Disturbed spermatogenesis associated with thickened lamina propria of seminiferous tubules is not caused by dedifferentiation of myofibroblasts

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Introduction

Male infertility is predominantly associated with impaired spermatogenesis and morphological alterations of the lamina propria (LP) of seminiferous tubules, such as thickening with accumulation of extracellular matrix (ECM) (Albrecht, 2009). In addition, tubules with massive increase in their diameter (dilatation) are also frequently characterized by disturbed spermatogenesis. The structural composition of the LP of these dilated tubules has not yet been described.

Myofibroblasts (‘peritubular cells’) represent the main cell type of the LP. These cells are large, flat cells, and their processes often split up into two or more lamellae. They are braced in a network of microfibrils and collagen fibrils in the tubular wall and arranged in discontinuous cell layers with interposed layers of ECM (Holstein et al., 1996). In contrast with rodents, where only a single layer of flat contractile (‘myoid’) cells occurs, in the human testis, there are three to five layers of myofibroblasts, each separated by ECM. One or two outer layers of fibroblasts form the external border of...
semifluorescent tubules (Davidoff et al., 1990; Holstein et al., 1996). The overall physiological thickness of the LP is <10 μm, although the precise thickness varies among investigators (Tonutti et al., 1960; Schütte, 1984).

In the human LP, myofibroblasts express α-smooth muscle actin (SMA), desmin, myosin heavy chain (MYH 11), GB 42, vimentin, CD90 as well as collagen I, IV, XVIII, fibronectin and laminin (Davidoff et al., 1990; Kohnen et al., 1995; Holstein et al., 1996; Albrecht et al., 2006; Schell et al., 2010). In addition, secretion of molecules with yet unknown function, such as monocoyte chemotactic protein-1, interleukin-6, nerve growth factor and glial-derived neurotrophic factor, has been described (Schell et al., 2008; Spinnler et al., 2010).

Peritubular myofibroblasts have been characterized as contractile cells (Roosen-Runge 1951; Davidoff et al., 1990; Albrecht, 2009). They are suggested to play a fundamental role in testicular sperm transport and in the release of spermatozoa from the germinal epithelium to the tubular lumen (Davidoff and Middendorff, 2000; Middendorff et al., 2000; Albrecht, 2009).

Motions of semifluorescent tubules were first described by Roosenerunge (1951) in rats and dogs. Contractility of isolated cells and tubules, respectively, has repeatedly been suggested in further species, including man, and molecules such as prostaglandin F2α, endothelin, angiotensin II, noradrenalin and oxytocin (Miyake et al., 1990). In further studies, thin sections, testicular biopsies were fixed in 5.5% glutaraldehyde in cacodylate buffer and embedded in low viscosity epoxy resin (Richard et al., 1990). For azan-anti-SMA-double staining, they were stained by the azan trichrome method according to Heidenhain (Kiernan, 2008) using azocarmine G followed by a solution containing orange G and aniline blue, providing dark red nuclei, light red cytoplasm and blue collagen fibres. For azan-anti-SMA-double staining, azan staining was performed following anti-SMA immunostaining (see above).

In elderly men, modification of regular morphology through myofibroblast to fibroblast transformation, with progression from the inner myofibroblast layers to the peripheral ones (Davidoff et al., 1990), and loss of contractile markers in thickened LP (Arenas et al., 1997) have been described.

It is not known, however, whether dedifferentiation of myofibroblasts is responsible for disturbed spermatogenesis associated with LP alterations. This question is addressed in the present study.

### Materials and Methods

#### Human testicular tissue

Testicular biopsy was indicated according to Bergmann and Kiescher 2010. After written informed consent, testicular biopsies were taken under general anesthesia. The reported study has been approved by the Ethic’s committee of the Medical Faculty of the Justus-Liebig University Gießen (decision 75/00 and 56/05). In detail, Bouin-fixed and paraffin-embedded tissue from 35 infertile men (cryptorchidism, n = 7; orchitis, n = 3; testicular feminization, n = 3; cancer of the prostate, n = 3; after vasectomy, n = 13, from the border area of seminomas, n = 3; from transsexuals, n = 3) was investigated. For seminithin and ultra-thin sections, testicular biopsies were fixed in 5.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 2 h and embedded in Epon 812. Frozen tissue was used for laser microdissection, RT–PCR and western blot analyses.

#### Immunohistochemistry

Either a combination of the peroxidase anti-peroxidase (Dako, Hamburg, Germany) and avidin–biotin peroxidase complex (Vector, Burlingame, CA, USA) method or the Envision kit (Dako) was used on paraffin sections. Peroxidase activity was visualized by the nickel-glucose-oxidase approach (Davidoff et al., 1995). Double staining was performed using the Envision-double staining kit (Dako). For negative controls, sections were used, in which primary antibodies were replaced by phosphate-buffered saline.

The following primary antibodies were employed: monoclonal mouse anti-SMA (Serotec, Düsseldorf, Germany, 1:100), -CD34 class II (Dako, 1:200), -CD90 (Thy-1, Dianova, Hamburg, Germany, 1:50) and -MYH 11 (Sigma, St Louis, MO, USA, 1:500), monoclonal rabbit anti-calponin-1 (Cal, Epitomics, Burlingame, CA, USA, 1:100) and polyclonal rabbit anti-SMA (Serotec, Düsseldorf, Germany, 1:100), -CD90 (Thy-1, Dianova, Hamburg, Germany, 1:50) and -PHosphodiesterase 5 (PDE5, Cell Signaling, Beverly, MA, USA, 1:200).

#### Azan staining and azan-anti-SMA-double staining

Paraffin sections were stained by the azan trichrome method according to Heidenhain (Kiernan, 2008) using azocarmine G followed by a solution containing orange G and aniline blue, providing dark red nuclei, light red cytoplasm and blue collagen fibres.

#### Morphometry

Generally, test probes, i.e. points, lines and planes, are projected to randomly sampled micrographs of a biological system. The number of test points hitting a structure of interest, the number of intersections with the boundary of a structure and the number of transsects of an object sampled in an unbiased counting frame are proportional to the volume, surface and length of the structure of interest, respectively (Mühlfeld et al., 2010). The resulting parameters are expressed as ratios or densities relating the volume, surface or length to a reference volume. These should be used to calculate the total values by multiplication with the reference volume whenever possible. In the case of human biopsies, only ratios...
can be obtained and need to be interpreted with care. Additionally, surface and length estimations actually require the use of isotropic sections, which was not the case here. However, with the contorted seminiferous tubules and the parameters investigated here, one can safely assume that anisotropy does not dominate the results.

Fields of view were obtained from 5 μm azan-SMA-double-stained tissue slices by systematic uniform random sampling and investigated morphometrically with the newCAST system (Visiopharm, Horsholm, Denmark). A grid of line segments was projected onto the fields of view. Using standard morphometry procedures (Weibel, 1979), we estimated the volume fractions of the tubule lumen (including the epithelium) and LP in relation to total tubule volume, the volume fractions of myofibroblasts and ECM in relation to LP volume and the arithmetic mean barrier thickness of the LP and its components.

Moreover, spermatogenesis and qualitative morphological classification (LP group, see below) of individual tubules were investigated and later on correlated to each other, to the thickness of LP components and to the tubular diameter. To analyse significant differences between LP groups, the Kruskal–Wallis ANOVA and the Mann–Whitney U-test were used.

The thickness of LP in cross-sectioned seminiferous tubules >10 μm was defined as ‘thickened LP’ according to the literature (Christl et al., 1988). Seminiferous tubules displaying a diameter >400 μm in cross-sections were defined as ‘dilated tubules’.

**Laser capture microdissection and RT–PCR**

For laser capture microdissection (LCM), the PALM Robo Microlaser System (Carl Zeiss MicroImaging, Munich, Germany) was used. Cryosections of 10 μm were mounted on RNase-free PALM membrane slides (Carl Zeiss), stained for 3 min in haematoxyline, dehydrated in ascending concentrations of ethanol and air-dried for 10 min.

Individual areas of human tissue (regular LP, thickened LP, lung tissue) were microdissected and catapulted into sterile oil-covered caps of 0.5 ml tubes. In every single cap ~50–60 chippings from LP were collected. Human lung tissue served as a positive control. For RNA isolation, chippings were dissolved in RLT buffer (Qiagen, Hamburg, Germany) with 10% β-mercaptoethanol and frozen at −20°C. Total RNA was extracted from individual caps using the Qiagen RNeasy micro kit employing DNase on column digestion. Subsequently, total RNA was reverse transcribed using MuLV reverse transcriptase (Gene Amp RNA PCR kit, Applied Biosystems, CA, USA). PCR amplification was performed on a Mastercycler gradient (Eppendorf, Hamburg, Germany) in a final volume of 25 μl. PCR mixture consisted of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 10 mM dNTPs, 1.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 0.4 μM/μl of exon spanning oligo-dT PDESA primers (5’TCTA TTCTGGGTGCTTTGTGTA-3′, forward and 5’TGGTCTTTTGT GTCTTGAGGTTGTA-3′, reverse; fragment of 245 bp). SMA-specific primers (5’TGT TCA CCA TCA TCA GTT GC-3′, forward and 5’AGG GCC AG GAA GAC TA GG-3′, reverse) were used as positive controls for contractile cells, resulting in a 198 bp fragment. β-Actin served as a housekeeping gene (5’TGT GTC CCA TCC TCG GTA AG-3′, forward and 5’GAG TAC TGG TTC GCA GGA GAG-3′, reverse; fragment of 199 bp). Human testis RNA without MuLV reverse transcriptase and water were used as negative controls. The samples were denaturated for 12 min at 95°C, followed by 45 cycles consisting of 30 s at 95°C, 20 s at 59°C and 30 s at 73°C. Finally, PCR samples were kept at 73°C for 7 min and stored at 4°C. Amplified products were separated by electrophoresis on 2% agarose gels.

### Protein preparation and immunoblotting

Isolation of seminiferous tubules and testicular blood vessels as well as preparation of cytosolic and membrane proteins were performed as described previously (Müller et al., 2004).

After separation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis under reducing conditions, proteins were transferred to nitrocellulose membranes as described previously (Müller et al., 2004). After staining with Ponceau S (P7170, Sigma) and blocking (Müller et al., 2004), blots were exposed to antibodies directed against SMA (Serotec, I:7500), soluble guanylyl cyclase β1 (sGC, Cayman, I:5000), PKG I (Calbiochem, I:1000) or PDE5 (Cell Signaling, I:2000). Following incubation with goat anti-mouse or anti-rabbit IgG, linked to peroxidase (Pierce, Rockford, IL, I:2000), immunoreactive bands were visualized by enhanced chemiluminescence.

### Results

#### Regular morphology of the peritubular LP of the human testis

Normally, the LP of the human testis (Fig. 1) comprises 5–7 cell layers with interposed ECM (Fig. 1A–D). The latter could be visualized in semithin (Fig. 1B and C) and azan-stained paraffin sections (Fig. 1D, blue colour). Most of the peritubular cells represent myofibroblasts characterized by the expression of both smooth muscle cell (SMA, Fig. 1E) and fibroblast (CD90, Fig. 1F) marker proteins as described before (Albrecht et al., 2006). In the outermost layers, true fibroblasts are also present. These cells were characterized as SMA-negative cells (Fig. 1G, asterisk) in azan-stained sections additionally treated with anti-SMA antibodies. This newly developed staining procedure allowed the simultaneous detection of SMA-positive cells (black cytoplasm), SMA-negative cells (red cytoplasm) and ECM (blue staining), and thus their distribution pattern in detail.

The vast majority of tubules with normal LP (91.3%, see below) display intact spermatogenesis (Fig. 1B), whereas a regular LP is rarely found in tubules with impaired spermatogenesis (Fig. 1C, <10%, see below).

#### Presence of myofibroblasts in each tubule with thickened peritubular LP

An involvement of the myofibroblast cell population in the development of germ cell defects is conceivable especially in tubules with thickened LP (Figs 2 and 3) and dilated tubules (Fig. 4). First, seminiferous tubules with thickened LP were investigated (Figs 2 and 3). The presence and localization of myofibroblasts and their relationship to ECM were characterized using semithin sections (Fig. 2B, E and H) and azan-anti-SMA-double staining (Fig. 2C, F and I). All tubules, even with severe alterations of the LP, displayed SMA-positive cells within the LP (Fig. 2C, F and I). Smooth muscle cells of testicular blood vessels, taken as an internal positive control, were also SMA-immunonegative (Fig. 2C, arrow). The germinal epithelium, Sertoli and Leydig cells were immunonegative. Comparison of SMA (Fig. 2J) and CD90 (Fig. 2K) staining revealed that contractile cells represent myofibroblasts also in tubules with thickened LP.
Three different types of thickened LP

Focusing on the relationship between the myofibroblast cell population and ECM, three groups of morphological alterations, Groups 2–4, were categorized (Fig. 2). Intact LP morphology (Fig. 1) was defined as Group 1. Categories are exclusively based on morphological alterations of the LP and are independent of potential disturbances in spermatogenesis.

Group 2 (Fig. 2A–C): Group 2 (scheme, Fig. 2A) contains tubules displaying slight accumulation of ECM in between a regular network of SMA-positive myofibroblasts (Fig. 2C). Overall, it comprises changes, which are not classified into Groups 1, 3 or 4.

Group 3 (Fig. 2D–F): the LP of tubules, which comprises three layers, is categorized as Group 3 (scheme, Fig. 2D). Here, the LP consists of two rings of myofibroblasts and one layer of ECM of variable thickness between them. The first layer of myofibroblasts is attached to the germinal epithelium, whereas the second one borders the tubule towards the interstitium. Staining intensity of myofibroblasts for SMA did not differ from Group 1 (Fig. 1).

Group 4 (Fig. 2G–K): in comparison to Group 3, Group 4 (scheme, Fig. 2G) lacks the inner ring of myofibroblasts. The germinal epithelium is followed by ECM (Fig. 2I), which is surrounded by one ring of myofibroblasts, characterized by SMA- (Fig. 2J) and CD90- (Fig. 2K) immunoreactivity at the outer surface. ECM is of variable thickness among individual tubules of this group (Fig. 2H–K).

In summary, myofibroblasts were found in all groups of structural alterations of the LP. Seminiferous tubules without myofibroblasts in the LP could not be detected. Electron microscopic investigations confirmed LP changes and the defined groups (data not shown).

Pattern of LP alterations is independent of the clinical background

The pattern of changes of the LP was independent of the clinical background of testicular alterations. All patients showed fibrotic changes of all subtypes of thickened LP. There were only differences in the relative number of the single groups described. For example, tubules displaying pathological alterations of LP Groups 3 and 4 were more often found in patients with cryptorchidism than in vasectomized patients.

However, the structure of LP changes gradually. In the case of testicular feminization, for example, testes of 1-year-old children, different to those of adults, are without any thickening of the LP, although the molecular defect is already evident (data not shown).

Myofibroblasts express proteins indicating their functional integrity

To get further hints on the functional integrity of these myofibroblasts, we extended the investigations of the contractile cell-specific structural protein SMA (Fig. 3A–C) to explorations of MYH 11 and Cal, known as markers for differentiated contractile cells (Owens et al., 2004; Li et al., 2009). MYH 11 (Fig. 3D–F) and Cal (Fig. 3G–I) were found in peritubular myofibroblasts of all groups and in vascular smooth muscle cells. MYH 11-negative LP was also detectable in some tubules of certain cases (data not shown). Lack of this staining, however, was independent of the LP group and disturbances of spermatogenesis and might be due to the treatment of testicular tissue after surgery prior to fixation or by insufficient fixation of inner tissue segments.

In addition, components of the relaxation-mediating cGMP pathways, which were shown to be of special importance for functional activity of contractile cells (Middendorff et al., 1997; 2002; Mewe et al., 2006a,b) were investigated. In particular, PKG I (Fig. 3J–L), which mediates the cGMP-dependent relaxation, and PDE5 (Fig. 3M–O), which degrades cGMP and thereby controls the duration of cGMP action, were studied. In addition to vascular smooth muscle
cells (data not shown), myofibroblasts of the peritubular LP (Fig. 3J–O) were PKG I- and PDE5-immunopositive. PDE5-immunoreactivity was also present in Leydig cells (Fig. 3M, arrow).

SMA-, MYH11-, Cal-, PKG I- and PDE5-immunoreactivity were shown in all groups of the LP (Groups 1/2: Fig. 3A, D, G and M; Group 3: Fig. 3B, E, H and N, Group 4: Fig. 3C, F, I, L and O).

In agreement with immunohistochemical (IHC) data, RT–PCR analyses revealed PDE5 transcripts both in laser-microdissected regular (Fig. 3P) and thickened LP (Fig. 3Q). In addition, western blot analyses showed protein expression of SMA, PKG I, PDE5 and the cGMP-generating sGC (Fig. 3R) in isolated seminiferous tubules and testicular blood vessels.
Functional integrity of the peritubular LP of dilated tubules

All dilated tubules (diameter ≥ 400 μm in cross-sectioned tubules) investigated displayed impaired spermatogenesis. Azan staining and SMA-IHC (Fig. 4A and B) revealed a regular structural composition of the LP. In agreement with the results for tubules with thickened LP, myofibroblasts of dilated tubules displayed no changes in the expression of marker proteins for differentiated contractile cells, such as SMA (Fig. 4B), MYH 11 (Fig. 4C), Cal (Fig. 4D), PKG I (Fig. 4E) and PDE5 (Fig. 4F), suggesting that disturbances of myofibroblast function...
might not be responsible for impaired spermatogenesis in the dilated tubules.

**LP morphology correlates with spermatogenesis**

Next, we were interested in the relationship of the morphology of LP and germ cell epithelium. First, the ratio of the diameter of the lumen (incl. epithelium) and LP thickness was examined in tubules of individual LP groups and compared with one another (Fig. 5A). The diameter of seminiferous tubules is known as a useful parameter in pathology and clinical andrology, indirectly indicating the quality of spermatogenesis (Holstein et al., 1988). The diameter (lumen)/LP ratio steadily decreased from Groups 1 to 4.

In agreement, the percentage of tubules with intact spermatogenesis decreased from Groups 1 to 4 (Fig. 5B). In Group 1, 91.3% of the tubules showed intact spermatogenesis, i.e. only a minority of tubules with intact LP displayed disturbances of germ cell development. In Group 2, about 55.6%, in Group 3, 46.3% and in Group 4, only 5.8% of tubules comprised intact spermatogenesis (Fig. 5B).

Thus, the LP group might be an indicator for the probability of intact spermatogenesis.

**In LP of Group 4, which displays highest correlation to disturbed spermatogenesis, thickness of LP, ECM and myofibroblast layers shows maxima**

To obtain deeper insight into morphologically defined LP groups, the thickness of LP as well as of ECM and myofibroblast layers was analysed by stereological investigations in LP Groups 1–4 each and compared between individual groups.

The overall thickness of the LP differed significantly between all LP groups. From Groups 1 to 4, the thickness of LP and ECM was steadily
increasing (Fig. 6A). The thickness of myofibroblast layers also differed between Groups 1 and 4. Surprisingly, Group 4 comprises the thickest myofibroblast layers (Fig. 6A). Hence, increase in LP thickness is based on both increase in ECM and myofibroblast thickness.

Myofibroblasts of the thickened LP as newly developed cells?

Next, we wanted to obtain information on the origin of the myofibroblast cell population, since myofibroblasts are present in all tubules investigated, and the thickness of myofibroblast layers especially increases in the strongest changes of LP structure (Fig. 6A, Group 4). It was of interest whether these myofibroblasts represent delocalized members of the original myofibroblast population of the LP or whether they represent newly developing myofibroblasts originating either from cell proliferation or (as found in fibrosis of other organs) from interstitial fibroblasts and extra-testicular stem/progenitor cells, respectively (Epperly et al., 2003).

To address the question of proliferation in areas of thickened tubular LP, expression of the proliferation marker proliferating cell nuclear antigen (PCNA) was analysed (Fig. 6B and C). As expected, PCNA-immunoreactivity was detectable in dividing germ cells. PCNA-positive cells, however, were barely detectable within intact or thickened LP. Interstitial tissue in the neighbourhood of thickened LP was also without any increase in PCNA staining. Thus, local cell proliferation might not be responsible for thickened myofibroblast layer of the LP.

To evaluate the potential origin of myofibroblasts of the thickened LP from outside the LP, CD34 (Fig. 6D and E), indicating haematopoietic stem cells and progenitor cells in lung fibrosis (Epperly et al., 2003), was used as a marker. CD34+ cells were found both surrounding the LP directly (Fig. 6D and E) and spreading across the interstitial space (Fig. 6D). Co-localization of SMA and CD34 in LP cells, however, was barely detectable (Fig. 6E). In contrast, direct proximity of SMA+ and CD34+ cells was found in the neighbourhood of each tubule. These data could favour CD34+ cells, as precursor cells of newly developing tubular myofibroblasts.

Discussion

The present study shows: (i) that myofibroblasts are present and fully differentiated in the LP of all seminiferous tubules; (ii) that there are three different types of thickened LP showing increasing pathology; (iii) that the pattern of LP alterations is independent of the clinical background; (iv) that LP morphology correlates with spermatogenesis; (v) that in the group with thickest LP, the thickness of myofibroblast layers shows a maximum; (vi) and that there is evidence for newly developed myofibroblasts in thickened LP.

Morphological alterations of the LP of seminiferous tubules are generally accepted to be associated with impaired spermatogenesis (Schell et al., 2010). The simultaneous demonstration of myofibroblasts and ECM by combination of a classic histological technique and IHC analyses in combination with stereological methods allowed us to reveal different categories of LP alterations, which show a gradual increase in pathology from Groups 2 to 4 but regularly comprise myofibroblasts.

The degree of LP pathology correlates well with disturbances of spermatogenesis allowing us to use LP structure as an indicator for the degree of germ cell defects. Germ cell defects are found in <10% of tubules with intact LP (Group 1), whereas intact spermatogenesis can be nearly completely excluded in tubules of LP Group 4. In agreement, it was recently suggested that the thin LP might be one of the best indicators of complete spermatogenesis (Sato et al., 2008). Searching for irregularities of the myofibroblast cell population, we detected myofibroblasts in the LP of all seminiferous tubules of 

![Figure 5](https://academic.oup.com/humrep/article-abstract/26/6/1450/2914538/1457)
Arenas et al. to our initial hypothesis, there was no hint on dedifferentiation of myo-Sertoli cell-only syndrome, myofibroblasts could be found. In contrast even in tubules with severe morphological alterations. Even in testicular biopsies of infertile men with impaired spermatogenesis, additional studies would be helpful to verify that myofibroblast function is sustained in its cellular environment.

Thickening of LP was found to be independent of the clinical background of testicular alterations and can be suggested as a general predictive factor for disturbed spermatogenesis in agreement with Sato et al. (2008). In the past, a series of investigations were performed to search for correlations between thickened LP and well-defined disturbances, such as cryptorchidism, varicocele or Klinefelter’s syndrome (Paniagua et al., 1990; Martin et al., 1992; Santoro et al., 2000), suggesting that thickening of the LP might be a specific characteristic for one of these disturbances. We studied tubules of infertile patients with cryptorchidism, orchitis, testicular feminization, carcinoma of the prostate or after vasectomy, and tubules in the border line of seminomas as well as tubules from transsexuals. Fibrotic LP changes, occurring in all clinical groups, were revealed as a common pathological reaction for one of these disturbances. We studied tubules of infertile patients with cryptorchidism, orchitis, testicular feminization, carcinoma of the prostate or after vasectomy, and tubules in the border line of seminomas as well as tubules from transsexuals. Fibrotic LP changes, occurring in all clinical groups, were revealed as a common pathological reaction pattern of the testis rather than a disease-specific one.

In the human testis, the cGMP-degrading PDE5 could be localized for the first time. Beside peritubular cells and testicular blood vessels, the enzyme is present in Leydig cells comparable to the rat (Scipioni et al., 2005). Therefore, chronic treatment with PDE5 inhibitors, such as sildenafil (Viagra®), as used in patients suffering from pulmonary hypertension (Galie et al., 2005; Ghofrani et al., 2006), might affect myofibroblast and Leydig cell function. In latter cells, cGMP

testicular biopsies of infertile men with impaired spermatogenesis, even in tubules with severe morphological alterations. Even in Sertoli cell-only syndrome, myofibroblasts could be found. In contrast to our initial hypothesis, there was no hint on dedifferentiation of myofibroblasts into fibroblasts, as suggested by Davidoff et al. (1990) and Arenas et al. (1997), who described that in specimens of elderly patients, myofibroblasts dedifferentiate to fibroblasts. In contrast to these data, myofibroblasts were found in all tubules and even attached to the basement membrane in tubules with thickened LP of Groups 2 and 3. Thus, loss of myofibroblasts in seminiferous tubules with thickened LP is not a cause of their impaired spermatogenesis.

In all tubules examined, including dilated tubules and all subtypes with thickened LP, myofibroblasts express contraction- and relaxation-associated proteins in the LP. Especially, MYH 11 and Cal, established markers for differentiated contractile cells (Li et al., 2009; Schell et al., 2010), were found beside SMA in all subtypes of thickened LP. Demonstration of these proteins was completely independent of the quality of spermatogenesis in the same seminiferous tubule. In agreement with Schell et al. (2010), we sometimes found loss of MYH 11 staining in (some regions of) our sections from men with impaired spermatogenesis. But, this loss of MYH 11-immunoreactivity did not correlate with LP groups or the quality of spermatogenesis in individual tubules, suggesting that methodical reasons were responsible. It had been shown previously that the quality especially of human testicular sections greatly depends on the treatment of human testis specimens between surgery and fixation (Feng and Holstein, 1990).

In addition to these proteins, enzymes of cGMP pathways, which had been previously shown to be of relevance for relaxation of contractile cells in the male reproductive tract (Middendorff et al., 1997; 2002; Meuw et al., 2006a,b), were regularly found. Detailed analyses of the localization of PDE5, shown to slow down spontaneous contractions of isolated seminiferous tubules in rat (unpublished results), and PKG I revealed these proteins in all groups of LP. In myofibroblasts of seminiferous tubules, these enzymes are expressed in addition to molecules, known to mediate seminiferous tubule contraction, such as endothelin and PGF 2a (Miyake et al., 1986; Yamamoto et al., 1987; Tripiciano et al., 1996, 1998, 1999). Thus, neither loss of myofibroblasts nor loss of function might be responsible for impaired spermatogenesis. Despite preserved contractility of individual myofibroblasts, lack of tubular contraction due to increase in ECM remains to be elucidated. In dilated tubules with impaired spermatogenesis, additional studies would be helpful to verify that myofibroblast function is sustained in its cellular environment.

**Figure 6** Thickness, proliferation and potential origin of the myofibroblast layers of LP. (A) Morphometric investigation of the thickness of LP (dark blue), of myofibroblast layers (black) and of ECM (light blue) in LP Groups 1–4. The thickness of ECM and LP significantly differed among all individual LP groups, resulting in a gradual increase towards Group 4. The thickness of myofibroblast layers significantly differed between 1 versus 4, 2 versus 4 and 3 versus 4 (a–i, m, n: P ≤ 0.001; j, k, l, P ≤ 0.05). (B and C) PCNA immunostaining, indicating cell proliferation, in seminiferous tubules with regular and thickened (see asterisks) LP. Staining was nearly exclusively found in germ cells. (D and E) IHC demonstration of CD34, marking haematopoeitic stem cells and progenitor cells (red staining) together with SMA (brown staining) in seminiferous tubules with thickened LP (collagen layers are indicated by asterisks, D: Group 3, E: Group 4). Bars = 12 μm.
pathways have been repeatedly described to modulate testosterone secretion (Middendorff et al., 2000).

In mouse models of peritubular cell-specific knockouts of the androgen receptor, produced by two different approaches, oligozoospermia (Zhang et al., 2006) and azoospermia (Welsh et al., 2009), respectively, were described. The latter study showed that beside disturbances of Sertoli cell-specific gene expression and formation of the basement membrane, reduced expression of SMA and the smooth muscle cell-specific intermediate filament protein desmin, especially in peritubular cells. Thus, defects in contractility of these cells could have contributed to the germ cell defects found in this model. Our studies indicate, however, that patients suffering from testicular feminization, who lack androgen effect, and transsexuals pre-treated by estrogens display no differences in myofibroblast localization and protein expression compared with other patients. This argues against a negative effect of androgen depletion (or imbalance of androgen and estrogen) on myofibroblast function in man.

Thickening of the LP of seminiferous tubules describes accumulation of collagen fibres and further extracellular components, which is referred to as fibrosis and well described in other organs, e.g. the lung, liver, heart and kidney (Dempsey et al., 2006; Rosenblom et al., 2010). In the lung, some consistent pathological events of pulmonary fibrosis at cellular and molecular levels are established. In general, dysregulated epithelial–mesenchymal communication might represent the start point in the development of fibrosis (Thannickal et al., 2004). Alveolar epithelial cells display increased apoptosis, dysregulated proliferation and ineffective migration. Afterwards, these changes affect the surrounding fibroblasts. They ‘differentiate’ to myofibroblasts, show increasing resistance to apoptosis, enhanced ECM secretion, increased migration in early stages and potentially increased proliferation (Thannickal et al., 2004). In testicular tissue, initial germ cell defects could represent the first step in the development of fibrotic changes, followed by alternations of the surrounding LP. Therefore, it cannot be ignored that thickening of the LP could develop secondary to impaired spermatogenesis. Alternatively, both the decrease in spermatogenesis and thickening of the LP may have a common cause, i.e. decreased gas (O2) transport and/or increased oxidative damage. In this context, findings of vasculature-associated niches for undifferentiated spermatogonia (Yoshida et al., 2007) and of hypoxic conditions (Fonseca et al., 2011) as well as changes of microvasculature (Strieter and Mehrad, 2009) early in fibrosis are of special importance.

Since myofibroblasts remain detectable in the LP of all tubules analysed, it seemed likely, at first glance, that these cells, in cases of thickened LP, represent the original population of cells, which only became delocalized by the increase in ECM. The increase in thickness of myofibroblast layers especially in heavily damaged LP suggests that these cells are, at least in part, newly developed cells as described for pulmonary fibrosis. Evidence for increased cell proliferation, which is hypothesized for pulmonary fibrosis, however, was not visible within or in the neighbourhood of the LP, neither in tubules with regular or thickened LP. But myofibroblasts of the LP could stem from transforming growth factor β-activated (Rosenblom et al., 2010) fibroblasts (of the LP or the interstitium) or from cells of extra-testicular origin. Cells of haematopoetic origin, characterized by the expression of the membrane-bound glycoprotein CD34, are presumed to contribute to the development of myofibroblast cell population in fibrosis (Hashimoto et al., 2004). Epperly et al. (2003) revealed that 20–50% of cells in fibrotic foci in irradiation-induced pulmonary fibrosis are bone marrow-derived cells. In the adult testis, CD34+ cells were described in interstitial tissue (Kuroda et al., 2004) and isolated testicular CD34+ cells are used for stem and progenitor cell culturing, facilitating the generation of multipotent stem cells (Kim et al., 2008). Some of these cells, however, might represent testicular mast cells, known to be CD34+, too (Metcalfe, 2008). Our data indicate localization of CD34+ cells in close proximity to thickened LP, suggesting that these cells, which might be derived from the haematopoetic lineage, are one source of myofibroblasts of thickened LP. In consideration of these elucidations, it seems possible that the above-described systems of one and two rings of functionally active myofibroblasts must be seen as an active system fed by infiltrating cells rather than a fixed structure developed only by the rearrangement of original LP components.

Authors’ roles

J.V. did most of the morphological experiments including stereology and wrote the paper together with R.M. D.M. performed western blot experiments. C.F. performed LCM and RT–PCR. M.B. and S.K. chose the testis biopsies suitable for this study. C.M. organized the stereological studies. R.M. conceptualized and directed the project and wrote the paper together with J.V.

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