H).

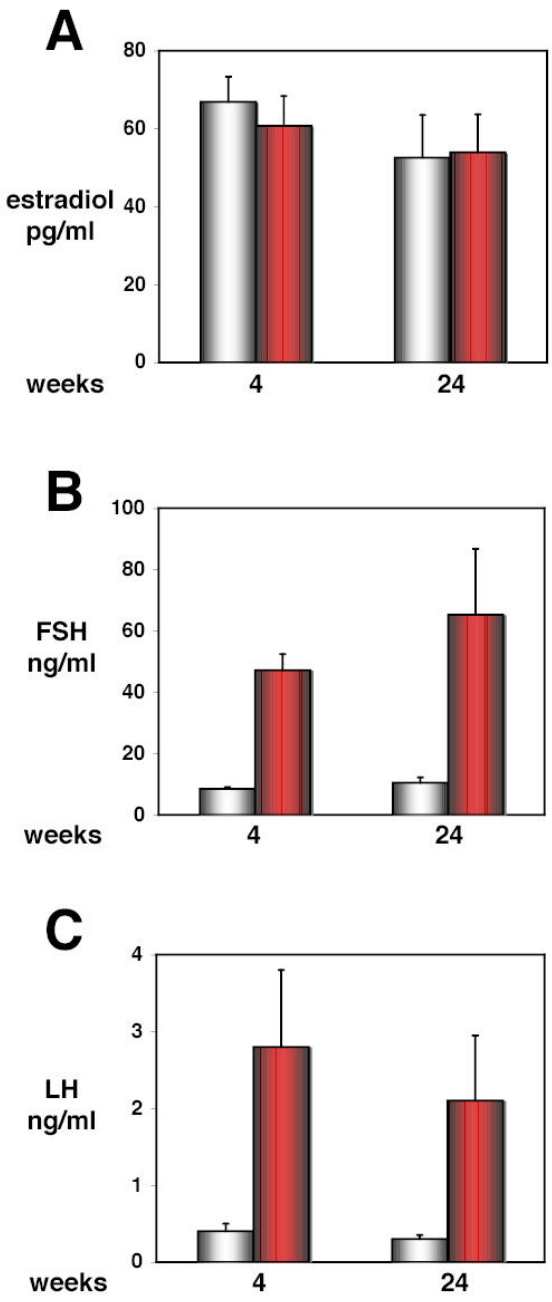

Figure 8. FSH and LH levels in GLP-1 null mice.

Estradiol (A), FSH (B), and LH (C) serum levels were measured in wild-type and GLP-1^{lacZ} null animals at the time points indicated. Estradiol levels were unchanged (A) but levels of FSH and LH were significantly elevated in GLP-1^{lacZ} null animals (B and C). Gray columns:wild-type mice; red columns:GLP-1^{lacZ} null mice.

Table 1

Number of matings that generated offspring over a two year period-reproductive capacity of GLP-1^{lacZ} +/+, +/− and −/− mice

Genotypes	Female+/+	Female+/−	Female−/−
Male+/+	6/6	6/6	0/6
Male+/−	7/7	10/10	0/6
Male−/−	0/6	0/6	0/6

Table 2

Testes to body weight ratio

Age (weeks)	WT	GLP-1 null
2	0.43±0.15	0.32±0.01
5	0.72±0.09	0.38±0.06**
6	0.74±0.09	0.29±0.05**
24	1.00±0.34	0.28±0.15*

* P value < 0.05

** P value < 0.01