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Molecular and ultrastructural characteristics of adhering junctions and cytoskeletons in cells of mammalian testes

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Dedicated to my grandfather

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List of Abbreviations

α-SMA	smooth muscle α-actin
aa	amino acids
AJ(s)	adherens junction(s)
ATCC	American Type Culture Collection
ARVCF	“armadillo repeat” gene deleted in the Velo-Cardio-Facial syndrome
BL	basal lamina
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco’s Modified Eagle’s Medium
DP	desmoplakin
Dsc	desmocollin
Dsg	desmoglein
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EM	electron micrographs
FCS	fetal calf serum
GJ(s)	gap junction(s)
gp	guinea pig
HEPES	2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethane sulfonic acid
HRP	horseradish peroxidase
ID(s)	intercalated disk(s)
IF(s)	intermediate-sized filament(s)
IFM	immunofluorescence microscopy
IgG	immunoglobulin G
LSMC	lamellar smooth muscle cell
mcl	monoclonal
MPM	minimal plaque material
MW	molecular weight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
pcl	polyclonal
PG	plakoglobin
Pkp	plakophilin
PVDF	polyvinyliden fluoride
rb	rabbit
RT	room temperature
sd	significance not decidable yet
SDS	sodium dodecyl sulfate
SMC	smooth muscle cell
TJ(s)	tight junction(s)

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Summary

The molecular and ultrastructural characteristics of the epithelial cells in the seminiferous tubules as well as the peritubular wall cells of mammalian testes have been subject to controversial debates for several decades that have not been clarified yet. Using biochemical as well as light and electron microscopical methods, in particular immunolocalization techniques, I have studied sexually mature testes from several mammalian species (man, bull, boar, guinea pig, rat and mouse). The analyses included direct interspecies comparison of the tubular and peritubular structures with epithelial tissues of the adjacent excurrent duct system, including the epididymis, and in addition other comparative epithelial and muscular tissue controls.

The present study of cytoplasmic filaments of Sertoli cells in seminiferous tubules confirmed the presence of vimentin intermediate-sized filaments (IFs) and the absence of cytokeratin IFs. Furthermore, my analyses of the corresponding cell-cell adhering junctions (AJs) of Sertoli cells and germ cells validated the absence of desmosomes or "desmosome-like" junctions. In addition, epithelial molecules such as E-cadherin or EpCAM were absent. In contrast, AJs present in the seminiferous tubules are based on N-cadherin clusters anchored in cytoplasmic plaques. These plaques contain α- and β-catenin, plakoglobin, proteins p120 and p0071 as well as a protein of the striatin family.
My findings revealed that the intratubular Sertoli cells are interconnected with adjacent Sertoli cells as well as with germ cells by a novel type of AJs: Specific N-cadherin-based AJs, i.e., variously-sized, often very large cell-cell contacts ("***areae adhaerentes***"). In addition, in certain regions of bovine Sertoli cells, I have found small clusters of sieve-like cell-cell contacts perforated by cytoplasm-to-cytoplasm channels 5–7 nm in luminal diameter ("**cribelliform junctions**"). They are generally associated and laterally connected by tight junction-like membrane-membrane contacts.

The cells of the seminiferous tubules are surrounded by a well-developed basal lamina. However, I could show that the basal lamina is not attached to the Sertoli cells by any hemidesmosomal structures and lacks hemidesmosomal marker molecules such as the integrin α6/β4 complex, protein HD230/233 (bullous pemphigoid antigen, BPA 230) and tetraspanin CD151.

The basal lamina of the seminiferous tubules is enclosed by a lamellar encasement structure, the peritubular walls. These are composed of a bandage system of monolayers of peritubular smooth muscle cells (SMCs) interspersed with layers of extracellular matrix (ECM). The peritubular SMCs are for the most part polyhedral and very flat (down to ca. 30–100 nm cytoplasmic thickness). The number of these lamellar monolayers can vary from one to six per bandage system in different species, developmental stages and regions. They contain

smooth muscle α-actin, the corresponding myosin light and heavy chains, tropomyosin, α-actinin, smoothelin, desmin, vimentin, talin, filamin A, dystrophin, drebrin, caveolin, caldesmon, calponin and protein SM22α, i.e. a typical molecular complement of SMCs. In addition, the peritubular SMC cytoplasm is enriched with myofilament bundles and other typical SMC structures such as "dense bodies", plasma membrane-associated “focal adhesions” and caveolae. Hence, these peritubular cells represent a novel kind of smooth muscle cells and tissue. In this study, I refer to these cells as “**lamellar smooth muscle cells**” (**LSMCs**) and not as they have been described in the literature as “myoid cells”, “myofibroblasts” or “myoepithelial cells”. Furthermore, within a monolayer, the LSMCs are connected by end-to-end junctional contacts and laterally by numerous, vertical AJs located in variously-sized, closely overlapping cell processes (“***processus alter supra alterum***”). The major transmembrane glycoprotein present in the AJs of LSMCs is cadherin-11, often in addition with P-cadherin in some species. These AJ cadherins are anchored in cytoplasmic junction plaques formed by β-catenin, plakoglobin, occasionally protein p0071 and also the 54 kDa plaque protein myozap as well as protein LUMA.

Using endothelial cell type marker molecules such as VE-cadherin, claudin-5, protein PE-CAM (CD31), protein LYVE-1 and podoplanin, I could show that the peritubular wall structure of all species examined is not surrounded by a continuous lymphatic endothelium, as it has often been claimed in the literature for rodent testes.

The novel types of AJ structures are compared with the AJs hitherto known. The special cell type characters of the seminiferous tubule cells and the LSMCs of the peritubular wall are discussed with respect to their architectonic and physiological functions as well as to structural changes during development, aging and in pathogeneses. Finally, my data contributes to the understanding of molecular characteristics of the male genital tract and can be used for the diagnostic pathology of genital tumors.

Zusammenfassung

Der molekularbiologische und ultrastrukturelle Charakter der epithelialen Zellen in den Hodenkanälchen sowie der Zellen der peritubulären Wand („peritubular wall“) ist bisher kontrovers diskutiert und noch nicht ausreichend untersucht worden. Unter Verwendung von biochemischen sowie licht- und elektronenmikroskopischen Methoden, insbesondere der Immunlokalisation, wurden in der vorliegenden Arbeit Hodengewebe von geschlechtsreifen Säugetieren mehrerer Spezies untersucht (Mensch, Rind, Schwein, Meerschwein, Ratte und Maus). Die Untersuchungen wurden im direkten Vergleich mit epithelialen Geweben des anschließenden Nebenhodens sowie weiteren epithelialen Kontrollgeweben durchgeführt.

Die im Rahmen dieser Arbeit durchgeführten Untersuchungen haben für die Sertoli-Zellen das Vorhandensein des Intermediärfilament-Proteins Vimentin sowie die Abwesenheit von Cytokeratinen bestätigt. Außerdem waren ebenfalls Desmosomen, desmosomen-ähnliche Zellverbindungen und desmosomen-spezifische Moleküle sowie bestimmte andere Zellverbindungsmoleküle wie E-cadherin und EpCAM in den Hodenkanälchen abwesend. Die Zellverbindungsstrukturen zwischen Sertoli-Zellen, wie auch die zwischen Sertoli-Zellen und Keimzellen, umfassen das Transmembran-Glykoprotein N-cadherin sowie cytoplasmatische Plaque-Proteine wie α- and β-Catenin, Plakoglobin, Protein p120, sowie Protein p0071 und Striatin. In den Sertoli-Zellen wurden dabei zwei neue Zellverbindungstypen gefunden: 1. Die „***areae adhaerentes***”, welche sich über verschieden große Areale zwischen den Sertoli-Zellen und auch zwischen Sertoli-Zellen und Keimzellen erstrecken. 2. Die „***areae cribelliformes***“, welche siebartige Strukturen mit cytoplasmatischen Kanälchen mit einem Durchmesser von etwa 5–7 nm darstellen.
Die Basallamina der Hodenkanälchen weist keine Hemidesmosomen oder Strukturen spezifisch für hemidesmosomale Marker wie beispielsweise den Integrin-α6/β4-Komplex, Protein HD230 oder Tetraspanin CD151 auf.

Die Hodenkanälchen werden von einer peritubulären Wand umgeben, welche sich aus einem System von einzelnen Schichten extrazellulärer Matrix und lamellärer Glattmuskelzellen aufbaut. Diese Schichten stellen sehr flache und lange Zelllagen dar (teilweise 30–100 nm innerer Membran-Membran-Abstand). Die Anzahl der jeweiligen Zellschichten unterscheidet sich zwischen den Spezies von eins (z.B. Nagetiere) bis sechs (z.B. Mensch, Rind). Die peritubulären Glattmuskelzellen weisen typische Glattmuskelmarker auf wie Glattmuskel-α-Actin, Myosin (leichte und schwere Kette), α-Actinin, Tropomyosin, Smoothelin, Desmin, Vimentin, Filamin A, Talin, Dystrophin, Drebrin, Caveolin, Caldesmon, Calponin sowie Protein SM22α. Sie besitzen Myofilamentbündel, oft in parakristalliner Form, sowie glattmuskel-typische Strukturen wie

Verdichtungszonen („dense bodies“), „focal adhesions“ und Caveolen. Zusätzlich sind diese Zellen über Zellverbindungsstrukturen verbunden, die überwiegend das Glykoprotein Cadherin-11 enthalten sowie teilweise auch P-Cadherin. Die dazugehörigen Plaque-Strukturen umfassen β-Catenin, Plakoglobin sowie die Proteine Myozap und LUMA. Zusammengefasst kann die Aussage getroffen werden, dass diese Zellen alle typischen Merkmale von Glattmuskelzellen aufweisen. Sie werden deshalb hier als lamelläre Glattmuskelzellen (“**lamellar smooth muscle cells**”, **LSMCs**) bezeichnet, anstatt wie bisher in der Literatur überwiegend lediglich als „glattmuskel-ähnliche“ bzw. „myoide“ Zellen, „Myofibroblasten“ oder „myoepitheliale“ Zellen.

Unter Verwendung von endothelialen Markern (VE-Cadherin, Claudin-5, Protein PE-CAM, Protein LYVE-1 und Podoplanin) konnte zusätzlich gezeigt werden, dass die peritubuläre Wand nicht von einem sogenannten „lymphatischen Endothel“ umgeben ist, wie es bisher in der Literatur vor allem für Nagetiere beschrieben worden ist.

Der spezielle Zelltypcharakter der Hodenkanälchen und der LSMCs der peritubulären Wand werden hinsichtlich ihrer möglichen physiologischen Funktion und der molekularen sowie strukturellen Änderung bei der Entwicklung, Alterung und Pathogenese diskutiert. Es wird vor allem auch der Wert dieser Moleküle als Zelltyp-Identifizierungsmerkmale für die pathologische Diagnostik, u.a. von männlichen Genitaltumoren, hervorgehoben.

1 Introduction

The understanding of regulatory and mechanistic processes involved in the development of mammalian epithelia is as important for cell and molecular biology as it is for medical research, in particular processes leading to epithelial diseases, including carcinomas. For the ultrastructural architecture and molecular composition of certain epithelial tissues the cytoskeletal and cell-cell junctional assemblies of specific proteins and glycoproteins are essential. The major cell-cell junctions of epithelial cells and tissues have been identified since more than half a century ago and still dominate current cell biology textbooks (summarized in Table 1). These are the tight junctions (*zonulae occludentes*), the gap junctions (*nexus*), and the two forms of cadherin-based junctions, the adherens junctions (*zonulae adhaerentes*, *fasciae adhaerentes* and *puncta adhaerentia*) and the desmosomes (*maculae adhaerentes*). In this context, it should be mentioned that a series of further cell-cell connecting structures have been described as cell-cell junctions (Franke et al. 2009; for a historical review see also Franke 2010; for a list of these additional types of cell-cell junctions see Table 2). However, the general awareness of these junctions seems to depend on their possible importance in functions and in diseases associated with genetic or epigenetic alterations.

1.1 The male reproductive system

Epithelia are denoted as primary body tissues covering cavities, lumina and surfaces of the whole vertebrate body, either as “simple” (i.e. one cell layer), “complex” or “stratified” epithelia. These cells are mostly orientated in a basal-apical polarity and are attached to a lamina layer at the basal surface. A unique epithelium-type tissue, in which somatic differentiation processes are directly correlated with meiotic divisions and spermatogenesis, is the seminiferous tubule of mammalian testes. Here the basal lamina-grounded somatic cells, the "Sertoli cells" (named after the Italian cell biologist Enrico Sertoli 1842–1910; for origin see von Ebner 1888; for a review see França et al. 2016), are connected with multiple cell-cell attachment structures to each other and to germ cells (see, e.g., Dym and Fawcett 1970; Dym 1975; Franke et al. 1978a; Holstein 1985; Russell and Peterson 1985; Schulze and Holstein 1993; Pelletier 2001). Moreover, these elongated, non-proliferative sustentacular cells form a tight-fitting barrier, the “blood-testis barrier” (BTB), on the one hand to control paracellular movements and translocations of molecules and particles into the tubular lumen, and on the other hand to support germ cell differentiation in association with the Sertoli cells (e.g., Chiquoine 1964; see also Dym and Fawcett 1970; Fawcett et al. 1970; Fawcett 1975; Setchell and Waites 1975; Dym and Cavicchia 1977; Holstein and Roosen-Runge 1981; Russel and Peterson 1985; Cheng

and Mruk 2012). The Sertoli cells finally harbor the spermatid heads in a series of apical indentations ("pockets") before germ cells are released into the excurrent duct system for final maturation and autonomous motility (Fawcett 1994; for schematic overviews see Figs. 1 and 2).

Table 1 Molecular components of "classic" cell-cell junctions (Farquhar and Palade 1963; Franke 2010; see also Nelson and Fuchs 2010; Schulzke et al. 2012)

Cell-Cell Adhesion Type	Occurrence (Examples)	Associated Cytoskeletal Filaments	Transmembrane Proteins or Glycoproteins (Examples)	Cytoplasmic Plaque Proteins (Examples)
Tight Junction				
Zonula occludens	Epithelial cells Endothelial cells	Microfilaments (Actin)	Occludin, Tricellulin, Claudins 1–24	Proteins ZO-1, ZO-2, ZO-3, Cingulin, Symplekin
Adherens Junction				
Zonula adhaerens *Fascia adhaerens* *Punctum adhaerens*	Epithelial cells Endothelial cells Many other cell types	Microfilaments (Actin)	Cadherins (e.g. E-cadherin, N-cadherin, P-cadherin, VE-cadherin, M-cadherin, Cadherin-11)	*Armadillo*-type proteins: Plakoglobin, β-catenin, Proteins p120, p0071, ARVCF, Neurojungin Myofilament-associated proteins: α-catenin, Striatin, Myozap
Desmosome				
Macula adhaerens	Epithelial cells Cardiomyocytes Meningeal cells Dendritic reticulum cells	Intermediate-sized filaments (e.g. Desmin, Cytokeratins, Vimentin)	Desmogleins 1–4, Desmocollins 1–3	Plakoglobin, Desmoplakin 1+2, Plakophilins 1–3
Gap Junction				
Nexus	Diverse kinds of tissue-forming cells		Connexins 1–21	Proteins ZO-1, ZO-2, ZO-3

Table 2 Further kinds of cell-cell adhering junctions (discoveries after 1990)

Cell-Cell Adhesion Type	Occurrence	Transmembrane Proteins or Glycoproteins (Examples)	Cytoplasmic Plaque Proteins (Examples)
Complexus adhaerens			
Schmelz et al. 1990, 1994; Schmelz and Franke 1993; Hämmerling et al. 2006[1]	Endothelial and virgultar cells of lymph node sinus and other lymphatic vascular endothelia	N-cadherin, VE-cadherin, Claudin-5, Protein JAM-A	α-catenin, β-catenin, Protein p120, Plakoglobin, Desmoplakin, Protein ZO-1, Afadin
Area composita			
Borrmann et al. 1999, 2000, 2006; Borrmann 2000; Franke et al. 2006[2]	Cardiomyocytes of maturing and adult hearts, parts of Purkinje fibers	N-cadherin, Cadherin-11, Desmoglein-2, Desmocollin-2, Protein PERP[3]	α-catenin, β-catenin, Protein p120, Protein p0071, Protein ARVCF, Plakoglobin, Desmoplakin, Plakophilin-2, Proteins ZO-1–3, Vinculin, Striatin, Protein LUMA[4], Ankyrin
Contactus adhaerens			
Rose et al. 1995; Bahjaoui-Bouhaddi et al. 1997; Hollnagel et al. 2002	Granular cells of cerebellar glomeruli	M-cadherin, N-cadherin	α-catenin, β-catenin, Plakoglobin
Zonula limitans externa			
Paffenholz et al. 1999	Heterotypic: Photoreceptor, Müller glia cells	N-cadherin	Neurojungin, α-catenin, β-catenin, Protein p120, Vinculin, Symplekin, Protein ZO-1, Plakophilin-2
Cortex adhaerens			
Straub et al. 2003	Eye lens interior	N-cadherin, Cadherin-11	α-catenin, β-catenin, Protein p120, Plakoglobin, Vinculin, Ezrin, Periaxin, Periplakin
Colligatio permixta			
Boda-Heggemann 2005; Boda-Heggemann et al. 2009	Glia and glioma cells	N-cadherin, Cadherin-11 (VE-cadherin)	α-catenin, β-catenin, Protein p120, Protein ARVCF, Plakophilin-2, Proteins ZO-1–2, Cinculin, Afadin, Vinculin

Continuation of Table 2

Cell-Cell Adhesion Type	Occurrence	Transmembrane Proteins or Glyco-proteins (Examples)	Cytoplasmic Plaque Proteins (Examples)
Manubrium adhaerens			
Wuchter et al. 2007; Rickelt et al. 2009	Mesenchymal culture cells	N-cadherin, Cadherin-11	α-catenin, β-catenin, Protein p120, Protein p0071, Protein ARVCF, α-Actinin, Afadin, Plakoglobin, Vinculin, Ezrin
Cis-E-N-cadherin heterodimer junctions			
Straub et al. 2011	Endoderm-derived cells, in particular hepatocytes, hepatocyte-like and pancreatic duct cells	Cis-E-N-cadherin	α-catenin, β-catenin, Protein p120, Protein p0071, Protein ZO-1, Plakoglobin
Tessellate junctions			
Franke et al. 2013	Almost all stratified epithelia	Occludin, E-cadherin, Claudin-1, Claudin-4	β-catenin, Protein PERP

[1] For further references see Supplement Literature Collection No. 1

[2] For further references see Supplement Literature Collection No. 2

[3] Ihrie et al. 2005; Franke et al. 2013

[4] The molecular arrangement of protein LUMA has been described by various authors as a transmembrane protein TMEM43 (Dreger et al. 2001; Bengtsson and Otto 2008; Merner et al. 2008; Christensen et al. 2011b; Liang et al. 2011; Rajkumar et al. 2012; Baskin et al. 2013; Haywood et al. 2013; Hodgkinson et al. 2013). However, results obtained in the laboratory of Prof. Dr. Werner W. Franke have led to the conclusion that this protein is most likely membrane-associated as a component of the cytoplasmic plaque (see also, e.g., Franke et al. 2014).

1.2 Molecular organization of cytoskeletal and cell-cell adherens junction structures of the seminiferous tubules

Although *prima facie* the cell layer in the seminiferous tubules containing mature Sertoli cells looks like a typical epithelium, it differs from the majority of other epithelial cells with respect to several morphological and molecular components. These differences include in the absence of intermediate-sized filaments (IFs) of the cytokeratin type, generally considered to be a hallmark of epithelial cells, and the presence of vimentin IFs (for cytokeratin IFs see, e.g., Franke et al. 1978c, e, 1979a–d, 1981a; Sun et al. 1979; Bannasch et al. 1980, 1981; Moll et al. 1982a; Moll 1993; for reviews see Chu and Weiss 2002; Moll et al. 2008; Franke et al. 2010; for vimentin IFs see, e.g., Franke et al. 1978c,

1979c; see also Spruill et al. 1983; Miettinen et al. 1985; Ramaekers et al. 1985; Paranko and Virtanen 1986; Mali et al. 1987; Stosiek et al. 1990; Aumüller et al. 1992; Steger and Wrobel 1994; Steger et al. 1996; for a report of the additional presence of neurofilaments in Sertoli cells of some species see Davidoff et al. 1999).

Sertoli cells with adjacent Sertoli cells as well as with germ cells are connected by gap and tight junctions as well as by various types of specific adhering junctions. Originally, the early generation of transmission electron microscopists described certain kinds of adherens junction (AJ)-type cell-cell contact structures in the seminiferous tubules as typical desmosomes, “desmosome-related” junctions or “desmosome-like” junctions (e.g. Nicander 1967; Altorfer et al. 1974; for further references see Table 3). In contrast, authors of the laboratory of Prof. Dr. Werner W. Franke have repeatedly reported for more than three decades a total absence of specific desmosomal structures and desmosomal marker molecules in Sertoli cells of mature mammals active in spermatogenesis (e.g., Franke et al. 1979c, 1982b, 1983, 1989; Mueller and Franke 1983; Moll et al. 1986; Schmelz et al. 1986a; Theis et al. 1993; Schäfer et al. 1994; Nuber et al. 1995; Mertens et al. 1996; Domke 2013; confirmed, e.g., by Pelletier and Byers 1992). However, other authors have continued to claim the presence of desmosomes or desmosome-like junctions and desmosome-specific molecules in Sertoli cells of mature mammalian testes (Table 3). Based on this long and still ongoing debate, I decided to devote a part of my doctoral thesis to the final clarification of this controversy and to the analysis of the AJs in the *tubuli seminiferi* of diverse mammalian species in detail.

In addition, in some initial studies (Domke 2013) I noticed that the cytoskeletal and junctional protein composition and organization of the thin encasement structure surrounding the seminiferous tubules, the peritubular wall, had not yet been sufficiently analyzed. Moreover, the nomenclature indicated that the cell type classification of these cells forming the peritubular wall was not clear: In numerous publications, over the last 60 years, these cells had been called “myoid cells”, “myofibroblasts”, “contractile-type cells” or just “lamina propria cells” (for definition of “myofibroblasts” as modified fibroblasts see, e.g., Supplement Literature Collection No. 3). To clarify this, I decided to determine in my doctoral thesis the complete cell type-specific molecular composition and ultrastructural architecture of the peritubular wall layer system surrounding the seminiferous tubules in diverse mammalian species, using immunocytochemical and immunoelectron microscopical as well as biochemical methods. In view of the potential diagnostic importance of cytoskeletal and junctional molecules as markers in histology and pathology and also with respect to the worldwide interest in the development and conceptualization of male contraceptives based on the interference with cell-cell interactions in the testis, I have performed this study in a highly controlled mode.

Table 3 List of some references claiming the presence of desmosomes and/or hemidesmosomes in Sertoli cells of mammalian seminiferous tubules

	Desmosomes[1]	Hemidesmosomes
Altorfer et al. 1974*	+	/
Alves et al. 2013	+	/
Bergmann et al. 1984*	+	/
Chen et al. 2017	+	/
Cheng and Mruk 2002, 2011, 2012	+	+
Cheng et al. 2011, 2013	+	+
Connell 1978*	+	+
Dierichs and Wrobel 1973*	+	/
França et al. 2016	+	/
Goossens and van Roy 2005	+	+
Holthöfer et al. 2007	+	/
Johnson and Boekelheide 2002a,b	+	/
Kopera et al. 2010	+	+
Lee and Cheng 2004	+	+
Lee et al. 2009	+	+
Li MW et al. 2009, 2011	+	+,/
Li N et al. 2015	+	/
Lie et al. 2009a,b, 2010, 2011, 2013	+	+,/,/,+,+
Mahoney et al. 2002	+	/
Mok et al. 2013	+	+
Morrow et al. 2010	+	/
Mruk and Cheng 2004a, b[2], 2011, 2015	+	+,+,/,+
Mruk et al. 2014, 2017; Mruk 2016	+	/
Mulholland et al. 2001	+	+
Nagano and Suzuki 1978*	+	/
Nicander 1967*	+	+
Osman 1978*	+	/
Osman and Ploen 1978*	+	/
Qian et al. 2013	+	/
Russell 1977a, b*	+	/
Russell and Peterson 1985*	+	/
Schulze 1984*	+	/
Su et al. 2013	+	+
Vogl et al. 1993, 2000, 2008, 2013	+	/,+,/
von Kopylow et al. 2010	+	/
Wen et al. 2018	+	/
Whittock 2003	+	/
Wine and Chapin 1999	+	/
Wong and Cheng 2005	+	+
Wong et al. 2005	+	/
Wrobel et al. 1979*	/	+
Xia et al. 2005	+	/
Yan et al. 2007	+	+
Yan and Cheng 2005	+	/

Continuation Table 3

For a German textbook claim of the occurrence of desmosomes see, e.g., Lüllmann-Rauch (2015)

* References marked with an asterisk (*) are primarily based on electron microscopy; + claiming the presence of desmosomes or hemidesmosomes; / not mentioned

[1] Desmosomes and desmosome-like junctions

[2] See here, however, the specific footnote in Table 1 of Mruk and Cheng (2004)

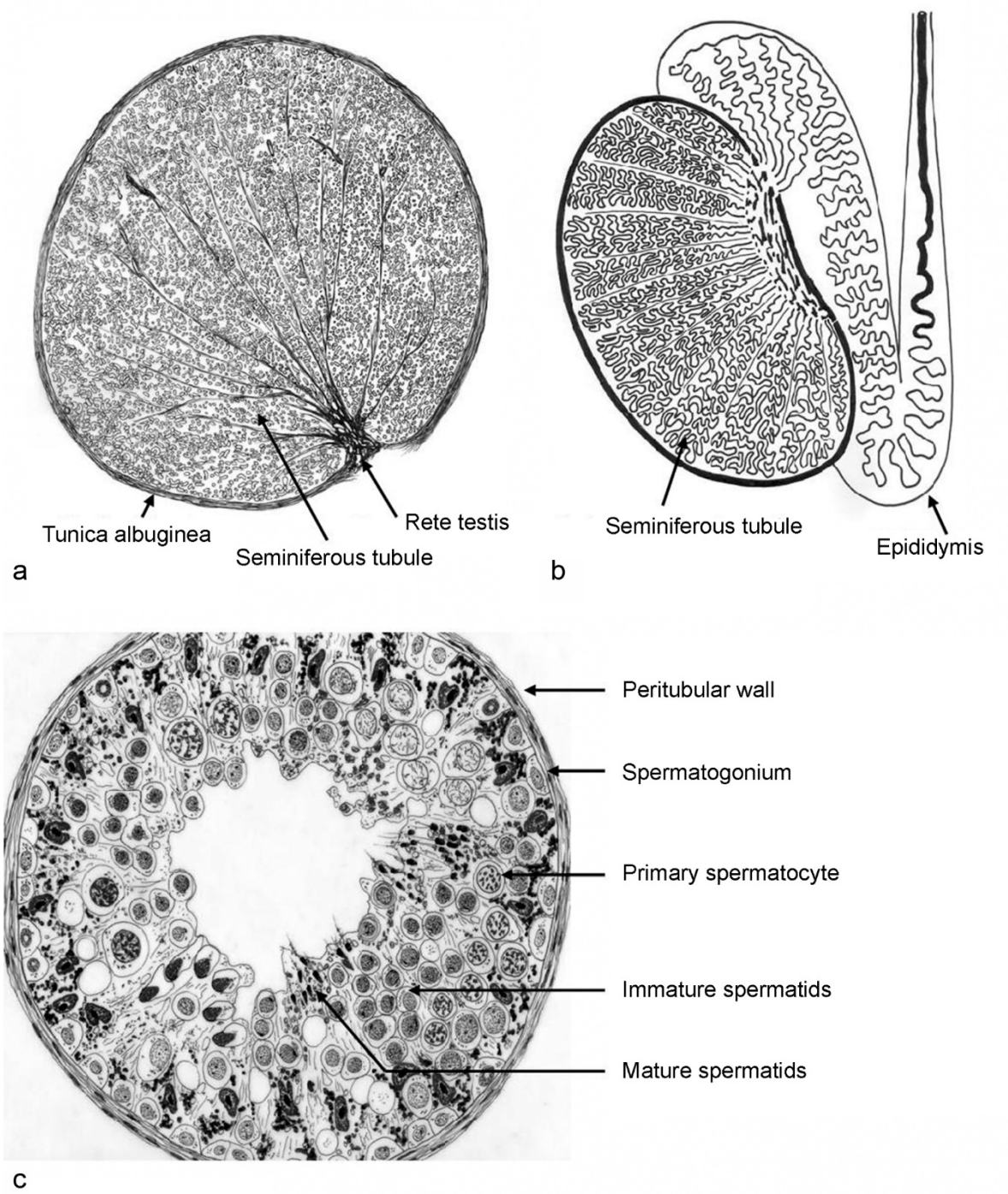
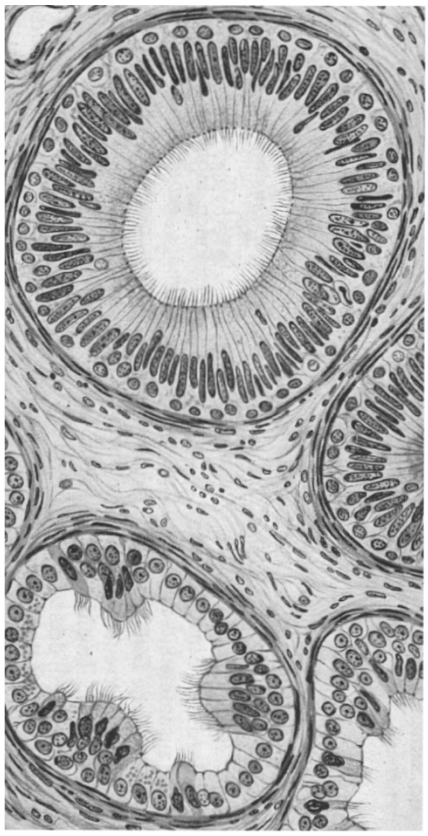
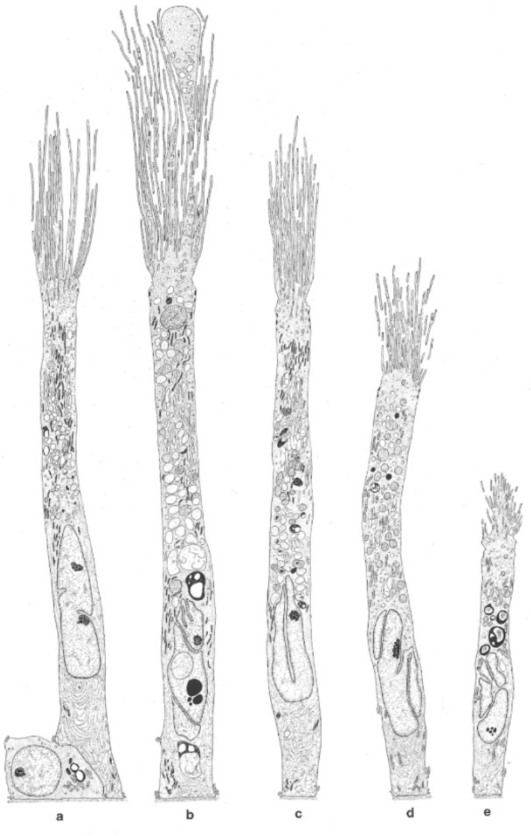


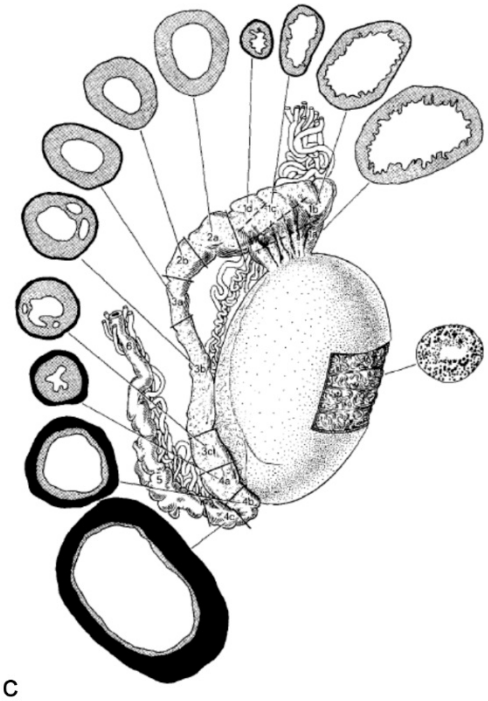
Fig. 1 Schematic overview demonstrating the seminiferous tubules and the excurrent duct system, including the epididymis, of the human testis. (**a**, **b**) Seminiferous tubules are connected with the rete testis system from which sperm and testicular fluid can pass into the excurrent duct system. (**c**) Spermatogenesis takes place in the seminiferous tubule as shown in a cross-section (modified from Holstein et al. 2003).



a



b



c

Fig. 2 Schematic overview of the human epididymis. (**a**) Cross-section of a ductus epididymidis (top) with stereocilia and "basal cells" and of a *ductulus efferens* (bottom; Bargmann 1977). (**b**) Drawing of the principal and basal cells in human epididymis with decreasing epithelial height as well as decreasing height of stereocilia and increasing infoldings of the nuclear envelope (Weiss 1988). (**c**) The black labelling on the outer side of the duct system illustrates the increasing thickness of the additional subepithelial smooth muscular layer (modified from Baumgarten et al. 1971).

2 Materials

2.1 Tissues

Rat and mouse tissue samples were obtained from the animal laboratory facilities of the German Cancer Research Center (DKFZ, Heidelberg, Germany). Bovine tissues of freshly slaughtered animals were obtained from a regional slaughterhouse (Mannheim, Germany). Porcine tissue samples of freshly slaughtered boars were provided by Prof. Dr. Heiner Niemann, head of the Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health (Mariensee, Germany). Human tissues, including tumor samples taken and prepared in compliance with the regulations of the Ethics Committees of the Universities of Heidelberg and Marburg, were obtained from clinical cases (Germany; see, e.g., Franke et al. 2006; Langbein et al. 2003; Moll et al. 2009). In particular the following tissues were used: testis, testicular excurrent ducts, liver, intestine, tongue mucosa, esophagus, heart, bladder and bovine muzzle.

2.2 Cell culture lines

Most cell lines used in this study had been obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) or from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cell culture lines and media used are listed in Tables 4 and 5.

2.3 Antibodies

2.3.1 Primary antibodies

The monoclonal and polyclonal antibodies used for immunoblot analyses of polypeptides separated by gel electrophoresis as well as for immunofluorescence and immunoelectron microscopy are listed in Table 6.

2.3.2 Secondary antibodies

For indirect immunofluorescence microscopy primary antibody complexes were visualized with secondary antibodies conjugated with fluorochromes. These Cy3- (Dianova, Hamburg, Germany) or Alexa 488- (Invitrogen, Karlsruhe, Germany) conjugated secondary antibodies directed against mouse, rabbit, guinea pig or rat immunoglobulins had generally been generated in goats (see Table 7). Antibodies used were diluted according to the manufacturer's recommendations.

Immunoblot analyses were performed with horseradish peroxidase (HRP)-conjugated secondary antibodies (Dianova) against mouse, rabbit or guinea pig immunoglobulins using an enhanced chemiluminescence (ECL)-System ("Western blotting" Luminol Reagent, Santa Cruz Biotechnology, CA, USA).

For immunoelectron microscopy nanogold-conjugated secondary antibodies (Nanoprobes, Yaphank, NY, USA) were used. Coupled antibody structures were subsequently enhanced and enlarged by a silver reaction with a "HG Silver™ Enhancement Kit" (Nanoprobes).

Table 4 Cell culture media and supplements

Media and supplements	Source reference
Claycomb Medium	Sigma-Aldrich (Taufkirchen, Germany)
Dulbecco's Modified Eagle's Medium (DMEM) with glutamine	Life Technologies GmbH (Darmstadt, Germany)
Fetal calf serum (FCS)	Biochrome GmbH (Berlin, Germany)
Ham's F-12 liquid medium	Biochrome
L-glutamine 200 mM (100x)	GIBCO, Thermo Fisher Scientific (Bonn, Germany)
Modified Eagle's Medium (MEM)	Biochrome
Non-essential amino acids (aa, 100x)	Biochrome

Table 5 Cell culture lines

Cell culture line	Origin	Reference	Main growth medium used
B1	Bovine dermal fibroblasts	Cowin et al. 1986	80 % DMEM + 20 % FCS
HL-1	Murine cardiomyocytes	Claycomb et al. 1998	87 % Claycomb medium,+ 10 % FCS + 100 units/ml penicillin/streptomycin + 100 µM norepinephrin, + 4 mM L-glutamine
Caco-2	Human colon adenocarcinoma (ATCC, HTB-37)	Fogh et al. 1977	85 % MEM (+ non-essential aa) + 15 % FCS
HaCaT	Human keratinocytes	Boukamp et al. 1988	90 % DMEM + 10 % FCS
PLC	Primary human liver carcinoma (ATCC CRL-8024)	Alexander et al. 1976	90 % DMEM + 10 % FCS
SV80	SV40-transformed human WI38 fibroblasts (ATCC CCL-75.1)	Girardi et al. 1966	90 % DMEM + 10 % FCS
3T3	Murine endothelial cell line (ATCC CCL-92)	Todaro and Green 1963	90 % DMEM + 10 % FCS

Table 6 Primary antibodies

Antigen*	Species, Clonality	Source	References
Cadherins			
E-cadherin (cadherin-1, CDH1)	mcl[1], m[3] (cl. 36)	BD Transduction Laboratories (Lexington, KY, USA), 610182	Vestweber and Kemler 1984, 1985
	mcl, rb[4] (cl. EP700Y)	Abcam (Cambridge, UK), ab40772	
N-cadherin (cadherin-2, CDH2)	mcl, m (cl. 32)	BD Transduction Laboratories, 610921	Takeichi 1990
	pcl[2], rb	QED Biosciences (San Diego, CA, USA), 42031	Bhowmick et al. 2001; Nürnberger et al. 2002
P-cadherin (cadherin-3, CDH3)	mcl, rat (cl. PCD-1)	Thermo Fisher Scientific (Waltham, MA, USA), 13-2000Z	Nose and Takeichi 1986
VE-cadherin (cadherin-5, CDH5)	pcl, rb	Cayman Chemical Company (Ann Arbor, MI, USA), 160840	Lampugnani et al. 1995; Dejana et al. 2000
	mcl, m (cl. BV9/1B5)	Kindly provided by Elisabetta Dejana (University of Milan, Milan, Italy)	Hämmerling et al. 2006
K-cadherin (cadherin-6, CDH6)	mcl, m (cl. 2B6)	Progen Biotechnik (Heidelberg, Germany), 16111	Shimazui et al. 1998
OB-cadherin (cadherin-11, CDH11)	mcl, m (cl. 5B2H5)	Thermo Fisher Scientific, 32-1700	Okazaki et al. 1994
	mcl, m (cl. 16A)	Progen Biotechnik, 16113	Tomita et al. 2000
	pcl, rb	Thermo Fisher Scientific, 71-7600	
M-cadherin (cadherin-15, CDH15)	pcl, rb	Abcam, ab129078	Donalies et al. 1991; Hollnagel et al. 2002
Desmoglein-1 (Dsg-1)[6]	mcl, m (cl. 27B2)	Thermo Fisher Scientific, 32-6000	Kurzen et al. 1998
	mcl, m (cl. P23)	Progen Biotechnik, 651110	Kurzen et al. 1998
Desmoglein-1+2[6]	mcl, m (cl. DG3.10)	Progen Biotechnik, 61002	Schmelz et al. 1986a, b
Desmoglein-2 (Dsg-2)[6]	pcl, rb (cl. rb 5)	Progen Biotechnik, 610121	Schäfer et al. 1996; Peitsch et al. 2014
	mcl, m (cl. 6D8)	Thermo Fisher Scientific, 32-6100	
	pcl, rb	Atlas Antibodies (Bromma, Sweden), A57749	von Kopylow et al. 2010
	pcl, goat	R&D Systems (Minneapolis, MN, USA), AF947	
	mcl, m (cl. 10G11)	Progen Biotechnik, 61059	Schäfer et al. 1994, 1996
	mcl, m (cl.141409)	R&D Systems, MAB947	

Continuation of Table 6

Antigen	Species, Clonality	Source	References
Desmoglein-3 (Dsg-3)[6]	mcl, m (cl. 5G11)	Thermo Fisher Scientific, 32-6300	Schäfer et al. 1996; Kurzen et al. 1998
Desmocollin-1 (Dsc-1)[6]	mcl, m (cl. U100)	Progen Biotechnik, 65192	Nuber et al. 1995, 1996
Desmocollin-2 (Dsc-2)[6]	pcl, rb (cl. rb 36)	Progen Biotechnik, 610120	Nuber et al. 1995
	pcl, gp[5] (cl. R1A/B)	Laboratory of Prof. Dr. Werner W. Franke	
	pcl, gp	Progen Biotechnik, GP542	Nuber et al. 1995
Desmocollin-3 (Dsc-3)[6]	mcl, m	Progen Biotechnik, 65193	Nuber et al. 1996
Transmembrane tight junction proteins			
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, 2000
	mcl, m, Alexa 488	Thermo Fisher Scientific, 33-1500	Moroi et al. 1998
	pcl, rb	Abcam, ab31721	
Tricellulin-α	pcl, rat	Kindly provided by Profs. Drs. Shoichiro and Sachiko Tsukita	Ikenouchi et al. 2005
Claudin-1	mcl, m (cl. 2H10D10)	Thermo Fisher Scientific, 37-4900	Furuse et al. 1998
	pcl, rb	Thermo Fisher Scientific, 51-9000	
Claudin-2	mcl, m (cl.12H12)	Thermo Fisher Scientific, 32-5600	Furuse et al. 1998
Claudin-3	pcl, rb (cl. Z23.JM)	Thermo Fisher Scientific, 34-1700	Tsukita and Furuse 1998
	pcl, rb	Abcam, ab15102	
Claudin-4	pcl, rb	Thermo Fisher Scientific, 36-4800	
	mcl, m (cl. 3E2C1)	Thermo Fisher Scientific, 32-9400	
Claudin-5	mcl, m (cl. 4C3C2)	Thermo Fisher Scientific, 35-2500	Morita et al. 1999b
	mcl, m (cl. 4C3C2	Thermo Fisher Scientific, 18-7364	
Claudin-6	pcl, rb	Abcam, ab75055	
Claudin-11	pcl, rb	Thermo Fisher Scientific, 36-4500	Bronstein et al. 1996
	pcl, rb	Abcam, ab53041	Fink et al. 2009

Continuation of Table 6

Antigen	Species, Clonality	Source	References
Other transmembrane proteins and glycoproteins			
Protein PERP[6]	mcl, m (cl. 26.3.30,26.2. 22,8.2.9)	Laboratory of Prof. Dr. Werner W. Franke	Ihrie et al. 2005; Franke et al. 2013
	pcl, gp (cl. 4A)	Laboratory of Prof. Dr. Werner W. Franke	Franke et al. 2013
	pcl, rb	Abcam, ab5986	
HD 230/233	mcl, m (cl. HD233)	Kindly provided by Prof. Dr. Katsushi Owaribe (University of Nagoya, Japan)	Owaribe et al. 1990, 1991
Jam-A (JAM-1)	pcl, rb (cl. CD321)	Thermo Fisher Scientific, 36-1700	Xia et al. 2005
	mcl, m (cl.43/JAM-1)	BD Transduction Laboratories, 612120	
Jam-B (Jam-2)	mcl, m (cl. 1G4)	Biomol (Hamburg, Germany), WA-AT2578a	Ebnet et al. 2004
Jam-C (JAM-3)	pcl, rb	Thermo Fisher Scientific, 40-8900	
	pcl, rb	Bethyl Laboratories (Montgomery, TX, USA), A303-761A	
EpCAM	mcl, m (cl. HEA 125)	Progen Biotechnik, 61004	Momburg et al. 1987
	mcl, m (cl. VU 1D9)	Progen Biotechnik, 16114	Litvinov et al. 1994a, b, 1997
	pcl, rb	GeneTex (Irvine, CA, USA), GTX54034	
Nectin-1	mcl, m (cl. CK8)	Thermo Fisher Scientific, 37-5900	Takai et al. 2003
Nectin-2	pcl, rb (cl. H-108)	Santa Cruz Biotechnology, sc-28638	Irie et al. 2004
	pcl, rb EPR6717	Abcam, ab135246	
α6-Integrin	pcl, rat (cl. GoH3)	Progen Biotechnik, 10709	Sonnenberg et al. 1991; Bosman et al. 1993
	pcl, rb	Abcam, ab97760	
	mcl, rb (cl. EPR18124)	Abcam, ab181551	
ß1-Integrin	mcl, rat (cl. CD29)	BD Transduction Laboratories, 550531	Palombi et al. 1992b
	mcl, m (cl. TS2/16)	Thermo Fisher Scientific, MA2910	
	mcl, m (cl. 18/CD29)	BD Transduction Laboratories, 610468	

Continuation of Table 6

Antigen	Species, Clonality	Source	References
β4-Integrin	mcl, rat (cl. 346-11A)	Abcam, ab25254	Sonnenberg et al. 1991
	mcl, rb (cl. EPR8559)	Abcam, ab133682	
	pcl, rb	Proteintech (Rosemont, IL, USA), 21738-1-AP	
Tetraspanin CD151	mcl, m (cl. 11G5a)	Acris Antibodies (Herford, Germany), SM1209P	Sterk et al. 2000
	pcl, rb	Abcam, ab185684	
Connexin 43	pcl, rb	Thermo Fisher Scientific, 71-0700	Goodenough et al. 1996
Protein LUMA			
Protein LUMA (TMEM43)[6]	pcl, gp (cl. 2A)	Laboratory of Prof. Dr. Werner W. Franke	Franke et al. 2014
	pcl, gp (cl. 4B)	Laboratory of Prof. Dr. Werner W. Franke	Franke et al. 2014
	mcl, m (cl. E1)	Santa Cruz Biotechnology, sc-365298	
	pcl, rb (cl. N1C2)	GeneTex (Irvine, CA, USA), GTX110229	
	mcl, m (cl. F3)	Santa Cruz, sc-271887	
***Armadillo* repeat proteins**			
β-catenin	mcl, m (cl. 14)	BD Transduction Laboratories, 610154	Ozawa et al. 1990
	mcl, rb (cl. E247)	Abcam, ab32572	
	pcl, rb	Sigma-Aldrich, C2206	McCrea et al. 1991
Protein p120	mcl, m (cl. 98/pp120)	BD Transduction Laboratories, 610134	Reynolds et al. 1994, 1996a, b
	pcl, rb	Sigma-Aldrich, P1870	
Protein p0071[6]	mcl, m (cl. SEPP 7.7.9)	Progen Biotechnik, 651165	Hofmann et al. 2008, 2009
	pcl, gp (cl. GP71)	Progen Biotechnik, GP71	
ARVCF[6]	mcl, m (hARVCF)	Prof. Dr. Ilse Hofmann (DKFZ, Heidelberg, Germany)	Sirotkin et al. 1997; Borrmann et al. 2000

Continuation of Table 6

Antigen	Species, Clonality	Source	References
Plakoglobin[6]	mcl, m (cl. PG 5.1.7.2)	Progen Biotechnik, 65105	Cowin et al. 1986
	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	
Plakophilin-1[6]	mcl, m (cl. 5C2)	Progen Biotechnik, 65160	Heid et al. 1994
	mcl, m (cl. 2D6)	Progen Biotechnik, 65161	Heid et al. 1994
Plakophilin-2[6]	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, rb	Acris Antibodies, APO1493PU-N	
	pcl, gp (cl. SR2A)	Laboratory of Prof. Dr. Werner W. Franke	Rickelt et al. 2010
	pcl, gp (cl. GP-PP2)	Progen Biotechnik, GP-PP2	Rickelt et al. 2010
Plakophilin-3[6]	mcl, m (cl. Paul 270.6.2)	Progen Biotechnik, 651113	Schmidt et al. 1999
	mcl, m (cl. Paul 310.9.1)	Progen Biotechnik, 651114	Schmidt et al. 1999
Other plaque proteins			
α-E-catenin (CTNNA1)	pcl, rb	Sigma-Aldrich, C8114	Herrenknecht et al.1991
	mcl, m	Thermo Fisher Scientific, 13-9700	Troyanovsky et al. 2011
	pcl, rb	Cell Signalling (Danvers, MA, USA), 3236	Kobielak and Fuchs 2004
α-N-catenin (CTNNA2)	pcl, rb	Sigma-Aldrich, C8239	Hirano et al. 1992
α-T-catenin (CTNNA3)	mcl, rat (cl.1159_12A 4S4)	Kindly provided by Prof. Dr. Frans van Roy (Flanders Interuniversity Institute of Biotechnology, University of Gent, Gent, Belgium)	Janssens et al. 2001, 2003; Goossens et al. 2007a, b
	pcl, rb (cl. 952)	Kindly provided by Prof. Dr. Frans van Roy	Goossens et al. 2007a

Continuation of Table 6

Antigen	Species, Clonality	Source	References
α-T-catenin (CTNNA3)	mcl, m (cl. 892_24D2S)	Kindly provided by Prof. Dr. Frans van Roy	Janssens et al. 2001
	pcl, rb (cl. Ag5008)	Proteintech, 13974-1-AP	
	mcl, m (cl. 892_24D2S)	Abcam, ab2499	Janssens et al. 2001
	mcl, m (cl. 892_24D2S)	Thermo Fisher Scientific, MA1-06311	Janssens et al. 2001
	mcl, m (cl. B-6)	Santa Cruz Biotechnology, sc-398138	
	mcl, m (cl. 892_24D2S)	Santa Cruz Biotechnology, sc-59943	Janssens et al. 2001
	pcl, rb	Abcam, ab131250	
	pcl, rb	Novus Biologicals (Wiesbaden, Germany), NBP1-82728	
Desmoplakin 1+2[6]	mcl, m (cl. Mix-2.15, -2.17, -2.20)	Progen Biotechnik, 65146	Franke et al. 1982a; Cowin et al. 1985
Desmoplakin 1[6]	pcl, gp (cl. Gp495)	Progen Biotechnik, DP-1	Köser 1999
Vinculin/ Metavinculin	mcl, m (cl. Vin-11-5)	Sigma-Aldrich, V4505	Geiger 1979, 1980
	pcl, rb	Sigma-Aldrich, V4139	
l/s-Afadin	pcl, rb	Sigma-Aldrich, A0224	Mandai et al. 1997
l-Afadin	pcl, rb	Sigma-Aldrich, A0349	Mandai et al. 1997
ZO-1	mcl, m (cl. ZO1-1A12)	Thermo Fisher Scientific, 33-9100	Stevenson et al. 1986; Anderson et al. 1989
ZO-2	pcl, rb	Thermo Fisher Scientific, 38-9100	Kirschner et al. 2011
ZO-3	mcl, m	Chemicon (Hofheim; Germany), MAB3260	Haskins et al. 1998; Itoh et al. 1999
Myozap[6]	mcl, m (cl. 517.67)	Progen Biotechnik, 651169	Pieperhoff et al. 2012
	pcl, gp (cl. 2A)	Laboratory of Prof. Dr. Werner W. Franke	Rickelt et al. 2011a
Plectin	mcl, m (cl.31)	BD Transduction Lab, 611348	Wiche et al. 1983
	mcl, m (cl. 7A8)	Novus Biologicals, NB120-11220	
	pcl, gp	Progen Biotechnik, GP21	Liu et al. 1996
	plc, gp	Progen Biotechnik, GP20	Liu et al. 1996
	pcl, rb	Abcam, ab83497	

Continuation of Table 6

Antigen	Species, Clonality	Source	References
Cingulin	mcl, m	Progen Biotechnik, 651122	Citi et al. 1989
Striatin[6]	mcl, m	BD Transduction Laboratories, 610838	Castets et al. 1996; Franke et al. 2015
	pcl, gp (cl. 301 B)	Laboratory of Prof. Dr. Werner W. Franke	Franke et al. 2015
	plc, gp (cl. hNT B)	Laboratory of Prof. Dr. Werner W. Franke	Franke et al. 2015
	pcl, rb	Millipore, AB5779	Castets et al. 1996
	pcl, rb	Sigma-Aldrich, S0696	
Microfilament and microfilament-associated proteins			
Actin	mcl, m (cl. 2G2)	Progen Biotechnik, 651132	Gonsior et al. 1999
Smooth muscle α-actin (α-SMA)	mcl, m (cl. ASM-1)	Progen Biotechnik, 61001	Skalli et al. 1986
	Pcl, rb	Abcam, ab5694	
Cardiac α-actin[6]	mcl, m (cl. Ac1-20.4.2)	Progen Biotechnik, 61075	Franke et al. 1996
Actin α-skeletal/ cardiac	mcl, m (cl. 22D3)	Novus Biologicals, NBP1-97725	Gunning et al. 1983
α-Actinin	mcl, m (cl. BM-75.2)	Sigma-Aldrich, A5044	Abd-el-Basset et al. 1991
	pcl, rb	Sigma-Aldrich, A2543	
	mcl, m (cl. EA-53)	Sigma-Aldrich, A7811	Lazarides and Burridge, 1975
Myosin cardiac (heavy chain)	mcl, m (cl. BA-G5)	Abcam, ab50967	Krenz et al. 2007
Myosin (skeletal+smooth)	pcl, rb	Sigma-Aldrich, M7648	
Myosin (smooth muscle; heavy chain)	mcl, m	Sigma-Aldrich, M7786	Babij et al. 1991
Myosin (smooth muscle; light chain 2)	pcl, rb	Cell Signalling, 3672	Kumar et al. 1989
Myom 2 (Myomesin)	pcl, rb	Acris Antibodies, AP01485PU-N	
Smoothelin	mcl, m (cl. R4A)	Millipore, MAB3242	van der Loop et al. 1996
	pcl, rb	Abcam, ab204305	
Tropomyosin	mcl, m (cl. TM311)	Sigma-Aldrich, T2780	Gimona 1997; Boyd et al. 1995
	pcl, rb	Sigma-Aldrich, T3651	
Filamin A (c-term)	mcl, rb	Abgent, AJ1299a	Wang et al. 1975

Continuation of Table 6

Antigen	Species, Clonality	Source	References
Intermediate-sized filament proteins			
Glia filament protein[6] (GFAP)	mcl, m (cl. GF12.24)	Progen Biotechnik, 61011	Gould et al. 1990
	pcl, rb	Millipore, ab5804	Uyeda et al. 1972
Vimentin[6]	mcl, m (cl. 3B4)	Progen Biotechnik, 65113	Franke et al. 1978c Heid et al. 1988
	pcl, gp (cl. Gp53)	Progen Biotechnik, GP53	Herrmann et al. 1996
Desmin[6]	mcl, m (cl. D9)	Progen Biotechnik, 10519	van Muijen et al. 1987
	mcl, m (cl. DE-R-11)	Dako, Aligent (Santa Clara, CS, USA), M0724	
	pcl, rb	Progen Biotechnik, 10570	Garcia-Martinez et al. 1986
Cytokeratin 4[6]	mcl, m (cl. 6B10)	Progen Biotechnik, 10525	van Muijen et al. 1986
Cytokeratin 7[6]	mcl, m (cl. Ks7.18)	Progen Biotechnik, 65025	Bartek et al. 1991
Cytokeratin 8[6]	mcl, m (cl. 17.2)	Progen Biotechnik, 65130	Magin et al. 1990
	mcl, m (cl. M20)	Progen Biotechnik, 10526	Magin et al. 1990
	mcl, m (cl. Ks8.1)	Laboratory of Prof. Dr. Werner W. Franke	
	mcl, m (cl. Ks8.7)	Progen Biotechnik, 65138	Moll et al. 1982a
Cytokeratin 9[6]	mcl, m, (cl.Ks9.70+ Ks9.216)	Progen Biotechnik, 651104	Langbein et al. 1994
	pcl, gp (cl. GPHK9-TY1)	Progen Biotechnik, GP-CK9	Langbein et al. 1994
Cytokeratin 15[6]	pcl, gp (cl. 15.2)	Progen Biotechnik, GP-K15	Leube et al. 1988
	pcl, gp (cl. 15.1)	Progen Biotechnik, GP-CK15	Jih et al. 1999
Cytokeratin 18[6]	mcl, m (cl. 174.14.11)	Laboratory of Prof. Dr. Werner W. Franke	
	mcl, m (cl. 18.04/214)	Progen Biotechnik, 61028	Bartek et al. 1991
	mcl, m (cl. RGE 53)	Progen Biotechnik, 10500	Ramaekers et al. 1983
	mcl, rb (cl. E431-1)	Abcam, ab32118	

Continuation of Table 6

Antigen	Species, Clonality	Source	References
Cytokeratin 8/18[6]	pcl, gp	Progen Biotechnik, GP11	Bader et al. 1988
Cytokeratin 19[6]	pcl, gp (cl. GP68)	Progen Biotechnik, GP-CK19	Bader et al. 1988
	mcl,m (cl. KS19.2)	Progen Biotechnik, 65129	Karsten et al. 1985
Cytokeratin 20[6] (c-term)	pcl, gp (cl. K20.2)	Progen Biotechnik, GP-K20	Moll et al. 1992
	mcl, m (cl. IT-Ks20.10)	Progen Biotechnik, 65154	Moll et al. 1982a, b
Pan-Cytokeratin[6]	mcl, m (cl. C22, 5+8)	Progen Biotechnik, 65131	Bartek et al. 1991
	pcl, rb	Progen Biotechnik, 10550	Ramaekers et al. 1985
Extracellular matrix proteins			
Elastin	mcl, m (cl. BA-4)	Sigma-Aldrich, E4013	Wrenn et al. 1986
Collagen IV	pcl, rb	Progen Biotechnik, 10760	Khoshnoodi et al. 2008
Collagen VI	pcl, rb (cl. COL6A1)	Acris Antibodies, R1043	
Pro-Collagen I	mcl, m (cl. PCID G10)	Millipore, MAB1913	McDonald et al. 1986
Fibronectin	mcl, m	Dianova	Singer et al. 1984
	mcl, m	Sigma-Aldrich	
Other proteins or glycoproteins			
Ankyrin G	mcl, m (cl. 4G3F8)	Thermo Fisher Scientific, 33-8800	Bennett 1992
Eplin (Lima-1)	pcl, rb	Sigma-Aldrich, HPA023871	
Talin	mcl, m (cl. TA205)	Sigma-Aldrich, SAB4200041	Otey et al. 1990
Troponin T	mcl, m (cl. JLT-12)	Sigma-Aldrich, T6277	Katus et al. 1991
Moesin	mcl, m (cl. 38)	BD Transduction Laboratories, 610401	Furthmayr et al. 1992
Laminin	pcl, rb	Progen Biotechnik, 10765	Christensen et al. 1992
Laminin α-5	mcl, m (cl. 4B12)	Millipore, MABT39	
Caldesmon	mcl, m (cl. E89)	Abcam, ab32330	Frid et al. 1992
Calponin	mcl, m (cl. hCP)	Sigma-Aldrich, C2687	Gimona et al. 1990

Continuation of Table 6

Antigen	Species, Clonality	Source	References
Caveolin-1	mcl, rb (cl. D46G3)	Cell Signaling, 3267	Okamoto et al. 1998
Dystrophin	pcl, rb	Abcam, ab15277	Ellis et al. 1990
SM22α	pcl, rb	Abcam, ab155272	Lees-Miller et al. 1987
	pcl, rb	Abcam, ab14106	
Drebrin[6]	pcl, gp	Progen Biotechnik, GP254	Peitsch et al. 2001, 2003, 2005
Synemin	pcl, rb	Sigma-Aldrich, S9075	Granger and Lazarides 1980
Paxillin	pcl, rb	Abcam, ab32084	Veith et al. 2012
Sperm cell proteins			
Calicin[6]	mcl, m (Susi 46.1.5/5.1.05)	Laboratory of Prof. Dr. Werner W. Franke	Longo et al. 1987; Paranko et al. 1995; von Bülow et al. 1995
Cylicin I[6]	mcl, m (cl. X 144.3.2,5/09)	Laboratory of Prof. Dr. Werner W. Franke	Hess et al. 1993
	pcl, gp	Laboratory of Prof. Dr. Werner W. Franke	
Cylicin II[6]	pcl, gp	Laboratory of Prof. Dr. Werner W. Franke	Hess et al. 1995
Arp-T1[6]	pcl, gp	Laboratory of Prof. Dr. Werner W. Franke	Heid et al. 2002
Arp-T2[6]	pcl, gp	Laboratory of Prof. Dr. Werner W. Franke	Heid et al. 2002
Actin-binding protein CP β3[6]	pcl, gp	Laboratory of Prof. Dr. Werner W. Franke	von Bülow et al. 1997
Endothelial marker proteins			
LYVE-1	plc, rb	Acris Antibodies, DP3500PS	Banerji et al. 1999
CD31 (PE-CAM-1)	mcl, m (cl. MEM-05)	Thermo Fisher Scientific, 37-0700	Simmons et al. 1990
Podoplanin	mcl, m	Dako, Aligent, M3619	Cîmpean et al. 2007
	mcl, m (cl. 18H5)	Acris Antibodies, DM3500P	
Factor VIII (von Willebrand factor)	mcl, m	Dako, Aligent	Hämmerling et al. 2006; Moll et al. 2009
VE-cadherin	(see Cadherins)		

[1] mcl monoclonal antibodies, [2] pcl polyclonal antibodies, [3] m mouse, [4] rb rabbit, [5] gp guinea pig,
[6] Antibodies have been originally generated in the laboratory of Prof. Dr. Werner W. Franke
* Antibody reactions not mentioned in the Results section are not of controlled positive significance to the themes of this thesis. In addition, antibodies of a human pathology diagnostic list have been used for special purposes (Moll 1993).

Table 7 Secondary antibodies

Name of antibody	Species	Source
Alexa 488	m	Invitrogen, A11029
Alexa 488	rb	Invitrogen, A11008
Alexa 488	gp	Invitrogen, A11073
Cy3	m	Dianova, 115-165-068
Cy3	rb	Dianova, 111-165-045
Cy3	gp	Dianova, 111-165-003
Cy3	rat	Dianova, 112-165-044

2.3.3 Other fluorescent markers

For specific staining of F-actin, Alexa Fluor®488- or Alexa Fluor®594-coupled phalloidin (Thermo Fisher Scientific) was used. In addition, visualization of the nuclear chromatin was obtained using 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, München, Germany).

2.4 Buffers, media and solutions

All chemicals used in this study were purchased from Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Roche Diagnostics (Mannheim, Germany), SERVA (Heidelberg, Germany), Sigma-Aldrich or Thermo Fisher Scientific if not otherwise indicated.

Table 8 Buffers or other solutions used for immunofluorescence microscopy

Buffer or solution	Composition and concentration	
Triton-X-100 solution	0.1 % (1 g) ad 1 L PBS, pH 7.4	Triton-X-100
Formaldehyde solution (2 %)	2 % (20 g) ad 1 L PBS, pH 7.4	Paraformaldehyde
Ammoniumchlorid buffer	50 mM (2.67 g) ad 1 L PBS, pH 7.4	NH_4Cl
Saponin solution	0.1 % (1 g) ad 1 L PBS, pH 7.4	Saponin
Citrate buffer	**Solution A:** 21.01 g ad 1 L H_2O	Citric acid
	Solution B: 29.41 g ad 1 L H_2O_{dest}	Sodium citrate
	9 mL A + 41 mL B ad 500 mL H_2O_{dest}, pH 6	
Tris-HCl buffer	**Stock solution:**	
	20.18 g ad 1L H_2O_{dest}, pH 7,4	Tris-HCl
	Working solution:	
	150 mL 100 mL pH 10.2 (or dependent on antibody)	Stock solution H_2O_{dest}

Table 9 Buffers or other solutions used for biochemical experiments

Buffer or solution	Composition and concentration
1x PBS	140 mM (80 g) NaCl 2.7 mM (2 g) KCl 1.7 mM (2 g) KH_2PO_4 8.1 mM (16 g) $Na_2HPO_4 \times H_2O$ ad 1 L H_2O_{dest}, pH 7.4
PBS-T	140 mM (80 g) NaCl 2.7 mM (2 g) KCl 1.7 mM (2 g) KH_2PO_4 8.1 mM (16 g) $Na_2HPO_4 \times H_2O$ 0.1 % (1 g) Tween®20 ad 1 L H_2O_{dest}, pH 7.4
Electrophoresis running buffer	92 mM (2.8 g) Tris-HCl 760 mM (14.3 g) Glycine 0.2 % (2.0 g) SDS ad 1 L H_2O_{dest}, pH 8.8
2x SDS buffer ("Laemmli buffer")	250 mM (30.29 g) Tris-HCl 20 % (200 mL) Glycerol 10 % (100 g) SDS 0.2 % (2 g) Bromphenol blue 40 mM (6.17 g) DTT ad 1 L H_2O_{dest}, pH 6.8
Transfer buffer 1	300 mM (36.3 g) Tris-HCl 20 % (200 mL) Isopropanol ad 1 L H_2O_{dest}, pH 10.4
Transfer buffer 2	25 mM (3.025 g) Tris-HCl 20 % (200 mL) Isopropanol ad 1 L H_2O_{dest}, pH 10.4
Transfer buffer 3	40 mM (5.24 g) Norleucin 25 mM (3.025 g) Tris-HCl ad 1 L H_2O_{dest}, pH 9.4
20 % Borate transfer buffer	0.4 M (24.7 g) Boracic acid 20 mM (7.4 g) EDTA ad 1 L H_2O_{dest}, pH 8.8 (with NaOH)
Coomassie staining solution	40 % (400 mL) Isopropanol 7 % (70 mL) Acetic acid 0.2 % (2 g) Coomassie brilliant blue R-250 ad 1 L H_2O_{dest}
PVDF membrane destaining solution	40 % (400 mL) Isopropanol 7 % (70 mL) Acetic acid ad 1 L H_2O_{dest}
Membrane stripping buffer	6.25 mM (6.25 mL) Tris-HCl (1 M) 20 mM (3.05 g) DTT 2 % (20 g) SDS ad 1 L H_2O_{dest}, pH 6.7
Blocking buffer	5 % (50 g) Milk powder ad 1 L PBS-T, pH 7.4

Table 10 Electron microscopy

Buffer, solution or diverse mixtures	Composition and concentration
Sodium cacodylate buffer	50 mM (10.7 g) Cacodylic acid ad 1 L $H_2O_{aqua\ ad.\ inj.}$, pH 7.2
Glutardialdehyde (2.5 %)	2.5 mL Glutardialdehyde (25 %) 1.25 mL (50 mM) KCl (1 M) 62.5 µL (2.5 mM) $MgCl_2$ (1 M) 2.5 mL Sodium cacodylate buffer (0,5 M) ad 25 mL $H_2O_{aqua\ ad.\ inj.}$, pH 7.2
Uranylacetate solution (0.5 %)	125 mg (0.5 %) Uranylacetate 25 mL $H_2O_{aqua\ ad.\ inj.}$
Osmium tetroxide (4 %)	1 g OsO_4 ad 25 mL $H_2O_{aqua\ ad.\ inj.}$
HEPES buffer	50 mM (5.95 g) HEPES ad 50 mL $H_2O_{aqua\ ad.\ inj.}$, pH 5.8
Lead citrate solution	2.02 mM (0.67 g) Lead(II) nitrate 2.99 mM (0.88 g) Sodium citrate 5 mL NaOH (1M) ad 25 mL $H_2O_{aqua\ ad.\ inj.}$, pH 12
Sucrose solution	13.6g (200 mM) Sucrose 200 mL HEPES (50 mM), pH 5.8
Sodium thiosulfate pentahydrate solution	250 mM (12.4 g) Sodium thiosulfate pentahydrate 200 mL HEPES (50 mM), pH 5.8
Epon mixture [Both components (A and B) were mixed 3:2 (w/v) before use. Epon mix (10 mL) was supplemented with 2,4,6-Tris(dimethyl-aminomethyl)-phenol (DMP30, 0.2 mL) as catalyst.]	**Solution A:** 100.2 g 2-Dodecenyl succinic anhydride (DDSA) 74.4 g Epon 812/Glycidether **Solution B:** 54.25 g Methyl nadic anhydride (MNA) 60.0 g Epon 812/Glycidether

2.5 Technical equipment

The equipment used in this study is listed in Table 11.

Table 11 Technical equipment

Instruments	Name	Manufacturer
Blotting chamber		cti GmbH (Idstein, Germany)
Centrifuge	5415R	Eppendorf Vertrieb Deutschland GmbH (Wesseling-Berzdorf, Germany)
Confocal laser scanning microscope	LSM 510, 700, 800, 880	Carl Zeiss Microscopy GmbH (Oberkochen, Germany)
Cryotome	Leica CM 3050 S	Leica Camera AG (Wetzlar, Germany)
1K Slow scan CCD Camera	Typ 7888	TRS (Moorenweis, Germany)
Developer	Optimum Typ TR	MS Laborgeräte (Heidelberg, Germany)
Electron microscope	EM 10, EM 900	Carl Zeiss Microscopy GmbH
Electrophoresis power supply	Phero-stab. 500	Biotec-Fischer (Reiskirchen, Germany)
Fluorescence microscope	Axiophot	Carl Zeiss Microscopy GmbH
Fluorescence microscope camera	Axio Cam HRc/MRc	Carl Zeiss Microscopy GmbH
Gel electrophoresis chamber	X Cell Sure Lock	Novex (San Diego, CA, USA)
Heating cabinet		Memmert (Schwabach, Germany)
Heating block	Thermomixer 5436	Eppendorf Vertrieb Deutschland GmbH (Wesseling-Berzdorf, Germany)
Incubators	Function Line	Heraeus Holding GmbH (Hanau, Germany)
Magnetic stirrer	Ika Combimag RCO	IKA®-Werke GmbH & CO. KG (Staufen, Germany)
Microtome	HM 355 S	Microm International GmbH (Walldorf, Germany)
Microtome blades	Leica 819	Leica Camera AG
Microwave instrument	Medite RHS Rapid Microwave Histoprocessor	Milestone S.r.l. (Sorisole, Italy)
pH meter	765 Calimatic	Knick Elektronische Meßgeräte GmbH & Co (Egelsbach, Germany)
Scales	PB 3002-S, PB 153-S	Mettler-Toledo (Giessen, Germany)
Scanner	Epson Perfection 4870	Epson America, Inc. (Long Beach, CA, USA)
Shaker	Silent Rocker	cti GmbH
Sterile laminar flow bench	SterilGardHood Class II Type A/B3	The Baker Company. Inc. (Sanford, ME, USA)
Ultramicrotome	Reichert Ultracut	Leica Camera AG
Ultramicrotome diamond knives, 45°	DiATOME ultra	Diatome AG (Biel, Switzerland)
Vortex	REAX 2000	Heidolph Instruments GmbH & Co. KG (Schwabach, Germany)
Water bath	W6	Labortechnik Medingen (Arnsdorf, Germany)

3 Methods

3.1 Preparations of tissue samples

Tissues of organs were removed from the animals and processed immediately to obtain optimal preservation: Organs were cut into small pieces (ca. 0.5 cm) in PBS and subsequently processed by rapid freezing for cryostat analyses or fixed with formaldehyde and embedded in paraffin (see below).

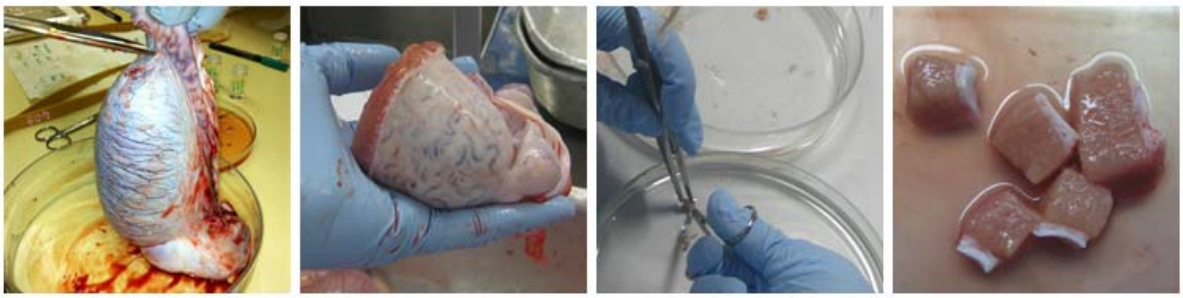


Fig. 3 Preparation of tissue samples from bull testis. For optimal preservation, the tissues of the required organs were removed from the animals and processed immediately.

3.1.1 Snap-frozen tissue

Tissue samples used were shock-frozen in isopentane (pre-cooled to the temperature of liquid nitrogen) for a few minutes. The duration was dependent on the specific size of the samples to allow instant and complete freezing. Specimens were subsequently stored in plastic vials with 5 mL isopentane at -80°C.

3.1.2 Paraffin-embedded tissue

Alternatively, tissue samples were fixed in 4 % (w/v) formaldehyde in PBS (pH 7.4), freshly prepared from paraformaldehyde powder, for 24 h at 4°C. After passage through an ethanol dehydration series of increasing concentrations, the dehydrated tissue samples were embedded in low-melting paraffin, kindly realized in the laboratory of Prof. Dr. Hermann-Josef Gröne (Division of cellular and molecular pathology, German Cancer Research Center).

3.2 Cell cultures

All cell cultures used were treated and passaged either as recommended by the manufacturer or the distributor (see Table 5). In general, monolayer cultures were passaged after the cells had grown to ~ 80–100 % confluency to ensure optimal cell-cell junction patterns. Cells were rinsed with 37°C warm 0.02 % (w/v) EDTA in PBS to remove residual culture medium and then incubated with trypsin (0.25 %) / EDTA (0.02 %) in PBS until the cells were dissociated. Then cells and medium were neutralized with culture medium, suspended in fresh culture medium and plated on coated or uncoated cell culture dishes supplied with fresh DMEM medium containing 10 % FCS and 1 % glutamine. Cells

were finally incubated at 37°C in 5 % CO_2. For microscopic examination cells were plated on glass coverslips coated with poly-L-lysine as a non-specific substratum.

3.3 Biochemical methods

For the identification of specific proteins present in cultured cells or tissues, cell lysates were prepared. Molecules or stable molecule complexes were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by immunoblot analyses using specific antibodies (Weber and Osborn 1969; Towbin et al. 1979; for a methodological review see Hames 1998).

3.3.1 Preparation of cell culture and tissue lysates

Cultured cells grown to confluency in culture dishes (5 or 10 cm in diameter) were gently washed twice with PBS (cooled on ice) to remove cell debris, dead cells and residual medium. The cells were covered with 200–500 µL 2x “Laemmli buffer” (Laemmli 1970) in 5 cm culture dishes (or 500–1000 µL for 10 cm culture dishes). The buffer contained dithiothreitol (DTT) as a reducing agent and SDS for cell lysis to dissociate protein complexes and proteins into polypeptides by reducing disulfide bridges and to charge the polypeptides negatively. The solution was supplemented with 1 µL benzonase and incubated for 5 min at room temperature (RT) to remove DNA and RNA and thus to reduce viscosity. The cells were then scraped off and collected using a cell scraper, transferred into an Eppendorf reaction tube and heated at 97°C for 5 min under agitation to denature and SDS-couple the polypeptides. The samples were centrifuged at 16,000 *g* for 5 min at RT and the resulting supernatants were either used directly or stored at -20°C.

Frozen tissue samples were cut into ca. 10 µm thin sections at -20°C (for freezing procedures sections see chapter 3.1.1; Moll et al. 1982b), collected in pre-cooled (-20°C) Eppendorf reaction tubes and supplemented with 200 µL 2x Laemmli buffer containing 1 µL benzonase. The suspensions from ca. 50 sections each were homogenized with a Dounce glass homogenizer, heated to 97°C for 5 min to resolve secondary and tertiary structures, and centrifuged at 16,000 *g* for 5 min. The resulting supernatant was used directly or was stored at -20°C.

3.3.2 Fractionation of proteins

For enrichment of proteins, frozen tissue samples were cut in ca. 10 µm thin sections at -20°C and homogenized on ice. After stepwise centrifugation (13,000 *g*, 2 min at 4°C) each resulting supernatant was separated and collected to extract proteins. In the first supernatant, soluble proteins of the cytoplasm were present and solubilized with “Complete Mini Protease Inhibitor” (Roche Diagnostics) in PBS. In the next step, samples were centrifuged in PBS with 1 % Triton-X-100 to dissolve proteins soluble in detergent,

especially IF and membrane proteins. In the third step, samples were homogenized in a solution of 1 % Triton-X-100 and 0.5 M NaCl in PBS. The remaining pellet contained enriched proteins and glycoproteins which were still insoluble after treatment with high ionic strength and detergent solution, notably junction proteins.

The supernatants obtained and the remaining pellets were treated overnight with methanol (fourfold volume) at -20°C. The next day, separation by centrifugation, drying of the pellet and treatment with 1x Laemmli buffer with benzonase were accomplished.

3.3.3 Protein gel electrophoresis

A modified method according to Laemmli (1970) was applied to separate proteins and polypeptides of the prepared cell or tissue lysates using gel electrophoresis. Thereby, polypeptides coupled with SDS move dependent on their molecular weight and in unfolded conformation in an electric field through a gel matrix (4–20 %, gradient tris-glycine gels, Anamed Elektrophorese GmbH, Groß-Bieberau/Rodau, Germany). For this, samples (15 µL) were loaded next to a protein marker solution (New England BioLabs, Frankfurt, Germany) containing 13 polypeptides of known molecular weight (see Table 12) and separated for 2 h at 20 mA.

Table 12 Reference polypeptides as molecular weight markers

Calculated MW[1] in kDa	Polypeptides	Source
212	Myosin, heavy chain	Rabbit muscle
158	MBP2[2]-β-galactosidase	*E. coli*
116	β-galactosidase	*E. coli*
97.2	Phosphorylase b	Rabbit muscle
66.4	**Serum albumin[3]**	**Bovine**
56.6	Glutamic dehydrogenase	Bovine liver
42.7	MBP2	*E. coli*
34.6	Thioredoxin reductase	*E. coli*
27	**Triosephosphate isomerase[3]**	***E. coli***
20	Trypsin inhibitor	Soybean
14.3	Lysozyme	Chicken egg white
6.5	Aprotinin	Bovine lung
3.4	Insulin A, β-chain	Bovine pancreas

[1]MW molecular weight, [2]MBP maltose-binding-protein, [3]Serum albumin and triosephosphate isomerase were added at double concentration to serve as reference points.

3.3.4 Transfer of polypeptides onto a membrane

3.3.4.1 Semi-dry transfer

Polypeptides separated in SDS-PAGE (see chapter 3.3.1) were transferred from the gel onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Merck Millipore, Billerica, MA, USA) using a semi-dry transfer method (Kyhse-Anderson 1984). For this, the “transfer sandwich” (3 layers of filter paper – 1 gel – 1 membrane – 5 layers of filter paper)

was placed horizontally between two plates with electrodes. To this end, PVDF membranes were rehydrated in isopropanol and moistened in transfer buffer 2. The gel was equilibrated in transfer buffer 3. Whatman® 3MM-Paper sheets (Sigma-Aldrich) were soaked in transfer buffer 1, 2 and 3, respectively, and placed with the gel and the PVDF membrane in the following arrangement on the anode of the "graphite-transfer chamber" (see scheme below). The semi-dry blot was carried out at 250 mA for 1.5 h. For components of the buffers see Table 9.

Cathode
3 Whatman-Paper in transfer buffer 3
SDS-gel in transfer buffer 3
PVDF-membrane in transfer buffer 2
2 Whatman-Paper in transfer buffer 2
3 Whatman-Paper in transfer buffer 1
Anode

Subsequently, PVDF membranes were incubated in Coomassie brilliant blue staining solution for 1 min to visualize separated polypeptide-containing bands. Background staining was reduced by washing the PVDF membrane in a destaining solution (Table 9). After drying and documentation, membranes were used for immunoblot (Western blot) analyses.

3.3.4.2 Wet transfer

For a wet transfer, in particular of large polypeptides, onto a membrane, the gel was equilibrated in 1 x borate transfer buffer and placed into a transfer sandwich (1 filter paper – gel – ethanol rehydrated membrane – 1 filter paper), encompassed by sponge pads and pressed together in a case. The gel sandwich was placed vertically in a tank filled with transfer buffer between electrodes. The transfer was carried out under constant stirring of the buffer (50 mA for 5 min, 75 mA for 5 min, 10 mA for 10 min and 125 mA for 2 h).

3.3.5 Immunoreaction analyses

Detection of polypeptides bound on the PVDF membrane was ensued using an indirect enzyme-immunoassay. Unspecific binding reactions were minimized by moistening the PVDF membrane with ethanol and incubation in a blocking solution with 5 % milk powder in PBS-T (0.05 % Tween®20) for ca. 1–2 h at RT. After saturation of free binding capacity, membranes were incubated with specific primary antibodies on a shaker for at least 1 h at RT for immunodetection (Western blot) of bound proteins. The antibodies used were diluted in the blocking solution according to the manufacturer's recommendation. In general, hybridoma cell culture supernatants were diluted 1:10 and purified antibodies 1:500–1:1000. For removal of

unbound primary antibodies, membranes were washed three times with PBS-T for 5 min each. Subsequently, membranes were incubated with secondary antibody solution on a shaker for 1 h at RT. The secondary antibodies used (mostly in a dilution of 1:5,000 in PBS-T) were species-specific antibodies coupled to HRP for immunoblot detection. After removal of unbound secondary antibodies and diminishing background reactions (three washes with PBS for 5 min each), antibody-HRP complexes were visualized using a chemiluminescent immunoblotting substrate containing luminol (ECL). This ECL-system enables emission of low intensity light at 428 nm which was detected on films (Konica Minolta, Langenhagen, Germany). Exposed films were then documented and processed using Adobe® Photoshop® CS6 (Adobe Systems Software Irelands Limited, Republic of Ireland).

3.4 Fluorescence microscopy

For confirmation of the presence of specific molecules detected by polypeptide analyses in tissues or cultured cells, immunofluorescence microscopic analyses were performed. Samples were incubated with specific primary and secondary antibodies before they were analyzed by immunofluorescence microscopy.

3.4.1 Fixation of cultured cells

For optimal adhesion and growth cultured cells were grown on glass coverslips coated with poly-L-lysine. Cells were gently washed twice with 37°C pre-warmed PBS to remove residual medium, floated cells and cell aggregates. Thereafter, cells were fixed either by incubation with -20°C cold methanol and acetone or in 2 % formaldehyde, freshly prepared from paraformaldehyde powder, in PBS, pH 7.4 (see Table 8).

3.4.1.1 Methanol/acetone fixation

Cells grown on coverslips and washed twice with PBS were fixed either with -20°C cold acetone for 10 min or with -20°C cold methanol for 5 min and subsequently in -20°C cold acetone for 30 sec or in a -20°C cold methanol/acetone mixture (1:1) for 10 min. After fixation, cells were air-dried and either used directly for immunofluorescence microscopic analyses or stored at -20°C.

3.4.1.2 Formaldehyde fixation

After two washing steps with PBS, cells grown on coverslips were incubated for 3–5 min in 2 % formaldehyde in PBS (w/v). Fixed cells were washed twice with PBS for 5 min and used immediately for immunofluorescence microscopy. For this, the cells were incubated twice in 50 mM NH_4Cl in PBS to saturate free reactive aldehyde groups (for subsequent immunofluorescence microscopy, see chapter 3.4.4).

3.4.2 Preparation and fixation of snap-frozen tissue

For localization of antigens in frozen tissue samples, frozen specimens were cut with a cryotome into ca. 4–7 µm thin sections at -20°C. Immediately thereafter the sections were placed on Menzel Super Frost glass object slides (Thermo Fisher Scientific) and air-dried at RT for about 1 h. For an initial histological investigation some sections were treated with 0.5 % methylene blue (w/v), washed with H_2O_{dest} and controlled in a light microscope for quality of the chosen tissue sample. For immunofluorescence microcopy sections were fixed with either acetone or a methanol/acetone mixture (1:1; -20°C), air-dried at RT for 5 min and then used directly. Alternatively, samples were fixed with formaldehyde as described (chapter 3.4.1.2), depending on antigen and antibody accessibility.

For tissue fixation, different methods were tested to find the specific optimal condition for immunofluorescence microscopy, considering antibody accessibility as well as quality of tissue sections and specimens. In most cases optimal condition for preservation of testicular tissue structures was obtained after treatment with a -20°C cold methanol/acetone mixture (1:1) for 10 min.

3.4.3 Preparation of paraffin-embedded tissue samples

In parallel to the preparation of cryostat sections, formaldehyde-fixed and paraffin-embedded samples were used. For this, paraffin-embedded tissue samples were pre-cooled at -20°C for 1–2 h and cut into ca. 5 µm thin sections using a microtome, placed on Menzel Super Frost glass object slides and dried overnight at 37°C in a heating chamber. Thereafter sections were either used directly or stored in a dark and dry place at RT.

After formaldehyde fixation and paraffin-embedding of tissue samples, the tissue structures in general are relatively well preserved but the accessibility of their antigens may be reduced. To overcome this problem, different methods for antigen-demasking exist to reveal protein epitopes to the antibodies (for “antigen retrieval”; see, e.g., Shi et al*.* 1991; Giberson and Demaree 2001; Rickelt et al*.* 2010). To achieve this, tissue sections were deparaffinated twice with xylene and rehydrated in a decreasing ethanol series (2x 100 %, 1x 95 %, 1x 80 %, 1x 70 % and 1x 50 % ethanol, each step for 5 min). Following a washing step with H_2O_{dest}, sections were transferred into PBS. Using a microwave-assisted method, sections were treated in a special microwave pressure cooking pot with appropriate buffer and temperature (e.g., Tris-HCl buffer at pH 10.2 for 20 min at 120°C). After treatment, the pot was cooled down for 12 min with cold water. Samples were transferred into PBS again for subsequent immunofluorescence labeling (see chapter 3.4.4).

For documentation of paraffin-embedded tissue samples, hematoxylin-eosin (HE) staining (Mayer 1891) was performed and analyzed in a light microscope. This staining method enabled investigations of tissue preservation and morphology. To this end, tissue

sections were deparaffinated, rehydrated in a decreasing alcohol series and then incubated for 5 min in hematoxylin staining solution (Chroma, Köngen, Germany). After a washing step with H_2O_{dest}, samples were rinsed in 10 % acetic acid, washed again and incubated for 3 min in eosin staining solution (Chroma). After additional washing, samples were dehydrated in an increasing ethanol series and treated with xylol before the samples were embedded in Eukitt. Documentation was realized with an Axiophot microscope.

In cases of special difficulties with epitope masking methodological procedures as described for cell type identification pathology have been applied (for details see Moll 1993).

3.4.4 Immunofluorescence microscopy and documentation

Fixed sections from snap-frozen tissue samples and cultured cells were rinsed with PBS after air-drying. For optimal epitope accessibility sections were treated with 0.1 % Triton-X-100 in PBS (w/v) or 0.1 % saponin in PBS (w/v) for 4–5 min to permeabilize the cells. In contrast, dehydrated and antigen retrieval-treated sections were incubated in 2 % milk powder with 0.1 % Triton-X-100 in PBS (w/v) for 20 min, respectively.

After further 2–3 washing steps with PBS (5 min each) at RT, specimens were incubated with the primary antibody solution for 1 h at RT in a humid chamber (purified antibodies were diluted according to the manufacturer's recommendation, supernatants were used undiluted). Unspecifically bound antibodies were removed by three washings with PBS (5 min each). Subsequently, tissue samples were incubated with secondary antibodies conjugated with fluorochromes for 45 min (Cy3: 1:500, Alexa 488: 1:250) specific for the species of the primary antibodies used. In addition, nuclear chromatins were often counterstained using DAPI (1:10,000 dilution) along with the secondary antibodies. For double-immunofluorescence microscopy, the primary and the secondary antibodies were used in double concentration, respectively. Unbound antibodies were removed by three washes with PBS (5 min each). Specimens were rinsed in H_2O_{dest} to remove salt crystals which had remained from PBS. Then the tissue sections were dehydrated in 100 % ethanol, air-dried and finally mounted with Fluoromount G (Southern Biotech; obtained via Biozol Diagnostica, Eching, Germany) using cover glasses and air-drying overnight under protection from light.

For documentation of immunofluorescence microscopy an Axiophot microscope as well as a confocal laser scanning microscope LSM880 with Argon-laser (488 nm) and a Helium-Neon-laser (534 nm) were used to localize specific antigens in defined optical sectional planes. Micrographs were visualized with an AxioCam MRc-camera. Comprehensive analysis and image processing were ensued using ZEN 2012 microscope software (Version 8.1, Carl Zeiss Microscopy GmbH) and Adobe® Photoshop® CS6 software. Images were partially combined using the Tile scan tool.

3.5 Electron microscopy

For ultrastructural analyses transmission electron microscopy (TEM) of ultrathin tissue sections was used (cf. Rickelt et al. 2011b; Pieperhoff et al. 2012).

3.5.1 Conventional transmission electron microscopy

Immediately after preparation, freshly obtained tissue samples (about 1 mm^3) were fixed in 2.5 % glutardialdehyde solution (in sodium cacodylate buffer, v/v, pH 7.2; Serva, Heidelberg, Germany; Table 10) for 30 min at 4°C. After three washings (5 min each) in sodium cacodylate buffer (50 mM, pH 7.2), an incubation in 2 % osmium tetroxide solution in sodium cacodylate buffer (v/v) was followed for 2 h at 4°C in order to fix lipids, in particular lipid membranes (for reviews see Hayat 1970; Glauert and Reid 1974). Specimens were washed three times with $H_2O_{aqua\ ad.\ injectabila}$ ($H_2O_{aqua\ ad.\ inj.}$; B. Braun Melsungen AG, Melsungen, Germany) for 5 min and incubated in 0.5 % uranyl acetate in $H_2O_{aqua\ ad.\ inj.}$ (w/v) overnight at 4°C. After another washing specimens were dehydrated in an increasing ethanol concentration series (50 %, 70 %, 80 %, 90 %, 96 % ethanol, 30 min each at 4°C). Then samples were dehydrated twice with 100 % ethanol and propylene oxide at RT (30 min each). For embedding into the artificial resin Epon 812 (Serva) tissue pieces were infiltrated with a 1:1 (v/v) mixture of Epon and propylene oxide at RT overnight. Tissue samples were rotated to ensure complete and rapid penetration of the resin into the tissue, while the propylene oxide slowly evaporated enabled by an open lid. The following day, tissue pieces were incubated in fresh Epon solution for another 4–6 h. Embedded tissue samples were put in silicon rubber molds (Plano, Wetzlar, Germany) filled with fresh Epon solution and incubated for 24 h at 60°C and thereafter for additional 48 h at 60°C after release of the silicon rubber mold for polymerization and maximal hardening of the resin.

For the initial histological investigations semi-thin sections were stained before ultrathin sectioning in order to define quality and area of the chosen tissue sample. For this, semi-thin sections were treated with staining solution (1 % toluidine blue, 1 % sodium borate, 1 % azure II [w/v] in $H_2O_{aqua\ ad.\ inj.}$), heated on a hot plate until a silver-green staining was visible around the sample and washed with $H_2O_{aqua\ ad.\ inj.}$ before the light microscopic observations started.

Using an ultramicrotome, ultrathin sections of 50–70 nm of chosen samples were prepared, put onto small copper grids, gently dried with a cloth and finally air-dried. The grids were covered with a thin film of 1 % pioloform in chloroform (w/v) to ensure adhesion and stability of the tissue sections. For “contrasting”, a method according to Reynolds et al. (1963) was applied: Sections were incubated in 2 % uranyl acetate in methanol for 15 min. After several washing steps (1x in methanol, 1x in methanol with $H_2O_{aqua\ ad.\ inj.}$ [1:1], and 8 short washing steps in $H_2O_{aqua\ ad.\ inj.}$) an incubation in lead citrate

solution followed for 5 min. Finally, the grids with the sections were washed again eight times with $H_2O_{aqua\ ad.\ inj.}$, gently dried with a cloth and air-dried.

Ultrathin sections were observed with an electron microscope EM10 or EM900 (Zeiss) at 80 kV. For documentation Kodak-electron microscopy negative films (No. 4489; 3.25 x 4 inch; Sigma-Aldrich) were developed (developer D19 Kodak and fixer Agefix Agfafoto 51) in a darkroom and processed using Adobe® Photoshop® CS6. Digital images were recorded by a Slow Scan CCD camera (Typ 7888, TRS).

3.5.2 Immunoelectron microscopy

For immunoelectron microscopy analysis, a “pre-embedding” method was performed. To this end, appropriate snap-frozen tissue sections (about 7 µm) were prepared, placed on coverslips and air-dried at RT for about 1 h. Specimens were fixed with freshly prepared 2 % formaldehyde in PBS (pH 7.4) for 5–10 min at RT and then washed with PBS. Free aldehyde groups were saturated by immediate incubation in 50 mM NH_4Cl in PBS (w/v) twice for 5 min each. Thereafter, samples were permeabilized by detergent treatment with 0.1 % saponin in PBS for 5 min and washed three times with PBS for 5 min before incubation with the specific primary antibodies in a humid chamber for 3 h at RT took place using dilutions as in immunofluorescence microscopy. After removal of unbound antibodies with three PBS washing steps (5 min each), an incubation followed with a Nanogold-coupled secondary antibodies (Nanoprobes, Yaphank, NY, USA) specific for the species of the primary antibodies used (overnight at 4°C). Tissue sections were then fixed with 2.5 % glutardialdehyde solution (RT) for 30 min (cultured cells for only 15 min) at 4°C. Then the specimens were shortly washed with cacodylate buffer (50 mM) and incubated twice in a sucrose solution for 3 min each. A silver enhancement reaction with gold particles followed using a “HG Silver™ Enhancement Kit“, choosing three different time points (in most cases 6 min, 7 min and/or 8 min). This enhancement step was stopped by two washing steps for 5 min each with sodium thiosulfate pentahydrate solution (250 mM sodium thiosulfate pentahydrate in 50 mM HEPES) followed by washes with $H_2O_{aqua\ ad.\ inj.}$. To enhance membrane contrast, incubation followed in 0.3–3.0 % osmium tetroxide in $H_2O_{aqua\ ad.\ inj.}$ for 30 min at 4°C. After two additional gentle washing steps in $H_2O_{aqua\ ad.\ inj.}$ specimens were dehydrated in an increasing ethanol series (compare chapter 3.5.1) and then embedded into Epon, using gelatin capsules filled with Epon which were placed over the coverslip so that the tissue sections were covered with Epon. After incubation and polymerization overnight at 60°C, coverslides were removed from the tissue sections by a temperature gradient after freezing in liquid nitrogen for a few minutes (cf. Franke et al. 1978b). Specimens were polymerized for two further days and then sectioned for TEM (compare chapter 3.5.1).

4 Results

In my initial studies of the adherens junctions (AJs) in the seminiferous tubules of various mammalian species several results differed fundamentally from the published results in the majority of other research reports in this field (see also Introduction). Consequently, in respect to this controversy my doctoral thesis work comprised a profound and systematical analysis of the molecular composition and ultrastructural organization of the isotypic AJs connecting the cells in the seminiferous tubules as well as the cells that form the peritubular walls in different mammalian species (human, bovine, porcine, guinea pig, murine, i.e. rat and mouse). For direct comparison of the seminiferous tubule tissue with "true" epithelial tissues, the adjacent excurrent duct tissue, including the epididymis, was used. In addition, various other epithelial tissues (bladder, intestine, liver, tongue mucosa and bovine muzzle) and different types of smooth muscle tissues (e.g., those of the bladder, intestine, stomach, oesophagus and blood vessel walls) were used as control tissues.

For an initial morphological overview of testicular tissues of the different mammalian species tested, hematoxylin-eosin (HE) staining of thin sections of frozen tissues was used as a basis for further analyses. Main methods included light and electron microscopical immunolocalization of diverse structural proteins using specific antibodies. The sensitivity and specificity of the antibodies used were analyzed by SDS-PAGE and immunoblotting of protein lysates from dissected tissue samples or subcellular fractions. The molecular composition of testicular tissues of the species studied was in most points identical, unless mentioned otherwise.

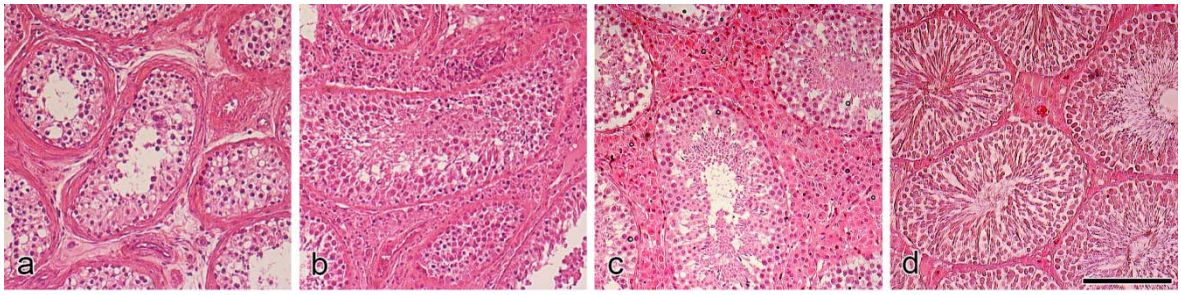


Fig. 4 Light microscopical overviews of cross-sections through seminiferous tubules of different species visualized by hematoxylin-eosin (HE) staining. Slightly different histological architecture is seen here in the tubules of (**a**) man, (**b**) bull, (**c**) boar and (**d**) rat testis. Bar 200 µm.

4.1 Histological aspects of the testicular tissues

Light microscopical analyses of testicular tissue samples of the mammalian species examined show an overview of seminiferous tubules containing Sertoli cells which protrude from the basal lamina into the lumen of the tubules and are associated with germ cells of different spermatogenic stages (see also Fig. 1). The basal lamina is tightly

associated with the monolayers of the cells of the peritubular wall which are interspersed with layers of extracellular matrix (ECM) material. The peritubular wall borders on the interstitial compartment with its various cell types (Fig. 4; for details see chapter 4.6).

4.2 Biochemical identification of proteins and glycoproteins in testicular tissues

For the identification and characterization of proteins and glycoproteins of cell-cell junctions as well as of cytoplasmic filaments and other cytoskeletal elements, whole tissue lysates were analyzed by SDS-PAGE and immunoblotting (Western blot). In a number of cases, specifically to exclude false positive or negative results because of limited availability or reactivity of epitopes or cross-reactive epitopes, tissue pieces were further fractionated and the specific enriched fractions were separately examined by SDS-PAGE.

For biochemical and immunolocalization analyses different cell culture lines were used as positive controls in addition to the epithelial tissues listed above. These included epithelial cell culture lines such as human HaCaT keratinocytes, PLC hepatocellular carcinoma cells and Caco-2 intestinal cells as well as normal and SV40-transformed fibroblast cells (SV80).

Major results obtained are presented in Fig. 5 (and summarized in Table 13 in chapter 4.4), confirming results of parts of my initial studies (Domke 2013). Reactions specific for the cadherin glycoprotein families of desmogleins and desmocollins, i.e. in particular for Dsg-2 and Dsc-2, as well as for the desmosomal plaque proteins desmoplakin and plakophilin Pkp-2 were negative in the tissue samples of the seminiferous tubules but positive in the excurrent duct tissues, including the epididymis. Vimentin, as the major intermediate-sized filament (IF) component of Sertoli cells but also present in interstitial and peritubular wall cells, was detected in all tissue samples of the seminiferous tubules analyzed. Likewise, antibody reactions against AJ plaque proteins, including α- and β-catenin, proteins p120 and/or p0071, or against the AJ transmembrane glycoproteins cadherin-11 and N-cadherin were intensely positive in whole tissue lysates of seminiferous tubules. Particularly, positive antibody reactions against the mesenchymal marker protein N-cadherin were seen in seminiferous tubules but were absent in excurrent duct epithelial tissues. In comparison, antibodies against E-cadherin, characteristic of homotypical adhesion between AJs of simple epithelial cells, showed no reaction in seminiferous tubule tissues but were positive in tissues of excurrent ducts. In general, smooth muscle marker proteins such as smooth muscle α-actin (α-SMA), smoothelin, desmin, light and heavy chains of smooth muscle myosin, vinculin and talin as well as proteins myozap and LUMA were markedly positive in all samples tested (see also chapter 4.3).

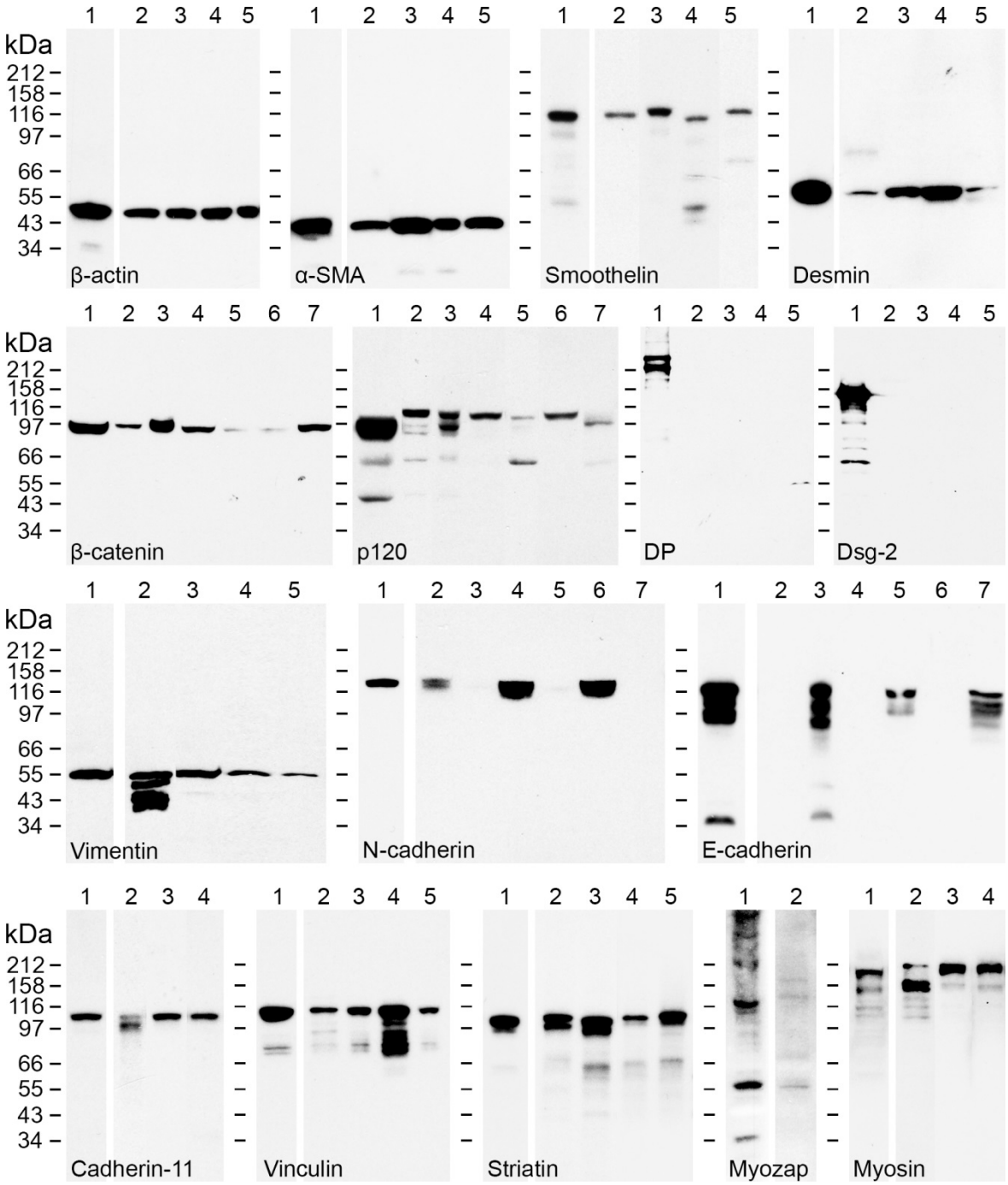


Fig. 5 Biochemical identification of proteins in mammalian testicular tissue samples (seminiferous tubules, including the peritubular wall structures) and the corresponding control tissues, using SDS-PAGE of total protein lysates, followed by immunoblot analyses with specific antibodies (for continuation see next page).

Continuation of Fig. 5

Antibody reactions against the cytoskeletal control protein **β-actin** (MW ca. 43 kDa), as reference for equal loading of proteins, reveal a single polypeptide band in cultured human HaCaT keratinocytes (lane 1) and in the tissue samples of seminiferous tubules of man (lane 2), bull (lane 3), boar (lane 4) and rat (lane 5).

The same is true for the smooth muscle markers **smooth muscle α-actin** (**α-SMA**) and **smoothelin** (MW ca. 99 kDa) in all samples of tissues containing seminiferous tubules (lanes 2–5, as afore) as well as for bovine bladder tissue examined for control (here in lane 1). **Desmin** is as well present in the tissue samples containing seminiferous tubules (lanes 2–5), even though in different quantities, and in cultured adult mouse cardiomyocyte-derived cells of line HL-1 (lane 1).

The adherens junction (AJ) plaque protein **β-catenin** is found in all samples tested, including human HaCaT keratinocytes (lane1), tissues of bull testis (lane 2) and bull epididymis (lane 3), boar testis (lane 4) and boar epididymis (lane 5), rat testis (lane 6) and rat excurrent duct tissue (lane 7), although in different relative amounts (note here low reaction intensities in lanes 5 and 6). Similarly, the AJ plaque **protein p120** is detected in all samples tested containing human HaCaT keratinocytes (lane1), bull testis (lane 2) and proteins of bull epididymis (lane 3) as well as of boar testis (lane 4), boar epididymis (lane 5), rat testis (lane 6) and rat excurrent duct tissue (lane 7).

In comparison to human HaCaT keratinocytes (lane 1), **desmoplakin** (DP) cannot be recognized in tissue samples containing molecules of seminiferous tubules of bull (lane 2), rat (lane 3), mouse (lane 4,) and man (lane 5). Likewise, **desmoglein-2** (**Dsg-2**) is not found in bull (lane 2), rat (lane 3), mouse (lane 4) and human (lane 5) testicular tissues containing seminiferous tubules, in contrast to its abundance in human HaCaT keratinocytes (lane 1). Negative results have also been obtained with Dsg-1 and Dsg-3 and for corresponding desmocollins (not shown).

Antibodies against **vimentin** (MW ca. 54 kDa) show positive reactions in tissue materials of seminiferous tubules of human (lane 2), bovine (lane 3), rat (lane 4) and mouse (lane 5) testes as well as in proteins of cultured human SV80 cells of mesenchymal origin (lane 1) examined in parallel as control protein preparation. Antibodies against the AJ protein **N-cadherin** reveal single polypeptide bands in human HaCaT keratinocytes and in testicular tissue protein samples from seminiferous tubules of bull (lane 2), boar (lane 4) and rat (lane 6) but not in appreciable amounts of epididymis tissue lysates of bull (lane 3), boar (lane 5) and rat (lane 7) origin. In comparison, **E-cadherin** is shown here to be exclusively detected in human epithelial HaCaT cells (lane 1) and excurrent duct tissues of bull (lane 3), boar (lane 5) and rat (lane 7) but not in the enriched testicular tissue samples of seminiferous tubules (lanes 2, 4 and 6 of bull, boar and rat). **Cadherin-11** (**CDH-11**) is identified in the SDS-PAGE results of proteins from seminiferous tubules of man (lane 2), bull (lane 3) and boar (lane 4) as well as in several samples of excurrent duct tissues (lane 1, boar).

The microfilament-associated protein **vinculin** is present in the tissue samples from seminiferous tubules of man (lane 2), bull (lane 3), boar (lane 4) and rat (lane 5) as well as in human heart tissue (lane 1). Protein **striatin** is identified in the tissues of seminiferous tubules of man (lane 2), bull (lane 3), boar (lane 4) and rat (lane 5) origin as well as in fractions of bovine bladder tissue (lane 1). Protein **myozap** (MW ca. 52 kDa) is detected in the testicular tissue of excurrent ducts (lane 1, boar) as well as very weakly in tissue samples containing seminiferous tubules and interstitial tissue of several species (e.g., lane 2, human).

The smooth muscle marker **smooth muscle myosin heavy chain** (**myosin**) is present in the bull excurrent duct system (lane 1) and in the corresponding samples of seminiferous tubules and peritubular as well as interstitial tissue of man (lane 2), bull (lane 3) and boar (lane 4).

4.3 Control tissues: Light and electron microscopical immunolocalization results

For comparison of cells in true epithelia with cells of the seminiferous tubules, samples of adjacent excurrent duct tissues, including the epididymis, of the same species were analyzed. These tissue samples, known to contain simple or columnar epithelia, were examined in this study in addition to various other epithelial tissues such as bladder, intestine, liver, tongue mucosa or bovine muzzle. They were used for the determination of specificity and sensitivity of the antibodies as well as for general controls of methods applied.

As expected, these epithelial control tissues showed a positive reaction for cytokeratin intermediate-sized filaments (IFs) and an absence of vimentin IFs as well as the presence of simple epithelium-type desmosomal proteins such as desmoplakin, plakoglobin, plakophilin-2, desmoglein-2 and desmocollin-2 which revealed complete or far-reaching co-immunolocalization (Figs. 6, 15 and 16). Different localization of E-cadherin and N-cadherin was seen in epithelial tissues in the form of a total absence of N-cadherin and the general presence of E-cadherin in AJs of excurrent duct cells (Fig. 7). Some of the cytoplasmic plaque proteins of epithelial cell-cell junctions were found to occur in all AJ structures of the *zonula adhaerens* and *fascia adhaerens* type as well as in *puncta adhaerentia*, including α- and β-catenin (e.g., Figs. 8 and 9), and protein p120. In contrast, certain other plaque proteins such as protein myozap (Figs. 8 and 9), protein PERP and certain members of the striatin family appeared to be present only in the plaques of the subapical *zonula adhaerens* but were absent in lateral punctate AJs. In some tissue samples of the excurrent ducts another cell junction type containing the glycoprotein EpCAM was detected (Fig. 10).

The epithelial excurrent ducts are surrounded by a basal lamina and in addition in the anterior portion by a smooth muscle wall of increasing thickness in *ductuli efferentes* and *ductus epididymidis* (Fig. 2c; cf. Baumgarten et al. 1971). These muscle cells were positive for major smooth muscle markers, including smooth muscle α-actin (α-SMA) and the corresponding myosin light and heavy chains as well as α-actinin, tropomyosin, smoothelin, desmin, vinculin, filamin A, talin, calponin and SM22α (e.g., Figs. 11–14; for more details see chapter 4.6; for general references see chapter 5.5). In addition, protein LUMA showed positive reactions in smooth muscle wall cells of excurrent duct tissues (Figs. 15–16).

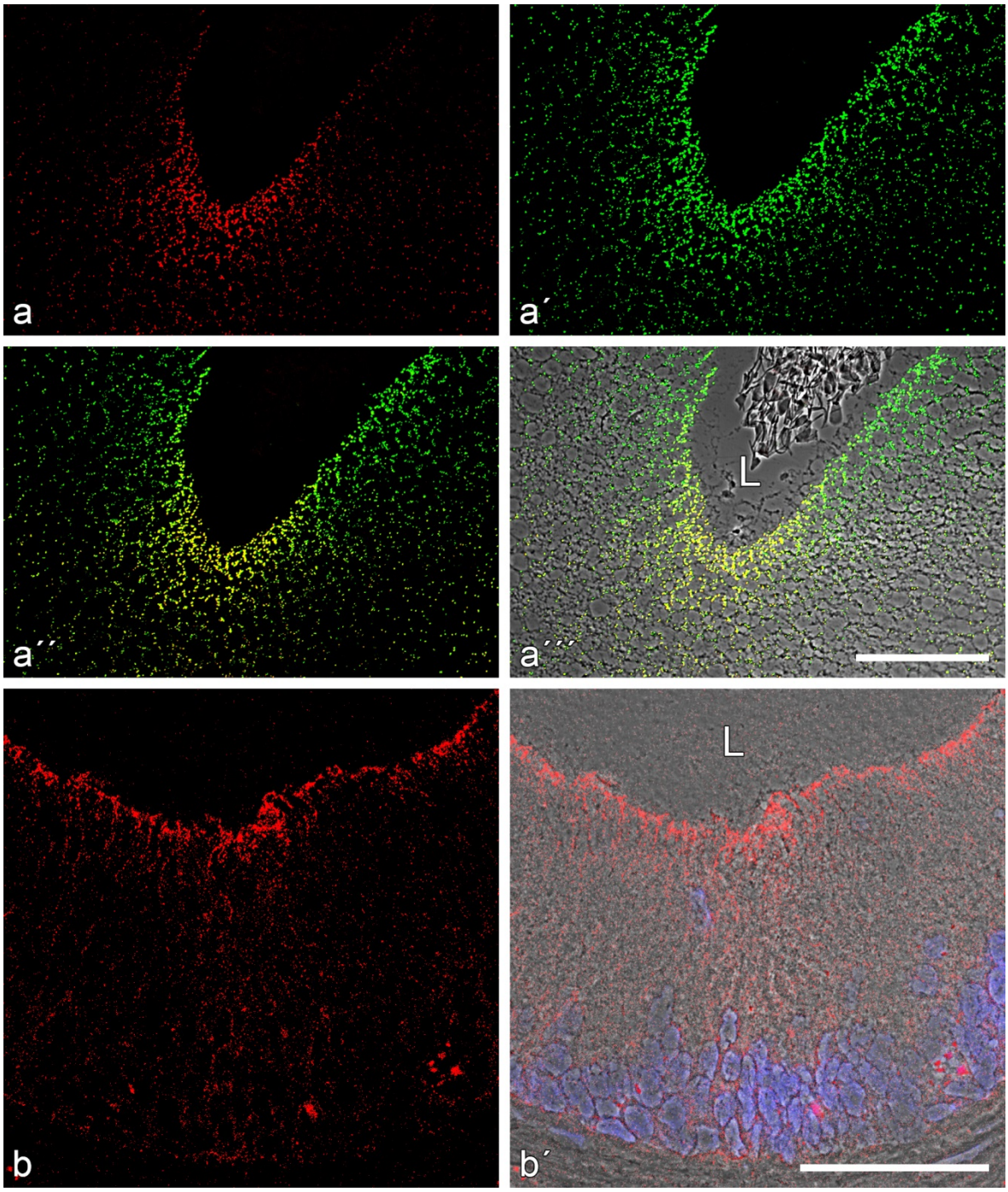


Fig. 6 Double-label immunofluorescence microscopy of an oblique cryotomy cross-section through parts of the excurrent duct system in frozen bull testis, displaying positive immunostaining for **desmoglein-2** (**Dsg-2**; **a**, **a''**, **a'''**; *red*; monoclonal mouse antibody) and **desmoplakin** (**DP**; **a'**–**a'''**; *green*; rabbit antibodies) with colocalization of both desmosomal marker proteins (*yellow* merger colour; reactions are shown on a phase-contrast background in **a'''** and **b'**). Note the spermatozoa in the lumen (L). The monoclonal mouse antibody against **plakophilin-2** (**Pkp-2**; **b**, **b'**; *red*) shows also positive immunostaining of desmosomal structures in the excurrent duct epithelium. Nuclei have been stained *blue* with DAPI. *Bars* 50 µm.

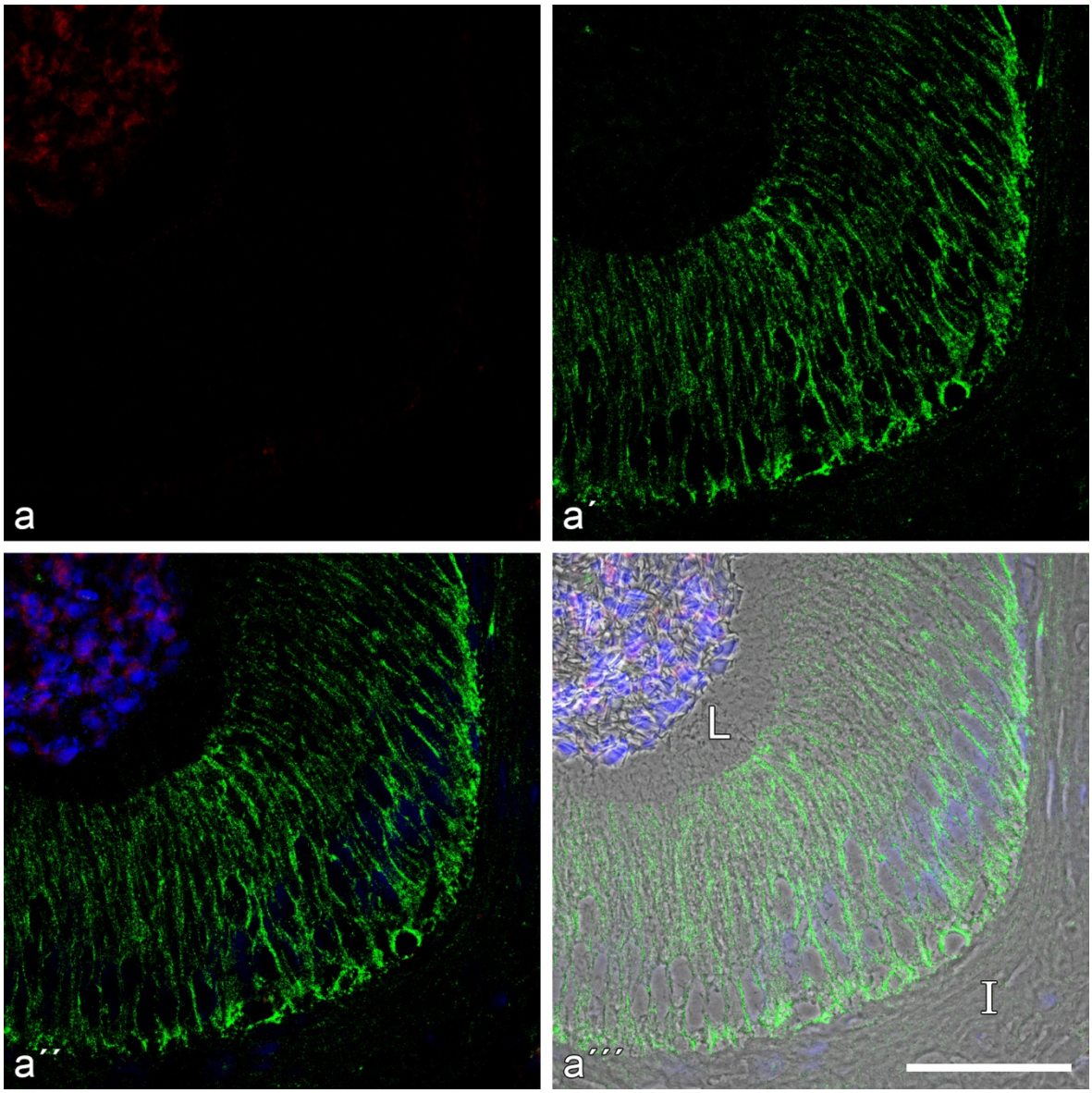


Fig. 7 Double-label immunofluorescence microscopy of a cross-section through the excurrent duct system of bull testis after reactions with antibodies against the adherens junction (AJ) proteins **N-cadherin** (**a**, **a''**, **a'''**; *red*; monoclonal mouse antibody) and **E-cadherin** (a'–a'''; *green*; rabbit antibodies). All tubular epithelial cells are intensely positive (*green*) for E-cadherin but totally negative for N-cadherin (*red*). Nuclei have been stained *blue* with DAPI. I, interstitial space; L, lumen. *Bar* 50 µm.

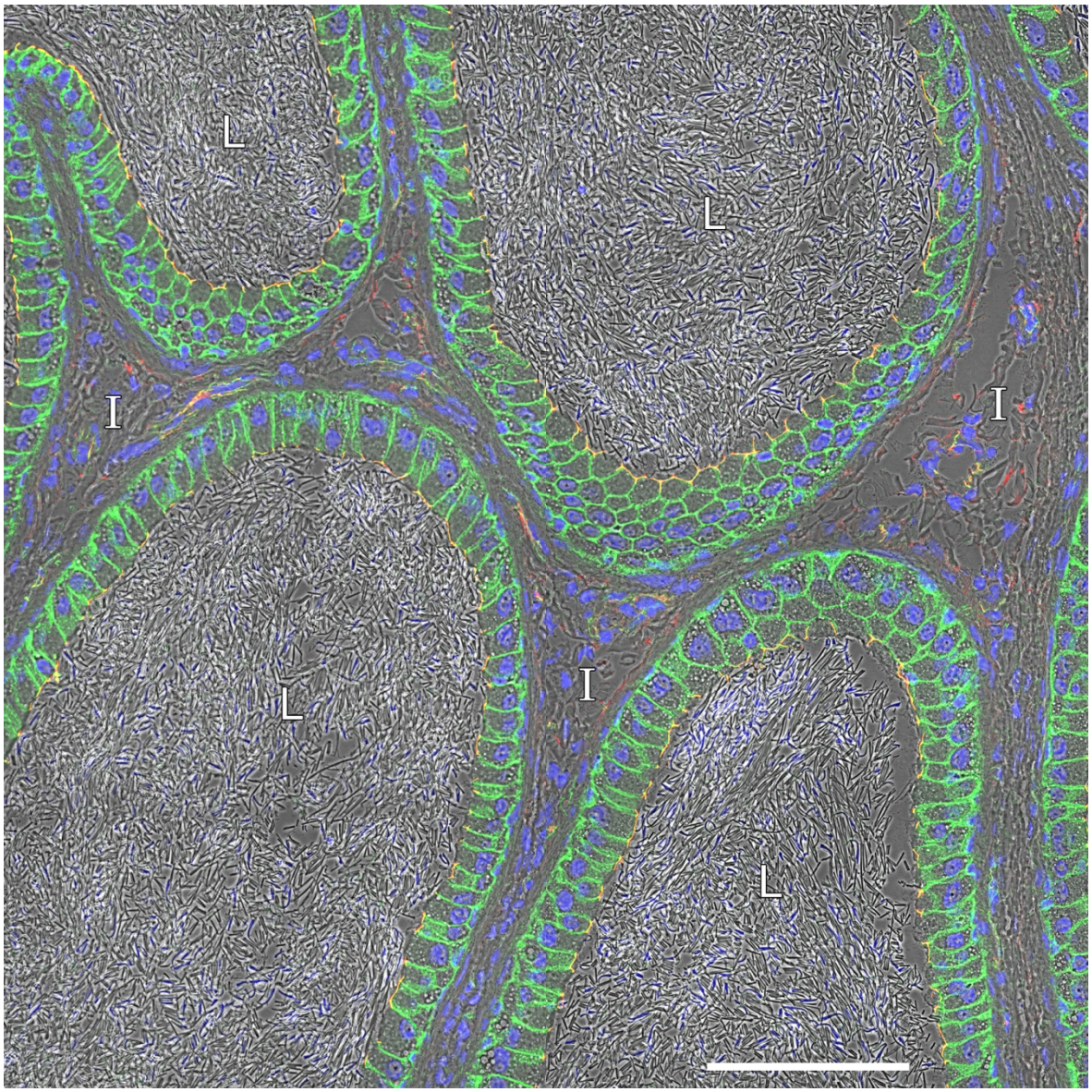


Fig. 8 Double-label immunofluorescence microcopy of a cryostat cross-section through frozen rat testis tissue, showing excurrent ducts after reactions with antibodies to protein **myozap** (*red*; monoclonal mouse antibody) and to the *armadillo* plaque protein **ß-catenin** (*green*; rabbit antibodies). Protein myozap reaction is seen in the subapical *zonula adhaerens* of the epithelial cells (seen here in *yellow* merge colour) and in some cells of the interstitial tissue (I) but is not reliable detectable in lateral membrane junctions of the epithelia. Note that the ductal lumen (L) is filled with masses of aggregated spermatozoa. The general structure is revealed on a phase-contrast background. Nuclei have been stained *blue* with DAPI. *Bar* 100 µm.

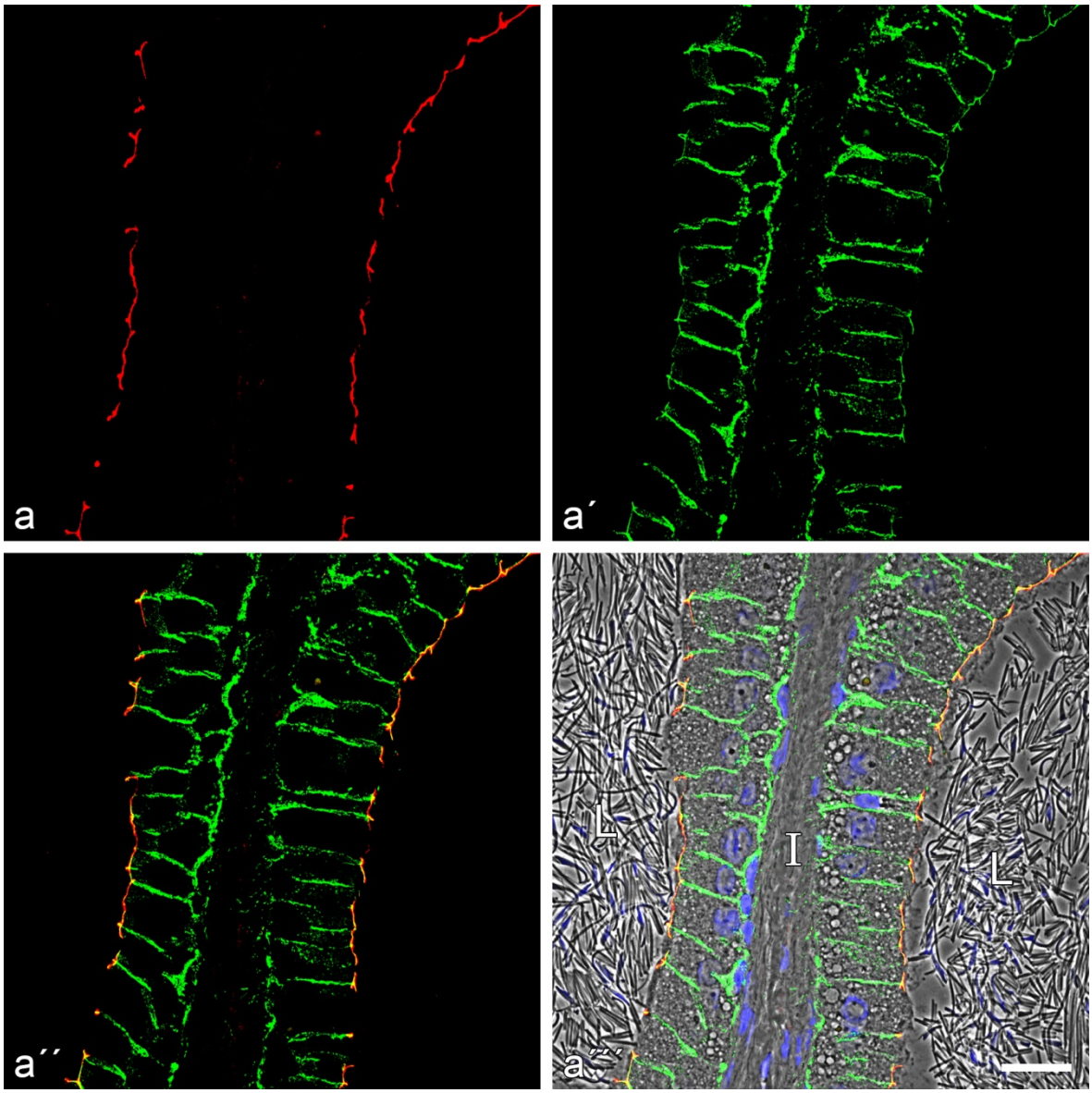


Fig. 9 Double-label immunofluorescence microscopy displaying the specific immunostaining of the adherens junctions (AJs) connecting the excurrent duct epithelial cells of rat testis. Immunoreactions with protein **myozap** (**a**, **a''**, **a'''**; *red*; monoclonal mouse antibody) and the *armadillo* plaque protein **β-catenin** (**a'**–**a'''**; *green*; rabbit antibodies) indicate that ß-catenin is present in both the subapical *zonula adhaerens* and in the numerous AJs along the lateral membrane-membrane contacts. By contrast, protein myozap is seen only in the subapical zonula but is not detectable in the lateral membrane-membrane junctions. Nuclei have been stained *blue* with DAPI. I, interstitial space; L, lumen. *Bar* 20 µm.

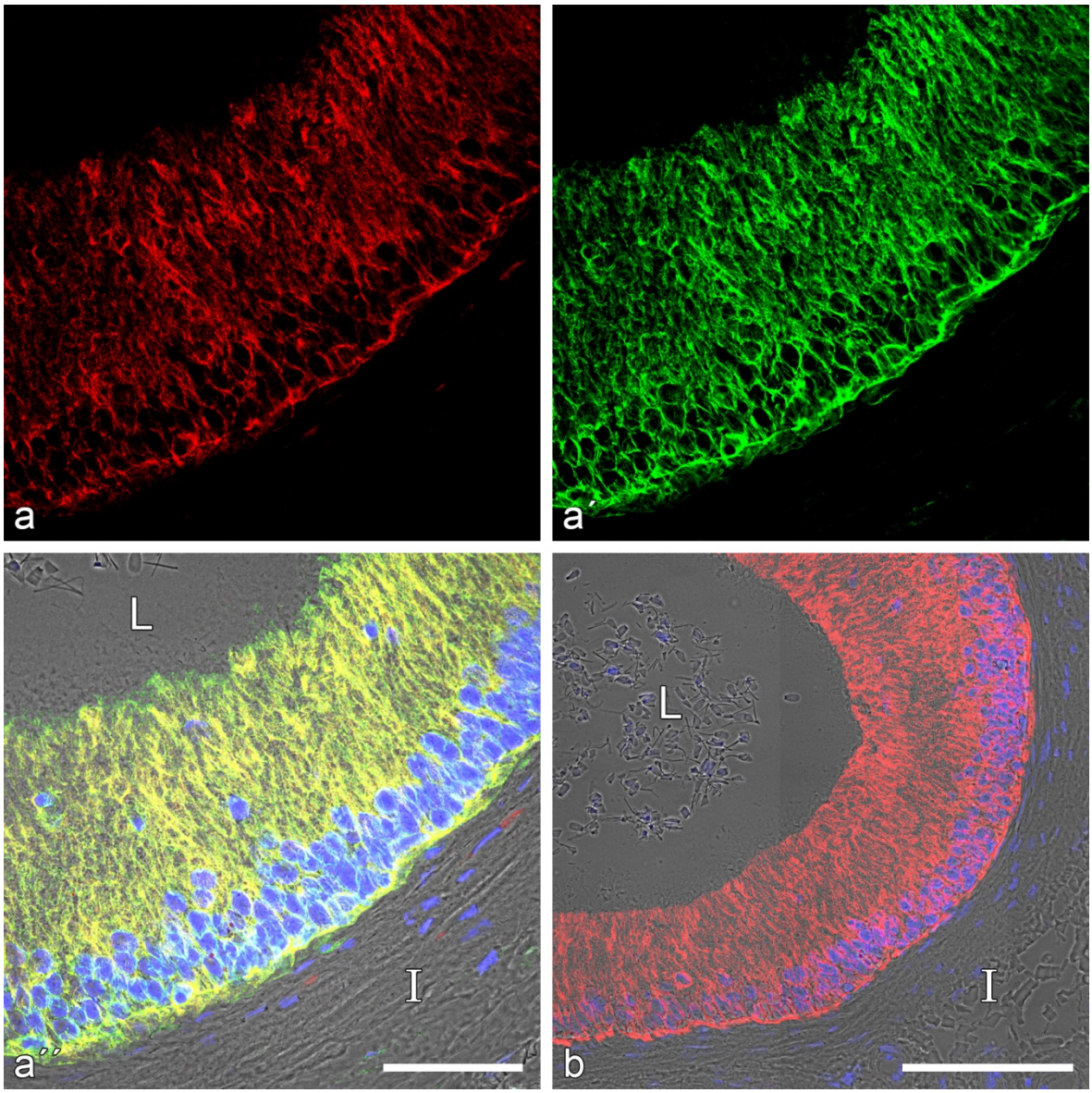


Fig. 10 Double-label immunofluorescence microscopy of cryostat cross-sections through a frozen bull testis tissue region containing excurrent duct epithelia, after reaction with antibodies to **EpCAM** (**a**, **a''**; *red*; monoclonal mouse antibody) and two different antibodies to **cytokeratins 8 and 18** (**a'**, *green*; guinea pig antibodies; **b**, *red*; monoclonal mouse antibody). Note the colocalization in all epithelial cells of the excurrent duct system (*yellow* merge colour). (**a''**, **b**) Cell structures are seen on a phase-contrast background. Nuclei have been stained *blue* with DAPI. Note also the aggregates of spermatozoa in the lumen (L; **a''**, **b**). I, interstitial space. Nuclei have been stained *blue* with DAPI. *Bars* (**a**–**a''**) 50 µm and (**b**) 100 µm.

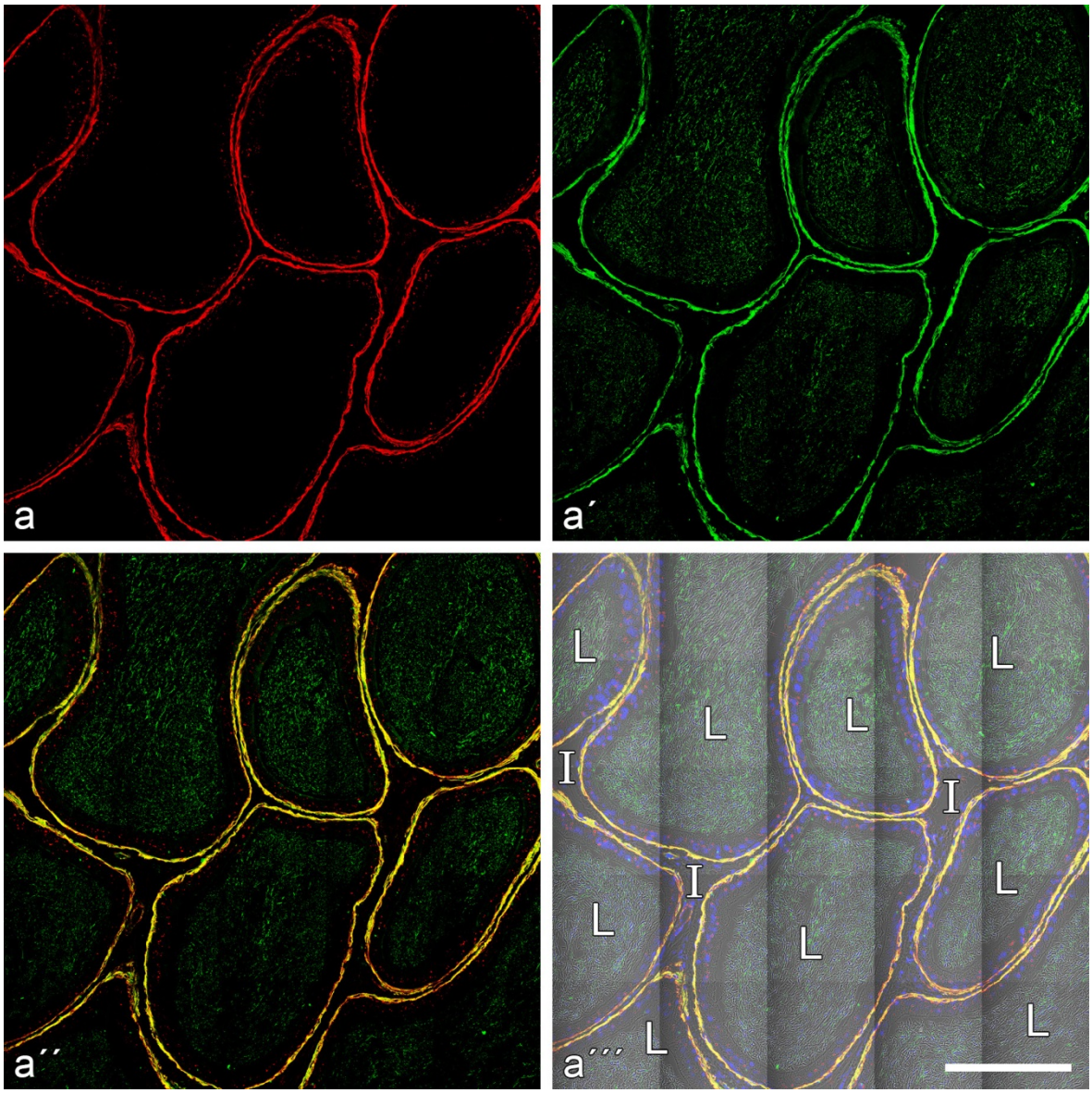


Fig. 11 Double-label immunofluorescence microscopy of a cryostat cross-section through the epithelium of parts of the excurrent duct system of frozen rat testis visualized in a tile scan survey arrangement. Antibody reactions with the smooth muscle cell (SMC) marker **smooth muscle α-actin** (**α-SMA**; **a**, **a**'', **a**'''; *red*; monoclonal mouse antibody) and the general muscle cell marker **tropomyosin** (**a'–a'''**; *green*; rabbit antibodies) are presented. Both markers show positive immunoreactions in the peritubular wall LSMCs encasing the tubules ("colocalization" is indicated by *yellow* merge colour). Note the accumulation of maturing spermatozoa in the tubule lumen (L). I, interstitial space. *Bar* 200 µm.

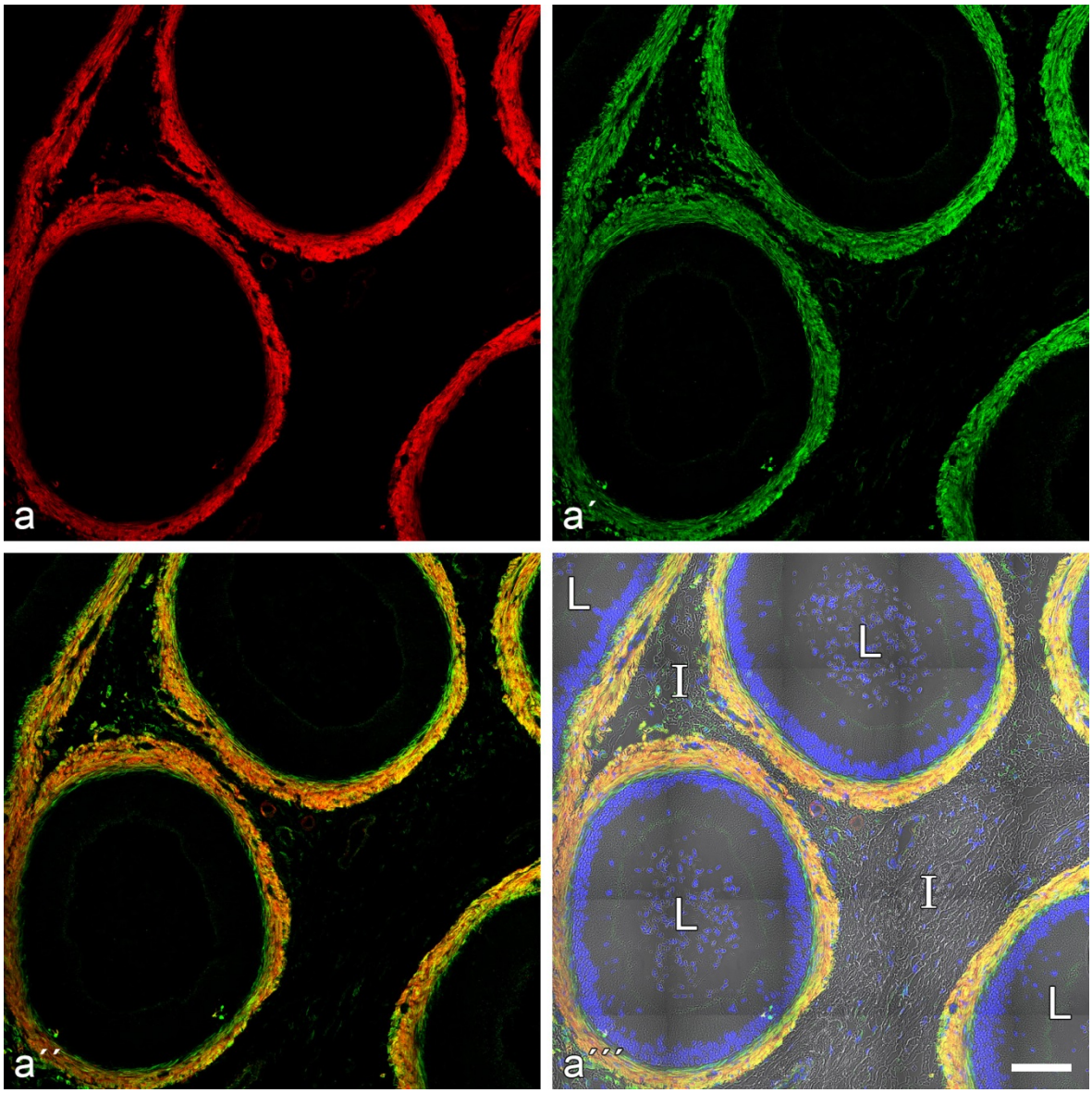


Fig. 12 Tile scan survey of double-label immunofluorescence microscopy of a cryostat cross-section through an anterior portion of the excurrent duct system of frozen bull testis. Immunoreactions with the smooth muscle cell (SMC) markers **smoothelin** (**a**, **a**'', **a**'''; *red;* monoclonal mouse antibody) and **desmin** (**a'**–**a'''**, *green*; rabbit antibodies) show immunostaining of both SMC markers in the peritubular wall cells encasing the excurrent ducts (colocalization is indicated by *yellow* merge colour). Nuclei have been stained with DAPI (*blue*). I, interstitial space; L, lumen. *Bar* 100 µm.

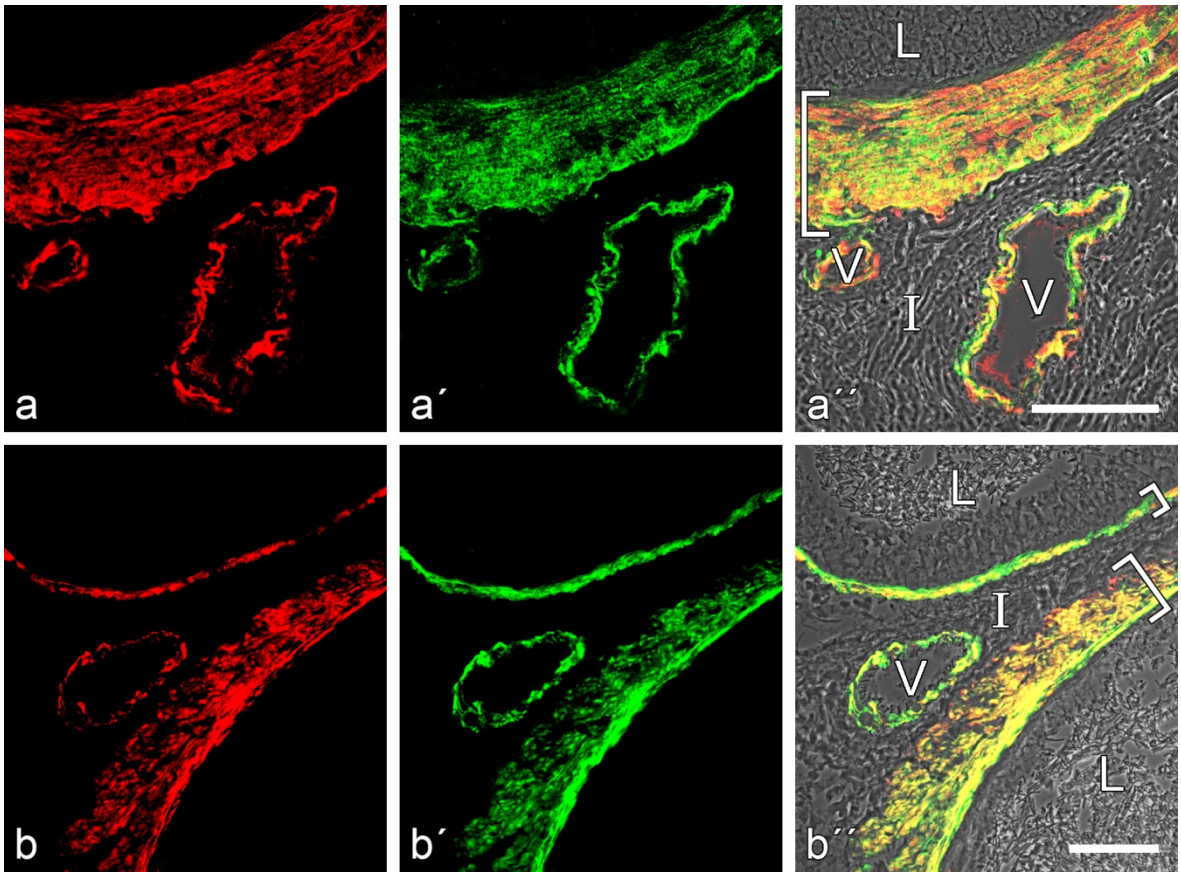


Fig. 13 Double-label immunofluorescence microscopy of a cryostat cross-section through a subsequent part of the excurrent duct system of frozen (**a**) bull and (**b**) rat testis. Positive "colocalization" for **smooth muscle myosin heavy chain** (**a**–**a''**; *red*; monoclonal mouse antibody), **caldesmon** (**b**–**b''**; *red*; rabbit antibodies) and **smooth muscle α-actin** (**α-SMA**; **a'**–**a''**, *green*; rabbit antibodies, **b'**–**b''**; *green;* monoclonal mouse antibody) is seen in the rather thick peri-epithelial SMC walls (denoted by brackets) and the perivascular (V, vessel lumen) wall cells of the interstitial region (I). L, lumen. *Bars* 50 µm.

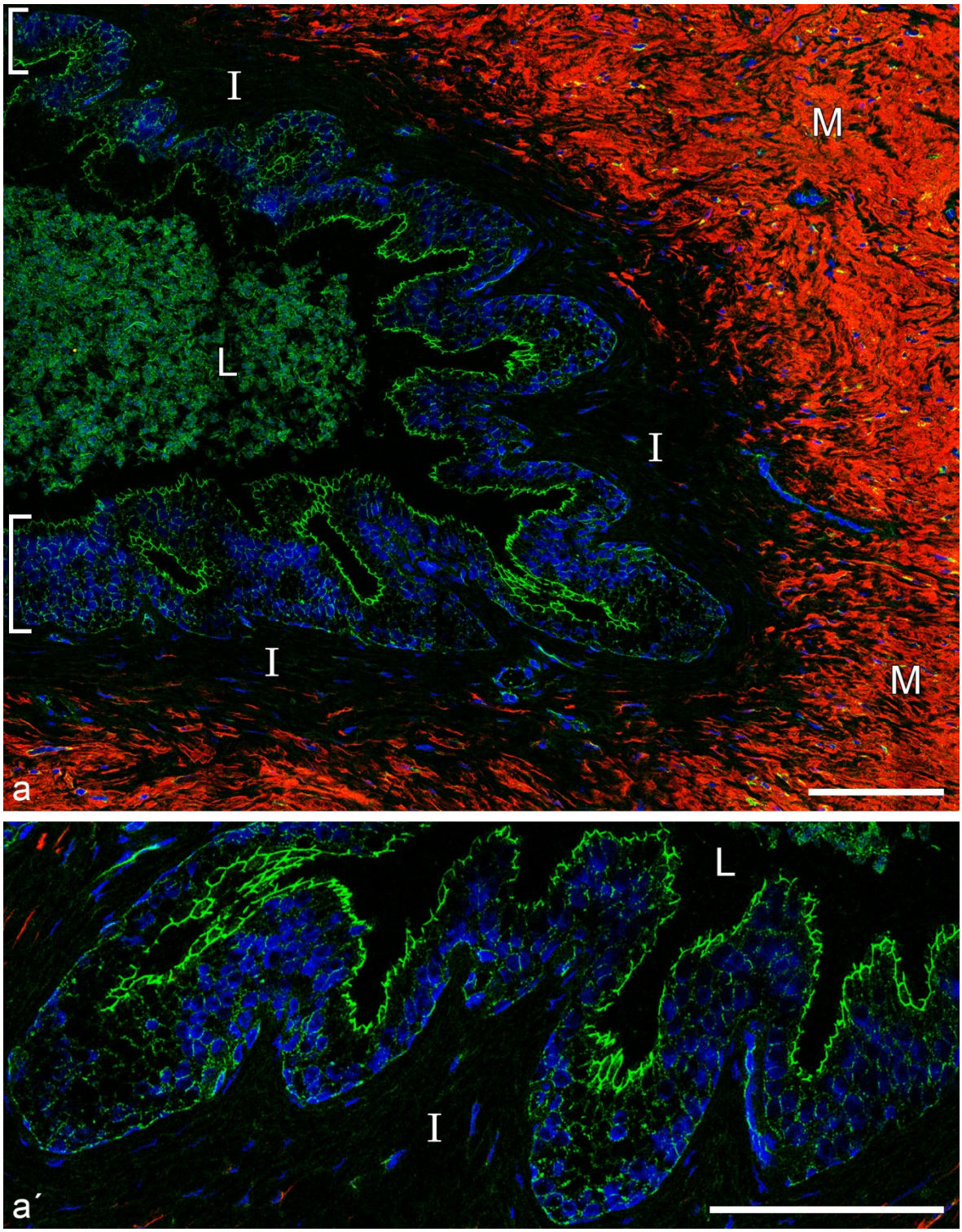


Fig. 14 Double-label immunofluorescence microscopy of a cryotomy cross-section through an anterior epididymal part of the excurrent duct system of frozen boar testis after immunolocalization reactions against **smoothelin** (**a**, **a'**; *red*; monoclonal mouse antibody) and protein **myozap** (**a**, **a'**; *green*; guinea pig antibodies). Note the intense and extended positive reaction of smoothelin in the thick smooth muscle (M) wall tissue separated from the epithelium (brackets) by a mesenchymal cell-rich *lamina propria* and an interstitial (I) region, here in comparison with the specific myozap immunostaining of the *zonula adhaerens* of the excurrent duct epithelium. Note also the aggregates of spermatozoa in the ductal lumen (L). *Bars* 100 µm.

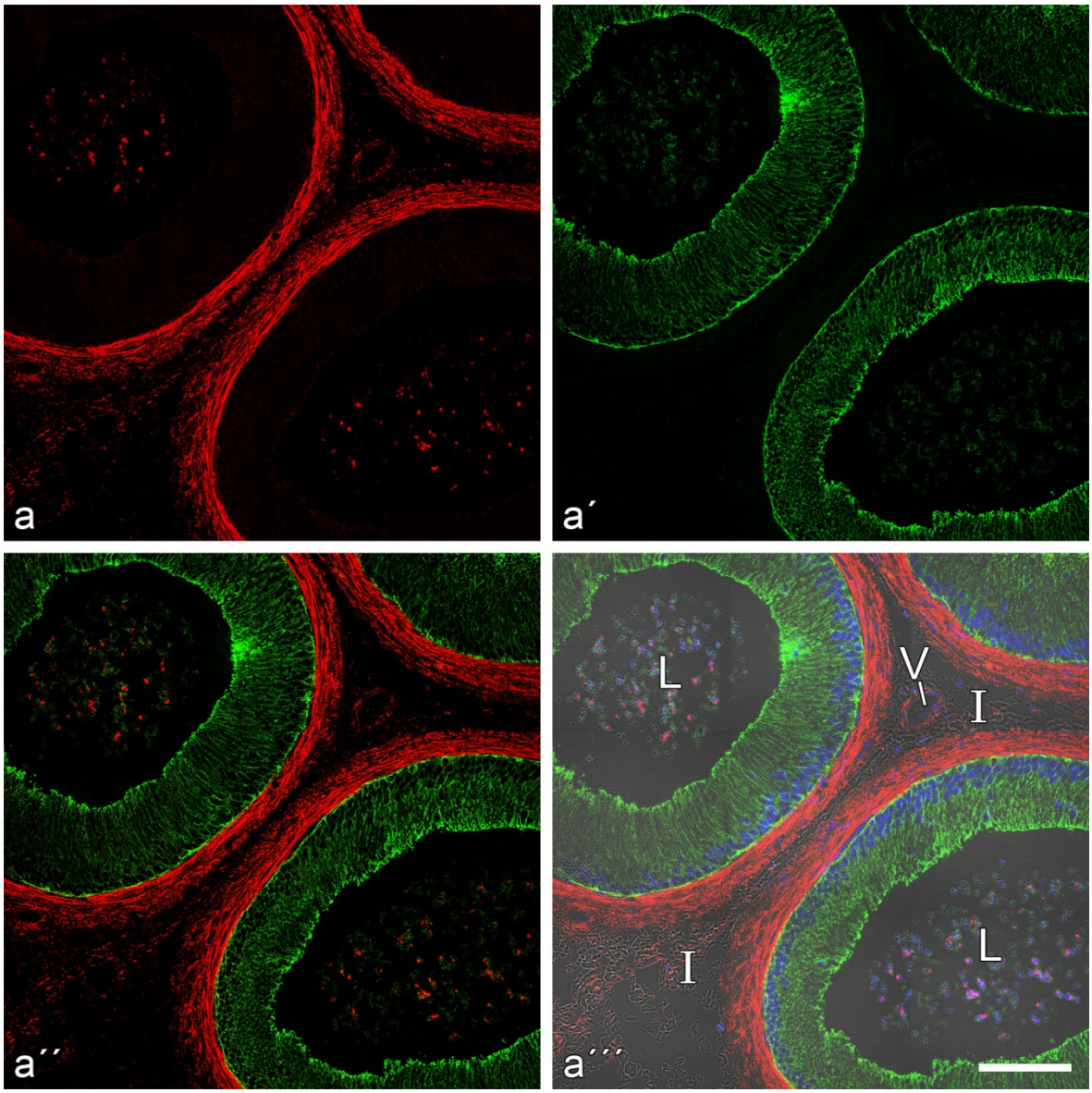


Fig. 15 Double-label immunofluorescence microscopy of a cryostat cross-section through an anterior portion of the excurrent duct system of frozen bull testis. After immunoreactions to **LUMA** (**a**, **a''**, **a'''**; *red;* guinea pig antibodies) and to **desmoplakin** (**a'**–**a'''**; *green*; monoclonal mouse antibody) all epithelial cells (L, lumen) show positive reaction for desmoplakin but are totally negative for LUMA. In contrast, peritubular wall cells encasing the excurrent ducts and the vascular (V) walls show positive reaction for LUMA. I, interstitial space. *Bar* 100 µm.

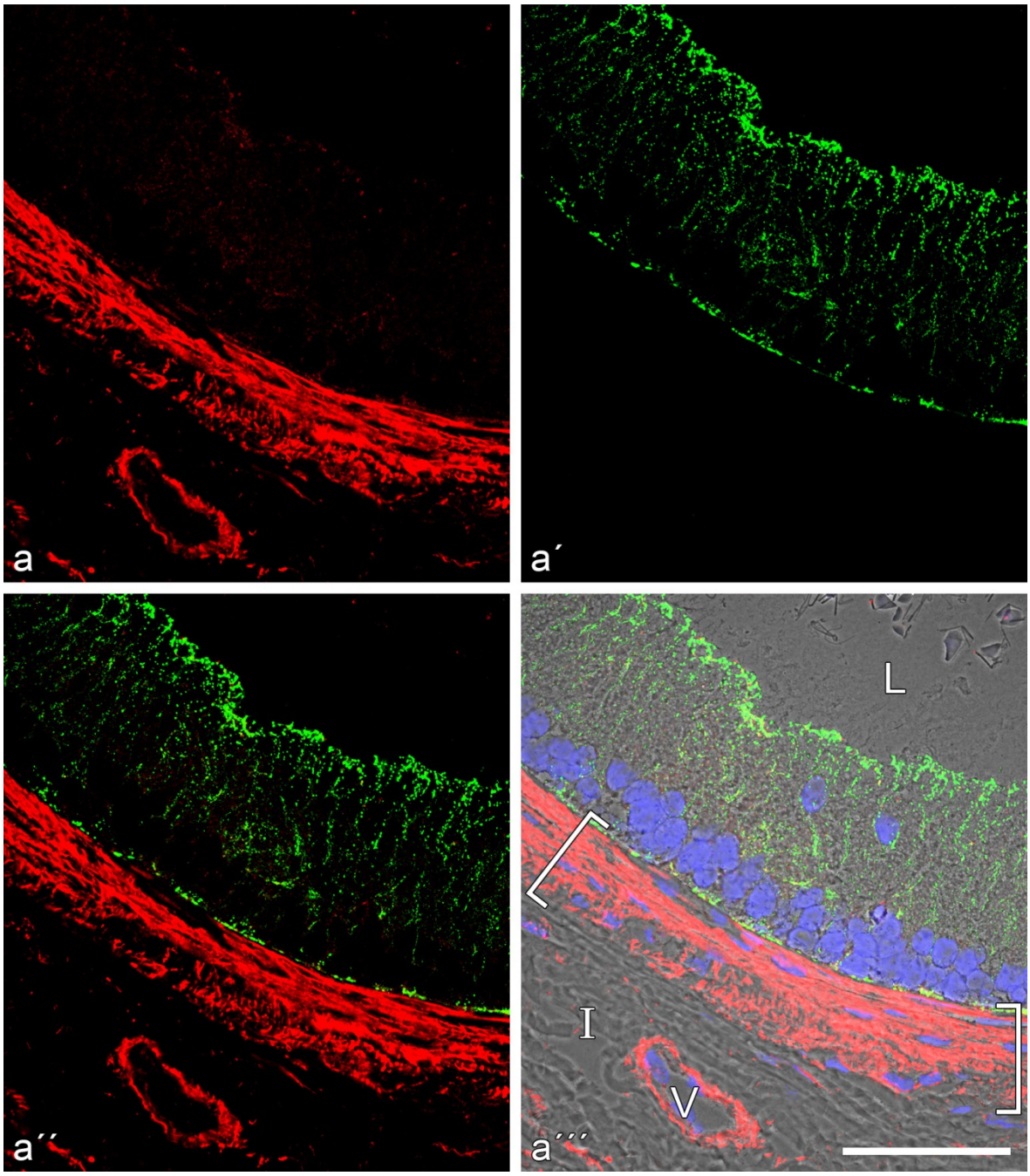


Fig. 16 Double-label immunofluorescence microscopy of a cryostat cross-section through a subsequent part of the excurrent duct system of frozen bull testis. After immunoreactions to **LUMA** (**a**, **a''**, **a'''**; *red*; guinea pig antibodies) and to **desmoplakin** (**a'**–**a'''**; *green*; monoclonal mouse antibody) positive reaction for LUMA is seen in the rather thick peri-epithelial SMC walls (denoted by brackets) and the perivascular (V, vessel lumen) walls in the interstitial region (I). Desmoplakin-positive reaction is seen in luminal epithelial cells (L, lumen). *Bar* 50 µm.

4.4 The seminiferous tubules: Cytoskeletal and cell-cell junctional molecules of Sertoli cells

4.4.1 Immunofluorescence analyses of adhering and adherens junctions

The somatic cells of the mature and active seminiferous tubules, the Sertoli cells, reach from the basal lamina to the adluminal compartment. They enclose the germ cells and form a major part of the so-called “blood-testis barrier”. This thesis has focused on the elucidation of the molecular nature and cell structure architecture of the various cell-cell junctions of the AJ category in the seminiferous tubules, in direct comparison with the AJs of the epithelia of the excurrent duct system and several non-testicular tissues.

In agreement with the biochemical results, cells lining the seminiferous tubules do not contain cytokeratin but only vimentin IFs (Franke et al. 1979c; for further references see Introduction) and no E-cadherin-based but exclusively N-cadherin-based AJs (Figs. 17 and 18). These results are in concordance with those of other reports (e.g., Cyr et al. 1992, 1993; Newton et al. 1993; Byers et al. 1994; Domke 2013). Reactions for all other cadherins examined were negative in seminiferous tubules of mature testes, including P-cadherin, VE-cadherin and cadherin-11 (see also Cyr et al. 1992).

In all N-cadherin-based AJs of Sertoli cells the typical AJ plaque proteins have been seen in colocalization with N-cadherin, although often with different reaction intensities (see, e.g., Figs. 17 and 19; for β-catenin as well as protein p0071 and striatin see also Figs. 6, 7 and 8 of Domke et al. 2014). The plaque protein myozap has been absent in Sertoli cells of bovine, porcine and human testes, whereas some positive myozap reactions have been noted in rodent Sertoli cells. Reactions of nectin antibodies have also been negative in lateral AJs of Sertoli cells, in contrast to the presence of nectin in the apical indentations containing spermatid heads and – together with protein myozap – in the *zonula adhaerens* of the epithelial cells of the excurrent ducts. Negative reactions have been obtained in all species examined for proteins PERP and EpCAM. A complete list of the AJ plaque proteins detected in N-cadherin-based isotypical cell-cell AJ structures is presented in Table 13.

In confirmation with my initial results (Domke 2013), the results of the present thesis using diverse antibodies for desmosome-specific marker molecules were negative in the seminiferous tubules examined and in the entire interstitial tissue region. Examples of such negative results have been shown for desmogleins and desmocollins, i.e. for Dsg-2 and Dsc-2, as well as for desmoplakin and plakophilin Pkp-2 (Fig. 20). In comparison, the desmosome-specific antibodies used showed positive reactions in excurrent duct epithelia (see, e.g., chapter 4.3).

Examinations of the presence of T-catenin in AJs of the seminiferous tubules have not yet given conclusive results (see also Janssens et al. 2001, 2003; Goossens et al. 2007a; van Hengel et al. 2013, for a review see Chiarella et al. 2018).

Table 13 Reactions of adhering junction molecules in mature mammalian testes

Antibodies specific for	Sertoli and germ cells[1]	Peritubular wall cells
Transmembrane Glycoproteins (cadherins)		
E-cadherin	–	(+)[2]
VE-cadherin	–	–
N-cadherin	+	sd[3]
P-cadherin	–	(+)[4]
Cadherin-6	–	–
Cadherin-11	–	+
Desmoglein-1 (Dsg-1)	–	–
Desmoglein-2 (Dsg-2)	–	–
Desmoglein-3 (Dsg-3)	–	–
Desmocollin-1 (Dsc-1)	–	–
Desmocollin-2 (Dsc-2)	–	–
Desmocollin-3 (Dsc-3)	–	–
Other Transmembrane Molecules		
EpCAM	–	–
Protein PERP	–	–
Cytoplasmic Plaque Proteins		
Desmoplakin 1+2	–	–[4]
Plakophilin-1	–	–
Plakophilin-2	–	–
Plakophilin-3	–	–
β-catenin	+	+
Protein p120	+	sd
Protein p0071	+	sd
Plakoglobin	+	(+/–)
α-catenin	+	+
Protein ZO-1	+	–
Cingulin	+	–
Myozap	(+/–)[6]	(+/–)
Plectin	+	–
Striatin[7]	+	sd
Protein LUMA	–	+

Continuation Table 13

[1] Junctions connecting Sertoli cells with Sertoli cells and Sertoli with germ cells

[2] Only one specific monoclonal antibody solution against E-cadherin (BD Transduction Laboratories, 610182) showed positive reaction in LSMCs. Other antibodies used showed complete negative reaction in LSMCs.

[3] sd Significance was not decidable yet.

[4] A monoclonal antibody solution against P-cadherin (Thermo Fisher Scientific, 13-2000Z) was only positive in LSMCs of bovine testes.

[5] One of the desmoplakin guinea pig antisera (Progen, DP-1) has shown occasionally cross-reactions in bovine LSMCs but the monoclonal antibody used (Progen, 65146) has not. This reaction difference needs to be further examined.

[6] Some antibodies against plaque protein myozap (Progen, 651169, and antibody clone cl. 2A) have shown immunolocalization in AJs of Sertoli cells of rodent testes but not in the other species examined. Protein myozap has been identified in bovine LSMCs but not in those of the other species tested.

[7] Whether it is striatin or a closely related member in the striatin family of proteins has not yet been determined.

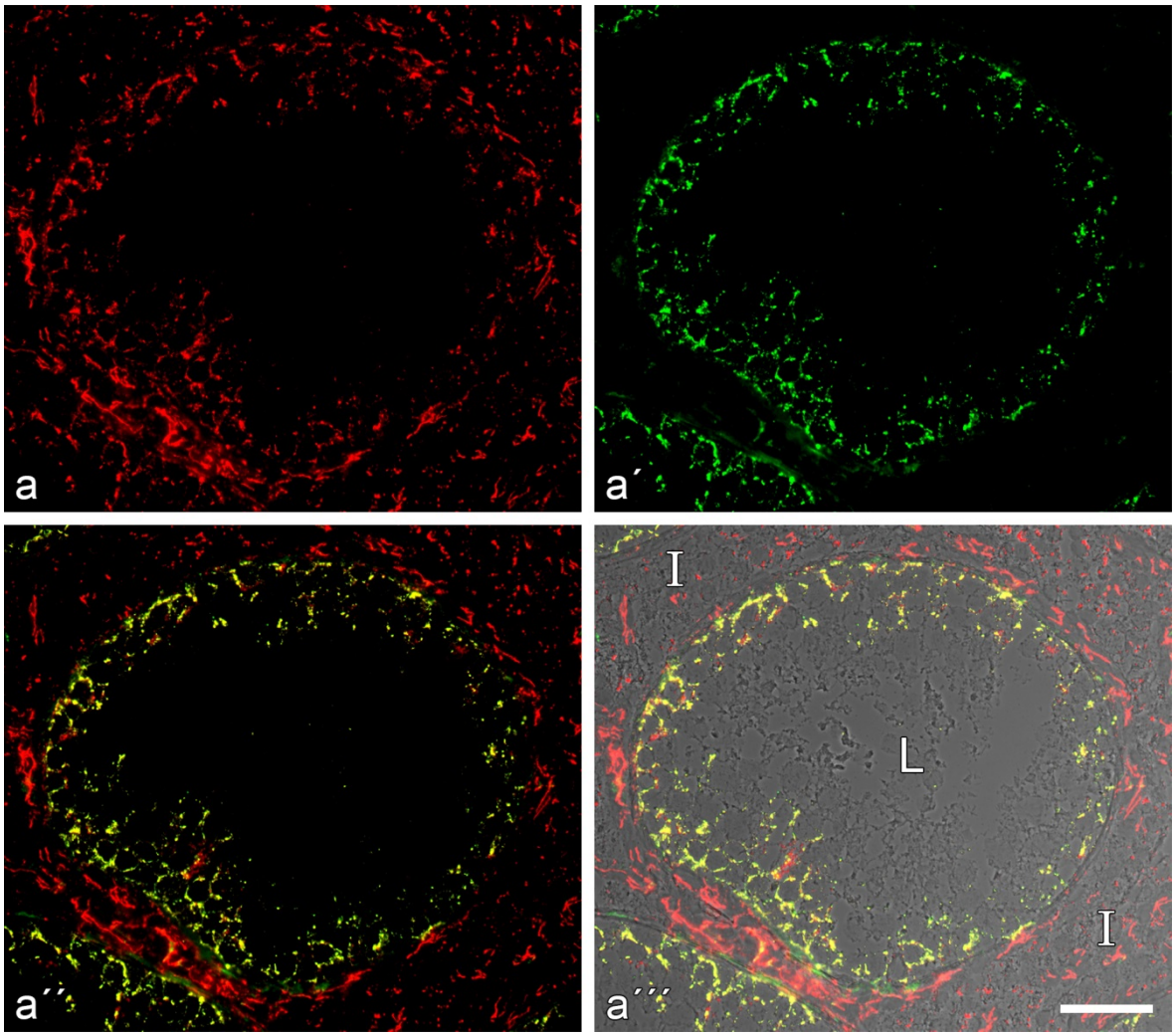


Fig. 17 Double-label immunofluorescence microscopy of a cryostat cross-section through a seminiferous tubule in boar testis using antibodies against the *armadillo* protein **β-catenin** (**a**, **a''**, **a'''**; *red*; monoclonal mouse antibody) and the transmembrane glycoprotein **N-cadherin** (**a'**–**a'''**; *green*; rabbit antibodies). Both AJ-marker molecules colocalize in the seminiferous tubule (*yellow* merger colour). In addition, some β-catenin-positive cells are also seen in the interstitial space (I). L, lumen. *Bar* 50 µm.

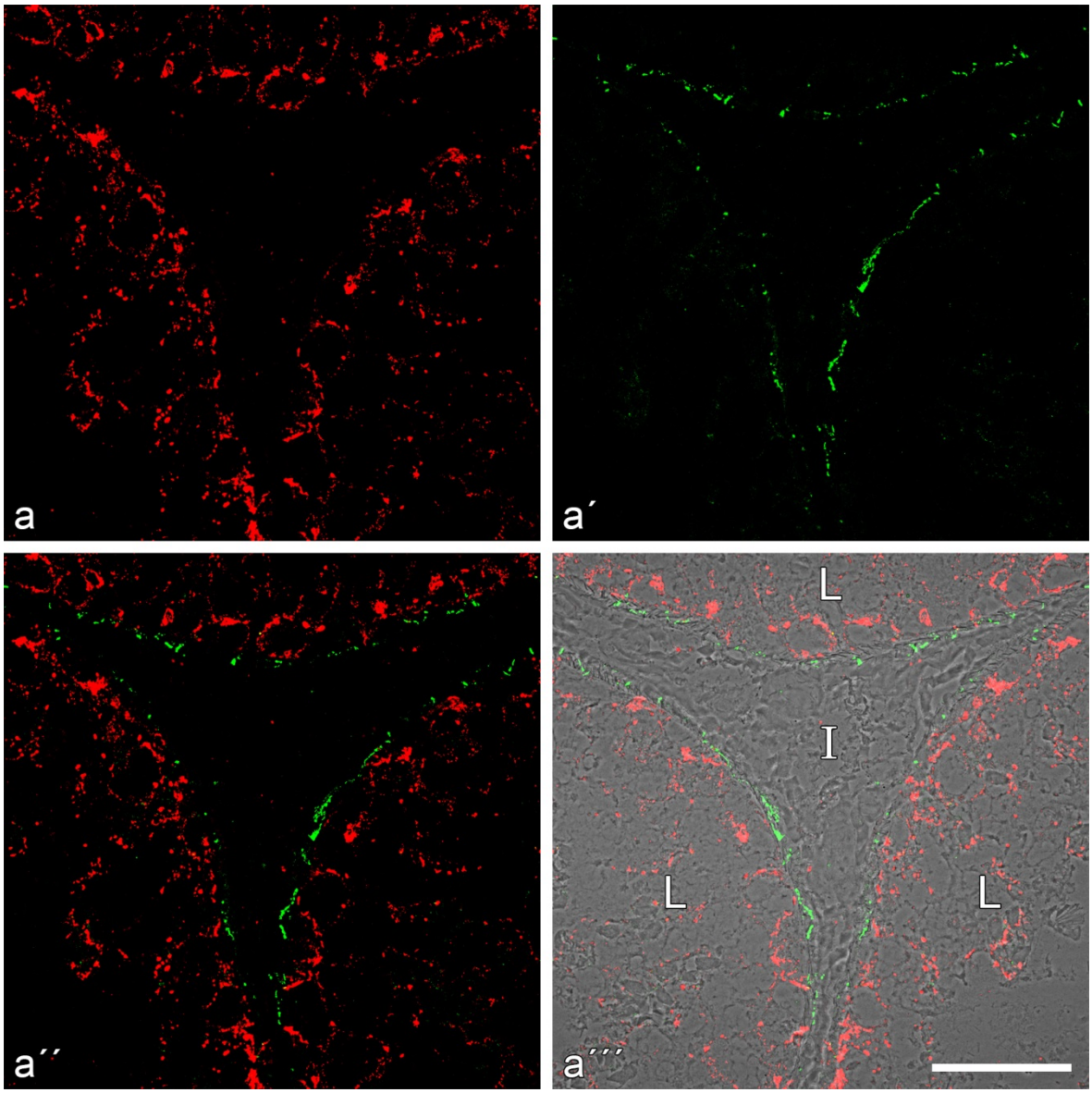


Fig. 18 Double-label immunofluorescence microscopy of a cryostat cross-section through seminiferous tubules of frozen testis tissue of a sexually mature bull with antibodies against the AJ glycoproteins **N-cadherin** (**a**, **a''**, **a'''**; *red*; rabbit antibodies) and **E-cadherin** (**a'**–**a'''**; *green*; monoclonal mouse antibody). N-cadherin-positive AJs are present (*red*) in all seminiferous tubules (L, lumen) but no E-cadherin-containing structures. Only a few cells in the peritubular wall show here a positive immunostaining reaction for E-cadherin (*green*). I, interstitial space. *Bar* 50 µm.

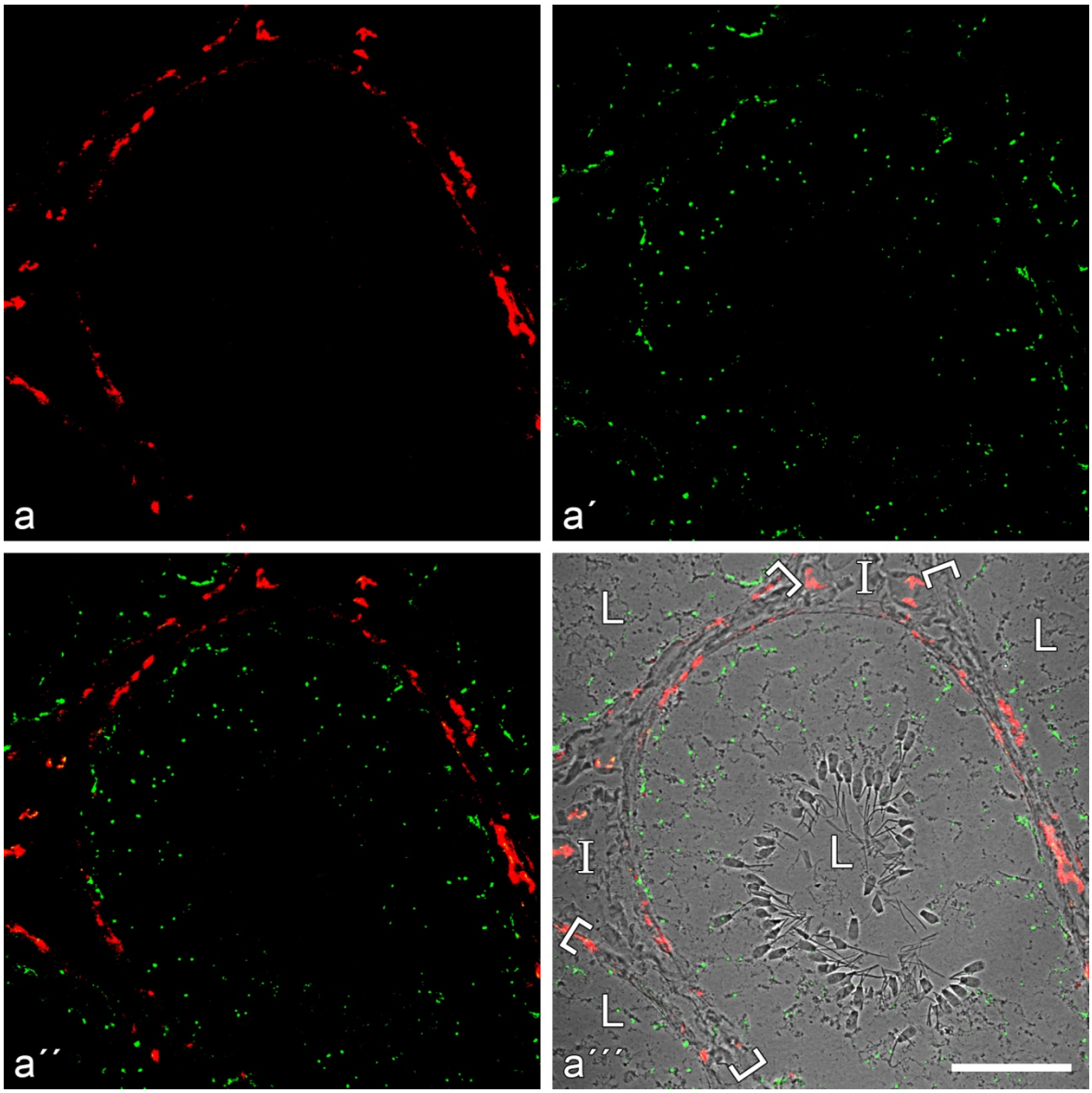


Fig. 19 Double-label immunofluorescence microscopy of a cryotomy cross-section through a seminiferous tubule of frozen bull testis, showing immunoreactions for protein **myozap** (**a**, **a''**, **a'''**; red; monoclonal mouse antibody) and the *armadillo* protein **β-catenin** (**a'**–**a'''**; *green*; rabbit antibodies). Note that all Sertoli cells are positive for β-catenin-rich AJs but negative for protein myozap. Note also some positive myozap-staining in peritubular wall (brackets) and endothelial cells (I, interstitial space). (**a'''**) Note spermatids in association with the apical indentations (L, lumen) and lateral plasma membranes of Sertoli cells. *Bar* 50 µm.

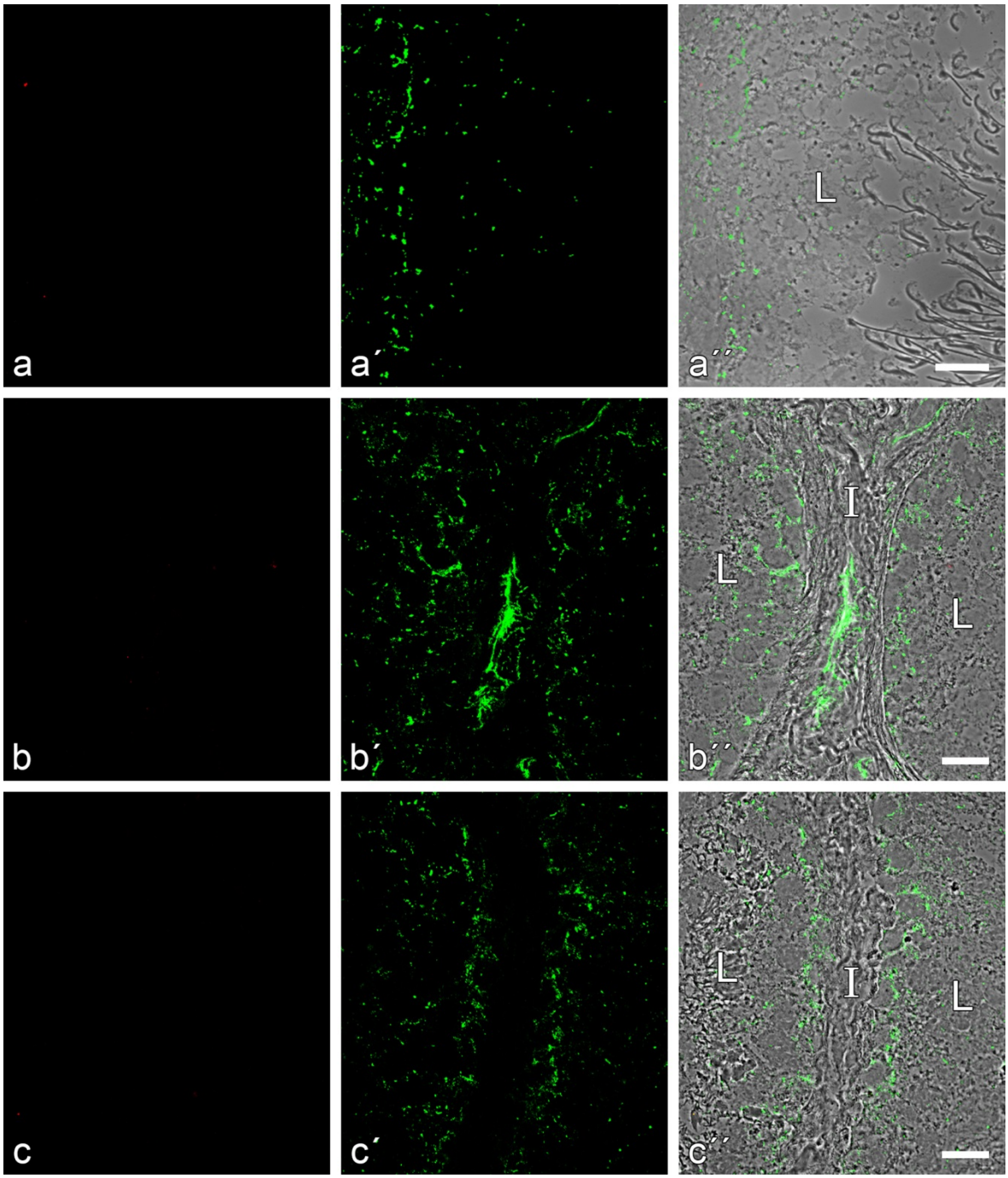


Fig. 20 Double-label immunofluorescence microscopy of cryostat cross-sections through frozen rat (**a**–**a''**) and bull (**b**–**b''** and **c**–**c''**) testes after reactions with monoclonal mouse antibodies against the desmosomal markers **desmoplakin** (**DP**; **a**, **a''**; *red*), **desmoglein-2** (**Dsg-2**; **b**, **b''**; *red*) and **plakophilin-2** (**Pkp-2**; **c**, **c''**; *red*) as well as rabbit antibodies against the AJ marker **β-catenin** (**a'**–**a''**, **b'**–**b''**; *green*) and **N-cadherin** (**c'**–**c''**; *green*). Note in the seminiferous tubules a positive staining of β-catenin (*green*) but totally negative immunostaining for DP, Dsg-2 and Pkp-2. I, interstitial space; L, lumen. *Bars* 20 µm (see also Domke et al. 2014).

4.4.2 Electron and immunoelectron microscopy of cytoskeletal structures and cell-cell adherens junctions

For ultrastructural analyses, tissue samples were electron microscopically examined using ultrathin sections of tissue samples fixed in glutaraldehyde. Fig. 21 presents a survey of a part of a typical Sertoli cell characterized by a large nucleus, the outer nuclear membrane of which is associated with vimentin IFs, including here a very large, almost paracrystalline bundle (bottom part), and a close plasma membrane connection of two Sertoli cells with variously-sized AJs of the *punctum adhaerens* type.

Sertoli cell-cell contacts in the seminiferous tubules can vary in size, including small as well as very large and complex AJ structures, usually associated with a cytoplasmic plaque of locally variable area and thickness (Figs. 22 a–g). Often cell-cell junction-like plasma membrane structures can be seen over extended regions of diameters of more than 10 µm (e.g., Fig. 22 a; for higher magnifications of very large cell-cell AJs see also Figs. 22 a'''–g). These structures appear in close and parallel order with plasma membrane-to-membrane distances of 5–15 nm and diameters or lateral lengths up to several micrometers (Figs. 22–24), occupying very large cell-cell junction regions. Such gigantic and close AJ-type cell-cell contacts often begin near the basal plasma membrane and extend over almost the entire lateral cell-cell contact regions (see, e.g., Figs. 22 a–c). They have therefore been subsumed under the special junction category of ***areae adhaerentes***. At higher resolution (Figs. 22 d–g) some of these junction intercepts are covered by cytoplasmic plaque material of various shapes and densities. Many of them reveal intermembrane ("mesoglea-like") periodical punctate arrays (Figs. 22 f and g; see also Fig. 1 of Franke et al. 1982b).

Sertoli cell-cell junctions of the adherens type frequently also show characteristic paracrystalline actin myofilament bundles close and parallel to the plasma membrane ("ectoplasmic specializations"; e.g. Figs. 23 a–f and 24 a and a'; see also Nicander 1967; Dym and Fawcett 1970; Franke et al. 1978a; Vogl et al. 2000; Mruk and Cheng 2004a, b; Wong et al. 2005). These paracrystalline myofilament bundle specializations often appear to be connected to the plasma membrane by very thin lateral cross-bridges (Figs. 23 b–g and 24 a'). In addition, in some regions very close contacts of the plasma membranes of two adjacent cells are visible, resembling "kissing points" of a tight junction-like type (see, e.g., arrowheads in Fig. 23 a' and arrows in Fig. 24 a''; see also Fawcett 1981, Franke et al. 1982b).

Cribelliform junctions (***areae cribelliformes***) of bovine seminiferous tubules are characterized by membrane-bound cytoplasm-cytoplasm pore channels of an internal cytoplasmic luminal diameter of 5–7 nm and a total length of 6–9 nm, often closely associated with myofilament bundles (see, e.g., Figs. 23 d–h; for a schematic presentation

see Fig. 25). However, these structures mostly occur in clusters of two to six cribelliform junctions (see, e.g., Figs. 23 d–e).

Immunoelectron microscopical analyses have confirmed the absence of desmosomal structures and desmosome-specific marker molecules such as desmoglein-2, desmocollin-2, desmoplakin and plakophilin-2. *Vice versa*, immunogold labelling showed variously-sized, often extended regions positive for N-cadherin as well as for the *armadillo* protein β-catenin and for a striatin along the cell-cell contacts between Sertoli cells as well as between Sertoli and germ cells (Figs. 26 a–f).

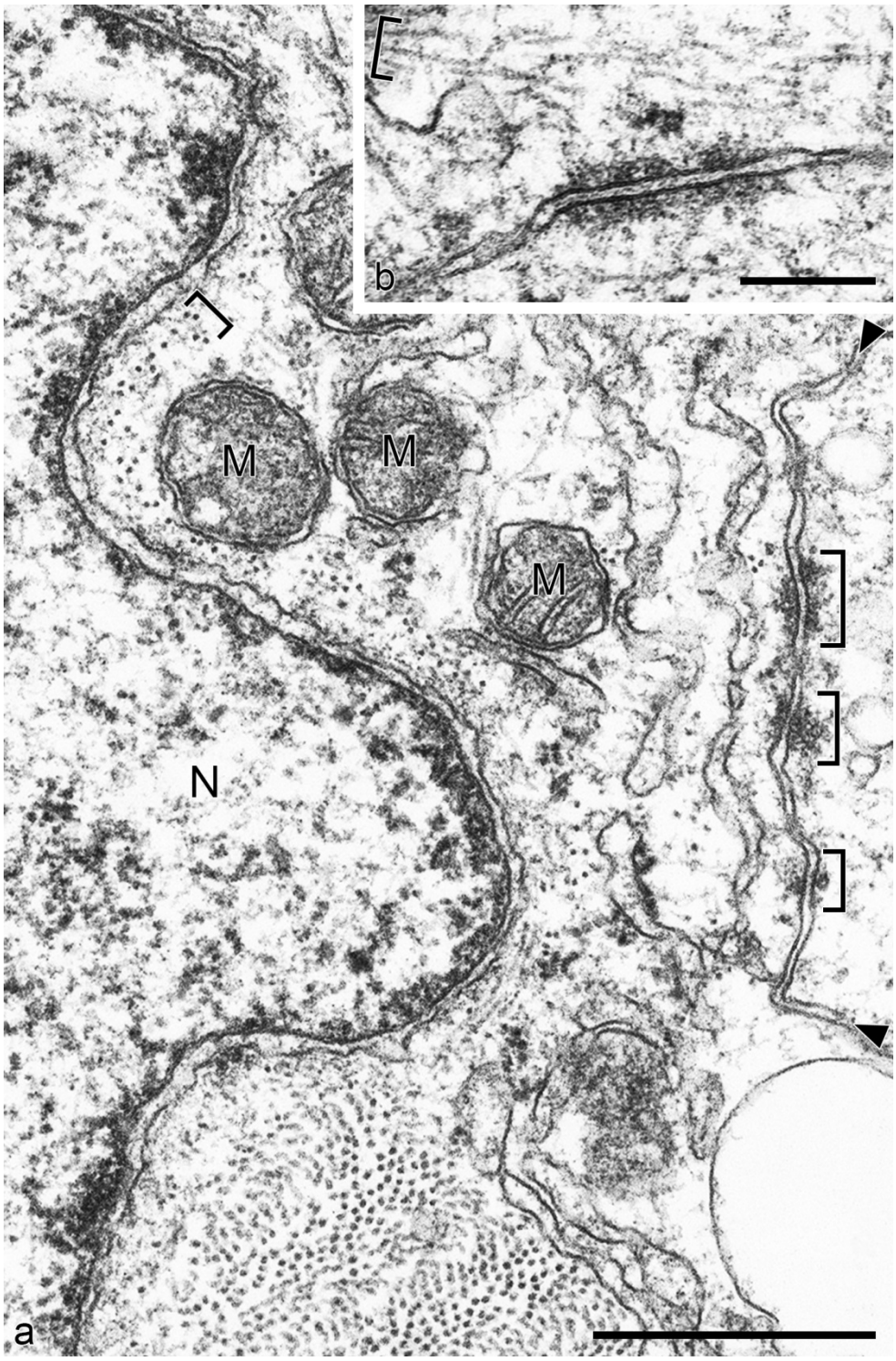


Fig. 21 Electron micrographs of ultrathin sections through a Sertoli cell in a seminiferous tubule of bull testis. (**a**) Sertoli cell (N, nucleus), showing close associations of the nuclear envelope with vimentin intermediate-sized filament (IF) bundles (see also the IFs indicated by a bracket in the upper left), mitochondria (M) and endoplasmic reticulum cisternae. An extended plasma membrane cell-cell junction region with plaque-bearing adherens junction (AJ) structures is highlighted by arrowheads (three AJs of the *punctum adhaerens* type are denoted by brackets on the right hand side). (**b**) A cell-cell contact with a very small AJ punctate "midline" structure and a plaque structure of heterogeneous thickness is approached by a bundle of vimentin filaments which comes close to the plaque of the junction but is not directly attached to it (bracket in the upper left). *Bars* (**a**) 500 nm and (**b**) 200 nm (see also Domke et al. 2014).

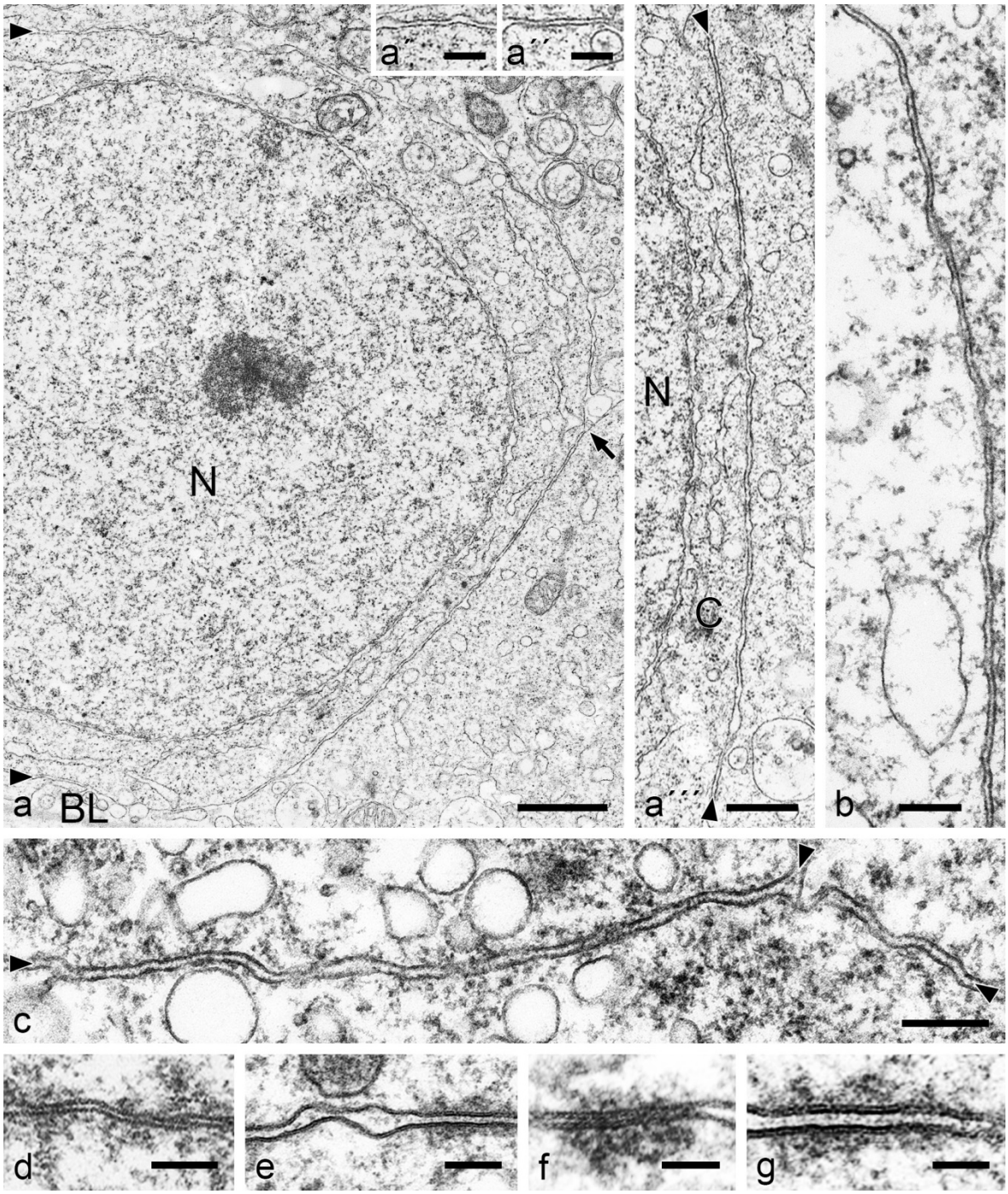


Fig. 22 Electron micrographs of ultrathin sections through seminiferous tubules of boar (**a**–**a'''**) and bull (**b**–**d**) testis. (**a**) Survey pictures show adjacent Sertoli cells with details of the very extended, narrow-spaced (5–15 nm) plasma membrane connections (*area adhaerens* junctions; denoted by arrowheads). (**a'**–**a'''**) Details at higher magnification. (**b**–**d**) In such extended, narrow-spaced plasma membrane connections locally limited "minimal plaque material" (MPM) AJs are seen with thin and loosely arranged plaque-like structures. (**c**) Some junctions show narrow intermembrane distances (5–20 nm) associated with plaque material of different sizes and configurations. (**e**) Other small and rather narrow junctions (ca. 5–6 nm intermembrane distance) which here reveal asymmetrical cytoplasmic coating with irregularly shaped plaque material. (**f** and **g**) An AJ with a continuous planar order of 6–7 nm-thick membranes shows serially arranged "punctate midline" granules of 2–3 nm diameter but rather loose cytoplasmic plaque coverage (**g** partial magnification). BL, basal lamina; C, cytoplasm. *Bars* (**a**) 1 µm, (**a'''**) 500 nm, (**a'**, **a''**, **b**, **c**) 200 nm, (**d**, **e**) 100 nm and (**f**, **g**) 50 nm (see also Domke et al. 2014).

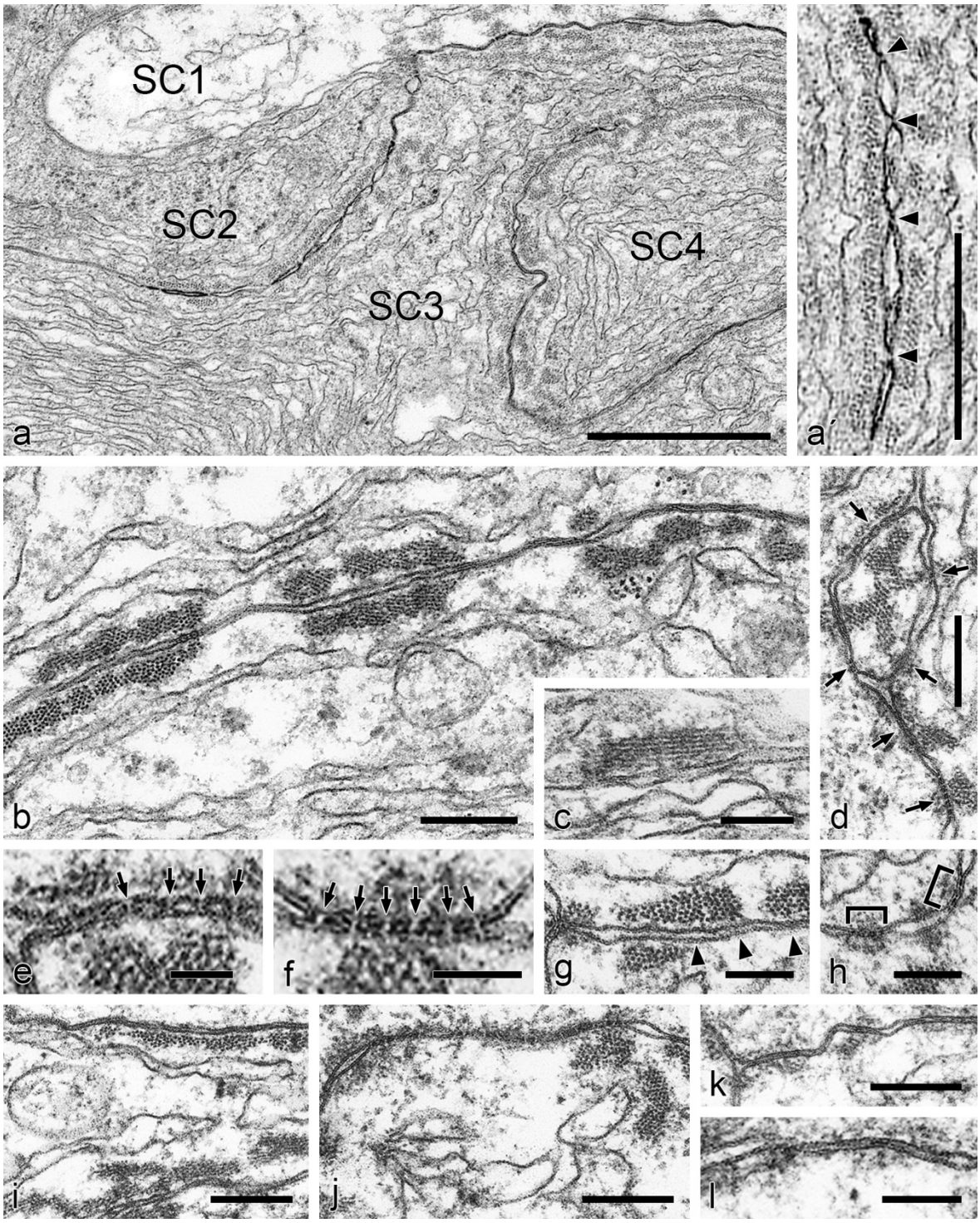


Fig. 23 Electron micrographs of ultrathin sections through seminiferous tubules of bull testicular tissue. (for continuation see the next page).

Continuation of Fig. 23 Electron micrographs of ultrathin sections through seminiferous tubules of bull testicular tissue. (**a**) Overview of four adjacent Sertoli cells (SCs) with adherens junction (AJ) regions and adjacent myofilament paracrystals parallel to the plasma membrane ("ectoplasmic specializations") as well as some regions with rather small cribelliform junctions. Sertoli cells of a specific subtype are characterized by a high packing density of endoplasmic reticulum. (**a'**) Some regions show interspersed cells with small, tightly adpressed membrane junctions suggesting even direct molecular interaction (arrowheads). (**b**, **c**) Other cortical regions are dominated by typical junctions associated with parallel paracrystalline actin myofilament bundles very close to the plasma membrane ("ectoplasmic specializations"). (**d**–**f**) Sertoli cell contacts with distinct narrow channels (cribelliform junctions) appearing as sieve-plate junctions between the cytoplasms are denoted by arrows. (**e**, **f**) Higher magnification of cribelliform junctions (*areae cribelliformes*) with channel-like sections are seen with an inner "pore" diameter of 5–7 nm and a total length of 6–9 nm. These junctions are often characterized on one side or on both sides of the channel by electron-dense, plaque-like structures (brackets in **h**). (**c**, **g**, **i**, **j**) Often plasma membrane contacts are associated with adjacent actin filament bundles, which are at some sides cross-bridged to the plasma membrane by short structures (see also arrowheads in **g**). (**j**–**l**) The parallel and narrow junction-like structures of adjacent cells are coated irregularly with loose cytoplasmic dense materials. *Bars* (**a**) 1 µm, (**a'**) 500 nm, (**b**, **d**, **i**, **j**, **k**) 200 nm, (**c**, **g**, **h**, **l**) 100 nm and (**e**, **f**) 50 nm (see also Domke et al. 2014).

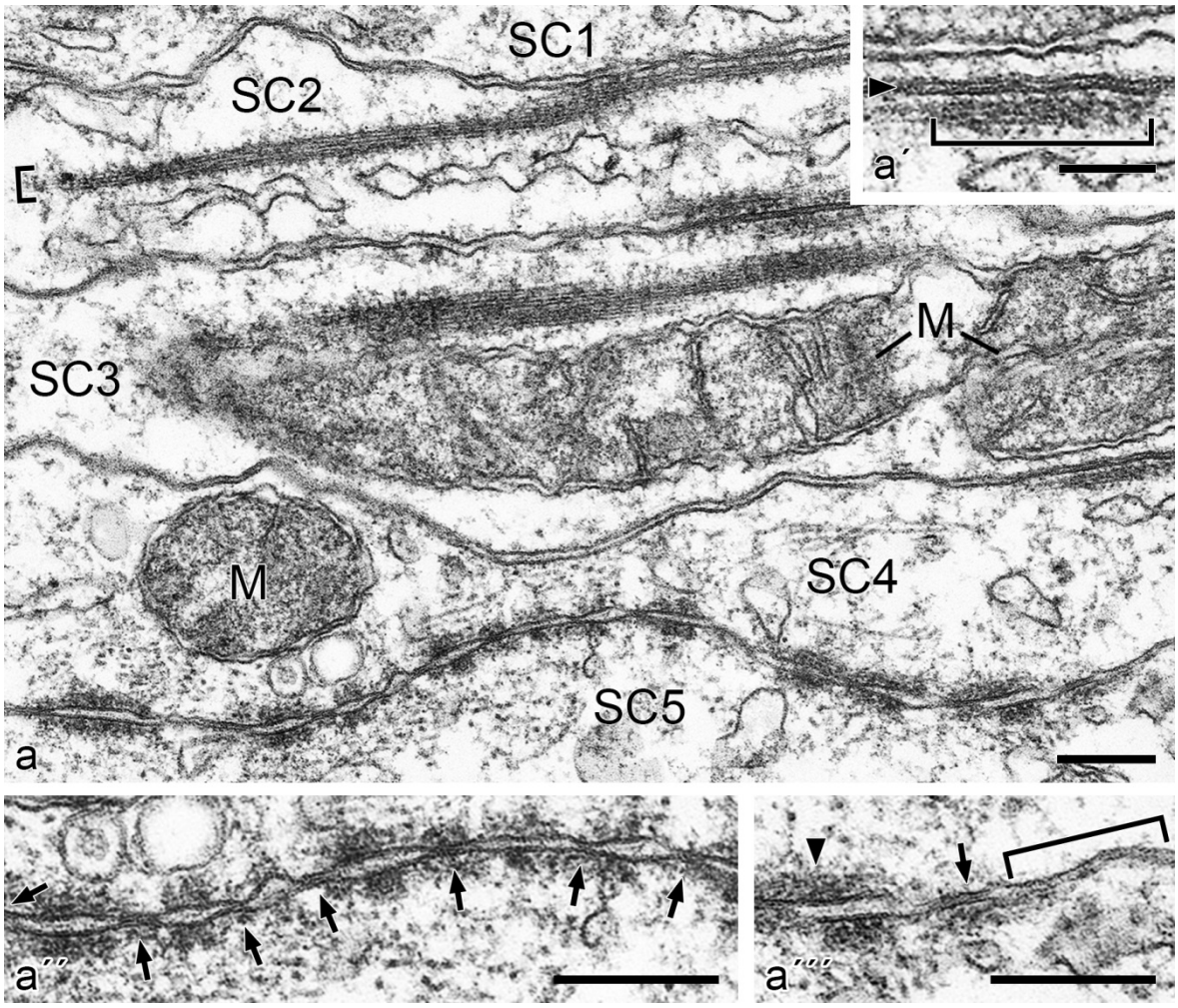


Fig. 24 Electron micrographs of ultrathin sections through seminiferous tubules of bull testes. Cell-cell junctions of Sertoli cells (SCs) represent specific subtypes of AJs. (**a**) Overview showing parts of five tight-associated Sertoli cells (numbered 1–5) with extended cell-cell contact regions (*areae adhaerentes*). Often lateral cross-bridges are present between the plasma membrane and bundles of cortical actin myofilament paracrystals (up to 4 nm thick; denoted by brackets in the insert **a'**). One can further recognize two distinct junction subtypes in rather regularly alternating arrangements: rather short and close contacts (partially shown at higher magnification and denoted by arrows in **a''** and **a'''**) and usually wider (membrane-to-membrane distances between 8 and 25 nm), often with distinct midline granules (cf. cells nos. 1 and 2 in **a**) as well as rather dense and thick cytoplasmic plaques (denoted by an arrowhead in **a'''**). In **a'''** note also the adjacent occurrence of three different junction types side-by-side: a *punctum adhaerens*-type junction (arrowhead), a very tightly adpressed membrane junction (arrow), and a junction of the "minimal plaque material" (MPM) AJ type with ca. 8–18 nm intermembrane distance and very thin, often hardly recognizable cytoplasmic plaque (bracket). M, mitochondria. *Bars* (**a**, **a''**, **a'''**) 200 nm and (**a'**) 100 nm (see also Domke et al. 2014).

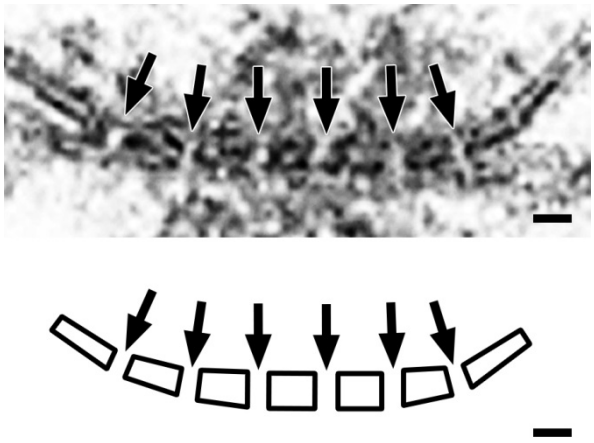


Fig. 25 Higher magnification of a cribelliform junction (*area cribelliformis*) in a seminiferous tubule of bull testes. This sieve-like junction is characterized by channel-like cytoplasm-cytoplasm continuities of an inner pore diameter of 5–7 nm and a total length of 6–9 nm (arrows). Usually, these junctions occur in clusters of rather regularly and closely spaced cell-cell junctions in distinct regions. The lower picture shows a schematic drawing of a cribelliform junction. *Bar* 10 nm.

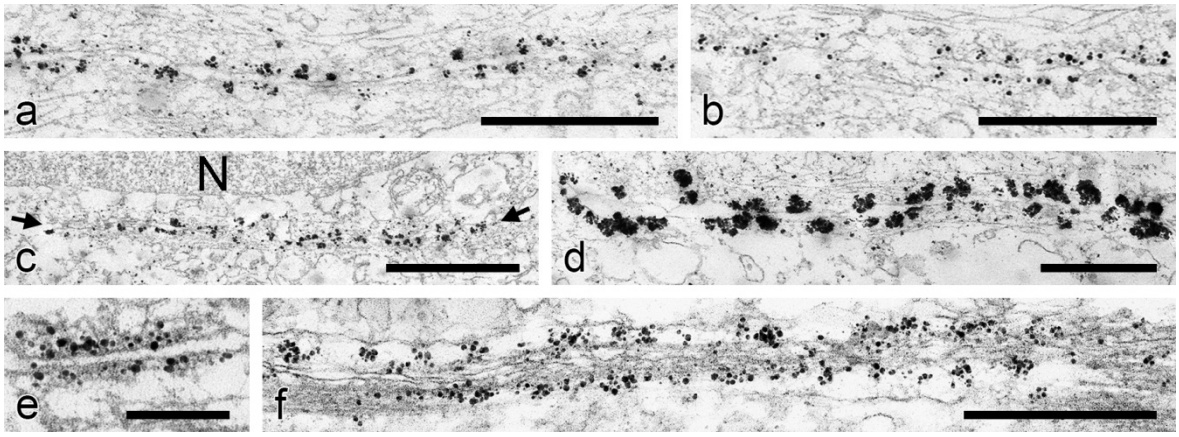


Fig. 26 Immunoelectron microscopy of seminiferous tubules of bull testicular tissue using antibodies against **β-catenin** (**a**–**d**), **N-cadherin** (**e**) and **striatin** (**f**). (**a**–**d)** After silver enhancement the immunogold grains show specific binding of β-catenin in extended regions of Sertoli cell contacts with neighboring cells, including very long (4–6 µm) and continuously labelled regions (*areae adhaerentes*). (**e**) All types of Sertoli-Sertoli cell junctions are also positive for N-cadherin. (**f**) In addition, other adherens plaque proteins such as striatin are seen. *Bars* (**c**) 1 µm, (**d**, **f**) 500 nm and (**a**, **b**, **e**) 200 nm (see also Domke et al. 2014).

4.5 The basal plasma membrane and the basal lamina

The cells of the seminiferous tubule are attached to a well-developed basal lamina (BL) which appears as a partly stratified structure separating the tubule from the surrounding peritubular wall tissue beginning with the first layer of extracellular matrix (ECM) material.

As desmosomes have repeatedly been claimed to laterally connect cells of the seminiferous epithelium, Sertoli cell hemidesmosomes have been postulated to be involved in the basal connection with peritubular wall cells or ECM structures (for references see Table 3). However, my studies of the Sertoli cells and the BL have revealed a total absence of hemidesmosomal structures as well as of hemidesmosomal hallmark molecules (for general references of such structures and molecules see chapter 5.2). In particular, the analyses showed that the basal plasma membrane of the cells of the seminiferous tubules does not contain hemidesmosomal marker molecules such as protein HD230/233 (bullous pemphigoid antigen; BPA 230), the integrin α6β4 complex, tetraspanin CD151 and plectin. The significance of these negative results has been controlled by positive immunostaining reactions of all these hemidesmosomal markers in the adjacent epithelia of the excurrent ducts and in diverse other epithelia. As a representative example, the reaction of glycoprotein HD 230/233 is shown in the cell type comparison of Fig. 27: While this molecule is totally missing in Sertoli cells it is present in the hemidesmosomes on the basal plasma membrane of the excurrent duct epithelial cells in all species examined. On the other hand, specific laminins have been seen in intimate association with the BL and with peritubular wall cells and ECM structures as well as in the walls of blood vessels located in the interstitial space (Figs. 28 and 29).

The BL can reach thicknesses up to 1.5 µm in bull testis and shows in some regions up to 2.5 µm deep indentations into the Sertoli cells (Fig. 53). These occur often in an almost regular pattern with lateral distances of ca. 2–3 µm (see, e.g., also Wrobel et al. 1979, for human testes see Chakraborty et al. 1976). In other species, notably boar, such indentations have also been noted although mostly at much lower frequencies (not shown).

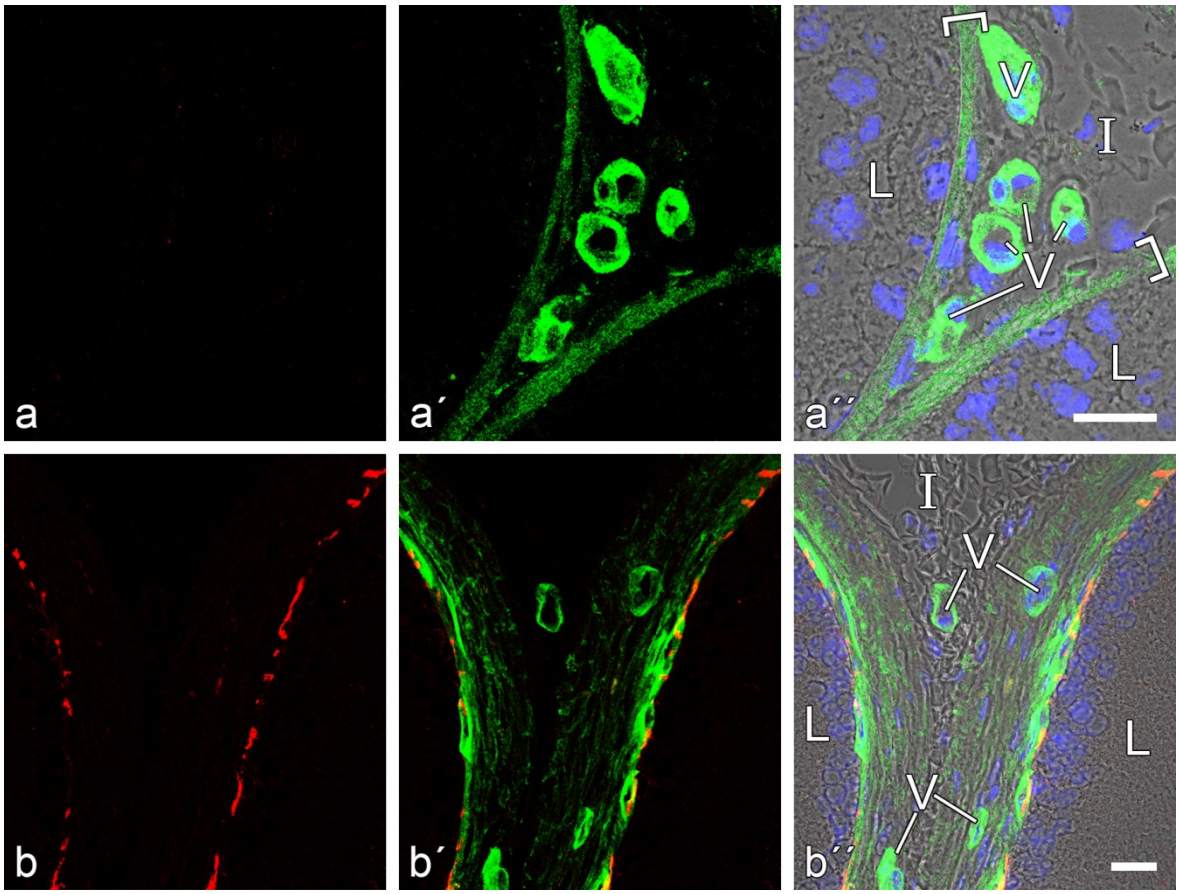


Fig. 27 Double-label immunofluorescence microscopy of cryostat cross-sections through seminiferous tubules (**a**–**a''**) and excurrent ducts (**b**–**b''**) of frozen bull testis. Immunoreactions with the hemidesmosomal (HD) marker protein **HD230/233** (**a**, **a''**, **b**, **b''**; *red*; monoclonal mouse antibody) and the extracellular matrix (ECM) marker protein **collagen IV** (**a'**–**a''**, **b'**–**b''**; *green*; rabbit antibodies) show in the Sertoli cells as well as in the perivascular walls (V, vessels) and in the peritubular walls (brackets) of the seminiferous tubule cells totally negative reactions for the HD marker protein but a positive immunoreaction of collagen IV (**a**–**a''**). In contrast, an intensely positive reaction for HD marker molecules at distinct hemidesmosomal structures is seen at the basal plasma membrane of the excurrent duct epithelium. Reactions of collagen IV are also distributed in the ECM material of the peritubular walls and the walls of blood vessels (V) of the interstitium (I; **b**–**b''**). Nuclei have been stained *blue* with DAPI (**a''**, **b''**). L, lumen. *Bars* 20 µm.

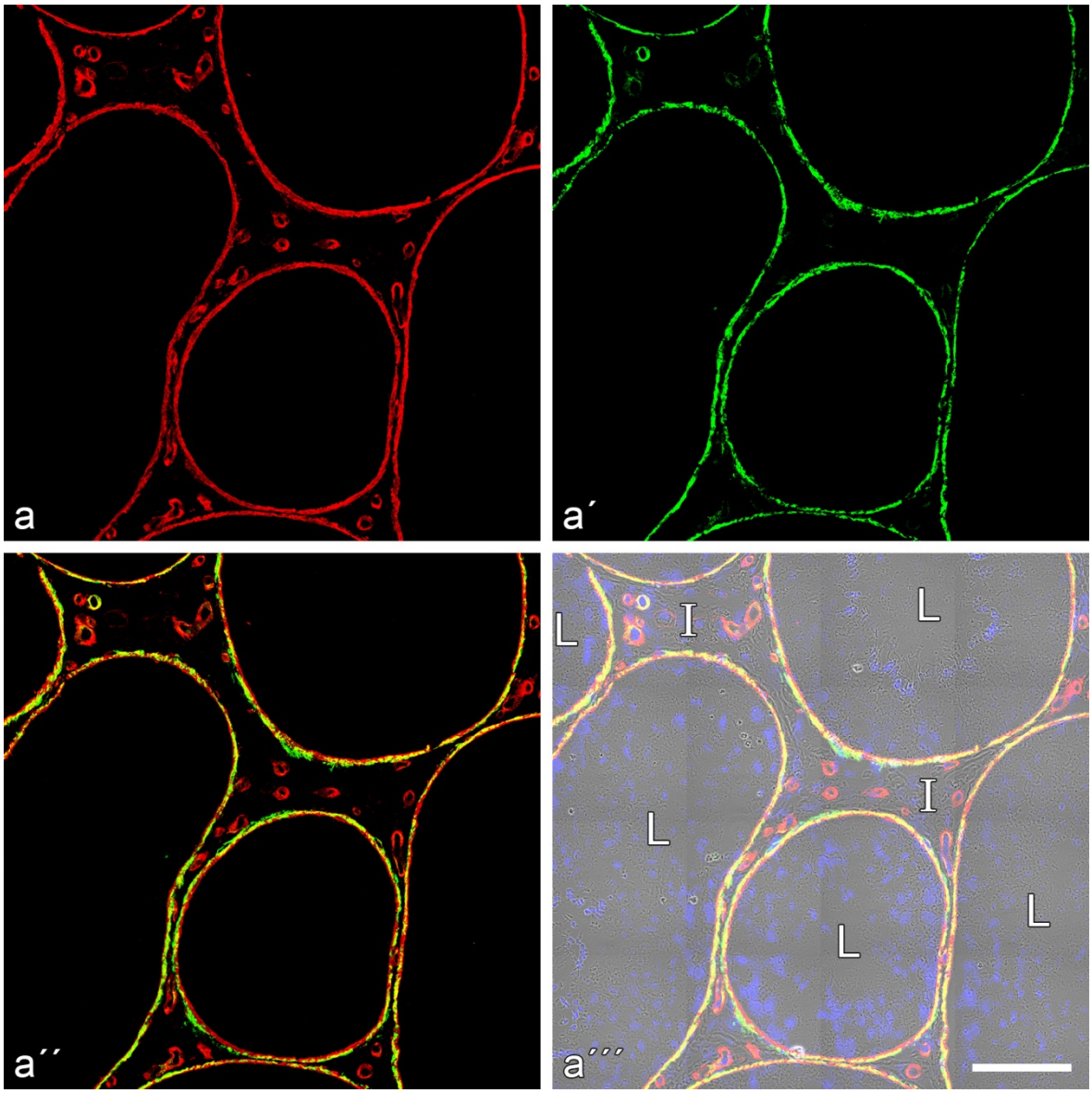


Fig. 28 Tile scan survey of double-label immunofluorescence microscopy of a cryostat cross-section through seminiferous tubules in frozen bull testis tissue. Immunostaining with antibodies against **laminin** (**a**, **a**'', **a**'''; *red*; rabbit antibodies) and **smooth muscle α-actin** (**α-SMA**; **a'**–**a'''**; *green*; monoclonal mouse antibody) shows colocalization or partially overlapping reactions in peritubular smooth muscle cell (SMC) layers. Note however, that here most of the very small blood vessels in the interstitial region (I) are positive only for α-SMA. Nuclei have been stained *blue* with DAPI (**a'''**). L, lumen. *Bar* 100 µm.

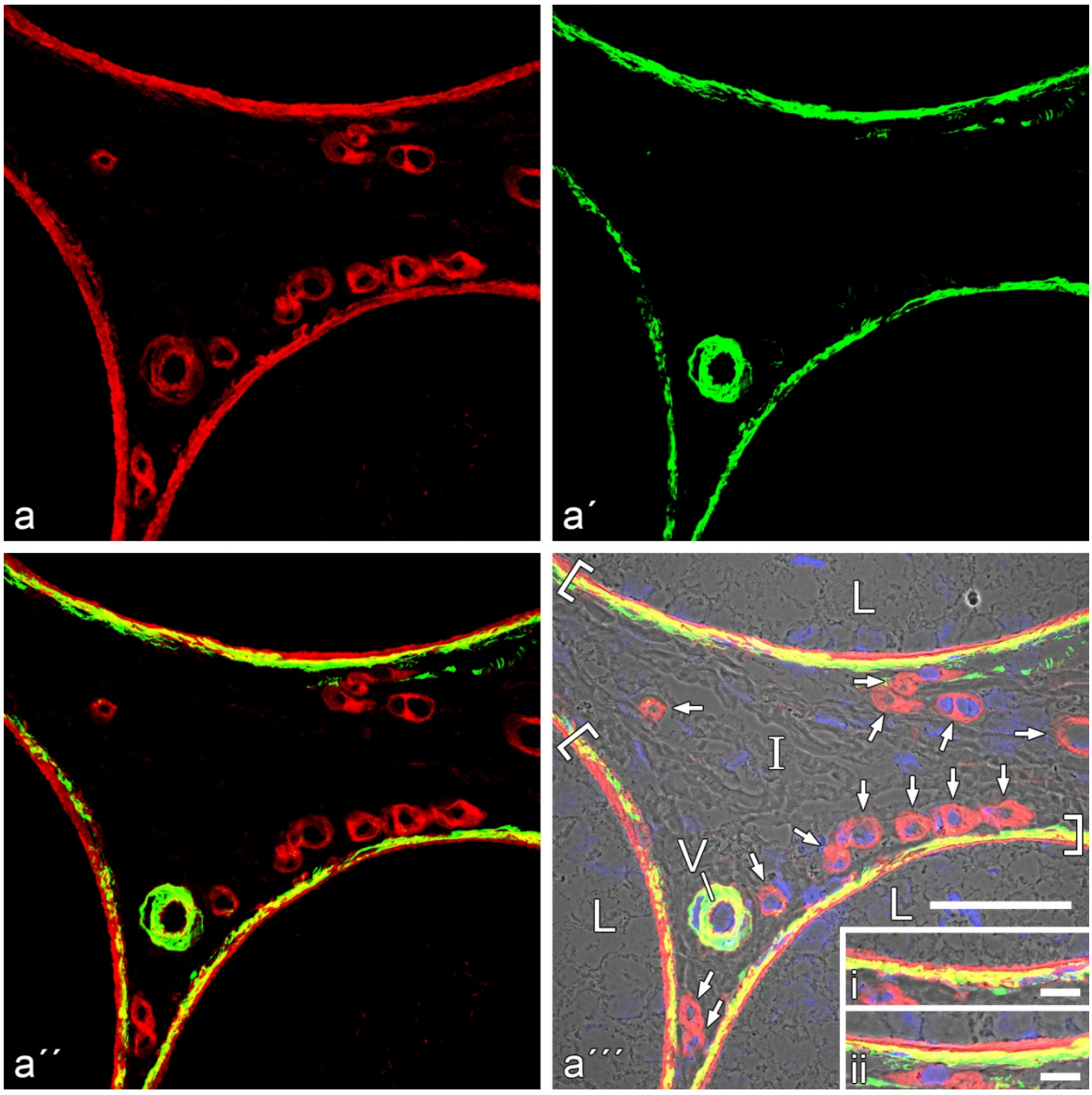


Fig. 29 Double-label immunofluorescence microscopy of a cryostat cross-section through seminiferous tubules of frozen bull testis. After immunoreactions of antibodies against the basal lamina component **laminin** (**a**, **a''**, **a'''**; *red;* rabbit antibodies) and the smooth muscle cell (SMC) marker **smooth muscle α-actin** (**α-SMA**; **a'**–**a'''**; *green*; monoclonal mouse antibody) laminin-staining (*red*) is seen along the basal lamina line at the bottom of the Sertoli cells and α-SMA-staining (*green*) of SMC layers in the peritubular walls (denoted in **a'''** by brackets) as well as in blood vessel (V) walls. In most regions the basal lamina is followed by a SMC layer positive for both α-SMA and laminin structures (very close localization or even partial "colocalization" of both markers is indicated by *yellow* merge colour). The two inserts (**i**, **ii**) in the lower right of **a'''** present higher magnifications showing that laminin is not restricted to the basal lamina. Here it is also seen in parts of the ECM layers located between the SMC layers. In addition, laminin is present in perivascular walls of most of the very small blood vessels which here are negative for α-SMA (small arrows). Nuclei have been stained *blue* with DAPI. I, interstitial space. L, lumen. *Bars* (**a**–**a'''**) 50 µm and (**i**, **ii**) 10 µm.

4.6 The peritubular walls: Cell type-characteristics and cell-cell junctions of the lamellar smooth muscle cells (LSMCs)

The cells of the special tissue surrounding the BL of seminiferous tubules in mature testes have been examined using immunofluorescence light and immunoelectron microscopy, including tile scanning methods and lateral as well as vertical tomographies with respect to the corresponding tubule.

The results show that the tubules are tightly surrounded by cell bandage sheets which represent monolayers of very thin (in some regions down to ~ 30 nm cytoplasmic membrane-membrane interspace) and broadly extended polyhedral cells. These cells have a fully developed smooth muscle character and mainly appear as flattened lamellae. Based on their molecular characteristics these cells can be classified as lamellar SMCs (LSMCs). They are organized as stacks of laterally tightly connected LSMC monolayers interspersed with ECM layers and are wrapped around the seminiferous tubules (cf., e.g., Leeson and Leeson 1963; De Kretser et al. 1975; Bustos-Obregon 1976; Wrobel et al. 1979; Maekawa et al. 1996). Rodents display an exception as their peritubular wall usually consists only of one LSMC layer.

4.6.1 Immunofluorescence microscopical analyses of cytoskeletal and adherens junction molecules

In this study, immunocytochemical analyses have revealed that peritubular wall cells are fully developed SMCs rich in smooth muscle α-actin (α-SMA), corresponding myosin light and heavy chains, smoothelin, α-actinin, tropomyosin, desmin, vimentin, vinculin, talin, filamin A, drebrin, dystrophin, calponin, protein SM22α and caldesmon (Figs. 30–41, Table 14; for general references and reviews of these marker molecules see chapter 5.5).

In most cases, the mentioned smooth muscle marker molecules have not only been detected in the LSMCs surrounding the tubules but also in the blood vessel walls of the interstitial space of all species examined which thus are internal positive SMC-type controls (Figs. 30, 32, 37–38). For these experiments again the excurrent ducts were used as another control cell and tissue type (see chapter 4.3; Figs. 11–16). Table 14 presents a comparative list of the SMC-typical molecules tested and identified in cryostat cross-sections by immunofluorescence microscopy using specific antibodies.

The LSMCs are connected by single – or clusters of – AJs containing cadherin-11, a type II cadherin (Figs. 42, 45), as well as the *armadillo*-type cytoplasmic plaque protein β-catenin, plakoglobin and occasionally protein p0071. In sexually mature bull testes, the LSMCs as well as the smooth muscle cells of blood vessel walls often also contain P-cadherin (Figs. 43–45). In addition, the plasma membrane glycoprotein E-cadherin has been selectively detected in some LSMCs (Figs. 18, 44, 46). Negative findings will have to

be carefully examined and controlled using antibodies binding to different epitopes or scaffold complexes of all species studied.

While Sertoli cells are negative for the AJ plaque protein myozap, a number of cell-cell contact sites of LSMCs, blood vessel walls and endothelia have shown positive reactions for this protein (Fig. 47). The same was seen for protein LUMA which showed positive reactions in LSMCs of bull and human testes (Fig. 48) as well as in cells of the outer smooth muscle walls of excurrent duct tissues (Figs. 15–16 in chapter 4.3).

4.6.2 Intermediate-sized filament proteins in the LSMCs

Desmin and vimentin were generally detected in LSMCs. However, the intensity and distribution patterns of these IF proteins differed in certain regions within one LSMC or between different LSMCs and often also in different species (for general references see chapter 5.5). In addition, the occurrence of IF proteins identified as cytokeratins 8 and 18 was unexpectedly recognized in LSMC layers of bull and boar testes (Figs. 49–51; Table 15). In the peritubular LSMCs, desmin and cytokeratins 8 and 18 are in some parts in very close contact whereas other cell parts show separate localizations of cytokeratin and desmin (see, e.g., Fig. 51).

Table 14 Smooth muscle marker molecules identified and localized in peritubular and blood vessel wall SMCs of mammalian testes

Antibodies specific for	Peritubular wall cells (LSMCs)	Blood vessel SMCs
Smooth muscle α-actin (α-SMA)	++	++
Myosin (smooth muscle; heavy chain)	++	++
Myosin (smooth muscle; light chain 2)	++	++
Myosin (skeletal and smooth muscle)	+	+
Desmin	++	++
Drebrin[1]	+	+
Vimentin	++	++
Smoothelin	++[2]	+++
Protein SM22α (SM22α)	++[3]	++
Calponin	+[4]	++
Caldesmon	+	+
α-Actinin	++	++
Tropomyosin	+	+
Talin	+	++
Filamin A	+	++
Vinculin/Metavinculin	+	+
Dystrophin	+	+
Caveolin-1	+	+

[1] With the antibodies used (Peitsch et al. 2001, 2003, 2005) drebrin has been seen in the LSMCs of peritubular walls as well as in SMCs of blood vessel walls whereas reactions on other testicular cells were negative or rather weak and diffuse. As the literature contains other results, in particular specific reactions with actin-rich regions of Sertoli cells (Li et al. 2011; Su et al. 2013; Chen et al. 2017) and specific regions of neuronal cells (Shirao and Obata 1986) this discrepancy will be dealt with in a later publication of the laboratory of Prof. Dr. Werner W. Franke.

[2] Partly different smoothelin antibody reactions have been noted in peritubular walls: Polyclonal smoothelin antibodies used (Abcam, ab204305) have shown a positive reaction in peritubular walls of all species while the monoclonal smoothelin antibody (Millipore, MAB3242) has reacted positively only with LSMCs of bull and boar peritubular wall tissues but not in human and rodent peritubular walls. However, all antibodies have been generally positive in SMCs of blood vessel walls. Whether this is due to the presence of a different amino acid sequence or protein modifications or epitope “masking” by certain scaffold or other complex formations remains to be examined.

[3] Partly different SM22α antibody reactions have been noted in peritubular walls: Some polyclonal SM22α antibodies (Abcam, ab155272) have shown positive reactions on blood vessel walls of all species but no positive reaction in peritubular walls. Other polyclonal SM22α antibodies (Abcam, ab14106) react positively only in bovine peritubular wall LSMCs but not in peritubular walls of the other species examined, whereas positive reactions have been seen in blood vessel walls of all species tested.

[4] With the specific antibodies used calponin has only been detected in bull and human peritubular walls but not in peritubular walls of other species. Again, it needs to be examined in future experiments whether this is based on antibody specificities or specific epitope-containing scaffolding complexes, or on one specific absence of calponin in certain other species.

Table 15 Immunolocalization results with additional structural marker molecules in reactions with peritubular walls and blood vessel walls of diverse mammalian species

Antibodies specific for	Peritubular wall cells (LSMCs)	Blood vessel wall SMCs
Laminin 5[1]	–	+
Laminin mix	++	++
HD 230/233	–	–
Pro-Collagen	+	+
Collagen-IV	++	+
Elastin	++	+
α-cardiac Actin	–	–
α-skeletal/cardiac Actin	–	–
Myosin Cardiac (heavy chain)	–	–
Cytokeratin 8	(+)[2]	–
Cytokeratin 18	(+)[2]	–
α6-Integrin	(+)[3]	+
β4-Integrin	(+)	+
Fibronectin	(+)	+

[1] The laminin 5 antibodies used (Progen, 10765) are human-specific.

[2] The antibodies against cytokeratins 8 and 18 used are positive only with LSMCs of bull and boar tissues (compare also with Table 16 in the Discussion).

[3] The α6- and β4-Integrin antibodies used react differently, maybe based on species specificity of the antibodies used or on epitope "masking". However, colocalization as is typical for hemidesmosomes has not been seen.

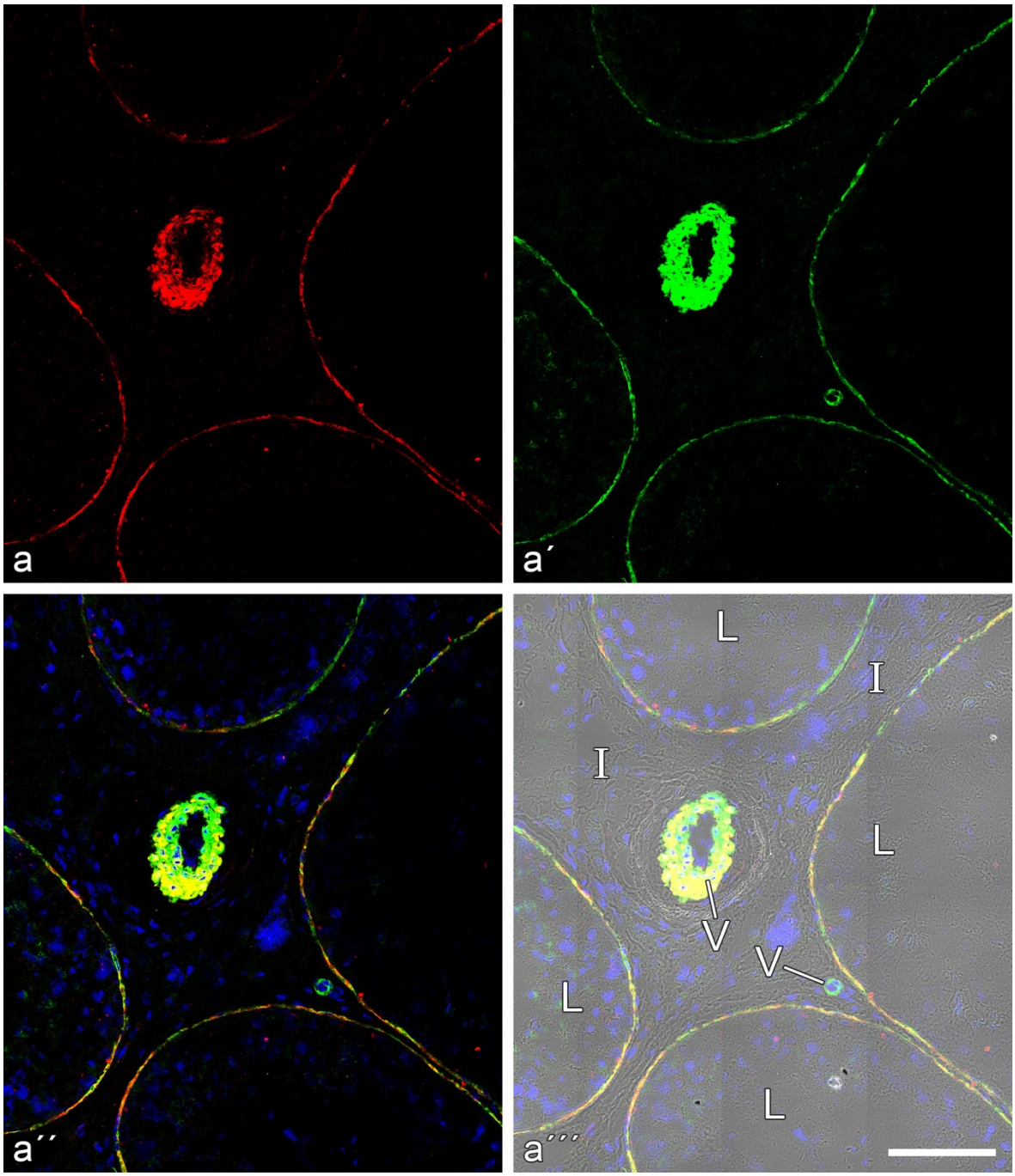


Fig. 30 Tile-scan overview showing double-label immunofluorescence microscopy of frozen bull testis tissue presenting a cryostat cross-section through seminiferous tubules. Immunoreactions of **smoothelin** (**a**, **a''**, **a'''**; *red*; monoclonal mouse antibody) and **desmin** (**a'**–**a'''**; *green*; rabbit antibodies) show colocalization of both smooth muscle marker proteins in the peritubular wall structures, the peritubular lamellar smooth muscle cells (LSMCs), around the seminiferous tubules (L, lumen) as well as in the perivascular smooth muscle cell layer of blood vessels (V) in the interstitial space (I). Nuclei have been stained *blue* with DAPI (**a''**–**a'''**). *Bar* 100 µm (see also Domke and Franke 2018, in revision).

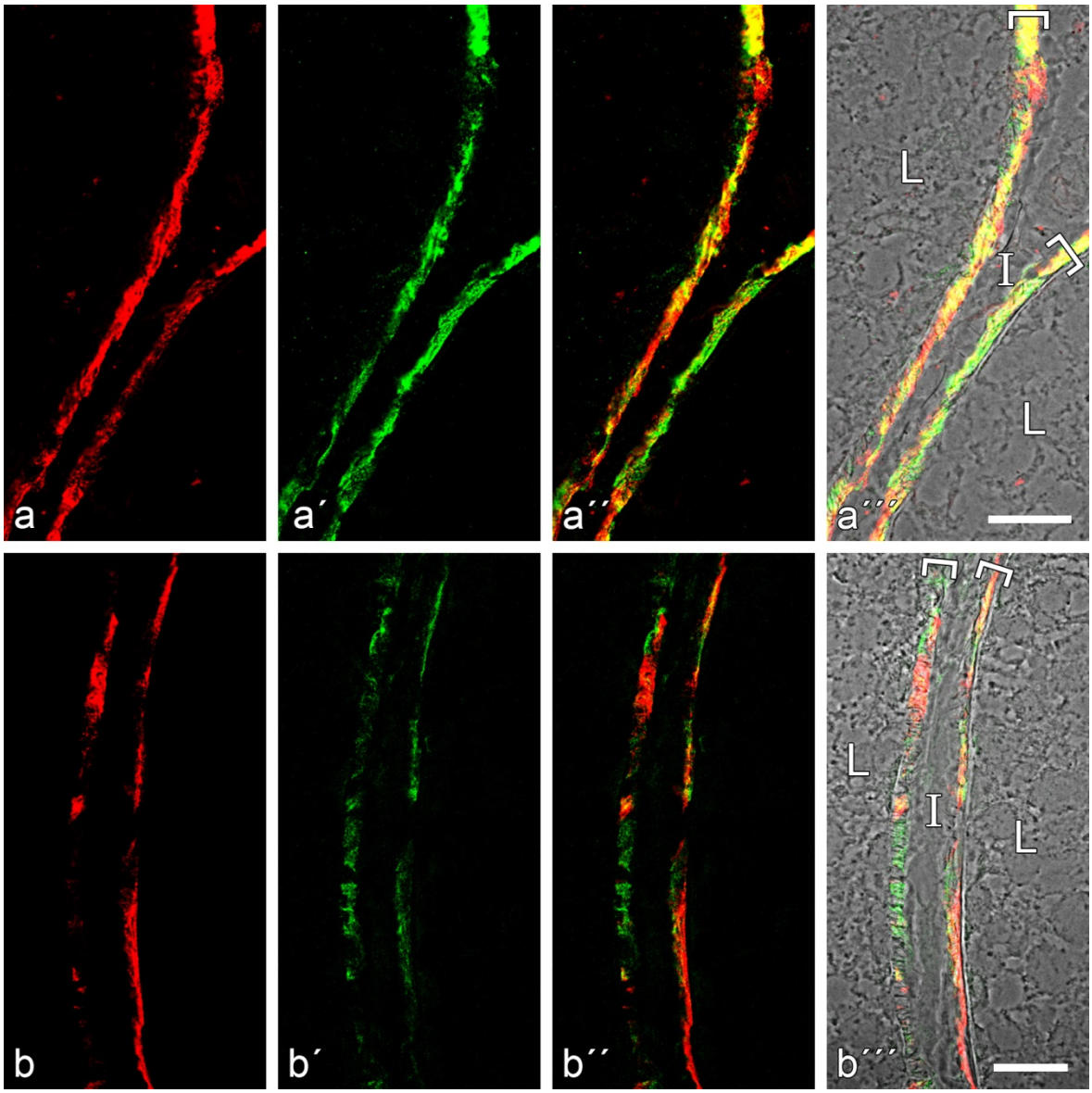


Fig. 31 Double-label immunofluorescence microscopy of cryostat cross-sections through frozen bull testis tissue. Immunoreactions show in the peritubular LSMC layers (brackets) colocalization of **smoothelin** (**a**, **a’’**, **a’’’**, **b**, **b’’**, **b’’’**; *red*; monoclonal mouse antibody) and **desmin** (**a’**–**a’’’**, **b’**–**b’’’**; *green*; rabbit antibodies) in many regions (*yellow* merge colour, with phase contrast background in **a’’’**, **b’’’**) but clearly not in all. At higher magnification (**b**–**b’’’**) another region shows the local differences of both smooth muscle proteins. Many fibrils are seen in different orientations – some longitudinal, some in a cross-striated appearance. L, lumen; I, interstitial space. *Bars* 20 µm (see also Domke and Franke 2018, in revision).

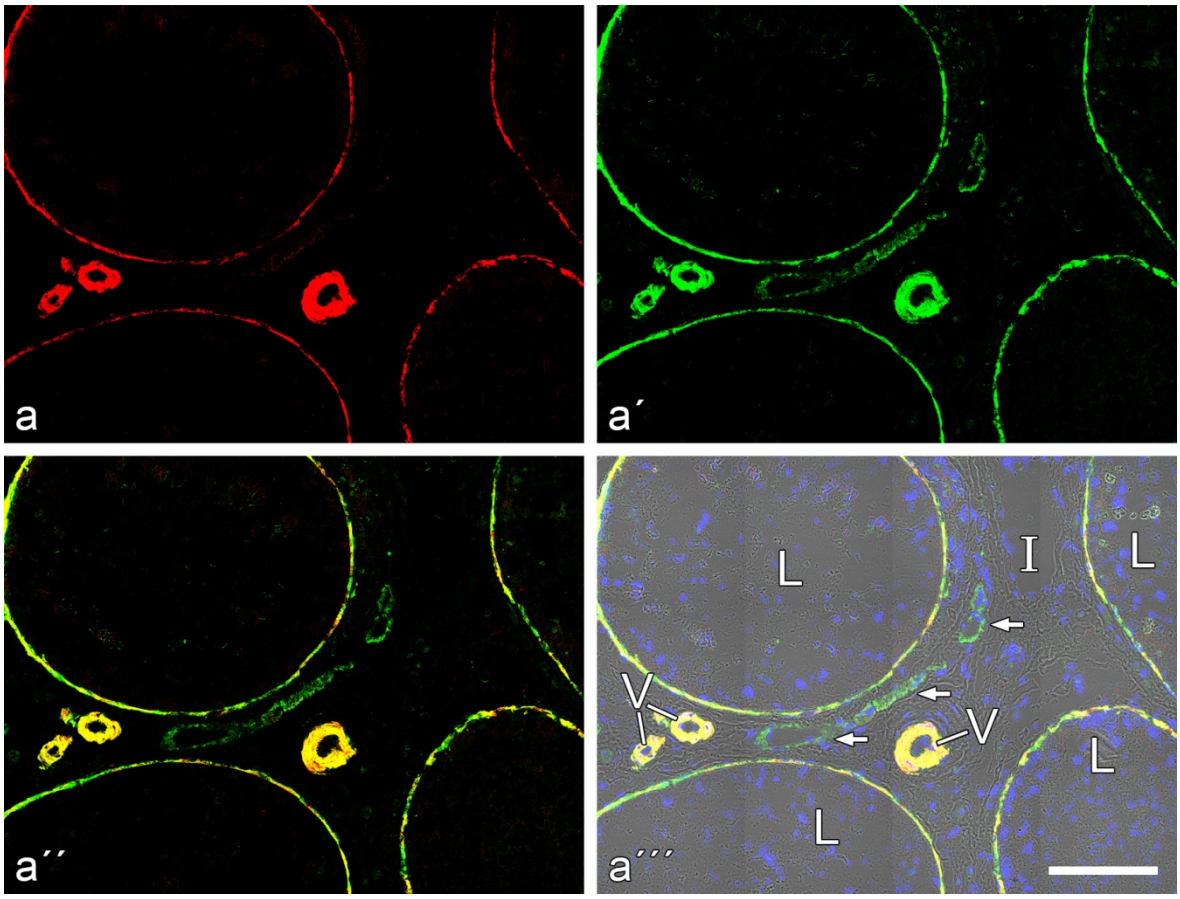


Fig. 32 Tile-scan survey showing double-label immunofluorescence microscopy of a cryostat cross-section through seminiferous tubules in frozen bull testis tissue. Immunoreactions to **smoothelin** (**a**, **a''**, **a'''**; *red*; monoclonal mouse antibody) and **smooth muscle α-actin** (**α-SMA**; **a'**–**a'''**; *green*; rabbit antibodies) show colocalization of both smooth muscle marker proteins in the peritubular LSMC structures around the seminiferous tubules (L, lumen) and in some perivascular smooth muscle cell layers of blood vessels (V) whereas a certain type of thin blood vessel walls is only positive for α-SMA (denoted by arrows in **a'''**, on optical phase-contrast background). Nuclei have been stained *blue* with DAPI. I, interstitial space. *Bar* 100 µm.

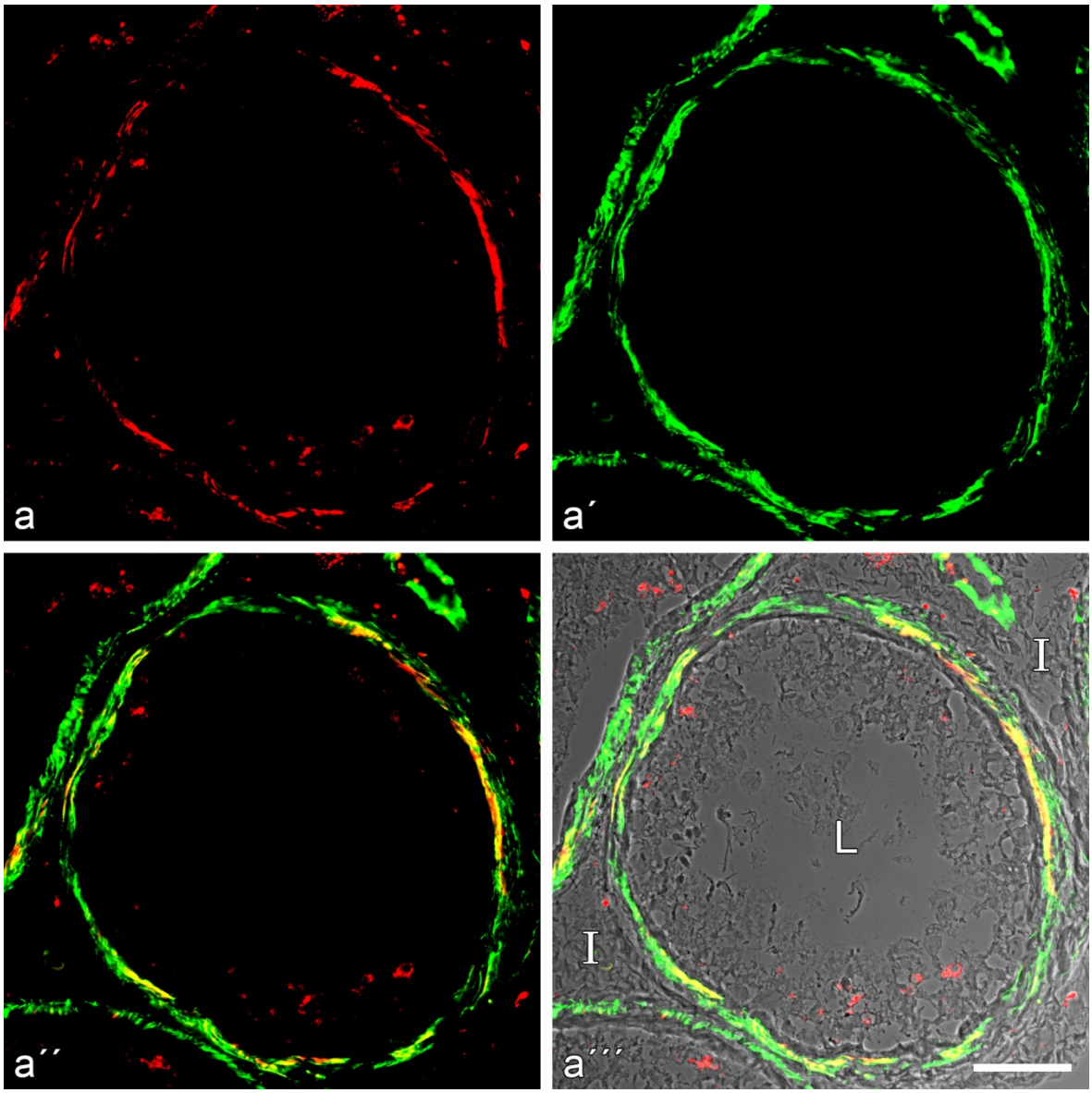


Fig. 33 Double-label immunofluorescence microscopy of a cryostat cross-section through frozen human testis tissue. Immunoreactions of the smooth muscle markers **smoothelin** (**a**, **a''**, **a'''**; *red*; rabbit antibodies) and **smooth muscle α-actin** (**α-SMA**; **a'**–**a'''**; *green*; monoclonal mouse antibody) show that colocalization of both smooth muscle marker proteins (*yellow* merger colour) is seen only in some regions of the peritubular LSMC structure around the seminiferous tubules but not in all (L, lumen). I, interstitial space. *Bar* 50 µm.

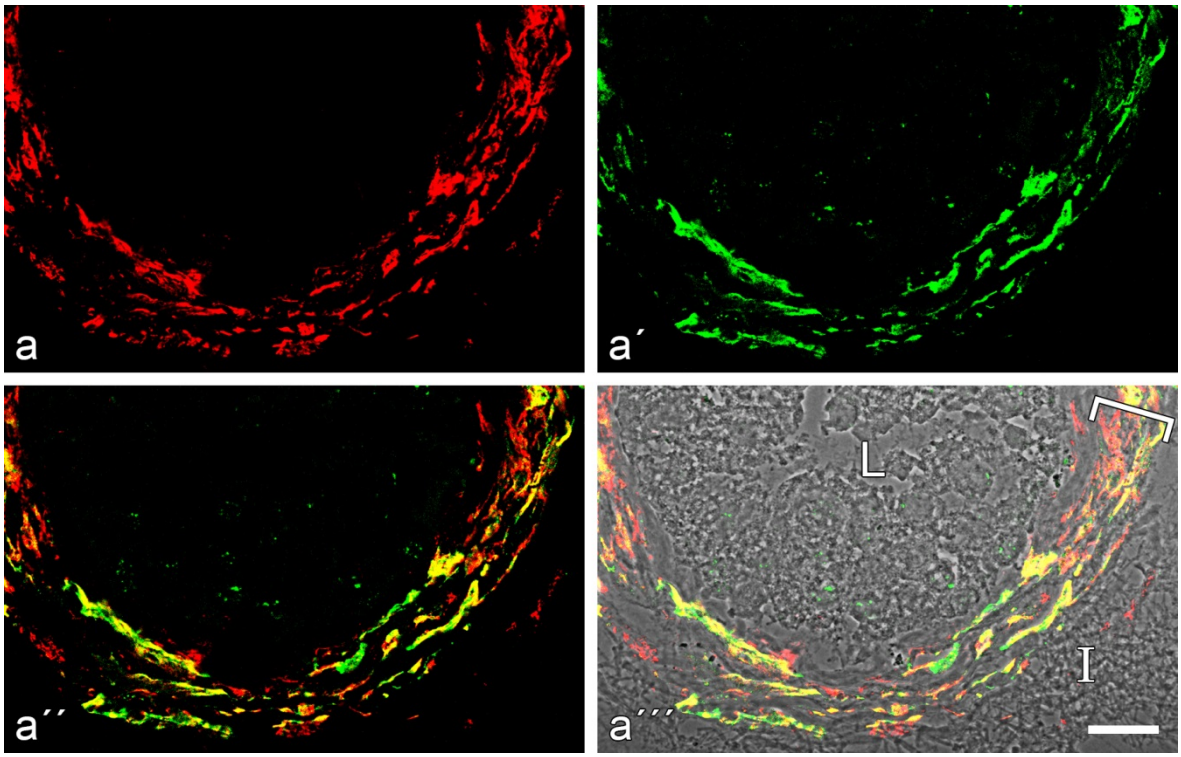


Fig. 34 Double-label immunofluorescence microscopy of a cryostat cross-section through a part of a seminiferous tubule of frozen human testis, containing here rather thick SMC walls (denoted by bracket). These LSMCs are positively immunostained for **smooth muscle myosin heavy chain** (**a**, **a''**, **a'''**; *red*; monoclonal mouse antibody) and **smooth muscle α-actin** (**α-SMA**; **a'**–**a'''**; *green*; rabbit antibodies). I, interstitial space; L, lumen. *Bar* 20 µm (see also Domke and Franke 2018, in revision).

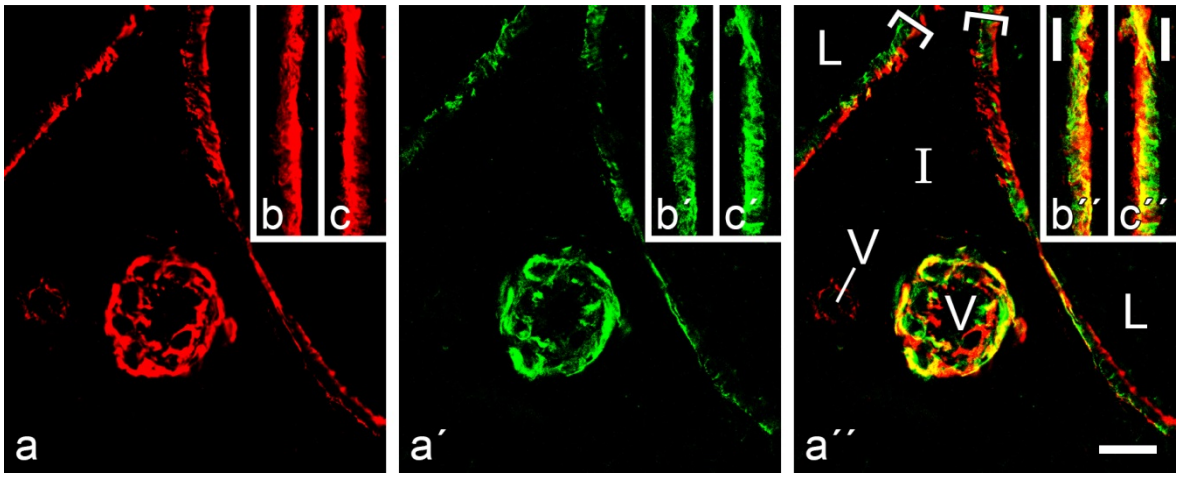


Fig. 35 Double-label immunofluorescence microscopy of a cryostat cross-section through seminiferous tubules (L, lumen) of a frozen bull testis. Immunoreactions of antibodies to **smooth muscle α-actin** (**α-SMA**; **a**, **a''**; *red*; monoclonal mouse antibody) and to **desmin** (**a'**–**a''**; *green*; rabbit antibodies) show colocalization regions (*yellow* merger colour) in the peritubular LSMC layers (**a''**; brackets) as well as in the perivascular (V, vessel) SMCs but also some regions which are positive for either α-SMA or desmin. (**b'**, **c'**) Note a small vessel (left V) which is positive only for α-SMA. The inserts (**b**–**b''**, **c**–**c''**) demonstrate orientation differences of protein immunostaining in different peritubular wall layers or intercepts. I, interstitial cells. *Bars* (**a**–**a''**) 20 µm and (**b**–**b''**, **c**–**c''**) 10 µm (see also Domke and Franke 2018, in revision).

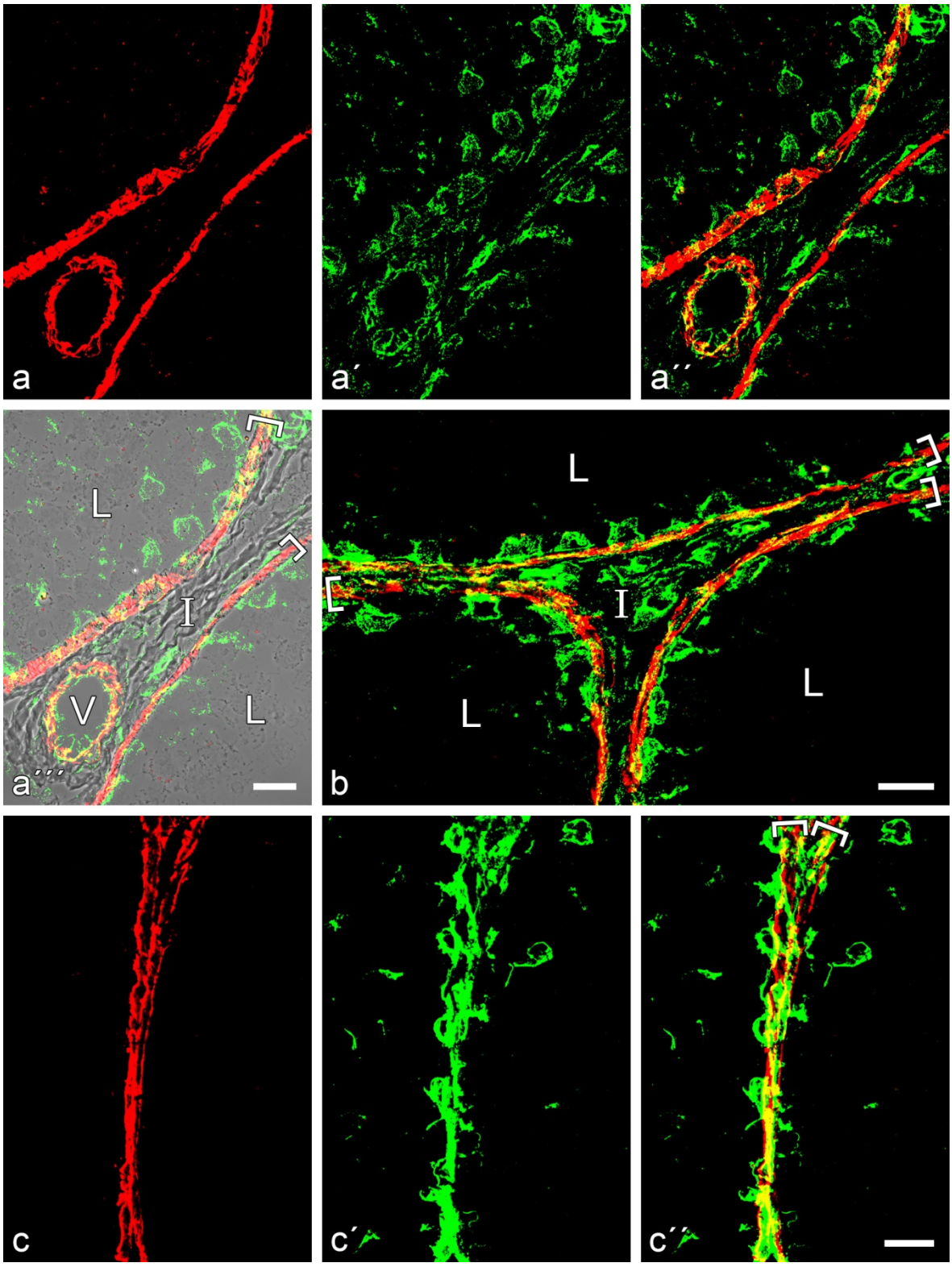


Fig. 36 Double-label immunofluorescence microscopy of a cryostat cross-section through seminiferous tubules of mature (**a**, **b**) bull and (**c**) rat testes. After immunoreactions to **smooth muscle α-actin** (**α-SMA**; **a**, **a''**, **a'''**, **b**, **c**, **c''**; *red;* monoclonal mouse antibody) and to the intermediate-size filament protein **vimentin** (**a'**–**a'''**, **b**, **c'**, **c''**; *green*; guinea pig antibodies) all Sertoli cells (L, lumen) show positive reaction for vimentin filament bundles but are totally negative for α-SMA. In contrast, peritubular LSMCs (brackets) show regions of colocalization or optical overlapping of α-SMA and vimentin (**a''**, **a'''**, **b** and **c**; *yellow* merge colour). I, interstitial space. *Bars* 20 µm.

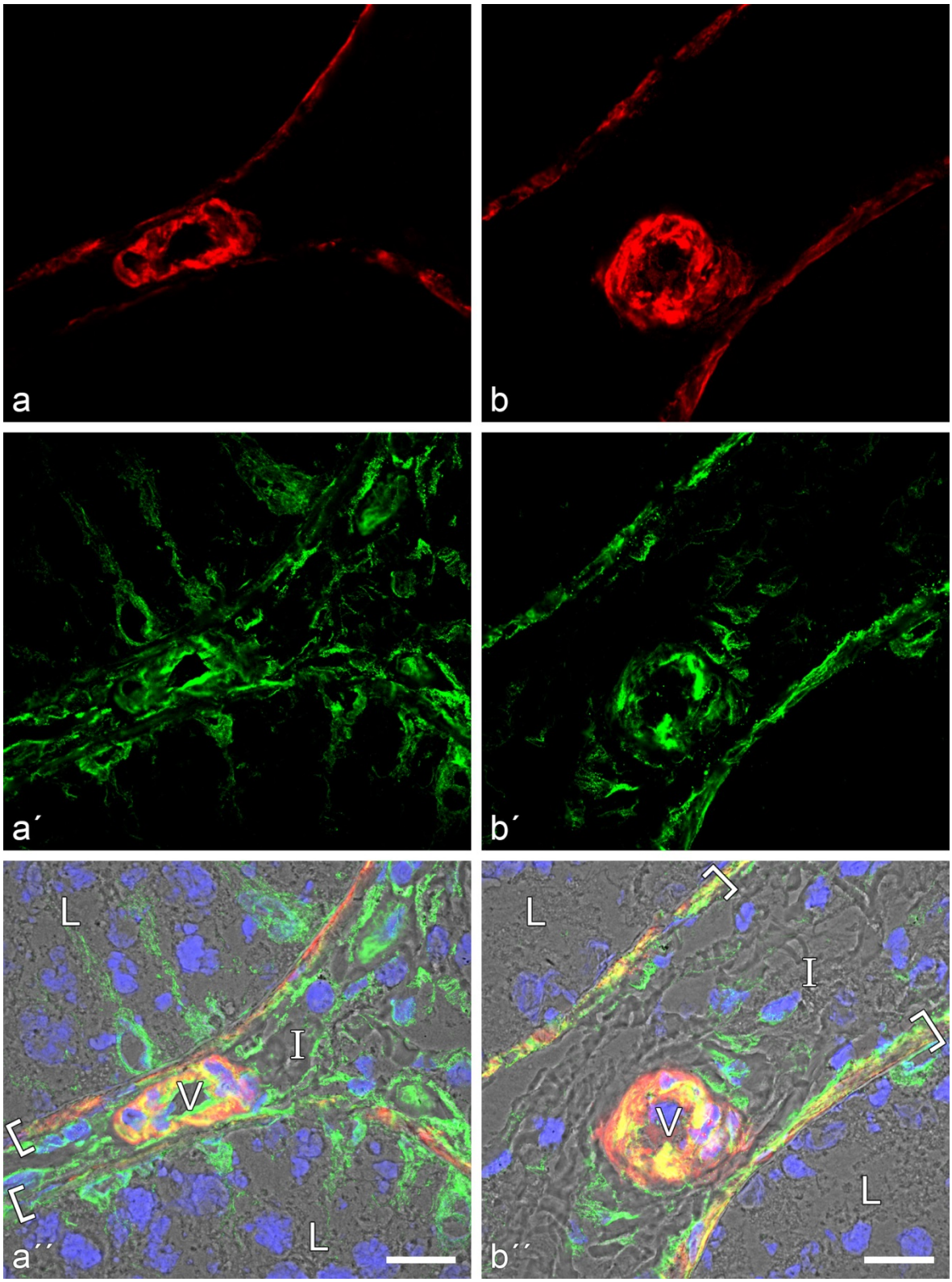


Fig. 37 Double-label immunofluorescence microscopy of cross-sections through seminiferous tubules of frozen bull testis tissue, showing that antibodies against **smoothelin** (**a**, **b**; *red*; monoclonal mouse antibody) and **desmin** (**b'**, **b''**; *green*; rabbit antibodies) react positively in parts of LSMCs surrounding the tubules (L, lumen) and blood vessels (V) and that the **vimentin** filament bundles (**a'**, **a''**; *green*; guinea pig antibodies) are structures which occur in both the Sertoli cells as well as in various cells of the interstitial space (I). Nuclei have been stained *blue* with DAPI. *Bars* 20 µm.

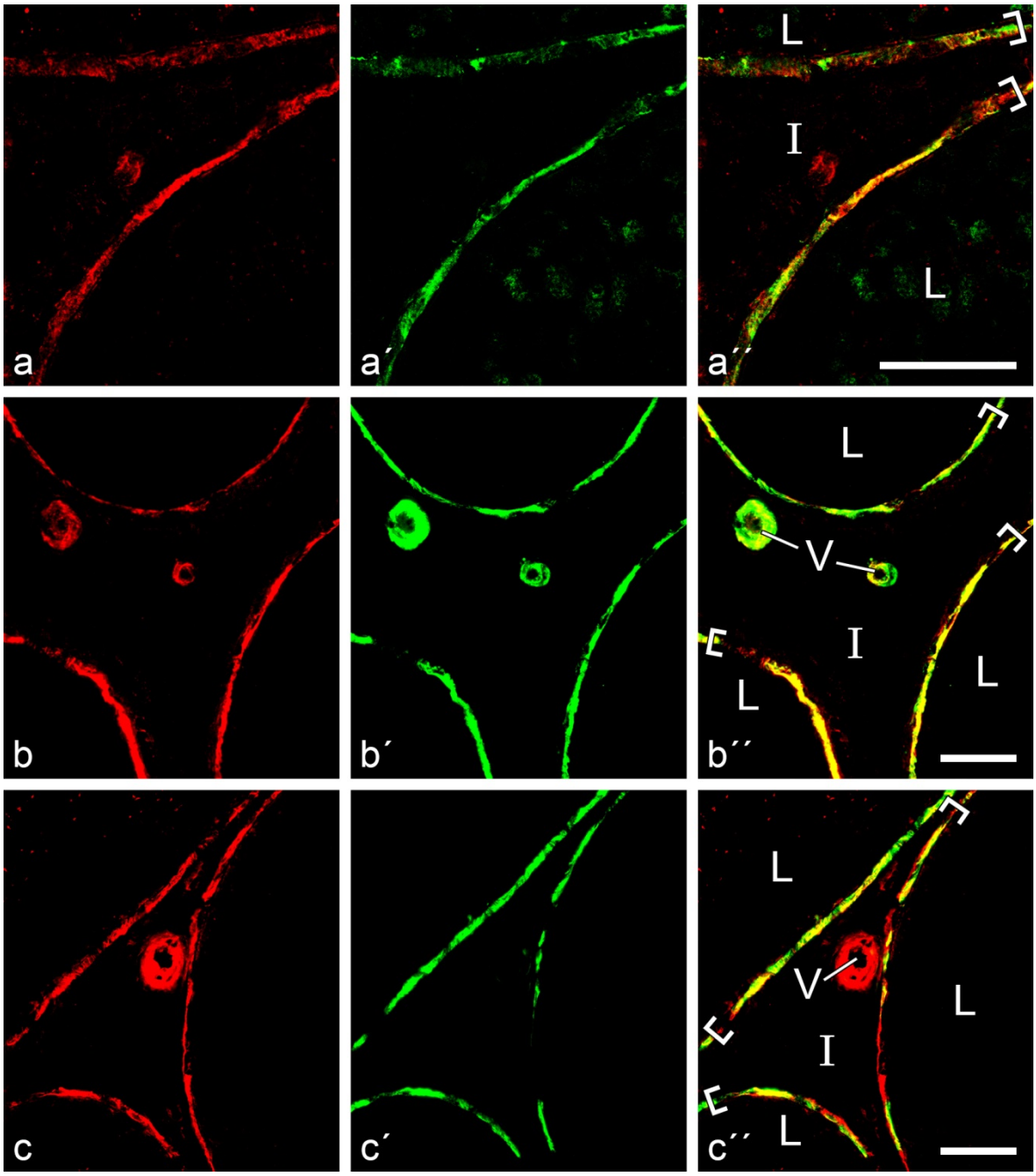


Fig. 38 Double-label immunofluorescence microscopy of cryotomy cross-sections through frozen bull testes after immunoreactions with antibodies against the protein **talin** (**a**, **a''**; *red*; monoclonal mouse antibody), protein **calponin** (**b**, **b''**; *red*; monoclonal mouse antibody), protein **SM22α** (**c**, **c''**; *red*; rabbit antibodies) and against the SMC marker **smooth muscle α-actin** (**α-SMA; a'–a''**, **b'–b''**; *green*; rabbit antibodies) as well as **desmin** (**c'–c''**; *green*; monoclonal mouse antibody). Note the positive staining in extended regions of the LSMCs (denoted by brackets). Immunoreactions show positive results, partly appearing optically as colocalization, in the peritubular LSMCs surrounding the seminiferous tubules (L, lumen). SMCs of a blood vessel (V) in the interstitial space (I) indicate that these are here only positive for SM22α, α-SMA and calponin. *Bars* 50 µm.

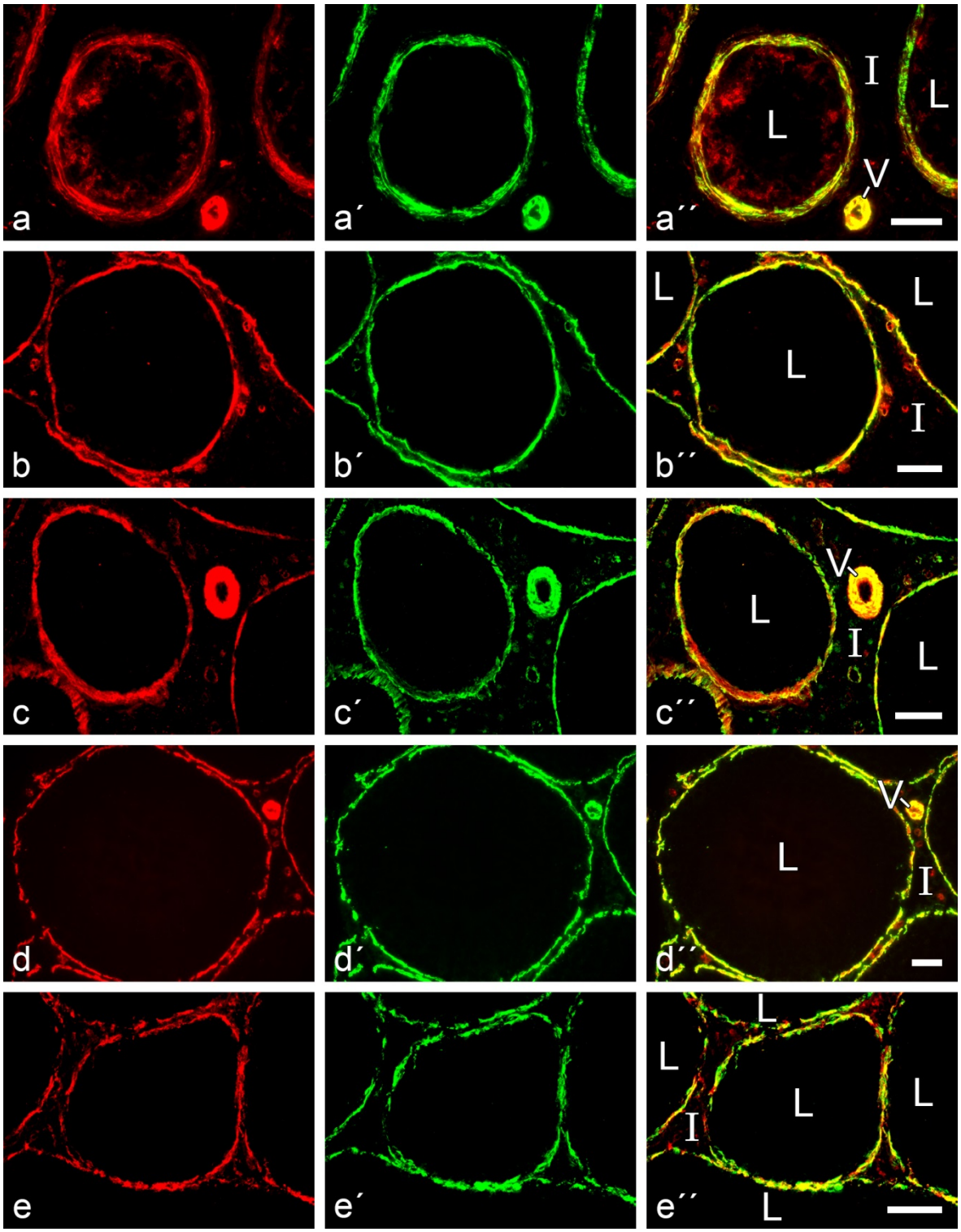


Fig. 39 Double-label immunofluorescence microscopy of cryostat cross-sections through seminiferous tubules of frozen (**a**) human, (**b**) bovine, (**c**) porcine, (**d**) rat and (**e**) guinea pig testes after reactions with rabbit antibodies against **filamin A** (**a**, **a''**, **b**, **b''**, **c**, **c''**, **d**, **d''**, **e**, **e''**; *red*) and a monoclonal mouse antibody to **smooth muscle α-actin** (**α-SMA**; **a'**–**a''**, **b'**–**b''**, **c'**–**c''**, **d'**–**d''**, **e'**–**e''**; *green*). Both filamin A and α-SMA are localized in the LSMCs of the peritubular and the vascular walls (V, blood vessels), often optically giving the impression of colocalization (*yellow* merge colour) in all species. I, interstitial space; L, lumen. *Bars* 50 µm.

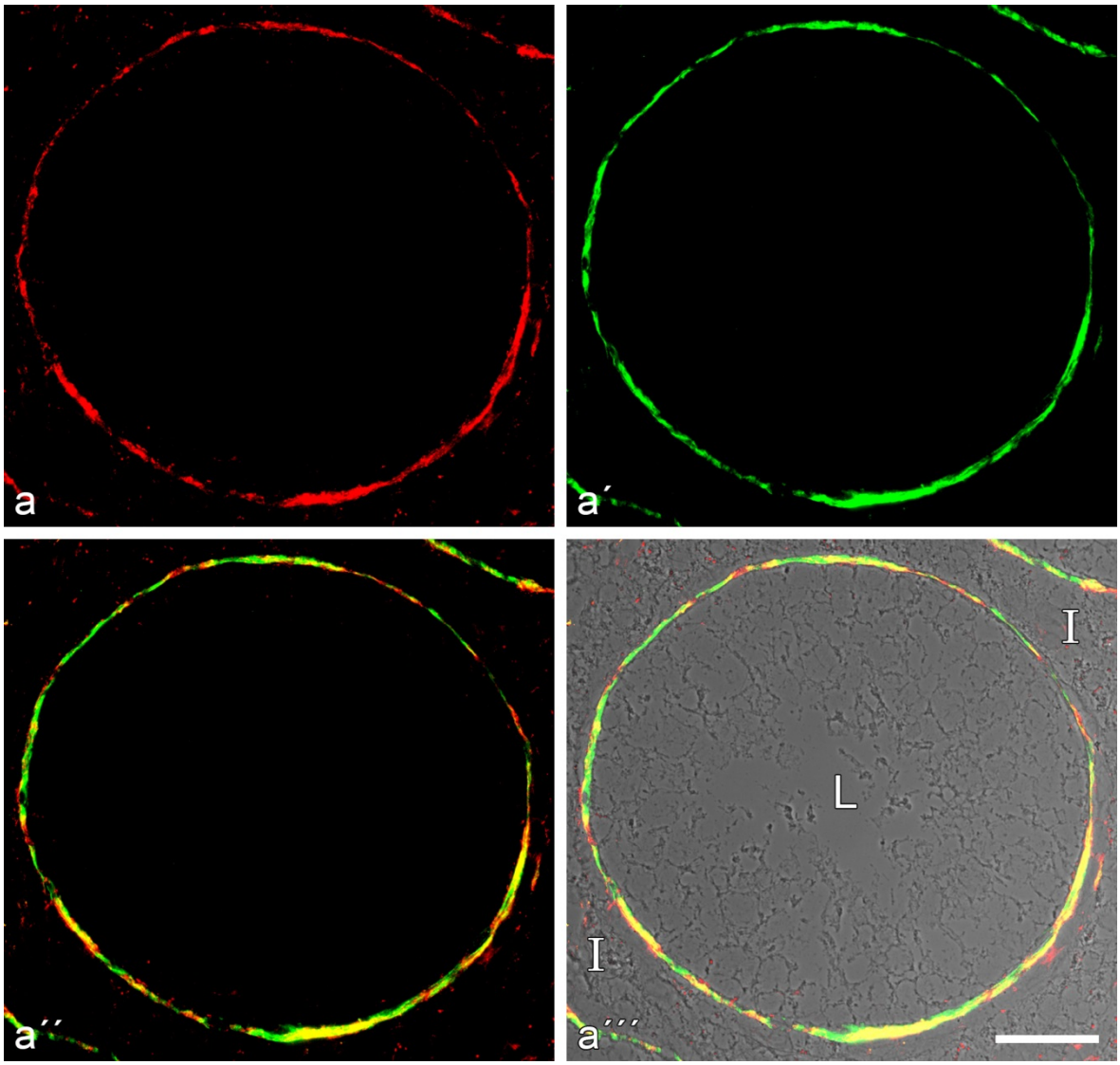


Fig. 40 Double-label immunofluorescence microscopy of a cryostat cross-section through frozen boar testis tissue. Immunoreactions of **dystrophin** (**a**, **a''**, **a'''**; *red*; rabbit antibodies) and **smoothelin** (**a'**–**a'''**; *green*; monoclonal mouse antibody) show extended colocalization of both marker proteins (*yellow* merger colour) in the LSMCs of the peritubular wall structure. I, interstitial space; L, lumen. *Bar* 50 µm (see also Domke and Franke 2018, in revision).

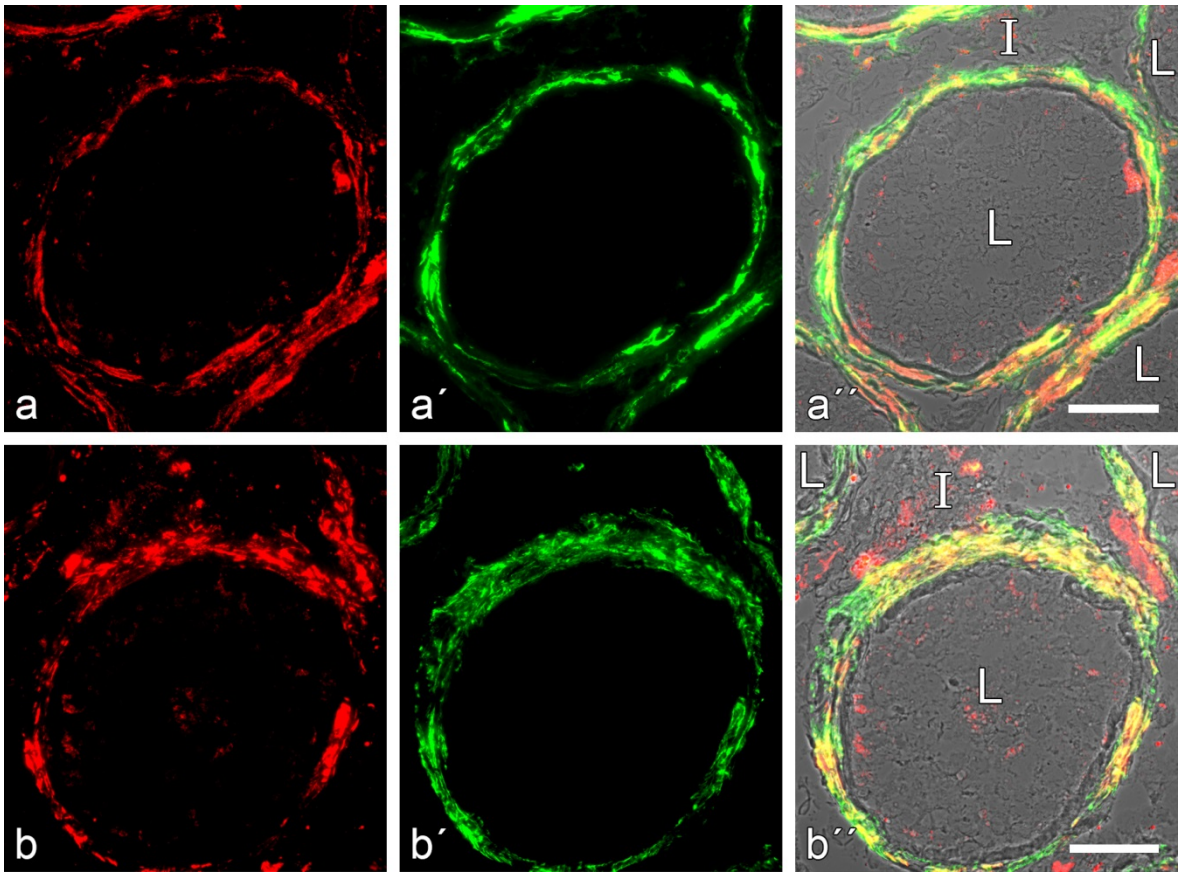


Fig. 41 Double-label immunofluorescence microscopy of a cryostat cross-section through frozen human testis tissue. Immunoreactions of **dystrophin** (**a**, **a''**; *red*; rabbit antibodies) and **desmin** (**a'**–**a''**; *green*; monoclonal mouse antibody) as well as **caldesmon** (**b**, **b''**; *red*; rabbit antibodies) and **smooth muscle α-actin** (**α-SMA**; **b'**–**b''**; *green*; monoclonal mouse antibody) show colocalization of both marker proteins (*yellow* merger colour) only in some regions of the peritubular wall but not in all (L, lumen). I, interstitial space. *Bars* 50 µm.

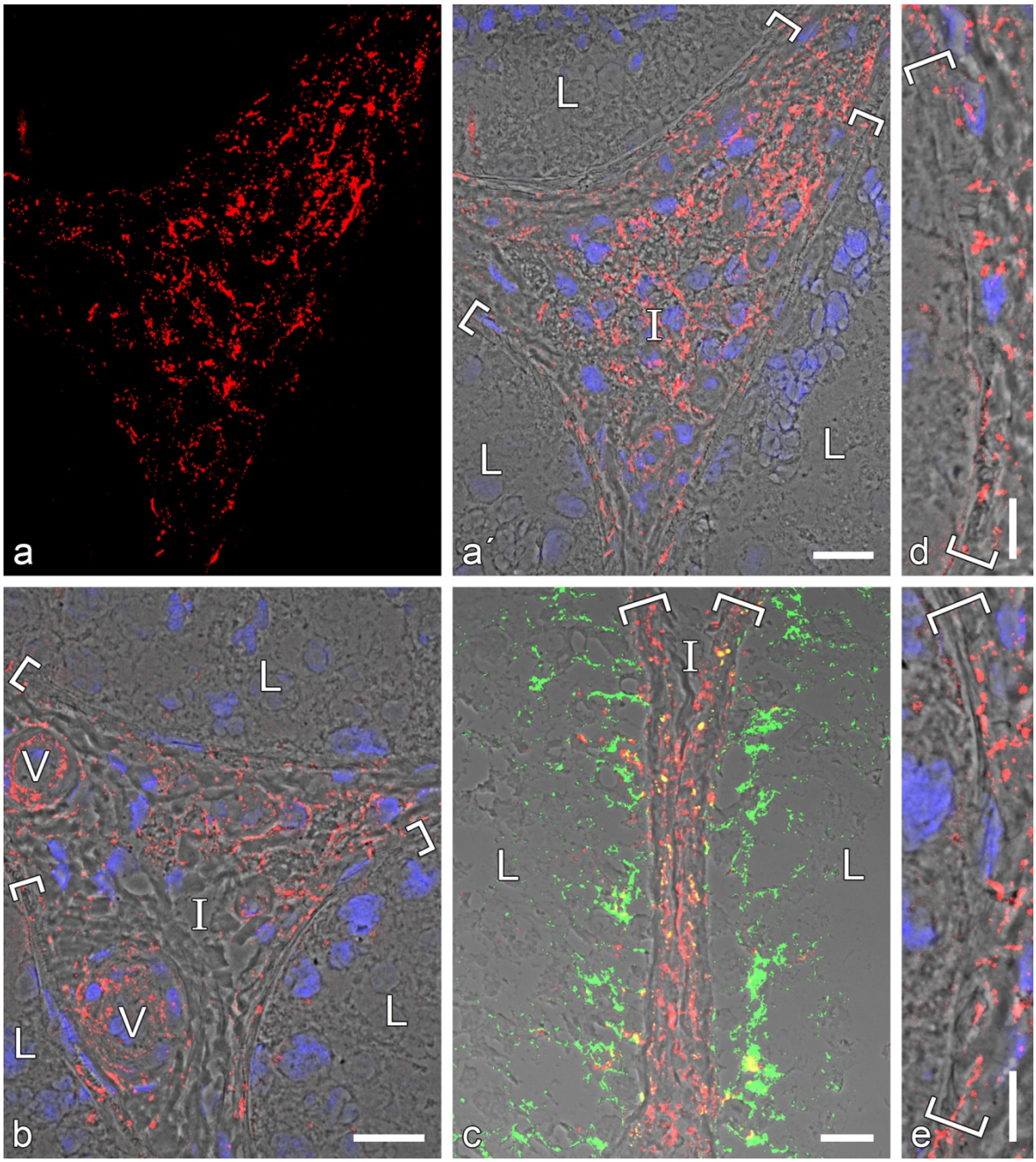


Fig. 42 Double-label immunofluorescence microscopy of a cryotomy cross-section through seminiferous tubules of frozen bull (**a**, **b**, **d**, **e**) and human (**c**) testes show immunoreactions of **cadherin-11** (**a**, **a'**, **b**, **c**, **d**; *red*; monoclonal mouse antibody) and **N-cadherin** (**c**; *green*; rabbit antibodies). (**a**, **b**, **d**, **e**) Positive punctate reaction sites of cadherin-11 are seen in regions of the peritubular wall structures including LSMCs (brackets; higher magnification images are shown in **d** and **e**) and of blood vessel (V) walls as well as in some other cells of the interstitial space (I). (**c**) As in the bovine Sertoli cells (**a**–**b**) cadherin-11 is usually also not recognized in human Sertoli cells which, however, are rich in N-cadherin-positive AJs. Nuclei have been stained *blue* with DAPI. L, lumen. *Bars* (**a**, **b**, **c**) 20 µm and (**d**, **e**) 10 µm (see also Domke and Franke 2018, in revision).

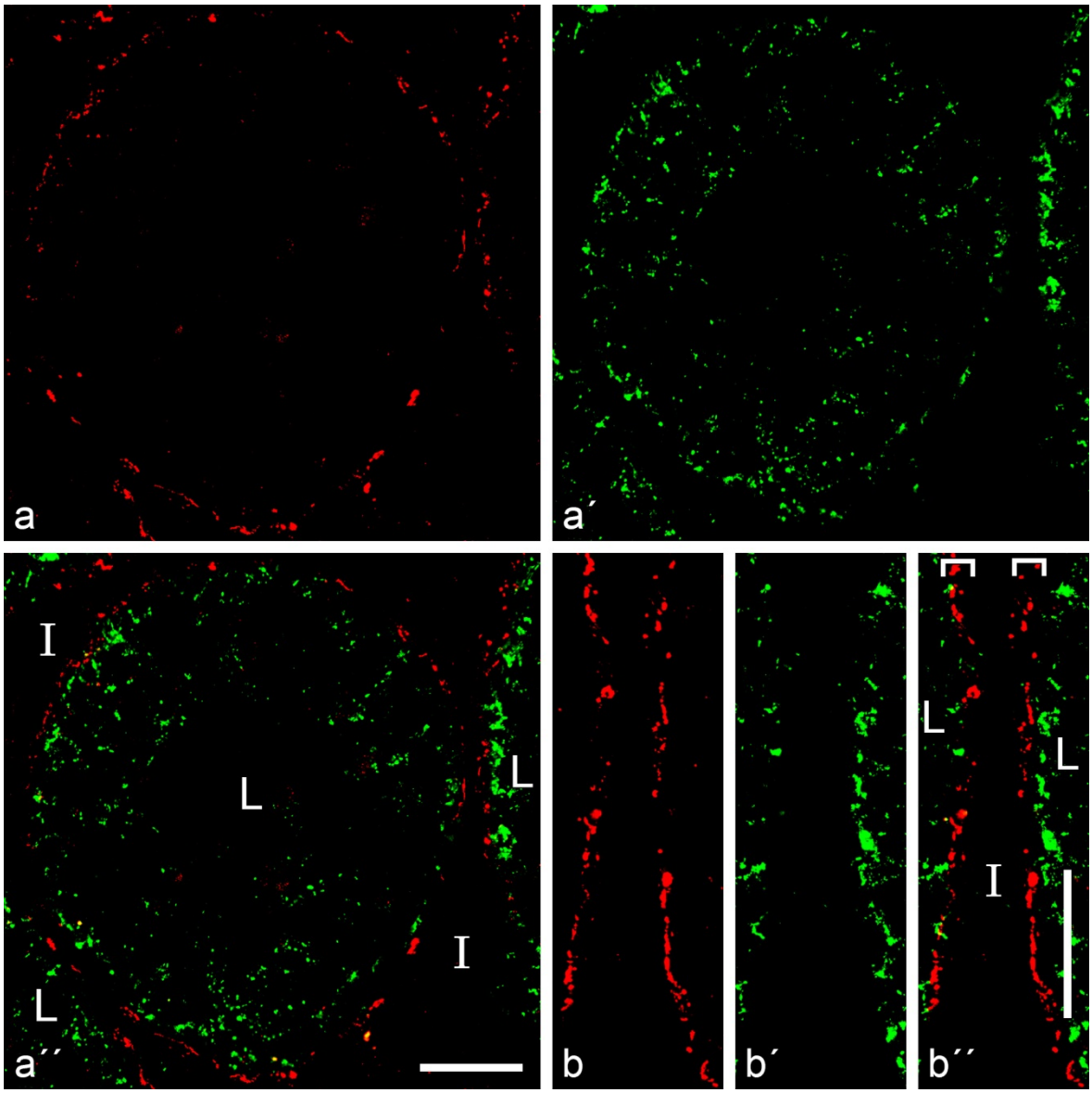


Fig. 43 Double-label immunofluorescence microscopy of a cryotomy cross-section through seminiferous tubules of frozen bull testis show immunoreactions of **P-cadherin** (**a**, **b**, **a''**, **b''**; *red*; rat antibodies) and **N-cadherin** (**a'**, **b'**, **a''**, **b''**; *green*; monoclonal mouse antibody). (**a**, **b**) Positive reactions of P-cadherin are seen in regions of the peritubular wall structures (LSMCs; brackets in **b''**). N-cadherin is seen in the tubules (L) and if any sparsely in the LSMCs. I, interstitial space. *Bars* (**a**, **b**) 50 µm.

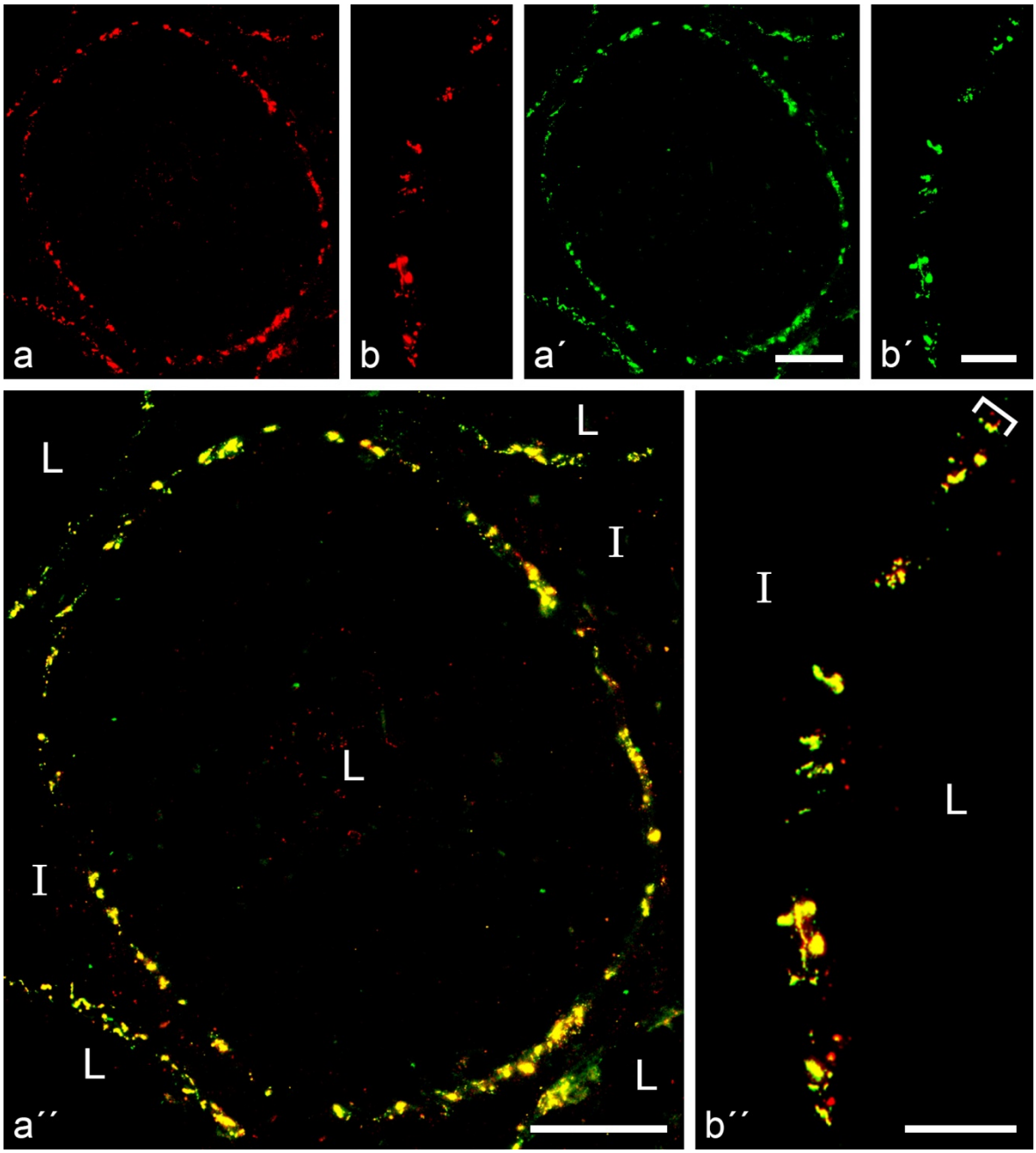


Fig. 44 Double-label immunofluorescence microscopy of a cryotomy cross-section through seminiferous tubules of frozen bull testis show immunoreactions of **P-cadherin** (**a**, **b**, **a''**, **b''**; *red*; rat antibodies) and **E-cadherin** (**a'**, **b'**, **a''**, **b''**; *green*; monoclonal mouse antibody). (**a**, **b**) Colocalizations of P-cadherin and E-cadherin are seen in regions of the peritubular wall structures (LSMCs; brackets in **b''**). L, lumen; I, interstitial space. *Bars* (**a**) 50 µm and (**b**) 20 µm.

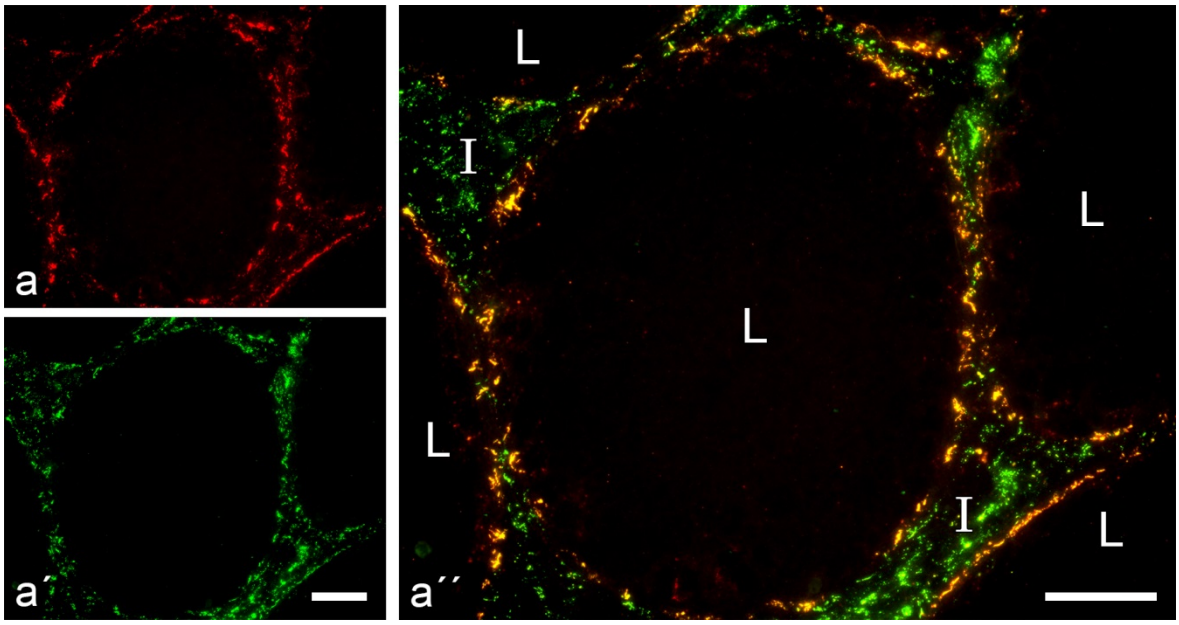


Fig. 45 Double-label immunofluorescence microscopy of a cryotomy cross-section through seminiferous tubules of frozen bull testis show immunoreactions of **cadherin-11** (**a**, **a''**; *red*; monoclonal mouse antibody) and **P-cadherin** (**a'**, **a''**; *green*; rat antibodies). Reaction sites of cadherin-11 and P-cadherin are seen in regions of the peritubular LSMCs. In addition, some other cells of the interstitial space (I) are positive for cadherin-11. L, lumen. *Bars* 50 µm.

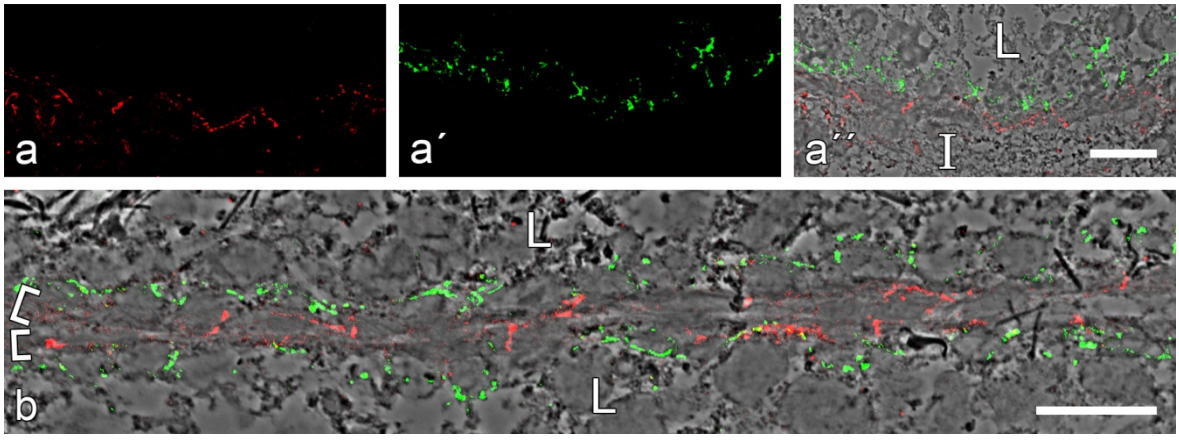


Fig. 46 Double-label immunofluorescence microscopy of cryostat cross-sections through seminiferous tubules of frozen rat testes. After immunoreactions against **E-cadherin** (**a**, **a''**, **b**; *red*; monoclonal mouse antibody) and **N-cadherin** (**a'**–**a''**, **b**; *green*; rabbit antibodies) Sertoli cells are positive for N-cadherin (*green*) but negative for E-cadherin. A positive immunoreaction for E-cadherin (*red*) is only seen in some peritubular wall cells (**b**; brackets). *Bars* 20 µm.

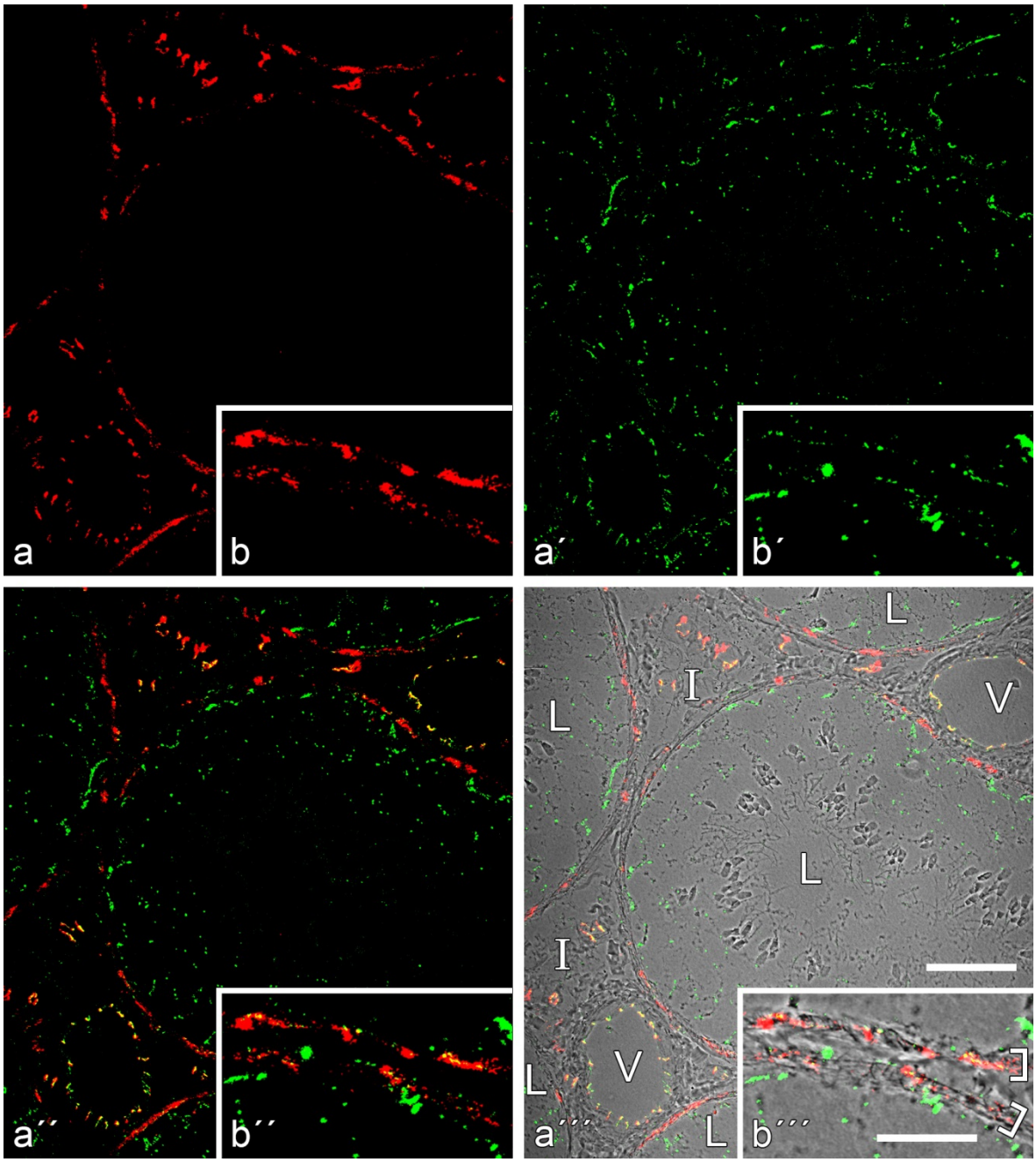


Fig. 47 Double-label immunofluorescence microscopy of a cryotomy cross-section through seminiferous tubules (L, lumen) of bull testis. After immunoreactions of protein **myozap** (**a**, **a''**, **a'''**; *red*; monoclonal mouse antibody) and the *armadillo* protein **β-catenin** (**a'**–**a'''**; *green*; rabbit antibodies) all Sertoli cells and blood vessel (V) endothelial cells are positive for β-catenin whereas Sertoli cells are negative for protein myozap. In contrast, both myozap-positive and β-catenin-positive AJs are recognized in cells of the peritubular walls and distinct colocalization of both, myozap and β-catenin, is characteristic for the AJs of vascular endothelial cells (note the *yellow* merger colour). I, interstitial space. *Bars* (**a**) 50 µm and (**b**) 20 µm (see also Domke and Franke 2018, in revision).

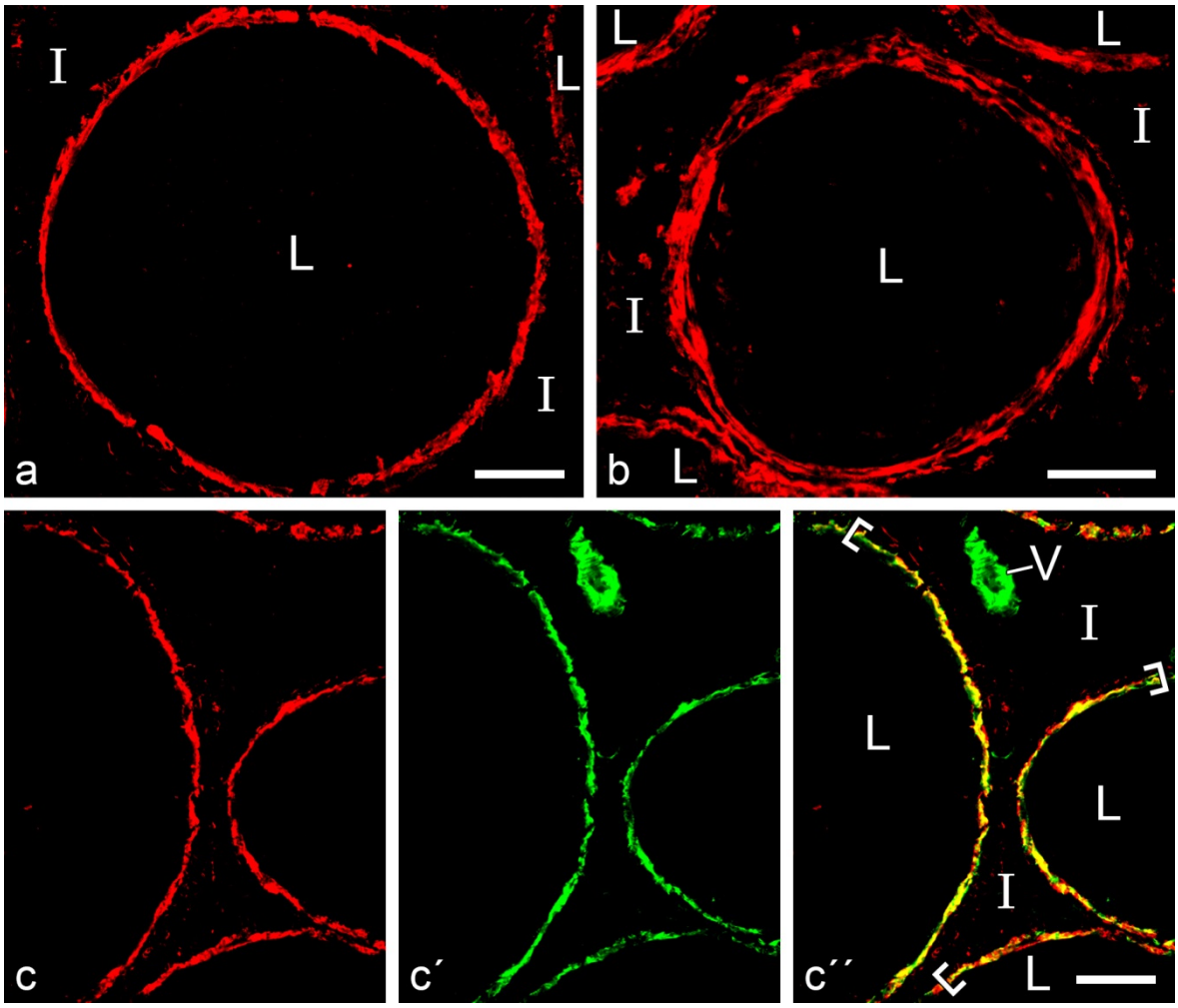


Fig. 48 Double-label immunofluorescence microscopy of a cryostat cross-section through frozen (**a**, **c**) bull and (**b**) human testicular tissue. Immunoreactions of **LUMA** (**a**–**c**, **c''**; red; guinea pig antibodies) and **α-SMA** (**c'**, **c''**; green; monoclonal mouse antibody) show positive reactions in the LSMCs (brackets in **c''**) of the peritubular wall structure. L, lumen; I, interstitial space; V, blood vessel. *Bars* 50 µm.

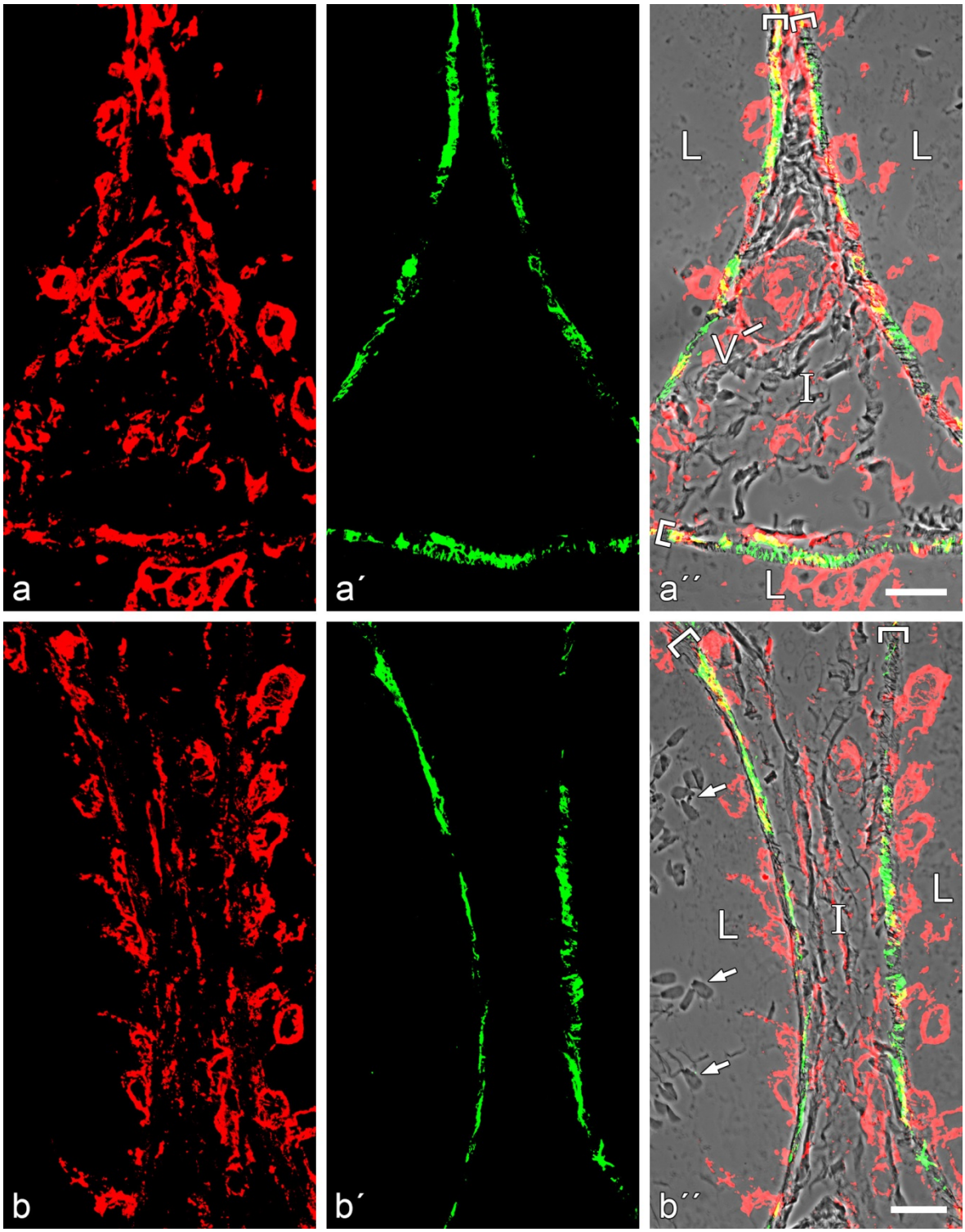


Fig. 49 Double-label immunofluorescence microscopy of cross-sections through a bovine testicular tissue containing seminiferous tubules and interstitial cells using antibodies against **vimentin** (**a**, **a''**, **b**, **b''**; *red*; monoclonal mouse antibody) and antibodies against **cytokeratins 8 and 18** (**a'**–**a''**, **b'**–**b''**; *green*; guinea pig antibodies). All Sertoli cells are intensely positive (*red*) for vimentin filament bundles but totally negative for any of the cytokeratins (*green*). By contrast some cytokeratin-positive LSMCs (brackets in **a''** and **b''**) occur in the peritubular wall (note the *yellow* merger colour, indicative of partial "colocalization" of both IF proteins in these LSMCs). Groups of spermatids in the lumen (L) are denoted by arrows. I, interstitial space. *Bars* 20 µm.

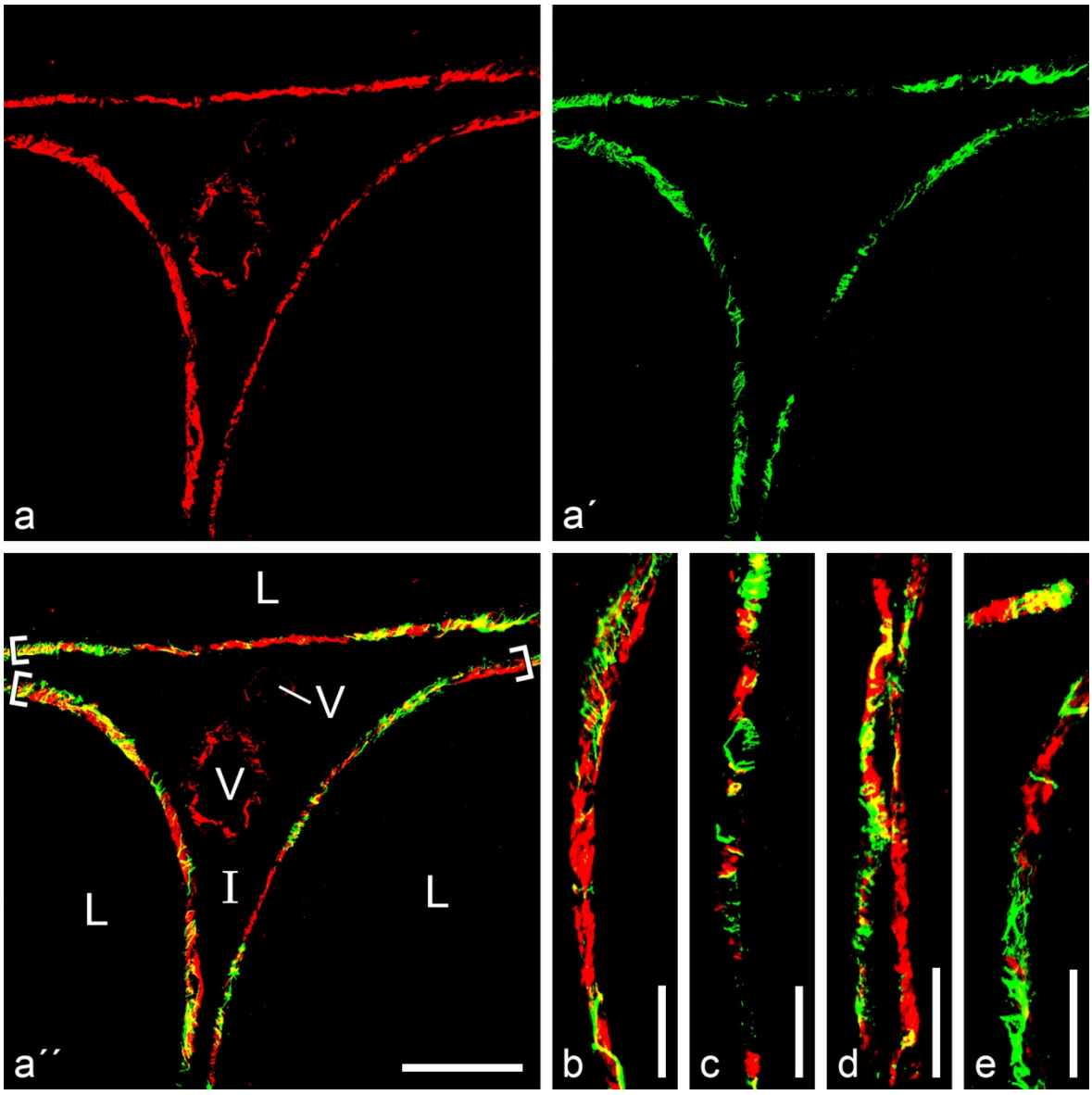


Fig. 50 Double-label immunofluorescence microscopy of cryostat cross-sections through bull testis, showing that peritubular walls surrounding the seminiferous tubules (L, lumen; walls are denoted by brackets in **a''**) contain cell regions positive for **smooth muscle α-actin** (**α-SMA**; **a**, **a''**, **b**–**e**; *red*; monoclonal mouse antibody) and others containing intermediate-sized filaments of **cytokeratins 8 and 18** (**a'**–**e**; *green*; guinea pig antibodies). Some limited regions give the impression of small overlap reactions of both vimentin and cytokeratins (*yellow* merge colour). The perivascular walls (V, vessels) are exclusively positive for α-SMA. Various fibrillar patterns of cytokeratin positivity are seen, including regions with oblique and cross-striation structures (higher magnifications in **b**–**e**). I, interstitial space. *Bars* (**a**) 50 µm and (**b**, **c**, **d**, **e**) 20 µm (see also Domke and Franke 2018, in revision).

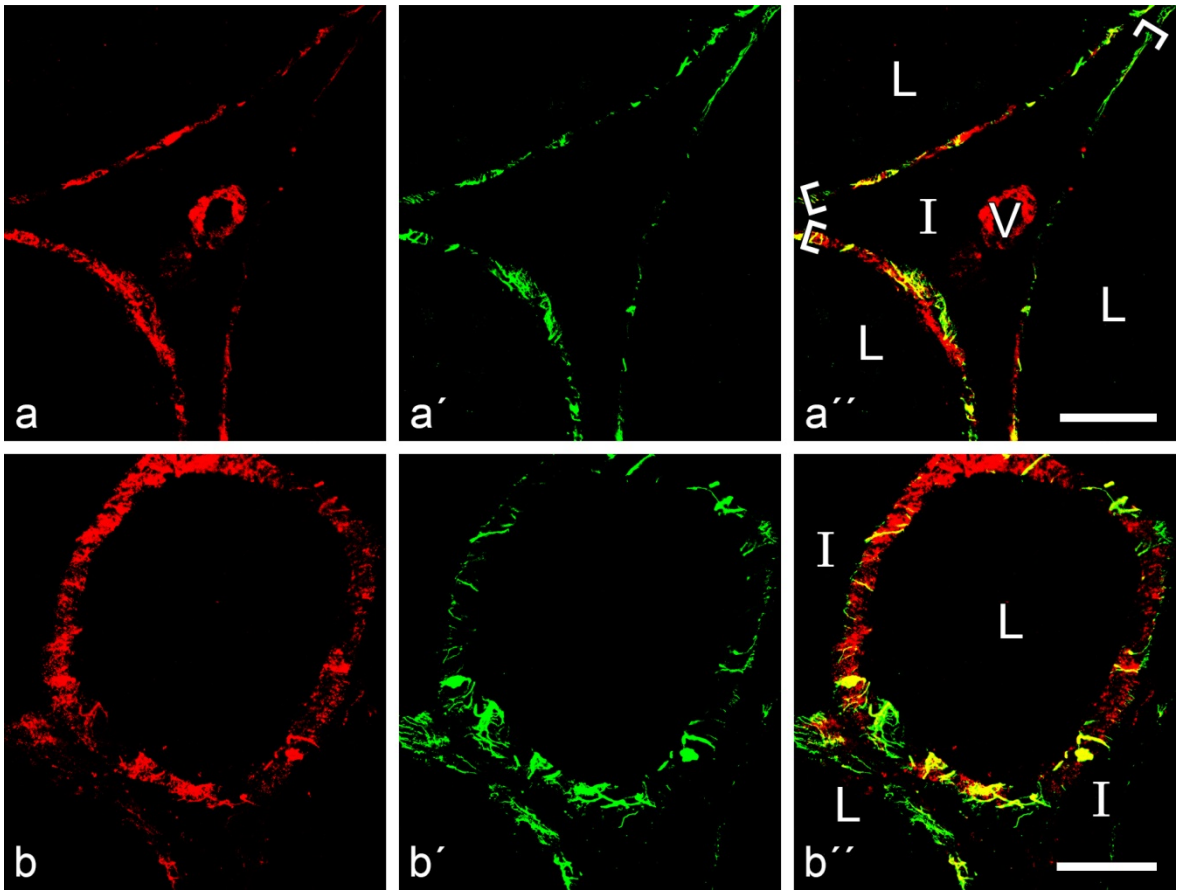


Fig. 51 Double-label immunofluorescence microscopy of cryostat cross-sections through seminiferous tubules of bull testes. After immunoreaction to the intermediate filament (IF) protein **desmin** (**a**, **a''**, **b**, **b''**; *red*; monoclonal mouse antibody) and to **cytokeratins 8 and 18** (**a'**, **a''**, **b'**, **b''**; *green*; guinea pig antibodies) all Sertoli cells are totally negative for both markers but the peritubular wall LSMCs (brackets in **a''**) are positive, partly in substructures, suggesting "colocalization" or topological overlap (*yellow* merger colour). I, interstitial space; L, lumen. *Bars* 50 µm.

4.6.3 Ultrastructural and immunoelectron microscopical analyses of the LSMCs

The peritubular wall is characterized by thin alternating monolayers of LSMCs and layers of ECM rich in collagen fibrils (Fig. 52 c). The Sertoli cells of the seminiferous tubule are attached to the BL which is followed by an ECM layer containing closely packed bundles of collagen fibrils, followed by a LSMC monolayer, which is again followed by another ECM layer and another LSMC layer. The number of such LSMC layers per tubule varies between species and regions (cf. Christl 1990). In certain species such as man, boar and bull there are regions with three pairs of LSMC-ECM layers (Fig. 52) and other regions with up to four, five or six LSMC-ECM layers.

Thus the peritubular wall tissue represents a bandage system of monolayers of SMCs which in the most part have a lamellar form. These LSMC monolayers have sizes and shapes completely different from those of all other kinds of SMC tissues hitherto known. The cytoplasm of these LSMCs is enriched with myofilament bundles, often in high packing densities, locally even in paracrystalline fiber arrays, mostly in coaxial orientation with respect to the corresponding seminiferous tubule and with identical orientation in a given cell (Figs. 53–55). These cells are also rich in other typical SMC structures such as cytoplasmic "dense bodies" of varying sizes (mostly up to 1 µm long and up to 0.5 µm thick in centripetal direction), plasma membrane-associated “focal adhesions” and clusters of caveolae. Cytoplasmic plaque-bearing focal adhesions occur in some regions in high frequency, similar to those present in other kinds of SMCs (e.g., Fig. 53 d). Such frequencies and high packing densities of filament bundles, dense bodies and caveolae are also known from other SMC types.

The thickness of the collagen fibril-dominated ECM layers can vary remarkably. Also the thickness of the LSMC monolayers varies in different cell regions (see, e.g., Figs. 53 c and d). In bull testis, for example, the LSMCs are very thin in some regions (down to ca. 30 nm cytoplasmic membrane-to-membrane thickness) whereas in other regions, in particular the perinuclear one, the cytoplasm can exceed 1.5 µm in thickness. Over extended areas the thin lamellar regions of the LSMCs do not contain any mitochondria, large vesicles or other large cell structures.

While the LSMC monolayers are separated by ECM layers, LSMCs within the same monolayer are usually tightly connected at the cell ends or in overlapping cell processes by AJs (Figs. 55–57). The end-on-end form of the AJ-like junctions often contains only hardly detectable plaque structures (Fig. 56 f). Some of the overlapping cell protrusions of the *processus adhaerens* type (*alter supra alterum*) are filiform or serial interdigitating while others appear broader in variously-sized lamellar forms. The AJs often occur in clusters.

Typical AJ components in such overlapping thin cell protrusions have also been identified by immunoelectron microscopy, including cadherin-11 together with ß-catenin and protein myozap. Using immunoelectron microscopy, it often appears that such membranes rich in AJ molecules extend over areas larger than the specific, clearly visible cytoplasmic plaque structure (e.g. Fig. 58).

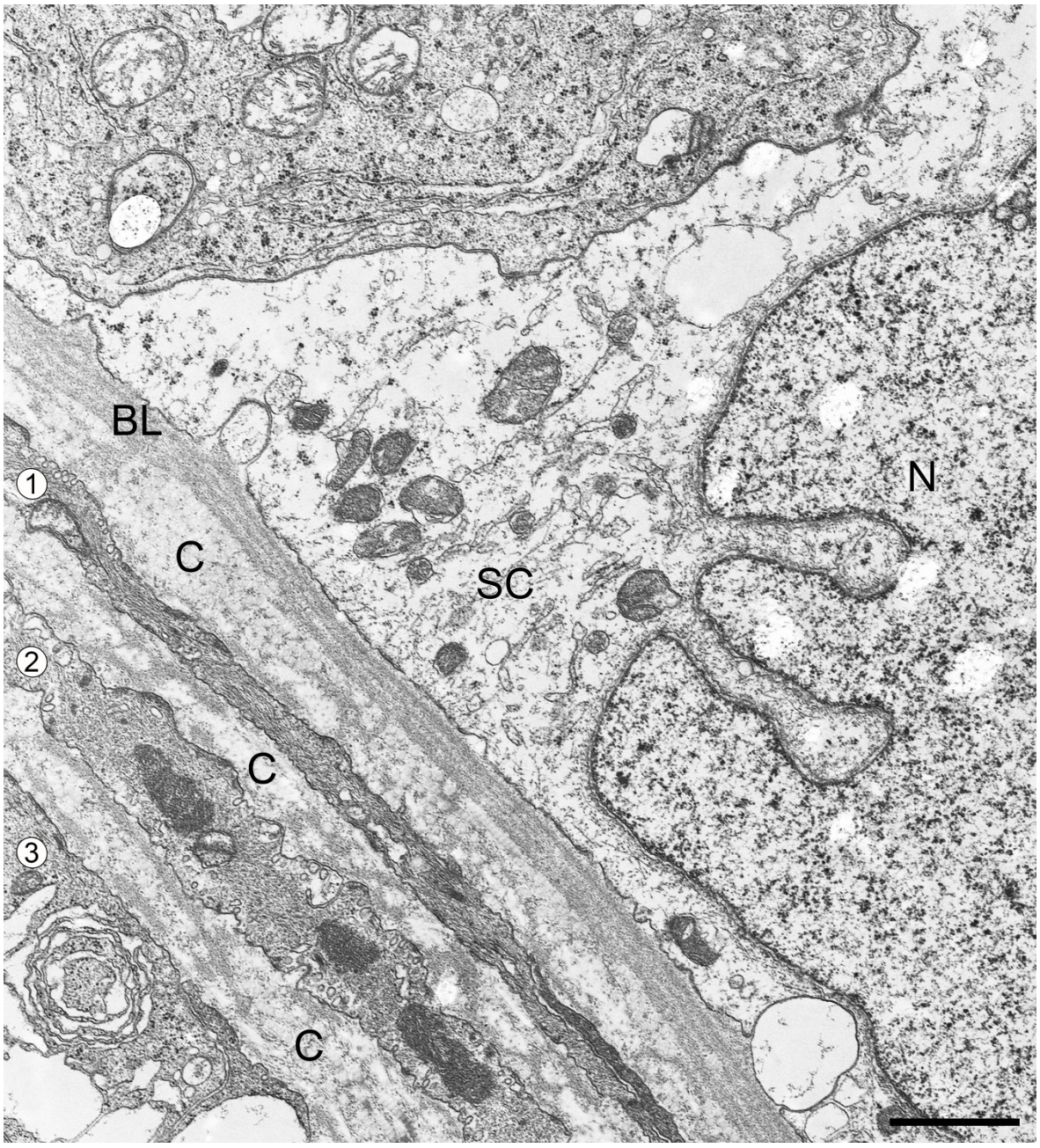


Fig. 52 Electron micrograph of an ultrathin cross-section through a seminiferous tubule of glutaraldehyde-fixed bull testis. Here the basal region of a tubule with Sertoli cells (SCs) and germ cells is shown which is connected to structures of the interstitial space by the basal lamina (BL). In the peritubular wall the flat LSMC layers (nos. 1–3, numbered on the left margin) alternate with extracellular matrix (ECM) layers rich in collagen (C). The peritubular wall cells are rich in filament bundles, focal adhesions and dense bodies. N, nucleus. *Bar* 1 µm.

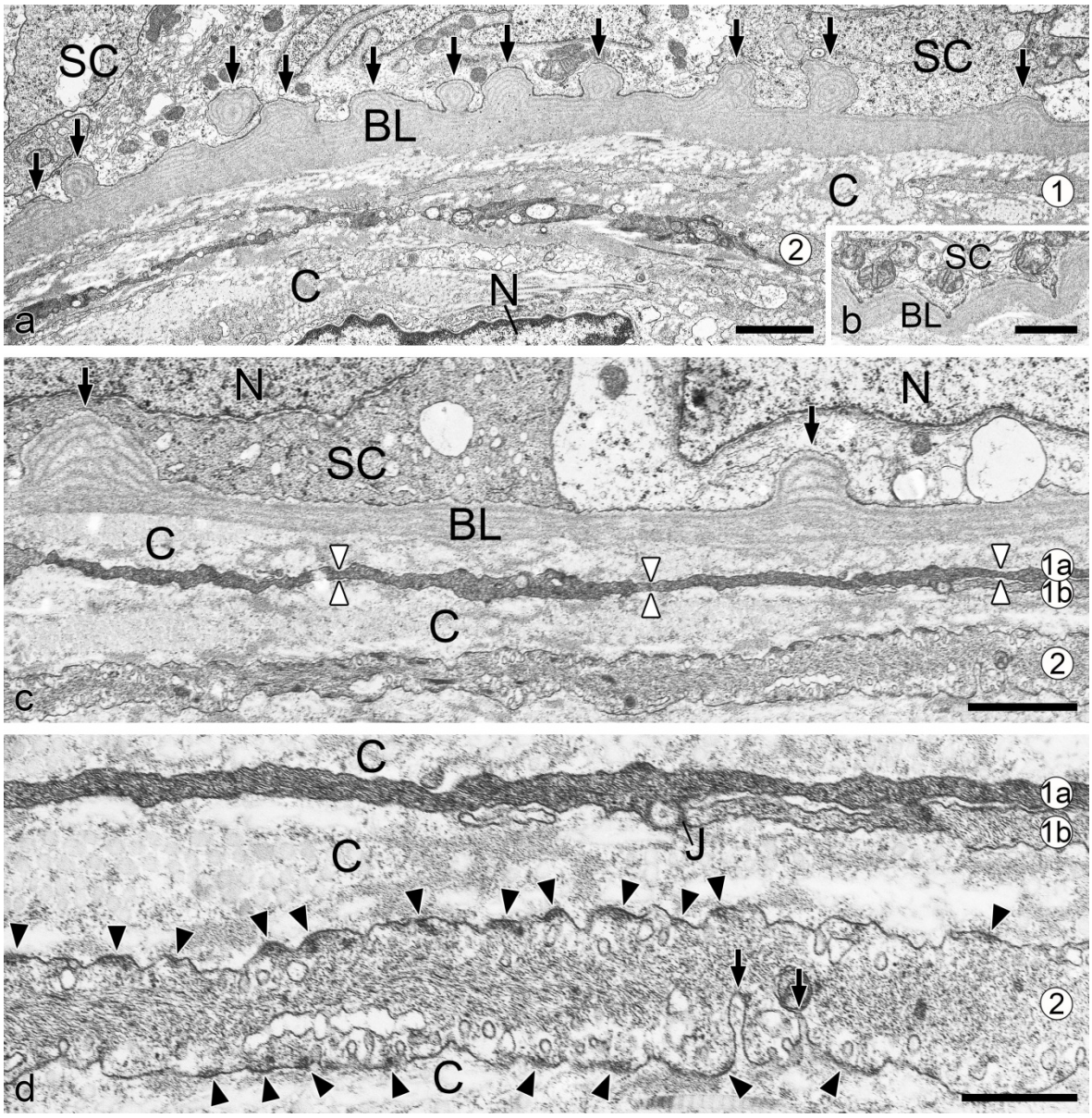


Fig. 53 Electron micrographs of ultrathin cross-sections through peritubular wall structures encasing seminiferous tubules of bull testis. (**a**) The basal lamina (BL) presents in many regions rather closely spaced indentations of up to 1.5 µm (arrows) into the cytoplasm of the Sertoli cells (SCs). However, the BL is for the most part on the other side nearly even-surfaced and borders on a collagen (C) fiber-rich extracellular matrix (ECM) layer, which is followed by a first LSMC layer (numbered on the right hand margin: no. 1). In addition, another ECM layer and the second LSMC layer (no. 2) are seen. (**b**) In some other regions the SC shows small and short acute projections into the BL. (**c**) Fili- and/or lamelliform processes of LSMCs (numbered on the right hand margin) surrounding SCs (arrows denote BL indentations of the type described in **a**) are associated with layers of collagen (C) fiber-rich ECM. In such regions the smooth muscle cytoplasms are very thin (in some places down to ca. 30 nm; denoted by white arrowheads) and characterized by densely packed actin and actomyosin filaments. (**d**) Higher magnification of a part of the region shown in **c**, shows much closer packed filament bundles in the upper LSMC layer (no. 1) than in the lower LSMC layer (no. 2). In LSMC layer no. 1 a cell-cell junction (J) in an overlapping region is denoted. The numerous focal adhesions of LSMC layer no. 2 are highlighted by arrowheads and two narrow plasma membrane invaginations are denoted by arrows. Note also the numerous caveolae and vesicles at the plasma membrane of the lower LSMC (no. 2). N, nuclei. *Bars* (**a**) 2 µm, (**b**, **c**) 1 µm and (**d**) 500 nm (see also Domke and Franke 2018, in revision).

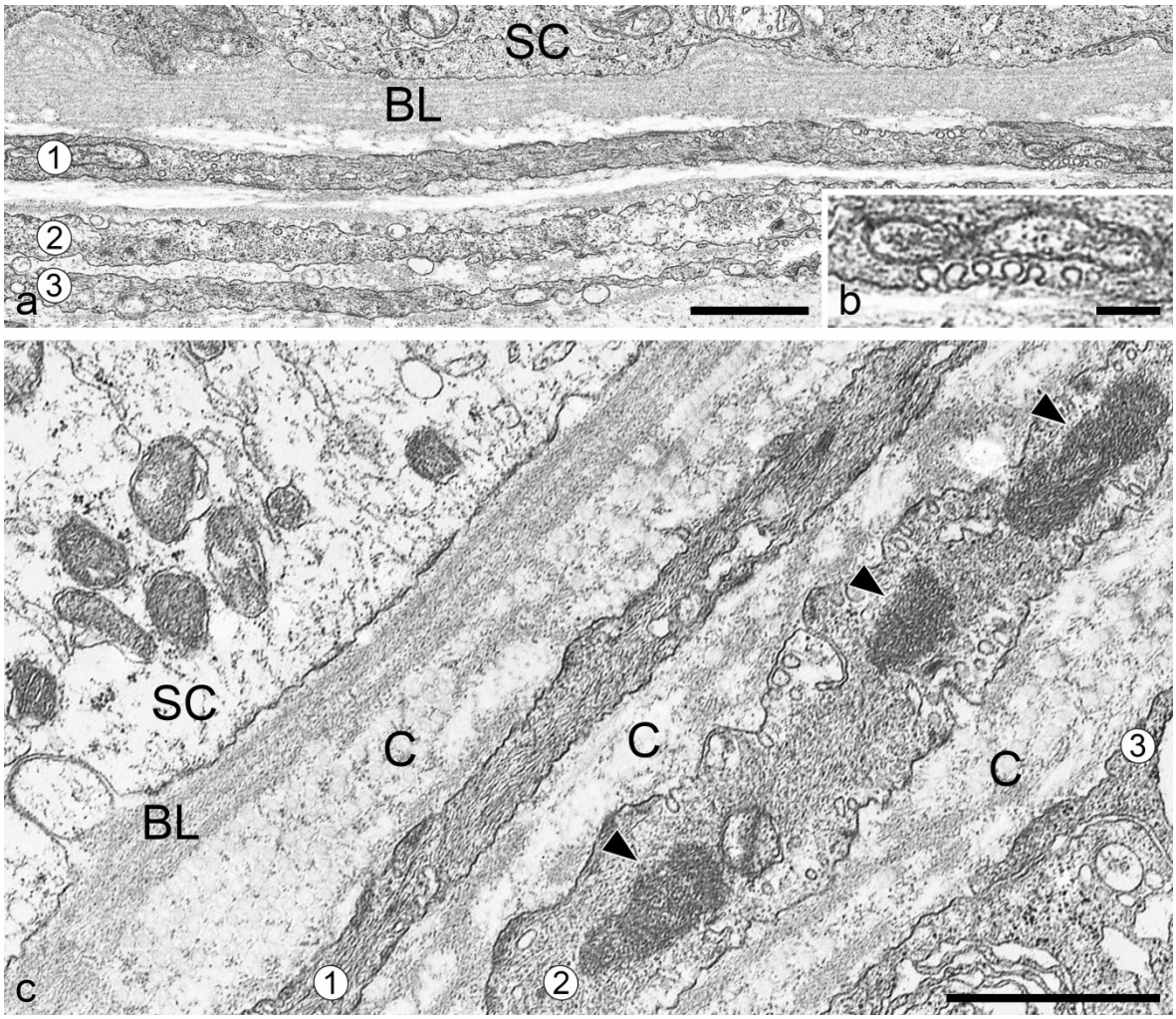


Fig. 54 Electron micrographs of ultrathin sections through bull testicular tissue. (**a**) Further details of the LSMC layers of the peritubular wall encasing the seminiferous tubules, interspersed by collagen (C) fiber-rich ECM layers (note that almost all collagen fibers are cross-sectioned, i.e., coaxial with the seminiferous tubule). (**b**, insert) Higher magnification of a group of plasma membrane caveolae is seen at the right hand margin of a LSMC layer (no. 1). (**c**) A Sertoli cell (SC) borders on the basal lamina (BL) which is attached to the first collagen (C)-rich ECM layer. The following three layers of LSMCs shown here (nos. 1–3) are rich in myofilaments, intermediate-sized filaments, cytoplasmic "dense bodies" (arrowheads) and caveolae. Note that the filaments in LSMC layer no. 1 extend in a direction different from that of the filaments seen in LSMC layer no. 2. *Bars* (**a**, **c**) 1 µm and (**b**) 200 nm (see also Domke and Franke 2018, in revision).

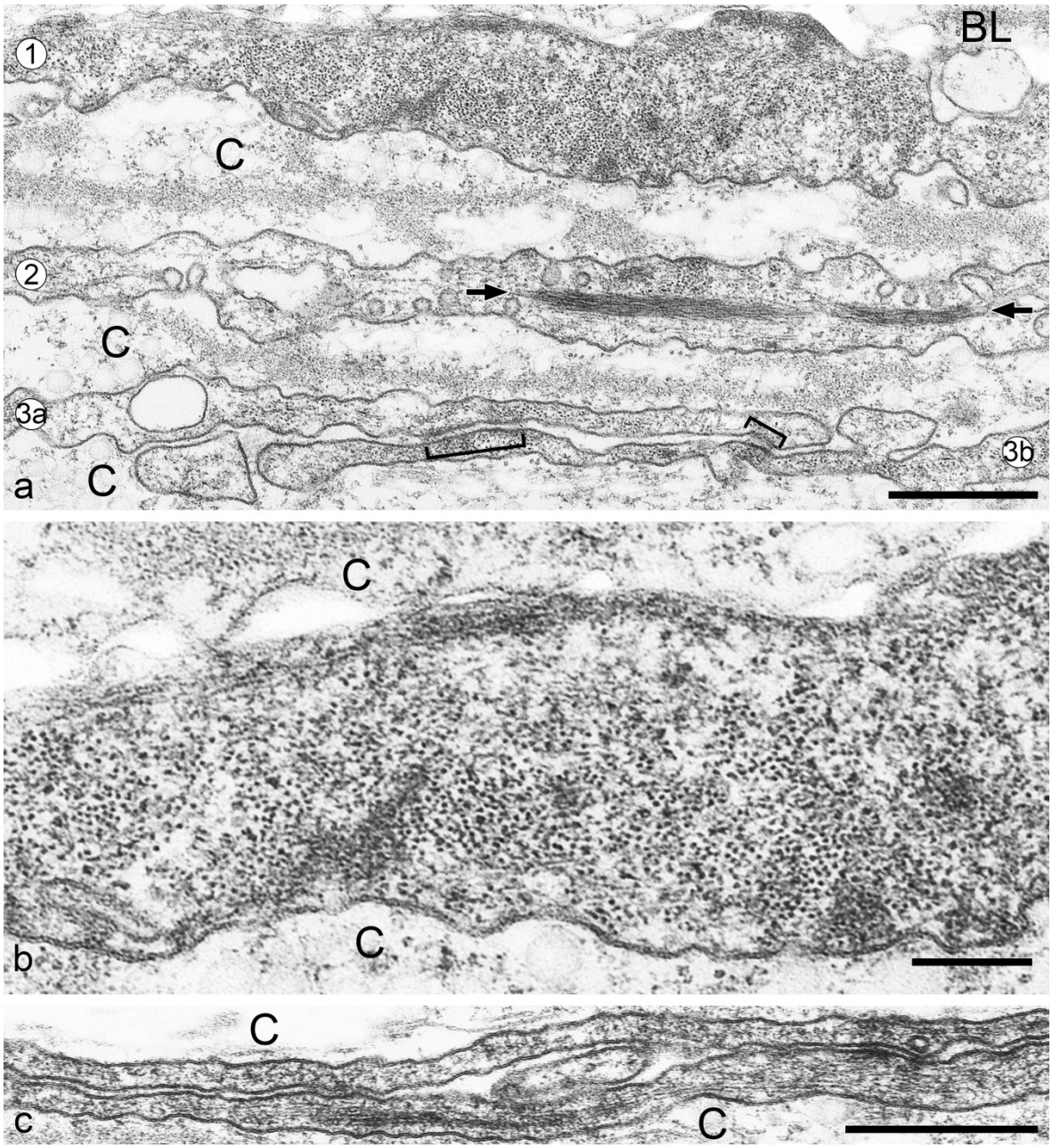


Fig. 55 Electron micrographs of ultrathin cross-sections through bull testis tissue revealing details of the LSMC monolayers. (**a**) LSMCs interspersed by collagen (C) fiber-rich ECM layers (here almost all cross-sectioned) are rich in intermediate-sized filaments and myofilaments (running here in same direction as the specific seminiferous tubule). LSMC layer no. 2 contains in addition a dense packed peritubular fiber bundle (arrows). Layer no. 3 shows the connection of two overlapping LSMC protrusion ends connected here by three *puncta adhaerentia* resembling AJ structures (denoted by brackets). (**b**) Higher magnification of LSMC monolayers no. 1 is shown. Note mostly paracrystalline arrays of actin filaments in densely packed arrays (ca. 6 nm thick) and myosin filaments (ca. 12 nm thick). Such filaments occur here in close association with focal adhesions and cytoplasmic plaques of cell-cell junctions (denoted by brackets in LSMC layer no. 3). (**c**) Two LSMCs in very close association indicate extended cell-cell contact regions, partly with AJ structures. BL, basal lamina. *Bars* (**a**, **c**) 500 nm, (**b**) 200 nm (see also Domke and Franke 2018, in revision).

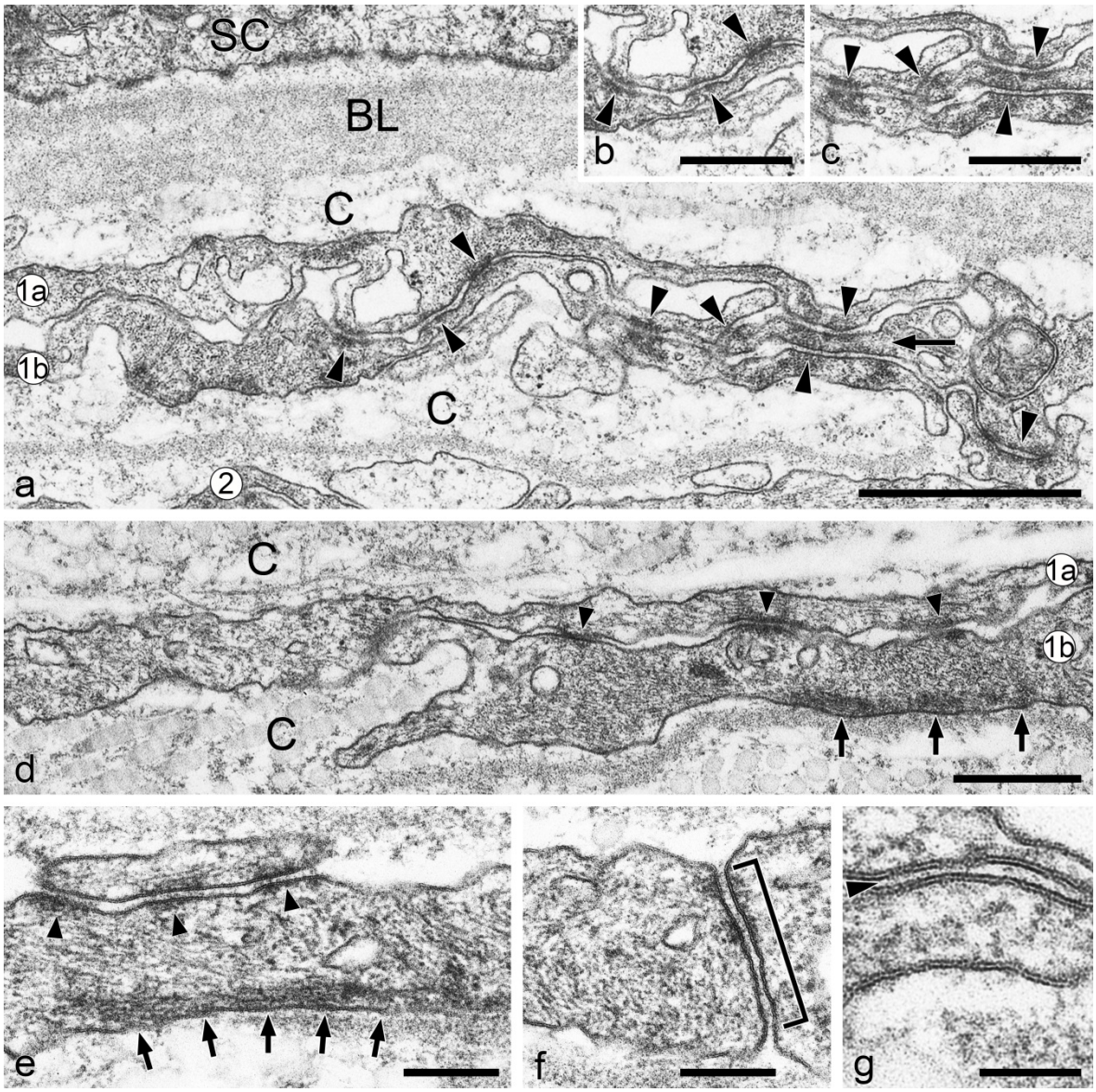


Fig. 56 Electron micrographs of ultrathin cross-sections of peritubular walls of seminiferous tubules of bovine testes, showing overlap regions of LSMC layers with various ultrastructural forms of cell-cell junctions of the adherens junction (AJ) type. (**a**–**c**) The basal lamina (BL) associated with the Sertoli cell (SC) and the first ECM layer (C, collagen fibrils) is seen, followed by the first LSMC (nos. 1a and 1b) layer. Note the numerous AJs (arrowheads) connecting LSMCs (nos. 1a and 1b) which may also occur within cell-cell invaginations (e.g. at the thin arrow in the right). (**d**) Other forms of thin LSMC layers show clusters of vertical AJs (arrowheads) as well as of focal adhesions (arrows) and individual dense bodies (thin arrow). (**e** and **f**) Different subforms of AJs (denoted by arrowheads in the upper left region) as well as a more extended AJ (bracket in the right hand part of **f**) are seen as well as an extended focal adhesion-rich region (arrows). (**g**) Here, a cell-cell contact region with very little electron-dense cytoplasmic plaque material is seen. *Bars* (**a**) 1 µm, (**b**, **c**, **d**) 500 nm, (**e**, **f**) 200 nm and (**g**) 100 nm (see also Domke and Franke 2018, in revision).

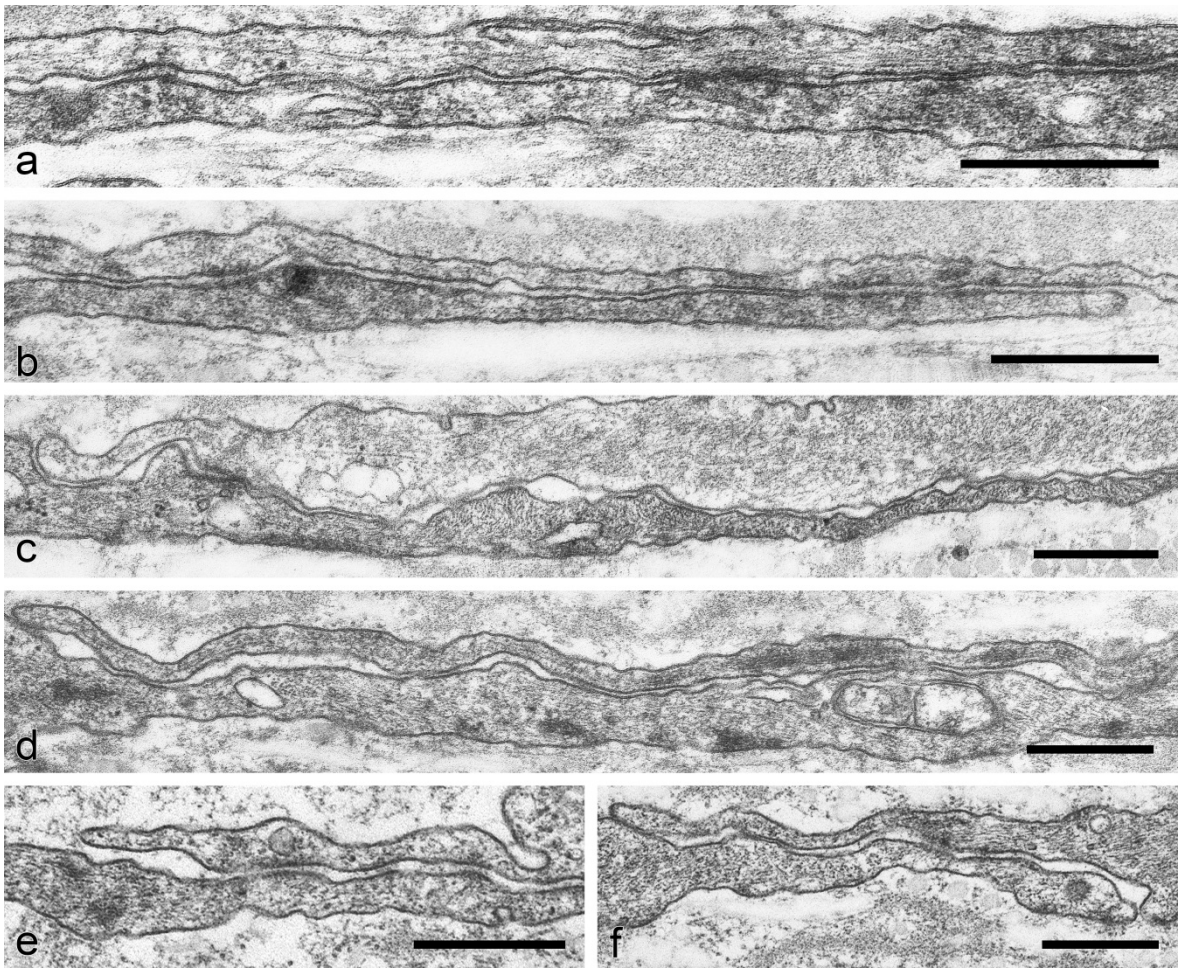


Fig. 57 Electron micrographs of ultrathin sections through peritubular walls of seminiferous tubules of bovine testes: Various ultrastructural forms of vertical cell-cell junctions of the adherens junction (AJ) type are present in the peritubular wall LSMC layers in extended overlapping regions (*processus alter supra alterum*). Adjacent monolayer cells are connected by these membrane-membrane complexes contributing to closure of the peritubular wall structure against translocations of cells, particles and large molecular complexes. *Bars* 500 nm (see also Domke and Franke 2018, in revision).

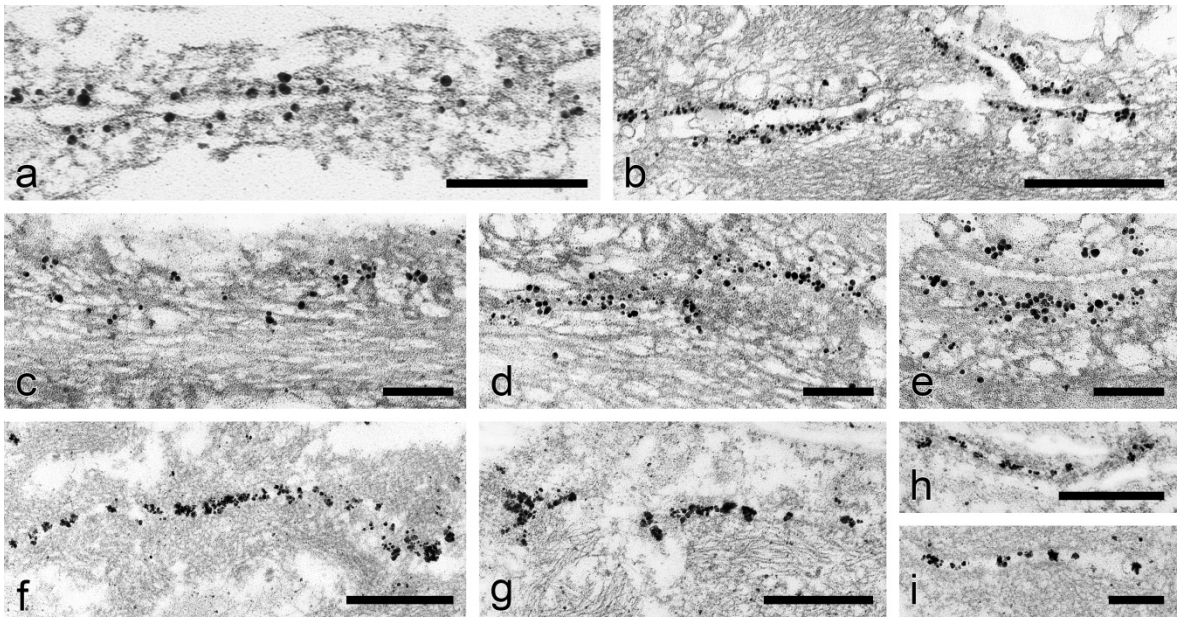


Fig. 58 Immunoelectron micrographs of ultrathin sections through bull testicular tissue. Extended AJ-rich regions between LSMCs in peritubular walls are immunogold-labelled (with silver enhancement) on the plaque-bearing, i.e. cytoplasmic side with antibodies against **β-catenin** (**a** and **b**), protein **myozap** (**c**–**e**) and **cadherin-11** (**f**–**i**). Note also the frequent dense associations of the thin cytoplasmic AJ plaques with myofilaments. *Bars* (**a**, **c**, **d**, **e**, **i**) 200 nm and (**b**, **f**, **g**, **h**) 500 nm (see also Domke and Franke 2018, in revision).

4.7 Immunocytochemical examination of the presence of a lymphatic endothelial cell layer covering the peritubular wall

Over the past half century various authors have emphasized that, at least in rodent testes, the peritubular wall layers are intimately covered by a continuous, rather thin cell layer classified as a “lymphatic endothelium” (e.g., Fawcett et al. 1969; for further references see chapter 5.6). However, a direct cell type characterization of these cells with typical endothelial markers has not yet been published.

Therefore, in this thesis work the peritubular wall structure and the adjacent region of the interstitial space have been systematically examined by immunocytochemistry using established endothelial cell type markers such as VE-cadherin, claudin-5, podoplanin, factor VIII (von Willebrand factor), platelet endothelial cell adhesion molecule (PE-CAM; CD31) and lymph vessel endothelium hyaluronan receptor 1 (LYVE-1; for references of endothelial cell type markers see chapter 5.6). In these experiments the excurrent ducts as well as the blood and lymph vessels in adjacent testicular regions were used as controls.

My immunocytochemical studies have shown that in all species examined neither the excurrent duct epithelia nor the LSMC layer system of the peritubular wall are associated with an extended single cell layer with characteristics of a lymphatic endothelium (Figs. 59–61). Usually there are only very few and mostly very small lymphatic vessels in the interstitial space, some in close and many in greater distance from the peritubular wall. These results show that there is no direct coverage of the peritubular LSMC-ECM stacks by a lymphatic endothelium.

4.8 Application of immunocytochemical antigen retrieval techniques

Considering the possible value of antibodies to determine the specific cell types or cell type-derivations during development and certain pathological processes, but also for tumor diagnoses, aldehyde-fixed and paraffin-embedded tissue material have been routinely used after “antigen retrieval” treatment (for details see chapter 3.4.3). While some of the results obtained were very similar to those observed with snap-frozen tissue samples mentioned, a major part of the antigen retrieval immunostaining experiments were negative, apparently as artefacts resulting from epitope inactivation due to the specific treatment. Other immunostaining results (examples are shown in Figs. 62 and 63) demonstrate that here the specific treatments have resulted in accessibilities of the specific antigens in agreement with the results obtained with frozen tissue sections (compare, e.g., Figs. 38–39). Additional antibodies with different epitope binding properties need to be generated to find diagnostically useful reagents for immunocytochemical tests on aldehyde-fixed and paraffin-embedded tissues.

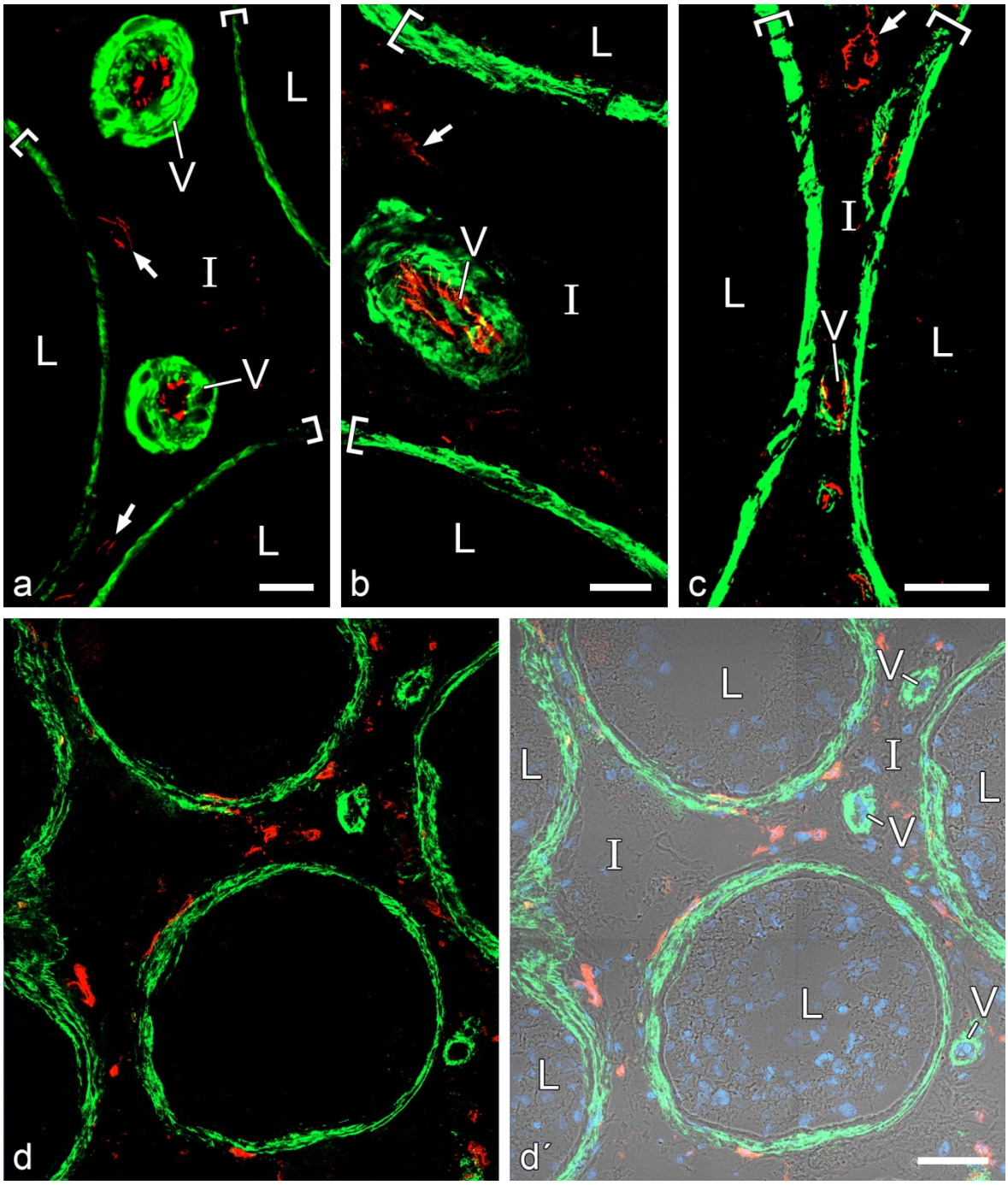


Fig. 59 Double-label immunofluorescence microscopy of cryostat cross-sections through seminiferous tubules of frozen bull (**a**, **b**), boar (**c**) and human (**d**, **d'**) testes. Immunostaining reactions of peritubular LSMCs as well as blood and lymph vessels (V) are shown with a monoclonal mouse antibody against **smooth muscle α-actin** (**α-SMA**, **a**–**d'**; *green*). For comparison, the vascular endothelial markers **VE-cadherin** (**a**–**c**; *red*; rabbit antibodies) and **LYVE-1** (**d**, **d'**; *red;* rabbit antibodies) have been chosen for comparison to visualize specifically the vascular endothelium (*red* in vessels; lymph vessels are denoted by arrows and the peritubular walls by brackets). Note that even the endothelia of the smallest lymph and blood vessels are positive for both endothelial markers but negative for the SMC markers. Note in particular the complete absence of an endothelial marker reaction in the outermost cell layer of the peritubular LSMC wall. L, lumen; I, interstitial space. *Bars* (**a**, **b**, **c**) 20 µm and (**d**) 50 µm.

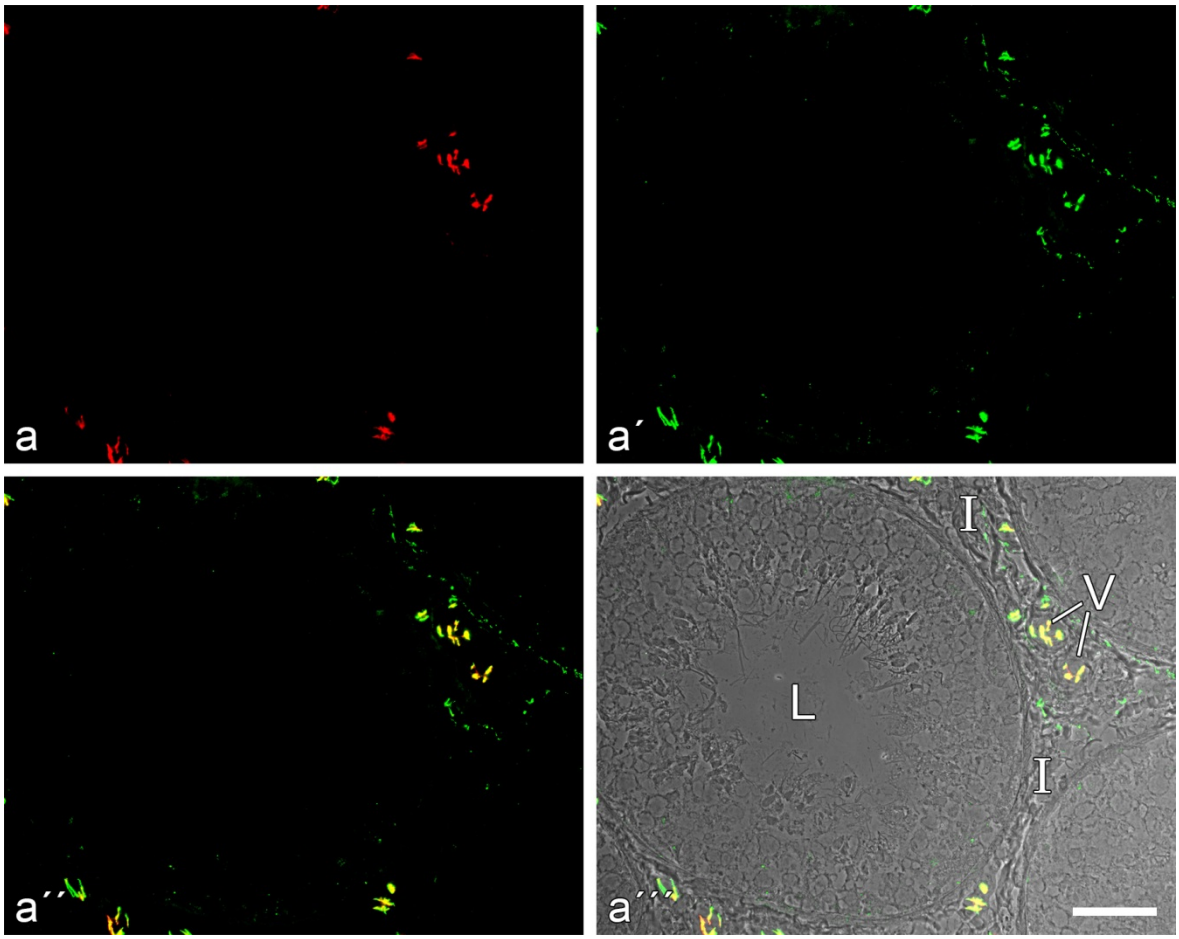


Fig. 60 Double-label immunofluorescence microscopy showing a cryotomy cross-section through seminiferous tubules of bull testis after reactions with antibodies to **claudin-5** (**a**, **a''**, **a'''**; *red*; monoclonal mouse antibody) and **VE-cadherin** (**a'**–**a'''**; *green*; rabbit antibodies). Note the exclusive occurrence of both junction marker proteins in blood vessel endothelial cells (V, vessels) and their absence in seminiferous tubules (L, lumen) and peritubular wall LSMCs (I, interstitial space). Note the partially very close localization of both endothelial markers (*yellow* merger colour). *Bar* 50 µm.

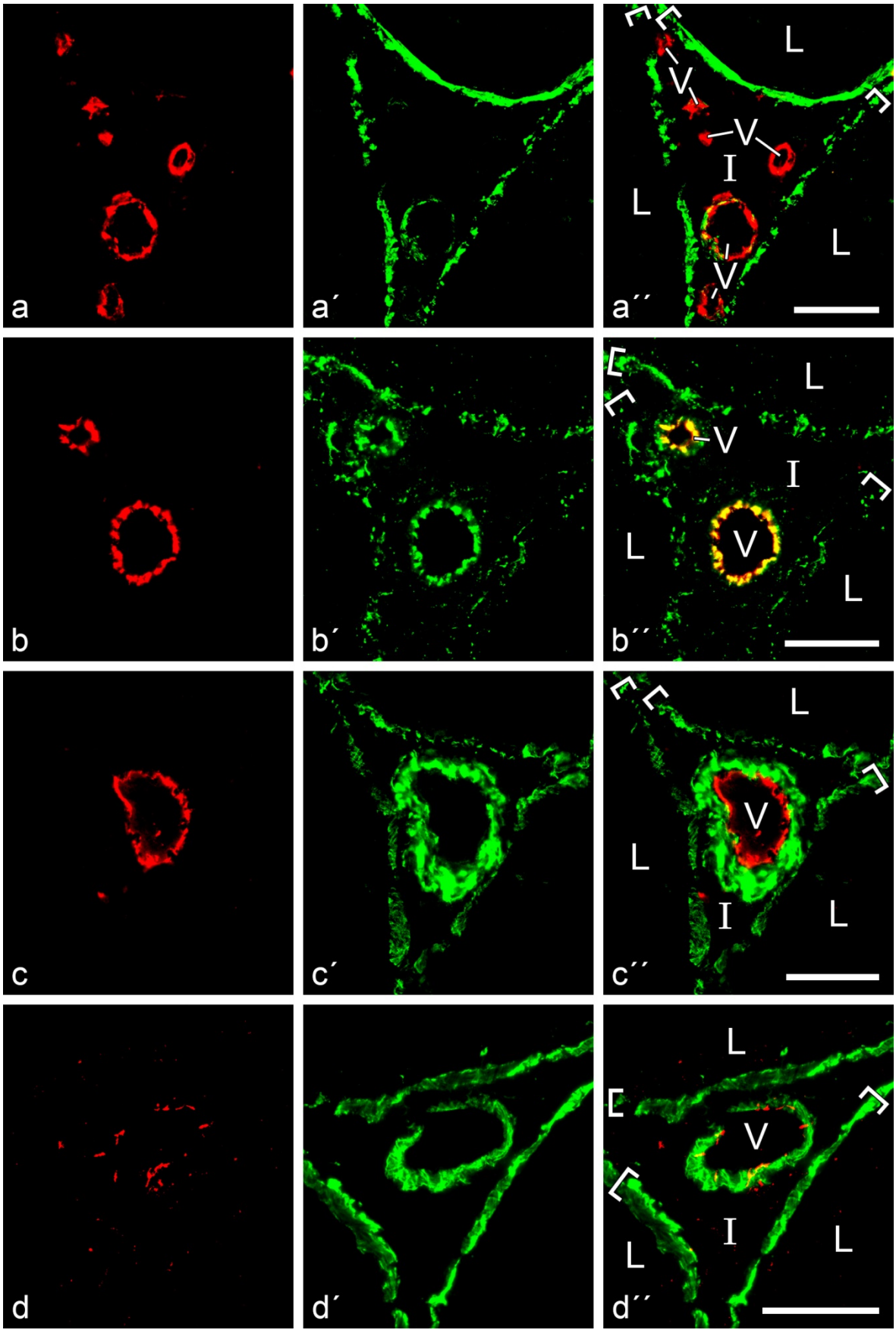


Fig. 61 Double-label immunofluorescence microscopy of cryostat cross-sections through seminiferous tubules of frozen rat testes. (for continuation see the next page).

Continuation of Fig. 61 Immunostaining reactions of peritubular LSMCs as well as blood and lymph vessels (V) are shown with antibodies to **claudin-5** (**a**, **a''**, **b**, **b''**, **c**, **c''**; *red*; monoclonal mouse antibody), VE-cadherin (**d**, **d''**; *red*; monoclonal mouse antibody) and **smooth muscle α-actin** (**α-SMA**; **a'**, **a''**, **d'**, **d''**; *green*; rabbit antibodies), **β-catenin** (**b'**, **b''**; *green*; rabbit antibodies) as well as **desmin** (**c'**, **c''**; *green*; rabbit antibodies). Note again that the smaller lymph and blood vessel (V) endothelia are positive for the endothelial marker but negative for SMC markers. Note also the complete absence of an endothelial marker reaction in the cell layer covering the peritubular LSMC wall. L, lumen; I, interstitial space. *Bars* 50 µm.

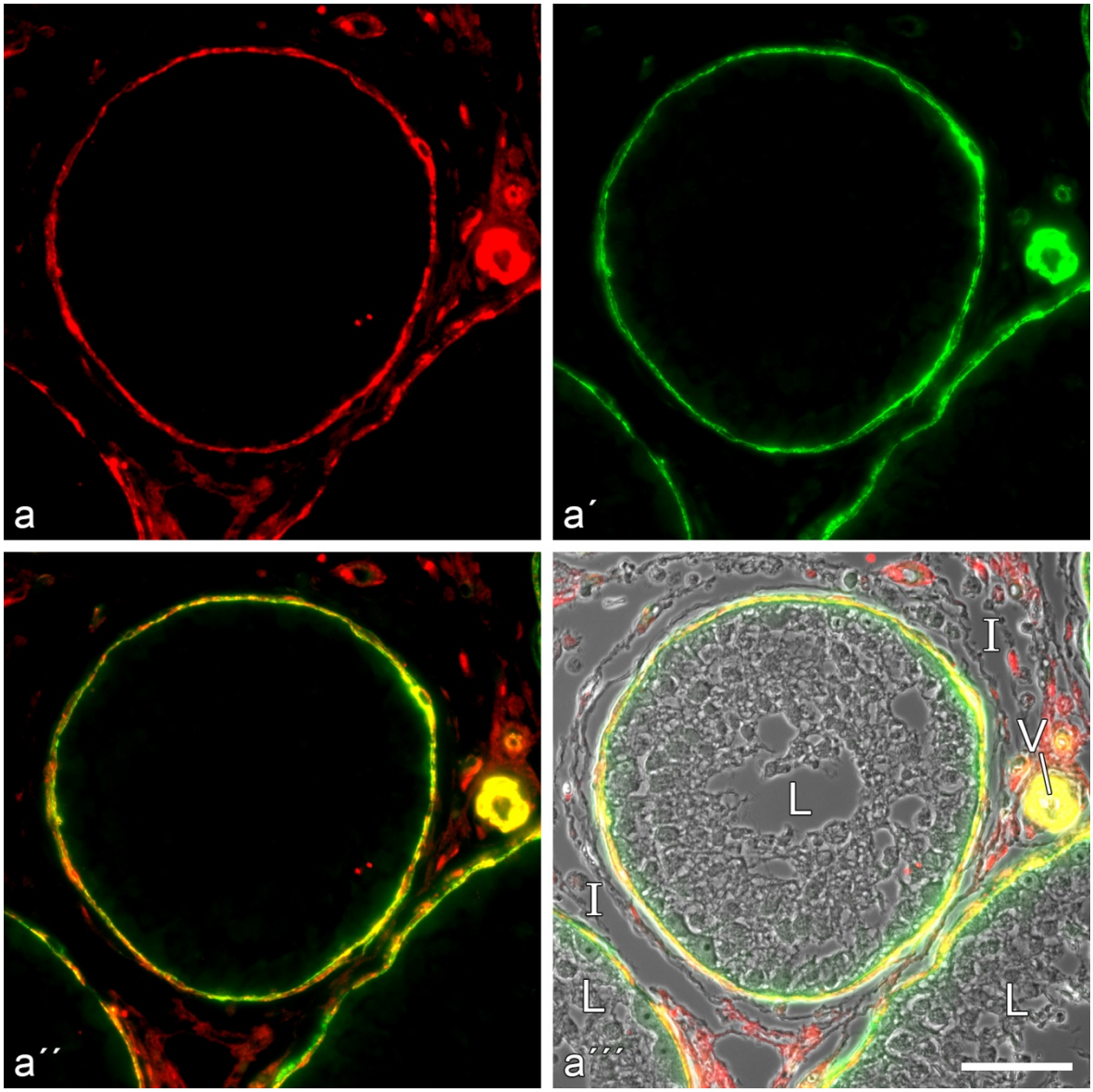


Fig. 62 Double-label immunofluorescence microscopy of a formaldehyde-fixed paraffin embedded cross-section through bull testis after antigen retrieval treatment and immunoreactions with antibodies against the smooth muscle cell (SMC) marker proteins **SM22α** (**a**, **a''**, **a'''**; *red*; rabbit antibodies) and **smooth muscle α-actin** (**α-SMA**; **b'**–**b''**; *green*; monoclonal antibody). Note the positive staining in extended regions of the peritubular wall LSMCs and in the SMCs of a blood vessel (V) in the interstitial space (I). L, lumen. *Bar* 50 µm.

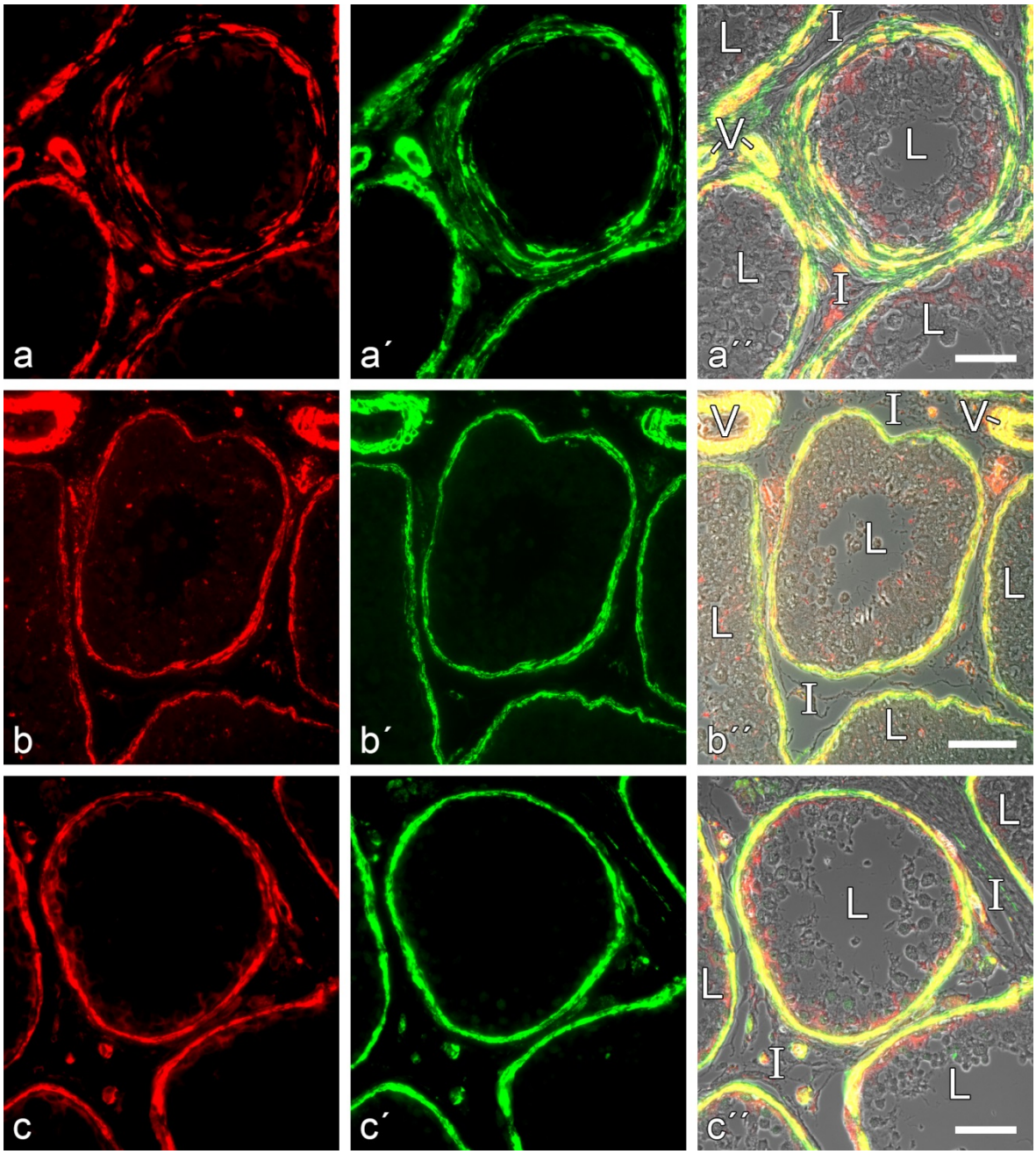


Fig. 63 Double-label immunofluorescence microscopy of a formaldehyde-fixed paraffin embedded cross-section through human testis after antigen retrieval treatment and immunoreactions with antibodies against the smooth muscle cell (SMC) marker proteins **SM22α** (**a**, **a''**; *red*; rabbit antibodies), **caldesmon** (**b**, **b''**; *red*; rabbit antibodies), **filamin** A (**c**, **c''**; *red*; rabbit antibodies) and **smooth muscle α-actin** (**α-SMA; a'–a''**, **b'–b''**, **c'–c''**; *green*; monoclonal antibody). Note the positive staining in extended regions of the peritubular wall LSMCs and in the SMCs of blood vessel walls (V) in the interstitial space (I). L, lumen. *Bar* 50 µm.

5 Discussion

Since decades controversial reports have been published on the cytoskeletal and cell junctional components in the epithelium of the mature seminiferous tubules and the peritubular wall tissue of mammalian testes (for references see, e.g., Introduction and Table 3). Starting from some unexpected findings in my bachelor thesis work (Domke 2013) I decided to further elucidate in my doctoral thesis the cell and molecular biological composition of these cells and thus to determine their specific cell type in a highly controlled form.

5.1 Absence of desmosomes, desmosome-like junctions and desmosome-specific molecules in the seminiferous tubules

The first group of results of this thesis work (chapter 4.4) confirmed and extended the concept that the epithelium of the mature and active *tubuli seminiferi* of mammalian testes is very different from all other mammalian epithelia examined so far. In particular, the absence of cytokeratins in contrast to the exclusive presence of vimentin intermediate-sized filaments (IFs) in mature and healthy Sertoli cells was proven (cf. Franke et al. 1979c, 1982b; Spruill et al. 1983; Paranko and Virtanen 1986; Bergmann and Kliesch 1994; Romeo et al. 1995; for further references see Introduction). The present study could validate the statement (not only “proposal” as the conclusion was called by Vazquez-Levin et al. 2015) that the normal mature seminiferous tubule epithelium does not contain desmosomes (*maculae adhaerentes*) or desmosome-like junctions, and not even any desmosome-specific molecules (e.g., Franke et al. 1981b, 1982a; for further references see also Domke et al. 2014). In addition, this epithelium lacks E-cadherin-based adherens junctions (AJs) and EpCAM-containing cell-cell junctions (see also, e.g., Cyr et al. 1992, 1993; Newton et al. 1993; Byers et al. 1994; Domke et al. 2014).

In this context, it should be emphasized that these cells do not contain any of the desmosomal components which have been detected in certain cells also outside of desmosomal structures. For example, plakophilin-2 (Pkp-2) occurs not only in desmosomes but also in specific AJs of certain mesenchyme-derived cells and cardiac tumors (e.g., Barth et al. 2009, 2012; Rickelt et al. 2010). The E- or N-cadherin-based “meningioma junctions” also have a plaque structure with α- and β-catenin, plakoglobin and protein p120 as well as plakophilin-2 (Pkp-2; Akat et al. 2008). Desmoplakin is present in addition to AJ molecules also in the *complexus adhaerentes* of some lymphatic endothelia (Schmelz et al. 1990, 1994; Schmelz and Franke 1993; see also Table 2 and Supplement Literature Collection No. 1), and dispersed desmoglein-2 (Dsg-2) glycoprotein has also been found in non-junction-containing regions of the surfaces of certain types of melanoma cells (e.g., Schmitt et al. 2007; Rickelt et al. 2008). Finally, the composite

junctions (*areae compositae*) of mammalian cardiomyocytes contain all simple epithelial desmosome molecules (desmoplakin, plakophilin-2, desmoglein-2 and desmocollin-2) in a large, cell type-specific complex together with AJ components and other specific molecules (e.g., Borrmann et al. 1999; Borrmann 2000; Franke et al. 2006, 2009; see also Table 2 and Supplement Literature Collection No. 2).

From the controlled experiments of this thesis, involving a wide range of antibodies and six different species, it is now clear that the seminiferous tubules of mature and healthy mammalian testes do not contain known desmosome-specific components and thus no desmosomes or desmosome-like junctions. This should finally lead to a general correction in cell biology textbooks and references (see also a comment of Pelletier and Byers 1992: “Therefore, the term desmosome-like is seemingly inappropriate to designate these junctions…”).

5.2 Absence of hemidesmosomes and hemidesmosomal marker molecules in the seminiferous tubules

Following the controversy concerning the presence or absence of desmosomes in mature seminiferous tubules, I have also examined the presence of hemidesmosomes in and at the basal plasma membrane of the Sertoli and germ cells. Here, the controlled analyses have also revealed a total absence of hemidesmosomes and specific hemidesmosomal marker molecules in contrast to published results of other authors (e.g., Wrobel et al. 1979; Cheng and Mruk 2012). The list of missing hemidesmosomal marker components includes transmembrane components of hemidesmosomes such as the bullous pemphigoid antigen 230/233 (BP230; Stanley et al. 1981; Klatte et al. 1989; Owaribe et al. 1990, 1991; Sawamura et al. 1991; Hopkinson et al. 1995; Koster et al. 2003; for comparison with a positive control see Fig. 21), tetraspanin CD151 (Sterk et al. 2000), the transmembrane integrin α6β4 complex, assumed to be required for anchoring IFs (Sonnenberg et al. 1991; Sawamura et al. 1991; for reviews see, e.g., Schwarz et al. 1990; Borradori and Sonnenberg 1999; Koster et al. 2004; Kligys et al. 2008; Walko et al. 2015) and the plaque protein plectin (Garrod 1999). This again emphasizes the special nature of the epithelium of the seminiferous tubules. Consequently, as hemidesmosomal anchorages of cytoskeletal filaments and hemidesmosomal attachments to the basal lamina do not exist in Sertoli cells, additional characterizations are required to determine if and how these cells form stable IF associations with the basal plasma membrane and cell attachments with basal lamina structures (see, e.g., Jones et al. 1998; Borradori and Sonnenberg 1999). Clearly, the basal lamina of these cells as well as other inter- and extracellular regions of the peritubular wall contain networks of polymers of type IV

collagen, certain laminins and several other components, similar to the corresponding structures in other epithelia.

5.3 Tight and gap junctions in the seminiferous tubules

Tight and gap junctions connecting Sertoli cells or Sertoli cells with germ cells have been studied by a series of researchers in various mammalian species. The structures and proteins of the tight junctions in the intratubular cell layers of mature, spermatid-producing mammalian testes have been subjects of many studies at the ultrastructural and the molecular level, in particular the forms associated with the so-called “blood-testis barrier” (see, e.g., Dym and Fawcett 1970; Gow et al. 1999; Morita et al. 1999a; Saitou et al. 1997; Pelletier 2001; Southwood and Gow 2001; Morrow et al. 2010; McCabe et al. 2016). Moreover, specific close (“tight”) and linear arrays of tight junction-related series of “dots” and/or “strands” have been described in such and other epithelia in a number of reports (e.g., Gilula et al. 1976; Furuse et al. 1993; Pelletier 2001; Schneeberger and Lynch 2004; Anderson and Van Itallie 2009; Furuse 2010; for review see, e.g., Tsukita et al. 2001). Special gene knockout experiments have already been performed that have given functional insights (see, e.g., results with Osp/claudin-11 null mice of Gow et al. 1999 and Southwood and Gow 2001).

Correspondingly, the gap junctions of the seminiferous epithelium, including both the transmembrane connexin forms, i.e. connexin 43, as well as associated cortical proteins in a plaque-equivalent position, have also been repeatedly described in the literature (for relevant references on gap junctions of Sertoli and other cells see, e.g., Unwin 1987; Risley et al. 1992; Goodenough et al. 1996; Steger et al. 1999; Koval 2008; Goodenough and Paul 2009; for generally relevant reviews see in particular Laird 2006, 2017; Nielsen et al. 2012; Zhou and Jiang 2014; Kidder and Cyr 2016). Additionally, next to already described structures new cell-cell contacts were noted in the present study.

5.4 Two novel cell-cell junctions in the seminiferous tubules

Junctions of the Sertoli cells and germ cells are dynamic structures that are regulated with respect to the testicular development and functions. In this study, two generally novel types of cell-cell junctions have been recognized in seminiferous tubules, namely the often very large “*areae adhaerentes”,* which are N-cadherin-based adhering junctions (AJs), and the special type of cribelliform (sieve-like) junctions (“*areae cribelliformes*”) which are prominent in bull testes and characterized by cytoplasmic cell-to-cell channels of 5–7 nm in luminal diameter (see also Domke et al. 2014). The novel cell-cell junctions are added to the list of novel types of adhering junctions in mammalian tissues (Table 17).

5.4.1 *Areae adhaerentes*

A number of cell-to-cell AJs of Sertoli cells, with each other and with germ cells, occur in different long-known morphological forms, the *fascia adhaerens* and the *punctum adhaerens* (for general reviews see Farquhar and Palade 1963 and Franke 2010). The newly defined additional AJ structures, the *areae adhaerentes*, include variously-sized, often very extended forms and may appear with continuous lengths of more than 10 µm (see, e.g., Figs. 22 a–c and 24 a) and cell-cell contact areas larger than 30 μm^2. This group of junctional structures (*areae adhaerentes*) includes some of the largest stable cell-cell junction contacts so far found not only in mammalian tissues but in the overall vertebrate cell biology. Moreover, the close and stable membrane-to-membrane distance over very large regions indicates a continuous molecular cell-cell interaction. This suggests continuous AJ-like structures, although in many parts neither distinct intercellular bridges nor specific cytoplasmic plaque structures are recognized.

The list of proteins and glycoproteins localized in *areae adhaerentes* of Sertoli-Sertoli cell and Sertoli-germ cell contacts includes N-cadherin clusters mostly anchored in cytoplasmic plaques containing α- and β-catenin, plakoglobin, protein p120, protein p0071 and at least one protein of the striatin family (for this protein family see, e.g., Castets et al. 1996, 2000; Gaillard et al. 2001; Blondeau et al. 2003; Franke et al. 2015; for reviews see Benoist et al. 2006; Bobik 2012; Hwang and Pallas 2014). It has, however, to be emphasized that this list cannot yet be considered to be complete. This may be attributable to local differences of the molecular composition or to molecules that are cytochemically undetectable because appropriate antibodies for reactions with specific epitopes are not yet available. Interestingly, although N-cadherin is known as the most predominant, perhaps even only cadherin of these AJs, N-cadherin gene knockout studies by Jiang et al. (2015) led to meiotic defects and failure in spermatogenesis but not to total cell dissociations and infertility. Thus, AJ-related structures and their possible functions need to be examined in further detailed genetic studies.

5.4.2 *Areae cribelliformes*

In bovine testes the newly described cribelliform junctions have been found as clusters of regularly and closely spaced cell-cell junctions in distinct regions of Sertoli cells, generally in association with some of the “ectoplasmic specializations”, i.e. paracrystalline-like arrays of actin microfilament bundles which appear to be linked with short lateral cross-bridges to the plasma membrane (for ectoplasmic specializations see, e.g., Russell 1977b; Franke et al. 1978a, 1982b; Vogl and Soucy 1985; Vogl et al. 1986, 1993, 2000; Grove and Vogl 1989; Mruk and Cheng 2004a; Li et al. 2015). Compared to gap junctions,

which contain pores of 1.5–2 nm in luminal diameter, the cell-to-cell channels of the cribelliform junctions are clearly much wider (5–7 nm, see, e.g., Figs. 24 and 25).

In future studies further research analyses of the newly described cribelliform junctions should include studies of different species with the additional use of different tight and gap junction markers (for relevant references see, e.g., the collections of reviews edited by Cereijido and Anderson 2001; LaFlamme and Kowalczyk 2008; Nelson and Fuchs 2010; Fromm and Schulzke 2012).

5.5 A novel and special kind of smooth muscle cells in peritubular walls

The results of the biochemical, immunocytochemical and electron microscopical experiments have shown that the cells of the peritubular wall encasing the seminiferous tubules are a special form (“lamellar”) of fully developed smooth muscle cells (SMCs) with a laterally extended and for the most part very flattened, polyhedral cell shape. The peritubular SMCs are organized as bandage-like peritubular monolayers interspersed with ECM layers as described in chapter 4.6.3.

5.5.1 Molecular, structural and functional aspects of the LSMCs

The peritubular SMCs, referred to as “lamellar smooth muscle cells” (LSMCs), contain indeed a full complement of SMC-typical molecules and structures (Table 14 and Figs. 30–41). They represent a novel morphologically special category of SMCs, which fundamentally differ in their shape and tissue organization from all other kinds of reported SMCs (cf., e.g., Gabella 1994). In the literature these LSMCs of the peritubular wall are often classified as “myoid cells”, “myofibroblasts”, “myoepithelial cells”, “special fibroblasts” or “undifferentiated mesenchymal cells” (e.g., Fawcett et al. 1969; Böck et al. 1972; Hadley and Dym 1987; Tung et al. 1984; Palombi et al. 1992a; Maekawa et al. 1994, 1996; Losinno et al. 2012, 2016; for an early classification as SMCs based only on electron microscopy see Ross and Long 1966). However, these cells are not just “myoid” cells (meaning “a cell looking alike a muscle cell” but not a real muscle cell) or “myofibroblasts” (for the specific and correct definition of myofibroblasts as modified fibroblasts by Gabbiani and colleagues see Gabbiani et al. 1971; Majno et al. 1971; Ryan et al. 1974; Eddy et al. 1988; Hinz et al. 2012; see also Supplement Literature Collection No. 3).

Previous authors have already called the peritubular wall cells “SMCs” on the basis of positive reactions of single – or very few selected – SMC marker molecules such as desmin or α-SMA (e.g., Virtanen et al. 1986; Palombi et al. 1992a; Romano et al. 2007). This, however, is insufficient for cell identification and classification since the synthesis of just one or a few individual major proteins alone does not allow to define a specific cell type.

This thesis work has shown that LSMCs are indeed SMCs *sensu stricto*: In addition to SMC-characteristic molecules they also contain all major SMC-typical structures such as myofilaments, often even organized in muscle-type paracrystalline bundle arrays, “dense bodies” (cf. Bond and Somlyo 1982), “focal adhesions” (for reviews see, e.g., Jockusch et al. 1995; Critchley 2004, Dubash et al. 2009) and caveolae (locally often in clusters; for references and reviews see, e.g., Supplement Literature Collection No. 6). In molecular terms the LSMCs contain all major SMC hallmark proteins, including smooth muscle specific α-actin (α-SMA; see, e.g., Skalli et al. 1986) and the corresponding myosin light and heavy chains (see, e.g., Babij et al. 1991; for review see also Krendel and Mooseker 2005), desmin (cf. Small and Sobieszek 1977; Hubbard and Lazarides 1979; Sparn et al. 1994) and smoothelin (cf. van der Loop 1996, 1997; Wehrens et al. 1997; Niessen et al. 2005; van Eys et al. 2007; Lepreux et al. 2013). As summarized in Table 14 almost all known SMC marker molecules are present in LSMCs (compare also references of the Supplement Literature Collection No. 4). In addition, throughout the present study strong immunoreactions for the smooth muscle markers found in LSMCs were also seen in SMCs of the adjacent blood vessel walls, which therefore provided optimal positive controls (see, e.g., Figs. 30 and 32).

A high frequency of caveolae can also be found in the testicular LSMCs and the vascular SMCs. Caveolae are formed as a special type of lipid rafts and represent small invaginations (60–100 nm) of the plasma membrane (see, e.g., Ross and Long 1966, for references of caveolae and caveolin-1 see, e.g., Supplement Literature Collection No. 6; specifically for peritubular SMCs see Oliveira et al. 2016). In addition, Ca^{2+} binding and regulator proteins such as calponin, calponin-related protein SM22α and caldesmon are present in LSMCs of diverse species (for references see Supplement Literature Collection 5; for reviews see Winder and Walsh 1996; Wang 2001). However, it remains to be examined which specific isoforms of such proteins can be localized in the peritubular wall or whether some local or cell type-specific differences are based on special isoforms or different scaffolding forms and perhaps different epitope accessibilities. In some species, certain differences have been detected even between the peritubular wall and the SMCs of nearby located blood vessels (for a different immunostaining pattern of a calponin isoform in Sertoli cells of rat testes tissue see, e.g., Zhu et al. 2004).

In general, SMCs have a multifunctional potential, including contraction, proliferation, synthesis, and secretion of extracellular components, such as cytokines and growth factors. In adult animals, mature SMCs can show plasticity, phenotypical changes and responses to environmental signals unlike skeletal and cardiac muscle, which are terminally differentiated (e.g., Schwartz et al. 1990; Thyberg et al. 1990; Owens 1995, Owens et al. 2004). Vascular SMCs are also involved, e.g., in the control of blood

pressure and blood flow. The range of potential functions of the LSMCs of the peritubular walls of mammalian testes should be further studied on the basis of their molecular composition and structures, including the special forms of AJs (see chapter 5.5.4; Table 17).

Smoothelin, for example, is known to be exclusively expressed in fully differentiated and contractile SMCs whereas cardiac and skeletal muscle cells do not contain this protein (van der Loop et al. 1996, 1997). In particular, smoothelin isoform B represents a potential marker for true smooth muscle cells (see, e.g., van Eys et al. 2007). In addition, Gan et al. (2007) have shown that the transcriptional regulatory element for another SMC gene, encoding α-SMA, in true SMCs is different from the transcription regulatory system for its expression in myofibroblasts, i.e. a special form of fibroblast cells (Tomasek et al. 2002).

The frequency of myofilaments in LSMCs, with identical orientation in a given cell, is quite striking (for rodent peritubular walls, however, see also contrasting statements of Losinno et al. 2012, 2016). Furthermore, these myofilaments have a coaxial orientation corresponding to the respective seminiferous tubule, indicative of the specific direction of the contraction of the tubule. In testicular tissues this contractile nature was first shown by Roosen-Runge (1951) and Clermont (1958) and was also found for early non-mammalian vertebrates by Unsicker and colleagues (Unsicker 1974, 1975; Unsicker and Burnstock 1975). The regulation and functional roles of these peritubular wall cell contractions have been studied for decades (see, e.g., Fawcett et al. 1969; Dym and Fawcett 1970; Fawcett et al. 1973). In many previous studies of the specific contractility it has also been a major question whether and how signaling molecules may be transported from the LSMCs into seminiferous tubules or vice versa. For technical reasons, a high proportion of these experimental studies have been performed with cultured cells or combinations of cultured peritubular wall cells and Sertoli cells (see, e.g., Tung and Fritz 1980, 1986a, 1986b; Tripiciano et al. 1996; 1997; Romano et al. 2005, 2007; Albrecht et al. 2006; for the influence of losses of contractility markers in infertility patients see, e.g., Welter et al. 2013).

5.5.2 Cytoskeletal heterogeneities of the LSMCs

While some of the SMC tissues contain desmin as the by far predominant, often seemingly exclusive IF protein, many types of SMCs contain the IF-protein vimentin, alone or together with desmin. Indeed, vimentin is long-known as a constitutive cytoskeletal IF component of various SMC types, notably vascular wall SMCs and certain SMC-derived tumors and cell cultures, either alone or together with desmin (e.g., Small and Sobieszek 1977; Hubbard and Lazarides 1979; for further references see also Supplement Literature Collection No. 4.1; for a review see Rensen et al. 2007; for testis, in particular for

developmental stages, see van Vorstenbosch et al. 1984; Virtanen et al. 1986; van Muijen et al. 1987; Tung and Fritz 1990).

The presence of a further IF-protein in LSMCs of bulls and boars, namely cytokeratins of a simple epithelial character, i.e. cytokeratins 8 and 18, may seem disturbing in view of the predominant cell type specifications of IF-protein type syntheses. However, In the literature the occurrence of cytokeratin IFs in certain SMC tissues has been reported for various mammalian species, notably myometrial and perivascular SMCs, cell cultures as well as certain SMC tumors (leiomyomas and leiomyosarcomas) and other pathogenic forms (for a list of references see Table 16). Now this thesis adds certain bovine and porcine testicular LSMCs to this list.

Additionally, the presence of E-cadherin together with cytokeratins 8 and 18 is detectable in testicular LSMCs of certain species such as bull and boar. Generally, E-cadherin is known to occur regularly and only in epithelial and certain epithelium-derived cells as an epithelial hallmark protein (for references see, e.g., Vestweber and Kemler 1984; Takeichi 1988, 1990; Perez and Nelson 2004; Strumane et al. 2004; Meng and Takeichi 2009). The regulations of the synthesis and the functions of these marker molecules in LSMCs of these species are still not clear.

5.5.3 Special morphological aspects of the LSMCs

The extended flat flaps of the LSMCs, their stacked arrangements in monolayers alternating with ECM layers, and their bandage-like, tight encasement of the seminiferous tubules show their morphological difference from other smooth muscle tissues (see, e.g., Gabella 1994). The most striking difference from all other SMC tissues is their flattened cell shape in many areas. Large parts of the LSMCs appear as thin regions in which the cytoplasmic membrane-to-membrane thickness is very low (30–100 nm), and consequently such regions are free of any large cell organelles, vesicles or sizeable aggregates. In particular, mitochondria are generally excluded from such cell portions which suggest special local metabolic conditions. Most likely, these very thin LSMC regions are among the thinnest lamellar cytoplasmic structures that have been found in vertebrate cells *in situ*, comparable for example, only to some endothelial areas and short transition regions of oligodendrocytes to the myelin sheaths. Morphologically, very thin but mostly filiform, interdigitating projections and lamellipodial processes have also been found in special fibroblastic cells of heart valves (e.g., Filip et al. 1986; Wuchter et al. 2007; Barth et al. 2009, 2012; for review see Franke et al. 2009), and interstitial cells of Cajal cells (e.g., Faussone-Pellegrini and Thuneberg 1999). Only in certain cell cultures, still much thinner cytoplasmic regions have been found with membrane-to-membrane cytoplasmic structures which in that case, however, are stabilized by columella-like, short cytoplasmic bridges (11–16 nm; Franke et al. 1978d).

Table 16 Examples of reports of the presence of cytokeratins 8 and 18 in specific human smooth muscle cells (in addition to vimentin or desmin or both; 1987-1993)

Reference*	Smooth muscle tissues and/or cell cultures	Developmental stage or pathological form
Huitfeldt and Brandtzaeg 1985	Myometrium	Normal adult tissue
Brown et al. 1987	Myometrium	Normal adult tissue, tumors (leiomyomas, leiomyosarcomas)
Jahn et al. 1987	Blood vessels in synovial tissue and umbilical cord	Normal adult tissue
Norton et al. 1987	Smooth muscle in adult and fetal small intestine, esophagus, blood vessels, myometrium	Normal adult and fetal tissue, tumors (leiomyomas, leiomyosarcomas)
van Muijen et al. 1987	Stroma of umbilical cord and placental villi, small intestine, tongue, blood vessels	Normal fetal tissue
Bader et al. 1988	Vascular walls of umbilical cord, cultured cells derived therefrom	Normal adult tissue
Gown et al. 1988	Cell cultures of myometrium, fetal intestine and heart, some tumors	Normal adult and fetal tissue, tumors (leiomyomas, leiomyosarcomas)
Kasper et al. 1988	Vascular cells and stellate cells of umbilical cord	Normal adult tissue
Kuruc and Franke 1988	Vascular walls of some cardiac blood vessels	Normal adult and fetal tissue
Miettinen 1988	Some soft tissue tumors	Tumors (leiomyomas, leiomyosarcomas)
Ramaekers et al. 1988	Myometrium, blood vessels	Tumors (leiomyomas, leiomyosarcomas)
Turley et al. 1988	Myometrium	Normal adult tissue
Bozhok et al. 1989	Fetal allantois, urogenital sinus, Wolffian and Müllerian ducts, mesentery, urinary bladder and certain regions of colon, rectum and atrium cordis walls, cell cultures	Normal tissues in fetal stages
Franke et al. 1989	Certain fetal organs, including blood vessels, adult myometrium	Normal adult and fetal tissue, atherosclerotic tissues, tumors (leiomyomas, leiomyosarcomas)
Knapp et al. 1989; Knapp and Franke 1989	Diverse cell culture lines derived e.g. from smooth muscle tissue, fibroblasts	Normal adult tissue
Jahn and Franke 1989	Arteries	Normal adult tissue, atherosclerotic lesions
Tauchi et al. 1990	Soft tissue tumors	Tumors (leiomyomas, leiomyosarcomas)
Glukhova et al. 1991	Aortic walls	Normal fetal tissue, atherosclerotic plaques
Jahn et al. 1993	Coronary artery, peripheral vascular walls, myointimal cells	Normal fetal and neonatal tissues, vein graft disease, tissue transplantation-associated atherosclerosis
Miettinen et al. 1993	Myometrium	Normal adult tissue

* For an early review see Gusterson 1987. For specific later reports see Jahn 1997; Johansson et al. 1997, 1999; Slomp et al. 1997; Yu and Bernal 1998; Chu and Weiss 2002; for cytokeratins in non-epithelial cells of amphibia and fishes see Markl and Franke 1988; Ferretti et al. 1989.

5.5.4 The novel cell-cell junctions connecting the LSMCs

In a healthy state, the peritubular wall with its bandage system of LSMC monolayers appears impermeable for translocations of large particles and molecule complexes. Thus, it may represent an additional “outer barrier” around the seminiferous tubule (e.g., Fawcett 1994). Therefore, it is necessary to analyze the cell-cell connections and molecular compositions of these LSMCs.

Within a given LSMC monolayer the cells are laterally connected in two ways: (i) Directly end-to-end (see, e.g., Fig 56 f) or (ii) by usually more frequent, closely-spaced vertical AJs located in overlapping LSMC processes (collations adhaerentes) of the type *processus alter supra alterum*, an apparently novel architectonic form (Figs. 55 a, d and 56 a–e; Table 17). Similar serial AJ structures have been shown in mesenchymal stem cells and the filopodia connecting cardiac valve fibrocytes (Wuchter et al. 2007; Barth et al. 2009, 2012). An indication of the occurrence of such two types of AJ-type junctions between LSMCs had already been noted by Dym and Fawcett (1970) in rat testis although mostly in much smaller forms (see Figs. 3-7 in Dym and Fawcett 1970). Such serial vertical AJ-type junction formations for lateral connections also appear related to the serial invagination and interdigitating connections of certain mammalian endothelial cells (see, e.g., Figs. 1-3 in Franke et al. 1988).

The predominance of cadherin-11, a type II cadherin, in immunolabelling experiments at the light (Figs. 36 and 39) and electron (Fig. 57 f–i) microscopic level is in agreement with their SMC character (see chapters 4.6.1 and 4.6.3; for rat testes see also Johnson and Boekelheide 2002a,b), as this glycoprotein is well-known to be involved in the development and maintenance of diverse types of SMCs, osteogenic and tendon-associated cells as well as other mesodermally-derived cells (e.g., Okazaki et al. 1994; Hoffman and Balling 1995; Kimura et al. 1995; Simmoneau et al. 1995; Alimperti et al. 2014; Row et al. 2016; for further references and reviews see Supplement Literature Collection No. 7).

In addition, P-cadherin, known as a major component present in some proliferative tissues (Hirai et al. 1989) and certain epithelia (e.g., the basal cell layers of certain stratified epithelia such as bovine tongue mucosa; Nose and Takeichi 1986; Johnson et al. 1993; Wu et al. 1993; Furukawa et al. 1997), has been identified in LSMCs of mature bull testes (see, e.g., Figs. 37–39) but not at all in Sertoli cells of mature animals. On the other hand, however, this glycoprotein has also been reported by other authors to occur in Sertoli cells of fetal, postnatal and very young bulls. Perhaps, this can be explained by a correlation with the transition from an early to a mature state in testis development. Previously, this glycoprotein has been reported to occur in Sertoli cells of rats during pre- and perinatal stages but then to gradually disappear postnatally, while in advanced states

of maturation it has then been detected in LSMCs (see, e.g., Wu et al. 1993; Lin and DePhilip 1996). Since P-cadherin-deficient mice are fertile (Radice et al. 1997), it needs to be analyzed which functional role P-cadherin plays in the stabilization, intercellular adhesion and maintenance of integrity of the peritubular LSMCs.

The 54 kDa plaque protein myozap has been discovered in certain regions of LSMCs as well as in endothelial cells and some interstitial mesenchymal cells of bovine and human testes. It also appears to be present in the *zonula adhaerens* of the excurrent duct epithelia of all examined testicular tissues. Myozap is a cytoskeletal protein first found by Seeger et al. (2010) in intercalated disks of cardiomyocytes (composite junctions, *areae compositae*) as a special plaque component. In addition, it has also been identified in the endothelial AJs of blood and lymph vessels (Pieperhoff et al. 2012) and in the *zonula adhaerens* junctions of E-cadherin-based epithelia (Rickelt et al. 2011a). In this study, protein myozap has not been seen in AJs of Sertoli cells in seminiferous tubules of bovine, boar and man but has been noted in Sertoli cells of rodents. However, as protein myozap has been detected in this study as a major component of the cytoplasmic AJ plaques of bovine and human LSMCs in the peritubular wall as well as in blood vascular SMCs, it appears to offer another potential valuable cell type marker for diagnostic immunocytochemistry.

Protein LUMA (TMEM43) known to occur in the composite junctions of heart tissue (e.g., Franke et al. 2014) has also been detected in LSMCs of testicular tissues and in certain blood vessel walls but not in Sertoli cells. In respect to the known involvement of certain LUMA-mutations in cardiomyopathies (see, e.g., Christensen et al. 2011a; Liang et al. 2011; Larsen et al. 2012; Baskin et al. 2013; Haywood et al. 2013; Hodgkinson et al. 2013), the functional role of protein LUMA in testicular LSMCs needs to be further examined.

5.6 Absence of a lymphatic endothelium

The results of this thesis have shown that in rodents, as in all other mammalian species studied, the peritubular wall structure is not tightly covered by an extended lymphatic endothelium or a single endothelial cell layer as it has been claimed to the contrary in numerous publications and textbooks (e.g., Fawcett et al. 1969, 1970, 1973; Dym and Fawcett 1970; Dym 1975, 1994; Clark 1976; Söderström 1981; Maekawa et al. 1996; Yazama et al. 1997; Losinno et al. 2012, 2016). Only occasionally, rather small lymphatic vessels, located in the interstitium i.e. away from the peritubular wall, are positive for established endothelial cell type markers (see chapter 4.7; for references of endothelial cell type markers applied see, e.g., Newman et al. 1990; Lampugnani et al. 1992, 1995; Banerji et al. 1999; Morita et al. 1999b; Dejana et al. 2000; Prevo et al. 2001; Sleeman et al. 2001;

Karkkainen and Alitalo 2002; Miettinen 2003; Dejana 2004; Hämmerling et al. 2006; Cîmpean et al. 2007; Baluk et al. 2007; Lampugnani and Dejana 2007; Pfeiffer et al. 2008; Moll et al. 2009; Noda et al. 2010; Bravi et al. 2014; for specific human endothelial markers see Podgrabinska et al. 2002; Miettinen 2003). On the other hand, the very sensitive immunocytochemistry methods applied have also revealed the regular occurrence of small lymphatic capillaries in the interstitium between the seminiferous tubules, in contrast to the negative results of other authors who have stated that a testicular lymphatic system is restricted to the septula testis and absent in most of the – or even the entire – interstitium (e.g., Holstein et al. 1979; Hirai et al. 2012; Svingen et al. 2012; DeFalco et al. 2015).

Negative results with the prototype of the *complexus adhaerens* subtype of lymphatic endothelia, which contain the plaque protein desmoplakin (Schmelz et al. 1990, 1994; Schmelz and Franke 1993), have led to the conclusion that the lymphatic vessels of the testicular system are generally of the *complexus adhaerens*-free category (for *complexus adhaerens*-positive lymphatic endothelia see, e.g., Valiron et al. 1996; Gallicano et al. 1998; Kowalczyk et al. 1998; Sawa et al. 1999; Ebata et al. 2001a, b; Zhou et al. 2004; Hämmerling et al. 2006; Baluk et al. 2007; Pfeiffer et al. 2008; Moll et al. 2009).

However, the cytochemical and ultrastructural results which have been obtained in the course of this doctoral thesis still do not allow the typological identification of the outermost lamellar cells specifically in rodent testes. They are to some extent compatible with the originally electron microscopically-derived interpretation of Ross and coworkers (e.g., Ross 1967; Bressler and Ross 1972) that this outermost cell layer of the peritubular wall consists of laterally connected fibroblasts or fibrocytes or may even present a yet still unknown cell type. The origin of these cells, however, needs to be examined with additional cell type-specific markers.

5.7 Comparison with studies using cell cultures containing Sertoli and LSMC-derived cells

In the last four decades a huge number of data obtained from cell cultures of Sertoli cells and/or peritubular wall-derived cells has been published, including enriched or “purified” cell types as well as “co-cultures”. In particular LSMC-containing cultures, grown in various cell culture media, have been used for biochemical and physiological studies (Mayerhofer 2013; for further examples of references see Supplement Literature Collection No. 8). Such studies have led for example to discoveries of the synthesis and effects of, e.g., endothelin-1, decorin, biglycan and fibronectin, procollagenase IV and collagenase IV, plasminogen activators and their inhibitors.

However, the cell biological value of such cultures is still questionable. For example, the LSMCs growing in cell culture are very different to the typical LSMC morphology *in situ*. In all cell cultures used in previous reports, the cells have not a polyhedral shape and have not been shown to be connected by the kind of cell-cell junctions active in living tissues. In all these cultures the LSMCs do not grow in the form embedded in both sides with ECM structures and material which is characteristic for the monolayer cell bandage stack system of the peritubular LSMCs *in situ*. Obviously, cell culture systems need to be developed to allow Sertoli cells on the one hand and LSMCs on the other to grow in a mode close to their *in situ* organizations.

5.8 Species differences, seasonal differences and evolution

To date only limited cell biological and molecular information is available for non-mammalian vertebrates and seasonal differences of different mammalian species. So far, only a few studies have dealt with the regulation of seasonal fertility changes, for example by changes of the outer temperature (see Supplement Literature Collection No. 9).

However, in early cell biological studies similarities of smooth muscle-like cells were noted in peritubular walls of certain other vertebrates (for lower vertebrates, in particular birds, reptiles and amphibia, see, e.g., Rothwell and Tingari 1973; Rothwell 1975; Unsicker 1975; Unsicker and Burnstock 1975; see also Supplement Literature Collection No. 9). So far, however, only a limited portion of the antibodies used in this thesis work appears to be cross-reactive and thus applicable for such studies. For future analyses, further species-specific antibodies need to be developed.

Table 17 Novel types of adhering junctions in mammalian tissues (discoveries after 1990; see also Table 2)

Type		Reference
Complexus adhaerens (some specific endothelia, virgultar cells of lymph nodes, lymphatic retothelium)	1990	Schmelz and Franke 1993; Schmelz et al. 1990, 1994; Hämmerling et al. 2006
Contactus adhaerens (granular cells of cerebellar glomeruli)	1995	Rose et al. 1995; Bahjaoui-Bouhaddi et al. 1997; Hollnagel et al. 2002
Zonula limitans externa (heterotypic: photoreceptor ⇔ Müller glia cells)	1999	Paffenholz and Franke 1997, Paffenholz et al. 1999
Area composita (composite junctions; intercalated disks of cardiomyocytes)	1999, 2006	Borrmann 2000; Borrmann et al. 2000, 2006; Franke et al. 2006
Iunctura structa (specific stratified epithelia)	2002	Langbein et al. 2002
Cortex adhaerens (eye lens)	2003	Straub et al. 2003
Colligatio permixta (astrocytes, astrocytoma)	2004	Boda-Heggemann 2005
Incl. *Meningeal junctions* (meningioma)	2008	Akat et al. 2008
Puncta adhaerentia minima (specific mesenchymal cells)	2007	Wuchter et al. 2007; Barth et al. 2009
Processus adhaerens (specific mesenchymal cells)	2007	Wuchter et al. 2007
Manubrium adhaerens (specific mesenchymal cells in culture)	2007	Wuchter et al. 2007; Rickelt et al. 2009
Coniunctio adhaerens (mesenchymal cells of high proliferative activity)	2009	Barth et al. 2009; Rickelt et al. 2009
cis-E:N-Cadherin heterodimer junctions (hepatocytes, hepatomas, certain pancreatic cells)	2011	Straub et al. 2011
Area tessellata (tessellate junctions)	2013	Franke et al. 2013
Area adhaerens (seminiferous tubules of testes, N-cadherin-based)	2014	Domke et al. 2014; present study
Area cribelliformis (Sertoli cells in seminiferous tubules of bovine testes)	2014	Domke et al. 2014; present study
Collatio adhaerens (overlapping lamelliform processes of the type *processus alter supra alterum*, with vertical AJs for lateral cell-cell connections, in peritubular walls of testes, Cadherin-11-based)	2018	Present study

6 Conclusions and Outlook

The present systematic study of isotypic cell-cell adhering junctions (AJs) and cytoskeletal filaments of Sertoli and germ cells of the seminiferous tubules has confirmed the absence of desmosomes and "desmosome-like" junctions as well as the absence of several other epithelial AJ molecules. In addition, this thesis presents the finding of two novel types of cell-cell junctions in seminiferous tubules: (i) The N-cadherin-based AJs of the category "***areae adhaerentes***", including very large cell-cell junctions, and (ii) the "***areae cribelliformes***" (cribelliform junctions), clusters of sieve-plate-like junctions perforated by small cytoplasm-to-cytoplasm channels (5–7 nm).

The analyses of the basal lamina of the seminiferous tubules have revealed the lack of hemidesmosomes and hemidesmosomal markers. Furthermore, I have proven the absence of a continuous lymphatic endothelium surrounding the peritubular walls of rodent testes in contrast to a widespread claim in the literature.

Detailed investigations of the peritubular wall cell monolayers enwrapping the seminiferous tubules have demonstrated a smooth muscle cell character and a novel kind of smooth muscle cell and tissue, the "**lamellar smooth muscle cells**" (**LSMCs**). In addition, a novel architectonic cell-cell connection system has been characterized: The LSMCs are laterally connected within their specific monolayer by sparse end-to-end AJs as well as by groups of vertical AJs located in closely overlapping cell processes (collationes adhaerentes) of the type "***processus alter supra alterum***".

In summary, the results of my thesis provide an ultrastructural and molecular basis of testicular cell structures and will support the finding of additional potential "markers" for the characterization of testes-derived cells and cell lines in basic research, studies of developmental stages and aging, as well as immunocytochemical diagnoses of male genital tumors and other diseases. In particular, the availability of a series of cell type specific markers will be important for tumor diagnostics (The World Health Organization Classification of Tumours of the Urinary System and Male Genital Organs; eds. Moch et al. 2016).

The isotypic AJs presented in this thesis represent only one category of cell-cell connections. Additional analyses of heterotypic cell-cell junction molecules, notably those involved in the embedding of the spermatid heads in the specific apical indentations, are needed to complete the list of candidate molecules for cell type diagnoses as well as for the development of possible direct male contraceptives, starting with the results of the Heidelberg laboratory (Prof. Dr. Werner W. Franke; see, e.g., Franke et al. 1978a; Longo et al. 1987; Hess et al. 1993, 1995; von Bülow et al. 1995; Heid et al. 2002) and of the Osaka laboratory (Prof. Dr. Yoshimi Takai; see, e.g., Takai et al. 2003; Irie et al. 2004; for further references see Supplement Literature Collection No. 10).

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Lisa Maria Domke