E-Cadherin Regulates Human Nanos1, which Interacts with p120ctn and Induces Tumor Cell Migration and Invasion

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Abstract

Down-regulation of the epithelial cell-cell adhesion molecule E-cadherin is frequently associated with tumor formation and progression. Besides its role in physical cell-cell adhesion, E-cadherin is also thought to be involved in intracellular signaling in normal epithelial cells. In these cells, the Armadillo catenin p120ctn binds to the cytoplasmic domain of E-cadherin and stabilizes the adhesion complexes. On loss of E-cadherin, cytoplasmic p120ctn might accumulate and contribute to tumor malignancy. We used suppression subtractive hybridization to search for genes regulated by E-cadherin expression. We isolated human Nanos1 as a transcript of which levels decrease on E-cadherin reexpression in a human breast cancer cell line. The hNanos1 protein bears a COOH-terminal (CCHC)2 zinc finger domain and belongs to a human colorectal DLD1 cancer cells functionally abolished cell-cell adhesion. It induced cytoplasmic translocation of p120ctn, as well as strong migratory and invasive properties. We also found that the NH2-terminal domain of hNanos1, which is conserved only among mammals, interacts with p120ctn. hNanos1 counteracted the stimulatory effect of p120ctn on cell protrusion formation. Together, these findings describe a new function for hNanos1 as a downstream effector of E-cadherin loss contributing to tumor progression. Targeting hNanos1 might be a promising strategy in the treatment of E-cadherin—negative tumors in particular. (Cancer Res 2006; 66(20): 10007-15)

Introduction

E-Cadherin is a major epithelial cell-cell adhesion molecule that functions as a tumor suppressor (1). Moreover, E-cadherin deficiency plays a causative role in tumor cell invasion and metastasis (2–4). The extracellular domains of the transmembrane E-cadherin molecules form homophilic adhesion dimers stabilized by direct binding to cytoplasmic Armadillo catenins (5). β-Catenin interacts tightly via its Armadillo repeats with the catenin binding domain of the COOH-terminal tail of E-cadherin and via its NH2-terminal domain with α-catenin. This is essential for the formation of actin cytoskeleton-coupled cell junctions, although the detailed mechanism is a matter of debate (6). In addition, p120 catenin (p120ctn) associates via its Armadillo repeat region with the juxtamembrane domain of the E-cadherin cytoplasmic tail. The interactions of E-cadherin with p120ctn and β-catenin are necessary for dynamically regulating adequate cell-cell adhesion by modulating events such as cadherin clustering and the strength of the connection with the actin cytoskeleton (reviewed in ref. 5). One major function of p120ctn is to stabilize cadherin complexes at the cell membrane by controlling the entry of cadherins into the degradative endocytic pathway (reviewed in refs 7, 8).

The molecular basis of the suppressive signals that are conferred by functional expression of E-cadherin on cancer cells has not been fully elucidated. Loss of E-cadherin might induce signaling by catenins released from the cell membrane. Indeed, β-catenin is also a major component of the canonical Wnt signaling pathway (reviewed in refs. 5, 9). When the Wnt signaling pathway is turned on (e.g., by certain gene mutations in cancer), cytoplasmic β-catenin becomes protected from proteasomal degradation, translocates to the nucleus, binds LEF/Tcf, and acts as an oncogenic transcription cofactor. However, in the absence of such mutations or of a Wnt signal, the β-catenin that is released from the membrane on cadherin loss is rapidly degraded. Cadherins are both necessary and sufficient for localization of p120ctn at the cell membrane (10). Cadherin deficiency does not significantly alter p120ctn levels but leaves it stranded in the cytoplasm and/or the nucleus (10–12). In the cytoplasm, p120ctn interacts with Rho GTPases (reviewed in refs. 7, 8, 13). It directly binds, via its Armadillo repeats, to RhoA and inhibits its activity by acting as a guanine nucleotide dissociation inhibitor. p120ctn also indirectly activates Rac1 and Cdc42 through guanine nucleotide exchange factors such as Vav-2. The combined effects of cytoplasmic p120ctn promote cell migration and, consequently, invasion and metastasis. In the nucleus, p120ctn can interact with the transcription factor Kaiso, and it is likely that this also contributes to the malignant phenotype (14, 15).

Here, we describe the identification of human Nanos1 as a transcript that is down-regulated by E-cadherin. The human Nanos 1 gene encodes a (CCHC)2 zinc finger protein that is highly homologous to the Nanos orthologues in Drosophila and mouse, which are known to have an evolutionarily conserved function in embryonic patterning and germ line development (16–22). Our finding that E-cadherin regulates the expression of hNanos1 in...
carcinoma cells prompted us to seek a functional link between E-cadherin and hNanos1 in epithelial cells. We generated cell systems with inducible expression of hNanos1 and found that hNanos1 impairs E-cadherin function in cell-cell adhesion and promotes cell motility and invasion. Interestingly, we found that hNanos1 interacts with the Armadillo protein p120ctn and counteracts the effect of its overexpression on cell protrusion formation.

Materials and Methods

Suppression subtractive hybridization and cloning of hNanos1. Polyadenylated mRNA, obtained from subconfluent human E-cadherin–negative MDA-MB-231 breast cancer cells and a derivative transfected cell line reexpressing E-cadherin, was isolated with the Fast Track 2.0 Kit (Invitrogen, Merelbeke, Belgium), in accordance with the recommendations of the manufacturer. cDNA subtraction was done using the PCR-select cDNA subtraction kit (BD Biosciences Clontech, Erembodegem, Belgium). The subtracted cDNA libraries, generated by subcloning the PCR products in the pGEM-T vector (Promega, Madison, WI), were screened for differentially expressed cDNAs using plus/minus colony hybridization. Finally, the differential expression of isolated clones was confirmed by Northern blotting.

The 538-bp hNanos1 cDNA fragment isolated in the suppression subtractive hybridization (SSH) screen was used to probe a fetal kidney phage cDNA library (Stratagene). The full-length sequence of the hNanos1 cDNA was obtained by sequencing the isolated specific cDNA clones and several EST clones that were identified by BLAST analysis. To identify the 5' and 3' ends of the hNanos1-specific transcripts, rapid amplification of cDNA ends (RACE) was done on RNA isolated from SW480 human colon cancer cells. Additional genomic sequence data were obtained by isolation and characterization of a hNanos1-specific genomic PAC clone. A 16-kb HindIII fragment containing the single-exon hNanos1 gene was subcloned into the pGEM-1Zf(+) vector (Promega) to produce pGEM11-hNanos1PAC74-HindIII.

Northern blot analysis. Total RNA was isolated from various human cell lines with the RNeasy kit (Qiagen, Chatsworth, CA) according to the instructions of the manufacturer. Total RNA (30 μg) was glyoxylated, size fractionated in a 1% agarose gel, and transferred to a Hybond-N membrane (Amersham Pharmacia Biotech, Rainham, United Kingdom). Hybridization was done following standard procedures. Probes were radioactively labeled by random priming (RadiPrime labeling kit, Gibco BRL Life Technologies, Paisley, United Kingdom). The hNanos1-specific probe used was the 538-bp insert of the pGEM-T clone obtained from the SSH analysis. The other probe was an 800-bp fragment (exons 14-16) of the human hNanos1-specific genomic PAC clone. A 16-kb HindIII fragment containing the single-exon hNanos1 gene was subcloned into the pGEM-1Zf(+) vector (Promega) to produce pGEM11-hNanos1PAC74-HindIII.

Cell culture and reagents. The human E-cadherin-negative breast cancer cell line MDA-MB-231 (American Tissue Culture Collection, Manassas, VA) and its E-cadherin--transfected derivative were maintained in L-15 medium (Gibco BRL Life Technologies) supplemented with 10% FCS, 2 mMol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The HEK293T cell line was obtained from the American Cell Type Culture Collection (Rockville, MD) and maintained in DMEM supplemented with 5% FCS, 5% newborn bovine serum, 2 mMol/L L-glutamine, 0.4 mMol/L sodium pyruvate, 100 units/mL penicillin and 100 μg/mL streptomycin. DLD1TTR2-Teton cells, also called DLD1-Tet, were kindly provided by Dr. H. Clevers (Hubrecht Laboratory, Utrecht, the Netherlands) and cultured in RPMI with 10% FCS. SW480 and transfected derivatives, kindly provided by Dr. Schwarte-Waldhoff (Ruhr-Universität Bochum, Bochum, Germany) (24, 25), were maintained in DMEM supplemented with 10% FCS and 100 units/mL penicillin and 100 μg/mL streptomycin.

Generation of stable inducible cell systems. DLD1-Tet cells were stably transfected with linearized pCDNA4/TO-Myc-hNanos1 by electroporation. Clones were isolated after 2 weeks of selection in 500 μg/mL zeocin and 10 μg/mL blasticidin (Invitrogen). Expression was induced with doxycycline (1 μg/mL; Sigma, St. Louis, MO) for 48 hours.

Immunofluorescence assays. Methanol fixation and immunofluorescence were done following standard procedures (11). The specific antibodies used were mouse monoclonal antibody (mAb) 9E10 against the Myc-tag (dilution 1:500 Sigma), mouse mAb clone 36 against E-cadherin (1:100; Transduction Laboratories, Lexington, United Kingdom), mouse mAb clone 14 against β-catenin (1:500; Transduction Laboratories), and mouse mAb pp120ctn against p120ctn (1:500; Transduction Laboratories). The secondary antibody used was Alexa 594–coupled antimmunoglobulin globulin (1:200; Molecular Probes, Eugene, OR). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes). Samples were examined with a Zeiss Axiosplan microscope (Zeiss, Le Pecq, France).

Immunoblotting. Cells were lysed in buffer [50 mMol/L Tris-HCl (pH 7.4), 150 mMol/L NaCl, 1% (v/v) Igepal, 1% (w/v) sodium deoxycholate, 5 mMol/L iodoacetamide, 0.1% (w/v) SDS] containing protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Proteins (0.5 μg) were separated by 10% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA). After blocking with 5% nonfat dry milk in TBS containing 0.1% Tween 20, the membranes were incubated with primary antibody. The following mAbs were used: mAb clone 36 against E-cadherin (1:2,500), mAb clone 14 against β-catenin (1:500), mAb pp120ctn against p120ctn (1:1,000), mAb clone 9E10 against the Myc tag (1:500), and mouse mAb against GAPDH (1:200; Chemicon International, Temecula, CA). The secondary antibody used was horseradish peroxidase (HRP)–conjugated antimouse immunoglobulin antibody (1:1,000; DakoCytomation, Glostrup, Denmark). The proteins were visualized with an enhanced chemiluminescence plus detection kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Coinmunoprecipitation assays. Cells were rinsed with PBS and lysed in buffer [0.5% NP40, 150 mMol/L Tris-HCl (pH 7.4), 50 mMol/L NaCl, 1 μmol/L leupeptin, 0.3 μmol/L aprotinin, 5 μmol/L Pefabloc], lysates (400 μg of protein) were incubated overnight at 4°C with 1 μg of the relevant antibody, and then for 2 hours with 50 μL of 50% protein G-Sepharose beads or protein A-Sepharose beads (Amersham Pharmacia Biotech). Adsorbed immunoprecipitates were washed five times with lysis buffer and boiled for 5 minutes in sample buffer [60 μmol/L Tris-HCl (pH 6.8), 1.7% SDS, 6% glycerol, 0.1 mol/L DTT, 0.002% bromophenol blue]. Eluted proteins were separated by 10% SDS-PAGE (for detection of p120ctn and α-catenin) or 12.5% SDS-PAGE (for detection of Myc-hNanos1 fusion proteins), followed by immunoblotting as described above. An anti-Myc HRP–coupled antibody (1:5,000; Invitrogen) was used for detection of Myc-tagged hNanos1. Other primary antibodies were mAb HEC1D-1 against E-cadherin (1:1,000; Takara, San Diego, CA), clone 14 against β-catenin (1:1,000), and pp120ctn against p120ctn (1:150). The secondary antibody was HRP-conjugated secondary antibody (1:3,000; Amersham Pharmacia Biotech).

Cell aggregation assay. Single-cell suspensions were prepared according to an E-cadherin saving procedure (26). Cells were incubated for 30 minutes with continuous shaking in an isotonic buffer containing 1.25 mMol/L Ca2+. Rat mAb DECA-1 (Sigma) was used to functionally block E-cadherin, starting 30 minutes before aggregation at 4°C and continuing throughout aggregation at 37°C. Particle diameters were measured with a Coulter particle size counter LS200 (Coulter Electronics).
Collagen type I invasion assay. Cells were seeded on top of a jellified collagen type I solution (0.09%; Upstate Biotechnology, Lake Placid, NY). Invasion was scored on living cultures using a microscope with a computer-controlled stage as described (27).

Cell dispersion assay. The spatial distribution of cells was characterized and quantified with an algorithmic program of cellular sociology based on three geometric models: Voronoï’s partition, Delaunay’s graph, and minimum spanning tree (28). Details are described in Supplementary data.

In vitro cell migration assay. For each cell type, 10^5 cells were seeded in culture medium in a 22-mm dish with a 6-mm restriction ring placed inside. Twenty-four hours after plating, the ring was removed, the cells were washed twice with PBS and then covered with culture medium with or without doxycycline. In this model, the cells migrate as an outgrowth from the confluent area initially delineated by the ring. Five hours after removing the ring, phase-contrast images of outgrowth areas were recorded at a ×40 magnification every 15 minutes during 1 hour. Cell migration was characterized and quantified with a previously described software program (29) that measures trajectories of peripheral cell nuclei and cell migration speeds.

Results

Cloning of hNanos1 mRNA as an E-cadherin–suppressed transcript. To identify genes the expression of which is modulated by E-cadherin expression, we conducted comparative transcriptome analysis of E-cadherin–negative and E-cadherin–positive cancer cell lines. Through SSH analysis of the closely related human breast cancer cell line MDA-MB-231 and its E-cadherin–expressing counterpart, MDA2BE5.36 (11, 28), we isolated a 538-bp cDNA fragment the expression of which was lower in the E-cadherin–expressing cells. Completion of the 4,035-nucleotide (nt) full-length cDNA sequence (GenBank accession no. AF458985),
which we originally called hEC-Rep1a for human E-cadherin repressed 1a, showed that the originally cloned cDNA fragment was part of the 3’ untranslated region (UTR) of the human Nanos1 transcript (Fig. 1A), an orthologue of the Drosophila Nanos gene (16). According to the UCSC human genome browser (NCBI Build 35),5 hNanos1 is a single-exon gene located on chromosome 10q26.11. Northern blot analysis confirmed the differential expression of hNanos1 in the MDA-MB-231 cell line relative to its E-cadherin expressing derivative MDA2BE5.36, and also revealed hybridization to two transcripts, one of 1,810 nt and one of 4,035 nt (Fig. 1A). These transcripts differ only in the size of their 3’-UTR sequences due to the use of an alternative polyadenylation signal (AAAAAA) at position 1,764 of the transcript (Fig. 1A), which was also confirmed by 3’ RACE experiments (data not shown).

Alignment of the predicted hNanos1 ORF of 292-amino-acid residues with those of other Nanos-related proteins revealed a high degree of conservation of the COOH-terminal (CCHC)2 zinc finger domain of 52 amino acids (Fig. 1C and Supplementary Fig. S1). In contrast, the sequences and lengths of their NH2-terminal parts were poorly conserved (data not shown), as had been described for several Nanos orthologues (30).

Restricted mRNA expression profile of hNanos1. Northern blot analysis of hNanos1 mRNA expression in a panel of E-cadherin–negative and E-cadherin–positive cell lines revealed that this transcript was not ubiquitously expressed. hNanos1 was expressed at high levels only in the E-cadherin–deficient cell lines SW480 (colon cancer), SK-BR-3 and MDA-MB-231 (breast cancer), SK-N-AS (neuroblastoma), and WERI-Rb-1 (retinoblastoma; Fig. 1D). Moderate expression levels were detected in the A431 (epidermoid), MDA2BE5.36 (breast cancer), and IMR32 (neuroblastoma) cell lines. hNanos1 transcripts were rare or absent in several other cell lines tested. In conclusion, high levels of hNanos1 mRNA were only seen in cell lines expressing low levels of E-cadherin mRNA. All hNanos1-positive cell lines analyzed expressed both the 1,810-nt and 4,035-nt transcripts.

hNanos1 and E-cadherin expression are inversely related. E-Cadherin expression can be modulated in the hNanos1-positive human colon cancer cell line SW480 by treatment with phorbol ester, by transfection with Smad4 cDNA, or by growing the cells to confluency (24, 25). Treatment of SW480 cells with the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) led to a gradual decrease in hNanos1 mRNA levels during the first 6 hours of treatment, after which hNanos1 mRNA levels were reestablished (Fig. 2A). At the end point of TPA treatment (24 hours), hNanos1 mRNA levels were even higher than in untreated cells. E-Cadherin mRNA levels had an inverse profile, as they increased during the first 6 hours of treatment and then decreased to zero after 24 hours of TPA treatment. In Smad4-transfected SW480 clones D1 and D14, E-cadherin mRNA expression was induced and hNanos1 mRNA expression was reduced (Fig. 2B) in comparison with E-cadherin–deficient mock-transfected SW480 clones K5 and K6. Finally, the inverse correlation between E-cadherin and hNanos1 mRNA expression was shown by culturing these cells at increasing cell densities, which resulted in induction of E-cadherin and reduction of hNanos1 with increasing cell density in both Smad4- and mock-transfected SW480 cells (Fig. 2C).

hNanos1 disturbs E-cadherin–dependent cell-cell adhesion. To determine the intracellular localization and function of hNanos1, we generated a human epithelial cancer cell system with inducible expression of Myc-tagged hNanos1. Addition of doxycycline induces tagged hNanos1 in DLD1-Teton-hNanos1 clones, but not in DLD1-Teton-mock clones, as shown by immunoblotting (55-kDa band in Fig. 3A). This induction was associated with an obvious morphotypic switch: cells lost the ability to form compact colonies and showed more and longer cytoplasmic protrusions (Fig. 3A), although total levels of the cell junctional proteins E-cadherin, p120ctn, and β-catenin were not decreased (Fig. 3C).

In a cell aggregation assay, noninduced DLD1-Teton-hNanos1 cells formed large aggregates within 30 minutes (Fig. 3D, −dox N30). Aggregation required functional E-cadherin, as it was prevented by addition of an antibody (DEMA-1) that functionally blocks E-cadherin. When hNanos1 was induced by addition of doxycycline, the cells failed to form aggregates and their particle size distribution curve resembled that of cells treated with DECA-M-1 (Fig. 3D, +dox N30). DLD1-Teton-mock cells showed aggregation after 30 minutes irrespective of doxycycline treatment (Fig. 3D). In summary, induction of hNanos1 in epithelial tumor cells interfered with their ability to form E-cadherin–dependent three-dimensional aggregates, although total levels of E-cadherin were not decreased.

Figure 2. Modulation of E-cadherin mRNA levels in SW480 human colorectal cancer cells induces inverse changes in hNanos1 transcripts. Transcripts were analyzed by Northern blot using GAPDH hybridization as RNA loading control. Columns, mRNA expression levels obtained after quantification and normalization to GAPDH. hNanos1 and E-cadherin transcript levels were compared in (A) subconfluent SW480 cells treated with TPA (250 ng/mL) for the indicated durations; (B) mock-transfected SW480 clones (K5 and K6) and Smad4-transfected clones (D1 and D14; ref. 24); (C) control K5 clone and Smad4-transfected D1 clone of SW480 cells that were either grown to confluency (C) or as a dense monolayer (M).
Immunofluorescence analysis revealed that induced Myc-tagged hNanos1 in DLD1-Teton-hNanos1 cells was expressed as cytoplasmic dots with perinuclear concentration (Fig. 3E, e). E-Cadherin, β-catenin, and p120ctn were highly enriched at cell-cell contacts in DLD1-Teton-mock cells not treated with doxycycline (data not shown), and a similar situation was seen in DLD1-Teton-hNanos1 cells treated with doxycycline (Fig. 3E, a-d). However, expression of Myc-tagged hNanos1 induced less intense staining at the cell contacts for each of the three proteins investigated (Fig. 3E, f-h), and in the case of p120ctn, even prominent cytoplasmic staining was seen although nuclei remained always unstained (Fig. 3E, h). These results indicate that strong expression of hNanos1 interferes with the function of the E-cadherin junctional complex in human tumor cells by relocating the junctional proteins without altering their expression levels.

hNanos1 induces cell migration, cell dispersion, and invasion of epithelial cells. An in vitro cell migration model was used to determine whether induction of hNanos1 influences cell migration. We monitored the migration of DLD1-Teton-mock and DLD1-Teton-hNanos1 cells (with or without doxycycline) growing out from a confluent monolayer into adjacent free space. Phase-contrast images showed that 6 hours after the start of the experiment, induced DLD1-Teton-hNanos1 cells (+Dox) clearly had migrated a longer distance than untreated DLD1-Teton-hNanos1 cells and DLD1-Teton-mock cells (with or without doxycycline; Fig. 4A). Migration was characterized quantitatively in this assay.
Migration trajectories of individual cells showed that the movements of DLD1-Teton-mock cells (untreated and treated) and untreated DLD1-Teton-hNanos1 cells were restricted and random, in contrast to the strikingly directional migration of the induced DLD1-Teton-hNanos1 cells (Fig. 4B). In addition, the migration speed was significantly increased by 81% on induction of hNanos1 expression in the DLD1-Teton-hNanos1 cells (29.23 ± 7.56 µm/h; Fig. 4C) compared with the untreated DLD1-Teton-hNanos1 cells (16.14 ± 3.99 µm/h; Fig. 4C). No difference in cell migration speed between the untreated and treated DLD1-Teton-mock cells was observed. In conclusion, we show that hNanos1 induction in DLD1-Teton-hNanos1 cells results in a rapid and directional cell migration.

To further evaluate whether hNanos1 expression correlates with invasion into a three-dimensional matrix, we did a collagen type-I invasion assay. Expression of hNanos1 strongly induced invasiveness in DLD1-Teton-hNanos1 cells, similar to that induced by the E-cadherin–blocking antibody DECMA-1 (Fig. 4D).

**hNanos1 interacts with the Armadillo catenin p120ctn.** In *Drosophila*, Nanos protein is recruited by Pumilio to the Nanos response elements in the 3′-UTR of target mRNAs (31). The crystal structure of the Pumilio Puf domain, consisting of eight so-called PUM repeats forming a positively charged superhelix, resembles that of the Armadillo repeats (32). Based on the Nanos-Pumilio interaction and the topological similarities of the PUM and Armadillo repeats, we investigated the possibility that hNanos1 interacts with human Armadillo proteins. Myc-tagged Nanos indeed formed a complex with endogenous p120ctn in induced (+dox) DLD1-Teton-hNanos1 cells (Fig. 5A) but did not associate with the non-Armadillo repeat protein α-catenin (Fig. 5A) or with E-cadherin (data not shown), whereas α-catenin associated with E-cadherin as expected (not shown). These findings were
consolidated and extended in Madin-Darby canine kidney-Tetoff-hNanos1 cells with inducible expression of hNanos1: reciprocal coimmunoprecipitations showed that hNanos1 interacts with both endogenous β-catenin and endogenous p120ctn (data not shown). To identify which part of hNanos1 mediated this interaction with p120ctn, we extended our analysis to HEK293T cells transiently cotransfected with constructs encoding full-length p120ctn (isoform 3A) and Myc-tagged hNanos1 (full-length protein, NH2-terminal or COOH-terminal fragment). Interestingly, we found that the hNanos1-p120ctn interaction was mediated by the NH2-terminal part of hNanos1, which lacks the evolutionarily conserved zinc finger domain (Fig. 5B).

hNanos1 abolishes arborization caused by p120ctn overexpression. The observed interactions between hNanos1 and Armadillo proteins indicate that hNanos1 might affect the functions of these interaction partners. Because p120ctn is known to function in cell migration by modulating Rho GTPases in the cytoplasm (reviewed in ref. 13), we analyzed the effect of hNanos1 expression on this function. Overexpression of p120ctn in epithelial cells increases lamellipodia formation. This effect is particularly striking in fibroblasts, in which overexpressed p120ctn induces dramatic arborization (33). Therefore, we analyzed the effect of hNanos1 expression on p120ctn functionality in transfected NIH 3T3 fibroblasts. Exogenous p120ctn and hNanos1 both resided in the cytoplasm in these cells. Coexpression of either full-length or an NH2-terminal fragment of hNanos1 in NIH 3T3 cells expressing high levels of ectopic p120ctn diminished formation of dendrite-like structures (Fig. 5C, b and c), whereas cotransfection of a backbone vector did not interfere with the phenotype induced by p120ctn (Fig. 5C, a). Notice in c and f that a cell that expresses EGFP-p120ctn but not the hNanos1 fragment still exhibits arborization, in contrast to a cell coexpressing both proteins.

Discussion

E-Cadherin expression is frequently down-regulated in many different types of tumors (34). Because E-cadherin is a key cell-cell adhesion protein in epithelial cells, loss of its expression or function diminishes cell-cell contacts and contributes to tumor progression and invasion. However, as E-cadherin inactivation may also occur early in certain precursor lesions, its loss is thought to induce intracellular signaling that promotes tumor development and progression. Because little is known about such signaling pathways, we screened for transcripts that are differentially expressed on E-cadherin reexpression in the breast cancer cell line MDA-MB-231. We isolated a partial cDNA that was down-regulated in cells reexpressing E-cadherin. The completed full-length cDNA sequence was highly homologous to the evolutionarily conserved gene Nanos.

All the Nanos and Nanos-related proteins that have been characterized in several organisms contain a conserved COOH-terminal RNA-binding (CCHC)2 zinc finger domain (Fig. 1C and Supplementary Fig. S1; ref. 20, 21, 30, 35). In Drosophila, Nanos cooperates with Pumilio to repress translation by binding to Nanos.
response elements located in the 3′-UTR of target transcripts (e.g., hunchback and cyclin B mRNAs; refs. 36, 37). Nanos regulates several processes in Drosophila, including differentiation of the anterior-posterior body axis (16), control of neuronal dendrite morphogenesis (38), establishment of germ cell identity (39), self-renewal of germ line stem cells (40), migration and survival of primordial germ cells (17, 18), oogenesis and spermatogenesis (19). Mice deficient in the individual Nanos genes are viable (20, 21). Nanos1 knockout mice are fertile and exhibit no obvious phenotypic abnormalities. In contrast, Nanos2 is essential for germ cell development in male mice, and Nanos3 is important in both male and female sexual differentiation. A human Nanos protein was reported to interact with human Pumilio-2 and to be abundant in germ line stem cells (22).

In Drosophila, Nanos expression is regulated at the translational level (41). Whereas most Nanos mRNA is translationally repressed in the Drosophila embryo, translational activation occurs at the embryonic posterior pole. This switch from translational repression to activation is mediated by cis-acting signals in the 3′-UTR of the Nanos mRNA (41, 42). Whether Nanos expression can be translationally regulated also in mammals needs further investigation. Our identification of two hNanos1 transcripts that differ only in the lengths of their 3′-UTR might be relevant to this. In E-cadherin-deficient cancer cells, hNanos1 expression seems to be regulated at the transcriptional level. We cloned the minimal hNanos1 promoter in a luciferase reporter construct and found that reporter gene activity in MDA-MB-231 cells is higher than in the E-cadherin transfected.6 This implies the presence of E-cadherin-sensitive regulatory sequences.

We found that expression of the hNanos1 transcript was reduced following E-cadherin reexpression in a human cancer cell line. Analysis of a series of cell lines showed an inverse correlation between E-cadherin and hNanos1 expression. In the human colorectal cancer cell line SW480, hNanos1 expression was inversely regulated by various conditions modulating E-cadherin expression. On TPA treatment, hNanos1 expression diminished during the first 6 hours, then increased during the next 18 hours; this is in line with the attribution of different effects to TPA depending on the length of treatment. Indeed, despite the strong activation of protein kinase C by short-term TPA treatment, down-regulation of this important kinase by sustained TPA treatment is well known.

We then sought to identify the function that hNanos1 might have in cancer cells. Using functional assays, we showed that hNanos1 expression impairs the function of E-cadherin in cell-cell adhesion. Levels and extracellular exposure of E-cadherin protein were not changed on hNanos1 induction in the epithelial cells analyzed, indicating that hNanos1 interferes with the functionality of E-cadherin rather than with its expression. Furthermore, hNanos1 expression promoted cell migration and invasion of epithelial cells in vitro. Together, these data indicate that hNanos1 has a function in cancer cells deficient in E-cadherin.

Intriguing in this respect is our discovery that hNanos1 binds the Armadillo protein p120ctn and triggers its translocation from cell-cell contacts to the cytoplasm. The hNanos1-p120ctn association is correlated with abolishment of the arborization phenotype induced in fibroblasts by overexpression of p120ctn (13, 33). This extensive branching of cellular protrusions is known to result from p120ctn-induced reduction in Rho activity with concomitant activation of Rac1 and Cdc42. This indicates a link between hNanos1 and Rho GTPase signaling via cytoplasmic p120ctn. The underlying mechanisms deserve further investigation. Unfortunately, several attempts to convincingly show an effect of hNanos1 on the activity of small GTPases of the Rho family were unsuccessful because the doxycycline treatment itself had a strong negative effect on the levels of GTP-bound Rac and Rho (data not shown). Nonetheless, it is worth mentioning that cytoplasmic p120ctn has been observed in different types of tumors (43–46). The cytoplasmic localization of hNanos1 induced in our epithelial tumor cell system supports the possibility that hNanos1 has a function in the migratory effects mediated by cytoplasmic p120ctn (7). It is tempting to speculate on a similar mechanism to explain the role of Nanos in particular normal cell types, such as in primordial germ cell migration (17) and neuronal dendrite morphogenesis (38), especially because we saw an obvious increase in cytoplasmic p120ctn on hNanos1 induction in the tumor cell system we analyzed.

The Armadillo protein p120ctn interacts with the NH₂-terminal part of hNanos1, which is conserved only among mammals. The more broadly conserved COOH-terminal part of Nanos and Nanos-related proteins, containing the (CCHC)₂ zinc finger domain, is thought to represent the ancestral function of Nanos proteins in germ line development and survival in various invertebrate and vertebrate species (47, 48). One can speculate that other specialized functions of Nanos genes were generated later in evolution in weakly conserved domains (30, 35, 48). p120ctn is dispensable for cadherin stability and adherens junction assembly in Drosophila and Caenorhabditis elegans (49, 50), but it is critically required in mammals (reviewed in refs. 7, 8). The combination of these data supports the possibility that the p120ctn-hNanos1 interaction has an important function in mammals but not in evolutionarily distinct organisms, such as flies and worms.

In summary, our data show that hNanos1 is a downstream target gene of E-cadherin capable of interfering with the proadhesive and anti-invasive functions of E-cadherin and of interacting with Armadillo proteins including p120ctn. Further research should investigate the following: (i) how hNanos1 transcript levels are regulated; (ii) how it can interfere with E-cadherin functionality; (iii) how it activates directional cell migration and invasion; (iv) what the molecular and biological consequences are of its interaction with Armadillo proteins; and (v) whether hNanos1 is an appropriate molecular target for therapy of certain cancers in which the function of E-cadherin is defective.

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6 Our unpublished data.

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