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A Thesis Submitted to the
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ABSTRACT

This study aimed to assess the hypothesis that the growth factor receptor c-kit regulates apoptosis in the prepubertal mouse ovary. Employing an in vivo mouse model, c-kit translation was locally blocked by intra-ovarian bursal injection of oligonucleotides antisense to c-kit in 20-day-old gonadotropin primed female mice. Effects of the antisense treatment were assessed using DNA fragmentation analysis and the terminyl deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) technique and compared to effects of missense oligonucleotide treatment. DNA laddering, a hallmark of apoptosis, was induced in ovaries by the c-kit antisense treatment six hours post-treatment and was significant compared to minimal laddering induced six hours following missense treatment. No significant differences between treatment groups were observed 2, 4, 8, 18, and 24 hours following treatment. To determine whether these results could be confirmed by an alternative method of apoptosis detection, the in situ TUNEL technique was applied, however a functional assay could not be established. The results of this study indicate that apoptosis may be induced in prepubertal mouse ovaries six hours following local blockade of c-kit using antisense oligonucleotides, although confirmatory studies are required.

ACKNOWLEDGEMENTS

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INTRODUCTION

The c-kit growth factor receptor functions as an essential but uncharacterized regulator of mammalian ovarian follicle development. C-kit is a proto-oncogene at the White spotting (W) locus on mouse chromosome 5 and human chromosome 4 and encodes a transmembrane tyrosine kinase receptor that is a member of the platelet-derived growth factor receptor subfamily.[1-3] The ligand for the receptor is a glycoprotein termed kit ligand (KL),[4] steel factor (SlF), stem cell factor (SCF),[5, 6] and mast cell growth factor (MGF),[7, 8] and is encoded at the Steel (Sl) locus on mouse chromosome 10 and human chromosome 12.[3, 9-11] The significance of c-kit in oocyte development was recognized when mutations in the loci encoding c-kit or its ligand resulted in defects in gametogenesis in mice.[1, 9, 10, 12] Mice with double dose mutations at either the W or Sl loci generally exhibit sterility, in addition to hypoplastic anemia, mast cell deficiency, and cutaneous melanocyte deficiency.[9, 10] The sterility in females with Steel loci mutations is manifest by a decrease in oocyte number and developmental arrest at the one-layer follicular stage.[12] It has since been established that KL and c-kit are important regulators in embryonic oogenesis and that KL is required for the in vitro survival and proliferation of primordial follicular cell complexes in culture.[13-16] Further, blockade of c-kit function in utero, through the administration of a c-kit specific antibody, results in severely impaired early development of existing primordial follicles in fetal and neonatal mice.[17-19]

Throughout development, it appears that the level of c-kit and KL expression in the ovary correlates with the rate of oocyte growth. C-kit and KL expression is highest in oocytes that are growing rapidly and is lowest in oocytes where growth has been suspended or has ceased.[20, 21] C-kit mRNA is first detected at low levels in primordial germ cells as early as 8 days post coitum and at increasing levels in germ cells as they migrate to the genital ridge and differentiate into male and female gonads.[22] During the perinatal period, most oocytes have entered the quiescent prophase stage of meiosis, and c-kit expression is no longer detectable.[20, 21] In the postnatal ovary, expression of KL and c-kit resumes in the central developing portion of the ovary. By postnatal day 5, growing 1- and 2-layer follicles demonstrate high levels of c-kit and KL expression which progressively increases in growing follicles up to day 17.[21, 23] Thereafter, KL expression is limited to the outer rim of multi-layer follicles. In atretic follicles, c-kit and KL expression fall to minimal levels.[21] These temporal expression patterns of c-kit and KL correspond to their function in postnatal folliculogenesis. Stepwise in vivo antibody blockade of c-kit function in newborn and juvenile mice significantly impairs primordial follicle development, primary follicle growth, follicular fluid formation, and preovulatory follicle maturation.[24]

In the postnatal ovary, oocytes and thecal cells have been shown to express the c-kit receptor while granulosa cells and stromal derived cells express KL.[21, 23, 25-27] In vitro oocyte growth of follicles derived from juvenile mice is stimulated by KL in a dose dependent manner.[28] Further, growth of oocytes in vitro is inhibited by a functional c-kit blocking antibody.[28] Thecal cells in bovine follicular cultures are also stimulated by the

addition of KL which results in cell growth, development, and steroid production.[26] C-kit and KL may therefore mediate a signaling pathway between oocytes, granulosa cells, and thecal cells in growing and developing follicles in the postnatal ovary. It is likely that KL, produced by granulosa cells, acts as an oocyte and thecal cell growth factor by phosphorylating downstream targets via the c-kit receptor, although the location and function of these targets are not known.

Limited evidence suggests that activation of c-kit and KL may function to inhibit apoptosis. Well established roles for apoptosis have been defined in ovarian prenatal development and postnatal tissue homeostasis. Apoptosis is as an active process of cell death that is gene directed and occurs to fulfil a biological function.[29, 30] Morphological features characterizing apoptosis include cell shrinkage, cytoplasm collapse, condensation of chromatin, nuclear fragmentation, and membrane blebbing.[29-31] Apoptosis can be further distinguished from other forms of cell death by the characteristic activation of internucleosomal nucleases producing cleaved DNA fragments that are multiples of approximately 185 basepairs in length.[32]

The rapid prenatal attrition of female germ cells may be explained by the induction of apoptosis due to an inadequate source of KL during the perinatal period. This is supported by studies where apoptosis in cultured primordial germ cells or fetal ovaries is repressed by the addition of KL.[33, 34] However, a role for c-kit in postnatal follicular cell death and homeostasis has not been addressed. In the postnatal ovary, the majority of follicles that undergo endocrine dependent development ultimately undergo atresia and luteal regression following apoptosis in granulosa cells.[31, 35-37] In prepubertal

rats stimulated with gonadotropins, apoptotic granulosa cells can be detected in atretric follicles within 48 hours of treatment and by five days following treatment, all atretic follicles demonstrate apoptotic granulosa cells.[36] In adult rat ovaries, apoptosis in atretic follicles can be observed in granulosa cells and occasional thecal cells throughout the estrous cycle.[36] All follicles may contain a program to undergo apoptosis and progression toward ovulation may be due to constant suppression of apoptosis in follicular cells. The presence or withdrawal of factors likely modulate the induction of apoptosis and subsequent follicular atresia in the ovary.[31, 37] For example, gonadotropin withdrawal in rats using an in vivo treatment of an anti-equine chorionic gonadotropin antibody rapidly induces apoptosis in antral follicles.[38] Factors which have been proposed to modulate apoptosis in the ovary include epidermal growth factor, transforming growth factor α, basic fibroblast growth factor, insulin like growth factor I, keratinocyte growth factor, hepatocyte growth factor, vasoactive intestinal peptide, gonadotropin releasing hormone, transforming growth factor ß, tumor necrosis factor α and dehydroepiandrosterone (DHEA).[35, 36, 39]

Because apoptosis is a process under gene control, these potential regulatory factors must function by up- or down-regulating specific apoptotic inducer and repressor gene products. One theory maintains that gonadotropins and other growth factors may regulate apoptosis in the postnatal ovary by altering the balance of members of the bcl-2 gene family. Overexpression of bcl-2 in transgenic mice is associated with decreased apoptosis in follicular cells and enhanced folliculogenesis.[40] Specifically, gonadotropins may inhibit apoptosis in the developing rat ovary by increasing levels of apoptosis

suppressor genes bcl-2 and bcl-x^{short}, while decreasing levels of expression of apoptosis inducer genes bax and bcl-x^{long}.[41] Gonadotropins and other factors may alternatively mediate apoptosis by modulating levels of the death inducer gene p53.[42, 43] Interestingly, the mechanism of p53 mediated induction of apoptosis in the ovary may be via up-regulation of the bax gene or down-regulation of the bcl-2 gene.[42, 44] Additionally, increased levels of fas and fas ligand have been observed in mouse and rat atretic follicular cells and may also be involved in the induction of apoptosis in the mammalian ovary.[45-47]

Although the mechanism and function of c-kit and KL have not been defined, considerable evidence suggests that this ligand-receptor pair regulate the expression of apoptotic inducer and/or repressor genes.[33, 48, 49] In cultures of natural killer cells, the addition of KL results in an up-regulation of the apoptotic suppressor gene product, bcl-2, and a subsequent inhibition of apoptosis.[49] KL also reversibly rescues IL-3-deprived mast cells from apoptosis in vitro. However, in mast cells, KL does not inhibit apoptosis by inducing the expression of bcl-2, but by down-regulating expression of the apoptotic inducer gene product, bax.[50-52] In Friend erythroleukemia cells, KL inhibits apoptosis via suppression of a p53 mediated pathway.[53] The observation that apoptosis is inhibited in primordial germ cell cultures by the addition of KL supports the concept that c-kit and KL function to repress apoptosis during oocyte development.[33, 34]

Thus, an essential role for c-kit in prenatal and postnatal ovarian folliculogenesis has been firmly established. While some evidence indicates that c-kit may function to

inhibit apoptosis in natural killer cells, mast cells, and primordial germ cells, the mechanism of function of c-kit in the postnatal mammalian ovary is not known.

STATEMENT OF PURPOSE

This study was designed to determine if c-kit's function in postnatal folliculogenesis can be explained by repression of apoptosis in developing follicular cells. To assess this, an in vivo mouse model was employed to assess the effects of blocking ovarian c-kit expression by intra-ovarian bursal injection of oligonucleotides antisense to murine c-kit cDNA. Apoptosis was subsequently assessed using DNA fragmentation analysis as well as an in situ apoptotic detection methodology. The specific hypothesis addressed is: Does in vivo blockade of c-kit function induce apoptosis in developing prepurbertal mouse ovaries?

METHODS

All methods described below were planned, optimized, and performed by the student independently with the exception of the mouse in vivo intra-ovarian bursal injections which were performed jointly by the student and Dr. Hugh Taylor.

Oligonucleotides.

To block c-kit function in mouse ovaries in vivo, 25 base pair antisense oligonucleotides were synthesized to target a region near the translation start codon of murine c-kit mRNA corresponding to nucleotides 120-146. [3] The sequence of the antisense c-kit oligonucleotide was 5' GGA TGG ATG GCG GAG ACG GCT CCC C 3'. This oligonucleotide sequence was selected based on its previously demonstrated blockade of c-kit protein expression and function.[54] When microinjected into rat oocytes, this sequence significantly decreased cell-surface expression of c-kit and inhibited oocyte germinal vesicle breakdown.[54] To provide a control oligonucleotide treatment, all nucleotides of the antisense oligonucleotides were synthesized in a random order to create a missense oligonucleotide. The c-kit missense sequence was 5' CTG GCT GAC CTG CCC CAC GTG GAC C 3'.[54] Oligonucleotides were synthesized, phosphorothioate modified, and OPC cartridge purified by the Program for Critical Technologies in Molecular Medicine of the Yale University School of Medicine Department of Pathology. Oligonucleotides were dried under vacuum and resuspended in sterile PBS at a concentration of 600 μM.

Animals and Surgical Procedure

Prepubertal CD-1 female mice (Charles River Laboratories, Wilmington, MA) ranging from twenty to twenty-three days of age were included in the study and maintained in accordance with the guidelines of Yale Animal Care and Use Committee (YACUC). Mice were anesthetized by intraperitoneal injection with a mixture of 100 mg/kg body weight ketamine and 20 mg/kg xylazine provided by the Yale Animal Care Resources. Ovaries were exposed by laparotomy using a ventral vertical incision and isolated under a dissecting microscope. A method of intrabursal administration of DNA in rat ovaries described by Piontkewitz *et al.* was adopted for this study.[55] Twenty microliters of 600μM antisense or missense oligonucleotides in PBS were loaded into a 100-microliter syringe (Hamilton Co., Reno, Nevada) and slowly injected through a 32 gauge needle into the ovarian bursa of each ovary. Care was taken to inject the treatment in the space between mesothelium surrounding the ovary and the ovarian surface and to avoid puncture of the ovary. If the ovary was punctured and bleeding ensued the mouse was marked and not included in the analysis. Both ovaries in each mouse received the same treatment solution. Peritoneal and skin incisions were closed using fine sutures. To initiate follicular growth 90 minutes following surgery, mice received 5 IU of pregnant mare's serum gonadotropin (PMSG) (Sigma-Aldrich, St. Louis, MO) administered by subcutaneous injection using tuberculin needles. One to 24 hours following gonadotropin treatment ovaries were collected following anesthetization and repeat ventral laparotomy. Following ovary collection and preparation, mice were sacrificed in accordance with the guidelines of YACUC.

DNA Extraction

Ovaries were dissected free from associated ovarian mesothelium, fallopian tube and adipose tissue. Ovaries were quickly cut into small pieces using a sterile disposable blade and immediately frozen in polyethylene 2.0 ml cryotubes on dry ice. Genomic DNA was extracted using the QIAamp Tissue Kit (Qiagen Inc., Santa Clarita, CA) according to the manufacturer's instructions. To obtain RNA-free genomic DNA, samples were digested with RNAse A (DNAse free, Boehringer-Mannheim Corp.) at 10 μg/ml for 10 minutes at room temperature either following tissue lysis or nucleic acid elution steps based on a protocol adapted from Palumbo and Yeh, 1994.[39] The quality and purity of each nucleic acid sample was determined by measuring optical densities at A260 and A280 nm.

DNA Fragmentation Analysis

A biochemical characteristic of apoptosis is the activation of an intracellular endonuclease activity. This results in inter-nucleosomal cleavage of genomic DNA into fragments of multiples approximately 185 base pairs in length.[32, 56] Apoptosis can therefore be detected by a distinctive ladder pattern following size fractionation by agarose gel electrophoresis.[32] To analyze samples for DNA fragmentation, equal quantities of purified RNA-free DNA (2 μg) of each sample were added to 4 μl gel loading buffer and double distilled water to total 35 μl. Samples were electrophoresed on 1.8% agarose gels containing ethidium bromide (0.5 μg/ml) submerged in TAE buffer (40mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.2) for 50 minutes at 90 V. Four micrograms of a 123 base pair DNA ladder (GibcoBRL, Life Technologies, Inc.,

Gaithersburg, MD) was run simultaneously for size comparison. Gels were visualized and photographed on an UV transilluminator.

Histology

For morphological analysis, ovaries were isolated and removed in association with surrounding mesothelium, tube, and adipose tissue. Ovaries were immediately placed in cassettes (Tissue Tek) and fixed in 10% formalin overnight at 4°C. Ovaries were then paraffin-embedded, sectioned 4-6 μm, and mounted on poly-L-lysine-coated slides as routine by the Yale University School of Medicine Department of Surgical Pathology. Sections were heated for 30 minutes at 60°C and deparaffinized through three 5-minute washes of xylene, then rehydrated through 3-minutes washes of 100%, 90%, and 75% ethanol, and two 1-minute washes in double distilled water. Sections were stained with hematoxylin for 45 seconds, set in aqueous mounting medium (Crystal/Mount, Biomeda Corp., Foster City, CA), and examined and photographed under a light microscope. Follicle diameter was measured using a calibrated ocular micrometer. Follicles were classified as small, medium, or large using the methodology described by Pedersen and Peters, 1968.[57]

In situ apoptosis detection

DNA fragmentation distinctive of apoptosis was detected in situ by using a modified protocol of the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) technique.[58] Apoptotic breaks in nuclear DNA were detected by labeling free 3'OH ends of DNA with fluorescein modified nucleotides using terminal

deoxynucleotidyl transferase.[58] To detect in situ apoptosis, a commercially available kit was used with modifications of the manufacturer's protocol (In Situ Cell Death Detection Kit, Boehringer-Mannheim, Corp.). Ovaries were isolated and removed in association with surrounding tissues as above and fixed in 10% formalin, 4% paraformaldehyde (prepared from 16% stock solution) or 4% paraformaldehyde, pH 7.2, freshly prepared from solid for ½, 1, 1½, 6, or 12 hours at 4°C. Tissue was embedded in paraffin, sectioned, mounted and deparaffinized as above. The assay was initially performed on archival normal adult mouse ovary paraffin embedded sections to test the efficacy of the technique. For proteolytic digestion, tissue was treated with 20 or 200 μg/ml proteinase K (Sigma-Aldrich, St. Louis, MO; 10 mM TrisHCl, pH 7.6) for 30 minutes at room temperature or 37°C. Additionally, some samples were permeabilized by treating with 0.5% Triton X-100 (Rohm & Haas, Philadelphia, USA) in 0.1% sodium citrate for 2 minutes on ice. To alternatively achieve proteolysis, tissue was subjected to 1-minute microwave irradiation at 750 W (high) in 0.1 M citrate buffer, pH 6.0, followed by gentle cooling and washing in PBS, pH 7.4.[59] For samples that were labeled with horseradish peroxidase (POD), endogenous peroxidase activity was blocked by incubating samples in 0.3% hydrogen peroxide in methanol for 20 minutes at room temperature. A hydrophobic marker (Pap Pen, Research Products International Corp., Mount Prospect, IL) was used to create a barrier encircling tissue. To reduce non-specific binding, samples were blocked with 10% fetal bovine serum (FBS) for 30 minutes at room temperature and washed twice with PBS. DNA fragments were 3'-OH-labeled using deoxynucleotidyl transferase diluted 1:5 in a reaction buffer containing fluorescein modified nucleotides as directed by the manufacturer or at additional dilutions of 1:10, 1:50, 1:100, 1:1,000 and 1:10,000. To

label nuclear DNA, 35-50 µl of the enzyme/buffer solution was added to each tissue section, coverslips were applied to avoid evaporative losses, and slides were incubated at 37°C for 30 minutes or 1 hour. Slides were washed with three 1-minute PBS washes. Signal conversion of samples was achieved by applying 35-50 µl of a anti-fluorescein antibody, fab fragment from sheep, conjugated with alkaline phosphatase (AP) or horseradish peroxidase (POD) non-diluted or at a 1:2 or 1:5 dilution in blocking solution. Coverslips were applied and slides were incubated at 37°C for 30 minutes, then washed three times with PBS. To develop sections labeled with AP, slides were flooded with an AP developing solution (Histomark, Ph Thalo Red Solution, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) prepared according to the manufacturer's instructions and incubated for 10 minutes at room temperature and then washed in distilled water. To develop sections labeled with POD, a DAB/metal concentrate (Boehringer Mannheim Corp.) was prepared at a 1:10 dilution in peroxide buffer (Boehringer-Mannheim Corp.), applied to slides for 30-90 seconds after which the reaction was stopped by washing slides in distilled water. Slides were counterstained, mounted, and visualized as above. To prepare a positive control, DNA nicks with free 3'OH ends were induced by treating tissue with 1 mg/ml DNAse I (Boerhinger-Mannheim, Inc.; 1mg/ml BSA, 1 mM $MgCl_2$, 50mM TrisHCl, pH 7.5) for 10 minutes at room temperature. Substitution of the TdT enzyme with buffer solution served as a negative control.

RESULTS

Effect of RNAse treatment on DNA Fragmentation Analysis

Non-RNA-free DNA was extracted from ovaries collected 6 and 18 hours following in vivo intrabursal treatment with c-kit antisense (AS-6h, AS-18h) or missense (MS-6h, MS-18h) oligonucleotides and PMSG priming without RNAse treatment. Ratios of optical densities at A260 and A280 ranged from 1.605 to 1.851 and confirmed purity of nucleic acid samples. DNA (2 μg) from each sample was fractionated by electrophoresis for DNA fragmentation analysis. In all fractionated DNA samples, a nucleic acid smear with weak laddering was observed (Figure 1). To determine if nucleic acid smear was attributable to an overlay of RNA, 2 μg each of MS-6h, MS-18h, and AS-18h DNA was treated with RNAse. The RNAse-treated samples were then electrophoresed simultaneously with an additional 2 μg of MS-6h DNA that was not treated with RNAse. The nucleic acid smear present in the non-RNAse-treated MS-6h DNA was absent in the RNAse treated MS-6h DNA and in the RNAse-treated MS-18h and AS-18h DNA (Figure 2). Laddering was visible in the AS-18h DNA, and weakly visible in the MS-18h DNA. The size of the DNA fragments was estimated to be 1.5 times the length of the simultaneously run 123 base pair DNA ladder fragments or approximately 185 base pairs.

DNA fragmentation analysis 6, 18, and 24 hours post treatment

DNA extracted from ovaries 6, 18, or 24 hours following antisense (AS) or missense (MS) oligonucleotide treatment was purified of RNA through RNAse treatment. 2 μg of each RNA-free DNA sample was fractionated by electrophoresis for fragmentation analysis. Strong laddering was observed in AS-6h DNA, less strong laddering in the AS-18 h DNA, and weak laddering in AS-24h and all MS treated ovarian DNA (Figure 3).

DNA fragmentation analysis 2, 4, and 8 hours post treatment

To further define the time course of DNA fragmentation following treatments, DNA was extracted from ovaries 2, 4, and 8 h following AS or MS oligonucleotide treatment. RNAse was added to ovarian tissue samples following proteolysis but prior to DNA purification and elution to increase yield. 2 μg of DNA was fractionated by electrophoresis. At 2h, both MS and AS treated ovaries demonstrated strong laddering, although laddering of AS-2h was slightly more pronounced than MS-2h (Figure 4). Weak laddering was observed in all other DNA, however MS-4h laddering was slightly less than MS-8h and AS-4h, and laddering in AS-8h was slightly greater than AS-4h. DNA fragmentation analysis in untreated mice or mice treated with PBS alone were not conducted and therefore these results are unavailable for comparison.

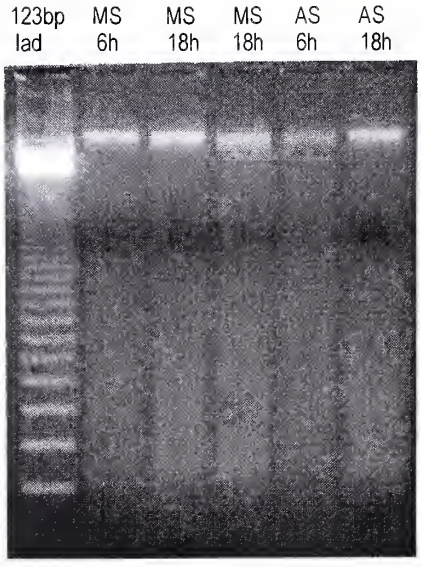


Figure 1. Fractionated DNA purified from ovaries 6 or 18 h post MS or AS treatment without RNAse Treatment. 2 ug DNA per lane.

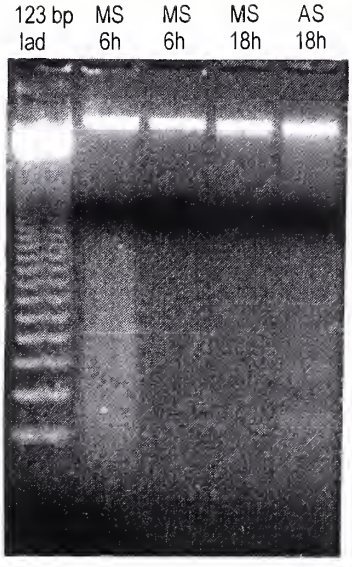


Figure 2. Fractionated DNA purified from ovaries 6 or 18 h post MS or AS treatment. Lane 1: no RNAse. Lane 2-4: RNA-free DNA. 2 ug DNA per lane.

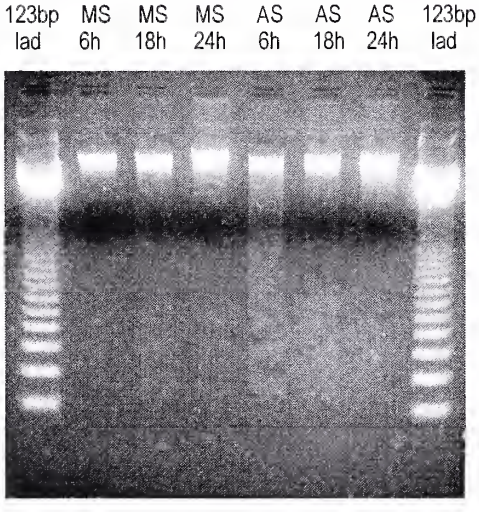


Figure 3. RNA-free DNA purified from ovaries 6, 18, or 24 h post MS or AS treatment. 2 ug DNA per lane.

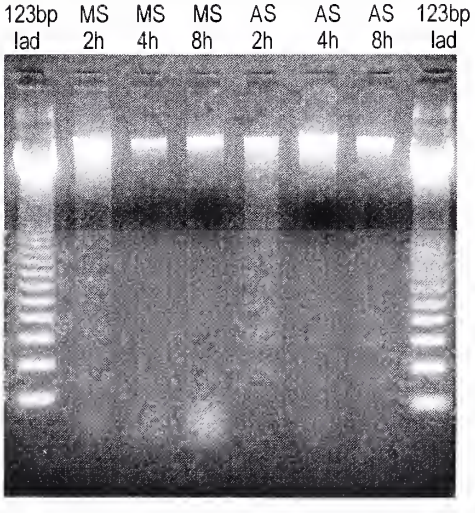


Figure 4. RNA-free DNA purified from ovaries 2, 4, or 8 h post MS or AS treatment. 2 ug DNA per lane.

In situ detection of apoptosis by TUNEL technique

To detect apoptosis in situ, the TUNEL technique was applied to paraffin embedded sections of ovaries 6, 18, and 24 hours following AS and MS oligonucleotide treatment. Initially, to test the efficacy of the technique, TUNEL was applied to archival paraffin-embedded mouse ovarian tissue. In this tissue, staining was specific to nuclei of granulosa cells near the antrum of a large atretic follicle indicative of a functional assay (Figure 8). Also, no staining was observed in the negative control and nuclei were stained in the positive control (DNAse-treated) slide, indicative of an efficacious technique (not shown). However, efficacy of the technique could not be demonstrated when repeated in the experimental antisense and missense treated ovarian tissue and high background unspecific staining was consistently observed. Figure 5 demonstrates this diffuse non-specific TUNEL nuclear staining in MS-18h treated ovaries fixed in formalin and developed with POD/DAB although some preferential staining of granulosa cells can be discerned. However, no differences in staining were seen in the similarly processed AS-18h treated ovaries shown in Figure 6 and Figure 7 (higher magnification). A methodical trouble shooting approach was undertaken to test effects on fixation, enzyme concentration, converter concentration, and developing method. However, a functional non-specific TUNEL assay could not be established. The best of all protocols tested employed an alternative proteolysis using microwave irradiation and citrate buffer as shown in MS-24h treated ovaries in Figure 9. Using this protocol, increased staining of granulosa cells of some follicles was observed, but background nuclear staining persisted (Figure 9).

Fig. 5.

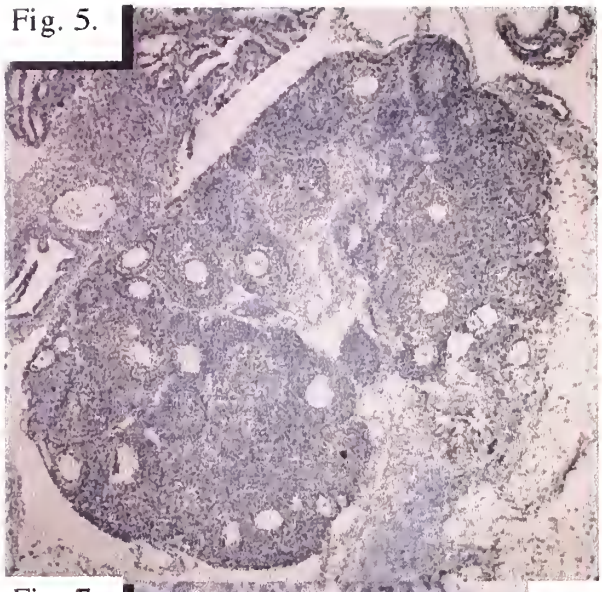


Fig. 6.

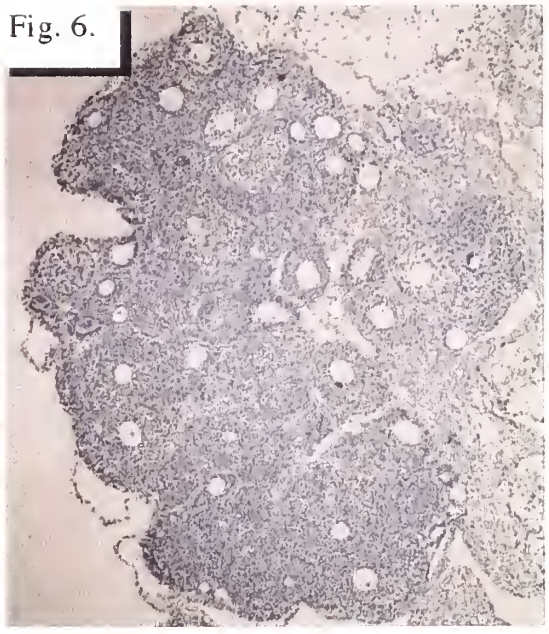


Fig. 7.

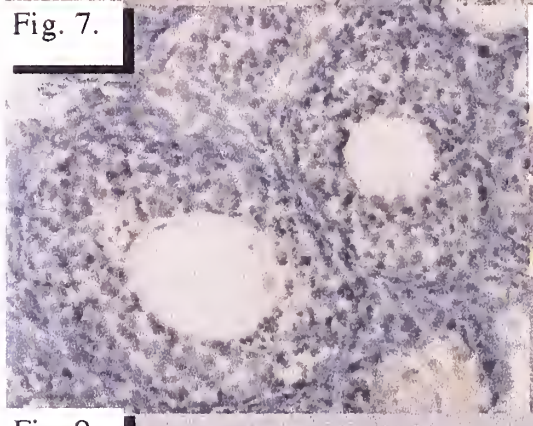


Fig. 8.

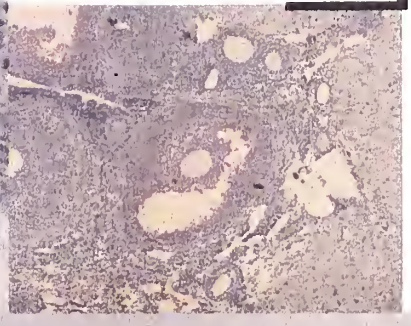
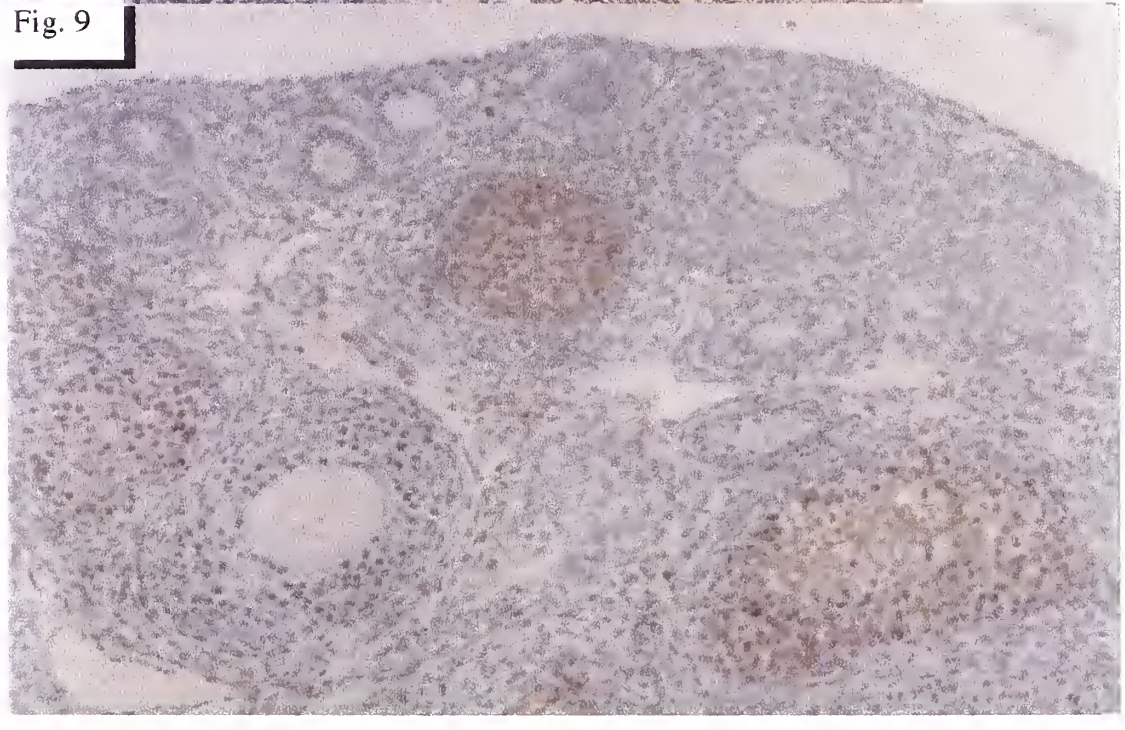


Fig. 9



Morphology

Paraffin embedded, hematoxylin stained, 4-6 μm sections of ovaries 6, 18, and 24 hours following AS and MS oligonucleotide treatment were examined for morphology under a light microscope. No differences in gross ovarian morphology, follicular development, or follicular diameter were observed between ovaries treated with MS and AS oligonucleotides. Figure 5 (MS-18h) and Figure 6 (AS-18h) demonstrate these findings. By 24 h, no atretic follicles were observed in either treatment group. Electron microscopy is beyond the scope of this project and therefore morphological evidence of apoptosis could not be assessed.

DISCUSSION

In this study, local blockade of c-kit translation in ovaries of gonadotropin primed mice was performed to characterize the role, if any, of c-kit in the mediation of apoptosis in developing postnatal ovarian follicles. By directly adapting the treatment protocol from prior published reports, the efficacy of c-kit blockade was assumed but not directly examined. The sequence used to synthesize oligonucleotides antisense to mouse c-kit cDNA in the present study had been previously shown by Ismail et al to impair the surface expression of c-kit when injected into rat oocytes.[54] Further, antisense treatment impaired a presumable c-kit-mediated function in meiosis resumption.[54] Additionally, the missense treatment had no effect on c-kit expression or function.[54] The c-kit antisense oligonucleotides used in this study and in the present study were synthesized to target the start region of mouse c-kit mRNA which is highly conserved in rats.[3,5,54] The route of administration differed in the present study and was not intracellular. Because the goal in the present study was to examine the effects of local c-kit blockade in the ovary, an intra-ovarian bursal administration was used. The intrabursal technique employed was directly adapted from work by Piontkewitz et al where expression and function of CCAAT enhancer binding protein-α was effectively blocked when oligonucleotides antisense to that cDNA message were injected into rat ovarian bursas in vivo.[55] The Piontkewitz et al study is the only one to demonstrate effective local blockade of mRNA expression in the mammalian ovary using antisense oligonucleotides. Therefore, the intra-bursal administration of oligonucleotides was adapted for the present study, as opposed to alternative and undocumented possible methods of intraperitoneal,

intravenous, or intra-ovarian injection of oligonucleotides. The present study is limited by a lack of direct evidence that the treatment impaired c-kit translation. While speculation that c-kit expression and function are impaired in the current model is reasonable, a direct examination of the time course of c-kit expression and potential blockade in response to treatment are needed. In aims to study the time-course response of c-kit protein expression following putative c-kit mRNA translation blockade, considerable efforts were undertaken to establish a functional immunohistochemistry methodology specific for murine c-kit. These efforts, however, were unsuccessful. As an alternative method to evaluate putative blockade of c-kit, efforts are now in progress to apply the reverse-transcriptase-polymerase chain reaction to amplify c-kit transcripts in ovaries following treatment with antisense and missense c-kit oligonucleotides. Successful quantification of c-kit mRNA transcripts following treatments would provide evidence to assess the efficacy of c-kit blockade as well as provide an assessment of the time-course of c-kit down-regulation. To further validate the efficacy of c-kit blockade, the present study would benefit from studies to localize the cellular targets of the antisense and missense oligonucleotide treatments. This could be easily achieved by labeling the oligonucleotides with an identifiable label, such as biotin, and performing immunohistochemistry using an anti-biotin antibody to verify distribution of oligonucleotides within the ovarian tissue.

The DNA fragmentation analyses suggest that the antisense oligonucleotide treatment is associated with increased levels of apoptosis in gonadotropin primed mouse ovaries. When DNA of treated ovaries was purified and fractionated by electrophoresis, evidence of apoptosis was established by the distinctive laddering pattern made by

internucleosomal fragments of multiples of 185 base pairs in length. Six hours following the antisense treatment, strong laddering of DNA was observed, and this was less intense but significant at 18 hours, and minimal by 24 hours. In contrast, weak laddering was observed at 6, 18, and 24 hours following missense treatment. These findings were consistent with the hypothesis that blockade of c-kit removed apoptosis suppression. However, when this experiment was repeated to examine the time-course effect around six hours, insignificant differences were observed between antisense and missense treatments at 2, 4, and 8 hours following treatment. Further, significant laddering was observed at 2 hours following both antisense and missense treatment. These findings raise the concern that apoptosis could be induced by the trauma of the intrabursal injection procedure or by non-specific effects of oligonucleotide treatment. It is therefore imperative that these experiments be repeated with the inclusion of a non-treated control and a vehicle (PBS) only control to assess the possible confounding affects of the treatment technique. However, it is also possible that differences in levels of apoptosis can not be discerned using a qualitative DNA fragmentation assay. It therefore may be necessary to quantify DNA fragmentation using 3' terminal transferase to attach a quantifiable label, such as radioactive dideoxynucleotides, to free 3' hydroxyl DNA termini.[40, 46, 60] Further, while c-kit blockade could potentially affect apoptosis in all ovarian cell types, it may be necessary to dissect oocyte and granulosa follicular cells from thecal cells to enable the detection small quantifiable effects in DNA laddering in specific cell populations that cannot be appreciated in the examination of the total genomic DNA of whole ovaries.

The time course of c-kit function in relation to PMSG-priming warrants further exploration in this study. Levels of c-kit and KL mRNA and protein expression correlate with primordial germ cell growth during prenatal development, and with oocyte and follicular cell growth during postnatal follicle development. In the postnatal ovary, an increase in c-kit and KL expression is seen in follicles progressing from primordial to large growing antral follicles. However, little or no expression is observed in atretic follicles.[21, 23] Further, c-kit appears to play an essential function in early follicular development as follicles in mice bearing the *Steel* panda mutations are arrested at the one-layer cuboidal stage.[12] In this study, treatment to block c-kit expression was given to prepubertal mice primed with PMSG shortly following treatment to initiate development in a large cohort of primordial follicles. Thus, the effect of blocking c-kit expression on apoptosis could be assessed in early postnatal follicle growth assuming that c-kit function is important soon after PMSG-priming. A prior study demonstrated that intraperitoneal injection of a c-kit functional blocking antibody induces follicular atresia and ovulation failure when administered 12 and 24 hours before gonadotropins but not when injected earlier or later.[24] While the present study provides some evidence for inhibition of apoptosis in ovaries 6 hours following oligonucleotide treatment, it is not known whether c-kit expression is in fact effectively blocked at that time-point. The intrabursal technique of administering oligonucleotides antisense to C/EBPα protein by Piontkewitz et al included gonadotropin priming 2 hours following surgery. They were able to demonstrate decreased C/EBPα protein 24 and 48 hours following surgery as well as an effect of the antisense treatment on follicle development.[55] The Ismail et al study where

antisense and missense mouse c-kit oligonucleotides were injected directly into rat oocytes, demonstrated decreased oocyte surface c-kit protein expression at 4 hours following treatment and enhanced meiosis resumption at 20-24 hours following treatment. In the present study, the effects of c-kit blockade using intrabursal injection of antisense oligonucleotides were presumed to occur between 4 and 24 hours based on these related studies. However, without direct examination the efficacy and time-course of the treatment-induced blockade of c-kit expression, interpretation of DNA fragmentation analyses at 2, 4, 6, 18, and 24 hours is limited. Therefore, an examination of the time-course of c-kit mRNA expression and blockade following the oligonucleotide treatment and PMSG priming is essential and would allow correlation of blockade of c-kit expression with effects, if any, on apoptosis.

Also important to note are the potential confounding effects of gonadotropins on c-kit expression. One study of 6-week-old female mice demonstrated a substantial reduction of c-kit RNA levels in thecal cells 90 minutes following intraperitoneal injection of 5 IU PMSG. Thecal cell c-kit RNA was undetectable by 4 hours following PMSG and remained undetectable at 8 and 45 hours. PMSG had no effect on oocyte c-kit RNA expression. In the present study, inhibition of c-kit expression may have been mediated by PMSG, therefore negating attempts to study the specific effect of blocking c-kit using oligonucleotides antisense to c-kit. Further, the 5 IU dose of PMSG used is recommended for adult mice and may represent a supraphysiologic dose in 20-day-old mice. Thus, the strong DNA laddering observed 2 hours following surgery in both AS and MS oligonucleotide groups may in part be explained by a gonadotropin-mediated inhibition of c-kit expression in both the treatment and control groups and subsequent

loss of c-kit suppression of apoptosis. However, physiological levels of gonadotropins are not associated with the induction of apoptosis in the mammalian ovary. Rather, withdrawal of gonadotropin support is associated with the induction of apoptosis in granulosa cells and follicular atresia.[37, 38] Subsequent study of the effects of c-kit antisense oligonucloetide treatment in the absence of PMSG should be conducted to remove the confounding effects of exogenous gonadotropins.

Ultimately, assessment of apoptosis induction by a methodology in addition to DNA fragmentation analysis would be helpful in assessing validity of results. In this study, considerable efforts were taken to establish a protocol to detect apoptosis in situ using the TUNEL technique. Unfortunately, after manipulation of each step in the protocol and consultation with experienced researchers, a functional assay could not be developed. Additionally, it was planned to include an analysis of effects of antisense c-kit oligonucleotide treatment on the expression of apoptosis markers through Northern analysis of extracted RNA. As in vitro evidence in other cell lineages suggests that c-kit may regulate bcl-2, bax, or p53 expression, examining mRNA levels of these apoptotic markers after of blocking c-kit in vivo could provide useful quantifiable data regarding c-kit function in the ovary.[49-53]

In summary, the findings of this study indicate that induction of apoptosis in prepubertal mouse ovaries may be induced by the intra-bursal administration of c-kit antisense oligonucleotides and that this effect may be maximal six hours following treatment. Confirmatory evidence for these findings was not demonstrated, and therefore future studies are necessary to adequately assess the validity of the hypothesis that c-kit inhibits apoptosis in the post-natal mouse ovary. To validate the efficacy of the treatment

method, studies using reverse-transcriptase polymerase chain reaction should be performed to determine whether c-kit mRNA expression is down-regulated following treatment with antisense but not missense oligonucleotides. Further, c-kit mRNA expression following oligonucleotide treatment should be quantified at time-points between 4 and 48 hours to describe the time-course of the effects of the treatment and allow correlation with possible effects on apoptosis. Interpretation of DNA fragmentation analysis could be facilitated by labeling DNA fragments and quantifying the amounts of laddering induced by c-kit antisense compared to missense oligonucleotide treatment. To remove possible confounding effects of gonadotropins, DNA fragmentation analysis could be performed in ovaries treated in vivo with c-kit antisense oligonucleotides in the absence of PMSG. Finally, an alternative method to evaluate the effects of c-kit blockade on apoptosis in the ovary could be employed to validate the results, as efforts to utilize the TUNEL technique for this purpose proved unsuccessful. To this end, levels of apoptosis related gene products bcl-2, bax, and p53 could be isolated and quantified by RNA extraction and Northern analyses and compared between c-kit antisense and missense oligonucleotide treated ovaries. Pursuit of the proposed future studies may further assess the hypothesis posed in the present research and elucidate the mechanism of c-kit function in the post-natal mammalian ovary.

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