

# MT1-MMP in rat testicular development and the control of Sertoli cell proMMP-2 activation

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## SUMMARY

**Metalloproteases (MMPs) are likely to be involved in the restructuring events occurring in the testis throughout development. We here demonstrate that membrane-type 1 (MT1)-MMP, a physiological activator of proMMP-2 under TIMP-2 control, is present within the testis together with MMP-2 and TIMP-2. In the prepubertal testis MT1-MMP immunoreactivity was uniformly distributed, whereas in the adult it was confined to the apical compartment of the tubules, where meiosis and spermiogenesis occur. We further showed that the two cell lineages (somatic and germinal) expressed MT1-MMP and TIMP-2, whereas MMP-2 was of somatic origin. To get a better picture into proMMP-2 activation, use was made of a model of cultured Sertoli cells treated with FSH or co-cultured with germ**

**cells to mimic an immature or a mature developmental period, respectively. We found that follicle-stimulating hormone enhanced the expression of MMP-2 and TIMP-2 but not of MT1-MMP, and promoted the activation of proMMP-2. In co-cultures, a tremendous elevation and activation of MMP-2 was observed, which might relate to the processed MT1-MMP form solely detected in germ cells. That MMP-2 synthesis and activation are under local (germ cells) and hormonal (follicle-stimulating hormone) regulation emphasizes the importance of MMPs in testicular physiology.**

Key words: Metalloprotease, Testis, FSH, Sertoli cell, Germ cell

## INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of structurally related proteins that degrade most if not all components of the extracellular matrix and basement membranes in a zinc-dependent manner at a physiological pH. They have been implicated in extracellular matrix remodeling in relation to embryonic developmental processes, inflammation and tumor invasion and metastasis (Birkedal-Hansen, 1995; Werb, 1997; Shapiro, 1998; Nagase and Woessner, 1999). Among them, MMP-2 is a pivotal MMP in remodeling of basement membrane, pericellular, and cell attachment proteins. It is secreted from cells as an inactive zymogen, proMMP-2. Thus, the activation of MMP-2 is one of the critical steps involved in controlling its activity. Activation occurs by a membrane-linked process that involves membrane-type 1 (MT1)-MMP (Sato et al., 1994) and tissue inhibitor of metalloproteinase-2 (TIMP-2; Strongin et al., 1995; Wang et al., 2000). MT1-MMP, like the five other MT-MMPs currently identified (Velasco et al., 2000), differs from all other MMPs by having in addition to the three basic domains characterizing MMPs (i.e. a candidate leader sequence, a propeptide region, and a zinc-coordinated catalytic domain), a transmembrane domain which localizes the enzyme to the plasma membrane (Sato et al., 1994). As a result, MT1-MMP complexed to TIMP-2 serves as a cell surface receptor for MMP-2. Cleavage of proMMP-2 is accomplished by other MT1-MMP molecules (i.e. not complexed to TIMP-2) and a fully active enzyme is generated

after autoproteolytic activation of MMP-2. Free TIMP-2 (i.e. not complexed to MT1-MMP) inhibits the reaction. Thus, the relative proportions of MT1-MMP and TIMP-2 are critical in governing proMMP-2 activation (Itoh et al., 1998; Stanton et al., 1998; Kurschat et al., 1999; Hernandez-Barrantes et al., 2000; Wang et al., 2000).

The testis is an organ in which a series of radical remodeling events occurs during development. It is composed of two main compartments: the interstitium, containing the steroidogenic Leydig cells, and the seminiferous tubules, composed of germ cells, Sertoli cells and peritubular cells. Sertoli cells play key roles in spermatogenesis: they are targets for follicle-stimulating hormone (FSH) and testosterone, the hormones responsible for the initiation and maintenance of spermatogenesis, and they form the tubule and provide structural and nutritional support for the developing germ cells (Ritzen et al., 1981; Skinner, 1991; Jégou, 1993; Parvinen, 1993; Sharpe, 1994; Griswold, 1995). Interestingly, the seminiferous tubule undergoes extensive restructuring in a cyclic fashion. As germ cells develop, they progress towards the tubular lumen without perturbing the structural integrity of the tubule. To accomplish this, germ cells, which have established intricate relationships with Sertoli cells, have to pass the blood-testis barrier. This barrier is formed at the initiation of puberty by a tight-junctional complex facing adjacent Sertoli cells. Once germ cells have passed the barrier, they enter the meiotic process and continue their migration towards the tubular lumen while differentiating into sperm

cells. The last step in spermatogenesis is spermiation, or extrusion of sperm cells into the lumen, accompanied by Sertoli cell phagocytosis of the residual bodies (shed cytoplasts) (Russell, 1980). To permit these movements while maintaining integrity of the tubule, there must be a fine tuning of the different proteases and antiproteases present in the seminiferous tubules (Fritz et al., 1993). Previous reports have consistently suggested that serine and cysteine proteases could play a role in these restructuring events (Fritz et al., 1993; Mruk et al., 1997; Le Magueresse-Battistoni et al., 1998; Sigillo et al., 1999; Wong et al., 2000). Although several MMPs are known to be secreted by Sertoli cells, including MMP-2 and its inhibitor TIMP-2 (Fritz et al., 1993; Hoeben et al., 1996), no study to date has provided evidence of their involvement.

These observations prompted us to examine the *in vivo* and *in vitro* occurrence of MT1-MMP in the testis. The time course of the appearance of MT1-MMP transcript and protein, its cellular localization, the spatiotemporal distribution of TIMP-2 and MMP-2, and the predicted role of MT1-MMP strongly suggest an involvement of MT1-MMP in proMMP-2 activation within the testis throughout development. As the seminiferous tubule is the site of extensive remodeling, we next focused our study on the control of MMP-2 expression and activation in Sertoli cells. Using 20-day-old rat cultured Sertoli cells, we demonstrated that proMMP-2 activation is under both systemic (FSH-mediated) and local (exerted by germ cells) control.

## MATERIALS AND METHODS

### Tissue and cell preparation

Whole testes were collected from 17.5 days post coitum (d.p.c.) and 1-, 10-, 20-, 60- and 90-day-old Sprague-Dawley rats, immediately stored at  $-70^{\circ}\text{C}$  and then processed for RNA. The interstitial cells removed by collagenase-dispase (0.05%) digestion of 20-day-old rat testes will be referred to as the Leydig-enriched fraction (60-70% enrichment), even though this fraction is contaminated with macrophages and peritubular cells. Peritubular and Sertoli cells were isolated from 20-day-old rats and cultured in Ham's F12:DMEM (Gibco) at  $32^{\circ}\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$  as previously described (Mather and Phillips, 1984; Le Magueresse-Battistoni et al., 1994). Peritubular cells were cultured in 10% serum until confluent. This usually required 5 days. At that time, the purity of the cultures was estimated to be close to 90%. Sertoli cells were seeded in 60  $\text{cm}^2$  dishes (for northern blots) or 20  $\text{cm}^2$  dishes (for western blots and zymography) at a ratio of  $1 \times 10^6$  viable cells per 5  $\text{cm}^2$ . Contamination by germ cells (spermatogonia and early spermatocytes) ranged between 10% and 15% at the time of plating, declined continuously, and was reduced to 2-3% after 5 days of culture (assessed by flow cytometry and cell staining studies; Le Magueresse and Jégou, 1988). Contamination by peritubular cells (assessed by alkaline phosphatase staining) was 1% and 3-5% of the total population in 1- and 5- day-old Sertoli cell cultures, respectively (Le Magueresse-Battistoni et al., 1998). In some instances, FSH (US National Institutes of Health FSH o-19) or 8-bromo-cAMP (Sigma, France) was included in the Sertoli cell culture medium for the duration indicated.

Spermatogenic cells were isolated from 60- to 90-day-old rat testes by trypsinization (Meistrich et al., 1981) or a mechanical procedure (Aravindan et al., 1996). The resulting crude germ cell population (containing germ cells from all developmental steps but no somatic cells) was submitted to a centrifugal elutriation using a Beckman JE-6 rotor as described previously (Meistrich et al., 1981). Two fractions were harvested, the pachytene spermatocyte fraction

(enrichment of 80-85%; contaminated primarily by early spermatids) and the early spermatid fraction (steps 1-8; enrichment 75-80% with primary contamination by both spermatocytes and elongated spermatids). The purity of cell types was assessed as previously described on air-dried smears stained with periodic acid Schiff (PAS) and hematoxylin (Meistrich et al., 1981; Le Magueresse et al., 1986), and by flow cytometry (Le Magueresse and Jégou, 1988). After collection, the different cell populations were either processed for RNA or protein analysis, or used for co-culture experiments. For culture studies,  $2.5 \times 10^6$  germ cells (crude suspension or elutriated cells) were added to the 5-day-old cultured Sertoli cells (dish area 20  $\text{cm}^2$ ) for 48 hours.

### RNA extraction

Total RNA was isolated using acid-guanidium thiocyanate-phenol-chloroform extraction in a single step procedure as reported previously (Chomczynski et al., 1987). The polyadenylated (poly(A)<sup>+</sup>) RNA was isolated using  $\mu$ MACS mRNA Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

### Reverse-transcriptase polymerase chain reaction

3  $\mu\text{g}$  total RNA were reverse-transcribed in 10  $\mu\text{l}$  of reaction mixture consisting of 0.2 mM dNTPs (Sigma), 10 U  $\text{ml}^{-1}$  of MMLV reverse transcriptase (Gibco BRL), 0.01 M DTT (Gibco BRL) and 5  $\mu\text{M}$  random hexamers (Sigma) in 20 mM Tris-HCl buffer (Gibco BRL). Reverse transcription (RT) was carried out for 60 min at  $37^{\circ}\text{C}$  and  $100^{\circ}\text{C}$  for 5 min. 10  $\mu\text{l}$  of water was added per sample at the end of the RT. Primers for MMP-2 were: sense (5'-CTATTCTGTGTCAGC-CTTTGG-3') and antisense (5'-CAGACTTGGTTCTCCAACCTT-3'), with an expected product of 309 bp (Wells et al., 1996). Primers for MT1-MMP were: sense (5'-GCTCATTCATGGGTAGCGAT-3') and antisense (5'-AGTAAAGCACTCGCTTGGGT-3'), with an expected product of 329 bp. Primers for TIMP-2 were: sense (5'-ATCAGAGCCAAAGCAGGTGAGCG-3') and antisense (5'-GGTA-ATGTGCATCTTGCCATCTCC-3'), with an expected product of 243 bp. Primers for GAPDH were: sense (5'-TCCCACCACCCTG-TTGCTGTA-3') and antisense (5'-ACCACAGTCCATGCCATCAC-3') with an expected product of 455 bp. All primers were purchased from Oligoexpress (Montreuil, France).

PCR was carried out using 2  $\mu\text{l}$  of the RT reaction, 1  $\mu\text{M}$  of the designed specific primers in the presence of Taq polymerase (0.01 U  $\mu\text{l}^{-1}$ , Eurobio, France), dNTPs (100  $\mu\text{M}$ ; Sigma),  $\text{MgCl}_2$  (1.5 mM; Eurobio) and 50 mM Tris-HCl (Eurobio) in a final volume of 20  $\mu\text{l}$ . The polymerase chain reaction amplification was performed using a Mastercycler gradient (Eppendorf). The optimal temperature of annealing was of  $55^{\circ}\text{C}$  for MMP-2,  $59^{\circ}\text{C}$  for MT1-MMP and GAPDH, and  $70^{\circ}\text{C}$  for TIMP-2. Briefly, after a first denaturation at  $94^{\circ}\text{C}$  for 4 minutes, samples were submitted to 35 cycles (GAPDH, 30 cycles) of denaturation ( $94^{\circ}\text{C}$ , 30 seconds), annealing (optimal temperature, 30 seconds) and elongation ( $72^{\circ}\text{C}$ , 60 seconds). Final elongation was performed at  $72^{\circ}\text{C}$  for 5 minutes. Negative controls contained water instead of cDNA. Amplified cDNAs were visualized in a 1.5% agarose gel stained with ethidium bromide. A DNA ladder (Gene Ruler, Fermentas, St Leon-Rot, Germany) was loaded on each gel. PCR with no RT reactions gave no band, eliminating the possibility of a genomic DNA contamination in the RNA preparations (not shown).

### Northern blot analysis

The probes used for hybridization were the PCR products for MMP-2 (309 bp), MT1-MMP (329 bp) and GAPDH (455 bp), a 700 bp *HindIII-XhoI* fragment of human TIMP-2 complementary DNA (cDNA) (D. R. Edwards, Calgary University, Canada), an 1100 bp *BamHI-EcoRI* fragment of rat 18S cDNA (A. Ferguson, University of Texas, USA). Probes were labeled using the RTS RadPrime DNA Labeling System (Life Technologies, Gaithersburg, UK). Northern blot analysis was performed as previously described (Le Magueresse-Battistoni et al., 1994), using either total RNA (10-20  $\mu\text{g}$  as measured

by absorbance at 260 nm) or poly(A)<sup>+</sup> (3 µg). A 0.28 kb to 9.5 kb Promega RNA ladder was used to determine the size of the transcripts.

### Western blot analysis

Primary antibodies used in this study were a rabbit anti-MMP-2 (1:500), a rabbit anti-TIMP-2 (1:1000) (Chemicon International, Temecula, CA) and a rabbit anti-MT1-MMP (1:1000) (Sigma). SDS-PAGE and western blotting were carried out using (1) tenfold concentrated cell culture media (Centriprep, cut-off at 10 kDa; Amicon, Beverly, MA, USA) for the analysis of TIMP-2, (2) a immunoprecipitated proteins from concentrated cell culture media (MAGmol protein A MicroBeads, Miltenyi Biotec) for the analysis of MMP-2 or (3) cell lysates prepared using PBS containing 1% NP-40 and 5 mM EDTA for the analysis of MT1-MMP (Okada et al., 1997).

Proteins (exactly 40 µg), measured by BCA protein assay (Pierce/Interchim, Montluçon, France), were separated by electrophoresis performed on polyacrylamide gels (7.5% for MMP-2 and MT1-MMP; 15% for TIMP-2) under reducing conditions. The proteins were then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Biorad). After treatment with a blocking solution (5% bovine serum albumin (BSA) in Tris-buffered saline (TBS)) for 3 hours, the membrane was incubated overnight with the primary antibody at 4°C. The PVDF membrane was washed and incubated with peroxidase-conjugated goat anti-rabbit IgG antibody (Covalab, Lyon, France). After washing in TBS containing 0.1% Tween-20, proteins were detected using a chemiluminescent detection system, ECL+ (Amersham Pharmacia Biotech).

### Gelatin zymography

Cell culture media were concentrated tenfold using Centriprep (Amicon, Beverly, MA, USA). 40 µg of proteins were electrophoresed at 4°C on 10% polyacrylamide gels containing 1 mg ml<sup>-1</sup> gelatin (Sigma) in the absence of any reducing agent. Following electrophoresis, SDS is removed from the gel by exchange in Triton X-100 (two washes of 30 minutes at room temperature in 2.5% Triton X-100 followed by two washes of 20 minutes in distilled water). The gel is subsequently incubated at 37°C for 48 hours in 100 mM Tris-base, pH 7.6 containing 15 mM CaCl<sub>2</sub>. In these conditions, gelatinases present in the samples renatured and autoactivated. White zones of lysis indicating gelatin degrading activity were revealed by staining with Coomassie Brilliant Blue R-250.

### Immunohistochemistry

Testes were fixed in AFA (acetic acid/formaldehyde/alcohol) or in Bouin's liquid. Tissues were then dehydrated and embedded in paraffin. Sections (4 µm thick) were applied to 3-aminopropyl triethoxysilane-coated slides. After deparaffinization and rehydration, nonspecific binding of antibodies was blocked with phosphate-buffered saline (PBS) containing 1% BSA for 2 hours. Endogenous peroxidases were blocked with H<sub>2</sub>O<sub>2</sub> for 10 minutes at room temperature. Sections were then incubated with the rabbit anti-MT1-MMP antibody (used in western-blotting studies) for 1 hour at room temperature. Anti-MT1-MMP antibody was diluted (1:200) using DAKO antibody diluent (Dako, Trappes, France). The diluent alone was used to prepare negative controls. Such diluent is particularly useful for reducing background staining while maintaining specific reactivity. After rinsing with 0.05 M Tris-HCl, pH 7.6, containing 0.15 M NaCl, sections were incubated with goat anti-rabbit immunoglobulin conjugated to peroxidase (Envision+, Dako, Trappes, France) for 30 minutes at room temperature and the immunohistochemical reaction was carried out using diaminobenzidine (DAB). Sections were next stained with PAS, counterstained with Harris hematoxylin and mounted. Sections were observed under an Axioplan microscope (Zeiss).

### Data analysis

All experiments were repeated at least three times using independent

cell preparations and triplicate dishes; a representative experiment from each series is presented here. The band densities obtained in northern blotting analyses were determined by scanning densitometric analysis (Alcatel TITN Answare, Massy, France). The amount of RNA in each lane of each northern blot was internally standardized within a blot by assessing the amount of GAPDH or 18S mRNA per lane. The significance of the results was determined by Student's *t* test when comparing data from two groups. Differences are accepted as significant at *P*<0.05.

## RESULTS

### Developmental expression of MT1-MMP protein in the testis

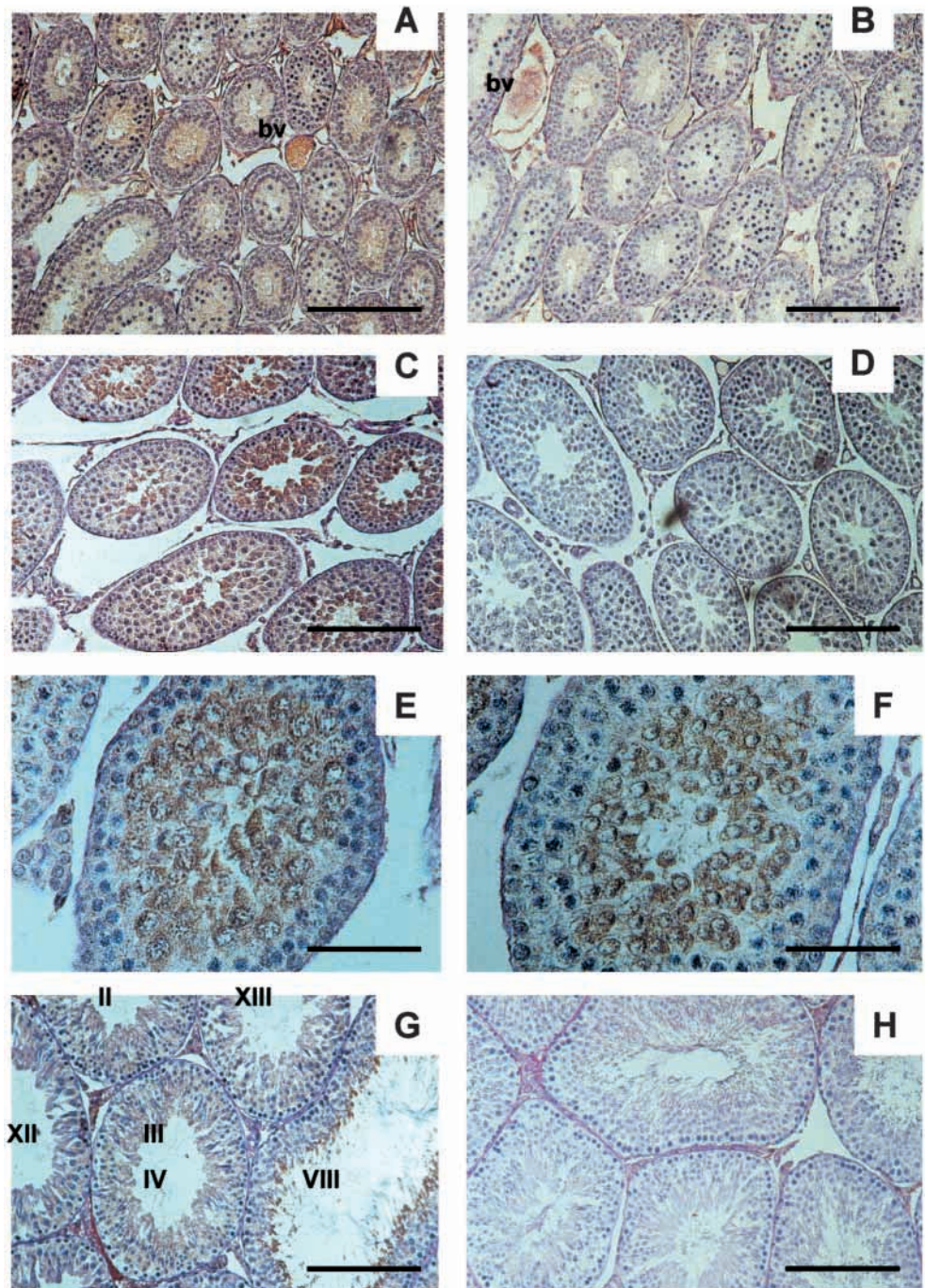
Expression of MT1-MMP immunoreactivity was examined throughout testicular development. We found homogeneous staining in all cells in the cords and in the interstitial area up to the mid-pubertal period (Fig. 1A, a 19-day old rat testis cross-section). At that age, early pachytene spermatocytes are the most advanced germ cells. Two weeks later, when early spermatids largely populate tubules, immunostaining was concentrated in the apical portions of the seminiferous tubules (Fig. 1C); late pachytene spermatocytes (Fig. 1E) and early spermatids (Fig. 1F) were intensely labeled. Immunostaining then increased as spermatids were elongating and differentiating into sperm cells (Fig. 1G; Fig. 2). Spermiogenesis, which refers to the morphological transformation of spermatids into spermatozoa, involves 19 steps in the rat, with 14 stages defined from I to XIV (Leblond and Clermont, 1952). At low magnification (Fig. 1G), a distinct pattern of immunostaining in germ cells could be observed depending on tubular cross-sections. Sperm cells in stage VIII (Fig. 1G; Fig. 2A) were the most intensively stained cells whereas tubular cross-sections at stages X-XIII (Fig. 1G; Fig. 2C) showed strong staining in large late pachytene spermatocytes and elongating spermatids. An intermediate situation is observed in stages II-III (Fig. 2E) and III-V (Fig. 2G,H) with a progressive and selective accumulation of the immunoreactivity in the cytoplasm of elongated spermatids. Sertoli cells also showed immunostaining. However, not all tubule cross-sections exhibited positive immunostaining in Sertoli cells and, within a single tubule, immunostaining was not detected in all Sertoli cells (Fig. 2). Control testicular cross-sections remained unstained.

### Western-blot analysis of MT1-MMP in isolated testicular cells

To confirm and extend immunohistochemistry (IHC) data, lysates corresponding to the different seminiferous tubular cell types were analyzed by western blot using the same anti-MT1-MMP antibody. Two proteins that migrated at 63 kDa and 60 kDa were observed in cultured peritubular and Sertoli cells, in freshly isolated Sertoli cells (all recovered from 20-day-old rats) and in freshly isolated germ cells recovered from adult rat testes. The germ cells analyzed consisted of a crude fraction or of pachytene spermatocyte and early spermatid fractions enriched by centrifugal elutriation. In germ cell extracts, we detected a band of 45 kDa in addition to the doublet around 60 kDa. This band was present whether or not trypsin was used in the germ cell isolation procedure (Fig. 3).



**Fig. 1.** Immunohistochemical localization of MT1-MMP in 19-day-old (A,B), 33-day-old (C-F) and 60-day-old (G,H) rat testis. MT1-MMP was uniformly distributed in 19-day-old testis (A) but it concentrated in the apical portions of the seminiferous tubules in 33-day-old testis (C). Late pachytene spermatocytes (E) and early spermatids (F) are intensely stained in 33-day-old rats. In adult rats (G), immunoreactivity differed according to the stages indicated in roman numerals, with sperm cells in stage VIII being the most strongly labeled. Controls in B, D and H show no staining. Bar, 350  $\mu$ m for A-D,G,H and 110  $\mu$ m for E,F. Abbreviation: bv, blood vessel.



### Expression of MT1-MMP mRNA during testicular development and in purified cells

Total RNA was prepared from whole testes collected from prenatal (17.5 d.p.c.), newborn (1 day), prepubertal (10 days), midpubertal (20 days) and adult (60 days) rats, from Leydig, Sertoli or peritubular cells (recovered from 20-day-old rats), and from germ cells. RT-PCR analysis was performed and a PCR product of the expected size (329 bp) and sequence (not shown) was obtained with every RNA sample (Fig. 4A,B). The PCR product was next used to probe a northern blot to identify the size of the MT1-MMP transcript in various testis samples including whole testis RNA, cultured Sertoli or peritubular cells, and germ cells. We found a single 4.5 kb band with every sample (Fig. 4C).

### Expression of MMP-2 and of TIMP-2 RNAs in whole testis and in purified cells

We next used RT-PCR to investigate whether MMP-2 and TIMP-2 were co-expressed with MT1-MMP during testicular development and within the different testicular cell types. A PCR product corresponding to MMP-2 and TIMP-2 was detected at all ages (Fig. 5A) and in every somatic cell analyzed (Fig. 5B). By contrast, germ cells exhibited mRNAs for TIMP-2 but not for MMP-2 (Fig. 5B). These data provide evidence that the germ cell preparations were not contaminated with somatic cells.

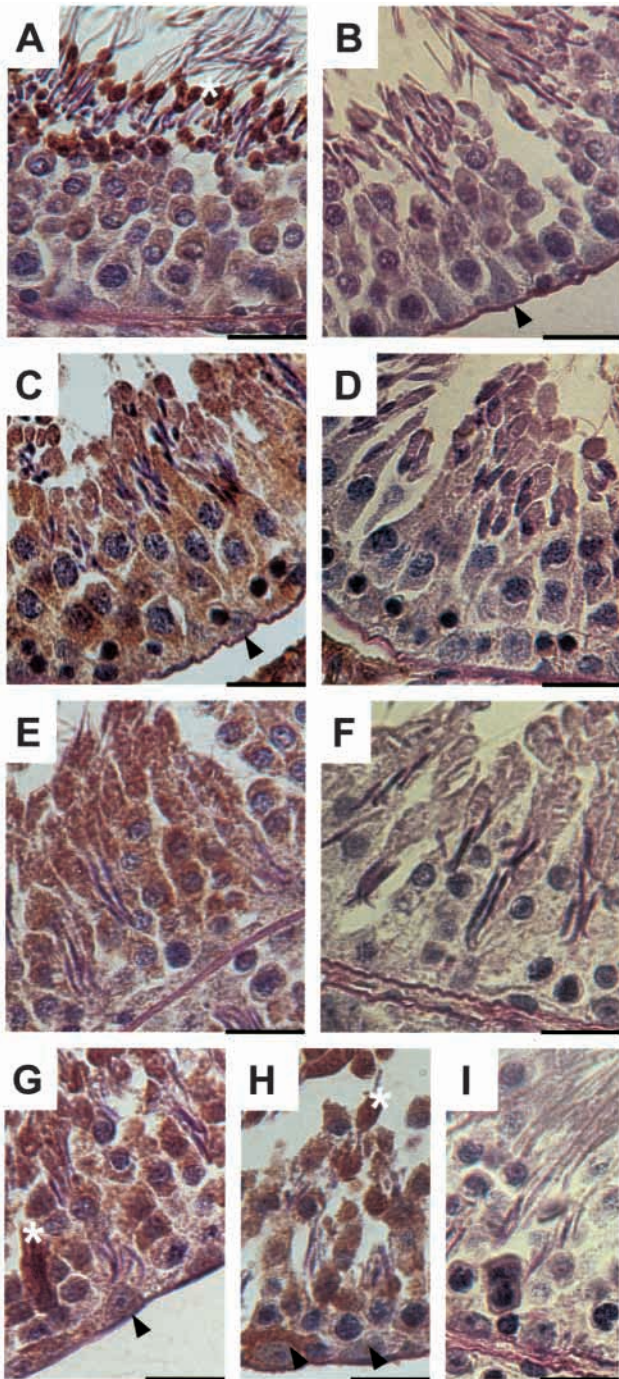
### FSH regulation of MMP-2, TIMP-2 and MT1-MMP expression in cultured Sertoli cells

Exposure of Sertoli cells to FSH (50 ng ml<sup>-1</sup>) for 48 hours from the time of plating resulted in a stimulation of the MMP-2

mRNA level by approximately fourfold ( $P < 0.05$ ;  $n = 3$ ). A cAMP analog (8-bromo-cAMP, 0.1 mM) was even more effective than FSH, confirming involvement of the protein kinase A pathway in gonadotropin action (Fig. 6A). No action of FSH on MMP-2 antigen production was detected at 24 hours and 48 hours of treatment (not shown). However after 72 hours of treatment (Fig. 6B), we consistently detected a slight increase in MMP-2 antigen level. It increased approximately 50% per densitometric analysis in the three experiments performed. Culturing Sertoli cells with FSH induced no significant variations in the levels of either MT1-MMP mRNA (Fig. 7A) or MT1-MMP antigen (Fig. 7B).

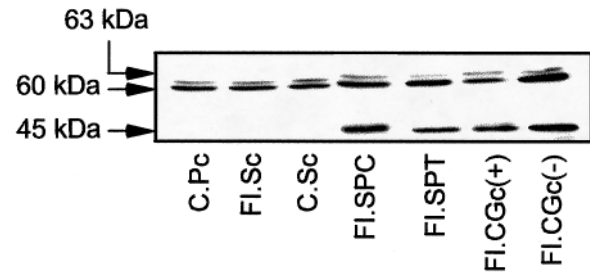
TIMP-2 regulation by FSH was next deeply investigated



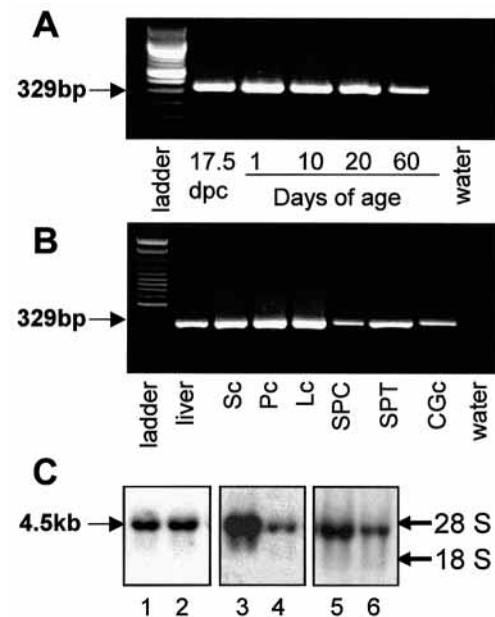


**Fig. 2.** Immunohistochemical localization of MT1-MMP in adult rat testis at stages VIII (A,B), XIII (C), X (D), II-III (E), II (F), III-IV (G), IV-V (H,I). Controls are B, D, F and I. White asterisks indicate immunostained elongated spermatids; arrowheads point to Sertoli cells. Bar, 50  $\mu$ m.

(Fig. 8). Contrasting results have been previously published with either FSH stimulating (Ulisse et al., 1994) or having no action (Grima et al., 1996) on Sertoli cell TIMP-2 expression. In our hands, FSH acted in a dose-dependent fashion on TIMP-2 mRNA levels (Fig. 8A), especially on the 0.9 kb mRNA species of TIMP-2. The half-maximum effect was observed with 10 ng ml<sup>-1</sup>, and the plateau value was at 50 ng ml<sup>-1</sup> at



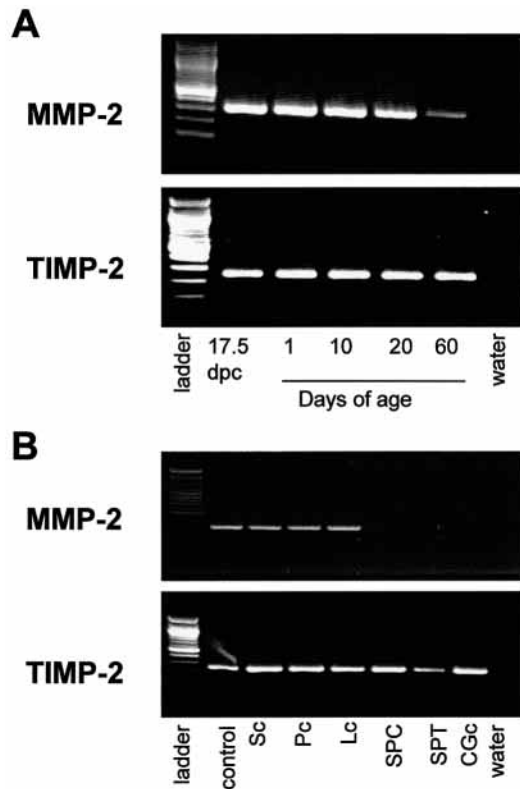
**Fig. 3.** Western blot analysis of MT1-MMP in purified testicular cells. Cell lysates of 5-day-old cultured peritubular (C.Pc) and Sertoli (C.Sc) cells, and of freshly isolated Sertoli cells (FI.Sc), spermatocytes (FI.SPC), spermatids (FI.SPT), or mixed germ cells (FI.CGc) were prepared. 40  $\mu$ g of proteins were loaded on to 7.5% SDS-PAGE gels. (+) and (-) indicate whether or not trypsin has been used in the course of the germ cell isolation.



**Fig. 4.** Analysis of MT1-MMP gene expression during testicular development and in purified testicular cells by RT-PCR (A,B). Total RNA was prepared from (A) whole testis at various ages (17.5 dpc and 1, 10, 20, and 60 days old) and (B) from adult rat liver (positive control), Sertoli cells (Sc), peritubular cells (Pc), Leydig cells (Lc), spermatocytes (SPC), spermatids (SPT) and crude germ cells (CGc). The expected size of the PCR product is 329 bp. A DNA ladder has been included in the gels. (C) Northern blot analysis of MT1-MMP in 10-day-old (lane 1) and 15-day-old (lane 2) rat testis, in cultured Sertoli (lanes 3, 4) or peritubular cells (lane 5), and in crude germ cells (lane 6), using total RNA (10-20  $\mu$ g) except in lane 3 (3  $\mu$ g of polyA(+)). The positions of the 28S and 18S are indicated.

48 h of treatment (approximately a 3.8-times increase;  $P < 0.05$ ). The response to these low doses supports the concept of a physiological rather than a pharmacological action of FSH. The action of FSH on TIMP-2 mRNA levels was also time dependent, with a maximum at 48 hours of treatment (not shown).

The kinetics of TIMP-2 production in the presence or the absence of FSH was next monitored by western blotting (Fig. 8B). We first observed that, in conditions under which MT1-

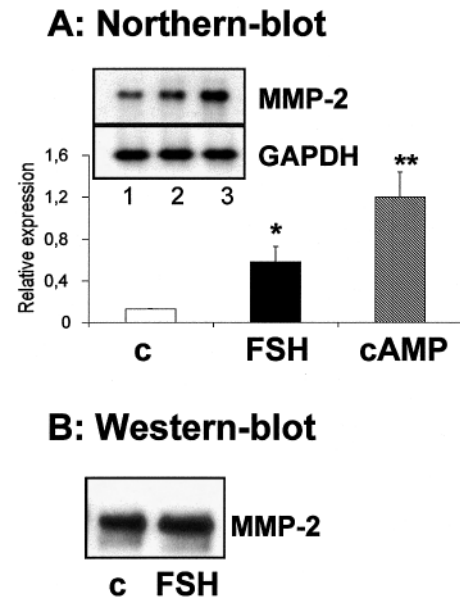


**Fig. 5.** RT-PCR analysis of MMP-2 and TIMP-2 expression during testicular development (A) and in purified testicular cells (B). Total RNA was prepared from whole testes recovered from rats of different ages and from various purified testicular cells. Symbols are as used in Fig. 4. Rat uterus was used as a positive control. A DNA ladder has been included in the gels.

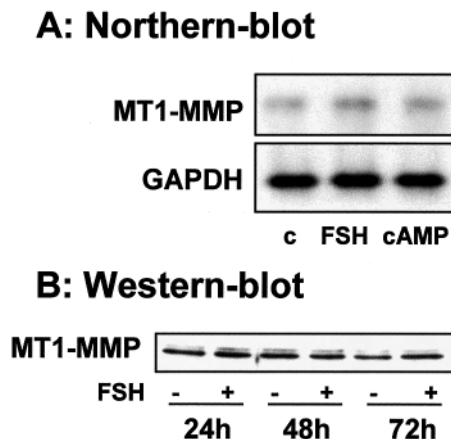
MMP did not fluctuate (Fig. 7B), TIMP-2 antigen levels were found to increase highly in culture. Second, FSH stimulation of TIMP-2 antigen levels (Fig. 8B) was first detected at 24 hours of treatment and the maximum stimulation was observed at 48 hours. The effects lasted for up to 72 hours of treatment. Data obtained by densitometric analysis of the western blots corresponding to the three different experiments performed showed increases of ~100% after 48 hours and 50% after 72 hours when compared with time-matched controls.

#### Role of FSH in MMP-2 activation in cultured Sertoli cells

Functional demonstration of the impact of FSH on MMP-2 was provided by gelatin zymography. This technique allows discrimination between active and inactive gelatinases, as they migrate differently. Two groups of gelatinolytic bands were detected in Sertoli cell culture media (Fig. 9A). One group migrated at or near 92 and 84 kDa and the second group migrated as a triplet around 72 kDa, 66 kDa and 62 kDa. Based on these sizes and on previous data from the testis (Hoeben et al., 1996) and in other systems (Birkedahl-Hansen, 1995; Stanton et al., 1998; Hernandez-Barrantes et al., 2000; Wang et al., 2000), these bands are likely to correspond to MMP-9 (the pro- and active forms) and MMP-2 (the pro-, intermediate and active forms), respectively. The temporal production of



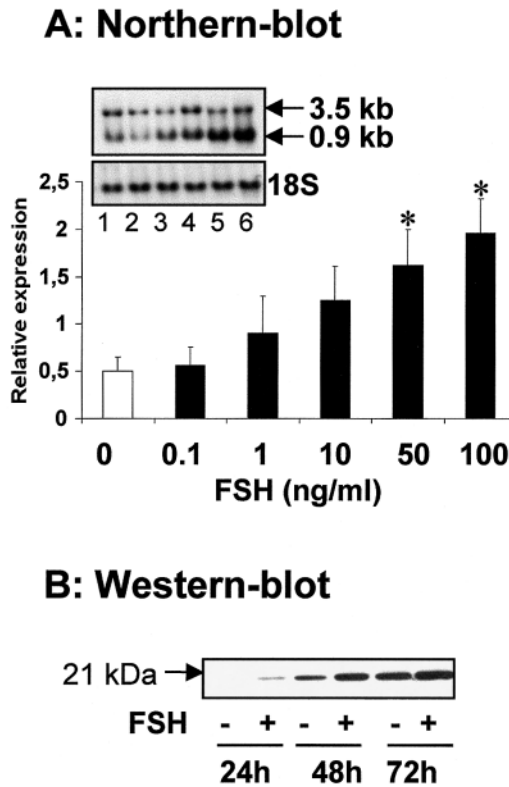
**Fig. 6.** FSH and MMP-2 gene expression. Sertoli cells were treated or not (control (c)) with FSH ( $50 \text{ ng ml}^{-1}$ ) or 8-bromo-AMP ( $10^{-3} \text{ M}$ ) for 48 hours, after which RNA was extracted and  $3 \mu\text{g}$  of PolyA(+) were analyzed by northern blot (A). Membranes were successively probed with MMP-2 and GAPDH. A representative blot is shown in the inset (lane 1, control; lane 2, FSH; lane 3, 8-bromo-cAMP). Data yielded by scanning three independent blots were normalized to the GAPDH signal and expressed as mean  $\pm$  s.e.m. of three dishes. *P* values against the control: \*, 0.05; \*\*, 0.01. (B) MMP-2 antigen levels were analyzed by western blot in the 72-hour culture media of Sertoli cells treated with or without FSH.



**Fig. 7.** FSH and MT1-MMP gene expression. Sertoli cells were treated or not (control (c)) with FSH ( $50 \text{ ng ml}^{-1}$ ) or 8-bromo-AMP ( $10^{-3} \text{ M}$ ) for 48 hours, after which RNA was extracted and  $3 \mu\text{g}$  of polyA(+) were analyzed by northern blot (A). Membranes were successively probed with MT1-MMP and GAPDH. A representative blot is shown. (B) MT1-MMP antigen levels were analyzed by western blot in the lysates of Sertoli cells treated with FSH (+) or not (-) for 24, 48 or 72 hours.

MMP-2 was next examined in culture in the presence or absence of FSH (Fig. 9B). In the absence of FSH, production of the 72 kDa proMMP-2 declined with the duration of culture, the intermediate 66 kDa band stayed constant in intensity and



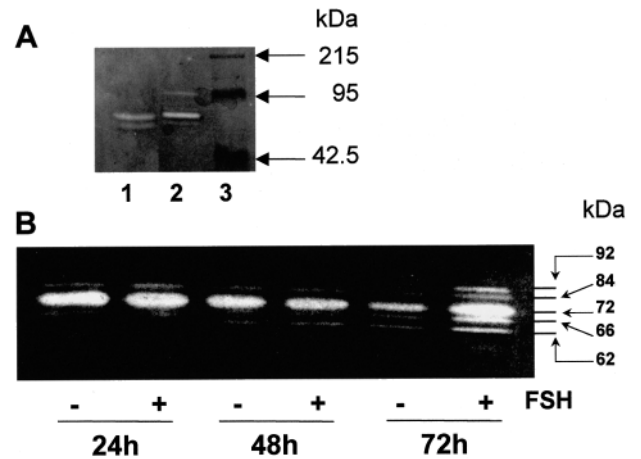


**Fig. 8.** FSH and TIMP-2 gene expression. Sertoli cells were treated or not treated with increasing doses of FSH ranging from 0.1 ng ml<sup>-1</sup> to 100 ng ml<sup>-1</sup>, after which total RNA was extracted and 20 µg were analyzed by northern blots (A). Blots were hybridized successively with the TIMP-2 and 18S probes, and quantified. A representative blot is shown in the inset (lanes 1-6 correspond to cultures exposed to 0-100 ng ml<sup>-1</sup> FSH for 48 hours). Data are expressed as mean±s.e.m. of triplicate dishes; control is lane 1, with no FSH. *P* value against the control: \*, 0.05. (B) TIMP-2 antigen levels were analyzed by western blot in the culture media of Sertoli cells treated with (+) or without (-) FSH for 24, 48 or 72 hours.

the active 62 kDa form first appeared at 48 hours and increased in intensity at 72 hours of culture. Addition of FSH (50 ng ml<sup>-1</sup>) resulted in an enhancement of the three MMP-2 lytic bands after 72 hours of treatment. Note that after 48 hours of treatment with FSH, a slight but reproducible (*n*=3) increase of the 62 kDa band could be observed. The lytic bands corresponding to MMP-9 were also increased by FSH after 72 hours of treatment.

**ProMMP-2 activation in Sertoli cells co-cultured with germ cells**

We also explored whether germ cells, which express MT1-MMP but not MMP-2 (Fig. 5B) and produce TIMP-2 at a level comparable to the Sertoli or peritubular cell production (Fig. 10A), could modulate Sertoli cell MMP-2 when in co-culture. Using western blots, we observed that addition of either a crude germ cell fraction (Fig. 10B, lane 2) or enriched fractions of pachytene spermatocytes (lane 3) or early spermatids (Fig. 10B, lane 4) dramatically stimulated MMP-2 antigen levels after 48 hours of coculture. No effects were detected after 24 hours of co-culture (not shown). Zymographic analysis revealed a dramatic increase in the intensity of the MMP-2 lytic



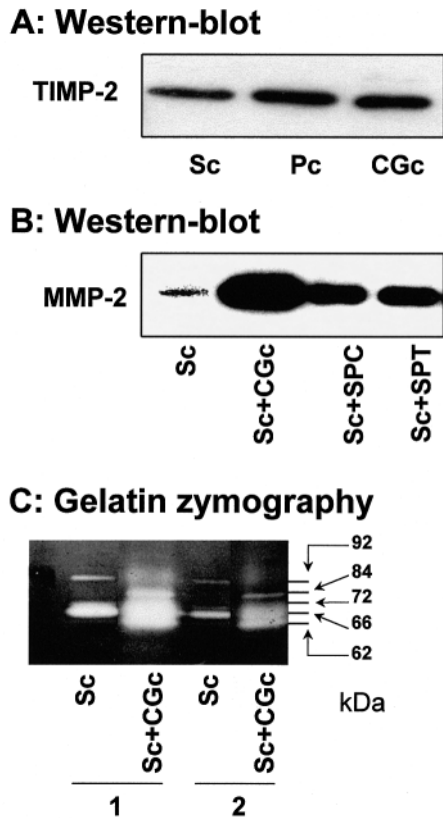
**Fig. 9.** Gelatin zymography of Sertoli cell culture media (A) and the action of FSH (B). (A) Purified human MMP-2 (lane 1, 1 ng) and *M<sub>r</sub>* standards (lane 3) were used to determine the size of the lytic bands present in a 3-day-old Sertoli cell culture media (lane 2). (B) Sertoli cells were treated with FSH (50 ng/ml) (+) or not treated (-) for 24, 48 and 72 hours, after which culture medium was collected and 40 µg of proteins were analyzed by gelatin zymography. The sizes of the lytic bands are indicated on the right.

bands, including the 62 kDa band in the 48-hour co-culture media compared to the time-matched control. In addition there was an almost total switch from the 92 kDa to the 84 kDa lytic band, meaning that germ cells also activated MMP-9 (Fig. 10C).

**DISCUSSION**

The results of this study clearly indicate that MT1-MMP is expressed within the testis from the late embryonic up to the adult stage, together with MMP-2 and TIMP-2. This suggests that TIMP-2 and MT1-MMP could cooperate to produce active MMP-2. We also provide evidence for the roles of FSH and of germ cells in that process. Developmental expression of MT1-MMP was first assessed by means of IHC. We observed a ubiquitous pattern of MT1-MMP immunoreactivity in cross-sections of prepubertal testes. Such a pattern fits with the widespread restructuring known to occur at that time, including the FSH-dependent growth of the seminiferous cords (Russell, 1980; Gondos and Berndston, 1993; Meehan et al., 2000). Later in development, when early spermatids differentiate and populate tubules, we observed a switch in the pattern of expression, and MT1-MMP immunoreactivity accumulated preferentially in the apical compartment of the seminiferous epithelium. In adult rats, in which spermatogenesis is cyclical, we found that MT1-MMP immunoreactivity was also cyclical. The strongest immunoreactivity was associated with stages VII-VIII, which are characterized by important remodeling events including translocation of germ cells through the blood-testis barrier, extrusion of sperm cells into the lumen and phagocytosis of the shed cytoplasts by Sertoli cells (Russell, 1980).

To confirm and extend these findings, we used isolated testicular cells (somatic and germ cells) and northern and western blotting. We confirmed the previous finding (Sato et al.,



**Fig. 10.** MMP-2 activation in Sertoli cells co-cultured with germ cells. (A) Western blot analysis of TIMP-2 in Sertoli cells (Sc), peritubular cells (Pc) and crude germ cells (CGc). 40  $\mu$ g of proteins were deposited per lane on 15% SDS-PAGE. (B) Western blot analysis of MMP-2 in Sertoli cells (Sc) and in Sertoli cells co-cultured with crude germ cells (CGc), spermatocytes (SPC) or spermatids (SPT). 40  $\mu$ g of proteins were analyzed per lane on 7.5% SDS-PAGE. (C) Sertoli/crude germ cells co-culture media were analyzed by gelatin zymography. 80  $\mu$ g of proteins were analyzed per lane in lanes 1 and 40  $\mu$ g in lanes 2. The sizes of the lytic bands are indicated on the right.

1994) of a single 4.5 kb transcript and the protein migrating as a doublet of 63–60 kDa. An additional band of 45 kDa was detected in germ cell extracts only. Such band might correspond to the 44 kDa processed MT1-MMP lacking the N-terminal portion of the molecule and the entire catalytic domain (Stanton et al., 1998; Maquoi et al., 2000; Hernandez-Barrantes et al., 2000). We indeed used an antibody directed against the hinge domain located in the C-terminal part of the molecule. However, the reason why Sertoli cells and peritubular cells did not exhibit the processed band is currently unknown.

Collectively, our data are consistent with the idea that MT1-MMP could be one of the proteases involved in the extensive remodeling that occurs during the prepubertal period and in the adult rat testis at specific stages of the seminiferous cycle. MT1-MMP has been described as an interstitial collagenase (Ohuchi et al., 1997). However, this protease is known for its ability to activate proMMP-2 (Sato et al., 1994) in the presence of TIMP-2 (Caterina et al., 2000; Wang et al., 2000). As we observed that MMP-2 and TIMP-2 were transcribed together with MT1-MMP throughout testicular development and in somatic cells, we speculated that one role for MT1-MMP in

testes might be to activate proMMP-2 in the presence of TIMP-2. To substantiate our hypothesis further, use was made of a model of cultured Sertoli cells treated with FSH or co-cultured with germ cells to mimic an immature (Griswold, 1995) or a mature (Le Magueresse and Jégou, 1988; Han et al., 1993; Wong et al., 2000) developmental period, respectively.

We first determined the kinetics of expression of the three partners (i.e. MMP-2, TIMP-2 and MT1-MMP) in Sertoli cells treated with FSH for up to 72 hours or not treated. Our data showed that MT1-MMP gene expression was high and constitutive (no fluctuation with time nor in the presence of FSH), whereas TIMP-2 protein levels increased with time and were significantly enhanced by FSH. Therefore the ratio of TIMP-2 to MT1-MMP increased as a function of time during the culture period analyzed (72 hours). Interestingly, in FSH-treated cultures at 48 hours and in control cultures at 72 hours, TIMP-2 antigen levels were more or less the same. A careful examination of the zymograms at these time points revealed the appearance of a distinct band at 62 kDa, corresponding to the active form of MMP-2. This 62 kDa lytic band increased further at 72 hours in FSH-treated cells, as did TIMP-2 antigen levels. These data are consistent with the literature documenting that proMMP-2 activation is highly sensitive to the stoichiometric ratios of TIMP-2 to MT1-MMP (Strongin et al., 1995; Shofuda et al., 1998). Whether TIMP-2 becomes inhibitory at a particular high concentration in the testis, as shown elsewhere (Hernandez-Barrantes et al., 2000; Wang et al., 2000), remains to be determined. Finally, we demonstrated that FSH not only promoted activation of proMMP-2 but also augmented MMP-2 expression at the mRNA and protein levels.

In a second part of this study, we examined germ cell control of Sertoli cell proMMP-2 activation. Spermatogenesis is known to depend on intricate and intimate relationships between Sertoli cells and germ cells (Jégou, 1993; Le Magueresse-Battistoni, 1993; Parvinen, 1993; Sharpe, 1994). We thus predicted that MMP-2 of Sertoli cell origin would aid the progressive movement of germ cells towards the tubular lumen during their differentiation into sperm cells. In turn, germ cells would control the rate and extent of proMMP-2 activation. Our prediction was also based on the finding of a processed MT1-MMP form in germ cells. Indeed, this form has been previously associated with binding to TIMP-2 and proMMP-2 activation (Hernandez-Barrantes et al., 2000). We thus examined whether germ cells that do not express MMP-2 (Fritz et al., 1993; this study) expressed TIMP-2. We found, in contrast to a previous RT-PCR study (Grima et al., 1996) that germ cells contained TIMP-2 transcripts and produced the 21 kDa TIMP-2 protein. At present, we cannot explain this discrepancy with the previous report.

When Sertoli cells and germ cells were co-cultured, we detected a dramatic increase in the accumulation of the MMP-2 antigen. The enhancement in MMP-2 production was even more evident with a crude germ cell suspension than with either pachytene spermatocytes or early spermatids. This probably resulted from the fact that the crude fraction contains elongated spermatids, which we found strongly immunoreactive for MT1-MMP. Using gelatin zymography, we extended these data and showed that germ cells could also enhance the intensity of the 62 kDa lytic band, corresponding to the active form of MMP-2. The second gelatinase (probably MMP-9) was also activated by germ cells.



The fact that MMP-2 levels declined for 72 hours from the time of plating was consistent with the time-dependent decrease in the number of contaminating germ cells in the Sertoli cell cultures and with the enhancement in MMP-2 production in co-culture. By contrast, we demonstrated that germ cells expressed TIMP-2 and MT1-MMP, and that TIMP-2 levels increased with time in culture whereas MT1-MMP levels did not change. We also observed that the germ cell specific MT1-MMP form was not present in the fresh Sertoli cell preparation, although it was contaminated with 10-15% germ cells. It appears therefore that the contaminating germ cells present in the fresh preparations of Sertoli cells were either not sufficient in number to reproduce the co-culture effects or that the contaminating spermatogonia and early spermatocytes do not influence the Sertoli cell MMP-2 machinery as more developed germ cells do. The possibility of isolating highly enriched populations of spermatogonia for co-culture with Sertoli cells should clarify the point.

Whether germ cell effects require cell-cell contact or whether they can occur with germ cell conditioned media will be addressed in future studies. Another issue to be resolved is identification of the possible intraepithelial substrates for MMPs. MMPs are known to cleave not only interstitial collagens and basement membrane components but also non-matrix components, with significant biological ramifications (Nagase and Woessner, 1999). Indeed, MMPs, or a cascade of proteolytic events activated by MMPs, can lead to cleavage and release of a variety of active molecules from cell surfaces, including bFGF. This is secreted by germ cells (Han et al., 1993) and is a potent activator of MMPs. Additionally, the junctional proteins that compose the blood-testis barrier (Byers et al., 1993) are potential targets for MMPs, as shown in other systems for integrin (Von Bredow et al., 1997), occludin (Wachtel et al., 1999) and cadherin (Steinhusen et al., 2001). Finally, it has been demonstrated that protease-sensitive elements hold spermatids and Sertoli cells together (Russell, 1980), but the nature of these protease-sensitive elements is at yet unknown.

In conclusion, we demonstrated that FSH and germ cells could enhance MMP-2 gene expression and levels of active MMP-2, with germ cells having a substantially greater effect than FSH under the conditions employed. Active MMP-2 might then be involved in the extensive remodeling that occurs within the seminiferous epithelium throughout development. MMP-2 is certainly not the sole protease involved. Mice with a single deficiency in various proteases and antiproteases including MMP-2 and TIMP-2 are overtly normal, viable and fertile. MT1-MMP-deficient mice, which displayed severe runting, wasting and increased mortality, can still reproduce (Carmeliet and Collen, 1998; Holmbeck et al., 1999). Serine- and cysteine-proteases are thought to be involved in the germ cell migratory route (Fritz et al., 1993; Mruk et al., 1997; Le Magueresse-Battistoni et al., 1998; Sigillo et al., 1999; Wong et al., 2000). Finally, four other MT-MMPs can activate proMMP-2 in a TIMP-2-sensitive fashion (Murphy et al., 1999; English et al., 2000). This suggests that the different proteases and antiproteases in the testis exert overlapping functions.

A challenge for the future will be to identify the full complement of proteases and their regulatory mechanisms. This will enable the design of additional studies to define precisely the role and relative importance of each in the

complex steps of spermatogenesis. Then, the phenotypic effects in gene knockout experiments can be interpreted with knowledge of their integrated roles and potential for compensatory action.

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