Am J Physiol Cell Physiol 289: C1002–C1014, 2005.
First published June 1, 2005; doi:10.1152/ajpcell.00175.2005.

Inducible expression of Snail selectively increases paracellular ion permeability and differentially modulates tight junction proteins

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Submitted 12 April 2005; accepted in final form 30 May 2005

Carrozzino, Fabio, Priscilla Soulié, Denise Huber, Noury Mensi, Lelio Orci, Amparo Cano, Eric Féraille, and Roberto Montesano. Inducible expression of Snail selectively increases paracellular ion permeability and differentially modulates tight junction proteins. *Am J Physiol Cell Physiol* 289: C1002–C1014, 2005. First published June 1, 2005; doi:10.1152/ajpcell.00175.2005.—Constitutive expression of the transcription factor Snail was previously shown to trigger complete epithelial-mesenchymal transition (EMT). The aim of this study was to determine whether inducible expression of *Snail* could modify epithelial properties without eliciting full mesenchymal conversion. For this purpose, we expressed mouse *Snail* (mSnail) cDNA in Madin-Darby canine kidney (MDCK) cells under the control of a doxycycline-repressible transactivator. Inducible expression of *Snail* did not result in overt EMT but induced a number of phenotypic alterations of MDCK cells, the most significant of which was the absence of fluid-filled blisterlike structures called "domes." To understand the mechanisms responsible for dome suppression, we assessed the effect of mSnail expression on epithelial barrier function. Although mSnail did not alter tight junction (TJ) organization and permeability to uncharged solutes, it markedly decreased transepithelial electrical resistance. In light of these findings, we evaluated the ability of MDCK cell monolayers to maintain ionic gradients and found that expression of mSnail selectively increases Na^+ and Cl^- permeability. Analysis of the expression of claudins, transmembrane proteins that regulate TJ ionic permeability, showed that mSnail induces a moderate decrease in claudin-2 and a substantial decrease in claudin-4 and -7 expression. Together, these results suggest that induction of mSnail selectively increases the ionic permeability of TJs by differentially modulating the expression of specific claudins.

epithelium; Madin-Darby canine kidney cells; claudin; dome

THE DEFINING CHARACTERISTIC of epithelial cells is their ability to form continuous sheets that constitute a structural and functional interface between distinct body compartments. The integrity of epithelial tissues requires the establishment and maintenance of junctional complexes (22), a set of specialized intercellular contacts that comprise tight junctions (TJs), adherens junctions, desmosomes, and gap junctions. The TJ consists of a beltlike network of anastomosing strands that encircle the cells at the boundary between the apical and basolateral membrane domains. Each TJ strand is composed of a row of intramembrane proteins and pairs with a similar strand on an adjacent cell to obliterate the intercellular space. TJs serve as a regulated barrier that restricts the diffusion of solutes through the paracellular pathway (46, 67). Recent evidence indicates that transmembrane proteins of the claudin family are essential components of TJ strands and determine their selective permeability properties (2, 72, 73).

Despite their highly differentiated and apparently static phenotype, epithelial cells are endowed with a remarkable degree of plasticity. Thus, in specific developmental processes, as well as in adult life during tumor progression, epithelial cells escape from the rigid structural constraints imposed by intercellular junctions and adopt a migratory behavior. Epithelial plasticity is variable in degree (30), ranging from epithelial-mesenchymal transition (EMT), which is characterized by disruption of intercellular contacts, loss of epithelium-specific proteins, switch to a mesenchymal gene expression pattern, and gain of invasive properties (70), to more limited phenotypic changes that are not associated with full mesenchymal conversion. Thus, during biological processes that involve coordinated cell rearrangements and tissue remodeling, such as branching morphogenesis and organ regeneration, epithelial cells transiently downmodulate or relocalize junctional proteins without losing their distinguishing phenotypic characteristics (33, 80). The molecular mechanisms responsible for epithelial plasticity and EMT have only recently begun to be elucidated (30, 70).

Increasing evidence indicates that the transcription factor Snail acts as a key regulator of EMT (7, 13, 14). Snail belongs to a family of zinc finger-containing transcriptional repressors and was originally identified as a regulator of mesoderm formation in developing *Drosophila* (34, 50). Snail has subsequently been shown to be critical for developmental processes in mouse that require EMT, such as the emigration of neural crest cells from the neural tube. Thus it has been reported that *Snail*-null embryos die at the gastrulation stage because of failure of EMT (14). When ectopically expressed in epithelial cells, Snail represses the transcription of *E-cadherin* and triggers a complete EMT with the acquisition of invasive and tumorigenic properties (7, 10, 13, 54, 55).

The ability to tightly control gene expression with inducible systems has greatly facilitated the analysis of gene function (9, 28, 46, 64). Unlike constitutive transfection procedures, inducible systems provide an opportunity to assess the short-term

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effects of a given gene during defined time periods and to verify the reversibility of the observed changes. We hypothesized that the extreme phenotypic switch triggered by stable transfection of *Snail* (7, 13) could mask or overshadow more subtle effects of the transcription factor on epithelial properties. We therefore expressed mouse *Snail* (mSnail) cDNA in Madin-Darby canine kidney (MDCK) cells with a tetracycline-inducible expression system with a view to identifying novel downstream targets of Snail. We report here that inducible expression of *Snail* does not result in overt EMT but selectively alters TJ barrier function. Specifically, we found that mSnail differentially modulates the expression of specific claudins and increases paracellular ionic conductance without affecting TJ permeability to uncharged solutes, resulting in dome disappearance. These findings suggest that in addition to its well-established role in EMT during embryogenesis and tumor progression, Snail may act as a regulator of epithelial permeability in physiological and pathological settings.

MATERIALS AND METHODS

Cell culture and transfections. MDCK II Tet-Off cells, which stably express the tetracycline-repressible transactivator (tTA) (29), were obtained from Clontech (Palo Alto, CA). The cells were grown in DMEM supplemented with 4 mM L-glutamine, 5% FCS (Life Technologies, Gaithersburg, MD), 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma, St. Louis, MO), and 1 μg/ml puromycin (Sigma) to maintain the selection pressure for tTA-expressing cells. Mouse *Snail* (mSnail) complete cDNA was cloned into the puHD10–3 plasmid (13, 29) at the *Not*I-*Xba*I sites. Transfections of subconfluent cultures of MDCK II Tet-Off cells were performed with Effectene (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. The cells were either cotransfected with puHD10–3 and pNT-Hygro (which carries the hygromycin-resistance gene) or transfected with pNT-Hygro alone. After selection in DMEM containing 300 μg/ml hygromycin (Sigma) plus 2 μg/ml doxycycline (Dox; Clontech) to turn off the expression of the transfected gene, surviving colonies were isolated and the resulting cell lines were grown in DMEM supplemented with 5% tetracycline-free FCS (Tet-free FCS; Clontech), 150 μg/ml hygromycin, 1 μg/ml puromycin, and 2 μg/ml Dox (this medium is hereafter referred to as "complete medium"). For experiments, cells were harvested by trypsinization from confluent stock cultures that had been maintained in the presence or absence of 2 μg/ml Dox for at least 4 days. *Clone 10*, which exhibited a robust induction of mSnail (see RESULTS), was predominantly used in this study. Analysis of *clones 1* and *12* was included in a number of experiments and provided results similar to those with *clone 10*. MDCK/Snail cells, a MDCK II cell line constitutively overexpressing mSnail (13), were grown in DMEM supplemented with 10% FCS and 400 μg/ml G418 (GIBCO-Invitrogen, Carlsbad, CA) to maintain the selection pressure for mSnail expression. Subconfluent cultures of MDCK/Snail cells were transfected with Effectene with the pPGKE-cad-hyg expression plasmid (a generous gift of Dr. L. Larue, Institut Curie, Orsay, France), which contains the full-length murine E-cadherin cDNA and a hygromycin-resistance sequence (12, 40). Stable transfectants were selected in 200 μg/ml hygromycin B. Surviving colonies were isolated, and the resulting MDCK/Snail/E-cadherin cell lines (referred to as MSE-10 cells) were grown in medium supplemented with 150 μg/ml hygromycin and 400 μg/ml G418.

RT-PCR. RNA was extracted from cells with TRIzol (Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed with random hexadeoxynucleotides (Promega, Madison, WI) and Superscript II (Life Technologies). Amplification of mSnail gene was performed with the following primers: forward 5′-AAGCCCAACTATAGCGAGCTG-3′, reverse 5′-CTTCGGATGTGCATCTTCAGAG-3′ (66). An endogenous 398-bp canine β-actin sequence was amplified as a control of RNA quality with the following primers: forward 5′-GACATCAAGGAAGAAGCTCTGC-3′ and reverse 5′-CCTCCGATCCACACAGAATACT-3′.

RNase protection assay. RNase protection assay (RPA) was performed as previously described (57). [^{32}P]dUTP cRNA probes were generated for mSnail, and the housekeeping gene acidic ribosomal phosphoprotein P0 was used as an internal control (56).

Western blot. Cells were incubated in lysis buffer (in mM: 50 Tris·HCl, pH 8.0, 150 NaCl, and 5 EDTA, pH 8.0, with 1% Triton X-100 and 1% Nonidet P-40) containing 1 mM PMSF, 2 μg/ml leupeptin, and 1 μg/ml pepstatin A for 30 min on ice. Afterwards, the lysed cells were scraped, transferred to an Eppendorf tube, and centrifuged. The supernatant was collected, and protein content was measured with a bicinchoninic acid (BCA) protein assay kit (BCA*, Pierce, Rockford, IL). Equal amounts of total extracts (25 μg) were separated by 7.5% or 12% SDS-PAGE before transfer onto polyvinylidene difluoride membranes (Bio-Rad, Reinach, Switzerland). To control for differences in loading, proteins were both stained with Coomassie blue and immunoblotted for actin. Membranes were blocked 90 min at room temperature in PBS containing 0.4% (vol/vol) Tween 20 (PBS-Tween) and 5% (wt/vol) nonfat milk powder and then incubated either overnight at 4°C or for 2 h at room temperature with primary antibodies: anti-E-cadherin rat monoclonal (Sigma; 1:2,000); anti-E-cadherin rabbit serum (gift of D. Bosco, University of Geneva; 1:3,000); anti-β-catenin rabbit serum (Sigma; 1:4,000); anti-fibronectin rabbit serum (Life Technologies; 1:3,000); anti-total actin rabbit serum (gift of G. Gabbiani, University of Geneva; 1:1,000); mouse monoclonal anti-Na^+-K^+-ATPase α_1-subunit (clone C464.6, Upstate, Lake Placid, NY; 0.05 μg/ml) and β_1-subunit (clone 464.8, Upstate; 0.5 μg/ml); rabbit sera (all from Zymed Laboratories, South San Francisco, CA) against zonula occludens (ZO)-1 (0.5 μg/ml), occludin (1 μg/ml), claudin-1 (not cross-reacting with claudin-3; 1 μg/ml), claudin-2 (0.5 μg/ml), claudin-3 (1 μg/ml), and claudin-7 (1 μg/ml); and mouse monoclonal anti-claudin-4 (clone 3E2C1, Zymed; 1 μg/ml). After extensive washing in PBS-Tween, the membranes were incubated for 45 min at room temperature with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Otelfingen, Switzerland, or Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:3,000. Membranes were then washed extensively in PBS-Tween, and antigen-antibody complexes were detected by enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

Hanging drop assay of cell aggregation. Cells were trypsinized and resuspended at 5×10^5 cells/ml in complete medium with or without 2 μg/ml Dox. Four 20-μl drops of cell suspension were placed on the inside of the lid of a 35-mm culture dish (Nunc, Roskilde, Denmark). The lid was then replaced on the dish so that the drops were hanging from the lid. To prevent drop evaporation, 2 ml of PBS was placed in the bottom of each dish. After incubation for 24–48 h at 37°C to allow cell aggregation at the drop meniscus (37, 38, 60), loose cell clusters were dissociated by gently passing them 10 times through a 200-μl Gilson pipette tip (71), and the residual tight aggregates were photographed with a Nikon TMD inverted microscope. To ascertain that cell aggregation was mediated by E-cadherin, duplicate samples were resuspended in a 1:1 mixture of culture medium and hybridoma supernatant containing a function-blocking E-cadherin monoclonal antibody (anti-Arc-1, generously provided by Dr. B. Imhof, University of Geneva) (8, 36).

Chemotaxis. Chemotaxis was performed according to Falk et al. (21) in 48-microwell chemotaxis chambers (Neuro Probe, Gaithersburg, MD), using 8-μm pore-size polyvinylpyrrolidone-free polycarbonate membranes (Neuro Probe) coated with 100 μg/ml type I collagen prepared as described previously (48). Cells were resuspended in DMEM with 0.1% BSA (serum-free medium, SFM) and added to the upper chamber of the wells. Each well was filled with 50

μl of cell suspension (10^6 cells/ml). The lower compartment of each chamber was filled with 28 μl of SFM supplemented with hepatocyte growth factor (HGF; gift from Dr. R. Schwall, Genentech, San Francisco, CA) at varying concentrations as a chemoattractant. SFM was used as a control for random unstimulated migration. Each experimental condition was performed in sextuplicate. The chambers were incubated for 6 h at 37°C in a humidified 5% CO_2 atmosphere. The filters were then removed, and the cells were fixed with 100% ethanol and stained with toluidine blue. Cells that had not migrated were removed from the upper surface of the membrane with a filter paper. Migration was measured by densitometric analysis with Scion Image software (Scion, Frederick, MD).

Cyst formation in collagen gels. Cells were sandwiched between two collagen gels for 7 days, fixed, dehydrated, and embedded in Epon 812 as described previously (49). Semithin sections (1 μm thick) were cut with a LKB ultramicrotome (LKB Instruments, Gaithersburg, MD), stained with 1% methylene blue, and photographed under transmitted light with a photomicroscope (Zeiss, Oberkochen, Germany).

Dome formation. MDCK cells were seeded at confluent density (4×10^6 cells/dish) into 60-mm dishes (Falcon, Becton Dickinson Labware Europe, Le Pont de Cloix, France) coated with 30 μg/ml type I collagen. The cultures were subsequently monitored by phase-contrast microscopy over a period of 14 days to assess dome formation.

Cyst formation in suspension culture. Cell culture-tested agarose, 0.5% (wt/vol) in distilled water, was autoclaved, dispensed into a six-well multiwell plate (Corning Costar, Cambridge, MA; 2 ml/well), and allowed to gel at 4°C. Cells were suspended at a concentration of 5×10^4 cells/ml in a 1:1 mixture of DMEM and F12 medium supplemented with 1% ITS$^+$ Premix (BD Biosciences, Two Oak Park, Bedford, MA) with or without 2 μg/ml Dox. Three milliliters of cell suspension was then poured on top of the agarose gel to allow formation of floating cell clusters. After 1 wk, the aggregates were fixed as described previously (77) and subsequently included in 2.5% agar-agar (GIBCO) to preserve cyst architecture and prevent lumen collapse. The agar blocks were then dehydrated and embedded in Epon 812. Semithin sections were prepared as described above.

Measurement of $^{86}Rb^+$ uptake. The transport activity of Na^+-K^+-ATPase was measured by ouabain-sensitive $^{86}Rb^+$ uptake under initial rate conditions (25). Confluent MDCK cells grown on polycarbonate semipermeable Transwell filters (12-mm diameter, 0.4-μm pore size; Corning Costar), were preincubated in serum-free DMEM with or without ouabain (2 mM) for 60 min at 37°C. Measurements were performed in quadruplicate after the addition of 50 μl of medium containing tracer amounts of ^{86}RbCl (Amersham, 100 nCi/sample) for 3 min. Incubation was stopped by cooling on ice, rapid aspiration of the incubation medium, and three washes with ice-cold washing solution (in mM: 150 choline chloride, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 2 $BaCl_2$, and 5 HEPES, pH 7.4). Cells were lysed in 750 μl of Triton X-100 (1% wt/vol), and radioactivity was measured by liquid scintillation counting of 400-μl samples. Protein content was determined in parallel with the BCA assay. Ouabain-sensitive $^{86}Rb^+$ uptake was calculated as the difference between the mean values measured in quadruplicate samples incubated with or without 2 mM ouabain and was expressed as mean ± SE picomoles of rubidium per microgram of protein per minute. Preliminary experiments showed that the rate of $^{86}Rb^+$ uptake was linear for at least 5 min at 37°C and that ouabain-sensitive $^{86}Rb^+$ uptake accounted for approximately two-thirds of the total $^{86}Rb^+$ uptake.

Ca^{2+} switch. The Ca^{2+} switch assay was performed as previously described (31, 51, 69). MDCK II Tet-Off cells, as well as MDCK-Snail *clone 10* cells that had been grown for 4 days in the presence or absence of Dox, were plated at confluent density (5.5×10^5 cells/cm^2) on Transwell filters in normal-Ca^{2+} (NC) medium (DMEM + 5% FCS, 1.8 mM Ca^{2+}) and allowed to attach at 37°C for 4 h. The cells were then gently washed four times with low-Ca^{2+} (LC) DMEM (3 μM Ca^{2+}) and kept overnight in LC medium containing 5% FCS that had been dialyzed extensively against Ca^{2+}-free DMEM, with or without 2 μg/ml Dox, to allow disassembly of cell junctions. The cells were then switched back to physiological Ca^{2+} conditions by replacing the LC medium with NC medium. At selected time points, TJ reassembly was monitored either by measuring transepithelial electrical resistance (TER) or by electron microscopy after glutaraldehyde fixation, as described below.

Measurement of TER. Cells were plated at confluent density (5.5×10^5 cells/cm^2) on Transwell filters in complete medium with or without 2 μg/ml Dox. At the indicated time points, TER was measured with a Millicell-ERS volt-ohm meter (Millipore, Billerica, MA), and monolayer TER values (expressed as $\Omega \cdot cm^2$) were obtained by subtracting blank (cell free) filter readings (31, 45).

Mannitol flux. Determination of [^{3}H]mannitol flux was carried out as previously described (4, 63). Briefly, 1.7×10^6 cells/cm^2 were plated on polyethylene terephthalate cell culture inserts (Falcon/BD, 0.4-μm pore size, 0.9-cm^2 surface area). After 24 h, media were replaced with fresh media with or without Dox. The apical media were supplemented with [^{3}H]mannitol (2 μCi/ml, NEN-Dupont, Wilmington, DE), and after 3 h incubation at 37°C in a humidified 5% CO_2 atmosphere apical and basolateral media were collected and radioactivity was counted with a liquid scintillation counter (Betamatic V; Kontron Instruments, Zurich, Switzerland).

Mannitol permeability. MDCK cells were plated at confluent density on Transwell filters. Apical or basolateral media were supplemented with 200 mosM mannitol, and after 6 h incubation at 37°C in a humidified 5% CO_2 atmosphere apical and basolateral media were collected and osmolality was measured. Results were expressed as mean ± SE mosmol/kgH_2O.

Unidirectional ion flux. MDCK cells plated at confluent density on Transwell filters were transferred to NaCl-containing defined medium [in mM: 120 NaCl, 5 KCl, 4 $NaHCO_3$, 1 $CaCl_2$, 1 $MgSO_4$, 0.2 NaH_2PO_4, 0.15 Na_2HPO_4, 5 glucose, 10 lactate, 1 pyruvate, and 20 HEPES, pH 7.4, with essential and nonessential amino acids and vitamins (GIBCO)]. For experiments, apical or basolateral NaCl-containing defined medium was substituted with either nominally Na^+-free medium (in which NaCl was replaced by *N*-methyl-D-glucamine-Cl and other Na^+ salts were replaced by K^+ salts) or nominally Cl^--free medium (in which NaCl was replaced by Na-gluconate, KCl by K-gluconate, and $CaCl_2$ by $CaSO_4$). After 24- to 48-h incubation at 37°C under a CO_2-air atmosphere, Na^+ and Cl^- content of apical and basolateral media was measured with ion-specific electrodes in a Synchroton LX20 apparatus (Beckman-Coulter). Results were expressed as mean ± SE millimoles per liter.

Immunofluorescence. Staining for E-cadherin was performed as previously described (12, 17). Cells grown on glass coverslips were incubated for 2 h at room temperature with an anti-E-cadherin rat monoclonal antibody (Sigma, 1:300). After 1-h incubation with a FITC-labeled rabbit anti-rat secondary antibody (Nordic Immunological Labs, Tilburg, The Netherlands; 1:300), cells were counterstained with 0.03% Evans blue, mounted on glass slides, and photographed with an epifluorescence microscope (Axiophot, Zeiss). Negative controls consisted of omission of primary antibody.

Thin-section electron microscopy. For conventional ultrastructural analysis, MDCK II Tet-Off cells, as well as MDCK-Snail *clone 10* cells from cultures pretreated for 4 days in the presence or absence of Dox, were seeded onto Transwell filters and subjected to the calcium switch protocol described above. Eight hours after switching to NC medium, the monolayers were fixed in 2.5% glutaraldehyde in 100 mM cacodylate buffer (pH 7.4) for 90 min. After extensive washing in cacodylate buffer, each filter was removed from its support with a scalpel and cut into four pieces. These were postfixed for 20 min in 1% osmium tetroxide in Veronal acetate buffer, stained en bloc with uranyl acetate, dehydrated in a graded series of ethanol, and embedded in Epon 812. For tracer studies, the medium in the upper compartment of Transwell filter cultures was replaced by 2.5% glu-

taraldehyde in 100 mM cacodylate buffer containing 2% Alcian blue 8 GS (Fluka, Buchs, Switzerland). After postfixation in osmium tetroxide, pieces of filters were treated quickly (1–2 min) with 1% tannic acid (Mallinckrodt, St. Louis, MO) in 50 mM cacodylate buffer, pH 7.0, washed, stained en bloc with uranyl acetate, dehydrated, and embedded in Epon 812. Thin sections were cut with an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Philips EM10 electron microscope.

Freeze-fracture electron microscopy. Cells pretreated for 4 days in the presence or absence of Dox were plated into 60-mm dishes (Falcon) at confluent density (4×10^6 cells/dish) in NC medium (1.8 mM Ca^{2+}). Attached cells were rinsed four times with LC medium (3 μM Ca^{2+}) 2 h after being plated and incubated overnight in the same medium. After being switched to NC medium, cells were fixed at various time points with 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4) for 1 h, washed in cacodylate buffer, gently scraped with a rubber policeman, and pelleted in a plastic centrifuge tube. The pellet was cryoprotected by infiltration with 30% (vol/vol) glycerol in 100 mM phosphate buffer, mounted on gold specimen holders, and quickly frozen in the liquid phase of partially solidified Freon 22 cooled with liquid nitrogen. Fracturing was carried out at a stage temperature of −100°C under a vacuum of 10^{-7} Torr in a freeze-fracture unit (BAF400; Balzers High Vacuum, Balzers, Liechtenstein). After evaporation of platinum and carbon, replicas were cleaned by sequential treatment with sodium hypochlorite and chloroform-methanol (2:1), picked up on Parlodium-coated 150-mesh copper grids, and examined in a Philips EM10 electron microscope. Morphometric analysis of the number of TJ strands was performed on micrographs of freeze-fracture replicas. A line parallel to the main axis of each TJ was traced, and a series of perpendicular lines was drawn at 400-nm intervals. The number of strands in a given TJ segment was defined as the number of intersections with the perpendicular line (5).

RESULTS

Inducible expression of mSnail in stably transfected MDCK cells. The initial objective of this study was to determine whether inducible expression of *Snail* in MDCK cells could increase epithelial plasticity without causing complete and irreversible EMT. We subcloned mSnail into the tetracycline-responsive pUHD10–3 plasmid (28) and transfected the resulting construct into MDCK II Tet-Off cells, which express the tetracycline-repressible transactivator tTA (6, 29). This resulted in the establishment of stably transfected cell clones expressing mSnail on withdrawal of the tetracycline derivative Dox. Induction of mSnail in the absence of Dox was confirmed by RT-PCR and RPA in several clones of transfected MDCK II Tet-Off cells (data not shown). *Clone 10*, which exhibited a robust induction of mSnail (Fig. 1*A*), was used predominantly, but analysis of *clones 1* and *12* was included in some experiments (see below).

Induction of mSnail expression results in mild phenotypic changes in MDCK cells. Examination of sparse cultures of MDCK-Snail *clone 10* cells grown in the presence or absence of Dox showed that mSnail expression was associated with the acquisition of a spindlelike morphology and cell scattering (Fig. 1*C*). However, on reaching confluence, mSnail-expressing MDCK cells resumed an epithelium-like shape and formed a continuous monolayer of closely apposed polygonal cells (not shown). Western blot analysis showed that mSnail downregulates the epithelial marker protein E-cadherin and strongly upregulates the mesenchymal protein fibronectin (Fig. 1*B*), as previously reported (7, 13). Similar results were obtained with *clones 1* and *12* (not shown).

To determine whether E-cadherin downregulation affected cell-cell adhesion, we evaluated the effects of mSnail on cell aggregation with a hanging drop assay. mSnail expression inhibited cell aggregation to a similar extent as a function-blocking anti-E-cadherin antibody (Fig. 1*D*). Inhibition of cell aggregation was most prominent after 24–48 h of treatment but became less apparent during subsequent incubation, possibly because of incomplete downregulation of E-cadherin (not shown).

Because MDCK II Tet-Off cells that inducibly express mSnail are scattered in low-density cultures (see above) and MDCK II cells constitutively overexpressing mSnail exhibit increased motility (13), we examined the migratory properties of MDCK-Snail *clone 10* cells in a microchamber chemotaxis assay. mSnail expression was associated with significantly enhanced migration in response to a chemoattractive stimulus provided by HGF (Fig. 1*E*).

Finally, we examined the effect of mSnail on the ability of MDCK cells to form multicellular cysts in three-dimensional collagen gels. Analysis of several clones showed that expression of mSnail causes MDCK cells to form irregularly shaped, flattened structures that contain multiple small lumenlike spaces instead of a single, wide lumen (Fig. 1*F* and data not shown). The cumulative findings described above indicated that induction of mSnail expression in epithelial cells causes subtle phenotypic changes instead of the full EMT seen with constitutive mSnail expression (7, 13).

Induction of mSnail expression abrogates dome formation. Examination of postconfluent cultures of MDCK-Snail *clone 10* cells disclosed an additional and impressive effect of mSnail expression, the complete suppression of dome formation. Domes are turgid blisterlike structures that form in postconfluent monolayers of polarized epithelial cells as a result of localized fluid accumulation between the basal cell surface and the plastic substratum (41). Whereas MDCK-Snail *clone 10* cells grown in the presence of Dox formed a monolayer crowded with domes, as observed in untreated MDCK II Tet-Off cells, cells grown without Dox formed a cobblestone-like monolayer that was virtually devoid of domes (Fig. 2*A*). Dome suppression occurred in all the clones examined. In addition, the effect of mSnail was fully reversible: in confluent cultures in which mSnail was previously expressed, domes appeared within 24 h of Dox addition and ensuing mSnail repression (data not shown).

The reversible ability of mSnail to completely inhibit dome formation prompted us to investigate the molecular basis of this phenomenon. Dome formation is thought to depend on three important physiological characteristics of epithelial cells: *1*) vectorial (i.e., apical to basal) fluid transport (41, 42, 82), *2*) cell-substratum adhesion (58), and *3*) sealing function of TJs (15). Inhibition of dome formation therefore provided a useful model to investigate the effect of mSnail on each of these physiological parameters.

Na^+-K^+-ATPase, a basolaterally localized Na^+ pump, provides the driving force for vectorial fluid transport in polarized epithelial cells (19, 23), and its activity is required for dome formation (41). We assessed the effect of mSnail on the level of Na^+-K^+-ATPase α_1- and β_1-subunit expression by Western blot. Unexpectedly, induction of mSnail increased the expres-

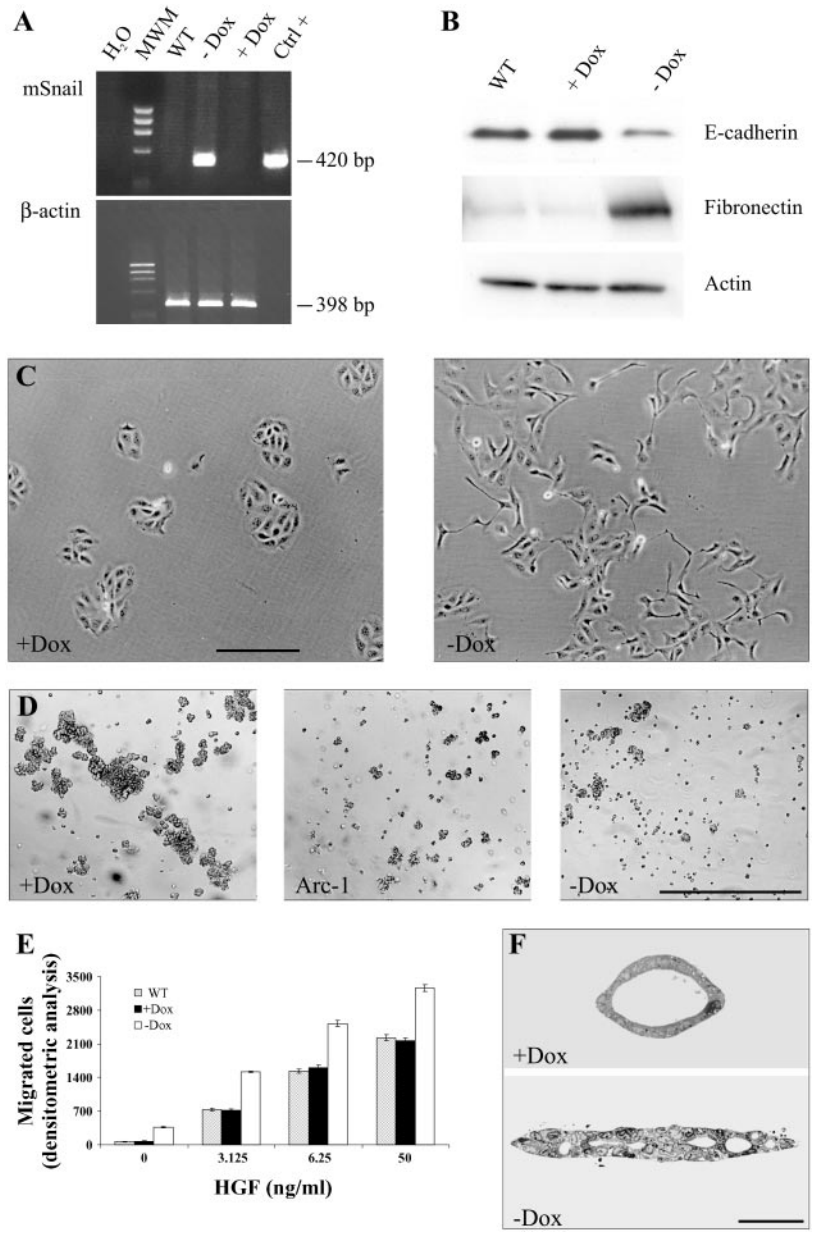

Fig. 1. Inducible expression of mouse *Snail* (mSnail) induces mild phenotypic changes in Madin-Darby canine kidney (MDCK) cells. *A*: induction of mSnail on omission of doxycycline (Dox) from the culture medium. With RT-PCR amplification, a transcript for mSnail was detected in MDCK-Snail *clone 10* cells cultured for 4 days in the absence of Dox (−Dox) but not in the same cells cultured in the presence of Dox (+Dox) nor in parental MDCK II Tet-Off cells (WT), which served as a negative control. The puHD10–3 plasmid, which carries mSnail cDNA, was used as a positive control (Ctrl+). β-Actin amplification of the same RNA samples was used as a control of RNA quality. Template-free reactions (H_2O) were run in parallel as negative controls. ϕX174 RF-DNA/*Hae*III fragments (Invitrogen) were used as molecular weight markers (MWM). *B*: expression of mSnail downregulates the epithelial marker E-cadherin and upregulates the mesenchymal marker fibronectin. Total extracts of confluent wild-type MDCK II Tet-Off cells (WT) and mSnail-transfected cells, cultured for 4 days in the presence (+Dox) or absence (−Dox) of Dox, were immunoblotted with antibodies to E-cadherin and fibronectin. +Dox cells exhibit levels of E-cadherin and fibronectin that are comparable to those of wild-type MDCK II Tet-Off cells. In contrast, −Dox cells show reduced levels of E-cadherin and upregulation of fibronectin. Uniform loading of lanes was verified by immunoblotting of total actin. *C*: expression of mSnail (*right*) results in cell elongation and scattering in sparse cultures. In contrast, uninduced control cells (*left*) form compact colonies of closely opposed cells (phase-contrast microscopy). Bar = 200 μm. *D*: cell aggregation assay. *Left*: MDCK-Snail *clone 10* cells cultured for 4 days in the presence of Dox and grown in suspension for a further 24 h form discrete cell aggregates. *Middle*: uninduced control cells suspended in a 1:1 mixture of culture medium and hybridoma supernatant containing function-blocking E-cadherin antibody (Arc-1) show minimal aggregation. *Right*: MDCK-Snail *clone 10* cells cultured for 4 days in the absence of Dox and grown in suspension for a further 24 h; mSnail expression inhibits cell aggregation to a similar extent as the E-cadherin antibody. Bar = 500 μm. *E*: mSnail enhances chemotaxis of MDCK-Snail *clone 10* cells toward hepatocyte growth factor (HGF). The migration of mSnail-expressing MDCK cells (−Dox) is significantly higher than that of either uninduced control cells (+Dox) or wild-type MDCK II Tet-Off cells (WT). The assay was carried out in sextuplicate and repeated 3 times. $P < 0.001$ by unpaired Student's *t*-test. *F*: mSnail disturbs lumen formation in a cyst formation assay. *Top*: when sandwiched between two collagen gels, MDCK-Snail *clone 10* cells maintained in the presence of Dox form multicellular structures enclosing a wide lumen. *Bottom*: in the absence of Dox, MDCK-Snail *clone 10* cells generate flattened structures that contain multiple small lumen-like spaces. Bar = 200 μm.

sion of both α_1- and β_1-subunits (Fig. 2*B*). Immunofluorescence confocal microscopy indicated that the increase in whole cell Na^+-K^+-ATPase expression seen by Western blot was associated with increased cell surface expression (data not shown). To assess the functional activity of Na^+-K^+-ATPase, we then measured ouabain-sensitive $^{86}Rb^+$ uptake in confluent MDCK cell monolayers. Consistent with the increased protein expression, mSnail caused an ~1.5-fold increase in cation transport activity of Na^+-K^+-ATPase (Fig. 2*C*). In addition, bumetanide-sensitive $^{86}Rb^+$ uptake, which measures rubidium transport by the Na^+-K^+-$2Cl^-$ exchanger, was not altered by expression of mSnail (data not shown), implying that mSnail specifically modulates Na^+-K^+-ATPase activity through increased expression of both subunits of the enzyme. These findings indicated that the dome-suppressing activity of mSnail was not due to inhibition of transepithelial ion transport.

Because domes arise in regions of the monolayer in which pressure from accumulated fluid exceeds the strength of cell-substrate adhesion (58), mSnail could inhibit dome formation by modulating the adhesion of MDCK cells to the underlying culture dish. To indirectly address this question, we took advantage of the ability of MDCK cells to form floating cysts enclosing a fluid-filled lumen when grown in suspension culture (76). In floating cysts, the apical cell surface is oriented toward the outside of the cyst, and fluid is transported through the cyst wall in an apical-to-basolateral direction, similar to what occurs in domes of postconfluent monolayers. If mSnail inhibited dome formation by altering the adhesion of MDCK cells to the substrate, one would expect to observe unperturbed lumen formation in suspension cultures. On the contrary, mSnail-expressing MDCK cells formed solid cell aggregates devoid of a fluid-filled lumen when grown in suspension (Fig.

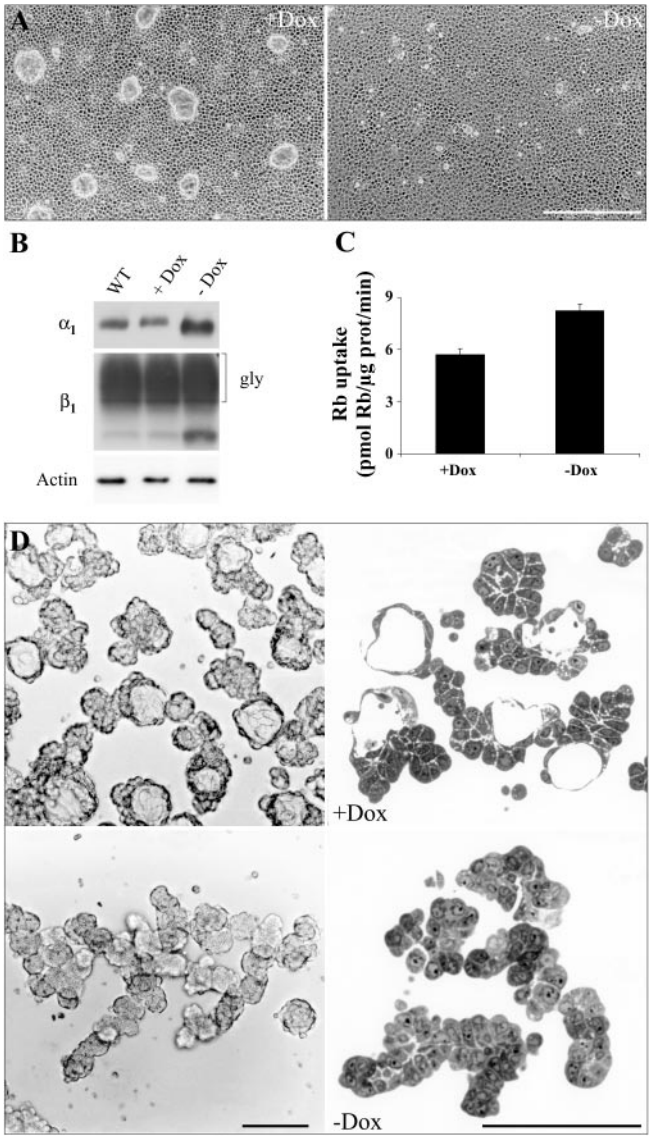

Fig. 2. *A*: inducible expression of mSnail inhibits dome formation in postconfluent monolayer cultures of MDCK cells. *Left*: MDCK-Snail *clone 10* cells grown to postconfluence in the presence of Dox form numerous domes. *Right*: MDCK-Snail *clone 10* cells maintained for the same time period in the absence of Dox form a dome-free cobblestone-like monolayer. Bar = 300 μm. *B*: Western blot analysis of Na^+-K^+-ATPase α_1- and β_1-subunits in total extracts of confluent wild-type MDCK II Tet-Off cells (WT), uninduced controls (+Dox), and induced cells (−Dox). Induction of mSnail increases the expression of both α_1- and β_1-subunits. The broad band above 50 kDa (gly) represents the mature glycosylated β_1-subunit. The lower 44-kDa band represents the core peptide. Uniform loading of lanes was verified by immunoblotting of total actin. *C*: Na^+-K^+-ATPase cation transport activity. Filter-grown MDCK cells were preincubated in serum-free DMEM with or without ouabain (2 mM) for 60 min at 37°C. Ouabain-sensitive $^{86}Rb^+$ uptake was calculated as described in MATERIALS AND METHODS as the difference between the mean values measured in quadruplicate. mSnail causes an ~1.5-fold increase in Na^+-K^+-ATPase cation transport activity. Data represent means ± SE of 4 independent experiments and were compared with Student's unpaired *t*-test; $P < 0.001$. *D*: mSnail inhibits fluid accumulation and lumen formation in aggregates of suspension-grown MDCK cells. *Top*: +Dox MDCK-Snail *clone 10* cells grown on agarose for 7 days form floating aggregates, many of which contain a fluid-filled central cavity, as seen by phase-contrast microscopy of living cultures (*left*) or by examination of semithin sections after Epon embedding (*right*). *Bottom*: −Dox MDCK-Snail *clone 10* cells form compact aggregates devoid of a lumen. *Left,* phase-contrast microscopy of living cultures; *right*, semithin section. Bars = 100 μm.

2*D*). Lumen formation was induced in the aggregates on addition of Dox to repress mSnail expression (data not shown). These results imply that mSnail-induced dome suppression was not mediated by alterations of cell-substratum adhesion.

Induction of mSnail expression modifies functional properties of TJs. On the basis of findings described above, we next investigated whether mSnail inhibits dome formation by interference with the barrier function of TJs. A classic experimental protocol used to study potential alterations of TJs is the "Ca^{2+} switch" assay. This is based on the observation that TJs are disrupted if epithelial cells are grown in medium containing subphysiological Ca^{2+} concentrations (<5 μM). Subsequent restoration of physiological levels of Ca^{2+} (1.8 mM) results in rapid and synchronous de novo assembly of TJs (26, 27). To analyze the potential effects of mSnail on the sealing properties of TJs, MDCK-Snail *clone 10* cells were grown overnight on Transwell filters in LC medium in the presence or absence of Dox and then switched back to NC medium. TJ development was monitored by measuring TER, a reliable indicator of TJ barrier function, at selected times after the Ca^{2+} switch. TER in control cells gradually increased after the Ca^{2+} switch and within 8 h reached ~160 $\Omega \cdot cm^2$, a value reported for MDCK II cells (26). In contrast, TER of mSnail-expressing MDCK cells reached only ~30 $\Omega \cdot cm^2$. These experiments demonstrated a marked impairment in the establishment of TER in Snail-expressing MDCK cells (Fig. 3*A*).

By thin-section electron microscopy, TJs are visualized as focal sites of close apposition of the lateral membranes of adjacent cells, where the intercellular space is obliterated. To establish whether the observed decrease in TER correlated with impaired assembly of TJs, filter-grown MDCK-Snail *clone 10* cells were processed for electron microscopy after Ca^{2+} switch. Cells grown in either the presence or the absence of Dox and subjected to Ca^{2+} switch for 8 h showed focal appositions of the adjacent plasma membranes typical of TJs (data not shown). When plasma membranes are observed en face by freeze-fracture electron microscopy, TJs appear as a network of branching and anastomosing strands that encircle each cell at the boundary between the apical and lateral plasma membrane domains. Comparison of TJ organization in freeze-fracture replicas of MDCK cells subjected to Ca^{2+} switch for 8 h did not show major differences in the network pattern of TJ strands between cells maintained in the presence or absence of Dox (Fig. 3*B*). In addition, the mean ± SE number of strands in cells grown without Dox (2.86 ± 0.15) was comparable to that measured in control cells grown in the presence of Dox (2.49 ± 0.16) ($P > 0.1$ by unpaired Student's *t*-test). These findings indicated that expression of mSnail does not reduce TER by decreasing the structural complexity of TJs.

To determine whether the functional integrity of TJs was maintained in mSnail-expressing MDCK cells, we next performed tracer experiments using electron-dense dyes that are unable to cross intact TJs. Ruthenium red and Alcian blue are cationic heavy metal conjugates that bind to and visualize cell surface-associated carbohydrate residues ("cell coat" or "glycocalyx"). When these dyes are added to the primary fixative and applied to the apical surface of an intact epithelial monolayer, their diffusion to the intercellular space is prevented by TJs (26). We chose to use Alcian blue as an electron-dense tracer because in our hands it produced a more uniform staining of the cell coat than the more widely used ruthenium

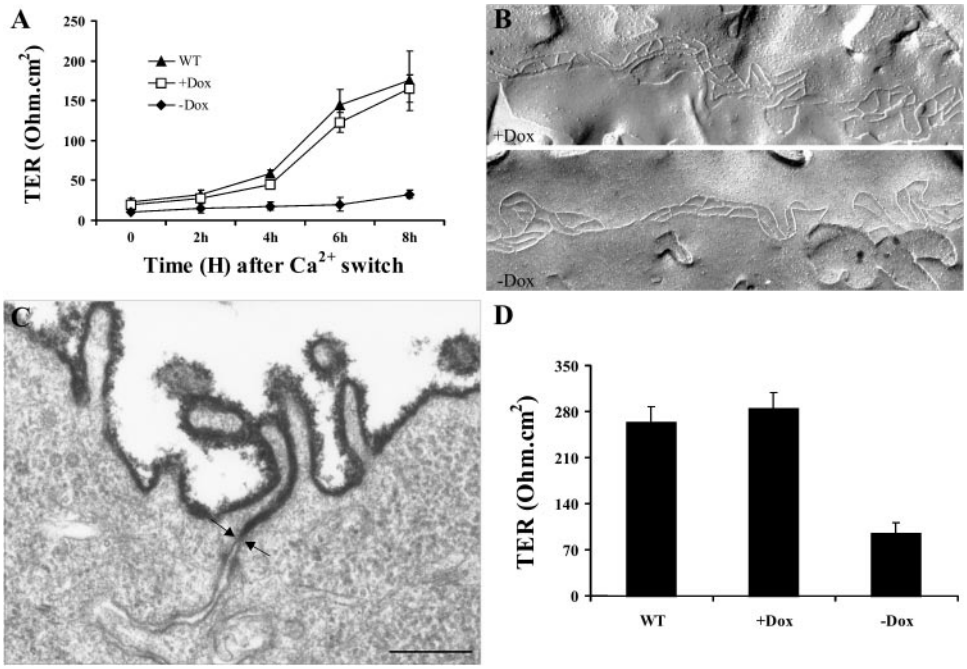

Fig. 3. Tight junctions (TJs) remain intact while transepithelial electrical resistance (TER) is reduced. *A*: mSnail inhibits the establishment of TER in the "Ca^{2+} switch" assay. MDCK II Tet-Off cells (WT), as well as MDCK-Snail *clone 10* cells that had been cultured for 4 days in the presence or absence of Dox, were plated at confluent density on filters and subsequently incubated overnight in low-Ca^{2+} medium to allow disassembly of cell junctions. The cells were then switched back to normal-Ca^{2+} medium. At various time points after the Ca^{2+} switch, the monolayers were subjected to TER measurements to monitor TJ reassembly. TER in control cells gradually increased after the Ca^{2+} switch and within 8 h reached ~160 $\Omega \cdot cm^2$. In contrast, TER in mSnail-expressing MDCK cells reached only ~30 $\Omega \cdot cm^2$. *B*: structural organization of TJs is not grossly altered by mSnail expression. Filter-grown MDCK Snail *clone 10* cells were processed for freeze-fracture electron microscopy 8 h after Ca^{2+} switch. TJs of cells grown in the presence (*top*) or absence (*bottom*) of Dox do not show major differences in the number and complexity of intramembrane strands. Bar = 500 nm. *C*: expression of mSnail does not interfere with the ability of TJs to prevent the diffusion of electron-dense tracers. MDCK-Snail *clone 10* cells cultured for 4 days in the absence of Dox were plated on filters at confluent density and subjected to the Ca^{2+} switch protocol. Eight hours later, culture medium in the upper Transwell compartment was replaced by fixative containing the cationic dye Alcian blue. The electron-dense stain labels the apical domain of the monolayer but does not penetrate into the lateral intercellular space (arrows indicate the location of the TJ). Bar = 300 nm. *D*: mSnail causes a sustained drop in TER. MDCK Tet-Off cells (WT), as well as MDCK-Snail *clone 10* cells cultured 4 days in the presence or absence of Dox, were plated on filters at confluent density. TER was measured 24 h after seeding. −Dox cells exhibit a marked decrease in TER compared with control cells (WT); $P < 0.01$ by unpaired Student's *t*-test. TER of +Dox cells is not significantly different from control values. The assay was carried out 6 times in quadruplicate.

red (R. Montesano, unpublished observations). Confluent monolayers of MDCK-Snail *clone 10* cells on Transwell filters were either subjected to Ca^{2+} switch or maintained in Ca^{2+}-depleted medium as a control. Eight hours after the Ca^{2+} switch, the medium in the upper Transwell compartment was replaced by Alcian blue-containing fixative solution, and the monolayers were processed for thin-section electron microscopy. In control cultures maintained in Ca^{2+}-depleted medium, Alcian blue stained both the apical and lateral membranes of MDCK cells (not shown). In contrast, in cultures subjected to Ca^{2+} switch, Alcian blue staining was restricted to the apical cell surface and stopped abruptly at the level of the TJ, irrespective of whether the cells were grown in the presence (not shown) or in the absence (Fig. 3*C*) of Dox. These findings corroborated the conclusion that mSnail expression does not result in gross alterations of TJ integrity.

Together, the Ca^{2+} switch experiments described above indicated that mSnail interferes with the establishment of TER without overtly disturbing the structural organization and overall integrity of TJs. To substantiate the alterations of TER observed in the Ca^{2+} switch assay, and to exclude the possibility that the impaired development of TER was a transient phenomenon, we next performed TER measurements on monolayers of MDCK cells grown on filters in standard culture medium. Under these conditions, mSnail expression was associated with a pronounced decrease of TER (Fig. 3*D*), a result that confirmed the findings obtained with the Ca^{2+} switch assay.

mSnail selectively increases paracellular ion permeability without altering junctional permeability to uncharged molecules. In addition to restricting the paracellular flow of ions, as measured by TER, TJs also regulate the diffusion of nonionic molecules. To assess whether expression of mSnail disrupts the ability of TJs to maintain concentration gradients of small uncharged solutes, we measured the diffusion of mannitol across monolayers of MDCK cells grown on filters. Measurement of both transepithelial [^{3}H]mannitol fluxes (data not shown) and ability to maintain a transepithelial osmotic gradient generated by addition of mannitol to either apical or basolateral medium (Fig. 4*A*) did not reveal significant differences between cultures of MDCK-Snail *clone 10* cells grown in the presence or absence of Dox. Therefore, mSnail expression does not modify the ability of TJs to retard the diffusion of mannitol and water in the apical-to-basal or basal-to-apical direction.

Given the lack of correlation between the effect of mSnail on TER and mannitol flux, we hypothesized that regulated expression of mSnail selectively alters TJ permeability to ions. To

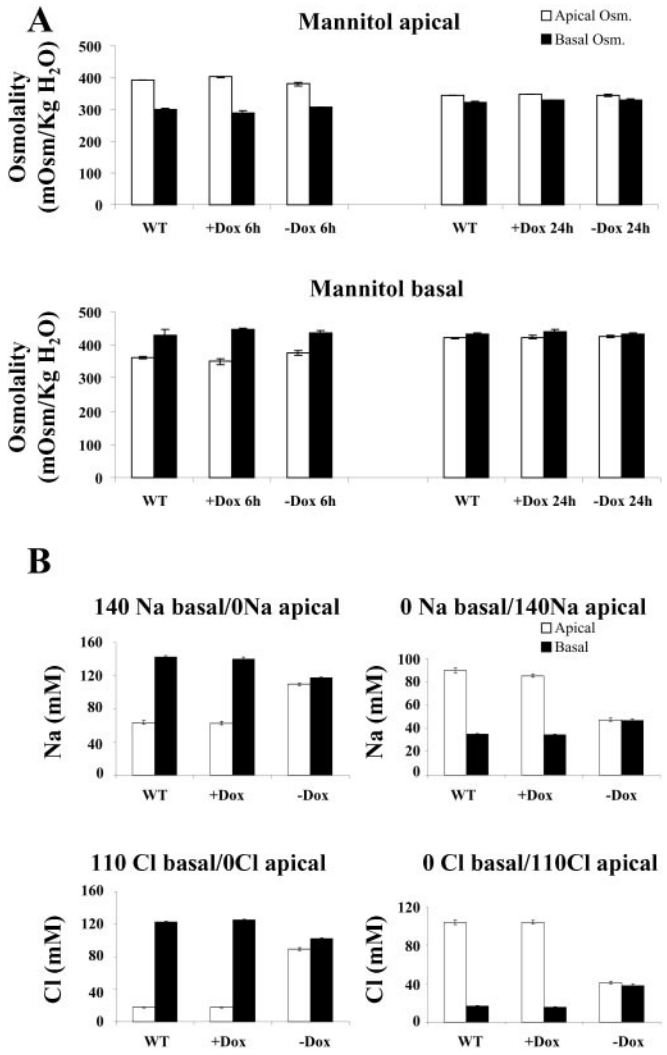

Fig. 4. mSnail selectively alters the ionic permeability of TJs. *A*: transmonolayer diffusion of mannitol is not affected by mSnail. MDCK cells were plated at confluent density on Transwell filters. Apical (*top*) or basolateral (*bottom*) medium was supplemented with 200 mosM mannitol. After 6- and 24-h incubation at 37°C, apical and basolateral media were collected and osmolality was measured. Results are expressed as means ± SE in mosmol/kgH_2O. *B*: expression of mSnail increases paracellular Na^+ and Cl^- permeability. MDCK cells were incubated in asymmetric incubation solution before measurement of Na^+ and Cl^- concentrations of the apical and basolateral medium. MDCK II Tet-Off cells (WT) and MDCK-Snail *clone 10* cells incubated with Dox maintain robust transepithelial ion gradients in the presence of either apical or basolateral Na^+- or Cl^--free medium. In contrast, Na^+ and Cl^- transepithelial gradients are abolished in MDCK-Snail *clone 10* cells incubated without Dox in the presence of either apical or basolateral Na^+- or Cl^--free medium for 48 h. Similar results were obtained after a shorter incubation (24 h). Results are expressed means ± SE.

test this, we evaluated the ability of monolayers of MDCK cells grown on filters to maintain transepithelial ionic gradients. For this purpose, MDCK cells were incubated in asymmetric incubation solution before measurement of Na^+ and Cl^- concentrations of the apical and basolateral medium. MDCK-Snail *clone 10* and *clone 1* cells incubated with Dox, as well as MDCK II Tet-Off cells, maintained robust transepithelial ion gradients in the presence of either apical or basolateral Na^+- or Cl^--free medium. In contrast, transepithelial gradients of Na^+ and Cl^- were abolished in MDCK-Snail *clone 10* and *clone 1* cells incubated without Dox in the presence of either apical or basolateral Na^+- or Cl^--free medium for 24–48 h (Fig. 4*B* and data not shown). These results therefore showed that expression of mSnail increases TJ permeability to Na^+ and Cl^-.

mSnail differentially modulates expression of specific claudins. TJs are composed of at least three families of transmembrane proteins (occludin, claudins, and junctional adhesion molecules) and of numerous "cytoplasmic plaque" proteins such as ZO-1, ZO-2, and ZO-3 (46, 67). Among these proteins, claudins have emerged as essential components of TJ strands and as key regulators of their selective permeability properties (2, 72, 73). In particular, different claudins have been shown to regulate paracellular permeability to specific ions (1, 18, 74). In addition to claudins, occludin has also been proposed to regulate the barrier function of TJs (5, 16, 47, 78). To determine whether mSnail alters the paracellular ionic permeability of MDCK cells by modulating specific TJ components, we wanted to determine its potential effect on the expression of occludin and different claudins. Because measurement of claudin RNA levels was hindered by the fact that only the sequences of canine claudin-2 and -3 are known at present, we decided to assess claudin protein expression by Western blot analysis of MDCK-Snail *clone 10* and *clone 1* cells. We found that expression of mSnail is associated with a decrease in the expression of occludin and claudin-2, and with an almost total suppression of the expression of claudin-4 and -7 (Fig. 5 and data not shown). Importantly, the levels of claudin-1 and -3 were only slightly affected by mSnail expression, which demonstrates the specificity of the observed effects.

Restoration of E-cadherin in MDCK cells that constitutively overexpress mSnail restores epithelial morphology but is not sufficient to reestablish claudin expression. Snail is a potent repressor of *E-cadherin* expression (7, 13), and E-cadherin has been implicated in the process of TJ assembly (32). If Snail were to decrease claudin-2, -4, and -7 via repression of *E-cadherin*, then one could predict that introduction of the *E-cadherin* gene in mSnail-transfected cells would reestablish normal claudin levels. Because induced MDCK-Snail *clone 10* cells show only a moderate decrease in the expression of *E-cadherin* (Fig. 1*B*) whereas MDCK cells constitutively overexpressing mSnail (MDCK/Snail) are totally depleted of E-cadherin (13), we considered it more appropriate to reexpress the *E-cadherin* gene in constitutive MDCK/Snail cells. In E-cadherin-transfected MDCK/Snail cells (MSE-10 cells), E-cadherin expression and cell surface association were restored to levels equivalent to those seen in wild-type MDCK II Tet-Off cells (Fig. 6). E-cadherin expression was associated with a partial reversal of MDCK/Snail cells, which have been shown to undergo complete EMT (13), from a fibroblast-like to an epithelium-like phenotype (Fig. 6*A*). Despite these changes, claudin expression remained dramatically downregulated in *E-cadherin*-transfected cells (Fig. 6*B*). These results suggest that mSnail modulates claudin expression independently of E-cadherin repression.

DISCUSSION

The transcription factor Snail plays a crucial role in developmental processes that require EMT (14, 34, 50). When stably transfected in epithelial cells, *Snail* triggers full EMT associ-

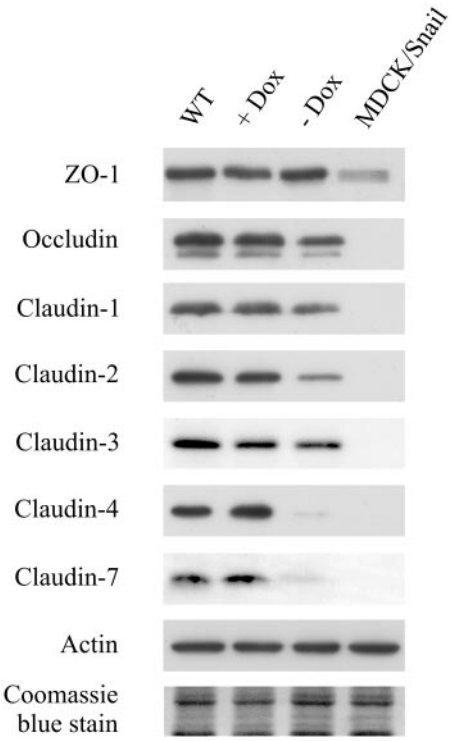

Fig. 5. Inducible expression of mSnail differentially modulates the expression of TJ proteins. Total extracts of confluent wild-type MDCK II Tet-Off cells (WT), mSnail-transfected MDCK II Tet-Off cells, either uninduced (+Dox) or induced (−Dox), and MDCK II cells constitutively overexpressing mSnail (MDCK/Snail) were electrophoresed and blotted. Expression of zonula occludens (ZO)-1, occludin, and claudin-1, -2, -3, -4, and -7 was detected with specific antibodies. Constitutive overexpression of mSnail (MDCK/Snail) represses both occludin and claudin expression and moderately downregulates ZO-1 expression. In contrast, inducible expression of mSnail (−Dox) does not affect ZO-1 expression and is associated with a slightly decreased expression of claudin-1 and -3, a more evident downregulation of the expression of occludin and claudin-2, and a pronounced decrease in the expression of claudin-4 and -7. Uniform loading of lanes was verified both by immunoblotting of total actin and Coomassie blue staining.

ated with the acquisition of invasive and tumorigenic properties (7, 10, 13, 54, 55). Using an inducible system, we have shown here that conditional expression of *Snail* in MDCK cells does not result in overt EMT but elicits more subtle alterations of the epithelial phenotype. The most striking effect of mSnail was the suppression of domes, blisterlike structures that form in postconfluent cultures as a consequence of fluid accumulation between the monolayer and the underlying substratum. Mechanistic analysis of mSnail-induced inhibition of dome formation demonstrated a selective increase in paracellular ionic permeability, without detectable disturbance of TJ structure and overall barrier function. Interestingly, these permeability changes were accompanied by a differential modulation of TJ transmembrane protein expression, and most notably of claudin-2 and claudin-4, which were shown recently to control paracellular ion conductance (1, 2, 74). These results imply that, in addition to promoting EMT during embryogenesis and tumor progression, Snail may act as a regulator of epithelial permeability in physiological and pathological settings.

In previous studies, constitutive overexpression of Snail triggered the conversion of MDCK and other epithelial cell lines into elongated, mesenchyme-like cells devoid of extensive intercellular contacts and growing in a disordered criss-cross pattern (7, 13). In contrast, we found in the present study that inducible expression of *Snail* in MDCK cells under the control of a tetracycline-repressible promoter results in milder phenotypic changes. Thus, although mSnail-expressing MDCK cells exhibit certain characteristics (e.g., E-cadherin downregulation, reduced cell-cell adhesion, increased migration and cell scattering in low-density cultures) that are

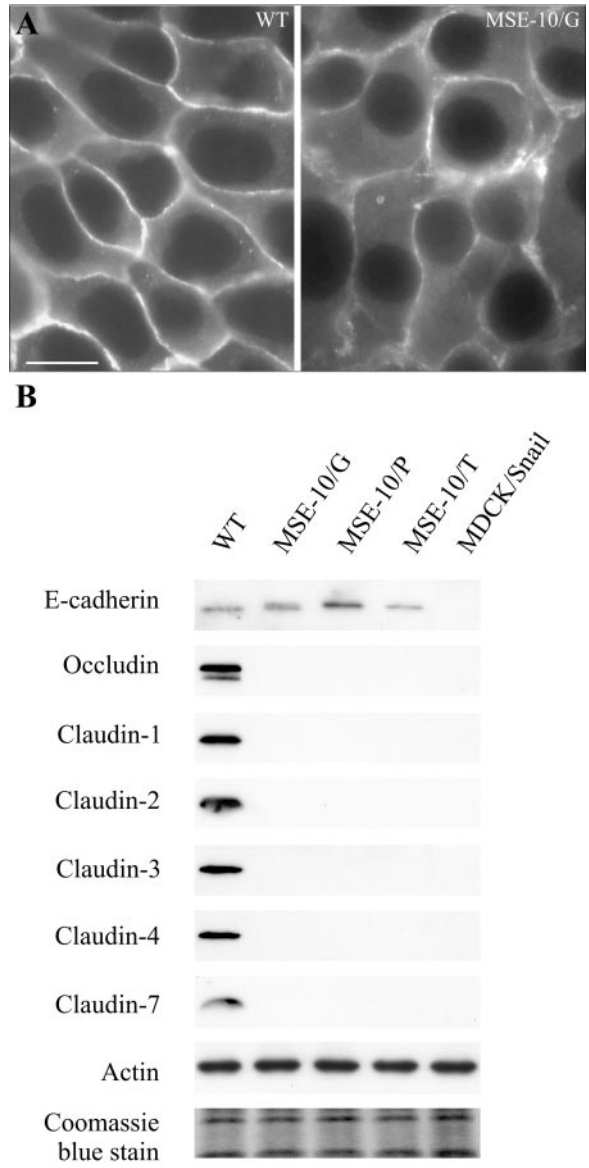

Fig. 6. Expression of E-cadherin in MDCK II cells constitutively overexpressing mSnail partially restores the epithelial phenotype but does not reinduce claudin expression. *A*: immunofluorescence for E-cadherin. In MSE-10/G cells (*right*), E-cadherin is reexpressed at the cell surface to a level comparable to that seen in wild-type MDCK II Tet-Off cells (*left*). Note that MSE-10/G cells have a polygonal epithelium-like morphology. Bar = 20 μm. *B*: Western blot analysis of E-cadherin and TJ proteins in total extracts of confluent wild-type MDCK II Tet-Off cells (WT), 3 clones of MDCK II cells constitutively overexpressing both mSnail and E-cadherin (MSE-10/G, -10/P, and -10/T), and MDCK II cells constitutively overexpressing mSnail (MDCK/Snail). Reexpression of E-cadherin in the MSE clones does not restore expression of occludin and claudins-1, -2, -3, -4, and -7. Uniform loading of lanes was verified by both immunoblotting of total actin and Coomassie blue staining.

consistent with partial EMT, they nonetheless form a continuous monolayer of closely packed polygonal cells on reaching confluence. The reasons for these differences between the present and previous studies are presently unclear. The lack of overt EMT in our system is unlikely to be due to transient expression of Snail, because long-term induction (up to 3 wk) did not result in further phenotypic alterations (F. Carrozzino and R. Montesano, unpublished observations). We had initially hypothesized that the lack of complete EMT could be due to the relatively low levels of transgene expression that are often achieved by conditional expression systems (61). However, a comparative analysis showed that inducibly transfected MDCK cells produce levels of mSnail mRNA that are similar to if not higher than those obtained in stably transfected MDCK cells (F. Carrozzino and C. Di Sanza, unpublished observations). Nonetheless, in light of the recent demonstration that glycogen synthase kinase-3 regulates *Snail* transcription, posttranslational degradation, and nuclear export (3, 79, 81), it is conceivable that differences in Snail protein stability account in part for the observed discrepancies between the constitutive and inducible systems. Further studies will be required to address these issues.

A striking observation made during the preliminary phenotypic characterization of our inducible transfectants was that postconfluent monolayers of Snail-expressing MDCK cells were consistently devoid of domes. Because dome formation requires transepithelial transport of ions and water in an apical-to-basolateral direction (41, 42, 82), we initially investigated whether Snail could affect the expression or function of Na^+-K^+-ATPase, a membrane pump that provides the driving force for the vectorial fluid transport necessary for dome formation (41). To our surprise, mSnail increased both expression levels and transport activity of Na^+-K^+-ATPase. Moreover, the basolateral localization of Na^+-K^+-ATPase was unaltered by mSnail expression. Exchange of intracellular Na^+ for extracellular K^+ by the basolateral Na^+ pump maintains a low intracellular Na^+ concentration that drives passive Na^+ entry through the apical membrane. Our results therefore imply that transcellular apical-to-basolateral Na^+ transport and the ensuing water transport are increased in cells expressing inducible mSnail. These findings contrast with a recent report indicating that stable expression of *Snail* represses Na^+-K^+-ATPase β_1-subunit expression in MDCK cells (20). The reasons for this discrepancy are not known. However, it is possible that the increase in Na^+-K^+-ATPase expression and activity observed in our system is not a direct effect of mSnail but results from a secondary regulatory mechanism aimed at compensating mSnail-induced alterations of ionic permeability. Such a compensatory mechanism is unlikely to be operative in constitutively transfected cells, which as a consequence of EMT have completely lost their TJs and show drastically reduced levels of Na^+-K^+-ATPase. Whatever the reasons for the above-mentioned discrepancy, the findings obtained in our experimental model argue against disturbances of transcellular ion transport systems being primarily responsible for the dome-suppressing activity of mSnail.

To generate and maintain the hydrostatic pressure necessary for focal detachment of the monolayer and dome formation, MDCK cells must not only express functional ion transporters in the plasma membrane but also have intact TJs endowed with normal sealing properties (15). Thin-section and freeze-fracture electron microscopy of synchronous junction reassembly after a Ca^{2+} switch protocol did not reveal clear defects of TJ organization and overall barrier function in mSnail-expressing MDCK cells. However, despite the structural integrity of TJs, expression of mSnail caused a consistent and substantial drop in TER. Remarkably, the decrease in TER was not accompanied by changes in the paracellular flux of uncharged molecules (e.g., water and mannitol), indicating a selective alteration of TJ ionic permeability. TER is an instantaneous measurement that reflects ionic permeability at a given time point (46). To determine whether mSnail disrupts the ability of MDCK cells to maintain ionic gradients over a longer period of time, and to determine the potential ion selectivity of the paracellular diffusion barrier, we measured unidirectional ion fluxes and found that expression of mSnail increases Na^+ and Cl^- permeabilities. This indicates that mSnail expression selectively alters the bidirectional ionic permeability of TJs, allowing backflux of reabsorbed solutes, which may at least in part account for the absence of dome formation.

What is the molecular basis of mSnail-induced changes in TJ ionic permeability? Recent evidence supports the idea that members of the claudin family of transmembrane proteins are critical regulators of TJ permeability (2, 72, 73). In particular, it has been proposed that different claudins regulate the permeability to specific ion species (1, 18, 74) and that overall paracellular permeability is determined by the combination and ratios of claudin species (72). In attempting to understand the molecular mechanisms by which mSnail selectively increases paracellular ion permeability, we examined its potential effect on the expression of various claudins. We found that mSnail barely affects the expression levels of claudin-1 and -3 while moderately downregulating claudin-2 and dramatically decreasing claudin-4 and -7 expression. The net result of this modulation is a marked increase in claudin-2-to-claudin-4 and claudin-2-to-claudin-7 ratios. Interestingly, claudin-2 and -4 have been shown to exert opposing effects on paracellular ion permeability: claudin-2 reduces TER and promotes the formation of cation-selective channels (1, 24), whereas claudin-4 selectively decreases Na^+ conductance (74). With respect to claudin-7, no information is currently available concerning its potential role in the regulation of paracellular ion permeability. Given this knowledge, a high claudin-2-to-claudin-4 ratio is expected to be associated with increased paracellular Na^+ conductance, as we have observed on mSnail expression. Our findings, albeit correlative, therefore suggest that mSnail increases the ionic permeability of MDCK cells by pushing the claudin-2/claudin-4 balance in favor of claudin-2. Signals that regulate the expression of specific claudins have only recently begun to be identified. Thus Singh and Harris (68) reported that EGF inhibits claudin-2 expression while simultaneously increasing the expression of claudin-1, -3, and -4, whereas Lipschutz et al. (44) reported that the ERK1/2 signaling pathway selectively decreases claudin-2 expression. Together with the works of Singh and Harris (68) and Lipschutz et al. (44), the present study suggests that differential modulation of claudin isoform expression plays a key role in the regulation of epithelial permeability.

While this work was in progress, two papers were published describing effects of Snail on claudin expression. In the first study, Ikenouchi et al. (35) reported that overexpression of Snail in epithelial cells directly represses the expression of

occludin as well as claudin-3, -4, and -7, while at the same time disrupting TJs and adherens junctions. In the second study, Ohkubo and Ozawa (53) reported that constitutive overexpression of *Snail* represses the expression of occludin at the transcriptional level and reduces the expression of claudin-1 and ZO-1 by posttranscriptional mechanisms. In these two studies, however, claudins were downregulated in a nonselective manner, and their suppression was associated with full EMT and complete loss of TJs. Therefore, our report is the first to demonstrate that *Snail* can differentially regulate the expression of specific claudins in the absence of gross alterations of TJ structure and barrier function. The differences between our results and those of Ikenouchi et al. (35) and Ohkubo and Ozawa (53) may be accounted for by the fact that the latter studies were based on a constitutive expression of *Snail*.

Because Snail is a potent repressor of *E-cadherin* expression, we wanted to determine whether Snail-induced claudin downregulation could be reversed by forced expression of *E-cadherin*. We found that repletion of E-cadherin expression in MDCK cells constitutively transfected with mSnail (13) induced partial reversion to an epithelial phenotype but did not restore claudin expression. In agreement with the results of Ohkubo and Ozawa (53), these findings suggest that mSnail modulates claudin expression directly, and not via repression of *E-cadherin*.

What is the significance of our findings? A fundamental function of epithelia is to create a diffusion barrier between the luminal environment and the internal milieu and to regulate the exchange of solutes between these two compositionally different compartments. Movement of solutes, ions, and water across the epithelial barrier occurs through both the transcellular pathway, owing to the asymmetric distribution of membrane pumps and channels, and the paracellular pathway, via TJs. Whereas the contribution of the transcellular route has been characterized in considerable detail, the molecular mechanisms that regulate TJ permeability are less well understood. Although a variety of cytokines and intracellular signaling molecules have been reported to influence the assembly and function of TJs (46, 75), the factors responsible for the fine-tuning of paracellular permeability are still largely unknown. Our findings raise the interesting possibility that induction of *Snail* provides a mechanism for modulating TJ permeability in response to changing physiological needs to maintain the homeostasis of the internal milieu.

An additional role of Snail in the physiological regulation of paracellular permeability is suggested by the heterogeneous distribution of claudin isoforms in epithelia of different organs and even in distinct regions of the same organ (e.g., along the length of the nephron) (39, 43, 59, 62). How claudins are regulated in a tissue-specific and segment-specific manner is still largely unknown, but emerging evidence points to the involvement of transcription factors (52, 65). It is therefore tempting to speculate that regional variations in *Snail* expression may contribute to the generation of tissue-specific patterns of claudin isoforms.

A variety of pathological settings are characterized by disruption of TJ barrier function and increased epithelial permeability (73). Thus, in collagenous colitis, an inflammatory bowel disease, a decrease in claudin-4 expression causes leakage of ions and water into the intestinal lumen, resulting in diarrhea (11). It will be interesting in future studies to establish whether inappropriate expression of *Snail* plays a role in pathological conditions associated with epithelial barrier dysfunction.

In conclusion, although caution must be exercised when extrapolating information gained from in vitro systems to the whole organism, the results of this study raise the possibility that Snail participates in the regulation of epithelial permeability. Such a role seems to depart sharply from Snail's established function in EMT. However, this apparent contradiction may be reconciled by considering that Snail expression may have different biological outcomes, depending on the environmental context as well as the nature and timing of the inducing stimulus. Thus sustained expression of Snail may result in a drastic phenotypic switch characterized by loss of cell-cell adhesion and acquisition of mesenchymal traits, as is observed during embryogenesis and tumor progression. On the other hand, transient induction of Snail may modulate the physiological properties of epithelial cells without disrupting normal tissue architecture.

ACKNOWLEDGMENTS

We thank J. Rial-Robert, M. Eissler, P. Couleru, M. Rousselot, F. Layzeras, M. Quayzin, A. Widmer, and D. Caille for excellent technical assistance, C. Di Sanza, Dr. A. Calabrese, and Dr. V. Serre for helpful advice, N. Dupont for secretarial work, Dr. L. Larue for providing the pPGKE-cad-hyg plasmid, and Dr. J. Harrison for helpful advice and critical reading of the manuscript.

GRANTS

This study was supported by Grants 3100A0-101734 and 3100-067878 from the Swiss National Science Foundation (to R. Montesano and E. Féraille, respectively) and Grant SAF2001-02819 from the Spanish Ministry of Science and Technology (to A. Cano).

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