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Original Article

Differential distribution of laminin chains in the development and sex differentiation of mouse internal genitalia

KIM FRÖJDMAN[1], PETER EKBLOM[2], LYDIA SOROKIN[3], AHMED YAGI[4] and LAURI J. PELLINIEMI[1]*

[1]*Laboratory of Electron Microscopy, University of Turku, Turku, Finland,* [2]*Department of Animal Physiology, Uppsala University, Uppsala, Sweden,* [3]*Max-Planck-Gesellschaft, Arbeitsgruppen für Rheumatologie, Erlangen, Germany and* [4]*Department of Anatomy, University of Turku, Turku, Finland*

ABSTRACT The distribution of laminin chains and basement membranes (BMs) in the ontogenesis and sex differentiation of male and female mouse gonads and mesonephros was studied by conventional and immunocytochemical light and electron microscopy. The α1 (synonymous to A) chain was recognized with MAbs against fragment E3, and three chains of laminin with PAbs raised against EHS-laminin. BMs, which formed around the mesonephric duct, the mesonephric tubules, and the paramesonephric duct, contained the laminin α1 chain. The α1 chain appeared with epithelial differentiation in the developing gonads in both sexes. The α1 chain was first evident around the embryonic gonadal cords and remained, after development, in the BMs of the testicular cords and ovarian follicles. The laminin α1 chain was also detected in BMs of the myoid cells around the epithelial rete cords, and transiently in the surface epithelium and in the corpus luteum. Laminin β-γ chains were found in many locations where the α1 chain was not detected. These included the mesenchyme of the early mesonephros, the BMs of blood vessels and surface epithelium in the differentiated testis and ovary, between the theca cells in the ovary, and in some corpora lutea. The morphological differentiation of the BMs of the embryonic testicular cords proceeded rapidly. In contrast, the BM of the ovarian cords remained relatively poorly differentiated during the prenatal phases, and developed concomitantly with the differentiation of the follicles. The results show that BMs in the differentiating internal genitalia are heterogeneous with respect to their laminin chains, and suggest that all known laminin chains must be analyzed in the differentiation of gonadal epithelia for a complete role of the BMs in gonadal sex differentiation.

KEY WORDS: *laminin, mice, gonads, testis, ovary*

Introduction

Laminins, prominent glycoproteins in basement membranes (BMs), are large complexes composed of a heavy α chain, and the light ß and γ chains (Burgeson *et al.*, 1994). There are several laminin variants. In the present study the laminin α1 chain, and laminin recognized with PAbs against mouse EHS tumor, will be studied. The α1 chain, a component of laminin-1 and laminin-3, is almost exclusively found in epithelial cell basement membranes, whereas the laminin ß1-γ1 chains have a wider distribution (Klein *et al.*, 1990). Laminin binds to different cell membrane receptors, also in the testis (Davis *et al.*, 1991), it binds other extracellular matrix compounds, and it forms complexes with itself (Paulsson, 1992). A number of studies have shown that laminin-1 supports attachment and migration of a variety of cells as well as being crucial for epithelial cell development in the mouse kidney (Klein *et al.*, 1988; Ekblom, 1989; Ekblom *et al.*, 1990).

In the embryonic testis, the early gonadal cords are surrounded by extracellular matrix composed of laminin (Fröjdman *et al.*, 1992a,b), fibronectin (Paranko *et al.*, 1983), type I, III, IV and V collagens, and heparan sulfate proteoglycan (Pelliniemi *et al.*, 1984; Paranko, 1987). Laminin is a component in BMs on the cells of the mesonephric duct and tubules, testicular cords, myoid cells, endothelial cells and the Leydig cells in the male (Agelopoulou and Magre, 1987; Hadley and Dym, 1987; Gelly *et al.*, 1989; Kuopio and Pelliniemi, 1989; Kuopio *et al.*, 1989; El Ouali *et al.*, 1991; Fröjdman *et al.*, 1992b). Laminin probably mediates connection between the Sertoli cells and the BM, since antibodies to laminin inhibit Sertoli cell attachment to reconstituted BM (Tung and Fritz, 1993). This attachment is important for the morphology of the Sertoli cells and for the *in vitro* differentiation of cords composed of Sertoli cells (Hadley *et al.*, 1990). In pre- and postnatal rat ovaries, laminin is

Abbreviations used in this paper: ECM, extracellular material; BM(s), basement membrane(s); MD, mesonephric duct; MT, mesonephric tubule; pc, post coitum; pn, post natum; MAbs, monoclonal antibodies; PAbs, polyclonal antibodies.

***Address for reprints:** Laboratory of Electron Microscopy, University of Turku, Kiinamyllynkatu 10, FIN-20520 Turku, Finland. FAX: 358-21-6337380.

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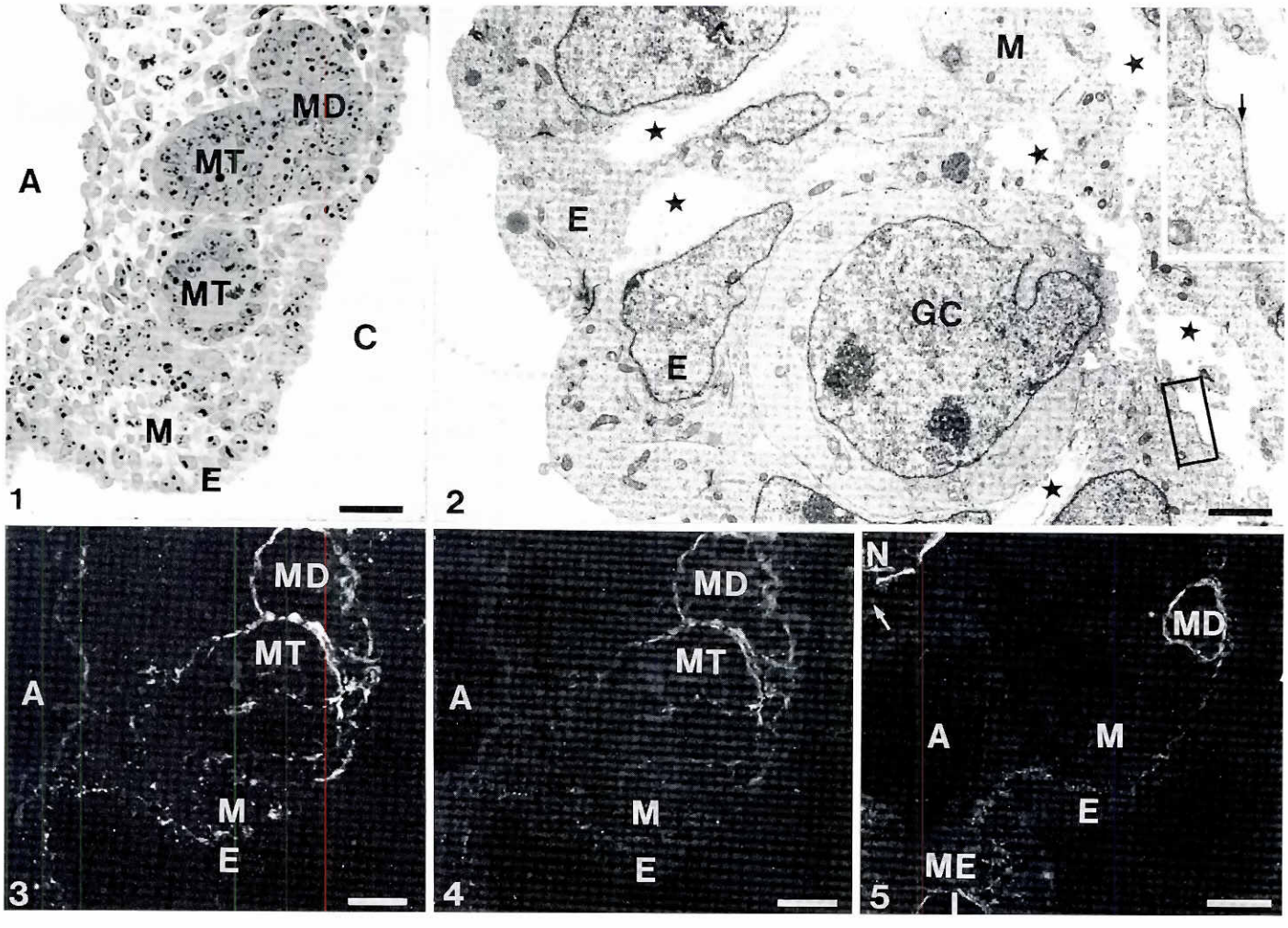


Figs. 1-5. The asexual phase. **(1)** *Light micrograph of the mesonephros of a 10-day-old X chromatin-positive female mouse embryo at the level of the future gonad. The mesonephric duct (MD) and the mesonephric tubules (MT) form elongated epithelial structures. In the ventral aspect of the mesonephros, the surface epithelium (E) and the underlying mesenchyme (M) are loose and there is no recognizable gonad. A, aorta; C, coelom. Scale bar, 30 μm.* **(2)** *Electron micrograph of the ventral mesonephros of a 10-day-old X chromatin-negative male embryo. Large extracellular spaces (asterisks) are seen between the primitive cells of the surface epithelium (E) and in the mesenchyme (M). A primordial germ cell (GC) is seen in association with the surface epithelium. Scale bar, 2 μm. The rectangle represents the area seen at higher magnification in the inset which shows the basal part of a surface epithelial cell with a patch of a basement membrane (arrow).* **(3)** *Distribution of all laminin chains in the mesonephros of a 10-day-old (pc) male embryo. Prominent reactions are seen around the mesonephric duct (MD) and the distal mesonephric tubules (MT), whereas more ventrally the distribution of the laminin chains is irregular and punctate under the surface epithelium (E) and in the mesenchyme (M). The basal surface of the endothelial cells of the aorta (A) are also labeled with the PAbs. Scale bar, 2 μm.* **(4)** *Double immunoreaction of the laminin α1 chain in the same section as in Fig. 3 shows the localization around the mesonephric duct (MD) and distal mesonephric tubules (MT). More ventrally, only a faint reaction for the α1 chain is seen under the surface epithelium (E) and in the mesenchyme (M). No reaction is seen around the aorta (A). Scale bar, 2 μm.* **(5)** *Immunoreaction for the laminin α1 chain in a 10-day-old male embryo at a caudal level of the mesonephros. Reaction is present around the mesonephric duct (MD) and as a faint string under the surface epithelium (E). The intestine (I) and the neural tube (N) are also surrounded by α1 chain. A punctate reaction is also seen among the mesenchymal cells of the mesentery (ME). No reaction for the α1 chain is found in the mesonephric mesenchyme (M) or in the notochord (arrow), which is positive for the ß-γ chains (not shown). A, aorta. Scale bar, 3 μm.*

found around the follicles and between the interstitial theca cells (Bagavandoss *et al.*, 1983; Leardkamolkarn and Abrahamson, 1992; Fröjdman *et al.*, 1993). The heterogeneity in the components of laminin in the human genitourinary tract was suggested by the application of monoclonal antibodies (Leu *et al.*, 1986). Recently, northern blot analysis of human tissues revealed that both the α1 and α2 (M) chains are expressed in fetal testis (Vuolteenaho *et al.*, 1994).

The differentiation of the intragonadal epithelia in the developing testis and ovary is guided into male and female directions by a regulatory system (Pelliniemi *et al.*, 1993a,b). Our earlier findings on the changes in cytoskeletal components and membrane bound integrins in the epithelial cells (Fröjdman *et al.*, 1992b, 1993; Fröjdman and Pelliniemi, 1994, 1995) suggest that the basement membranes may show sexual dimorphism. The new fragment-specific antibodies to laminin chains offer now possibilities to

investigate their expression and distribution in the developing epithelial cells of the testis and ovary.

Results

The interpretation of the present results relies first on monoclonal antibodies (MAbs) against the E3 fragment of the laminin α1 chain, and second, on polyclonal antibodies (PAbs) which apparently reveal the presence of three laminin chains. Since the analysis is based on double immunoreactions with MAbs and PAbs, ß-γ chains (ß1 or γ1 or both) are reported in locations where the α1 chain was not detected. Differences observed in the intensity of immunoreactions with the PAbs may well be due to the presence or absence of the laminin α1 chain.

The asexual phase of mesonephric development

The mesonephroi at the pregonadal phase at the age of 10 days were similar in male and female embryos (Fig. 1), identified by X chromatin analysis. The epithelial mesonephric duct (MD) and the distal mesonephric tubules (MTs) were surrounded by prominent basement membranes (BMs). At the proximal end of the MTs towards the ventral part of the mesonephros, however, no BM was found. In the dorsal parts of the mesonephros, close to the MD, a BM was found under the surface epithelium whereas in the ventral part of the mesonephros only few patches of BM were found below the surface epithelial cells (Fig. 2). The loose mesenchyme beneath the surface epithelium contained large extracellular spaces and relatively small amounts of extracellular material (ECM). The paramesonephric duct (PMD) developed in the 12- to 13-day old embryos and became gradually surrounded by a prominent BM.

The PAbs recognizing all chains of laminin (Fig 3), and the α1 chain specific MAbs (Figs. 4 and 5), all showed prominent reactions around the MD and distal MTs in 10-day-old embryos. Accumulations of ß-γ chains (Fig. 3) were found in the mesenchyme of the ventral mesonephros, but only occasional weak reactions for the α1 chain were evident in this region (Fig. 4). More caudally in the organ, a weak labeling of the laminin α1 chain was found under the surface epithelium in the ventral part of the mesonephros (Fig. 5).

The localization of the laminin α1 chain was similar in the mesonephros of both sexes in 11 to 13-day-old embryos. The α1 chain specific MAbs reacted weakly with the BM underlying the surface epithelium of the mesonephros dorsal to the gonad in 11-day-old embryos (Fig. 7), while PAbs showed a strong reaction. The reaction for the α1 chain gradually increased around the proximal part of the MTs which had established contact with the intragonadal rete cords. In contrast, the reaction around the distal parts of the MTs and MD (Fig. 7) became weaker in both sexes. With the formation of the PMD, a weak reaction for the α1 chain surrounded it. In 13- to 14-day-old embryos, the reaction for the α1 chain under the mesonephric surface epithelium was strong close to the PMD but weaker elsewhere.

The testes

The gonadal blastema phase of the testis

In 11- to 12-day-old male embryos, the gonad was composed of a dense blastema tissue (Fig. 6). Within this blastema, early epithelial cell precursors of testicular cords, possessing differentiating BMs (Fig. 8), were found. Accumulations of electron-dense ECM were frequently detected in the interspace between the BMs and the pericordal cells.

Laminin chains were found on the surface of the early cords (Figs. 7, 10 and 11). However, there were several locations where laminin ß-γ chains, (Fig. 10) but not the α1 chain, were present (Fig. 11). ECM, which contained the laminin α1 chain, was found to demarcate the region of the gonadal blastema (Figs. 7 and 11) at these early phases of testicular differentiation.

Gonadal cords and seminiferous tubules

The testicular cords and the rete cords became gradually surrounded by prominent BMs, as seen by electron microscopy in the 14-day old embryo (Fig. 9). Between the differentiating BM of the Sertoli cells and the developing pericordal myoid cells, a relatively wide zone containing electron-dense ECM was found. In the old male the BM of the seminiferous tubules thickened and became fuzzy or stratified (Fig 15).

During fetal and postnatal stages of development, the BMs of the cords reacted strongly with the α1 specific MAbs (Figs. 13 and 17) and the PAbs recognizing all chains of laminin (Figs. 12 and 16). In some locations of the gonad of the 21-day-old (pn) male, the immunoreaction for α1 chain was prominently localized in the lamina lucida of the BM (Fig. 14). The reactions for the PAbs and MAbs in the BMs of the seminiferous tubules remained strong (Figs. 18 and 19) and were also prominent at the ages of 8 and 10 months.

The surface epithelium and tunica albuginea

In the early gonad of the 11-day-old male embryo, no continuous sheet of ECM was found by electron microscopy under the most superficial cells of the gonad. During the following day of development, ECM accumulated on the basal surface of the differentiating surface epithelium, and in the 13-day-old (pc) mouse testis, a continuous BM was found under the one to two cell layer thick surface epithelium. This subepithelial BM gave reactions for ß-γ chains at all stages studied (Fig. 16). In contrast, the differentiating surface epithelium in the 13-day-old (pc) mouse testis gave only a weak and locally restricted reaction for the laminin α1 chain. The reactivity for the α1 chain gradually declined in the subepithelial region and was no longer detectable in 18-day-old (pc) or older male mice (Fig. 17). With the differentiation of the elongated cells of the tunica albuginea under the surface epithelium in 13- to 14-day-old (pc) males, large extracellular spaces appeared with seemingly irregularly arranged ECM. Between the differentiating cells of the tunica, reactions for ß-γ chains (Fig. 16), but not for the α1 chain (Fig. 17), were noted.

The interstitium

The differentiating myoid cells acquired BMs during the first postnatal week. In the 6-day-old mouse testis, the outer surface of the myoid cells already possessed a relatively well-differentiated BM. At this age, and in the 18-day-old mouse, the inner surfaces of these cells were covered by irregularly arranged ECM. The BMs on the outer surface of the myoid cells gave prominent reactions for the ß-γ chains (Fig. 12). In contrast, the reaction for the α1 chain on the outer surface of the myoid cells was locally restricted (Figs. 13 and 14).

In other interstitial locations, moderate reactions for the ß–γ chains of laminin were seen in many locations where the α1 chain was not found. Immunolabeling electron microscopy of the early postnatal mouse testis showed reactions for the ß-γ chains in the BMs of the endothelial and muscle cells of the blood vessels (Fig. 12 and 16). Also some individual cells in the vicinity of the Leydig

cells were almost totally surrounded by the ß-γ chains of laminin (Fig. 12). In other places, as on the surfaces of the Leydig cells and the lymph endothelial cells, irregular local reaction for the ß-γ chains but not for α1 chain were found.

The ovary

The prenatal ovary

In the 11-day-old female embryo, the developing gonad consisted of a tight tissue of somatic cells and germ cells (Fig. 20). Electron microscopy showed accumulations of ECM on the surfaces of some cells and fragments of BMs (Fig. 21). Gradually, the gonadal cords became surrounded by primitive BMs. The space between the BM of the epithelial cells and the neighbor cells was in many locations extremely thin (Fig. 24) as compared with the corresponding zone in the testis (Fig. 9). With the differentiation of the fetal gonad, the cords in the medulla remained in loose irregular continuity with the smaller cords in the cortex of the ovary.

In the 11-day-old female embryo, a reaction for the laminin α1 chain was detected between the cells or groups of cells in the blastema. In the mesonephric zone adjacent to the gonad, there were frequent reactions for the ß-γ chains, but not for the α1 chain (Figs. 22 and 23). Caudally in the gonad, where no continuity was established with the MTs, the thickened gonadal ridge was separated from the mesonephric mesenchyme by a layer of ECM positive for the α1 chain. With the growth of the fetal gonad, the α1 chain remained present around the gonadal cords and the rete cords (Fig. 25).

The surface epithelium

In the 11-day-old female embryo, no BM of the surface epithelium was recognized. With the differentiation of the ovarian tissues, cortical cords in the ventral side of the ovary remained in places continuous with the surface epithelium (Fig. 25). The reaction for the α1 chain was positive in the BMs of the thickened surface epithelium which also contained germ cells. However, in the dorsolateral parts of the gonad (close to the hilum and without direct contact to the cords), the reaction for laminin α1 chain gradually declined and was negative in the mesovarium region of the 18-day-old female fetus (Fig. 25).

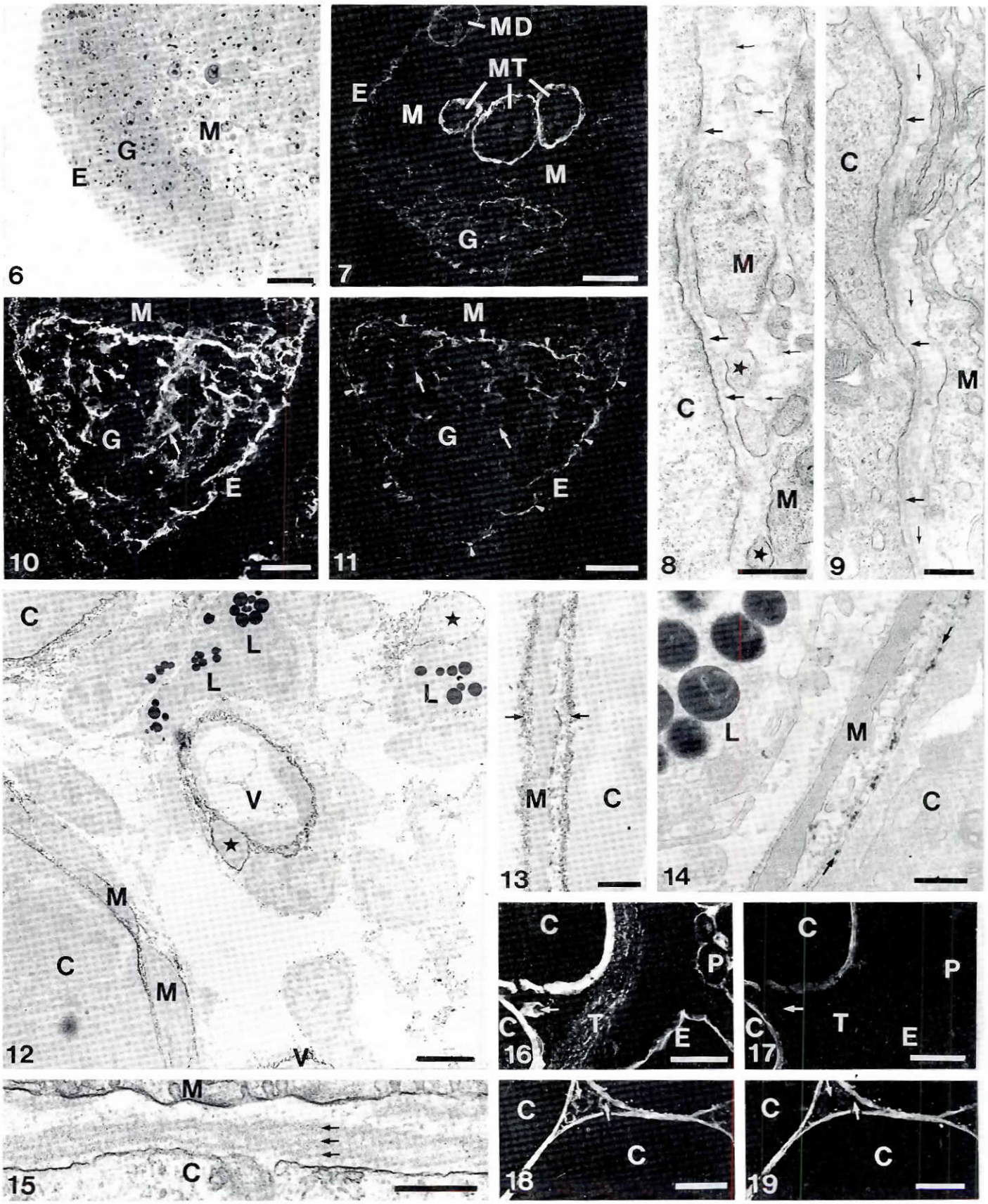
With the formation of the follicles, the continuity between the follicles and the surface epithelium was disrupted. In some instances the BM of the surface epithelium was close to that of primordial follicles (Fig. 26). The reaction for the α1 chain was first strong in the BM of the ovarian surface cells. Thereafter, and concomitantly with the differentiation of a proper surface epithelium, the reaction for α1 chain gradually declined and was mainly absent in the 17- and 18-day-old (pn) mouse ovaries. In the adult mouse, some variation in the reactivity for the α1 was observed under the surface epithelium (Figs. 27 and 32). The reaction for the ß-γ chains in the subepithelial BM was, in general, continuous along the BM (Fig. 31). Few negative areas apparently matched those which lacked the lamina densa in electron microscopy.

Follicles

With the differentiation of the ovarian cords into follicles, they became surrounded by prominent BMs (Figs 26 and 28). In some locations, BMs of the neighboring follicles were found close to each other without intervening cells (Fig. 28). In the adult, there were structural variations in the follicular BMs (Fig. 29).

Primordial, as well as primary and antral follicles, all showed prominent reaction for the laminin α1 chain (Figs. 30 and 32). Immunolabeling electron microscopy revealed the α1 chain in both the lamina densa and lamina lucida of the follicles (Fig. 30). The reaction for the α1 chain remained around the follicles of the adult mouse. Around atretic follicles, reactions of different intensity were seen for the α1 chain. Within some larger follicles, a punctate reaction for laminin was also found in some locations, probably corresponding

Figs. 6-19. The testis. (6) *Light micrograph of the early gonad in an 11-day-old male embryo. The gonadal blastema (G) between the surface epithelium (E) and the loose mesonephric mesenchyme (M) is dense. Scale bar, 30 μm.* **(7)** *Immunoreaction for the α1 chain of laminin in an 11-day-old male embryo is prominent around the mesonephric tubules (MT), whereas the reaction around the mesonephric duct (MD) and under the surface epithelium (E) of the mesonephros is weak. The α1 chain is also found in the periphery and inside the newly formed gonad (G). The mesenchyme of the mesonephros (M) is negative for the α1 chain. Scale bar, 40 μm.* **(8)** *Electron micrograph of the peripheral compartment of an early testicular cord (C) in an 11-day-old male embryo. A prominent basement membrane (thick arrows) underlies the Sertoli cell. Outside the basement membrane of the cord, pericordal cells (M) and cell extensions (asterisks) are seen. The extracellular space contains large amounts of matrix (thin arrows). Scale bar, 0.5 μm.* **(9)** *Electron micrograph of the basement membrane of a testicular cord (C) in a 14-day-old male embryo is composed of a lamina densa (thick arrows) and lamina lucida. Outside the basement membrane a large zone of reticular lamina is recognized (thin arrows). M, pericordal cell. Scale bar, 0.3 μm.* **(10)** *Immunolabeling light microscopy of the testis from a 12-day-old male embryo. Laminin chains, as detected with the PAbs, were frequently found inside (arrows) and around the gonad (G). E, surface epithelium; M, mesonephros. Scale bar, 30 μm.* **(11)** *Double immunoreaction for the laminin α1 chain in the same section as in Fig. 10. Reaction is seen around (arrowheads) the gonadal blastema tissue (G) towards the mesonephros (M) and under the surface epithelium (E). Several spots positive for the ß-γ chains are negative for the α1 chain (arrows). Scale bar, 30 μm.* **(12)** *Immunolabeling electron microscopy with the PAbs of an 8-day-old (pn) mouse testis shows laminin chains around the testicular cords (C), on the surface of the myoid cells (M) and vascular endothelial cells of the blood vessels (V). Some cells associated with the blood vessels and the Leydig cells (L) also show prominent reactions (asterisks). Scale bar, 5 μm.* **(13)** *Immunolabeling electron microscopy of a 21-day-old (pn) male testis shows reaction for the α1 chain in the basement membranes (arrows) of the cords (C) and of the myoid cells (M). Scale bar, 1 μm.* **(14)** *Immunolabeling electron microscopy of the testes from a 21-day-old (pn) male. Reaction for laminin chain α1 is seen in the lamina lucida of the basement membrane (arrows) on the surface of the testicular cord (C). M, myoid cell; L, Leydig cell. Scale bar, 0.5 μm.* **(15)** *Electron microscopy of the basement membrane of a seminiferous tubule (C) from an 8-month-old mouse. The basement membrane is thickened and contains two to three sheets of lamina densa (arrows) intervened by lamina lucida-like layers. M, myoid cell. Scale bar, 0.5 μm.* **(16)** *Immunoreaction for all laminin chains in the testis from an 18-day-old (pn) mouse. Reaction is found around the testicular cords (C), under the surface epithelium (E), in the tunica albuginea (T), and in the testis-associated fat pad (P). Also the blood vessels (arrow) contain laminin in their BMs. Scale bar, 50 μm.* **(17)** *Double immunoreaction for the laminin α1 chain in the same section as in Fig. 17. Only the surface of the testicular cords (C) gives reaction for the α1 chain, whereas the surface epithelium (E), tunica albuginea (T), the blood vessels (arrow) and the fat pad (P) are negative. Scale bar, 50 μm.* **(18)** *Immunoreaction for laminin chains, as revealed by the PAbs, in the testis of an 11-week-old male. Laminin chains are found in the ECM of the lamina propria of the seminiferous tubules (C), and in the walls of the interstitial blood vessels (arrows). Scale bar, 50 μm.* **(19)** *Immunoreaction for the laminin α1 chain in the same sections as seen in Fig. 18. α1 chain is present in the ECM of the lamina propria of the seminiferous tubules (C) whereas the blood vessels (arrows) are negative. Scale bar, 50 μm.*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to Call-Exner bodies (Figs. 31 and 32). Some small cell groups of the adult ovary, which were positive for the laminin α1 chain and possessed BM-like material by electron microscopy, may represent remnants of atretic follicles, rete cords, or corpora lutea.

Corpus luteum

In the corpus luteum of the cycle, BMs were found on the surface of epithelial cell clusters by electron microscopy (Fig. 33). The BMs showed prominent reactions for the laminin α1 chain inside and around some corpora lutea (Y1 in Fig. 35). In corpora lutea with less regular outer borders, a reaction was mainly found only for the ß-γ chains of laminin and not for the α1 chain (Y2 in Figs. 34 and 35). In mice which had been pregnant for 10, 12 or 15 days, reactions for the ß-γ chains, but not for the α1 chain, were observed in the corpora lutea.

The postnatal interstitium

Prominent BMs were seen underlying endothelial and muscle cells of the blood vessels. With the differentiation of the interstitial cells of the postnatal mouse ovary, ECM accumulated between the cells of the theca.

The laminin ß-γ chains were frequently found on the cells of the interstitium (Fig. 31), including the theca cells and the endothelial and muscle cells of the blood vessels. No reaction for α1 chain was observed on these cells (Fig. 32). Likewise, the tunica albuginea of the ovary showed no reaction for the laminin α1 chain.

Immunoblotting

Ovarian and testicular proteins from 10- and 19-day-old (pn) mice, respectively, contained three chains reactive with the PAbs against laminin (Fig. 36). The reactions for two light chains were prominent, whereas the reactions for the heavy chain were weaker (Fig. 36).

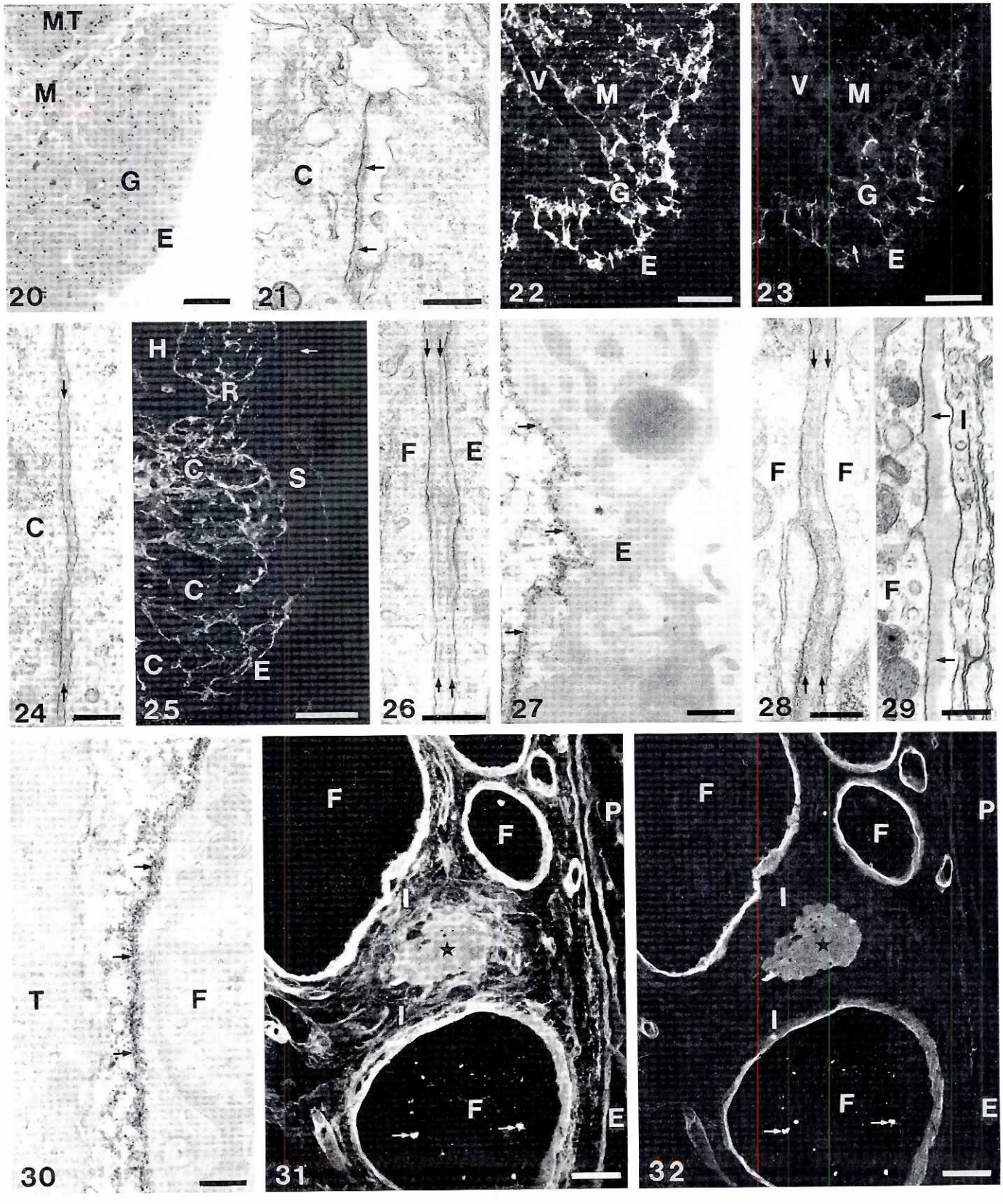
Discussion

The appearance of the laminin α1 chain associated with the differentiating epithelial cells of the testicular and ovarian cords, rete cords, follicles, myoid cells, corpora lutea, mesonephric tubules, mesonephric duct and the paramesonephric duct, is apparently related to the formation of the BMs of the respective epithelia. The absence of reactions for the laminin α1 chain in the BMs and BM-like coats related to the mesonephric mesenchyme, the endothelial and muscle cells of the blood vessels, the Leydig cells, and the theca cells, suggests that these cells do not possess laminin-1, as defined as the α1-ß1-γ1 complex. Immunoreactions with the PAbs in these situations may indicate the presence of several different laminin-like complexes, since the γ1 chain, recognized by the PAbs, is present in several different laminin complexes (Burgeson *et al.*, 1994).

The formation of the gonads begins by proliferation and transformation of primitive mesenchymal and surface epithelial cells of the mesonephros into a blastema cell population. The inducer of this transformation is not known. The formation of this blastema tissue includes changes in the expression of intermediate filaments in the cells (Fröjdman *et al.*, 1992b, 1993), and an increased cellular adhesion and expression of the α_6 subunit of integrins (Fröjdman and Pelliniemi, 1994, 1995). According to the present results, this transformation is accompanied by the expression of the ß1 or γ1 or both laminin chains. The polarized appearance of the laminin α1 chain on the basal surfaces of differentiating epithelial cells, as in the developing kidney (Sorokin and Ekblom, 1992; Sorokin *et al.*, 1992), include another transformation process, and correlates temporally with the appearance of polarized cytokeratin in the basal cytoplasm of these early gonadal cord cells (Fröjdman *et al.*, 1992b). The reorganization of the epithelial cells into elongated testicular cords (Fröjdman *et al.*, 1992b) apparently caused a delay in α1 chain expression in the medulla of the male gonad.

The laminin α1 chain is expressed in many organs during the early differentiation of the epithelial BMs (Klein *et al.*, 1988, 1990). In adult organs, the α1 chain is often lost or weakly expressed (Ekblom *et al.*, 1990; Goodman, 1992). It has been postulated that the synthesis of Sertoli cell laminin is down-regulated in the postnatal testis (El Ouali *et al.*, 1991). The presence of the α1 chain in the BMs of the seminiferous tubules was therefore unexpected.

Figs. 20-32. The ovary. (20). *Light micrograph of the early gonad of an 11-day-old female embryo. The tight gonadal blastema (G) is continuous with the surface epithelium (E). MT, mesonephric tubules; M, mesonephric mesenchyme. Toluidine blue staining. Scale bar, 30 μm.* **(21)** *Electron micrograph from the gonadal blastema in an 11-day-old female embryo shows patches of primitive basement membrane (arrows) underlying occasional somatic cells (C). Scale bar, 0.5 μm.* **(22)** *Immunoreaction to all laminin chains is found in the early gonad (G) and mesonephros (M) of an 11-day-old female embryo. Some cell clusters are devoid of internal laminin (arrows). E, surface epithelium; V, blood vessel. Scale bar, 40 μm.* **(23)** *Immunoreaction for the laminin α1 chain in the same section as in Fig. 22. Chain α1 is found discontinuously surrounding some cell clusters (arrows) and under the surface epithelium (E) of the gonad (G). The mesonephric mesenchyme (M) is virtually negative for the α1 chain, and no reaction is seen in the blood vessel (V) BMs. Scale bar, 40 μm.* **(24)** *Conventional electron micrograph of the primitive BM (arrows) between somatic cord cells (C) in the ovary from a 14-day-old embryo. Scale bar, 0.3 μm.* **(25)** *Laminin α1 chain in the ovary and hilus from an 18-day-old fetus. The α1 chain is found around the gonadal cords (C) and discontinuously under the surface epithelium in the ventral side of the gonad (E). In the hilus (H), the reaction under the surface epithelium is weak or negative (arrow). The cords in the medulla are via the α1 chain positive rete cords (R) connected to the mesonephric tubules. The stroma (S) between the medullary cords and the surface epithelium is negative for the α1 chain. Scale bar, 30 μm.* **(26)** *Conventional electron micrograph of the intercellular space between the surface epithelium (E) and the cell of a primordial follicle (F) illustrates well-differentiated BMs (arrows). Scale bar, 0.5 μm.* **(27)** *Immunolabeling electron micrograph in the surface epithelium (E) of a 3.5-month-old female shows the laminin α1 chain in the BM (arrows) of the epithelia. Scale bar, 0.5 μm.* **(28)** *In the 6-day-old (pn) female, the BMs (arrows) of two adjacent primordial follicles (F) face each other without intervening cells. Scale bar, 0.3 μm.* **(29)** *Conventional electron microscopy of the follicular wall in a 5-month-old female. The BM (arrows) on the follicular granulosa cell (F) is thickened and no lamina lucida is detectable. I, interstitial cell. Scale bar, 0.5 μm.* **(30)** *Immunolabeling electron microscopy of the periphery of an early antral follicle (F) shows prominent reaction to the laminin α1 chain in the basement membrane (arrows) of the follicle in an 8-day-old (pn) female. T, perifollicular cell. Scale bar, 0.5 μm.* **(31)** *Immunolabeling light microscopy for chains of laminin in the ovary from a 17-day-old (pn) female. A prominent reaction is seen around the follicles (F). A weaker reaction is found in several locations in the interstitium (I), under the surface epithelium (E) and in the fat pad (P). A reaction is also seen in the follicular Call-Exner bodies (arrows). The asterisk shows a tangentially cut BM of a follicle. Scale bar, 30 μm.* **(32)** *Double immunoreaction to the laminin α1 chain in the same section as seen in Fig. 31. Only the basement membranes of the follicles (F) and the Call-Exner bodies (arrows) are positive, whereas the interstitium (I), the surface epithelium (E) and the fat pad (P) are negative. Asterisk: tangentially cut follicular BM. Scale bar, 30 μm.*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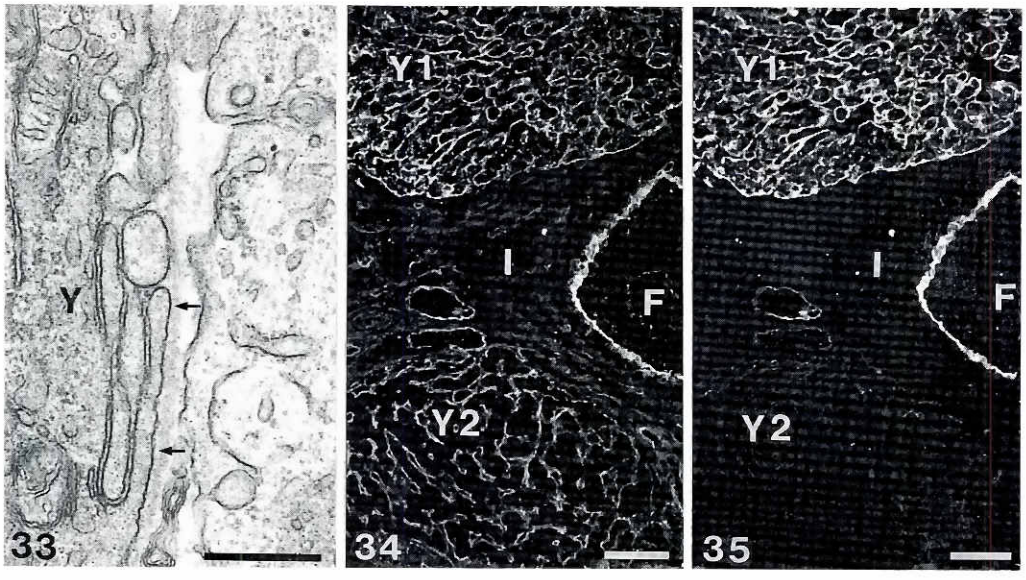

Figs. 33-35. The corpora lutea. **(33)** *Electron micrograph of the corpus luteum (of the cycle) from a 5-month-old female. A BM (arrows) underlies an epithelial cell (Y) of a cell cluster. Scale bar, 0.5 μm.* **(34)** *Immunolabeling light microscopy in the ovary of a non-pregnant 5-month-old female. Immunoreaction with the PAbs in two corpora lutea show a strong reaction for laminin inside and around one corpus (Y1), whereas in the other one (Y2) the reaction is moderate and discontinuous. F, follicle, I, interstitium. Scale bar, 50 μm.* **(35)** *Double immunoreaction for the α1 chain of laminin in a 5-month-old female (same section as in Fig. 34). A strong reaction is found inside and around one corpus luteum (Y1), whereas only a rudimentary reaction is seen in the other one (Y2). The follicle (F) is surrounded by a prominent reaction to the α1 chain. I, interstitium. Scale bar, 50 μm.*

In the present study, we did not observe any local differences in the immunoreactivity with the different laminin antibodies to testicular cord BMs, as have been observed in the human testis (Leu *et al.*, 1986).

The presence of the laminin α1 chain in the gonadal surface epithelium of both sexes, disappearing earlier in the male than in the female, are likely to be related to the earlier differentiation of the surface epithelium in the male (Fröjdman *et al.*, 1993). A positive reaction for the α1 chain also in some locations in the ovarian surface epithelium in the adult female may indicate that laminin-1 is involved in cyclic changes of the surface epithelium (Auersperg *et al.*, 1991), and that more than one laminin-like complexes may be expressed in a balanced system by these cells, as has been seen in endothelial cells (Tokida *et al.*, 1990; Glukhova *et al.*, 1993).

The presence of groups of cells surrounded by the laminin α1 chain containing BMs in some corpora lutea indicates that the transformation of granulosa cells into lutein cells includes epithelial aggregation and expression of epithelial cell characteristics by the cells. The aggregation of fetal-type Leydig cells (Kuopio and Pelliniemi, 1989; Kuopio *et al.*, 1989) and the cell groups of Langerhans (van-Deijnen *et al.*, 1992), both possessing basement membranes, are other examples of glandular islets with similar features. The absence of the laminin α1 chain in some corpora lutea of the cycle and in those of late pregnancy, suggests that with the maturation of the corpora, the expression of the α1 chain is downregulated. The transient expression of the α1 chain in the corpus luteum may offer the means for distinguishing its different endocrine phases.

The earlier maturation of the BMs around the testicular cords, as compared with the thin and irregular BMs during the embryonic and fetal phases in the female, suggests a sex-specific regulatory system. The delayed formation of ovarian cord BMs, also noted in the rat (Grund and Pelliniemi, 1987), is probably not due to the expression of the α1 chain, since this chain appeared at the same age around the gonadal cords in both sexes. The poor differentiation of the zone between the BM of the cords and the pericordal interstitial cells in the fetal ovary is probably related to the indifferent nature of the ovarian interstitium (Paranko, 1987). The structural variation in the BMs of the follicles may be due to the dynamics and growth of the individual follicles. Thickening of the BMs of the seminiferous tubules with age has also been reported in the old rat (Ichihara *et al.*, 1993).

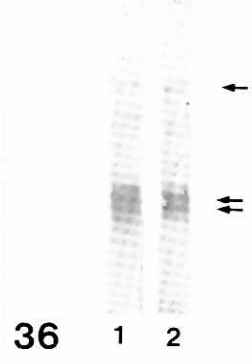

Fig. 36. Immunoblots of laminin chains in gonads from 10-day-old (pn) males (lane 1) and 19-day-old (pn) females (lane 2) *show weak bands for the α1 chain (single arrow), and two bands (double arrows) at approximately Mr 200 k, corresponding the light chains.*

Although our observations on BM-like coats of laminin between the theca cells are similar to those obtained by others (Leardkamolkarn and Abrahamson, 1992), the absence of the laminin α1 chain in these locations indicates different complexes of laminins than those found in the classical laminin-1 (Burgeson *et al.*, 1994). The presence of laminin ß-γ chains but not the α1 chain in the BMs of the endothelial cells in the mesonephros and the gonads throughout the study is in agreement with similar findings in several other organs (Klein *et al.*, 1990).

The uniform and even distribution of the classical EHS-laminin in the BMs of developing gonads has now proved to be based on the multivalency of the previously used PAbs. The present results on laminin-1 chains in the differentiating internal genitalia suggest that all different laminin subtypes and their component chains (Aumailley *et al.*, 1990; Burgeson *et al.*, 1994) have to be tested for a complete understanding of the putative role of BMs and laminin containing ECM in the sexual differentiation and development of the gonads.

Materials and Methods

Tissues

Mesonephroi and gonads were collected from male and female embryos at ages from 10 to 14 days, from fetuses at the age of 18 days, newborn animals, from 6-, 10-, 18-, 19-, and 21-day-old, and from 11 week-old and older Balb/c strain mice. The day after copulation was considered as day 0 of embryonic age. The sex of the 10- to 14-day-old embryos was identified by X chromatin analysis (Fröjdman *et al.*, 1992b) and the older males and females were identified using anatomical criteria.

Conventional microscopy

For conventional light and electron microscopy, the tissues were fixed in 5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.16 mol/L s-collidine buffer (pH 7.4) with or without 0.5% alcian blue (Gurr, BDH Chemicals Ltd., Poole, England). The tissues were then post-fixed with potassium ferrocyanide-osmium fixative, embedded in epoxy resin, and processed for light and electron microscopy as described earlier (Fröjdman *et al.*, 1992b). Semithin sections for light microscopy were stained with 0.5% toluidine blue and thin sections for electron microscopy with uranyl acetate and lead citrate. The specimens were analyzed with a Leitz Diaplan light microscope (Ernst Leitz, Wetzlar, Germany) and with Jeol JEM-100SX and JEM-1200EX electron microscopes (Jeol Ltd., Tokyo, Japan).

Antibodies and immunocytochemistry

Monoclonal antibodies (MAbs) to fragment E3 of the laminin α1 chain, obtained from the Engelbreth-Holm-Swarm (EHS) tumor, have been derived from hybridization of induced spleen cells from Lou rats with Y-Ag.1.2.3. rat myeloma cells (Sorokin *et al.*, 1992). The epitopes recognized by the MAbs are restricted to the globular domain G4 of fragment E3. Four different antibodies (clones 198, 200, 201 and 207, Sorokin *et al.*, 1992) gave similar results in immunocytochemisty. The polyclonal laminin antibodies (PAbs), a courtesy of Dr. J-M. Foidart, were raised in rabbit against purified laminin-1 isolated from mouse EHS tumor (Foidart *et al.*, 1980). Other PAbs raised in rabbit against the P1 fragment of human laminin-1 (Risteli and Timpl, 1981) gave similar results as the PAbs to mouse laminin (Foidart *et al.*, 1980).

For light microscopic immunocytochemistry, fresh tissues frozen in liquid nitrogen were cut into 2 to 6 µm thick sections with a cryostat microtome, transferred onto slides, air-dried, and fixed in acetone for 7 min at -20°C. The immunocytochemical reactions were made by incubation with the MAbs, and the reactions were visualized with fluorescein isothiocyanate-conjugated goat anti-rat IgG (Cappel Lab., Malvern, PA, diluted 1:50). Tetramethylrhodamine isothiocyanate-coupled goat anti-rabbit IgG (Cappel), diluted 1:50 was used for double immunofluorescence to detect the PAbs (diluted 1:1000). The specificity of the antibodies has been tested before (Foidart *et al.*, 1980; Sorokin *et al.*, 1992). The reactions were controlled by omitting primary or secondary antibodies, using non-related primary or secondary antibodies, using serial dilutions of the primary antibody, and by using known positive and negative control tissues.

For electron microscopic immunocytochemistry, the gonads were cut into small pieces, incubated for 25 min in culture medium (MEM, GIBCO, Paisley, Scotland, UK) containing 0.1% sodium azide. The tissues were then incubated for 6 to 8 h at 4°C with the antibodies to laminin, followed by the Vectastain kit (Vector Laboratories, Burlingame, CA, USA) procedure as described earlier (Fröjdman and Pelliniemi, 1994). The reactions were visualized with diaminobenzidine tetrahydrochloride (Polysciences, Warrington, PA, USA) using H_2O_2 as substrate. After the immunocytochemical procedure, the specimens were thoroughly washed and fixed in 5% glutaraldehyde in 0.16 mol/L s-collidine buffer. The tissues were then further fixed in 2% osmium tetroxide in water for 2 h, dehydrated and embedded in epoxy resin (Fröjdman *et al.*, 1992b). Thin sections were examined unstained.

Immunoblotting

Testes and ovaries from 10- and 19-day-old (pn) mice were collected. The samples were washed and reduced by boiling them for 5 min in Laemmli buffer containing 4% SDS and 10% 2-ß-mercaptoethanol. The proteins were separated on a 5% homogenous SDS-polyacrylamide gel and transferred to nitrocellulose. A blocking solution of 0.3% of fish skin gelatine was used. Incubation with the primary antibodies was followed by biotinylated secondary antibodies and the ABC-complex according to the procedure of the Vectastain ABC kit (Vector Laboratories). The reactions were visualized with 4-chloro-1-naphtol using H_2O_2 as substrate.

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