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Paracrine regulation of Sertoli cell proliferation

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Stockholm 2003

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7349-443-7

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Cover photos
Front: Cultured Sertoli cells prepared from 8 to 9-day-old rat. Giemsa staining.
Back: Histological section of testis from 8 to 9-day-old rat. Carmalume staining.

ABSTRACT

The Sertoli cell is a highly differentiated somatic cell in the seminiferous epithelium of the testis. This cell is of prime importance for both testicular development and spermatogenesis. Sertoli cells proliferate up to the start of puberty and, due to their nursing functions, the final Sertoli cell number is well correlated to the amount of germ cells produced by the adult testis. The physiological regulation of Sertoli cell growth is not fully understood, but follicle stimulating hormone (FSH) from the pituitary and testicular paracrine factors are known to be important.
Testicular function may be harmed by local or general inflammatory diseases. Inflammatory stimuli induce testicular production of proinflammatory cytokines, which may be of pathogenic importance. Sertoli cells are possible targets of these proinflammatory cytokines.
The aim of the present thesis was to set up a Sertoli cell proliferation assay, suitable for screening of putative mitogens, and use it to study the effects of growth factors and cytokines, thought to be of physiological or pathophysiological relevance.

Sertoli cells from 8 to 9-day-old rats were isolated and cultured under serum-free conditions. Proliferation was assessed by three different methods; ^{3}H-thymidine incorporation, BrdU immunocytochemistry and MTT supravital staining.
Transforming growth factor-α (TGF-α) and epidermal growth factor (EGF) were found to dose-dependently stimulate Sertoli cell proliferation, while insulin-like growth factor (IGF)-I and IGF-II, acidic and basic fibroblast growth factors (FGFa, FGFb) were negative in the system.
In search for Sertoli cell growth factors in testicular tissue, proteins from testes of immature (5-day-old), pubertal (25-day-old) and adult (50-day-old) rats were purified by HPLC gel permeation chromatography. Pubertal and adult testes contained a 17 kDa protein with potent stimulatory effect on immature Sertoli cell multiplication *in vitro*. This protein was identified as interleukin-1α (IL-1α), which is constitutively produced by the adult testis. Immature rat testis produced a Sertoli cell growth stimulating factor(s) in the 45 kDa size range that still remains to be identified.
The recombinant proinflammatory cytokines IL-1α, IL-1β and TNF-α showed potent Sertoli cell mitogenic effects. It was further demonstrated that the IL-1-induced Sertoli cell proliferation was mediated by the p38 MAPK intracellular signal transduction pathway.
IL-1α and IL-1β, but not TNF-α, also produced a striking morphological change which may represent a change in Sertoli cell differentiation state. There was a synergistic increase in Sertoli cell proliferation by IL-1 or TNF-α together with the important endocrine Sertoli cell regulator FSH. Furthermore, the cytokines IL-6 and IFN-γ modulated the FSH effect in different ways. IL-6 increased, while IFN-γ inhibited FSH-induced Sertoli cell proliferation. Bacterial endotoxin directly stimulated Sertoli cell growth.
Taken together, the results show that TGF-α is a Sertoli cell growth factor and that proinflammatory cytokines and bacterial endotoxin interfere with Sertoli cell proliferation *in vitro*. TGF-α may be a physiological paracrine Sertoli cell growth regulator while cytokines and endotoxin may potentially disturb testicular cytoarchitecture and development during inflammatory and infectious diseases *in vivo*.

LIST OF PUBLICATIONS

The present study is based on the following papers, which will be referred to by their Roman numerals

I. Scarpino S., Morena A.R., **Petersen C**., Fröysa B., Söder O., Boitani C.
A rapid method of Sertoli cell isolation by DSA lectin, allowing mitoitic analyses
Molecular and Cellular Endocrinology 1998, 146: 121-127

II. **Petersen C**., Boitani C., Fröysa B., Söder O.
Transforming growth factor-α stimulates proliferation of rat Sertoli cells
Molecular and Cellular Endocrinology 2001, 181: 221-227

III. **Petersen C**., Boitani C., Fröysa B., Söder O.
Interleukin-1 is a potent growth factor for immature rat Sertoli cells
Molecular and Cellular Endocrinology 2002, 186: 37-47

IV. **Petersen C**., Svechnikov K., Fröysa B., Söder O.
p38 MAPK pathway mediates interleukin-1-induced Sertoli cell proliferation
Submitted manuscript

V. **Petersen C**., Fröysa B., Söder O.
Endotoxin and proinflammatory cytokines modulate Sertoli cell proliferation *in vitro*
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

a.a.	Amino acid
AEC	Amino ethyl carbazole
BrdU	Bromodeoxyuridine
BSA	Bovine serume albumine
DAB	Diaminobenzidine
DSA	Datura stramonium agglutinin
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GA3PDH	Glyceraldehyd-3-phosphate dehydrogenase
HPLC	High pressure liquid chromatography
HRP	Horse-radish peroxidase
3βHSD	3β hydroxysteroid dehydrogenase
ICE	IL-1β converting enzyme
IFN	Interferon
IGF	Insulin-like growth factor
IL-1	Interleukin-1
IL-1ra	Interleukin-1 receptor antagonist
IL-1R	Interleukin-1 receptor
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
JNK/SAPK	Jun amino-terminal/stress-activated protein kinase
kDa	Kilodalton
LH	Lutenizing hormone
LPS	Lipopolysackaride
MAPK	Mitogen-activated protein kinase
MIH	Müller inhibiting hormone
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue
PBS	Phosphate buffered saline
PVDF	Polyvinylidene difluoride
RT-PCR	Reverse-transcriptase polymerase chain reaction
SCF	Stem cell factor
SDS/PAGE	Sodium dodecyl sulphate/polyacrylamide gel electrophoresis
TGF-α	Transforming growth factor-α
TIR	Toll/IL-1R
TNF-α	Tumour necrosis factor-α
TNFR	TNF receptor

1 INTRODUCTION

1.1 THE TESTIS

The male gonad has two main functions – the testosterone synthesis and the production of germ cells. Germ cell differentiation, or spermatogenesis, is located to the seminiferous tubules while testosterone is produced in the interstitial tissue between the tubules. The seminiferous tubules consist of the developing germ cells, the Sertoli cells and outside the basal membrane the peritubular or myoid cells. The interstitium harbours testosterone producing Leydig cells and resident testicular macrophages. Regarding the permeability of nutrients and growth factors, the testis is divided by the so called blood-testis barrier into two compartments. The barrier is formed by tight junctions between adjacent Sertoli cells and divides the testis into the basal and the adluminal compartments. The Sertoli cell tight junctions separate meiotic spermatocytes and spermatids in the adluminal compartment from the spermatogonia and early spermatocytes in the basal compartment (Clermont, 1993; Weinbauer et al., 2001) (Fig. 1B). Thus, the interstitium, the peritubular cells, the diploid germ cells and the basal parts of the Sertoli cells are in contact with substances either produced by the interstitial tissue or reaching the testis via the blood circulation.

Lutenizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary are the main endocrine regulators of testicular functions. LH stimulates testosterone production by the Leydig cells and FSH acts on Sertoli cells. LH function and subsequent high intratesticular testosterone concentration is required to drive spermatogenesis while the role of FSH in this matter is more obscure (Griswold, 1998; Weinbauer et al., 2001).

The testis is often described as an “immunologically privileged” site and it is shown to constitutively produce both immunosuppressive factors (Head and Billingham, 1985; Pöllänen et al., 1988; Wyatt et al., 1988) and proinflammatory cytokines (De et al., 1993; Jonsson et al., 1999).

In the immature testis of the 8 to 9-day-old rat, spermatogenesis has not yet started and the seminiferous tubules, or more correctly since there is no lumen, the seminiferous cords, only contain three cell types: spermatogonia, peritubular cells and Sertoli cells (Fig. 1A).

1.2 THE SERTOLI CELL

The Sertoli cell, named after Enrico Sertoli who described the cell in 1865, is essential for spermatogenesis. The fully differentiated adult Sertoli cell has a complicated three-dimensional structure and extends from the basal membrane towards the lumen of the seminiferous tubule. The Sertoli cell harbours clones of differentiating germ cells in cytoplasmic crypts and moves them upwards through the tight junctions of the blood-testis barrier. Apart from its obvious supportive role for the seminiferous epithelium, the Sertoli cell also provides the germ cells with nutrients and growth factors throughout their development. The meiotic germ cells above the blood-testis barrier are thus totally dependent upon this transport. It is also established that the number of

Sertoli cells correlates well to the amount of germ cells produced in a testis (Orth et al., 1988; Hess et al., 1993).
The Sertoli cell is the only cell type in the testis with FSH receptors and it responds with inhibin production following FSH stimulation (Clermont, 1993; Griswold, 1998). Clinically, inhibinB is used as a measure of adult Sertoli cell function (Andersson et al., 1997; Andersson et al., 1998). Sertoli cells, but not germ cells, express androgen receptor and are thus the target for testosterone action in the seminiferous epithelium (Gondos and Berndston, 1993).

1.2.1 Immature Sertoli cells

Before puberty and final differentiation the Sertoli cell is quite different from the adult cell type. The immature Sertoli cell differs both in morphology and biochemical activity compared to the fully differentiated cell.
Fetal Sertoli cells are essential for testis formation and male sex differentiation. The sex-determining gene on the Y-chromosome, *sry*, is only expressed in the supporting cell lineage of the primitive gonad and drives this cell type into Sertoli cell differentiation. Furthermore, the Sertoli cell is the producer of Müller inhibiting hormone (MIH) and thus responsible for the inhibition of development of female inner genitalia. Later on in embryonic development, Sertoli cells together with peritubular cells are needed for testis cord formation (Griswold, 1998; McLaren, 2000).
Postnatally, Sertoli cells are diffusely distributed in the seminiferous cords but gradually acquire properties of adult Sertoli cells. At the start of puberty, the tight junctions of the barrier are formed and the Sertoli cells display more differentiated biochemical activities, e.g. production of transferrin (Skinner and Griswold, 1980) and androgen binding protein (Tindall et al., 1975).

1.2.2 Sertoli cell proliferation

Sertoli cells proliferate during fetal life up to puberty and then cease to divide. In the rat, Sertoli cells stop proliferating at postnatal day 16-18 (Steinberger and Steinberger, 1971; Nagy, 1974; Orth, 1982) which also coincides with the establishment of the blood-testis barrier (Vitale et al., 1973) and the formation of the tubular lumen (Gondos and Berndston, 1993). After this time no further replication occurs and the Sertoli cell number is considered to be stable throughout life (Steinberger and Steinberger, 1971; Nagy, 1974). However, there are some indications that Sertoli cells could occasionally multiply later in life (Nagy, 1972; Miranda et al., 2002).
It has been known for a long time that FSH is an important mitogen for immature Sertoli cells both *in vivo* and *in vitro* (Griswold et al., 1975; Griswold et al., 1977; Orth, 1984; Meachem et al., 1996; Singh and Handelsman, 1996). Later studies have confirmed that FSH is important, though not the only factor needed, for Sertoli cell proliferation. In the hypogonadal (hpg) mouse, which lacks circulating FSH (and LH), the number of Sertoli cells is reduced by 35% and comparable results are found in the FSH β subunit knockout mouse (reduction by 30-39%) (Baker and O´Shaughnessy, 2001; Wreford et al., 2001). Humans without a functional FSH receptor have smaller testes than normal, a strong indication of reduced Sertoli cell number, and impaired spermatogenesis, though some of them are fertile (Tapanainen et al., 1997).
Considering that Sertoli cells seem to be able to divide considerably even in the absence of FSH action it is suggested that paracrine factors are important to obtain an adequate Sertoli cell number. *In vitro* studies have shown mitogenic effect on Sertoli

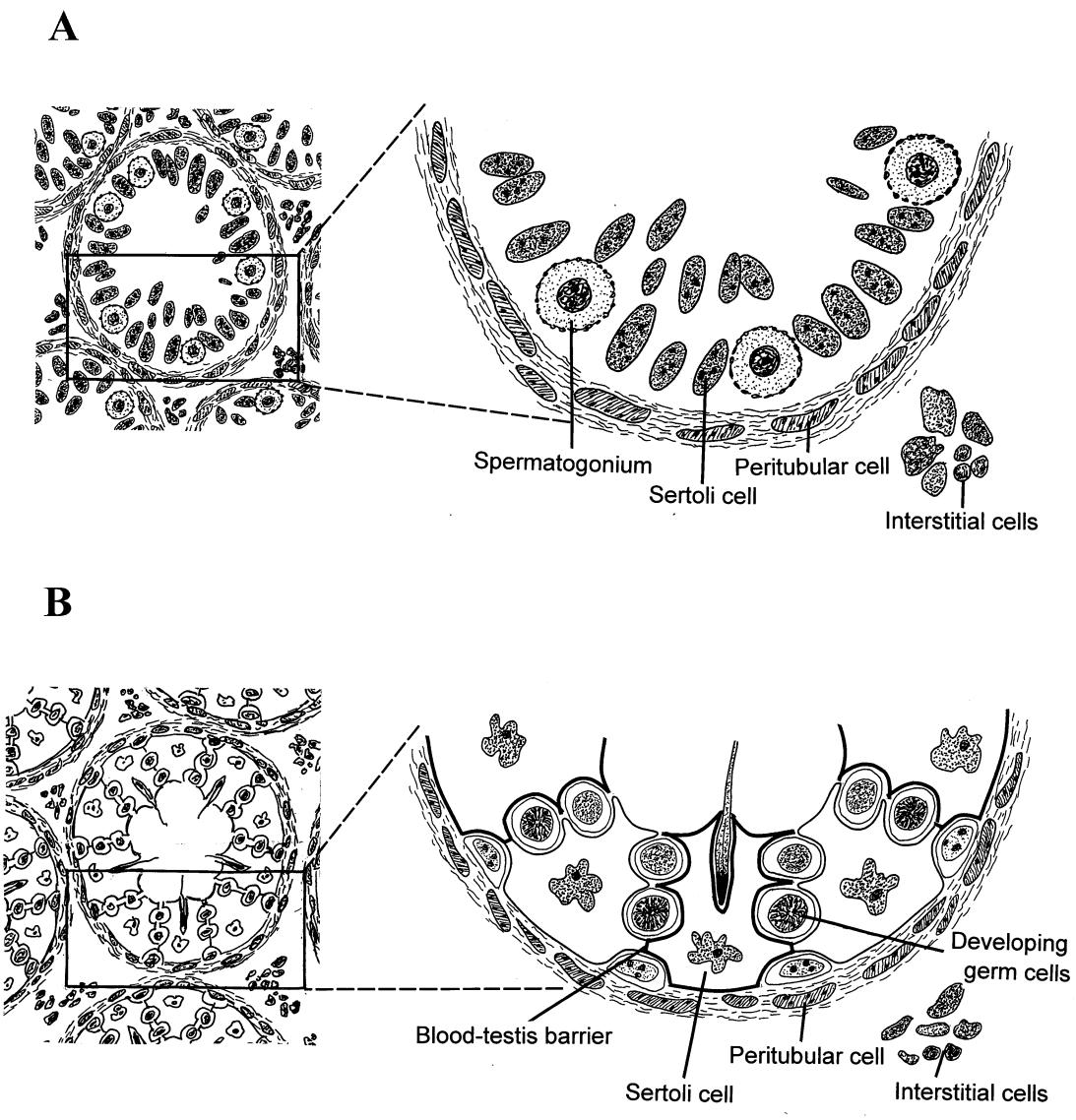

Figure 1 Illustration of testicular histology. A: Immature testis. B: Adult testis. Illustration © Eva Hall

cells by some of these factors (Borland et al., 1984; Jaillard et al., 1987; Boitani et al., 1995; Hu et al., 1999).

1.3 TESTICULAR PATHOLOGY AND THE SERTOLI CELL

Testicular function may be harmed by local testicular inflammation but also by more general inflammation and systemic infections (Hales et al., 1999; Handelsman, 2001; Nieschlag et al., 2001). Disturbed testicular function may also be a consequence of a maldeveloped testis. Recently, it was suggested that impaired spermatogenesis, cryptorchidism (retentio testis), testicular cancer and hypospadias should be looked upon as one entity – the testicular dysgenesis syndrome (TDS). TDS could be mild (impaired spermatogenesis) or severe (malformations) but still reflects a maldeveloped testis. A disturbed Sertoli cell function is suggested to be important in the pathogenesis of TDS (Skakkebaek et al., 2001). Indeed, poorly differentiated Sertoli cells are found in human testes with spermatogenic dysfunction (Steger et al., 1996; Steger et al., 1999). Sertoli cells with immature morphological features are also present in cryptorchid testes and in the close vicinity of testicular cancer (Schulze and Holstein, 1993). The incidence of TDS is increasing and this may be due to the influence of environmental xenobiotics, so called endocrine disrupters (Skakkebaek et al., 2001). Immature Sertoli cells are one of several possible targets of these environmental substances. It is well recognised that Sertoli cells are targets for other toxicants, which harm testicular function (Boekelheide, 1993), such as the plasticizers known as phtalates.

Proinflammatory cytokines may be important modulators of various kinds of testicular damage. Some endocrine disrupters are shown to induce the production of proinflammatory cytokines by macrophages (Yamashita et al., 2002) and both experimental systemic infection and phtalate exposure cause induction of proinflammatory cytokines in the testis (Creasy et al., 1983; Granholm et al., 1992; Jonsson et al., 2000; Jonsson et al., 2001; Gerdprasert et al., 2002). It is also suggested that proinflammatory cytokines are important in the pathogenesis of autoimmune orchitis (Yule and Tung, 1993; Matsuzaki et al., 1997).

1.4 PROINFLAMMATORY CYTOKINES

Proinflammatory cytokines are produced by activated macrophages and act as mediators of inflammation. They also link the specific immune system with the inflammatory reaction. The pleiotropic functions of proinflammatory cytokines are to a large extent redundant and the biological function of one cytokine can often be compensated for by another.
Interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are the major proinflammatory cytokines produced by activated macrophages, but are also produced by many other cell types. The biological effects of these proinflammatory cytokines are often interrelated. As an example IL-1, TNF-α and IL-6, are all involved in fever induction, catabolic effects and pathogenesis of autoimmune diseases. In addition, IL-6 is also a major product of IL-1- or TNF-α-stimulated cells. Proinflammatory cytokines are often shown to have a dual role in inflammatory

response. On the one hand they may have protective effects against certain infections and on the other they may cause pathological complications themselves (Hirano, 1998; Idriss and Naismith, 2000; Durum, 2001; Feldmann and Brennan, 2001; Feldmann and Saklatvala, 2001).
In the testis, proinflammatory cytokines play two major roles. Apart from being inflammatory mediators of pathophysiological importance, they also function as growth and differentiation factors during normal physiological events (Hales et al., 1999).

1.4.1 IL-1

The IL-1 family consists of two biologically active ligands, IL-1α and IL-1β, and a naturally occurring receptor antagonist, IL-1ra. There are two main receptors; the signalling type I receptor, IL-1RI, and the scavenger receptor type II, IL-1RII. The IL-1 receptors may be released as soluble receptors without any signalling capacity. Thus, the IL-1 system comprises proteins that mediate the IL-1 effect (namely IL-1α, IL-1β and membrane bound IL-1RI) and proteins that antagonize the same effect (IL-1ra, IL-1RII and soluble IL-1RI). IL-18 is also a member of the IL-1 family but acts through its own receptor (Nakamura et al., 1989; Dinarello, 1994; Martin and Falk, 1997). In addition, several other family members, both ligands and receptors are described (Born et al., 2000; Smith et al., 2000).

1.4.1.1 IL-1α

IL-α is synthesized as a precursor protein, which is cleaved by calpain into the mature 17 kDa form (Kobayashi et al., 1990; Dinarello, 1994). Both the precursor and the mature 17 kDa proteins are biologically active (Mosley et al., 1987; Dinarello, 1994). IL-α is a true proinflammatory cytokine and when administered systemically, produces amongst others fever, headache, arthralgias and, in higher doses, hypotension.
The protein lacks a signal peptide and therefore is not considered to be secreted, but is thought to exert its effects *in vivo* in a membrane bound form. However, mature IL-1α may be released even in the absence of cell death (Dinarello, 1994; Martin and Falk, 1997; Dinarello, 2001a). The other calpain cleavage product, the N-terminal 16 kDa protein may also be of biological importance since it has been shown to act as a transforming nuclear oncoprotein (Stevenson et al., 1997).
Besides its proinflammatory action, IL-1α is constitutively produced by various epithelial tissues under non-inflammatory conditions and it is known to be a growth factor for both epithelial non-malignant and malignant cells (Pöllänen et al., 1989; Granholm and Söder, 1991; Ito et al., 1993; Dinarello, 1994; Furuya et al., 2000).
In addition to the 17 kDa IL-1α, there is a 24 kDa splice variant, which lack the calpain cleavage site (Sultana et al., 2000).

1.4.1.2 IL-1β

IL-1β is, like IL-1α, synthesized as a precursor protein but this precursor is not biologically active. The precursor is cleaved by an intracellular protease, IL-1β converting enzyme (ICE), into the mature and biologically active 17.5 kDa form (Black et al., 1988).
IL-1β may be secreted from the cell even if it, like IL-1α, lacks a signal peptide (Dinarello, 1994).

IL-1β is the major IL-1 isoform produced by activated macrophages and exerts the same proinflammatory effects as IL-1α. Unlike IL-1α, IL-1β does not seem to be constitutively produced by epithelial non-inflamed healthy tissues.

1.4.1.3 IL-1ra

Interleukin-1 receptor antagonist, IL-1ra, is synthesized as a precursor protein with a signal peptide and the cleaved mature 17.7 kDa form is secreted (Eisenberg et al., 1990).
Three intracellular isoforms, lacking the signal peptide, has also been identified (Haskill et al., 1991; Muzio et al., 1995; Malyak et al., 1998).
IL-1ra is, similar to IL-1α, produced by many different cell types. There is a constitutive production of the intracellular forms in epithelial cells while secreted IL-1ra is induced in peripheral blood, spleen, lung and liver by proinflammatory stimuli (Mantovani et al., 1996; Gabay et al., 1997; Arend et al., 1998).
IL-1ra binds to IL-1RI without inducing any intracellular signalling response. It is a true receptor antagonist without any agonist activity (Dripps et al., 1991; Granowitz et al., 1992; Dinarello, 1994). Indeed, it is shown that the antagonizing effect of IL-1ra plays an important role *in vivo*. IL-1ra mutant mice are more susceptible to lethal endotoxinemia than wild-type mice and the transgenic IL-1ra overexpressing mice are protected from lethal endotoxin effects (Hirsch et al., 1996).

1.4.1.4 IL-1 receptors

The type I receptor, IL-1RI, is a 80 kDa transmembrane glycoprotein with a 219 amino acid C-terminal intracellular domain. There is considerable homology within the intracellular domains of IL-1RI and another group of receptors known to be important for innate immunity – the toll-like receptors (TLR). Based on these homologous TIR-domains, TLRs and IL-1RI, together with some other proteins in the IL-1 signalling system, form the IL-1/toll receptor family (Daun and Fenton, 2000) (Sims et al., 1988; Sims et al., 1989; O'Neill, 2000).
IL-1RI associates with another transmembrane protein, the IL-1 receptor accessory protein (IL-1R AcP), to form a high affinity receptor complex with the ligand (Greenfeder et al., 1995; Daun and Fenton, 2000).
All three ligands, IL-1α, IL-1β and IL-1ra bind to IL-1RI, but there are differences in binding affinities depending on the cell type studied. Interestingly, it seems that IL-1α and IL-1β are differentially dependent on IL-1R AcP for their binding affinity to IL-1RI (Cullinan et al., 1998; Daun and Fenton, 2000).
Il-1RI is the signalling IL-1 receptor that mediates the biological responses of IL-1 (Sims et al., 1993). After IL-1 binding, the IL-1RI - IL-1R AcP – complex associates with the serin/threonine kinase IRAK (IL-1 receptor associating kinase) through the adapter protein MyD88. IRAK is then heavily phosphorylated and further intracellular signalling cascades are elicited (Cao et al., 1996; Wesche et al., 1997; Burns et al., 1998; Daun and Fenton, 2000).
The type II receptor, IL-1RII, is a 60 kDa transmembrane protein with a considerable homology to IL-1RI, but with a very short (29 a.a.) intracellular domain, that lacks signalling capabilities (and a TIR-domain). It binds both IL-1α and IL-1β (it has very low binding affinity for IL-1ra) with similar affinities as IL-1RI (McMahan et al., 1991; Daun and Fenton, 2000). IL-1RII is considered to be a decoy receptor working as a functional IL-1 inhibitor (Colotta et al., 1993; Re et al., 1994).

1.4.1.5 IL-1 and the testis

IL-1RI is expressed in the murine, rat and human adult testis (Cunningham et al., 1992; Gomez et al., 1997). It has been localized to Sertoli cells and interstitial cells (Cunningham et al., 1992; Gomez et al., 1997; Wang et al., 1998).
IL-1RII is shown to be expressed in several cell types in the testis including the Sertoli cells (Gomez et al., 1997).
The ligand IL-1α is constitutively produced by the adult testis (Khan et al., 1987; Khan et al., 1988; Jonsson et al., 1999; Sultana et al., 2000; Gustafsson et al., 2002) and it is shown to be produced by the Sertoli cells (Gerard et al., 1991; Gerard et al., 1992; Cudicini et al., 1997; Jonsson et al., 1999). In the rat, the IL-1α production starts at postnatal day 20 (Syed et al., 1988; Jonsson et al., 1999; Wahab-Wahlgren et al., 2000) and it is suggested to act as a spermatogonial growth factor (Pöllänen et al., 1989; Parvinen et al., 1991; Söder et al., 1991). However, IL-1 action is not an absolute pre-requisite for murine male fertility (Cohen and Pollard, 1998).
IL-1α has also been shown to influence testicular function by modulating Leydig cell testosterone production (Verhoeven et al., 1988; Fauser et al., 1989; Calkins et al., 1990; Hales, 1992; Mauduit et al., 1992; Svechnikov et al., 2001) and different Sertoli cell functions, for instance transferrin and lactate production (Karzai and Wright, 1992; Nehar et al., 1998).
In contrast, IL-1β is not constitutively produced by the testis but there is an induced production by testicular macrophages after immunostimulation (Hales et al., 1992; Xiong and Hales, 1994; Kern et al., 1995; Jonsson et al., 2001).
IL-1β is shown to stimulate Sertoli cell transferrin production and to interfere with FSH-induced aromatase activity (Khan and Nieschlag, 1991; Hoeben et al., 1996). It is also shown to stimulate Leydig cell proliferation (Khan et al., 1992b).
IL-1ra is not constitutively produced by the rat testis (Jonsson et al., 2001) but its expression has been demonstrated in isolated murine Sertoli cells (Zeyse et al., 2000). Like IL-1β, IL-1ra is induced in the testis by proinflammatory stimuli (Jonsson et al., 2001).

1.4.2 IL-6

IL-6 is a glycosylated protein with a molecular mass ranging from 21 kDa to 28 kDa depending on different posttranslational modifications (Haegeman et al., 1986; Hirano et al., 1986; May et al., 1986; Zilberstein et al., 1986; May et al., 1988). Together with other proinflammatory cytokines, though not IL-1 and TNF-α, IL-6 is a member of a certain cytokine subfamily. This IL-6 related cytokine subfamily is composed of cytokines with structural similarities, functional redundancy, and a shared receptor subunit (Hirano, 1998).
IL-6 is produced by many cell types both within and outside the immune system (Tovey et al., 1988; Northemann et al., 1990; Van Snick, 1990). IL-6 is a well-known growth and differentiation factor for T and B-cells, but it can also stimulate proliferation of other non-malignant and malignant cells (Hirano, 1998; Lazar-Molnar et al., 2000). IL-6 has both proinflammatory and anti-inflammatory features. In many cell types it is induced by IL-1 and TNF-α and works as a mediator in the inflammatory response. On the other hand, IL-6 downregulates TNF-α production and also antagonizes IL-1 effects (Aderka et al., 1989; Hauptmann et al., 1991; Feldmann and Saklatvala, 2001).

The IL-6 receptor complex consists of an 80 kDa transmembrane protein (IL-6Rα) that binds IL-6 and the receptor subunit gp130 that works as a signal transducer (Yamasaki et al., 1988; Taga et al., 1989; Hibi et al., 1990). As a consequence, and different to the IL-1system, the soluble IL-6 receptor (the IL-6Rα subunit) works as an agonist. The soluble IL-6Rα binds the ligand and may thereafter associate with the signalling gp130 subunit (Hirano, 1998).
Il-6R is expressed in the testis and both IL-6Rα and gp130 are present in Sertoli cells (Okuda and Morris, 1994; Fujisawa et al., 2002). It is clear that testicular IL-6 production is induced by inflammatory stimuli *in vivo* and *in vitro* (Syed et al., 1993; Cudicini et al., 1997; Stephan et al., 1997; Jonsson et al., 2000; Gerdprasert et al., 2002). Although IL-6 is shown to be present in isolated cells and tubular cultures (Syed et al., 1993; Cudicini et al., 1997), it is not expressed in intact rat testis (Jonsson et al., 1999).
IL-6 has effects on testicular cells *in vitro*; it is shown to stimulate germ cell proliferation and Sertoli cell transferrin production (Hakovirta et al., 1995; Hoeben et al., 1997).

1.4.3 TNF-α

TNF-α is a member of the TNF superfamily of cytokines. The family includes about ten ligands, which activate a corresponding family of structurally related receptors (Jupp et al., 2001). TNF-α is synthesized as a precursor protein and then cleaved to its 17.3 kDa secreted form (Fransen et al., 1985; Pennica et al., 1985). It is produced by a variety of hematopoetic and non-hematopoetic cells both normal and malignant (Idriss and Naismith, 2000). TNF-α has multiple biological effects, which include immunostimulation, resistance to infections, resistance to tumours and involvement in sleep regulation (Idriss and Naismith, 2000). TNF-α can induce both necrotic and apoptotic cell death as well as cell proliferation and differentiation (Tartaglia and Goeddel, 1992; Beyaert and Fiers, 1994; Idriss and Naismith, 2000; Jupp et al., 2001). There are two TNF receptors, the TNFR1 (p55 or p60 receptor) and the TNFR2 (p75 or p80 receptor) (Dembic et al., 1990; Himmler et al., 1990; Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990). There is no homology between the intracellular regions of the two receptors (Tartaglia and Goeddel, 1992). TNFR1 is expressed in almost all tissues while TNFR2 expression is predominantly found in hematopoetic and endothelial cells (Jupp et al., 2001). The ligand TNF-α binds as a trimer to three receptor molecules before cell signalling can occur (Idriss and Naismith, 2000). Upon ligand binding, TNFR1 associates with TNFR1-associated death domain protein (TRADD) and, depending on which intracellular proteins are further recruited, it promotes apoptosis and inhibits inflammation or *vice versa*. TNFR2 associates with other adaptor proteins and mediates anti-apoptotic and proinflammatory effects (Baud and Karin, 2001). Soluble TNF receptors are present *in vivo* and, similar to the IL-1 system, function as inhibitors of TNF-α action (Seckinger et al., 1989; Nophar et al., 1990).
TNF receptors are expressed in the testis and have been localized to Leydig cells and Sertoli cells (Mauduit et al., 1991; De et al., 1993; Mauduit et al., 1996; Pentikainen et al., 2001). In the adult testis, TNF-α is constitutively produced by germ cells (De et al., 1993). Proinflammatory stimuli may also induce testicular TNF-α production by interstitial cells (Xiong and Hales, 1993a; Xiong and Hales, 1994; Jonsson et al., 2000).

TNF-α is shown to inhibit Leydig cell testosterone production (Mealy et al., 1990; Mauduit et al., 1991; Xiong and Hales, 1993b; Hales et al., 1999) and to influence several Sertoli cell functions (Boockfor and Schwarz, 1991; Mauduit et al., 1993; Riccioli et al., 1995; Besset et al., 1996; Boussouar et al., 1999).

1.4.4 IFN-γ

Interferon-γ (IFN-γ) is a member of the interferon family of proteins that also comprises IFN-α and IFN-β. Interferons have well-documented antiviral, antiproliferative and immunomodulatory effects.
IFN-γ is a 15-16 kDa protein, which is synthesized with a signal peptide and further secreted. It is produced by T-lymphocytes and natural killer (NK) cells and the production is stimulated by antigens or cytokines. IFN-γ itself is a macrophage stimulator and may induce IL-1 production, though it is also an endogenous inhibitor of IL-1 action (Dijkema et al., 1985; Sen and Lengyel, 1992; Dinarello, 2001a; Dinarello, 2001b; Durum, 2001; Feldmann and Brennan, 2001; Feldmann and Saklatvala, 2001). IFN-γ binds as a dimer to its dimerized receptor, which consists of two ligand binding α-chains and two β-chains required for signalling (Stroud et al., 2001).
In the testis, there is a constitutive production of IFN-γ, which has been localized to the early spermatids (Dejucq et al., 1995). Both IFN-γ receptor subunits are expressed in Sertoli cells and Leydig cells (Kanzaki and Morris, 1998). Besides the constitutive expression of IFN-γ, there is an induced production in the testis following viral infection (Dejucq et al., 1998).
IFN-γ is shown to inhibit Leydig cell testosterone production and also to affect Sertoli cells (Stephan et al., 1995; Hales et al., 1999; Riccioli et al., 2000).
Increased IFN-γ is demonstrated to be a pathogenic factor in experimental autoimmune orhitis and chronic treatment with IFN-γ results in reduced testis weight and delayed sexual development. By contrast, beneficial effects of systemic IFN-γ treatment are seen in humans with mumps orchitis (Hales et al., 1999).

1.5 LPS

Lipopolysackaride (LPS) or endotoxin is a major component of the outer membrane of gram-negative bacteria. It is a potent immuno-stimulator and induces local and generalized inflammatory reactions in both humans and experimental animals (Wiese et al., 1999). LPS induces the production of proinflammatory cytokines, mainly IL-1, TNF-α and IL-6, in immune cells (Muzio and Mantovani, 2000; Feldmann and Saklatvala, 2001). LPS acts through the toll-like receptor 4 (TLR4), which has an intracellular TIR-domain and thus is a member of the IL-1/toll receptor family (see IL-1 receptors) (Medzhitov et al., 1997; Poltorak et al., 1998; Takeuchi et al., 1999; Daun and Fenton, 2000; Modlin, 2002). TLR4 intracellular signalling is similar to that of the IL-1 system and activated TLR4 associates with both MyD88 and IRAK (Daun and Fenton, 2000). TLR4 expression is mainly found in spleen and lymphocytes (Medzhitov et al., 1997; Daun and Fenton, 2000) but there are no studies as yet exploring its expression in the testis.
Systemic LPS administration causes an inflammatory response in the testis, with locally induced proinflammatory cytokine production, and leads to spermatogenic damage (Jonsson et al., 2000; O'Bryan et al., 2000; Gow et al., 2001; Jonsson et al., 2001; Gerdprasert et al., 2002). The induced testicular production of proinflammatory cytokines has been localized to monocytes/macrophages in the interstitium (Jonsson et

al., 2001; Gerdprasert et al., 2002), but *in vitro* studies have also demonstrated induced cytokine production in Sertoli cells (Stephan et al., 1997; Zeyse et al., 2000; Huleihel and Lunenfeld, 2002).

1.6 TESTICULAR GROWTH FACTORS

A number of paracrine growth factors have been identified in the postnatal testis; e.g. acidic and basic fibroblast growth factors (FGFa or FGF-1 and FGFb or FGF-2), insulin-like growth factors I and II (IGF-I and IGF-II), transforming growth factor-α (TGF-α), epidermal growth factor (EGF) and stem cell factor (SCF) (Hansson et al., 1989; Mullaney and Skinner, 1992a; Koike and Noumura, 1994; Koike and Noumura, 1995; Caussanel et al., 1996; Munsie et al., 1997; Shoba et al., 1999; Cancilla et al., 2000; Cupp and Skinner, 2001). SCF is a spermatogonial growth and survival factor produced by Sertoli cells (Mauduit et al., 1999), while the other above-mentioned growth factors are shown to be produced by and act on several testicular cell types. Besides being involved in cell proliferation and differentiation, these growth factors are shown to modulate different testicular functions, e.g. Leydig cell steroidogenesis and Sertoli cell protein production (Mullaney and Skinner, 1991).
Receptors for FGFs, IGFs and TGF-α/EGF (see below) are present in Sertoli cells and FGFb, IGF-I, IGF-II and EGF are shown to stimulate Sertoli cell proliferation (Borland et al., 1984; Jaillard et al., 1987; Le Magueresse-Battistoni et al., 1994; Cancilla and Risbridger, 1998).

1.6.1 TGF-α /EGF

TGF-α and EGF are peptide growth factors within the EGF superfamily. They are both synthesized as transmembrane precursors that may be proteolytically cleaved and released from the cell membrane (Riese and Stern, 1998). TGF-α and EGF are produced by a variety of epithelial cells and are shown to be involved in cell proliferation, differentiation and cancer development (Wells, 1999; Olayioye et al., 2000). TGF-α and EGF both bind to and act through the EGF receptor (EGFR or erbB1) – one of four members of the ErbB receptor family. EGFR is a transmembrane 170 kDa glycoprotein with a cytoplasmic tyrosine kinase domain. Ligand binding results in receptor dimerization, autophosphorylation and further intracellular signalling cascades (Carpenter, 1987; Riese and Stern, 1998; Olayioye et al., 2000). EGFR is expressed in all epithelial and stromal cells and it is upregulated in many different human tumours (Wells, 1999).
EGFR is also expressed in the testis and has been localized to both somatic and germ cells (Mullaney and Skinner, 1992b; Caussanel et al., 1996; Nakazumi et al., 1996; Cupp and Skinner, 2001). Likewise, the ligands TGF-α and EGF are produced by the testis of several species (Mullaney and Skinner, 1992b; Radhakrishnan et al., 1992; Caussanel et al., 1996; Nakazumi et al., 1996; Cupp and Skinner, 2001) and there are many studies on TGF-α /EGF effects in the testis. A lack of as well as an excess of EGF is demonstrated to impair murine spermatogenesis (Tsutsumi et al., 1986; Wong et al., 2000). EGF is also shown to stimulate several Sertoli cell functions, e.g. lactate and inhibin production, and Leydig cell steroidogenesis (Mallea et al., 1986; Perez-Infante et al., 1986; Verhoeven and Cailleau, 1986; Morris et al., 1988; Gonzales et al., 1989; Sordoillet et al., 1991; Syed et al., 1991; Nehar et al., 1993). Furthermore, EGF is

demonstrated to stimulate both Sertoli, Leydig and germ cell proliferation (Jaillard et al., 1987; Khan et al., 1992a; Wahab-Wahlgren et al., 2003).
TGF-α is produced by the rat testis and several studies have focused on its role in early testicular development where it is thought to regulate testicular growth (Mullaney and Skinner, 1992b; Levine et al., 2000; Cupp and Skinner, 2001).

1.7 INTRACELLULAR SIGNAL TRANSDUCTION

When an extracellular ligand, e.g. a growth factor or a cytokine, binds to its cell surface receptor, there is a subsequent stimulation of intracellular enzymes. These intracellular proteins propagate and amplify the signal from the activated receptor to intracellular targets, which include transcription factors that regulate gene expression. Intracellular signal transduction pathways thus connect the cell surface to the nucleus. The complex signalling network may function independently or in concert with other pathways. The signalling systems thus integrate incoming signals to produce all types of cellular responses. The role of signal transduction pathways in disease pathology is currently studied and signalling proteins are obvious potential targets for pharmacological interventions in the future.

1.7.1 Mitogen-activated protein kinase (MAPK) pathways

Mitogen-activated protein kinases (MAPKs) are important components of the intracellular signalling machinery. MAPK signalling is based upon a cascade of three sequentially activated protein kinases. A MAPK kinase kinase (MAPKKK/MAP3K/MAP2KK/MEKK) activates a MAPK kinase (MAPKK/MAP2K/MKK/MEK), which in turn activates a MAPK (Fig.2). Many MAPKKKs and MAPKKs are described and though certain MAPKK are known to specifically activate different MAPKs, the MAPKKs themselves may be activated by several MAPKKKs. MAPK signalling is a complex and diverse system, which transduce and integrate signals from a large number of different stimuli resulting in a

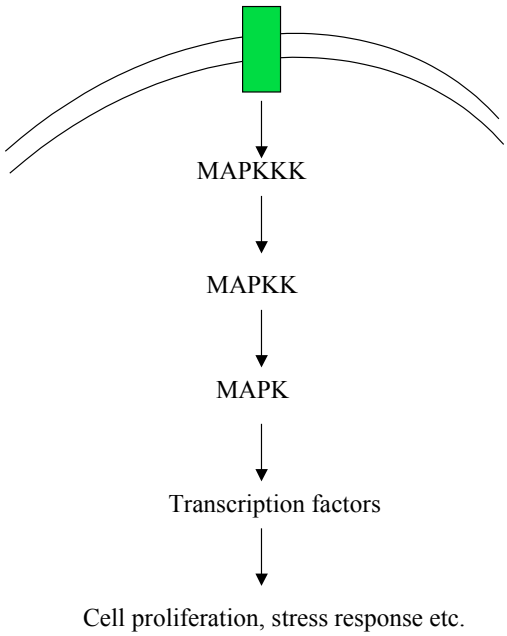

Figure 2 Schematic and oversimplified illustration of MAPK signalling. Green box represents an activated receptor at the cell membrane

variety of physiological responses. MAPK signalling is known to be involved in cell proliferation, differentiation, development, inflammation and apoptosis.
Activated MAPKs may translocate to the nucleus and regulate gene expression by targeting transcription factors. Furthermore, MAPKs may have cytoplasmic targets as well, by which they regulate gene expression at a post-transcriptional level. Indirectly, MAPKs are also involved in translational control.
There are three main MAPK families in mammalian cells; extracellular signal-regulated kinases (ERK), jun amino-terminal kinases/stress-activated protein kinases (JNK/SAPK) and p38 kinases. The ERK pathway is activated by many growth factors and is considered to regulate cell proliferation and cell survival. By contrast, JNK/SAPK and p38 are linked to apoptosis and both pathways are known to be activated by stress stimuli and cytokines. However, the roles of each MAPK pathway are highly dependent on cell type and context (Wilkinson and Millar, 2000; Chang and Karin, 2001; Zhang and Liu, 2002).
There are specific inhibitors of ERK and p38 MAPK cascades, but no available inhibitor of the JNK pathway.
ERK, JNK/SAPK and p38 MAPK pathways may all be activated in immature Sertoli cells (De Cesaris et al., 1998; Crepieux et al., 2001). Furthermore, IL-1 signalling may occur through all three main MAPK cascades (Daun and Fenton, 2000).

2 AIMS

Previous *in vitro* studies on Sertoli cell proliferation have been hampered by methodological problems. It has been difficult to get reproducible replicate primary cultures of Sertoli cells, due to cell aggregates and subsequent contact inhibition (Schlatt et al., 1996). Under these culture conditions it is hard to evaluate the effects of growth regulating factors and especially difficult to screen for growth factor activity.

Therefore, the first aim of this thesis was:

- To set up a Sertoli cell proliferation assay, suitable for screening of putative growth factors

The following aims were:

- To examine the effect of testicular growth factors present in the immature testis on Sertoli cell proliferation
- To search for Sertoli cell growth factors in testicular extracts from different developmental ages
- To investigate the effect of proinflammatory cytokines on Sertoli cell proliferation

The last aim (that developed from the results) was:

- To identify what MAPK signalling pathways are mediating IL-1-induced Sertoli cell proliferation

3 MATERIALS AND METHODS

This section is a brief description of the materials and methods used in the present thesis. For details and original references, please consult the corresponding papers. Paper I describes in detail the Sertoli cell isolation method, which is the basis of the present thesis. This method is further refined and used in subsequent papers. The isolation method is based on the ability of immature Sertoli cells to selectively bind to the lectin Datura Stramonium Agglutinin (DSA).

3.1 EXPERIMENTAL ANIMALS

Sprague-Dawley and Wistar rats were used as testis donors and were sacrificed by decapitation or cervical translocation (5 to 10-day-old rats) or CO_2 inhalation (15 to 50-day-old rats) before testis removal.

3.2 TEST SUBSTANCES

Recombinant human growth factors TGF-α, EGF, IGF-I, IGF-II, FGFa, FGFb and SCF (Biosource International, CA, US) were used in paper II.
Recombinant rat cytokines IL-1α, IL-1β, TNF-α, IL-6 and IFN-γ (R&D systems, Abingdon, UK) were used in paper III- V.
LPS serotype 0111:B4 was used in paper V.
Ovine FSH from NIH, Bethesda, MD, was used in paper I, while ovine FSH from Sigma, Missouri, US, was used in papers II – V.

3.3 SERTOLI CELL ISOLATION AND CULTURE

Testes from 8 to 9 day-old rats were decapsulated and subjected to three sequential enzymatic digestions. The mixed cell suspension was seeded out in Petri dishes coated with DSA lectin. After 24 h of culture at 37° C, the Petri dishes were carefully washed and the adherent cells were detached by trypsin treatment. The cells were then counted, diluted and replated in either 96-well culture dishes, on round plastic cover slips in 24-well plates or in new Petri dishes.
Cells were treated with test material for 48 h before harvest, fixation or lysation (see section 3.5 and Fig.3). In studies of MAPK signal transduction pathways, cells in Petri dishes were treated with test material for 30 min to 1 h before lysation (see section 3.9).

3.4 ASSESSMENT OF PURITY OF CELL PREPARATION

Cells attached to DSA lectin were analysed by 3βHSD staining or alkaline phosphatase activity to visualize possible contaminating Leydig or peritubular cells respectively. Immunocytochemistry with antibodies against rat monocytes/macrophages ED1 were used to identify testicular macrophages. Sertoli cells were positively identified by anti-cytokeratin immunocytochemistry.
Isolated and cultured cells were analysed by alkaline phosphatase activity and anti-cytokeratin immunocytochemistry. A human anti-adrenal autoimmune serum cross-

reacting with rat Leydig cells was used to detect possible contamination of Leydig cells.

3.5 ASSAYS OF SERTOLI CELL PROLIFERATION

Sertoli cell proliferation was assessed by three separate methods (Fig. 3). All three methods have their specific advantages and disadvantages. The assays are complementary to each other and thus important for the overall interpretation of the results.

3.5.1 ^{3}H-thymidine incorporation (I – V)

Sertoli cell DNA-synthesis, at the end of culture, was measured by incorporation of ^{3}H-thymidine. The method readily detects an increase in cell proliferation since the spontaneous Sertoli cell DNA-synthesis in this culture system is very low. One drawback is that the method is sensitive to increases in cell density over a certain level since contact-inhibited cells stop dividing (see section 3.5.3.). Another disadvantage is that it may detect not only replicative DNA-synthesis but also DNA repair.
Cells in 96-well plates were pulsed for the last 4 h of culture with ^{3}H-thymidine and then harvested after 48 h of the last incubation period. Incorporated radioactivity was measured in a scintillation spectrometer as counts per minute (cpm). FSH or testicular extract were used as positive controls.

3.5.2 BrdU incorporation (II, V)

The thymidine analogue BrdU was used to visualise proliferating Sertoli cells at the end of culture. The method was used to confirm results from the ^{3}H-thymidine incorporation assays. It is more time- and cell-consuming than the other two methods and thus not suitable for screening purposes. Furthermore, the cells need to be clearly separate from one another on the slide, which makes it difficult to study factors with cell aggregating effects. As all immunohistochemical procedures the method is depending on several independent factors, e.g. antibody penetration and enzyme activity.
Cells on cover slips in 24-well plates were labelled with BrdU for the last 4 h of the final 48 h incubation time. Cells were then fixed and stained for possible alkaline phosphatase activity (to identify peritubular cells) before immunocytochemistry using a primary anti-BrdU antibody and a HRP-linked second antibody was performed. BrdU positive cells were identified after reaction with DAB. Labelling indices (i.e. the percentage of BrdU positive and alkaline phosphatase negative cells) were calculated.

3.5.3 Supravital staining (II – V)

An MTT-assay was used to estimate the number of living Sertoli cells at the end of culture. The thiazolyl blue dye MTT is converted to a coloured formazan product by enzymes of mitochondria and endoplasmatic reticulum of living cells and the absorbance of the formazan colour may be detected by a spectrophotometer (Dirami et al., 1995). The results from this assay thus reflect the changes in cell number during the total incubation period. Furthermore the method is useful for evaluation of results from ^{3}H-thymidine incorporation assays, since it clearly demonstrates the impact of contact inhibition. If the two assays are run in parallel it is easy to detect if a lack of stimulated DNA-synthesis is due to an excessive cell proliferation, and subsequent contact

inhibition. The disadvantage of the MTT-assay is that it is not as sensitive as ^{3}H-thymidine incorporation measurements and that increased cellular enzyme activity may interfere with the analyses. Besides assessing proliferation, supravital staining with MTT is also a useful tool to detect cytotoxic effect of test material.
Cells in 96-well plates were pulsed with MTT either before test material was added or after 44 h of incubation with test material. After 4 h of incubation with MTT, the cells were lysed and absorbance at 570 nm was measured approximately 18 h after cell lysation. Data was expressed as absorbance units (AU). FSH or testicular extracts were used as positive controls.

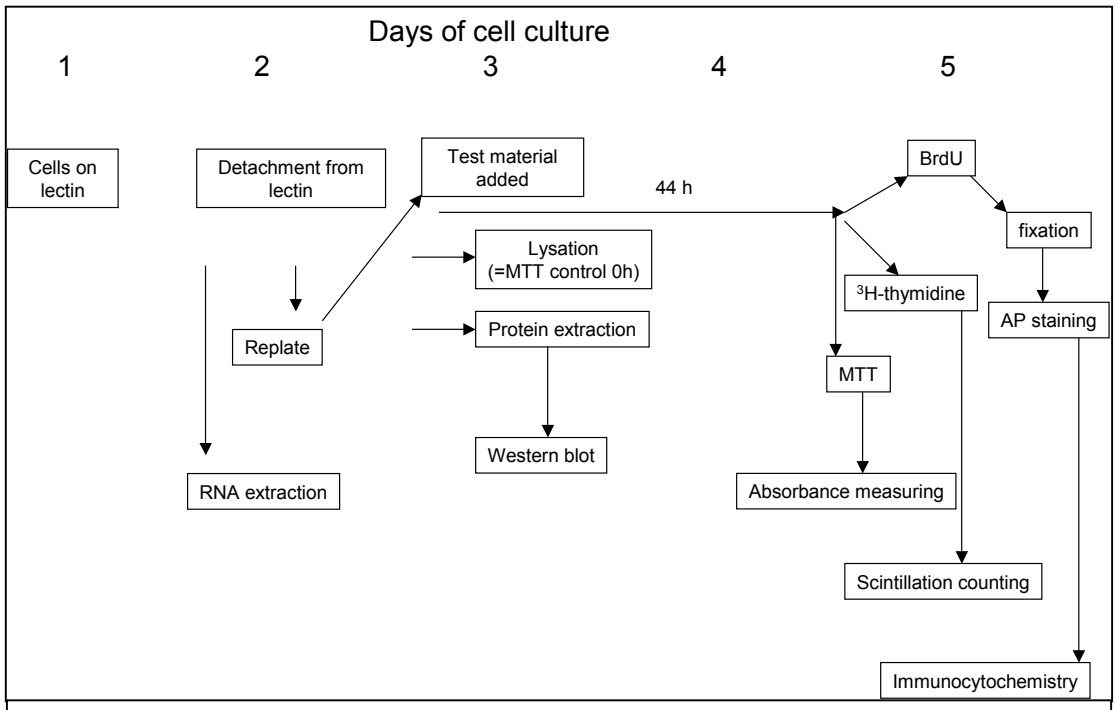

Figure 3 Flow-sheet demonstrating the Sertoli cell culture and the assays used

3.6 PREPARATION OF TESTICULAR PROTEIN EXTRACT (III)

In order to search for Sertoli cell growth factors, aqueous testicular extracts were prepared.
Testes from rats of different ages were decapsulated and extracted in 0.15M NaCl. After one freeze-thaw cycle and centrifugation at high speed, the supernatants were passed through a desalting column in order to recover the high molecular weight fraction. Protein concentration was measured by the Bradford method.

3.7 HPLC GEL PERMEATION CHROMATOGRAPHY (III)

In order to characterize the detected Sertoli cell stimulatory activity, testicular extracts from 5-, 25- and 50-day-old rats were subjected to gel permeation chromatography. Samples were run through a gel filtration column in a HPLC system with PBS as elution buffer. Molecular size markers ranged from 66.4 kDa to 17.6 kDa. Absorbance was measured at 280 nm.

3.8 RT-PCR (III, V)

To detect expression of cytokine and receptor gene expression at the mRNA level, RT-PCR was performed. Total RNA was extracted either from isolated Sertoli cells, cultured for 24 h with or without LPS treatment *in vitro*, or total intact testes of 8 to 9-day-old rats. Total RNA from inflammation-induced peritoneal exudates cells was used as positive control. In paper III specific primers for the cytokines and receptors were used for the RT reaction, while in paper V an oligo (dT) primer was used. The PCR reaction was performed with the specific primers and conditions as indicated in the separate papers. The housekeeping gene GA3PDH and ribosomal protein S27 were used as positive controls.

3.9 PAGE/WESTERN ANALYSES IN MAPK SIGNALLING STUDIES (IV)

PAGE/Western technique was used to demonstrate activation of different MAPKs by detecting their phosphorylated forms in Sertoli cell lysates.
Cells cultured in Petri dishes were pre-treated for 30 min with or without MAPK inhibitor followed by 30 min incubation with or without IL-1. Cells were then lysed and protein was extracted. Cell protein, measured by the Bradford method, was subjected to SDS-PAGE and transferred to a PVDF membrane. Immunoblotting was performed with primary antibodies against total or phosphorylated MAPKs followed by HRP-conjugated second antibodies. Signals were detected by chemiluminescence.

3.10 STATISTICAL ANALYSES

One-way analysis of variance (ANOVA) with Tukey´s post hoc test or Student´s t-test on raw data were used as statistical methods. $P < 0.05$ was considered significant.

4 RESULTS AND DISCUSSION

4.1 DEVELOPMENT OF A SERTOLI CELL PROLIFERATION ASSAY (I, II, III)

Isolation of Sertoli cells from immature rat testis was performed by repeated enzymatic digestions and selective lectin binding under serum-free conditions. Sertoli cells bound to DSA lectin were detached, replated and used for three different cell proliferation assays. Apart from in paper I, all cell preparations were isolated from testes of 8 to 9-day-old donor rats. The cell preparations contained highly purified Sertoli cells. There were virtually no contaminating Leydig cells, as assessed by 3βHSD staining and Leydig cell specific immunostaining, no macrophages or germ cells, and only a low percentage of peritubular cells. Before detachment from lectin the cell preparation contained approximately 4% of peritubular cells, detected by alkaline phosphatase activity, while the percentage of this contaminating cell type decreased to less than 0.5% after replating and culture. The majority of cells were cytokeratin positive, which further characterized the cell preparation as being predominantly early postnatal Sertoli cells. The presence of functioning Sertoli cells was also demonstrated by the response to FSH treatment. FSH stimulated both cAMP production and cell proliferation in a dose dependent manner.

After 48 hours incubation with test material, the cell cultures were pulsed with either ^{3}H-thymidine, MTT or BrdU. DNA synthesis was measured by ^{3}H-thymidine incorporation, assessed by scintillation counting. Proliferating Sertoli cells were identified by immunocytochemistry against incorporated BrdU together with the absence of alkaline phosphatase activity. An MTT-method of supravital staining was used to assess the cell viability together with the dose-dependent and net cell number changes during culture with test material. There was a positive and linear correlation between Sertoli cell number and the absorbance unit values (Fig.4).

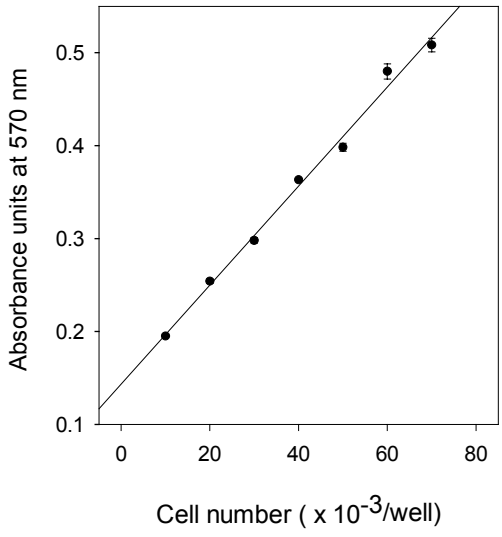

Figure 4 Cell number calibration curve in MTT-assay

Independent cell preparations of Sertoli cells from different ages demonstrated that the ability of the cells to proliferate decreased with the age of the donor animal, in agreement with their capacity to divide *in vivo*. The cells from 8 to 9-day-old rat displayed a very low proliferation when treated with medium only, but were still able to respond with significant proliferation to a crude source of testicular growth factors (i.e. testis extract from 5-day-old rat).
In summary, the cell preparation of easily counted, non-aggregated and FSH-responsive Sertoli cells, cultured under serum-free conditions, provided us with a useful tool for studying Sertoli cell growth stimulating activity.

4.2 GROWTH FACTOR EFFECT ON SERTOLI CELL PROLIFERATION (II)

TGF-α, EGF and betacellulin, all three ligands of the same receptor (EGFR), dose-dependently stimulated immature Sertoli cell DNA-synthesis, as assessed by incorporation of ^{3}H-thymidine. Treatment with TGF-α or EGF also increased the BrdU positive labelling index of Sertoli cells (Fig.5).
In the presence of TGF-α, the estimated living cell number was increased compared to the controls, although there was no actual increase in cell number compared to the start of the incubation period. There was no synergistic effect on Sertoli cell DNA synthesis when Sertoli cells were co-incubated with TGF-α and different FSH concentrations. TGF-α did not produce any apparent morphological changes apart from its capacity to increase cell density in culture.
The growth factors SCF, IGF-I, IGF-II, FGFa and FGFb had no statistically significant stimulatory effect on Sertoli cell proliferation.
Although TGF-α failed to produce a net increase in living cell number, we concluded that it acted as a Sertoli cell mitogen, as shown by ^{3}H-thymidine and BrdU incorporation *in vitro*, even if a possible survival effect of TGF-α may have contributed to the results. We suggested that cross-reacting members of this growth factor family might contribute to the paracrine regulation of Sertoli cell growth *in vivo*.

At the time, our results were only supported by one previous study that showed mitogenic action of EGF on porcine Sertoli cells (Jaillard et al., 1987). By contrast, another study demonstrated that TGF-α had no effect on rat Sertoli cell proliferation (Mullaney and Skinner, 1992b). However, the same group subsequently published results on embryonic and postnatal Sertoli cells that were coherent with our data, where they reported that TGF-α stimulates Sertoli cell growth and that FSH, the endocrine mitogen for Sertoli cells, increases the expression of EGFR (Levine et al., 2000; Cupp and Skinner, 2001).
Our results were also in contrast with earlier studies showing that FGFb, IGF-I and II had proliferative effect on immature Sertoli cells (Borland et al., 1984; Jaillard et al., 1987; Mullaney and Skinner, 1992a). In fact, we also demonstrated a tendency to stimulate Sertoli cell proliferation by FGFs, but the effects were not statistically significant. The dissimilar results may be due to differences in species, culture conditions, purity and regarding IGFs, a possible interference with IGF binding proteins in the cell cultures.

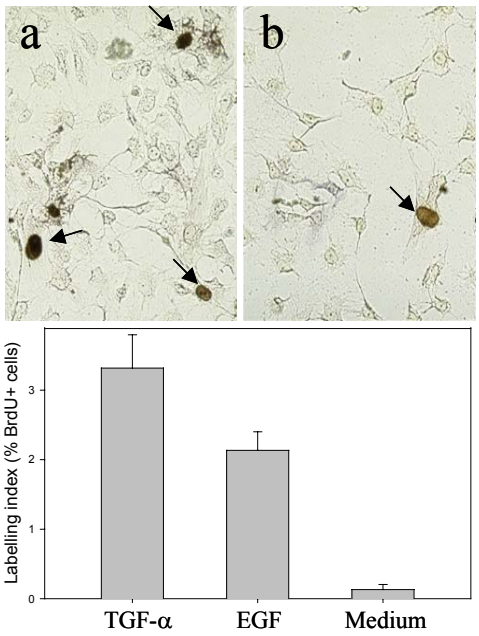

Figure 5 Stimulatory effects of TGF-α and EGF on BrdU incorporation in Sertoli cells. Upper panel: TGF-α treated cells (a) and control cells (b). Arrows indicate BrdU-positive cells. Lower panel: Increased BrdU labelling index in TGF-α and EGF treated cells compared to control cells.

4.3 IDENTIFICATION OF A SERTOLI CELL GROWTH FACTOR IN TESTICULAR EXTRACT (III)

We demonstrated that testicular extract from rats of different developmental ages increased Sertoli cell proliferation, as assessed by ^{3}H-thymidine incorporation and supravital staining. BSA at equal concentrations had no stimulatory effect. Testicular extracts from immature (5-day-old), pubertal (25-day-old) and adult (50-day-old) rats produced a significantly higher mitogenic activity of Sertoli cells than did the extracts from 10- and 15-day-old rats. Gel permeation chromatography demonstrated that the early postnatal testicular extract (i.e. testis from 5-day-old rat) contained a Sertoli cell mitogenic factor that eluted at an apparent molecular size of 45 kDa. By contrast, the peak stimulatory activity in testes from 25- and 50-day-old rats had an apparent molecular size of 17.5 – 20 kDa. In the 50-day-old rat there was also a slight activity in the 45 kDa size range. The peak stimulatory activity in 25- and 50-day-old rat testicular extracts had an apparent molecular size and developmental expression pattern as the constitutively produced testicular IL-1α. Since IL-1ra completely neutralized this mitogenic activity of pubertal and adult testis, but not the growth stimulatory activity of early postnatal testis, we concluded that the main Sertoli cell growth factor in these extracts was identical to IL-1α.

The Sertoli cell growth stimulatory activity in testis from 5-day-old rat remains to be identified.

4.4 EFFECT OF PROINFLAMMATORY CYTOKINES ON SERTOLI CELL PROLIFERATION (III AND V)

The proinflammatory cytokines IL-1α, IL-1β and TNF-α were all potent stimulators of Sertoli cell proliferation (Fig.6 and 7). All three cytokines increased Sertoli cell DNA-synthesis dose-dependently, as assessed by incorporation of ^{3}H-thymidine. The maximal response was equal to, or higher than, the response produced by FSH. The stimulatory effects of these cytokines were abolished by IL-1ra and neutralizing TNF-α binding antibody respectively. IL-1α and IL-1β also increased the estimated number of living cells in a dose-dependent fashion (Fig.6), while TNF-α had no significant effect on living cell number. Coincubation with FSH and IL-1α or TNF-α produced a synergistic stimulation of Sertoli cell DNA-synthesis. There was also a synergistic increase of living cell number when Sertoli cells were treated with IL-α and FSH simultaneously.

Interestingly, IL-1 (both α and β), but not TNF-α, produced a striking morhphological change in Sertoli cells, which was reversed by IL-1ra. After a few hours of IL-1 treatment the cells stretched out and aggregated into clusters. We interpreted this phenotypic modulation as a differentiation of the Sertoli cells from a single-cell phase to an aggregated stage. Because of this phenotypic change we could not study DNA-synthesis by BrdU incorporation and immunocytochemistry in IL-1 treated cells. This was however possible in TNF-α treated cell cultures where we found a significantly increased labelling index compared to control cells.

We also showed that immature isolated Sertoli cells express the signalling IL-1RI (RT-PCR and Western immunoblot) and the scavenger receptor IL-1RII (RT-PCR) and both TNFRs (RT-PCR). Conversely, only IL-1RI and TNFRs, but not IL-1RII, were detected in total intact testis from the same donor age (RT-PCR).

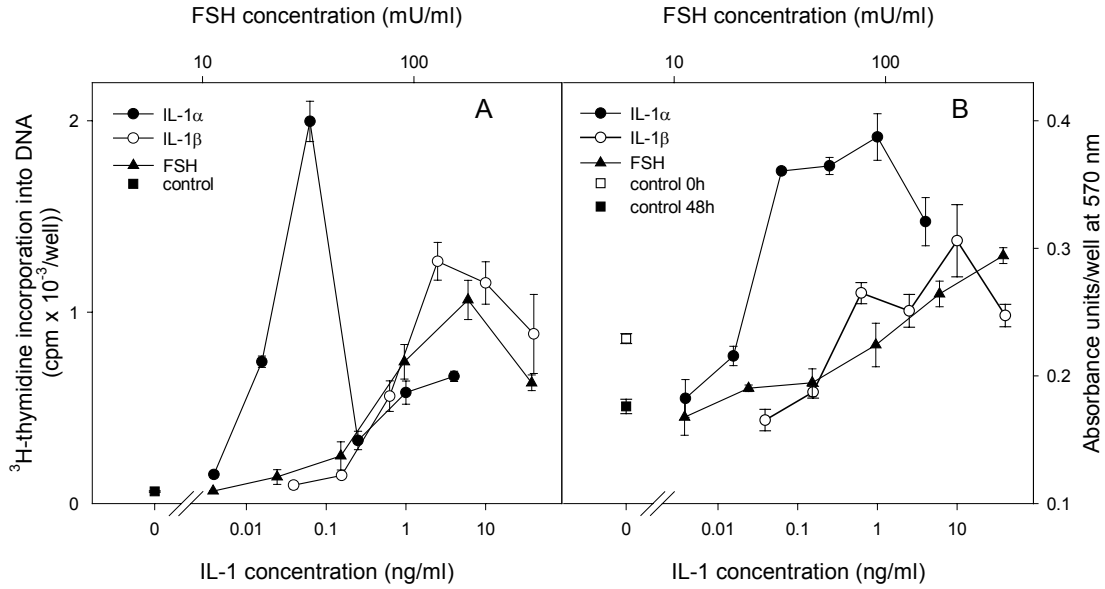

Figure 6 Stimulatory effects of IL-1α, IL-1β and FSH on Sertoli cell proliferation in vitro. (A) DNA synthesis as measured by incorporation of tritiated thymidine. (B) Number of living cells in cultures measured by supravital staining

In contrast to the effects of IL-1 and TNF-α, the proinflammatory cytokines IL-6 and IFN-γ failed to stimulate Sertoli cell growth (Fig.7). However, in combination with FSH both these cytokines modulated Sertoli cell DNA-synthesis in different ways. IL-6 increased, while IFN-γ inhibited, FSH-induced Sertoli cell proliferation.
We concluded that IL-1 and TNF-α are potent stimulators of Sertoli cell proliferation *in vitro* and that IL-6 and IFN-γ may modulate FSH action on immature Sertoli cells. We suggested that since these cytokines are readily induced in the immature testis by proinflammatory stimuli, though not present under healthy conditions, they might be of pathophysiological importance.

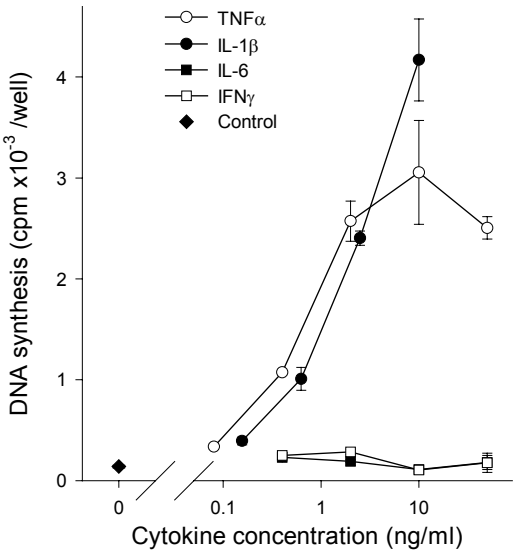

Figure 7 Effect of proinflammatory cytokines on Sertoli cell proliferation

There are no previous reports on IL-1 or TNF-α- induced Sertoli cell proliferation, although both cytokines are shown to stimulate proliferation of Leydig cells (Khan et al., 1992b). The expression of IL-1- and TNF receptors in Sertoli cells confirmed the results of earlier studies and it is well established that these cytokines have multiple effects on Sertoli cells (Boockfor and Schwarz, 1991; Khan and Nieschlag, 1991; Karzai and Wright, 1992; De et al., 1993; Mauduit et al., 1993; Besset et al., 1996; Hoeben et al., 1996; Mauduit et al., 1996; Nehar et al., 1998; Hellani et al., 2000; Riccioli et al., 2000; Pentikainen et al., 2001).

4.5 EFFECT OF LPS ON SERTOLI CELL PROLIFERATION (V)

The bacterial endotoxin LPS also dose-dependently stimulated Sertoli cell proliferation *in vitro*. LPS increased Sertoli cell DNA-synthesis and the estimated number of living cells in culture. The IL-1 and TNF-α inhibitors, IL-1ra and neutralizing TNF-α antibody, did not inhibit the proliferative effect of LPS. The LPS effect was higher than (DNA-synthesis) or equal to (estimated living cell number) the FSH growth stimulatory effect on Sertoli cells.

LPS induced the expression of the proinflammatory cytokines IL-1α, IL-1β, TNF-α and IL-6 in isolated Sertoli cells, as revealed by RT-PCR, while there were no changes in the expression level of IL-1ra or IL-1- and TNF receptors.
We concluded that LPS is a potent stimulator of Sertoli cell proliferation *in vitro* and of possible pathophysiological importance *in vivo*. Considering that IL-1- and TNF-inhibitors failed to inhibit the LPS effect, we suggested that LPS, in spite of the induced cytokine production, directly stimulated Sertoli cell proliferation.
Our results on induced cytokine production in Sertoli cells by LPS treatment confirmed earlier published data, though there were some discrepancies (Gerard et al., 1992; Stephan et al., 1997; Huleihel and Lunenfeld, 2002). There are no previous reports on LPS mitogenic effect on Sertoli cells.

4.6 MAPK SIGNALLING IN IL-1-INDUCED SERTOLI CELL PROLIFERATION (IV)

IL-1α treatment activated the p38 and JNK/SAPK MAPK pathways, as assessed by Western analyses using phospho-specific antibodies against the respective MAPKs. By contrast, ERK was not activated. The p38 inhibitor SB203580 inhibited IL-1-induced Sertoli cell proliferation, as assessed by ^{3}H-thymidine incorporation (Fig.8) and estimated number of living cells, while, as expected, the ERK inhibitor UO126 had no effect. Since there is no available selective inhibitor of the JNK/SAPK pathway, we were not able to test if this MAPK cascade mediated some of the IL-1 mitogenic effect.

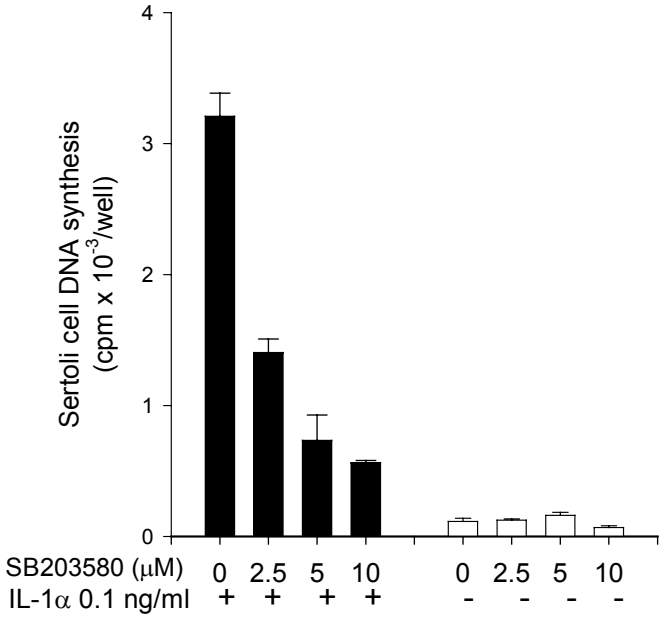

Figure 8 Inhibitory effect of p38 MAPK inhibitor SB203580 on IL-1-induced Sertoli cell proliferation measured by ^{3}H-thymidine incorporation

We showed that the SB203580 inhibitory effect was non-toxic since there was no decrease in viability by SB203580 treatment alone.
We concluded that the p38 MAPK pathway is the major activator of IL-1-induced Sertoli cell proliferation, although we could not exclude that JNK/SAPK contributed to the proliferative effect of IL-1α on Sertoli cells.
Even though p38 MAPK activation is predominantly linked to apoptosis and inflammatory responses, our data on p38 MAPK mediated Sertoli cell proliferation is supported by other studies showing mitogenic signalling by this pathway (Campos et al., 2002; Kuemmerle and Zhou, 2002; Zhao et al., 2002).
It was unexpected that ERK was not involved in Sertoli cell proliferation stimulated by IL-1. ERK is the major pathway of growth factor mediated cell proliferation and IL-1 is known to activate ERK (together with the other main MAPKs) in other cell systems (Daun and Fenton, 2000). Furthermore, immature Sertoli cells are known to have the ERK cascade present (De Cesaris et al., 1998; Crepieux et al., 2001).

4.7 GENERAL DISCUSSION

The Sertoli cell proliferation assay was efficient and with this preparation of purified and, in the culture dish, evenly distributed Sertoli cells, we overcame the problem of local contact inhibition that made Schlatt and coworkers conclude that, concerning studies on Sertoli cell growth, it is "nearly impossible to use proliferation assays like the incorporation of tritiated thymidine for the detection of mitogenic changes" (Schlatt et al., 1996). Furthermore, in studies of cell proliferation it is an advantage to exclude serum components from the culture system, since the mixture of unknown growth factors may interfere with the assay. We chose the 8 to 9-day-old rat as Sertoli cell donor, since the cells from this age showed very low spontaneous proliferation in culture (negative control) but still responded well to mitogenic stimuli.
One argument against using *in vitro* methods for studying physiological *in vivo* processes is always that a cell culture *per definition* is an unphysiological system. Our cell preparation is by no means exactly mirroring the immature Sertoli cells *in vivo*, but the different spontaneous and stimulated mitogenic activity of cell preparations from different donor ages, in accordance with their expected physiological activity, demonstrates that the cells retain some of the *in vivo* properties *in vitro*. The cells mitogenic response to FSH, which is a well-established *in vivo* function, further supports the usefulness of the primary culture system in studies of Sertoli cell growth. Moreover, the cell preparation was found to be valuable in studies of intracellular signalling. Most studies on MAPK-mediated cell proliferation are using transformed cell lines, i.e. cells with perturbed growth regulation, and in that respect a primary culture may represent a more physiological situation.
The usefulness and specificity of the Sertoli cell proliferation assay was further demonstrated when we screened for mitogenic activity of recombinant putative Sertoli cell growth factors and found that TGF-α/EGF stimulated Sertoli cell proliferation, while there were no effects of the other growth factors tested.
The role of TGF-α as a physiological paracrine Sertoli cell growth factor is supported by the studies of Cupp and Skinner (Cupp and Skinner, 2001) on embryonic and early postnatal testicular growth. Using organ culture, they showed that TGF-α increases ^{3}H-thymidine incorporation in early postnatal rat testis and that inhibition of TGF-α action decreases testicular embryonic growth by 25-30%. Considering that the Sertoli cells are

the predominant cell type in the testis at these developmental stages, it is reasonable to assume that a diminished Sertoli cell number is the major contributor to the latter result. We also used the Sertoli cell proliferation assay to purify growth factors from testicular extracts and managed to identify one of them, the 17.5 – 20 kDa factor in adult testis, as IL-1α. IL-1α should not be considered as a physiological Sertoli cell growth regulator. There is no IL-1α present in the testis at this age and when constitutive IL-1α production starts, the Sertoli cells have stopped dividing. However, IL-1α may, as other proinflammatory cytokines, be produced by testicular macrophages following proinflammatory stimuli. Possibly, though only supported by *in vitro* studies (ours and others), immature Sertoli cells may also be IL-1α producers in response to gram-negative bacterial components or other stressful stimuli (i.e. cell isolation). Furthermore, a few studies (Nagy, 1972; Miranda et al., 2002) report that Sertoli cells (in the rat) may be proliferating beyond the time point when they are generally believed to have lost the capacity to divide. If this is true, Sertoli cells may possibly respond and proliferate by IL-α-stimulation even after start of puberty. It is also conceivable that IL-1α may stimulate Sertoli cell growth in the adult testis during tissue repair *in vivo*.
Apart from IL-1α, other inducible proinflammatory cytokines were shown to stimulate, or in other ways interfere with, Sertoli cell growth. Considering the redundancy and cytokine inducing capacity by proinflammatory cytokines it may be difficult to evaluate the specific effect of each cytokine. However, some specificity of these cytokines was demonstrated during the study. While IL-1α and IL-1β together with their proliferative effect also induced a striking morphological change in cultured Sertoli cells, the TNF-α effect was purely mitogenic. Furthermore, IL-1ra abolished the IL-1 effect but failed to inhibit TNF-α-induced Sertoli cell proliferation. Our data shows that the IL-1 effect is not due to TNF-α induction or *vice versa*.
The growth stimulatory effect of LPS also has to be discussed in the context of cytokine induction in cell culture. In our culture system, LPS treatment induced IL-1α, IL-1β, TNF-α and IL-6 at least at the mRNA level. Even if we showed that the LPS mitogenic effect was not due to IL-6, IL-1 or TNF-α induction (i.e. the major cytokines induced by LPS), there is of course still the possibility that other induced cytokines in fact exerts this mitogenic effect.

Finally, what is the possible relevance of our findings? It is important for male fertility to achieve an adequate number of Sertoli cells before puberty in order to be able to produce enough germ cells during adult life. TGF-α may be one of the paracrine factors that regulate Sertoli cell growth *in vivo*, although considering the data from the TGF-α and EGFR mutant mice (Levine et al., 2000), it is not essential for Sertoli cell proliferation.
If it is a good thing to have many Sertoli cells, what is then the relevance of our findings regarding cytokine- and LPS-induced Sertoli cell proliferation? Do inflammatory or infectious diseases increase the Sertoli cell number and are thus good for sperm production? One has to keep in mind that none of the studied cytokines are present in the healthy immature testis. Interference with the physiological growth regulation during an inflammatory/infectious disease may disturb the development of normal testicular cytoarchitechture and subsequently impair spermatogenesis.
The effect of cytokines, and especially IL-1, may also be discussed in the light of TDS – the testicular dysgenesis syndrome (see Introduction). The increasing incidence in testicular cancer and reduced semen quality is suggested to be the result of a disturbed

Sertoli cell function. It is also known that poorly differentiated Sertoli cells are often present in patients with testis cancer. Endocrine disrupters are believed to play a role in the etiology of TDS and several endocrine disrupters are shown to induce the production of proinflammatory cytokines, including IL-1. The morphological change in cultured IL-1-treated Sertoli cells that we found *in vitro* may represent a change in differentiation state, and one may speculate that interference with Sertoli cell differentiation by IL-1 causes disturbed germ cell development and subsequent malignant transformation of the same cells.

4.8 CONCLUSIONS

The conclusions based on the results of this thesis are:

- Our Sertoli cell proliferation assay is a useful tool to screen for putative Sertoli cell growth factors
- The growth factors TGF-α/EGF stimulate Sertoli cell proliferation.
- The proinflammatory cytokines IL-1 and TNF-α are potent growth factors for immature Sertoli cells
- IL-1-induced Sertoli cell proliferation is mediated by the p38 MAPK pathway
- IL-1 is a phenotypic modulator of immature Sertoli cells
- The major Sertoli cell growth factor in adult testicular extract is identical to IL-1α
- The cytokines IL-6 and IFN-γ modulate FSH action on immature Sertoli cells
- The bacterial endotoxin LPS is a potent stimulator of Sertoli cell proliferation

The interference of proinflammatory cytokines in Sertoli cell growth, and possibly differentiation, *in vitro* may contribute to the understanding of the pathophysiological actions of proinflammatory cytokines in the immature testis, both in the context of local or generalized infections and inflammatory diseases, as well as the action of endocrine disrupters.

5 ACKNOWLEDGEMENTS

It has been great fun working at the Pediatric Endocrinology Unit during these years! I always felt that it was joyful to go to the lab - the excitement about new results, planning of the next experiment and always the possibility of interesting discussions. Sometimes there was of course also tedious work and discouraging results, but the lab was never ever boring! All my friends and colleagues at "Barnendokrin lab" have contributed to that. I want to thank all of you!

A special thank to:

Olle Söder, my supervisor, for your enthusiasm, inspiration, kindness, criticism and support! It is difficult to thank you because there is too much to thank for! In spite of all your other projects, you always had time when I needed it. You provided the frame for all my research work and still left me a comfortable amount of independence. I really do appreciate that! You also made me lecture for students, which is something I used to fear somewhat, but now truly enjoy! Thank you!

Martin Ritzén for creating "Barnendokrin lab", which obviously was a pre-requisite for this thesis. Your "creation" is really a very nice place to work in!

Berit Fröysa for teaching me everything I know about cell culture and bioassays. Our shouts of joy when experiments worked as well as our anger and sadness when they did not - I will never forget those moments in the lab! We really shared the same interest: "our Sertoli cells" were our little babies and we were proud as parents when they behaved well. How will I manage without your "magic hands"?!

Carla Boitani for everything you taught me about lab technique and planning of experiments during your stay in Stockholm and thereafter! Without you, there would not have been a "Sertoli cell proliferation assay"! Apart from that, your kindness, warmth, patience and willingness to help have contributed more than you may think to this thesis;

Yvonne Löfgren for all your excellent RT-PCRs and all your good laughs. If the Swedish saying "a good laugh makes your life longer" is true, you have given me a couple of extra years!

Aida Wahlgren for the company along this journey that we both started a long time ago. Thank you for all your concerns and also for all the parties and other social events that you have arranged or taken the initiative for. Those activities have really been good for "the spirit of the lab"!

Cecilia Zetterström for fruitful discussions, both from a scientific, clinical and philosophical point of view! Your enthusiasm for science is contagious!

Konstantin Svechnikov for good advice and help with Western blots. I think I will always need you to do the transfer!

Dionisisos Chrysis for valuable criticism at seminars, your social competence and sense of humour;

Kirsi Jahnukainen for your enthusiasm, inspiring working-capacity and for being such a good model of a female scientist;

Brian Setchell for your helpfulness and modesty. It is truly an honour having had the privilege of working with you!

Lars Hagenäs for your originality, curiosity and interest in things that really do matter;

Elisabeth Hedström for producing new and exciting "Sertoli cell results", which makes me eager to start experimental work after the dissertation;

other former or present lab members *Christine Carlsson-Skwirut, Maria Ahlsén, Helena Eklund, Susanne Hallberg, Nina Martinelle, Mona-Lisa Strand, Ulrika Berg, Ola Nilsson, Taranum Sultana, Andrei Chagin, Giedre Grigelioniene, Svetlana Lajic, Monique Assmus, Hector Urien-Rodriguez, Eugenia Colón, Sara Gustafsson, Mi Hou, Mikko Huhtanen, Cissi Bornestaf, Arif Siddiqui, Katarina Mårtensson, Lars Sävendahl, Peter Bang, Mikael Holst, Gunnie Westerholm, Tina Granholm and Mikael von Euler* for being the specific characters that you are and thereby creating the special atmosphere of the lab (now and before);

Eva Hall for your beautiful illustrations;

Viktoria Dixon (one of my little-sisters) for reading the manuscript of the thesis and commenting on the language;

Christian, my husband, for accepting this odd testicular interest of mine as a part of me.

This work was supported by grants from the Swedish Medical Research Council, the Children´s Cancer Fund, CTRF, the Cancer and Allergy Fund, the Wera Ekström Foundation for Paediatric Research, the Jerring Foundation, the Märta and Gunnar V Philipson Foundation, the Åke Wiberg Foundation, the Bergvall Foundation, the Foundation Frimurare Barnhuset in Stockholm, H.R.H. Crown Princess Lovisa Society of Pediatric Health Care, the Society for Child Care, the Samariten Foundation, the Swedish Society of Medicine, the Swedish Society for Medical Research and Karolinska Institutet.

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