

## 293 cells express both epithelial as well as mesenchymal cell adhesion molecules

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**Abstract.** The 293 cell line, used extensively in various types of studies due to the ease with which these cells can be transfected, was thought to be derived by the transformation of primary cultures of human embryonic kidney cells with sheared adenovirus type 5 DNA. Although the 293 cells were assumed to originate from epithelial cells, the exact origin of these cells remains unknown. Previous attempts to characterize these cells combined immunostaining, immunoblot analysis and microarray analysis to demonstrate that 293 cells express neurofilament subunits,  $\alpha$ -internexin, and several other proteins typically found in neurons. These findings raised the possibility that the 293 cell line may have originated from human neuronal lineage cells. Contrary to this suggestion, in this study, we found that the 293 cells expressed N-cadherin and vimentin, which are marker proteins expressed in mesenchymal cells. Furthermore, the 293 cells also expressed E-cadherin, cytokeratins 5/8 and desmoglein 2, which are epithelial cell markers. When the cells, primarily cultured from the kidneys of Clawn miniature swine and passaged 10-15 generations [termed porcine kidney epithelial (PKE) cells] were examined, they were found to be positive for the expression of both mesenchymal and epithelial markers. Thus, transformation by adenovirus was not necessary for the cells to express N-cadherin. Occludin and zonula occludens (ZO)-1, two components of tight junctions in epithelial and endothelial cells, were detected in the 293 and the PKE cells. Thus, the findings of the present study demonstrate that 293 cells retain several characteristics of epithelial cells.

### Introduction

The 293 cells were thought to be derived from primary human embryonic kidney cells by transformation with sheared fragments of adenovirus 5 (Ad5) DNA and contain nucleotides 1-4344 of Ad5, comprising the early region 1 (E1) transforming sequences integrated into chromosome 19 (1,2). Since the isolation of these cells over 30 years ago, the 293 cell line has been widely used for the production of E1-deleted Ad vectors and in a number of transfection studies (3).

Although 293 cells express cytokeratins, which are epithelial cell markers, a previous study used a combination of immunostaining, immunoblot analysis and microarray analysis to demonstrate that 293 cells express neurofilament (NF) subunits,  $\alpha$ -internexin, and several other proteins typically found in neurons. These findings raised the possibility that the 293 cell line was derived from human neuronal lineage cells transformed by adenovirus (4). Although the presence of keratin proteins is characteristic of the early stage differentiation of epithelial cells and is unusual in neurons of the human or the rodent central nervous systems, these proteins are found in transformed lines of undoubted neuronal origin. For example, the rat PC12 line, derived from the adrenal gland, and NTera-2 cells derived from human embryonal carcinoma cells, express keratins, all three NF triplet protein subunits and vimentin (5-6). Human tumors, which contain NFs and keratins, have also been described (8).

Despite extensive analysis of neural cell marker expression in 293 cells, the expression of epithelial cell markers other than cytokeratins has not yet been studied, to the best of our knowledge. Herein, we examined the expression of E-cadherin, zonula occludens (ZO)-1, occludin and desmoglein 2, which are proteins involved in the formation and maintenance of cell-cell junctions (9-20). These proteins are components of the specialized junctional structure, consisting of the tight junction (occludin and ZO-1), the adherens junction (E-cadherin), and the desmosome (desmoglein 2). These structures are found at the apical area of lateral cell-cell contacts (9). Tight junctions are located in the most apical contact region, which constitutes the transepithelial permeability barrier. This unique junction is formed by the association of claudins and occludin (two transmembrane components of tight junctions (10,11) with ZO-1 and other cytoskeletal proteins (12,13). The adherens junction is located proximal to the tight junction. The main adhesion receptors

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*Abbreviations:* Ad5, adenovirus 5; E1, early region 1; MDCK, Madin-Darby canine kidney; NF, neurofilament; PBS, phosphate-buffered saline; PKE, porcine kidney epithelial

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within the adherens junctions are the classic cadherins (14), the cytoplasmic domain of which is associated with  $\beta$ -catenin (15,16).  $\beta$ -catenin, in turn, associates with  $\alpha$ -catenin to generate a three protein complex (17,18). Desmosomes are multi-unit, protein hetero-complexes that contain desmocollin and desmoglein, two glycoproteins of the cadherin family (19,20). They are located basal to the adherens junction and are associated with intermediate filaments. In simple epithelia, these three junction structures are typically aligned in the order described above, although desmosomes are also independently distributed throughout other areas of the cell membrane (19,20).

In the present study, we found that the 293 cells expressed N-cadherin, a cell adhesion protein expressed in neural cells. However, the 293 cells also expressed cytokeratins 5/8, as well as desmoglein 2, which are epithelial cell markers. Low expression levels of E-cadherin were detected in the 293 cells using immunoblot analysis, but not by immunostaining. The cells primarily cultured from the kidneys of Clawn miniature swine and passaged 10-15 generations [termed porcine kidney epithelial (PKE) cells], tested positive for the expression of cytokeratins and N-cadherin. Thus, transformation by adenovirus was not necessary for the cells to express N-cadherin. Occludin, an integral component of tight junctions in epithelial and endothelial cells, was detected in both the 293 and the PKE cells. Thus, the findings of the present study demonstrate that 293 cells retain several characteristics of epithelial cells.

## Materials and methods

**Cells and transfection.** The 293 cells were provided by Dr Tatsuhiko Furukawa (Department of Molecular Oncology, Kagoshima University, Kagoshima, Japan). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. DLD1, a human colorectal adenocarcinoma cell line, was provided by Dr Shintaro T. Suzuki (Kwansei Gakuin University, Kobe, Japan). The PKE cells, provided by Dr Takami Matsuyama (Department of Immunology, Kagoshima University), were cells primarily cultured from the kidneys of Clawn miniature swine [a swine strain established by Japanese scientists (21)] and passaged 10-15 generations. Madin-Darby canine kidney (MDCK) cells were provided by Dr Yasushi Daikuhara (Kagoshima University Dental School, Kagoshima, Japan). The expression vector encoding hemagglutinin (HA)-tagged full-length E-cadherin was prepared as previously described (22). The vector contains neo gene, which confers G418-resistance. As a control, an empty vector without E-cadherin gene was used yielding nH-2 and nH-6 clones. Transfection of 293 cells with the HA-tagged E-cadherin vector resulted in EH-5 and EH-13 clones. The cells ( $5 \times 10^5$ ) were transfected with the expression vector (10  $\mu$ g) using the calcium-phosphate transfection method as previously described (15), and stably transfected cells were selected in G418-containing medium. Isolated G418-resistant clones were tested for the expression of the transfected construct by immunofluorescence microscopy and immunoblot analysis.

**Antibodies and reagents.** Mouse monoclonal antibodies (mAbs) against E-cadherin (catalogue no. 610182),  $\beta$ -catenin (catalogue no. 610153), fibronectin (catalogue no. 610077) and plakoglobin ( $\gamma$ -catenin; catalogue no. 610253) were obtained

from BD Transduction Laboratories (Lexington, KY, USA). Pan-cadherin mAb (catalogue no. C1821-100UL) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal anti-occludin (catalogue no. 71-1500), anti-ZO-1 (catalogue no. 61-7300) and mouse monoclonal anti-vimentin (catalogue no. 18-0052) antibodies were purchased from Zymed Laboratories (South San Francisco, CA, USA). A mouse mAb specific for cytokeratins 5/8 (catalogue no. MAB3228) was acquired from Merk Millipore Ltd. (Tokyo, Japan). A mouse mAb against desmoglein 2 was obtained from Progen Biotechnik GmbH (Heidelberg, Germany). A rat mAb (3F10; catalogue no. 11867423001) directed against HA was purchased from Roche Applied Science (Mannheim, Germany). All secondary antibodies (fluorescein-, rhodamine- and peroxidase-conjugated) were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

**Immunoblot analysis.** Immunoblot analysis was performed essentially as previously described (22). Briefly, the cells were lysed by boiling in SDS sample buffer for 5 min. Proteins (30-50  $\mu$ g) were separated by SDS-PAGE and then transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were incubated with the appropriate primary antibodies diluted at 1:1,000, followed by incubation in horseradish peroxidase-conjugated secondary anti-mouse (catalogue no. 315-036-045) or anti-rabbit (catalogue no. 111-036-045) antibodies (Jackson ImmunoResearch Laboratories, Inc.) diluted at 1:1,000. Proteins were visualized using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

**Immunofluorescence staining.** Immunofluorescence staining of the cells was performed as previously described (23) with some modifications. The cells were cultured on coverslips for 48 h prior to fixation. The cells were then fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. Following 3 washes in PBS containing 50 mM  $\text{NH}_4\text{Cl}$ , the cells were permeabilized using 0.1% Triton X-100 in PBS for 5 min. After washing in PBS, the cells were soaked in blocking solution (PBS containing 5% fetal calf serum) for 15 min, and then incubated for 30 min with primary antibodies diluted in blocking solution. After washing 3 times in PBS, the cells were incubated with rhodamine- or fluorescein-conjugated secondary antibodies. The cells were analyzed as previously described (24) using an Olympus microscope.

## Results

**Compared with MDCK cells, PKE and 293 cells express reduced amounts of E-cadherin.** E-cadherin is a major cell adhesion molecule in epithelial cells and an epithelial cell marker (25). Although the immunofluorescence staining of MDCK cells, a typical epithelial cell line, with E-cadherin antibodies revealed strong membrane staining, the PKE and the 293 cells exhibited no membrane staining (Fig. 1A, upper panels). In a control experiment, we noted clear membrane staining for  $\beta$ -catenin, a cytoplasmic subunit of the transmembrane cell adhesion cadherin complex, in these 3 cell lines (Fig. 1A, lower panels).

The immunoblot detection of E-cadherin in these cells revealed that E-cadherin expression in the PKE and the

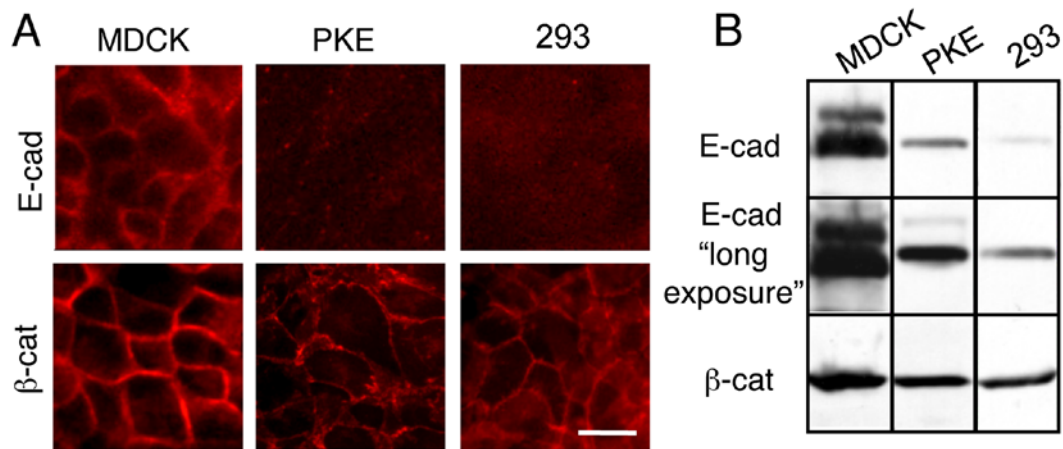


Figure 1. Detection of E-cadherin (E-cad) and β-catenin (β-cat) in Madin-Darby canine kidney (MDCK), porcine kidney epithelial (PKE) and 293 cells. (A) MDCK, PKE and 293 cells were cultured on coverslips for 24 h and then examined for E-cad or β-cat using immunofluorescence microscopy. Scale bar, 25 μm. (B) Cells were directly lysed in SDS-sample buffer, subjected to SDS-PAGE, and examined by immunoblot analysis with E-cad and β-cat antibodies. The lower panel of E-cad shows a longer exposure time (by a factor of five) as compared with the upper panel, to allow the detection of small amounts of E-cad in PKE and 293 cells.

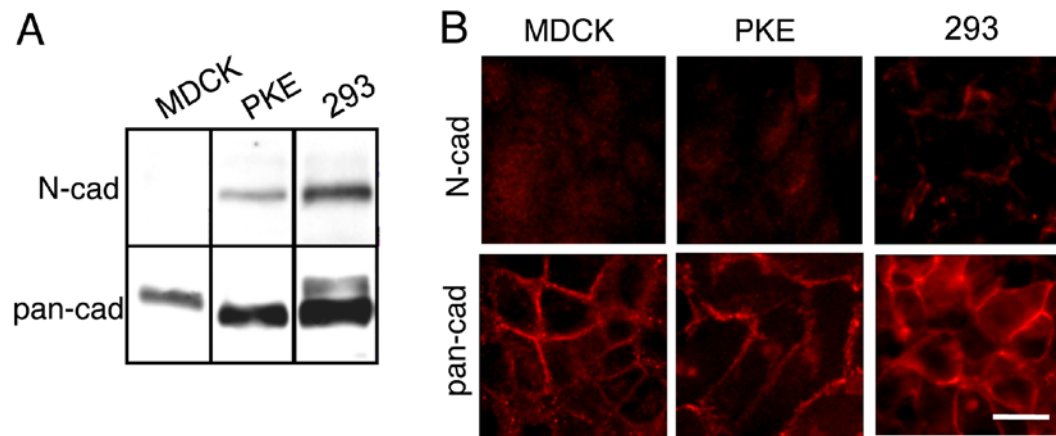


Figure 2. Detection of N-cadherin (N-cad) and pan-cadherin (pan-cad) in Madin-Darby canine kidney (MDCK), porcine kidney epithelial (PKE) and 293 cells. (A) Cells were directly lysed in SDS-sample buffer, subjected to SDS-PAGE, and examined by immunoblot analysis with N-cad or pan-cad antibodies. (B) Cells were cultured on coverslips for 24 h and then examined for N-cad and pan-cad antibodies using immunofluorescence microscopy. Scale bar, 25 μm.

293 cells was markedly decreased compared with its expression level in the MDCK cells (Fig. 1B).

**293 and PKE cells express N-cadherin.** The immunoblot detection of N-cadherin in the PKE and the 293 cells revealed high expression levels of N-cadherin (Fig. 2A). The MDCK cells, on the other hand, did not express N-cadherin (Fig. 2A), which was in agreement with previous findings (26). Immunoblot analysis using pan-cadherin antibodies raised against a synthetic peptide corresponding to the C-terminal amino acids of chicken N-cadherin, revealed that the PKE and the 293 cells expressed a protein with the same electrophoretic mobility as N-cadherin (Fig. 2A). Although the MDCK cells do not express N-cadherin, they express a protein that migrates slowly relative to N-cadherin. This protein has been shown to be K-cadherin (23,27). The 293 cells also expressed a protein with a slightly slower electrophoretic mobility (Fig. 2A). Although this protein has not been identified definitively, we conjecture that this protein is the precursor form of N-cadherin, containing the prosequence.

The immunofluorescence staining of the cells with pan-cadherin antibodies revealed clear membrane staining (Fig. 2B). Thus, the proteins recognized by the pan-cadherin antibodies were present on the membrane. No heterogeneity of expression within the cell lines was noted. These results are consistent with a previous observation that N-cadherin is expressed endogenously at cell-cell contact sites in 293 cells (28).

**293, PKE and MDCK cells express cytokeratins 5/8.** The MDCK cells exhibited strong and marked cytoplasmic and filamentous staining with a pan-keratin antibody mix containing mAb specific for keratin 5 and mAb specific for keratin 8 (Fig. 3A, upper panels). This staining pattern was also observed in the PKE and the 293 cells (Fig. 3A, upper panels). No heterogeneity of expression within the cell lines was noted as expected for typical kidney-derived cell lines and the 293 cells.

**293, PKE and MDCK cells express ZO-1 and occludin.** Although the decreased expression of E-cadherin and the unexpected expression of N-cadherin argue against the

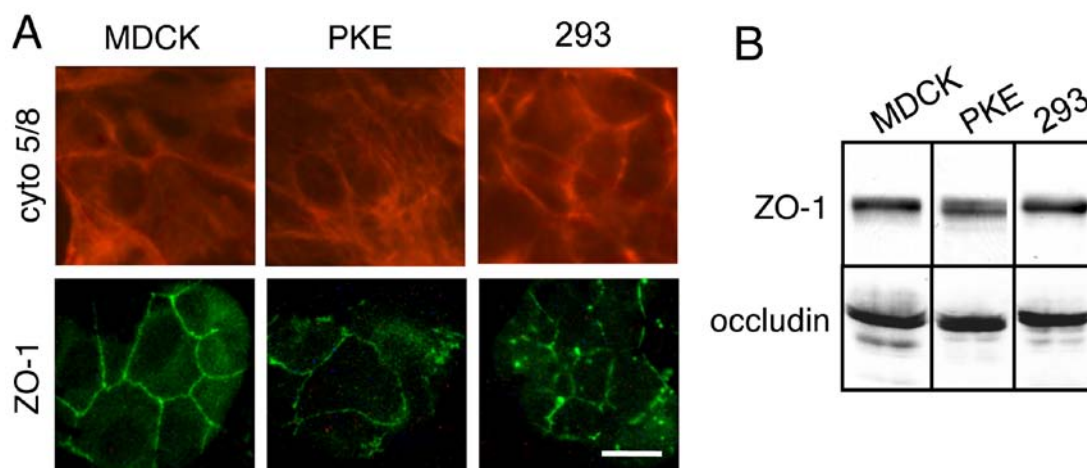


Figure 3. Detection of cytokeratin 5/8 (cyto 5/8), zonula occludens (ZO)-1, and occludin in Madin-Darby canine kidney (MDCK), porcine kidney epithelial (PKE) and 293 cells. (A) Cells were cultured on coverslips for 24 h and then examined for cyto 5/8 or ZO-1 using immunofluorescence microscopy. Scale bar, 25  $\mu$ m. (B) Cells were directly lysed in SDS-sample buffer, subjected to SDS-PAGE, and examined by immunoblotting with ZO-1 and occludin antibodies.

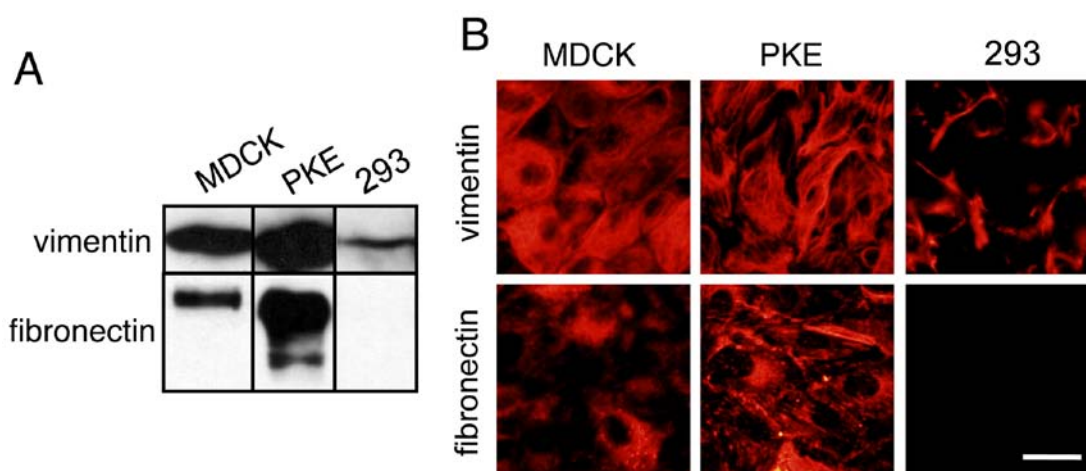


Figure 4. Detection of vimentin and fibronectin in Madin-Darby canine kidney (MDCK), porcine kidney epithelial (PKE) and 293 cells. (A) Cells were directly lysed in SDS-sample buffer, subjected to SDS-PAGE, and examined by immunoblot analysis with vimentin or fibronectin antibodies. (B) Cells were cultured on coverslips for 24 h and then examined for vimentin or fibronectin antibodies by immunofluorescence microscopy. Scale bar, 25  $\mu$ m.

epithelial cell origin of 293 cells, the expression of keratin 5/8 supports the notion that they are epithelial cells. To examine the epithelial nature of 293 cells, we determined the expression levels of ZO-1 and occludin in these cells. These proteins are components of tight junctions in epithelial and endothelial cells (11). Immunoblot analysis of the cells revealed that the PKE and 293 cells, as well as the MDCK cells, expressed ZO-1 and occludin (Fig. 3B).

Immunofluorescence staining of these cells with ZO-1 antibodies revealed strong membrane staining (Fig. 3A, lower panels). Thus, transport of the tight junction component, ZO-1, from its site of biosynthesis occurred. No heterogeneity of expression within the cell lines was noted. Immunostaining for occludin revealed similar membrane staining patterns (data not shown).

*MDCK and PKE cells express the mesenchymal markers, vimentin and fibronectin.* The immunoblot detection of vimentin in the MDCK and the PKE cells revealed high expression levels of vimentin (Fig. 4A). The 293 cells, on the

other hand, expressed vimentin at a reduced level (Fig. 4A). Immunoblot analysis of fibronectin revealed that the PKE cells expressed a large amount of fibronectin (Fig. 4A). Although the MDCK cells expressed a small amount of fibronectin, the 293 cells did not express detectable amounts of fibronectin.

Consistent with the results of the immunoblot analysis, immunofluorescence staining of the cells with vimentin and fibronectin antibodies revealed that the PKE cells expressed these proteins (Fig. 4B). The 293 cells expressed vimentin at a low level.

*Although desmoglein 2 is expressed in the 293 cells, it remains localized in intracellular compartments.* The immunoblot detection of desmoglein 2 in the 293 cells revealed high expression levels (Fig. 5A). Immunofluorescence staining of the 293 cells, however, revealed no membrane staining, although the staining of the DLD1 cells revealed clear membrane staining (Fig. 5B). Thus, desmoglein 2 remained localized in intracellular compartments. No heterogeneity of expression within the cell lines was noted.



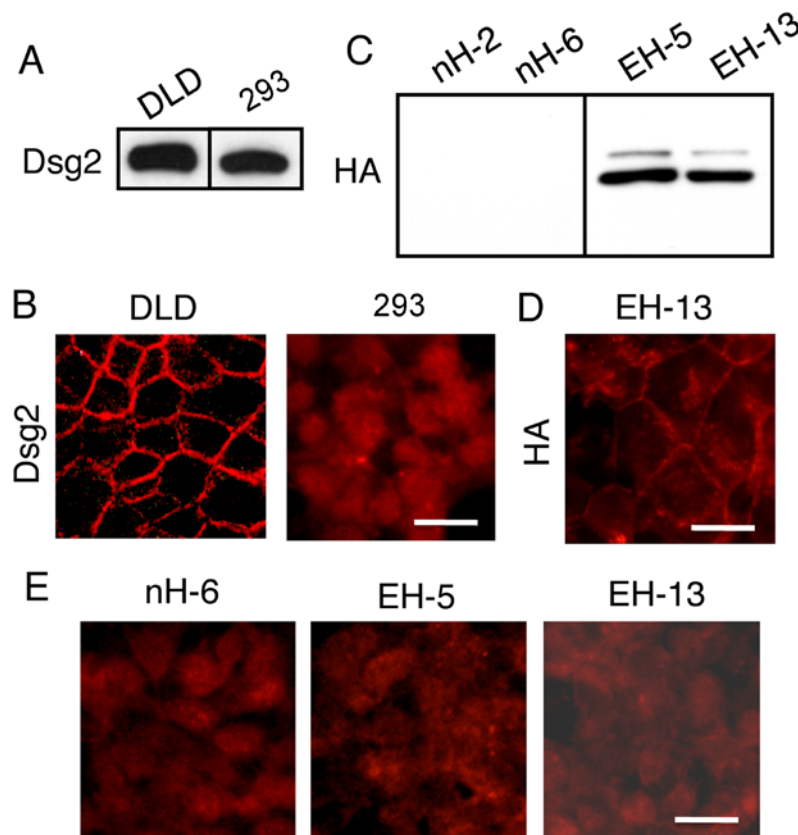


Figure 5. Detection of desmoglein 2 (Dsg2) in 293 cells. (A) DLD1 (colon cancer cell line) or 293 cells were directly lysed in SDS-sample buffer, subjected to SDS-PAGE, and examined by immunoblotting with Dsg2 antibodies. (B) Cells (DLD1 and 293) were cultured on coverslips for 24 h and then examined by immunofluorescence microscopy with Dsg2 antibodies. (C) 293 cells were transfected with an hemagglutinin (HA)-tagged E-cadherin expression vector. 293 cell clones that stably expressed HA-tagged E-cadherin (EH-5 and EH-13), and clones that were negative for E-cadherin (nH-2 and nH-6) were isolated. These clones were subjected to (C) immunoblot analysis or (D) immunofluorescence staining with anti-HA antibodies. (E) These cells were cultured on coverslips for 24 h and then examined with Dsg2 antibodies by immunofluorescence microscopy. Scale bars, 25  $\mu$ m.

These observations suggested that low E-cadherin expression levels were responsible for the failure of desmosome assembly. In an attempt to restore the localization of desmosomes in the membrane through increased E-cadherin expression, the 293 cells were transfected with the expression vector for HA-tagged E-cadherin and stable transfectants were isolated following selection with G418 (Fig. 5C). In these cells, E-cadherin was detected mainly on the surface membrane as revealed by staining with anti-HA antibodies (Fig. 5D). Immunofluorescence staining of these E-cadherin-expressing cells with desmoglein 2 antibodies revealed no membrane staining (Fig. 5E). Thus, E-cadherin expression in 293 cells is insufficient for the cell surface localization of desmoglein 2.

## Discussion

A thorough analysis of the 293 cells, thought to have been derived from human embryonic kidney cells that had been transformed by adenovirus 5 (Ad5), notably revealed that these cells express a variety of proteins (such as NF subunits) that are typically found in neural cells (4). The transformation of cells with Ad5, including the early region 1 (E1), has generated several human embryonic retinal cell lines (29), suggesting that Ad5 E1 may preferentially transform human neural lineages. Previous research has demonstrated that the efficient transformation of primary human amniocytes with

the E1 gene of human Ad5 yielded stable cell lines which exhibited the morphological features of epithelial cells (30); a thorough immunocytochemical analysis confirmed the expression of epithelial cell markers and the analysis also revealed the expression of neuronal and glial marker proteins, such as nestin, vimentin, A2B5 and glial fibrillary acidic protein (GFAP) (30). In agreement with previous studies on 293 cells, these results suggest that epithelial and neuronal marker proteins are co-expressed in E1-transformed human amniotic fluid-derived cells. 293 cells exhibit chromosomal abnormalities, containing less than three times the number of chromosomes of a normal diploid human cell (31). Structural genomic alterations produced during cultivation for decades in different laboratories have been proposed to underlie the sometimes different conclusions drawn from experimentation with 293 cell lines (31). Thus, these differences may be the reason why we obtained the data demonstrating that 293 cells have characteristics of epithelial lineage cells.

In the present study, we examined the expression of epithelial marker proteins in 293 cells. Moreover, epithelial features were also investigated in non-transformed PKE cells, as well as in MDCK cells as a control cell population. Our data revealed that the 293 and PKE cells homogeneously expressed the neural cell marker, N-cadherin. These cells were also found to express various epithelial marker proteins, cytokeratins 5/8, ZO-1, occludin and desmoglein 2. Strictly speaking, desmoglein 2 is

not a specific marker of epithelial cells, as it is expressed not only in epithelial cells, but also in various non-epithelial cells, such as myocardial and Purkinje fiber cells of the heart (32). However, desmoglein 2 is found in all cell types that possess desmosomes and is the only desmoglein detected in diverse tissues, such as simple and transitional epithelia (33). Non-epithelial cell lines, including human fibroblast, rhabdomyosarcoma and glioma cell lines, do not express desmoglein 2. To the best of our knowledge, there is no evidence at present to suggest that neural cells express desmoglein 2. As demonstrated by the present study for the first time to the best of our knowledge, the epithelioid characteristics of 293 cells were confirmed by the homogenous expression of the epithelial markers, cytokeratins 5/8, as well as by the expression of the epithelial-specific contact proteins, ZO-1 (33) and occludin (10). Although endothelial cells express ZO-1 and occludin, they do not express E-cadherin, which is detected in 293 cells.

In the assembly of epithelia, surface interactions between adhesion molecules of the cadherin superfamily nucleate a cascade of protein-protein interactions that leads to the formation of additional junctions including desmosomes and tight junctions (34). It is generally accepted that before the extracellular domains of cadherins are capable of mediating adhesion, the cytoplasmic domains must first bind to catenins inside the cell (16,35). The formation of this molecular complex confers adhesive strength by linking cadherins to the actin cytoskeleton and by clustering cadherin molecules, thus increasing the avidity of their interactions. We have previously demonstrated that an E-cadherin-expressing human colon carcinoma cell line lacking  $\alpha$ -catenin expression failed to organize desmosomes, and that the expression of  $\alpha$ -catenin in these cells by transfection resulted in the reorganization of desmosomes (36). Thus, the formation of desmosomes is dependent on the integrity of E-cadherin-catenin complexes.

N-cadherin has been shown to rescue some, but not all, functionalities of E-cadherin during selective embryogenic events (37); gene replacement experiments have revealed that the strength of cellular adhesion provided by N-cadherin is sufficient to mediate morula compaction; however, it is insufficient for the subsequent formation of a fully polarized functional trophectoderm. A previous study also demonstrated that the first desmosomes in mouse embryos are formed between trophectoderm cells in early cavitating blastocysts (38), and, therefore, we conjectured that N-cadherin may not act as a substitute for E-cadherin during desmosome formation. However, the attempted rescue by the exogenous expression of E-cadherin in 293 cells, failed to induce desmosome formation. Thus, it is clear that other factors are required for induction. In conclusion, the findings of the present study have established that 293 cells, by means of their epithelioid properties described herein, are suitable for use in studies on desmosome formation.

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