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GP130, the shared receptor for the LIF/IL6 cytokine family in the mouse, is not required for early germ cell differentiation, but is required cell-autonomously in oocytes for ovulation

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SUMMARY

GP130 is the shared receptor for members of the IL6 family of cytokines. Members of this family have been shown to enhance the survival of migratory (E10.5) or postmigratory (E12.5) murine primordial germ cells (PGCs) in culture; however, it is uncertain what role these cytokines play during PGC development in vivo. We have examined PGC numbers in E13.5 GP130-deficient mouse embryos and found that males exhibited a slight decrease in PGC numbers; females were normal. Also, we used the Cre-loxP system to inactive GP130 specifically in germ cells and found that this resulted in a fertility defect in females. These animals were found to have a slight reduction in the number of primary follicles and a major defect in ovulation. This data suggests that GP130 is required in female germ cells for their normal function, but is dispensable in male germ cells.

Key words: GP130, LIF, Germ cells, Oogenesis, Ovulation, Mouse

INTRODUCTION

Primordial germ cells (PGCs) are the embryonic precursors of the gametes. In the mouse embryo at 7.5 days of gestation (E7.5), a founding population of approximately 45 PGCs is induced to form at the junction between the posterior epiblast and the allantois (Lawson and Hage, 1994). The PGCs then migrate and proliferate until 3000 PGCs have colonized the gonads by E11.5 (Tam and Snow, 1981). PGCs continue to proliferate within the developing gonads and initiate the process of sexual differentiation. By E14.5, there are approximately 20,000 PGCs present in both male and female embryos. In the male, the germ cells have undergone mitotic arrest and have organized into testis cords. In the female, the PGCs have entered meiosis and have arrested at the diplotene stage of meiosis I (reviewed by McLaren, 2001). Mitotic arrest is maintained in the male until three days postpartum, when the process of spermatogenesis is initiated. In the mature female, a small number of oocytes initiate growth, complete meiosis I and are ovulated every four days until the resting pool of oocytes is depleted (reviewed by Hogan et al., 1994).

Germ cell survival and proliferation are regulated at multiple stages of development. This regulation is vital because errors in germ cell proliferation cause profound effects on fertility and result in the formation of germ line tumors. Several growth factors have been identified that can affect PGC survival/proliferation in culture (reviewed by Wylie, 1999). Specifically, factors controlling the survival of migratory PGCs have been well defined and a mixture of bFGF, Kit-ligand (KITL) and LIF has been shown to immortalize PGCs in culture (Matsui et al., 1992; Resnick et al., 1992). KIT is necessary for PGC survival in vivo based on the observations that mutations in *Kit* and *Kitl* cause a compete loss of PGCs by E9.5 (Besmer et al., 1993). However, the in vivo roles of bFGF and LIF in germ cell development are uncertain. bFGF-knockout animals are viable, fertile and have no obvious defects in gametogenesis (Ortega et al., 1998). LIFR-knockout animals have defects in multiple organ systems and die shortly after birth; however, they appear to have normal numbers of PGCs (Ware et al., 1995). LIF-knockout females are infertile because of a defect in implantation; however, these animals have normal numbers of oocytes, and these oocytes are ovulated, fertilize and develop normally when transplanted into a wild-type uterine environment (Stewart et al., 1992).

The lack of an obvious PGC defect in LIF- and LIFR-knockout animals is surprising considering the profound effect LIF has on PGC survival in culture. However, LIF is a member of a family of cytokines that exhibit overlapping functions (reviewed by Taga and Kishimoto, 1997). In the mouse, the IL6 family consists of six members [IL6, IL11, LIF, OSM, CNTF and CT1 (SLC6A8 – Mouse Genome Informatics)]. Members of this family signal through receptor complexes that are dimers (or multimers) comprising high affinity growth factor specific receptors [e.g. LIFR, OSMR, IL6R (IL6Rα – Mouse Genome Informatics)] and a low affinity common receptor (GP130; IL6ST – Mouse Genome Informatics). Binding of the IL6 ligands to their receptor complexes results in the activation of members of the JAK family, and subsequent

phosphorylation of STAT3 (signal transducers and activator of transcription). Alternatively, GP130-mediated signals can be transduced through the RAS/MAPK pathway (reviewed by Taga and Kishimoto, 1997).

These cytokines share common receptors and common signal transduction machinery, and this might explain the relatively mild phenotypes resulting from inactivation of a single family member or a single high affinity receptor. Ablation of the common receptor (GP130), or a common cytoplasmic component (STAT3), should affect all IL6 family members and result in stronger phenotypes. This appears to be the case. STAT3-knockout animals die by E7.5 (Takeda et al., 1997), and GP130-null animals die of cardiac and hematological disorders by E15.5 (Yoshida et al., 1996), or at birth (Kawasaki et al., 1997), depending on the genetic background. Intriguingly, GP130-null animals have been reported to have fewer numbers of PGCs (T. Taga, unpublished).

In order to clarify the role of the IL6 family in PGC development, we examined PGC numbers in GP130-deficient males and females. In addition, we have used the Cre-loxP system to generate germ-cell-specific ablations of GP130. Surprisingly, our data demonstrate that GP130-mediated signaling is not required for the early stages of PGC development, but reveal a novel role for GP130-mediated signaling late in oogenesis.

MATERIALS AND METHODS

Mouse strains and genotyping

The TNAP-Cre, GP130Flox and Oct4ΔPE:GFP lines have been described previously (Anderson et al., 1999; Betz et al., 1998; Lomeli et al., 2000). For breed tests, CD1 males and females were purchased from Charles River. Genotyping of animals was performed by PCR. DNA was isolated from tail snips (adults) or from heads (embryos) using the Wizard Genomic DNA purification kit (Promega). PCR was performed on 1-100 ng of genomic DNA using RedMix Plus (PGC Scientific) or Platinum PCR Supermix (Invitrogen) as a source of Taq, buffer and dNTPs. Final primer concentrations were 0.4 μM. PCR consisted of: an initial denaturing step of 5 minutes at 95°C; followed by 5 cycles of 30 seconds at 95°C, 1 minute at 65°C and 30 seconds at 72°C; followed by 35 cycles of 30 seconds at 95°C, 1 minute at 60°C and 30 seconds at 72°C; followed by a 10 minute extension step at 72°C. Primers for genotyping the Oct4ΔPE:GFP strain are described by Anderson et al. (Anderson et al., 1999). Primers for the TNAP-Cre line (forward: GGCTCTCCTCAAGCGTATTCAAC; reverse: CAAACG-GACAGAAGCATTTTCCAG) generate a 300 base pair (bp) fragment spanning the junction between the IRES and Cre sequences. Primers for GP130 (forward: ACGTCACAGAGCTGAGTGATGCAC; reverse: GGCTTTTCCTCTGGTTCTTG) generate a 400 bp fragment from the GP130 wild-type allele and a 600 bp fragment from the GP130Flox allele. Absence of a fragment is diagnostic of the GP130Δ allele. Presence of this allele was confirmed by Southern blotting using a PCR generated probe (Betz et al., 1998).

RT-PCR

PGC-containing tissue was dissected from E10.5 and E12.5 animals, and digested in 500 μl 0.25% trypsin (37°C for 15 minutes). The tissue was triturated into a single cell suspension and filtered through a nylon mesh. The mesh was washed with 1 ml 2% BSA in PBS, and the resulting 1.5 ml suspension was sorted using a FACS Vantage. GFP-positive cells (typically 98% pure) were spun down (10 minutes, 1,000 ***g***), and lysed in 300 μl TriZol Reagent (Invitrogen). RNA was isolated as per the manufacturer's instructions using 5 μg of linear polyacrylamide (Sigma) as a carrier. 15 ng RNA (from 3000 cells) was reverse transcribed (1 hour, 42°C) in a 10 μl volume containing 1×buffer (Invitrogen), 100 ng oligo dT, 2 mM DTT, 0.5 mM dNTPs, 10 U RNAsin (Promega) and 200 U Superscript II (Invitrogen). PCR was performed on 1 μl of the RT reaction using RedMix Plus as a source of Taq, buffer and dNTPs. Cycling was performed as described above. Primers were:

GP130 (forward: CGTGGGAAAGGAGATGGTTGTG; reverse: AGGGTTGTCAGGAGGAAGGCTAAG);

OSMR (forward: CACGATGGGCTATGTTGTGGAC; reverse: TCTGAGGTGATGGTGGTGCTTG); and

LIFR (forward: ATTTCCCCAGTTGCTGAGC; reverse: TC-TTCCTCTGCTTTGGCTTGC).

Primers for the PGC marker gene *Kit*, and the somatically expressed gene *Kitl* (*Steel*), were used as positive and negative controls, respectively (Anderson et al., 1999).

Immunostaining

Whole-mount GP130 staining was performed on PGC-containing tissue dissected from E10.5 embryos. Embryos were dissected in PBS/2% bovine serum, and tissues were fixed in 4%PFA/PBS for 20 minutes at room temperature. Tissues were washed for five minutes (3×) with PBS and stored overnight in PBS+0.1% TX-100 (4°C, with rocking). Two GP130 antibodies were used; a goat anti-GP130 (gαGP130) (R and D Systems), raised against the extracellular domain, and a rabbit anti-GP130 (M20) (Santa Cruz) raised against the cytoplasmic domain. When using the goat primary, tissues were blocked overnight at 4°C in 2% donkey serum in PBS. For the rabbit primary, tissues were blocked in 2% horse serum/2% BSA. Tissues were incubated overnight at 4°C with primary antibody at a concentration of 2 μg/ml in the appropriate blocking buffer. Tissues were washed for 1 hour (5×) in PBS/0.1% TX-100 at room temperature. Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, and used at 15 μg/ml in the appropriate blocking buffer (4°C, overnight). Tissues were washed as described and mounted in 75% glycerol on Lab-Tek chambered coverglass (NalgeNunc International). Images were captured using a Zeis LSM 510 confocal system.

For later stage embryos or adults, frozen sections were prepared. The gonads were dissected and fixed in 4% PFA/PBS for either 20 minutes at room temperature (embryos), or overnight at 4°C (adults). Adult testes were cut in half to allow better penetration of the fixative. The gonads were washed for 5 minutes (3×) in PBS, and then sunk in sucrose (20% sucrose) overnight at 4°C. Gonads were embedded in OCT medium and 12 μm sections cut. Sections were blocked (1 hour at room temperature in the appropriate blocking buffer) and then incubated with 2 μg/ml primary in blocking buffer. Slides were washed for 5 minutes (3×) with PBS and incubated with secondary antibodies (15 μg/ml cy5-conjugated secondary antibodies in blocking buffer for 1 hour at room temperature). Slides were washed as described above and mounted in 75% glycerol with 100 μg/ml DABCO (Sigma).

For western blotting, extracts of embryonic gonads were prepared in NP-40 buffer (1% NP-40 (Sigma) in 10 mM HEPES, 150 mM NaCl, 1.5 mM EDTA) plus a 1:50 dilution of Protease Inhibitor Cocktail (Sigma) and 1 mM PMSF. Membranes were blocked in 5% milk (for M20 staining) or with 2% IgG-free BSA (Jackson ImmunoResearch Laboratories) (for goat anti-GP130 staining) in PBS/0.1% Tween-20. Primary antibodies were used at a concentration of 1 μg/ml in the appropriate blocking solution.

Histology

For Hematoxylin and Eosin staining, ovaries were fixed in 4%PFA/PBS at 4°C overnight. They were then dehydrated and processed for paraffin sectioning. 12 μm sections were cut and stained with Harris Hematoxylin and Eosin (Sigma).

Ovulation tests

Natural breeds were set up between CD1 and GP130Flox/ΔTNAP-Cre/+ animals. Females were sacrificed by CO_2 inhalation, and ovulated eggs were collected at noon (E0.5) after observing a copulation plug. Eggs were collected and cultured in M2 medium (Hogan et al., 1994).

Germ cell counts

Individual gonads from E13.5 embryos were digested in 50 μl trypsin (37°C for 15 minutes). The digestion was stopped by the addition of 5 μl 50 mg/ml soybean trypsin inhibitor (Sigma). Tissues were triturated into a single cell suspension and gfp-positive cells were counted on a hemacytometer. Three fields were counted and averaged to determine the concentration of gfp-positive cells. Total PGCs= concentration×55 μl.

RESULTS

GP130 is expressed in PGCs and in growing oocytes and spermatocytes

Saito et al. have reported that the gp130 message is ubiquitously expressed (Saito et al., 1992) and we have verified its expression in E10.5 and E12.5 germ cells using RT-PCR (Fig. 1A). We were unable to detect expression of the LIF receptor (LIFR) at E10.5, but *Lif* mRNA was present at E12.5. The OSM receptor (OSMR) transcript was not detected in PGC mRNA at either stage; however, *Stat3* mRNA was present at both E10.5 and E12.5. We have examined GP130 protein expression at various stages of germ cell development by using immunocytochemistry. We used two antibodies directed against GP130 (M20 and gαGP130). Both antibodies recognized a single 150 kDa protein band in extracts prepared from E14.5 male and female gonads (Fig. 1B; data not shown). Using the gαGP130 antibody we detected GP130-surface staining on PGCs at E10.5 and at E15.5 (Fig. 1C-F). At all embryonic stages, staining in the PGCs appeared weaker than staining in the surrounding somatic tissue. GP130 was also expressed in follicle cells (both granulosa and thecal) and oocytes of the adult ovary (Fig. 2A-C). Staining in primordial stage oocytes was weak and diffuse (Fig. 2B), whereas cell surface staining became more obvious in growing oocytes (2C). The appearance of staining in the adult testes varied from tubule to tubule, suggesting that GP130 expression is also stage specific during spermiogenesis (Fig. 2D-F). Membrane staining was evident in primary spermatocytes in tubules exhibiting a few elongating spermatids (Fig. 2E). Intense membrane staining was visible within all spermatocytes and spermatids of tubules containing mature sperm (Fig. 2F). The M20 antibody also stained germ cells from embryonic and adult stages, but it appeared to detect mainly a cytoplasmic pool of the protein (Fig. 2G,H).

GP130 signaling is not required for PGC survival in the early embryo

$GP130^{-/-}$ embryos survive until E15.5 (Yoshida et al., 1996), or until birth (Kawasaki et al., 1997) depending on the genetic background. On either background, these animals exhibit defects in cardiac development and hematopoesis, and are reported to have reduced numbers of germ cells (T. Taga, unpublished). We have attempted to confirm this finding and pinpoint how GP130 might function in PGC development. GP130Flox/+ animals (Betz et al., 1998) were crossed to TNAP-Cre animals (Lomeli et al., 2000) to inactivate one allele of GP130. GP130Flox/+ TNAP-Cre/+ animals were crossed with Oct4ΔPE:GFP animals (Anderson et al., 1999) to generate $GP130\Delta/+$ $GFP^{+/-}$ double heterozygotes. The efficiency of

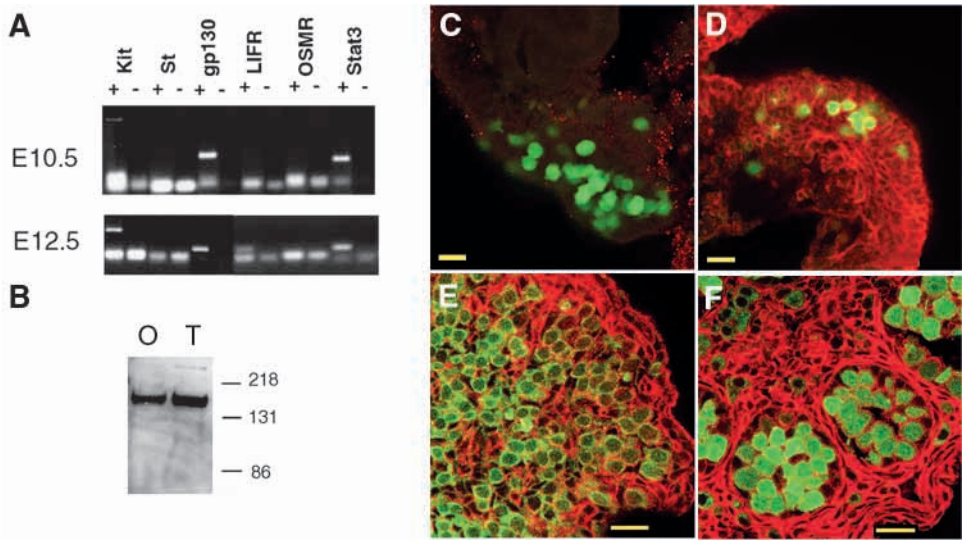

Fig. 1. GP130 is expressed in PGCs throughout early development. (A) RT-PCR showing expression of GP130 and STAT3 in PGCs at E10.5 and E12.5. LIFR message is present at E12.5. The PGC marker gene *Kit* is expressed at both stages, and the somatic marker *Kitl* (St) is absent. '+' indicates RT+ and '–' indicates RT- controls. (B) GP130 protein is present in extracts prepared from E14.5 testes (T) and ovaries (O). The equivalent of 1/3 of a gonad was loaded per lane. Blotting was performed using the M20 antibody. (C,D) Whole mounts stained with goat anti-GP130 (gαGP130; red). (C) Control (E10.5; no primary antibody). PGCs express GFP (green) driven by the Oct4ΔPE promoter. (D) GP130 is expressed on the cell surface of PGCs at E10.5. Cell surface expression is evident on somatic as well as germ cells. (E,F) 12 mm frozen sections from E15.5 embryos stained with gαGP130. GP130 is expressed in the ovary (E) and testis (F) at E15.5. Cell surface staining is much stronger in the somatic cells than in the PGCs. Scale bars: 20 μm.

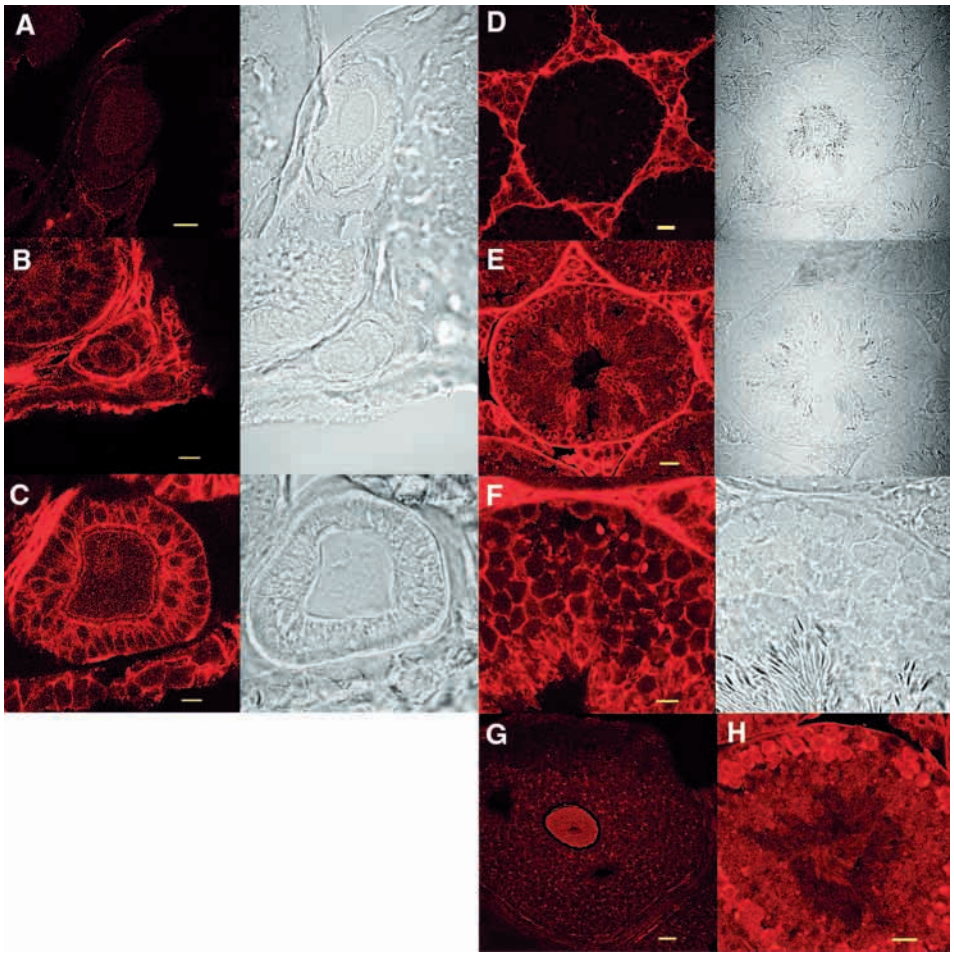

Fig. 2. GP130 is expressed in the adult ovary and testis. (A) Control. (B) Primordial follicle stained with gαGP130. Staining is weak and diffuse in the oocyte. (C) Growing follicle stained with gαGP130. Cell surface staining is evident at the surface of the oocyte, and in the thecal and granulosa cells of the follicle. (D) Seminiferous tubule stained with naïve goat IgG. Staining at the periphery of the tubule and in Leydig cells is nonspecific. (E) Seminiferous tubule stained with gαGP130. This tubule contains elongating spermatids but no mature sperm. Cell surface staining is most prominent in the primary spermatocytes, which also exhibit a strong perinuclear spot of GP130 staining. (F) Seminiferous tubule stained with gαGP130. This tubule contains mature sperm. GP130 is strongly expressed on the surface of all spermatocytes and spermatids. (G,H) The M20 antibody also stains growing oocytes (G) and primary spermatocytes (H); however, staining is more diffuse. Scale bars: 20 μm for A,D,E,G,H; 10 μm for B,C,F.

excision was high in male and female GP130Flox/+ TNAP-Cre/+ parents based on transmission of the GP130Δ allele (32 out of the 58 offspring examined were GP130Δ/+). The GFP marker was bred to homozygosity and GP130Δ/+ $GFP^{+/+}$ animals were crossed to generate GP130Δ/Δ animals carrying the GFP germ cell marker. No GP130Δ/Δ offspring survived until birth in these crosses. However, the normal Mendelian ratio of GP130Δ/Δ animals was observed at E13.5 (in 4 litters, 14 out of 48 pups were GP130Δ/Δ). At this stage most GP130Δ/Δ animals were indistinguishable from their littermates (5 out of 14 were visibly runted). However, GP130Δ/Δ collected at later stages (E15.5) were smaller than their littermates and visibly anemic.

As the reduced numbers of PGCs observed by Taga (T. Taga, unpublished) could be a secondary effect caused by hematopoietic and/or other disorders in GP130-null animals, we have chosen to examine PGC numbers only in those E13.5 embryos that appeared overtly normal. Gonads were dissected from E13.5 embryos, sectioned and stained for GP130 expression. The loxP sites in the GP130Flox allele flank exon 16 (1852-1917 nucleotides of the coding sequence), which encodes for the transmembrane domain of GP130. Hence, excision of the GP130Flox allele should result in the formation of a non-functional secreted form of GP130 (Betz et al., 1998). However, antibody staining of GP130Δ/Δ gonads revealed a reduction in overall GP130 staining as opposed to a relocalization of the protein (Fig. 3A-D). This is similar to the reduction of overall GP130 levels observed by Hirota et al. (Hirota et al., 1999) in MLC2vCreKI/+ GP130Flox/GP130Flox hearts. The small, GFP-positive structures (punctate staining) seen in the GP130Δ/Δ ovary (Fig. 3A) are probably apoptotic germ cells. We have performed PARP staining on similar ovary sections, and have observed PARP-positive (apoptotic) cell fragments occurring at similar frequencies in both GP130Δ/Δ and wild-type ovaries (data not shown). Also, despite reduced GP130 staining, both male and female GP130Δ/Δ gonads still appeared to have normal numbers of PGCs. To quantitate PGC numbers, individual gonads were dissected from E13.5 embryos and dissociated in trypsin; GFP-positive cells were then counted as described in the Materials and Methods. GP130Δ/Δ males exhibited a slight, but statistically significant reduction in germ cell numbers; however, females were normal (Fig. 3E). Hence, GP130 does not appear to play an essential role in the early development of PGCs.

Germ cell specific ablation of GP130 caused a fertility defect in females

GP130 is expressed in growing oocytes and in spermatocytes (Fig. 2), and may have a role during gametogenesis. To examine the role of GP130-mediated signaling in oogenesis/spermatogenesis, TNAP-Cre/+ GP130Flox/+ animals were crossed to GP130Flox/+ animals to generate animals with a germ cell-specific loss of GP130 function (GCKO). The

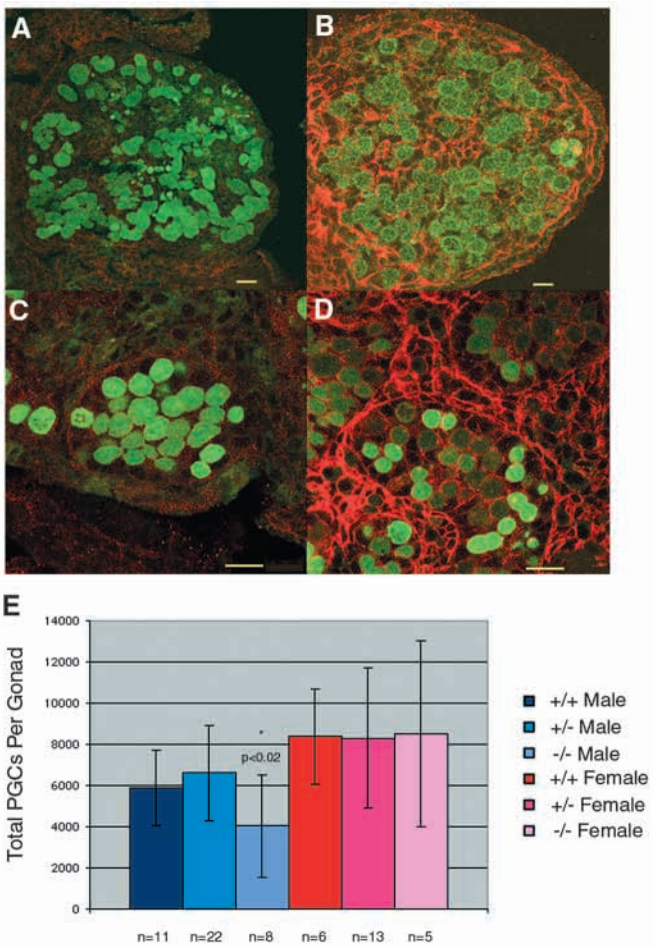

Fig. 3. GP130-deficient embryos have almost normal numbers of PGCs. PGCs express the gfp marker (green), and GP130 staining (red) was performed using the gαGP130 antibody. Staining and counts were performed on gonads dissected at E13.5. (A-D) GP130 expression in a Δ/Δ ovary (A), a wild-type ovary (B), a Δ/Δ testis (C), and a wild-type testis (D). Both male and female Δ/Δ gonads exhibit reduced staining. (E) Germ cell counts in Δ/Δ, Δ/+ and +/+ littermates. PGCs were counted in individual testes or ovaries as described (Materials and Methods). The males exhibit a slight reduction in PGC numbers, which is statistically significant in the case of the Δ/Δ (–/–) littermates (Student's *t*-test, $P<0.02$). Error bars show standard deviations. Scale bars: 20 μm.

normal Mendelian ratio of GP130Flox/Δ TNAP-Cre/+ animals (22/169) was recovered in these crosses, and these animals were similar in size to their heterozygous and homozygous littermates. Four-month-old GCKO females or males were crossed with CD1 animals in order to test their breeding performance. When possible, Flox/Δ Cre-siblings were selected as controls; however, when siblings of this genotype were not available +/Δ Cre+ or +/+ Cre+ animals were used. When compared with their siblings, the breeding performance of GCKO females declined in their second and third litters (when females were 5 months and 6 months old, respectively; Fig. 4A). Several GCKO females (n=3) produced no pups in multiple breeding attempts. The breeding performance of GCKO males was indistinguishable from their littermates (n=4; data not shown).

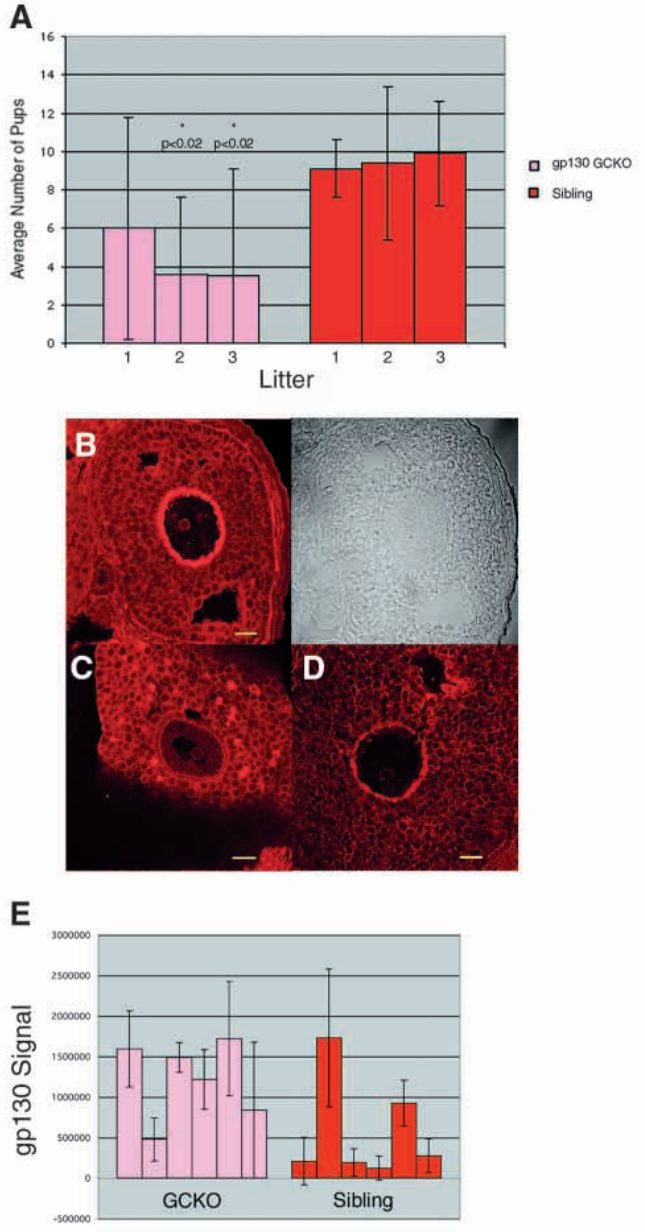

Fig. 4. Germ cell specific ablation of GP130 causes a breeding defect in females. (A) Breeding performance of GCKO females and their wild-type or heterozygous littermates. Four-month-old females were bred three times and the number of pups in each litter were recorded. GCKO females (n=8) produced statistically fewer pups in their second and third litters (Student's *t*-test) as compared with their littermates (n=8). Error bars show standard deviations. (B-E) Cre-mediated excision of GP130 results in accumulation of a secreted form of the protein. GP130 staining (red) was performed using the gαgp130 antibody. (B) Staining in an antral-stage oocyte from a GP130Flox/GP130Δ Cre+ (GCKO) animal. The surface of the oocyte can be seen in the light transmitted image (right). GP130 staining fills the zona. (C) Staining in an antral-stage oocyte from a wild-type female. (D) Staining in an antral-stage oocyte from a GP130+/GP130Δ Cre-female. (E) Summary of staining data from six GCKO ovaries and six heterozygous sibling controls. Staining intensity was quantified by summing the pixel intensities within the secreted ring of GP130. Three oocytes per ovary were quantified and their staining intensity was averaged. Error bars represent standard deviations. Scale bars: 20 μm.

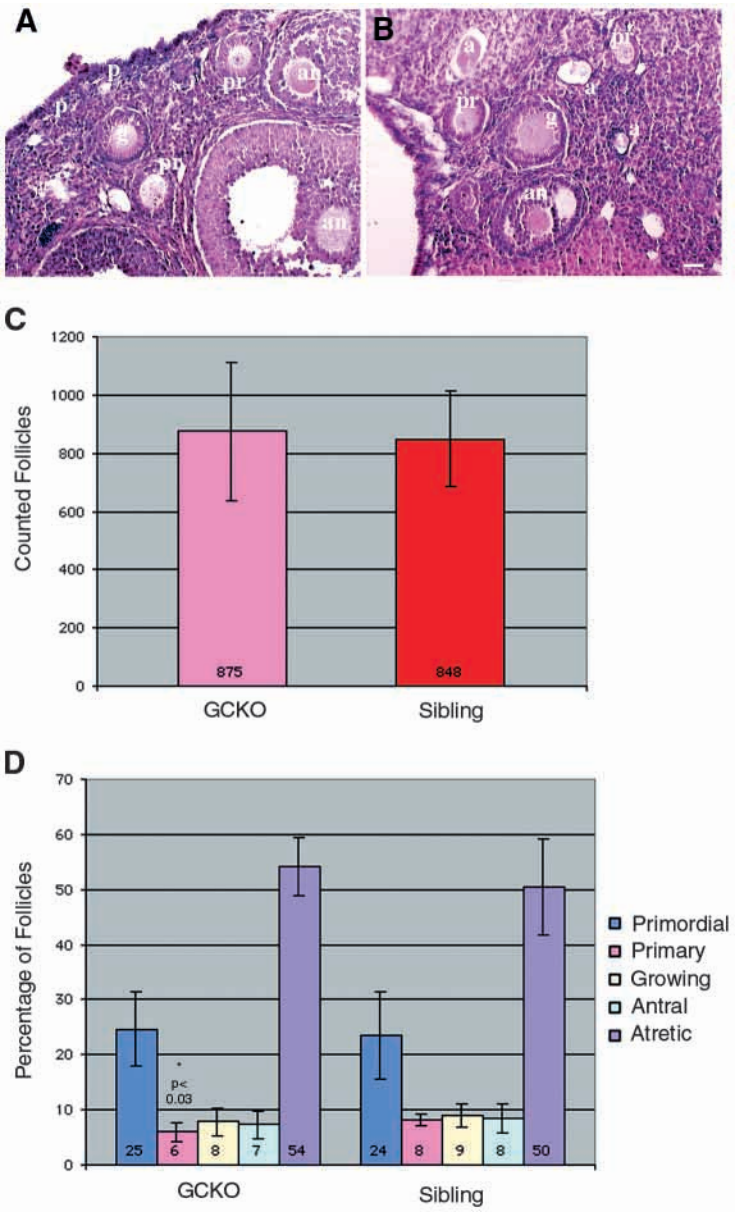

Fig. 5. Ovaries from GCKO females exhibit no gross morphological defects. (A,B) Hematoxylin and Eosin stained sections from a GCKO ovary (A) and a GP130Flox/GP130+ Cre+ sibling (B). Follicles at various stages are evident. Primordial (p), primary (pr), growing (g), atretic (a) and antral (an) stage follicles are indicated. (C) GCKO females (n=6) have normal numbers of total follicles relative to their siblings (n=5). Follicles were counted in every fifth section. (D) Counted follicles were staged based on morphology. GCKO females have a slight, but statistically significant reduction in the percentage of follicles in the primary stage (Student's t-test, P<0.03). Error bars show standard deviations. Scale bars: 30 μm.

Germ cell excision of the first allele of GP130 was efficient based on the transmission of the GP130Δ allele to offspring (see above). To examine excision efficiency of the second allele, ovaries from GCKO animals were fixed, sectioned and stained for GP130. Growing and antral-stage oocytes from GCKO ovaries were surrounded by an intense ring of GP130 staining (Fig. 4B). Secreted GP130 filled the developing zona, and it was not possible to distinguish any membrane staining at the surface of the oocyte. This staining was dramatically different from wild type (Fig. 4C) and was distinct from GP130 heterozygotes (Fig. 4D). Heterozygous littermates typically exhibit a less intense ring of secreted GP130. Staining intensity of 18 growing or antral-stage GCKO oocytes (from six females), and 18 growing or antral-stage heterozygous sibling oocytes (from six females) were quantified by summing the pixel intensities within the secreted ring of GP130 using NIH Image (http://rsb.info.nih.gov/nih-image/index.html), and then subtracting the staining intensity of the IgG control. GCKO oocytes had an average stain of 1,222,860±632,660. Controls had an average stain of 575,716±688,605. Hence, taken as a population, GCKO females exhibit twice the staining of their sibling controls (n=18, Student's t-test P<0.006); however, staining variations were large both between animals and within individual ovaries (see Fig. 4E).

Inactivation of GP130 in germ cells does not have a dramatic effect on ovary morphology

Morphometric analysis was performed on GCKO ovaries to determine whether loss of GP130 function affects oocyte survival and/or maturation. After breed testing, ovaries from seven-month-old females were fixed, serially sectioned, and then stained with Hematoxylin and Eosin (Fig. 5). Follicles were counted in every fifth section (i.e at 60 μm intervals). GP130 GCKO ovaries had normal total numbers of follicles (Fig. 5C); however, they had a slight reduction in the percentage of primary follicles (statistically significant, P<0.03, Student's t-test), and a slight increase in the percentage of atretic follicles (not significant; Fig. 5D). Hence, loss of GP130 function did not dramatically perturb oocyte survival.

GP130 is required in germ cells for ovulation

In humans, LIF is expressed in the follicular fluid and the amount of LIF increases dramatically prior to ovulation (reviewed by Lass et al., 2001). To test whether GP130-mediated signaling is necessary for ovulation and/or fertilization, GP130 GCKO animals and sibling controls were mated with CD1 males. Ovulated eggs were collected at E0.5 and cultured to examine development. Fig. 6 shows a summary of the ovulation data. GP130 GCKO females (n=5) released fewer eggs than their littermates (n=6). Two GCKO females released no eggs at all. Additionally, of the three GCKO animals that were able to ovulate, two produced eggs that appeared normal but did not cleave well in culture. We conclude that the fertility defect seen in GCKO females is caused by a defect in oocyte maturation/ovulation.

DISCUSSION

GP130 is expressed in germ cells in the male and female throughout much of development. In the adult female, GP130 expression is weak in primordial follicles but is upregulated in growing oocytes. GP130 expression exhibits a similar pattern

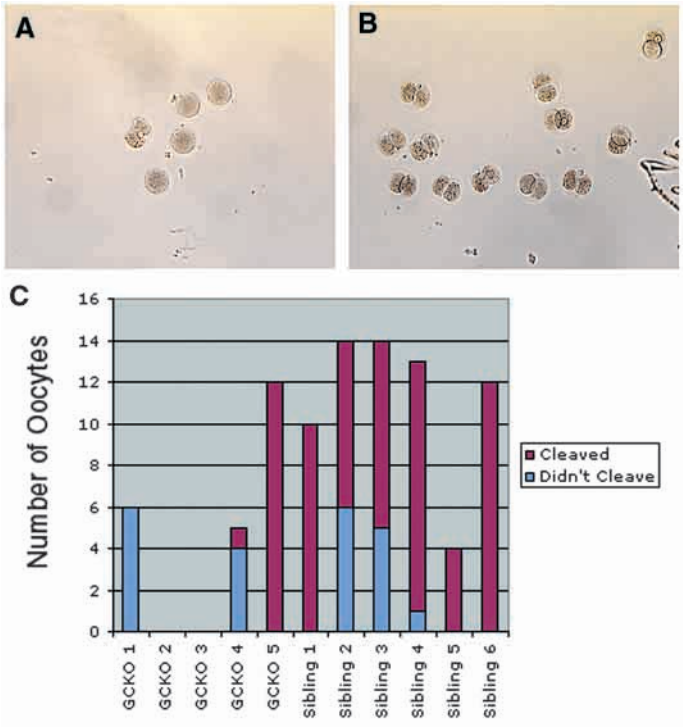

Fig. 6. (A) Eggs ovulated from a GCKO female after one day in culture. (B) Eggs ovulated from a GP130Flox/Δ Cre-sibling control. (C) Summary of ovulation data. Typically, GCKO females release fewer eggs than their siblings, and few of those eggs, apart from those released from GCKO 5, cleave in culture.

in the male, being absent in spermatogonia but strongly expressed in spermatocytes. This expression pattern resembles that of KIT (Manova et al., 1990), and, like KIT, GP130-mediated signaling may have multiple roles in germ cell formation and function.

Data from culture experiments led us to expect that GP130 signaling would be important for mediating germ cell survival during the period in which PGCs are migrating and colonizing the gonads. Consequently, we have analyzed PGC numbers in male and female E13.5 GP130-deficient embryos. The males were found to have a slight but statistically significant decrease in PGC numbers, whereas the females were normal. This suggests that an IL6 family member maybe required in the developing testis to support either PGC survival or proliferation. However, germ cell specific ablation of GP130 had no effect on male fertility or testis morphology in the adult (data not shown). This suggests that GP130 signaling is not necessary in germ cells in the male. Hara et al. have shown that OSM is a potent mitogen for Sertoli cells (Hara et al., 1998). We speculate that our GP130-deficient males may have a defect in Sertoli cell development that indirectly affects PGC numbers. Additionally, LIF is believed to promote PGC survival in culture by blocking apoptosis (Pesce et al., 1993); however, we did not observe an increase in apoptotic PGCs in GP130-deficient testes (data not shown). Finally, Chuma and Nakatsuji have recently reported that LIF can prevent male PGCs from undergoing meiosis in culture (Chuma and Nakatsuji, 2001). It is possible that LIF might control this process in vivo and that ablation of GP130 could indirectly affect PGC numbers in the male by altering their meiotic state; however, on the basis of nuclear morphology, it does not appear that PGCs in GP130-null testes are initiating meiosis (data not shown).

Contrary to the potent effects described for LIF on cultured PGCs, signaling by LIF/IL6 family members appears to have only a slight role (in the male) during the early stages of PGC development. However, GP130 signaling is required for germ cell function in the female. GCKO females produce either small litters or no litters at all. Ovaries from GCKO females have normal numbers of follicles, but a slight reduction in the percentage of primary stage follicles. Consistent with this, Nilsson et al. have demonstrated that LIF can promote the primordial to primary transition in cultured rat follicles (Nilsson et al., 2002). bFGF (Nilsson et al., 2001) and KITL (Packer et al., 1994) have also been shown to promote growth in cultured follicles. In addition, certain mutations in *Kitl* (Sl^{pan} and Sl^{t}) can impair oocyte growth in vivo (Huang et al., 1993; Kuroda et al., 1988). However, unlike KIT, GP130-mediated signaling is not obligatory for this transition, as the GCKO females still have follicles present at all stages.

GCKO females exhibit a dramatic and significant reduction in the number of oocytes released during natural matings, and the oocytes that are released rarely cleave. Hence, we propose that the fertility defect observed in these animals is caused by a defect occurring late in oocyte growth. By contrast, Ernst et al. (Ernst et al., 2001) have reported that females homozygous for a mutated form of GP130 that lacks the STAT3 binding domain (GP130ΔSTAT3) are not impaired in ovulation but instead exhibit a defect in implantation similar to the defect described for $LIF^{-/-}$ females (Stewart et al., 1992). We are uncertain as to why our GCKO animals and the GP130ΔSTAT3 animals have defects at different stages. GP130ΔSTAT3 homozygous animals are viable, unlike GP130Δ homozygotes, indicating that the ΔSTAT3 allele retains some signaling function (perhaps through the RAS/MAPK pathway), and perhaps this activity is sufficient to rescue oocyte maturation/ovulation. We have generated a limited number of STAT3 GCKO animals (see below), and the females have a phenotype similar to that of our GP130 GCKO females (data not shown). This suggests that STAT3 is required for this step. Also, it rules out any possible dominant-negative effect resulting from the large amount of secreted GP130 produced by our mutant animals.

Our results suggest that GP130 signaling is dispensable in the male germ line but has an unexpected function in female germ cells. This conclusion depends on the efficiency and tissue specificity of the TNAP-Cre line. This line has been previously described (Lomeli et al., 2000). Briefly, Lomeli et al. observed ~60% excision of a reporter gene by E13.5 (Lomeli et al., 2000). In addition, transmission of the GP130Δ allele indicates that excision is highly efficient and can reach 100% in adults. Hence, Cre-mediated excision appears to be efficient at this locus. Lomeli et al. have reported that some animals of the TNAP-Cre line can exhibit non-tissue specific expression of Cre, particularly if the TNAP-Cre allele is inherited from the mother (Lomeli et al., 2000). We were able to obtain a normal Mendelian ratio of GP130 GCKO animals using either male or female carriers for Cre. This suggests that the leakiness of this Cre line is not extensive enough to result in lethality. However, we recovered very few GCKO STAT3

animals (2 out of 134 pups), indicating that TNAP-Cre mediated excision was not entirely effective at bypassing lethality in this case. Clearly, the efficiency of excision and the consequence of leaky Cre expression need to be evaluated on a case-by-case basis.

In summary, we have clarified the role of GP130-mediated signaling in PGC development by examining both PGC numbers in GP130-deficient animals and breeding performance in GP130 GCKO adults. Surprisingly, our evidence suggests that IL6 signaling is not vital for either PGC proliferation or survival in early embryos. We suggest that GP130-mediated signaling is necessary to control some aspect of oocyte growth. Nichols et al. have recently reported that GP130-mediated signaling plays a subtle role in maintaining the survival/pluripotency of blastocysts arrested in diapause (Nichols et al., 2001). Perhaps GP130 signaling performs a similar function in growing oocytes, which must maintain pluripotency and meiotic arrest during a long period of growth. Future work will focus on trying to understand what aspect of oocyte growth is controlled by GP130 signaling.

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