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Original Article

Molecular profiling of the basement membrane of pluripotent epiblast cells in post-implantation stage mouse embryos

Sugiko Futaki [1], Itsuko Nakano, Miwa Kawasaki [2], Noriko Sanzen [2], Kiyotoshi Sekiguchi*

Laboratory of Matrixome Research and Application, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka, 565-0871, Japan

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ABSTRACT

Introduction: The basement membrane (BM) is a sheet-like extracellular matrix (ECM) lining the basal side of epithelial and endothelial cells. The molecular composition of the BM diversifies as embryonic development proceeds, providing optimized microenvironments for individual cell types. In post-implantation stage embryos, the embryonic BMs are essential for differentiation of the epiblast, a layer of multipotent embryonic stem cells, and subsequent embryogenesis. To better understand the role of BMs and cell–BM interactions in early embryogenesis, it is imperative to accumulate information on the molecular entities of the embryonic BMs.

Methods: We analyzed the expressions and localizations of 20 major BM proteins (11 laminin subunits, 6 type IV collagen subunits, nidogen-1 and -2, and perlecan) and other ECM-related proteins such as fibronectin and integrins in post-implantation stage embryos by immunohistochemistry.

Results: We found that a set of BM proteins, laminin α5, β1, and γ1 (comprising laminin-511), type IV collagen α1 and α2 (yielding type IV collagen $\alpha1_2\alpha2$ [IV]), nidogen-1 and -2, and perlecan, were consistently present in the epiblast/ectoderm BMs throughout the early post-implantation stages. In contrast, laminin α1 was detected in the epiblast BM at E5.5 but decreased in later stages, suggesting that laminin-511 is a major laminin isoform in the early embryonic BM. In addition, fibronectin, a mesenchymal ECM protein, was enriched in the endoderm BM, indicating that the BM compositions differ between the ectoderm and the endoderm. Consistent with these observations, integrin α5, a high-affinity receptor for fibronectin, was localized in the endoderm, while integrin α6, a receptor for laminin-511, was localized in the ectoderm.

Conclusions: The embryonic BMs underlying the epiblast/ectoderm contain a common toolkit comprising laminin-511, type IV collagen ($\alpha1_2\alpha2$ [IV]), nidogen-1 and -2, and perlecan, providing a physiological basis for the utility of laminin-511 as a culture substrate for pluripotent stem cells. The distinctive association of laminin-511 and fibronectin with endodermal and ectodermal cells, together with the differential expression of integrin α5 and α6 in these cells, suggests that the ectodermal and endodermal cells rely on their integrin-dependent interactions with laminin-511 and fibronectin, respectively, to ensure their fate specification in embryonic development.

1. Introduction

The basement membrane (BM) is a sheet-like extracellular matrix (ECM) composed of a repertoire of glycoproteins and proteoglycans. BMs are formed at the basal side of epithelial and endothelial cell sheets and surround individual muscle fibers, muscle satellite cells, adipocytes, vascular pericytes, and Schwann cells. BMs provide cell-adhesive substrates and evoke signals that regulate cell survival, differentiation, and migration via cell-surface ECM receptors such as integrins. BM molecules and soluble factors

Abbreviations: BM, Basement membrane; ECM, Extracellular matrix.

* Corresponding author. Fax: +81 6 6105 5936.
E-mail address: sekiguch@protein.osaka-u.ac.jp (K. Sekiguchi).

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[1] Present address: Anatomy and Cell Biology, Osaka Medical College, 2–7 Daigaku-machi, Takatsuki, Osaka 569–8686, Japan.
[2] Present address: RIKEN Center for Biosystems Dynamics Research (BDR), 2–2–3 Minatojima-minamimachi, Chuo-ku, Kobe 6500047, Japan.

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captured on BMs vary by tissue types and developmental stages, and provide customized microenvironments for individual cells. In general, BMs contain laminin, type IV collagen, nidogen, and perlecan, comprising a molecular toolkit for BM construction [1,2]. The molecular composition of a BM determines its biological properties. For example, BMs contain one or several laminin isoforms, and the combination of these isoforms affects their cell-adhesive activities because laminin isoforms have different affinities for integrin receptors [3]. Thus, elucidation of the molecular entities of BMs is imperative to understand the mechanisms for tissue organization and development.

During early embryonic development in mice, a BM is indispensable for differentiation of the epiblast, an epithelial layer of pluripotent cells in peri-implantation stage embryos. Disorders of BM formation and cell–BM interactions cause lethality at this embryonic stage [4–6]. Epiblast cells give rise to three germ layers, the ectoderm, endoderm, and mesoderm. Epiblast BM formation and remodeling are also involved in the development of these germ layers. The ectoderm is a simple columnar epithelial cell layer supported by the ectodermal BM at its basal side. The ectoderm layer subsequently differentiates into the neural ectoderm and epidermal ectoderm. The mesoderm appears during gastrulation, a coordinated migration of differentiating cells from the epiblast through the primitive streak at the posterior midline of the epiblast layer. During gastrulation, local degradation of the posterior epiblast BM is critical for primitive streak formation. The endoderm, either primitive, embryonic, or extraembryonic, also forms an epithelial structure that requires an underlying BM. The definitive endoderm, from which endodermal organs such as the digestive system and lungs develop, arises from gastrulating cells that reach and settle in the visceral endoderm layer with a BM. Differentiation, growth, and migration of the cells in these three germ layers are critical for further embryonic development.

We previously investigated the differential localization of BM components at the organogenic stage of mouse embryos [7,8], and demonstrated that the expression patterns of BM proteins diversified as embryonic development proceeded, resulting in varied BM compositions depending on tissue types, cell types, and developmental stages. This diversification of BM compositions suggests that BMs are customized to provide optimal microenvironments for individual cells.

Here we focused on the development of post-implantation stage embryos, and analyzed the distribution of the BM toolkit, laminins, type IV collagens, nidogens, and perlecan. Laminin is a heterotrimeric protein consisting of α, β, and γ subunits. Laminin isoforms are composed by selected combinations of five α, three β, and three γ subunits, and are named by three digit numbers referring to their subunit compositions. For example, laminin-111 is composed of α1, β1, and γ1 [9,10]. Type IV collagen is composed of three α subunits selected from subunits α1 through α6. Combinations of two α1 and one α2 ($\alpha1_2\alpha2$ [IV]), heterotrimers of α3, α4, and α5 (α3α4α5 [IV]), and two α5 and one α6 ($\alpha5_2\alpha6$ [IV]) have been reported [11]. Laminins and type IV collagens both self-assemble to form the basal architecture of BMs. Nidogen-1 and -2 are monomeric glycoproteins that interact with other BM proteins through three globular domains. Perlecan, a heparan sulfate proteoglycan, is widely distributed in BMs as well as in cartilages [12]. In this study, we performed a comprehensive immunohistochemical survey of 20 major BM proteins (11 laminin subunits, 6 type IV collagen subunits, nidogen-1 and -2, and perlecan) at embryonic day (E) 5.5 through 7.5. Our results show that the early epiblast BM comprises laminin-511, type IV collagen ($\alpha1_2\alpha2$ [IV]), nidogen-1 and -2, and perlecan. Such comprehensive studies on the BMs in early post-implantation embryos may provide insights into the ECM milieu optimized for early embryonic cell differentiation.

2. Materials and methods

2.1. Mouse embryos

Post-implantation stage embryos were collected from ICR mice at E5.5, E6.5, and E7.5. Embryos with decidua, a thickened endometrial tissue surrounding an individual embryo, were excised and immediately frozen in Tissue-Tek OCT compound (Sakura Finetek) using liquid nitrogen. The frozen tissues were sectioned horizontally at 7–8-μm thickness. Serial sections from the proximal to distal end of the embryonic cylinders were obtained. Every one section from 2 or 3 serial sections of the same embryos was stained with hematoxylin and eosin for morphological validation. Only the set of sections at the correct angle and embryonic stages [13–15] were used for subsequent immunohistochemical analyses. The mice were kept in a specific pathogen-free environment under stable conditions of temperature and light (12-h/12-h light/dark cycle). All mouse experiments were performed in compliance with our institutional guidelines and were approved by the Animal Care Committee of Osaka University.

2.2. Antibodies and immunohistochemistry

The primary antibodies used for immunohistochemistry are listed in Table 1. Production and evaluation of the 20 BM protein antibodies were described elsewhere [7,8,16]. Antibodies against Oct-3/4 (Santa Cruz Biotechnology), Gata6 (R&D Systems), integrin α5 (MFR5) (BD Biosciences), and integrin α6 (GoH 3) (BD Biosciences) were commercially available. An anti-fibronectin polyclonal antibody was produced in our laboratory as described previously [17]. The procedure for immunohistochemical staining of frozen sections was described previously [7,8]. Briefly, embryos were freshly frozen and sectioned without pre-fixation. The sections were post-fixed with an appropriate fixative, such as 4% paraformaldehyde or acetone, depending on the individual primary antibodies. After inactivation of endogenous peroxidase by H_2O_2 treatment, the sections were sequentially incubated with a blocking solution, primary antibodies, and horseradish peroxidase-

Table 1
List of primary antibodies.

Category	Protein	Antibody	Production/Reference
BM protein	laminin α1	5B7-H1	Matrixome Project [8]
BM protein	laminin α2	4H8-2	SIGMA Aldrich (#L0663)
BM protein	laminin α3	M35-N3-B9	Matrixome Project [8]
BM protein	laminin α4	M49-N7-F3	Matrixome Project [8]
BM protein	laminin α5	M5N8-C8	Matrixome Project [8]
BM protein	laminin β1	1B5-D12	Matrixome Project [8]
BM protein	laminin β2	B24-N8-D6	Matrixome Project [8]
BM protein	laminin β3	B31-N8-G8	Matrixome Project [8]
BM protein	laminin γ1	A5	Chemicon International
BM protein	laminin γ2	C21-N1-C9	Matrixome Project [8]
BM protein	laminin γ3	C83-N4-F4	Matrixome Project [8]
BM protein	collagen α1(IV)	H11	Sado et al. [16]
BM protein	collagen α2(IV)	H22	Sado et al. [16]
BM protein	collagen α3(IV)	H31	Sado et al. [16]
BM protein	collagen α4(IV)	RH43	Sado et al. [16]
BM protein	collagen α5(IV)	H53	Sado et al. [16]
BM protein	collagen α6(IV)	B66	Sado et al. [16]
BM protein	nidogen-1	ELM1	NeoMarkers (#RT797)
BM protein	nidogen-2	nd2c	Matrixome Project [8]
BM protein	Perlecan	A7L6	Chemicon International
	Oct-3/4	H-134	Santa Cruz Biotechnology (#sc-9081)
	Gata6		R&D Systems (#AF1700)
	integrin α5	5H10-27 (MFR5)	BD Biosciences
	integrin α6	GoH 3	BD Biosciences
	fibronectin		Kozaki et al. [17]

Table 2
Target genes and their primers for quantitative RT-PCR.

Target gene	Gene symbol	Primer 1	Primer 2
Laminin α1	Lama1	tggagacggtggacagtgacct	cagccactgccaagtctatagca
Laminin α2	Lama2	cagtcagaagatggatggaatgg	gtcgtttgtatcagctgatgtcga
Laminin α3	Lama3	gggaaggtcacgacctctatga	Aatgagttccacacagggagtgt
Laminin α4	Lama4	Agaatctctgtgatggcagatgg	gcagctttactgaagctcacagg
Laminin α5	Lama5	tggctcctacctggatggcag	ctccacacgcaccaacacacg
GAPDH	Gapdh	tcctgcaccaccaactgcttagc	tggatgcagggatgatgttctgg

conjugated secondary antibodies. Finally, the sections were colorized with diaminobenzidine solution and counterstained with hematoxylin. To examine the possible local differences in BMs in detail, and to minimize artificial differences, serial sections of the entire embryonic tissue were immunostained and colorized simultaneously. For whole-mount immunostaining, embryos at E6.5–E7.5 were dissected from the decidua and fixed in 4% paraformaldehyde in PBS for 60 min on ice. The subsequent procedures were performed at 4 °C. After washing with PBS, the embryos were serially incubated in PBS containing 0.1% Triton-X100 and 0.1% Tween-20 (PBST) for 60 min, PBST containing 2% bovine serum albumin (BSA-PBST) for 60 min, and BSA-PBST containing primary antibodies for 16 h. The embryos were then washed with PBST three times for more than 30 min per time, and incubated with secondary antibodies in BSA-PBST for 16 h. Finally, the embryos were stained with DAPI, and observed under an LSM510 confocal laser scanning microscope (Zeiss).

2.3. Quantitative RT-PCR

Embryos from E6.5 to E16.5 were dissected in PBS. Extraembryonic membranes were removed as much as possible, especially from early embryos, to avoid contamination. Total RNA was extracted using an RNeasy Kit (Qiagen) and reverse-transcribed with SuperScript™ II Reverse Transcriptase (Invitrogen) and random primers. Quantitative PCR was performed with Power™ SYBR™ Green Master Mix (Applied Biosystems) and an ABI PRISM 7000 (Thermo Fisher Scientific). The target genes and their primers are listed in Table 2. The expression levels of the target genes were normalized by those of glyceraldehyde-3-phosphate

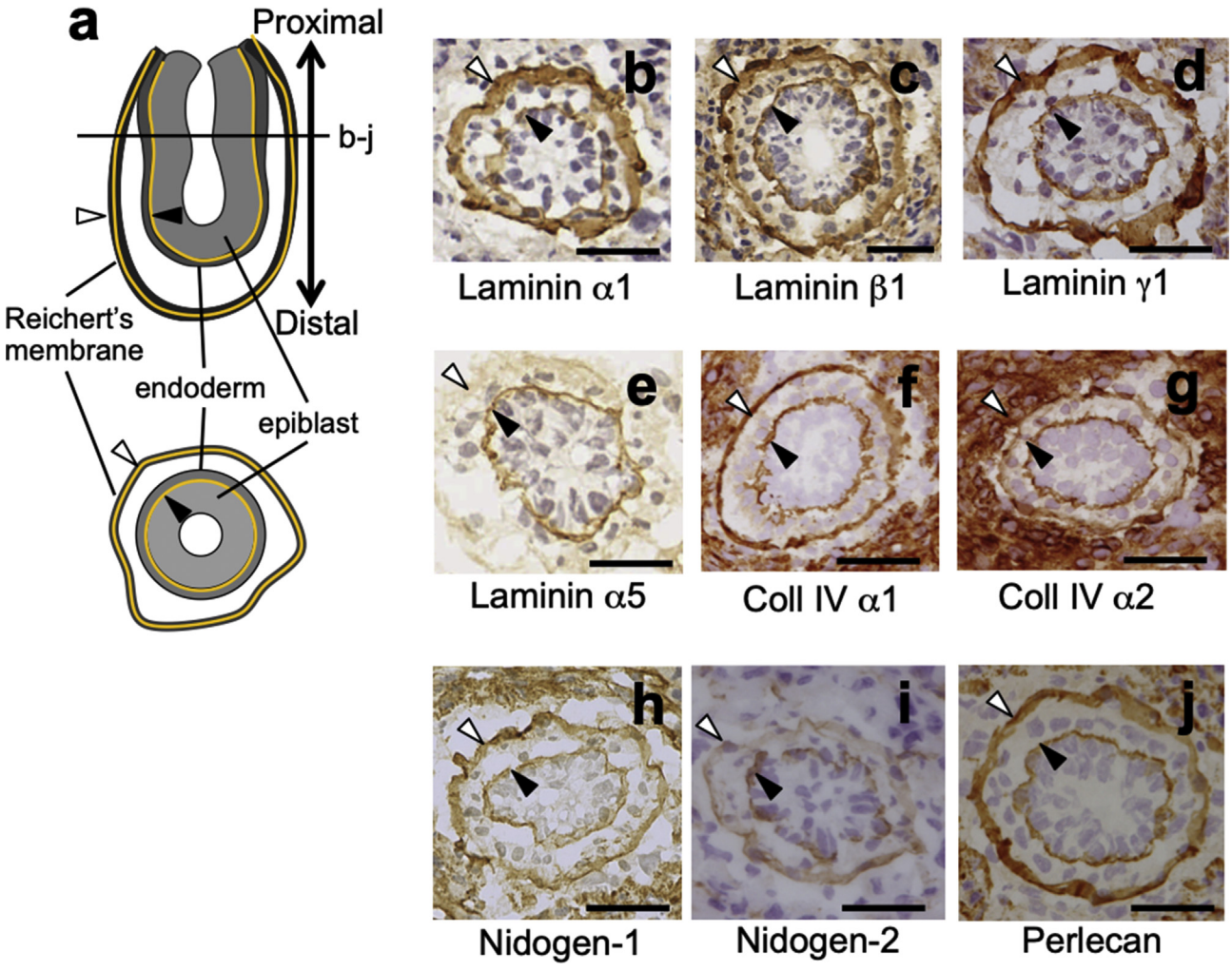

Fig. 1. Immunostaining of epiblast BMs and Reichert's membrane in E5.5 embryos. (a) Schematic diagram of an E5.5 mouse embryo sectioned vertically (upper panel) and horizontally (lower panel). The line marked "b–j" indicates the position at which the E5.5 embryos were sectioned for the immunostaining shown in panels (b–j). The yellow lines indicate BMs. In the lower panel, the inner circle (black arrowhead) is the embryonic BM and the outer layer (white arrowhead) is Reichert's membrane (b–j) Representative images of immunostaining for laminin subunits α1 (b), β1 (c), γ1 (d), and α5 (e), type IV collagen (Coll IV) α1 (f) and α2 (g), nidogen-1 (h) and −2 (i), and perlecan (j). The black and white arrowheads in (b–j) indicate the embryonic BM and Reichert's membrane, respectively. Note that laminin α5 (e) is clearly detected in the embryonic BM, but hardly present in Reichert's membrane. Scale bars: 50 μm.

dehydrogenase (GAPDH). The relative expression levels were indicated with the highest level in the analyzed stages set at 100.

3. Results

3.1. Localization of BM proteins in epiblast stage embryos (E5.5)

We performed a panel of immunohistochemical analyses for the 20 BM proteins (Table 1) at three post-implantation stages of early mouse embryos (E5.5, E6.5, and E7.5). The E5.5 embryo has the appearance of an elongated sac with a bilayer of cells (Fig. 1a). The inner cell layer, the epiblast, surrounds a blastocoel cavity. The outer cell layer is the endoderm and is separated from the epiblast layer by an embryonic BM. In a transverse section, two layers of BMs are observed in the E5.5 embryo. The inner layer is the embryonic BM (Fig. 1a, black arrowheads), and the outer layer is Reichert's membrane (Fig. 1a, white arrowheads), an extraembryonic membrane composed of BM molecules and parietal endoderm cells.

Nine proteins comprising laminin α1, β1, γ1, and α5, type IV collagen α1 and α2, nidogen-1 and -2, and perlecan were detected in the embryonic BM (Fig. 1b–j, black arrowheads). Most of them were also positive in Reichert's membrane (white arrowheads) except for laminin α5 that was hardly detectable in Reichert's

Table 3
Summary of immunohistochemical staining intensities of 20 BM proteins in early embryos.

	Embryonic			Extra-embryonic	
	E5.5	E6.5	E7.5	E7.5	
	Epiblast/Ectoderm			Amnion	Chorion
Laminin α5	++	++	++	++	++
Laminin β1	++	++	++	++	++
Laminin γ1	++	++	++	++	++
Collagen(IV) α1	++	++	++	++	++
Collagen(IV) α2	++	++	++	++	++
Nidogen-1	++	++	+	+	++
Nidogen-2	++	++	++	++	++
Perlecan	++	++	++	++	++
Laminin α1	+	+/-	+/-	-	+
Laminin α3	-	-	-	+	+
Laminin α4	-	-	-	(+)	(+)
Laminin β2	-	-	-	-	+/-
Laminin α2	-	-	-	-	-
Laminin β3	-	-	-	-	-
Laminin γ2	-	-	-	-	-
Laminin γ3	-	-	-	-	-
Collagen(IV) α3	-	-	-	-	-
Collagen(IV) α4	-	-	-	-	-
Collagen(IV) α5	-	-	-	-	-
Collagen(IV) α6	-	-	-	-	-

++	positive, clear, continuous
+ , (+)	positive, faint, (partly or fragmented)
+/-	ambiguous
-	undetectable

membrane (Fig. 1e). These results indicate that the embryonic BM at the epiblast stage contains both laminin-111 and -511, while Reichert's membrane mainly contains laminin-111. The other subunits of laminin and type IV collagen were not detected in E5.5 embryos (Suppl. fig. 1). Table 3 summarizes the immunoreactivities of the 20 BM proteins in the epiblast and early ectodermal BMs.

3.2. BM composition in gastrulation stage embryos (E6.5): formation of the ectoderm, endoderm, and mesoderm

At E6.5, the posterior part of the embryonic BM (designated ectodermal BM hereafter) became fragmented and disappeared locally (Fig. 2b–f, arrows). Differentiating mesodermal cells appeared between the epiblast and endoderm layers (Fig. 2, asterisks).

The overall immunoreactivities of the 20 BM proteins in the ectodermal BM in E6.5 embryos were similar to those in E5.5 embryos, showing positivity for laminin α1, α5, β1, and γ1, type IV collagen α1 and α2, nidogen-1 and -2, and perlecan (Table 3). Representative images of laminin β1, γ1, α1, and α5, and perlecan are shown in Fig. 2. Notably, the immunostaining signals for laminin α1 in the ectodermal BM at E6.5 were less pronounced than those at E5.5 (Fig. 2d, black arrowhead), while the signals in Reichert's membrane were increased (Fig. 2d, white arrowhead). Laminin α5 was clearly detected in the ectodermal BM, but hardly present in Reichert's membrane (Fig. 2e). Immunostaining for laminin β1 and γ1 produced slightly higher signals in Reichert's membrane than in the ectodermal BM (Fig. 2b and c). These results indicate that the ectodermal BM at E6.5 mainly contains laminin-511 as a major laminin isoform, while Reichert's membrane contains laminin-111.

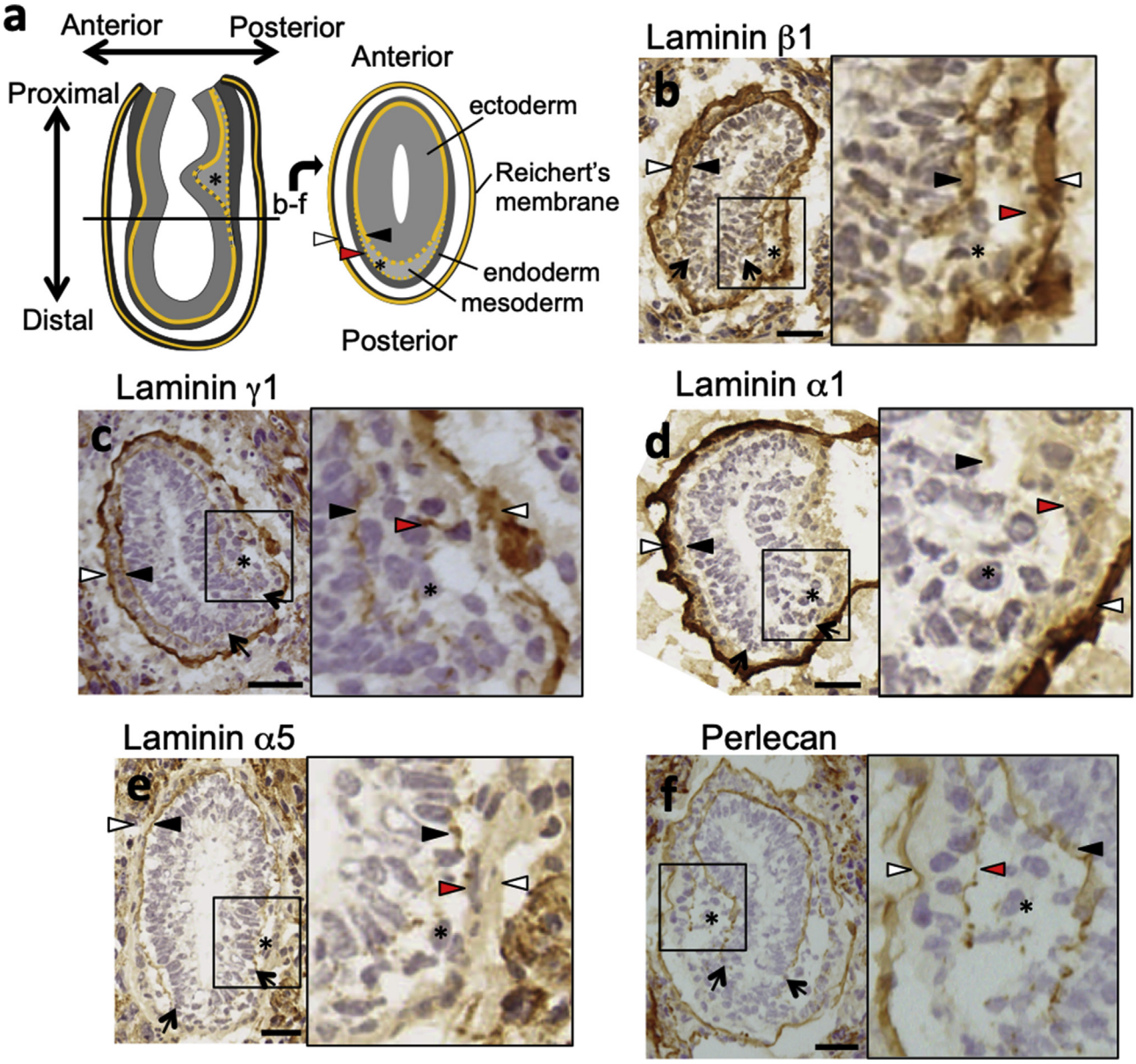

Fig. 2. Immunostaining of E6.5 embryos at the gastrulation stage. (a) Schematic diagram of an E6.5 mouse embryo sectioned vertically (left panel) and horizontally (right panel). (b–f) Horizontal sections of E6.5 embryos immunostained for laminin subunits β1 (b), γ1 (c), α1 (d), α5 (e), and perlecan (f). The arrows indicate the posterior region where the BMs break down. Posterior regions of the sections (squares) are magnified in the right panels. The asterisks indicate mesodermal cells migrating between the epiblast and endoderm layers. The ectodermal BM (black arrowheads) and Reichert's membrane (white arrowheads) are indicated. Scale bars: 50 μm.

As the formation of the mesoderm layer proceeded, the BM structure was detected with the endoderm layer. However, the endodermal BM was thin and often fragmented in E6.5 embryos. The magnified images in Fig. 2 indicate the endodermal BM (red arrowheads) and migrating mesoderm (asterisks), respectively. The components detected in the endodermal BM were similar to those in the ectodermal BM. In addition, the endodermal BM was found to contain fibronectin as described below.

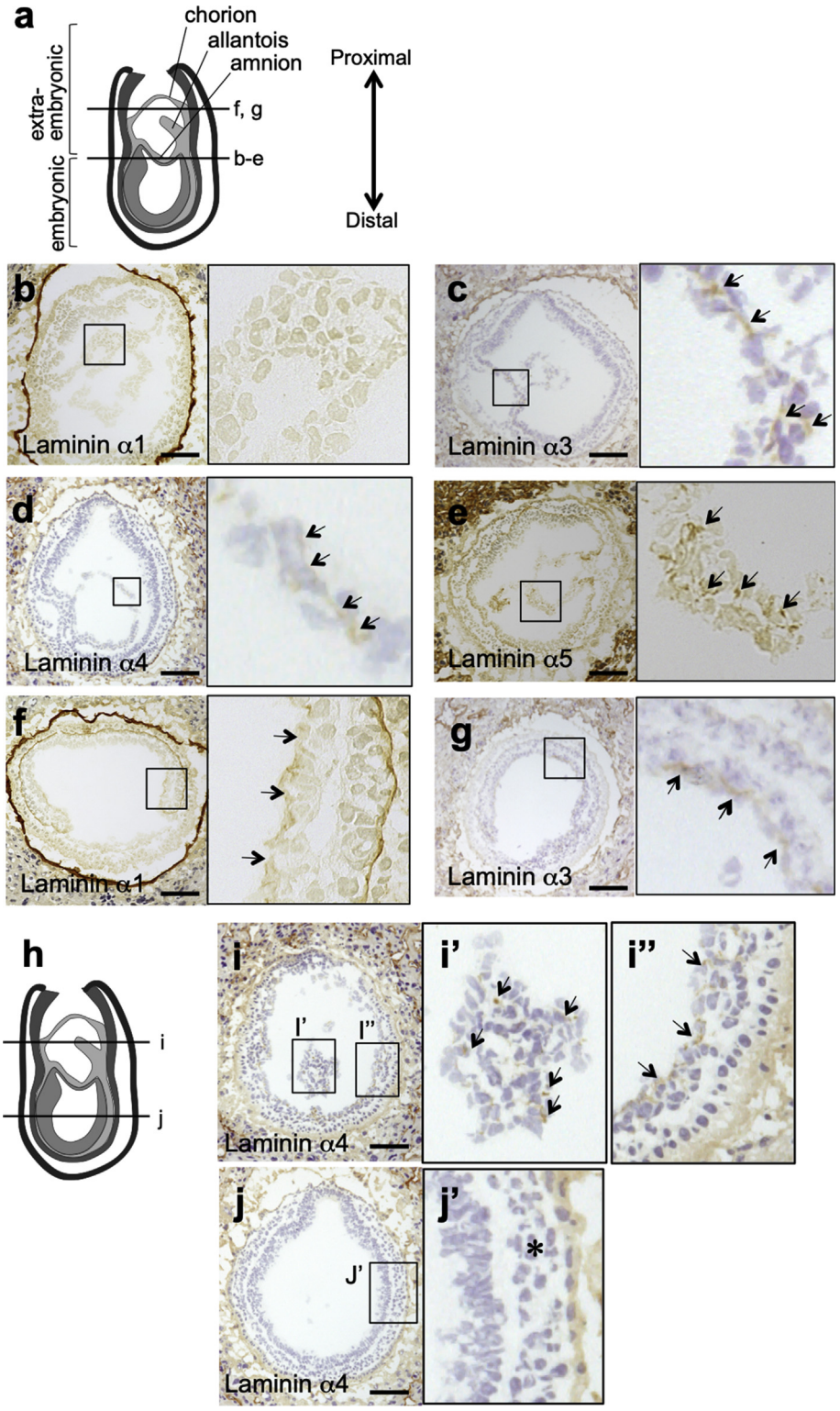

Fig. 3. Immunoreactive BM proteins in the extraembryonic region of E7.5 embryos. (a) Schematic diagram of an E7.5 embryo showing the extraembryonic structures and the positions of the sections shown in panels (b–g). (b–e) Immunostaining of laminin subunits in the amniotic BM (arrows) where laminin α1 is negative. (f, g) Immunostaining of laminin α1 and α3 in the chorionic BM (arrows). (h) Schematic diagram of an E7.5 embryo indicating the positions of the sections shown in panels (i–j). (i–j) Laminin α4 is detected in the extraembryonic mesoderm (i, arrows), but not in the embryonic region (j). The asterisk in (j') indicates the embryonic mesoderm. Scale bars: 100 μm.

3.3. Differentiation of the extraembryonic region in E7.5 and diversification of BM components

Extraembryonic structures such as the amnion, allantois, and chorion are formed by E7.5 (Fig. 3a). The amnion and chorion are composed of two cell layers separated by a BM. Laminin β1 and γ1, type IV collagen α1 and α2, nidogen-1 and -2, and perlecan were detected in the BMs of these extraembryonic structures, similar to the case for the ectodermal BM in the embryonic region (Table 3, data not shown). For the other laminin subunits, we found that the amniotic BM lacked laminin α1 (Fig. 3b) and was partially positive for laminin α3 and α4 (Fig. 3c and d). It also contained laminin α5 (Fig. 3e). The chorionic BM retained a faint laminin α1 signal (Fig. 3f) and was positive for laminin α3 (Fig. 3g). Laminin α4 was detected in the extraembryonic mesoderm (Fig. 3i), but not in the embryonic region (Fig. 3j), suggesting that laminin-311 and -411 were expressed in the extraembryonic tissues prior to their expression in the embryonic region.

In contrast to the extraembryonic regions, the complexity of the ectodermal BM at E7.5 was similar to those at the earlier stages (Table 3). Fig. 4 shows the immunostaining signals in the ectodermal BMs. The ectodermal BM at E7.5 contained laminin-511 as a major laminin isoform.

We further confirmed the differential localization of laminin α1 and α5 at E7.5 by whole-mount immunofluorescence staining (Fig. 5). Embryonic ectodermal cells were marked by Oct-3/4 immunostaining. Laminin α5 signals were detected in the BMs of both embryonic and extraembryonic regions, while laminin α1 signals were restricted to the proximal exocoelomic wall. These results indicate that the BMs in the embryonic region, especially the epiblast/ectodermal BMs, contain laminin-511 rather than laminin-111 as the major laminin isoform.

We also confirmed the relative expression levels of RNA transcripts of laminin α subunits from E6.5 to E16.5 (Fig. 6). Laminin α1 and α5 showed the highest expression levels at E6.5, while the other three α subunits were undetectable at E6.5, supporting the conclusion that the early embryonic BM is composed of laminin isoforms containing α1 and α5 subunits.

3.4. Ectodermal and endodermal cells interact with their underlying BMs through distinct integrins

The endodermal BM becomes distinguishable from the epiblast/ectodermal BMs as the mesoderm layer is formed between the ectoderm and the endoderm. As exemplified in Fig. 2, the immunostaining signals for BM proteins in the endodermal BM were weak and fragmented. We found that fibronectin, an ECM protein typically distributed in the mesenchymal matrix, was detected in the endodermal BM zone of gastrulating embryos (Fig. 7a and c). The fibronectin immunoreactivity was higher in the endodermal BM than in the ectodermal BM (Fig. 7a and c), while the perlecan signals were more pronounced in the ectodermal BM compared

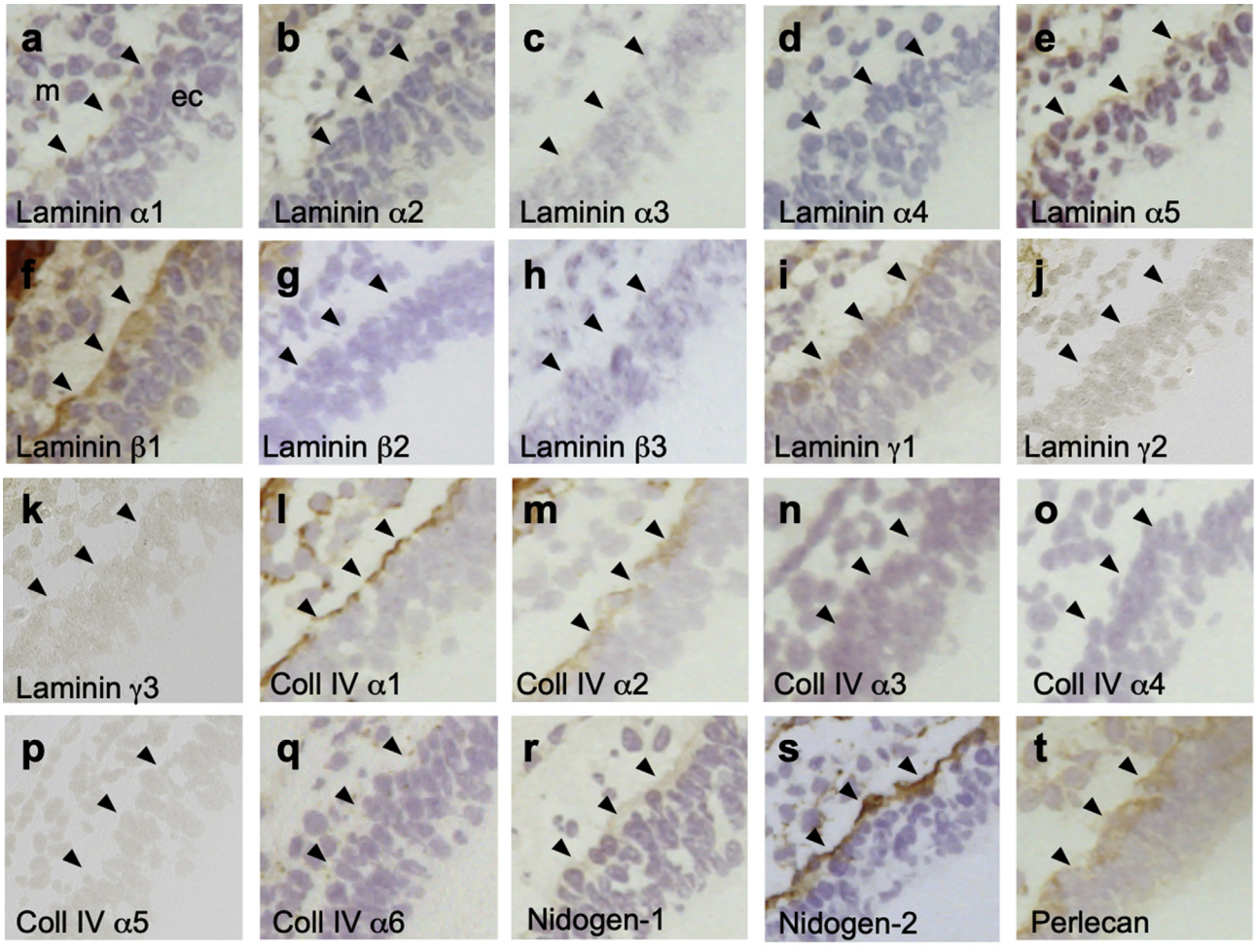

Fig. 4. Immunostaining for BM proteins in the ectodermal BMs of E7.5 embryos. The arrowheads indicate the positions of the ectodermal BMs. The positions of the ectoderm (ec) and mesoderm (m) are indicated in (a). Scale bar: 50 μm.

with the endodermal BM (Fig. 7b and c). These findings suggest that the endoderm and ectoderm cells are differentially attached to their extracellular environments through distinct adhesive components.

The differential localizations of fibronectin and BM proteins between the ectoderm and endoderm prompted us to investigate the expression of integrins that bind to fibronectin (integrin α5β1) and laminin-511 (integrin α6β1) (Fig. 8). Integrin α5 was selectively expressed at the basolateral surface of the endodermal cells in the E6.5 embryo, while integrin α6 was only detected in the ectodermal cells (Fig. 8a and b). These results were consistent with the differential expression of fibronectin and laminin-511 in the two cell layers, indicating that the ectoderm cells preferentially interact with laminin-511 in the ectodermal BM through integrin α6β1 and the endoderm cells interact with fibronectin in the endodermal BM via integrin α5β1.

4. Discussion

The epiblast BM is the fundamental microenvironment for the establishment and maintenance of pluripotent epiblast cells. We found that laminin α5, rather than α1, was the primary laminin α subunit in the epiblast/ectodermal BMs of early post-implantation stage embryos. Expression of laminin α1 and α5 in early embryonic BMs was reported previously [18,19]. Consistent with our observations, Miner et al. [17] reported that laminin α5 was the major laminin α subunit comprising the embryonic BM. Because laminin β1 and γ1 were continuously detected in the epiblast/ectodermal BMs, it is conceivable that laminin-511 consisting of α5, β1, and γ1 subunits is the major isoform in the epiblast/ectoderm BMs. Although laminin-111 has often been considered a major laminin in the early embryonic BM, the laminin α1 signals became weaker in the epiblast/ectodermal BMs and were mainly detected in Reichert's membrane.

Our results show that the molecular composition of the epiblast/ectodermal BMs is rather simple and remains unaltered during post-implantation developmental stages until E7.5. Thus, laminin α5, β1, and γ1, type IV collagen α1 and α2, nidogen-1 and -2, and perlecan were detected in the epiblast/ectodermal BMs in all stages examined. Local differences in BM composition were not detected in the epiblast/ectodermal BMs, suggesting that the contents of the epiblast/ectodermal BMs remain constant throughout the early

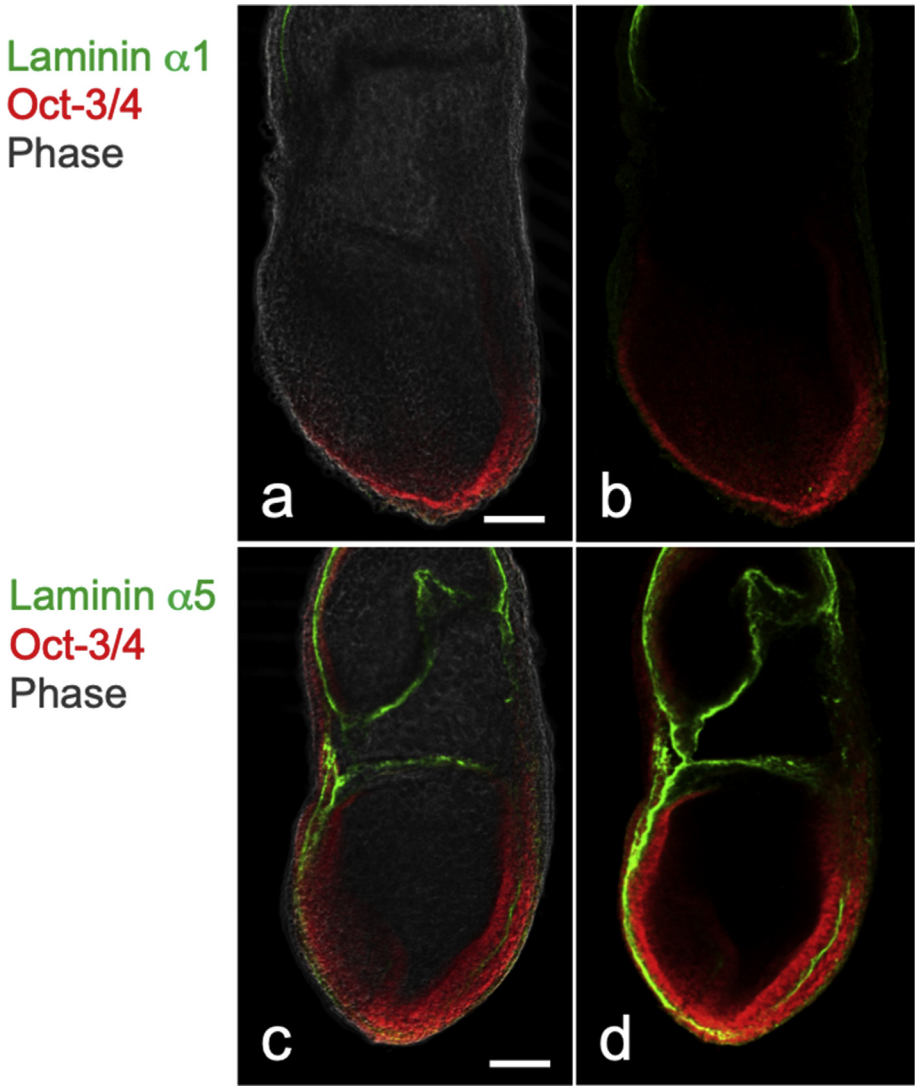

Fig. 5. Differential localization of laminin α1 and α5 in E7.5 embryos. Whole-mount immunostaining of E7.5 embryos for laminin α1 (a and b, green) and laminin α5 (c and d, green). Embryonic regions are labeled with an antibody against Oct-3/4 (red). Images of the whole embryos were taken by confocal microscopy. (a, c) Single optical planes of the confocal microscopy images merged with phase-contrast images. (b, d) Projection images of five optical planes of the confocal microscopy images. Scale bars: 50 μm.

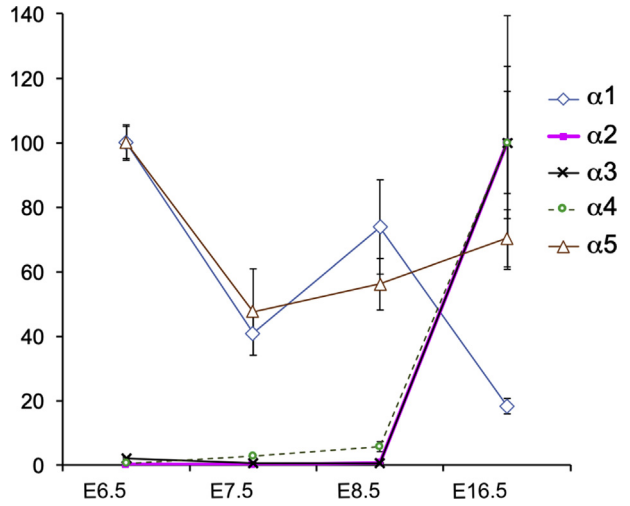

Fig. 6. Developmental changes in RNA transcript levels of laminin α subunits. The expression levels of RNA transcripts for five laminin α subunits (α1 to α5) in E6.5, E7.5, and E8.5 embryos were quantified by RT-PCR. The transcript levels were normalized by those of GAPDH, and are presented with the highest value set at 100 for each gene. The levels at E16.5 were included as a reference. Laminins α1 and α5 show their highest expression at E6.5, while Laminins α2, α3, and α4 are highest at E16.5 and hardly detected in the early stages.

post-implantation stages. Taken together, the obtained results suggest that the BMs of the epiblast and ectoderm are composed of a common toolkit comprising laminin-511, type IV collagen ($\alpha1_2\alpha2$ [IV]), nidogen-1 and -2, and perlecan.

Despite the restricted composition of the epiblast/ectodermal BMs, the BM complexity increased in the extraembryonic regions between E6.5 and E7.5. Our results showed that laminin α3 and α4 subunits, which were undetectable in the embryonic region, were detected in the amnion, allantois, and chorion. Because β1 and γ1 were the dominant laminin β and γ subunits detected in these extraembryonic regions, it is conceivable that laminin α3 and α4 expressed in these regions comprise laminin-311 and laminin-411, the latter of which may well localize in the region of early vasculogenesis [20]. In addition, these extraembryonic structures were located proximal to the ectoplacental cone where the embryo attaches to the uterine wall. The maternal tissue and/or ectoplacental cone may have an impact on the differentiation of extraembryonic cells as well as the diversification of BM components. The other laminin and type IV collagen subunits were undetectable in these early stages and appeared in later stages from E8.5 to E10.5, i.e., early organogenic stages (unpublished observations).

Stem cells require a specialized environment, so-called niche, to maintain their stemness. In early embryos, the epiblast maintains pluripotency and stands on the epiblast BM composed of the fundamental BM toolkit including laminin-511. The interactions of cells with BMs are mediated by cell surface receptors, particularly integrins. Integrin-mediated adhesive interactions of cells with BMs are a prerequisite for establishment and maintenance of the epiblast. Lack of either laminin γ1 or integrin β1 resulted in failure of epiblast differentiation *in vitro* and early embryonic lethality *in vivo* [4,6,21–23]. Moreover, ablation of laminin–integrin interactions by replacement of a single amino acid in laminin γ1 critical for integrin binding also caused early post-implantation lethality [5]. We found that integrin α6, which comprises the laminin-binding integrin α6β1, was selectively expressed in the ectoderm, but not the endoderm, of E6.5 embryos. These results strongly suggest that the epiblast/ectoderm cells interact with laminin-511 in their BMs through integrin α6β1. In line with the *in vivo* situation, pluripotent stem cells including multiple lines of human embryonic stem cells and induced pluripotent stem (hiPS) cells express integrin α6 as a major integrin species and can be stably passaged on recombinant laminin-511 without losing their pluripotency [24,25]. A recombinant laminin-511 fragment retaining the full integrin-binding activity has also been widely used for culture of hiPS cells under feeder-free and xeno-free conditions [26,27].

In contrast to the integrin α6 expression in the ectodermal cells, the endodermal cells at E6.5 predominantly expressed integrin α5. We also found that fibronectin was localized in the BM zone of the endoderm rather than in the ectoderm, suggesting that the endodermal cells utilize fibronectin as their substrate via integrin α5β1. Liu et al. [27] reported that endodermal cells derived from mouse embryonic stem cells spread on fibronectin and formed typical focal adhesions, a complex of cell adhesion molecules including integrin α5β1. Given these results, fibronectin appears to be a favored substrate for differentiation and maintenance of early endodermal cells *in vitro*.

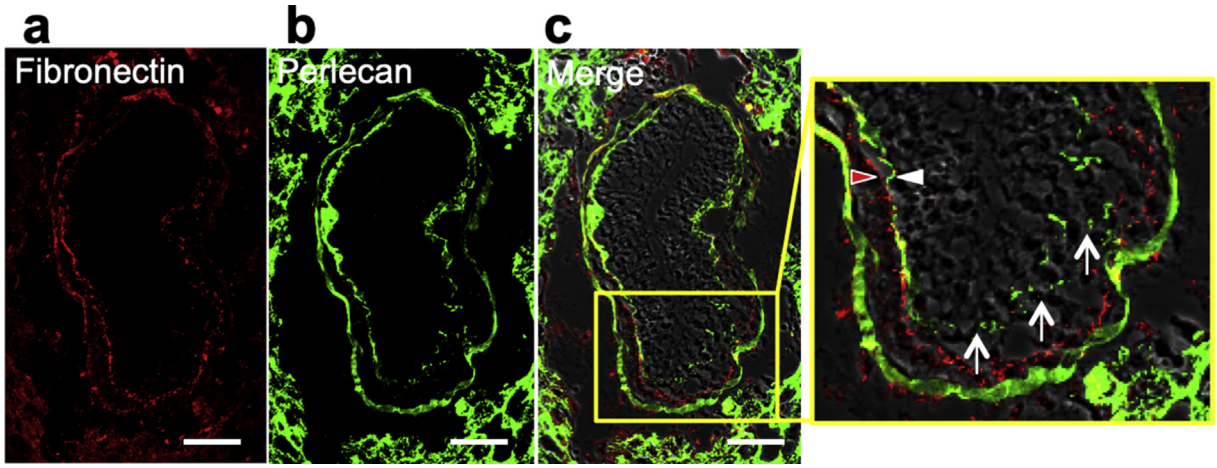

Fig. 7. Distinct BM compositions between the ectoderm and endoderm during gastrulation. (a, b) Horizontal sections of an E6.5 embryo immunostained for fibronectin (a) and perlecan (b). Panel (c) shows the merged image. A magnified view of the boxed area is shown on the right. Perlecan and fibronectin were separately detected in the posterior region in (c). The arrows indicate breakdown of the ectodermal BM in the posterior gastrulation region. The white and red arrowheads indicate the ectodermal and endodermal BMs, respectively. Scale bars: 50 μm.

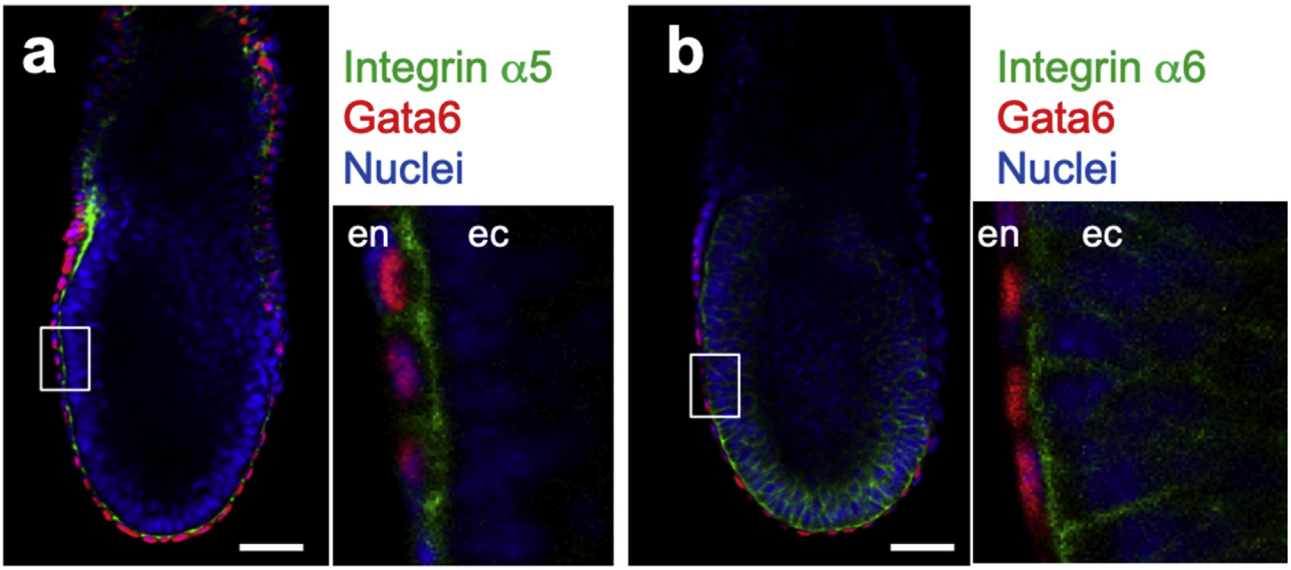

Fig. 8. Distinctive expression of integrins in the ectoderm and endoderm. (a, b) Confocal microscopic images of whole-mount immunohistochemical staining of E6.5 embryos for integrin α5 (a) and α6 (b). Gata6 immunostaining labels the endodermal cell nuclei. en: endoderm; ec: ectoderm. Scale bars: 50 μm.

In summary, our comprehensive profiling of embryonic BMs revealed that the BMs underlying the epiblast/ectoderm are comprised of a restricted repertoire of BM proteins including laminin-511, type IV collagen ($\alpha1_2\alpha2$ [IV]), nidogen-1 and -2, and perlecan, which define a toolkit for embryonic BMs. The dominant expression of laminin-511 in the epiblast/ectoderm BMs provides a physiological basis for the utility of laminin-511 and its functionally active fragment as culture substrates that support efficient expansion of human pluripotent stem cells. Further detailed profiling of BMs in organogenic stages and adult tissue stem cells will provide information that facilitates better understanding of the environmental requirements for stem cell differentiation and maintenance in their niche.

Declarations of interest

K. Sekiguchi is a cofounder and shareholder of matrixome Inc. The other authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

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References

[1] Pozzi A, Yurchenco PD, Iozzo RV. The nature and biology of basement membranes. Matrix Biol 2017;57–58:1–11.
[2] Yurchenco PD. Basement membranes: cell scaffoldings and signaling platforms. In: Yurchenco PD, editor. Extracellular matrix Biology. Cold Spring Harbor Laboratory Press; 2011.
[3] Nishiuchi R, Takagi J, Hayashi M, Ido H, Yagi Y, Sanzen N, et al. Ligand-binding specificities of laminin-binding integrins: a comprehensive survey of laminin-integrin interactions using recombinant alpha3beta1, alpha6beta1, alpha7-beta1 and alpha6beta4 integrins. Matrix Biol 2006;25:189–97.
[4] Fassler R, Meyer M. Consequences of lack of beta 1 integrin gene expression in mice. Genes Dev 1995;9:1896–908.
[5] Kiyozumi D, Taniguchi Y, Nakano I, Toga J, Yagi E, Hasuwa H, et al. Laminin γ1 C-terminal Glu to Gln mutation induces early postimplantation lethality. Life Sci Alliance 2018;1:e201800064.
[6] Smyth N, Vatansever HS, Murray P, Meyer M, Frie C, Paulsson M, et al. Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. J Cell Biol 1999;144:151–60.
[7] Manabe R, Tsutsui K, Yamada T, Kimura M, Nakano I, Shimono C, et al. Transcriptome-based systematic identification of extracellular matrix proteins. Proc Natl Acad Sci U S A 2008;105:12849–54.
[8] The life science database archive The matrixome project, mouse basement membrane bodymap. National Bioscience Database Center (NBDC); 2008. http://dbarchive.biosciencedbc.jp/archive/matrixome/bm/home.html.
[9] Aumailley M, Bruckner-Tuderman L, Carter WG, Deutzmann R, Edgar D, Ekblom P, et al. A simplified laminin nomenclature. Matrix Biol 2005;24:326–32.
[10] Hohenester E, Yurchenco PD. Laminins in basement membrane assembly. Cell Adhes Migrat 2013;7:56–63.
[11] Khoshnoodi J, Pedchenko V, Hudson BG. Mammalian collagen IV. Microsc Res Tech 2008;71:357–70.
[12] Breitkreutz D, Koxholt I, Thiemann K, Nischt R. Skin basement membrane: the foundation of epidermal integrity–BM functions and diverse roles of bridging molecules nidogen and perlecan. BioMed Res Int 2013;2013:179784.
[13] Abari E, Kociok N, Hartmann U, Semkova I, Paulsson M, Lo A, et al. Alterations in basement membrane immunoreactivity of the diabetic retina in three diabetic mouse models. Graefes Arch Clin Exp Ophthalmol 2013;251:763–75.
[14] Kaufman MH. The atlas of mouse development. Academic Press; 1992.
[15] Theiler K. The house mouse. Springer-Verlag Berlin Heidelberg; 1989.
[16] Sado Y, Kagawa M, Kishiro Y, Sugihara K, Naito I, Seyer JM, et al. Establishment by the rat lymph node method of epitope-defined monoclonal antibodies recognizing the six different alpha chains of human type IV collagen. Histochem Cell Biol 1995;104:267–75.
[17] Kozaki T, Matsui Y, Gu J, Nishiuchi R, Sugiura N, Kimata K, et al. Recombinant expression and characterization of a novel fibronectin isoform expressed in cartilaginous tissues. J Biol Chem 2003;278:50546–53.
[18] Miner JH, Li C, Mudd JL, Go G, Sutherland AE. Compositional and structural requirements for laminin and basement membranes during mouse embryo implantation and gastrulation. Development 2004;131:2247–56.
[19] Gersdorff N, Muller M, Otto S, Poschadel R, Hubner S, Miosge N. Basement membrane composition in the early mouse embryo day 7. Dev Dynam 2005;233:1140–8.
[20] Kinder SJ, Loebel DA, Tam PP. Allocation and early differentiation of cardiovascular progenitors in the mouse embryo. Trends Cardiovasc Med 2001;11:177–84.
[21] Fassler R, Pfaff M, Murphy J, Noegel AA, Johansson S, Timpl R, et al. Lack of beta 1 integrin gene in embryonic stem cells affects morphology, adhesion, and migration but not integration into the inner cell mass of blastocysts. J Cell Biol 1995;128:979–88.

[22] Fujiwara H, Hayashi Y, Sanzen N, Kobayashi R, Weber CN, Emoto T, et al. Regulation of mesodermal differentiation of mouse embryonic stem cells by basement membranes. J Biol Chem 2007;282:29701–11.
[23] Murray P, Edgar D. Regulation of programmed cell death by basement membranes in embryonic development. J Cell Biol 2000;150:1215–21.
[24] Miyazaki T, Futaki S, Hasegawa K, Kawasaki M, Sanzen N, Hayashi M, et al. Recombinant human laminin isoforms can support the undifferentiated growth of human embryonic stem cells. Biochem Biophys Res Commun 2008;375:27–32.
[25] Rodin S, Domogatskaya A, Strom S, Hansson EM, Chien KR, Inzunza J, et al. Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. Nat Biotechnol 2010;28:611–5.
[26] Miyazaki T, Futaki S, Suemori H, Taniguchi Y, Yamada M, Kawasaki M, et al. Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells. Nat Commun 2012;3:1236.
[27] Nakagawa M, Taniguchi Y, Senda S, Takizawa N, Ichisaka T, Asano K, et al. A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. Sci Rep 2014;4:3594.