Alterations in ovarian function of mice with reduced amounts of KIT receptor

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The KIT receptor, present on oocyte and theca cells in ovarian follicles, and its ligand, KIT LIGAND, produced by granulosa cells, are encoded at the *Kit* gene and the *Mgf* gene, respectively. Both *Kit* and *Mgf* mutations affect oogenesis and folliculogenesis. In this study, the ovarian function of heterozygous mice with a mutation *Kit*^{W-lacZ} was examined. Firstly, the amounts of KIT and KIT LIGAND proteins in the ovaries of mice at different ages were determined. Secondly, *in vivo* and *in vitro* folliculogenesis of wild type and heterozygous mice were compared. Western blotting showed that the amounts of both KIT and KIT LIGAND proteins were decreased in mutant mice. Ovarian follicle populations were counted and more type 5a follicles and fewer type 5b (preantral folli-

cles) were present in ovaries from *Kit*^{W-lacZ/+} ovaries. Furthermore, the relationships between oocyte size and follicle size differed between wild type and heterozygous mice. This finding may be a consequence of altered proliferation of granulosa cells or of altered oocyte growth in mutant mice. Other features of folliculogenesis, such as initiation of follicular growth, total follicle population and follicular atresia, were not affected by the mutation. Analysis of *in vitro* folliculogenesis did not reveal other differences between wild type and mutant mice. It is concluded that the *Kit*^{W-lacZ} mutation affects the expression of KIT and KIT LIGAND proteins, resulting in alterations in granulosa cell proliferation and/or oocyte growth in preantral follicles.

Introduction

KIT receptor and its ligand, KIT LIGAND, are essential for three main developmental processes: haematopoiesis, melanogenesis and gametogenesis. In ovaries, KIT is expressed in the primordial germ cells, and later in the primordial and growing oocytes, whereas KIT LIGAND is produced by the surrounding somatic cells, firstly, by the cells along the migratory pathway of the primordial germ cells during embryonic life and later by the granulosa cells. The *Kit* gene encoding the transmembrane tyrosine kinase receptor KIT maps to the mouse *W* locus (Chabot *et al.*, 1988; Geissler *et al.*, 1988). KIT LIGAND (Huang *et al.*, 1990), which is also known as stem cell factor (SCF; Zsebo *et al.*, 1990) or mast cell growth factor (MGF; Anderson *et al.*, 1990), is encoded by the *Mgf* gene at the *Steel* (*Sl*) locus (Zsebo *et al.*, 1990).

Many mutant alleles are known at the *Kit/W* and *Mgf/Sl* loci in mice (Mouse Genome Informatics; <http://www.informatics.jax.org/>), most of which are associated with reduced fertility. Some mutations, such as *Kit*^{W-e} and

Mgf^{Sl-d}, induce sterility because the primordial germ cells fail to survive early oogenesis (Mintz and Russell, 1957; Godin *et al.*, 1991; Buehr *et al.*, 1993). In other cases, such as *Mgf*^{Sl-t} and *Mgf*^{Sl-pan}, primordial follicles are present in the ovary, but follicular growth is arrested at the primary stage (Kuroda *et al.*, 1988; Huang *et al.*, 1993), thus implying that functional KIT–KIT LIGAND interactions are necessary for normal folliculogenesis. However, because of this blockade at the primary stage, these mutant models fail to provide information on the relevance of KIT–KIT LIGAND interactions for later events of folliculogenesis.

Other approaches have also documented a role of KIT–KIT LIGAND for folliculogenesis. Firstly, using *in vivo* injections of a blocking antibody, Yoshida *et al.* (1997) demonstrated that KIT and KIT LIGAND are required for antrum formation, granulosa cell proliferation and follicle survival. Secondly, using *in vitro* culture of intact mouse follicles from the preantral stage to ovulation, Reynaud *et al.* (2000) identified antrum formation, steroidogenesis and oocyte survival as stages of folliculogenesis that are dependent on KIT–KIT LIGAND interactions. Finally, Parrott and Skinner (1999) cultured neonatal ovaries in the presence of anti-KIT antibody and demonstrated that KIT

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and KIT LIGAND play a role in the initiation of primordial follicle development. However, a common limitation of these studies is that it is impossible to estimate the magnitude of KIT–KIT LIGAND blockade when antibodies are added either *in vivo* (Yoshida *et al.*, 1997) or *in vitro* (Parrott and Skinner, 1999; Reynaud *et al.*, 2000).

In the present study, mice with a novel allele (*Kit^{W-lacZ}*) at the *Kit* locus that had been generated by targeted insertion of the *LacZ* gene in the first *Kit* exon (Bernex *et al.*, 1996) were used. The magnitude of the reduction in ovarian amounts of KIT receptor and KIT LIGAND was measured by comparing ovaries from heterozygous (*Kit^{W-lacZ/+}*) and wild type (*Kit^{+/+}*) mice, as mice homozygous (*Kit^{W-lacZ/W-lacZ}*) for this null mutation die at birth. Follicle populations, the kinetics of oocyte and follicular growth, steroidogenesis *in vitro* and follicle survival *in vitro* were compared between these two genotypes. The results presented in this study demonstrate for the first time that the amounts of KIT and KIT LIGAND proteins are affected in *Kit^{W-lacZ/+}* mutants, and that haploinsufficiency at the *W* locus results in some alterations in ovarian function.

Materials and Methods

Transgenic model

In 129/Sv mice carrying the *Kit^{W-lacZ}* allele, the *Kit* gene is inactivated and the first exon of *Kit* is replaced by a *nlslacZ-neo* cassette. Full details on the construction and procedures used have been described by Bernex *et al.* (1996). Homozygous *Kit^{W-lacZ/W-lacZ}* mice cannot be studied as they die at birth from severe anaemia. Heterozygous mice were easily identified by their white tails, which result from a defect in melanogenesis. When ovaries from heterozygous mice were incubated overnight at 32°C in an X-Gal substrate (5-bromo 4-chloro 3-indolyl β -galactoside), all oocytes stained blue as was expected from the data of Manova *et al.* (1990). As soon as theca cells appear (day 14 after birth), a fraction of these cells are also stained.

Experiment 1: detection of KIT and KIT LIGAND proteins

One-dimensional electrophoresis and western blotting. Ovaries from wild type (*Kit^{+/+}*) and heterozygous mice, identified by their white tails, were collected at day 10 after birth, day 20 after birth and from adult mice. The ovaries were frozen at –20°C in 100 μ l lysis medium (10 mmol KCl l^{-1} , 10 mmol Tris l^{-1} , 0.5 mmol EDTA l^{-1}) containing protease inhibitors (1 mmol phenyl methyl sulphonyl fluoride (PMSF; Sigma, St Louis, MO) l^{-1} , 100 mmol *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma) l^{-1} and 100 mmol *N*- α -p-tosyl-L-lysine chloromethyl ketone (TLCK; Sigma) l^{-1}).

Ovaries from each genotype (either *Kit^{+/+}* or *Kit^{W-lacZ/+}*) were pooled, crushed and centrifuged at 3300 *g* for 30 s, and the protein concentration in the supernatants was

assessed (Bradford, 1976). Equal amounts of proteins were used for western blotting. The samples were heated at 90°C for 1 min in electrophoresis sample buffer (1.25 mol Tris l^{-1} containing 15% (v/v) glycerol and 10% (w/v) SDS) and subjected to electrophoresis under non-reducing conditions using polyacrylamide SDS gels (5–15% gradient). Proteins were transferred overnight at 4°C on a nitrocellulose membrane (wet transfer using 1.2% (w/v) glycine, 0.25% (w/v) Tris and 20% (v/v) methanol as a buffer). After transfer, the efficiency of transfer was checked by staining the nitrocellulose with 0.2% (w/v) Ponceau S stain (Sigma, St Louis, MO). The membranes were washed with TBS (10 mmol Tris l^{-1} and 0.1% (v/v) Tween 20 (Sigma), pH 7.4) and incubated with blocking buffer (1 mol Tris l^{-1} with 5% (w/v) milk powder, 0.2% (v/v) Igepal (Sigma) and 10% non-immune serum, pH 7.4). The membranes were incubated with primary antibodies at 37°C for 2 h and washed with TBS. The primary antibodies used were: monoclonal rat antibody against KIT receptor at 1:20 dilution (ACK₂; Ogawa *et al.*, 1991) and polyclonal rabbit antibody raised against mKIT LIGAND at 1:400 dilution (Genzyme, Cambridge). Incubation with the primary antibodies was followed by incubation with peroxidase-labelled secondary antibodies: anti-rat for KIT receptor (Biosys) and anti-rabbit for mKIT LIGAND (Sanofi Diagnostics Pasteur). Bands were visualized using chemiluminescence (ECL; Amersham Pharmacia Biotech, Little Chalfont) and quantified by image analysis with Kepler Software (Large Scale Biology, Rockville, MD) (Reynaud *et al.*, 1999). These experiments were conducted three times.

The blots were reprobed (after deshybridization) with a monoclonal mouse antibody raised against actin at 1:1000 dilution (Amersham, Les Ulis) to confirm that the loads used were similar between samples. The secondary antibody was peroxidase-labelled anti-mouse antibody (Biosys).

Experiment 2: characterization of folliculogenesis *in vivo*

Ovulation rate and uterine weights. Ovaries from *Kit^{+/+}* mice ($n = 25$) and *Kit^{W-lacZ/+}* adult mice ($n = 26$) aged 5–7 months were obtained and the corpora lutea from both ovaries were counted to determine ovulation rate. Uterine weights were also recorded.

Histological counts

Primordial follicles. Ovaries of mice aged 9 ($n = 3$ mice per genotype) and 20 ($n = 4$ mice per genotype) days, and adult mice aged 5–7 months ($n = 3$ per genotype) were embedded in paraffin wax. All sections from one ovary per mouse were cut (5 μ m thickness) and mounted, and primordial follicles were counted on one section out of every ten. The actual numbers of primordial follicles per ovary were obtained using Abercrombie's correction (Abercrombie, 1946).

Follicular population and atresia. Ovaries from 20 day old and adult mice were removed ($n = 4$ mice per age and

per genotype) and embedded in paraffin wax. All sections were observed and the numbers of preantral and antral follicles were counted on the section on which the nucleolus was found. The follicles were classified according to the classification system of Pedersen and Peters (1968) as class 4, 5a, 5b, 6 or 7 follicles. Type 4 follicles correspond to a growing oocyte surrounded by a ring of 20–60 granulosa cells. Type 5a follicles are typical preantral follicles with the oocyte surrounded by three layers of granulosa cells (100–200 cells on the largest section). Type 5b follicles are formed of almost fully grown oocytes surrounded by many layers of granulosa cells (200–400 cells on the largest section) and show no evidence of antrum formation. Type 6 follicles contain 400–600 granulosa cells on the largest section and scattered areas containing follicular fluid. Type 7 follicles contain > 600 granulosa cells on the largest section and have a large antral cavity. Follicles were classified as atretic when at least five pycnotic bodies were present on the section studied (Driancourt *et al.*, 1985).

Antrum formation. Development of an antral cavity within follicles was assessed in ovaries of 20 day old and adult mice ($n = 3$ mice per age and per genotype) by measuring the proportion of follicles with three, four, five or six layers of granulosa cells with an antral cavity > 1000 μm^2 .

Relationship between oocyte and follicle diameters. Ovaries from mice aged 9 and 20 days were examined ($n = 2$ per age and per genotype). In ovaries from mice aged 9 days, 40 and 30 oocyte–follicle pairs were measured from *Kit^{+/+}* and *Kit^{W-lacZ/+}* ovaries, respectively. In ovaries from mice aged 20 days, 44 and 56 oocyte–follicle pairs were measured in *Kit^{+/+}* and *Kit^{W-lacZ/+}* ovaries, respectively. For each follicle, follicle and oocyte diameter were measured on the section where the nucleolus of the oocyte was visualized and plotted for each mouse at each age to generate a regression line linking both parameters.

Experiment 3: characterization of folliculogenesis in vitro

Folliculogenesis in vitro: follicle isolation and culture conditions. The culture conditions described by Cortvrindt *et al.* (1996) were used. Prepuberal mice (aged 16 days, $n = 2$ mice per genotype) were killed by cervical dislocation and ovaries were collected in L15 Leibovitz medium containing 10% fetal calf serum (FCS), 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1} (Gibco BRL). Preantral follicles ($n = 48$ per genotype) were dissected mechanically from both ovaries. These follicles were class 5a follicles (Pedersen and Peters, 1968). The follicles were cultured individually for 12 days in 40 μl medium covered by oil in 96-well half area plates (Costar Corporation, Cambridge). The culture medium was α -MEM supplemented by ITS (5 μg insulin ml^{-1} , 5 μg transferrin ml^{-1} and 5 ng selenium ml^{-1} ; Sigma), 100 mIU r-FSH ml^{-1} (Gonal-F, Serono, Geneva), 10 mIU LH ml^{-1} (Lhadi, Serono) and

5% heat-inactivated FCS. The follicles were cultured at 37°C, under 5% CO_2 . One day after initiation of culture, each follicle was measured and the presence of theca cells was recorded. Subsequently, the follicles were scored for development and 20 μl culture medium was removed for steroid analysis (see below) at 3 day intervals. A 20 μl aliquot of fresh medium was added to replace the aliquot removed for analysis on each occasion.

At day 12 of incubation, ovulation was induced by addition of 1.5 U hCG ml^{-1} (Ovidrel; Serono) and 5 ng recombinant epidermal growth factor ml^{-1} (EGF; Boehringer Mannheim, Meylan). Mucification of cumulus–oocyte complexes was observed after 16 h. The diameters of the oocytes were measured and nuclear maturation (germinal vesicle, germinal vesicle breakdown or polar body extrusion) was assessed after visual inspection under a microscope and Hoechst staining.

The parameters recorded were: survival rate, development of an antral cavity, basal steroidogenesis and luteinization after addition of hCG, oocyte growth and the ability of each oocyte to resume meiosis.

Hoechst staining procedure. Oocytes were rinsed in phosphate buffered saline (PBS) and fixed on a slide overnight in 70% (v/v) EtOH and H_2O . The oocytes were stained with Hoechst solution (1 μg Hoechst 33342 ml^{-1} in 2.3% (w/v) trisodium citrate solution; Sigma). Nuclear maturation was observed using fluorescent microscopy.

Steroid assays. In all experiments, 20 μl medium was removed from each well at day 3, 6, 9, 12 and 13 of incubation, and stored at -20°C . At days 3, 6 and 9 of incubation, aliquots of 12 follicles (one line) were pooled for assay, whereas at days 12 and 13 of incubation, medium from individual follicles was analysed.

Oestradiol, progesterone and testosterone concentrations were quantified in these media, using validated radioimmunoassays (Terqui *et al.*, 1988; Hochereau-de Reviers *et al.*, 1990; Saumande, 1991). The minimum detectable values were 20 pg ml^{-1} , 0.1 ng ml^{-1} and 0.2 ng ml^{-1} for oestradiol, progesterone and testosterone, respectively. The intra-assay coefficients of variation were 6.9 and 7.6% for 0.1 and 5 ng oestradiol ml^{-1} , respectively, 15.5 and 2.2% for 0.5 ng and 2 ng progesterone ml^{-1} , respectively, and 1.4 and 6.6% for 0.4 and 10 ng testosterone ml^{-1} , respectively.

At days 3, 6, 9 and 12 of incubation, the steroid outputs in medium was for 72 h, whereas at day 13 of incubation, steroid accumulation was for 16 h only. Thus, output per follicle per hour was calculated for days 12 and 13 of incubation so that outputs at the end of culture could be compared.

Statistical analysis

Effects of genotype and time on steroid production were analysed by ANOVA for repeated measurements. Effects of

age and genotype on KIT and KIT LIGAND proteins were detected by a two-way ANOVA. Differences in percentages were checked by chi-squared analysis. Data are mean $\pm$ SEM.

Results

Experiment 1: detection of KIT and KIT LIGAND proteins

KIT protein was detected by western blotting in ovarian homogenates obtained at days 10 and 20 of age as well as in adult mice, using ACK₂ as the primary antibody (Fig. 1). A 145 kDa band was detected and was present at the three stages studied. When the amounts of KIT protein were compared by ANOVA, a significant age effect was detected. Equal amounts of KIT protein were present in ovaries collected from mice aged 10 days and from adult mice, but the amounts of KIT protein were significantly decreased in ovaries collected at day 20 after birth from *Kit*^{+/+} and in *Kit*^{W-lacZ/+} mice ($P < 0.05$). A significant effect of genotype was observed in day 20 ovaries ($P < 0.05$; $135\,100 \pm 19\,500$ arbitrary units in *Kit*^{+/+} ovaries versus $65\,200 \pm 14\,000$ in *Kit*^{W-lacZ/+} ovaries) and there was a tendency towards a significant effect of genotype in adult ovaries ($203\,600 \pm 8\,800$ in *Kit*^{+/+} ovaries versus $176\,400 \pm 12\,200$ in *Kit*^{W-lacZ/+} ovaries). Ovaries from heterozygous mice contained amounts of KIT protein which were reduced by 15–50% compared with ovaries from wild type mice.

Western blotting using an anti-KIT LIGAND antibody revealed a 35 kDa band, regardless of age (Fig. 2). Analysis of the amount of the band showed a significant effect of age for *Kit*^{+/+} ($P < 0.01$) and heterozygous ($P < 0.05$) mice, as amounts of KIT LIGAND increased with time (Fig. 2). In wild type ovaries, as well as in heterozygous ovaries, KIT LIGAND was present in reduced amounts in day 10 ovaries compared with ovaries from mice of other ages (*Kit*^{+/+} ovaries: $P < 0.01$; *Kit*^{W-lacZ/+} ovaries: $P < 0.05$). The amounts of KIT LIGAND were similar in day 20 and adult ovaries. A significant effect of genotype was also detected, as heterozygous mice contained decreased amounts of KIT LIGAND protein ($P \leq 0.05$; $41\,583 \pm 4\,322$ in *Kit*^{W-lacZ/+} and $48\,419 \pm 2\,911$ in *Kit*^{+/+} ovaries). No significant interaction between age and amounts of KIT LIGAND was detected.

Probing of the western blots with anti-actin antibody (Fig. 3) confirmed that the loads were adequate and that the differences observed in amounts of KIT and KIT LIGAND were not artefacts.

Experiment 2: characterization of ovarian function in vivo

Ovulation rate and uterine weights. The distribution of ovulation rates (Fig. 4) was unaffected by genotype. Mean ovulation rates were 10.7 ± 0.6 and 12.0 ± 0.8 for *Kit*^{+/+} and *Kit*^{W-lacZ/+}, respectively. Uterine weights were also

Fig. 1. Representative western blots obtained after probing the membrane with ACK₂, an antibody against KIT receptor. The signals obtained after loading 50 μ g of ovarian proteins from mice aged 10 or 20 days, or from adult mice, are shown.

Fig. 2. Representative western blots obtained after probing the membrane with an antibody against KIT LIGAND. The signals obtained after loading 50 μ g of ovarian proteins from mice aged 10 or 20 days, or from adult mice, are shown.

Fig. 3. Representative blots obtained after probing the membrane with an anti-actin antibody. The signals obtained after loading 50 μ g of ovarian proteins from mice aged 10 or 20 days, or from adult mice, are shown.

similar between *Kit*^{+/+} (153.3 ± 24.5 mg) and *Kit*^{W-lacZ/+} mice (161.7 ± 26.8 mg).

Histological counts

Primordial follicles. Primordial follicles were counted on sections obtained from mice aged 9 and 20 days, and from adult mice. A significant effect of age was detected ($P < 0.001$), as the size of the primordial follicle population decreased with increasing age of the mice. In mice aged 9 days, 1061 ± 96 and 1067 ± 112 primordial follicles were present in *Kit*^{+/+} and *Kit*^{W-lacZ/+} mice, respectively, whereas in mice aged 20 days, the populations

Fig. 4. Distribution of *in vivo* ovulation rates of *Kit^{+/+}* (□) and *Kit^{W-lacZ/+}* mice (■).

of primordial follicles decreased to 481 ± 17 and 304 ± 79 follicles in *Kit^{+/+}* and *Kit^{W-lacZ/+}* mice, respectively. In adult ovaries, only 87 ± 31 (*Kit^{+/+}*) and 99 ± 46 (*Kit^{W-lacZ/+}*) primordial follicles could be counted. No effects of genotype or genotype by age interactions were found.

Total growing follicles. Total follicular populations were similar between genotypes. In ovaries from mice aged 20 days, the mean total numbers of follicles (types 4–7 inclusive) were 163 ± 11 and 194 ± 15 follicles in *Kit^{+/+}* and *Kit^{W-lacZ/+}* mice, respectively. In adult ovaries, 102.2 ± 8.4 and 94.2 ± 29.5 follicles were present in ovaries from *Kit^{+/+}* and *Kit^{W-lacZ/+}* mice, respectively. This decrease associated with ageing was significant ($P < 0.05$).

When the ovarian populations of each follicle size (types 4, 5a, 5b, 6 and 7) from day 20 (Fig. 5a) and adult (Fig. 5b) mice were analysed by ANOVA, a significant effect of genotype was detected for type 5a follicles ($P \leq 0.05$), as ovaries from *Kit^{W-lacZ/+}* mice contained more type 5a follicles than did ovaries from *Kit^{+/+}* mice. An effect of genotype was also observed for type 5b follicles ($P \leq 0.01$), as ovaries from *Kit^{W-lacZ/+}* mice contained fewer follicles than did ovaries from *Kit^{+/+}* mice. For all other classes of follicle (type 4, 6 and 7), the follicle numbers were similar in ovaries from *Kit^{+/+}* and *Kit^{W-lacZ/+}* mice.

Atretic follicles. No effect of genotype on follicular atresia was observed in ovaries of any age. At day 20, 7.1 ± 5.6 and $14.9 \pm 5.7\%$ of follicles were atretic in ovaries from *Kit^{+/+}* and *Kit^{W-lacZ/+}* mice, respectively. In ovaries from adult mice, $27 \pm 6.4\%$ of type 6 and type 7 follicles were atretic in ovaries from *Kit^{+/+}* mice versus $17 \pm 4.5\%$ in ovaries from *Kit^{W-lacZ/+}* mice.

Relationship between follicle and oocyte growth

The regression lines linking follicle and oocyte diameters in ovaries from *Kit^{+/+}* and *Kit^{W-lacZ/+}* mice at days 9 and

Fig. 5. Follicle populations in *Kit^{+/+}* (□) or *Kit^{W-lacZ/+}* ovaries (■) from (a) mice aged 20 days or (b) adult mice. Follicles were classified according to Pederson and Peters (1968). ^{ab}Different superscripts indicate significant differences between *Kit^{+/+}* and *Kit^{W-lacZ/+}* ovaries.

20 are shown (Fig. 6a,b). At day 9, the regression lines linking follicular diameter (*y*) and oocyte diameter (*x*) in ovaries from *Kit^{+/+}* mice were $y = 2.22x - 11.24$ ($r = 0.96$) and $y = 2.48x - 18.88$ ($r = 0.82$), whereas the regression lines were $y = 1.67x - 2.43$ ($r = 0.93$) and $y = 1.63x - 5.57$ ($r = 0.89$) in ovaries from *Kit^{W-lacZ/+}* mice. The slopes of the lines were clearly different in samples from *Kit^{+/+}* and *Kit^{W-lacZ/+}* females (2.22 and 2.48 versus 1.67 and 1.63), indicating that oocyte growth was altered in *Kit^{W-lacZ/+}* mice.

At day 20, the slopes of the regression lines linking follicular and oocyte diameters were also different (Fig. 6b). The regression lines were $y = 3.40x - 72.15$ ($r = 0.80$) and $y = 3.56x - 87.70$ ($r = 0.82$) in ovaries from *Kit^{+/+}* mice, whereas in ovaries from *Kit^{W-lacZ/+}* mice, the regression lines were $y = 2.57x - 28.17$ ($r = 0.79$) and $y = 2.43x - 31.78$ ($r = 0.89$). Again, the slopes of the regression lines were not as steep for ovaries from *Kit^{W-lacZ/+}* mice as for *Kit^{+/+}* mice.

Fig. 6. Correlation between follicle and oocyte diameters in individual *Kit*^{+/+} (○ and □) and *Kit*^{W-lacZ/+} (● and ■) ovaries from mice aged (a) 9 or (b) 20 days. Regression lines calculated for *Kit*^{+/+} (-----) and *Kit*^{W-lacZ/+} mice (—) are represented.

Antrum formation

At day 20, the proportion of follicles with three or four layers of granulosa cells and an antral cavity was 59% (42 of 71) in *Kit*^{+/+} mice versus 44% (40 of 90) in *Kit*^{W-lacZ/+} mice. This difference was not significant. The proportion of follicles with an antral cavity increased to 60 (12 of 20) and 80% (39 of 49) for follicles from *Kit*^{+/+} mice with five or six layers of granulosa cells, respectively, and to 71 (27 of 38) and 79% (30 of 38) for follicles from *Kit*^{W-lacZ/+} mice with five or six layers of granulosa cells, respectively. In adult mice, a similar increase in the proportion of antral follicles with increasing size was observed in the two genotypes.

Experiment 3: characterization of folliculogenesis in vitro

Follicular development, antrum formation and survival. At the start of culture, mean follicle diameters were 174.6 ± 2.7 and 170.9 ± 2.9 μm in the *Kit*^{+/+} ($n = 48$) and *Kit*^{W-lacZ/+} ($n = 48$) groups, respectively. Theca cells were

Fig. 7. *In vitro* antrum formation (percentage of follicles with a cavity) in *Kit*^{+/+} (○) and *Kit*^{W-lacZ/+} (●) follicles during culture.

found surrounding 94% of the *Kit*^{+/+} and 92% of the *Kit*^{W-lacZ/+} follicles. At day 3 of culture, most of the follicles were attached to the bottom of the well ('diffuse' stage, with a disruption of the basal membrane and a layer of cells forming on the bottom of the well). Follicles of both genotypes were similar at this stage. At days 6 and 9, follicles progressively formed an antral-like cavity (visualized as translucent areas within the granulosa cell mass) (Fig. 7). When the proportion of follicles forming an antrum at days 6 and 9 was analysed by chi-squared analysis, there was no evidence for an effect of genotype on antrum formation. At the end of culture, > 60% of follicles had reached the 'antral' stage (74% for *Kit*^{+/+} versus 62% for *Kit*^{W-lacZ/+}). Although more follicles originating from *Kit*^{+/+} ovaries had formed an antrum by the end of culture, this difference was not significant.

At the end of culture (day 12), follicle survival was high and unaffected by genotype (96% in *Kit*^{+/+} group and 98% in *Kit*^{W-lacZ/+} group).

Steroid outputs. Steroid outputs from day 3 to day 9 are shown (Table 1). No effect of genotype and no genotype by time interactions were identified for all steroids. In contrast, the time effect was highly significant ($P < 0.001$), as the output of each steroid increased throughout culture (Table 1). At days 12 and 13, when steroid analysis was expressed per hour, a significant effect of time ($P < 0.001$; Table 2) was detected as oestradiol, progesterone and testosterone outputs per hour increased further after induction of ovulation by hCG + EGF.

Oocyte features. After addition of hCG and EGF, mucification of cumulus cells was unrelated to the genotype and occurred in 77 and 69% of the follicles from *Kit*^{+/+} and *Kit*^{W-lacZ/+} mice, respectively.

Table 1. Steroid output of follicles from *Kit^{+/+}* and *Kit^{W-lacZ/+}* mice at days 3, 6 and 9 of culture

	Day 3		Day 6		Day 9	
	<i>Kit^{+/+}</i>	<i>Kit^{W-lacZ/+}</i>	<i>Kit^{+/+}</i>	<i>Kit^{W-lacZ/+}</i>	<i>Kit^{+/+}</i>	<i>Kit^{W-lacZ/+}</i>
Testosterone (ng ml ⁻¹ h ⁻¹)	0.020 ± 0.005 ^a	0.050 ± 0.009 ^a	0.73 ± 0.13 ^b	0.82 ± 0.12 ^b	4.48 ± 1.25 ^c	4.65 ± 0.85 ^c
Progesterone (ng ml ⁻¹ h ⁻¹)	0.037 ± 0.007 ^d	0.032 ± 0.004 ^d	0.083 ± 0.009 ^e	0.076 ± 0.007 ^e	0.148 ± 0.0616 ^f	0.133 ± 0.014 ^f
Oestradiol (ng ml ⁻¹ h ⁻¹)	0.109 ± 0.012 ^g	0.123 ± 0.011 ^g	1.33 ± 0.14 ^h	1.56 ± 0.25 ^h	1.35 ± 0.16 ⁱ	2.13 ± 0.31 ⁱ

Values with different superscripts in the same row are significantly different.

Table 2. Steroid output of follicles from *Kit^{+/+}* and *Kit^{W-lacZ/+}* mice at days 12 and 13 of culture

	Day 12		Day 13	
	<i>Kit^{+/+}</i>	<i>Kit^{W-lacZ/+}</i>	<i>Kit^{+/+}</i>	<i>Kit^{W-lacZ/+}</i>
Testosterone (ng ml ⁻¹ h ⁻¹)	0.77 ± 0.27 ^a	0.37 ± 0.15 ^a	2.79 ± 0.95 ^b	2.42 ± 0.71 ^b
Progesterone (ng ml ⁻¹ h ⁻¹)	0.26 ± 0.04 ^c	0.32 ± 0.05 ^c	2.02 ± 0.17 ^d	1.89 ± 0.17 ^d
Oestradiol (ng ml ⁻¹ h ⁻¹)	2.60 ± 0.40 ^e	2.06 ± 0.46 ^e	6.28 ± 1.22 ^f	5.36 ± 0.96 ^f

Values with different superscripts in the same row are significantly different.

Oocyte diameters were similar between genotypes (82.6 ± 1.5 µm in *Kit^{+/+}* oocytes and 85.4 ± 1.3 µm in *Kit^{W-lacZ/+}* oocytes). Nuclear maturation was unaffected by genotype, as 70.3% of *Kit^{+/+}* oocytes resumed meiosis and reached metaphase II (polar body extruded) compared with 63.3% for *Kit^{W-lacZ/+}* oocytes.

Discussion

In the present study, ovarian function of heterozygous *Kit^{W-lacZ/+}* mice was examined. The amounts of KIT receptor and KIT LIGAND in the ovaries of these mice at different ages were first evaluated. Folliculogenesis *in vivo* and *in vitro* in wild type and heterozygous mice were then compared. The decreased amounts of KIT and KIT LIGAND found in heterozygous mice were associated with an altered growth pattern of oocytes and with alterations of granulosa cell proliferation and antrum formation in pre-antral follicles.

Western blotting of ovarian samples, using ACK₂ as a primary antibody, revealed a band at 145 kDa, as was expected from earlier reports (Kauma *et al.*, 1996; Tanikawa *et al.*, 1998). High amounts of KIT were detected in ovaries from mice aged 10 days, which is in agreement with previous studies in which large numbers of *Kit*-expressing oocytes were observed at this age (Manova *et al.*, 1990; Yoshida *et al.*, 1997). At day 14 of age, theca cells appear in the largest follicles and *Kit* is also expressed in this layer (Manova *et al.*, 1990). The finding of decreased amounts of KIT at day 20 was unexpected. It may be explained by the 50% reduction in the number of primordial follicles between day 10 and day 20 (see Results), and this reduction not being fully compensated for by the presence of KIT among theca cells, as few follicles only have reached the large preantral or early antral stage at day 20. The amounts of KIT were increased at the adult stage. This

finding may be due to the expression of KIT in the theca or stromal cells at this stage. A fraction of the large number of theca cells present in large antral and preovulatory follicles expresses KIT and could compensate for the reduction in the number of primordial follicles observed between day 20 and adult mice. The amounts of KIT LIGAND protein increased between day 10 and day 20 in *Kit^{+/+}* and *Kit^{W-lacZ/+}* mice. This observation agrees with earlier data showing that expression of KIT LIGAND is high in granulosa cells of growing follicles (Manova *et al.*, 1993), which are numerous at day 20 (see Results). Large amounts of KIT LIGAND in adult ovaries may be produced by the additional production of KIT LIGAND by corpora lutea, as has been reported for mice and sheep (Motro and Bernstein, 1993; Gentry *et al.*, 1996).

The present study is the first in which the effects of a null mutation of the *Kit* gene on the amounts of the KIT and KIT LIGAND proteins in ovaries of heterozygous mice have been simultaneously quantified. The amounts of KIT were lower by 15–50% in *Kit^{W-lacZ/+}* mice at day 20 and in adults compared with wild type mice. In addition, amounts of KIT LIGAND were decreased by 15% in the heterozygous versus wild type mice. This finding may indicate that there may be a regulatory loop between the amounts of KIT and KIT LIGAND in the ovaries, as has been suggested for mast cells (Baghestanian *et al.*, 1996). Heterozygous *Mgf^{SL-pan/+}* mice display a reduced expression (from 20 to 60%) of mRNA coding for KIT LIGAND, depending on the organ studied (Huang *et al.*, 1993), but the effects on KIT were not assessed. Such a genotype would be of interest to confirm the existence of a regulatory loop.

Three main events of folliculogenesis, namely oocyte growth, granulosa cell proliferation and antrum formation, were disrupted in the heterozygous *Kit^{W-lacZ/+}* mice with reduced amounts of KIT and KIT LIGAND.

The slopes of the regression lines linking oocyte and follicular diameters were reduced in *Kit^{W-lacZ/+}* mice compared with wild type mice. This finding indicates that throughout the preantral stage, oocyte and follicular growth were affected by the reduction in the amounts of KIT and KIT LIGAND observed. The altered slopes of the regression lines may be the consequence of altered oocyte growth, in which case, the implication is that KIT and KIT LIGAND interact to regulate oocyte growth. This contention is in good agreement with data obtained by Packer *et al.* (1994) who cultured oocytes in collagen gel for 6 days and showed that the addition of KIT LIGAND increased the growth rate of oocytes. Alternatively, the alterations in granulosa cell proliferation discussed below could also contribute to alter the slopes of the regression lines linking oocyte and follicle diameters. The final sizes of the oocytes were similar in ovaries from *Kit^{+/+}* and *Kit^{W-lacZ/+}* mice, which indicates that these early alterations were corrected later during folliculogenesis.

When the distribution of growing follicles in size classes was observed, more type 5a and fewer type 5b follicles were observed in ovaries from heterozygous mice compared with wild type mice. Granulosa cells of types 5a and 5b proliferate actively and the changes in numbers observed in the present study may indicate an altered granulosa cell proliferation, although it was not quantified in the present study. This observation is in agreement with the earlier results of Yoshida *et al.* (1997) who reported a strong blockade of granulosa cell proliferation after injection of a blocking anti-KIT antibody. As *Kit* is not expressed by granulosa cells (Manova *et al.*, 1990), this effect must be indirect. Other proteins present in oocytes (such as GDF-9; Elvin *et al.*, 1999) or in theca cells (such as KGF; Parrott and Skinner, 1998), the amounts of which are related to the amounts of KIT or KIT LIGAND, could indirectly affect proliferation of granulosa cells.

Histological examination revealed that in *Kit^{W-lacZ/+}* follicles, the pattern of antrum formation was altered at day 20 of age compared with wild type follicles, as fewer preantral follicles with three or four layers of granulosa cells had developed an antral cavity. However, such a difference was not found in adult ovaries. This finding may indicate that the process of antrum formation is altered in these *Kit^{W-lacZ/+}* mice. Earlier results supporting a role of KIT and KIT LIGAND in antrum formation *in vivo* were reported by Yoshida *et al.* (1997), where antrum formation and accumulation of follicular fluid were disturbed after *in vivo* injection of an antibody blocking KIT–KIT LIGAND interactions (ACK₂). Furthermore, addition of an another blocking antibody (SC1494) to mouse follicles cultured *in vitro* for 12 days also severely inhibited antrum formation (Reynaud *et al.*, 2000).

Four other events of folliculogenesis appeared unaffected by the reduced amounts of KIT and KIT LIGAND in heterozygous *Kit^{W-lacZ/+}* mice, namely initiation of follicular growth, follicular populations, follicular atresia and steroidogenesis. Characterization of folliculogenesis *in*

vivo in wild type and heterozygous mice did not reveal differences in the populations of primordial follicles, whatever the age of the mouse, indicating that the *Kit^{W-lacZ}* mutation did not affect the exit from the pool of primordial follicles at the heterozygous state. The extent of atresia was not different between *Kit^{+/+}* and *Kit^{W-lacZ/+}* mice, which indicates that follicle survival was not affected in the heterozygous mice. This finding was confirmed *in vitro*, where follicles from *Kit^{+/+}* and *Kit^{W-lacZ/+}* mice survived similarly.

Both results may initially appear to contradict the data reported by Parrott and Skinner (1999) and Yoshida *et al.* (1997), which showed that the KIT–KIT LIGAND interaction is a key factor for initiation of folliculogenesis and that *in vivo* injections of a blocking antibody strongly reduced survival of type 5a and 5b follicles. The discrepancy between these results and the results of the present study can be explained by noticing that follicles still express at least half the normal level of wild type receptor in *Kit^{W-lacZ/+}* mice, whereas the magnitude of the decrease in KIT bioactivity is probably higher after antibody injection.

In the present study, the *Kit^{W-lacZ}* mutation induced decreased amounts of KIT and KIT LIGAND proteins and haploinsufficiency at the *W* locus resulted in some alterations in ovarian function. Graded requirements of ovarian function for a fully functional KIT–KIT LIGAND interaction were also demonstrated, ranging from high (antrum formation, granulosa cell proliferation and oocyte growth) to low (initiation of follicular growth, follicle atresia and follicle survival).

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