Differentiation of columnar epithelia: the hensin pathway

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Summary

Epithelia, the most common variety of cells in complex organisms exist in many shapes. They are sheets of polarized cells that separate two compartments and selectively transport materials from one to the other. After acquiring these general characteristics, they differentiate to become specialized types such as squamous columnar or transitional epithelia. High density seeding converts a kidney-derived cell line from flat 'generic' epithelial cells to columnar cells. The cells acquire all the characteristics of differentiated columnar cells, including microvilli, and the capacity for apical endocytosis. The high seeding density induces the deposition of a new protein termed hensin and polymerization of hensin is the crucial event that dictates changes in epithelial phenotype. Hensin is widely expressed in most epithelia. Its deletion in mice leads to embryonic lethality at the time of generation of the first columnar epithelium, the visceral endoderm. Moreover many human cancers have deletions in the hensin gene, which indicates that it is a tumor suppressor.

Key words: Hensin, DMBT1, Epithelial terminal differentiation, Columnar epithelia

Introduction

For organisms composed of a sphere of cells, growth is limited by the diffusion of oxygen. This was the likely reason for the appearance of epithelia, which allowed organisms to trap a small portion of the primitive oceans and to alter its composition to make the first *milieu interieure*. Hence, along with germ cells, epithelia must have been one of the first differentiated cell types to evolve in multicellular organisms. Genetic analysis suggests that sponges might represent the first metazoans, although some researchers have proposed that *Trichoplax* is closer to the 'Urmetazoan' than are sponges (Schierwater, 2005). Sponges and *Trichoplax* have a simple body plan, containing only a few types of epithelial and germ cells. A brief look at the catalogue of cell types in humans reveals a few hundred cell types, perhaps as many as three quarters of them being epithelia.

During embryonic development, the rudiments of most organs contain what look like generic epithelia, flat cells that are connected by tight junctions and adherens junctions rest on a basement membrane and exhibit apical-basal polarity. We can term these cells proto-epithelia in part because all other epithelia originate from them. In mature organs, the shape of cells is quite different and characteristic of the organ in question. There are 'simple' epithelia, i.e. epithelia composed of a sheet of single cells. Some of these are squamous (such as endothelia), others are cuboidal (e.g. in the kidney tubules) or columnar (e.g. in the intestine). There are also multi-layered epithelia such as transitional (e.g. urinary bladder) and stratified squamous epithelia (e.g. skin).

In some cases these terminally differentiated cells and their less differentiated precursors exist simultaneously. In the small and large intestine, the epithelial sheet is organized into characteristic folds to form crypts and villi. Crypts contain stem cells that can generate all four types of epithelial cells of the intestine (Marshman et al., 2002). In the prostate, basal cells also thought to be stem cells co-exist with luminal cells that are their differentiated progeny. Similarly in the skin, basal cells generate the squamous epithelium that is destined to be cornified. In each organ, the morphology of the less differentiated cell is clearly different from that of the terminally differentiated one and their gene expression patterns of course differ. It is difficult to define characteristics common to all terminally differentiated epithelia but not their precursors. However, at least in simple epithelia, we can provide a preliminary list. Perhaps the most obvious difference is in the apical compartment of the cell. For instance, embryonic pancreatic acinar cells or mucus secreting goblet cells have no secretory granules but fully differentiated mature ones do. Another obvious difference is the presence of brush border microvilli in the intestine: crypt cells have few microvilli whereas the absorptive epithelia of the villus (or surface in the colon) have exuberant microvilli. Microvilli contain cytokeratins, actin and actin-binding proteins such as villin. Although some crypt cells express these proteins, their organization into a subapical terminal web is a characteristic of absorptive cells.

Since all of these cell types originate from proto-epithelia, one can posit a pathway of epithelial differentiation that goes from proto-epithelia to terminally differentiated epithelia. Defining this pathway is an important goal, because many cancer biologists believe that a block in terminal differentiation of epithelial cells can be a root cause of cancer. Various

molecules play a role, including members of the Wnt, FGF and BMP gene families. Here we discuss a key driver of this process, the extracellular matrix protein hensin.

Intercalated cells in the kidney

The collecting tubule of the nephron is composed of two epithelial cell types: the majority are principal cells responsible for transport of Na^+ and water; and the remainder are the intercalated cells dedicated to H^+ transport. Recent studies have shed significant light on the mechanism determining the epithelial cell phenotype. At one extreme is the β-intercalated cell, which has an apical Cl^-:HCO_3^- exchanger and a basolateral H^+ ATPase; hence this cell secretes HCO_3^- into the lumen of the tubule. The other extreme is the α-intercalated cell in which the H^+ ATPase is located in the apical membrane whereas the Cl^-:HCO_3^- exchanger is located in the basolateral membrane. Remarkably, the β-intercalated cell converts to an α phenotype when the tubule segment (or the animal in vivo) is exposed to an acid medium (Schwartz et al., 1985). The trans-epithelial transport of this segment also reverses direction from secretion of HCO_3^- to secretion of acid, thereby playing an important role in the regulation of the pH of the blood.

The β-intercalated cell can be established as an immortalized clonal cell line. Confluent epithelia formed by cells from this line secrete HCO_3^- from the apical side. Seeding them at low density on filters generates flat, large cells with minimal surface microvilli. These show no apical endocytosis and have no actin network underneath the apical membrane. Remarkably, when the same cells are seeded at high density and allowed to proceed to confluence, they assume a completely different phenotype: they form columnar cells that are twice as tall and develop robust apical endocytosis and a thick sub-apical actin cytoskeleton. The low-density cells are 'real' epithelia; i.e. they have polarized apical and basolateral membranes that contain different proteins (van Adelsberg et al., 1994) and lipids (van't Hof et al., 1997). They contain lateral and tight junctions but have no sub-apical actin and do not express villin or cytokeratin 19. By contrast, the high-density cells have sub-apical actin, and express cytokeratin 19 and villin (Vijayakumar et al., 1999) (Fig. 1). In vivo, α-intercalated cells have vigorous apical endocytosis whereas the β form cells have none. The β form has essentially no microvilli whereas the α form exhibits prominent apical folds and microvilli. In addition, examination of electron micrographs of the cortical collecting tubule shows that α-intercalated cells are taller, they jut into the tubule lumen whereas the β forms are more flat. High seeding density thus induces a transformation of β-intercalated cells similar to that seen in vivo when they convert into α-intercalated cells. Note that all of these studies were performed in a cell culture system where the inducing event is seeding cells at high density. During epithelial differentiation in vivo, all cells touch each other and hence they are at a constant density. Hence, the high density seeding must be spuriously activating a pathway that is normally acting using another signaling event.

Hensin, a phenotypic regulator of epithelial cells

How does simple seeding at high density cause this transformation? A factor located in the extracellular matrix of the high density cells converts them to columnar cells. This can be captured on filters and makes low-density cells acquire the same features as high density cells; they become taller and have sub-apical actin and also begin to express cytokeratin 19 and villin Fig. 1. The protein responsible for this change has been purified and is termed hensin.

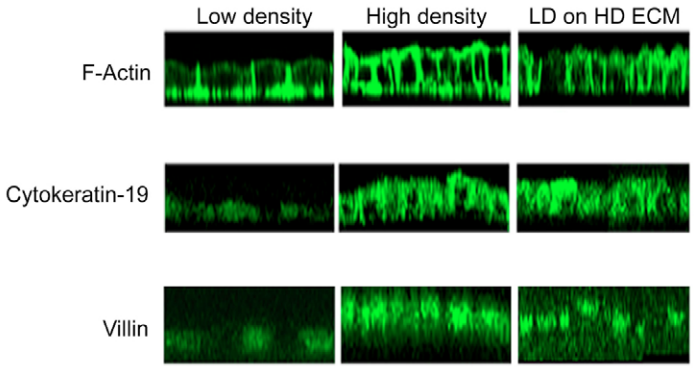

Fig. 1. Distribution of cytoskeletal proteins in low-density and high-density cells. *X-Z* Optical sections of the intercalated cell line cultured at low density, high density and at low density but on the extracellular matrix of high-density cells. The cells were stained by phalloidin and with antibodies to villin and cytokeratin 19. Note the cell height in high-density cells and in cells seeded on high-density matrix.

Hensin is a 180 kDa secreted glycoprotein that is expressed in all epithelia tested (Takito et al., 1999). It has a large number of alternatively spliced isoforms that vary in size up to an apparent molecular weight of 340 kDa (Fig. 2). In many organs, the more caudal a tissue, the higher is the level of expression; for instance, in the gastro-intestinal tract the highest level of expression is in the colon. In the kidney, hensin is expressed only in the ureteric bud lineage (also caudal) but there is no expression in the epithelia derived from the metanephric mesenchyme. Hensin is also expressed in the brain, in the lung, in the skin and even in macrophage derivatives such as osteoclasts.

Hensin contains three types of domain: SRCR (scavenger receptor cysteine rich), CUB (Clr-Uef-BMP1) and Zp (zona

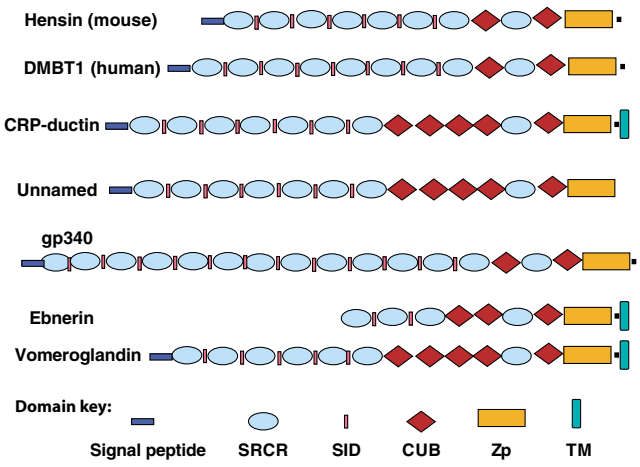

Fig. 2. Domain structure of hensin isoforms.

pellucida) (Fig. 2). The most abundant are the SRCR domains, which comprise 100-110 residues and are present in a large number of proteins in all metazoans, including sponges; but no specific function has been assigned to them (Sarrias et al., 2004). CUB domains are also ~110 residues long and were first identified in the complement components C1r and C1s. They are present in a large number of proteins but remain of unknown function (Bork and Beckmann, 1993). Zp domains (for Zona pellucida) were first identified as sperm receptors and are composed of about 280 residues. They are present in a wide array of organisms and proteins and they are thought to be involved in protein polymerization (Jovine et al., 2005).

The genomic sequence of hensin encodes 18 SRCR domains, 6 CUB domains and 1 ZP region, as well as a transmembrane domain. Each epithelium seems to express a specific splice form. The cDNAs of some of these isoforms reveal the presence of a membrane anchor, but in the intestine where this sequence is present all hensin forms are nevertheless secreted (Cheng et al., 1996); in other cases (such as the salivary form gp340) (Holmskov et al., 1999), hensin lacks the anchor and clearly is secreted. There have been some suggestions that secreted forms of hensin play a role in immune defense by binding to bacteria (Madsen et al., 2003), surfactants or trefoil factors (Thim and Mortz, 2000). However, most of these are based on in vitro binding studies, and given that many of the domains of hensin are protein-protein interaction domains it is difficult at present to decide whether these studies actually reveal physiological roles of hensin. Antibodies that recognize SRCR domains block the ability of hensin to induce formation of columnar epithelia, which suggests that these domains play a crucial role in either binding to receptors or to the formation of the hensin fibril (see below).

Polymerization of hensin

Remarkably, the low-density cells abundantly synthesize hensin and secrete it in a polarized manner from the basolateral side. However, they secrete only monomers of hensin whereas high-density cells produce an array of hensin forms from monomers to very-high-order polymers, only the highest order of which precipitate in the ECM (Hikita et al., 1999). Studies suggested that only these insoluble forms of hensin in the ECM are capable of converting cells displaying the low-density phenotype to columnar epithelia.

Thus, like other extracellular matrix proteins, hensin is synthesized as a soluble monomer but it is only the multimers that deposit in the basement membrane and mediate its functions. Some ECM proteins such as collagen need to be specifically proteolyzed to generate the insoluble forms. Others, such as fibronectin, require the cell to participate in the formation of the complex. In the case of hensin, at least three other proteins are required to form the active multimers. The first one identified was galectin 3. Galectin is a member of a large family of lectins that bind to β-galactosides. It can convert hensin monomers to dimers. Moreover, extraction of galectin 3 from insoluble hensin removes its ability to convert cells to columnar epithelia and adding galectin3 back restores this ability (Hikita et al., 2000). This suggests that galectin 3 not only stimulates formation of the hensin fibril, perhaps by making hensin dimers, but also might maintain these multimers in the correct orientation. Galectin 3 unlike other galectins contains a protein-protein interaction domain in addition to the carbohydrate-recognition domain. Hence, it is likely to induce multimeric association of glycoproteins. Interestingly the galectin-3-knockout mice are viable and fertile and do not seem to have any anomalies in epithelial organs (Colnot et al., 1998). All galectins are defined by the presence of homologous carbohydrate-binding domains; hence if their role in hensin polymerization is mediated by binding of the lectin to carbohydrate moieties, other members of this large family of proteins could substitute for galectin 3 in hensin polymerization.

A cis-trans peptidyl prolyl isomerase is also necessary for hensin polymerization and this could be a member of the cyclophilin family of prolyl isomerases (Watanabe et al., 2005). In addition, recent studies suggest that integrins need to be activated and that tyrosine phosphorylation of β1 integrin is crucial (our unpublished results). The order in which these three proteins act in hensin polymerization is not yet clear.

Conversion of β- to α-intercalated cells is mediated by hensin

The collecting tubule is a major site of regulation of acid-base transport in the kidney. When animals are fed an alkaline ash diet, it secretes HCO_3^- into the lumen. Feeding these animals an acid diet reverses the direction of the flux to absorption. This is accompanied by a decrease in the number of β-intercalated cells that secrete HCO_3^- and an increase in the number of acid-secreting α-intercalated cells, which absorb HCO_3^-. By measuring the intracellular pH of individual β-intercalated cells, one can demonstrate that the same cells that secrete HCO_3^- through an apical Cl^-:HCO_3^- exchanger before acid exposure begin to secrete acid and develop a basolateral Cl^-:HCO_3^- exchanger. Since the cells internalize the apical exchanger, they must also develop apical endocytosis (Schwartz et al., 2002). The cells that change their phenotypic state in this way also start laying down hensin in the extracellular matrix. Addition of anti-hensin antibodies prevents the acid-induced changes in the location of the Cl^-:HCO_3^- exchangers. Hensin antibodies also prevent the switch from the HCO_3^- secretion to HCO_3^- absorption seen on exposure to acid. Hensin thus not only can cause formation of columnar epithelia in culture but also drive a physiologically relevant change in the phenotype of intercalated cells. It is interesting that examination of published electron micrographs of β and α intercalated cells shows that the α intercalated is much taller than the β form suggesting that this process is also a columnarization event.

General role of hensin in differentiation in vivo

Since hensin is broadly expressed in various epithelial cells, it might play a general role controlling their phenotype. In the intestine, for example, proliferating cells that ascend the crypt wall differentiate as they go towards the surface. As they reach the villi (in the small intestine) or the surface (in the colon) they become terminally differentiated, acquiring all the characteristics of absorptive intestinal cells, including microvilli and a variety of markers of specific markers of differentiation (Crosnier et al., 2006). Hensin in differentiated cells is present in the villus, adopting a pattern similar to that of extracellular matrix proteins. By contrast, in the crypt cells it is cytoplasmic. Generation of conditional knockout alleles should reveal the details its role in these tissues, as well as the functions of hensin in other epithelia.

Hensin in cancer

Screens of a variety of human tumors have revealed that hensin is deleted in the majority of epithelial cancers. Much of this work was based on the discovery that certain malignant brain tumors lack the gene encoding DMBT1 (deleted in malignant brain tumor 1) (Mollenhauer et al., 1997), which was later found to be a human orthologue of hensin (Takito et al., 1999). The proportion of tumors lacking hensin/DMBT1 varies, in glioblastomas as many as half of them exhibit it, whereas in others the incidence is lower. Deletions have been found in skin, breast, oral, esophageal, colon and lung cancers by a variety of methods. Some of these studies have used cancer cell lines, but most also included resected tumors. There is a general idea in cancer biology that tumorigenesis requires that terminal differentiation of the epithelia be abrogated and, hence, the widespread deletion of hensin/DMBT1 in several tumors suggests it might be a tumor suppressor gene.

Hensin in embryonic development

A global deletion of hensin is embryonic lethal. Homozygous mutants fail to survive beyond embryonic day (E) 4.5, but blastocysts (E3.5) are obtained in appropriate mendelian proportions. Incorporating β-galactosidase into the hensin locus reveals that hensin begins to be expressed in the trophectoderm and is present at a low level in the embryonic stem (ES) cells. A few hours later the primitive endoderm begins to express hensin at a high level. This is a layer of epithelial cells that covers the ES cells in late blastocyst development; it eventually forms the visceral endoderm, an epithelial layer that surrounds the whole embryo, and parts of it form the placenta (Fig. 3). The first cells that express hensin are these extra-embryonic tissues. The primitive endoderm is crucial for assuring the normal development of ES cells into the embryo proper. In hensin-deficient mice it has a large number of apoptotic cells. The death of the embryo might thus be due to a defect in ES cell survival resulting from abnormal primitive endoderm development. Culturing blastocysts in vitro confirms this: the mutant embryos all degenerate whereas those taken from the wild-type and heterozygous mice survive (Takito and Al-Awqati, 2004).

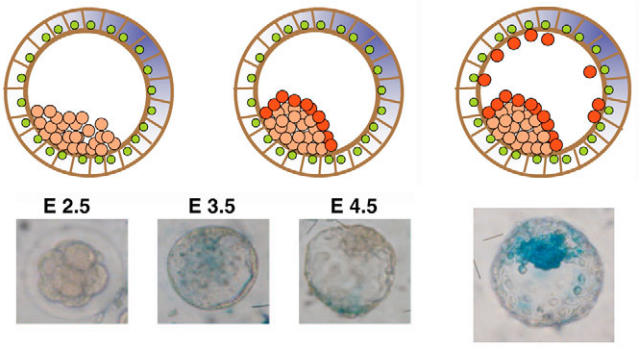

Fig. 3. Development of the mouse embryo between E3 and E4. The upper panels are a schematic of the blastocyst development. A layer of cells (in red) covering the ES cells (orange) becomes the epithelium of the primitive endoderm. Later, some of its cells will populate the inside layer of the trophectoderm to form the parietal endoderm whereas that covering the ES cells will form the visceral endoderm. In the lower panels are embryos taken from mice with *LacZ* inserted into the hensin locus. The embryo on the right was removed at E3.5 and cultured for 24 hours in vitro. Hensin expression begins in the ES cells (E3.5) and eventually is concentrated in the primitive endoderm.

Once the blastocyst is implanted, the ES cells begin to divide and the embryo elongates to form the egg cylinder stage. Hensin is expressed in the visceral endoderm (VE). The top part of this is the extra-embryonic VE. The bottom half is the embryonic VE, which regulates the epiblast – i.e. the embryo proper (Fig. 4). The extra-embryonic VE is a columnar epithelium. The embryonic VE is a squamous epithelium except at the tip, where a few cells are columnar. Hensin is expressed only in the columnar epithelial cells. Remarkably, these few columnar cells (distal VE) migrate upwards and within a few hours become squamous epithelia (anterior VE) and cause that region of the epiblast to become the head (Srinivas et al., 2004). Blocking the movement of VE by knocking out the transcription factor Hex, which is normally expressed in these cells, results in truncation of the head elements (Martinez-Barbara, 2000).

Hensin might thus be responsible for generation of columnar epithelia in the early embryo in vivo. ES cells seeded on filters coated with fibronectin and collagen IV grow as a monolayer expressing some epithelial proteins but not in a pattern that suggests polarized differentiation. By contrast, when grown on laminin and hensin, the ES cells adopt a morphogenetic pattern similar to embryoid bodies, forming hemi-spheres on the filters. The surface epithelium of the hemi-spheres is covered by a polarized epithelium that exhibits an apical cytoskeleton composed of actin, cytokeratin 19 and villin. The surface epithelia also display vigorous apical endocytosis, whereas ES cells grown on fibronectin and collagen IV do not internalize the marker (Takito and Al-Awqati, 2004).

These studies suggest that seeding ES cells on hensin or laminin results in a differentiation event. However, the surface epithelium of the hensin-grown hemispheres is columnar and thus similar to the intercalated cell line described above. Remarkably, the cells on the surface of the hemispheres seeded on laminin are instead flat, low and squamous. Both of these epithelia (but not those seeded on collagen IV or fibronectin) express VE markers such as BMP2 and transthyretin. However, cells seeded on laminin express α-fetoprotein, another marker of the VE. Another difference between these two types of epithelia is the expression of desmosomal proteins. Desmocollin is expressed in hemispheres grown on hensin; those grown on laminin instead express desmoglein. In embryos at the egg cylinder stage desmoglein is seemingly expressed in all VE cells. Note also that the hemispheres seeded on laminin are easily detached whereas those seeded on hensin are much more firmly attached. Since the columnar epithelia in the distal VE migrate to form the anterior VE, which is rather squamous during migration, the cells might switch signaling from hensin polymers to laminin polymers? If so, how does this occur? They could degrade hensin and lay down laminin or replace one receptor by another or alter some other signaling event.

Future prospects

The study of the biology of hensin promises to uncover a crucial aspect of epithelial cell biology. The conversion of proto-epithelium to a differentiated epithelium under the

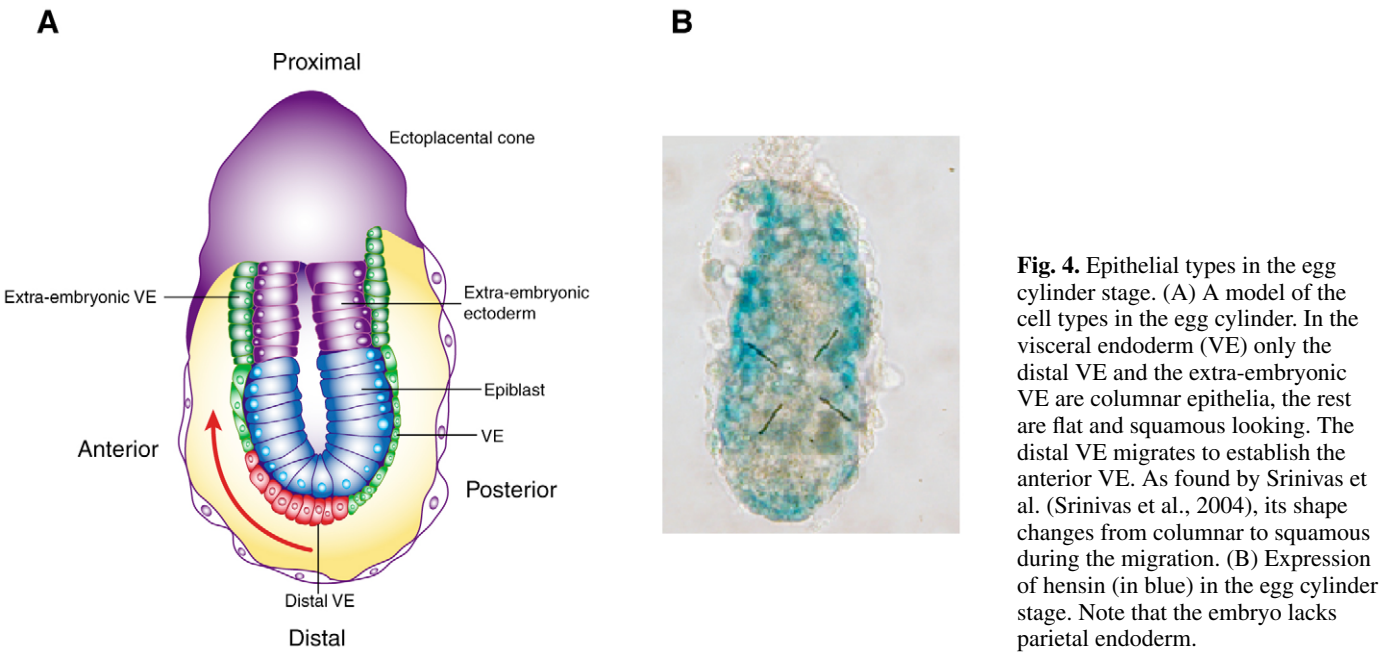

Fig. 4. Epithelial types in the egg cylinder stage. (A) A model of the cell types in the egg cylinder. In the visceral endoderm (VE) only the distal VE and the extra-embryonic VE are columnar epithelia, the rest are flat and squamous looking. The distal VE migrates to establish the anterior VE. As found by Srinivas et al. (Srinivas et al., 2004), its shape changes from columnar to squamous during the migration. (B) Expression of hensin (in blue) in the egg cylinder stage. Note that the embryo lacks parietal endoderm.

influence of hensin is probably the first such pathway discovered. Because hensin is also expressed in the skin (squamous epithelia), the urinary bladder and ureter (transitional epithelium), it may be involved in a more fundamental process of differentiation than conversion of simple proto-epithelia into columnar epithelial cells. That hensin seems to be deleted in a variety of epithelial cancers suggests that it has a dominant role in forcing the epithelium to maintain its differentiated state. All of these studies suggest that hensin plays a general role in epithelial differentiation; hence the identification of the signaling pathways involved acquires a new urgency. Hensin signaling can be divided into that which causes polymerization of hensin and that which involves binding of polymeric hensin to its receptor. A central question is whether hensin is central to a common pathway of differentiation or is a common intermediate in a more diverse set of pathways.

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