The NEDD8 system is essential for cell cycle progression and morphogenetic pathway in mice

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NEDD8/Rub1 is a ubiquitin (Ub)-like molecule that covalently ligates to target proteins through an enzymatic cascade analogous to ubiquitylation. This modifier is known to target all cullin (Cul) family proteins. The latter are essential components of Skp1/Cul-1/F-box protein (SCF)–like Ub ligase complexes, which play critical roles in Ub-mediated proteolysis. To determine the role of the NEDD8 system in mammals, we generated mice deficient in Uba3 gene that encodes a catalytic subunit of NEDD8-activating enzyme. Uba3−/− mice died in utero at the periimplantation stage. Mutant embryos showed selective apoptosis of the inner cell mass but not of trophoblastic cells. However, the mutant trophoblastic cells could not enter the S phase of the endoreduplication cycle. This cell cycle arrest was accompanied with aberrant expression of cyclin E and p57Kip2. These results suggested that the NEDD8 system is essential for both mitotic and the endoreduplicative cell cycle progression. β-Catenin, a mediator of the Wnt/wingless signaling pathway, which degrades continuously in the cytoplasm through SCF Ub ligase, was also accumulated in the Uba3−/− cytoplasm and nucleus. Thus, the NEDD8 system is essential for the regulation of protein degradation pathways involved in cell cycle progression and morphogenesis, possibly through the function of the Cul family proteins.

Introduction

In eukaryotes, there exist multiple ubiquitin (Ub)*-like proteins that covalently bind to target protein through a reaction analogous to ubiquitylation (Hochstrasser, 2000; Jentsch and Pyrowolakis, 2000). Unlike Ub chain linkage, which primarily acts as a degradation signal, the roles of the Ub-like molecules are largely unknown. Among Ub-like molecules, NEDD8 (a mammalian homologue of the budding yeast Rub1, related to Ub1) has the highest homology to Ub and is conserved in various organisms such as yeast, plant, and mammal (Kamitani et al., 1997; Yeh et al., 2000). Isopeptide linkage of NEDD8 to target proteins is catalyzed by the APP-BP1–Uba3 heterodimer complex and Ubc12 as the E1 (activating)-like and E2 (conjugating)-like enzymes, respectively (Lammer et al., 1998; Liakopoulos et al., 1998; Osaka et al., 1998). The NEDD8 systems are essential in fission yeast (Osaka et al., 2000) but not in budding yeast (Lammer et al., 1998; Liakopoulos et al., 1998), whereas their importance in mammalian organisms has not yet been determined.

The targets of NEDD8 are unknown apart from cullin (Cul) family proteins, which are also conserved across species (Liakopoulos et al., 1998; Osaka et al., 1998; Hori et al., 1999; Gray and Estelle, 2000). Cul-1 is a common subunit of a class of Skp1/Cul-1/F-box protein (SCF) complexes responsible for ubiquitylation of a multitude of proteins that regulate various biologically important processes such as cell cycle progression and signal transduction (Deshaies, 1999; Weissman, 2001; Pickart, 2001). Recently, other Cul family proteins have also been suggested to constitute a large family of distinct Ub protein ligase complexes (E3-enzymes), based on their interaction with a recently identified ROC1–Rbx1–Hrt1, a RING finger protein, which is essential for the catalytic activity of SCF (Deshaies, 1999; Kamura et al., 1999; Ohta et al., 1999). Indeed, Cul-2 and Cul-5, each complexed with elongin B and elongin C, are reported to have Ub protein ligase activities.
The modification of SCF by NEDD8 is essential in fission yeast. Pcu1<sup>K720R</sup>, a Pcu1 (Cul-1 homologue) mutant defective for NEDD8ylation cannot complement the lethal phenotype of Δpuc1, and overexpression of Pcu1<sup>K720R</sup> results in accumulation of SCF complex substrates, Rum1, a cyclin-dependent kinase (CDK) inhibitor (CKI) (Osaka et al., 2000). In contrast, the budding yeast homologue Cdc53 does not require NEDD8 modification for its function (Lammer et al., 1998). In Arabidopsis thaliana, Axr1, an APP-BP1 homologue was identified from screening of mutants resistant to auxin response. However, Axr1-1 cells, which have no NEDD8-modifying activity remain viable (Gray and Estelle, 2000). Thus, the NEDD8 system plays a synergistic role with the SCF complex in various organisms, but its requirement may significantly differ among species.

Biochemical studies have shown that the NEDD8 modification of the SCF complex enhances the Ub ligase activity by recruiting E2 to the complex efficiently without affecting the stability, assembly, or substrate binding capacity of the SCF complex (Kawakami et al., 2001). However, the in vitro ubiquitylation activity of the SCF complex is still evident without its modification by NEDD8 (Podust et al., 2000; Read et al., 2000; Wu et al., 2000; Kawakami et al., 2001). Furthermore, it was reported recently that p<sup>9<sup>581<sup>Cls/1</sup>Cls1</sup></sup> but not NEDD8 promotes the ubiquitylation activity of SCF<sup>CDK1</sup> (Ganoth et al., 2001). Thus, although NEDD8 modification is essential for Cul function in fission yeast its precise molecular action remains to be unraveled.

To investigate the role of NEDD8 in mammalian organisms in vivo, we generated the Uba3-deficient mouse, which lacks the catalytic subunit of NEDD8 activating enzyme. Analysis of Uba3-deficient mice showed that the NEDD8 system is essential for cell cycle progression including the endoreduplication cycle. The endoreduplication cycle is an unusual mode of cell cycle that results in duplication of the chromosome without intervening mitosis (Varmuza et al., 1988; Edgar and Orr-Weaver, 2001). Although, this cycle is mutually exclusive with mitotic cell cycle, the two processes share common mechanisms such as fluctuation of CDKs activity (Teras et al., 1998). We found that Uba3-deficient trophoblastic cells could not enter S phase, and this cell cycle arrest was accompanied with the high expression of cyclin E and p57<sup>Kip2</sup>. Furthermore, β-catenin, a mediator of the Wnt/wingless (Wg) signaling pathway, accumulated in the cytoplasm and nuclei of mutant cells, suggesting that the SCF complex and its modification by NEDD8 are essential for β-catenin degradation. Since the Wnt/Wg signaling pathway regulates the orientation of cell polarity axis and tissue specific gene expression (Bedington and Robertson, 1999; Bellaiche et al., 2001), we propose that the regulation of the NEDD8 system may coordinate the cell cycle progression and cell polarity axis formation in the development of multicellular organism.

**Results**

**Cloning and targeting of mouse Uba3 gene**

Mouse Uba3 cDNA (Genbank AY029181) consists of 2,117 nucleotides with 94% homology to human cDNA. Deduced 441 amino acids were 99% identical to their human counterpart. Mouse Uba3 genomic DNA was obtained by screening C57BL/6J mouse genomic bacterial artificial chromosome (BAC) library using cDNA as a probe. Two independent BAC clones were obtained, and their structures were determined (Fig. 1 A). Mouse Uba3 gene was encoded by 14 exons that spanned ~14 kb length genomic DNA. The active site cysteine residue essential for NEDD8 activation was encoded by exon 6, and the targeting vector was designed to delete exons 5–7 (Fig. 1 A). After electroporation of targeting vector into TT2 embryonic stem (ES) cells, the homologously recombined ES cells were screened by PCR and genomic Southern blot. Two independent lines of heterozygous ES cells were then transmitted into germ line.

**Embryonic lethality of Uba3-deficient mice**

Uba3 heterozygous mice were born healthy and fertile without any noticeable pathological phenotypes compared with wild-type littermates at least within the 2-yr observation period. However, subsequent intercrossing of heterozygous mice has so far failed to produce any viable homozygous (Uba3<sup>-/-</sup>) mice (Table I). To further characterize Uba3<sup>-/-</sup> mice, embryos in utero at various stages of development were dissected out and their genotypes analyzed by Southern blot or PCR (Fig. 1, B and C). Although Uba3<sup>-/-</sup> mice were evident at embryonic day (E)3.5 with normal appearance, we noticed that ~13% of total embryos had defects in blastocyst formation and hatching. These embryos were ho-

![Figure 1. Targeted disruption of the Uba3 gene.](image-url)
mozygous mutants as revealed by PCR genotyping. Thus, it was likely that half of the *Uba3*−/− embryos die before implantation (Table I). The phenotype may vary according to the amount of Uba3 maternally provided because weak Uba3 immunoreactivity was observed in every blastocyst examined (unpublished data). We next examined the histology of embryos between E5.5 and E7.5 (Fig. 2, A–H). In E6.0–6.5 embryos, a cylinder-like two-layered cellular structure was observed in normal embryos (Fig. 2, A–D). However, Uba3-deficient embryos, which could be identified at this stage by the absence of Uba3 immunoreactivity (Fig. 2 E), lacked such cylinder-like structure (Fig. 2, F and G). By E7.5, these disorganized embryos could not be found; rather, only completely absorbed embryonic tissues could be identified in the decidua (Fig. 2 H and Table I). We next characterized the nature of disorganized embryos at E6.0 by immunohistochemistry using several tissue-specific markers. Cytokeratin-endoA antibody (TROMA-1) stains the trophoblast cell lineage (Brulet et al., 1980). TROMA-1 stained the extraembryonic tissue of wild-type embryos but not embryonic ectoderm (Fig. 3, A and B). In Uba3-deficient embryos, TROMA-1 reacted to most cells at E6.0, suggesting that these cells were composed mostly of extraembryonic tissue (Fig. 3, C and D), and thus these embryos lacked embryonic ectoderm.

**Selective apoptosis of inner cell mass in Uba3-deficient mice**

In the next step, we investigated whether such regression in *Uba3*−/− mice was due to cell death. Apoptotic cells could not be observed in either wild-type or mutant embryos at E6.0–6.5 (unpublished data). Rather, apoptotic cells were observed at earlier stage of the mutant embryos (Fig. 4 C) but not of wild-type (Fig. 4 A), which had just implanted to the uterus as judged by hematoxylin and eosin (H&E) staining (Fig. 4, B and D). These results indicate that most *Uba3*−/− cells undergo apoptosis, whereas mutant extraembryonic tissues escape this death pathway and remain viable as late as E6.5.

**Impaired cell cycle progression in Uba3-deficient cells**

To further analyze the cellular defects of Uba3 deficiency, we next transferred the blastocysts into in vitro cultures (Fig. 5, A–F). After hatching, the wild-type blastocysts grew on the gelatin-coated glass. Trophoblastic cells with large nuclei spread over the glass, whereas the inner cell mass (ICM), which forms the future embryonic ectoderm, increased in size at the center of the sheet of trophoblastic cells (Fig. 5, A and B). Whereas half of the *Uba3*−/− blastocysts could hatch and outgrow, neither could expand after 72 h to a size similar to that seen with *Uba3*+ cells (Fig. 5, D and E; note the bar). Specifically, *Uba3*−/− ICM (Fig. 5 F) underwent apoptosis and detached from the culture dish, consistent with the cell death noted in histological studies. In this regard, all efforts to obtain ES cell lines from homozygous embryos were unsuccessful.

**Table I. Genotype analysis of *Uba3*+/- intercross progeny**

| DNA source           | +/- | +/- | --/- | ND | Resorbed |
|----------------------|-----|-----|------|----|----------|
| Tails (4-wk old)     | 97  | 163 | 0    | 0  |           |
| Yolk sacs (E11.5–17.5)| 3   | 7   | 0    | 5  |           |
| Embryos (E7.5–E8.5)  | 5   | 12  | 0    | 3  |           |
| Blastocysts (E3.5)   | 17  | 30  | 5    | 4  |           |
| Blastocysts (E3.5 cultured) | 2 | 6 | 0 | 2 |           |
| Normal ICM           | 2   | 6   | 0    | 2  |           |
| Atrophied ICM        | 0   | 0   | 2    |    |           |

Genotype analysis of *Uba3* heterozygous intercross progeny. Embryos were genotyped by Southern blotting or PCR analysis (Fig. 1, A–C). Empty decidua were scored as “Resorbed.” Some blastocysts harvested at E3.5 were grown in culture before recovering DNA for genotyping. Phenotype data of cultured blastocysts represents typical data from one set of experiments. ND, not determined.

**Figure 2. Comparison of *Uba3*+ and *Uba3*−/− embryos in utero.**

*Uba3* expression was analyzed by immunohistochemical stainings using anti-*Uba3* antibody in *Uba3*+ (A) or *Uba3*−/− (E) embryos at E6.0. *Uba3*+/− (B–D) and *Uba3*−/− (F–H) embryos at various stages as indicated were sagittally sectioned and stained with H&E. A cylinder-like two-layered cellular structure observed in normal embryos (B and C) was not seen in *Uba3*−/− embryos (F and G). By E7.5, *Uba3*−/− embryos could not be found; rather, only completely absorbed embryonic tissues could be identified in the decidua (H). EE, embryonic ectoderm; EPC, ectoplacental cone; EXE, extraembryonic ectoderm. Bars, 100 μm.
Uba3 is required for S phase entry and endoreduplication of the trophoblasts

To identify cell cycle defects in Uba3-deficient cells, we added BrdU to the in vitro culture medium and tested whether Uba3−/− cells can enter the S phase. In trophoblastic cells, Uba3+/− (Fig. 6 A) but not Uba3−/− (Fig. 6 B) could enter S phase. In the ICM, both Uba3+/− (Fig. 6 C) and Uba3−/− (Fig. 6 D) could enter this phase. DNA staining revealed that most Uba3−/− trophoblastic cells contained small nuclei (Fig. 6 F), whereas Uba3+/− cells contained large nuclei (Fig. 6 E), a morphological hallmark of endoreduplication (Varmuza et al., 1988). To assure the S phase entry is indeed impaired in vivo, we injected BrdU in 5.75 d postcoitum pregnant female and analyzed BrdU incorporation in the embryos. In wild-type embryos, BrdU-positive cells were observed in most parts of the embryonic ectoderm and extraembryonic tissue (Fig. 6, G and I). In contrast, mutant embryos showed few BrdU-positive cells (Fig. 6, H and J). Since the endoreduplication cycle requires fluctuation of CDKs activity (Traas et al., 1998), we next investigated whether any cell cycle regulators are dysregulated in trophoblasts. Cyclin E, a member of G1 cyclins, has been reported to be expressed periodically in trophoblastic cells, and this periodicity is essential for endoreduplication (MacAuley et al., 1998; Weiss et al., 1998). In Uba3+/− cells, cyclin E expression was evident in a few trophoblasts consistent with the cell cycle-regulated expression pattern of cyclin E (Fig. 7 A), whereas overexpression of cyclin E was observed in most of Uba3−/− trophoblasts (Fig. 7 B). A similar accumulation of
cyclin E was also observed in the ICM of Uba3⁻⁻⁻ embryos (Fig. 7 D) compared with Uba3⁺⁺⁺ embryos (Fig. 7 C). Immunostaining of other cyclins revealed that cyclin D3, another member of G1 cyclins, was highly expressed compared with wild-type littermates. Since overexpression of cyclin E normally assists in overriding the G1-S checkpoint rather than G1-S arrest (Lukas et al., 1997), we next tested the expression of CKIs that negatively regulate CDK activity. Among the member of CKIs, the expression of p57⁰⁰² is known to be induced upon differentiation of trophoblasts, and its level fluctuates during the endoreduplication cycle (Hattori et al., 2000). Indeed, p57⁰⁰² was expressed abundantly in most Uba3⁻⁻⁻ trophoblasts (F) but only in part of Uba3⁺⁺⁺ cells (E). p57⁰⁰² was expressed in a few cells in the ICM of both Uba3⁺⁺⁺ and Uba3⁻⁻⁻ embryos (G and H). Bars, 100 μm.
phase enters the G2-like phase of the endoreduplication cycle (Hattori et al., 2000). No significant accumulation of other members of CKI, p21Cip1, and p27Kip1 was noted in trophoblasts or ICM (unpublished data). Thus, S phase entry of Uba3−/− trophoblasts is blocked in part due to accumulation of p57Kip2, or alternatively trophoblasts are arrested before the point that p57Kip2 degrades. Intriguingly, cyclin E accumulated in Cul-3−/− trophoblasts as well, which failed to endoreduplicate (Singer et al., 1999), suggesting that Cul-3 function might be impaired in Uba3−/− mice.

**Accumulation of β-catenin in nuclei of Uba3-deficient cells**

The above studies suggested the functional impairment of Cul family proteins in Uba3−/− mice. In the next step, we examined the expression of putative substrates of the SCF complex. One such substrate is β-catenin. In *Drosophila*, SCFslimb complex regulates not only cell cycle progression but also axis formation of developing embryos through the regulation of several signaling pathways, including Wnt/Wg and NF-κB/dorsal pathways (Jiang and Struhl, 1998; Spencer et al., 1999). The mammalian homologue of SCFslimb complex, SCFTrCP1, binds to phosphorylated β-catenin for ubiquitylation (Kitagawa et al., 1999; Winston et al., 1999). β-Catenin was localized at the plasma membranes of Uba3+ cells (Fig. 8, A–C). On the other hand, a high expression of β-catenin was observed in the nuclei and cytoplasm of Uba3−/− cells (Fig. 8, D–F), indicating that cytoplasmic degradation of β-catenin is diminished significantly in Uba3−/− cells. These results suggest that the NEDD8 system is essential to control the cytoplasmic level of β-catenin, and SCFTrCP1 plays a critical role for β-catenin degradation.

**Discussion**

The NEDD8 system had been demonstrated to cooperate with SCF function in a variety of organisms (Hochstrasser, 1998). Despite its evolutionary conservation, the requirement of the NEDD8 system differs significantly among species. Here, we provide the first evidence that the NEDD8 system is indeed essential for cell cycle progression and development of mammalian embryos. Analysis of Uba3−/− embryos revealed that the NEDD8 system is essential for both the mitotic and endoreduplicative cell cycle. The latter cycle is known to occur in several tissues in mammals, such as trophoblastic giant cells, hepatocytes, and megakaryocytes (Varmuza et al., 1988; Zimmet and Ravid, 2000). The role of the endoreduplication cycle is not well understood but has been proposed to be involved in cell differentiation, cell expansion, metabolic activity, and resistance against DNA-damaging agents (Nagl, 1976; MacAuley et al., 1998). Although the mechanisms that allow cells to switch from mitosis to endoreduplication are not well understood, it seems that the endoreduplication cycle uses the same machinery as mitotic cell cycle (Traas et al., 1998; Edgar and Orr-Weaver, 2001). Among these regulators, anaphase-promoting complex/cyclosome (APC/C), another Ub ligase complex essential at the mitotic phase, is important for the transition to endoreduplication in *Drosophila* (Sigrist and Lehner, 1997). Although APC/C contains Cul-like subunit APC2, APC2 lacks the consensus domain for NEDD8 modification. Thus, NEDD8 does not seem to regulate APC/C. The essential role of the NEDD8 system in endoreduplication is probably through the modification of Cul-3. Indeed, Cul-3−/− trophoblastic cells failed to enter the endoreduplication cycle (Singer et al., 1999). In contrast, Cul-I−/− trophoblasts actively entered the cycle (Wang et al., 1999), suggesting that Cul-1 and Cul-3 may act in an opposite manner in the cycle. Enhancement of polyploidization of the cells is also...
observed in mutants of F-box proteins, pop1 and pop2 in fission yeast (Kominami and Toda, 1997), and Skp2 in mice (Nakayama et al., 2000). These facts suggest that certain SCF complexes are involved in the negative regulation of switching from normal cell division cycle to the endoreduplication cycle. In Uba3−/− embryos, residual cells in the extraembryonic tissue may have been actively committed to the endoreduplication cycle by loss of Cul-1 function. However, those may have failed to enter the next S phase by loss of Cul-3 function. These suggest that Cul-1 and Cul-3 may act in a sequential manner during the cell cycle progression.

We also showed aberrant expression of p57kip2 in Uba3−/− trophoblasts. Consistent with this finding, it is reported that fluctuations of p57kip2 level are essential for the entry to S phase of the endoreduplication cycle (Hattori et al., 2000). However, our results do not exclude the possibility that Uba3−/− trophoblasts arrest just before the degradation of p57kip2 by other cell cycle defects. It is also possible that the presence of dysregulated Wnt/Wg signaling pathway might interfere with the endoreduplication cycle. It was reported recently that knirps and knirps-related transcription factors are regulated by the Wnt/Wg signaling pathway, and both inhibit endoreduplication in a spatio-temporal manner (Fuss et al., 2001). Furthermore, in Drosophila wing primordia Wnt/Wg induces arrests of the cells at G2 phase by down-regulating mitosis-inducing phosphatase, Cdc25 (Johnston and Edgar, 1998).

Our results also showed that loss of the NEDD8 system led to selective apoptosis of ICM although the exact mechanism could not be identified. Since Uba3−/− ICM but not trophoblasts can enter the S phase, the apoptotic process may be linked to a DNA replication checkpoint. Based on previous findings and the results of our study, we postulate that loss of the NEDD8 system leads to impaired replication and/or repair of the DNA for the following reasons. First, subunit of global genomic repair protein xeroderma pigmentosus group E (XP-E) interacts with Cul4A (Shiyanov et al., 1999). XP-E is induced by UV irradiation and binds to UV-damaged DNA (Tang et al., 2000). XP-E activity may be relevant to the essential role of the NEDD8 system in DNA replication and repair and possible dissociation from the apoptotic pathway, it is worth noting that accumulation of cyclin E and β-catenin, which was observed in Uba3−/− mice, has been otherwise implicated in carcinogenesis (Nielsen et al., 1998; Spruck et al., 1999; Polakis, 2000). Moreover, one might expect that loss of the NEDD8 system should also lead to the dysfunction of tumor suppressor, pVHL (von Hippel-Lindau syndrome gene product), which acts as Ub ligase together with Cul-2. Thus, we propose that attenuation of the NEDD8 system may link to carcinogenesis or the unusual characters of cancer cells.

It is likely that NEDD8 modification is essential for all of the Cul family members because Uba3-deficient mice appear to have both phenotypes induced by loss of Cul-1 and Cul-3 and thus even severer than each Cul-1−/− and Cul-3−/− mouse. Why is NEDD8 modification essential for Cul function? NEDD8-ylation of Cul-1 recruits Ub-E2 to the SCF complex (Kawakami et al., 2001) and stimulates in vitro ubiquitylation of IkBα and p27kip1 by SCFrskp2 and SCFp21, respectively (Podust et al., 2000; Read et al., 2000; Wu et al., 2000). However, such NEDD8-ylation was not strictly required, at least for in vitro ubiquitylation activity (Ganoth et al., 2001). Thus, the NEDD8 pathway may have other roles in addition to recruit the E2 enzyme to the SCF complex. Since NEDD8-ylated SCF complexes are spatially temporally regulated during centrosome duplication and DNA replication, the NEDD8 modification may control the regulation in those processes. Alternatively, the NEDD8 system may be regulated in a spatio-temporal manner.

Finally, the NEDD8 system, which seemed to be diverged from the Ub system, is a regulator of the protein degradation machinery. However, unlike Ub NEDD8 targets a highly conserved family of Cul-based E3 enzymes for activation of their ultimate functions. This pathway may coordinate the regulation of cell cycle progression and morphogenetic pathway.

Materials and methods

Gene targeting of Uba3

Two genomic clones that cover the entire Uba3 gene were isolated from a mouse B6C37BL/BAC library by hybridization screening (Incyte Genomics). The Uba3 targeting vector was constructed by inserting neomycin-resistant gene instead of the 2-kb segment, which encodes the conserved cysteine residue necessary for activation of NEDD8, and is flanked by 5- and 2-kb of Uba3 genomic sequences (Fig. 1 A). We electroporated the targeting vector into mouse TTE ES cells, selected with G418 (200 µg/ml), and then screened for homologous recombinants by PCR and Southern blot analysis. PCR primers were as follows: 5′-GTAAGTGGCCCTGGACTCTATCA-3′, 5′-GCCCATCTACATCATTGAATACCAGG-3′, and 5′-GCCAGTCTAGCGGAATGCTG-3′. Southern blot analysis was performed by digesting genomic DNA with SpeI and hybridization with a probe shown in Fig. 1 A to detect 7- and 6-kb bands that correspond to wild-type and mutant alleles, respectively. Genotyping of mice by PCR was performed using the following three primers: 5′-GTAAGTGGCCCT GGACTCTATCA-3′ (a), and 5′-TGCGAGCCTGTTTTCGAGAG-
GAAGC-3’ (b), and 5’- GCCCTCTGTCCCCCCCATCTATCA-3’ (c). These three primers were used to detect the wild-type and mutant alleles simultaneously, whereas only two primers (Fig. 1a, a and c) were used to detect only the wild-type allele. The genotypes of the blastocysts were analyzed mostly by the latter condition and depicted as Uba3+/– or Uba3−/–. Mice were housed in an environmentally controlled room, and the experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Tokyo Metropolitan Institute of Medical Science.

Histology and immunofluorescence

Embryos in utero were dissected at various stages of development, fixed in 4% paraformaldehyde (PFA), paraffin embedded, and sectioned sagittally. Sections were stained by Meyer’s H&E. For immunofluorescence analysis, the following primary and secondary antibodies were used: anti-cytokeratin-endoA antibody (TROMA-1; Developmental Studies Hybridoanal Bank), anti-cyclin E antibody (M-20; Santa Cruz Biotechnology, Inc.), anti-Uba3 antibody raised in rabbits in our laboratories, anti-p57Kip2 antibody (provided by D. H. Toyoshima, Tiskuba University, Ibaraki, Japan), anti-Brdu antibody (Bu20a, Dako), anti-β-catenin antibody (clone14; Transduction Laboratories) and Alexa 488 nm anti-rabbit and Alexa 594 nm anti-mouse antibodies (Molecular Probes). Nuclei were counterstained with TOTO3 (Molecular Probes) or Hoechst 33342 (Molecular Probes). Fluorescence images and differential interference contrast images were obtained using a confocal laser scanning microscope (LSM510; ZEISS) or a fluorescence microscope (AX70; Olympus) equipped with cooled charge-coupled device camera (Senys/OI; Photometrics). Pictures were taken using IPLab image software (solution systems).

Blastocysts in vitro culture

Eight-cell embryos or blastocysts were flushed out and collected from pregnant female uteri at E2.5 or E3.5, then cultured in vitro in M16 medium (Sigma-Aldrich) at 37°C with 5% CO2. After washing, individual blastocysts were transferred to ES cell medium and spread onto 0.1% gelatin-coated chambered coverslip (Lab-Tek; Nalgene).

BrdU incorporation

Expanded blastocysts were cultured for 48 h and labeled with 20 μM BrdU (Sigma Aldrich) for 30 h before fixation by 70% ethanol overnight at 4°C. Embryos were treated with 4N HCl and 0.5% Triton X-100 in PBS for 30 min at room temperature. After washes with PBS, embryos were subjected to immunocytochemistry as described above. In vivo experiments, BrdU (100 μg/g of body weight) was injected i.p. to pregnant females at 5.75 d postcoitus. The females were killed 2 h after injection; the decidua were fixed in 4% PFA at 4°C overnight. Sagittal sections were treated with 2N HCl for 45 min, incubated in proteinase K (20 μg/ml) for 10 min at room temperature, and then processed for immunohistochemistry.

TUNEL assay

Apoptotic cells were detected by Tdt-mediated dUTP-biotin nick end labeling (TUNEL) assay using Apoptag kit (Intergen). The assay was performed as per manufacturer’s instructions. In brief, the sections and PFA-fixed blastocysts were treated by proteinase K (20 μg/ml) for 15 min at room temperature. After equilibration, the sections were incubated with digoxigenin-dUTP under the TdT enzyme drop for 1 h at 37°C. After stopping reaction, the tissues were incubated covered by antidigoxigenin conjugate for 30 min at room temperature. The digoxigenin conjugate was detected immunochromically by FITC-labeled antibody.

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