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Requirement for Heparan Sulphate Proteoglycans to Mediate Basic Fibroblast Growth Factor (FGF-2)-induced Stimulation of Leydig Cell Steroidogenesis

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This study reports that, in contrast to previous findings, basic fibroblast growth factor (FGF-2) stimulates immature Leydig cell steroidogenesis in the absence of luteinizing hormone (LH). Heparan sulphate proteoglycans (HSPGs) are essential for this action of FGF-2 and the data suggest that HSPG/FGF-2 interactions have a significant role in the maintenance of immature Leydig cell steroidogenesis. Culture conditions were established for the maintenance of immature rat Leydig cells steroidogenesis *in vitro* for at least 2 days. Under these conditions the effect of exposure to FGF-2 at doses ranging from 0.1–10 ng/ml was shown to cause a significant stimulation of basal, but not LH-stimulated, 5α-androstane 3α,17β-diol production over 24h in culture. This stimulatory action on basal steroidogenesis is mediated through HSPG, as it was blocked by the addition of heparin (100 μg/ml), sodium chlorate (25mM) and protamine sulphate (5 μg/ml). These data demonstrate the involvement of HSPG in regulating FGF-2 action on Leydig cells and a potential role for Leydig cell HSPG in mediating paracrine regulatory actions of other heparin binding growth factors.

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INTRODUCTION

The heparin binding growth factor family consists of a family of at least nine polypeptides which include basic fibroblast growth factor (FGF-2) [1]. FGF-2 has proliferative, differentiating and angiogenic activities in many cell lines, and in the testis has been shown to affect Leydig cell steroidogenesis [2–7]. The precise effect of either stimulating or inhibiting steroidogenesis is dependent on the *in vitro* Leydig cell culture systems studied; nevertheless these studies have led to the hypothesis that FGF-2 can potentially act as a paracrine factor and alter Leydig cell steroidogenesis within the testis.

The biological activities of FGF-2 are mediated through high affinity cell surface receptors; at least two of the four members of the FGF receptor (FGFR) family are known to have a high affinity for FGF-2, i.e. FGFR-1 and FGFR-2 [8]. As well as the FGFRs, FGF-2 also interacts with low affinity binding sites on heparan sulphate proteoglycans (HSPGs) on the cell surface or in the extracellular matrix [9]. This study examined the action of FGF-2 on 5α-androstane 3α,17β-diol production by immature rat Leydig cells in maintenance culture conditions similar to those described previously [10]. The results contrast with previous findings and the role of HSPGs in mediating these effects of FGF-2 was determined.

MATERIALS AND METHODS

Animals

20–21-day-old male Sprague–Dawley rats were obtained from the Central Animal House at Monash University. Animals were killed in a CO_2-charged chamber.

Reagents

Percoll and Dextran T70 were obtained from Pharmacia (Uppsala, Sweden). Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient mixture (F12) were obtained from GIBCO (Grand

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Island, NY). Medium 199 with Hanks' balanced salts and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (M199H), penicillin and streptomycin sulfate were purchased from Commonwealth Serum Laboratories (Melbourne, Australia). Bovine serum albumin (BSA), porcine heparin sodium salt, deoxyribonuclease 1, bovine lipoproteins (lot 110H00662) and 5α-androstane 3α,17β-diol were purchased from Sigma (St Louis, MO). Collagenase type 1 was purchased from Worthington (Freehold, NJ). Activated charcoal was obtained from Merck (Darmstadt, Germany), EDTA from BDH/Merck (Australia) and Ultima Gold scintillation fluid from Packard (Canberra, Australia). Tritiated 5α-androstane 3α,17β-diol was purchased from New England Nuclear-Du Pont (Boston, MA). Antiserum to 5α-androstane 3α,17β-diol was generously supplied by Drs C. Nancarrow and M. Tetsuka of the Commonwealth Scientific and Industrial Research Organisation (Australia), and rat LH-I-6/7 was obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD). Bovine recombinant FGF-2 was obtained from Boerhinger Mannheim (Castle Hill, Australia), protamine sulphate was obtained from The Boots Co. (Australia) and sodium chlorate from Fluka (Buchs, Switzerland).

Leydig cell preparation and purification

Interstitial cells were prepared from decapsulated testes which were incubated in M199H containing 0.5 mg/ml collagenase and 5 mg/ml BSA in a shaking (90 cycles/min) water bath at 32C. Leydig cells were prepared from interstitial cells as previously described [11] with minor modifications. The elutriation medium (M199H) contained only 0.5% BSA, 0.9 mM EDTA and 12 mg/l DNAase as additives. The discontinuous Percoll gradient used to purify Leydig cells consisted of seven layers with densities as follows: 1.060 (5ml), 1.064 (8ml), 1.067 (5ml), 1.069 (8ml), 1.070 (5ml), 1.075 (8ml) and 1.096 (5ml). Cells taken from the centrifugal elutriator were layered onto the gradient which was spun at 800 ***g*** for 30 min. Leydig cells were collected from the bottom of the gradient with 2.5ml waste being taken first followed by 17ml of Leydig cells (1.069–1.096).

Culture of cells

Cells were cultured as previously described [11] in DMEM-F12 plus 0.1% BSA containing 0.1 mg/ml bovine lipoprotein. Cells were plated down for 2 h in DMEM-F12 before media were removed and factors added in DMEM-F12 (0.1% BSA) for a further 22 h.

Radioimmunoassay (RIA) for 5α-androstane 3α,17β-diol

Media were assayed for 5α-androstane 3α,17β-diol using a RIA as described by Risbridger and Davies [11]. The antiserum was obtained from Drs Nancarrow and Tetsuka and showed significant cross reactivity with a number of androgens including 5β-androstane 3α,17β-diol (87.7%), 5β-androstan-3α-ol-17-one (51.1%) and 5α-dihydrotestosterone (33.5 %) but less than 6% cross reactivity with testosterone. 5α-androstane 3α,17β-diol was measured as it is the major androgen produced by the immature rat testis[12].

Statistical analysis

Regression analysis and paired *t*-tests were carried out using SAS (SAS Institute Inc, Cary, NC) statistical software.

RESULTS

Culture conditions for immature rat Leydig cells

Figure 1 shows that the immature rat Leydig cells, isolated using the methods described herein, retain their responsiveness to LH and steroidogenic capacity over 2 days in culture. An approx. 20-fold response to a maximum dose of LH (16ng/ml) was observed on both day 1 and 2, so that levels of approx. 20ng 5α-androstane 3α,17β-diol/10^6 cells/h were produced *in vitro*. The following experiments were performed on day 1 of culture under these stable conditions.

Effect of FGF-2 on Leydig cell steroidogenesis

Regression analysis showed that addition of FGF-2 at doses ranging from 0.05 to 10 ng/ml significantly stimulated ($P < 0.01$, $r = 0.9866$) 5α-androstane 3α,17β-diol production *in vitro* in a dose dependent manner in the absence of LH [Fig. 2(a)]. In the presence of a maximally stimulating dose of LH

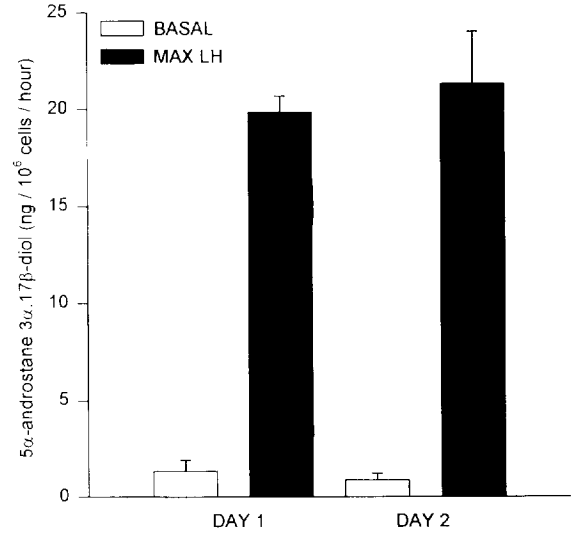
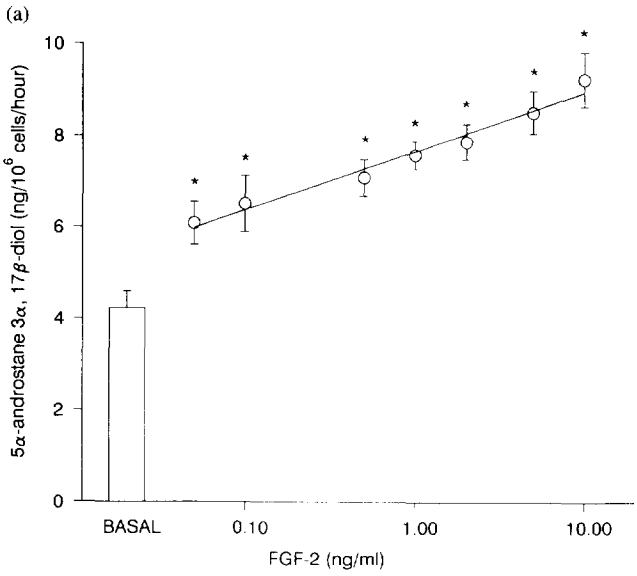


Fig. 1. The response of immature rat Leydig cells, *in vitro*, to stimulation by maximum levels of LH (16 ng/ml) in the presence of 0.1 mg/ml bovine lipoprotein over 2 days of culture. Basal response (0 ng/ml LH) is also shown. Each bar represents the mean ± SD obtained from five replicate wells.



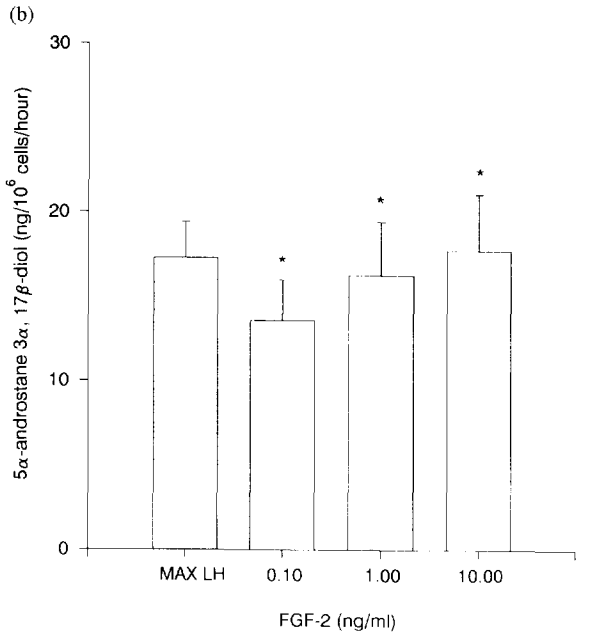


Fig. 2. (a) The response of immature rat Leydig cells to increasing doses of FGF-2 (0.05–10 ng/ml) at basal (0 ng/ml) LH levels. FGF-2 significantly stimulated (*$P < 0.01$) steroidogenesis above basal levels at each dose. Each value represents the mean ± SD obtained from five replicate wells. (b) The response of immature rat Leydig cells to increasing doses of FGF-2 (0.1–10ng/ml) at maximum (16 ng/ml) LH levels. FGF-2 had no significant effect (*$P > 0.05$) on steroidogenesis, above or below control LH stimulated levels, in the presence of LH at any dose. Each value represents the mean ± SD obtained from five replicate wells.

(16ng/ml) no significant effect ($P > 0.05$) on steroidogenesis was observed [Fig. 2(b)].

The effect of FGF-2 on steroidogenesis was examined further in order to determine whether HSPGs play a role in mediating the response of immature Leydig cells to FGF-2.

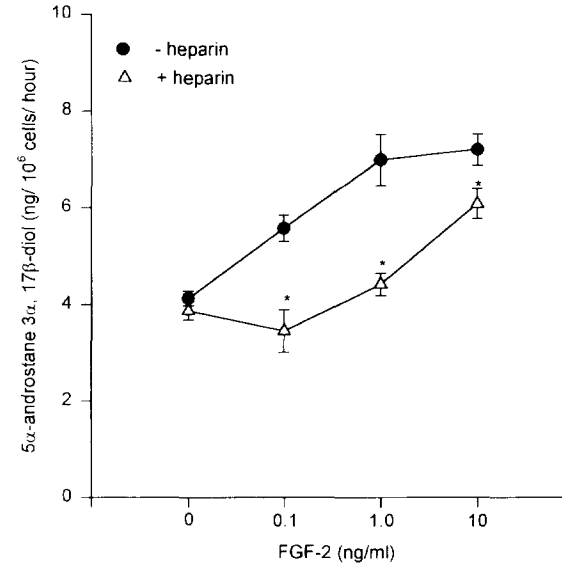


Fig. 3. The response of immature rat Leydig cells to increasing doses of FGF-2 (0.1–10 ng/ml) in the presence (△) and absence (●) of sodium chlorate (25 mM) in the absence of LH. Sodium chlorate significantly inhibited (*$P < 0.01$) FGF-2 stimulated steroidogenesis, when compared with controls, at each dose of FGF-2. Each value represents the mean ± SD obtained from five replicate wells.

Role of HSPGs in FGF-stimulated steroidogenesis

The formation of new fully sulphated heparan sulphate proteoglycans was disrupted by the addition of 25mM sodium chlorate, which significantly inhibited ($P < 0.01$) FGF-2 stimulated steroidogenesis in the

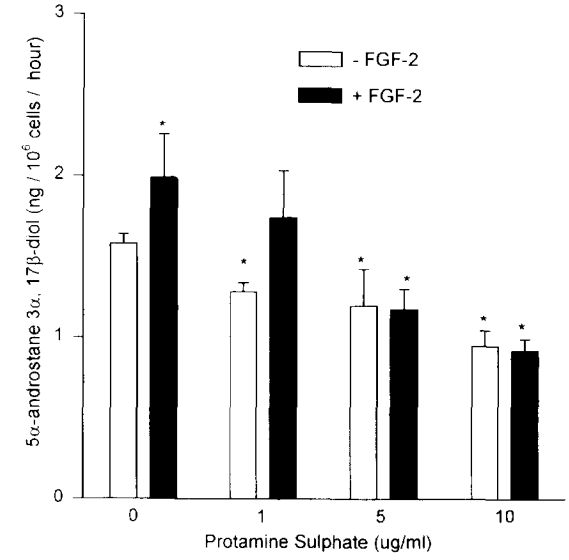


Fig. 4. The response of immature rat Leydig cells to increasing doses of protamine sulphate (1–10 μg/ml) in the presence (■) and absence (□) of FGF-2 (0.1 ng/ml). Protamine sulphate significantly ($P < 0.05$) inhibited basal steroidogenesis, when compared to the basal control at each dose shown and also significantly ($P < 0.05$) blocked the FGF-2 stimulated ($P < 0.05$) increase in steroidogenesis at doses ⩾5 μg/ml. Each value represents the mean ± SD obtained from four replicate wells and (*) indicates a significant difference ($P < 0.05$) from the basal control.

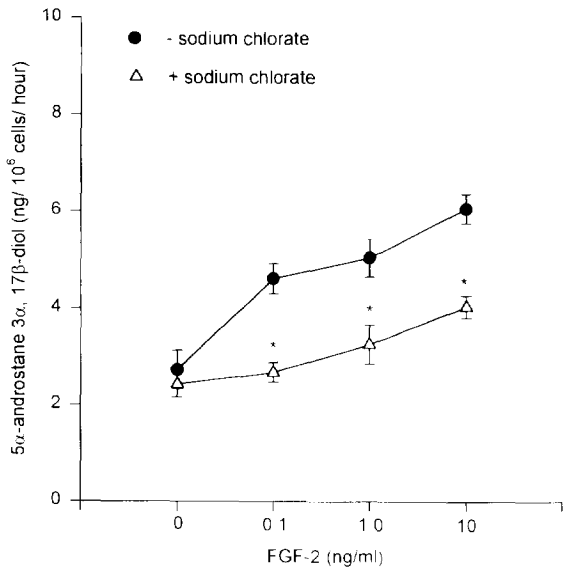


Fig. 5. The response of immature rat Leydig cells to increasing doses of FGF-2 (0.1–10 ng/ml) in the presence (△) and absence (●) of heparin (100 μg/ml). Heparin significantly inhibited (*$P < 0.01$) FGF-2 stimulated steroidogenesis, to below control levels, at each dose of FGF-2. Each value represents the mean ± SD obtained from five replicate wells.

presence of 0.1–10 ng/ml FGF-2, but had no effect on steroidogenesis in the absence of FGF-2 (Fig. 3). The addition of 0.1 ng/ml FGF-2 significantly stimulated ($P < 0.05$) steroidogenesis; the concomitant addition of protamine sulphate at 5 or 10 μg/ml significantly ($P < 0.05$) blocked the FGF-2 stimulated increase in steroidogenesis (Fig. 4). A dose of 1μg/ml protamine sulphate did not inhibit FGF-2 stimulated steroidogenesis although it lowered basal steroidogenesis in the absence of FGF-2 ; no further significant ($P < 0.05$) decrease in basal steroidogenesis was observed with the addition of higher doses of protamine sulphate.

Heparin (100 μg/ml), when added to immature Leydig cells in the absence of FGF-2, was not significantly ($P > 0.05$) different to controls, however in the presence of FGF-2 (0.1–10 ng/ml) heparin was able to significantly inhibit ($P < 0.01$) the FGF-2 induced increase in 5α-androstane 3α,17β-diol production (Fig. 5).

DISCUSSION

These results show that FGF-2 can increase basal androgen production by immature rat Leydig cells *in vitro*, in contrast to a previous report by Murono *et al.* [5]. The effect is dose dependent and requires HSPG to mediate this action.

The data show that immature Leydig cells are maintained in culture for 2 days and over that time the capacity of the Leydig cells to produce 5α-androstane 3α,17β-diol is stable and a 10-fold response to LH (16 ng/ml) stimulation is consistently observed. The levels of androgen produced *in vitro* (2–20 ng/h/10^6 cells) are consistent with previous reports [13,14] and are within the levels of steroid estimated to be present *in vivo*, as the total content of endogenous steroid in the immature rat testis (which contains 2.3×10^6 Leydig cells [15]) is approx. 20 ng steroid [16]. The effect of FGF-2 at doses ranging from 50 pg/ml to 10 ng/ml is to increase steroidogenesis and this represents an augmentation of basal levels and not a rescue from declining steroidogenesis; this effect contrasts with the inhibitory action on steroidogenic enzyme activities (5α-reductase and 3β-HSD) in immature rat Leydig cells [17,18].

It remains to be determined how FGF-2 stimulates steroidogenesis, but as the FGFRs have tyrosine kinase activity it is reasonable to postulate that signal transduction mechanisms involving protein tyrosine kinases are involved. However, although FGF-2 acts through a membrane bound receptor it also binds to HSPGs which are considered to be equally important for biological activity and for eliciting the appropriate biological response [9]. Different species of HSPG and heparin have been associated with binding to FGF-2 [19]. Kan *et al.* [20] have shown that there is an essential heparin-binding domain within the FGF receptor kinase, indicating that a complex of FGF-2, high affinity receptor and HSPG may be required for full response of a cell to FGF-2. Roghani *et al.* [21] proposed that heparin or HSPGs stabilise the ligand–receptor complex of FGF-2 and its high affinity tyrosine kinase receptor. Our results concur with the above literature in that they show that HSPGs are essential for the full biological activity of FGF-2 *in vitro*.

Sodium chlorate was used to block the formation of fully sulphated HSPGs and we have shown, as in a number of different systems [22], that this inhibited the actions of FGF-2 and blocked the FGF-2 induced increase in steroidogenesis. The degree of sulphation of HSPGs, along with the sequence of the polysaccharide residues, can provide the specificity by which HSPGs selectively bind different heparin binding growth factors including FGF-2 [22,23].

Protamine sulphate is a functional inhibitor of FGF-2 action [24] which acts by preferentially competing with FGF-2 for binding sites and disrupting binding of the ligand, high affinity receptor and HSPG. Protamine sulphate blocked the stimulation by FGF-2. The doses of protamine sulphate used in these studies inhibited basal and LH stimulated steroidogenesis (data not shown), whilst this might indicate a toxic effect, this is unlikely because the doses of protamine sulphate used are identical to those used with no toxic effects in many other systems [24]. An alternative interpretation is that an endogenous factor, sensitive to protamine sulphate, is present on the Leydig cells which contributes to the mainten-

ance of androgen production by immature Leydig cells in culture but this remains to be determined.

Binding of FGF-2 ligand to HSPGs can be displaced or blocked by addition of heparin (10μg/ml) [25]. Our results show that the addition of heparin blocks the FGF-2 induced increase in steroidogenesis, although it was noted that in the absence of FGF-2, this dose of heparin had no effect on steroidogenesis.

FGF-2 action can be tightly and rapidly controlled by HSPGs in addition to regulation by the FGFR phenotype. This has been convincingly demonstrated in embryonic neural tissues where there is a change in the glycosylation of HSPG which alters the binding specificity of the FGF-2 ligand [26]. This type of regulatory mechanism does not rely on a change in cell surface receptors or an alteration in the amount of ligand produced, but nonetheless permits a rapid change in cell signalling. In the testis, where expression of FGF-2 mRNA has been detected in several cell types, including the Sertoli, Leydig and the peritubular cells, and at differential developmental ages [27], this provides an additional mode of regulating the paracrine actions of FGF-2 within the testis. The types of HSPG on Leydig cells and any changes or modifications to HSPGs that might alter ligand activity are currently unknown. The action of FGF-2 might require the presence of activating and inhibitory sequences of HSPGs as suggested by Guimond *et al.* [22] or alternatively FGF-2 action might involve sequestration in the HSPGs until required and released by endogenous heparanases [28] so that FGF-2 and HSPG become available to the high affinity receptor in soluble forms. Whatever the mechanism of action, full biological activity of FGF-2 requires HSPG.

It is apparent from this study, that growth factors such as FGF-2 which have an essential requirement for substances such as HSPG (located in extracellular matrix and/or on cell surfaces) can have diverse effects simply as a result of the presence or absence of HSPGs. These growth factors may be present endogenously or exogenously added to the Leydig cell cultures. *In vivo* the interaction between the ligand, the receptors and the HSPGs has the potential to add another degree of rapid and tight control on the paracrine action of not only FGF ligands in the testis but also other heparin binding growth factors.

Further work is required to investigate the nature and roles of cell surface proteoglycan–growth factor interactions.

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REFERENCES

1. Basilico C. and Moscatelli D.: The FGF family of growth factors and oncogenes. *Adv. Cancer. Res.* **59** (1992) 115–165.
2. Fauser B.C.J.M., Baird A. and Hsueh A.J.W.: Fibroblast growth factor inhibits luteinizing hormone-stimulated androgen production by cultured rat testicular cells. *Endocrinology* **123** (1988) 2935–2941.
3. Sordoillet C., Savona C., Chauvin M.A., de Peretti E., Feige J.J., Morera A.M. and Benhamed M.: Basic fibroblast growth factor enhances testosterone secretion in cultured porcine Leydig cells: site(s) of action. *Molec. Cell. Endocr.* **89** (1992) 163–171.
4. Murono E.P., Washburn A.L., Goforth D.P. and Wu N.: Biphasic effect of basic fibroblast growth factor on ^{125}I-human chorionic gonadotrophin binding to cultured immature Leydig cells. *Molec. Cell. Endocr.* **92** (1993) 121–126.
5. Murono E.P., Washburn A.L., Goforth D.P. and Wu N.: Evidence that both receptor- and heparan sulfate proteoglycan-bound basic fibroblast growth factor are internalised by cultured immature Leydig cells. *Molec. Cell. Endocr.* **98** (1993) 81–90.
6. Murono E.P., Washburn A.L., Goforth D.P. and Wu N.: Basic fibroblast growth factor induced increase in ^{125} I-human chorionic gonadotrophin binding to luteinizing hormone receptors in cultured immature Leydig cells is mediated by binding to heparan sulfate proteoglycans. *Molec. Cell. Endocr.* **97** (1993) 109–114.
7. Burgess W.H. and Maciag T.: The heparin-binding (fibroblast) growth factor family of proteins. *A. Rev. Biochem.* **58** (1989) 575–606.
8. Johnson D.E., Lee L.P., Lu J. and Williams L.T.: Diverse forms of a receptor for acidic and basic fibroblast growth factors. *Molec. Cell. Biol.* **10** (1990) 4728–4736.
9. Rapraeger A.C., Krufka A. and Olwin B.B.: Requirement of heparan sulfate for bFGF—mediated fibroblast growth and myoblast differentiation. *Science* **252** (1991) 1705–1708.
10. Risbridger G.P. and Hedger M.: Adult rat Leydig cell cultures: minimum requirements for maintenance of luteinizing hormone responsiveness and testosterone production. *Molec. Cell. Endocr.* **83** (1992) 125–132.
11. Risbridger G.P. and Davies A.: Isolation of rat Leydig cells and precursor forms after administration of ethane dimethane sulfonate. *Am. J. Physiol.* **266** (1995) E975–E979.
12. Chubb C. and Ewing L.L.: Steroid secretion by sexually immature rat and rabbit testes perfused *in vitro*. *Endocrinology* **109** (1981) 1999–2003.
13. Verhoeven G. and Cailleau J.: Influence of coculture with Sertoli cells on steroidogenesis in immature rat Leydig cells. *Molec. Cell. Endocr.* **71** (1992) 239–251.
14. van Haren L., Flinterman J.F., Orly J. and Rommerts F.F.: Luteinizing hormone induction of the cholesterol side-chain cleavage enzyme in cultured immature rat Leydig cells: no role for insulin-like growth factor 1? *Molec. Cell. Endocr.* **87** (1992) 57–67.
15. Hardy M.P., Zirkin B.R. and Ewing L.L.: Kinetic studies on the development of the adult population of Leydig cells in testes of the pubertal rat. *Endocrinology* **124** (1989) 762–770.
16. Tapainen J., Kuopio T., Pelliniemi L.J. and Huhtaniemei I.: Rat testicular endogenous steroids and number of Leydig cells between the fetal period and sexual maturity. *Biol. Reprod.* **31** (1984) 1027–1035.
17. Murono E.P. and Washburn A.L.: Fibroblast growth factor inhibits 5-reductase activity in cultured immature Leydig cells. *Molec. Cell. Endocr.* **68** (1990) R19–R23.
18. Murono E.P. and Washburn A.L.: Basic fibroblast growth factor inhibits delta 5-3 beta-hydroxysteroid dehydrogenase-isomerase activity in cultured immature Leydig cells. *Biochem. Biophys. Res. Commun.* **168** (1990) 248–253.
19. Aviezer D., Levy E., Safran M., Svahn C., Buddecke E., Schmidt A., David G., Vlodavsky I. and Yayon A.: Differential structural requirements of heparin and heparan sulfate proteoglycans that promote binding of basic fibroblast growth factor to its receptor. *J. Biol. Chem.* **269** (1994) 114–121.
20. Kan M., Wang F., Xu J., Crabb J.W., Hou J. and Mc Keehan W.L.: An essential heparin-binding domain in the fibroblast growth factor receptor kinase. *Science* **259** (1993) 918–1921.
21. Roghani M., Mansukhani A., Dell'era P., Bellosta P., Basilico C., Rifkin D.B. and Moscatelli D.: Heparin increases the affinity of basic fibroblast growth factor for its receptor but is not required for binding. *J. Biol. Chem.* **269** (1994) 3976–3984.

22. Guimond S., MaccaranaM., Olwin B.B., Lindahl U. and Rapraeger A.C.: Activating and inhibitory heparin sequences for FGF-2 (basic FGF). *J. Biol. Chem.* **268** (1993) 23,906–23,914
23. Maccarana M., Casu B. and Lindahl U.: Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor. *J. Biol. Chem.* **268** (1993) 23,898–23,905.
24. Neufeld G. and Gospodarowicz D.: Protamine sulfate inhibits mitogenic activities of the extracellular matrix and fibroblast growth factor, but potentiates that of epidermal growth factor. *J. Cell. Physiol.* **132** (1987) 287–294.
25. Moscatelli D.: Metabolism of receptor-bound and matrix-bound basic fibroblast growth factor by bovine capillary endothelial cells. *J. Cell. Biol.* **107** (1988) 753–759.
26. Nurcombe V., Ford M.D., Wildschut J.A. and Bartlett P.F.: Developmental regulation of neural response to FGF-1 and FGF-2 by heparan sulfate proteoglycans. *Science* **260** (1993) 103–106.
27. Mullaney B.P. and Skinner M.K.: Basic fibroblast growth factor (bFGF) gene expression and protein production during pubertal development of the seminiferous tubule: follicle-stimulating hormone-induced Sertoli cell bFGF expression. *Endocrinology* **131** (1992) 2928–2934.
28. Vlodavsky I., Bar-Shavit R., Ishai-Michali R., Bashkin P. and Fuks Z.: Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism. *TIBS* **16** (1991) 268–271.