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REGULAR ARTICLE

The cell–cell junctions of mammalian testes. III. Absence of an endothelial cell layer covering the peritubular wall of the seminiferous tubules—an immunocytochemical correction of a 50-year-old error in the literature

Werner W. Franke[1] · Lisa M. Domke[1] · Yvette Dörflinger[1] · Ralf Zimbelmann[1]

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Abstract

In the molecular biological and ultrastructural studies of the peritubular wall cells encasing the seminiferous tubules of mammalian testes, we found it necessary to characterize the outermost cell layer bordering on the interstitial space in detail. For half a century, the extremely thin cells of this monolayer have in the literature been regarded as part of a lymphatic endothelium, in particular in rodents. However, our double-label immunofluorescence microscopical results have shown that in all six mammalian species examined, including three rodent ones (rat, mouse, guinea pig), this classification is not correct: the very attenuated cells of this monolayer are not of lymphatic endothelial nature as they do not contain established endothelial marker molecules. In particular, they do not contain claudin-5-positive tight junctions, VE-cadherin-positive adherens junctions, "lymph vessel endothelium hyaluronan receptor 1" (LYVE-1), podoplanin, protein myozap and "von Willebrand Factor" (vWF). By contrast and as controls, all these established marker molecules for the lymphatic endothelial cell type are found in the endothelia of the lymph and—partly also—blood vessels located nearby in the interstitial space. Thus, our results provide evidence that the monolayer cells covering the peritubular wall do not contain endothelial marker molecules and hence are not endothelial cells. We discuss possible methodological reasons for the maintenance of this incorrect cell type classification in the literature and emphasize the value of molecular analyses using multiple cell type–specific markers, also with respect to physiology and medical sciences.

Keywords Vascular endothelia · Lymphatic vessels · Cell junctions · Cell type markers · Immunocytochemistry

This article is written in respect of the cell biology work of Donald W. Fawcett, who certainly would have been the first to correct his former conclusions based only on the electron microscopical appearance of the testicular histology of rodents.

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✉ Werner W. Franke
w.franke@dkfz.de

Lisa M. Domke
l.domke@dkfz.de

Yvette Dörflinger
y.doerflinger@dkfz.de

Ralf Zimbelmann
r.zimbelmann@dkfz.de

[1] Helmholtz Group for Cell Biology, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

Abbreviations

AJ(s)	Adherens junction(s)
ECM	Extracellular matrix
LSMC(s)	Lamellar smooth muscle cell(s)
PW	Peritubular wall
SM	Smooth muscle
SMC(s)	Smooth muscle cell(s)
ST(s)	Seminiferous tubule(s)

Introduction

Historically, cell types and tissues have long been given names based on morphological or putative functional aspects or with the use of the name of the assumed discoverer. In the original microscopical studies of testicular tissues, the peritubular wall (PW) cells encasing the seminiferous tubules (STs) have been given a variety of terminological names of

which "myoid" has apparently been the mostly used one (for a recent report showing that these cells even represent cells of a novel kind of smooth muscle (SM) tissue, the "lamellar smooth muscle cells" (LSMCs), see Domke and Franke 2019). However, very early on a special exception was introduced and has been repetitively asserted in the literature: the outermost monolayer of very thin and broad cells covering the PW and bordering on the interstitial space has been widely referred to—in particular for rodent testes—as part of a "lymphatic endothelium," i.e., an endothelial cell layer, thus contributing to the formation of a special category of "lymphatic vessels" (e.g., Fawcett et al. 1969, 1970, 1973; Dym and Fawcett 1970; Dym 1975, 1988, 1994; Clark 1976; Connell 1976; Wrobel et al. 1979, 1981; Söderström 1981; Hadley and Dym 1987; Maekawa et al. 1996; Yazama et al. 1997; Losinno et al. 2012, 2016). To demonstrate the constant reiteration and wide distribution of this concept of a lymphatic endothelial cell layer covering the PW, we present a historical selection list of 15 examples in a Supplementary Literature and Document Collection (SLDC; Online Resource 1). This classification as "lymphatic endothelium" has also special weight as the supporters include some of the widely known lymphatic vessel experts (e.g., Leak and Burke 1968; Fawcett et al. 1969, 1970; Leak 1970, 1971, 1976; Holstein et al. 1979; Weiss 1983). Only very few authors have named these cells "fibrocytes" or "fibroblasts" but also without any specific structural or molecular evidence (e.g., Bressler and Ross 1972). To clarify this cell type, we felt obliged to examine and characterize the cells of this layer using established markers for the lymphatic endothelial cell type.

Material and Methods

Tissues and antibodies

Testes of animals of six mammalian species (man, bull, boar, rat, mouse and guinea pig) were used as described for snap-frozen tissue samples or chemically fixed tissues in previous publications (see, e.g., Domke et al. 2014; Domke 2018; Domke and Franke 2019). For controls, the following tissues were used in parallel: STs and testicular excurrent ducts, liver, intestine, tongue mucosa, esophagus, heart and bladder.

Antibodies used for immunoblot analyses of polypeptides separated by gel electrophoresis as well as for immunofluorescence microscopy are listed in Tables 1 and 2 of Domke and Franke (2019) as well as in Table 6 of Domke (2018). Antibodies used in particular for the identification or exclusion of possible endothelial marker molecules and special other typical endothelial components are listed in Table 1 of our present article. For the antibody binding methods applied, see also Domke et al. (2014), Domke (2018) and Domke and Franke (2019). For certain aspects, we have also used serial ultrathin sections in tomography analyses.

Results

Electron microscopy

In all species studied, the STs (seminiferous tubules; *tubuli seminiferi*) were tightly encased by the PW structure consisting of monolayers of polyhedral, rather flat LSMCs, on both sides associated with layers of extracellular matrix (ECM) structures, dominated by collagen filament bundles oriented parallel to the specific ST (denoted *C* in Fig. 1a, d). In the different species examined, the number of such LSMC monolayers varies between one to five or six (in rodents, mostly one). In general, this PW appears to be surrounded by an additional, very attenuated monolayer of polyhedral cells in which SM structures are usually not detectable. Figures 1, 2, and 3 present in cross-sections the electron microscopical appearance of PW structures in different mammalian species (Fig. 1a: bovine testis; for an example of an anomalously thin boar testis PW with only one to two LSMC layers, see Figs. 1b, 2 and 3 rodents).

In all species examined, the cells of this outermost layer are extremely flat and broad, down to 10–25-nm inner membrane-to-membrane distance (see, e.g., Fig. 1b), i.e., in large regions even more attenuated than the LSMC regions described in our recent paper (Domke and Franke 2019). In Figs. 2 and 3, this is specifically shown for testes of two rodent species, i.e., rat and mouse. Here, the outermost cell layer shows remarkable regional differences of the course and ECM material associations. In some regions, it is rather closely associated with the subjacent ECM layer but there are also regions in which variously sized parts of the uppermost layer are widely dissociated from the subjacent ECM and protrude into the interstitial space (e.g., Figs. 2c–e and 3c–e). From our electron microscopic studies, it is also clear that rather large regions of this outermost cell layer are not stably attached to an ECM structure and also do not bear a distinct apical extracellular glycocalyx (Figs. 1, 2, and 3). Electron microscopy of serial ultrathin sections has further shown that these outermost, very attenuated cells commonly do not contain *caveolae* structures and that their basal plasma membrane is not attached to other cells or ECM structures via tangles of "anchoring filaments," i.e., two ultrastructural criteria characteristic for several types of lymphatic vessels (for various subtypes and for lymphangiogenesis, see, e.g., Leak and Burke 1968; Leak 1970, 1976; Daróczy 1988; Jussila and Alitalo 2002; Luttun et al. 2004; Baluk and McDonald 2008; Karpanen and Alitalo 2008; Bruyère and Noël 2010).

Table 1 Antibodies used for the identification of endothelial cells in lymphatic or blood vessels

Antigen	Type, clonality	Source	References
N-cadherin (cadherin-2, CDH2)	mcl, m (cl. 32)	BD Transduction Laboratories, 610921	Nose and Takeichi 1986; Takeichi 1990; Navarro et al. 1998
	pcl, rb	QED Biosciences, 42031	
VE-cadherin (cadherin-5, CDH5)	pcl, rb	Cayman Chemical Company, 160840	Lampugnani et al. 1992, 1995; Dejana et al. 2000
	mcl, m (cl. BV9/1B5)	Kindly provided by Elisabetta Dejana (University of Milan, Italy)	Hämmerling et al. 2006; Giannotta et al. 2013
Smooth muscle α-actin	mcl, m (cl. 1A4/ASM-1)	Progen Biotechnik, 61001	Skalli et al. 1986
	pcl, rb	Abcam, ab5694	
Desmin	mcl, m (cl. D9)	Progen Biotechnik, 10519	van Muijen et al. 1987
	mcl, m (cl. DE-R-11)	Dako Agilent, M0724	
	pcl, rb	Progen Biotechnik, 10570	
β-Catenin	mcl, m (cl. 14)	BD Transduction Laboratories, 610154	Ozawa et al. 1990
	mcl, rb (cl. E247)	Abcam, ab32572	
Plakoglobin	mcl, m (cl. PG 5.1.7.2)*	Progen Biotechnik, 65105	Cowin et al. 1985a, b, 1986; Franke et al. 1987
	mcl, m (cl. PG 11E4)	Kindly provided by Prof. Dr. Margaret J. Wheelock (University of Nebraska, Omaha, NE, USA)	
	mcl, m (cl. 15)	BD Transduction Laboratories, 610253	
	pcl, gp (cl. GP57)*	Progen Biotechnik, GP57	
Protein p120	mcl, m (cl. 98/pp120)	BD Transduction Laboratories, 610134	Reynolds et al. 1994, 1996; Iyer et al. 2004
	pcl, rb	Sigma-Aldrich, P1870	
Protein p0071	mcl, m (cl. SEPP 7.7.9)*	Progen Biotechnik, 651165	Hofmann et al. 2008, 2009
	pcl, gp (cl. GP71)*	Progen Biotechnik, GP71	
Plakophilin-2	mcl, m (cl. 2-518)*	Progen Biotechnik, 651167	Rickelt et al. 2010
	mcl, m (cl. MIX-CM -62,-86,-150)*	Progen Biotechnik, 651101	Mertens et al. 1996
	pcl, gp (cl. GP-PP2)*	Progen Biotechnik, GP-PP2	
	pcl, rb	Acris Antibodies, APO1493PU-N	
	pcl, gp (cl. SR2A)*	Authors' laboratory	
Protein myozap	mcl, m (cl. 517.67)*	Progen Biotechnik, 651169	Rickelt et al. 2011 Pieperhoff et al. 2012
	pcl, gp (cl. GP2A/B)*	Authors' laboratory	
Protein CD34	mcl, m (cl. QBEnd/10)	Progen Biotechnik, 16109	Fiedler et al. 2006; Nielsen and McNagny 2008
	pcl, rb (cl. N2C3)	GeneTex, GTX111632	
Desmoplakin I+II	mcl, m (cl. Mix-2.15, -2.17, -2.20)*	Progen Biotechnik, 65146	Franke et al. 1981, 1982; Cowin et al. 1985b
	pcl, gp (cl. Gp495)*	Progen Biotechnik, DP-1	Koeser et al. 2003
Desmoglein-2	pcl, rb (cl. rb 5)*	Progen Biotechnik, 610121	Schäfer et al. 1994, 1996
	mcl, m (cl. 6D8)	Thermo Fisher Scientific, 32-6100	
	pcl, goat	R&D Systems, AF947	
	mcl, m (cl. 10G11)*	Progen Biotechnik, 61059	Koeser et al. 2003
	mcl, m (cl. 141409)	R&D Systems, MAB947	
Claudin-5	mcl, m (cl. 4C3C2)	Thermo Fisher Scientific, 35-2500	Morita et al. 1999
	mcl, m	Zytomed, 18-7364	Vorbrodt and Dobrogowska 2004
Occludin	mcl, m (cl. OC-3F10)	Thermo Fisher Scientific, 33-1500	Furuse et al. 1993
	mcl, rat (cl. MOC37)	Kindly provided by Profs. Drs. Shoichiro and Sachiko Tsukita (Kyoto/Osaka, Japan)	Saitou et al. 1997
	mcl, m, Alexa 488	Thermo Fisher Scientific, 33-1500	Moroi et al. 1998

Table 1 (continued)

Antigen	Type, clonality	Source	References
	pcl, rb	Abcam, ab31721	
	pcl, rb	Zymed Laboratories, San Francisco, CA, USA	Vorbrodt and Dobrogowska 2004
LYVE-1 (lymph vessel endothelium hyaluronan receptor 1)	pcl, rb	Acris Antibodies, DP3500PS	Banerji et al. 1999; Prevo et al. 2001; Podgrabinska et al. 2002; Ji et al. 2007
Podoplanin	mcl, m mcl, m (cl. 18H5)	Dako, Aligent, M3619 Acris Antibodies, DM3500P	Schacht et al. 2005; Cîmpean et al. 2007; Ji et al. 2007; Noda et al. 2010; Suzuki et al. 2015
Factor VIII (von Willebrand factor)	mcl, m	Dako, Agilent	Hämmerling et al. 2006; Moll et al. 2009
Vimentin	mcl, m (cl. 3B4)*	Progen Biotechnik, 61013	Franke et al. 1978, 1979
	pcl, gp*	Progen Biotechnik, GP53	

mcl monoclonal, *pcl* polyclonal, *gp* guinea pig, *m* mouse, *rb* rabbit

*Originally from Authors' laboratory

Double-label immunofluorescence microscopy

When cryostat sections through testicular tissues of bulls, boars, or men were immunostained for two different specific antigens, their entire PWs were intensely positive for SM cell type marker molecules such as desmin, SM α-actin and smoothelin as were the vascular SM walls, in some places also for vimentin (for some examples see, e.g., Figs. 4a–d and 5; see also Domke and Franke 2019). By contrast, the endothelial structures of all testicular blood and lymph vessels, located nearby in the interstitial space, were the only positive cells for the specific endothelial marker molecules used such as claudin-5, VE-cadherin[1], protein LYVE-1 and podoplanin (Figs. 4, 5, and 6) as well as protein myozap and von Willebrand factor VIII. These lymphatic endothelia were mostly very thin or appeared—in grazing sections—as extended structures (e.g., Figs. 4a and 5a). In cross-sections through rather small lymphatic vessels, near-colocalizations were seen for different endothelial junction markers that are known to be closely located to each other (see, e.g., Fig. 5b–b‴ for claudin-5 and VE-cadherin in yellow merger color), in certain lymphatic endothelial regions even "completely" colocalizing (see, e.g., also Baluk et al. 2007). No immunostaining of endothelial markers was seen along—and in—cells of the specific outermost monolayer of the PWs (for an example of more widely reacting molecules see below). This general difference of intense SM protein positivity in PW cells on the one hand and distinct endothelium-specific marker labelling of vascular endothelia on the other was especially striking in some sections through porcine testes that showed a very high frequency of blood as well as lymph vessels in the interstitial space (Fig. 6).

The same pattern of immunostaining results has been found for all three rodent species examined. Essentially no endothelial marker reactions have been detected in the outermost layers of the PWs but only in the separate—generally much smaller—lymph and blood vessels in the interstitial space (Fig. 7 presents examples of rat testis after reactions with claudin-5 and VE-cadherin; for a contrasting report in murine testis see, e.g., Hirai et al. 2012). Figure 8 shows differences of intensities between blood and lymph vessels. While small lymph vessels are very positive for endothelial markers, here claudin-5, they are mostly negative for SM markers (see, e.g., the SM α-actin reaction in Fig. 8a–a″). Another frequent view is that seen in the cross-sectioned vessels of Fig. 8b–b″. As both tight and adherens junctions (AJs) are located side-by-side, the limited light microscopic resolution often suggests colocalization, as also indicated by the small structures showing yellow merger color for claudin-5 and β-catenin (for similar observations, see also Baluk et al. 2007). By contrast, a far-reaching vessel wall versus endothelium reaction is seen for claudin-5 and desmin in Fig. 8c–c″.

In principle, the immunostaining results are the same in the rodents examined as in the other mammalian species. Figure 9 shows, for example, in a guinea pig testis, relatively wide ST lumina with very narrow interstitial regions containing only rather small vascular structures that, however, are fully positive for the endothelial markers used. And in Fig. 10a–a‴, an extended section through a rather large part of a human testis is seen, showing a lymphatic vessel with an endothelium positive for both podoplanin (red) and LYVE-1 (green), here accompanied by some extremely small vascular structures positive for either one or the other.

[1] VE-cadherin has also been noted—often only in local cell groups or colonies—in certain kinds of other cells (Boda-Heggemann et al. 2009).

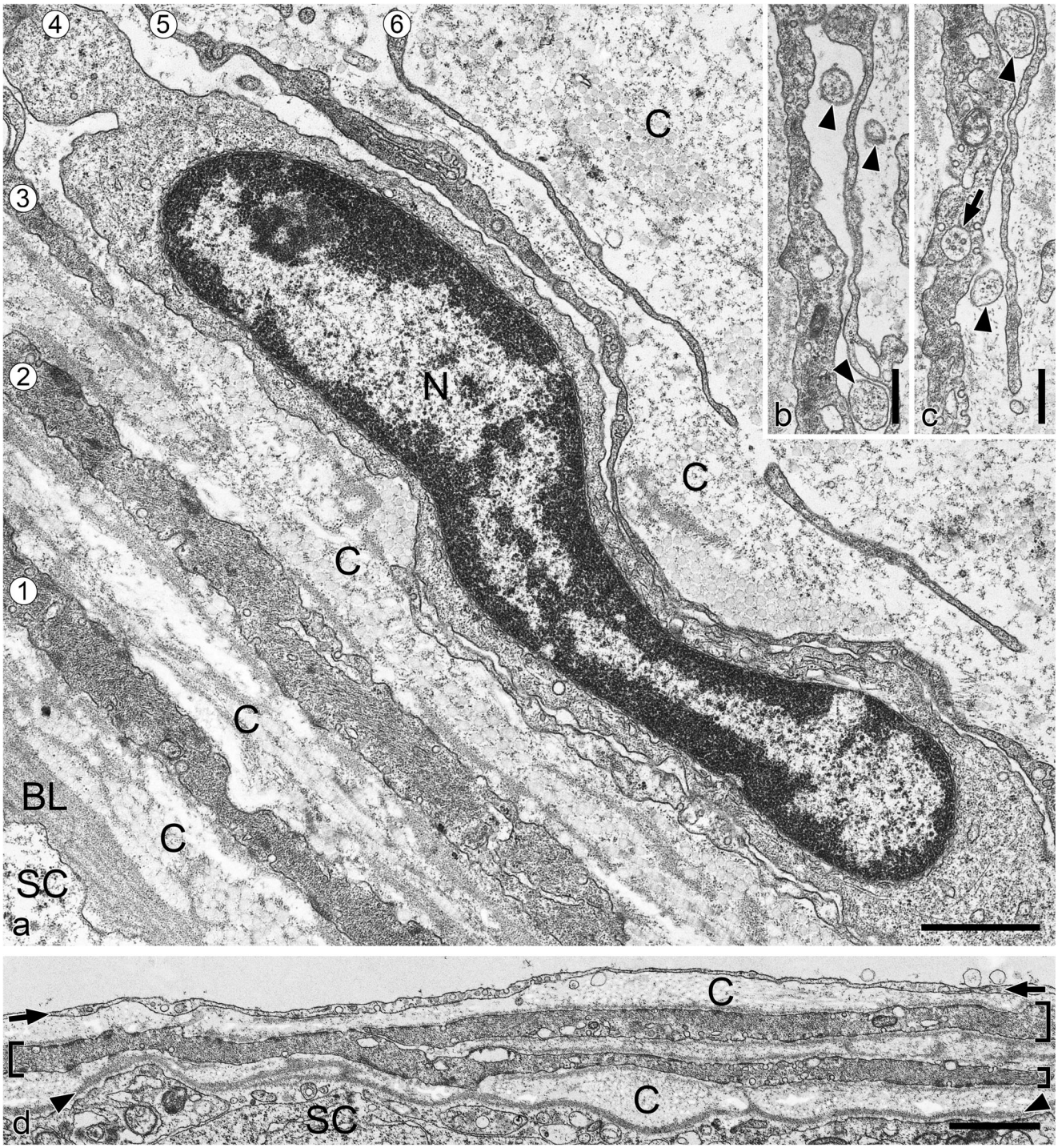
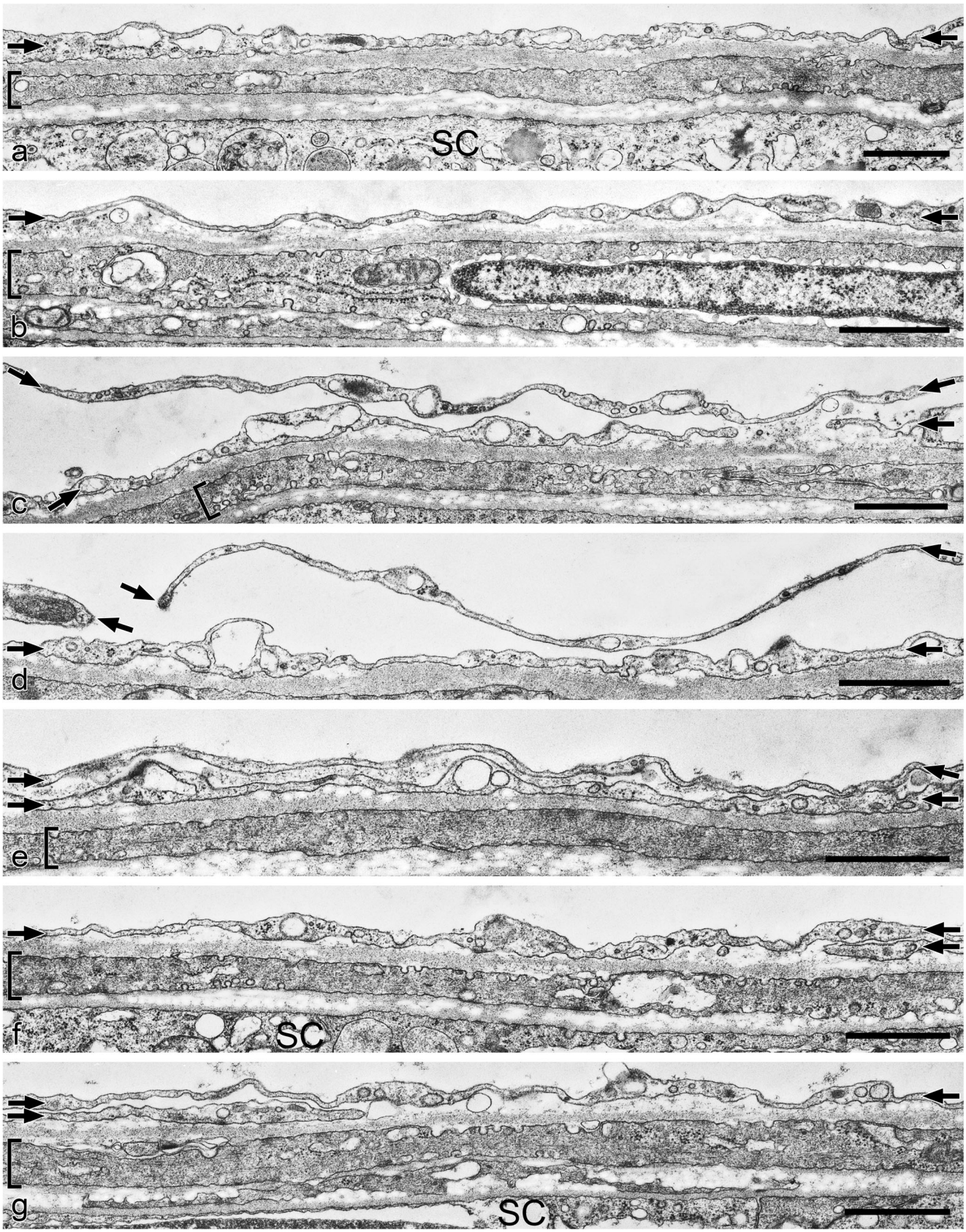


Fig. 1 Electron micrographs of ultrathin cross-sections through the peripheral regions of seminiferous tubules (ST) of bull (**a**, **b**, **c**) and boar (**d**) testes, showing the peritubular wall (PW) and its lamellar smooth muscle cell (LSMC) layers (nos. 1–6 in **a**; denoted by brackets in **d**), interspersed with layers of collagen (C)-rich extracellular matrix (ECM) the first of which is attached to the basal lamina (BL; denoted by arrowheads in **d**) associated with the Sertoli cells (SC). Note that in such sections, the cells of the outermost layers are very attenuated (labelled nos. 5 and 6 in **a** and denoted by arrows in **d**; see also **b** and **c**) revealing a very wide lamellar or very thin filopodial shape. For special details of the latter see the insert figures **b** and **c** in which such tubular or filopodial cell protrusions are denoted by arrowheads; the arrow in **c** denotes a very thin intracytoplasmic membrane-surrounded structure containing extremely small vesicles. N, nucleus. Bars 1 μm (**a**, **d**), 500 nm (**b**, **c**)

As already reported in previous publications, endothelial AJs are also markedly positive for protein myozap (e.g., Pieperhoff et al. 2012) as well as for proteins p120 and plakoglobin (for reviews, see Franke et al. 1987, 1988; Franke 2009; for endothelial structures and functions, see also, e.g., Iyer et al. 2004) and these proteins have also been


a
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◀ **Fig. 2** Electron micrographs of ultrathin sections through the outer attenuated lamellar cells of PWs surrounding STs of rat testes, showing the specific monolayers of the very thin outermost cells (arrows), followed by a layer of ECM material, a single monolayer of LSMCs (brackets; note the densely packed myofilament bundles) and another ECM layer associated with the basal lamina and Sertoli cells (SC). Note the very large and very attenuated outermost cell layer regions and their locally variable protrusions into the interstitial space. N, nucleus. Bars 1 μm

found in the AJs of the LSMC monolayers of PWs (see, e.g., Domke and Franke 2019). Again, we have not discovered them in the outermost PW-associated layer. Finally, desmoplakin-positive *complexus adhaerens* structures as they occur in certain special endothelial tissues (e.g., Schmelz and Franke 1993; Schmelz et al. 1990, 1994; Valiron et al. 1996; Kowalczyk et al. 1998; Ebata et al. 2001; Hämmerling et al. 2006; Pfeiffer et al. 2008; Moll et al. 2009) have not been identified in any type of testicular blood or lymph vessel.

Special controls using antibodies against proteins CD34 and β-catenin

Immunofluorescence and immunoelectron microscopy for the detection of cytoskeletal and cell–cell junctional

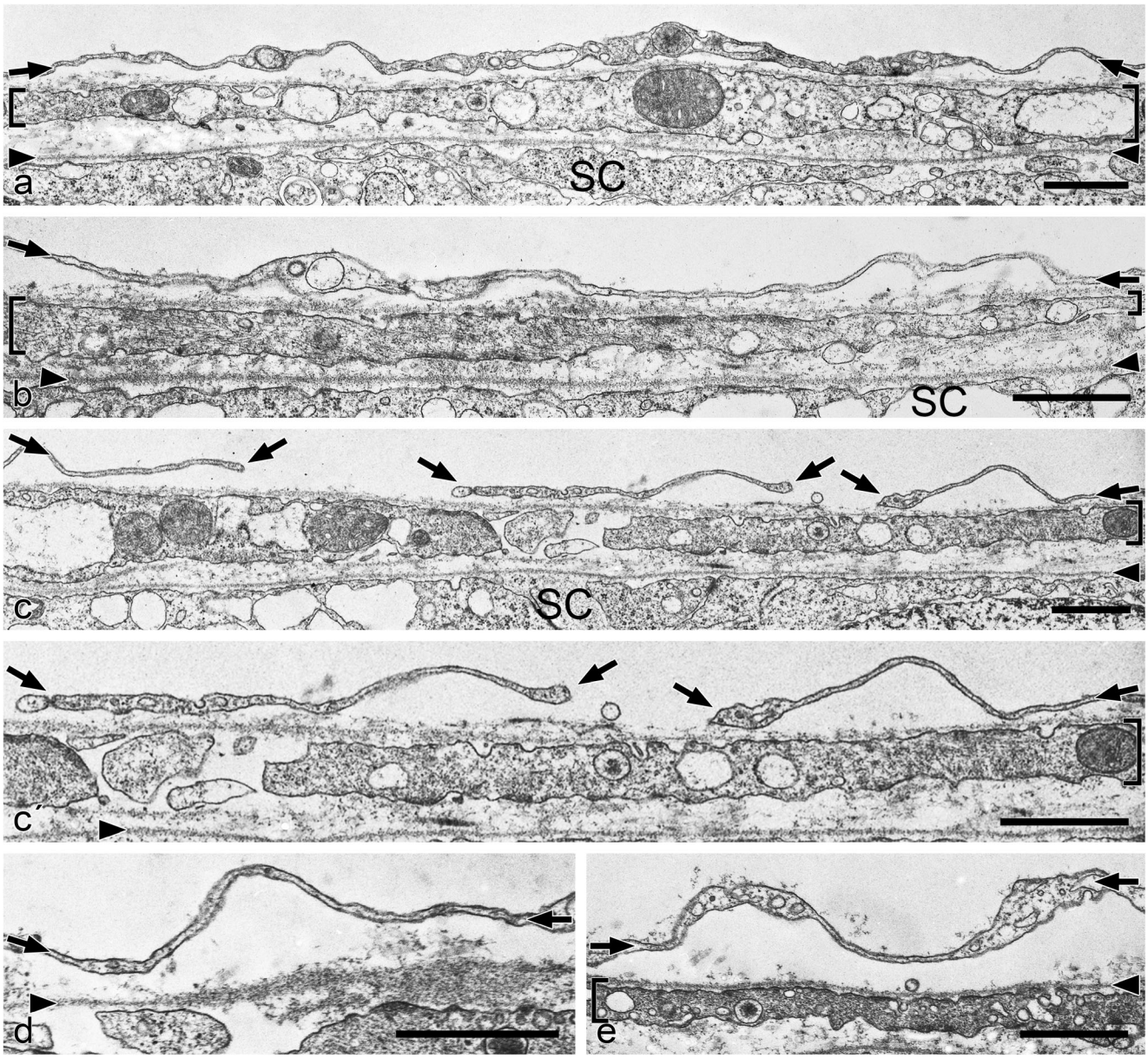

Fig. 3 Electron micrographs of ultrathin sections through mouse testicular tissue, showing details of the LSMC monolayers (brackets) and the thin cell outermost monolayers (arrows) of the PW encasing the STs (SC, Sertoli cell). Note also the interspersed, relatively thin ECM layers and the basal lamina associated with the Sertoli cells (denoted by arrowheads). Note also the two forms of the thin lamellar cells, i.e., the LSMCs and the very attenuated outermost monolayer cells with regions protruding into the interstitial space. Bars 1 μm

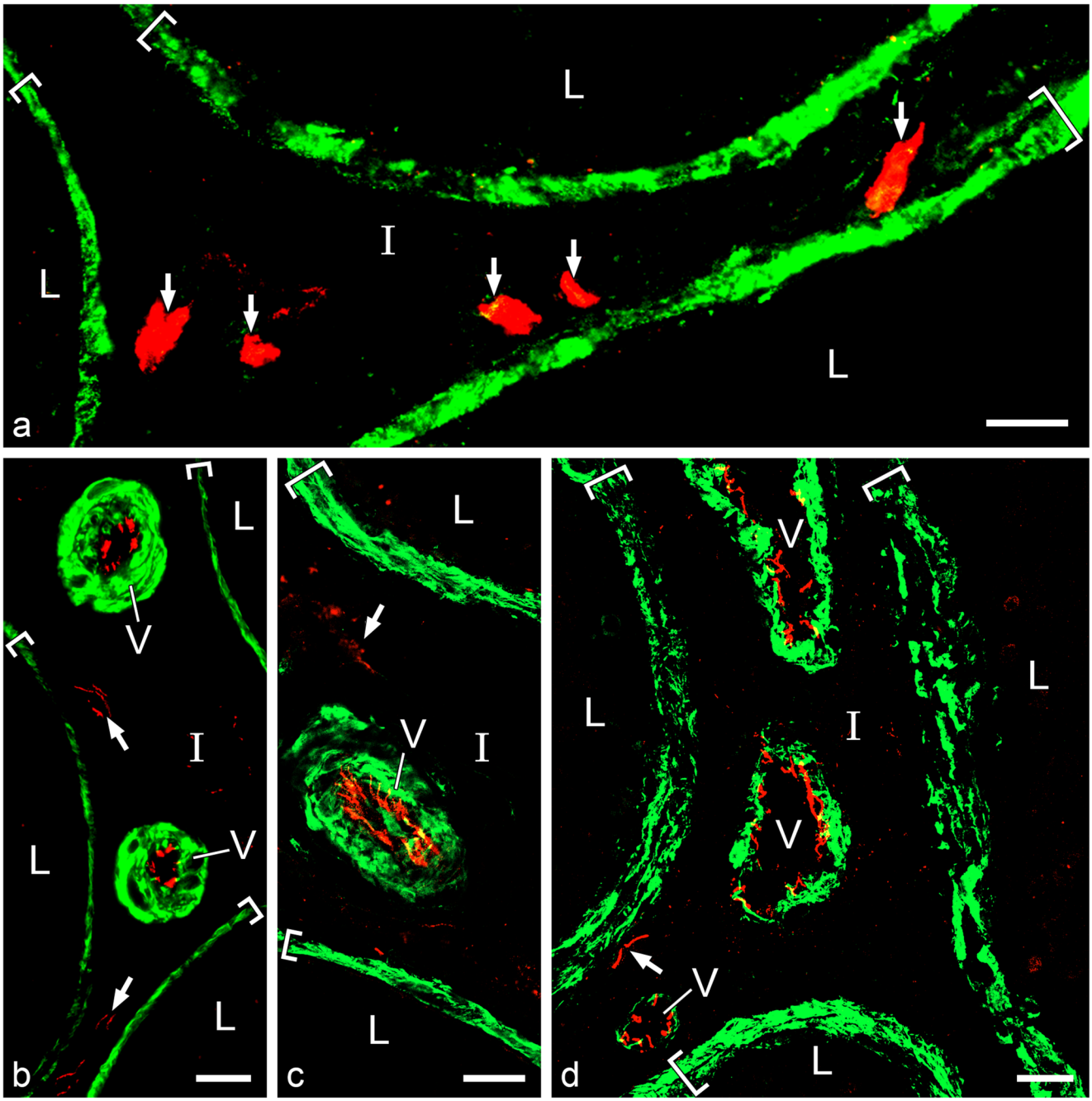

Fig. 4 Double-label immunofluorescence microscopy of cryostat cross-sections through STs of frozen bull (**a**–**c**) and human (**d**) testes. Immunostaining reactions are shown with antibodies against the smooth muscle (SM) cell markers desmin (**a**; green) and smooth muscle α-actin (α-SMA, **b**–**d**; green), in comparison with the endothelial cell markers claudin-5 (**a**; red) and VE-cadherin (**b**–**d**; red). The peritubular LSMCs as well as the cells of the blood vessel (V) walls in the interstitial space (I) are positive for the SMC markers but negative for the blood and lymph vessel endothelial markers that, on the other hand, are positive on all vascular endothelial cells, including compact and small SMC-free small lymphatic vessels (some are denoted by arrows). Note that the entire peritubular LSMC walls on their outer wall show an absence of the endothelial marker reaction. L, lumen. Bars 20 μm

molecules in both endothelial as well as a wide range of mesenchymally derived cell types also gave the expected positive localization reactions in the endothelial cells of the blood and lymph vessels as described in previous publications (Franke et al. 1978, 1979, 1987, 1988; Pieperhoff et al. 2012). As an example, we show here the reaction of protein CD34 (for a relevant review, see Fiedler et al. 2006) in the outermost layer of the PWs but not in the SMCs (Fig. 10b–b‴). A similar but much weaker reaction was seen with antibodies to β-catenin.

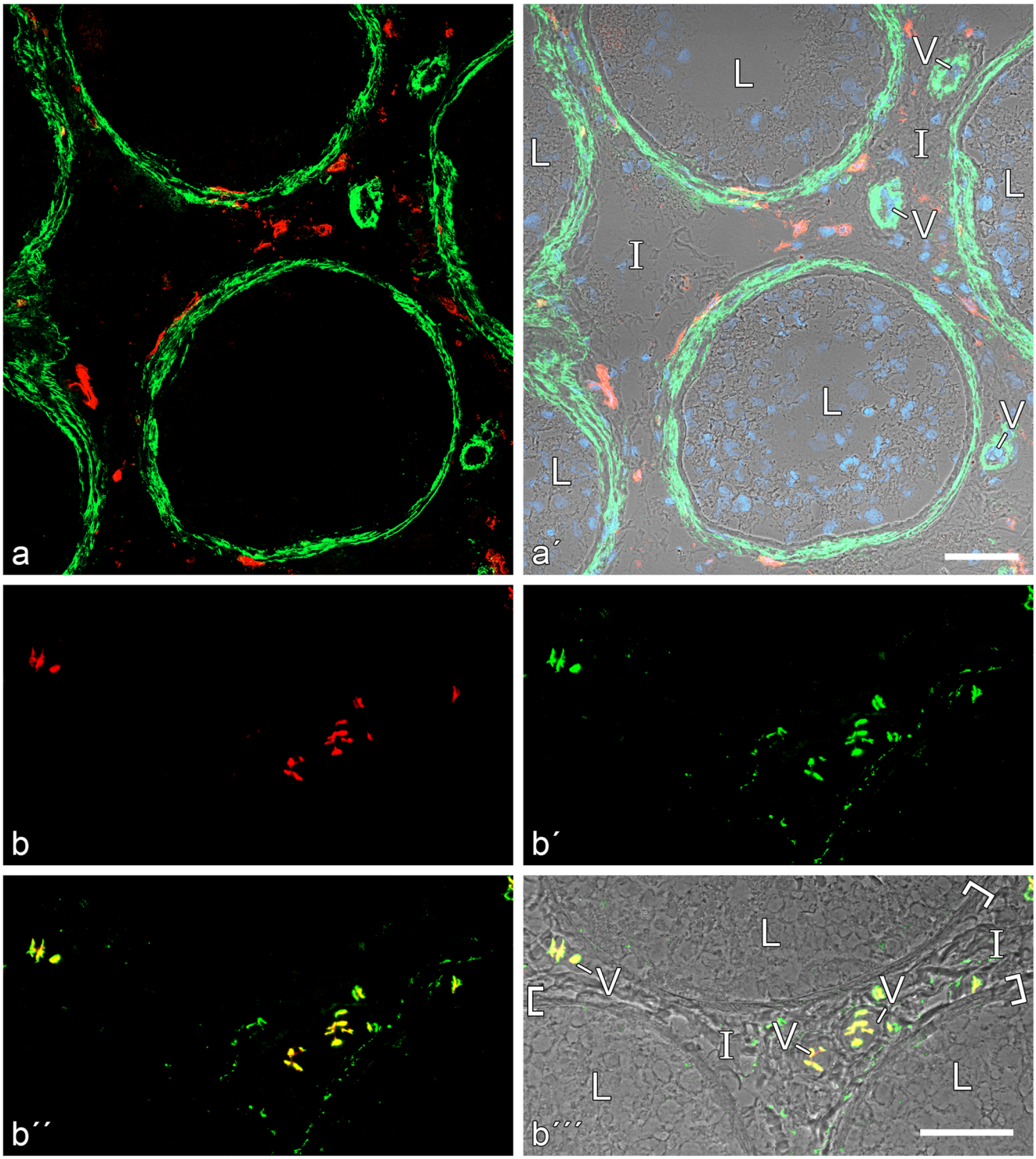

Fig. 5 Double-label immunofluorescence microscopy of cryostat cross-sections through STs of frozen human (**a**, **a′**) and bovine (**b, b‴**) testes (**a′** and **b‴** present the specific reactions on a phase contrast background). Immunostaining reactions of peritubular LSMCs (brackets) as well as blood vessel (V) walls are shown for the SMC marker smooth muscle α-actin (α-SMA, **a**, **a′**; green). In comparison, the vascular endothelial junction markers LYVE-1 (**a**, **a′**; red), claudin-5 (**b**, **b″**, **b‴**; red) and VE-cadherin (**b′**, **b‴**; green) react with the endothelium of the blood vessels (V) and lymph vessels (red contours and punctate reactions). Note that even the smallest lymphatic vessels are positive for the endothelial markers but that Sertoli and spermatogonial cells are totally negative for both. Note also the near-colocalization of both endothelial junction markers. L, lumen; I, interstitial space. Bars 50 μm

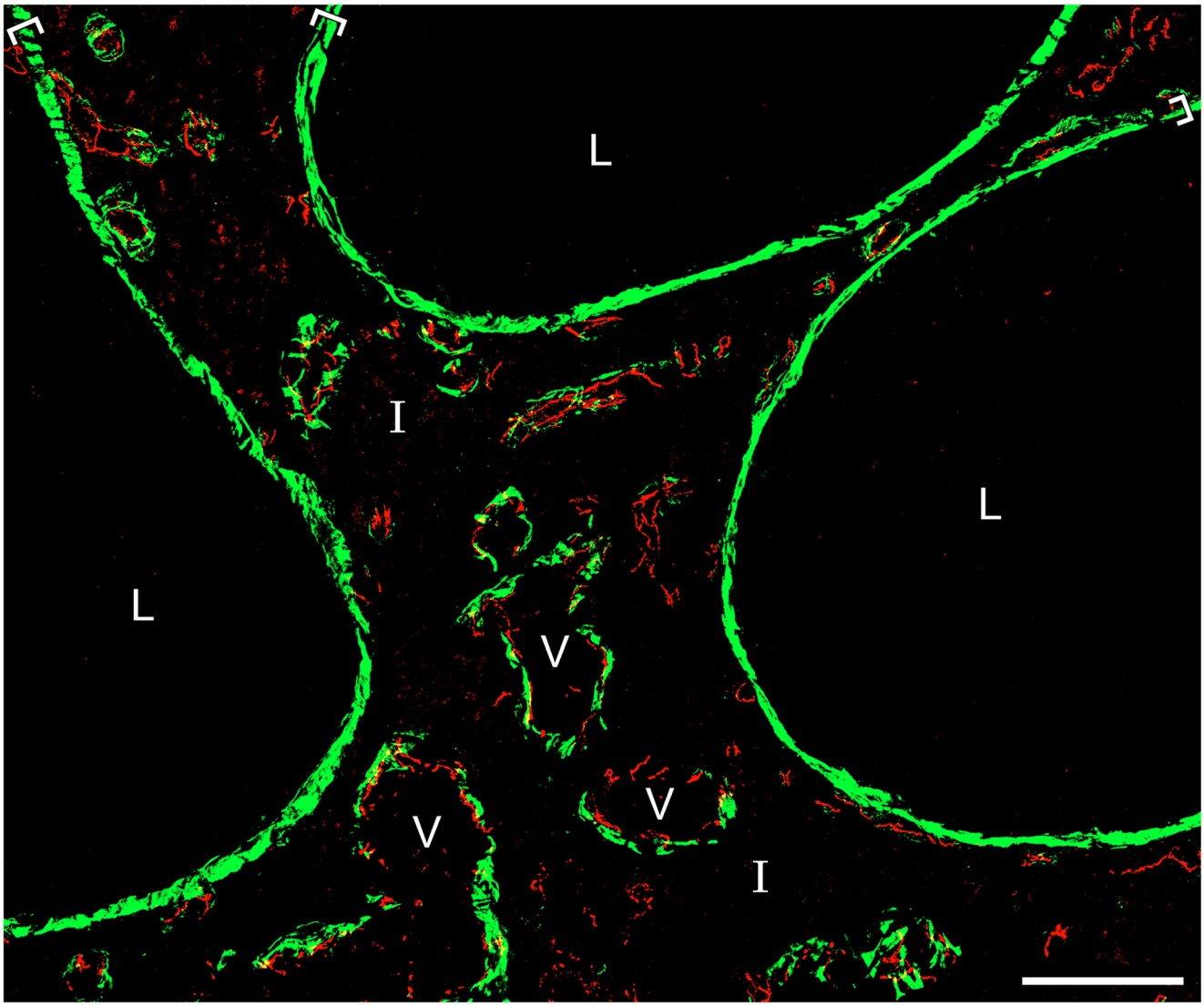

Fig. 6 Double-label immunofluorescence microscopy of a cryostat cross-section through STs of frozen boar testis, showing immunostaining with antibodies against VE-cadherin (red) and smooth muscle α-actin (α-SMA; green). The vascular endothelial marker again visualizes specifically cell–cell adherens junctions (AJs) of the numerous blood vessels (some are denoted by V) and the endothelial cell structures of the numerous small lymphatic vessels (purely red structures). Peritubular LSMC walls (denoted by brackets) and vascular walls are totally negative for VE-cadherin but totally positive for α-SMA. L, lumen; I, interstitial space. Bar 50 μm

Discussion

The clear conclusion of this study is that the very attenuated cells of the outermost layer associated with the PW of the STs of rodents and other mammalian species are not cells of a lymphatic endothelium and are not even endothelium-like cells in ultrastructural and molecular terms. As we have directly controlled our results by positive reactions of the endothelial cells located in the adjacent blood and lymph vessels in the interstitial space, the significance of our negative conclusion is obvious, for rodent testes as well as for all other mammalian species examined. The cells of this outermost PW-associated monolayer are also remarkable as they represent—to the best of our knowledge—in large parts the thinnest lamellar cells of the mammalian body in situ (11–25-nm inner cytoplasmic thickness), i.e., similar to the 9–11-nm inner cytoplasmic membrane-to-membrane thickness in the most attenuated cultured cells shown so far (Franke et al. 1978; for extended, very thin cell protrusions—often also with cell–cell junctions of the AJ type—in certain mammalian tissues see also Barth et al. 2009, 2012).

Consequently, it is also clear that for conclusions like this, the identification of cell type–specific major architectonic molecules by immunolocalization in situ is decisive, if based on reliably good antibodies. As such, immunolocalization-suitable antibodies have been developed only in the last few decades, earlier tissue and cell type nomenclature has been based on the appearance, position, or assumed function. The

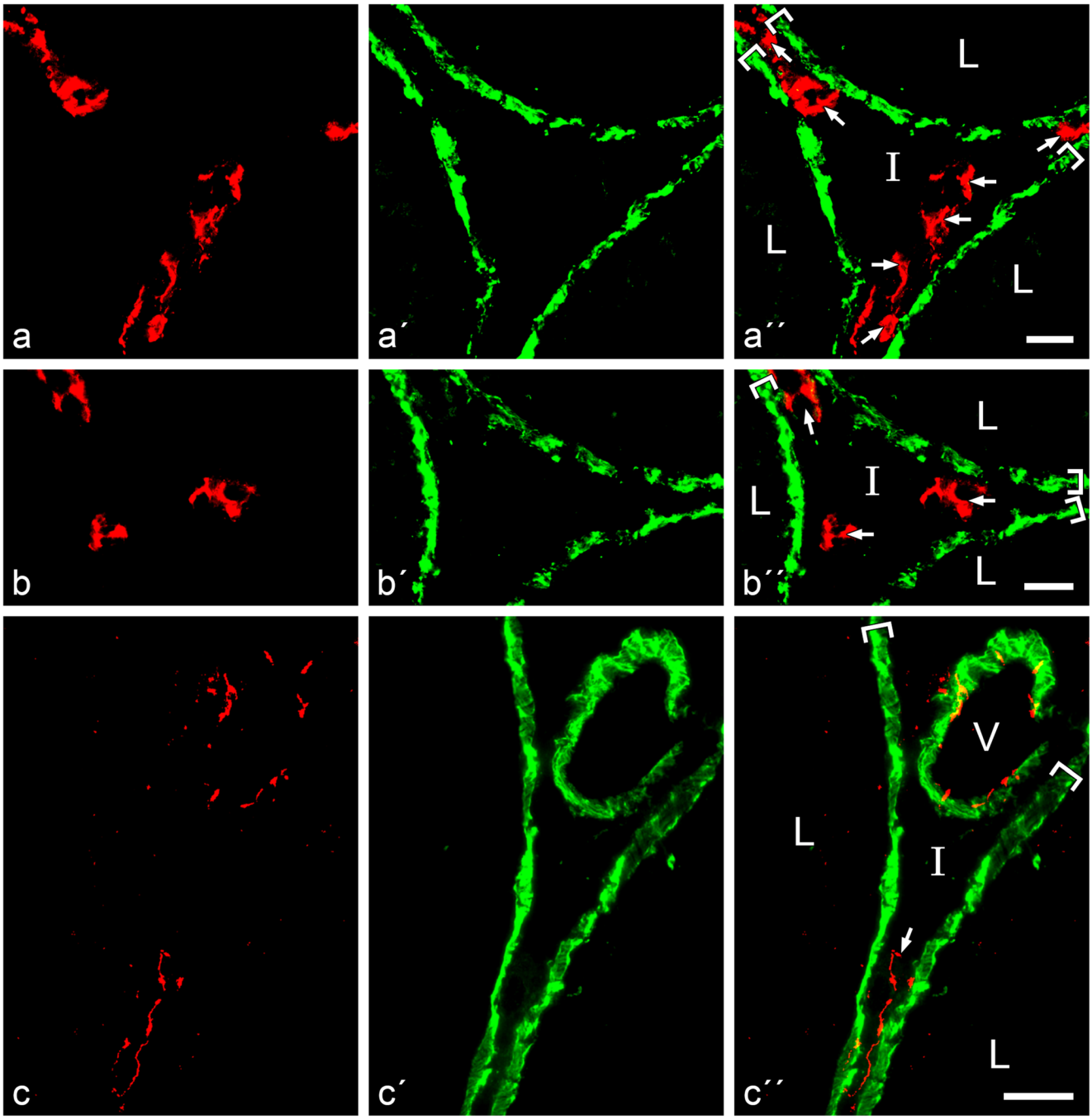

Fig. 7 Double-label immunofluorescence microscopy of cryostat cross-sections through STs of rat testis. Immunostaining reactions with antibodies to claudin-5 (**a**, **a″**, **b**, **b″**; red) and VE-cadherin (**c**, **c″**; red) show the exclusive occurrence of both marker molecules in vascular endothelia (arrows; V, vessel) and their complete absence in cells of the PW, shown by smooth muscle α-actin (α-SMA, **a′**, **a″**, **b′**, **b″**, **c′**, **c″**; green). L, lumen; I, Interstitial space. Bars 20 μm

blood and lymph vessel system, for example, has interestingly been given function or position-oriented names, beginning with "sucking vessel" ("Saugader," Hildebrandt 1802), "blood vessel" ("Blutader," Virchow 1858) and finally "endothelium" (Arnold 1876) and "reticulo-endothelial system" (Aschoff 1924). In contrast, major architectonic, cell type–characteristic and thus diagnostically reliable molecules have been isolated, chemically examined and made detectable by specific antibodies as cytoskeletal or junctional elements only several decades later, i.e., far after 1980 (for relevant reviews on endothelial cells, see, e.g., Simionescu et al. 1982; Daróczy 1988; Gotlieb and Wong 1988; Jaffe 1988; Larson 1988; Palade 1988; Wagner 1988; Zetter 1988). Consequently, the basis for the identification and localization of stable and

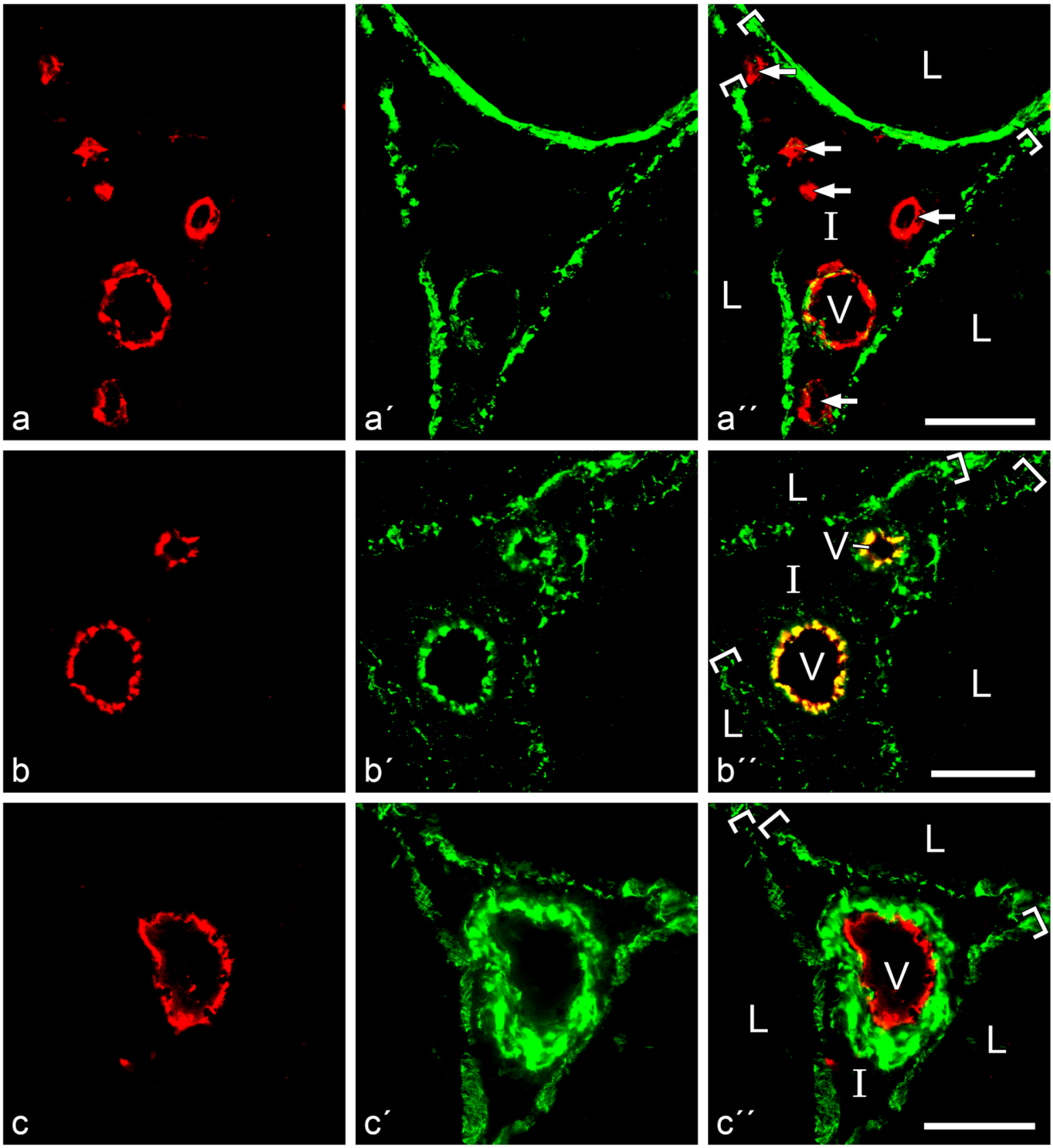

Fig. 8 Double-label immunofluorescence microscopy of cryostat cross-sections through STs of frozen rat testes after reactions of blood vessels (V) and lymph vessels (arrows) with antibodies to claudin-5 (**a**, **a″**, **b**, **b″**, **c**, **c″**; red) in comparison with antibodies against smooth muscle α-actin (α-SMA; **a′**, **a″**; green), β-catenin (**b′**, **b″**; green) and desmin (**c′**, **c″**; green). Note that the smaller lymph and blood vessel (V) endothelia are positive for the endothelial marker claudin-5 but negative for all three SMC markers. Note also the complete absence of an endothelial marker reaction in a cell layer covering the peritubular LSMC wall (4). L, lumen; I, interstitial space. Bars 50 μm

specific endothelial cells was only prepared in the 1990s, i.e., after the discovery of cell type–specific marker molecules such as VE-cadherin (Lampugnani et al. 1992, 1995; Navarro et al. 1998; Dejana 2004; Ferreri and Vincent 2008; Bravi et al. 2014), claudin-5 (Morita et al. 1999), protein LYVE-1 (Banerji et al. 1999; Prevo et al. 2001), podoplanin (Schacht et al. 2005; Cîmpean et al. 2007) and protein myozap (Seeger et al. 2010; Rickelt et al. 2011; Pieperhoff et al. 2012),

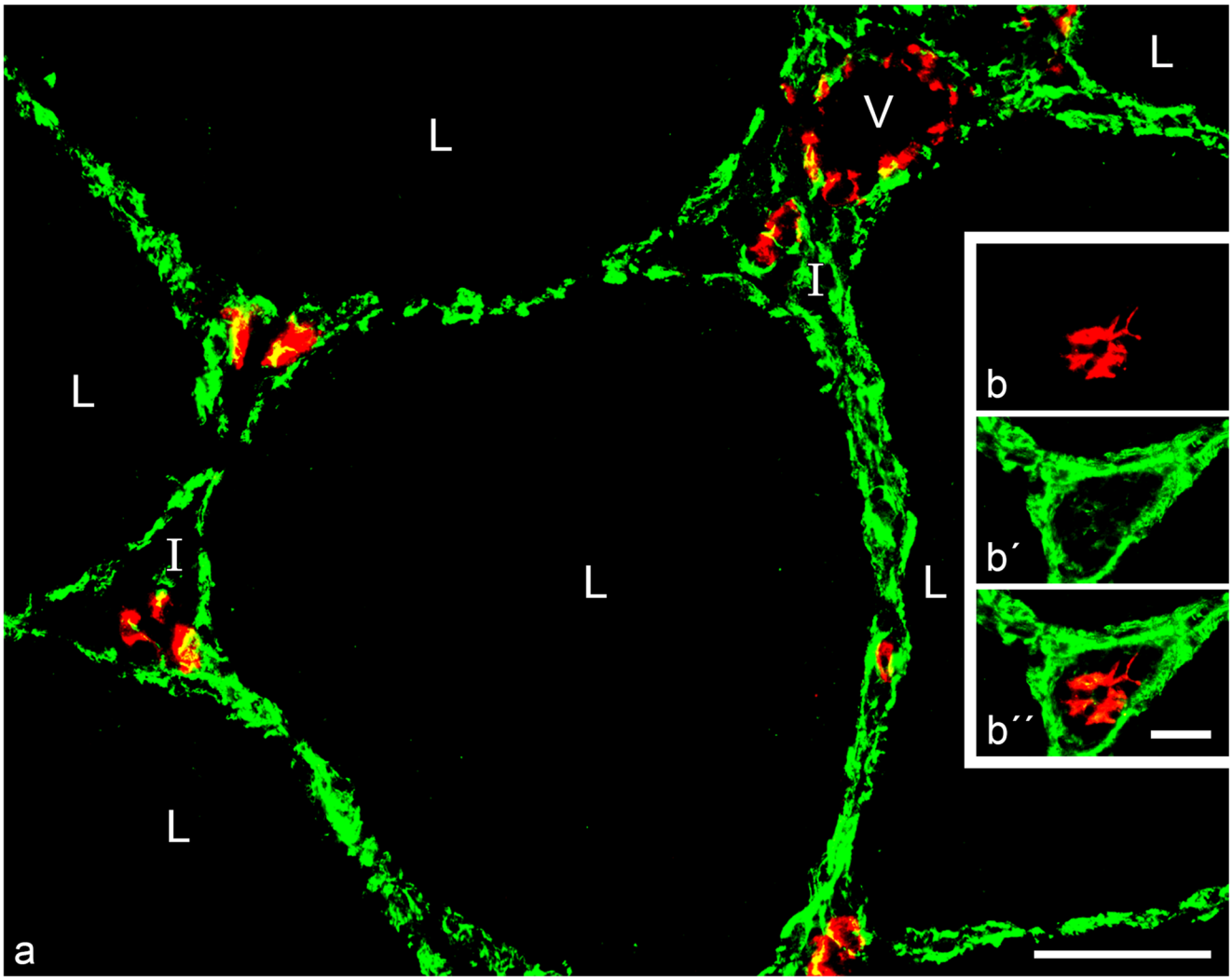

Fig. 9 Double-label immunofluorescence microscopy of a cryostat cross-section through STs of frozen guinea pig testis, showing immunostaining reactions of the specific endothelial tight junction marker claudin-5 (**a**, **b**, **b″**; red) and the SMC marker desmin (**a**, **b′**, **b″**; green). Note that the endothelia of both the—here rather small—lymphatic and blood (V) vessels are positive for the endothelial marker but negative for the SMC marker. Note again the complete absence of the endothelial marker reaction in the outermost cell layer covering the peritubular LSMC wall. L, lumen; I, interstitial space. Bars 50 μm (**a**), 20 μm (**b″**)

which have laid the foundation for a systematic and reliable molecular diagnosis of lymphatic endothelial cells in situ (see, e.g., also Mäkinen et al. 2001; Sleeman et al. 2001; Podgrabinska et al. 2002; Baluk et al. 2007; Baluk and McDonald 2008; Noda et al. 2010).

On the other hand, the positive molecular classification and diagnosis of the cells of the outermost PW-covering monolayer ("fibrocytes" sensu Bressler and Ross 1972) has not yet been finished. As already mentioned, these cells are so extremely thin that special electron microscopy immunolocalization protocols had to be developed. We hope that these methods will soon allow an improved molecular biological identification of these cells.

Finally, we have to discuss some central problems of the previous articles of this series (Domke et al. 2014; Domke and Franke 2019) and the present one. In particular, we need to start a serious general discussion of publications in cell biology. The reason is that we had to learn that published incorrect results are not corrected by the responsible authors but just repeated again and again for several decades. In the field of male genital cell biology, for example, we had explicitly published the regular absence of desmosomes and desmosome-like structures as junctions connecting the Sertoli cells in the STs of mammalian testes (e.g., Franke et al. 1981, 1982, 1983; for recent reviews, see Franke 2009 and the Introduction in the report of Domke et al. 2014). Nevertheless, the list of these incorrect claims has been again repeated and even extended in recent years (see Introduction and Table S1 in Electronic Supplementary Material of Domke and Franke 2019). Now in the present report, we have cited some examples of

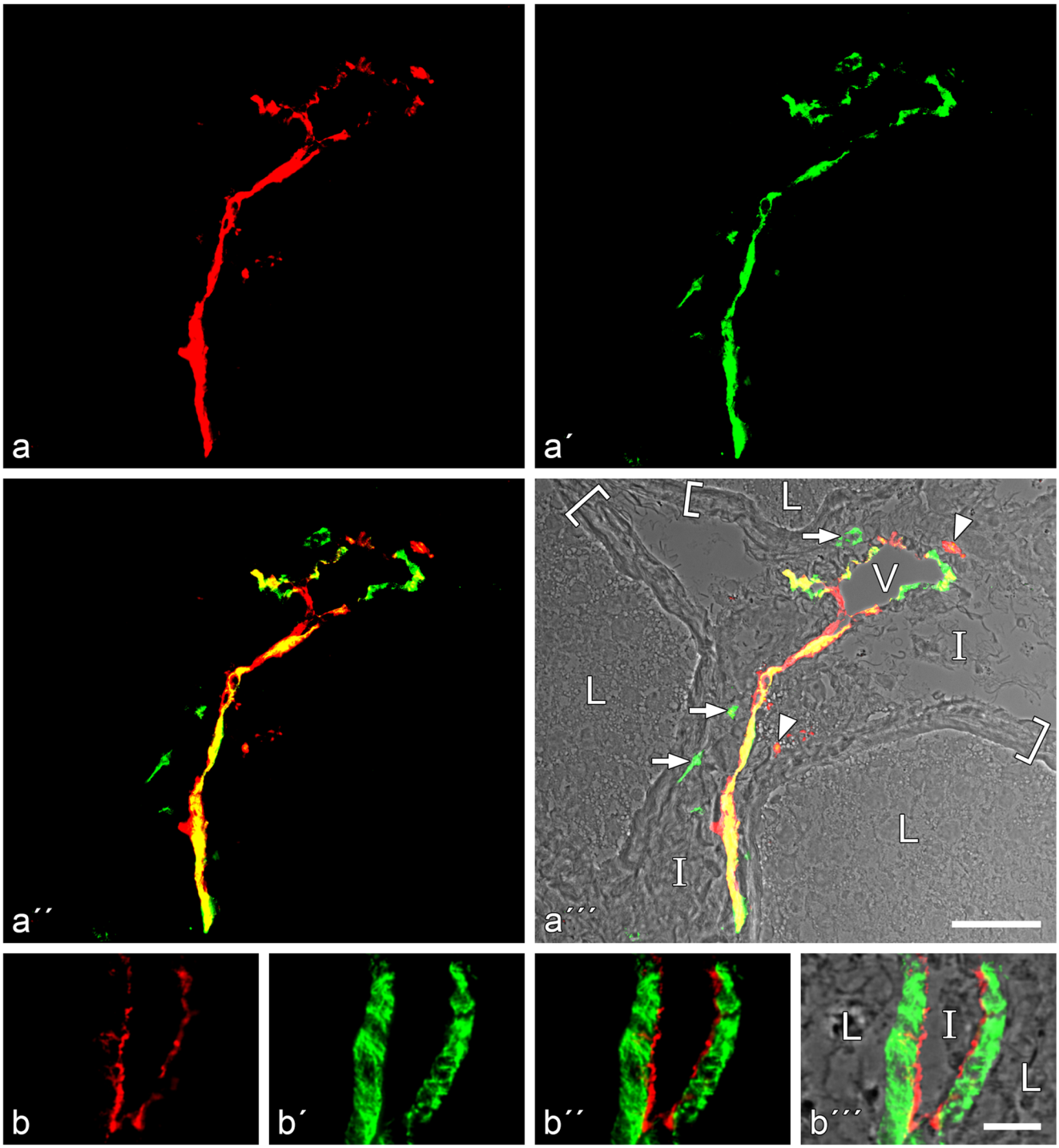

Fig. 10 Double-label immunofluorescence microscopy of a cryostat section through STs of frozen human testis, showing positive immunostaining for vascular endothelium with both podoplanin (**a**, **a″**, **a‴**; red) and LYVE-1 (**a′**, **a‴**; green) in a relatively large vascular wall structure (V) whereas some very small vascular structures are only LYVE-1-positive (arrows). The arrowheads in **a‴** denote a special kind of very small lymphatic endothelial structures, which here prominently react with podoplanin (red). Note the complete absence of both markers in the cells of the PW (brackets). As some sort of control the reaction of protein CD34 is shown in **b**–**b‴** (red), in direct comparison with the LSMCs (green). The specific positive CD34 reaction on the PW surface cells demonstrates again that this protein is not endothelium-specific but occurs in a wide range of diverse mesenchymally and hematopoietically derived cells (for a review, see, e.g., Nielsen and McNagny 2008). L, lumen; I, interstitial space. Bar 50 μm

incorrect results claiming the close and extended association of a lymphatic endothelium with the outermost SM cell layer of the PWs of the STs in several mammalian species, notably rodents, which as we have now extensively demonstrated is

also incorrect. As the authors of such incorrect reports apparently do not correct their claims, the question arises how can one here protect the importance of the truth in science, in basic research as well as in physiology and medical research, including diagnosis and therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and institutional guidelines for the care and use of animals were followed in the procurement of testicular tissue samples from rats and mice (source: Central Animal Laboratory of the German Cancer Research Center, Heidelberg, Germany), from bulls (source: local slaughterhouse in Mannheim, Germany) and from boars (source: Institute of Farm Animal Genetics, Friedrich-Loeffler-Institute, Mariensee, Germany). Cryopreserved and aldehyde-fixed human testis samples were obtained from surgical material taken, examined for diagnostic pathology and processed in compliance with the regulations of the Ethics Committee of the University of Heidelberg, Germany, in accordance with the ethical standards of the Federal Research Committee of Germany and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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