

Cadherin switching: essential for behavioral but not morphological changes during an epithelium-to-mesenchyme transition

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Summary

Epithelium-to-mesenchyme transitions (EMTs) are characterized by morphological and behavioral changes in cells. During an EMT, E-cadherin is downregulated while N-cadherin is upregulated. The goal of this study was to understand the role cadherin switching plays in EMT using a classical model system: transforming growth factor β 1 (TGF- β 1)-mediated EMT in mammary epithelial cells. We showed that stress fibers and focal adhesions are increased, and cell-cell junctions are decreased in response to TGF- β 1. Moreover, these changes were reversible upon removal of TGF- β 1. Downregulation of E-cadherin and upregulation of N-cadherin were both transcriptional.

Neither experimental knockdown nor experimental overexpression of N-cadherin interfered with the morphological changes. In addition, the morphological changes associated with EMT preceded the downregulation of E-cadherin. Interestingly, TGF- β 1-induced motility in N-cadherin-knockdown cells was significantly reduced. Together, these data suggest that cadherin switching is necessary for increased motility but is not required for the morphological changes that accompany EMT.

Key words: Cadherin, TGF- β , Epithelium-to-mesenchyme transition, Motility

Introduction

Epithelial cell-cell junctions provide tissue integrity and promote cellular polarity (Perez-Moreno et al., 2003). The junctional complex is composed of tight junctions, adherens junctions and desmosomes (Tsukita et al., 2001; Wheelock and Johnson, 2003b). The adherens junction plays a pivotal role in regulating the activity of the entire junctional complex, and the major adhesion molecules in the adherens junctions are the cadherins (Nagafuchi, 2001; Wheelock and Johnson, 2003a; Wheelock and Johnson, 2003b; Wheelock et al., 2001). In some situations, including the dynamic cellular rearrangements that are integral to embryonic development, tissue integrity must be disrupted so that cells can migrate from their original position to establish new structures (Pla et al., 2001; Savagner, 2001; Shook and Keller, 2003). For example, during *Drosophila* gastrulation, prospective mesodermal cells (which originally express E-cadherin and form adherens junctions) lose their expression of E-cadherin, become disperse and begin to migrate (Oda et al., 1998). Likewise, when epithelial cells change their relative position within a tissue, they convert to motile fibroblastic cells. This phenomenon is referred to as the epithelium-to-mesenchyme transition (EMT) (Affolter et al., 2003; Hay, 1995). During the EMT, cell-cell junctions are disrupted, the actin cytoskeleton is extensively reorganized and the cells acquire increased migratory characteristics (Boyer et al., 2000; Savagner, 2001). It has been proposed that, when cancer cells invade adjacent tissues, they use a mechanism

much like the EMT that is involved in normal developmental processes (Gotzmann et al., 2004; Thiery, 2002; Thiery, 2003).

Transforming growth factor β (TGF- β) suppresses the growth of many normal epithelial cells. In some cancers, TGF- β signaling is genetically disrupted, which might contribute to unregulated cell growth (Brattain et al., 1996; de Caestecker et al., 2000; Massague et al., 2000; Waite and Eng, 2003). Interestingly, TGF- β also has tumor-promoting activity, owing to its ability to induce EMT in some cell types (Akhurst and Derynck, 2001; Cui et al., 1996; Derynck et al., 2001; Oft et al., 2002; Roberts and Wakefield, 2003; Siegel and Massague, 2003; Wakefield and Roberts, 2002). During TGF- β -mediated EMT, E-cadherin expression is decreased while N-cadherin expression is increased (Bhowmick et al., 2001; Grande et al., 2002; Miettinen et al., 1994; Piek et al., 1999). The expression of E-cadherin is regulated by several transcription factors, including snail, slug, E12/E47, SIP1 and delta EF1/ZEB-1, each of which has been reported to repress its transcription (Battle et al., 2000; Bolos et al., 2003; Cano et al., 2000; Comijn et al., 2001; Conacci-Sorrell et al., 2003; Grooteclaes and Frisch, 2000; Hajra et al., 2002; Perez-Moreno et al., 2001; van Grunsven et al., 2003). Snail expression is induced by TGF- β in rat hepatocytes (Gotzmann et al., 2002; Spagnoli et al., 2000; Valdes et al., 2002) and in Madin-Darby canine kidney (MDCK) cells (Peinado et al., 2003). However, SIP1 but not snail has been reported to be induced when mouse mammary epithelial

(NMuMG) cells are treated with TGF- β 1 (Comijn et al., 2001). In addition, overexpression of these transcriptional repressors alone induces EMT in some experimental systems (Batlle et al., 2000; Bolos et al., 2003; Cano et al., 2000; Comijn et al., 2001; Savagner et al., 1997).

E-Cadherin is expressed by most epithelial tissues and some epithelium-derived cancer cells have been shown to lose E-cadherin expression (Batlle et al., 2000; Cano et al., 2000; Comijn et al., 2001; Nieman et al., 1999). N-Cadherin is typically expressed by mesenchymal cells. However, some cancer cells inappropriately express N-cadherin, which promotes motility and invasion (Hazan et al., 2000; Islam et al., 1996; Nieman et al., 1999). Thus, the loss of E-cadherin expression and gain of N-cadherin expression in cancer cells is reminiscent of the cadherin switching that is seen during normal embryonic EMT (Cavallaro et al., 2002; Christofori, 2003). One could hypothesize that the function of cadherin switching during EMT is to allow epithelial cells to separate from one another and to acquire a motile phenotype, as occurs during gastrulation, for example. Studies presented here were designed, in part, to address this hypothesis.

To investigate the role of cadherin switching during EMT, we used the classical model system, TGF- β 1-induced EMT in mammary epithelial cells. Several laboratories have investigated cadherin expression during TGF- β -mediated EMT in the mouse mammary epithelial cell line NMuMG, with conflicting results. Some studies reported that the expression level of E-cadherin did not change in response to TGF- β 1 (Bakin et al., 2000; Bhowmick et al., 2001), whereas others showed that E-cadherin was downregulated (Miettinen et al., 1994; Piek et al., 1999). In addition, there are some reports that TGF- β 1 induces EMT in MCF10A human mammary epithelial cells (Seton-Rogers et al., 2004; Tang et al., 2003). In the present study, we defined the role cadherin switching plays in the morphological and behavioral changes that are fundamental to EMT using these two model systems: NMuMG mouse mammary epithelial cells and MCF10A human mammary epithelial cells.

Materials and Methods

Reagents, antibodies and cultured cells

All reagents were from Sigma-Aldrich (St Louis, MO) or Fisher Chemicals (Fairlawn, NJ) unless otherwise indicated. Mouse monoclonal antibody (mAb) against the extracellular portion of human E-cadherin (HECD1) (Shimoyama et al., 1989) was a kind gift from M. Takeichi (RIKEN Center for Developmental Biology, Kobe, Japan). Mouse mAbs against the cytoplasmic portion of E-cadherin (4A2), N-cadherin (13A9) and β -catenin (6F9) have been described previously (Johnson et al., 1993; Knudsen et al., 1995). 4A2 was used to detect mouse E-cadherin and HECD1 was used to detect human E-cadherin. A rabbit polyclonal antibody (pAb) against E-cadherin (Wheelock et al., 1987) was used for double staining. Rat mAb against the extracellular portion of mouse E-cadherin (ECCD2), mouse anti- α -catenin mAb, rabbit anti-ZO-1 pAb (Zymed, San Francisco, CA), rabbit anti fibronectin pAb (recognizes mouse fibronectin; Sigma-Aldrich), mouse anti-human-fibronectin mAb (Takara, Madison, WI), mouse anti-glyceraldehyde-3-phosphate-dehydrogenase (anti-GAPDH) mAb (Abcam, Cambridge, MA) were used. Mouse NMuMG cells (CRL-1636) and human MCF10A cells (CRL-10317) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Subclones of NMuMG cells, clones NMuMG/E9 and NMuMG/E11 cells, were prepared by limiting dilution in flat-

bottomed 96-well plates. NMuMG cells and their subclones were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 4.5 g l⁻¹ glucose and 10 μ g ml⁻¹ insulin. MCF10A cells were maintained in a 1:1 mixture of DME and Ham's F12 medium supplemented with 5% horse serum (Invitrogen-Gibco, Carlsbad, CA), 20 ng ml⁻¹ epidermal growth factor (EGF), 100 ng ml⁻¹ cholera toxin, 10 μ g ml⁻¹ insulin and 500 ng ml⁻¹ hydrocortisone. TGF- β 1 (R&D Systems, Minneapolis, MN) treatment was done without serum deprivation. For long-term treatment with TGF- β 1 or TGF- β 1+EGF, the supplements were replenished every 3 days.

Manipulation of cadherin expression

Myc-tagged human N-cadherin (Kim et al., 2000; Salomon et al., 1992) was subcloned into LZRS-MS-neo (Iretton et al., 2002) and used to generate virus. Retroviral production and infection was performed as described on the Nolan lab website (<http://www.stanford.edu/group/nolan/>). Briefly, Phoenix 293 cells (Grignani et al., 1998) were transfected using calcium phosphate (Stratagene, La Jolla, CA) and selected with puromycin. To produce N-cadherin-overexpressing NMuMG/E9 and MCF10A cells, freshly prepared conditioned medium containing the recombinant retrovirus and supplemented with 4 μ g ml⁻¹ polybrene was added to the cells and infected cells were selected with G418 (Cellgro, Mediatech, Herndon, VA). Myc-tagged human E-cadherin, the gene encoding which was inserted into the pLKneo vector (Kim et al., 2000) was transfected into NMuMG/E9 cells and NMuMG/E11 cells using Lipofectamine 2000 Reagent (Life Technology, Rockville, MD) and selection was done with G418. Untagged human E-cadherin was transduced into NMuMG/E9 cells using the retrovirus system.

N-cadherin-knockdown NMuMG/E9 cells (NMuMG/E9 Δ Ncad cells) were generated by inserting 19 nucleotides of N-cadherin (GACTGGATTTCCTGAAGAT; nucleotides 431-449 of mouse N-cadherin GenBank[®] accession AB008811, which is identical to nucleotides 215-233 of human N-cadherin GenBank accession BC036470) into the pSilencerTM1.0-U6 vector (Ambion, Austin, TX). The recombinant plasmid was co-transfected with a neomycin-resistance plasmid (using Lipofectamine 2000) into NMuMG/E9 cells and selection was done with G418. N-Cadherin-knockdown MCF10A cells were generated by inserting the same 19 nucleotides of N-cadherin into pSUPER.retro.puro vector (OligoEngine, Seattle, WA) and transfecting Phoenix 293 cells to produce retroviral particles. Infected MCF10A cells were selected with puromycin. As a control small interfering RNA (siRNA), 19 nucleotides of the gene encoding enhanced green fluorescent protein (eGFP) (CGATGC-CACCTACGGCAAG; nucleotides 786-804 of eGFP GenBank accession U55762) were inserted into pSilencerTM1.0-U6 vector and pSUPER.retro.puro vector.

Transwell motility assays

2 \times 10⁵ MCF10A cells or 5 \times 10⁵ NMuMG/E9 cells were plated in the upper chamber of uncoated polyethylene terephthalate membranes (BD BioCoatTM control culture inserts, six-well plates, pore size of 8 μ m; Becton Dickinson, San Jose, CA). When TGF- β 1 was used, it was added to the stock culture 2 days before plating on the membranes and to both the upper and the lower chamber during the assay. After plating on membranes, the cells were incubated for 24 hours, and the cells that did not migrate through the membrane were removed with cotton swabs. Cells traversing the membrane were stained with eosin y-methylene blue using HEMA3. Cells in nine random fields of view at 100 \times magnification were counted and expressed as the average number of cells per field of view. Three independent experiments were performed and the data were represented as an average with the

standard deviation. The statistical analysis was by one-way analysis of variance followed by Scheffe's *F* test.

In vitro wound healing assays

Cells were plated on a dish with a 2 mm grid 3 days before the initiation of the assay so that they would be confluent on the day of the experiment. TGF- β 1 treatment was initiated 2 days before the assay. Monolayers of confluent cultures were lightly scratched with a pipette tip and were observed at timed intervals for up to 27 hours. Quantification was done by measuring the number of pixels in the wound area using Adobe® Photoshop® and calculating the decrease in the area by subtracting the number of pixels from the number of pixels in the corresponding wound area at the 0 hour time point. Statistical analysis was done as above.

Detergent extraction, SDS-PAGE and immunoblots

Monolayers of cultured cells were washed with ice-cold PBS and extracted on ice with TNE buffer (10 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride). Extracts were centrifuged at 20,000 *g* for 15 minutes at 4°C and the supernatant was collected. Protein concentration was determined using the DC protein assay (Bio-Rad, Hercules CA). Cell extracts were resolved by sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and immunoblotted as described previously (Johnson et al., 1993).

Immunoprecipitation

A 500 μ l aliquot of cell extract (500 μ g) was incubated with 300 μ l 4A2 (against the E-cadherin cytoplasmic domain) hybridoma supernatant for 30 minutes at 4°C. 50 μ l packed anti-mouse IgG affinity gel (ICN Pharmaceuticals, Costa Mesa, CA) was added, and mixing continued for 30 minutes. Immune complexes were washed with TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20). After the final wash, the packed beads were resuspended in 2 \times sample buffer and boiled for 5 minutes, and the proteins were resolved by SDS-PAGE and immunoblotted.

Immunofluorescence microscopy

Cells were processed as previously described (Kim et al., 2000). When double staining included TRITC-conjugated phalloidin, the cells were fixed in 3.7% formaldehyde for 15 minutes, permeabilized with 0.2% Triton X-100 in PBS for 15 minutes and blocked in 10% goat serum in PBS. To confirm E-cadherin expression on the cell surface after TGF- β 1 treatment, the cells were fixed in 3.7% formaldehyde for 15 minutes without permeabilization and probed with the

ECCD2 antibody, which recognizes the extracellular portion of E-cadherin. Cells were examined on a Zeiss Axiovert 200M microscope (Göttingen, Germany) equipped with an ORCA-ER digital camera (Hamamatsu, Houston, TX). Images were collected and processed using OpenLab software (Improvision, Boston, MA).

Conventional RT-PCR

Total RNA was extracted with TRI reagent and analysed by reverse-transcription polymerase chain reaction (RT-PCR) using a TITANIUM One-Step RT-PCR kit (Becton Dickinson, San Jose, CA) and previously reported forward and reverse primers for snail (Gotzmann et al., 2002), SIP1 (Cacheux et al., 2001), slug (Zhao et al., 2002), E12/E47 (Perez-Moreno et al., 2001), E-cadherin (Tegoshi et al., 2000), N-cadherin (Chung et al., 1998) and GAPDH (Xu et al., 2000). β -Actin primers were supplied with the kit. The conditions for PCR reactions were: 94°C for 30 seconds, 65°C for 30 seconds and 68°C for 1 minute for 28–30 cycles for snail, SIP1, slug, E12/E47, E-cadherin and β -actin; 94°C for 1 minute, 62°C for 2 minutes and 72°C

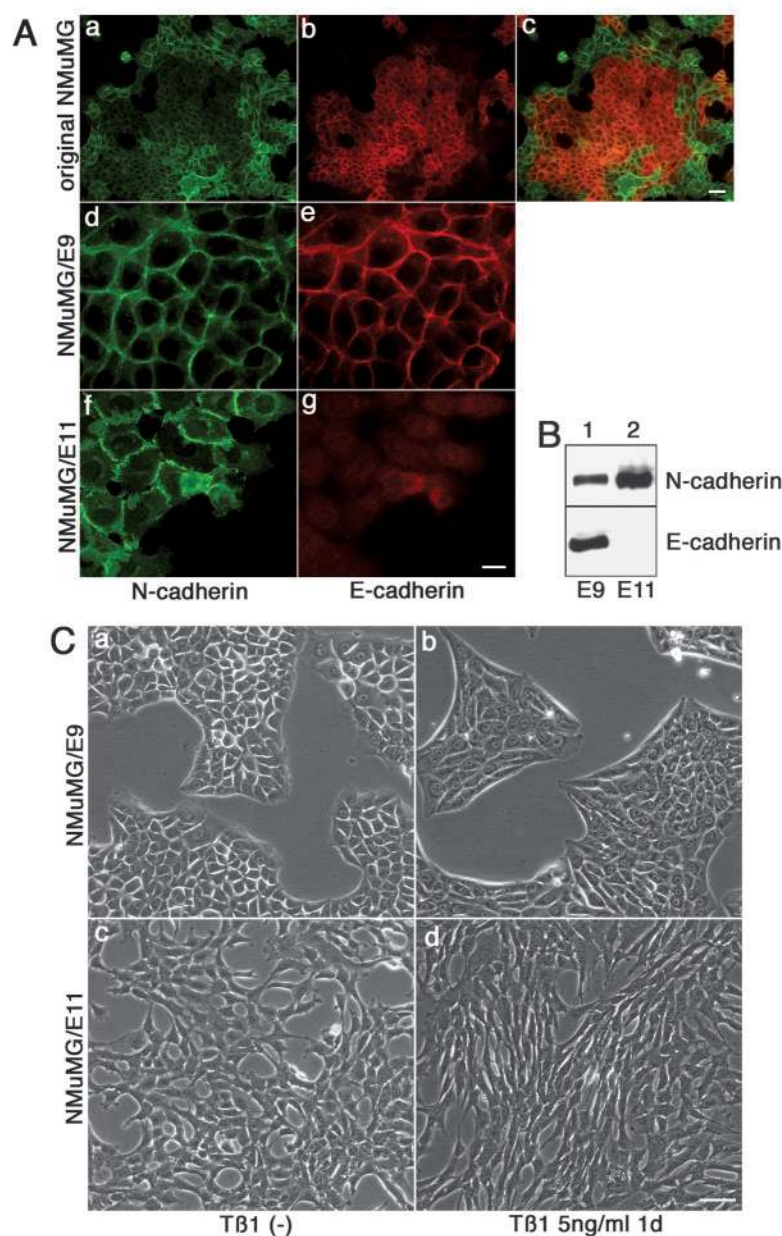


Fig. 1. Subcloning NMuMG cells. (A) Parental NMuMG cells (a-c), clone NMuMG/E9 (d,e) and clone NMuMG/E11 (f,g) were stained for N-cadherin (a,d,f) and E-cadherin (b,e,g). (c) A merged picture of a,b. (a-c) Photographs were taken using a 10 \times objective; scale bar, 50 μ m. (d-g) Photographs were taken using a 40 \times dry objective; scale bar, 10 μ m. (B) TNE extracts of NMuMG/E9 (lane 1) and NMuMG/E11 (lane 2) were analysed for N-cadherin (top) and E-cadherin (bottom) by immunoblots. (C) Both clones underwent morphological changes (elongation of cell shape, b,d) in response to TGF- β 1 [T β 1; 5 ng ml⁻¹ for 1 day (d)]. Photographs were taken of living cells using a 10 \times objective; scale bar, 50 μ m.

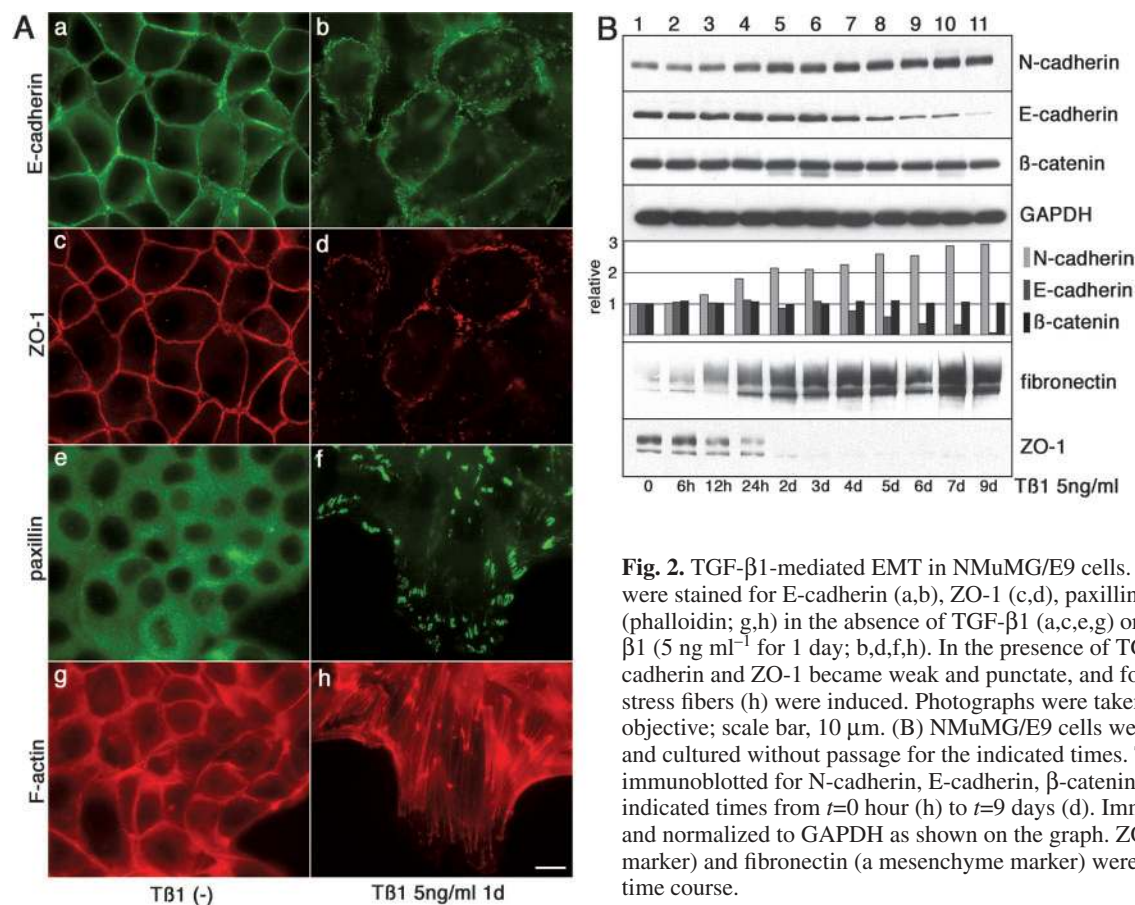


Fig. 2. TGF- β 1-mediated EMT in NMuMG/E9 cells. (A) NMuMG/E9 cells were stained for E-cadherin (a,b), ZO-1 (c,d), paxillin (e,f) and F-actin (phalloidin; g,h) in the absence of TGF- β 1 (a,c,e,g) or in the presence of TGF- β 1 (5 ng ml⁻¹ for 1 day; b,d,f,h). In the presence of TGF- β 1, the signals for E-cadherin and ZO-1 became weak and punctate, and focal adhesions (f) and stress fibers (h) were induced. Photographs were taken using a 63 \times oil objective; scale bar, 10 μ m. (B) NMuMG/E9 cells were treated with TGF- β 1 and cultured without passage for the indicated times. TNE extracts were immunoblotted for N-cadherin, E-cadherin, β -catenin and GAPDH at the indicated times from $t=0$ hour (h) to $t=9$ days (d). Immunoblots were quantified and normalized to GAPDH as shown on the graph. ZO-1 (an epithelium marker) and fibronectin (a mesenchyme marker) were examined in the same time course.

for 3 minutes for 25 cycles for N-cadherin; 94°C for 45 seconds, 60°C for 30 seconds and 72°C for 90 seconds for 30 cycles for GAPDH. PCR products were analysed by electrophoresis on 1-2% agarose gels.

Quantitative real-time RT-PCR

Total RNA was analysed using an Mx3000P™ Real-Time PCR System (Stratagene, La Jolla, CA). Combinations of primers and probes for snail, SIP1 and human 18S rRNA (for endogenous control) were purchased from Applied Biosystems (Foster City, CA).

Results

Establishment of NMuMG clones

NMuMG cells purchased from ATCC were heterogeneous in both morphology and cadherin expression. Some cells expressed similar levels of both E- and N-cadherin, whereas others expressed high levels of N-cadherin and low levels of E-cadherin (Fig. 1A). When the cells were sparsely plated and allowed to form individual colonies from single cells, we found that, in some colonies, all of the cells expressed both cadherins and were compact like typical epithelial cells but, in other colonies, all of the cells expressed only N-cadherin and looked like fibroblasts (data not shown). Thus, we concluded that the heterogeneity in cadherin expression was due to clonal differences in the cells and not to culture conditions. To eliminate the heterogeneity in the NMuMG cell culture, we established subclones of cells using limiting dilution. One clone, NMuMG/E9, had an epithelial morphology and

expressed both E- and N-cadherin. Another clone, NMuMG/E11, had a fibroblastic morphology and expressed high levels of N-cadherin and barely detectable levels of E-cadherin (Fig. 1A,B). These cells did express a very small amount of E-cadherin, because we could detect it by immunoblot upon extended exposure or by immunofluorescence in very dense cultures (data not shown). Interestingly, even though these two cell lines had very different morphologies, they both became elongated in response to TGF- β 1, and these morphological changes required at least 12 hours of TGF- β 1 exposure (Fig. 1C), as previously reported for the parental NMuMG cells (Piek et al., 1999). Because cellular elongation is one hallmark of EMT, we assumed that both clones retained their ability to undergo EMT in response to TGF- β 1. Our goal in isolating clones of NMuMG cells was to generate a cell line that not only was homogeneous with regard to cadherin expression but also had a typical epithelial morphology when cultured under standard conditions. Thus, we used the NMuMG/E9 clone for the remainder of this study.

NMuMG/E9 cells undergo classical TGF- β 1-mediated EMT

Under normal growth conditions, NMuMG/E9 cells were very compact and had well-organized cell-cell junctions. E-Cadherin was located at cell-cell borders and ZO-1 was restricted to a narrow segment of the cell-cell borders,

corresponding to a typical tight junction localization (Fig. 2Aa,c). Phalloidin staining showed that F-actin distribution was mainly at cell-cell borders and very few stress fibers were evident (Fig. 2Ag). When the cells were treated with TGF- β 1, the staining patterns for E-cadherin and ZO-1 became intermittent and jagged, indicating a breakdown of cell-cell junctions (Fig. 2Ab,d). Simultaneously, F-actin distribution dramatically changed from cell-cell borders to stress fibers and staining for paxillin showed that many focal adhesions had formed (Fig. 2Ah,f, respectively). The expression level of N-cadherin protein in NMuMG/E9 cells began to increase 12 hours after TGF- β 1 addition and gradually increased up to ~2.9 times (Fig. 2B). By contrast, E-cadherin levels remained approximately the same during the first 3 days and then gradually decreased. β -Catenin levels were not affected by treatment with TGF- β 1 (Fig. 2B). Cadherin switching was accompanied by increased fibronectin expression (a mesenchyme marker) and decreased ZO-1 expression (an epithelium marker); changes in the expression of these two markers became clear 12 hours after TGF- β 1 addition (Fig. 2B). Notably, dissolution of cell-cell junctional complexes (as evidenced by altered morphology, changes in the cytoskeleton and loss of ZO-1 from cell borders) was initiated at least 2 days before E-cadherin was downregulated. Indeed, 1 day after TGF- β 1 treatment, NMuMG/E9 cells showed morphological changes accompanied by N-cadherin and fibronectin induction, yet E-cadherin was still localized to the cell surface, as evidenced by immunostaining of unpermeabilized cells with ECCD2, which recognizes the extracellular domain of E-cadherin (Fig. 3A). Moreover, E-cadherin was still complexed with α - and β -catenins, similar to untreated NMuMG/E9 cells (Fig. 3B). To ensure the cadherins were functional, we performed aggregation assays, which did not show any difference in the ability of cells to interact with one another with or without TGF- β 1 treatment (data not shown). These data suggest that E-cadherin remained functional for some time after morphological EMT was induced.

Several transcription factors (including snail, SIP1, slug and E12/E47) have been reported to downregulate E-cadherin expression (Batlle et al., 2000; Bolos et al., 2003; Cano et al., 2000; Comijn et al., 2001; Conacci-Sorrell et al., 2003; Hajra et al., 2002; Perez-Moreno et al., 2001; van Grunsven et al., 2003). Accordingly, we used conventional RT-PCR to determine whether the mRNA levels of any of these factors changed in NMuMG/E9 cells in response to TGF- β 1. Snail and SIP1 levels increased 2 days after TGF- β 1 addition (Fig. 4A). Changes in slug and E12/E47 were less apparent. We further investigated the time course of changes in the expression levels of *snail* and *SIP1* mRNAs with conventional RT-PCR (Fig. 4B) and quantitative real-time RT-PCR (Fig. 4C). Snail and SIP1 levels gradually increased sevenfold and 16-fold, respectively (Fig. 4C). Thus, in our system, snail and SIP1 are both likely candidate transcription factors responsible for regulating E-cadherin expression when the cells are treated with TGF- β 1. This partially supports the report by Comijn et al. (Comijn et al., 2001), who showed that SIP1, but not snail, was induced in NMuMG cells in response to TGF- β 1.

Cadherin switching is reversible in NMuMG/E9 cells

We were interested in determining whether the biochemical

changes that accompany TGF- β 1-induced EMT were reversible upon removal of TGF- β 1. To address this, we wanted to treat the cells with TGF- β 1 for a prolonged time to ensure complete change from epithelial cells to mesenchymal

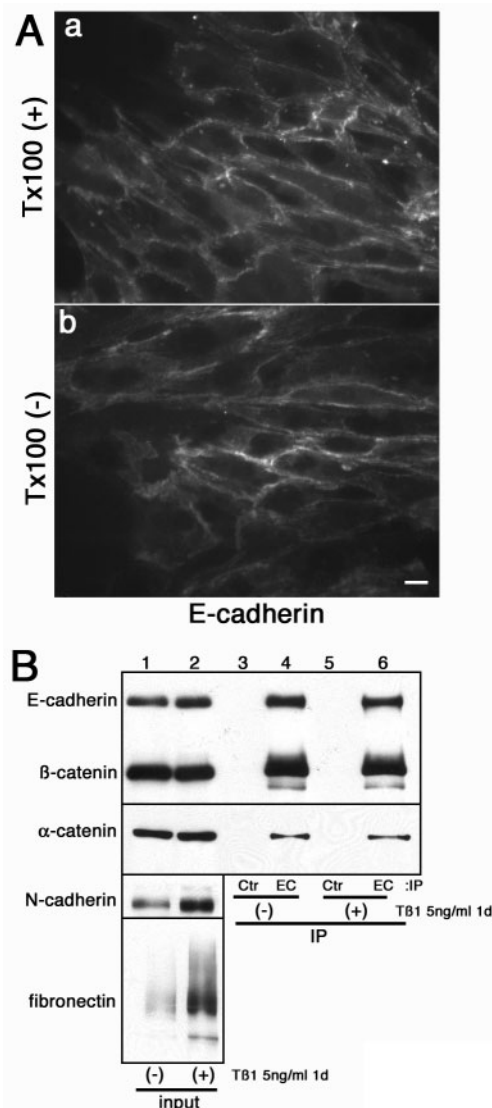


Fig. 3. E-Cadherin continued to function after morphological EMT was induced. (A) Permeabilized (0.2% Triton X-100, Tx100; a) and unpermeabilized (b) NMuMG/E9 cells that had been treated with 5 ng ml⁻¹ TGF- β 1 for 1 day were stained with antibodies against the extracellular portion of E-cadherin (ECCD2). E-Cadherin was localized to the cell surface (b). Photographs were taken using a 40 \times dry objective; scale bar, 10 μ m. (B) Extracts of untreated NMuMG/E9 cells (lanes 3, 4) or NMuMG/E9 cells treated with 5 ng ml⁻¹ TGF- β 1 for 1 day (lanes 5, 6) were immunoprecipitated (IP) with anti-E-cadherin (EC; lanes 4, 6) or control antibodies (Ctr; lanes 3, 5) and immunoblotted for E-cadherin, β -catenin and α -catenin. Comparable amounts of β -catenin and α -catenin were coimmunoprecipitated with E-cadherin in treated (lane 6) and untreated (lane 4) NMuMG/E9 cells, indicating that E-cadherin junctional complexes were functional. Lanes 1, 2 are immunoblots of treated and untreated cells to show N-cadherin and fibronectin were induced in this experiment, as expected, and to show the input levels of E-cadherin, β -catenin and α -catenin in the immunoprecipitations.

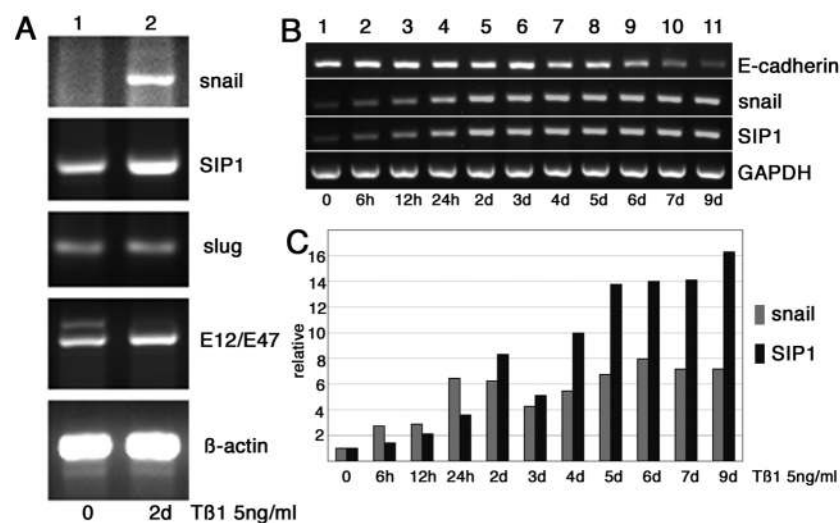


Fig. 4. Snail and SIP1 were induced during TGF-β1-mediated EMT in NMuMG/E9 cells. (A) RT-PCR using total RNA extracted from untreated (lane 1) and TGF-β1-treated (5 ng ml⁻¹ for 2 days) NMuMG/E9 cells (lane 2) was done for snail, SIP1, slug, E12/E47 and β-actin (as a control). The identity of the slower migrating band in the E12/E47 lane is not known. (B) RT-PCR using total RNA extracted from NMuMG/E9 cells over the same time course as Fig. 2B. E-cadherin, snail, SIP1 and GAPDH were analysed. (C) The *snail* and *SIP1* mRNA levels were analysed by quantitative real-time RT-PCR using the same total RNA as in B.

cells. However, lengthy treatment of NMuMG/E9 cells with TGF-β1 resulted in elevated levels of apoptosis. To circumvent this problem, we investigated the possibility of including EGF in the culture medium, because it has been reported to act as a survival factor for mammary epithelial cells and might thus prevent TGF-β1-induced apoptosis (Rosfjord and Dickson, 1999). When we added EGF at the same time as we added TGF-β1, the apoptotic effect was diminished, which enabled us to observe long-term treatment of NMuMG/E9 cells. EGF

treatment alone did not affect cadherin expression, nor did it have any effect on the actin cytoskeleton (data not shown). Moreover, there was no obvious difference, other than cell viability, between cells treated with TGF-β1 alone and cells treated with TGF-β1+EGF (data

not shown). Treatment of NMuMG/E9 cells with 1 ng ml⁻¹ TGF-β1 together with 20 ng ml⁻¹ EGF for more than 14 days converted the cells to a fully fibroblastic phenotype with well-organized stress fibers (Fig. 5A). By 30–60 days of treatment, the cells showed significant loss of both E-cadherin protein and mRNA and had fully upregulated expression of N-cadherin (Fig. 5B,C). However, upon removal of both TGF-β1 and EGF, the cells gradually regained their original phenotype, including establishment of cell-cell junctions (Fig. 5A). Indeed, 7 days after removal of TGF-β1 and EGF, expression levels of E- and N-cadherin protein and mRNA were restored approximately to basal levels (Fig. 5B,C). The *snail* and *SIP1* mRNA levels increased in the presence of TGF-β1+EGF and also returned to basal levels upon removal of the inducing agents. Thus, changes in snail and SIP1 production were coupled to cadherin switching (Fig. 5C). This reversibility was also seen in expression levels of the mesenchyme marker fibronectin (Fig. 5B).

Modulation of N-cadherin expression in NMuMG/E9 cells had no effect on morphology in the presence or absence of TGF-β1

When NMuMG/E9 cells were treated with TGF-β1 E-cadherin

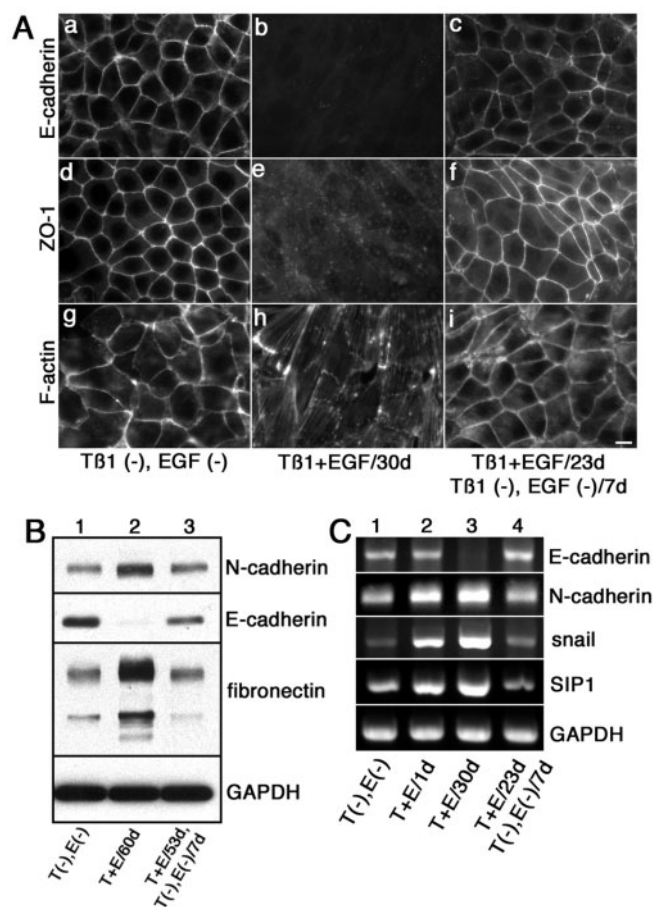


Fig. 5. Cadherin switching was reversible in TGF-β1-treated NMuMG/E9 cells. (A) Untreated NMuMG/E9 cells (a,d,g), NMuMG/E9 cells treated with 1 ng ml⁻¹ TGF-β1 and 20 ng ml⁻¹ EGF for 30 days (b,e,h), and NMuMG/E9 cells treated with TGF-β1 and EGF for 23 days and subsequently cultured upon removal of both reagents for an additional 7 days (c,f,i) were stained for E-cadherin (a-c), ZO-1 (d-f) or F-actin (phalloidin) (g-i). Photographs were taken using a 40× dry objective; scale bar, 10 μm. (B) TNE extracts from untreated NMuMG/E9 cells (lane 1), TGF-β1- and EGF-treated NMuMG/E9 cells for 60 days (lane 2) or reversibly treated NMuMG/E9 cells (lane 3) were examined for N-cadherin, E-cadherin, fibronectin and GAPDH by immunoblot. (C) RT-PCR was performed using total RNA extracted from untreated NMuMG/E9 cells (lane 1), TGF-β1- and EGF-treated NMuMG/E9 cells (for 1 day, lane 2, or for 30 days, lane 3) or reversibly treated NMuMG/E9 cells (lane 4) to examine the levels of mRNAs encoding E-cadherin, N-cadherin, snail, SIP1 and GAPDH.

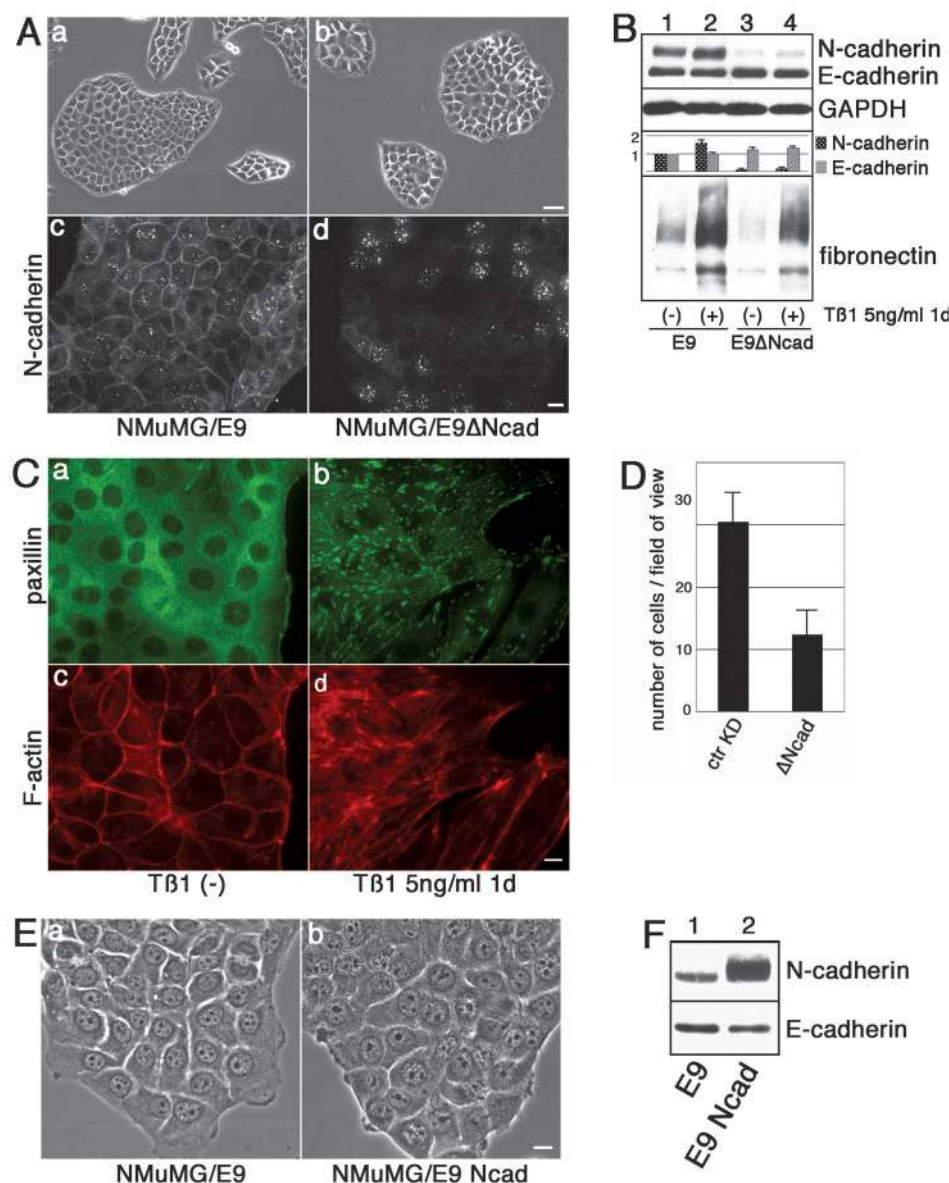


Fig. 6. Manipulation of N-cadherin expression in NMuMG/E9 cells did not interfere with TGF-β1-induced morphological change. (A) N-Cadherin-knockdown NMuMG/E9 cells (NMuMG/E9ΔNcad cells) showed similar morphology to parental NMuMG/E9 cells in phase-contrast microscopy (a,b). Photographs were taken of living cells using a 10× phase objective; scale bar, 50 μm. Parental NMuMG/E9 cells and NMuMG/E9ΔNcad cells were stained with N-cadherin mAb in the absence of TGF-β1 (c,d). Cell-cell border staining of N-cadherin was significantly reduced. Photographs were taken using a 40× dry objective; scale bar, 10 μm. (B) TNE extracts of NMuMG/E9 cells and NMuMG/E9ΔNcad cells in the absence and presence of TGF-β1 (5 ng ml⁻¹ for 1 day) were immunoblotted for N-cadherin, E-cadherin, GAPDH and fibronectin. N-Cadherin and E-cadherin levels were quantified and normalized to GAPDH as shown on the bar graph. (C) NMuMG/E9ΔNcad cells in the absence (a,c) or presence of TGF-β1 (5 ng ml⁻¹ for 1 day; b,d) were stained for paxillin (a,b) and F-actin (c,d). Photographs were taken using a 40× dry objective; scale bar, 10 μm. (D) Transwell motility assays were done using control knockdown NMuMG/E9 cells (ctr KD) and NMuMG/E9ΔNcad cells in the absence of TGF-β1 showed that NMuMG/E9ΔNcad cells were significantly less motile than control cells ($P < 0.05$). (E) NMuMG/E9 cells expressing 2×-Myc-tagged human N-cadherin (NMuMG/E9 Ncad cells) showed similar morphology to parental NMuMG/E9 cells in phase-contrast microscopy (a,b). Photographs were taken using a 40× dry objective; scale bar, 10 μm. (F) TNE extracts of parental NMuMG/E9 cells and NMuMG/E9 Ncad cells were immunoblotted for N-cadherin (top) and E-cadherin (bottom).

expression did not significantly change until day 3. However, the morphological change was clearly evident within 1 day, suggesting that continued expression of E-cadherin did not interfere with initiation of the morphological changes that are characteristic of EMT. By contrast, upregulation of N-cadherin occurred on the same timescale as the morphological changes. Together, these data led us to speculate that downregulation of E-cadherin was not necessary for morphological EMT but that induction of N-cadherin might be essential for the transition to occur. To determine whether induction of N-cadherin expression was essential for TGF-β1-induced morphological changes, we established NMuMG/E9ΔNcad cells using pSilencer siRNA. In the absence of TGF-β1, the morphology of NMuMG/E9ΔNcad cells was almost identical to that of parental NMuMG/E9 cells (Fig. 6A), in spite of a significant reduction in N-cadherin expression (~90%) and almost complete removal from cell borders (Fig. 6B). The level of E-cadherin expression increased only slightly (up to 1.2-fold) in

the NMuMG/E9ΔNcad cells compared with parental NMuMG/E9 cells (Fig. 6B). Upon addition of TGF-β1, the N-cadherin level in NMuMG/E9ΔNcad cells increased but was still much lower than in parental NMuMG/E9 cells (Fig. 6B). In fact, N-cadherin increased 1.6-fold in both the parental and knockdown cells in response to TGF-β1, although the absolute level of N-cadherin in the knockdown cells was substantially lower than in the parental cells. Surprisingly, in spite of the low level of N-cadherin expression, TGF-β1-treated NMuMG/E9ΔNcad cells underwent a morphological change that was similar to parental NMuMG/E9 cells, together with induction of stress fibers and focal adhesions (Fig. 6C). These data suggest that a high level of N-cadherin expression is not essential for the morphological changes that accompany TGF-β1-induced EMT. To investigate further the effect of N-cadherin expression on cell morphology, we established N-cadherin-overexpressing NMuMG/E9 cells using retroviral transduction. In spite of the presence of highly expressed

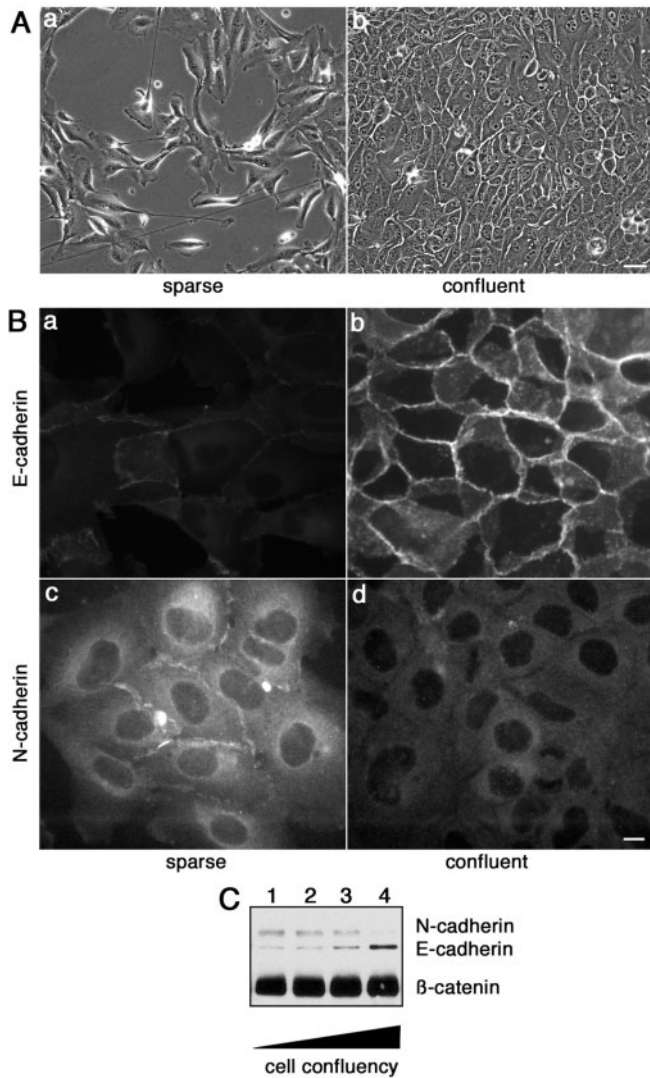


Fig. 7. E-Cadherin and N-cadherin expression in MCF10A cells was dependent on cell confluence. (A) Phase-contrast micrographs of MCF10A cells in sparse culture (a) or confluent culture (b). Photographs were taken of living cells using a 10× objective; scale bar, 50 μm. (B) MCF10A cells in sparse culture (a,c) or confluent culture (b,d) were stained for E-cadherin (a,b) or N-cadherin (c,d). Photographs were taken using a 40× dry objective; scale bar, 10 μm. (C) TNE extracts of MCF10A cells in increasing confluence were immunoblotted for N-cadherin (top), E-cadherin (middle) and β-catenin (bottom).

exogenous N-cadherin, which was accompanied by a slight reduction in E-cadherin level (Fig. 6F), these cells were still compact and did not show any morphological features of EMT (Fig. 6E). Together, these data indicate that two prominent features of EMT (morphological changes and upregulation of N-cadherin) are independent events.

Upregulation of N-cadherin contributes to TGF-β1-induced cell motility

The most obvious phenotypic change in epithelial cells as they undergo EMT is the change from an epithelial morphology to

a more fibroblastic morphology. However, an equally important aspect of EMT is the behavioral change from relatively non-motile cells to highly motile cells. Because upregulation of N-cadherin during TGF-β1-mediated EMT was not essential for the morphological changes, we speculated that it might be important in modulating cellular behavior. This idea is consistent with previous studies showing that exogenous expression of N-cadherin by some tumor cells increases their motility and invasive characteristics (Hazan et al., 2000; Islam et al., 1996; Nieman et al., 1999). Indeed, the NMuMG/E9 cells expressing siRNA directed against N-cadherin (NMuMG/E9ΔNcad cells) showed significantly slower motility than parental NMuMG/E9 cells (Fig. 6D).

NMuMG cells are a classical model system for investigating TGF-β1-induced EMT in mammary epithelial cells. However, they are very sensitive to TGF-β1-induced apoptosis, even in the presence of EGF, making it difficult for us to establish reliable quantitative results in motility assays when we treat with TGF-β1, especially given that we could not be guaranteed that different sublines of these cells had the same apoptotic rates in response to TGF-β1. Thus, we turned to a different model system to pursue this question. The human mammary epithelial cells MCF10A showed a fibroblast-like spindle morphology in sparse conditions and an epithelial-like compact morphology in dense cultures, suggesting they might also be susceptible to EMT (Fig. 7A). Like NMuMG/E9 cells, MCF10A cells expressed both E- and N-cadherin, but expression levels of E- and N-cadherin varied depending on the level of culture confluence (Fig. 7B,C). In sparse culture, MCF10A cells formed loose cell-cell contacts and expressed N-cadherin together with low levels of E-cadherin. As the culture became confluent, N-cadherin levels decreased and E-cadherin levels increased (Fig. 7C). In fully confluent cultures, MCF10A cells formed compact epithelial cultures and cell border staining for N-cadherin was undetectable by immunofluorescence microscopy (Fig. 7B).

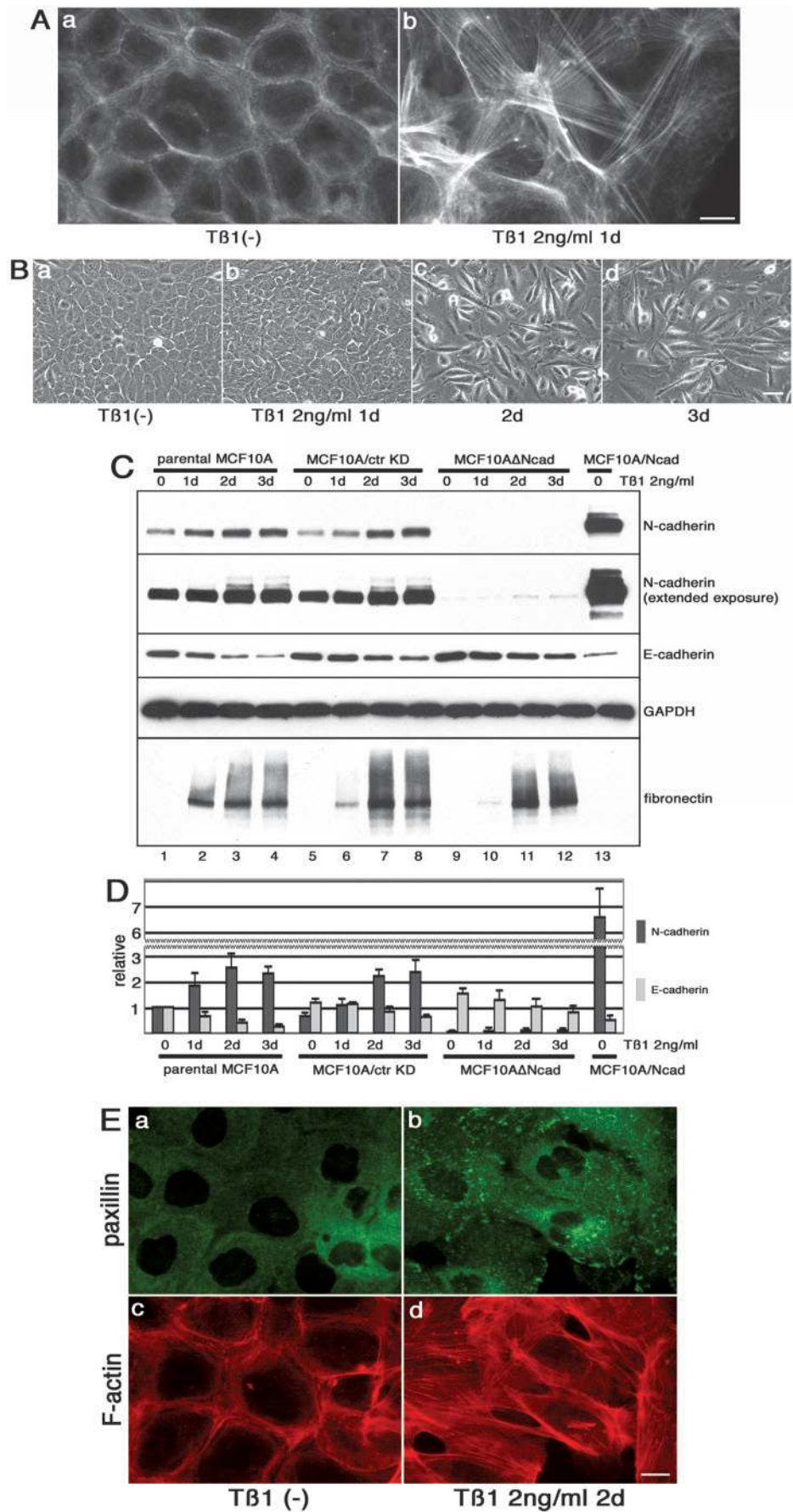
To determine whether MCF10A cells underwent EMT in response to TGF-β1, we treated them as we had the NMuMG cells and showed that they underwent a characteristic morphological change including stress fiber formation (Fig. 8A). Fig. 8B shows that TGF-β1 treated MCF10A cells lost their cell-cell contacts, converted to a fibroblastic phenotype and were not compact even in dense cultures. Moreover, in TGF-β1-treated MCF10A cells, the switch from E-cadherin to N-cadherin expression took place much more quickly than in NMuMG cells (Fig. 8C, lanes 1-4). E-Cadherin decreased to 0.3-fold and N-cadherin increased to 2.3-fold by 3 days after TGF-β1 addition (Fig. 8D). Importantly, MCF10A cells did not become apoptotic in response to TGF-β1, and so they provided an appropriate system in which to quantify motility.

Once we established that the MCF10A cell line was appropriate for motility assays, we generated several cell lines to investigate the role of N-cadherin in cell motility induced by TGF-β1. We prepared N-cadherin knockdown cells (MCF10AΔNcad cells) and control knockdown cells (MCF10A/ctr KD) using retroviral delivery. The use of retroviral delivery allowed us to work with a population of virally transduced cells, thus avoiding the complications of clonal variation. MCF10A/ctr KD cells showed the same morphology and cadherin levels as parental MCF10A cells in the absence or presence of TGF-β1 (Fig. 8C,D, lanes 5-8, and

Fig. 8. TGF- β 1-mediated EMT in MCF10A cells. (A) MCF10A cells in the absence (a) or presence (b) of TGF- β 1 [2 ng ml⁻¹ for 1 day (d)] were stained for F-actin. Photographs were taken using a 63 \times oil objective; scale bar, 10 μ m. (B) Phase-contrast micrographs of TGF- β 1-treated MCF10A cells ($t=0-3$ days). Photographs were taken of living cells using a 10 \times objective; scale bar, 50 μ m. (C) TNE extracts of TGF- β 1-treated parental MCF10A cells ($t=0-3$ days; lanes 1-4) control knockdown cells (ctr KD cells) ($t=0-3$ days; lanes 5-8), N-cadherin knockdown cells (Δ Ncad cells) ($t=0-3$ days; lanes 9-12) and untreated N-cadherin overexpressing cells (Ncad cells) (lane 13) were immunoblotted for N-cadherin (regular and longer exposure), E-cadherin, GAPDH and fibronectin. The slower migrating bands in the N-cadherin immunoblot are pro-region-containing precursor forms of N-cadherin. (D) Quantification of N- and E-cadherin levels using normalization with GAPDH. (E) MCF10A Δ Ncad in the absence (a,c) or presence (b,d) of TGF- β 1 (2 ng ml⁻¹ for 2 days) were co-stained for paxillin (a,b) and F-actin (c,d). Photographs were taken using a 63 \times oil objective; scale bar, 10 μ m.

data not shown). In the absence of TGF- β 1, MCF10A Δ Ncad cells showed the same morphology as parental MCF10A cells, and they underwent a similar morphological change in response to TGF- β 1 (Fig. 8E). Downregulation of E-cadherin took place (up to 0.5-fold), albeit not as extensively as in parental MCF10A cells. Importantly, upregulation of N-cadherin was suppressed (Fig. 8C, lanes 9-12). To separate the upregulation of N-cadherin from other TGF- β 1-induced mechanisms, we established cells that would overexpress N-cadherin without TGF- β 1 treatment using retroviral transduction (MCF10A/Ncad cells). MCF10A/Ncad cells expressed 6.6-fold the N-cadherin as did untreated parental MCF10A cells and 2.8-fold the N-cadherin as did parental MCF10A cells treated with TGF- β 1 for 3 days while maintaining approximately the same level of E-cadherin as the treated parental cells (Fig. 8C,D, lanes 4, 13).

The MCF10A, MCF10A/ctr KD, MCF10A Δ Ncad and MCF10A/Ncad set of cell lines provided an excellent system in which to test the idea that upregulation of N-cadherin was necessary for the increased cell motility that is characteristic of EMT. We used two complementary assays to compare



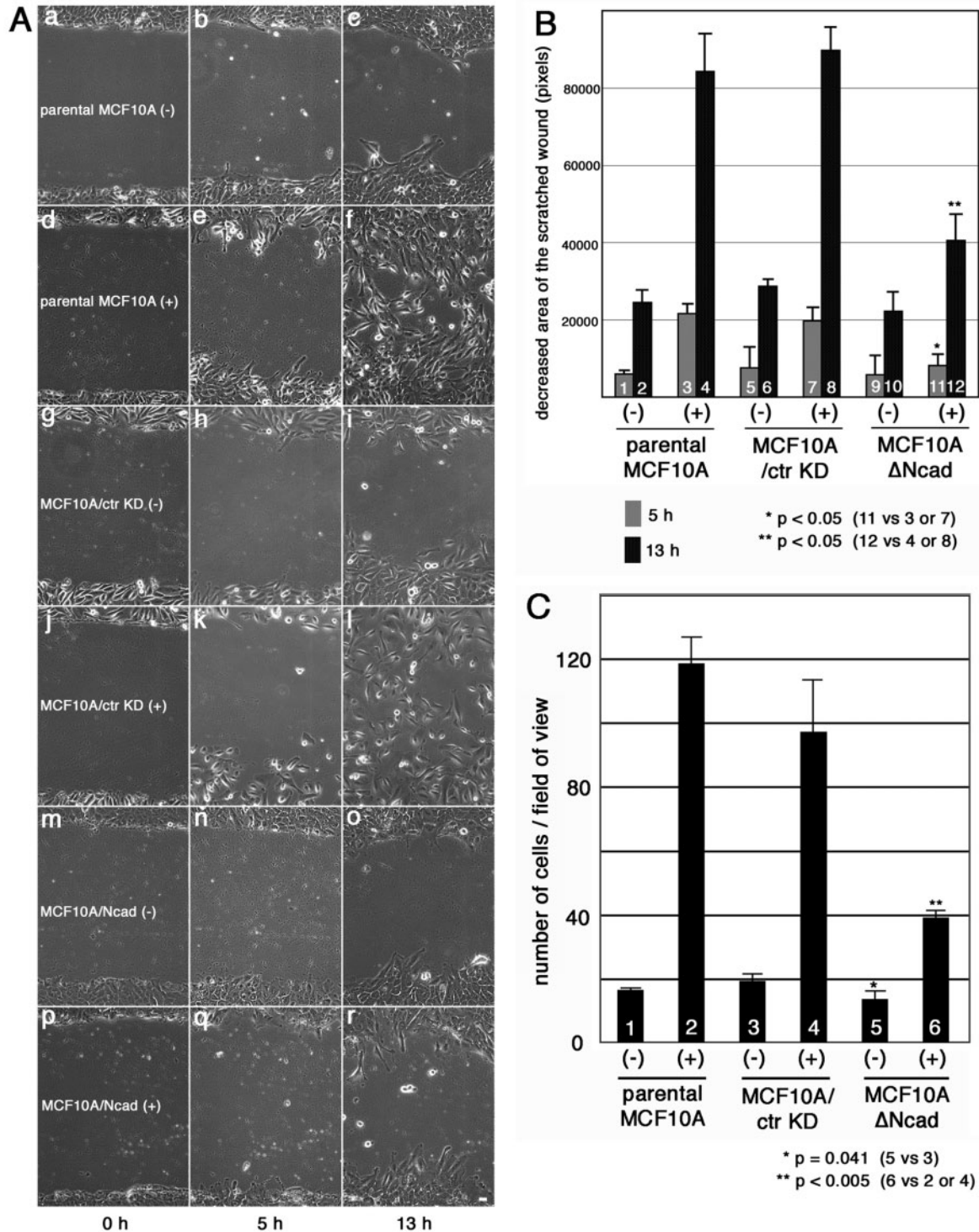


Fig. 9. Knockdown of N-cadherin in MCF10A cells decreased cell motility. (A) Wound-healing assays. Phase-contrast micrographs of living cultures of parental MCF10A cells (a-f), MCF10A/ctr KD cells (g-l) and MCF10AΔNcad cells (m-r) in a wound-healing assays performed in the absence (a-c,g-i,m-o) or presence (d-f,j-l,p-r) of TGF-β1 (2 ng ml⁻¹). TGF-β1 treatment began 2 days before initiation of the assays. Photographs were taken just after the incision (0 hour) (a,d,g,j,m,p) and at 5 hours (b,e,h,k,n,q) and 13 hours (c,f,i,l,o,r) after incision using a 10× objective; scale bar, 50 μm. (B) Motility was quantified by measuring the decrease in the denuded area at 5 hours and 13 hours, and presented as the average decrease in the number of pixels with standard deviation in three independent experiments. TGF-β1 treatment increased motility in all cell lines tested. In the presence of TGF-β1, MCF10AΔNcad cells were significantly slower than parental or control knockdown cells at 5 hours (h) and 13 hours. (C) Transwell motility assays of parental MCF10A cells, MCF10A/ctr KD cells and MCF10AΔNcad cells in the absence (-) or presence (+) of TGF-β1 (2 ng ml⁻¹). TGF-β1 treatment began 2 days before initiation of the assays. Data are presented as the average number of migrating cells in nine random fields of view in three independent experiments. In the presence of TGF-β1, MCF10AΔNcad cells were significantly less motile than parental or control knockdown cells.

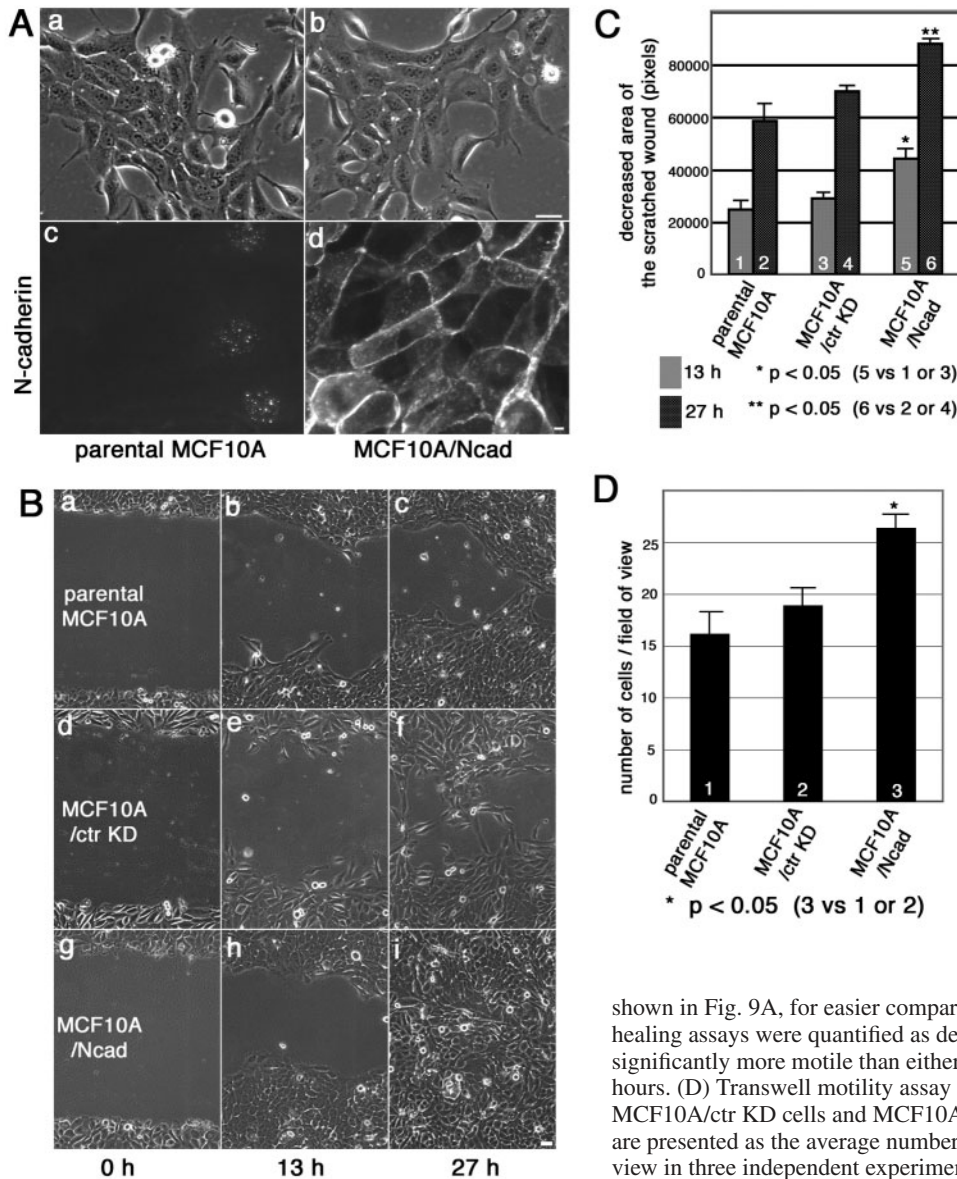


Fig. 10. N-Cadherin overexpression promoted motility in MCF10A cells. (A) Phase-contrast micrographs of living cells (a,b; 10 \times objective; scale bar, 50 μ m) and N-cadherin immunostaining micrographs (c,d; 40 \times objective; scale bar, 10 μ m) of parental MCF10A cells (a,c) or MCF10A cells overexpressing 2 \times -Myc-tagged human N-cadherin (MCF10A/Ncad; b,d). (B) Phase-contrast micrographs of living cultures of parental MCF10A cells (a-c), MCF10A/ctr KD cells (d-f) or MCF10A/Ncad cells (g-i) in a wound-healing assay performed in the absence of TGF- β 1. Photographs were taken immediately after incision (0 hour; a,d,g) and at 13 hours (b,e,h) or 27 hours (c,f,i) after incision using a 10 \times objective; scale bar, 50 μ m. Some of these panels are the same as those shown in Fig. 9A, for easier comparison to MCF10A/Ncad cells. (C) Wound-healing assays were quantified as described in Fig. 9. MCF10A/Ncad cells were significantly more motile than either control cell line at 13 hours and at 27 hours. (D) Transwell motility assay comparing parental MCF10A cells, MCF10A/ctr KD cells and MCF10A/Ncad cells in the absence of TGF- β 1. Data are presented as the average number of migrating cells in nine random fields of view in three independent experiments.

TGF- β 1-mediated motility in the MCF10A Δ Ncad cell line to that in control knockdown cells and parental MCF10A cells. A wound-healing assay (Fig. 9A) was quantified by measuring the decreased wound area at 5 hours and 13 hours after scratching (Fig. 9B). This assay showed that parental MCF10A cells and MCF10A/ctr KD cells responded to TGF- β 1 by filling the wound within 13 hours. By contrast, MCF10A Δ Ncad cells treated with TGF- β 1 were significantly retarded in their ability to migrate into the denuded area. A transwell motility assay showed similar results (Fig. 9C). TGF- β 1 treatment increased the motility of parental MCF10A cells and MCF10A/ctr KD cells 7.3-fold and 5.1-fold, respectively. Untreated MCF10A Δ Ncad cells showed slightly lower motility than untreated parental MCF10A cells or untreated MCF10A/ctr KD cells. TGF- β 1-treated MCF10A Δ Ncad cells were more motile than untreated MCF10A Δ Ncad cells, but TGF- β 1-treated MCF10A Δ Ncad cells were significantly less motile than TGF- β 1-treated parental MCF10A cells or TGF- β 1-treated MCF10A/ctr KD cells. That is, TGF- β 1-induced

motility in MCF10A Δ Ncad cells was 2.9-fold rather than the 7.3-fold seen in the parental MCF10A cells. These data indicate that upregulation of N-cadherin contributes to TGF- β 1-induced motility in MCF10A cells. It is likely that changes in the cytoskeleton and in focal contacts also contribute to the increased motility that is associated with EMT and it is these changes that mediate the differences we see between TGF- β 1-treated and untreated MCF10A Δ Ncad cells.

The above data suggest that upregulation of N-cadherin expression upon induction of EMT plays a significant role in the increased motility of MCF10A cells. However, it is also clear that TGF- β 1-induced mechanisms other than upregulation of N-cadherin also contribute to increased motility. To investigate the role that N-cadherin plays in motility in these cells without the complication of TGF- β 1 treatment, we compared motility in MCF10A/Ncad cells with that of control cells and parental cells in both the scratch assay and the transwell assay in the absence of TGF- β 1. Fig. 10A shows that MCF10A/Ncad cells have a morphology similar to

parental MCF10A cells (Fig. 10A,a,b) and that they express N-cadherin in the absence of TGF- β 1 and localize it to cell-cell interfaces, as expected (Fig. 10Ad). In a scratch assay, MCF10A/Ncad cells filled the denuded area significantly more efficiently than did the parental cells or the transfection controls, and this is especially apparent at 27 hours (Fig. 10B,C). In addition, the MCF10A/Ncad cells were significantly more motile in a transwell assay than were the control cells (Fig. 10D). Thus, it is clear that N-cadherin contributes to motility in MCF10A cells and that its induction is essential for the increased motility that is an important component of EMT in these cells.

Discussion

Heterogeneity in NMuMG cells

We established two subclones of mouse mammary NMuMG cells, one with an epithelial morphology (NMuMG/E9) and the other with a fibroblastic morphology (NMuMG/E11). It is likely that NMuMG/E9 cells are representative of the original parental NMuMG cells, whereas NMuMG/E11 cells have been converted to a more mesenchymal phenotype. The NMuMG/E11 cells still express a very small amount of E-cadherin, consistent with their epithelial origin. NMuMG/E11 cells grew much faster, were more motile and were more resistant to TGF- β 1-induced apoptosis than were NMuMG/E9 cells (data not shown). The heterogeneity in parental NMuMG cells might contribute to some of the inconsistencies we see in the literature concerning EMT in this cell line. Van den Broecke et al. characterized subclones of NMuMG cells and reported that the epithelioid clones expressed E-cadherin homogeneously and were not invasive whereas the fibroblastic clones were E-cadherin-negative and more invasive than the epithelioid clones (Van den Broecke et al., 1996). NMuMG/E9 cells and NMuMG/E11 cells are similar to the clones in this earlier report. Interestingly NMuMG/E11 cells express more *snail* mRNA than NMuMG/E9 cells (data not shown), which might contribute to downregulation of E-cadherin and conversion to the mesenchymal phenotype (Batlle et al., 2000). Assuming the original parental NMuMG cells were homogeneously epithelial when established, the changes that happen as cells convert from epithelial (as in the NMuMG/E9 clone) to fibroblastic (as in the NMuMG/E11 clone) is reminiscent of TGF- β 1-induced EMT in these cells. However, the phenotype of NMuMG/E11 is not reversible, as is authentic TGF- β 1-induced EMT (Fig. 5).

Cadherin switching during TGF- β 1-mediated EMT

Snail is a member of a superfamily of zinc-finger transcriptional factors and induces EMT in many experimental systems (Nieto, 2002). One report using NMuMG cells suggested that the downregulation of E-cadherin was not caused by snail but was rather mediated by SIP1 (Comijn et al., 2001). In our studies of NMuMG/E9 cells, both snail and SIP1 expression correlated with downregulation of E-cadherin. In our studies, downregulation of E-cadherin protein was much slower than the induction of *snail* or *SIP1* mRNA (Fig. 2); this discrepancy is still not understood, but it might be due to the stability of E-cadherin protein or mRNA. Another possible

explanation is that, for efficient negative regulation of E-cadherin, the cell needs to acquire a certain threshold level of snail or SIP1 to function effectively. Both snail and SIP1 have been reported to induce EMT when experimentally overexpressed (Batlle et al., 2000; Cano et al., 2000; Savagner et al., 1997). In these studies, expression levels of the transcriptional regulators might have been extraordinarily high. In a more physiological situation like the TGF- β 1 treatment shown in our study, the induction of snail or SIP1 is lower and it might take longer to achieve maximal repression. It remains to be determined whether snail or SIP1 induction alone is sufficient to explain TGF- β 1-induced morphological EMT. Upregulation of N-cadherin was also transcriptional but the transcriptional regulators that influence its expression have yet to be identified. Analysis of gastrulation in the *Drosophila* embryo showed that twist was an activator of N-cadherin (Oda et al., 1998), and overexpression of twist has been reported in gastric cancer cells that have abnormally high N-cadherin levels (Rosivatz et al., 2002). In addition, twist was recently shown to be upregulated in a mouse model for metastatic breast carcinoma and to be essential for the cells to metastasize from the breast to the lung (Yang et al., 2004). However, we did not see any correlation between TGF- β 1-induced upregulation of N-cadherin and twist production (data not shown).

Although cadherin switching is an integral component of EMT, upregulation of N-cadherin is not essential for all aspects of TGF- β -mediated morphological EMT. For example, TGF- β 1 treatment made the fibroblastic NMuMG/E11 cells more elongated, even though N-cadherin, which was already highly expressed, did not increase further (data not shown). Another interesting finding in this study is that cadherin switching is slow in some mammary epithelial cells (NMuMG) and much more rapid in others (MCF10A). Whether this is due to species differences or to other differences between these cell lines is not known.

Reversibility of EMT

TGF- β 1-induced EMT in NMuMG cells was fully reversible, including changes in cellular morphology, cell-cell junctions, the actin cytoskeleton and expression levels of cadherins, snail and SIP1, and mesenchymal markers such as fibronectin. The reversibility of EMT is clearly important in normal embryonic development. Some steps in cancer progression are EMT like but are often not reversible, because they are due to genetic alterations. However, there is evidence to support the hypothesis that, when cancer cells invade adjacent tissues, they undergo a reversible EMT. Cancer cells at the leading edge of invasion often lose E-cadherin expression. However, once the cells have reached their metastatic destination, they recover E-cadherin expression, which might provide an advantage for tumor survival in the new region (Brabletz et al., 2001).

What role does cadherin switching play in EMT?

Downregulation of E-cadherin in TGF- β 1-treated NMuMG/E9 cells was much slower than the initiation of morphological changes. E-Cadherin protein levels began to decrease 4 days after TGF- β 1 addition. By contrast, the morphological changes and stress fiber reorganization began 12 hours after TGF- β 1 addition and were prominent within 24 hours. Although the

amount of E-cadherin protein remained approximately the same during the first 24 hours, its staining pattern changed in concert with the morphological changes, suggesting that adherens junctions were being disturbed. Recent reports have pointed out the significance of the internalization of E-cadherin as one mechanism cells use to disrupt E-cadherin function (Ivanov et al., 2004; Palacios et al., 2001; Palacios et al., 2002; Paterson et al., 2003). However, in our studies, internalization of E-cadherin was not observed and comparable amounts of E-cadherin remained on the cell surface and were concentrated at cell-cell interfaces in TGF- β 1-treated NMuMG/E9 cells, suggesting that E-cadherin itself does not interfere with the process of EMT and that downregulation of E-cadherin is not essential for morphological EMT to occur. One possibility is that N-cadherin is a competitor of E-cadherin and that its relatively rapid induction after TGF- β 1 treatment suppresses E-cadherin function, allowing EMT to occur. However, E-cadherin remained in a complex with catenins even after morphological changes were evident, so it is unlikely that N-cadherin suppressed E-cadherin function.

N-Cadherin knockdown in NMuMG/E9 cells or in MCF10A cells did not interfere with TGF- β 1-mediated morphological changes including actin cytoskeleton reorganization. Thus, it appears that upregulation of N-cadherin is not essential for the morphological changes associated with EMT. Moreover, forced expression of N-cadherin in NMuMG/E9 cells did not induce any morphological changes (Fig. 6). In addition, forced expression of E-cadherin in the fibroblastic NMuMG/E11 cells did not convert them to an epithelial phenotype, nor did forced expression of E-cadherin in NMuMG/E9 cells interfere with TGF- β 1-induced morphological changes (data not shown). Taken together, these data suggest that, even though cadherin switching is a common feature of EMT, it is not the driving force behind the morphological changes and actin cytoskeleton reorganization.

Cadherin switching and cell confluence

Interestingly, MCF10A cell morphology is dependent on the confluence of the monolayer. That is, in sparse cultures the cells are fibroblastic but, in confluent cultures, they become compact, form tight cell-cell junctions and appear epithelial (Fig. 7A). Moreover, cadherin status is also dependent on cell confluence. As the culture becomes dense, E-cadherin levels increase and N-cadherin levels decrease. This relationship between cell morphology and cadherin status is reminiscent of that observed in TGF- β 1-mediated EMT and one could imagine that there is a common mechanism for EMT-associated cadherin switching and cell-density-associated cadherin switching. Recently, Conacci-Sorrell et al. (Conacci-Sorrell et al., 2002) showed that the transcription factor slug regulates E-cadherin expression in SW 480 colon-cancer cells in a cell-density-dependent manner. However, in our study, the degree of density-associated cadherin switching was not comparable to that of EMT-associated cadherin switching (data not shown).

N-Cadherin knockdown and motility

Our lab and others have reported that N-cadherin promotes motility when expressed by epithelial cells (Hazan et al., 2000;

Islam et al., 1996; Nieman et al., 1999), and increased motility is also an important characteristic of EMT. These observations prompted us to investigate the relationship between TGF- β 1-induced motility and cadherin switching. As is the case for other mammary epithelial cell lines, N-cadherin overexpression in MCF10A cells promoted motility in the absence of TGF- β 1 (Fig. 10). We used siRNAs to establish N-cadherin knockdown MCF10A cells (MCF10A Δ Ncad). These cells responded to TGF- β 1 by undergoing a morphological change and downregulating E-cadherin. However, they were prevented from upregulating N-cadherin in response to TGF- β 1 by the siRNA. Interestingly, there was a significant disruption in the ability of MCF10A Δ Ncad cells to become motile in response to TGF- β 1, suggesting that N-cadherin is at least partly responsible for the increase in motility that is an important component of EMT. This is in agreement with a recent study from the Mareel laboratory showing that N-cadherin plays a crucial role in TGF- β -stimulated invasion and wound-induced migration in myofibroblasts (De Wever et al., 2004). This study further showed that N-cadherin induction by TGF- β is mediated by Jun N-terminal kinase (JNK) activation.

In one study, increased motility associated with TGF- β -mediated EMT was reported to involve RhoA activation and the phosphoinositide-3-kinase pathway (Bhowmick et al., 2001). In another study, RhoA activation by TGF- β 1 was shown to require the activity of the guanine-nucleotide-exchange factor NET1 (Shen et al., 2001). The mechanism by which N-cadherin induction promotes motility has yet to be elucidated. We have recently shown that the closely related protein R-cadherin promotes motility by activating Rho-family small GTPases (Johnson et al., 2004) and are investigating the possibility that N-cadherin acts in a similar manner.

TGF- β 1-mediated EMT can be divided into at least two aspects: a morphological change including actin cytoskeleton reorganization and disruption of cell-cell junctions; and increased cell motility. These two aspects might be related to one another but the former is independent of cadherin switching, whereas the latter is modulated by cadherin switching. Here, we separate the roles cadherins play in these two distinct aspects of EMT and present the first evidence that N-cadherin plays a significant role in the increased cell motility that is an integral part of EMT. We conclude that cadherin switching is involved in TGF- β 1-mediated EMT and that upregulation of N-cadherin is indispensable for TGF- β 1-induced motility.

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