# Stabilization of β-catenin in the mouse zygote leads to premature epithelial-mesenchymal transition in the epiblast

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## Summary

**Many components of the Wnt/β-catenin signaling pathway are expressed during mouse pre-implantation embryo development, suggesting that this pathway may control cell proliferation and differentiation at this time. We find no evidence for a functional activity of this pathway in cleavage-stage embryos using the Wnt-reporter line, BAT-gal. To further probe the activity of this pathway, we activated β-catenin signaling by mating a zona pellucida3-cre (Zp3-cre) transgenic mouse line with a mouse line containing an exon3-floxed β-catenin allele. The result is expression of a stabilized form of β-catenin, resistant to degradation by the GSK3β-mediated proteasome pathway, expressed in the developing oocyte and in each cell of the resulting embryos. Nuclear localization and signaling function of β-catenin were not observed in cleavage-stage embryos derived from these oocytes. These results indicate that in pre-implantation embryos, molecular mechanisms independent of the GSK3β-mediated ubiquitination and proteasome degradation pathway inhibit the nuclear function of β-catenin. Although the mutant blastocysts initially developed normally, they then exhibited a specific phenotype in the embryonic ectoderm layer of early post-implantation embryos. We show a nuclear function of β-catenin in the mutant epiblast that leads to activation of Wnt/β-catenin target genes. As a consequence, cells of the embryonic ectoderm change their fate, resulting in a premature epithelial-mesenchymal transition.**

Key words: Epithelial-mesenchymal transition, Mouse pre-implantation embryo, Wnt/β-Catenin, Gastrulation

## Introduction

E-cadherin, α- and β-catenin are present in the unfertilized mouse egg, as proteins assembled into an adhesion complex, and as maternal mRNA (Ohsugi et al., 1996). Analysis of transcription, protein synthesis, and membrane localization of the E-cadherin-catenin adhesion complex components during pre-implantation development indicated that they are accumulated for use at compaction and trophectoderm formation. Indeed, null mutants of E-cadherin and α-catenin are characterized by defective trophectoderm (Larue et al., 1994; Riethmacher et al., 1995; Torres et al., 1997), and embryos lacking β-catenin exibit early gastrulation patterning defects (Haegel et al., 1995; Huelsken et al., 2000).

β-Catenin is a central component of the cadherin-catenin adhesion complex, linking cadherins to α-catenin, which anchors the adhesion complex to the actin-based cytoskeletal network (Aberle et al., 1996). β-catenin is also a crucial component in the Wnt/β-catenin signaling pathway controlling the expression of specific target genes that regulate cell proliferation, cell fate and differentiation (Wodarz and Nusse, 1998). Upon Wnt signaling, and after several intermediate steps leading to an inactivation of GSK3β (glycogen synthase kinase 3β), β-catenin accumulates in the cytoplasm and translocates to the nucleus. Here it regulates the transcription of target genes in concert with members of the Lef1/TCF family of transcription factors. In the absence of Wnt signaling the cytoplasmic level of β-catenin is controlled by a multi-protein complex, including GSK3β, APC (adenomatous polyposis coli), CSNK1A1 (casein kinase Iα), and axin, which prepares the protein for ubiquitination and proteasome degradation (Huelsken and Behrens, 2000).

Interestingly, when levels of the cadherin-catenin adhesion complex components in the cleavage-stage embryos were compared, a surplus of uncomplexed cytoplasmic β-catenin was found, reminiscent of the stabilization of β-catenin upon Wnt signaling (Ohsugi et al., 1996). Although expression of several components of the pathway as well as of those of the β-catenin degradation machinery has been described in cleavage-stage embryos (Knowles et al., 2003; Lloyd et al., 2003; Wang et al., 2004), involvement of the Wnt/β-catenin signaling pathway during pre-implantation development has not yet been shown.

To query whether the Wnt/β-catenin pathway could be involved in regulating cell fate in pre-implantation development, we performed a series of genetic experiments. We anticipated that an inactivation of the maternal and zygotic

β-catenin gene cannot lead to informative results about the Wnt pathway because of the requirement of β-catenin for E-cadherin-mediated cell adhesion and compaction. Therefore, we chose to generate a stabilized form of β-catenin in the unfertilized egg using a cre-*loxP* strategy. Mice carrying a β-catenin exon 3 floxed allele were crossed to a transgenic line containing the *cre-recombinase* gene under control of the Zona pellucida 3 promoter (de Vries et al., 2000; Harada et al., 1999), resulting in females from this cross expressing an exon 3-deleted β-catenin allele in their oocytes. As exon 3 harbors the GSK3β phosphorylation motifs (Aberle et al., 1997), the protein will therefore not be subject to degradation by the standard ubiquitination pathway. Furthermore, the embryos arising from crossing these females and wild-type males express both the stabilized and wild-type form of β-catenin in all their cells. Our results based on analysis of these embryos indicate that the Wnt/β-catenin pathway is not required for mouse pre-implantation development, and the first detectable changes are in the embryonic ectoderm of early post-implantation embryos.

## Materials and methods

### Mice and embryos

Mice carrying the floxed exon 3 allele of β-catenin (Harada et al., 1999) were kept on a 129-SvJ.C57BL/6 mixed background. The transgenic line C57BL/6-Tg(Zp3-cre)93Knw/J (*Zp3-cre*) was produced in C57BL/6J (de Vries et al., 2000); the Wnt-reporter line, BAT-gal, is on a B6D2F1 background (Maretto et al., 2003). In all experiments the time when the vaginal plug was detected was taken as E0.5 of embryonic development.

Morula aggregation experiments were performed between 129-*Gt(ROSA)26Sor*/J (ROSA26 (Soriano, 1999)) and *β-catΔEx3/+* embryos, and, after overnight culture, blastocysts were transferred into pseudopregnant females.

To produce teratomas, genotyped E6.5 embryos were transferred under the kidney capsule of F1 (C57BL/6x129) mice and the resultant teratomas were processed for histological examination 6 weeks later.

Genotyping was done by PCR analysis of tail tips, single oocytes, pre-implantation embryos, or material scraped from paraffin sections following lysis in buffer containing proteinase K. DNA was precipitated with isopropanol and resuspended in Tris-EDTA buffer. The *cre* transgene was detected using as primers: CreS: 5′CAA-GTTGAATAACCGGAAATG3′ and CreAS: 5′GCCAGGTATCTCT-GACCAGA3′. The β-catenin exon 3 floxed allele was detected using primers Ex2S: 5′GACACCGCTGCGTGGACAATG3′ and Ex3AS: 5′GTGGCTGACAGCAGCTTTTCT3′. Recombination to give the exon3-less allele in isolated oocytes and embryos was detected using primers: C-F: 5′GCTGCGTGGACAATGGCTAC3′ and C-R: 5′TGA-GCCCTAGTCATTGCATAC3′.

### lacZ staining and in situ hybridization

Embryos were fixed in 1% formaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, 0.02% NP-40 for 5 min (blastocysts) to 15 min (E5.5 to E7.5 embryos) at room temperature. After two washes in PBS, they were transferred into freshly prepared X-Gal staining solution (Maretto et al., 2003), and stained for 4-6 hours at 37°C in the dark. After rinsing with PBS, embryos were post-fixed in 2% paraformaldehyde and examined under an Axiovert 200 microscope (Zeiss) for photography with an Axiocam digital camera (Zeiss).

Whole-mount in situ hybridization using digoxigenin-labeled riboprobes for *Nanog*, *Otx2*, *Hex* and *Bmp4* genes was performed as described (Knecht et al., 1995; Parr et al., 1993). In situ hybridization for *Snai1* mRNA on sections was done as described (Lescher et al., 1998).

### Antibodies and immunoblotting

Antibodies used were: E-cadherin polyclonal affinity-purified rabbit antibody, anti-gp84 (Vestweber and Kemler, 1984); β-catenin mouse monoclonal antibody (C19220, Transduction Laboratories); and Oct4 mouse monoclonal antibody (sc-5279, Santa Cruz Biotechnology). Secondary tagged antibodies to detect the primary antibody complexes were an Alexa Fluor 488 goat anti-rabbit (A-21045, Molecular Probes) and an Alexa Fluor 633 goat anti-mouse (A-21052, Molecular Probes).

R1 ES cells and mutant ES-cell lines were homogenized in lysis buffer, and cell lysates were probed with β-catenin mouse monoclonal antibody at a 1:3000 dilution as described (Boussadia et al., 2002).

### Histology and immunohistochemistry

Blastocysts were incubated in acid Tyrode's solution (T-1788, Sigma) to remove the zona pellucida. After two washes in M2 medium (M-7167, Sigma), embryos were fixed in 2% paraformaldehyde in PBS at room temperature for 10 min, followed by 0.25% Triton X-100/PBS treatment for 8 min. After pretreatment with 1% heat-inactivated sheep serum in PBS (antibody incubation buffer) for 30 min, embryos were incubated with the primary antibody for 30 min at 37°C, then washed twice in incubation buffer for 30 min. Embryos were next incubated for 30 min at 37°C with the fluorescent-tagged secondary antibodies, washed in incubation buffer and mounted in a drop of Prolong Antifade reagent (P-7481, Molecular Probes). A Leica SP2 UV confocal microscope (Leica Microsystems) equipped with a HCX PLAPO CS 63?/1.2 water immersion lens was used to visualize the antibody complexes. Image acquisition parameter settings were kept constant for different samples to enable signal intensity comparison.

For immunohistological analysis, embryos were fixed in 4% paraformaldehyde/PBS, dehydrated, embedded in paraffin and sectioned at 7 μm. Sections were dewaxed, rehydrated and stained with E-cadherin antibody (1:50 dilution), β-catenin antibody (1:50), Oct4 antibody (1:50), anti-Lef1 (a gift from R. Grosschedl, 1:50) and T-Brachyury antibody (a gift from B. Herrmann, 1:50). The EnVision Plus System (Dako, Germany) with DAB peroxidase substrate (Sigma, Germany) was used to detect and amplify the signals.

## Results

### Normal pre-implantation development of mutant embryos

To determine the effect of the mutant, stabilized form of β-catenin, we assessed its localization in oocytes, pre-implantation embryos, and post-implantation embryos. To do this we crossed females heterozygous or homozygous for the β-catenin exon 3 floxed allele (Harada et al., 1999) to *Zp3-cre* transgenic males carrying *cre-recombinase* under control of the *Zp3* promoter (de Vries et al., 2000). Females heterozygous or homozygous for the β-catenin exon 3 floxed allele and hemizygous for the *Zp3-cre* transgene (*β-catEx3flox/+;cre/+* or *β-catEx3flox/β-catEx3flox;cre/+*) were mated with wild-type C57BL/6 males to ensure that the resulting mutant embryos (*β-catΔEx3/+*) inherited a wild-type β-catenin allele.

In accordance with our previous results (Ohsugi et al., 1996) a faint but clear cytoplasmic distribution of β-catenin was observed in oocytes of wild-type ovaries (Fig. 1A). Enhanced cytoplasmic localization of β-catenin was found in *β-catΔEx3* (mutant) oocytes (Fig. 1B), but no nuclear localization of β-catenin was detected in either wild-type or mutant oocytes. When single unfertilized eggs from heterozygous females were genotyped, 50% exhibited the deleted exon 3 allele (*β-catΔEx3*), confirming the high efficiency of the *Zp3-cre*

transgene for recombining floxed sequences during oocyte maturation (Fig. 1C).

Mutant and wild-type embryos were morphologically indistinguishable, and mutant embryos developed to blastocysts. Interestingly, in *β-catΔEx3/+* blastocysts β-catenin was only found associated with the cell membrane, and no cytoplasmic or nuclear localization was detected (Fig. 1F, wild type in E). However, since the staining procedure involved permeabilization of the embryos, a possible nuclear localization of the stabilized β-catenin may be below detection level. The β-catenin *ΔEx3* allele is expressed during pre-implantation development as monitored by RT-PCR analysis from mutant blastocysts (not shown). Further support that the stabilized form of β-catenin is probably synthesized during pre-implantation development comes from immunoblot experiments of extracts of ES cells isolated from mutant blastocysts (Fig. 1D). Mutant and wild-type blastocysts were also indistinguishable when stained for E-cadherin and Oct4 in double immunofluorescence experiments (Fig. 1G,H). From these results it is concluded that the presence of the dominant β-catenin *ΔEx3* allele had no obvious effect on pre-implantation development.

In contrast, when post-implantation stages at E6.5 were examined, mutant embryos exhibited a morphologically distinct phenotype (Fig. 1J,K); the embryonic portion was less expanded and structured and often appeared as a disorganized mass of cells (Fig. 1K). But we found no difference in the extra-embryonic region and in the ectoplacental cone of wild-type and mutant embryos. These results suggest that the expression of the stabilized form of β-catenin from the beginning of embryonic development specifically affects the embryonic portion at early gastrulation.

### Stabilized β-catenin affects the embryonic ectoderm

To determine the portion of the embryo affected by stabilized β-catenin, E6.5 embryos within their decidua were sectioned and examined histologically (Fig. 2A,B). Compared to wild-type embryos the mutant embryos showed specific alterations in the embryonic ectoderm, where the cell layers appeared disorganized and cells were loosely attached (Fig. 2B). In serial sections through to E6.5 mutant embryos, condensed cell clusters of presumably intact embryonic ectoderm were surrounded by scattered cells, suggesting that not all cells of the embryonic ectoderm cell layer were equally affected. Indeed the extra-embryonic ectoderm, the ectoplacental cone and the visceral endoderm (VE) appeared normal, although the embryonic VE did appear thicker (Fig. 2B, arrow), a likely consequence of the morphological changes in the epiblast. Immunostaining of β-catenin in E5.5 and E6.5 embryos revealed that in both wild-type and mutant embryos the protein was localized largely to cell membranes (Fig. 2C-F). Interestingly, only the embryonic portion of the mutant embryos showed enhanced cytoplasmic staining for β-catenin; and no clear-cut nuclear localization was found here (Fig. 2D,F).

### Nuclear function of β-catenin in mutant ectoderm

Enhanced cytoplasmic localization of β-catenin in the mutant embryo epiblast suggested that the phenotype is due to a signaling function of mutant β-catenin. To test this possibility, wild-type and *β-catEx3flox/β-catEx3flox;cre/+*

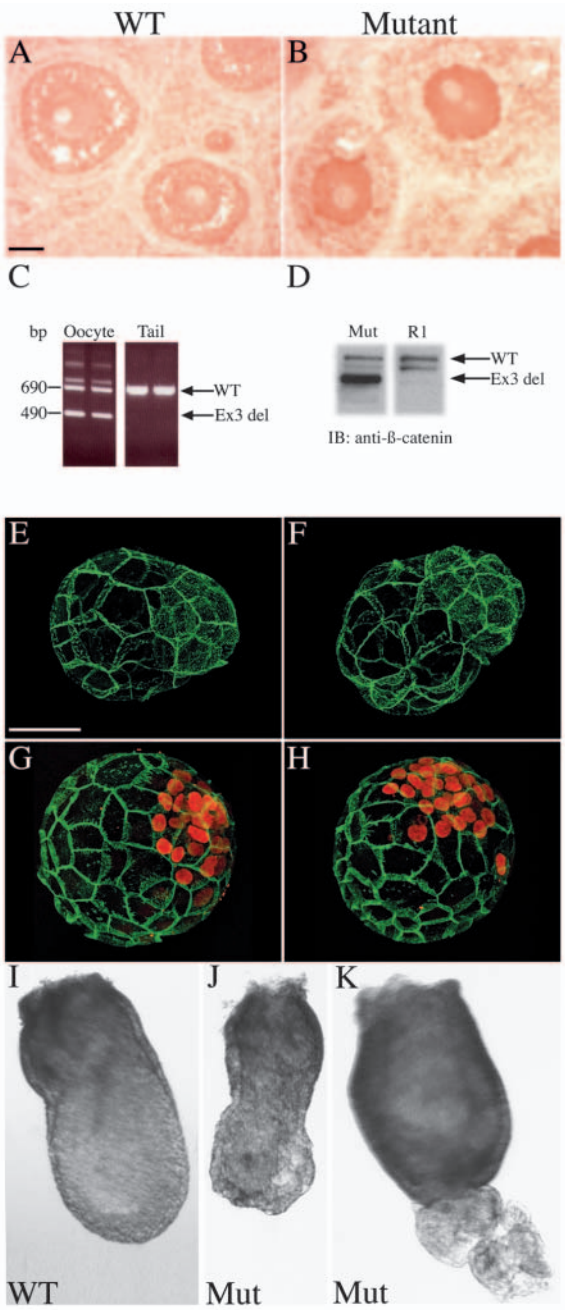


**Fig. 1.** Mutant embryos develop to blastocysts but exhibit a phenotype at early gastrulation. Immunohistochemical detection of β-catenin in wild-type (A) and mutant (B) ovaries revealed enhanced cytoplasmic localization of β-catenin in mutant oocytes. (C) Genotyping of single oocytes from *β-catEx3flox/+;cre/+*females by PCR demonstrates the efficient deletion of the floxed exon 3 of the β-catenin gene by the oocyte-specific cre-recombinase; tail DNA from the same females was used as the control. (D) Western blot of cell lysates from mutant and wild-type (R1) ES cells shows the expression of the stabilized form of β-catenin in the mutant cells. (E-H) Immunofluorescent detection of β-catenin in wild-type (E), and mutant (F) blastocysts, and simultaneous detection for E-cadherin (green) and Oct4 (red) (G, wild type; H, mutant) revealed no differences between these embryos. (I-J) Phenotype of mutant (J,K) and wild-type (I) E6.5 embryos revealed that the epiblast of mutant embryos varied, being small or disorganized. Scale bar: 50 μm.

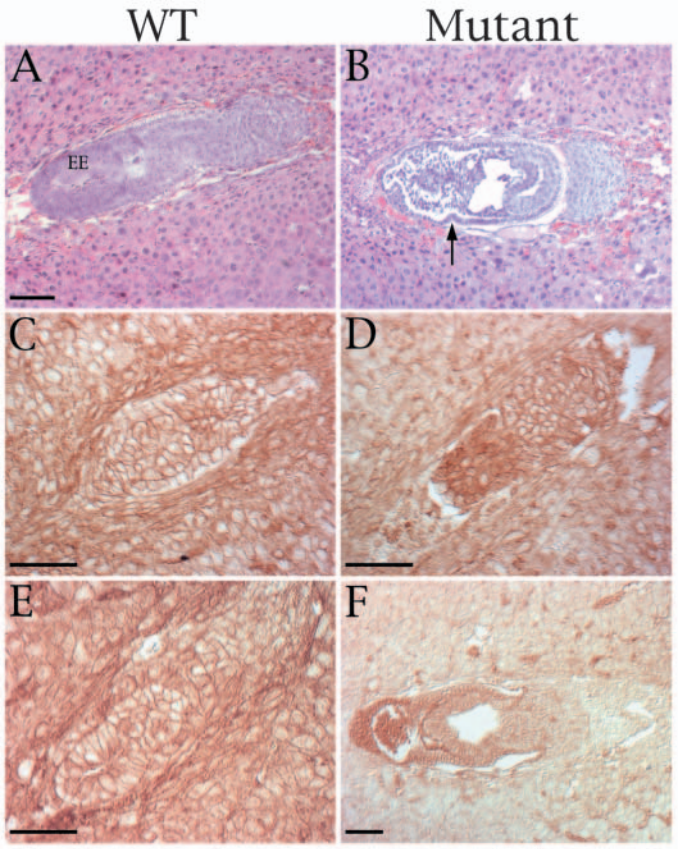


**Fig. 2.** Expression of the stabilized form of β-catenin leads to an ectodermal phenotype. (A,B) Histological sections of E6.5 wild-type and mutant embryos that were Hematoxylin and Eosin-stained. (C,D) Immunohistochemical analysis of sections from E5.5 embryos to detect β-catenin. (E,F) Immunohistochemical analysis of sections from E6.5 embryos to detect β-catenin. β-catenin was localized at the cell membrane in wild-type embryos, whereas in the mutant epiblast, β-catenin was enhanced and distributed throughout the cells. All embryos are oriented with the embryonic part to the left. EE, embryonic ectoderm. Arrow indicates thickened visceral endoderm in the mutant. Scale bar: 100 μm.

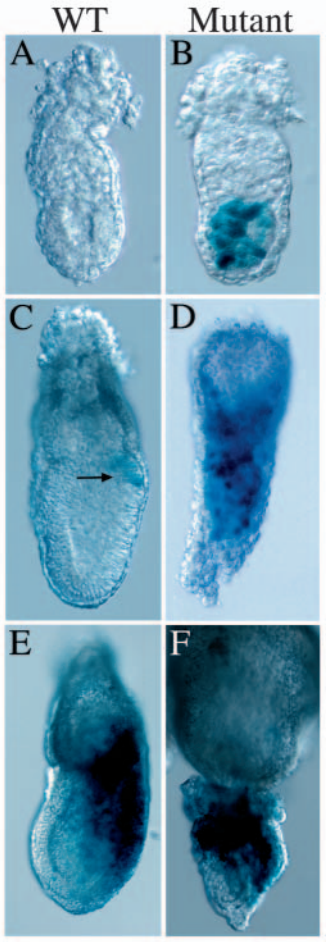


**Fig. 3.** Nuclear function of β-catenin in mutant epiblast. Males of the Wnt-reporter line, BAT-gal, were crossed to wild-type or *β-catEx3flox/β-catEx3flox;cre/+* females to obtain embryos at E5.5 (A,B), E6.5 (C,D), and E7.5 (E,F). In wild type, β-galactosidase-positive cells can first be detected on the posterior side of the proximal epiblast at E6.5 (C, arrow), and at E7.5 the β-galactosidase expression domain extended throughout the posterior part of the embryo (E) (Maretto et al., 2003). In contrast, in mutant embryos, β-galactosidase-positive cells were first found scattered throughout the epiblast at E5.5, and this positive staining became enhanced at E6.5 and E7.5, with no obvious pattern to the staining. Arrow: β-galactosidase-positive cells in wild-type E6.5 embryos.

females were crossed with males of a Wnt-reporter line (BAT-gal), which carries the *lacZ* gene driven by Wnt-responsive elements (Maretto et al., 2003). No β-galactosidase-positive cells were observed during pre-implantation development in either wild-type or mutant embryos, even with prolonged enzymatic reaction to enhance sensitivity (not shown). These results indicate that Wnt/β-catenin signaling is not active during pre-implantation development and that the stabilized form of β-catenin can only exert its nuclear function after E4.5.

Interestingly, in post-implantation development, already at E5.5 mutant embryos exhibited clusters of β-gal-positive cells in the epiblast (Fig. 3B), which also showed accumulated β-catenin in the cytoplasm (compare Fig. 2D and Fig. 3B). In contrast, we never observed β-gal-positive cells in wild-type E5.5 embryos (Fig. 3A). Wnt/β-catenin signaling in wild-type embryos can first be seen at E6.5, and, in agreement with a previous report (Maretto et al., 2003), β-gal-positive cells became apparent in the posterior side of the proximal epiblast (arrow in Fig. 3C) at the junction between embryonic and extra-embryonic ectoderm. In E7.5 wild-type embryos, probably due to Wnt3 signaling (Liu et al., 1999), the β-gal expression domain became extended in the posterior part of the embryo (Fig. 3E). In contrast to wild-type embryos, β-gal-positive cells were distributed over the entire E6.5 and E7.5 mutant embryos (Fig. 3D,F). These results clearly demonstrate the nuclear signaling function of β-catenin in mutant embryos as early as E5.5.

## Mutant embryos prematurely express mesenchymal markers in the epiblast

Phenotypic and histological analysis of mutant embryos pointed to specific alterations in the embryonic ectoderm cell layer. To further characterize these embryos, expression of RNA or protein products of *Oct4*, *T-Brachyury*, *Snai1*, *Lef1* and *Cdh1* (E-cadherin) were analyzed on serial sections from E5.5 and E6.5 wild-type and mutant embryos.

In both wild-type and mutant E5.5 embryos β-catenin exhibited mostly membrane localization (Fig. 4A,B). However, the epiblast cells of mutant embryos also showed enhanced cytoplasmic localization (Fig. 4B). No differences were seen between wild-type and mutant embryos in expression of Oct4, a pluripotency marker; the nuclei and cytoplasm of epiblast cells were positive for Oct4 protein (Fig. 4C,D). Although *T-Brachyury*, a mesoderm-specific transcription factor, is not expressed in the epiblast of wild-type E5.5 embryos, we

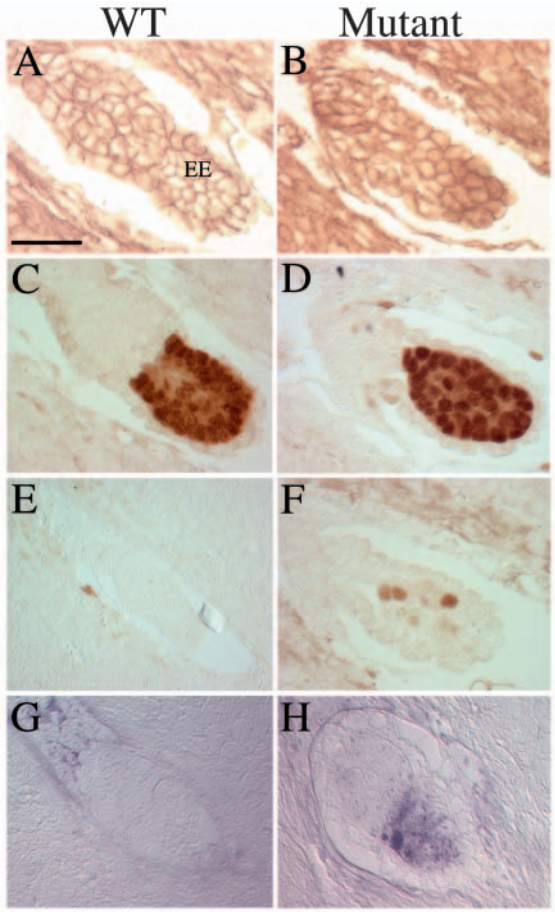


**Fig. 4.** Expression of Wnt/β-catenin target genes in the mutant epiblast. Immunohistochemical analysis of sections from wild-type (A) and mutant (B) E5.5 embryos revealed an enhanced cytoplasmic localization of β-catenin in cells of the mutant epiblast. No difference between wild-type and mutant epiblast was observed for Oct4 (C,D), while some cells in the mutant epiblast expressed T-Brachyury protein (E,F). In situ hybridization analysis of the expression of *Snai1* in wild-type (G) and mutant (H) embryos revealed expression in some cells of the mutant epiblast. All embryos are oriented with the embryonic part to the right. Scale bar: 100 μm.

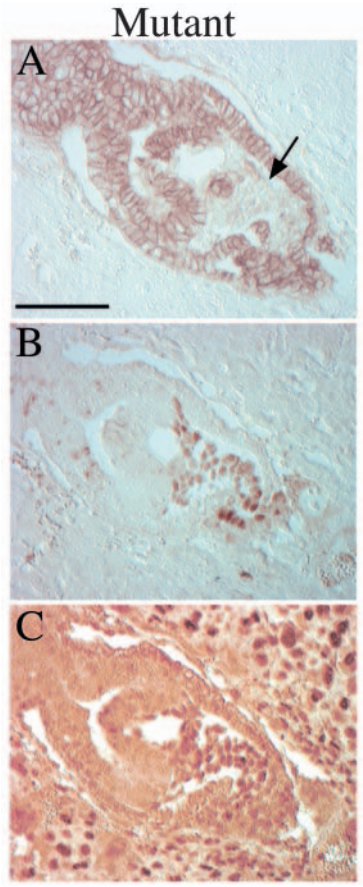


**Fig. 5.** Premature epithelial-mesenchymal transition in the epiblast of mutant embryos. Immunohistochemical analysis of E6.5 mutant embryo serial sections showed that some cells of the embryonic ectoderm (arrow) that were negative for E-cadherin (A) expressed T-Brachyury (B) and Lef1 (C), demonstrating their change from an epithelial to a mesenchymal cell fate. Arrow indicates cells that changed cell fate. Scale bar: 100 μm.

observed some cells containing nuclear T-Brachyury in mutant E5.5 embryos (Fig. 4E,F). Similarly, when in situ hybridization was performed to detect mRNA of another mesoderm-specific transcription factor, *Snai1*, it was found to be expressed in cells of the epiblast of E5.5 mutant embryos, but not in the epiblast of wild-type embryos (Fig. 4G,H). Taken together, these results clearly demonstrate that some cells in the mutant epiblast express mesoderm-specific genes by E5.5.

This claim is further substantiated by staining serial sections of mutant and wild-type embryos at E6.5 for E-cadherin, Lef1, and T-Brachyury protein (Fig. 5). In wild-type embryos, E-cadherin typically marked the epithelial cell layer of the embryonic ectoderm, while T-Brachyury and Lef1 protein expression began in the presumptive mesoderm during primitive streak formation (not shown). Remarkably, in mutant embryos, cells detached from the embryonic ectoderm layer were negative for E-cadherin staining (Fig. 5A, arrow), but instead expressed T-Brachyury and Lef1 (Fig. 5B,C). These results suggest that the epithelial cells of the embryonic ectoderm in mutant embryos take on a mesodermal fate.

To test the consequence of β-catenin signaling in the epiblast on embryonic patterning, expression of *Bmp4*, *Hex*, *Nanog*, *Nodal* and *Otx2* mRNAs was determined in situ in E6.5 mutant and wild-type embryos (Fig. 6). No difference in *Bmp4* expression in the extra-embryonic ectoderm was observed between wild-type and mutant E6.5 embryos, further indicating that the extra-embryonic part of mutant embryos is normal or less affected. At E6.5, Otx2 and Nanog are markers for embryonic ectoderm. *Nanog* mRNA was expressed in wild-type, but not in mutant embryos, in contrast to *Otx2* which was expressed in both wild-type and mutant embryos (Fig. 6). The expression of *Otx2* in mutant embryos is consistent with the known expression of *Otx2* in the embryonic ectoderm and the delaminating mesoderm (Ang et al., 1994). The expression of *Nodal* mRNA varied and was not very informative (not shown). Interestingly, Hex, a marker for the distal visceral endoderm (DVE), was absent in mutant embryos, suggesting that the mutant epiblast negatively influenced the DVE activity required for anterior-posterior (A-P) axis determination (Fig. 6, arrow). Thus, cellular changes in the epiblast due to the nuclear activity of β-catenin result in complex alterations in marker gene expression.

## Mutant embryos have restricted differentiation potential

Mutant embryos were recovered up to E9.5. Although the extra-embryonic cells appeared normal, the embryonic portion

had ceased growing and became increasingly disorganized and pycnotic (not shown). To analyze the differentiation potential of these mutant embryos, two independent sets of experiments were performed.

First, ROSA26 morulae were aggregated with either wild-type or mutant morulae, and resultant post-implantation chimeric embryos at E7.5 were sectioned after staining for β-galactosidase to label the ROSA26 cells. Wild-type morulae intermixed well with the ROSA26 partner cells, and descendants of both parts contributed equally to derivatives of all three germ layers and to extra-embryonic tissues (not shown). In contrast, when mutant morulae were combined with ROSA26 morulae, an unequal distribution of cells was observed; mutant cells contributed little to the embryonic ectoderm and preferentially colonized the visceral endoderm, mesoderm, and extra-embryonic tissues. In some extreme cases the embryonic ectoderm cell layer was exclusively composed of ROSA26 cells, while mutant cells contributed to visceral endoderm, mesoderm and extra-embryonic tissues (Fig. 7A,B).

Second, to further test the differential potential of the mutant epiblast, teratomas were induced by transplanting the embryonic part of wild-type and mutant E6.5 embryos under the kidney capsule. Wild-type embryos developed into typical fully differentiated teratomas or teratocarcinomas (Damjanov et al., 1971; Solter et al., 1970) composed of a large variety of different tissues and cell types and containing nests of embryonal carcinoma cells (Fig. 7C). In contrast, tumors derived from mutant embryos were very small (the largest was 3 mm in diameter) and were composed of cuboidal cells forming gland-like or tubular structures (Fig. 7E, arrowhead). The overall architectural features and the irregular shape of cells and nuclei suggested a malignant phenotype (Fig. 7E, star). The presence of a hyaline membrane (Fig. 7E, arrow) is reminiscent of Reichert's membrane typically seen in murine yolk sac tumors (Damjanov and Solter, 1973), thus classifying it as a yolk sac carcinoma. Sections of wild-type and mutant embryo-derived teratomas were stained for β-catenin (Fig. 7D,F). In the wild-type, β-catenin was largely localized at the

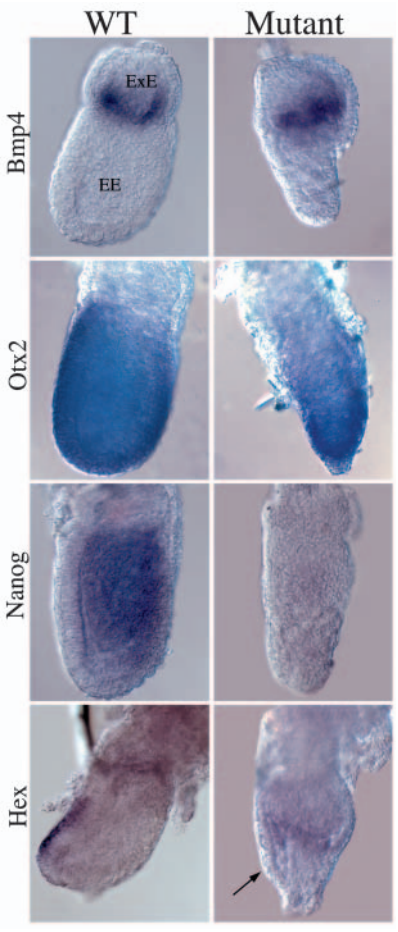


**Fig. 6.** Embryonic patterning is changed as a result of stabilized β-catenin expression. The expression of embryonic patterning genes was analyzed by whole-mount in situ hybridization of E6.5 wild-type and mutant embryos. In wild-type and mutant embryos *Bmp4* had a similar expression pattern in the extra-embryonic ectoderm. The expression domain of *Otx2* was less extensive in the mutant embryo epiblast in comparison to that of the wild-type embryo. No *Nanog* expression was found in the mutant epiblast. The mutant visceral endoderm (arrow) expressed no *Hex*. Arrow indicates mutant visceral endoderm negative for *Hex*; EE, embryonic ectoderm.

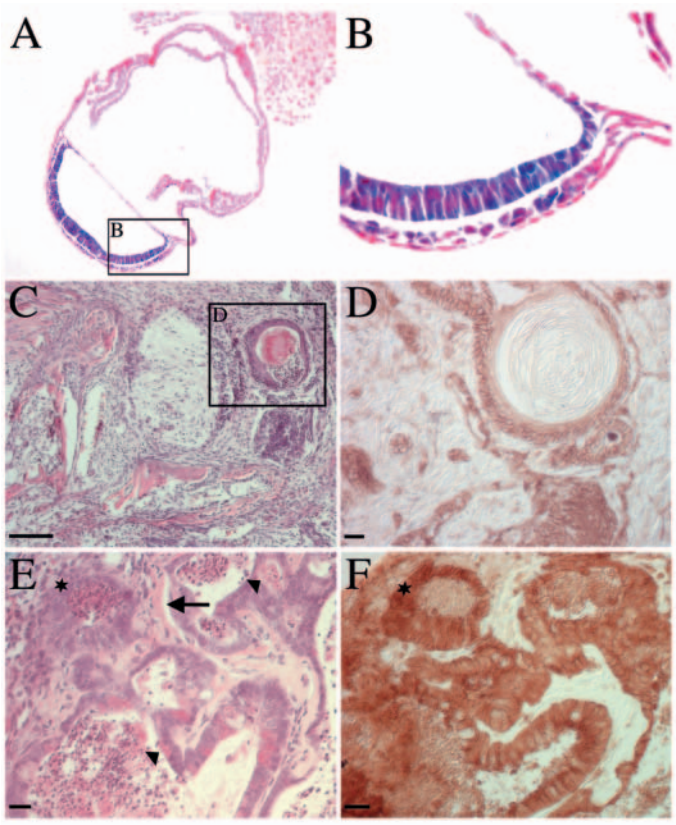


**Fig. 7.** Mutant embryos exhibit a restricted differentiation potential. (A,B) Determination of β-galactosidase expression in sections from E7.5 embryos that developed from aggregation chimeras between ROSA26 and mutant morulae revealed that mutant cells contributed mostly to extra-embryonic tissues and were only occasionally found in the embryonic ectoderm of the chimeras. In some cases the embryonic ectoderm cell layer was exclusively composed of ROSA26 cells (A,B). (C-E) Analysis of wild-type and mutant E6.5 embryos transplanted under the kidney capsule to induce teratomas. (C) Teratocarcinoma produced by transplantation of wild-type embryo. Several well differentiated tissues are visible, including bone, muscle, neural tissue and skin epithelium. (D) Adjacent section stained with antibody to β-catenin. Higher magnification of area marked in panel C shows expression of β-catenin in the membranes of the epithelial cells. (E) Histological section of tumor derived from transplanted mutant embryo. Cells similar to those present in extra-embryonic endoderm form small glandular structures (arrowhead). Hyalin substance corresponding to Reichert's membrane (arrow) fills extracellular spaces. Certain gland-like structures display substantial cellular and nuclear polymorphism (star). (F) Adjacent section stained with antibody to β-catenin. There is intensive membrane and cytoplasmic staining. Cells exhibiting polymorphism (star) also show nuclear staining. Scale bar: 100 μm.

cell membrane of epithelial cells (Fig. 7D). Sections from mutant teratomas showed rather uniform and enhanced staining in the cytoplasm of most cells as well as in the nuclei of some cells (Fig. 7F, star), indicating that the stabilized form of β-catenin is maintained during tumor growth.

## Discussion

The best known functions of β-catenin are involvement in the cadherin–catenin cell adhesion complex, and in the Wnt/β-catenin signaling pathway. The detection of members of the Wnt/β-catenin pathway in pre-implantation embryos using different approaches (Evsikov et al., 2004; Knowles et al., 2003; Lloyd et al., 2003; Wang et al., 2004) suggested a regulatory role for the Wnt/β-catenin pathway in cleavage-stage embryos.

We found no evidence for active Wnt/β-catenin signaling during pre-implantation development using the Wnt-reporter line BAT-gal (Maretto et al., 2003). We then designed genetic experiments to study the nuclear signaling function of β-catenin in pre-implantation embryos. In *β-catEx3flox/+, cre/+* females the stabilized form of β-catenin is efficiently generated in the growing oocyte. By several criteria, pre-implantation development of mutant embryos derived from these oocytes proceeded normally. More importantly, no nuclear signaling function of β-catenin could be detected in mutant embryos using the Wnt-reporter line BAT-gal. In accordance with this, we observed no nuclear localization of β-catenin in mutant oocytes or embryos.

These results suggest mechanisms independent of GSK3β-mediated phosphorylation and β-Trcp-mediated proteolysis must come into play to prevent action of the stabilized form of β-catenin in the blastomere nuclei. One alternative pathway, able to degrade both wild-type and mutant β-catenin, has been described (Liu et al., 2001; Matsuzawa and Reed, 2001). This pathway, activated by p53, involves the ubiquitin ligase SIAH1, which, in collaboration with several interacting proteins, SIP, APC, and Skp-Cullin-F-box complexes, leads to degradation of β-catenin by the proteasome. The presence of this pathway in pre-implantation embryos is plausible, since APC is expressed in these developmental stages (de Vries et al., 2004), and SIAH2, a protein closely resembling SIAH1, is also expressed (W.N.dV., unpublished). Furthermore, the other molecules of this pathway, as well as those of the proteasome complex, are abundantly expressed in full-grown oocytes and two-cell stage embryos (A. V. Evsikov, unpublished) (Evsikov et al., 2004).

However, mechanisms disrupting TCF/β-catenin interaction may also be involved in preventing the nuclear action of β-catenin. Expression sequence tags (ESTs) for ICAT, an 81-amino acid peptide that interferes with Wnt signaling (Tago et al., 2000), have been found in a two-cell stage cDNA library (Evsikov et al., 2004; Knowles et al., 2003). Secreted Frizzled-related proteins (SFRPs) can also attenuate Wnt signaling in colorectal cancer cells, even in the presence of downstream stabilizing mutations, such as those found in β-catenin and APC (Suzuki et al., 2004). SFRPs are expressed in cleavage-stage embryos, and a similar mechanism could therefore silence the stabilized form of β-catenin in our experiments.

As β-catenin is able to interact with a large variety of molecules (Huelsken and Behrens, 2000), the nuclear activity of β-catenin in pre-implantation embryos could be regulated by the interplay of a relative excess of repressors that will inhibit the transcriptional activity of β-catenin and/or the developmentally regulated expression of co-activators during early cleavage-stages. Repressors interacting with β-catenin may act in concert with the protein degradation pathways to robustly control β-catenin nuclear activity during pre-implantation development. Finally, one has to consider differences in the regulation of transcription of β-catenin during development. Although β-catenin is rather ubiquitously expressed, little is known about a fine-tuning of transcription that could result in different amounts of protein being synthesized. A β-catenin dose-dependent differentiation of ES cells carrying mutations in APC has been reported recently (Kielman et al., 2002). A certain threshold level of β-catenin may be required for its nuclear function, and reaching this might well depend on the transcriptional activity of the gene.

Although pre-implantation development progressed normally, post-implantation mutant embryos exhibited a specific phenotype in the embryonic ectoderm, while the morphology of the extra-embryonic portion was normal. As our mating scheme ensured that every cell in the developing mutant embryo would express the stabilized form of β-catenin, this suggests that cells of extra-embryonic tissues have mechanisms similar to those in pre-implantation embryos to control the nuclear activity of the stabilized form of β-catenin.

Cells of the embryonic ectoderm prematurely expressed direct Wnt/β-catenin target genes such as *T-Brachyury* and *Lef1*, a likely direct effect of the nuclear functioning of β-catenin. Indeed, T-Brachyury was detected as early as E5.5 in some cells of the epiblast when most cells still express the pluripotency marker Oct4. Mutant epiblast cells not only express the mesodermal-specific genes *T-Brachyury* and *Lef1*, they also lose E-cadherin expression and thus adopt a mesenchymal fate. Hence, besides acting positively on the transcription of *T-Brachyury* and *Lef1*, mutant β-catenin signaling may also repress E-cadherin transcription, a mechanism proposed for the normal epithelial-mesenchymal transition during streak formation (Huber et al., 1996) and demonstrated for E-cadherin repression in hair follicles (Jamora et al., 2003). Alternatively or in combination, *Snai1* may also be involved in the repression of E-cadherin transcription, as *Snai1* mRNA can be detected in E5.5 mutant embryos. This premature epithelial-mesenchymal transition leads to disintegration of the embryonic ectoderm cell layer and is probably the cause of inefficient embryonic patterning.

The fact that the embryonic ectoderm is largely devoid of mutant cells in morulae aggregation experiments suggests a cell-autonomous event induced by the mutant form of β-catenin. Mutant cells probably segregate from the epiblast because of the downregulation of E-cadherin. However, it may well be that stabilized β-catenin induces other adhesive mechanisms that act to separate wild-type and mutant cells in the epiblast of chimeric embryos.

Mutant embryos transplanted under the kidney capsule give rise to yolk sac carcinomas, indicating that the embryonic ectoderm cells have lost their growth and/or pluripotent capacity. Tumors derived from mutant embryos were similar to those produced by transplanting the extra-embryonic part of the egg-cylinder (Solter and Damjanov, 1973), suggesting that embryonic endoderm, in the absence of functional ectoderm,

changes its differentiation pattern. This observation, together with the absence of *Hex* expression in embryonic endoderm of mutant embryos, indicates the importance of normal signaling from the epiblast in regulating patterning and differentiation of embryonic visceral endoderm.

In conclusion, we found no evidence that Wnt/β-catenin signaling plays a role in regulation of mouse pre-implantation development. The observed presence of components of the pathway in early cleavage-stages may simply indicate they are in place for use in primitive streak formation, or that they have an alternative function in the pre-implantation embryo. More importantly, pre-implantation embryos and some cells of post-implantation embryos apparently control intracellular β-catenin levels by a mechanism independent of the GSK3β-mediated ubiquitination and proteasome degradation pathway. In contrast, cells of the epiblast are unable to regulate the stabilized form of β-catenin. In these cells β-catenin is able to exert its nuclear function, and this results in a premature epithelial-mesenchymal transition. It will be of future interest to determine the molecular mechanisms responsible for this difference in control of the Wnt/β-catenin pathway.

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