Secretome analysis of testicular peritubular cells as a clue for solving the puzzle of the human spermatogonial stem cell niche

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Abbreviations

α-SMA	α-smooth muscle actin
ANGPTL2	angiopoietin-related protein 2
BMP4	bone morphogenetic protein 4
CCL2	C-C motif chemokine 2
CDH13	cadherin-13
cGKI	cGMP-dependent protein kinase 1
CSF-1	colony stimulating factor 1
CSF1R	colony stimulating factor 1 receptor
CTGF	connective tissue growth factor
CXCL12	stromal cell-derived factor 1
CXCR4	C-X-C chemokine receptor type 4
DCN	decorin
DFG	Deutsche Forschungsgemeinschaft
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	endoplasmatic reticulum
FBXW7	F-box/WD repeat-containing protein 7
FGF2	fibroblast growth factor
FLBN2	fibulin-2
FSH	follicle-stimulating hormone
FST	follistatin
GATA4	GATA-binding protein 4
GDNF	glial cell line-derived neurotrophic factor
GFRα-1	GDNF family receptor α1
GO	Gene Ontology
GPER	G protein-coupled oestrogen receptor
HMGB-1	high-mobility group box 1
HSD3B	3 beta-hydroxysteroid dehydrogenase
HTPC	human testicular peritubular cell
HTPC-F	human testicular peritubular cell fibrosis
HUVEC	human umbilical vein endothelial cell
ICAT	isotope-coded affinity tags
IGF-1	insulin-like growth factor 1
IGF-1R	insulin-like growth factor 1 receptor

IL-1/6	interleukin-1/6
INHBA	inhibin beta A
iTRAQ	isobaric tags for relative and absolute quantitation
LAMA1/2/4	laminin alpha-1/2/4
LAMC1	laminin gamma-1
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LIFR	leukemia inhibitory factor receptor
LTBP-1	latent-transforming growth factor beta-binding protein 1
MCP-1	monocyte chemoattractant protein-1
MS	mass spectrometry
MYH11	myosin heavy chain 11
NGF	nerve growth factor
NR5A1	nuclear receptor subfamily 5, group A, member 1
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDGFRA	platelet-derived growth factor receptor-A
PEDF	pigment epithelium-derived factor
POU5F1	POU domain class 5 transcription factor 1
PTX3	pentaxin 3
SASP	senescence-associated secretory phenotype
SA-β-GAL	senescence-associated β-galactosidase
SCF	stem cell factor
SCX	peptide strong cation exchange
SILAC	stable isotope labeling with amino acids in cell culture
SMC	smooth muscle cell
SPARC	secreted protein acidic and rich in cysteine
SSC	spermatogonial stem cell
STAR	steroidogenic acute regulatory protein
TESE	testicular sperm extraction
TGF-β	transforming growth factor beta
TMT	tandem mass tags
TNF-α	tumor necrosis factor-alpha
TSP-1/2/3	thrombospondin 1/2/3
VEGF-C	vascular endothelial growth factor C
VEGFR	vascular endothelial growth factor receptor
WHO	World Health Organization

1   Introduction

"Infertility is the inability of a sexually active, non-contracepting couple to achieve spontaneous pregnancy in one year" (World Health Organization, 2000)

World Health Organization (WHO)

Approximately 15 % of all couples are unable to achieve pregnancy, whereby in about half of the individual cases, the unfulfilled desire to have children is attributed to the male factor (Male Infertility Best Practice Policy Committee of the American Urological Association and Practice Committee of the American Society for Reproductive Medicine, 2006; Patel and Niederberger, 2011)

Prerequisites for fertility in man are intact spermatogenesis, sperm transport and accessory sex gland function. In this context, male factor infertility is normally accompanied with reduced sperm counts, frequently combined with reduced semen quality parameters (Nieschlag et al., 2010). Diverse factors can cause male infertility, ranging from hormonal imbalances, congenital abnormalities, infections, genetic disorders, sexual dysfunction (Cates et al., 1985; O'Flynn O'Brien et al., 2010) to emerging influences through environmental factors, obesity and excessive stress (de Kretser, 1996; Feichtinger, 1991; Giwercman and Bonde, 1998; Shukla et al., 2014).

For a diagnostic evaluation of male infertility, the semen analysis is a central assessment and should follow the WHO Laboratory Manual for the Examination and Processing of Human Semen (World Health Organization, 2010). The semen analysis provides information about the fluid volume, total sperm number, sperm vitality, motility and morphology.

Complementary, hormonal determinations, genetic studies or a testicular biopsy can be indispensable (Jungwirth et al., 2015; World Health Organization, 2000). In case of azoospermia, i.e. the full absence of sperms in the ejaculate, a testicular biopsy can be used for sperm retrieval techniques (testicular sperm extraction, TESE) (Berookhim and Schlegel, 2014; Schwarzer et al., 2003). Thereby, a histological investigation can be a

predictive tool to discriminate between different degrees of spermatogenic disorder. Cases of non-obstructive azoospermia range from complete atrophy of seminiferous tubules, which are only lined by Sertoli cells (Sertoli cell-only syndrome) (Anniballo et al., 2011) through "mixed atrophy" with a simultaneous occurrence of both tubules containing germ cells and degenerated tubules (Sigg and Hedinger, 1981) to germ cell arrest, an interruption of spermatogenesis without further production of spermatozoa (Gat et al., 2010).

Although recent advances in andrological diagnosis have been achieved, in around 40 % of men with disturbed sperm production, no defined etiological factor can be assigned (de Kretser, 1997). Thus, a greater understanding of the complex mechanisms of spermatogenesis remains necessary.

The present work aims to explore the role of peritubular cells surrounding seminiferous tubules in controlling fundamental testicular functions. Peritubular cells have direct borders with spermatogonial stem cells (SSCs), which form the basis of lifelong spermatogenesis, and are considered to contribute to the SSC niche microenvironment via secreted factors. Therefore, a comprehensive proteomic characterization of the secretome of cultured human testicular peritubular cells (HTPCs) was performed. Nano-liquid chromatography tandem mass spectrometry (nano-LC-MS/MS) combined with extensive data mining and bioinformatics was used to provide detailed insights into the secretome of HTPCs, and moreover into the SSC niche in man.

2   Literature review

2.1   Spermatogenesis

The process of spermatogenesis is continuous throughout the adult life and comprises all steps from the mitotic division of spermatogonial stem cells (SSCs) to the formation of mature spermatozoa. The initial phase of spermatogenesis takes place in the basal region of the seminiferous tubules of the testis and regulates the balance of self-renewal and differentiation (Figure 1).

In humans, SSCs are divided into resting spermatogonia (A_{dark}) and active spermatogonia (A_{pale}) (Oatley and Brinster, 2012; Schlatt and Weinbauer, 1994). The active type A spermatogonia can either differentiate into type B spermatogonia or, in this steady-state spermatogenesis, perform a self-renewal process to maintain the SSC population (Ebata et al., 2011; Oatley and Brinster, 2012). The detailed mechanisms of SSC division in mammalian testes are poorly understood and remain a contentious issue (Oatley and Brinster, 2008).

The type B spermatogonia divide mitotically into primary spermatocytes which undergo meiosis and produce secondary spermatids. In the second meiotic division, secondary spermatocytes divide into haploid spermatids. During spermiogenesis, the final stage of spermatogenesis, spermatids are transformed in a multi-step process to mature spermatozoa. Mature spermatozoa are finally released from the germinal epithelium (spermiation) and transported through the lumen of the seminiferous tubule to the rete testis and further to the epididymis.

In man, the entire process of spermatogenesis takes approximately 10 weeks, followed by additional two weeks during post-testicular maturation of spermatozoa in the epididymis (Amann, 2008; Nieschlag et al., 2009).
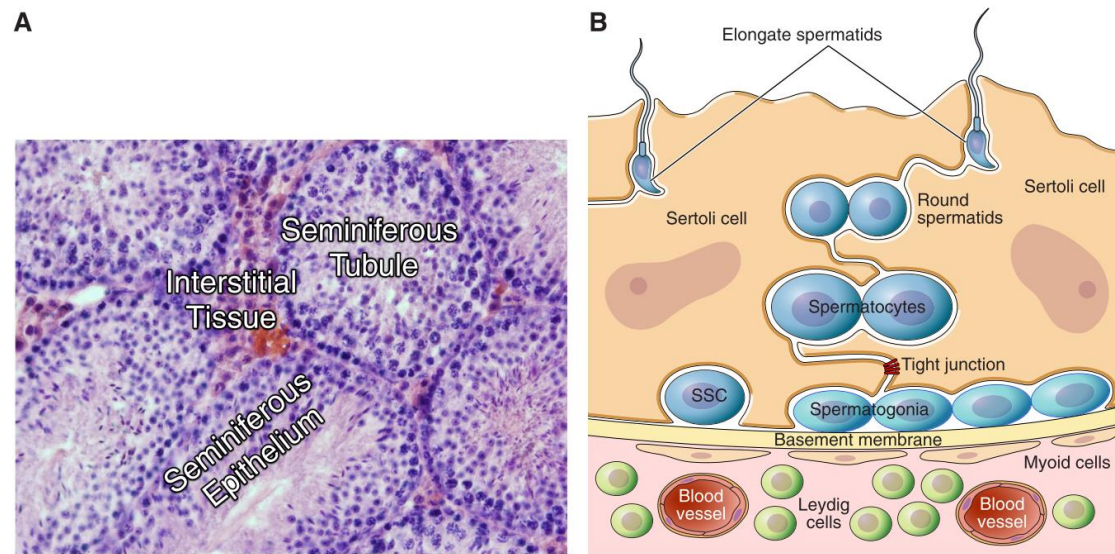
Figure 1. Spermatogenesis in mammals. (A) Cross-section through seminiferous tubules from an adult mouse testis. (B) Schematic overview of the testicular compartments showing seminiferous epithelium and interstitial tissue. During the formation of elongate spermatids from undifferentiated spermatogonia, germ cells gradually move from a basal compartment to the adluminal compartment of the seminiferous epithelium. At all stages of maturation, they are accompanied by Sertoli cells. Peritubular myoid cells form the wall of the seminiferous tubules and lay on the outside of the basement membrane. Leydig cells are located in the interstitial tissue and are embedded in the vasculature network. Modified from Oatley and Brinster (2012).

2.2   Spermatogonial stem cell niche

In 1978, Schofield proposed a concept, where stem cells reside in specific anatomic locations, 'niches', which are essential for the regulation of stem cell behavior (Schofield, 1978). Over the last decades, the niche theory has been extended to a variety of self-renewing tissues, including bone marrow (Chen et al., 2016a; Kiel et al., 2005; Schofield, 1978; Zhang et al., 2003), brain (Doetsch et al., 1999; Shen et al., 2004; Song et al., 2012), intestine (Barker et al., 2007; Farin et al., 2016; Sailaja et al., 2016), skeletal muscle (Sherwood et al., 2004; Yin et al., 2013), skin (Blanpain and Fuchs, 2006; Fuchs and Segre, 2000; Hsu et al., 2014) and testis (Chen et al., 2005; de Rooij, 2009; Kanatsu-Shinohara et al., 2012; Oatley and Brinster, 2012; Yoshida et al., 2007). In various model organisms, it could be shown that niches are far more than the historically described anatomic stem cell location. Instead, they serve as a specialized microenvironment composed of surrounding cellular components and a complex milieu of signal

molecules emitted from support cells to regulate stem cell maintenance, differentiation, self-renewal and localization (Jones and Wagers, 2008; Li and Xie, 2005).

For SSCs, the knowledge about their niche properties and molecular mechanisms controlling stem cell functions is limited. The location of SSCs along the periphery of seminiferous tubules adjacent to the basement membrane ((Chiarini-Garcia et al., 2003), see Figure 1 B) suggests that surrounding somatic support cell populations can make a contribution to the SSC niche. It is not surprising that Sertoli cells, the somatic cells within the tubules, are regarded as the major contributor to the SSC niche in the mammalian testis (de Rooij, 2009; Oatley and Brinster, 2012). However, taking the observation of Yoshida et al., who revealed a preferred, non-random localization of undifferentiated spermatogonia close to the vascular network and interstitial cells (Yoshida et al., 2007), it raises questions about the extent of the contribution by other testicular somatic cells, including peritubular myoid cells, Leydig cells and the surrounding vasculature network.

Several studies described age-related changes in male fertility and a decrease in reproductive functions, e.g. (Kuhnert and Nieschlag, 2004; Paul and Robaire, 2013; Plas et al., 2000; Wu et al., 2010b). Aging can cause alterations of the SSC niche which are manifested by a decline in the number of stem cells and stem cell activity. Transplantation studies imply an impaired capacity of the SSC niche environment of old males to support colonization of transplanted SSCs indicating a reduced potential to regenerate spermatogenesis (Zhang et al., 2006c).

2.2.1 Growth factors enhancing SSC self-renewal

Differentiation and self-renewal of the SSCs can be stimulated by local and systemic factors. Recent studies indicate a major influence of niche factors on these fundamental stem cell activities (Hofmann, 2008; Meng et al., 2000; Oatley et al., 2009; Oatley et al., 2011; Yoshida et al., 2007).

Significant scientific successes in unraveling the niche growth factor milieu could be achieved by *in vitro* and *in vivo* rodent studies and by developing strategies for the

cultivation of undifferentiated murine spermatogonia. Taking these approaches, multiple studies could demonstrate the essential role of the niche growth factors glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor (FGF2) and colony stimulating factor 1 (CSF-1) in the regulation of SSC self-renewal. It is a particularly conspicuous aspect, that these factors show similar functional characteristics in other self-renewing tissues.

Throughout the years, GDNF emerged to be a key player in the maintenance of SSCs by regulating their self-renewal (Chen and Liu, 2015; Kanatsu-Shinohara et al., 2004; Kubota et al., 2003; Naughton et al., 2006; Oatley and Brinster, 2008). In 2000, Meng et al. made a striking observation, where GDNF-deficient mutant mice showed a severe decline of stem cell reserves and, contrarily, an accumulation of undifferentiated spermatogonia after a GDNF overexpression (Meng et al., 2000). In progeny-deficient mice testes, GDNF secretion by Sertoli cells was reported to be controlled by the stimulation of follicle-stimulating hormone (FSH) (Tadokoro et al., 2002). Moreover, it was shown that tumor necrosis factor-alpha (TNFα) also induced GDNF production by Sertoli cells (Simon et al., 2007). The presence of GDNF could be proven for multiple species, in testes of mice and rats (Buageaw et al., 2005; Kubota et al., 2004; Ryu et al., 2005), bovine (Aponte et al., 2006; Oatley et al., 2004), hamster (Kanatsu-Shinohara et al., 2008a) and primates (Hermann et al., 2007; Maki et al., 2009). Further studies confirmed that SSCs in mice testis (Buageaw et al., 2005; Hofmann, 2008) as well as in primate (Maki et al., 2009) and human testes (Wu et al., 2009) express a membrane-bound receptor for GDNF, the GDNF family receptor α1 (GFRα-1).

While for a long time the general attention was paid solely on Sertoli cell produced GDNF, Spinnler et al. could demonstrate GDNF production also by human testicular peritubular cells (Spinnler et al., 2010). Most recently, it could be shown that expression of GDNF in mouse peritubular cells is controlled by testosterone stimulation and that transplantation of testosterone-treated peritubular cells to germ cell depleted mice could restore spermatogenesis (Chen et al., 2016b). These findings clearly support the conclusion that other testicular cells, beside Sertoli cells, may be a part of the stem cell niche.

Besides GDNF/GFRα-1 signaling, which has a leading role in controlling SSC activities, there are additional mechanisms involved in the regulation of SSC self-renewal and proliferation (Figure 2). Sertoli cell produced FGF2 and the epidermal growth factor (EGF) seem to be closely interlocked with GDNF-regulated self-renewal actions and promote, when co-supplemented with GDNF, the expansion of the SSC pool in long-term culture studies (Kanatsu-Shinohara et al., 2005; Kanatsu-Shinohara et al., 2008a; Kubota et al., 2004). However, since FGF2 and EGF enhance - beside SSC proliferation - also growth of non-stem cell spermatogonia, they act in a non-specific manner with respect to self-renewal (Lee et al., 2007a; Oatley and Brinster, 2012).
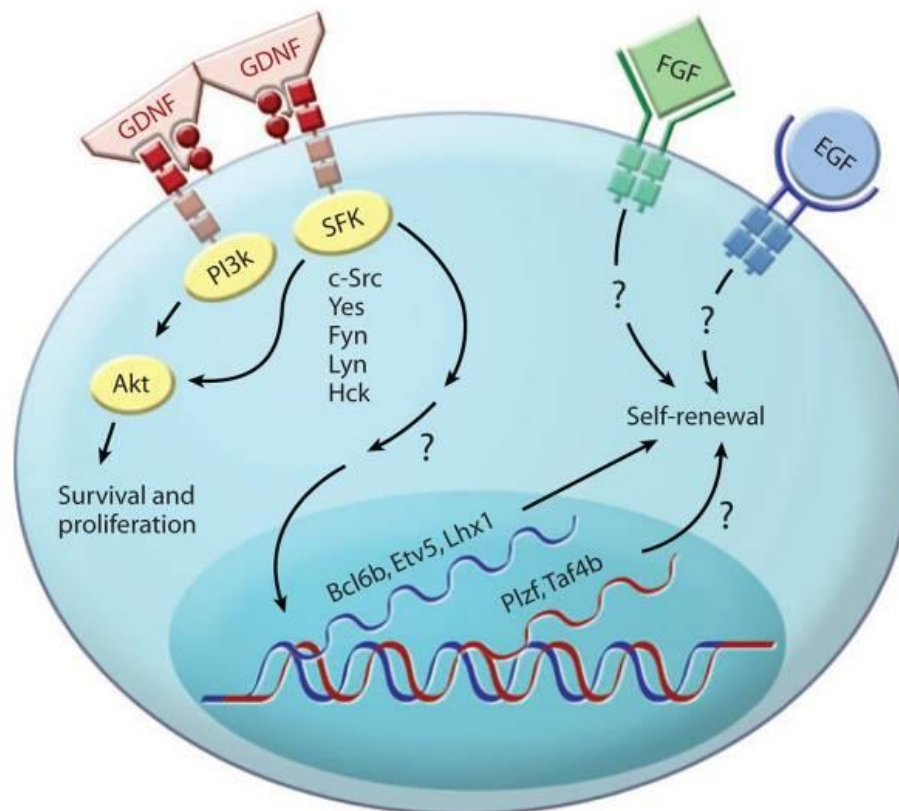
Figure 2. Signaling pathways of GDNF, FGF2 and EGF in self-renewal regulation of murine spermatogonial stem cells (SSCs). Known intrinsic molecular mechanisms and downstream activation processes are shown. Figure from Oatley and Brinster (2008).

Gene expression profiling of mouse spermatogonia revealed an overexpression for *Csfr1*, the receptor gene of the colony stimulating factor 1 (CSF1), whereof a role for CSF1 as potential stimulator for SSC self-renewal has been concluded (Kokkinaki et al.,

2009; Oatley et al., 2009). Remarkably, CSF1 was detected in interstitial Leydig cells as well as peritubular myoid cells, which expanded the pool of supporting cells that may be part of the SSC niche microenvironment (Oatley et al., 2009). Additional experiments using cultured mouse SSCs could show that insulin-like growth factor 1 (IGF1) supplemented medium increased the SSC pool (Kubota et al., 2004) and enhances SSC proliferation in a pathway different from the essential GDNF and FGF2 signaling (Wang et al., 2015). Interestingly, Kanatsu-Shinohara et al. recently showed the first negative regulator of SSC self-renewal, F-box/WD repeat-containing protein 7 (FBXW7), that counteracts with positive self-renewal signals (Kanatsu-Shinohara et al., 2014).

2.2.2   Growth factors initiating differentiation of SSCs

For a continuous spermatogenesis and fertility, a stable balance of self-renewal and differentiation has to be maintained. Studies on cultivated isolated mouse SSCs revealed that stimulation with Activin A and Bone morphogenetic protein 4 (BMP4) reduced the number of SSCs, indicating an influence on germ cell differentiation (Nagano et al., 2003). Complementary studies also demonstrated an increased expression of c-Kit, an early marker for differentiating spermatogonia, after BMP4 addition to undifferentiated spermatogonia (Pellegrini et al., 2003). More recently, it was proposed that BMP4-induced differentiation affects cell adhesion pathways (Carlomagno et al., 2010) and leads to an upregulation of Sohlh2, an assumed regulator of c-Kit expression (Li et al., 2014). Finally, the Sertoli cell-produced stem cell factor (SCF) can initiate germ cell differentiation through the transmembrane receptor c-Kit (de Rooij et al., 1999; Filipponi et al., 2007; Schrans-Stassen et al., 1999).

Besides rather established factors and pathways involved in SSC self-renewal and differentiation that are addressed above, there are upcoming targets and mechanisms including immunoglobulin kappa J region (RBPJ) (Garcia et al., 2014), FGF8-FGFR1 signaling (Hasegawa and Saga, 2014) and Wnt/β-catenin signaling (Takase and Nusse, 2016) that may contribute to the regulation of stem cell activity.

Taken together, the spermatogonial stem cell niche is constituted by a complex interplay of growth factors and provides structural support for the maintenance and regulation of stem cell activity. Over the years, considerable progress could be made in disclosing niche components contributed by support cells which provide a first insight into the mosaic of niche factors. However, also because of limited access to human SSCs, experiments were mainly conducted in rodent models. It is therefore necessary to examine the transferability of these scientific findings to the human reproduction system. Moreover, the production of known SSC key regulators (e.g. GDNF, CSF1) by Leydig cells and peritubular myoid cells supports the hypothesis that besides the established role of Sertoli cells, additional somatic cell populations have the potential to affect SSC fate.

2.3   Peritubular cells of the testis

Peritubular myoid cells are the primary cellular component of the wall of the seminiferous tubules in testes of all mammalian species. Their organization in the peritubular compartment is however strikingly different between species. In man, they are arranged in five to seven layers of very flat, elongated, spindle-shaped cells surrounding the seminiferous tubules, contrary to laboratory rodents where only a single cell layer is observed (Maekawa et al., 1996; Mayerhofer, 2013; Ross and Long, 1966; Schell et al., 2008; Welsh et al., 2009).

2.3.1   Phenotypic characteristics of testicular peritubular cells

Using immunocytochemistry, different phenotypes between the inner and outer cellular layers in man could be determined. Inner cellular layers showed positive immunoreactivity for desmin, indicating a smooth muscle-like phenotype, whereas outermost layers were positive for vimentin, suggesting a connective tissue phenotype (Davidoff et al., 1990). The expression of smooth muscle cell-specific markers (e.g. α-smooth muscle actin (α-SMA), desmin-type intermediate filaments, myosin heavy chain 11 (MYH11)) further underlines their smooth muscle-like character (Albrecht et al., 2006; Huang et al., 2012; Tung and Fritz, 1990; Virtanen et al., 1986).

Based on these phenotypic characteristics, these cells play a vital role in the transport of mature, yet immotile sperm towards the rete testis. It is assumed that cycles of contraction and relaxation of the peritubular cells circularly arranged around seminiferous tubules generate propulsive impulses important for the transport of immotile sperm and fluid (Ellis et al., 1981; Hargrove et al., 1977; Losinno et al., 2016; Romano et al., 2005; Ross and Long, 1966). The significance of peritubular myoid cell contractility for the regulation of spermatogenesis and male fertility could be highlighted in previous mouse studies (Welsh et al., 2009; Zhang et al., 2006a). More recently, in patients with mixed atrophy, a loss of contractile marker proteins, MYH11 and calponin, and of cGMP-dependent protein kinase 1 (cGKI) – a molecular marker for relaxation – could be associated with male infertility (Welter et al., 2013).

Peritubular cell function and their smooth muscle-like phenotype starts to take shape during puberty, whereby differentiation of peritubular cells appears to be critically regulated by androgens with an additive role of follicle stimulating hormone (FSH) (Schlatt et al., 1993). Starting in the same stage of sexual development, also the receptor for G protein-coupled oestrogen (GPER) is detectable in testicular peritubular cells of pubertal up to adult non-human primates. It is noteworthy in this context that a reduction up to a complete loss of GPER was observed in testes of men with impaired spermatogenesis (Sandner et al., 2014).

A recent study proposed an additional steroidogenic phenotype of human testicular peritubular cells and could prove the expression of pluripotency markers, namely POU domain class 5 transcription factor 1 (POU5F1) and GATA-binding protein 4 (GATA4) as well as of various steroidogenic markers including nuclear receptor subfamily 5, group A, member 1 (NR5A1), steroidogenic acute regulatory protein (STAR), CYP11A1 and CYP17A1 and 3 beta-hydroxysteroid dehydrogenase (HSD3B). In addition, the expression of markers for putative stem Leydig cells, leukemia inhibitory factor receptor (LIFR) and platelet-derived growth factor receptor-A (PDGFRA) suggest that the peritubular compartment of the testis may serve as a safe shelter for cells of the adult Leydig cell lineage (Landreh et al., 2014).

2.3.2 Studies on cultured human testicular peritubular cells (HTPCs)

The development of a relevant culture model for peritubular cells from the adult human testis finally facilitated the investigation of this rather ill-explored cell type also in the human system (Albrecht et al., 2006). Peritubular cells were isolated from man undergoing reconstructive surgery resulting in obstructive azoospermia but normal spermatogenesis (HTPCs) and patients with impaired spermatogenesis, non-obstructive azoospermia, and testicular fibrosis (HTPC-Fs) (Mayerhofer, 2013). The cells were derived by an explant culture method and were characterized regarding their phenotype by expression of above mentioned smooth muscle specific markers (e.g. α-SMA, MYH11, calponin, cGKI) (Schell et al., 2008; Welter et al., 2013).

In addition to its contractile nature (Schell et al., 2010), recent studies could also give a brief insight in the repertoire of secretory products. First, HTPCs show a predominant production of extracellular matrix (ECM) and basement membrane components, such as collagen type I, IV and XVIII, laminin, secreted protein acidic and rich in cystein (SPARC), fibronectin and the proteoglycan decorin (Adam et al., 2012a; Adam et al., 2011; Albrecht et al., 2006). Interestingly, decorin secretion is significantly higher in HTPC-Fs, which show impaired testicular function and fibrosis (Adam et al., 2012b).

The secretory character of HTPCs/-Fs is further mirrored by the secretion of various signaling molecules, such as nerve growth factor (NGF) and pro-inflammatory cytokines, e.g. monocyte chemotactic protein 1 (MCP1) and interleukin 6 (IL6) (Mayerhofer, 2013). The production of these paracrine factors can be regulated by the mast cell produced TNF-α (Schell et al., 2008).

Finally, one key finding was that HTPCs/-Fs produce the neurotrophic factor GDNF, which holds, as discussed in chapter 2.2, a central position in the paracrine regulation of SSCs self-renewal (Spinnler et al., 2010). Most recent mice studies showed that GDNF secretion seems to be critically regulated by testosterone stimulation. It was reported that testosterone-dependent GDNF secretion by peritubular cells could significantly enhance the colonization of transplanted SSCs into germ-cell depleted mice and could restore spermatogenesis (Chen et al., 2014; Chen et al., 2016b).

Considering thereby the anatomical location of peritubular cells in close proximity to SSCs, spatially separated only by a thin basal lamina, paracrine communication between both neighboring cells seems very well conceivable. Moreover, the findings outlined above led to the hypothesis that peritubular cells are an essential contributor to the SSC niche microenvironment and therefore play an essential role in human male fertility.

2.4 Quantitative proteomics for investigation of the cell secretome

Secreted proteins play an integral role in the cell-to-cell communication in multicellular organisms. Among them are growth factors, hormones and cytokines that may act as autocrine/paracrine intercellular messengers as well as components of the extracellular matrix. This complex set of molecules is often referred to as 'secretome', describing the entirety of proteins secreted by cells, tissues and organisms at a certain time and condition (Blanco et al., 2012; Hathout, 2007; Uhlen et al., 2015). Thus, the secretome reflects the current status of a cell in a particular environment and exerts a strong influence on physiological and pathological processes (Finoulst et al., 2011; Makridakis and Vlahou, 2010).

2.4.1 Mechanisms of protein secretion

Protein secretion in eukaryotic cells follows, in most cases, a well-defined process referred to as the classical endoplasmatic reticulum (ER)/Golgi secretory pathway (Baines and Zhang, 2007; Lee et al., 2004; Nickel and Rabouille, 2009; Nickel and Wieland, 1998; Osborne et al., 2005; Park and Loh, 2008). Secretory proteins containing N-terminal or internal signal peptides are targeted to the ER and the newly synthesized proteins are further directed across specialized ER membrane domains. Packaged into vesicular carriers, the synthesized proteins are transported to the Golgi apparatus where they undergo post-translational modification and processing. The export to the extracellular space or cell surface requires a dock and fusion of membrane-bound secretory vesicles with plasma membrane structures (porosomes) and vesicle swelling for a regulated expulsion of intravesicular contents (Anderson, 2006; Kelly et al., 2004).

Furthermore, there is growing evidence that certain secretory proteins use unconventional, non-classical secretory pathways (Nickel and Rabouille, 2009; Nickel and Seedorf, 2008; Prudovsky et al., 2008). Several soluble cytoplasmic and nuclear proteins lacking a guiding signal peptide sequence have been described that are transported to the cell surface through ER- and Golgi-independent pathways. Among them are, e.g. interleukin 1β, galectin 1, fibroblast growth factor 2 (FGF2), thioredoxin, and high-mobility group box 1 (HMGB-1) (Nickel and Rabouille, 2009; Nickel and Seedorf, 2008).

2.4.2   Secretome sample preparation and processing

Recent advances in proteomics techniques, including quantitative mass spectrometry (MS) and bioinformatics, open up new opportunities for a comprehensive analysis of cell secreted proteins. Secretome analyses using MS-based proteomics thereby require appropriate sample processing steps (Brown et al., 2012; Chevallet et al., 2007; Hathout, 2007; Kupcova Skalnikova, 2013; Makridakis and Vlahou, 2010; Rocha et al., 2014; Stastna and Van Eyk, 2012). A straightforward workflow starts with cell isolation and characterization, typically using immunoassays against cell-specific markers. Cells are further cultured in medium, complemented with ~10 % bovine serum. To avoid potential contamination and masking of cell secreted proteins by serum proteins present in high amounts in the culture medium, extensive washing of cells in either phosphate-buffered saline (PBS) or serum-free medium is essential. After cultivation for additional 12-48 h in serum-free medium, conditioned medium containing the secreted proteins is collected and centrifuged to remove detached cells and debris (Brown et al., 2012). Secretome proteins can then be concentrated using centrifugal filtration devices with molecular weight cutoff (3-10 kDa).

One major challenge in secretome proteomics is the low concentration of secreted proteins groups, such as cytokines, in a complex background of other proteins present in huge excess (Chevallet et al., 2007; Meissner et al., 2013). To counteract this problem, commonly referred to as "undersampling", pre-fractionation steps are used to facilitate identification of low abundant proteins and increase the overall proteome coverage by

reducing the sample complexity. Pre-fractionations can be carried out either on the protein level or on the peptide level after enzymatic digestion. While in the beginning of MS-based secretome analysis two-dimensional gel electrophoresis and protein identification by MS was frequently applied, many modern approaches commonly use a combination of a first-dimension separation and subsequent C_{18}-based liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Kupcova Skalnikova, 2013). A recent study compared three pre-fractionation strategies carried out prior to nano-LC-MS/MS analysis regarding their performance in secretome analyses (Piersma et al., 2010). One-dimensional gel electrophoresis followed by LC-MS/MS was superior compared to strong cation exchange (SCX) chromatography at the peptide level and reversed phase chromatography using tC2 cartridges at the protein level, with regard to the number of identified proteins, reproducibility of protein identification and throughput.

2.4.3 Mass spectrometry-based quantitative proteomics

The development of comprehensive, unbiased and quantitative analysis strategies in recent years made MS-based proteomics the method of choice for large-scale investigation of protein secretion (Cox and Mann, 2011; Hathout, 2007; Mallick and Kuster, 2010; Meissner et al., 2013). There are two main strategies in quantitative MS-based proteomics, either based on the incorporation of stable isotope labels or a label-free approach using spectral counting or precursor ion intensity measurements (Gao et al., 2008; Ong and Mann, 2005; Walther and Mann, 2010).

Chemical and metabolic isotopic labeling methods for relative quantification include isotope-coded affinity tags (ICAT) (Gygi et al., 1999), tandem mass tags (TMT) (Thompson et al., 2003), isobaric tags for relative and absolute quantitation (iTRAQ) (Ross et al., 2004)) and stable isotope labeling with amino acids in cell culture (SILAC) (Ong et al., 2002). SILAC is a metabolic labeling strategy that gained popularity in cell culture based comparative secretome analysis (Barderas et al., 2013; Chen et al., 2012; Marimuthu et al., 2013; Polacek et al., 2010; Rocha et al., 2014). The technique relies on the incorporation of non-radioactive, "heavy" isotope-labeled amino acids into all newly

synthesized proteins in cell culture systems. This allows a metabolic encoding of whole cell proteomes ("light" and "heavy" forms), which can be distinguished by MS. A direct sample mixing prior further processing steps minimizes sample-handling errors and increases the accuracy and robustness of quantification (Harsha et al., 2008; Ong and Mann, 2007). Notwithstanding the clear advantages, SILAC faces difficulties with cultures of primary cells that show limited division in cell culture, since comprehensive incorporation of the label requires a minimum of five cell divisions in the labeling medium (Blagoev and Mann, 2006; Stastna and Van Eyk, 2012). Moreover, the finite number of isotope labels that are available in label-based methods severely limits the multiplexing capacity indispensable for large-scale experiments (Dephoure and Gygi, 2012).

Their excellent scalability and the more simple and economical nature made label-free quantitation methods an attractive alternative for quantitative proteomics studies in recent years (Feltcher et al., 2015; Meissner et al., 2013; Soleilhavoup et al., 2016). This upswing is based on the empirical observations that chromatographic peak heights and peak areas (Andersen et al., 2003; Bondarenko et al., 2002; Chelius and Bondarenko, 2002; Wang et al., 2003) or the number of spectra (Colinge et al., 2005; Liu et al., 2004) correlate linearly to the protein abundance in the sample. Relative quantification by precursor ion intensity measurement is done by comparing the peak intensity of each peptide of a protein in different LC-MS datasets originating from different samples (Zhu et al., 2010). The spectral counting approach uses the number of identified MS/MS spectra from peptides of the same protein as estimation of relative protein abundance between different samples (Lundgren et al., 2010; Zhu et al., 2010). Compared to label-based methods, spectral counting is inferior in quantification accuracy but outstanding in proteome coverage and dynamic range (Arsova et al., 2012; Li et al., 2012; Mallick and Kuster, 2010; Merl et al., 2012).

2.4.4 Bioinformatics tools predicting subcellular localization and secretory pathways

A key challenge in secretome studies remains the discrimination between truly secreted proteins and those released due to cell leakage and cell death. For the determination of the subcellular localization, several internet-accessible tools have been developed. Frequently used platforms are the Gene Ontology (GO) annotation database (Ashburner et al., 2000), UniProt (http://www.uniprot.org/), the DAVID platform (Huang da et al., 2009) and Ingenuity (http://www.ingenuity.com/). Most popular tools for the prediction of classical secretory pathways by proofing the presence of a signal peptide in the amino acid sequence are SignalP (Petersen et al., 2011), Phobius (Kall et al., 2004) and SPOCTOPUS (Viklund et al., 2008). Non-classically secreted proteins can be predicted using SecretomeP, based on queries of features providing information on various post-translational and localization aspects (Bendtsen et al., 2004). A combination of different tools and algorithms for the classification of potentially secreted proteins can be advantageous for large-scale experiments (Gao et al., 2008; Le Bihan et al., 2012; Uhlen et al., 2015).

3   Publication

Secretome Analysis of Testicular Peritubular Cells:

A Window into the Human Testicular Microenvironment and the

Spermatogonial Stem Cell Niche in Man

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Abstract

Spermatogonial stem cells (SSCs) are vital for lifelong spermatogenesis in man. In their niches, a special growth factor milieu and structural support by surrounding cells are thought to ensure their maintenance. In man, the cells of the wall of seminiferous tubules, human testicular peritubular cells (HTPCs), are considered to contribute to this microenvironment and the overall testicular microenvironment via secreted proteins. Therefore, the secretome of cultured HTPCs from five individual men was analyzed by LC-MS/MS. Quantification and comparison to the proteome of HTPC lysates revealed 263 out of 660 identified secretome proteins to be at least 5-fold enriched in the culture media. To obtain additional evidence for secretion, signal peptide and gene ontology (GO) enrichment analyses were applied. The latter revealed—besides extracellular matrix (ECM) components—a significant over-representation of chemokines and growth factors acting in signaling pathways that appear critical for SSC maintenance. Immunohistochemistry, performed with human testicular sections, depicted expression of selected proteins in vivo. The significant enrichment of proteins related to cell adhesion and migration may indicate their involvement in SSC regulation. Our data strongly support the hypothesis of a crucial role of HTPCs in the composition of SSC niches in man.

Keywords

Introduction

Seminiferous tubules of the testis represent a special compartment, in which spermatogenesis takes place. Once started at puberty, this process occurs throughout life and is fueled by the division and differentiation of stem cells, called spermatogonial stem cells (SSCs). This cell type resides within a niche that is thought to be built by Sertoli cells (1). Yet, other testicular cells are likely also involved physically and functionally in the formation of this niche. In this context, Yoshida et al. observed a nonrandom orientation of undifferentiated spermatogonia along the seminiferous tubules toward the vascular network and accompanying interstitial cells (2). The ordered location of SSCs adjacent to the interstitium suggests that cell types other than Sertoli cells make a valuable contribution to the composition of a SSC niche.

The somatic cells of the tubular wall, peritubular cells, are such cells that also may have a say. They are separated from SSCs only by a basal lamina and thus certainly form a morphological part of the niche. These cells are poorly explored, especially in humans (3, 4). In man, several layers of very thin, spindle-shaped, peritubular cells together with extracellular matrix (ECM) proteins form this compartment, whereas only a single cell layer of such cells forms the tubular wall in rodents (4, 5). Peritubular cells in the adult express markers for smooth muscle-like cells (e.g., smooth muscle actin) and for connective tissue cells (e.g., vimentin) (6, 7), and thus they are regarded to be contractile, smooth muscle-like cells. They appear to be involved in the transport of immotile sperm and fluid in seminiferous tubules (8).

Recently, a culture method for human testicular peritubular cells (HTPCs) was established, which allows a systematic characterization of this type of cell (7). It uses as a starting point very small testicular tissue samples from adult patients with obstructive azoospermia and normal spermatogenesis (7, 9-13). By showing typical markers for peritubular cells, including smooth muscle actin (7, 10), and by documenting the absence of markers for Leydig cells (LH-receptor) or Sertoli cells (FSH-receptor) (11), the purity of the explant cell cultures over several passages could be verified. As an important result stemming from the initial characterization, HTPCs were shown to be a

source of several neurotrophic factors, including glial cell line derived neurotrophic factor (GDNF) (11, 14). This factor is regarded to be crucial for the renewal of SSCs throughout species (1, 15). HTPCs produce GDNF in a constitutive fashion, as shown by ELISA measurements (11). Thus in man, GDNF is a product of peritubular cells as well as Sertoli cells. As a result of the proximity of peritubular cells to SSCs, peritubular cells via GDNF may regulate SSCs in a paracrine way.

These results have led to the hypothesis that peritubular cells of the tubular wall contribute directly to the SSC niche in the human testis (11). In addition, peritubular cells may also be involved in the general regulation of testicular functions by producing factors which may regulate Sertoli cells and Leydig cells, for example. Recent mouse studies provide evidence for this notion (4, 16-18).

To further explore these novel ideas and to expand the knowledge about the role of HTPCs for the human testis, we performed a proteomic analysis of the HTPC secretome using nano-LC-MS/MS technology. On the basis of five individual human donors, we present here the first comprehensive data set of proteins secreted by HTPCs.

Experimental procedures

Human Testicular Samples, Isolation and Cultivation of HTPCs

Isolation and culture of HTPCs from human testicular biopsies were performed, as described previously (7, 10, 12, 14, 19-21). The cells were isolated from five patients with azoospermia but normal spermatogenesis due to vasectomy. The local ethics committee approved the study and the use of these biopsies and cells (permission no. 3051/11). All cells were derived by explant culture, which allows us to monitor outgrowth of peritubular wall cells. They were initially screened for smooth muscle cell markers including smooth muscle cell actin (SMA) (7, 10) and for the absence of contamination by Sertoli cells and Leydig cells by examining expression of receptors for FSH and LH (11). The specific cells studied in this project stem from five men (age 29–53 years). They were passaged and propagated over 7–9 passages in Dulbecco's modified Eagle's medium (DMEM) high glucose with phenol red + 10% fetal calf serum (FCS; both from PAA GmbH, Cölbe, Germany). All cells used were verified to express SMA by RT-PCR, immunocytochemistry, or Western blot.

Protein Sample Preparation

For proteomic analysis, human testicular peritubular cells from five patients (passages 7–9) were seeded to a cell culture dish (60 × 15 mm, 21 cm^2; Sarstedt, Nümbrecht, Germany) and were allowed to grow under normal in vitro conditions (DMEM high glucose with phenol red + 10% FCS). When cells reached a confluence of 90%, the medium was removed. Cells were washed twice with 2 mL of medium without FCS and without phenol red, and fresh medium was added (without FCS and phenol red; 2 mL/dish). After 24 h, the conditioned medium was collected, centrifuged for 3 min at 1000g and stored at −80 °C, until processing for proteomic analysis. Cell pellets were harvested and likewise stored.

Proteins contained in the conditioned medium were concentrated for 40 min at 3000g on an Amicon Ultra-4 centrifugal filter with a 3 kDa molecular weight cutoff (Millipore, Schwalbach, Germany). The remaining supernatant was dried in a vacuum centrifuge,

and the protein pellet was resuspended in 15 µL Laemmli sample buffer prior to electrophoresis. For cell lysate samples, the protein concentration was determined using the Pierce 660 nm Protein Assay (Pierce/Thermo Scientific, Rockford, IL) (22).

SDS-PAGE

SDS-PAGE was performed using a 4% stacking gel and a 12% separation gel (overall gel size 7 cm (L) × 8.5 cm (W) × 0.75 mm) on a mini-Protean II system (Bio-Rad, Hercules, CA). Samples were run for 15 min at a constant voltage of 80 V and for 70 min at 100 V. After electrophoresis, gels were stained overnight by Coomassie R-250 and destained in 5% methanol with 7% acetic acid.

Gel Slicing and In-Gel Digestion

Gels were washed twice in water, and gel lanes were cut into 12 bands for trypsin digestion and subsequent LC-MS/MS analysis. Fractionated bands were further minced into small pieces and subjected to in-gel trypsin digestion. In brief, proteins were equilibrated twice with 50 mM ammonium bicarbonate (ABC), and cysteine residues were reduced during a 45 min incubation in 10 mM dithioerythritol/50 mM ABC at 56 °C. Alkylation was carried out in 55 mM iodoacetamide/50 mM ABC for 30 min at room temperature in darkness. After they were washed in 50 mM ABC, gel pieces were dehydrated in 60% acetonitrile (ACN) and dried by vacuum centrifugation (vacuum concentrator, Bachofer, Reutlingen, Germany). Subsequent enzymatic digestion was performed overnight in 50 mM ABC with 100 ng sequencing-grade porcine trypsin (Promega, Madison, WI) per gel slice. The generated peptides were extracted twice with 5% formic acid in 50% ACN and once with 100% ACN. The peptide-containing solution was dried using a vacuum concentrator and stored at −80 °C until LC-MS/MS analysis.

LC-MS/MS Analysis

Prior to LC-MS/MS, peptide samples were dissolved in 40 µL of solvent A consisting of 0.1% formic acid and centrifuged for 15 min at 18 000*g*. Peptides were separated by nano reversed-phase liquid chromatography on an Ettan MDLC system (GE Healthcare, Munich, Germany) with a 15 cm separation column (ReproSil-Pur 120 C18 AQ, 3 µm bead size, 75 µm i.d., Dr. Maisch, Ammerbuch-Entringen, Germany). Peptide mixtures were injected and trapped at 10 µL/min on a guard column packed with C18 PepMap 100, 5 µm, 300 µm × 5 mm (LC Packings/Dionex, Idstein, Germany) and separated at a constant flow rate of 280 nL/min with a gradient from 0 to 30% B (0.1% formic acid and 84% ACN) in 80 min followed by a second ramp to 60% B in 30 min. The LC system was coupled online to an Orbitrap XL instrument (Thermo Scientific, San Jose, CA) via a distal coated SilicaTip (FS-360-20-10-D-20, New Objective, Woburn, MA), and the electrospray ionization was operated at a needle voltage of 1.6 kV. Mass spectra were acquired in cycles of one MS scan from m/z 300–2000 and up to five data dependent MS/MS scans of the most intensive peptide signals with charge state ≥2 at a collision energy of 35%. Dynamic exclusion was activated for 30 s to minimize repeated precursor selection.

Database Searching and Data Analysis

MS RAW data were processed using MASCOT Daemon and MASCOT Server version 2.4 (Matrix Science, London, U.K.) (23). Peak lists were searched against the IPI.HUMAN.v3.87 database augmented with the MaxQuant contaminant database (25 Jun 2012, http://maxquant.org/downloads.htm), resulting in 91 711 entries in the forward peptide sequence database including common contaminants. Applied search parameters are listed next. Fixed modifications: carbamidomethyl (C); variable modifications: oxidation (M); decoy database: checked; max missed cleavages: 1; peptide charge: 2+ and 3+; peptide tol. ± 2 Da; MS/MS tol. ± 0.8 Da.

Scaffold version 3.6.5 was used to obtain a list of protein identifications with a false discovery rate (FDR) ≤ 0.9%, requiring at least two individual peptides per protein with a minimum peptide probability of 90% (24, 25).

Normalized spectral counts, in Scaffold denoted as "quantitative value", were exported to Microsoft Excel and used for quantitative comparison between secretome and cell lysate data. A similar strategy has been applied by Piersma et al. (26). Prior to further data analysis, mapped contaminants were eliminated from the list of identified proteins.

Comparison of the secretome and cell lysate data sets was performed in a proportional Venn diagram provided by Google Image Charts (https://developers.google.com/chart/image/docs/chart_wizard).

Subcellular Localization and Pathway Prediction

The freely accessible SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict signal peptide cleavage sites in FASTA sequences of the identified secretome and cell lysate proteins, applying "sensitive" D-cutoff values >0.34 (27). Regarding the subcellular localization, we used annotation mapping incorporated in UniProt (http://www.uniprot.org/) with respect to the gene ontology (GO) term "cellular component" (28).

To get a specific and detailed functional profile of the analyzed HTPC secretome, we first performed a DAVID functional annotation clustering (http://david.abcc.ncifcrf.gov/home.jsp) in order to cluster enriched annotation groups within the set of identified secretome proteins (29, 30). Groups comprising similar biological function terms were filtered for most significant terms above an enrichment score of 1.3 (p-value <0.05). Complementary, this functional profile was visualized in a grouped network using the Cytoscape 3.0.0 plug-in ClueGO v2.0.3 (31, 32). At a network specificity displaying GO terms from tree levels 2 to 6 and a minimum number of 10 genes per term, the right-sided hypergeometric test was used to calculate enrichment for terms and groups. Functional grouping was based on κ-score (>0.3). Additionally, GO term fusion was selected for redundancy reduction to aspire an insightful view on the functional profile of the HTPC secretome.

Immunohistochemistry

Immunohistochemical staining was performed as published elsewhere (7, 33) using testicular samples of five men. For staining, specific mouse monoclonal antibodies against collagen I (1:100, c = 2 µg/mL; AF5610-1) and collagen IV (1:100, c = 1 µg/mL; AF5910; both ACRIS antibodies, Aachen, Germany) were used. A rabbit polyclonal antiserum against fibronectin (1:500, c = 1 µg/mL; cat. no. F3648; Sigma, Taufkirchen, Germany) and a rat monoclonal antibody against PTX3 (1:100, c = 1 µg/mL; cat. no. ab90806; Abcam, Cambridge, United Kingdom) were used. Corresponding negative controls consisted of mouse IgG (I5381; Sigma, Taufkirchen, Germany), rabbit IgG (PP64; Millipore, Schwalbach, Germany), rat IgG (MCA1123R; AbD SeroTec, Puchheim, Germany) instead of the specific antibodies/antiserum. Sections were counterstained with hematoxylin.

Western Blot

For immunoblotting, lysates of peritubular cells were used. Western blotting was performed as described before (20), using the same monoclonal antibody against PTX3, as for immunohistochemistry (1:100, c = 1 µg/mL; cat. no. ab90806; Abcam, Cambridge, United Kingdom). Secondary goat antirat antibody (1:10.000, c = 80 ng/mL; 112-035-167; Dianova, Hamburg, Germany) conjugated with peroxidase was added to detect bands with chemiluminescent solutions (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce, Thermo Scientific, Rockford, IL).

Results

To characterize the secretome of human testicular peritubular cells, we analyzed cell culture supernatants of HTPCs isolated from five individual donors using nano-LC-MS/MS. To reduce undersampling and to increase the number of identified proteins, the proteins were prefractionated using 1D SDS-PAGE (12 slices per donor; Figure 1), and fractions were analyzed individually by LC-MS/MS. To obtain a high-confidence data set, only identifications with at least two individual peptides were accepted, and using a decoy database approach (34), an FDR $\leq$ 0.9% was determined. The entire protein list can be viewed in Supplementary Table 1. In total, 813 proteins could be identified in the cell supernatants, from which 271 were found in samples of all five individual donors, 382 in at least four, 494 in at least three, and 619 in at least two donors.
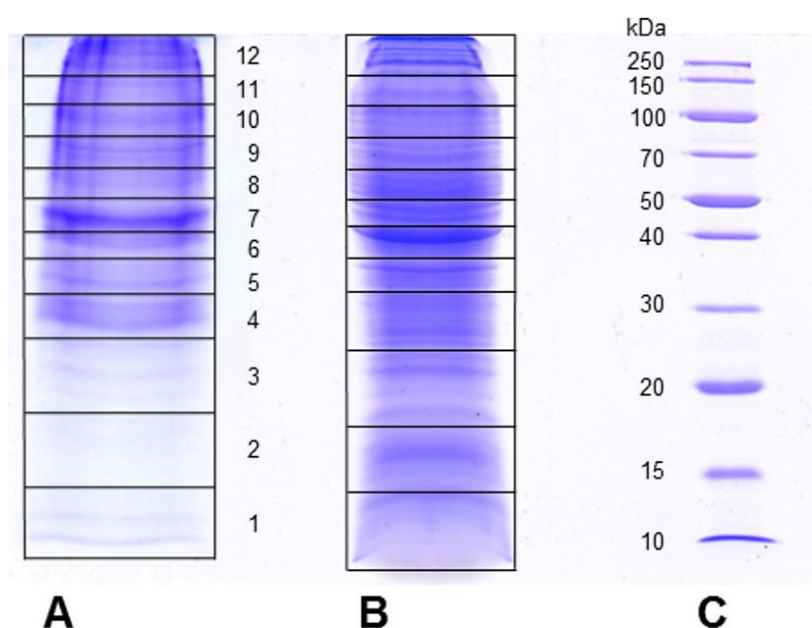

Figure 1. One-dimensional SDS gels (12% polyacrylamide) stained by Coomassie brilliant blue R-250. (A) HTPC proteins enriched by ultrafiltration from 2 mL serum-free cell culture supernatant and (B) 50 μg of corresponding cell lysate proteins. Boxes in A and B indicate fractions individually analyzed by LC-MS/MS. (C) Molecular weight marker ranging from 10 to 250 kDa.

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To check if the presence of proteins identified in the cell culture supernatant is due to secretory activity rather than cell degradation or cell lysis, we additionally analyzed two corresponding HTPC lysates, following the same analytical strategy as described above. From the cell lysates, a total of 1570 proteins were identified (Supplementary Table 2). Supernatant and cell lysate data sets show an overlap of 617 proteins, whereas 196 were exclusively detected in the cell culture supernatants, and 953 were exclusively detected in cell lysates (Figure 2).
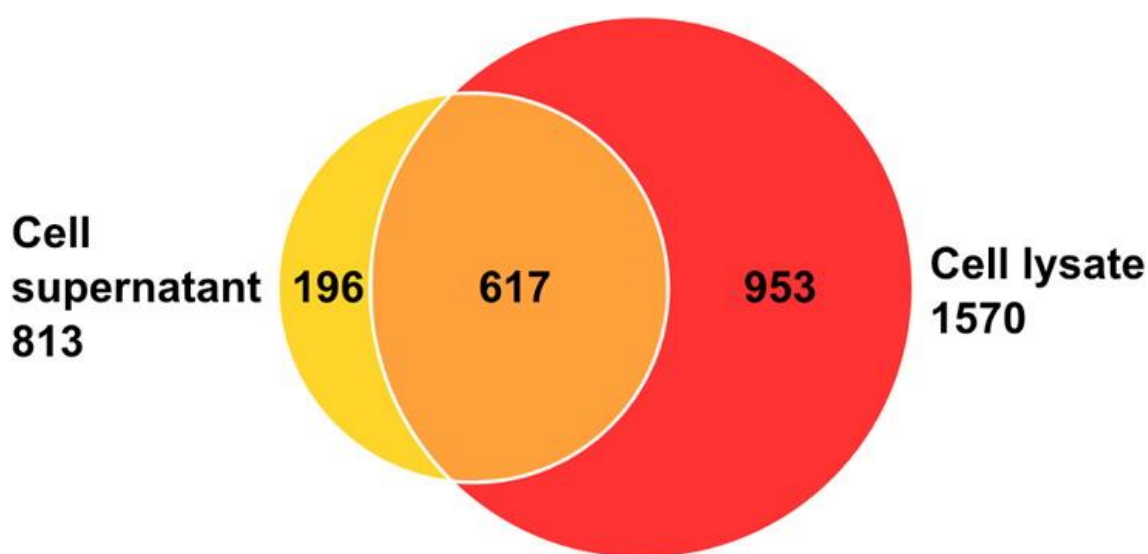
Figure 2. Proportional Venn diagram of identified HTPC proteins in cell culture supernatant and cell lysate data sets.

Protein abundance between cell culture supernatants and cell lysate was quantitatively compared applying a spectral counting approach (35). Proteins detected in both cell culture supernatants and corresponding cell lysates were considered to be secreted by HTPCs if they fulfilled the following enrichment criteria: (i) normalized spectral counts need to be at least 5 times higher in cell culture supernatants compared to corresponding cell lysate data and (ii) at least 10 spectra per protein in at least one sample need to be acquired. One hundred ninety-six proteins were exclusively detected in the supernatant, thus exceeding by far the first secretion criterion. Sixty-seven proteins, detected in both supernatant and cell lysate, were enriched in cell

supernatants, and therefore, we consider a total of 263 proteins identified from the cell culture medium to be secreted by HTPCs. This data set is referred to as the HTPC secretome data set and was used for the following analyses. In Table 1, the 25 most prominent proteins of the HTPC secretomes are listed. All these proteins were detected in all individual donors and sorted in descending order with respect to their average spectral counts. The entire set of HTPC secretome proteins is available as Supplementary Table 5. The data set contained a noticeable number of proteins related to structural components of the ECM like fibronectin or collagens, as well as multiple cytokines and growth factors such as vascular endothelial growth factor (VEGF) and pigment epithelium derived factor (PEDF).

Table 1. Top 25 of 263 proteins of the HTPC Secretome Data Set with Corresponding Spectral Counts[a]

| Protein Name | accession number | Average Spectral Counts | | ratio cell supernatant/ cell lysate[b,c] | signal peptide[d] |
		cell supernatant	cell lysate		
Isoform 1 of Fibronectin	IPI00022418 (+1)	1312.0	23.9	54.8	YES
Collagen alpha-2(I) chain	IPI00304962	932.2	13.8	67.4	YES
cDNA FLJ53292, highly similar to Homo sapiens fibronectin 1 (FN1), transcript variant 5, mRNA	IPI00922213	622.8	0.0	n.d. in CL	NO
Collagen alpha-1(I) chain	IPI00297646	602.7	36.6	16.5	YES
Isoform 1 of Glia-derived nexin	IPI00009890	429.2	28.8	14.9	YES
72 kDa type IV collagenase	IPI00027780	384.8	2.9	134.7	YES
Plasminogen activator inhibitor 1	IPI00007118	306.8	3.6	85.6	YES
Collagen alpha-1(VI) chain	IPI00291136	291.9	17.5	16.6	YES
Metalloproteinase inhibitor 1	IPI00032292	286.7	2.1	134.4	YES
Isoform 1 of Collagen alpha-1(III) chain	IPI00021033	244.7	1.2	204.7	YES
Insulin-like growth factor-binding protein 7	IPI00016915 (+1)	244.5	5.4	45.4	YES
cDNA FLJ54471, highly similar to Complement C1r subcomponent	IPI00956148	236.3	0.6	403.3	YES
Laminin subunit gamma-1	IPI00298281	235.0	2.6	91.2	YES
Isoform A of Decorin	IPI00012119	221.3	0.0	n.d. in CL	YES
Isoform 1 of EGF-containing fibulin-like extracellular matrix protein 1	IPI00029658 (+3)	210.4	0.0	n.d. in CL	YES
Pentraxin-related protein PTX3	IPI00029568	194.4	2.7	72.8	YES
Lumican	IPI00020986	192.0	0.6	315.0	YES
Thrombospondin-2	IPI00018769	177.4	0.3	631.2	YES
Biglycan	IPI00010790 (+1)	176.6	0.0	n.d. in CL	YES
Laminin subunit beta-1	IPI00013976 (+1)	169.2	0.3	602.3	YES
Pigment epithelium-derived factor	IPI00006114	168.9	0.0	n.d. in CL	YES
Isoform 1 of Sulfhydryl oxidase 1	IPI00003590	168.4	0.0	n.d. in CL	YES
Isoform 1 of Coiled-coil domain-containing protein 80	IPI00260630 (+1)	160.4	0.0	n.d. in CL	YES
ABI family, member 3 (NESH) binding protein	IPI00419966 (+1)	146.0	0.0	n.d. in CL	YES
Complement C1s subcomponent	IPI00017696	145.7	0.0	n.d. in CL	YES

[a]All listed proteins are detected in five of five individual donors and sorted in descending order with respect to their average spectral counts.

[b]Ratio cell supernatant/cell lysate: ratio according to spectral counting.

[c]n.d. in CL: the protein is only detected in cell supernatant and not in cell lysate.

[d]Signal peptide?: signal peptide prediction status.

To strengthen evidence for secretion, two independent bioinformatics tools were used. We subjected both sets of secretome proteins and cell lysate proteins to a signal peptide prediction analysis, disclosing the proteins which enter the classical endoplasmatic reticulum–Golgi secretory pathway. For 81% of the secretome proteins, the presence of a signal peptide could be predicted using the SignalP 4.1 server (27). In contrast, a signal peptide was predicted for only 16% of the proteins from the cell lysate data set. For further validation, we performed a comparative GO analysis using the term "cellular component" to disclose the subcellular location of proteins in the secretome and cell lysate data set (Figure 3). As expected, the vast majority from the secretome data set (250 out of 263 identifiers) and from the cell lysate data set (1541 out of 1570 identifiers) could be mapped to the GO term "cellular component". Strikingly, 31% of the secretome proteins were related to "extracellular space" (a location definitely requiring secretion), while only a small fraction of 3% of cell lysate proteins matched this subterm.
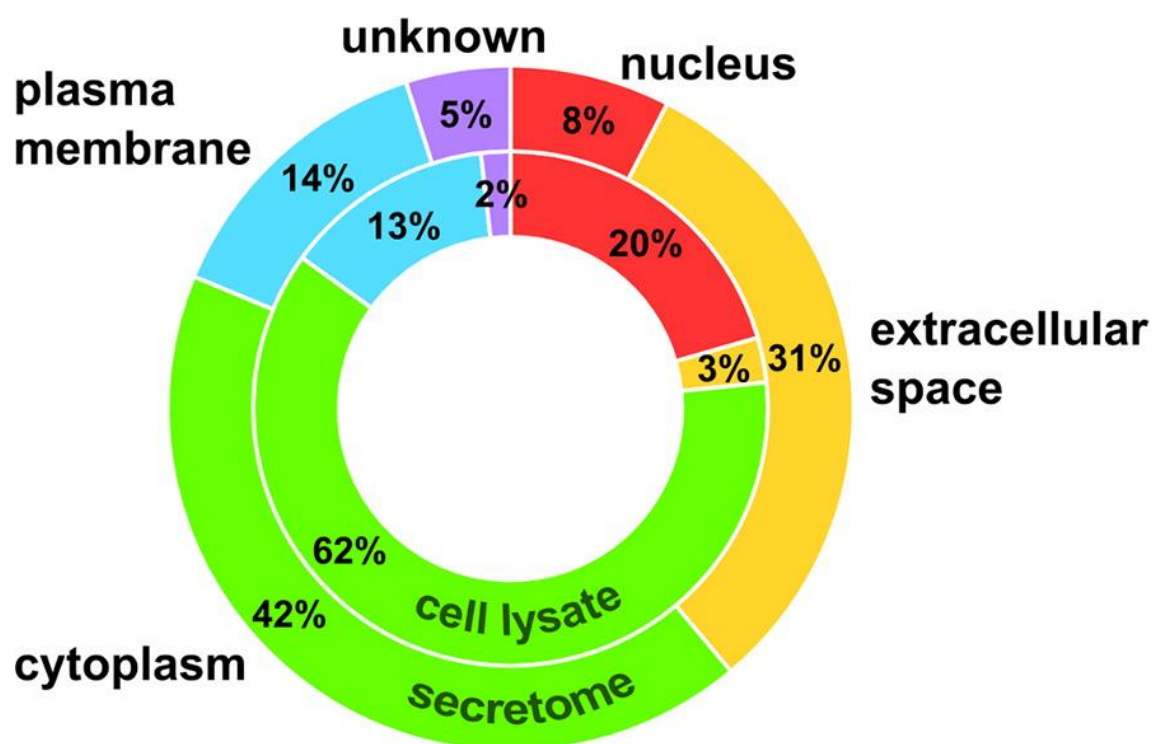
Figure 3. GO analysis of secretome and cell lysate proteins according to the term "cellular component".

Secretome proteins were functionally clustered using the DAVID platform (29, 30) and filtered for significant terms exceeding an enrichment score of 1.3 (p-value <0.05). The 263 secretome proteins clustered into 24 terms of the GO "biological process" database (Table 2). Proteins related to GO terms "skeletal system development", "vasculature development", "cell motion", "response to wounding", "wound healing", and "regulation of cell adhesion" were strongly over-represented in the secretome. Proteins mapped to the obtained functional clusters are listed in Supplementary Table 6. A ClueGO analysis was performed to visualize the most relevant biological processes related with the HTPC secretome proteins in a functionally grouped network (31, 32). Applying the tool "GO Term fusion" as a redundancy reduction feature, the network analysis revealed functionally grouped terms related to "skeletal system development", "vasculature development", "cell motility", and "cell adhesion" (Figure 4).

Table 2. DAVID Analysis of HTPC Secretome Proteins According to the GO Term "Biological Process"[a]

GO term	enrichment score
skeletal system development	8.47
vasculature development	6.25
cell motion	5.30
response to wounding/defense response	5.06
wound healing	4.88
regulation of cell adhesion	3.38
proteoglycan metabolic process	2.93
enzyme linked receptor protein signaling pathway	2.90
multicellular organismal metabolic process	2.88
positive regulation of cell migration	2.49
regulation of proteolysis	2.32
cell morphogenesis/cell morphogenesis involved in differentiation	2.11
skeletal system morphogenesis/face/head/body morphogenesis	2.03
skeletal system morphogenesis/cartilage development	1.94
response to organic substance/response to steroid hormone stimulus	1.92
tube development/respiratory tube development	1.92
lipid localization/vacuole organization	1.92
tube development/tube morphogenesis	1.83
regulation of inflammatory response/negative regulation of response to stimulus	1.82
aminoglycan metabolic process/polysaccharide metabolic process	1.63
glycoprotein metabolic process	1.62
regulation of blood coagulation	1.46
lipid transport	1.38
response to extracellular stimulus	1.32

[a]The 24 over-represented GO terms are shown. Lowest enrichment score corresponds to a p-value <0.05.
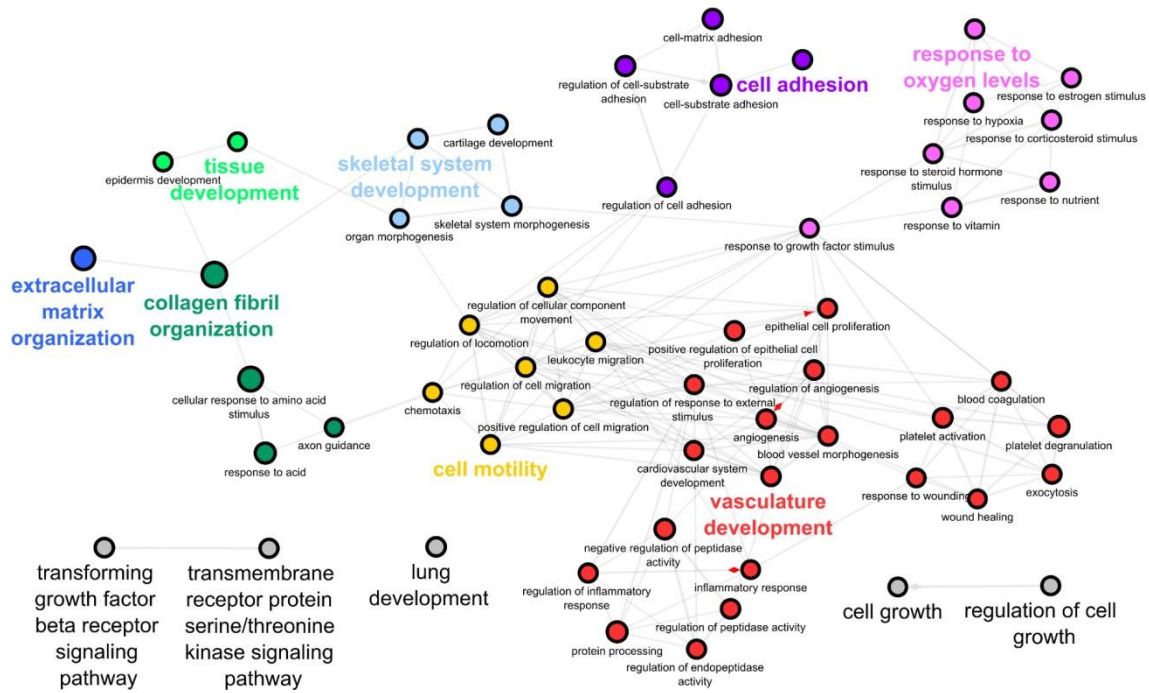
Figure 4. ClueGO network analysis of HTPC secretome proteins according to the GO term "biological process". Associated GO terms are depicted as nodes, where the node size indicates the particular enrichment significance. Functional grouping is based on κ-score (>0.3). Ungrouped terms are displayed in gray.

Immunohistochemical staining of human testicular sections was used to demonstrate in vivo expression of four proteins identified in the secretome analysis in cultured HTPCs. The four proteins chosen were all clearly detected in the peritubular compartment and belong to different classes of secreted proteins: fibronectin is a noncollagenous member of ECM proteins, collagen I and IV are prototype ECM proteins, and PTX3 is a secreted factor related to immune function. All proteins were readily identified in the wall of seminiferous tubules, both in the cytoplasm of peritubular cells and in the ECM (Figure 5, arrows). Immunoreactivity of the examined ECM proteins, as expected, was also seen in the interstitial areas of the testis, where ECM proteins are found. All negative control sections (Figure 5, bottom row) lacked specific immunoreactivity. The PTX3 antibody, besides strongly staining the perinuclear areas of peritubular cells, also reacted with cells of the germinal epithelium. To further examine the specificity of the PTX3 antibody, Western blotting with lysates of HTPCs was performed (Figure 5B).

Results revealed one single PTX3 band of the correct size and therefore support the conclusion that HTPCs in vitro and in vivo are producers of PTX3.
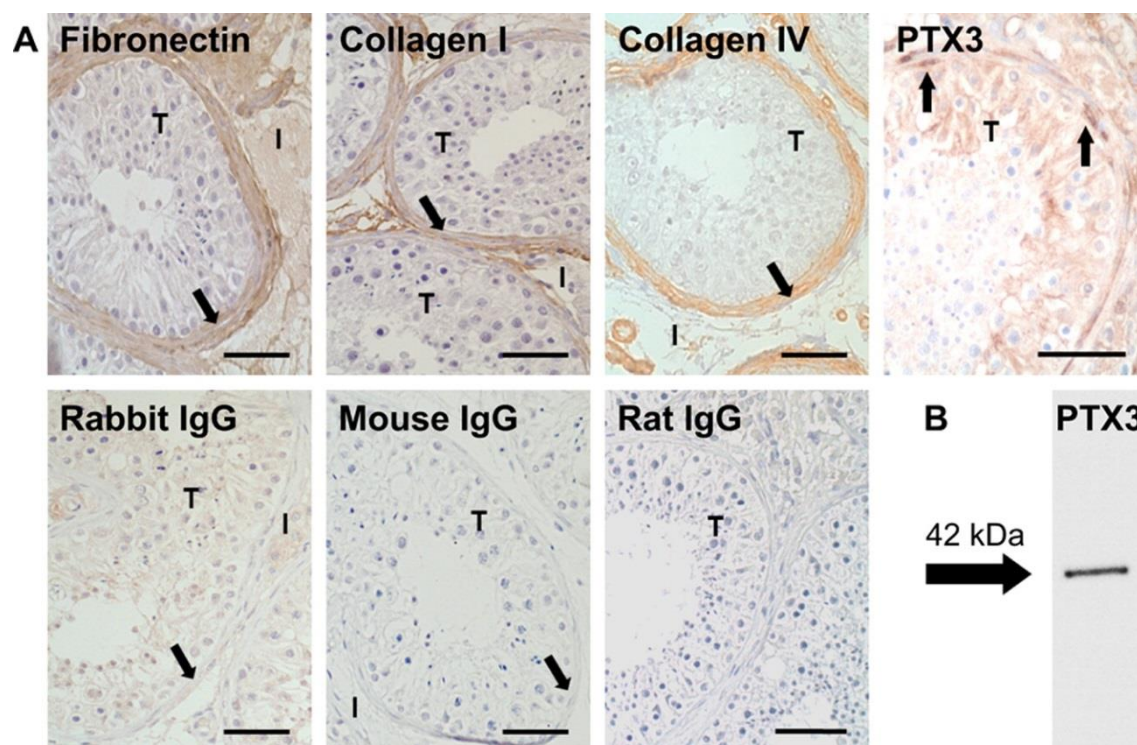
Figure 5. (A) Immunohistochemical detection of PTX3, fibronectin, collagen I and collagen IV, and corresponding controls, namely, rabbit IgG for fibronectin, mouse IgG for collagen I and collagen IV, and rat IgG for PTX3 is shown. Note that in all cases, peritubular cells and/or their immediate surrounding ECM (arrows) and the ECM of the interstitial areas (I) of the human testis are stained. The PTX3 antibody, besides specifically staining the cytoplasm of peritubular cells, also stained cells in the seminiferous tubules (T). The latter was reduced in corresponding control (rat IgG), and hence staining of peritubular cells and cells of the germinal epithelium has to be regarded as a specific reaction. Haematoxylin staining was used to counterstain nuclei. Bars represent 50 μm. (B) Immunoreactive PTX3, with a molecular weight of 42 kDa (arrow) was detected in a HTPC cell lysate by Western blot analysis.

Discussion

General Remarks

We performed a comprehensive analysis of proteins secreted by isolated testicular peritubular cells (7) in an attempt to decipher their contribution to the testicular microenvironment—specifically to the molecular composition of the SSC niche. Culturing primary cells derived from five individual human donors, we generated five secretome samples in individual experiments. We chose 1D SDS-PAGE combined with LC-MS/MS and spectral counting for relative quantification, an approach successfully applied for the in-depth characterization of secretomes from various cell types (26, 36-39). The presence of cellular proteins in cell culture supernatants can arise from secretory events or can be the result of cell lysis or degradation. To discriminate between these phenomena, we additionally analyzed cell lysates and performed a quantitative comparison of protein abundances using spectral counting. We categorized proteins as "secreted" when an abundance ratio of >5 (according to spectral counts) in cell culture supernatants versus cell lysates was fulfilled, and thereby received a data set of 263 identified secretome proteins. To confirm the validity of this data set, two independent bioinformatics approaches were performed: (i) In a GO analysis based on "cellular component" database, 31% of these proteins match the GO term "extracellular space", corresponding to a 10-fold enrichment compared to cell lysate proteins (3%). (ii) Using the signal peptide prediction algorithm "SignalP", signal peptide sequences could be predicted from 81% of the secretome proteins but only 16% of proteins identified from cell lysates. Remaining proteins in our data set which lack a signal peptide might be released into the secretome through mechanisms frequently referred to as "unconventional protein secretion" (40). Taken together, the combination of quantitative LC-MS/MS results with gene ontology and signal peptide prediction analyses clearly emphasizes the high confidence of our secretome data set.

DAVID annotation clustering and the Cytoscape visualization tool ClueGO were used to functionally cluster secretome proteins according to their GO "biological process" terms. Both tools characterized proteins related to the leading functional terms "skeletal

system development", "vasculature development", "cell motion", and "cell adhesion", as predominantly enriched in the secretome. In the following paragraphs, we focus our discussion on the potential function with respect to the SSC niche of individual members of these clusters, all of which were detected in secretome samples of at least three of five donors.

Secreted Proteins Comprising Factors Playing a Major Role in the Skeletal System and Vasculature Development

Previous studies described a predominant production of ECM components by testicular peritubular cells (5, 41). It was shown that they secrete individual ECM proteins like fibronectin, collagens, and proteoglycans (7, 12, 42-44). The results obtained by our study mirror these findings. Structural proteins primarily consisting of ECM as well as basement membrane components were strongly over-represented in the secretome. In detail, we found a distinct enrichment of ECM proteins such as fibronectin, collagens I, III, IV, V, VI, XI, XII, XIV, XV, XVI, XVII, SPARC, and fibrillin-1, partially involved in skeletal system and vasculature development. To confirm that secretion of these proteins by HTPC cultures reflects the situation in the human testis, we performed immunohistochemistry, which depicts fibronectin, collagen I, and collagen IV. Therefore, HTPCs contribute to the formation of the basement membrane (45) and the overall architecture of the tubular wall and thus the formation of SSC niches along the basement membrane.

The enriched functional cluster "vasculature development" contains 21 secretome proteins, among them are connective tissue growth factor (CTGF) and vascular endothelial growth factor C (VEGFC). The latter is a member of the VEGF family, which resembles important signaling proteins for the regulation of neovascularization. VEGFC is mainly active in angiogenesis, lymphangiogenesis, and endothelial cell proliferation and induces the permeability of blood vessels (46).

The significant enrichment of proteins related to GO term "vasculature development" may reflect a notable contribution of HTPC-secreted proteins to the formation of a vasculature-oriented niche for undifferentiated spermatogonia (2). Furthermore, the

secretion of various growth factors like CTGF and VEGFC indicates a possible contribution of HTPCs to the niche growth factor milieu.

HTPC Secretome Enriched for Proteins Associated with Cell Motion and Cell Adhesion

GO analysis of secretome proteins revealed a significant enrichment of proteins taking part in the movement of cellular components. We observed an enrichment of proteins like versican, the multifunctional laminins LAMA1 and LAMC1, as well as C–C motif chemokine 2 (CCL2) and stromal cell-derived factor 1 (CXCL12), two chemokines associated with cellular component movement. Chemokines play a major role in stem cell mobilization, migration, and homing (47, 48), thus providing a further indication for the potential role of HTPCs contributing to the SSC niche. The detection of CXCL12 in the HTPC secretome is consistent with a recent study of Yang et al., who found CXCL12 in Sertoli cells as well as clearly focused at the basement membrane of seminiferous tubules in adult mice testis (49). They further described a contribution of CXCL12 to the regulation of SSC self-renewal by signaling via the C-X-C chemokine receptor type 4 (CXCR4) expressed by undifferentiated spermatogonia, which pointed to a possible role in the regulation of SSC homing. In a testis cell culture system with reconstituted mouse SSC niches, CXCL12 was moreover demonstrated to take part in SSC chemotaxis into the niche microenvironment (50). In addition, the CXCL12-CXCR4 signaling pathway seems to be important in retaining undifferentiated spermatogonia in their niche (51). These findings may imply chemotactic properties of HTPC-secreted proteins, which are possibly influencing SSC movement to the basal membrane niche.

Furthermore, cell adhesion proteins were clearly enriched in the HTPC secretome, among them the ECM proteins cadherin-13 (CDH13), fibulin-2 (FLBN2), laminin alpha-1 (LAMA1), laminin alpha-2 (LAMA2), and laminin alpha-4 (LAMA4). Laminins are ligands of alpha-6- and beta-1-integrin, which are surface markers on SSCs (52). More recently, beta-1-integrin and its ability to bind laminin were reported to be crucial in SSC homing to their regular location at the basal membrane (53, 54). We hypothesize

that the secretion of the ECM laminins by HTPCs may support the attachment of SSCs to the basement membrane and retain them within the basal compartment of the seminiferous tubules.

HTPCs Secrete Cell-Signaling Proteins Related to Inflammatory Response

DAVID analysis of the secretome data set revealed an enrichment of cell signaling molecules such as interleukin 6 (IL6) and pentraxin 3 (PTX3). This is surprising, because the latter is known to be produced in response to inflammatory signals, e.g. toll-like receptor (TLR) engagement, interleukin 1 beta (IL1B), and tumor necrosis factor alpha (TNF-α) (55). In human testis, TNF-α is released by macrophages and mast cells, which are located in the tubular wall close to the peritubular cells.(9) PTX3 further plays a role in the resistance against pathogens (55, 56) and the control of autoimmunity (57). Performing an additional immunohistochemical staining, the presence of PTX3 in the peritubular compartment could be proven (Figure 5). Interestingly, our results confirm the ones previously described in a study of Doni et al. (58), in which a different antibody was used to identify PTX3 in the human male genital tract. The authors focus their description of the testicular expression on germ cells of the testis. However, in Figure 1c of their manuscript, they show unequivocally the presence of immunoreactive PTX3 in peritubular cells, yet they do not comment on this site of expression. Most likely this is due to the inconspicuous nature of this testicular compartment (4), which may be related to the fact that little is known about the cells composing this compartment. These findings represent a further example for interaction between HTPCs and adjacent cell types and indicate an active participation of HTPC in the paracrine signaling network in human testis.

Secreted Proteins from HTPCs in Multicellular Signaling Pathways Potentially Directing SSC Fate

DAVID analysis addressing KEGG pathways revealed the transforming growth factor beta (TGF-β) signaling pathway to be significantly enriched for the secretome (Supplementary Table 7). This group included the secretome proteins thrombospondin

THBS1, THBS2, and THBS3, latent transforming growth factor beta binding protein 1 (LTBP1), inhibin beta A (INHBA), and follistatin (FST), as well as decorin (DCN), as shown previously (12) (Figure 6). DCN is a secreted proteoglycan located in the peritubular wall of the testis (59). It possibly interacts with several growth factors (including TGF-β and EGF), and in this way it likely disrupts the balance between testicular paracrine signaling pathways (12). It has been shown recently that DCN production in the testis is stimulated by TNF-α and related with fibrotic changes emerging from a testicular dysfunction (13).
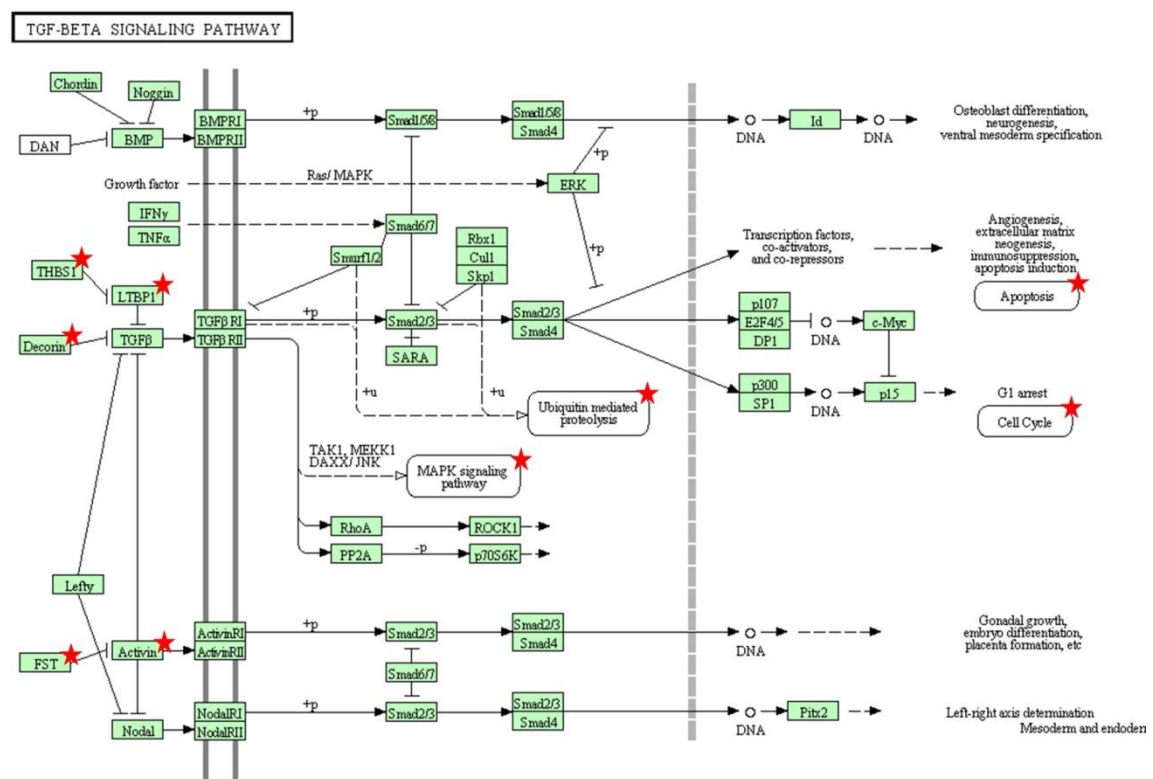
Figure 6. TGF-β signaling pathway is enriched in HTPC secretome. Involvement of HTPC secretome proteins in TGF-β signaling pathway and interlocked pathways is marked with a red star. Encircled biological processes are other pathway maps. Pathway information was generated from KEGG.

INHBA and FST are both part of the inhibin–activin–follistatin axis. Activin and inhibin are members of the TGF-β superfamily, whereas the autocrine glycoprotein FST is an antagonist of the TGF-β subfamily and counteracts the signaling of activing (60). In their interplay, INHBA and FST are involved in the regulation of follicle-stimulating

hormone (FSH) secretion (61). Besides testosterone, FSH is a major hormonal regulator of spermatogenesis and acts via FSH receptors expressed on Sertoli cells (62). Systemically, in hypogonadal rodents, FSH was shown to increase the number of spermatogonia during the initial stages of spermatogenesis and, indirectly, the number of Leydig cells (63). Furthermore, FSH is supposed to play a major role in the testicular development prior to puberty, which was observed in mice lacking receptors for FSH and androgen (64). In coculture experiments of Sertoli cells and type A spermatogonia, FSH led to secretion of glial cell line derived neurotrophic factor (GDNF) by Sertoli cells, which stimulated the proliferation of spermatogonia (65). GDNF is a well-established niche factor mainly produced by Sertoli cells (1, 15, 66, 67). However, recent studies provide first evidence that HTPCs also produce and secrete GDNF (28-56 pg/mg cellular protein within 24 h) and thereby complement the Sertoli cells in the contribution to the human SSC niche (11).

We hypothesize that HTPCs are involved in the development and maintenance of testicular functions by secreting several proteins that act through the multistage TGF-β signal transduction pathway in close interplay with surrounding Sertoli and Leydig cells.

Conclusion

Our analysis demonstrates the extensive secretory activity of a human cell type, which up to now has been regarded as a simple structural cell at the interface of the germinal epithelium and the interstitial cells of the testis. The secretome of HTPCs provides the most comprehensive information available to date of the assumed microenvironment of the human testis, especially the one prevailing in the wall of the seminiferous tubules. The structural nature of HTPCs is mirrored by the enrichment of ECM components involved in skeletal system and vasculature development. Yet the HTPC secretome contains chemokines and growth factors with the potential of influencing stem cell activity. Moreover, secreted proteins are involved in key signaling pathways. HTPCs are thus able to interact via secreted proteins with the surrounding testicular cell types, namely, Leydig cells and Sertoli cells, as well as germ cells, including SSCs.

While the study of any of these interactions may lead to a better understanding of the human testis, the results are of special relevance for the understanding of the microenvironment of the niches, in which human SSCs reside. The interest in human SSCs is due, in part, to their potential to treat male infertility, including infertility due to gonadotoxic cancer treatment of boys (68, 69). Adequate culture conditions and the ability to propagate human SSCs are prerequisites for possible therapies, which include transplantation back to the testis (69). The knowledge about the repertoire of factors secreted by HTPCs (i.e., structural and functional niche cells) offers now the unprecedented opportunity to test relevant factors for their ability to regulate human SSCs and thus to optimize culture conditions.

Supporting Information

Supplementary tables. This material is available free of charge via the Internet at http://pubs.acs.org.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Abbreviations

ABC	ammonium bicarbonate
ACN	acetonitrile
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
FCS	fetal calf serum
FDR	false discovery rate
GO	gene ontology
HTPC	human testicular peritubular cell
SSC	spermatogonial stem cell

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4   Discussion

Seminiferous tubules in human testis are surrounded by several layers of spindle-shaped, peritubular smooth muscle cells that are responsible for propelling immotile sperm through cycles of contraction and relaxation. Besides this established role in sperm transport, which is of undisputed importance for male fertility, astonishingly little is known about their role in the complex interplay of testicular cells regulating core functions of the testis. The anatomical proximity to Sertoli cells and SSCs, which reside on the inside of the seminiferous tubules, and Leydig cells in the interstitial compartment, depicts a strategic position in the paracrine signaling network of the testis.

The recent establishment of a method for the isolation and cultivation of human peritubular cells in the lab of Artur Mayerhofer allowed for the first time a systematic characterization of this cell type in the human system (Albrecht et al., 2006). In follow-up studies, they could give a first impression of the secretory potential of HTPCs (Adam et al., 2012a; Adam et al., 2011; Schell et al., 2008). Consequently, they made the significant finding that GDNF, a key factor in the regulation of SSC proliferation and renewal, is constitutively produced by HTPCs (Spinnler et al., 2010). These findings encouraged the speculation that peritubular cells via secreted factors are involved in the paracrine regulation of testicular functions, and in particular, in the regulation of the SSC niche in man.

To pursue this hypothesis, this study was designed as cooperation project between our group and the group of Artur Mayerhofer from the Department for Cell Biology (Faculty of Medicine, LMU Munich).

We used quantitative proteomics to comprehensively define the secretome of cultured HTPCs. Primary cells derived from five men were isolated and cultivated according to a standard procedure in the lab of Artur Mayerhofer (performed by Stefanie Windschüttl) as described (Albrecht et al., 2006). An extensive protein pre-fractionation using 1D SDS-PAGE along with nano-LC-MS/MS in combination with a label free quantification

by "spectral counting" was applied. Comprehensive proteomes of proteins in conditioned media and – as a control – in HTPC cell lysates were established.

4.1 Defining the secretome: Methodical aspects of secretome analysis

Among the major challenges faced in MS-based secretome studies are sample purity, complexity and the detection of low abundant proteins, such as immune-modulatory cytokines within a high protein dynamic range (Mallick and Kuster, 2010; Meissner et al., 2013). Our methodical approach was designed to address all these obstacles with the aim of achieving an extensive and reliable secretome profile of HTPCs.

Sample purity in mammalian culture models is strongly influenced by the serum supplementation during cultivation. A commonly used strategy to circumvent contamination with bovine serum proteins and potential masking of cell secreted proteins is repeated washing with PBS and cultivation in serum-free medium prior to collection of conditioned medium (Barderas et al., 2013; Brown et al., 2012; Chaker et al., 2013; Chen et al., 2012). However, even extensive washing is often not sufficient to remove serum proteins from conditioned media to a concentration below the detection limit of the MS system applied. We therefore used an augmented database including possible bovine serum contaminants to minimize erroneous cross-species identifications (Bunkenborg et al., 2010). The contaminants list matched to 20 out of 1786 total identifications, which were excluded for further data processing.

To reduce proteome complexity and coverage, we applied pre-fractionation at the protein level by 1D SDS-PAGE, which outperformed protein-LC separation techniques as a first-dimension separation strategy (Piersma et al., 2010). Combined with reversed phase LC and MS/MS-based spectral counting for relative quantification, this approach is highly attractive and has been implemented in various fields of secretome investigation (Gao et al., 2008; Piersma et al., 2010; Valimaki et al., 2013; Wu et al., 2010a). The great potential of this orthogonal protein-peptide separation method in handling dynamic range issues could be demonstrated by the detection of various low

abundant cytokines and growth factors in the presence of highly concentrated components of the extracellular matrix (ECM) within the secretome of HTPCs.

An additional potential risk of contamination of the secreted proteins in the conditioned media is constituted by intracellular proteins, which are released due to cell lysis and cell death. To exclude the latter from the final "secretome" dataset, we quantitatively compared protein abundance in conditioned media with corresponding cell lysates. A spectral count ratio > 5 in conditioned media compared to cell lysate was taken as prerequisite to be categorized as secreted. Taking into consideration that quantification accuracy is decreased for proteins with only few peptides identified (Gao et al., 2008; Mallick and Kuster, 2010), a minimum of 10 spectra per protein in one sample was required for relative quantification. Normalization between samples to compensate differences in the protein content was performed based on the total number of all observed spectra to furthermore increase the robustness and reliability of the quantification. In summary, stringent consecutive filtering for contaminants from remaining cell culture supplemented bovine serum proteins and intracellular proteins arisen from dead cells, yielded a total of 263 HTPC secreted proteins.

The subcellular location among the secretome proteins is distributed to 31 % onto the category "extracellular space", which represents 10 times more than in cell lysate data (3 %). A comparable enrichment of 28 % extracellular space annotations in the secretome vs. 2 % in the cell lysate could be observed in a secretome study of H460 non-small cell lung cancer cells using similar experimental conditions and secretion criteria (Piersma et al., 2010). The remaining fraction of matches to other cellular component ontologies ("cytoplasm", "plasma membrane" and "nucleus") for the secretome can be partly explained by the possibility of multiple annotations to different categories in GO classifications. This phenomenon is very vividly illustrated in the recently published "Tissue-based map of the human proteome" (Uhlen et al., 2015). In a whole proteome scan, the authors found a large overlap between different location categories for the human secretome, which could be traced back to the observation that two thirds of all genes encoding human secreted proteins show multiple splice variants with alternative locations. A second computational approach using the SignalP algorithm for signal

peptide prediction was applied to the HTPC dataset. Among secretome proteins, 81 % were confirmed to follow the classical secretory pathway. Remaining proteins without a predicted signal peptide may be released by alternative, non-classically secretion pathways (Nickel and Rabouille, 2009).

In conclusion, quantitative proteomics combined with computational prediction of subcellular locations and secretion mechanisms offers great potential for the establishment of a reliable and comprehensive secretome dataset derived from cell culture experiments.

4.2   Contribution of HTPCs to the architecture of the tubular wall

To investigate the functional nature of the HTPC secretome, we performed statistical enrichment analyses using the DAVID Bioinformatics Resources (Huang da et al., 2009) and the Cytoscape plug-in ClueGO, a tool for visualization of functionally grouped networks (Bindea et al., 2009). Dominant functional categories were indicated by both tools as enriched GO "biological process" terms as follows: "skeletal system development", "vasculature development", "cell motion", and "cell adhesion". These sets of enriched secretome proteins are discussed in the following chapters regarding their potential contribution to overall testicular functions and in particular to the SSC niche in men.

The wall enclosing seminiferous tubules in human testes is comprised by several layers of peritubular myoid cells and extracellular matrix (ECM) proteins. In our study, we detected a massive secretion of various ECM components by HTPCs. The large clusters related to skeletal system and vasculature development contained various forms of collagens, fibronectin, fibrillin-1 and SPARC among others. This is in agreement with previous studies, which also reported an extensive production of ECM proteins including collagens, fibronectin and proteoglycans by testicular peritubular cells (Adam et al., 2012a; Mayerhofer, 2013; Schell et al., 2008; Skinner et al., 1985). The predominance of ECM components also mirrors the situation in secretomes of

phenotypically and functionally similar smooth muscle cells (SMCs). Thereby, a broad fraction of collagenous and non-collagenous ECM proteins was measured in conditioned media from SMCs derived from human adipose tissue (Justewicz et al., 2012) as well as vascular SMCs isolated from Sprague-Dawley rat aorta (Gao et al., 2008), resulting in secretome datasets comprised of 31 and 212 putatively secreted proteins, respectively.

The observations in the human testis are particularly interesting in connection with cases of sub- or infertility with spermatogenesis disorders, which frequently show a strong fibrotic remodeling of the peritubular wall (Davidoff et al., 1990; Meineke et al., 2000; Welter et al., 2013). Tubular fibrosis in the human testis is characterized by irregular deposits of ECM components between the individual layers of peritubular myoid cells and is a strong indicator for male infertility (Davidoff et al., 1990; Gulkesen et al., 2002; Mayerhofer, 2013). In this context, peritubular cells from fibrotically remodeled human testis (HTPC-F) show, beside morphological changes, an altered secretion behavior which may lead to an imbalance of paracrine signaling in the testis (Adam et al., 2011). Both these alterations together suggest changes in the cellular behavior concerning the contractile and secretory abilities of peritubular cells in the state of testicular fibrosis (Mayerhofer, 2013; Welter et al., 2013). This tends to agree with the conviction in classical SMCs, where a phenotypic switching towards a more secretory phenotype is accompanied with a decline of the contractile function (Owens et al., 2004; Rose and Babensee, 2007).

To complement our MS-based secretome data, immunohistochemical analyses were carried out in the lab of Artur Mayerhofer and could confirm the *in vivo* expression of collagen I and IV and fibronectin in testis tissue slices of healthy men. It is worth mentioning that collagen is used as coating material or as gel matrix in artificial systems for the aspired *in vitro* cultivation of SSCs to stimulate their differentiation and colonization (De Chiara et al., 2014; Lee et al., 2007b; Shiva et al., 2016)

Collectively, peritubular cells secrete integral elements for the formation and potential remodeling of the basal membrane and the tubular wall and thus provide an architectural basis for ongoing spermatogenesis in fertile men.

4.3 Role of the HTPC secretome in the testicular vascular network

A substantial proportion of HTPC secretome proteins were significantly annotated to the term "vasculature development", among them the connective tissue growth factor (CTGF), vascular endothelial growth factor C (VEGF-C) and angiopoietin-related protein 2 (ANGPTL2). CTGF belongs to the matricellular proteins of the CCN family, has associated functions in angiogenesis, and is a key mediator and marker in various fibrotic diseases (Brigstock, 2010; Dendooven et al., 2011; Hall-Glenn and Lyons, 2011; Phanish et al., 2010). VEGF family members play a crucial role in the regulation of angiogenesis in mammals. VEGF-C in particular is reported to be the key paracrine factor required for lymphatic development (Karkkainen et al., 2004; Makinen et al., 2001). Furthermore, VEGF-C has been shown to be involved in proliferation and migration of endothelial cells and the regulation of vascular permeability (Ergun et al., 1994; Karkkainen et al., 2004; Lohela et al., 2009; Makinen et al., 2001; Yamazaki and Morita, 2006). Prominent angiogenesis inhibitors among the HTPC secretome dataset are thrombospondin-2 and pigment epithelium-derived factor (PEDF). The former seems to be a potential inhibitor of endothelial cell proliferation, migration and capillary tube formation, and is discussed as anti-angiogenetic therapeutic agent (Bouis et al., 2006; Vailhe and Feige, 2003). However, particularly PEDF has gained an established position as potent inhibitor of angiogenesis (Bouck, 2002; Chuderland et al., 2014; Dawson et al., 1999). There is emerging evidence that PEDF antagonizes VEGF in its driving force for neovascularization in tissues and thus it is important in the maintenance of an angiogenic balance (Chuderland et al., 2014; Pollina et al., 2008; Zhang et al., 2006b). The anti-angiogenic power of PEDF was impressively demonstrated in studies on diabetic retinopathy, where PEDF effectively suppressed both the expression as well as the receptor binding of pro-angiogenic VEGF (Zhang et

al., 2006b). This has given rise to the assumption that HTPCs via secreted proteins are involved in the maintenance of the avascular nature of the seminiferous epithelium. In the human testis, the vascular network is strictly limited to the peritubular compartment and the interstitium, whereas the tubular compartment is not penetrated by blood vessels and lymphatic channels (Ergun et al., 1994). Strikingly, the vascular formation in the interstitial space seems to affect the establishment of SSC niches in murine testis, which were observed to be preferentially located adjacent to the vascular network and interstitial cells (Yoshida et al., 2007).

Building on the results of our secretome study, Windschüttl and coworkers examined the role of HTPC secreted PEDF in the establishment and maintenance of the avascularity of seminiferous tubules (Windschuttl et al., 2015). In co-culture experiments using individual cell tracking, they observed a repulsion of human umbilical vein endothelial cells (HUVECs) by HTPCs, which could be blocked by the addition of a PEDF antiserum. This indicates a predominant anti-angiogenetic influence of HTPC-derived PEDF on preventing the vascularization of seminiferous tubules in men. They furthermore revealed an enhanced expression of PEDF in HTPCs after dihydrotestosterone treatment, with a simultaneous decrease of the pro-angiogenic proteins VEGF-C and ANGPTL2 (Windschuttl et al., 2015). Hence it is assumed that locally controlled PEDF secretion, in coordination with angiogenic stimulators, shapes the complex vascular network of the seminiferous tubules and in consequence contributes to the human SSC niche.

4.4 Participation of HTPC secretome proteins in the molecular control of SSC homeostasis

For a substantial number of proteins of the HTPC secretome, roles in processes underlying cell migration have been reported. Examples include versican, the laminins alpha-1 (LAMA1) and gamma-1 (LAMC1), as well as the chemotactic factors C-C motif chemokine 2 (CCL2) and stromal cell-derived factor 1 (CXCL12). The successful detection of these low-abundant chemokines illustrates again the high sensitivity of the applied MS-based strategy. Chemokines are awarded a central role in the mobilization

and homing of stem cells, as reviewed for hematopoietic and mesenchymal stem cells (Lapidot and Petit, 2002; Wu and Zhao, 2012).

In particular CXCL12 is reported to be a key factor in stem cell migration and moreover to be essential for stem cell maintenance (Ding and Morrison, 2013; Greenbaum et al., 2013; Khurana et al., 2014; Ratajczak et al., 2013; Sugiyama et al., 2006). In accordance with our secretome data, CXCL12 could be detected in testes from adult mice, concentrated at the basement membrane of seminiferous tubules (Yang et al., 2013). Its receptor, C-X-C chemokine receptor type 4 (CXCR4), is expressed on undifferentiated spermatogonia and is triggered for SSC migration and expansion upon transplantation (Ara et al., 2003; Kanatsu-Shinohara et al., 2012; Niu et al., 2016; Yang et al., 2013). In this context, CXCL12-CXCR4 signaling is of crucial relevance for SSC homing to cognate niches and consequently for the re-establishment of spermatogenesis. Inhibition of the CXCL12-CXCR4 axis in studies on cultured undifferentiated mouse spermatogonia leads to a loss of SSCs, and in adult mice to an impaired SSC maintenance, ending with a complete loss of the germline (Yang et al., 2013). To summarize, CXCL12-CXCR4 signaling may influence SSC activities in a variety of ways and CXCL12 therefore constitutes a valuable contribution to the niche growth factor milieu in mammalian testes.

Many of the secreted proteins were linked to cell adhesion regulation processes. This enriched cluster included multifunctional ECM components of the basement membrane, such as laminin alpha-1 (LAMA1), alpha-2 (LAMA2), alpha-4 (LAMA4) and fibulin-2 (FLBN2) and other well-studied adhesion molecules, e.g. cadherin-13 (CDH13). Cell-to-cell adhesion is an intensely discussed topic in SSC homing, which is defined as the migration and retention of stem cells in cognate niches (Oatley and Brinster, 2012). In the male reproductive system, SSC homing to the basement membrane niche is a crucial prerequisite for ongoing spermatogenesis. SSCs express the transmembrane proteins alpha-6- and beta-1-integrin, known adhesion receptors for laminin molecules and thus may enable an interaction with the nearby basement membrane (Shinohara et al., 1999). Impaired beta-1-integrin expression on mouse SSCs inhibited the attachment to basement membrane associated laminin and prevented the

reestablishment of spermatogenesis after transplantation (Kanatsu-Shinohara et al., 2008b). Thus, HTPC secreted laminins likely play an important role for retaining SSCs in their dedicated locations at the basement membrane and thereby ensure physical stem cell maintenance inside their niches.

DAVID computational enrichment analysis disclosed cell signaling molecules related to inflammatory response to be significantly enriched in the HTPC secretome. Interleukin–6 (IL-6) and pentraxin 3 (PTX3) represent prominent examples for this functional cluster. The latter was among the most abundant proteins in the secretome dataset according to spectral counting. This is all the more unexpected, since very limited knowledge exists so far about its role within the testis. Nevertheless, expression of PTX3 in the testis has already been demonstrated (Doni et al., 2009) and could be confirmed by the group of Artur Mayerhofer within the framework of the HTPC secretome studies. Using immunohistochemical staining of human testicular biopsies, the expression in the peritubular compartment could be verified. PTX3 belongs to the group of soluble pattern recognition receptors and is part of the innate immune system. It is primarily known to be involved in resistance against selected pathogens and microorganisms, and in protection against autoimmunity (Baruah et al., 2006; Garlanda et al., 2005; Garlanda et al., 2002). Production of PTX3 is stimulated by toll-like receptor engagement and primary proinflammatory signals, as for example interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) (Garlanda et al., 2005). Sources of TNF-α in the human testis are mast cells and macrophages, which can be found within the interstitial, but also within the peritubular compartment (DeFalco et al., 2015). Interestingly, it was also shown that TNF-α increased the production of inflammatory markers by HTPCs, including the cytokines monocyte chemoattractant protein-1 (MCP-1) and IL-6, suggesting a local regulation of peritubular cells by adjacent immune cells (Schell et al., 2008). Similarly, with regard to PTX3, a rapid and local activation under inflammatory conditions was proposed (Garlanda et al., 2005). Whether and to what degree this knowledge is transferable to the prevailing situation in human testis and in particular to HTPCs remains to be elucidated. Collectively, the secretion of multiple signaling molecules, including locally regulated inflammatory markers,

reinforces the impression that numerous paracrine interactions between HTPCs and their surrounding testicular cells may occur.

A key finding of the secretome study could be obtained from a KEGG pathway screening based on DAVID Bioinformatics Resources, which indicated a significant enrichment of the transforming growth factor beta (TGF-β) signaling pathway for the HTPC secretome. This is particularly interesting since TGF-β signaling plays a pivotal role in the regulation of core functions during testis development and in the maintenance of adult spermatogenesis (Fan et al., 2012; Loveland and Hime, 2005; Miles et al., 2013; Moreno et al., 2010; Young et al., 2015). Among secretome proteins involved in this signaling pathway were decorin (DCN), latent-transforming growth factor beta-binding protein 1 (LTBP-1), thrombospondins (TSP-1, TSP-2, and TSP-3), inhibin beta A (INHBA), and follistatin (FST).

DCN is a family member of small leucine-rich proteoglycans and has already been described in the peritubular wall of the human testis some time ago (Ungefroren et al., 1995). More recent studies revealed an enhanced secretion of DCN by HTPCs in response to TNF-α and tryptase stimuli, both known products of macrophages and mast cells in the testis (Adam et al., 2011; Adam et al., 2012b). In conformity with this, an increase in DCN levels could also be observed in testes of men with impaired spermatogenesis along with tubular fibrosis, which is well known to be accompanied by an increased number of immune cells in the peritubular compartment (Adam et al., 2011; Adam et al., 2012b). DCN interacts with a variety of growth factors, as described for TGF-β (Hildebrand et al., 1994) and platelet-derived growth factor (PDGF) (Nili et al., 2003). Additionally, it can itself bind to several growth factor receptors, some of which are expressed by multiple testicular cell types, including peritubular cells, Leydig cells, Sertoli cells and SSCs (Basciani et al., 2010; Foresta et al., 1991; Mayerhofer, 2013; Wang et al., 2015). Well documented DCN receptors represent the epidermal growth factor receptor (EGFR) (Schaefer and Iozzo, 2008), insulin-like growth factor 1 receptor (IGF-1R) (Schonherr et al., 2005) and vascular endothelial growth factor receptor (VEGFR) (Iacob et al., 2008). Thus, altered DCN levels in male infertility are likely to

lead to an imbalance in growth factor signaling in the testis and may affect the SSC niche in a direct and indirect manner.

Other HTPC secretome proteins involved in TGF-β signaling were INHBA and FST, which are both negative regulators of activin A activity. Activin A and inhibin belong to the TGF-β superfamily and were first discovered for their negative regulation of follicle-stimulating hormone (FSH) secretion by pituitary gonadotropes (Ling et al., 1986; Robertson et al., 1986). FSH directly influences Sertoli cell function and induces local growth factor signaling (i.e., GDNF), and thus indirectly influences SSC fate (reviewed in (Barakat et al., 2012; de Kretser et al., 2001; Hedger and Winnall, 2012)). Later studies suggest more and more local actions of inhibin, activin and follistatin on fundamental testicular processes, during onset of spermatogenesis in the developing testis (Barakat et al., 2008; Fan et al., 2012; Miles et al., 2013; Mithraprabhu et al., 2010; Moreno et al., 2010) and in the adult testis in spermatogenesis and immunoregulation (reviewed in (Hedger and Winnall, 2012)). A recently published study showed in an impressive way, that TGF-β inhibits proliferation of tubule-associated stem Leydig cells and has inhibitory effects on differentiation, whereas activin stimulated Leydig cell differentiation (Li et al., 2016). The superior role of peritubular cells in the regeneration of Leydig cells (Landreh et al., 2014; Li et al., 2016; O'Shaughnessy et al., 2008) may thereby hint at a participation of HTPC-released factors in the local control of Leydig cell maintenance, thus ensuring essential testosterone supply in males.

The critical role of TGF-β signaling for the development and regulation of testicular functions is well-known. Due to the striking accumulation of secretome proteins involved in the complexity of TGF-β signaling, HTPCs may likely contribute to the molecular control of SSC homeostasis, in a direct dialogue with adjacent Sertoli and Leydig cells.

4.5   Possible impact of HTPCs on aging of the SSC niche

A hallmark of virtually all living organisms is the gradual decline of structural and functional integrity with increasing age. Aging in all imaginable variations has

fascinated and preoccupied people for millennia. Reproductive aging has become an emerging issue with the trend towards delayed childbearing in advanced countries. While age-associated alterations of the human ovary ultimately lead to a complete omission of oocyte function, testicular aging is much less pronounced. Nevertheless, a decline in male reproductive functions has become evident in man with advancing age (reviewed in (Johnson et al., 2015; Kuhnert and Nieschlag, 2004; Plas et al., 2000; Zitzmann, 2013)). Advanced paternal age often results in a reduced reproductive potential, which is characterized by a decline in semen quality. In this context, various studies report a decline in semen volume, sperm motility and normal morphology, and a higher DNA fragmentation with increasing male age (reviewed in (Johnson et al., 2015)). Other investigations highlight the association between paternal age and offspring health, with an accumulation of birth defects and diseases in the children of older fathers (Kovac et al., 2013; Sartorius and Nieschlag, 2010). Testicular aging also includes a lower testosterone production in older men. Lower testosterone levels can be traced back to reduced Leydig cell functions with advancing age and are often accompanied by an altered body composition, reduced energy level, muscle strength and sexual function, decreased cognitive functions and depressed mood (Matsumoto, 2002; Zirkin and Tenover, 2012). Lower testosterone levels may, however, also lead to testicular alterations with advancing paternal age, including germ cell loss and an increased rate of Sertoli cell-only syndrome in older men (Paul and Robaire, 2013). Therefore, it appears probable that aging affects spermatogenesis and underlying SSC functions. However, the diverse causes and mechanisms of SSC aging are still poorly understood.

Initial transplantation experiments of aged SSCs in rodent testes imply that both SSCs and their testicular environment including supporting niche cells are affected by aging (Zhang et al., 2006c). Further SSC transplantation studies suggest that particularly the age-dependent decline of niche components may cause a deterioration of the SSC niche and therefore has a main responsibility for infertility in old men (Ryu et al., 2006).

In this context, the question arises whether HTPCs are affected by aging, and whether and how HTPCs could contribute to aging of the SSC niche in man. As a test for this

hypothesis, a follow-up project funded by the Deutsche Forschungsgemeinschaft (DFG) could be initiated, building on the illuminating insights obtained from functional *in-vitro* experiments and proteomics studies on cultured HTPCs, which are discussed in this work.

To date, descriptive studies on testicular tissue samples of aged men could already show age-related alterations of the tubular wall, such as thickening of the basement membrane. Stereological analyses indicated a decline of peritubular cells in testes of older men (Xia et al., 2012). Additionally, in animal studies on aged hamsters (Morales et al., 2004), aged horses (Fukuda et al., 2001) and brown Norway rats (Wang et al., 2002) distinct fibrotic changes of the wall of seminiferous tubules could be observed and indicated important functional alterations of testicular peritubular cells with aging. Notwithstanding their stereotyped and tissue-specific behavior, almost all cells, including stem and progenitor cells, can undergo phenotypic changes both *in vivo* and *in vitro* that are closely associated with aging. This phenomenon, cellular senescence, is manifested by a permanent cell-cycle arrest upon a limited number of divisions and is regarded as a critical hallmark of aging (Campisi and d'Adda di Fagagna, 2007; Lopez-Otin et al., 2013). *In vitro*, a basic distinction can be drawn between telomere-dependent replicative senescence and a stress-induced premature senescence (Sikora et al., 2014). Common biomarkers of senescent cells *in vitro* and *in vivo* are the increase in senescence-associated β-galactosidase (SA-β-GAL) activity (Debacq-Chainiaux et al., 2009; Dimri et al., 1995) and a senescence-associated secretory phenotype (SASP) (Kuilman et al., 2010; Rodier and Campisi, 2011). The SASP is characterized by an altered secretome of senescent cells, which is particularly distinguished by an enrichment of inflammatory molecules (e.g. pro-inflammatory cytokines) (Freund et al., 2010), as well as growth factors (Coppe et al., 2006; Rodier and Campisi, 2011). An increased knowledge about senescent cells and the SASP is suggested to open up new therapeutic opportunities for the delay and prevention of age-related diseases (Tchkonia et al., 2013). This idea could presumably be applicable to the testis, as well.

The investigation of testicular aging is as yet challenged by the lack of reliable markers for senescence (Kuilman et al., 2010) and relevant cellular models for the human testis,

which could be beneficial for decoding mechanisms of senescence (Campisi, 2005). These limitations may be a reason, why to the best of my knowledge no studies on cellular senescence were conducted in any specific cell type of the human testis and to my knowledge, only peritubular cells would be available for that purpose.

Peritubular cells in culture can be passaged over a rather long period of time until they show initial indications of senescence, namely a reduced proliferation rate, an increased cell size, and expression of β-galactosidase. Pilot studies serving as preliminary work for this project could already prove significant and expected senescence-related alterations of *in-vitro* propagated HTPCs, including reduced proliferation ability and conversely, an increase in mean cell size with advancing passage number. In addition, β-galactosidase expression could be clearly detected in obviously senescent HTPCs (Mayerhofer lab, data not shown). To evaluate global age-associated changes in the cellular proteome and the secretome, reflecting the SASP of HTPCs, a comprehensive proteomics analysis was performed on young and old HTPCs. Samples were compared from old (senescent, high passages) and young (pre-senescent, low passages) HTPCs, stemming from identical donors. First evaluations already indicate extensive alterations in the cellular proteome and the SASP of senescent HTPCs (Flenkenthaler et al., unpublished). Both the cellular proteome as well as the SASP dataset contain promising candidate proteins that are considerably altered in abundance in older passages. Aging-related changes outlined by the SASP are reported to induce paracrine senescence in healthy cells (Acosta et al., 2013) and may lead to an altered intercellular communication, an established hallmark of aging (Lopez-Otin et al., 2013). These changes of secreted factors therefore imply a realignment of the paracrine signaling network of the testis and may affect the communication with surrounding testicular cell types, including SSCs. The SASP may lead to defects in SSC and niche function by systemic factors, resulting in an age-related decline of SSC activity. A detailed analysis of aging-related alterations in the SASP dataset can lead to further information, whether this age-related secretory repertoire of HTPCs may modulate SSC activity and thus contribute to aging of the SSC niche in man.

The comprehensive proteomics data obtained from ongoing studies are supposed to make substantial contributions to the understanding of the senescence of HTPCs, and will likely shed more light on aging of the human SSC niche and altogether on age-related infertility in man.

5 Conclusion

In summary, modern MS-based proteomics in combination with extensive bioinformatics data analysis could provide unprecedented insights in the repertoire of secretion products from cultured HTPCs. This study clearly underlines the important role of peritubular cells for human testis function and the SSC niche in particular.

The medical significance of human SSCs is founded in their great potential to treat testis-induced infertility, including the restoration of spermatogenesis in cancer survivors. However, there are still some obstacles on the way towards a clinical implementation of a regenerative SSC therapy. Appropriate culture conditions are thereby an indispensable requirement for a stable *in vitro* SSC propagation and subsequent SSC transplantation (Struijk et al., 2013).

These data provide now a comprehensive platform for further functional studies of HTPC secreted proteins investigating the regulation of testicular functions, and particularly the molecular regulation of the human SSC niche in context of testicular health and disease. Moreover, this knowledge may lead to further advances in the establishment of a reliable culture system for the long-term propagation of isolated human SSCs and might help in future treatment of male infertility.

6   Summary

The continuity of spermatogenesis throughout the adult life relies on a strictly regulated balance between self-renewal and differentiation of spermatogonial stem cells (SSCs). Their activities are strongly influenced by a complex interplay of extrinsic features provided by a specialized niche microenvironment within the seminiferous tubules of the testis. However, the composition and structure of SSC niches is still poorly understood. In mammals, surrounding testis somatic cell populations are supposed to direct the niche functions. Among them are peritubular myoid cells, which in men are organized in several layers around the seminiferous tubules, were recently indicated to influence the SSC niche and overall testicular function in men via secreted factors. Peritubular myoid cells are smooth muscle-like cells that build the wall of the seminiferous tubules encasing the tubular compartment with underlying Sertoli cells and SSCs. Beside their unique role in the transport of immotile sperm by peristaltic contractions, their overall importance for regulation of testicular function and especially for the SSC niche remains unclear.

Addressing this issue, the present work describes a comprehensive proteomic characterization of the secretome derived from cultured human testicular peritubular cells (HTPCs). Using modern nano-liquid chromatography tandem mass spectrometry in combination with rigorous data analysis and bioinformatics, this approach allowed previously unrivaled insights into the large secretory activity of peritubular cells in the human testis. Computational Gene Ontology enrichment analyses enabled a systematic dissection of the obtained rich secretome catalog and could clearly broaden the horizon of the formerly inconspicuous peritubular cells within the orchestration of testicular functions in male reproductive health and disease. The significant enrichment of extracellular matrix components related to skeletal system and vasculature development is thereby representative for the primary structural nature of HTPCs and moreover highlights the integral role of HTPCs in providing an architectural basis for the establishment of SSC niches in men. HTPCs seem capable to interact with nearly all testicular cell types and show a lively participation in the paracrine signaling network

controlling testicular functions by the secretion of numerous signaling molecules. It could be illustrated that HTPC secretory products act in key signaling pathways that contribute to the progression of spermatogenesis, and may thereby participate either directly or indirectly in the molecular control of SSC maintenance.

In summary, the presented work emphasizes the undervalued potential of peritubular cells as a decisive player for human male fertility and will advance the understanding of the human spermatogonial stem cell niche in reproductive health and disease.

7   Zusammenfassung

Sekretomanalyse von testikulären peritubulären Zellen als Ansatz, um die Funktion der humanen spermatogonialen Stammzellnische zu klären

Die kontinuierliche Aufrechterhaltung der Spermatogenese während des Erwachsenenlebens basiert auf einer präzise geregelten Balance zwischen Selbsterneuerung und Differenzierung von spermatogonialen Stammzellen (SSCs). Deren Aktivitäten werden stark von einem komplexen Zusammenspiel von extrinsischen Faktoren bestimmt, die von ihrer speziellen Mikroumgebung (Nische) innerhalb der Samenkanälchen des Hodens bereitgestellt werden. Die Zusammensetzung und Struktur dieser SSC Nischen ist noch weitestgehend unbekannt. Bei Säugetieren wird vermutet, dass die umliegenden somatischen Zellpopulationen die Funktionen der Nischen maßgeblich steuern. Dazu zählen die peritubulären myoiden Zellen, die beim Mann in mehreren Lagen um die Samenkanälchen angeordnet sind. Aktuelle Studien weisen bereits auf einen Einfluss dieser Zellen auf die SSC Nische und die Regulation der Spermatogenese hin. Peritubuläre Zellen besitzen einen Glattmuskelzell-ähnlichen Phänotyp und sind ein Hauptbestandteil der Wand der Samenkanälchen, die das tubuläre Kompartiment, das auch die Sertoli-Zellen und SSCs beherbergt, umschließt. Neben ihrer erwiesenen Rolle beim Transport von noch unbeweglichen Spermien durch peristaltische Kontraktionen, ist ihre Gesamtbedeutung für die Regulation von testikulären Funktionen und insbesondere für die SSC Nische noch unklar.

Zu dieser Thematik wurde in der vorliegenden Arbeit eine umfassende proteomische Charakterisierung des Sekretoms von kultivierten humanen testikulären Peritubulärzellen (HTPCs) durchgeführt und beschrieben. Durch die Kombination von moderner nano-Flüssigkeitschromatographie mit Tandem-Massenspektrometrie, gefolgt von einer statistischen Datenanalyse und bioinformatischen Auswertungen, ermöglichte dieser Ansatz einen bisher unerreichten Einblick in die enorme Sekretionsaktivität von peritubulären Zellen im Hoden. Mittels computergestützten „Gene Ontology enrichment" Analysen konnte eine systematische funktionelle Analyse

des umfassenden Sekretomprofils durchgeführt werden. Dabei konnte das Potential der bisher wenig beachteten peritubulären Zellen für die Steuerung von testikulären Funktionen in Fertilität und Infertilität deutlich aufgezeigt und erweitert werden. Die signifikante Anreicherung von extrazellulären Matrixproteinen im Sekretom reflektiert die grundlegende strukturelle Natur von HTPCs und unterstreicht ihre wesentliche Rolle als Strukturgeber von SSC Nischen. HTPCs scheinen in der Lage zu sein, mit beinahe allen testikulären Zelltypen zu kommunizieren und zeigen durch die Sekretion von zahlreichen Signalmolekülen eine sehr aktive Teilnahme am parakrinen Signalnetzwerk, das für die Steuerung von wichtigen testikulären Funktionen verantwortlich ist. Es konnte gezeigt werden, dass von HTPC sezernierte Proteine in Signalwegen involviert sind, die zum Fortgang der Spermatogenese von entscheidender Bedeutung sind. Es besteht daher eine hohe Wahrscheinlichkeit, dass diese Proteine entweder direkt oder indirekt zur Erhaltung von SSCs beitragen.

Zusammengefasst zeigt die vorliegende Arbeit die wichtige und bisher oft unterschätzte Rolle von peritubulären Zellen für die männliche Fertilität. Sie verbessert das Verständnis über die Funktion der Stammzellennische im menschlichen Hoden und ist daher von hoher Relevanz für die Reproduktionsmedizin.

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9   Appendix

9.1   Supporting information

Supplementary tables from (Flenkenthaler et al., 2014):

Supplementary table 1:   Proteins identified in HTPC cell supernatant and cell lysate samples including mapped contaminants.

Supplementary table 2:   Proteins identified in HTPC cell lysate samples and normalized spectral counts.

Supplementary table 3:   Proteins identified in HTPC cell supernatant samples and normalized spectral counts.

Supplementary table 4:   HTPC cell supernatant vs. cell lysate normalized spectral count ratio and SignalP analysis.

Supplementary table 5:   HTPC secretome proteins and SignalP analysis.

Supplementary table 6:   DAVID Functional Annotation Clustering of HTPC secretome proteins.

Supplementary table 7:   DAVID Functional Annotation Chart of HTPC secretome proteins regarding KEGG pathways.

This material is available at http://pubs.acs.org/doi/suppl/10.1021/pr400769z

9.2 Publications

9.2.1 Original articles

Frohlich, T.; Kemter, E.; <u>Flenkenthaler, F.</u>; Klymiuk, N.; Otte, K.; Blutke, A.; Krause, S.; Walter, M. C.; Wanke, R.; Wolf, E.; Arnold, G. J. Progressive muscle proteome changes in a clinically relevant pig model of Duchenne muscular dystrophy. *Sci. Rep.* **2016**, 6, 33362.

Windschuttl, S.; Kampfer, C.; Mayer, C.; <u>Flenkenthaler, F.</u>; Frohlich, T.; Schwarzer, J. U.; Kohn, F. M.; Urbanski, H.; Arnold, G. J.; Mayerhofer, A. Human testicular peritubular cells secrete pigment epithelium-derived factor (PEDF), which may be responsible for the avascularity of the seminiferous tubules. *Sci. Rep.* **2015**, 5, 12820.

<u>Flenkenthaler, F.</u>; Windschuttl, S.; Frohlich, T.; Schwarzer, J. U.; Mayerhofer, A.; Arnold, G. J. Secretome Analysis of Testicular Peritubular Cells: A Window into the Human Testicular Microenvironment and the Spermatogonial Stem Cell Niche in Man. *J. Proteome Res.* **2014**, 13 (3), 1259-1269.

Nynca, J.; Arnold, G.J.; Frohlich, T.; Otte, K.; <u>Flenkenthaler, F.</u>; Ciereszko, A. Proteomic identification of rainbow trout seminal plasma proteins. *Proteomics* **2014**, 14, 133-140.

9.2.2 Oral presentations

Flenkenthaler, F.; Windschuttl, S.; Frohlich, T.; Schwarzer, J. U.; Mayerhofer, A.; Arnold, G. J. Secretome analysis of human testicular peritubular cells (HTPCs) indicates an impact on the spermatogonial stem cell niche. *48th Annual Conference Physiology and Pathology of Reproduction* **2015**, Zurich, Switzerland

Flenkenthaler, F.; Windschuttl, S.; Frohlich, T.; Schwarzer, J. U.; Mayerhofer, A.; Arnold, G. J. Secretome analysis of human testicular peritubular cells (HTPCs) indicates an impact on the spermatogonial stem cell niche. *ReproZentrum München* **2015**, Munich, Germany

Flenkenthaler, F., and Windschuttl, S. Secretome analysis of testicular peritubular cells: A window into the human testicular microenvironment and the spermatogonial stem cell niche in man. *3rd Workshop of the Research Unit 'Germ Cell Potential'* **2013**, Münster, Germany

Flenkenthaler, F., and Windschuttl, S. Peritubular factors and SSC. *2nd Workshop of the Research Unit 'Germ Cell Potential'* **2012**, Münster, Germany

9.2.3 Abstracts and poster presentations

Flenkenthaler, F.; Windschuttl, S.; Frohlich, T.; Schwarzer, J. U.; Mayerhofer, A.; Arnold, G. J. Secretome analysis of human testicular peritubular cells (HTPCs) indicates an impact on the spermatogonial stem cell niche. *48th Annual Conference Physiology and Pathology of Reproduction* **2015**, Zurich, Switzerland

Flenkenthaler, F.; Windschuttl, S.; Frohlich, T.; Schwarzer, J. U.; Mayerhofer, A.; Arnold, G. J. Secretome analysis of testicular peritubular cells: A window into the human testicular microenvironment and the spermatogonial stem cell niche. *Gene Center Retreat* **2014**, Wildbad-Kreuth, Germany

Flenkenthaler, F.; Windschuttl, S.; Frohlich, T.; Schwarzer, J. U.; Mayerhofer, A.; Arnold, G. J. MS Profiling of the HTPC Secretome to Explore their Contribution to the Spermatogonial Stem Cell Niche. *5. DVR-Kongress des Dachverbands Reproduktionsbiologie und –medizin e.V.* **2013**, Münster, Germany

Flenkenthaler, F.; Windschuttl, S.; Frohlich, T.; Mayerhofer, A.; Arnold, G. J. MS Profiling of the HTPC Secretome to Explore their Contribution to the Spermatogonial Stem Cell Niche. *Gene Center Retreat* **2013**, Wildbad-Kreuth, Germany

Awarded with prize for best poster presentation.

Flenkenthaler, F.; Windschuttl, S.; Frohlich, T.; Wolf, E.; Mayerhofer, A.; Arnold, G. J. Secretome Analysis of Human Peritubular Cells: Impact on Testicular Stem Cells and their Niches. *11ᵗʰ Annual World Congress of the Human Proteome Organization (HUPO)* **2012**, Boston, MA, USA

Flenkenthaler, F.; Windschuttl, S.; Frohlich, T.; Wolf, E.; Mayerhofer, A.; Arnold, G. J. Secretome Analysis of Human Peritubular Cells: Impact on Testicular Stem Cells and their Niches. *Gene Center Retreat* **2012**, Wildbad-Kreuth, Germany

9.3 Travel grants

Fellowship, Deutsche Gesellschaft für Proteomforschung (DGPF)

Flenkenthaler, F.; Windschuttl, S.; Frohlich, T.; Wolf, E.; Mayerhofer, A.; Arnold, G. J. Secretome Analysis of Human Peritubular Cells: Impact on Testicular Stem Cells and their Niches. *11ᵗʰ Annual World Congress of the Human Proteome Organization (HUPO)* **2012**, Boston, MA, USA

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