Lack of β-catenin affects mouse development at gastrulation

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SUMMARY

Molecular analysis of the cadherin-catenin complex elucidated the central role of β-catenin in this adhesion complex, as it binds to the cytoplasmic domain of E-cadherin and to α-catenin. β-catenin may also function in signalling pathways, given its homology to the gene product of the Drosophila segment polarity gene armadillo, which is known to be involved in the wingless signalling cascade. To study the function of β-catenin during mouse development, gene knock-out experiments were performed in embryonic stem cells and transgenic mice were generated. β-catenin null-mutant embryos formed blastocysts, implanted and developed into egg-cylinder-stage embryos. At day 7 post coitum, the development of the embryonic ectoderm was affected in mutant embryos. Cells detached from the ectodermal cell layer and were dispersed into the proamniotic cavity. No mesoderm formation was observed in mutant embryos. The development of extraembryonic structures appeared less dramatically or not at all affected. Our results demonstrate that, although β-catenin is expressed rather ubiquitously, it is specifically required in the ectodermal cell layer.

Key words: embryonic stem cells, mouse embryogenesis, knock-out mutant, cell adhesion, E-cadherin, β-catenin

INTRODUCTION

Evidence accumulating during recent years underlines the importance of cadherin cell adhesion molecules in the development of multicellular organisms (Takeichi, 1991; Kemler, 1992). Although the adhesive function of cadherins has largely been studied in cultured cells, their spatiotemporal expression pattern during early development suggests an essential role in morphogenetic events, such as germ layer formation, gastrulation or neural plate induction (Vestweber and Kemler, 1984; Nose and Takeichi, 1986; Hoffman and Balling, 1995). Specifically, the requirement of E-cadherin (uvomorulin) for the morphogenetic events, such as proliferation, adhesion and differentiation of cells.

Molecular cloning and primary sequence analysis revealed that catenins are homologous to other peripheral cytoplasmic proteins known to be localised in specific cell-cell and/or cell-substratum junctions (Kemler, 1993). β-catenin exhibits high homology to human plakoglobin, a component of desmosomal plaques and adherens junctions (Cowin et al., 1986; Franke et al., 1989), and to the product of the Drosophila segment polarity gene armadillo (Riggleman et al., 1990; Peifer et al., 1992). More recently found members of this protein family include band 6.1 (Hatzfeld et al., 1994), p120 (Reynolds et al., 1994), APC (Kinzler et al., 1991) and SRP1 (Yano et al., 1992). These proteins share a common repeated motif, called the arm repeat, which is thought to be important in mediating specific protein-protein interactions (Peifer et al., 1994a).

Studies on the molecular organisation of the E-cadherin-catenin complex in cultured cells (Ozawa and Kemler, 1992) and in vitro using recombinant proteins (Aberle et al., 1994) clearly demonstrated the central role of β-catenin in complex formation. In agreement with this, in the human epithelial cancer cell line, HSC 39, it has been shown that the lack of E-cadherin-catenin complex formation due to the expression of a truncated β-catenin correlates with the loss of cell adhesion (Oyama et al., 1994). Expression of β-catenin in these cells restores both complex formation and cell adhesion (Kawanishi et al., 1995). Plakoglobin is very likely identical to γ-catenin
and, like β-catenin, it binds to the cytoplasmic domain of E-cadherin (Knudsen and Wheelock, 1992; Aberle et al., 1994). Moreover, two distinct E-cadherin-catenin complexes can be found in the same cell, one composed of E-cadherin, α- and β-catenin, the other of E-cadherin, α-catenin and plakoglobin (Butz and Kemler, 1994; Hinck et al., 1994a). The biological significance of these two distinct cadherin-catenin complexes is not yet well understood, but these experiments suggest that β-catenin can be substituted for by plakoglobin.

Analysis of cadherin expression during early Xenopus (Schneider et al., 1993; Fagotto and Gumbiner, 1994) and mouse development (Butz and Larue, unpublished data) reveals that cadherins have a rather ubiquitous distribution, suggesting an association of cadherins with the various cell type-specific cadherins during development. More recently a possible signalling function of β-catenin was suggested by experiments studying the effect of overexpression and under-expression of β-catenin during Xenopus development (Heasman et al., 1994; Funayama et al., 1995). The possibility that β-catenin might act as a signalling molecule is supported by its homology to Drosophila Armadillo, which is known to participate in the signalling cascade initiated by Wingless that leads to establishing segment polarity during Drosophila embryogenesis (Riggleman et al., 1990; Nordemeier et al., 1994; Peifer et al., 1994b; Peifer, 1995). To gain insights into the function of β-catenin during mouse embryonic development, we have inactivated β-catenin by gene targeting experiments in embryonic stem (ES) cells and have generated transgenic mice. We describe here the phenotype of embryos deficient in β-catenin.

**MATERIALS AND METHODS**

**Construction of the targeting vector**

Genomic DNA corresponding to the β-catenin locus was isolated from a λ-EMBL3 phage library made from 129/Sv mouse DNA. A 7 kb EcoRI fragment containing exons 2 to 8 of β-catenin was subcloned in pBluescript KS+ (Stratagene). A restriction map was established, and exon-intron boundaries were determined by sequence analysis.

A 1.1 kb SphI fragment was deleted, removing exon 2 and part of exon 3 of β-catenin. It was replaced by the 4.5 kb XbaI fragment from plasmid IRES-βgeo (kind gift from Dr A. Smith and Dr P. Soriano), containing the translational initiator IRES (Pelletier and Sonenberg, 1988) placed upstream of the promoterless fusion gene β-galactosidase-neomycin (βgeo) (Friedrich and Soriano, 1991).

**Electroporation, selection and screening of ES cell clones**

R1 ES cells (Nagy et al. 1993) were cultured in the presence of neomycin-resistant embryonic fibroblasts as feeder cells in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal calf serum (FCS), 0.1 mM non-essential amino acids (Gibco), 0.1 mM β-mercaptoethanol, 50 U/ml penicillin, 50 μg/ml streptomycin and 1000 U/ml leukemia inhibitory factor (LIF) (ESGRO, Gibco). 3×10⁷ cells were electroporated with 30 μg of XbaI-linearized targeting construct using a Bio-Rad Gene Pulser (500 μF, 240 V). Recombinant neomycin-resistant clones were selected in the presence of 400 μg/ml G418 (Gibco) for 10 days, transferred into 24-well plates (Nunc) and expanded in 60 mm culture dishes (Falcon). ES cells were passaged on gelatin-coated dishes in 30% Buffalo Rat Liver (BRL) cell-conditioned medium (Smith and Hooper, 1987) without feeder cells, to screen for homologous recombination events by Southern blot analysis. The probes used were a XbaI-EcoRI fragment containing exons 7 and 8 and a BamHI-XhoI fragment containing the neomycin-resistance gene, as indicated in Fig. 1C.

**Generation of β-catenin knock-out mice**

ES cells which had undergone homologous recombination at the β-catenin locus were microinjected into blastocysts from (C57BL/6 × DBA/2) superovulated mice. Male chimeras, as judged from the coat color, were bred with C57BL/6 females to test for germline transmission. Tail DNA from agouti offspring was used to test for the presence of the mutation by Southern blot analysis. Germline chimeras were crossed with 129/Sv females to put the mutation on an inbred background. Offspring were routinely tested for the lacZ gene by X-Gal staining of ear-clips (Felthaus et al., 1994), and those that were used for maintenance of the mouse line were also genotyped by a Southern blot of tail DNA.

**PCR analysis of β-catenin genotypes**

Extracts were prepared from either neonate tails, dissected day 7.5 p.c. embryos, scraped paraffin sections or in vitro cultured blastocysts. Samples suspended in 10–20 μl of phosphate-buffered saline (PBS)/H₂O (1:1) were boiled for 10 minutes, cooled on ice and incubated for 3 hours to overnight in the presence of 1 μg/ml proteinase K (Boehringer). After an additional 10 minutes boiling, 5 μl of supernatants were subjected to PCR amplification.

Primers for the PCR reaction were from the neo gene (B: 5'-CTCTTCTCTGTCTTTACGGTA-3') and the 5' and 3' portions of exon 3 of β-catenin (A: 5'-ATATTGACGGCGATGCA-3' and C: 5'-TCACTGCCTGGATGGAT-3'), see scheme in Fig. 3A). The PCR mix consisted of 250 pM of each oligonucleotide, 400 μM dNTPs (Pharmacia), 1.5 mM MgCl₂ and 2.5 U Taq polymerase in Taq buffer (USB) in a final volume of 50 μl. Temperature cycling conditions were at 95°C for 40 seconds, 55°C for 30 seconds and 2 minutes at 72°C for 35 cycles. PCR products were resolved on 2% agarose gels.

**Histological analysis of mutant embryos and in situ hybridisation**

Embryos within their decidua were fixed with 4% paraformaldehyde in PBS overnight at 4°C, washed in PBS overnight at 4°C, dehydrated in graded alcohols, embedded in paraffin, sectioned at 7 μm, and stained with hematoxylin and eosin.

For in situ hybridisation, digoxigenin-labelled riboprobes were prepared from plasmids pMash-2 (gift from Dr F. Guillemot), a 273 bp Bluescript subclone of Otx2 cDNA (gift from Dr P. Brület) and a 1.8 kb Bluescript subclone of T-Brachyury (gift from Dr B. Herrmann), using a DIG-RNA Labelling Kit (Boehringer lot no. 1175025). In situ hybridisation was performed as previously described (Jostarndt and Puntschart 1994) using the DIG Nucleic Acid Detection Kit (Boehringer lot no. 1175041). Slides were lightly counterstained with eosin.

In vitro culture of preimplantation embryos **β-catenin+/−** offspring were crossed inter se after superovulation of 7- to 9-week-old females. At day 1.5 or 2.5 p.c. (vaginal plugs were scored as day 0.5 p.c.), embryos were collected from the oviducts in flushing medium (Spindle, 1980) and cultured in TE medium (Yamamura and Spindle, 1988) at 37°C in 5% CO₂ atmosphere. At day 3.5 p.c., embryos were transferred to R1 medium (ES cell culture medium) without LIF, in 10% CO₂ atmosphere. Photographs were taken every 6 hours using an Axiovert 35 phase-contrast microscope (Zeiss). Embryos at day 5.5 or 8.5 p.c. were fixed and immunostained with antibodies directed to β-catenin, E-cadherin or plakoglobin.

For blastocyst outgrowth experiments, embryos were collected at day 2.5 p.c., freed from their zona pellucida by treatment with tyrode acid, transferred on gelatin-coated Petriperm dishes (Heraeus) and individually cultured in ES cell culture medium without LIF in 10%
CO₂ atmosphere at 37°C. Sizes of trophectoderm outgrowths were measured, and embryos were fixed and genotyped by PCR.

Antibodies and indirect immunofluorescence tests
Antibodies directed against the extracellular part of E-cadherin (DECMA1; Vestweber and Kemler, 1985) and anti-peptide antibodies against β-catenin (α-M14M; Aberle et al., 1994) or plakoglobin (α-D15A; Butz and Kemler, 1994) were affinity-purified and used as previously described. Appropriate secondary antibodies conjugated with rhodamine were from Dianova.

Double staining experiments for β-catenin and E-cadherin were performed as follows: embryos were fixed with 0.5% paraformaldehyde in Hepes-buffered saline containing 2 mM CaCl₂, followed by cold (~20°C) methanol for 10 minutes and permeabilization in 0.5% Triton X-100 for 10 minutes. After a blocking step with 1% FCS in PBS for 30 minutes, the embryos were incubated with primary antibodies (DECMA1 and anti-β-catenin) and then with the respective fluorophore-conjugated secondary antibodies (1 hour each incubation step). Embryos were mounted in 50% glycerol/50% PBS containing 100 mg/ml 1,4-diazabicyclo[2.2.2]octane (Sigma), and observed under a fluorescence microscope (Zeiss) or a confocal krypton-argon laser scan microscope (Leica). Digital images were printed on Fujix Pictureography 3000 (Fuji Photo Film).

For staining of plakoglobin, samples were permeabilized in 0.5% Triton X-100 for 10 minutes, fixed in methanol for 10 minutes and subjected to immunofluorescence experiments using anti-plakoglobin antibodies as described above.

RESULTS

High frequency of β-catenin gene targeting in ES cells
To disrupt the β-catenin gene by homologous recombination, a genomic region was cloned from a DNA library of mouse strain 129/Sv, and the structure of the gene was partially established (Fig. 1B). A replacement-type targeting vector was constructed (pBCKO), in which exon 2 and a portion of exon 3 of the β-catenin gene were replaced by the IRES-geo cassette (Fig. 1A). The internal ribosome entry site (IRES) element (Oh and Sarnow, 1993; Mountford and Smith, 1995) allows translational initiation by ribosomal subunits 5’ of the AUG of the fusion gene βgeo (Friedrich and Soriano, 1991), thereby driving preferential translation of the βgeo sequence within the fusion transcript. The IRES-geo cassette contains neither promoter nor polyadenylation signal elements. After homologous recombination βgeo should come under the control of β-catenin transcriptional regulatory elements, which should reduce the overall number of neomycin-resistant ES cell clones and enrich for homologous recombination events.

After transfection and selection of 3×10⁷ ES cells, approximately 300 neomycin-resistant clones were obtained. Genomic DNAs from 125 of these clones were digested with EcoRI or SspI and analysed by Southern blotting with a genomic probe containing exons 7 and 8 of β-catenin (Fig. 1C). The mutated and wild-type alleles gave a 5.6 kb or a 7 kb fragment after EcoRI digestion (Fig. 1D), and a 12 kb or an 11 kb fragment after SspI digestion (not shown). The frequency of homologous recombination was remarkably high, with 80% of neomycin-resistant ES cell clones having integrated the targeting vector at the β-catenin locus. The presence of a single copy of pBCKO in homologously recombined clones was confirmed with a neo probe (Fig. 1C), which hybridised to a 5.6 kb fragment after EcoRI, and to a 12 kb fragment after SspI digestion (not shown).

Lack of β-catenin induces embryonic lethality
Targetted ES cell clones were injected into C57BL/6 × DBA/2 blastocysts and male chimeras were bred with C57BL/6 females to screen for germline transmission of the mutation. For 4 out of 8 injected ES cell clones, the targeted allele was transmitted through the germline. These founder animals were crossed with 129/Sv females to establish inbred mouse lines. Heterozygous animals (β-catenin+/–), genotyped by Southern blot analysis of tail DNA, appeared healthy and normal (not shown).

When β-catenin+/– animals obtained from the 4 transgenic mouse lines were intercrossed, no β-catenin–/– offspring were found. From a total number of 87 offspring, 33 turned out to be wild type and 54 to be β-catenin+/– genotype. These results demonstrate that loss of β-catenin induces embryonic lethality.

It has been shown previously that E-cadherin-null mutant embryos die around the blastocyst stage, when the trophoderm epithelium cannot form properly (Larue et al., 1994). Given the central role of β-catenin for E-cadherin-catenin
Fig. 2. Histological characterisation of β-catenin−/− embryos from blastocyst to day 8.5 p.c. The development of wild-type (A,D,F,H) and β-catenin−/− embryos (B,E,G,I) are compared. No differences were observed between wild-type (A) and mutant (B) blastocysts at day 4.5-p.c., and postimplantation (C) egg-cylinder stage embryos (transversal section) at day 6.5 p.c. The disruption of the ectodermal epithelium with detached cells in the proamniotic cavity (arrow head), is the primary defect detected in β-catenin−/− embryos at day 7.0 p.c. (E). A wild-type embryo at day 7.0 p.c. is shown (D). At day 7.5 p.c. embryonic structures are no longer recognisable in mutant embryos (G). A wild-type embryo at day 7.5 p.c. is depicted (F). When wild-type (H) and mutant (I) embryos at day 8.5 p.c. were compared, the latter appeared rather degenerated, including necrotic areas (arrow head); al, allantois; am, amniotic cavity; ec, ectoderm; en, endoderm; ep, ectoplacental cavity; epc, ectoplacental cone; ex, exocoelomic cavity; icm, inner cell mass; me, mesoderm; pa, proamniotic cavity; pen, parietal endoderm; re, Reichert’s membrane; tr, trophectoderm; ven, visceral endoderm; ven-l, visceral endoderm-like; xec, extraembryonic ectoderm; yc, yolk cavity; zp, zona pellucida. No morphological differences were found between β-catenin+/+ and +/− embryos. Scale bars: A,B, 27 μm; C-E, 65 μm; F,G, 130 μm; H,I, 195 μm.
complex formation, it seemed quite possible that β-catenin mutant embryos would exhibit a similar phenotype during preimplantation development. Therefore, embryos obtained from β-catenin null intercrosses were cultured individually, photographed at regular intervals and immunostained for β-catenin. At day 3.5 postcoitum (p.c.), null mutant embryos could not be unambiguously distinguished from their littermates by immunostaining, due to the presence of residual maternal β-catenin (Ohnuki et al., unpublished data). However, between days 4.5 and 5.5 p.c., β-catenin staining was clearly reduced or negative in 15 (19%) of 78 blastocysts. No obvious morphological differences could be observed between embryos positive or negative for β-catenin (Fig. 2A,B), and embryos of all genotypes hatched in vitro and attached to the culture plates. These results suggest that embryos lacking zygotic gene activity for β-catenin can develop normally to the end of preimplantation development.

**Lack of β-catenin affects embryonic ectoderm at the gastrulation stage**

To determine unambiguously the developmental potential of β-catenin null embryos during postimplantation development, embryos were genotyped individually by PCR, as outlined in Fig. 3 (top panel), either as dissected out of their deciduae, or using embryonic material peeled off from paraffin sections. At day 6.5 p.c., all embryos had developed to normal egg-cylinder-stage embryos, containing embryonic and extraembryonic parts and proamniotic cavities. Specifically, the embryonic ectodermal cell layer was typically composed of high columnar epithelial cells surrounded by visceral endodermal cells (Fig. 2C). First signs of a defective phenotype became apparent around day 7.0 p.c. in the embryonic ectodermal cell layer (Fig. 2E). Cells detached from the ectodermal cell layer and were dispersed into the proamniotic cavity (Fig. 2E). At day 7.5 p.c., the phenotype of β-catenin null embryos became more dramatic and clearly visible when embryos were dissected out of their deciduae (Fig. 3A,B). The mean size of homozygous mutant embryos (0.8 mm) was half of that of their wild-type or heterozygous littermates (1.7-1.8 mm). Of 52 embryos, 12 (23%) were β-catenin null, reflecting a Mendelian distribution and full penetrance of the mutation (Table 1). Histo logical sections from day 7.5 p.c. embryos revealed that the three cavities (ectoplacental, exocoelomic and amniotic) had not formed in β-catenin null embryos (Fig. 2G). Instead, a condensed mass of cells was located in the region adjacent to the ectoplacental cone (EPC), which occasionally contained a small cavity (compare Fig. 2F,G). The embryos appeared disorganised; the three germ cell layers and the amniotic folds as well as the allantois had all failed to form. In particular, the typical epithelial organization of embryonic ectoderm was entirely lost. Extraembryonic structures seemed to develop normally, because trophoblast derivatives including the EPC were comparable to those of normal embryos. Parietal endoderm and Reichert’s membrane had also formed and delimited a large yolk sac cavity filled with amorphous material. However, the cellular morphology of parietal endodermal cells was different in mutant embryos (Fig. 2G).

The condensed mass of cells located under the EPC was largely composed of extraembryonic cells as monitored by in situ hybridisation for Mash-2. Mash-2 transcripts are expressed in extraembryonic and trophoblast derivatives (Fig. 4A) (Guilleminot et al., 1994). In mutant embryos, a Mash-2 probe hybridised to trophoblast derivatives and to the cell aggregate located under the ectoplacental cone (Fig. 4B). Only the cell population facing the yolk sac cavity did not contain Mash-2 mRNA. Hence, it may have been derived from the embryo proper. The gene Otx2 is normally expressed in the embryonic epiblast at day 6.5 p.c. (Fig. 4C) and in the anterior neuroectoderm of day 7.5 p.c. embryos (Simeone et al., 1993). Otx2 mRNA was hardly detected in β-catenin null embryos, only a weak, diffuse signal being observed occasionally (Fig. 4D). No expression of T-Brachyury mRNA, a mesodermal cell marker (Wilkinson et al., 1990), was detected in β-catenin null embryos (not shown).

**Table 1. β-catenin null embryos show an abnormal phenotype at day 7.5 p.c.**

<table>
<thead>
<tr>
<th>Genotype of embryos</th>
<th>Number of embryos</th>
<th>Mean size (mm)</th>
<th>Morphology</th>
</tr>
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<tbody>
<tr>
<td>β-catenin+/−</td>
<td>11 (21%)</td>
<td>1.6±0.1</td>
<td>normal</td>
</tr>
<tr>
<td>β-catenin−/−</td>
<td>29 (56%)</td>
<td>1.7±0.3</td>
<td>normal</td>
</tr>
<tr>
<td>β-catenin−/−</td>
<td>12 (23%)</td>
<td>0.8±0.2</td>
<td>abnormal</td>
</tr>
</tbody>
</table>

*Whole day 7.5 p.c. embryos were dissected from the deciduae and genotyped according to the PCR strategy shown in Fig. 3. Sizes of the embryos were measured after removal of Reichert’s membranes.

Fig. 3. Abnormal development of β-catenin null embryos at day 7.5 p.c. β-catenin null embryos dissected out of their deciduae appeared smaller than their wild-type littermates (see also Table 1). Particularly the embryonic structures were less developed. (A) Wild-type embryo; (B) homozygous mutant. The PCR strategy for genotyping such embryos is shown on the upper left panel. Using oligonucleotides hybridising to the 5’ region of exon 3 (A−), to β-geo (B−) and to the 3’ region of exon 3 (C−) allows simultaneous amplification of wild-type and mutant alleles. Exons are depicted as hatched boxes, introns as horizontal lines, and IRES β-geo as a grey box. DNA fragments of 100 bp (wild-type allele) and 290 bp (mutant allele) were separated according to size on a 2% agarose gel (upper right panel). Corresponding genotypes are indicated. *, oligonucleotides remaining after the PCR reaction.
At day 8.5 p.c., \( \beta \)-cat \(-/-\) embryos remained disorganised and increasing cell death was observed in several areas (Fig. 2I). Surrounding visceral endoderm cells with apical vacuoles, as well as parietal endoderm and Reichert’s membrane, appeared to be less affected. Signs of necrosis and large quantities of red blood cells were found in the decidual tissue surrounding the embryos. Resorption continued at day 9.5 p.c., by which time all remaining organised structure had almost completely disappeared (not shown).

Taken together, these results show that \( \beta \)-cat \(-/-\) embryos are able to develop normally until day 6.5 p.c. and form embryonic and extraembryonic structures at the early egg-cylinder stage. At the early primitive streak stage (day 7.0 p.c.), when gastrulation normally occurs, embryonic structures cannot form properly. In particular, the further development of the ectodermal cell layer seems to be specifically impaired, while extraembryonic tissues (ectoplacental cone, parietal and visceral endoderm) are less affected.

**In vitro development of \( \beta \)-cat \(-/-\) embryos**

Blastocysts from \( \beta \)-cat \(+/−\) intercrosses were either subjected to indirect immunofluorescence tests or were cultured to examine whether a mutant phenotype also becomes apparent during blastocyst outgrowth. After morphological examination or immunostaining, individual embryos were genotyped by PCR. As already mentioned, low abundant maternal \( \beta \)-catenin protein can still be detected in day 4.5 p.c. embryos (Ohsugi et al., unpublished data) but progressively disappears in \( \beta \)-cat \(-/-\) expanded blastocysts (Fig. 5B). Staining for E-cadherin gave a comparable pattern in \( \beta \)-cat \(-/-\) and littermate blastocysts, in that the protein was localized at the lateral membrane of trophodermal epithelial cells and at the surface of the inner cell mass (Fig. 5C,D). Staining intensity for E-cadherin appeared to be slightly weaker in \( \beta \)-cat \(-/-\) embryos compared to heterozygous or wild-type littermates (Fig. 5C,D). A potentially more interesting difference was observed for plakoglobin. It has been reported that plakoglobin localises in the newly formed desmosomes by the blastocyst stage (Fleming et al., 1991). This might be reflected by the punctate staining in the trophodermal cell layer of wild-type embryos (Fig. 5E). In \( \beta \)-cat \(-/-\) blastocysts, however, a more uniform and enhanced staining for plakoglobin was consistently observed over the entire lateral membrane of trophodermal epithelial cells (Fig. 5F). The membrane localisation of plakoglobin appeared superimposable on that of E-cadherin. These results suggest a compensatory mechanism whereby plakoglobin could substi-
tute for β-catenin in the cadherin-catenin complex of β-cat −/− embryos and enable mutant embryos to form a functional trophectoderm.

In another series of experiments, blastocysts were cultured for 4-6 days to examine the mutant phenotype during in vitro culture. Trophoblast outgrowth appeared equally well developed in mutant and wild-type embryos (Fig. 6). However, in the developing ICMs of β-cat −/− embryos cells lost their contacts with time and became dissociated; groups of loosely associated cells detached from the trophoblast, occasionally continuing to proliferate in distant areas of the culture dish. This β-cat −/− phenotype during development in vitro correlates with that of postimplantation embryos.

**DISCUSSION**

We describe here the inactivation of the gene encoding for β-catenin by homologous recombination in ES cells and the generation of transgenic mice. We show that lack of β-catenin results in embryonic lethality; more specifically, our analysis points to a primary defect in the embryonic ectoderm cell layer of day 6.5-7.5 p.c. embryos.

During early mouse embryonic development, two true epithelial cell layers are formed: the trophectoderm at the blastocyst stage and the embryonic ectoderm at the egg-cylinder stage. Trophectoderm formation is not affected in β-cat −/− embryos, most likely due to the persistence of maternal β-catenin, which might to some extent compensate the lack of zygotic gene activity. In addition, we observed an enhanced localisation of plakoglobin at the lateral membrane of trophectodermal epithelial cells, which suggests that plakoglobin can substitute for β-catenin to assure a functional E-cadherin-catenin complex at this developmental stage. It remains unclear whether the increase of plakoglobin is due to increased transcriptional activity or prolonged protein stability.

Our in vivo and in vitro data on β-cat −/− embryos indicate that the embryo develop into egg cylinders composed of embryonic and extraembryonic ectoderm and of parietal and visceral endoderm. Histological examination, as well as the expression of cell-type-specific genes Mash-2 and Otx-2, points to a primary defect in the maintenance of embryonic ectoderm. Extraembryonic structures, including trophoblast, ectoplacental cone and visceral and parietal endoderm, appear normal and/or are only affected in consequence of the primary defect. At early gastrulation stage, the embryonic ectodermal cell layer is subjected to increased cell proliferation and morphogenetic movements, such as induction of mesoderm, primitive streak formation, and the establishment of the anterior-posterior axis. This all requires a continuous integration of rapidly dividing cells into the epithelial cell layer. On the cellular level, a constantly occurring transition between polarised and dividing, non-polarised epithelial cells has to be assured with a precise control of cell adhesion, cytoskeletal organisation, and cell polarity.

A possible explanation of the phenotype in β-cat −/− embryos is that the lack of β-catenin affects the adhesive properties of E-cadherin in embryonic ectodermal cells, leading to an improper integration of dividing cells into the ectodermal cell layer. This view is supported by the central role β-catenin plays in complex formation, binding both E-cadherin and α-catenin (Ozawa and Kemler, 1992; Aberle et al., 1994). Although plakoglobin has been reported to bind to E-cadherin and α-catenin (Butz and Kemler, 1994; Hinck et al., 1994a), it apparently can not substitute for the function of β-catenin in embryonic ectoderm. Thus, in spite of the high degree of homology between β-catenin and plakoglobin, each protein most likely exhibits unique properties, some yet to be discovered.

Given the homology of β-catenin and plakoglobin to armadillo and the known involvement of armadillo in the wingless (Wnt) pathway, these proteins may well be part of signalling pathways. First support for such ideas came from recent findings that elevated expression levels of β-catenin or plakoglobin in response to wnt-1 expression can increase cadherin-mediated adhesion in cultured cells (Bradley et al., 1993; Hinck et al., 1994b). However, since the embryonic phenotype of wnt-1 deficiency, which leads to defects in brain development (McMahon and Bradley, 1990), and the β-cat −/− phenotype reported here are distinct, we do not currently favor a direct molecular interaction of both proteins. It is possible that in embryonic ectoderm β-catenin is the target for
another member of the family of wnt signalling molecules. Additional support for a possible signalling function of β-catenin comes from studies of early Xenopus development. Overexpression of β-catenin in the ventral side of early Xenopus embryos induced the formation of a secondary body axis (Funayama et al., 1995); analysis of β-catenin deletion constructs suggested that axis duplication might be induced by an intracellular signalling capacity of β-catenin. More informative for the interpretation of our results is the work of Heasman and colleagues on β-catenin depletion, which led to an inhibition of dorsal mesoderm formation during Xenopus development. Mesoderm formation was also inhibited in β-catenin−/− mouse embryos, as monitored by the absence of T-Brachyury gene expression. Since in Xenopus, depletion of β-catenin had no apparent effect on the calcium-dependent aggregation of isolated blastomeres, the effect on dorsal mesoderm induction was explained by a role of β-catenin in signalling pathways (Heasman et al., 1994). It may well be that β-catenin exerts a signalling function in the mouse embryonic ectoderm in addition to its role in cadherin-catenin complex formation. This could explain why lack of this widely expressed protein results in specific defects in the embryonic ectodermal cell layer. The availability of β-catenin-negative ES cell lines established from β-catenin−/− embryos will be helpful for further analysis of β-catenin function.

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