

Epithelial-Mesenchymal Transition Events during Human Embryonic Stem Cell Differentiation

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Abstract

Epithelial-mesenchymal transition (EMT) occurs during embryonic development and may also be associated with the metastatic spread of epithelial tumors. During EMT, E-cadherin is down-regulated and this correlates with increased motility and invasion of cells. We show that differentiation of human embryonic stem (ES) cells in monolayer culture is associated with an E- to N-cadherin switch, increased vimentin expression, up-regulation of E-cadherin repressor molecules (Snail and Slug proteins), and increased gelatinase (matrix metalloproteinases; MMP-2 and MMP-9) activity and cellular motility, all characteristic EMT events. The 5T4 oncofetal antigen, previously shown to be associated with early human ES cell differentiation, is also part of this process. Abrogation of E-cadherin-mediated cell-cell contact in undifferentiated ES cells using neutralizing antibody (nAb) SHE78.7 resulted in increased cellular motility, altered actin cytoskeleton arrangement and a mesenchymal phenotype together with presentation of the 5T4 antigen at the cell surface. nAb-treated ES cells remained in an undifferentiated state, as assessed by OCT-4 protein expression, and did not express EMT-associated transcripts. Removal of nAb from ES cells resulted in the restoration of cell-cell contact, absence of cell surface 5T4, decreased mesenchymal cellular morphology and motility, and enabled the differentiation of the cells to the three germ layers upon their removal from the fibroblast feeder layer. We conclude that E-cadherin functions in human ES cells to stabilize the cortical actin cytoskeletal arrangement and this prevents cell surface localization of the 5T4 antigen. Furthermore, human ES cells represent a useful model system with which to study EMT events relevant to embryonic development and tumor cell metastasis. [Cancer Res 2007;67(23):11254–62]

Introduction

Epithelial-mesenchymal transition (EMT) occurs during normal embryo development and might also be associated with tumor cell invasion and metastasis (1–3). A defining characteristic of EMT is the loss of cadherin-1 (E-cadherin)-mediated cell-cell contacts and the acquisition of a mesenchymal phenotype (1, 4). During mouse embryonic development, EMT is required for epiblast cells to

facilitate their ingression within the primitive streak, with loss of cell surface E-cadherin and gain of cadherin-2 (N-cadherin) observed in this process (1, 5). Similarly, metastasis of primary epithelial tumor cells, which is the main cause of death in cancer patients, is associated with loss of E-cadherin-mediated cell-cell contacts and gain of a mesenchymal phenotype, potentially facilitating cellular motility and invasion (1, 6–9). The role of E-cadherin as a metastasis repressor is well established (1, 3). For example, loss of E-cadherin in epithelial cells leads to the abrogation of cell-cell contact and increased motility (3, 10), with forced expression of wild-type E-cadherin protein in carcinoma cell lines sufficient for reversal of this phenotype (1, 3, 4, 11). In contrast to other tumor suppressors (e.g., p53), E-cadherin is rarely mutated in carcinomas (1, 12) and transcriptional inactivation of E-cadherin by E-box binding proteins (e.g., Snail and Slug) correlates with tumor cell metastasis (3, 13, 14). However, E-cadherin can also be regulated at the protein level, including proteolytic degradation of the cell surface protein (15) or its internalization via the c-met receptor pathway (16–18).

Although loss of cell surface E-cadherin is considered a prerequisite for EMT, many other cellular events are required to impart increased cellular motility and invasion. For example, altered matrix recognition and adhesion, up-regulation of promigratory molecules, and expression of extracellular proteases are critical components of EMT (1). Regulation of cell matrix recognition and adhesion is partly governed by the selection of proteins displayed at the plasma membrane (1). For example, N-cadherin is up-regulated on breast, prostate, bladder, thyroid, and squamous cell carcinomas and imparts migration and invasion of the cells (19). The matrix metalloproteinase (MMP) family, in particular MMP-2 and MMP-9, are known to influence invasion during development and metastasis (20–24). MMP family proteins degrade extracellular matrix and cell surface proteins, leading to the release of stored promigratory factors which aids the migration and invasion of cells within tissues. Thus, EMT is a complex event requiring the correct spatiotemporal expression, interaction, and modification of a range of cellular and extracellular factors to allow cellular motility and invasion to proceed.

Snail genes are associated with all EMT processes studied to date (25, 26) and they function by binding to the E-cadherin promoter leading to the repression of gene transcripts (1, 13, 27). However, Snail family members also function as potent survival factors (26), for example, both Snail and Slug can induce cell survival in the absence of EMT (26). In addition, it has been shown that Slug expression is associated with resistance to chemotherapeutic agents in mesotheliomas and could induce cell survival in leukemias. Slug, Snail, and SIP1 (another E-box binding protein) are also associated with the ingression of epiblast cells within the primitive streak during early development (28–30). Similarly, Slug

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protein expression is associated with chick epiblast ingression and its expression in neural crest cells is associated with their emergence from the neural tube (29). Slug is likely to be a key regulator of EMT in the developing embryo because its repression can block both the emergence of mesoderm from the primitive streak and EMT within the neural crest (29, 30).

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass/epiblast of preimplantation embryos (31–33). ES cells can be cultured in an undifferentiated state over extended passages *in vitro* and can be induced to differentiate into the three primary germ layers *in vitro* and *in vivo* (31–33). We have previously shown that spontaneous differentiation of mouse and human ES cells is associated with cell surface expression of the 5T4 oncofetal antigen (34, 35). The 5T4 antigen is a glycoprotein up-regulated in colorectal, gastric, and ovarian carcinomas and this expression correlates with poorer clinical outcome in these patients (36–39). Moreover, forced expression of h5T4 in mouse ES cells leads to loss of cell-cell contacts and, in epithelial cells, membranous E-cadherin is down-regulated and associated with alterations in the actin cytoskeleton (34, 40). Therefore, we hypothesized that upon ES cell differentiation, mechanisms associated with altered cellular motility and invasion would occur, with loss of E-cadherin critical for this event.

Materials and Methods

Human ES cell culture and differentiation. HES4 hES cells (31) were cultured as previously described (35). The medium was changed daily. Cells were passaged after 7 to 10 days by gently cutting and teasing the morphologically undifferentiated cells using a yellow pipette tip and transferring colony pieces to a fresh culture dish. For differentiation studies, colony pieces were transferred to gelatin-treated organ culture dishes in the absence of a fibroblast feeder layer in ES cell medium unless otherwise stated.

Antibody-induced loss of E-cadherin-mediated cell-cell contacts. E-cadherin-mediated cell-cell contacts were abrogated by culture of HES4 ES cells in 2 $\mu\text{g}/\text{mL}$ of mouse anti-E-cadherin SHE78.7 antibody (Invitrogen Corporation) for 24 h prior to experimental analysis. Mouse IgG_{2a} antibody was used as a control in all experiments (Invitrogen Corporation).

Immunofluorescent imaging of ES cells. Human ES cells were cultured on Nunc plastic slide flask chambers (Nalge, Nunc International) coated with feeders and grown for various times. Cells were prepared as previously described (35). Primary antibodies were as follows: mouse anti-human 5T4 (1 $\mu\text{g}/\text{mL}$; ref. 41); rabbit anti-OCT-4 (1:100 dilution; Santa Cruz Biotechnology); mouse anti-human E-cadherin (clone SHE78.7; 1:100 dilution; Invitrogen), rabbit anti-N-cadherin (1:100 dilution; Santa Cruz Biotechnology); goat anti-Slug (1:100 dilution; Santa Cruz Biotechnology); rabbit anti-Snail (1:100 dilution; Santa Cruz Biotechnology) and anti-E12/E47 (1:100 dilution; Santa Cruz Biotechnology). Actin cytoskeleton was detected using phalloidin-Texas red conjugate (1:1,000 dilution; Sigma). The cells were viewed on an Olympus BX51 fluorescence microscope and/or a Zeiss Laser Scanning Confocal Microscope. Images were overlaid using Adobe Photoshop version 6.0 where described.

Fluorescent flow cytometry analysis of ES cells. Human ES cells were treated with dissociation buffer (Invitrogen), washed once in 900 μL of PBS and resuspended in 100 μL of 0.2% bovine serum albumin in PBS (fluorescence-activated cell sorting buffer) containing the primary antibody. Primary antibodies were as follows: mouse anti-human E-cadherin (1:100 dilution; Invitrogen); rabbit anti-N-cadherin (1:100 dilution, Santa Cruz Biotechnology), mouse anti-human 5T4 (1 $\mu\text{g}/\text{mL}$; ref. 41) and incubated for 1 h on ice. Cells were washed once in 900 μL of PBS, resuspended in 100 μL of fluorescence-activated cell sorting buffer containing appropriate phycoerythrin-conjugated secondary antibody (all 1:100 dilution; Santa Cruz Biotechnology), and incubated for 30 min on ice. The cells were washed once in 900 μL of PBS and fixed in 400 μL of 1% formaldehyde. Cell

fluorescence was analyzed using a Becton Dickinson FACScalibur. Viable cells were gated using forward and side scatter and the data represent cells from this population.

Reverse transcription-PCR. Total RNA was extracted from cells using RNazol B according to the manufacturer's instructions (Biogenesis) and synthesis of cDNA from mRNA was performed as previously described (34). Reverse transcription-PCR (RT-PCR) was done using 1 μL of the cDNA solution for 45 cycles. Samples were run on 2% agarose gels containing 400 ng/mL of ethidium bromide and visualized using an Epi Chemi II Darkroom and Sensicam imager with Labworks 4 software (UVP). Primers used were as follows (read 5' to 3'; forward, F; reverse, R; all at 60°C annealing): β -Tub—F, GGAACATAGCCGTAAGTGC; R, TCACTGTGCCTGAACTTACC, 317 bp; OCT-4—F, AGAAGGAGCTAGAACAGTTTGC; R, CCGTTACAGAACCATACTCG, 415 bp; E-cadherin—F, TCGACACCCGATTCAAAGTGG; R, TTCCA-GAAACGGAGGCCTGAT, 194 bp; N-cadherin—F, CCGACGAATGGATGAAA-GACC; R, TTGCAGCCTATGCCAAAGC, 438 bp; Snail—F, TATGCTGCCTCC-CAGGCTTG; R, ATGTGCATCTTGAGGGCACC, 145 bp; Slug—F, TCGGACC-CACACATTACC; R, TCTCTCAATCTAGCCATCAGC, 528 bp; SIP1—F, ACACACAGGAAAAAGACC; R, ATGAACAGCTTAACACAGC, 671 bp; E12/E47—F, ATCCACACCTCACACACC; R, GAACAGCAGCAGAAATAGC, 596 bp; MMP-2—F, AGATCTTCTTCTCAAGGACCGGTT; R, GGCTGGTCAGTGGC-TTGGGGTA, 225 bp; MMP-9—F, GCGGAGATTGGGAACCAGCTGTA; R, GACGGCCTGTGTACACCCACA, 208 bp; tissue inhibitors of metalloproteinase-1 (TIMP-1)—F, ACCATGGCCCCCTTGAGCCCCCTG; R, TCAGGC-TATCTGGGACCCGAGGA, 627 bp; and TIMP-2—F, CTCGGCAGTGTGTGGGGTC; R, CGAGAACTCCTGCTGGGG, 364 bp.

Western blotting. Cells were trypsinized, washed twice in PBS and resuspended in lysis buffer (2×10^7 cells/mL in 0.5 mol/L Tris, 1.5 mol/L NaCl, 0.5% v/v NP40, and 0.2 mmol/L phenylmethylsulfonyl fluoride) on ice for 20 min. SDS-PAGE and Western blotting was performed as previously described (42). Primary antibodies were as follows: rat anti-mouse E-cadherin (1:200 dilution, DECM-1; Sigma), anti-N-cadherin (1:200 dilution), anti-Slug (1:200 dilution), anti-Snail (1:200 dilution), and anti-E2A (1:200 dilution; all from Santa Cruz Biotechnology). The membrane was probed using horseradish peroxidase-conjugated immunoglobulins (DAKO) as described previously (42). Western blot images were captured using an Epi Chemi II Darkroom and Sensicam imager with Labworks 4 software (UVP).

Zymogram analysis. HES4 human ES cells were cultured in synthetic serum-containing medium in the presence of an irradiated fibroblast feeder layer (undifferentiated) or differentiated in the absence of a feeder layer on gelatin-treated plates for various times in ES cell medium or N2B27 medium (43). Protease activity was determined using a precast 10% gelatin gel (Bio-Rad) and visualized according to the manufacturer's instructions as previously described (42). Images were captured using an Epi Chemi II Darkroom and Sensicam imager with Labworks 4 software (UVP).

Cell motility assay. Costar Transwell 24-well plates exhibiting 5- μm pore size were used for all motility assays. The transwells were immersed overnight in gelatin solution (0.1% in PBS) and rinsed in PBS. Transwells were blocked in ES cell medium for 30 min at 37°C/5% CO₂ and washed in PBS. Differentiated ES cells were cultured on gelatin-treated plates for 10 days in the absence of a feeder layer. The cells were transferred to the transwell plates and incubated overnight at 37°C/5% CO₂. The transwell was washed gently in PBS and cells were removed from within the transwell using a dry cotton bud followed by two washes in PBS. This procedure was repeated twice. The transwell was stained with crystal violet for 10 min, washed in water and allowed to air-dry. Cells present on the underside of the transwell (i.e., migrated cells) were counted by microscopy. The number of cells on the bottom of the plate (i.e., cells which had migrated through the pores and had become detached from the transwell) were also assessed (none were observed). Error bars show the SE of all samples.

Results

Human ES cell differentiation is associated with a membranous E- to N-cadherin switch. E- and N-cadherin protein expression was assessed in undifferentiated and differentiating

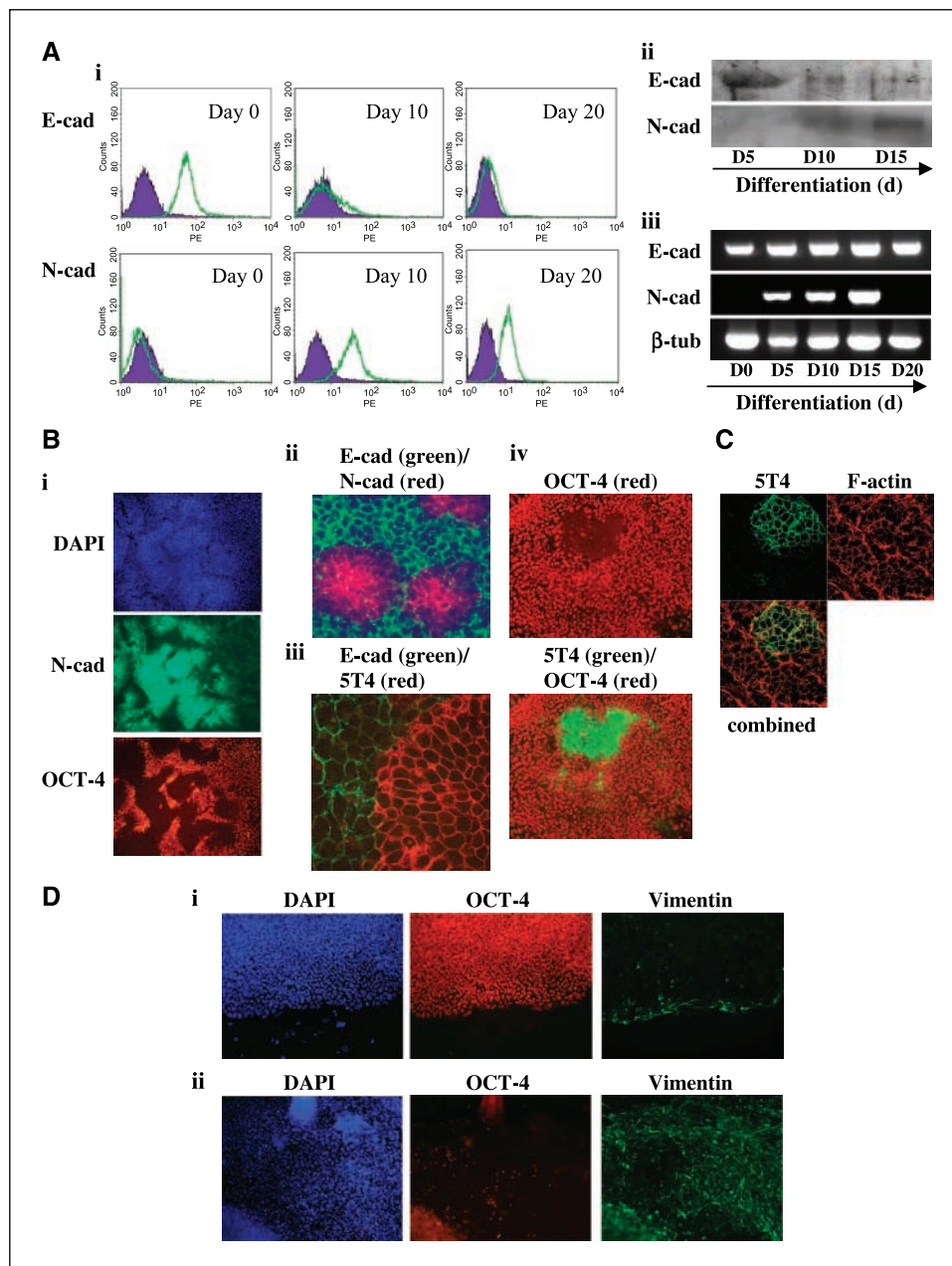


Figure 1. Human ES cell differentiation is associated with loss of cell surface E-cadherin and gain of cell surface N-cadherin and 5T4 proteins. HES4 ES cells were maintained in an undifferentiated state by culture in ES cell medium in the presence of a fibroblast feeder layer, and were differentiated in monolayer culture by transfer of the cells to gelatin-treated plates, as described previously (35). **A**, (i) cell surface E-cadherin (*E-cad*) and N-cadherin (*N-cad*) proteins were assessed in undifferentiated HES4 ES cells (day 0) and cells differentiated for 10 and 20 d by fluorescent flow cytometry in a Becton Dickinson FACScalibur. E-Cadherin or N-cadherin (*open population*); isotype cAbs (*closed population*). Viable cells were gated using forward and side scatter and the data represents cells from this population. (ii) Western blot analysis was done to assess total cellular E-cadherin (*E-cad*) or N-cadherin (*N-cad*) proteins in HES4 ES cells differentiated for 5, 10, and 15 d (as described above). (iii) RT-PCR analysis of E-cadherin (*E-cad*), N-cadherin (*N-cad*), and β -tubulin (β -*tub*—control) transcript expression was assessed in undifferentiated HES4 ES cells (day 0) and in cells differentiated for 5, 10, 15, and 20 d as described above. **B**, (i) immunofluorescent microscopy analysis of N-cadherin (*N-cad*) and OCT-4 protein expression in HES4 ES cells cultured in ES cell medium for 12 d to promote spontaneous differentiation. Note that N-cadherin expression correlates with the absence of the pluripotent marker OCT-4. (ii) HES4 ES cells were differentiated as described in **B** (i) and assessed for E-cadherin (*green*) and N-cadherin (*red*) expression using fluorescent microscopy. Note discrete areas of N-cadherin protein expression in the absence of E-cadherin. (iii) Confocal microscopy image of HES4 cells cultured as described in **B** (i) and assessed for expression of E-cadherin protein (*green*) and 5T4 antigen (*red*) showing discrete cell surface expression of these two proteins. Note that very low levels of 5T4 antigen were detected in the cytoplasm of E-cadherin-positive cells. (iv) HES4 ES cells were differentiated as described in **B** (i) and assessed for 5T4 antigen (*green*) and OCT-4 protein (*red*) expression using fluorescent microscopy. Note that expression of 5T4 protein was associated with loss of the pluripotent marker OCT-4. **C**, confocal microscopy analysis of HES4 cells cultured as described in **B** (i) and assessed for filamentous actin (*red*) and 5T4 antigen (*green*). Note the discrete cell surface expression of 5T4 in differentiated cells. **D**, (i) immunofluorescent microscopy analysis of vimentin (*green*) and OCT-4 protein (*red*) expression in undifferentiated HES4 ES cells. Note that OCT-4-positive cells exhibit little expression of vimentin. (ii) Immunofluorescent microscopy analysis of vimentin and OCT-4 protein expression in HES4 ES cells cultured in ES cell medium for 12 d to promote spontaneous differentiation. Note that ES cell differentiation is associated with increased expression of vimentin and loss of OCT-4.

HES4 cells using fluorescent flow cytometry analysis (Fig. 1A, *i*). Undifferentiated HES4 ES cells cultured on irradiated embryonic fibroblasts (feeder layer) expressed cell surface E-cadherin on >95% of the population (Fig. 1A, *i*; E-cad, day 0), whereas N-cadherin was absent from the majority of the cells (Fig. 1A, *i*; N-cad, day 0). At day 10 following differentiation of the cells by their removal from the feeder layer, a significant proportion of the population (>90%) lacked cell surface E-cadherin (Fig. 1A, *i*; E-cad, day 10) and had gained N-cadherin protein expression (Fig. 1A, *i*; N-cad, day 10). Twenty days following the induction of differentiation, a significant proportion of the cells exhibited low levels of cell surface E-cadherin protein (Fig. 1A, *i*; E-cad, day 20) with N-cadherin protein expression detected in >95% of the population (Fig. 1A, *i*; N-cad, day 20). Determination of E-cadherin and N-cadherin proteins was also assessed by Western blot analysis of total cell lysates (Fig. 1A, *ii*). Total N-cadherin protein was absent from ES cells differentiated for 5 days and subsequently detected at 10 and 15 days following differentiation of the cells (Fig. 1A, *ii*; N-cad). In contrast, E-cadherin protein was detected in HES4 ES cells differentiated for 5 days, whereas lower levels were observed at days 10 and 15 following induction of differentiation (Fig. 1A, *ii*; E-cad). Analysis of transcript expression in the undifferentiated and differentiating HES4 population was assessed by RT-PCR analysis (Fig. 1A, *iii*). N-cadherin transcripts were absent from undifferentiated ES cells (day 0) and rapidly up-regulated upon differentiation of the cells (day 5), with levels peaking at day 15 and absent at day 20. In contrast, E-cadherin transcripts were detected in undifferentiated ES cells (day 0) and throughout the differentiation time points analyzed (days 5–20).

HES4 ES cells were cultured for 12 days on a feeder layer to induce spontaneous differentiation and assessed for expression of N-cadherin (*green*) and OCT-4 (*red*) proteins by immunofluorescent microscopy (Fig. 1B, *i*). OCT-4 protein was absent from a proportion of the cells within the colony, showing that spontaneous differentiation had occurred, with N-cadherin protein expression correlating with loss of OCT-4. Examination of E-cadherin (*green*) and N-cadherin (*red*) proteins under these culture conditions showed discrete expression of the two proteins (Fig. 1B, *ii*), showing that loss of cell surface E-cadherin is associated with gain of cell surface N-cadherin. Confocal analysis of E-cadherin (*green*) and 5T4 antigen (*red*) showed discrete expression of the two proteins (Fig. 1B, *iii*), similar to that observed for E-cadherin and N-cadherin. Low levels of 5T4 antigen were detected in the cytoplasm of E-cadherin-positive cells (Fig. 1B, *iii*), similar to that reported for mouse ES cells (42, 44). Furthermore, determination of 5T4 (*green*) and OCT-4 (*red*) proteins in spontaneously differentiating HES4 ES cells showed that 5T4 expression correlated with loss of OCT-4 (Fig. 1B, *iv*). Therefore, HES4 ES cell differentiation is associated with loss of E-cadherin and expression of both N-cadherin and 5T4 proteins.

To assess changes in the actin cytoskeleton upon spontaneous differentiation of hES cells, we assessed 5T4 expression and filamentous actin arrangement in HES4 ES cells using confocal microscopy (Fig. 1C). Although discrete areas of 5T4 expression (*green*) were observed, this did not seem to be associated with altered actin cytoskeleton arrangement (*red*) under these conditions. To assess the likelihood of an EMT event occurring during hES cell differentiation, we assessed the expression of vimentin, a specific subunit of intermediate filaments in mesenchymal tissue. Undifferentiated HES4 ES cells exhibited low levels of vimentin expression at the periphery of the colonies (Fig. 1D, *i*). In contrast,

differentiating HES4 ES cells exhibited loss of OCT-4 protein, and this was associated with increased expression of vimentin (Fig. 1D, *ii*), suggesting that an EMT event occurs during this process.

ES cell differentiation is associated with up-regulation of E-cadherin repressor proteins. To determine whether down-regulation of cell surface E-cadherin protein upon ES cell differentiation was associated with the expression of E-cadherin transcript repressors, we assessed the expression of Snail, Slug, and E12/E47 transcripts using RT-PCR (Fig. 2A). Undifferentiated HES4 ES cells lacked transcripts encoding Slug and exhibited low levels of Snail transcripts (Fig. 2A; day 0). Upon differentiation of HES4 ES cells in the absence of a feeder layer, both Snail and Slug transcripts were increased, with significantly lower levels of the latter detected at day 20 (Fig. 2A; days 5–20). E12/E47 transcripts were expressed at similar levels in both undifferentiated and differentiating HES4 ES cells. Western blot analysis of Snail, Slug, and E12/E47 proteins in whole cell lysates (Fig. 2B) showed the absence of Slug and low levels of Snail proteins in undifferentiated HES4 ES cells (day 0). Following induction of differentiation, both Snail and Slug proteins were significantly increased (day 15). E12/E47 protein was detected at similar levels in both undifferentiated and differentiated HES4 ES cells (Fig. 2B). Nuclear localization of Snail (Fig. 2C, *i*) and Slug (Fig. 2C, *ii*) proteins were observed in a proportion of cells within

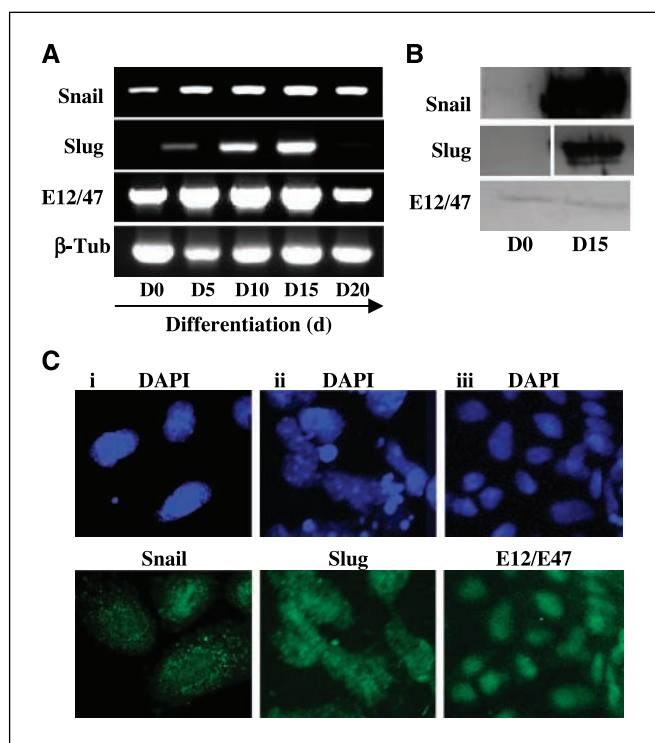


Figure 2. Human ES cell differentiation is associated with the expression of Snail and Slug proteins. HES4 ES cells were maintained in an undifferentiated state by culture in ES cell medium in the presence of a fibroblast feeder layer and differentiated in monolayer culture by transfer of the cells to gelatin-treated plates. A, transcript expression of Snail, Slug, E12/E47, and β -tubulin (β -tub—control) was determined by RT-PCR in undifferentiated HES4 ES cells (day 0) and in cells differentiated for 5, 10, 15, and 20 d. B, HES4 ES cells were differentiated for 15 d and total cell lysates were assessed for Snail, Slug, and E12/E47 proteins by Western blot analysis. C, HES4 ES cells were differentiated for 10 d and assessed for (i) Snail, (ii) Slug, and (iii) E12/E47 proteins using immunofluorescent microscopy. 4',6-Diamidino-2-phenylindole (DAPI) shows the total cells within the field of view.

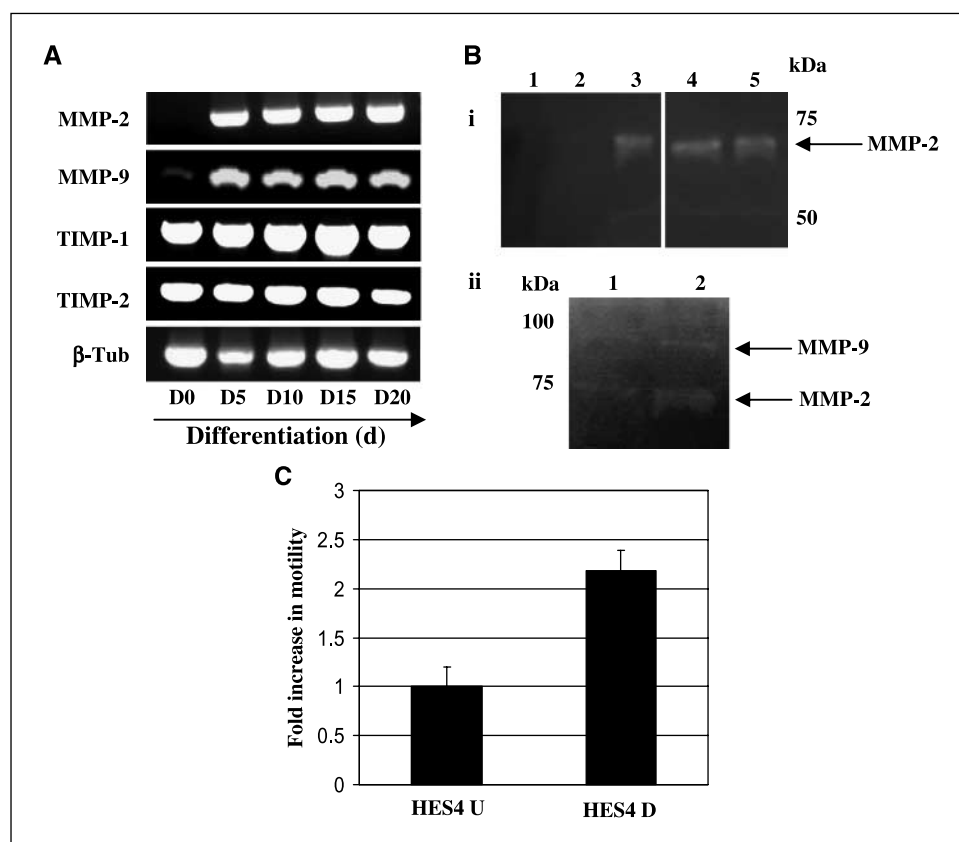


Figure 3. Human ES cell differentiation is associated with MMP activity and increased cellular motility. HES4 ES cells were maintained in an undifferentiated state by culture in ES cell medium in the presence of a fibroblast feeder layer and differentiated in monolayer culture by transfer of the cells to gelatin-treated plates. *A*, transcript expression of MMP-2, MMP-9, TIMP-1, TIMP-2, and β -tubulin (β -tub—control) was determined by RT-PCR in undifferentiated HES4 ES cells (day 0) and in cells differentiated for 5, 10, 15, and 20 d. *B*, (i) gelatin zymogram analysis was performed to determine MMP-2 and MMP-9 activity within the culture supernatants of undifferentiated and differentiating HES4 ES cells. *Lane 1*, ES cell medium alone; *lane 2*, supernatant from undifferentiated HES4 ES cells; *lanes 3 to 5*, supernatant from HES4 ES cells differentiated for 5, 10, and 15 d, respectively. *Arrow*, size of the active MMP-2 (65 kDa). (ii) Gelatin zymogram analysis in HES4 ES cells differentiated for 5 d (*lane 1*) and 10 d (*lane 2*) in neurobasal N2B27 medium. *Arrow*, size of the active MMP-2 (65 kDa) and MMP-9 (92 kDa). *C*, cellular motility of undifferentiated (HES4 U) and differentiating (10 d; HES4 D) HES4 ES cells was assessed using Costar Transwell 5 μ m pore size plates. Data represents the fold change in motility compared with undifferentiated cells. Note that differentiated HES4 ES cells exhibit increased motility compared with undifferentiated cells.

the differentiating HES4 population. In contrast, E12/E47 protein was detected in the nucleus of the majority of the population of both undifferentiated (data not shown) and differentiating HES4 cells (Fig. 2C, *iii*). Up-regulation of SIP1 transcripts were also detected following differentiation of the cells (data not shown). Overall, these results show that during ES cell differentiation, transcripts and proteins encoding known E-cadherin repressor molecules are associated with a cell surface E- to N-cadherin protein switch.

ES cell differentiation is associated with MMP proteolytic activity and increased cellular motility. To determine whether other EMT-like processes were occurring during ES cell differentiation, we assessed the expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 transcripts in HES4 ES cells (Fig. 3A). Undifferentiated HES4 ES cells lacked MMP-2 transcripts and exhibited low levels of MMP-9 transcripts (Fig. 3A; day 0). Following the induction of differentiation of the cells in the absence of a feeder layer, both MMP-2 and MMP-9 transcripts were up-regulated and detected for up to 20 days (Fig. 3A; days 5–20). TIMP-1 and TIMP-2 transcripts were detected at similar levels throughout the differentiation period. Zymogram analysis of gelatinase activity showed that undifferentiated HES4 cells lacked MMP-2 and MMP-9 activity (Fig. 3B, *i*, *lane 2*; *lane 1* shows media without ES cells). Induction of differentiation by removal of HES4 ES cells from a fibroblast feeder layer for 5, 10, and 15 days resulted in proteolytic activity associated with the 65 kDa active form of MMP-2 (Fig. 3B, *i*, *lanes 3, 4, and 5*, respectively). Although MMP-9 proteolytic activity was not detected following differentiation of HES4 ES cells in the absence of a feeder layer in ES cell medium, both MMP-2 and MMP-9 were detected following differentiation of HES4 ES cells in

the neurobasal medium N2B27 [Fig. 3B, *ii*; day 5 (*lane 1*) and day 10 (*lane 2*)]. To further exemplify the EMT event during human ES cell differentiation, we assessed the cellular migration of undifferentiated and differentiated (10 days) HES4 ES cells using 5- μ m pore size Transwell plates (Fig. 3C). ES cells differentiated for 10 days in the absence of a fibroblast feeder layer exhibited >2-fold increased motility compared with undifferentiated cells.

Loss of E-cadherin-mediated cell-cell contact in ES cells induces reversible actin cytoskeleton rearrangement in the absence of EMT-associated transcript expression. To determine whether loss of E-cadherin in HES4 ES cells could induce EMT-associated events, we treated the cells with the E-cadherin neutralizing antibody (nAb) SHE78.7 and assessed actin cytoskeleton arrangement and EMT-associated transcript expression (Fig. 4). Control antibody (cAb)-treated cells maintained cell-cell contact (Fig. 4A, *i*; cAb) and cortical actin cytoskeleton arrangement (Fig. 4A, *ii*; cAb). In contrast, treatment of HES4 ES cells with nAb promoted loss of cell-cell contacts (Fig. 4A, *i*; nAb) and polarized actin cytoskeleton arrangement (Fig. 4A, *ii*; nAb). Abrogation of E-cadherin cell-cell contacts using nAb resulted in loss of E-cadherin protein (data not shown), but the majority of the cells maintained the expression of OCT-4 (Fig. 4B), showing that inhibition of E-cadherin-mediated cell-cell contact does not induce differentiation of the cells. RT-PCR analysis of EMT-associated transcripts in cAb- and nAb-treated HES4 ES cells showed no difference between the two treatments (Fig. 4C), showing that loss of E-cadherin alone is insufficient to induce a full EMT event in ES cells. Removal of SHE78.7 from HES4 ES cells restored both cell-cell contact and cortical actin cytoskeleton arrangement within 7 days (Fig. 4D, *i* and *ii*, respectively) and OCT-4 protein was expressed in

the majority of the population (Fig. 4*D*, *iii*). To determine the differentiation potential of HES4 ES cells treated with nAb, we cultured the cells in nAb for 7 days followed by removal of nAb and overgrowth of the cells for 28 days in ES cell medium (Fig. 4*D*, *iv*). HES4 ES cells treated with nAb for 7 days prior to differentiation exhibited a similar transcript expression profile to HES4 ES cells cultured under normal conditions (Fig. 4*D*, *iv*; day 0; ref. 35). By contrast, removal of nAb and overgrowth of the cells resulted in loss of OCT-4 transcripts and up-regulation of markers representative of the three germ layers and extra embryonic endoderm (Fig. 4*D*, *iv*; day 28). These observations show that inhibition of E-cadherin-mediated cell-cell contacts in HES4 ES cells does not induce the differentiation of the cells or up-regulation of EMT-associated transcripts. Furthermore, the effect of nAb-treatment of HES4 ES cells is reversible, allowing subsequent differentiation of the cells under appropriate conditions.

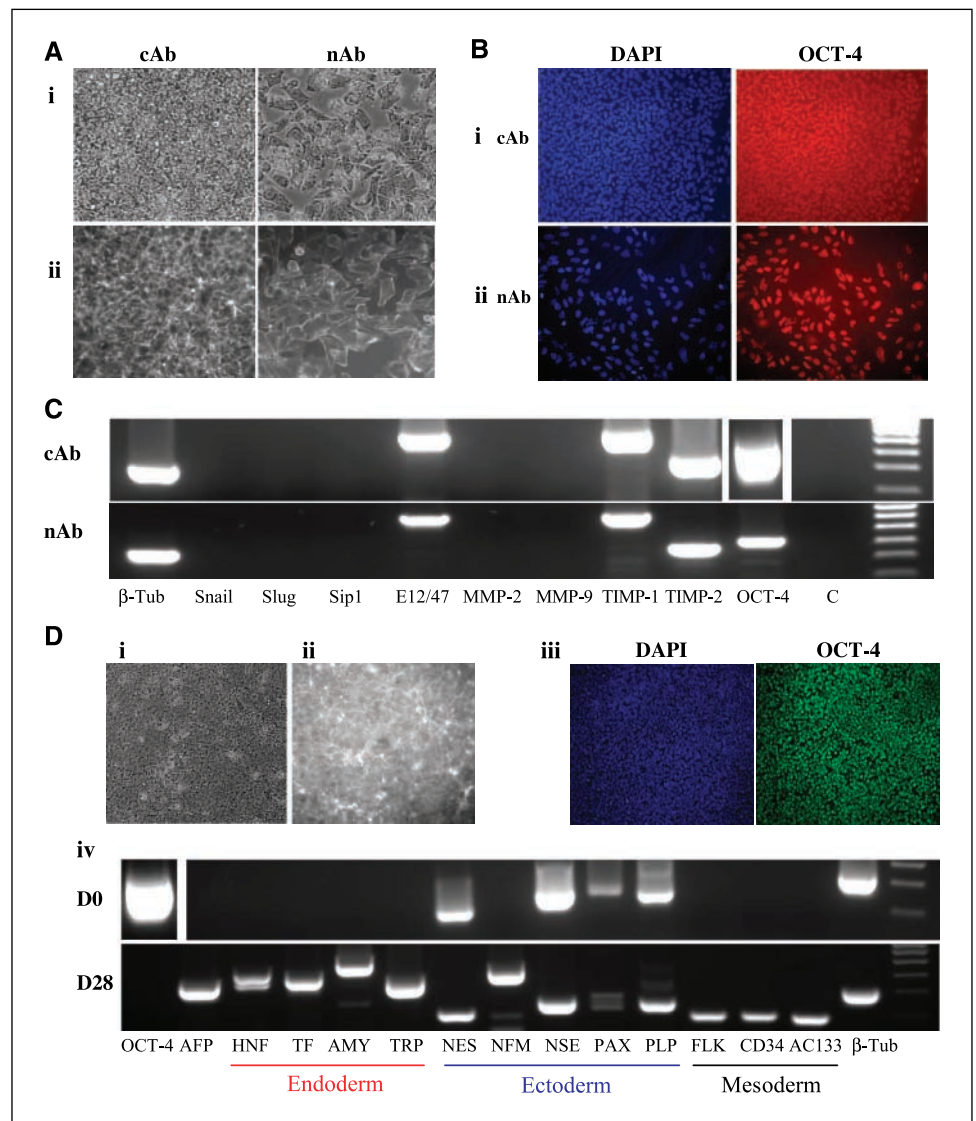
Inhibition of E-cadherin-mediated cell-cell contacts increases cellular motility but decreases proliferation of HES4 ES cells. To determine whether loss of E-cadherin in HES4 ES cells affected cellular motility, we assessed cAb- and nAb-treated HES4 ES cells in a transwell assay (Fig. 5*A*). cAb-treated HES4 ES

cells exhibited low motility, similar to that observed in undifferentiated cells (Fig. 5*A*; HES4 +cAb and HES4 U, respectively). In contrast, nAb-treated HES4 ES cells (HES4 +nAb) exhibited 2.5-fold increased motility compared with cAb-treated (HES4 +cAb) ES cells. Removal of nAb from HES4 ES cells for 5 days (HES4 -nAb d5) resulted in decreased motility that was not significantly altered from that observed in either undifferentiated (HES4 U) or cAb-treated (HES4 cAb) ES cells. Analysis of HES4 ES cell proliferation following treatment of the cells with cAb or nAb for 7 days showed significantly decreased cell numbers in the latter (Fig. 5*B*). Subsequent removal of nAb from the cells (Fig. 5*B*; nAb-) resulted in restoration of cellular proliferation that was similar to that observed in cAb-treated HES4 ES cells.

5T4 antigen is localized at the plasma membrane following inhibition of E-cadherin-mediated cell-cell contact in ES cells.

To determine whether loss of E-cadherin could induce changes in protein localization at the plasma membrane in HES4 ES cells, we treated the cells with either cAb or nAb for 8 h and assessed expression of the 5T4 oncofetal antigen using immunofluorescent microscopy (Fig. 6*A*). Inhibition of E-cadherin cell-cell contacts using nAb seemed to induce cell surface localization of 5T4

Figure 4. Loss of E-cadherin-mediated cell-cell contact in ES cells induces reversible actin cytoskeleton rearrangement in the absence of up-regulation of EMT-associated transcripts. **A**, HES4 ES cells cultured in ES cell medium in the presence of a fibroblast feeder layer were treated with either cAb or E-cadherin nAb SHE78.7 for 24 h and assessed for (i) cellular morphology using phase contrast microscopy and (ii) actin cytoskeleton arrangement using phycoerythrin-conjugated phalloidin and fluorescent microscopy. Note that SHE78.7-treated cells exhibit altered actin cytoskeleton arrangement and a mesenchymal-like morphology. **B**, immunofluorescent analysis of OCT-4 protein expression in HES4 ES cells treated with (i) cAb and (ii) nAb for 7 d. DAPI shows the total number of cells (nuclei) within the field of view. Note that loss of E-cadherin did not affect OCT-4 protein expression. **C**, RT-PCR analysis of EMT-associated transcripts in HES4 ES cells treated with either (i) cAb or (ii) nAb antibody for 7 d (C, control; β -tub, β -tubulin). Note that no significant differences were observed between cAb- and nAb-treated ES cells. **D**, HES4 ES cells were cultured for 7 d in nAb followed by removal of nAb for 2 d and assessed for (i) cellular morphology, (ii) actin cytoskeleton arrangement, and (iii) OCT-4 protein expression. DAPI shows the total number of cells (nuclei) within the field of view. (iv) HES4 ES cells were cultured for 7 d in nAb followed by removal of the antibody and differentiation of the cells induced by overgrowth for 28 d in ES cell medium. Note that the differentiated cells lacked OCT-4 transcript expression and exhibited up-regulation of transcripts associated with all three germ layers.



(Fig. 6A, ii), whereas cAb-treated cells exhibited significantly lower levels of the antigen (Fig. 6A i). To confirm that 5T4 was localized at the cell surface in nAb-treated HES4 ES cells, we assessed the expression of the antigen using fluorescent flow cytometry (Fig. 6B, i). Localization of the 5T4 antigen at the cell membrane was significantly increased following treatment of HES4 ES cells with nAb compared with cAb-treated cells. Removal of nAb from HES4 ES cells for 5 days resulted in decreased 5T4 expression at the cell surface similar to that observed in cAb-treated cells (Fig. 6B, ii). Therefore, abrogation of E-cadherin-mediated cell-cell contacts in HES4 ES cells results in reversible plasma membrane localization of the 5T4 antigen.

Discussion

The study of EMT events has provided insights into embryonic development and the metastatic spread of epithelial tumor cells. However, this research is impeded by the lack of a naturally regulated *in vitro* model system allowing the study of spatial and

temporal events associated with this process (7). In this study, we have shown that human ES cells represent a versatile model system with which to study EMT events in real-time using a monolayer differentiation assay. As proof-of-principle, we have shown a novel role for E-cadherin in preventing cell surface localization of the 5T4 oncofetal antigen in human ES cells. Recently, it has been shown that EMT occurs within individual colonies of primate and human ES cells (45, 46). In the human ES cell study (45), it was shown that EMT events were associated with the peripheral cells of the colony when cultured under feeder-free conditions in conditioned medium. Our method differs in that the cells are induced to differentiate by their removal from the fibroblast feeder layer and that EMT events are observed in the entire population of differentiating ES cells, as defined by loss of E-cadherin and gain of N-cadherin. Therefore, rather than EMT being associated with a subpopulation of differentiating cells, it seems to be a definitive step in human ES cell differentiation, at least under the conditions we describe. We have recently shown an EMT event during mouse ES cell differentiation (42), similar to that described in this manuscript. Together, these data confirm that ES cells are a useful *in vitro* model system with which to elucidate the mechanisms associated with EMT during human development and may be useful to model molecular and biochemical events that occur during metastasis.

The similarities observed between the invasion properties of placental trophoblast and tumor cells led to the identification of the 5T4 oncofetal antigen (41). Antibodies raised against syncytiotrophoblast plasma membrane glycoproteins resulted in the isolation of a monoclonal antibody recognizing a cell surface antigen, subsequently identified as 5T4 (41). 5T4 expression is associated with increased motility and decreased adhesion in epithelial cells. For example, forced expression of 5T4 cDNA in murine epithelial cells resulted in loss of cell-cell contacts, down-regulation of E-cadherin, and reorganization of the actin cytoskeleton (47). Furthermore, 5T4 antigen expression has been detected in various cancers and correlates with poorer clinical outcome in breast, colorectal, and ovarian carcinomas (36, 38, 41). Although E-cadherin expression has been shown to regulate cell surface localization of the EphA2 receptor (48), we are not aware of any reports correlating loss of E-cadherin with the presentation of promigratory factors at the plasma membrane of epithelial cells. Our data suggests that E-cadherin exhibits multiple functions in ES cells; maintaining cell-cell contact and cortical actin cytoskeleton arrangement as well as inhibiting plasma membrane localization of the 5T4 antigen.

The HES cell EMT model system allows the elucidation of the function of individual molecules within this process. For example, we show that loss of cell surface E-cadherin is sufficient to induce certain characteristics associated with a mesenchymal phenotype in the absence of up-regulation of EMT-associated transcripts. Therefore, although E-cadherin down-regulation plays a pivotal role in EMT, this event is unable to induce a full EMT phenotype in hES cells. We have recently shown that loss of E-cadherin in mouse ES cells also results in plasma membrane localization of the 5T4 antigen (42). Furthermore, neither E-cadherin down-regulation nor N-cadherin were required for the up-regulation of EMT-associated transcripts, suggesting that these proteins do not actively induce a full EMT event. However, both 5T4 and N-cadherin-null mouse ES cells exhibited significantly decreased motility following EMT, showing that these proteins act as promigratory factors during this process in ES cells. The similarities between our observations in

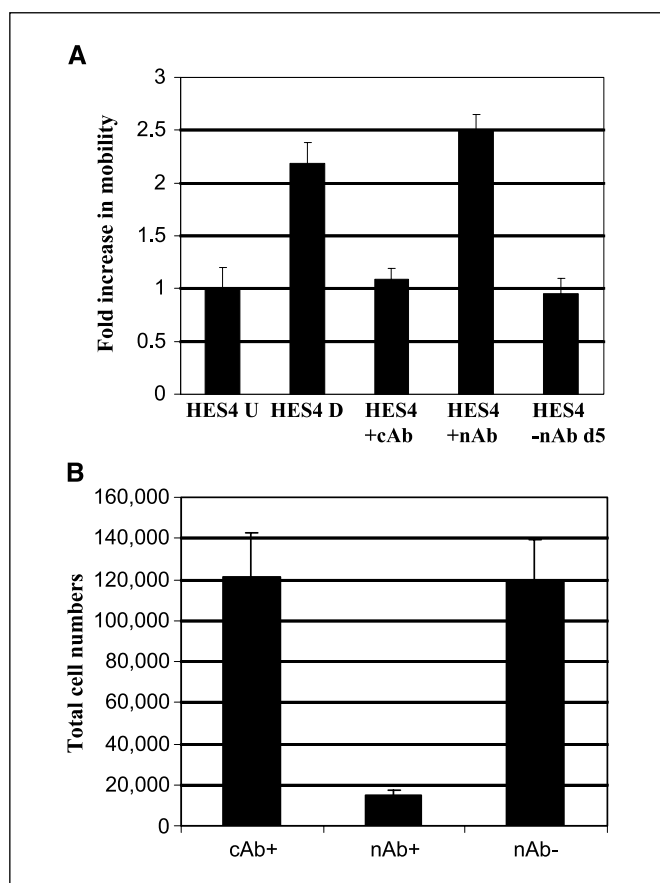
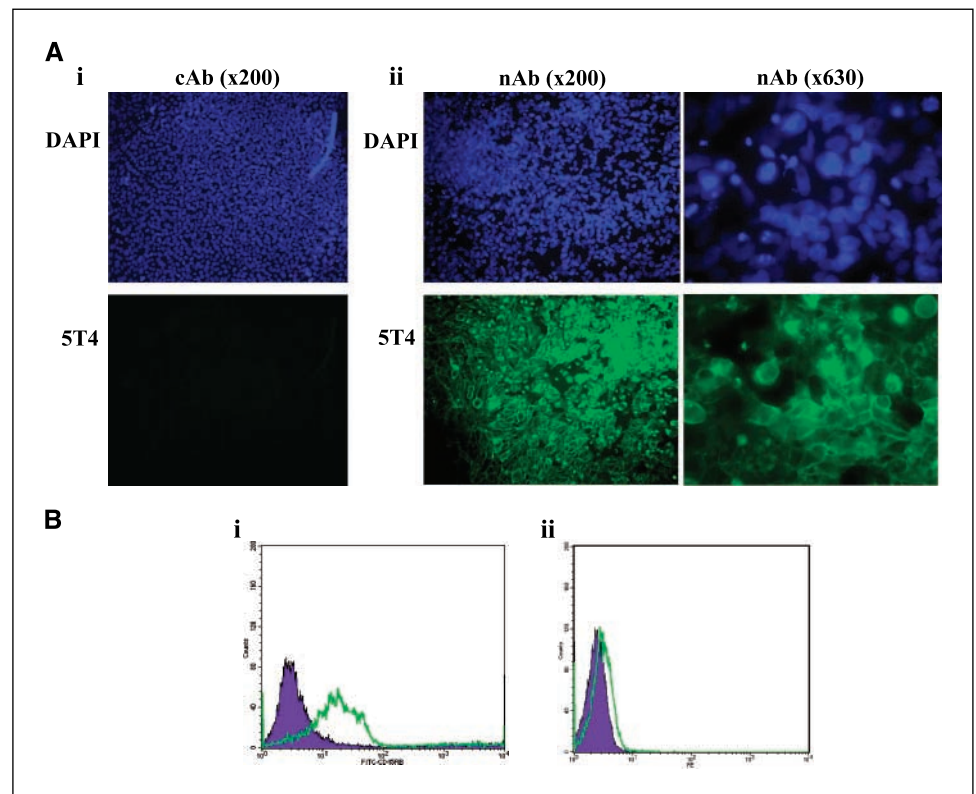


Figure 5. Inhibition of E-cadherin-mediated cell-cell contacts increases cellular motility but decreases proliferation of hES cells. *A*, cellular motility of undifferentiated and differentiating HES4 ES cells and following treatment of the cells with E-cadherin nAb (SHE78.7) was assessed using Costar Transwell 5 μ m pore size plates. Data represents the fold change in motility compared with undifferentiated cells. *HES4 U*, undifferentiated cells; *HES4 D*, ES cells differentiated for 10 d; *HES4 +cAb*, ES cells treated with cAb; *HES4 +nAb*, undifferentiated ES cells treated with nAb SHE78.7; *HES4 -nAb d5*, removal of nAb from undifferentiated HES4 ES cells for 5 d prior to motility analysis. *B*, cellular proliferation was assessed in undifferentiated HES4 ES cells treated with cAb (*cAb+*), nAb (*nAb+*), and following removal of nAb for 7 d (*nAb-*). Note that nAb-treated cells exhibited decreased proliferation and that this was reversed following removal of the antibody.

Figure 6. 5T4 antigen is localized at the plasma membrane following abrogation of E-cadherin-mediated cell-cell contact. **A**, undifferentiated HES4 ES cells were treated with (i) cAb or (ii) E-cadherin nAb SHE78.7 (nAb) for 8 h and assessed for DAPI (total number of cells, nuclei, within the field of view) and 5T4 antigen expression by immunofluorescent microscopy (magnification, $\times 200$ and $\times 630$). **B**, (i) undifferentiated HES4 ES cells were treated with control (cAb; filled population) or nAb (open population) and assessed for cell surface expression of the 5T4 antigen using fluorescent flow cytometry. Viable cells were gated using forward and side scatter and the data represents cells from this population. Note that 5T4 antigen is detected at the cell surface of nAb-treated ES cells. (ii) Analysis of cell surface 5T4 expression in HES4 ES cells treated with either cAb (closed population) or nAb (open population) and subsequent removal of the antibodies for 5 d was assessed using fluorescent flow cytometry. Viable cells were gated using forward and side scatter and the data represents cells from this population. Note that removal of nAb resulted in similar levels of 5T4 antigen at the cell surface compared with cAb-treated cells.



mouse and human ES cells are striking and we hypothesize that 5T4 and N-cadherin function in a similar manner in both species, that is, by imparting increased cellular motility upon ES cell differentiation.

Snail genes have been observed in all EMT processes studied to date (25, 26), and they function by binding to the E-cadherin promoter resulting in gene transcript repression (26). The observation that these transcripts and proteins are up-regulated during hES cell differentiation suggests that they may function to inhibit E-cadherin transcript expression. However, Snail and Slug can also function to inhibit apoptosis (26). During ES cell differentiation, significant apoptosis is observed and Snail proteins may also impart cellular survival during this process. MMPs have also been reported to regulate cell surface E-cadherin protein by proteolytic cleavage (1, 7), resulting in a soluble 80 kDa form of E-cadherin which is often detected in tumor cell lines and biopsy samples (1, 7). It has been shown that the 80 kDa soluble form of E-cadherin can induce MMP-2 and MMP-9 expression in tumor cell lines (1, 7). Therefore, several mechanisms may act during hES cell EMT to regulate cell surface E-cadherin protein expression. The ES cell model therefore provides a useful *in vitro* system with which to study transcriptional and proteolytic regulation of EMT-associated molecules.

Our data shows that an EMT event occurs during hES cell differentiation and that this model system may provide an insight into this process. Furthermore, we show a novel function for

E-cadherin in preventing cell surface localization of the 5T4 antigen. The similarities between the hES cell EMT and known processes during cancer metastasis are striking. Patient mortality following the metastatic spread of epithelial tumors is significantly increased and these account for at least 80% of all cancers. The ES cell model system may allow the study of EMT events *in vitro* and the subsequent comparison with tumor biopsies/cell lines to elucidate critical events associated with the onset of tumor cell spread. However, although it is clear that events associated with EMT occur during tumor cell metastasis, it is still unclear whether EMT is a prerequisite for tumor cell spread or is simply associated with this process. For example, there is recent evidence that tumor cells could spread in the absence of EMT (49, 50). Therefore, ES cell EMT constitutes a useful *in vitro* model system to study the effects of specific molecules during this process to determine their function in cellular motility and invasion.

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