Maternal β-catenin and E-cadherin in mouse development

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Summary

The oocyte to embryo transition in metazoans depends on maternal proteins and transcripts to ensure the successful initiation of development, and the correct and timely activation of the embryonic genome. We conditionally eliminated the maternal gene encoding the cell adhesion molecule E-cadherin and partially eliminated the β-catenin gene from the mouse oocyte. Oocytes lacking E-cadherin, or expressing a truncated allele of β-catenin without the N-terminal part of the protein, give rise to embryos whose blastomeres do not adhere. Blastomere adhesion is restored after translation of protein from the wild-type paternal alleles: at the morula stage in embryos lacking maternal E-cadherin, and at the late four-cell stage in embryos expressing truncated β-catenin. This suggests that adhesion per se is not essential in the early cleavage stage embryos, that embryos develop normally if compaction does not occur until the morula stage, and that the zona pellucida suffices to maintain blastomere proximity. Although maternal E-cadherin is not essential for the completion of the oocyte-to-embryo transition, absence of wild-type β-catenin in oocytes does statistically compromise developmental success rates. This developmental deficit is alleviated by the simultaneous absence of maternal E-cadherin, suggesting that E-cadherin regulates nuclear β-catenin availability during embryonic genome activation.

Key words: Mouse, Maternal, E-cadherin, β-catenin, Embryonic genome, Adhesion, Compaction

Introduction

In metazoans, the first phase of embryonic development occurs during a period of transcriptional silence when the embryo is dependent on stored maternal RNAs and proteins. Maternal RNAs are either translated and degraded (Huarte et al., 1987), or deadenylated and stored for later translation (Oh et al., 2000). In the pre-implantation mammalian embryo the genomes of two differentiated cells, the sperm and egg, are combined and reprogrammed to give rise to a new totipotent embryo. Once this is accomplished, cleavage divisions reduce the size of each cell until the blastomeres become polarized and flatten against each other maximizing intercellular contact, which in mice occurs at the 8-cell stage (Fleming and Johnson, 1988; Johnson, 1996).

Null mutants of both E-cadherin and β-catenin exhibit an early embryonic lethal phenotype. E-cadherin null embryos fail to form an intact trophectoderm cell layer (Larue et al., 1994). Lack of β-catenin results in early gastrulation lethality, with no mesoderm formation and a block in anterior-posterior axis formation and head development (Haegel et al., 1995; Huelsken et al., 2000). It was postulated that these null embryos developed as far as they did because stable residual maternal proteins or new synthesis from maternal transcripts could partially rescue the phenotype. However, these studies did not query the outcome of embryo development in the absence of the maternal protein.

E-cadherin (uvomorulin), the prototype and founding member of the cadherin superfamily of calcium-dependent cell adhesion molecules, plays a central role in cell adhesion and determination of cell shape (Kemler et al., 1977; Yagi and Takeichi, 2000). E-cadherin has an extracellular domain that allows homophilic interaction with E-cadherin molecules on neighboring cells. Stable and functional adherens junctions are formed by interaction of the cytoplasmic tail of E-cadherin with the catenins, which in turn interact with the actin cytoskeleton (Nagafuchi, 2001). E-cadherin mediates compaction of the individual blastomeres in the 8-cell stage embryo, and this adhesion triggers the development of the trophectoderm and other epithelial cell layers at later developmental stages (Gumbiner, 1996). In embryos, mutation of E-cadherin, perturbation of the function of molecules interacting with E-cadherin, or disturbance of the compaction process, all lead to the relocation of E-cadherin, and of the molecules interacting with it (Clayton et al., 1999; Ohsugi et al., 1997; Pey et al., 1998).

β-catenin is one of several intracellular mediators necessary for the maintenance and function of E-cadherin in cell-cell interactions, as well as being a central player in the WNT signal transduction pathway (Kemler et al., 1989). This dual role is defined by its cellular localization and protein-binding partners (Gottardi and Gumbiner, 2001; Miller and Moon, 1996).

β-catenin binds to the cytoplasmic domain of E-cadherin (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989), and to α-catenin, which connects the E-cadherin-catenin adhesion complex with the actin filament network. β-catenin is also bound by dynein and may thus tether microtubules at adherens

junctions, ensuring the interactions between microtubule and actin networks thought to be crucial for mechanical and signaling events in the cell cortex (Ligon et al., 2001).

In somatic cells, β-catenin is the central component of the WNT/β-catenin signal-transduction pathway. Upon WNT receptor-ligand binding and several intermediate steps, β-catenin translocates to the nucleus, and, in association with transcription factors of the TCF/LEF1 family, controls the expression of target genes. In the absence of a WNT signal cytosolic β-catenin associates with a multimeric protein complex, consisting of APC (adenomatous polyposis coli), GSK3B (glycogen synthase kinase 3β), CSNK1A1 (casein kinase Iα) and AXIN, in which it is phosphorylated and marked for degradation by the ubiquitin-proteasome pathway (Gottardi and Gumbiner, 2001; Liu et al., 2002). β-catenin is also key in a number of intracellular pathways: β-catenin binds to transcriptional co-activators and to a component of the SWI/SNF chromatin-remodeling complex to activate transcription either by recruiting general transcription factors to target gene promoters, or by changing chromatin structure (Barker et al., 2001; Hecht et al., 2000; Miyagishi et al., 2000; Nielsen et al., 2002; Takemaru and Moon, 2000).

To determine the role of E-cadherin and β-catenin during the oocyte to embryo transition, we used the oocyte specific *Zp3-cre* transgene (de Vries et al., 2000), in combination with proven *loxP*-tagged (floxed) alleles of these molecules. The E-cadherin floxed allele has been used to determine the role of E-cadherin in the lactating mammary gland, the adherens junctions in the epidermis, hair follicle formation and the peripheral nervous system (Boussadia et al., 2002; Young et al., 2002; Young et al., 2003). The floxed β-catenin allele has been successfully used to delineate a role for β-catenin in brain development, in development of the ectodermal ridge and neural crest, in vascular development, and in the embryonic endoderm (Barrow et al., 2003; Brault et al., 2001; Cattelino et al., 2003; Hari et al., 2002; Lickert et al., 2002; Machon et al., 2003). We now report that deletion of this floxed β-catenin allele only partially deletes the β-catenin gene, resulting in a truncated protein without its N-terminal part.

Using combinations of the *Zp3-cre* transgene and floxed alleles, we found that these molecules are crucial for maintaining blastomere adhesion, but that such cell contact is not essential for initiation of development. These results also suggest that the WNT/β-catenin signaling pathway is probably not functional at this time in development. Interestingly, the absence of maternal E-cadherin in combination with the partially deleted β-catenin allele results in rescue of the loss-of-embryo phenotype found in females whose oocytes express truncated β-catenin. A role for E-cadherin-β-catenin interaction during the oocyte to embryo transition is suggested.

Materials and methods

Mice and genotyping

B6.129-*Catnb*^{tm2Kem} mice (Brault et al., 2001) containing the floxed β-catenin allele (β^F), as well as B6.129-*Cdh1*^{tm2Kem} mice (Boussadia et al., 2002) containing the floxed E-cadherin allele (E^F), were backcrossed to C57BL/6J for ten generations and intercrossed to generate mice homozygous for each floxed allele. To generate embryos lacking either maternal β-catenin or E-cadherin, or both, mice were crossed as described in Fig. 1.

Genotyping of all mice was done by PCR using DNA extracted from tail snips of 21-day-old mice using a MasterPure™ DNA purification kit (Epicentre, catalog number MCD85201). To determine the presence of the *Zp3-cre* transgene, a primer pair specific for cre-recombinase was used: 5′-TGATGAGGTTCGCAAGAACC-3′/5′-CCATGAGTGAACGAACCTGG-3′. Primer pairs used to detect the different alleles of β-catenin and E-cadherin were as described (Boussadia et al., 2002; Brault et al., 2001).

Embryo isolation

All embryos were obtained from timed matings of four-week old females, as described (Evsikov et al., 2004), ensuring collection of embryos as synchronized in their development as possible.

Whole-mount immunofluorescence

Whole-mount immunofluorescence was performed as described (Evsikov et al., 2004). The primary antibodies were: a monoclonal antibody that recognizes an epitope in the N-terminal part of β-catenin (catalog number 610153, BD Transduction Laboratories); a rabbit polyclonal β-catenin antibody raised against the C-terminal part of the protein (Sigma catalog number C-2206); and polyclonal rabbit antiserum against E-cadherin (GP84) (Vestweber and Kemler, 1984). Secondary antibodies were: Cy™3-conjugated donkey anti-rabbit and Cy™3-conjugated AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, catalog numbers 711-165-152 and 715-165-150, respectively).

Proteasome inhibition

Two-cell embryos from mutant E-cadherin and control females were flushed 24 hours after mating. Some of the embryos were immediately fixed and processed for whole-mount immunofluorescence. The rest of the embryos were cultured in vitro in M16 medium, with or without 5 μM N-CBZ-LEU-LEU-AL (MG132; Sigma, catalog number C-2211; stock solution was 5 mM in DMSO), a membrane permeable inhibitor of the proteinase activity of the 20S proteasome subunit, at a concentration known to inhibit polar body extrusion (Josefsberg et al., 2000; Mellgren, 1997). After 10 hours of culture at 37°C, 5% CO_2, the embryos were fixed and processed for immunodetection of β-catenin using the monoclonal β-catenin antibody.

RNA purification and RT-PCR

RNA purification was performed as described (Oh et al., 2000). For RT-PCR, RNA was resuspended in water containing 40U rRNasin® (Promega), and DNA removed using the DNA-free™ kit (Ambion, catalog number 1906). Reverse transcriptase reactions were carried out using the SuperScript™ Preamplification System (Invitrogen, catalog number 11904-018) according to the supplier's instructions.

Primer pairs for both β-catenin and E-cadherin were designed to span an intron-exon boundary:

β-catenin, 5′-AAGGAAGCTTCCAGACATGC-3′/5′-AGCTTGC-TCTCTTGATTGCC-3′; and

E-cadherin, 5′-AAGTGACCGATGATGATGCC-3′/5′-CTTCTCT-GTCCATCTCAGCG-3′.

PCR reactions were carried out using aliquots of cDNA containing equal amounts of RNA, determined by a preceding control PCR on two embryo equivalents of cDNA using mitochondrial ATP synthase (*mt-Atp6*) primers (5′-TTCCACTATGAGCTGGAGCC-3′/5′-GGTA-GCTGTTGGTGGGCTAA-3′). PCR products were usually detected using ethidium bromide staining of agarose gels. However, to ensure that PCR products were detected when a given transcript was at its lowest level, Southern hybridization (Sambrook et al., 1989) was carried out using E-cadherin or β-catenin α-[^{32}P]-dCTP labeled cDNA probes.

To determine whether or not the C-terminal coding sequences of β-catenin were expressed in embryos lacking the N-terminal part of β-catenin, a primer pair situated in these sequences was used: 5′-GAACAGGGTGCTATTCCACG-3′/5′-GAAAGCCGCTTCTTGTA-ATCC-3′.

Primer pairs used to determine the presence of transcripts of proteins that interact with β-catenin were as follows:

Apc, 5′-AGTCCTTCCACGTCGAAGAC-3′/5′-AAGCGTGTTC-TGAATCTGGC-3′;

Gsk3b, 5′-ACCGAGAACCACCTCCTTTG-3′/5′-TCACAGGGA-GTGTCTGCTTG-3;

Smarca4 (*Brg1*), 5′-TCTCTACGGCAGTGTGATCG-3′/5′-CACT-GCTTCCCTCCTTCTTC-3′;

Sdccag33l, 5′-ATCCACTGGCGATGCTTTAC-3′/5′-TTCTGCTG-TCTCTCGAAGGC-3′;

Ruvbl1 (Pontin52), 5′-GTCATCTTTGCATCCAACCG-3′/5′-AGC-TGCACCGAATACCTCAG-3′; and

Tcf3, 5′-GTCAACGAATCGGAGAATCAG-3′/5′-ACAGCTCAG-ATGGATGAGGG-3′.

All PCR conditions were optimized using the Epicentre FailSafe™ PCR Premix Selection Kit (catalog number FS99060), according to the manufacturer's instructions.

Western blotting

Livers were dissected from 6-week-old male mice that were either heterozygous for the β-catenin floxed allele (β^{F}/β) or the β-catenin deleted allele ($\beta^{F\text{-}del}/\beta$), and immediately homogenized in chilled RIPA buffer [25 mM Tris (pH 8.0), 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% IGEPAL, 1 mM EDTA] containing protease inhibitors (Calpain Inhibitor I, catalog number 1086090; Calpain Inhibitor II, catalog number 1086103; Bestatin, catalog number 874515; Pefabloc SC Plus, catalog number 1873601; Roche Applied Science), at concentrations according to the manufacturer's instructions. One hundred and fifty micrograms of extract was separated on an 8% SDS-PAGE gel, transferred to a Hybond™ ECL™ nitrocellulose membrane (Amersham, Catalog number RPN2020D), and incubated in Tris buffered saline (TBS, pH 7.6) containing 5% non-fat dried milk (NFDM; Carnation brand) and 0.1% Tween 20 for 1 hour at room temperature. All subsequent steps were carried out using TBS containing 5% NFDM and 0.1% Tween 20. The blotted membrane was incubated with the rabbit polyclonal β-catenin antibody for 16 hours at 4°C. After multiple washes at room temperature, the membrane was incubated with a secondary antibody supplied with the ECL™ Western Blotting Analysis System (Amersham, catalog number RPN2108) for 1 hour at room temperature. Protein was detected using the ECL™ Western Blotting Analysis System.

Development of embryos containing a maternal and paternal ⁻N-β-catenin allele

β^{F}/β^{F};*cre*/Ø (mutant) and β^{F}/β^{F};Ø/Ø (control) females were crossed with $\beta^{F\text{-}del}/\beta$;Ø/Ø males. Females and males were either caged together overnight (natural matings), or for 2 hours 13 hours after injection of human chorion gonadotropin (superovulation). Embryos were flushed at the 2-cell stage, and cultured to the blastocyst stage. Experiments with embryos from natural matings were repeated three times, whereas those from superovulation were only done once.

Statistical methods

Analysis of variance (ANOVA) was used to test the difference among the mean number of pups per litter from the mutant β-catenin and control females ([β^{F}/β^{F};*cre*/Ø], [β^{F}/β;*cre*/Ø], and [β^{F}/β^{F};*cre*/Ø]). Raw data were used in the ANOVA, as they appeared to meet the assumptions of normality and homogeneous variances. Where the null hypothesis was rejected, Tukey's W was used to determine which groups were significantly different from each other. Significance tests were performed at α=0.05. The χ^2 and proportion tests were done as described (Devore and Peck, 2001).

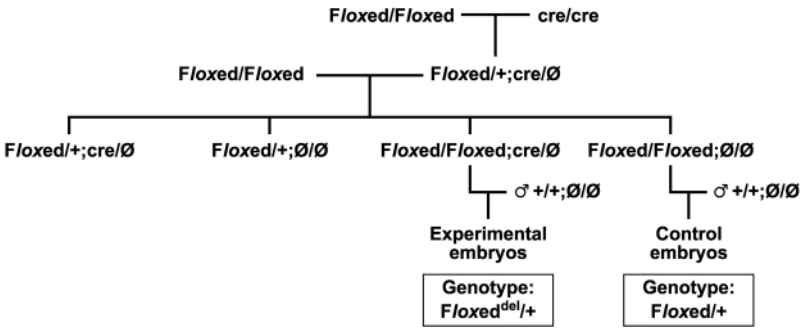

Fig. 1. Mating scheme used to generate embryos lacking either maternal E-cadherin or β-catenin. To generate females whose oocytes would be deficient in either β-catenin or E-cadherin, homozygous floxed females (Floxed/Floxed: [β^{F}/β^{F}] or [E^{F}/E^{F}]) of each line were crossed with homozygous C57BL/6-Tg(Zp3-cre)93Knw males (cre/cre) containing a *cre*-recombinase transgene under control of the *Zp3* promoter. Males hemizygous for the *Zp3-cre* transgene and heterozygous for either floxed allele (Floxed/+;cre/Ø: [β^{F}/β;*cre*/Ø] or [E^{F}/E;*cre*/Ø]), were backcrossed to females homozygous for the β-catenin or E-cadherin floxed alleles (Floxed/Floxed: [β^{F}/β^{F}] or [E^{F}/E^{F}]). This generated females homozygous for either floxed allele and heterozygous for *Zp3-cre* (Floxed/Floxed;cre/Ø: [β^{F}/β^{F};*cre*/Ø] or [E^{F}/E^{F};*cre*/Ø]) that produced oocytes deficient in either β-catenin or E-cadherin. These females were crossed to wild-type C57BL/6J males to generate embryos lacking maternal β-catenin or E-cadherin, but with a wild-type paternal allele (Floxed^{del}/+; del=deleted floxed). Control females homozygous for either floxed allele, but not carrying the *Zp3-cre* transgene (Floxed/+: [β^{F}/β^{F};Ø/Ø] or [E^{F}/E^{F};Ø/Ø]) were mated to wild-type C57BL/6J males (+/+; Ø;Ø) to generate control embryos. To determine whether presence of the *Zp3-cre* transgene would influence the outcome of embryo development, control females heterozygous for either floxed allele and hemizygous for *Zp3-cre* (Floxed/+;cre/Ø: [β^{F}/β;*cre*/Ø] or [E^{F}/E;*cre*/Ø]) were also mated to wild-type C57BL/6J males. Mice homozygous for both E-cadherin and β-catenin floxed alleles were bred by intercrossing mice homozygous for either β-catenin or E-cadherin floxed alleles ([β^{F}/β^{F}] or [E^{F}/E^{F}]), and crossing their offspring inter se. To obtain females homozygous for both floxed alleles and hemizygous for the *Zp3-cre* transgene, females homozygous for both floxed alleles were crossed to males homozygous for both floxed alleles, and hemizygous for the *Zp3-cre* transgene [β^{F}/β^{F};E^{F}/E^{F};*cre*/Ø]. This cross produced [β^{F}/β^{F};E^{F}/E^{F};*cre*/Ø] females that gave rise to oocytes deficient in both β-catenin and E-cadherin.

Results

Oocyte specific deletion of the floxed alleles

To determine the effect of the loss of maternal β-catenin or E-cadherin on pre-implantation embryo development, we used the zona pellucida glycoprotein 3 promoter to express cre-recombinase early in the oocyte growth cycle (de Vries et al., 2000). In this mating scheme (Fig. 1), females homozygous for either the β-catenin or E-cadherin floxed allele, and hemizygous for the *Zp3-cre* transgene ([β^{F}/β^{F};*cre*/Ø] or [E^{F}/E^{F};*cre*/Ø]), were crossed to wild-type C57BL/6J males. Embryos derived from such oocytes are devoid of the maternal sequences encompassed by the *loxP* sites, and give rise to embryos of the genotype [$\beta^{F\text{-}del}/\beta$] or [$E^{F\text{-}del}/E$], having inherited a wild-type allele from the male. As controls, females containing the homozygous

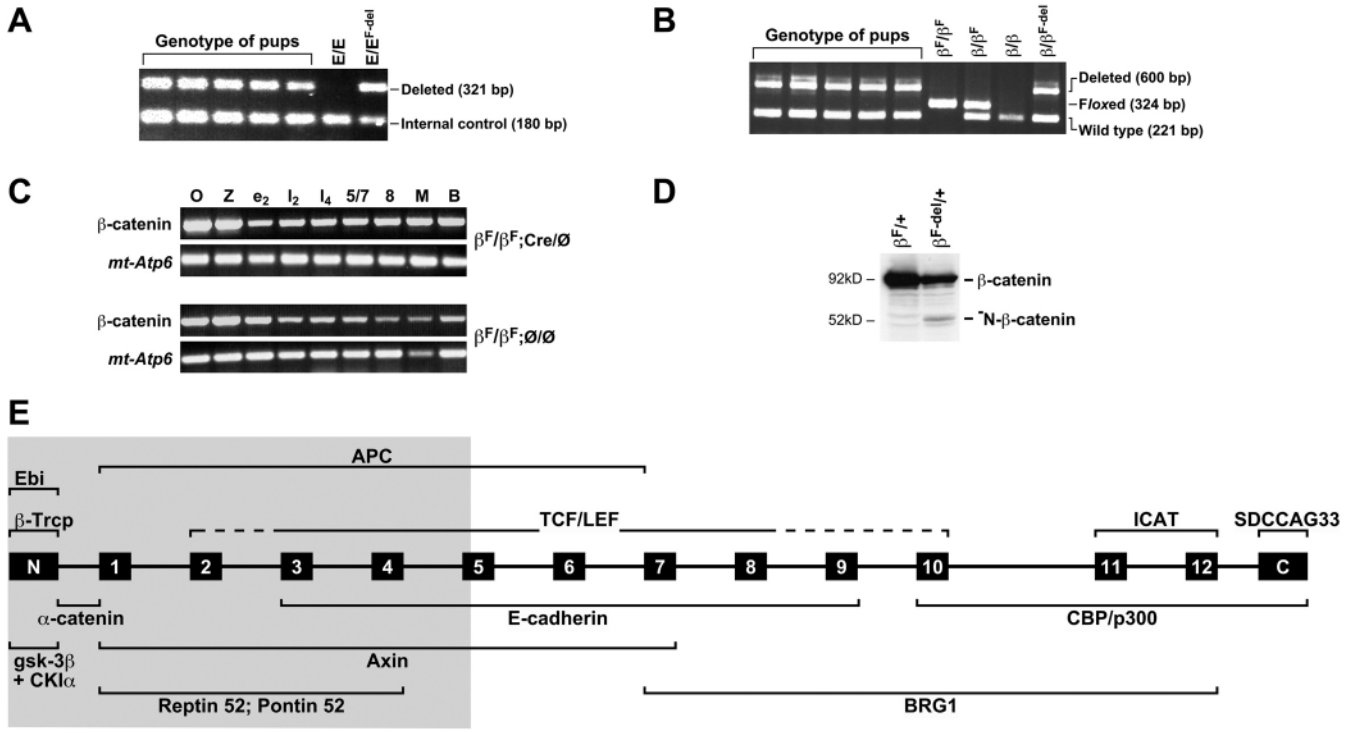

Fig. 2. Effective elimination of E-cadherin and β-catenin sequences encompassed by *loxP* sites in oocytes, and characteristics of the floxed allele of β-catenin. (A) Genotypes of some pups from females producing oocytes lacking E-cadherin. The PCR product characterizing the floxed-deleted E-cadherin allele inherited from the mother is indicated. The internal control is an unrelated wild-type product used as an indicator of successful PCR reactions. Genotypes of the control DNA are indicated at the top of each lane. (B) Genotypes of some pups from females producing oocytes lacking β-catenin. The upper, floxed-deleted β-catenin allele inherited from the female is indicated, as well as the lower wild-type β-catenin allele inherited from the male. The middle product representing the floxed allele is clearly absent in the DNA of the pups analyzed. Genotypes of control DNA are indicated at top of each lane. (C) RT-PCR using primers specific for the 3′ sequences of β-catenin encoding the C-terminal part of the protein. A PCR replicon is detectable in all the embryos ostensibly lacking maternal β-catenin (i.e. O, Z and $^{e}2$; β^F/β^F; *cre*/Ø; top panel), as well as in the control embryos (β^F/β^F; Ø/Ø; bottom panel). Mitochondrial ATP synthase (*mt-Atp6*) primers were used as a control in both cases. O, ovulated oocyte; Z, zygote; $^{e}2$, early two-cell embryo; $^{l}2$, late two-cell embryo; $^{l}4$, late four-cell embryo; 5/7, five- to seven-cell embryo; 8, eight-cell embryo; M, morula; B, blastocyst. (D) Western blot analysis using the polyclonal β-catenin antibody recognizing the C-terminal part of the protein. β^F/+ indicates extract obtained from a control animal containing one floxed β-catenin allele and one wild-type allele. $\beta^{F\text{-}del}$/+ indicates extract from a heterozygous animal containing a deleted-floxed allele and a wild-type allele. β-catenin, wild-type β-catenin; $^{-}$N-β-catenin, 52 kDa protein recognized by the polyclonal β-catenin antibody. (E) Schematic representation of the interaction of β-catenin with different binding partners (not to scale). The N-terminal and C-terminal are depicted by rectangular black boxes with an N or C, respectively. The 12 armadillo repeats are depicted by square, numbered black boxes. The regions where specific proteins interact with β-catenin are depicted by brackets, with the name of the protein indicated. The large gray box indicates the part of β-catenin absent in the floxed β-catenin ($^{-}$N-β-catenin or truncated) allele. Ebi, Ebi; β-Trcp, β-transducin repeat containing protein; Gsk3β, glycogen synthase kinase 3β; CKIα, casein kinase Iα; APC, adenomatous polyposis coli; BRG1, SMARCA4; XSox3/17, *Xenopus* Sox3 or 17; ICAT, CATNBIP1, catenin beta interacting protein 1; CBP/p300, CREBBP, CREB binding protein/E1A binding protein p300; SDCCAG33, serologically defined colon cancer antigen 33.

floxed allele, but not the *Zp3-cre* transgene ([β^F/β^F;Ø/Ø] or [E^F/E^F;Ø/Ø]), as well as females heterozygous for either floxed allele and hemizygous for the *Zp3-cre* transgene ([β^F/β;*cre*/Ø] or [E^F/E;*cre*/Ø]), were mated to wild-type males. Females homozygous for floxed alleles of both β-catenin and E-cadherin, and hemizygous for the *Zp3-cre* transgene, were also mated with wild-type males. Oocytes produced by these females are devoid of maternal floxed sequences of β-catenin and E-cadherin, and give rise to embryos of the genotype [$\beta^{F\text{-}del}/\beta;E^{F\text{-}del}/E$].

Live-born pups were obtained from embryos lacking maternal β-catenin or E-cadherin. To confirm that the intervening sequences between two *loxP* sites in floxed alleles were removed, PCR analysis was performed on DNA extracted from these progeny. Tail DNA from 122 pups derived from embryos lacking maternal E-cadherin contained the deleted-floxed E-cadherin allele ($E^{F\text{-}del}$) (Fig. 2A), and DNA from 116 pups derived from embryos lacking maternal β-catenin contained the deleted-floxed β-catenin allele ($\beta^{F\text{-}del}$) (Fig. 2B). These and subsequent data demonstrate complete and effective elimination of floxed sequences by this *Zp3-cre* transgene.

Partial deletion of the floxed β-catenin allele

As live born pups were obtained in spite of the effective elimination of the floxed sequences, we reexamined the constructs and found that the floxed β-catenin allele contains an in-frame ATG contained within a Kozak consensus in exon 7, the first exon following deleted exons 2 to 6. RT-PCR using primers situated in the 3′ sequences of the β-catenin gene, coding for the C-terminal part of the protein, revealed that a

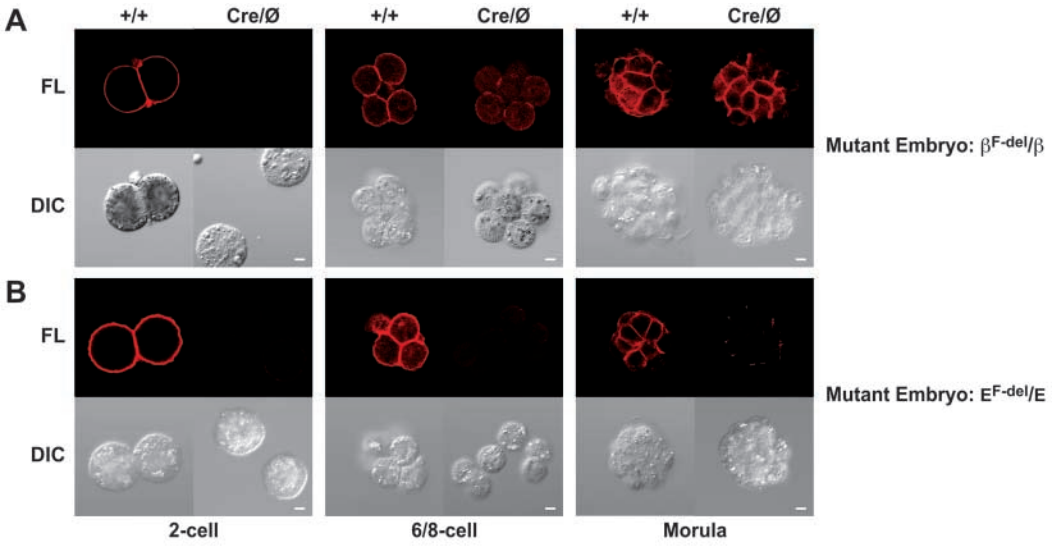

Fig. 3. (A) No β-catenin is found in embryos expressing truncated β-catenin, using an N-terminal specific antibody, until the paternal allele is activated. A monoclonal β-catenin antibody that recognizes an epitope in the N-terminal part of the protein was used. Cre/Ø indicates embryos expressing truncated β-catenin; +/+ indicates control embryos. β-catenin synthesized from the paternal allele is first detected on the surface of embryos expressing truncated β-catenin at the 4- to 8-cell stage transition (6/8-cell stage). (B) E-cadherin is absent in embryos prior to activation of the paternal allele. A polyclonal antibody against E-cadherin was used. E-cadherin is first detected on the surface of embryos lacking maternal E-cadherin at the late morula stage. Cre/Ø indicates embryos lacking maternal E-cadherin; +/+ indicates control embryos. The stage of embryonic development is indicated below the figure; fluorescent (FL) or Nomarski Differential Interference Contrast (DIC) images are indicated on the left. Scale bar: 10 μm.

transcript is indeed present in oocytes and embryos containing the floxed β-catenin allele (Fig. 2C). Western blot analysis using a polyclonal antibody recognizing the C-terminal part of the β-catenin protein showed that a truncated protein of the predicted size is translated from the transcript originating from the $^{-}$N-β-catenin allele (Fig. 2D). The truncated protein contains armadillo repeats 6 to 12 as well as the C terminus, thus retaining the binding sites for CATNBP1 (ICAT), SMARCA4 (BRG1), SDCCAG33 and CREBBP/p300, as well as a portion of the binding sites for APC, E-cadherin and TCF/LEF (Fig. 2E).

β-catenin and E-cadherin in blastomere adhesion

The N-terminal part of β-catenin contains the binding site for α-catenin and some part of the binding site for E-cadherin (armadillo repeats 3-5). Immunofluorescent analysis of β-catenin in embryos expressing truncated β-catenin, using a monoclonal antibody reacting with the N-terminal part of the protein, was carried out. Although β-catenin was detected at the surface of control blastomeres, the N-terminal part of the protein is not present at the surface of 2-cell stage blastomeres. Intact β-catenin translated from paternal transcripts was first demonstrated at the 4- to 8-cell stage transition (Fig. 3A).

Removing the zona pellucida from these cleavage stage embryos resulted in a compelling functional assay: individual blastomeres of 2-cell stage embryos from the experimental group immediately dissociated (Fig. 3A), but by the 4- to 8-cell stage β-catenin, translated from the activated paternal allele, was detected and rescued blastomere adhesion (Fig. 3A). The levels of fluorescence suggest that the amount of β-catenin on the blastomere surface of morulae was similar in control embryos and those heterozygous for the $^{-}$N-β-catenin allele. This result clearly demonstrates that truncated β-catenin is not capable of supporting adhesion. However, such embryos do survive until protein synthesized from the paternal allele reaches the blastomere surface.

Immunofluorescent detection of E-cadherin in embryos lacking maternally derived protein revealed that there was no detectable E-cadherin at the surface of 2- or 6- to 8-cell stage embryos. The first pinpoints of E-cadherin from the paternal allele were only demonstrable at the morula stage (Fig. 3B). Removing the zona pellucida from these cleavage stage embryos, as in the case of the embryos expressing truncated β-catenin, resulted in immediate dissociation of individual blastomeres of embryos up to the 8-cell stage. Adhesion of the maternal E-cadherin-deficient embryos did not occur until the morula stage, coinciding with the first detectable protein (Fig. 3B). The levels of fluorescence indicated that less E-cadherin was present in morulae that lacked maternal E-cadherin. Nonetheless, compaction occurred in these embryos, albeit an entire cell division later than in normal embryos.

β-catenin-E-cadherin interaction and effects on protein localization

E-cadherin localization in embryos expressing truncated β-catenin

To determine whether absence of intact β-catenin influenced localization of E-cadherin on the blastomere surface, 2-, 4- and 8-cell stage embryos expressing truncated β-catenin were examined using E-cadherin antibody (Fig. 4A). E-cadherin is immunodetected at the surface of 2- and 4-cell stage embryos expressing truncated β-catenin, although the zone of detection is not as sharply demarcated as in wild-type embryos, and adhesion does not occur. Interestingly, E-cadherin could also be detected in the cytoplasm of 2- and 4-cell stage embryos expressing truncated β-catenin. In 8-cell stage embryos containing truncated and intact β-catenin, E-cadherin is detectable at the cell surface in a normal pattern (Fig. 4A).

Nuclear localization of β-catenin in embryos lacking maternal E-cadherin

To determine whether absence of E-cadherin influences the localization of β-catenin, zygotes, and 2- and 8-cell stage embryos, were examined using a polyclonal antibody that

detects epitopes on truncated β-catenin. In control embryos, β-catenin was detected at the surface and in the pronuclei of zygotes, and at the surface of 2- and 8-cell stage embryos. However, in embryos lacking maternal E-cadherin, β-catenin was detected solely in the pronuclei and nuclei of zygotes and 2-cell stage embryos, respectively. This result demonstrates that detectable quantities of β-catenin are present in the nuclei of 2-cell stage embryos that lack E-cadherin.

To determine whether the truncated β-catenin protein is also able to translocate to the nucleus, we made use of embryos from $E^F/E^F;\beta^F/\beta^F;cre/Ø$ females. These embryos, which lack maternal E-cadherin and express truncated β-catenin, were analyzed by immunofluorescence using the polyclonal antibody to β-catenin. This showed that truncated β-catenin is found in the pronuclei and nuclei of zygotes and 2-cell stage embryos (Fig. 4C). This result was confirmed using another polyclonal antibody (AbCam, catalog number ab6302), which recognizes the C-terminal part of the protein. Comparison of

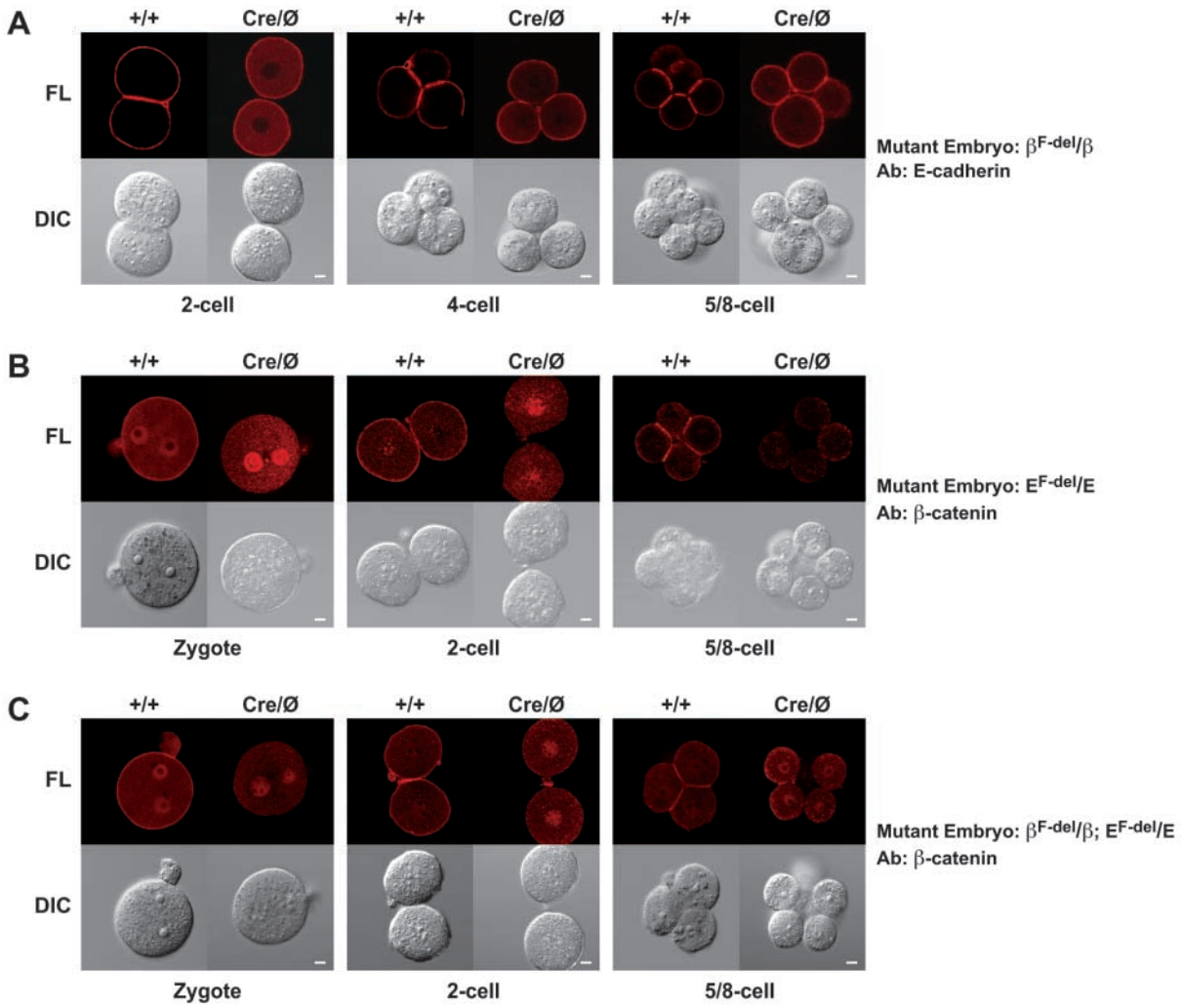

Fig. 4. Localization of E-cadherin and β-catenin in embryos lacking the binding partner. (A) E-cadherin is detectable on the blastomere surface of embryos expressing truncated β-catenin. E-cadherin is visible on the surface of the blastomeres of both the control embryos (+/+) and the embryos expressing truncated β-catenin (Cre/Ø). E-cadherin is also detectable in the cytoplasm of 2-cell and 4-cell stage embryos expressing truncated β-catenin. (B) β-catenin is present in the nucleus of early embryos lacking maternal E-cadherin. β-catenin was detected in control embryos (+/+) and embryos lacking maternal E-cadherin (Cre/Ø), using the polyclonal β-catenin antibody recognizing the C terminus of the protein. β-catenin is visible on the blastomere surface of control zygotes, 2-cell stage embryos and 8-cell stage embryos, with some β-catenin also visible in the cytoplasm of control zygotes and 2-cell stage embryos. By contrast, β-catenin is visible in the pronuclei and cytoplasm of zygotes, as well as in the nuclei and cytoplasm of 2-cell stage embryos lacking maternal E-cadherin. Only a small amount of β-catenin is detectable in 8-cell embryos lacking maternal E-cadherin. (C) Truncated β-catenin is present in the nucleus of embryos lacking maternal E-cadherin. β-catenin was detected in control embryos (+/+), and in embryos expressing truncated β-catenin and lacking maternal E-cadherin (double mutant) embryos (Cre/Ø), using the polyclonal β-catenin antibody that detects the C-terminal part of the protein. β-catenin is detected in the pronuclei of control and double-mutant zygotes, on the surface and in the cytoplasm of control 2-cell stage embryos, in the nuclei and cytoplasm of double-mutant 2-cell stage embryos, and on the surface of 4 to 8-cell stage control and double-mutant embryos. Note for B and C, blastomeres of 8-cell stage embryos lacking maternal E-cadherin do not adhere to each other. Consequently all manipulations during the immunostaining process were carried out with extreme caution to maintain blastomeres of embryos in a clump. No fluorescence was detected in embryos where the primary antibody was omitted (data not shown). Scale bars: 10 μm.

the pattern of staining between embryos lacking maternal E-cadherin and expressing wild-type β-catenin, and that of embryos lacking both maternal E-cadherin and expressing truncated β-catenin reveals a similar staining pattern.

Regulation of β-catenin levels in embryonic blastomeres

Degradation of β-catenin via the proteasome in pre-implantation embryos

To determine whether β-catenin concentration in early embryos could be controlled by the same mechanism as in somatic cells, we made use of embryos lacking maternal E-cadherin. As the somatic mechanism involves the proteasome, 2-cell stage embryos were incubated with the proteasome inhibitor MG132 and β-catenin was detected using the monoclonal β-catenin antibody (Fig. 5A). β-catenin is visible at the cell surface and in the cytoplasm of a control embryo (Fig. 5A, first panel). β-catenin protein is detectable in the cytoplasm and especially the nucleus, but not at the surface of the blastomeres, of an embryo lacking maternal E-cadherin that was incubated in the proteasome inhibitor (Fig. 5A, second panel). Low levels of β-catenin, slightly more than background staining, are visible in the nucleus and cytoplasm of an MG132-free, control embryo lacking maternal E-cadherin (Fig. 5A, compare panels three and four).

Are the components regulating β-catenin levels present in the early embryo?

The results obtained above suggest that the multimeric cytoplasmic protein complex, which assures degradation of β-catenin in somatic cells, not only exists, but is also functional at this stage. To determine whether these molecules, as well as other molecules interacting with β-catenin, are present in early embryos, we initially turned to the annotated 2-cell stage library (Evsikov et al., 2004). Expressed sequence tags (ESTs) for two molecules involved in the regulation of β-catenin are present in the 2-cell stage library: *Apc* and casein kinase Iα (*Csnk1a1*). ESTs for other molecules interacting with β-catenin were also found: *Smarca4* (*Brg1*), *Sdccag33* (*Tsh*), and *Catnbip1* (*ICAT*). To confirm the presence of these, and other molecules interacting with β-catenin in early embryos, RT-PCR was performed (Fig. 5B). These assays revealed that transcripts of at least two molecules, *Gsk3b* and *Apc*, which are involved in regulating intracellular levels of the molecule, are present from the full-grown oocyte to the 4- to 8-cell stage. Transcripts of a number of molecules capable of interacting with β-catenin in the nucleus to influence transcription by acting as co-activators, or by changing chromatin structure, are also present from the full-grown oocyte to the blastocyst stage: *Smarca4* (*Brg1*), *Sdccag33* (*Tsh*), *Ruvbl1* (Pontin52) and *Tcf3*.

Table 1. Development of embryos derived from females expressing truncated β-catenin in their oocytes crossed to males heterozygous for the deleted-floxed β-catenin allele

	2-cell stage embryos	Expanded/hatched blastocysts
Natural matings*		
Mutant (β^F/β^F; cre/Ø × $\beta^{F\text{-}del}/\beta$)	52	43 (83%)
Control (β^F/β^F; Ø/Ø × $\beta^{F\text{-}del}/\beta$)	30	28 (93%)
Superovulation†		
Mutant (β^F/β^F; cre/Ø × $\beta^{F\text{-}del}/\beta$)	79	71 (90%)
Control (β^F/β^F; Ø /Ø × $\beta^{F\text{-}del}/\beta$)	20	19 (95%)

*χ^2=1.85; *P*=0.173. Proportion test: z=–4.81.
†χ^2=0.508; *P*=0.476. Proportion test: z=–7.0.

Expression of truncated β-catenin influences embryo development

To determine whether embryo development was adversely affected by the expression of a maternal and paternal $^{-}$N-β-catenin allele, we crossed β^F/β^F;*cre*/Ø females with $\beta^{F\text{-}del}/\beta$;Ø/Ø males, giving rise to embryos of the genotype $\beta^{F\text{-}del}/\beta^{F\text{-}del}$ and $\beta^{F\text{-}del}/\beta$. As controls, β^F/β^F;Ø/Ø females were crossed with $\beta^{F\text{-}del}/\beta$;Ø/Ø males. Embryo development to the blastocyst stage was monitored (Table 1). Using the chi-squared (χ^2) test, we determined that embryos obtained from β^F/β^F;*cre*/Ø females developed less efficiently than those from control females. Hypothetically, if the expression of two deleted-floxed β-catenin alleles is lethal, at least 50% of embryos should die. This hypothesis was rejected using a

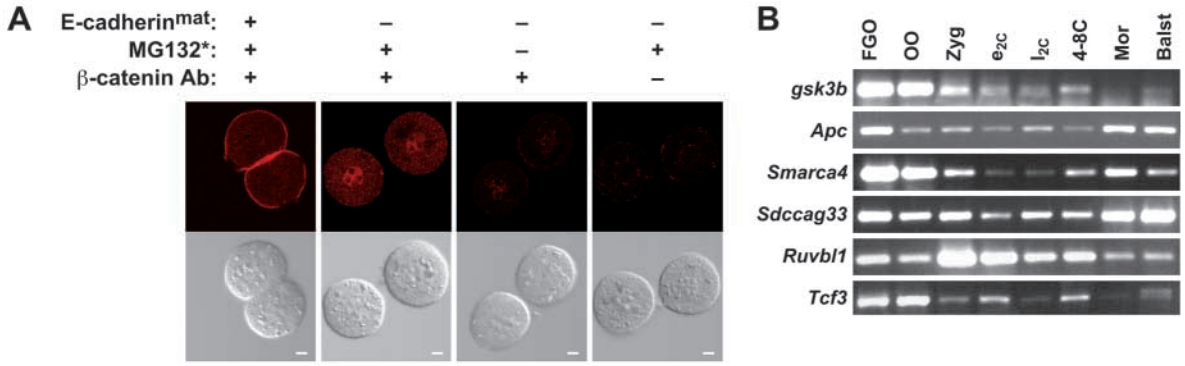

Fig. 5. The level of β-catenin in 2-cell stage embryos is controlled by the same mechanism as in somatic cells. (A) Involvement of the proteasome. The presence (+) or absence (–) of maternal E-cadherin (E-cadherin^{mat}) in the embryos analyzed is indicated, as is the presence (+) or absence (–) of the proteasome inhibitor MG132 (5 μM). The monoclonal β-catenin antibody (β-catenin Ab) was used for staining (+), except where it was omitted (–) to determine background levels of staining. Secondary antibody was used in every staining. Scale bar: 10 μm.
(B) Transcripts for proteins interacting with β-catenin in the cytoplasm or nucleus are present in oocytes and early embryos. Names of the transcripts are indicated to the left of the figure.

proportion test, and therefore the loss of embryos from β^F/β^F;*cre*/Ø females cannot be ascribed to the expression of two deleted-floxed β-catenin alleles. These data suggest that full-length β-catenin is not a requirement for pre-implantation development to blastocyst in vitro.

To determine the effect of the absence of maternal E-cadherin or the expression of truncated β-catenin on embryo development, the number of live born and weaned pups obtained from females producing oocytes lacking E-cadherin, or expressing truncated β-catenin, was recorded. No loss of embryos was indicated in females producing oocytes lacking maternal E-cadherin: the mean number of pups per litter born to these females was the same as for control females (6.0 pups per litter in both cases). However, loss of embryos was indicated in females producing oocytes expressing truncated β-catenin, as the mean number of pups per litter from these females was less than the mean number of pups per litter from control females (Fig. 6A). Females producing oocytes expressing truncated β-catenin had a mean of 5.0 pups per litter, significantly smaller than the 6.8 pups per litter from β^F/β^F;Ø/Ø control females, and the 6.5 pups per litter from β^F/β;*cre*/Ø control females (Tukey's $W=1.64$; $0.01<P<0.05$). Progeny derived from oocytes expressing truncated β-catenin were phenotypically normal, and lived the normal expected life span of a C57BL/6J mouse without any signs of disease. Taking the size of the data set and the results in Table 1 into account, embryos are probably lost before or during the blastocyst stage. Interestingly, the loss-of-embryo phenotype was rescued by the absence of maternal E-cadherin in embryos expressing truncated β-catenin. No difference was found between the number of pups per litter obtained from females lacking both maternal E-cadherin and expressing truncated β-catenin in their oocytes ($E^F/E^F;\beta^F/\beta^F$;*cre*/Ø), and control females ($E^F/E^F;\beta^F/\beta^F$;Ø/Ø). These results suggest that E-cadherin, in its role as regulator of cytoplasmic β-catenin, may play a role in embryo development.

The whole-mount immunofluorescence staining suggested that the paternal allele of β-catenin is expressed earlier than that of E-cadherin (Fig. 3). To accurately determine the time of activation of the respective paternal alleles, RNA was prepared from control embryos, and embryos lacking maternal E-cadherin or expressing truncated β-catenin. RT-PCR was performed using primers situated within the sequences encompassed by the *loxP* sites. In contrast to controls where maternal transcripts of both β-catenin and E-cadherin are clearly detectable at all time points (Fig. 6B,C; lower panels), the RT-PCR product for β-catenin is not seen until 34 hours after mating, at the late 2-cell stage, whereas the RT-PCR product for E-cadherin is not seen until 48 hours after mating, at the late 4-cell stage (Fig. 6B,C; top panels). This clearly demonstrates that β-catenin mRNA produced from the activated paternal allele is available for translation at least 14 hours prior to that of paternal E-cadherin.

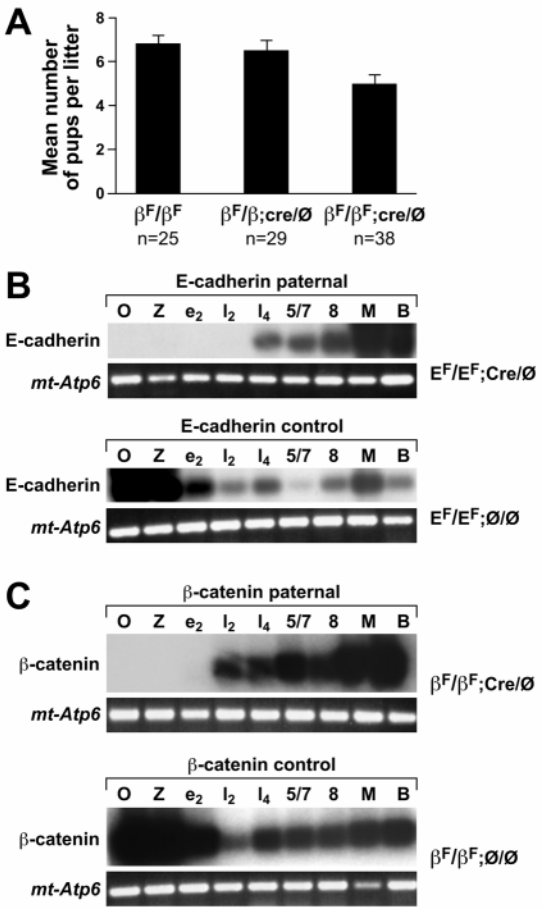

Fig. 6. (A) Embryos expressing truncated β-catenin do not develop optimally, as indicated by fewer pups per litter. Bar graph depicting the mean number of pups per litter obtained from females producing oocytes expressing truncated β-catenin (β^F/β^F;cre/Ø) and control females (β^F/β^F and β^F/β;cre/Ø). The number of litters recorded (*n*) in each group is indicated at the bottom of the graph. (B,C) The paternal alleles of β-catenin and E-cadherin are activated sequentially. (B) Expression of E-cadherin in embryos lacking maternal E-cadherin (top) and control embryos (bottom). (C) Expression of wild-type β-catenin in embryos expressing truncated β-catenin (top) and control embryos (bottom). The expression of both genes was monitored by RT-PCR and subsequent Southern blot analysis of the PCR products using a ^{32}P-labeled E-cadherin cDNA probe. Wild-type β-catenin was monitored using primers situated in sequences coding for the N-terminal part of the protein. The gene product monitored is indicated on top of the figure; the genotype of the females from which the embryos were isolated is indicated on the right. Mitochondrial ATP synthase (*mt-Atp6*) was used as a control for the RT-PCR, and was detected by ethidium bromide staining. Embryo stages are the same as in Fig. 2.

Discussion

E-cadherin and β-catenin are required for blastomere adhesion

In normal embryos compaction starts at the late 8-cell stage, continues in the morula, and is accompanied by changes in cell polarity that lead to the formation of the first differentiated tissue in the embryo, the trophectoderm epithelium (Fleming and Johnson, 1988). The distribution of E-cadherin on the surface of blastomeres changes throughout this process (Ohsugi et al., 1996; Vestweber et al., 1987). Remodeling of the E-cadherin-β-catenin-α-catenin complexes is required for the rearranging of cadherin-mediated cell-cell adhesion, the grouping of cells into appropriate structures, and cell migration (McNeill, 2000; Ozawa et al., 1989). Interestingly, E-cadherin-

specific morpholino oligonucleotides arrest embryos at the 2-cell stage (Kanzler et al., 2003), leading these authors to postulate that 2-cell arrest is caused by an altered distribution of β-catenin, resulting in an aberrant transcription of genes that can be activated by β-catenin. However, we find that genetic ablation of all maternal E-cadherin allows translocation of β-catenin to the nucleus but does not result in 2-cell arrest. Indeed, the only effect of the loss is that adhesion of blastomeres is ablated until the late morula stage (Fig. 7A,B). The fact that compaction can occur a cell division later than in normal embryos indicates that confinement within the zona pellucida enables adequate blastomere contact for compaction to ensue once paternal E-cadherin reaches the blastomere surface. Understanding the function of specific genes and dynamic interactions at the initiation of mammalian development thus rests on the knowledge of in vivo analysis of the phenotype of mice bearing null and hypomorphic alleles of specific genes.

Our results further show that removal in the oocyte of the N-terminal part of β-catenin, containing the binding site for α-catenin and a part of the binding site for E-cadherin, eliminates blastomere adhesion (Fig. 7C). Although E-cadherin is present at the blastomere surface of embryos expressing truncated β-catenin, these blastomeres do not adhere to each other, apparently as a result of the inability to form an adhesion complex. Furthermore, E-cadherin is mislocalized to the cytoplasm in 2- and 4-cell stage embryos, a likely result of the inability of truncated β-catenin to bind E-cadherin. This mislocalization is reversed during the 4- to 8-cell transition, when β-catenin synthesized from the paternal allele interacts with E-cadherin, reaches the blastomere surface, and enables blastomere adhesion. Embryos remain intact from fertilization to the 8-cell stage because their blastomeres are kept in close proximity to each other by the zona pellucida. This result underscores the importance of interaction of E-cadherin with the catenins and other members of the adhesion complex to maintain cell-cell adhesion.

Although maternal E-cadherin was previously found to be present in the E-cadherin null embryos, the membrane localization of α-catenin, β-catenin and ZO-1 (TJP1 – Mouse Genome Informatics), all of which are involved in interacting with E-cadherin to establish proper cell-cell interaction and organize the cortical actin filament, was abnormal (Ohsugi et al., 1997). E-cadherin null embryos, while containing maternal E-cadherin, are not able to maintain compaction and form an intact trophectoderm layer (Larue et al., 1994). Embryos lacking maternal E-cadherin undergo compaction and subsequent cavitation as soon as the paternal protein is expressed. E-cadherin is thus not required for early embryo development, but synthesis and post-translational modification of E-cadherin, translated from the newly activated embryonic genome, as well as its interaction with β-catenin, is required for the dynamic changes necessary for blastomere-blastomere adhesion during compaction and trophectoderm formation.

β-catenin and pre-implantation embryo development

The floxed β-catenin allele we used in this study has been used to delineate the role of β-catenin and the WNT pathway in development of the brain, neural crest and embryonic endoderm (Brault et al., 2001; Hari et al., 2002; Lickert et al., 2002; Machon et al., 2003). Our analysis revealed that excision

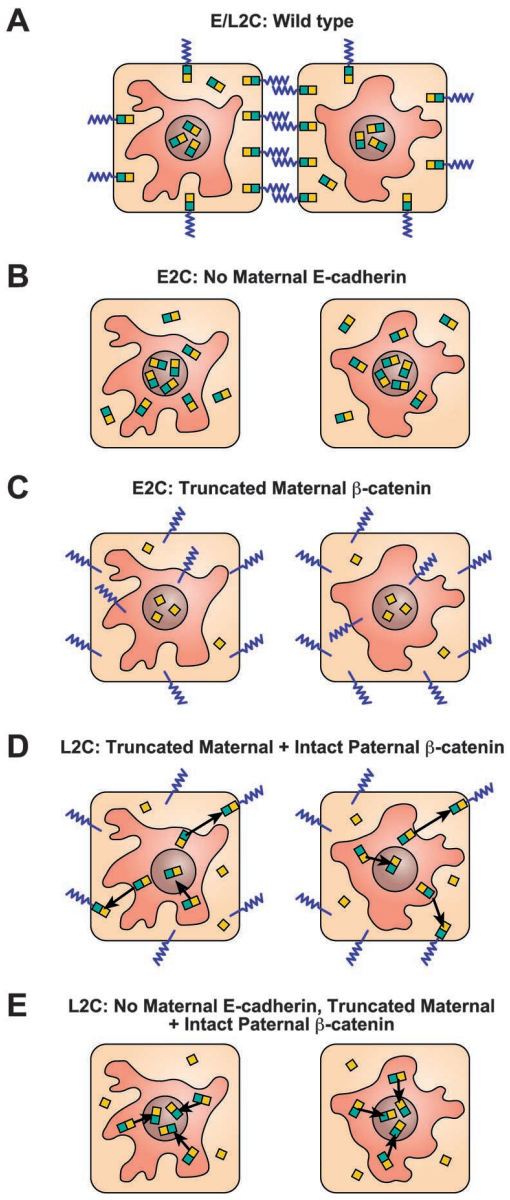

Fig. 7. Schematic representation of the phenotypes obtained for embryos either expressing truncated β-catenin, lacking maternal E-cadherin, or expressing truncated β-catenin as well as lacking maternal E-cadherin. (A) A control early/late 2-cell stage embryo (E/L2C) where interaction of β-catenin (yellow and blue rectangles) and E-cadherin (blue zig-zag lines) ensures adhesion of the two blastomeres. (B) In early 2-cell stage embryos (E2C) lacking maternal E-cadherin, the adhesion complex cannot form, resulting in blastomeres that are not able to adhere. Consequently larger amounts of β-catenin translocate to the nucleus. (C) In early 2-cell embryos expressing truncated β-catenin (yellow squares), the blastomeres fail to adhere, even though E-cadherin is present on the blastomere surface. (D) In late 2-cell embryos expressing truncated β-catenin, the wild-type paternal β-catenin allele is activated. Protein translated from this allele is sequestered by E-cadherin to form the adhesion complex (yellow and blue rectangles with blue zig-zag lines attached), and a lesser amount is translocated to the nucleus. (E) By contrast, in late 2-cell stage embryos expressing truncated β-catenin and also lacking maternal E-cadherin, all newly synthesized β-catenin is able to translocate to the nucleus because no E-cadherin is present to sequester it to the adhesion complex.

of the floxed sequences removes the N-terminal part of the protein, leaving the C-terminal part intact. The N-terminal part contains binding sites for α-catenin, GSK3B, CSNK1A1, Reptin52 and RUVBL1 (Pontin52). From the best estimates in the literature, the binding sites for E-cadherin, TCF/LEF, APC and AXIN are partially removed, but in the cases cited above the WNT/β-catenin signaling pathway is non-functional. Because oocytes expressing truncated β-catenin undergo normal maturation, fertilization and early 2-cell stage development, we suggest that the WNT/β-catenin signaling pathway is not functional at this time in development. Further support for this is gained from the fact that β-catenin translocates to the nucleus in embryos lacking maternal E-cadherin, in essence mimicking an overexpression of β-catenin, which in systems responsive to the WNT/β-catenin pathway causes detrimental effects. Nonetheless, these embryos develop normally.

However, it cannot be ignored that the C-terminal part of the protein, containing binding sites for CREBBP/p300, CATNBP1 (ICAT), SMARCA4 (BRG1) and SDCCAG33, is still intact. In the nucleus, transcriptional co-activators p300/CREBBP may bind β-catenin to activate transcription either by recruiting general transcription factors to target gene promoters, or by changing chromatin structure (Hecht et al., 2000; Miyagishi et al., 2000; Takemaru and Moon, 2000). β-catenin can also recruit the chromatin-remodeling factor BRG1 to TCF-responsive promoters, forming a complex to remodel chromatin and facilitate transcriptional activation (Barker et al., 2001; Nielsen et al., 2002). Although this truncated protein might not be active, it is reasonable to speculate that the C-terminal part of β-catenin is needed for the initial changes in chromatin restructuring during nuclear reprogramming that take place during the oocyte to embryo transition. Truncated β-catenin, like the wild-type protein, translocates to the pronuclei of zygotes and the nuclei of 2-cell stage embryos (Fig. 7C). This may indicate that the truncated protein is able to interact with different factors, for which transcripts have been shown to be present in oocytes and early embryos, during this time of nuclear reprogramming, a hypothesis that remains to be tested.

Females with oocytes that express truncated β-catenin produce fewer pups per litter than controls, but this loss-of-embryo phenotype is rescued if oocytes also do not express maternal E-cadherin (Fig. 7D,E). It is known that β-catenin forms a complex with E-cadherin soon after being synthesized in the endoplasmic reticulum, and some β-catenin is left in the free cytoplasmic form (McCrea and Gumbiner, 1991; Ozawa and Kemler, 1992). We postulate that in the situation where intact β-catenin is newly synthesized from the paternal allele at the late 2-cell embryo stage, it is preferentially sequestered by E-cadherin, resulting in insufficient amounts of free β-catenin available for nuclear translocation. However, in embryos lacking maternal E-cadherin and expressing the truncated β-catenin allele, newly synthesized β-catenin is not sequestered by E-cadherin, which is absent from the oocyte and which is activated only at the late 4-cell stage. Intact β-catenin is thus available in the free form, and can be channeled to the nucleus (Fig. 7E).

These results give an intriguing glimpse into the interplay between maternal and newly synthesized E-cadherin and β-catenin during the oocyte to embryo transition. Early cleavage embryos can therefore be seen as a system that is both robust and able to tolerate quite large changes, while at the same time being dependent on the meticulous timing of new transcription and translation.

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