FEBS Letters 442 (1999) 178–182 FEBS 21392

Dermal fibroblast proliferation is improved by β-catenin overexpression and inhibited by E-cadherin expression

C. Soler*, C. Grangeasse, L.G. Baggetto, O. Damour

Institut de Biologie et Chimie des Protéines, CNRS, 7 passage du Vercors, 69367 Lyon Cedex 07, France

Received 4 October 1998; received in revised form 4 December 1998

Abstract Several recent studies have shown that proteins of the cadherin-catenin complex are not only involved in cell-cell adhesion but also in the proliferation and differentiation processes. For the first time, we investigated the effect of the quantity of cytoplasmic β-catenin on dermal fibroblast proliferation by overexpressing human β-catenin in human dermal fibroblasts. Our results show that dermal fibroblasts overexpressing normal β-catenin or a stabilized β-catenin mutant have a higher growth rate than control fibroblasts. Moreover, when confluence is reached, the number of fibroblasts is increased when the cells overexpress β-catenin suggesting a role for β-catenin in the regulation of contact growth arrest. Finally, by comparing proliferation in normal dermal fibroblasts and dermal fibroblasts expressing E-cadherin we observed a negative regulatory effect of E-cadherin expression on fibroblast proliferation. These data demonstrate the involvement of β-catenin and cadherin in the dermal fibroblast proliferation process and in contact growth arrest.

Key words: Catenin; Cadherin; Proliferation; Fibroblast

1. Introduction

Catenins are cytoplasmic proteins involved in intercellular junction. They allow a linkage between several proteins of the cadherin superfamily and actin cytoskeleton. Cadherins are transmembrane proteins with an extracellular fragment which binds to another cadherin on an adjacent cell, and an intracellular fragment which can bind to β or γ-catenin. By linking to α-catenin or α-actinin, two actin binding proteins, β or γ-catenins make the link between cadherins and the actin cytoskeleton. Like cell-matrix adhesion proteins, cell-cell adhesion molecules of the cadherin superfamily and catenins play a critical role in cytoskeletal organization leading to cell-junction formation and epithelial polarization [1]. These cadherins are morphogenetic regulatory proteins while morphogenetic signaling is mediated by catenins [2,3]. β-Catenin binding to the intracellular segment of cadherins regulates the formation of the cadherin/catenin complex and its linkage to the actin cytoskeleton. The binding of the cadherin/catenin complex with actin is also regulated by phosphorylation, particularly on tyrosine residues [4–7], but also on serine/threonine residues [8]. In addition, cadherins and catenins are not only structural proteins, they are also involved in cell signaling [9].

Several studies have shown the involvement of cadherins in cell growth, migration, differentiation and death. In particular, Hermiston and Gordon [10] have shown that embryonic mouse cells transformed with a dominant-negative N-cadherin cDNA lead to general disturbances in enterocyte proliferation, migration and death. Moreover, overexpression of E-cadherin in enterocyte cells led to a lower cell growth [11]. Furthermore, VE-cadherin (cadherin-5) was demonstrated to exert an inhibitory effect on cell growth at confluence. Transfection experiments in CHO (Chinese hamster ovary) cells led to inhibitory growth arrest in these cells. However, if the VE-cadherin cDNA transfected to these cells encodes a truncated protein unable to bind the catenins, no cell growth arrest is observed, suggesting a role for the catenins in the cell growth arrest process [12].

Other reports have shown the importance of the amount of cellular β-catenin in the proliferation of colon and melanoma cells. In colon polypoid cells, the amount of β-catenin is higher because of a mutation of the APC (adenomatous polyposis coli) gene or a mutation of the β-catenin gene stabilizing the β-catenin protein and leading to an accumulation in cell-cell junctions, as well as in the cytoplasm and the nucleus. A complex between β-catenin and the transcription factor Tcf-4 can then be formed inducing the regulation of several genes [13,14]. Similarly, β-catenin has been shown to be more stable after a mutation of the N-terminal domain in several melanoma cell lines [15].

In this study, we investigated, for the first time, the effect of the amount of cytoplasmic β-catenin on dermal fibroblast proliferation by directly overexpressing human β-catenin in human dermal fibroblasts. Our results show that dermal fibroblasts overexpressing normal β-catenin or a stabilized β-catenin mutant have a higher growth rate than control fibroblasts. Moreover, when confluence is reached, the number of fibroblasts is increased when cells overexpress β-catenin, suggesting a role for β-catenin in the regulation of contact growth arrest. Finally, by comparing proliferation in normal dermal fibroblasts and in dermal fibroblasts expressing E-cadherin we observed a negative regulatory effect of E-cadherin expression on fibroblast proliferation. These data demonstrate the involvement of β-catenin and cadherin in the dermal fibroblast proliferation process and in the contact growth arrest.

2. Materials and methods

2.1. Cell culture

Human skin fibroblast cultures were initiated in the laboratory by explant outgrowth from the dermis of neonatal foreskin. Fibroblasts were subcultured and maintained in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal calf serum, 200 μM glutamine, and antibiotics (penicillin and gentamicin). Cells were passaged using 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline, pH 7.4 (PBS). Cells were used for experiments between passages 3 and 10. Cell culture reagents were from Sigma (St-Quentin Fallavier, France) and plasticware was from Falcon (Dutscher, Brumath, France).

*Corresponding author. COLETICA, 32 rue St-Jean de Dieu, 69007 Lyon, France. Fax: (33) 4 78586425.

PII: S0014-5793(98)01648-2

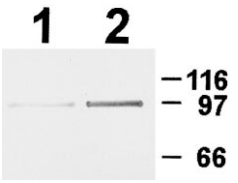

Fig. 1. β-Catenin immunoblot analysis in total cell extracts. Cells were transfected with pCIneo (lane 1) or pCIneoβcat (lane 2). Molecular mass markers of 116, 97 and 66 kDa are indicated by bars on the right.

2.2. Cell transfection experiments

Human fibroblasts were plated 1 day before the transfection experiments in culture dishes (200 000 cells per 60 mm dish). Plasmid DNA (2.5 μg) was complexed with 10 μl of polycation Superfect (Qiagen, France) by vortexing in 150 μl of cell free culture medium (without serum and antibiotics) during 15 s. The complex was allowed to form for 10 min and 1 ml of complete medium was added. Cells were washed with PBS and fibroblasts were incubated in the polycation/DNA complex for at least 2 h at 37°C in 5% CO_2. Then, cells were washed three times with PBS and fresh culture medium was added. After 24 h, the cells were trypsinized and plated in 24 well plates with 500 μl medium containing 400 μg/ml geneticin (Sigma, St-Quentin-Fallavier, France) to isolate stably transformed fibroblasts. These cultures were performed for at least 3 weeks and individual clones were trypsinized and used for proliferation studies after protein expression was demonstrated by Western blotting.

2.3. Expression vectors

The human β-catenin expression vector pCIneoβcat was constructed to overexpress β-catenin in fibroblasts. For this, full-length β-catenin cDNA was removed from the plasmid pBATβ (a generous gift of Dr. Hülsken [16]) by restriction using *Sal*I and *Xba*I and then recloned in plasmid pCIneo (Promega, France) restricted by *Sal*I and *Xba*I.

The truncated human β-catenin expression vector pCIneoΔβcat was constructed to express a N-terminal 89 amino acid deletion mutant of the human β-catenin. The plasmid pCIneoβcat was restricted by *Xho*I to remove a 494 bp fragment encoding the 89 first amino acids and the plasmid was ligated. An ATG start codon located just after the *Xho*I restriction site allowed the initiation of translation.

The mouse E-cadherin expression vector pCIneoE-cad was constructed to overexpress E-cadherin in human fibroblasts. Full-length mouse E-cadherin cDNA was removed from pBATEM2 (a generous gift of Prof. Takeichi [17]) by *Hin*dIII/*Bgl*II restriction and blunt-ended with the Klenow fragment. Then, the cDNA was subcloned in pCIneo (Promega) restricted by *Sma*I.

The cDNAs for β-catenin, N-terminal truncated β-catenin and E-cadherin were placed under the control of the human cytomegalovirus (CMV) strong promoter in pCIneo. The latter encodes the geneticin (G-418) resistance gene allowing selection of stable transformants using geneticin in the cell culture medium.

2.4. Antibodies

For immunocytochemistry experiments, a mouse monoclonal antibody against human β-catenin was purchased from Transduction Laboratories (Interchim, Montluçon, France). The monoclonal antibody DECMA-1 against mouse E-cadherin was purchased from Sigma (St-Quentin-Fallavier, France). CY3-labeled anti-mouse and anti-rat antibodies were from Chemicon (Euromedex, France). For immunoblotting experiments, alkaline phosphatase antibodies were purchased from BioSys (Compiègne, France).

2.5. Immunocytochemistry

Cells were rapidly washed with Tris-buffered saline (TBS) and fixed for 20 min at room temperature in 2% paraformaldehyde. Then, cells were rinsed three times for 5 min with TBS and permeabilized by incubation with 0.2% Triton X-100 in TBS for 4 min at room temperature. After washing, cells were incubated with the first antibody in TBS for 1 h at room temperature in a humidified chamber, washed and stained with CY3 anti-mouse or anti-rat antibodies for 1 h in the dark. Then, cells were washed and mounted with FluoPrep (Biomérieux, Craponnes, France). Fluorescence was detected with a Carl Zeiss microscope (Germany) equipped with epifluorescence and photographed using T-max 400 film (Kodak). Pictures were taken using a ×100 immersion objective.

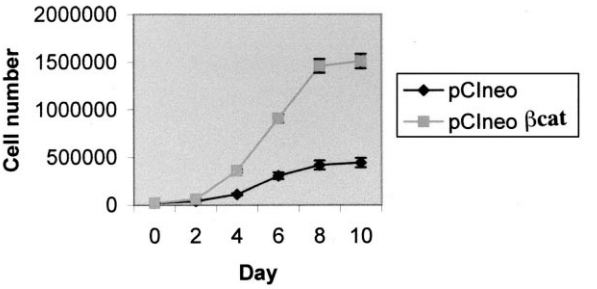

Fig. 2. Proliferation study of transfected fibroblasts with pCIneo alone or pCIneoβcat. Results are expressed as the average values obtained from six different measurements ± S.E.M.

2.6. Cell extracts

Monolayers of human fibroblasts were washed with ice-cold TBS and scraped at 4°C with a rubber policeman in 10 mM Tris, 0.5% Nonidet P-40, 0.5% Triton, 1 mM Ca^{2+}, 5 mM *N*-ethylmaleimide and 10 μg/ml phenylmethylsulfonyl fluoride. The lysate was centrifuged for 30 min at 4°C and the supernatant containing the soluble proteins was recovered.

2.7. Electrophoresis and immunoblotting

Polyacrylamide slab gel electrophoresis in the presence of SDS was performed according to the procedure of Laemmli [18] with a 7.5% resolving gel and a 3% stacking gel. Resolved proteins from cell extracts were transferred electrophoretically to a nitrocellulose membrane with 0.45 μm pores (Bio-Rad, Ivry sur Seine, France) for 2 h at 80 V. The nitrocellulose was blocked in TBS containing 1 mM Ca^{2+} and 3% BSA overnight at 4°C. Nitrocellulose was incubated with the previously defined buffer for 2 h at room temperature. Then the nitrocellulose was washed four times for 5 min in TBS containing 0.05% Tween and 1 mM Ca^{2+}, and incubated in the second antibody (alkaline phosphatase conjugated) for 1 h. Visualization was performed using an alkaline phosphatase conjugated assay kit (Bio-Rad, Ivry sur Seine, France).

2.8. Proliferation studies

Stably transformed fibroblasts expressing β-catenin, Δβ-catenin or E-cadherin, or pCIneo alone, were trypsinized, plated in 6 well plates (2000 cells per cm^2) in DMEM containing 10% fetal calf serum in the presence of geneticin (400 μg/ml). Cells were harvested and trypsinized every 2 days and counted with a Malassez cell. Each reported value was the average of the measurements made in six different wells ± S.E.M.

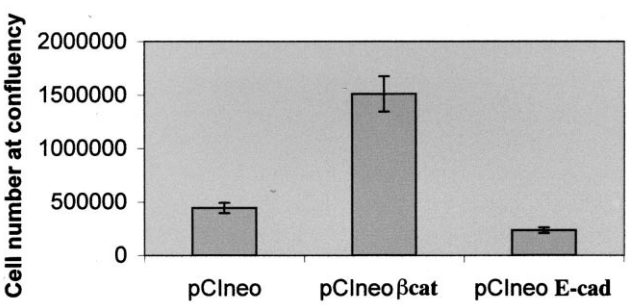

Fig. 3. Number of fibroblasts at confluence. Fibroblasts transfected with pCIneo, pCIneoβcat and pCIneoE-cad were counted after 10 days of culture. Results are expressed as the average values obtained from six different measurements ± S.E.M.

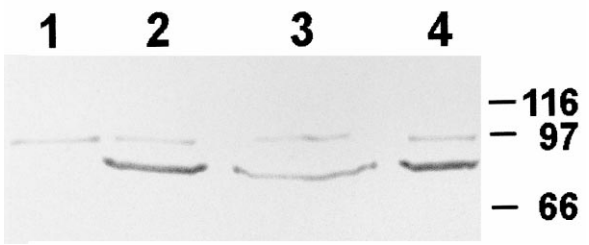

Fig. 4. Immunoblot analysis of β-catenin in total cell extracts. Cells were transfected with pCIneo (lane 1) or pCIneoΔβcat (lanes 2–4). Molecular mass markers of 116, 97 and 66 kDa are indicated by bars on the right.

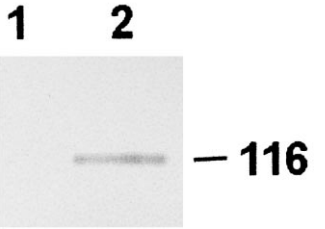

Fig. 6. E-cadherin immunoblot analysis in total cell extracts. Cells were transfected with pCIneo (lane 1) or pCIneoE-cad (lane 2). Molecular mass markers of 116, 97 and 66 kDa are indicated by bars on the right.

3. Results

3.1. Overexpression of normal β-catenin in human dermal fibroblasts

To evaluate the role of the cellular β-catenin amount on fibroblast proliferation, we overexpressed normal β-catenin in human dermal fibroblasts. For this purpose, fibroblasts were transformed with the expression vector pCIneoβcat which encodes the human β-catenin full-length cDNA under the transcriptional control of the strong human CMV promoter. Control fibroblasts were transformed with the empty vector pCIneo. One clone overexpressing β-catenin was isolated and analyzed. Immunocytochemical studies did not reveal major differences in the distribution of β-catenin in the transformed cells (data not shown). However, densitometric analysis of immunoblots showed that the amount of β-catenin was four times higher in pCIneoβcat transformed cells than in pCIneo transformed cells (Fig. 1). Analysis of proliferation (Fig. 2) showed that the clone overexpressing β-catenin had a higher growth rate than the control one (pCIneo alone). Moreover, when confluence was reached, the number of fibroblasts was four times higher in pCIneoβcat transformed cells than in pCIneo transformed cells (Fig. 3) suggesting a role of the cellular β-catenin amount in cell contact growth arrest.

3.2. Overexpression of a N-terminal deletion mutant of β-catenin

An expression vector encoding a 89 amino acid N-terminal deletion mutant of the human β-catenin, which encodes a more stable protein than normal β-catenin [19], was constructed and overexpressed in human fibroblasts. The pCIneoΔβcat vector was transfected in dermal fibroblasts and three stably transformed clones were shown to express this truncated β-catenin (Fig. 4). Proliferation assays were performed by using these three clones and showed that these clones had a growth rate similar to that of pCIneoβcat transformed cells (Fig. 5). At confluence the number of cells transformed with pCIneoΔβcat and with pCIneoβcat were similar (Fig. 5) and four times higher than the number of control cells transformed with pCIneo.

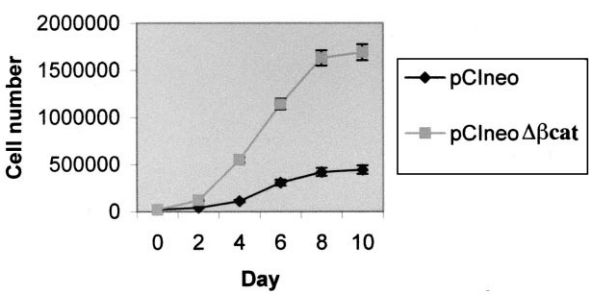

Fig. 5. Proliferation study of transfected fibroblasts with pCIneo alone or pCIneoΔβcat. Results are expressed as average values obtained from six different measurements ± S.E.M.

3.3. Expression of mouse E-cadherin in dermal fibroblasts

In order to reduce the cytoplasmic amount of β-catenin by linking it to E-cadherin, we expressed mouse E-cadherin in dermal fibroblasts. Human β-catenin was shown to bind

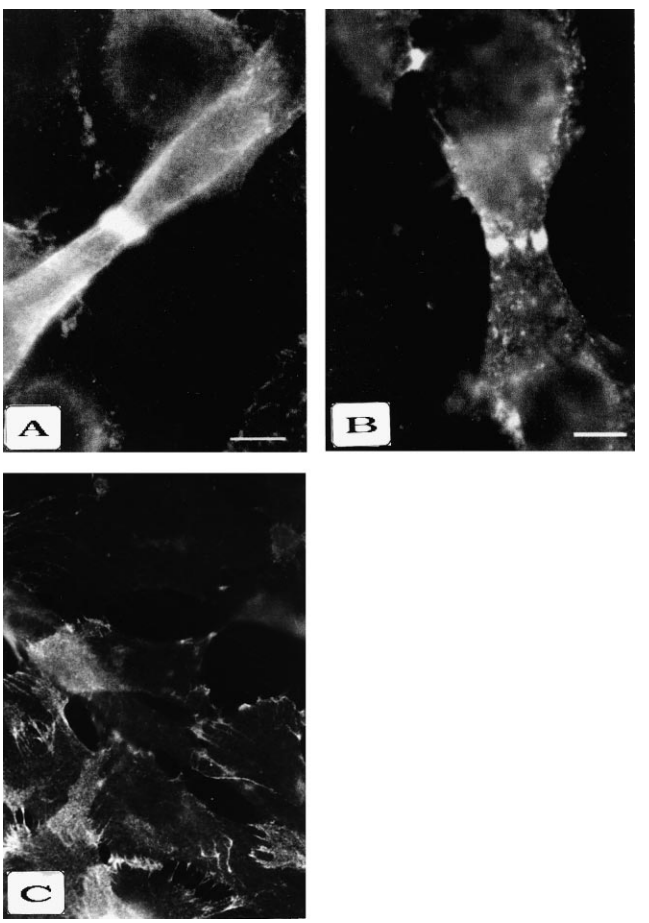

Fig. 7. Immunostaining of E-cadherin (A) and β-catenin (B) from dermal fibroblasts transformed with pCIneoE-cad and immunostaining of β-catenin in cells transformed with the empty vector pCIneo (C).

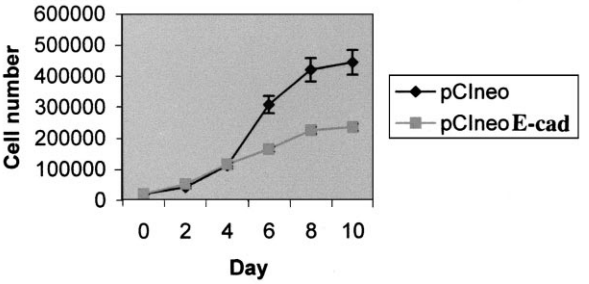

Fig. 8. Proliferation study of transfected fibroblasts with pCIneo alone or pCIneoE-cad. Results are expressed as the average values obtained from six different measurements ± S.E.M.

mouse E-cadherin [16]. The pCIneoE-cad expression vector was constructed as described in Section 2, and dermal fibroblasts were transfected. One stably transformed clone was isolated (Fig. 6) and analyzed by immunocytochemistry. Fig. 7 shows that in pCIneoE-cad transformed fibroblasts, the β-catenin labeling was mainly localized in cell-cell contacts (Fig. 7b) together with E-cadherin (Fig. 7a), suggesting that most endogenous β-catenin was bound to exogenous E-cadherin. As a control, fibroblasts transfected with the empty vector pCIneo did not show such specific structures (Fig. 7c). A proliferation assay showed that pCIneoE-cad transformed fibroblasts had a lower growth rate than control fibroblasts (pCIneo alone) (Fig. 8). Moreover, at confluence we obtained a lower number of cells with pCIneoE-cad transformed cells than in control cells (Fig. 3).

4. Discussion

Transfection of normal dermal fibroblasts with cDNAs under the transcriptional control of a strong promoter is a powerful system which allowed us to raise significantly the cellular amount of normal human β-catenin in dermal fibroblasts. Also, with this system, we expressed a stabilized N-terminal deleted β-catenin (Δβ catenin is stabler than normal β-catenin), and E-cadherin in order to link a significant amount of the endogenous β-catenin and thus lower the amount of free β-catenin. With this approach the growth rate of dermal fibroblasts was shown to be regulated by the amount of β-catenin.

In addition, it has been observed that all the stably transformed clones overexpressing β-catenin or Δβ-catenin had a higher growth rate than control clones stably transformed with the empty vector pCIneo. These results are consistent with those of Behrens et al. [20] and Molenaar et al. [21], who demonstrated an association between β-catenin and the transcription factors Tcf-1 and LEF-1 which transfer cell proliferation signals to the nucleus. Moreover, the presence of an abnormally elevated β-catenin cellular amount in colorectal adenoma and melanoma cells and the β-catenin/APC/Tcf association in these cells support the hypothesis that β-catenin plays a major role in the regulation of proliferation and in apoptosis [13–15].

In order to lower the endogenous β-catenin level of non-transformed fibroblasts, we transfected cells with an expression vector encoding E-cadherin (pCIneoE-cad). Stably transformed cells expressing E-cadherin showed a colocalization of E-cadherin and β-catenin suggesting a linkage between these two proteins. In such cells, the growth rate was shown to be lower than in control cells transformed with the pCIneo alone. These results indicate that lowering the free pool of cytoplasmic β-catenin leads to a lower β-catenin cell proliferation signal. This finding is consistent with those of Heasman et al. [22] and Fagotto et al. [23] who demonstrated that E-cadherin is able to antagonize cell signaling mediated by β-catenin.

Our study demonstrates the involvement of the amount of cellular β-catenin in the regulation of the cell growth arrest process. At confluence, the number of dermal fibroblasts overexpressing β-catenin was higher than the number of control fibroblasts. This observation is in agreement with the fact that exogenous expression of VE-cadherin in CHO cells inhibits cell growth at confluence. However, when a truncated VE-cadherin unable to bind β-catenin was transfected to the CHO cells, the growth arrest process could not be achieved [12]. We obtained a similar result in transforming fibroblasts with E-cadherin. In this case, the number of cells that reached confluence was lowered as if the cell growth arrest were reached with fewer cells. We suggest that cadherins directly regulate the free pool of β-catenin available for cell proliferation signaling and cell growth arrest. Our study and others concur in demonstrating the occurrence of a link between cell-cell adhesion proteins and cell signaling.

Acknowledgements: This work was supported by a grant from the Ministère de l'Enseignement Supérieur et de la Recherche to Christophe Soler, a grant from Direction de la Recherche et des Etudes Techniques to Christophe Grangeasse, and a grant from the Centre National de la Recherche Scientifique. We thank Prof. A. Cozzone for reading the manuscript and gratefully acknowledge Prof. Birchmeier and Prof. Takeichi for providing us with β-catenin and E-cadherin cDNAs, respectively.

References

[1] Drubin, D.G. and Nelson, W.J. (1996) Cell 84, 335–344.
[2] Cunningham, B. (1995) Curr. Opin. Cell Biol. 7, 628–633.
[3] Takeichi, M. (1995) Curr. Opin. Cell Biol. 7, 619–627.
[4] Behrens, J., Vakaet, I., Friis, R., Winterhager, E., Van Roy, F., Mareel, M.M. and Birchmeier, W. (1993) J. Cell Biol. 120, 757–766.
[5] Hamaguchi, M., Matsuyoshi, N., Ohnishi, Y., Gotoh, B., Takeichi, M. and Nagai, Y. (1993) EMBO J. 12, 307–314.
[6] Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Takeichi, M. and Ito, F. (1994) Cell. Adhes. Commun. 1, 295–305.
[7] Soler, C., Rousselle, P. and Damour, O. (1998) Cell. Adhes. Commun. 5, 13–25.
[8] Serres, M., Grangeasse, C., Haftek, M., Durocher, Y., Duclos, B. and Schmitt, D. (1997) Exp. Cell Res. 231, 163–172.
[9] Gumbiner, B.M. (1995) Curr. Opin. Cell Biol. 7, 634–640.
[10] Hermiston, M.L. and Gordon, J.I. (1995) Science 270, 1203–1207.
[11] Hermiston, M.L., Wong, M.H. and Gordon, J.I. (1996) Genes Dev. 10, 985–996.
[12] Caveda, L., Martin-Padura, I., Navarro, P., Breviario, F., Corada, M., Gulino, D., Lampugnani, M.G. and Dejana, E. (1996) J. Clin. Invest. 98, 886–893.
[13] Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Volgestein, B. and Clevers, H. (1997) Science 275, 1784–1787.
[14] Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K.W. (1997) Science 275, 1787–1790.
[15] Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E. and Polakis, P. (1997) Science 275, 1790–1792.
[16] Hülsken, J., Birchmeier, W. and Behrens, J. (1994) J. Cell Biol. 127, 2061–2071.

[17] Nose, A., Nagafuchi, A. and Takeichi, M. (1988) Cell 54, 993–1001.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Munemitsu, S., Albert, I., Rubinfeld, B. and Polakis, P. (1996) Mol. Cell. Biol. 16, 4088–4094.
[20] Behrens, J., von Kries, J.P., Kuhl, M., Bruhn, D., Wedlich, R., Grosschedl, R. and Birchmeier, W. (1996) Nature 382, 638–642.
[21] Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O. and Clevers, H. (1996) Cell 86, 391–399.
[22] Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., Mc Crea, P., Kintner, C., Noro, C.Y. and Wylie, C. (1994) Cell 79, 791–803.
[23] Fagotto, F., Funayama, N., Glück, U. and Gumbiner, B.M. (1996) J. Cell Biol. 132, 1105–1114.