Endocrine, *vol. 26, no. 2, 169–176, March 2005* 0969–711X/05/26:169–176/$30.00

Alterations in Reproductive Function in Src Tyrosine Kinase Knockout Mice

Katherine F. Roby,[1] Deok-Soo Son,[2] Christopher C. Taylor,[2] Valerie Montgomery-Rice,[3] Jeremy Kirchoff,[2] Sandy Tang,[2] and Paul F. Terranova[2,3]

Departments of [1]Anatomy & Cell Biology, [2]Molecular & Integrative Physiology, and [3]Obstetrics & Gynecology, Center for Reproductive Sciences, University of Kansas Medical Center, Kansas City, Kansas 66160

The role of Src tyrosine kinase in regulating reproductive processes in female mice was investigated using Src wild-type (+/+), heterozygous (+/–), and knockout (–/–) mice. Ovarian Src kinase activity transiently increased in Src +/+ mice following eCG administration. Src knockout mice were infertile. Estrous cycles and vaginal opening in Src knockouts were variable and altered compared with Src +/+ and +/– mice. Follicle development was compromised in Src knockout mice as evidenced by reduced numbers of large preantral and antral follicles compared to Src +/+ mice. Corpora lutea were not observed in the ovaries of Src knockout mice; however, administration of eCG and hCG did result in ovulation. Serum LH and FSH on d 40 and 52 of age did not differ between Src wild-type and knockout females. Results from these studies reveal that female Src knockout mice are infertile due to reduced follicle development and anovulation.

Key Words: Src kinase; fertility; follicle development.

Introduction

Src tyrosine kinase is a non-receptor kinase involved in regulating cellular responses to stimuli. Src is involved in a variety of cell signaling events, regulating both cell growth *(1,2)* and differentiation *(3)*, cell death, and migration *(4)*. Many different substrates have been identified for Src *(5)*; however, the cellular functions of Src and the proteins with which Src directly interacts in many cell types are still poorly defined.

Previous studies have shown that blockade of Src tyrosine kinase using dominant negative and pharmacological approaches alters ovarian *(6–8)* and adrenal steroidogenesis *(9,10)*. Treatment of rat ovarian thecal-interstitial cells (TIC) with herbimycin, a Src-selective inhibitor, led to increased accumulation of cAMP in the culture media *(8)*. Similar results have been observed in mice using two Src inhibitors, herbimycin and geldanimycin *(11)*. Additional studies using Leydig cell lines (MA10 and TM3) and primary cultures of rat TIC revealed that blockade of Src with either herbimycin or Src-dominant negatives increased progesterone and androstenedione secretion *(6,7)*. A similar increase in thecal progesterone and androgen secretion was observed in herbimycin-treated porcine TIC given luteinizing hormone (LH) and platelet-derived growth factor (PDGF) *(12)*. In addition, stimulation of Src activity using temperature-sensitive Src mutants reduced steroid secretion in thecal cells and Leydig cell lines *(6,7)*. Stimulation of Src activity using PDGF also reduced gonadotropin-stimulated steroidogenesis in porcine TIC *(12,13)*. Thus, it is clear that Src regulates ovarian steroidogenesis, and gonadotropins utilize Src as a downstream effector because FSH stimulation of ERK activity in rat granulosa cells could be inhibited by blocking Src tyrosine kinase activity *(14)*.

Because of the potential role of Src in regulating steroidogenesis and gonadotropin action, reproductive function was investigated in Src knockout mice. Based on in vivo studies Src knockout mice exhibited reduced ovarian follicular development and infertility.

Results

Src Tyrosine Kinase Expression and Activity in the Mouse Ovary

Expression of Src kinase mRNA was assessed by RT-PCR of total RNA isolated from ovaries collected from wild-type and knockout mice on d 30 of age. As expected, a 657-bp band representing Src message was amplified in +/+ ovaries but not in –/– ovaries (Fig. 1). SYF fibroblast cell lines expressing Src and knockout for Src were used as controls; as expected, a 657-bp band representing Src message was amplified in the +/+ fibroblast but not the –/– fibroblast cells.

Ovarian Src kinase activity, as assessed by immunecomplex kinase assay and the phosphorylation of enolase, increased transiently in response to eCG administration to wild-type mice. Phosphorylation of enolase increased 1 h after the injection of eCG, reached a peak after 2 h, and

Received January 27, 2005; Revised February 16, 2005; Accepted February 22, 2005.

Author to whom all correspondence and reprint requests should be addressed: Katherine F. Roby, PhD, Department of Anatomy and Cell Biology, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, Kansas 66160, USA. E-mail: kroby@kumc.edu

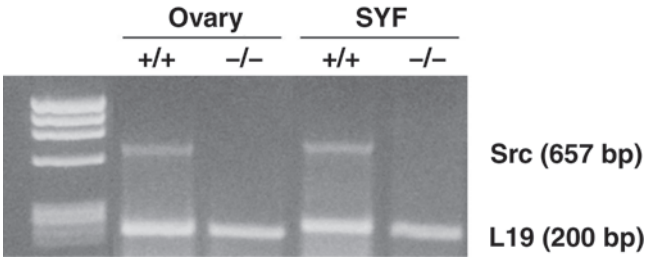

Fig. 1. Src mRNA expression in the ovary. RT-PCR of total RNA from ovaries collected from +/+ and –/– littermates. Src PCR product was not detected in –/– ovaries. Primers for L19 amplifying a 200-bp fragment were included in each reaction tube as an internal control. RNA from Src knockout, SYF –/– and Src transfected, SYF +/+ fibroblast was subjected to RT-PCR and served as negative and positive controls, respectively. Src primers used amplified a 657-bp fragment.

declined thereafter to basal levels at 24 and 48 h (Fig. 2). In addition, a low level of Src autophosphorylation was detected. Changes in Src autophosphorylation exhibited the same pattern as phosphorylation of enolase, although the overall levels were lower (Fig. 2).

Characteristics of Fertility and Cyclicity in Src Knockout Mice

Female Src knockout mice were infertile and did not produce litters when paired with male +/+, +/–, or –/– mice. Similarly, Src knockout male mice were infertile and did not produce litters when paired with female +/+, +/–, or –/– mice. Extensive breeding of the Src knockout females was tested in at least 10 breeding pairs in each of those groups for periods of at least 8 mo each. No pups were born in any of those breeding pairs. In a separate experiment the ability of wild-type fertile male mice to mate female knockout mice was assessed. Copulatory plugs were never observed in knockout females, whereas all wild-type females were plugged. Pairing of +/+ females with +/+ males produced numerous litters and Src +/– females paired with +/– males also produced numerous litters. In fact, Src heterozygous mice exhibited enhanced fertility, based on litters produced over time when compared to wild-type mice. Src +/– mice produced 7.38 ± 0.30 litters per month per 10 pairs compared to 4.91 ± 0.57 for +/+ mice ($p < 0.001$; Table 1). The number of pups born per litter and the weaned to born ratio were not different between Src heterozygous and wild-type mice (Table 1). Of those pups weaned from Src heterozygous breeding pairs, the percentage of knockout mice was lower than expected: 10.4% –/– compared to approx 25% expected based on 1(+/+):2(+/–):1(–/–) ratios. Sixty percent of weaned (–/–) mice were females and 40% were males. Fewer Src knockout mice than the anticipated 25% was previously reported *(15)*, and, although not investigated, *in utero* loss was suggested.

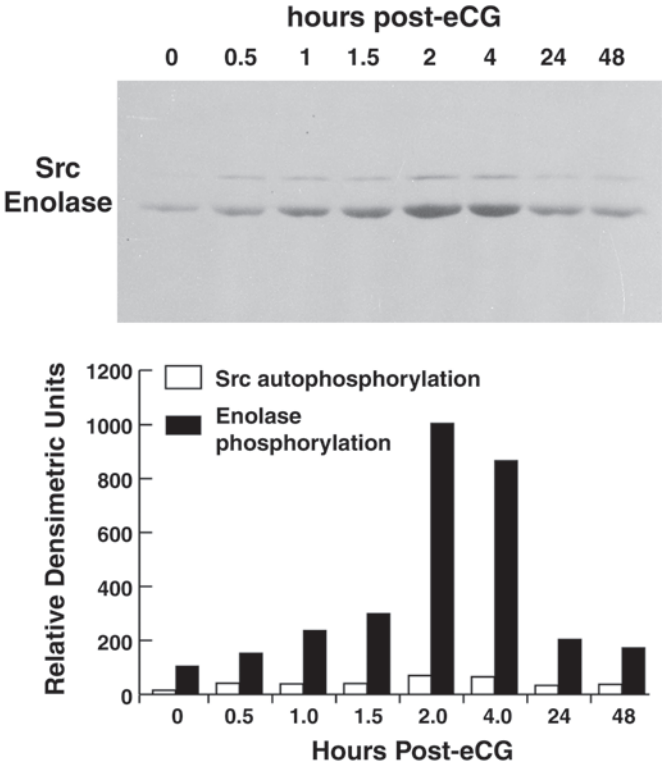

Fig. 2. Src kinase activity in the mouse ovary. Ovaries were collected from immature Src wild-type mice after 2.5 IU eCG administration on d 25 of age. Src kinase activity was analyzed by immune-complex kinase assay using enolase as a substrate and [$^{32}\gamma$P]ATP. Reaction products were separated by PAGE and analyzed by phosphoimaging. Src kinase activity (enolase) in the ovary increased transiently after eCG administration. Autophosphorylation of Src (Src) was also detected and increased transiently in response to eCG. Relative densitometric units from one experiment representative of three are illustrated in the lower panel.

Table 1
Fertility of Src +/– and+/+ Mice[a]

	Litters born / month / 10 pairs	Pups / litter	Wean:born (%)
Src +/–	7.38 ± 0.30*	6.76 ± 0.22	703:937 (75.0%)
Src +/+	4.91 ± 0.57	7.02 ± 0.26	485:597 (76.7%)

Src knockout female and knockout male mice were infertile.

[a]Litters born, pups/litter, and the wean to born ratio were determined. Mice were weaned on day 20.

Data are compiled from 28 different breeding pairs from each genotype over a 12 month period.

*$p < 0.001$ Src +/– compared to Src +/+; Src (+/–) female mice were bred to Src (+/–) male mice; and female (+/+) mice were bred to male (+/+) mice.

Vaginal opening and estrous cycles in Src knockout mice were variable and altered when compared with Src wild-type and heterozygous mice. Twelve mice from each genotype were observed daily to assess for vaginal opening beginning at 25 d of age. Forty percent of Src knockout mice never exhibited vaginal opening. Of the remaining 60% of

Table 2
Vaginal Cyclicity in Src Wild-Type, Src Heterozygous, and Knockout Mice Was Determined by Assessment of the Cells Obtained by Daily Vaginal Lavage

	Src wild-type	Src heterozygous	Src knockout
Number of cycles in 30 d	4.4 ± 0.1	5.4 ± 0.4	2.7 ± 0.6*
Percentage of days in each phase			
Estrus	29.7 ± 1.5	29.5 ± 3.5	26.6 ± 5.2
Diestrus	48.4 ± 1.2	48.1 ± 4.5	59.9 ± 6.6
Proestrus	21.9 ± 1.4	22.4 ± 0.5	13.5 ± 1.7*

$n = 4$ mice per genotype with 42–77 consecutive days of vaginal lavage; Src knockout mice were those that had undergone vaginal opening; 40% of Src knockout mice did notundergo vaginal opening. *$p < 0.05$ Src –/– compared to Src +/+ and Src +/–.

Src knockout mice, vaginal opening was delayed occurring between 40 and 55 d of age as compared to Src heterozygous and wild-type mice where vaginal opening occurred in all animals by d 30 of age. Four mice from each genotype, Src knockout mice that exhibited vaginal opening, wild-type, and heterozygous mice, were subjected to vaginal lavage for 42–77 consecutive days. Src –/– mice exhibited 2.7 ± 0.6 cycles per 30 d whereas +/+ and +/– mice had 4.4 ± 0.1 and 5.4 ± 0.4 cycles, respectively, in the same time period. Even though some vaginal cyclicity was evident in the Src knockout mice, corpora lutea were never observed during histological examination of their ovaries. The quality of the vaginal cycles was also assessed by determining the percentage of days each mouse spent on diestrus, proestrus, and estrus. No differences were observed in percentage of days spent on diestrus or estrus (Table 2). However, a lower percentage of days ($p < 0.05$) were spent on proestrus in Src knockout mice (13.5 ± 1.7%) when compared with +/+ (21.9 ± 1.4%) and +/– (22.4 ± 0.5%) mice.

Follicular Development in the Src Knockout Mouse

Follicular development was quantitatively assessed in every fourth section of serially sectioned ovaries from Src –/– and Src +/+ mice on d 12, 20, 26, 40, and 52 of age. As determined by two-way ANOVA, the numbers of type 1 primordial and type 2/3 primary follicles were not affected by age or genotype and there was no interaction effect of genotype and age (Fig. 3 A,B). Although the numbers of type 4 follicles decreased with increasing age ($p < 0.0001$ by two-way ANOVA), there was no effect of genotype (Fig. 3C). An effect of genotype (Src +/+ vs –/–) became significant ($p < 0.0001$ by two-way ANOVA) with type 5 follicles, those with three or more layers of granulosa cells but no antrum; numbers of type 5 follicles were significantly lower in Src knockout mice compared with wild-type mice (Fig. 3D). Numbers of atretic follicles in wild-type and Src knockout ovaries were not different for any preantral stage at any age examined.

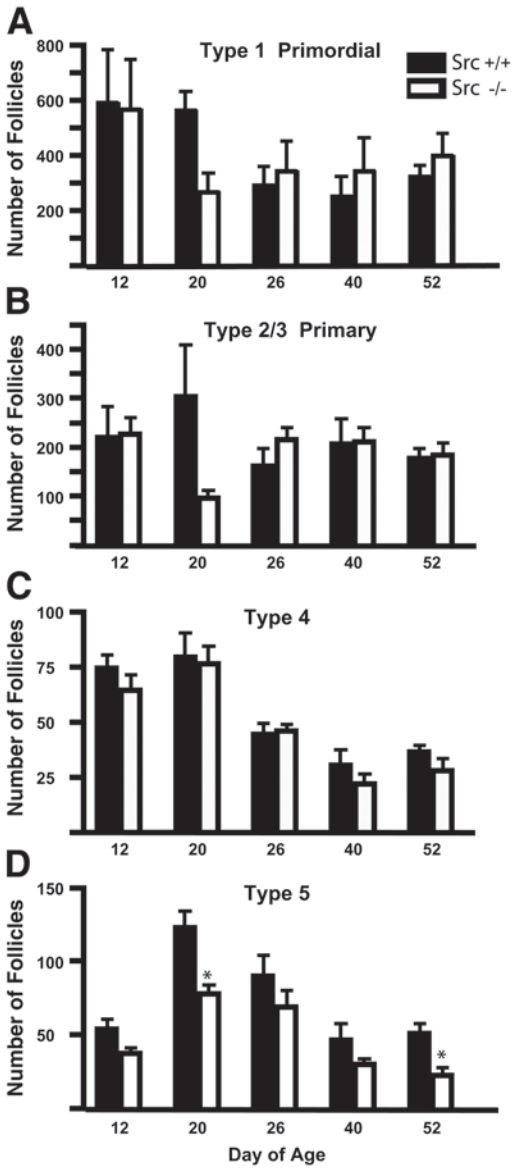

Fig. 3. Preantral follicle development in Src –/– and Src +/+ ovaries. The number of preantral follicles was enumerated in every fourth section of one serially sectioned ovary collected from Src +/+ and Src –/– ovaries on the day of age as indicated. Data were analyzed by two-way ANOVA. No effects of genotype (+/+ vs –/–), age, or interactions were detected for type 1 or type 2/3 follicles ($p > 0.1$). A significant effect of day of age only ($p < 0.0001$) was detected for type 4 follicles. A significant effect of both genotype ($p < 0.0001$) and age ($p < 0.0001$) was detected for type 5 follicles. Data are presented as means ± SEM of the total number of follicles counted in every forth section of one ovary from each animal. The number of ovaries was as follows (+/+: –/–): d 12, 4:4; d 20, 5:4; d 26, 5:5; d 40, 6:7; and d 52, 4:4. *$p < 0.05$ +/+ vs –/– within the same day of age.

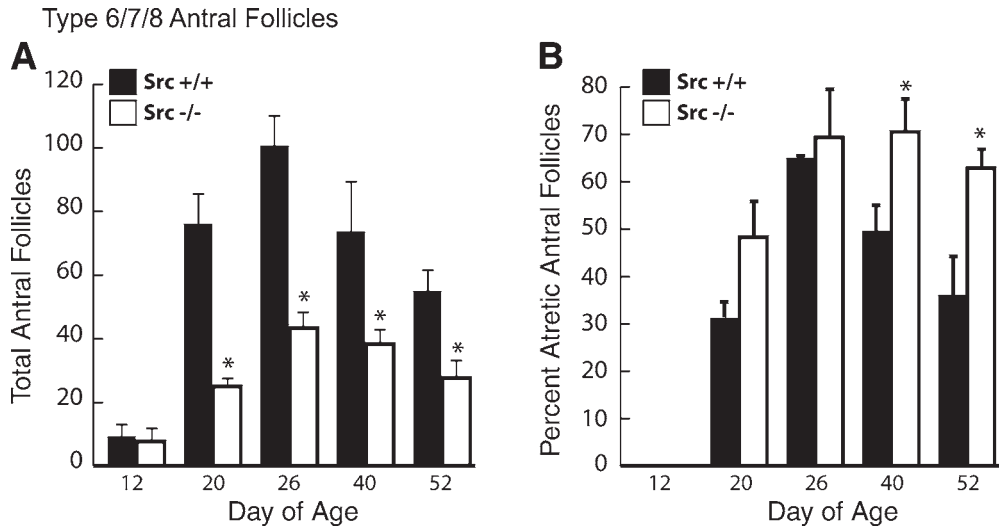

Fig. 4. Antral follicle development in Src +/+ and Src –/– ovaries. The total number of antral follicles (**A**) was reduced in Src –/– ovaries compared to +/+ ovaries ($p < 0.0001$). The percentage of atretic antral follicles (**B**) was higher ($p < 0.0001$) in Src –/– ovaries. There were no atretic antral follicles on d 12 of age. Data are presented as means ± SEM of the total number of antral follicles or the percentage of atretic antral follicles counted in every fourth section of one ovary from each animal. The number of ovaries was as follows (+/+: –/–): d 12, 4:4; d 20, 5:4; d 26, 5:5; d 40, 6:7; and d 52, 4:4. Data were analyzed by two-way ANOVA. *$p < 0.05$ +/+ vs –/– within the same day of age.

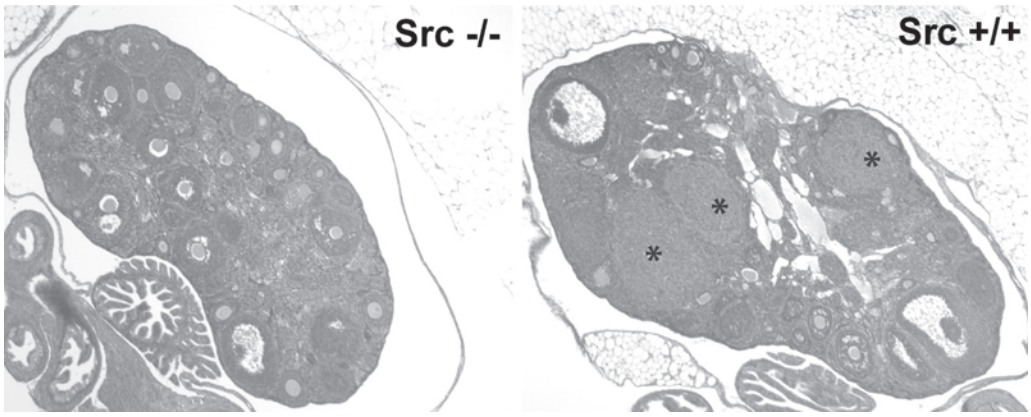

Fig. 5. Representative ovaries from Src –/– and +/+ mice on d 52 of age. Ovaries from Src wild-type mice contain corpora lutea (*) and follicles at all stages of development. Ovaries from Src knockout mice had no corpora lutea and limited development of healthy follicles into the antral stages.

The total number of antral follicles (type 6/7/8) was significantly lower in the Src –/– ovaries when compared with the wild-type littermates ($p < 0.0001$ by two-way ANOVA, Fig. 4A). Together with fewer total numbers of antral follicles in Src knockout mice, the percentage of those antral follicles that were atretic was greater in Src knockout mice compared to wild-type mice ($p < 0.0001$ by two-way ANOVA, Fig. 4B). There were no atretic antral follicles on d 12 of age in either genotype. A representative example of an ovary on d 52 of age from a wild-type and Src knockout mouse is shown in Fig. 5. Of note is the absence of corpora lutea (Fig. 5 and Table 3) and the lack of large preovulatory follicles in the Src knockout ovary (Fig. 5).

Serum Gonadotropin Levels in Female Src +/+ and Src –/– Mice

No differences in serum concentrations of LH and FSH were observed between wild-type and Src knockout mice on d 40 and 52 of age (Table 3), a time when antral follicular development was reduced and antral follicular atresia was increased in the knockout ovaries compared to the wild-type ovaries.

Ovulatory Responsiveness to Gonadotropin Administration

Src wild-type, Src heterozygous, and Src knockout female mice on d 29 of age were administered eCG to induce fol-

Table 3
Serum LH and FSH Concentrations in Src Wild-Type and Knockout Littermates

	LH (ng/mL)	FSH (ng/mL)	Percentage ovaries with CL
d 40			
Src wild-type	0.180 ± 0.050 (7)	6.75 ± 0.84 (7)	86 (6/7)
Src knockout	0.159 ± 0.061(6)	5.82 ± 1.50 (3)	0 (0/7)
d 52			
Src wild-type	0.171 ± 0.050 (8)	10.64 ± 2.05 (8)	100 (8/8)
Src knockout	0.146 ± 0.043 (6)	7.93 ± 1.39 (3)	0 (0/6)

Values are means ± SEM, number of animals in parentheses. For corpora lutea (CL), number of animals with CL/total animals examined is indicated.

Table 4
Ovulatory Response to Gonadotropin Administration in Src Wild-Type, Src Heterozygous, and Src Knockout Mice[a]

	Src wild-type	Src heterozygous	Src knockout
Number ovulating/ treated (%)	8/8 (100)	10/10 (100)	5/8 (63)
Number of ova shed per animal	15.8 ± 3.1	20.1 ± 3.0	10.3 ± 4.0

[a]Mice, d 29 of age, were administered eCG and 52 h later hCG. The morning following hCG oviducts were irrigated and the shed ova enumerated.

licular development followed 52 h later with an ovulatory dose of hCG. In response to gonadotropin administration 5 of 8 (approx 63%) Src knockout mice ovulated as evidenced by ova flushed from the oviduct on the morning following hCG (Table 4). Ovulation was 100% in both the wild-type (8 of 8) and heterozygous (10 of 10) groups (Table 4). The number of ova shed was not statistically different between each genotype (Table 4).

Discussion

Data in the present study indicate that in vivo activation of Src kinase occurred in response to the exogenous gonadotropin (eCG). It is well known that eCG increases ovarian cAMP. Whether there was a direct relationship between elevated cAMP and activation of Src kinase in ovarian cells is not clear. In fact it is not known which cells within the ovary —theca–interstitial, granulosa, or others—are responsible for the increased Src activity. In vitro studies in other systems have shown that cAMP phosphorylates and activates Src and that direct interactions between Src- and the inhibitory G-protein α (Gαi) and between Src and the stimulatory G-protein α (Gαs) occur at the catalytic domain of Src (not the SH2/SH3 domains) *(16)*. Furthermore, stimulation of Gαi or Gαs and expression of constitutively active mutants of Gαi or Gαs increased Src kinase activity *(16)*. Growth regulators such as platelet-activating factor (PAF) that bind to specific G-protein coupled receptors on endothelial cells increased intracellular cAMP levels, required adenylate cyclase and PKA, and also led to phosphorylation of Src *(17)*. Additional in vitro studies have shown that isoproterenol activation of G-protein coupled beta-adrenergic receptors increased cAMP which activated Src and transactivated the EGF receptor *(18–20)*. Thus, in several non-ovarian in vitro systems, a link between G-protein coupled receptors and/or increased cAMP and activation of Src has been demonstrated. The present study provides the initial in vivo findings that administration of a gonadotropin such as eCG increased Src kinase activity in the ovary.

The downstream effects of Src activation in the ovary are still under investigation; however, it appears that Src functions at a physiological level, at least in part, by regulating ovarian steroidogenesis *(6–8)* and follicular development. An important consideration of the role of Src kinase in ovarian function is the emerging understanding of the interactions of the estrogen receptor (ER) and Src. Recent studies have indicated that the genomic and non-genomic activities of the ER are interrelated and there is significant cross talk with Src *(21)*. Estrogen-induced activation of the Src/ERK phosphorylation cascade triggers vital cellular functions, including cell proliferation and differentiation and Src can phosphorylate ER *(22)*. Early studies indicated estrogen was important in ovarian follicular development since exogenous estrogen administered to immature mice increased the number of large preantral and small antral follicles *(23)*. More recent studies have further illustrated that the growth of follicles is dependent on estradiol *(24,25)*. In the absence of estradiol receptor-α follicles grow only to the preantral stage *(24,26)*. This growth is limited due to a loss of estrogen action on the granulosa cell via reduced cyclin D2 *(27)*. In addition, the aromatase knockout mouse exhibits impaired follicle development past the preantral stage *(28)*. Thus, the data from these two knockout mice clearly define a role of estradiol in growth of the follicle past the preantral or early antral stages (for review see ref. *24*).

Ovaries from Src knockout mice contained no preovulatory follicles. In addition 50–70% of the antral follicles observed were atretic (Fig. 4B) and the ovaries never contained corpora lutea. Thus, in the absence of fully functional Src kinase, normal follicle development was disrupted. Possibly, the disrupted follicle development observed in the Src knockout ovary is, at least in part, due to the loss of Src-ER interaction, limited activity of ER, and reduced granulosa cell proliferation. The question remains as to whether the total lack of Src in the knockout mouse interferes with the ability of endogenous estradiol to stimulate follicular development. The lack of estrogen action (due to the lack of Src) may be the cause of infertility (lack of follicle development and an LH surge) in the Src knockout mouse.

Total lack of Src resulted in several reproductive phenotypes in females. First, vaginal opening either did not occur or was delayed in Src knockout mice. This observation may reflect a loss of estrogen action as vaginal opening in mice occurs in response to increasing estradiol produced by maturing follicles around at the time of the first ovulatory cycle *(29)*. In Src knockout mice where limited follicle development is observed, it would be expected that estradiol levels are reduced. Thus, if Src does not play a direct role in the mechanism of vaginal opening, disruptions in vaginal opening observed in the Src knockout mouse may be a result of limited follicle growth and resultant reduced estradiol levels. Why some Src knockout mice did undergo vaginal opening is not clear. Those knockout mice that underwent vaginal opening did not exhibit normal estrous cycles (Table 2). Fewer total cycles occurred over time with significantly less time spent in proestrus likely due to the fewer numbers of antral follicles in ovaries of Src knockout mice. Thus, a waxing and waning of small antral follicles (without ovulation) in the Src knockout mice could have resulted in vaginal cyclicity.

Limited follicle development may also be a result of an ineffective gonadotropin signal. LH and FSH levels in Src knockout and wild-type mice were not different on d 40 and 52 of age, although they both tended to be lower in knockout mice. Possibly, the pulsatile secretion of LH and FSH are altered in the knockout mice and thus the ovaries do not receive the appropriate regimen of LH and FSH. In mice, owing to their small size, it would be difficult to assess LH/FSH pulsatility. Previous studies have implicated Src in GnRH regulation of the glycoprotein α subunit promoter *(30–32)*. Measurement of LH and FSH in sera at various times of the day and cycle may provide insight into whether the Src knockout mice are gonadotropin deficient. Gonadotropin with altered biological activity and/or a reduced responsiveness of the ovary to gonadotropin may also explain limited follicle development in the presence of "normal" serum levels of LH and FSH in Src knockout mice. Administration of exogenous gonadotropin-induced ovulation in some Src knockout mice. Again the penetrance of the knockout phenotype was not complete as some mice did not respond to eCG/hCG with ovulation. Thus, exogenous gonadotropin could, at least in part, rescue the ovulatory defect in Src knockout mice. Thus, it would appear that the endogenous gonadotropin in Src knockout mice may have lacked full biological activity. Whether this is due to modifications in the gonadotropin itself, the ability to respond to the endogenous hormone, or an alteration in LH/FSH pulsatility is not yet known.

In summary, the results indicate that female Src knockout mouse are infertile and exhibit reduced antral follicular development and anovulation. The reduced follicular development and delayed vaginal opening indicate a lack of estrogen and/or gonadotropin action. Future studies are aimed at more detailed analysis of gonadotropins and the secretion pattern in the Src knockout mouse.

Materials and Methods

Animals

Src tyrosine kinase heterozygous mice on a C57BL6 background were obtained from Jackson Laboratories (Bar Harbor, ME), and were established as a breeding colony in our laboratory. Targeted disruption of Src in this line of mice was originally carried out by Dr. Philippe Soriano and has been thoroughly described *(15)*. Breeding pairs, a single +/– male and a single +/– female were paired at the time of weaning, 20 d of age (d 0 was considered the day of birth), and were housed together continuously. Animals were checked daily for parturition, and the date and number of pups born were carefully monitored. Breeding pairs produced +/+, +/–, and –/– pups. All pups were weaned on d 20 of age and the sex and phenotype were recorded. Genotype was confirmed by PCR as described by Jackson Laboratories and the Soriano Lab (http://www.fhcrc.org/labs/soriano/protocols/pcrgen.html). Phenotype always reflected the genotype as originally described *(15)*: wild-type mice had a black coat and were normal size; heterozygous mice had an agouti coat, were normal size, and incisors erupted; knockout mice had agouti coats, were runted from birth, and incisors did not erupt. Soft chow was provided to all knockout mice at the time of weaning. Runted knockout mice were healthy and as vigorous as wild-type and heterozygous mice. Mean weight (in grams) for females on d 21 of age was: +/+, 7.2 $\pm$ 0.4 (n = 16); +/–, 7.3 $\pm$ 0.4 (n = 29); –/–, 3.7 $\pm$ 0.4 (n = 14), on d 55 +/+, 27.0 $\pm$ 1.2 (n = 5); +/–, 26.4 $\pm$ 01.3 (n = 5); –/–, 14.0 $\pm$ 0.9 (n = 5).

To assess vaginal opening and cyclicity, female mice were housed four per cage beginning on the day of weaning and checked daily between 0800 and 0900 h. The day of vaginal opening was recorded. Vaginal lavage was initiated on d 45 for +/+ and +/– mice and at least 10 d after vaginal opening in Src knockout mice. Vaginal lavage was carried out for at least 42 consecutive days. Day of the estrous cycle

was assigned according to that described by Allen *(33)*. Vaginal opening and cyclicity were assessed in 4 to 12 mice of each genotype.

To assess fertility of knockout mice breeding pairs were established as follows: female –/– and male –/–; female –/– and male +/–; female –/– and male +/+; female +/– and male –/–; and female +/+ and male –/–. Breeding pairs were housed together continuously for 8 mo beginning at the time of weaning. Mice were monitored daily to assess for the delivery of pups. Mating of knockout female mice was assessed by placing a female knockout mouse in the cage of a proven fertile +/+ male mouse at 1700 h just before lights out; +/+ males were paired with +/+ female mice as controls. The presence or absence of a copulatory plug was determined at 0900 h the following morning. All mice were provided food and water *ad libitum*, exposed to a 12 h light: 12 h dark schedule and housed under pathogen-free conditions. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Ovulation Induction

At 0900 h on d 29 of age female mice were administered 0.36 IU eCG per gram body weight subcutaneously to induce follicle development. Dose of eCG was based on body weight due to the weight disparity between +/+, +/–, and –/– mice. Fifty-two hours later an ovulatory dose (2.5 IU) of hCG was administered. The following morning mice were sacrificed, oviducts were flushed with saline, and the shed ova enumerated.

Follicle Assessments

Ovaries were collected from +/+ and –/– mice on d 12, 20, 26, 40, and 52 of age, fixed in Bouins' solution overnight, and processed and embedded in paraffin using standard procedures. One ovary from each mouse was serially sectioned at 8 μm, placed on glass microscope slides, and stained with hematoxylin and eosin. Healthy and atretic follicles were enumerated in every fourth section according to criteria of Pedersen and Peters *(34)* and Byskov *(35)*. Only follicles containing the nucleus of the oocyte were counted. Follicles from between four and seven ovaries from different animals were counted on each day of age as listed above. Ovaries were photographed using a digital camera on an inverted Olympus 1X71 microscope with a 4× objective.

Src Immune Complex Kinase Assay

The Src kinase assay was carried out as previously described *(7,36)*. Briefly, immature Src +/+ mice (d 25 of age) were injected with 2.5 IU eCG. Ovaries were collected at various time points, as indicated, homogenized and immunoprecipitated with Src-specific antibodies (1 μg; AB327; Oncogene Science Inc., Cambridge, MA). Immunoprecipitates were collected and Src kinase activity was determined by the addition of [γ-^{32}P]ATP and enolase as substrate. Reaction products were subjected to PAGE on a 10% SDS gel, visualized, and analyzed by phosphoimaging (Packard BioScience, Meriden, CT).

RT-PCR

Total RNA was isolated using TRIzol® reagent according to manufacturer's instructions. Total RNA (3 μg) was treated with DNase I and each sample was divided into two (one without M-MLV as a control and one with M-MLV as the specific) prior to RT using random primers with M-MLV. A portion of the RT reaction was used for PCR with the following primers; forward 5' TCAACGCCGAGAAC CCGAGAG 3' and reverse 5' GTTCATCCGCTCCACAT AGGC 3' resulting in amplification of a 657-bp fragment (mouse Src mRNA GenBank 6678128). In addition L19 was amplified in each tube with the primers 5' CTGAAGG TCAAAGGGAATGTG 3' and 5' GGACAGACTCTTGA TGATCTC 3' and served as a control.

Total RNA isolated from Src knockout (SYF) and Src expressing (SYF+c-Src) fibroblast cell lines was used as control for RT-PCR experiments. SYF and SYF+c-Src cell lines were purchased from ATCC (CRL-2459 and CRL-2498, respectively) and were cultured according to the methods described by ATCC.

RT-PCR products were separated on 1.5% agarose gels containing ethidium bromide and photographed under UV illumination. RT-PCR experiments were repeated at least three independent times using total RNA from ovaries collected from different mice.

Mouse LH and FSH Analysis

Serum concentrations of mouse LH and FSH were determined in the Ligand Assay & Analysis Core Laboratory at the University of Virginia Center for Research in Reproduction (www.healthsystem.virginia.edu/internet/crr/ligand.cfm) using species specific ELISA.

Statistics

Data were analyzed by one-way or two-way analysis of variance (ANOVA) as appropriate using GraphPad Prism (www.graphpad.com). Differences were considered significant if $p \leq 0.05$.

Acknowledgments

The authors thank the Ligand Assay & Analysis Core Laboratory at the University of Virginia for radioimmunoassay of LH and FSH. The Virginia Center for Research in Reproduction is supported by U54-HD28934. This work was supported by a Specialized Cooperative Centers Program in Reproduction Research (HD33994-08, PFT).

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