Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 8263–8267, August 1994
Developmental Biology

E-cadherin null mutant embryos fail to form a trophectoderm epithelium

(homologous recombination/preimplantation embryos/epithelium formation)

LIONEL LARUE, MAMI OHSUGI, JENS HIRCHENHAIN, AND ROLF KEMLER

Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-79108, Freiburg, Germany

Communicated by C. Nüsslein-Volhard, May 16, 1994 (received for review April 6, 1994)

ABSTRACT **The cell adhesion molecule E-cadherin mediates the compaction process of mouse preimplantation embryos and is important for the maintenance and function of epithelial cell layers. To determine precisely the role of E-cadherin in epithelial biogenesis we monitored the developmental potential of embryos homozygously negative for E-cadherin that were derived from E-cadherin heterozygous transgenic mice. The homozygous negative embryos died around the time of implantation, although they did undergo compaction like their littermate controls, largely due to the presence of residual maternal E-cadherin. At the blastocyst stage, E-cadherin-negative embryos failed to form a trophectodermal epithelium or a blastocyst cavity. These results demonstrate the pivotal role of E-cadherin in one of the most basic morphogenetic events in the development of multicellular organisms, the biogenesis of an epithelium.**

Mouse preimplantation embryos represent a unique model for studying the biogenesis of an epithelium from a nonpolarized cell, the fertilized egg (1, 2). During preimplantation development, loosely attached blastomeres maximize their cell–cell contacts and form a compact morula as a prerequisite for the generation of two distinct cell types, those of the trophectoderm epithelium and those of the inner cell mass (ICM). The first morphological changes in spherical blastomeres occur at the 8-cell stage and result in cytocortical asymmetry, including apical membrane microvilli and polarization of the actin cytoskeleton (2). This cellular asymmetry is maintained in the outer cell layer that surrounds the nonpolar inner cells at the 16-cell morula stage. Compaction is mediated by the cell adhesion molecule E-cadherin/uvomorulin (3–5), which is expressed on the cell surface of oocytes and of all cells in early embryos. *De novo* synthesis of E-cadherin due to zygotic gene activity begins at the late 2-cell stage (6–8). Initially, E-cadherin is uniformly distributed on the cell surface of cleavage-stage embryos; redistribution and clustering at cell contact sites occur in 8-cell stage embryos and are concordant with the appearance of the cytocortical asymmetry (6). This redistribution is a developmentally regulated process since it occurs only in cells destined for epithelial differentiation.

To study the requirement for E-cadherin during development more precisely, we inactivated the gene encoding E-cadherin by homologous recombination in embryonic stem (ES) cells and generated mice heterozygous for E-cadherin (E-cad +/−).

MATERIALS AND METHODS

ES Cell Cultures and Transfections. Embryonal stem cell line ES-D3 was derived from 129/Sv blastocysts and was routinely maintained on an inactivated embryonic fibroblast feeder layer (9, 10). Gene transfer experiments were carried out using the electroporation method, and transfected clones were isolated after 10–14 days of culture in medium containing 400 μg of Geneticin (GIBCO) per ml.

The screening of homologous recombined ES cells was performed on 1000 independent G418-resistant ES cell clones using the polymerase chain reaction (PCR). Ten percent of the clones were homologously recombined and the proper integration of the targeting vector was confirmed by Southern blot analyses.

Construction of the Targeting Vector. To construct the pKOUNS3 plasmid, the 5′ *Nsi* I–*Sac* I fragment (positions −991 to +96) and the 3′ *Sac* I–*Acc* I fragment (positions +96 to 2106) of E-cadherin (11) were cloned into the pGN vector (a gift from P. Brulet, Institut Pasteur, Paris) (12). The right arm of the targeting vector contained a mRNA destabilizing sequence (12) of 102 nucleotides (5′-UAA UAU UUA UAU AUU UAU AUU UUU AAA AUA UUU AUU UAU UUA UUU UUA UAA UAA UAU UUA UAU AUU UAU AUU UUU AAA AUA UUU AUU UAU UUA UUU UUA UAA-3′) with two *Ssp* I sites in the 3′ fragment at the *Bam*HI restriction site (position +1929). The HSV-tk cassette was inserted into the construct at the *Acc* I site. The plasmid was linearized with either *Xho* I or *Nsi* I. After homologous recombination, β-galactosidase was under the control of the E-cadherin gene promoter, and the E-cadherin AUG was present downstream of the stop codon of the chimeric neo–E-cadherin mRNA.

Southern Blot Analysis of Homologous Recombination Events. The PCR analyses were performed on DNA derived from about 100 cells. Briefly, cells were rinsed in phosphate-buffered saline (pH 7.3; PBS), Ca^{2+} and Mg^{2+} free, pelleted, resuspended in 10 μl of PBS/double-distilled water (vol/vol = 1:1), and boiled for 10 min. After 5 min on ice, cell extracts were incubated for 30 min at 55°C in the presence of 5 μg of proteinase K, followed by boiling and cooling to 4°C. Nested PCR was performed from extracts using as a first set of β-galactosidase primer LL19 (5′-GGG GAT GTG CTG CAA GGC GAT TA-3′) and E-cadherin primer LL20 (5′-GGA ACA GGA GAG CTT GAG TTC TG-3′) and as a second set of β-galactosidase primer LL18 (5′-GGT TTT CCC AGT CAC GAC GTT G-3′) and E-cadherin primer LL17 (5′-AAA CCT GAT GGA TGT GGG ATG-3′). Ten microliters of the 20-μl boiling extract and 5 μl of the first PCR amplification were mixed with 2.5 μl of dimethyl sulfoxide, and 37.5 μl of PCR mix reaction was added after 10 min. Amplifications were carried out for 33 cycles each of 40 sec at 94°C, 40 sec at 53°C, and 2 min at 72°C.

ES cell DNA was isolated (13), digested with restriction enzymes, separated by agarose gel electrophoresis, transferred on Hybond-N (Amersham), and hybridized with a random-primed probe. Conditions for hybridization were in

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ES, embryonic stem; E, embryonic day; ICM, inner cell mass.

2× sodium citrate buffer (SSC)/1% sodium dodecyl sulfate (SDS)/10% dextran sulfate/0.5% (wt/vol) low fat milk at 65°C, overnight. Filters were washed twice in 2× SSC/0.1% SDS at room temperature for 10–15 min and twice in 0.2× SSC/0.1% SDS at 65°C for 40 min. For hybridization, two exogenous probes were used as depicted in Fig. 1*A* (probe a, *Eco*RI–*Ear* I fragment, positions −1493 to −1333; probe b, *Acc* I–*Xba* I fragment, positions +2106 to +3300). Probe a hybridized to a 6.0-kb fragment of the wild-type allele and the 4.6-kb fragment of the mutant allele from *Eco*RI-digested DNA. Probe b hybridized to a 6.5-kb fragment of the wild-type allele and the 4.8-kb fragment of the mutant allele from *Hin*dIII-digested DNA. The results of the PCR analysis for homologously recombined clones could all be confirmed by Southern blot analysis. To exclude the possibility of additional random integrations of the targeting vector, *Eco*RI-digested DNA was probed with a 3000-bp fragment of the *lacZ* gene (c) and with the HSV-tk gene. No additional integrations were observed.

Embryo Culture and Establishment of ES Cell Lines. ES cells carrying a homologous recombination event were injected into C57BL/6 blastocysts as described (14) and germline chimeric mice were generated. The presence of the mutation in chimeras and progeny was controlled by PCR and Southern blot analysis of *Hin*dIII-digested tail DNA using probe b. Mice heterozygous for E-cadherin were intercrossed. Females, 6–8 weeks old, were superovulated and the day of vaginal plug was taken as day 0.5 (E0.5) of embryonic development. Embryos were collected at day E1.5 or E2.5 and cultured in egg culture medium (15) under mineral oil at 37°C in the presence of 5% CO_2.

Embryos flushed at E2.5 were cultured for 2 days and the zona pellucida was removed with tyrod acid (16). ES cell lines were established on embryonic fibroblast feeder cells in standard ES cell medium (9, 10), which had been supplemented with 1000 international units of leukemia inhibitory factor per ml (ESGRO, GIBCO). DNA was prepared from cells cultured in the absence of feeder cells and subjected to Southern blot analysis.

Indirect Immunofluorescence Tests. Embryos were fixed at room temperature for 30 min in freshly prepared 2.5% paraformaldehyde in saline buffer (pH 7.5) and permeabilized for 10 min with 0.25% Triton X-100. ES cells grown on gelatin-coated coverslips were fixed with cold methanol. An indirect immunofluorescence test was performed with affinity-purified rabbit anti-E-cadherin (anti-gp84), and bound antibodies were revealed with dichlorotriazinylaminofluorescein (DTAF)-conjugated anti-rabbit IgG (Dianova, Hamburg, F.R.G.).

Immunofluorescence tests on live embryos were performed with monoclonal antibody DE-1 (3) at E2.5, and embryos were cultured to determine their developmental potential. The examination had no effect on the growth of embryos but did confirm that only embryos that exhibited a strong staining for E-cadherin developed into blastocysts.

RESULTS AND DISCUSSION

Loss of E-Cadherin Leads to Embryonic Lethality. After electroporation of vector DNA in ES-D3 cells and selection with G418, individual colonies were tested for homologous recombination by nested PCR using the oligonucleotides I-I′ and II-II′ successively (Fig. 1). Ten percent of clones after G418 selection were already found to be homologously recombined, and therefore the selection with ganciclovir was omitted. Integration of the targeting vector into the E-cadherin gene was analyzed by digestion of the genomic DNA with *Hin*dIII, which generated fragments of 6.5 kb from wild-type and 4.8 kb from homologously recombined DNA when hybridized with probe b. The 20 clones identified using nested PCR were all homologously recombined as verified by Southern blotting (Fig. 1*D*). The difference in the hybridization intensity between the 6.5-kb and 4.8-kb bands in E-cad +/− ES cells seen in Fig. 1*D* is due to residual DNA from embryonic fibroblast feeder cells.

Targeted ES cells were injected into C57BL/6 blastocysts and coat color chimeras were bred for germ-line transmission of the targeted gene. Germ-line transmission with two independent targeted ES cell clones was obtained. Mice heterozygous for E-cadherin (E-cad +/−) were healthy without any apparent defect. The heterozygotes were intercrossed and none of the offspring was found to be homozygous (Table 1).

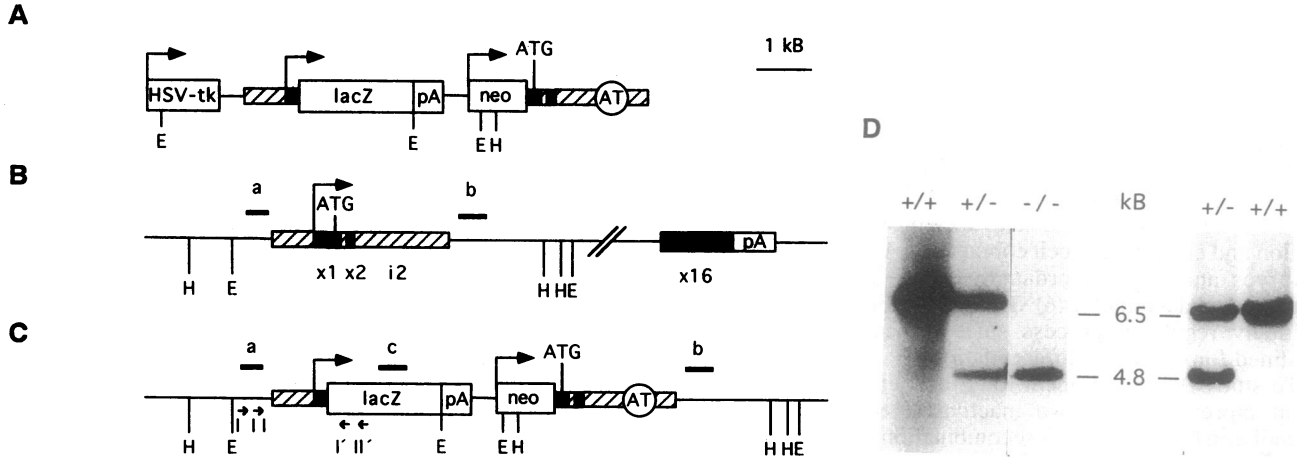

FIG. 1. Disruption of the E-cadherin gene by homologous recombination. (*A*) Targeting vector used for homologous recombination. A HSV-tk cassette was inserted in the same transcriptional orientation as the E-cadherin gene. The left arm of the targeting vector (1 kb) contains the basic E-cadherin promoter, the transcription start site, and the 5′ untranslated region. This fragment was placed upstream of *lacZ* cDNA. The exogenous fragment contains two parts: (*i*) *lacZ* cDNA with its own ATG and stop codon, followed by the poly(A) site of simian virus 40 and (*ii*) a neomycin cassette lacking a poly(A) site. The right arm of the targeting vector (2 kb) contains the 3′ part of exon 1 (x1), including the translation start site, intron 1, exon 2 (x2), and part of intron 2 (i2). A mRNA destabilizing sequence (AT) was placed near the 3′ part of the targeting vector. (*B*) Structure and partial restriction map of the wild-type E-cadherin gene consisting of 16 exons (x3–x15 are not shown). (*C*) Predicted structure of the E-cadherin gene after targeted integration of the vector. Probes used for Southern blot analysis of ES cells and mice (a–c) and positions of the primers used for PCR screening (I, I′, II, II′) are indicated. (*D*) Southern blot analysis with probe b of *Hin*dIII-digested DNA isolated from wild-type (+/+), heterozygous (+/−), and embryo-derived homozygous (−/−) ES cells (*Left*) and wild-type and heterozygous mice (*Right*).

The ratio of heterozygous to wild-type mice was 62.5% to 37.5%, respectively. This result demonstrates that loss of E-cadherin leads to prenatal lethality.

E-Cadherin-Negative Embryos Fail To Form a Trophectodermal Cell Layer. The developmental potential of preimplantation embryos from heterozygous intercrosses was studied since the antibody blocking experiments pointed to the importance of E-cadherin during the compaction process at the morula stage (3–5). Two-cell embryos were cultured individually and photographed every 6 hr (Fig. 2). The developmental potential of each embryo was correlated with the presence of E-cadherin as scored by indirect immunofluorescence tests. Around 25% of the embryos exhibited weak but significant staining for E-cadherin (Table 1 and Fig. 3*B*) and all had a defect in blastocyst formation. The remaining were strongly labeled for E-cadherin (Fig. 3*A*) and had developed into normal blastocysts (Table 1). Embryos genotyped as E-cad −/− by the level of E-cadherin in immunofluorescence were cultured and two ES cell lines negative for E-cadherin were established, while from seven embryos with strong E-cadherin staining, wild-type or E-cad +/− ES cell lines were generated (see below).

At early cleavage stages, no morphological differences were observed and cell divisions were unaltered. E-cad −/− embryos compacted at the 8- to 16-cell stage and were indistinguishable from their littermate embryos (Fig. 2 *E* and *F*). Confocal microscopy revealed the presence of residual maternal E-cadherin, which likely mediates the compaction process (Fig. 3*B*). Residual maternal E-cadherin was also detected later in embryonic development at E4.5 (Fig. 3*D*). These experiments provide evidence of the stability of a maternal E-cadherin in development. At E4.5, when hatching of control blastocysts was observed, E-cad −/− embryos were still in the zona pellucida and the earlier compacted morphology had become looser (Fig. 2*G*). E-cad −/− embryos clearly exhibited a defect in hatching, which likely represents one cause for the prevention of further development. When the zona pellucida was removed at the 8-cell stage, E-cad −/− embryos still compacted, but at E4.5 they lost cell–cell contacts more dramatically (Fig. 2*I*). Possibly, the zona pellucida keeps embryos with reduced adhesiveness together mechanically.

In E4.5 E-cad −/− embryos, an outer cell layer separating from the inner cells and the formation of small cavity-like cysts were occasionally observed (Fig. 3*D*). In the outer cell layer, maternal E-cadherin exhibited the typical basolateral membrane distribution with an enrichment in the most apical part of the lateral membrane. The outer cells were cubical and not elongated as in normal embryos. Their reduced adhesive strength might lead to leakiness in vectorial fluid transport during the formation of the blastocyst cavity.

Loss of E-Cadherin Does Not Cause General Cell Lethality. Forty-seven embryos from four heterozygous intercrosses were freed of their zona pellucida at E2.5 and cultured in embryo medium. At E4.5, 33 had formed blastocysts, while 14 lacked blastocyst formation (Fig. 2 *I* and *J*). When transferred on an embryonic fibroblast feeder layer, all embryos attached and all blastocysts were positive for E-cadherin; for the remaining 14 embryos that did not form blastocysts, 13 were E-cadherin negative and one was E-cad-

Table 1. Analysis of E-cad −/− embryos

Cross	Age of progeny	Mean no. of pups per litter	Genotype +/+	+/−	−/−
(+/−) × (+/−)	Newborn	4.5	57	95	0
(+/−) × (+/+)	Newborn	7.0	407	410	—
(+/−) × (+/−)	E3.5–E4.5	—	165*		54

Analysis of E-cadherin genotypes in offspring generated from heterozygous mice. DNA was isolated from 4-week-old animals and analyzed by Southern blotting. +/+, Wild-type mice; +/−, heterozygotes; −/−, homozygotes. Embryos were scored for their developmental potential to form blastocysts and by immunolabeling with affinity-purified anti-E-cadherin antibodies at E3.5–E4.5. All embryos with weak staining for E-cadherin had failed to grow to blastocysts.

*Wild-type and heterozygous embryos were not discriminated.

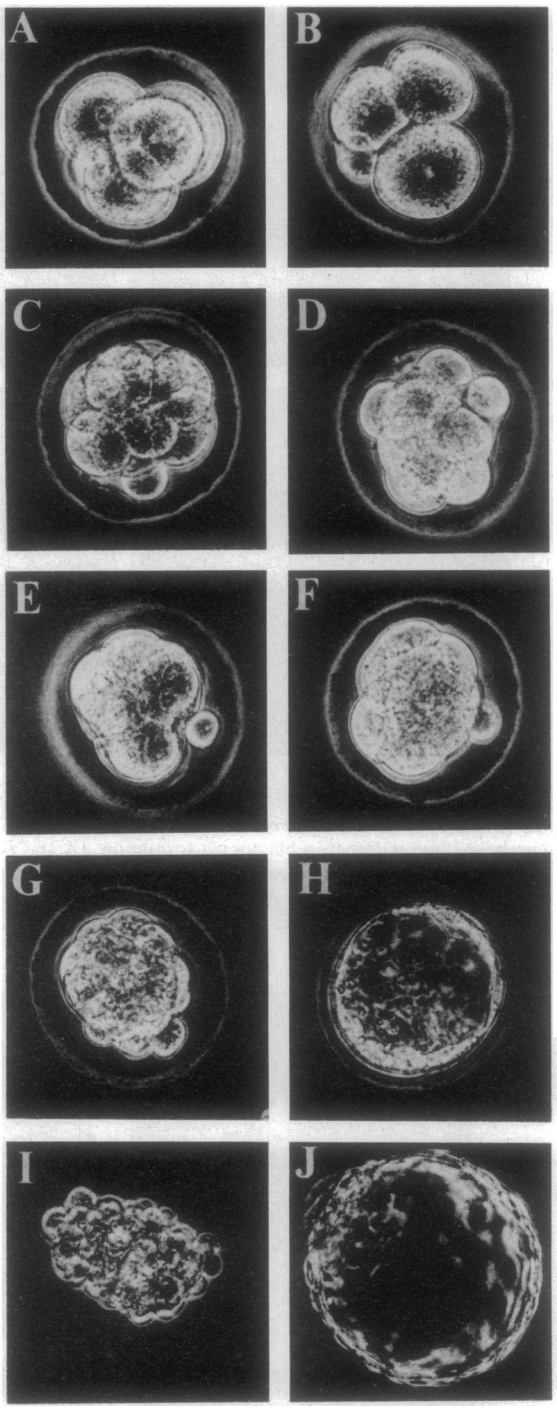

FIG. 2. Preimplantation embryos from mice heterozygous for E-cadherin (+/−) crossed *inter se*. Embryos were collected at E1.5, cultured individually, and photographed every 6 hr. The developmental potential of each embryo was correlated with the presence of E-cadherin at E3.5 and E4.5 (*A*, *C*, *E*, *G*, and *I*) E-cad −/− embryos. (*B*, *D*, *F*, *H*, and *J*) E-cad +/+ or E-cad +/− embryos. (*A* and *B*) Four-cell stage, E1.0. (*C* and *D*) Eight- to 16-cell stage, E2.0. (*E* and *F*) Compacted morula, E2.5. (*H*) Early blastocyst, E3.5. (*J*) Expanded blastocyst, E4.5; the zona pellucida was removed at E2.5. (×270.)

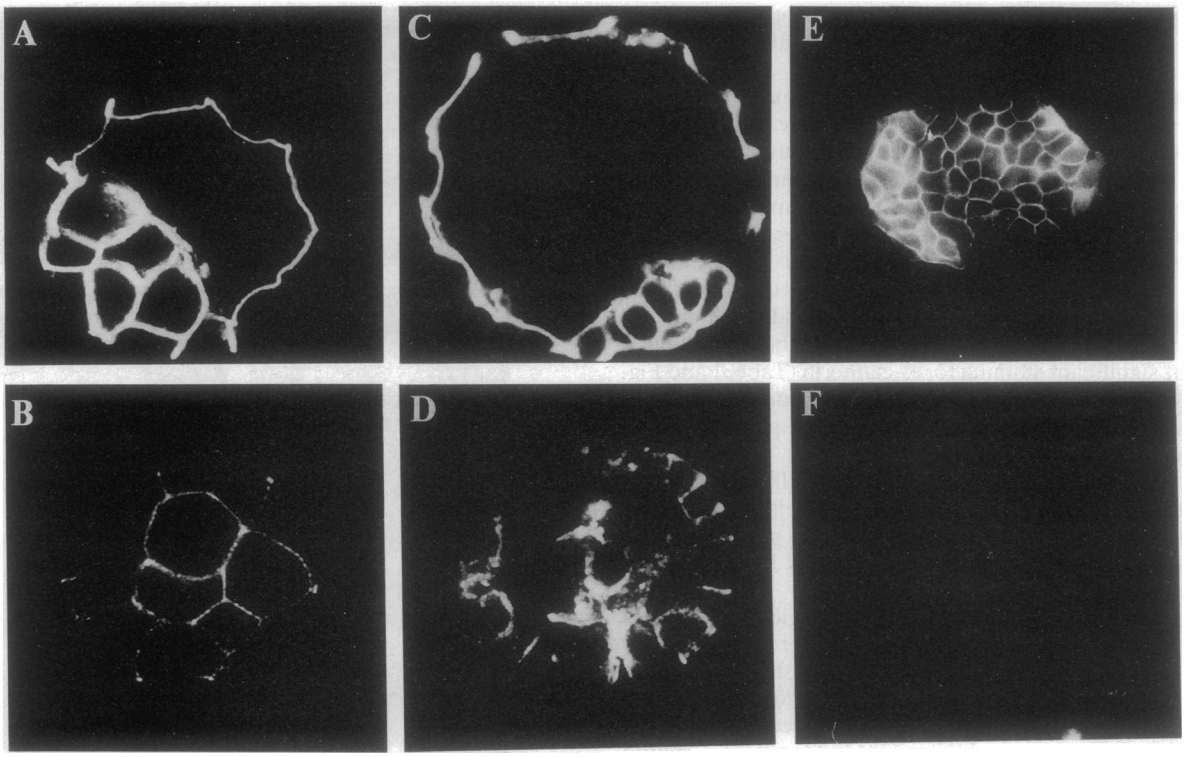

FIG. 3. Immunolocalization of E-cadherin on embryos derived from E-cad +/− intercrosses (*A–D*) and on E-cad +/− and −/− ES cells (*E* and *F*). Embryos (*A–D*) were examined by confocal microscopy. The low abundant E-cadherin in *B* and *D* is maternally derived protein. These embryos had a defect in blastocyst formation. (*A* and *B*) Embryos at E3.5. (*C* and *D*) Embryos at E4.5. (*A–D*) Confocal micrographs. The relative difference in fluorescence intensity in *B* and *D* was due to different exposure times when the digitized images were photographed. Exposure times were as follows: *A*, 0.5 sec; *B*, 3 sec; *C*, 0.25 sec; *D*, 10 sec. (*E* and *F*) Comparative staining with anti-E-cadherin antibodies of ES cells E-cad +/− (*E*) and E-cad −/− (*F*). (*A–D*, ×450; *E* and *F*, ×540.)

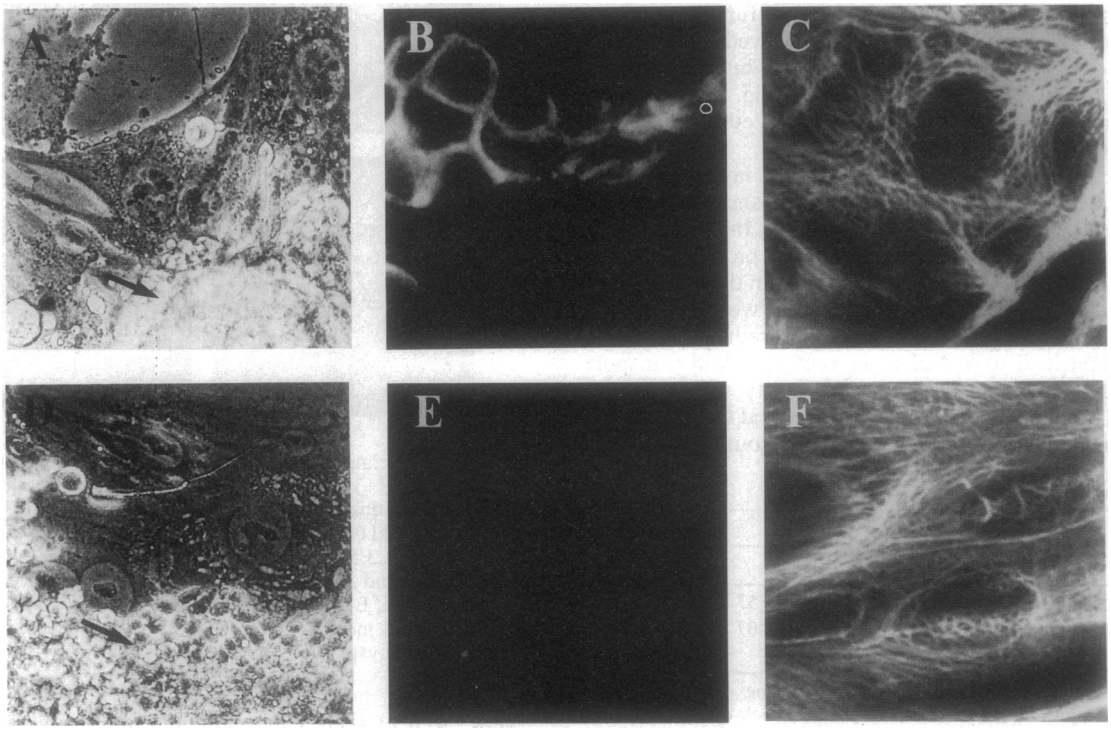

FIG. 4. *In vitro* outgrowth of embryos derived from E-cad +/− intercrosses. Embryos at E4.5 without zona pellucida were cocultured for 3 days on an embryonic feeder layer. (*A–C*) E-cad +/+ or +/− embryos. (*D–F*) E-cad −/− embryos. (*A* and *D*) Phase contrast. (*B* and *E*) Staining for E-cadherin. (*C* and *F*) Staining for cytokeratin filaments. E-cad −/− attached embryos are negative for E-cadherin (*E*) but express cytokeratin polypeptides (*F*). The ICM of E-cad −/− embryos grows more dispersed compared to the compacted mass of cells in controls (*A* and *D*, arrows). The confocal micrographs in *B* and *E* are taken from the optical plane indicated by the arrows in *A* and *D*. (*A* and *D*, ×100; *B*, *C*, *E*, and *F*, ×450.)

herin positive (Fig. 4). Interestingly, with E-cadherin-negative embryos trophectodermal outgrowth was observed, which was, however, less extended than in control embryos. These attached cells expressed cytokeratin polypeptides as monitored with monoclonal antibody TROMA-1 (9), which indicates that at least part of the differentiation program for epithelial ontogenesis occurs in E-cad −/− embryos (Fig. 4 *C* and *F*). Embryonic fibroblast feeder cells were negative for TROMA-1. Compared to littermate control embryos, which exhibited a compacted mass of ICM cells (Fig. 4*A*), the ICM of E-cad −/− embryos was composed of loosely attached cells (Fig. 4*D*). These cells do not express E-cadherin (Fig. 4*E*). From this loose mass of cells, ES cell lines were established that did not grow as typical ES-cell aggregates but, instead, exhibit reduced cell–cell adhesiveness. Immunofluorescence (Fig. 3*F*) and immunoblot experiments (not shown) showed that these cells were negative for E-cadherin. The genotype of these cells was controlled by Southern blot analysis (Fig. 1*D*). These results demonstrate that differentiation programs for trophectodermal and ICM cells occur in E-cad −/− embryos.

Taken together, these experiments demonstrate that E-cadherin is of central importance during the first morphogenetic events of mammalian development. Our results are consistent with the notion that the relative abundance of E-cadherin is crucial in providing both cell polarity and the tightness and integrity of the trophectoderm epithelium. It is likely that E-cadherin molecules necessary for terminal epithelial differentiation accumulate in sufficient amounts during early cleavage stages. Some of these are maternally contributed, which might be sufficient for the compaction process, but additional zygotic E-cadherin is required for the proper initiation and maintenance of epithelial subcellular structures—i.e., organization of the zonula adherens. Since phosphorylation of E-cadherin correlates with compaction, it is possible that post-translational modifications are involved in terminal differentiation of epithelial cells. In addition, a coordinated assembly of the cadherin–catenin complex with the actin filament network plays a central role in this process (17), so the functional role of catenins must also be considered (18).

We thank Julia Felthaus for technical assistance, Lore Lay for the photographs, and Rosemary Schneider for typing the manuscript. Special thanks go to Davor Solter for his helpful comments and to Randy Cassada for critically reading the manuscript. L.L. is supported by a Human Frontier Science Program long-term fellowship.

1. Ekblom, P., Vestweber, D. & Kemler, R. (1986) *Annu. Rev. Cell Biol.* **2**, 27–47.
2. Fleming, T. P. & Johnson, M. H. (1988) *Annu. Rev. Cell Biol.* **4**, 459–485.
3. Hyafil, F., Morello, D., Babinet, C. & Jacob, F. (1980) *Cell* **21**, 927–934.
4. Johnson, M. H., Maro, B. & Takeichi, M. (1986) *J. Embryol. Exp. Morphol.* **93**, 239–255.
5. Vestweber, D. & Kemler, R. (1985) *EMBO J.* **4**, 3393–3398.
6. Vestweber, D., Gossler, A., Boller, K. & Kemler, R. (1987) *Dev. Biol.* **124**, 451–456.
7. Sefton, M., Johnson, M. H. & Clayton, L. (1992) *Development (Cambridge, U.K.)* **115**, 313–318.
8. Kemler, R., Gossler, A., Mansouri, A. & Vestweber, D. (1990) in *Morphoregulatory Molecules*, eds. Edelman, G. M. & Thiery, J.-P. (Wiley, New York), Vol. 41, pp. 41–56.
9. Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W. & Kemler, R. (1985) *J. Embryol. Exp. Morphol.* **87**, 27–45.
10. Robertson, E. J. (1987) in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, ed. Robertson, E. J. (IRL, Oxford), pp. 71–112.
11. Ringwald, M., Baribault, H., Schmidt, C. & Kemler, R. (1991) *Nucleic Acids Res.* **19**, 6533–6539.
12. Le Mouellic, H., Lallemand, Y. & Brulet, P. (1992) *Cell* **69**, 251–264.
13. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), p. 545.
14. Gossler, A., Doetschman, T., Korn, R., Serfling, E. & Kemler, R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9065–9069.
15. Yamamura, H. & Spindle, A. (1988) *J. Exp. Zool.* **248**, 45–54.
16. Hogan, B., Costantini, F. & Lacy, E. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), p. 332.
17. Kemler, R. (1993) *Trends Genet.* **9**, 317–321.
18. Ozawa, M., Ringwald, M. & Kemler, R. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4246–4250.