Laminin α5 influences the architecture of the mouse small intestine mucosa

Zhen X. Mahoney1, Thaddeus S. Stappenbeck 2 and Jeffrey H. Miner1,3,*

1Department of Internal Medicine/Renal Division, 2Department of Pathology and Immunology, and 3Department of Cell Biology and Physiology, Washington University School of Medicine, St Louis, MO 63110, USA

*Author for correspondence (e-mail: minerj@wustl.edu)

Accepted 13 May 2008
Journal of Cell Science 121, 2493-2502 Published by The Company of Biologists 2008
doi:10.1242/jcs.025528

Summary
The mammalian intestine displays two distinct patterns of mucosal organization. The small intestine contains mucosal epithelial invaginations (the crypts of Lieberkühn) that are continuous with evaginations (villi) into the lumen. The colon also contains crypts of Lieberkühn, but its epithelial surface is lined by flat surface cuffs. The epithelial cells of both organs communicate with the underlying mesenchyme through a basement membrane that is composed of a variety of extracellular matrix proteins, including members of the laminin family. The basement membranes of the small intestine and colon contain distinct laminin subtypes; notably, the villus basement membrane is rich in laminin α5. Here, we show that the diminution of laminin α5 in a mouse model led to a compensatory deposition of colonic laminins, which resulted in a transformation from a small intestinal to a colonic mucosal architecture. The alteration in mucosal architecture was associated with reduced levels of nuclear p27Kip1 – a cell-cycle regulator – and altered intestinal epithelial cell proliferation, migration and differentiation. Our results suggest that laminin α5 has a crucial role in establishing and maintaining the specific mucosal pattern of the mouse small intestine.

Introduction
The mouse and human gastrointestinal tubes are divided into stomach, small intestine and colon. In each of these organs, the inner mucosal surface is composed of a continuous simple epithelium that is in contact with both the lumen and the underlying mesenchymal components, including blood vessels, myofibroblasts and immune cells, which collectively form the lamina propria. At the base of the mucosa is a thin layer of smooth muscle, the muscularis propria. Each organ establishes a specific mucosal organization that is crucial to the function of that particular organ. For example, to increase its absorptive surface, the small intestine contains a multitude of finger-like structures, called villi, that project into the lumen. Villi are covered by an epithelium that is supplied by four to eight adjacent pit-like invaginations that are called crypts of Lieberkühn. By contrast, the absorptive load of the colonic mucosa is far less than that of the small intestine, and it contains no villi. Colonic crypts supply the relatively flat surface epithelium (Sancho et al., 2004). It is not well understood how the gastrointestinal tube develops and maintains specific mucosal patterns along the cephalocaudal axis.

The epithelium of the intestinal mucosa undergoes rapid and perpetual renewal throughout life (reviewed in Sancho et al., 2004; Radtke and Clevers, 2005). In the small intestine, epithelial stem cells reside near the base of crypts and give rise to epithelial progenitor cells (Potten et al., 1997; Booth and Potten, 2000). Epithelial progenitor cells are transit-amplifying cells, which exit the cell cycle at the junction of crypts and villi, and differentiate into four distinct types of cell. Three of these, the absorptive enterocytes, the mucus-secreting goblet cells and the hormone-secreting enteroendocrine cells, complete their differentiation as they migrate upwards onto adjacent villi in an ordered column. These three types of cell reach the villus tip in two to five days and undergo programmed cell death and sloughing into the intestinal lumen. By contrast, Paneth cells, the fourth type of cell, migrate downwards to the base of the crypts while completing terminal differentiation. The epithelium of the mouse colon is organized similarly, except that villi and Paneth cells are not present. Colonic epithelial cells complete terminal differentiation during migration through the upper crypt and onto the flat epithelial surface (Stappenbeck et al., 1998; Radtke and Clevers, 2005).

At the interface of the epithelium and lamina propria lies a thin sheet of specialized extracellular matrix, the basement membrane (BM). BMs are widely distributed in the body, surrounding all epithelia, endothelia, muscles, nerves, and fat cells (Kalluri, 2003). Besides a structural role in maintaining tissue integrity and compartmentalization, BMs also promote cell adhesion, survival, proliferation and differentiation, and serve as a highway for cell migration. Laminin, type IV collagen, nidogen and sulfated proteoglycans are the four main components of all BMs (Timpl, 1996; Timpl and Brown, 1996). Laminins are αβγ heterotrimeric proteins that form a family of five α-, four β- and three γ-chains that can assemble into at least 15 unique isoforms (Miner and Yurchenco, 2004). In vitro studies show that different laminin isoforms exhibit different properties in terms of regulating cell adhesion, proliferation, differentiation and migration (Vachon and Beaulieu, 1995; Siler et al., 2000; Pouliot et al., 2002; Turck et al., 2005). Distinct phenotypes from various laminin-chain knockout mice also indicate a functional divergence among laminins (Yurchenco et al., 2004). The distribution of laminin isoforms within tissues is tightly controlled and often developmentally regulated (Miner, 1998; Simon-Assmann et al., 1998; Lefebvre et al., 1999). The functional implications of such a wide and dynamic distribution of laminin isoforms in tissues have only begun to be explored.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/121/15/2493/DC1

Key words: Basement membrane, Small intestine, Intestinal epithelial cell, Lutheran (Bcam), p27Kip1 (Cdkn1b)
With regard to the intestine, a large number of studies over the last two decades using cell culture models have attempted to address the roles of individual extracellular matrix molecules in regulating intestinal cell behavior (Simon-Assmann et al., 2007). Although much important progress has been made, the lack of a bona fide BM and the correct cellular and vascular microenvironments in most cases leads to questions regarding applicability to the in vivo situation. Here, we report the generation of mutant mice that lack the laminin α5 (LAMA5) chain and, thus, the laminin α5β1γ1 trimer (LM-511 in the new nomenclature) (Aumailley et al., 2005), in the intestinal subepithelial BM in vivo. This resulted in a shift of the laminins in the villus BM to the colonic-type isoforms (LM-111 and LM-411), which caused a colonic transformation of the mutant small intestine. These data reveal that the specific combinations of laminin isoforms that are deposited in the subepithelial BM contribute to patterning the intestinal tube into small intestine and colon. We further demonstrate that laminins are instructive for pattern formation by influencing intestinal epithelial cell behavior, probably through p27Kip1 (also known as Cdkn1b), a cell-cycle regulator that is also involved in regulating differentiation, migration and apoptosis.

Results

A laminin α5 transgene widely expressed during embryonic development is silenced in adult mouse small intestine mucosa

Mice that lack laminin α5 (Lama5−/− mice) die in late gestation, exhibiting growth retardation and multiple developmental defects (Miner et al., 1998; Miner and Li, 2000; Nguyen et al., 2002; Bolcato-Bellmin et al., 2003; Fukushima et al., 2006; Rebustini et al., 2007). Defective placental vascularization is probably the defect that causes embryonic lethality (Miner et al., 1998), which has hindered further study of the roles of laminin α5 in vivo beyond fetal development. Previously we produced two independent lines of transgenic mice expressing a full-length mouse laminin α5 transgene (called Mr5; see Fig. 1A). Miw, the regulatory element used, contains a Rous sarcoma virus long terminal repeat inserted into the chicken β-actin promoter, a combination that is widely active in transgenic mice (Suemori et al., 1990). The Mr5 transgene in both lines directs widespread deposition of full-length laminin α5 in BMs such that transgene-derived protein rescues all known developmental defects of Lama5−/− mice (Kikkawa and Miner, 2006). Thus, during embryonic development, transgene-derived laminin α5 accumulates in BMs wherever it is necessary, and it is functional, such that Lama5-knockout–Mr5-transgene (hereafter referred to as KO/Tg) mice are viable and fertile.

While surveying the deposition pattern of Mr5-derived protein in postnatal KO/Tg animals, we discovered that levels were low in the small intestine, kidney and lung (Fig. 1, and data not shown). In contrast to the abundant laminin α5 staining in control Lama5+/−/−; Mr5 (hereafter referred to as Het/Tg) mice, which are phenotypically indistinguishable from wild-type mice (supplementary material Fig. S1 and data not shown), α5 levels were greatly reduced in the villus subepithelial BM throughout the length of the KO/Tg small intestine. The reduction was first obvious in mice that were around 1 month old, and most of the laminin α5 was lost in mice that were over 3 months old (n=32) (Fig. 1D-I). This reduction was confirmed by three additional antibodies that recognize distinct epitopes along the α5 protein (data not shown). Interestingly, KO/Tg small intestines did contain laminin α5 in mesenchymal structures within the villi and in the intestinal smooth muscle at all ages (Fig. 1D-I).

The observed reduction in laminin α5 levels was consistent in more than 40 KO/Tg mice examined from both independent lines. Furthermore, in an unrelated transgenic line in which fatty acid transport protein 4 was expressed under the same Miw regulatory element that is present in Mr5, transgene-derived protein was also greatly reduced in the adult small intestine (data not shown) (Moulson et al., 2007). To investigate whether laminin α5 was reduced at the mRNA level, we performed quantitative real-time reverse transcriptase (RT)-PCR and found that laminin α5 mRNA was greatly diminished in KO/Tg intestinal epithelial cells and mucosa (Table 1). Together, these data demonstrate that after embryonic development, the expression of the Mr5 transgene wanes, leading to the eventual loss of α5 from the intestinal epithelial BM.

Laminin α5 is required for both establishing and maintaining small intestine crypt-villus architecture

The reduction of laminin α5 in the subepithelial BM during postnatal life led to dramatic changes in the architecture of the small intestine mucosa. Whole-mount analysis of Het/Tg and KO/Tg small intestine showed that the normally slender, finger-shaped individual villi (Fig. 2A) appeared to coalesce to varying degrees in the KO/Tg small intestine. These alterations included what appears to be simple fusion of adjacent villi (Fig. 2B), fusion of multiple consecutive villi in a ‘cerebroid’ pattern (Fig. 2C), and complete loss of individual villi in a ‘mosaic’ pattern (Fig. 2D). The latter strongly resembles the surface of the mouse colon (Fig. 3H). This abnormal mucosal structure was also apparent by scanning electron microscopy (Fig. 2E-G).
The villus coalescence first appeared in the distal small intestine, but spread to proximal and middle small intestine segments in mice of advanced age (supplementary material Fig. S2). No apparent changes in the colon were observed (Fig. S2), despite the fact that laminin α5 was also reduced in KO/Tg colon with concomitant upregulation of laminin α1 (supplementary material Fig. S1). It is not clear why the distal small intestine suffered more severe villus fusion compared with the more proximal parts, despite the fact that laminin α5 levels were greatly reduced throughout the length of the KO/Tg small intestine (Fig. 1). In the following studies we chose to focus on the distal small intestine, as it displayed an early-onset and consistent phenotype. In the distal small intestine, signs of villus coalescence were first observed around 2 months of age (four out of five mice), and a more advanced stage of villus coalescence (the ‘cerebroid’ pattern) occurred in mice at least 9 months of age (23 out of 23 mice). At the same age, female mice tended to exhibit a more severe phenotype than male mice. All mice were maintained on a mixed 129-C57BL/6J-CBA/J background.

To gain a better understanding of the basis for villus coalescence, we carefully obtained histological sections perpendicular (Fig. 3A-D) or parallel (Fig. 3E,F) to the intestinal muscle wall. The histology of KO/Tg sections correlated with the findings in whole-mount views and, in addition, revealed extended crypts between joined villi (Fig. 3B,C,F). In severe cases of villus coalescence, the deep-crypt, flat surface epithelium architecture in KO/Tg distal small intestine (Fig. 3C) largely resembled a colon (Fig. 3D). Thus, following the reduction of laminin α5 levels, the KO/Tg small intestine gradually loses the crypt-villus architecture of a normal small intestine and adopts a colon-like structure. Our data suggest that laminin α5 is required for the maintenance of a normal small intestine pattern in adult mice. One point worth noting here is that because villus coalescence seems to occur in a linear zigzag fashion (Fig. 2C,F; supplementary material Fig. S2), in two-dimensional sections perpendicular to the intestinal wall, the majority of villi appear to be un-fused, even though in three-dimensional whole-mount views the majority of villi are fused.

We next asked whether laminin α5 is also required to establish a normal small intestine pattern, rather than to merely maintain it. To address this question, we studied embryonically lethal Lama5–/– mice. To overcome the problem that small intestine crypt-villus structure is not established until 1 month after birth, we grafted intestines from Lama5–/– and control embryos under the dorsal skin of nude mice. After 1 month, control small intestine grafts developed a typical crypt-villus architecture (Fig. 3I). However, the Lama5–/– small intestine graft developed an abnormal crypt-villus architecture (Fig. 3J) and, in severe cases (Fig. 3K), resembled a grafted normal colon (Fig. 3L). Combining the data from both Lama5–/– mice and KO/Tg mice, we conclude that laminin α5 is required for both the establishment and maintenance of small intestine crypt-villus architecture.

KO/Tg distal small intestine undergoes colonic transformation

The architectural resemblance between the distal small intestine of KO/Tg mice and the colon of wild-type mice prompted us to

### Table 1. Lama5 mRNA levels are reduced in the small intestine of KO/Tg mice

| Age (in months) | RNA source               | Het/Tg ΔCT (± s.d.) | KO/Tg ΔCT (± s.d.) | Average fold change (range) |
|-----------------|--------------------------|---------------------|--------------------|-----------------------------|
| 1               | Scraped mucosa           | 11.03±0.16          | 13.54±0.13         | −5.7 (−5.2 to −6.2)         |
| 6               | Isolated intestinal epithelial cells | 12.35±0.12 | 19.66±0.16         | −158.7 (−142.0 to −177.3) |
| 10              | Isolated intestinal epithelial cells | 12.01±0.31 | 19.05±0.18         | −131.6 (−116.2 to −149.1) |

Fig. 2. Villus coalescence in adult KO/Tg distal small intestine. (A-D) Whole-mount views of distal small intestine mucosa. Compared with the villi of Het/Tg mice (A), the KO/Tg villi (B-D) showed varying degrees of villus coalescence, from a widened phenotype (B) to a ‘cerebroid’ pattern (C) to a ‘mosaic’ pattern (D). (E-G) Scanning electron micrographs confirmed the findings in (A-D). Bars, 200 μm.

Fig. 3. Crypt-villus architecture is disrupted in adult KO/Tg distal small intestine. (A-D) H&E-stained sections of distal small intestines. KO/Tg showed varying degrees of loss of normal crypt-villus architecture (A-C) and resembled colon (D). (E, F) Villus coalescence was clearly seen in cross sections. Note the crypt-like structures trapped in the center (arrow in F). The approximate positions of cross-sectioning are indicated by dashed lines in A and C. (G,H) Whole-mount views of intestine mucosa. KO/Tg small intestine (G) develops local flat epithelial surfaces (boxed area) with visible crypt mouths (arrow) that were also observed in normal colon (arrow and boxed area in H). (I-L) H&E stained sections of intestinal grafts. Lama5–/– small intestine grafts (J,K) failed to develop a normal crypt-villus architecture (I) but instead presented a colon-like architecture (L). Bars, 100 μm.
determine whether there was any additional evidence that this alteration was a transformation of one tissue type (small intestine) into another (colon). To do this we examined the subtypes of mucin secreted by goblet cells. A transition in mucin subtypes has been used in diagnosing colonic transformation in patients (Mimura et al., 1999; Di Tonno et al., 2001). In mice, sulfomucin-containing goblet cells are predominant in the small intestine, whereas those containing sialomucin are only found in the colon (Deplancke et al., 2000). These two subtypes can be readily distinguished by high iron diamine staining, which stains sialomucin blue and sulfomucin brown. We observed a substantial number of sialomucin-containing goblet cells in the KO/Tg distal small intestine, even in areas without the dramatic crypt-villus architecture changes noted above \( (n=4) \) (Fig. 4A-C).

Transmission electron microscopic analysis revealed additional evidence of colonic transformation. Goblet cells in the normal Het/Tg small intestine contained small (1.2 μm diameter on average; 20 granules from two mice were measured) electron-dense granules (Fig. 4D), whereas goblet cells in the KO/Tg small intestine contained larger (2.2 μm diameter on average; 20 granules from two mice were measured), more electron-lucent granules (Fig. 4E). The latter goblet-cell granules are characteristic of those found in the normal mouse colon (Fig. 4F). Indeed, the differences in the expression of other related family members (Miner et al., 1998; Bolcato-Bellemin et al., 2003), we next assessed the deposition of laminin α-chains in the KO/Tg small intestine. In the control, the BM underlying the villus epithelium contained primarily laminin α5, with less detectable laminin α1 and no laminin α4 (Fig. 5A,C,E). In the absence of laminin α5, laminins-α1 and -α4 accumulated ectopically in the villus subepithelial BM (Fig. 5B,D,F). We did not observe any alterations in laminin composition in the crypt subepithelial BM (Fig. 5), and there were no apparent changes in laminin α2 and laminin α3 in the KO/Tg small intestine (data not shown). Therefore, given that laminin β1 is the major β-chain and laminin γ1 the major γ-chain in the intestinal subepithelial BM, the KO/Tg small intestine contains primarily laminin-111 (LM-111; α1β1γ1) (Aumailley et al., 2005) and LM-411 (α4β1γ1) rather than LM-511 in the villus subepithelial BM. Additional immunohistochemical studies demonstrated that nidogen-1, type IV collagen, and perlecan were deposited appropriately in the KO/Tg small intestine (data not shown). Ultrastructural analysis of the KO/Tg subepithelial BM did not reveal any differences from control (data not shown). Thus, increased expression of laminin α1 and/or laminin α4 are likely to compensate for the loss of laminin α5 by maintaining the integrity of the BM. Interestingly, similar laminin

![Image](63x144 to 281x338)

**Fig. 4.** KO/Tg distal small intestines lose small intestine features and exhibit characteristics of colon. (A-C) High iron diamine staining of intestine. Most control distal small intestine goblet cells contained sulfomucin (dark brown in A), whereas most KO/Tg distal small intestine goblet cells contained sialomucin (blue in B), which is characteristic of colonic goblet cells (blue in C). (D-F) Transmission electron micrographs of goblet cells. The goblet cell in the KO/Tg distal small intestine (E) and wild-type colon (F) are similar; both contain larger and more electron lucent granules than those in goblet cells from normal distal small intestine (D). Bars, 50 μm (A-C); 4 μm (D-F).

![Image](316x216 to 565x458)

**Fig. 5.** The laminin composition of KO/Tg small intestine subepithelial BMs resembles that of the normal colon. Intestine sections were stained with antiserum directed against laminin α-chains, as indicated. (A) Laminin α5 was detected in the control subepithelial BM of villi but not of crypts. (B) Laminin α5 staining was greatly reduced in villus BM (arrow) of KO/Tg mice but was detectable in both the mesenchymal structures within villi and the intestinal smooth muscle wall. (C) Low levels of laminin α1 were detected in the control subepithelial BMs of both villi and crypts. (D) Levels of α1 were increased in KO/Tg villus BM. (E) In controls, high levels of laminin α4 were detected in the endothelial BM of blood vessels, but not in the villus subepithelial BM (arrowhead in inset). (F) In KO/Tg, in addition to endothelial BM, laminin α4 was deposited in the villus subepithelial BM (arrowhead in inset). (G-L) Compared with WT distal small intestine, laminin α5 was only weakly deposited in the subepithelial BMs of WT colon (arrow in H), whereas laminins α1 and α4 were abundant. Arrowheads in the insets of K and L point to the position of epithelial BMs. Dashed horizontal lines indicate the crypt-villus boundary. Bars, 100 μm.
isoform compensation occurred in the Lama5<sup>−/−</sup> embryonic intestinal graft (supplementary material Fig. S3).

The colonic transformation of the KO/Tg small intestine led us to hypothesize that normal colonic BMs contain less LM-511 but more LM-111 and LM-411 as compared with small intestine villi. Indeed, compared with villus BMs, laminin α5 was expressed at very low levels in the colonic epithelial BMs, except for those that are immediately underneath the surface epithelium (Fig. 5G,H). In addition, by immunostaining, high levels of laminins α1 and α4 were detected in the colonic epithelial BM (Fig. 5I-L). This distribution is similar to the small intestinal villus BM of KO/Tg mice, but opposite to that observed in WT small intestine (Fig. 5). Thus, mouse small intestine and colon normally contain different laminins, and the laminin composition in KO/Tg small intestine has switched from a small intestinal type (primarily LM-511 and LM-411) to a colonic type (primarily LM-111 and LM-411). And because this switch preceded the morphological changes in the KO/Tg small intestine, our data suggest that the identity of laminins in the subepithelial BM provides instructions for patterning the intestinal mucosa, either towards small intestine crypt-villus units or towards colon crypt-surface units.

Adult KO/Tg mice display aberrant epithelial cell behavior in the small intestine

To gain insights into the cellular mechanisms that underlie tissue patterning and remodeling in the KO/Tg small intestine, we examined cell proliferation, migration, differentiation and apoptosis in these mice. Cellular phenotypes from relatively normal looking KO/Tg villi were studied to try to avoid any phenotypes potentially secondary to morphological changes in the villi.

**Proliferation**

Immunofluorescence using antibodies directed against Ki67, an antigen expressed in proliferating cells, showed a statistically significant 1.5-fold increase on average in the length of the proliferative compartment in the KO/Tg small intestine compared with control (four mice per group, ten crypts measured per mouse; \(P<0.001\)) (Fig. 6A,B,G). An alternative method, labeling of proliferating cells with 5-bromodeoxyuridine (BrdU), revealed a 1.6-fold increase on average in the number of proliferating cells per crypt in the KO/Tg small intestine (four mice per group, 20 crypts analyzed per mouse; \(P<0.001\)).

**Migration**

We initially assayed the migration of intestinal epithelial cells up the villus by administering a single intraperitoneal injection of BrdU and analyzing the position of BrdU-labeled cells 24 hours later. After 24 hours, the leading edge of BrdU-labeled KO/Tg enterocytes was typically within five to ten cells of the villus tip (in well-oriented sections), whereas the leading edge of the BrdU-positive enterocytes in Het/Tg mice was only three to five cells from the crypt opening (Fig. 6C,D). Quantification of the distance from the crypt-villus

![Figure 6.](https://example.com/fig6.png)
junction to the top BrdU-positive cell revealed that KO/Tg epithelial cells migrate significantly faster than control cells (Fig. 6H). This finding was validated in one pair of mice by performing a staggered chlorodeoxyuridine (CldU)/iododeoxyuridine (IdU) double labeling experiment in which the distance that cells migrated after exit from the crypt in 12 hours was calculated by measuring the distance between the highest CldU-labeled cell and the highest IdU-labeled cell on the same villus (Fig. 6E,F). The KO/Tg epithelial cells migrated significantly faster than Het/Tg cells, at a rate of about 6 μm/hour, versus 3 μm/hour for the control (35 villi measured from each mouse, P<0.001).

**Differentiation**

We next investigated whether KO/Tg epithelial cells exhibited correct differentiation along the four intestinal epithelial cell lineages. Immunostaining for sucrase-isomaltase, a marker for differentiated enterocytes in the distal small intestine, did not reveal any difference between KO/Tg and Het/Tg, even in morphologically disturbed villi (Fig. 6I-K). Similarly, there was no difference in sucrase-isomaltase at the mRNA level (Fig. 6L). Immunostaining for chromogranin A, a marker for enteric neurons, showed that the number and distribution of this cell type were similar in KO/Tg and controls (supplementary material Fig. S4A,B). By contrast, there appeared to be elevated numbers of Paneth cells and goblet cells in the KO/Tg small intestine, based on immunostaining for lysozyme and periodic acid-Schiff stain, respectively (supplementary material Fig. S4C–F).

In addition to cell-lineage-specific marker changes, at both light- and electron-microscope levels we noted a conspicuous population of cells in the KO/Tg small intestine that were intermediate in appearance between goblet and Paneth cells (Fig. 6M–O). Such intermediate cells can occasionally be observed at crypt-villus junctions in wild-type small intestine, and they probably represent the common progenitor cells of the goblet- and Paneth-cell lineages (Troughton and Trier, 1969; Kamal et al., 2001). In KO/Tg mice, the number of intermediate cells was dramatically increased (Fig. 6P), and these cells were ectopically located either on sides of villi or at the bases of crypts, where only terminally differentiated cells should be found (Fig. 6N and data not shown).

**Apoptosis**

We investigated whether the apoptosis rate was altered in the KO/Tg small intestine by counting the number of cells with apoptotic bodies in hematoxylin and eosin (H&E)-stained paraffin sections. In four KO/Tg-Het/Tg littermate pairs at various ages, there were no significant differences in apoptosis noticed either in the crypts or on the villi (data not shown). The increase in proliferation without a concomitant increase in apoptosis may be responsible in part for the observed lengthening of the crypts.

_Lutheran_, a cell surface receptor for laminin α5, is downregulated in KO/Tg small intestine

To investigate the molecular mechanisms whereby lamins regulate the behavior of intestinal epithelial cells, we examined the localization and expression levels of known laminin receptors, including integrin αβ1, integrin αβ3, integrin α6β4, dystroglycan, and the Lutheran blood group glycoprotein/basal cell adhesion molecule (Lu/B-CAM, also known as Beam) (Miner and Yurchenco, 2004). By immunostaining, we did not find any significant changes in integrin α3, α6, β1, β4, or dystroglycan in the KO/Tg small intestine (supplementary material Fig. S5). Immunostaining with an antibody specifically recognizing the activated form of integrin β1 also did not reveal any changes, suggesting that activation of β1-containing integrins is not impacted by the altered BM composition (supplementary material Fig. S5). Immunostaining for Lu/B-CAM revealed that it is expressed at relatively low levels in the intestinal mucosa compared with the intestine smooth muscle. In the mucosa, Lu/B-CAM was highly expressed in the mesenchymal structures in the villus core, but only very weak expression was found at the basal surface of the intestinal epithelial cells (Fig. 7A). This basal staining was never observed in the KO/Tg small intestine (Fig. 7B). Lu/B-CAM expression at the basal surface was higher in the proximal small intestine, but this staining was reduced in KO/Tg mice (Fig. 7C,D).

p27Kip1, a cell cycle inhibitor, is downregulated in KO/Tg small intestine

The increased cell proliferation and reduced differentiation in KO/Tg mice prompted us to examine the localization and expression level of p27Kip1, a cell-cycle inhibitor that is known to inhibit S phase entry, promote cell-cycle exit, and maintain cells in a differentiated
state (Tian and Quaroci, 1999; Kaldis, 2007). In control small intestine, p27Kip1 expression was mainly localized to the nuclei of differentiated cells at the bottom of crypts and along the villi (Fig. 7E). In KO/Tg small intestine, the nuclear localization of p27Kip1 was dramatically reduced (Fig. 7F); this was shown with two different antibodies in five pairs of mice.

**Impaired lipid absorption in KO/Tg mice**

The dramatic loss of distal small intestine surface area in the KO/Tg mice suggests that these mice should exhibit absorption defects, particularly in regard to lipids, which are normally absorbed in the distal small intestine. We assayed dietary lipid absorption by feeding control and KO/Tg mice a high-fat diet (21% fat by weight, 42% of calories from fat). Food intake and feces output were determined daily, and total fecal lipids were extracted and quantified. We found that feces from KO/Tg mice contained twice as much lipid as control mice (Table 2). This finding translates into a 3% reduction in lipid absorption in KO/Tg mice as compared with Het/Tg mice ($n=3$). These data suggest a functional deficit that reflects the morphological defects.

**Discussion**

KO/Tg mice as a model system to study the roles of laminins in intestal morphogenesis

In the adult intestine, five different laminin isoforms (laminin-111, -211, -332, -411 and -511) predominate. Importantly, these isoforms are distributed in specific patterns along the crypt-villus axis of the intestine and are developmentally regulated (Simon-Assmann et al., 1998; Lefebvre et al., 1999; Teller et al., 2007) (our study). Functional studies of laminins in the adult intestine have been hampered by early embryonic lethality or functional redundancy. Mice deficient for laminin α1, β1 or γ1 all die prior to E7, before intestinal development begins (Smyth et al., 1999; Miner et al., 2004; Alpy et al., 2005; Scheelee et al., 2005). Even though laminin-α2- or laminin-α4-deficient mice are able to survive to adulthood, no apparent intestinal defects are seen in these mice, perhaps because of compensation by laminin α1 (Simon-Assmann et al., 1994) (our unpublished data). Laminin-α3-knockout mice die neonatally, and the intestinal phenotype has not been examined (Ryan et al., 1999). Laminin-α5-knockout mice do not survive beyond E17; they exhibit smooth muscle defects in the embryonic small intestine, but no obvious alterations in villus organization were reported (Miner et al., 1998; Bolcato-Bellemim et al., 2003). Here, KO/Tg mice represent a mouse model in which laminin α5 deposition is specifically lost in the subepithelial BM of the small intestine in postnatal mice. A similar model cannot be conveniently created using a conditional knockout strategy, as laminin α5 in the small intestine is secreted by both epithelial and mesenchymal cells (Lefebvre et al., 1999; Bolcato-Bellemim et al., 2003). Therefore, common small-intestine-specific Cre transgenes, such as Fabp-Cre or villin-Cre, which are only expressed by epithelial cells, should not effectively eliminate laminin α5 from the subepithelial BM.

In vivo and in vitro functions of laminins in intestinal epithelial cells

The dramatic alterations in cell proliferation, migration and differentiation in KO/Tg mice are consistent with in vitro data showing that different laminin isoforms exhibit distinct properties in regulating these and other processes. We were unable to delineate individual contributions from each laminin isoform in vivo because of the nature of compensation by laminin family members. Our data do suggest that laminins, in the context of the BM, regulate tissue patterning and remodeling by fine-tuning behavior of intestinal epithelial cells. The crypt-restricted deposition of laminin α2 is consistent with its function in promoting cell proliferation in vitro (Dowigiet et al., 2004). The abrupt onset of laminin-α5 deposition at the crypt-villus junction and its abundance in the villus subepithelial BM suggest a role in inducing cell differentiation. In the absence of laminin α5, the proliferative compartment expanded, suggesting a delay in initiating differentiation, despite the upregulation of laminin α1 and laminin α4. Enterocytes differentiated normally in the absence of laminin α5, probably because of upregulation of laminin α1, which has been shown to induce Caco2 cell differentiation into mature enterocyte-like cells (Turck et al., 2005). However, the terminal differentiation of goblet cells was affected by the absence of laminin α5 and/or the increased expression of laminin α1 and α4, as shown by the increased numbers of intermediate cells and alteration of mucous granules towards the colon type. Finally, the faster cell migration observed in KO/Tg small intestine could result from the combined reduction in laminin α5 and the increased α4 in the subepithelial BM, because LM-511 was found to be the most adhesive substrate in vitro compared with other isoforms (Siler et al., 2000; Poulö et al., 2002; Turck et al., 2005), and LM-411 potently promotes cell migration (Fujiiwara et al., 2001). Further studies will be needed to confirm these proposed functions for laminin isoforms.

**Laminins as regulators of tissue patterning**

The gastrointestinal tube is regionalized into stomach, small intestine and colon with distinct patterns of mucosal folding. This regionalization is governed by a combination of signaling molecules and transcription factors (Rossant and Tam, 2002; Kerszberg and Wolpert, 2007). We have now provided evidence that laminins serve as positional cues during the regionalization of the intestinal tube into small intestine and colon. We showed that mouse small intestine and colon normally contain different combinations of laminin isoforms; a transition in laminin composition from a small intestine type to a colon type leads to remodeling of small intestine mucosa to a colon-like mucosa. In addition, our grafting experiments suggest that the correct combination of laminin isoforms is also required for patterning the small intestine mucosa during early postnatal development. Laminin exhibits several features that make it an attractive candidate to provide positional information. (1) There are over 15 different laminin heterotrimers identified so far (Miner and Yurchenco, 2004), and combinations of these isoforms create even more heterogeneity, accommodating the need for multiple patterns. (2) Different laminin isoforms have distinct effects on cell proliferation, migration and differentiation, making them particularly useful for multiple purposes (Vachon and Beaulieu, 1995; Siler et al., 2000; Poulö et al., 2002; Turck et al., 2005). The combination of various isoforms could therefore facilitate the fine-tuning required for specific tissue patterns. (3) BMs are widely distributed in the body, surrounding all epithelia, endothelia, muscles, fat cells and peripheral nerves.
Materials and Methods

Generation and genotyping of Lama5-knockout and Mr5 transgenic mice

The generation and characterization of Lama5−/− and full-length laminin α5 (Mr5) transgenic mice have been described previously (Miner et al., 1998; Moulson et al., 2001). Mice were maintained on a mixed 129-C57BL/6J-CBA/J background. Studies were approved by the Washington University Animal Studies Committee. For genotyping, DNA extracted from tail biopsies was amplified by PCR using the following primer pairs to detect the three alleles: Lama5 (+): 5′-GGAGTCCTGTATTCGGGCACTCCTG-3′ and 5′-CAACATCTGCAAGAGTTGTTGG-3′; Lama5 (−): 5′-CGTTGACCTTGGACATGGTCTGG-3′ and 5′-GCCATATTGTTGGTGCTAGCC-3′; Mr5: 5′-CTCTAGAGCGGATCACGGAGG-3′ and 5′-CCATAGGTTGGCCCAATGGAGC-3′.

Antibodies, immunostaining and histology

The following antibodies and reagents were used: rat anti-laminin-α1 and anti-laminin-β1 (Abrahamson et al., 1989; St John et al., 2001) (clones 8B3 and 5A2, respectively, gifts from Dale Abrahamson, University of Kansas Medical Center, Kansas City, KS); rat anti-laminin-α2 (Schuler and Sorokin, 1995) (clone 4H8-2, Alexis Biochemicals/Axxora); rabbit anti-LM-332 (Marinkovich et al., 1992) (a gift from M. Peter Marinkovich, Stanford University, Stanford, CA); rabbit anti-laminin-α4 (Sasaki et al., 2001) and anti-Lu/B-CAM (gifts from Takako Sasaki, Portland, OR); rabbit anti-laminin-α5 (Miner et al., 1997) and anti-rabbit-laminin-β1 (MAB1914, Chemicon); rabbit anti-p27Kip1 (gift from Kwo-yih Yeh, Louisiana State University Health Sciences Center, Shreveport, LA); rabbit anti-chromogranin A (InnolnuStaar); rabbit anti-lysozyme (LabVision); rabbit anti-iκB-α (Novoceastra); mouse anti-BrDU (BD Biosciences); rabbit anti-integrin-α3 (a gift from C. Michael Diperico, Albany Medical College, Albany, NY); rabbit anti-integrin-β1 (Chemicon); rabbit anti-Lu/B-CAM (Chemicon); rat anti-integrin-β4 (BD Pharmingen); mouse anti-dystroglycan (clone 7D11, Developmental Studies Hybridoma Bank); rat anti-activated integrin-β1 (9EG7; BD Pharmingen); rabbit anti-p27Kip1 (Zymed); mouse anti-p27Kip1 (BD Biosciences); Hoechst 33342 (Sigma); Alexa-Fluor-488-conjugated anti-mouse IgG1 (Molecular Probes); FITC-conjugated anti-rat and Cy3-conjugated anti-rabbit (Chemicon).

For cryo-sections, mouse small intestine and colon were prepared as reported (Stappenbeck et al., 2002). Briefly, intestines were dissected and flushed with ice-cold PBS (pH 7.4) to remove the luminal contents. The lumen was then infused with OCT compound (VWR) prior to freezing in 2-methylbutane cooled in a dry ice-ethanol bath. Frozen sections were cut at 7 μm in a cryostat and air dried on gelatin-coated slides.

To prepare paraffin-section tissues, intestines were removed and flushed first with ice-cold PBS, then with ice-cold 4% PFA in PBS. The intestines were then opened up along the mesenteric side, pinned on wax (villus-side up), and fixed in 4% PFA in PBS at 4°C for 4-6 hours. After rinsing in PBS and dehydration in graded ethanol, the intestines were oriented in 2% agar prior to paraffin embedding. 5-μm-thick sections were cut parallel to either the cephalocaudal axis or the crypt-villus axis.

For scanning electron microscopy, 4×4-mm tissue fragments were fixed in 2% PFA/2.5% glutaraldehyde in 0.1 M cacodylate buffer and post-fixed in 1% osmium tetroxide. After rinsing in H2O, the tissue was treated with 1% OsO4, post-fixed with 0.1% osmium tetroxide, dehydrated in ethanol, and critical-point-dried before being sputter coated with gold/palladium.

Immunostaining and transmission electron microscopy were carried out as described previously (Kikkawa et al., 2003). Immunostained sections were examined through a microscope equipped for epifluorescence (Eclipse E800; Nikon). Images were captured with a Spot 2 cooled color digital camera (Diagnostic Instruments) using Spot Software Version 3.5.9. Digital electron microscopic images were captured using a Hitachi H7300 transmission electron microscope or a Hitachi S-3000H scanning electron microscope. Images were imported into Adobe Photoshop 7.0 for processing and layout.

Isolation of intestinal epithelial cells and real-time RT-PCR

Intact intestinal epithelia cells were isolated similarly as described (Bjerke and Cheng, 1999). Briefly, mice were anesthetized and the intestinal lumen was flushed in situ with Ca2+/Mg2+-free Hank’s balanced salt solution (CMF-HBSS) to remove fecal contents. After intravenous perfusion of 30 mM EDTA in CMF-HBSS, the intestines were oriented in 2% agar prior to paraffin embedding. Aliquots of the intestinal lumen were in situ with Ca2+-Mg2+-free Hank’s balanced salt solution (CMF-HBSS) to remove fecal contents. After intravenous perfusion of 30 mM EDTA in CMF-HBSS, the intestines were oriented in 2% agar prior to paraffin embedding. Aliquots of the intestinal lumen were
Determination of the migration rate of intestinal epithelial cells

The CldU/IdU double-labeling method utilizes two different BrdU monoclonal antibodies with different affinities for CldU and IdU. With this method (see Vega and Peterson, 2005) CldU-labeled cells and IdU-labeled cells can be discriminated using rat anti-BrdU (BU1/75 ICR1, Abcam) and mouse anti-BrdU (clone B44, BD biosciences), respectively, without crossreactivity. Lamin5+/-; Mx5 (Het/Tg) mice were labeled with CldU and IdU for 24 and 12 hours, respectively. KO/Tg mice were labeled with CldU and IdU for 24 and 12 hours, respectively. These differences were chosen to ensure linear measurements of cell migration on the villi. The distance migrated in 12 hours was calculated as the distance between the first CldU-labeled cell and the first IdU-labeled cell.

High-iron diamine staining

High-iron diamine staining was performed as described previously (Spicer, 1965). Sections were deparaffinized, rehydrated, and then immersed overnight (24 hours) in high-iron diamine solution, which contains 120 mg of N,N-dimethyl-m-phenylenediamine dihydrochloride and 20 mg of N,N-dimethyl-p-phenylenediamine hydrochloride dissolved in 50 ml distilled water and then mixed with 1.4 ml of fresh 60% (w/v) ferric chloride. After washing in running water, sections were stained with 1% alcian blue in 3% acetic acid (pH 2.5) for 5 minutes and counterstained with 0.5% aqueous Neutral Red for 2-3 minutes. After washing in running water, sections were dehydrated, cleared, and mounted.

Lipid absorption analysis

Mice were maintained on a high-fat western diet (Harlan) for three weeks before being transferred to individual metabolic cages to allow collection of feces and measurement of food consumption. Fecal lipid content and lipid absorption were measured as described previously (Newberry et al., 2006).

Statistical analyses

Two-tailed, unequal-variance Student’s t-tests were used throughout this study for determining statistical significance.

We thank Jeanette Cunningham, Gloriosa Go, Jennifer Richardson and Cong Li for technical assistance; Dale Abrahamson, Peter Marinovich, Takako Sasaki, Rupert Timpl and Kwo-yih Yeh for antibodies; the Pulmonary Morphology Core (supported by NIH P01HL029594) and the Digestive Diseases Research Core Center Morphology Core (supported by NIH P30DK052574) for histology services; the Microscopy and Digital Imaging Core in the Research Morphology Core (supported by NIH P30DK079333) for electron microscopy support; and Brent Polk for helpful insights. Mice were housed in a facility supported by NIH grant C06RR015502. This work was supported by grants from the NIH (R01GM060432 and P01HL029594) and the Digestive Diseases Research Core Center for Auditory and Vestibular Studies (supported by NIH P30DK052574) and the Digestive Diseases Research Center (supported by NIH P30DK052574) for histology services; the Microscopy and Digital Imaging Core in the Research Center for Auditory and Vestibular Studies (supported by NIH P30DK052574) and the Washington University Center for Kidney Disease Research (supported by NIH P30DK097933) for electron microscopy support; and Brent Polk for helpful insights. Mice were housed in a facility supported by NIH grant C06RR015502. This work was supported by grants from the NIH (R01GM060432 and R01DK064687) and the March of Dimes (#1-FY02-192) to J.H.M. J.H.M. is an Established Investigator of the American Heart Association.

References

Abrahamson, D. R., Irwin, M. H., St John, P. L., Perry, E. W., Accavitti, M. A., Heck, L. W. and Couchman, J. R. (1989). Selective immunoreactivities of kidney basement membranes to monoclonal antibodies against laminin: localization of the end of the long arm and the short arms to discrete micronodules. J. Cell Biol. 109, 3477-3491.

Aly, F., Jivkov, V., Sorokin, L., Klein, A., Arnold, C., Huss, Y., Keding, M., Simon-Assmann, P. and Lefebvre, O. (2005). Generation of a conditionally null allele of the laminin α5 gene. Genes Dev. 19, 59-70.

Ammaiano, M., Bruckner-Tuderman, L., Carter, W. G., Deutzmann, R., Edgar, D., Abrahamson, D. R., Irwin, M. H., St John, P. L., Perry, E. W., Accavitti, M. A., Heck, L. W. and Couchman, J. R. (1989). Selective immunoreactivities of kidney basement membranes to monoclonal antibodies against laminin: localization of the end of the long arm and the short arms to discrete micronodules. J. Cell Biol. 109, 3477-3491.

Aly, F., Jivkov, V., Sorokin, L., Klein, A., Arnold, C., Huss, Y., Keding, M., Simon-Assmann, P. and Lefebvre, O. (2005). Generation of a conditionally null allele of the laminin α5 gene. Genes Dev. 19, 59-70.

Ammaiano, M., Bruckner-Tuderman, L., Carter, W. G., Deutzmann, R., Edgar, D., Abrahamson, D. R., Irwin, M. H., St John, P. L., Perry, E. W., Accavitti, M. A., Heck, L. W. and Couchman, J. R. (1989). Selective immunoreactivities of kidney basement membranes to monoclonal antibodies against laminin: localization of the end of the long arm and the short arms to discrete micronodules. J. Cell Biol. 109, 3477-3491.

Aly, F., Jivkov, V., Sorokin, L., Klein, A., Arnold, C., Huss, Y., Keding, M., Simon-Assmann, P. and Lefebvre, O. (2005). Generation of a conditionally null allele of the laminin α5 gene. Genes Dev. 19, 59-70.

Ammaiano, M., Bruckner-Tuderman, L., Carter, W. G., Deutzmann, R., Edgar, D., Abrahamson, D. R., Irwin, M. H., St John, P. L., Perry, E. W., Accavitti, M. A., Heck, L. W. and Couchman, J. R. (1989). Selective immunoreactivities of kidney basement membranes to monoclonal antibodies against laminin: localization of the end of the long arm and the short arms to discrete micronodules. J. Cell Biol. 109, 3477-3491.

Aly, F., Jivkov, V., Sorokin, L., Klein, A., Arnold, C., Huss, Y., Keding, M., Simon-Assmann, P. and Lefebvre, O. (2005). Generation of a conditionally null allele of the laminin α5 gene. Genes Dev. 19, 59-70.

Ammaiano, M., Bruckner-Tuderman, L., Carter, W. G., Deutzmann, R., Edgar, D., Abrahamson, D. R., Irwin, M. H., St John, P. L., Perry, E. W., Accavitti, M. A., Heck, L. W. and Couchman, J. R. (1989). Selective immunoreactivities of kidney basement membranes to monoclonal antibodies against laminin: localization of the end of the long arm and the short arms to discrete micronodules. J. Cell Biol. 109, 3477-3491.
Nguyen, N. M., Miner, J. H., Pierce, R. A. and Senior, R. M. (2002). Laminin alpha5 is required for lobar septation and visceral pleural basement membrane formation in the developing mouse lung. Dev. Biol. 246, 231-244.

Nguyen, L., Besson, A., Heng, J. I., Schuurmans, C., Teyoul, L., Parras, C., Phippot, A., Roberts, J. M. and Guillenot, F. (2006). p27kip1 independently promotes neuronal differentiation and migration in the cerebral cortex. Genes Dev. 20, 1511-1524.

Parsons, S. F., Lee, G., Spring, F. A., Willig, T. N., Peters, L. L., Gim, J. A., Tanner, M. J., Mohandas, N., Anstee, D. J. and Chasis, J. A. (2001). Lutein blood group glycoprotein and its newly characterized mouse homologue specifically bind alpha5 chain-containing human laminin with high affinity. Blood 97, 312-320.

Potten, C. S., Booth, C. and Pritchard, D. M. (1997). The intestinal epithelial stem cell: the mucosal governor. Int. Exp. Pathol. 78, 219-243.

Pouliot, N., Saunders, N. A. and Kaur, P. (2002). Laminin 10/11: an alternative adhesive ligand for epidermal keratinocytes with a functional role in promoting proliferation and migration. Exp. Dermatol. 11, 387-397.

Radlak, F. and Clevets, H. (2005). Self-renewal and cancer of the gut: two sides of a coin. Science 307, 1904-1909.

Rahuel, C., Filipe, A., Ritie, L., El Nemer, W., Patey-Mariaud, N., Eladari, D., Cartron, J. P., Simon-Assmann, P., Le Van Kim, C. and Colin, Y. (2005). Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. J. Cell Biol. 141, 151-160.

Rebustini, I. T., Patel, V. N., Stewart, J. S., Layvey, A., Georges-Labouesse, E., Miner, S., Puch, S., Richards, A., Torok-Storb, B., Muller, C. A., Sorokin, Sherr, C. J. and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Meth. Enzymol. 356, 167-196.

Sancho, E., Batlle, E. and Clevers, H. (2005). Signaling pathways in intestinal development and cancer. Annu. Rev. Cell Dev. Biol. 21, 695-723.

Rahuel, C., Filipe, A., Ritie, L., El Nemer, W., Patey-Mariaud, N., Eladari, D., Cartron, J. P., Simon-Assmann, P., Le Van Kim, C. and Colin, Y. (2005). Intestinal epithelial cell differentiation. J. Cell Biol. 167, 702-709.

Simon-Assmann, P., Lefebvre, O., Bellissant-Waydelich, A., Olsen, J., Orian-Rousseau, V. and De Arcangelis, A. (1998). The laminins: role in intestinal morphogenesis and differentiation. Ann. N. Y. Acad. Sci. 859, 46-64.

Simon-Assmann, P., Turck, N., Sidhom-Jenny, M., Gradwohl, G. and Kedinger, M. (2007). In vitro models of intestinal epithelial cell differentiation. Cell Biol. Toxicol. 23, 241-256.

Smyth, N., Vatanasey, H. S., Murray, P., Meyer, M., Frie, C., Paulsson, M. and Edgar, D. (1999). Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. J. Cell Biol. 141, 151-160.

Spicer, S. S. (1965). Diamine methods for differentialising mucosubstances histochemically. J. Histochem. Cytochem. 13, 211-234.

St John, P. L., Wang, R., Yin, Y., Miner, J. H., Robert, B. and Abrahamson, D. R. (2001). Gliomelic laminin isoform transitions: errors in metamorphic culture are corrected by grafting. Am. J. Physiol. Renal Physiol. 280, F695-F705.

Stappenbeck, T. S., Wong, M. H., Saam, J. R., Mysorekar, I. U. and Gordon, J. I. (1998). Notes from some crypt watchers: regulation of renewal in the mouse intestinal epithelium. Curr. Opin. Cell Biol. 10, 702-709.

Stappenbeck, T. S., Hooper, L. V., Manchester, J. K., Wong, M. H. and Gordon, J. I. (2002). Laser capture microdissection of mouse intestine: characterizing miRNA and protein expression, and profiling intermediary metabolism in specified cell populations. Proc. Natl. Acad. Sci. USA 102, 310-317.

Suemori, H., Kadodawala, Y., Goto, K., Araki, I., Kondoh, H. and Nakatsuiji, N. (1990). A mouse embryonic stem cell line showing pluripotency of differentiation in early embryos and ubiquitous beta-galactosidase expression. Cell Differ. Dev. 29, 181-186.

Teller, I. C., Ausclair, J., Herring, E., Gauthier, R., Menard, D. and Beaulieu, J. F. (2007). Laminins in the developing and adult human small intestine: relation with the functional absorptive unit. Dev. Dyn. 236, 1980-1990.

Tian, J. Q. and Quaroni, A. (1999). Involvement of p21(WAF1/Cip1) and p27(Kip1) in intestinal epithelial cell differentiation. Am. J. Physiol. 276, C1245-C1258.

Timpl, R. (1996). Macromolecular organization of basement membranes. Curr. Opin. Cell Biol. 8, 618-624.

Timpl, R. and Brown, J. C. (1996). Supramolecular assembly of basement membranes. BioEssays 18, 125-132.

Troughton, W. D. and Trier, J. S. (1969). Paneth and goblet cell renewal in mouse duodenal crypts. J. Cell Biol. 41, 251-268.

Turck, N., Gross, I., Gendry, P., Stuttmann, J., Freund, J. N., Kedinge, M., Simon-Assmann, P. and Launay, J. F. (2005). Laminin isoforms: biological roles and effects on the intracellular distribution of nuclear proteins in intestinal epithelial cells. Exp. Cell. Res. 303, 494-503.

Vachon, P. H. and Beaulieu, J. F. (1995). Extracellular heterotrimeric laminin promotes differentiation in human enterocytes. Am. J. Physiol. 268, G857-G867.

Vega, C. J. and Peterson, D. A. (2005). Stem cell proliferative history in tissue revealed by temporal halogenated thymidine analog discrimination. Nat. Methods 2, 167-169.

Vurchenko, P. D., Amenta, P. S. and Patton, B. L. (2004). Basement membrane assembly, stability and activities observed through a developemental lens. Matrix Biol. 22, 521-538.