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Cellular and molecular partners involved in gut morphogenesis and differentiation

M. Kedinger*, **O. Lefebvre, I. Duluc, J. N. Freund and P. Simon-Assmann**

INSERM Unit 381, Development and Pathology of the Digestive Tract, 3 Avenue Molière, 67200 Strasbourg, France

The intestinal mucosa represents an interesting model to study the cellular and molecular basis of epithelial–mesenchymal cross-talk participating in the development and maintenance of the digestive function. This cross-talk involves extracellular matrix molecules, cell–cell and cell–matrix adhesion molecules as well as paracrine factors and their receptors. The cellular and molecular unit is additionally regulated by hormonal, immune and neural inputs. Such integrated cell interactions are involved in pattern formation, in proximodistal regionalization, in maintenance of a gradient of epithelial proliferation and differentiation, and in epithelial cell migration.

We focus predominantly on two aspects of these integrated interactions in this paper: (i) the role of basement membrane molecules, namely laminins, in the developmental and spatial epithelial behaviour; and (ii) the importance of the mesenchymal cell compartment in these processes.

Keywords: intestine; epithelial–mesenchymal cell interactions; laminins; *Cdx* homeobox genes

1. INTRODUCTION

To be able to define the principal cell interactions that occur during development of the gut and in the adult organ, it is important to note the following basic morphofunctional characteristics of the gut. The gut derives from the association of the embryonic endoderm to the splanchnic mesoderm. At early developmental stages, the intestinal tube is formed by the central stratified and undifferentiated endoderm surrounded by the undifferentiated mesenchyme. Intestinal morphogenesis comprises (i) the formation of the central lumen and the outgrowth of villi accompanied by a progressive formation of a single epithelium, and later on (ii) the formation of crypts in the intervillus zones. From this stage onwards the gut epithelium is compartmentalized: the crypts correspond to the stem cell and proliferative cell compartment, and the villi to the differentiation compartment. The cells coming out of the crypts migrate vertically along the villus axis; they are finally extruded in the lumen. In parallel, the mesenchyme differentiates into the mucosal and submucosal connective tissue and the outer muscle layers. Thus, the functional unit of the gut is formed by the epithelial villus–crypts entity and by the connective tissue core comprising the mesenchyme-derived subepithelial myofibroblasts, fibroblasts and muscle fibres, in addition to lymphocytes, blood vessels/capillaries and nerve fibres/terminations. The intestinal stem cells differentiate into various cell types among which the absorptive cells or enterocytes represent the major cell phenotype. They are characterized by a sophisticated polarization, with (i) an apical membrane, or brush border, which forms folds comprising a cytoskeletal core and carries the digestive and absorptive functions, and (ii) a basolateral domain, which faces the basement membrane (BM) at the basal pole of the cells. For additional details, there are comprehensive reviews on these various aspects in Potten & Loeffler (1990), Hermiston *et al.* (1994) and Kedinger (1994). Finally, the gut exhibits a longitudinal regionalization that is characterized by a functional gradient along the small intestine and by marked changes in the morphological pattern and functional properties between the small intestine and the colon. In parallel, a series of homeobox genes are found to express gradient or specific patterns along the proximodistal axis of the gut (James & Kazenwadel 1991; Walters *et al.* 1997). It is also worthy to note that only the distal part of the intestinal tube, the colon, develops tumours.

The importance of the epithelial–mesenchymal entity in the intestine has been demonstrated experimentally by the use of co-cultures or of various intestinal recombinants. These have enabled us to show that: (i) contact between both epithelial and mesenchymal cells is required for cell differentiation; (ii) the two cell compartments exert reciprocal permissive or inductive interactions; (iii) these cell interactions are functional not only during development but also in the adult tissue; and (iv) tissue integrity is not required, i.e. cultured cells can be used instead of tissues (Kedinger 1994). The next two sections will emphasize the importance of the subepithelial BM in the epithelial differentiation resulting from heterologous cell interactions (§ 2), and the involvement of the mesenchymal cell compartment (§ 3).

*Author for correspondence (michele.kedinger@inserm.u-strasbg.fr).

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2. EXPRESSION AND ROLE OF LAMININ ISOFORMS IN THE INTESTINE

(a) *The basement membrane: specific expression of laminin variants in the developing and adult organ*

The intestinal BM is visualized by electron microscopy between the endoderm/epithelium and the mesenchyme/connective tissue at the foetal stages and along the crypt-to-villus axis. The BM is either regular (early foetal stages, crypts in the adult organ) or interrupted (morphogenetic steps, upper third of the villi). Developmental or spatial variations in the expression or localization of various constituent BM molecules have been reported in several immunocytochemical studies, suggesting that cell–matrix interactions may play important inductive actions. The distribution within human and rodent developing intestinal mucosa of ubiquitous BM components, such as type IV collagen, laminin, perlecan and nidogen, are described in comprehensive reviews (Simon-Assmann *et al.* 1995; Beaulieu 1997). Among extracellular matrix molecules, laminins, which are major components of the BMs, are of particular interest as they display a high variability. Laminins are cross- or T-shaped, heterotrimeric proteins composed of three chains named α (long arm chain), β and γ (short arm chains) (Burgesson *et al.* 1994). Up to now, at least 11 laminin variants have been described; they are often expressed in a tissue- and developmental-specific manner, which suggests that they might generate functional diversity (Miner *et al.* 1997; Simon-Assmann *et al.* 1997).

The first laminin molecule that was identified in the intestinal basement membrane corresponds to laminin-1; this molecule, isolated from the Engelbreth–Holm–Swarm (EHS) tumour, comprises three subunits—α_1, β_1 and γ_1—encoded by three distinct genes. Using specific monoclonal antibodies, we showed that, whereas the β_1/γ_1 chains are homogeneously distributed in the crypt and villus subepithelial basement membrane in rodent intestine, the α_1 chain is restricted to the crypt zone. However, in the adult human intestine the α_1 chain (visualized with the 4C7 mAb, which recognizes the α_5 chain in addition to, or instead of, α_1 (Miner *et al.* 1997)) presented a decreasing gradient of intensity from the tip of the villus to the crypt mouth; no α_1 chain was expressed in crypt basement membranes in the jejunum and ileum (Beaulieu & Vachon 1994; Simon-Assmann *et al.* 1994*a*). Interestingly, the α_2 chain, which associates with β_1 and γ_1 chains to form the laminin-2 variant, was exclusively present in the basement membrane that surrounds the bottom of crypts in both rodent and human intestines. Thus, laminin α_1 and α_2 chains exhibit a complementary expression, at least in the human intestine. In addition, although the α_1 chain is present early in the foetal intestinal anlagen, the α_2 chain is expressed only around birth when crypt downgrowth occurs in both species. Changes in the level and molecular forms of laminin-1 are obvious as a function of intestinal development: a maximal biosynthetic activity of laminin-1 and a higher relative proportion of α_1 chains versus β_1 and γ_1 chains were observed at stages corresponding respectively to villus emergence and individualization of the smooth muscle layers (Simo *et al.* 1991).

Laminin-5, which consists of α_3, β_3 and γ_2 chains, has been described mainly in the skin where it is found to be associated to hemidesmosomal structures, forming stable adhesion devices and contributing to the interaction between the keratinocytes and the underlying connective tissue (Rousselle *et al.* 1991). In the human intestine, the three constituent chains of laminin-5 have been detected in the subepithelial basement membrane concomitant with laminin-1, as early as 8 weeks of gestation. In parallel to the formation of villi, and up to the adult stage, laminin-5 is progressively expressed according to an increasing gradient from the crypt mouth to the villus tip, and is absent from the BM lining the proliferative cells in the crypts (Orian-Rousseau *et al.* 1996; Leivo *et al.* 1996). The distribution of laminin-5 individual chains in the mouse intestine is somewhat different from that found in human: at early stages of development only γ_2 and β_3 chains are found; in the adult intestine α_3 and β_3 chains are restricted to the very top of the villi, where the most differentiated cells that will be extruded into the lumen are found. Among the hemidesmosomal components described in the skin, only the HD1 protein and the $\alpha_6\beta_4$ integrin are expressed in the intestinal cells and are found co-distributed with laminin-5 (Orian-Rousseau *et al.* 1996).

Two other α chains—α_4 and α_5—have been discovered recently; they are identified in four novel laminin heterotrimers: laminin-8 ($\alpha_4\beta_1\gamma_1$), laminin-9 ($\alpha_4\beta_2\gamma_1$), laminin-10 ($\alpha_5\beta_1\gamma_1$) and laminin-11 ($\alpha_5\beta_2\gamma_1$) (Miner *et al.* 1997). Although only few data are available concerning their tissue localization, both α_4 and α_5 transcripts have been detected in the gut (Livanainen *et al.* 1995; Miner *et al.* 1997). Using specific rat monoclonal antibodies raised by Dr L. Sorokin (Erlangen, Germany), we found a regular deposition of α_5 chains at the endodermal–mesenchymal interface; these were found in the undifferentiated mouse intestine and, later on, at the base of the epithelial cells, with an increasing gradient of intensity from the crypt mouth to the villus tip in the adult intestine (O. Lefebvre, M. Kedinger, L. Sorokin and P. Simon-Assmann, unpublished data).

As more than ten different laminin gene products are contributing to basement membrane assembly, and owing to their specific patterns along the crypt–villus axis, we need to understand how these proteins, which belong to an ever-growing family, are assembled and how they can direct specific biological functions.

(b) *Epithelial–mesenchymal complementarity in the formation of the basement membrane*

To approach the physiological significance of the developmental and spatial microheterogeneities in the intestinal BM composition, we analysed the cellular origin of individual BM components as a function of the developmental stage of the tissue. For this purpose, we used a strategy that has previously made a great contribution to the study of the nature of the epithelial–mesenchymal cell interactions in the gut (Haffen *et al.* 1989). Interspecies (chick–rodent), embryonic, epithelial–mesenchymal tissue recombinants are developed as grafts in the chick embryo for various time periods, and the deposition of individual BM components in the hybrid intestines is detected using species-specific antibodies.

Table 1. *Epithelial and/or mesenchymal origin of BM components or constituent chains of laminin variants deposited at the epithelial–mesenchymal interface*

(Hybrid intestines composed of cross-associations between chick and rodent epithelial–mesenchymal foetal anlagen (chick mesenchyme/rodent endoderm or vice versa) were grafted for 5, 9 and 13 days and analysed immunocytochemically with species-specific antibodies to the different proteins. For details, see Simon-Assmann *et al.* (1988, 1989, 1990, 1997), Simo *et al.* (1992*a*) and Orian-Rousseau *et al.* (1996). Key: ep, epithelial; mes, mesenchymal; coll IV, type IV collagen. Constituent chains of the laminin variants analysed: laminin-1 ($\alpha_1\beta_1\gamma_1$); laminin-2 ($\alpha_2\beta_1\gamma_1$); laminin-10 ($\alpha_5\beta_1\gamma_1$); laminin-5 ($\alpha_3\beta_3\gamma_2$).)

					laminin constituent chains					
		coll IV	perlecan	nidogen	laminin-1 α_1	laminin-2 α_2	laminin-10 α_5	laminin $\beta_1\gamma_1$	laminin-5 β_3	laminin-5 γ_2
5 days	ep	+	+	—	+	—	+	+	—	+
	mes	+	—	+	—	+	+	+	+	—
9 days	ep	—	+	—	+	—	+	+	—	+
	mes	+	—	+	—	+	+	+	+	+
13 days	ep	—	+	—	+	—	+	+	—	—
	mes	+	—	+	+	+	+	+	+	+

This technique allowed us to show that perlecan deposition is achieved by the epithelial compartment, whereas collagen type IV and nidogen are mostly mesenchymal products (Simon-Assmann *et al.* 1988, 1989, 1990; for review, see Simon-Assmann *et al.* 1995). The production of the various laminin isoforms is even more complex as individual α, β and γ chains are produced by different cell types according to a sequential chronology (for review, see Simon-Assmann *et al.* 1997). The detailed data are summarized in table 1.

It appears that although the β_1 and γ_1 chains, shared in particular by laminins-1, -2 and -10, are deposited at the epithelial–mesenchymal interface by both epithelial and mesenchymal cells, the α_1 chain constituent of laminin-1 is produced by the epithelial cells during the whole development of the hybrids and by the mesenchymal cells only at advanced differentiated states (Simo *et al.* 1992*a*). The α_2 chain, characteristic of laminin-2, is deposited exclusively by the mesenchymal cells (O. Lefebvre, M. Kedinger, L. Sorokin and P. Simon-Assmann, unpublished data, using antibodies raised by Schuler & Sorokin (1995)); this observation confirms former *in situ* hybridization studies (Vuolteenaho *et al.* 1994). The α_5 chain, constituent of laminin-10, has a dual epithelial and mesenchymal origin (O. Lefebvre, M. Kedinger, L. Sorokin and P. Simon-Assmann, unpublished data). These data are strengthened by *in situ* hybridization in the embryonic gut anlagen (Sorokin *et al.* 1997).

The laminin-5 γ_2 chain is of particular interest, as it presents a shift in its cellular expression. Indeed, this chain is deposited by undifferentiated epithelial cells at early stages of development, and by the mesenchymal cells at later stages giving rise to the adult pattern (Orian-Rousseau *et al.* 1996). In contrast, the β_3 chain, at present found only in laminin-5, is produced exclusively by the mesenchymal cells whatever the development stage of the grafts (V. Orian-Rousseau and P. Simon-Assmann, unpublished data). No conclusion can be drawn for the constituent α_3 chain, due to the lack of immunostaining during early phases of intestinal mouse development with the antibodies used.

Altogether, these results show that epithelial or mesenchymal cells are able to produce several laminin isoforms simultaneously and to deposit them in the adjacent basement membrane. Epithelial cells produce the laminin-1 and laminin-10 isoforms, and the mesenchymal cells laminin-2 and laminin-10 at any stage of differentiation. In addition, mesenchymal cells display a delayed expression/deposition of α_1 and γ_2 chains, which suggests the requirement of inductive cell interaction processes resulting in their differentiation *in situ*. The requirement of a close tissue cooperation for the assembly of the BM is also strengthened by the findings related to the formation of the laminin–nidogen complex (for a review, see Dziadek (1995)).

(c) *Attempts to define the biological role of laminin isoforms*

To determine the physiological significance of laminin variants, adequate *in vitro* or *in vivo* experimental models have been used. The current knowledge on the function of the basement membrane molecules and of their receptors has been growing recently, owing to the analysis of spontaneous mutations and to the development of targeted mutations/deletions in mice. These models have brought a wealth of information mostly in the field of integrins (for a review, see Fassler *et al.* (1996)); at present only limited data are available for laminins.

Several lines of evidence indicating that laminin-1 is intimately involved in triggering differentiation of intestinal epithelial cells were provided by *in vitro* models. First, the addition of antilaminin-1 antibodies to intestinal endodermal/fibroblastic co-cultures inhibited the expression of lactase, which is an apical differentiation marker (Simo *et al.* 1992*b*). Second, laminin-1, used as substratum, has been found to increase significantly the expression of both lactase and sucrase (two enterocytic differentiation markers) in contrast to laminin-2 (Vachon & Beaulieu

1995). Third, the capacity of two cell lines of human colonic cancers (HT29 and Caco-2 cells) to produce the laminin α_1 chain is correlated with their differentiation degree and ability to form a monolayer and a well-organized BM when cultured on fibroblastic cells (Bouziges *et al.* 1991, De Arcangelis *et al.* 1994). These data were confirmed by using an antisense RNA strategy, which allowed us to analyse the direct involvement of the α_1 chain in laminin-1 assembly, basement membrane formation and cell differentiation (De Arcangelis *et al.* 1996). The inhibition of α_1 chain expression in the Caco-2 cells leads to: (i) an incorrect secretion of the two other constituent chains of laminin, the β_1 and γ_1 chains; (ii) the lack of basement membrane formation in the co-culture model, despite the production of type IV collagen and nidogen by the mesenchymal cells; and (iii) alterations in the structural and functional polarity of the cells (rare microvilli, absence of apical sucrase expression). However, in the inverse experiment, it was disappointing to find that induction of the α_1 chain expression in the α_1-deficient HT29 cell population failed to induce their differentiation and to restore a basement membrane formation in co-cultures (A. De Arcangelis, O. Lefebvre and P. Simon-Assmann, unpublished data). The absence of any significant effect of the laminin α_1 chain in this experiment could be linked to the lack, in the HT29 cells, of complementary laminin chains required in the process of basement membrane assembly and subsequently of cell differentiation, or to the abnormal expression of cell–cell adhesion molecules. Therefore, the demonstration of a specific role of laminin-1 in differentiation still remains to be ascertained for definite. Up until now, no inhibition of laminin-1 *in vivo* in mouse has been recorded. However, in *Drosophila*, mutation of the laminin α chain is embryonically lethal, owing to defects in numerous tissues (Henchcliffe *et al.* 1993; Yarnitzki & Volk 1995; Garcia-Alonso *et al.* 1996). In addition, knockout of the ubiquitously expressed LAMC1 gene coding for the γ_1 subunit leads to early embryonic lethality (Smyth & Edgar, unpublished data). Owing to the expected lethality of α_1 knockout animals, our intention is to use intestine-specific, gene targeting with the Cre-lox P system to analyse the role of the α_1 chain *in vivo.*

An opportunity to define the potential role of the laminin α_2 chain in the intestine was provided by the finding that the natural mutant mouse *dy* lacks the α_2 chain (Xu *et al.* 1994). The major phenotype of homozygous mutant *dy*/*dy* mice is a severe muscular dystrophy. Because expression of the α_2 chain in the intestine coincides with the formation of crypts, it was considered possible that this chain might play a role in their genesis and/or in the maintenance of a defined extracellular microenvironment specific for the stem cell compartment. However, analysis of the intestine of the *dy* mouse did not provide any evidence for such a role. Furthermore, the lack of the α_2 chain did not prevent differentiation of the stem cells into the various epithelial cell phenotypes (Simon-Assmann *et al.* 1994*a*). Since the crypt basement membrane in normal mouse contains additionally the α_1 chains, their presence may compensate for the lack of α_2 chains.

Concerning laminin-5, its specific role in the skin has been highlighted by the marked consequences of mutations in human. Mutations in any of the three genes encoding for laminin-5 chains give rise to the blistering skin disease called junctional epidermolysis bullosa (for review, see Uitto & Pulkkinen 1996). Although pyloric atresia have been associated with subtypes of junctional epidermolysis bullosa, due to mutations in the α_6- or β_4-integrin subunits, until now the potential involvement of laminin-5 in gut alterations in such pathologies has never been analysed. Nevertheless, the following observations indicate that, in the gut, laminin-5 could play a role in cell migration along the crypt–villus axis. First, laminin-5 is localized in the BM lining the villus enterocytes, where active cell migration occurs, but absent in the crypts (Orian-Rousseau *et al.* 1996). Second, laminin-5 co-localizes with the $\alpha_6\beta_4$ integrin and the protein HD1 in type II hemidesmosome structures (Simon-Assmann *et al.* 1994*b*; Orian-Rousseau *et al.* 1996). Interestingly, the fact that two other hemidesmosome components—BP180 (bullous pemphigoid) and BP230 (Orian-Rousseau *et al.* 1996)—are not expressed in the intestine, suggests that type II hemidesmosomes do not function in the gut as anchorage devices like in the skin. Third, the fact that parental HT29 and Caco-2 cells exhibit differences when injected in nude mice or in immunosupressed neonatal rats—important in growth and formation of metastasis for the former cells but not for the latter—can be correlated to the expression of laminin-5, integrin $\alpha_6\beta_4$ and HD1 in HT29 and to their absence in Caco-2 cells (De Arcangelis *et al.* 1994; Simon-Assmann *et al.* 1994*b*; Fontao *et al.* 1997; Orian-Rousseau *et al.* 1998). Fourth, in experimental conditions (described in the following section), in which Caco-2 cells are induced to overexpress integrin $\alpha_6\beta_4$, HD1 and laminin-5 γ_2 chain, the tumours raised in nude mice from these cells are significantly larger than the controls (Lorentz *et al.* 1997). These observations are in accordance with the finding of a preferential expression of laminin-5 γ_2 chain in invasive malignant colon cancer cells (Pyke *et al.* 1995). In addition, laminin-5 is a primary target of the matrix metalloprotease-2, which cleaves the γ_2 chain allowing a subsequent activation of cryptic sites that trigger cell motility (Giannelli *et al.* 1997). Related to these data, it will be of particular interest to know if this truncated form of the γ_2 chain is present in the developing intestine that undergoes intensive remodelling and in the mature organ where active cell migration occurs.

(d) *Molecular mechanisms involved in LN1-dependent differentiation*

The role of laminin-1 in intestinal cell differentiation has been emphasized by arguments detailed above and the molecular mechanisms involved in the differentiating effect of laminin-1 are under investigation. The caudal-related *Cdx-2* homeobox gene was a good candidate as mediator of the effect of laminin because: (i) the Cdx-2 protein is mostly expressed in enterocytes lining the villi (James *et al.* 1994); (ii) it binds cis-elements in the promoters of various enterocyte differentiation genes (Suh *et al.* 1994); and (iii) *Cdx-2* overexpression stimulates polarization and differentiation of undifferentiated, rat intestinal IEC cells (Suh & Traber 1996). The implication of *Cdx-2* in laminin-mediated differentiation has been confirmed by the two following observations. First, *Cdx-2* is stimulated when Caco-2 TC7 cells (Chantret *et al.* 1994) are cultured on laminin-1 coatings. Second, in laminin-α_1

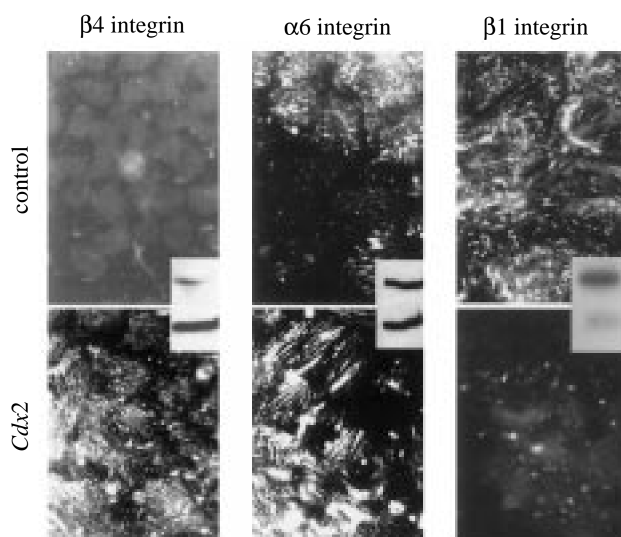

Figure 1. Shift from the $\alpha_6\beta_1$ to the $\alpha_6\beta_4$ integrin linked to *Cdx-2* overexpression in Caco-2 TC7 cells. The immunocytochemical detection of the three integrin subunits with specific antibodies was performed on control and *Cdx-2*-transfected cells cultured on glass coverslips for 5 days, fixed and permeabilized. Inserts: the expression of the corresponding proteins was analysed by Western blotting (Lorentz *et al.* 1997).

antisense Caco-2 cell clones, we found a correlation between the level of the remaining endogeneous α_1 gene expression and the level of *Cdx-2* expression. Furthermore, the molecular changes induced by laminin-1 coatings are strictly parallel to those resulting from the overexpression of *Cdx-2* by transfection of the full-length cDNA in Caco-2 TC7 cells (Lorentz *et al.* 1997). These changes recapitulate various characteristics of differentiated enterocytes: they include increased sucrase, and to a lesser extent lactase, transcripts and activity, as well as modifications in cell–cell and cell–matrix adhesion proteins (Lorentz *et al.* 1997). An obvious effect concerns the significant stimulation of the expression of the integrin-β_4 subunit, which is almost undetectable in the control cells, and its localization as patches at the ventral side of the cells grown on laminin coatings or overexpressing *Cdx-2* (figure 1). This induction goes along with a clearcut membrane segregation at the basal pole of the cells of the α_6-integrin subunit (without changes in the total amount of either the mRNA or the protein), which co-localizes with the β_4 subunit (figure 1). In parallel, the expression and basal staining of the β_1-integrin subunit becomes weak (figure 1). Thus both laminin-1 coating and *Cdx-2* overexpression induce a shift from the integrin $\alpha_6\beta_1$ to $\alpha_6\beta_4$ and most probably the formation of type II hemidesmosomes. Indeed, the hemidesmosomal protein, HD1, is also induced in the experimental cells. Interestingly, co-culture of Caco-2 cells on mesenchymal cells also induces expression of the basal β_4-integrin subunit (see Kedinger *et al.* 1997). Another important consequence of the overexpression of *Cdx-2* is the downregulation of *Cdx-1*. *Cdx-1* is a caudal-related gene that is found in the crypt cells (Silberg *et al.* 1997). Taken together, these data provide substantial evidence that the extracellular matrix signals contribute to the phenotypic changes occurring at the crypt–villus junction via the stimulation of key genes, including *Cdx-2*, which control the expression of differentiation markers and turn off other genes, characteristic of the crypt compartment.

The importance of a tight control of these regulatory genes is strengthened by the fact that: (i) the disruption of *Cdx-2* gene in mouse leads to morphological alterations of the distal gut, where this gene exhibits its higher expression *in situ*, and even to the formation of colonic adenocarcinomas (Chawengsaksophak *et al.* 1997); and (ii) a decreased expression of *Cdx-1* and *Cdx-2* genes is reported in human colonic cancers (Ee *et al.* 1995; Silberg *et al.* 1997). Linked to this, it was surprising to observe that the injection of *Cdx-2*-transfected cells in nude mice leads to the development of tumours significantly larger than those formed from the control cells. As discussed above (§ 2c), we think that this may result from the upregulation of the integrin-β_4 subunit, of the laminin γ_2 chain and from the formation of type II hemidesmosomes.

3. ROLE OF THE MESENCHYMAL COMPARTMENT

Former experiments *in vitro* and in grafts demonstrated the requirement of the mesenchyme for morphogenesis and epithelial cell differentiation (Haffen *et al.* 1989; Kedinger 1994). We also showed that the mesenchyme dictates the form (epithelial ridges versus villi) in chick–rodent hybrid intestines. Our major interest in this cellular compartment arises from recombination experiments that have demonstrated: (i) the inductive role of the small intestinal mesenchyme, which induces the colonic endoderm to develop small intestinal structures and cytodifferentiation (Duluc *et al.* 1994); this point is interesting as the colonic tumours display small intestinal-type cytodifferentiation; and (ii) the mesenchymal origin of major proteins of the subepithelial BM: type IV collagen, nidogen and specific constituent chains of laminin (see § 2*b*). In addition, various soluble factors that control epithelial growth, motility and morphogenesis are expressed in the gut by the mesenchymal cell compartment—in the vicinity of the epithelium—during the foetal and perinatal period (e.g. hepatic growth factor/scatter factor (HGF/SF), neuregulin (NGF) and keratinocyte growth factor (KGF)). These factors function via tyrosine kinase receptors that are expressed exclusively or predominantly by epithelial cells (respectively *c-met*, c-erb B3 and B4, FGFR2- IIIb; for a review, see Birchmeier *et al.* (1995)). On this basis, our aim is to acquire a better knowledge of the phenotypic and metabolic characteristics of the mesenchymal cells, of their inductive properties on the epithelial cell behaviour and of their own regulation.

(a) *Phenotypic characteristics of mesenchymal clonal cell lines, and inductive properties on epithelial cells*

Two morphologically distinct cell clones, A1F1 and F1G9, derived from primary cultures of ileal lamina propria from postnatal rats, appeared to be particularly interesting because they display clearcut differences. These specific characteristics concern their morphology, their expression of cytoskeletal and surface markers, their sensitivity to cytokines and their effect on epithelial cells (Fritsch *et al.* 1997; see table 2). Concerning the latter point, F1G9 cells induce the differentiation of the endodermal cells in

Table 2. *Characteristics of two intestinal mesenchymal cell lines*

(The clonal lines F1G9 and A1F1 are derived from intestinal lamina propria from postnatal rats (Fritsch *et al*. 1997). VM, vimentin; α-SM: α-smooth muscle actin; DM, desmin; CK: cytokeratin; CD45, leucocyte marker; $TGF\beta_1$, transforming growth factor β_1; IL2, interleukin 2. (*a*) Intestinal endodermal microexplants from 14-day foetal rats were co-cultured on confluent cultures of the mesenchymal cell lines; differentiation was assessed by apical lactase expression. (*b*) Foetal intestinal endoderms were enveloped with F1G9 or A1F1 cell sheets, grafted in the coelom of chick embryos and recovered 10–13 days later. Key: +, positive; −: negative; downward-pointing arrow, inhibition; 0, no response; upward-pointing arrow, increased; *ca*. −, almost negative.)

	F1G9	A1F1
morphology	epithelioid	stellate
phenotypic differences		
VM	+	+
α-SM	5%	*ca*. −
DM	−	−
CK	−	−
Thy 1-1	+	−
CD45	*ca*. −	+
laminin	++	+
coll IV	+	+
proliferative response to		
$TGF\beta_1$	↘	0
IL2	0	↘
differentiation response to		
$TGF\beta_1$	α-SM+	0
(*a*)		
inductive effect of the cell lines on epithelial cells		
proliferation	limited	↗
differentiation	+ (lactase +)	− (lactase −)
(*b*)		
inductive effect of the cell lines on morphogenesis and differentiation	crypt/villus structures (lactase +)	glands (lactase −)

co-culture and a crypt–villus morphogenesis in grafts, whereas the A1F1 cells stimulate proliferation rather than differentiation of the endodermal cells *in vitro* and induce a gland morphogenesis *in vivo* (table 2). Interestingly, the level of epithelial *Cdx-1* and *Cdx-2* gene expression in the grafted recombinants comprising F1G9 or A1F1 cells reflected the morphological proximodistal gradient: higher *Cdx-1* and *Cdx-2* expression in the A1F1 (flat surface/deep glands, colon-like) grafts than in the F1G9 (small intestinal-like) grafts (Duluc *et al*. 1997). The different inductive properties of the two mesenchymal cell lines can be correlated, although no causative link has been established, with the following observations: (i) the F1G9 cell line produces twice as many laminin as the A1F1 cells; and (ii) $TGF\beta_1$ induces F1G9 but not A1F1 cells to differentiate into myofibroblasts (Fritsch *et al*. 1997). In order to better understand the properties of the mesenchymal cells along the gut axis, we established homogeneous clonal cell lines derived from the subepithelial myofibroblast cell layer of three different proximodistal segments (proximal jejunum, distal ileum and proximal colon). The cell lines exhibited some variations in the expression of specific proteins: HGF/SF, $TGF\beta_1$ and epimorphin (a mesenchymal protein essential for epithelial morphogenesis (Hirai *et al*. 1992)) and retinoic acid receptors, in accordance with the corresponding proximodistal expression patterns *in situ* (Plateroti *et al*. 1998). The physiological significance of these differences remains to be analysed.

In parallel, human mesenchymal clonal cell lines derived from normal duodenal biopsies have been established and characterized. Interestingly, two of them (C9 and C11) display differences similar to those described for F1G9 and A1F1 as regards to their sensitivity to cytokines and their induction of epithelial proliferation, assessed on endodermal or Caco-2 cells (C. Fritsch and M. Kedinger, personal communication).

From these data, one can conclude that phenotypically different mesenchymal cell clones have the ability to induce variable morphogenesis, proliferation versus differentiation rates and also homeobox gene expression in the associated epithelial cells. These results can be related to two recent papers (Hentsch *et al*. 1996; Kaestner *et al*. 1997) that report the expression in the intestinal mesenchyme of the homeobox gene *Hlx* and the transcription factor *Fkh6*, and their ability to regulate the mitogenic signal of the visceral endoderm during development. Indeed, targeted disruption of *Hlx* genes is associated with a deficient gut elongation and looping, while disruption of *Fkh6* is associated with expanded, branched crypts and lengthening of the villi with an overall altered architecture and cytodifferentiation. Thus, these two regulatory genes appear, respectively, to control positively and negatively the epithelial cell proliferation. According to Kaestner *et al*. (1997), BMP2 and BMP4 (bone morphogenetic proteins, which are members of the TGFβ family) could be the targets of *Fkh6*, that are implicated in the signalling pathway from the mesenchyme to the epithelium. Moreover, it is worth noting that TGFβ produced by fibroblasts induce T84 cells, derived from a colon carcinoma lung metastasis, to form organized luminal structures composed of polarized cells (Halttunen *et al*. 1996).

Owing to the differential inductive properties on the epithelial cell behaviour of subsets of mesenchymal cells (see above), to their sensibility to cytokines or to other stimuli, as well as their subsequent ability to induce proliferation of lymphocyte populations (Griebel *et al*. 1993; Hogaboam *et al*. 1997; Roberts *et al*. 1997), one can hypothesize that the epithelial–mesenchymal cross-talk is regulated, at least partly, by the interactions between mesenchymal cells and lymphocytes. Indeed, an imbalanced cytokine rate may lead to changes in the relative proportion of various mesenchymal cell phenotypes, which could provoke impaired interactions with the epithelial cells and, as a consequence, tissue injury. The marked increase of the inflammatory cytokines in chronic inflammatory bowel disease (Crohn's disease (CD) and ulcerative colitis) could be at the origin of such an impaired mesenchymal-cell steady state. This

hypothesis is currently being tested in the laboratory by studying the effect of cytokines produced by CD biopsies on mixed cultures of two types of differentially sensitive, human mesenchymal cell lines (C9 and C11) labelled with two different mutants of the green fluorescent protein.

Changes in tissue homeostasis resulting from phenotypic modifications of the mesenchymal cell compartment can be correlated with variations in the mesenchymal cell production/degradation of extracellular matrix molecules (collagens and other connective tissue proteins) under the influence of various cytokines in inflammatory bowel disease (for a review, see Kedinger *et al.* (1996)). These processes may contribute to the epithelial lesions as well as to the fibrotic complications found in these pathologies. Alternatively, changes in the expression of extracellular matrix or adhesion molecules, as well as overexpression of regulatory peptides such as TGFβ_1 and KGF by the lamina propria cells in inflammatory bowel disease (Babyasky *et al.* 1996; Brauchle *et al.* 1996), may favour the epithelial repair by increasing cell migration or proliferation (Podolsky *et al.* 1996). The role, and regulation by cytokines, of (myo)fibroblasts in the alteration of the extracellular matrix microenvironment during colon tumour formation and invasion represent also a major field of interest for the understanding of pathological states linked to impaired epithelial–mesenchymal interactions (Martin *et al.* 1996).

(b) *Mesenchyme-mediated regulation of epithelial cell behaviour and of morphogenesis*

It is well known that the developmental programme of the gut may be influenced by hormones; in particular, glucocorticoids (GC) have been shown to induce precocious maturation of the foetal and postnatal intestines (for reviews, see Kedinger *et al.* (1989) and Henning *et al.* (1994)). In previous experiments, we showed that GC-induced, precocious lactase expression in 14-day foetal endodermal cells occurred only when the cells were co-cultured with intestinal or skin fibroblastic cells; in these conditions GC induced a precocious deposition of basement membrane molecules. These experiments allowed us to conclude that, at least at these early stages of development, the effect of GC on epithelial maturation was mediated by an action of these hormones on the ability of the mesenchymal cell to organize cell-surface laminin molecules (Simo *et al.* 1992*b*), or that mesenchymal cells make the epithelial cells competent to respond to GC. The involvement of thyroid hormones in the development of the small intestine has also been suggested in various experimental models (see Henning *et al.* 1994). Recently their involvement has been demonstrated more directly: the homozygous inactivation in mice of the T3Rα gene encoding a thyroid hormone receptor results in a strongly delayed intestinal maturation (Fraichard *et al.* 1997). The most important effect was found in the distal small intestine where both the height of the crypt–villus unit and the production of digestive enzymes was significantly reduced, and the depth of the muscle layers was strongly altered. Interestingly, TR3α-receptor expression was highest in the mesenchyme-derived muscle coat of the distal intestine (Fraichard *et al.* 1997), suggesting that the mucosal failure may be mesenchyme mediated.

We were also interested by the potential effect of retinoic acid (RA) on intestinal characteristics. Indeed, although the differentiating and morphogenetic properties of retinoids during development are well known (Gudas 1994), they have not been documented in the gut up until now. We showed that RA treatment of 14-day foetal rat intestines maintained in organ culture or developed as grafts resulted, respectively, in precocious formation of villi and epithelial differentiation, and in an increase of crypt cell proliferation and a thickening of the outer muscle layers (Plateroti *et al.* 1997). These effects of RA were paralleled by a stimulation in the expression of several cytoplasmic and nuclear receptors, among which some were found to be expressed in the mesenchyme (CRBP-I and RARγ), and of laminin α_1 and β_1 constituent chains. An *in vitro* comparison of the effect of RA on (i) co-cultures of foetal intestinal endodermal cells and intestinal myofibroblastic cells, and (ii) both cell types cultured in isolation, allowed us to observe that RA stimulated the polarization of the epithelial cells as well as the expression of lactase and *Cdx-1* genes only in the co-cultures; in addition, RA increased the expression of RA receptors and of laminin α_1 and β_1 chains in the co-cultures as well as in the mesenchymal cells cultured in isolation. Taken together, these data indicate that the differentiating and morphogenetic effects of RA in the gut may involve the mesenchymal cell compartment, which again exhibits its ability to modulate the pattern of epithelial homeobox gene expression (Kedinger *et al.* 1997). Concerning the mesenchymal involvement in the response to RA, it is also worth noting that, in a completely different system, i.e. arterial repair after injury, CRBP-I has been found to be a marker of smooth muscle cell activation and may play a role in tissue repair (Neuville *et al.* 1997).

4. CONCLUDING REMARKS

The gut development and mature steady state involve reciprocal inductive interactions between both epithelial and mesenchymal cells that allow pattern formation, maintenance of the stem cell population and regulated proliferation and differentiation. As in other organs that are under similar cell control, we are at the very beginning of the understanding of the molecular nature of the cellular cross-talk. It appears clear that the epithelial–mesenchymal unit is controlled by paracrine, hormonal and exogeneous factors. The major findings that have been reported in the past few years include: (i) specific mesenchyme or epithelial homeobox genes/transcription factors control epithelial cell proliferation; (ii) the TGFβ-BMP family of proteins may be involved in the control of mesenchymal cell differentiation on the one hand, and of epithelial cell proliferation/differentiation and migration on the other; and (iii) basement membrane molecules (shown for laminin), produced by both epithelial and mesenchymal cells, act via key regulatory genes, i.e *Cdx-2*, on the expression of cell–cell and cell–matrix adhesion molecules, and of differentiation and functional proteins. These key genes may also regulate the expression of other homeobox genes; this is exemplified by the *Cdx-2*-induced downregulation of *Cdx-1*. The challenge for the future will be to use the cellular and *in vivo* models to study further the link between the different regulatory pathways and to

integrate the experimental observations and our knowledge of the morphofunctional characteristics of the gut to understand some pathological alterations and to propose new therapies.

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