Reproductive Biology 17 (2017) 25–33

Contents lists available at ScienceDirect

Reproductive Biology

journal homepage: www.elsevier.com/locate/repbio

Original article

Developmental effects of imatinib mesylate on follicle assembly and early activation of primordial follicle pool in postnatal rat ovary

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ARTICLE INFO

Article history:
Received 12 August 2016
Received in revised form 25 November 2016
Accepted 28 November 2016
Available online 28 December 2016

Keywords:
Imatinib
Folliculogenesis
Oogenesis
Rat
Ovary

ABSTRACT

Imatinib mesylate is an anti-cancer agent that competitively inhibits several receptor tyrosine kinases (RTKs). RTKs play important roles in the regulation of primordial follicle formation, the recruitment of primordial follicles into the pool of growing follicles and maturation of the follicles. In the present study, we investigated the effects of the tyrosine kinase inhibitor imatinib on primordial follicle assembly and early folliculogenesis in postnatal rats. Female Sprague-Dawley rats were treated with either imatinib (150 mg/kg) or placebo (water) on postnatal days 2–4. Bilateral ovariectomy was performed on postnatal day 2 and 5. Histology, immunohistochemistry, and mRNA analysis were performed. Imatinib treatment was associated with increased density of the multi-oocyte follicles ($P < 0.01$), oogonia ($p < 0.01$) and germline clusters ($P < 0.05$), decreased activation of primordial follicles, increased expression of c-Kit and AMH, and decreased protein expression of Kit-ligand and GDF9 when compared to age-matched controls. In conclusion, imatinib affects folliculogenesis in postnatal rat ovaries by delaying the cluster breakdown, follicular assembly and early activation of the primordial follicle pool.
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1. Introduction

The primordial follicle assembly and the recruitment of primordial follicles into the pool of growing follicles initiate folliculogenesis in mammalian species. Oocytes originate from primordial germ cells (PGCs) [1,2]. During embryonic development, PGCs migrate to the genital ridge, undergo a phase of mitotic proliferation, and are surrounded by epithelial/mesenchymal cell layers to create germline clusters [3,4]. After cessation of mitotic divisions, PGCs cells enter meiosis and give rise to oogonia [5]. The transition of germline clusters into individual follicles occurs when the cluster breaks down [6]. During this process, most of the oogonia are lost through apoptotic cell death [7]. The remaining oocytes are surrounded by pre-granulosa cells and a basement membrane to constitute the primordial follicle pool [6,8].

The control factors in this critical process that determines the reproductive life span and fertility of female mammals are largely undefined. C-Kit, a receptor tyrosine kinase (RTK) and its ligand, have demonstrated a regulatory role during primordial follicle formation [9,10]. The Kit ligand is highly expressed at the oocyte membrane, specifically during the window of cyst breakdown and primordial follicle formation [11]. Furthermore, peptide inhibition of Kit signaling during ovarian culture is known to reduce the formation of primordial follicles whereas Kit ligand

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http://dx.doi.org/10.1016/j.repbio.2016.11.003
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supplementation enhances it [11]. Kit signaling also regulates the recruitment of primordial follicles into the pool of growing follicles, which are thereafter destined to ovulate or degenerate through atresia [12].

The tyrosine kinase inhibitor imatinib mesylate (STI517, Glivec®) is a 2-phenylaminopyrimidine derivative that can block the activity of several RTKs including cytoplasmic Abelson tyrosine kinase (c-Abl), tyrosine kinase c-Kit, macrophage colony- stimulating factor (c-fms), and platelet-derived growth factor (PDGFR) [13,14]. This agent is used as treatment for multiple cancers and is also proposed as an agent to prevent primordial follicle loss in mice ovaries during chemotherapy based on its role as a c-Abl kinase inhibitor via PI3K/PTEN/Akt signaling pathways [15,16].

There is limited information about the impact of imatinib mesylate on the formation of the ovarian follicle pool. In the present study, we investigated the effects of the tyrosine kinase inhibitor imatinib on primordial follicle assembly in the postnatal rat ovary, with focus on the underlying cellular and molecular mechanisms.

2. Materials and methods

2.1. Animals and study design

All experiments were performed with female Sprague Dawley rats at postnatal day 2 or 5. The animals (n = 85) were born and maintained under standard life conditions for 2–5 days at the Animal Center of the Norwegian Institute of Public Health, Oslo, Norway, or the University of Turku, Finland. Permission for drug treatment was obtained from the

Norwegian Animal Research Authority, Oslo, Norway. The distribution of animals in the four experimental groups is shown in Table 1.

From day 2 to 4, the treatment groups were injected daily with 150 mg/kg imatinib mesylate (Glivec ®, STI571, Novartis Pharmaceuticals Corporation, Switzerland), while a placebo group was injected daily with distilled water to control the stress responses in postnatal rats. A control group with no treatment was also added. The imatinib-treated rats were compared with 2-day-old control, 5-day-old placebo control and 5-day-old non-injected control animals (Table 1). The rats treated with a daily dose of imatinib weighed 8–14 g. The dose selection was based on scaling of toxicokinetic parameters from rat to human, resulting in daily doses of 280–340 mg/m^2 [17]. Daily intracavitary injections of imatinib dissolved in water, or only water, were administered into the stomach with respect to the rats' weight (1.2–2.1 mg, 80–150 μL) [18,19]. Body weight was recorded daily. All rats were sacrificed at postnatal day 2 or 5 by decapitation, after which bilateral ovariectomy was performed. The ovaries were fixed in 10% formalin or Bouin's fluid, or frozen directly in liquid nitrogen. The frozen materials were stored at −80°C until further analyses.

2.2. Morphology and density of oogonia and follicles

The ovaries used for morphological analysis were collected from all study groups (day 2 control: n = 11, day 5 control: n = 11, placebo: n = 7, and imatinib-treated group: n = 28). All samples were fixed with Bouin's fluid for 24 h and subsequently kept in 70% alcohol at room temperature. They were clarified, dehydrated, embedded in paraffin wax, sectioned (4 μm), and stained with hematoxylin and eosin. Oogonia and ovarian follicles were evaluated by light microscopy (Nikon Eclipse E400) at a magnification of ×40. The number of oogonia and follicles at each early developmental stage (primordial, primary and secondary) was counted in every 10th section in 3 parallel sections for each sample. To avoid double counting, follicles were counted in the sections where their oocyte nucleus was observed. Two persons counted the number of follicles in all sections at each developmental stage. They were controlled by interobserver variation.

Table 1
Distribution of animals in the experimental groups; rats from postnatal day 2–5 with or without injection of distilled water (placebo) or imatinib (150 mg/kg).

Experimental rats	Day 2	Day 3	Day 4	Day 5
Day 2 control (n = 18)	Sacrificed	–	–	–
Day 5 control (n = 17)	–	–	–	Sacrificed
Day 5 placebo (n = 11)	Injection	Injection	Injection	Sacrificed
Day 5 Imatinib (n = 39)	Injection	Injection	Injection	Sacrificed

The quality of follicles was evaluated based on the morphological integrity of the oocyte, granulosa cells and basement membrane as previously described [20], and classified as intact or degenerated. The multi-oocyte follicles (MOFs) and clusters were separately evaluated. Healthy and early atretic follicles at different developmental stages containing ≥2 oocytes were counted as MOFs to avoid misinterpretation with advanced atretic oocytes [21]. A group of oogonia lacking a layer of granulosa cells and surrounded by an epithelial/mesenchymal cell layer or pregranulosa cells was counted as a cluster (Fig. 1).

The density of oogonia and each type of follicle in the ovaries was calculated as the total number of oogonia or follicles divided by the total tissue volume and expressed as the number of oogonia or follicles/mm^3 of ovarian tissue [22]. The volume of analyzed ovarian sections was calculated by summing the number of sections of area multiplied by the thickness of each section. The areas were measured by using Nikon's NIS-Elements software with ×4 magnification.

2.3. Quantitative real time polymerase chain reaction (RQ-PCR)

2.3.1. RNA extraction and cDNA synthesis

Total RNA from the study groups (day 2 control: n = 7, day 5 control: n = 6; placebo: n = 4; imatinib-treated: n = 11) was extracted using the Trizol reagent as per protocol provided (Invitrogen, Carlsbad, CA, USA) with the help of the Tissue Lyser II (Qiagen Inc., Valencia, CA, USA). The concentration and purity of total RNA were assessed spectrophotometrically using a NanoDrop 1000™ (Thermo Scientific, Waltham, MA, USA) at 280 nm, and 1 μg of extracted total RNA from each sample was reverse transcribed with the iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The obtained cDNA was diluted to a final concentration of 30 ng/mL. Primers (Table 2) derived from the *Rattus norvegicus* gene; National Center for Biotechnology Information (NCBI) GenBank; were designed according to literature or developed using the NCBI Primer designing tool. A NCBI basic local alignment search tool (BLAST) ensured the specificity of primer sequences for rat, and the primers were commercially produced by Eurogentec (Maastricht, the Netherlands). The primers were tested for efficiency by qPCR analysis of a dilution series of pooled cDNA at a temperature gradient (55–65 °C) for primer-annealing and subsequent melting curve analysis. The reaction mixture for the qPCR contained 6 μL diluted cDNA, 7.5 μL iQSYBR Green Supermix (Bio Rad Laboratories Inc.), forward (0.6 μL) and reverse (0.6 μL) primers (final concentration of 0.4 pmol/μL for each primer), and sterile water (0.3 μL) according to the manufacturer's instructions.

2.3.2. Polymerase chain reaction amplification

RQ-PCR was performed by using important gene markers involved in early folliculogenesis and primordial follicle assembly encoding c-kit [23], KL-1 (kit-ligand 1) [23], FOXL2 (forkhead

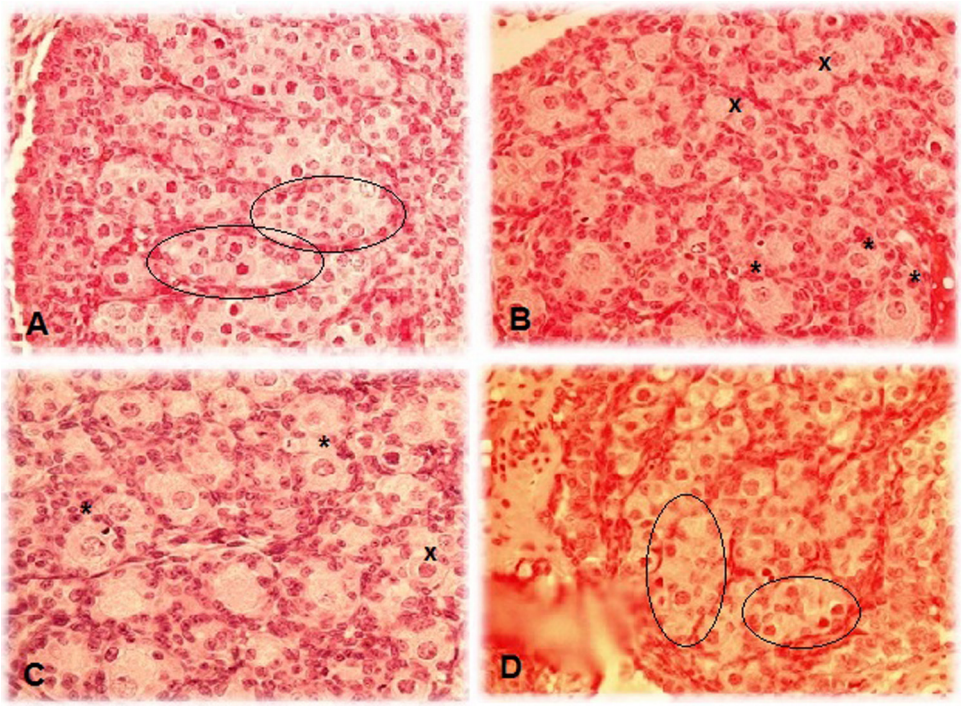

Fig. 1. Images of ovarian tissue from 2-day-old control (A), 5-day-old control (B), 5-day-old placebo-treated (C) and 5-day-old imatinib-treated (D) rats. Ovaries of 2-day-old and 5-day-old imatinib-treated rats showed clusters of oogonia (A, D; black ovals). 5-day-old control and placebo-treated rats presented primordial (x) and primary follicles (*).

transcription factor L2) [24], FIGLA (factor in the germline alpha), NOBOX (newborn ovary homeobox protein), PDGFC (platelet-derived growth factor C), AMH (anti-Müllerian hormone) [24], GDF9 (growth differentiation factor 9) and StAR (steroidogenic acute regulatory protein) [24]. The thermal cycler CFX96 real-time PCR detection system (Bio-Rad) and CFX Manager System Software Version 3.0 (Bio Rad) were used. The mRNA quantity was calculated relative to the expression of reference genes (Table 2). For this, data were analyzed using the efficiency corrected Delta-Delta-Ct method [25]. The fold-change values of the genes were normalized by using the geometric average of the fold-change values of two reference genes: HPRT and SDHA [26].

2.4. Immunohistochemical and immunofluorescence labeling

The ovaries from the study groups (day 2 control: n = 3, day 5 control: n = 3; placebo: n = 3; imatinib-treated: n = 3) were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned in 4 μm, and deparaffinized. The endogenous peroxidase activity was blocked with 0.3% H_2O_2 (Merck) in methanol for 30 min at room

Table 2
Primers used for the mRNA quantification of housekeeping genes (HKG; stably expressed) and genes of interest (GOI).

Genes	Accession no.	Primer	Product size	Reference
HKG				
HPRT	NM_012583.2	F-GCTGAAGATTTGGAAAAGGTG R-AATCCAGCAGGTCAGCAAAG	157	Hvid et al. [26]
SDHA	NM_130428.1	F-AGACGTTTGACAGGGGAATG R-TCATCAATCCGCACCTTGTA	160	Hvid et al. [26]
GOI				
AMH	NM_012902.1	F-ACAGCTATGAGCATGCCTTC R-GAGGCTCCCATATCACTTCA	195	Ahn et al. [24]
c-Kit	D12524.1	F-CTTTTGCGCAAGCTTTTGT R-ATCCCCCGCTCCAAAGTAT	145	Madden et al. [23]
FIGLA	XM_575589.5	F-AGCAGGTGCAAAACCCTCG R-CTCCTCCAACACCTCAGCTTG	111	This manuscript
FOXI2	XM_003750571.3	F-TCGCTAAGTTCCCGTTCTAC R-GTAGTTGCCCTTCTCGAACA	176	Ahn et al. [24]
GDF-9	NM_021672.1	F-TGCCTGGCTGTGTCTTCTTATT R-CCATCTACAGGCAACAGCAAG	123	This manuscript
KL-1	NM_021843.4	F-GGCCTACAATGGACAGCAAT R-TCAACTGCCCTTGTAAGACT	113	Madden et al. [23]
NOBOX	NM_001192013.1	F-AGGAAAATGCGGGGCCAGGAA R-AGGACAGCTTGTCTGGGTCAC	115	This manuscript
PDGFC	NM_031317.1	F-ATTGTGCCTGTTGCCTCCAT R-TCGGTGAGCGACTTATGCAA	130	This manuscript
StAr	NM_031558.3	F-TCAAGGAAATCAAGGTCCTG R-TGTTCAGCTCTGATGACACC	208	Ahn et al. [24]

temperature and rehydrated in a graded ethanol series to phosphate buffer saline (PBS). For antigen retrieval, the slides were boiled for 10 min in 10 mM citrate buffer (pH 6.0) in a microwave. The slides were cooled down to room temperature, rinsed with PBS and blocked with 5% goat or rabbit serum, depending on the secondary antibody composition (c-kit, KL-1 and Ki67–goat serum; AMH and GDF-9–rabbit serum), for 30 min at room temperature.

For the immunohistochemical staining, the tissue sections were incubated overnight (4 °C) with the primary antibodies: AMH, GDF-9, c-Kit or KL (1:450, Santa Cruz) and Ki67 (1:250, Abcam), followed by incubation with the biotinylated secondary antibodies (1:200, Dakocytomation). The biotinylated proteins were visualized with streptavidin-biotin complex/horseradish peroxidase (Vector Laboratories) and 0.05% diaminobenzidine solution. The sections were counterstained with Mayer's hematoxylin (Merck). Images were acquired using the Olympus BX50 microscope equipped with a Leica 320 digital camera.

Immunohistochemical analysis is a qualitative investigation method which gives valuable information on tissue processes without standardization [27]. Therefore, protein expression is classified as not expressed (−), weakly expressed (+), clearly expressed (++) or strongly expressed (+++). To evaluate Ki67 immunostaining, the total and labeled numbers of primordial follicles and the total and labeled numbers of granulosa cells per primary and secondary follicles were counted in each study group.

For the immunofluorescence labeling, the tissue sections were incubated overnight (4 °C) with the primary antibodies: AMH, GDF-9, c-Kit or KL (1:50, Santa Cruz) and Ki67 (1:50, Abcam), followed by incubation with Alexa-Fluor conjugated secondary antibodies (1:200, Life Technologies) for 1 h at room temperature. Nuclear counterstaining was performed by incubating the samples for 5 min with Hoechst 33342 (1:2000; Invitrogen). The slides were mounted with FluorSave Reagent (Calbiochem). Immunolocalization was visualized and images were acquired using the Keyence BZ-9000 microscope. Fluorescence intensity area calculation was performed automatically with the help of the image processing and analysis software Image J (NIH, USA).

In both methods, the negative controls lack the primary antibodies. The isotypes normal goat IgG (Santa Cruz) and normal rabbit IgG (Abcam) were included. At least three samples were evaluated in each of the groups studied.

To quantify the protein expression, we applied immunofluorescence and measured labeling intensity by means of digital image analysis (Image J software, NIH, USA). This method allows us to quantitatively analyze protein immunostaining. However, as the ovary is formed by heterogeneous cell types, and immunofluorescence did not allow us to differentiate oogonia from follicles and follicular developmental stages, we present the total intensity, not staining intensity, per follicular class.

2.5. Statistical analyses

Animal weight and daily weight gain were compared between placebo and imatinib-treated groups and among each day (2–5) with two-way ANOVA. Mean percentages and densities of oogonia and preantral follicles (total and morphologically normal), as well as clusters and MOFs were compared between all study groups with one-way ANOVA and Tukey's multiple comparisons as *post hoc* test. Likewise, differences in mRNA expression (fold-change) and IF intensity (fold-change) were compared by applying one-way ANOVA and Tukey as *post hoc* test. The mean percentage of Ki67-positive primordial follicles and the mean percentage of proliferating granulosa cells in all follicles was calculated in each study group and compared by applying one-way ANOVA and Tukey as *post hoc* test.

In all cases, the statistical tests were performed by using Prism 6 software (GraphPad). All data are presented as mean values ± SEM, the standard errors being calculated from the variance between samples. P-values < 0.05 indicated statistical significance.

3. Results

3.1. Animal weight

No statistically significant difference was observed in the weight of animals at postnatal day 2 before any treatment (placebo: 7.3 ± 0.45 g; imatinib: 7.13 ± 0.23 g). After treatment, the weight gain in imatinib-treated rats was significantly lower than in the placebo group at postnatal days 4 and 5 (Fig. 2).

3.2. Cell density, distribution and morphology

Comparison of morphological analysis between the study groups was based on mean densities of total and degenerated oogonia, preantral follicles, germline clusters, and MOFs (Table 3). The samples of age-matched controls, 5-day non-injected controls and placebo-treated animals showed comparable morphology with nearly completed primordial follicle assembly, with only a few degenerated oogonia and maturing follicles up to the secondary stage, whereas the ovaries of 5-day-old imatinib-treated rats resembled those of 2-day-old rats, with a high number

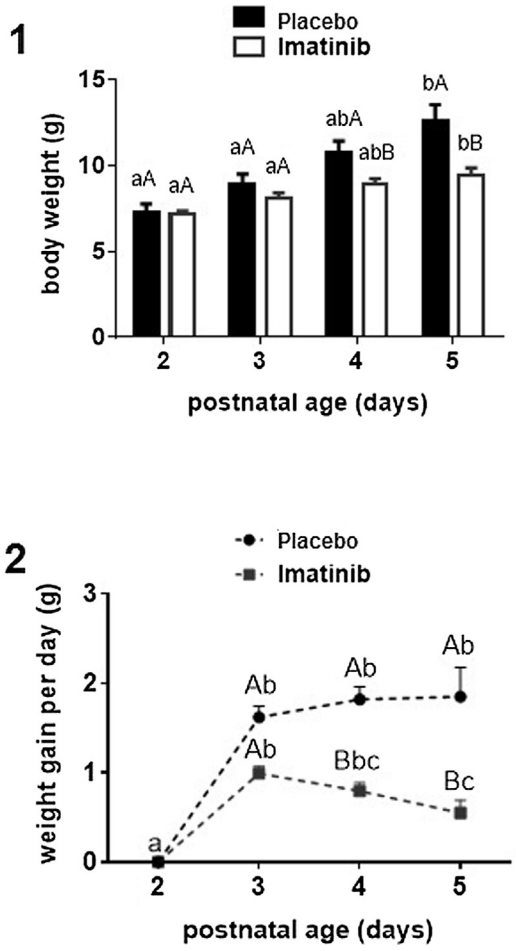

Fig. 2. Body weight (1) and daily weight gain (2) in placebo and imatinib treated animals. A, B – Different upper case letters indicate significant differences between placebo and imatinib treatments. a-c different superscripts indicate significant differences among days of age within each study group; $P < 0.05$.

Table 3
Mean (±SEM) densities/mm^3 of oogonia, ovarian follicles, multi oocytes follicles and germ cell clusters.

	2 day control N = 11	5 day control N = 11	Placebo N = 7	imatinib N = 28
Total oogonia and follicles				
Total	8184 ± 457	5804 ± 501	5767 ± 833	8466 ± 384
Intact	5899 ± 418	4528 ± 357	4872 ± 726	6327 ± 319
Degenerated	2284 ± 171^a	1276 ± 211^b	895 ± 124^b	2138 ± 183^a
Oogonia				
Intact	2605 ± 508^a	89 ± 41^b	24 ± 24^b	1061 ± 263^a
Degenerated	991 ± 161^a	35 ± 23^b	60 ± 60^b	412 ± 94^a
Primordial follicles				
Intact	2488 ± 545	3195 ± 256	3829 ± 593	3490 ± 240
Degenerated	989 ± 124^{ab}	1057 ± 192^a	687 ± 65^a	1460 ± 134^b
Primary follicles				
Intact	386 ± 82	792 ± 130	715 ± 270	569 ± 78
Degenerated	156 ± 45	120 ± 35	118 ± 47	198 ± 41
Secondary follicles				
Intact	0 ± 0^a	155 ± 32^b	133 ± 52^b	100 ± 23^b
Degenerated	0 ± 0	0 ± 0	0 ± 0	13 ± 9
Multi oocyte follicles	265 ± 86^a	175 ± 52^a	102 ± 31^a	542 ± 64^b
Germ cell clusters	490 ± 51^a	25 ± 12^b	12 ± 12^b	217 ± 41^c

$^{a-c}$different superscripts indicate significant differences among the studied groups within each row; $P < 0.05$.

of both intact and degenerated oogonia and ongoing follicle assembly (Fig. 1, Table 3). A higher density of intact and degenerated oogonia, germline clusters and MOFs was more frequently observed in the ovaries of imatinib-treated rats than in the ovaries of age-matched controls. The number of degenerated primordial follicles was higher in the imatinib-treated group, but significant differences were only observed between the imatinib-treated group and placebo controls. No difference in the densities of primary and secondary follicles was observed between the imatinib-treated group and the age-matched control groups.

3.3. Relative mRNA expression of selected markers

The mRNA expression levels of 9 genes are depicted in Fig. 3. The mRNA expression of FIGLA decreased (>5-fold), that of NOBOX decreased (>8-fold) and that of StAR increased (> 30-fold) from day 2 to day 5 in the control groups. In the 5-day-old imatinib-treated group, FIGLA and NOBOX mRNA expression did not show significant differences from the 2-day control group. StAR mRNA expression appeared to be lower in the ovaries of imatinib-treated rats than in age-matched control groups and showed a similarity to the low expression in 2-day-old samples (>20-fold). Imatinib treatment significantly increased c-Kit, KL-1, GDF9 and AMH mRNA expression and decreased mRNA expression of PDGFC when compared to age-matched controls. An up-regulation of FOXL2 mRNA expression was observed in all study groups at day 5.

3.4. Immunohistochemical and immunofluorescence analysis

The protein expression of c-Kit, KL, AMH, GDF9 and Ki67 proteins is depicted in Fig. 4 and Table 4. Evaluation of Ki67 immunostaining showed that the mean proportion of activated primordial follicles with proliferating granulosa cells increased significantly from day 2 to day 5 (Table 4). Imatinib significantly decreased the proportion of activated primordial follicles when compared to age-matched controls (Table 4). No differences were observed in the proliferation activity of granulosa cells in developing follicles (Table 4).

C-Kit protein was strongly expressed in the majority of oogonia in day 2 control and in imatinib-treated ovaries. There was an accumulation of c-kit protein in the growing follicles from age-matched groups, but not in those from the imatinib-treated group. KL protein expression in granulosa cells was weaker in follicles from imatinib-treated ovaries when compared to the day 2 controls and age-matched controls. Compared with other study groups, strong AMH expression was observed in granulosa cells from developing follicles in imatinib-treated ovaries. GDF9 protein expression ranged from absent to weak in oocytes from imatinib-treated rats, whereas its expression was clearly observed in the oocytes from other study groups, especially in oocytes from primary and secondary follicles in age-matched controls.

Fluorescence intensity area calculations confirmed the findings from conventional immunohistochemistry. AMH staining was significantly stronger in imatinib-treated ovaries, whereas expression of KL and GDF9 was significantly weaker in imatinib-treated ovaries than in the other study groups (Table 5).

4. Discussion

The assembly of the primordial follicles and their further development until the ovulation of healthy mature oocytes are critical for ovarian function. Any disruption of these processes can affect the size of the primordial follicle pool and the future of female fertility. Our observations showed that treatment with imatinib during 2–5 postnatal days delayed the germline cluster breakdown. Morphological analysis showed insufficient invasion and encapsulation of oocytes by pregranulosa cells. This led to higher numbers of intact and degenerating oogonia and increased formation of MOFs. Furthermore, imatinib treatment increased the degeneration of the newly formed primordial follicles and inhibited their activation. The present study indicates that imatinib delays follicle assembly and inhibits primordial follicle activation in the postnatal rat ovary.

Physiological degeneration and apoptosis of oogonia and ovarian follicles peak at the time of cluster breakdown in the rat ovary at birth [7]. The excess production and subsequent culling of germ cells is supposed to represent a means of 'germ cell selection', in which only oocytes of the highest quality can develop further into viable gametes [2]. These remaining oocytes, which are surrounded by pre-granulosa cells, constitute the primordial follicle pool and make up the ovarian reserve for adult life in mammalians [28].

This was also supported by the present observation of a high density of both intact and degenerating oogonia in 2-day-old ovaries and low density in age-matched control groups. Treatment with imatinib led to a delay in the cluster breakdown and apoptosis of oogonia. Significantly higher numbers of both intact and degenerating oogonia and MOFs were observed in the imatinib-treated group, whereas the number of primordial follicles was not changed compared to age-matched controls. However, a higher proportion of primordial follicles showed degenerative morphology.

The density of primary and secondary follicles was not significantly affected by treatment with imatinib. However, when the number of activated primordial follicles was analyzed, significantly decreased recruitment of primordial follicles into the pool of growing follicles was observed after treatment with imatinib. The inhibiting effect of imatinib on follicle activation is supported by a previous study that showed significantly decreased numbers of primary follicles, developing Graafian follicles and developed Graafian follicles in adult imatinib-treated rats [29].

Imatinib targets several tyrosine kinase receptors including c-Kit, PDGF, c-Abl, and c-fms [13,14]. Many of them have multiple functions in the ovarian folliculogenesis. C-fms and its interaction

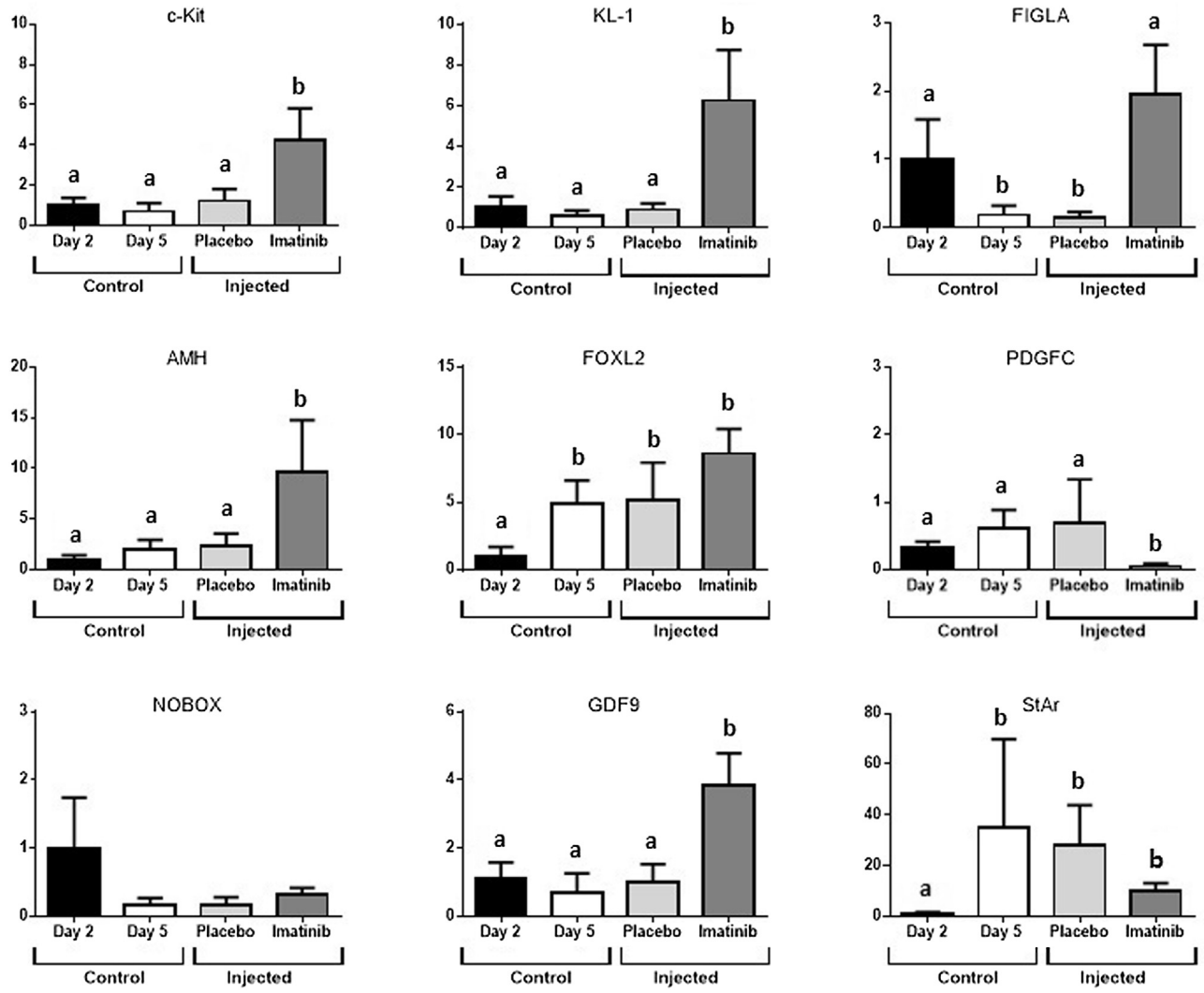

Fig. 3. The fold-change in mRNA expression levels of c-Kit, KL, FIGLA, AMH, FOXl2, PDGFC, NOBOX, GDF9, and StAR genes in rat ovaries from all study groups. a-b different superscripts indicate significant differences among the studied groups; $P < 0.05$.

with macrophage colony-stimulating factor are associated with ovarian follicular maturation [30]. C-Abl has a role in the maintenance of genomic integrity in both meiotic and mitotic oocytes [31]. Activation of c-Abl by cytotoxic insult is known to phosphorylate TAp63, which promotes the death of germ cells, whereas pharmacological inhibition of c-Abl by imatinib counteracts the effect of chemotherapy on the ovarian reserve in mice [16,31]. The observed delay in the formation of primordial follicles in the postnatal rat and the decreased activation of primordial follicles may potentially increase the ovarian reserve and fertility potential after puberty. On the basis of the limited data in the literature, we hypothesize that tyrosine kinase inhibitors may provide an innovative approach for the development of new treatment strategies for fertility preservation. This can be achieved if they are able to protect the primordial follicles by avoiding their activation and apoptosis during cancer treatment.

Imatinib inhibits the c-Kit protein, which is expressed in oogonia and oocytes during early ovarian follicular development in rat, human and several other species [32–34]. KL, which binds to the c-kit receptor, is expressed in granulosa cells [35–37]. Their interaction is required for the survival and development of follicles [9,10]. Inactivating mutations of c-Kit and KL cause defects in germ cell migration and proliferation [38,39]. Disturbed onset of primordial follicle development, primary follicle growth, follicular fluid formation in pre-antral follicles, and the ultimate stage of follicle maturation before ovulation have been described in animal models with mutated c-kit or KL [39]. In the present study, high expression of c-Kit mRNA was detected in 2-day-old control and 5-day-old imatinib-treated rats with a high density of oogonia, which is in line with the physiologically high expression of c-kit in oogonia [40–42]. We were also able to show that treatment with imatinib disturbed the onset of primordial follicle development. This supports the observations from animal models with inactivating mutations of c-Kit and KL [38,39].

In the present study, treatment with imatinib also led to increased mRNA expression of KL-1 and decreased production of KL protein. The high transcription at RNA level probably occurred as a response to the low synthesis or increased degradation of the KL protein. Previous studies on mice ovaries with anti-c-kit antibody that specifically block KL/c-Kit interaction have shown a very similar ovarian phenotype as observed after imatinib therapy in the present study [12,39]. Administration of an anti-c-Kit antibody increased the number of oogonia/oocytes, reduced the number of primary follicles, and decreased the activation of primordial follicles [12,39]. The comparable morphological effects of c-kit antibody and imatinib suggest that a large part of the effect

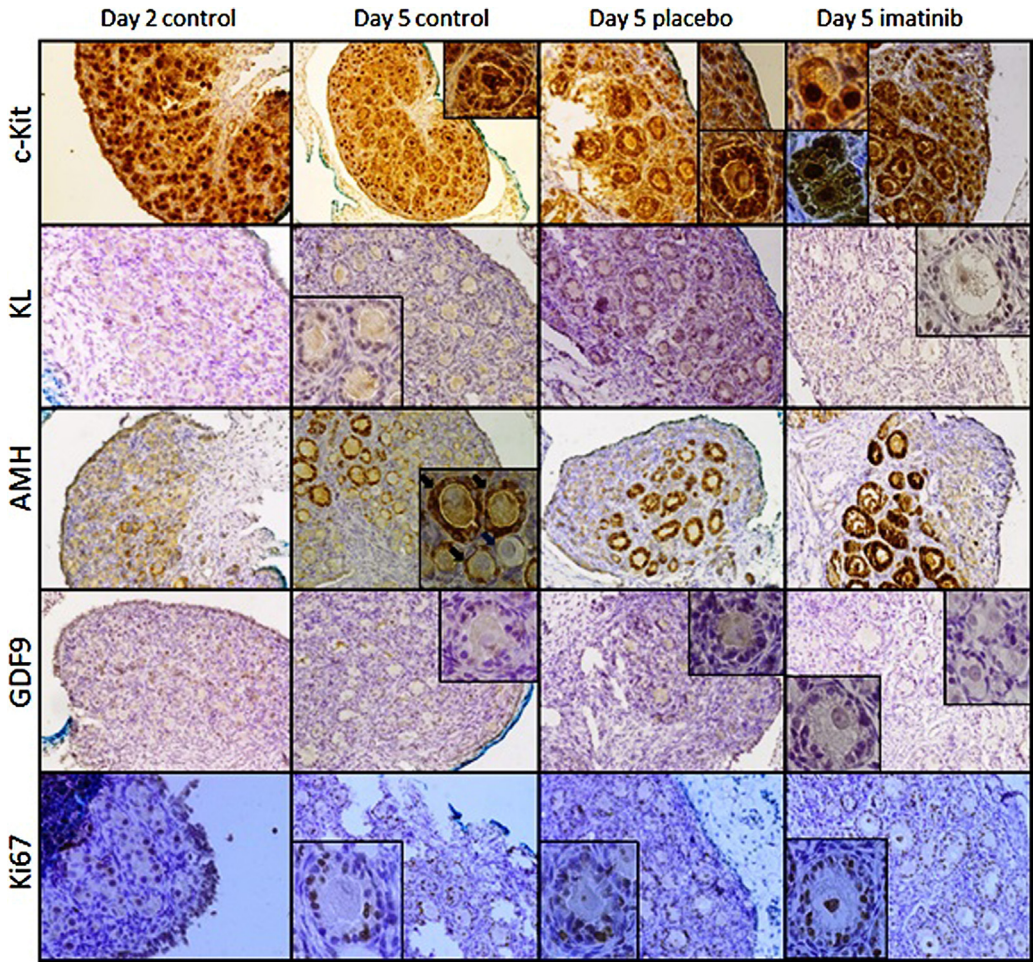

Fig. 4. Representative immunohistochemical images of 2-day-old, 5-day-old controls, placebo and imatinib-treated 5-day-old rat ovaries. Strong immunolabeling of c-kit was observed in oocytes and oogonia, KL in granulosa cells, GDF9 in oocytes, Ki67 in proliferating granulosa cells, AMH in granulosa cells from developing follicles (black arrows), and negative immunolabeling of AMH in oogonia and granulosa cells from primordial follicles (blue arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of imatinib is mediated by specific inhibition of c-kit/KL interaction. However, it does not exclude the possibility that imatinib also targets other RTKs.

In rat, protein expression of PDGF ligands and receptors has been identified in oocytes of primordial, primary and developing follicles [43,44]. PDGFA and B display high expression in the oocytes of primordial follicles, whereas PDGFC and D are expressed in the granulosa and theca cells [45]. Expression of PDGFC is also observed in germline clusters and in primordial follicles shortly after birth in rat ovary [43]. The exact function of PDGFC in early folliculogenesis is not clear, but PDGF is known to regulate the transition of the primordial follicle to primary stage by stimulating KL expression in granulosa cells [44]. In the current study, imatinib downregulated the ovarian expression of PDGFC mRNA, and this was associated with the low protein expression of KL. This suggests a possible role of PDGF-receptor inhibition in the decreased activation of primordial follicles after treatment with imatinib.

Several other proteins/factors are involved in cluster breakdown, primordial follicle formation and subsequent formation of the primordial follicle pool. Inappropriate function of the transcription factors FOXL2, FIGLA, and NOBOX is known to influence the timing of cluster breakdown and to lead to premature or delayed formation of primordial follicles [46]. NOBOX plays an important role in oogenesis and ovarian development, while FIGLA regulates germ cell-specific genes, being crucial in the formation and maintenance of primordial follicles [47]. In the present study, only FIGLA was affected by imatinib treatment. High expression of FIGLA was observed both in the 2-day control and in the imatinib-treated group. This may reflect the delayed follicle assembly with a high number of oogonia and clusters, probably in the early stage of follicle formation.

Defected cluster breakdown is known to lead to follicular degeneration or the formation of MOFs [8]. The mechanisms involved in MOFs formation are not yet clear, but GDF-9 has been proposed as one of the involved potential factors [48]. Wang and Roy have shown that GDF-9 can promote the formation of primordial follicles and their subsequent growth in neonatal hamster ovaries [49]. They have also suggested that the actions of GDF-9 at specific stages of follicle development may be mediated through altering the expression of KL [49,50]. This theory is supported by the low protein expression of GDF-9 and KL in imatinib-treated rats with a delay in cluster breakdown and formation of primordial follicles in the current study.

The decrease in GDF-9 expression in the follicles and the increased number of MOF in the imatinib-treated group is also in agreement with the previous morphological observation from GDF-9-deficient mice [48] and supports the role of GDF-9 deficiency in the formation of MOF. Yoshida et al. have also suggested that GDF-9 may be a candidate for a granulosa cell-proliferating factor produced by oocytes activated via c-Kit [39]. The observation that follicular defect in early ovarian folliculogenesis is similar in GDF-9-deficient mice, in mice treated with

Table 4
Summary of the qualitative immunolabeling of c-Kit, KL, AMH, and GDF9 proteins and the mean (±SEM) percentages of primordial follicles with ki67-positive granulosa cells (activated primordial follicles) and mean percentages of Ki67-positive granulosa cells in primary and secondary follicles.

Factors	2 day control N = 7	5 day control N = 6	Placebo N = 4	imatinib N = 11
c-Kit				
Oogonia	+++	+/++	+/++	+++
Primordial follicles	++	+/++	+/++	++
Primary follicles	++	+++	+++	++
Secondary follicles	NA	+++	+++	++
KL				
Oogonia	++	++	++	+
Primordial follicles	++	++	++	+
Primary follicles	++	++	++	+
Secondary follicles	NA	++	++	+
AMH				
Oogonia	–	–	–	–
Primordial follicles	–	–	–	–
Primary follicles	+	+	+	++
Secondary follicles	NA	++	++	+++
GDF9				
Oogonia	–	–	–	–
Primordial follicles	+	+	+	–
Primary follicles	++	++	++	–/+
Secondary follicles	NA	++	++	–/+
Ki67				
Oogonia	NA	NA	NA	NA
Primordial follicles	13.0% (±0.5)[a]	25.0% (±2.5)[b]	22.0% (±5.9)[b]	12.0% (±0.1)[a]
Primary follicles	31.8% (± 0.7)	32.2% (±0.1)	33.8% (±0.1)	51.1% (±0.1)
Secondary follicles	NA	24.0% (±0.9)	35.6% (±0.3)	45.1% (±0.5)

(–) not expressed; (+) weakly expressed; (++) clearly expressed; (+++) strongly expressed.
NA: Not applicable no secondary follicles were observed in the ovaries of 2-days rats.
[a–b] different superscripts indicate significant differences among the studied groups within each row; $P < 0.05$.

anti-c-Kit antibody (ACK2) and in the present study in rats treated with imatinib, further supports the relationship between GDF-9 and c-Kit.

AMH, a member of the transforming growth factor beta (TGFß) family, is known to have inhibitory actions on ovarian primordial follicle assembly [51]. This is in agreement with the observed high expression of AMH in imatinib-treated ovaries and delay in primordial follicle assembly. Increased AMH expression is synergic with the increased expression of FOXL2 expression that was also observed in this study [52]. AMH and FOXL2 are known to collaborate in reserving ovarian follicles [52]. While the reason for upregulation of these proteins is not fully known, the present results suggest that RTK inhibition can upregulate AMH expression in the postnatal rat ovary.

Steroidogenesis is important for the maturation of ovaries and plays a role in folliculogenesis. In early folliculogenesis, cholesterol must be translocated from the outer to the inner mitochondrial membrane. This process is mediated by the StAR [53]. Low expression of StAR is reported in postnatal rat ovaries younger than 6 days; it increases with maturation of the ovaries [53]. We observed that the age-dependent increase in StAR mRNA expression was inhibited by the imatinib treatment. The delay in the follicular development observed in imatinib-treated rats could be linked to the lower expression of StAR, which may reduce the steroidogenic activity.

In line with the present observation of reduced weight gain in imatinib-treated rats, decreased absolute and relative body and organ weights, as well as nose-to-tail length have been previously reported in postnatal rats [18,19]. Poor postnatal growth has shown to associate with disturbed RTK-dependent bone modeling at the metaphyseal osteochondral junction [18].

In general, the results of this study revealed that imatinib affects the early folliculogenesis in postnatal rat ovary by a delay in primordial follicle assembly, increasing the formation of MOFs and decreasing the activation of the primordial follicle pool. This was associated with decreased expression of KL and GDF9 proteins and increased expression of c-Kit and AMH proteins. Further studies are required to clarify how the delayed formation of the primordial follicle pool affects long-term ovarian function and fertility.

Table 5
Summary of the mean (±SEM) values of fluorescence intensity for c-Kit, KL, AMH, GDF9, and Ki67 proteins detected in ovarian sections.

Factors	2 day control N = 7	5 day control N = 6	Placebo N = 4	imatinib N = 11
c-Kit	1.49 ± 0.26	1.99 ± 0.15	2.28 ± 0.02	2.51 ± 0.14
KL-1	1.03 ± 0.12[a]	0.70 ± 0.04[a]	0.67 ± 0.08[a]	0.30 ± 0.10[b]
AMH	1.00 ± 0.32[a]	1.13 ± 0.01[a]	1.12 ± 0.02[a]	2.18 ± 0.20[b]
GDF9	1.00 ± 0.07[a]	2.02 ± 0.01[b]	2.26 ± 0.23[b]	0.49 ± 0.12[c]
Ki67	1.24 ± 0.22	1.25 ± 0.07	1.42 ± 0.08	2.57 ± 0.31

[a–c]different superscripts indicate significant differences among the studied groups within each row; $P < 0.05$.

Conflicts of interest

None declared

Sources of funding

This research was supported by grants from the Norwegian National Advisory Unit on Women's Health, Oslo University Hospital, Norway, Turku University Hospital special research grants, The Academy of Finland and The Sigrid Jusélius Foundation, Finland

Acknowledgment

We would like to thank Trude Olsen at the Norwegian Institute of Public Health, Oslo, Norway, for her help at the animal house and the Research Institute for Internal Medicine at Oslo University Hospital, Rikshospitalet, Oslo, Norway, for microscopy assistance.

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