

A role for cadherins in tissue formation

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SUMMARY

We have produced null mutant mouse embryonic stem cells for the cell adhesion molecule E-cadherin. Such E-cadherin^{-/-} ES cells are defective in cell aggregation; this defect can be corrected by transfection with cDNA for either E-cadherin or N-cadherin driven by a constitutive promoter. The presence (or absence) of E-cadherin regulates the expression of the transcription factor T-brachyury, indicating that cadherins play a role in linking cell surface receptors and gene expression. Comparative analysis of the parental and the genetically altered ES cell lines was performed to examine cell differentiation and the capability to form organized tissues. While differentiating

E-cadherin^{-/-} ES cells are still able to express various early and late differentiation markers, they show a clear-cut deficiency in forming organized structures. This phenotype can be rescued by constitutive expression of E-cadherin, which results exclusively in formation of epithelia. In contrast, rescue transfectants expressing N-cadherin show no epithelial structures, instead forming neuroepithelium and cartilage. These results provide the first evidence that specific cadherins directly stimulate differentiation into certain types of tissues.

Key words: cadherin, ES cell, mouse histogenesis, epithelia

INTRODUCTION

Cell-cell adhesion has long been recognized as one of the primary processes in embryonic development and tissue formation (Edelman and Crossin, 1991; Takeichi, 1991). Considerable progress has been made in elucidating the structure and function of cell adhesion molecules (CAMs) which mediate specific cell-cell interactions (for review, Hynes, 1992). Cadherins comprise a family of Ca²⁺-dependent CAMs which in general interact with each other homophilically. The classical cadherins, including E- (uvomorulin), N- and P-cadherin, are the best-studied members of the cadherin family. Most notable for understanding cadherin function have been the recent determination of high-resolution structures of the amino-terminal extracellular domains of two cadherins (Shapiro et al., 1995; Overduin et al., 1995) and the identification of catenins as the cytoplasmic anchorage proteins for cadherins. In particular, catenins have recently been shown not only to regulate cadherin-mediated adhesion (Kemler, 1993), but also to participate most likely in signaling pathways (Gumbiner, 1995).

Several lines of evidence implicate cadherins in developmental processes. First, the expression patterns of cadherins correlate with distinct morphogenetic events. For example, E-cadherin is expressed during mouse gastrulation and in epithelial tissues (Vestweber and Kemler, 1984; Butz and Larue, 1995), N-cadherin in neuroepithelium, myocardium and osteoblasts (Hatta and Takeichi, 1986; Miyatani et al., 1989), and R-cadherin

exhibits a distinct expression pattern in the developing nervous system (Matsunami and Takeichi, 1995; Takeichi, 1995).

Second, antibodies blocking the adhesive function of cadherins affect developmental and histogenic processes. Anti-E-cadherin antibodies block compaction and blastocyst formation during mouse preimplantation development (Kemler et al., 1977; Vestweber and Kemler, 1985) and histogenic events in chicken lung and skin organ cultures (Hirai et al., 1989), while antibodies to L-CAM perturb feather patterning and structure in chicken (Gallin et al., 1986).

Third, a dominant-negative genetic approach, involving heterotypic expression of wild-type or mutated cadherin proteins during *Xenopus* development, confirmed the importance of cadherins for cell adhesion and in several developmental processes (Kintner, 1992; Levine et al., 1994; Dufour et al., 1994; Holt et al., 1994; Lee and Gumbiner, 1995). Targeting of the E-cadherin gene in mouse embryonic stem (ES) cells generated null mutant embryos that are deficient in forming a trophoblast epithelium (Larue et al., 1994).

Although all these results are consistent with the notion of cadherins being important in vertebrate development, in most cases, the effects of cadherin perturbation can be interpreted in the light of their roles in cell adhesion, cell sorting, and cell migration. In the present report, we provide new information about cadherin function. We show that specific cadherins are more directly involved in the differentiation of certain types of tissues and provide evidence that the presence or absence of cadherins influences specific gene activity. Our results strongly

suggest that cadherins participate directly in a signaling pathway that regulates spatial patterning in mouse embryos.

MATERIALS AND METHODS

Targeting and expression vectors

The pKOUNS3 plasmid used to target the E-cad gene by homologous recombination was described previously (Larue et al., 1994). Expression vectors were constructed using the plasmids pPGK β geopA (Soriano et al., 1991) and pSPGKhyg (a gift from A. Berns, The Netherlands Cancer Institute, Amsterdam). The murine PGK-1 (phosphoglycerate kinase) promoter has been shown to drive widespread but non-uniform expression in transgenic mice (McBurney et al., 1994).

The murine full-length E-cad cDNA, excised from pSKEcad (Ozawa et al., 1989), replaced the β geo chimeric gene at the *Sma*I, *Xba*I sites of pPGK β geopA to generate the pPGKEcad and pPGKEcadAS (antisense) plasmids. The 6.5 kb *Not*I fragment containing the expression cassettes of the pPGKEcad and pPGKEcadAS plasmids was inserted in the *Hind*III-*Cl*aI site of the pSPGKhyg plasmid to generate the pPGKEcad-PGKhyg and pPGKEcadAS-PGKhyg plasmids.

The 1.8 kb blunted *Cl*aI-*Hind*III hygromycin cassette fragment isolated from pSPGKhyg was inserted into the dephosphorylated, *Not*I linearized pPGK β geopA vector to generate the pPGK β geopA-PGKhyg plasmid. The blunted *Eco*RI-*Eco*RI murine and *Eco*RV-*Sma*I chicken N-cadherin cDNA fragments, obtained from the pMNcad and pckNcad plasmids (gifts from M. Takeichi, Kyoto University), replaced the β geo chimeric gene of pPGK β geopA-PGKhyg at the *Xba*I, *Sma*I sites to generate the pPGKmNcad-PGKhyg and pPGKckNcad-PGKhyg plasmids.

ES cell culture and transfection

Embryonal stem cell line D3 (Doetschman et al., 1985) and heterozygous (E-cad^{+/-}) ES cell lines (Larue et al., 1994) were routinely maintained on an inactivated feeder cell layer, or in 60% BRL-conditioned medium (Robertson, 1987). Gene transfer experiments were carried out as previously described (Larue et al., 1994). The pPGKEcad-PGKhyg, pPGKmNcad-PGKhyg, pPGKEcadAS-PGKhyg, pPGKhyg and pPGKckNcad-PGKhyg plasmids were linearized with *Hind*III, *Ap*aI, *Hind*III, *Xho*I and *Sal*I, respectively. Selection was performed with 2 mg/ml G418 to obtain E-cad^{-/-} ES cells, and with 600 μ g/ml hygromycin to generate the other transfectants from E-cad^{-/-} ES cells.

E-cad-rescued (resc) ES cells were screened *in vivo* using an affinity-purified rabbit anti-E-cad antibody and Dynabeads M-280 affinity-coated with purified sheep anti-rabbit IgG (Dynal, Oslo, Norway). The E-cad-positive ES cell clones were easily recognizable by their phenotype and having attached beads on the surface of the cells. Using the same immunological technique and screening for the absence of attached beads, E-cad^{-/-} ES cell lines were isolated. N-cad-rescued transfectants were pre-selected by morphological changes of the E-cad^{-/-} ES cell phenotype and characterized subsequently by Southern blot analysis. Southern blot analyses were also performed to establish the unique and proper integration of the different plasmids used for control transfection experiments.

ES cell chimeras and semiquantitative PCR analysis

Cells from two independent E-cad^{+/-} (HT1 and HT2), two E-cad^{-/-} (HM1 and HM3) and two resc E-cad (E-cad resc 1 and E-cad resc 2) or N-cad (N-cad resc 1 and N-cad resc 2) ES lines were injected into C57BL/6 or NMRI blastocysts and, after transfer into pseudopregnant females, embryos and extraembryonic tissues were isolated at day E10.5.

The yield of purified DNA (Maniatis et al., 1982) was similar from

one embryo preparation to another, with the maximal difference being 3-fold. To compensate for variable DNA yields, the amount of DNA was standardized by using the endogenous *IL-3* gene as an internal control. The *IL-3* primers amplified a fragment of 545 bp (Keller et al., 1993). For quantification of ES cell contribution to individual chimeric embryos, the exogenous *lacZ* gene was present in all ES cells used. The *lacZ* primers (5'-GCG TTG GCA ATT TAA CCG CC-3' and 5'-CAG TTT ACC CGC TCT GCT AC-3') amplified a fragment of 469 bp. To standardize the quantitation, DNA from wild-type and heterozygous transgenic embryos at E10.5 was mixed to generate standards containing 100%, 10%, 1%, 0.1%, 0.01% or 0% DNA from heterozygous embryos. After amplification with *IL-3* primers and *IL-3* calibration, these templates were amplified with the *lacZ* primers. The *lacZ*-PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, transferred to Hybond-N, and hybridized with a *lacZ* probe. The signals were quantified using a phosphorimager (Fujix, Bas 1000). No significant differences were ever observed between different ES cell lines of the same genotype.

Teratoma production

ES cells were injected subcutaneously into the back of 4- to 6-week-old male 129, 129/Sv or nude mice. ES cells were injected either as single-cell suspensions (1, 5 or 15 \times 10⁶) or as preformed aggregates from hanging-drop culture. Tumors approximately 1.5 cm in diameter were split into three parts to prepare DNA, for cryosectioning and for paraffin embedding. Southern blot analysis was performed to verify the genotypes of the tumor DNAs.

In situ hybridizations

In situ hybridizations were carried out as described by Wilkinson and Green (1990). ³⁵S-labeled probes in both orientations were derived by T3, T7 and SP6 transcription. The cadherin-11 clone E plasmid (a gift from I. Hoffmann and R. Balling) contains a cDNA fragment from bases 3170 to 3651 of the *cadherin-11* gene (Hoffmann and Balling, 1995). This plasmid was digested with *Xho*I or *Not*I for unidirectional transcription with T7 or T3 RNA polymerase to produce the sense and antisense RNA probes. Probes were generated accordingly for E-cadherin (Ringwald et al., 1987), N-cadherin (Miyatani et al., 1989), T-brachyury (Herrmann, 1991), *mox-1* (Candia et al., 1992), *MyoD* (Edmonson and Olson, 1989), *pax3* (Gruss and Walther, 1992), histone H3 (Plumb et al., 1983) and NCAM (Lyons et al., 1992).

Antibodies

Affinity-purified rabbit antibodies against the extracellular part of E-cad, α -catenin and β -catenin, used in all experiments, have been described (Butz and Kemler, 1994). TROMA-1 is a rat monoclonal antibody (Brüet et al., 1980). Rabbit antisera against neurofilaments-L,M,H (Affiniti, Nottingham, UK) and pan-cadherin (Sigma, Deisenhofen, Germany) were used. FITC goat anti-rabbit IgG and FITC goat anti-rat second antibodies were from Dianova (Hamburg, Germany).

Immunological tests

Immunoblots and immunoprecipitations were performed as described previously (Butz and Kemler, 1994). Briefly, immunoprecipitations were carried out with 30 μ g β -catenin antibodies in 70 μ l cell lysate from 2 \times 10⁶ cells in the presence of 50 μ l Protein A-Sepharose at 4°C. Immunocomplexes were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose, and bound proteins were detected by subsequent incubation with antibodies against E-cad, α -catenin, β -catenin (each 5 μ g/ml) or anti-pan-cad (12 μ g/ml). Immunoblots were stained by chemiluminescence with the ECL kit system (Amersham). Indirect immunofluorescence tests were carried out as described (Butz and Larue, 1995).

Northern blot and RT-PCR analysis

10 μ g of total RNA was separated electrophoretically on a 1% agarose gel containing formaldehyde (Ausubel et al., 1988), transferred on

Hybond-N membranes (Amersham) and hybridized with specific random primer probes for T-brachyury (Herrmann, 1991), Mox-1 (Candia et al., 1992) and GAPDH (Fort et al., 1985).

Semiquantitative RT-PCR was performed on mRNA from the genetically altered ES cell lines using the Quick-Prep mRNA purification kit (Pharmacia). The amount of mRNA was determined by UV measurement, cDNAs were synthesized using oligo(dT)₁₈ and Superscript reverse transcriptase (Gibco) for 45 minutes at 42°C and standardized with HPRT (hypoxanthine phosphoribosyltransferase). PCR was performed in 50 µl on a Perkin-Elmer 2400 thermo cycler. cDNA was denatured for 2 minutes at 94°C, 30 cycles were performed (94°C for 5 seconds, 55°C for 5 seconds, 72°C for 5 seconds) and the reaction was completed at 72°C for 2 minutes. Primer pairs for amplification were, for T-brachyury: 5' primer 5' TGC TGC CTG TGA ATA AT 3' and the 3' primer 5' ACA AGA GGC TGT AGA ACA TG 3' to generate a 502 bp fragment; for cadherin-11: 5' ACG CTG AAG CCT ACA TCC TG 3' and 5' GAG TCA TCA TCA AAA GTG TC 3' to generate a 574 bp fragment; for α -catenin: 5' ATG ACT GCC GTC CAC GCA 3' and 5' ATC TGC CAT GTC AGC CAG 3' to generate a 437 bp fragment; for myogenin: 5' CAA CCA GGA GGA GCG CGA TC 3' and 5' CTC CAT CTT TCT CTC CTC AG 3' to generate a 495 bp fragment; for goosecoid: 5' GCA CCA TCT TCA CCG ATG AG 3' and 5' CAG CAG TCC TGG GCC TGT AC 3' to generate a 430 bp fragment; for oct-4: 5' GAG TAT GAG GCT ACA GGG AC 3' and 5' AAT GAT GAG TGA CAG ACA GG 3' to generate a 379 bp fragment, and for nodal: 5' GGA GTT TCA TCC TAC CAA CC 3' and 5' TCC TGC CAT GCC ACG GTA GC 3' to generate a 386 bp fragment.

RESULTS

Cadherins control ES cell aggregation

To disrupt both copies of the E-cadherin (E-cad) gene in ES cells, two consecutive rounds of homologous recombination were performed. Two independent, heterozygous ES cell lines (E-cad^{+/-}), already described and previously used to produce transgenic mice, were electroporated with a linearized targeting vector (Larue et al., 1994). After selection in 2 mg/ml G418 and screening for the absence of E-cadherin by antibodies, 43 independent transfectants were isolated. In ten of these E-cad^{-/-} ES cell lines (HM1 to HM10) further characterized by Southern and northern blot analysis, no random non-homologous integration of the targeting vector was observed (data not shown). E-cad^{-/-} ES cells exhibited a uniform, clear cell-adhesion defect (Fig. 1C). While wild-type (Fig. 1A) or E-cad^{+/-} (Fig. 1B) ES cells grew as typical clumps of cell aggregates, E-cad^{-/-} ES cells grew rather dispersed, although their cell-substratum adhesion seemed not to be altered. These results demonstrate that E-cadherin is the crucial cell-cell adhesion molecule in ES cells.

To rescue cell-cell adhesion, three of the E-cad^{-/-} ES cell lines (HM1, HM2 and HM4) were transfected with plasmids carrying both the hygromycin resistance cassette and cDNA coding either for E-cad or N-cadherin (mNcad). As controls, plasmids encoding E-cad cDNA in the opposite orientation (antisense) or the β -geo chimeric cDNA (see Materials and Methods) were included in the experiments. All cDNAs were

driven by the constitutive phosphoglycerate kinase (PGK-1) promoter. Thirty-seven independent E-cad-expressing ES cell clones (E-cad resc 1 to 37) were isolated by screening for cell surface expression of the protein using antibodies and confirmed by Southern analysis to have unique integration of the plasmid. Among transfectants expressing N-cad, 86 ES cell clones (N-cad resc 1-86) were isolated and characterized by Southern and northern blot analysis (not shown), as well as for the presence of N-cad protein (see below).

Expression of either E-cad or N-cad reversed the phenotype of E-cad^{-/-} ES cells in that the transfectants grew as cell aggregates similar to wild-type or E-cad^{+/-} ES cells. Representative transfected cell lines, E-cad resc 1 and N-cad resc 1, are depicted in Fig. 1D,E. No induction of cell aggregation was observed in control transfectants expressing E-cad antisense or β -geo (not shown). Altogether, these experiments demonstrate that ES cell aggregation depends on the presence of E-cad and that N-cad can substitute for a lack of E-cad in ES cell aggregation.

To study the expression of the exogenous E-cad and N-cad and their association with catenins, immunoprecipitation and subsequent immunoblot experiments were performed (Fig. 1F). Cell lysates from wild-type, E-cad^{+/-}, E-cad^{-/-}, E-cad resc 1 and N-cad resc 1 ES cells were immunoprecipitated with affinity-purified anti- β -catenin antibodies, and the molecular composition of the cadherin-catenin complex obtained was monitored in subsequent immunoblots with anti-E-cad, anti- α -catenin and anti- β -catenin and anti-pan-cad antibodies. As expected, the E-cadherin-catenin complex was detected in wild-type and E-cad^{+/-} ES cells (Fig. 1F, lanes 1 and 2). In E-cad^{-/-} cells, heterodimers between α - and β -catenin were formed, while the E-cad introduced in E-cad resc 1 cells again assembled with catenins (Fig. 1F, lanes 3 and 4). In cell lysates from N-cad resc 1 ES cells anti-pan-cad antibodies detected a 140×10³ M_r protein corresponding to N-cadherin (Fig. 1F, lane 5) and an 88×10³ M_r protein corresponding to β -catenin (Fig. 1F, lane 6). Indirect immunofluorescence tests revealed membrane localization of all components of the cadherin-catenin complex in E-cad resc 1 and N-cad resc 1 ES cells (not shown). All these experiments demonstrate that in E-cad resc 1 and N-cad resc 1 ES cells, the introduced cadherins associate with endogenous catenins to form the known adhesion complex.

The degree of cell adhesion correlates with the relative amount of the introduced cadherin in E-cad^{-/-} cell lines, as depicted in Fig. 2. Of the 37 E-cad resc ES cell lines, two expressed a relatively high level of E-cad (E-cad resc 1 and 2) and grew as typical ES cell aggregates (Fig. 2C and D, lane 3). 10 ES cell lines (E-cad resc 3-12) with an intermediate level of E-cad expression formed adherent monolayers (Fig. 2B and D, lane 2) while the rest, which expressed only traces of E-cad, more closely resembled E-cad^{-/-} ES cells in their phenotype (Fig. 2A and D, lane 1). Even the two E-cad 'high-expressing' transfectants only expressed about 1/10 the amount of E-cad of wild-type ES cells. In N-cad transfectants, a similar correlation between the amount of N-cad and the reversion of the E-cad^{-/-} ES cell phenotype was observed, using semiquantitative RT-PCR analysis or anti-pan-cad antibodies (not shown).

E-cadherin affects T-brachyury expression

E-cad is expressed during early mouse embryonic development

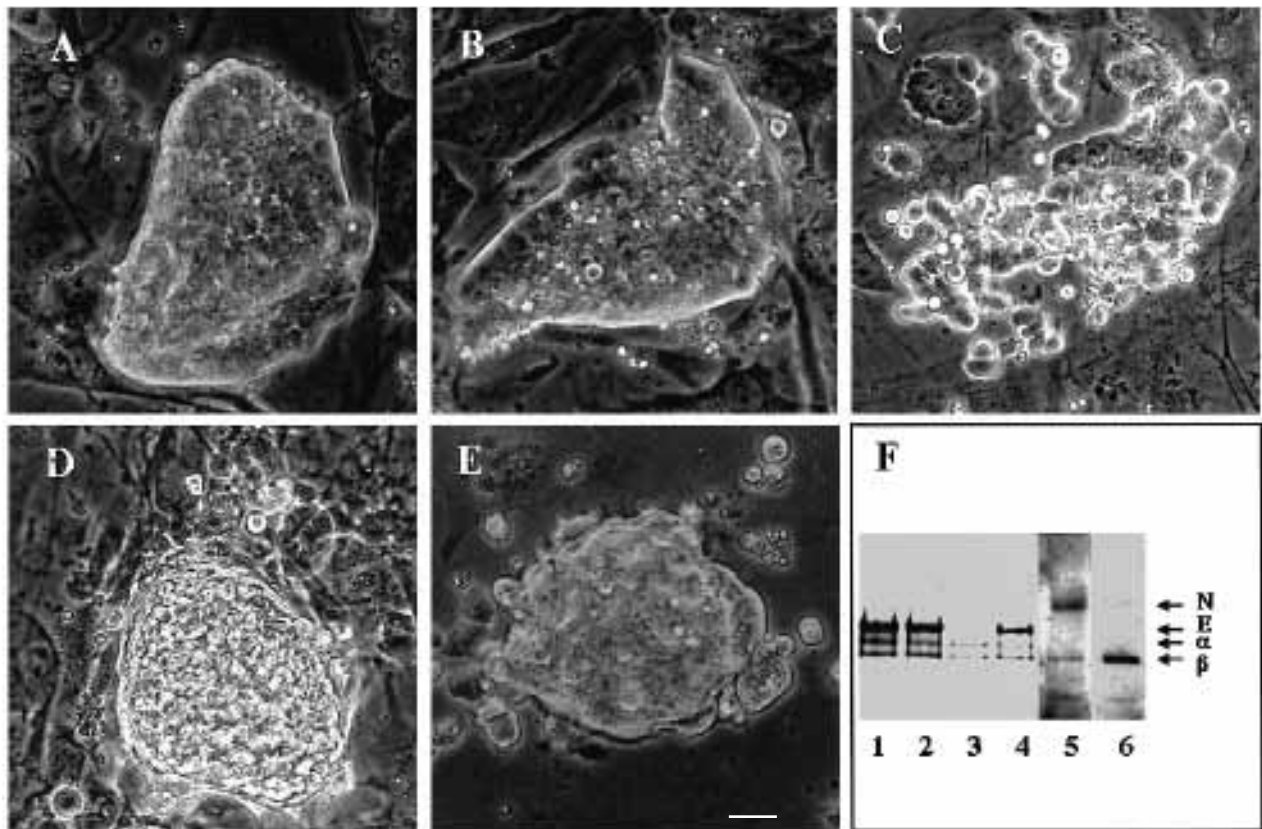


Fig. 1. ES cell aggregation is cadherin dependent. Phenotypes of the genetically altered ES cells are presented in A-E. Wild-type (A) and E-cad^{+/−} (B) ES cells exhibit an identical morphology, while a representative E-cad^{−/−} ES cell line HM1 is deficient in cell adhesion (C). Cell aggregation is restored in representative E-cad resc 1 (D) and N-cad resc 1 (E) ES cells. (F) Immunoblot analysis of cadherin adhesion complexes immunoprecipitated with anti β-catenin antibodies, successively incubating with anti-E-cad, anti-α-catenin and β-catenin (lanes 1-4); anti-pan-cad (lane 5); anti-β-catenin (lane 6) antibodies. Arrows indicate the positions of N, N-cad; E, E-cad; α, α-catenin; β, β-catenin. Lane 1, wild-type ES cells; lane 2, E-cad^{+/−} cells; lane 3, E-cad^{−/−} cells; lane 4, E-cad resc ES cells; lanes 5 and 6, N-cad resc ES cells. Scale bar, 25 μm.

in all cell types; it first becomes inactivated in mesoderm cells at gastrulation stage (Vestweber and Kemler, 1984; Damjanov et al., 1986; Butz and Larue, 1995), which then express mesoderm-specific genes such as *T-brachyury* (Herrmann, 1991). To study whether similar changes in gene expression at the RNA level could also be monitored in the genetically altered ES cells, semiquantitative RT-PCR and northern blot analysis were performed with the mesoderm-specific genes *nodal*, *goosecoid*, *T-brachyury*, *mox-1* and *myogenin*. All cells tested expressed an equal amount of transcription factor oct-4 mRNA, indicating the pluripotential phenotype of these cells (Schöler, 1991). No expression of goosecoid, myogenin or mox-1 was observed in any cells tested, while nodal exhibited a low expression in all cell types only detectable by RT-PCR (Table 1). However, T-brachyury mRNA was clearly present

in E-cad^{−/−} ES cells, both in RT-PCR and northern blot, and was absent in wild-type or E-cad^{+/−} cells (Fig. 3, Table 1).

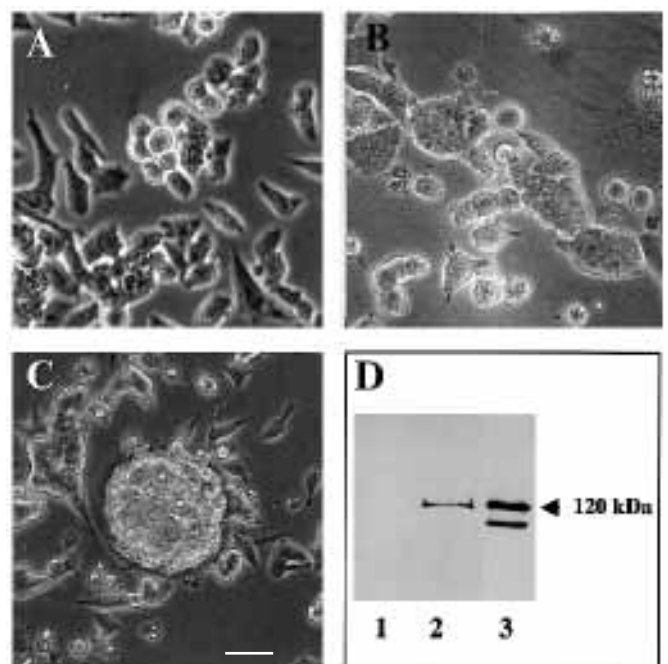


Fig. 2. ES cell aggregation depends on the amount of E-cad expression. Three independent E-cad-rescued ES cell lines, E-cad resc 13 (A), E-cad resc 3 (B) and E-cad resc 1 (C), exhibiting different degrees of cell aggregation. Cell aggregation correlates with the amount of E-cad expressed in these cells (D). Whole cell lysates of 4×10^5 cells from E-cad resc 13 (lane 1), E-cad resc 3 (lane 2) and E-cad resc 1 (lane 3) were immunoblotted with anti-E-cad antibodies. The position of E-cad ($120 \times 10^3 M_r$) is indicated. Scale bar, 25 μm.

Table 1. Summary of comparative, semiquantitative RT-PCR analyses of mRNA expression in the genetically altered ES cell lines

Amplified product	ES cell genotype				
	+/+	+/-	-/-	-/-E	-/-N
α -catenin	+++	+++	+++	+++	+++
oct-4	++	++	++	++	++
nodal	(+)	(+)	(+)	(+)	(+)
myogenin	-	-	-	-	-
T-brachyury	-	-	+++	-	++
goosecoid	-	-	-	-	-

Poly (A)⁺ RNA was subjected to RT-PCR analysis using primers for the genes indicated (see Materials and Methods).

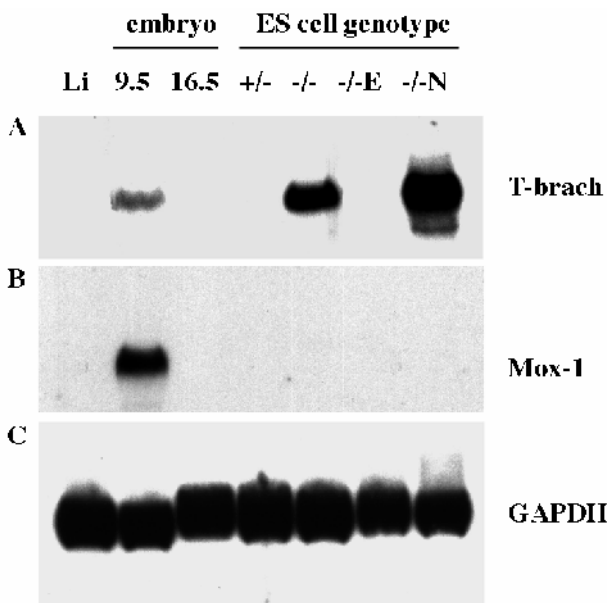


Fig. 3. Expression of T-brachyury is down-regulated by the presence of E-cad. Total RNA from adult liver, mouse embryos day E 9.5 and day E 16.5, as well as from genetically altered ES cell lines was probed for expression of T-brachyury (A), *mox-1* (B) and a housekeeping marker GAPDH (C) by northern blot analysis. T-brachyury is highly expressed in E-cad^{-/-} ES cells but becomes repressed in E-cad-rescued ES cells (see also Table 1).

Remarkably, expression of E-cad, but not of N-cad, led to a repression of T-brachyury mRNA synthesis. These results strongly suggest that the presence of E-cad can negatively regulate T-brachyury expression.

Formation of chimeric embryos

The genetically altered ES cells were injected into blastocysts to analyze quantitatively their ability to participate in the formation of chimeric embryos. The degree of chimerism of each embryo at day E10.5 was determined by performing semiquantitative PCR analysis. For all cell types injected, the percentage of embryos recovered was the same and no embryos exhibited obvious morphological alterations. The results, summarized in Fig. 4, show that E-cad^{-/-} ES cells contributed very poorly to chimeras, most likely due to their deficiency in cell adhesion. Similar results were obtained with N-cad resc ES cells, although these cells contributed more frequently to

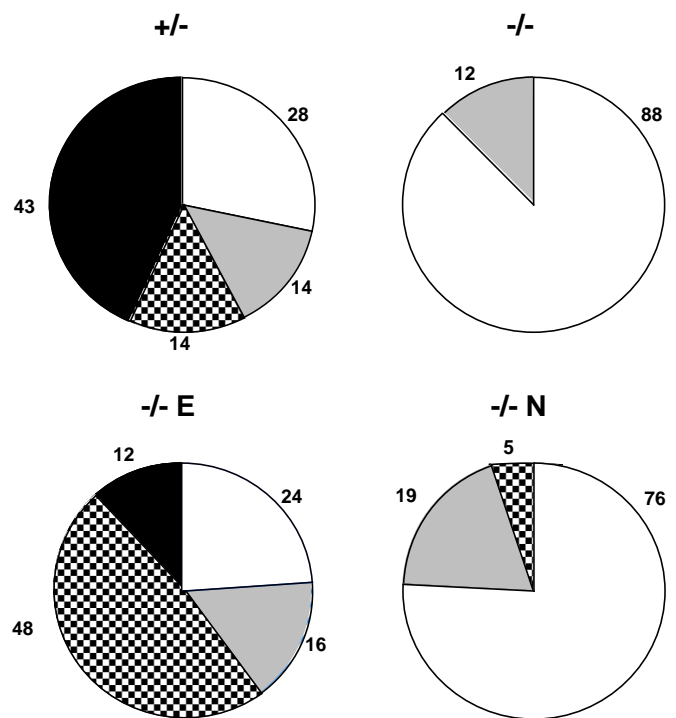


Fig. 4. Genetically altered ES cells lacking E-cad contribute poorly to chimeric embryos. Diagrammatic comparison of the efficiency of contributing to chimerism as determined on day E10.5 embryos. +/- = E-cad^{+/-}; -/- = E-cad^{-/-}; -/- E = E-cad resc 1 and -/- N = N-cad resc 1 ES cell lines. For each ES cell line about 30 day E10.5 embryos were analyzed individually by semiquantitative PCR; the degree of chimerism in each embryo was classified into one of four categories: non-chimeric (less than 0.01%, white), low chimerism (0.01-0.1%, grey), intermediate chimerism (0.1-1%, checked) and high chimerism (greater than 1%, black). The percentage of embryos in each category is indicated.

chimeras. With E-cad resc ES cells, the percentage of chimerism was comparable to that obtained with E-cad^{+/-} ES cells (about 75% of embryos). The low contribution of E-cad resc ES cells compared to E-cad^{+/-} ES cells might be explained by the fact that they express E-cad under a constitutive promoter. In any case, these experiments show that E-cad is required for a significant participation of ES cells in chimeric embryos.

Cadherins can direct tissue formation

When injected subcutaneously into syngenic hosts, ES cells form benign, solid teratomas, a well-established method to study the differentiation potentiality of these cells. Under such experimental conditions, ES cells not only differentiate into derivatives of all three germ layers, they additionally manifest to a high degree a tissue-like organization, i.e. form epithelium, muscle, cartilage, bone, neuroepithelium, teeth and hair follicles. Teratomas were produced and subjected to histological analysis for the following ES cell lines: Wild-type ES-D3 cells, three independent E-cad^{+/-} ES cell lines (HT1 to HT3), five independent E-cad^{-/-} ES cell lines (HM1 to HM4), five E-cad resc lines (E-cad resc 1 to 5) and three E-cad^{-/-} lines rescued with N-cad (N-cad resc 1 to 3). None of the tumors was invasive or metastatic. Tumors from wild-type and E-

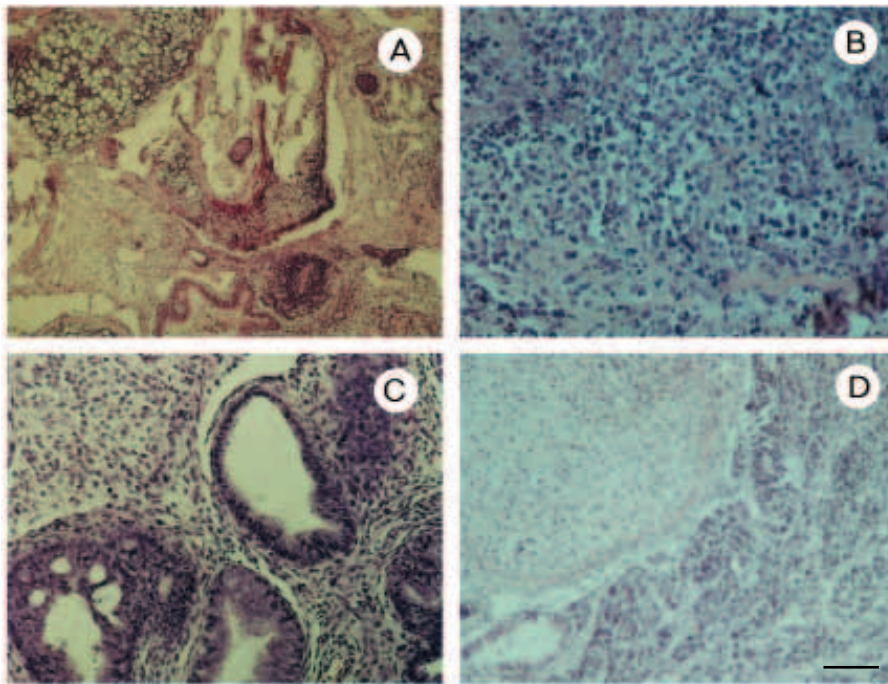


Fig. 5. Cadherins can regulate tissue formation in teratomas. ES cells (2×10^6) were injected subcutaneously into syngenic mice; teratomas were removed after 3–4 weeks and subjected to histological examination. Wild-type (A) and E-cad^{+/-} ES cells differentiate into a variety of different normal looking structures. No organized structures were found in tumors from E-cad^{-/-} ES cells (B). Tumors derived from E-cad resc ES cells contained only epithelia (C) and those from N-cad resc ES cells only cartilage and neuroectoderm (D). Scale bar in A, 250 μ m; in B, C, 50 μ m; in D, 125 μ m.

cad^{+/-} ES cells were indistinguishable and all exhibited the known, chaotically disarranged distribution of various normal-looking tissues (Fig. 5A). In contrast, no organized structures were observed in tumors derived from E-cad^{-/-} ES cells (Fig. 5B). E-cad resc ES cells exclusively formed epithelia and no other organized structures (Fig. 5C). The frequency of epithelium formation was higher in tumors derived from E-cad high-expressing ES cells (E-cad resc 1 and 2) than in those expressing intermediate levels of E-cad (E-cad resc 3 and 4). In contrast, with tumors derived from N-cad resc ES cells (Fig. 5D), instead of epithelia, only cartilage and neuroepithelium were seen as organized structures in tumors derived from three independent rescue lines. These experiments, summarized in Table 2, clearly show that ES cells lacking E-cad are deficient in forming organized structures, and that constitutive re-expression of E-cad leads to epithelial formation, while N-cad induces cartilage and neuroepithelium. To extend these findings, E-cad^{-/-} ES cells were transfected with chicken N-cad cDNA using the same expression vector system. Three independent transfected cell lines (cN-cad resc 1 and 3) were established and injected into nude mice. As in the case of murine N-cad, expression of chicken N-cad resulted in the formation of cartilage and neuroepithelium (not shown, but see Table 2).

Several controls were included in these experiments; E-cad^{-/-} ES cells expressing E-cad antisense or β geo were unable to form organized structures and their tumors resembled those of E-cad^{-/-} cells. Wild-type ES cells constitutively expressing E-cad or N-cad were still able to differentiate into full teratomas, although in these tumors an unusually high proportion of epithelia in the case of E-cad and of cartilage and neuroepithelium in N-cad transfectants was observed. The results, shown in Table 2, were independent of how many cells were injected (1, 5 or 15×10^6 cells) or whether single-cell suspensions or preformed aggregates (hanging-drop culture) were injected. In summary, these experiments show that removing

or adding cadherins in ES cells results in striking differences in the types of differentiated structures these cells are capable of forming.

Cadherins and cell differentiation

The different tissue-forming abilities of the genetically altered ES cells produced here raised several interesting questions. Particularly the absence of organized structures in E-cad^{-/-} teratomas called for further clarification of the differentiation state of these cells. Therefore, the expression of a variety of early and late differentiation markers was analyzed by immuno-histochemistry or by in situ hybridization of specific transcripts on sections of teratomas obtained from the genetically altered ES cells. Probes used for gene expression included T-brachyury, mox-1, pax-3, cad-11, E-cad, N-cad, N-CAM, histone H3, myogenin, cytokeratins and neurofilaments. In E-cad^{-/-} tumors, expression of all these differentiation markers was observed, much as depicted in Fig. 7D for cad-11. Most importantly, expression of all marker transcripts was always confined to single cells or small clumps of cells scattered through the tumor. No structural organization was delineated in E-cad^{-/-} ES cell tumors by any expression pattern.

Of particular interest was the reactivity of the monoclonal antibody TROMA-1, which recognizes cytokeratin 8, a marker specific for epithelial cell differentiation. TROMA-1 stained multiple single cells or small cell aggregates in E-cad^{-/-} tumors and specifically labeled epithelia in E-cad-rescued and wild-type cell tumors (Fig. 6A–C). In N-cad-rescued cell tumors, TROMA-1 recognized clumps of cells that exhibited no epithelial organization as judged by histological examination (Fig. 6D).

Cad-11 is first detected in mesodermal cells at gastrulation and is highly expressed in mesenchymal cells surrounding developing organs (Hoffmann and Balling, 1995; Fig. 7A). A similar expression pattern of cad-11 transcripts was observed

Table 2. Teratomas derived from genetically altered ES cell lines

Genotype of ES cells	Cell line	No. of tumors/ No. of hosts	Type of tissues
+/+	D3	14/14	epithelia, muscle, cartilage, bone, neuroepithelium
+/-	HT1	3/3	epithelia, muscle, cartilage, bone, neuroepithelium
	HT2	1/1	
	HT3	2/2	
-/-	HM1*	8/8	no tissue formation
	HM2	1/3	
	HM3	3/8	
	HM4	0/3	
-/- + E-cadherin	Ecad1	5/5	epithelia
	Ecad2	1/1	
	Ecad3	4/4	
	Ecad4	4/4	
	Ecad5	2/2	
-/- + N-cadherin	Ncad1	16/17	cartilage, bone, neuroepithelium
	Ncad2	3/3	
	Ncad3	3/3	
-/- + cN-cadherin	cNcad1	4/4	cartilage, bone, neuroepithelium
	cNcad2	4/4	
-/- + E-cadherin antisense	AS1	3/3	no tissue formation
	AS2	3/3	
+/+ + E-cadherin	D3E1	3/3	epithelia†, muscle, cartilage, bone, neuroepithelium
	D3E2	3/3	
	D3E3	3/3	
+/+ + N-cadherin	D3N1	3/3	epithelia, muscle, cartilage†, bone, neuroepithelium
	D3N2	3/3	
	D3N3	3/3	

ES cells ($1-2 \times 10^6$) were injected subcutaneously into 129/Sv mice and tumors were examined after 3-7 weeks. For each of the genetically altered ES cells, independently established cell lines gave comparable results. Tumor growth of E-cad^{-/-} cells was delayed in some lines but was similar to those of the other cell lines when 15×10^6 cells were injected (*). In control experiments with wild-type ES cell transfectants, the relative proportion of epithelia (for E-cad transfectants) or cartilage and neuroepithelium (for N-cad transfectants) appeared higher (†) than in tumors obtained with wild-type ES cells.

in wild-type ES cells (Fig. 6C) while, in tumors from E-cad^{-/-} cells, cad-11 exhibited a low and diffuse expression (Fig. 7D). High expression of cad-11 transcripts was observed in cells surrounding organized structures in tumors from E-cad and N-cad-rescued E-cad^{-/-} ES cells (Fig. 7E,F). When the expression of E-cad or N-cad transcripts was examined in tumors derived from E-cad resc and N-cad resc ES cells, the corresponding transcript was found highly expressed in epithelia for E-cad, and in osteoblasts and neuroepithelium for N-cad, but only weakly and heterogeneously in non-organized cells (not shown).

DISCUSSION

Analysis of the onset of organogenesis during mouse development is hampered by the relative inaccessibility of the embryo after implantation. Mainly for this reason, much effort has been put into developing in vitro models to study cell differentiation and morphogenesis. Embryonic stem (ES) cell lines are widely used in basic and medical research to generate transgenic mice

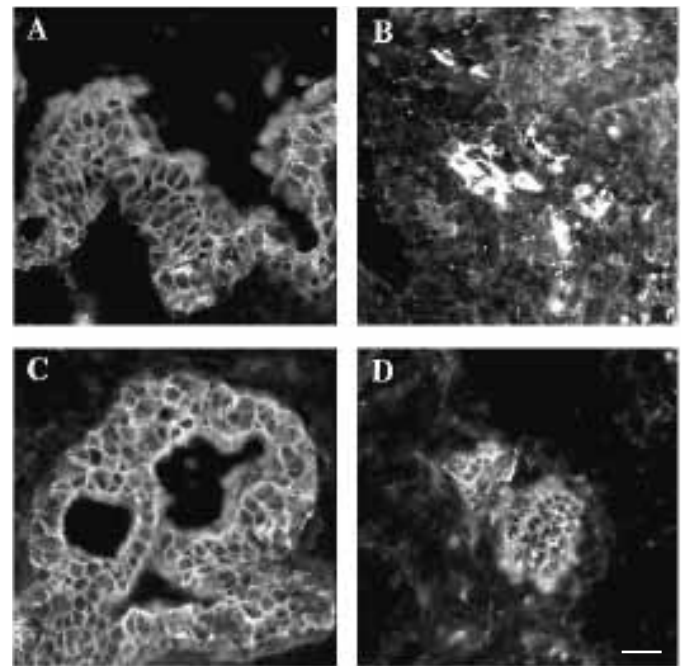


Fig. 6. Expression of cyokeratin 8 in tumors derived from genetically altered ES cell lines. Cryostat sections (7 μ m) from tumors of wild-type (A), E-cad^{-/-} (B), E-cad resc (C) and N-cad resc (D) ES cells were subjected to immunofluorescence tests with monoclonal antibody TROMA-1, which recognizes the epithelial marker cyokeratin 8. TROMA-1 stains epithelia (A,B), but only single cells or clumps of cells in tumors derived from E-cad^{-/-} ES cells (B). The cell aggregates that are positive for TROMA-1 in tumors from N-cad resc ES cells produce no recognizable epithelial structure (D). Scale bar, 25 μ m.

(Evans and Kaufman, 1981) with a predetermined genotype (Baribault and Kemler, 1989). Their differentiation potentiality in vitro allows the analysis of stem cell development, e.g. differentiation into cells of the hematopoietic or lymphoid cell lineage (Doetschmann et al., 1985; Potocnik et al., 1994). They form organ-like structures and tissues during the growth of benign teratomas when injected into syngenic hosts, differentiating into a variety of tissues including muscle, epithelia, cartilage, bone, hair follicles and others (Robertson, 1987). Thus ES cells are well suited to study either cell differentiation in vitro or the formation of tissues during tumor growth, or to manipulate gene expression in transgenic mice. We have used the ES cell system to address questions about the role of cadherins in cell differentiation and tissue formation. Since the classic work by Townes and Holtfreter (1955) on the reorganization of dissociated sea urchin embryonic cells, adhesion molecules have been recognized as important regulators in animal morphogenesis. This led to the challenging hypothesis that CAMs themselves might represent morphoregulatory molecules (Edelman, 1984; Takeichi, 1995). Although the requirement of E-cad for the plasticity of epithelial cell phenotypes has been shown in cultured cells (Marrs et al., 1995), a more direct involvement in patterning and histogenesis is less well established. Here we provide the first experimental evidence that cadherins can directly regulate morphogenetic events and that cadherins can direct tissue formation; ES cells expressing E-cad constitutively are only capable of forming

epithelia, while cells constitutively expressing N-cad instead differentiate into cartilage and neuroepithelium. We also find that E-cad, but not N-cad, can reduce the expression of the transcription factor T-brachyury, suggesting that cadherins directly influence gene expression and are involved in a molecular dialogue between the cell surface and the nucleus.

ES cells lacking E-cad

The inactivation of E-cad results in adhesion-defective ES cells. These E-cad^{-/-} ES cells grow loosely attached to each other and to the substratum, indicating that minor cell-cell adhesion and cell-substratum adhesion mechanisms are still functioning. We found that differentiating E-cad^{-/-} cells express various early or late differentiation markers, confirming a pluripotentiality similar to that of their wild-type counterparts. However, when injected into blastocysts, E-cad^{-/-} ES cells are defective in forming chimeric embryos. Perhaps the absence of E-cad prevents an efficient recruitment of these cells into the inner cell mass. Most strikingly, E-cad^{-/-} ES cells are unable to form any organized structures during tumor growth. This result was obtained with several independently isolated E-cad^{-/-} ES cell lines and appears not to be influenced by number of cells or passages. We would have predicted that E-cad^{-/-} ES cells would be deficient in forming epithelia because of our previous finding that E-cad^{-/-} embryos are deficient in forming an intact trophoblast epithelium (Larue et al., 1994). We had expected that E-cad-negative ES cells would preferentially differentiate into the mesoderm lineage and eventually form muscle or cartilage. We reasoned that genes for other cadherins (or more generally other CAMs) should be unaffected in E-cad^{-/-} cells and should still mediate cell adhesion during differentiation. Indeed, although we observed rather high expression of N-CAM and N-cad and also of cad-11 mRNA during tumor growth of E-cad^{-/-} cells, these molecules were apparently unable to induce cell patterning or the organization of structures under these experimental conditions. One explanation for these results is that E-cad is required for early cell condensation, which might be a prerequisite for subsequent inductive events leading to controlled cell patterning and the generation of differentiated structures.

Cadherins and tissue formation

The lack of cell adhesion in E-cad^{-/-} ES cells can be rescued by the constitutive expression of either E-cad or N-cad. Rescue of the E-cad^{-/-} phenotype was dependent on the amount of cadherin expressed, indicating that both E-cad and N-cad can mediate the typical condensed phenotype of ES cell aggregates. We have established and analyzed a large number of transfected cell

lines expressing either cadherin to rule out any possible selection of predetermined transfected ES cells which could be developing in a restricted differentiation pathway. This was not the case, since our results were all consistent and did not depend on cell line or on cell number.

Our most striking observation is that cadherins can direct histogenesis, since different genetic manipulations of cadherin expression result in the formation of different tissues. E-cad resc ES cells form only epithelia; interestingly, several types of epithelia, i.e. columnar or multilayer, were observed. In contrast, constitutive N-cad expression results in the formation of only neuroepithelium and cartilage, both tissues that express N-cad during normal embryonic development (Hatta and Takeichi, 1986). Several interesting questions arise from these results. For example, why do not all E-cad resc ES cells form epithelia during tumor growth? One possibility is that the expression of E-cad might become variable during tumor growth due to the absence of any selection, and only those cell aggregates exhibiting high E-cad expression will form epithe-

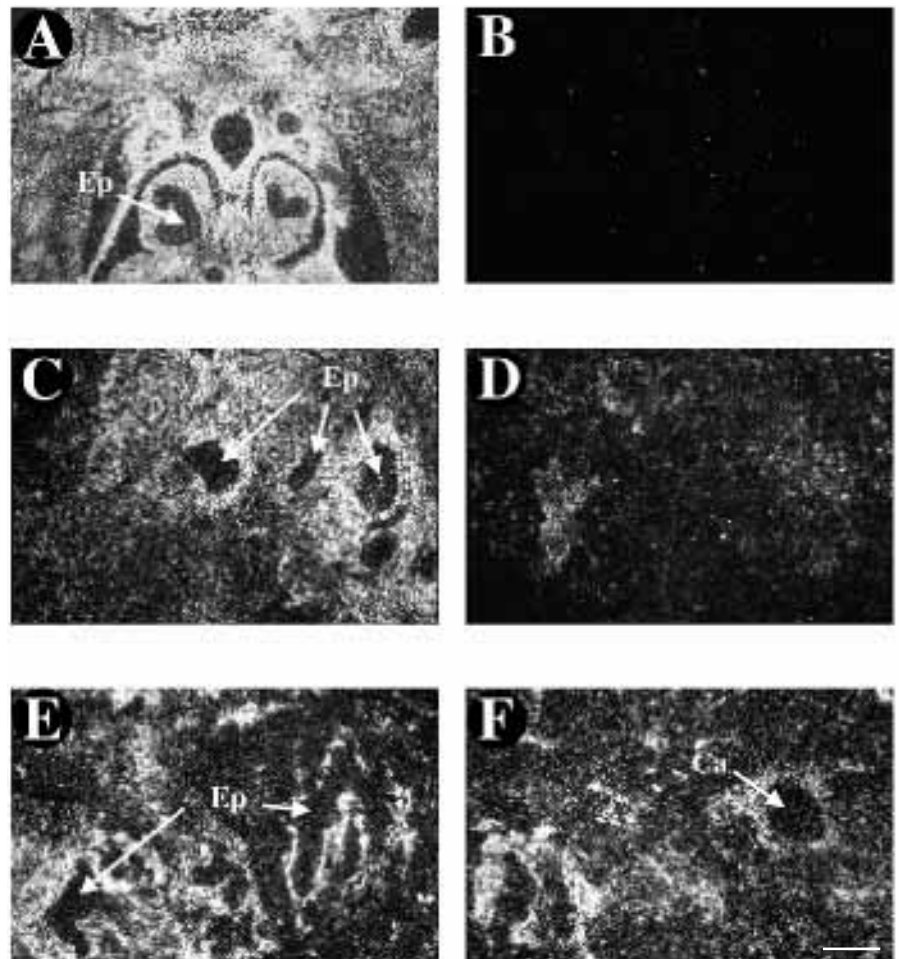


Fig. 7. Expression of cad-11 in tumors derived from genetically altered ES cell lines. In situ hybridization analysis of cad-11 mRNA in tumors derived from wild-type (C), E-cad^{-/-} (D), E-cad resc (E) and N-cad resc (F) ES cells. Cad-11 is diffusely expressed in E-cad^{-/-} tumors (D), but is highly expressed in cells surrounding organized structures (E,F). Epithelia (Ep) and cartilage (Ca) are negative for cad-11 transcripts. Controls included transverse thoracic sections of day E 12.5 embryos using cad-11 antisense (A), or sense (B) probes. Scale bar, 250 μ m.

lial structures successfully. Such a view is supported by results of in situ hybridization experiments, which detected high amounts of E-cad mRNA in epithelia and only low and scattered expression in the intermingling cells. The latter express a variety of different cell markers, similar to what was observed in E-cad^{-/-} tumors. The resolution of the in situ hybridization experiments did not allow us to distinguish whether a single cell expresses only one or multiple markers. Importantly, these same differentiation markers are not expressed in epithelial structures. Similarly, in tumors derived from N-cad resc ES cells, cells that were not organized expressed little N-cad mRNA but exhibited a scattered expression of other differentiation markers. One might conclude from these results that the relative amount of a given cadherin is critical to induce morphogenetic events. During tumor growth and seemingly arbitrarily under the experimental conditions here, it appears that only cells expressing a sufficient amount of E-cad (or N-cad) are able to condense and induce a tissue-specific program of gene expression.

That the amount of E-cad on the cell surface is important has also been considered for E-cad knock-out mice where maternal E-cad is sufficient to mediate the compaction process at the morula stage, while zygotic gene activity is required for the formation of a trophectoderm epithelium (Larue et al., 1994). Similarly, a certain threshold level of cadherin may be required and could explain the difference between E-cad^{-/-} cells and N-cad resc ES cells. Constitutive expression of E-cad or N-cad not only results in the formation of specific tissues, but, moreover, these tissues seem to mediate inductive events similar to those during embryonic development. Cad-11 is expressed in mesenchymal cells surrounding organ anlagen in the embryo (Hoffman and Balling, 1995). We found high expression of cad-11 mRNA in E-cad resc cell tumors in cells surrounding epithelia, and in cartilage in N-cad resc cell tumors, while in E-cad^{-/-} tumors cad-11 expression was rather low and only scattered.

E-cad and T-brachyury expression

We have studied gene expression in the genetically altered ES cell lines generated, reasoning that the basis of the differences in differentiation might already become apparent in the ES cell phenotype. We concentrated on mesoderm-specific genes as good candidates for expression in E-cad^{-/-} ES cells. Indeed, we found high expression of T-brachyury mRNA in such cells. Strikingly, expression of T-brachyury becomes repressed by the constitutive expression of E-cad, but not of N-cad. At this point, we can only correlate the expression of T-brachyury with the presence or the absence of E-cad protein (or RNA) and we cannot exclude the possibility that, by some unknown mechanism, E-cad negatively controls an independent pathway, thus resulting in T-brachyury expression. Although this is possible, it seems more likely that E-cad is more directly involved in regulating T-brachyury expression. We show that E-cad is complexed with catenins in E-cad resc ES cells. β -catenin and plakoglobin are homologues to the segment polarity gene *armadillo* in *Drosophila*. Armadillo is itself a component of the *Drosophila* E-cadherin adhesion complex (Oda et al., 1994), but also of the wingless signaling pathway (Riggleman et al., 1990). β -Catenin and/or plakoglobin may well exhibit similar signaling function (Gumbiner, 1995). In such a scheme, this function would be regulated by E-cad.

Although very attractive, these ideas still need more thorough investigations. Particularly, it remains enigmatic why N-cad is unable to repress T-brachyury expression, although it complexes with catenins in N-cad resc ES cells. The experimental system presented here should allow us to elucidate the molecular interactions that cadherins might be regulating.

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