The role of peritubular cells in testicular functions and aging

Nina Maria Schmid

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Zweitgutachter: Prof. Dr. Gisela Grupe

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München, 11.11.2019

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Content

Abbreviations

3D	Three-dimensional
AROM	Aromatase
ASC	Apoptosis associated speck-like protein containing a caspase activation and recruitment domain
ATP	Adenosine 5´triphosphate
Bcl-2	B-cell lymphoma 2
BPA	Bisphenol A
BTB	Blood testis barrier
CAPS	Cryopyrin-associated periodic syndromes
CARD	Caspase activation and recruitment domain
CCF	Cytoplasmic chromatin fragments
CCL2	C-C motif chemokine ligand 2
CDKi	Cyclin-dependent kinase inhibitor
CEBPβ	CCAAT/enhancer binding protein-β
CINCA	Chronic infantile neurological cutaneous articular syndrome
CLEM	Correlative light electron m
COX	Cyclooxygenase
CXCL	C-X-C motif chemokine ligand
CXCR4	C-X-C motif chemokine receptor 4
DAMP	Danger-associated molecular patterns
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DPP4	Dipeptidyl peptidase 4
ECM	Extracellular matrix
EM	Electron microscopy
ER	Endoplasmic reticulum
FCAS	Familial cold auto-inflammatory syndrome
FGF	Fibroblast growth factor
FIB	Focused ion beam
G-phase	Gap-phase
GDNF	Glial cell line-derived neurotrophic factor
GH	Growth hormone
GSDMD	Gasdermin D
HeLa	Henrietta Lacks
HGF	Hepatocyte growth factor

hTERT	Human telomerase
HTPC	Human testicular peritubular cells
IGF-1	Insulin-like growth factor-1
IL	Interleukin
INF-γ	Interferon gamma
K	Potassium
LH	Luteinizing hormone
LM	Light microscopy
LPS	Lipopolysaccharides
MA	Mixed atrophy
MAM	Mitochondria associated membranes
MERC	Mitochondria-ER contacts
MIF	Macrophage migration inhibitory factor
MKTPC	Monkey testicular peritubular cells
MMP	Matrix metallopeptidases
MS	Mass spectrometry
mtDNA	Mitochondrial DNA
MWS	Muckle-Wells Syndrome
NF-κB	Nuclear factor kappa B
NLR	Nucleotide-binding oligomerization domain-like receptor
NLRP3	NLR family pyrin containing 3
nm	Nanometer
NOA	Non-obstructive azoospermia
NOMID	Neonatal-onset multisystem inflammatory disorder
$p16^{INK4A}$	Cyclin-dependent kinase inhibitor 2A
$p21^{CIP1}$	Cyclin-dependent kinase inhibitor 1A
P2X	Purinergic receptor
p53	Tumor protein p53
PAMP	Pathogen-associated molecular patterns
PAR	Protease-activated receptor
PEDF	Pigment epithelium-derived factor
PTX3	Pentraxin 3
PYD	Pyrin-domain
qPCR	Quantitative real time polymerase chain reaction
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
S-phase	Synthesis phase
SA β-gal	Senescence-associated β-galactosidase

SAHF	Senescence-associated heterochromatin foci
SASP	Senescence-associated secretory phenotype
SCO	Sertoli cell only
SEM	Scanning electron microscopy
SOD1	Superoxide dismutase 1
SSC	Spermatogonial stem cell
TLR	Toll-like receptors
TNF	Tumor necrosis factor
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
μm	Micrometer

Publications and declaration of contribution as a co-author

Publication I

NLRP3 in somatic, non-immune cells of rodent and primate testes.

Lena Walenta*, **Nina Schmid***, J. Ullrich Schwarzer, Frank-Michael Köhn, Henryk F. Urbanski, Rüdiger Behr, Leena Strauss, Matti Poutanen, Artur Mayerhofer
* equal contribution

L. Walenta and **N. Schmid** performed the cellular experiments and downstream analysis. L. Walenta and **N. Schmid** evaluated the results. J.U. Schwarzer and F.-M. Köhn provided human samples, H.F. Urbanski and R. Behr provided non-human primate samples, L. Strauss and M. Poutanen provided mouse samples, as well as conceptual input. A. Mayerhofer perceived of the study, directed the investigations and supervised the experiments. L. Walenta, **N. Schmid** and A. Mayerhofer drafted this manuscript. All authors participated to and approved the final version.

Reproduction; September 2018

We hereby confirm the above statement concerning publication

Nina Schmid	Dr. Lena Walenta
Prof. Dr. Artur Mayerhofer	PD Dr. Lars Kunz

Publication II

Insights into replicative senescence of human testicular peritubular cells.

Nina Schmid, Florian Flenkenthaler, Jan B. Stöckl, Kim-Gwendolyn Dietrich, Frank M. Köhn, J. Ullrich Schwarzer, Lars Kunz, Manja Luckner, Gerhard Wanner, Georg J. Arnold, Thomas Fröhlich, Artur Mayerhofer

N. Schmid executed the majority of the cellular experiments and evaluated results. K.G. Dietrich was involved in cell culture experiments. M. Luckner performed rOTO and ultra-thin embedding. **N. Schmid** and G. Wanner performed FIB/SEM-tomography and 3D-reconstruction. F. Flenkenthaler, J.B. Stöckl, T. Fröhlich and G.J. Arnold performed the proteomic studies and evaluated the results. J.U. Schwarzer and F.-M. Köhn, supplied testicular tissues and provided together with L. Kunz conceptual input. A. Mayerhofer conceived of the study, supervised the experiments. All authors provided helpful comments. **N. Schmid**, G. Wanner and A. Mayerhofer drafted the manuscript, and all authors contributed to the final version.

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Nina Schmid

Prof. Dr. Artur Mayerhofer

PD Dr. Lars Kunz

Publication III

Characterization of a non-human primate model for the study of testicular peritubular cells— comparison with human testicular peritubular cells.

N. Schmid*, J.B. Stöckl*, F. Flenkenthaler, K.-G. Dietrich, J.U. Schwarzer, F.-M. Köhn, C. Drummer, T. Fröhlich, G.J. Arnold, R. Behr, A. Mayerhofer
*equal contribution

N. Schmid performed the majority of the cellular experiments and K.G. Dietrich participated in these experiments. The proteomic studies were implemented by J.B. Stöckl, F. Flenkenthaler, T. Fröhlich. and G.J. Arnold; they also evaluated the results. J.U. Schwarzer, F.-M. Köhn, C. Drummer and R. Behr provided the testicular tissues, as well as conceptual input. A. Mayerhofer conceived of the study, directed the work and supervised the experiments. **N. Schmid** and A. Mayerhofer drafted this article. All authors contributed and approved the final version.

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Nina Schmid	Jan. B. Stöckl
Prof. Dr. Artur Mayerhofer	PD Dr. Lars Kunz

Summary

Peritubular cells are the cellular components of the wall of the seminiferous tubules in the testis. Previous studies indicated that they play important roles for the spermatogonial stem cell niche, immune surveillance and overall testicular functions. A functional decline, accompanied by testicular changes is observed in men with impaired spermatogenesis and idiopathic infertility. In many cases the peritubular wall compartment is affected by fibrotic thickening and signs of sterile inflammation. These alterations, amongst others, are particularly attributed to elderly men. As structural and functional changes in the human testis are generally difficult to examine, investigations require adequate models. Isolated human testicular peritubular cells (HTPCs) represent such a model, as these cells can be cultured and propagated. Therefore, they allow the investigation of human testicular inflammation and its mechanisms as well as aspects of human testicular aging.

Inflammatory events within the testis are one possible cause for male infertility. A novel testicular pro-inflammatory molecule is NLR family pyrin containing 3 (NLRP3), which is a component of the multimeric NLRP3 inflammasome. NLRP3 was found in somatic testicular cells, more precisely in HTPCs, as well as peritubular and Sertoli cells of humans and non-human primates. In testes of men suffering from idiopathic infertility, prominent NLRP3 presence was observed in tubules with impaired spermatogenesis, indicating a role of NLRP3 in male infertility and a contribution to testicular immune regulation.

The investigation of senescence, a hallmark of aging, in cultured HTPCs, revealed signs of replicative senescence like: i) cell cycle arrest; ii) increased cell size; iii) expression of senescence-associated β-galactosidase; and iv) telomere attrition. During aging a chronic, sterile, low-grade inflammation – termed inflammaging – develops, which is indicated e.g. by elevated macrophage migration inhibitory factor (MIF). A comparative approach revealed striking cellular alterations of senescent HTPCs concerning the mitochondrial network and the abundance of lysosomes. These findings correspond to previously detected changes of mitochondrial, lysosomal and nuclear proteins in senescent cells, implying that HTPCs age in a particular manner and thereby may contribute to testicular aging.

All previous studies utilizing HTPCs revealed a degree of heterogeneity, most probably due to age, lifestyle, and medical history of the individual human donors. To evade

these issues, a translational model for HTPCs was established. Monkey testicular peritubular cells (MKTPCs) from the common marmoset monkey (*Callithrix jacchus*) were isolated. The comparison of MKTPC and HTPC proteome revealed a large overlap, supporting the relevance of the marmoset monkey as a translational model for the human and enabling further investigations on peritubular cell function and regulation.

Zusammenfassung

Peritubuläre Zellen sind die zellulären Bestandteile der Wand der humanen Hodenkanälchen. Sie spielen eine wichtige Rolle für die Nische der spermatogonialen Stammzellen, immunologische Prozesse und für die generelle Hodenfunktion. Bei Männern mit eingeschränkter Fertilität und idiopathischer Infertilität treten unter anderem, vermehrt Veränderungen der Wand der Hodenkanälchen auf. Anzeichen fibrotischer Verdickungen der Wand und/oder sterile Entzündungen sind dafür charakteristisch. Derartige Veränderungen werden auch für Hoden älterer Männer beschrieben. Geeignete zelluläre Modelle sind von großer Wichtigkeit, da strukturelle und funktionelle Veränderungen des humanen Hoden schwer zu untersuchen sind. Isolierte humane testikuläre peritubuläre Zellen (HTPCs) stellen ein relevantes Modellsystem dar. Sie können sowohl kultiviert als auch passagiert werden und ermöglichen somit die Untersuchung der Entzündungsmechanismen und zusätzlich Aspekte der Alterung des Hodens.

Als mögliche Ursache für die männliche Unfruchtbarkeit werden Entzündungsprozesse im Hoden angesehen. Daran beteiligt könnte das pro-inflammatorische Molekül NLRP3 sein, der Sensorteil des NLRP3 Inflammasom Multimers. Die Expression von NLRP3 konnte, sowohl in somatischen Hodenzellen, in HTPCs, sowie in peritubulären und Sertoli-Zellen von humanen und nicht-humanen Primaten nachgewiesen werden. Bei Männern mit subfertilen Pathologien konnte in den Hodenkanälchen mit beeinträchtigter Spermatogenese ein vermehrtes Auftreten von NLRP3 gezeigt werden; dies legt somit einen Zusammenhang zwischen NLRP3 und entzündlichen Prozessen im Hoden nahe.

Seneszenz ist ein Kennzeichen des Alterns. Anzeichen replikativer Seneszenz konnten in kultivierten HTPCs durch: i) Zellzyklusarrest; ii) Zunahme der Zellgröße; iii) Expression Seneszenz-assoziierter β-Galactosidase und iv) Telomerverkürzung nachgewiesen werden. Chronische, sterile Entzündungen entwickeln sich oft während des Alterungsprozesses. Sie sind zum Beispiel durch erhöhtes Auftreten des pro-inflammatorischen Zytokins MIF (*macrophage migration inhibitory factor*) gekennzeichnet. Auffällige zelluläre Veränderungen von seneszenten HTPCs hinsichtlich ihres mitochondrialen Netzwerks und der Abundanz von Lysosomen, konnten durch vergleichende Ansätze nachgewiesen werden. Diese zytologischen Veränderungen entsprechen den, zuvor durch Proteomics gezeigten Veränderungen mitochondrialer, lysosomaler und nukleärer Proteine seneszenter Zellen und impliziert,

dass HTPCs sich in spezifischer Weise verändern und somit zur Hodenalterung beitragen können.

Vorhergehende Studien an HTPCs zeigten bereits ihre generelle Heterogenität. Sie ist vermutlich auf das Alter, den Lebensstil und die Krankengeschichte der individuellen Spender zurückzuführen. Um diese Parameter auszuschließen bzw. einzugrenzen, wurde ein Modell für HTPCs etabliert. Peritubuläre Zellen konnten erstmals aus Hodengewebe von Weißbüschelaffen (*Callithrix jacchus*) (MKTPCs) isoliert werden. Der Vergleich der Proteome von MKTPCs und HTPCs zeigt eine große Übereinstimmung und damit die Relevanz des Weißbüschelaffen als taugliches Humanmodell. MKTPCs ermöglichen dadurch weiterführende Untersuchungen zur Funktion und Regulation von peritubulären Zellen.

1 Introduction

1.1 Fertility – a social topic

In modern life, there is a general trend (primarily in developed countries) to have children later in life. In 1979, women were 24 and men 29 years old, when they had their first child, today, the reproductive phase is postponed (Statistisches Bundesamt, 2018). In 2016, women were 30 years and men (only married men are registered) were 34 years when they became parents (Statistisches Bundesamt, 2016, Statistisches Bundesamt, 2018). Primary socioeconomic reasons, like the opportunity for women to reach higher education, financial independency and professional careers, choose them to delay childbearing. Further, the increased accessibility to assisted reproductive technologies allows older couples with limited natural conception to conceive children (Balasch and Gratacos, 2012). The impact of maternal age on fertility and on the health of the offspring is well documented with several studies, showing increased risk of infertility, complications during pregnancy, abortion and congenital anomalies for women over the age of 35 (Niessen et al., 2017). By interest the consequences of increased paternal age are considerably less studied and there is no definition of “advanced” paternal age. However, paternal age gained increased importance since there is cumulative evidence for a negative influence on possible pregnancy and higher risk of e.g. congenital birth defects, genetic and neurocognitive disorders, and childhood leukemia for the offspring (D’Onofrio et al., 2014, Lian et al., 1986, Sharma et al., 2015).

1.2 Male fertility: from stem cell to epididymis

Male fertility requires functional spermatozoa in an adequate number. The testis is the central organ of male reproductive function through its generation of spermatozoa and steroid hormones (mainly testosterone). A layer of connective tissue, the *tunica albuginea*, encloses the lobular organized testis, which is structurally separated into two distinct compartments, the interstitial and the tubular compartment (Figure 1). Within the interstitial compartment, the steroid hormone producing Leydig cells reside among resident immune cells, as well as blood and lymph vessels. Leydig cells synthesize testosterone (besides other androgens) dependent on luteinizing hormone (LH) stimulation, and estradiol *via* transformation of testosterone by aromatase (Nieschlag et al., 2009). Yet, testosterone is the essential factor for maintaining lifelong spermatogenesis and thus, male fertility (Smith and Walker, 2014).

The tubular compartment consists of very thin, coiled tubules, the seminiferous tubules, which are arranged in lobules. The seminiferous tubules are surrounded by a basement membrane, extracellular matrix and concentrically arranged layers of peritubular myoid cells, referred to as *lamina propria* (Bustos-Obregon, 1976). Inside the seminiferous tubules, the spermatogonial stem cells (SSCs) reside at the basement membrane in a highly organized manner, embedded into cytoplasmic niches of Sertoli cells, which provide structural and nutritional support. Sertoli cells are orientated from the basement membrane towards the tubular lumen (Mruk and Cheng, 2004, Heller et al., 1948). Tight junctions between neighboring Sertoli cells form the blood-testis barrier (BTB), which develops with the rising androgen production during puberty (Wong and Cheng, 2005). The BTB of the testis is foremost the physical barrier between seminiferous tubules and blood vessels in the interstitial compartment and shields the developing germ cells from the immune system (Mruk and Cheng, 2015).

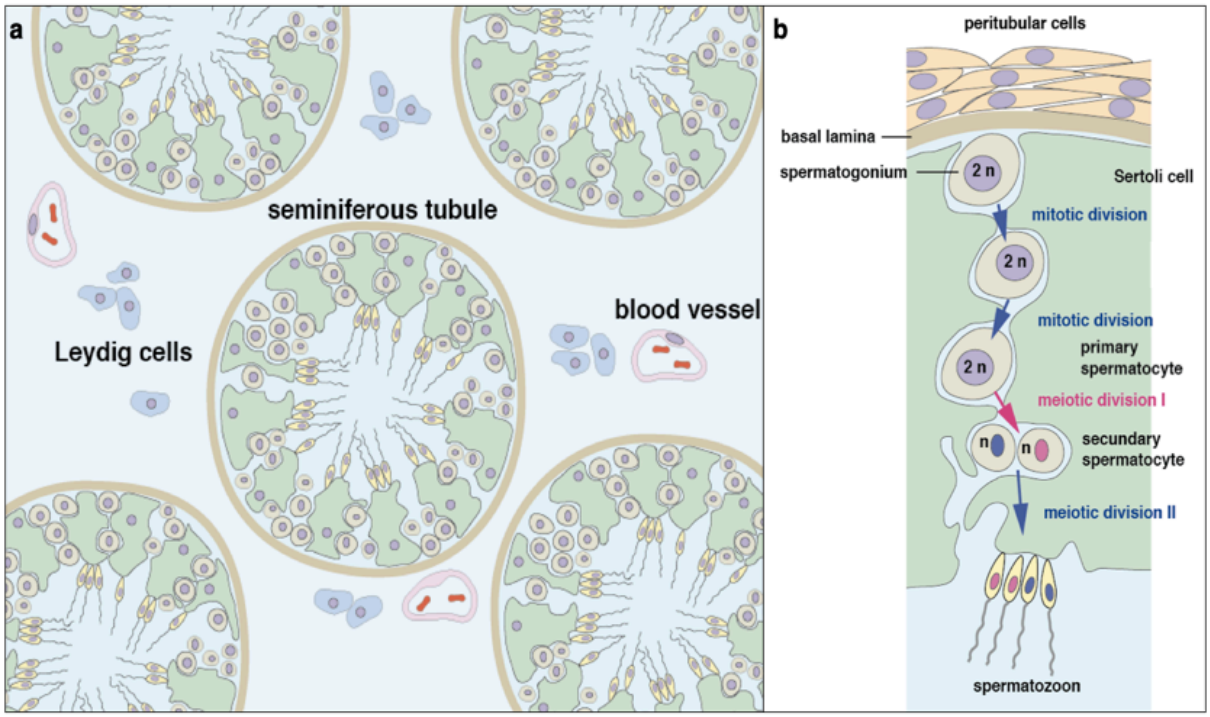

Figure 1 The seminiferous tubule

(a) Schematic cross section of seminiferous tubules, comprising the seminiferous epithelium (Sertoli cells (green), germ cells in different development stages (yellow)), surrounded by the peritubular wall (brown). Leydig cells (blue) and blood vessels (red) are located in the interstitial tissue. (b) Detailed scheme of seminiferous epithelium (cross section). Steps of germ cell differentiation and migration from basal compartment to the tubular lumen. Sertoli cells are located on the peritubular wall, consisting of peritubular cells and basal lamina, embedding spermatogonium and consequential spermatocytes and spermatids.

The SSCs enable permanent generation of spermatozoa and classify as reposing (type) A_{dark} and active (type) A_{pale} spermatogonia (Clermont, 1966, Goossens and

Tournaye, 2013). The active A_{pale} spermatogonia either develops into type B spermatogonia, which undergo spermatogenesis, or self-renew and thereby restore the stem cell pool. Stem cell maintenance is depending on the stem cell niche, a specific microenvironment of signaling molecules and growth factors supplied by vicinal somatic cells, which protects the SSC from differentiation (Goossens and Tournaye, 2013, Li and Xie, 2005, Jones and Wagers, 2008).

During the approximately ten week long procedure of human spermatogenesis, type B spermatogonia divide mitotically at the basement membrane of the seminiferous tubules and the emerging diploid primary spermatocytes undergo meiotic division (Ehmcke and Schlatt, 2006). Two haploid secondary spermatocytes arise, which again divide meiotically into haploid round spermatids. They further mature into spermatozoa and are released into the lumen of seminiferous tubules (O'Donnell et al., 2011). The immotile spermatozoa are transported from the testicular lumen *via* the *rete testis* into the epididymis (Hargrove et al., 1977, Romano et al., 2005). During the transit through the coiled epididymis, which spans about six meters, the final maturation occurs within additional two weeks. The spermatozoa remain stored in the epididymis until ejaculation (Robaire and Hermo, 1988).

1.2.1 Functions of the peritubular wall

In the human testis, the wall of the seminiferous tubules consists of multiple layers (five to seven) of elongated, spindle-shaped peritubular cells and extracellular matrix proteins (Bustos-Obregon, 1976). In contrast, rodents possess just one layer of peritubular cells and thus differ considerably from the human peritubular wall (Nakata et al., 2015). Both Sertoli cells and peritubular cells ensure the synthesis of the extracellular matrix components like collagens, fibronectins, laminins and proteoglycans thereby building up and stabilizing the tubular wall (Skinner et al., 1985). Peritubular cells are contractile, have a smooth muscle-like phenotype and are therefore also called peritubular myoid cells. They express smooth muscle proteins like smooth muscle actin, calponin and myosin (Mayerhofer, 2013). Their ability to contract is essential for the transport of the immotile spermatozoa from the lumen of the seminiferous tubules to the *rete testis*. The cellular layers of the peritubular wall can be separated into an inner myofibroblastic-like phenotype, whilst the outer layer has a pure fibroblast-like character (Davidoff et al., 1990). The location of the peritubular cells, directly at the basement membrane and in close vicinity to the SSCs, Sertoli cells and Leydig cells, places them in a strategically super-position to interfere with these surrounding cells (Skinner and Fritz, 1985, Verhoeven et al., 2000, Welsh et al., 2012). The importance of the paracrine activity of peritubular cells is reflected by

the secretion of growth factors. They, for instance, secrete the growth factor glial cell line-derived neurotropic factor (GDNF), which is essential for spermatogenesis (Chen et al., 2014, Chen et al., 2016, Spinnler et al., 2010).

1.3 Male infertility: inflammation, aging and senescence

The World Health Organization describes infertility as “a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after twelve months or more of regular unprotected sexual intercourse” (Zegers-Hochschild et al., 2009). Various factors can be held responsible for male infertility. Besides erectile dysfunction, insufficient semen quality and obstruction of the ductal system, the integrity of the testis is of great importance (Cooke and Saunders, 2002). Spermatogenesis is a highly organized process and can be disturbed at each level, eventually leading to infertility. Dysfunctional spermatogenesis is diagnosed as non-obstructive azoospermia (NOA) implying that no sperm is present in the ejaculate (Chiba et al., 2016). Causes for NOA can be genetic abnormalities, large varicoceles (defined as abnormal dilation of veins in the pampiniform plexus of the spermatic cord) and hormonal disorders concerning the hypothalamic-pituitary-gonadal axis (Jarow et al., 1989, Wosnitzer et al., 2014). Especially the malfunctioning of Sertoli and somatic cells, which crucially support spermatogenesis, is typical in male infertility. However, the majority of causes is idiopathic and poorly understood.

Common phenotypes of NOA include mixed atrophy (MA) syndrome, germ cell arrest syndrome and the Sertoli cell only (SCO) syndrome (Behre et al., 2000). SCO patients show local or complete germ cell aplasia and reduced tubular diameters accompanied by thickened basal lamina and possible immune cell infiltration (Tsai et al., 2012, Silber et al., 1995). Locally, tubules may contain germ cells but these do not progress beyond the spermatogonial stage. The MA phenotype is characterized by simultaneous presence of intact and SCO type of tubules or even tubules merely consisting of the *lamina propria*. Tubules of MA patients can be fibrotic and with appearance of different spermatogenic defects (Sigg and Hedinger, 1981). The germ cell arrest syndrome is characterized by interruption of spermatogenesis at different levels leading to incomplete development of spermatozoa (Del Castillo et al., 1947).

Fibrosis is a typical attribute of the infertile testicular phenotype. It represents a pathophysiological repair mechanism as response to stressors or an injury (Kisseleva and Brenner, 2008) and is characterized by extensive extracellular matrix (ECM) production, deposition and thus, tissue remodeling. Frequently, the tubular wall

compartment is affected by fibrotic remodeling under pathological conditions, which is indicated by thickening of the wall. A massive presence of abnormally arranged collagen fibers within the peritubular cell layers and a thickening of the basal lamina occurs (Soderstrom, 1986). Peritubular cells can become hypertrophic, lose their myofibroblastic phenotype and may be transformed into fibroblasts, which produce huge amounts of collagen fibers and ECM proteins, resulting in extensive changes of the ECM composition and impaired spermatogenesis (Haider et al., 1999, Schell et al., 2008).

1.3.1 Inflammation and infertility

Inflammatory processes within the testis are a major cause of male infertility, which often remains asymptomatic. Mainly two types of inflammation appear: a pathogen induced (viral, bacterial), which is very rare, and a sterile one (Schuppe et al., 2010).

Sterile inflammation can be caused by testicular torsion and other mechanical traumata or autoimmune reactions resulting in the secretion of cytokines and chemokines by resident immune cells and somatic cells (e.g. Sertoli cells, peritubular cells) (Sarkar et al., 2011). Cytokine secretion can serve as chemoattractant and thus initiates immune cell infiltration. In the testis, inflammatory cytokines, such as IL1β and TNFα, negatively influence steroidogenesis, resulting in low testosterone levels, which ultimately lead to impaired spermatogenesis (Li et al., 2006). Increased cytokine presence may also be involved in reactive oxygen species (ROS) generation, which is responsible for permanent peroxidative damage of spermatozoa, thus having a negative impact on their fertilizing capacity (Fraczek and Kurpisz, 2015). Furthermore, pro-inflammatory cytokines affect the BTB permeability; if they enter germ cells of the seminiferous tubules, apoptosis of the SSCs can be triggered (Jacobo et al., 2011).

In the healthy human testis, immune cells especially macrophages and mast cells occur physiologically (Zhao et al., 2014, Frungieri et al., 2002a, Meineke et al., 2000). However, increased numbers of immune cells are a typical symptom of male subfertility or infertility. Testicular immune cells have essential regulatory functions e.g. maintenance of the testicular immune privilege and protection of the immunogenic germ cells by setting up an immune suppressive environment (Loveland et al., 2017). If this complex immunological environment becomes imbalanced, circulating immune cells can migrate and provoke inflammatory processes.

Macrophages

Testicular macrophages mainly occur within the interstitial compartment, in close vicinity to the Leydig cells and occasionally in the tubular wall compartment (Heinrich and DeFalco, 2019). Increased numbers and altered distribution of macrophages are associated with infertile pathologies (Mahran et al., 2017). Consequently, testicular macrophages can contribute to the regulation of steroidogenesis, Sertoli cell function and germ cell survival (Meinhardt et al., 2018). They can also foster pathologies of male infertility *via* phagocytosis or paracrine actions e.g. the secretion of pro-inflammatory factors (IL1β and TNFα) (Frungieri et al., 2002a). In addition, macrophages are a major source of ROS, which can cause damage of the spermatozoic DNA and membrane, resulting in infertility and decreased motility (Tremellen, 2008).

Mast cells

Besides macrophages, an increased number of testicular mast cells is linked to human sub- and infertility. In pathologies like MA, SCO and germ cell arrest higher abundance and changed distribution towards the tubular wall can be observed (Meineke et al., 2000). Secreted mast cell products are able to stimulate fibroblast proliferation and thus relate mast cells to pathogenic fibrosis of the tubular wall. One unique mast cell product is the serine protease tryptase, which is known to activate the protease-activated receptor 2 (PAR2) (Iosub et al., 2006). This receptor is expressed by macrophages, germ cells and peritubular cells in the testis. Activation of PAR2 causes proliferation of fibroblasts and leads to overexpression of cyclooxygenase-2 (COX2), a key enzyme in the synthesis of inflammation-mediating prostaglandins (Frungieri et al., 2002b). The mast cell product chymase is able to cleave angiotensin I to angiotensin II, leading to IL6 secretion by peritubular cells (Welter et al., 2014). Additionally, mast cells are a source of extracellular ATP. This nucleotide acts *via* purinergic receptors (P2X) and thus, enhances the expression of pro-inflammatory molecules like IL6 by peritubular cells (Walenta et al., 2018). These mast cell products coincide with fibrotic and inflammatory changes in the testis and are increased in infertile patients due to the increased mast cell number.

Lymphocytes

In general, lymphocytes rarely occur within the human testis (Pollanen and Niemi, 1987). Their infiltration of the testis was detected in patients with infertile pathologies showing signs of fibrosis and dysfunctional germinal epithelium (Suominen and Soderstrom, 1982, el-Demiry et al., 1987).

1.3.1.1 The NLRP3 Inflammasome

The NLRP3 inflammasome is part of the innate immune system. This protein complex forms to facilitate host immune defense against microbial infection and cellular damage (Franchi et al., 2012). Inflammasomes are protein oligomers consisting of a sensor, an adapter and an effector protein, which are formed in the cytosol of immune cells (He et al., 2016). Their activation mediates inflammatory caspase activation, cytokine production (IL1β, IL18) and the initiation of a lytic form of cell death, pyroptosis (Martinon et al., 2002).

Intracellular sensor proteins, so-called pattern-recognition receptors (PRR), are part of the innate immune system and recognize several pathogen- and host-derived danger signals (Takeuchi and Akira, 2010). There are various sensor proteins described and the majority belongs to the nucleotide-binding oligomerization domain-like receptor (NLR) family (Platnich and Muruve, 2019). NLR family pyrin domain containing 3 (NLRP3) is one of them and part of the NLRP3 inflammasome (Franchi et al., 2009). Canonical activation of the NLRP3 inflammasome occurs in two steps, a priming and an activating step (Figure 2) (Sharma and Kanneganti, 2016). The first step is induced by pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), which result in nuclear factor kappa B (NF-κB) downstream signaling, leading to upregulation of NLRP3, pro-IL1β and pro-IL18 transcription (Wang et al., 2002, Mariathasan et al., 2006, Sha et al., 2014). Usually, this priming signal is received through toll-like receptors (TLRs), IL1 receptor or TNFα receptor (Broz and Dixit, 2016).

Following this priming step, a second activating signal is induced by different PAMPs like bacterial pathogenic components (e.g. lipopolysaccharides (LPS)), viral products or pore-forming toxins (Schroder and Tschopp, 2010). Furthermore, DAMP signals like crystals, particles or extracellular ATP, which provokes purinergic receptor P2X7 pore formation and subsequent K^+ efflux, can induce inflammasome activation (Petrilli et al., 2007, Piccini et al., 2008, Franchi et al., 2007). Calcium sensing receptor (CASR) signaling resulting in elevated intracellular Ca^{2+} concentration can evoke inflammasome assembly (Lee et al., 2012, Murakami et al., 2012). It has also been shown that oxidized mitochondrial DNA (mtDNA) and ROS, as result of mitochondrial dysfunction, trigger inflammasome oligomerization (Jo et al., 2016) in a cascade-like manner. These are some examples among the inflammasome activation triggers. After the second activation step NLRP3 monomers provoke the oligomerization and associate with the pyrin-domain (PYD) of the adapter protein ASC (apoptosis associated speck-like protein containing a caspase activation and recruitment domain)

(Bryan et al., 2009). ASC, in turn recruits the effector protein pro-caspase 1 *via* the recruitment domain caspase activation and recruitment domain (CARD) (Fernandes-Alnemri et al., 2007). Active caspase 1 leads to maturation of pro-IL1β and pro-IL18 into IL1β and IL18 or the cleavage of Gasdermin D (GSDMD), which induces pyroptosis (Sborgi et al., 2016).

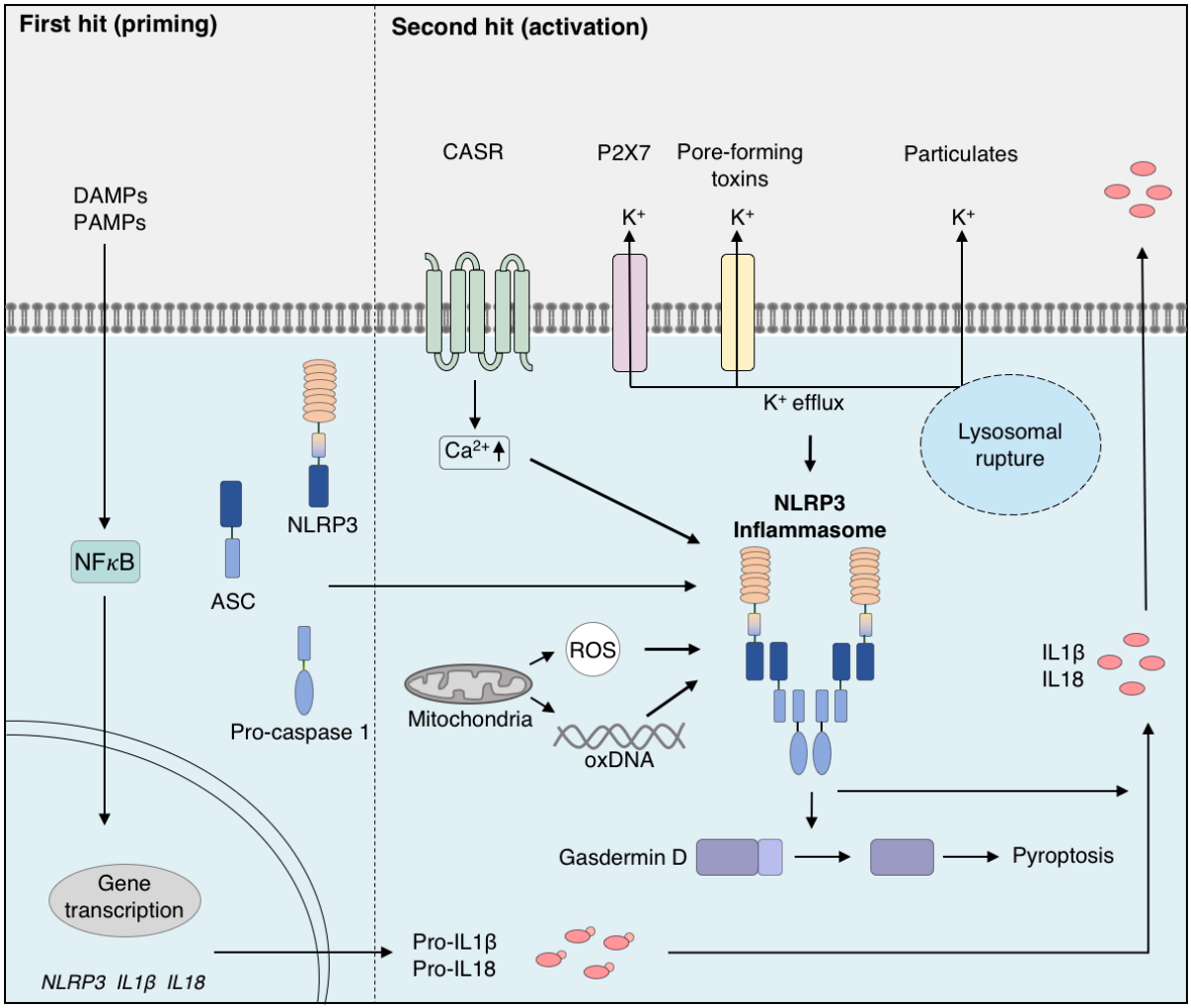

Figure 2 Schematic drawing of NLRP3 inflammasome activation

NLRP3 inflammasome activation requires two hits. Priming hit is triggered by DAMPs and PAMPs, which stimulate NFκB dependent transcription of *NLRP3, IL1β* and *IL18*. Second hit induces NLRP3 inflammasome assembly. CASR signaling induces NLRP3 inflammasome activation by intracellular Ca^{2+} increase. Activation is triggered by K^+ efflux due to P2X7, pore-forming-toxins, and particulates. Further, oxidized mitochondrial DNA and ROS induce NLRP3 inflammasome assembly. Finally, caspase 1 cleaves Gasdermin D leading to pyroptosis and processes pro-IL1β and pro-IL18 into their mature forms. Modified on the basis of (Franchi et al., 2012, Kim et al., 2017a).

The non-canonical activation of the NLRP3 inflammasome is initiated by the recognition of LPS within the cytosol by human caspase 4 and caspase 5 (Kayagaki

et al., 2011). This process initiates the oligomerization of caspase 4 and 5 and thus their activation (Shi et al., 2014). GSDMD is subsequently cleaved by active caspase 4 and 5 resulting in a pyroptosis-induced K^+ efflux which in turn activates canonical NLRP3 inflammasome assembly and caspase 1-dependent IL1β maturation (Miao et al., 2010).

As a result of inflammasome activation mature IL1β mediates the recruitment of innate immune cells (Chen and Nunez, 2010). Parallel with IL18, it is involved in the modulation of the adaptive immune response. Additionally, IL18 plays an important role in activation of natural killer cells and T cells as well as in the production of interferon gamma (INF-γ) (Labzin et al., 2016). Finally, the innate and the adaptive immune response resolve the cellular damage in collaboration (Sims and Smith, 2010, Dinarello, 2009). However, if the NLRP3 inflammasome activity is dysregulated, various pathogenic inflammatory disorders (e.g. arteriosclerosis (Duewell et al., 2010)) can arise. Therefore, the NLRP3 inflammasome activation has to be regulated precisely to prevent overexpression and ensure an adequate immune response

Hyper-activation of NLRP3 inflammasomes is demonstrated in various autoimmune diseases e.g. rheumatoid arthritis and systemic *lupus erythematosus* (Ozkurede and Franchi, 2012). Additionally, it has been implicated that chronic inflammation due to abnormal NLRP3 inflammasome activation contributes to metabolic pathologies like obesity, type 2 diabetes and gout (Grant and Dixit, 2013). Harmful gain-of-function mutations in the NLRP3 gene are responsible for a group of auto-inflammatory disorders termed cryopyrin-associated periodic syndromes (CAPS) (Booshehri and Hoffman, 2019). CAPS are characterized by steady NLRP3 inflammasome activity resulting in excessive IL1β production causing familial cold auto-inflammatory syndrome (FCAS), neonatal-onset multisystem inflammatory disorder (NOMID)/ chronic infantile neurological cutaneous articular syndrome (CINCA) and the Muckle-Wells Syndrome (MWS) (Cuisset et al., 2011). These three syndromes share symptoms like fever, blood neutrophilia, tissue-specific inflammation in joints, skin and conjunctiva. Hearing loss and kidney amyloidosis may develop in MWS and NOMID, whereas inflammation of the central nervous system specifically occurs in NOMID (Broderick et al., 2015). The MWS is of special interest, since it relates to impaired spermatogenesis and infertility in men (Tran et al., 2012, Fan et al., 2017).

1.3.2 Aging

Aging is generally accepted as a time-dependent decline of the physiological functions, which concerns the majority of organisms. It is a natural process and accompanied by

age-progressive cellular damage triggered by several endogenous and exogenous stressors (Kirkwood, 2005). Cells possess various repair mechanisms and can thus partly cope with the damage. In case of irreversibility, damage leads to cell cycle arrest (senescence) or programmed cell death (apoptosis) (Hoeijmakers, 2009). Aging represents the major risk factor for various diseases like cancer, neurodegenerative disorders, and cardiovascular problems and finally ends with death (Niccoli and Partridge, 2012).

Aging is a very complex, interrelated and individual process, which is characterized by a variety of hallmarks (Lopez-Otin et al., 2013). One of them is genomic instability, the accumulation of genetic damage during lifetime. The DNA integrity and stability are constantly challenged by endogenous (e.g. DNA replication errors, ROS) and exogenous (e.g. chemical, physical and biological agents) factors (Niedernhofer et al., 2018). The resulting genetic lesions comprise point mutations, translocations, chromosomal alterations, telomere attrition and gene disruption. To minimize these lesions and to counteract the constant increase of endogenous genotoxins with age, different DNA repair mechanisms have evolved (Lord and Ashworth, 2012). However, the DNA repair capacity decreases with age (Burtner and Kennedy, 2010). Thus, DNA damage and mutations increase, leading to the activation of the DNA damage response (DDR), which consists of a network of pathways that monitors DNA integrity and activates DNA repair mechanisms and cell cycle check points (Ou and Schumacher, 2018).

Telomeres, the repetitive regions at the ends of mammalian chromosomal DNA, which protect the chromosomal DNA from degradation, shorten with each cell division. This process is one explanation for the limited proliferative capacity of mammalian cells (Blackburn et al., 2006, Olovnikov, 1996). Telomere shortening induces the DDR and thus, cell cycle arrest, differentiation or apoptosis (Fumagalli et al., 2012). The mtDNA is a major target for genetic damage and aging-associated mutations, since mtDNA lacks protective histones and possesses limited DNA repair mechanisms (Shigenaga et al., 1994). Mitochondrial dysfunction is another hallmark of aging. The accumulation of dysfunctional mitochondria results in diminished respiratory chain activity, electron leakage, reduced ATP generation and increased ROS production (Green et al., 2011, Harman, 1965).

Throughout lifetime epigenetic alterations like DNA methylation, chromatin remodeling and posttranslational histone modifications have impact on all cells and tissues (Fraga and Esteller, 2007). The epigenetic alterations during aging (Wilson and Jones, 1983),

e.g. altered histone methylation or acetylation, distinct DNA methylation or chromatin modification lead to changed accessibility of the genome and thus contribute to the aging process (Talens et al., 2012).
The loss of protein homeostasis – or proteostasis – is also linked to aging (Koga et al., 2011). Chaperones and two proteolytic systems, the ubiquitin proteasome system (UPS) and autophagy are responsible for the maintenance of intracellular proteostasis (Powers et al., 2009). During aging, endogenous and exogenous stressors impair proper folding of proteins. Mis- or unfolded proteins usually get degraded by proteolytic systems, which become impaired with age (Tomaru et al., 2012). As a consequence, unfolded proteins aggregate and accumulate, finally leading to proteotoxic effects (van Ham et al., 2010).

Nutrient sensing is the ability of cells to sense and respond to the abundance of e.g. sugars, amino acids and lipids (Efeyan et al., 2015). Deregulated nutrient sensing is another hallmark of aging and affects the somatotrophic axis (Barzilai et al., 2012). Central components of this axis are the growth hormone (GH), its mediator, insulin-like growth factor-1 (IGF-1), and insulin signaling. Both factors transmit the availability of glucose to the cells (Barzilai et al., 2012, Rincon et al., 2004). Upcoming insulin resistance and decreased GH and IGF-1 levels (Rudman et al., 1981, Sonntag et al., 2000) during aging may accelerate the aging process and are associated with an increased risk for type 2 diabetes, cardiovascular diseases and stroke (Colman et al., 2009).

Aging at the cellular level is characterized by cellular senescence, a stable cell cycle arrest including typical phenotypic changes (Campisi and d'Adda di Fagagna, 2007). In addition stem cell exhaustion is acknowledged as hallmark of aging, leading to a reduced regenerative potential of tissues (Janzen et al., 2006, Gruber et al., 2006). For example, the decline in hematopoiesis with age, due to hematopoietic stem cell exhaustion leads to decreased immune cell generation and an increased incidence of anemia and myeloid malignancies with age (Shaw et al., 2010).

Finally, aging generally leads to an altered intercellular communication and consecutively, to an age-associated decline of cellular function (Russell and Kahn, 2007, Rando and Chang, 2012). A major change in intercellular communication is “inflammaging” (Franceschi et al., 2000), a term that describes a chronic low-grade inflammatory phenotype with altered peri- and extracellular milieu during the aging process. This altered environment can cause accumulation of pro-inflammatory tissue damage, disturbed function of the immune system, secretion of pro-inflammatory

cytokines and enhanced activation of nuclear factor-κB (NF-κB) transcription (Zhang et al., 2013, Salminen et al., 2012). Indicating, that the aging process is not only a cell-biological phenomenon, but rather linked to altered intercellular communication, thus offering possibilities to modulate aging at this level (Green et al., 2011).

1.3.2.1 The aging testis

Whereas the female reproductive capacity completely ceases with the menopause and the risk factors for the offspring with increasing maternal age are well documented (Allen et al., 2009), the reproductive capacity in men is controversially discussed. In contrast to woman, male fertility does not end completely; men may have children throughout their lifetime. However, male age has an impact on fertility and the impact of paternal age for the offspring is poorly understood.

Aging of the male reproductive system is a highly disputed topic: on the one hand, there is evidence for a functional decline of the testis with age (Paniagua et al., 1987), on the other hand, normal testicular morphology and ongoing spermatogenesis is described during aging (Nieschlag et al., 1982). The changes observed in the testis during aging are very heterogenous and supposedly stem from a combination of various factors including lifestyle, general health conditions and age itself. Age-related changes comprise a decline in total sperm count and sperm motility (Kuhnert and Nieschlag, 2004, Handelsman and Staraj, 1985). These alterations are paralleled by structural changes such as fibrosis and thickening of the basal membrane. They are accompanied by complete or partial arrest of spermatogenesis and decreased numbers of Sertoli cells and cells undergoing spermatogenesis (Paniagua et al., 1987, Paniagua et al., 1991, Honore, 1978). These findings are a matter of debate, as the selection criteria of the donors seem to be inconsistent: confounding issues like infertility of donors might have influenced the studies. A study with donors with proven fertility disagreed and revealed no aging effect on both, sperm quality and number or hormonal disorders (Nieschlag et al., 1982). Additionally, a recent light microscopic study disproved alterations of the tubular wall (Pohl et al., 2019).

There is evidence for age-related changes of the stem cell niche (Paul and Robaire, 2013) and thus a decreased germ cell number. Besides peritubular myoid cells, in particular Sertoli cells contribute to the stem cell niche and could be predisposed for age-dependent alterations since they barely proliferate and have no regenerative capacity (Sharpe et al., 2003). Alterations in number (Jiang et al., 2014) and increased nuclear and nucleolar size of Sertoli cells appear with age (Pohl et al., 2019). During lifetime, an increasing amount of stem cell mutations occur, yielding mutated

spermatozoa. These mutations can be passed on to the offspring. There is increasing evidence that the paternal age is a risk factor for congenital disorders of the offspring. Negative effects of advanced age were reported for the physical and mental health of the offspring later in life (Herati et al., 2017). Children of fathers older than 45 years have a threefold risk of schizophrenia and autistic disorders in contrast to those of fathers aged 20 to 24 years (Malaspina et al., 2015). The epigenetic heritable gene expression modification and genomic alterations, which occur during aging, seem to be responsible for this increased disease risk (Curley et al., 2011). Natural conception may also be negatively influenced by reduced spermatozoic integrity, due to DNA mutations and/or fragmentation of chromatin packing (Spano et al., 2000, Zitzmann, 2013).

Aging also affects the testosterone producing Leydig cells. Testosterone is the central anabolic hormone and has a pivotal role in maintaining spermatogenesis and sexual function (Kaufman and Vermeulen, 2005). An age-related decline of the testosterone level (Wu et al., 2008) can be due to a reduced number of Leydig cells and/or disorders of the hypothalamic-pituitary-gonadal axis, which regulates spermatogenesis on the hormonal level. In summary, diminished generation of sex hormones with age can lead to hypogonadism, a restricted functional activity of the gonads, which is paralleled by impaired fertility (Grossmann, 2010).

A further accompanying factor of aging is a low-grade pro-inflammatory state. There is evidence in elderly men for an elevated number of testicular macrophages and mast cells and thus for increased expression of pro-inflammatory cytokines like IL1β and TNFα, which can negatively influence male fertility (Lloberas and Celada, 2002). These cytokines have a negative impact on steroidogenesis and additionally upregulate cyclooxygenase-2 expression and thus elevate prostaglandin production (Matzkin et al., 2010). This scenario is also reflected in adult men affected by inflammatory-associated idiopathic infertility (Frungieri et al., 2018).

1.3.3 Senescence

A central hallmark of aging is cellular senescence characterized by an irreversible cell cycle arrest. This phenomenon was first described by Leonard Hayflick and Paul Moorhead in 1961 on the basis of human fibroblasts in culture (Hayflick and Moorhead, 1961). They observed that these cells lose their proliferative capacity over time and reach their proliferative limit, known as the “Hayflick limit”. Initially, senescence was thought to be a cell culture effect/artifact. However, *in vivo* senescence occurs during embryogenesis (Storer et al., 2013), serves as anti-cancer mechanism (Collado and

Serrano, 2010) and is involved in tissue remodeling and wound healing (Demaria et al., 2014, Sharpless and Sherr, 2015). The accumulation of senescent cells contributes to aging and age-related disease.

There is no universal biomarker for cellular senescence; however, a combination of various hallmarks is used to identify senescent cells. Besides the cell cycle arrest the most commonly used marker is senescence-associated β-galactosidase (SA β-gal) at pH 6 (Dimri et al., 1995, Kurz et al., 2000), which indicates an increased lysosomal content with age (Lee et al., 2006). This increased lysosomal content in senescent cells could be due to aggregation of old lysosomes or increased biogenesis of lysosomes (Barbosa et al., 2018). The morphology of senescent cells is typically altered, the cells appear enlarged, irregularly shaped, flattened and vacuolated (Druelle et al., 2016, Lloyd, 2013, Loffredo et al., 2013). Loss of the nuclear lamina protein Lamin B1 results in destabilization of the nucleus, decreased heterochromatin condensation and emergence of cytoplasmic chromatin fragments (CCFs) (Sadaie et al., 2013). A senescence-mediated gene expression profile is related to a local redistribution of heterochromatin into senescence-associated heterochromatin foci (SAHF), which can also be used as senescence marker (Narita et al., 2003).

Cellular senescence is provoked by different stress signals. Elevated concentrations of ROS can occur in aged cells and cause damage to DNA, proteins and lipids, which subsequently result in a senescent phenotype (Davalli et al., 2016). Another possible cause of cellular senescence is telomere attrition, which triggers DNA damage response (DDR) when they reach a critical length (d'Adda di Fagagna et al., 2003). The DDR induces cell cycle arrest until the damage is eliminated (Galbiati et al., 2017).

The major mechanisms that implement senescence and the connected cell cycle arrest involve the tumor suppressor pathways p53 and $p16^{INK4A}$-pRb (Beausejour et al., 2003). Activation of p53 controls an anti-proliferative transcriptional program *via* the induction of the cyclin-dependent kinase inhibitor (CDKi) $p21^{CIP1}$ leading to inhibited CDK2 activity and subsequent hypo-phosphorylated retinoblastoma-protein (Rb) and cell cycle arrest (d'Adda di Fagagna et al., 2003). CDKs have important regulatory roles in cell cycle progression. The CDK inhibitor p16 blocks CDK4 and CDK6, two proteins that are necessary for G1- to S-phase progression, and thus p16 promotes cell cycle arrest (Mirzayans et al., 2012).

Mitochondrial dysfunction is also characteristic for senescent cells. Consistent biogenesis of mitochondria and selective mitophagy are required for the preservation

of functional mitochondria with age (Sun et al., 2016). Ongoing competition between fission and fusion processes of mitochondria explains their occurrence from fragmented to large networks (Sugiyama et al., 1993, Fannin et al., 1999). Diminished mitophagy, possibly due to decreased autophagy in senescent cells or mtDNA mutation, results in a defect mitochondrial network (Seo et al., 2010). The accumulation of dysfunctional mitochondria provokes ROS-induced senescence (Moiseeva et al., 2009). A typical hallmark of senescent cells is a chronic sterile inflammatory state. There is evidence that mtDNA is able to activate an innate immune response and to induce various factors, which are associated with a distinct, so called senescence-associated secretory phenotype (SASP) (Sun et al., 2016, Wiley et al., 2016). Senescent cells are still metabolically active and secrete a plethora of factors referred to as SASP. The transcription factors NF-κB and CCAAT/enhancer binding protein-β (CEBPβ) mediate this secretory phenotype (Coppe et al., 2010). The activated NF-κB pathway can induce NLRP3 inflammasome assembly and thus contribute to a pro-inflammatory environment by IL1β, TNFα and IFN-γ secretion (Bauernfeind et al., 2009). The senescent cells influence the surrounding tissue *via* the SASP in a beneficial or harmful way. The SASP includes interleukins (IL1α, IL1β, IL6), chemokines (CXCL8, CXCL1), growth factors (β-FGF, HGF) and proteases (MMP-1, -3, -13) (Young and Narita, 2009). This pro-inflammatory milieu can recruit immune cells which may foster further inflammatory processes and the elimination of senescent cells, respectively (Greten and Eggert, 2017).

Apoptosis resistance is another feature of senescent cells and might explain why they do not die in culture (Childs et al., 2014). It is assumed that the upregulation of anti-apoptotic B-cell lymphoma 2 (Bcl-2) family proteins is responsible for apoptosis resistance, since the inhibition of Bcl-2 induces apoptosis in senescent cells (Hampel et al., 2005, Ryu et al., 2007, Chen et al., 2008).

Various factors like oxidative stress, mutations and the absence of chaperones trigger endoplasmic reticulum (ER) stress during senescence and result in accumulation of proteins (Chadwick and Lajoie, 2019). To compensate this stress, the ER evokes the unfolded protein response (UPR) that results in reduced protein synthesis, ER enlargement and export of defective proteins (Denoyelle et al., 2006). Increased UPR is possibly due to enhanced protein synthesis by the SASP (Druelle et al., 2016, Cormenier et al., 2018).

1.4 Comparison of different cell culture models

Cell cultures have been implemented in biological research around 1900 (Jedrzejczak-Silicka, 2017). This approach enables the cultivation of cells from different organisms and different tissues from donors of all ages. Cells in culture can easily be manipulated and treated depending on the respective questions. Furthermore, cell culture systems help to avoid animal studies and provide a relevant tool for mechanistic studies but cannot mirror the *in vivo* situation, which makes it difficult to translate the results to a holistic system.

1.4.1 Cell culture models for aging (replicative senescence)

The first cell culture experiments concerning aging were already implemented in 1912, when Alexis Carrel studied aging processes using isolated and sub-cultivated chicken cells (Carrel, 1912). His conclusions that single cells were immortal and aging is a multicellular phenomenon were disproved by Leonard Hayflick and Paul Moorhead 50 years later, in 1961. They showed that single cells have a limited proliferation capacity in culture (Hayflick and Moorhead, 1961). This discovery was the first hint that senescence might be correlated to aging and was the initiation for many following *in vitro* studies for the investigation of aging and senescence (de Magalhaes et al., 2004, Serrano et al., 1997, Shelton et al., 1999). Nowadays replicative senescence triggered by serial passaging of somatic cells in culture is an established cell culture model to study changes of cells through the aging process (Chen et al., 2013). The term “replicative senescence” is justified by the fact that senescent cells reach their replicative life span and end up in a cell cycle arrest (Hayflick, 1965). This was presumed for a long time as a cell culture artifact, but we now know about aging from cells in culture that tumor cells proliferate without limitation and that senescence might suppress tumorigenesis (McHugh and Gil, 2018). Cell culture experiments gave the first indication that non-immortal cells age in culture, reach a proliferation limit and therefore might contribute to aging (Campisi, 2001, Lidzbarsky et al., 2018, Gil, 2019). Another 60 years later, research in senescence is still of high interest. It was shown that the elimination of senescent cells increases healthy life span and with the INK-ATTAC mouse model, a versatile tool to investigate the causative role of senescent cells in various diseases was developed (Baker et al., 2011).

1.4.2 HTPCs – a cellular model for the peritubular wall

Preceding investigations of testicular peritubular cells indicated that their role in the human testis goes beyond sperm transport and includes paracrine and immunological regulation of the testis (Mayerhofer, 2013). Human Testicular Peritubular Cells

(HTPCs) are a unique cell culture model, which enables studies in the human system. HTPCs are isolated from small testicular tissue samples originating from donors with obstructive azoospermia but normal spermatogenesis *via* explant culture (Albrecht et al., 2006). HTPCs can be cultivated and sub-cultivated and therefore represent an established cell culture model for mechanistic studies (Albrecht, 2009). Yet, the cells can only be passaged in culture for a certain period of time (up to one year) until signs of replicative senescence appear. These time-dependent alterations allow for investigation of aging-associated processes.

HTPCs express androgen receptors (Mayer et al., 2018), have contractile abilities, and express smooth muscle genes (e.g. smooth muscle actin, myosin and calponin) as do their *in vivo* counterparts (Schell et al., 2010). Further characterization of HTPCs revealed their secretory functions including dominant production of ECM molecules (e.g. collagens, fibronectins, laminins, decorin and biglycan) (Flenkenthaler et al., 2014, Adam et al., 2012). The secretion of pro-inflammatory molecules like C-C motif chemokine ligand 2 (CCL2), IL6 and pentraxin 3 (PTX3) demonstrates their participation in testicular inflammatory processes (Schell et al., 2008). The expression of Toll-like receptors, as key regulators of innate immune responses, was detected in HTPCs (Mayer et al., 2016). Among others, TLRs are activated by biglycan, thereby inducing inflammatory cytokine production (Mayer et al., 2016). TLRs are functionally linked to the purinergic receptors P2X4 and P2X7, which can be activated by biglycan or extracellular ATP, presumably originating from mast cells (Babelova et al., 2009, Walenta et al., 2018). Besides TLRs and purinergic receptors, HTPCs also express the associated receptors for mast cell products like chymase, tryptase and TNFα. This fact demonstrates the interaction of HTPCs with testicular immune cells (Adam et al., 2011).

During previous years, evidence accumulated stating that peritubular cells are involved in male (in)fertility by secreting important factors for the SSC niche like GDNF (Spinnler et al., 2010) and C-X-C motif chemokine ligand 12 (CXCL12) (Flenkenthaler et al., 2014). Besides peritubular cells, Sertoli cells express these factors; however, a specific peritubular knock-out study in mice verified that GDNF from peritubular cells is essential for SSC maintenance (Chen et al., 2014, Chen et al., 2016).

HTPCs also possess steroidogenic capacity, which can be activated in culture (Landreh et al., 2014). In addition, peritubular cells may contribute to the avascularity of the seminiferous tubules and the BTB in the human testis by the secretion of the anti-angiogenic molecule pigment epithelium-derived factor (PEDF) (Windschuttl et al.,

2015). In summary, the previous studies attribute peritubular cells an important role in overall testicular functions.

1.4.3 A translational model for testicular peritubular cells (MKTPCs)

Previous studies employing HTPCs showed a high degree of heterogeneity presumably due to individual testicular tissue donors, because lifestyle, age, nutrition and medical history of patients may also affect HTPCs. To control these confounding issues, a translational cellular model for the study of human peritubular cells had to be established. As the structure of rodent and primate seminiferous tubular wall and of peritubular cells differs (e.g. in the number of layers of peritubular cells), rodents represent a suboptimal model for studying peritubular cells (Cooke and Saunders, 2002). Hence, the common marmoset monkey (*Callithrix jacchus*) served as model organism for the cultivation of monkey testicular peritubular cells (MKTPCs). Marmosets are small (adult, 350-400 g) new world primates, part of the primate family Callitrichidae and native to the Brazilian rainforest (t Hart et al., 2000). They are sexually mature at around 18 month and in general give birth to twins. Marmosets have in average a lifespan of 16 years and show age-related pathologies comparable to humans, e.g. cancer, and are therefore used as non-human primate model for aging (Tardif et al., 2011). Marmosets are also implemented as model organism in neuroscience, toxicology and infectious diseases (Mansfield, 2003, Okano et al., 2012, Zuhlke and Weinbauer, 2003). They share many biologically relevant aspects concerning germ cell development and organization of spermatogenesis with humans, and are thus widely used as a model in reproductive research (Holt and Moore, 1984, Millar et al., 2000, Hearn et al., 1978). Using a similar approach as for HTPCs, MKTPCs were isolated from testicular tissue fragments and sub-cultured. MKTPCs in culture express characteristic peritubular cell markers, like smooth muscle markers and androgen receptor but lack markers for Leydig cells (LH-receptor) and Sertoli cells (FSH-receptor), identical to their equivalents *in vivo*. A considerable overlap between secreted and cellular MKTPC and HTPC proteins was detected *via* mass spectrometry (MS), supporting the relevance of the marmoset monkey as a translational model for the human (Schmid et al., 2018).

2 Aims of the thesis

Spermatogenesis is a highly organized process and can easily be disrupted at every level, resulting in male sub- or infertility, but the majority of contributing factors remains poorly understood. Inflammatory events within the testis are often involved in infertility and in many cases fibrotic alterations of the testicular peritubular wall appear. Similar changes were observed in the testis of elderly men. Yet, the precise molecular mechanisms were only slightly explored. Therefore, the objectives were:

1. The exploration of the possible role of NLRP3, the sensor part of the NLRP3 inflammasome, in inflammation-associated testicular pathologies. NLRP3 expression should be investigated in samples of men with functional spermatogenesis and infertile pathologies and non-human primates (common marmoset monkey, rhesus macaque). Further, the *AROM*$^{+}$ mouse, as a systemic infertility mouse model of sterile inflammation will be investigated.

2. The examination of replicative senescence in a human cellular model (HTPCs) to gain insights into human testicular aging. Aging should be monitored by serial passaging of HTPCs in culture. The analysis of cellular alterations should include correlative light and electron microscopy, focused ion beam/scanning electron microscopy (FIB/SEM) tomography, mass spectrometry and gene expression studies.

3. To bypass confounding factors of human samples like nutrition, smoking, physical activity and general medical history, a translational model for HTPCs should be established. Therefore, testicular peritubular cells, should be isolated from tissue of marmoset monkeys (MKTPC). MKTPCs should be examined in regard to their suitability as model system for HTPCs implementing a comparative proteomic approach and subsequently used for mechanistic, regulative and functional investigations of peritubular cells with predictable confounders.

3 Results

3.1 Publication I

NLRP3 in somatic non-immune cells of rodent and primate testes.

Lena Walenta*, **Nina Schmid***, J. Ullrich Schwarzer, Frank-Michael Köhn, Henryk F. Urbanski, Rüdiger Behr, Leena Strauss, Matti Poutanen, Artur Mayerhofer

* equal contribution

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Abstract

NLRP3 is part of the NLRP3 inflammasome and a global sensor of cellular damage. It was recently discovered in rodent Sertoli cells. We investigated NLRP3 in mouse, human and non-human primate (marmoset and rhesus macaque) testes, employing immunohistochemistry. Sertoli cells of all species expressed NLRP3, and the expression preceded puberty. In addition, peritubular cells of the adult human testes expressed NLRP3. *NLRP3* and associated genes (*PYCARD*, *CASP1*, *IL1B*) were also found in isolated human testicular peritubular cells and the mouse Sertoli cell line TM4. Male infertility due to impairments of spermatogenesis may be related to sterile inflammatory events. We observed that the expression of NLRP3 was altered in the testes of patients suffering from mixed atrophy syndrome, in which tubules with impairments of spermatogenesis showed prominent NLRP3 staining. In order to explore a possible role of NLRP3 in male infertility, associated with sterile testicular inflammation, we studied a mouse model of male infertility. These human aromatase-expressing transgenic mice (*AROM*$^{+}$) develop testicular inflammation and impaired spermatogenesis during aging, and the present data show that this is associated with strikingly elevated *Nlrp3* expression in the testes compared to WT controls. Interference by aromatase inhibitor treatment significantly reduced increased *Nlrp3* levels. Thus, throughout species NLRP3 is expressed by somatic cells of the testis, which are involved in testicular immune surveillance. We conclude that NLRP3 may be a novel player in testicular immune regulation.

REPRODUCTION
RESEARCH

NLRP3 in somatic non-immune cells of rodent and primate testes

Lena Walenta[1,*], Nina Schmid[1,*], J Ullrich Schwarzer[2], Frank-Michael Köhn[3], Henryk F Urbanski[4], Rüdiger Behr[5], Leena Strauss[6], Matti Poutanen[6] and Artur Mayerhofer[1]

[1]Cell Biology – Anatomy III, Biomedical Center Munich (BMC), Ludwig-Maximilians-Universität München, Martinsried, Germany, [2]Andrology Center, Munich, Germany, [3]Andrologicum, Munich, Germany, [4]Division of Neuroscience, Oregon National Primate Research Center, Beaverton, Oregon, USA, [5]Platform Degenerative Diseases, German Primate Center, Göttingen, Germany and [6]Institute of Biomedicine, Research Center for Integrative Physiology and Pharmacology and Turku Center for Disease Modeling, University of Turku, Turku, Finland

Correspondence should be addressed to A Mayerhofer; Email: Mayerhofer@lrz.uni-muenchen.de

*(L Walenta and N Schmid contributed equally to this work)

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Introduction

NLRP3 (NLR family pyrin domain containing 3) is the molecular sensor of the NLRP3 inflammasome, which is primarily known to be expressed as an essential part of the innate immune response (Sharma & Kanneganti 2016). Inflammasomes are multimeric protein complexes that form in the cytoplasm according to a two-hit hypothesis. In the first step, the priming step, cells are challenged by pathogen-associated molecular patterns or danger-associated molecular patterns (DAMPs) (Patel *et al.* 2017). In the second step, NLRP3 is activated in response to a wide variety of stimuli. Diverse modes of activation have been identified (Jo *et al.* 2016). Hence, NLRP3 is being regarded as a global sensor of cellular damage. Activation allows NLRP3 oligomerization and subsequent recruitment of the adaptor protein PYCARD (PYD and CARD Domain Containing) and the effector protein Pro-caspase1 in a cascade-like assembly. Thereby, Pro-caspase1 becomes activated enabling processing of Pro-IL1β/IL18 to mature IL1β and IL18 and cleavage of Gasdermin D. In turn, released interleukins are able to promote inflammatory processes and contribute essentially to the immune response, while cleaved Gasdermin D fosters a cell death form termed pyroptosis (Broz & Dixit 2016). Thus, the NLRP3 inflammasome has been proven crucial for the removal of pathogens or damaged cells.

Deregulated inflammasome activation is, however, also considered a central driver of autoimmune diseases as well as neurologic and metabolic disorders with an inflammatory component activated mostly by endogenous DAMPs. Among them are chronic inflammatory diseases like atherosclerosis or diabetes (Guo *et al.* 2015). A special case is Muckle-Wells syndrome, an auto-inflammatory disorder based on

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NLRP3 gain-of-function mutations. Besides general sterile inflammatory symptoms due to unrestrained NLRP3 inflammasome activation, Muckle-Wells syndrome has been associated with impaired spermatogenesis and infertility (Tran *et al.* 2012, Tran 2017).

This may link the NLRP3 to the human testis, where sterile inflammatory events have been associated with impaired spermatogenesis (Mayerhofer 2013, Mayer *et al.* 2016, Walenta *et al.* 2018). Testicular sterile inflammation is witnessed among others by increased numbers of immune cells and changes in the architecture of the wall of seminiferous tubules and possibly in the functions of its cellular building blocks, peritubular cells (Mayerhofer 2013).

NLRP3 has also been described to act independently of the inflammasome. Some studies reported inflammasome-independent actions of an inflammatory nature (Shigeoka *et al.* 2010, Mizushina *et al.* 2015); yet, NLRP3 expression in non-immune cells has been established and was assigned to diverse functions. NLRP3 has especially been found in many epithelial cell types and been attributed a role in preserving epithelial barrier integrity, for instance, in lung and kidney (Pulskens *et al.* 2014, Kostadinova *et al.* 2016).

Recently, NLRP3 was described in a testicular epithelial cell type, the Sertoli cell (Hayrabedyan *et al.* 2015, 2016). Besides a possible implication in epithelial barrier, i.e. blood–testis barrier, function, functionality of the NLRP3 inflammasome including IL1β production and release in murine Sertoli cells was shown.

Sertoli cells line the seminiferous tubules and upon the onset of puberty form the blood–testis barrier, which is essential to the immune privilege of the testis and crucial to spermatogenesis (Franca *et al.* 2016). Sertoli cells also secrete immunoregulatory factors and thus, actively modulate the testicular immune response (Kaur *et al.* 2014). Sertoli cells interact with the neighboring peritubular cells in many ways (Tung *et al.* 1984, Skinner *et al.* 1985, Oatley & Brinster 2012). Our previous studies in human testicular peritubular cells (HTPCs) revealed that these express Toll-like receptors (TLRs) as well as purinergic receptors and produce a variety cytokines (Mayer *et al.* 2016, Walenta *et al.* 2018). Thus, they play an essential role in immune surveillance of the testis and presumably in male infertility, by complementing Sertoli cell functions.

NLRP3 expression and functionality in mouse testis, in Sertoli cells in particular, has been established (Hayrabedyan *et al.* 2015, 2016, Minutoli *et al.* 2015), and there is evidence for *NLRP3* expression in the whole human testis (Lech *et al.* 2010); yet, the cellular sites are not known. We examined NLRP3 expression and its expression sites in human and non-human primate testes. We further located testicular expression during postnatal development and regulation of NLRP3 in a mouse model of male infertility (*AROM+*).

Materials and methods

Human samples

Sections from fixed paraffin-embedded testis samples with normal spermatogenesis and samples from men with idiopathic infertility, showing mixed atrophy, were studied as described previously (Welter *et al.* 2014, Mayer *et al.* 2016). The local ethical committee (Ethikkommission, Technische Universität München, Fakultät für Medizin, München, project number 5158/11) approved the study.

Non-human primates

Specimens form the common marmoset monkeys (*Callithrix jacchus*) were obtained from the self-sustaining marmoset monkey colony at the German Primate Center (Deutsches Primatenzentrum; DPZ, Göttingen), and marmoset monkey housing has been described elsewhere (Debowski *et al.* 2015). Testes were received from marmoset monkeys killed for scientific purposes or castrated for colony management purposes. Veterinarians executed castration and killing, and organ extraction was carried out in accordance with relevant guidelines and regulations of the German Animal Protection Act. There is no additional ethical approval for the killing of animals for organ collection necessary.

Rhesus monkey (*Macaca mulatta*) samples were obtained from Oregon National Primate Research Center (ONPRC) Tissue Distribution Program as described previously (Frungieri *et al.* 2000, Adam *et al.* 2012). Rhesus monkeys were housed by the ONPRC, subjected to the regulations of the National Research Council's Guide for the Care and Use of Laboratory Animals. Testes fragments of both species were fixed with Bouin's solution and embedded in paraffin for immunohistochemistry and/or used for RNA extraction.

Infertility mouse model

The transgenic mouse line expressing human P450 aromatase under control of the ubiquitin C promoter (*AROM+*) has been previously generated (Li *et al.* 2001, 2003). *AROM+* mice in FVB/N genetic background and their WT littermates were used in this study. Aromatase inhibitor (AI, Finrozole, Vetcare, Finland) was used to treat 4-week-old mice for 6 weeks (Aguilar-Pimentel *et al.*, unpublished data). The mice were fed soy-free natural ingredient food pellets (Special Diets Services, Witham, UK) and tap water *ad libitum* and were housed in specific pathogen-free conditions at Central Animal Laboratory, University of Turku (Finland). Animals were handled under a license by the Finnish Animal Ethics Committee and by the institutional animal care policies of the University of Turku (Finland), which fully met the requirements defined in the NIH Guide for the care and use of laboratory animals. Testes of *AROM+* and age-matched WT mice at 2, 5 and 10 months of age were used for qPCR studies. In addition, further testes samples from *AROM+* and WT mice (2.5 months) treated with AI and placebo, respectively, were examined. Testicular tissue samples for the study were fixed in Bouin's fluid and embedded in paraffin and used for immunohistochemistry.

Immunohistochemistry

Immunohistochemical staining of testicular tissue samples from mouse, monkey and human was carried out as described previously (Mayer *et al.* 2016). For each species, a minimum of four and up to 14 different sections were examined. Polyclonal rabbit anti-NLRP3 IgG (R30750, NSJ Bioreagents, San Diego, CA, USA) was used as primary antibody to stain for NLRP3. Negative controls consisted of omission of the primary antibody, of incubation with rabbit IgG or non-immune serum instead of the primary antiserum. Sections were counterstained with hematoxylin and visualized using a Leica DM2500 microscope equipped with a DMC2900 CMOS camera or an Zeiss Axiovert microscope with an Insight Camera (18.2 Color Mosaik) and Spot advanced software 4.6 (SPOT Imaging Solutions, Sterling Heights, MI, USA).

Human peritubular cell isolation and culture

HTPCs were isolated from small, human testicular tissue samples exhibiting normal spermatogenesis as described (Albrecht *et al.* 2006, Schell *et al.* 2008). All patients (undergoing reconstructive surgery of the vas deferens, aged 36–52 years) had granted written informed consent for scientific purposes. The local ethical committee (Ethikkommission, Technische Universität München, Fakultät für Medizin, München, project number 5158/11) approved the study. All experiments were performed in accordance with relevant guidelines and regulations. Cells were cultivated in DMEM High Glucose (Gibco) supplemented with 10% fetal bovine serum (Capricorn Scientific, Ebsdorfergrund, Germany) and 1% penicillin/streptomycin (Biochrom, Berlin, Germany) at 37°C, 5% (v/v) CO_2. Purity of cell isolations was assessed as described previously (Walenta *et al.* 2018).

TM4 cell culture

The Sertoli cell line TM4 originated from immature mouse Sertoli cells (American Tissue Culture Collection (ATCC CRL1715), Riversville, MD, USA) was cultured as described elsewhere (Rossi *et al.* 2016).

Reverse transcription (RT) PCR/quantitative (q) PCR

RNA from whole human and monkey testis samples was isolated via the RNeasy FFPE Kit (Qiagen). RNA from human peritubular cells was extracted using the RNeasy Micro Kit (Qiagen). SuperScriptII (Invitrogen) and random 15mer primers were used for reverse transcription of human and monkey RNA. RNA from mouse testis was isolated using TRIsure reagent according to the manufacturer`s instructions (Bioline, Bioline reagents Ltd., London, UK). RNA was treated with deoxyribonuclease I (DNase I Amplification Grade Kit, Invitrogen Life Technologies) and RT-PCR was carried out using the SensiFAST cDNA Synthesis Kit (Bioline). For qPCR studies the QuantiFast SYBR Green PCR Kit (Qiagen) was applied using following protocol in a LightCycler 96 System (Roche Diagnostics): Pre-incubation (95°C, 5 min), 40 cycles denaturation (95°C, 10 s) and annealing/extension (60°C, 30 s) followed by melting (65–97°C) and cooling-down (37°C, 30 s). Oligonucleotide primers for amplification were the following: human and rhesus *NLRP3* (143 bp amplicon) 5′-GTGTTTCGAATCCCACTGTG-3′ (forward) and 5′-TCTGCTTCTCACGTACTTTCTG-3′ (reverse), marmoset *NLRP3* (143 bp amplicon) 5′-GTGCTTCTAATCACACTGTG-3′ (forward) and 5′-TCTGCTTCTCACATGTCTTCT-3′ (reverse), murine *Nlrp3* (144 bp amplicon) 5′-TCTCCACAATTCTGACC CACA-3′ (forward) and 5′-ACATTTCACCCAACTGTAGGC-3′ (reverse), human *PYCARD* (117 bp amplicon) 5′-AAGCCAG GCCTGCACTTTAT-3′ (forward) and 5′-CTGGTACTGCTCATC CGTCA-3′ (reverse), murine *Pycard* (154 bp amplicon) 5′-CATTGCCAGGGTCACAGAAG-3′ (forward) and 5′-GCAG GTCAGGTTCCAGGAT-3′ (reverse), human *CASP1* (83 bp amplicon) 5′-GTTTCAGTCACACAAGAAGGGAG-3′ (forward) and 5′-GGAACGGATAAACAGCTTTCTCTT-3′ (reverse), murine *Casp1* (90 bp amplicon) 5′-GACATCCTTCATCCTCAGAAA CA-3′ (forward) and 5′-AAGGGCAAAACTTGAGGGTC-3′ (reverse), human *IL1B* (127 bp amplicon) 5′-CTTGGTGATG TCTGGTCCATATG-3′ (forward) and 5′-GGCCACAGGTATTTT GTCATTAC-3′ (reverse), murine *Il1b* (101 bp amplicon) 5′-TGAAGTTGACGGACCCCAAA-3′ (forward) and 5′-TGATG TGCTGCTGCGAGATT-3′ (reverse), human *RPL19* (199 bp amplicon) 5′-AGGCACATGGGCATAGGTAA-3′ (forward) and 5′-CCATGAGAATCCGCTTGTTT-3′ (reverse), murine *Rpl19* (199 bp amplicon) 5′-AGGCATATGGGCATAGGGAA-3′ (forward) and 5′-CCATGAGGATGCGCTTGTTT-3′ (reverse), human *HPRT* (163 bp amplicon) 5′-CCTGGCGTCGTGATT AGTGA-3′ (forward) and 5′-GGCCTCCCATCTCCTTCATC-3′ (reverse), murine *Hprt* (110 bp amplicon) 5′-CTGGTGAA AAGGACCTCTCGAA-3′ (forward) and 5′-CTGAAGTCATCA TTATAGTCAAGGGCAT-3′ (reverse). Amplicon identity was verified by agarose gel electrophoresis and consecutive sequence analysis (GATC, Konstanz, Germany). Quantitative results were calculated according to the $2^{-\Delta\Delta Cq}$ method and normalized to *RPL19* and *HPRT* as reference genes. Results are depicted as means ± S.E.M. Statistical analyses were obtained via unpaired *t*-tests (two-tailed) or one-way ANOVA with Newman–Keuls post-test for multiple comparisons of $-\Delta\Delta C_q$ values using GraphPad Prism 6.0 Software (GraphPad Software Inc.). $P<0.05$ was considered statistically significant.

Results

In testicular sections of mouse, human and non-human primate (marmoset monkey, rhesus macaque), immunoreactive NLRP3 was found in Sertoli cells (Fig. 1A, B, C and D). Typically, the cytoplasm of Sertoli cells was stained completely, reaching from the contact site of the basal lamina to the luminal site, while the nuclear area remained spared (Fig. 1E and F). Sertoli cell staining was also observed in immature primate (Fig. 1G) and mouse testes (not shown). All controls performed were negative. *NLRP3* expression in human, mouse and primate testes was confirmed in whole testes lysates via RT-PCR (Fig. 2A) followed by sequence analyses.

In human testis samples, NLRP3 staining was also found in peritubular myoid cells of the seminiferous

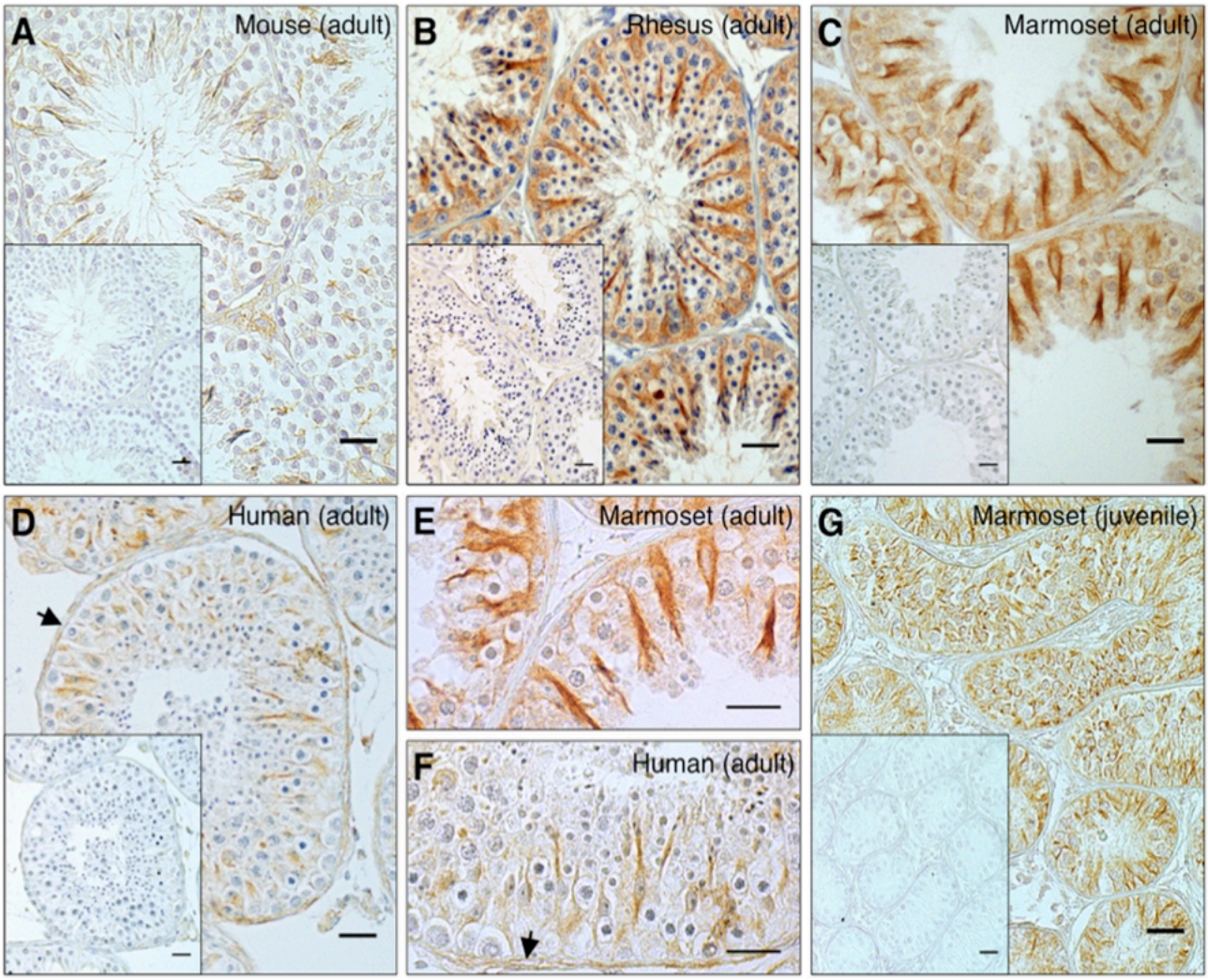

Figure 1 NLRP3 is expressed in Sertoli cells of mouse and primate testes and in peritubular cells of the human testis. Immunohistochemistry for NLRP3 revealed staining in mouse ($n=14$; A: adult, 3 months) rhesus macaque ($n=4$; B: adult, 18 years), common marmoset ($n=7$; C: adult, 11 years; G: new-born) and human testes ($n=8$; D: adult, 48 years). Sertoli cells expressed NLRP3 in all samples examined. Expression in peritubular cells (arrows) of the seminiferous tubule was solely detected in human samples. Higher magnifications of adult marmoset (E) and human (F) sections confirmed these findings. Inlays: negative controls. Bars=25 µm.

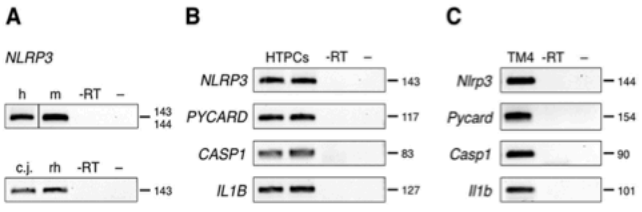

Figure 2 Expression of NLRP3 in human, nonhuman, primate and mouse testes. (A) In human (h), mouse (m), marmoset monkey (cj) and rhesus macaque (rh) *NLRP3* transcripts indicated expression in whole testis lysates. (B) *NLRP3* transcripts were detected in cultured human testicular peritubular cells (HTPCs; $n=2$). Samples stem from men exhibiting normal spermatogenesis. Inflammasome-associated gene expression of *PYCARD*, *CASP1* and *IL1B* was identified in HTPCs as well. (C) Mouse Sertoli cell line TM4 exhibited expression of *Nlrp3*, *Pycard*, *Casp1* and *Il1b* transcripts. Negative controls consisted of non-reverse transcribed RNA as template (–RT) and a non-template reaction (–). Labels indicate amplicon lengths.

tubular wall (Fig. 1D and F). In isolated HTPCs, *NLRP3* expression was confirmed. In addition, expression of the components of the NLRP3 inflammasome cascade, *PYCARD*, *CASP1* and *IL1B*, was detected (Fig. 2B). The mouse cell line TM4 was employed as a Sertoli cell model. *Nlrp3*, *Pycard*, *Casp1* and *Il1b* transcript expression in TM4 cells was documented (Fig. 2C).

Human testicular sections from patients suffering from mixed atrophy syndrome (MA; Fig. 3) were studied. In these sections, tubules with normal spermatogenesis and impaired spermatogenesis were observed next to each other. NLRP3 was detected predominantly in peritubular cells of fibrotically altered, thickened sectors of the tubular wall when spermatogenesis was impaired. Absence of cellular staining in the tubular compartment was seen in some cases.

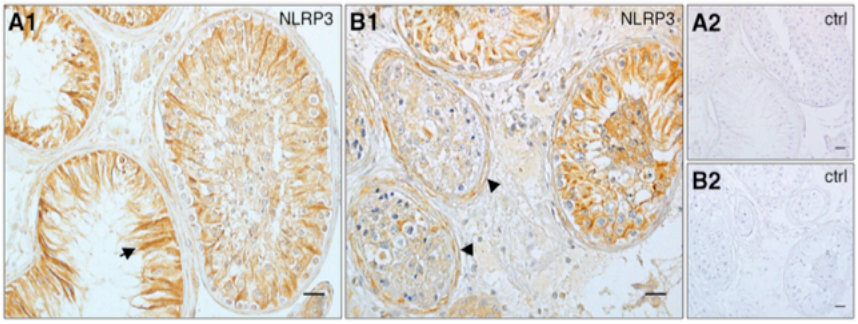

Figure 3 NLRP3 expression in human testes is associated with phenotypic characteristics in subfertility patients. (A1) In patients suffering from mixed atrophy (MA) syndrome ($n=5$) staining in Sertoli cells remained prominent, especially in tubules with impaired spermatogenesis (arrow). (B1) Staining of peritubular cells and the tubular wall (arrowheads) intensified corresponding to thickened sectors of the tubular wall, which are associated with MA pathology. Tubular walls stained most intensely in seminiferous tubules, which lacked Sertoli cell staining. (A2, B2) Negative controls corresponding to A1 and B1, respectively. Bars = 25 µm.

To explore the possibility of testicular NLRP3 involvement in male infertility, we used *AROM+* mice as a model of inflammation-associated male infertility (Li *et al.* 2001, 2003). These transgenic mice express human aromatase under the ubiquitin C promoter that causes an increased estrogen/testosterone ratio. Importantly, the mice develop testicular sterile inflammation and impaired spermatogenesis with age, a phenotype resembling inflammation-associated human male infertility (Li *et al.* 2006). The *Nlrp3* transcript levels measured at 2, 5 and 10 months of age were increased in the *AROM+* testes compared to WT, albeit a statistically significant increase in this group of mice was detected solely at 5 months and 10 months of age (Fig. 4A, B and C).

To explore the possibility of a phenotypic rescue through AI treatment as shown previously (Li *et al.* 2004), a separate group of mice at 2.5 months of age was used. At 2.5 months, there was a statistically significant increase in *Nlrp3* transcript levels in *AROM+* testes. Prepubertal AI treatment reduced the elevated *Nlrp3* levels in *AROM+* mice approximately two-fold compared to placebo treated *AROM+* mice, although the initial basal levels of WT (±AI) mice were not completely restored (Fig. 5A). In WT mice, AI treatment did not have any effect on *Nlrp3* levels. Testicular morphology of WT and *AROM+* mice illustrated that at the age of 2.5 months, vacuolization of the germinal epithelium was already emerging (Fig. 5B).

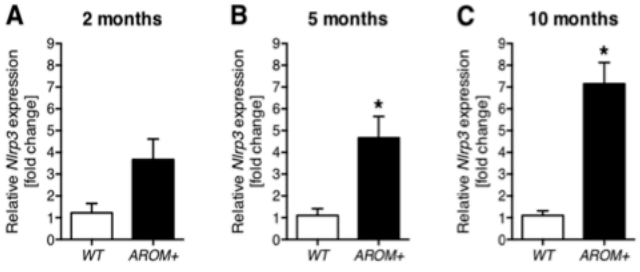

Figure 4 Testicular *Nlrp3* levels are elevated in an infertility mouse model with increasing age. (A, B and C) *Nlrp3* transcript expression was elevated ~three-fold in 2 months old *AROM+* mice ($n=4$) compared to WT mice ($n=3$), ~four-fold in 5 months old *AROM+* mice ($n=5$) compared to WT mice ($n=3$) and ~seven-fold in 10 months old *AROM+* mice ($n=8$) compared to WT mice ($n=7$). Data represent means ± S.E.M. normalized to WT control. Asterisks (*) denote statistical significance, $P<0.05$ (unpaired *t*-test).

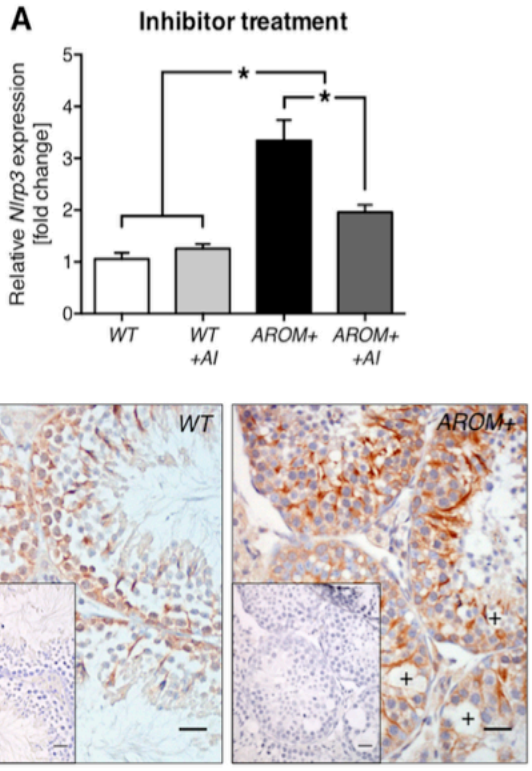

Figure 5 Elevated testicular *Nlrp3* levels can be reduced by an intervention strategy. (A) When treated with an aromatase inhibitor (AI) *Nlrp3* transcript levels in 2.5 months old *AROM+* ($n=7$) decreased from significant ~three-fold elevation in comparison to WT ($n=8$) almost by half ($n=6$). AI treatment of WT mice ($n=8$) did not show any differences from WT. Data represent means ± S.E.M. normalized to WT control. Asterisks (*) denote statistical significance, $P<0.05$ (ANOVA with Newman–Keuls post-test). (B) Staining for NLRP3 in WT and *AROM+* testicular sections (2.5 months) depicted positively stained Sertoli cells and revealed alterations of the testicular phenotype in *AROM+* compared to WT mice, e.g. vacuolization (+). Inlays: negative controls. Bars = 25 µm.

Discussion

While immunoreactive NLRP3 in the human testis was not reported in a previous study (Kummer *et al.* 2007), evidence for NLRP3 expression was established in the testis (Lech *et al.* 2010, Hayrabedyan *et al.* 2015, 2016, Minutoli *et al.* 2015). More recent studies indicated a testicular role of NLRP3, mostly based on transcript and protein expression analysis (Fan *et al.* 2017, Bazrafkan *et al.* 2018, Hajipour *et al.* 2018, Lin *et al.* 2018); yet, the cellular sites of expression *in situ* remain unknown. The results of our study confirm that Sertoli cells *in situ* are the major sites of NLRP3 expression in the testes of rodent and, as we found, also primate species. Furthermore, the expression precedes the onset of puberty. Surprisingly, NLRP3 was found in a further somatic cell type of the human testis, the peritubular cell. Previous studies indicated that this cell type has multiple roles in testicular immune surveillance and that it may contribute to sterile inflammation related to male infertility (Mayer *et al.* 2016, Walenta *et al.* 2018). Changes in the expression pattern of NLRP3 were indeed observed in testes of infertile men. Increased levels of NLRP3 also accompanied an age-dependent development of inflammation and infertility in *AROM+* mice, a model for male infertility. The effectiveness of AI treatment to block increased NLRP3 levels in *AROM+* argues for a role of NLRP3 in male infertility.

Sertoli cells, the major sites of NLRP3 in the testis, form the blood–testis barrier. Sertoli cells also exert a number of immune functions and express for example TLRs (Zhao *et al.* 2014). The observed NLRP3 expression in these cells *in situ* correlates with the recent report showing NLRP3 in isolated rodent Sertoli cells (Hayrabedyan *et al.* 2016) and NLRP3 functionality was shown in this study. In this report, IL1β secretion by isolated mouse Sertoli cells required priming for example with the endotoxin LPS. Since bacterial infections of the testis are rare, it is likely that organ-relevant activation may occur through other mechanisms. As NLRP3 can be activated by a variety of agents (Jo *et al.* 2016), this will require an extensive investigation.

We studied NLRP3 expression in immature testes. Sertoli cells become fully functional only at puberty under the influence of FSH and androgens (Stanton 2016). At that time, the blood–testis barrier forms, which is essential for the immune privilege of the testis and spermatogenesis (Mayerhofer & Bartke 1990, Franca *et al.* 2016). Since NLRP3 was expressed by Sertoli cells already in the neonate and infantile monkey testis, this questions hormonal influences on NLRP3. It argues for a role before the onset of sexual maturity and speaks for a non-hormonal regulation of its expression. In this context, it is important to acknowledge the possibility that NLRP3 could serve an altogether inflammasome-independent purpose in the testis.

Peritubular cells are the immediate neighbors of Sertoli cells and form the tubular wall. In rodents, only one layer of these slender, elongated peritubular cells exists. In contrast, in the human testis, there are several layers. These smooth muscle-like cells contract thereby transporting sperm, and they also produce extracellular matrix. Previous studies identified additional properties, which indicate that they collaborate with Sertoli cells and complement several of their functions (Mayerhofer 2013, Flenkenthaler *et al.* 2014). An example is GDNF, a growth factor secreted by both cell types, which is crucial for spermatogonial stem cell renewal (Spinnler *et al.* 2010, Chen *et al.* 2016), another example relates to immunological actions. Peritubular cells possess immunological properties, including TLRs, purinergic receptors and they produce cytokines (Mayer *et al.* 2016, Walenta *et al.* 2018). Thus, they may play a role in immune surveillance of the testis. This is of importance in human male infertility, in which sterile inflammatory events are being recognized as crucial. For instance, the extracellular matrix factor biglycan is secreted by peritubular cells of the human testis, acts via TLRs and leads to the secretion of pro-inflammatory cytokines (Mayer *et al.* 2016). A recent study pinpointed involvement of NLRP3 in glial cells and sterile inflammation of the brain (Freeman *et al.* 2017). We now report the expression of the damage sensor NLRP3 in HTPCs and human Sertoli cells. Furthermore, expression of *PYCARD*, *CASP1* and *IL1B* was verified in isolated HTPCs.

While putative induction and roles of the NLRP3 inflammasome in both, human peritubular cells and Sertoli cells, remain to be studied, our immune staining suggests a correlation of NLRP3 expression with idiopathic male infertility. The NLRP3 pattern in Sertoli cells and peritubular cells changed in human testicular sections from MA samples. MA samples were used in order to stain specimens exhibiting tubules of both, normal and impaired spermatogenesis. Thus, unaffected tubules were used to control potential artifacts of fixation and processing of testicular samples. NLRP3 staining in Sertoli cells of the tubules with impaired spermatogenesis was either markedly increased or was found to be absent, combined with enhanced staining in peritubular cells. While additional studies are needed to verify this observation, it may imply a dynamic regulation, possible complementary actions and, importantly, functional involvement in the events associated with male infertility. Male infertility is often characterized by signs of sterile inflammation, including increased numbers of immune cells, mainly mast cells in the walls of seminiferous tubules, fibrotic thickening of the walls and a phenotype switch of peritubular cells (Meineke *et al.* 2000, Mayer *et al.* 2016), and our data suggest that NLRP3 expression is involved.

The involvement of NLRP3 in the pathophysiology of male infertility and sterile testicular inflammation is

further supported by the results obtained in the *AROM+* mouse model, a model for male infertility. In *AROM+* mice sterile testicular inflammation is a consequence of hormonal imbalances eventually resulting in increased testicular TNFα levels, massive accumulation of macrophages and increased levels of TLRs, to name only few of the changes (Li *et al.* 2003, 2006, Mayer *et al.* 2016). Our results show that testicular *Nlrp3* levels increase likewise. An intervention, such as AI treatment, restored the phenotype, as shown previously (Li *et al.* 2004), and as we found this intervention also blocked testicular *Nlrp3* elevation. The results suggest activation of NLRP3 as a yet unknown player in male infertility and imply non-infectious modes of activation, presumably through endogenous DAMPs (Jo *et al.* 2016).

In summary, the data show expression of NLRP3 in the testes and implicate NLRP3 in the regulation of male (in)fertility. The possible involvement of NLRP3 in deranged testicular functions is also supported by recent data on male fertility in Muckle–Wells syndrome. This auto-inflammatory disorder is caused by an activating *NLRP3* gene mutation and interestingly impaired spermatogenesis and male sub- or infertility was reported (Tran *et al.* 2012, Tran 2017).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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3.2 Publication II

Insights into replicative senescence of human testicular peritubular cells.

Nina Schmid, Florian Flenkenthaler, Jan B. Stöckl, Kim-Gwendolyn Dietrich, Frank M. Köhn, J. Ullrich Schwarzer, Lars Kunz, Manja Luckner, Gerhard Wanner, Georg J. Arnold, Thomas Fröhlich, Artur Mayerhofer

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Abstract

There is evidence for an age-related decline in male reproductive functions, yet how the human testis may age is not understood. Human testicular peritubular cells (HTPCs) transport sperm, contribute to the spermatogonial stem cell (SSC) niche and immune surveillance, and can be isolated and studied *in vitro*. Consequences of replicative senescence of HTPCs were evaluated to gain partial insights into human testicular aging. To this end, early and advanced HTPC passages, in which replicative senescence was indicated by increased cell size, altered nuclear morphology, enhanced β-galactosidase activity, telomere attrition and reduced mitochondrial DNA (mtDNA), were compared. These alterations are typical for senescent cells, in general. To examine HTPC-specific changes, focused ion beam scanning electron microscopy (FIB/SEM) tomography was employed, which revealed a reduced mitochondrial network and an increased lysosome population. The results coincide with the data of a parallel proteomic analysis and indicate deranged proteostasis. The mRNA levels of typical contractility markers and growth factors, important for the SSC niche, were not significantly altered. A secretome analysis identified, however, elevated levels of macrophage migration inhibitory factor (MIF) and dipeptidyl peptidase 4 (DPP4), which may play a role in spermatogenesis. Testicular DPP4 may further represent a possible drug target.

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Insights into replicative senescence of human testicular peritubular cells

Nina Schmid[1], Florian Flenkenthaler[2], Jan B. Stöckl[2], Kim-Gwendolyn Dietrich[1], Frank M. Köhn[3], J. Ullrich Schwarzer[4], Lars Kunz[5], Manja Luckner[6], Gerhard Wanner[6], Georg J. Arnold[2], Thomas Fröhlich[2] & Artur Mayerhofer[1*]

There is evidence for an age-related decline in male reproductive functions, yet how the human testis may age is not understood. Human testicular peritubular cells (HTPCs) transport sperm, contribute to the spermatogonial stem cell (SSC) niche and immune surveillance, and can be isolated and studied *in vitro*. Consequences of replicative senescence of HTPCs were evaluated to gain partial insights into human testicular aging. To this end, early and advanced HTPC passages, in which replicative senescence was indicated by increased cell size, altered nuclear morphology, enhanced β-galactosidase activity, telomere attrition and reduced mitochondrial DNA (mtDNA), were compared. These alterations are typical for senescent cells, in general. To examine HTPC-specific changes, focused ion beam scanning electron microscopy (FIB/SEM) tomography was employed, which revealed a reduced mitochondrial network and an increased lysosome population. The results coincide with the data of a parallel proteomic analysis and indicate deranged proteostasis. The mRNA levels of typical contractility markers and growth factors, important for the SSC niche, were not significantly altered. A secretome analysis identified, however, elevated levels of macrophage migration inhibitory factor (MIF) and dipeptidyl peptidase 4 (DPP4), which may play a role in spermatogenesis. Testicular DPP4 may further represent a possible drug target.

Aging of any given organ is characterized by a decline of its function. This may apply also for the male gonad. It was postulated that with increasing age, an overall decline of male reproductive functions occurs, which involves reduced sperm counts and androgen production, paralleled by structural changes[1,2]. Several investigations indicate that structural and functional changes in the testis of men in advanced age involve the androgen-producing Leydig cells and the Sertoli cells, which are regarded as the nurse cells for male gem cells[3–7]. In addition, age-related alterations of the tubular wall compartment (also called *tunica propria*) in men were reported. These changes include progressive enlargement and sclerosis, accompanied by impaired spermatogenesis and (in some cases) complete tubular sclerosis[2,5,6]. In contrast, a study in men with proven fertility, who were examined later in life, suggested that aging of the human testis may not necessarily be associated with structural/functional changes, in general[8]. Hence, there is debate about the selection criteria of men in the mentioned studies, as patients with pre-existing infertility and accompanying structural changes may have been included[2,5,6]. In addition, all these investigations in elderly men have limitations with respect to statistically relevant amounts of samples. Conceivably, age-related alterations of testicular structure and function in men are likely a consequence of many factors acting together in the long-lived human species. Besides age itself, medical conditions, use of drugs and overall lifestyle can affect both, the regulation of testicular functions and/or the male gonads itself. These issues cannot be well separated in human and there is no established, long-lived animal model to adequately mirror this situation.

A possible approach to investigate testicular aging would be the examination of senescence of testicular cells, yet most human testicular cells cannot be propagated *in vitro*, with the exception of peritubular cells. Human testicular peritubular cells form several layers within the wall surrounding the germinal epithelium and morphological studies suggested that they may change during testicular aging[2,5,6]. Peritubular cells of the adult testis are contractile, smooth muscle-like cells and secrete extracellular matrix (ECM) proteins. Imbalances between secretion/maintenance of the ECM and cellular contractile abilities are documented in the testes of men with impaired

[1]LMU München, Biomedical Center (BMC), Anatomy III – Cell Biology, 82152, Planegg-Martinsried, Germany. [2]LMU München, Gene Center, Laboratory for Functional Genome Analysis (LAFUGA), 81377 München, Germany. [3]Andrologicum München, 80331, München, Germany. [4]Andrologie Centrum München, 81241, München, Germany. [5]LMU München, Department Biology II, Division of Neurobiology, 82152, Planegg-Martinsried, Germany. [6]LMU München, Department Biology I, Ultrastructural Research, 82152, Planegg-Martinsried, Germany. *email: Mayerhofer@lrz.uni-muenchen.de

spermatogenesis[9,10]. The resulting fibrosis of the tubular wall is furthermore accompanied by accumulation of immune cells, implying a sterile type of inflammation in the tubular wall of infertile men[10–12]. As mentioned, human testicular peritubular cells (HTPCs) can be isolated and cultured from human testicular tissue[13]. HTPCs produce important growth factor molecules, e.g. glial cell line derived factor (GDNF) and C-X-C motif chemokine ligand 12 (CXCL12) and thereby most likely contribute to the spermatogonial stem cell (SSC) niche of the testis and to life-long spermatogenesis[14–16]. Initially, Sertoli cells were known as sole producers of GDNF and an overall important part of the SSC niche. Yet the importance of complementary GDNF, derived from peritubular cells, was confirmed in a systemic animal model[17]. The GDNF-mutant mice lost their fertility with increasing age. This observation links aging to a function of peritubular cells and concurs with the concept that impairments of the SSC niche may be part of testicular aging[18]. In this context a recent study of the human testes by Pohl *et al.*[19] documented the occurrence of diminished spermatogenic efficiency, increased amount of proliferating A_{dark} spermatogonia and altered nuclear morphology of Sertoli cells with increasing age. This suggests alterations of Sertoli cell function. It does not rule out changes in peritubular cell functions, although obvious structural alterations of the tubular wall or peritubular cells with increasing age in men were not described.

HTPCs also secrete cytokines, express Toll-like receptors, purinergic receptors and produce reactive oxygen species (ROS)[20]. Previous work implies that peritubular cells are involved in sterile inflammation, associated with some cases of male infertility[10,21,22]. Inflammation may also be part of aging. Indeed the term "inflammaging"[23] was coined. A possible functional involvement of peritubular cells in aging of the human testis was not examined yet. We reasoned that replicative senescence, triggered by serial passaging of cultured HTPCs, may allow the investigation of their contribution to testicular aging. Replicative senescence is an established cell culture model for research in aging and senescence[24,25]. It describes a cellular response, which limits proliferation of aged or damaged cells, in association with restricted cellular function[26] and a characteristic secretory phenotype, i.e. senescence-associated secretory phenotype (SASP)[27]. The consequences of replicative senescence in early and advanced passages of cultured HTPCs were investigated employing complementary approaches, including proteomics, qPCR studies and light microscopy (LM) combined with focused ion beam scanning electron microscopy (FIB/SEM) tomography, which allowed 3D reconstructions of various cellular compartments.

Results

Replicative senescence in HTPCs. HTPCs divide in culture with a doubling time of 2–3 days and can be propagated until cell division ceases after approximately one year. Cells from the same donors without (low passage number) and with signs of replicative senescence (advanced passage number) were compared. In addition to growth arrest, the following criteria were used to define replicative senescence of HTPCs: a change of the spindle-like morphology to an irregularly shaped and flattened morphology (Fig. 1a), increased β-galactosidase activity (Fig. 1b), increased cell size (Fig. 1c) and reduced telomere length (Fig. 1d). Cells fulfulling these criteria were further examined. Furthermore, we found that mtDNA was reduced in advanced passges, compared to the corresponding early passages (Fig. 1e).

FIB/SEM tomography of early vs. advanced passages of HTPCs. Ultrathin embedding of HTPCs allowed identification of target cells in SEM due to the coordinate system engraved on the slides and the specific cell topography. Superposition of LM (phase contrast) and SEM images allowed rapid correlation of fine structural details in the nanometer range. HTPCs of early passages are elongate, spindle-shaped cells, with a prominent nucleus and several nucleoli (Figs 1a, 2a,b). In phase contrast, granular substructures dispersed within the cytoplasm were observed in all cells. At one side of the nucleus the cytoplasm appeared to be devoid of larger substructures over a distance of 10–20 µm (Fig. 2a). In contrast, cells of advanced passages exhibited an abounding number of granules, either bright or dark in phase contrast, evenly distributed within the cytoplasm (Fig. 2a'). For better insight into the distribution of cellular substructures in relation to the nucleus, longitudinal sections were chosen for FIB/SEM analysis of both passages (Fig. 2c,c',d,d').

Cells in early passages were rather flat with a height in the range of 3 µm (Fig. 2c). The nucleus was lens shaped with parallel envelope membranes. Nuclear pores could be visualized by volume rendering of the 3D-data set. Several strands of ER were typically connected to the outer nuclear membrane. The ER formed: i) large, parallel sheets densely packed with ribosomes, ii) smaller sheets of fenestrated rough ER, randomly orientated and iii) bulb shaped dilatations with either electron translucent or fine granular lumen (Fig. 2c,d). Mitochondria were up to 15 µm long and very variable in their diameter, ranging from 0.3 µm to 0.1 µm, without any cristae. Only in the 3D context could they be recognized as mitochondria (Fig. 2c,d,f). Most mitochondria were branched and formed a complex network including numerous loops. Characteristic for all mitochondrial networks were multiple intimate contact sites with sheets of rough ER (Fig. 2g). The mitochondrial tubes were locally tightly enwrapped so that the membranes of ER and the outer mitochondrial membrane could not be discriminated, even at the resolution limit of FIB/SEM (2 nm isovoxel). At the site of contact, the ER was depleted of ribosomes. There was a population of roughly globular organelles composed of two components: a very electron dense matrix of packed/and or fused membranes and electron translucent areas resembling vacuoles, ranging from approximately 200 nm to 2 µm in diameter (Fig. 2c). When present in higher densities, they were interconnected. They were regarded as lysosomes, rather than autophagosomes or autophagolysosomes, as there was no indication for the presence of membranous phagophores enwrapping cell organelles[28], e.g. mitochondria. When examining lysosomes in a 3D context, it became evident that the ER lumen was in continuity to both, the vacuolar, and the membrane part of the lysosomes. In early passages lysosomes were not distributed randomly within the cell but rather accumulated in one half of the cell, typically 10–15 µm distal from the nucleus. This concurs with the granular structures observed in the phase contrast images (Fig. 2a, a').

HTPCs in advanced passages lost their original spindle-like shape and flatten out to irregular shapes (Fig. 2a', b', c'). Although the nucleus still appeared to be roughly lens shaped in LM, deduced from DAPI images (Fig. 2a'),

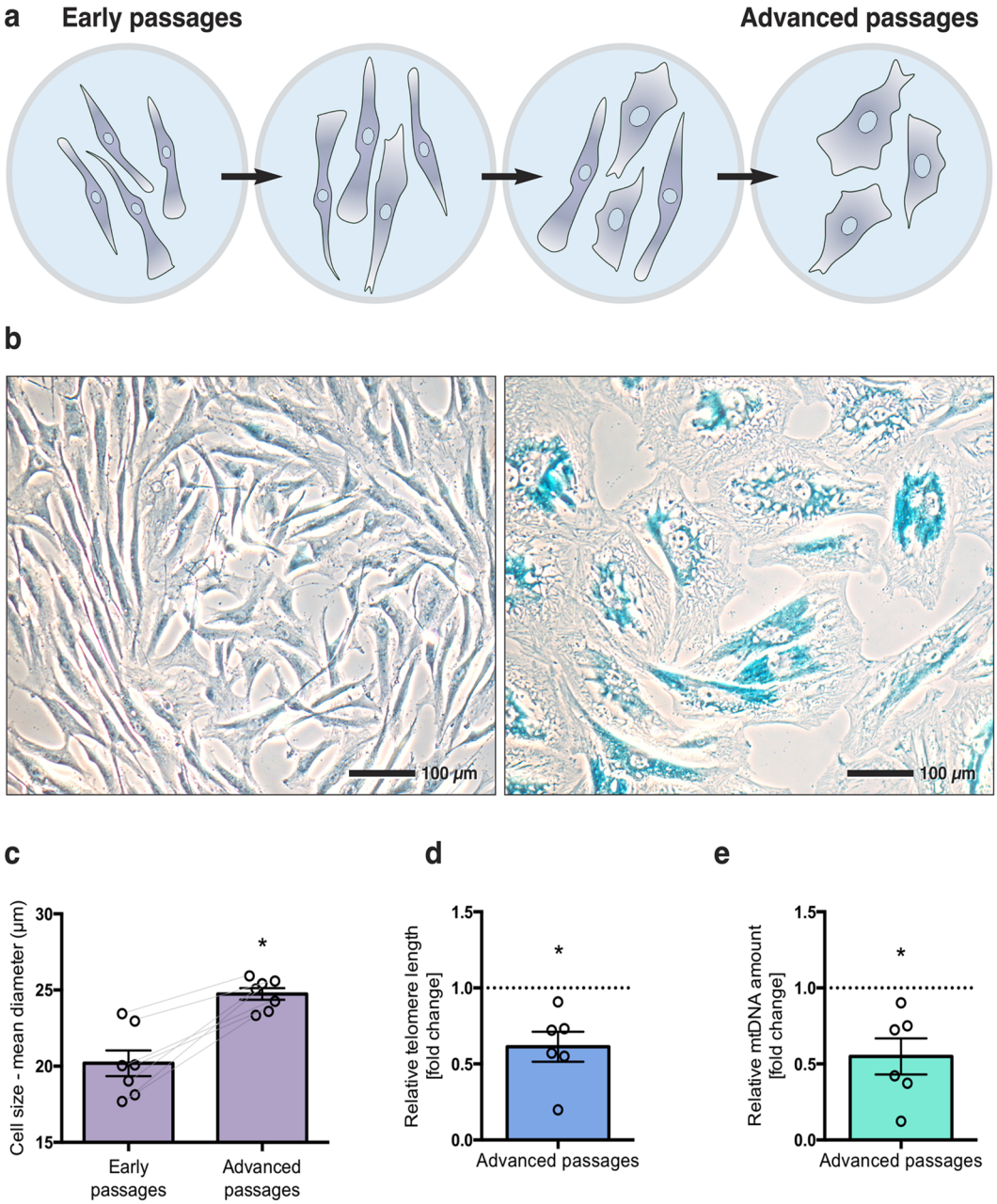

Figure 1. Changes associated with replicative senescence in HTPCs. Schematic presentation of HTPCs in transition from early to advanced passages (**a**). Light micrographs of senescence associated β-galactosidase staining of HTPCs: in contrast to HTPCs from early passage (passage = P; P5, left), β-galactosidase activity is prominent in advanced passage (P15, right) (**b**). Measurement of cell sizes revealed a significant increase in diameter in advanced passages from the same donor (n = 7), statistics were calculated with a paired two-sample *t*-test (**c**). Quantification of the relative telomere length from isolated gDNA from advanced passages of HTPCs from the same donor (n = 5) with significant telomere attrition, normalized to early passages (**d**). Quantification of mtDNA copy number by qPCR indicates significantly decreased amount of mtDNA in advanced HTPC passages (**e**). Statistical analysis was done with an one-sample *t*-test (**d**,**e**), mean ± SEM are given, asterisks indicate statistical significance, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

the 3D analysis revealed numerous cytoplasmic invaginations (Fig. 2c'). Nuclear pores were still visible with volume rendering of the 3D data set. The mitochondrial networks were pertained (Fig. 2f'), however, their number decreased in relation to the corresponding cytoplasmic volume (normalized to 100 µm^3) (Table 1). Deducing from statistical measurements, the mitochondria (network) became smaller in diameter (0.22 µm *vs.* 0.18 µm) but extended in length (5.8 µm *vs.* 8.8 µm) (Fig. 2f'). However, the total mitochondrial surface in a given volume of 100 µm^3 was reduced to approximately 25% compared to cells of early passages. The characteristic contact sites between mitochondria and ER were preserved, however, the shape of ER changed from large parallel sheets to smaller slabs with random orientations (Fig. 2g'). There was indication for at least partial degradation of mitochondria or mitochondrial segments, which became visible upon large volume serial block face sectioning: the mitochondrial matrix became locally electron translucent, formed blebs, with electron dense membrane fragments, likely residual cristae (Fig. 3a,b). The 3D reconstruction revealed that several segments of the

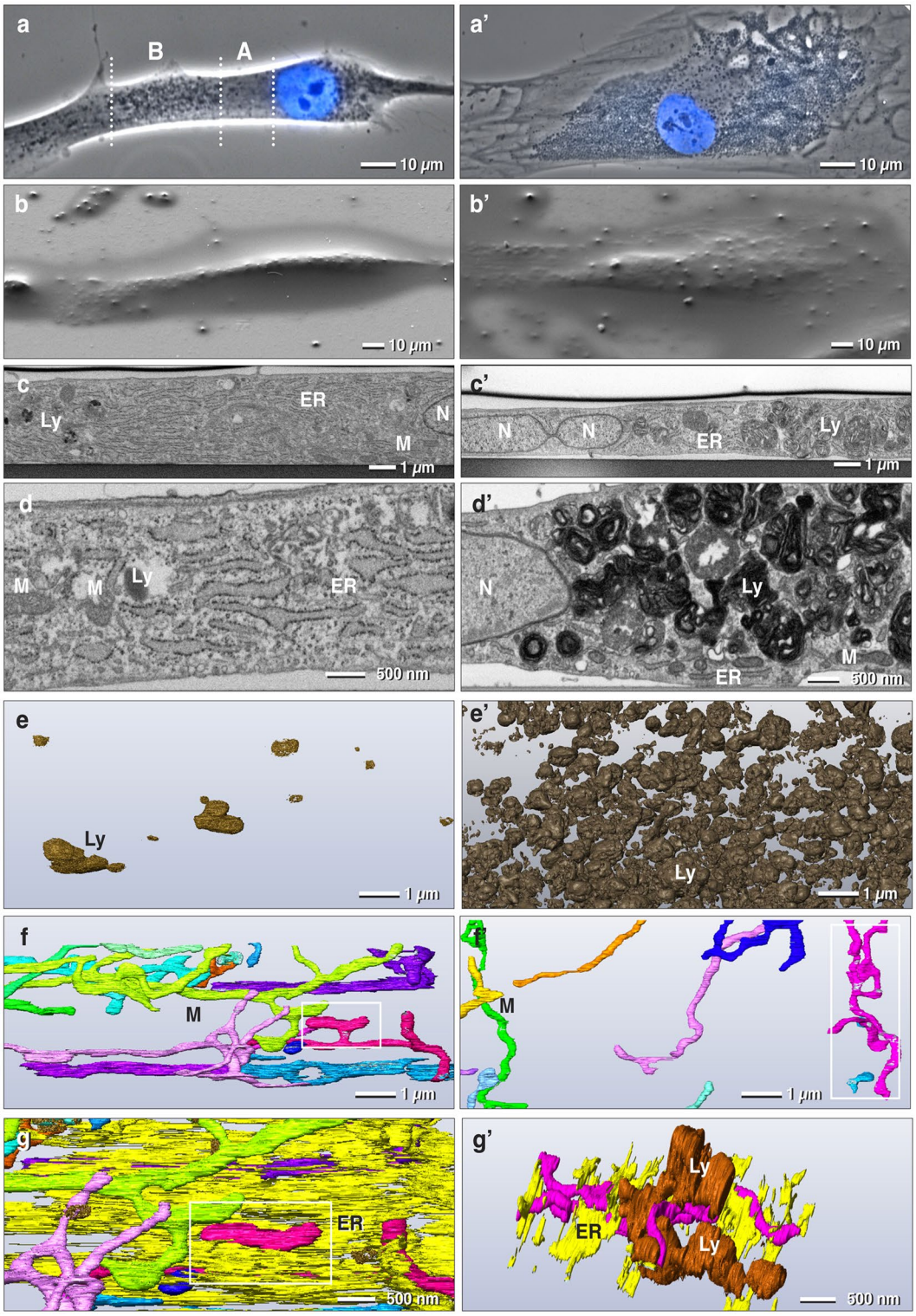

Figure 2. Focused ion beam scanning electron microscopy (FIB/SEM) tomography of early *vs*. advanced passages of HTPCs. Correlative LM and FIB/SEM tomography of ultrastructural changes of HTPCs (early passages: **a**–**g**; advanced passages: **a'**-**g'**). In early passages the cells are spindle-shaped with a rather homogeneous region of cytoplasm proximal to the nucleus (**a**: A) and a distal, granular region (**a**: B). Senescent cells flatten out and their cytoplasm becomes packed with granules (**a'**). Selected cells from LM were re-localized with SEM (**b** and **b'**) and sectioned with FIB longitudinally (**c**,**c'**; **d**,**d'**). In early passages the homogeneous part of the cytoplasm is characterized by large, parallel sheets of rough endoplasmic reticulum (ER) and granules, which are classified as lysosomes (Ly) (**c**–**e**), moderate electron dense after osmium fixation (**c**,**c'**) and very electron dense after rOTO-staining (**d**,**d'**). In senescent cells lysosomes accumulate in enormous numbers (**c'**,**d'**,**e'**). Mitochondria are elongated and form branched larger networks, independent of the passage (**f**,**f'**; individual mitochondria are differently colored). However, in senescent cells the portion of mitochondria related to the cytoplasm is significantly reduced (**f**,**f'**). The ER is densely packed with ribosomes during all stages and is associated with both, mitochondria (**g**,**g'**) and lysosomes (**g'**). M = mitochondrion; N = nucleus

	Early passages	Advanced passages
Number of mitochondria/100 μm^3 *	20	6
Mean volume of mitochondrion	0.22 μm^3	0.22 μm^3
Mean surface area of mitochondrion	7.5 μm^2	6.5 μm^2
Average diameter of mitochondrion	0.22 µm	0.18 µm
Calculated length of mitochondrion (L = V/A) V = Volume, A = Area	5.8 µm	8.8 µm
Volume portion of lysosomes*	7%	60%
Volume portion of ER*	31%	4%

Table 1. Morphological changes of cell organelles. *Related to cytoplasmic volume.

mitochondrial network can be interconnected by vacuolated blebs (Fig. 3d'; Supplementary Fig. 1). Endosomes and small vesicular structures were in close vicinity to both, ER and mitochondria (Fig. 3a,c,d). Within the sheets of rough ER, small lens-shaped vacuoles were frequently observed (Fig. 3a') and several vacuoles of different sizes can be interconnected *via* rough ER (Fig. 3c'). The most striking structural alteration was an enormous accumulation of lysosomes (Fig. 2d,d'; e,e', 3a'). Their volume portion increased almost tenfold (from 7% to 60%). 3D reconstructions revealed that most lysosomes, are interconnected, either directly by their electron dense and electron translucent components or by short strands of ER (Fig. 2e', 3a'). The ER was in luminal connection to the lysosomes (Fig. 3a'; Supplementary Fig. 1). The intimate contact of the mitochondria with sheets of rough ER was pertained, as shown by 3D reconstructions (Fig. 3c,c', d).

Proteomic approach for comparison of early vs. advanced HTPC passages. A quantitative proteome analysis of early and advanced passages of cultured HTPCs (n = 6) and their conditioned media (CM) was performed to explore potential molecular alterations of replicative senescence of the HTPC proteome and secretome. A total of 3692 proteins were identified in HTPC-lysates at a false discovery rate (FDR) < 0.01. All identified proteins in early and advanced HTPC passages are listed in the Supplementary Table 1. Proteome analysis of CM identified 860 proteins, including 569 proteins with annotated extracellular localization, defining the secretome dataset. The identified proteins in CM of early and advanced HTPC passages are listed in the Supplementary Table 2. Hierarchical clustering and principal component analyses demonstrated passage-associated segregation of cellular proteomes and secretomes from early and advanced passages (Supplementary Fig. 2a,b). Label-free quantification (LFQ) revealed that 208 proteins were significantly different in quantity (*t*-test, FDR < 0.05) in cellular proteomes (Fig. 4c, Supplementary Table 3) and 131 proteins in the secretomes (Fig. 4d, Supplementary Table 3), between HTPCs from early and advanced passages, respectively. A large number of nuclear and mitochondrial proteins were decreased in contrast to increasing lysosomal proteins (Fig. 4a,b).

The secretome analysis of advanced passages showed, among others, a significant increase of the dipeptidyl peptidase 4 (DPP4) (Fig. 4d). As DPP4 expression has not been identified before in testicular peritubular cells, we explored its presence *in situ*. DPP4 staining was carried out with human testicular sections from middle-aged men (48–50 years). DPP4 was absent in many of the peritubular cells of the seminiferous tubular wall. However, in half of the samples examined (3 out of 6 examined) immuno-reactive peritubular cells were found *in situ*. DPP4 was also found in cells of the interstitial space (Fig. 4e). The gene set enrichment analysis (GSEA) showed substantial alterations between early and advanced passages in cell lysates and CM. The gene sets enriched in early passages were primarily related to mitochondrial and chromosomal functions, and more specifically related to RNA splicing, chromatin organization and DNA repair (Fig. 4a), while secretory, lysosomal, and metabolic processes were enriched in advanced passages (Fig. 4b). In the HTPC CM, gene sets related to extracellular matrix were enriched in early passages, while gene sets related to different binding and signaling processes were enriched in advanced passages. The detailed results of the GSEA are listed in Supplementary Tables 5, 6 and are visualized as functional networks in Supplementary Fig. 3.

qPCR study of characteristic HTPC marker genes. For complementation of the study, possible alterations between early and advanced passages were examined by qPCR. Transcription levels of *AR*, *ACTA2*, *CNN1*, *StAR* and *PTGS1* remained stable (Fig. 5a). Inflammatory genes, namely *IL6*, *IL8*, *PTGS2* and *PTX3* were also not affected by passaging except for *CCL2*, which was increased about 2-fold (Fig. 5b). However, ELISA-measurements did not confirm such a change at the protein level (Supplementary Fig. 5). Expression levels of the growth factors *CXCL12* and *GDNF* varied between the different donors, although without detectable tendency (Fig. 5c). ELISA-measurements of CXCL12 did not indicate a significant age-associated change, but rather inter-individual alterations (Supplementary Fig. 4).

Discussion

In man, aging of many organs, including the testis, cannot be readily assessed. Information about cellular senescence of human testicular cells is likewise missing. HTPCs are the only human testicular cell type, which can be cultured and propagated until replicative senescence is witnessed. The present study combines ultrastructural 3D data from HTPCs during senescence with a proteomic analysis of cellular and secreted proteins and a qPCR analysis and provides a detailed picture of senescence-associated changes in these human testicular cells.

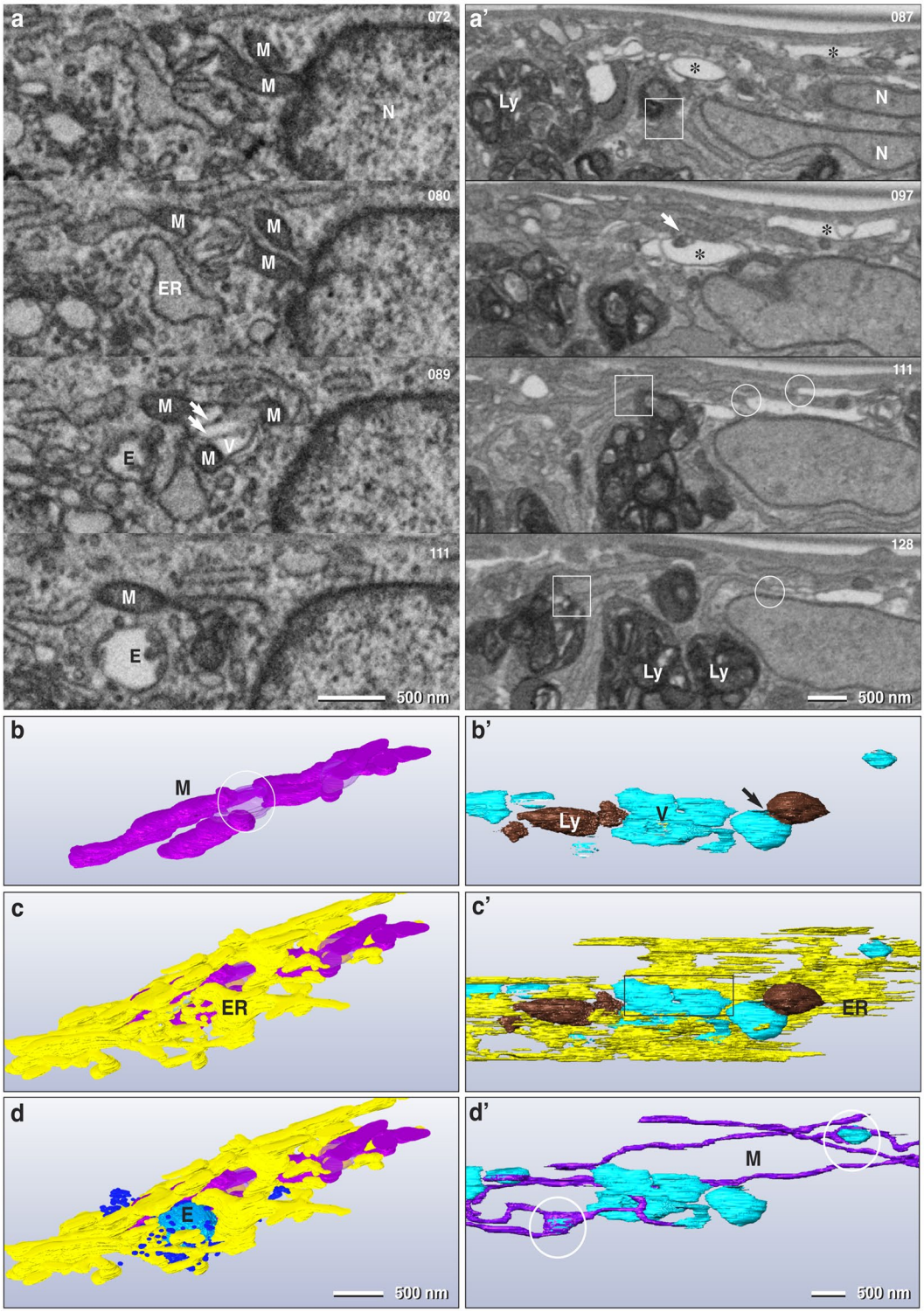

Figure 3. Selected FIB/SEM micrographs of a series of HTPCs from early (**a**–**d**) and advanced passages (**a'**–**d'**). The numbers (upper right) indicate the selected micrographs from the series. In the early passage the HTPC shows contact sites of ER with a branched mitochondrion (M) with several cross sections within one micrograph (4 nm isovoxel). Vacuoles (V) form within the mitochondrial matrix, leading to swellings (**b**; circle/transparent magenta) with some cristae still visible (**a**, arrows). Sheets of ER are attached to the mitochondrial network, noticeable after 3D reconstruction (**c**,**d**). The advanced passage exhibits numerous lens shaped vacuoles (**a'**; asterisks; voxel size: 7.5 × 7.5 × 14 nm). Strands of rough ER are connected at both ends to the vacuoles (**a'**, circles). Electron dense inclusions within the vacuole were identified as lysosomes (**a'**, arrow), when following the FIB/SEM series. Lysosomes (Ly) are connected to strands of ER at multiple sites (**a'**; squares). 3Dreconstruction of vacuoles (V) and lysosomes (Ly) show that they form clusters connected to each other (**b'**; arrow). The ER forms large sheets, best visible in top view (**c'**) compared to the FIB/SEM block face micrographs (**a'**). The ER membrane is in continuity with the vacuole membrane (**c'**; rectangle). The mitochondrion (M) shown is also locally fused with smaller and larger vacuoles (**d'**; circles).

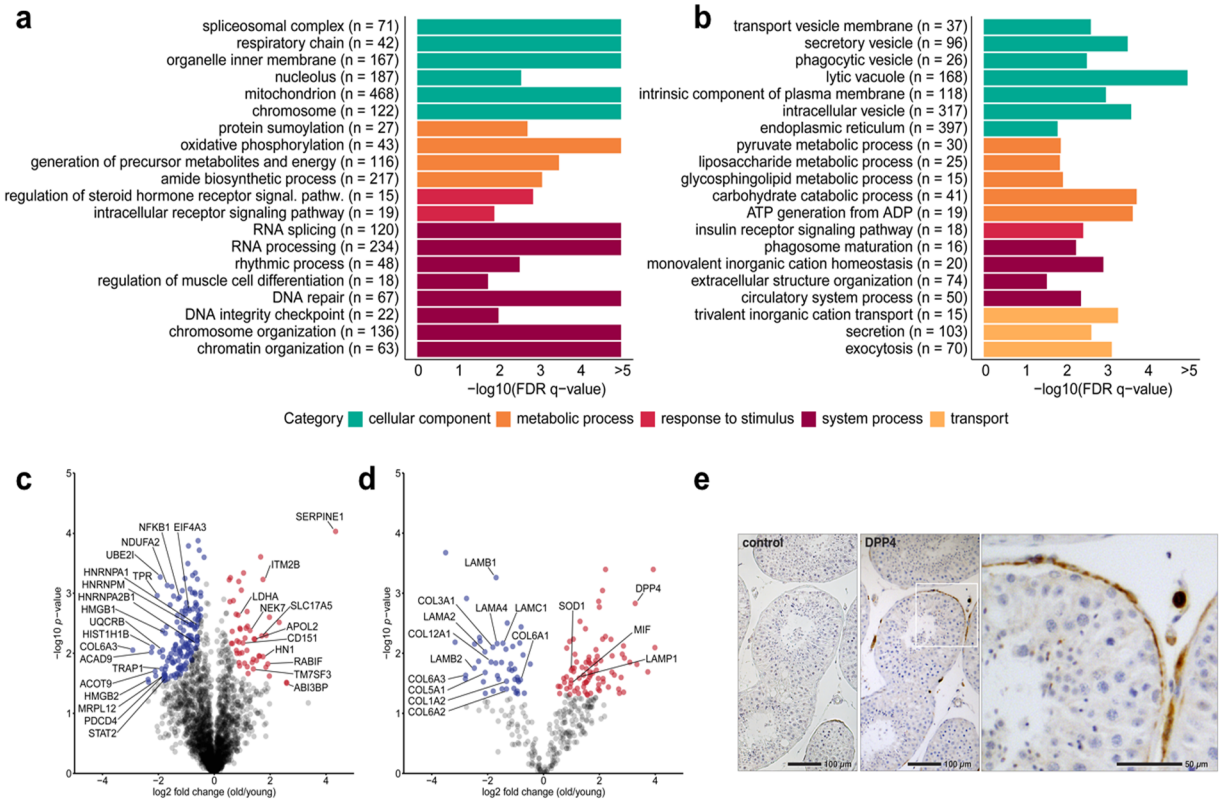

Figure 4. Proteomic analysis of advanced compared to early passages of HTPCs. Gene set enrichment analysis (GSEA) revealed significantly enriched gene sets (FDR q-value ≤ 0.05) and were summarized using REVIGO by clustering semantically similar GO terms. Each of the 20 characteristic gene sets enriched in early (**a**) and advanced passages (**b**) of HTPCs are shown. Color-coding refers to the corresponding highest GO hierarchy level. The x-axis shows the enrichment significance resulting from the GSEA and is depicted as –log10 (FDR q-value). The number of quantified proteins per gene set is shown in brackets. Volcano plots of intracellular and extracellular proteins, which are more abundant in passaged HTPC cellular proteomes (**c**) and secretomes (**d**) are depicted as red dots and proteins less abundant are shown as blue dots, respectively. Selected proteins with significant difference in abundance are labeled. P-values were calculated by a paired two-sample *t*-test. DPP4 expression in testicular peritubular cells (**e**). Light micrographs of immunohistochemical staining of human testicular sections. DPP4 is detected in several peritubular cells and cells of the interstitial space. Right micrograph: detail of the DPP4 staining (framed area). The negative control is without staining.

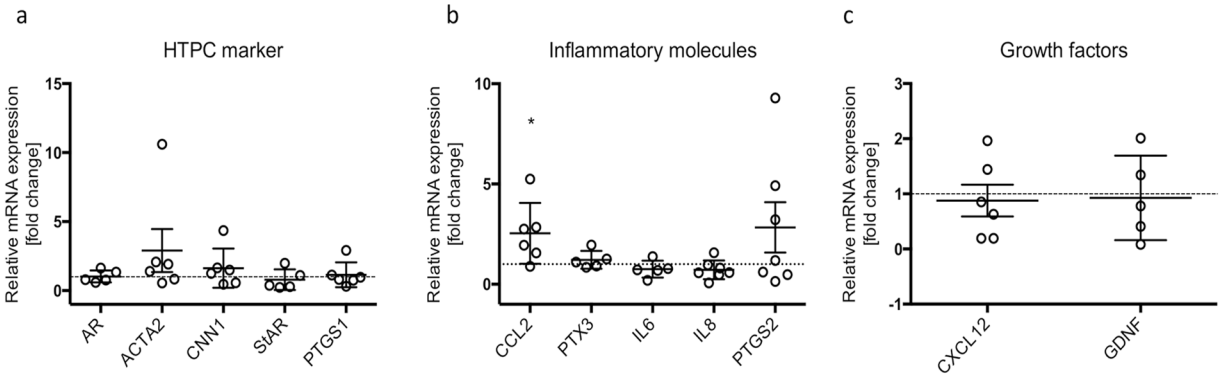

Figure 5. qPCR study of typical genes expressed in peritubular cells. mRNA levels of characteristic HTPC marker genes like *AR*, *ACTA2*, *CNN1*, *StAR* and *PTGS1* (**a**). Inflammation-associated genes show significantly increased mRNA level of *CCL2*. *PTX3*, *IL6*, *IL8* and *PTGS2* are not changed (**b**). mRNA expression of growth factors, *CXCL12* and *GDNF* (**c**). Graphs represent individual measurements and means ± SEM. Statistical analysis was executed with one-sample *t*-test, Asterisks show statistical significance, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

In general, replicative senescence of cells is characterized by cell cycle arrest[29,30], changes in the cellular phenotype and telomere shortening[31]. As there is no universal marker, the combination of different hallmarks is typically used to characterize senescent cells[32]. All HTPCs analyzed in this study showed a combination of impaired proliferative competence, reduced telomere length, increased cell size, and increased β-galactosidase activity,

i.e. the general hallmarks of senescent cells[29,31,33,34]. The study of these cells allowed us to identify HTPC-specific changes, which may be of relevance for the functions of the testis.

Detailed insights into cellular compartments of HTPCs, based on 3D reconstruction, identified three significant ultrastructural changes, associated with cellular senescence in HTPCs: i) a significant decrease of rough ER ii) a dramatic increase of lysosomes, iii) a decrease in the number of mitochondria. Ultrastructural reconstructions revealed that the ER is central in all these changes. It is connected to all lysosomes (Fig. 3a'), is in intimate contact to all mitochondria (Figs. 2g,g': 3c), and involved in vacuole formation (Fig. 3a'). The ER was packed with ribosomes in a surprisingly high density in early and advanced passages, hence an efficient protein synthesis is visibly maintained in senescent cells. Both *in vivo* and *in vitro* (human) testicular peritubular cells secrete a plethora of proteins, mainly ECM components[15]. The proteomic data supported the general capacity for protein synthesis during all passages, however secreted ECM proteins are significantly decreased, concurring with the structural reduction of the ER from 31% to 4% of the cytoplasmic volume (Table 1). These results are in line with impaired protein homeostasis (proteostasis) in senescent HTPCs, which is associated with aging in many cells[35].

The striking increase of lysosomes, which make up 60% of the cell volume in advanced passages, further argues for impaired proteostasis as a central event. The 3D reconstructions showed that in HTPCs lysosomes were connected to the ER in early and advanced passages (Fig. 3a'; Supplementary Fig. 1). Only in early passages, cellular polarity was observed with respect to a region located at one side of the nucleus, which was almost free of lysosomes and occupied by accumulation of parallel-arranged large sheets of rough ER (Fig. 2c,d). This cellular polarity was lost gradually in advanced passages. The massive accumulation of lysosomes reduced the space available for rough ER and implies steric hindrance of formation of rough ER. Similar data were recently published for large volume FIB/SEM reconstructions of HeLa cells: the dictyosomes, endosomes, lipid bodies and lysosomes form an interconnected system for Golgi degradation and reconstitution[36]. The massive increase of lysosomes, both in number and volume, may have different reasons. Thus, together with the proteomic data (Fig. 4b) and published physiological *in vivo* data[37] the results indicate impaired proteostasis.

Small vacuoles are initially visible within rough ER sheets as lens shaped structures (Fig. 3a') and subsequently, larger, spherical structures, still in luminal contact with ER, were found. They were considered to be nascent lysosomes and it seems likely that the formation of the vacuolar part of the mature lysosomes is a consequence of direct involvement of ER membranes and ER lumen. Similar autophagolysosomes/autophagosomes, degrading mitochondria, are described in podocytes of rats after acute ischemia[38] and in hexa KO cells, shown in serial 3D reconstruction, and also indicateinvolvement of ER[39].

The contact sites of ER with mitochondria are being discussed for Ca^{2+} exchange[40] but also as a supply site of membrane components from the ER to the outer mitochondrial membrane[41]. Changes of the mitochondrial network and the reduction in surface area of mitochondria by a factor of four was qualitatively paralleled with a reduction of the rough ER (Table 1). The investigation of lysosomes revealed that the majority is composed of an electron dense matrix, which is, at least in part, formed by an aggregation of membranes. However, when looking at the mitochondria with large volume reconstruction, there are characteristic features: empty spaces, lacking cristae, within the mitochondrial matrix, similar in appearance to data from Szento *et al.*[38] and contacts of mitochondria with lysosomes (Fig. 2g'; Supplementary Fig. 1) even at several segments of individual mitochondria, which may explain the reduction of mitochondrial volume by degradation (Fig. 3a**;** Supplementary Fig. 1). Compared to typical autophagy of cell organelles, as reviewed by Feng *et al.*[42], where entire mitochondria or other cell organelles are engulfed by membranes to form an autophagosome[43], the observed mechanism in HTPCs seems to be different. The mitochondrial vacuoles may enable fusion with ER-derived vacuoles and/or existing lysosomes. Mitochondria and rough ER are in direct contact to each other, as well as to lysosomes, thus forming a common, interconnected system shifting with progressing senescence to lysosome formation (Fig. 6).

Mass spectrometry revealed further striking changes of the cellular protein pattern, including the ubiquitous transcription factor NFκB1 that showed lower levels in senescent HTPCs. It was reported that a loss of NFκB1 may lead to early onset aging[44]. Furthermore, a reduced abundance of the RNA binding protein HNRNPA1 was found. It controls cellular senescence and the SASP *via* sirtuin1. Loss of HNRNPA1 induces a senescent phenotype in human diploid fibroblasts[45] also due to its crucial role in telomere protection[46]. With increasing age, energy production is reduced as reviewed by Barzilai, *et al.*[47]. The GSEA results of the proteome suggest that the energy metabolism changes in senescent cells from an oxidative metabolism to anaerobic glycolysis, which correlates with decreased mtDNA, structural changes within the mitochondria as reviewed by Bratic *et al.*[48] and increased LDHA abundance in advanced HTPC passages[49]. Senescent cells show changes in chromosome and chromatin architecture in general, as reviewed by Sun *et al.*[50]. GSEA showed that chromosome and chromatin organization are changed in advanced passages (Fig. 4a,b). This is paralleled by alterations of the shape of the nucleus, seen in many HTPCs (Fig. 3a'). In addition, the reduced DNA repair capacity in advanced passages, in combination with age-related increased levels of DNA damage, could reinforce cellular senescence[51].

ROS production by HTPCs was described in previous studies[20]. The analysis of the secretome revealed that a number of factors, e.g. superoxide-dismutase 1 (SOD1), an antioxidative factor, were elevated in advanced passages. This may be a reaction to higher ROS levels, which can cause DNA damage, leading to cellular senescence in human fibroblast cell lines[52]. MIF, a pro-inflammatory cytokine, increased in senescent HTPCs, is expressed by immune cells and several other cell types, including smooth muscle cells[53] (Fig. 4d). MIF is released into the extracellular space in response to various stimuli (e.g. mitogens and pro-inflammatory cytokines), and elevated levels indicate a pro-inflammatory milieu. MIF binds to cell surface receptors, either CXC chemokine receptors (CXCRs) or the CD74 receptor[54]. The G-protein–coupled chemokine receptors CXCR2, −4, and −7 are linked to chemotaxis of immune cells, in general. MIF could thereby contribute to an inflammatory environment within the human testis, also *via* suppression of the anti-inflammatory effect of glucocorticoids[55]. MIF actions may lead to enriched cytokine levels and can activate important pathways in senescence and aging, namely p53 and NFκB as reviewed by Salminen *et al.*[56].

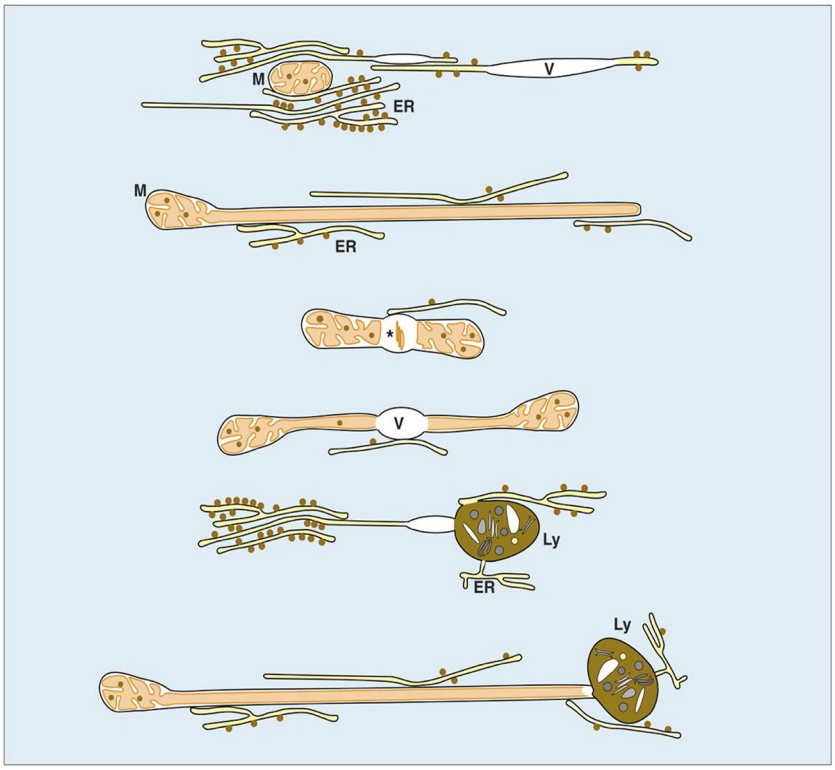

Figure 6. Schematic representation of the network of endoplasmic reticulum (ER), mitochondria (M), lysosomes (Ly), vacuoles (V) and their mutual relations based on electron microscopy data. All partners can be connected *via* ER. Sheets of rough ER enwrap mitochondria. Small lens shaped vacuoles form within the ER lumen. Mitochondria are elongated and range up to 15 µm. They have a minimal diameter of approx. 100 nm, without any cristae present. With progressing senescence, vacuoles form within the mitochondrial matrix, sometimes with degenerating cristae visible (asterisk). Both, mitochondria and rough ER are in direct contact to lysosomes forming a common, interconnected system.

Within the testis, a special situation may exist, since MIF is a ligand for the CXCR4, expressed by SSCs, and is essential for postnatal maintenance of the SSCs in marmosets and mice[57]. If indeed applicable to the testis, higher levels of MIF may influence the SSC niche *via* CXCR4 binding. CXCR4-expressing cells in the testis may also be indirectly influenced by DPP4, which was recently described as a senescence marker for human fibroblasts[58]. DPP4 was significantly elevated in the secretome of advanced HTPC passages (Fig. 4d) and detected *in vivo* in human testicular samples in peritubular cells (Fig. 4e). DPP4 is able to cleave several neuropeptides but also chemokines, including CXCL12. This is of importance in hematopoiesis, angiogenesis, and stem cell homing. With respect to the testis, it was shown that at least in mice and marmosets CXCL12-CXCR4 signaling is required for postnatal maintenance of SSCs, SSC propagation and that it prevents SSC differentiation. As CXCL12 truncation by DPP4 can reduce CXCL12-CXCR4 signaling significantly[59], DPP4 within the testis may have a negative impact on spermatogenesis and may antagonize other chemokine- and neuropeptide actions. The expression of DPP4 in the human testis, not shown before, raises the possibility that inhibitors for DPP4 may be potentially useful for therapy of diseases of the testis or age-related changes. In this respect, gliptins, i.e. established anti-diabetic drugs[60] may be of interest, as they are inhibitors of DPP4 and were shown to affect stem cell niches[61].

The levels of several ECM factors, including various laminins and collagens, are reduced in the secretome of advanced passages (Fig. 4d). They represent the major fractions of secreted proteins of HTPCs[15], and reduced levels indicate an overall diminished secretory activity and/or impaired proteostasis of senescent HTPCs. Yet a reduced secretion is not in line with reported increased ECM-deposits in the tubular wall of elderly men[6] or the changes seen in old rodent testes[62]. The human data is, however, being debated and questioned. It is possible that men with pre-existing infertility may have been included. As fibrosis of the peritubular wall is a hallmark of male infertility, such an inclusion may conceivably have biased the conclusion. If the results of the present study correlated with the *in vivo* situation, fibrosis of the peritubular wall is not be expected in testes of healthy elderly men without confounding issues[8]. Indeed, a recent light microscopic study by Pohl *et al.*[19] did not detect differences in the tubular wall compartment from young and elderly men. Further, peritubular cells are smooth muscle-like cells, comparable with other smooth muscle cells of the body. Of note, senescent vascular smooth muscle cells, like senescent HTPCs, also showed reduced ECM protein secretion[63].

Several distinct factors of importance for HTPCs (AR and GDNF), were not readily detectable by LC-MS/MS, but could be analyzed by qPCR (Fig. 5). *AR* was not significantly altered in advanced passages. AR-activation enhances the smooth muscle-phenotype and thereby may regulate contractility of HTPCs[16]. In full accordance with the proteomic data, the smooth muscle factors *ACTA2* and *CNN1* also remained stable, thus HTPCs keep their smooth muscle-like phenotype. GDNF contributes to spermatogenesis in mice[14,17] and was also unchanged in advanced passages, like protein (LC-MS/MS) and mRNA levels of *PTGS1*. This enzyme is involved in the

generation of prostaglandins and thereby in the regulation of GDNF[64]. CXCL12 also contributes to spermatogenesis and the mRNA and protein levels (Supplementary Fig. 4) were likewise not significantly altered. For all these transcript levels, considerable variations between individual human samples were, however, observed.

Several pro-inflammatory factors were also examined (*IL6*, *IL8*, *CCL2*, *PTGS2* and *PTX3)*, only *CCL2* expression was slightly, but statistically significantly (about 2-fold) elevated in senescent HTPCs. This cytokine was identified in HTPCs[21,22] and is involved in attracting monocytes and thus is considered a pro-inflammatory factor[65]. A related smooth muscle cell type in blood vessels showed higher levels of CCL2 with age, causing an inflammatory environment[66]. However, ELISA-measurements showed that not necessarily CCL2 content was changed in the cell culture media of HTPCs (Supplementary Fig. 5).

In summary, the results describe details of cellular senescence in human testicular cells. Senescence in HTPCs is not associated with a decreased expression of crucial HTPC genes, including contractility markers, *AR* and the growth factors *GDNF* and *CXCL12*. However, striking morphological changes in senescent HTPCs are accompanied by altered cellular protein levels, indicating impaired proteostasis. The secretome analysis revealed that specifically ECM-components are reduced. In contrast, inflammatory factors are increased. The HTPC-specific SASP includes MIF and DPP4, which both, directly or indirectly could influence the SSCs. In particular, DPP4 may be involved in degrading CXCL12. Thereby it could negatively influence the SSC niche. If so, it may serve as a potential drug target.

Reliable data on testicular aging and associated functional and structural changes in man are missing to the very day. Hence, the full *in vivo*-relevance of the changes in the proteome and secretome of HTPCs, in combination with ultrastructural large volume 3D-data, for the understanding of testicular aging in man remains to be shown.

Methods

HTPCs were isolated from testicular tissue fragments as described earlier[13,21]. The study was approved by the local ethical committee (Ethikkommission, Technische Universität München, Fakultät für Medizin, project number 309/14). For the scientific use of the tissue samples, the donors had granted written declaration of informed consent. HTPCs were cultured and propagated in DMEM High Glucose (Gibco, Paisly, UK) added with 10% fetal bovine serum (Capricorn Scientific, Ebsdorfergrund, Germany), 1% penicillin/streptomycin (Biochrom, Berlin, Germany) at 37 °C and 5% (v/v) CO_2. Purity of the cell cultures was shown previously[10]. The cells studied derived from donors, with obstructive azoospermia and normal spermatogenesis. The age of the donors ranged from 39 to 55 years. HTPCs underwent serial passaging until the typical signs of senescence appeared (growth arrest, increase in size, β-galactosidase expression, telomere shorting). All these samples were compared with the respective early passage from the same donor. The number of individual patient-derived cells for the different experiments is provided below. All experimental methods were implemented in accordance with relevant guidelines and regulations (including all biosafety and laboratory regulations).

β-galactosidase staining. Cultured HTPCs were seeded onto coverslips. Senescence-associated β-galactosidase staining was performed using a commercial kit (Senescence β-Galactosidase Staining Kit, Cell Signaling Technology #9860, Danvers, MA, USA), according to manufacturer's instructions. Staining was examined with a Zeiss Axiovert microscope (Zeiss GmbH, Oberkochen, Germany). HTPCs in early (P5 - P7) and advanced (P12 - P15) passages from 5 different donors were used for this experiment.

Cell size measurement. Cell size was determined using the CASY® Cell Counter on basis of variances in electrical resistance (Schärfe Systems, Reutlingen, Germany). HTPCs (from n = 7 individual donors, early (P3 – P7) and advanced (P11 – P14) passages) were trypsinized, centrifuged, resuspended in PBS and the measurement was implemented as described before[67]. For statistical analysis paired *t*-test (two-tailed) was applied.

DNA extraction. Total DNA extraction, from HTPCs in early (P3 – P8) and advanced (P12 – P20) passages (n = 6 different donors) was carried out with Wizard® SV Genomic DNA Purification System (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions.

Relative telomere length quantification. Total DNA from HTPCs (n = 6 donors, early (P3 – P8) and advanced (P12 – P20)) was analyzed with the Relative Telomere Length Quantification qPCR Assay Kit, (Science Cell, Carlsbad, CA, USA) according to the manufacturer's instructions. The kit contains 2 primer sets, one for the recognition and amplification of the telomere sequence and a reference primer set for data normalization. qPCR was carried out with QuantiFast® SYBR Green PCR Kit (Qiagen, Hilden, Germany). A total amount of 5 ng DNA from cultured HTPCs was used in duplicates in a LightCycler® 96 System (Roche Diagnostics GmbH, Penzberg, Germany) with following conditions: Initial denaturation (95 °C, 10 min) and 32 cycles of denaturation/annealing/extension (95 °C, 20 s/52 °C 20 s/72 °C 45 s). Quantification was implemented with comparative ΔΔCq method. Statistical analysis was done *via* one-sample *t*-test.

Mitochondrial DNA copy number quantification. The mtDNA copy number was quantified by qPCR (n = 6 different donors, early (P3 – P8) and advanced (P12 – P20)). A mitochondrial and a nuclear locus were compared as described elsewhere[68]. qPCR was executed with QuantiFast® SYBR Green PCR Kit (Qiagen) using 5 ng DNA and two different primer sets (for nuclear receptor coactivator three (NCOA3) and mtDNA) (Supplementary Table 7). qPCR conditions: 5 min, 95 °C preincubation, 40 cycles of amplification including denaturation at 95 °C for 10 s, annealing temperature 60 °C for 30 s and a melting step by heating from 65 °C to 95 °C, followed by a cool down to 37 °C for 30 s in a LightCycler® 96 System (Roche). Comparative ΔΔCq method was used for mtDNA quantification and statistically analyzed with one-sample *t*-test.

FIB/SEM. Cells were seeded on laser marked slides[69]. For light microscopic investigations cells were fixed in cacodylate buffer and stained with DAPI, as described by Luckner and Wanner[69]. From each passage 10 representative interphase cells were selected in phase contrast light microscopy according to their cell size and shape and documented with a CCD camera with the corresponding epifluorescence DAPI image. Cells were post-fixed/stained with either osmium tetroxide or reduced osmium-TCH-osmium (=rOTO) and ultra-thin embedded with epoxy resin as described[36,69]. After polymerization, the cells were documented again by bright field light microscopy for visualization and control of the heavy metal staining again. For high resolution SEM and FIB milling, cells were processed as described in detail by Luckner and Wanner[69]. Images were recorded with 3072 × 2048 pixel. The resulting data sets were aligned using Amira™ (Thermo Fisher Scientific, Waltham, MA, USA), first automatically with the module "align slices" and corrected with the "shear" function. The quality of the alignment had to be verified manually by fine correction. Image stacks were segmented and reconstructed in Amira™ (Thermo Fisher Scientific) and/or processed with a volume-rendering algorithm (*volren*) for direct visualization. 3D reconstructions/correlations were performed with Amira™. Dependent on the scientific demand for 3D reconstruction pixel sizes from 7.5 µm in x/y down to 2 nm isovoxel were chosen.

Nano LC-MS/MS. Cultured HTPCs in early (P3 – P7) and advanced (P11 – P14) passages (n = 6 different donors) were washed five times in serum-free DMEM to remove FBS and cell debris, and incubated for additional 24 h in serum-free DMEM. Cell pellets were harvested, suspended and homogenized in 8 M urea and 50 mM ammonium bicarbonate, as described previously[70]. The conditioned media (CM) were collected and centrifuged at 1,000 *g* for 3 min. The supernatant was transferred to Amicon 3 kDa centrifugal filter devices (Millipore) to desalt and concentrate the secreted proteins. The remaining concentrate solute was dried in a vacuum centrifuge. Protein concentration was determined using the Pierce 660 nm Protein Assay (Thermo Fisher Scientific)[71]. Protein samples from cells (20 µg) and their CM (10 µg) were digested in consecutive incubation steps with Lys-C (enzyme/substrate: 1:100; Wako) for 4 h at 37 °C and trypsin (enzyme/substrate: 1:50; Promega) overnight at 37 °C, as described earlier[70]. Peptide samples were analyzed by nano-LC-MS/MS on an UltiMate™ 3000 RSLCnano system (Thermo Scientific) coupled to a TripleTOF® 5600 + mass spectrometer (Sciex). 2.5 µg of peptides were separated at a flow rate of 200 nl/min with an analytical column (Acclaim™ PepMap™ RSLC C18, 75 µm × 50 cm, 2 µm, Thermo Fisher Scientific) in consecutive linear gradients: 5–25% solvent B (0.1% formic acid in acetonitrile) in 255 min and 25–50% B in 60 min. MS data were acquired in scan cycles of one survey scan (m/z 400–1250) followed by 70 data dependent CID fragmentation scans. MS raw data were processed using MaxQuant (v. 1.6.1.0). Database search parameters were set to SCIEX TOF instruments and protein identification was performed using the human Swiss-Prot subset (release 2018–10) and the MaxQuant common contaminants database at a false discovery rate of 1%. Label-free quantification (LFQ) was used as quantification strategy with a LFQ min. ratio count of 1 and the match between runs feature enabled. Data analysis and statistics was done in Perseus[72] and R[73]. Gene Ontology (GO) and KEGG pathway annotations were retrieved from UniProt. Protein identifications in the conditioned media data were filtered for extracellular locations to define the secretome protein dataset. The presence of a signal peptide position and the keyword "secreted" in UniProt were used as indication for classical secretion. Paired *t*-tests were used to identify significantly differentially abundant proteins with an s0 value of 0.1[74]. The FDR was controlled to be < 0.05. Gene set enrichment analysis (GSEA) was done using the Reactome and KEGG databases, as well as GO categories (molecular function, biological process, cellular component). Permutation type was set to gene type, enrichment statistic was weighted and as metric a *t*-test was used, while the rest of the settings were kept at their defaults. Enrichment maps were generated in Cytoscape[75] using the Enrichment map[76] and clusterMaker2[77] apps. The following settings were used for the enrichment maps: FDR q-value was 0.05 and as metric for edge generation an overlap index of 0.5 was used. REVIGO[78] was used to summarize significant gene sets by clustering similar ontology terms.

Immunohistochemistry. Immunohistochemical staining was performed as published previously[67]. Sections from patients with normal spermatogenesis (n = 6, age 48–50 years) were studied (Ethikkommission, Technische Universität München, Fakultät für Medizin, project number 309/14). Primary polyclonal goat anti-human DPP4 antibody (1:40, R&D Systems, Minneapolis, MN, USA) was used. For negative control purposes, the primary antiserum was omitted and replaced by non-immune serum. Hematoxilin was used to counterstain the sections. Examination of the sections was done with a Zeiss Axiovert light microscope (Zeiss GmbH).

Reverse transcription and qPCR. RNeasy Plus Micro Kit (Qiagen) was used for total RNA isolation from HTPCs in early (P3 – P8) and advanced (P12 – P16) passages. 200 ng RNA were reverse transcribed, random 15mer primer and SuperScript™ II (Invitrogen, Darmstadt, Germany). A LightCycler® 96 System (Roche) and QuantiFast® SYBR Green PCR Kit (Qiagen) were used. Primer are listed in Supplementary Table 7 Results were analyzed according to $2^{-\Delta\Delta Cq}$ method, mRNA expression was normalized to HPRT and RPL19, which served as endogenous references. Results were depicted as means ± SEM. Statistical analysis was done with one-sample *t*-test of $\Delta\Delta C_q$ values using GraphPad Prism 6.0 Software (GraphPad Software, San Diego, CA, USA). Negative controls consisted of non-reverse transcription and non-template reactions.

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Author contributions

N.S. performed the majority of the cellular experiments and evaluated results. K.G.D. was involved in the culture and characterization of cells and cellular experiments. M.L. performed rOTO and ultra-thin embedding. N.S. and G.W. performed FIB/SEM-tomography and 3D-reconstruction. F.F., J.B.S., T.F. and G.A. performed the proteomic studies and evaluated the results. J.U.S., F.M.K. provided testicular tissues and provided together with L.K. conceptual input. A.M. conceived of the study, supervised the experiments. All authors provided helpful comments. N.S., G.W. and A.M. drafted the manuscript, and all authors contributed to the final version.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to A.M.

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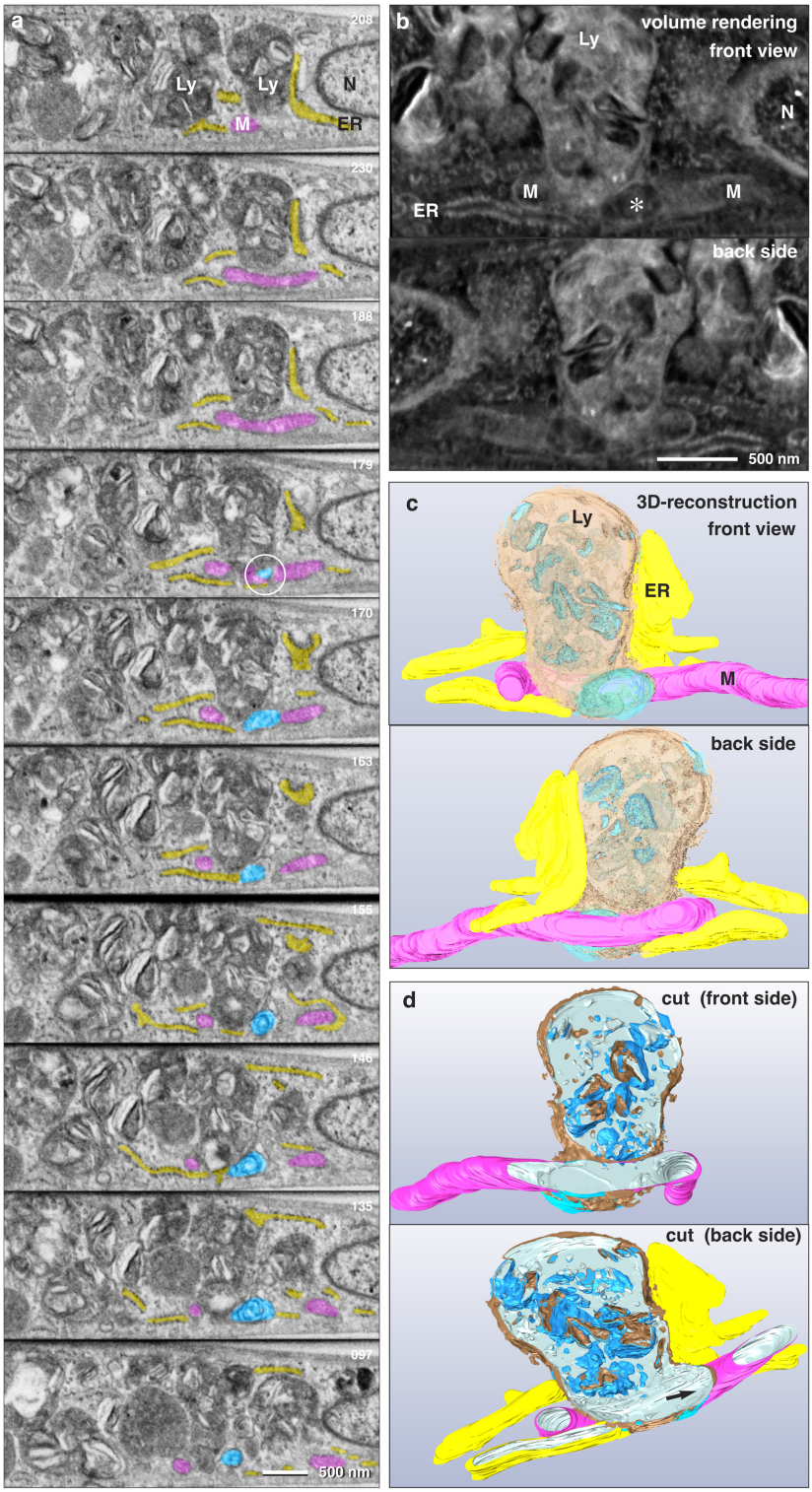
Supplementary information

Supplementary Figure 1

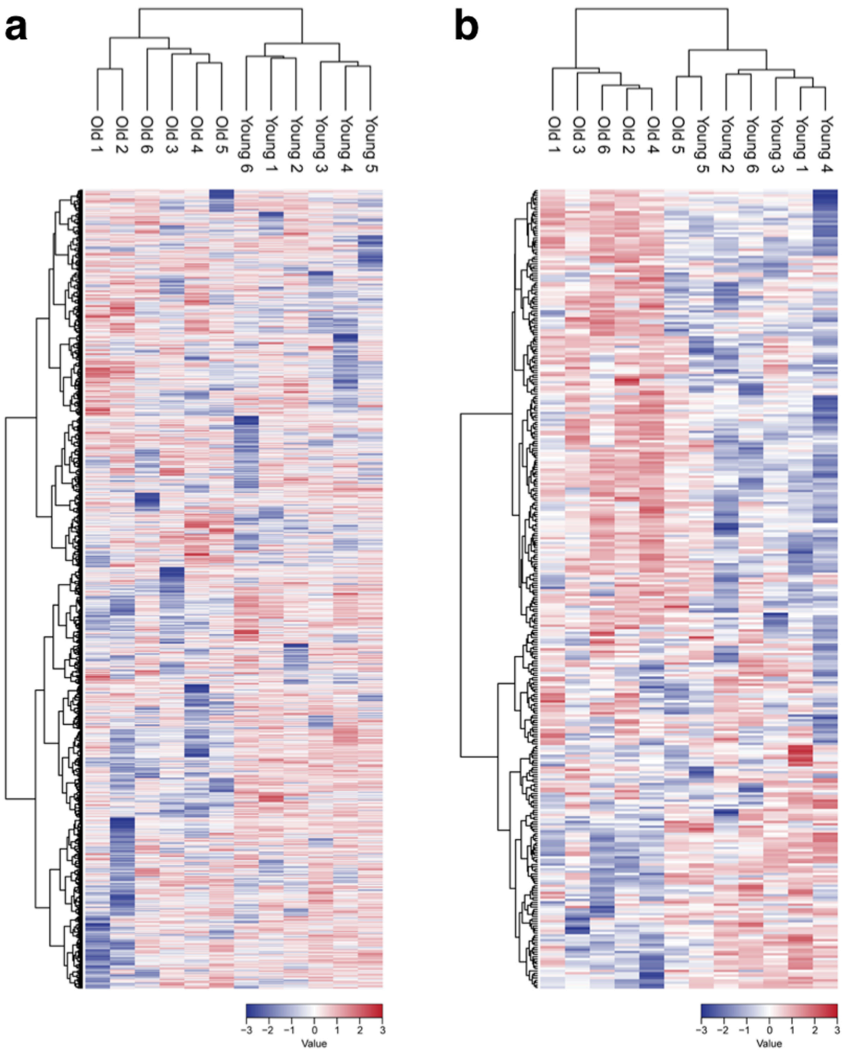


Supplementary Figure 1

Selected FIB/SEM micrographs (a), volume rendering (b) and corresponding 3D-reconstruction (c, d) of a high-resolution series (4 nm isovoxel) of an advanced passage of HTPC. The numbers (a; upper right) indicate the selected micrograph from the series. A

lysosome (Ly) is in contact with several strands of rough endoplasmic reticulum (ER) and a segment of a long mitochondrion (M; pink). The point of fusion of the mitochondrion with the lysosome (a; circle) is a vacuole-like structure (blue) within the mitochondrion. Volume rendering of the contact site: the vacuole within the mitochondrion is fused with the lysosome and a strand of ER is attached to the mitochondrion (b). Segmentation of the series (c) shows the interaction between lysosome, mitochondrion, vacuole, and ER both in front view and from the backside. When cut, luminal connection between the lysosome, vacuole and the mitochondrion is visible (d, arrow).

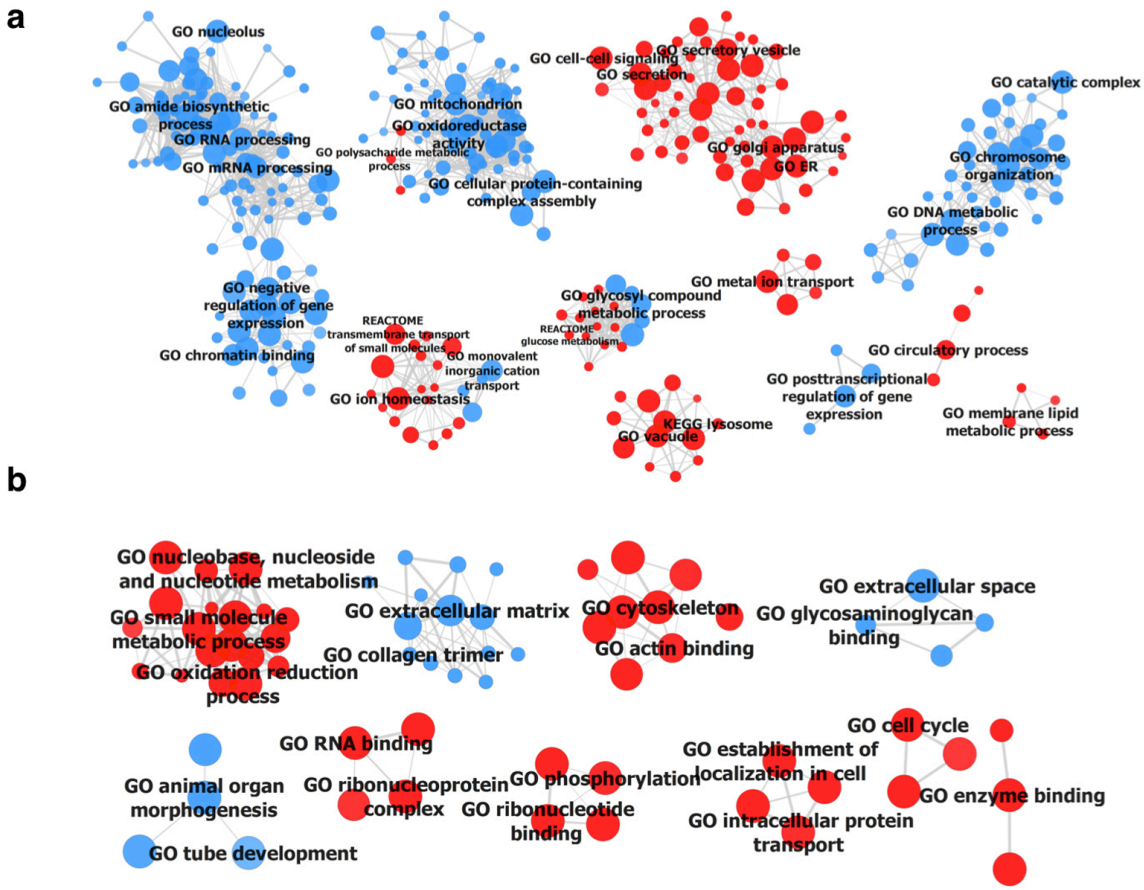
Supplementary Figure 2



Supplementary Figure 2

Unsupervised hierarchical clustering indicating separation of cellular proteomes (a) and secretomes (b) from early and advanced HTPC passages. LFQ intensity values are z-score normalized and color-coded according to the expression values.

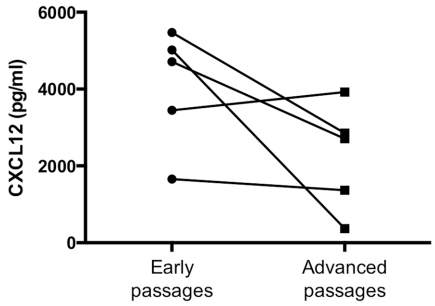
Supplementary Figure 3



Supplementary Figure 3

Functional networks of gene sets disturbed in advanced compared to early HTPC passages. GSEA results from HTPC cellular proteomes (a) and secretomes (b) from early and advanced passages are visualized as networks with nodes representing significantly (FDR q-value ≤ 0.05) enriched gene sets and edges representing mutual overlap of at least 0.5. Node size scales with gene set size while edge width represents extent of overlap between gene sets. Red node color shows enrichment in advanced passages, while blue color represents enrichment in early passages. Annotation databases are indicated as GO (Gene Ontology), REACTOME or KEGG.

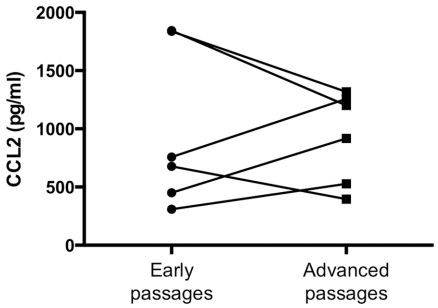
Supplementary Figure 4



Supplementary Figure 4

ELISA-measurement of CXCL12. ELISA was performed using cell culture media of early and advanced passages of HTPCs, connected by a line; cells were incubated for 24 h.

Supplementary Figure 5



Supplementary Figure 5

CCL2 ELISA-measurement. CCL2 analysis in cell culture media using cell culture media of early and advanced passages of HTPCs, connected by a line; cells were incubated for 24 h.

Supplementary Tables

Supplementary Table 1–6

https://www.nature.com/articles/s41598-019-51380-w

Supplementary Table 1.

Proteins identified in early and advanced passages of HTPCs.

Supplementary Table 2.

Proteins identified in the conditioned media of early and advanced passages of HTPCs.

Supplementary Table 3:

Proteins significantly different in abundance (paired t-test, FDR < 0.05) between early and advanced passages of HTPCs. HTPCs are marked with a '+'. Positive log2 fold changes indicates higher abundancy in the senescent group.

Supplementary Table 4:

Proteins significantly different in abundance (paired t-test, FDR < 0.05) between secretomes of early and advanced passages of HTPCs are marked with a '+'. Positive log2 fold changes indicates higher abundancy in the senescent group.

Supplementary Table 5:

Gene set enrichment analysis from cellular proteomes from advanced vs. early passages of HTPCs. Enriched gene sets in senescent cells are listed in table A, while gene sets decreased in senescent cells are shown in table B.

Supplementary Table 6:

Gene set enrichment analysis from secretomes from advanced vs. early passages of HTPCs. Enriched gene sets in secretomes of senescent cells are listed in table A, while gene sets decreased in secretomes of senescent cells are shown in table B.

Supplementary Table 7

Oligonucleotide primer for PCR studies

Gene	Reference ID	Nucleotide sequence	Amplicon size
ACTA2	NM_001613.2	5´-ACA ATG AGC TTC GTG TTG CC-3´ 5´-GAG TCA TTT TCT CCC GGT TGG-3´	90
AR	NM_001011645.3	5´-AGC CTC AAT GAA CTG GGA GA-3´ 5´-TCC TGG AGT TGA CAT TGG TG-3´	175
CCL2	NM_002982	5'-CAG CCA GAT GCA ATC AAT GCC-3' 5'-TGG AAT CCT GAA CCC ACT TCT-3'	190
CNN1	XM_005257058.4	5´-CGA AGA CGA AAG GAA ACA AGG T-3´ 5´-GCT TGG GGT CGT AGA GGT G-3	186
CXCL12	NM_000609.6	5´-TCA GCC TGA GCT ACA GAT GC-3 5´-CTT TAG CTT CGG GTC AAT GC-3	161
DPP4	NM_001935.3	5´-TGG TCT CCA AAC GGC ACT TT-3´ 5´-TGC CCA TGT CAC ATC ACA CA-3	273
GDNF	NM_000514.3	5´-GCA GAC CCA TCG CCT TTG AT-3´ 5´-ATC CAC ACC TTT TAG CGG AAT G-3´	93
HPRT	NM_000194.2	5´-CCT GGC GTC GTG ATT AGT GA-3´ 5´-GGC CTC CCA TCT CCT TCA TC-3´	163
IL6	NM_000600.4	5´-AAC CTG AAC CTT CCA AAG ATG G-3´ 5´-TCT GGC TTG TTC CTC ACT ACT-3´	159
IL8	NM_000584.4	5´-TCT TGG CAG CCT TCC TGA-3´ 5´-GAA TTC TCA GCC CTC TTC-3´	271
mtDNA	NC_012920.1	5´-GCCACAGCACTTAAACACATCTCT-3´ 5´-TAGGATGGGCGGGGGT-3´	186
NCOA3	NC_000020.11	5´-CCTCTGGGCTTTTATTGCGAC-3´ 5´-CGGTCATCAGAAGAACAGGTAAGT-3´	188
PTGS1	NM_000962.4	5'-TCC ATG TTG GTG GAC TAT GG-3' 5'-GTG GTG GTC CAT GTT CCT G-3'	96
PTGS2	NM_000963.3	5´-CTT ACC CAC TTC AAG GGA-3´ 5´-GCC ATA GTC AGC ATT GTA AG-3	132
PTX3	NM_002852.3	5´-TAG TGT TTG TGG TGG GTG GA-3´ 5´-TGT GAG CCC TTC CTC TGA AT-3´	110
RPL19	NM_000981.3	5´-AGG CAC ATG GGC ATA GGT AA-3´ 5´-CCA TGA GAA TCC GCT TGT TT-3´	199
StAR	NM_000349	5´-ACG TGG ATT AAC CAG GTT CG-3´ 5´-CAG CCC TCT TGG TTG CTA AG-3´	149

3.3 Publication III

Characterization of a non-human primate model for the study of testicular peritubular cells — comparison with human testicular peritubular cells.

Nina Schmid*, Jan B. Stöckl*, Florian Flenkenthaler, Kim-Gwendolyn Dietrich, Johannes U. Schwarzer, Frank-Michael Köhn, Charis Drummer, Thomas Fröhlich, Georg J. Arnold, Rüdiger Behr, Artur Mayerhofer
*equal contribution

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Abstract

STUDY QUESTION: Are monkey testicular peritubular cells (MKTPCs) from the common marmoset monkey (Callithrix jacchus) a suitable translational model for the study of human testicular peritubular cells (HTPCs)?

SUMMARY ANSWER: MKTPCs can be isolated and propagated in vitro, retain characteristic markers for testicular peritubular cells and their proteome strongly (correlation coefficient of 0.78) overlaps with the proteome of HTPCs.

WHAT IS KNOWN ALREADY: Smooth-muscle-like peritubular cells form the wall of seminiferous tubules, transport sperm, are immunologically active, secrete a plethora of factors and may contribute to the spermatogonial stem cell niche. Mechanistic studies are hampered by heterogeneity of human samples.

STUDY DESIGN, SIZE, DURATION: We established a culture method for MKTPCs and characterized these cells from six young adult animals (2-3 years). To examine whether they qualify as a translational model we also examined HTPCs from seven men and compared the proteomes of both groups.

MAIN RESULTS AND THE ROLE OF CHANCE: We established a method for isolation and cultivation of MKTPCs, and provide a comprehensive analysis of their protein repertoire. The results let us conclude that MKTPCs are suitable as a non-human primate model to study peritubular cell functions.

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ORIGINAL ARTICLE

Characterization of a non-human primate model for the study of testicular peritubular cells—comparison with human testicular peritubular cells

N. Schmid[1,†], J.B. Stöckl[2,†], F. Flenkenthaler[2], K.-G. Dietrich[1], J.U. Schwarzer[3], F.-M. Köhn[4], C. Drummer[5], T. Fröhlich[2], G.J. Arnold[2], R. Behr [5], and A. Mayerhofer [1,*]

[1]Cell Biology—Anatomy III, Biomedical Center Munich (BMC), Ludwig-Maximilians-Universität München, Großhaderner Strasse 9, 82152 Martinsried, Germany [2]Laboratory for Functional Genome Analysis LAFUGA, Gene Center, LMU München, Fedor-Lynen-Strasse 25, 81377 Munich, Germany [3]Andrology Center, Lortzingstrasse 26, 81241 Munich, Germany [4]Andrologicum, Burgstrassse 7, 80331 Munich, Germany [5]Platform Degenerative Diseases, German Primate Center, Kellnerweg 4, 37077 Göttingen, Germany

*Correspondence address. Cell Biology—Anatomy III, Biomedical Center Munich (BMC), Ludwig-Maximilians-Universität München, Großhaderner Strasse 9, 82152 Martinsried, Germany. E-mail: Mayerhofer@lrz.uni-muenchen.de orcid.org/0000-0002-9388-4639

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PARTICIPANTS/MATERIALS, SETTING, METHODS: We used explant cultures to obtain MKTPCs, which express smooth muscle markers (calponin (*CNN1*), smooth muscle actin (*ACTA2*)), lack FSH-receptors (*FSHR*) and LH-receptors (*LHCGR*), but possess androgen receptors (*AR*). MKTPCs can be passaged at least up to eight times, without discernable phenotypic changes. Mass-spectrometry-based analyses of the MKTPC and HTPC proteomes were performed.

MAIN RESULTS AND THE ROLE OF CHANCE: We established a method for isolation and cultivation of MKTPCs, and provide a comprehensive analysis of their protein repertoire. The results let us conclude that MKTPCs are suitable as a non-human primate model to study peritubular cell functions.

LARGE SCALE DATA: List of identified proteins in MKTPCs by liquid chromatography–tandem mass spectrometry is accessible at the ProteomeXchange (identifier PXD009394).

LIMITATIONS, REASON FOR CAUTION: This is an *in vitro* cellular non-human primate model used to provide a window into the role of these cells in the human testis.

[†]Authors contributed equally.

WIDER IMPLICATIONS OF THE FINDINGS: Previous studies with HTPCs from patients revealed a degree of heterogeneity, possibly due to age, lifestyle and medical history of the individual human donors. We anticipate that the new translational model, derived from young healthy non-human primates, may allow us to circumvent these issues and may lead to a better understanding of the role of peritubular cells.

STUDY FUNDING AND COMPETION OF INTEREST(S): This work was supported by grants from the Deutsche Forschungsgemeinschaft (MA 1080/27-1; AR 362/9-1; BE 2296/8-1). The authors declare no competing financial interests.

Key words: testis / cellular model / proteome / marmoset monkey / non-human primate

Introduction

The human testis in health and disease cannot be readily studied. Cellular models, however, allow insights and human testicular peritubular cells (HTPCs) derived from individual patients represent such a model. These cells form the wall of seminiferous tubules and can be isolated via explant culture from very small fragments obtained during surgical procedures (Albrecht *et al.*, 2006; Schell *et al.*, 2008; Mayerhofer, 2013). They can be passaged and examined by cellular and molecular techniques. The results obtained, in combination with studies on human testicular sections, led to the insight that HTPCs play an important role in the human testis in health and disease. These smooth-muscle-like cells are associated with the transport of immotile sperm, but also possess secretory functions. Glial cell line-derived neurotrophic factor (GDNF) is a factor secreted by these cells, which is required for renewal of spermatogonial stem cells. This indicates a contribution to the spermatogonial stem cell niche (Spinnler *et al.*, 2010), a view supported by recent studies in mice (Chen *et al.*, 2014, 2016). HTPCs also secrete extracellular matrix components, including decorin (DCN) and biglycan (BGN) (Flenkenthaler *et al.*, 2014). The latter is increased in infertile patients and, as we showed, can activate Toll-like receptors (TLRs) of HTPCs. Thereby it induces inflammatory reactions, namely secretion of C–C motif chemokine ligand 2 (CCL2), pentraxin 3 (PTX3) or interleukin 6 (IL6) (Mayer *et al.*, 2016). Taken together, the results support important roles of HTPCs in male fertility and infertility.

HTPCs were also shown to be plastic cells and their ability to differentiate towards a steroidogenic, presumably Leydig cell type, became evident (Landreh *et al.*, 2014). The last-mentioned study showed inter-individual differences in the basal and the forskolin-stimulated steroidogenic capacities. Further studies (Welter *et al.*, 2014; Mayer *et al.*, 2016), which examined immunological aspects, revealed that HTPCs derived from individual patients are heterogeneous with respect to the amounts of secreted factors. This may be due to the differences in age, lifestyle and medical history of the patients. While this heterogeneity is instructive and may be of interest in view of an improved understanding of inter-individual differences and personalized treatment option in the future, these differences also make general in-depth mechanistic studies difficult.

To circumvent these issues, and to be able to study peritubular cell function and regulation in greater detail, we sought to establish a translational model. In search for suitable model organism, we turned to the common marmoset monkey (Michel and Mahouy, 1990; Mansfield, 2003; Zuhlke and Weinbauer, 2003; Li *et al.*, 2005). Marmosets are non-human primates, which are often used in reproductive research.

Here we describe the isolation, cultivation and proteomic characterization of monkey testicular peritubular cells (MKTPCs). We propose that they are an apt model for HTPCs because of their similarity to human and the possibility to control confounding lifestyle issues.

Materials and Methods

Animals

Common marmoset monkeys (*Callithrix jacchus*) stem from the self-sustaining marmoset monkey colony of the German Primate Center (Deutsches Primatenzentrum; DPZ, Göttingen). All animals used for this study were between 2 and 3 years old, i.e. young adult, sexually mature healthy animals (Li *et al.*, 2005). Marmoset monkey testes were obtained from animals euthanized for scientific purposes unrelated to this study or castrated for colony management purposes. Euthanasia and castration were performed by experienced veterinarians. Parts of the testes were fixed in Bouin's fixative and embedded in paraffin, for later sectioning and immunohistochemistry, other parts were used for isolation of MKTPCs by explant culture.

Ethical approval

Organ retrievals from *Callithrix jacchus* were carried out in accordance with relevant institutional guidelines and legal regulations, namely the German Animal Protection Act. The local ethical committees (Ethikkommission, Technische Universität München, Fakultät für Medizin, München, project number 5158/11) approved the study with human tissues. All experiments were performed in accordance with relevant guidelines and regulations.

Isolation and cultivation of MKTPCs

Isolation and cultivation of MKTPCs was performed as described in detail previously for human samples (Albrecht *et al.*, 2006). In brief, small pieces of testicular tissue were seeded onto cell culture dishes. The explant cultures were incubated under humidified conditions (37°C, 5% CO_2) until the cells started to grow out of the tubular wall. They were cultivated and propagated in Dulbecco's modified Eagle's medium (DMEM) high glucose (Gibco, Paisley, UK) containing 10% fetal calf serum (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA). MKTPCs in passages 2–3 were used for proteomic studies, for RT-PCR cells from early (3) and advanced passages (8) were used.

RNA isolation and RT-PCR

Total RNA from cultured MKTPC was prepared as described earlier (Welter *et al.*, 2014) using the RNeasy microkit (Qiagen, Hilden, Germany). In brief, a total amount of 200 ng of RNA was subjected to reverse transcription, using random primers (15-mer) and SuperScript II Reverse Transcriptase, 200 U/µl (Invitrogen GmbH, Darmstadt, Germany). Intron-spanning primer pairs amplified specific products for *ACTA2*, *CNN1*, *AR*, *GDNF*, *FSHR*, *GATA4*, *LHCGR*, *INSL3*, *DCN*, *BGN*, *CCL2*, *PTX3*, *IL6*, *CD3e*, *TPSG1* and *CMA1* (Table I). PCR consisted of 35 cycles of denaturing (at 95°C for 60 s) annealing (at 60°C) and extension (at 72°C for 45 s). PCR products were visualized by midori green

Table I Oligonucleotide primers used for PCR studies

Gene	Gene name	Reference ID	Nucleotide sequence	Amplicon size
RPL19	Ribosomal Protein L19	NM_001330200.1	5′-AGG CAC ATG GGC ATA GGT AA-3′ 5′-CCA TGA GAA TCC GCT TGT TT-3′	199
ACTA2	Actin, Alpha 2, Smooth Muscle, Aorta	NM_001613.2	5′-ACC CAG TGT GGA GCA GCC C-3′ 5′-TTG TCA CAC ACC AAG GCA GT-3′	100
CNN1	Calponin 1	XM_017026289.1	5′-CGA AGA CGA AAG GAA ACA AGG T-3′ 5′-GCT TGG GGT CGT AGA GGT G-3′	183
AR	Androgen Receptor	XM_008989422.2	5′-GCC CCT GAT CTG GTT TTC AA-3′ 5′-CCA CTG GAA TAA TGC TGA AGA GT-3′	163
GDNF	Glial Cell Derived Neurotrophic Factor	NM_000514.3	5′-GCA GAC CCA TCG CCT TTG AT-3′ 5′-ATC CAC ACC TTT TAG CGG AAT G-3′	93
FSHR	FSH Receptor	NM_000145.2	5′-CTG CTC CTG GTC TCT TTG CT-3′ 5′-GGT CCC CAA ATC CTG AAA AT-3′	208
GATA4	GATA Binding Protein 4	NM_002052	5′-TCC AAA CCA GAA AAC GGA AG-3′ 5′-CTG TGC CCG TAG TGA GAT GA-3′	187
LHCGR	Luteinizing Hormone/Choriogonadotropin Receptor	NM_001301843.1	5′-CTG GAT GCC ACG CTG ACT-3′ 5′-ACG CAC TCT GTC CAC TCT-3′	100
INSL3	Insulin Like 3	XM_017968209.1	5′-ACT TCT CAC CAT CATCGC CA-3′ 5′-GAG GGT CAG CAG GTCTTG TT-3′	96
CD3e	CD3e Molecule	XM_009006841.1	5′-ATCTGCATCACTCTGGGCTT-3′ 5′-TGGGCTCATAGTCTGGGTTG-3′	160
TPSG1	Tryptase Gamma 1	NM_001204310.1	5′-CAT TGT GAG CTG GGG TGA AG-3′ 5′-AGA CCA GCA GAA GGA AGA GG-3′	182
CMA1	Chymase 1	XM_002753692.4	5′-AAG TTG AAG GAG AAA GCC AGC-3′ 5′-TTC AAC ACA CCT GTT CTT CCC-3′	125
DCN	Decorin	XM_002752816.3	5′-ATG AGG CTT CTG GGA TAG GC-3′ 5′-GTC CAG CAA AGT CGT GTC AG-3′	170
BGN	Biglycan	XM_008990069.2	5′-GAG ACC CTG AAC GAA CTC CA-3′ 5′-TTG TTG TCC AAG TGC AGC TC-3′	176
CCL2	C–C Motif Chemokine Ligand 2	NM_002982	5′-CAG CCA GAT GCA ATC AAT GCC-3′ 5′-TGG AAT CCT GAA CCC ACT TCT-3′	190
PTX3	Pentraxin 3	NM_002852.3	5′-TAG TGT TTG TGG TGG GTG GA-3′ 5′-TGT GAG CCC TTC CTC TGA AT-3′	110
IL6	Interleukin 6	XM_017975106.1	5′-AAG AGG TAG CTG CCC CAA AT-3′ 5′-AGT GCC TCT TTG CTG CTT TC-3′	145

(NIPPON Genetics EUROPE GmbH, Düren, Germany) staining in agarose (Biozym Scientific GmbH, Oldendorf, Germany) gels. Positive controls consisted of *Callithrix jacchus* whole testis lysate cDNA (+). Negative controls were performed by excluding reverse transcriptase (−RT), and a non-template reaction (−). The identities of all PCR products were verified by sequencing.

Immunohistochemistry

Immunohistochemistry was performed as described (Schell *et al.*, 2010). Parts of the testicular tissue, which were also used for explant cultures were fixed and embedded in paraffin. Sections (5 µm) of marmoset monkey testicular tissue were incubated with monoclonal mouse antibody against ACTA2 (#A5228, 1:1000; Sigma, St. Louis, MO, USA), monoclonal rabbit antibody against Calponin-1 (C-term) (#1806-1, 1:250; Epitomics, Cambridge, UK) and AR (#5153, 1:400; Cell Signaling, Cambridge, UK). Controls consisted of incubation with non-immune normal goat serum instead of specific antibodies. Hematoxylin counterstained the cell nuclei. Sections were examined with a Zeiss Axiovert microscope, an Insight Camera (18.2 Color Mosaik) and Spot advanced software 4.6 (both from SPOT Imaging Solutions, Sterling Heights, MI, USA).

Immunofluorescence

MKTPCs were seeded onto cover slips and incubated overnight. They were fixed with 3.7% formaldehyde (Sigma) for 10 min, washed with 0.1% Triton X-100/phosphate buffered saline (PBS) (Sigma) and permeabilized with ice cold 0.2% Triton X-100/PBS for 10 min. Cells were blocked with 0.1% Triton X-100/PBS + 5% goat normal serum (Sigma). The same primary antibodies as for the immunohistochemistry were used and were diluted in 0.1% Triton X-100/PBS + 5% goat normal serum, CNN1 1:100 (Epitomics), ACTA2 1:200 (Sigma), AR 1:100 (Cell Signaling) and incubated for 2 h at room temperature. Secondary antibody for CNN1 and AR, goat anti-rabbit alexa flour 488 1:1000 (Thermo Fisher Scientific, Waltham, MA, USA) for ACTA2, goat anti-mouse alexa fluor 555 1:1000, were incubated for 1.5 h at room temperature. Cells were washed and counterstained with 1.5 µg/ml DAPI for 5 min. For the control, normal goat serum was used instead of the primary antibody. Examination was performed with a fluorescence microscope (Zeiss, Oberkochen, Germany).

Human peritubular cell isolation and culture

The procedure involving human peritubular cells (HTPCs) isolation and culture from human testicular tissue samples exhibiting normal

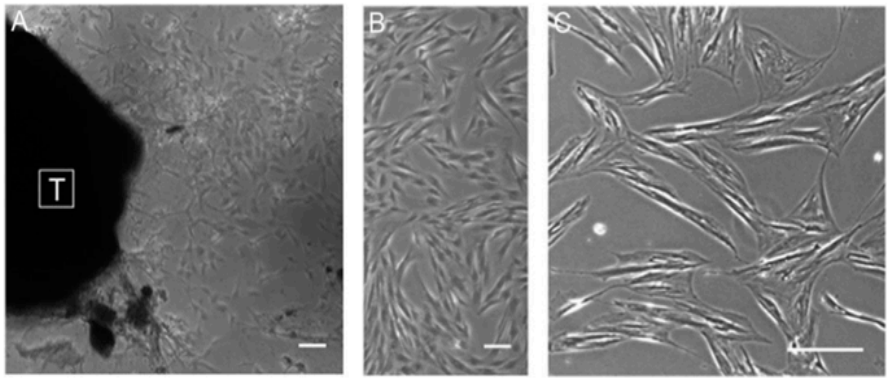

Figure 1 Testicular tissue-explant culture of MKTPCs. (**A**) Small pieces of testicular tissue consisting of tubules (depicted with (T)) were placed onto cell culture dishes. The cells grow out from the walls of the seminiferous tubules within 7–14 days, picture was captured on day 10. (**B**) Cells in passage 1, 8 weeks after tissue extraction. (**C**) MKTPCs in passage 4, the cells were sub-cultured and propagated for several passages. (Scale bars represent 25 µm).

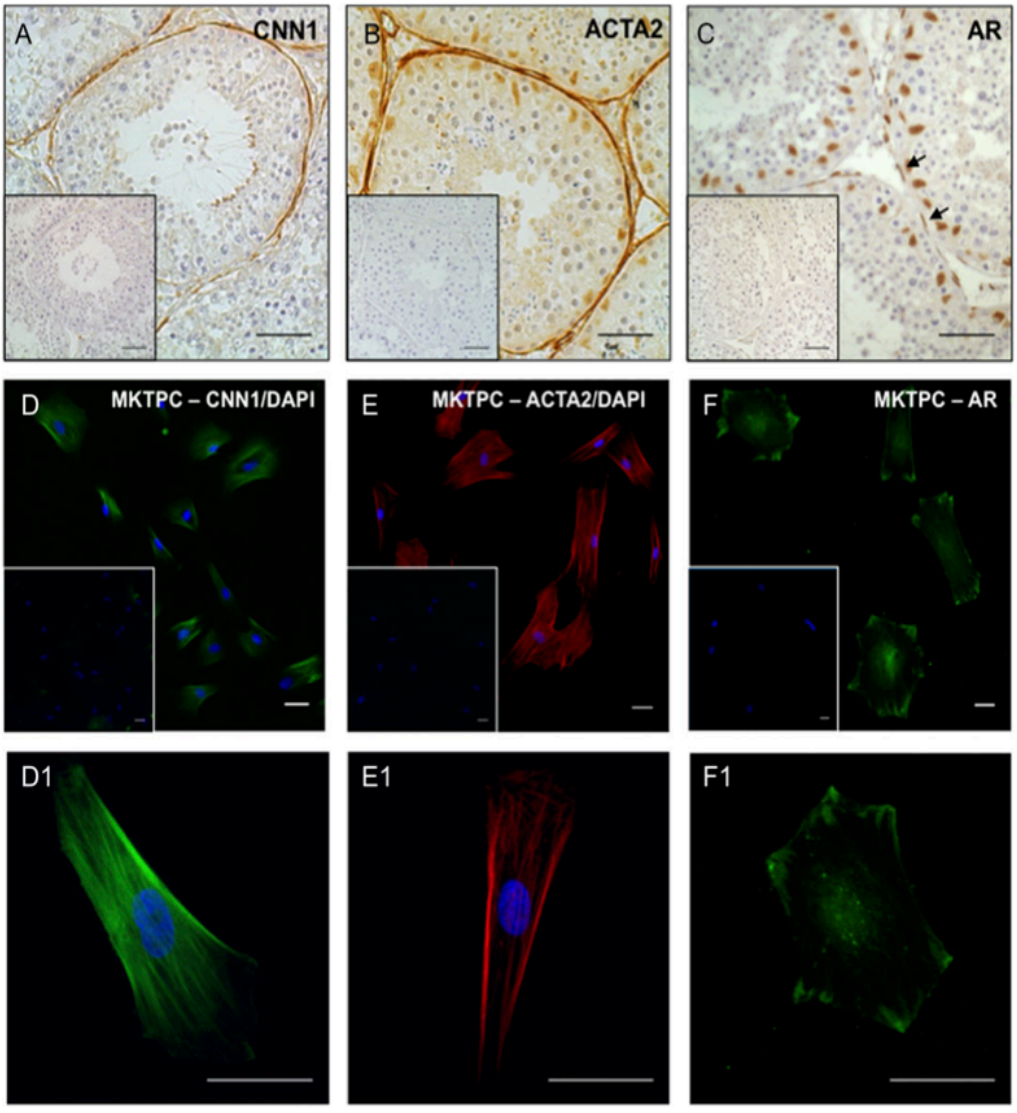

Figure 2 Expression of peritubular cell markers *in situ* and in cultured MKTPCs shown by immunohistochemistry. (**A**–**C**). Smooth muscle (SM) markers (A), CNN1, (B), ACTA2 and (C), AR were detected in individual testicular tissue sections from MKTPC donor animals (scale bar 50 µm). (**D**–**F**) Immunofluorescence of SM-markers, CNN1 (MKTPCs, passage 6) and ACTA2 (MKTPCs, passage 6), and AR (MKTPCs, passage 3) (**D1**, **E1**, **F1**). Higher magnifications, corresponding to **D**–**F**, respectively. Insets in A–F show negative controls.

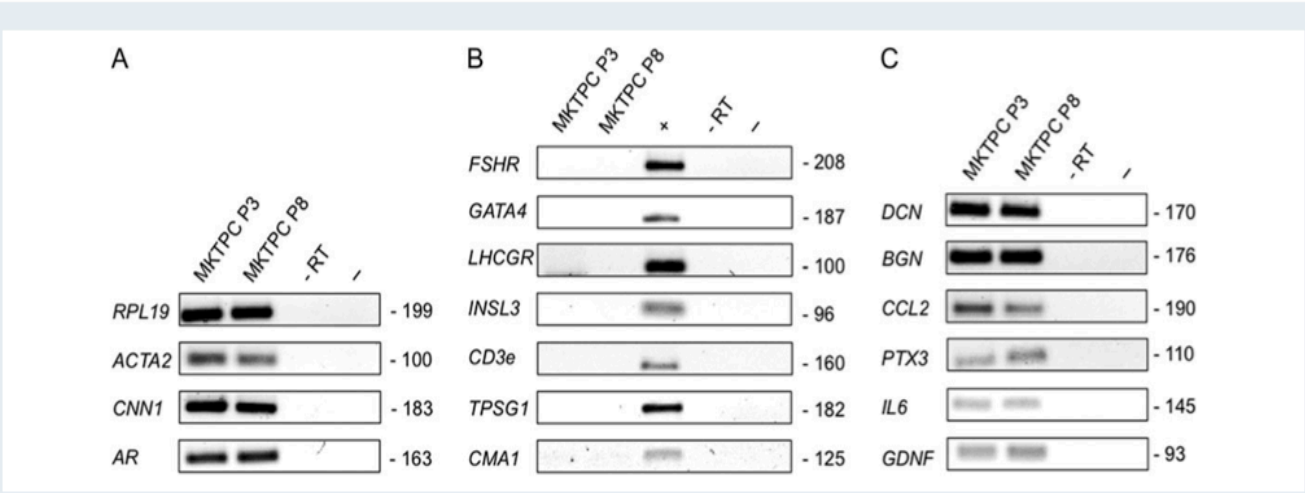

Figure 3 Expression analysis by RT-PCR. (**A**) Cultured MKTPCs express characteristic markers, SM-markers, e.g. *ACTA2*, *CNN1* and the *AR*, (**B**) but lack markers for Leydig cells (*LHCGR*, *INSL3*) and Sertoli cells (*FSHR*, *GATA4*), which were found in the positive control (+), cDNA from *Callithrix jacchus* whole testis lysate. (**C**) Extracellular matrix, *DCN* and *BGN*, the inflammatory markers *CCL2*, *PTX3* and IL6 and the neurotrophic factor *GDNF* were also expressed by MKTPCs. Expression of *RPL19* was used as reference gene. Note that immune cell markers (*CD3e*, *TPSG1*, *CMA1*), expressed in the testis, were not found in MKTPCs.

spermatogenesis was described previously (Albrecht *et al.*, 2006; Schell *et al.*, 2008). The patients ($n = 7$; aged 39–55 years) had granted written informed consent for scientific purposes. Cells were cultivated in DMEM High Glucose (Gibco) supplemented with 10% fetal bovine serum (Capricorn Scientific) and 1% penicillin/streptomycin (Biochrom, Berlin, Germany) under humidified conditions (37°C, 5% CO_2). Cells in passages 3–7 were harvested for proteomic procedures, as described (Flenkenthaler *et al.*, 2014).

Nano-liquid chromatography–tandem mass spectrometry

Cell pellets were resuspended in 8 M Urea in 50 mM ammonium bicarbonate, sonicated for 5 min at 4°C and homogenized using QIAshredders (Qiagen, Hilden, Germany) at 2500 *g* for 1 min. Protein concentration was determined using the Pierce 660 nm assay (Thermo Scientific, San Jose, CA). The 10 µg protein were reduced in 4 mM dithiothreitol (DTT) and 2 mM tris(2-carboxyethyl)phosphine for 30 min at 56°C followed by an alkylation step in 8 mM iodoacetamide (IAA) at room temperature in the dark for 30 min. Remaining IAA was quenched at a final concentration of 10 mM DTT. The samples underwent a first digestion step with Lys C (enzyme/substrate: 1/100; Wako, Neuss, Germany) at 37°C for 4 h, were diluted with ammonium bicarbonate to a concentration of 1 M urea and digested overnight with porcine trypsin (enzyme/substrate: 1/50; Promega, Madison, WI, USA) at 37°C. The 2.5 µg of peptides dissolved in 0.1% formic acid (FA) were subjected to liquid chromatography-electrospray ionization–tandem mass spectrometry (LC–MS/MS) analysis. LC was performed on an Ultimate 3000 RS system (Dionex, Sunnyvale, CA, USA). Peptide samples were first trapped on a C18 trap column (µ-Precolumn, C18 PepMap 100, 5 µm, Thermo Scientific, San Jose, CA) at a flow rate of 30 µL/min and separated on a C18 nano-flow column (Acclaim PepMap RSLC, 2 µm, 75 µm × 50 cm) at a flow rate of 0.2 µL/min using the following consecutive gradients: 5–25% B for 285 min and 25–50% B for 30 min (A: 0.1% FA in water, B: 0.1% FA in acetonitrile). Electrospray ionization was done with an uncoated SilicaTip (FS360-20-10-N-20-C15; New Objective, Woburn, MA) and a needle voltage of 2.3 kV. For MS data acquisition, a data dependent top 70 CID method was performed on a 5600+ mass spectrometer (Sciex, Concord, Canada).

Data analysis

Mass spectrometry (MS) raw data were processed using the MaxQuant software package (version: 1.6.0.1) (Cox and Mann, 2008). The data were searched separately for both species, using the UniProt subset for *Callithrix jacchus* for MKTPCs and the human Swiss-Prot subset for HTPCs (both: Release 2017/06), each augmented with the MaxQuant common contaminants database. Identification was performed with the 'match between run' feature enabled and a target decoy search strategy (resulting in a false discovery rate of 1%). For quantification, the MaxQuant label-free quantification strategy was applied. Data analysis and statistics were performed with Perseus (version: 1.5.8.5). For the scatterplot of MKTPC and HTPC datasets, the protein families contained in both datasets were merged into one matrix using the associated gene name. The 'circoletto' graph was done for the 100 most abundant protein identifications of each species with the corresponding online tool hosted by the bioinformatics analysis team (http://tools.bat.infspire.org/circoletto) using the following deviations from the default settings: e-value cutoff 10^{-15}; e-value for coloring; use (score-min)/(max-min) ratio to assign colors. With the blastp online tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) the top 25 proteins were blasted against the human subset of the Swiss-Prot database. For the spectral counting approach, MS data were searched with Mascot (V 2.4.0) and analyzed with Scaffold (V 4.1.1) using the same databases as for the MaxQuant analysis. For abundance ranks, the averaged spectral counts were used. Functional classification of the proteins was done with the Panther GO analysis online tool (http://pantherdb.org) using gene names and the GO 'biological process' and 'cellular process' database.

Results

Isolation and initial characterization

Using a similar approach as employed for HTPCs previously (Albrecht *et al.*, 2006), we were able to isolate and culture MKTPCs. Explant cultures of marmoset testicular tissue fragments, small pieces of tissue (1–2 mm), were seeded onto cell culture dishes. The cells started growing out of the walls of the seminiferous tubules within 7–14 days,

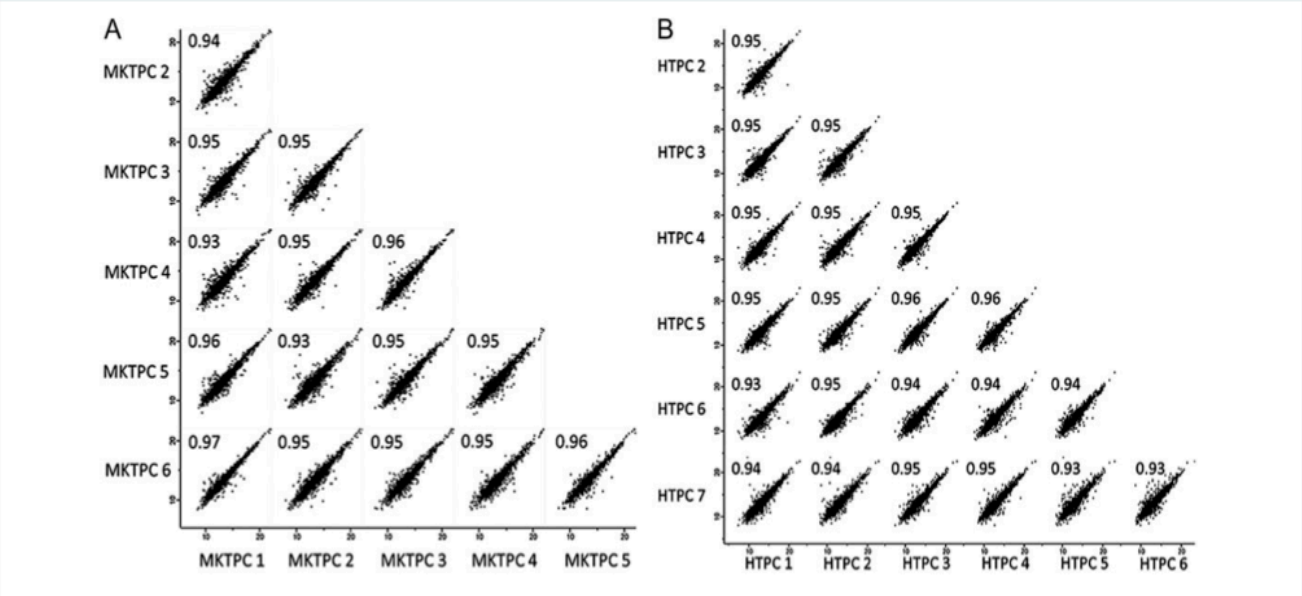

Figure 4 (**A**) Multi-scatter plot of log_2 Max-Quant LFQ protein intensity values of MKTPCs from six donors. The number in the upper left corner of each individual scatterplot shows the Pearson correlation. (**B**) Multi-scatter plot of log_2 Max-Quant LFQ protein intensity values of HTPCs from seven donors.

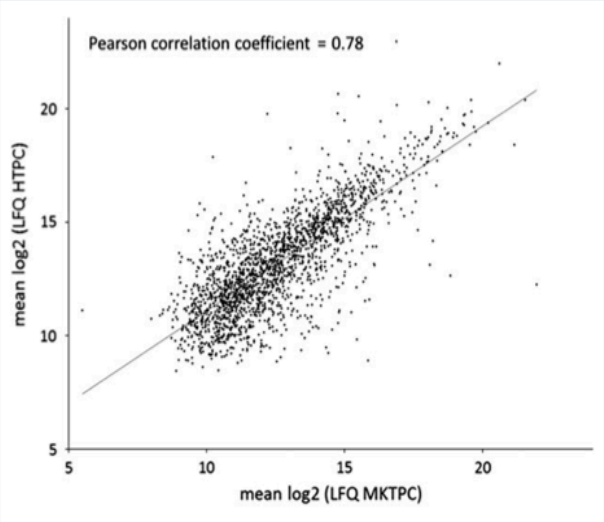

Figure 5 Scatter plot of averaged log_2 LFQ protein intensity values of MKTPCs and HTPCs.

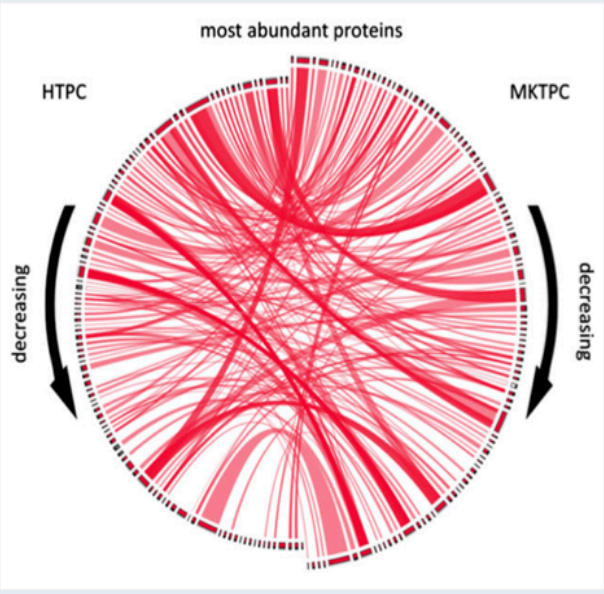

Figure 6 Circoletto analysis of the top 100 protein identifications found in HTPC and MKTPC. The left half circle shows HTPC proteins sorted by their abundance from highest at the top to the lowest at the bottom. The right half circle lists the top 100 proteins identified in MKTPC in the same order. Both groups are compared with respect to protein sequence similarity. Proteins are represented by boxes on the circle. The length of the boxes indicates the length of the protein sequences. Sequence similarities between two corresponding proteins (MKTPC vs HTPC) are represented by a ribbon connecting the two boxes (cutoff e-value 10^{-15}). Different ribbon color shades were used for better distinction.

and small spindle-like cells became visible. They proliferated and cells were propagated for several passages (Fig. 1).

As shown by immunohistochemistry, sections of the corresponding testicular tissue, from which explant cultures were derived, contain ACTA2-, CNN1- and AR-positive peritubular cells. The corresponding MKTPCs *in vitro* are likewise positive for CNN1, ACTA2 and AR as shown by immunofluorescence (Fig. 2).

Further, as shown by RT-PCR, MKTPCs (all from passages 3 and 8) are positive for smooth muscle (SM) cell markers, including *CNN1*, *ACTA2*, and for the peritubular cell marker *AR*. The cells lack markers for Leydig cells (*LHCGR*, *INSL3*) or Sertoli cells (*FSHR*, *GATA4*). Mast cell markers like *tryptase* (*TPSG1*) and *chymase* (*CMA1*) and the T-cell marker *CD3e* were not detected. Like HTPCs, they express *GDNF*, extracellular matrix components including *DCN* and *BGN*, also the inflammatory molecules *CCL2*, *PTX3* or *IL6* (RT-PCR) and are thus

similar to HTCPs. The cells can be passaged and the characteristic markers for MKTPCs remain stable for at least up to eight passages (Fig. 3).

Proteome analysis from MKTPCs and comparison with HTPCs

Proteins of MKTPCs from six individual donors were investigated by LC–MS/MS. In total 2437 protein groups were identified (FDR < 1%) in MKTPCs of which 1825 protein groups could be quantified in at least three individual samples. The mass spectrometry proteomics data were deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD009394. The inter-individual variance was investigated by a multi-scatter plot analysis of all protein label-free quantification (LFQ) intensities between all MKTPC individuals (Fig. 4A). The Pearson correlation coefficient ranged between 0.93 and 0.97.

Additionally, a set of HTPCs from seven individual human donors was analyzed using exactly the same parameters and compared to the results of the MKTPCs. From 3374 protein groups, which were identified in HTPCs, 2137 are contained in the MKTPC dataset, demonstrating that 88% (2137 out of 2437) of the proteins in MKTPCs are represented in the HTPC data. The inter-individual variance was again assessed using a multi-scatter plot between all individual donors, which results in a Pearson correlation coefficient between 0.93 and 0.96 (Fig. 4B).

The means of the MaxQuant LFQ values were scatter plotted against each other, which results in a correlation coefficient of 0.78 (Fig. 5) between human and marmoset cells. To investigate the variability of this inter-species comparison, each individual of MKTPCs were plotted against all individual HTPC donors. The mean Pearson correlation coefficient was 0.75 with no significant outliers (Grubbs' test for outliers; 99% confidence) and therefore showing a very homologous correlation pattern (Supplementary Information Fig. S1).

From both datasets the 100 most abundant proteins were retrieved and analyzed with the circoletto tool to visualize sequence homology of highly expressed proteins between HTPC and MKTPC (Fig. 6). The resulting network shows sequence homologies (<e-value 10^{-15}) between the most abundant proteins found in both species, of which a vast majority shows high degree of similarity. Furthermore, a BLAST

Table II The top 25 most abundant proteins in MKTPC. Sequence similarities with corresponding human proteins were determined by BLAST analysis and are indicated as '% identity'.

Callithrix jacchus UniProt accession	*Callithrix jacchus* Gene name	*Callithrix jacchus* protein name	Human UniProt accession	% Identity
F7IH64	ACTA2	Actin, alpha 2, smooth muscle, aorta	P62736	100
B0KWW5	FLNA	Filamin-A isoform 1,2	P21333	98.79
F7BQY8	VIM	Vimentin	P08670	99.36
F6ZZ90	MYH9	Myosin heavy chain 9	P35579	99.49
F7I1Q9	TAGLN	Transgelin	Q01995	99.5
F7I9U1	TPM2	Tropomyosin beta chain isoform 2	P07951	87.32
F7HTQ3	CALD1	Caldesmon 1	Q05682	95.33
F7I0W8	LOC100409006	Uncharacterized protein	P63261	100
U3EQM9	ACTN4	Alpha-actinin-4	O43707	99.89
F6YS84	TPM1	Tropomyosin 1	P09493	85.92
U3BZ94	TUBB4B	Tubulin beta chain	P68371	99.33
U3CYB6	ENO1	Alpha-enolase isoform 1	P06733	99.31
F7I318	LGALS1	Galectin	P09382	99.07
F7HZP3	TUBA1B	Tubulin alpha chain	P68363	100
F6RMA3	ANXA2	Annexin	P07355	99.71
F7IHK8	PRDX1	Peroxiredoxin 1	Q06830	97.99
U3EKQ0	ACTN1	Actinin alpha 1	P12814	99.78
F7CXD3	EEF1A1	Elongation factor 1-alpha	P68104	100
F6SQW1	PPIA	Peptidyl-prolyl cis-trans isomerase	P62937	99.78
U3FXT1	HSPA5	78 kDa glucose-regulated protein	P11021	99.39
F7HVD2	HSPA8	Heat shock protein family A (Hsp70) member 8	P11142	99.85
F7FP14		Uncharacterized protein	Q8N386	62.38
F6YTV2	LMNA	Lamin A/C	P02545	98.65
F6XL35	PKM	Pyruvate kinase	P14618	99.06
U3D5Z3	TLN1	Talin-1	Q9Y490	99.21

analysis of the 25 most abundant MKTPC using the Human Swiss-Prot database reveals that 22 are showing sequence identities >95% (Table II). Two further proteins could be matched to their human counterparts with >85% identity and represent members of the tropomyosin family, and only one single protein (F7FP14; uncharacterized protein) could not unambiguously be assigned to a human protein.

To review if the most abundant proteins in MKTPCs are also highly abundant in HTPCs, the identified MKTPC and HTPC protein groups were sorted according to their MS/MS spectral count values, a measure for their abundance. Comparison of both datasets revealed that 21 out of the top 25 MKTPC protein groups are contained in the top 50 HTPC protein groups (Table III). Only transgelin (TAGLN), caldesmon (CALD1) and the tropomyosins TPM1 and TPM4 ranked noticeably lower in HTPCs compared to MKTPC.

To assess similarities between MKTPCs and HTPCs at the functional level, the identified proteins were analyzed with the PANTHER analysis tool using the 'Biological Process' and 'Cellular Process' Gene Ontology Databases. The proteins of both MKTPC and HTPC show highly similar distributions concerning related biological and cellular processes, suggesting strong biochemical and functional similarities between MKTPC and HTPC (Fig. 7).

Table III The top 25 most abundant MKTPC protein groups and their number of group members. The abundance ranks of MKTPC protein groups are compared to the ranks of the corresponding protein groups in HTPCs.

Rank in MKTPC	Rank in HTPC	Protein group	# Proteins in group
1	2	Actin, cytoplasmic 1	11
2	4	Filamin-A	3
3	7	Myosin 9	5
4	9	Alpha-actinin-4	7
5	1	Vimentin	45
6	3	Tubulin beta	19
7	4	Uncharacterized protein homologue to human neuroblast differentiation-associated protein AHNAK	3
8	63	Transgelin	1
9	93	Tropomyosin alpha-4 chain	5
10	42	Myosin-10	3
11	239	Tropomyosin alpha-1 chain	4
12	23	Filamin-C	2
13	26	Alpha-enolase	6
14	12	Talin-1	1
15	14	Heat shock protein family A (Hsp70) member 5	1
16	113	Caldesmon	3
17	44	Filamin-B	4
18	6	Plectin	2
19	19	Clathrin heavy chain	2
20	11	Tubulin alpha chain	7
21	15	Heat shock protein 90	4
22	21	Cytoplasmic dynein 1 heavy chain 1	1
23	8	Pyruvate kinase	5
24	20	Lamin isoform A	3
25	27	Heat shock protein family A (Hsp70) member 8	3

Discussion

The previous studies in HTPCs provided new insights in to the functions of these testicular cells (Spinnler *et al.*, 2010; Mayerhofer, 2013; Landreh *et al.*, 2014; Welter *et al.*, 2014; Mayer *et al.*, 2016). While a previous proteomic study of HTPCs revealed that they are rather homogeneous with respect to their cellular proteome and their repertoire of secreted factors (Flenkenthaler *et al.*, 2014), further studies showed that their ability to secrete steroids (Landreh *et al.*, 2014) or to respond to stimuli with cytokine secretion (Mayer *et al.*, 2016) occur in a patient-specific fashion. It is likely that the differences are due to age, lifestyle and or medical history, which are beyond control.

We hypothesized that cells from a non-human primate species, specifically with a controlled lifestyle, may be an ideal additional model in which to study TPCs. We chose the common marmoset monkey (*Callithrix jacchus*), which is a well-established model organism for reproductive research, for this model. The testicular structures of marmosets are comparable in several biologically relevant aspects with humans, including characteristics of germ cell development and function (Michel and Mahouy, 1990; Millar *et al.*, 2000; Mansfield, 2003; Zuhlke and Weinbauer, 2003). Importantly, the architecture of the tubular wall shows several layers and therefore comes close to the human situation. Both contrast to rodent testes, in which the wall of the seminiferous tubules consists of a single cell layer of peritubular cells. Finally, the whole genome from *Callithrix jacchus* was sequenced (Sato *et al.*, 2015), which enables further investigation on the genomic and proteomic level.

We successfully isolated and cultured cells from the wall of seminiferous tubules of the testis of young adult, healthy *Callithrix jacchus*, using the same approach as for HTPCs, namely explant culture. The cells obtained upon initial characterization could be clearly identified as pure peritubular cells. Virtually all MKTPCs were immunoreactive for the smooth muscle markers, CNN1, ACTA2, which were detected only by peritubular cells *in situ*. In addition, the combination of smooth muscle markers, expression of *AR* and *GDNF* and the absence of Sertoli cell markers (*FSHR*, *GATA4*) and Leydig cell markers (*LHCGR*, *INSL3*) allows this conclusion. Importantly, the expression of the characteristic factors remains stable over at least eight passages.

In human testes tryptase-immunoreactive mast cells are found close to the tubular wall (Meineke *et al.*, 2000) and within the layers of peritubular cells and we also detected tryptase-immunoreactive mast cells by immunohistochemistry in *Callithrix jacchus* testes (data not shown). We therefore explored a possible contamination with immune cells. RT-PCR for mast cell markers *tryptase*, *chymase* or the T-cell marker *CD3e* yielded, however, negative results in MKTPC.

We further compared the MKTPC proteome data with a published human macrophage proteome data (Eligini *et al.*, 2015). We found that five proteins coincide, namely Chloride intracellular channel protein 1, Elongation factor 2, Plastin 3, Tubulin alpha 1 chain, Vimentin. These proteins are, however, not specific for macrophages but

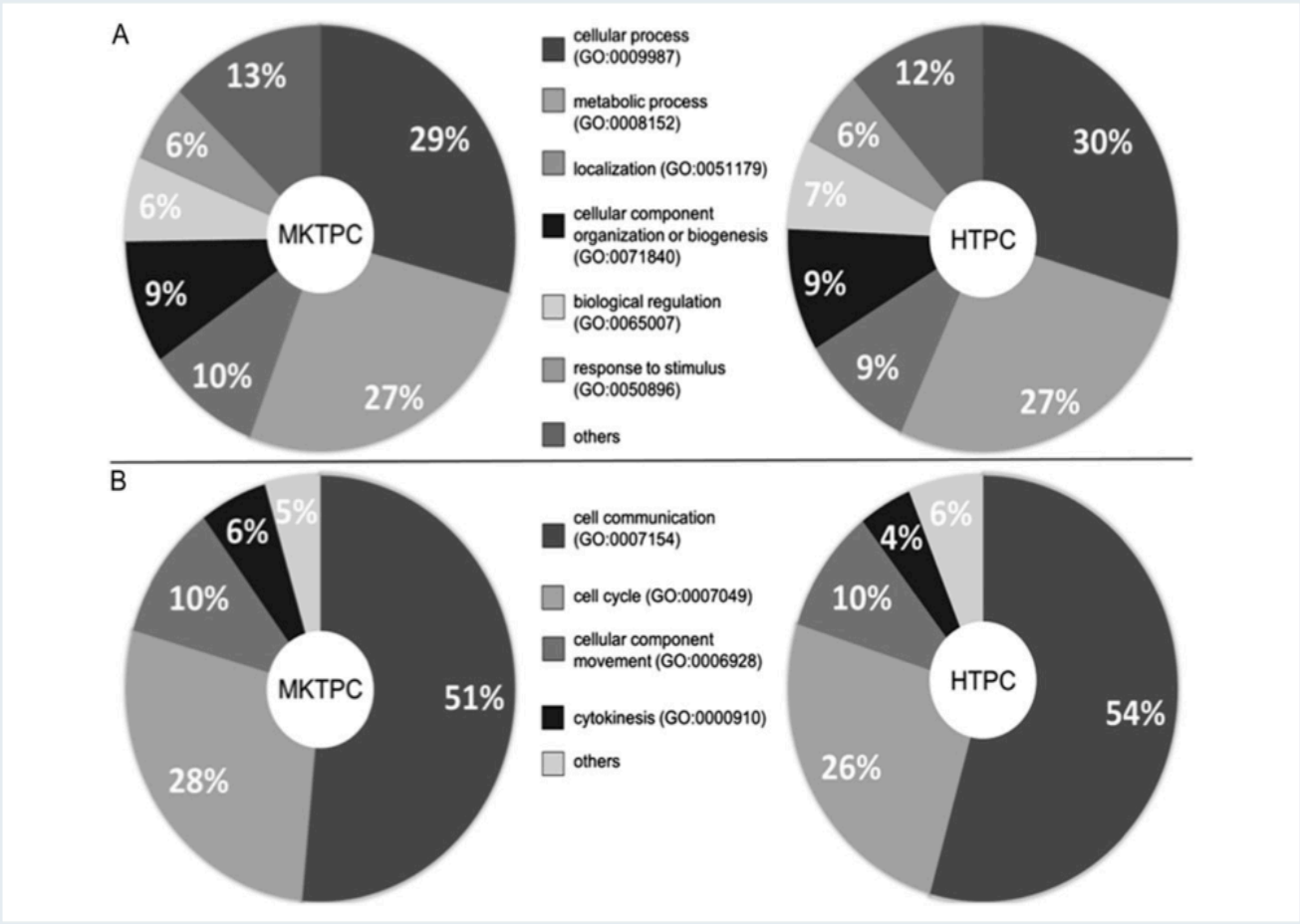

Figure 7 (**A**) GO analysis using the 'biological process' database for MKTPC (left) and HTPC (right) proteomes. (**B**) GO analysis regarding the 'cellular process' subset for MKTPC (left) and HTPC (right).

represent ubiquitously occurring proteins. Apolipoprotein B receptor, which is considered a characteristic macrophage receptor (Hassel *et al.*, 2017), is not detected in our proteome analysis. Thus, isolation of MKTPCs is a practical way to obtain pure testicular peritubular cells of a non-human primate species.

We initially tested whether they further resemble HTPCs, and found that they produce *DCN* and *BGN*, *CCL2*, *PTX3* and *IL6* as well as *GDNF*. Hence, they resemble HTPCs in this respect (Spinnler *et al.*, 2010; Mayer *et al.*, 2016; Walenta *et al.*, 2018).

To further characterize MKTPCs at the protein level, a proteome study of peritubular cells obtained from six individual young adult *Callithrix jacchus* donors was performed. In order to focus on the most abundant proteins being easily assessable with a single-run LC–MS/MS method, we kept the proteomics workflow as simple as possible and did not use any pre-fractionation at the protein or the peptide level. Nevertheless, the analysis of the acquired mass spectra led to the identification of 2437 MKTPC protein groups (FDR < 1%). For the chosen approach, this represents a fairly high number of protein IDs and reflects the suitability of the *Callithrix jacchus* database for LC–MS based proteome analysis of MKTPC samples. Additionally, a multi-scatter plot analysis between the donors revealed very reproducible protein expression patterns, demonstrating the robustness of isolation and cultivation methods as well as a low inter-individual variability between the individual donors.

For the suitability of the animal model, the similarity between MKTPCs and HTPCs at the proteome level is an important indicator. To assess this, HTPC proteomes from seven human donors were analyzed using exactly the same methodology. Inter-individual correlation analysis shows clear homogeneity and reproducibility similar to MKTPCs, with the latter one being more accessible and generated under monitored conditions. The inter-species scatter plot analysis of protein intensity values between MKTPC and HTPC showed a Pearson correlation coefficient of 0.78 indicating similar abundance patterns of MKTPC and HTPC proteins. A further MKTPC vs HTPC multi-scatter plot analysis at the level of individuals showed in all cases very similar Pearson correlation coefficients with no outliers. Taken together the correlation analyses reveal a clear conformity between MKTPCs and HTPCs on the level of protein expression patterns and a high degree of inter-individual reproducibility of MKTPCs.

A circoletto network analysis as well as a BLAST analysis of the 25 most abundant proteins showed high sequence homology between HTPC and MKTPC proteins, indicating a high degree of functional similarity. Using a spectral count quantification approach combined with homology based protein grouping, we could further demonstrate that the broad majority of the 25 most abundant MKTPC protein groups are also highly expressed in HTPCs. Only transgelin (TAGLN), caldesmon (CALD1) and the two tropomyosins (TPM1 and TPM4) showed lower spectral counts in MKTPC. Since all of these four proteins bind to actin this finding may reflect slight differences in the cytoskeleton between MKTPC and HTPCs. Finally, the PANTHER GO analysis of MKTPC and HTPC proteins lead to almost identical results, suggesting

a strong resemblance at the level of biological and biochemical processes between MKTPCs and HTPCs. The high similarity at the proteome level elaborated here further approves MKTPC as an excellent non-human primate model to study the biology of HTPCs.

In summary, isolation of MKTPCs is a feasible way to obtain primate peritubular cells, which resemble their human counterparts. They are derived from young adults raised under controlled conditions and provide an opportunity to explore functions and regulation of testicular peritubular cells without unknown confounding issues like lifestyle, age, nutrition and the medical history of patients. We anticipate that this may lead to a better understanding of the role of peritubular cells in male (in)fertility, including their role in the spermatogonial stem cell niche and their plasticity.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

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Authors' roles

N.S. performed the majority of the cellular experiments and K.G.D. participated in these experiments. J.B.S, F.F., T.F. and G.A performed proteomic studies and evaluated the results. J.U.S, F.-M.K., C.D. and R.B. provided testicular tissues, as well as conceptual input, A.M. conceived of the study, directed the work and supervised the experiments. N.S. and A.M. drafted the article. All authors contributed to and approved the final version. This work was performed in partial fulfillments of the requirements of doctoral degrees (N.S. and J.B.S.) at LMU.

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Conflicts of interest

The authors declare no competing financial interests.

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Supplementary information

Figure S1

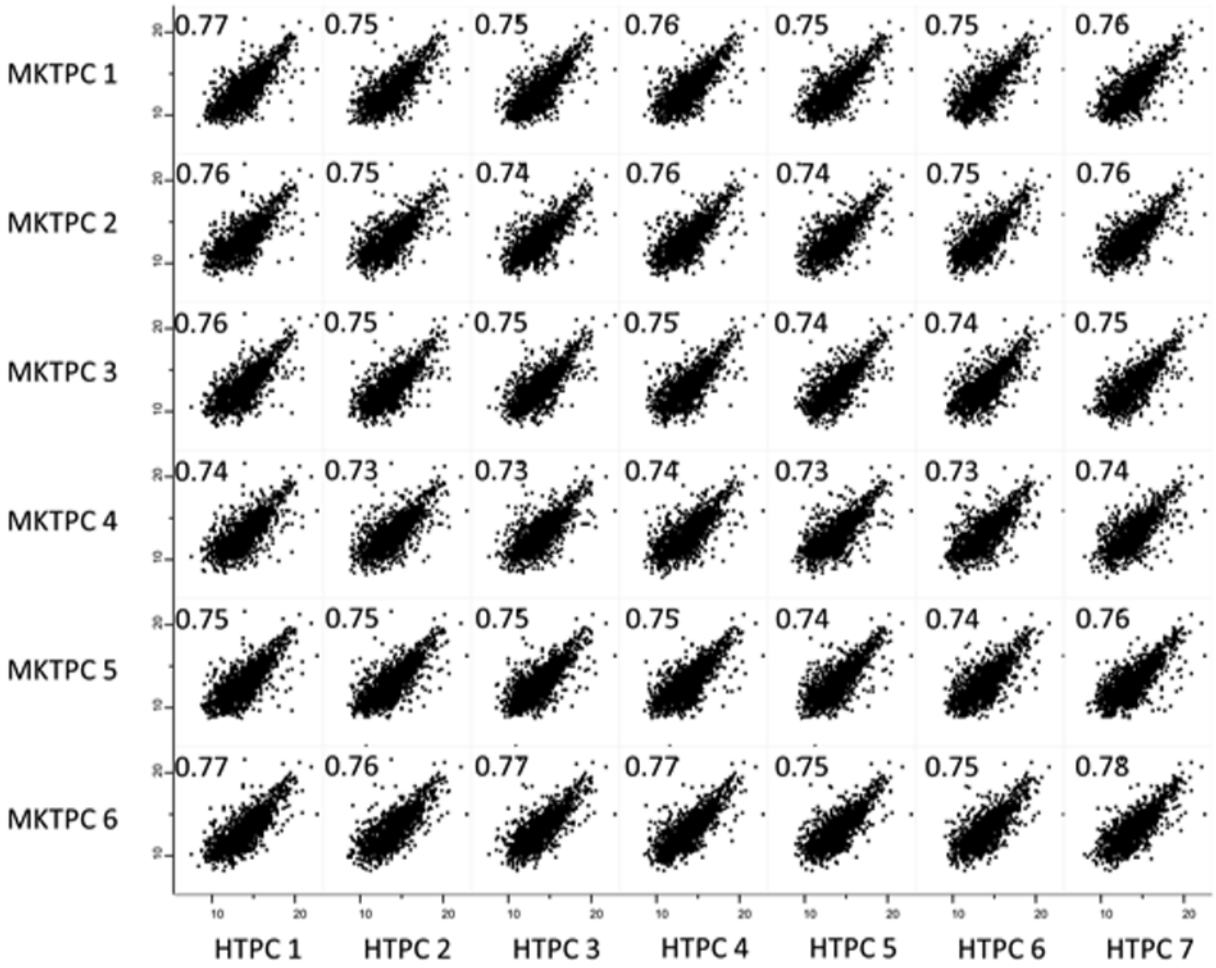

Figure S1

Multi scatter plot showing individual MKTPCs compared against individual HTPCs. The number in the upper left corner of each individual scatter plot shows the Pearson coefficient.

4 Discussion

4.1 The NLRP3 inflammasome in testicular inflammation and senescence?

Inflammatory processes within the testis can be responsible for male subfertility or infertility. The sensor part NLRP3 of the NLRP3 inflammasome was validated in testis samples of human, other primates (common marmoset, rhesus macaque), and an sterile inflammation-associated infertile *AROM*$^{+}$ mouse model. NLRP3 was detected in Sertoli cells of all employed species and additionally in peritubular cells of the human samples from infertile patients. Both peritubular cells and Sertoli cells were previously associated with immunoregulatory functions in the testis, providing a possible connection to NLRP3.

Cellular senescence is associated with a chronic secretion of inflammatory factors. NLRP3, due to its broad variety of activators, is an interesting factor for further investigation regarding aging of the testis. The canonical NLRP3 inflammasome activation pathways are characterized by a decreased intracellular K^{+} level, which could be e.g. mediated by rupture of the highly abundant lysosomes in senescent HTPCs (Hornung et al., 2008). In addition, damaged mitochondria, ROS (Zhou et al., 2010, Zhou et al., 2011), lipids and aggregated proteins (Sheedy et al., 2013, Ralston et al., 2017), which occur during aging and in senescent cells, can trigger inflammasome activation and thus contribute to inflammaging. Since various damage-related proteins accumulate in senescent cells, it is more likely that NLRP3 inflammasome activation is a result of multiple triggers and can not attributed to a specific ligand. Further, the NLRP3 inflammasome is involved in multiple hallmarks of aging and senescence. NLRP3 inflammasomes have been identified to participate in autophagosome formation; interestingly, the inhibition of autophagy results in elevated inflammasome activity, whereas the induction of autophagy limits inflammasome activity (Shi et al., 2012). Triggering of caspase 1-dependent mitochondrial damage and decreased mitophagy has also been attributed to NLRP3 inflammasomes (Yu et al., 2014). If NLRP3 plays a role in human testicular aging, still remains to be evaluated. Yet, the limited access to testicular tissue samples of elderly men and appropriate controls hamper functional studies within the human system. Therefore, different rodent models were used to study alterations of testicular aging (Salomon et al., 2013, Zhao et al., 2013, Matzkin et al., 2016). A recent study using the Syrian hamster as a model, revealed significantly increased levels of NLRP3 and fibrosis of the peritubular

wall in testes of aged animals (Matzkin et al., 2019). Yet, in men, fibrosis of the tubular wall is associated with infertility but not necessarily with aging, which was currently confirmed by a light microscopic study from (Pohl et al., 2019). They could not detect fibrotic alterations of the peritubular wall in tissue samples of elderly men. These results are in line with the proteomic data of HTPCs, which showed significantly reduced ECM secretion of senescent cells. In contrast to rodents, this does not indicate fibrotic changes of the peritubular wall with age, but rather a general reduction of the secretory activity of senescent HTPCs. Therefore, species-dependent variations of the aging process must be considered when choosing a model for the investigation of aging in the testis.

4.2 Cytological and proteomic aspects of HTPCs

Electron microscopy (EM) allows fundamental insights into cellular architecture. Despite the striking resolution in ultrathin sections of about 2 nm in xy-direction, the resolution in z-direction was restricted to a section thickness of approximately 70 nm. Large volume reconstructions were not possible, due to acrobatic serial sectioning in the range of 100-200 sections at maximum. The three-dimensional (3D) reconstructions were mainly based on interpretation of several single sections, thus much basic structural information e.g. from small cellular structures like the cytoskeleton (actin filaments, 7 nm in diameter) could not be obtained (Peddie and Collinson, 2014). Single investigations indicated that the 3D structure of cell organelles did not match with text book knowledge – until now (Wei et al., 2012, Vihinen et al., 2013). From the actual point of view, it is necessary to compare any cytological changes at a 3D level and, if possible, quantitatively. Although time-consuming and expensive, only reconstruction of a relevant number of cells in 3D can give sufficient information for a profound comparison of ultrastructural changes at EM level.

Three modern large volume EM techniques enable new possibilities for 3D reconstruction: i) Array tomography using the ATUMtome, generating serial sections, which are collected automatically and investigated with scanning electron microscopy (SEM). This technique has low resolution in z, but is non-destructive; ii) 3View® enables serial block face sectioning, with low resolution in z but it is very economic and suitable for very large volumes and iii) FIB/SEM tomography with by far highest resolution in z (2 nm). In general, the challenge of all techniques is the correlation of 3D LM with 3D EM data sets (Luckner and Wanner, 2018a).

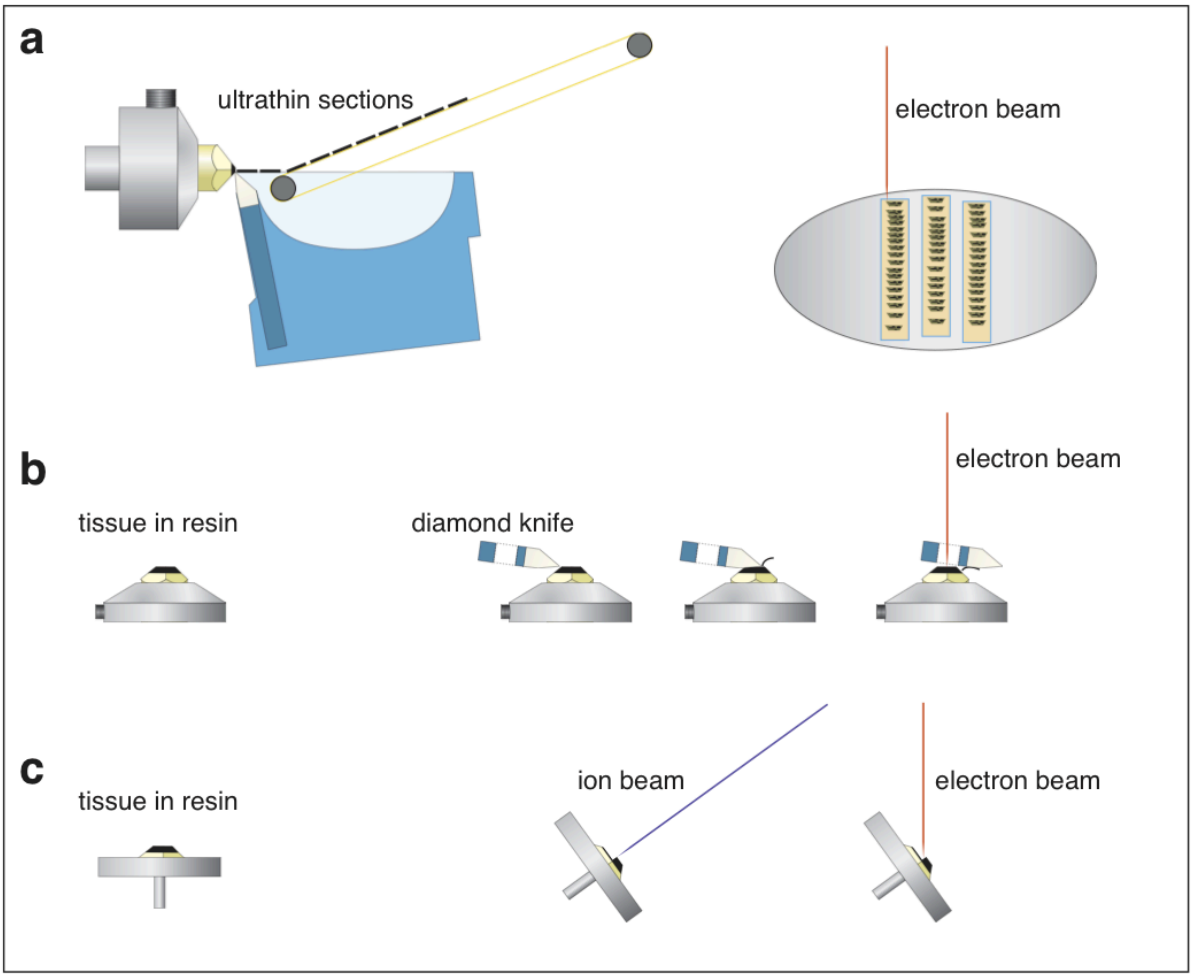

Figure 3 Schematic drawings of large volume electron microscopic techniques

(a) ATUMtome: serial sectioning with automated collection onto adhesive tape, investigated with SEM, for array-tomography. (b) 3View®, an ultramicrotome built in SEM with subsequent imaging of the block face. (c) FIB/SEM tomography: thin slices of material are removed by an ion beam (blue) and the block face is imaged by SEM at an angle of 36° (orange).

A new technique, facilitating correlative light and electron microscopy (CLEM) for routine application (by thin and ultrathin embedding) made FIB/SEM economic for CLEM (Luckner and Wanner, 2018a). CLEM could be successfully adopted for HTPCs. The evaluation of structural changes related to senescence in HTPCs required an immortal cell line as reference. Therefore, HeLa, which was intensively studied at all stages of cell cycle, including cellular rearrangements during mitosis (Luckner and Wanner, 2018b), was used for the interpretation of the EM data from senescent HTPCs regarding morphology and cytology.

Simplified, HTPCs of all passages can be categorized as interphase cells. However, in contrast to HeLa interphase cells, the mitochondrial network is more or less evenly distributed throughout the cytoplasm, thus resembling mitochondria in HeLa

anaphase. The central metabolic role of the Golgi, visible as dominant structure of prominent, interconnected cisternae is obviously reduced or even lost in HTPCs as it was not observed in any of the investigated cells (n = 14). However, rudimentary Golgi presence may be deduced from the occurrence of aggregated stacks with few cisternae observed in some cells.

The obviously highly dynamic ER of HeLa in all cell cycle stages present in irregular shaped sheets forming an elaborate 3D structure (Luckner and Wanner, 2018b) differs from ER of HTPCs. In early passages of HTPCs, ER is locally concentrated in packages of in parallel arranged sheets. The extraordinary density of attached ribosomes during all passages suggests massive proteins synthesis. A possibly significant part of proteins is produced for secretion. High-resolution 3D reconstruction with volume rendering gives a clear image of the interconnected ER sheets and the arrangement of polysomes. However, due to the dense packaging of all cellular components it was not possible to discern a mechanism involving vesicle formation within the confusing arrangement of subcellular structures. In HeLa, endosomes were abundant in all cell cycle stages, whereas HTPCs rather possessed vesicles, which were distributed throughout the cytoplasm but predominantly observed in the basal region of the cells. In early and advanced passages, vesicles were abundant, indicating the preserved secretory function of senescent HTPCs.

A secretome analysis of HTPCs before and during senescence revealed that the composition of the secretome significantly changed (Schmid et al., 2019). These changes may have a huge impact on neighboring cells, due to the location of peritubular cells (Maekawa et al., 1996); e.g. SSCs, Sertoli cells, and Leydig cells can be affected. The secretory activity of senescent HTPCs is reduced, but still present, which can be ascribed to the abundance of rough ER. Secreted inflammatory molecules (e.g. MIF) could generate a pro-inflammatory milieu with potential recruitment of immune cells leading to inflammation and thus tissue destruction (Bernhagen et al., 2007). Such a modification of the secretome and the subsequent inflammatory milieu is an explanation for the aging-associated alterations of the testis (Salminen and Kaarniranta, 2011).

DPP4 is a promising example for the potential impact of the altered secretome of senescent HTPCs in the testis. This ubiquitously expressed protease can occur in membrane-bound or soluble form (Bloom and Bennett, 1966). DPP4 cleaves multiple proteins/peptides including CXCL12, which is an essential factor for the SSCs and other stem cells (e.g. hematopoietic stem cell) (Christopherson et al., 2004). The

interaction between CXCL12 and its cognate receptor CXCR4, which is expressed on the SSC, is essential for SSC homing and SSC pool preservation (Heckmann et al., 2018). Thus truncation of CXCL12 due to increased DPP4 levels in the testis could negatively influence the SSC niche, leading to SSC differentiation and SSC loss. It was shown that the inhibition of DPP4 preserves CXCL12, encouraging regenerative stem/progenitor cell accumulation and homing to ischemic tissue (Jungraithmayr et al., 2012, Xie et al., 2018, Kanatsu-Shinohara et al., 2012). DPP4 inhibitors are widely used in diabetes therapy, thus multiple drug candidates are available for translational application to testicular pathologies. Furthermore, an involvement in fibrotic (Soare et al., 2019) and inflammatory processes (Wronkowitz et al., 2014) is attributed to DPP4. Since these processes are also related to infertility, DPP4 should be further investigated concerning testicular pathologies.

Mitochondria in HeLa changed their shape during mitosis from large clusters in interphase to bunches of threadlike single mitochondria in meta- to anaphase. In HTPCs the mitochondria also built up a huge network maintained in advanced passages, albeit a MitoTracker™ staining indicated fragmentation of mitochondria during senescence. The ultrastructural investigation and 3D reconstruction rebutted this perception and revealed a complex mitochondrial network hidden within clustering lysosomes. However, quantitative analysis disclosed a reduced number of mitochondria with smaller diameter and volume in advanced passages, implying diminished mitochondrial function.

The mitochondria are in massive contact to strands and sheets of ER. These so-called mitochondria associated membranes (MAMs) or mitochondria-ER contacts (MERCs) play important roles in several signaling pathways in cell physiology and pathology (Rizzuto et al., 1998, Storer et al., 2013, Giacomello and Pellegrini, 2016). MAMs are very dynamic structures and the numbers and characteristics of mitochondria-ER contact sites change during senescence and aging. Furthermore, MAMs are involved in the development of age-related diseases (e.g. Alzheimer´s, Parkinson´s disease) (Krols et al., 2016, Danese et al., 2017). In senescent HTPCs, the number of mitochondria, the amount of ER and the contact sites between mitochondria and ER are altered. Such alterations affect cells in regard to glucose homeostasis, lipid exchange, lipid biosynthesis, autophagy, calcium homeostasis and likely ROS production, which may also be the case for HTPCs (Janikiewicz et al., 2018).

For HeLa it was shown that endosomes/lysosomes are in luminal contact to ER and form – together with lipid bodies – an integrated system, discussed to function as a

membrane carrousel for degradation and reconstitution of the Golgi during the cell cycle (Luckner and Wanner, 2018b). HTPCs only possess a residual Golgi, if even, and no lipid bodies. Their lysosomes are similar in size (0.5-1 μm), however, more complex in composition. They form large aggregates, attached to mitochondria and are in luminal contact to ER at multiple sites. Although a membrane turnover can be postulated for HTPCs being cultivated for almost a year with active protein secretion, a homologue membrane carrousel as in HeLa with periodically complete recycling of cell organelles is not likely for HTPCs due to the lack of both, lipid bodies and an elaborate Golgi.

Among others, two main functions of HTPCs *in situ* can be simplified as secretion (e.g. collagens) and contraction by actin and myosin, both very energy costly processes. As *in vitro* contraction of HTPCs is promoted by external stimuli e.g. ATP, their main function remains secretion. One could speculate that the reduction of mitochondrial cristae especially in long segments of ultrathin mitochondria (100 nm diameter without any cristae) results in a reduced ATP level. Drastic changes in cellular protein composition were revealed by a proteome analysis: lysosome-associated protein levels were increased, which is in accordance with the ultrastructural data, whereas mitochondrial protein levels were reduced indicating their diminished function. This was accompanied by elevated antioxidant protein levels (e.g. SOD1), which counteract higher ROS levels appearing in senescent cells.

A small circulation within the endomembrane system might exist in HTPCs, yet with lysosomes continuously accumulating due to lysosome production being favored over degradation. From 3D data, it can be concluded that a fraction of mitochondria fuses with lysosomes. Further CLEM investigations on very early passages of HTPCs should concentrate on investigations with a combination of MitoTracker™ and lysosomal markers to elucidate, if degradation or turnover is likely. The absence of lipid bodies in HTPCs is striking, however, they are discussed to function as a transient membrane storage in HeLa (Luckner and Wanner, 2018b) and plant cells (Wanner and Köst, 1984), which is an unnecessary feature in stagnating/senescent cells.

Appropriate autophagy regulation is essential for the physiological quality control and reduced efficiency of autophagy has been implicated in various age associated human pathologies (Leidal et al., 2018). Autophagy is characterized by constitutive turnover of cellular proteins as well as of intact cell organelles, which are degraded and recycled by lysosomes – obviously necessary for maintenance of vital cells (Mizushima, 2007). Lysosomes are the basic module for autophagous processes. The massive

accumulation of lysosomes in senescent cells does not become clear at the first glance. Two scenarios seem likely:

a) Lysosomes are formed by degradation of cell organelles. The more organelles are degraded, the more lysosomes are formed, resulting in a disappearance of all mitochondria and ER in very late passages. However, this was not the case. Despite significant reduction, prominent numbers of mitochondria and ER were still present.

b) Lysosomes are formed by degradation of cell organelles but the lysosomes then are recycled again to intact organelles. These two processes become imbalanced during late passages: the lysosomes accumulate as they were not recycled. This would explain the significant reduction of mitochondria and ER, revealed by ultra-structural data.

The progressive decline in autophagy and the high abundance of lysosomes during senescence (confirmed by a proteome analysis of HTPCs) likely contribute to the accumulation of defect proteins and damaged organelles (Barbosa et al., 2018).

In order to complement the study a gene expression analysis was implemented. Partly, typical genes/proteins of HTPCs could not be measured by MS. For instance, interleukins are difficult to detect by MS, due to their small size and their low abundance. To overcome these limitations, a qPCR study was implemented, to complement the biochemical results. The analyzed genes were very heterogenic in expression because they were derived from different human donors. The expression levels of characteristic HTPC genes were not significantly altered implying that the cells retain their smooth muscle-like phenotype during senescence.

4.3 Simplified model for senescence in HTPCs

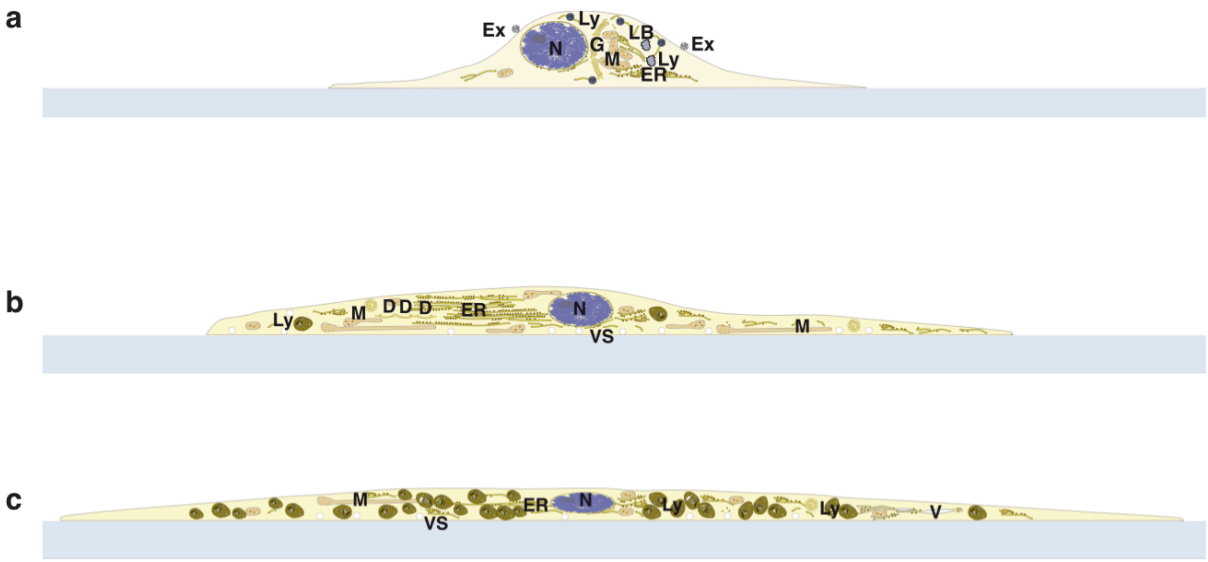

Figure 4 Simplified model for senescence in HTPCs

Schematic drawings of HeLa cell in interphase (a), HTPC of an early passage (b) and advanced passage (c). HeLa cells have a dominant Golgi (G) in vicinity of nucleus (N), clusters of mitochondria (M) an elaborate endoplasmic reticulum (ER), many endolysosomes (Ly) and lipid bodies (LB). Exosomes (Ex) are regularly observed. In early passages HTPCs exhibit a massive accumulation of ER sheets occupied by abundant ribosomes. Mitochondria are very elongated, forming several networks. The Golgi is present as an aggregation of rudimentary dictyosomes (D). Lysosomes are present in low number. Small vesicles (VS) are abundant, mainly at the basal region of the cell. In advanced passages mitochondria and ER in HTPCs are still present, although reduced in size and number. An abundance of lysosomes occupies a large proportion of the cell. Manny vacuoles (V) form in the lumen of the ER fusing with both, lysosomes and mitochondria.

4.4 Future of investigations of senescence

Therapeutic strategies for senescence-associated diseases are under investigation. There are different implications, but currently two main approaches are under development: i) the elimination of senescent cells by senolytics; and ii) the inhibition of the senescence-associated secretory phenotype (SASP) (Soto-Gamez and Demaria, 2017, Hickson et al., 2019, Kirkland and Tchkonia, 2015).

Senolytics were tested for therapy with the aim to eliminate senescent cells in tissues and thus improve the healthy life span (Hickson et al., 2019). The strategy to eliminate senescent cells is to induce cell death in the affected cells. Thus, the target of senolytics are mechanisms concerning the apoptosis resistance of senescent cells.

Two possible agents are ABT263 and ABT737. These molecules inhibit the anti-apoptotic proteins of the Bcl-2 family and thereby induce apoptosis in senescent cells (Zhu et al., 2016). The majority of these senolytic drugs have intrinsic toxicities, which currently limits their use for human purposes.

Another approach to inhibit senescence-associated inflammatory processes by reducing the negative effects of the SASP e.g. by targeting NF-κB, the major driver of the SASP (Kirkland and Tchkonia, 2015). In addition, the neutralization of distinct pro-inflammatory SASP members is an effective strategy and a possible therapy for aging- and inflammatory-associated pathologies in the testis. However, these drugs are non-specific for senescent cells and known anti-inflammatory drugs are not suitable for long term use because of their serious side effects.

Recently, dipeptidyl peptidase 4 (DPP4) was identified to be up-regulated on the plasma membrane of senescent cells (Kim et al., 2017b) as well as in the secretome of senescent HTPCs (Schmid et al., 2019). It additionally represents a possible target to identify and eliminate senescent cells in aged tissue.

4.5 Examination of the impact of aging on testis – a challenge

Due to the long lifespan of several decades, *in vivo* studies on human aging are very difficult. A promising alternative for aging research are cell culture models. Their major advantage is, that these systems can be implemented with human cells – a reasonable approach, since human aging has a cellular basis. We tried to translate the cell culture model of “replicative senescence” to HTPCs to gain insights into the possible role of peritubular cells in human testicular aging. As anticipated, the cell culture system has its limitations. In culture, cells lose the cross talk with other cells and their specific environment. Also, changed oxygen and carbon dioxide levels lead to cell culture artifacts (Cristofalo et al., 2004), which additionally limit the translation to the *in vivo* situation. A further limitation of senescent cells is the scarce number of cells available for experiments due to the cell-cycle arrest. However, senescent HTPCs could provide insights into human testicular aging and the related alterations. Yet, to which extent these results could be translated to the *in vivo* situation needs further investigation.

4.6 What can MKTPCs provide?

The fundamental issue with human samples is the drastic heterogeneity. HTPCs stem from different donors with their own unique lifestyle. Merely the age and the reason for

the surgery of the donors is known. However, the tissue samples are rare and valuable, therefore one cannot choose the donors. To overcome this limitation and to provide an additional cellular model for the peritubular wall, a non-human primate model for HTPCs was established, using the common marmoset monkey. The basic characterization of the cells, MKTPCs, and a comprehensive proteomic approach supports their relevance as model for HTPCs and the *in vivo* situation (Schmid et al., 2018). The whole life of the marmosets is monitored, thus there are no issues like different nutrition or "lifestyle". They are all treated equally and a well-documented medical history is accessible for better comparability. Even the possibility to select the age or rather the developmental stage (e.g. pubescent, adult, elderly) is given, which enables for instance the isolation of peritubular cells from young and old monkeys to gain insights, if peritubular cells age *in vivo*. Another advantage for non-human primates as a model organism was shown by a recent gene expression analysis across mammalian organ development. The study revealed the largest percentage difference in gene expression between the murine and human testis compared to other organs, forcing the mouse as model organism for testicular research to take backseat (Cardoso-Moreira et al., 2019).

Somatic cells, e.g. HTPCs, *in vitro* are of limited use for research applications due to their finite proliferative capacity, resulting in replicative senescence (Hayflick and Moorhead, 1961). The main reason for replicative senescence is the progressive telomere shortening due to mitotic divisions (Allsopp et al., 1995). Yet, senescence can be bypassed by the preservation of telomere length by telomerase, an enzyme, which elongates telomeres (Kim et al., 1995). Various studies demonstrated that overexpression of exogenous human telomerase (hTERT) leads to immortalization (Bocker et al., 2008, Ouellette et al., 2000, Cui et al., 2002). Recently marmoset fibroblasts were immortalized with transposon-integrated transgenic hTERT (Petkov et al., 2018). Thus, immortalization of MKTPCs provides the possibility for generating a permanent cell line.

4.7 Perspective and outlook

Male infertility is a widespread issue and, as already mentioned, the causes often remain unknown. Within the last decades various studies reported a constant decline in sperm quality and number (Carlsen et al., 1992, Auger et al., 1995, Lackner et al., 2005). Recently, Levine et al. (2017) presented statistical data of a decline in sperm count from men in Europe from 1973 till 2011 by approximately 50%. Looking ahead fearlessly, the data imply sterility of western societies within the next fifty years.

Putative causes are most likely self-inflicted: environmental influences, tobacco, alcohol, unhealthy diets and lack of physical exercise. Recently, the influence of microplastic or rather the widespread plasticizers e.g. Bisphenol A (BPA) on male fertility has drawn attention (Cariati et al., 2019) and needs further investigation concerning its reproductive toxicity.

A better understanding of the mechanisms of testicular aging to avoid congenital defects of the offspring for example is highly relevant. The trend to delay family planning and thus the impact of the paternal age on the offspring is of great common interest. As life expectancy increases, healthy aging is a major topic, also in terms of testicular aging. The effects of aging on the testis have attracted growing attention, since age-associated alterations entail comorbidities e.g. due to a lower testosterone level.

When discussing aging and male infertility in a larger context, it is illuminating to elucidate historical and social aspects as well as the biological importance. The perception about life and death has changed during human history. In former times dying "young" (from our point of view) was normal, due to rough living conditions and rudimentary medical practices. Throughout history, medical care improved and individual life span expanded. Thus, diseases could gradually be cured, attitudes to death have changed and death is no longer readily accepted (San Filippo, 2006). Till the end of the 19th century death was rather abrupt and often caused by acute infections (Stannard, 1975). Today, treatment options for chronic disease and life-sustaining techniques lengthen this process. The limited life expectancy was probably constant over some millenniums till industrial era and the onset of new insights in surgery, microbiology, immunology and pharmacy. Successively the global population became older (Morrison, 2005). Since 1950 the life expectancy in Europe raises from 64 to 78 years (United Nations, 2019). Human aging is becoming a problem in 21st century, the proportion of people older than 65 rises from approximately 7% to 20% at the middle of the 21st century worldwide (United Nations, 2019). Aging is now a severe risk factor for many diseases like e.g. cancer and neurodegenerative diseases. Within less than fifty years the life motto changed from “struggle for life” to “work for life” and nowadays to easy living (fun society). Consequently, the attitude to aging and death has changed fundamentally. Nowadays people want to become very old and instead of fear of death, there is fear of aging. The actual desire is “growing old without aging”.

From a biological perspective concerning aging, the evolutionary program is clear: aging, and in consequence death, are a requirement for keeping a population dynamic,

to adapt to environmental changes and optimizing the gene pool. For an individual, the inborn instinct of self-preservation (essential for survival and raising of offspring) stands in contrast to a limited lifespan for a population. It is a rather new aspect from a social point of view, that individual egoism has to be adapted to benefit a population (limited resources, climate change, regenerative energies). The ongoing elongation of lifespan due to medical advancements has its basis on the individual expectations. However, the trend to become parents later in life is burdened with increasing risk for offspring health. If this tendency is ongoing, plausible individual interests inevitably will come in conflict with ethical considerations. From a biological point of view, raising offspring requires parents, who remain mentally and physically fit till children reach autonomy.

The evaluation of male infertility concerning both, the individual and the population, is clearly a detriment for both. Its prevention has therefore a high priority. Although the main causes for sterile inflammation and environmental factors are known, the mechanisms remain unclear. The alarming increase of evidence of environmental factors being responsible for sub/infertility demands comprehensive investigation. It is expected that by radical reduction of any pollutant, infertility can possibly be prevented. The situation for infertility caused by sterile inflammation is more complex. Although the scenario has been known for several years, the discussion about its triggers is in part still speculative. However, the recent finding that accumulation of NLRP3 in testis of infertile patients could be responsible for sterile inflammation may offer new possibilities for diagnosis and therapy. Further investigations should concentrate on NLRP3 as putative target for specific drugs – a promising strategy.

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7 Appendix

7.1 Additional publication

Insights into the Role of Androgen Receptor in Human Testicular Peritubular Cells

Mayer C, Adam M*, Walenta L*, **Schmid N***, Heikelä H*, Schubert K*, Flenkenthaler F*, Dietrich KG*, Gruschka S, Arnold GJ, Fröhlich T, Schwarzer JU, Köhn FM, Strauss L, Welter H, Poutanen M, Mayerhofer A

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7.2 Scientific posters

Schmid N, Flenkenthaler F, Dietrich K, Fröhlich T, Arnold GJ, Behr R, Mayerhofer A. A non-human primate model for the study of testicular peritubular cells (Poster Award, 28. Jahrestagung der Deutschen Gesellschaft für Andrologie (DGA), Saarbrücken 09/2016)

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7.3 List of figures