Endometrial Receptivity for Trophoblast Attachment: Model Studies Using Cell Lines

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SUMMARY

Implantation of the mammalian embryo confronts us with many unanswered questions with respect to its cell biological aspects. Embryo implantation is initiated by a cell-to-cell interaction between the receptive endometrium and the blastocyst which involves contact formation between uterine epithelium and trophoblast via their apical cell membranes. This epithelium-to-epithelium interaction leading to definitive adhesion of the embryo to the uterine wall is far from trivial and has been called a cell biological paradox. It has been proposed that some of the molecular events involved in epithelium-to-mesenchyme transition might play a role in development of adhesiveness of uterine epithelium for trophoblast and, vice versa, in development of trophoblast adhesiveness for uterine cells.

To get insight into molecular steps of development of uterine epithelial adhesiveness, we report experimental data obtained with human cell lines used as an in vitro model for human receptive and non-receptive uterine epithelium, respectively, i.e. RL cells allowing trophoblast cells to adhere to their apical plasma membrane and HEC cells not allowing trophoblast to adhere.

We show that adhesiveness of epithelial RL cells for trophoblast is correlated with a non-polarized phenotype of cells, with a modulation of their junctional complexes, and expression of adhesion-related molecules at their luminal surface. By extrapolation of the data, we suggest that restructuring of the epithelial organisation from a polarized to non-polarized architecture might be a prerequisite for adhesiveness of the luminal surface of uterine epithelial cells for human trophoblast.

INTRODUCTION

Implantation of the mammalian embryo is not only of fundamental importance for the establishment of pregnancy but it also exerts great fascination to basic scientists since it confronts us with many unanswered questions with respect to its cell biological mechanisms. Embryo implantation is a complex process involving a sequence of events including hatching and attachment of the blastocyst to the endometrium, followed in the human and many other species by invasion of the uterine wall. Over the last years, a number of parameters involved in implantation have been identified. Without reviewing all events, however, we will concentrate on endometrial receptivity. Our aim is not to provide a comprehensive review but to highlight aspects of how the surface epithelium of the endometrium allows adhesion of the blastocyst in the implantation initiation phase.

ADHESION AT THE APICAL SURFACE OF EPITHELIUM - A CELL BIOLOGICAL PARADOX

When examining the interaction between the receptive endometrium and the blastocyst in more detail, we are confronted with the fact that uterine epithelium and trophoblast epithelium make their first contact via their apical cell membranes (Fig. 1). However, apical cell membranes of such simple epithelia are supposed to be non-adhesive.

It is a typical property of simple epithelia to possess an apical-basal cell polarity. As one aspect of this, epithelial cells exhibit three distinct membrane domains, i.e. the apical, the lateral, and the basal plasma membrane domains. While basal and lateral membranes are studded with adhesion molecules so that they can mediate cell-to-cell and cell-to-matrix adhesion, apical plasma membranes normally lack most of these molecules and lack adhesive properties. Thus, the initial contact between uterine and embryonic tissues may be called a cell biological paradox.
Solutions for the paradox are found when taking a side view to processes in embryology that involve interaction of two epithelia, e.g. the fusion of palatal shelves or the fusion of endocardial cushions. At least some of these are combined with epithelium-to-mesenchyme transition [23, 24], for literature on other such processes see [13, 16]. Epithelium-to-mesenchyme transition is also discussed to be involved in tumor cell invasion [2]. It has been proposed that some of the molecular events involved in epithelium-to-mesenchyme transition may also be found in both, the acquisition of receptivity by the uterine epithelium and the expression of the invasive phenotype by the trophoblast [13, 14, 15, 16]. It has been argued, therefore, that the uterine epithelium when entering the state of receptivity and the trophoblast when acquiring invasiveness might modify their program of epithelial differentiation and downregulate at least part of it, most importantly the typical polar organisation of a simple epithelium.

CELL BIOLOGICAL ASPECTS OF ENDOMETRIAL EPITHELIUM IN VIVO

In several investigations it has been tried to define molecular changes in the composition of the plasma membranes of the uterine epithelium at receptivity. Peculiar properties that the uterine epithelium exhibits at receptivity comprise not only the apical cell pole but all domains of the plasma membrane and also the cytoskeleton.

The apical plasma membrane of the receptive uterine epithelium shows changes in lectin binding properties, displays a reduction of the thickness of the glycocalyx, a loss and/or a downregulation of marker enzymes for the apical plasma membrane domain, an increased density of intramembranous protein particles so that the values equal those typically found at the basolateral membrane domain, and an acquisition of receptors for matrix or cell surface molecules, for example HSPG [for reviews [14, 16, 31]. More interestingly, at the apical plasma membrane, the receptive uterine epithelial cells show the remarkable ability to form junctions that are otherwise typically found in the basolateral membrane compartments: reflexive gap junctions [32] and hemidesmosome-like junctions [12]. Formation of hemidesmosome-like junctions could be observed in a peculiar experimental condition in the rabbit, i.e. when implantation initiation was blocked by administering proteinase inhibitors into the uterine lumen. By this treatment the enzymatic dissolution of the extracellular blastocyst coverings was blocked. Therefore, remnants of blastocyst coverings remained interposed between the trophoblast and the uterine epithelium, and, surprisingly, the apical plasma membrane of the uterine epithelium formed hemidesmosome-like junctions with this peculiar type of extracellular matrix material that normally would have been dissolved.

The changes listed above allow to detect a loss of characteristics of the apical plasma membrane of polarized epithelia and a gain of properties that are more typical for the basolateral plasma membrane domain. This view can also be applied to changes seen in the lateral plasma membrane domain. For example, in the prereceptive phase rabbit uterine epithelium shows the typical polar organisation with respect to the localisation of tight junctional strands in the subapical junctional belt region. At receptivity, these strands proliferate, however, towards the basal cell pole while at the same time free apical protrusions develop [14]. The α6-integrin subunit is found to show a change of polar distribution in the opposite direction, i.e. from a predominantly basal localization apically to include the whole of the lateral plasma membrane, in the human [1]. Uvomorulin (E-cadherin) which tends to be maximally concentrated in the subapical junctional belt of polarized epithelia, shows a more equal distribution over the whole lateral plasma membrane around receptivity, and in certain areas of the rabbit implantation chamber it even becomes concentrated maximally at a very unusual location, i.e. at basal cytoplasmic processes that penetrate the basal lamina [17, 18]. The desmosome-associated protein desmoplakin shows the same changes [11].

At the basal pole of uterine epithelial cells, a reduction in strength of adhesion to the basement membrane is since a long time known to occur at receptivity in rats and mice [8, 10, 38, 40], but has originally not been related to changes in cell polarity. Both in
the human and the rabbit basal cytoplasmic processes of the uterine epithelium are seen to penetrate the basal lamina focally, in the rabbit most pronounced in the vicinity of the blastocyst but in the human already in the absence of a blastocyst, i.e. in the cycle around receptivity.

There is evidence that the changes in the uterine epithelium at receptivity are indeed very fundamental, since they do not only involve membrane phenomena of which one could immediately see a connection with trophoblast attachment, but also profound changes in the organization of the cytoskeleton. The uterine epithelium is one of the few epithelial types that normally express vimentin in addition to cytokeratins. Vimentin expression is strongly increased in the rabbit implantation chamber, and here the intracellular location changes from predominantly basal to predominantly apical.

These observations have led to the concept that receptivity represents a change in and/or a loss of the expression of the general epithelial phenotype of the uterine epithelial cells, similar to the molecular events involved in epithelium-to-mesenchyme transition in embryology. This latter process profoundly influences cell behaviour and may be governed by master genes which still have to be identified. Alternatively, post-transcriptional or even post-translational events may be even more relevant. However, it must be pointed out that application of this concept to uterine receptivity is still very hypothetical. Loss of polar organization along the apico-basal axis appears to be a common theme for all those systems. Changes in molecular parameters appear to be less consistent as far as data are available. The changes seen in uterine epithelium at receptivity do not seem to comprise the complete set of parameters typical for epithelium-to-mesenchyme transition. For example, loss of α6- and β4-integrin subunits and expression of α5- and β1-integrin subunits is found in epithelium-to-mesenchyme transition but is not seen in the uterine epithelium. However, the latter does show changes in expression of other integrin subunits and changes in the polar distribution of integrins. Up-regulation of vimentin is found in epithelium-to-mesenchyme transition and respective uterine epithelium. E-cadherin was reported to be down-regulated in epithelium-to-mesenchyme transition as well as in invasive tumor cells. Such down-regulation, however, is not seen in the uterine epithelium at receptivity. Data on other relevant parameters are still very incomplete for uterine epithelium or in case of syndecan and perlecain, partially contradictory.

**ADHESIVENESS OF ENDOMETRIAL EPITHELIUM FOR TROPHOBLAST IN VITRO**

Molecular steps leading to development of adhesiveness of uterine epithelial cells are difficult to investigate experimentally in vivo, and data on the in vivo situation in the human remain descriptive at best. Here, we use a cell culture system that may be useful for the study of development of adhesiveness of human uterine epithelial cells.

1. AN IN VITRO ASSAY FOR THE STUDY OF APICAL ATTACHMENT COMPETENCE OF EPITHELIAL CELLS

In our laboratory we established a centrifugal force-based adhesion assay that allows selection of uterine cells with different affinities for trophoblast. Using this assay, we have selected human endometrial cell lines which were classified apically non-adhesive (HEC cells) vs. adhesive (RL cells) for trophoblast-type cells. HEC cells and RL cells, respectively, were cultured to confluency on a coverslip. Multicellular spheroids were formed of human JAR choriocarcinoma cells which were used as the malignant counterpart of trophoblast. JAR cell spheroids were delivered onto the apical (luminal) surface of confluent uterine monolayers. After incubation, the confrontation culture was centrifuged, attached spheroids were counted, and the results expressed as the percentage of the number of spheroids added initially (Fig. 2). JAR cells attached with low efficiency to HEC monolayers but with high efficiency to RL monolayers either in the presence or the absence of serum. JAR cell attachment to HEC cells was comparable to attachment in the controls, i.e. human dermal fibroblast (HDF) monolayers or poly-D-lysine coated glass. JAR cells also failed to attach to MDCK cells (an epithelial cell line derived from kidney) and AN cells (a human endometrial tumor cell line with a non-epithelial phenotype) pointing to specificity of apical adhesiveness of RL cells in this assay (Fig. 2). These results suggest that monolayers which allow JAR cells to attach in this assay need to express certain epithelial properties (in contrast to AN and HDF) but that additional features are also needed since not all epithelial show this property (HEC and MDCK).

2. A NON-POLARIZED EPITHELIAL PHENOTYPE AS PREREQUISITE OF APICAL ATTACHMENT COMPETENCE

To get insight into the program underlying uterine epithelial apical (luminal) adhesiveness we characterized parameters of the epithelial phenotype of HEC and RL cells. Here, we argue that adhesiveness for trophoblast might be correlated with a non-polarized epithelial phenotype (RL cells) while non-adhesiveness might be correlated with a polarized epithelial
Fig. 2 - Apical adhesiveness of RL (RL95-2 cells), HEC (HEC-1-A cells), MDCK (MDCK cells), AN (AN3 CA cells), HDF (human dermal fibroblasts), and lysine coverslips (poly-D-lysine coated glass coverslips) for human trophoblast (JAR choriocarcinoma spheroids) as determined in the centrifugal force-based adhesion assay. The origin of cells and their ultrastructural phenotype is indicated. Adhesiveness in the presence and absence of fetal calf serum is expressed as the percentage of the number of spheroids seeded. Values are mean ± SEM.

phenotype (HEC cells).

To prove the epithelial nature of non-polarized RL cells we performed cell typing with respect to the expression of intermediate filament proteins. SDS-PAGE of the cytoskeletal preparations showed four major polypeptides of molecular weight 54000 Da, 52000 Da, 45000 Da, and 40000 Da. In Western blots with cytokeratin antibodies cytokeratins 7 (54000 Da), 8 (52000 Da), 18 (45000 Da), and 19 (40000 Da) were identified. Vimentin also gave a weak band (57000 Da).

The same pattern of cytokeratin polypeptides and also vimentin was found in cytoskeletal preparations from polarized HEC cells. Thus, a cytokeratin polypeptide pattern consisting of components 7, 8, 18, 19 as well as vimentin has been found for both, non-polarized RL cells and polarized HEC cells, indicating their origin from single-layered endometrial epithelium known to contain these cytoskeletal proteins.

In non-adhesive HEC monolayers the single cells showed a polarized epithelial phenotype with respect to the distribution of organelles and to membrane organization (Fig. 3). Nuclei were located at the base of the cells whereas mitochondria, endoplasmic reticulum and Golgi apparatus were located predominantly at the supranuclear region. The cells showed laterally closely apposed plasma membranes with tight junctions in the subapical region and adherens junctions and desmosomes scattered along the lateral membranes. The apical surface was cove-

red with microvilli which were relatively short. In contrast, adhesive RL cells showed ultrastructural features indicating lack of epithelial polarization (Fig. 4).

Nuclei were located in the center of the cell and organelles tended to pile up perinuclearly. Cells formed primitive adherens junctions but no tight junctions. The free surface of the cells appeared dome-like and was free of microvilli.

Lack of apical-basal cell polarity is also reflected in a peculiar arrangement of the cytoskeleton. After staining with TRITC-phalloidin, HEC cells showed spot-like actin staining associated with the microvilli of the apical plasma membrane, peripheral bands which surrounded the margin of the cells as well as stress fibers which ran longitudinally at the base of the cells (Fig. 5). A different actin organization was observed in RL cells (Fig. 5). In contrast to HEC cells, microvilli-associated staining as well as stress fibers could not be observed in RL cells. Nevertheless, cells showed actin staining along the entire cell surface and diffuse staining within the cytoplasm.

3. MODULATION OF JUNCTONAL COMPLEXES IN CONJUNCTION WITH APICAL ADHESIVENESS

Loss of epithelial cell polarity might be correlated with changes in cell contacts. We have thus mapped homotypic intercellular junctions (e.g. tight junctions, adherens junctions, and desmosomes) and functionally related proteins (e.g. ZO-1, E-cadherin, α-catenin, β-catenin, plakoglobin, and desmoplakin) in non-adhesive HEC cells and adhesive RL cells.

The lateral plasma membranes of adjacent HEC cells are aligned in parallel and form in their subapical part characteristic tight junctional membrane contacts consisting of a series of fusion spots (Fig. 6). In freeze fractures, the tight junction structures appeared as smooth strands on the protoplasmic face of the replica and as furrows on the exoplasmic face of the replica. Using immunohistochemistry, the tight junction protein ZO-1 was likewise found at the subapical part of the lateral border of adjacent cells. Ruthenium red added to the apical side of HEC monolayers stained intensely the apical cell surface including the membrane of luminal vesicles and the content of many apparently cytoplasmic vesicles, but no staining was observed beyond the level of the junctional contact points thus proving that these junctions were indeed an effective penetration barrier.

In contrast to HEC cells, no characteristic tight junctions were seen at the membrane contacts of adjacent RL cells in transmission electron microscopy (Fig. 6). Also in freeze fractures tight junction strands were never observed. In keeping with this, the tight junction-associated protein ZO-1 was not demonstrable in RL cells by immunohistochemistry. The tracer ruthenium red was found to be able to pe-
Fig. 3 - Transmission electron micrograph of HEC cells cultivated on poly-D-lysine coated glass. Cells show an apical-basal polarized phenotype with numerous microvilli at the apical cell pole. Asterisk: growth medium; arrows: microvilli; N: nucleus; OO coverslip. Bar: 1 μm.

Fig. 4 - Transmission electron micrograph of RL cells cultivated on poly-D-lysine coated glass. Cells lack apical-basal polarity. Asterisk: growth medium; N: nucleus; OO coverslip. Bar: 1 μm.

netrate in the lateral intercellular space from the apical compartment beyond the subapical region, indicating the lack of a fully developed tight junction barrier.

Moreover, adhesiveness might not only be correlated with presence or absence of tight junctions but also with adherens junctions. Conventional immunofluorescence microscopy revealed that E-cadherin, α-catenin, β-catenin and plakoglobin are expressed by HEC monolayers. Confocal microscopy showed that E-cadherin, α-catenin, β-catenin, and plakoglobin were confined to cell-to-cell contacts. When cell lysates from metabolically labelled HEC cells were subjected to immunoprecipitation using antibodies specific for E-cadherin, four major protein bands of molecular weight 120000 Da, 102000 Da, 88000 Da, and 80000 Da were detected. When such anti-E-cadherin immunoprecipitate gels were blotted and subsequently stained with anti-E-cadherin, anti-α-catenin, anti-β-catenin, and anti-plakoglobin, it was possible to identify the major bands as E-cadherin (120000 Da), α-catenin (102000 Da), β-catenin (88000 Da), and plakoglobin (80000 Da). Immunoprecipitates with antibody specific to α-catenin and subsequent immunostaining with appropriate antibodies revealed that E-cadherin, α-catenin, β-catenin,

Fig. 5 - Actin staining of HEC cells (a, b) and RL cells (c) with TRITC-conjugated phalloidin. HEC cells show fluorescence associated with microvilli (a; focussed at the apical plasma membrane), peripheral bands and stress fibers (b; focussed more deeply). RL cells show fluorescence localized within a submembrane cortex (c). Bars: 10 μm.
and plakoglobin were present as α-catenin complexes. Immunoprecipitates collected with anti-β-catenin or plakoglobin differed, however, insofar as either β-catenin or plakoglobin was not detectable in the alternative immunoprecipitate. This indicates that two different E-cadherin-catenin complexes are present in HEC cells, one composed of E-cadherin, α-catenin and β-catenin, the other of E-cadherin, α-catenin and plakoglobin. Both complexes were confined to cell-to-cell contacts. RL cells showed like HEC cells the presence of E-cadherin, α-catenin, β-catenin and plakoglobin. Confocal microscopy revealed that E-cadherin, α-catenin and plakoglobin were colocalized in all plasma membrane domains of these cells. On contrast, β-catenin was localized only in lateral membrane domains of adjacent cells in spite of the fact that otherwise these cells are non-polarized (see above). When cell lysates from metabolically labeled RL cells were subjected to immunoprecipitations with antibodies against E-cadherin, α-catenin, β-catenin and plakoglobin the same pattern of bands was obtained as with HEC cells. Thus, the comparison of the two cell lines leads to the conclusion that two different E-cadherin-catenin complexes are present in both cell types, one complex containing E-cadherin and α-catenin in association with β-catenin, the other E-cadherin and α-catenin in association with plakoglobin. However, the apically adhesive RL cells showed differential distribution of E-cadherin/plakoglobin complexes and E-cadherin/β-catenin complexes while the apically non-adhesive HEC cells did not.

Important questions also remain about whether and how distribution and formation of desmosomes could be related to acquisition of adhesiveness. In HEC cells, transmission electron microscopy showed typical desmosomes localized basally to the tight junctions (Fig. 6). By immunohistochemistry, desmoplakin could be identified in punctate arrays, restricted along cell-to-cell contacts, i.e. at the lateral plasma membrane. The apical plasma membrane was unlabelled. In adhesive RL cells, lateral plasma membranes usually lacked typical desmosomes which were only occasionally found (Fig. 6). Nevertheless, desmoplakin was demonstrable immunohistochemically and, in contrast to HEC cells, was not restricted to cell-to-cell contacts but also seen in the apical (luminal) and basal membranes.

In summary, RL cells (non-polarized) showed a lack of morphologically discernible tight junctions and of ZO-1 expression, tracer leakiness of the paracellular pathway, atypical features of adhesions junctions as well as a lack of well-formed desmosomes while HEC cells (polarized) showed integrity of the junctional complexes.

Thus, development of adhesiveness for trophoblast might be correlated with a modulation of tight junctions and/or remodeling of adhesions junctions and/or modulation of desmosomes, connected with the expression of a non-polarized phenotype as typical for RL cells.

**ADHESION-RELATED MOLECULES AT THE APICAL (LUMINAL) CELL SURFACE**

Successful binding between uterine cells and trophoblast must be expected to require not only that appropriate adhesion molecules are present but also that these are accessible to their ligand. The relative ability of cell surface molecules to gain access to their targets might be related to the level of sterical hindrance by the glycalyx. Figure 7 illustrates that HEC and RL cells show conspicuous differences in glycalyx thickness as shown by ruthenium red precipitation, i.e. a thick glycalyx in apically non-adhesive HEC cells contrasting with RL cells.

These observations imply that adhesiveness might be correlated with a reduction in thickness of the glycalyx. One component of the glycalyx is the cell surface-associated mucin MUC1. It has been proposed that a reduction of MUC1 at the cell surface may be correlated (and possibly causally connected) with the development of the receptive phenotype, at least in rodents, while increased MUC1 expression seen during the secretory phase in the human in vivo seems to contradict this. The present results obtained with endometrial cell lines are, on the other hand, consistent with such a view: MUC1 is expressed at high levels on HEC cells (Fig. 7) but

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**Fig. 6** - Membrane contacts of HEC cells (a, c) and RL cells (b, d) cultivated on poly-D-lysine coated glass. HEC cells but not RL cells show closely apposed plasma membranes with proper formation of tight junctions (t), adherens junctions (a) and desmosomes (d). Asterisk: growth medium; H1, H2 HEC cells; R1, R2 RL cells. Bars: 1 μm (a, b), 0.2 μm (c, d).

**Fig. 7** - Histograms of thickness of glycalyx of HEC cells and RL cells (a). Transmission electron micrograph of the surface of HEC cells (b) and RL cells (c) stained with ruthenium red to show the glycalyx. Conical vertical image of HEC monolayer (d) stained by antibody to mucin MUC1. Asterisk: growth medium; OD coverslip. Bars: 0.5 μm (b, c), 10 μm (d).

**Fig. 8** - Confocal images of HEC monolayers (a) and RL monolayers (b) after staining with antibody to α6-integrin subunit. Vertical sections reveal that HEC cells are labelled at sites of cell-to-cell contact, while RL cells are labelled at the entire plasma membrane. Similar distribution patterns were obtained after staining with other antibodies mentioned in the text. Arrows mark the position of cell-to-cell contacts. Asterisk: growth medium; OD coverslip. Bars: 10 μm.

**Fig. 9** - Apical adhesiveness of RL cells cultured on poly-D-lysine coated coverslips before (medium - CD) and after (medium + CD) perturbation of actin cytoskeleton via cytochalasin D. Adhesiveness in the presence of fetal calf serum is expressed as the percentage of the number of human trophoblast (JAR choriocarcinoma spheroids) seeded at the apical surface. For comparison, adhesiveness of poly-D-lysine coated coverslips for spheroids is shown. Values are mean ± SEM.
not on RL cells.

Integrins are discussed as candidate molecules that may mediate cell-to-cell binding between uterine epithelium and trophoblast 2, 41, 42. We analyzed the localization of integrin subunits α6, β1, and β4-44 which are also expressed in human uterine epithelium in vivo 1. We demonstrate that the apical (luminal) plasma membrane of adhesive RL cells contains integrins functionally linked to the cytoskeleton.

In HEC monolayers integrin staining was confined to the sites of cell-to-cell contacts and was absent from the apical cell pole (Fig. 8). In RL monolayers, integrin staining was evenly distributed over the whole cell membrane (Fig. 8). However, since even confocal light microscopy yields only limited details about integrin distribution within the plane of the membrane or about membrane confinement, we went to the electron microscopy level in order to search for microdomains. Integrins were found evenly distributed over the whole luminal RL cell surface at low density. Only occasionally, integrins were associated in small clusters. However, clusters revealed no ordered distribution within the plasma membrane 44.

More interestingly, adhesiveness of RL cells for trophoblast may be dependent on proper linkage of integrins to the actin cytoskeleton. Upon treatment with cytochalasin D the actin network was perturbed but general cell morphology and distribution of cell surface integrins remained unchanged. Adhesiveness of RL cells for trophoblast, however, was lost (Fig. 9). Thus, not only a proper organization of the actin cytoskeleton (Fig. 5) but also an intact interaction between cytoskeleton and integrins appears to be important for adhesiveness of the apical (luminal) surface of RL cells for trophoblast.

CONCLUDING REMARKS

Although many questions remain, we extrapolate from these in vitro data that development of adhesiveness of the apical (luminal) surface of human uterine epithelial cells for trophoblast in vivo might need - (i) an overall restructuring of the epithelial phenotype from a polarized to a non-polarized architecture, specifically: (ii) a non-polar arrangement of the actin cytoskeleton, - (iii) an apical localization of cell adhesion molecules linked to an intact actin cytoskeleton, - and (iv) a reduction in the thickness of the glycoalyx.

These data give further support to the hypothesis that, in vitro, reorganization of the epithelial phenotype of uterine cells prepares the apical (luminal) cell pole for cell-to-cell contact with trophoblast. It will be interesting to see whether these complex processes might be coordinated by master genes as known to be involved also in epithelium-to-mesenchyme transition, or to what extent they might depend on regulation at the post-transcriptional levels.

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