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Short Communication

α-Catenin-Deficient F9 Cells Differentiate into Signet Ring Cells

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It has been demonstrated that α-catenin is frequently lost in diffuse type adenocarcinomas. We have isolated α-catenin-deficient mouse teratocarcinoma F9 cells by gene targeting. Wild-type F9 cell aggregates cultured in the presence of retinoic acid differentiated into embryoid bodies with an outer layer of epithelial cells. In contrast, cell aggregates of α-catenin-deficient cells did not develop outer layers under the same conditions. The outer surface cells of α-catenin-deficient cell aggregates, however, differentiated into epithelial cells as determined by their expression of epithelial marker proteins. These differentiated cells scattered from aggregates and showed signet ring cell morphology, which is frequently observed in diffuse type adenocarcinomas. We have provided clear evidence that a single mutation in the α-catenin gene may be a direct cause not only of the scattered properties of cells but also of signet ring cell formation in diffuse type adenocarcinoma. *(Am J Pathol 1999, 154:1323–1328)*

α-Catenin associates with the carboxy-terminal region of the cadherin cytoplasmic domain via β-catenin to form a functional cadherin-catenin cell adhesion complex.[1] It has been demonstrated that α-catenin is frequently lost in diffuse type adenocarcinomas.[2–5] Loss of α-catenin expression has also been observed in some human adenocarcinoma cell lines.[6–11] The epithelial morphology and rigid cell adhesion activity were lost in these α-catenin-deficient carcinoma cells but restored by the exogenous expression of α-catenin.[11–14] These observations strongly suggest that loss of α-catenin function is involved in the scattered phenotype of diffuse type adenocarcinoma cells.[15]

Signet ring cell carcinomas have been described in most but not all series of diffuse type gastric carcinomas.[16] Frequent loss of α-catenin expression was observed in gastric carcinomas with scattered cell growth.[5] Loss of α-catenin expression was also observed in a signet ring cell gastric carcinoma cell line.[7, 17] However, the loss of α-catenin expression in colon cancer cell lines did not cause a morphological change of cells to signet ring cells.[10] Thus, it is not clear whether loss of α-catenin expression is involved in signet ring cell formation.

F9 is a clonal line of mouse teratocarcinoma-derived embryonal carcinoma cells that shows no epithelial cell morphology under standard culture conditions. However, F9 cultures treated with retinoic acid when they are in the form of small aggregates differentiate epithelial cells on the outer surface of aggregates.[18] Due to these properties, F9 cells are regarded as a good *in vitro* model system for the study of epithelium formation mechanisms. The present study was performed to explore the effects of loss of α-catenin expression on epithelial cell morphogenesis using α-catenin-deficient F9 cells.

Materials and Methods

Targeting and Expression Vectors

The 17-kb mouse α-catenin genomic clone (p16-2) was isolated from a 129/Sv mouse genomic library. For construction of the targeting vector, the PSIBP trap-selection cassette replaced the *Sma*I-*Xho*I fragment of the p16-2

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clone, which contains the 5′ half of an exon encoding β catenin binding site. In the PSIBP trap-selection cassette, splicing acceptor,[19] IRES,[20] and β-geo[21] sequences are tandemly connected and inserted between two lox P sequences.[22] For negative selection, the DTA gene replaced the 4.5-kb fragment following unique *Bst*PI site of the p16-2 clone.[23]

For construction of the α-catenin expression vector (pEFαFL), the 3.7-kb full-length α-catenin cDNA[24] replaced the *Xba*I-*Xba*I stuffer sequence of pEF-MC1neo.[25]

Gene Targeting

Mouse F9 EC cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum in gelatin-coated (0.2%) culture dishes. Cell aggregates, initially consisting of 2.5×10^3 cells per aggregate, were formed by the hanging drop culture method.[26] For induction of visceral endoderm differentiation, 5×10^{-8} M retinoic acid were added to the medium from the beginning of aggregate formation.

The targeting vector (25 μg) was linearized with *Sal*I and electroporated at 250 V and 960 μF using a gene pulser into 2×10^7 F9 cells in 0.4 ml of HEPES-buffered saline. Cells were subjected to G418 selection at 370 μg/ml for 1 week and at 100 μg/ml for another 12 days. For isolation of $F9^{D\alpha(-/-)}$ cells, the heterozygous clone $F9^{S\alpha(+/-)}$ (clone 13) was re-electroporated with the targeting vector as described above and then subjected to G418 selection at 650 μg/ml for 4 days, 360 μg/ml for 5 days, and 150 μg/ml for 5 days. To obtain $\alpha F9^{D\alpha(-/-)}$ cells, 20 μg of pEFαFL expression vector were electroporated into $F9^{D\alpha(-/-)}$ as described above, then subjected to G418 selection at 900 μg/ml for 14 days. Resistant clones were picked after selection. DNA was isolated from cultured cells as described[27] and tested for integration at the targeted locus by Southern blotting of *Hind*III digests. The probe was a 1-kb *Bst*PI-*Hind*III fragment located immediately downstream of the region of homology of the targeting vector (see Figure 1).

Antibodies

The following primary antibodies were used: function-blocking rat anti-mouse E-cadherin mAb (ECCD-1),[28] rat anti-mouse E-cadherin mAb (ECCD-2),[29] rat anti-mouse α-catenin mAb (α18)[30]; rat anti-mouse occludin mAb (MOC37),[27] mouse anti-β-catenin mAb (Transduction Lab., Lexington, KY), and mouse anti-cytokeratin 18 mAb (Ks 18.04, Progen Biotechnik, Heidelberg, Germany). As the secondary antibody, FITC-conjugated goat anti-rat IgG (Tago, Inc., Burlingame, CA) and rhodamine-conjugated goat anti-mouse IgG (Amersham Pharmacia Biotech, Buckinghamshire, UK) were used. Immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblotting analysis, and immunostaining were performed as described.[24]

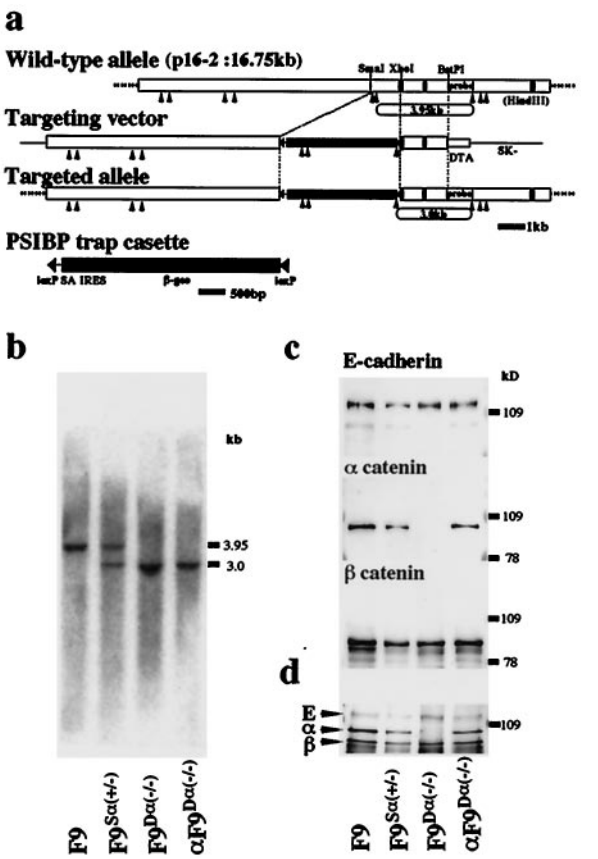

Figure 1. Targeted inactivation of the α-catenin gene in mouse F9 cells. a: The structures of the wild-type allele, targeting vector, targeted allele, and the PSIBP trap-selection cassette are shown together with the pertinent restriction sites. Closed boxes represent the three α-catenin exons in clone p16-2. Insertion of the PSIBP trap cassette resulted in generation of a new *Hind*III fragment of 3.0 kb in contrast to the 3.9-kb wild-type allele. b: Southern blotting analysis of DNA derived from each cell clone. Genomic DNA was digested with *Hind*III and analyzed using the *Bst*PI-*Hind*III fragment indicated in a as a probe. c: Western blotting analysis of each cell clone with anti-E-cadherin, anti-α-catenin and anti-β-catenin mAbs. d: Immunoprecipitation analysis of each cell clone with anti-E-cadherin mAb. E, E-cadherin; α, α-catenin; β, β-catenin.

Cell Dissociation and Aggregation Assay

Cell dissociation and aggregation assays were performed as described previously.[30, 31] In some cell aggregation experiments, ECCD-1 was added to the culture medium.

Partial Purification of Signet Ring Cells and Histochemical Analysis

Day 10 embryoid body culture medium of $F9^{D\alpha(-/-)}$ cells was settled in a centrifuge tube to remove large cell aggregates and the supernatant was then moved to another tube and centrifuged at 1000 $\times$ *g* for 5 minutes. The cell pellet with signet ring cells was washed and resuspended in HEPES-buffered magnesium-free saline. This signet ring cell-rich fraction was smeared on glass slides, air-dried, and fixed with 10% formaldehyde. Slides were stained with hematoxylin and periodic acid-Schiff.

Ultrathin Section Electron Microscopy

Embryoid bodies and pellets of signet ring cells were prepared for ultrathin sectioning as described previously.[27] Ultrathin sections were cut with a diamond knife, double-stained with uranyl acetate and lead citrate, and then examined using a 1200EX electron microscope (JOEL, Tokyo, Japan) at an acceleration voltage of 100 kV.

Results

F9 cells targeted at one α-catenin allele ($F9^{S\alpha(+/-)}$) were generated by replacing part of the α-catenin genomic DNA with the PSIBP trap-selection cassette in sense orientation (Figure 1a, see Methods). To isolate F9 cells homozygous for this replacement ($F9^{D\alpha(-/-)}$), $F9^{S\alpha(+/-)}$ cells were retransfected with the α-catenin-targeting vector by electroporation, then cultured in the presence of increasing concentrations of G418. Heterozygous and homozygous genotypes were demonstrated by Southern blotting (Figure 1b), and the loss of expression of α-catenin protein in $F9^{D\alpha(-/-)}$ cells was confirmed by immunoblotting with the anti-α-catenin monoclonal antibody (mAb) α18 (Figure 1c). Immunoblotting with anti-E-cadherin (ECCD-2) and anti-β-catenin mAbs showed that the levels of expression of these molecules in $F9^{D\alpha(-/-)}$ cells were comparable to those in parental F9 or $F9^{S\alpha(+/-)}$ cells (Figure 1c). Immunoprecipitation with anti-E-cadherin mAb showed that E-cadherin formed a complex with α- and β-catenin in wild-type F9 or $F9^{S\alpha(+/-)}$ cells (Figure 1d). Even in $F9^{D\alpha(-/-)}$ cells, E-cadherin formed a complex with β-catenin despite the absence of α-catenin (Figure 1d). Southern blotting analysis of DNA derived from $F9^{D\alpha(-/-)}$ cells using the neo fragment as probe showed that a single copy of the targeting vector was inserted in the α-catenin locus and random integration of the targeting vector did not occur (data not shown). To confirm that $F9^{D\alpha(-/-)}$ cells have no severe mutations other than the lack of α-catenin expression, we introduced an expression vector encoding full-length α-catenin into $F9^{D\alpha(-/-)}$ cells, then isolated cells re-expressing similar amounts of α-catenin to wild-type F9 cells, $\alpha F9^{D\alpha(-/-)}$. The expressed α-catenin was derived from the introduced expression vector because the targeted locus in $\alpha F9^{D\alpha(-/-)}$ cells retained the homozygous genotype (Figure 1b). In these $\alpha F9^{D\alpha(-/-)}$ cells, expressed α-catenin formed a complex with E-cadherin and β-catenin (Figure 1c). Because these $\alpha F9^{D\alpha(-/-)}$ cells were indistinguishable from the wild-type F9 cells in all assays performed here (see below), one of the clones, $\alpha F9^{D\alpha(-/-)}$-4-4, was used as a representative line of normal F9 cells throughout subsequent experiments.

In low density monolayer culture, $\alpha F9^{D\alpha(-/-)}$ and $F9^{D\alpha(-/-)}$ cells showed different morphologies. $\alpha F9^{D\alpha(-/-)}$ cells formed small and compact colonies (Figure 2a) similar to those of parental F9 cells. In contrast, $F9^{D\alpha(-/-)}$ cells showed a scattered morphology, suggesting dysfunction of their cadherin-catenin adhesion system (Figure 2f). In fact, cell dissociation assay showed

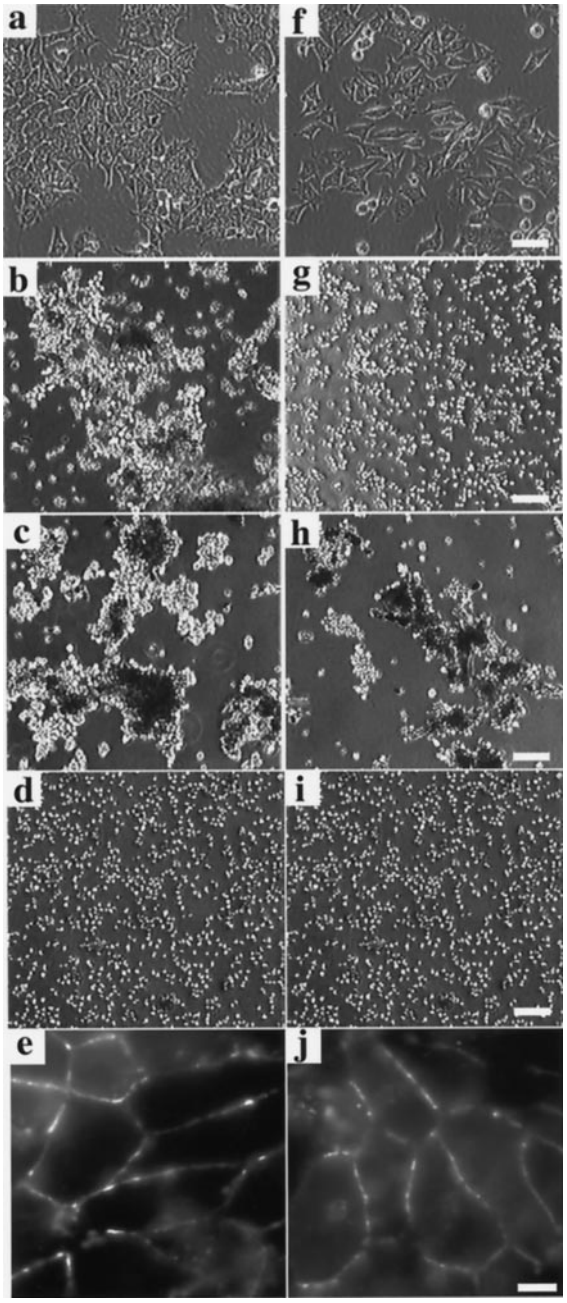

Figure 2. Cell adhesion properties of α-catenin-deficient cells. **a–e**: $\alpha F9^{D\alpha(-/-)}$, α-catenin-positive control cells. **f–j**: $F9^{D\alpha(-/-)}$, α-catenin-deficient cells. **a** and **f**: Phase contrast images. Bar, 100 μm. **b** and **g**: Cell dissociation assay. Bar, 100 μm **c**, **d**, **h**, and **i**: Cell aggregation assay. Bar, 100 μm. In **d** and **i**, function-blocking anti-E-cadherin mAb, ECCD-1, was added to culture medium. **e** and **j**: Immunostaining with the anti-E-cadherin mAb ECCD-2. Bar, 25 μm.

that $\alpha F9^{D\alpha(-/-)}$ cells were hardly dissociated into single cells, while $F9^{D\alpha(-/-)}$ cells were readily dissociated (Figure 2, b and g). Cell aggregation assay, however, showed that both $\alpha F9^{D\alpha(-/-)}$ and $F9^{D\alpha(-/-)}$ cells formed cell aggregates of a similar size and in similar numbers (Figure 2, c and h). After 1 day of aggregation, both types of cells formed spherical aggregates (data not shown). A function-blocking monoclonal anti-E-cadherin antibody (ECCD-1) inhibited this aggregate formation (Figure 2, d

and i). When cells were immunocytochemically stained for E-cadherin (Figure 2, e and j) or β-catenin (data not shown), the antigens were found to be concentrated in the boundaries between cells. These results demonstrated that the strong state of cell adhesion activity mediated by E-cadherin-catenin complex was dependent on α-catenin, whereas the weak state was not.

Because cell-cell adhesion has been implicated in the differentiation of F9 cells into visceral endoderm with an epithelial structure,[32] we examined the differentiation of $\alpha F9^{D\alpha(-/-)}$ and $F9^{D\alpha(-/-)}$ cell aggregates grown in the presence of retinoic acid. After culture in the presence of 5×10^{-8} mol/L retinoic acid for 5 days $\alpha F9^{D\alpha(-/-)}$ cell aggregates were surrounded by an outer layer with a smooth surface (Figure 3 (top) a). In contrast, $F9^{D\alpha(-/-)}$ cell aggregates were irregularly surrounded by round-shaped cells and did not develop a clear outer layer (Figure 3 (top) e). Electron microscopic analysis showed that the outer surface cells of $\alpha F9^{D\alpha(-/-)}$ cell aggregates consisted of polarized epithelial cells, characterized by numerous microvilli on the outer (apical) surface and well-developed junctional complex at cell-cell boundaries[18] (Figure 3 (top), b and c). The outer surface cells of $F9^{D\alpha(-/-)}$ cell aggregates, however, did not show such epithelial morphology (Figure 3 (top) f). These cells were spherical, had microvilli sparsely distributed over the entire cell surface, and were morphologically indistinguishable from the inner cell mass. No junctional complex was found at cell-cell boundaries of these cells (Figure 3 (top) g).

We next used immunohistochemical analysis to examine expression of the epithelial marker proteins cytokeratin 18 and occludin. Cytokeratin 18 is one of the constituents of intermediate filaments and is expressed mainly in epithelial cells.[32] Occludin is a major membrane protein of tight junctions and is predominantly expressed in epithelial and endothelial cells.[33] The outer layer cells of $\alpha F9^{D\alpha(-/-)}$ cell aggregates expressed both cytokeratin 18 and occludin (Figure 3 (top) d). The expressed occludin was clearly localized at cell-cell adhesion sites, probably at tight junctions, in a linear pattern. Cytokeratin 18 and occludin expression were also observed in the outer surface cells of $F9^{D\alpha(-/-)}$ cell aggregates (Figure 3 (top) h). Occludin, however, was localized in an irregular dot-like pattern. α-Fetoprotein, a visceral endoderm-specific product,[18] was also detected in both $F9^{D\alpha(-/-)}$ and $\alpha F9^{D\alpha(-/-)}$ cell aggregates after 8 days of culture in the presence of retinoic acid (data not shown). These results indicated that, in the presence of retinoic acid, α-catenin-deficient cells did not show epithelial morphology but differentiated into visceral endoderm as determined by the expression of epithelial marker proteins.

On culture in the presence of retinoic acid, many cells were scattered from $F9^{D\alpha(-/-)}$ cell aggregates. Most of these scattered cells expressed epithelial marker proteins (data not shown, see Figure 3 (bottom) d). Some of the scattered cells showed a unique signet ring cellular configuration (Figure 3 (bottom) a). Electron microscopic examination demonstrated that these cells had an intracytoplasmic lumen pushing the nucleus to one side (Figure 3 (bottom) b). Numerous microvilli were often ob-

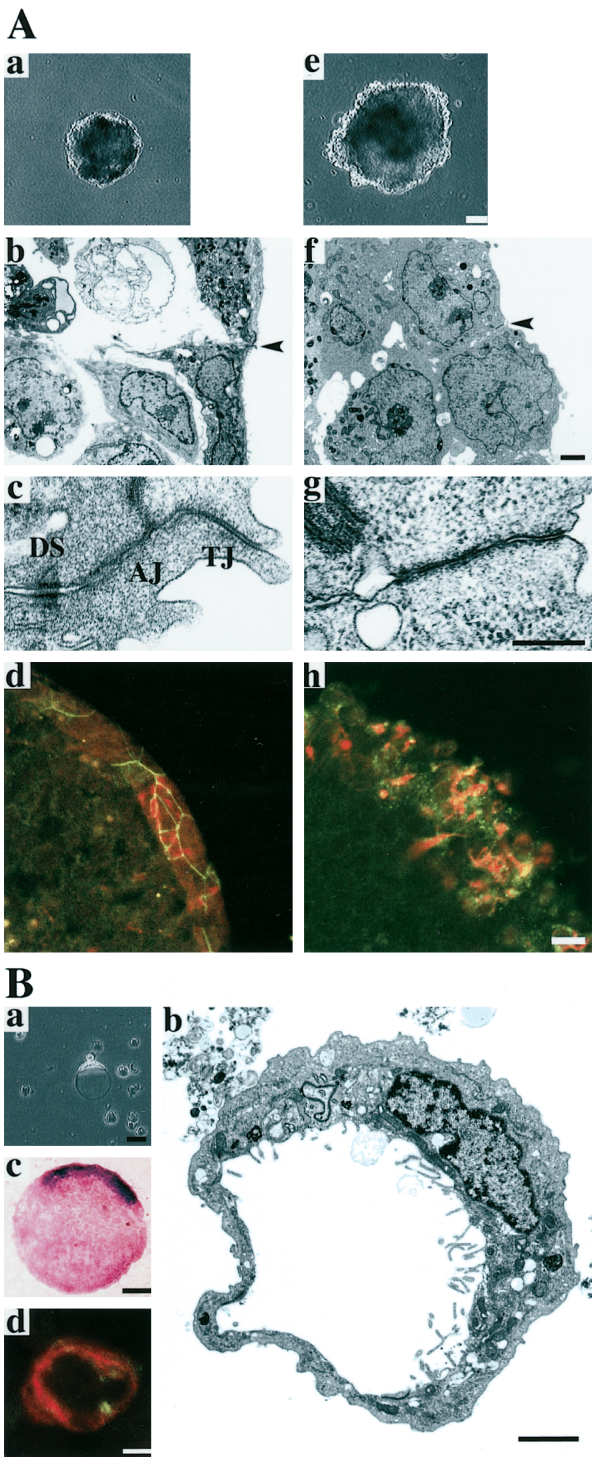

Figure 3. Top (A): Visceral endoderm differentiation in $\alpha F9^{D\alpha(-/-)}$ and $F9^{D\alpha(-/-)}$ cell aggregates. a–d: $\alpha F9^{D\alpha(-/-)}$ cell aggregates. e–h: $F9^{D\alpha(-/-)}$ cell aggregates after 5 days in culture in the presence of 5×10^{-8} M retinoic acid. a and e: Phase contrast images. Bar, 100 μm. b and f: Electron micrographs. Bar, 5 μm. c and g: Higher magnification of cell-cell contact sites indicated by arrowheads in b and f, respectively. Bar, 500 nm. d and h: Immunostaining with anti-occludin and anti-cytokeratin 18 mAbs. Expression of both occludin (green) and cytokeratin 18 (red) was detected in outer surface cells of both aggregates. Bar, 20 μm. Bottom (B): Signet ring cells derived from differentiated $F9^{D\alpha(-/-)}$ cell aggregates. a Phase contrast image. b Electron micrograph. c Periodic acid-Schiff and hematoxylin staining. d Immunostaining with anti-occludin and anti-cytokeratin 18 mAbs. Expression of both occludin (green) and cytokeratin 18 (red) was detected in signet ring cells. Bar, 20 μm.

served on the surface of this intracytoplasmic lumen. Signet ring cells have been reported to be present in mucin-producing carcinomas. Signet ring cells derived from $F9^{D\alpha(-/-)}$ cell aggregates showed positive staining with periodic acid-Schiff, a routine mucin staining method (Figure 3 (bottom) c), and, for cytokeratin 18 and occludin, epithelial marker proteins (Figure 3 (bottom) d). Signet ring cells were hardly produced from $F9^{D\alpha(-/-)}$ cell aggregates cultured in the absence of retinoic acid (data not shown). These observations indicated that the signet ring cells originated from $F9^{D\alpha(-/-)}$ cells differentiated into visceral endoderm.

Discussion

Loss of α-catenin expression is thought to be one of direct causes of the scattered phenotype of diffuse type adenocarcinoma cells.[15] Consistent with this hypothesis, α-catenin-deficient F9 cells lost their adhesiveness and scattered from cell aggregates when cultured under conditions suitable for epithelial differentiation. Because these scattered cells expressed epithelial marker proteins, they were differentiated into epithelial cells but failed to form epithelium. Undifferentiated F9 cells, however, showed the weak state of cadherin-dependent cell adhesion activity and formed spherical aggregates in suspension culture. These observations suggested that E-cadherin/β-catenin complex without α-catenin is not sufficient for the maintenance of cell-cell adhesion between F9 cells differentiated into epithelial cells, although it mediates the weak state of cell adhesion between undifferentiated F9 cells. α-Catenin plays critical roles in junctional complex formation between epithelial cells.[14] This junctional complex formation may be important for the maintenance of cell-cell adhesion between epithelial cells.

Morphological analysis showed that α-catenin-deficient F9 cells formed a signet ring cell configuration when they were differentiated into epithelial cells. Because these cells were positively stained with periodic acid-Schiff, they were thought to resemble a type of traditional signet ring cell carcinoma. Numerous microvilli were often observed on the intracytoplasmic luminal surface of these signet ring cells. This observation suggested the maintenance of epithelial cell polarity in these cells, even though they grew as single round cells. This is consistent with a previous observation obtained by electron microscopic analysis of signet ring adenocarcinoma cells in serous effusions.[16]

Several human gastric carcinoma cell lines with signet ring cell properties, such as HSC-43[17] and HSC-39,[34] have been established. In these cell lines, abnormalities of the cadherin-catenin system were frequently observed. For example, the expression of α-catenin was not detected in HSC-43.[7] In HSC-39, β-catenin molecules lacking the α-catenin-binding domain were expressed and E-cadherin/β-catenin complex did not interact with α-catenin.[35] The present results also demonstrated that the exogenous expression of α-catenin in α-catenin-deficient F9 cells prevented signet ring cell formation. These observations strongly suggested that dysfunction of α-catenin in epithelial cells is a direct cause of signet ring cell formation. It remains unclear how α-catenin-deficient cells form signet ring cellular configurations. Further studies addressing this question may provide important information regarding the oncogenic mechanisms of signet ring cell formation and the molecular mechanisms of epithelial cell morphogenesis.

We have provided clear evidence that a single mutation in the α-catenin gene may be a direct cause not only of the scattered properties of cells but also of signet ring cell formation in diffuse type adenocarcinoma. Because signet ring cell formation was regulated by addition of retinoic acid in the α-catenin-deficient F9 cell system, these cells are a promising new *in vitro* model system for studying oncogenic mechanisms and for diagnosis of signet ring cell carcinoma.

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