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Peritubular myoid cells have a role in postnatal testicular growth

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Keywords: peritubular myoid cells, testis growth, imatinib, FSH, hemicastration, postnatal, rat

Abbreviations: ATP, adenosine triphosphate; BrdU, 5-bromo-2-deoxyuridine; DAPI, 4',6'-diamidino-2'-phenylindole; FCS, fetal calf serum; FSH, follicle stimulating hormone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDNF, glial cell line-derived neurotropic factor; HCA, hemicastration; HPRT1, hypoxanthine guanine phosphoribosyl transferase 1; HTPCs, human testicular peritubular cells; PDGF, platelet-derived growth factor; PFA, paraformaldehyde; PBS, phosphate-buffered saline; SCF, stem cell factor; S26, ribosomal protein S26; WT1, Wilms' tumor suppressor gene 1

FSH stimulates testicular growth by increasing Sertoli cell proliferation and elongation of seminiferous cords. Little is known about the peritubular myoid cells in testicular development. In order to investigate the role of peritubular myoid cells in early testicular growth in rodents, two traditional models to induce testicular growth were used: FSH treatment and hemicastration. In order to affect proliferation of peritubular myoid cells, both treatments were combined with imatinib, a tyrosine kinase inhibitor. In addition, effects of imatinib on human testicular peritubular cell proliferation were investigated. Testicular weight, diameter and length of seminiferous cords, numbers of germ, Sertoli and BrdU-positive cells and FSH-levels were measured. FSH treatment and hemicastration increased length of the seminiferous cords and testicular weight by increasing first the early proliferation of peritubular myoid cells and later also the proliferation of the Sertoli cells. Imatinib blocked the FSH and hemicastration-induced testicular hypertrophy and decreased the proliferation of PDGF-stimulated human testicular peritubular cells in vitro. Present results provide new evidence that peritubular myoid cells have an important role in postnatal testicular growth.

Introduction

Postnatal increase in the number of Sertoli cells is believed to determine final testicular size and later total sperm output. Follicle-stimulating hormone (FSH) is known to stimulate the proliferation of Sertoli cells and to induce testicular growth. Indeed, mice lacking FSH receptor show decreased testicular growth[1] and also humans who have non-functional FSH receptor have small testicular size.[2] In agreement with these findings Sertoli cell proliferation[3-6] and testicular growth[7,8] can be stimulated by FSH administration during postnatal period. Similarly, postnatal hemicastration (HCA), which is known to increase pituitary FSH secretion by reducing inhibin B production, stimulates testicular hypertrophy of the contralateral testis.[9-11] Adequate postnatal testicular growth is important for future fertility since the final number of Sertoli cells in the adult testis correlates with total sperm output.[12,13]

Less is known about the role of peritubular myoid cells in testicular growth. Proliferation of the peritubular myoid cells[14] coincides with the expansion of the Sertoli cells during postnatal period which ceases at the postnatal day 15 in the rat.[15,16] Supporting their close interaction during development, communication between the peritubular myoid and the Sertoli cells is required for the formation of basal lamina during postnatal development.[17,18] Thus, peritubular myoid cell proliferation may play a more important role in the regulation of testicular growth than earlier appreciated.

In order to investigate the role of the peritubular myoid cells in testicular growth in rats, we used two traditional models to induce testicular growth: FSH treatment and hemicastration. Since we have earlier shown that the inhibition of peritubular myoid cell proliferation during postnatal period is able to decrease final testicular size[19] we combined FSH administration or hemicastration with imatinib mesylate treatment (Glivec®, STI571, Novartis Pharma). FSH treatment and hemicastration increased the length of the seminiferous cords and testicular weight by increasing the early proliferation of peritubular myoid cells and later also the proliferation of the Sertoli cells. Imatinib blocked the FSH and hemicastration-induced testicular hypertrophy and

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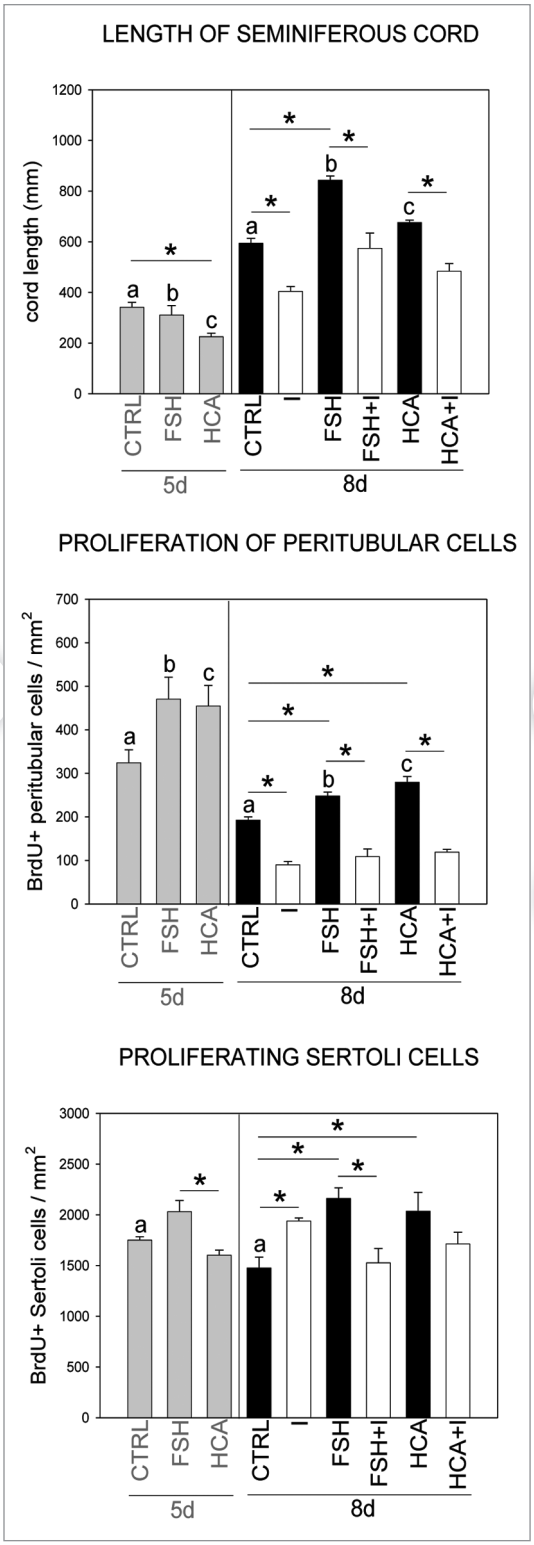

Figure 1. The effects of FSH administration (FSH), hemicastration (HCA) and imatinib treatment (I) on the seminiferous cord length and the number of BrdU-positive Sertoli and peritubular myoid cells. Results are means + SEM * and $^{a-c}$ indicate significant differences ($p < 0.05$) between the experimental groups.

was also able to decrease the proliferation of the human testicular peritubular cells (HTPCs) in vitro.

Results

FSH administration and hemicastration induced testicular growth. Hemicastration and FSH treatment increased plasma FSH levels, resulting in testicular growth at the postnatal age of 8 d (**Table 3**). FSH treatment caused 45% and hemicastration 9% increase in testicular weight when compared with the control value, but the body weights did not change. Faster longitudinal outgrowth of seminiferous cords and increased proliferation rate of Sertoli cells and peritubular myoid cells (**Figs. 1 and 2**) were evident in both the hemicastrated group and FSH-treated group. Proliferation of the peritubular myoid cells increased earlier (day 5) than that of the Sertoli cells (day 8), suggesting a role in the FSH-induced testicular growth (**Table 3 and Fig. 1**). No significant changes in germ or Sertoli cell numbers per area, in the proliferation rate of germ cells or in the diameter of seminiferous cords were detected after FSH administration or hemicastration when compared with controls at the postnatal age of 8 d indicating that the testicular growth was due to lengthening of the testicular seminiferous cords/tubules.

To further study the mechanism behind FSH- and hemicastration-induced testicular hypertrophy, we analyzed transcript levels of SCF, c-kit, GDNF, Ret, PDGF ligands and receptors. Significant ontogenetic changes in the levels of these transcripts were found. Transcript levels of SCF, c-kit, PDGFB and Ret were significantly increased and PDGFA significantly decreased from postnatal age of 5 d to postnatal age of 8 d (**Fig. 3**) at these age points. FSH administration and hemicastration did not significantly affect the expression pattern of SCF, c-kit, Ret or PDGF ligands (**Fig. 3**). Only PDGFR-α expression was significantly increased after hemicastration on postnatal day 8 (**Fig. 3**) and GDNF expression significantly decreased after FSH-treatment and hemicastration on postnatal day 5 (**Fig. 3**).

Imatinib decreased postnatal testicular growth. Imatinib treatment alone significantly decreased plasma levels of FSH, testicular size, length of the seminiferous cords and proliferation of the peritubular myoid cells on postnatal day 8 (**Table 3 and Fig. 1**). Instead, imatinib increased the number of the proliferating Sertoli cells significantly and led to a significantly increased number of Sertoli cells per area (**Table 3 and Figs. 1 and 2**). Imatinib treatment did not affect the total number or the number of proliferating germ cells on postnatal day 8.

Imatinib abolished testicular growth induced by FSH treatment and hemicastration. Imatinib treatment was able to abolish both the FSH- and hemicastration-induced longitudinal growth of seminiferous cords and significantly decreased peritubular myoid cell proliferation in both of these treatment groups (**Fig. 1**). Proliferation of the Sertoli cells was significantly decreased in FSH-treated group but the decrease in the hemicastrated group was more modest and did not reach statistical significance. Imatinib treatment in combination with FSH administration or hemicastration had not significant effect on the total number of Sertoli cells per area (**Table 3**). The diameter of the seminiferous

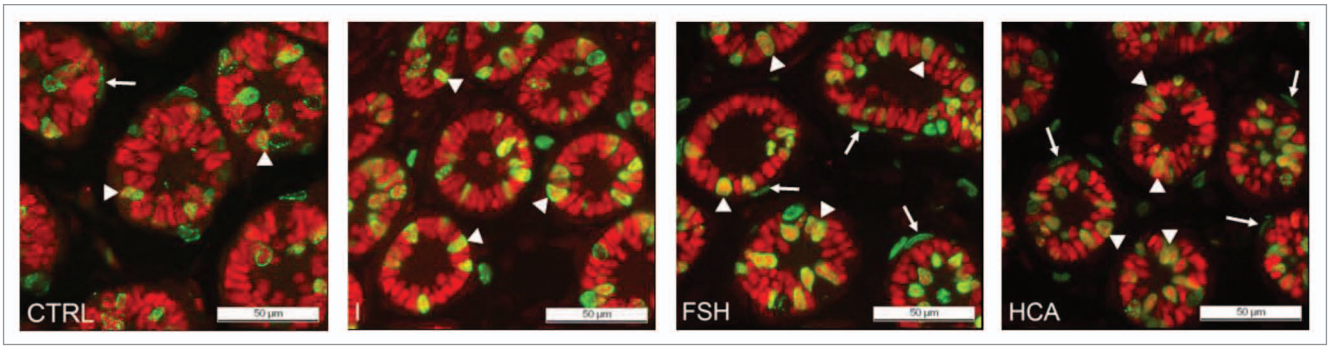

Figure 2. The immunohistochemical detection of BrdU incorporation (green fluorescence) and WT-1 positive Sertoli cells (red fluorescence) in the testis of control (CTRL), imatinib-treated (I) FSH-treated (FSH) and hemicastrated (HCA) rats at the age of 8 d. Proliferating peritubular myoid cells are identified according to BrdU staining and peritubular location (→) and proliferating Sertoli cells by double staining with BrdU and WT-1 (►).

cords increased after combined administration of FSH and imatinib (**Table 3**). Imatinib treatment in combination with FSH administration or hemicastration did not significantly affect the number of germ cells.

Imatinib inhibited the proliferation of the human testicular peritubular cells (HTPCs) in vitro. Effects of increasing concentrations of imatinib on viability of human testicular peritubular cells are shown in **Figure 4A**. Viability of the cells was not affected by the concentrations ≤ 10 μM but was dose dependently reduced with higher concentrations. Imatinib concentration of 10 μM was therefore used for further analysis. Exposure of human peritubular cells to 10 μM imatinib had no effect on their proliferation or number per ml when compared with control values. However, when HTPCs were stimulated with 5 ng/ml of recombinant human PDGF, the natural ligand of the PDGFα and β receptors,[31] a significant increase in cell proliferation (**Fig. 4D**) and cell number/ml (**Fig. 4C**) was detected. Treatment with 10 μM imatinib effectively blocked this PDGF-induced proliferation and increase in HTPC numbers (**Fig. 4B and C**).

Discussion

The aim of the present study was to investigate the role of peritubular myoid cells in postnatal testicular growth. Therefore two traditional models to stimulate Sertoli cell proliferation, FSH administration and hemicastration, were compared and combined with imatinib treatment. Our present data indicate that peritubular myoid cells play a more critical role in determination of final testicular size than earlier thought.

Interestingly, both FSH administration and hemicastration increased first peritubular myoid cell proliferation, already on the postnatal day 5. Three days later an increase in the number proliferating Sertoli cells was seen and accordingly the length of the seminiferous cords and testis weight increased. Since Sertoli cells are the only cells expressing FSH receptors in the testis,[32,33] FSH administration and hemicastration most probably affected the proliferation of peritubular myoid cells through paracrine factors like PDGF ligands. Increased expression level of PDGF receptor α was detected after hemicastration, and there was a tendency to higher PDGFβ, suggesting that hemicastration and FSH treatments may affect the PDGF signal cascade. Our finding is not in agreement with the previous study by Gnessi and coworkers where they showed that FSH dose dependently inhibits PDGF production.[34] Possible explanation is that our work was done in vivo, and Gnessi and coworkers investigated the effects of FSH on PDGF production in in vitro cultures.

To further study the mechanism behind FSH- and hemicastration-induced testicular hypertrophy, we analyzed also transcript levels of SCF, c-kit, GDNF and Ret. However, pathways analyzed here seemed not to be similarly affected. An obvious limitation of the present study is that the measurements of the steady-state mRNA levels were done in the whole testes and therefore may not properly reflect changes at the cellular level.

Since we had earlier shown that tyrosine kinase inhibitor imatinib caused inhibition of peritubular myoid cell proliferation during the postnatal period and decreased final testicular size,[19] we used imatinib to test, whether it could interfere in the communication of Sertoli cells and peritubular myoid cells, particularly PDGF signaling. Sertoli cells produce PDGFs that act on myoid cells harboring PDGF receptors.[34,35] Imatinib is a small molecular analog of ATP that inhibits the Bcr-Abl, PDGFRα, PDGFRβ, c-Fms, Arg and c-kit tyrosine kinases.[36-39] PDGFs stimulate gonocyte and peritubular myoid cell proliferation.[40-42] C-kit receptor, in turn is known to be expressed on differentiating spermatogonia and Leydig cells,[43-45] and its ligand SCF is produced by Sertoli cells.[44,46] Treatment with imatinib alone led to a significant decrease in the proliferation of the peritubular myoid cells, without decreasing the number of proliferating Sertoli cells, which are known to be c-kit and PDGF receptor-negative. Present data confirms our earlier observations showing that a short 3-d postnatal imatinib treatment and inhibition of peritubular myoid cell proliferation is able to affect the final testicular size in adult rats.[19]

The present study demonstrates that imatinib treatment is able to block the FSH-stimulated testicular growth and hemicastration-induced compensatory hypertrophy of the contralateral testis. When imatinib was combined with FSH administration or hemicastration, especially the early proliferation of peritubular myoid cells, which were shown to initiate the testicular hypertrophy, decreased significantly. This blocked the FSH-induced

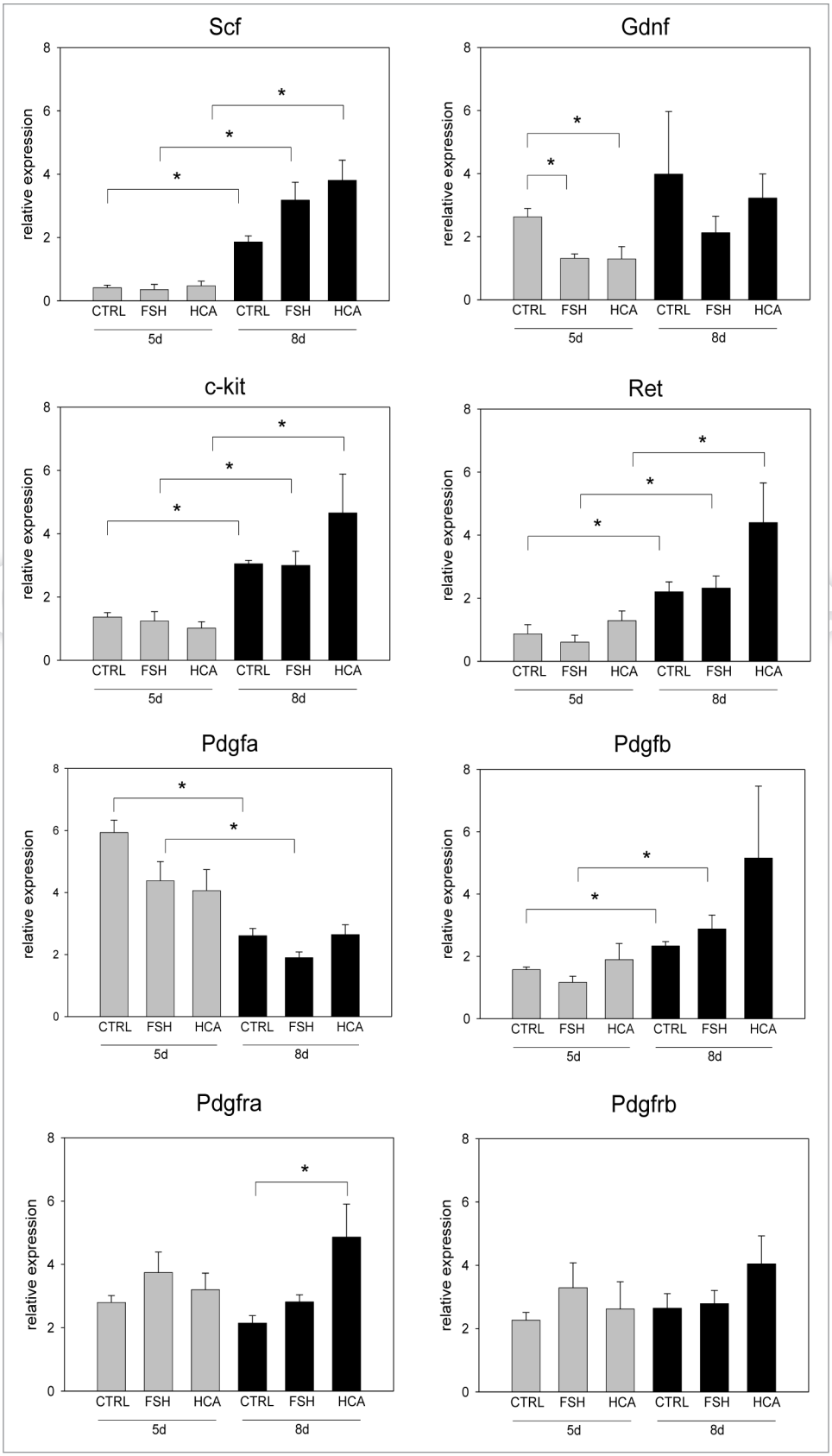

Figure 3. The effects of FSH administration and hemicastration on the relative levels of testicular SCF, c-kit, GDNF, Ret, PDGF-A, PDGF-B, PDGFRα and PDGFRβ gene expression in 5- and 8-d-old rats. Results are means + SEM *depicts the statistically significant difference.

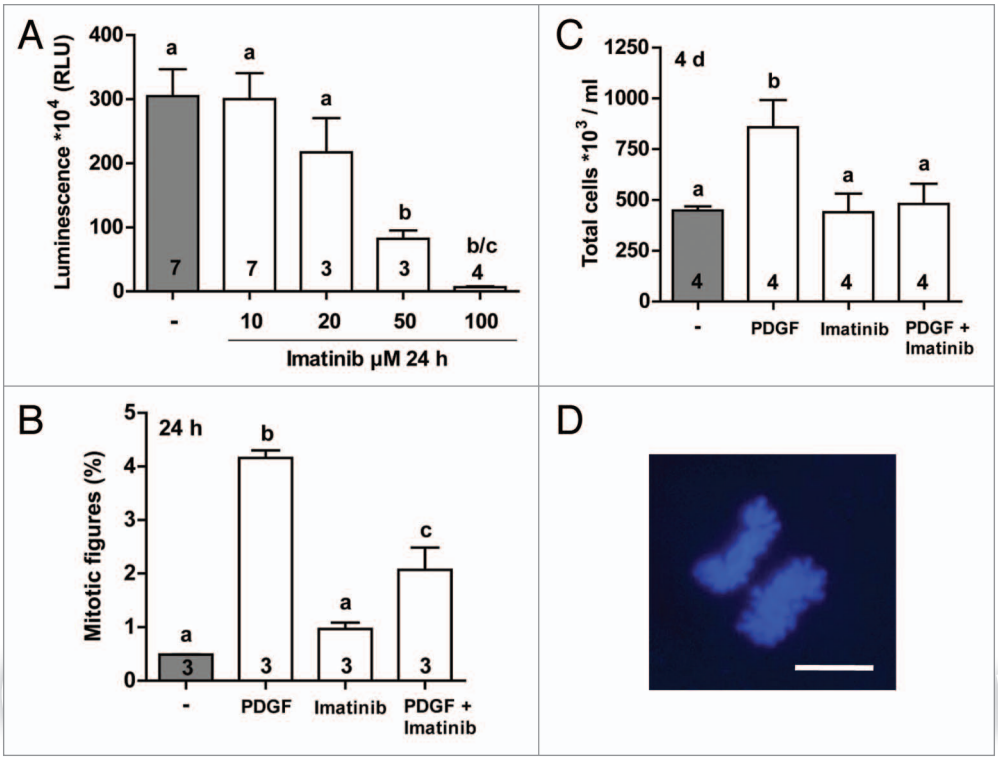

Figure 4. The effect of imatinib in cultured human testicular peritubular cells (HTPCs) on (A) cell viability (ATP assay), (B) the proportion of proliferating cells showing a mitotic figure (%) and (C) total cell counts. Imatinib blocked the PDGF-induced increase in cell proliferation of HTPCs. (D) Example of a mitotic figure stained with DAPI indicating a proliferating HTPC. Bar: 3 µm. Results are means + SEM. Different letters indicate significant differences. Numbers in columns indicate the number of patients used for cell harvesting. RLU, relative luminescence units.

accelerated tubular outgrowth and prevented the hypertrophy of the testis. Observation suggests that peritubular myoid cell proliferation is a significant determinant of longitudinal growth of the seminiferous cords and the final testicular size.

The inhibition of myoid cell proliferation was seen also in human peritubular cells. Imatinib affected the cells that were actively induced to proliferate by PDGF stimulation. The observation confirms that imatinib exerts its function in peritubular cells by interfering with active protein tyrosine kinase cascade. Thus, activity of imatinib in a particular cell should be selective in terms of how active this cascade is in the cell. During postnatal testicular development such ligand stimulation and cascade activation is known to occur in peritubular myoid precursor and germ cells.

In conclusion, the present study provides new evidence that peritubular myoid cells have an important role in the longitudinal growth of seminiferous cords in the postnatal rat testis. Thus, Sertoli cell proliferation is not the only determinant of postnatal testicular growth but peritubular myoid cells participate in this regulation and affect the growth of basement membrane lined by Sertoli cells.

Materials and Methods

Animals and treatments. Sprague-Dawley rats were housed in plastic cages (Tecniplast) in a climate-controlled room at 21 ± 3°C with a relative humidity of 55 ± 15% at the Animal Center of Turku University. Aspen chips (Tapvei Co.) were used as bedding material and animals were maintained on a 12 h light/12 h dark cycle (lighted from 07 to 19 h). Animals had free access to tap water and standard laboratory animal feed [Commercial RM3 (E) SQC, Special Diet Service].

The day of birth was designed as day 0. Two days after birth, the size of each litter was reduced by random selection to 8. Litters were divided into nine treatment groups (**Table 1**) and every group included pups from two or more litters.

In order to induce the growth of the contralateral testis, one group of animals were unilaterally castrated, i.e., hemicastrated (HCA) a day after birth (day 1) using cooling as analgesia.[20] Another group of male pups was exposed with lyophilized recombinant human FSH (Puregon) dissolved in water (200 IU/kg, s.c.) daily from day 1 onwards to stimulate testicular hypertrophy. Part of the hemicastrated and FSH-exposed pups and one group of intact pups were treated with imatinib mesylate (150 mg/kg; Glivec®, STI571, Novartis Pharmaceuticals Corporation) dissolved in water and injected (150 µl) intracavitally into the stomach once daily on postnatal days 5–7 as described in reference 19. Control animals were injected in the same manner with water alone. The experimental groups and the timeline of the experiment are presented in **Table 1**. In 30% of experimental animals combined administration of FSH and imatinib caused lethal reaction up to 24 h after treatment. Animals became lethargic and did not survive the planned experiment. The reason for mortality remained

Table 1. The experimental groups and the time-line for treatment of immature male rats

Age	Group	Treatment							
			1 d	2 d	3 d	4 d	5 d		
5 d	CTRL	Control							
	FSH	FSH	x	x	x	x	DEATH		
	HCA	Hemicastration	x						
			1 d	2 d	3 d	4 d	5 d	6 d	7 d	8 d
8 d	CTRL	Control								
	I	Imatinib					x	x	x	
	FSH	FSH	x	x	x	x	x	x	x	
	FSH + I	FSH	x	x	x	x	x	x	x	DEATH
		Imatinib					x	x	x	
	HCA	Hemicastration	x							
	HCA+I	Hemicastration	x							
		Imatinib					x	x	x	

unsolved. Substitute animals were injected to maintain the group size large enough.

The animals were sacrificed by decapitation at the age of 5 or 8 d. The weight of the body and testis were recorded and blood and testicular samples were collected. All animal experiments were approved by the Turku University Committee on the Ethics of Animal Experimentation.

Analysis of the diameter and length of seminiferous cords. In order to calculate the diameter and the length of the seminiferous cords, Bouin-fixed 4-μm thick testicular sections (n = 4 per group) were stained with hematoxylin and eosin. Morphometric analysis involved the measurement of cord diameter and the proportion of testicular tissue made by interstitial tissue. Cord length was calculated by using a formula:[21] length of the cord (m) = {weight of the testis (mg) x cord area (%)}/{π x [diameter of the cord (μm)/2)]^2}. All analyses were performed utilizing a morphometric program (Leica IM500 Version: 4.0; Leica Microsystems Imaging Solutions Ltd.).

The immunohistochemical detection of BrdU incorporation and the double staining with WT-1. BrdU (Roche Applied Science) (50 mg/kg) was injected intra peritoneally three hours prior to sacrificing the animal. The samples of testicular tissue were fixed overnight with 4% paraformaldehyde-fixative, embedded in paraffin and cut to 4-μm thick sections. To detect BrdU-positive peritubular myoid cells, immunohistochemistry was performed for the testis samples following manufacturer's instructions (Roche Diagnostics Corp.), as described in reference 19. Shortly, monoclonal anti-BrdU (6 μl/100 μl PBS, Roche Diagnostics Corp.) was used as the primary antibody and the secondary antibody treatment and visualization were performed utilizing the PowerVision immunohistochemistry kit (PowerVision + TM Poly-HRP IHC Kit Biotin-free, anti-mouse/rabbit, ImmunoVision Technologies, Co.), according to the manufacturer's instructions.

Proliferating Sertoli and germ cells were detected using double fluorescense immunohistochemistry with antibodies against BrdU and WT-1 as described in references 22 and 23. A rabbit polyclonal antibody against WT-1 (180) (Santa Cruz; 1:100 dilution) was used for identifying Sertoli cells and an anti-BrdU antibody (a mouse monoclonal antibody, Roche Diagnostics Corp., 6 μl/100 μl TBS) was utilized to label proliferating cells. Cell nuclei were stained with DAPI (0.5 ng/ml, Sigma). After secondary antibody incubation the slides were examined under fluorescence microscope.

All Sertoli and germ cells as well as BrdU-positive peritubular myoid, Sertoli and germ were counted from the minimum area of 300,000 μm^2 per testis section (n = 4 per group).

Hormone assays. Plasma levels of FSH were measured employing immunofluorometric assay as described in reference 24. For the FSH assay, the sensitivity was 0.1 μg/L and the intra- and interassay coefficients of variation 4.4% and 10.4%, respectively, at a level of 4.8 μg/L.

The FSH levels of eight animals in each experimental group were measured.

Extraction of RNA and performance of quantitative RT-PCR. Total RNA was extracted from testicular tissue using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions and treated with the DNase I Amplification Grade Kit (Invitrogen) as described in reference 25. Briefly, quantitative RT-PCR was performed employing the DyNAmo SYBR Green 2-Step qRT-PCR Kit (Finnzymes), with the inclusion of controls without reverse transcriptase. For each set of conditions four independent samples were analyzed in triplicate. The mRNA levels of platelet derived growth factors *Pdgfa* and *Pdgfb* and their receptors *Pdgfra* and *Pdgfrb*, *c-kit*, Stem cell factor (*Scf*), Glial cell line-derived neurotropic factor (*Gdnf*) and its receptor *Ret* were analyzed relative to the levels of *glyceraldehyde-3-phosphate dehydrogenase (Gapdh), hypoxanthine guanine phosphoribosyl* trans*ferase 1 (Hprt1)* and *ribosomal protein S26 (S26)* mRNAs. The primers and annealing temperatures used in this regard are presented in **Table 2**.

Culture of human peritubular cells. Isolation of human testicular peritubular cells (HTPC) was performed as previously described in references 26–28. HTPCs originate from

Table 2. The primers used in qRT-PCR analysis

Primers for quantitative RT-PCR				
Gene	**Primer sequence**	**Annealing temperature**	**Product length**	**GenBank accession number**
Gapdh	5'-AGA CAG CCG CAT CTT CTT GT-3' 5'-CTT GCC GTG GGT AGA GTC AT-3'	55°C	207 bp	NM_017008
Hbrt1	5'-AAG CTT GCT GGT GAA AAG GA-3' 5'-CCG CTG TCT TTT AGG CTT TG-3'	54°C	185 bp	NM_012583
S26	5'-AAG GAG AAA CAA CGG TCG TG-3' 5'-GCA GGT CTG AAT CGT GGT G-3'	57°C	300 bp	XM_001066146
c-kit	5'-GGC CTA GCC AGA GAC ATC AG-3' 5'-CAT TCG GAA ACC TTC CTT GA-3'	59°C	234 bp	D12524
Scf	5'-CAA AAC TGG TGG CGA ATC TT-3' 5'-GCC ACG AGG TCA TCC ACT AT-3'	61°C	217 bp	NM_021843
Pdgfra	5'-ACG TTC AAG ACC AGC GAG TT-3' 5'-CAG TTT GAT GGA CGG GAG TT-3'	64°C	225 bp	XM_214030
Pdgfrb	5'-ACC TGG TGG ACT ACC TGC AC-3' 5'-TGT CCG CGT ATT TGA TGT GT-3'	63°C	234 bp	NM_031525
Pdgfa	5'-GAG ATA CCC CGG GAG TTG AT-3' 5'-AAA TGA CCG TCC TGG TCT TG-3'	63°C	244 bp	NM_012801
Pdgfb	5'-GTC GAG TCG GAA AGC TCA TC-3' 5'-CAC TGC ACA TTG CGG TTA TT-3'	60.5°C	212 bp	XM_001075973
Ret	5'-ACA GCC TTC CGT CTG AAA GA-3' 5'-AAG CCC CGT ACA ACT TGA TG-3'	58°C	157 bp	NM_012643
Gdnf	5'-GCC GAG ACA ATG TAC GAC AA-3' 5'-CTG GAG CCA GGG TCA GAT AC-3'	57°C	206 bp	NM_019139

Table 3. The effects of FSH administration and hemicastration (HCA) with or without imatinib treatment (I) on the serum levels of FSH and the morphological findings of postnatal testicular growth in rats

Parameter	**Age and treatment group**								
	5d			**8d**					
	CTRL	**FSH**	**HCA**	**CTRL**	**I**	**FSH**	**FSH + I**	**HCA**	**HCA+I**
Body weight (n = 8)	13.7 ± 0.8	11.6^{a} ± 0.2	13.5 ± 0.4	20.3^{c} ± 0.8	12.8^{d} ± 0.2	21.0^{c} ± 0.4	14.1^{d} ± 0.6	20.8^{c} ± 0.9	14.0^{d} ± 0.9
FSH (ng/ml) (n = 8)	3.8 ± 0.4	26.1^{a} ± 1.3	11.4 ± 1.4	5.3 ± 0.8	2.9^{d} ± 0.5	15.6^{bc} ± 0.7	50.0^{d} ± 6.3	16.2^{bc} ± 1.3	15.7 ± 1.6
Testis weight (mg) (n = 8, except in HCA and HCA+I n = 16)	9.4 ± 0.04	7.8 ± 0.03	8.7 ± 0.04	17.5^{c} ± 0.05	13.7^{d} ± 0.04	25.3^{bc} ± 0.08	21.0^{d} ± 0.16	19.1^{ce} ± 0.05	15.9^{d} ± 0.06
Cord diameter (μm) (n = 4)	54.2 ± 0.9	47.1^{a} ± 1.8	52.9^{e} ± 0.5	55.4 ± 0.9	58.0 ± 1.4	57.1^{c} ± 0.6	61.1^{d} ± 1.4	56.5^{c} ± 0.9	60.4 ± 1.4
Sertoli cells/mm^2 (n = 4)	6689 ± 283	6900 ± 430	6029 ± 172	7372 ± 440	9428^{d} ± 383	8498^{c} ± 354	9099 ± 670	8512^{c} ± 439	9374 ± 576
Germ cells/mm^2 (n = 4)	180 ± 18	133 ± 11	181 ± 25	511^{c} ± 91	299 ± 42	412^{c} ± 77	275 ± 27	539^{c} ± 116	251 ± 67
BrdU+ germ cells/mm^2 (n = 4)	99.8 ± 5.0	45.3 ± 19.7	50.5 ± 15.8	219.1^{c} ± 31.3	147.6 ± 17.4	229.4^{c} ± 43.8	149.6 ± 17.2	268.3^{c} ± 51.3	114.5^{d} ± 33.0

The values presented are means ± SEM, significant difference ($p < 0.05$) ^{a}compared with control value at the age of 5-d-old, ^{b}compared with control value at the age of 8-d-old, ^{c}compared with value after similar treatment at the age of 5-d old, ^{d}compared with value after similar treatment without imatinib at the age of 8-d-old and ^{e}HCA compared with FSH at the same age.

patients displaying normal spermatogenesis. The cells, passages 3–10, were maintained in DMEM supplemented with 10% fetal calf serum (FCS; both from PAA GmbH). All participants granted written informed consent. The local ethics committee of Technical University of Munich approved the study.

To determine viability of cells in culture the CellTiter-Glo® Luminescent Cell Viability Assay (Promega GmbH) was used as described before in reference 29. Cells were seeded in quadruplicates in 24-well tissue culture plates and incubated for 24 h with/without imatinib (10, 20, 50 and 100 μM). The kit reagents were added directly to the cells and luminescence, as a marker of cell viability, was measured with FLUOstar OPTIMA (BMG LABTECH GmbH).

For further analysis cells were seeded on 60-mm dishes and treated with 5 ng/ml PDGF-BB (human recombinant; Sigma) or/and 10 μM imatinib for 4 d. As the PDGF stock was prepared in 4 mM HCl containing 0.1% BSA, a 1:1,000 dilution of this stock without PDGF was added to the media as a control. Cells were trypsinized and numbers determined with an automated cell counting device (CASY-system, Casy, Schärfe Systems).[28]

Cell proliferation was evaluated by counting anaphase, metaphase and telophase mitotic figures in HTPCs, stained with DAPI (4',6-diamidino-2-phenylindole; 1.5 μg/ml; Vectashield mounting medium, Vector Laboratories). Labeling of nuclei was done as described previously in reference 30. Mitotic events were expressed as percentage of DAPI stained nuclei. At least 200 nuclei per slide were counted.

Statistical analysis of the data. The means and SEM values obtained in independent experiments were calculated. The Mann-Whitney Rank Sum test or t-test (pairwise comparison) was used for single statistical comparison of independent groups of samples. ANOVA followed by the Tukey's test was employed for multiple comparisons. A p value of less than 0.05 was considered to indicate a statistically significant difference between groups.

Disclosure of Potential Conflicts of Interest

The authors have nothing to declare. The part of this study dealing with human peritubular cells was done in partial fulfilment of the requirements of a Dr. rer. nat. thesis at LMU of Marion Adam.

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