Glial cell line-derived neurotrophic factor is constitutively produced by human testicular peritubular cells and may contribute to the spermatogonial stem cell niche in man

K. Spinnler 1, F.M. Köhn 2, U. Schwarzer 3, and A. Mayerhofer 1,*

1Institute for Cell Biology, Anatomy and Center for Integrated Protein Science Munich (CIPSM), Ludwig Maximilian University, Biedersteiner Strasse 29, D-80802 Munich, Germany 2Andrologicum, Burgstrasse 7, D-80331 Munich, Germany 3Practice for Urology and Andrology, D-85356 Freising, Germany

*Correspondence address. Institute for Cell Biology, Anatomy, Center for Integrated Protein Science Munich (CIPSM), University of Munich, Biedersteiner Strasse 29, D-80802 Munich, Germany. Tel: +49-89-4140-3150; Fax: +49-89-397035; E-mail: mayerhofer@lrz.uni-muenchen.de

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Introduction

Peritubular cells are very thin, spindle-like myofibroblastic cells, which in rodents form a single layer around the seminiferous tubule. The peritubular wall of human testes, in contrast, is composed of several layers of peritubular cells (see Schell et al., 2010). To date, human peritubular cells have been rather ill-explored in many respects, possibly owing to the lack of adequate cellular models. We recently developed an appropriate model for the study of human testicular peritubular cells (HTPCs). They were isolated from testes of men with obstructive azoospermia (but normal morphology), and also from fibrotically remodelled human testes (HTPC-Fs). Results of our previous studies have provided insights not only into the contractile nature of HTPCs (Schell et al., 2010) but also into the repertoire of secretory products: the latter includes a prototype neurotrophin, namely nerve growth factor (NGF; Schell et al., 2008). In testes of infertile men, mast cells and macrophages are significantly increased, especially within the peritubular cell compartment (Meineke et al., 2000; Frungieri et al., 2002a, b). When we examined whether interactions may occur, we found that tumour necrosis factor-α (TNF-α), i.e. a product of mast
cells and macrophages, enhanced the NGF secretion (Schell et al., 2008).

Spermatogonial stem cells (SSCs) represent an adult stem cell population residing within the testis and provide the foundation of life-long spermatogenesis (Hofmann, 2008; Oatley and Brinster, 2008; Maki et al., 2009; Mizrahi et al., 2010). SSCs reside along the basement membrane of the seminiferous tubule and it is thought that self-renewal and differentiation of the SSCs are regulated by systemic and by local factors (Yoshida et al., 2007; Hofmann, 2008; Maki et al., 2009): the latter implies a crucial importance of surrounding cells for the stem cell niche. In mammalian tests, Sertoli cells are thought to be the major contributor to the SSC niche. Whether and, if so, to what degree contributions by other testicular somatic cells, including peritubular myoid cells, Leydig cells and the vascular network of the testis, may occur is not known (Yoshida et al., 2007; Oatley and Brinster, 2008).

How do Sertoli cells control SSCs? Available data from in vivo and in vitro studies clearly indicate that a secreted factor, glial cell line-derived neurotrophic factor (GDNF), is a key player responsible for maintaining SSCs by regulating their self-renewal (see for example Meng et al., 2000; Naughton et al., 2006; Oatley and Brinster, 2008). GDNF is a member of the transforming growth factor-β superfamily. Sertoli cell production of GDNF is enhanced by FSH, at least in cultures of cells from post-natal mice (Tadakoro et al., 2002). Interestingly, it was reported that TNF-α also increased the GDNF production by Sertoli cells (Simon et al., 2007).

GDNF has been found in testes of different species, including mouse and rat (Kubota et al., 2004; Bugaw et al., 2005; Ryu et al., 2005) bovine (Oatley et al., 2004) and in primates (rhesus monkey; Hermann et al., 2007; Maki et al., 2009). That GDNF is also present in human testes, is likely, but to our knowledge, this has not been demonstrated: it can be assumed because recent studies demonstrated that SSCs of mice (Hofmann, 2008), primates (Maki et al., 2009) and human (Wu et al., 2009) express a receptor for GDNF, GFR-α1. The glycosylphosphatidylinositol-anchored binding molecule GFR-α1 and the transmembrane tyrosine kinase molecule rearranged in transformation are the functional receptor complex for GDNF (Airaksinen and Saarma, 2002; Oatley and Brinster, 2008). It has been postulated that GFR-α1 expression can be taken as a good marker for the identification of SSCs in monkey and other species (Maki et al., 2009), including human (Gassei et al., 2010).

The expression of GFR-α1 in SSC, located at the basal part of the seminiferous tubule adjacent to the peritubular cells, raises the question of whether testicular peritubular cells may secrete the ligand. Like Sertoli cells, testicular peritubular cells are also in close proximity to SSCs and indeed their possible contribution to the SSC niche has been discussed (Hofmann, 2008; Oatley and Brinster, 2008). To our knowledge, however, this possibility has not yet been fully addressed. A close examination of immunohistochemical staining for GDNF in newborn rat testes, presented in a previous study, may lend some support to this assumption (see Katoh-Semba et al., 2007).

These observations and the ability of peritubular cells to produce NGF led us to hypothesize that human peritubular cells may also produce other factors, namely GDNF, and that this may be regulated by products of mast cells or macrophages. The recently established culture method for human peritubular cells allows us, for the first time, to directly and unambiguously test this hypothesis.

### Materials and Methods

#### Isolation of HTPC/-Fs, cell culture and testicular samples

Isolation of HTPC/-Fs was performed as previously described (Albrecht et al., 2006; Schell et al., 2008, 2010). All participants gave a written informed consent. The local ethics committee has approved the study and the use of the cells. Patients displayed either normal spermatogenesis or impaired spermatogenesis and testicular fibrosis based on histological analyses. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) + 10% foetal calf serum (FCS; both from PAA GmbH, Colbe, Germany). Treatment with 0.5 IU FSH (Sigma Aldrich, Deisenhoven, Germany), 10 IU hCG (Sigma Aldrich), 50 μM forskolin (Sigma Aldrich), 5 ng/ml human recombinant TNF-α (Sigma Aldrich) or 100 ng/ml human recombinant skin β-tryptase (Promega, Mannheim, Germany) was performed as detailed below. For all experiments, freshly isolated or cryopreserved cells from passages 4 to 13 were used. For the immunohistochemistry, testicular samples from fertile and infertile men obtained from the archives of the Department of Dermatology and Allergy were used (fixed and embedded in paraffin; see Meineke et al., 2000; Frungieri et al., 2002a,b; Schell et al., 2010).

#### Immunohistochemistry

Immunohistochemistry was performed as described elsewhere (Mayerhofer et al., 1999; Albrecht et al., 2006), using cells from at least 10 different patients for the immunostaining. A polyclonal antibody against GFR-α1 (Abcam, Cambridge, UK: 1:200) was used for the study. Controls consisted of incubation with non-immune normal serum (rabbit 1:2000) instead of specific antibodies or omission of the primary antibody.

#### Laser microdissection

Human testicular biopsies embedded in paraffin were cut into sections and mounted on a polyethylene naphthalene membrane, as previously described (Frungieri et al., 2002a,b). Dissection of three cellular compartments of the testis (tubular, peritubular and interstitial) was performed using a laser microdissection (LMD) device (P.A.L.M. GmbH Mikrolaser Technologie, Bernried, Germany). The samples obtained were kept frozen (−80°C) until the RT–PCR assays were performed. In order to ensure that the sections contained suitable RNA for this procedure, the whole section was also scraped from the slide, and RNA was extracted using the RNeasy FFPE kit (QIAEN GmbH, Hilden, Germany) and subjected to RT–PCR using oligo-dT primers.

#### Isolation of RNA and RT–PCR for cells in culture

Cells from at least six patients per experiment were grown to sub-confluence, washed twice with phosphate-buffered saline (PBS) and suspended in RLT buffer (QIAEN GmbH) containing 1% β-mercaptoethanol (according to the manufacturer’s protocol). Isolation of RNA was performed with the QIAEN RNaseq kit (QIAEN GmbH, Hilden, Germany) and subjected to RT–PCR using oligo-dT primers.
Table I Information about oligonucleotide primers used for RT-PCR experiments and PCR conditions.

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Primer-sequence (5′–3′)</th>
<th>Amplicon size (bp)</th>
<th>Annealing temperature (°C)/cycles</th>
<th>GenBank accession no.</th>
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<td>TGG CAG TGC TTC CTA GAA GAG</td>
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<td></td>
<td></td>
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<tr>
<td>Semi-nested GDNF</td>
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<td>138</td>
<td></td>
<td></td>
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<tr>
<td>GFR-α1 Forward</td>
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<td>160</td>
<td>56/35</td>
<td>NM_005264</td>
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<tr>
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<td>TTT TTG CAA ATG TCG TCG AG</td>
<td></td>
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<td></td>
</tr>
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<td>376</td>
<td>52/35</td>
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<td></td>
</tr>
<tr>
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<tr>
<td>TNFR2 Forward</td>
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<tr>
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<tr>
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<tr>
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</table>

GDNF, glial cell line-derived neurotrophic factor; GFR-α1, GDNF family co-receptor-α1; PAR-2, receptor for tryptase; TNFR1/2, receptors for tumour necrosis factor (TNF); LHR, LH-receptor; FSHR, FSH-receptor.

Primer sequences and PCR details are listed in Table I. Identities of PCR products were verified by sequencing (Frungieri et al., 2002a, b).

cAMP assay

Cells were seeded (10^5/well) on a six-well plate (Nunc, Langenselbold, Germany) and stimulated for 30 min with hCG (10 IU; Sigma), porcine FSH (0.5 IU; Sigma) or forskolin (50 μM; Sigma) in DMEM without FCS: the concentrations and preparations were chosen to match those which effectively alter cAMP levels in rat or human granulosa cells (see Grosse et al., 2000; Sommersberg et al., 2000; Mayerhofer et al., 2006). After the incubation, the cells were washed once with 10 mM PBS and then stored at −80°C until use. Measurements of the cAMP concentrations in cellular lysates of HTPC/-Fs were performed using a commercial cAMP Assay kit (R&D Systems, Inc., Minneapolis, MN, USA). Briefly, reagents, samples and standards were prepared according to the manufacturer’s instructions (assay sensitivity: minimum detection 0.58–3.00 pmol/ml cAMP). Absorbance was measured at 450 nm with the correction wavelength set at 544 nm using a Fluostar photometer (Fluostar Optima; BMG Labtech, Offenburg, Germany). All measurements were performed at least in triplicate.

GDNF enzyme-linked immunosorbent assay

Cells were cultured, and stimulated with TNF-α (5 ng/ml) or tryptase (100 ng/ml) in DMEM containing 5% FCS. These concentrations were chosen because they effectively stimulate the NGF secretion or induce increases in intracellular calcium levels (Albrecht et al., 2006; Schell et al., 2008). Supernatants were collected after 24 and 72 h. All samples were stored at −20°C until use. Enzyme-linked immunosorbent assay (ELISA) measurements of secreted GDNF levels were performed using the GDNF E\textsubscript{max} immunoAssay System (Promega, Madison, WI, USA) according to the manufacturer’s protocol (assay sensitivity: minimum detection 15.6 pg/ml GDNF). Briefly, Nunc maxi-sorp 96-well plates (Nunc) were coated with polyclonal GDNF antibodies overnight. The following day, samples were added and captured by a monoclonal antibody against GDNF, followed by signal detection. Absorbance was measured at 450 nm using a Fluostar photometer (Fluostar Optima; BMG Labtech). All measurements were performed at least in duplicate with cells from three to seven different patients and were normalized to quantity of cellular protein (Frungieri et al., 2002a, b; Schell et al., 2008).

Statistical analyses

Data analysis and statistics were performed using PRISM 4.0 (Graph-Pad Software, Inc., San Diego, CA, USA). Statistical analysis was performed using the Mann–Whitney test. Data shown are the mean ± SEM. Statistical significance was set at P < 0.05.
Cells expressing GFR-α1 in human testis are in close proximity to the cells of the peritubular wall

RT–PCR of commercial human testes cDNA followed by sequencing indicated that GFR-α1 is expressed in human testes, as expected (Fig. 1A). Sections of human testicular biopsies (n = 10) displaying normal, as well as impaired spermatogenesis, contain GFR-α1-positive spermatogonial cells in the basal compartment of the seminiferous tubules bordering on the peritubular wall (Fig. 1B). Controls with non-immune normal serum instead of specific antibodies or omission of the primary antibody yielded negative results.

Identification of GDNF expression in cellular compartments of the human testis

The RT–PCR analysis followed by sequencing of samples obtained by LMD showed that not only cells of the tubular compartment (i.e. Sertoli and germ cells, as expected) but also cells forming the peritubular wall express GDNF (Fig. 2A–C). GDNF was not found in interstitial cellular compartments. Evidence for GDNF mRNA was also found in sections from whole testes, which had been scraped from the slide.

Characterization of HTPCs and HTPC-Fs: identification of GDNF and receptors

RT–PCR experiments revealed that cultured HTPCs (n = 6 patients), as well as HTPC-Fs (n = 8 patients), possess GDNF mRNA, which was also found in human commercial cDNA testes (Fig. 3A). The identity of GDNF was verified by sequencing.

Furthermore, HTPCs, as expected, also express PAR-2, i.e. the receptor for mast cell tryptase, and TNFR1/2. Functionality of these receptors was examined by studying initial signalling, namely calcium influx or phosphorylation of MAP-kinases (see Albrecht et al., 2006; Schell et al., 2008). Importantly, HTPCs and HTPC-Fs both lacked detectable expression of FSH- and LH-receptors (Fig. 3A), indicating that neither Sertoli cells nor Leydig cells contaminated the cellular preparations. The latter point is corroborated by experiments (Fig. 3B) in which FSH and HCG failed to stimulate cAMP in HTPCs at concentrations that are effective in granulosa cells (see Grosse et al., 2000; Sommersberg et al., 2000; Mayerhofer et al., 2006). However, forskolin, used as the positive control, strongly increased cAMP.

GDNF secretion by isolated HTPCs and HTPC-Fs is constitutive and is not affected by TNF-α or tryptase

To investigate whether HTPCs and HTPC-Fs secrete GDNF, we measured the GDNF levels in the culture medium using an ELISA. We found that with time (24–72 h) GDNF accumulated in the cell culture medium under basal conditions (Fig. 4A) indicating constitutive expression and secretion. Levels of 300–400 pg/mg cellular protein were found after 72 h. No difference between the levels of secreted GDNF was apparent in HTPCs versus HTPC-Fs. When TNF-α (5 ng/ml) or tryptase (100 ng/ml) were added to the cultures for 24 h (not shown) and 72 h, GDNF levels in the culture medium did not change (Fig. 4B and C).

Discussion

Our study provides a clear evidence that peritubular cells of the human testis are a source of GDNF. As GFR-α1-positive cells are found close to the peritubular wall, we propose that in human testes, peritubular wall cells contribute to the SSC niche.

It was previously shown that Sertoli cells are the main producers of testicular GDNF, e.g. in mice (Meng et al., 2000; Katoh-Semba et al., 2007; Hofmann, 2008). As GDNF mRNA was found in the human tubular compartment using our LMD/RT–PCR approach, we assume that human Sertoli cells are producing this factor. Although Sertoli cells are certainly present in all samples analysed (see Fig. 2A), exact excision of complexly shaped Sertoli cells by this method is unfortunately not possible. Hence, we did not attempt to perform LMD/RT–PCR experiments to distinguish between Sertoli cells and germ cells.

The lack of antibodies suitable for the use on human paraffin-embedded sections kept us from localizing GDNF by immunohistochemistry in testicular sections. A previous study in rat (Katoh-Semba et al., 2007) provided evidence for the Sertoli cell localization of GDNF. Interestingly, GDNF was present most likely also in...
Peritubular cells of newborn rat testes (see Fig. 4 in Katoh-Semba et al., 2007), a result that was not discussed further by those authors. In the present study, we turned our attention to these cells of the tubular wall, which in contrast to interstitial cells, at least in our LMD/RT–PCR study, also expressed GDNF mRNA. Peritubular cells of the human testis, which build this wall, have not been extensively studied, but the use of primary cultures of HTPCs has provided insights into their nature. Thus, peritubular cells express characteristics of smooth muscle (including smooth muscle actin and others; for details see Albrecht et al., 2006; Schell et al., 2010) and importantly, they are a source of several important signalling molecules, e.g. interleukin 6, monocyte chemo-attractant protein 1 and the prototype neurotrophin, NGF. Indeed, peritubular cells produce large amounts of NGF under basal conditions in a constitutive fashion and the NGF secretion can be enhanced by TNF-α (Schell et al., 2008). We now find that they also produce and secrete the neurotrophin GDNF. Production in both HTPCs and HTPC-Fs occurred in a constitutive fashion. Hence, the LMD/RT–PCR study, together with the cellular studies, allows us to conclude that human peritubular cells in vivo and in vitro express and secrete GDNF.

Furthermore, the levels of GDNF constitutively produced by HTPCs were similar to the levels secreted by HTPC-Fs. HTPC-Fs are derived from testes of patients with impaired spermatogenesis and testicular peritubular fibrotic remodelling. In our LMD/RT–PCR experiments, we have also used peritubular walls of normal, and fibrotically remodelled, human seminiferous tubules and found GDNF expression irrespective of the state of spermatogenesis. Hence, it is possible that peritubular cells in health and disease may not differ with respect to their ability to produce GDNF, but this point clearly requires further investigation.

HTPCs do not express FSH- or LH-receptors, but possess receptors for tryptase (PAR-2) and for TNF-α (TNFR1/2; see Albrecht et al., 2006; Schell et al., 2008 and the present study): the same is true for HTPC-Fs (the present study). Therefore, peritubular cells are likely to be subjected to regulation by paracrine factors originating from immune cells. This point is of considerable interest, because the numbers of mast cells and macrophages, i.e. sources of these signalling molecules, are significantly increased in patients with impaired spermatogenesis, and are found in close proximity to the cells of the peritubular wall (Meineke et al., 2000; Frungieri et al., 2002a, b; Spiess et al., 2007). Hence, we previously examined the possible roles of these immune cell-derived factors on HTPCs. We found that TNF-α strongly increased the NGF production and secretion by HTPCs (Schell et al., 2008). In contrast, as documented in the present study,
TNF-α and tryptase failed to affect GDNF secretion. This result, obtained in HTPCs and HTPC-Fs, as well as whole testis samples, (—), sample without cDNA. HTPCs and HTPC-Fs express GDNF. HTPCs and HTPC-Fs lack FSH-receptor (FSHR) or LH-receptor (LHR), but express the receptor for mast cell tryptase, PAR-2, and the tumour necrosis factor (TNF) receptors, TNFR1/2. (B) Absence of functional LHRs and FSHRs, linked to cAMP, in HTPC-Fs: measurement of cAMP levels showed that only forskolin, but not KCG or FSH affected cAMP levels (mean ± SEM, n = 3). Note that cAMP levels in the forskolin group of this experiment were beyond the upper limit of the assay and are therefore not given as exact value.

In the present study, we also confirmed localization of the cellular targets for GDNF in sections of human testes employed for the LMD experiments. GFR-α1 is an accepted SSC marker and part of the signalling complex in SSC in all species examined, including human and non-human primates (Maki et al., 2009; Wu et al., 2009; Gassei et al., 2010). Immunoreactive GFR-α1 is detected (as expected) in cells resembling spermatogonial cells of the human testis, which resides in the basal compartment of the seminiferous tubules, i.e. in a location which is in close proximity to both the peritubular wall and to Sertoli cells. We conclude that this anatomical
position indicates that a GDNF–GFRα1 system exists in the human testis and involves testicular peritubular cells, which are newly recognized producers of GDNF. Secretion of GDNF occurs in a constitutive manner and is not affected by the immune cell-derived factors, TNF-α and tryptase, at least in vitro. Testicular peritubular cells therefore appear to complement the actions of Sertoli cells in supporting the human SSC niche by providing GDNF.

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