

PREPUBERTAL ONSET OF SPERMATOGENESIS IS UNDER THE DIRECT CONTROL OF FGFs FAMILY

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ABSTRACT. The onset of spermatogenesis in prepubertal males is marked by reentering mitosis of quiescent prospermatogonia. Since Sertoli cells at this time are nonmature and do not mediate hormonal regulation, it is hypothesized that this event is under the direct control of paracrine growth factors Fibroblast Growth Factor (FGF) 1 or FGF 2. We have used a Day-2 mouse testis organ culture assay to identify factors that may control this process. Recombinant FGF 1, FGF 2, FGF 7 as well as heparin-binding proteins isolated from 0 and 4-day-old mice testes were tested. The DNA synthesis in quiescent prospermatogonia was identified immunocytochemically by using Cell Proliferation kit. In control testes the percentage of labeled germ cells was 10%, while after the application of 100 pg/ml FGF-2 the mitogenic activity of prospermatogonia increased up to 57%. Similar effect was recorded after addition of 9 µg/ml of heparin binding protein isolated from 0 day-old mice testes. It is concluded that the prepubertal onset of spermatogenesis is highly coordinated event involving cascade of growth factors and proteins. Heparin-binding proteins, including FGF's family play a key role in paracrine regulation of prepubertal spermatogonial proliferation in mammals.

KEY WORDS. prepubertal spermatogenesis, FGF's family

INTRODUCTION

Different factors are known to stimulate DNA synthesis, proliferation and/or differentiation of germ cells in the adult testes. In the rat activin A increases ³H-thymidine incorporation by differentiating spermatogonia (Mather et al, 1990), whereas inhibin decreases their number (Moore et al, 1994). Il-1 α , IGF-I and IGF-II stimulate ³H-thymidine incorporation into differentiating spermatogonia in adult seminiferous tubules in vitro (Nakayama et al, 1999). Thus several growth factors

appear to regulate the proliferation of spermatogonia in adult testes but none of these data demonstrate the stimulation of DNA synthesis by the “totipotent” prospermatogonial cells of neonatal animals (Kubota et al, 2004). Previously by using Sertoli cell secreted media, isolated from rat prepubertal Sertoli cells we have stimulated prospermatogonial proliferation up to 5-10 fold over the controls in organ culture (Kancheva et al, 1990). We hypothesized that Sertoli cells secrete growth factors, probably FGFs family acting as a local mitogenic factors on germ cells.

Fibroblast Growth Factors (FGFs) make up a large family of polypeptide growth factors that are found from nematodes to humans. In vertebrates the 22 members of FGFs family range in molecular mass from 17 to 34 kDa and share 13-71% amino acid identity. FGFs have a high affinity for heparin sulfate proteoglycans and require heparin sulfate to activate one of four cell-surface FGF receptors (Ornitz and Itoh, 2001). FGFs are involved in many biological processes acting through kinase receptors.

To date based on recent evidence, FGF and TGF β family members are the only growth factors implicated in regulation of DNA synthesis in prospermatogonial stem cells during the initiation of spermatogenesis in postnatal testicular development.

The aim of the present work is to study the effect of some members of FGFs family: recombinant FGF 1, FGF 2 and FGF 7 on mouse prospermatogonial cell proliferation and to compare this effect with heparin-binding proteins from testicular extract of 0- and 4-day-old mice.

MATERIAL AND METHODS

Materials

Male conventional mice 2 days old were supplied by animal-breeding farm of the Bulgarian Academy of Sciences (Sofia, Bulgaria). Dulbecco's modified Eagle's medium (DMEM) and bovine serum albumin (BSA) (Sigma); Bacto-Agar (Difco Laboratories); OCT Compound (Miles, Scientific); Procelloidin (Fluka Chemica-Biochemica); Organ culture dishes (Falkon, Becton-Dickinson); Cell proliferation kit (Amersham) were used. Purified recombinant Fibroblast Growth Factors 1, 2 and 7 and Heparin-binding proteins isolated from 0-day- and 4-day-old mice were kindly provided by Prof. Anthony R. Bellve (Columbia University, NY, USA).

Tissue culture

Tissue cultures were prepared as described previously (Martinova et al, 2001). Briefly, 2-day-old mouse testes were cut into 2 segments, placed on permeable celloidine mambranes and overlaid with 2% low-melting-point agar in 0.9%NaCl (vol/vol). Each preparation was placed in an organ culture dish containing DMEM supplemented with 2% BSA and 5-bromo-2-deoxyuridine (BrdU) 1:500 (negative control).

In the first group of experiments, the medium was additionally supplemented with FGF 1, 2 or 7 in doses of 1, 3, 10, 30, 100 and 300pg/ml.

In the second group of experiments, the medium was supplemented with heparin-binding protein isolated from 0 or 4-day-old mice testes in a doses 0.1, 0.33, 1, 3, 9, 27 and 80 μ g/ml.

The explants were cultured 24h at 37°C with 5% CO₂. At the end of cultivation period the segments were immersed in OCT Compound, snap-frozen in liquid nitrogen and cryosectioned at 5µm.

Immunocytochemistry

Sections mounted on slides were fixed in Carnoy's fixative and incubated in 0.03% H₂O₂ for 30 min to block endogenous peroxidase activity. Detection of incorporated BrdU was achieved with monoclonal antibody incubation to BrdU followed by anti-mouse IgG2a antibodies conjugated with horse radish peroxidase and stained with DAB/substrate/intensifier. The sections were counterstained with Harris's heamatoxylin, dehydrated and mounted in Canada balsam.

Statistical analysis

Labeled and unlabeled prospermatogonial nuclei were counted in control and experimental groups. At least 100 cells were counted in three different testicular segments, each done in triplicate. Statistical analysis was performed by Student's t-test. Photographs were taken on an Opton microscope.

RESULTS

On day 2 post partum (p.p.) mouse testis is composed of seminiferous cords and interstitial tissue. In the cords two cell types are well distinguished: Sertoli cells and prospermatogonia. Nonmature Sertoli cells are situated on the basal membrane. They are cylindric in shape, small in size (5-7µm in diameter) with dark nuclei. In 2-day-old mouse testis Sertoli cells actively proliferate and markedly incorporate BrdU. Prospermatogonia are localized centrally in the seminiferous cords. They are big, round cells (about 20µm in diameter) with pale nucleus (Fig.1 and 2 arrows).

In control testes of all groups the percentage of labeled prospermatogonia was about 10% (Graphics 1 and 2).

The effect of FGF 1, 2 and 7 is shown on Graphic 1.

Dose-response curve of FGF 1 application have shown stimulation of prospermatogonial germ cell proliferation up to 27% at dose 100pg/ml. FGF-2 stimulates BrdU incorporation up to 57 % at dose 100pg/ml. The effect of FGF 7 is similar to FGF 1 with maximum stimulation up to 31 % at a dose of 30pg/ml.

The effect of heparin-binding proteins isolated from 0- and 40day-old mouse testis is shown on Graphic 2.

The application of heparin-binding protein extracted from 0-day-old mouse testis stimulates DNA synthesis in prospermatogonia in dose-response manner with maximum stimulation up to 59 % in dose 9µg/ml. Heparin-binding protein from 4-day-old mice testes stimulates prospermatogonial proliferation up to 37 % in dose 1µg/ml.

In the testicular cords after incubation with maximally effective doses of FGF 2 and HBP-0 many prospermatogonial cells were localized between Sertoli cells on the basal membrane.

DISCUSSION

It is known that growth factors in general stimulate cell proliferation and/or differentiation by interacting with cell-surface receptors. Using RT-PCR we showed

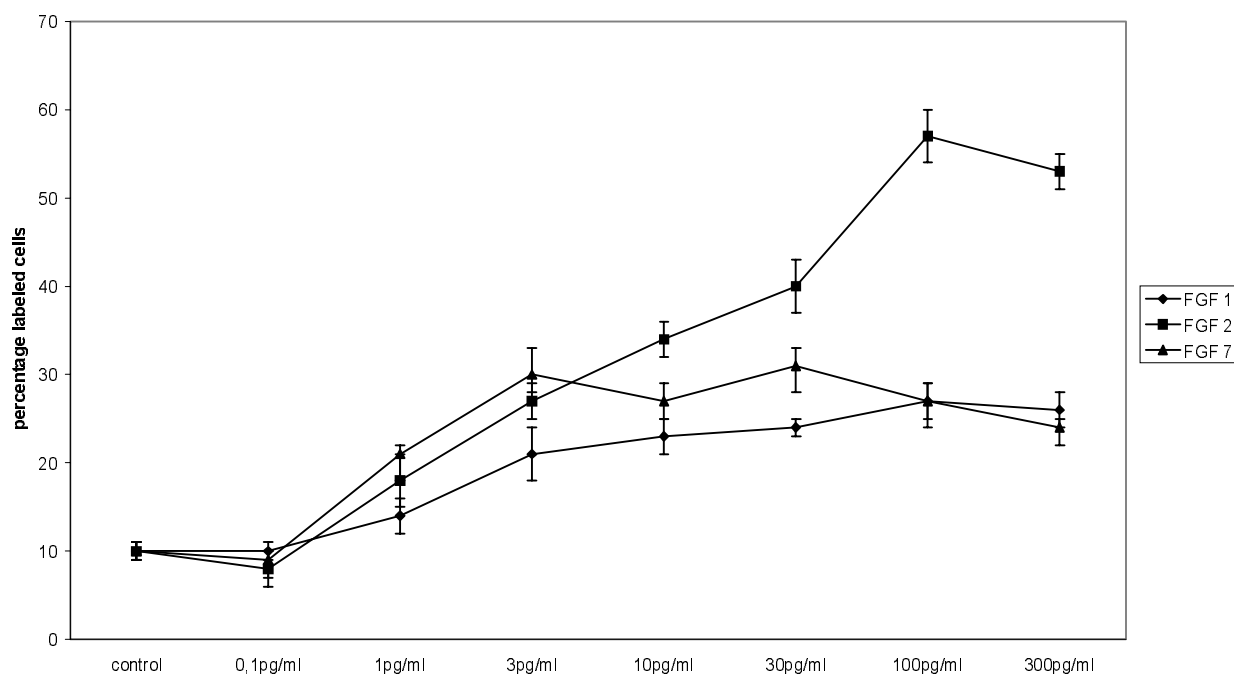
previously that FGF-1 and FGF-2 mRNA is detected in Day-4 mouse testes. We suggested that at least two FGFs are present in the mouse testis at the onset of spermatogenesis (FGF-1 and FGF-2) and prospermatogonia have at least one FGF receptor prior to the onset of spermatogenesis – FGFR-2 (Seidensticker et al, 1996). Lately it was found an expression of mRNA for FGFR-1, 2, 3 and 4 in fetal, immature and adult testis (Cancilla and Risbridger, 1998). It is suggested that ligands FGFs 1-5 and 8 can signal through these receptors (Cancilla et al, 2000). Our results indicated that not only FGF-1 and FGF-2 but FGF-7 as well can stimulate prospermatogonial DNA synthesis and probably acts through the above mentioned receptors. In addition FGF-7 stimulates DNA synthesis in exocrine pancreatic cells in diabetic and control rats in vitro (Ogneva and Martinova, 2002), thus being a universal mitogen for a wide variety of cell types.

Increased proliferative activity of prospermatogonia was registered not only after treatment with recombinant FGFs but after incubation of testicular tissue with heparin-binding proteins. Mitogenic activity of germ cells was stronger expressed after application of HBP isolated from 0-day-old mouse testis. Our results confirm the hypothesis that bFGF gene expression is predominantly early in prepubertal testicular development and decreases with sexual maturity (Gnessi et al, 1997). It is known that FGF 2 is integrated into an insoluble substrate such as extracellular matrix as FGF 2-heparin-sulfate proteoglycan complexes (Gospodarowicz, 1990). Heparin sulfate binds FGF 2 and protects it from denaturation and proteolytic degradation. Hydrolysis of the extracellular matrix results in the liberation of heparin-sulfate-FGF complexes which can be biologically active (Gospodarowicz, 1986). Since the testicular tissue is very rich in extracellular matrix synthesized by peritubular cells, it can be concluded that increased mitogenic activity of 2-day-old mouse prospermatogonia in vitro is due to biologically activated FGF 2-heparin-sulfate complexes as well as to applied recombinant FGF 1, 2 and 7.

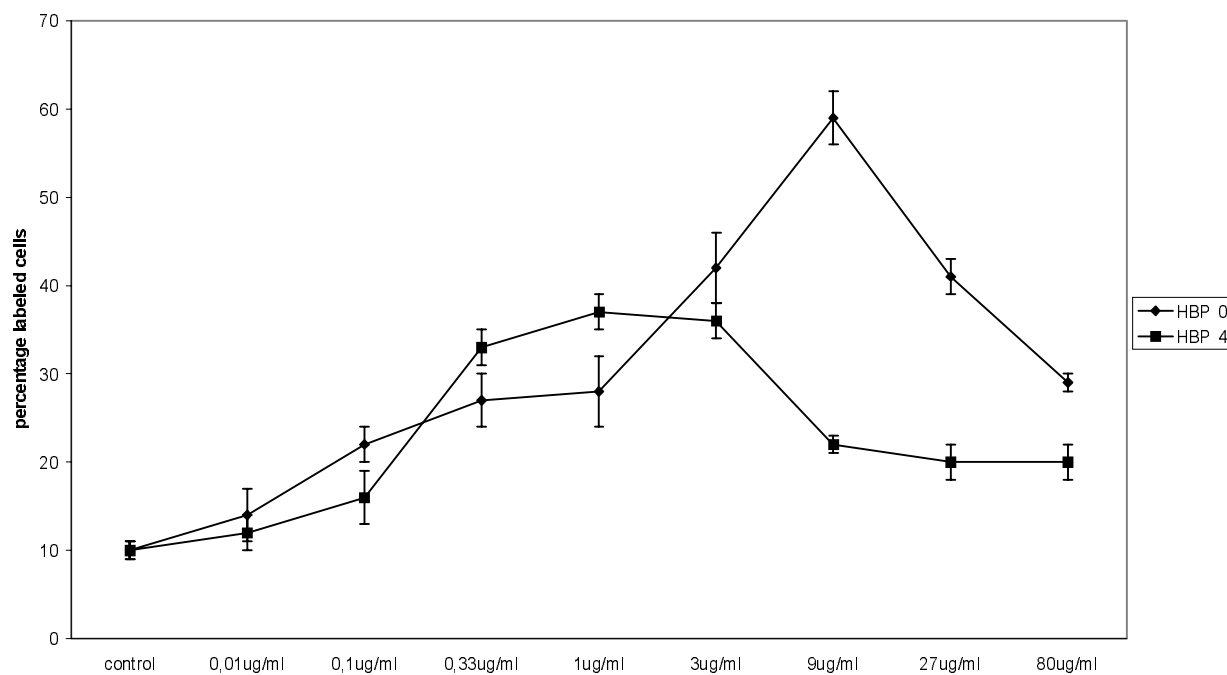
REFERENCES

- CANCILLA, B., G. P. RISBRIDGER 1998. Differential localization of fibroblast growth factor receptor -1, -2, -3 and -4 in fetal, immature and adult rat testes. *Biol.Reprod.*,58,1138-1145.
- CANCILLA, B., A. DAVIES, M. FORD-PERRISS, G. P. RISBRIDGER 2000. Discrete cell- and stage-specific localization of fibroblast growth factors and receptor expression during testis development. *J. Endocrinol.*, 164, 149-159.
- GNESSI, L., A. FABBRI, G. SPERA 1997. Gonadal peptides as mediators of development and functional control of the testis: An integral system with hormones and local environment. *Endocrine Reviews*, 18, 541-595.
- GOSPODAROWICZ, D., G. NEUFELD, L. SCHWEIGERER 1986. Molecular and biological characterization of fibroblast growth factor: An angiogenic factor which also controls the proliferation and differentiation of mesoderm and neuroectoderm derived cells. *Cell Differ.* 19, 1-
- GOSPODAROWICZ, D., 1990. Fibroblast growth factor : chemical structure and biologic function. *Clin. Orthopaed. Related Res.*, 256,231-248.
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- KANCHEVA, L., Y. MARTINOVA, V. GEORGIEV 1990. Prepubertal Sertoli cells secrete a mitogenic factor(s) that stimulates germ and somatic cell proliferation. *Mol. Cell. Endocrinol.*, 69, 121-127.
- KUBOTA, H., M. R. AVABROCK, R. L. BRINSTER 2004. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc.Natl.Acad.Sci.USA* 101, 16395-16396.
- MARTINOVA, Y., D. NIKOLOVA, A. BELLVE 2001. Mullerian inhibitory substance down-regulates seminiferous growth factor-stimulated rat prespermatogonial cell proliferation in vitro. *Acta Morphol.Anthropol.*, 6, 45-50.
- MATHER, J. P., K. ATTIE, T. WOODRUFF, G. RICE, D. PHILLIPS 1990. Activin stimulates spermatogonial proliferation in germ-Sertoli cell cocultures from immature rat testis. *Endocrinology*, 127, 3206-3214.
- MOORE, A., L. KRUMMEN, J. MATHER 1994. Inhibins, activins, their binding proteins and receptors – interactions underlying paracrine activity in the testis. *Mol. Cell. Endocrinol.*, 100, 81-86.
- NAKAYAMA, Y., T. YAMAMOTO, S. ABE 1999. IGF-I, IGF-II and insulin promote differentiation of spermatogonia to primary spermatocytes in organ culture of newt testes. *Int.J.Dev.Biol.*, 43, 343-347.
- OGNEVA, V., Y.MARTINOVA, I. IVANOV, M. INCHOVSKA, A. NERONOV 2002. Effects of fibroblast growth factors FGF2 and FGF7 on the proliferation of insuloma cell line RIN 38. *C.R.Acad.bulg.Sci.*, 55, 99-102.
- ORNITZ, D., N. ITOH 2001. Fibroblast growth factors. *Genome Biol.*, 2, REWIEVS3005.
- SEIDENSTICKER, M., Y.MARTINOVA, A. BELLVE 1996. Fibroblast growth factor and the onset of spermatogenesis. *Proc. Northeast Reg. Confer. on Developmental Biology*, Marine Biological Lab. In Woods Hole, MA, USA, pp 148-151.



Graphic 1. Dose-response curves for prospermatogonial cell proliferation stimulated by FGF-1, 2 and 7 in organ culture of 2-day-old mouse testis. The data derived from triplicate cultures and are expressed as mean \pm standart deviation (SD).



Graphic 2. Dose-response curves for prospermatogonial cell proliferation stimulated by HBP 0 and HBP 4 in organ culture of 2-day-old mouse testis. The data derived from triplicate culture and are expressed as mean \pm standart deviation (SD).