E-Cadherin Promotes Intraepithelial Expansion of Bladder Carcinoma Cells in an *in Vitro* Model of Carcinoma *in Situ*

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ABSTRACT

High-grade transitional cell carcinomas (TCCs) of the urinary bladder are frequently associated with carcinoma *in situ*, which may replace large areas of the mucosa of the urinary tract. The invasive component of TCCs often reveals a loss of expression of the cell-cell adhesion molecule E-cadherin, but the role of E-cadherin in the development and expansion of intraepithelial neoplasia is unknown. To study the underlying mechanism of intraepithelial expansion (IEE), we have developed an IEE assay. Human TCC cell lines were investigated in this IEE assay for their capacity to replace the surrounding normal murine urothelial cells. *In vitro* IEE appeared to be prominent in three (SD, RT112, and 1207) of the four E-cadherin-positive cell lines. Although the two E-cadherin-negative cell lines (T24 and J82) were able to penetrate surrounding normal urothelium as single cells, they largely lacked the capacity of IEE. These results prompted us to investigate whether the cell-cell adhesion molecule E-cadherin is an important determinant for IEE. T24 cells that were transfected with full-length mouse *E-cadherin* cDNA displayed an enhanced IEE rate. Transfection did not influence their proliferative capacity, their pattern and level of integrin expression, or their ability to expand in the absence of surrounding urothelium. The data suggest that E-cadherin-mediated cohesiveness is an important factor in the IEE of bladder carcinoma cells. These observations argue for a dual, paradoxical role of E-cadherin in bladder tumorigenesis. On the one hand, E-cadherin promotes the expansion of intraepithelial neoplasia; on the other hand, its loss correlates with invasive behavior.

INTRODUCTION

High-grade TCCs[2] of the urinary tract are frequently accompanied by carcinoma *in situ* (1). Carcinoma *in situ* of the urinary tract is characterized by the replacement of the normal lining urothelium by dysplastic cells, which show a variety of cellular and molecular changes. Carcinoma *in situ* may replace large areas of the urinary tract mucosa, extending even to the urethra and, in males, to the prostatic ducts and glands (2). In general, carcinoma *in situ* is regarded as a precursor lesion for invasive bladder carcinoma (3).

Patients with carcinoma *in situ* in the flat peripheral urothelium adjacent to tumors have a higher probability of tumor recurrences and/or invasion (4, 5). Clinical and experimental data suggest that bladder tumor recurrences could be the consequence of an IEE of the transformed cells from the original tumor or shedding and subsequent reattachment of bladder cells, particularly to traumatized areas in the bladder mucosa (6–11). Lateral expansion of the attached tumor cells can then lead to the replacement of normal urothelium by cancer cells. The mechanisms involved in lateral (*i.e.,* intraepithelial) expansion of bladder tumor cells are only partially understood. A cocultivation model established recently in our laboratory permits the direct visualization of attachment and subsequent IEE of bladder tumor cells at the expense of surrounding normal urothelium (10, 11). In this assay, a tumor cell suspension is inoculated on confluent mouse urothelial cultures containing de-epithelialized areas of a standard size. Implantation of tumor cells occurs predominantly in these de-epithelialized areas, and the time course of IEE can be assessed by selective immunostaining of the tumor cells. Previously, we have shown in this model that exposure to growth factors and culture on substrates coated with particular extracellular matrix proteins could influence IEE (10, 11).

E-cadherin is a member of a family of transmembrane glycoproteins involved in intercellular adhesion. E-cadherin function is mediated by the interaction with the cytoplasmatic α-, β-, and γ-catenins. These catenins connect E-cadherin with the cytoskeleton. In model systems, loss of E-cadherin expression is associated with the gain of the invasive phenotype in tumors (12, 13, 14). Similarly, it was reported that loss of the invasion suppressor molecule E-cadherin or catenins is associated with deeply invasive bladder cancer and is predictive for poor survival of patients with bladder cancer (15–17). The E-cadherin/catenin complex also contributes to a variety of physiological functions like cell growth, differentiation, wound healing, cell motility, morphogenesis, and organogenesis (18). However, its role in the expansion of carcinoma *in situ* of the bladder has not yet been studied.

In this study, initial experiments comparing E-cadherin-positive and -negative human TCC cell lines suggested a potentially enhancing role of E-cadherin in IEE. By use of T24 cells, stably transfected with an E-cadherin cDNA construct, we could confirm that this molecule indeed contributes to IEE, most likely by conveying increased cohesiveness to the TCC cells.

MATERIALS AND METHODS

Cell Culture. The human bladder cancer cell lines T24, SD, RT112, JON, and J82 were kindly provided by Dr. J. A. Schalken (Urological Research Laboratory, University Hospital Nijmegen, the Netherlands) and have been characterized previously (19, 20). Human bladder carcinoma cell line 1207 was obtained from Dr. W. I. De Boer (GETU Service d'Urologie, Paris, France; Ref. 21). Cell lines were maintained in the same standard medium as the primary bladder explant cultures.

Primary Explant Cultures of Murine Urothelium. Murine urinary bladders were dissected from female C3H/He mice, 6–8- weeks of age, and cut into halves. The mucosa of the bladder was stripped from its underlying muscle layer and subsequently spread on a collagen type IV (25 μg/ml human collagen type IV)-coated Cyclopore membrane (Becton Dickinson Labware, Bedford, MA) with the submucosa facing the culture support. Standard culture medium consisted of a 1:1 mixture of DMEM and Ham's F10, supplemented with 10% heat-inactivated FCS, 10 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenite, 10 μM HEPES, 50 nM hydrocortisone, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The explant cultures were grown at 37°C in a humidified atmosphere of 5% CO_2. These murine explant cultures on porous membranes mimic the *in vivo* situation; the cultured urothelium shows a polarized multi-layering and differentiation into umbrella cells (22).

IEE Assay. The cocultivation model to study IEE of bladder carcinoma cells was described previously (11). Briefly, in confluent murine explant cultures, four standardized circular areas were denuded in the periphery of the primary cultures by cautious imprinting with a 3-mm diameter biopsy punch (Stiefel, Offenbach am Main, Germany). The urothelium in the injured areas

[2] The abbreviations used are: TCC, transitional cell carcinoma; IEE, intraepithelial expansion.
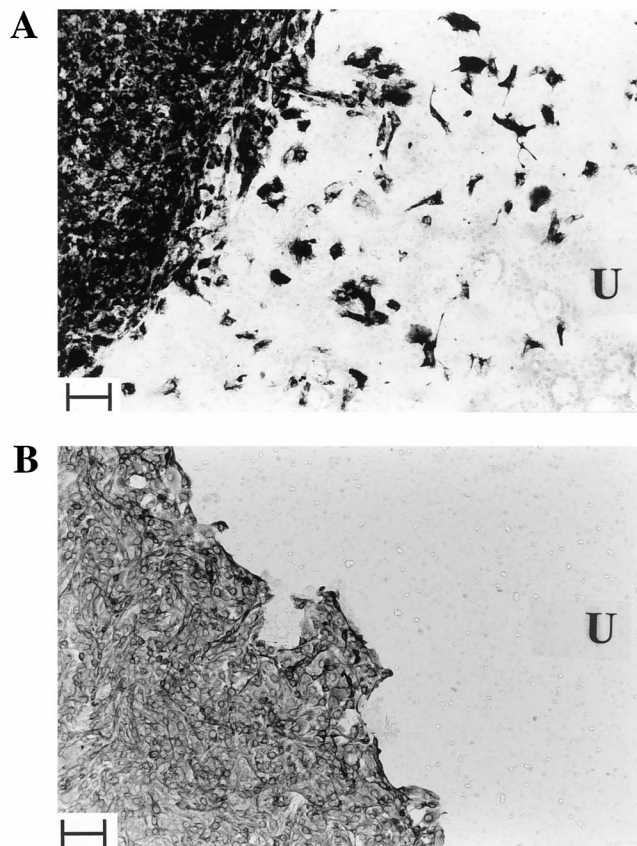
Fig. 1. Low-power overviews of human bladder carcinoma cells adjacent to primary normal mouse urothelium: *A,* T24 cells; and *B,* RT112 cells. Bladder carcinoma cells were selectively stained with RCK108. *Bar,* 100 μm. *U,* unstained normal surrounding mouse urothelium.

was scraped away from the Cyclopore membrane with a micropipette tip. Subsequently, the cultures were washed twice with PBS, followed by seeding of 10^5 tumor cells in 1.5-ml standard medium on the murine urothelial explant cultures. The bladder tumor cells were allowed to attach to the injured areas in the explant cultures for 24 h; nonadherent cells were then washed away with PBS, and the cultures were either terminated or continued for another 4, 7, or 14 days in standard medium. Each experiment was performed twice in triplicate. The cultures were terminated by fixing them in 70% ethanol and stored at 4°C until immunohistochemistry; selective identification of the human bladder carcinoma cells was performed.

Monoclonal, human-specific antibodies, RCK108 (Eurodiagnostica, Arnhem, the Netherlands) or DC10 (Beckman Coulter, Fullerton, CA), directed against cytokeratin 19 were used to distinguish the human bladder carcinoma cells from murine urothelium (10). Monoclonal antibody DC10 was used for staining J82 cells, whereas the other cell lines were stained with monoclonal antibody RCK108. Nearly 100% of the bladder carcinoma cells of each cell line was labeled with RCK108 or DC10. The secondary antibody was horseradish peroxidase-conjugated goat antimouse immunoglobulins (Dako, Glostrup, Denmark). Peroxidase activity was visualized with 0.03% H_2O_2 and 0.02% 3,3,-diaminobenzidine tetrahydrochloride (Fluka, Basel, Switzerland) diluted in PBS.

The immunostained areas were quantitated with a Hitachi CCTV camera equipped with the KS400 image analysis software package (Kontron Elektronik, Eching, Germany) and expressed in mm^2.

Construction of Stable Transfectants. The E-cadherin-negative cell line T24 was cotransfected with plasmids pBATEM2 and pSVneo, which harbors the neomycin resistance gene. Marion Bussemakers (Urological Research Laboratory, University Hospital Nijmegen) generously provided plasmid pBATEM2, containing the full-length mouse *E-cadherin* cDNA, originally constructed by Nagafuchi *et al.* (23). For transfection, cultures were cotransfected with a total of 20 μg of DNA (pBATEM2:pSVneo, 20:1) using the DNA-calcium phosphate method (24), and transfected cells were selected with 800 μg/ml G418. E-cadherin expression in these transfected cell lines was detected as follows. Clones were grown to confluence on multichamber slides (Nunc, Naperville, IL) and fixed in methanol at −20°C. Cells were incubated overnight at 4°C with monoclonal antibody DECMA-1 (Sigma Chemical Co.,

Fig. 2. IEE of six different bladder tumor cell lines. Graphs display the area (mm^2) occupied by human bladder tumor cells in peripheral lesions measured on different time points. Data are expressed as means (n = six cultures, with four peripheral lesions each); *bars,* SD. Note the difference in the scale of the *Y axis* in the various graphs.
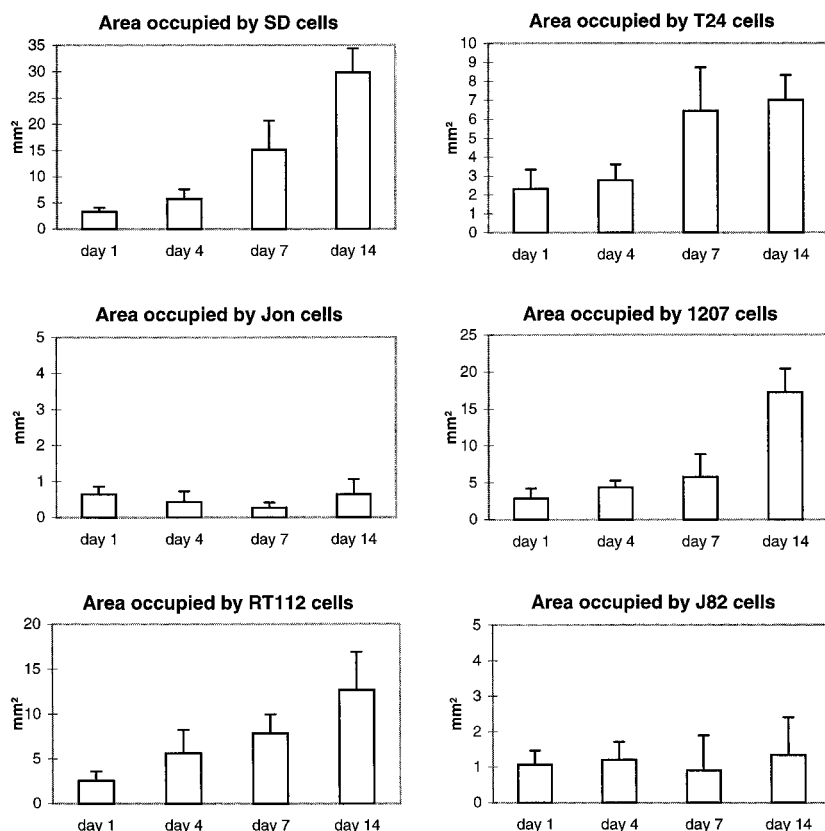
Table 1 *Characteristics of bladder carcinoma cell lines*

TCC cell lines were tested in an *in vitro* model of IEE. IEE was characterized by tumor expansion (tumor area in mm^2) and by the pattern that TCC cell lines displayed during infiltration of the normal surrounding urothelium.

Cell line	E-cadherin expression (*in vitro*)	IEE	
		Tumor expansion	Infiltration pattern
SD	+	++	Sharp demarcation
T24	−	−/+	Single cells
RT112	+	+	Sharp demarcation
J82	−	−	Single cells
JON	+	−/+	Sharp demarcation
1207	−/+	+	Single cells

St. Louis, MO), followed by a biotinylated rabbit antirat immunoglobulins (Dako) and fluorescein-conjugated streptavidin (Dako). The different clones retained their resistance to G418 and were stable in their E-cadherin expression during the entire course of the described experiments.

Flow Cytometric Analysis of E-Cadherin and Integrin Expression. Cells were harvested by a short trypsinization of confluent monolayers. Cell suspensions were made in PBS containing 0.5% BSA, 0.1% NaN_3, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$ at a concentration of 1×10^6 cells/ml. E-cadherin expression of a selection of nine different, transfected T24 clones was examined with the DECMA-1 antibody, followed by biotinylated rabbit antirat immunoglobulins and fluorescein-conjugated streptavidin. Integrin expression was studied with the use of specific antibodies against α1-integrin (HP2B6; Beckman Coulter, Fullerton, CA), α2-integrin (NCL-CDW49b; Novacastra, Newcastle upon Tyne, United Kingdom), α3-integrin (NCL-CDW49c; Novacastra), α4-integrin (P4G9; Dako), α5-integrin (P1D6; Dako), α6-integrin (NCL-CDW49f; Novacastra), β1-integrin (TDM29; Sanbio, Uden, the Netherlands), and β3-integrin (NCL-CD61; Novacastra). As a negative control, the primary antibody was omitted and replaced by a PBS/BSA/azide solution. Data acquisition and analysis were performed on duplicate samples on a FACScan flow cytometer using CELLQuest software (Becton Dickinson, San Jose, CA).

Immunoblotting. Confluent cultures were lysed in 2× sampling buffer [4% SDS, 200 mM DTT, 100 mM Tris (pH 6.8), 20% glycerol, and 2% Triton X-100], and equal quantities of protein (30 μg) were run on a 7.5% SDS-PAGE. The MDCK cell line was used as a positive control for E-cadherin expression. After electroblotting, blots were immunostained with the DECMA-1 antibody, followed by biotinylated rabbit antirat immunoglobulins. Next, an alkaline phosphatase-conjugated streptavidin label (Biogenex, San Ramon, CA) was applied. Finally, bound antibody was visualized by histochemical staining with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Roche Diagnostics, Basel, Switzerland).

***In Vitro* Invasion Assay.** Chicken heart invasion assays were performed as described by Mareel *et al.* (26). Briefly, a selection of *E-cadherin*-transfected T24 clones was confronted with precultured rounded fragments of embryonic chicken heart on soft agar for 24 h. Next, fragments with attached bladder tumor cells were kept in suspension culture under gyrotory shaking (120 rpm; 37°C; 5% CO_2) for 6 days in MEM REGA 3 medium (Life Technologies, Inc., Breda, the Netherlands) containing 10% FCS. Fragments were fixed in 4% phosphate buffered formalin and embedded in paraffin. Invasion was scored on serial histological sections stained with H&E. The tumor cells were distinguished from the heart tissue by their morphological features.

Wound Colonization Assay. T24 Clones were grown to confluence in 10-cm diameter culture dishes. With a plastic pipette tip, cells were scraped away in the shape of a cross. The width of the lesion was ~5 mm. After 16 h, the movement of cells into the wound was monitored and photographed.

Expansion of T24 Clones on Collagen Type IV-coated Cyclopore Membranes. With a 3-mm diameter biopsy punch (Stiefel, Offenbach am Main, Germany), a superficial circular imprint was made on the cell culture inserts, coated with collagen type IV. A cell suspension of 10^5 cells/2 μl of standard medium was pipetted within the borders of the circular imprint with a 10-μl micropipette. The cells in the drop of medium attached into the circular area within 24 h. Every 2 days, medium on top of the membranes was refreshed, taking care that the medium only covered the area of tumor cells. Medium underneath the membrane was refreshed twice a week. The circumferences of the outgrowing sheet of tumor cells were drawn daily. From these drawings, the area (mm^2) of outgrowth was determined.

[^{3}H]Thymidine Incorporation. To assess the potential differences in proliferation of the different T24 clones, a cell kinetic study was performed as described earlier (11). Briefly, T24 clones grown in 75-cm^2 culture flasks were synchronized in serum-free culture medium for 24 h. Subsequently, 10^5 cells/were seeded in collagen type IV-coated 96-well dishes and were cultured in standard medium for 4 consecutive days. Proliferative activity was determined at 24, 48, 72, and 96 h. During the final 16 h of culture, cells were incubated with 0.5 μCi of [^{3}H]thymidine/well. The incorporated [^{3}H]thymidine in harvested cells was counted in a BetaPlate scintillation counter (LKB-Pharmacia, Woerden, the Netherlands).

RESULTS

IEE of Human Bladder Carcinoma Cells. The IEE of a panel of six different TCC cell lines was studied. After the seeding of tumor cells on wounded confluent murine urothelial cultures, the tumor cells preferentially attached to the de-epithelialized areas. Within 24 h, these areas were covered both by tumor cells and regenerating urothelium. Control experiments showed that in the absence of inoculated tumor cells, the de-epithelialized areas became entirely covered by regenerating normal urothelial cells within 24 h (11). The areas covered by tumor cells at 24 h were of the same size for four of the
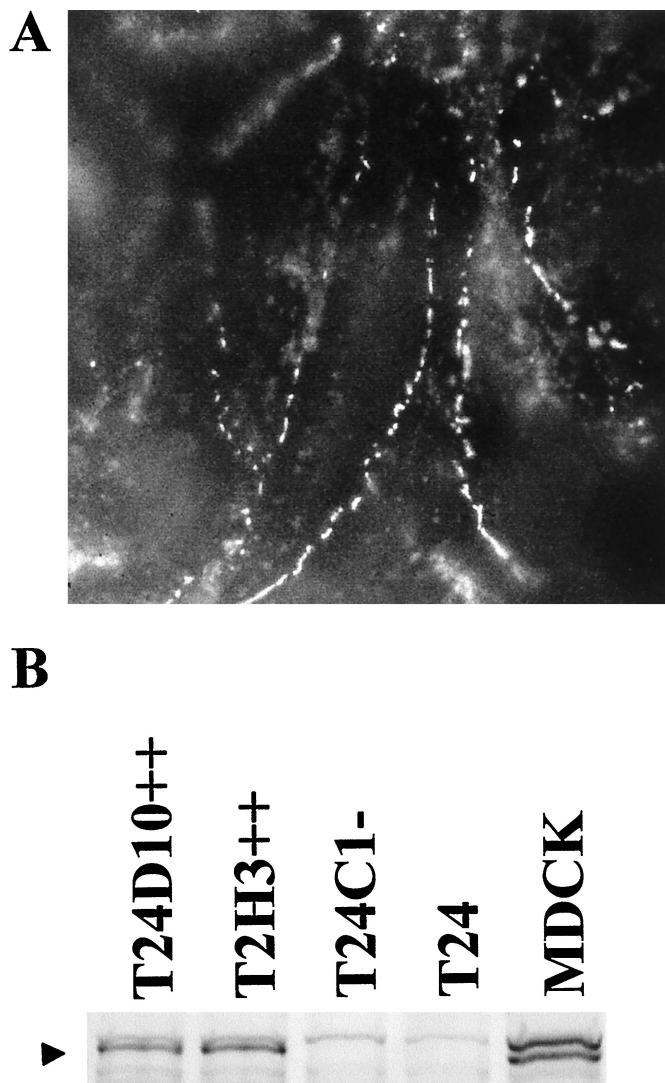
Fig. 3. Analysis of E-cadherin expression in transfected T24 clones. *A*, immunofluorescent staining for E-cadherin in T24D10++ (×100). *B*, Western blotting of total lysates of MDCK, T24, and T24 transfectants.
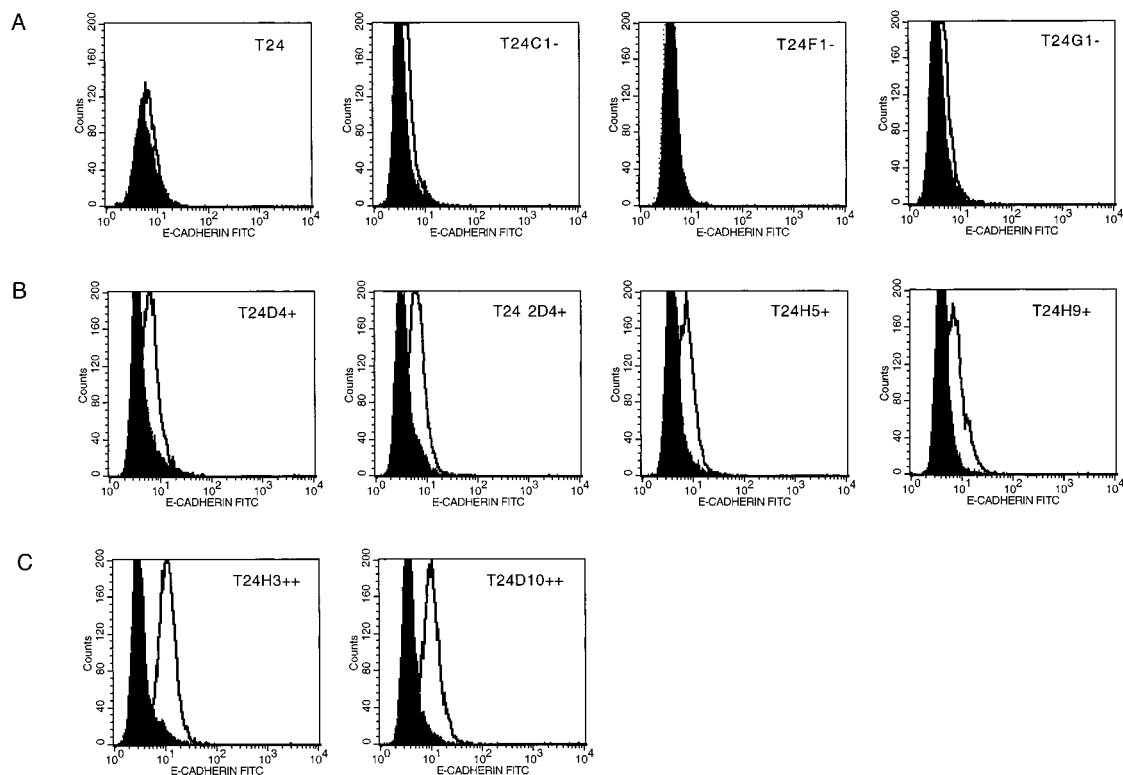
Fig. 4. Flow cytometry analysis of E-cadherin expression in: *A,* native T24 cells and the negative controls T24C1−, T24F1−, T24G1−; *B,* clones T24D4+, T24 2D4+, T24H5+, and T24H9+; and *C,* clones T24D10++ and T24H3++.

six examined tumor cell lines, but implantation of JON or J82 cells was less effective. As described previously, E-cadherin expression influenced the pattern of IEE (10, 11). Bladder tumor cell lines with no (T24 and J82) or a heterogeneous (1207) expression of E-cadherin were able to infiltrate the surrounding normal urothelium as single cells (Fig. 1*A*), whereas bladder tumor cell lines with a homogeneous (SD, RT112, and JON) expression of E-cadherin displayed a sharp demarcation between the tumor cells and the normal urothelium (Fig. 1*B*). The six tumor cell lines further differed with respect to their subsequent IEE rate (Fig. 2 and Table 1). The IEE of bladder carcinoma cells was most pronounced for the SD cells and less for the 1207 and RT112 cells, whereas T24, JON, or J82 displayed hardly any or no expansion. Therefore, three of four E-cadherin-positive bladder carcinoma cell lines had a better IEE rate than the two E-cadherin-negative bladder carcinoma cell lines. These results suggested a positive effect of E-cadherin on intraepithelial expansion.

Transfection of T24 and J82 with Full-Length Mouse *E-Cadherin* cDNA. To investigate whether E-cadherin is involved in the IEE of bladder tumors, we transfected T24 cells with the mouse *E-cadherin* gene. Earlier, it was shown that T24 cells express α- and β-catenin (25). We obtained 19 G418-resistant T24 cell clones, with membrane-bound E-cadherin expression (Fig. 3*A*). Clones varied with regard to their levels of immunohistochemically detected E-cadherin expression. Expression levels of E-cadherin of nine different T24 transfectants were analyzed by flow cytometry. Clones T24H9+, T24 2D4+, T24D4+, and T24H5+ had low to moderate expression levels (Fig. 4*B*), whereas the clones T24H3++ and T24D10++ had a high expression level of E-cadherin (Fig. 4*C*). As a negative control for E-cadherin expression, we used the clones (T24C1−, T24F1−, and T24G1−) that were G418 resistant but had no detectable expression of E-cadherin, either by immunofluorescence, immunoblotting, or flow cytometry (Fig. 4*A*). Immunoblotting with the DECMA-1 antibody on different cell lysates of T24 clones showed a characteristic

band at M_r 120,000 that was typical of the E-cadherin-positive clones (Fig. 3*B*). At the ultrastructural level no difference in the presence of desmosomes between the nontransfected (E-cadherin-negative) and transfected, E-cadherin-positive T24 cells was noted (data not shown).

Wound Colonization Assay. The capacity to repair lesions in a confluent cell culture was monitored for the different clones. Strikingly, untransfected T24 cells and the T24 clones T24C1−, T24F1−, and T24G1−, with no E-cadherin expression, migrated into the denuded area as single cells (Fig. 5*A*). SD and the T24 clones with a high or moderate E-cadherin expression filled up the lesions by moving as a cohesive sheet (Fig. 5*B* and Table 2). However, no difference in time required for the repair of the lesions was seen.

***In Vitro* Embryonic Chicken Heart Invasion Assay.** *In vitro* invasion of bladder carcinoma cells was examined after 6 days of cocultivation with embryonic chicken heart fragments. As a negative control, we used the E-cadherin-positive SD cell line. Tumor cell adhesion to chicken heart fragments varied from 100% for T24D10++ and T24H3++ cells to 65% for the T24 cells (Table 2). Untransfected T24 cells exhibited the strongest invasive capacity. After successful adhesion, T24 tumor cells infiltrated nearly all embryonic chicken heart fragments (87%). SD cells were unable to invade the embryonic chicken heart fragments at all. Transfection of E-cadherin in T24 cells resulted in a significant reduction of invasion for T24D10++ and T24H3++ (Table 2). Both cell lines had a high expression of E-cadherin. Clones T24H9+, with a moderate expression of E-cadherin, T24C1− and T24G1−, with absent E-cadherin expression, exhibited a moderate to high invasive capacity into the embryonic chicken heart fragments. These results revealed that the levels of E-cadherin expression of the transfected T24 clones corresponded with their functionality.

IEE of Transfected T24 Cells. The SD cell line was used as a positive control, because it had the highest expansion rate of the TCC cell lines (Fig. 2). Transfection of T24 cells with mouse *E-cadherin*

cDNA stimulated IEE of two independently obtained clones, T24D10++ and T24H3++, both with a high expression of the E-cadherin protein (Fig. 6). Clones with a moderate/low expression level of E-cadherin (*e.g.*, T24H9+) did not show a significant stimulation of IEE. The implantation (defined by the tumor area on day 1) of the different T24 transfectants was comparable, indicating that possible differences in IEE could not be attributed to differences in attachment of the T24 transfectants to the wounded area. The expression of integrins was determined by flow cytometry in T24, T24H3++, T24D10++, and T24C1−. In the four tested clones, no differences were observed in expression levels of α1-, α2-, α3-, α4-, α5-, α6-, β1-, or β3-integrins (data not shown).

Expansion of T24 Clones on Collagen Type IV-coated Cyclopore Membranes in the Absence of Surrounding Urothelium. We tested whether the observed differences in IEE rate between the tested T24 clones could be explained by an altered expansion rate (expansion defined as the outgrowth of a sheet of cells) or proliferative capacity. The expansion of different T24 clones in the absence of surrounding urothelium was assessed (Fig. 7). These experiments revealed no differences in the outgrowth or in the [^{3}H]thymidine incorporation (data not shown) of the various T24 clones. The moderately E-cadherin-expressing T24 2D4+ had a lower expansion rate.

DISCUSSION

The IEE assay used here is considered an experimental model relevant for the study of the mechanisms underlying the *in vivo* expansion of carcinoma *in situ* of the bladder, because in this model,
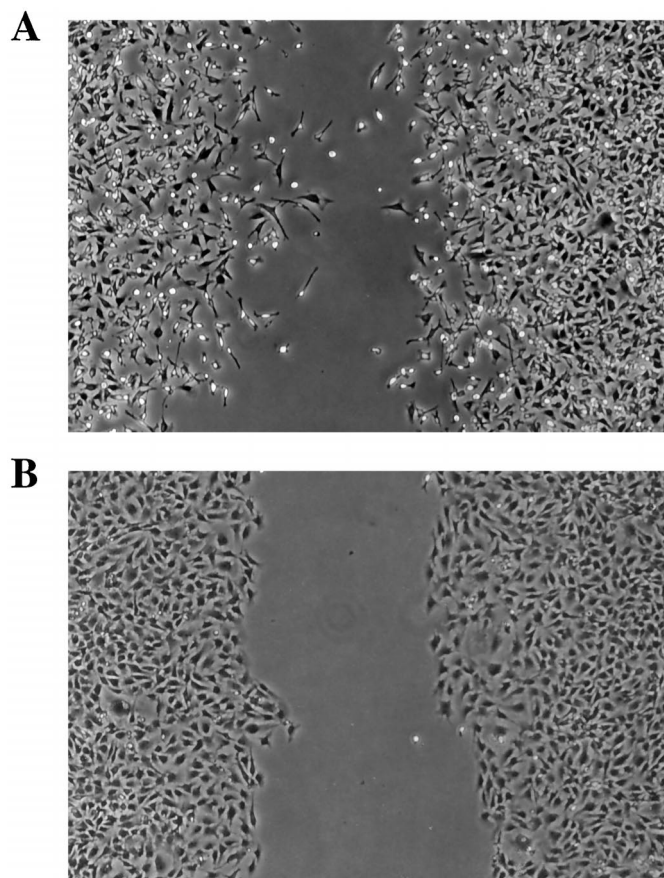
A

B

Fig. 5. Wound colonization of transfected T24 cells. The photographs illustrate the difference in wound-filling capacity between the E-cadherin-negative T24 clone, T24G1− (*A*) and an E-cadherin-positive clone, T24D10++ (*B*).

Table 2 *In vitro* invasion of *E-cadherin*-transfected T24 cells

Cell line	E-cadherin expression of cultured cells	*In vitro* confronting cultures		Wound colonization
		Adherent[a]	Invasive[b]	
SD	++	20/23 (87%)	0/20 (0%)	Cohesive sheet
T24	−	15/23 (65%)	13/15 (87%)	Single cells
T24D10++	++	34/34 (100%)	3/34 (9%)	Cohesive sheet
T24H3++	++	11/11 (100%)	2/11 (18%)	Cohesive sheet
T24H9+	+	21/22 (95%)	7/21 (33%)	Cohesive sheet
T24G1−	−	21/21 (100%)	11/21 (52%)	Single cells
T24C1−	−	20/21 (95%)	15/20 (75%)	Single cells
T24H5+	+	11/12 (92%)	3/11 (27%)	Cohesive sheet

[a] Number of adherent/total number of confronted embryonic chicken heart cultures.
[b] Number of invasive/number of confronted cultures with adherent tumor cells.

IEE is the outcome of the balance between the regenerative potential of normal urothelial cells and the growth of transformed cells. Previous studies have shown that this *in vitro* IEE may be influenced by exposure to growth factors and by culture on substrates composed of particular extracellular matrix proteins (10, 11). These earlier studies suggested that modulation of IEE was largely the consequence of the interaction of these factors with the normal urothelium, rather than a direct effect on the bladder carcinoma cells. The observed considerable variation among the six tested TCC cell lines in their capacity to expand at the expense of normal urothelium implies that factors intrinsic to these cell lines also determine the outcome of the cocultivations.

Previously, we have shown that the pattern of infiltration of TCC cells into the surrounding normal urothelium is determined by E-cadherin expression (10, 11). E-cadherin-negative TCC cells infiltrate the normal urothelium as individual cells, whereas tumor cells with a homogeneous expression of E-cadherin exhibit a sharp demarcation with the normal urothelium. The 1207 TCC cell line with a heterogeneous expression of E-cadherin *in vitro* also displays the capacity to infiltrate the normal urothelium as individual cells. Staining of these cocultivations with a human specific E-cadherin antibody revealed that the infiltrative single cells of the 1207 cell line had a reduced or absent expression of E-cadherin as compared with the high homogeneous expression of the primary tumor at the implantation site (data not shown). These results indicate that a reduced or absent expression of E-cadherin results in infiltration of the surrounding normal urothelium as single cells.

Comparison of the six TCC cell lines revealed that the two constitutively E-cadherin-negative bladder carcinoma cell lines (T24 and J82) hardly showed any IEE, whereas three of four E-cadherin-positive cell lines (SD, RT112, and 1207) did. We hypothesized that a functional E-cadherin-catenin complex is required for effective IEE of TCC cells. To further test this hypothesis, we established stable transfectants of T24 cells expressing the full-length mouse *E-cadherin* cDNA. Several clones of E-cadherin-expressing T24 cells were obtained. Functionality of the transfected T24 clones was analyzed with the embryonic chicken heart *in vitro* invasion assay (12, 26) and a wound colonization assay (27).

The E-cadherin-negative cell line T24 was generally capable of invasion into the embryonic chicken heart, whereas only the T24 transfectants with high expression of E-cadherin (T24H3++ and T24D10++) had a significantly reduced invasive capacity, implying a functional E-cadherin-catenin complex. The T24 clones with a moderate/low but homogeneous expression of E-cadherin (*e.g.*, T24H9+) retained part of their invasiveness (Table 2). This observa-

Fig. 6. IEE of T24 transfectants. Graphs display the area (mm^2) occupied by human bladder tumor cells in peripheral lesions measured on different time points. Data are expressed as means (n = four or five cultures, with four peripheral lesions each).
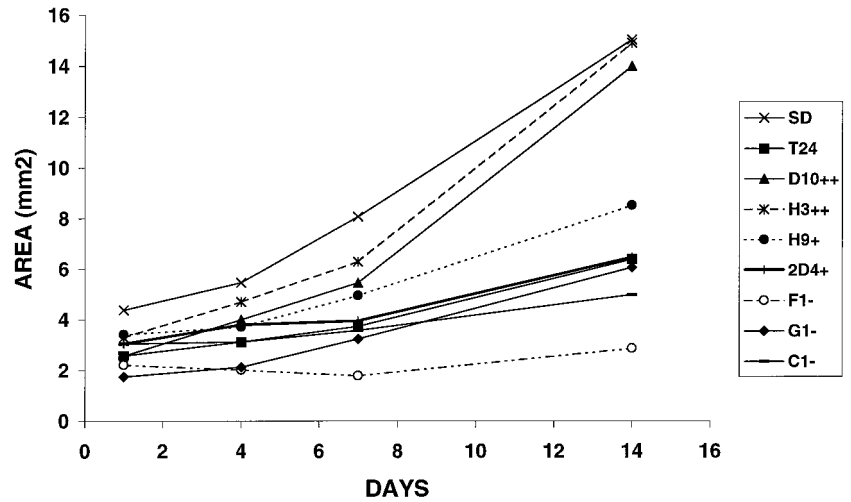
tion is in accordance with those of Vleminckx *et al.* (12), who suggested that a threshold expression of E-cadherin has to be reached to prevent *in vitro* invasion into the embryonic chicken heart fragments. Similarly, in IEE assays a significant enhanced expansion rate was only observed for the two T24 clones with high E-cadherin expression but not for the T24 clones with a moderate or low expression level, suggesting that the same E-cadherin-mediated mechanism induces both suppression of *in vitro* invasion and promotion of IEE. The enhanced E-cadherin-mediated expansion rate became manifest only under the cocultivation conditions of IEE. Thus, increased expression of E-cadherin by transfected T24 cells was neither associated with an increased expansion rate on collagen type IV-coated membranes nor with an increased proliferative activity or wound repair. Several reports documented E-cadherin-mediated suppression of cell motility and inhibition of proliferation (27–29). This discrepancy with our findings on T24 cells may be explained by the use of fibroblast cells in these studies rather than epithelial cells. Furthermore, the motility assays used in these reports (27–29) were based on single cell

assays, whereas our expansion assay (Fig. 7) monitors the outgrowth of a cohesive sheet of cells.

Recently, Pignatelli (30) speculated on the possibility of a molecular cross-talk between cadherins and integrins in cancer cells. Because integrins are involved in motility and could be a regulator in IEE (7), we examined whether E-cadherin expression levels in transfected T24 cells would correlate with expression levels of integrins. Flow cytometric analysis of $\alpha 1$-, $\alpha 2$-, $\alpha 3$-, $\alpha 4$-, $\alpha 5$-, $\alpha 6$-, $\beta 1$-, or $\beta 3$-integrin expression levels did not point to such a mechanism in *E-cadherin*-transfected T24 cells. It could still be argued that E-cadherins could lead to an altered localization or affinity of integrins on the cell membrane. Because we did not find any altered migratory behavior of the transfected T24 cells in our expansion assay in the absence of surrounding normal urothelium, E-cadherin-integrin cross-talk in our transfected T24 cells seems an unlikely mechanism for the observed IEE. We strongly feel that cohesion of transformed T24 cells is the most important E-cadherin-mediated determinant for IEE.

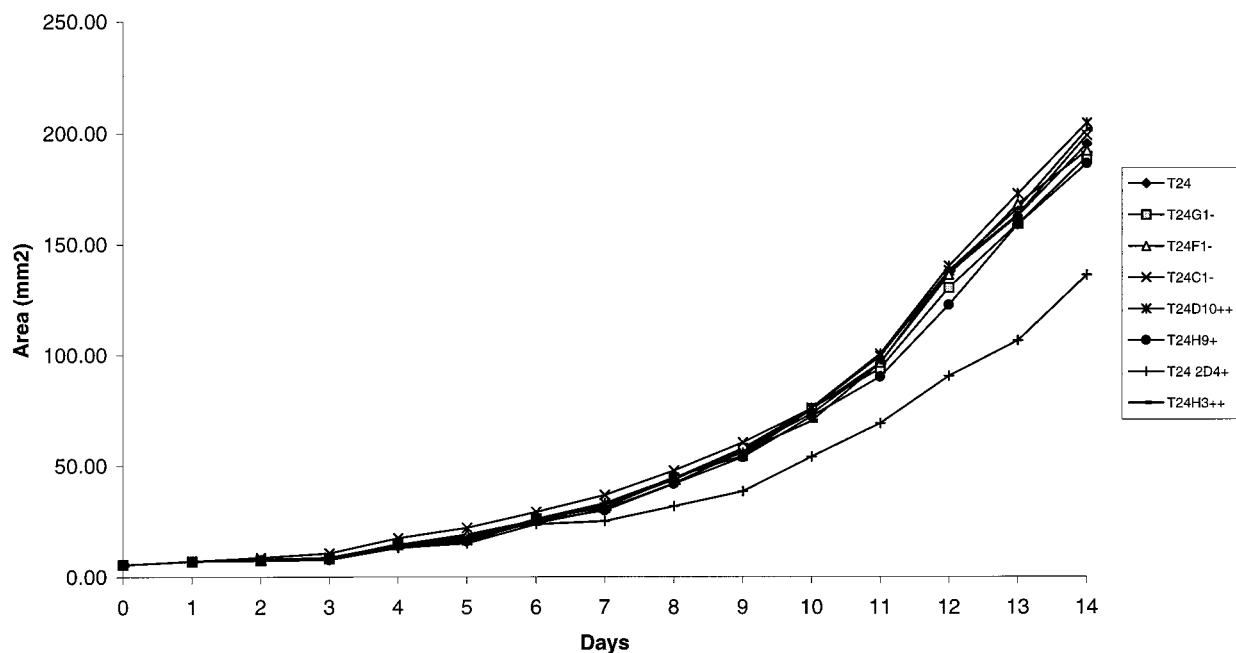Our study suggests that E-cadherin is an important molecule for

Fig. 7. Expansion of T24 clones in the absence of surrounding normal urothelium. Data are expressed as means (n = four cultures).

IEE of TCC *in vitro*, but it does not prove that this view holds true for bladder carcinoma *in situ*. Although a number of studies analyzed E-cadherin immunoreactivity during different stages of bladder cancer progression, none reported on the E-cadherin expression in carcinoma *in situ* of the bladder in detail (15–17, 31). In a preliminary study, we stained paraffin sections with carcinoma *in situ* of 10 patients for E-cadherin. All of these 10 lesions had a normal, homogeneous, membranous expression of E-cadherin, confirming our hypothesis on the contributory role of E-cadherin in the intraepithelial propagation of bladder carcinomas.

For carcinogenesis *in vivo*, our observations would imply that on the one hand, E-cadherin promotes expansion of carcinoma *in situ* and on the other hand, opposes invasiveness of the transformed cells. Generalizing, E-cadherin-mediated cohesiveness may represent a major property of transformed clones, allowing carcinoma *in situ* to expand at the expense of surrounding normal epithelial cells *in vivo*.

REFERENCES

1. Kakizoe, T., Matumoto, K., Nishio, Y., Mikinobu, O., and Kishi, K. Significance of carcinoma *in situ* and dysplasia in association with bladder cancer. J. Urol., *133:* 395–398, 1985.
2. Hudson, M. A., and Herr, H. W. Carcinoma *in situ* of the bladder. J. Urol., *153:* 564–572, 1995.
3. Lamm, D. L. Carcinoma *in situ*. Urol. Clin. N. Am., *19:* 499–508, 1992.
4. Althausen, A. F., Prout, G. R., and Daly, J. J. Non-invasive papillary carcinoma of the bladder associated with carcinoma *in situ*. J. Urol., *116:* 575–580, 1976.
5. Weinstein, R. S., Coon, J. S., Schwartz, D., Miller, A. W., III, and Pauli, B. U. Pathology of superficial bladder cancer with emphasis on carcinoma *in situ*. Urology, *26* (Suppl.)*:* S2–S10, 1985.
6. Boyd, P. J. R., and Burnard, K. G. Site of bladder-tumor recurrence. Lancet, *2:* 1290–1292, 1974.
7. Hinman, F. Recurrence of bladder tumors by surgical implantation. J. Urol., *75:* 695–696, 1956.
8. See, W. A., Miller, J. S., and Williams, R. D. Pathophysiology of transitional tumor cell adherence to sites of urothelial injury in rats: mechanisms mediating intravesical recurrence due to implantation. Cancer Res., *49:* 5414–5418, 1989.
9. Soloway, M. S., and Masters, S. Urothelial susceptibility to tumor cell implantation: influence of cauterization. Cancer (Phila.), *46:* 1158–1163, 1980.
10. Rebel, J. M. J., Thijssen, C. D. E. M., Vermey, M., Delouvée, A., Zwarthoff, E. C., and Van Der Kwast, T. H. E-cadherin expression determines the mode of replacement of normal urothelium by human bladder carcinoma cells. Cancer Res., *54:* 5488–5492, 1994.
11. Bindels, E. M. J., Vermey, M., Rebel, J. M. J., Zwarthoff, E. C., and Van Der Kwast, T. H. *In vitro* modulation of implantation and intraepithelial expansion of bladder tumor cells by epidermal growth factor. Exp. Cell Res., *235:* 395–402, 1997.
12. Vleminckx, K., Vakaet, L., Mareel, M., Fiers, W., and Van Roy, F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor. Cell, *66:* 107–119, 1991.
13. Behrens, J., Mareel, M. M., Van Roy, F. M., and Birchmeier, W. Dissecting tumor cell invasion: epithelial cells acquire invasive properties following the loss of uvomorulin-mediated cell-cell adhesion. J. Cell Biol., *108:* 2435–2447, 1989.
14. Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M., and Birchmeier, W. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/β-catenin complex in cells transformed with a temperature-sensitive v-*SRC* gene. J. Cell Biol., *120:* 757–766, 1993.
15. Bringuier, P. P., Umbas, R., Schaafsma, H. E., Karthaus, H. F. M., Debruyne, F. M. J., and Schalken, J. A. Decreased E-cadherin immunoreactivity correlates with poor survival in patients in bladder cancer. Cancer Res., *53:* 3241–3245, 1993.
16. Otto, T., Bex, A., Schmidt, U., Raz, A., and Rübben, H. Improved prognosis assessment for patients with bladder carcinoma. Am. J. Pathol., *150:* 1919–1923, 1997.
17. Syrigos, K. N., Krausz, T., Waxman, J., Pandha, H., Rowlinson-Busza, G., Verne, J., Epenetos, A. A., and Pignatelli, M. E-cadherin expression in bladder cancer using formalin-fixed, paraffin-embedded tissues: correlation with histopathological grade, tumor stage and survival. Int. J. Cancer, *64:* 367–370, 1995.
18. Syrigos, K. N., Harrington, K. J., and Pignatelli, M. Role of adhesion molecules in bladder cancer: an important part of the jigsaw. Urology, *53:* 428–434, 1999.
19. Masters, J. R. W., Hepburn, P. J., Walker, L., Highman, W. J., Trejdosiewisz, L. K., Povey, S., Parkar, M., Hill, B. T., Riddle, P. T., and Franks, L. M. Tissue culture model of transitional cell carcinoma: characterization of twenty-two human urothelial cell lines. Cancer Res., *46:* 3630–3636, 1986.
20. Paulie, S., Hansson, Y., Lundblad, M. L., and Perlmann, P. Lectins as probes for identification of tumor associated antigens on urothelial and colonic carcinoma cell lines. Int. J. Cancer, *31:* 297–303, 1983.
21. De Boer, W. I., Houtsmuller, A. B., Izadifar, V., Muscatelli-Groux, B., Van Der Kwast, T. H., and Chopin, D. Expression and function of EGF, FGF and TGFβ-growth factor family members and their receptors in invasive human transitional-cell-carcinoma cells. Int. J. Cancer, *71:* 284–291, 1997.
22. De Boer, W. I., Rebel, J. M. J., Vermey, M., De Jong, A. A. W., and Van Der Kwast, T. H. Characterization of distinct functions for growth factors in murine transitional epithelial cells in primary organotypic culture. Exp. Cell. Res*., 214:* 510–518, 1994.
23. Nagafuchi, A. Shirayoshi, Y., Okazaki, K., Yasuda, K., and Takeichi, M. Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. Nature (Lond.), *329:* 341–343, 1987.
24. Chen, C., and Okayama, H., High efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol., *7:* 2745–2752, 1987.
25. Giroldi, L. E., Bringuier, P. P., Shimazui, T., Jansen, K., and Schalken, J. A. Changes in cadherin-catenin complexes in the progression of human bladder carcinoma. Int. J. Cancer, *82:* 70–76, 1999.
26. Mareel, M., Kint, J., and Meyvisch, C. Methods of study of the invasion of malignant C3H mouse fibroblasts into embryonic chicken heart *in vitro*. Virchows Arch. B Cell Pathol., *30:* 95–111, 1977.
27. Chen, H., Paradies, N. E., Fedor-Caiken, M., and Brackenbury, R. E-cadherin mediates adhesion and suppresses cell motility via distinct mechanisms. J. Cell Sci., *110:* 345–356, 1997.
28. Soler, C., Grangeasse, C., Baggetto, L. G., and Damour, O. Dermal fibroblast proliferation is improved by β-catenin overexpression and inhibited by E-cadherin expression. FEBS Lett., *442:* 178–182, 1998.
29. Llorens, A., Rodrigo, I., Lopez-Barcons, L., Gonzalez-Garrigues, M., Lozano, E., Vinyals, A., Quintanilla, M., Cano, A., and Fabra, A. Down-regulation of E-cadherin in mouse skin enhances a migratory and invasive phenotype linked to matrix metalloproteinase-9 gelatinase expression. Lab. Investig., *78:* 1131–1141, 1998.
30. Pignatelli, M. Integrins, cadherins, and catenins: molecular cross-talk in cancer cells. J. Pathol., *186:* 1–2, 1998.
31. Shimazui, T., Schalken, J. A., Giroldi, L. A., Jansen, C. F. J., Akaza, H., Koiso, K., Debruyne, F. M. J., and Bringuier, P. P. Prognostic value of cadherin-associated molecules (α-, β-, γ-catenins and p120^{cas}) in bladder tumors. Cancer Res., *56:* 4154–4158, 1996.