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White spotting variant (Wv) mouse as an experimental model for ovarian aging and menopausal biology

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Abstract

Objective—Menopause is a unique phenomenon in modern women, as most mammalian species possess a reproductive period comparable to their lifespan. Menopause is caused by the depletion of germ cell-containing ovarian follicles, and in laboratory studies is usually modeled in animals in which the ovarian function is removed by ovariectomy or chemical poisoning of the germ cells. Our objective was to explore and characterize the white spotting variant (Wv) mice that have reduced ovarian germ cell abundance, a result of a point mutation in the c-kit gene that decreases the kinase activity, as a genetic model for use in menopausal studies.

Methods—Physiological and morphological features associated with menopause were determined in female Wv/Wv mice compared to age-matched wildtype controls. Immunohistochemistry was used to evaluate the presence and number of follicles in paraffin-embedded ovaries. Bone density and body composition were evaluated using the PIXImus X-ray densitometer, and lipids, calcium, and hormone levels were determined in serum using antigen-specific EIAs. Heart and body weight were measured, and cardiac function was evaluated by transthoracic echocardiography.

Results—The ovaries of the Wv/Wv females have a greatly reduced number of normal germ cells at birth compared to wildtype mice. The remaining follicles are depleted by around 2 months, and the ovaries develop benign epithelial lesions that resemble morphological changes that occur during ovarian aging, whereas a normal mouse ovary has numerous follicles at all stages of development and retains some follicles even in advanced age. Wv mice have elevated plasma gonadotrophins and reduced estrogen and progesterone levels, a significant reduction in bone mass density, and elevated serum cholesterol and lipoprotein levels. Moreover, the Wv female mice have enlarged hearts and reduced cardiac function.

Conclusions—The reduction of c-kit activity in Wv mice leads to a substantially diminished follicular endowment in newborn mice and premature depletion of follicles in young mice, though the mutant females have a normal lifespan after cessation of ovarian function. The Wv female mice exhibit consistent physiological changes that resemble common features of postmenopausal

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women. These alterations include follicle depletion, morphological aging of the ovary, altered serum levels of cholesterol, gonadotropins, and steroid hormones, decreased bone density, and reduced cardiac function. These changes were not observed in male mice, either age-matched male Wv/Wv or WT mice, and are unlikely caused by global loss of c-kit function. The Wv mouse may be a genetic, intact-ovary model that mimics closely the phenotypes of human menopause to be used for further studies to understand mechanisms of menopausal biology.

Keywords

menopause; ovarian follicles; germ cells; c-kit; Wv mice; mouse model

Introduction

Menopause is defined as the permanent cessation of menstruation resulting from depletion of germ cells and loss of ovarian follicular activity, and it is accepted to be a by-product of, or at least more obvious because of, modern health advances and the extension of lifespan that occurred in the last century¹⁻³. The perimenopausal period commences when the first features of menopause begin until at least 1 year after the final menstrual period, and lasts an average of 5 years. In humans, the transition to menopause is a set of gradual changes, in which ovarian function, reproductive capacity, and hormonal status are altered before menses stops completely. Menopause generally occurs between 45–55 years of age, and the symptoms and their severity vary among women³⁻⁶.

In postmenopausal women, circulating levels of estrogen and progesterone produced by the ovary are low, whereas the serum levels of gonadotropins released from the pituitary are high due to the loss of feedback inhibition by the ovarian steroid hormones^{1-3,7}. These changes in hormones affect various metabolic activities in women and increase the risk of significant health problems, including bone loss, or osteoporosis, increased body fat deposition and altered metabolism, and cardiovascular diseases⁸⁻¹⁰. Moreover, ovarian tissues undergo aging and morphological changes¹¹⁻¹³, and the risk of ovarian cancer increases dramatically during the perimenopausal and postmenopausal periods¹⁴⁻¹⁷.

The main cause of menopause-associated health problems has generally been attributed to the reduction in estrogen. Estrogen exerts protective effects on developing certain kinds of heart disease, most likely by regulating cholesterol and lipoprotein levels, and suppresses bone turnover¹⁸⁻²³. However, production of androgens and inhibins by the remaining ovarian tissue and elevated serum levels of gonadotropins released by the pituitary could also exert independent or synergistic effects on these physiological functions in menopause^{2,22-25}. For example, although most evidence is consistent with a central role for a reduced estrogen level^{18,20,24,26,27}, FSH, independently of estrogen, has been shown to act on osteoclasts to activate bone resorption, causing hypogonadal bone loss^{22,23,28,29}. Thus the contribution of other factors to menopausal-associated disorders can be explored more fully by an ovarian aging/menopause model.

Since most mammals -- unlike humans -- retain ovarian follicles throughout their lifespan, there are no simple animal models to study the physiology and pathology of menopause¹. One laboratory procedure is to remove the ovary (oophorectomy), but this does not produce a perimenopausal period or retain ovarian tissue, thus limits understanding of the complex and often gradual changes that lead to the menopausal phenotype, and certainly menopausal influences on ovarian morphology and ovarian cancer risk cannot be evaluated. Chemical toxins, such as busulphan and 4-vinylcyclohexene diepoxide (VCD), have been used to target and kill germ cells and follicles³⁰⁻³², but leave the ovarian tissue otherwise intact. Mutant mice such as the follitropin receptor knockout (FORKO) and dioxin/aryl

hydrocarbon (AhR) knockout also exhibit some phenotype of ovarian failure and have been proposed as potential models to study menopause³³.

The W (White Spotting) series of mice are characterized by their lack of pigment in some areas of their body coat³⁴. The defect was later identified to be the deleting mutation in c-kit, which is required for development of the mast cells, pigment cells, and germ cells^{35–39}. Due to the dominant-negative phenotype in pigment cell development and migration in heterozygous mutants, the W/+ mice are spotted dorsally and ventrally on their body coats³⁹. A number of W alleles have been independently isolated and described that vary in the overall severity of the mutant phenotype³⁸. Some homozygous W mutations are lethal, with embryos dying near birth or soon after, whereas other alleles have relatively mild phenotype³⁸. The Wv mutants, which contain a point mutation in the conserved ATP binding domain of tyrosine kinases that reduces the c-kit receptor tyrosine kinase activity to less than 5%, has a similar but less severe phenotype as the W^{38,39}, and is often used in studies reported in the literature^{40,41}. The kit receptor functions in kit-mediated signaling in primordial germ cells, which are derived from the primordial streak and then migrate to the genital ridges, and in kit-mutant mice the migration and proliferation of the germ cells is suppressed, such that the ovaries of the Wv/Wv mice have about 1–5% of germ cells at birth compared to normal mice^{35,36}. The few germ cells present in the ovaries of Wv mice are depleted soon after birth. Consequently, ovulation ceases to occur and the release of pituitary gonadotropins is elevated^{40,42}, due to a lack of feedback inhibition that is normally mediated through the formation of the progesterone-producing corpus luteum^{1–3}. As such, the Wv mouse may be a unique model for menopause investigations.

In addition to oogenesis, spermatogenesis is also affected by the Wv/c-kit mutation, and male Wv mice are sterile^{43–45}. Although c-kit is required for proliferation and migration of primordial germ cells during embryogenesis, kit signaling is not required for proliferation of male germ cells, and in fact, spermatogonial stem cells from male Wv/Wv mice are capable of self-renewal and proliferation^{44,45}. However, germ cell self renewal does not rescue the sterility defect in Wv males, since c-kit activity is particularly important in the process of spermatogonial differentiation, and mature spermatocytes are not produced.

In the current study, our objective was to investigate the physiological changes in the Wv mice and explore the possibility to use the Wv mice as a genetic model of menopause.

METHODS

Mice Maintenance and Breeding

The Wv mouse (C57BL/6J-Kit W-v) breeding pairs were originally obtained from Jackson Laboratory (Bar Harbor, ME) in 2002. The Wv mouse colony was maintained in the University of Miami Miller School of Medicine Laboratory Animal Facility (LAF) and propagated by inbreeding, with routine backbreeding into the C57BL/6J wildtype background. Mice were housed in micro-isolated cages with free access to autoclaved water and Purina 5001 standard mouse chow in barricaded viral pathogen-free rooms, maintained at 72–76°F and under a 12 hour light-dark cycle. The mice were genotyped by coat color: wildtype, +/+, have a black or agouti coat; heterozygotes, Wv/+, have ventral or dorsal white spots, light belly, and a gray-black coat; homozygotes, Wv/Wv, have black eyes and a white coat. All animal procedures were approved by the University of Miami Miller School of Medicine IACUC committee and followed NIH guidelines.

Histology

Ovaries were fixed in 10% buffered formalin and embedded in paraffin. The paraffin blocks were cut into 5-µm-thick sections that were placed on positively charged slides, and sections

were dewaxed in xylene and hydrated through graded ethanol. Heat-induced antigen retrieval was then performed in 10 mM sodium citrate (pH 6.0) in a boiling water bath for 40 min, and cooled at room temperature for 30 min before proceeding. Slides were either processed for H&E staining or for immunohistochemistry. The endogenous peroxidase activity was blocked by immersing the slides in 3% H₂O₂ in methanol for 15 min. After blocking in 1% BSA for 30 min, the slides were incubated with goat polyclonal anti-Stella/Dppa3/PGC7 (R&D Systems) at 1:2000 dilution, at 4°C overnight. After washing, the slides were incubated with a polyclonal rabbit anti-goat IgG bridging antibody for 35 min, followed by washing, and finally with a secondary horseradish peroxidase-labeled polymer anti-rabbit antibody (DAKO, Carpinteria, CA; or Vector Laboratory, Burlingame, CA) for 35 minutes at room temperature. Diaminobenzidine was used as the chromogen for the immunoperoxidase reaction. The slides were counterstained with hematoxylin and mounted in 50:50 xylene/Permount.

Serum Analysis

Serum was collected and stored at -80°C until analyzed. Serum analysis required 70 µl and was analyzed by the UM Comparative Pathology Facility for lipids (cholesterol, LDL, VLDL, HDL, triacylglycerides) and electrolytes (calcium, potassium, creatine kinase-MB). FSH, beta-estradiol, and progesterone levels in serum were determined using ELISA-based spectrophotometric immunoassay kits from ALPCO Diagnostics (Salem, NH).

Bone Density and Body Composition Measurements

The analysis of bone mass and body composition metabolism used the mouse densitometer dual energy X-ray absorptiometry (PIXImus small animal) DEXA or DXA system (GE-Lunar, Madison, WI) to measure body mass and bone density⁴⁶. The PIXImus mouse densitometer uses X-ray scanning designed for the estimation of bone mineral density (BMD) and body composition for small animals such as mice, and the variation for BMD is <1% coefficient of variations for whole body scanning⁴⁷. Full body scans and X-ray absorptiometry data were processed with manufacturer supplied software (Lunar PIXImus 2, vers. 2.1; <http://piximus.com/>).

Three-point biomechanical testing was performed on a miniature material-testing machine on both the right and left femurs^{48,49}. All bones for mechanical testing were collected and stored at -20°C, and were rehydrated in saline on the day of testing. All measurements were performed at room temperature. The load was applied at a constant rate of 1.0 mm per minute over a span of 10 mm. Force displacement data was collected every 0.02 sec during the test and imported into an Excel spreadsheet to generate load to failure (N), stiffness (N/mm), and energy to failure (J). Additionally, the 4th and 5th whole vertebrae were harvested and tested individually to failure using a crush test of the vertebral body. Samples were compressed at a cross-head speed of 0.05 mm/s using a servohydraulic materials testing machine. Posterior elements were left intact, and the endplates of the vertebrae were minimally shaved flat. Data was processed as described above.

Cardiac Function Analysis

Transthoracic echocardiography was performed on 3-month-old female and male Wv/Wv and wildtype mice (each, n=6), using the VisualSonics Vevo 770 high-resolution in vivo microimaging system (Toronto, Canada). Mice were anesthetized with 1% isoflurane inhalation and placed on a heated platform. Two-dimensional imaging was recorded with a 40-hertz transducer to capture long- and short-axis projections with guided M-Mode, B-Mode and PW Doppler recorded. The average reading for each parameter measured was recorded from at least ten frames from each animal and the standard deviation calculated⁵⁰.

Data Analysis

Basic analytical procedures used in similar studies were used to establish the statistical significance of the data^{51,52}. Student's unpaired *t*-test was used to compare the differences in means between two groups, with $P < 0.05$ considered significant. We confirmed significance using web-based calculators for Wilcoxon-Mann-Whitney non-parametric and one-way ANOVA tests. Only results meeting all levels of significance are indicated.

RESULTS

Ovarian germ cell/follicle deficiency in Wv mice

We obtained the original Wv/+ breeding pairs from Jackson Laboratory and have maintained and studied a colony of Wv mice in our lab for the last 9 years⁴⁰. Housekeeping practices include routine backcrossing of heterozygous Wv mice into the CB57BL/6 background to reduce possible selective breeding pressures. The mice with Wv/Wv genotype are viable, white coated with black eyes, sterile, with normal lifespans similar to wildtype littermates, and predisposed to ovarian neoplasms known as tubular adenomas^{40,41}. The Wv/Wv mice are milder in all phenotypes than other W strains but are suitable for us to study the ovarian phenotypes^{37–39}. Wildtype ovaries at all ages contain numerous follicles (Fig. 1A). Newborn ovaries are virtually full of primary germ cells. Reproductively mature wildtype ovaries contain follicles at all stages of development, including primary, secondary, antral, and corpora lutea. Ovaries from older (1 year) and very aged (2 years) mice show signs of ovarian aging, including stromal and surface irregularities, and reduced follicle numbers, though follicles are still present even at 2 years.

In comparison, ovaries from newborn Wv/Wv mice contain only a few germ cells, shown by staining with the germ cell marker, PGC7 (Fig. 1B). By about 6 weeks of age when female mice become reproductively mature, most follicles have been depleted, and the Wv/Wv ovary contains a limited number of pre-antral and antral follicles and corpora lutea, which demonstrates that some follicles can mature normally⁴³. However, by 3 months, follicles are completely absent and the ovary is infiltrated with epithelial-like components (Fig. 2B; ref. 40). The lesions are known as tubular adenomas⁴¹, benign epithelial growths that resemble morphological changes that occur during ovarian aging in humans^{11,12}. We have analyzed ovaries from Wv mice up to 2 years of age when the entire ovaries were completely permeated with the tumor cells. The lesions are somewhat more complex and intense in older mice but the tumor cells have not further expanded into large masses or acquired malignant features⁴⁰.

In the more than 400 Wv/Wv females examined, we observed a very small variation between individual animals of the same age, suggesting the ovarian phenotypes are stable and reproducible.

Altered plasma hormone levels in Wv mice

Ovarian follicles are still present even in very aged (2 years old) wildtype mice (Fig. 1A), indicating that mouse ovaries do not identically present at a stage that corresponds to menopause⁵³. The Wv female mice, however, do show an early, complete depletion of ovarian follicles, and we confirmed that serum FSH, a key gonadotropin, increases approximately 10-fold in 3–4 month old Wv/Wv females^{40,42} (Fig. 2A). The magnitude of FSH increase is very similar to the elevation found in menopausal women^{54,55}. The Wv/Wv mice also have reduced serum progesterone (Fig. 2B) and beta-estradiol (Fig. 2C). The mice were not synchronized in the estrous cycle, and the wildtypes showed considerable variation in serum estrogen and progesterone. Thus, it appears that the Wv/Wv mice model closely

the changes in ovarian physiology (follicle depletion), endocrine factors (hormonal increase) and ovarian pathology (morphological aging) in menopausal women.

Changes in serum lipid profiles in female and male Wv mice

The Wv/Wv female mice had significant changes in serum cholesterol and high density (HDL) (Fig. 3). Although the changes in low density lipoprotein (LDL) levels were found to be borderline statistically significant, serum LDL levels tended to be higher in the Wv/Wv females. Triglycerides and very low density lipoprotein (VLDL) levels were similar in wildtype and Wv/Wv females (Fig. 3). Serum lipids did not differ between male wildtype and Wv/Wv mice, and LDL was undetectable in the male mice. Levels of calcium, potassium, and creatine kinase-MB were equal in wildtype and Wv/Wv mice of the same gender and within normal range (data not shown).

Decreased bone density in female (but not male) Wv mice

One generally accepted consequence of menopause is osteoporosis, which is characterized by low bone mineral density (BMD) and changes in the architecture of trabecular bone²⁰. One out of every two postmenopausal women will likely experience a bone fracture due to osteoporosis⁵⁶, making this a serious and widespread public health concern. We examined the bone mass of the Wv and wildtype mice using the PIXImus small animal DEXA system to estimate BMD and body composition of 3-month-old wildtype and Wv/Wv mice (Fig. 4A). The Wv/Wv female mice were consistently smaller (~23% body weight) than WT females (Fig. 4A). Although body fat typically increases in menopause, we did not find in these young mice an increase in percent body fat in the Wv/Wv; rather the body fat composition correlated well with the reduced total body weight and size of the Wv/Wv females. WT female mice tended to have a greater percent body fat than the Wv/Wv females, but the difference was not statistically significant.

Significant changes were observed in some bone characteristics. BMD was reduced 17% in Wv/Wv female mice (0.0470 g/cm²) compared to WT females (0.0565 g/cm²) ($P < 0.05$), and whole animal bone area calculated from the PIXImus scans was reduced approximately 23% in the Wv/Wv females (6.76 $\pm$ 0.60 cm² versus 8.75 $\pm$ 0.60 cm² in WT). We further examined bone strength of femurs and lumbar regions in the three month Wv/Wv and WT females using a three point biomechanical fracture test, which measures structural strength, stiffness, and toughness, or the energy of failure⁵⁰. Only stiffness of the femurs of the Wv/Wv differed from control WT (Fig. 4C). This change agrees well with the reduced BMD, which is directly related to material stiffness of the bone⁴⁹. No significant differences were found between male wildtype and Wv/Wv mice for any of the measurements, which supports that the changes are specific to Wv/Wv females and most likely related to the loss of ovarian function.

Reduced cardiac function in female Wv mice

A second major health risk associated with menopausal women is cardiovascular disease, which is in fact the greatest single cause of death in women of this age group⁵⁷. As reported from the large Framingham Study, post-menopausal women have more than a 2-fold age-adjusted increase in risk for coronary heart disease compared to age-matched premenopausal women⁵⁸. The change in serum cholesterol levels in the Wv/Wv females, considered a predictive risk for cardiac dysfunction, suggested that the Wv/Wv might also display a cardiac phenotype associated with menopause.

Despite their smaller body size, the Wv female mice had surprisingly larger hearts than their wildtype counterparts (Fig. 5A). At 3 months of age, WT littermates (n=5) had an average heart weight to body weight (HW/BW) ratio of 4.22 $\pm$ 0.32, whereas Wv/Wv littermates at

the same age had a HW/BW ratio of 5.48 ± 1.35 , which because of the greater variation was not significant. Age, however, augmented the difference, since at 6 months the HW:BW ratio was approximately 50% greater in Wv/Wv females and by 12 months double or more that of their WT counterparts ($P = 0.0001$) (Fig. 5A, B). As shown in Fig. 5B, as an example, a 1-year-old Wv/Wv heart can be extremely large (212 mg; HW/BW ratio = 9.2), and 3X the size of a normal wildtype heart (109 mg; HW/BW ratio = 2.73).

Enlargement of the heart often indicates that the heart itself does not work properly. To investigate heart function, we performed transthoracic echocardiography^{59,60}, and determined the left ventricle ejection fraction (EF) of 3-month, age-matched male and female mice (Fig. 6A,B). EF, a measure of the percentage of blood that is pumped out of a filled ventricle with each heartbeat⁵⁰, averaged 80% for wildtype females, wildtype males, and Wv/Wv males, which is normal for an adult mouse heart and showed minimal deviation between animals (Fig. 6B). Wv/Wv females had a mean ejection fraction of 63%, or a 21% decrease from normal, and the greatest range of measured ejection fractions (57–68%). The change in ejection fraction agreed well with the decrease in fractional shortening (FS), which measures the ratios and changes in the diameter of the left ventricle between contracted and relaxed states as a determinant of the pumping ability of the heart (Fig. 6A). Stroke volume (SV), or the volume of blood pumped from the left ventricle with each cardiac output, was not altered (Fig. 6A). Thus, the Wv female, but not male mice, exhibit reduced cardiac function.

DISCUSSION

Human females have an extended postmenopausal lifespan that is not matched as an age-dependent process in laboratory animal or mouse models. Studies have previously relied on surgical removal of the ovaries to mimic the hormonal and endocrine changes that occur in menopause. However, the majority of women transition into menopause as a consequence of age-related, gradual changes occurring in the hypothalamus-pituitary-ovary interactions resulting in follicle depletion. Most women retain the residual ovarian tissue in menopause; less than 13% of women undergo surgical menopause⁶¹.

Although most laboratory rodents (mice and rats) undergo reproductive senescence, the change is referred to as estropause, and is not a true menopause^{62,63}. In estropause, the rodent is in an extended estrous due mostly to neuronal changes in the hypothalamus-pituitary axis⁶³. The rodents are anovulatory but with intermediate or low, though not undetectable, levels of estrogen; progesterone levels may be increased, and the mouse or rat ovaries retain follicles. In human menopause, the physiological alterations occur in the hypothalamus-pituitary-ovarian axis, and the primary mechanism that results in reproductive senescence and diminished circulating hormones is follicle depletion^{51,63}. Ovarian estrogen and progesterone levels in menopause are generally very low. Thus while the aging mouse model can provide insights to the mechanisms or consequences of menopause, the basic mechanism responsible for reproductive senescence differs from human menopause.

The Wv/Wv female mice, however, reach reproductive senescence at an early age, due to an irreversible and complete loss of ovarian germ cells and follicles. The loss of ovarian function precipitates changes in hormonal and metabolic activity that model many features of human menopause, and include nearly undetectable steroid hormones, elevated serum gonadotropins, ovarian morphological aging, and health problems that associate with menopause, such as decreased bone density, elevated serum cholesterol, and altered cardiac function. We did not find in the age-matched male Wv/Wv mice similar changes in these physiological parameters, thus the c-kit mutation itself is not likely responsible. Rather the changes are specific to the female Wv/Wv mice, and the cessation of ovarian function

appears to be responsible for the altered phenotype in the females. Thus, the Wv/Wv mice may be suitable to investigate menopausal-associated disorders independent of aging-related effects.

In our studies we examined two major menopause-associated disorders, bone loss and cardiac dysfunction. The bone mineral density and stiffness of Wv/Wv long bones was decreased compared to WT littermates, as might be predicted in a menopausal state. Even more striking, the changes in the Wv/Wv heart weight relative to body weight and indices of left ventricle function model ventricular hypertrophy, which is a cardiac abnormality found in post-menopausal women commonly associated with estrogen reduction^{52,64,65}. Many models of cardiac ventricular hypertrophy introduce a pressure overload by surgical implantation of an aortic constriction following ovariectomy⁶². The changes found in the female Wv/Wv mice occur as a natural course of ovarian failure, in the presence of an intact ovary, and can be found early during the mouse lifetime. As far as we are aware, no cardiac or bone abnormalities have been noted in mice bearing mutations in c-kit or the kit ligand, SCF, genes⁶². The kit protein is expressed on the cell surface of putative cardiac stem cells⁶⁶, and on hemangioblast precursors to hematopoietic stem cells and endothelial progenitor cells, where the ckit mutation in hematopoietic stem cells seems to affect cell mobilization into the peripheral circulation⁶². While we cannot completely discount the genetic mutation in effecting changes observed in the bone and heart, we did not observe similar changes in the male Wv/Wv mice. Thus, the menopause-like state of the female Wv/Wv mice seems to be the direct cause of such abnormalities.

Although the Wv/Wv mice are sterile for most of their lives, the Wv/Wv ovary does, in fact, mature and contain follicles that develop into dominant follicles and finally corpora lutea. Oocytes in the Wv/Wv mice do undergo ovulation, and Wv mice have been reported to be able to get pregnant and deliver full term live pups^{41,42}. The depletion of the ovarian follicles in the Wv/Wv females models the cause of menopause, independently of aging. In most cases, women have a 30–35 year reproductive lifespan, marked by monthly hormonal/menstrual cycles and possible extended periods of anovulation during pregnancy. An average normal wildtype female mouse can be expected to be reproductively competent for 8–12 months, or until 14 months old, and undergo continuous estrous cycles. The longterm effects of the hormonal and metabolic cycles on the physiological changes in menopause may not be accounted for in the Wv/Wv mice. For example, pregnancy reduces the risk of ovarian cancer, and the reduction of risk has been attributed to the elevated levels of progesterone⁶⁷. Although we detected a range of levels for progesterone in wildtype mice, progesterone, as well as estrogen, levels were consistently low in Wv/Wv mice, whereas FSH levels were more than 10-fold greater.

Nevertheless, the Wv model may be useful in addressing important questions and mechanisms in post-menopausal biology. Although post-menopausal bone problems have been thought to be the result of the reduction in estrogen levels, FSH may act directly on bone to reduce bone density²⁸. The dramatic elevations in FSH may be more or as much pertinent to age-related bone loss as estrogen, which might be explored by such a model as the Wv/Wv mice. Moreover, it may be that other factors of the aged ovary may participate in physiological changes.

CONCLUSIONS

The c-kit mutation in Wv mice leads to the reduction of ovarian germ cells and follicles. The Wv female mice live a normal lifespan in the absence of ovarian follicles, modeling the cause of menopause. We found that the Wv females exhibit several physiological changes, including reduction in bone density, enlargement of the heart and decreased heart function,

and altered hormone levels, which are common features of postmenopausal women. The Wv mice are amenable and reasonable ovary-intact models for menopause, with features that closely mimic the cause and phenotypes of human menopause.

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Abbreviations

BMD	bone mineral density
BSA	bovine serum albumin
BW	body weight
DEXA	dual energy X-ray absorptiometry
EF	ejection fraction
EIA	enzyme-linked immunoassay
FS	fractional shortening
FSH	follicle stimulating hormone
H&E	hemotoxylin and eosin
HW	heart weight
SV	stroke volume
WT	wildtype
Wv	white spotting variant

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Figure 1. Ovarian germ cell phenotype in *Wv/Wv* mice

Ovaries were collected from wildtype and *Wv/Wv* mice at ages ranging from newborn (1 day) to 2 years. **(A)** Wildtype ovaries retain follicular structures in advanced age. Ovaries were collected from wildtype females at the indicated ages and stained with H&E. A newborn ovary is shown at 200X magnification; other images are shown at 40X magnification. **(B)** Ovaries from wildtype and *Wv/Wv* mice were collected and stained with the germ cell marker, PGC7. Representative images are shown for newborn (200X magnification) and 1.5 and 3 months (40X). The far right panel enlarges on follicles, including primary and secondary, present in 3-month wildtype ovaries. *Wv/Wv* ovaries at this age completely lack follicles.

Figure 2. Serum hormone levels in wildtype and Wv/Wv mice

Blood was collected from wildtype and Wv/Wv mice at 3 months of age to determine hormone levels: **(A)** FSH (ng/mL) ($n=5$) (mean $\pm$ s.d.), **(B)** progesterone (ng/mL) (WT, $n=8$; Wv/Wv, $n=5$), and **(C)** beta-estradiol (pg/mL) (WT, $n=7$; Wv/Wv, $n=8$). The means are indicated for progesterone and beta-estradiol. Significant difference (*) between Wv/Wv and WT was calculated using Student's *t*-test and Wilcoxon-Mann-Whitney analysis and set as $P < 0.05$. All hormone levels were determined by specific ELISA based assays.

Figure 3. Serum cholesterol and lipoprotein levels in Wv/Wv female and male mice

Serum cholesterol (**CHOL**), triglycerides (**TG**), high density lipoprotein (**HDL**), very low density lipoprotein (**VLDL**), and low density lipoprotein (**LDL**) levels were determined in serum samples from 3 month old female and male Wv/Wv and WT mice. Samples from 4 animals in each group were analyzed. Results are reported as mean $\pm$ s.d., with significance set as $P < 0.05$ (*). LDL levels were not detectable (N.D.) in female WT or any male mice.

Figure 4. Body composition and bone density in wildtype and Wv/Wv mice

The PIXImus small animal DEXA system (PIXImusTM, Fitchburg, WI) was used to assess whole body area for body composition of wildtype and Wv/Wv mice at 3 months of age. (A) Representative examples of wildtype (n=5) and Wv/Wv (n=6) females are shown. (B) Both female and male mice (n=5–6) were measured for body weight (g), percent body fat (%), and bone mineral density (BMD) (g/cm²). Results are reported as mean $\pm$ s.d., with $P < 0.05$ (*), calculated using unpaired Student's *t*-test, and confirmed using one-way ANOVA and Wilcoxon-Mann-Whitney analyses. The variation of the PIXImus for BMD is $<1\%$. BMD is 0.0565 g/cm² in wildtype and 0.0470 g/cm² in Wv/Wv. Wv/Wv females had significantly lower body weight and BMD than wildtype controls at 3 months. Male wildtype and Wv/Wv males showed no significant differences in body composition. (C) Stiffness of femurs and 4th and 5th lumbar from Wv/Wv and wildtype females was determined using a biomechanical fracture test. Wv/Wv femurs had approximately 23% reduced stiffness.

Figure 5. Heart enlargement in Wv/Wv mice

(A) The ratio of heart weight (mg) to body weight (g) [HW:BW ratio] was determined for 5–6 female mice at 3, 6, and 12 months of age. Values are expressed as mean $\pm$ s.d. Significant difference (* $P < 0.05$) was determined using both the unpaired Student's t -test and Wilcoxon-Mann-Whitney test, and was $P = 0.0001$ at 12 months. (B) Examples of representative HW:BW for 12 month female wildtype and Wv/Wv mice. The mean HW:BW ratios for WT ($n=5$) and Wv/Wv ($n=6$) were 3.5 ± 0.5 and 7.5 ± 1.0 , respectively. Hearts were photographed at equal magnification using a Zeiss stereodissection microscope.

Figure 6. Cardiac function in Wv/Wv mice

Transthoracic echocardiography was performed on 3 month-old female and male Wv/Wv and wildtype mice (n=6 mice, each) to determine basic cardiac function. In (A), heart function was assessed as left ventricle ejection fraction (EF), fractional shortening (FS), and stroke volume (SV). Wv/Wv females had significantly lower EF and FS (mean $\pm$ s.d., $P < 0.05$). In (B), the range of % ejection fractions determined is shown for female and male mice. The mean ejection fraction percent for wildtype females, wildtype males, and Wv/Wv males was 80%, with minimal variation, whereas the mean ejection fraction percent for Wv/Wv females was 63%, and values ranged from 57–68%.